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Mass Spectroscopic Identification of Cytokinins

GLUCOSYL ZEATIN AND GLUCOSYL RIBOSYLZEATIN FROM *VINCA ROSEA* CROWN GALL[1]

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ROY O. MORRIS
Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

Mass spectrographic and chemical studies of the permethyl and trimethylsilyl ethers of two new cytokinins isolated from *Vinca rosea* crown gall callus cultures by Peterson and Miller (Plant Physiol 59: 1026-1028) indicate that they are 6-(4-0-β-D-glucopyranosyl-3-methyl-*trans*-but-2-enylamino) purine (glucosyl zeatin) and 9-β-D-ribofuranosyl-6(4-0-β-D-glucopyranosyl-3-methyl-*trans*-but-2-enylamino)purine (glucosyl ribosylzeatin). The nature of the mass spectra of the permethylated cytokinins suggests that these derivatives may have considerable utility in the detection of low levels of cytokinins in plant material.

In the preceding paper, Peterson and Miller (11) describe the isolation and identification of two new cytokinin-active compounds from *Vinca rosea* crown gall tissue grown in culture. On the basis of spectroscopic, chromatographic, and enzymic evidence, these were considered to be glucosides of *trans*-zeatin (factor II) and *trans*-ribosylzeatin (factor I), respectively. Evidence was also presented which strongly suggested that in both compounds the glucose moieties were attached to the isoprenoid side chain.

We present here chemical and mass spectroscopic evidence as to the identity of the two factors and we show that it is indeed the terminal hydroxyl group of the isoprenoid chain of zeatin or ribosylzeatin which bears the glucosyl substituent.

MATERIALS AND METHODS

Pure samples of factors I and II and the products resulting from each upon hydrolysis by β-glucosidase were utilized as received from Peterson and Miller (11). Factor I was subjected to further purification by high performance liquid chromatography (10) upon μBondapak/C_{18} (Waters Associates, Milford, Mass.) and found to contain only one major UV-absorbing species. Its purity was estimated to be greater than 95%.

Preparation of Derivatives. Preparation of the permethyl derivatives of factors I and II and of their hydrolysis products was accomplished by a modification of the dimethylsulfinyl carbanion-methyl iodide procedure of Hakomori (3). In a typical experiment, 10 μg of each cytokinin was dried in a stream of nitrogen at room temperature, dissolved in an appropriate amount of dry 1.5 M dimethylsulfinyl carbanion solution in dimethylsulfoxide (a 5-fold molar excess over the expected number of hydroxyl groups present) and, after 30 min at room temperature, an excess of purified methyl iodide (or, where appropriate, perdeuteromethyl iodide, CH_3I-d_3)[2] was added and the mixture was incubated at room temperature for 60 min. It was necessary to exclude traces of moisture from the reaction mixture up to this point, and all manipulations of reagents were done under nitrogen in V-vials (Kontes Glass Co., Vineland, N.J.) sealed with Teflon-backed silicone rubber septa (Analabs, New Haven, Conn.). Reactions were terminated by the addition of a 5-fold excess of water (v/v), and the permethyl derivatives were extracted into chloroform, washed with water to remove any excess of base and dimethylsulfoxide, and examined directly by combined GLC-MS. Trimethylsilyl derivatives were prepared by reaction with BSTFA +1% TMCS (Regisil, Regis Chem. Co., Morton Grove, Ill.) for 2 hr at 60 C as described earlier (1).

Mass Spectra. Mass spectra of the permethyl and trimethylsilyl derivatives were recorded at 70 ev on a Varian MAT CH-7 spectrometer equipped with a System 150 data system (System Industries, Sunnyvale, Calif.). Samples were admitted to the spectrometer source either by direct probe or via a GLC column and single stage glass jet separator. Column conditions were: glass column, 3% Dexsil 300 on Anachrom A, 28 cm × 0.18 cm i.d.; He carrier, 15 ml min^{-1}; 150 C isothermal for 3 min then 8 C min^{-1} to 350 C; Separator temperature 240 C.

