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Oxidation of Stigmasterol in Heated Triacylglycerols

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ABSTRACT

A triacylglycerol mixture containing 5% stigmasterol by weight and devoid of other unsaponifiables was heated at 180 ± 5°C for several hours. Oxidation products from stigmasterol were separated and isolated by column chromatography and preparative thin-layer chromatography. Characterization of individual compounds was based on chromatographic mobilities, specific colour reactions, UV and IR spectroscopy and fragmentation patterns from the mass spectra of the purified oxidation products. A hydrocarbon (stigmasta-3,5,22-triene), a triunsaturated ketosteroid (stigmasta-3,5,22-trien-7-one), a diunsaturated ketosteroid (stigmasta-4,22-dien-3-one) and a pregnane derivative (Δ^5-pregnen-3β-ol-2-one) were identified. There is also evidence for the presence of more polar compounds such as hydroxy and epoxy derivatives.

INTRODUCTION

Stigmasterol is a widely distributed phytosterol and occurs in soybean oil and other vegetable oils and fats (Itoh *et al.*, 1973; Seher & Vogel, 1976). Such oils are often subjected to conditions which are suitable for the oxidation of acylglycerols and unsaponifiables. It is particularly important to identify the oxidation products formed by heating of sterols because some of these products have been found to be angiotoxic and/or carcinogenic in experimental animals (Smith & Kulig, 1975; Finocchiaro & Richardson, 1983).

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So far research work has been carried out mainly with cholesterol. This steroid is prevalent in food systems of animal origin and during autoxidation and thermal processing of foods yields a mixture of oxidation products, some of which have been isolated and their biological properties studied (Smith, 1981; Ryan *et al.*, 1981). Attempts have also been made to detect autoxidation products from β-sitosterol, the major phytosterol. Yanishlieva *et al.* (1980) detected 10 major products in autoxidized β-sitosterol. More recently, Daly *et al.* (1983) characterized many oxidation products of the same compound. These products were structurally similar to those of heated cholesterol. Both these studies were conducted by directly heating β-sitosterol in the pure state.

The present research was designed to detect and characterize oxidation products from stigmasterol and it is additional to previous work on the role of diunsaturated sterols in heated oils (Boskou & Morton, 1976; Blekas & Boskou, 1986, 1988). In order to obtain more realistic conditions, the sterol was not heated directly but a 5% mixture in oil was used for the heating experiments. Natural oils contain sterols and other unsaponifiable constituents. In this work a triacylglycerol mixture, devoid of sterols and other unsaponifiables, was used, which was prepared from commercial trioleylglycerol by column chromatography on active silicic acid.

MATERIALS AND METHODS

Materials

Commercial triolein was purchased from Fluka. Silicic acid for the separation of lipids was obtained from BDH. Stigmasterol (Fluka purum) was recrystallized from methanol. The product (m.p. 168–170°C) was checked by gas chromatography and was found to contain more than 97% stigmasterol.

Methods

Column chromatography

Thirty-gram portions of triolein were loaded onto a column packed with 299 g of silicic acid. Compounds less polar than triacylglycerols were eluted with hexane while triglycerides were recovered with hexane containing 2% diethyl ether. Only those fractions were collected which contained the triglycerides. The purity of those fractions was checked by thin-layer chromatography (Silica Gel G plates were used which were sprayed with sulfuric acid). The purified triacylglycerol mixture had the following fatty

acid composition (as methylesters per cent): Palmitic 3·4, palmitoleic 3·9, oleic 86·0, linoleic 4·9 and others 1·8.

Heating

Portions of 30 g of the purified triglyceride mixture containing 5% stigmasterol were heated in a salt bath connected to an energy regulator for 48 h. The bath was kept at 180 ± 5°C for 8 h during the day and switched off overnight.

Fractionation of unsaponifiables and thin-layer chromatography

The non-saponifiable fraction which contained the stigmasterol and its oxidation products was obtained by saponification and extraction with diethyl ether. After evaporation of the solvent the unsaponifiables were loaded to a column packed with Silica Gel G and eluted successively with hexane:diethyl ether 3:1, 1:1 and 1:3. Fractions were checked with TLC on Silica Gel GF_{254} plates and were divided conventionally into more polar and less polar according to their mobilities relative to stigmasterol.

Ordinary chromatograms were developed in heptane-ethyl acetate 1:1 VanLier & Smith, 1968), dried and sprayed with 50% H_2SO_4, followed by heating for a few minutes at 110°C. Reproducibility was established by repeating the foregoing procedures several times. Additional chromatograms were developed in chloroform and diethyl ether (Yanishlieva *et al.*, 1980). Because of structural similarities, R_f values were compared to those reported in the literature for cholesterol and β-sitosterol oxidation products (VanLier & Smith, 1968; Yanishlieva *et al.*, 1980; Daly *et al.*, 1983).

