Hydroxygeranyllinalool Glycosides from Tobacco Exhibit Antibiosis Activity in the Tobacco Budworm [*Heliothis virescens* (F.)]

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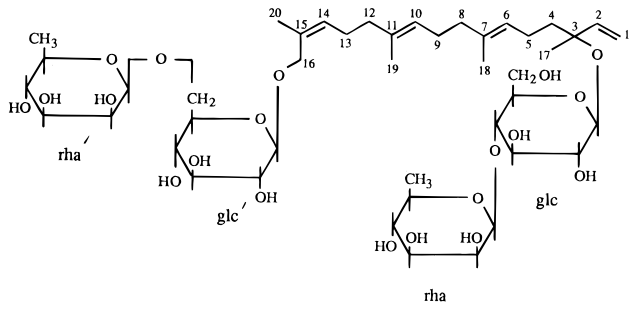
Leaves of Tobacco Introduction TI-165 were found to be resistant to tobacco budworm [*Heliothis virescens* (F.)] attack. HPLC profiles of leaf extracts showed that TI-165 had relatively high levels of two components (**A** and **B**) that were absent in susceptible varieties. Compounds **A** and **B** were isolated from TI-165 by a combination of preparative C_{18}, silicic acid column, and centrifugal thin-layer chromatography. They were identified as diterpene glycosides: compound **A**, 16-hydroxygeranyllinalyl-3-*O*-[α-L-rhamnopyranosyl(1→4)]-*β*-D-glucopyranoside-16-*O*-[α-L-rhamnopyranosyl-(1→6)]-*β*-D-glucopyranoside; compound **B**, 16-hydroxygeranyllinalyl-3-*O*-[α-L-rhamnopyranosyl(1→4)]-*β*-D-glucopyranoside-16-*O*-[α-L-rhamnopyranosyl(1→6)]-[α-L-rhamnopyranosyl(1→4)]-*β*-D-glucopyranoside (hydroxygeranyllinalool glycosides). Budworm bioassays with whole tobacco leaves and purified mixtures of **A** and **B** showed significant correlation between larval weights and levels of **A** and **B**. HPLC analyses of freeze-dried leaves of 68 *Nicotiana* species indicated that 26 species had high levels of diterpene glycosides identical to or related to **A** and **B**.

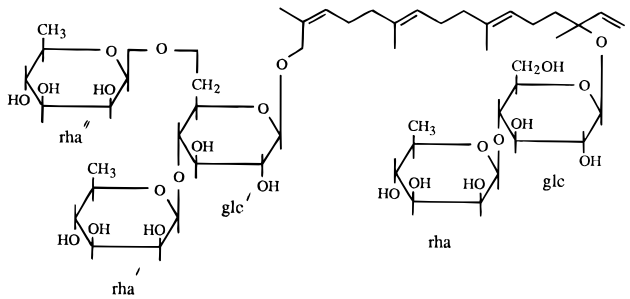
Keywords: *Nicotiana; Heliothis virescens (F.); antibiosis; 16-hydroxygeranyllinalool glycosides; diterpene glycosides*

INTRODUCTION

Tobacco budworm [*Heliothis virescens* (F.)] infests tobacco, cotton, and other crops. The economic cost to South Carolina tobacco farmers (leaf damage and control) in 1994 was >$6 million (Gooden et al., 1996). Budworm-resistant tobacco genotypes have been identified; these exhibit various modes of resistance. One mode of resistance to budworm attack in tobacco is ovipositional nonpreference. Chemicals, such as α- and *β*-4,8,13-duvatriene-1,3-diols and their corresponding mono-ols and sucrose esters, associated with the leaf cuticular waxes, have been shown to be ovipositional stimuli (Jackson et al., 1984, 1986; Johnson and Severson, 1982, 1984; Severson et al., 1984, 1985). These compounds were found to be absent from the resistant TI-1112 cultivar (Severson et al., 1984). A second mode of resistance involves moth flight orientation due to certain volatile chemicals emitted by tobacco leaves. Mitchell et al. (1991) and Tingle et al. (1990) demonstrated that susceptible tobacco cultivars (e.g., NC2326) emit attracting volatiles, while Tingle et al. (1990) showed that budworms do not fly toward the resistant variety, TI-1112, which apparently lacks the attracting compounds. A third mode of budworm resistance is antibiosis. Cultivars identified with this type of resistance were TI-163, TI-165, TI-168, and TI-170 (Johnson and Severson, 1984; Johnson et al., 1992, 1995). However, the antibiosis mode of resistance in these lines was unknown.



Compound A



Compound B

Figure 1. Structures of compounds **A** and **B** (16-hydroxygeranyllinalool glycosides).

This paper reports the isolation from TI-165 and the subsequent identification and HPLC analyses of two diterpene glycosides (designated compounds **A** and **B**, Figure 1) that have been shown to correlate with *H. virescens* resistance both in field-grown tobacco and in laboratory dietary bioassays. We also report the results of an HPLC survey for related diterpene glycosides in the leaves of the *Nicotiana* species.

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MATERIALS AND METHODS

All solvents were analyzed reagent grade. All tobacco cultivars were grown at the Pee Dee Research and Education Center, Clemson University, Florence, SC, under normal cultural practices. *Nicotiana* species were grown at the Department of Crop Science, North Carolina State University, Oxford, NC.

Leaf Sampling Procedure. Tobacco leaves were sampled when they reached 12−15 cm in length. Five 2-cm disks were removed with a cork borer, one disk/leaf from five plants. For leaf position sampling studies, one disk was removed from leaves of the appropriate leaf length from five different plants. The disks were weighed and placed in a scintillation vial (fitted with a Teflon cap-liner), 15 mL of methanol was added, and the vials were stored at 0 °C. Thymol (50 μL containing 0.125 mg of thymol in methanol) was added to the samples as an internal standard. The disks were then cut into small pieces and ground with a Virtis blade grinder (Gardiner, NY). Samples were filtered into autoinjector vials for HPLC analysis. *Nicotiana* species samples (obtained from a previous study; Snook et al., 1986) consisted of field-grown plants from which mature leaves were obtained, freeze-dried, and ground in a Wiley mill to pass a 40-mesh screen. Ten milliliters of MeOH was added to 50 mg of leaf material. Thymol was added as an internal standard as above. Samples were extracted by ultrasonication for 20 min and then filtered before analysis.

HPLC Analyses. The ground methanol leaf extracts were analyzed on a Beckman Ultrasphere C_{18} reversed-phase column: isocratic solvent 60% MeOH/H_2O (0.1% H_3PO_4) for 25 min, 1.5 mL/min flow rate. The column was flushed with 100% MeOH for 10 min and recycled at 60% MeOH/H_2O for 12 min. Retention time for Compounds **A** and **B** was dependent on the age of the HPLC column. The column effluent was monitored at 210 nm. Quantitation was done according to the internal standard method, using thymol, on a Hewlett-Packard 1090 HPLC equipped with a diode array detector.