Position of the Hexose Moiety. Permanganate oxidation of permethylated factor I to determine the position of the hexose fragment was carried out in aqueous acetone. Factor I (10 μg) was permethylated as described above, dissolved in 100 μl 80% aqueous acetone and mixed with 100 μl potassium permangate solution (0.1% in 50 mM K-phosphate buffer, pH 7.5). After 2 hr at room temperature, the oxidation products were extracted into chloroform, washed, evaporated under a stream of dry N_2, and re-permethylated. The second permethylation was done with CH_3I-d_3 specifically to label the N-6 position of the adenosine ring with a perdeuteromethyl group.

Hexose Identification. The method of Sweeley *et al.* (14) as modified by Clamp *et al.* (2) was used. The hexose and pentose moieties were split from factor I by methanolysis in 1 M methanolic HCl at 100 C for 3 hr and converted to their trimethylsilyl ethers by treatment with BSTFA. Identification was by means of GLC and comparison with retention times of known carbohydrate standards. Dexsil 300 was utilized as the stationary phase for GLC.

RESULTS AND DISCUSSION

Preliminary studies indicated that the mass spectrum of underivatized factor I had abundant ions at m/e 135, 136, 119, and 108 which characterized it as an adenine derivative and additional ions at m/e 202, 188, 160, and 148 which were almost

[1] Technical Paper No. 4388 from the Oregon State University Agricultural Experiment Station.

[2] Abbreviations: CH_3I-d_3, perdeuteromethyl iodide; BSTFA, bis(trimethylsilyl)trifluoracetamide; TMCS, trimethylchlorosilane; TMS, trimethylsilyl.

identical to those reported by Shannon and Letham (13) for zeatin. The spectrum did not contain a recognizable molecular ion either at m/e 219 (zeatin) or m/e 351 (ribosylzeatin) and for this reason, the spectra of the permethyl and trimethylsilyl derivatives of both factors I and II were examined.

Glucosyl Zeatin (Factor II). Spectrum of the Hydrolysis Product. The β-glucosidase hydrolysis product of factor II gave upon permethylation—a derivative, the mass spectrum of which is shown in Figure 1. The spectrum was characterized by an apparent molecular ion at m/e 261 and major fragment ions at 230, 216, 188, 162 to 164, and 133 to 135. It was identical to the spectrum obtained from the permethyl ether of zeatin. The molecule contained three methyl groups as indicated from the mass of its molecular ion and by the increase of the mass of the molecular ion to m/e 270 upon perdeuteromethylation. Since adenosine derivatives do not, in general, form the imino methyl derivatives found upon methylation of guanine (15), the structure of methylated zeatin is as shown in Figure 1, with attachment of methyl groups to the side chain hydroxyl group, the exocyclic nitrogen and position 9 of the ring.

The fragmentation pathway is consistent with the above structure and is strictly analogous to that proposed for free zeatin (13). The base peak at m/e 230 originates by favorable loss of $OCH_3^{\cdot}$ from the side chain of the molecular ion—a process analogous to loss of $OH^{\cdot}$ in free zeatin. Peaks at m/e 216 (loss of $CH_2OCH_3^{\cdot}$), m/e 162 (loss of the side chain), and m/e 133 (loss of the side chain and N-6 nitrogen atom giving the 9-methylpurinyl cation) arise by processes analogous to those in free zeatin. Partial side chain loss followed by ring closure to give the tricyclic ion, m/e 188, analogous to that described by Shannon and Letham (13) is also a facile pathway. Assignments were confirmed by perdeuteromethyl labeling, and we conclude that zeatin is a hydrolysis product of factor II.

Mass Spectra of Derivatives of Factor II. Spectra of the permethyl and trimethylsilyl derivatives of factor II itself are shown in Figure 2. The spectrum of the permethyl derivative (Fig. 2A) was very similar to that of permethyl zeatin with prominent fragment ions at m/e 230, 188, 162, 164, 133, and 134. It had, in addition, peaks at m/e 45, 88, and 101 characteristic of methylated saccharides (8) plus a weak but identifiable molecular ion at m/e 465. Perdeuteromethylation increased the mass of the molecular ion to m/e 483 indicating the presence of six methyl groups of which two at least must be on the zeatin moiety. Factor II itself thus has a mol wt of 381. This and the presence of four methyl groups in the molecule other than those associated with the zeatin fragment are consistent with the presence of a hexose substituent (Fig. 2).

