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Isolation and Characterization of Esters of Indole-3-Acetic Acid from the Liquid Endosperm of the Horse Chestnut (*Aesculus* species)[1]

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ABSTRACT

Esters of indole-3-acetic acid were extracted and purified from the liquid endosperm of immature fruits of various species of the horse chestnut (*Aesculus parviflora, A. baumanni, A.pavia rubra, and A. pavia humulis*). The liquid endosperm contained, at least 12 chromatographically distinct esters. One of these compounds was purified and characterized as an ester of indole-3-acetic acid and *myo*-inositol. A second compound was found to be an ester of indole-3-acetic acid and the disaccharide rutinose (glucosyl-rhamnose). A third compound was partially characterized as an ester of indole-3-acetic acid and a desoxyaminohexose.

Shantz and Steward (26) demonstrated that a substance isolated from the vesicular embryo sac of immature fruits of *Aesculus woerlitzensis* greatly stimulated cell division of carrot root phloem explants. They purified this material to homogeneity and characterized its hydrolysis products as IAA, and a disaccharide composed of glucose and rhamnose. The instrumentation available did not permit characterization of the intact compound. An earlier study from FC Steward's laboratory demonstrated that coconut milk was a stimulatory adjunct to tissue culture media (3) and additional studies showed that the liquid endosperm from the walnut (*Juglans regia*) and from immature grains of *Zea mays* were alternative sources of the stimulatory adjunct (26).

Our studies of the indolylic compounds of kernels of *Z. mays* resulted in the characterization of several esters of IAA and *myo*-inositol and *myo*-inositol glycosides (*e.g.* Refs. 6, 19, 27, 28). IAA-*myo*-inositol esters were also found to be present in other members of the *Zea* tribe, in rice (*Oryza sativa*) (14), and in vegetative tissues of maize (4). We also examined the indolylic compounds of horse chestnut liquid endosperm and observed a number of esters of IAA (A Schulze, RS Bandurski, unpublished data, 1976).

This work, and a previously published abstract of a portion of this work (W Domagalski, A Schulze, RS Bandurski 1985, Plant Physiol 77:S-3, abstract), showed IAA-*myo*-inositol (IAInos)[3] to be present in horse chestnut. Aharoni and Cohen showed the compound to be present in IAA treated tobacco (N Aharoni, JD Cohen 1986, Plant Physiol 80:S-34, abstract), and thus we know that IAInos is widely distributed in nature.

The present studies arose from our prior interest in esters of IAA (6, 19, 28) and from the studies of FC Steward's laboratory (3, 21, 26) indicating that endosperm factors provided valuable adjuncts to plant tissue culture media. Thus, the data presented here extend the taxonomic distribution of esters of IAA and *myo*-inositol, increases the number of known esters, and provides knowledge of stimulatory adjuncts for plant tissue culture.

Table I. *Thin Layer Chromatographic Profile of IAA Esters in Aesculus sp*

Chromatography was on Silica Gel 60 using ethylacetate: acetonitrile:ethanol:water (5:3:1:1) as solvent. Detection was with the Ehmann reagent (9). Ammonolysis of these compounds under mild condition yields IAA and IAA-amide indicating the compounds are IAA esters. Migration is expressed relative to that of the B_1 (11, 19) indole-3-acetyl-*myo*-inositol ester (R_{IAInos}) where the migration of IAInos = 1.0.

R_{IAInos}	*A. parviflora*	*A. baumanni*	*A. pavia humulis/A. pavia rubra*
0.32	+		
0.41	+		+
0.57		+	
0.62		+	
0.70		+	
0.84			+
0.87	+		
0.93	+	+	+
1.00	+	+	+
1.04		+	
1.07	+	+	+
1.10		+	+
1.12		+	
1.14	+	+	+
1.22	+		
1.46		+	
1.52	+		
1.61–66	+	+	
1.88	+		
2.20	+		

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[3] Abbreviations: IAInos, indole-3-acetyl-*myo*-inositol; R_{IAInos}, thin layer chromatography migration relative to indole-3-acetyl-*myo*-inositol. FAB, fast atom bombardment; LH-20, lipophyllic Sephadex: EI, electron impact; m+, molecular ion; m/z, mass per number of charges; TMS, trimethyl silyl.

