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STEROIDAL SAPOGENINS FROM *CORDYLINE CANNIFOLIA* LEAVES

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Abstract—From three separate collections of *Cordyline cannifolia* leaves, three main sapogenins were obtained and identified as cordylagenin (1β,3α-dihydroxy-5α,25β-spirostane), its 3β,25α-isomer, brisbagenin; and a new sapogenin, cannigenin, shown to be the 25α epimer of cordylagenin. In addition, small quantities were found of smilagenin and yamogenin and TLC indicated the presence of ruscogenin/neoruscogenin. The dihydroxy sapogenin content of *C. cannifolia* leaves was found to vary from 0.02% to 0.43% of the dry weight. In a cultivation trial, this content of the leaves fell markedly over a two year period.

INTRODUCTION

Our previous work on the leaves of *Cordyline cannifolia* has been reported [1–3], and this paper deals with the isolation and characterisation of a new sapogenin, cannigenin, (1β,3α-dihydroxy-5α,25α-spirostane), and of other known sapogenins.

RESULTS AND DISCUSSION

The saponins of *Cordyline cannifolia* leaf were extracted, hydrolysed and the sapogenins separated and isolated by column chromatography and by PLC. From the original bulk collection of leaf obtained from Bailey's Creek (originally incorrectly quoted as Dawson's Creek [2]), three sapogenins were isolated and designated as compounds A, B and C. Compound A co-chromatographed with diosgenin and yamogenin and gave the same pinkish–purple colour with 50% sulphuric acid. The acetate and trifluoroacetate derivatives of compound A co-chromatographed with the corresponding derivatives of yamogenin, but separated from those of diosgenin. The sapogenin was obtained as needles, mp 180° (lit. value [4] for yamogenin 184–185°). The IR spectrum was identical with that of yamogenin.

Compound B co-chromatographed with smilagenin and sarsasapogenin and gave the same yellow colour with 50% sulphuric acid. The acetate and trifluoroacetate derivatives of compound B co-chromatographed with the corresponding derivatives of smilagenin, but separated from those of sarsasapogenin. The sapogenin B crystallised as needles, mp 199–201° (lit. value [4] for smilagenin 182°). The IR and MS data were identical with those of smilagenin. Only a small quantity of compound B was isolated which did not allow further study of the sapogenin, but it was assumed to be smilagenin, although no explanation can be given for the high mp obtained. Compound C was identified as cordylagenin [2].

A second collection of *C. cannifolia* leaves obtained near Eubenangee Swamp, when processed in a manner similar to that of the first collection, yielded the same three sapogenins as found in the first collection, plus brisbagenin [3].

An additional collection of *C. cannifolia* leaves from Davis Creek was extracted in an attempt to obtain a supply of cordylagenin for further chemical studies. When attempting to convert the isolated sapogenin to 5α,25β-spirosta-1-en-3-one (cordylagenone) [2], 5α,25α-spirosta-1-en-3-one (brisbenone) was obtained. This suggested that from this collection of leaves, the 25α-epimer of cordylagenin had been isolated, and it is given the trivial name cannigenin.

Cannigenin, $C_{27}H_{44}O_4$, (M^+, *m/e* 432), mp 215.5–217°, $[\alpha]_D^{23}$ −58.03 (c, 0.52; $CHCl_3$) crystallised as needles from methanol. Its IR spectrum showed absorption due to hydroxyl groups (ν_{max} 3420, 1070 and 1055 cm^{-1}) and a spiroketal moiety (ν_{max} 983, 921, 900 and 864 cm^{-1}); the absorption at 900 cm^{-1} was of greater intensity than that at 921 cm^{-1} (25α-spirostane). The NMR spectrum showed resonances for two tertiary methyl groups (3H, *s*, δ 0.76 and 0.83), two secondary methyl groups (3H, *d*, $J \sim 7$Hz; 0.78 and 0.95), two protons on carbon atoms bearing hydroxyl groups (1H, *m*, 3.79 and 4.06) and three protons on carbon atoms bearing ether oxygen atoms (1H, *m*, 4.36 and 2H, *m*, 3.41). The MS showed a strong ion at *m/e* 139, thus indicating that the hydroxyl groups in cannigenin were not located on the spiroketal moiety.

