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ARGENTATION HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF GIBBERELLIN ESTERS

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SUMMARY

Pairs of gibberellins, differing from each other only by the presence or absence of a double bond, were separated in the form of their *p*-nitrobenzyl esters on a silver nitrate-impregnated silica column by high-pressure liquid chromatography and identified by mass spectrometry.

INTRODUCTION

High-pressure liquid chromatography (HPLC) seems ideally suited to the analysis of individual radioactive gibberellins, but so far only one group of investigators has applied this technique[1,2]. One of the difficulties encountered is the lack of separation of pairs of gibberellins differing from each other only by the presence or absence of a double bond. Argentation chromatography has solved this problem in the lipid field[3], but gibberellins are too strongly adsorbed on silver nitrate-impregnated silica. Adsorption was therefore decreased by converting them to the *p*-nitrobenzyl esters. This derivatization has the added advantages that it facilitates the detection by enhanced ultraviolet absorption and the identification by characteristic mass spectra.

The structures of the gibberellins used in this work are shown in Fig. 1. Each compound in the bottom row differs from the one above it by containing an additional double bond. GA_4 and GA_7 are isomers of GA_{20} and GA_5, respectively, having a hydroxyl group at C-13 instead of C-3. GA_1 and GA_3 have hydroxyl groups at both C-3 and C-13.

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components.

* Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product named to the exclusion of others which may also be suitable.

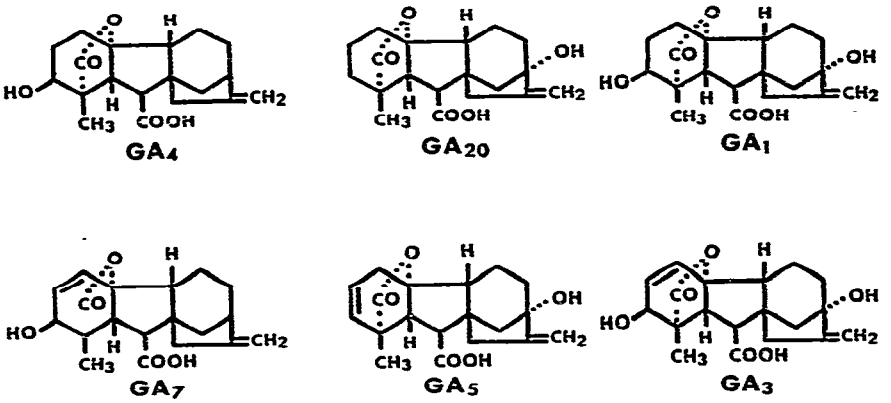

Fig. 1. Gibberellin structures.

The pump was of the dual-piston reciprocating type, constaMetric II (Laboratory Data Control, Riviera Beach, Fla., U.S.A.), and the detector was a Hitachi variable-wavelength spectrometer, Altex (Berkeley, Calif., U.S.A.) Model 155-30, equipped with a flow-cell having a 10-mm pathlength and a 20-μl volume. A single-channel recorder, Model 355 (Linear Instr., Irvine, Calif., U.S.A.), was attached to the output of the detector. The sample injector was a Model 706 sample valve (Disc Instr., Costa Mesa, Calif., U.S.A.) with sample loops of various sizes.

The chromatographic column was assembled from 2-ft. sections of 7 mm I.D. stainless-steel tubes, capped with 10-μm end fittings (Waters Assoc., Milford, Mass., U.S.A.). Each section was packed with Porasil A, 37–75 μm, by pouring small portions into the tube and tapping its end against the table top. Eight sections were connected by 0.009 in. I.D. stainless-steel tubes with Swagelok nuts and ferrules (Oakland Valve & Fitting Co., Pleasant Hill, Calif., U.S.A.) making the total column length 16 ft. A forecolumn, 6 cm in length, was first washed by passing 250 ml of methanol through it at the rate of 2 ml/min. The silica was then impregnated with silver nitrate by passing 250 ml of a 0.5% (w/w) solution of $AgNO_3$ in methanol through and then recycling 500 ml more of this solution overnight at the same rate. The column was finally rinsed by the passage of 200 ml of methanol.

All solvents used in our experiments were spectroquality (Burdick & Jackson, Muskegon, Mich., U.S.A.). The column effluent was collected with a Radirac Model 3400B (LKB Produkter, Bromma, Sweden) fraction collector, operated with a 15-ml syphon.

