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STRESS COMPOUNDS IN THE LEAVES OF *NICOTIANA UNDULATA* INDUCED BY TMV INOCULATION

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Key Word Index—*Nicotiana undulata*; Solanaceae; tobacco; sesquiterpenoid; stress compounds.

Abstract—In leaves of *Nicotiana undulata* inoculated with TMV, 19 sesquiterpenoids were detected as stress compounds. Among them, dehydrocarissone, capsidiol 3-acetate, aubergenone, 4-*epi*-aubergenone, *trans*-dihydrocarissone, 1,2-dehydro-α-cyperone and 3-*epi*-3-hydroxysolavetivone are novel tobacco stress compounds.

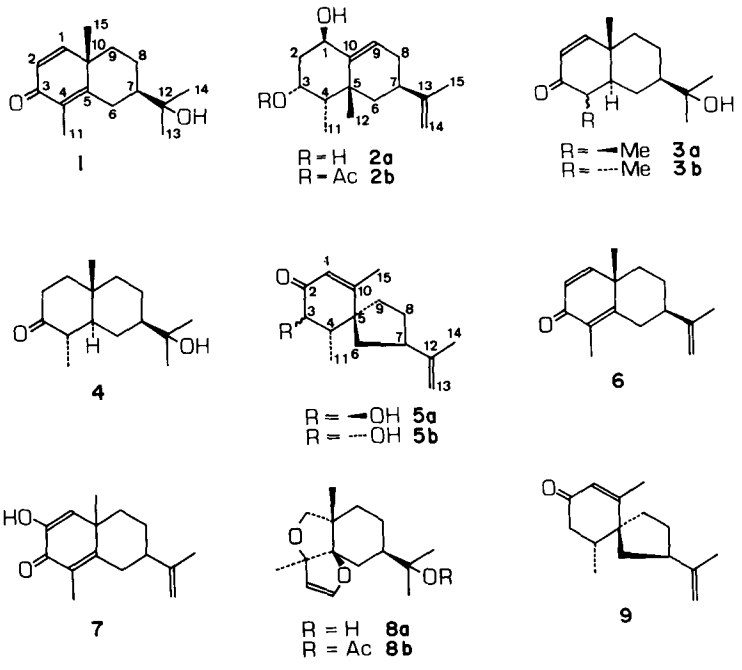
INTRODUCTION

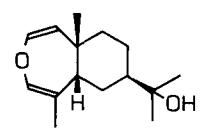
Tobacco leaves produce stress compounds in response to virus or bacterial infection or ethrel treatment. Up to now, 20 sesquiterpenoids have been isolated from various *Nicotiana* species [1–4]. In our continuing study on tobacco stress compounds, we found 19 stress compounds in TMV-infected leaves of *Nicotiana undulata*. The main compound was phytuberin. In addition to 11 known tobacco stress compounds, seven novel ones were detected: dehydrocarissone, capsidiol 3-acetate, aubergenone, 4-*epi*-aubergenone, *trans*-dihydrocarissone, 3-*epi*-3-hydroxysolavetivone and 1,2-dehydro-α-cyperone.

RESULTS AND DISCUSSION

Induced stress compounds in TMV-inoculated leaves of *N. undulata* were initially examined by capillary GC and capillary GC-MS analysis. These analyses revealed many sesquiterpenoidal stress compounds, 19 of which were identified. Seven compounds, **1**, **2b**, **3a**, **3b**, **4**, **5b**, and **6**, were novel tobacco stress compounds. On the basis of their MS spectra and GC retention times, in comparison with those of authentic samples, the other 12 compounds appeared to be capsidiol **2a**, 3-hydroxysolavetivone **5a**, 2-keto-α-cyperone **7**, phytuberol **8a**, phytuberin **8b**, solavetivone **9**, occidenol **10**, occidentalol **11**, occidol **12a**, occidol acetate **12b**, occidol isomer-1 **13** and occidol isomer-2 **14**, respectively. The latter compounds have already been identified as tobacco stress compounds.

