Isolation of a Novel Tetrasaccharide, Bemisiotetrose, and Glycine Betaine from Silverleaf Whitefly Honeydew

Yuan-an Wei,[†] Donald L. Hendrix,*[,‡] and Ronald Nieman[§]

Carbohydrate Research Laboratory, Industrial Testing and Experimental Center, Guangxi University, Nanning, Guangxi 530004, People's Republic of China, Western Cotton Research Laboratory, ARS, U.S. Department of Agriculture, 4135 East Broadway Road, Phoenix, Arizona 85040, and Department of Chemistry, Arizona State University, Tempe, Arizona 85287

Oligosaccharides in the honeydew secreted by the silverleaf whitefly (*Bemisia argentifolii* Bellows and Perring) feeding upon cotton (*Gossypium hirsutum* L.) were isolated by gel permeation chromatography followed by gradient elution HPLC employing an amine column and an evaporative light-scattering detector. A tetrasaccharide was collected from the HPLC eluate and characterized by mass spectral and methylation analysis and by 1D and 2D NMR. The results of these analyses are consistent with a highly symmetrical tetrasaccharide: *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔1)-*O*-α-D-glucopyranosyl-(4←1)-α-D-glucopyranoside. In addition, a significant peak of glycine betaine (*N*,*N*,*N*-trimethylglycine) was detected in the HPLC chromatograms of sugars from this honeydew.

Keywords: *Insect; carbohydrate; HPLC; cotton*

INTRODUCTION

The silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring, causes losses of hundreds of millions of dollars annually to agricultural productivity in subtropical regions. *B. argentifolii* withdraws phloem sap from plant leaves and excretes a concentrated sugar solution termed honeydew. A serious problem results when honeydew falls on cotton fiber. This leads to "sticky" and sooty mold-contaminated cotton, which makes the fiber difficult to impossible to process in textile mills and therefore of lower commercial value.

The honeydew of *B. argentifolii* contains a number of uncommon sugars, which differ from sucrose, the primary sugar in phloem sap of the cotton plant (Tarczynski et al., 1992; Ziegler, 1975). For instance, while glucose, fructose, and sucrose occur in this honeydew, the unusual disaccharide trehalulose [*O*-α-D-glucopyranosyl-(1→1)-*β*-D-fructoside] makes up nearly half of this sugar mixture. The trisaccharide melezitose [*O*-α-D-glucopyranosyl-(1→3)-*O*-*β*-D-fructofuranosyl-(2←1)-α-D-glucopyranoside], its metabolite turanose [*O*-α-D-glucopyranosyl-(1→3)-D-fructofuranoside] (Hendrix et al., 1992), and a number of larger oligosaccharides (Hendrix and Wei, 1994) also occur in this secretion. All of the sugars in this mixture are created from dietary sucrose within the insect, possibly in biochemical reactions involving symbiotic microbes (Davidson et al., 1994).

It has been suggested that the stickiness of honeydew-contaminated cotton could be remedied by chemical or enzymatic treatment (Hendrix et al., 1995); however, in order to ameliorate cotton fiber stickiness in this manner it is essential to know the chemical nature of these sugars. A program was therefore initiated to isolate and characterize the sugars in *B. argentifolii* honeydew. A literature survey showed that previous honeydew sugar research dealt almost exclusively with the identification of smaller saccharides. In part, this might be because smaller saccharides are easily demonstrated to be constituents of insect honeydews.

For many years, the HPLC method of choice for analyzing sugar mixtures depended upon refractive index detection. However, these methods are unable to resolve complex oligosaccharide mixtures because they cannot be used with gradient elution. Evaporative light-scattering detectors (ELSD) are insensitive to solvent composition, which allows their use with gradient elution. In addition, this detector responds only to the mass of solute in eluted peaks, which results in a more accurate quantitation of analyzed materials for which standards are not available. Using this detector, it has been found that oligosaccharides larger than trisaccharides constitute a substantial fraction of *Bemisia* honeydew carbohydrates (greater than 13% peak area), and from their relative abundance it is likely that these sugars play a significant role in the physical and chemical characteristics of this secretion.

