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DETECTION OF BRASSINOLIDE AND CASTASTERONE IN *ALNUS GLUTINOSA* (EUROPEAN ALDER) POLLEN BY MASS SPECTROMETRY/MASS SPECTROMETRY

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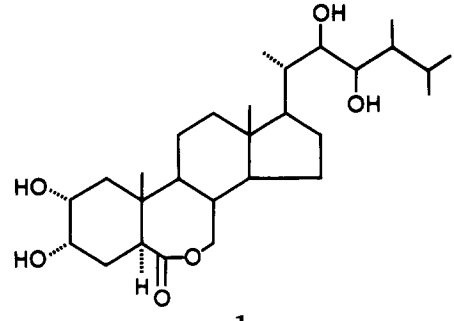
Since the initial report of the isolation and structure elucidation of the novel plant-growth-promoting steroid brassinolide (**1**) from rape pollen (*Brassica napus* L.) (1), this compound has been subsequently detected in seed and sheaths of Chinese cabbage (*Brassica campestris* var. *pekinensis*) (2), chestnut (*Castanea crenatia*) insect galls (3), seed of labab (*Dolichos labab*) (4), and green tea (*Thea sinensis*) leaves (5). Castasterone (**2**), the probable biogenetic precursor of **1**, has been found in these same plants (2-6) plus seed of *Phaseolus vulgaris* (7) and pollen of *Pinus thunbergii* (8). Among several other biologically active brassinolide analogs also found in these plants, reports of 2-deoxycastasterone or typhasterol in cattail (*Typha latifolia*) pollen (9) and pine pollen (8) are noteworthy because they represent other pollen sources of these growth regulators. The amounts of 2-deoxycastasterone isolated (0.068 and 0.09 mg/kg of pollen) are near the quantities of brassinolide (0.1 mg/kg) obtained from rape pollen (1). These levels are considerably higher than those found in other plant sources, therefore indicating that pollens are relatively rich sources of steroidal growth regulators.

Selective ion monitoring of the (MH+) protonated molecule produced by gc/cims of methylboronate ester derivatives has been employed to detect these compounds in active fractions (2, 3, 5, 10, 11). An alternative technique for analysis of target compounds in complex biological mixtures is that of tandem mass spectrometry or ms/ms (12). In this procedure, the first mass filter is used to select the ion of interest from all the other ions produced from the matrix. This ion is usually the molecular ion in the electron impact mode or the protonated molecule (MH+) in the chemical ionization mode. These selected ions then undergo collisionally activated dissociation (CAD) to produce daughter ions which are then separated by a second mass filter and analyzed. In this note we report the application of this technique to the detection of **1** and **2** in partially refined fractions of European alder [*Alnus glutinosa* (L.) Gaertn.] pollen.



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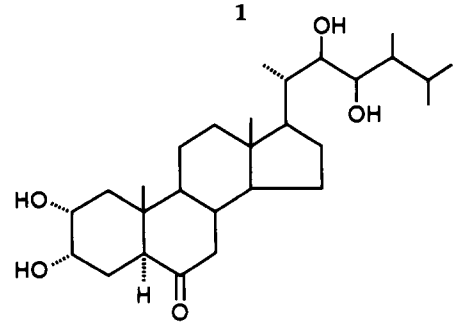

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Rape pollen was used as a known source of brassinolide for evaluation of the ms/ms technique. European alder (*A. glutinosa*) pollen was chosen for this study because extracts from it exhibited activity that was comparable to rape pollen in the bean second internode bioassay 13. Black locust (*Robinia pseudoacacia* L.) pollen, which showed slight activity (13), and oat (*Avena sativa* L.) and corn (*Zea mays* L.) pollens, which showed no activity, were also analyzed.

Because pollen presents a very complex sample matrix and the expected amount of brassinolide in the samples may be quite small, a sample clean-up procedure was designed. The extraction and fractionation procedure was adapted from the original procedure used to isolate brassinolide (1). At each stage in the procedure an aliquot was collected and saved for ms/ms analysis.

Brassinolide is a moderately polar molecule with four free hydroxyl groups. It does not elute from a gc column unless it is derivatized. No molecular ion is observed in eims. However, cims, using isobutane as reagent gas, produces an abundant MH^+ at *m/z* 481 with smaller fragments observed at *m/z* 463, 445, and 427, showing the successive losses of three molecules of H_2O from the protonated molecule. The MH^+ ion at *m/z* 481 was chosen as the parent for CAD daughter ms/ms experiments. Underivatized brassinolide was not an ideal molecule for ms studies for two reasons: (1) the ions produced in ei were not useful for ms/ms because of the lack of a parent ion, (2) in the ci mode, brassinolide produces useful ions, but sensitivity was not particularly good. Approximately 50 ng of pure material was required to produce enough ions at MH^+ (*m/z* 481) to obtain full CAD daughter spectra with an acceptable signal-to-noise ratio. The CAD daughter spectrum (Figure 1) was quite complex with many decomposition pathways.