Isolation and Identification of Compounds A and B. *A. Isolation of Compounds **A** and **B**.* Approximately 1.5 kg of fresh TI-165 leaves (all leaves were 15 cm or less in length) were slurried in a Waring blender with 12 L of MeOH and filtered, and the extract was evaporated until approximately 300 mL remained. The concentrated extract was treated with CH_2Cl_2 (3 × 200 mL) to remove chlorophyll and then taken to 100 mL. The final concentrate was submitted (3 × 33 mL) to preparative reversed-phase column chromatography. Approximately 100 g of the packing material from a Waters PrepPAK 500 C_{18} cartridge (Millipore Corp., Milford, MA) was repacked into a smaller glass chromatography column (54 × 2.54 cm, 15 psi of nitrogen pressure used to aid flow), washed with MeOH, and recycled to H_2O. The column was eluted with 1 L of H_2O, 1 L of 40% MeOH/H_2O, and finally 1 L of 65% MeOH/H_2O. The 65% MeOH/H_2O effluent from all three runs was combined and evaporated to dryness. The residue was dissolved in MeOH, 50 g of silicic acid (SA, Mallinckrodt, 100 mesh, washed with methanol and activated at 155 °C for 1 h) added and evaporated to dryness to produce a SA/sample deposited mixture and submitted to SA column chromatography. The column (3 × 40 cm) was packed (100 g of SA) in ethyl acetate and, after the sample was applied to the top of the column (as a SA/sample deposited mixture), eluted with the following solvents: 1 L of ethyl acetate; 1 L of ethyl acetate/acetone (1:1 v/v); 2.5 L of acetone; 2 L of MeOH/acetone (1:9 v/v). **A** and **B** were found in the last two eluting solvents. The purity of **A** and **B** in the acetone fraction was sufficient for studies involving a mixture of the two compounds.

*B. Separation of Compound **A** from Compound **B**.* Pure samples of compound **A** and compound **B** were obtained by preparative, centrifugally accelerated, radial, thin-layer chromatography (Harrison Research Chromatotron, Palo Alto, CA). Plates were coated with silica gel 60, PF_{254} w/gypsum (EM Science, Gibbstown, NJ) to give a 2-mm thickness, washed with MeOH, and dried at 70 °C for 1 h. Samples of **A** + **B** (100 mg/2 mL of MeOH) were then applied in a narrow band to the center of the plate with a syringe, and the MeOH was evaporated at 70 °C. After the spinning plate was wetted with CH_2Cl_2, a linear solvent gradient was performed from 13.5% MeOH/CH_2Cl_2 to 16% MeOH/CH_2Cl_2 over 220 min (3 mL/min flow rate; 5-mL fractions collected). Fractions were monitored by HPLC (after the CH_2Cl_2 was evaporated and the residue was redissolved in MeOH). Pure **A** was obtained from a single Chromatotron run. Pure **B** required two or three Chromatotron separations. Evaporation of CH_2Cl_2/MeOH solvents from compound **A** or **B** produced a clear, glasslike residue, probably due to tightly bound water. This water was conveniently displaced by dissolving the residue in MeOH and adding an equal amount of acetonitrile. Upon evaporation of this solution, an amorphous white powder was obtained.

*C. Fast Atom Bombardment (FAB) Mass Spectrometric (MS) Analyses on Compounds **A** and **B**.* All MS were obtained using a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10-kV accelerating potential. Ions were produced by FAB with xenon using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired for the first MS are averaged profile data of three scans as recorded by a JEOL complement data system. These spectra were acquired from *m*/*z* 200 to 2000 at a rate that would scan from *m*/*z* 0 to 2500 in 1 min. A filtering rate of 100 Hz and an approximate resolution of 1000 (a 10% valley) were used in acquiring these spectra. Samples were dissolved in a thioglycerol matrix for analysis.

*D. Acid Hydrolysis of Compounds **A** and **B**.* Compounds **A** and **B** (1 mg each) were treated with 1 mL of 0.05 N HCl and heated at 100 °C for 1, 2, and 4 h. At each interval, 250 μL was removed and blown to dryness under a stream of nitrogen. Liberated sugars were analyzed by conversion to their silyl derivatives by adding 10 μL each of dimethylformamide and BSTFA and heating at 75 °C for 30 min. Derivatized sugars were analyzed by gas chromatographic analyses on an immobilized SE-54 (30 m × 0.3 mm i.d.) capillary column prepared according to the method of Arrendale et al. (1988): injector, 250 °C; detector, 350 °C, temperature program, 100−300 at 8 °C/min. Only rhamnose and glucose were found in the acid hydrolysates. Appropriate response factors were determined for rhamnose and glucose. Compound **A** gave a rhamnose/glucose ratio of 1.16:1, while compound **B** gave a ratio of 3.2:2.

*E. Sugar Linkage Analyses of Compounds **A** and **B**.* Linkage analysis of the sugar residues on **A** and **B** were carried out by Dr. Russell W. Carlson (Complex Carbohydrate Research Center, University of Georgia, Athens, GA) (Hakamori, 1964; York et al., 1986). The procedure entailed permethylation of 1 mg of **A** or **B** with MeI-DMSO, hydrolysis with trifluoroacetic acid, $NaBD_4$ reduction, acetylation with Ac_2O/pyridine, and analysis of the liberated sugar moieties by capillary GC/MS. The linkage analyses (together with the FAB/MS data) showed that in compound **A** there were originally two terminally linked rhamnoses, one 6-linked glucose, and one 4-linked glucose (see Figure 1). Compound **B** contained three terminally liked rhamnoses, one 4-linked glucose, and one 4,6-linked glucose.

F. ^1H and ^{13}C NMR Analyses. All NMR data were acquired on a Bruker AMX400 spectrometer (400.13 MHz, ^1H) using 30 mg of each of the compounds dissolved in 0.5 mL of dimethyl-d_6 sulfoxide (DMSO-d_6). Samples were also prepared with a drop of deuterium oxide (D_2O) in the DMSO-d_6 solvent. ^1H and ^{13}C chemical shifts at 25 °C were referenced to TMS, via the DMSO resonance frequency at 2.49 and 39.5 ppm, respectively. 2D ^1H COSY (correlation spectroscopy; Aue, et al., 1976) and TOCSY (total correlation spectroscopy; Bax and Davis, 1985b) were carried out at 25 °C using a spectral width of 2.8 kHz for both dimensions, while 2D ROESY experiments (rotation frame nuclear Overhauser enhancement spectroscopy; Bax and Davis, 1985a) were accquired with a spectral width of 4.8 kHz. For 2D TOCSY experiments, a spin lock field of 10 kHz was used during DIPSI-2 (decoupling in the presence of scalar interactions; Shaka et al., 1988) mixing time of 60 ms, which includes 1-ms trim pulses. ROESY spectra were recorded with a spin lock field of 1.8 kHz during the 300-ms mixing time. For 2D $^1H-^{13}C$ heteronuclear experiments, a spectral width of 16 kHz was used in the ^{13}C dimension for HMQC (heteronuclear multiple quantum coherence; Bax et al., 1983) and HMQC-TOCSY (Lerner and Bax, 1986); a 20.8-kHz spectral width was used for HMBC (heteronuclear multiple

bond correlation; Bax and Summers, 1986). $^{1}H-^{13}C$ coupling constants of 150 and 10 Hz were used in HMQC and HMBC experiments, respectively. Quadrature detection in the indirectly observed dimensions was obtained using States−TPPI methods (Marion et al., 1989) for all 2D experiments. From the above data, compounds **A** and **B** were determined to be 16-hydroxygeranyllinalyl-3-*O*-α-L-rhamnosyl(1→4)-β-D-glucoside-16-*O*-α-L-rhamnosyl(1→6)-β-D-glucoside (compound **A**) and 16-hydroxygeranyllinalyl-3-*O*-α-L-rhamnosyl(1→4)-β-D-glucoside-16-*O*-[α-L-rhamnosyl(1→6)]-[α-L-rhamnosyl(1→4)]-β-D-glucoside (compound **B**).

