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The sterols and tetracyclic triterpenoids of *Digitalis purpurea* L. seeds

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Three types of sterols were isolated from the non-saponified fraction of *D. purpurea* seed oil. These were the 4,4′-dimethylsterols, the 4α-methylsterols and the 4-desmethylsterols. By combination of t.l.c., g.l.c. and mass spectrometry, the first group were found to consist of cycloartenol and 24-methylenecycloartenol, the second group of 24-methyl-lophenol, 24-ethyl-lophenol, 24-methylene-lophenol, 24-ethylidenelophenol, obtusifoliol, 4α-methylzymosterol and cycloeucalenol. The third group of true phytosterols consisted of cholesterol, campesterol, stigmasterol, β-sitosterol, fucosterol, 28-isofucosterol and 24-methylenecholesterol. Possibly present also in this group were 7-ene-cholestenol, 7-ene-campestenol, 7-ene-22-dihydrostigmastenol, 7-ene-24-methylenecholestenol and 7-ene-isofucostenol.

Interest in the phytosterols of *Digitalis* species has been stimulated by the discovery that cholesterol (Caspi, Lewis & others, 1966; Tschesche & Lilienweiss, 1966) and β-sitosterol (Bennett, Heftmann & Winter, 1969) are implicated in the biosynthesis of cardenolide steroids, and that cholesterol is similarly implicated in sapogenin biosynthesis (Tschesche & Hulpke, 1966). β-Sitosterol has also been converted to stigmasterol by *D. lanata* (Bennett & Heftmann, 1969).

The sterols of *Digitalis canariensis* were identified as cholesterol, campesterol, stigmasterol and β-sitosterol (Linde, Ergene & Meyer, 1965). Earlier work on the phytosterols of *Digitalis purpurea* (Cowley, Evans & Ginman, 1971), showed that it was possible to separate the sterols of both the leaves and the seeds into a glycoside and a lipid fraction. The composition of the glycoside fraction was found to be identical to that reported earlier by Jacobsohn & Frey (1967); however, the sterols of the lipid fraction were much more complex. I now describe an investigation of the non-saponified fraction of the seed oil, using a combination of chromatographic methods together with mass-spectrometry.

METHODS AND RESULTS

Extraction. Crushed seeds (350 g) were extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus. Removal of solvent gave an oil filtered in acetone through Celite. The residue, after removal of acetone, was refluxed (3 h) with 6% potassium hydroxide in 90% ethanol (400 ml). The cooled solution was diluted to 2 litres with 1% aqueous ammonium sulphate and the non-saponified fraction removed by ether extraction (300 ml). After removal of the solvent, the residue (1·8 g) was separated into three main fractions on a column of alumina, eluting with ether–light petroleum (b.p. 40–60°) (Williams, Goad & Goodwin, 1967). The eluate was monitored by t.l.c. (Silica gel H, 250 μm, activated 110° 30 min, developed with chloroform–isopropyl ether (98 : 2), visualized by 10% antimony trichoride in

Table 1. *The t.l.c. of the acetates of reference sterols and sterols from* D. purpurea *on silver nitrate/silica gel.*

Compound	R_F value*
Cycloartenol acetate	0·59
24-Methylenecycloartenol acetate	0·41
Fraction 1 acetates	0·77; 0·59; 0·41
24-Ethylidenelophenol acetate	0·46
Cycoeucalenol acetate	0·37
24-Methylenelophenol acetate	0·27
Fraction 2 acetates	0·68; 0·55; 0·46; 0·37; 0·27
7-Ene-cholestenol acetate	0·90
Cholestanol acetate	0·90
Sitosteryl acetate	0·84
Stigmasterol acetate	0·82
Fucosteryl acetate	0·70
Isofucosteryl acetate	0·60
24-Methylenecholesteryl acetate	0·55
Cholesteryl acetate	0·84
Campesteryl acetate	0·84
Fraction 3 acetates	0·90; 0·84; 0·70; 0·60; 0·55

* T.l.c. on air-dried silica gel H plates containing 20% w/w of silver nitrate. Plates were developed in benzene–hexane 1 : 1.

benzene). These fractions were collected. 1. 4,4′-Dimethylsterols (114 mg). 2. 4-Monomethylsterols (110 mg). 3. 4-Desmethylsterols (200 mg).

