Phytochemistry, 1972, Vol. 11, pp 2073 to 2076. Pergamon Press. Printed in England.

SHORT COMMUNICATION

THE RESOLUTION OF 25α- AND 25β-SPIROSTA-3,5-DIENES FROM SAPOGENIN YIELDING PLANTS

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(*Received* 2 *November* 1971, *in revised form* 30 *December* 1971)

Abstract—The separation of the artefacts, 25α- and 25β-spirosta-3,5-dienes, is described and their individual characters are recorded. Following acid hydrolysis of plant material the ratios of diene to sapogenin and of 25α- to 25β-diene varied with different species and morphological parts. The 25α-diene commonly predominated as did diosgenin over yamogenin. Diene production is reduced when, before undergoing acid hydrolysis, the plant material is incubated with water for increased sapogenin yield. The label from acetate-2-^{14}C was found predominantly in the 25α-diene from such an incubation-acid hydrolysis experiment.

INTRODUCTION

THE CHIEF raw material for the steroid industry is diosgenin (25α-spirosta-5-en-3β-ol)[1] which is released from its glycosides by hydrolysis *in situ* of the plant material.[1] In the early work, ethanolic acid was used for hydrolysis and it was found that some of the diosgenin was dehydrated in ring A to yield 25α-spirosta-3,5-diene.[2] This dehydration was found to be favoured by increasing concentrations of acid and of ethanol and by long heating times.[3,4] The availability of sapogenin with a relatively high yamogenin content and a successful chromatographic procedure have enabled the 25β-spirosta-3,5-diene and its 25α-epimer to be prepared and separated and for both to be fully described. (The term '25β-diene' will be used to describe the former compound and '25α-diene' its epimer; a mixture of the two will be referred to as 'diene'.)

RESULTS AND DISCUSSION

A natural mixture of diosgenin (63%) and yamogenin (37%), isolated from the fruit wall of *Balanites orbicularis* Sprague,[5] when refluxed with 4 N HCl in 50% EtOH for 8 hr gave diene (yield 67%). This contained 18% of 25β-diene (about half of the theoretical amount) thus confirming the earlier observations that some epimerization of the 25β- to the 25α-form occurs under such drastic hydrolysis conditions.[6] It was also observed that repeated TLC (6×) of a given sample of either 25α- or 25β-diene on silica gel caused some decomposition resulting in a dumbell shaped area.

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The 25α-diene, m.p. 165°, did not form an acetate or digitonide. Strong absorption near 900 cm^{-1} indicated the 25α-configuration. When the diene was treated with the antimony trichloride reagent a purple colour developed without heating thus suggesting the presence of a conjugated diene system. The UV spectrum showed absorption maxima at 245, 234 and 228 nm very similar to those given by cholesta-3,5-diene. A comparison of the NMR spectrum with those of other 25α-spirostans confirmed the 25α-configuration in this spirosta-3,5-diene.[7] The structure of the 25β-diene, m.p. 181°, was substantiated in a similar manner.

We have found that diene formation is minimised to 5–7% of the sapogenin isolated,[8] when hydrolysis is effected by refluxing the plant material with 2 N HCl for 2 hr. Diene, when so produced from the fruit wall of *B. orbicularis* (yield 0·061%) was resolved into 25α-diene and 25β-diene in a ratio of 1·5:1. Examination of the diene fractions isolated after acid hydrolysis of the tubers of species of *Dioscorea* and the seed from species of *Balanites* showed the ratios of 25α- to 25β-diene varied, although the sapogenin isolated in these experiments contained more than 90% 25α-sapogenin (Table 1). The 25α-diene usually predominated and different ratios of the two epimeric dienes would account for the variations in the m.ps previously reported for 25α-diene.

TABLE 1. SAPOGENIN AND DIENE ISOLATED FROM THE TUBERS OF SPECIES OF *Dioscorea* AND FROM THE SEEDS OF SPECIES OF *Balanites*

Species	Sapogenin yield, %	Ratio, 25α-diene:25β-diene
D. floribunda	3·8	5·3:1
D. deltoidea	4·5	3 8:1
D. esculenta	Trace	0·8:1
D. racemosa	Trace	Trace of 25α-diene
B. orbicularis	0·80	4·1:1
B. pedicellaris	0·62	6·2:1

Steroids were isolated after 2 N HCl hydrolysis without prior incubation. Sapogenin yields (%, moisture free basis) were estimated by IR spectrometry. The sapogenin, of the parts of all of the species listed, comprised more than 90% 25α-sapogenin.

