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DEBNEYOL, A FUNGICIDAL SESQUITERPENE FROM TNV INFECTED *NICOTIANA DEBNEYI*

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Key Word Index—*Nicotiana debneyi*; Solanaceae; sesquiterpene; phytoalexin; debneyol; tobacco necrosis virus; fungitoxicity.

Abstract—An antifungal sesquiterpene diol, debneyol, has been isolated from tobacco necrosis virus-inoculated leaves of *Nicotiana debneyi* and a structure is proposed from chemical, spectroscopic and biogenetic evidence. In contrast to the related phytoalexin capsidiol which is fungistatic, debneyol appears to exhibit genuine fungicidal activity.

INTRODUCTION

Tobacco species have been used as test plants for the detection and identification of virus diseases for a considerable time but only comparatively recently have the effects of virus infections on the secondary metabolism of the host been investigated. Several structurally related antifungal sesquiterpenes have now been shown to be induced in virus-inoculated *Nicotiana* species [1–9]. Some excellent reviews discuss these compounds in the wider context of Solanaceous phytoalexins and stress metabolites [10–12].

Here we describe the isolation of debneyol, a new antifungal sesquiterpenoid diol from *Nicotiana debneyi* L. infected with tobacco necrosis virus. A structure for debneyol is proposed from chemical and spectroscopic evidence and the nature of its antifungal activity is described.

RESULTS AND DISCUSSION

Isolation and structure

Debneyol was isolated from tobacco necrosis virus-infected leaves by chromatography of the toluene extract followed by crystallisation, in a typical yield of 25–30 mg/kg fr. wt. It did not exhibit a parent ion in the mass spectrum but a prominent ion $C_{15}H_{24}O$ $[M-H_2O]^+$ at *m/z* 220.1814 (43%) indicated that the molecular formula was $C_{15}H_{26}O_2$.

The ^{1}H NMR spectrum revealed the presence of one secondary methyl group (δ0.98, *d*, J = 7.1 Hz), two tertiary methyls (δ1.10 and 1.18, each *s*), a non-equivalent methylene adjacent to oxygen (δ3.41 and 3.58, each *d*, J = 10.9 Hz) and a trisubstituted double bond (one vinyl proton δ5.48, *dt*, J_1 = 6.4, $J_2 = J_3$ = 1.7 Hz). The ^{13}C spectrum showed the expected 15 carbon resonances of which the most easily assignable signals were those at δ141.6 (weak, olefinic quaternary carbon), 120.1 (olefinic carbon), 74.7 (weak, quaternary carbon adjacent to oxygen) and 68.6 (carbon adjacent to oxygen). There was no resonance assignable to a carbonyl group. Debneyol readily formed a monoacetate (acetic anhydride–pyridine, room temperature) in which the non-equivalent $-CH_2-O-$ protons underwent downfield shifts to δ3.99 and 4.06 (*d*, J = 11.2 Hz) indicating that a primary alcohol group was present in debneyol. That this was part of a 1,2-dihydroxyisopropyl group was inferred from the mass spectrum which showed fragment ions at *m/z* 220.1814 (43%) $[M-H_2O]^+$, 207.1733 (16) $[M-CH_2OH]^+$, 202.1693 (11) $[M-H_2O-H_2O]^+$, 189.1623 (99) $[M-H_2O-CH_2OH]^+$, 162.1385 (27) $[M-MeCH(OH)CH_2OH]^+$ and 161.1330 (58) $[M-MeCH(OH)CH_2OH-H^-]^+$.

