ARTICLES

Structure of the Tomato Glycoalkaloid Tomatidenol-3-β-lycotetraose (Dehydrotomatine)

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Experiments were carried out to establish the structure of the monosaccharides comprising the carbohydrate side chain of the tomato glycoalkaloid dehydrotomatine. This was accomplished by (a) isolating dehydrotomatine and α-tomatine from commercial tomatine, (b) derivatizing the monosaccharides formed on acid hydrolysis of commercial tomatine, dehydrotomatine, and α-tomatine to alditol acetates and methylated alditol acetates, and (c) determining the structures of the galactose, glucose, and xylose derivatives by gas chromatography−mass spectrometry. Both dehydrotomatine and α-tomatine have the same tetrasaccharide side chain. They differ only by the presence in dehydrotomatine of a double bond at carbon atoms 5 and 6 of ring B and its absence in α-tomatine. Dehydrotomatine, or tomatidenol-3β-lycotetraose, appears to represent a new class of glycoalkaloids in which a lycotetraose carbohydrate side chain is attached to an aglycon containing a double bond. Biosynthetic pathways leading to the formation of dehydrotomatine and α-tomatine and the significance of the results to host-plant resistance and the diet are discussed.

Keywords: *Carbohydrate analysis; dehydrotomatine; gas chromatography; glycoalkaloids; HPLC; mass spectrometry; tomatidenol; tomatidine; α-tomatine; tomatoes*

INTRODUCTION

We previously reported that commercial tomatine consisted of a mixture of the known tomato glycoalkaloid α-tomatine and a new glycoalkaloid, which we named dehydrotomatine (Friedman et al., 1994). In that study, the two chromatographic peaks from commercial tomatine were collected from the HPLC column. Mass spectral analysis of the compound that produced the smaller HPLC peak revealed a molecular ion peak that was 2 Da less than the molecular ion of the α-tomatine signal. These observations, and additional studies on the structure of the respective aglycons derived from acid hydrolysates of the two glycoalkaloids, were the basis for our suggestion that the structure of dehydrotomatine is analogous to that of α-tomatine, except that the former molecule has a double bond between carbon atoms 5 and 6, as shown in Figure 1.

Although Bushway and Perkins (1995) made similar observations, a question about the nature and number of monosaccharide residues making up the carbohydrate side chain of dehydrotomatine remained unresolved. The objective of this study was to answer this question with the aid of a combination of chemical and analytical methods.

MATERIALS AND METHODS

Materials. Commercial tomatine was obtained from Sigma Chemical Co., St. Louis, MO. Glucose was obtained from Fisher Scientific Co., St. Louis, MO; galactose and xylose were from Eastman Kodak, Rochester, NY; and mannitol was from Katayama Chemical Co., Japan. All other compounds and reagents were purchased from Aldrich, Milwaukee, WI.

Separation of Commercial Tomatine into Dehydrotomatine and α-Tomatine. Commercial tomatine was resolved into α-tomatine and dehydrotomatine by preparative high-performance liquid chromatography (HPLC) using UV detection. Conditions were as follows: 3 mL of eluent/min was passed through a 25 cm × 10 mm, 5 μm particle Supelco LC-ABZ column; eluent consisted of 25% acetonitrile and 100 mM ammonium phosphate, pH 3. Commercial tomatine (2.5 mg) in 50% methanol and 0.1% acetic acid was applied to the column; the two peaks were collected from the UV detector and monitored at 200 nm. This procedure was repeated 10 times with fresh samples of commercial tomatine. The fractions from the different runs for each compound were pooled, and the acetonitrile was evaporated with the aid of a water aspirator. The solutions were made basic with NH_4OH and extracted into butanol. The butanol was then evaporated, and the residues were used for the chemical studies described below.

Acid Hydrolysis of Dehydrotomatine and α-Tomatine to Corresponding Aglycons. Dehydrotomatine (0.45 mg) or α-tomatine (0.29 mg) was dissolved in 1 mL of 1 N HCl and then heated in a 5 mL vial with a sealed Teflon cap at 95−100 °C for 70 min. After cooling, the mixture was neutralized with 1 N NH_4OH and partitioned five times with 2 mL of benzene. The combined benzene solutions were washed five times with 2 mL of H_2O. The benzene was then evaporated to dryness and the residue dissolved in 1 mL of benzene. Aliquots of this solution were used for gas chromatography−mass spectrometry (GC−MS) of tomatidenol and tomatidine.

