Stages in the Biosynthesis of the Epoxy Lactol Side Chain of Nic-1, Insect Antifeedant Steroid of *Nicandra physaloides*

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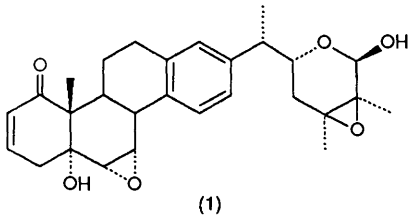
Isotopic labelling experiments with *Nicandra physaloides* plants show that the insect antifeedant steroid Nic-1 **1** is formed from 24(28)-methylenecholesterol **2a**; in the double bond isomerisation to 24-methylcholesta-5,24(25)-dien-3β-ol **3a**, the 25-(*pro-R*) methyl becomes C-27, the *pro-Z* methyl in **3a**. Hydroxylations lead through the diol **4a** to the triol **6a**, and hence to the lactol **7a**, with retention of the 26-hydrogen.

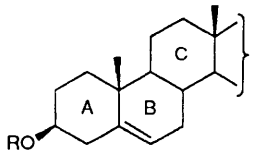
The Solanaceae is a large plant family containing around 90 genera, some of which are economically important *e.g. Solanum* (potato), *Lycopersicon* (tomato) and *Capsicum* (pepper); a number of species show interesting biological activities, *e.g.*, *Withania*. A large group of plant steroids, the withanolides,[1] have been isolated from members of the Solanaceae, all based on the 24-methylcholestane skeleton and these are characterised by extensive oxidative modifications.



(1)

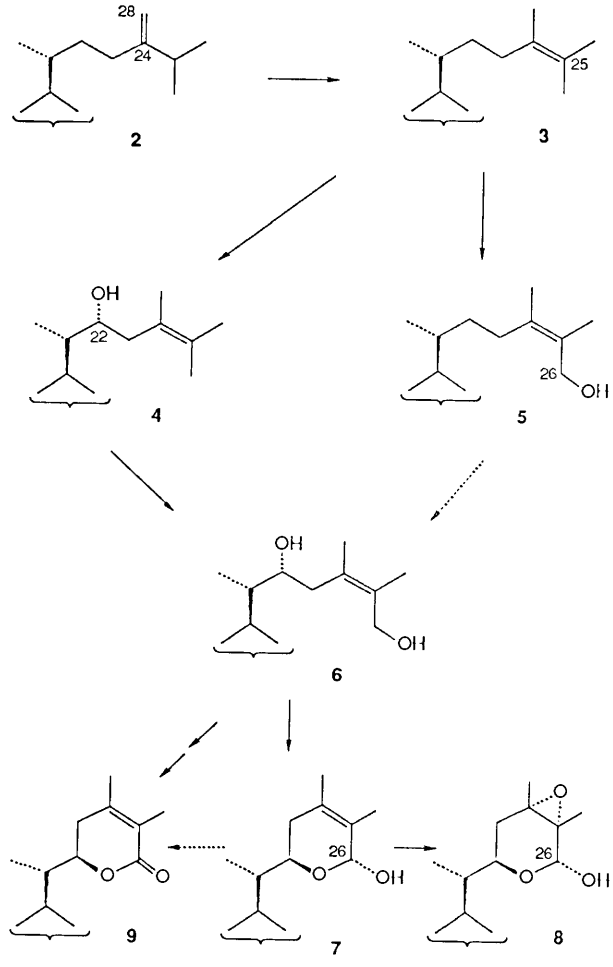
A distinctive subset has been found in *Nicandra physaloides* (the Peruvian shoo-fly plant), including the insecticidal and insect antifeedant sterol Nic-1 **1**.[2] Nic-1 displays a number of interesting structural features and in a previous paper [3] we have elucidated the biogenetic origins of the aromatic ring-D. We now turn our attention to the unusual epoxy lactol side chain.† This unit also appears in Nic-3 and in modified form, in other nicandrenoids.

Structural comparisons within the group suggest that side chain modifications may either precede ring A/B oxidative development or be partly independent of it, as in a metabolic grid with enzymes of low substrate specificity. In this paper, we report experiments which confirm this view and which define stages in the oxidative development of the epoxy lactol function. Selectivity in the functionalisation of the diastereotopic C-25 methyl groups of the 24-methylenecholesterol precursor is also revealed.



a; R = H
b; R = Ac
c; R = TBDMS

A reasonable hypothetical sequence of oxidative development is shown in Scheme 1, starting from 24(28)-methylenecholesterol **2a**, a known metabolite of Solanaceae.[5] Isomerisation to the 24(25)-isomer **3a** could be followed by either 22α- or 26-hydroxylation, forming **4a** or **5a**, respectively. A second hydroxylation then would lead to the 22α, 26-diol **6a**. Oxidation at the primary alcohol site would then afford the unsaturated lactol **7a**, finally epoxidised to the Nic-1 side chain **8a**. Further