Fraction 1. Thin-layer chromatography of the derived acetates of this fraction (Table 1) gave spots with the same R_F values as authentic specimens of cycloartenol and 24-methylenecycloartenol acetates. Gas chromatography of the free sterols and steryl acetates indicated the presence of the same two components (Table 2). Separation of the acetates by p.l.c. (Table 1) gave three zones.

Zone 1 (*Upper zone*). This zone which gave no colour with the Liebermann Burchard reagent (Cowley & others, 1971), was converted to the free alcohol and recrystallized from hexane (yield 30 mg). A mass spectrum gave ions at m/e 326, 308, 228, corresponding to $CH_3[CH_2]_n$–CH_2OH minus water, where n = 20, 22 and 24. A series of fragment ions appeared at M–18–$(14)_n$ indicative of a long chain primary alcohol, a characterization in agreement with the infrared spectrum. G.l.c. of the acetates (SE30) gave a series of peaks. This non-steroidal zone was not further investigated.

Zone 2. This gave a yellow colour (λ_{max} 460 nm) with the Liebermann-Burchard reagent. G.l.c. of the free sterols and steryl acetates (Table 2) indicated the presence of cycloartenol. This was confirmed by the mass spectrum which exhibited a molecular ion at m/e 468 with significant fragmentation ions at m/e 453, 408, 393, 365, 357, 339, 315, 297, 286, 271, 175 and 155. This corresponded to that from authentic cycloartenol acetate (I) (Benveniste, Hirth & Ourisson, 1966; Audier & others, 1966).

Zone 3. The Liebermann-Burchard reaction was similar to zone 2, but t.l.c. (Table 1) and g.l.c. (Table 2) indicated the presence of 24-methylenecycloartenol (II). A mass spectrum had a molecular ion at m/e 482 and fragmentation ions at

Table 2. *The g.l.c. of reference 4-4'-dimethylsterols and sterols from* D. purpurea.

Compound	Formula No.†	Relative retention* A	Relative retention* B	Relative retention* of acetates A**
Lanasterol		3·69	10·3	5·66
Cycloartenol	I	3·78	10·6	5·74
24-Methylenecycloartenol ..	II	4·34	11·9	6·11
Fraction 1. Zone 2		3·78	10·6	5·74
Zone 3		4·34	11·9	6·11

* Relative to cholestane.

A. 6 ft × ¼ in (glass column) S.E.30. 1·5%. Free sterols analysed at 218°, N_2 flow of 25 ml/min. Acetates analysed at 230°, N_2 flow of 40 ml/min.

B. 6 ft × ¼ in glass column NGS. 1% at 225°, N_2 60 ml/min.

** Mass spectra were obtained on about 10 μg of steryl acetates condensed from g.l.c. with the SE30 column. An A.E.I. (MS-902) instrument was used with an inlet temperature of 212°.

† See Figs 1 & 2.

m/e 467, 422, 407, 379, 353, 357, 335, 315, 313, 300, 297, 295 and 165. This is similar to the fragmentation of 24-methylenecycloartenol acetate (II) (Benveniste & others, 1966; Audier & others, 1966).

Fraction 2. The acetates of this fraction produced five spots by t.l.c., three of which had similar R_F values to standard references (Table 1). G.l.c. of the free sterols gave four peaks (SE30) and five peaks (NGS) (Table 3). The acetates were separated into four zones by p.l.c. (Table 1; Goad & Goodwin, 1967).

Zone 1 (Upper zone). The Liebermann-Burchard reaction was typical of a 7-ene sterol. T.l.c. (Table 1) and g.l.c. (Table 3) indicated the presence of 24-methyl- and 24-ethyl-lophenols. The mass spectrum of the acetate of the major component (80%) had a molecular ion at m/e 456, and significant fragmentation ions at 441, 396, 381, 315, 273, 255 and 213. This is consistent with the fragmentation pattern of 24-methyl-lophenol acetate (III) (Goad & Goodwin, 1967). The minor com-

Table 3. *The g.l.c. of reference 4α-methylsterols and sterols from* D. purpurea.

