Ring D Expansion and Aromatisation in the Biosynthesis of Nic-1, an Antifeedant Steroid from *Nicandra physaloides*

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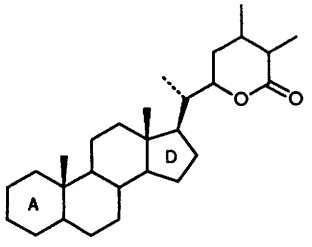
Isotope administration experiments with *Nicandra physaloides* plants using [3'-C^2H_3]- and [3'-$^{14}C_3$]-mevalonic acid, analysed by ^2H NMR and by degradation, respectively, show that the aromatic ring-D of Nic-1 **2** is formed by ring-D expansion in a steroid precursor with oxidative inclusion of the C/D angular methyl.

The plant family Solanaceae is a rich source of phytosteroids based on the 24-methylcholestane skeleton, frequently displaying highly oxidised structures. A major group is formed by the withanolides,[1] based on template **1**; more than 100 examples



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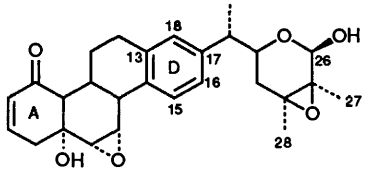
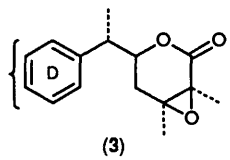


(2)

have been characterised in the genera *Withania*, *Acnistus*, *Datura*, *Jaborosa*, *Lycium*, *Nicandra* and *Physalis*. A varied spectrum of biological properties is displayed. *Nicandra physaloides*, the Peruvian 'Shoofly' plant, has been shown to contain insect antifeedant susbstances,[2] as its popular name suggests. The chief of these is Nic-1 **2**,[3] which exhibits both antifeedant and insecticidal properties *versus* the tobacco hornworm; this biological activity is of particular interest in that this larva is a specific feeder on Solanaceae, a family including important commercial vegetables.

The most remarkable structural feature of Nic-1 is the aromatic ring-D carrying a side chain displaced from its customary site adjacent the C/D junction in typical steroids. Four other *Nicandra* metabolites[3] share this feature *i.e.* Nic-1 lactone **3**, Nic-17 **4**, Nic-12 **5** and Nic-10 **6**. The acetal **2** and lactone **3** are related through redox processes and the series **4**, **5** and **6** might plausibly be derived in Nature through oxidative degradation. Thus Nic-1 is probably the first ring-D aromatic steroid formed in a biogenetic sequence unique to *N. physaloides*.

The formation of Nic-1 from a simple steroid precursor, *e.g.* 24-methylenecholesterol, must involve oxidative modification of both the A/B fragment and the side chain, as well as ring-D aromatisation. Since Nic-3 **7**[3] also occurs in *N. physaloides*, and no similar ring D aromatic steroids have been found in plants,



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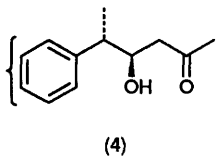

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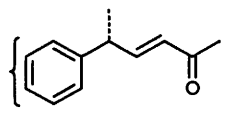

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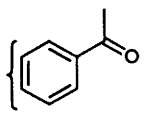

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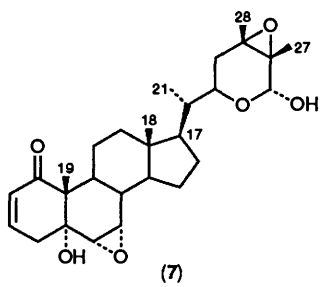

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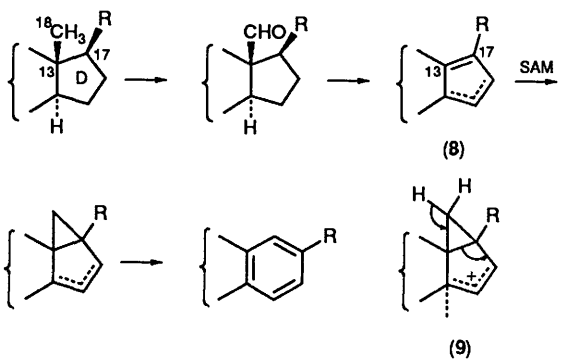


(8)

(9)