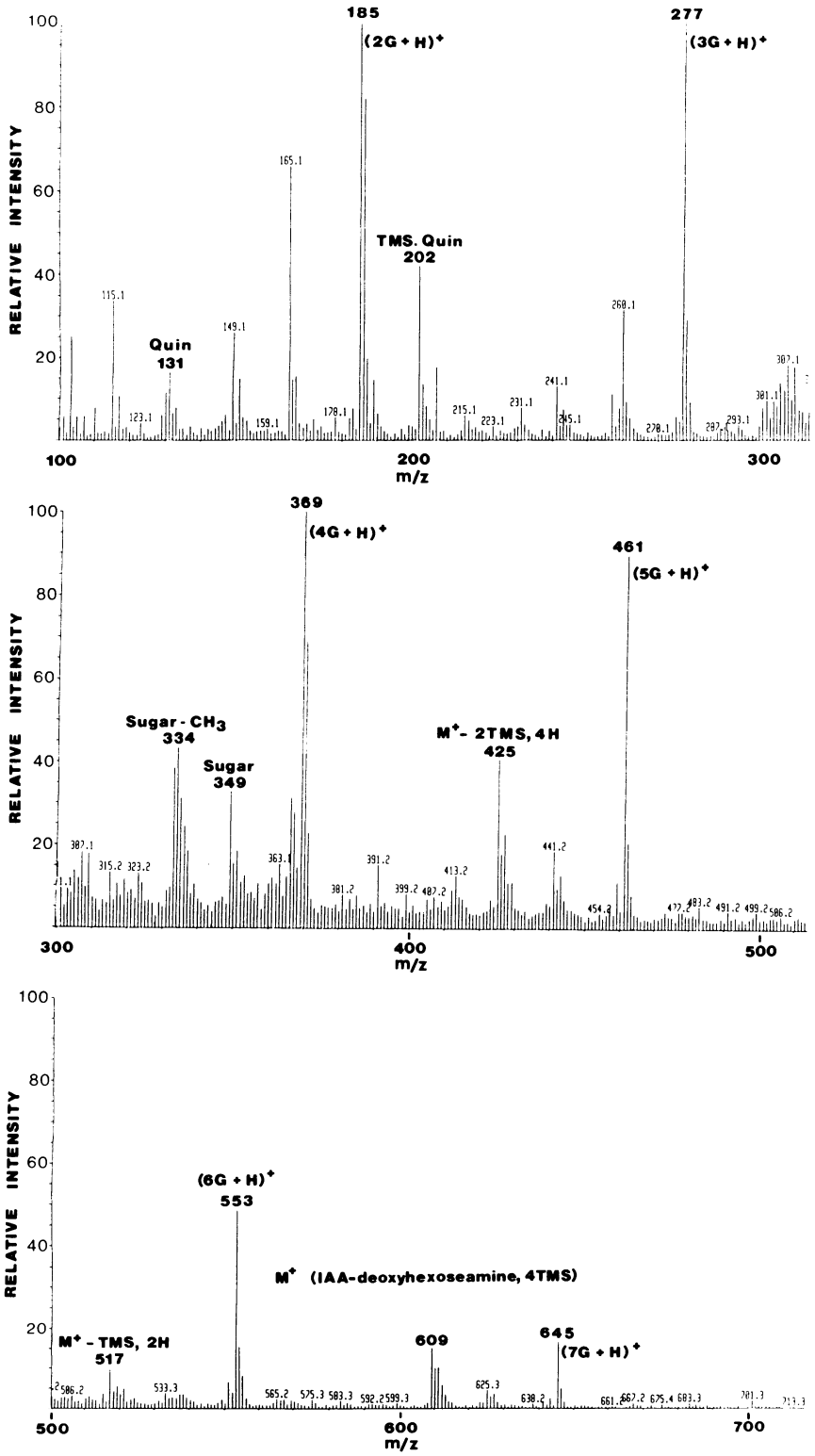

FIG. 1. FAB spectrum of a putative IAA-desoxyaminohexose isolated from *A. parviflora*. The molecular ion, M^+ is at 609. Glycerol ions are designated as G and shown at m/z = 645, 553, 461, 369, 277, and 185. Diagnostic ions are labeled in the figure using the minus symbol to indicate loss of that fragment by the parent ion.

