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THE STEROLS OF GRAPEFRUIT PEEL

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Abstract—The following sterols have been identified in grapefruit peel: β-sitosterol, stigmasterol, campesterol, cyclocucalenol, 24-methylene lophenol, 24-ethylidene lophenol (citrostadienol), cycloartenol and 24-methylene cycloartanol. In addition several unidentified 4α-methyl sterols have been demonstrated, two of these are probably 24ξ-methyl lopenol and 24ξ-ethyl lophenol, respectively.

INTRODUCTION

PREVIOUS investigations have demonstrated the presence of β-sitosterol and citrostadienol (4α-methyl$\Delta^{7,24(28)}$ stigmastadien-3β-ol; 24-ethylidene lophenol) in grapefruit peel.[1,2] The presence of the latter sterol is of particular interest and it has been suggested that the various 4α-methyl sterols found in plants may be intermediates in the biosynthesis of the major phytosterols such as β-sitosterol.[2–9] Since citrostadienol was reported to be present in grapefruit peel oil in comparatively large amounts this source was investigated in the present work to obtain a sample of this compound. However as the isolation procedure progressed it became evident that the sterol composition of grapefruit peel was far more complex than had previously been reported. Studies were consequently extended to characterize some of the additional sterols observed. A preliminary report of part of this work has been published;[10] the present communication illustrates the complexity of the sterol mixture obtained from a plant tissue.

RESULTS AND DISCUSSION

The non-saponifiable material obtained from grapefruit peel was chromatographed on alumina (Brockmann grade III) and the limonene and other hydrocarbons (up to 80 per cent of the non-saponifiable lipid) eluted with light petroleum. Elution with diethyl ether then gave a yellow oil (10 per cent of non-saponifiable lipid) which contained the sterols. This latter fraction was rechromatographed on alumina and developed with increasing concentrations of diethyl ether in light petroleum to provide fractions which were shown by thin-layer

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Table 1. Relative retention times* of grapefruit peel 4,4′-dimethyl and 4α-methylsterols

Stationary phase	Grape fruit peel		Cycloartenol	24-Methylene cycloartanol	Citro-stadienol	24-Methylene lophenol	Cyclo-eucalenol
	4,4′-dimethyl sterols	4α-methyl sterols					
5% QF-1	4·90, 5·95	4·25, 4·44, 5·00, 5·44	4·90	5·95	5·44	4·44	—
3% SE-30	3·75, 4·61	3·00, 3·35, 4·60, 4·82	3·75	4·61	4·82	3·35	3·35
1% Hi-EFF 8B	—	8·00, 9·00, 10·00, 11·11, 14·44	—	—	14·44	11·11	10·00

* Relative to cholestane.

chromatography to contain the 4,4'-dimethyl sterols, the 4α-methyl sterols and the 4-desmethyl sterols respectively.

Identification of the 4-Desmethyl Sterols

Gas–liquid chromatography (GLC) with 3% SE-30 as stationary phase revealed three components with retention times identical to β-sitosterol (60%), stigmasterol (16%) and campesterol (24%).