As expected, preliminary experiments analyzing crude rape pollen in the mass spectrometer yielded very little signal at *m/z* 481; the parent ion for brassinolide and usable daughter spectra could not be obtained. Therefore, all further experiments used partially refined pollen extracts. Table 1 shows the major ions in the CAD daughter spectrum of *m/z* 481 for pure brassinolide (100 ng) and the CH_2Cl_2 solubles and 70% MeOH reverse phase chromatography fractions of rape and alder pollens inlet into the mass spectrometer via the probe. The presence of brassinolide is

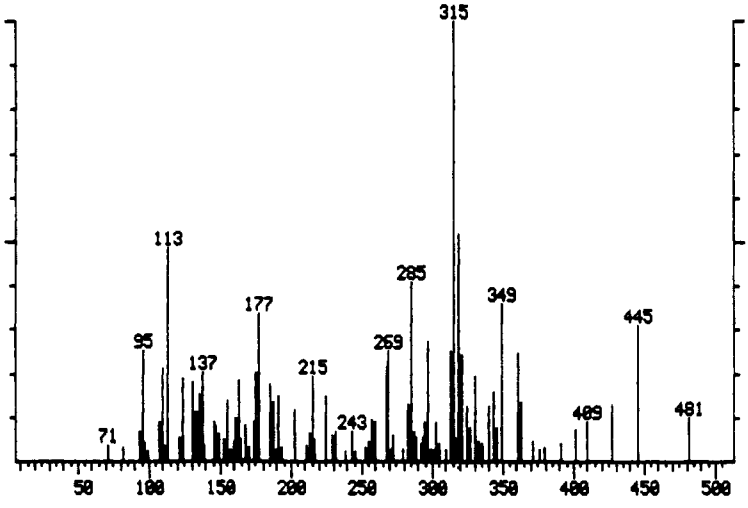

FIGURE 1. Collisionally activated dissociation (CAD) daughter spectrum of MH^+ (*m/z* 481) from cims of brassinolide.

TABLE 1. Ms/ms Daughter Ions of Brassinolide *m/z* 481[a]

Daughter ions *m/z*	Relative Abundance[b]				
	Brassinolide standard	Rape CH_2Cl_2	Rape 70% MeOH	Alder CH_2Cl_2	Alder 70% MeOH
463	1	100	41	100	28
445	51	18	100	37	66
427	11		12		25
409	2		4		13
361	28		24	11	44
349	40		49	15	65
333	9		12		26
321	28		35		49
319	35		5		48
315	100	21	96	26	100
297	16	7	13	14	36

[a]Produced by positive ion chemical ionization with isobutane and collisionally activated dissociation with argon.

[b]Intensities are normalized against the most abundant daughter ion.

suggested in the relatively unrefined CH_2Cl_2 fractions for both rape and alder pollens by the presence of daughter ions of *m/z* 481 at 445 and 315. Because these spectra were quite weak and the relative daughter ion abundances varied from those of the standard sample, these data were not sufficient to identify brassinolide.

No *m/z* 445 or 315 daughters were observed in corn, oat, or black locust pollens. These results were obtained from the analysis of ca 1 mg of sample matrix, a large amount of sample matrix to introduce into the source of the mass spectrometer and expect to maintain good ci conditions. Therefore, the analysis of larger aliquots of sample by probe/ms did not look promising, and further clean-up was needed for positive detection of brassinolide. After the first chromatographic step (silica gel), ms/ms analysis of the 10% EtOH eluates gave spectra similar to those of the CH_2Cl_2 soluble fraction. However, after the second chromatography (reverse phase C18), the 70% MeOH eluates gave *m/z* 481 daughter spectra that were quite similar to that of the standard material (Table 1). Even after solvent partition and two chromatographic fractionations, the concentration of brassinolide in the sample was still quite low, being estimated on the order of 50 ng/mg. No *m/z* 315 daughters, the base daughter for brassinolide, were detected in any of the corn, oat, or black locust pollen fractions.

To verify that, indeed, brassinolide was being measured, the methylboronate derivative was formed and analyzed by gc/ms. The ci mass spectrum of brassinolide *bis*-methylboronate is shown in Figure 2a. The MH^+ ion is at *m/z* 529. A fragment at *m/z* 469 arises from the loss of $CH_3B(OH)_2$ (60 daltons). The CAD daughters of *m/z* 529 are shown in Figure 2b. This spectrum is rich in structural information. The intense daughters at *m/z* 373 and *m/z* 155 arise from cleavage at the methylboronate group on the side chain. Table 2 shows the major ion intensities in the ms/ms spectra of brassinolide *bis*-methylboronate and the alder 70% MeOH fraction. These spectra are virtually identical, demonstrating that the alder pollen fraction does, indeed, contain brassinolide. Chemical ionization gc/ms of the methylboronate derivatives has been reported (10, 13). The advantage of gc/ms over probe/ms is that the brassinolide is separated from matrix by the gc and eluates at the proper retention time.

