MACROLIDE ANTIBIOTIC STUDIES. XV*

THE AUTOXIDATION OF THE POLYENES OF THE FILIPIN COMPLEX AND LAGOSIN

R. W. RICKARDS and ROGER M. SMITH

Research School of Chemistry, Australian National University
Canberra, A. C. T. 2600, Australia

B. T. GOLDING**

Department of Chemistry, University of Manchester,
Manchester 13, England

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Macrolide antibiotics of the polyene antifungal subgroup undergo aerial autoxidation in methanol solution by a radical addition process which can be inhibited by antioxidants. The related pentaenes of the filipin complex, which has the main component (I), and lagosin (II) yield as the major primary products the corresponding tetraene epoxides (III) and (IV), which are probably mixtures of diastereoisomers. The structures of the products were established by spectroscopy and chemical degradation. Extended autoxidation leads to higher oxidation products and ultimately to polymeric materials.

Macrolide antibiotics[1] of the polyene subgroup[2] lack the antibacterial activity[3] of their non-polyene analogues[4], but are highly active against yeasts and fungi[5]. Many exhibit antiprotozoal activity[6], and some show considerable promise for controlling serum cholesterol levels, prostate malfunction, and obesity[6]. Several polyenes, notably nystatin, amphotericin B, and trichomycin, are important therapeutic agents, and of these three nystatin has been structurally defined.[7]*** Members of the subgroup as a whole are unstable, and exposure to acids, alkalis, heat, air or light is accompanied by decomposition and loss of biological activity. In particular, this sensitivity to air and light, which is primarily associated with the polyene chromophore, creates problems in storage prior to clinical use. Whilst the inactivation by aerial oxidation of the polyene macrolides pimaricin[8] and nystatin[9] has been studied qualitatively, and quantitative studies made in the cases of nystatin[9] and filipin[10], the detailed chemistry of the autoxidation processes themselves has remained undefined. In view of the clinical value of these polyene antibiotics[5,6], we have studied the autoxidation in solution of two of the simpler representatives, filipin (I) and lagosin (II), and present here evidence that the major primary autoxidation products are the corresponding epoxides (III) and (IV).

* Part XIV. MANWARING, D. G.; R. W. RICKARDS and R. M. SMITH : The total absolute configuration of methymycin. Tetrahedron Letters 1970-13 : 1029~1032, 1970.

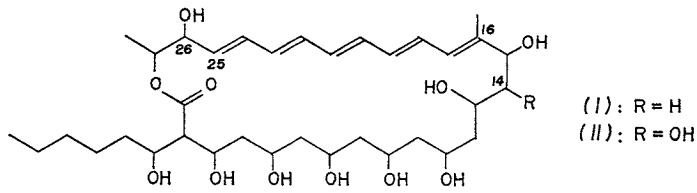
** Present address : School of Molecular Sciences, University of Warwick, Coventry, England.

*** A recent X-ray analysis[29] defines the structure of amphotericin B.

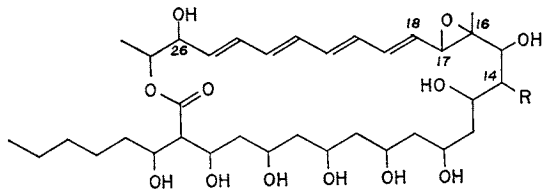
Autoxidation of the Filipin Complex

Filipin, originally isolated by WHITFIELD and colleagues[11] from *Streptomyces filipinensis*, was assigned the $C_{35}H_{58}O_{11}$ structure (I) independently by CEDER and RYHAGE[12] and by GOLDING, RICKARDS and BARBER[13], both groups extending the earlier studies of DJERASSI[14] and DHAR[15] and their co-workers. During this work GOLDING[16] observed that filipin samples from *S. filipinensis* required extensive purification since they contained a number of related components, including higher molecular weight materials, as judged by thin-layer chromatography and mass spectrometry of trimethylsilylated preparations. Subsequently BERGY and EBLE[17] have resolved the fermentation material of WHITFIELD *et al.*[11] into three major pentaenoid components, designated filipins II, III and IV and present to the extent of 25, 53 and 18 % respectively, the residual filipin I (4 %) being a mixture of at least five components*. The major components have identical molecular weights after hydrogenation,[17]** and from ultraviolet and infrared data[17] all carry a similar trisubstituted pentaene chromophore whose longest wavelength maximum is in good agreement[2,18] with an all-*trans* stereochemistry. Furthermore, the allylic methyl group apparent in proton magnetic resonance spectra[17] of these main components must in each case be at the end of the chromophore as in (V), since the only branched dicarboxylic acids obtained by DJERASSI *et al.*[14] from nitric acid oxidation of perhydro-filipin were 2-methyl-substituted acids. The total, unseparated mixture will be referred to as the filipin complex.

