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Steryl esters of *Digitalis purpurea* L. herb

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The non-polar fraction sterols of *Digitalis purpurea* flowering plants were separated into a steryl ester and free sterol fraction. The ester fraction consisted of cycloeucalenol, obtusifoliol, 24-methylenelophenol, 24-ethylenelophenol, cholesterol, campesterol, stigmasterol, sitosterol, isofucosterol and 24-methylenecholesterol. These compounds were esterified with an $\alpha\beta$-unsaturated C10 fatty acid, whereas the glycerides from the same plant consisted of a complex of acids predominated by C18 and C16 types. The free sterols consisted of cycloartenol, 24-methylenecycloartenol, cholesterol, campesterol, stigmasterol, sitosterol, fucosterol and 24-methylenecholesterol. The relative proportions of each of these steroids in the two fractions were recorded.

Phytosterols have both physiological (Evans, 1972a; Cowley & Evans, 1972) and metabolic (Tschesche & Lilienweiss, 1966; Bennett, Heftmann & Winter, 1969; Tschesche & Hulpke, 1966) functions in *Digitalis* species. It has been demonstrated that these compounds occurred as polar glucosides (Jacobsohn & Frey, 1967) and as non-polar lipids (Cowley, Evans & Ginman, 1971) an observation of possible physiological interest. The non-polar lipids from the seeds consisted of 4,4′-dimethylsterols, 4 mono-methylsterols and 4-desmethylsterols (Evans, 1972b). This communication describes an investigation of the relative composition of the steryl esters and free sterols of *D. purpurea* herb.

METHOD AND RESULTS

Second year *D. purpurea* in flower were harvested above the soil line and dried at 55° for five days.

Extraction. One kg of powdered plant material was extracted with light petroleum (40–60°) in a Soxhlet. The solid residue (200 g) was dissolved in acetone, filtered through celite and evaporated to an oily liquid. This was separated into three fractions by gradient elution on alumina (Brockmann grade III) using light petroleum–ether mixtures. The eluates were monitored by thin layer chromatography (t.l.c.) on silica gel G 250 μm activated 120° 30 min and developed with either isopropyl ether–acetic acid (24:1) or with light petroleum–ether–acetic acid (90:10:1). Fraction 1 (1·5 g of a red-brown oily liquid) consisted of steryl esters, carotenoids and alkanes; fraction 2 (50 mg) of triglycerides, diglycerides and monoglycerides; fraction 3 (500 mg) of free sterols and a green pigment.

Fractions 1 *and* 2. Both fractions were separately purified by preparative layer chromatography (p.l.c.) using chloroform as solvent on silica gel H 1 mm layers. The lipids were detected under ultraviolet light after spraying with Rhodamine 6G and the steryl ester zone (R_F 0·7) and glyceride zone (R_F 0·5) eluted with dry ether. They were each evaporated under nitrogen to produce about 40·0 mg of yellow-brown oils. These were refluxed for 3 h with 6% KOH in 90% ethanol. From

fraction 1 a non-saponified matter was extracted with ether and the fatty acid esters from both fractions were recovered with ether after adjusting to a pH of 4·0 with 1N HCl.

Examination of the fatty acids. The methyl esters were synthesized by refluxing for 2 h in 10 ml of 5% HCl in dry methanol. The esters were precipitated by the addition of water and extracted with ether, dried over anhydrous sodium sulphate and evaporated to give 5 mg of a yellow oil (fraction 1) and 10 mg of a brown oil (fraction 2). These esters produced only one spot by t.l.c. using silica gel G and developing with chloroform–methanol (99:1). T.l.c. on silver nitrate 10%, silica gel G with light petroleum–ether (80:20) produced one spot (R_F 0·56) for fraction 1 and three spots (R_F 0·59; 0·53 and 0·25) for fraction 2. Gas liquid chromatography (g.l.c.) on polyethylene glycol adipate and Apiezon M (Table 1) indicated that

Table 1. *Composition of the fatty acid-methyl-esters from the steryl ester and glyceride fractions.*

	Carbon No.	Retention time (min) PEGA*	Apiezon†
Triglyceride fraction			
Capric	10	6·9	2·8
Lauric	12	12·7	5·4
	14	23·5	11·8
Palmitic	16	43·8	26·0
Stearic	18	83·1	57·5
	18/Br	75·5	—
	16/Br	36·5	—
Oleic	18/1	92·7 }	48·0
Linoleic	18/2	111·5 }	
Linolenic	18/3	142·3	34·0
	16/1	50·0	23·0
Steryl ester fraction			
	10/1	—	2·4

* 5 ft × ¼ inch polyethylene glycol adipate 10% on Chromosorb W (100–120) at 190° with nitrogen at 40 ml min^{-1} on a Pye 104 GC Unit.