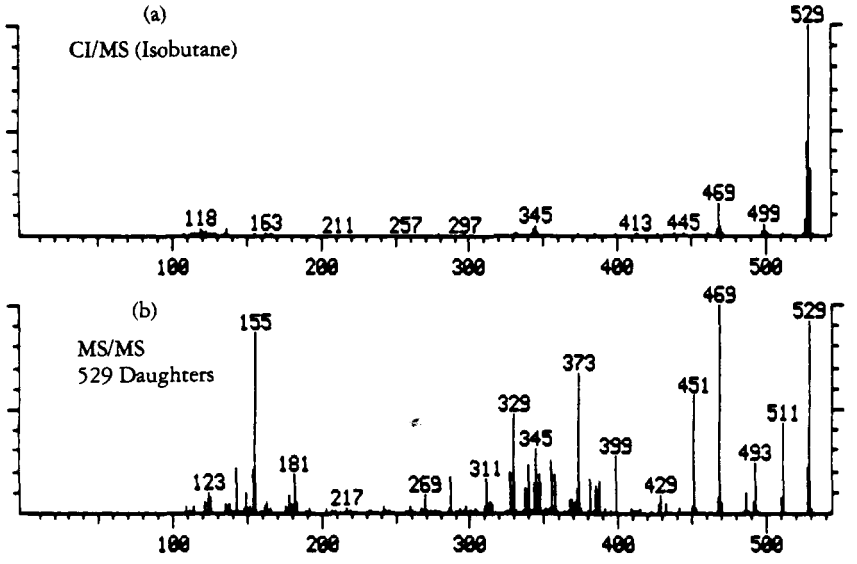

FIGURE 2. Chemical ionization mass spectrum of brassinolide *bis*-methylboronate (a) and the CAD daughter spectrum the MH^+ ion (b).

Thus, gc/ms gives two dimensions of information, i.e., a retention time plus the occurrence of the parent (MH^+) ion. Ms/ms also gives two dimensions: separation and occurrence of the MH^+ ion and structural confirmation from its daughters.

Using the gc/ms/ms daughter experiment, three dimensions of information-retention time, the parent ion, and structural information for the MH^+ ion—are obtained. With gc/ms/ms, which has this extra information (i.e., retention time), one can obtain increased sensitivity by using selected reaction monitoring (SRM-the ms/ms equivalent of conventional ms selected ion monitoring) and monitor only specific daughter ions for a given parent without giving up the selectivity needed for conclusive identification. Using SRM of the methylboronate derivative, MH^+ (*m/z* 529), gc/ms/ms monitoring daughters at *m/z* 155 and 469, the same ratio of daughters at *m/z* 151 and 469 was observed in the derivatized standard and crude CH_2Cl_2 soluble fractions from the rape and alder pollens. No *m/z* 529 parent that produced detectable daughters of either *m/z* 155 or 469 was observed in the oat, alder, or corn pollen-extract derivatives. Thus, brassinolide was detectable in the CH_2Cl_2 fractions using gc/ms/ms of the *bis*-methylboronate derivatives.

TABLE 2. Gc/ms/ms of Brassinolide *bis*-Methylboronate[a] from Rape Pollen

m/z	Relative abundance	
	Standard	70% MeOH
529	54	88
511	28	39
469	86	100
451	51	58
399	18	23
373	74	80
355	26	30
345	32	35
339	26	26
329	47	54
155	100	100

[a]Produced by positive ion chemical ionization with isobutane and collisionally activated dissociation with argon.

Castasterone, (**2**), the probable biological precursor of brassinolide, was not available as an authentic standard. It would be expected to have an MH^+ ion at *m/z* 465. Daughter experiments on *m/z* 465 from the second chromatog-

raphy fraction of the alder pollen showed that daughters had been formed from *m/z* 465, but without an authentic standard castasterone sample, positive identification was not possible. The methylboronate of castasterone has a MH^+ ion at *m/z* 513. Gc/ms of the second chromatography fraction of the alder pollen showed a weak 513 ion that eluted about 2 min before brassinolide. This is about where the *bis*-methylboronate of castasterone would be expected to elute, based on the report of Ikekawa *et al* (14). Table 3 presents the major ions observed in the gc/ms/ms daughter scan of *m/z* 513 from the methylboronate of the alder fraction and the gc/ms/ms daughter spectrum of *m/z* 527 from the methylboronate of homocastasterone which was available in pure form. Homocastasterone, which has one more methylene group than castasterone on the side chain, gives daughters which have strikingly similar intensitites to those for the *m/z* 513 component in the alder fraction, supporting the tentative identification of castasterone made from the gc retention time and the presence of an ion at the proper mass for the MH^+ ion. The castasterone concentration was estimated to be about 10% of the brassinolide concentration in both gc/ms and ms/ms experiments.