Identification of the 4α-Methyl Sterols

The alumina column fractions containing the 4α-methyl sterols gave an immediate blue colour with the Liebermann–Burchard reagent (Δ^7 or Δ^8 sterol). These sterols were completely precipitated with digitonin and careful chromatography of the regenerated sterols on alumina provided the pure 4α-methyl sterol fraction for subsequent investigation. GLC with QF-1 or SE-30 as stationary phase indicated four components but with Hi-EFF 8B five peaks were observed (Table 1). The major component had retention data on all columns corresponding to citrostadienol; a second component corresponded to 24-methylene lophenol. The separation of sterols and triterpenes on silver nitrate-impregnated silica gel has been reported previously[11–14] and this method has been used successfully in the present work. Separation of the components of the 4α-methyl sterol fraction following acetylation was achieved by preparative thin-layer chromatography on silica gel impregnated with 10% silver nitrate. The sterol acetates were resolved into four component bands, which were eluted and rechromatographed to effect a further purification. The fractions have been designated as A, B, C and D in order of decreasing R_f value. B co-chromatographed with citrostadienol acetate (VI); C with cycloeucalenol acetate (III) and D with 24-methylene lophenol acetate (V): Analysis of these fractions by GLC on 1% Hi-EFF 8B and 1% QF-1 columns gave the results presented in Table 2; the relative amounts of the different components are included but variations have been noted with different batches of grapefruit. Fraction A showed two major and three minor components. An immediate blue colour was obtained with the Liebermann–Burchard reagent whilst the i.r. spectrum was consistent with that of a 4α-methyl sterol such as lophenol.[15] The mass spectrum had parent ions at m/e 456 and 470 and fragmentation peaks at m/e 441 and 455 [M-CH_3]; 396 and 410 [M-acetate]; 381 and 395 [M-(CH_3 + acetate)]; 329 [M-side chain (*a*)]; 287 [M-side chain and part of ring D (*b*)]; 269 [M-(*a* + acetate)]; and 227 [M-(*b* + acetate)]. These fragmentations indicate[16] that one major component has a saturated 9-carbon side-chain whilst the other has a 10-carbon saturated side-chain. These data are consistent with the presence of 24ξ-methyl- and 24ξ-ethyl lophenol acetates, GLC retention data showed that the former predominated. The mobility of these compounds on silver nitrate–silica gel thin layers is also in accord with these structures. Evidence for the presence of 24ξ-methyl lophenol in sugar-cane leaf waxes has been reported previously.[5]

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TABLE 2. RELATIVE RETENTION TIMES* OF GRAPEFRUIT 4α-METHYL STEROL ACETATES

Fraction	1% Hi-EFF 8B	1% QF-1
A	6·89 (17), 8·22 (14), 9·11 (42), 11·22 (27)	6·48 (2), 7·34 (49), 7·86 (11), 8·87 (36), 9·38 (2)
B	11·29 (5), 13·22 (95)	8·06 (5), 9·28 (95)
C	7·89 (57), 9·28 (19), 10·67 (4), 12·89 (20)	6·42 (0·5), 7·11 (51), 8·37 (38), 8·94 (5·5), 10·10 (5)
D	8·35 (22), 10·74 (78)	6·68 (4), 7·34 (81), 8·26 (10), 9·38 (5)
Cycloeucalenol acetate	†	8·37
24-Methylene lophenol acetate	10·74	7·34
Citrostadienol acetate	13·22	9·28

* Relative to cholestane.
† Cycloeucalenol acetate appeared to be unstable on this column.
Figures in parenthesis indicate the approximate percentage compositions of each fraction.

One predominant sterol was observed upon GLC of fraction B and this was shown to be the acetate of citrostadienol (VI) which was first isolated from grapefruit peel oil.[2] It gave an immediate blue colour with the Liebermann–Burchard reagent and the i.r. spectrum had a peak at 817 cm^{-1} indicating a trisubstituted double bond. The mass spectrum had a molecular peak at m/e 468 and other peaks at m/e 453 [M-CH_3]; 408 [M-acetate]; 393 [M-(CH_3+acetate)]; 370 [M-part of side chain][6,17,18]; 327 [M-(side chain+2H)]; 310 [M-(acetate+part of side chain)]; 269 [M-(side chain+acetate)]; 267 [M-(side chain+2H+acetate)] and 227 [M-(side chain and part of ring D+acetate)]. This fragmentation pattern is the same as that published for 24-ethylidene lophenol acetate obtained from tobacco tissue cultures.[6]

Fraction C co-chromatographed with cycloeucalenol acetate (III) on silver nitrate–silica gel thin layers. GLC on QF-1 revealed two major components (Fig. 1), peak (*b*) had a retention time identical to authentic cycloeucalenol acetate. The i.r. spectrum had pronounced peaks at 883 and 1635 cm^{-1} showing the presence of a methylene group in one or more of the compounds present in fraction C. Reaction with the Liebermann–Burchard reagent produced a clear yellow colour (λ_{max} 462 nm) which reached a maximum intensity after 35 min and then faded. There was no development of a blue or green colour which is the more typical response of Δ^7 or Δ^5 sterols respectively to this reagent.[19] However the response obtained was identical to that given by lanosterol[20] and treatment of macdougallin[21] and cycloeucalenol with the Liebermann–Burchard reagent also produced a similar yellow colour although the intensity was much less than that observed with lanosterol. Both lanosterol and macdougallin have in common a Δ^8 bond and a 14α-methyl group. By contrast, zymosterol with a Δ^8 bond but no 14α-methyl group is reported to give a blue colour with the Liebermann–Burchard reagent.[22] These facts seem to suggest that the presence of a 14α-methyl group in a sterol may result in the production of a yellow colour with the

