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THE BIOSYNTHESIS OF SESQUITERPENOID PHYTOALEXINS IN SUSPENDED CALLUS CULTURES OF *NICOTIANA TABACUM**

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; elicitation; biosynthesis; callus cultures; ^{13}C-labelling; ^{13}C NMR; phytoalexin; debneyol.

Abstract—Suspended callus cultures of *Nicotiana tabacum* treated with commercial cellulase (*ex Trichoderma viride*) incorporated sodium [1, 2-$^{13}C_2$]acetate to give a ^{13}C-enrichment of *ca* 1.6% of the carbon content of debneyol. ^{13}C NMR data indicated that the angular methyl group in debneyol arises by migration from the C-10 position of a eudesmane-type intermediate. ^{13}C enrichments of 0.8% and 1.3% respectively were observed in phytuberol and phytuberin isolated from the cultures.

INTRODUCTION

We have previously reported the elicitation of sesquiterpenoid phytoalexins in suspended callus cultures of *Nicotiana tabacum* L. by a commercial preparation of cellulase *ex Trichoderma viride* [1]. One of the four principal metabolites was the vicinal diol debneyol (1) which has the same carbon skeleton as capsidiol—a major terpenoid component elicited in the tobacco cultures. In earlier work it was demonstrated, via incorporation of sodium [1, 2-$^{13}C_2$]-acetate, that the angular methyl group of capsidiol arises by methyl migration from the C-10 position of a eudesmane-type intermediate [2, 3]. We now report a similar result obtained when the biosynthesis of debneyol is effected with incorporation of sodium [1, 2-$^{13}C_2$]acetate.

RESULTS AND DISCUSSION

In our earlier work [1], we observed incorporation of sodium [2-^{14}C]acetate into debneyol at *ca* 2.4%. Incorporation was maximal if acetate was added *ca* 12 hr after the culture had been treated with cellulase. This procedure was repeated, sodium [1, 2-$^{13}C_2$]acetate (90 atom % $^{13}C_2$) being added at the level of 1 mg per flask of 100 ml of culture. The combined media from 80 such flasks yielded, after extraction and purification, 10 mg of debneyol. The intensity of the satellites in the ^{13}C NMR spectrum indicated ^{13}C enrichment of *ca* 1.6% of the carbon content of the molecule. The ^{13}C NMR spectrum of the doubly labelled debneyol contained several overlapping lines, and the editing capability of DEPT experiments [4] was particularly useful for resolving these. Figure 1 shows the low-frequency region in the DEPT spectrum of ^{13}C-enriched debneyol, and Table 1 lists the measured ^{13}C shieldings and coupling constants: these data agree with those we reported previously [1]. The positions of the intact acetate-derived units are indicated in structure 2. Only five intact acetate units survive (as in capsidiol [3]) and the absence of coupling between C-5 and C-15 indicates that these atoms originated from

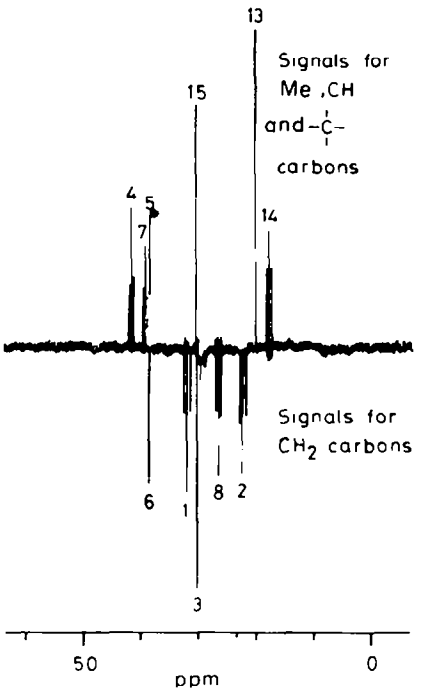

Fig. 1. The complex low frequency region of the DEPT spectrum (θ = 135°) of ^{13}C-enriched debneyol (20 mg/ml in $CDCl_3$).* The signal for the quaternary carbon 5 is from the proton-decoupled spectrum.