Acetylation of cannigenin gave a diacetate, $C_{31}H_{48}O_6$ (M^+, *m/e* 516), which would not crystallise, $[\alpha]_D^{23}$ −23.86° (*c*, 0.46; $CHCl_3$), $\nu_{max.}$ 1738 and 1242 cm^{-1}. The NMR spectrum showed the presence of 1β- and 3α-acetoxy groups (3H, *s*, 1.97 and 2.07) and (1H, *q*, 4.88 and 1H, *m*, 5.93); two tertiary methyl groups (3H, *s*, 0.77 and 0.95); two secondary methyl groups (3H, *d*, $J \sim 7$Hz, 0.80 and 0.95); and three protons on carbon atoms bearing ether oxygen atoms (1H, *m*, 4.38 and 2H, *m*, 3.41).

Partial hydrolysis of cannigenin diacetate yielded the monoacetate as needle crystals, $C_{29}H_{46}O_5$, (M^+, *m/e* 474), mp 245–246.5°, $[\alpha]_D^{23}$ −48.5° (*c*, 0.57; $CHCl_3$), ν_{max}

1715 and 1268 cm^{-1}. The NMR spectrum contained resonances characteristic of a 1β-acetoxy (3H, *s*, 1.97 and 1H, *q*, $J \sim 10.5$ and 5.5 Hz, 4.93) and a 3α-hydroxyl (1H, *m*, 4.07) group located on a 5α,25α-spirostane skeleton.

Jones oxidation of the monoacetate afforded a compound, mp 204.5–206°, undepressed on admixture with brisbenone. The IR spectrum was identical with that of brisbenone, with strong absorption at 1680 cm^{-1} (1-en-3-one) and at 980, 920, 898 and 865 cm^{-1} (spiroketal), with the intensity at 898 cm^{-1} being greater than that at 920 cm^{-1} (25α-spirostane). Catalytic hydrogenation of the compound over Adams' catalyst furnished a sapogenin characterised as tigogenin by its mp 201–202°, undepressed on admixture with tigogenin, its identical IR spectrum, and the identical chromatographic characteristics of the sapogenin, sapogenin acetate and sapogenin trifluoroacetate with equivalent derivatives of tigogenin.

The above data indicated a 1β,3α-dihydroxy-5α,25α-spirostane structure for cannigenin. Application of the Tori and Aono rules [5] for determining the chemical shifts of the C-10 and C-13 methyl signals in steroidal sapogenins to cannigenin, cannigenin monoacetate and cannigenin diacetate confirmed the structure; the observations of Bridgeman *et al.* [6] on the chemical shifts and coupling constants of 1- and 3-substituted steroids provided additional support.

Panova *et al.* [7] have recently isolated, along with ruscogenin and neo-ruscogenin, a new steroidal sapogenin from the roots and rhizomes of *Ruscus hypoglossum*. The sapogenin and sapogenin acetate have mps 213–15° and 170–173° respectively and the IR spectrum showed that the absorption at 900 cm^{-1} was greater than that at 920 cm^{-1}, indicating a 25α-structure. There is a distinct possibility that this compound could be cannigenin.

Many different leaf samples of *C. cannifolia* were separately processed and examined by two-way TLC for the presence of steroidal sapogenins. Most of the samples produced spots corresponding to smilagenin, yamogenin, cordylagenin/cannigenin and brisbagenin. In the solvent systems used, cordylagenin and cannigenin were not separated and no attempt was made to differentiate between them in this survey.

Cordylagenin/cannigenin was the predominant sapogenin in the majority of leaf samples tested, but in a few, brisbagenin was the major compound. In a few samples, the quantities of both cordylagenin/cannigenin and brisbagenin were substantial. In most of the samples examined by TLC, two additional spots were detected. One spot had an R_f value intermediate between that of cordylagenin and brisbagenin using the dichloromethane–methanol–formamide solvent system and an R_f value greater than both cordylagenin and brisbagenin using the ethyl acetate solvent system. The compound co-chromatographed with ruscogenin and produced the same brown–purple colour with 50% sulphuric acid. The acetylated derivative co-chromatographed with ruscogenin diacetate and, after partial hydrolysis, the monoacetate co-chromatographed with ruscogenin-1-acetate. Hydrogenation of the sapogenin using Adams' catalyst produced a compound which co-chromatographed with brisbagenin and which gave the same yellow colour with 50% sulphuric acid. The evidence obtained suggests that the sapogenin in *C. cannifolia* leaf extracts is ruscogenin or neo-ruscogenin. The compound was only a trace component and it was not possible to determine whether the sapogenin was either ruscogenin or neo-ruscogenin or a mixture of both.

