Characterization of the Sucrose Ester Fraction from *Nicotiana glutinosa*

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Investigation of green leaf cuticular chemicals revealed that several of the 65 *Nicotiana* species produced sucrose ester mixtures varying widely in both the composition of the acid moieties and the position of attachment of the acid moieties to the hydroxyl groups of the sucrose molecule. The sucrose ester (SE) fraction from one of the more interesting species, *Nicotiana glutinosa*, was examined in detail. The leaf surface chemicals of green, field-grown plants were removed with methylene chloride, and this whole leaf wash was partitioned between hexane/80% methanol–water to yield a crude SE fraction. Chromatography of the crude polar fraction on Sephadex LH-20 yielded two SE-containing fractions, designated fractions I and II SE. Fractions I and II SE were analyzed by capillary GC/MS after conversion to their trimethylsilyl (TMS), *tert*-butyldimethylsilyl (TBDMS), and peracetate derivatives. The acid distributions of the two SE fractions were determined by capillary GC analyses of the free acids, liberated by saponification. The fractions were also analyzed by fast atom bombardment mass spectrometry (FABMS) and ^{13}C NMR. The structures of fraction I SE contained one acetic acid group esterified to the fructose moiety in addition to three other aliphatic acids on the glucose portion of the sucrose, while fraction II SE only contained three aliphatic acids esterified to hydroxyl groups on the glucose moiety of the sucrose molecule.

Investigations on the cuticular waxes from green tobacco leaf of *Nicotiana tabacum* cultivars revealed three major classes of compounds (Severson et al., 1984a, 1985a,b). These included hydrocarbons, diterpenes, and sucrose esters (SE). The hydrocarbons consisted of C_{25}–C_{36} normal- and branched-chain aliphatic isomers, while the diterpenes were of the duvane and/or labdane types. The third class of major cuticular components, the SE, were first isolated from a tobacco introduction (TI), TI-165, which demonstrated resistance to the tobacco budworm (*Heliothis virescens* F.) (Severson et al., 1985a; Johnson and Severson, 1984). TI-165 produces a series of SE (six groups differing by 14 Da due to additional methylene groups in the acid moieties) in which the hydroxyl on carbon 6 of glucose is acetylated (esterified with acetic acid) and the hydroxyl groups on carbons 2, 3, and 4 are esterified with three molecules of C_3–C_7 aliphatic acids (Table I). The genome responsible for the production of the SE type produced by *N. tabacum* appears to have been inherited from the progenator species *Nicotiana tomentosiformis*, which also produces this same SE type. Subsequently, SE were found in the green leaf cuticular waxes of many commercial and experimental cultivars of *N. tabacum* (Severson et al., 1985b). Different types of SE have also been identified in trichome secretions of wild potato species (King et al., 1987, 1988). Commercial flue-cured and burley tobaccos produce low levels of SE, with C_4 and C_5 fatty acids as predominant esterifying groups, but cigar and Oriental cultivars produce higher levels and a wider variety of esters, with the most abundant acyl groups being C_5 and C_6 isomers. It is now widely accepted that SE are the precursors of the 6-*O*-acetyltriacylglucopyranosides (glucose esters) isolated from cured Turkish tobaccos. Along with these glucose esters, SE are the precursors of 3-methylvaleric and 3-methylbutyric acids, which are important flavor components of Turkish tobacco smoke (Schumacher, 1970; Rivers, 1981; Severson et al., 1985a). High

Table I [a]

Nicotiana species	sucrose ester structure	type
N. tabacum, *N. tomentosiformis*	glucose: 6 CH_2-O-C(=O)-CH_3; 2,3,4-OR; fructose: 1 CH_2OH, 6 CH_2OH, 3-HO	SE-I
N. glutinosa (fraction II)	glucose: 6 CH_2OH; 2,3,4-OR; fructose: 1 CH_2OH, 6 CH_2OH, 3-HO	SE-II
N. glutinosa (fraction I)	glucose: 6 CH_2OH; 2,3,4-OR; fructose: 1 CH_2OH, 6 CH_2OH, 3-O-C(=O)-CH_3	SE-III

[a] R = C_3–C_9 acids.

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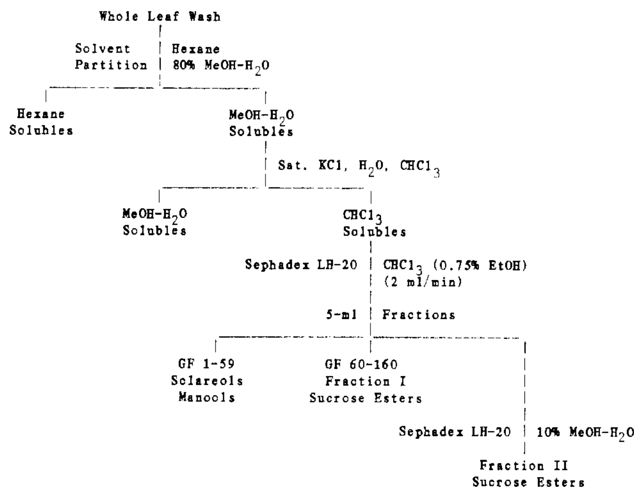

Figure 1. Isolation scheme for SE from *N. glutinosa*.

levels of SE in the cuticular waxes of *N. tabacum* cultivars have been implicated in resistance of these cultivars to the tobacco budworm by an antibiosis factor (Johnson and Severson, 1984). Recent studies have demonstrated that the SE from *N. tabacum* possess both antibiotic and plant growth regulating activities (Cutler et al., 1986).

Recent studies on the green leaf cuticular chemicals of the remaining *Nicotiana* species revealed several species that also produce SE (Severson et al., 1984b, 1987; Arrendale and Chortyk, 1985; Arrendale et al., 1987a,b). We anticipated some variation in SE structures but were surprised to find a wide diversity in both composition of acid moieties and positions of attachment of the acid moieties to the sucrose molecule. Also, many of the 65 *Nicotiana* species produce more than one type of SE, unlike *N. tabacum*, which produces only one basic type (Table I). We examined in detail the SE fraction of one of these species, *Nicotiana glutinosa*.

MATERIALS AND METHODS

Materials. Sources: hexane and methanol (distilled-in-glass grade), Burdick and Jackson (Richmond, CA); chloroform (0.75% ethanol by volume) and methylene chloride (resi-analyzed grade), J. T. Baker (Phillipsburg, NJ); dimethylformamide (DMF), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluroacetamide (MTBSTFA) (silylation grade), Pierce Chemical Co. (Rockford, IL); acetic anhydride, Mallinckrodt (St. Louis, MO); 4-(dimethylamino)pyridine, Aldrich (Milwaukee, WI). *N. glutinosa* plants used in these investigations were grown at the Crops Research Laboratory, Oxford, NC.

Isolation of Sucrose Ester Fractions. Detailed procedures for the extraction (whole-leaf wash) of the cuticular waxes from green plants and the isolation of the SE from this whole leaf wash may be found elsewhere (Severson et al., 1984a, 1985a). Only the salient features of this methodology are presented here.

The cuticular waxes were removed from green *N. glutinosa* plants by dipping them into methylene chloride to yield the whole-leaf wash (WLW). The procedure used for the isolation of fractions I and II SE from the cuticular waxes (WLW) is shown in Figure 1.

The WLW was solvent-partitioned between hexane and 80% methanol–water. The methanol–water solubles, containing the SE, were solvent-partitioned between 80% methanol–water and chloroform. The chloroform-soluble fraction, which now contained diterpenes and SE, was chromatographed on Sephadex LH-20 in chloroform. Gel fractions (GF) 1–59 contained diterpenes (sclareols and manools), and GF 60–160 contained a crude fraction of SE, designated as fraction I. Elution with 10% methanol–water yielded a second fraction of SE (fraction II).