Confirmation of the presence of a hexose fragment was found in the spectrum of the TMS derivative. A series of weak fragment ions at 361, 331 to 332, 319, 305, 291, 217, 204, 191, and 103 were all characteristic (6) of a trimethylsilylated hexose. In addition, the ratio of the fragment ions 204:217 was high, indicating the presence of a hexopyranose ring (12). Further, the TMS derivative had a molecular ion at m/e 741 indicating the presence of five trimethylsilyl substituents as opposed to six methyl groups in the permethylated derivative. The difference has been noted before (7, 15) for those purines which contain an exocyclic nitrogen.

Unfortunately, apart from minor variations in the relative intensity of the $TMSOTMS^+$ peak at m/e 147, all TMS-hexopyranose isomers give identical spectra (5), and it is not possible to conclude from the spectra the identity of the hexose. The enzymic analyses of Peterson and Miller (11) show that in this case, it is glucose.

Two pieces of evidence allow the conclusion that the glucose is attached to the terminal hydroxyl of the side chain. First, the zeatin fragment ion m/e 230 contains two methyl groups (Fig. 2) which the fragmentation patterns show to be located on the N-6 and the 9 position of the ring. These two positions are thus eliminated as hexose attachment points leaving the side chain hydroxyl group as the most obvious point of attachment. Further, no major fragment ions occur in the spectrum of the permethylated derivative at m/e 231 or 232. The presence of such ions, derived by H transfer to the base, is characteristic of ring-attached hexose or pentose derivatives such as 9-ribosylzeatin (15; and see the spectrum of ribosyl zeatin below). We conclude therefore that such structures are absent from factor II and that it is 6-(4-0-β-D-glucosyl-3-methyl-but-2-enylamino) purine. Facile elimination of the permethylglucosyl fragment causing the base peak to be at m/e 230 supports this conclusion.

Glucosyl Ribosylzeatin (Factor I). The mass spectra of the permethyl derivatives of the hydrolysis product of factor I and of factor I itself are shown in Figure 3, A and B. The spectrum of the permethylated β-glucosidase hydrolysis product was identical to that of authentic permethylated ribosylzeatin and this, when considered with the chromatographic and UV spectral data of Peterson and Miller (11), establishes its identity as *trans*-ribosylzeatin. The spectrum was characterized by a moderately intense molecular ion at m/e 421 and prominent fragment ions at 390, 348, 246, 216 (base peak), 202, 174, 101, and 45. Perdeuteromethylation showed that there were five methyl groups in the molecule.

The fragmentation pattern paralleled that of free zeatin, after appropriate allowance was made for the presence of the ribose