Compound	Formula No.†	Relative retention* A	Relative retention* B	Relative retention of acetates A**
Obtusifoliol	VII	2·65	6·7	4·41
24-Methylenelophenol ..	IX	3·10	9·9	4·63
Cycloeucalenol	VIII	3·47	7·8	5·33
24-Ethylidenelophenol ..	VI	4·63	12·2	6·11
Crude Fraction 2 ..		2·65; 3·10; 3·47	6·7; 7·8; 9·9	—
		4·63	12·2	
Fraction 2. Zone 1 ..		2·93; 4·56	9·74; 12·1	3·52; 4·56
Zone 2 ..		4·40	11·3	
		4·63	12·2	4·11; 6·11
Zone 3 ..		2·65	6·7	
		3·47	7·8	4·41; 5·33; 6·48; 7·22
Zone 4 ..		3·10	9·9	4·63

* Relative to cholestane.

A. 6 ft × ¼ in glass column SE30. 1·5%. Free sterols analysed at 218°, N_2 flow of 25 ml/min. Acetates analysed at 230°, N_2 flow 40 ml/min.

B. 6 ft × ¼ in glass column NGS. 1% at 225°, N_2 60 ml/min.

** Mass spectra were obtained on about 10 μg of steryl acetates condensed from the SE30 column. An A.E.I. (MS-902) instrument was used with an inlet temperature of 212°.

† See Figs 1 & 2.

ponent (20%) had a molecular ion at m/e 470 and fragmentation ions at m/e 455, 410, 395, 329, 269, 287, 227, this being similar to the fragmentation of 24-ethyllophenol (IV).

Zone 2. G.l.c. (Table 3) gave compounds with similar Rt values to 4α-methylzymosterol and 24-ethylidenelophenol. The identity of the acetate of the major component (60%) was confirmed by its mass spectrum, which gave a molecular ion at m/e 454 and significant fragmentation ions at m/e 439, 394, 379, 287, 329, 269, 227. This is identical to the mass spectrum of 4α-methylzymosterol acetate (V) (Rees, Goad & Goodwin, 1968). The minor component had a molecular ion in the mass spectrum at m/e 468 and fragmentation ions at m/e 453, 408, 393, 370, 327, 310 and 267. This is similar to the fragmentation pattern of 24-ethylidenelophenol (VI) (Goad & Goodwin, 1966; Benveniste & others, 1966).

Zone 3. This had a similar R_F value to cycloeucalenol acetate (Table 1). It produced a yellow colour with the Liebermann-Burchard reagent (λ_{max} 460 nm). G.l.c. of the acetates of this fraction gave four peaks (Table 3), the major component having a similar Rt value to obtusifoliol acetate, and a second major peak to cycloeucalenol acetate. The mass spectrum of the first compound had a molceular ion at m/e 468, and fragmentation ions at m/e 453, 408, 393, 385, 329, 325, 269, 267, 287 and 227. This is similar to the fragmentation pattern of obtusifoliol acetate (VII) (Barrera, Bretón & others, 1967). The second major component had a molecular ion in the mass spectrum at m/e 468 and fragmentation ions m/e 408, 453, 393, 383, 365, 353 and 300, corresponding to cycloeucalenol acetate (VIII) (Audier & others, 1966).

Zone 4. The Liebermann-Burchard reaction indicated that this fraction consisted of a 7-ene sterol. T.l.c. (Table 1) and g.l.c. (Table 3) indicated the presence of 24-methylenelophenol. This was confirmed by the mass spectrum which had a molecular ion at m/e 454 and fragmentation ions at m/e 439, 394, 379, 371, 327, 287, 267 and 227. This is similar to the fragmentation of 24-methylenelophenol (IX) (Bergman, Lindgreen & Svohn, 1965).

Fraction 3. T.l.c. (Table 1) of the acetates produced six spots, five of which had R_F values similar to sitosteryl acetate, stigmasteryl acetate, fucosterylacetate, isofucosteryl acetate and 24-methylene cholesterol. The sixth spot had a similar R_F value to cholestanol and 7-ene-cholestenol. G.l.c. (Table 4) indicated the presence of cholesterol, 24-methylenecholesterol, campesterol, stigmasterol, β-sitosterol, fucosterol, isofucosterol and 7-ene-isofucostenol. P.l.c. of the acetates produced three zones (Table 1).