MATERIALS AND METHODS

Plant Material. Collections of fluid from the vesicular embryo sac (liquid endosperm) were made during late August to early September during the summers of 1976, 1977, 1978, 1984, and 1985. The fruits of *Aesculus parviflora* are small and fluid could only be obtained from vesicular sacs in which the embryo had aborted, thus preventing the liquid endosperm from being absorbed by the developing cotyledons (26). From the remaining species it was possible to obtain about 0.05 to as much as 0.5 ml of fluid, usually 0.1 to 0.2 ml, from each immature vesicle sac. The fruits were cut approximately in half and the fluid aspirated with a syringe and collected into an iced beaker. After about 20 ml of fluid had been collected, an equal volume of acetone was added. The 1:1 fluid:acetone mixture then could be stored at −20°C for periods of up to 10 years without serious loss of the ester conjugates. Collections were made from the Michigan State

Table II. *A Comparison of the 70 eV Mass Spectral Fragmentation Pattern of Acetylated Putative Indole-3-Acetyl-myo-Inositol from A. parviflora with Authentic Indole-3-Acetyl-myo-Inositol from Z. mays*

m/z	*A. parviflora*	*Z. mays*	
	abundance		
589	0.1	0.2	Hexaacetyl IAInos
547	5.0	4.0	Pentaacetyl IAInos
374	0.7	1.4	547–174
175	1.5	1.5	IAA
174	3.7	4.6	IAA-H
169	3.2	4.6	
157	82.3	83.2	IAA fragment
145	1.4	2.7	Inositol fragment
130	100.0	100.0	IAA quinolinium ion
77	3.8	5.8	Benzene ring

University campus and from the following species, *A. parviflora*, *A. hippocastanum*, *A. pavia humulis*, and *A. pavia rubra*.

Chromatography. Dowex 50-X2-400 (Sigma) was prepared by cycling the resin through 1 M NaOH, water, 1 M HCl, water, 1 M NaOH, and then water until the eluate was neutral. The purified resin was then stored in 50% ethanol-water (v/v) and washed briefly just prior to use. The column dimensions were 0.9 × 19 cm and elution was with 50% aqueous ethanol using a flow rate of 2 ml·h^{-1} collecting 1 ml fractions (10).

LH-20 Chromatography. Lipophyllic Sephadex (LH-20) was washed extensively with alcohol and water and then stored in 50% ethanol water. Column dimensions were 2.0 × 23 cm and elution was with 50% aqueous ethanol using a flow rate of 2.0 ml·h^{-1} and collecting fractions of 2.5 ml.

Thin Layer Chromatography. TLC was on precoated plates of Silica Gel 60 (Merck Darmstadt, Brinkman) using ethyl acetate:methyl ethyl ketone:ethanol:water (5:3:1:1) as solvent. Indole compounds were detected by dipping the plates in Ehmanns' reagent, blotting dry, and heating at 100°C. Following washing, the plates may be kept indefinitely with retention of the characteristic blue color for indoles (9).

HPLC Chromatography. A Varian model 5000 gradient liquid chromatograph equipped with a Rheodyne high pressure loop injector and a UV detector was used for HPLC analysis. Column dimensions were 4 × 250 mm and flow rates of 1 ml·min^{-1} were used. Reverse phase columns were packed with C_{18} Partasil 10-ODS. Straight phase chromatography was on Partasil 10. The straight phase solvent system used was ethyl acetate: acetonitrile:EtOH: H_2O (65:21:7:7) or as indicated. ODS solvent systems are as described.

Mass Spectrometry. FAB spectra were obtained with a VG-ZAB-HF mass spectrometer (Shell Development, Modesto, CA). The sample was inserted into the probe in a glass capillary with glycerol and manually sublimed using resistance heating and scanning over the mass range 100 to 1200. EI probe spectra were obtained with that same instrument. The spectra for the hepataacetyl rutinose were obtained with a Hewlett-Packard 5992A using a 11 m CP Sil-19 CB-WCOT, fused silica capillary column. Spectra of the IAA-*myo*-inositols were obtained at 70 eV using a Hewlett-Packard 5985 quadrapole and 60 cm × 3 mm 3% OV-17, column.

Chemicals. Chemicals were of the best available grade. Pyridine was distilled over NaOH and stored under N_2 in a desiccator over calcium sulfate at −20°C. Dowex-50, LH-20 Sephadex, heptaacetyl rutinose were from Sigma Chemical Co.; 4-dimethyl aminopyridine was from Aldrich Chem. Co. C_{18} Partasil 10 ODS and Partasil 10 were from Whatman, Clifton, NJ. TLC plates were from Brinkman, Westbury, NY manufactured by Merck-Darmstadt.

