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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PROTON NUCLEAR MAGNETIC RESONANCE OF ELEVEN ISOMERS OF METHYL RETINOATE*

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SUMMARY

Methyl all-*trans*-retinoate was isomerized with fluorescent light in either heptane, acetonitrile or dimethyl sulfoxide. Eleven isomers of methyl retinoate were produced during irradiation in dimethyl sulfoxide. The isomers were purified by high-performance liquid chromatography (HPLC) using reversed-phase columns and were characterized by proton nuclear magentic resonance and chemical methods. They were identified as the methyl esters of a 13-*cis*-(5 → 10)-photocyclized isomer, the 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 7,13-di-*cis*, 13-*cis*-, 9,13-di-*cis*-, a 13-*trans*-(5 → 10)-photocyclized isomer, the 11-*cis*-, 9-*cis*-, 7-*cis*- and all-*trans*-isomers of retinoic acid. After prolonged irradiation in polar solvents, the two photocyclized isomers accumulated until they were the only two observable isomers in the HPLC pattern. The rate of disappearance of the all-*trans*-isomer increased as solvent polarity increased. Only seven isomers were produced by irradiation in heptane.

INTRODUCTION

The isomerization of vitamin A compounds is of theoretical and practical importance. The *cis–trans* isomers form upon exposure to heat or light, and each isomer possesses different biological activity. To date, the most efficient procedure for the separation of these isomers is by high-performance liquid chromatography (HPLC)[1–7]. The formation, separation, and identification of seven geometric isomers of methyl retinoate, which had been formed by irradiation of methyl all-*trans*-retinoate in heptane, have been reported[1,2]. The isomers were identified by spectroscopic and HPLC methods as the 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-isomers of methyl retinoate. In addition, an unidentified isomer, which had the molecular weight of methyl retinoate but an atypical UV absorption,

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was reported[1]. There was some uncertainity about the identification of the 9,11,13-tri-*cis*-isomer, which had not been previously reported. Also, the proton nuclear magnetic resonance (NMR) spectrum of the 11,13-di-*cis*-isomer was not visually similar to the published data for 11,13-di-*cis*-retinaldehyde. The objectives of the present investigation were (1) to identify the unknown isomer, (2) to confirm the identification of the seven geometric isomers previously reported, and (3) to identify isomers produced by irradiation in polar solvents.

The structures of the eleven isomers of methyl retinoate are shown in Fig. 1.

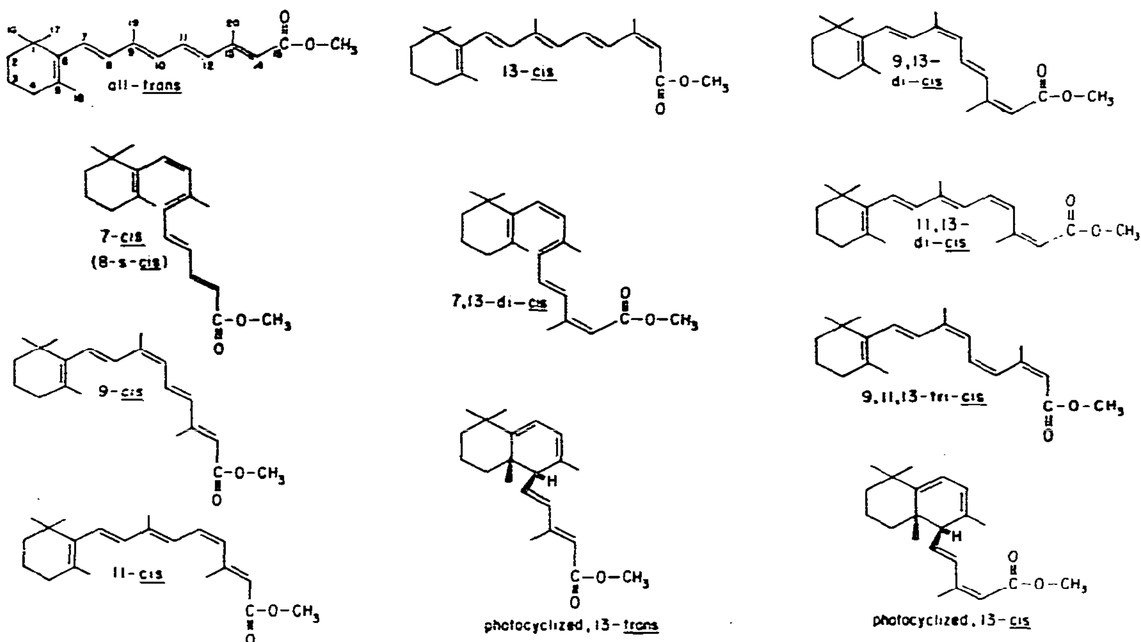

Fig. 1. Isomers of methyl retinoate.