Laboratory Bioassay Procedure. Purified samples of **A** and **B** or mixtures were dissolved in MeOH and deposited onto hydrolyzed Celufil (a non-nutritive cellulose; U.S. Biochemical Corp., Cleveland, OH) at 200, 100, 50, and 25 mg each on 2 g of Celufil. Solvent-treated Celufil was used as a blank control. The deposited samples were mixed with 20 g of diet composed of the following: water (600 mL), agar (9 g), Vanderzant wheat germ diet (68 g), cholesterol (0.25 g), methyl *p*-hydroxybenzoate (0.5 g), sorbic acid (0.5 g), tetracycline (30.8 mg), vitamins (6.6 g), and 1 N KOH (16 mL). The mixed diet was poured into soda straws (19.5 cm × 6 mm diameter). After solidification, the straws were cut into 3-cm sections and placed into 1-oz diet cups and one neonate budworm larva was added; four larvae/rep and 6 reps were used. Weights of larvae were recorded after 7 days and results plotted as percent of control Celufil diet.

A leaf disk bioassay was also performed. A 2-cm disk was removed from a 12−15-cm length of tobacco leaf with a cork borer and placed in a Petri dish. One neonate budworm larva was added, and larval weights were recorded as above.

RESULTS AND DISCUSSION

By comparing levels of numerous leaf chemical constituents (both surface and internal) of budworm-susceptible NC2326 and budworm-resistant TI-165, it was determined that TI-165 contained two compounds, revealed by HPLC monitoring at 210 nm and designated compounds **A** and **B**, that were absent in NC2326 (Figure 2). Further, related resistant lines TI-163, TI-168, and TI-170 also were found to contain high levels of **A** and **B**. Typical HPLC chromatograms of budworm-susceptible NC2326 and budworm-resistant TI-165 tobacco are shown in Figure 2. The presence of **A** and **B** in various tobacco lines and their associated budworm resistance prompted us to isolate and identify these compounds.

Compounds **A** and **B** were isolated from young TI-165 tobacco leaves by a combination of solvent partitioning, preparative reversed-phase, and silicic acid column chromatography. Compound **A** was separated from compound **B** by preparative, centrifugally accelerated, radial, thin-layer chromatography (Chromatotron). Acid hydrolyses showed both compounds were glycosides containing only rhamnose and glucose. Sugar linkage analyses indicated compound **A** had a rhamnose/glucose ratio of 1:1, while compound **B** had a rhamnose/glucose ratio of 3:2. Determination of the molecular weights of the compounds by FAB/MS was difficult due to the formation of thioglycerol, ammonia, sodium, and potassium adducts (Figures 3 and 4). FAB/MS of hydrogenated **A** and **B** revealed the correct molecular weight of compound **A** as 922 and that of compound **B** as 1068. The difference in mass between the original compounds and their hydrogenated derivatives showed that both **A** and **B** contained four double bonds. It was also evident from the molecular weights and the rhamnose/glucose ratios that compound **A** had two rhamnoses and two glucoses, while compound **B** had three rhamnoses and two glucoses. Linkage analyses of the compounds showed that in compound **A**, there were two diglycosides with the configuration rhamnosyl(1→4)glucoside and rhamnosyl(1→6)glucoside. Similarly, compound **B** had two diglycosides with the configuration rhamnosyl(1→4)glucoside and [rhamnosyl(1→6)][rhamnosyl(1→4)]glucoside.

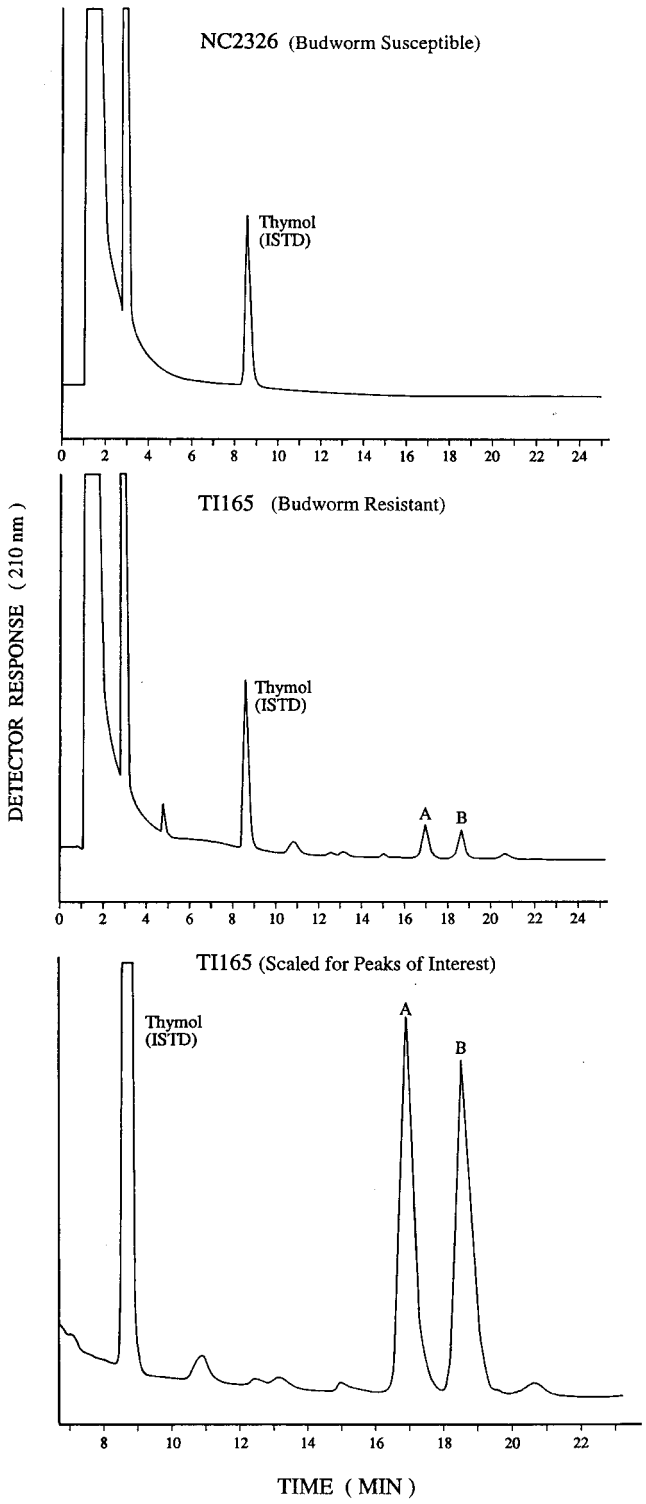

Figure 2. HPLC profiles of budworm-susceptible NC2326 tobacco and budworm-resistant TI-165 methanol leaf disk extracts.

NMR spectral assignments of the sugars were made on the basis of TOCSY (Figure 5), COSY, HMQC, HMQC-TOCSY (Figure 6), and ROESY (Figure 7) experiments. TOCSY spectra (Figure 5, compounds **A** and **B**) were used first to establish scalar coupled spin systems within sugar moieties and segments of the backbone. Four hexose sugar spin systems were observed in compound **A** (dissolved in DMSO-d_6/D_2O) in which protons of OH groups were exchanged with

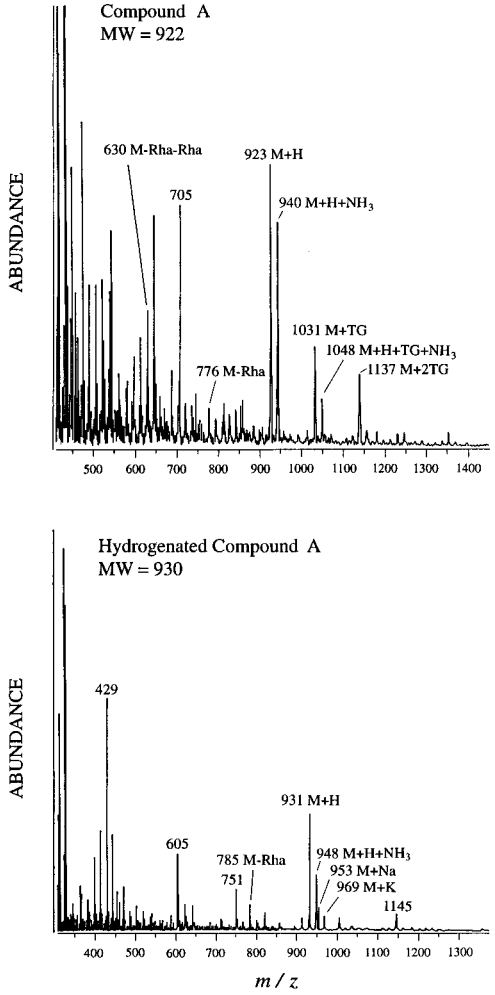

Figure 3. FAB mass spectra of compound **A** and hydrogenated compound **A**.