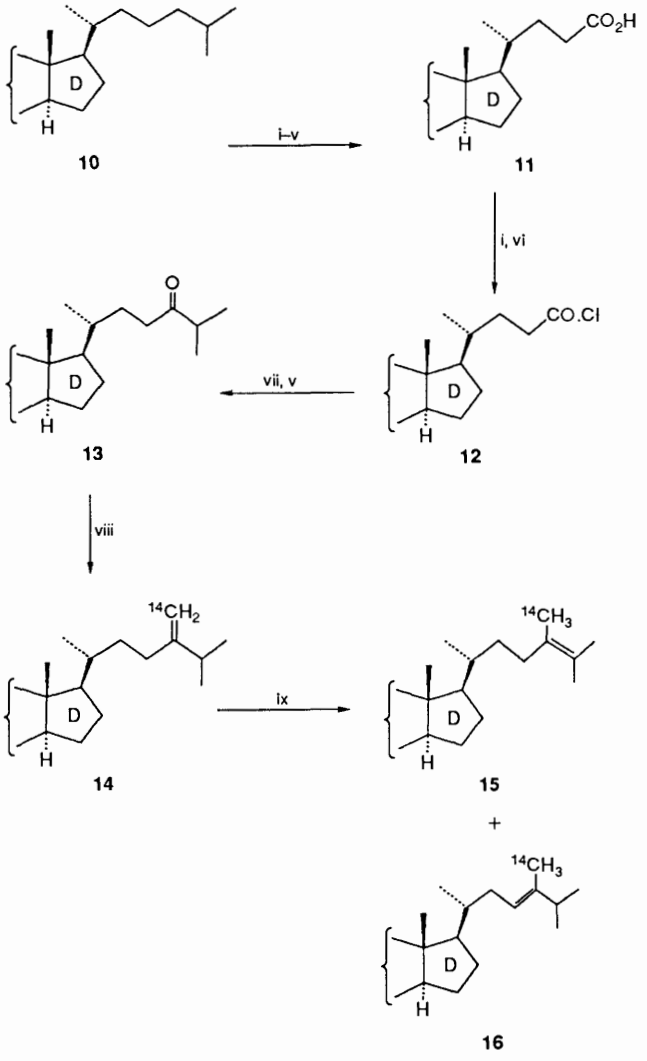


Scheme 1 Biosynthetic pathways to the Nic-1 side chain in *N. physaloides*

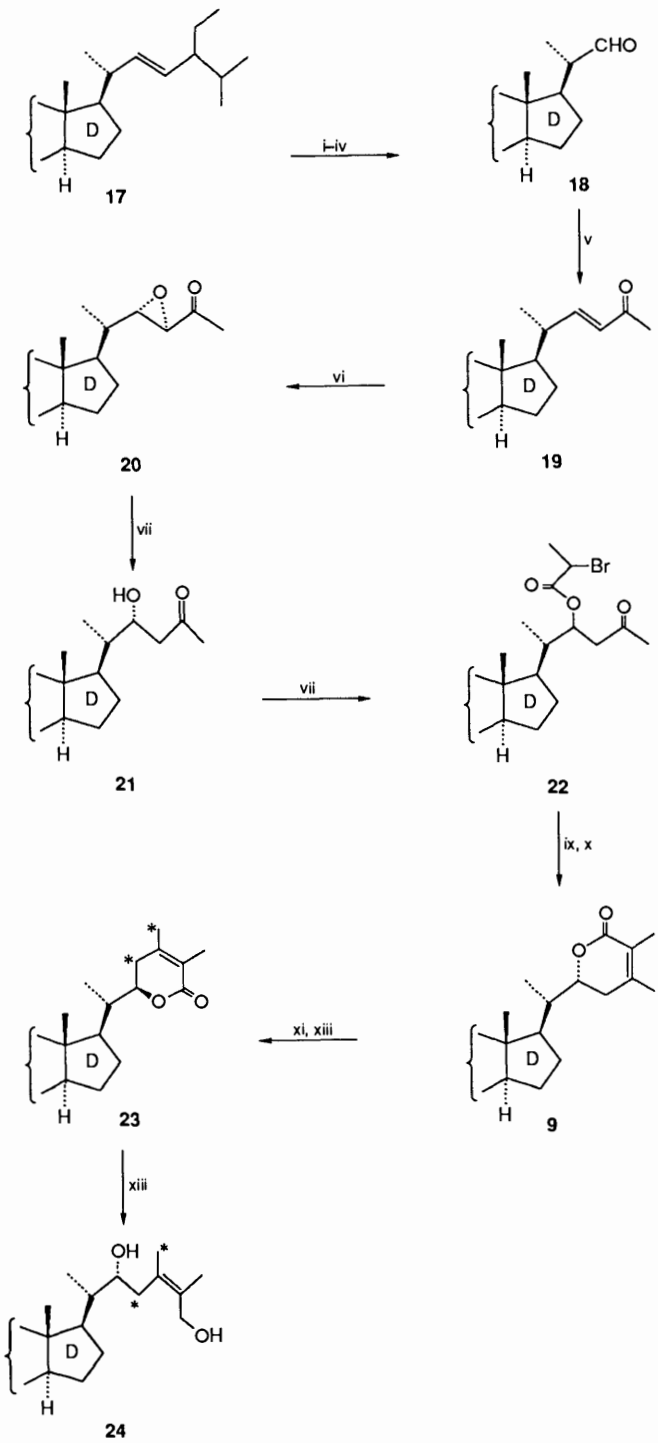
oxidation of the lactol **7a** to lactone oxidation level may provide the pathway to the withanolide type **9a**, in other solanaceous plants. It is possible, although perhaps less likely, that **9a**→**7a** reduction could take place under certain circumstances. A grid involving either alcohols **4a** or **5a** is possible. To test these notions we set out to synthesise several of these intermediates, suitably radiolabelled for biogenetic experiments.

