Phytochemistry, 1976, Vol. 15, pp. 689–694. Pergamon Press Printed in England.

BIOSYNTHESIS OF THE SESQUITERPENOID, CAPSIDIOL, IN SWEET PEPPER FRUITS INOCULATED WITH FUNGAL SPORES

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(*Received* 28 *August* 1975)

Key Word Index—*Capsicum frutescens*; Solanaceae; bell-pepper; *Monilinia fructicola*; biosynthesis; acetate-mevalonate pathway; ^{13}C NMR; methyl migration; phytoalexin; eremophilane; capsidiol.

Abstract—*Capsicum frutescens* fruits inoculated with spore suspensions of *Monilinia fructicola* incorporated 1–4% of sodium acetate-[2-^{14}C] or *RS*-mevalonolactone-[2-^{14}C] into the phytoalexin, capsidiol. Labelled capsidiol was characterized by GC–RC, TLC–RC, gel chromatography (in conjunction with liquid scintillation counting) and GC–MS. The mode of incorporation of sodium acetate-[1,2-$^{13}C_2$] into capsidiol, as indicated by the pattern of ^{13}C-^{13}C coupling from ^{13}C NMR data, supports the hypothesis that the angular methyl group of the capsidiol skeleton arises by migration from the C-10 position of a eudesmane-type intermediate.

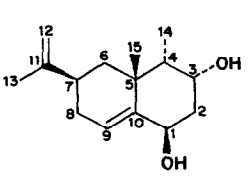
INTRODUCTION

In vivo studies of monoterpenoid and sesquiterpenoid biosynthesis in higher plant systems via incorporation of postulated precursors have been technically difficult. Various methods of administration of radioactive precursors have been used [1,2] but generally they have resulted in very low levels of incorporation [3,4]. One notable exception involved the feeding of *RS*-mevalonic acid-[2-^{14}C] to freshly opening rose flowers, which resulted in the appearance of 10.5% of the label in geraniol β-D-glucoside [5,6]; however, incorporation into sesquiterpenoids amounted to only about 0.1% [5]. Transport barriers, compartmentation effects [9,10], rapid catabolism of the desired product [5,11] and consumption of precursors by competing and more active pathways [12] are among the reasons put forward to explain the poor uptake of potential precursors. These aspects of biosynthesis have been discussed in some detail by Banthorpe *et al.* [4].

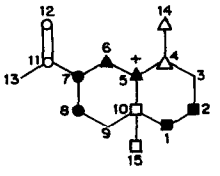
In other instances a particular biosynthetic system may have a naturally low activity. It would therefore be desirable to stimulate such a system so that production of compounds normally occurring in trace quantities is greatly enhanced. This latter phenomenon occurs during induction of post-infectional inhibitors (phytoalexins) by plants as a presumed defence against pathogenic attack. This topic has received considerable attention by virtue of its biochemical significance and its potential value in the development of antifungal or antibacterial agents [13–16]. A particularly striking effect has been reported to occur in potato tubers inoculated with the bacterium *Erwinia carotovora*, which produced the nor-sesquiterpenoid, rishitin, in amounts exceeding 1mg/g fr. wt [17]. Shih and Kuć [18] have demonstrated substantial incorporation of ^{14}C-labelled acetate and mevalonate into rishitin formed by potato tuber slices after inoculation with the fungus *Phytophthora infestans*.

The finding by Stoessl *et al.* [19] that production of the sesquiterpenoid capsidiol (**1**) from capsicum fruits inoculated with fungal spore suspensions is increased about 100-fold indicated that the biosynthesis of capsidiol from mevalonate is stimulated, or less probably that a more immediate (C_{15}) precursor is converted into capsidiol under these conditions. Fruits of *Capsicum frutescens* offer the advantage of possessing a sterile cavity into which large volumes of potential precursors can be injected aseptically together with fungal spore suspensions. Furthermore, since the capsidiol is secreted into the cavity of the pepper it may be insulated to some extent from catabolic processes.