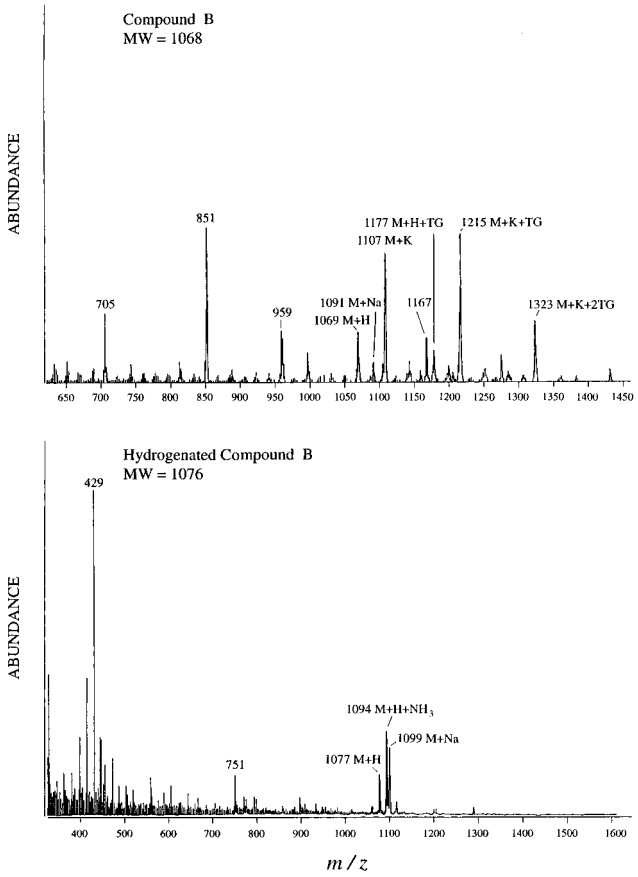

Figure 4. FAB mass spectra of compound **B** and hydrogenated compound **B**.

deuterium, while five hexose sugar spin systems were observed in sample B. COSY spectra (not shown) were then used to identify the primary scalar coupling within the spin systems. The assignments of the sugar proton resonances began with the anomeric signals in the chemical shift range of 4.0−4.7 ppm. Initiated at resonance frequency of 4.15 ppm of compound **A**, H-2 proton was assigned on the basis of the COSY cross peak to H-1 proton. A $^1H-^1H$ *J*-coupling constant of 9 Hz between H-1 and H-2 (J_{12}) indicates a β-D-linkage. H-3 through H-5 protons of the ring were also assigned on the basis of the COSY cross peaks from these protons to their vicinal protons and their vicinal *J*-coupling constants shown in Table 1 (proton−proton coupling constants of the sugars). CH_2 protons of C-6 were assigned on the basis of COSY correlations to H-5 and the correlations of these two protons to a single carbon in HMQC spectra. The assignment of this sugar to be glucose (glc) is made on the basis of the cross peak multiplicity of individual protons and their *J*-coupling constants with adjacent protons. This assignment is also supported by the strong ROESY cross peaks of H-1 to H-2 and H-5. Another glucose (glc′) with a β-D-linkage was assigned using a similar procedure. The differentiation in chemical shift patterns of these two glucose sugars indicates that they connect to other segments at different positions in the sugar rings. This was also supported by the analysis of ROESY spectra of the compounds (see below). Two rhamnosyl units were identified for compound **A** with H-1 proton resonance frequency of 4.54 and 4.67 ppm, while three rhamnosyl units were identified for compound **B** with H-1 proton resonance frequency of 4.58, 4.62, and 4.67 ppm. The H-5 protons of the rhamnoses were assigned by the COSY cross peaks to a methyl proton signal. The assignments of H-2, H-3, and H-4 of the rhamnoses were obtained using the same procedure as for the assignments of the glucose protons. The $^1H-^1H$ *J*-coupling constant of <2 Hz between H-1 and H-2 in all rhamnoses indicates that the sugars adopt an α-L-linkage.

The analysis of COSY, TOCSY, and HMQC-TOCSY spectra showed that there were three repeated butene units in both compounds **A** and **B** (Figure 6, HMQC-TOCSY spectra of compound **A**). The positions of methyl groups attached to the butene were assigned on the basis of HMBC cross peaks of the methyl protons to the quaternary butene carbon and ROESY cross peaks of the methyl protons to the butene proton (=CH proton) (Figure 7, ROESY spectra of compound **A**). The connections between these units were assigned on the basis of the HMBC cross peaks of the methyl protons to CH_2 carbons and ROESY cross peaks of the methyl protons to the geminal protons. The HMBC cross peak of C-15 to a pair of geminal protons and ROESY cross peaks of methyl H-20 to the same pair of protons assured the assignment of the H-16 protons. The methylpropylene segment starting the backbone was assigned by the COSY cross peak between propylene H-1 and H-2 protons and HMBC cross peaks of the quaternary carbon C-3 to the methyl and propylene protons. As a result, the backbone was determined to be 16-hydroxygeranyllinalool. The stereoconformation of the methypropylenes was determined on the basis of strong ROESY cross peaks observed for methyl protons (H-20) to H-14, geminal H-16 to geminal H-13, geminal