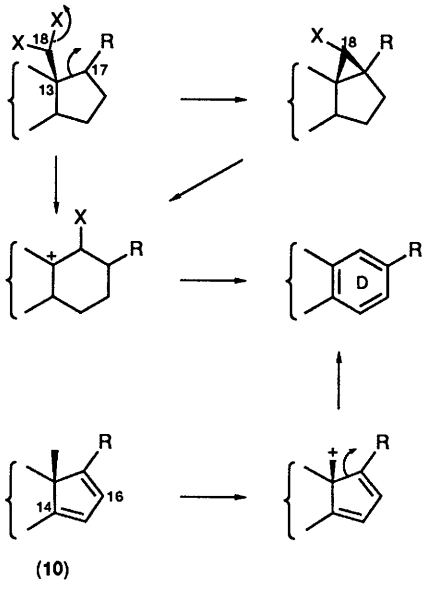
Scheme 1.

the aromatisation stage is likely to be late in the pathway, and Nic-3 may be a precursor to Nic-1.

Two plausible hypotheses on the aromatisation may be entertained. Thus, Scheme 1, the 13-methyl could be oxidised and eliminated following the pattern of the excision of the 14-

methyl in lanosterol.[4] The product 13(17)-ene **8** could then react with *S*-adenosylmethionine, perhaps in a similar way to cyclopropane fatty acid biosynthesis, with subsequent collapse of the bicyclo[3.1.0]hexane to the observed natural aromatic. Further ring-D oxidation is required at some stage, as shown in structure **8**; the aromatisation step could then be imagined as proceeding *via* intermediate **9**.

An alternative pathway, Scheme 2, involves oxidation of C-18 followed by 1,2-shift of C-17 to form a new six-membered ring, with or without a cyclopropyl fused intermediate. The additional oxidations necessary to aromatise ring-D could take place before or after (or both) the ring expansion. One mechanistically attractive hypothesis involves dehydrogenation of ring-D to a 14,16-diene **10**; cytochrome P_{450} oxidation at C-18 leads to a radical or cation intermediate which rearranges directly to the aromatic system.



(10)

Scheme 2.

These possibilities can in theory be readily distinguished by appropriate precursor incorporation experiments using labelled *S*-adenosylmethionine and mevalonic acid. Such experiments are the subject of this paper (see ref. 5 for a preliminary communication).

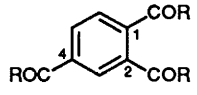
Results and Discussion

It was first necessary to establish isotope administration methods for *N. physaloides*. This plant is commonly grown from seed as a decorative annual. Although seeds are readily available, isolation work revealed wide variations in nicandrenoid content from different plant crops; it is very likely that a number of chemotypes exist within the species with different plant profiles. Such a situation has been demonstrated for *Withania somnifera* by the elegant studies of Lavie and his co-workers on the chemogenetics of the Solanaceae.[6] Seed was thus selected from a Nic-1 yielding strain grown in Nottingham, and a series of preliminary experiments were carried out using young plants. It has been shown previously that nicandrenoids are essentially confined to leaf tissue, and that they are not significantly produced in the first few weeks after germination. However 6 week old plants contain *ca.* 0.1–0.3% Nic-1 in leaf (dry weight). Table 1 shows the results of feeding [2-^{14}C]mevalonic acid (MVA) and [1-^{3}H]farnesol by different methods. The best results (absolute incorporations for MVA, 0.8%; for farnesol, 0.015%) were obtained through stem feeding with the precursors, and this procedure was adopted for subsequent experiments.

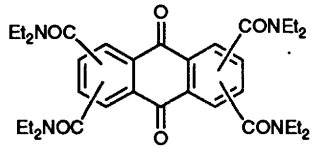
We then prepared [3′-$^{14}CH_3$]MVA, using literature procedures[7] developed for [3′-^{13}C]MVA slightly modified for the scaling down required in ^{14}C work, and this was administered to seven week old *N. physaloides* plants. *S*-[^{14}C]Adenosylmethionine (SAM) was similarly applied to a separate group of plants. Nic-1 was isolated in each experiment, diluted, and oxidised with potassium permanganate to trimellitic acid **11**. The acid was isolated and purified as its triester **12**.

Table 2 shows the results of these experiments. It is clear that [$^{14}CH_3$]SAM is incorporated into Nic-1, but no activity appears in ring-D; it is presumably all located at C-28. However on incorporation of [3′-^{14}C]MVA, 26% of the original activity appears in the trimellitic acid fragment. Since four sites (C-19, C-18, C-21 and C-26 or C-27) in a steroid precursor such as **7** would be expected to be labelled, this experiment is consistent with the retention of C-18 as part of the aromatic fragment.

