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# SESQUITERPENOID PHYTOALEXINS FROM SUSPENDED CALLUS CULTURES OF *NICOTIANA TABACUM**

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**Key Word Index**—*Nicotiana tabacum*; Solanaceae; tobacco; elicitation; callus cultures; structure; $^{13}$CNMR; GC/MS; sesquiterpenoids; cyclic derivatives.

**Abstract**—Treatment of suspended callus cultures of *Nicotiana tabacum* with commercial cellulase elicited four principal stress metabolites including the phytoalexin capsidiol and a second eremophilane-type diol, shown on the basis of chemical and spectroscopic evidence to be 4-epieremophil-9-ene-11ξ, 12-diol (without assignment of absolute configuration). This diol appears to be structurally identical with debneyol, isolated from *N. debneyi* (see accompanying paper). Among minor metabolites were an isomer and a dehydro-analogue of the diol. GC/MS of cyclic derivatives (boronates and di-*t*-butylsilylene derivatives) of vicinal diols was useful for their detection and characterisation. The remaining two major metabolites appeared to be phytuberol and phytuberin.

## INTRODUCTION

Plant tissue cultures are proving to be of increasing value as systems for the study of phytoalexin elicitation. Extensive investigations have been made of the induction of isoflavonoid phytoalexins in tissue cultures of *Phaseolus vulgaris* and *Glycine max* [1–4], and the induction of flavonoid phytoalexins in *Petroselenium hortense* [5, 6]. Elicitation of terpenoid phytoalexins has been reported in tissue cultures of *Ipomoea batatas* [7], *Solanum tuberosum* [8, 9], *Nicotiana tabacum* [10, 11] and *Capsicum annuum* [12] but neither the biochemistry nor the enzymology of these systems has yet been studied in depth.

The production of four sesquiterpenoid phytoalexins by *N. tabacum* callus in response to spores of *Phytophthora parasitica* was briefly reported in abstracts by Budde and Helgeson [10, 11]: these products were stated to be capsidiol, rishitin, epirishitin, and a diol ($M_r$ 238) considered to be an eremophilene-11,12-diol. No further details have been published, although Helgeson has contributed a general review of his work on the response of *Nicotiana* cultures to *P. parasitica* [13]. We now report the elicitation of several stress metabolites in suspended callus cultures of *N. tabacum* in response to treatment with commercial cellulase (from *Trichoderma viride*). Two of the four major metabolites have been characterised as capsidiol (**1**) and a vicinal diol which was shown on the basis of evidence presented below to have structure **2**. The latter diol has further been identified (with the possible exception of absolute configuration) with debneyol, isolated from *N. debneyi* by Burden *et al.* [14]. It seems probable that the same diol was isolated in the previous work by Budde and Helgeson [10] and in the studies by Fuchs *et al.* [15] on metabolites in tobacco leaves inoculated with tobacco mosaic virus.

## RESULTS AND DISCUSSION

Gas chromatography was used to analyse an extract which was obtained from the culture medium of cellulase-treated *N. tabacum* suspended callus cultures, then partially purified by vacuum sublimation, and treated to effect trimethylsilylation of reactive functional groups. Retention index ($R_I$) values and salient mass spectrometric data for the four main peaks are given in Table 1. Components (a) and (c) appear to be phytuberol and phytuberin respectively, on the basis of their mass spectra [16], but have yet to be compared directly with authentic samples. Component (b) is evidently the TMSi ether of (a): its mass spectrum shows the great preponderance of the ion of *m/z* 131 $[Me_2C{=}OSiMe_3]^+$ that is characteristic of the dimethylcarbinol TMSi ether grouping [17], and corresponds to the ion of *m/z* 59 present in the mass spectrum of the free alcohol.‡

Capsidiol was isolated from the mixture by column chromatography on Lipidex 5000, followed by HPLC. It was identified by comparison of mass spectra and $R_I$ data (on two capillary columns) with those of authentic material [18]. For example, $R_I$ values on the SE-54 phase for the di-TMSi ether, di-TBDMS ether and diacetate were respectively 1915 ± 1 (at 135°), 2401 ± 1 (at 170°) and 2112 ± 1 (at 150°). The proportion of capsidiol relative to diol **2** in the extracts varied from 20% to 50%.