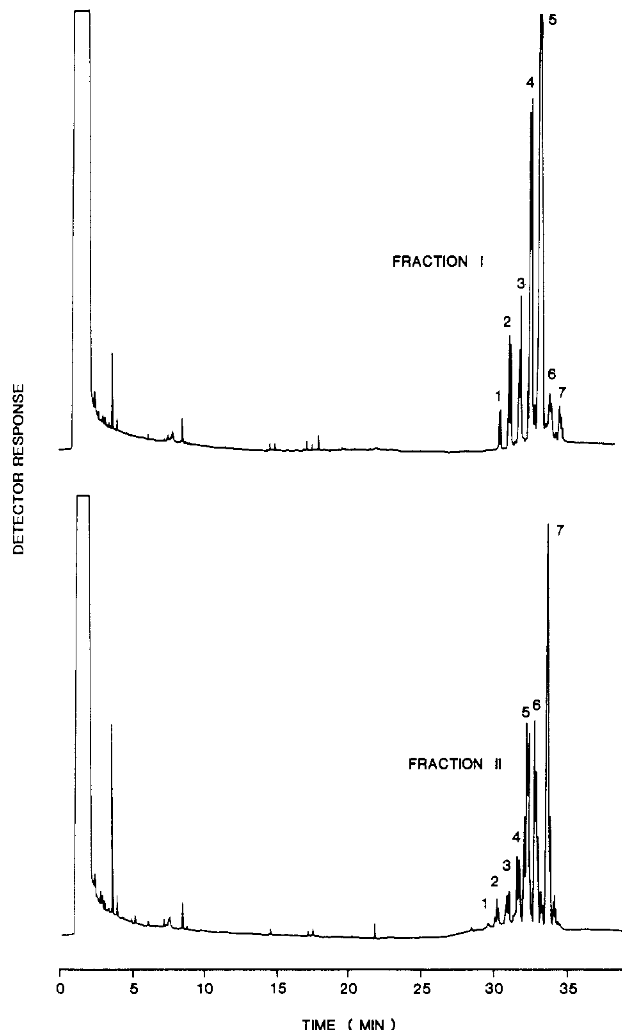

Figure 2. Gas chromatograms for the cold on-column injection capillary GC separation of the TMS derivatives of fractions I and II SE from *N. glutinosa*.

Table II. C_3–C_8 Acid Composition of Sucrose Ester Fractions (Mole Percent)[a] from *N. tabacum* cv. NC 2326 and TI 165 and *N. glutinosa*

	N. tabacum		*N. glutinosa*	
acid	NC 2326	TI 165	fraction I	fraction II
propanoic, C_3		0.3	0.7	0.2
isobutyric, C_4	10.0	2.7	9.9	2.8
butyric, C_4	1.0	1.0	0.3	0.5
2-methylbutyric, C_5	29.8	8.1	20.1	8.5
3-methylbutyric, C_5	54.7	13.9	3.1	1.3
valeric, C_5	0.1	0.7	1.0	0.7
3-methylvaleric, C_6	1.9	68.8	3.7	1.1
4-methylvaleric, C_6		1.4	4.7	2.4
hexanoic, C_6	0.3	0.7	1.3	0.7
4-methylhexanoic, C_7	0.3	1.2	27.5	42.3
5-methylhexanoic, C_7		0.8	32.0	30.6
heptanoic, C_7			0.2	1.4
me thylheptanoic, C_8		0.3	1.1	1.7
octanoic, C_8			0.3	0.6
mol acetic acid/ mol C_3–C_8 acids	1:3.1	1:3.0	1:2.9	

[a] Based on total C_3–C_8 acids. Determined by GC after hydrolysis of SE.

Cold On-Column Injection Capillary Gas Chromatography Analyses. Fractions I and II SE were separated by cold on-column injection (Arrendale and Chortyk, 1985) capillary GC on a SE-54-immobilized stationary-phase FS capillary column (Arrendale and Martin, 1988) as their trimethylsilyl ether (TMS),

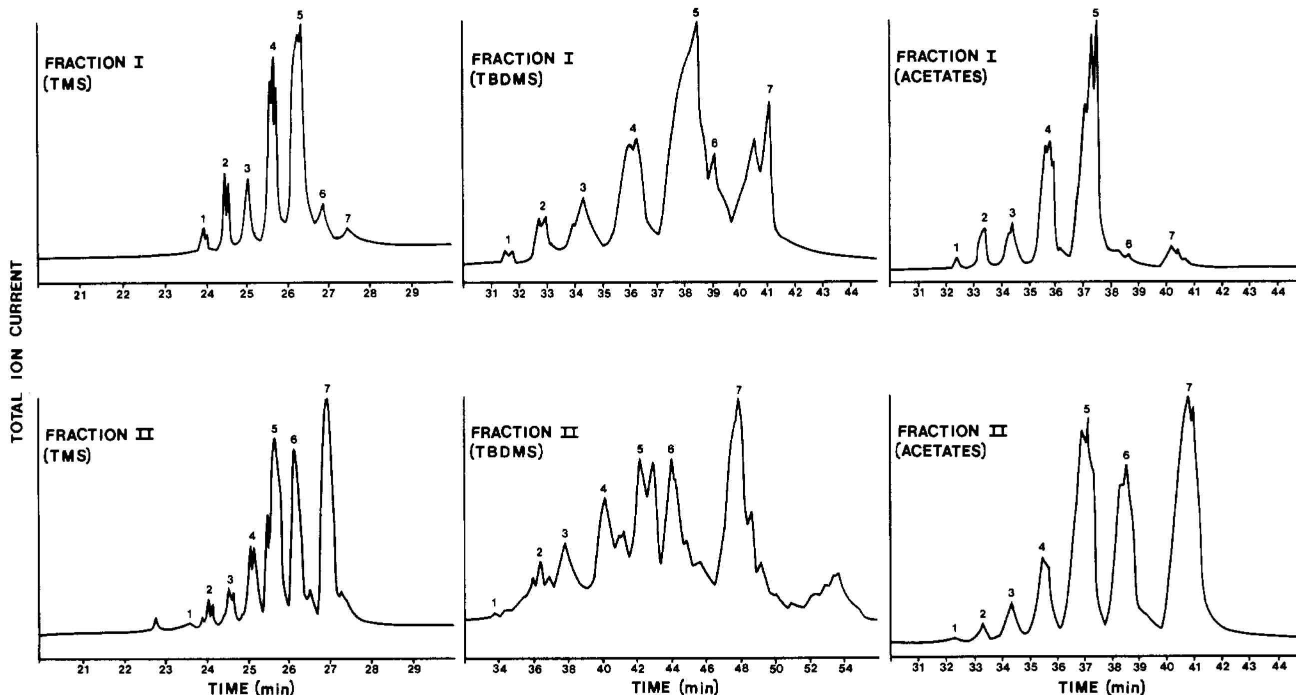

Figure 3. Total ion current (TI) chromatograms of the capillary GC/MS analyses of the TMS, TBDMS, and acetate derivatives of fractions I and II SE from *N. glutinosa*.

Table III. Sucrose Esters from *N. glutinosa* [Trimethylsilyl Ether Derivatives (GC/MS)]

		fraction I[a]			fraction II[b]		
			high-mass ions			high-mass ions	
group	acyl moieties/ glucose molecule	MW	glucose	fructose	MW	glucose	fructose
1	C_7-2 C_4	924	487	421, 407, 361	954	487	451, 437, 361
2	C_7-C_5-C_4	938	501	421, 407, 361	968	501	451, 437, 361
3	C_7-2 C_5	952	515	421, 407, 361	982	515	451, 437, 361
4	C_7-C_6-C_5	966	529	421, 407, 361	996	529	451, 437, 361
5[c]	2 C_7-C_5	980	543	421, 407, 361	1010	543	451, 437, 361
6	2 C_7-C_6	994	557	421, 407, 361	1024	557	451, 437, 361
7[d]	3 C_7	1008	571	421, 407, 361	1038	571	451, 437, 361

[a] Tetrakis(trimethylsilyl) ethers. [b] Pentakis(trimethylsilyl) ethers. [c] Most abundant group in fraction I. [d] Most abundant group in fraction II.

tert-butyldimethylsilyl ether (TBDMS), and peracetate derivatives, using a Hewlett-Packard 5840 gas chromatograph equipped with a flame ionization detector (FID). Chromatograms of the separation of the TMS derivatives of fractions I and II SE of *N. glutinosa* are shown in Figure 2. Note that each fraction can be subdivided into seven distinct groupings and that group 5 was the most abundant group in fraction I and group 7 was most prevalent in fraction II. Subsequent GC/MS analyses showed that each group within a fraction differs from the next by one methylene (CH_2, 14 Da) moiety. Fractions I and II SE both contained identical glucose moieties but differed by the presence (fraction I) or absence (fraction II) of an acetate moiety at carbon 3 of the fructose portion of the sucrose molecule (Table I). The TMS derivatives were prepared by heating the SE with a 1:1 mixture of BSTFA–DMF for 30 min at 76 °C (Severson et al., 1984a,1985a). Cold on-column injection capillary GC conditions for the separation of the TMS derivatives were as follows: capillary column, immobilized SE-54 FS 30 m × 0.3 mm (i.d.); liquid phase film thickness, 0.1 μm; linear velocity, 40 cm/s He; temperature program, 100 °C for 1 min, 100–300 °C at 6 °C/min; injection mode, cold on-column; injection volume, 1 μL; detector, FID.