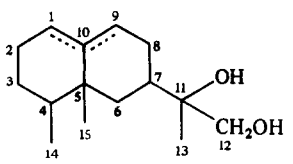
Chemical evidence for the presence of a 1,2-dihydroxyisopropyl function was obtained by the oxidation of debneyol with either chromium trioxide–pyridine or with activated manganese dioxide. Both reagents are known to oxidatively cleave the group, the latter under extremely mild conditions [13]. The product of oxidation in each case was a ketone $C_{14}H_{22}O$, the ^{1}H NMR spectrum of which exhibited a singlet at δ2.17 (methyl deshielded by adjacent carbonyl group) and had no resonances assignable to a $-CH_2OH$ group. A methine proton adjacent to carbonyl appeared at δ2.68 as a symmetrical nine line multiplet, suggestive of a $-CH_2-CHAc-CH_2-$ arrangement. The couplings of the methine proton, $J_1 \approx J_2 \approx$ 12 Hz and $J_3 \approx J_4 \approx$ 4 Hz, further suggested that the acetyl group occupied an equatorial position in an alicyclic ring with the axial hydrogen subject to two equivalent axial-axial and two equivalent axial-equatorial couplings.

The molecular formula together with the establishment of the functional groups requires debneyol to be bicyclic and the most biosynthetically feasible skeleton is that of an eremophilane. Alternative selinane structures are inconsistent with the ^{1}H NMR data. However, $\Delta^{1(10)}$ and Δ^9 structures appear equally possible (1), a problem of bridgehead double bond location which has arisen many times in eremophilane chemistry [14–18] and which has usually been solved only via extensive chemical transformations. Insufficient amounts of debneyol precluded this approach but we considered that the position might be deduced from a comparison of the mass spectral fragmen-

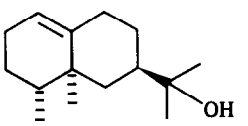
tation with that observed for suitable model compounds. Kusunol (**2**) and jinkoh-eremol (**3**) are such analogues [19] with mass spectra as listed in the Experimental Section. Kusunol is identical with the previously isolated valerianol [16, 17] and has essentially the same mass spectrum as that reported for this compound [20]. While the mass spectrum of **2** and **3** showed many common features, a major difference was the intensity of the ion at *m/z* 105. In jinkoh-eremol this was the base peak, while in kusunol it was very much reduced at 23% and of comparable intensity to many other peaks. The formation of the 105 ion in jinkoh-eremol can readily be rationalised in terms of loss of $[(CH_3)_2CHOH + H^{\cdot}]$ to give the ion at *m/z* 161 (98%) followed by cleavage of ring A at the allylic positions C-1,2 and C-4,5. Such an interpretation is not possible for kusunol (valerianol). As debneyol has as its base peak the ion C_8H_9 at *m/z* 105.0710 and furthermore has other fragment ions at 147 (28%) and 133 (14%) which are found in jinkoh-eremol but not kusunol, it is therefore assigned the Δ^9 structure.

The relative stereochemistry of the substituents in debneyol remains to be determined. Of the two tertiary methyl signals, at δ1.10 and 1.18, present in the ^{1}H NMR spectrum of debneyol, the latter is due to the angular methyl group (C-15) because a signal is present at the same chemical shift in the monoacetate and in the ketone derived by oxidative cleavage of the side chain. The doublet at δ0.98 in the ^{1}H NMR spectrum of debneyol can be unambiguously ascribed to the C-14 methyl group. Thus the chemical shifts of C-14 and C-15 are quite different from those of eremophilene-type sesquiterpenes having the methyl groups *cis* disposed. These occur in the range δ0.80–0.95 [14–19]. However, they compare well with the chemical shifts observed for the *trans* Δ^9 eremophilene, epi-capsidiol **4**, δ0.93 (C-14) and 1.12 (C-15) [21]. (In capsidiol (**5**) the angular methyl resonates at δ1.37 owing to additional deshielding by the 1,3-diaxial interaction with the OH group at C-1 [22].) Furthermore, application of standard substituent chemical shift parameters [23] to the observed chemical shift of the angular methyl in the *trans*-eremophilane **10** [24] gives a calculated chemical shift of δ1.27 for the corresponding *trans*-eremophilene derivative **9**, only slightly in excess of the observed values for debneyol and its derivatives. Thus there is evidence in the ^{1}H NMR spectrum for a *trans*-disposition of the two methyl groups in debneyol as in **6**.