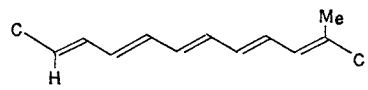
TINGSTAD and GARRETT[10] confirmed the rapid aerobic loss of biological activity of the solid filipin complex observed by WHITFIELD *et al.*[11], and correlated this with the decay of the ultraviolet absorption due to the pentaene chromophore at 355 nm. WHITFIELD and co-workers[11] also observed the conversion of the yellow filipin complex in concentrated alcoholic solution into a colourless crystalline degradation product, to



(I): R = H
(II): R = OH



(III): R = H
(IV): R = OH



(V)

* In view of the work of GOLDING[16] and BERGY and EBLE[17] it should be noted that mass spectra[16] of trimethylsilylatee impure filipin and perhydrofilipin showed no significant components corresponding to the $C_{37}H_{62}O_{12}$ formula proposed earlier for filipin by DJERASSI *et al.*[14]

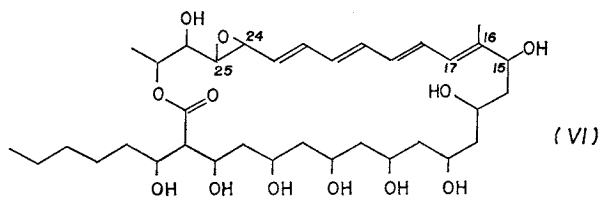
** A recent paper[30] provides further data on these components.

which was assigned the formula $C_{30}H_{50}O_{11}$ based on a then incorrect formula $C_{30}H_{50}O_{10}$ for filipin itself. This product lacked antifungal activity, and ultraviolet spectra and catalytic hydrogenation data indicated removal of one double bond from the polyene chromophore.

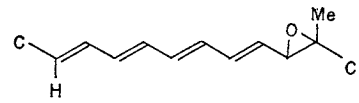
We observed[16] the same oxidative degradation using methanolic solutions of a partially purified filipin complex, estimated to contain approximately 3 % filipin I, 22 % II, 68 % III and 7 % IV, the major component of which has the formula $C_{35}H_{58}O_{11}$ and the structure (I)[13]. The crystalline product, isolated in 65 % yield has λ_{max} (in EtOH) 319, 304, and 291 nm, $\lambda_{inflex.}$ 282 nm, with infrared maxima (in Nujol) at 3370 (hydroxyl) and 1725 cm^{-1} (unconjugated lactone), and $[\alpha]_D^{27}$ 4.5±2° (in MeOH). These properties are in good agreement with those reported by WHITFIELD *et al.*[11], and the two products are clearly comparable. However, since most components of the filipin complex apparently undergo the reaction, as judged by decay of the pentaene absorption at 355 nm, the two preparations are probably both complexes, and may differ to some extent in their component ratios (see later).

The Structure of the Oxidofilipin Complex

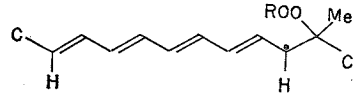
The reaction does not occur under nitrogen, and consequently represents an autoxidation process. The ultraviolet chromophore of the product complex is that of a typical tetraene, probably all-*trans* in configuration[2,18], in agreement with the uptake of four moles of hydrogen over platinum (recalculated from previous data[11] using the correct molecular weight). Mass spectrometry of the pertrimethylsilyl derivative, a procedure successfully applied in the determination of molecular weights of several polyene macrolides[7,13,17,19], showed the major (see later) molecular ion at *m/e* 1318. In view of the known $C_{35}H_{58}O_{11}$ structure (I) of the major component of the parent filipin complex[12,13], this ion must correspond to the nona-trimethylsilyl ether $C_{62}H_{130}O_{12}Si_9$ of a compound (or group of isomeric compounds) of formula $C_{35}H_{58}O_{12}$. The oxidation thus involves the addition of one atom of oxygen to the components of the filipin complex. The evidence described indicates that the oxidation product is a complex of tetraene epoxides corresponding to the pentaenes present in the parent complex, and hence including as a major component structure (III) or (VI), or possibly both. The total product will be referred to as the oxidofilipin complex.



