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TRITERPENOID AND STEROID CONSTITUENTS OF FLORIDA SPANISH MOSS

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Abstract—Both free and esterified sterols and triterpenes of Spanish Moss (*Tillandsia usneoides* L.; Bromeliaceae) from Florida were examined. Besides the eight sterols and triterpenes previously reported by others as present in Spanish Moss from Mexico, cycloeucalenol, 24-methylenecycloartanol and two unidentified triterpenes of the cycloartane group were detected by GLC and GLC-Mass Spectroscopy. Campesterol and stigmasterol were detected by these techniques and cholesterol was detected by GLC. Attempts to determine the specific nature of the esters of both triterpenes and desmethyl sterols were thwarted by the extreme complexity of the ester mixture. However, saturated fatty acids from C_{12} to C_{30} were detected in the sterol and triterpene ester fraction by GLC, with palmitic acid (25%) stearic acids (20%) predominating as the fatty acid moieties.

INTRODUCTION

THE FUNCTION of sterol esters in plants is not clearly understood,[1] and there is no information available regarding the function of triterpene esters. Recently in this laboratory a cyclopropane triterpene ester, cycloartenyl palmitate was isolated from two different plants.[1] This triterpene ester is capable of exhibiting the liquid crystal phenomenon (cholesteric mesophase).* The occurrence in plants of triterpenoid esters capable of exhibiting the liquid crystalline phase may be merely coincidental and may have no relationship to a specific function in plants, since there is no known biochemical role for the liquid crystalline property.[1] On the other hand, a more extensive search for sterol and triterpene esters capable of exhibiting this phenomenon and present as the solid mesophase in plants, may give some clue to the biochemical role of these fascinating compounds in plant tissues.

For this reason and others this laboratory has initiated an extensive search for sterol and triterpene esters in plants which may be capable of exhibiting the liquid crystal phenomenon. Since our initial observations were made on cyclopropane triterpene esters,[1,2] other established plant sources of these compounds seemed a logical place to begin further investigations. McCrindle and Djerassi have previously reported the presence of several cyclopropane triterpenoids in Spanish Moss (*Tillandsia usneoides* L.) of Mexican origin.[3] A re-investigation of this plant, with the specific objective of determining the triterpene ester content, was therefore initiated. The Djerassi laboratory isolated friedelin, β-sitosterol, cycloartenone (I), and cycloartenol (III)[3] from Spanish Moss. Later cycloart-23-ene-3β,25-diol (VI), cycloart-25-ene-3β,24 (α or β)-diol (V), 25-methoxycycloart-23-ene-3β-ol (VII), and 3β-hydroxycycloart-25-ene-24-one (IV) were isolated, the last two as the acetates[3] (Fig. 1). Our experiments were designed to expand these observations, and determine if possible, the esterified form of these compounds.

* The presence of the cyclopropane ring in the molecule does not seem to be a criterion for the formation of a cholesteric mesophase.

[1] F. F. KNAPP and H. J. NICHOLAS, *Mol. Cryst. Liq. Cryst.* **6**, 319 (1970).
[2] F. F. KNAPP and H. J. NICHOLAS, *Liquid Crystals and Ordered Fluids*, 147, Plenum Press (1970).
[3] C. DJERASSI and R. MCCRINDLE, *J. Chem. Soc.* 4034 (1962).

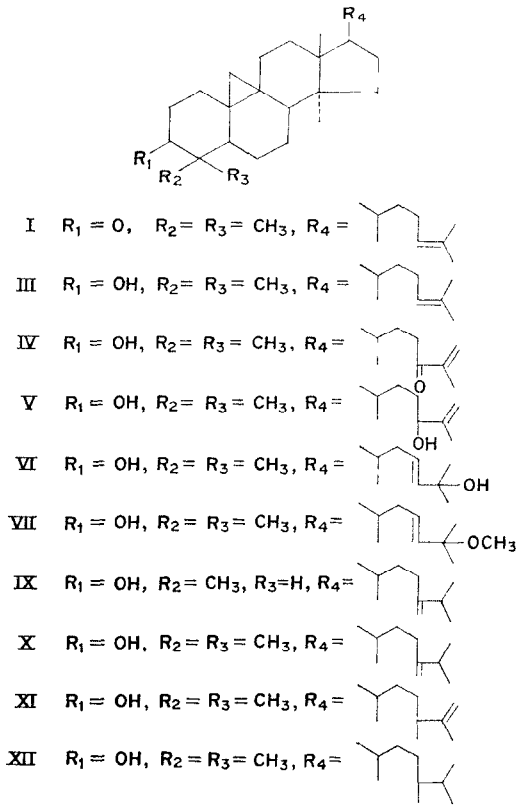

FIG. 1.