The larger saccharides in honeydew may play an important role in the regulation of the osmotic pressure of body fluids within honeydew-secreting insects (Fisher et al., 1984). Understanding the structure and biosynthesis of these larger saccharides may therefore be useful in developing strategies to control this serious agricultural pest.

Recently, we reported the identification of several saccharides in *Bemisia* honeydew which were isolated by gel permeation chromatography and HPLC (Hendrix et al., 1992; Hendrix and Wei, 1994). This work indicated that *B. argentifolii* honeydew consisted of at least 20 sugars including an unusual trisaccharide which was named bemisiose: *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔1)-α-D-glucopyranoside.

In carrying out the present saccharide analyses by HPLC, we observed a peak that did not exhibit elution behavior expected for a sugar. Analysis of the material that gave rise to this HPLC peak by infrared and mass

* Author to whom correspondence should be addressed.
[†] Guangxi University.
[‡] U.S. Department of Agriculture.
[§] Arizona State University.

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Table 1. HPLC Program 1

time (min)	% CH_3OH	% CH_3CN	% H_2O
0.0	0.0	78.0	22.0
2.1	0.0	78.0	22.0
5.0	0.0	75.0	25.0
7.0	3.0	72.0	25.0
10.0	5.0	70.0	25.0
30.0	5.0	70.0	25.0
33.0	10.0	65.0	25.0
40.0	20.0	55.0	25.0
45.0	72.0	0.0	28.0
50.0	72.0	0.0	28.0
55.0	0.0	78.0	22.0
60.0	0.0	78.0	22.0

Table 2. HPLC Program 2

time (min)	% CH_3OH	% CH_3CN	% H_2O
0.0	3.0	73.0	24.0
2.0	3.0	73.0	24.0
5.0	5.0	60.0	35.0
42.0	5.0	60.0	35.0
51.0	63.0	5.0	32.0
55.0	3.0	73.0	24.0
60.0	3.0	73.0	24.0

spectroscopy suggested it was glycine betaine, a quaternary amine formed from the oxidation of choline (Hanson and Scott, 1980). This compound appeared to have originated in the plant leaf since glycine betaine has been previously suggested to be a component of leaf phloem sap upon which this insect feeds (Ladyman et al., 1980).

EXPERIMENTAL PROCEDURES

Materials. Honeydew was recovered from the upper surface of upland cotton (*Gossypium hirsutum* L.) leaves harvested from a field heavily infested with *Bemisia argentifolii*. The sugars were removed from the leaves by washing in pans of deionized water. Debris in the water wash was removed by filtration and centrifugation. Water in the resulting sugar solution was then removed by lyophilization, resulting in a highly hygroscopic dark brown powder.

Methods of Oligosaccharide Purification and Analysis. A BioRad P-2 gel permeation column (4.8 × 120 cm) heated to 65 °C (Trenel et al., 1968) and eluted with deionized water was used to separate the sugars in this crude mixture by size. The eluant was removed from the eluted sugars by lyophilization. Sugars in the various fractions from the P-2 column were quantified by high-performance anion-exchange chromatography (HPAEC) using methods described previously (Hendrix and Wei, 1994). In addition, a preparative high-performance liquid chromatographic system was utilized to further purify the oligosaccharides eluted from the BioRad column. The preparative HPLC system consisted of a Dionex GP40 gradient pump, an Alltech Varex MKIII ELSD, a Spectra Physics Model AS3500 autosampler, and one Alltech Adsorbosphere NH_2 guard column (7.5 × 4.6 mm, 5 μm) and two Alltech Adsorbosil NH2 cartridge columns (250 × 4.6 mm, 5 μm) connected in series. An Alltech flow splitter was used to split and adjust the ratio of the effluent from the column to the ELSD and to an ISCO Model Foxy 200 fraction collector. The parameters of the ELSD were set as follows: drift tube temperature, 115 °C; nebulizing gas (nitrogen) flow, 2.0 standard liters/min. The output of the ELSD was processed by means of a Dionex Peaknet work station. A ternary solvent system was used which consisted of various ratios of methanol, acetonitrile, and water. Two relatively complex gradient programs were used, one for analysis of all components in honeydew (Table 1, program 1), and a second program for the analysis of a fraction from the P-2 gel column which appeared to be enriched in tetrasaccharides (Table 2, program 2). All the transitions between compositions listed in these programs were linear.