RESULTS

Fractionation of Extracts. The 1:1 endosperm fluid:acetone mixture was filtered and then concentrated *in vacuo* to about 3% of the original volume. This was applied to a Dowex-50 column and eluted with ethanol:water (1:1). The column eluate was monitored by TLC of the concentrated effluent using Ehmanns' reagent to visualize indoles including esters (9, 24). Usually the first 15 ml contained high concentrations of sugars and little or no indolylic compounds and was discarded. The ester fractions emerge between 18 to 36 ml, as judged by Ehmann-reactive material, except for the putative amino hexose ester, which emerged between 38 to 80 ml. The desired fractions were pooled, evaporated *in vacuo* to dryness, dissolved in ethanol:water and applied to a 2 × 23 cm column of LH-20 sephadex which had been equilibrated with 50% ethanol-water. Elution was with the same solvent. The successive aliquots were again monitored by TLC and the ester fraction eluted between 55 to 75 ml. The TLC profiles of all four species were similar and showed Ehmanns-reactive spots at R values relative to the B-1 spot of IAInos ($_{IAInos}$) as shown in Table 1.

Isolation and Partial Characterization of a Putative IAA-Aminodesoxy Hexose. An IAA ester was isolated from *Aesculus parviflora* which had characteristics suggestive of a desoxyaminohexose ester of IAA. The compound was present in only trace amounts and a complete characterization has not been possible. The preparation was as follows: 50 ml of endosperm fluid from *A. parviflora* was mixed with acetone and treated as described in "Materials and Methods." Esters eluted in the 18 to 36 ml fraction, but an Ehmann-reactive compound was observed in the 38 to 80 ml fraction. This fraction is approximately where tryptophan would elute, since an amino compound would be zwitterionic and would be slightly retained by a sulfonic acid resin. The 38 to 80 ml fractions were pooled and concentrated, and, owing to the minute amount of material available, the LH-20 column chromatography was omitted. The fraction was applied to a straight phase Partasil 10 column and eluted with ethyl acetate:acetonitrile:ethanol:water (50:20:15:15). With this solvent, the Ehmann-reactive compound eluted as a single peak at 9.1 min. The peak was concentrated, applied to a C_{18} column, and developed with 5% ethanol:95% water. The compound eluted at 9.5 min and the apparently homogeneous substance, was analyzed by FAB using the VG-ZAB instrument. The mass spectral fragmentation pattern is shown in Figure 1. The molecular ion is at 609 and this corresponds to a 4 TMS-IAA-desoxy-aminohexose. Glycerol matrix ions are identified as G + H. M^+ minus TMS and 2 H is at 517, M minus 2 TMS and 4 H is at 425 and major sugar ions are at 349 and 334. The TMS quinolinium ion, derived by cyclization and enlargement of the IAA (27) is at 202 and the quinolinium ion is shown at 131.

Isolation and Characterization of Esters of IAA and *myo*-Inositol. This compound was initially isolated from kernels of *Zea mays* (19) and in that tissue is the most abundant IAA ester. Endosperm fluid from *A. parviflora*, 38 ml was mixed with an equal volume of acetone, filtered, concentrated, and applied to a Dow 50 column. The eluate was monitored by TLC. Tubes 12 to 31 showed distinct Ehmann-reactive material at the proper position for the IAA inositols and were pooled and concentrated to an aqueous phase and placed on an LH-20 column and eluted with 50% ethanol. Substances with a migration corresponding to that of the two spots of IAInos on TLC emerged at 26 and 27 ml and were pooled and concentrated to dryness *in vacuo*. This material was dissolved in 50% ethanol and chromatographed on a C_{18} column using 5% ethanol-95% water as solvent and showed 280 nm absorbency at 4.8, 8.5, 10.0, and 13.7 min. Authentic IAInos gave an identical elution pattern. The peaks from the plant sample were pooled and chromatographed a second time as above. The same peaks were collected. pooled, dried, and