Compound **1** was isolated by preparative GC from the 100% ether fraction obtained on a silica gel column. The mass spectrum of this compound showed no significant molecular ion, but characteristic fragment ions at *m/z* 216 and 219 resulting from loss of water and methyl. This finding suggested that the M_r of **1** was 234 probably





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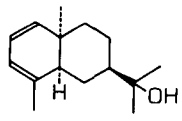

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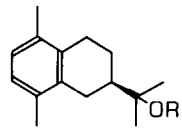
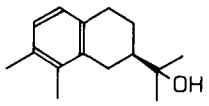


R = H 12a
R = Ac 12b



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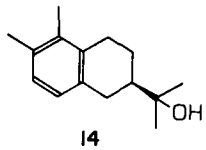

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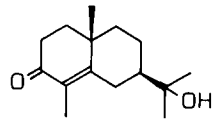

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corresponding to $C_{15}H_{22}O_2$. The absorption band at 1665 cm^{-1} in the IR spectrum suggested the presence of a partial structure of α,β and α',β'-unsaturated ketone. Among the ^1H NMR signals, the peaks of methyl on quaternary carbon at δ1.23 (3H, *s*), olefinic methyl at δ1.93 (3H, *s*) and two olefinic methines at δ6.23 and 6.75 (each 1H, *d*, J=9.8 Hz) were very similar to the corresponding signals of 1,2-dehydro-α-cyperone **6** (δ1.25, 1.92, 6.24 and 6.76), which is one of the volatile compounds of *Lycium chinense* [5]. Therefore, **1** was assumed to have the skeleton of 1,4a-dimethyl-5,6,7,8,-tetrahydro-2(4*a*H)-naphthalenone, which was supported by the homonuclear correlation (COSY) spectra.

In COSY spectra, a cross peak attributing to the 'W-conformation' long-range coupling between **6e** (δ2.97) and **8e** (δ1.79) protons was found. The characteristic fragment ion at *m/z* 59 (100%), the IR absorption band at 3400 cm^{-1} and ^1H NMR signal at δ1.28 (3H, *s*) and 1.29 (3H, *s*) indicated the presence of an isopropanol group. The above data suggest that **1** is dehydrocarissone having an isopropanol instead of an isopropenyl group. The production of carissone **15** and *trans*-dihydrocarissone **4** upon catalytic reduction of **1** supports the proposed structure. The ^{13}C NMR spectrum showed the expected 15 carbon resonances, which were easily assigned as mentioned in Experimental. The coupling of H-7 signal at δ1.37, J=13 and 3.5 Hz, further suggested that the isopropanol group occupied an equatorial position subject to two equivalent axial-axial and two equivalent axial–equatorial couplings. C-10 methyl seems to be located axially because of the similar chemical shift at δ1.23 to that of **6** at δ1.25. Compound **1** is first reported not only as a stress compound but also as a natural product.

Compound **2b** was isolated from the hexane: Et_2O (1:1) and Et_2O 100% eluted fraction. The difference in the NMR spectra of **2b** compared with **2a** was the appearance of the ^1H signal at δ2.04 (3H, *s*) of acetate methyl, the downfield shifts of the C-3, C-11 and H-3 signals (**2a**: δ65.3, 8.9, 4.59; **2b**: δ69.4, 10.0, 5.63) and the upfield shifts of the C-2 and C-4 signals (**2a**: δ36.3, 47.7; **2b**: δ33.1, 44.4) The COSY experiment of **2b** suggest that this compound is capsidiol 3-acetate. Proof for the assumed structure was furnished by acetylation of **2a**. Reaction of **2a** with Ac_2O in dry pyridine for 1.5 hr produced 3-monoacetate and 1,3-diacetate (*ca* 1:1). The MS and ^1H NMR spectra of prepared 3-monoacetate were in agreement with those of **2b**.