The identity of the eluted gibberellin esters was established without further purification of HPLC fractions by low-resolution electron-impact mass spectrometry (70 eV, direct probe sample introduction), using a double-focussing magnetic sector mass spectrometer, Model MM-70/70F (V. G. Micromass, Altrincham, Great Britain). Exact masses of molecular ions were measured on a CEC 21-110A mass spectrometer (DuPont, Wilmington, Del., U.S.A.) by peak matching. The ion source temperature was 195° $\pm$ 5° for all samples.

The preparation of the *p*-nitrobenzyl esters was carried out in a Reacti-Vial system (Pierce, Rockford, Ill., U.S.A.), consisting of an electrically heated metal

block (Reacti-Therm heating module) and 1-ml vials with conical well (Reacti-Vials), which were sealed with PTFE-lined screw caps. A 0.1 *M* solution of O-*p*-nitrobenzyl-N,N'-diisopropylisourea in dichloromethane (*p*-Nitrobenzyl8; Pierce) was added to the dry gibberellin (2 μl of reagent per μg) in the vial, which was tightly capped and heated at 80° for 2 h (ref. 4). The reaction is shown in Fig. 2.

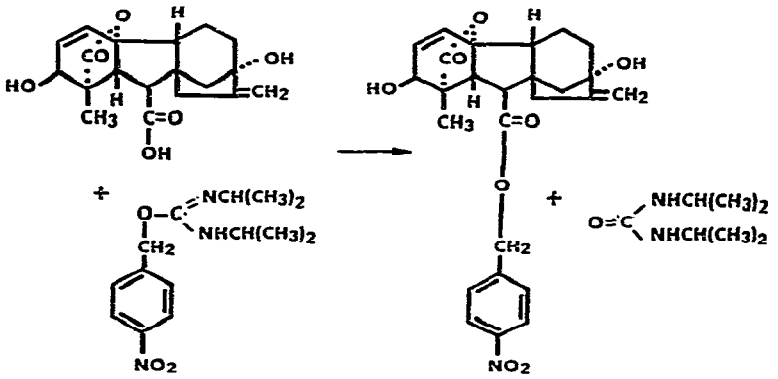

Fig. 2. Preparation of the GA_3 *p*-nitrobenzyl ester.

RESULTS AND DISCUSSION

The *p*-nitrobenzyl esters of gibberellins, purified by HPLC, gave an absorption maximum at 265 nm. Consequently, the detector was set at that wavelength. When the detector range was set at 0.01 and the time constant at 1.0 and the recorder was at 10 mV, the limit of detection for the gibberellin esters with a signal-to-noise ratio of less than 2:1 was 100 ng.

Various solvent mixtures and elution gradients were tried. Because the difference in polarity between GA_1 and GA_3 on the one hand and GA_4, GA_7, GA_{20} and GA_5 on the other hand demands a long waiting period between the elution of the two groups of gibberellins, it was decided to use two different solvent systems for the two groups. However, as in our previous work[5], we deliberately sacrificed speed for resolution. The chromatogram shown in Fig. 3 took 14 h. Complete separation of GA_4 from GA_7 and of GA_{20} from GA_5 was obtained with a mixture of *n*-hexane–methanol–dichloromethane (91:8:1). The gibberellins with a 3-hydroxyl group were eluted before the ones with a 13-hydroxyl group and, in every case, the more saturated analog was eluted before the less saturated one. For the separation of GA_1 from GA_3 (Fig. 4) we used *n*-hexane–methanol–isopropanol (85:13:2) and, again, the more saturated GA_1 was eluted before the less saturated GA_3.

The eluate was collected with a fraction collector and the fractions were evaporated and then subjected to mass spectral analysis without any further purification. The peaks preceeding the gibberellin peaks were due to contaminants in the samples and could not be readily identified. The low-resolution mass spectra confirmed the identity and purity of the *p*-nitrobenzyl derivatives of the isolated gibberellins.