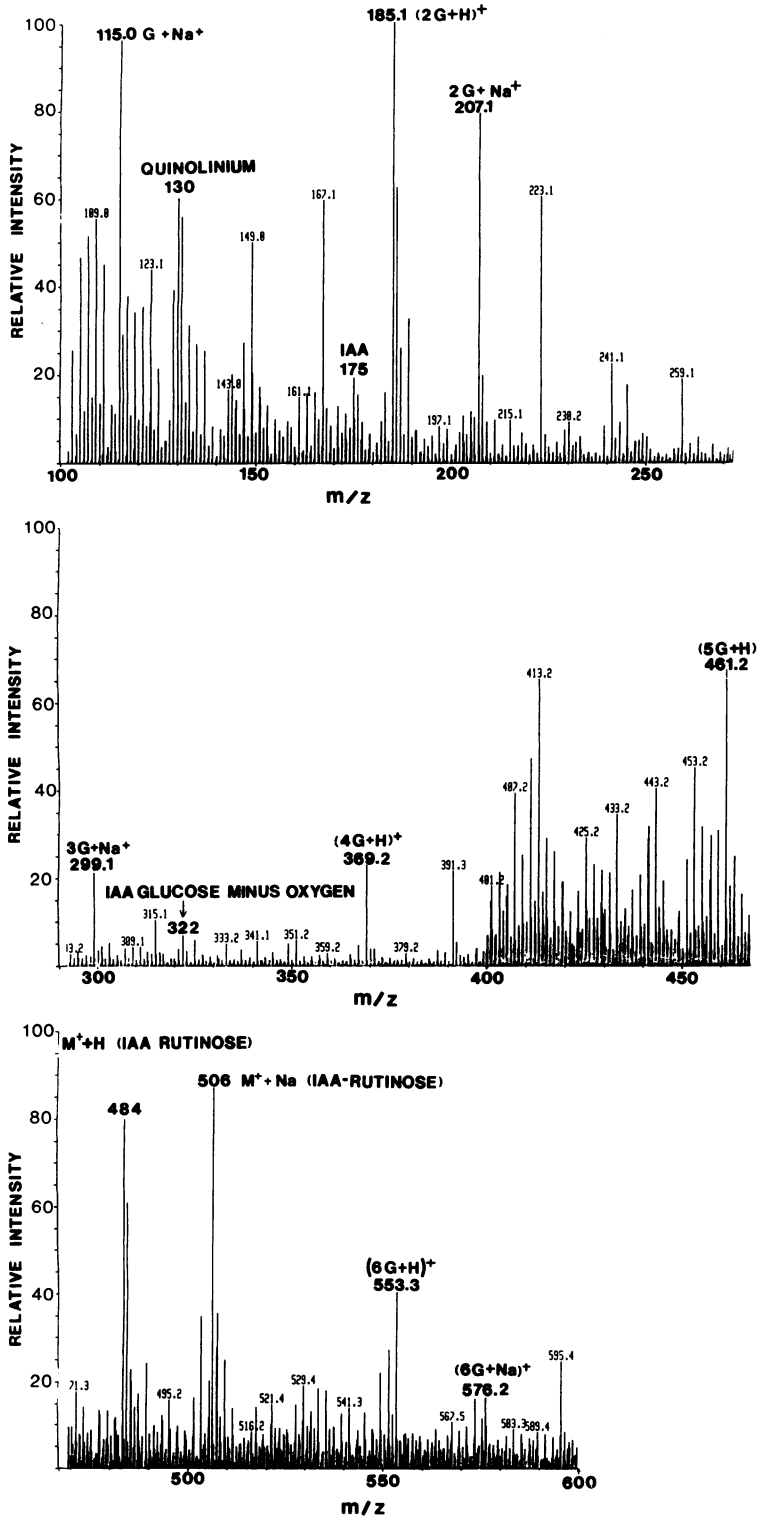

FIG. 2. FAB spectrum of the putative IAA-rutinose isolated from *A. parviflora*. The molecular ion plus a proton is at 484, and the molecular ion plus sodium is at 506. IAA-glucose minus oxygen is at 322. IAA is at 175 and the quinolinium ion of IAA is at 130.

acetylated using 50 μl of pyridine, containing 0.5% of *N*-dimethylaminopyridine (13). To this was added 50 μl of reagent grade acetic anhydride. Acetylation was for 10 min at 25°C, although the sample may be left at room temperature for several days. The sample was then dried in a stream of N_2 gas and dissolved in 25μl of ethylacetate for GC-MS. Mass spectra were obtained at 70 eV using a Hewlett-Packard 5985 quadrapole GC-MS with a 60 cm × 3 mm 3% OV-17 column. The initial column temperature was 200°C, held for 2 min and then programmed to 300°C at 20°C/min. An authentic IAInos sample yielded the characteristic four peaks of isomers with retention times of 8.1, 8.7, 9.5, and 10.2 min (11). The plant sample had not isomerized completely and yielded peaks at 8.1, 9.6, and 10.2 min. The 8.1 and 8.6 peaks are pentaacetyl IAInos and the 9.6 peak is a hexaacetyl IAInos having an acetyl group on the imino nitrogen. A comparison of the mass spectral fragmentation pattern of the putative IAInos from the horse chestnut with that of an authentic compound isolated from *Zea mays* is shown in Table II.