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Liebermann–Burchard reagent instead of the more usual blue or green colours. A more complete investigation of this point would be of interest. The mass spectrum of fraction C had a molecular ion at m/e 468 (cycloeucalenol acetate MW = 468). Thus whilst the evidence is consistent with one component (b) of fraction C being cycloeucalenol acetate the identity of the other major component (a) must at present remain obscure. However component (a) is probably an isomer of cycloeucalenol acetate, its mobility on silver nitrate–silica gel thin layers suggests the presence of a methylene group whilst the retention data on GLC may be consistent with a nuclear double bond rather than a cyclopropane ring.

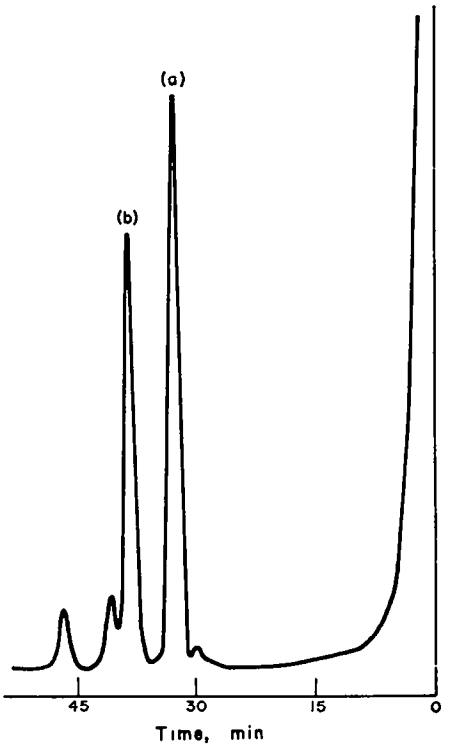

FIG 1. GAS–LIQUID CHROMATOGRAPHY ON 1% QF-1 OF FRACTION C OF THE GRAPEFRUIT 4α-METHYL STEROL ACETATES.

Fraction D co-chromatographed with 24-methylene lophenol acetate (V) on silver nitrate–silica gel and GLC showed a major component with a retention time identical to 24-methylene lophenol acetate. The Liebermann–Burchard reagent gave an immediate blue colour and the i.r. spectrum had peaks at 883 and 1635 cm^{-1} characteristic of a methylene group. The mass spectrum showed a mass peak at m/e 454 and other peaks at m/e 439 [M-CH_3]; 394 [M-acetate]; 379 [M-(CH_3+acetate)]; 370 [M-(part of side chain)]; 327 [M-(side chain+2H)]; 310 [M-(acetate+part of side chain)]; 287 [M-side chain and part of ring D]; 269 [M-(side chain+acetate)]; 267 [M-(side chain+2H+acetate)]; and 227 [M-(side chain and part of ring D+acetate)]. This is similar to the mass spectrum reported for 24-methylene lophenol acetate isolated from tobacco tissue cultures.[6] A second small mass peak was observed in the mass spectrum of fraction D at m/e 468. 24-Methylene lophenol

was first isolated from sugar-cane leaf waxes[5] and has since been reported in potato leaves,[7] tobacco tissue cultures,[6] tobacco leaves[23] and pea leaves.[8]