† 5 ft × ¼ inch Apiezon M 10% on Chromosorb W (100–120) at 195° and 60 ml min^{-1} of nitrogen.

the fatty acid methyl esters from the glycerides (fraction 2) were complex, consisting of saturated acids with chain lengths of C10; C12; C14; C16 and C18; C16 and C18 acids with branched chains possibly being present; mono-unsaturated C18:1 and C16:1; diunsaturated C18:2 and triunsaturated C18:3. The steryl ester fraction (fraction 1) produced a single component on both the polar and non-polar columns. On line g.l.c.-mass spectrometry of this ester was carried out through a two stage Ryhage separator. The line was at 275°, oven 145° and source 250°. Helium was carrier gas at 30 ml min^{-1} through a 5 ft × ¼ in glass column packed 10% SE-30 on Chromasorb W (Fig. 1). The mass spectrum had a molecular ion at *m/e* 184, a typical M-31 ion due to R CO^+ at *m/e* 153 and a series of fragment ions due to $CH_3O.CO(CH_2)_n^+$ at *m/e* 73; 87; 101; 115; 129; 143 and 157. The peak present at *m/e* 59 corresponded to the elimination of $(CH_3O.CO)^+$. An infrared spectrum of the oil obtained directly between discs exhibited a shift in the peak due to the ester moiety from 1740–50 to 1725 cm^{-1} suggesting a conjugated double bond. The remainder of fraction 1 (2 mg) was converted to the methoxy derivative

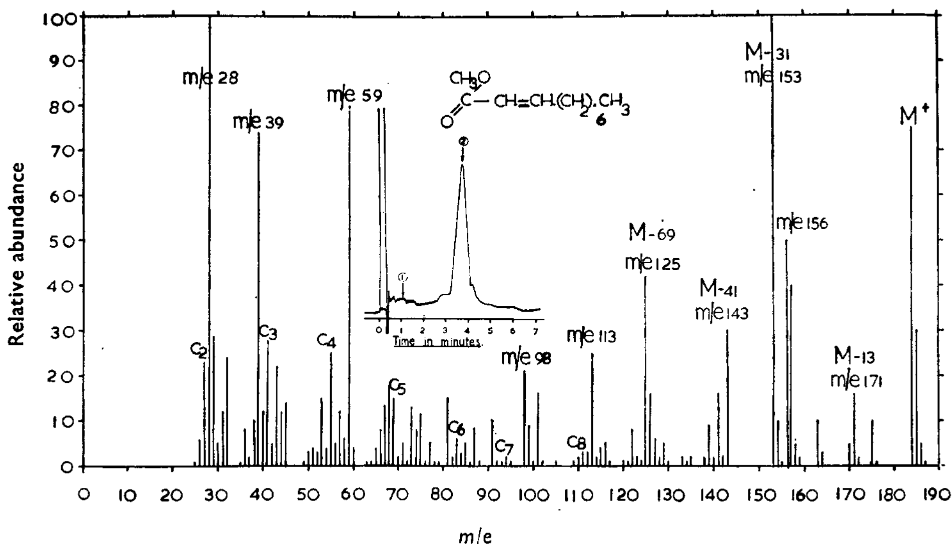


FIG. 1. On line g.l.c.-mass spectrum of the methyl ester of the fatty acid from the steroid fraction of *D. purpurea*.