For preparative work the fraction containing the less polar compounds was applied as a band across the bottom of the plates and developed in chloroform. When the solvent front had reached the top, the plate was removed, dried and the edges were sprayed with 50% H_2SO_4. Then the plate was heated at 100°C for a few minutes and the bands thus located were scraped off. Each band was subjected to further purification by successive applications to preparative thin-layer plates.

UV and IR spectroscopy

Ultraviolet light absorption measurements were carried out with ethanol solutions over the range of 220–340 nm (Williams & Fleming, 1973) using a Pye-Unicam, model SP 8000, spectrophotometer. Infrared spectra of purified oxidation products (1% solutions in CCL_4) were obtained with an IR Perkin–Elmer 281B spectrophotometer.

Gas chromatography–mass spectroscopy

GC–MS was conducted with a Hewlett–Packard 5992 B system. The column

temperature program was a 5 min hold at 250°C followed by a 7·5°C/min ramp to 280°C.

Additional analytical procedures

The suspected 5,6-epoxide was dissolved in ether, the solvent was evaporated and the resulting product was subjected to alkali picration. The absorption maxima of the chromophore was observed at 350 and 410 nm (Lee *et al.*, 1984). The suspected Δ^4-3-Ketone was sprayed with nicotinic acid hydrazide and the fluorescence of the reaction product was observed under UV radiation (Neher, 1969).

RESULTS AND DISCUSSION

The oxidation products of stigmasterol observed on TLC plates are shown in Fig. 1. The plates were developed in ethyl acetate-heptane 1:1 and sprayed with 50% H_2SO_4.

Eleven oxidation products were detected. Four of them were less polar than stigmasterol, six were more polar and one had an R_f value very close to that of stigmasterol.

When the analytical TLC plates were sprayed with sulfuric acid, spots 9 and 10 instantly became blue. This colour, which develops before heating of

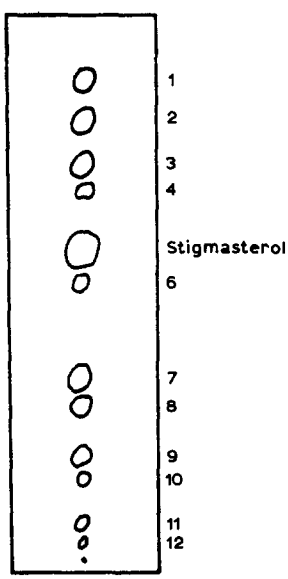

Fig. 1. Oxidation products of stigmasterol on TLC plates.

the plates, is typical for 7α- and 7β-hydroxysterols (Chicoye *et al.*, 1968; Smith & Hill, 1972; Daly *et al.*, 1983). The mobility values of spots 9 and 10 (Table 1) were very close to those reported for similar oxidation products from cholesterol (Smith & Hill, 1972). These findings indicate that spots 9 and 10 are probably epimeric 7-hydroxystigmasterols.

Spot 8 gave a yellow colour after alkali picration. This reaction is indicative of an epoxide (Daly *et al.*, 1983). The suspected area was scraped off the plate, dissolved in absolute diethyl ether and a picrate adduct was prepared. The adduct was isolated, dissolved in chloroform–methanol–water 10:10:3 and the UV absorption of the solution was examined. Two maxima were observed at 350 nm and 410 nm which are typical for chromophores obtained from epoxides (Lee *et al.*, 1984). These absorptions and the TLC data (Table 1) support the presence of a 5,6-epoxide.

Spot 12, by means of relative mobility versus reference standards and colour response to 50% sulfuric acid, was found to correspond to a triol, possibly Δ^{22}-stigmasten-3,5,6-triol. Such doubly oxidized products are characterized by their low mobilities in the TLC plates and have also been detected in autoxidized cholesterol (Smith *et al.*, 1967).

Other polar components such as hydroperoxides were not detected in the oxidation products. Hydroperoxides are reliably detected by spraying the plates with *N,N*-dimethyl-*p*-phenylenediamide dihydrochloride (Smith *et al.*, 1967). Apparently, such compounds are initially formed but they rapidly decompose to hydroxy derivatives under the conditions of heating (Yanishlieva *et al.*, 1980; Daly *et al.*, 1983).