We made some investigations into the feasibility of isolating C-3 of the trimellitic acid **11**. Our scheme involved lithiation of the tris(diethylamide) **13**, with the expectation that C-3 deprotonation would be preferred, with the metal stabilised by two *ortho* functions. *C*-Methylation would then have given 3-methyltrimellitic acid, and Kuhn–Roth oxidation would have yielded acetic acid containing C-3 of trimellitic acid (steroid C-18). However, the plan was frustrated by the rapid dimerisation of lithiated amide **13** to yield a mixture of the anthraquinone tetraamides **14** (M^+ 604). This observation demonstrated that lithiation was occurring unselectively. Changing the structure of the amide, the base and the methylating agent failed to afford any of the desired *C*-methylmellitic acid.



11; R = OH
12; R = OMe
13; R = NEt_2



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We thus required an alternative method to define the site in ring D labelled by the MVA methyl group, and we turned to deuterium labelling. Thus [3′-C^2H_3]MVA was prepared using the above methods, and a sample (408 mg) was administered to 23 *N. physaloides* plants. After 7 days, Nic-1 was isolated from the plants without carrier material. A ^{2}H NMR spectrum of this sample (160 mg; 12 680 scans; CH_2Cl_2–10% $CFCl_3$) showed a clear absorption at δ 6.99 (Fig. 1), with other expected signals and no ^{2}H impurities; this signal corresponds to that of 18-H(s) in the ^{1}H NMR spectrum (*cf.* 15-H, δ 7.41; 16-H, δ 7.10).

This evidence thus points to a biosynthetic pathway such as that shown in Scheme 2; either a cationic or radical mechanism would be plausible. An intermediate containing a 13,17-fused cyclopropane would also be possible, with parallels in the lanosterol–cycloartenol conversion.[8] In this context, the structures of the *Buxus* alkaloids are of interest since both compounds **15**, with a seven-membered ring-B and **16**, with cycloartenol A/B system,[9] co-occur. The biosynthesis of compounds **15** may plausibly be considered to arise by C-9/C-10 fission, analogous to one pathway for the *Nicandra* ring-D transformation.

Table 1. Administration of [1-^{3}H]farnesol and *RS*-[2-^{14}C]mevalonic acid to *Nicandra physaloides*.

	Method[a]	Dry leaf weight (g)	Nic-1 weight (mg)	Specific activity (dpm mmol^{-1})	Abs. incorp. (%)	Spec. incorp. (%)
[1-^{3}H]Farnesol[b]	A	4.2	9.2	3.2×10^7	1.5×10^{-2}	3.2×10^{-2}
	B	3.4	2.9	7.4×10^6	1.1×10^{-3}	7.3×10^{-3}
	C	8.6	6.8	9.5×10^6	3.4×10^{-3}	9.5×10^{-3}
[2-^{14}C]MVA	A	6.0	21.0	4.94×10^6	0.80	4.2×10^{-3}
	B	2.6	7.0	3.86×10^6	0.21	3.3×10^{-3}

[a] Method A, application to cut plant stems; method B, application to excised leaf stems; method C, application to intact leaf surface. [b] Mixture of geometric isomers containing 38% 2*E*,6*E*.

Table 2. Incorporation of ^{14}C-labelled species into *N. physaloides* plants, and Nic-1 degradation.

	No. of plants	Nic-1 weight (mg)	Abs. Incorp. (%)	Dilution factor	Specific activity of Nic-1 **2**, diluted (dpm mmol^{-1})	Weight of trimethyl mellitate **12** (mg)	Specific activity of trimethyl mellitate (dpm mmol^{-1})
[3′-^{14}C]MVA	10	12.0	0.1	6.55	444×10^3	15.0	116×10^3
[^{14}C]SAM	5	1.0	0.002	125.2	241×10^3	15.4	0