The *tert*-butyldimethylsilyl(TBDMS) derivatives were prepared by heating these with 1:1 MTBSTFA–DMF for 18 h at 76 °C. Cold on-column injection capillary GC conditions for the separation of the TBDMS derivatives were the same as those listed above for analyses of the TMS derivatives, except for the temperature program (100 °C for 1 min, 100–300 °C at 8 °C/min).

Fractions I and II SE were acetylated by mixing 100–200 mg of sample, 40 μL of methylene chloride, 60 μL of acetic anhydride, and approximately 5 μg of 4-(dimethylamino)pyridine (catalyst) in a screw-top tapered test tube and allowing this mixture to stand at room temperature for 1–2 h. Next, the solvent was removed with a stream of dry nitrogen, and the peracetate derivatives were redissolved in benzene. Conditions for capillary GC analysis of the peracetate derivatives were identical with those described above, except for the temperature program, which was 70 °C for 1 min, 70–300 °C at 8 °C/min.

Capillary GC/MS Analyses of Fractions I and II Sucrose Esters. The TMS, TBDMS, and peracetate derivatives of fractions I and II SE were analyzed by capillary GC/MS, on a Hewlett-Packard 5985B GC/MS system equipped with an open-split interface (Arrendale et al., 1984). Total ion current (TI) chromatograms of these analyses are given in Figure 3. The GC/MS interface zone temperature was 300 °C, the ion source temperature was 200 °C, and the electron impact (EI) ionization energy was 70 eV for each analyses. Other MS conditions for the analyses of the TMS derivatives: scan range, 40–850 Da; scan rate, 400 Da/s; electron multiplier voltage, 1800 V. Other MS conditions for the analyses of the TBDMS derivatives: scan range, 40–650 Da; scan rate, 266.7 Da/s; electron multiplier voltage, 2400 V. MS conditions for the analyses of the acetate derivatives: scan range 40–950 Da; scan rate, 266.7 Da/s; electron multiplier voltage, 2200 V.

Analyses of Sucrose Ester Acids. Approximately 30 mg of fractions I and II SE were saponified with 0.5 mL of 1.0 N KOH in 80% methanol–water at room temperature for 24 h.

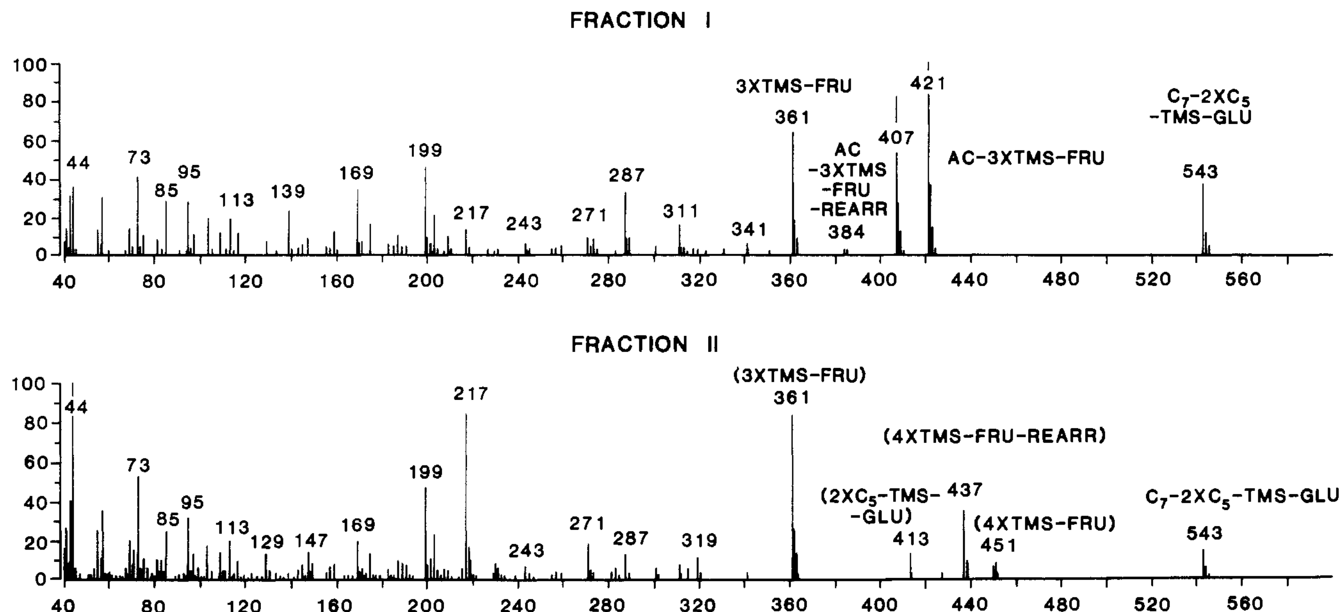

Figure 4. 70-eV EI mass spectra of the TMS derivatives of group 5 SE from fractions I and II of *N. glutinosa*.