*Part 4 in the series 'Elicitation of Terpenoid Stress Metabolites'. For Part 3 see Brooks, C. J. W., Watson, D. G. and Freer, I. M. (1986) *Phytochemistry* **25**, 1089.

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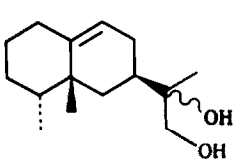

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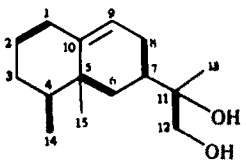

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Table 1. ^{13}C Shieldings and coupling constants of ^{13}C-enriched debneyol

C	δ_C (ppm)	C	δ_C (ppm)	$J(^{13}C-^{13}C)$ (Hz)
1	31.9	2	22.4	32.2
3	30.2*			
4	41.5	14	17.7	35.2
5	38.6	6	38.4	33
7	39.2	8	26.4	33.4
9	120.0*			
10	141.6*			
11	74.7	12	68.5	40.3
13	20.0*			
15	30.3*			

*Enhanced singlet.

separate acetate units. There was strong coupling between C-5 and C-6. These observations are consistent most simply with the occurrence of methyl migration in the biosynthesis of debneyol: however, the possibility of more complex routes, involving rearrangements via spiro intermediates, is not excluded. [It is noteworthy that we have observed the elicitation, in capsicum fruits treated with $CuSO_4$, of small amounts of the spiro compound solavetivone, accompanying the major eremophilanoid phytoalexin, capsidiol. The isolated compound matched authentic solavetivone in its retention index (I = 1787 on SE-54 phase at 125°), mass spectrum, and ^{13}C-NMR spectrum.]

The following additional points arise from the ^{13}C NMR data recorded for debneyol. (i) The coupling of C-11 to C-12 (but not C-13) indicates that in this side chain C-2 of mevalonate appears exclusively at C-13, as has been observed previously for other sesquiterpenoid phytoalexins from the Solanaceae [3, 5]. (ii) C-9 does show small satellites (1:16) from coupling to C-10; the coupling constant of 70.7 Hz is characteristic of two carbons involved in a double bond. Since C-9 also exhibits small (1:16) satellites from coupling to C-8 (J = 41.4 Hz) it is almost certain that this is due to over-enrichment rather than to the intermediacy of an abnormal isopentenyl pyrophosphate unit. (iii) The labelled carbons at C-5 and C-6 form an AB system in which the ratio of outer to inner lines is *ca* 1:15 and the latter are almost completely overlapping. It was possible to measure the coupling constant of 33 Hz from the C-6 signal in the DEPT spectrum, where there was no overlap with the C-5 signal.

Phytuberin and phytuberol, ^{13}C-enriched by 1.2% and 0.8% of the carbon content, respectively, were also isolated from the cellulase-treated *N. tabacum* cultures. These compounds have been previously isolated from *N. tabacum* cultures treated with *Pseudomonas* bacteria [6, 7]. The ^{13}C NMR spectra of the labelled compounds revealed five intact acetate units, as in ref. [5]. In addition, the acetate unit in phytuberin was doubly labelled, to a similar degree of enrichment as the rest of the molecule, exhibiting signals at 22.4 and 170.4 ppm with a coupling of 50 Hz. Similar incorporations and identical J values were observed for the 3-acetoxy group in the eremophilanoid PR-toxin, biosynthesised in the presence of [1, 2-$^{13}C_2$]acetate [8, 9]. It was previously noted [5] in the feeding of ^{13}C-acetate to fungally infected potato slices that phytuberin and phytuberol were labelled to a lesser degree than eight other sesquiterpenoid phytoalexins formed at the same time. In the present work, the incorporation of ^{13}C into phytuberin and phytuberol was also less than that into debneyol. It was considered in respect of the observations on potato that the lower level of labelling might reflect a separately-controlled biosynthetic pathway to phytuberin and phytuberol. There are a number of instances in tobacco and potato where these compounds accumulate as a result of some bacterial or fungal interactions but not of others [5–7; 10–12]. We have observed that phytuberin and phytuberol accumulate when tobacco cultures are treated with cellulase, but not when they are treated with a sterile extract from *Gliocladium deliquescens*; capsidiol and debneyol, however, accumulate in both instances. Further studies of the elicitation of sesquiterpenoids in *Nicotiana tabacum* cultures might provide insights into the mechanism of elicitor action.