MATERIALS AND METHODS

Materials and methods were as previously described[1]. Additionally, Partisil M9 10/50 reversed-phase octadecylsilane (ODS), Partisil M9 10/50 ODS-2 and Partisil PXS 10/25 ODS-2 columns (Whatman, Clifton, N.J., U.S.A.) were used for preparative HPLC separations. A Waters Assoc. (Milford, Mass., U.S.A.) system, consisting of a Model 660 solvent programmer, a Model 440 absorbance detector, two Model M 6000A chromatography pumps, a μBondapak C_{18} column and a Model U6K injector, was used with a Sargent-Welch Model XKR recorder (Sargent-Welch Scientific, Dallas, Texas, U.S.A.) for some HPLC separations. Solvents for isomer preparation (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) were purged with nitrogen gas for approximately thirty minutes before use.

NMR spectra were obtained either in deuterated chloroform, 100 atom % D, or in deuterated acetone, 100 atom % D (Sigma, St. Louis, Mo., U.S.A.). A trace of tetramethylsilane, 99.9%, NMR grade (Aldrich, Milwaukee, Wisc., U.S.A.), was included as an internal reference. All chemical shifts are reported as parts per

million downfield of tetramethylsilane (TMS). A Varian XL-100-15 NMR, interfaced to a Nicolet Technology Corp. TT-100A Fourier Transform accessory, was used for all NMR experiments. Tubes (5 mm; Wilmad Glass, Buena, N.J., U.S.A.) or capillary tubes, 1.7 × 90 mm (Kimble Products, Toledo, Ohio, U.S.A.) were used. Probe temperature was controlled at 20° with cooled nitrogen. Proton double resonance experiments were run in the gyrocode mode.

NMR spectra were matched using a version of LAOCN3 program[8,9] on an IBM 370/158 computer. The proton NMR spectra were matched assuming three isolated spin systems, namely H(7)–H(8), H(10)–H(11)–H(12) and H(14). Spin coupling constants over four or more bonds were set equal to zero for the computer simulation, as the resolution of our spectra did not allow determination of most long-range couplings. Adequate fits of theoretical and experimental spectra, except for some linewidths, were obtained. The proton NMR signals from hydrogens 10 and 12 could be differentiated in the isomers with 11-*trans* configuration, where $J_{11,12}$ was about 15 Hz and $J_{10,11}$ was 11 to 12 Hz. When the 11-bond was *cis*, then both $J_{11,12}$ and $J_{10,11}$ were 11 to 12 Hz. In these cases, the doublet with the broader linewidth was assigned to proton 10, as unresolved, long-range couplings are greater for proton 10 than proton 12 (refs. 10–12). Similarly, the proton NMR signal for hydrogen 7 was broader than the signal from hydrogen 8 in all isomers. This differential linewidth has also been attributed to unresolved, long-range couplings[10,11,13].

Iodine catalyzed re-isomerizations of the purified isomers were conducted in heptane in the dark. Each of the isomers was purified by HPLC, then dissolved in heptane. A portion of this solution was removed and saved as a control. A catalytic amount of iodine (1 μg) in heptane was added with mixing to the remaining solution. Aliquots were removed after 10 and 30 min, immediately dried under a stream of nitrogen in a warm water bath, then dissolved in methanol. Likewise, the control sample was dried and dissolved in methanol. The samples were analyzed by HPLC. Control solutions were typically 95–99% pure. Product peaks were identified by HPLC retention times, or by addition to known isomer samples and rechromatographed. The results and the reaction rates indicated that iodine in the dark rapidly converts an 11-*cis*-bond to 11-*trans*, slowly isomerizes the 13-bond to an equilibrium between *cis* and *trans* configurations, but does not affect the geometry of the 9-bond.