by stirring with 5 ml of 0·1M mercuric acetate for ¾ h at 25°. Three ml of 0·3M sodium ethoxide solution was added and the mixture stirred for 5 min, 0·5M sodium borohydride in 0·3M sodium methoxide was added and mercury removed by centrifugation. The clear supernatant was pipetted off, evaporated to dryness under nitrogen and the residue extracted with dry tetrahydrofuran. The mass spectrum of the residue from the tetrahydrofuran solution exhibited a base peak at *m/e* 31 and significant fragment ions at *m/e* 28; 45; 59 and 91. Fragmentation ions at *m/e* 73 possibly corresponding to $(CH_3O.CO.CH_2)^+$; at *m/e* 103 $(CH_3O.CO.CH.OCH_3)^+$; *m/e* 113 $(CH_2.(CH_2)_6.CH_3)^+$ and *m/e* 133 $(OCH_3.CH.(CH_2)_6.CH_3)^+$ confirm that the double bond in the original methyl ester was in the $\alpha\beta$-position. The fatty acid moiety of the steryl esters was therefore a C10$\alpha\beta$-unsaturated compound.

Examination of the non-saponified matter. The neutral fraction from fraction 1 was separated by column chromatography (Evans, 1972b) into two groups of steroids, the 4-monomethylsterols (15 mg) and 4-desmethylsterols (150 mg). Column fractions were monitored by t.l.c. (silica gel H 250 μm developed with chloroform–isopropyl ether 98:2). Both the monomethylsterols and phytosterols produced one spot by t.l.c. in the same system as above.

4-*Monomethylsterols.* T.l.c. of the acetates (pyridine–acetic anhydride 2:1) on silver nitrate (20%) silica gel developed with benzene–hexane 1:1, produced spots with R_F values identical to authentic samples of 24-ethylenelophenol acetate (R_F 0·46); cycloeucalenol and obtusifoliol acetates (R_F 0·37) and 24-methylenelophenol acetate (R_F 0·27). Three zones were collected by p.l.c. as above.

Zone 1 (upper zone). The Liebermann-Burchard reaction indicated a 7-enesterol (Cowley & others, 1971). G.l.c. (Table 2) indicated the presence of 24-ethylidenelophenol acetate. This was confirmed by the mass spectrum, which exhibited a molecular ion at *m/e* 468 and significant fragmentation ions at *m/e* 453; 408; 393; 327; 310; 260 and 267.

Table 2. *Comparison of free sterols and steryl esters by g.l.c.*

	Percentage of mixture		Relative retention*		
			A		B†
	Free	Esters	alcohols	acetates	acetates
4,4′-dimethyl sterols					
Cycloartenol	70	—	10·6	3·78	5·74
24-Methylenecycloartenol	30	—	11·9	4·34	6·11
4-Monomethylsterols					
Obtusifoliol	—	5	6·7	2·65	4·41
24-Methylenelophenol ..	—	5	9·9	3·10	4·63
Cycloeucalenol	—	80	7·8	3·47	5·33
24-Ethylidenelophenol ..	—	10	12·2	4·63	6·11
4-Desmethylsterols			Acetates	Alcohols	Acetates
Sitosterol	58·5 (Sitosterol, Isofucosterol, Fucosterol)	53·4 (Sitosterol, Isofucosterol, Fucosterol)	8·48	3·43	4·61
Isofucosterol			7·38	3·43	4·62
Fucosterol			8·95	3·43	4·62
Stigmasterol	46·6	1·4	7·29	2·86	3·92
Campesterol	1·0	2·8	6·91	2·57	3·90
24-Methylenecholesterol	0·6	21·3	7·07	2·21	3·42
Cholesterol	3·6	26·1	5·19	2·00	2·78

* Relative to cholestane.
A. 6 ft × ¼ in glass NGS 1·0% at 225, N_2 flow 60 ml min^{-1}.
B. 6 ft × ¼ in glass SE.30 1·5% 230°, N_2 40 ml min^{-1}.
† Mass spectra were obtained on about 10 μg of steryl acetates condensed from the SE.30 column. An A.E.I. (MS-902) was used with an inlet temperature of 212°.

Zone 2 produced a yellow colour (max 460nm) with Liebermann-Burchard reagent. G.l.c. suggested the presence of obtusifoliol and cycloeucalenol acetate (Audier, Beugelhams & Das, 1966). The mass spectrum of a condensed sample (10 μg) of the major component (Rt 5·33, relative to cholestane) had a molecular ion at *m/e* 468 and fragment ions at 453, 408, 393, 383, 365, 287, 267, 227, a similar spectrum to an authentic sample of cycloeucalenol acetate (Audier & others, 1966). The minor component (Rt 4·41) had a molecular ion in the mass spectrum at *m/e* 468 and fragmentation ions at *m/e* 453, 408, 393, 385, 329, 325, 287, 269, 267 and 227, an identical spectrum to obtusifoliol acetate (Barrera, Breton & others, 1967).