Acid Hydrolysis of Carbohydrate Side Chains of Glycoalkaloids. Dehydrotomatine (1.73 mg) was dissolved in 1 mL of 1 N HCl and heated in a 5 mL vial with a sealed Teflon cap for 70 min at 95−100 °C. After cooling, the solution was neutralized with 1 N NH_4OH. The sugar solution was then desalted by passage through columns of Organo cation (IR-120, H^+) and anion (CG-400, $HCOO^-$) exchange resins. An internal standard, 365 μg of mannitol, was added to the desalted solution. The solution was then evaporated to dryness on a water aspirator at 40 °C.

Alditol Acetate Derivatives. The desalted sugars were dissolved in 3 mL of H_2O to which was added 5 mg of $NaBH_4$.

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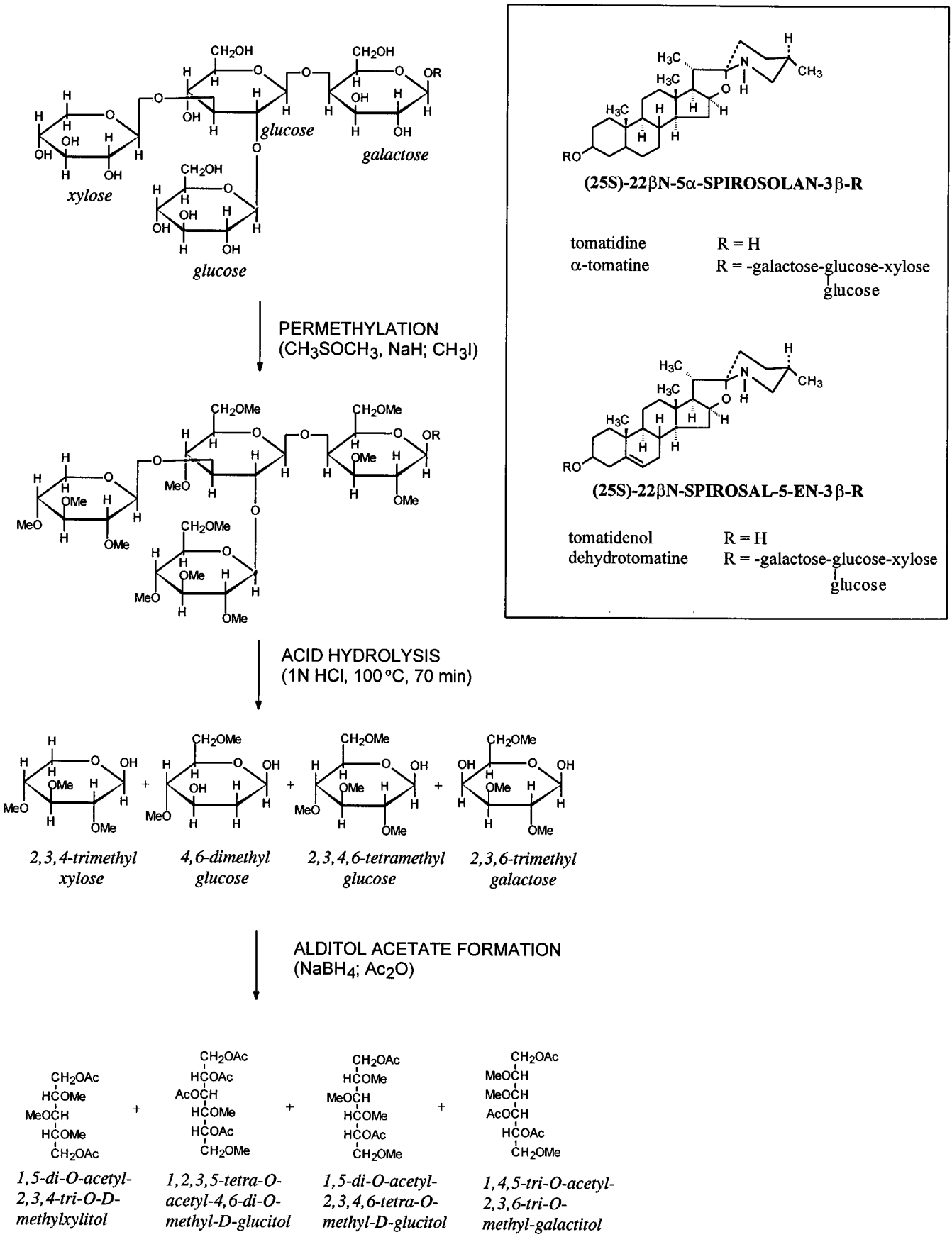

Figure 1. Elucidation of the structure of the tetrasaccharide side chain of α-tomatine and dehydrotomatine by the indicated chemical transformations.