Light catalyzed reisomerization of one of the purified cyclic isomers was carried out in nitrogen purged heptane. Two samples were irradiated, one for 10 min with a UVS 12 Mineralight (Ultraviolet Products, San Gabriel, Calif., U.S.A.), and the other sample for one hour with the fluorescent lamp used for the isomer production from methyl all-*trans*-retinoate.

The peaks, identified in the text as peaks 0, 1, 2, 2A, 6 and 7, were analyzed by low resolution mass spectroscopy[14]. All isomers exhibited molecular ions of *m/e* 314 and the expected fragmentation ions of methyl retinoates.

RESULTS AND DISCUSSION

Isomer identification

The reversed-phase HPLC pattern of methyl all-*trans*-retinoate after a 50-h

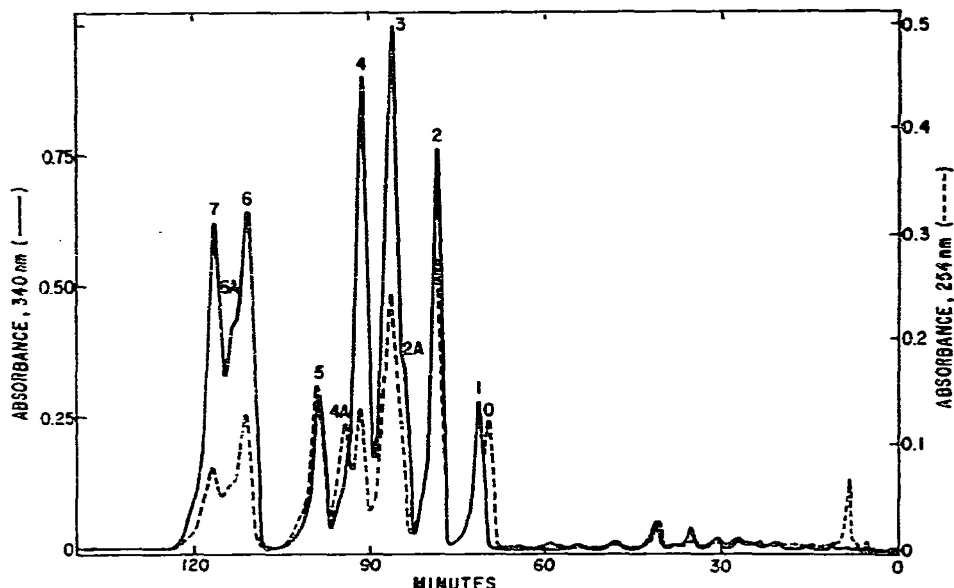

Fig. 2. Reversed-phase HPLC pattern of methyl all-*trans*-retionate after 50-h fluorescent-light irradiation in dimethyl sulfoxide. A total of 0.5 mg methyl retinoate was applied to a Whatman Partisil M9 10/50 ODS-2 column. A Waters Assoc. M 6000A pump delivered 3.0 ml/min of 90% methanol–10% water. Absorbance was detected at 254 and 340 nm with a Waters Assoc. Model 440 absorbance detector.

fluorescent-light irradiation in dimethyl sulfoxide is shown in Fig. 2. The peaks eluting between the void volume and 60 min include dimethyl sulfoxide and oxidation products formed during irradiation. At least eleven peaks follow which are attributed to methyl retinoate isomers. These isomers were related to those identified previously[1] by comparing the retention times and elution profile to isomer

TABLE I

METHYL PROTON CHEMICAL SHIFTS OF ISOMERS OF METHYL RETINOATE

In C^2HCl_3, the temperature was 20° unless otherwise noted. Shifts are ppm downfield of internal TMS. Estimated accuracies are ± 0.01 ppm for chemicals shifts.

Isomer	*HPLC peak*	*Methyl proton chemical shifts*				
		20CH₃	*19CH₃*	*18CH₃*	*16,17CH₃*	*OCH₃*
All-*trans*	7	2.37	2.01	1.72	1.03	3.73
7-*cis*	6A	2.34	1.90	1.52	1.04	3.71
9-*cis*	6	2.35	2.01	1.75	1.05	3.72
11-*cis**	5	2.35	2.00	1.71	1.03	3.66
13-*trans*-(5→10)-photocyclized	4A	2.31	1.67	0.97	1.13	3,71
9,13-di-*cis*	4	2.06	1.99	1.74	1.03	3.70
13-*cis*	3	2.07	1.99	1.72	1.02	3.72
7,13-di-*cis**	2A	2.09	1.96	1.54	1.06	3.65
11,13-di-*cis*	2	2.17	1.96	1.71	1.02	3.68
9,11,13-tri-*cis*	1	2.18	1.99	1.73	1.03	3.68
13-*cis*-(5→10)-photocyclized	0	2.04	1.69	0.97	1.14	3.69