Ammonlysis of the putative IAA-*myo*-inositols yielded IAA and IAA amide as determined by TLC. Gas chromatographic

Table III. *A Comparison of 70 eV Mass Spectral Fragmentation Pattern of Heptaacetyl Rutinose Derived by Hydrolysis of Putative IAA-rutinose from A. baumanni and A. parviflora with Authentic Sample of Heptaacetyl Rutinose*

m/z	Putative Heptaacetyl-Rutinose		Standard
	A. baumanni	*A. parviflora*	
	abundance		
533	4	3	6
532	5	5	13
531	11	10	17
375	8	6	11
317	73	67	65
273	84	62	69
228	22	24	27
197	44	43	54
184	93	93	87
169	40	38	37
157	100	100	100
155	96	98	92
127	40	43	44
109	73	76	73

analysis on an OV-1, 2%, 180 cm × 0.3 mm column showed an IAA peak at 9.5 min and an IAA amide peak at 12 min and a *myo*-inositol peak at 6.4 min. Measurement of the GC peak area of a sample which had been ammonolyzed with NH_4OH showed a stoichiometry of inositol to (IAA plus IAA amide) of 1.01 to 1.00.

Isolation and Characterization of IAA-Rutinose. Endosperm liquid from *A. parviflora*, 23 ml, was deprototeinized with 23 ml of acetone, filtered, concentrated, and chromatographed on a Dowex-50 column. Tubes 16 to 35 ml contained Ehmann's-reactive material and were pooled, concentrated, and chromatographed on an LH-20 column. Tubes 13 to 21 contained Ehmann's-reactive material and were pooled and concentrated. TLC disclosed 12 Ehmann-reactive spots as shown by the data of Table I. The sample was concentrated and streaked out for preparative TLC. Compounds migrating to an R_{IAInos} of 0.90 and 0.98 were pooled and eluted from the Silica Gel with 50% ethanol. This material was concentrated and chromatographed on a C_{18} column using 5.5% ethanol, 0.05% acetic acid, and 94.45% water. Two major peaks were obtained at retention times of 21.6 and 26.4 min. TLC disclosed an unknown indolylic compound at R_{IAInos} of 1.13, the putative IAA-rutinose at 1.07, and a mixture of IAInos and IAA-rutinose at 0.98. The 26.4 peak was also an IAA-rutinose. The 21.6 min peak was concentrated and chromatographed on a Partasil-10 column using ethyl acetate:acetonitrile:ethanol:water (65:21:7:7) and yielded peaks at 3.6, 5.3, and 7.2 min. The peak at 5.3 min was concentrated, dried, dissolved in 100 μl of 50% ethanol, and used for further characterization. FAB-MS disclosed a molecular ion at 483 which is detected as M^+ plus a proton as is shown in Figure 2. This corresponds to an IAA ester of glucosyl-rhamnose (IAA-rutinose). The exact mass, plus 1 proton, was measured as 484.1830 which agrees well with a calculated mass of 484.1819, for an empirical formula of $C_{22}H_{30}O_{11}N_1$. This is correct for IAA-glucosylrhamnose.

A portion of the 5.3 peak was ammonolyzed using 5 μl of sample, 5 μl of water, and 10 μl of concentrated ammonium hydroxide. Two Ehmann-reactive spots were obtained which corresponded in TLC migration to IAA and indole-3-acetamide. Hydrolysis under these mild alkaline conditions indicates that the compound is an ester, and formation of the amide indicates the involvement of the IAA carboxyl in the ester linkage.

A further 20 μl fraction was ammonolyzed, dried, and acetylated and chromatographed isothermally at 280°C on a 6 foot 3% OV-17 column. A peak emerged with a retention time of 3.9 min which corresponded exactly with that of authentic hepta acetyl rutinose. This same fraction, but prepared from *A. baumanni*, was examined by GC-MS and yielded a molecular ion at 533 and a fragmentation profile which corresponded exactly with that of an authentic sample of heptaacetyl rutinose as shown by the data of Table III.