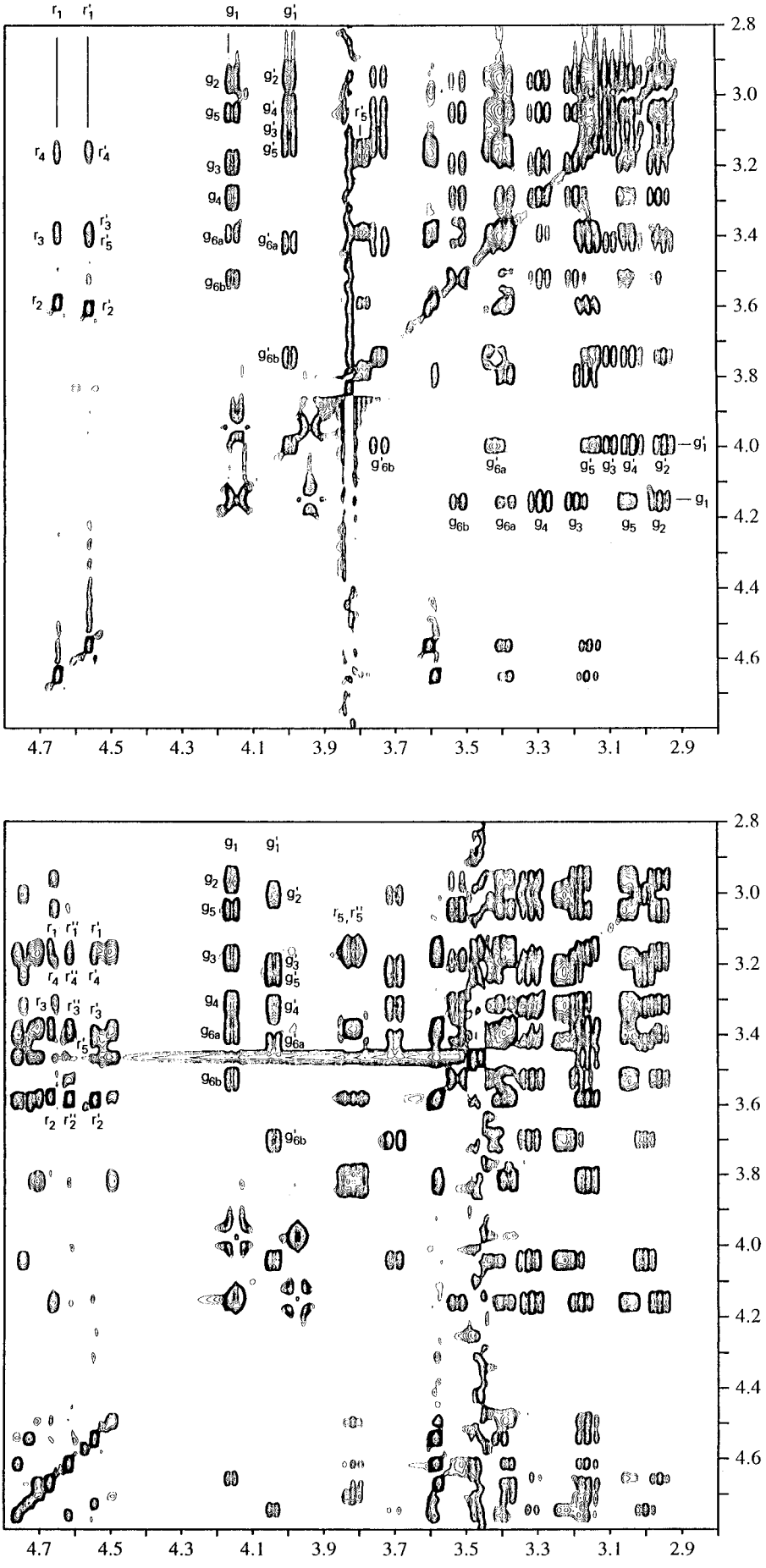

Figure 5. 2D TOCSY NMR spectra of compounds **A** (top) and **B** (bottom). The assigned cross peaks of sugar protons (r = rhamnose; g = glucose) are labeled for both compounds.

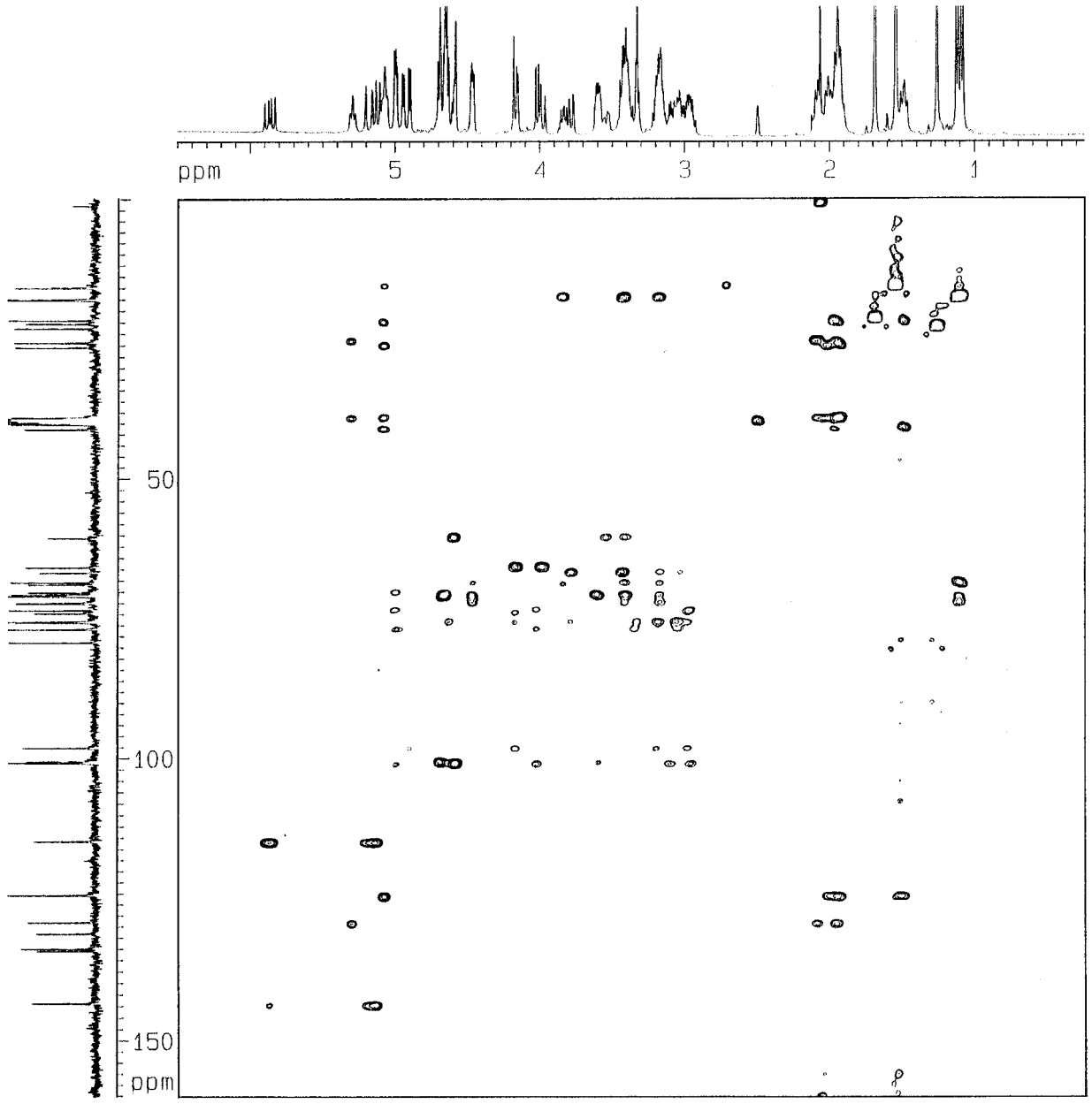

Figure 6. 2D HMQC-TOCSY NMR spectrum of compound **A**. Shown at the top and left margins of the 2D spectrum are 1D ^{1}H and ^{13}C spectra.

H-12 to H-10, methyl H-19 to geminal H-9, geminal H-8 to H-6, and methyl H-18 to geminal H-5.

Connectivity of the individual sugar and backbone structural units was determined. The long-range $^{1}H-^{13}C$ coupling of H-1 proton of glucose (glc) to the C-3 of the hydroxygeranyllinalool was observed in the HMBC spectra, and ROESY cross peaks were also observed for the H-1 of glucose (glc) to the protons attached to carbons at positions 1, 2, and 17 in both compounds **A** and **B**. These data indicate that the glc H-1 attaches to the hydroxygeranyllinalool at position 3. The linkage of the other glucose (glc′) to the backbone at position 16 was determined in both compounds, on the basis of the long range $^{1}H-^{13}C$ coupling of H′-1 of glc′ to the C-16 of the hydroxygeranyllinalool and strong ROESY cross peaks of the glc′ H′-1 proton to the protons attached to carbons at positions 16 and 20. The linkages between the sugars were determined by ^{1}H ROESY connectivities between sugar residues. The rhamnosyl linkages were determined to be (1→4) and (1′→6′) in compound **A**, on the basis of the strong ROESY connectivities of anomeric rha H-1 to glc H-4, and anomeric rha′ H′-1 to glc′ H′-6 and to be (1→4), (1′→4′), and (1″→6′) in compound **B**, on the basis of the strong ROESY connectivities of anomeric rha H-1 to glc H-4, rha′ H′-1 to glc′ H-4, and rha″ H″-1 to glc′ H-6. The rhamnosyl linkages are also supported by the results of the sugar linkage analysis. ^{1}H and ^{13}C chemical shifts and $^{1}H-^{1}H$ coupling constants are listed in Table 1 except for some rhamnosyl carbons of compound **B** due to severe degeneracy of their resonances along both the ^{1}H and ^{13}C dimensions in the HMQC spectrum.