Diol **2** was eluted from the Lipidex 5000 column before capsidiol, and was characterised by GC and GC/MS of the free diol, as well as its mono- and disilylated derivatives (TMSi and TBDMS ethers: Table 2). The mass spectra of these compounds showed no significant molecular ions, but, as expected [19], the diol readily afforded cyclic boronate esters (**3a**, **3b**) which yielded prominent molecular ions consistent with the composition $C_{15}H_{26}O_2$ for

*Part 2 in the series "Elicitation of terpenoid stress metabolites". For Part 1: see Watson, D. G. and Brooks, C. J. W. (1984) *Physiol. Plant Pathol.* **24**, 331.

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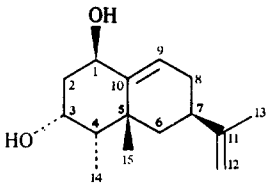
‡See note at end of text.



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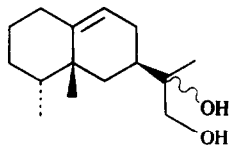

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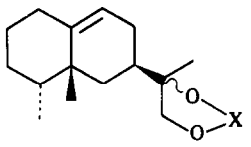
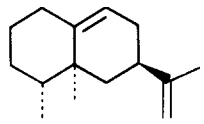


3a* X = BMe
3b X = BPh
3c X = Si$^t$Bu$_2$



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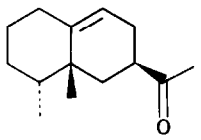

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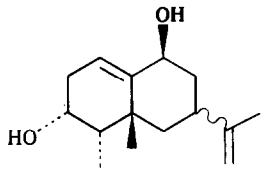

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* Absolute configurations are not yet confirmed

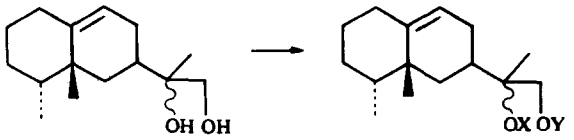
Table 1. Salient GC/MS data for trimethylsilylated extract from the culture medium of cellulase-treated *N. tabacum*

| Peak in GC | $R_I$ (SE-54) 135° | Mass spectrometric data (20 eV) (*m/z*) with relative abundance (%) in parentheses | | | | |
|---|---|---|---|---|---|---|
| | | M$^+$ | Base peak (above *m/z* 75) | Other major ions | | |
| (a) | 1731 | 252 (39) | 205 | 223 (30) | 149 (88) | 59 (66) |
| (b) TMSi ether of (a) | | 324 (< 1) | 131 | 266 (3) | 205 (4) | — |
| (c) | 1841 | 294 | 205 | 234 (32) | 189 (48) | 149 (45) |
| Capsidiol di-TMSi ether | 1915 | 380 (9) | 290 | 249 (14) | 222 (22) | 157 (8.5) |
| Diol **2** mono-TMSi ether | 1930 | (310) (−) | 162 | 292 (26) | 207 (32) | 189 (60) |

the free diol. It was also possible, by using Corey's reagent [20] to prepare the di-*t*-butylsilylene (DTBS) [21] derivative (**3c**) (M$^+$, *m/z* 378). The $^{13}$C NMR data for the diol (Table 3) confirmed the formula $C_{15}H_{26}O_2$ and indicated the presence of a trisubstituted double bond in a bicarbocyclic structure. The degree of substitution of the carbon atoms was determined from DEPT experiments [22]. The observed $^{13}$C chemical shifts for C-1, C-3 to C-5, C-9, and C-10, compared with those for capsidiol and for the 4α,5α-dimethyl compound (**4**) [18] were consonant with a 4-epieremophilane skeleton for the diol (see further below).