EXPERIMENTAL

Plant tissue cultures. The *Nicotiana tabacum* cultures were maintained as described previously [1] and were treated, 3 weeks after transfer to liquid medium, with commercial cellulase (10 μg/ml = 2×10^{-4} units/ml). The culture was incubated for 72 hr after addition of cellulase, and sodium [1, 2-$^{13}C_2$]acetate was added to the culture 12 hr after the addition of cellulase. The tissue and medium were extracted separately with EtOAc and the extracts dried over $MgSO_4$.

Materials. Solvents were Analar except for HPLC solvents (Rathburn Chemicals). Sodium [1, 2-$^{13}C_2$]acetate (90 atom % ^{13}C) was from M. S. D. Isotopes, Lipidex 5000 from Packard, and cellulase (*ex T. viride*) from BDH.

Column liquid chromatography. The extract from the culture medium was applied to a Lipidex 5000 column (40 × 1 cm) and eluted as described previously. Debneyol was further purified as described previously [1]. Phytuberin and phytuberol were eluted together in fractions 4–9 (15 ml fractions). Attempts to purify these compounds by reverse-phase HPLC in MeOH–H_2O mixtures resulted in their decomposition. The phytuberin/phytuberol mixture was applied to Silica gel G TLC plates (0.1 mm layer) which were developed with cyclohexane–EtOAc (1:1). Two cm bands were scraped from the plates to include the spots at R_f 0.30 and 0.57 corresponding to phytuberol and phytuberin respectively. Final purification was by vacuum sublimation (0.01 torr, 80°) on to a cold finger cooled by solid CO_2–Me_2CO.

NMR spectra. Solutions in $CDCl_3$ were used, spectra being recorded on a Bruker WP200SY instrument: the $CDCl_3$ carbon signal at δ77.0 ppm served as an int. standard.

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REFERENCES

1. Watson, D. G., Rycroft, D. S., Freer, I. M. and Brooks, C. J. W. (1985) *Phytochemistry* **24**, 2195.
2. Baker, F. C., Brooks, C. J. W. and Hutchinson, S. A. (1975) *J. Chem. Soc., Chem. Commun.* 293.
3. Baker, F. C. and Brooks, C. J. W. (1976) *Phytochemistry* **15**, 689.
4. Doddrell, D. M., Pegg, D. T. and Bendall, M. P. (1982) *J. Magn. Reson.* **48**, 323.
5. Stoessl, A., Stothers, J. B. and Ward, E. W. B. (1978) *Can. J. Chem.* **56**, 645.
6. Fujimori, T., Tanaka, H. and Kato, K. (1983) *Phytochemistry* **22**, 1038.
7. Tanaka, H. and Fujimori, T. (1985) *Phytochemistry* **24**, 1193.
8. Moreau, S., Lablache-Combier, A. and Biguet, J. (1981) *Phytochemistry* **20**, 2339.
9. Chalmers, A. A., de Jesus, A. E., Gorst-Allman, C. P. and Steyn, P. S. (1981) *J. Chem. Soc., Perkin Trans. 1* 2899.
10. Budde, A. D. and Helgeson, J. P. (1981) *Phytopathology* **71**, 206.
11. Budde, A. D. and Helgeson, J. P. (1981) *Phytopathology* **71**, 864.
12. Stoessl, A. (1982) *Physiol. Plant Pathol.* **20**, 263.