The mixture was kept at room temperature for 2 h. Five drops of acetic acid were then added to stop the reaction, and the solution was evaporated to dryness. The sodium borohydride-derived boric acid was then removed by codistillation with methanol (3 mL added five times). The residue was acetylated by treatment with acetic anhydride/pyridine (1:1.2 mL) for 10 min at 95−100 °C. The reaction mixture was left standing overnight. Methanol (5 mL) was then added, and the mixture was evaporated to dryness. Methanol addition−evaporation was repeated five times. Chloroform (3 mL) was then added,

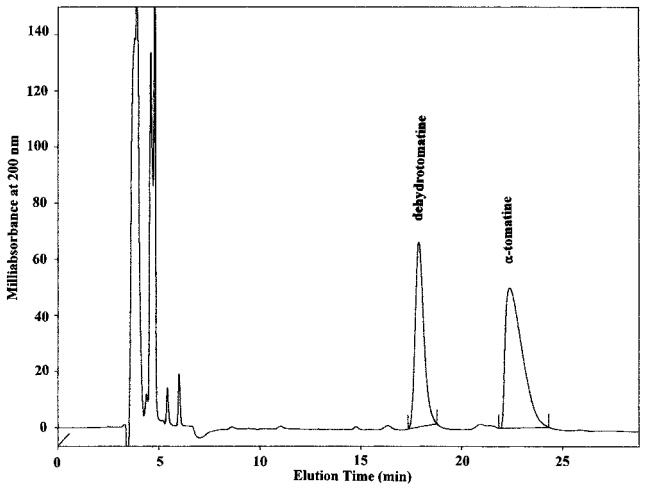

Figure 2. HPLC with UV detection of α-tomatine and dehydrotomatine.

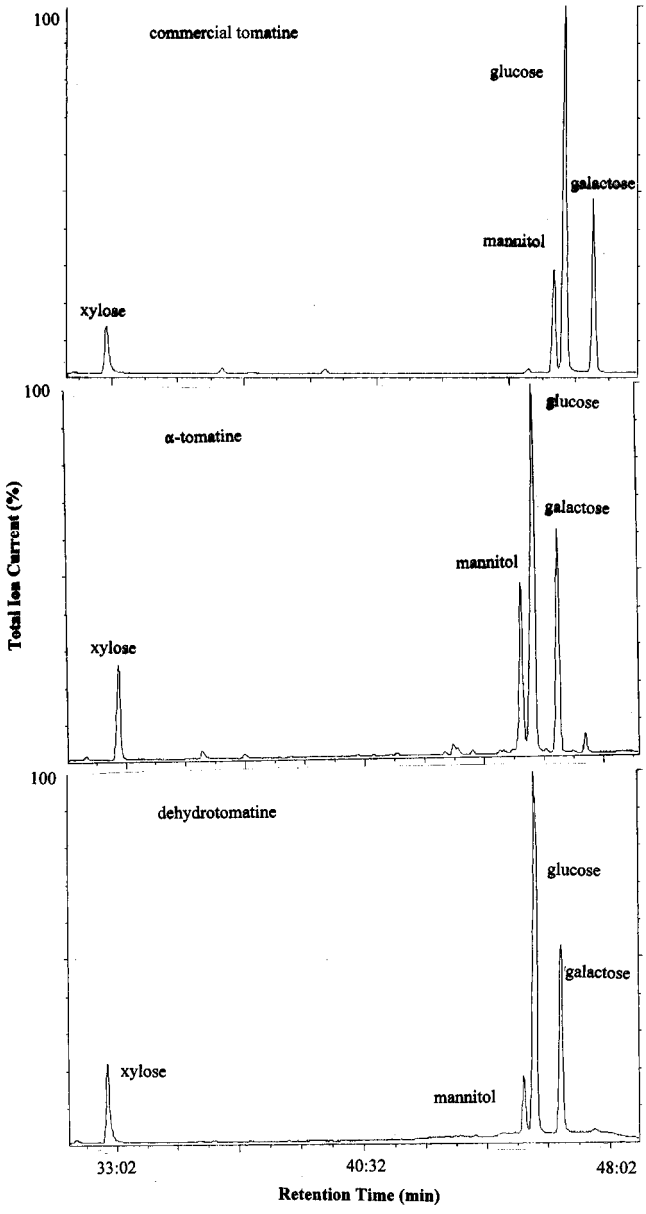

Figure 3. Gas chromatographic separation of alditol acetate derivatives of monosaccharides formed on acid hydrolysis of commercial tomatine, α-tomatine, and dehydrotomatine. Mannitol was used as an internal standard.