Identification of the 4,4'-Dimethyl Sterols

With the Liebermann–Burchard reagent the crude 4,4'-dimethyl sterol fractions gave a yellow–red colour (λ_{max} 462 nm) which increased in intensity for more than 3 hr. This response was identical to that obtained with cycloartenol and 24-methylene cycloartanol.[24] GLC on Q F-1 or SE 30 showed a major component (58 per cent) identical to 24-methylene cycloartanol (II) and a second component (42 per cent) corresponding to cycloartenol (I). There was no evidence of lanosterol. The two compounds were separated after acetylation by chromatography on a column of silver nitrate-impregnated silica gel and eluting with an increasing gradient of benzene in hexane. Two fractions were obtained and saponified to give compound E (eluted first) and compound F, both gave a yellow–red coloration with the Liebermann–Burchard reagent. Compound E had the same retention time as cycloartenol (I) on GLC and had an identical i.r. spectrum to cycloartenol. The mass spectrum of E showed a molecular ion at m/e 426 and other peaks at m/e 411 [M-CH_3]; 408 [M-water]; 393 [M-(CH_3 + water)]; 365; 339; 297 [M-(side chain + water)] and 286 [M-ring A]. This was the same as obtained for a sample of authentic cycloartenol and is comparable to the fragmentation data reported for cycloartenol acetate.[6, 25] Compound F had a retention time on GLC identical to 24-methylene cycloartanol (II). The i.r. spectrum was similar to that of cycloartenol except that the peak at 817 cm^{-1} was absent whilst peaks at 883 and 1635 cm^{-1} demonstrated the presence of a methylene group. The mass spectrum gave a mass peak at m/e 440 and other peaks at m/e 425 [M-CH_3]; 422 [M-water]; 407 [M-(CH_3 + water)]; 379; 353, 313; 300 [M-ring A] and 297 [M-(side chain + water)]. This fragmentation pattern is consistent with the identity of compound F with 24-methylene cycloartanol.[25] This compound was first isolated from rice bran oil[24] and has since been identified in several plant tissues.[6, 8, 17, 26]

The present results demonstrate the complexity of the sterol mixture obtained from a plant tissue. In particular the 4α-methyl sterol fraction of grapefruit peel has now been shown to contain several components, some unidentified, in addition to citrostadienol which was first isolated from this source. Very similar results have been obtained following the examination of the sterol complex from larch leaves.[27] The simultaneous presence in a plant tissue of all the compounds reported in this communication is relevant to phytosterol biosynthesis. The possible importance of the various 4,4'-dimethyl and 4α-methyl sterols now known to occur in plants has been discussed elsewhere.[3–9] In particular it has been suggested that cycloartenol may play a major role in phytosterol biosynthesis[6, 7] and may replace lanosterol, the intermediate in sterol production in animals and fungi. It is therefore perhaps significant that whilst cycloartenol (I) could be identified in grapefruit peel no evidence for lanosterol was obtained although this compound was carefully looked for. However, observations of this nature do not of themselves eliminate lanosterol from the phytosterol biosynthetic

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sequence and the isolation of lanosterol has in fact been reported from plant sources.[28–30] Of particular interest, in connexion with the possible involvement of cycloartenol, is the present identification of cycloeucalenol (III) accompanying these other suggested sterol intermediates. Cycloeucalenol was first reported in the woods of *Eucalyptus microcorys*[31] and *Swietenia mahagoni*[32] and can clearly be obtained from cycloartenol (I) by transmethylation to give first 24-methylene cycloartanol (II) followed by loss of one of the 4,4'-dimethyl groups. Cycloeucalenol (III) could then undergo further modification[8,9] by opening of the cyclopropane ring to give an intermediate (IV) such as that shown in Scheme 1. Loss of the 14α-methyl group would then give 24-methylene lophenol (V). A second transmethylation would produce citrostadienol (24-ethylidene lophenol; VI) which can be modified to give eventually β-sitosterol. 24ξ-Methyl or 24ξ-ethyl lophenol could of course arise by reduction of 24-methylene and 24-ethylidene lophenol respectively and support for the involvement of sterol intermediates containing a C24 methylene or ethylidene group in phytosterol biosynthesis has been obtained.[33] However a sequence such as that outlined in Scheme 1 remains to be proved at the enzyme level. One point of interest is that there is evidence that the various 4,4'-dimethyl and 4α-methyl sterols found in plants may to a large extent be esterified to fatty acids[17,34] and these esters become rapidly labelled with 2-^{14}C mevalonic acid.[9,35] In grapefruit the 4,4'-dimethyl and 4α-methyl sterols are also esterified (approximately 75 and 88 per cent of the total respectively).[36] The relevance of such observations to phytosterol biosynthesis is at present obscure.