* In $[^2H_6]$acetone, 20°.

mixtures produced in heptane and from the absorbance at 350 and 254 nm. For ease of discussion, the peak numbering system of McKenzie *et al.*[1], based upon the HPLC elution order, has been retained in the present study. New peaks observed in the present study eluted after peaks 0, 1, 2, 4, 5 and 6, and were designated 0A, 1A, 2A, 4A, 5A and 6A, respectively.

The methyl proton chemical shifts of the seven previously identified isomers of methyl retinoate from this study (Table I) agree with the corresponding methyl shifts previously reported[1]. Since the methyl shifts alone are not sufficient for positive identification of the isomers, the vinyl portions of the proton spectra, Fig. 3, were matched via computer simulation. These data are compiled in Table II. Visual matching of literature NMR spectra of all-*trans*-, 9-*cis*-, 11-*cis*-, 9,13-di-*cis*- and

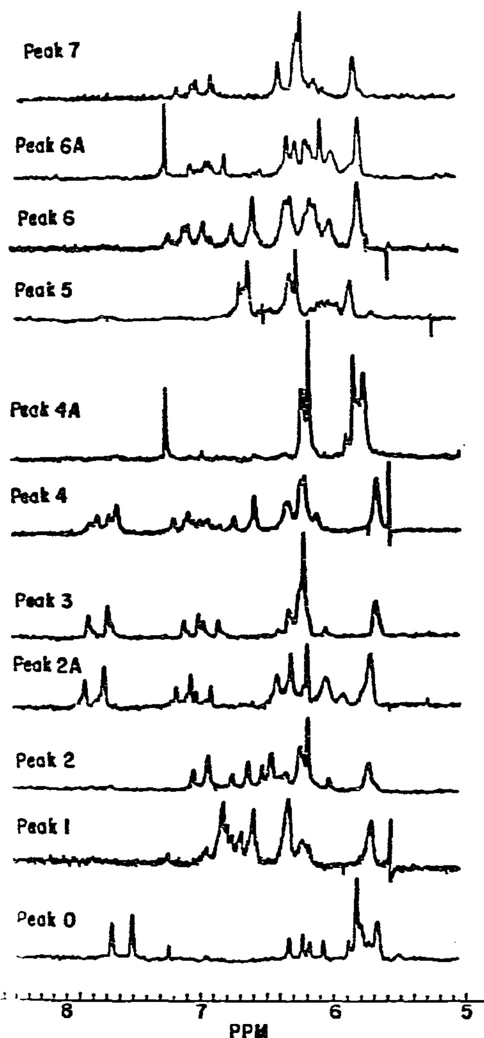

Fig. 3. The vinyl regions of the proton NMR spectra of eleven isomers of methyl retinoate. Peak numbers refer to HPLC peak elution order as shown in Fig. 2. The peak 4 NMR sample contained some peak 3 material. Peak at 7.25 ppm is from undeuterated chloroform. Sharp spikes, between 5 and 6 ppm are instrumental artifacts.

13-*cis*-retinoates and retinaldehydes provided identification of these isomers as HPLC peaks 7, 6, 5, 4 and 3, respectively. Computer simulation of the vinyl regions of the NMR spectra (Table II) of these also provided excellent agreement with literature values[15].

TABLE II

VINYL PROTON CHEMICAL SHIFTS AND COUPLING CONSTANTS OF ISOMERS OF METHYL RETINOATE

In C^2HCl_3, temperature 20° unless otherwise noted. Shifts are ppm downfield of internal TMS Estimated accuracies are ± 0.01 ppm for chemical shifts and ± 0.5 Hz for coupling constants