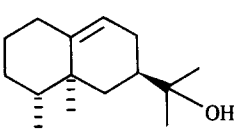
Finally the relative stereochemistry of the side chain at C-7 must be considered. From the ^{1}H NMR spectrum of



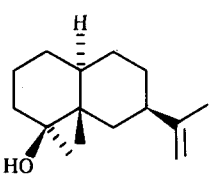
1



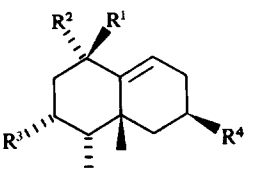
2



3



10



	R^1	R^2	R^3	R^4
4	H	OH	OH	$-C(=CH_2)-Me$
5	OH	H	OH	$-C(=CH_2)-Me$
6	H	H	H	$-C(Me)(OH)-CH_2OH$
7	H	H	H	$-C(Me)(OH)-CH_2OAc$
8	H	H	H	Ac
9	H	H	H	$-C(=CH_2)-Me$

the ketone **8** it is clear that in this compound the proton at C-7 is in the axial orientation (two large axial-axial couplings are seen) and hence the acetyl side chain must be in the equatorial position. Since debneyol was converted into ketone **8** by an extremely mild oxidation which would not be expected to lead to epimerisation of the proton α to the carbonyl group, it is safe to infer that the side-chain is also equatorial in debneyol itself. However, in the absence of information about the conformation of the rings it is not possible to deduce the stereochemical relationship of the dihydroxyisopropyl group at C-7 with the angular methyl. The obvious biosynthetic relationship with capsidiol does suggest, however, that a *cis* relationship as in **6** is the most plausible.

Debneyol was first isolated by us in 1979 [25] and subsequent reports suggest that an identical compound may have been isolated from TNV-inoculated leaves of *Nicotiana tabacum* [9] and from cultured cells of *N. tabacum* inoculated with *Phytophthora parasitica* var *Nicotianae* [26] or treated with extracts of *Gliocladium deliquescens* (Brooks, C. J. W., personal communication).

While eremophilane sesquiterpenes with a *cis* disposition of the vicinal methyls at C-14 and C-15 are relatively common natural products, the *trans*-dimethyl analogues are rare. Examples to date are capsidiol [21, 22], capsenone [21, 22], 1-deoxycapsidiol [27], two partially characterised diols isomeric with capsidiol [27] and the 4-hydroxy-eremophilane **10** from *Senecio amplexicalis* [24]. Debneyol can now be considered as an addition to this group.

A second antifungal compound accompanied debneyol in the extracts of TNV-infected *Nicotiana debneyi*. In the mass spectrum this showed a molecular ion at *m/z* 236, two mass units lower than debneyol but with a similar fragmentation pattern. This suggests that it may be structurally related.

Antifungal activity

The antifungal activity of debneyol was compared with that of two other sesquiterpene solanaceous phytoalexins, capsidiol and glutinosone [2, 3]. In the *Cladosporium cucumerinum*-TLC bioassay [2, 8], the minimum detectable quantities of debneyol, capsidiol and glutinosone were 5, 5 and 20 μg respectively.

In assays of spore germination, sporeling growth and mycelial growth of several fungi debneyol exhibited activity that was slightly less than that of capsidiol but considerably greater than that of glutinosone [29, 30]. ED_{50} values for capsidiol, debneyol and glutinosone estimated from effects on germination of spores were as follows: for *Cladosporium cucumerinum* 25–30, 50–75 and 100 μg/ml; for *Colletotrichum lindemuthianum* 10–25, 25–50 and 75–100 μg/ml; for *Ascochyta fabae* 50–75, 75–100 and 100 μg/ml; but for *Botrytis cinerea*, *Rhizopus stolonifer* and *Alternaria longipes* (a pathogen of tobacco) all ED_{50} values were greater than 100 μg/ml.