Recently, Shinozaki et al. (1996) reported the isolation and characterization of two hydroxygeranyllinalool glycosides from Burley tobacco. Previous to this paper, Tobita et al. (1993), at a Tobacco Chemists' Research Conference, reported on these two diterpene glycosides and a third, different but related, compound from *Nicotiana umbratica*. Their reported "compound II" appears to be the same as our compound B. However, our compound **A** has a sugar configuration different from any of their other reported structures.

HPLC analyses of tobaccos with different levels of **A** and **B** were compared to budworm larval weight (leaf

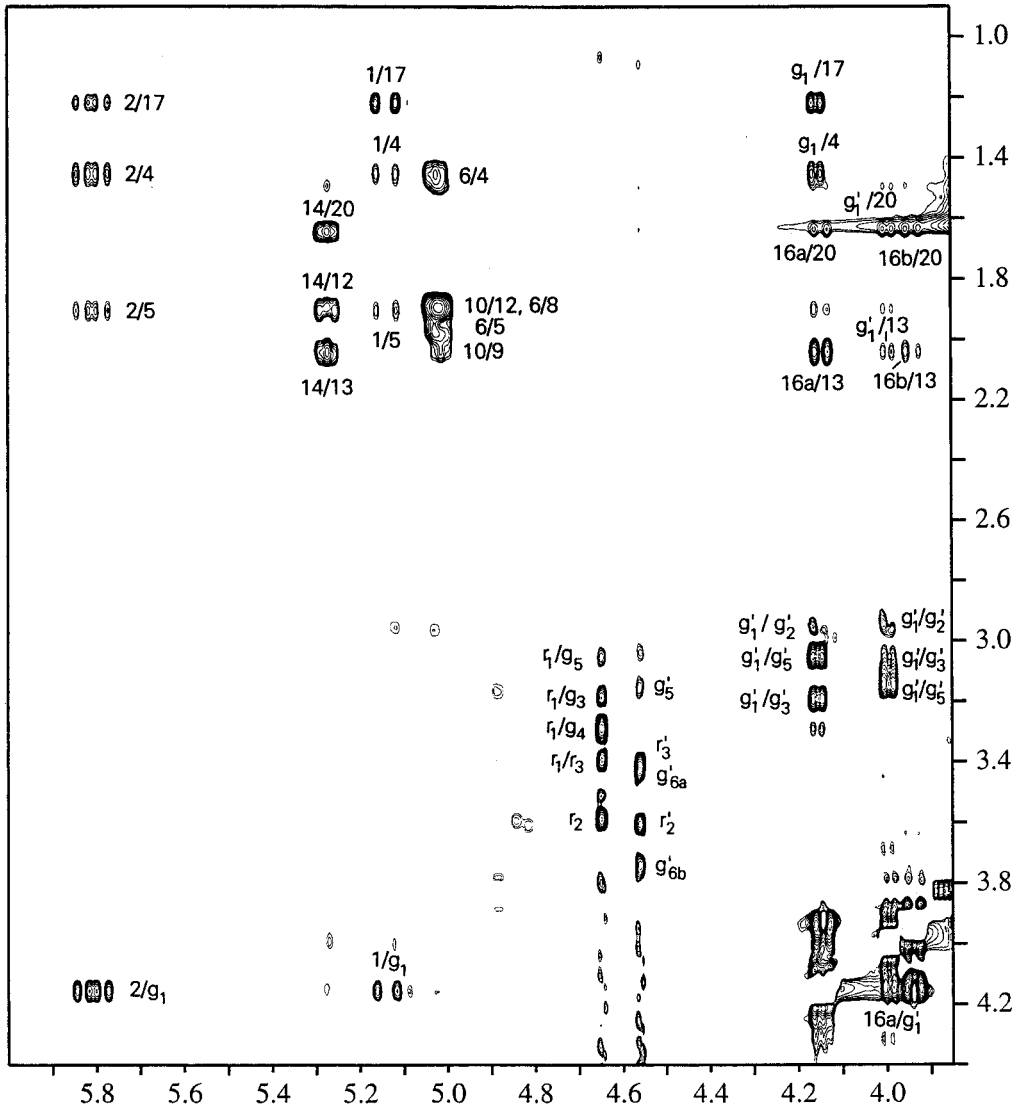

Figure 7. Portions of the 2D ROESY NMR spectrum of compound **A**. The assigned cross peaks are labeled as 7/9 representing the cross peak of H-7 to H-9 in the hydroxygeranyllinalool backbone; $2/g_1$ refers to the cross peak of H-1 to glucose H-1.

disk bioassay) (Table 2). A significant correlation was found between levels of **A** and **B** and larval weights (−0.804, compound **A**; −0.813, compound **B**; −0.826, compounds **A** + **B**). Table 3 shows that **A** and **B** were concentrated in the upper leaves of the tobacco plant. Female *H. virescens* moths prefer to lay eggs on or near the flowering or fruiting portions of tobacco plants (Jackson and Severson, 1989) and, therefore, the concentration of high levels of compounds **A** and **B** in the uppermost leaves is precisely what is needed by the plant to impart resistance.

The isolated hydroxygeranyllinalool glycosides were bioassayed by depositing the purified compounds onto Celufil and incorporating this mixture into a standard laboratory budworm diet. The results of these experiments are shown in Figure 8. A mixture of **A** and **B** almost completely inhibited growth of the larvae at only 9.2 mM concentration and exhibited a correlation ($r = -0.92$, $P < 0.01$) for reduced larval weights versus concentration of the mixture of A + B. Hydrogenation of the double bonds in the mixture of **A** and **B** resulted in a complete loss in activity, showing that the activity resides in the hydroxygeranyllinalool moiety of the molecule (Figure 8). Compound **A** appeared to be slightly more active than compound **B**. On the basis of millimolar concentrations, the activity of the individual compounds would be expected to equal the mixture of **A** and **B** since they differ only by one rhamnose. This extra rhamnose could make compound B slightly less active; however, it may be that the difference in activity of the individual compounds versus the mixture is due to the extra chromatographic separation required to obtain the pure compounds. The related aglycon of compounds **A** and **B**, geranyllinalool (obtained from Fluka, Ronkonkoma, NY), was tested in the laboratory diet bioassay. It was found to be approximately one-seventh less active than the mixture of A + B, requiring a concentration of 28 mM to achieve 75% growth inhibition versus only 4 mM concentration for a similar level of activity for A + B.