Isomer	*HPLC peak*	*Vinyl proton chemical shifts*						*Coupling constants*		
		H(14)	*H(12)*	*H(11)*	*H(10)*	*H(8)*	*H(7)*	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$
All-*trans*	7	5.79	6.30	7.01	6.15	6.16	6.26	16.0	11.4	15.0
7-*cis*	6A	5.78	6.26	6.93	6.22	6.11	5.95	13.0	11.8	14.6
9-*cis*	6	5.79	6.27	7.10	6.05	6.65	6.23	16.0	12.0	15.0
11-*cis**	5	5.84	6.00	6.67	6.63	6.21	6.35	16.0	11.9	10.8
13-*trans*-(5→10)--photocyclized	4A	5.74	6.12	6.21	2.78	5.84	5.77	6.0	9.7	14.9
9,13-di-*cis*	4	5.65	7.72	7.09	6.17	6.66	6.28	16.0	10.5	15.0
13-*cis*	3	5.65	7.78	7.00	6.26	6.17	6.26	16.5	11.4	15.2
7,13-di-*cis**	2A	5.69	7.82	7.07	6.36	6.23	6.00	13.2	11.2	15.2
11,13-di-*cis*	2	5.70	6.99	6.63	6.41	6.12	6.27	16.3	12.1	11.6
9,11,13-tri-*cis*-	1	5.70	6.90	6.71	6.30	6.68	6.29	16.0	11.1	11.8
13-*cis*-(5→10)--photocyclized	0	5.65	7.62	6.20	2.89	5.83	5.75	5.5	10.6	15.6

* In $[^2H_6]$acetone, 20°.

Peak 2A was a peak not seen in the previous study[1]. Use of more polar solvents, such as acetonitrile and dimethyl sulfoxide, during the irradiation of methyl all-*trans*-retinoate, produced sufficient peak 2A for isolation and identification. The absorbance ($\lambda_{max.}$ = 351 nm in methanol) and mass spectrum were consistent with the other methyl retinoates. The proton NMR spectrum of peak 2A resembled that of peak 3, methyl 13-*cis*-retinoate, except that the pair of doublets from hydrogens 7 and 8 had a spin-coupling constant of only 13.2 Hz, and signals from hydrogen 7 and the C-18 methyl group were shifted upfield to 6.00 and 1.54 ppm, respectively. From these data, peak 2A was identified as methyl 7,13-di-*cis*-retinoate. The corresponding 7,13-di-*cis*-retinaldehyde has been synthesized[16], as have methyl 7,9-di-*cis*-retinoate and methyl 7,9,13-tri-*cis*-retinoate[17], but none of these have been reported as photochemical irradiation products.

The unknown isomer, peak 0, was shown to have the same molecular ion and fragment ions as the other isomers, but its maximum absorption occurred at 263 nm, about 80 nm less than the other isomers[1]. Such a blue shift in the absorption maximum indicated a lesser degree of conjugation relative to the other isomers[18]. In the present study, peak 0 also became more prominent with increased irradiation time, and has now been found to accumulate more rapidly as solvent polarity increases in the solvent series heptane, acetonitrile and dimethyl sulfoxide. Integration of the proton NMR of the isomer in peak 0 indicated only five vinyl protons, one

less than the other methyl retinoates. The proton-decoupled carbon-13 NMR showed peaks from eight carbons in the vinyl region instead of the expected ten. This isomer did not react with iodine in the dark in heptane, but did provide an isomer pattern on HPLC after exposure to fluorescent and short wavelength UV light. These data indicated that the unknown isomer, peak 0, was a reversibly-photocyclized isomer of methyl retinoate. A double resonance NMR experiment was conducted to determine the mode of cyclization. Irradiation of the doublet of doublets at 6.20 ppm in the proton NMR (Table II) caused the doublets at 7.62 ppm (J = 15.6 Hz) and 2.89 ppm (J = 10.6 Hz) to collapse to singlets. These signals corresponded to hydrogens 11, 12 and 10, respectively, and the magnitude of $J_{11,12}$ indicated the 11-bond was *trans*. The carbon at position 10 was involved in the cyclization, as hydrogen 10 did not resonate in the vinyl region of the NMR spectrum. The coupling constant between hydrogens 7 and 8 was only 5.5 Hz, too small for either a *cis*- or *trans*-vinyl coupling. The methyl shifts (Table I) show a methyl group at 0.97 ppm, upfield of the geminal-methyls, C-16 and C-17, which resonate at 1.14 ppm. The NMR and other data are all consistent with the peak 0 isomer being a photocyclization product of methyl 7,13-di-*cis*-retinoate (Fig. 4) involving the 5, 7 and 9 double bonds; namely methyl [(1*R*, 8a*S*)-1,5,6,7,8,8a-hexahydro-2,5,5,8a-tetramethyl-1-naphthalenyl]-3-methyl-2*Z*,4*E*-pentadienoate. For brevity, this compound will be listed in the tables as the 13-*cis*-(5 →10)-photocyclized isomer.