the mixture was shaken to extract the alditol acetate sugars, and the chloroform was separated from the aqueous layer. This

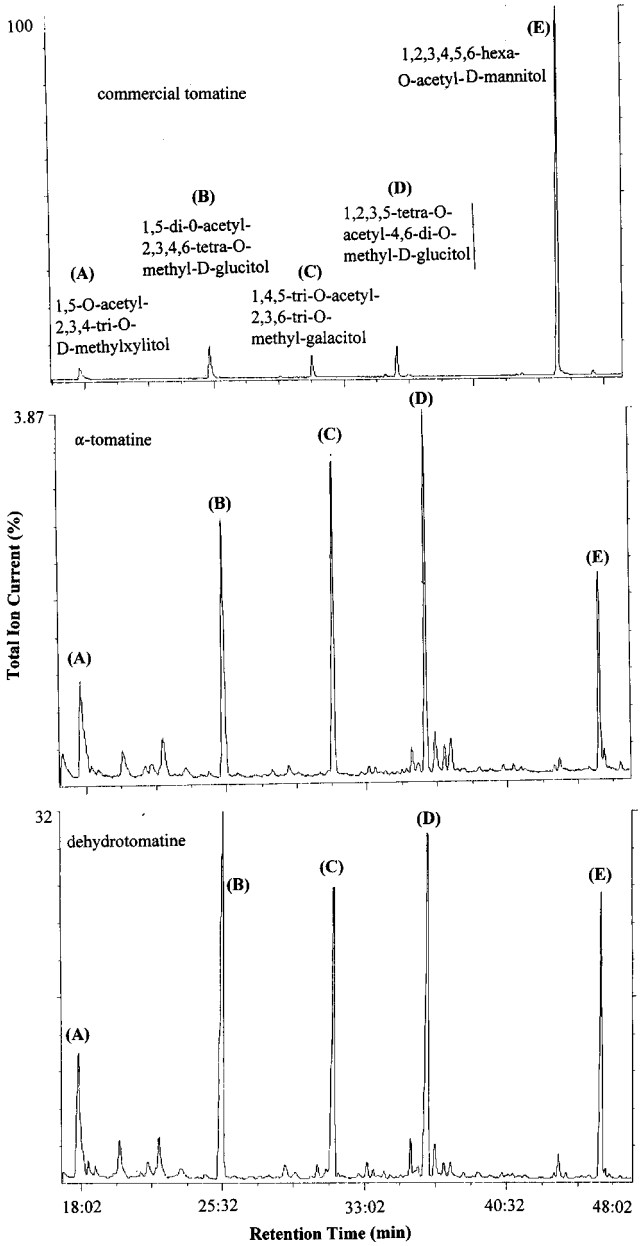

Figure 4. Gas chromatographic separation of partially methylated alditol acetate monosaccharides formed by acid hydrolysis of permethylated glycoalkaloids. Mannitol was used as an internal standard.

Table 1. Percent of Theoretical Amount of Monosaccharides Isolated after Acid Hydrolysis of Commercial Tomatine for Different Time Periods at 95−100 °C

	%		
time of hydrolysis (min)	xylose	glucose	galactose
15	77.5	22.5	0.0
30	55.0	39.1	5.9
45	32.2	52.9	14.9
70	9.9	62.0	28.1

procedure was repeated five times. The combined chloroform extracts were then washed five times with 5 mL of H_2O. The chloroform layers were transferred with a pipet, and the chloroform was evaporated by passing a stream of nitrogen over its surface. The residue was dissolved in 2 mL of chloroform. Aliquots of this solution were used for GC−MS.