The EI spectrum of the TMS derivative of the putative IAA-rutinose is shown in Figure 3. The spectrum corresponds with IAA-1-O-glucosyl-rhamnose. After the loss of rhamnose fragment the ions at 653 and 450 correspond with those previously observed for 1-O-IAA-glucose (8). Further evidence for the location of the IAA on the 1 position of glucose is provided by the failure of the intact IAA-rutinose to make the methoxime derivative (8, 20), and by the high ratio of amide to free IAA formed by ammonlysis. The sugars would appear to be in the pyranose configuration as judged by the large 204 relative to the 217 ion (6, 8, 17, 20 30). The ions 117, 129, 147, 204, 247, 291, 305, and 319 are TMS ethers of carbohydrates (7, 17, 30).

DISCUSSION

The present work extends the known distribution of IAA-*myo*-inositol since this compound had previously been described only from monocotyledonous graminae such as *Zea mays* and *Oryza sativa* (14, 19). IAInos is known to occur throughout the *Zea* tribe since Ehmann found it in sweet corn, field corn, popcorn, *Tesosinte*, and *Trypsicum* (6). It also occurs in vegetative tissues of maize (4). An insufficient number of plant species have been studied with respect to their IAA conjugates to understand distribution patterns and thus possibly to understand what factors determine the kind of conjugates synthesized and possibly to better understand their function.

The following conjugates have been found by methods involving extensive purification and mass spectral methods to establish the structure of the intact compound. We specifically mention mass spectral methods since these methods are capable of studying the intact molecule. This is important since other methods might result in a specious characterization of a mixture containing A-B and C-D, and erroneously concluding that a molecule composed of A-C or B-D occurred.

The following IAA conjugates have been found to be naturally occurring by the above criteria: indole-3-acetyl-*myo*-inositol (four isomeric forms) (6, 11, 19, 27); indole-3-acetyl-*myo*-inositol-arabinose (three isomeric forms) (6, 19, 27, 28); indole-3-acetyl-*myo*-inositol-galactose (three isomeric forms) (6, 27, 28); indole-3-acetyl-glucose (2, 4, and 6-0) (8, 22); [indole-3-acetyl]$_{2 \text{ and } 3}$]-*myo*-inositol (11); *N*-acetyl-indole-3-acetyl-lysine (16); indole-3-acetyl-aspartate (1, 5, 23); indole-3-acetyl-glutamate (2, 12, 23, 24); indole-3-acetyl-rutinose (21, 26, and this work). Conjugates formed following the exogenous application of IAA to the plant are not included in the above list. The 1-O-IAA glucose has been shown to be formed by an enzyme from *Z. mays* (22) and was among the first conjugates reported (29). A beta 1,4-IAA glucan is also known (25).

The compound to which IAA is linked in ester or amide bond is curious in that the plant does not use its most abundant constituent for conjugation. This indicates some specificity in the conjugating moiety. We have previously suggested that the conjugating moiety may act as a kind of zip code in determining transport or metabolism as has been found by Komoszynski and Bandurski (18). The observation that different IAA amino acids conjugates cause different tissue responses (15), may indicate that, at the cellular level, the conjugating moiety causes differences in the rate of hydrolysis of the conjugate or differences in its cellular location, and thus inducing a different response.

Although the characterization of the IAA-aminodesoxy hexose is tentative, several facts suggest this structure. There is first the

