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IDENTIFICATION OF GIBBERELLIN A_9 IN EXTRACTS OF NORWAY SPRUCE [*PICEA ABIES* (L.) KARST.] BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Gibberellin A_9 has been identified conclusively by combined gas chromatography–mass spectrometry of extracts of Norway spruce [*Picea abies* (L.) Karst.]. The clean-up of the 100-g samples were performed by poly-N-vinylpyrrolidone column chromatography and two different systems of high-performance liquid chromatography, and active fractions were detected by the Tan-qinbozu dwarf rice bioassay.

INTRODUCTION

The gibberellins (GAs) have received much attention in phytohormone research because of their unique regulating role in certain physiological processes. For a review of GA physiology and chemistry, see ref. 1. The progress of research in GA physiology has, however, been impeded by the fact that these compounds are very difficult to study. Conclusive identifications of gibberellins are difficult to perform as these substances are often present in small amounts, thus necessitating extensive purification of large amounts of plant material. The GAs lack properties that lend themselves to accurate determination by physico-chemical methods. The only alternative has often been to quantify the gibberellins or gibberellin-like substances by bioassays. The development of analytical techniques is, however, helping to increase the information obtained from GA investigations. Highly efficient separatory systems have been developed, including high-performance liquid chromatography (HPLC)[2–5] and gas chromatography (GC), with capillary columns and on-column injection systems[6]. The sensitivity of the mass spectrometer is increasing, which reduces the amount of GA necessary for identification. The cost of the equipment for combined

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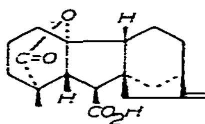
GC–mass spectrometry (MS) is falling, which makes the technique available for routine quantifications of GAs. In addition, quantification methods involving derivatization of GAs to UV-absorbing or fluorescing products have been developed[7–10].

Conifer species have proved more difficult to work with than many herbaceous species[11]. Solvent extracts of conifer species contain a large quantity of diterpenoid resin acids that are difficult to remove without separating the different GAs from one another. This is reflected by the fact that GAs have only been identified from two conifer species. Lorenzi *et al.*[12] identified a glucosyl ester of GA_9 in extracts of *Picea sitchensis*, which was an identification of GA_9 after enzymatic hydrolysis. In addition, a previously unknown isomer of GA_9, iso-GA_9, was identified[13]. The only other identification of endogenous conifer GAs was made by Kamienska *et al.*[14], who identified GA_3, GA_4 and GA_7 from germinating pollen of *Pinus attenuata*.



Gibberellin A_9

The purpose of the present investigation was to detect gibberellin-like substances in extracts of *Picea abies* by bioassay, to purify the active fractions and to identify them conclusively by GC–MS.

EXPERIMENTAL

Plant material

Seedlings of Norway spruce [*Picea abies* (L.) Karst.] were grown in a greenhouse under summer-climate conditions. They were watered daily and given Wallco nutrient solution twice a week. At the age of three months, the seedlings were harvested and immediately frozen in liquid nitrogen. The plant material was then stored at $-30°C$ until required for analysis.

Reagents

Methanol (E. Merck, Darmstadt, G.F.R.; analytical-reagent grade), ethyl acetate (May & Baker, Dagenham, England; analytical-reagent grade), light petroleum (b.p. 40–60°C; May & Baker; analytical-reagent grade), *n*-hexane (J. T. Baker, Deventer, The Netherlands; analytical-reagent grade), buffer chemicals (E. Merck; analytical-reagent grade), poly-N-vinylpyrrolidone (PVP, purchased as Polyclar AT Powder; GAF, New York, NY, U.S.A.), gibberellin A_3 (Sigma, St. Louis, MO, U.S.A.), gibberellin A_9 (Arne Dunberg, Umeå, Sweden), [^{14}C]gibberellin A_3 (specific activity 200 MBq/mmol, Amersham International, Amersham, Great Britain) and [^{3}H]gibberellin A_9 (specific activity unknown; Arne Dunberg) were used as supplied.