(VI)



(VII)



(VIII)

Mild hydrogenation of the crude complex afforded mainly an octahydro-derivative, $C_{35}H_{66}O_{12}$, which gave the expected molecular ion at *m/e* 1326 after per-trimethylsilylation. This octahydro-oxidofilipin complex was oxidised with chromic acid in acetic acid[20] and the resulting dicarboxylic acids

after methylation were examined by gas-liquid chromatography (glc). Comparison with standard straight-chain and 2-methyl-substituted diesters showed the presence in the oxidation mixture of a homologous series of straight-chain diacids up to decanedioic, but no 2-methyl-dicarboxylic acids. This necessitates that the major portion of the oxidofilipin complex has the 16, 17-oxido structure (III), the structurally significant decanedioic acid arising from the C 17~26 segment. A second, very minor homologous series of products revealed by glc may be the 2-methyl-2, 3-oxido-diesters formed from (III) by fission of the 14, 15-bond and of the unbranched C 18~26 chain. The alternative 24, 25-oxido structure (VI) would be expected[14,20] to yield after hydrogenation and oxidation a mixture of two homologous series of diacids, comprising 2-methyl-branched acids up to 2-methyldecanedioic (representing C 15~24) and straight-chain acids up to octanedioic (representing C 17~24). Furthermore, the absence of detectable quantities of undecanedioic acid in the oxidation mixture excludes any structures for the oxidofilipin complex which have nine adjacent unsubstituted carbon atoms, and also confirms that hydrogenolysis of the allylic epoxide bond in structure III has not occurred to an appreciable extent.

Confirmation that epoxidation has occurred mainly at the trisubstituted olefinic bond in (I) was obtained from proton magnetic resonance spectroscopy. The oxidofilipin complex lacked the C 16 allylic methyl resonance occurring at τ 8.00 in the filipin complex, and showed instead considerably enhanced absorption at τ 8.75, the region expected[21] for methyl attached to an epoxide ring.

The major components of the filipin complex undergo similar autoxidation, although probably at somewhat different rates, and the precise composition of the resulting oxidofilipin complex will depend upon the composition of the parent filipin, the conditions of autoxidation, and the extent of fractionation of the product. The present work defines the nature of the initial oxidation of the various filipins as a conversion of their chromophores from the pentaene (V) to the tetraene-epoxide (VII), and in conjunction with previous work[12~15] defines the structure (III) of a major portion of the oxidofilipin complex. The analytical methods used here are not sufficiently sensitive to completely exclude the presence in the complex of isomeric epoxides of the type (VI), but if present such compounds must be very minor components. Even that portion of the oxido-complex represented by the structure (III) is probably not a single entity but rather a mixture of stereoisomers, since the autoxidation itself may be non-stereospecific and furthermore the portion of the initial filipin complex represented[12,18] by structure (I) may from BERGY and EBLE'S work[17] be a mixture of stereoisomers. In connection with the steric course of the epoxidation, it is notable that although the various filipins themselves (and the filipin complex) all have large negative $[\alpha]_D$ values[11,17] the oxido-complex is only weakly optically active at the D-line, suggesting the presence of diastereoisomeric epoxide functions adjacent to the chromophore in the product (III).

The crude oxidofilipin complex also contains small amounts of more highly oxidised materials, notably compounds containing additional oxide functions, as shown by per-trimethylsilylation and mass spectrometry of the complex and its hydrogenation

product. Such compounds would not interfere with the chromic acid oxidation results above, since they would yield only smaller diacids than those from structure (III).

Oxidolagosin

Lagosin, $C_{35}H_{58}O_{12}$, isolated from a Nigerian *Streptomyces* specics[22], has the 14-hydroxyfilipin structure (II) without regard to stereochemistry[15,23], and may[15,17,24] be a stereoisomer of fungichromin[24]. The lagosin used in the present work wss homogeneous by thin-layer chromatography and mass spectroscopy[13]. Although much more stable than the filipin complex, lagosin in methanol solution underwent similar aerial autoxidation, the half-life in this case being days rather than hours. The resulting tetraene epoxide showed a typical all-*trans* ultraviolet chromophore[2,18] with λ_{max} (in EtOH) 319, 304 and 291 nm, $\lambda_{inflex.}$ 282 nm, infrared maxima (in Nujol) at 3370 (hydroxyl) and 1725 (unconjugated lactone), and, as with the oxidofilipin complex, a weakly positive optical rotation $[\alpha]_D^{27}$ 20 ± 4° (in MeOH) compared with the high negative rotation of the parent antibiotic. Mass spectrometry of the per-trimethylsilyl derivative showed the expected molecular ion at *m/e* 1406, corresponding to the deca-trimethylsilyl ether $C_{65}H_{138}O_{13}Si_{10}$ of oxidolagosin, $C_{35}H_{58}O_{13}$. Mild hydrogenation followed by oxidative degradation afforded a mixture of straight-chain dicarboxylic acids similar to that from the oxidofilipin complex.