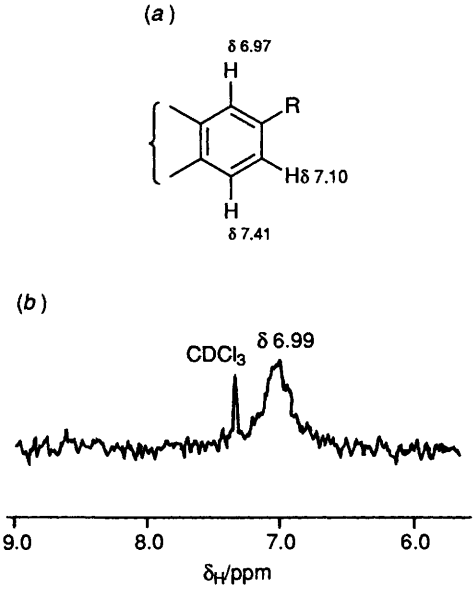
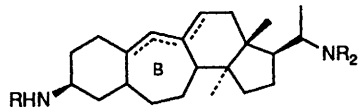


Fig. 1. (*a*), ^{1}H NMR data for Nic-1 **2** in $CDCl_2$–$CFCl_3$; (**b**), ^{2}H NMR of Nic-1 **2** [160 mg, undiluted, 12 680 scans in CH_2Cl_2–$CFCl_3$ (10%)] obtained after administration of [3′-C^2H_3]MVA.



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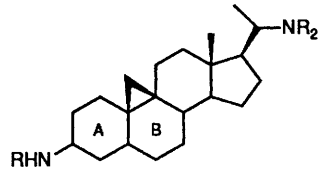

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Experimental

General.—M.p.s were determined with a Kofler hot-stage microscope, and are uncorrected. UV spectra were measured in ethanol; log ε follows λ_{max} IR spectra were recorded in chloroform (solids) or liquid films. Mass spectra were measured using electron impact ionisation on an AEI MS 902 or a VG 7070F spectrometer. ^{1}H NMR spectra were recorded at 90 MHz in deuteriochloroform, and ^{13}C NMR were determined at 25.15 MHz in the same solvent. Observed splittings (*J*) are quoted. Hydroxylic protons were identified by deuterium exchange. TLC used silica gel G (HF254) in 0.25 mm layers; preparative TLC employed 20 × 20 cm plates at 0.5 or 0.8 mm thickness. Plates were visualised in UV (254 nm) or by heating plates sprayed with 10% sulphuric acid. All solvents were appropriately dried and distilled before use. Organic solutions were dried over magnesium or sodium sulphate. Solvents were evaporated under reduced pressure. 'Extraction' implies the use of diethyl ether.

Precursor Incorporation Experiments.—*Nicandra physaloides* plants were grown from seed collected from selected plants used in structural studies, in sterile compost in an unheated greenhouse; administration to 6-week old plants was best effected in June–July when growth was most rapid. Plant stems were cut through just above soil level, and immersed in aqueous solutions of precursor in vials of minimum size. As solutions were taken up they were replaced with water over the experimental period (normally 5 d). Plant material was then air dried and crushed to a powder which was stirred in ether for 3 d. Filtration and evaporation of the solvent gave a green residue containing (TLC) two major components, Nic-1 and a mixture of simple phytosterols. These were separated on a silica gel dry column eluting with 30% and 70% light petroleum–ethyl acetate in succession. The crude Nic-1 (R_f 0.6) was purified by preparative TLC (70% ethyl acetate–hexane) and recrystallised from benzene to constant specific activity. Specific activities were determined by liquid scintillation counting (Intertechnique SL3050) calibrated using samples of standard quenching.

[1-^{3}H]*Farnesol.*—Farnesal was prepared by oxidation of commercial farnesol (using pyridinium chlorochromate in

dichloromethane at ambient temperature for 4.5 h) as a mixture containing 38% 2*E*,6*E*, 15% 2*E*,6*Z*, 24% 2*Z*,6*E* and 23% 2*Z*,6*Z* isomers (from ^{13}C NMR analysis). The aldehyde (82 mg) in ethanol (1 cm^3) was treated with sodium [^3H]borohydride (15.6 mg, 5.55×10^{10} dpm) in ethanol (2 cm^3). After 1 h, the solution was diluted with water and extracted with ether. The extracts gave [1-^3H]farnesol (74 mg, 87%, 3.29×10^{10} dpm $mmol^{-1}$) containing 38% 2*E*,6*E* isomer.