Permethylation of Dehydrotomatine. The methylsulfinylmethanide sodium salt ($CH_3SOCH_2^-Na^+$) was prepared by stirring a solution of NaH (1 g) in mineral oil (60% oil dispersion) in 15 mL of dimethyl sulfoxide (DMSO) for 3 h under an atmosphere of nitrogen. A 1-cm layer of mineral oil

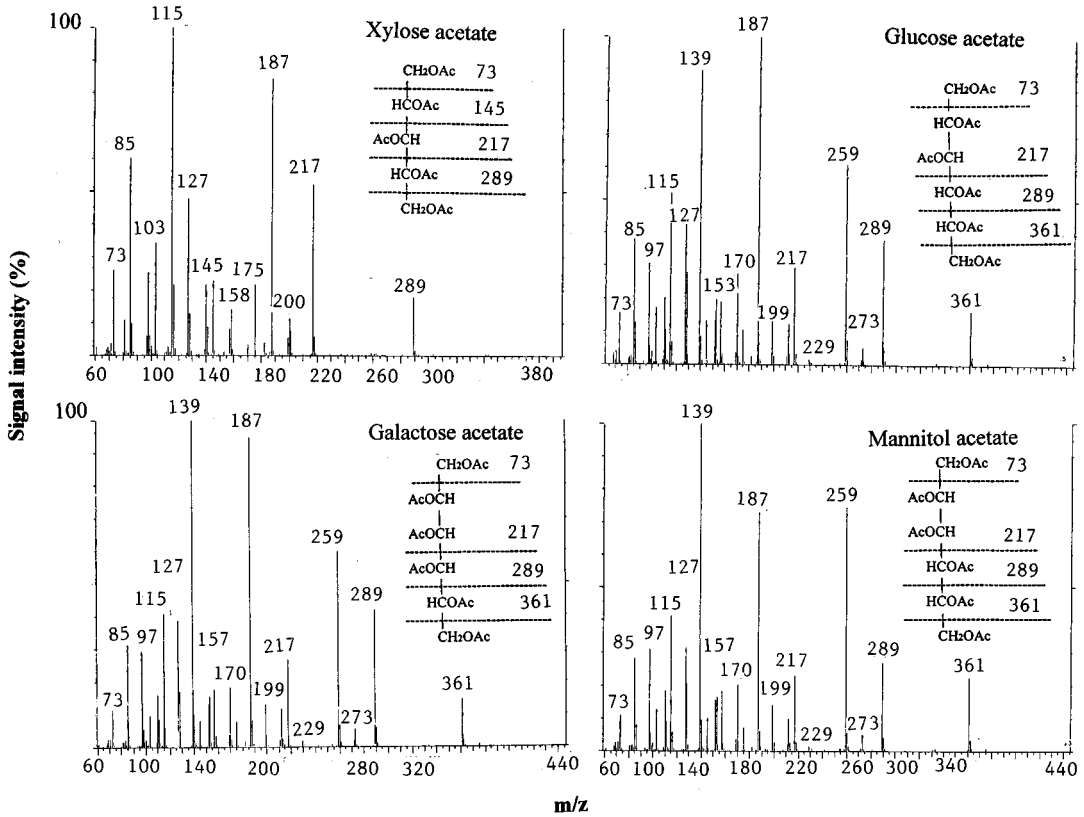

Figure 5. Mass spectra of alditol acetate derivatives of xylose, galactose, and glucose derived from acid hydrolysis and derivatization of the carbohydrate side chain of dehydrotomatine. The mannitol acetate derivative was used as an internal standard.

was added to protect the reagent from air and moisture. The reagent was kept in a refrigerator. Dehydrotomatine (0.88 mg) was dissolved in 1 mL of DMSO in a 5 mL vial sealed with a Teflon cap. Into this solution was injected a solution of methylsulfinylmethanide sodium in DMSO (0.5 mL). After 1.5 h of stirring at room temperature, methyl iodide (300 μL) was added dropwise with external cooling in ice water. The reaction mixture was left standing for 3 h with occasional shaking. The reaction was then stopped by addition of 2 mL of H_2O. The permethylated dehydrotomatine was extracted five times with 2 mL chloroform. The combined chloroform extracts were washed five times with 5 mL of H_2O. The chloroform layers were transferred each time with a small pipet into a 10 × 45 mm vial, and the chloroform was evaporated by passing a stream of nitrogen over its surface.

Hydrolysis of Permethylated Dehydrotomatine. The permethylated glycoalkaloid was dissolved in 1 mL of 1 N HCl. The solution was then heated at 95−100 °C for 70 min. After cooling, the hydrolysate was neutralized with 1 N NH_4OH and desalted by passing through cation and anion exchange columns, as described above. The internal standard mannitol (18.3 μg) was then added to the desalted solution, and the solution was evaporated to dryness. The methylated monosaccharides were acetylated to methylated alditol acetates as described above under Alditol Acetate Derivatives.