This evidence establishes the 16, 17-oxidolagosin structure (IV) for the major primary autoxidation product of lagosin. The main reaction is again a conversion of the pentaene system (V) into the tetraene-epoxide (VII). As with the oxidofilipin complex, the isomeric epoxide arising by attack at the less substituted end of the chromophore can only be present in very minor amount if at all, whilst optical rotation values indicate that the product (IV) may be a mixture of diastereoisomers arising by epoxidation from either face of the chromophore. The slower autoxidation of lagosin relative to the filipin complex may be due to the additional 14-hydroxyl substituent, to unknown steric or conformational factors, or simply to the high purity of the lagosin in contrast to the filipin complex. The latter contained only 72 % pentaene by ultraviolet absorption assay, and unknown impurities could be catalysing its autoxidation.

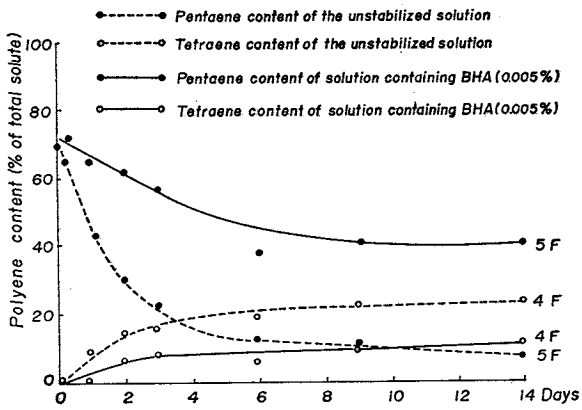
The Autoxidation Process

In agreement with the observations of WHITFIELD and co-workers[11], we find that very dilute methanolic solutions (*e.g.* 0.001 % w/v) of the filipin complex are stable for prolonged periods in air, whereas concentrated solutions in the absence of a nitrogen atmosphere were unstable even in the dark at 4°C. Concentrated solutions could be stabilized considerably by the addition of small amounts of antioxidants such as "butylated hydroxy-anisole" (BHA), a permissible foodstuff additive consisting of approximately 85 % 2- and 15 % 3-*t*-butyl-4-methoxyphenol[25]. Fig. 1 shows the decrease, measured spectroscopically, in the rates of loss of pentaene and of formation of tetraene caused by the addition of BHA (0.005 % w/v) to a solution of the filipin complex (0.8 % w/v) maintained in the dark at 8°C. There was also a decrease

in the concentration of peroxides as measured by titration.[26] In this particular experiment, some pentaene remains in the unstabilized solution even after 14 days, perhaps indicating different susceptibilities towards autoxidation of the various components of the complex (*cf.* also TINGSTAD and GARRETT[10]). Under less mild conditions complete decay of the pentaene chromophore occurs rapidly, followed by a progressive loss of tetraene absorption, and leading ultimately to polymeric oxidation products.

Fig. 1. Effect of antioxidant on the autoxidation of the filipin complex.

Methanol solutions (0.8% W/V) of the filipin complex, initially containing 72% pentaene and 0% tetraene, exposed to air in the dark at 8℃.



The degradation of these polyenes thus has all the characteristics of a radical chain autoxidation process, although the concentration of free radical intermediates was too low to permit their detection by electron spin resonance spectroscopy. The formation of epoxides during mono-olefin autoxidation is mechanistically complex, and has been reviewed recently[27]. Little work has been carried out with conjugated polyenoid systems. Of particular interest in the autoxidation of the filipin complex and of lagosin is the high preference for attack at a terminal double bond of the pentaene system, rather than at an allylic position. Hydrogen abstraction from an allylic position requires that a homoallylic carbon atom be brought into the plane of the polyene system to permit maximum stabilization of the developing allyl radical by π-orbital overlap. In a 28-membered lactone in which the all-*trans* pentaene already involves 12 co-planar carbon atoms, the high free energy of activation required for such abstraction may well favour the alternative of radical addition to the polyene terminus. Such addition, for example of a peroxy radical to give a less conjugated allyl radical intermediate of the type (VIII; R=H or alkyl) which subsequently collapses to the epoxide (VII)[27,28], would probably relieve steric constraints in the lactone ring. The high selectivity of addition to the most substituted terminal bond of these polyenes reflects the electrophilic character of the attacking radical, and may also be favoured by unknown steric factors.