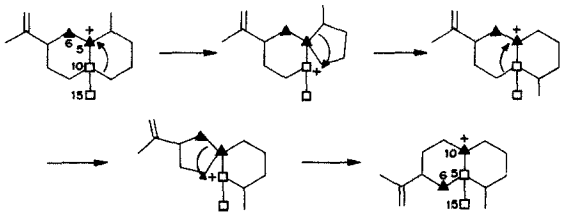
Capsidiol (**1**) is of particular interest since it is a 4-*epi*-eremophilane derivative. The eremophilane carbon skeleton is the classical example of a sesquiterpenoid structure that cannot be derived by simple coupling of isoprene units—or, in more modern terms, by direct cyclisation of farnesyl pyrophosphate [20,21]. During the course of biosynthesis of the eremophilane skeleton, the methyl group at C-5 is presumed to arise via methyl group migration from C-10 from a precursor of eudesmanoid skeletal type (**2**), as originally postulated by Robinson [22]. An alternative route proposed with particular reference to the stereoisomeric nootkatane (valencane) skeleton, involves a series of spiro-rearrangements [23]. One of several possible sequences is shown in Scheme 1. This latter proposal was of particular interest because several



1 Capsidiol



2 Eudesmane-type structure labelled from (1,2-$^{13}C_2$) acetate



Scheme 1. Hypothetical spiro-rearrangement. Symbols represent those pairs of ^{13}C atoms incorporated from sodium acetate-[1,2-$^{13}C_2$] which relate to a hypothetical mode of elaboration of the capsidane skeleton.

spiro-compounds [e.g. lubimin, oxylubimin, spirovetiva-1(10),11-dien-2-one and spirovetiva-1(10),3,11-trien-2-one] are known as phytoalexins produced by other members of the Solanaceae [24,25]. Studies by Brooks and Keates [26] on the biosynthesis of the eremophilane derivative petasin were consonant with Robinson's hypothesis, but afforded no strong evidence against the occurrence of spiro-rearrangement.

It was expected that preliminary experiments with *RS*-mevalonolactone-[2-^{14}C] or sodium acetate-[2-^{14}C] would provide information on the level of incorporation into capsidiol (**1**) and on the optimisation of incubation conditions. At the same time, it was hoped to develop studies of intermediates in the capsidiol biosynthetic pathway.

Although a distinction between the methyl shift and spiro-rearrangement pathways should be possible through incorporation of mevalonic acid labelled with ^{14}C at C-3′ it appeared that a more expeditious method, not requiring degradative procedures, could be based on the incorporation of sodium acetate-[1,2-$^{13}C_2$] followed by ^{13}C NMR spectrometry of the enriched capsidiol [27–30]. Of the six intact C_2 units incorporated through farnesyl pyrophosphate into a eudesmane type intermediate (**2**), one must be cleaved during the elaboration of the eremophilane or capsidane skeleton. If a simple methyl shift mechanism were in operation, the C-10: C-15 bond would be cleaved, resulting in the absence of coupling between the atoms ultimately occupying the C-5 and C-15 positions; however, in a rearrangement via spiro intermediates (Scheme 1), the atoms becoming C-5 and C-15 would remain paired, while C-6 would become isolated.

RESULTS AND DISCUSSION

Capsidiol (**1**) was isolated from whole fruits of *Capsicum frutescens* inoculated with *Monilinia fructicola* as described by Stoessl *et al.* [19] except that the cultivars used were Bell Boy, New Ace, and Propa. Yields of capsidiol were variable, but were highest from peppers at the intermediate ripening stage (green streaked with red) or from ripe (red) peppers as had been previously observed [19]. As far as was possible, incorporations of labelled precursors were carried out using peppers at this intermediate-ripening or ripe stage.

RS-*Mevalonolactone*-[2-^{14}C] as a capsidiol precursor

In preliminary experiments, aqueous solutions of *RS*-mevalonolactone-[2-^{14}C] (MVAL-[2-^{14}C]; 0.5 μCi/pepper) were injected into peppers 6 hr after inoculation with fungus spores. Following 48 hr incubation (from time of introduction of fungus) the diffusates were extracted with ether; aliquots of the ether extracts and aqueous (non-ether soluble) extracts were removed for liquid scintillation counting. Levels of incorporation of MVAL-[2-^{14}C] into capsidiol (Table 1) were of the order of 2–4% (4–8% of the *R*-isomer). PLC of the ether extracts on silica gel, followed by scanning, indicated that most of the radioactivity corresponded to the capsidiol band. Elution of the capsidiol band with Et_2O–MeOH (4:1 v/v) followed by repeated recrystallization from ether yielded pure capsidiol, mp 152–154°, specific radioactivity approximately 47000 dpm/mg. GC–RC of capsidiol, its diacetate and di-TMSi ether showed only one radioactive peak which corresponded to the mass peak and provided further evidence of its purity. Retention indices (1870, 2045 and 1912 respectively) agreed closely with those of unlabelled capsidiol and its derivatives. Combined GC–MS of the diol and its di-TMSi ether and diacetate derivatives gave molecular ions of *m/e* 236, 380 and 320 respectively, providing further confirmation of purity and identity.