Extraction and purification

A simplified picture of the extraction–purification–separation–detection procedure is shown in Fig. 1. Samples [each 100 g, (fresh weight)] of plant material were homogenized in 1000 ml of refrigerated methanol and extracted in darkness at 4°C

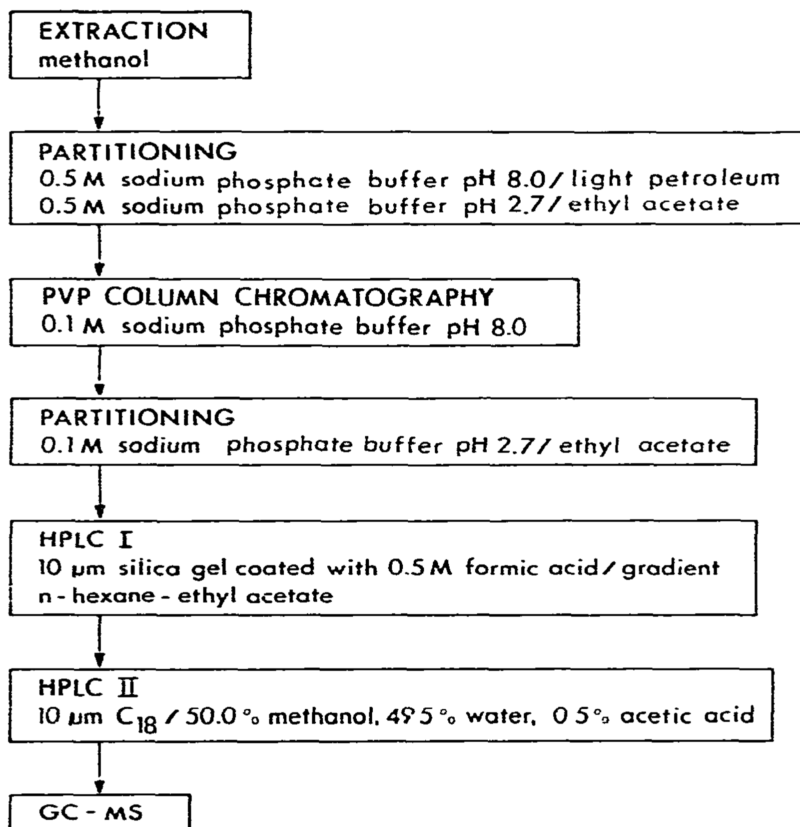


Fig. 1. Simplified picture of the procedure for extraction, purification, separation and detection of gibberellin-like substances in extracts of *Picea abies*.

for 2 h. The methanol was filtered off and the residue was washed carefully with 400 ml of refrigerated methanol. The methanol extract was evaporated to dryness at reduced pressure and 35°C. The extract was dissolved in 0.5 *M* sodium phosphate buffer (pH 8.0) and washed three times with equal volumes of light petroleum. The buffer phase was acidified to pH 2.7 with 6.0 *M* hydrochloric acid and extracted five times with half its volume of ethyl acetate. The ethyl acetate phases were combined, water was removed by freezing and filtering, and the ethyl acetate was evaporated to dryness at reduced pressure at 35°C. The extract was dissolved in 10 ml of 0.1 *M* sodium phosphate buffer (pH 11.0) and, after adjustment to pH 8.0, was applied to a 30 cm × 20 mm I.D. PVP column. The column was eluted with 0.1 *M* sodium phosphate buffer (pH 8.0) and the 0–200-ml fraction was collected, acidified to pH 2.7 and extracted five times with half its volume of ethyl acetate. The final residue was dissolved in 10 ml of ethyl acetate, transferred to a conical test-tube and evaporated to dryness under a stream of nitrogen.

HPLC equipment

HPLC system I consisted of a Spectra-Physics liquid chromatograph Model

3500 B connected via a Valco 100-μl loop injector to a 500 × 10 mm I.D. column packed with 10 μm RSIL (Alltech Assoc., Deerfield, IL, U.S.A.). The packing material was coated with 0.5 *M* formic acid which formed the stationary phase. The mobile phase was a gradient of *n*-hexane saturated with 0.5 *M* formic acid and ethyl acetate saturated with 0.5 *M* formic acid. The flow-rate was 2.0 ml min^{-1} and 10 ml fractions were collected.

HPLC system II consisted of a Milton Roy Minipump connected via a Valco 100-μl loop injector to a 40 × 4 mm I.D. pre-column packed with silica gel (120–160 mesh), followed by a 250 × 4 mm I.D. analytical column of 10 μm Nucleosil C_{18} (Skandinaviska Genetec, Kungsbacka, Sweden). The eluent was methanol–water–acetic acid (50.0:49.5:0.5) and the flow-rate was 1.0 ml min^{-1}. Fractions of 5 ml were collected and evaporated to dryness with a freeze dryer (Heto, Birkeröd, Denmark).