The protection from autoxidation which is afforded these pentaene and other polyene macrolides[9] by the addition of trace quantities of antioxidants could have application in increasing the stability of these antibiotics during storage before clinical use.

Experimental

General

Ultraviolet spectra were measured in ethanol on a Unicam SP800 spectrometer, infrared spectra were determined as Nujol mulls on Perkin-Elmer 237 or 257 instruments. Proton magnetic resonance spectra were recorded at 100 MHz for c. 10% solutions in pentadeutero-

pyridine containing tetramethylsilane as internal reference on a Varian HA-100 spectrometer. Mass spectra were measured on AEI MS9 or MS902 instruments at 70 eV, and optical rotations on a Bendix Ericsson ETL-NPL Automatic Polarimeter 143A. Gas liquid chromatography was carried out with a Perkin-Elmer 800 chromatograph. Methanol used in autoxidation studies was purified by distillation from magnesium methoxide.

The filipin Complex

Filipin complex, λ_{max} 357, 339, 323, and 307 nm ($E^{1\%}_{1cm}$ 1059, 1089, 700, and 366 respectively) and containing 72 % pentaene (lit.[15] records λ_{max} 356 nm, $E^{1\%}_{1cm}$ 1472), was analyzed by thin layer chromatography (t. l. c.) on Kieselgel H plates prepared in phosphate buffer[17] and developed with methylene dichloride - methanol (4 : 1). The component filipins I, II, III, and IV were assayed by photography of their fluorescence excited at 366 nm, followed by microdensitometric measurement of the film at the respective spot areas, to give the following composition: Rf 0.57, 3 % ; Rf 0.34, 22 % ; Rf 0.24, 68 % ; Rf 0.16, 7 %. An alternative ultraviolet assay at 357 nm of ethanol eluates of regions of another chromatogram gave the following composition of the total pentaenoid absorption: Rf 0.64~0.50, 4 %; Rf 0.50~0.36, 16 % ; Rf 0.36~0.28, 60 % ; Rf 0.28~0.14, 20 %. A different sample of the filipin complex[17] contained filipins I, II, III, and IV to the extent of 4 %, 25 %, 53 % and 18 % respectively.

Lagosin

After crystallization from methanol under nitrogen in the dark, the lagosin used had λ_{max} 357, 340, 324, and 309 nm ($E^{1\%}_{1cm}$ 1475, 1530, 1000, and 521 respectively) (lit.[15] records λ_{max} 356 nm, $E^{1\%}_{1cm}$ 1454), and showed only one component, Rf 0.15, in the t. l. c. system used for the filipin complex.

The Oxidofilipin Complex

The filipin complex (50 mg) in methanol (4 ml) was filtered under nitrogen and then allowed to stand in air in the dark at 6°C for 10 days until the pentaenoid chromophore was gone. T. l. c. of the solution in *n*-butanol - methyl acetate (1 : 2) showed only one spot, Rf 0.9, with blue ultraviolet fluorescence and giving a pink colour with concentrated sulphuric acid. In this solvent the filipin complex has Rf 0.8, with green ultraviolet fluorescence and a blue colour reaction. Removal of the methanol and chromatography of the residue in *n*-butanol - methyl acetate on silica gel gave a tetraene-containing fraction (33 mg), which was re-chromatographed on Florisil. Elution with methanol - methyl acetate (1 : 2) gave the crystalline oxidofilipin complex (23 mg).

In an alternative preparation, the filipin complex was dissolved under nitrogen in the minimum of boiling methanol and filtered in diffuse light. Colourless crystals separated from the cooling solution, and were collected after standing overnight at 0°C. Recrystallization from methanol afforded the oxidofilipin complex $[\alpha]^{27}_{D}$ 4.5±2°, $[\alpha]^{27}_{5461}$ 0±2° (in MeOH), λ_{max} 319, 304 and 291 nm, $\lambda_{inflex.}$ 282 nm, (lit.[11] records $[\alpha]^{20}_{D}$ 0°, λ_{max} 318, 303 and 290 nm, $\lambda_{inflex.}$ 281 nm), ν_{max} 3370 and 1725 cm^{-1}.