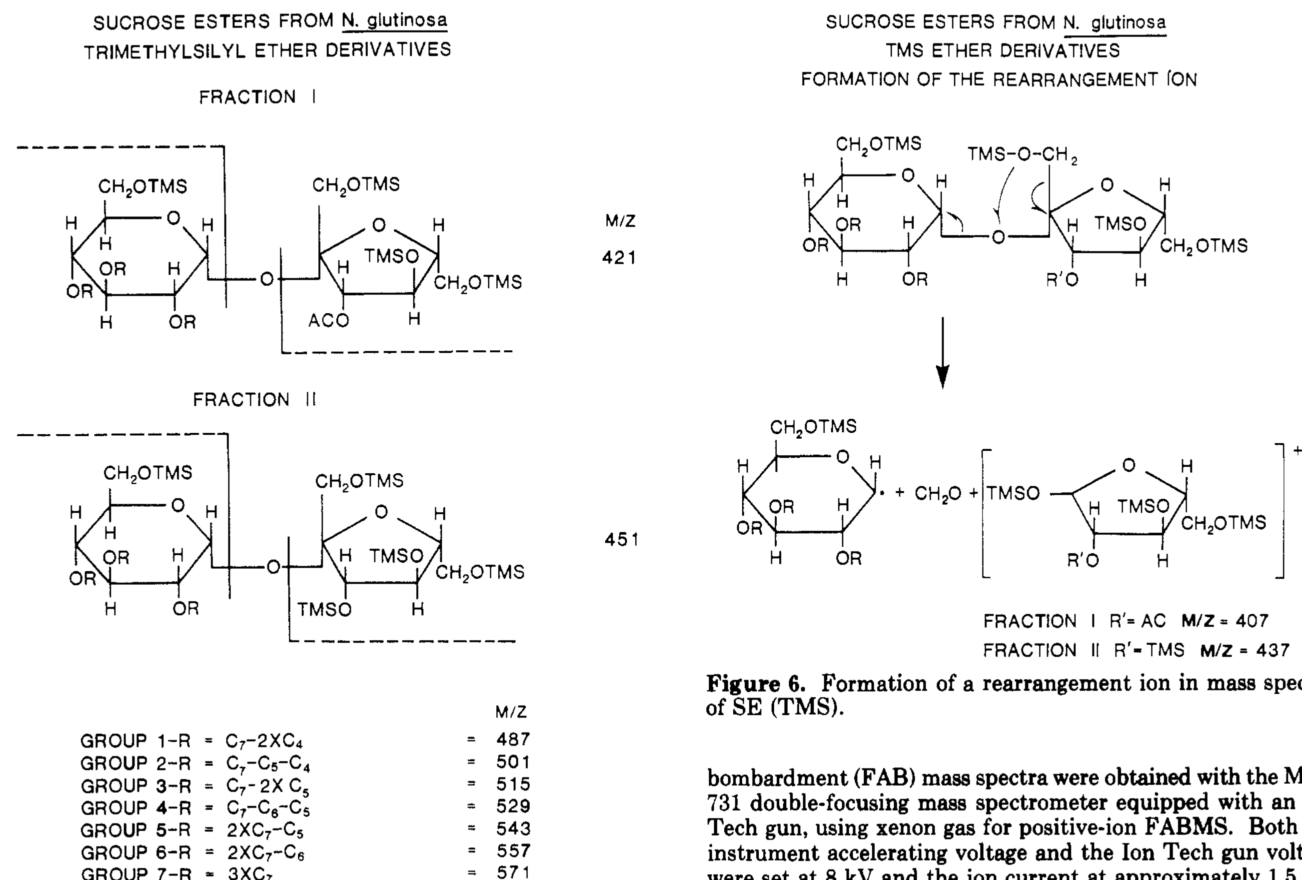

Figure 5. Molecular fragments responsible for the major high-mass ions in the mass spectra of the TMS derivatives of fractions I and II SE.

Figure 6. Formation of a rearrangement ion in mass spectra of SE (TMS).

Aliquots (30 μL) of the saponificates were transferred to 100-μL vials along with 30 μL of a 3:2 mixture of acetone–water. Next, 10 μL of 6 N HCl was added, resulting in the formation of a KCl precipitate. The free acids were analyzed by capillary GC under the following conditions: capillary column, FS OV-351, 27 m × 0.32 mm (i.d.); split injection mode, 2-μL injection; temperature program, 90–220 °C at 6 °C/min; linear velocity, 40 cm/s H_2; FID.

Fast Atom Bombardment Mass Spectrometry (FABMS) Analyses of Fractions I and II Sucrose Esters. Fast atom bombardment (FAB) mass spectra were obtained with the MAT 731 double-focusing mass spectrometer equipped with an Ion Tech gun, using xenon gas for positive-ion FABMS. Both the instrument accelerating voltage and the Ion Tech gun voltage were set at 8 kV and the ion current at approximately 1.5 μA. Low-resolution FAB mass spectra were obtained at 1:2000 resolution. Samples were dissolved in 1:1 methanol–glycerol for all FABMS analyses.

NMR Analyses of Fractions I and II Sucrose Esters. Recently, we discovered that our published ^{13}C NMR assignments for the ring carbons of the 6-*O*-acetyl-2,3,4-tris-*O*-(3-methylvaleryl)-α-D-glucopyranosyl β-D-fructofuranoside from *N. tabacum* were incorrect (Severson et al., 1985a). Reexamination of the molecule by modern two-dimensional NMR methods (to determine the connectivity of each ring carbon in the sucrose molecule) confirmed the inconsistencies and provided the correct C-13 assignments. Details of the methodology that were developed and used to obtain these data are beyond the scope of this work and are being published as a separate paper (Himmelsbach et al., 1990). The ^{13}C NMR assignments from

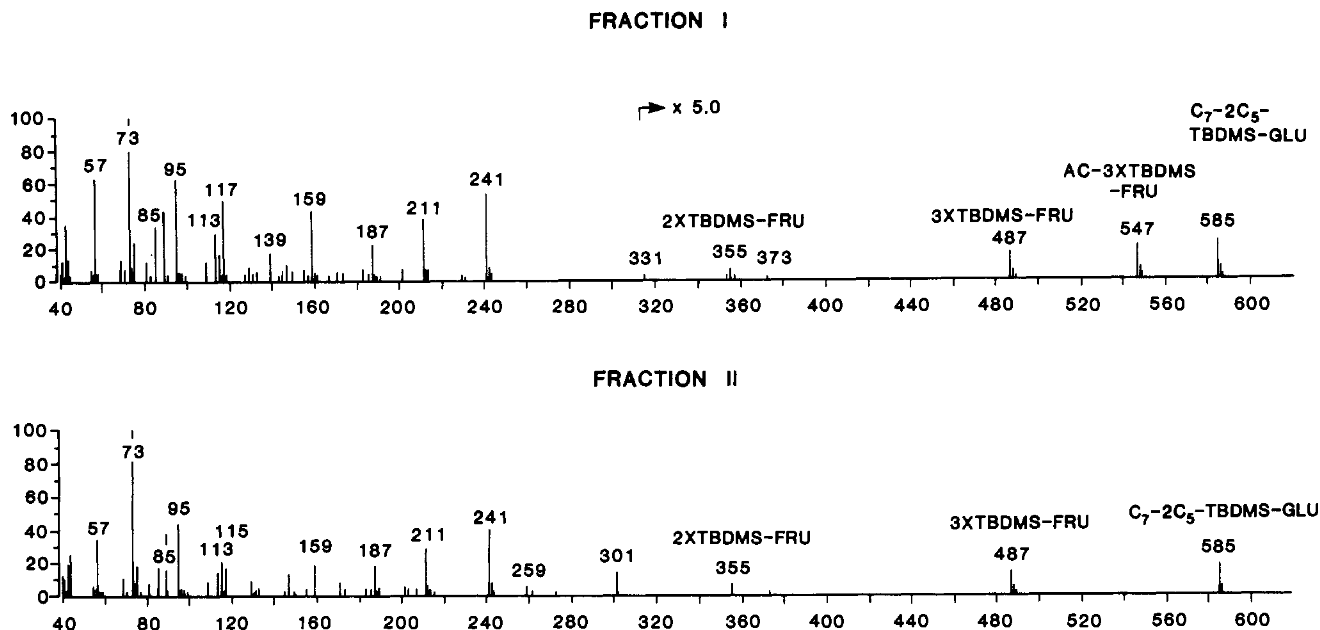

Figure 7. 70-eV EI mass spectra of the TBDMS derivatives of group 5 SE from fractions I and II.

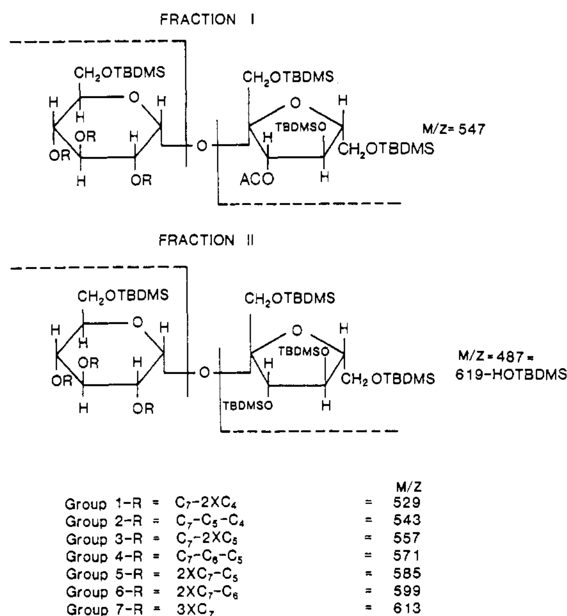

Figure 8. Molecular fragments responsible for the major high-mass ions in the mass spectra of the TBDMS derivatives of fractions I and II SE.

the sucrose carbons of fractions I and II SE from *N. glutinosa* and the corrected assignments for the SE from *N. tabacum* are discussed below.