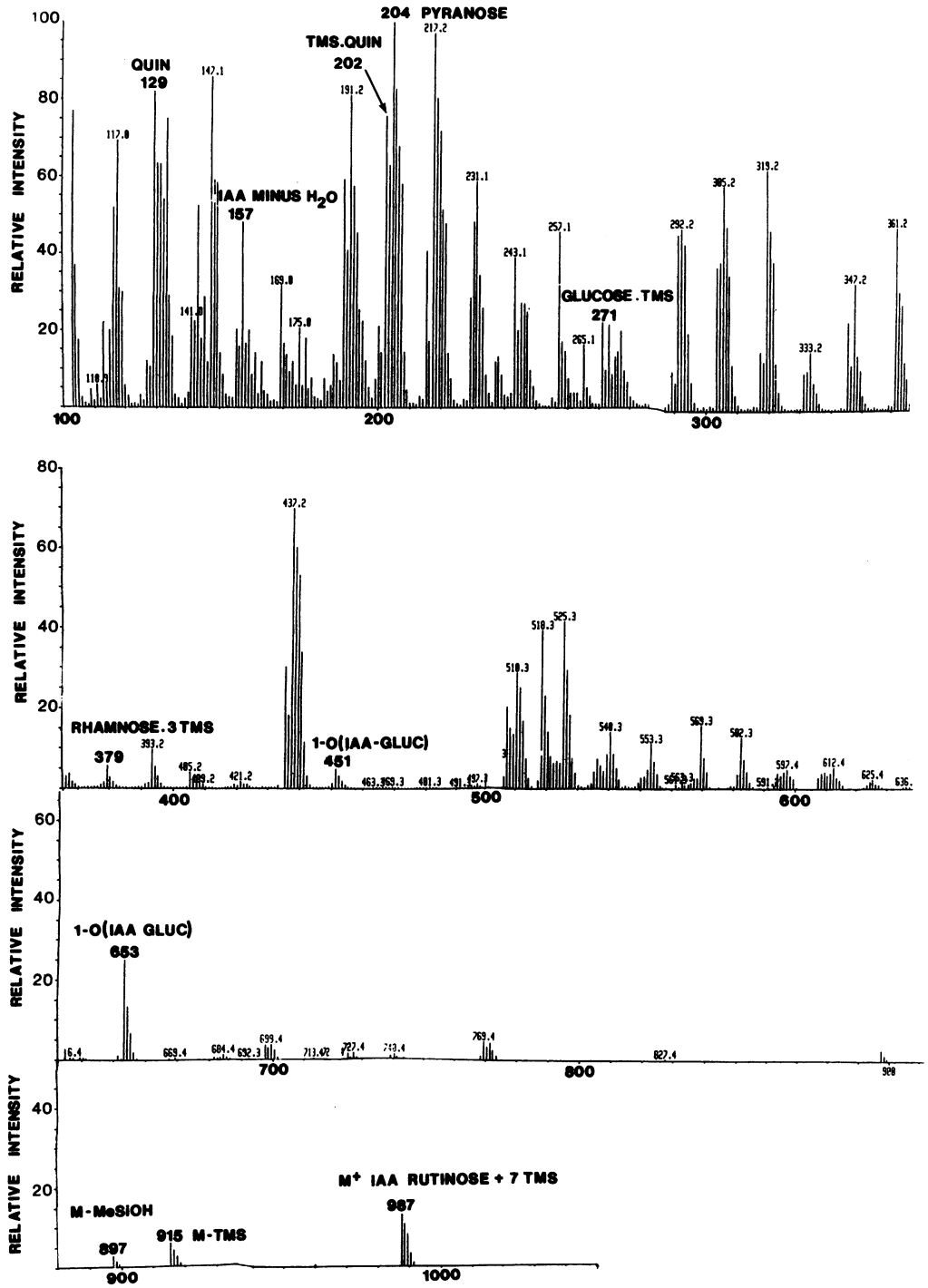

FIG. 3. 70 eV electron impact spectrum of the putative heptakis TMS IAA-rutinose isolated from *A. parviflora*. The molecular ion is at 987. The 4-TMS 1-O-IAA-glucose OH is at 451 and the 3 TMS rhamnose is at 379. IAA ions are at 202, 157, and 129. Other ions are as labeled in the figure.

long retention time on Dowex-50, longer than any of the esters, but rather coincident with tryptophan. Second, there is the low migration on TLC despite its being an IAA monosaccharide and, last, the characteristic mass spectral fragmentation pattern. If our assignment is correct, this would, to our knowledge, be the first reported occurrence of an aminodesoxoy sugar in plants.

Last, we wish to observe that greater knowledge of the kinds of IAA conjugates and their distribution in nature might provide insight into functions of the conjugates other than those previously described, such as, transport, storage, protective, and hormonal homeostatic roles (6).

Acknowledgments:—We are indebted to Dr. Axel Ehmann for the FAB and EI spectra of the IAA-rutinose and the FAB spectrum of the putative IAA-aminodesoxy-hexose and to Dr. Jerry Cohen for the EI spectrum of the heptaacetyl rutinose. The IAInos spectra were obtained at the MSU-DOE-NIH-mass spectral facility.

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