Bioassay

The Tan-ginbozu dwarf rice microdrop bioassay was used for the detection of gibberellin-like substances. It was performed according to the description given by Murakami[15]. Growing conditions in the growth chamber were: 32°C, 100% relative humidity and continuous light with a photon flux density of *ca.* 190 μE m^{-2} sec^{-1} (400–700 nm). Rice seeds were soaked in water for 2 days. Seeds with uniform length of coleoptile were planted in cylindrical bottles filled with 1% agar, eight in each bottle, and placed in the growth chamber for incubation for 45 h. The seedlings were selected for uniformity, leaving 6 seedlings per vial, and by means of a microsyringe 0.5 μl of 95% ethanol containing sample fractions, GA_3 standards or solvent blanks was added at the angle between the coleoptile and the first leaf. The seedlings were then incubated for another 3 days in the growth chamber and the lengths of the first and second leaves were measured with a ruler. The shortest seedling in each bottle was disregarded, and the mean length of the five remaining seedlings was calculated and taken as one observation of bioassay response. The plant extracts were dissolved in 50 μl of 95% ethanol throughout.

GC–MS equipment and identification of GA_9

The gas chromatograph was equipped with a 25 m × 0.25 mm I.D. capillary OV-101 quartz column (Hewlett-Packard). The temperature of the Grob-type injector was 250°C. The temperature of the column was 70°C for 1 min and subsequently increased by 25°C/min to a final value of 250°C, which was held for 20 min. The gas chromatograph was connected to a Finnigan Model 4021 mass spectrometer equipped with a INCOS computer system. The temperature of the interface and the ion source was 250°C. The electron multiplier voltage was 1600 V and the spectra were recorded at 20 eV. The extract fractions were methylated overnight in an ice-bath with diazomethane dissolved in diethyl ether and a few drops of methanol. After evaporation to dryness in a stream of nitrogen, the residue was redissolved in *ca.* 20 μl of dichloromethane for GC–MS analysis. Blank samples and standards were treated in the same way. The retention time of GA_9-methyl ester on GC was 13 min.

Gas chromatography

GC was carried out on a Pye 104 gas chromatograph with a flame ionization

detector (FID). The conditions were: 1% QF-1 (on 80–100 mesh Chromosorb) column (1.5 m × 4 mm) at 180°C with a carrier gas (nitrogen) flow-rate of 40 ml min^{-1}, injection temperature 250°C and detector temperature 300°C.

RESULTS AND DISCUSSION

The bioassay response of 54 10-ml fractions collected from HPLC system I is shown in Fig. 2 and reveals two regions of gibberellin-like activity. The first region (110–120 ml) has the same retention volume as [^{3}H]GA_9. The second region (300–320 ml) has the same retention volume as [^{14}C]GA_3. The retention volumes for [^{3}H]GA_9 and [^{14}C]GA_3 were determined between the different extract runs. No radioactive gibberellins were added to the extracts. The behaviour of the radioactive gibberellins was also used as an indicator of the condition of the chromatographic system. The purity of the active fractions was established by GC–FID after methylation and silylation, and it was concluded that further purification was necessary before any attempt to identify the active substances by GC–MS was made. New extracts were made and the two regions of gibberellin-like activity from HPLC system I were combined and chromatographed on a reversed-phase HPLC system. The mobile phase (methanol–water–acetic acid, 50.0:49.5:0.5) was chosen with regard to the polarity of the two detected gibberellin-like substances, and it was hoped to attain some retention of the more polar fraction (GA_3-like) and not too strong a retention of the non-polar fraction (GA_9-like). The bioassay of the fractions collected from this HPLC system is shown in Fig. 3, and it reveals only one active fraction having the same retention volume as [^{3}H]GA_9. No activity of the more polar fraction could be detected, leading us to believe at first that the activity was masked by inhibiting impurities eluting with the solvent front. Accordingly, another mobile phase was chosen (methanol–water–acetic acid, 20.0:79.5:0.5) for the more polar gibberellin-like substance from HPLC system I. No activity could be detected after this chromatographic system, however, indicating that some conditions before, during or after this run result in loss or degradation of this gibberellin-like substance. No further attempt to solve this problem was made. Work was instead concentrated on the identification of the less polar gibberellin-like substance. The active region from HPLC system II was analysed by GC–MS after methylation.

Electron impact (EI) spectra were recorded at 20 eV with a mass spectrometer sensitivity of 1 ng of GA_9 methyl ester. A comparison between the spectra obtained from standard GA_9 methyl ester and spruce extract, respectively (Fig. 4) shows a very high consistency. The combined amount of two 100-ng samples was sufficient to obtain complete spectra of GA_9-methyl ester in the spruce extracts and was estimated at *ca.* 1 ng/g fresh weight. Owing to a high injection temperature of the GC and a rapidly increasing column temperature, a slight variation in retention time was observed. Accordingly, the extracts were run on GC–MS, first in unspiked form and then spiked with a standard GA_9-methyl ester.