Zone 1 (Upper zone). The Liebermann-Burchard reaction indicated the presence of a mixture of 5-ene and 7-ene sterols and g.l.c. (Table 4) demonstrated the presence of β-sitosterol, campesterol, stigmasterol and cholesterol. Four compounds were collected by preparative g.l.c. The first had a molecular ion in the mass spectrum at m/e 428 and fragmentation ions at m/e 413, 368, 353, 315, 273, 255, 247, 223, 213, 145 and 119. This follows the expected fragmentation pattern for cholesteryl acetate (X) (Friedland, Lane & others, 1959). The presence of a molecular ion suggests that a trace of 7-ene-cholestenol (XI) was also present (Standifer, Derys & Barbier, 1968). The second peak was similarly identified as campesteryl acetate (XII) (mass spectrum, molecular ion at m/e 442, fragment ions at m/e 427, 382, 367, 315, 273,

Table 4. *The g.l.c. of reference 4-desmethylsterols and sterols from* D. purpurea.

Compound	Formula No.†	Relative retention* Column A	Relative retention* of derivatives: Acetates A**	Acetates B	TMS-ethers A	TMS-ethers C
β-Sitosterol	XV	3·43	4·61	8·48	4·30	4·12
Isofucosterol	XVIII	3·43	4·62	9·38	4·32	5·20
Fucosterol	XVII	3·43	4·62	8·95	4·30	5·00
Stigmasterol	XIV	2·86	3·92	7·29	3·65	3·32
24-Methylenecholesterol	XX	2·21	3·42	7·07	2·85	2·77
Campesterol	XII	2·57	3·91	6·91	3·20	3·19
Cholesterol	X	2·00	2·78	5·19	2·50	2·33
Crude Fraction 3		3·43; 2·86 2·57; 2·21 2·00	—	—	4·3; 3·65; 3·20 2·85; 2·50	—
Fraction 3. Zone 1		2·00; 2·57; 2·86 3·43	2·78; 3·15; 3·61; 3·92; 4·62	5·19; 6·29 6·91; 7·29 8·48; 9·57 10·62	2·50; 3·20 3·65; 4·30	4·12; 3·32; 3·19; 2·33
Zone 2		3·43; 2·22	3·15; 3·58; 4·63	5·14; 6·29 7·76; 8·95 9·38; 10·0; 11·33	4·32; 3·80	5·00; 5·20
Zone 3		2·21	3·42	7·05	2·85	2·77

* Relative to cholestane.

A. 6 ft × ¼ in glass column SE30. 1·5%. Free sterols and TMS-ethers analysed at 218°, N_2 flow 25 ml/min. Acetates at 230°, N_2 flow 40 ml/min.

B. 6 ft × ¼ in glass NGS. 1·0% at 225°, N_2 flow 60 ml/min.

C. 6 ft × ¼ in glass column EGSP. 2% at 201°, N_2 flow 20 ml/min.

** Mass spectra were obtained on about 10 μg of steryl acetates condensed from g.l.c. with the SE30 column. An A.E.I. (MS-902) was used with an inlet temperature of 212°.

† See Figs 1 & 2.

255, 269, 213, 261, 119 and 111), and a trace of 7-ene-campestenol acetate (XIII), the third peak was stigmasteryl acetate (XIV) (mass spectrum, molecular ion absent, fragment ion M-60 at m/e 394 and other significant fragmentation ions at m/e 379, 315, 273, 255, 281, 213, 145, 119 and 111). The final peak was identified as β-sitosterol acetate (XV) (mass spectrum, molecular ion at m/e 456, and fragment ions at m/e 441, 396, 381, 315, 275, 273, 283, 213, 119 and 111), also present was a small proportion of 7-ene-22-dihydrostigmastenol acetate (XVI). The bulk of zone 1 was reconverted to their alcohols and recrystallized from methanol (yield 15 mg, m.p. 138°) until only one product was produced by g.l.c. (98%). The infrared spectrum of this substance was identical to an authentic sample of β-sitosterol (XV).