Synthesis of Labelled Precursors.—Scheme 2 shows the formation of [28-^{14}C]-24(28)-methylenecholesterol **2a** and its

† A preliminary communication has been published.[4]



Scheme 2 *Reagents:* i, Ac_2O, Py; ii, Br_2; iii, CrO_3, AcOH; iv, Zn, AcOH; v, HO^-; vi, $SOCl_2$; vii, $(Me_2CH)_2Cd$; viii, $^{14}CH_2{=}PPh_3$; ix, I_2, PhH



Scheme 3 *Reagents:* i, Ac_2O, Py; ii, $PhIBr_2$; iii, O_3; iv, Zn, AcOH; v, $Ph_3P{=}CHCOMe$; vi, H_2O_2, HO^-; vii, Al–Hg; viii, MeCHBrCOBr; ix, $(EtO)_3P$; x, NaH; xi, HO; xii, ^{3}HHO, DBN; xiii, $LiAlH_4$. * denotes 3 H site.

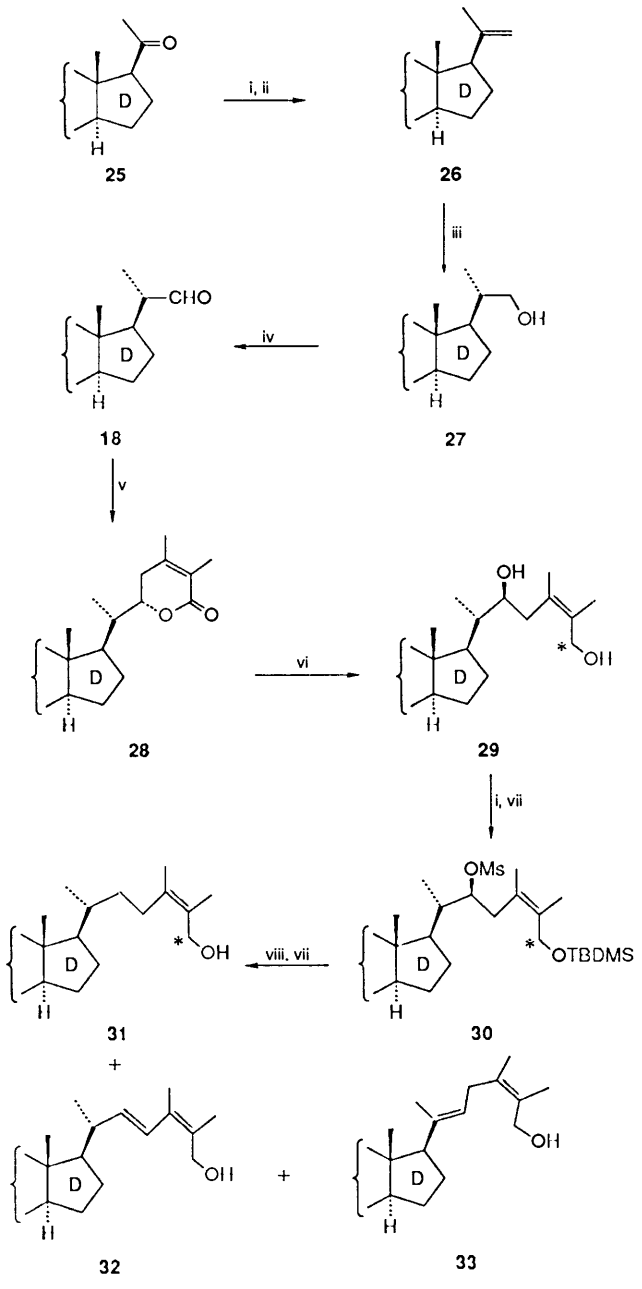
24(25)-isomer **3a**. A classical degradation[6] of cholesterol **10a** gave 3β-hydroxychol-5-enic acid **11a**. Treatment of the acid chloride **12b** with diisopropylcadmium provided 24-ketocholesterol **13a** which yielded the required product **14a** on reaction with [^{14}C]-methylenetriphenylphosphorane.[5a] Isomerisation of the side-chain double bond was effected with iodine in refluxing benzene to give **15a**; the 23-ene **16a** was also formed to a minor (20%) extent and could be separated by HPLC.