The second unidentified spot on TLC had an R_f value lower than that of either cordylagenin or brisbagenin using the dichloromethane–methanol–formamide solvent system and an R_f value higher than that of both these sapogenins, but similar to that of ruscogenin, in the ethyl acetate solvent system. The compound, which produced a pink colour with 50% sulphuric acid, was present in trace amounts and was not isolated.

For routine analysis of the cordylagenin/cannigenin and brisbagenin contents of *Cordyline cannifolia* leaf samples, a quantitative GLC method was developed. Increasing equal quantities of cordylagenin and brisbagenin were mixed with a constant quantity of 5α-cholestane and the mixtures, after preparation of the trimethylsilyl derivates, were examined by GLC. Analysis of the results gave regression equations for values of x (mg cordylagenin or brisbagenin) and values of y (ratio of integrated area of sapogenin to that of 5α-cholestane, which was used as the internal standard). For cordylagenin, $y = 0.385x - 0.0291$ and for brisbagenin, $y = 0.422x - 0.113$. Estimations from the regression lines were made and the predicted inverse tolerance limits obtained according to Williams [8]. If it be important that the confidence limits are close, then replicate determinations

Table 1. Predicted x values and tolerance limits from regression equations of the internal standard assay, using y values at the mean, lower and upper parts of the regression line

Sapogenin	y value observed	Mean of determine	Predicted x value	Limits $P = 0.95$
Cordylagenin	0.933	1	2.5	2.60 – 2.40 (±0.10, ±4.0%)
	0.933	4	2.5	2.55 – 2.45 (±0.05, ±2.0%)
	0.352	1	0.99	1.09 – 0.88 (±0.11, ±11.0%)
	0.352	4	0.99	1.05 – 0.93 (±0.06, ±6.1%)
	1.510	1	4.00	4.10 – 3.89 (±0.11, ±2.75%)
	1.510	4	4.00	4.06 – 3.93, (±0.07, ±1.75%)
Brisbagenin	0.942	1	2.5	2.71 – 2.29 (±0.21, ±8.4%)
	0.942	4	2.5	2.61 – 2.38 (±0.12, ±4.8%)
	0.317	1	1.02	1.24 – 0.79 (±0.22, ±21.5%)
	0.317	4	1.02	1.15 – 0.88 (±0.14, ±13.7%)
	1.554	1	3.95	4.17 – 3.73 (±0.22, ±5.5%)
	1.554	4	3.95	4.08 – 3.82 (±0.13, ±3.3%)

x = mg Cordylagenin or brisbagenin. y = Ratio of integrated area of sapogenin to that of 5α-cholestane (internal standard).

Table 2. Cordylagenin/cannigenin yields, at yearly intervals, of cultivated *Cordyline cannifolia* leaves

C.S.I.R.O. sample no.	Yield, % dry wt		
	At planting	After 1 yr	After 2 yr
9083	0.32	0.13	0.05
9084	0.37	—	0.09
9085	0.34	0.26	—

will greatly narrow them. In the results (Table 1), x values are quoted at the mean of y and also at values of y at the lower and upper limits of the regression line. Greater precision can be achieved by adjusting the experiment so that the regression line in and around the mean is employed during the assay.

Using the GLC analytical method, leaf samples from fifty *Cordyline cannifolia* plants collected from the Atherton Tablelands, North Queensland, in particular from Davis Creek and Wongabel State Forest, revealed a large variation in sapogenin content. The cordylagenin/cannigenin yield, calculated as cordylagenin, varied from 0.012 to 0.37% of the dry wt of leaf, and the brisbagenin yield varied from trace quantities to 0.43%. Out of the 50 different samples assayed, 29 gave yields of less than 0.1%.

From the highest yielding plants, cuttings were taken which were planted in a Rain Forest gully at the C.S.I.R.O. laboratories at Yerongpilly, Brisbane. Analysis of samples collected at yearly intervals revealed a steady decrease in steroid content (Table 2), which may be a result of the plants being cultivated outside their usual geographic distribution.