Zone 2. The Liebermann-Burchard reaction again indicated the presence of 5-ene- and 7-ene-sterols. The acetates by t.l.c. were found to be composed of fucosteryl acetate (XVII) and 28-isofucosteryl acetate (XVII) (Table 1). G.l.c. (SE30) produced three peaks and (NGS) five peaks, the major component corresponding to 28-isofucosteryl (Table 4), and a minor component tentatively identified as 7-ene-isofucostenol (XIX). The mass spectrum of the acetate had a molecular ion at m/e 454 and fragmentation ions at m/e 394, 379, 356, 313, 281, 296, 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_8H_{16} + 15 + 60. The relative intensities of the ions at m/e 296 and 55 indicated that the isomer 28-isofucosterol acetate formed the bulk of the mixture. The bulk of zone 2 was reconverted to their alcohols and recrystallized from methanol (yield 10 mg, m.p. 122°) until only one component was produced by g.l.c (95%). The infrared spectrum of the product was similar to an authentic sample of fucosterol, with the exception that the peak at 825 cm^{-1}

due to out of plane bending frequency of hydrogen at C_{28} was present at 812 cm^{-1} indicating the presence of the 28-isomer (XVIII).

Zone 3. By t.l.c. (Table 1) and g.l.c. (Table 4) 24-methylenecholesterol appeared to be present. This was confirmed by the mass spectrum which had a molecular ion at m/e 440, indicating the presence of a trace of 7-ene-24-methylenecholestenol acetate (XXI), and fragmentation ions at m/e 425, 380, 365, 313, 358, 296, 281, 253, 259, 267, 213, 119 and 111. This is similar to the fragmentation of an authentic sample of 24-methylenecholesterol acetate (XX) (Knights, 1968).

DISCUSSION

Three types of sterols were isolated from *Digitalis purpurea*, and were classified by means of the number of methyl groups at carbon four of ring A. The first group were the 4,4'-dimethylsterols, consisting of cycloartenol (I) and 24-methylene-cycloartenol (II). No trace could be found of the triterpenoids α and β-amyrin reported to be present in *D. purpurea* leaves by Tsukamoto, Yagi & others (1968). Cycloartenol is widely distributed in plants (Benveniste & others, 1964) (Schrieber & Osske, 1962), and is often accompanied by the 24-methylenecycloartenol (Goad & Goodwin, 1966). This observation has significance with regard to the bio-synthesis of sterols in *Digitalis* species because Goad (1967) has suggested that cycloartenol is the cyclization product of squalene in plants, and not lanasterol, as in animal tissues. Lanasterol could not be detected in *D. purpurea*, even though specifically searched for.

The second type of sterols are the 4α-methylsterols, which may be divided into four groups. The first group have a saturated side chain and consist of 24-methyl-lophenol (III) and 24-ethyl-lophenol (IV); previously these compounds have been reported in larch leaves (Goad & Goodwin, 1966). The second group are the

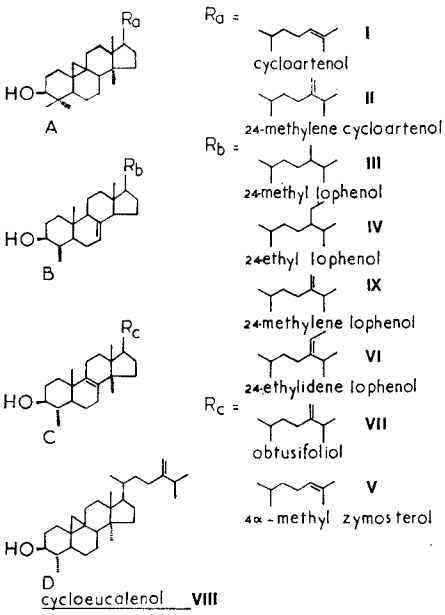

FIG. 1. The 4,4'-dimethyl- and 4-monomethylsterols of *Digitalis purpurea*.