Scheme 3 outlines the route to [^{3}H]-lactone **23a** and [^{3}H]-triol **24a**. A well known cleavage of stigmasterol **17a** afforded the aldehyde **18b**,[7] which was then elaborated to the lactone **9a** using literature methods.[8] The C-22 stereochemistry was controlled by the stereoselective epoxidation of **19b**. The reduction of the epoxide with aluminium amalgam was prone to give the product of carbonyl reduction as a contaminant. Heating lactone **9a** at reflux in tetrahydrofuran (THF) with tritiated water and diazabicyclononane gave the [^{3}H]-compound **23a**, which could then be reduced to the [^{3}H]-triol **24a**.

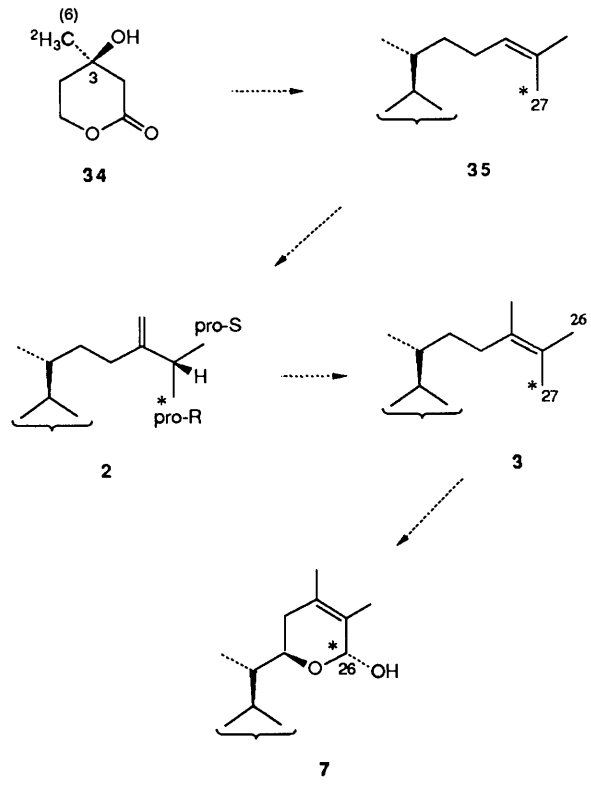
Scheme 4 displays the approach to the [^{3}H]-3,26-diol **3a**. A recent and efficient route[9] to the aldehyde **18c** from pregnenolone **25** was utilised. Condensation of the aldehyde with the anion derived from deprotonation of 2,3-dimethylbut-2-enoic acid gave the lactone **28c**, epimeric at C-22 with **9a**.[10] Reduction of the lactone with sodium [^{3}H]borohydride in THF–methanol gave the [^{3}H]-diol **29**. Protection of the primary alcohol, mesylation, reduction with lithium aluminium hydride and deprotection afforded the desired [^{3}H]-24(25)-en-26-ol **31a**. Inconveniently, almost equal quantities of the isomeric alcohols **32a** and **33a** were formed, but the mixture could be separated by HPLC to give the required compound.

Results and Discussion

The five sterols **14a**, **15a**, **23a**, **24a** and **31a** were administered in Tween 20–water–2–methoxyethanol to cut stems of 7-week old



Scheme 4 *Reagents:* i, TBDMSCl; ii, CH_2=PPh_3; iii, dicyclohexylborane; iv, PCC; v, Me_2C=$C(Me)CO_2Et$, BuLi–HMPA; vi, [^{3}H]$NaBH_4$, MeOH, THF; vii, MsCl; viii, $LiAlH_4$