Further experiments utilizing MVAL-[2-^{14}C] administered at various time intervals before and after fungal inoculation (Table 2) indicated that introduction of radioactive precursor into peppers 6 hr before fungal inoculation resulted in the best incorporation into the ether extract, and into capsidiol, although the *per cent* incorporation of ^{14}C was lower than previously obtained in all treatments. Increasing the fungus incubation period from 2 to 3 days resulted in an increased production of capsidiol, but little increased incorporation of MVAL-[2-^{14}C]. The data suggest that the transport of MVAL to the capsidiol-synthesising system is slow in relation to the time taken for induction of the biosynthetic system by the fungus spore suspension.

Sodium acetate-[2-^{14}C] *as a capsidiol precursor*

Sodium acetate-[2-^{14}C] introduced in the same manner as MVAL-[2-^{14}C] (at 0 and 6 hr respectively) showed incorporation levels of 2–3% into ether extracts.

Table 1. Distribution of ^{14}C after inoculation of peppers with fungal spores followed by incubation with MVAL-[2-^{14}C]

No. of peppers	% ^{14}C incorporated: Ether extract	% ^{14}C incorporated: Aqueous diffusate	Wt of crude capsidiol* (mg)	Capsidiol sp. act. after recrystallization (dpm/mg)
				$\times 10^{-3}$
23	3.3	43.4	10	47
66	2.7	46.3	37	46

* As determined by GLC of ether extracts at 150° on 1% SE-30.

Table 2. Effect of time of administration on incorporation of MVAL-[2-^{14}C] into peppers inoculated with fungus

No. of peppers per treatment	Time of administration* of MVAL-[2-^{14}C] (hr)	Wt of capsidiol in Et_2O extract (mg)	%^{14}C in Et_2O extract	%^{14}C in capsidiol†
18‡	0	3.5	2.2	1.4
18‡	6	3.3	1.7	1.2
18‡	18	2.7	1.1	0.7
10‡	−6	3.5	2.8	2.0
10‡	0	4.4	2.1	1.5
10§	−6	7.3	2.8	2.0
10§	0	6.4	2.8	1.8

* Hours after fungus inoculation. † Crude capsidiol after PLC. ‡ 2 day incubation. § 3 day incubation.

However, PLC of these extracts indicated 4 major peaks of radioactivity and the isolated capsidiol accounted for less than 1% of ^{14}C incorporated. Further experiments showed that introduction of acetate 18 hr after fungus inoculation favoured incorporation into capsidiol (Table 3) at the expense of other components of the ether extract. Furthermore, increasing the amount of administered sodium acetate-[2-^{14}C] resulted in correspondingly increased incorporation into capsidiol (not apparent when expressed as *per cent* incorporation). This is of particular significance in relation to the incubation with acetate-[1,2-$^{13}C_2$] in which degree of enrichment of capsidiol is more important than the *per cent* incorporation.

Sodium acetate-[1,2-$^{13}C_2$] as a capsidiol precursor

Following successful incorporation studies with radioactive acetate, experiments were conducted with sodium acetate-[1,2-$^{13}C_2$] (>90 atom % ^{13}C). Initially, two treatments—1 or 2 mg of sodium acetate-[1,2-$^{13}C_2$] per pepper 18 hr after fungal inoculation—were examined. Capsidiol, isolated from diffusates and purified by PLC as described previously, was examined by GC–MS. The abundance of the $[M + 2]^{+\cdot}$ ion (*m/e* 238) indicated that *ca* 8% of the molecules were doubly labelled. No differences were observed between capsidiol produced from 1 or 2 mg of injected acetate.

As large quantities (*ca* 50 mg) of capsidiol were required for ^{13}C NMR spectrometry, it was decided to conduct a further experiment and to isolate the total capsidiol from both diffusates and homogenates of whole peppers. The homogenates and diffusates from peppers inoculated with sodium acetate-[1,2-$^{13}C_2$] (1 mg per pepper, 18 hr after fungus infection), and incubated for 2 or 3 days at room temp, were extracted with Et_2O.

Although capsidiol had previously been purified by PLC, quantitative recovery was not always achieved; having regard to this, and to the large amount of crude material recovered from combined homogenates and diffusates, it was necessary to use an alternative capsidiol purification procedure. Gel-filtration on LH-20-50% Nedox 1114 with 5% isopropanol in toluene as eluent resulted in the elution of crude capsidiol at approximately 130 SEV units [31]. Reversed phase chromatography on the same gel, with methanol:heptane (9:1) afforded almost pure capsidiol, in good yield. The capsidiol was finally separated from a yellow contaminant by vacuum sublimation.