EXPERIMENTAL

Solvents

Light petrol (40–60°) was dried over sodium wire and redistilled. Diethyl ether was dried over sodium and redistilled over reduced iron.

Extraction of Non-saponifiable Lipid

Grapefruit were purchased locally and the centre portion removed. The peel was cut into strips, passed through a mincer and homogenized in methanol. One-fifth of the volume of 60% aqueous KOH was added and the mixture refluxed for 6 hr. After filtering, the residue was washed with methanol followed by diethyl ether. The bulked filtrates were diluted with water and the non-saponifiable lipids extracted into diethyl ether in the usual manner. The yield of non-saponifiable lipid, which was a yellow oil, was found to vary with different samples of grapefruit but was approximately 0·6–1·0 per cent of the peel wet weight.

Column Chromatography

The large amount of limonene and other hydrocarbons in the non-saponifiable lipid was found to interfere with the separation of the sterols. This material was therefore removed in a preliminary chromatography on alumina. In a typical separation 14·34 g of non-saponifiable lipid was chromatographed on 200 g of alumina (Brockmann grade III) and the hydrocarbons (11·4 g) eluted with 600 ml of light petroleum; the column was then eluted with 2 l. of diethyl ether to give a fraction (1·4 g) containing the sterols. The sterol fraction was then

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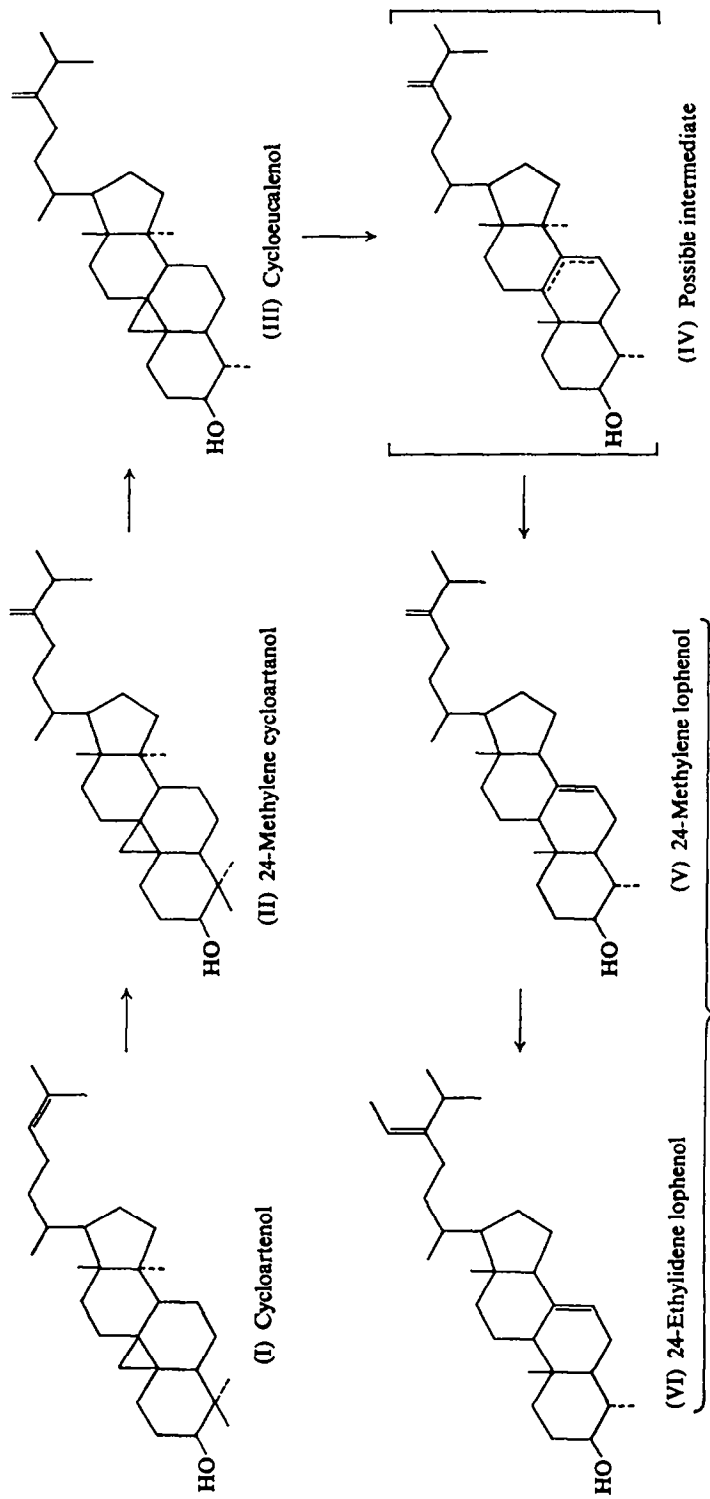