Compound **3a** was isolated from a fraction obtained after elution with Et_2O. The ^1H NMR spectrum showed the presence of an isopropanol group [(δ1.24 and 1.26 (each 3H, *s*)] and an α,β-unsaturated ketone [(δ5.86 and 6.77 (each 1H, *d*)]. The ^1H NMR spectral data of **3a** agreed with those of aubergenone **3a** [6] which is a known phytoalexin of eggplant. Furthermore, this fraction contained the compounds **3b** and **4**. The MS spectrum of **3b** was very similar to that of **3a**, which suggested that **3b** was a stereoisomer of **3a**. From the ^1H NMR results, it can be seen that only the angular methyl and H-4 shieldings differ significantly in **3a** and **3b**. The singlet at δ1.15 corresponding to the angular methyl and the H-4 methine signal at δ2.45 of **3a** shifted to δ1.06 and 2.26, respectively, in **3b**, difference being due to the methyl–methyl *syn*–axial interaction. Therefore, **3b** seems to be the 4-epimer of **3a**. The ^1H NMR spectrum of **3b** was similar to that of synthetic 4-*epi*-aubergenone as reported in ref. [7].

The MS spectral pattern of **4** resembled that of both **3a** and **3b**, except that the fragment ions of **4** above *m/z* 120 were 2 units greater than the corresponding ones in **3a** and **3b**. This suggests that **4** is the saturated form of **3a** or **3b**. The ^1H NMR signals of an angular methyl at δ1.07 (3H, *s*), a methyl attached to tertiary carbon at δ1.01 (3H, *d*) and two methyls at δ1.20 and 1.21 (each 3H, *s*) attached to carbon carrying oxygen. These data for **4** were agreement with those of *trans*-dihydrocarissone which is reported as a synthetic intermediate to 4-*epi*-aubergenone **3b** [7, 8].

In the capillary GC-MS chromatograms of hexane–ethyl ether (1:1) eluted fractions, two peaks with MS patterns resembling that of **5a** which has already been identified as tobacco stress compound were found. As the two peaks did not separate in preparative GC, the mixture of the two peaks was subjected to NMR analysis. In the ^1H and ^{13}C NMR spectra of this mixture, signals corresponding to **5a** and other additional signals corresponding to **5b** were observed. The most striking difference of the chemical shift in the ^1H and ^{13}C NMR between **5a** and **5b** were recognized at H-3 and C-4 methyl. An oxygen carrying methine proton of **5a** was measured at δ3.83; on the other hand the corresponding signal of **5b** resonanced at δ4.52. Furthermore, C-4 methyl signal of **5b** shifted to δ0.83 from 1.23 of the corresponding signal of **5a**. The ^{13}C NMR signal of C-4 methyl of **5b** was observed at δ8.1 as opposed to that at δ12.2 of **5a**. These findings suggest that **5b** is the 3-epimer of **5a**. The data are in agreement with those of 3-*epi*-3-hydroxysolavetivone obtained as the aglycone of glucoside from flue-cured Virginia tobacco

[9]. This is the first report of **5b** as a stress compound.

Compound **6** was obtained from the hexane–ethyl ether (4:1) eluted fraction. The MS fragmentation pattern and the GC retention time on capillary column of **6** was in accordance with those of authentic 1,2-dehydro-α-cyperone reported as volatile component of *Lycium chinense* [5].

The content of these 19 compounds in freeze-dried leaves, calculated from GC peak areas, is as follows: **1**; 31.1, **2a**; 5.4, **2b**; 33.2, **3a**; 9.5, **3b**; 53.8, **4**; 29.6, **5a**; 48.5, **5b**; 45.0, **6**; 2.2, **7**; 199.4, **8a**; 18.5, **8b**; 240.3, **9**; 21.0, **10**; 19.2, **11**; 2.7, **12a**; 53.7, **12b**; 4.7, **13**; 1.4 and **14**; 0.4 μg/g dry weight, respectively. These compounds were not detected in healthy leaves. Most belong to the eudesmane series of sesquiterpenoids.