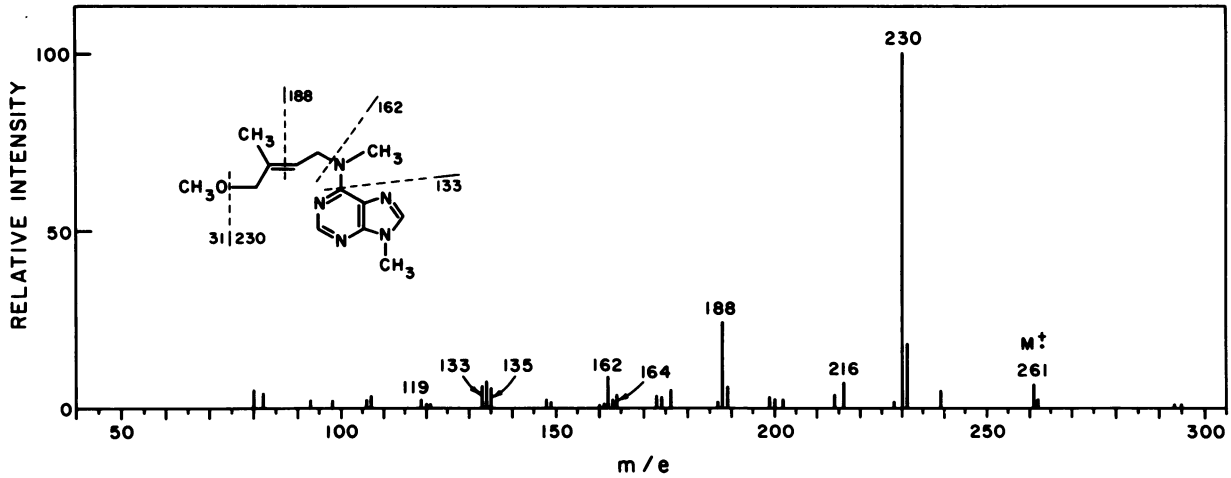

FIG. 1. Mass spectrum and structure of trimethyl zeatin derived from the β-glucosidase hydrolysis product of factor II. Major fragment ions arise as indicated by the broken lines.