Zone 3. The Liebermann reaction indicated a 7-ene sterol. G.l.c. relative retention was identical to 24-methylenelophenol acetate (Rt 4·63). The mass spectrum had a molecular ion at *m/e* 454 and fragment ions at *m/e* 493, 394, 379, 371, 327, 287, 267 and 227, similar to the fragmentation pattern of 24-methylenelophenol acetate (Bergmann, Lindgreen & Svohn, 1965).

4-*Desmethylsterols.* The Liebermann reaction indicated the presence of 5-ene sterols. The acetates by t.l.c. (silver nitrate 20%, silica gel developed with benzene–hexane 1:1) produced five spots. These corresponded with the acetates of standard references: sitosterol (R_F 0·84), stigmasterol (R_F 0·82), isofucosterol (R_F 0·60) and 24-methylene-cholesterol (R_F 0·55). G.l.c. (Table 2) demonstrated the presence of cholesterol, 24-methylene cholesterol, campesterol, stigmasterol and sitosterol. This fraction was separated into three zones as above by means of p.l.c.

Zone 1 (upper zone). G.l.c. suggested the presence of sitosterol, stigmasterol, campesterol and cholesterol acetates and 10 μg samples were collected by preparative g.l.c. The first peak (Rt 2·78) had a small molecular ion (*m/e* 428) and fragmentation

ions at *m/e* 413, 368, 353, 315, 273, 260, 255, 247, 223, 145 and 119, confirming the presence of cholesteryl acetate. The second peak (Rt 3·60) was identified as campesteryl acetate (M^+ *m/e* 442, fragment ions at *m/e* 427, 382, 367, 315, 274, 273, 269, 261, 255, 213, 119 and 111). The third peak (Rt 3·92) was stigmasteryl acetate (M^+ absent, fragment ions at M-60 *m/e* 394 and others at *m/e* 379, 315, 281, 273, 255, 213, 145, 119, and 111). The final peak (Rt 4·61) was sitosteryl acetate (M^+ *m/e* 456 and fragment ions at *m/e* 441, 396, 381, 315, 288, 283, 275, 273, 213, 119 and 111). The bulk of this zone was reconverted to the alcohols and repeatedly recrystallized from acetone to give 5 mg of sitosterol (m.p. 137° infrared identical to an authentic sample).

Zone 2. G.l.c. and t.l.c. indicated that this zone consisted of isofucosterol acetate (R_F 0·60, Rt 9·38) (Table 2). About 10 μg was collected by preparative g.l.c. for mass spectrometry. This exhibited a small molecular ion at *m/e* 454 and a large fragmentation ion at *m/e* 394, due to the M-60 ion. Significant ions occurred also at *m/e* 379, 356, 313, 296, 281, 255, 253, 173, 119 and 111. Ions were also present at *m/e* 327 and 267 corresponding to the elimination of $C_8H_{16} + 15$ and $C_6H_{16} + 15 + 60$. The relative intensities of ions at *m/e* 296 and 55 indicated the isomer 28-isofucosteryl acetate formed the bulk of the mixture. Zone 2 was reconverted to its alcohol and recrystallized from methanol to produce 2 mg of compound (98% pure by g.l.c.) (m.p. 122°) with an identical infrared spectrum to an authentic sample of isofucosterol.

Zone 3. T.l.c. (R_F 0·55) and g.l.c. (Rt 3·42) indicated that this zone was 24-methylenecholesteryl acetate. This was confirmed by the mass spectrum (M^+ at *m/e* 440 and fragmentation ions at *m/e* 425, 380, 365, 358, 296, 281, 267, 259, 253, 213, 119 and 111), which was identical to a standard sample (Knights, 1968).