RESULTS AND DISCUSSION

Our investigation of the cuticular chemicals from the 65 *Nicotiana* species revealed many species that produce SE and several that produce more than one type of SE (Severson et al., 1984b, 1987; Arrendale and Chortyk, 1985; Arrendale et al., 1987a,b). These variations in structure consisted of differences in both aliphatic acid compositions and positions of attachment of the acids to the sucrose molecule. Difficulties in describing and comparing these various structural types prompted us to begin a simplified classification scheme for SE, with assignments for the types of SE described for *Nicotiana* species thus far being given in Table I. Disregarding distributions of the C_3–C_9 aliphatic acids that are esterified to the glucose moiety, we have designated three SE types (SE-I, SE-II, SE-III) based upon the position of attachment of the acetyl moiety to the sucrose or fructose molecule (Table I). The presence or absence and location of attachment of the acetyl moiety was given high priority, as it appears to be controlled by a gene separate from that of all the other aliphatic acids. To this date, acetic acid is the only acid found attached to the fructose moiety of a cuticular SE molecule (Table I, type SE-III). For instance, the SE from *N. tabacum* were designated as type SE-I (Table I).

Table IV. Sucrose Esters from *N. glutinosa* [*tert*-Butyldimethylsilyl (TBDMS) Ether Derivatives (GC/MS)]

		fraction I[a]			fraction II[b]		
			high-mass ions			high-mass ions	
group	acyl moieties/ glucose molecule	MW	glucose	fructose	MW	glucose	fructose
1	C_7–2 C_4	1092	529	547, 487, 355	1164	529	487 (619 – (HO)TBDMS), 355
2	C_7–C_5–C_4	1106	543	547, 487, 355	1178	543	487 (619 – (HO)TBDMS), 355
3	C_7–2 C_5	1120	557	547, 487, 355	1192	557	487 (619 – (HO)TBDMS), 355
4	C_7–C_6–C_5	1134	571	547, 487, 355	1206	571	487 (619 – (HO)TBDMS), 355
5[c]	2 C_7–C_5	1148	585	547, 487, 355	1220	585	487 (619 – (HO)TBDMS), 355
6	2 C_7–C_6	1162	599	547, 487, 355	1234	599	487 (619 – (HO)TBDMS), 355
7[d]	3 C_7	1176	613	547, 487, 355	1248	613	487 (619 – (HO)TBDMS), 355

[a] Tetrakis(*tert*-butyldimethylsilyl) ethers. [b] Pentakis(*tert*-butyldimethylsilyl) ethers. [c] Most abundant group in fraction I. [d] Most abundant group in fraction II.

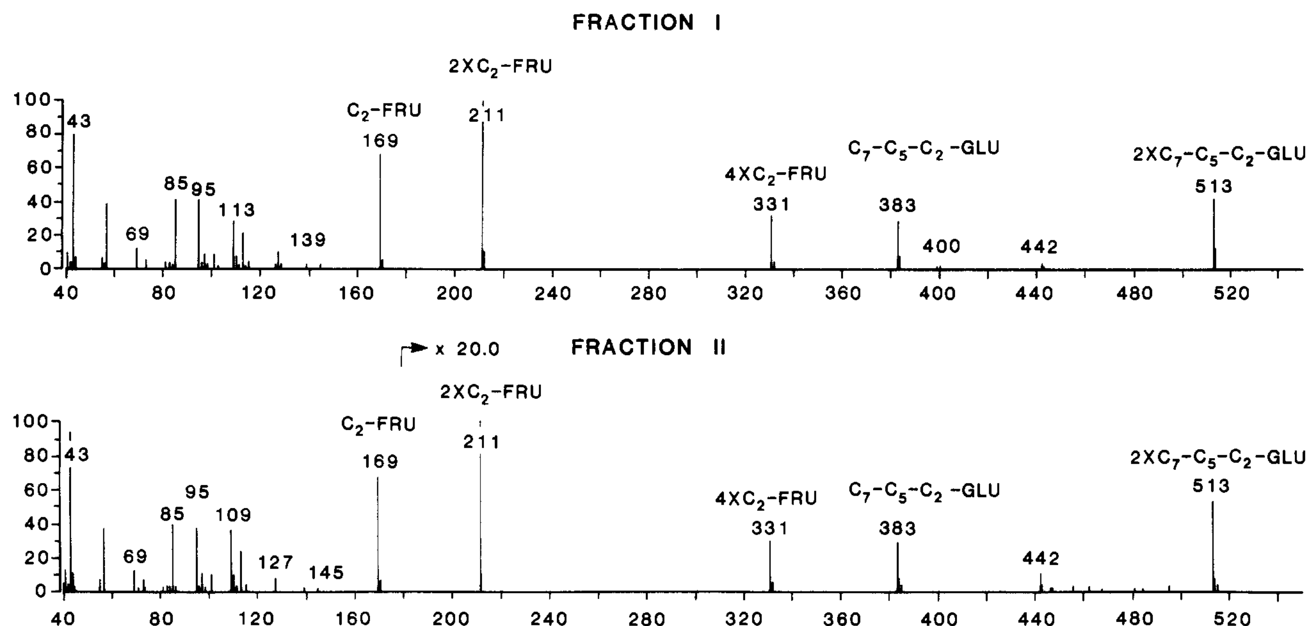

Figure 9. 70-eV EI mass spectra of the acetate derivatives of group 5 SE from fractions I and II.

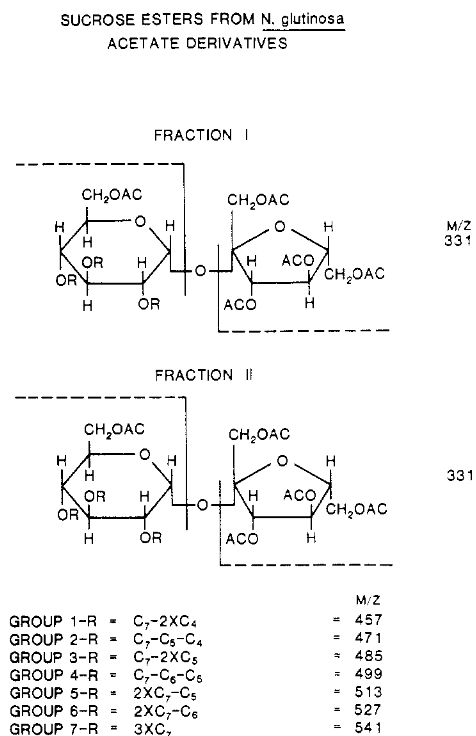

Figure 10. Molecular fragments responsible for the major high-mass ions in the mass spectra of the acetate derivatives of fractions I and II SE.

The presence of at least two different SE structural types in the cuticular waxes of *N. glutinosa* was first determined from the capillary GC/MS analyses of the trimethylsilyl ether derivatives of the $CHCl_3$ solubles of the whole-leaf wash (Figure 1). Subsequent separation of the two SE types on Sephadex LH-20 confirmed their presence and clearly indicated that a difference in polarity existed (Figure 1). Determination of the acid compositions of the two SE fractions and comparison with similar data from *N. tabacum* varieties served to further illustrate these differences (Table II). Although differences in the major acids are obvious even among *N. tabacum* cultivars, subtle differences in the ratio of acetic acid to the sum of all other acids are more important to structural elucidation. *N. tabacum* cultivars yield 1 mol of acetic acid to 3 mol of other acids, as do fraction I SE from *N. glutinosa*. From these data, one might expect that the structures of *N. tabacum* SE and fraction I SE from *N. glutinosa* were similar. However, comparison of the mass spectra of the trimethylsilyl ether derivatives of SE from *N. tabacum* (Severson et al., 1985a) and fraction I SE indicates that they are very different.