Tobita had reported that thin-layer chromatography results had indicated several *Nicotiana* species contained diterpene glycosides (Tobita et al., 1993). Thus, it was of interest to survey the *Nicotiana* species for diterpene glycosides. HPLC analyses of freeze-dried leaves indicated that a number of species had high levels of components with HPLC characteristics similar to those of **A** and **B**, indicative of diterpene glycosides. In addition to exhibiting peaks with the same HPLC retention times as **A** and **B**, at least four other compounds, which are undoubtedly related to **A** and **B**, were found in various species. Chromatographic examples

Table 1. ^1H and ^{13}C NMR Chemical Shift Assignments for Compounds A and B ([$^2H_6$]Dimethyl Sulfoxide)

^1H Assignments

	compound **A**				compound **B**				
	glc	glc'	rha	rha′	glc	glc′	rha	rha′	rha″
H-1	4.77	4.01	4.66	4.58	4.16	4.05	4.55	4.67	4.62
H-2	2.97	2.96	3.59	3.62	2.96	3.01	3.59	3.58	3.58
H-3	3.20	3.10	3.40	3.39	3.18	3.20	3.39	3.38	3.38
H-4	3.30	3.06	3.17	3.16	3.32	3.33	3.18	3.16	3.17
H-5	3.06	3.15	3.80	3.42	3.04	3.23	3.42	3.81	3.82
H-6	3.40	3.42	1.07	1.06	3.39	3.43	1.11	1.08	1.08
	3.53	3.75			3.53	3.70			

Backbone Carbon Numbers

C-1	5.13	C-2	5.83	C-4	1.49	C-1	5.14	C-2	5.85	C-4	1.47
C-5	1.96	C-6	5.07	C-8	1.94	C-5	1.94	C-6	5.05	C-8	1.92
C-9	2.01	C-10	5.07	C-12	1.95	C-9	1.99	C-10	5.05	C-12	1.92
C-13	2.09	C-14	5.30	C-16	3.98, 4.17	C-13	2.06	C-14	5.29	C-16	3.97, 4.15
C-17	1.25	C-18	1.54	C-19	1.54	C-17	1.25	C-18	1.52	C-19	1.52
C-20	1.68					C-20	1.67				

^{13}C Assignments

	compound **A**				compound **B**[a]				
	glc	glc′	rha	rha′	glc	glc′	rha	rha′	rha″
C-1	98.0	100.8	100.4	100.7	98.2	100.8	100.2	100.7	100.7
C-2	73.8	73.2	70.7	70.7	73.7	73.2	70.7	70.7	70.7
C-3	75.4	76.7	68.3	68.3					
C-4	76.7	70.7	71.9	72.0					
C-5	75.4	75.4	68.6	70.6					
C-6	60.3	66.5	15.8	15.7	60.5	66.5	16.1	16.1	16.1

Backbone Carbon Numbers

C-1	114.6	C-2	143.4	C-3	79.1	C-1	115.1	C-2	143.5	C-3	79.5
C-4	41.1	C-5	22.0	C-6	124.3	C-4	41.3	C-5	22.3	C-6	124.7
C-7	134.3	C-8	39.1	C-9	26.2	C-7	134.6	C-8	39.1	C-9	26.4
C-10	124.3	C-11	133.9	C-12	39.1	C-10	124.6	C-11	134.3	C-12	39.1
C-13	25.3	C-14	129.2	C-15	131.2	C-13	25.8	C-14	129.7	C-15	131.4
C-16	65.6	C-17	22.9	C-18	17.8	C-16	65.6	C-17	23.1	C-18	18.0
C-19	17.9	C-20	21.4			C-19	18.0	C-20	21.7		

[a] ^{13}C Sugar carbon resonances not listed due to severe degeneracy.

Table 2. Levels of Compounds A and B in Varieties and Various Breeding Lines versus Leaf Feeding by Budworms

variety or line	larval weights[a] (mg)	levels (μg/cm^2)		
		A	**B**	**A + B**
A	62	35	12	47
B	71	2	1	3
C	49	28	22	50
D	41	20	17	37
E	36	24	17	41
F	25	44	43	87
G	20	43	40	83
H	11	51	43	94
I	24	44	37	81
J	4	44	36	80
K	59	43	35	78
L	36	45	43	88
M	48	19	11	30
N	77	26	16	42
O	68	20	13	33
P	34	45	23	68
Q	41	30	17	47
NC95	80	0	0	0
NC2326	117	0	0	0
correlation[b]		−0.804	−0.813	−0.826

[a] Leaf disk bioassay; four disk/leaf; one neonate worm/disk; four leaves/rep; four reps. Weights are average of remaining live larvae. [b] Correlation between levels of **A** and **B** and larval weight ($P < 0.0001$) by simple linear regression analysis of variance.

Table 3. Concentration of Compounds A and B versus Leaf Position in TI-165

leaf position[a]	leaf length (cm)	levels[b] (μg/cm^2)		
		A	**B**	**A + B**
1	5−7.5	54	81	135
2	7.5−10	33	45	78
3	10−15	30	33	63
4	20−25	30	25	55
5	25−30	27	19	46
6	37−45	15	9	24
7	>50	4	1	5

[a] Position 1 = top bud leaf. [b] Average of four reps.

of these four types are shown in Figure 9. *N. cavicola* was found to have two compounds that elute after **A** and **B** (designated C and D), while *N. otophora* contained large amounts of the second of the two later eluting compounds. Analysis of *N. plumbaginifolia* leaves revealed a compound that eluted slightly earlier than **A** (designated **A′**), while the major diterpene glycoside of *N. umbratica* eluted between **A** and **B** (designated **A″**). Tobita et al. (1993) reported that *N. umbratica* contained one major hydroxygeranyllinalool glycoside, 16-hydroxygeranyllinalyl-3-*O*-β-D-glucoside-16-*O*-[α-L-rhamnosyl(1→6)][α-L-rhamnosyl(1→4)]-β-D-glucoside. This is an isomer of compound **A** in which the rhamnose is attached to the 16-*O*-glucose rather than the 3-*O*-glucose. One would expect an isomer of compound **A** to elute from the HPLC near **A**, and we in fact did observe a major peak eluting only 1 min after **A** (Figure 9, designated **A″**). Knowing the structures of **A**, **B**, and the compound from *N. umbratica* (**A″**), we can postulate the structures of the compounds in the other species. The compound from *N. plumbaginifolia* (**A′**) either has one fewer rhamnose than compound **A**

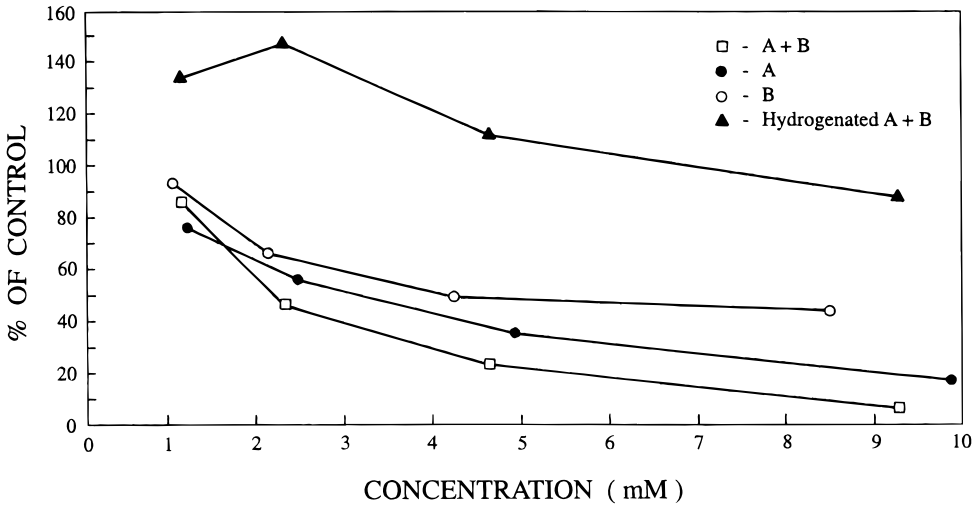

Figure 8. Concentration of compounds **A**, **B**, **A** + **B**, and hydrogenated **A** + **B** in laboratory bioassay diets versus budworm larval weights (7 day; expressed as percent of control). Control weights (average of remaining live larvae): **A** + **B** study = 45.5 mg; **A** study = 21.5 mg; **B** study = 20.9 mg; hydrogenated **A** + **B** study = 30.1 mg.