The less polar fraction contained ketosteroids which were additionally detected on Silica Gel GF_{254} plates by the quenching of fluorescence upon irradiation with UV light. Spot 3 exhibited a yellow fluorescence after spraying the TLC plates with isonicotinic acid hydrazide; this suggests the presence of a Δ^4-stigmasten-3-one (Neher, 1969). The relative mobility (Table 1) was similar to that of Δ^4-cholesten-3-one used as a reference standard and coincides with values reported for Δ^4-sitosterol-3-one (Daly *et al.*, 1983). The compound under investigation absorbs at 240 nm and its infrared spectrum had a strong band at the carbonyl frequencies (1675 cm^{-1}). These values characterize 4-en-3-ones (Jones *et al.*, 1950, 1952). GC–MS of the same compound showed peaks at *m/e* 410, 368, 325, 287, 269 and 124 (Table 2). The peaks at *m/e* 368 (M-42) and *m/e* 325 (M-85) should be attributed to the loss of a ketene from ring A (carbon atoms two and three) and loss of a ketene and of carbon atoms C_1, C_{10} and C_{19}, as suggested by Brown & Djerassi (1980) who elucidated the course of fragmentation of unsaturated 3-ketosteroids. The *m/e* 287 (M-123) and *m/e* 124 ions result from fission of the 6–7 and 9–10 bonds of ring B. They are characteristic and prevail in the mass spectra of 3-ketosteroids (Brown & Djerassi, 1980). The

TABLE 1

Oxidation Products of Stigmasterol Heated in Trioleylglycerol at 180°C for 48 h and Relative Mobilities of Analogous Products obtained from Cholesterol

	TLC relative R_f-values[a]	*Color*[b]	*Possible oxidation products of stigmasterol*	*TLC relative R_f-values*[a]	*Oxidation products of cholesterol*
1	1·54	Beige	Stigmasta-3,5,22-triene	1·52	Cholesta-3,5-diene
2	1·43	Yellow	Stigmasta-3,5,22-trien-7-one	1·44	Cholesta-3,5-dien-7-one
3	1·27	Brown–yellow	Stigmasta-4,22-dien-3-one	1·27	Cholest-4-en-3-one
4	1·23	Brownish	Stigmasta-4,6,22-trien-3-one	1·25	Cholesta-4,6-dien-3-one
6	0·95	Violet-blue	—	—	—
7	0·58	Brownish	—	—	—
8	0·51	Yellow	5,6-epoxy-stigmasterol	0·49	5,6-epoxy-cholesterol
9	0·34	Sky-blue[c]	Stigmasta-5,22-diene-3β,7β-diol	0·33	Cholest-5-ene-3β,7β-diol
10	0·30	Sky-blue[c]	Stigmasta-5,22-diene-3β,7α-diol	0·31	Cholest-5-ene-3β,7β-diol
11	0·12	Brownish	—	—	—
12	0·07	Brown–yellow	5α-stigmast-22-ene-3β,5,6β-triol	0·06	5α-cholestane-3β,5,6β-triol

[a] Developing solvent system: ethyl acetate–heptane 1:1 (v/v).
[b] After spraying TLC-plate with 50% sulphuric acid and heating 10 min at 110°C.
[c] Immediately after spraying TLC-plate with 50% sulphuric acid.

TABLE 2
Fragmentation Patterns from Mass Spectra of Stigmasterol and some of its Oxidation Products

Steroid	*m/e*	*Relative abundance*
Stigmasterol	412 (M^+)	100
	413 (M + 1)	30
	379 {—($H_2O + CH_3$)}	14
	369 {—(C_{25}—C_{27})}	15
	351 {—($H_2O + C_{25}$—C_{27})}	22
	271 {—(R + 2XH)}	14
	255 {—(R + H_2O)}	11
3β-Hydroxy-pregn-5-en-20-one	316 (M^+)	100
	317 (M + 1)	21
	301 (—CH_3)	9
	298 (—H_2O)	25
	283 {—($H_2O + CH_3$)}	25
	231 {—(H_2O + 67)}	21
	213 {—(R + H_2O + 42)}	62
	43 (CH_3CO^+)	92
Stigmasta-4,22-dien-3-one	410 (M^+)	9
	368 (—CH_2=C=O)	16
	325 {—(CH_2=C=O + 43)}	14
	287 (M–123)	8
	269 {—(R + 2XH)}	18
	124	26
	69	100
Stigmasta-3,5,22-trien-7-one	408 (M^+)	100
	409 (M + 1)	32
	269 (—R)	52
	267 {—(R + 2XH)}	18
	187	23
	174	40
	161	19
Stigmasta-3,5,22-triene	394 (M^+)	91
	395 (M + 1)	32
	379 (—CH_3)	26
	255 (—R)	74
	253 {—(R + 2XH)}	17
	213 {—(R + C_3H_6)}	19
	159	100

intense peak at *m/e* 269 corresponds to the loss of the side chain together with two hydrogen atoms from the steroid nucleus and it is a typical fragment for steroids with an unsaturated side chain (Wyllie & Djerassi, 1968). Therefore, the structure $\Delta^{4,22}$-stigmastadien-3-one is suggested for this compound.