The procedures described for dehydrotomatine were also applied to commercial tomatine and α-tomatine.

Thin Layer Chromatography (TLC) of Tomatidenol and Tomatidine. TLC of tomatidenol and tomatidine was performed on Merck precoated silica gel G plates, 0.25 mm thick. The plates were developed in a chamber saturated with chloroform/methanol/1% NH_4OH (2:2:1, v/v, bottom layer) (Filadelfi and Zitnak, 1983). Spots were visualized by spraying with 3% H_2SO_4 in 33% ethanol and heating for 140 °C for 30 min (Lehrfield and Goodwin, 1969).

Mass Spectrometry of Dehydrotomatine, α-Tomatine, Tomatidenol, and Tomatidine. The listed compounds were analyzed by liquid secondary ion mass spectrometry (LSIMS) as described previously (Friedman et al., 1993, 1994).

GC−MS of Carbohydrate Derivatives. GC−MS was performed on a GCQ gas−liquid chromatography−ion trap mass spectrometer (Finnigan, San Jose, CA). The split/splitless injector was operated at 240 °C. Injection was made in splitless mode. Chromatography was performed with a 0.25 mm × 30 m, 0.25 μm film, DB-5MS, fused silica column (J&W Scientific, Folsom, CA) with an average helium carrier gas flow set to a constant velocity of 40 cm/s. The column oven temperature was held at 120 °C for 1.5 min and then programmed to 195 °C at 1.5 °C/min. The mass spectrometer was operated in the electron ionization mode with a source temperature of 200 °C. Positive ions were monitored by scanning the analyzer from mass 50 to 650 every 0.75 s.

RESULTS AND DISCUSSION

Figure 1 illustrates the chemical transformations of α-tomatine and dehydrotomatine described in this study. Figure 2 shows separation of α-tomatine and dehydrotomatine peaks on an HPLC chromatogram. Figure 3 shows the gas chromatographic separation of alditol acetate derivatives of galactose, glucose, and xylose derived from acid hydrolysis of commercial tomatine, α-tomatine, and dehydrotomatine along with the internal standard mannitol. Figure 4 shows the gas chromatographic separation of partially methylated monosaccharides derived from commercial tomatine, α-tomatine, and dehydrotomatine. Figure 5 shows the mass spectra of alditol acetates for xylose, mannitol (internal standard), glucose, and galactose originating from dehydrotomatine. Analogous results (not shown) were obtained using commercial tomatine and α-tomatine. Figure 6 shows mass spectra of partially methylated alditols derived from dehydrotomatine along with the internal standard mannitol. Analogous results (not shown) were obtained for commercial tomatine and α-tomatine. Table 1 shows the time course of liberation and isolation of monosaccharides during acid hydrolysis of commercial tomatine, α-tomatine, and dehydrotomatine.

The mass spectra of α-tomatine and dehydrotomatine isolated by preparative HPLC exhibits molecular ion