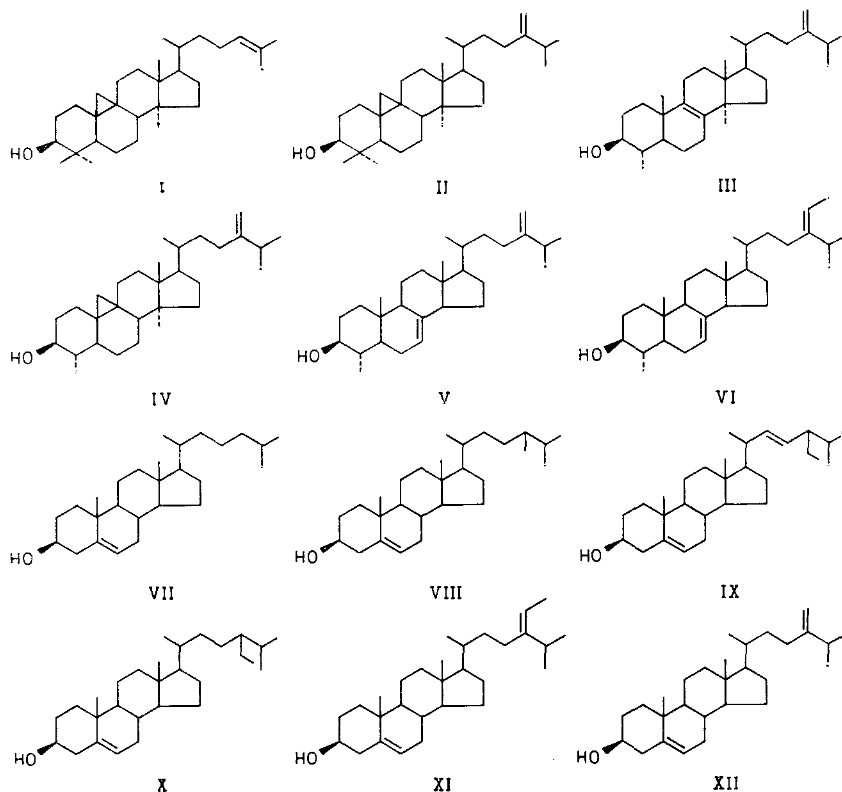
Fraction 3. This fraction was separated into two groups by column chromatography as previously described (Evans, 1972b). These were the 4,4′-dimethylsterols (60 mg) and the 4-desmethylsterols (250 mg). Both fractions by t.l.c. on silica gel using chloroform–isopropanol (97 : 3) as solvent were found to be free other groups of steroids.

4,4′-*Dimethylsterols*. T.l.c. of the derived acetates on silver nitrate–silica gel as before, suggested that this fraction consisted of cycloartenol (R_F 0·59) and 24-methylenecycloartenol (R_F 0·41), an observation confirmed by g.l.c. (Table 2). Two zones were separated by p.l.c. as above.

Zone 1 (upper zone). The Liebermann reaction produced a yellow-orange colour (max 460 nm) and a single component was shown to be present by g.l.c. and t.l.c. as above. The mass spectrum had a molecular ion at *m/e* 468 and significant fragmentation ions at *m/e* 453, 408, 393, 365, 357, 339, 315, 297, 286, 271, 175 and 155. The recrystallized material (2 mg) had an identical infrared spectrum to cycloartenol acetate.

Zone 2 also produced a yellow colour with the Liebermann reagent. T.l.c. and g.l.c. suggested 24-methylenecycloartenol acetate which was confirmed by the mass spectrum (M^+ *m/e* 482 fragment ions at *m/e* 467, 422, 407, 379, 357, 353, 335, 315, 313, 300, 297, 295 and 165) (Benveniste, Hirth & Ourisson, 1964). Insufficient material remained for an infrared spectrum.

4-*Desmethylsterols*. T.l.c. of the column fraction suggested that the free sterols were contaminated with pigments and a trace of 4-monomethylsterols. It was digested for 2 h in 6% KOH in 90% MeOH and the neutral fraction, free of green pigments, isolated with ether. The residue was purified by p.l.c. (silica gel G, chloroform–acetone, 9:1) and the 4-desmethyl zone (100 mg) eluted with chloroform. This



material produced a single spot in several solvent systems. T.l.c., g.l.c., mass and infrared spectroscopy of the derived acetates as before confirmed that the fraction of free phytosterols consisted of sitosterol, stigmasterol, campesterol, fucosterol, 24-methylene cholesterol and cholesterol (Table 2).