Conversion in high yields of the sapogenin diacetate to the sapogenin-1-acetate was achieved with cannigenin, cordylagenin and brisbagenin by reaction of the diacetate at room temp with either 0.25 M barium hydroxide for 15 min or with 1 M potassium hydroxide for 30 min. After these reaction times, in both cases, no trace of sapogenin diacetate was detectable on TLC examination, although traces of the sapogenins were detected. Ruscogenin diacetate, after reaction with 0.25 M barium hydroxide at room temp for 5 min, was hydrolyzed to form ruscogenin-1-acetate and ruscogenin, with the latter predominating. After reaction for 15 min, only a trace of the monoacetate was detected on TLC examination. Using 1 M potassium hydroxide and a reaction time of 5 min, no ruscogenin diacetate was detected and the monoacetate was predominant, but a substantial quantity of the sapogenin had been formed. After 15 min ruscogenin was predominant. The faster rate of hydrolysis of ruscogenin diacetate as compared to the diacetates of cannigenin, cordylagenin and brisbagenin must arise from conformational changes in ring A produced by the 5,6 double bond. The diacetate of the 2α,3β-dihydroxy sapogenin, gitogenin, was completely hydrolyzed on reaction for 5 min with either 0.25 M barium hydroxide or 1 M potassium hydroxide.

The monoacetates of cannigenin and brisbagenin, on oxidation with Jones' reagent, form brisbenone, and the monoacetate of cordylagenin forms cordylagenone, the reactions being almost quantitative. However, ruscogenin-1-acetate, mp 206–207.5° (lit. value [9] 178–180°), on Jones' oxidation produced a number of products, which were not isolated, but the lack of pronounced UV absorption at 244.5 nm [10] suggested the absence of the 1:4-dien-3-one.

EXPERIMENTAL

Plant materials. The first collection of *Cordyline cannifolia* R. Br. leaf was made from Bailey's Creek, north of the Daintree River, North Queensland, Australia. A voucher specimen, labelled WTJ 3874, has been lodged with the Herbarium Australiense, Canberra. A second collection of *C. cannifolia* leaf was made near Eubenangee Swamp, Babinda, North Queensland. A specimen, VKM 1191, originally misquoted as VKM 991 [3], has been deposited with the Herbarium Australiense. A third bulk collection of *C. cannifolia* leaf (VKM 994) was obtained from Davis Creek, near Mareeba, North Queensland. Further leaf material was supplied by the Royal Botanic Gardens, Kew, U.K. The aerial parts of *Ruscus aculeatus* L. were collected near Emsworth, Hampshire, U.K. From this material ruscogenin was isolated. Gitogenin was isolated from a commercial sample of *Trigonella foenumgraecum* L. seed.

Extraction of sapogenins. Leaves from the first collection of *C. cannifolia* (5 kg) were extracted by hot percolation with 85% aq. EtOH. The extract was conc to 3 l. under red press, H_2O (1 l.) was added and the mixture was shaken with 2 × 2 l. of Et_2O. To the aq. extract, combined with 2 × 1 l. aq. washings of the mixed Et_2O extracts, an equal vol of EtOH was added and the mixture conc under red press to 1 l. NaCl (100 g) was added, the pH adjusted to 4.5 by adding 1 M H_2SO_4, and the mixture extracted ×3 with equal vols of *n*-BuOH satd with H_2O. To the combined *n*-BuOH extracts was added an equal vol of H_2O and the mixture was conc to 800 ml. HCl was added to make the soln 4 M, the mixture was refluxed for 4 hr and filtered. The insoluble material, after drying, was dissolved in toluene (1 l.) and refluxed with 20% methanolic KOH soln (250 ml) for 1 hr before filtration. The insoluble material was washed with hot toluene and the filtrates combined. The toluene soln, after washing with 1 l. H_2O was evaporated to dryness to yield a mixture of crude sapogenins. This was shaken with ice-cold *n*-hexane for 30 min to give *n*-hexane insoluble (21 g) and *n*-hexane soluble (12 g) fractions. The *n*-hexane insoluble material was chromatographed on neutral Al_2O_3 using $CHCl_3$–EtOH (99:1) and EtOH as development solvents. The collected fractions were screened for sapogenins by TLC examination using Si gel, $CHCl_3$–EtOH (95:5) as development solvent, and 50% aq. H_2SO_4 as the locating reagent. The $CHCl_3$–EtOH eluate contained a mixture of sapogenins, but the EtOH eluate contained only sapogenin C. The $CHCl_3$–EtOH eluate was rechromatographed on Si gel using $CHCl_3$–EtOH (95:5) and EtOH. Material eluted with $CHCl_3$–EtOH, when rechromatographed on Al_2O_3 using $CHCl_3$–EtOH (97.5:2.5) was resolved into 2 fractions, one containing sapogenins A and B and the other containing sapogenin C. Sapogenins A and B were separated by PLC. The material from the EtOH eluate was rechromatographed on Al_2O_3, and elution with Et_2O–EtOH (97.5:2.5) yielded sapogenin A and elution with EtOH yielded sapogenin C. The *n*-hexane soluble material was chromatographed on neutral Al_2O_3 and the column developed successively with C_6H_6, C_6H_6–Et_2O (50:50), Et_2O, Et_2O–EtOH (97.5:2.5) and EtOH. Most of the Et_2O–EtOH eluate contained only sapo-