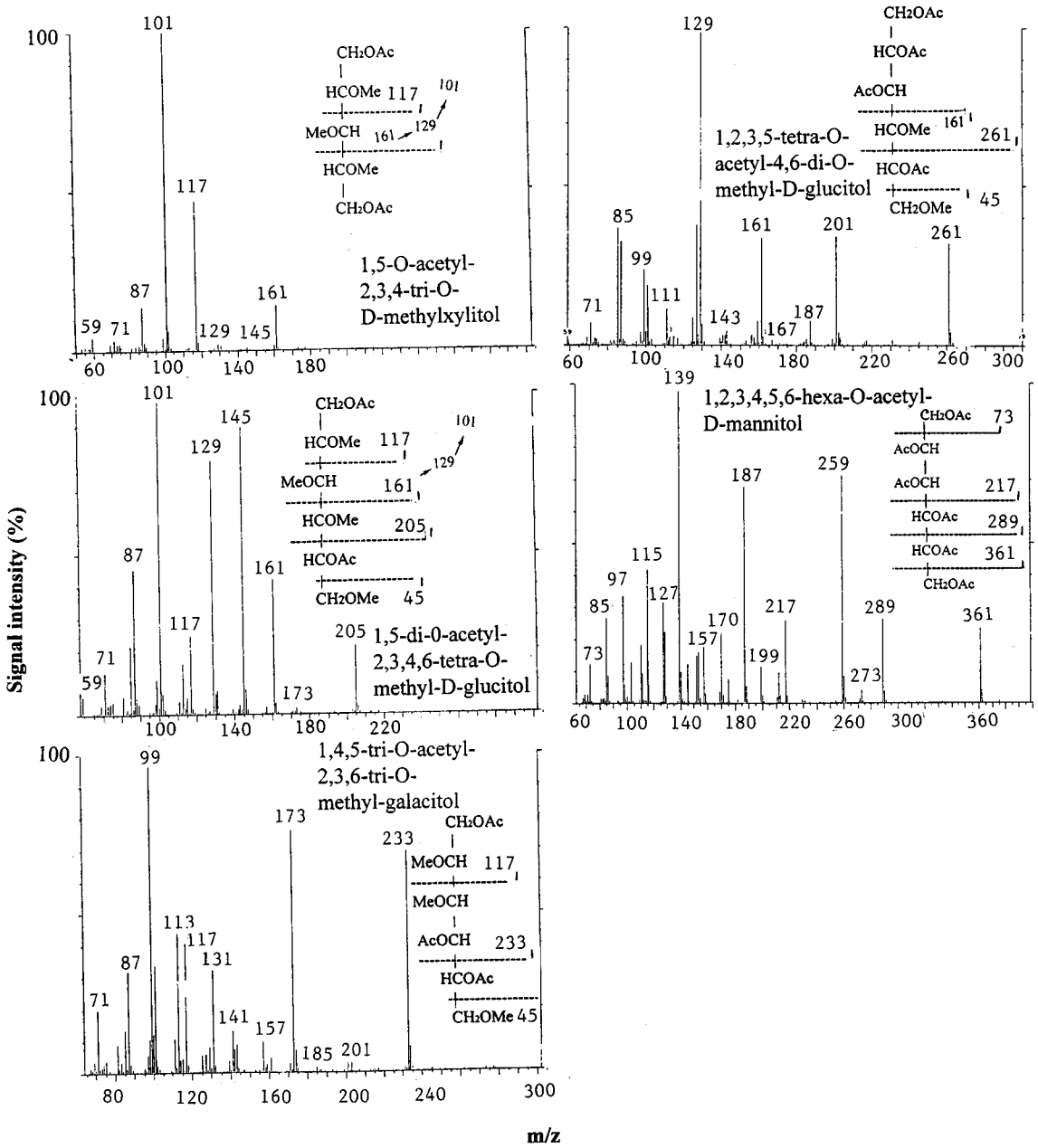

Figure 6. Mass spectra of partially methylated alditol acetate monosaccharides formed on acid hydrolysis of permethylated dehydrotomatine. The mannitol derivative was used an internal standard.

peaks that are in agreement with previously reported values (Bushway and Perkins, 1995; Friedman et al., 1994). This was also the case for the corresponding molecular ion peak for tomatidine, the aglycon derived from α-tomatine, and tomatidenol, the aglycon derived from dehydrotomatine.

The analytical data shown in Figures 3−6 were used to calculate the following molar ratios of alditol acetate derivatives of xylose, glucose, and galactose in dehydrotomatine: 1.00:2.39:1.10. The corresponding ratios for α-tomatine and for commercial tomatine were 1.00:2.06:0.97 and 1.00:2.77:1.25, respectively. Quantitation of the GC−MS data for the methylated alditol acetates revealed that 2,3,4-tri-*O*-methylxylose, 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylgalactose, and 4,6-di-*O*-methylglucose were, respectively, present in the following molar ratios in dehydrotomatine: 1.50:1.33:1.00:1.27. The corresponding values for α-tomatine and for commercial tomatine were 1.33:0.97:1.00:1.16 and 1.87:1.65:1.00:1.27, respectively.

The aglycons derived from acid hydrolysis of commercial tomatine, dehydrotomatine, and α-tomatine all had the same R_f value of 0.81 on TLC plates.

The main objective of this study was to characterize the monosaccharides comprising the carbohydrate side chain of dehydrotomatine. To accomplish this objective, we applied previously reported procedures for ascertaining the nature and number of individual monosaccharide groups in oligosaccharides associated with dehydrotomatine, α-tomatine, and commercial tomatine (Biermann, 1989; Carpita and Shea, 1989; Engelmaier et al., 1989; Herb et al., 1975; Lindberg, 1972; van Gelder, 1984; van Gelder et al., 1988).