EXPERIMENTAL

General. GC was performed on a Schimadzu 7A gas chromatograph. IR spectra were recorded as a film or KBr. ^{13}C NMR spectra were recorded at 25 MHz in $CDCl_3$ with TMS as int. standard. The multiplicities of ^{13}C NMR signals were determined by the INEPT method. ^{1}H NMR spectra and COSY spectra were recorded at 500 MHz in $CDCl_3$ with TMS as int. standard.

Extraction and isolation. Leaves of *N. undulata* were treated as described in ref. [10]. The virus used was TMV-OM (0.5 μg/ml in 0.1 M phosphate buffer, pH 7.0). From 2.3 kg of harvested leaves, 156.5 g of freeze-dried materials, 8.42 g of CH_2Cl_2 extract and 597 mg of Et_2O extract of the steam-distillate were obtained. The Et_2O extract of the steam-distillate was placed on a column of SiO_2 and eluted with a solvent gradient system from *n*-hexane to Et_2O to obtain 13 fractions. Each fraction was subjected to capillary GC-MS chromatography (Carbowax 20M fused-silica, 0.31 mm × 25 m, 100–210°, 2°/min). The compounds **1**, **2b**, **3a**, **3b**, **4** and **5b** were isolated from their respective fractions by preparative GC (5% FFAP, 2.6 mm × 1 m, 100–240°, 5°/min).

Dehydrocarissone (**1**). $C_{15}H_{22}O_2$; MS *m/z* (rel. int.): 234 $[M]^+$ (0), 219 (4), 216 (17), 201 (15), 173 (39), 161 (19), 159 (15), 145 (22), 135 (14), 133 (10), 131 (11), 119 (12), 105 (23), 91 (35), 79 (12), 77 (14), 59 (100), 53 (13), 43 (43), 41 (29), 39 (20); IR ν_{max}^{film} cm^{-1}: 3400, 1665, 1617, 1600, 1380, 1135, 825; ^{1}H NMR: δ1.23 (3H, *s*, H-15), 1.28 (3H, *s*)/1.29 (3H, *s*) (H-13/14), *ca* 1.30 (1H, *td*, H-9a), 1.37 (1H, *tt*, *J* = 13, 3.5 Hz, H-7a), 1.60 (1H, *qd*, *J* = 13, 3.5 Hz, H-8a), 1.79 (1H, *dm*, *J* = 13 Hz, H-8e), 1.87 (1H, *dt*, *J* = 13, 3 Hz, H-9e), 1.93 (3H, *s*, H-11), 2.04 (1H, *t*, *J* = 13 Hz, H-6a), 2.97 (1H, *d*, *J* = 13 Hz, H-6e), 6.23 (1H, *d*, *J* = 9.8 Hz, H-1), 6.75 (1H, *d*, *J* = 9.8 Hz, H-2); ^{13}C NMR: δ10.4 (C-11), 22.0 (C-8), 23.4 (C-15), 26.9/27.7 (C-13/14), 28.7 (C-6), 38.0 (C-9), 40.3 (C-10), 50.5 (C-7), 72.3 (C-12), 126.1 (C-2), 129.2 (C-4), 156.6 (C-1), 160.5 (C-5), 186.5 (C-3).