The gibberellin esters were easily identified and distinguished by mass spectra

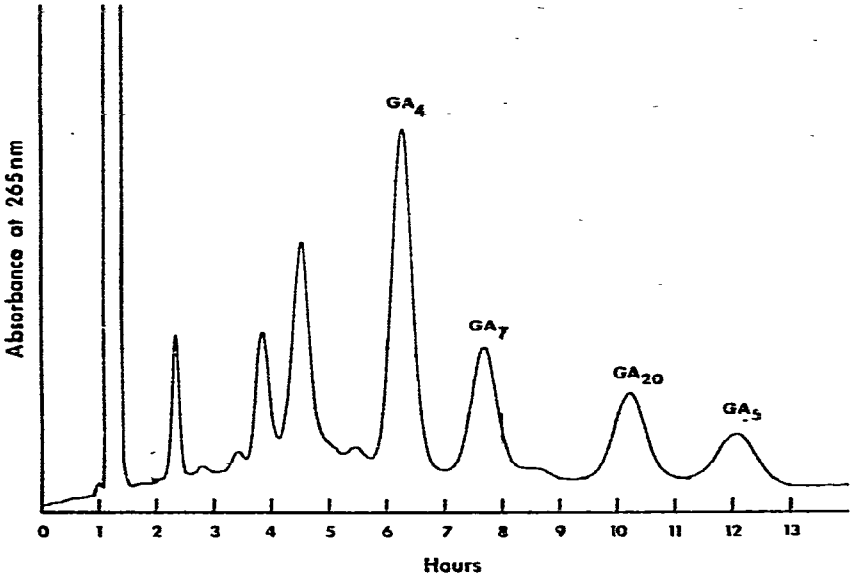

Fig. 3. Separation of 20 μg of each *p*-nitrobenzyl ester: GA_4, GA_7, GA_{20}, and GA_5. Column, 16 ft. × 7 mm I.D.; sorbent, Porasil A, 37–75 μm, impregnated with $AgNO_3$; eluent, *n*-hexane–methanol–dichloromethane (91:8:1); flow-rate, 2.5 ml/min at 500 p.s.i.; detector at 265 nm, range at 0.05, time constant at 1.0; recorder at 10 mV; chart speed, 2 cm/h.

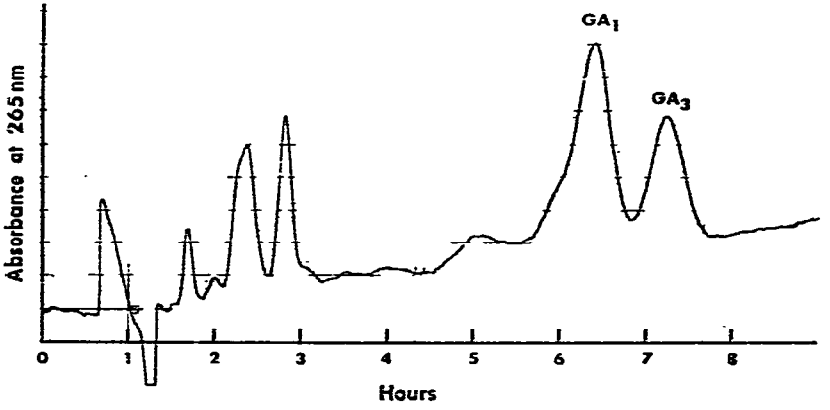

Fig. 4. Separation of 10 μg of each *p*-nitrobenzyl ester: GA_1 and GA_3. Column and sorbent, see Fig. 3; eluent, *n*-hexane–methanol–isopropanol (85:13:2); flow-rate, 3.0 ml/min at 800 p.s.i.; detector and recorder, see Fig. 3, except range which was at 0.02; chart speed, 3 cm/h.

obtained directly on HPLC fractions. Complete mass spectra for the GA_1 and GA_4 esters, shown in Figs. 5 and 6, are illustrative. Molecular ions were always observed, and their elemental compositions were confirmed by exact mass measurement (see Table I). The *p*-nitrobenzyl esters are less volatile than the corresponding methyl esters and require a direct probe temperature of 180° for volatilization. The reduced volatility of the *p*-nitrobenzyl esters in advantageous for detecting small amounts of

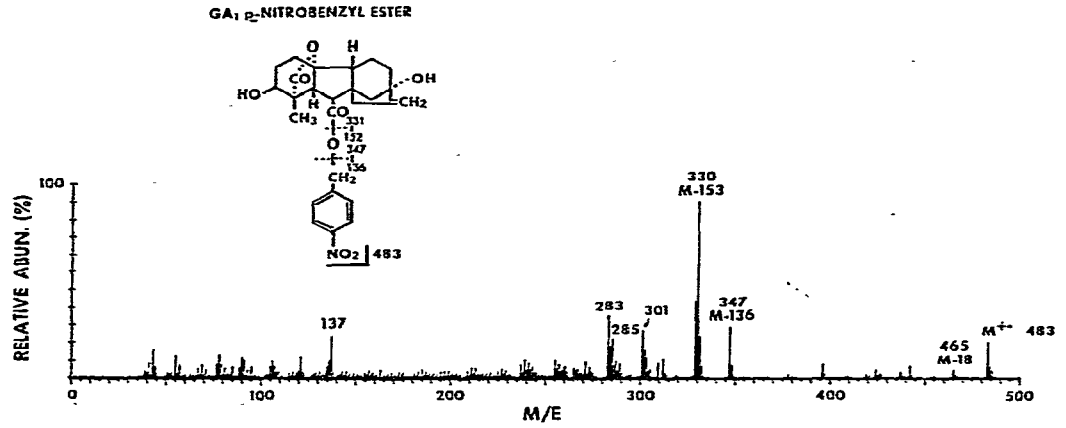

Fig. 5. Mass spectrum of GA_1 *p*-nitrobenzyl ester.