Mass Spectrometry. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) and fast atom bombardment mass spectrometry (FAB-MS) were applied to an aqueous solution (~10 μg/μL) of the native unknown saccharide. These MALDI-TOF analyses were performed on an LDI-1700 instrument, and FAB-MS analysis was carried out on a JEOL JMS-SX/SX-102A tandem mass spectrometer.

Methylation and GC−MS Analysis. A sample of the purified oligosaccharide was methylated by a modified Hakomori procedure (York et al., 1985) and then hydrolyzed, reduced, and partially acetylated. The resulting alditol acetates were analyzed by a Hewlett-Packard 5985 GC−mass spectrometer using a 30-m SP2330 capillary column (Supelco).

NMR Spectroscopy. A 6.5-mg sample of the tetrasaccharide was dissolved in D_2O (~0.7 mL) for the NMR experiments. Spectra were obtained at 25 °C on a Varian Unity-500 spectrometer equipped with a 5-mm indirect-detection gradient probe and operated at 499.0 Mhz for ^1H and 125.7 Mhz for ^{13}C. Chemical shifts were determined with acetone as an internal reference; assigned chemical shift values for acetone were 2.224 ppm for protons and 31.00 ppm for the methyl carbons. Phase-sensitive TOCSY, NOESY, and double-quantum filtered COSY (DQFCOSY) spectra were acquired using the method of States et al. (1982). The residual HDO signal was suppressed by presaturation. The mixing times for the NOESY and TOCSY spectra were 300 and 60 ms, respectively. Absolute value HMQC (Bax and Subramanian, 1986) and HMBC (Bax and Summers, 1986) spectra were acquired using pulsed-field gradients (Hurd and John, 1991) for coherence selection and artifact suppression, with a maximum gradient strength of 10 G/cm. The HMQC spectra were acquired with GARP decoupling of carbon during acquisition.

Analysis of Glycine Betaine. A portion of the isolated honeydew was fractionated using a BioRad P-2 column. One of the fractions from the P-2 column that was enriched in some of the larger oligosaccharides in this honeydew was analyzed by HPLC using gradient program 2. The material eluting from the column between 13 and 15 min was recovered from the HPLC solvent and incorporated into a KBr pellet for FT-IR analysis. An additional portion of the recovered solute was added to a matrix of 2,5-dihydroxybenzoic acid, and this mixture was analyzed by MALDI-TOF using a Vastec Lasertec research model instrument operated in the positive ion mode. A nitrogen laser supplying 3-ns pulses of light at 337 nm was used for sample desorption in these analyses.

RESULTS AND DISCUSSION

Figure 1 shows the result of gradient HPLC analysis of *B. argentifolii* honeydew collected from cotton leaves. Compared with earlier isocratic elution analysis of this honeydew (Hendrix et al., 1992), this analytical method exhibits a far better resolution of honeydew sugars. If isocratic elution is optimized to separate mono- and disaccharides, the larger saccharides in the mixture are retained so strongly on amine HPLC columns that their peaks spread beyond recognition. Moreover, the glycine betaine peak (3) in Figure 1 could not be separated from other honeydew components using isocratic elution (Hendrix et al., 1992). The eluate containing the peak 9 in Figure 1 was collected for further analysis. Results of MS and NMR analysis indicated that this peak was a novel tetrasaccharide which contained the structure of bemisiose (Hendrix and Wei, 1994). It was therefore named bemisiotetrose. The carbohydrate components in *Bemisia* honeydew that could be identified using this gradient technique and their relative abundance are listed in Table 3.