This experiment demonstrates the usefulness of ms/ms in the identification of brassinolide from partially refined extracts of pollens. Ms/ms techniques are a useful supplement to gc/ms by providing additional structural confirmation for the MH^+ ions generated in chemical ionization. The speed of the ms/ms experiment could be useful in studies in which plant scientists may want to assay large numbers of samples. Because relatively large samples (~1 mg) must be analyzed to detect brassinolide at ppm levels in these crude extracts, matrix problems would probably occur in the direct probe method, and therefore, accurate quantitative work would require gc/ms/ms of the methyl boronate derivatives.

TABLE 3. Gc/ms/ms of Homocastasterone *bis*-Methylboronate *m/z* 527 and *m/z* 513 Species from Methylboronated Alder Pollen Fraction[a]

Homocastasterone		Fission	Alder 70% MeOH	
m/z	Relative abundance		*m/e*	Relative abundance
527	100	MH^+	513	82
509	5	MH^+-H_2O	495	8
485	14	MH^+-42	471	42
467	82	$MH^+-CH_3B(OH)_2$	453	100
169	18	$C_{20}-C_{22}$	155	18

[a]Produced by isobutane positive ion chemical ionization and argon collisionally activated dissociation.

EXPERIMENTAL

SOURCES OF POLLEN.—Bee-collected *Brassica napus* (rape) pollen was obtained from the Zekorg Apiaries, Benito, Manitoba, Canada. Other pollen was hand-collected. *A. glutinosa* (European alder) pollen was provided and its identity was confirmed by the Beltsville Agricultural Research Center, Beltsville, MD. *Robinia pseudoacacia* (black locust), *Avena sativa* (oat), and *Zea mays* (corn) pollens were obtained from Ashland Farm Botanicals, Sedalia, Missouri.

EXTRACTION AND FRACTIONATION OF POLLENS.—A 10-g sample of each pollen was washed with H_2O (4×20 ml) and centrifuged. The residual pollen was extracted at room temperature with i-PrOH (3×50 ml). The extracts were filtered through Celite and concentrated at reduced pressure. The samples were defatted by partition between hexane and MeOH-H_2O (9:1). The methanolic phase was concentrated and partitioned between H_2O and CH_2Cl_2. These organic extracts were chromatographed in 50 times their weight of silica gel 60 (70-230 mesh, EM Laboratories) with toluene-absolute EtOH (19:10, 300 ml; 9:1, 500 ml). The 10% EtOH

eluates were chromatographed on columns of 100 times their weight of C(18) Bonded Hiflosil 80-100 mesh, Applied Science Laboratories) eluted with MeOH-H_2O (1:1, 100 ml; 7:3, 100 ml). Samples of CH_2Cl_2 solubles, 10% EtOH eluates, and 70% MeOH eluates were retained for ms/ms analysis.

GC/MS AND MS/MS.—All mass spectrometry experiments were performed with a quadrupole tandem mass spectrometer (Finnigan-MAT 4535/TSQ®). Conventional mass spectra were obtained by passing all ions through quadrupoles Q_1 and Q_2 in the Rf only mode and scanning Q_3. Isobutane was used as the reagent gas (0.25 torr) in ci experiments. The electron energy was 70 eV, and the source was maintained at 140°. Samples were either admitted into the source from the gc or with the insertion probe. A 6 ft × 2 mm ID glass column packed with 3% OV1 was used in the gc/ms experiments. The column was programmed from 230° to 290° at 4°/min. The carrier gas (helium) flow rate was 30 ml/min. For direct insertion, samples (1-2μl) in solution were placed into glass sample vials, the solvent allowed to evaporate, and then put into the mass spectrometer source on the standand sample probe, which was electrically heated in less than 3 min to 250°. For ms/ms experiments, Q_2 was used as a collision cell for CAD. Argon was the target gas at a measured pressure of 1-2 mtorr. The energy of the collisions was set at 20 v. Parents for ms/ms experiments were selected by setting Q_1 to pass only one selected *m/z* value. The daughter ions produced in the collision cell were then mass analyzed by scanning Q_3 and detected and recorded as the ms/ms daughter spectrum by the data system.

SAMPLE DERIVATIZATION.—Samples were derivatized with methylboronic acid in pyridine (1 mg/ml). A sample (10μg brassinolide or 1 mg of sample matrix) was dissolved in 100 μl of the methylboronic acid/pyridine solution and heated at 70° for 20 min. The resulting product mixture was analyzed by probe ms, gc/ms, or gc/ms/ms.

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