The $^1$H NMR spectrum of diol **2** showed an AB quartet ($\delta$ 3.40 and 3.57, $J$ = 10.9 Hz) attributable to an isolated $CH_2$ group adjacent to a hydroxyl group, as well as a single olefinic proton resonance at $\delta$ 5.47 (*dt*, $J$ = 6.3 and 1.8 Hz). Signals due to the protons of three methyl groups occurred at $\delta$ 1.09 (*s*), 1.16 (*s*) and 0.96 (*d*, $J$ = 7.1 Hz). The rest of the $^1$H NMR spectrum was complex, but oxidative cleavage of the vicinal diol grouping yielded the nor-

Table 2. GC/MS data for silyl ethers of diol 2




| Derivative | X | Y | $R_I$* OV-1 | $[M]^+$ (*m/z*) | $[M-XOH]^+$ (*m/z*) (%) | | Base peak (*m/z*) |
|---|---|---|---|---|---|---|---|
| Mono-TMSi | H | $SiMe_3$ | 1891 | (310) | 292 | 26 | 162 |
| Mono-TBDMS | H | $SiMe_2^tBu$ | 2130 | (352) | 334 | 25 | 162 |
| Di-TMSi | | $SiMe_3$ | 2065 | (382) | 292 | 27 | 279 |
| Di-TBDMS | | $SiMe_2^tBu$ | 2503 | (466) | 334 | 17 | 321 |

*Recorded on a 25 m × 0.32 mm fused silica column coated with OV-1: base peaks in the mass spectra (20 eV electron impact) were assigned from the ions above *m/z* 80.

Table 3. $^{13}C$ NMR data for diol 2, capsidiol, and aristolochene

| C | Diol 2 $\delta$* | Capsidiol (1) $\delta$ [18] | Aristolochene (4) $\delta$ [18] |
|---|---|---|---|
| 1 | 31.9 | 75.0 | 32.6 |
| 2 | 22.4 | 36.3 | 27.9 |
| 3 | 30.2 | 65.3 | 31.2 |
| 4 | 41.5 | 47.7 | 44.1 |
| 5 | 38.6 | 39.1 | 38.7 |
| 6 | 38.4 | 44.9 | 43.3 |
| 7 | 39.2 | 40.2 | 37.7 |
| 8 | 26.4 | 30.4 | 31.3 |
| 9 | 120.0 | 128.8 | 122.9 |
| 10 | 141.6 | 140.3 | 144.3 |
| 11 | 74.7 | 149.1 | 150.3 |
| 12 | 68.5 | 108.7 | 108.2 |
| 13 | 20.0 | 21.0 | 20.9 |
| 14 | 17.7 | 8.9 | 15.7 |
| 15 | 30.3 | 32.1 | 18.1 |

*Internal reference $CDCl_3$ at $\delta$77.0.

ketone (5) [$R_I$ = 1600 (SE-54, 110°); $M^+$ = 206] which afforded a more easily interpretable $^1H$ NMR spectrum. Methyl signals appeared at $\delta$1.17 (*s*), 2.16 (*s*) and 0.98 (*d*, *J* = 7.3 Hz) and an olefinic proton resonance at $\delta$5.51 (*dt*, *J* = 6.3 and 2.0 Hz). The signal at $\delta$2.67 (H-7) in (5) was extensively split (*dddd*) due to couplings of 13, 11, 5 and 4 Hz, and therefore axially oriented and flanked by methylene groups. Decoupling of this proton resonance produced a number of changes in the spectrum. A signal for one proton at $\delta$1.81 collapsed to a doublet of doublets (*J* = 14.0 and 2.5 Hz) upon removal of a 3.5 Hz coupling: this signal was due to H-6e, which also exhibited a geminal coupling* to H-6a (*J* = 14 Hz) and a long-range coupling to H-8e (*J* = 2.5 Hz) which was similar to that previously observed between H-6e and H-8e in compound (6) isolated from $CuSO_4$-treated capsicums [23]. The signal for H-6a, centred at $\delta$1.41, collapsed to a doublet (*J* = 14 Hz) upon irradiation of H-7 and consequent removal of a 13 Hz coupling. Decoupling of H-7 also affected two groups of complex signals in the region of $\delta$2.1 and 1.9. The same signals were affected by decoupling of the olefinic proton, and consequently were assigned to the two H-8 protons in a $\Delta^9$ rather than a $\Delta^{1(10)}$ structure. These data indicated that the diol had the eremophilane structure 2 except that the configurations at C-4, C-5, C-7 and C-11 had not been assigned. Nuclear Overhauser effect (NOE) difference experiments with the nor-ketone 5 were helpful: irradiation of the secondary methyl protons enhanced the resonance of H-7, and *vice versa* (the measured NOE enhancements were *ca* 2% and 4% respectively). Methyl-14 and H-7 were thus indicated to be close in space, and this is possible only if methyl-14 is $\alpha$, methyl-15 is $\beta$, and H-7 is $\alpha$ in 5 and hence also in 2. The configuration at C-11 has not yet been determined.