To isolate a sufficient amount of bemisiotetrose for structural analysis, 7.5 g of the honeydew powder was dissolved in water and clarified by centrifugation (100000*g*, 30 min). The resulting clarified supernatant

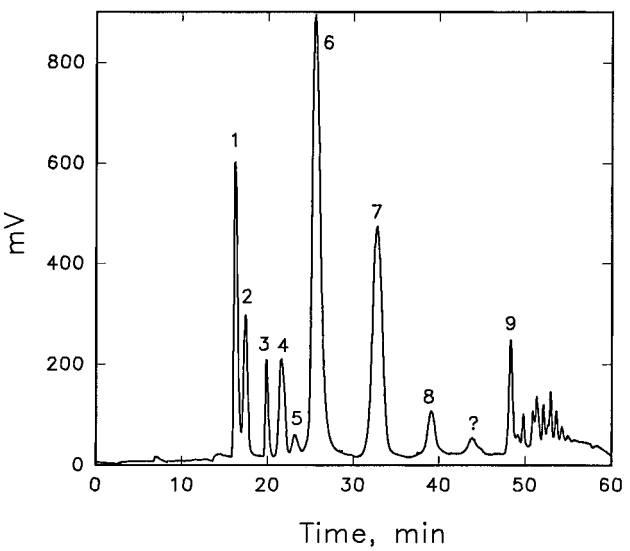

Figure 1. HPLC analysis of *Bemisia* honeydew collected from cotton leaves and chromatographed using gradient elution program 1. Peak identification: (1) fructose, (2) glucose, (3) glycine betaine, (4) sucrose, (5) turanose, (6) trehalulose, (7) melezitose, (8) bemisiose, (?) unknown tetrasaccharide, (9) bemisiotetrose.

Table 3. Elution of Honeydew Sugars from Amine HPLC Column

peak	retention time (min)	% total (peak area)[a]
fructose	16.22	10.48
glucose	17.42	5.63
glycine betaine	19.87	2.72
sucrose	21.63	5.38
turanose	23.13	1.29
trehalulose	25.53	36.59
melezitose	32.83	21.73
bemisiose	39.07	2.83
bemisiotetrose	48.33	4.67
all other peaks		8.68

[a] Determined from the output of the ELSD detector.

was loaded onto a BioRad P-2 gel column. The sugars in one of the resulting saccharide fractions from the P-2 column that contained mainly tetrasaccharides were further separated by preparative HPLC using gradient program 2 (Table 2). The result of this separation is shown in Figure 2. The eluate containing the peak corresponding to bemisiotetrose in Figure 2 was collected and the sugar recovered by lyophilization. HPAEC analysis of an acid hydrolysate of this peak revealed it consisted entirely of glucose. The FAB-MS spectra of this compound showed a $[M + H]^+$ ion at *m/z* 667, and the MALDI-TOF spectra showed two principal ions, one at *m/z* 686.8, which corresponds to the $[M + Na]^+$ ion, and a second ion at *m/z* 703.3, which is the ammonium form of the latter ion. GC−MS analysis of the permethylated alditol acetates of this compound showed the presence of only two types of hexosyl residues: terminal glucose and 4-linked glucose. From these data, it was concluded that this compound is a tetrasaccharide with molecular weight of 666, consisting only of glucose residues. In addition, these data are consistent with the following bonds between the glucose subunits: glucose-(1↔1)-glucose and glucose-(1→4)-glucose.