Capsidiol 3-acetate (**2b**). $C_{17}H_{26}O_3$; MS *m/z* (rel. int.): 278 $[M]^+$ (0), 200 (32), 185 (22), 157 (24), 144 (22), 143 (100), 132 (18), 129 (28), 128 (31), 117 (15), 115 (20), 105 (23), 91 (22), 43 (29), 41 (16); ^{1}H NMR: δ0.87 (3H, *d*, *J* = 7.3 Hz, H-11), 1.42 (3H, *s*, H-12), 1.73 (3H, *s*, H-15), 2.04 (3H, *s*, Ac-Me), 4.38 (1H, *m*, H-1), 4.69, 4.72 (each 1H, *d*, *J* = 1.5 Hz, H-14), 5.63 (1H, *dt*, *J* = 12.5, 4.5 Hz, H-3), 5.94 (1H, *dd*, *J* = 7.0, 2.0, H-9); ^{13}C NMR: δ10.0 (C-11), 21.4 (C-15 and Ac-Me), 30.3 (C-8), 32.0 (C-12), 33.1 (C-2), 39.1 (C-5), 40.1 (C-7), 44.4 (C-4), 44.7 (C-6), 69.4 (C-3), 74.6 (C-1), 108.8 (C-14), 129.1 (C-9), 140.0 (C-10), 149.3 (C-13). Ac-C=O was not detected.

Aubergenone (**3a**). $C_{15}H_{24}O_2$; MS *m/z* (rel. int.): 236 $[M]^+$ (< 1), 221 (2), 218 (1), 203 (2), 178 (22), 135 (12), 123 (14), 121 (17), 108 (11), 95 (14), 91 (11), 79 (12), 67 (13), 59 (100), 55 (11), 41 (12); ^{1}H NMR: δ1.14 (3H, *d*, *J* = 7.9 Hz, H-11), 1.15 (3H, *s*, H-15), 1.24/1.26 (each 3H, *s*, H-13/14), 2.45 (1H, *m*, H-4), 5.86 (1H, *d*, *J* = 10 Hz, H-1), 6.77 (1H, *d*, *J* = 10 Hz, H-2).

4-epi-*Aubergenone* (**3b**). $C_{15}H_{24}O$; MS *m/z* (rel. int.): 236 $[M]^+$ (< 1), 221 (2), 178 (26), 163 (13), 149 (10), 135 (17), 123 (21), 122 (16), 121 (18), 107 (12), 95 (13), 91 (11), 79 (11), 67 (12), 59 (100), 55 (14), 41 (15); IR ν_{max}^{film} cm^{-1}: 3440, 1662, 1380, 820; ^{1}H NMR: δ1.06 (3H, *s*, H-15), 1.14 (3H, *d*, *J* = 6.8 Hz, H-11), 1.22/1.23 (each 3H, *s*) (H-13/14), 1.59 (1H, *td*, *J* = 3, 12.6 Hz, H-5a), 1.85 (1H, *dm*, *J* = 13 Hz, H-6e), 2.26 (1H, *dq*, *J* = 6.7, 12.8 Hz, H-4), 5.85 (1H, *d*, *J* = 9.9 Hz, H-1), 6.71 (1H, *d*, *J* = 9.9 Hz, H-2); ^{13}C NMR: δ11.8 (C-11), 17.1 (C-15), 21.9 (C-8), 25.2 (C-6), 26.8/27.5 (C-13/14), 36.1 (C-10), 37.8 (C-9), 42.7 (C-4), 48.3/48.5 (C-5/7), 72.5 (C-12), 126.2 (C-2), 160.4 (C-1), 202.2 (C-3).