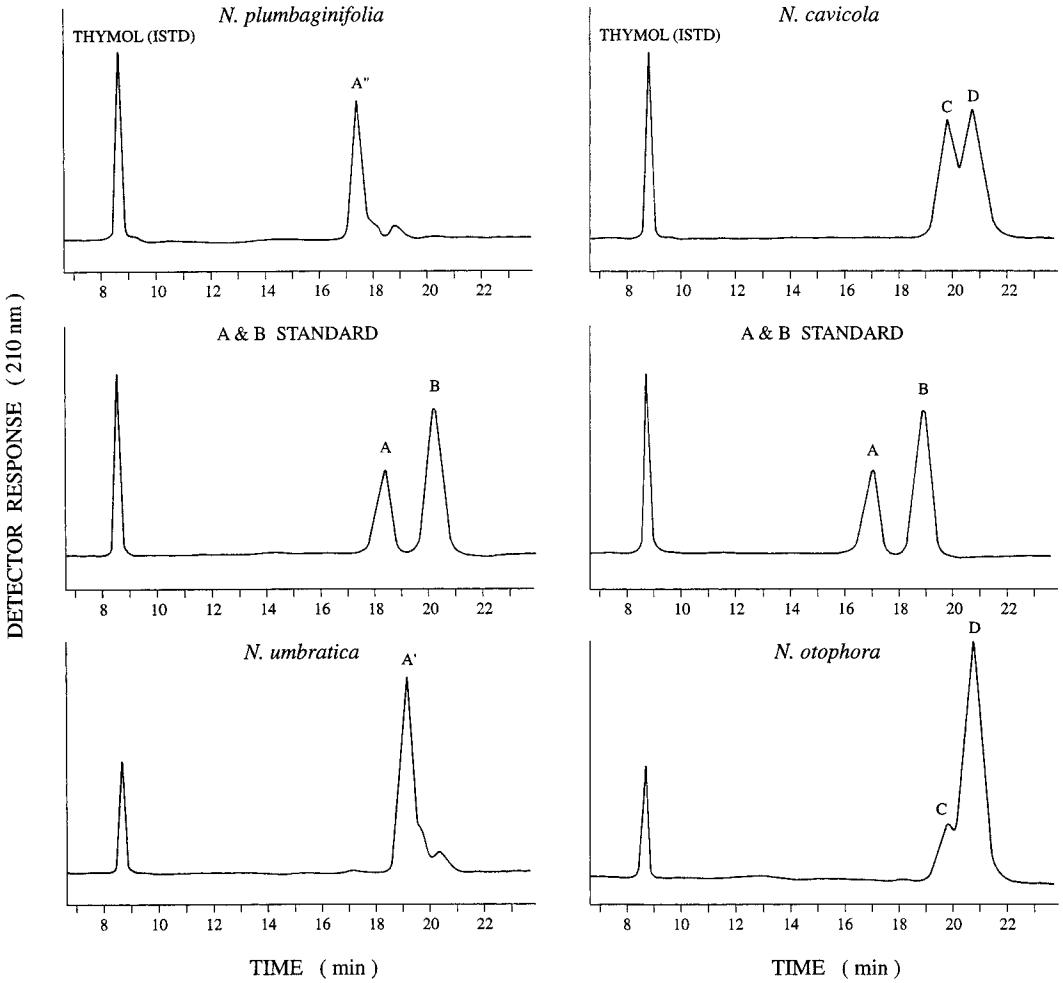

Figure 9. HPLC profiles of selected *Nicotiana* species containing different diterpene glycosides.

or one of the rhamnoses must be a glucose. The two compounds in *N. cavicola* (**C** and **D**) probably contain one more sugar residue than compound **B** with the earlier eluting peak possibly containing an extra glucose and the late eluting peak containing an extra rhamnose. The two peaks could, however, have identical sugar constituents but be different isomers, similar to the situation with compound **A** and the compound from *N. umbratica*.

Table 4 summarizes the results of our analyses for hydroxygeranyllinalool glycosides in the *Nicotiana* species. We found 26 species had appreciable levels of diterpene glycosides, while 29 species contained detectable levels of these compounds. Only 10 species were devoid of diterpene glycosides. It is anticipated that these diterpene glycoside isomers may be active against the budworm and the use of the *Nicotiana* species to introduce these diterpene glycosides into commercial tobacco or other crops would result in production of budworm-resistant plants.

Table 4. Results of *Nicotiana* Species Analyses for Diterpene Glycosides[a]

levels of diterpene glycosides[b] (% dry wt)		
>2.5%	trace to <2.5%	0%
N. acuminata[C,D]	*N. acaulis*[A]	*N. arentsii*
N. amplexicaulis[B]	*N. africana*[C]	*N. clevelandii*
N. attenuata[C,D]	*N. alata*[A]	*N. cordifolia*
N. benthamiana[A″,B]	*N. angustifolia*[C,D]	*N. eastii*
N. cavicola[C,D]	*N. benavidesii*[A,B]	*N. glauca*
N. debneyi (17D)[A,B]	*N. bigelovii*[A′,B]	*N. noctiflora*
N. excelsior[A″,B]	*N. bonariensis*[A′,A]	*N. rustica*
N. exigua[A,B,C]	*N. corymbosa*[C]	*N. solanifolia*
N. knightiana[A,C,D]	*N. forgetiana*[B]	*N. sylvestris*
N. glutinosa (24A)[C]	*N. fragrans*[B]	*N. wigandioides*
N. ingulba[A″,B]	*N. goodspeedii*[D]	
N. megalosiphon (32)[A,B,C,D]	*N. gossei*[A″,B]	
N. megalosiphon (32A)[A,A″,B]	*N. hesperis* (67A)[B]	
N. nudicaulis[A″,B]	*N. kawakamii*[C]	
N. occidentalis[A]	*N. langsdorffii*[C]	
N. otophora[C,D]	*N. linearis*[B]	
N. paniculata (40C)[C,D]	*N. longiflora*[A′,A]	
N. pauciflora[C,D]	*N. maritima*[C]	
N. plumbaginifolia[A′,A]	*N. miersii*[A,C]	
N. rosulata (53)[A,B]	*N. palmerii*[B,C]	
N. simulans[A,B,C]	*N. petunioides*[A′,A]	
N. tomentosa (58)[C,D]	*N. raimondii*[B,C]	
N. tomentosa (58A)[A′,A]	*N. repanda*[B]	
N. tomentosiformis[C,D]	*N. rotundifolia*[B,C]	
N. trigonophylla[C]	*N. sanderae*[B]	
N. undulata (61B)[C,D]	*N. setchellii*[C]	
N. umbratica[A″,B]	*N. spegazzinii*[A,C]	
N. velutina[B]	*N. stocktonii*[B]	
	N. suaveolens[C,D]	

[a] Numbers in parentheses are *Nicotiana* Species Seed Acquisition Numbers, Department of Crop Science, North Carolina State University, P.O. Box 1555, Oxford, NC 27565. [b] Letter codes A and B refer to peaks, observed in the chromatograms of the species, with the same HPLC retention times as compounds **A** and **B**. Other letter codes refer to postulated diterpene glycoside designations mentioned in the text and in Figure 9.

CONCLUSIONS

Hydroxygeranyllinalool glycosides were identified and implicated as the antibiosis factor in several budworm-resistant tobacco cultivars by correlational studies of leaf levels versus larval weights of budworm leaf feeding and larval weights versus compound concentration in laboratory diet bioassays.

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