The $^{13}C$ NMR data (Table 3) support the above conclusions. The resonances were assigned by comparison with the data of Birnbaum *et al.* [18] for aristolochene (4) and capsidiol (1), and other data for related compounds [23]. The methyl carbon resonance at $\delta$30.3 (C-15) is consistent only with a *trans*-diaxial relationship of the methyl groups at C-4 and C-5. The resonances of carbons 2, 6 and 8 showed the expected increase in shielding on going from 4 (methyl-14 equatorial, isopropenyl substituent at C-7) to 2 (methyl-14 axial, trisubstituted $sp^3$ carbon substituent at C-7).

Further evidence in support of structure 2 was obtained from NMR and mass spectrometric data for derivatives. The $^1H$ NMR spectrum of the benzeneboronate 3b showed the expected marked downfield shifts of signals for C-13 methyl (to $\delta$1.40) and for C-12 $CH_2$ (to $\delta$3.92 and 4.23). The base peaks in the mass spectra of the cyclic derivatives were ions retaining the three carbon atoms of the side chain. Ions at *m/z* 126 and *m/z* 168 from the methaneboronate and butaneboronate respectively were consistent with the expected boronate-containing fragments from retro-Diels–Alder cleavage of the cyclohexene

*These signals were more clearly seen in the 360 MHz spectrum recorded in Edinburgh.

ring. The presence of four exchangeable protons in the nor-ketone **5** was verified by treatment with KO$^t$Bu–$CD_3OD$ followed by GC/MS which yielded two chromatographic peaks having *ca* equal magnitude [$R_I$ = 1596, 1603 on SE-54, 110°] and giving similar mass spectra ($M^+$ = 210): the extra component is provisionally assumed to be the 7-epimer of **5**. In both spectra a major ion at *m/z* 164 $[M-CD_3CO]^+$ corresponded to that observed at *m/z* 163 in the parent ketone.

The selective formation of cyclic derivatives from the total extracts of tissue culture media (usually purified by sublimation to eliminate non-volatile materials) was particularly effective in revealing the presence of several additional vicinal diols related to diol **2**. Derivatives of two of these minor constituents (g) and (h) are indicated in Fig. 1, which shows a gas chromatogram of a sample of the extract that had been treated to convert suitable diols into cyclic DTBS derivatives. Peak (g)* corresponds to an isomer of diol **2**, and (h) to a dehydro-analogue: salient data were for (g), $R_I$ = 2294, $M^+$ = 368 and for (h), $R_I$ = 2299, $M^+$ = 366 (GC on OV-1 at 170°). Oxidation, of a mixture containing diol **2** together with these two diols, with lead tetra-acetate, and analysis by capillary GC/MS, gave two gas chromatographic peaks corresponding (by GC/MS) to nor-ketones [$M^+$ = 206 and 204]: a further scan by GC/MS, monitoring only the ions of *m/z* 206, revealed only one peak, suggesting that (g) might be the 11-epimer of **2**, yielding the same nor-ketone. With regard to the dehydro-analogue, it is of interest to note that an eremophila-1(10), 6-diene-11,12-diol has been isolated from a marine soft coral [24].

Correspondence of diol **2** with debneyol [14] was strongly indicated by comparisons of NMR spectra exchanged between the Bristol and Glasgow laboratories, and was confirmed—save for any evidence of absolute configuration—by direct experimental comparisons. We found that debneyol, supplied by Dr. Burden, and diol **2**, studied as methaneboronates and as benzeneboronates, had identical $R_I$ values (see Experimental) on OV-1 and SE-54 capillary columns, and that the mass spectra of the benzeneboronates were also identical.