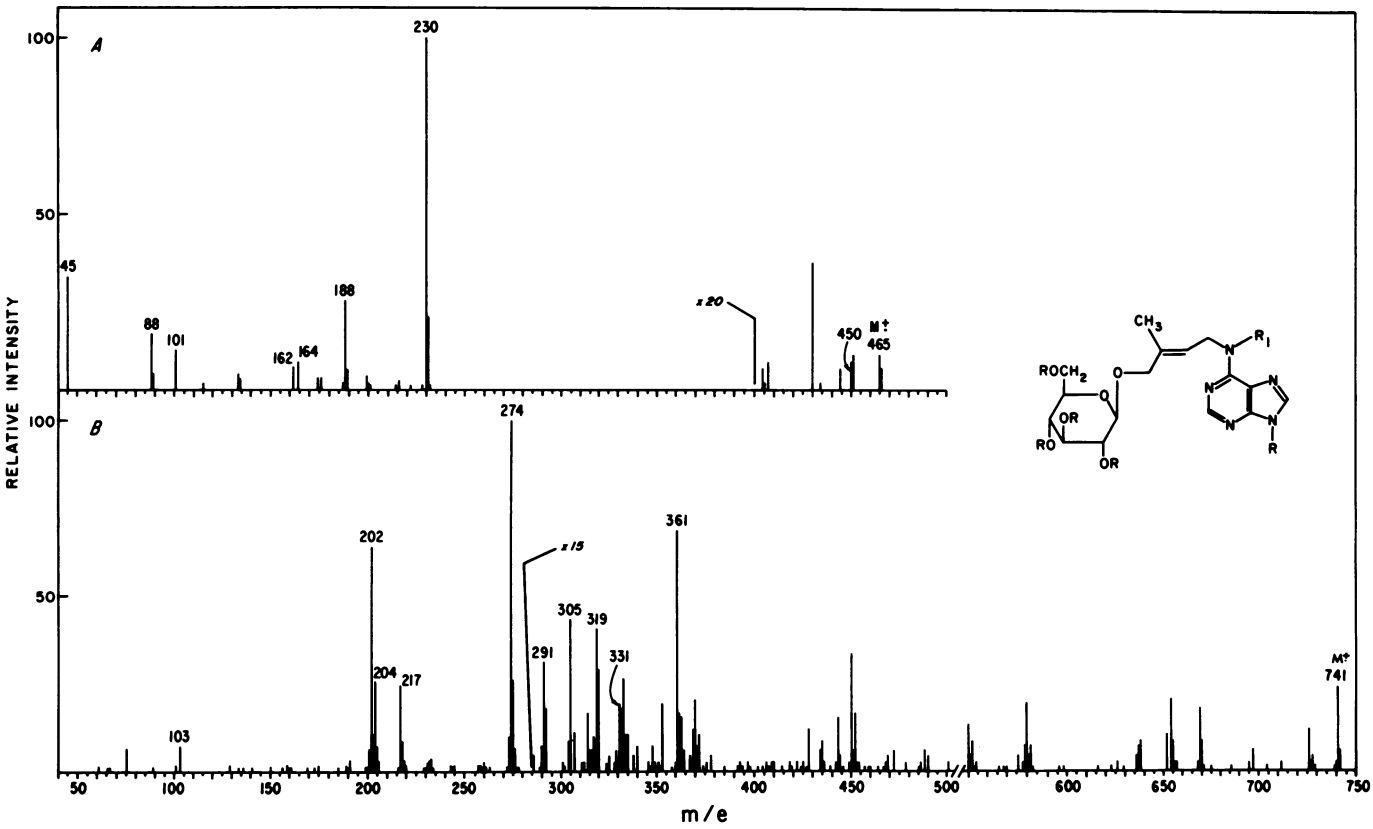

FIG. 2. Mass spectra of A: permethylated glucosyl zeatin (factor II) R,R_1 = CH_3; B: trimethylsilyl glucosyl zeatin R = $(CH_3)_3Si$, R_1 = H. Magnification of × 20 at m/e ≥ 400 (A) and × 15 at m/e ≥ 280 (B).