SCHEME 1. A POSSIBLE BIOSYNTHETIC SEQUENCE FOR STEROL PRODUCTION IN HIGHER PLANTS.

separated into fractions containing the 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols by chromatography on a second column of alumina (Brockmann grade III) (1 g of alumina/10 mg of lipid) and eluting with increasing concentrations of diethyl ether (E) in light petroleum (P) (usually 0, 2, 4, 6, 8, 15% E/P or 0, 2, 6, 9, 20% E/P; 10 ml/g of alumina). Column fractions were monitored by TLC on silica gel, 4,4'-dimethyl sterols appeared in the 4 and 6% E/P fractions; the 4α-methyl sterols in the 6 and 8 or the 9% E/P fractions and 4-desmethyl sterols in the 15 and 20% E/P fractions.

Thin-layer Chromatography

Kieselgel G (E. Merck A.-G., Darmstadt, Germany) thin layers were prepared incorporating Rhodamine 6G, and developed with chloroform. Sterol acetates were separated on thin layers of Kieselgel G incorporating 10% (w/w) silver nitrate with 40% benzene in hexane as developer. The sterol acetates were located by spraying with an acetone solution of Rhodamine 6G and viewing under u.v. light. Bands were scraped off the plates and eluted with dry diethyl ether.

Separation of 4,4-Dimethyl Sterol Acetates

Silicic acid (80–100 mesh, Mallinkrodt Chemical Works, St. Louis, U.S.A.) was impregnated with silver nitrate[14] and a slurry of this material in hexane packed into a 50 cm × 1 cm column. The sterol acetates were introduced as their solution in hexane and the column developed with an increasing gradient of benzene in hexane. Initially the mixing flask contained 20% benzene in hexane and the reservoir an equal volume of 50% benzene in hexane. 4–5 ml fractions were collected.

Gas–Liquid Chromatography

A Pye Argon Gas Chromatograph was used for most of this work. Columns were 4 ft × ¼ in. glass packed with either 5% QF-1, 3% SE-30 or 1% Hi-EFF 8B[37] on 80–100 mesh acid-washed silanized Gas Chrom P. The column temperature in all cases was 219°, argon flow was 60 ml/min. Cholestane was always run with the samples. For the analysis on 1% QF-1 (Table 1) a Varian Aerograph Model 1522-B fitted with hydrogen flame ionization detectors was used. The column was stainless steel (6 ft × ⅛ in.) and contained 1% QF-1 on 80–100 mesh acid washed silanized Chromosorb W. On-column injection was employed and temperatures were: column 233°; injector 245° and detector 235°, the nitrogen flow was 40 ml/min.

Digitonin Precipitation of Sterols

The digitonin-precipitable sterols were obtained by the method described previously.[30]

The Liebermann–Burchard Reaction

This was carried out as described previously.[30]

Infra-red Spectroscopy

Infra-red spectra were determined with samples as streaks on rock-salt discs using a Perkin–Elmer Infracord Spectrophotometer.

Mass Spectrometry

We are particularly grateful to Professor E. Lederer and Dr. H. E. Audier (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France), Dr. B. A. Knights (Dept. of Chemistry, Glasgow University) and Dr. W. Kelly (Unilever Research Laboratories, Colworth House, Bedford) for the determination of the mass spectra reported in this paper.

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