Propagules of *Colletotrichum lindemuthianum* whose growth had been completely inhibited by debneyol (200 μg/ml) in Czapek Dox agar failed to grow when they were transferred to fresh agar media lacking debneyol. However, propagules inhibited by the same concentration of capsidiol always resumed growth when removed from this inhibitor. Staining propagules with fluorescein diacetate confirmed that debneyol was fungicidal, killing cells within 5–10 min, whilst in agreement with other investigations [31, 32] capsidiol was fungistatic, the cells remaining alive for several days. These results imply that despite their obvious chemical and biosynthetic similarities, debneyol and capsidiol may have different modes of action.

EXPERIMENTAL

Mass spectra were obtained from direct insertion probe samples on a Finnigan 4021 MS-DS. Accurate molecular masses were obtained on a Kratos MS 80. 200 MHz ^{1}H and 22.5 MHz ^{13}C NMR spectra were recorded in $CDCl_3$ on JEOL FX 200 and JEOL FX 90Q instruments respectively. TLC was performed on Merck silica gel F_{254} plates. All compounds were shown to be TLC pure.

Isolation procedure. Leaves of *Nicotiana debneyi*, grown in a glasshouse, were inoculated with TNV. The inoculated leaves produced discrete necrotic lesions and after 7 to 10 days they were harvested, weighed and stored at −20°. Frozen tissue (2 kg) was extracted with toluene (3 ×) over several weeks at 5°. The combined extracts were evaporated, applied to a column of Kieselgel 40 and eluted with hexane containing increasing amounts of $CHCl_3$ until $CHCl_3$ alone was used. Antifungal activity in the fractions was monitored using a *Cladosporium cucumerinum* bioassay [28]. The antifungal fractions were collected and the solvent evaporated. TLC ($CHCl_3$–EtOH 24:1) revealed 3 zones after spraying with vanillin–H_3PO_4 reagent and heating. The compound (53 mg) of lowest R_f (0.25) gave a dark purple colour and was associated with most of the antifungal activity. Recrystallization from Et_2O-hexane gave debneyol, mp 50–52° (Kofler uncorr.), as feathery needles. The intermediate compound (R_f 0.28) was not antifungal but some activity was associated with the upper compound (4 mg, R_f 0.32) which gave a turquoise colour with vanillin. This gave an EIMS with ions *m/z* (rel. int.): 236 $[M]^+$ (6), 221 (47), 219 (21), 162 (23), 147 (28), 136 (13), 127 (20), 126 (21), 121 (30), 107 (32), 106 (32), 105 (82), 91 (84), 43 (100).

Debneyol (**6**). ^{1}H NMR: δ0.98 (3H, *d*, J = 7.1 Hz, H-14), 1.10 (3H, *s*, H-13), 1.18 (3H, *s*, H-15), 3.41 (1H, *d*, J = 10.9 Hz, H-12), 3.58 (1H, *d*, J = 10.9 Hz, H-12), 5.48 (1H, *dt*, J_1 = 6.4 Hz, $J_2 = J_3$ = 1.7 Hz, H-9); ^{13}C NMR: δ17.7, 20.1, 22.4, 26.4, 30.3, 30.3, 32.0, 38.5, 38.7 (w, C-5), 39.3, 41.6, 68.6 (C-12), 74.7 (w, C-11), 120.1 (C-9), 141.6 (w, C-10); EIMS *m/z* (rel. int.): 238 $[M]^+$ absent, 220 (43) [found 220.1814; $C_{15}H_{24}O$ requires 220.1827], 207 (16) [found 207.1733; $C_{14}H_{23}O$ requires 207.1749], 202 (11) [found 202.1693; $C_{15}H_{22}O$ requires 202.1721], 189 (99) [found 189.1623; $C_{14}H_{21}$ requires 189.1643], 187 (23), 163 (14), 162 (27) [found 162.1385, $C_{12}H_{18}$ requires 162.1408] 161 (59) [found 161.1330; $C_{12}H_{17}$ requires 161.1330], 147 (28), 145 (14), 133 (19), 121 (16), 119 (22), 107 (53), 105 (100) [found 105.0710; C_8H_9 requires 105.0704], 95 (22), 93 (33), 91 (40), 81 (39), 79 (23), 75 (32), 57 (23), 55 (28), 43 (73).