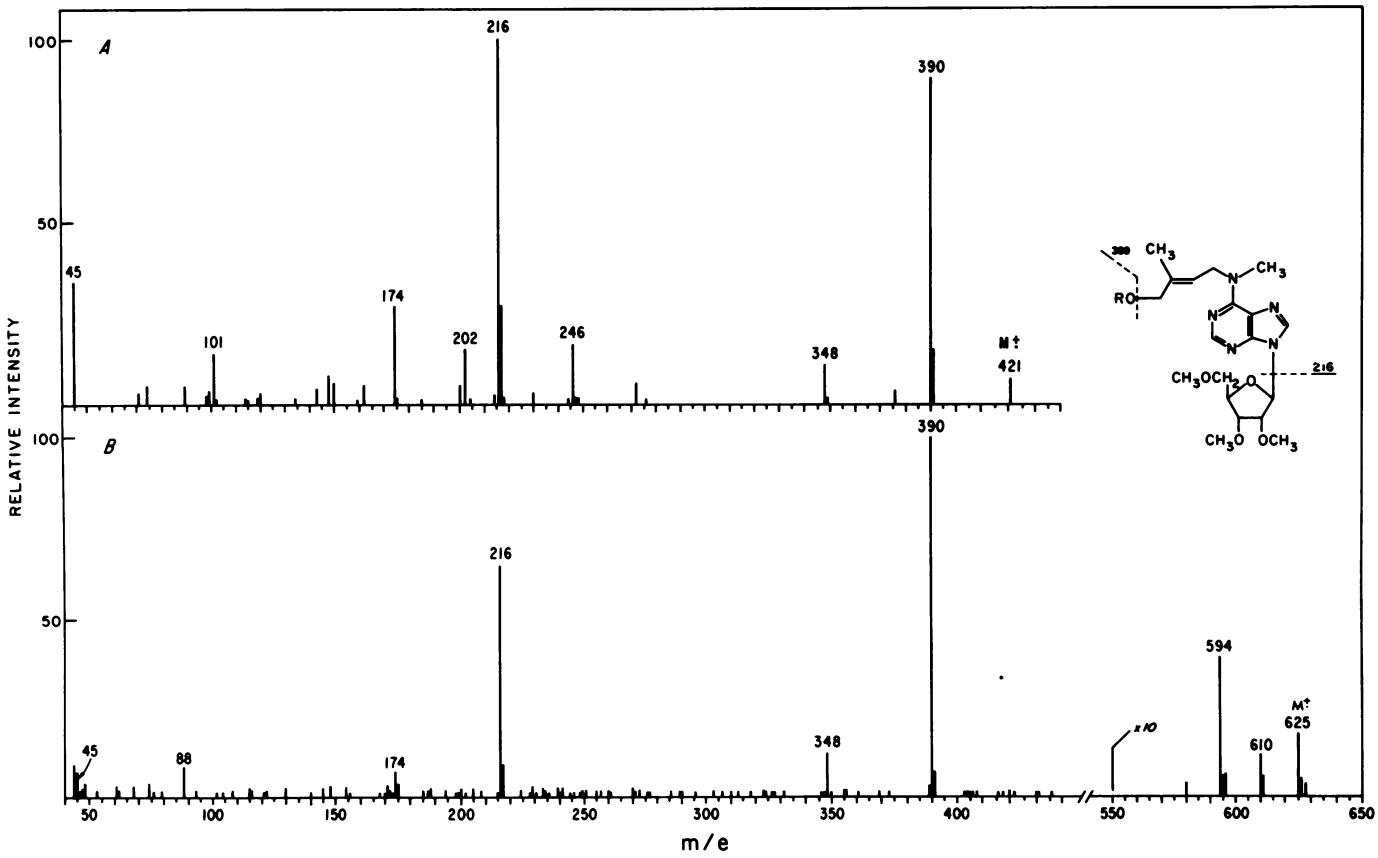

FIG. 3. Mass spectra of A: permethylated ribosylzeatin originating from factor I by β-glucosidase hydrolysis, R = CH_3; and B: permethylated glucosyl ribosylzeatin (factor I) R = 2',3',4',6'-tetramethyl glucosyl. Magnification × 10 at m/e ≥ 550.

moiety. Loss of $OCH^{\bullet}_3$ was favored and gave rise to m/e 390; partial side chain loss gave the riboside of the tricyclic structure m/e 348 analogous to that noted for free zeatin riboside (4). A common rearrangement in nucleosides mentioned earlier is fission of the base-ribose bond and transfer of one or more ribose protons to the base giving BH^+ or BH_2^+ (15). These fragments were present but not very intense (m/e 246–248). The rearranged fragment was represented by m/e 216 corresponding to loss of $OCH^{\bullet}_3$ from BH^+. Fragments at m/e 45, 71, and 101 are typical of permethylated ribose (15).

The spectrum of the permethylated parent compound, factor I (Fig. 3B) reflected the presence of the ribosylzeatin fragment with ions at m/e 390, 348, 216, 174, 101, and 45. It had a very weak molecular ion at m/e 625 (identified by loss of $CH^{\bullet}_3$ and $OCH^{\bullet}_3$ to give fragment ions at m/e 610 and 594). Perdeuteromethylation raised the mass of the molecular ion to m/e 649, indicating that the parent substance had a mol wt of 513 and that eight methyl groups were present. The masses of the prominent fragment ions at m/e 390 and 216 were increased to 402 and 219 by deuteromethylation indicating that five of the methyl groups were not attached to the ribosylzeatin fragment. Such information is again consistent with the presence of a hexose fragment in the molecule.