Benzyl [2-^{14}C]*Ethanoate, Benzyl* [2-$^2H_3$]*Ethanoate and Benzyl* [2-$^3H_3$]*Ethanoate.*—(*a*) Sodium [2-^{14}C]ethanoate (1 mCi) was mixed with ethanoic acid (212 mg, 3.53 mmol), benzyl chloride (461 mg, 3.64 mmol) and triethylamine (3 cm^3), and the mixture was heated at reflux for 4 h. The cooled mixture was mixed with water (3 cm^3) and extracted with ether (1×3 cm^3, 2×2 cm^3). The extracts were washed [dil. hydrochloric acid (2×3 cm^3), aq. sodium carbonate (2×3 cm^3), water (4×3 cm^3)], dried, and evaporated to yield benzyl [2-^{14}C]ethanoate as a pale yellow oil (490 mg, 90%). The material compared by TLC to an unlabelled sample, and was used without further purification.

(*b*) Benzyl [2-$^2H_3$]ethanoate was prepared from benzyl chloride (10 g), [$^2H_4$]ethanoic acid (11 g) and triethylamine (12 g) at reflux (2 h); the product was isolated as above, and distilled, b.p. 100–101 °C at 10 mmHg, *m/z* 153.188 (M^+, 44%) and 109.063 ($M - CD_2CO$, 100%).

(*c*) Lithium diisopropylamide was prepared at 0 °C in tetrahydrofuran (THF) (5 cm^3) from diisopropylamine (1 cm^3) and butyl-lithium (1.6M; 4.4 cm^3, 7.04 mmol). The solution was then cooled to −78 °C and benzyl ethanoate (1 g) in THF (3 cm^3) was added. After 5 min, ^3H_2O (0.1 cm^3) was added and the solution was allowed to warm to room temperature. After filtration and evaporation, the residue was distilled as above to yield the tritiated ester (0.7 g, 80 mCi).

4-*Hydroxy*-4-[$^2H_3$]*methylhepta*-1,6-*diene and* 4-*Hydroxy*-4-[^{14}C]*methylhepta*-1,6-*diene.*—(*a*) To magnesium turnings (240 mg) in ether (2 cm^3) and THF (2 cm^3) at 0 °C, was added benzyl[2-^{14}C]ethanoate (490 mg) and allyl bromide (1.1 g) in THF–ether (1:1) (4 cm^3), with stirring. The mixture was refluxed for 6 h, cooled, and treated with water (5 cm^3) and dil. hydrochloric acid before extraction with ether (2×5 cm^3). The extracts were washed, dried and evaporated to yield an oil which was purified by chromatography on silica gel [ethyl acetate–light petroleum (3:7)] to yield the required [^{14}C]diene alcohol (323 mg, 79%).

(*b*) A similar preparation using benzyl [2-$^2H_3$]ethanoate (12.1 g) gave the [$^2H_3$]diene alcohol (10 g), δ_H 2.25 (4, d, *J* 7 Hz) and 5.0–6.1 (6 H, m); *m/z* 88.089 ($M - C_3H_5$).

3-*Hydroxy*-3-[^{14}C]*methylglutaric Acid and* 3-*Hydroxy*-3-[$^2H_3$]*methylglutaric Acid.*—(*a*) The above [$^2H_3$]diene alcohol (10 g) in ethyl acetate (50 cm^3) at −78 °C was treated with ozone–oxygen until a permanent blue colour was obtained. Excess of ozone was purged with oxygen and the solution was allowed to warm to room temperature. The solvent was evaporated and the residue was heated at reflux with hydrogen peroxide (30%; 15 cm^3) and sulphuric acid (0.25 cm^3) in water (30 cm^3) for 2 h. The cooled mixture was neutralised with barium carbonate (1 g), filtered and evaporated to yield a white solid. The residue was extracted with acetone to yield, after evaporation and crystallisation from benzene, the [$^2H_3$]diacid (66 g, 52%), m.p. 110–111 °C, δ_H 2.73 (4 H, s) and 8.55 (3 H, br s).

(*b*) A parallel reaction using the [^{14}C]diene alcohol in dichloromethane (9 cm^3) and acetic acid (1 cm^3), and oxidizing the ozonide with acetic acid–30% hydrogen peroxide (3:4) gave the desired [^{14}C]diacid (307 mg), as a colourless solid.