DISCUSSION

The non-polar sterol fractions (Cowley & others, 1971) of *Digitalis purpurea* were found to consist mainly of free sterols together with a smaller proportion of steryl esters.

The free sterols were of two groups classified by means of the number of methyl groups on ring A of the nucleus. The smallest fraction consisted of 4,4′-dimethylsterols, namely cycloartenol (I) and 24-methylenecycloartenol (II). These tetracyclic triterpenoids are also present in the seeds (Evans, 1972b) of *D. purpurea* and in the aerial parts of several other plant species (Schrieber & Osske, 1962; Goad & Goodwin, 1966). Their presence in *D. purpurea* is in accordance with current theories on the biosynthesis of plant sterols (Goad, 1967). Most of the free sterol fraction (4-desmethylsterols) consisted of sitosterol (X), stigmasterol (IX), fucosterol (XI), campesterol (VIII), 24-methylene–cholesterol (XII) and cholesterol (VII). The C_{29} sterols composed the bulk of this fraction, sitosterol being the major component. These compounds are widely distributed in plants being considered the components of cell and organelle membranes (Evans, 1972a; Cowley & Evans, 1972; Kemp, Goad &

Mercer, 1967). Fucosterol is less widely distributed in higher plants but is a component of marine green algae (Gibbons, Goad & Goodwin, 1968) and 24-methylenecholesterol, present also in the flowers, fruits and seeds of *D. purpurea* (Evans, 1971), has previously been detected in the Cruciferae (Knights, 1968).

The steryl ester fraction differed considerably in composition from the free sterols. Two groups of steroids were present, the 4-monomethyl and 4-desmethyl groups. The 4,4′-dimethylsterols could not be detected as esters, but it is also of interest to note that only a trace of 4-monomethylsterols was present in the free form. These steroids were of three kinds: cycloeucalenol (IV) characterized by a 14α-methyl group and a 19β,9-cyclopropane bridge; obtusifoliol (III) by an 8-ene bond and a 14α-methyl group; 24-methylene and ethylidenelophenols (V and VI) characterized by the absence of a 14α-methyl group and the presence of a 7-ene double bond. The 4-desmethylsterols of the ester fraction were similar in composition to the free sterols but the proportions differed. The amount of sitosterol was similar to the free fraction but amounts of stigmasterol, the C-22 unsaturated homologue, were greatly reduced. Cholesterol and 24-methylenecholesterol on the other hand formed much more of the mixture. Fucosterol was absent from this fraction but the 28-isomer, isofucosterol (XI), was present in detectable quantities. Both of these compounds were present in the non-saponified matter of *D. purpurea* seeds (Evans, 1972b).

In *D. purpurea* these sterols were esterified with a single fatty acid component, which g.l.c. on a non-polar column indicated was a C10 compound. The mass spectrum of the methyl ester was typical of a C10 mono-unsaturated fatty acid ester with the exception that the base-peak normally present at *m/e* 74 due to the McLafferty rearrangement (McLafferty, 1963) and elimination of $(CH_3O{\cdot}C(OH) = CH_2)^+$ from the M^+ ion, was reduced. A large peak was at *m/e* 59 suggesting an αβ or βγ-unsaturated compound. The constitution of the conjugated double bond was confirmed by the shift in the infrared of the peak due to the carbonyl of the ester group from 1740 to 1725 cm^{-1} and also due to the ions at *m/e* 73, 203, 223 and 133 in the mass spectrum of the crude reaction products from a modified Markownikov reaction. The presence of the acid in the steryl esters was of interest as the glyceride acids from the same extracts consisted predominantly of C18 and C16 acids. The sterols of the intermediate and final stages of the biosynthetic route from squalene to sitosterol are therefore selectively esterified with an αβ-unsaturated C10 acid. Other workers (Kemp, Hammam & Goad, 1968) have shown that [^{14}C]labelled precursors are rapidly incorporated into ester sterols which are biosynthesized in the cytoplasmic and microsomal fractions of the cell. These esters are thought by Kemp & others, 1967) to represent an intercellular transportation system to the cell and organelle membranes where free sterols form essential components of the lipoprotein system. The composition of the ester fraction of *D. purpurea* is significantly different from the free sterols, an observation in accordance with their suggested functions in plant cells.

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