RESULTS

Monohydroxy Triterpenes

GLC showed that the free monohydroxy triterpene fraction was more complex than previously indicated.[3] The fraction could not be crystallized either as the free mixture or after acetylation, in consistence with the complexity exhibited by GLC. A GLC tracing of the components on a SE-30 (1%) column gave 5 peaks. Peaks 1, 2 and 3 had retention times identical to those of cycloeucalenol (IX), cycloartenol (III) and 24-methylenecycloartanol (X) respectively. Cycloartenol was previously reported to be the only monohydroxy triterpene identified in Spanish Moss.[3] We have found that it represents a minor component in Florida Spanish Moss and that 24-methylenecycloartanol is the major component. The ratio is 1:10 in favour of the latter triterpene. This same ratio was found in the triterpenes obtained by hydrolysis of the triterpene ester fraction. Combined GLC-MS of the first peak with RRT 3·98 (Table 1) gave a mass peak of 426 with fragments 411(M-CH_3); 408(M-HOH); 393(M-CH_3-HOH); 365, 300 and 283(M-side chain-HOH). This last peak indicated the presence of only one methyl at C-4 in the molecule as well as a C-14

methyl group.[4] Cycloeucalenol has a similar fragmentation pattern, as reported by other workers.[4]

Peak 2 with RRT 4·47 (Table 1) gave the same mass spectrum as authentic cycloartenol, already reported present in the plant.[3,4]

TABLE 1. RRT OF FREE AND ESTERIFIED STEROLS AND TRITERPENES IN SPANISH MOSS ON 3% OV 17 COLUMN AT 255°

	Occurrence	
	Free	Esterified
Cholestane	1	1
Cholesterol	—	2·21
Campesterol	2·97	2·91
Stigmasterol	3·22	3·17
β-Sitosterol	3·63	3·60
Cycloeucalenol (IX)	3·98	3·97
Cycloartenol (III)	4·47	4·41
24-Methylenecycloartanol (X)	4·90	4·88
Cycloart-23-ene-3β,25-diol (VI)	5·41	—
Triterpene alcohol 4	6·13	—
Triterpene alcohol 5	6·78	—
3β-Hydroxycycloart-25-ene-24-one (IV)	8·21	—
Cycloart-25-ene,3β,24-diol (V)	9·05	—

Peak 3 with RRT 4·90 (Table 1) gave a mass spectrum identical to that of authentic 24-methylenecycloartanol and to that reported in the literature for this compound.[4,5] The main peak (440) represents the molecular weight with fragments 425(M-CH_3); 422(M-HOH); 407(M-CH_3-HOH); 379; 353; 315; 313; 300(M-ring A) and 297(M-side chain-HOH). The co-occurrence of these 3 triterpenes in a plant is an additional support to the biosynthetic pathway of the 3 compounds proposed by Benveniste *et al.*[6]

Peak 4 with RRT 6·13 (Table 1) also represents a minor component. Its mass spectrum indicated a molecular weight 440 and the same fragmentation pattern, though different retention time, as 24-methylenecycloartanol and the 24-keto-alcohol (IV) (Table 1). Fragments are 425(M-CH_3); 422(M-HOH); 407(M-CH_3-HOH); 379; 353; 315; 313; 300(M-ring A) and 297(M-side chain-HOH). This similarity in fragmentation pattern indicates that this compound must belong to the cycloartane series of triterpenes.[4] It is logical to assume that the difference lies in the side chain, since the cycloartane skeleton is predominant in triterpenes of this plant. Even a difference in stereochemistry of the ring portion of the molecule would theoretically give the same fragmentation pattern.[4] We assume, therefore, that this compound represents a cycloartane type triterpene, the side chain of which may be either C_9H_{17} with no oxygen functions, or a $C_8H_{13}O$ with one oxygen function of undetermined position. A C_9H_{17} side chain would match the unsaturated side chain of cyclolaudenol (XI), yet the two compounds possess different retention times on GLC. The identity of this compound will be discussed in a subsequent publication.

[4] H. E. AUDIER, R. BEUGELMANS and B. C. DAS, *Tetrahedron Letters* 4341 (1966).
[5] F. F. KNAPP and H. J. NICHOLAS, *Phytochem.* **8**, 207 (1969).
[6] P. BENVENISTE, J. J. E. HEWLINS and B. FRITIG, *Europ. J. Biochem.* **9**, 526 (1969).