trans-*Dihydrocarissone* (**4**). $C_{15}H_{26}O_2$; MS *m/z* (rel. int.): 238 $[M]^+$ (0), 223 (2), 180 (19), 125 (13), 123 (16), 95 (13), 91 (11), 84 (11), 81 (18), 79 (12), 77 (11), 69 (13), 67 (17), 59 (100), 55 (31), 53 (13), 43 (26), 41 (40), 39 (16); IR ν_{max}^{film} cm^{-1}: 3430, 1695, 1450, 1380, 1140, 1160, 1090, 910; ^{1}H NMR: δ1.01 (3H, *d*, *J* = 6.6 Hz, H-11), 1.07 (3H, *s*, H-15), 1.20/1.21 (each 3H, *s*, H-13/14), 1.73 (1H, *ddd*, *J* = 2, 6.5, 13 Hz, H-1e), 1.80 (1H, *dm*, *J* = 15 Hz, H-6e), 2.22 (1H, *dq*, *J* = 6.6, 11 Hz, H-4a), 2.34 (1H, *ddd*, *J* = 2, 5, 15 Hz, H-2e), 2.52 (1H, *td*, *J* = 6, 15 Hz, H-2a); ^{13}C NMR: δ11.2 (C-11), 16.3 (C-15), 22.0 (C-8), 26.3 (C-6), 26.8/27.5 (C-13/14), 33.4 (C-10), 38.0 (C-9), 40.7 (C-2), 41.2 (C-1), 45.6 (C-4), 48.7 (C-7), 51.0 (C-5), 72.7 (C-12), 213.1 (C-3).

3-*Hydroxysolavetivone* (**5a**) *and* 3-epi-3-*hydroxysolavetivone* (**5b**). $C_{15}H_{22}O_2$; MS of the former r.t. compound *m/z* (rel. int.): 234 $[M]^+$ (1.5), 232 (1), 219 (1), 206 (1.5), 205 (3), 201 (1), 191 (1), 189 (2.5), 177 (13), 176 (100), 161 (14), 148 (13), 133 (21), 121 (8), 109 (45), 108 (39), 105 (10), 98 (10), 91 (13), 80 (9), 79 (13), 77 (9), 68 (27), 67 (9), 41 (8); MS of the latter r.t. compound *m/z* (rel. int.): 234 $[M]^+$ (1), 232 (1), 219 (1), 206 (1), 205 (4), 189 (2.5), 177 (13), 176 (100), 161 (13), 148 (14), 133 (22), 109 (46), 108 (42), 105 (8), 91 (13), 80 (10), 79 (13), 77 (8), 68 (29), 67 (9); ^{1}H NMR of the mixture of **5a** and **5b**: **5a** δ1.23 (3H, *d*, *J* = 6.8 Hz, H-11), 1.76 or 1.77 (3H, *s*, H-14), 2.02 (3H, *d*, *J* = 1.2 Hz, H-15), 3.83 (1H, *d*, *J* = 12.7 Hz, H-3), 4.75 (2H, *s*, H-13), 5.84 (1H, *q*, *J* = 1.2 Hz, H-1); **5b** δ0.83 (3H, *d*, *J* = 6.8 Hz, H-11), 1.76 or 1.77 (3H, *s*, H-14), 1.95 (3H, *d*, *J* = 1.2 Hz, H-15), 4.52 (1H, *d*, *J* = 5 Hz, H-3), 4.75 (2H, *s*, H-13), 5.81 (1H, *q*, *J* = 1.2 Hz, H-1); ^{13}C NMR of the mixture of **5a** and **5b**: **5a** δ12.2 (C-11), 21.4 (C-14), 22.6 (C-15), 32.8 (C-9), 31.4 (C-8), 40.2 (C-6), 47.3 (C-7), 48.0 (C-4), 74.0 (C-3), 109.0 (C-13), 122.7 (C-1). Other peaks could not be detected; **5b** δ8.1 (C-11), 20.3/21.2 (C-14/15), 32.0/36.1 (C-8/9), 45.6/46.4 (C-6/7), 109.2 (C-13), 126.0 (C-1). Other peaks could not be detected.

1,2-*Dehydro-α-cyperone* (**6**). $C_{15}H_{20}O$; MS *m/z* (rel. int): 216 $[M]^+$ (13), 201 (26), 187 (19), 173 (74), 159 (65), 145 (62), 134 (30), 133 (39), 131 (35), 119 (48), 105 (62), 91 (74), 82 (33), 81 (31), 79 (38), 77 (44), 53 (43), 43 (35), 41 (79), 39 (100).

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