Spot 2 was found to have a structure of an unsaturated 7-ketosteroid. The product exhibited a characteristic fluorescence under UV light. The ultraviolet spectrum showed a maximum at 278 nm and the IR spectrum had a strong peak at 1665 cm^{-1} which is due to the conjugated C=O stretching. These values are typical for 3,5-dien-7-ketosteroids (Jones *et al.*, 1950; Williams & Fleming, 1973). The mass spectrometry fragmentation pattern of this oxidation product of stigmasterol is also given in Table 2. Characteristic fragments resulted from the parent ion M (*m/e* 108) and the isotopic M + 1 (*m/e* 409) and loss of the side chain together with two hydrogen atoms (139 mass units). Other ions appeared at *m/e* 187, 174 and 161. These represent a cleavage of the most heavily substituted bond 8–14 followed by breakage of the 12–13, 11–12 and 9–11 bonds, respectively, and they are characteristic of $\Delta^{3,5}$-dien-7-ones (Budjikiewicz & Djerassi, 1962; Schiller, 1973). Thus, spot 2 was identified as $\Delta^{3,5,22}$-stigmastatrien-7-one.

Spot 1 was found to be a hydrocarbon and another minor, non-identified, product. The presence of the latter was evident when resolution was improved by multiple irrigation.

The suspected hydrocarbon when purified showed no fluorescence under UV light. The IR spectrum excluded the presence of a hydroxyl or carbonyl group. The UV absorption maximum at 234 nm was indicative of a conjugated diene (Williams & Fleming, 1973). The compound had a molecular ion at *m/e* 394 and ions at *m/e* 379 (M-CH_3), 255 (M-side chain), 253 (M-side-2H) and 213 (M-side chain-C_3H_6). The lack of an M-H_2O ion confirmed the absence of oxygen in the molecule. It is, therefore, concluded that stigmasterol upon heating is partly dehydrated to stigmasta-3,5,22-triene. Similar dehydration products were also identified in heated β-sitosterol (Yanishlieva *et al.*, 1980; Yanishlieva & Schiller, 1983).

The product, which initially migrated into the same region with the hydrocarbon, showed fluorescence under UV light. Its mass spectrum (Table 2) had a molecular ion at *m/e* 394 and other ions at *m/e* 379 (M-CH_3) and *m/e* 378 (M-H_2O). The ions at *m/e* 255 and *m/e* 237 are characteristic of the side chain of stigmasterol (Wyllie & Djerassi, 1968). Thus, it is concluded that this compound is probably a ketone with one carbon less than stigmasterol and an extra double bond.

Spot 6 was identified as Δ^5-pregnen-3β-ol-20-one. This compound was also detected in the autoxidation products of β-sitosterol and its mass spectrum has been described by Yanishlieva *et al.* (1982). In the mass

spectrometric analysis of this oxidation product, characteristic fragments resulted from the parent ion M^+ (316), loss of methyl (15 mass units), loss of H_2O (18 mass units), loss of H_2O and methyl (33 mass units) and loss of $CO–CH_3$ plus $C_{15}–C_{17}$ and H_2O (103 mass units). A peak at *m/e* 231 is also characteristic and it is due to the cleavage of ring D (Friedland & Lane, 1959). Finally, a strong peak at *m/e* 43 is typical for methylketones (Budjikiewicz & Djerassi, 1962). The IR spectrum of the investigated compound had a strong band at the carbonyl region (1710 cm^{-1}) which is characteristic of saturated ketosteroids (Jones *et al.*, 1952). The structure of Δ^5-pregnen-3β-ol-20-one is, therefore, suggested for this compound.

It is clear from the above that the nature of stigmasterol oxidation products is very complex and that this complexity requires a combination of analytical procedures. In this work an effort was made to characterize the major oxidation products which are formed when stigmasterol is heated in a triglyceride mixture. Undoubtedly, more research is needed to further clarify the structure of more oxidation products mainly arising from reactions of the unhindered double bond in the side chain which are as likely as reactions in the A and B ring of the sterol. This information might be useful to those interested in the biological effects of oxidized sterols and their health implications.

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