Peak 5 with RRT 6·78 (Table 1) has a molecular weight of 442 with fragments 427(M-CH_3), 424(M-HOH); 409(M-CH_3-HOH); 381; 315; 313; 302(M-ring A) and 297(M-side chain-HOH). This last fragment, again, indicates that this compound contains a cycloartane skeleton.[4] Two compounds present in Spanish Moss would give the same fragmentation pattern, and these are the two diols (V) and (VI) in addition to 24-methylcycloartanol (XII). All have different RRT than peak 5. Until sufficient material can be obtained for further investigation we assume that this compound is that of a 3β-hydroxy, 9,19-cyclopropane triterpene, the side chain of which is (a) similar but not identical to that of 24-methylcycloartanol and thus may possess a different stereochemistry at C_{17} or (b) may bear an oxygen function similar to that of (V) or (VI) yet in a different position or (c) a double bond may be in a different position in regard to an oxygen function. Such differences may account for the difference in retention time.

Dihydroxytriterpenes

The more polar fractions of the ethanol extract of Spanish Moss indicated the presence of diols (V) and (VI) previously reported present in this plant.[3] Thin-layer chromatography and GLC of cycloart-25-ene 3β,24-diol proved it to be pure. Its m.p. 188–190° was similar to that reported previously.[3] The mass spectrum of this compound gave a mass peak of 442 and a typical fragmentation pattern for a cyclopropane triterpene and was identical to that reported in the literature for this compound.[4] Cycloart-23-ene,3β-25-diol was identified by TLC and GLC by comparison with two samples of the authentic material kindly supplied by Professors Ourisson and Staratt. (The behaviour of their synthetic fatty acid esters with regard to formation of a cholesteric mesophase will be discussed in a subsequent publication.)

4-*Desmethyl Sterols*

The purified 4-desmethyl sterol fraction gave only one spot on TLC and a constant m.p. of 137°. GLC showed this to be a mixture consisting of campesterol, stigmasterol and β-sitosterol the last being reported previously present in Spanish Moss.[3] This was confirmed by GLC-MS. The fragmentation patterns of the three sterols were identical to those reported for campesterol, stigmasterol and β-sitosterol by others.[5,7]

Esterified Triterpenes and Sterols

The crude ester fraction isolated represented 0·07% of the original ethanolic extract. This crystalline fraction was saponified with alcoholic KOH. The nonsaponifiable components of the hydrolysate failed to crystallize from different solvents Gas–liquid chromatography of this fraction on a 3% OV-17 column showed 7 peaks. The RRT of peaks 1–7 were identical to those of authentic cholesterol (2·21), campesterol (2·91), stigmasterol (3·17), β-sitosterol (3·60), cycloeucalenol (3·97), cycloartenol (4·41) and 24-methylenecycloartanol (4·88). Diols (V) and (VI), 3β-hydroxy cycloart-25-ene-24-one and the two compounds corresponding to peaks 4 and 5 were absent in the ester fraction.

Fatty acids of the Ester Fraction

The acidic portion of the ester hydrolysate was examined by GLC on a 3% OV-17 column after methylation with boron trifluoride methanol reagent. Eighteen fatty acids

[7] B. A. KNIGHTS, *J. Gas. Chromatog.* 273 (1967)

of chain length from C_{12} to C_{30} were detected. The linear relationship between the log of the retention time and chain length indicated that the acids were chiefly saturated (C_{16} log RT = 0·00, C_{29} log RT = 2·00). Palmitic and stearic acids were the main components. Their percentage was 25 and 20 per cent respectively.

Column Chromatography of the Intact Ester Fraction

The original crystalline ester fraction of Spanish Moss was chromatographed on a column of $AgNO_3$, celite and silica gel G (4:10:10) column. Eluates monitored on TLC plates impregnated with $AgNO_3$ showed that no fractionation was achieved even after repeating the process on individual fractions.

Gas–liquid chromatography of the original ester mixture indicated its very complex nature. However, after repeated recrystallization from ether–methanol a less complex product was obtained and GLC indicated that 24-methylenecycloartanol palmitate was the major component of the ester fraction. A sample of authentic 24-methylenecycloartanol palmitate had the same retention on a 2 foot 3% OV-17 column at 300° as the major ester component. Cycloeucalenol palmitate was also detected. A sample of authentic cycloeucalenol palmitate was not available, but its retention time was calculated by assuming that the log of its retention time would be half-way between those of cycloeucalenol myristate and cycloeucalenol stearate. The log of the RT of the peak assumed to be cycloeucalenol corresponded to the calculated value exactly.