Scheme 5 Stereochemistry of the **2**→**3** conversion in *N. physaloides*

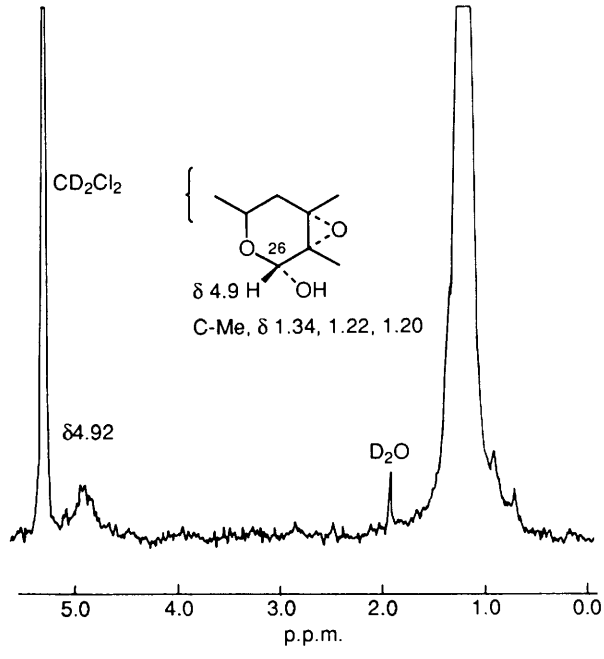


Fig. 1 ^{2}H NMR of Nic-1 from *N. physaloides* fed with [3′-CD_3]-MVA

Nicandra physaloides plants. After 4 days, the plants were dried and Nic-1 was isolated chromatographically and recrystallised to constant activity. The outcome of the experiments is shown in Table 1; absolute and specific incorporations are given and are based on sterol uptake. It can be seen that both 24-methylenecholesterol **14a** and its 24(25) double bond isomer **15a** are incorporated into Nic-1, at levels expected in such experiments. The triol **24a** shows a similar specific incorporation, but the putative intermediate alcohol **31a** was relatively poorly accepted. This suggests that the pathway from **3**→**6** preferentially uses intermediate **4** rather than **5**, with the last perhaps taking on a minor role. Clearly a competitive experiment between **4** and **5** is desirable to confirm this issue, but a viable synthesis of 22α-alcohol **4** is required for such a test. So far we have not been able to achieve this: the closest parallels are afforded by the recent syntheses of 22*R*-hydroxylanosterol and 22*R*-hydroxydesmosterol,[11] which employed an arsenic ylid to form the 22–23 bond. However our attempts to extend this work to the 24-methyl series were fruitless.

The lactone **23a** was a distinctly poorer precursor than the triol **24a**, although structurally closer to Nic-1. This suggests that the predominant pathway to the lactol **7** from the sterol **6** proceeds by way of a C-26 aldehyde, in preference to reduction of the lactone **9** (although the latter may constitute a minor route). This view is reinforced by the outcome of administration of [3′-C^2H]mevalonolactone **34** to *N. physaloides*. Examination

Table 1

	Weight of dry leaf/g	Weight of Nic-1/mg	Specific activity of Nic-1/dpm mol^{-1}	Absolute Incorporation (%)	Specific Incorporation (%)
Sterol					
14a	32.9	68	7.748×10^4	0.234	0.056
15a	31.4	66	8.825×10^4	0.158	0.068
23a	31.0	109	2.18×10^8	0.168	0.015
24a	30.0	95	5.45×10^8	0.50	0.057
31a	40.0	15	2.09×10^9	0.033	0.012

of the ^2H NMR spectrum of the resulting Nic-1, see Fig. 1, shows signals arising from (i), methyl groups C-19, C-21 as expected; (ii), 18-H, see ref. 3 and (iii), 26-H (δ 4.9). This indicates that C-26 of Nic-1 was derived from mevalonic acid C-6 with a substantial degree of retention of hydrogen, thus excluding lactone **9** from the dominant pathway.*

Scheme 1 thus summarises the present knowledge of the oxidative development of the Nic-1 side chain, from 24-methylenecholesterol **2**→**3**→**6** (chiefly *via* **4**→**7**→**8**).

A further stereochemical inference may be drawn from these experiments, as in Scheme 5. Mevalonic acid C-6 (3′-methyl) provides C-27 of desmosterol **35**. It has been shown [13] that, in the biosynthesis of 24-methylenecholesterol **2a** in *Physalis peruviana* cell cultures,† C-27 of **35** becomes C-26 (the *pro-R* methyl) of **2**. Since we have correlated C-26 of Nic-1 **7** and the C-27 of sterol **3**, with mevalonic acid C-6, it follows that the *pro-R* methyl of sterol **2** becomes the *pro-Z* methyl (C-27) of sterol **3**, implying β-face loss of 25-H. Net C-24 methylation of desmosterol **35** proceeds with retention of double bond geometry.

Experimental

For experimental generalisations see ref. 3. All *J* values are in Hz.

Preparation of [28-^{14}C]-24-*Methylenecholesterol*.—[^{14}C]-Methyltriphenylphosphonium iodide (2.22×10^8 dpm) in dry ethanol (1 cm^3) was syringed into a Reactivial and the solvent was removed under nitrogen. Unlabelled salt (0.2 g) was added and the mixture was suspended in THF (0.73 cm^3). Butyllithium 1.5 mol dm^{-3} (0.4 cm^3); was added with stirring; after 5 min a red solution formed, to which was added 24-ketocholesterol (0.1 g) in dry THF (0.73 cm^3). The reaction mixture was stirred overnight and then it was diluted with ether and washed with brine. Evaporation of the solvent afforded a crystalline solid which was purified on a silica column (ethyl acetate–hexane, 1:9–3:9), and then recrystallized to yield the title compound [5a] (4.55×10^7 dpm).