The spectra also agree with the main ions of spectra earlier published[16] which were obtained at 70 eV. However, spectra recorded at 70 eV also show a great number of ions with very low intensities as a result of fragmentation of the carbon frame. As expected, ions of higher masses in spectra of GA_9 are derived from the fragmentation of the lactone, ester and methylene group, as proposed by Binks *et al.*[16], with the molecular ion at m/e 330.

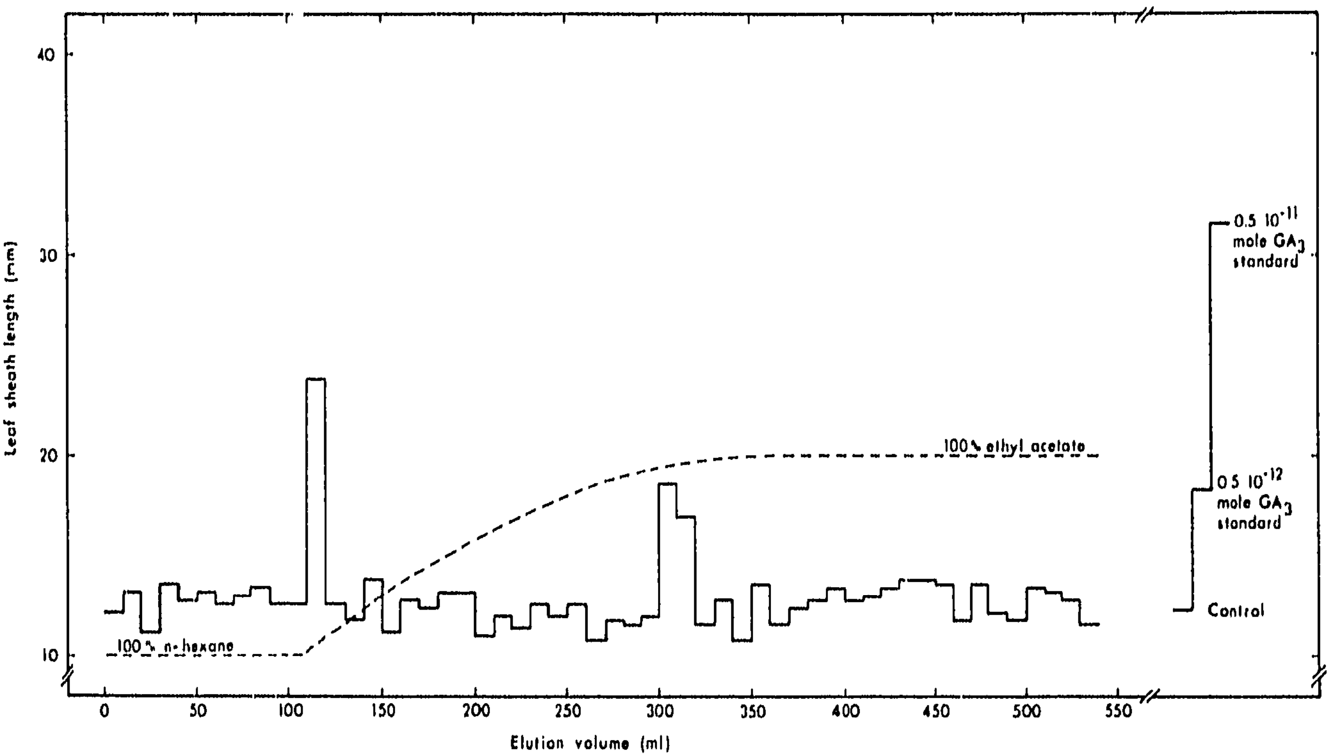


Fig. 2. Dwarf rice bioassay of *Picea abies* extract. Fractions collected from HPLC system 1. 1/100 of each fraction tested and compared with GA_3 standard. Stationary phase: 10 μm RSIL coated with 0.5 *M* formic acid. Mobile phase: gradient of *n*-hexane-ethyl acetate, as illustrated. Flow-rate, 2 ml/min; fraction size, 10 ml.