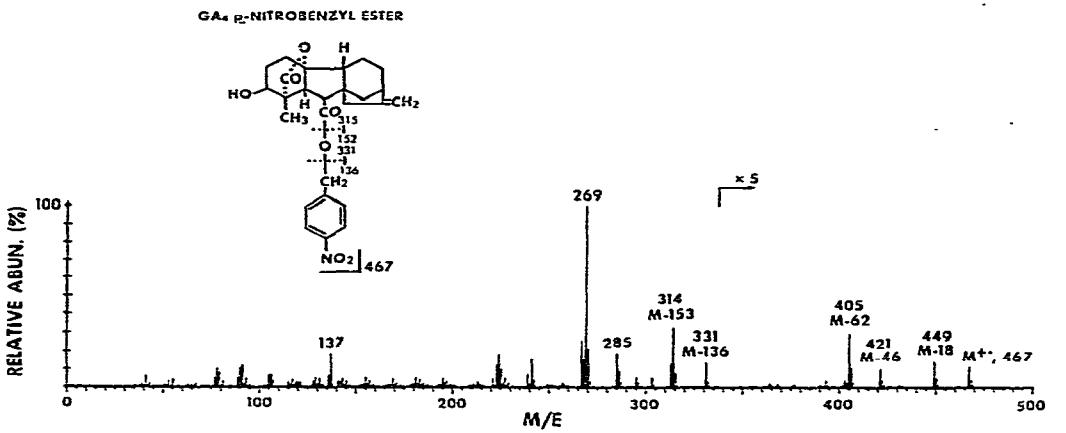

Fig. 6. Mass spectrum of GA_4 *p*-nitrobenzyl ester.

gibberellin esters directly in HPLC fractions, because impurities introduced with the HPLC solvents were more volatile, and could be distilled off the direct probe before the gibberellins began to volatize.

The mass spectra of gibberellin methyl esters have been reported in detail by MacMillan's group[6] and by Takahashi *et al.*[7], and a mass spectrum of the benzyl ester of GA_1 has recently been published without interpretation[8]. The spectra of the *p*-nitrobenzyl esters differ in several respects, and should complement the structural information obtainable from the methyl esters and trimethylsilyl methyl esters[6].

The reaction scheme shown in Fig. 7 accounts for the major ions present in the 70-eV spectra of the esters we have examined. For GA_1, GA_3, GA_4, GA_5, GA_7, and GA_{20}, the masses and abundances for all ions indicated in that scheme are given in Table I, along with the exact mass measurements of the molecular ions.

Loss of benzyl alcohol and benzyl radical from the molecular ions is characteristic in all of the compounds, and leads to prominent fragment ions at $(M-153)^+$.

TABLE I

MASSES AND RELATIVE ABUNDANCES OF MAJOR IONS IN THE 70-eV MASS SPECTRA OF GIBBERELLIN *p*-NITROBENZYL ESTERS

Measured (m) and calculated (c) masses: GA_1, m 483.1912; c for $C_{26}H_{29}O_8N$ 483.1893. (+ 4 ppm); GA_3, m 481.1729; c for $C_{26}H_{27}O_8N$ 481.1737 (−1.5 ppm), base peak *m/e* 136; GA_4, m 467.1970; c for $C_{26}H_{29}O_7N$ 467.1944 (+5.5 ppm); GA_5, m 465.1760; c for $C_{26}H_{27}O_7N$ 465.1787 (−5.6 ppm); GA_7, m 465.1753; c for $C_{26}H_{27}O_7N$ 465.1787 (−7.3 ppm); GA_{20}, m 467.1948; c for $C_{26}H_{29}O_7N$ 467.1943 (+0.8 ppm).