Diene formation can be further reduced by prior aqueous incubation of the plant material before adjustment to 2 N with HCl and refluxing for 2 hr. For example, incubation of the fruit wall of *B. pedicellaris* Millbr. and Schlecht[9] and of *B. orbicularis* with water at 37° resulted in an increase in sapogenin yield[10] whilst the diene yield fell (Table 2) and the same results were observed with the seed of *Trigonella foenum-graecum* L. and the tubers of species of *Dioscorea*. During incubation, endogenous saponases partially or wholly hydrolysed the saponins to sapogenins. The water soluble saponins would be more susceptible to acid dehydration at C-3 compared with the insoluble sapogenins and consequently a greater proportion of free sapogenin after incubation would result in a reduction in the diene yield from the subsequent acid hydrolysis stage.

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Diene, isolated after acid hydrolysis of fruit wall of *B. orbicularis*, was not epimerized by boiling with 2 N HCl for 2 hr but some epimerization to the 25α-form took place during incubation of the fruit wall (Table 2) and this probably occurred by a mechanism involving a $\Delta^{25(26)}$ compound, similar to that isolated from *Hosta kyosumiensis*.[11] When the *Balanites* fruit wall was incubated with acetate-2-^{14}C only the 25α-diene was significantly labelled (Table 3). This agrees with the work of Bennett and Heftmann[12] who obtained much higher incorporation of mevalonic acid into 25α-sapogenins than into 25β-sapogenins; also epimerization could have occurred. The results suggest the diene was formed from newly synthesised sapogenin. There is no evidence in the literature for the natural occurrence of diene in contrast to its status as an artefact.

TABLE 2. THE EFFECT OF AQUEOUS INCUBATION AT 37° ON THE YIELD OF DIENE FROM THE FRUIT WALL OF *B. orbicularis*

Incubation time (hr)	mg/100 g*	Ratio 25α-diene:25β-diene
0	79	0·98:1
12	66	1·36:1
24	71	1·15:1
48	28	1·15:1

* Isolation by continuous development PTLC. Estimation by weighing; adjusted for moisture free basis.

In an attempt to isolate diene without prior acid hydrolysis the following powders were extracted with light petroleum (b.p. 40–60°): the fruit wall and seed of *B. aegyptiaca* Del., *B. orbicularis* and *B. pedicellaris*. Only in the fruit wall of the latter species was found diene (0·08%) and free diosgenin (0·08%). The petrol exhausted tissue on acid hydrolysis yielded 5·51% of a mixture of diosgenin 95% and yamogenin 5%.[9] Diene with free diosgenin or yamogenin has been detected by TLC in a petrol extract of the powdered stem bark of *B. wilsoniana* Dawe and Sprague.[13]

TABLE 3. THE INCORPORATION OF ACETATE-2-^{14}C INTO SAPOGENIN AND DIENE ISOLATED FROM THE FRUIT WALL OF *Balanites orbicularis* AFTER INCUBATION FOR 48 hr

Isolation procedure	Activity of the diene, cpm/μm 25α	25β
Initial separation	28·1	5·4
PTLC with hexane-di-isopropyl ether	30·3	2·1
PTLC with hexane-ethyl acetate	31·0 ± 0·47	1·2 ± 0·09

Activity of the total sapogenin (diosgenin, 60%, yamogenin 40%) 2·39 ± 0·04 cpm/μm.

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EXPERIMENTAL

Materials. The fruits of *B. aegyptiaca*, *B. orbicularis* and *B. pedicellaris*, the seed of *T. foenum-graecum* and of the tubers of *D. deltoidea* were as previously used.[7,5,9,14] Those of other species of *Dioscorea* were obtained via the Tropical Products Institute. All yields are expressed on a moisture free basis. M.ps were determined on a Köfler block and are corrected.