Preliminary observations on biosynthetic aspects of the work have been made. The *de novo* synthesis of the four main sesquiterpenoid metabolites that were detectable by GC/MS after elicitation by cellulase was proved by addition of sodium[2-$^{14}$C]-acetate to *N. tabacum* cultures that were incubated for 48 hr: incorporation of label was maximal if labelled acetate was added *ca* 12 hr after treatment with cellulase. About 10% of the added radioactivity was recoverable in organic extracts of the combined tissue and culture medium. Analysis by GC–RC indicated maximal incorporation of *ca* 2.4% into diol **2**, and *ca* 0.4% into capsidiol, while for components (a) and (c) the incorporation was *ca* 2%. TLC–RC analysis revealed peaks of radioactivity in the extract from the culture medium at $R_f$ 0.28, 0.42 and 0.68 (mobile phase EtOAc), due respectively to capsidiol, diol **2** and a group of unresolved labelled compounds. There was very low background radioactivity in the TLC–RC scan of the extract from the medium, in contrast to that of the extract from the tissue, which showed peaks of radioactivity superimposed on a high background. The major part (*ca* 75%) of the labelled material that was extractable by organic solvents was recoverable from the culture medium.

In separate experiments, *N. tabacum* cultures were treated with a sterile aqueous extract from *Gliocladium deliquescens*, which had been found to act as a powerful elicitor of capsidiol in *C. annuum* fruits and tissue cultures [12, 23]. Analysis by GC/MS showed that a mixture of metabolites was produced that appeared to be markedly

*Symbols (d), (e) and (f) have been assigned to peaks observed in GC and GC/MS for the cyclic boronates of the same minor diols.

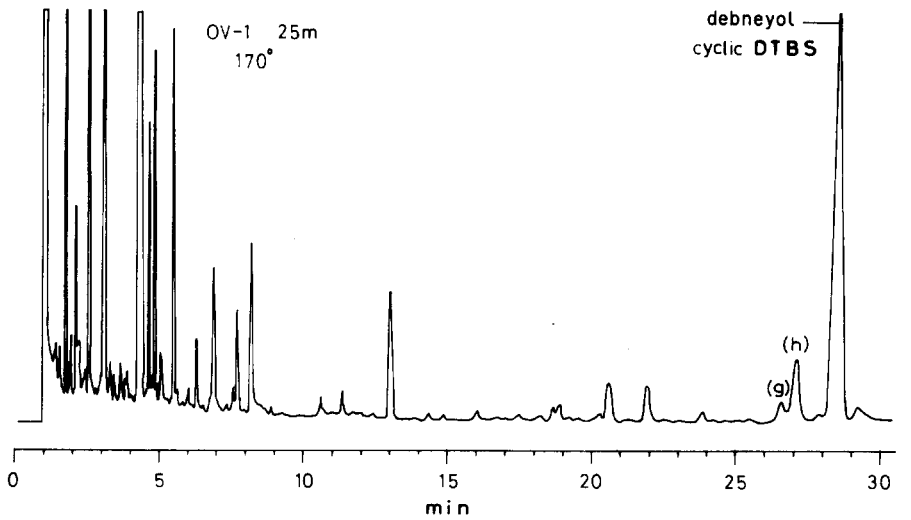



Fig. 1. Gas chromatography of di-*t*-butylsilylene derivatives. A gas chromatogram recorded for a sample of an extract (partially purified by vacuum sublimation) of tissue and culture medium of *N. tabacum* previously treated with cellulase: the sample was subjected to reaction with di-*t*-butylsilyl ditriflate in order to convert vicinal diols into DTBS derivatives. The 25 m column was as noted for Table 2.

different in composition from those produced by the action of cellulase: these await further investigation.

## EXPERIMENTAL

*Plant tissue cultures.* The *Nicotiana tabacum* L. cultures used had been established in Professor Overton's Plant Tissue Culture Unit for about 15 years: they were maintained on Murashige and Skoog's [26] medium with 0.2 mg/l kinetin and 1.0 mg/l IAA as growth hormones. In order to elicit phytoalexin accumulation, callus pieces were transferred to agitated liquid medium and allowed to grow for 3 weeks, then treated with cellulase (10 μg/ml $= 2 \times 10^{-4}$ units/ml) for 72 hr before extraction. The liquid medium was separated from tissue by filtration, and separate extractions of tissue and medium were effected with EtOAc (2 equal vols in each case): extracts were dried over $MgSO_4$.