The ^1H and ^{13}C NMR spectra of this compound revealed that this tetrasaccharide has a very symmetrical structure, since these spectra appear very similar to that of a disaccharide. Analysis of the NMR data revealed only two anomeric proton resonances (δ 5.42, d, 1 H, $J_{1,2}$ = 4.00 Hz; δ 5.18, d, 1H, $J_{1,2}$ = 3.50 Hz), which correlated from the HMQC spectrum (Figure 3) with two anomeric carbon resonances at δ 100.40 and 94.10 ppm, respectively. These data indicated that both are α-linked hexosyl residues and the carbon resonance at 94.10 ppm is due to an α-linked carbon in which the two hexosyl residues are α-1↔1-α-linked. This strong upfield displacement is typical of the resonance of the C-1 carbon of α-D-glucopyranosyl group residues which are anomerically linked to a hemiacetal rather than to an alcoholic "aglycon" moiety. This conclusion was confirmed by comparing these results to the ^{13}C spectra of α,α-trehalose [*O*-α-D-glucosyl-(1↔1)-α-D-glucopyranose], sucrose [*O*-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside], melezitose (Seymour et al., 1979), erlose [*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside] (Wei et al., unpublished), and bemisiose (Hendrix and Wei, 1994). However, it should be noted that the ^{13}C spectra of α,β-trehalose (Bock et al., 1984) does not follow this pattern. In the case of bemisiotetrose, the most convincing evidence for this assignment can be seen in the NMR data of its perbenzylated derivative (Wessel et al., 1991). In these data one can observe a disaccharide-like spectra and chemical shifts typical of anomeric carbons linked α-1↔1-α.

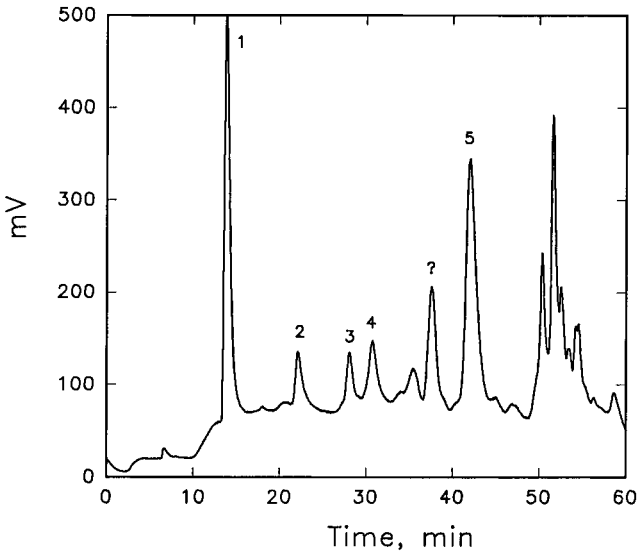

Figure 2. HPLC separation of a BioRad P-2 fraction of *Bemisia* honeydew sugars by gradient elution program 2. Peak identification: (1) glycine betaine, (2) trehalulose, (3) melezitose, (4) bemisiose, (?) unknown tetrasaccharide, (5) bemisiotetrose.

After the assignments of the resonances of anomeric protons and carbons were confirmed, they were used as a starting point to make the assignments in the coupling networks in the 2D DQFCOSY spectrum. Two sets of protons of two glucopyranose residues labeled a and b in Table 4 were clearly revealed in these spectra. In the TOCSY spectra, the anomeric signals also showed clear connectivites to the H-2, H-3, H-4 of their respective glucose rings. Two ring proton signals, H-4 (δ 3.42, t) of residue a and H-3 (δ 4.11, dd) of residue b, could easily be distinguished from their corresponding glucose ring resonances, and using these points as a reference, it was possible to trace the rest of the connectivites (H-2/H-3/H-4/H-5/ H-6). Using HMQC and HMBC for cross reference, all carbons and protons resonances were assigned as summarized in Table 4. The HMBC spectra was also helpful in assigning the H_a-6 and H_b-6 protons in each residue which experienced coupling to each other