The following general comments justify this approach. According to Lindberg (1972), methylation analysis is one of the most important methods in structural polysaccharide chemistry. It involves methylation of all hydroxyl groups, followed by acid hydrolysis to produce a mixture of partially methylated, stable sugars. The free hydroxyl groups formed after reduction by sodium borohydride, determined as alditol acetates, mark the

positions in which the sugar moieties are substituted. Generally, alditol acetates with the same substitution pattern give similar mass spectra typical of that substitution pattern.

The anomeric carbon atom is modified by reduction with the borohydride to the corresponding alditol to eliminate asymmetry that would otherwise result. This step avoids formation of mixtures of stereoisomers, thus minimizing the possibility of producing multiple peaks in chromatograms (Biermann, 1989; Carpita and Shea, 1989).

It is relevant to note that the use of NaH in DMSO to methylate sugars of glycoproteins may be accompanied by alkylation of amino acid residues and by reduction of disulfide bonds to sulfhydryl groups in the protein part of the glycoprotein (Friedman and Krull, 1970; Krull and Friedman, 1967). These aspects merit study.

Possible problems with this approach include degradation of carbohydrate moieties during acid hydrolysis and loss of volatile alditol derivatives, especially pentoses, during concentration of solutions. This may be the reason for the low recovery of xylose compared to the hexoses that is apparent from Figures 2 and 3. The susceptibility of the carbohydrate side chain to acid hydrolysis is also influenced by the structure of both the oligosaccharide (Friedman and McDonald, 1995; Friedman et al., 1993) and the aglycon (van Gelder, 1984).

Application of the cited approach to the determination of individual monosaccharides in the tetrasaccharide side chain of commercial tomatine, α-tomatine, and dehydrotomatine shows the following: (a) all three preparations produced the same galactose, glucose, and xylose methylated alditol acetate derivatives; and (b) the ratios of these sugars was also the same within experimental error of the multistep chemical transformations, with the possible exception of the higher than expected glucose content in commercial tomatine. We have no obvious explanation for this result.

On the basis of the above evidence, we conclude that dehydrotomatine and α-tomatine have the same oligosaccharide (lycotetraose) moiety. The two glycoalkaloids differ only by the presence of a double bond in ring B of the aglycon of dehydrotomatine and its absence in the aglycon of α-tomatine, as shown in Figure 1. These considerations suggest that dehydrotomatine be named tomatidenol-3β-lycotetraose; the corresponding name for α-tomatine is tomatidine-3β-lycotetraose.

The biosynthesis of dehydrotomatine and α-tomatine may arise by at least two different pathways. The first postulates that tomatidenol, the aglycon of dehydrotomatine which contains a double bond, arises from cholesterol, which also has a 5,6 double bond. By contrast, tomatidine, the aglycon of α-tomatine which lacks a double bond, arises from cholestanol, which also lacks a 5,6 double bond (Friedman and McDonald, 1997; Peterson et al., 1993). An alternative pathway postulates that the biosynthetic intermediate teinimine derived from cholesterol is partitioned; part of its double bond is hydrogenated by a hypothetical hydrogenase to tomatidine (Laurila et al., 1996), and the remainder forms tomatidenol. Since the present study has revealed that the two glycoalkaloids possess the same carbohydrate side chain, it is quite likely that tomatidenol and tomatidine are similarly glycosylated to the corresponding glycoalkaloids (Moehs et al., 1997; Zimowski, 1994). We are challenged to characterize the enzymes and intermediates involved in the biosynthesis of the two tomato glycoalkaloids.

The findings about dehydrotomatine have broad implications since published studies on the analysis of tomatine (Friedman and Levin, 1995; Kozukue et al., 1994; Stanker et al., 1994, 1996), its role in host-plant resistance (Duffey and Stout, 1996; Friedman, 1997; Lairini et al., 1996; Osbourn et al., 1996; Sandrock et al., 1996), and its dietary significance (Blankemeyer et al., 1997; Friedman et al., 1996, 1997; Roddick and Drysdale, 1984) assumed the presence of a single glycoalkaloid, α-tomatine. Future studies should address the distribution of both dehydrotomatine and α-tomatine in tomatoes at different stages of maturity and in processed tomato products, as well as the respective roles of the two tomato glycoalkaloids in the plant and in the diet.

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