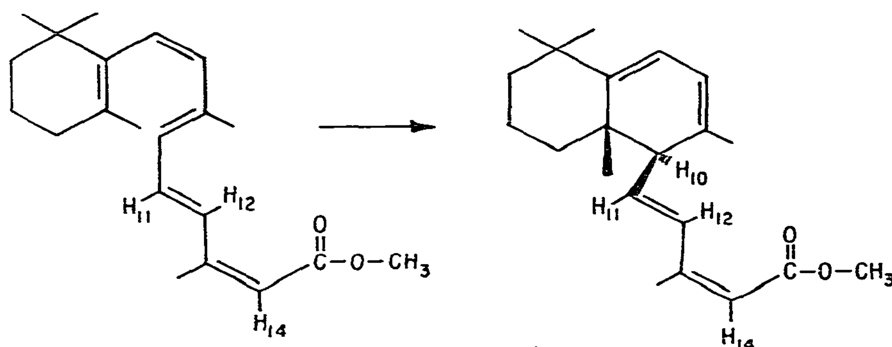

Fig. 4. Conversion of methyl 7,13-di-*cis*-retinoate (peak 2A) to a photocyclized isomer (peak 0).

Based upon the chemical shifts of the methyl group C-20 at 2.04 ppm, and of hydrogen 14 at 5.65 ppm, both of which are consistent with the corresponding shifts of 13-*cis*-isomers but not 13-*trans*-isomers, the configuration of the 13-bond is *cis* in the photocyclized isomer. The configurations at C-5 and C-10 are based upon theoretical and literature precedents[19–24]. This type of cyclization has been seen with vitamin A analogues[19–21] and with retinaldehyde[22–24].

Peak 4A was not recognized as a new peak at first, as it cochromatographed with peak 4. However, peaks 4 and 4A were resolvable at 90% methanol–10% water on a 50-cm ODS-2 column, as shown in Fig. 2. Peak 4A, like peak 0, increased upon prolonged irradiation, and had a $\lambda_{max.}$ of 267 nm. Based upon its proton NMR, Tables I and II, peak 4A was identified as the 13-*trans*-(5 → 10)-photocyclized isomer of methyl retinoate or methyl [(1*R*,8a*S*)-1,5,6,7,8,8a-hexahydro-2,5,5,8a-tetra-

methyl-l-naphthalenyl]-3-methyl-2*E*,4*E*-pentadienoate. A *cis–trans* isomerization would interconvert peaks 0 and 4A.

Peak 2 had been identified by McKenzie *et al.*[1] as methyl 11,13-di-*cis*-retinoate, but the literature NMR data for 11,13-di-*cis*-retinaldehyde[10] did not give a close approximation to their NMR spectrum of peak 2. From Table II, however, the chemical shifts and coupling constants of the vinyl hydrogens of peak 2 ($\lambda_{max.}$ = 346 nm in methanol) indicate the 11- and 13-bonds are *cis*, while the 7- and 9-bonds are *trans*. For example, the chemical shifts of hydrogen 8 is indicative of the configuration of the double bond between C-9 and C-10 (ref. 19). The chemical shift value for hydrogen 8 of about 6.2 ppm downfield from TMS is observed for the 9-*trans*-isomers (peaks 3, 5 and 7) while a shift of about 6.7 ppm is observed where the 9-bond is *cis* (peaks 4 and 6). The chemical shift value of hydrogen 8 in peak 2 is 6.12 ppm, consistent with a 9-*trans*-bond. From the shift of hydrogen 14 and the coupling constants $J_{7,8}$ and $J_{11,12}$, peak 2 can be identified as methyl 11,13-di-*cis*-retinoate. Isomerization of peak 2 by iodine in the dark produced peak 3, the 13-*cis*-isomer, supporting the assignment based upon NMR data.