Isolation and identification of the diene. Steroids were extracted in the usual way from the powdered plant materials following acid hydrolysis with 2 N HCl.[15] Diene, m.p. 141°, contained in the crude sapogenin extracts, was detected by TLC on 0·25 mm activated silica gel plates using hexane-acetone (4:1). Steroids were located with a 30% $SbCl_3$ in conc. HCl spray reagent. This gave a purple colour with spirostadiene in the cold; other steroids were visualized after heating at 110° for 15 min. 25α- and 25β-Dienes were resolved by TLC using *n*-hexane-di-isopropyl ether (20:1). The relative intensities of steroid spots were estimated from densitometric peak areas[16] and sapogenin was quantitated using IR spectrometry[15] (*S.D.* = 2·9%).

The powdered fruit wall of *B. orbicularis*, 1·24 kg, was refluxed with 8 l. 2 N HCl for 2 hr and the crude sapogenin was collected in the usual way.[15] The bulked mother liquors were chromatographed on an alumina column (40 × 5 cm Brockman activity 2–3) packed in *n*-hexane. Fractions of 25 ml were collected on elution with 150 ml cyclohexane, 50 ml cyclohexane:CCl_4 (1·1) and 150 ml CCl_4. The diene, located by TLC in fractions 9–11, was purified by PTLC on 1 mm layers of activated silica gel $PF_{254+366}$ (Merck) using hexane-di-isopropyl ether (32:1). Diene was located by spraying the edge of the plate with the $SbCl_3$ reagent, the diene viewed in UV and removed as a single band. This material (0·061% of the fruit wall) was resolved by continuous development PTLC[17] on 1 mm flourescent silica gel plates for 2 hr with hexane di-isopropyl ether (19:1). Individual dienes were recovered with $CHCl_3$ and recrystallized from *n*-hexane prior to physical analysis. Found for the upper diene band (25α-), R_f 0·85 (C, 82·6; H, 10·2. Calc. for $C_{27}H_{40}O_2$:C, 82·2; H, 10·1%). Found for the lower diene band (25β-), R_f 0·68 (C, 82·4; H, 10·2%). IR: solutions in $CHCl_3$ over the range 800–1000 cm^{-1}; NMR spectra: solutions in $CDCl_3$; UV spectra: solutions in methanol.

Partial synthesis of diene was effected by refluxing a sample of sapogenin, isolated from the fruit wall of *B. orbicularis*, with 4 N HCl in 50% EtOH for 8 hr. Individual epimers were obtained as above and estimated by weighing.

Incubation of plant material with water in the dark at 37° before hydrolysis with 2 N HCl gave sapogenins which were recovered and estimated as usual.[15] Samples, 50 g, of the powdered fruit wall of *B. orbicularis* were incubated for periods of up to 48 hr and the dienes similarly isolated and estimated by weighing (Table 2) In a separate experiment samples of the powdered fruit wall, 20 g, were incubated for 48 hr with 15 μc of NaOAc-2-^{14}C. The radioactivity of the sapogenin has been reported previously.[17] The 25α- and 25β-dienes were isolated, estimated by weighing and dissolved in toluene, containing 0·41% PPO and 0·01% POPOP, prior to scintillation counting. Diene did not cause quenching. The individual dienes were further purified by repeated PTLC using hexane–di-isopropyl ether (9:1) in the first separation and *n*-hexane–EtOAc (4:1) in the second separation. The purified dienes were examined in four TLC systems when autoradiographs prepared from the plates confirmed the absence of radioactive impurities (Table 3).

Without prior acid hydrolysis, diene was extracted from the fruit wall of *B. pedicellaris*, 100 g, with light petroleum, b p. 40–60°. The yellow fat so isolated was shown to contain 25α- and 25β-dienes using TLC. An aliquot of the unsaponifiable matter from this material was chromatographed as above and the diene fraction, 0 08%, was examined by IR spectroscopy.

Acknowledgements—The Tropical Products Institute, London, is thanked for their Research Studentships awarded to K.R.B. and C.N.W. Mr. Kenyon of the Institute and the Conservator of Forests, Entebbe are thanked for their assistance in obtaining plant material. We are also grateful to the National Research Development Corporation for its financial support.

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Key Word Index—Sapogenins; isolation; 25α-spirosta-3,5-diene; 25β-spirosta-3,5-diene.