Analyses of the capillary GC/MS data on the TMS derivatives of fractions I and II showed that the seven distinct groups within each fraction had identically substituted glucose moieties (Table III). The differences between fractions resided solely in the presence or absence of the acetate moiety on the fructose portion of the SE. For illustrative purposes, we chose group 5, as its concentration was at relatively high levels in both fractions

Table V. Sucrose Esters from *N. glutinosa* [Acetate Derivatives (GC/MS)]

group	acyl moieties/ glucose molecule	fraction I[a] MW	fraction I[a] high-mass ions glucose	fraction I[a] high-mass ions fructose	fraction II[a] MW	fraction II[a] high-mass ions glucose	fraction II[a] high-mass ions fructose
1	C_2-C_7-2 C_4	804	457	331, 211, 169	804	457	331, 211, 169
2	C_2-C_7-C_5-C_4	818	471	331, 211, 169	818	471	331, 211, 169
3	C_2-C_7-2 C_5	832	485	331, 211, 169	832	485	331, 211, 169
4	C_2-C_7-C_6-C_5	846	499	331, 211, 169	846	499	331, 211, 169
5[b]	C_2-2 C_7-C_5	860	513	331, 211, 169	860	513	331, 211, 169
6	C_2-2 C_7-C_6	874	527	331, 211, 169	874	527	331, 211, 169
7[c]	C_2-3 C_7	888	541	331, 211, 169	888	541	331, 211, 169

[a] Pentaacetate derivatives. [b] Most abundant group in fraction I. [c] Most abundant group in fraction II.

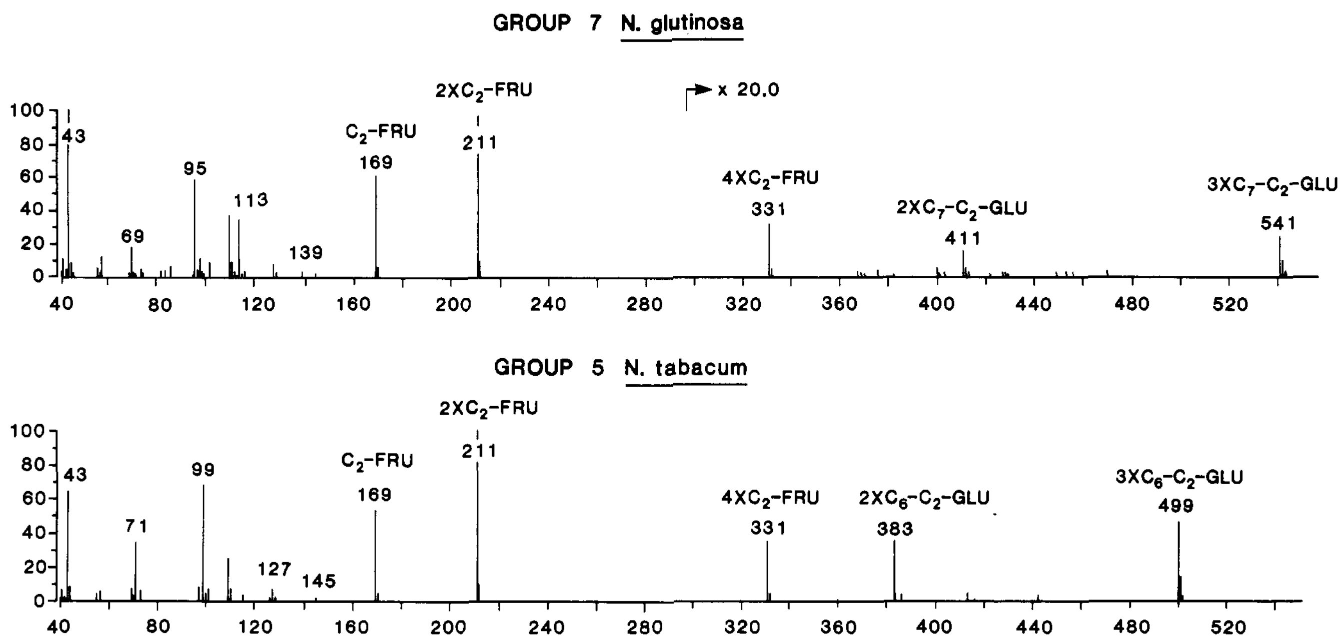

Figure 11. 70-eV EI mass spectra of the acetate derivatives of group 7 from the SE of *N. glutinosa* and group 5 from the SE of *N. tabacum*.

Table VI. ^{13}C **NMR Chemical Shifts[a] (ppm) for the Glucose (G) and Fructose (F) Carbons of Sucrose Esters**

	G1	G2	G3	G4	G5	G6	F1	F2	F3	F4	F5	F6
type SE-I[b] (*N. tabacum*)	89.2	70.5	69.3	67.8	68.7	61.6	64.3	104.7	78.3	73.8	82.1	60.8
type SE-II (*N. glutinosa*)[c]	88.7	71.5	70.7	68.7	69.5	61.5	64.2	104.5	77.8	73.1	81.4	60.6
(fraction II)	(−0.5)	(+1.0)	(+1.4)	(+0.9)	(+0.8)	(−0.1)	(−0.1)	(−0.2)	(−0.5)	(−0.7)	(−0.7)	(−0.2)
type SE-III (*N. glutinosa*)[c]	89.6	71.5	70.5	68.4	69.0	61.6	64.4	104.0	79.6	71.5	82.4	60.1
(fraction I)	(+0.4)	(+1.0)	(+1.2)	(+0.6)	(+0.3)	(0.0)	(+0.1)	(−0.7)	(+1.3)	(−2.3)	(+0.3)	(−0.7)

[a] Values obtained in $CDCl_3$ relative to TMS. [b] Assignments made by ^{13}C NMR connectivity experiments on a pure isolate (100 mg) of group V SE. See references. [c] Differences between the ^{13}C chemical shifts of *N. glutinosa* SE (types SE-II and SE-III) and those of *N. tabacum* SE (type SE-I).

I and II. The mass spectra of the group 5 SE (TMS) from fractions I and II are shown in Figure 4. The ions from the MS analyses of fraction I SE (TMS) can be logically obtained from a sucrose molecule having one acetate on the fructose moiety and three other acids esterified to the glucose portion as illustrated in Figure 5. The ion at m/z 543 was present in each spectrum and represents the glucose portion of each molecule, which had two C_7 acids and one C_5 acid esterified at carbons 2, 3, and 4 and a TMS group at carbon 6 (C-6) of glucose (Figure 5). The acetylfructose moiety of fraction I SE gave rise to an ion at m/z 421, and the fructose moiety of fraction II SE (TMS) yielded an ion at m/z 451, similar to that formed in the mass spectra of *N. tabacum* SE (TMS) (Severson et al., 1985a). The difference (30 Da) between these two fragment ions was of course the difference between a tetrakis(trimethylsilyl)fructose (for fraction II) and an acetyltris(trimethylsilyl)fructose (for fraction I), as one TMS group adds 72 Da and one acetyl adds 42 Da to the molecular weight. Each of these fructose fragment ions was also accompanied by ions of 14 Da less (407 and 437, respectively). These resulted from a rearrangement that commonly occurs with trimethylsilyl ether derivatives having a terminal D-fructofuranosyl group, whereby the TMS group on C-1 of fructose was transferred to the oxygen attached to C-2 of fructose, accompanied by cleavage of the sucrose molecule to yield the ion, a glucosyl radical, and a neutral molecule of formaldehyde (Figure 6) (Binkley et al., 1971). The presence of this rearrangement ion in MS data of fractions I and II SE (TMS) indicated that the acetate was not attached at C-1 of fructose, as this rearrangement would not occur when acetyl is attached to the oxygen at C-1. The high-mass ions resulting from the MS analyses of the SE (TMS) from fractions I and II are given in Table III.

The *tert*-butyldimethylsilyl ether derivatives were prepared, and the mass spectral data for group 5 of fractions I and II SE are presented in Figure 7. Each TBDMS group adds 114 Da; thus, the ions for the glucose moiety of the TBDMS group 5 SE were at m/z 585 or 42 Da higher than the TMS derivatives (m/z 543; Figure 4). The ion for the tris(*tert*-butyldimethylsilyl)acetylfructose of fraction I SE occurred at m/z 547. However, the tetrakis(*tert*-butyldimethylsilyl)fructose ion of fraction II SE was absent or so low in intensity as to be useless for structural characterization. It is possible that steric hindrance caused by the bulky *tert*-butyl groups resulted in rapid cleavage of a TBDMS group as the tris(*tert*-butyldimethylsilyl) ion at m/z 487 was present in the spectrum. This would also explain why the acetyl tris(*tert*-butyldimethylsilyl) ion at m/z 547 did occur in the spectrum of fraction I SE (Figure 8). Tabulated data from the capillary GC/MS analyses of SE (TBDMS) from fractions I and II are presented in Table IV, and the molecular fragments leading to these ions are illustrated in Figure 8.