The NMR data for peak 1 ($\lambda_{max.}$ = 339 nm in methanol) also confirms the assignment of McKenzie *et al.*[1], who identified peak 1 as methyl 9,11,13-tri-*cis*-retinoate. The only significant difference between the proton chemical shift data of peaks 2 and 1 in Tables I and II is the chemical shift of hydrogen 8. For peak 1, the chemical shift of hydrogen 8 is at 6.68 ppm, corresponding to a 9-*cis*-bond. Also, the isomerization with iodine in the dark converted peak 1 to peak 4, the 9,13-di-*cis*-isomer. Thus, peak 1 is methyl 9,11,13-tri-*cis*-retinoate.

The last major isomer, peak 6A, resolved as a shoulder on the trailing side of peak 6. The chemical shifts of the C-18 methyl group, 1.52δ, and H(7), 5.95δ, plus a 13.0-Hz coupling between protons H(7) and H(8), indicate the 7-bond is *cis*. The similarity of the rest of the NMR parameters to the all-*trans* values identifies peak 6A as methyl 7-*cis*-retinoate.

The identity of peaks 0A, 1A, and 5A (not visible in Fig. 2) in the HPLC pattern are, as yet, unknown. One of these might be methyl 9,11-di-*cis*-retinoate. The 9,11-di-*cis*-isomer of C_{20} retinoids has not been synthesized or identified in photo-irradiation mixtures. Alternatively, these peaks might be other photocyclized isomers, similar to peaks 0 and 4A, but with the 11-bond *cis*. Additional possibilities include the 7,9,13-tri-*cis*-isomer or the 7,11,13-tri-*cis*-isomer.

Solvent effects and isomer stabilities

The present observation of greater production of isomers with *cis*-bonds in solvents of high polarity agrees with reports that hindered retinaldehydes with 7-*cis*- or 11-*cis*-bonds increase in proportion to the other isomers when retinaldehyde is irradiated in polar solvents[3,5,7,25]. The rate of disappearance of the all-*trans*-isomer also increased in polar solvents, as comparable isomerization of the all-*trans*-isomer occurred after 28 h of irradiation in dimethyl sulfoxide, 50 h in acetonitrile and 100 h in heptane. However, not all of the eleven isomers were formed during irradiation in heptane, even after prolonged irradiation. As seen in Fig. 5B, only seven isomers were observed after 56 h of irradiation in heptane. No appreciable amounts of the 7-*cis*- (peak 6A), 11-*cis*- (peak 5), or 7,13-di-*cis*-isomers (peak 2A) were observed when heptane was used as the solvent. Maximal production of the 11-*cis*-isomer was

observed after 3 h in dimethyl sulfoxide, where it comprised nearly 30% of the HPLC pattern at 254 nm. In the heptane system, the 13-*cis*-isomer reached 30% of the HPLC pattern at 254 nm after 2 h. Thus, either of these isomers can be produced in maximal amounts by selection of solvent and by short irradiation time. Peaks 4 and 4A were not resolved, as seen from Fig. 5A, but the amount of peak 4A formed in heptane after 56 h would be no more than the amount of peak 0 present. The peak labeled "4" in Fig. 5A was shown by HPLC on another column to be composed of nearly equal absorbances, at 254 nm, from peaks 4 and 4A.

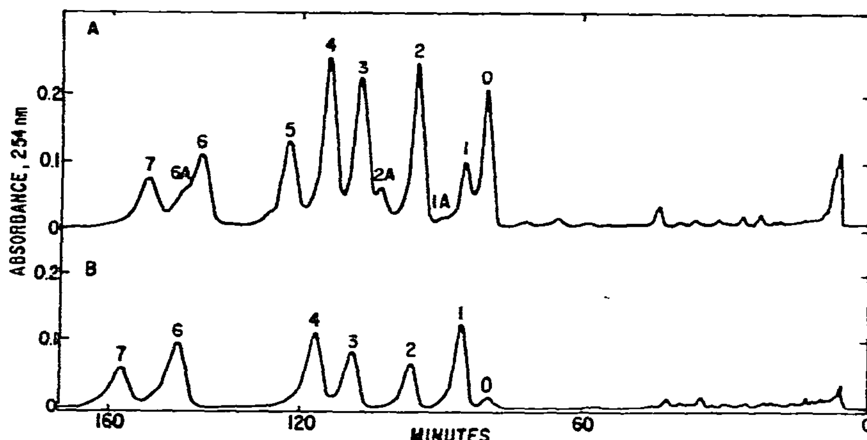

Fig. 5. Reversed-phase HPLC pattern of methyl all-*trans*-retionate irradiated 56 h in dimethyl sulfoxide and heptane. (A) Irradiation in dimethyl sulfoxide. (B) Irradiation in heptane. HPLC conditions were a Whatman Partisil PXS 10/25 ODS-2 column, 85% methanol–15% water, 0.7 ml/min: however room temperature was 27° for run A and 25° for run B.