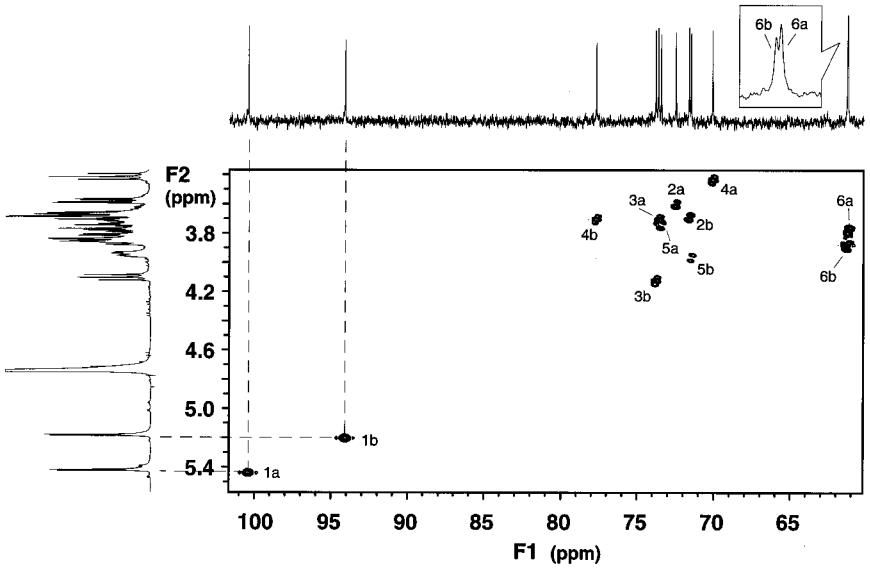

Figure 3. Gradient HMQC spectra of bemisiotetrose. The ^{1}H and ^{13}C NMR spectra of bemisiotetrose are shown on the F2 and F1 axes, respectively.

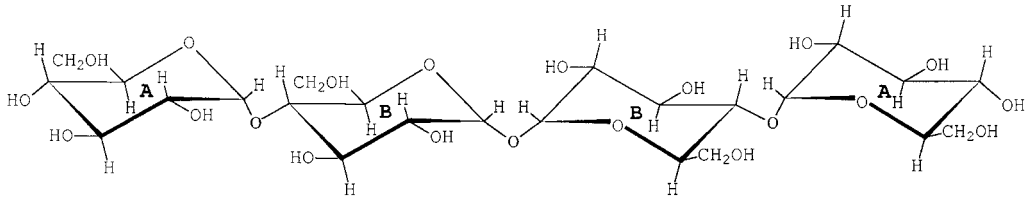

Figure 4. Structure of bemisiotetrose.

Table 4. NMR Data of Bemisiotetrose

proton	ppm[a]	assignm (*J*, Hz)[b]	carbon	ppm
H-1a	5.42	d (4.0)	C-1a	100.40
H-2a	3.58	dd (4.0; 10.0)	C-2a	72.49
H-3a	3.69	dd ≈ t (10.0; 9.5)	C-3a	73.63
H-4a	3.42	t (9.5; 9.5)	C-4a	70.11
H-5a	3.72	ddd (9.5; 5.0; 2.0)	C-5a	73.45
H_a-6a	3.85	dd (2.0; 12.0)	C-6a	61.25
H_b-6a	3.76	dd (5.0; 12.0)		
H-1b	5.18	d (3.5)	C-1b	94.10
H-2b	3.67	dd (3.5; 10.0)	C-2b	71.63
H-3b	4.11	dd (10.0; 9.0)	C-3b	73.80
H-4b	3.69	dd (9.0; 10.0)	C-4b	77.70
H-5b	3.95	ddd (10.0; 4.5; 2.0)	C-5b	71.50
H_a-6b	3.87	dd (2.0; 12.0)	C-6b	61.28
H_b-6b	3.80	dd (4.5; 12.0)		

[a] Chemical shifts in ppm refered to acetone as an internal reference. [b] Coupling constants (in hertz) are given in parentheses following each peak assignment.

and to H-5, since these data fell in a crowded region of the spectrum.