Preparation of [28-^{14}C]-24-*Methylcholesta*-5,24-*dien*-3β-*ol*.—[28-^{14}C]-24-Methylenecholesterol (2.78×10^7 dpm) and iodine (2 mg) were heated at reflux in benzene (1.25 cm^3) for 3 d. The mixture was evaporated and the residue was separated on a silica column using ethyl acetate (10–30%) in hexane, to afford the title compound (1.9×10^7 dpm) as white crystals, identified by TLC comparison with unlabelled material [14] made by the same method.

Preparation of (22R)-3β-*Hydroxyergosta*-5,24-*dien*-22,26-*olide*.—Sodium hydride (60% dispersion in oil; 24 mg); was added to a stirred solution of phosphonate (0.44 g) (from bromide **22**)[8] in dry THF (30 cm^3) under nitrogen. The solution was refluxed for 1 h, cooled and diluted with water (30 cm^3). The mixture was extracted with ether. The extracts were evaporated and the residue was chromatographed on silica [ethyl acetate (10–30%) in hexane] to yield the 3β-acetoxylactone **9b**, (0.25 g, 76%), m.p. 232–233 °C from ethanol (lit.,[8] m.p. 233 °C).

This sample was heated at reflux in 4% methanolic potassium hydroxide (40 cm^3) for 30 min. After acidification of the mixture, the product was isolated by ether extraction, to yield the 3β-hydroxy lactone **9a** (0.24 g), m.p. 219–222 °C from ethanol (Found: C, 78.3; H, 10.1. Calc. for $C_{28}H_{42}O_3$: C, 78.9; H, 9.9%); ν_{max}/cm^{-1} 3450 and 1697.

Preparation of [^{2}H]- *and* [^{3}H]-(22R)-3β-*Hydroxyergosta*-5,24-*dien*-22,26-*olide*.—The hydroxy lactone **9a** (25 mg) was dissolved in dry THF (1 cm^3) with diazabicyclononane (15 mg) and deuterium oxide (0.2 g) in a Reactivial and the solution was heated at 60 °C for 72 h. The product was diluted with ether and washed with water. Evaporation of the solvent gave the [$^2H_4$]hydroxy lactone (23 mg), *m/z* 430(5%, MD_4), 429(15, MD_3), 428(22, MD_2), 427(20, MD) and 426(12, M). A similar preparation using tritiated water (0.05 cm^3; 5.55×10^{11} dpm) in THF (0.45 cm^3) gave the [^{3}H] hydroxy lactone (24 mg, 2.38×10^8 dpm).

3β,22,26-*Trihydroxyergosta*-5,24-*diene*.—The hydroxy lactone **9a** (20 mg) was heated at reflux in dry THF (2 cm^3) with lithium aluminium hydride (20 mg) for 1 h. The cooled mixture was treated with ethyl acetate (10 cm^3) and the emulsion was washed into a Soxhlet thimble. Continuous extraction with chloroform and evaporation of the solvent gave the *title compound*, (18 mg, 89%), m.p. 127–129 °C (Found: C, 78.06; H, 10.32. $C_{28}H_{46}O_3$ requires C, 78.14; H, 10.70%); δ_H 0.72 (3 H, s, 18-H), 1.02 (3 H, s, 19-H), 1.71 and 1.81(6 H, s, 27-H and 28-H), 3.50 (1 H, 3α-H), 3.76 (2 H, m, 26-H), 4.36 (1 H, m, 22-H) and 5.38 (1 H, m, 6-H).

[^{3}H]-3β,22,26-*Trihydroxyergosta*-5,24-*diene*.—[^{3}H] Hydroxy lactone (20 mg) was reduced as in the preceding experiment, to yield the [^{3}H]-triol (19 mg), 1.84×10^8 dpm.

3β-(*t-Butyldimethylsilyloxy*)*ergosta*-5,24-*diene*-22,26-*olide*.—Butyllithium (1.6 mol dm^{-3} 0.9 cm^3); was added to diisopropylamine (0.3 cm^3) in dry THF (1 cm^3), and the resulting solution was stirred for 15 min and then cooled to −50 °C. A mixture of 2,3-dimethylbut-2-enoate (100 mg) and hexamethylphosphoramide (0.05 cm^3) was added and the resulting yellow solution was stirred at −50 °C for 3 h. 3β-(t-Butyldimethylsilyloxy)-23,24-dinorchol-5-enaldehyde [9] (210 mg) in dry THF (2.5 cm^3) was added and the resulting solution was maintained at

* Veleiro *et al.*[12] have isolated labelled withaferin A from *Acnistus breviflorus* fed with [2-^{14}C]-MVA. Degradation revealed ^{14}C at C-26 (lactone carbonyl), in discord with results reported here, but only 1.6% of the radioactivity of withaferin A was located at C-26; the authors consider that the biosynthesis of this withanolide involves side-chain degradation and resynthesis, with loss of label.