Acetylation of carbohydrates for GC and GC/MS analyses has been used successfully for many years. However, the use of this technique takes on a special significance in the case of the SE from *N. glutinosa*, as the major difference between fractions I and II was the presence of an acetyl moiety in fraction I. Thus, acetylation of fractions I and II should in fact render them identical

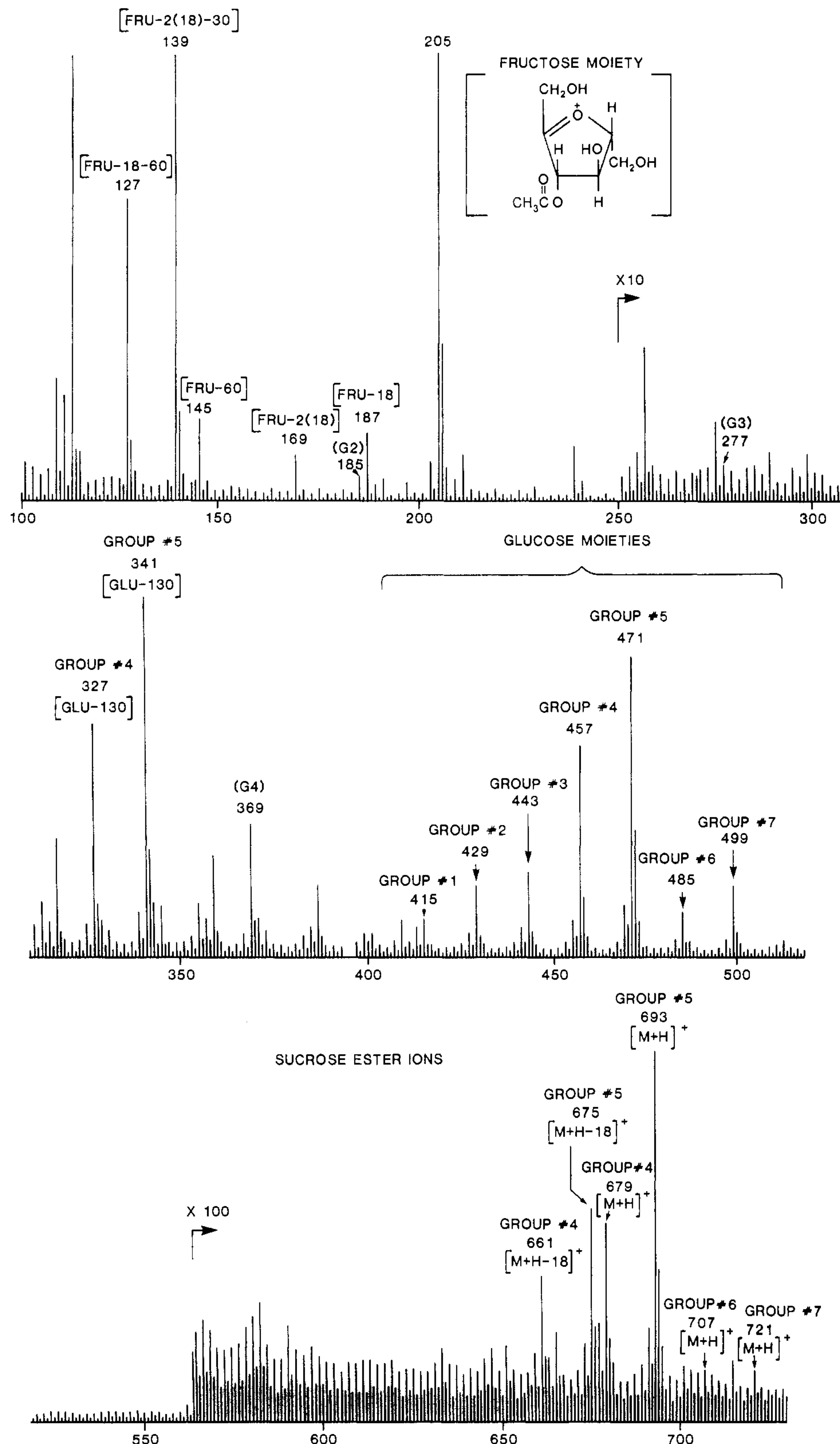

Figure 12. Positive-ion fast atom bombardment mass spectrum of fraction I SE from *N. glutinosa* [Gn = (Gn + H)$^+$].

since all free hydroxyls will then be esterified with an acetyl group. The TI chromatograms of the capillary GC/MS analyses of the acetate derivatives of fractions I and II (Figure 3) show that retention times of individual groups were identical and the mass spectral data of group 5 SE from fractions I and II (Figure 9) were also identical. Tabulated MS data for the acetate derivatives are given in Table V, and the molecular fragments leading to those ions are illustrated in Figure 10.

Additional proof of our hypothesis concerning the lack of an acetate group at C-6 of glucose in the SEs from *N. glutinosa*, the presence of an acetate on the fructose of fraction I SE, and its absence in fraction II can be obtained from a comparison of the peracetate derivatives of SE from *N. tabacum* and that of *N. glutinosa*. Figure 11 shows the mass spectrum of group 5 from *N. tabacum* (peracetate), which has an acetate at C-6 and three 3-methylvaleric acid moieties esterified at the 2-, 3-, and