DISCUSSION

Djerassi and McCrindle reported one sterol and seven triterpenes present in Spanish Moss plant material collected in Mexico. Of these, cycloartenol, in the form of several fatty acid esters, is known to be capable of exhibiting a cholesteric mesophase.[2] The present report indicates the presence of cycloeucalenol and cholesterol as additional substances capable of exhibiting a cholesteric mesophase when in the form of certain fatty acid esters.[2,8]

Unfortunately, the fatty acid constituents of the ester fraction consisted of even and odd compounds containing from 12 to 30 C, apparently mostly saturated. Although lauric, myristic, palmitic and stearic acids predominated, it was not possible to isolate individual triterpene esters, despite good techniques available for such isolations. However, the presence of cycloartenol, cycloeucalenol and cholesterol together with lauric, myristic and palmitic acids in Spanish Moss suggests that these triterpenes may be coupled with at least one of these acids, and therefore they may exist as naturally occurring liquid crystals.[2,8] The complexity of the mixture, coupled with the relatively small amount of esterified material present in the plant, must relegate this question to future studies.

The presence of 24-methylenecycloartanol as the major triterpene alcohol in Spanish Moss investigated in this report, in contrast to cycloartenol reported as the only monohydroxy triterpene present in Spanish Moss from Mexico[3] can be explained on the basis of the different sources of the plant materials or perhaps the stage of growth at which each plant material was collected. In our Spanish Moss collected in Florida, all of the compounds identified in Mexican Spanish Moss by Djerassi and McCrindle were detected except 25-methoxy-cycloart-23-ene-3β-ol (VII). Because of difficulty in separating cycloartenol and 24-methylenecycloartanol by crystallization only it seems possible that the cycloartenol reported by Djerassi and McCrindle may have been contaminated by a considerable amount of 24-methylenecycloartanol.

[8] G. W. GRAY, *Molecular Structure and the Properties of Liquid Crystals*, Academic Press, New York (1962).

While this manuscript was in preparation we became aware of the note by Schaefer and Ourisson commenting on the detection, in Spanish Moss, of cycloartanol 24,25-epoxide.[9] This compound may be identical with our compound 'triterpene alcohol 5' (Table 1).

EXPERIMENTAL

Melting points were recorded on a Fischer–Johns hot plate. All solvents were distilled before use and were of analytical grade supplied by Fischer and Mallinckrodt Chemical Works.

CC. Merck aluminium oxide was activated at 200° for 24 hr. Mixtures of light petroleum and Et_2O as indicated were used as eluents. For the separation of esters, a mixture of $AgNO_3$, celite and silica gel G (4:10:10) was used.

TLC. Silica gel G, (Merck) was used on 20 × 20 cm plates and usually 250 μ thickness and activated for 2 hr at 120° before use. Development of the plates was usually made with light petroleum–Et_2O (7:3). $AgNO_3$ plates (12%) were used for monitoring $AgNO_3$ columns and the eluent was hexane–Et_2O (93:7). Visualization in all cases was performed with anisaldehyde reagent (anisaldehyde–H_2SO_4–EtOH, (5·5:90)) and with Carr–Price reagent followed by heating the plates at 150° for 10 min.

GLC. GLC was performed on either a Barber–Colman 5000 or a Varian Aerograph Series 1700 gas chromatograph; both of which were equipped with H_2 flame ionization detectors. Sterols were analysed on glass 180-cm columns (i.d. 4 mm) on 1% SE-30 and 3% OV-17 on 100/120 Gas Chrom Q (Applied Science Lab., Inc.) at column temps. of 265° and 255° respectively. Argon and N_2 were used at 60 ml/min. The fatty acids of the sterol esters (as their methyl esters) were analysed on 3% OV-17 on 100/120 Gas Chrom Q at a column temp. of 200° and N_2 at 50 ml/min. Intact sterol esters were analysed on a 60-cm long glass column (i.d. 4 mm) packed with 1% SE-30 on 100/120 Gas Chrom Q. The column was conditioned (Thermal Stripping) for 12 hr at 320° with the carrier gas (N_2) at 120 ml/min. The column was operated at 290° with N_2 at 50 ml/min. The method of analysing intact sterol esters was previously described by Kuksis *et al.*[10]

GLC–MS. GLC–MS analysis were determined in an LKB Model 9000 single focusing gas chromatography–mass spectrometer. A 1% SE-30 column was used at a temp. of 265°; other conditions were as follows: He flow 30 ml/min, molecular separator 262°, ion source 240, ionizing energy 70 eV.