† In ref. 4, we quoted the original conclusions of Seo *et al.*;[13a] however these were reversed in a later paper,[13b] following reassignment of NMR signals.

−20 °C for 15 h. The product was partitioned between ether and water and the organic layers were dried and evaporated. Chromatography of the residue on silica (ethyl acetate–hexane 1:19) gave the *title compound* (110 mg, 43%), m.p. 203–204 °C from benzene; Found: C, 75.55; H, 10.5%; *m/z* 483.328. $C_{34}H_{56}O_3Si$ requires C, 75.50; H, 10.44%; M − C_4H_9, 438.329); ν_{max}/cm^{-1} 1690; δ_H 0.05 (6 H, s, 2 × Me), 0.62 (3 H, s, 18-Me), 0.83 (9 H, s, Bu^t), 0.92 (3 H, s, 19-Me), 0.98 (3 H, d, 21-Me), 1.72 (3 H, s, 27-H), 1.87 (3 H, s, 28-H), 2.65 (2 H, m, 23-H), 3.48 (1 H, m, 3-H), 4.41 (1 H, br d, 22-H) and 5.30 (1 H, m, 6-H).

3β-*t-Butyldimethylsilyloxy*)-22,26-*dihydroxyergosta*-5,24-*diene*.—The above lactone (1.4 g) was dissolved in dry THF (20 cm^3) and methanol (3.5 cm^3); sodium borohydride (0.9 g) was added and the resulting mixture was stirred at ambient temperature for 3 h. The mixture was diluted with water and extracted with ether. The extracts were dried and evaporated. Chromatography of the residue on silica (ethyl acetate–hexane, 1:4) gave the *title diol* (1.3 g, 92%), m.p. 195 °C from ethanol (Found: C, 74.7; H, 11.35; M, 544.431. $C_{34}H_{60}O_3Si$ requires C, 74.94; H, 11.11%; M^+, 544.431); δ_H 0.06 (6 H, s, 2 × Me), 0.70 (3 H, s, 18-Me), 0.89 (9 H, s, Bu^t), 0.96 (3 H, d, 21-Me), 1.01 (3 H, s, 19-Me), 1.71 and 1.81 (each 3 H, s, 27-H and 28-H), 2.75 (2 H, m, 23-H), 3.48 (1 H, m, 3-H), 3.73 (2 H, br, 22-H and 26-H), 4.33 (1 H, d, *J* 11.2, 26-H) and 5.31 (1 H, m, 6-H).

This compound, with acetic anhydride–pyridine, formed a *diacetate*, δ_H 1.99 and 2.06 (each 3 H, s, Ac).

In a radiochemical experiment, the lactone (200 mg was reduced with sodium [^{3}H]borohydride (7 mg, 250 mCi), to yield the [^{3}H]-diol (200 mg, 5.0 × 10^{16} dpm mol^{-1}).

3β,26-*Di*-(*t-butyldimethylsilyloxy*)-22-*hydroxyergosta*-5,24-*diene*.—The diol (410 mg) from the previous experiment was dissolved in dry DMF (10 cm^3) with imidazole (150 mg) and t-butyldimethylsilyl chloride (160 mg). The mixture was kept at ambient temperature for 12 h and then diluted with water. The mixture was extracted with ether and the extracts were washed, dried and evaporated to yield the *title alcohol* (490 mg, 98%), pure by TLC (ethyl acetate–hexane, 1:4) (Found: *m/z* 658.516. $C_{40}H_{74}O_3Si_2$ requires M^+, 658.517).

A parallel preparation with [^{3}H]-labelled diol afforded the [^{3}H]-alcohol.

3β-26-(*t-Butyldimethylsilyloxy*)-22-(*methylsulphonyloxy*)*ergosta*-5,24-*diene*.—The alcohol (700 mg) from the previous experiment in dry pyridine (10 cm^3) was cooled to 0 °C and methanesulphonyl chloride (0.63 cm^3) was added. The mixture was stirred at 0 °C for 10 min and then at ambient temperature for 5 min and then diluted with water. The product was extracted with ether. The extracts, after being washed, dried and evaporated, yielded the *title sulphonate*, (790 mg, 97%) as a colourless oil, pure by TLC, δ_H 0.05 and 0.08 (each 6 H, s, 2 × Me), 0.67 (3 H, s, 18-Me), 0.88 and 0.89 (each 9 H, s, Bu^t), 0.95 (3 H, s, 19-Me), 1.0 (3 H, d, 21-Me), 1.69 and 1.72 (each 3 H, s, 27- and 28-H_3), 2.94 (3 H, s, $MeSO_3$), 3.48 (1 H, m, 3-H), 4.13 (1 H, m, 26-H), 4.26 (1 H, d, *J* 11, 26-H), 4.84 (1 H, m, 22-H), 5.34 (1 H, m, 6-H); required number only of peaks in the ^{13}C NMR spectrum.