Ion		GA_1	GA_3	GA_4	GA_5	GA_7	GA_{20}
	M^+	483 (20)	481 (9)	467 (2)	465 (5)	465 (3)	467 (21
a	M−18	465 (4)	463 (4)	449 (3)	—	447 (2)	449 (2)
b	M−44	—	437 (1)	—	421 (2)	421 (2)	423 (1)
c	M−45	—	436 (2)	—	420 (1)	420 (4)	422 (2)
d	M−46	437 (3)	435 (4)	421 (2)	419 (1)	419 (3)	421 (4)
e	M−63	—	418 (30)	—	402 (1)	402 (71)	406 (1)
f	M−136	347 (29)	345 (20)	331 (14)	329 (8)	329 (23)	331 (26)
g	M−153	330 (100)	328 (20)	314 (33)	312 (20)	312 (34)	314 (100)
h	M−154	329 (43)	327 (33)	313 (13)	311 (5)	311 (23)	313 (30)
i	M−180	303 (12)	301 (25)	—	285 (34)	285 (27)	287 (29)
j	M−182	301 (26)	299 (27)	285 (19)	283 (13)	283 (37)	285 (74)
k	M−198	285 (21)	283 (32)	269 (100)	267 (15)	267 (100)	269 (10)
l	M−200	283 (35)	281 (19)	267 (27)	265 (4)	265 (21)	267 (16)
m	M−226	257 (8)	255 (58)	241 (16)	239 (100)	239 (86)	241 (27)
n	M−244	239 (11)	237 (74)	223 (12)	221 (28)	221 (57)	223 (4)

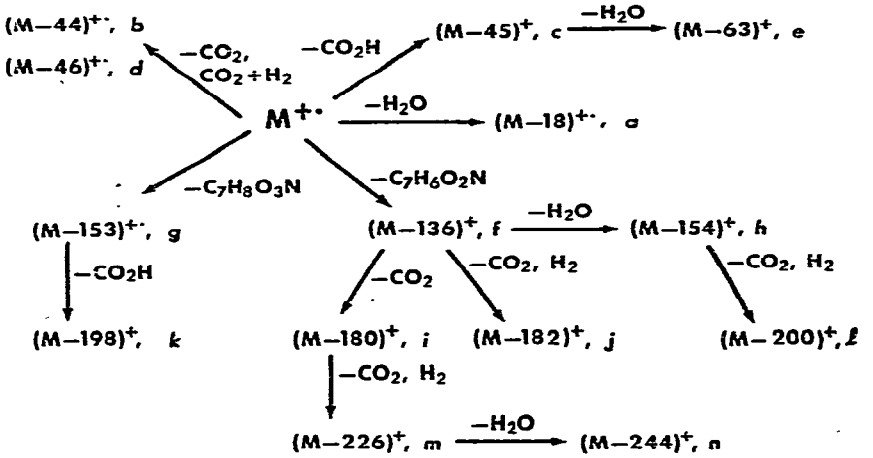

Fig. 7. Fragmentation scheme.

and $(M-136)^+$, respectively, which may have the structures indicated in Fig. 8 for GA_3. Ion *g* is analogous to the $(M-CH_3OH)^+$·ions in the spectra of the methyl esters, but it is much more prominent in the *p*-nitrobenzyl ester spectra. Ion *f* would be equivalent to $(M-CH_3)^+$ for the methyl esters, which is not observed[6]. Both ions, *f* and *g*, undergo extensive further decomposition. The abundances of ions produced by these reactions may yield some information about the structural features of the

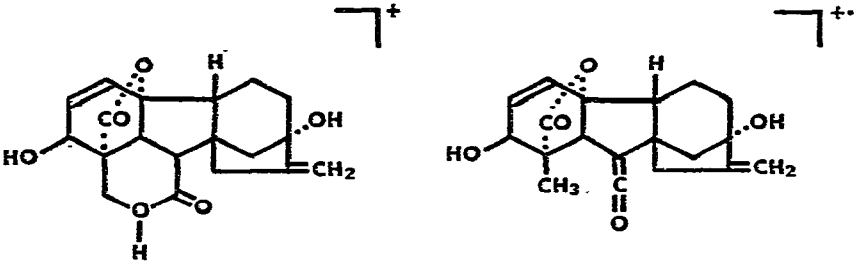

Fig. 8. Fragment ions of GA_3.

gibbane nucleus; *e.g.*, the presence of a double bond in ring A (GA_3, GA_5, and GA_7) appears to correlate with the high abundance of ions *m* and *n*. These ions are much less abundant in the mass spectra of GA_1, GA_4, and GA_{20}, in which the A ring is saturated. The spectra of the methyl esters do not show this correlation.

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