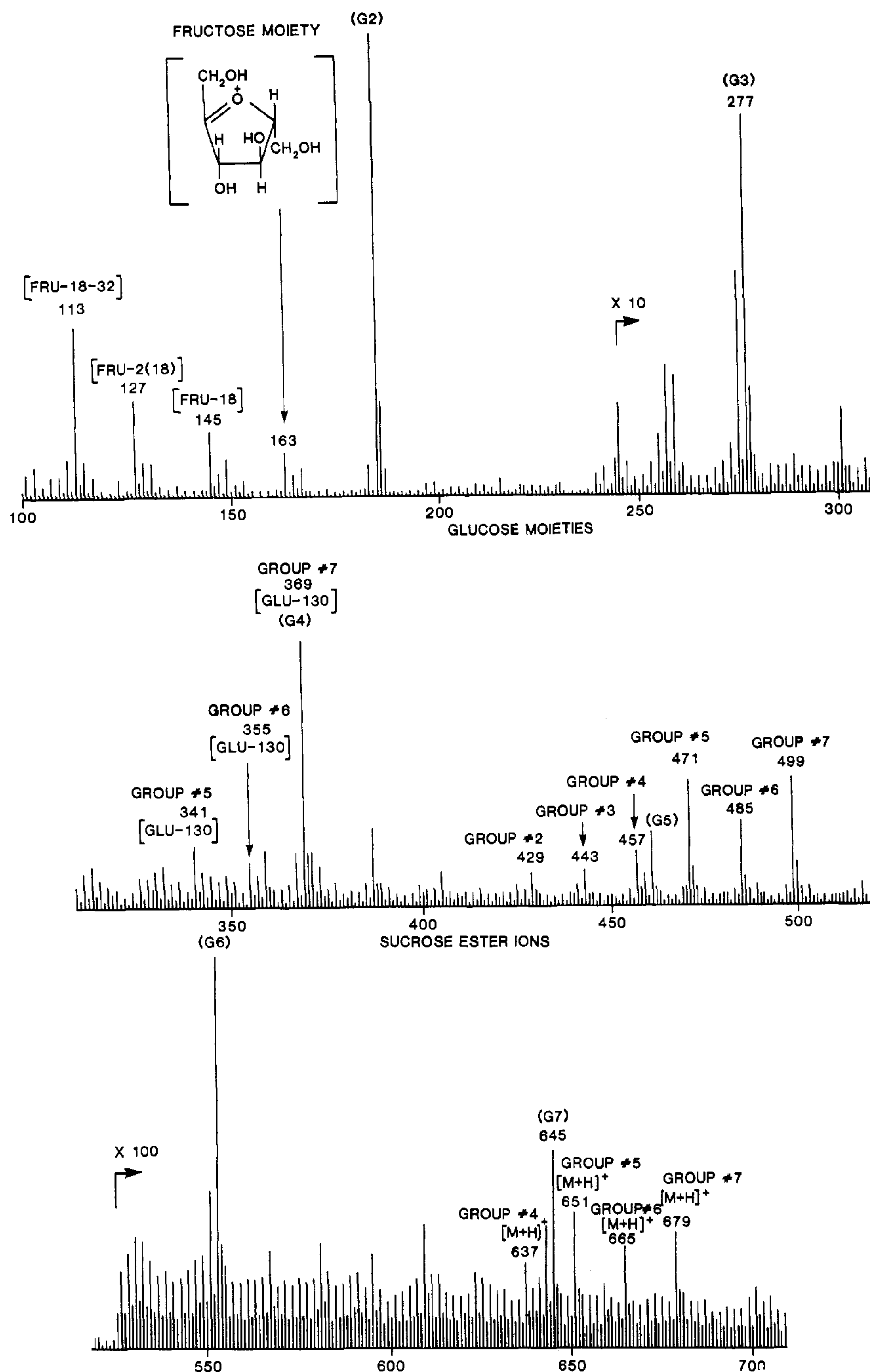

Figure 13. Positive-ion fast atom bombardment mass spectrum of fraction II SE from *N. glutinosa* [Gn = $(Gn + H)^+$].

4-carbons of glucose, along with the spectrum of group 7 SE from *N. glutinosa* which, as the peracetate derivative, now also has an acetate at C-6 of glucose and three C_7 acids at carbons 2, 3, and 4 of glucose. Ions from the acetylated fructose portion of each SE molecule are identical. The differences in the fragments from the glucose portion can be directly attributed to differences in the acid moieties at the 2-, 3-, and 4-carbons, respectively (C_6 acids from *N. tabacum* and C_7 acids from *N. glutinosa*). Thus, the positions of attachment of acetic acids are likely to be the same in each case, as are the positions of attachment of the C_6 and C_7 acids, respectively, indicating a free hydroxyl at C-6 of glucose of *N. glutinosa* SE and the presence of an acetyl group on fructose of fraction I SE from *N. glutinosa*.

Positive-ion fast atom bombardment mass spectra of fractions I and II SE are shown in Figures 12 and 13 and clearly indicate the molecular weights as calculated from our proposed structures for the SE in each fraction. Also, fragment ions for the glucose moieties showed that they are identical in each fraction. For instance, the protonated molecular ion for group 5 of fraction I was at m/z 693 and that of group 5 in fraction II was at m/z 651 or 42 Da less, which represents the presence of an acetyl on the fructose of fraction I SE and its absence in fraction II SE. In addition, the acetyl fructose moiety of fraction I SE was clearly indicated in the FAB-MS spectrum by an abundant ion at m/z 205, which was absent in the

spectrum of fraction II SE. Furthermore, an ion of low abundance from the fructose moiety of fraction II SE was present in the spectrum (m/z 163) and absent in that of fraction I SE.

Cuticular SE were first discovered from *N. tabacum* cultivars and thus have been the most thoroughly characterized. Confirmation of their structure was recently obtained (Wahlberg et al., 1986; Nishida et al., 1986) by 1- and 2-dimensional NMR techniques. Assignments of the ^{13}C chemical shifts of the sugar carbons of the type SE-I sucrose esters (*N. tabacum*) was difficult, and the NMR studies above were primarily conducted on the pentaacetate derivatives instead of the naturally occurring monoacetates. Investigation of fraction I (type SE-III) and fraction II (type SE-II) SE from *N. glutinosa* by NMR was complicated by the lack of a monoacetate on the glucose moieties and the presence of a monoacetate on the fructose moiety of fraction I SE (type SE-III).

Comparison of our published ^{13}C chemical shift assignments for the SE from *N. tabacum* (type SE-I) with those for fractions I and II SE from *N. glutinosa* revealed that many of our published sugar carbon (sucrose) assignments were incorrect (Severson et al., 1985a). We then initiated NMR studies on the *N. tabacum* SE by isolation of approximately 100 mg of group V SE, which contained an acetate at C-6 of glucose and three 3-methylvaleric acid groups attached at the 2-, 3-, and 4-carbons of glucose. The connectivity of each sucrose carbon in this SE was determined by newly developed NMR techniques. A complete description of this NMR methodology is beyond the scope of this discussion and therefore will be published elsewhere (Himmelsbach et al., 1990). However, the corrected ^{13}C chemical shift assignments for the group 5 SE from *N. tabacum* (type SE-I) are tabulated in Table VI, along with the assignments from fraction I (type SE-III) and fraction II (type SE-II) SE from *N. glutinosa*. The assignments for group 5 SE from *N. tabacum* are for the most part in agreement with those of Wahlberg et al. (1986), with the only exception being a reversal of the assignments for C-1 and C-6 of fructose, termed F1 and F6, respectively.

The assignment of the ^{13}C chemical shifts of the group 5 SE from *N. tabacum* by modern 2-dimensional NMR methods was essential, as the differences in the ^{13}C NMR chemical shifts for the three types of SE (Table VI) are subtle and their assignments directly from the 1-dimensional ^{13}C data were initially impossible. Comparison of the assignments for the glucose carbons of the group 5 *N. tabacum* SE (Type SE-I) with *N. glutinosa* fraction II SE (Type SE-II) revealed a general downfield shift, which maximized at carbon 3 of glucose (G3). These differences could result from differences in the acyl groups attached at the G2, G3, and G4 positions (Table II). The absence of the monoacetyl at G6 in the *N. glutinosa* fraction II SE appeared to make little difference in these data, and the chemical shifts for the fructose carbons were almost identical, showing only a minor upfield trend.

The chemical shifts for the glucose carbons of fractions I and II SE from *N. glutinosa* were, for all practical purposes, identical. As one might expect, ^{13}C assignments for the fructose carbons of fraction I SE or type SE-III (which have an acetate attached to the fructose moiety) differ in direction and magnitude from those of both *N. tabacum* and *N. glutinosa* fraction II SE. The major differences occurred at F3 and F4, which were shifted downfield and upfield, respectively, in the fraction I SE. An analogy may be drawn between these data for F3 and F4 of fraction I SE and ^{13}C chemical shifts of simple esters (Strothers, 1972). Studies have shown that the α- and β-carbons in the alkyl chains of acetates consistently yielded slight downfield shifts for α-carbons (+) and slight upfield shifts for β-carbons (−) when compared to the free alcohols. For instance, the differences in the ^{13}C NMR chemical shifts for the α- and β-carbons of ethyl acetate ($CH_3CH_2OCOCH_3$) were +2.8 and −3.6 ppm, respectively, when compared to those of ethanol (CH_3CH_2OH). Using this approach, one could conclude that F3 behaved as the α-carbon and was the location of attachment of the acetate in fraction I SE, as it was shifted downfield from the free alcohol (fraction II SE) by +1.3 ppm. By the same logic, F4 would correspond to the β-carbon, as it was shifted upfield from the free alcohol by −2.3 ppm. These data appeared to indicate that the acetate was attached at the F3 position for the fraction I SE from *N. glutinosa*. Results from recent NMR studies (Matsuzaki et al., 1988), using a completely different approach, were in agreement with our findings that the acetate of fraction I SE from *N. glutinosa* was attached to C-3 of the fructose molecule. However, there were differences in the assignment of the ^{13}C chemical shifts for the glucose carbons of SE from *N. glutinosa* (Table VI). Additional NMR studies will be necessary to clarify these discrepancies.

ACKNOWLEDGMENT

The MIT MS facility is supported by a grant from the NIH Division of Research Resources (RR00317) to K. Biemann.

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Received for review January 17, 1989. Accepted June 21, 1989.