*Materials.* Solvents were Analar except for HPLC solvents (Rathburn Chemicals). Petrol had bp range 40–60°. Cellulase (0.02 units/mg ex *Trichoderma viride*) was from BDH; N,O-bis(trimethylsilyl)trifluoroacetamide from Pierce; *t*-butyldimethylsilyl chloride from Lancaster Synthesis; and di-*t*-butylsilyl ditriflate from Aldrich. Sodium[2-$^{14}$C]-acetate (714 μCi/mg) was from the Radiochemical Centre, Amersham. $MeB(OH)_2$ was from Alfa; $PhB(OH)_2$ and $BuB(OH)_2$ were from Aldrich. Capsidiol was isolated from capsicum fruits treated with 0.1 M aq. $CuSO_4$ [23, 25].

*Methods of analysis.* Extracts from culture medium or tissue samples were sublimed at 0.01 torr (block temp. *ca* 80°) on to a cold finger cooled by solid $CO_2$–$Me_2CO$, and sublimates were dissolved in EtOAc. Packed-column GC (Perkin–Elmer F33 instrument) was done on a 6 ft × 3 mm i.d. glass column packed with 1% OV-1 and with $N_2$ as carrier gas (*ca* 25 ml/min) (FID). A Hewlett–Packard 5880A instrument was used for capillary GC, with 25 m × 0.32 mm i.d. fused silica WCOT columns, OV-1 and SE-54 (FID): He carrier gas (2 and 3 ml/min, respectively). GC/MS was done mainly on an LKB9000 instrument (EI, 20 eV) fitted with a 60 m × 0.32 mm i.d. fused silica bonded-phase DB-1 column (J & W Scientific): the He flow rate was *ca* 7 ml/min, with 25 ml/min as 'make-up' gas introduced between the column and the molecule separator. Samples were introduced by means of a falling-needle injector: solvent was purged for *ca* 2 min before injection. Temperatures were 190° for trimethylsilylated or methaneboronic acid-treated samples and 230° for samples treated with benzeneboronic acid, with *t*-butyldimethylsilyl chloride, or with the reagents required to make di-*t*-butylsilylene derivatives. GC–RC employed a Pye 104 instrument coupled to a Panax detector via a T-piece giving a 2:1 split ratio (FID: radiochemical detector). A Panax TLC–RC scanner was used for TLC plates. Total radioactivity in extracts was measured by scintillation counting; the fluor contained 4 g PPO and 0.2 g dimethyl POPOP per litre of toluene.

*Column liquid chromatography.* Typically, the combined extract from the medium and tissue derived from 20 flasks of cellulase-treated culture (tissue wet wt. *ca* 300 g) was applied to a 40 × 1 cm column of Lipidex 5000 (Packard) in 4 ml of petrol–EtOAc (3:2) and eluted with 250 ml of petrol–EtOAc (19:1), then 250 ml petrol–EtOAc (9:1) and finally 500 ml petrol–EtOAc (9:2). 15 ml fractions were collected. Diol **2** was eluted in fractions 25–30 and capsidiol in fractions 44–51. Typically, 300 *g* of cellulase-treated *N. tabacum* yielded (after sublimation of combined fractions) 2 mg of diol **2** of *ca* 90% purity, and *ca* 0.5 mg of impure capsidiol. Further purification was carried out by HPLC (Waters ALC 202) at 500 psi using a column 0.8 mm i.d. × 25 cm (Hypersil, Shandon) with refractive index detection. A sample (*ca* 1 mg) of diol was applied to the column in *ca* 100 μl hexane–EtOAc (7:3) for diol **2** or in EtOAc for capsidiol and eluted with hexane–EtOAc (7:3 for diol **2**, 1:1 for capsidiol): in both cases the diols eluted after *ca* 13 mins. The purity of the diols was assessed by capillary GC on SE-54 (135°) of the free diols (capsidiol $R_I = 1922$, diol **2** $R_I = 1918$) and where necessary, of derivatives cited in the text.

*Derivatives for GC and GC–MS.* Acetates and TMSi ethers of unhindered hydroxylic groups were prepared by conventional procedures at 80°; excess reagents ($Ac_2O$/pyridine; BSTFA) were evaporated under $N_2$. TMSi ethers of hindered OH groups were prepared by heating (6 hr) in BSA/BSTFA/TMCS (4:4:1).