GC–MS of this ^{13}C-^{13}C enriched capsidiol indicated that *ca* 5% of the molecules were doubly labelled. A total of 51 mg of ^{13}C-^{13}C enriched capsidiol from 103 peppers was examined by ^{13}C NMR spectrometry, in comparison with unlabelled capsidiol (62 mg). Relevant data are shown in Figs 1–3, and Table 4. The measured ^{13}C NMR chemical shifts for capsidiol agreed closely with those previously reported [32]. The satellites observed were due to ^{13}C-^{13}C coupling at C-1/C-2; C-4/C-14; C-5/C-6;

Table 3. Effect of time of administration on incorporation of sodium acetate-[2-^{14}C] into peppers inoculated with fungus

No. of peppers per treatment	Time of administration* of acetate-[2-^{14}C] (hr)	%^{14}C in Et_2O extract	Wt of capsidiol in Et_2O extract (mg)	Dpm in Et_2O extract ($\times 10^{-4}$)	Dpm in capsidiol† ($\times 10^{-4}$)
15‡	−6	2.0	5.5	57.3	13.3
15‡	0	2.0	5.6	57.1	18.9
10‡	0	2.5	4.0	50.9	16.1
10‡	6	2.2	4.1	45.1	17.3
10‡	18	1.9	3.6	39.0	19.6
10§	0	1.2	3.3	70.5	28.5
10§	6	2.0	3.2	119.0	37.2
10§	18	2.9	3.9	177.5	80.1

* Hours after fungus inoculation. † Crude capsidiol after PLC. ‡ 2 day incubation, 1.0 μCi per pepper. § 2 day incubation, 3.0 μCi per pepper.

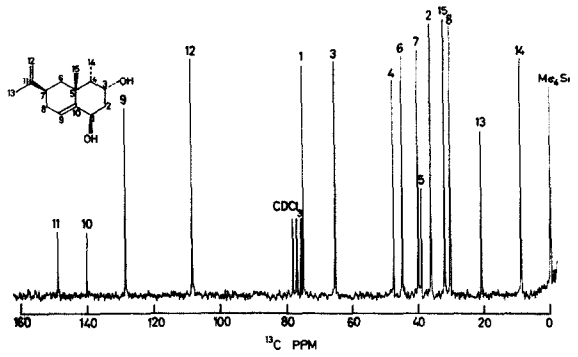


Fig. 1. Natural abundance ^{13}C NMR spectrum of capsidiol (62 mg) in $CDCl_3$:140710 accumulations.

C-7/C-8 and C-11/C-12. The absence of significant coupling between C-5 and C-15 indicated that these atoms originated from separate acetate units, as expected from the postulated migration of a methyl group from C-10 to C-5. This result, together with the strong coupling between C-5 and C-6, shows that no appreciable part of the isolated capsidiol was formed via spiro-rearrangement.

The ^{13}C NMR data provided some further information concerning the biogenesis of capsidiol:

(i) Coupling of C-11 to C-12 (but not to C-13) indicated that the identity of the carbon atom derived from C-2 of mevalonolactone is maintained at the terminal position (C-13). This is in harmony with the postulated

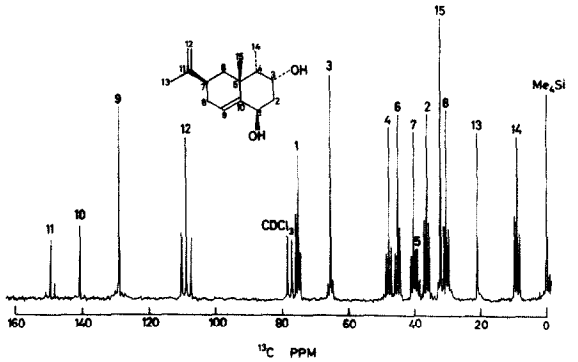


Fig. 2. ^{13}C NMR spectrum of ^{13}C-^{13}C enriched capsidiol (51 mg) in $CDCl_3$:388346 accumulations.