Debneyol monoacetate (**7**). Debneyol (12 mg) was dissolved in C_5H_5N (0.1 ml) and Ac_2O (0.1 ml) added. After leaving 24 hr at room temp the solvent was evaporated under a stream of N_2. TLC purification afforded debneyol monoacetate (**7**), R_f 0.48 ($CHCl_3$–EtOH, 39:1). ^{1}H NMR: δ0.97 (3H, *d*, J = 7.1 Hz, H-14), 1.15 (3H, *s*, H-13), 1.18 (3H, *s*, H-15), 2.10 (3H, *s*, CH_3CO_2–), 3.99 (1H, *d*, J = 11.2 Hz, H-12), 4.06 (1H, *d*, J = 11.2 Hz, H-12), 5.49 (1H, *d*, (*br*), J = 6.4 Hz); EIMS, *m/z* (rel. int.): 280 $[M]^+$ absent, 202 (54), 187 (33), 161 (18), 147 (32), 145 (26), 131 (21), 117 (33), 107 (26), 105 (58), 81 (29), 43 (100).

Ketone (**8**). Debneyol (15 mg) was dissolved in C_5H_5N (0.5 ml) and added to a complex of CrO_3 (50 mg) in C_5H_5N (0.5 ml) [33]. The mixture was left overnight, then diluted with H_2O and extracted with Et_2O. TLC ($CHCl_3$–EtOH 39:1) gave the ketone

(8) (9 mg, R_f 0.65). An identical product (6 mg) was obtained when debneyol (10 mg) in $CHCl_3$ (20 ml) was left to stand over activated MnO_2 (100 mg) prepared by the Attenborough procedure [34]. ^1H NMR: δ0.99 (3H, *d*, J = 7.3 Hz, H-14), 1.18 (3H, *s*, H-15), 2.17 (3H, *s*, H-13), 2.68 (1H, *dddd*, $J_1 \approx J_2 \approx 12$ Hz, $J_3 \approx J_4 \approx 4$ Hz, H-7), 5.52 (1H, *dt*, $J_1 = 6.1$ Hz, $J_2 = J_3 = 2.0$ Hz, H-9); EIMS *m/z* (rel. int.): 206 $[M]^+$ (16), 191 (8), 163 (28), 161 (13), 149 (12), 147 (11), 135 (8), 121 (13), 107 (35), 105 (32), 91 (33), 81 (27), 55 (23), 43 (100).

Kusunol (2). EIMS (Jeol D-300 mass spectrometer) *m/z* (rel. int.): 222 $[M]^+$ (7), 204 (48), 189 (18), 161 (100), 149 (25), 135 (29), 107 (17), 105 (23), 93 (18), 81 (18), 59 (70).

Jinkoh-eremol (3). EIMS (Jeol D-300 mass spectrometer) *m/z* (rel. int.): 222 $[M]^+$ (2), 204 (74), 189 (45), 161 (98), 147 (52), 133 (23), 119 (14), 105 (100), 93 (20), 91 (26), 59 (55).

Bioassays. The antifungal activity of debneyol, capsidiol and glutinosone was assessed by methods described previously [30, 35, 36]. Capsidiol and glutinosone were obtained from *Nicotiana clevelandii* and *Nicotiana glutinosa* respectively [1, 3].

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