*TBDMS ethers.* About 100 μg of extract was dissolved in 40 μl of *t*-butyldimethylsilyl chloride–imidazole soln. (each 1 M in DMF) and heated at 80° for 1 hr, then extracted with 2 × 40 μl of hexane; the hexane phase was separated, evaporated under $N_2$ and the residue taken up in EtOAc.

*DTBS derivatives* [20, 21]. About 100 μl of extract was dissolved in a mixture of MeCN (30 μl), N-Me morpholine (20 μl) 1-hydroxybenzotriazole (3 μl) and di-*t*-butylsilyl ditriflate (3 μl) and heated at 80° for 15 hr. Then 0.5 ml of satd aq. $NaHCO_3$ was added and the mixture was extracted into 3 × 0.5 ml of $Et_2O$ and dried over $MgSO_4$.

*Cyclic boronate esters.* To *ca* 100 μg of extract in 100 μl of EtOAc was added 100 μg of $MeB(OH)_2$, $PhB(OH)_2$ or $BuB(OH)_2$. Aliquots were taken for analysis after 10 min at room temp.

*Mass spectra of components (a) and (c).* The principal ions (20 eV) were: (a) *m/z* (rel. int.): 252 (36), 237 (15), 234 (15), 223 (30), 207 (59), 205 (100), 191 (36), 186 (34), 149 (88), 143 (72), 109 (36), 107 (37), 59 (66); (c) *m/z* (rel. int.): 294 (10), 249 (6), 234 (33), 205 (100), 189 (47), 149 (45), 147 (24), 107 (27), 95 (17), 93 (20), 43 (25). These mass spectral data were similar to those obtained by Uegaki *et al.* [16] for phytuberol and phytuberin, respectively.

*Comparison of 'Debneyol' and diol* **2**. The identity of 'debneyol' (*ex* Long Ashton) [14] and diol **2** was established by comparison on capillary GC of the methaneboronates ($R_I$ 1740, OV-1; 1783, SE-54; 135°) and benzeneboronates ($R_I$ 2324, OV-1; 2415, SE-54; 170°), by GC/MS of the benzeneboronates ($M^+$ 324, 161 (100)), and by the correspondence between the $^1$H and $^{13}$C NMR data recorded for these samples.

*Oxidative cleavage of diol* **2**. Pure diol **2** (4 mg), or the same amount of sublimed extract containing diol **2** together with minor vicinal diols, was dissolved in 0.5 ml 0.1 M $Pb(OAc)_4$–AcOH. After 10 min at room temp, excess KI–NaOAc was added and the liberated $I_2$ was at once reduced by addition of 0.1 M $Na_2S_2O_3$. The soln was diluted with $H_2O$ to 10 ml and extracted twice with 10 ml of $Et_2O$. The $Et_2O$ phase was washed with satd aq. $NaHCO_3$, then $H_2O$, and dried over $MgSO_4$. The residue was dissolved in EtOAc for GC. The product was sublimed before NMR spectrometry was carried out.

*Deuterium exchange.* The nor-ketone (*ca* 9 mg) derived from diol **2** was dissolved in 100 μl of 1 M KO$^t$Bu in $CD_3OD$. The soln was left at room temp. for 15 min and then 20 μl DOAc was added to discharge the yellow coloration. The soln was diluted with 2 ml $D_2O$ and extracted with 2 × 2 ml $Et_2O$ and dried over $MgSO_4$.

*NMR spectra.* Solns in $CDCl_3$ were used, spectra being recorded on a Bruker WP200SY instrument: the $CHCl_3$ proton at $\delta$7.25 served as internal standard. Additional $^1$H spectra for the nor-ketone were obtained at 360 MHz from the Edinburgh University/SERC high field NMR service (Dr. Ian Sadler). $^{13}$C NMR spectra were measured with the $CDCl_3$ carbon resonance ($\delta$77.0) as internal standard.

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## NOTE ADDED IN PROOF

Phytuberin and phytuberol were previously identified [27, 28] in tobacco callus challenged by *Pseudomonas* spp. The first report [27] was inadvertently overlooked in our original manuscript.