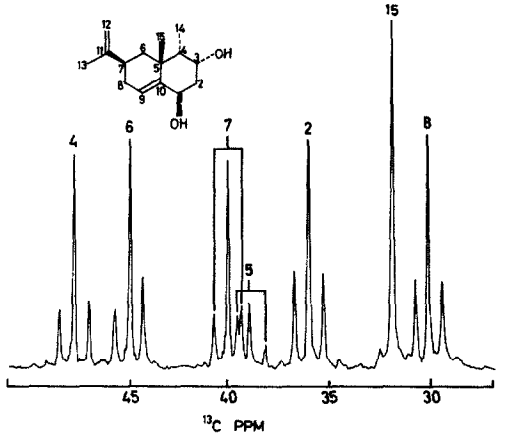
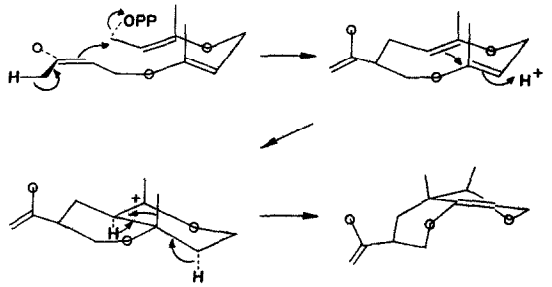


Fig. 3. Expanded portion of Fig. 2. showing satellites of C-5 and C-6.

mode of cyclisation of farnesyl pyrophosphate [e.g. to β-germacrene [20,21] Scheme 2].

(ii) Similarly, C-9 appeared to correspond wholly to C-2 of mevalonolactone; there was no indication of coupling to neighbouring atoms.

(iii) The remaining atom (C-3) of capsidiol that would be expected to originate from C-2 of mevalonolactone gave rise essentially to a singlet, but a minor pair of satellite signals was clearly visible. The question whether these denote coupling to C-2 or C-4 is presently under investigation (application of homonuclear decoupling [33] should be informative). C-3/C-4 coupling might imply the intermediacy of an "abnormal" isopentenyl pyrophosphate unit in which part of the terminal olefinic



Scheme 2. Postulated route for the conversion of farnesyl pyrophosphate to eremophilene. Small circles indicate carbon atoms derived from C-2 of mevalonate.

Table 4. ^{13}C Shieldings* and coupling constants of ^{13}C-^{13}C enriched capsidiol

Position	δC (ppm)	Position	δC (ppm)	J (^{13}C-^{13}C) (Hz)
1	75.1	2	36.3	35 ± 1
3	65.4	(2 or 4)	—	36
4	47.7	14	9.0	36
5	39.2	6	45.0	35
7	40.2	8	30.4	33
9	129.0			(singlet)
10	140.5			(singlet)
11	149.4	12	108.8	72
13	21.0			(singlet)
15	32.2			(singlet)

* Determined for $CDCl_3$ soln: tetramethylsilane internal reference.

carbon is derived from C-3′ of mevalonate. Such an apparent "exchange" of carbon between the methyl and methylene groups has been previously noted in both monoterpenoid [34] and sesquiterpenoid [11] biosynthesis, and the possible existence of a *cis*-isopentenyl pyrophosphate isomerase has been considered [11]. It may be mentioned that the incorporation of mevalonic acid-[2-^{14}C] into the eremophilanoid compound, isopetasol, led to one-third of the activity being present at C-3; however, the levels of radioactivity in that instance were such that a minor discrepancy might have escaped detection [26].

Salient features of this work have been briefly reported [35].

EXPERIMENTAL

Plant material. Capsicum fruits were the cultivars Propa, Bell Boy and New Ace of *Capsicum frutescens* L. and were obtained from the West of Scotland College of Agriculture at Auchincruive, Ayrshire.

Fungus spore-suspensions. Monilinia fructicola (Wint.) Honey was grown on 2% Oxoid agar No. 3 containing 2% of malt extract. Cultures of the fungus 11-30 days old showing a dark green covering of spore-forming bodies were used to prepare spore suspensions. 5–6 ml of the spore suspension containing approximately 10^6 spores per ml, were inoculated into each pepper by means of an automatic dispensing syringe. Injection was made just below the shoulder of the fruit, with the needle applied at an angle so that the suspension flowed directly into the cavity below.

Chemicals. RS-MVAL-[2-^{14}C] (sp. act. 138 μCi per mg) and sodium acetate-[2-^{14}C] (sp. act. 714 μCi per mg) were obtained from the Radiochemical Centre, Amersham. Sodium acetate-[1,2-$^{13}C_2$] (C-1, 91.9 and C-2, 90.4 atom *per cent* respectively) was obtained from Prochem, BOC Ltd. Solvents were of Analar grade, or were redistilled before use.