Extraction. Dried Spanish Moss (7 kg) was exhaustively extracted with hot EtOH. The 500 g of extract so obtained was digested in 3 l. of hot Me_2CO and the mixture was kept at 0° for 24 hr, then filtered. The precipitate was treated in the same manner and the process was repeated 3 times The combined Me_2CO extracts were evaporated and the residue extracted several times with boiling Et_2O The Et_2O-soluble material weighed 102 g and was subjected to column chromatography. Fractions (1 l.) were collected. The following eluates were obtained:

TABLE 2

Light petroleum–Et_2O	Number of fractions	Product
10–0	3	Hydrocarbons
10–0	5	Esters
9–1	7	Friedelin and cycloartenone
7–3	15	4,4-dimethyl and 4α-methyl triterpene alcohols
6–4	6	3β-hydroxycycloart-25-en-24-one
3–7	15	4-desmethyl sterol fraction
2–8	4	Cycloart-25-ene-3β,24-diol
0–10	3	Cycloart-23-ene-3β,25-diol

Preparation of the monohydroxy triterpene fraction. Elution of the column with light petroleum–Et_2O (7:3) gave a product which failed to crystallize from different solvents even after repeated column chromatography. The product was acetylated as usual and chromatographed on an aluminium oxide column (500 g). Elution with light petroleum gave a crystalline product 5·2 g. Repeated crystallization gave a product m.p. 105–112°

[9] R. HEINTZ, P. C. SCHAEFFER and P. BENVENISTE, *Chem. Commun.* 946 (1970).
[10] A. KUKSIS and M. J. MCCARTHUR, *Can. J. Biochem.* **40**, 679 (1962).

which gave one spot on TLC. Hydrolysis of this product to the free compound was effected by boiling with 15% alcoholic KOH for 3 hr. The product failed to crystallize and was chromatographed on a column. Elution with light petroleum–Et_2O (7:3) gave needles m.p. 95–105°.

Examination of the 4-desmethyl sterol fraction. The fraction from the column eluted with PE 3:7 was repeatedly crystallized from EtOH until constant m.p. of 137° (needles).

Examination of the dihydroxy triterpene fractions. Light petroleum–Et_2O (2:8) eluted cycloart-25-ene-3β, 24-diol. Eluates were evaporated to dryness whereby a white residue was obtained. After 2× recrystallization from EtOH the product 125 mg (needles) had m.p. 188–190°. The purity of the product was checked by GLC on 1% SE-30 column.

Pure ether eluted the fraction containing cycloart-23-ene-3β, 25-diol. Eluates were dried and gave a white powder residue (250 mg) m.p. 192–196° and which had the same R_f value as authentic sample of the diol supplied by Professors Ourisson and Staratt. No further purification was attempted at this time.

Examination of the ester fraction. Light petroleum–Et_2O 10–0 eluates were dissolved in a minimum amount of Et_2O and a product precipitated by dropwise addition of MeOH. This process was repeated several times until a faint yellow product was obtained m.p. 70–80°. TLC on silica gel G plate of this product gave one spot, but on silica G plates impregnated with 12% $AgNO_3$ it gave at least 13 spots of very close R_f values. Developing of the plate in this case was made with Hexane–Et_2O (93:7). Repeated column chromatography on silver nitrate–celite–silica gel G (4:10:10) was tried for the separation of the esters. This system combined with a TLC monitor on $AgNO_3$ plates showed that no separation took place and the isolation of individual esters could not be achieved.

Hydrolysis of the ester fraction. 100 mg of the ester fraction were hydrolysed with 15% alcoholic KOH. The neutral fraction was extracted and gave 2 main spots on TLC corresponding to the 4,4-dimethyl and 4-desmethyl sterols. The neutrol fraction failed to crystallize because of its complexity.

Preparation of the methyl esters of free fatty acids. The acidic portion obtained from the hydrolysis of the ester fraction was treated as usual and the fatty acids obtained were methylated using BF_3–MeOH (Applied Science Lab., Inc.). The free fatty acids were dissolved in 2 ml petrol, 5 ml of the reagent added, the mixture heated on a water bath for 15 min and then poured in H_2O. The mixture was extracted with Et_2O and the solvent evaporated.

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