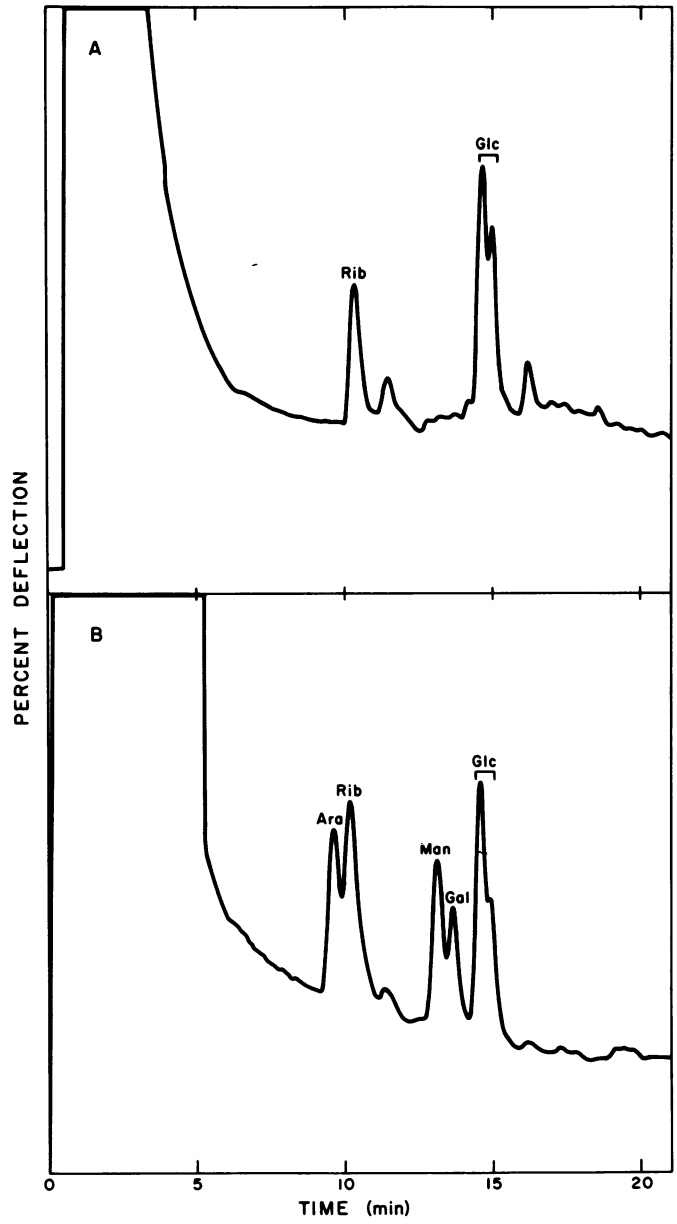

FIG. 4. Gas-liquid chromatograph of A: 2,3,5-tris(trimethylsilyl)-1-methyl ribose (Rib) and 2,3,4,6-tetrakis-0-(trimethylsilyl)-1-methyl glucose (Glc) derived by methanolysis and trimethylsilylation from factor I (5 μg); B: standard mixture of 0.5 μg each of the trimethylsilyl-1-methyl ethers of ribose (Rib), arabinose (Ara), mannose (Man), galactose (Gal), and glucose (Glc). All carbohydrates give multiple peaks by this procedure due to the formation of the α and β anomers or the presence of pyranose or furanose ring structures. This is particularly evident in the case of glucose. Column conditions: glass, Dexsil 300, 3% on Anakrom A; He carrier, 15 ml min^{-1}, program, 110 C for 6 min then linear increase at 8 C min^{-1}.

In order to confirm the enzymic identification of this hexose as glucose (11), factor I was subjected to methanolysis and the liberated sugars were identified by gas chromatography of their trimethylsilyl ethers. Figure 4 illustrates the GLC trace obtained. Clearly, the parent molecule contains both glucose and ribose.

Because of the presence of ribose on the 9 position of the purine ring, it was not possible to determine the position of attachment of the glucose fragment by inspection of the mass spectrum. This point was therefore established by first permethylating factor I and then subjecting it to mild permanganate oxidation to cleave the side chain (9). The resulting product was then permethylated again with CH_3I-d_3. If the glucose is attached to the side chain hydroxyl group as shown in Figure 3, this procedure should give rise to N-6-methyl-N-6-methyl-d_3-2′,3′,5′-trimethyl adenosine (Fig. 5). An authentic sample of this substance was prepared. It had a prominent molecular ion at m/e 340 and fragment ions at m/e 325 and 309. When the procedure was repeated with factor I, these fragment ions were observed. We therefore conclude on the basis of the mass spectroscopic evidence together with the chromatographic data presented earlier (11) that factor I is 9-β-D-ribofuranosyl-6(4-0-β-D-glucopyranosyl-3-methyl-*trans*-but-2-enylamino-purine – the side chain glucoside of *trans*-ribosylzeatin.

The studies presented here illustrate the utility of permethylation in identifying small quantities of cytokinin-active materials. The derivatives are readily resolved upon GLC and give spectra with well defined fragment ions. Most spectra recorded here were obtained on less than 2 μg of material. The derivatives have an added advantage over the TMS ethers in that they have lower mol wt and are stable in the presence of atmospheric moisture. Further, the nature of the spectra, having one or two prominent fragment ions, may make these derivatives useful for the quantitation of very low cytokinin levels in relatively crude extracts by mass spectroscopic multiple ion detection.

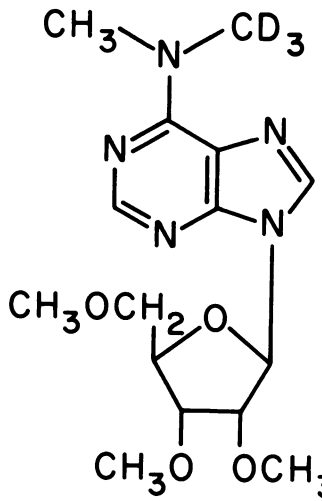

FIG. 5. N-6-Methyl-N-6-methyl-d_3-2′,3′,5′-trimethyl adenosine.

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