The oxidofilipin complex with hexamethyldisilazane and chlorotrimethylsilane in pyridine[13] gave the corresponding per-trimethylsilyl ether as a gum (Found: M, *m/e* 1318. $C_{62}H_{130}O_{12}Si_9$ requires M, *m/e* 1318).

16, 17-Oxidolagosin

Lagosin (50 mg) in methanol (6 ml) was maintained in air in the dark for 30 days at 6~8°C, then for 7 days at room temperature, when almost all the pentaenoid chromophore had gone. Evaporation and chromatography of the residue on Florisil in methanol - methyl acetate (1 : 2) gave 16, 17-oxidolagosin, $[\alpha]^{27}_{D}$ 20±4°, $[\alpha]^{27}_{5461}$ 25±5° (in MeOH), λ_{max} 319, 304 and 291 nm, $\lambda_{inflex.}$ 282 nm, ν_{max} 3370 and 1725 cm^{-1}. Trimethylsilylation[13] afforded the deca-trimethylsilyl ether as a gum (Found: M, *m/e* 1406. $C_{65}H_{138}O_{13}Si_{10}$ requires M, *m/e* 1406).

Degradation of the Oxidofilipin Complex

The unfractionated oxidofilipin complex formed from filipin (123 mg) was hydrogenated

over 10 % palladium on charcoal catalyst in methanol (20 ml) for 27 hours, to yield, after chromatography on silica gel, crude octahydro-oxidofilipin complex (105 mg), a gum which was transparent in the ultraviolet region. Mass spectroscopy of a sample after trimethylsilylation[13] showed mainly the nona-trimethylsilyl-octahydro derivative of the oxido-complex (Found : M, *m/e* 1326. $C_{62}H_{138}O_{12}Si_9$ requires M, *m/e* 1326), together with minor ions corresponding to compounds formed by reduction and silylation of more highly oxidized species.

The crude octahydro-oxidofilipin complex (100 mg) in acetic acid (10 ml) was oxidized with chromium trioxide (184 mg) for 2.5 hours at 65°C. After dilution, the solution was made alkaline, washed with ether, and then re-acidified and extracted with ether. The resulting acidic oil (27 mg) was methylated with diazomethane and fractionated by t. l. c. on Kieselgel G in ether - light petroleum (1 : 2) to give a mixture of diesters (8.2 mg). This mixture was analyzed by g. l. c. on 20 % polyethyleneglycol succinate at 210°C, 5 % butanediol succinate at 170°C, 10 % ethyleneglycol succinate at 190°C, 1.5 % XE-60 silicone gum at 140°C, and 5 % Carbowax 20M polyethyleneglycol at 170°C. Comparison with standard compounds showed the presence of a homologous series of straight-chain dimethyl esters from hexanedioate to decanedioate, together with a second homologous series, only about 15 % as intense as the first, which may represent 2-methyl-2, 3-oxido-diesters. No simple 2-methyl-diesters up to 2-methylundecanedioate could be detected.

Degradation of 16, 17-Oxidolagosin

Hydrogenation of 16,17-oxidolagosin to octahydro-16, 17-oxidolagosin (Found : M for the corresponding deca-trimethylsilyl ether[13], *m/e* 1414. $C_{65}H_{146}O_{13}Si_{10}$ requires M, *m/e* 1414), followed by oxidation with chromium trioxide and methylation of the resulting acids, as for the oxidofilipin complex, afforded a mixture of esters (0.7 mg) which was analyzed by g. l. c. on 20 % polyethyleneglycol succinate at 210°C and 5 % butanediol succinate at 170°C. The mixture was similar to that from the oxidofilipin complex, containing a series of unbranched dimethyl esters up to decanedioate.

Effect of Antioxidant on the Autoxidation of the Filipin Complex

Two methanolic solutions of the filipin complex (0.8 % w/v), one of which contained "butylated hydroxy-anisole" (0.005 % w/v)[25], were maintained in the dark in air at 8°C for 14 days. After correcting for solvent loss due to evaporation, the concentrations of pentaene and tetraene were determined by ultraviolet spectroscopy, assuming the pentaene intensity[15] at λ_{max} 356 and 320 nm to be $E^{1\%}_{1cm}$ 1472 and 930, respectively, and the tetraene intensity[11] at λ_{max} 320 nm to be $E^{1\%}_{1cm}$ 1050. The results are shown in Fig. 1. The peroxide values of the unstabilized solution after several days were up to 3 times that of the stabilized solution, as determined by the titration method of DAHLE and HOLMAN[26].

Acknowledgements

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