The C-4 of residue b appeared at a low field (δ 77.70; Table 4) showing that substitution occurred at this position. Moreover, a strong cross-peak between H-4 of residue b and C-1 of residue a was observed in the HMBC spectra. This interglycosidic correlation indicated that residue b was substituted at the C-4 position with residue a. More interestingly, the HMBC experiment showed long-range connectivites between carbon atoms and their coupled protons, indicating that there is a cross-peak between H-1 and C-1 of residue b. This indicates that there are two identical b residues and both of them were connected to a 1↔1 linkage. If the coupling between H-1 and C-1 came from the same b residue, i.e., directly bonded proton and carbon, the cross-peak would not be present in the HMBC spectrum. Therefore, the most reasonable explanation of these data is that the H-1 proton of one of the b residues is coupled through the C-1 carbon to another b residue through a bond due to a 1↔1 linkage between the two b residues.

Information concerning glycosidic linkages and the glycosyl sequence obtained from NMR experiments, combined with the results from mass spectra and methylation analysis, allowed us to conclude that bemisiotetrose is a highly symmetrical tetrasaccharide which consists of two identical disaccharides linked by their anomeric carbons. Each of the disaccharides in this molecule consists of a terminal glucose α-linked to the C-4 of the second glucose (i.e., maltose). The anomeric carbons of two disaccharides are also in the α configuration. The structure proposed for bemisiotetrose is given in Figure 4.

The glycine betaine peak in Figure 1 eluted from the P-2 column in a fraction that contained trisaccharides and tetrasaccharides. However, its elution behavior on the amine column indicated that it is neither a trisaccharide nor a tetrasaccharide, since it eluted from this column before the disaccharides sucrose and trehalulose (cf. Figures 1 and 2) and this column separates oligosaccharides strictly by size. This suggested that this peak was not due to a saccharide.

The FT-IR spectrum from this nonsaccharide (1626 cm^{-1} S; 1491, 1473 cm^{-1} M; 1415, 1394, 1332 cm^{-1} S; 985, 931, 891 cm^{-1} M) was very similar to that published for the ammonium hydroxide inner salt of glycine betaine (Pouchert, 1981). Standard samples of glycine betaine and the unknown isolated from the HPLC eluant eluted with identical retention times from the HPLC columns employed to chromatograph honeydew saccharides. In addition, the positive ion MALDI-TOF spectrum of this compound contained a parent ion at *m*/*z* 118.2 and an ion at *m*/*z* 234.9 which correspond

to a monomer and dimer of glycine betaine, respectively. Also, this spectrum contained a peak corresponding to an ion at *m*/*z* 74.1 that is equivalent to decarboxylated glycine betaine and a small peak at *m*/*z* 60.4, which corresponds to decomposition fragment of glycine betaine, trimethylamine. It was thus concluded that this compound was glycine betaine.

Glycine betaine did not appear in HPAEC chromatograms of this honeydew published earlier (Hendrix and Wei, 1994), since the detector used in the HPAEC analysis does not respond to glycine betaine. The ELSD, however, detected this compound since it responds to all nonvolatile materials eluted from the column. However, glycine betaine tended to coelute with certain honeydew oligosaccharides in isocratic separations, and it was only when gradient elution was employed with the ELSD detector that glycine betaine was observed.

This is the first demonstration of glycine betaine in insect honeydews. From the amount of material being secreted (2.7%) compared to the very tiny size of these insects, and the fact that glycine betaine is very abundant in water-stressed cotton leaves (Hanson, 1996), it is concluded that this must be coming from the phloem sap upon which the insect is feeding. However, animals and microorganisms are also capable of producing this compound (cf. references in Mar et al., 1995), so a direct analysis of phloem sap would be necessary to confirm this conclusion.

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