3-*Hydroxy*-3-[^{14}C]*methyl glutaric Anhydride and* 3-*Hydroxy*-3-[$^2H_3$]*methylglutaric Anhydride.*—The above [$^2H_3$]-diacid (0.5 g) was set aside in acetic anhydride (3.25 g) for 18 h. After evaporation under high vacuum, the residue was crystallised from dichloromethane to yield the desired anhydride (0.34 g, 76%), m.p. 102–103 °C, δ_H[$^2H_6$]acetone) 2.95 (4 H, m) and 4.64 (1 H, s, OH), ν_{max} 3350, 1813, 1760 and 1700 cm^{-1}. Best yields were obtained from a number of batches on this scale. The [^{14}C] analogue (84%) was very similarly prepared from the [^{14}C]diacid (307 mg).

RS-[3′-^{14}C]- *and* [3′-$^2H_3$]-*Mevalonolactone.*—The [$^2H_3$]-anhydride (500 mg) in propan-2-ol (3 cm^3) was added dropwise to a solution of 0 °C of sodium borohydride (320 mg) in propan-2-ol (2 cm^3). The solution was set aside at room temperature for 24 h when it was evaporated to dryness. The residue was dissolved in water (10 cm^3), acidified to pH 1 (hydrochloric acid) and the solution was continuously extracted with ether to yield the [3′-$^2H_3$]lactone as a colourless oil (218 mg, 42%), b.p. 100–101 °C at 10 mmHg; δ_H 1.95 (2 H, t, *J* 5 Hz), 2.62 (2 H, m), 3.90 (1 H, s, OH) and 4.50 (2 H, m); δ_D 1.39; *m/z* 133.080 (M^+, 4%); ν_{max} 3400 and 1701 cm^{-1}.

A parallel experiment with the [^{14}C]anhydride (230 mg) gave the [3′-^{14}C]mevalonolactone (87 mg), purified by chromatography (silica gel, ethyl acetate), 3.45×10^8 dpm $mmol^{-1}$, 155.4 μCi $mmol^{-1}$.

Oxidation of Nic-1 *to Benzene*-1,2,4-*tricarboxylic Acid.*—Nic-1 (57 mg) was heated at reflux in water (5 cm^3) containing potassium permanganate (200 mg) and sodium hydroxide (50 mg), for 5 h. The cooled mixture was acidified to pH 1 (sulphuric acid) and treated with sulphur dioxide to remove excess of oxidant. The solution was continuously extracted with ether for 48 h. The dried extract, on evaporation, gave a brown solid which was treated with an excess of diazomethane. The product was purified on silica gel [dry column, ethyl acetate–light petroleum (1:1)] to yield triemethyl benzene-1,2,4-tricarboxylate (9.1 mg) as a colourless oil, authenticated by comparison with a sample prepared by oxidation of pseudocumene.[10]

Administration of S-*Adenosyl*-L-[^{14}C]*methionine to* Nicandra physaloides.—Five *N. physaloides* plants (7 weeks old) were cut, and the stems were dipped into dilute aqueous nutrient (Phostrogen) containing [^{14}C]methyl-SAM (59 μCi), and maintained in a cool greenhouse for 7 d. Nic-1 was isolated as above, and the crude product (1 mg) was diluted with pure steroid (13.2 mg) before recrystallisation from benzene to 517 dpm mg^{-1}, 2.41×18^5 dpm $mmol^{-1}$, total 5.8 mg. Dilution of a sample of this product with more cold Nic-1 to 45.34 mg (total 2451 dpm) was followed by a permanganate oxidation to 1,2,4-trismethoxycarbonylbenzene (15.4 mg, 63%). A sample (9.2 mg) of this ester was radiochemically inactive, within experimental error (background *ca.* 20 dpm).

Administration of RS-[3′-^{14}C]*mevalonolactone to* Nicandra physaloides.—Ten *N. physaloides* plants (7 weeks old) were fed using a cotton wick through the stem, with *RS*-[^{14}C]-mevalonolactone (86 mg, 2.65×10^6 dpm mg^{-1}). The plants were dried and Nic-1 was isolated as above; the material was crystallised from benzene, to yield [^{14}C]Nic-1 (12 mg, 2.91×10^6 dpm $mmol^{-1}$). Permanganate oxidation of this product (9.8 mg) diluted with inactive material (54.4 mg) to 4.44×10^5 dpm $mmol^{-1}$ gave trimethyl benzene-1,2,4-tricarboxylate (15 mg, 43%), 1.16×10^5 dpm $mmol^{-1}$.

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Paper 0/02513E
Received 5th *June* 1990
Accepted 3rd *July* 1990