The [^{3}H]-methanesulphonate was similarly prepared.

3β,26-*Dihydroxyergosta*-5,24-*diene*.—The methanesulphonate (500 mg) from the previous experiment was dissolved in dry THF (10 cm^3) and lithium aluminium hydride (0.15 g) was added. The mixture was heated at reflux for 5 h and then it was cooled, quenched with water and filtered. The filtrate was extracted with ether and the precipitate was washed with the same solvent. The combined ethereal solutions were dried and evaporated. The residue showed a spot on TLC at R_f 0.95 and the corresponding fraction was isolated from a silica column (ethyl acetate–hexane, 1:19). This product was desilylated using tetrabutylammonium fluoride in THF, to yield material showing one TLC spot (ethyl acetate–hexane, 1:1) which was separated by HPLC on a μ-Porasil column (300 × 7 mm), (ethyl acetate–hexane, 1:4). The first compound that eluted was (E)-3β,26-*dihydroxyergosta*-5,22,24-*triene* (Found: *m/z* 412.334, 394.322. $C_{28}H_{44}O_2$ requires M^+, 412.334; M − H_2O 394.322); δ_H 0.55 (3 H, s, 18-Me), 1.0 (3 H, s, 19-Me), 1.67 and 1.77 (each 3 H, s, 27- and 28-H), 3.47 (1 H, m, 3-H), 4.25 (2 H, s, 26-H_2), 5.34 (1 H, m, 6-H), 5.52 (1 H, dd, *J* 8.8, 15.4, 22-H) and 6.44 (1 H, d, *J* 15.4, 23-H).

The second compound that eluted was the desired 3β,26-*dihydroxyergosta*-5,24-*diene* (Found: *m/z* 414.350, 396.342 and 314.258. $C_{28}H_{46}O_2$ requires M^+, 414.350; M − H_2O, 396.339; M − $C_6H_{12}O$, 314.261); δ_H 0.68 (3 H, s, 18-Me), 0.96 (3 H, d, *J* 7, 21-Me), 1.0 (3 H, s, 19-Me) 1.67 and 1.79 (each 3.H, s, 27-and 28.H), 3.53 (1 H, m, 3-H), 4.11 (2 H, s, 26-H), and 5.35 (1 H, m, 6-H). In a radiochemical preparation from [^{3}H]methanesulphonate, the [^{3}H]-diol was obtained (8.5 mg, 4.54 × 10^{13} dpm mol^{-1}).

The third compound that eluted was (E)-3β,26-*dihydroxyergosta*-5,20(22),24-*triene* (Found: *m/z* 412.334 and 394.322. $C_{28}H_{44}O_2$ requires M^+, 412.334, M − H_2O, 394.324); δ_H 0.56 (3 H, s, 18-Me), 1.0 (3 H, s, 19-Me), 1.68, 1.69 and 1.77 (each 3 H, s, 21, 27- and 28-H), 2.85 (2 H, d, *J* 7, 23-H), 3.53 (1 H, m, 3-H), 4.13 (2 H, s, 26-H), 5.06 (1 H, t, *J* 7,22-H) and 5.36 (1 H, m, 6-H); δ_C 18.05 (21-Me).

Administration Experiments with Nicandra physaloides *Plants*.—[28-^{14}C]-24-Methylenecholesterol (9 mg, 9.99 × 10^6 dpm) was dissolved 2-methoxyethanol (2.5 cm^3) and Tween 20 (40 mg); water (88 cm^3) was added in portions with sonication, to give a clear solution. Aqueous solutions of [28-^{14}C]-24-methylcholesta-5,24-dien-3β-ol (15 mg, 1.299 × 10^7 dpm), [^{3}H]-3β-hydroxyergosta-5,24-dien-22,26-olide (9.0 mg, 8.94 × 10^7 dpm), [^{3}H]-3β22,26-trihydroxyergosta-5,24-diene (10.0 mg, 9.7 × 10^7 dpm) and [26-^{3}H]-3β,26-dihydroxyergosta-5,24-diene (8 mg, 6.49 × 10^{11} dpm) were prepared in the same way.

Each solution was administered to the cut stems of *N. physaloides* plants, 6–7 weeks old; the solutions were taken up within a few hours. After 4 d, the plants were air dried and extracted with ether for 3 d at ambient temperature. Nic-1 was isolated from the evaporated extracts by chromatography on a silica column (ether–chloroform, 1:1), and recrystallised from benzene to constant activity. In each case, some unmetabolised starting sterol was recovered from the column and incorporation figures were corrected appropriately.

References

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