genin B, but the latter fractions yielded sapogenin A. The EtOH eluate contained trace amounts of sapogenin C.

TLC. Sapogenins were examined by TLC on air-dried Si gel layers using CH_2Cl_2-MeOH-formamide (93:6:1), cyclohexane –EtOAc–H_2O (600:400:1) and $CHCl_3$–EtOH (95:5) as development solvents. The sapogenins were located by spraying with 50% aq. H_2SO_4 and heating at 100° until the characteristic colours developed [11]. Sapogenin acetates and trifluoroacetates were examined on activated Si gel layers using $CHCl_3$–toluene (9:1). Sapogenins A and B were separated by PLC on air-dried Si gel layers, 500 μm thick, using $CHCl_3$–EtOH (95:5).

Preparation of acetates of sapogenin diols. Sapogenin diacetates were obtained by refluxing the sapogenins with Ac_2O for 30 min. The monoacetates of brisbagenin, cannigenin and cordylagenin were prepared by reaction of the diacetates with either 0.25 M methanolic $Ba(OH)_2$ for 15 min or with 1 M methanolic KOH for 30 min at room temp. Ruscogenin-1-acetate was prepared by reaction of ruscogenin diacetate with 1 M methanolic KOH for 1 min at room temp.

GLC assay of cordylagenin and brisbagenin. Standard solns (0.2%) of cordylagenin, brisbagenin and 5α-cholestane were prepared. Cholestane (1 ml) was added to each of 16 stoppered tubes along with varying equal volumes of the cordylagenin and brisbagenin solns (4 × 0.5 ml; 4 × 1 ml; 4 × 1.5 ml; 4 × 2 ml). The solvent in each tube was evaporated under a stream of N_2, the residue dissolved in dry C_5H_5N (1 ml) and 0.3 ml of Sylon-BTZ was added. After 8 hr, 10 μl of the soln of the TMS derivatives of the sapogenins was examined by GLC using dual glass columns (150 cm × 4 mm i.d.) packed with 3% OV 101 on acid-washed, silanised Chromosorb W, 80–100 mesh, column temperature 243°, carrier gas N_2 at 100 ml/min and dual FID. The peak areas of cordylagenin, brisbagenin and 5α-cholestane were obtained from an integrator. The retention time of 5α-cholestane, which was used as the internal standard, was 6.34 min, of cordylagenin 22.44 min and of brisbagenin 27.13 min.

Extraction, examination and assay of leaf samples. The leaf material examined was dried at 50° and powdered. A sample (10 g) was extracted by the method of Blunden *et al.* [12]. This entailed incubation of the plant material in H_2O for 24 hr at room temp, refluxing with 2 M HCl for 2 hr, separation of the acid-insoluble material by filtration and washing with H_2O, NH_3 soln and H_2O until neutral. The dried, acid-insoluble residue was extracted with petrol (40–60°) for 24 hr, the extract evaporated to dryness and the residue dissolved in 25 ml $CHCl_3$. The crude sapogenin extracts were examined by 2D-TLC on air-dried Si gel layers using double development in CH_2Cl_2–MeOH–formamide (93:6:1) in the first direction and EtOAc in the second. The steroidal compounds were located by spraying with 50% aq. H_2SO_4. The cordylagenin/cannigenin yields, calculated as cordylagenin, and the brisbagenin yields were determined by GLC. For this, 1, 2 or 4 ml of the sapogenin extract was added to a stoppered tube along with 1 ml 0.2% soln of 5α-cholestane. The mixture was then treated as described above and analysed by GLC.

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