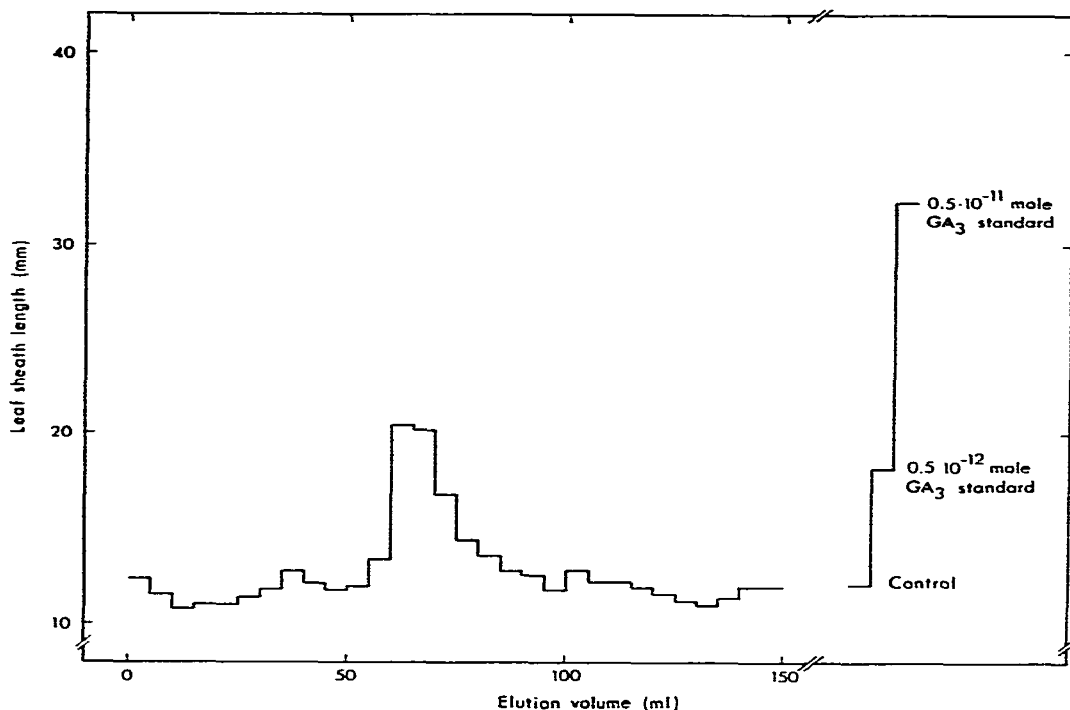


Fig. 3. Dwarf rice bioassy of *Picea abies* extract. Fractions collected from HPLC system II. 1/100 of each fraction tested and compared with GA_3 standard. Stationary phase: 10 μm Nucleosil C_{18}. Mobile phase: Methanol–water–acetic acid (50.0:49.5:0.5). Flow-rate, 1.0 ml/min; fraction size, 5 ml.

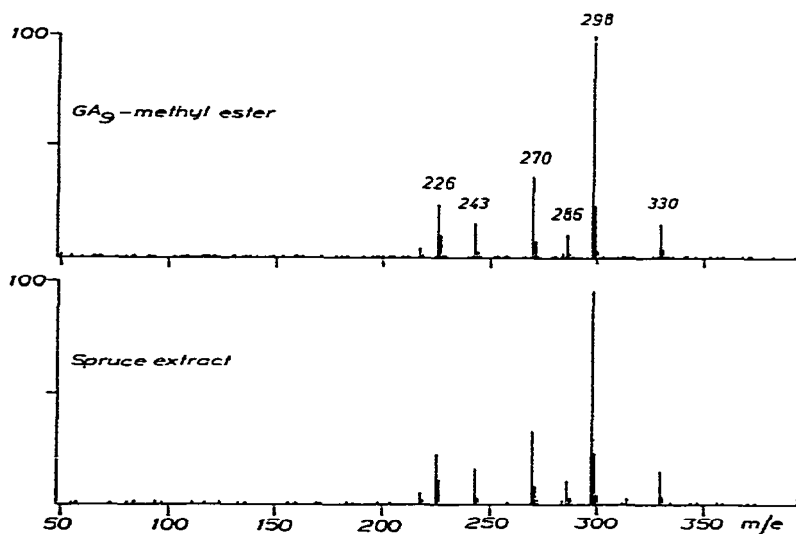


Fig. 4. Mass spectra (EI) of GA_9-methyl ester as reference compound and endogenously in spruce extract (M^+ = 330).

The behaviour of the less polar gibberellin-like substance in two HPLC systems and in capillary GC, together with the complete mass spectrum, indicates with high probability that GA_9 is present as a naturally occurring substance in extracts of *P. abies*. However, it is impossible to state that it occurs as an endogenous gibberellin in *P. abies*, since we do not know if it is released from a conjugate, *e.g.*, the glucosyl ester during the clean-up procedure. The probability that GA_9 only occurs as the glucosyl ester and not as the free acid is however very small, but remains to be investigated.

The amount of GA_9 in the material studied was *ca.* 1 ng/g fresh weight and is sufficient for quantification by multiple ion detection, thus enabling physiological studies of GA_9.

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