Incubation. According to the experiment, 0.5 μCi MVAL-[2-^{14}C], 1.0 or 3.0 μCi sodium acetate-[2-^{14}C], or 1 or 2 mg of sodium acetate-[1,2-$^{13}C_2$] per 0.5 ml H_2O were injected into each pepper via a hypodermic syringe. Peppers were incubated at room temp. in plastic trays for 2 or 3 days from the time of fungus inoculation; the time of precursor injection was varied, but the optimum for MVAL was found to be 6 hr prior to fungus inoculation and for acetate 18 hr after fungus inoculation.

Extraction of lipid material. Peppers were cut open and the diffusates and washings transferred to a separating funnel by means of a simple suction device. Et_2O-soluble material was isolated from these diffusates by 4 extractions with half-vols of Et_2O, each extract being washed in turn with the same 2 small portions of H_2O. The extracts were combined, dried (Na_2SO_4), filtered and evaporated to dryness. Whole fruits were extracted with three half volumes of Et_2O after removal of stalk, seeds, placenta and calyx, and maceration with ice-water in a Waring blender. Fleshy material which tended to cause emulsion formation was removed by filtration through glass wool after the first extraction.

Chromatography and purification of capsidiol. PLC (0.5 mm layer) was carried out using Si gel Camag DF5, with *t*-BuOH–EtOAc–HOAc (5:95:0.1) as the mobile phase. Plates were sprayed with 5% phosphomolybdic acid in EtOH followed by brief heating at 110°. Gel chromatography was carried out on the modified dextran gel N.1114-50%-LH-20 [31]. *Iso*-PrOH (5%) in toluene, and heptane (10%) in MeOH were used as liquid phases for straight-phase and reversed-phase partition chromatography, respectively. Capsidiol was further purified by recrystallisation from Et_2O or Et_2O–petrol as colourless, lustrous needles, mp 152–154°. Samples of capsidiol containing traces of yellow pigment were effectively purified by vac. sublimation at 60° and 0.05 Torr on to a cold-finger.

Methods of analysis and characterisation. GLC was carried out at 150° in an instrument fitted with dual FID and two 6-ft glass columns (3 mm i.d.) packed with 1% SE-30 and 1% QF-1 respectively. Flow rate of carrier gas (N_2) was 25 ml per min. GC–MS was carried out with a 10-ft glass column packed with 1% OV-1: the electron energy was normally 22.5 eV. GC–RC was carried out on a Pye/Panax instrument fitted with a 9-ft glass column (4 mm i.d.) packed with 1% SE-30. Radioactive components separated by PLC were monitored with a TLC radiochromatogram scanner. Radioactivity was measured by liquid scintillation counting, the fluor contained 4 g PPO and 0.2 g of dimethyl POPOP per l. of toluene. Scintillation counting of aq. sol was conducted using a mixture comprising sample (0.1 ml), MeOH (2.5 ml) and fluor (12.5 ml).

^{13}C NMR spectrometry. 51 mg of ^{13}C-^{13}C enriched capsidiol and 62 mg of 'natural' capsidiol were dissolved separately in 1 ml $CDCl_3$ in 10 mm sample tubes. ^{13}C NMR spectra were obtained with a Varian XL-100-12 NMR spectrometer fitted with a Varian XL-100 Fourier Transform accessory. The sweep width for full scans in Figs. 1 and 2 was 5120 Hz, and for the expanded scale scan (Fig. 3) 717.5 Hz (1.25 Hz per data point in each instance). The spectra of the natural abundance and ^{13}C-enriched capsidiol were obtained from 140,710 and 388,346 accumulations respectively.

Acknowledgements—We thank Dr. S. A. Hutchinson of the Botany Department for advice and for providing fungal cultures, Drs. D. S. Rycroft and D. D. MacNicol for ^{13}C NMR measurements and advice, Dr. A. Stoessl and his colleagues for support and for a sample of capsidiol, and Mrs. Juliet Borthwick for GC–MS runs. We would also like to thank Mr. R. E. Johnston and his colleagues at the West of Scotland College of Agriculture, Auchincruive for providing us with capsicum fruits. The LKB 9000 gas chromatograph-mass spectrometer was provided by S.R.C. grants No. B/SR/2398 and B/SR/8471, and research funds by grant No. B/RG/5986.7.

Note added in proof. It has just come to our attention that the preferred name for the annual sweet pepper used here and in previous references [19,35] is not *C. frutescens* but *C. annuum* L.

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