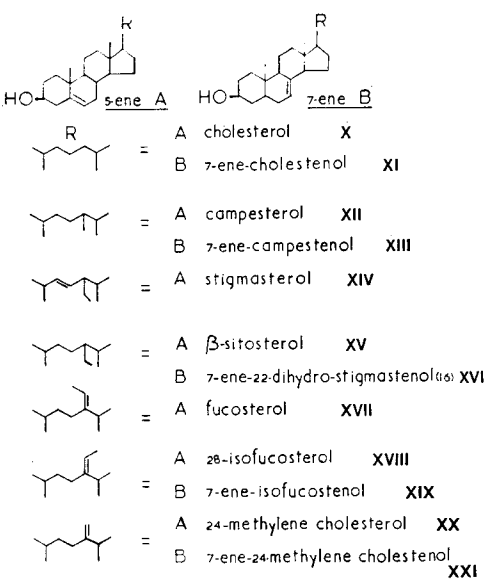

FIG. 2. The 4-desmethylsterols of *Digitalis purpurea*.

unsaturated homologues, 24-ethylidenelophenol (IV) which is known to be present in several plant tissues (Benveniste & others, 1966; Goad & Goodwin, 1967; Williams, Goad & Goodwin, 1967) and 24-methylenelophenol (IX), which also occurs in potato leaves and sugar cane wax (Osske & Schrieber, 1965). The third group of 4α-methylsterols are characterized by the presence of an 8-ene double bond and a 14α-methyl group. These are obtusifoliol (VII), known to occur in tobacco cultures (Benveniste & others, 1969), and 4α-methylzymosterol, which was isolated from potato leaves (Rees & others, 1968). Cycloeucalenol (VIII) was also present, and this compound is characterized by a 19β-9-cyclopropane bridge. The presence of this compound in plant tissues, together with the 8-ene, 14α,4α-dimethylsterols and the 7-ene-4-methylsterols, led Williams & others (1967) to propose a linear scheme of sterol biosynthesis. The presence together in *D. purpurea* of pairs of compounds (I) and (II), (III) and (IV), (VI) and (IX) and (VII) and (V) suggest, however, that the enzymes involved in side-chain methylation steps are not specific, but rather that methylation can occur at all stages of the sequence from cycloartenol. A scheme of biosynthesis involving non-specific methylation of this type would account for the accumulation of the great variety of compounds in *D. purpurea.*

The third type of sterols isolated were the 4-desmethylsterols (the true phytosterols). These consisted of mono- and di-unsaturated sterols, although it is possible that stanols were present also. The mono-unsaturated sterols were cholesterol (X), campesterol (XII) and β-sitosterol (XV). Possibly also present were 7-ene-cholestenol (XI), 7-ene-campestenol (XIII) and 7-ene-22-dihydrostigmastenol (XVI), as minor components. A complex mixture of 5-ene- and 7-ene-sterols have been identified also from oatseed (Knights & Laurie, 1967). The presence of 5-ene- and 7-ene-sterols in *D. purpurea* is of biochemical significance as the sequence of nuclear double bond rearrangements: 7-ene → 5-ene, 7-ene → 5-ene has been proposed by Dempsey, Seaton & others (1964) as a stage in sterol biosynthesis. Previously (Jacobsohn & Frey, 1967; Cowley & others, 1971) the 5-ene-sterols of this group were detected as glucosides in *D. purpurea* seeds and leaves. However, no trace was found of the 7-ene-sterols, indicating that glucoside formation occurs selectively after the biosynthesis of phytosterols is complete. This also suggests that enzymes involved in the formation of the glycoside bonds are extremely selective, utilizing only a few of the total available compounds. The physiological significance of this observation remains obscure at the present time. The di-unsaturated sterols consisted of stigmasterol (XIV), large amounts of 28-isofucosterol (XVII), together with traces of 7-ene-28-isofucostenol (XIX), small amounts of fucosterol (XVIII) and of 24-methylenecholesterol (XX) and possibly also 7-ene-24-methylenecholestenol (XXI). Fucosterol is a well known sterol of marine algae, and has been identified as a component of higher plants (Anders & Kranwach, 1957; Baisted, 1969). However, in plants it is the 28-isomer that predominates (Gibbons, Goad & Goodwin, 1968; Knights & Brookes, 1969). Hugel, Vetter & others (1964) have suggested that fucosterol is the immediate precurser of β-sitosterol. Later work by Van-Aller, Chikamatsu & others (1968) has similarly implicated 28-isofucosterol. The occurrence of these two isomers in marine green algae lead Gibbons & others (1968) to suggest that the transmethylation mechanism must involve an enzymatically controlled stereospecific hydrogen elimination, to produce one or other of these isomers. The presence together of fucosterol and 28-isofucosterol in *D. purpurea* indicates that a similar mechanism must be retained by higher plants.

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