It is somewhat surprising that the 9,11-di-*cis*-isomer was not observed, since the 11,13-di-*cis*-, 9,13-di-*cis*- and 9,11,13-tri-*cis*-isomers are all present in isomer mixtures produced in both heptane and dimethyl sulfoxide. As mentioned, this isomer might be one of the observed, but unidentified, peaks. This isomer might also cochromatograph with the 13-*cis*-isomer, as a shoulder has been observed on the trailing side of peak 3 at slow flow-rates. The NMR sample for peak 3 was produced by HPLC purification of an iodine catalyzed isomerization of the all-*trans*-isomer. Hence, the 9,11-di-*cis*-isomer might be present in peak 3 material produced by photoirradiation, but would not be present in samples produced by iodine catalysis.

The cyclized isomers, peaks 0 and 4A, predominated upon prolonged irradiation. As seen in Fig. 6, the HPLC-isomer pattern of methyl retinoate irradiated 550 h in acetonitrile, these are the only major peaks remaining in the methyl ester isomer region. According to mass spectral data, the early peaks in Fig. 6 are oxidation products, possibly similar to impurities identified in radioactive retinoic acid[26]. Probably, the cyclized isomers accumulate because they have low absorption in the emission region of fluorescent lamps[27].

After preparation of this manuscript, a study was received reporting the isolation and identification of eleven isomers of an aromatic analog of retinoic acid[28]. The NMR parameters for isomers of the aromatic retinoid agree with the parameters

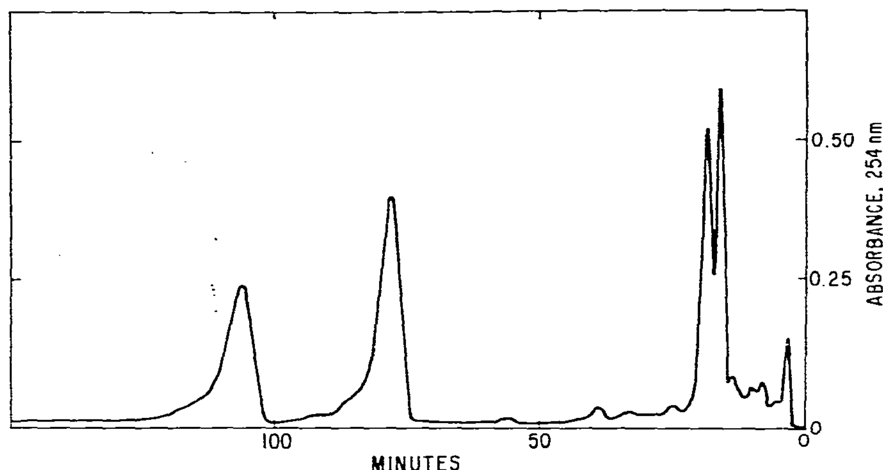

Fig. 6. HPLC pattern of methyl retinoate irradiated 550 h in acetonitrile. A sample of 0.1 mg methyl retinoate was chromatographed on a 25-cm Waters Assoc. μBondapak C_{18} column with 70% methanol–30% water at 1.3 ml/min from a Waters Assoc. M 6000A pump. Absorbance at 254 nm was monitored with a Waters Assoc. Model 440 absorbance detector. The early eluting peaks correspond to oxidized material, while peaks 0 and 4A are the major isomers in the methyl ester pattern. Note that the column and flow-rate are different from those in Figs. 2 and 5.

in Table II, except for the chemical shifts of protons H(7) and H(8). The greater downfield shifts in the aromatic retinoid for these protons, relative to those in retinoic acid, could be due to inductive effects or ring-current shielding from the aromatic ring. It is noteworthy that the 9,11-di-*cis*-isomer of the aromatic retinoid was not isolated from the photoisomerate mixture[28].

ACKNOWLEDGEMENTS

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