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ANALYSIS OF NATURAL BRASSINOSTEROIDS BY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The microanalysis of various kinds of brassinosteroids as their bismethaneboronate derivatives was investigated by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). GC analysis using a glass capillary column indicated that the brassinosteroid methaneboronates can be detected at the nanogram level. The fragmentation patterns of the bismethaneboronate derivatives of brassinosteroids were examined using GC–EI (electron impact)-MS and GC–CI (chemical ionization)-MS systems. Selected ion monitoring using the GC–CI-MS system was applied to the analysis of natural brassinosteroids in several plants. Brassinolide, castasterone, 28-norbrassinolide, brassinone, 24-ethylbrassinone and dolichosterone were identified in the immature seeds and sheaths of chinese cabbage, *Brassica campestris* L. var. pekinensis, the leaves of green tea, *Thea sinensis*, the insect galls of the chestnut tree, *Castanea crenata* L. Sieb. et Zucc., the insect galls and the leaves of *Distylium racemosum* Sieb. et Zucc. and the whole plant except the roots of the rice, *Oryza sativa* L. cv. Arborio J1. The results demonstrate that this microanalysis is useful for the screening of brassinosteroids in plants.

INTRODUCTION

Brassinolide (1), isolated from the pollen of rape (*Brassica napus* L.), is a new plant growth hormonal steroid having an unprecedented seven-membered lactone in ring B and two vicinal diols at both ring A and the side chain[1]. At very low concentration it promotes cell division, cell elongation and plant growth, and also shows a wide variety of responses in a number of bioassays for auxin, gibberellin and cytokinin[2–4]. Promising results of the application of brassinolide analogues in agriculture

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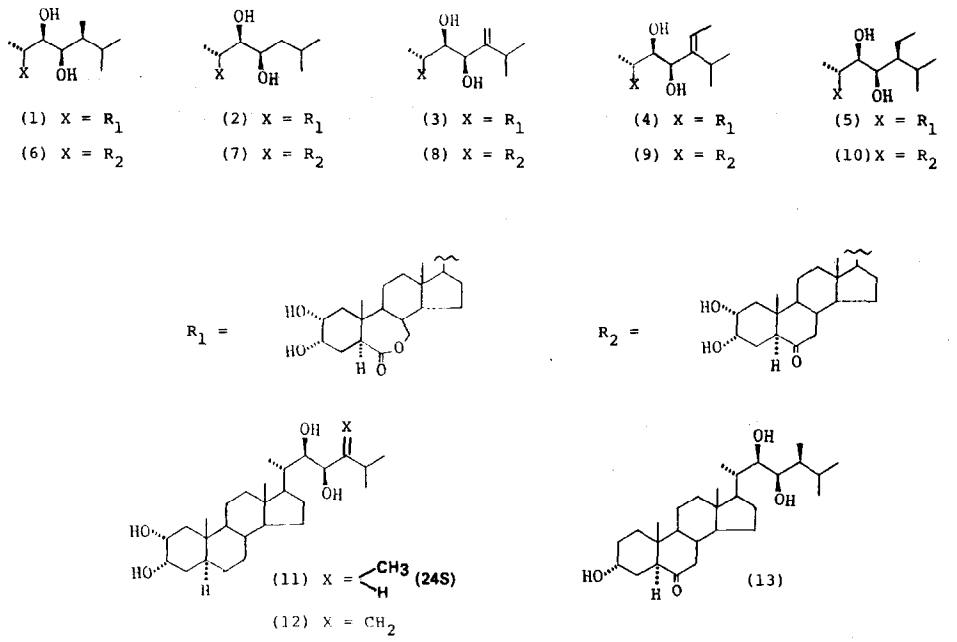


Fig. 1. Structures of brassinosteroids.

have also been reported[5]. Because of its scarcity, remarkable biological activities and novel structural features, much effort has been devoted to the chemical synthesis of brassinolide and related compounds and to study of their physiological properties. Our recent investigations[6–15] into the syntheses of these brassinosteroids and their structure–activity relationships disclosed the structural requirements for the plant-growth-promoting activity, and obtained some highly active brassinolide analogues.

A number of brassinolide related steroids, castasterone (6)[16], 28-norbrassinolide (2)[17], brassinone (7)[17], 24-ethylbrassinone (10)[17], dolicholide (3)[18], homodolicholide (4)[19], dolichosterone (8)[20], homodolichosterone (9)[20], 6-deoxocastasterone (11)[21] and 6-deoxodolichosterone (12)[21], have been isolated and identified in several higher plants[22]. These steroids possess two vicinal diol functions at the C-2,3 and C-22,23 positions. More recently, typhasterol (2-deoxycastasterone) (13) has been isolated from the pollens of *Typha Latifolia* L.[23] and *Pinus Thunbergii* Parl[24].

Since the discovery of brassinolide (1), our attention has focused on developing the microanalytical technique for these brassinosteroids and their identification in higher plants in order to demonstrate the ubiquitous distribution of these steroidal hormones in the plant kingdom. We have already reported that bismethaneboronates of brassinolide (1) and its related lactones are useful derivatives for gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)[25]. Because many natural brassinosteroids have been isolated and identified, further investigation of their microanalysis became necessary. In this paper we report our recent results on the GC and GC–MS analysis of brassinosteroids as their bismethaneboronate derivatives and the application to the identification of several brassinosteroids in some higher plants as a demonstration of the usefulness of our microanalytical technique.

EXPERIMENTAL

Samples and reagents

The following standard samples were synthesized in our laboratory: brassinolide (1)[6,7], 28-norbrassinolide (2)[8], 28-homobrassinolide (5)[9], dolicholide (3)[10,11], castasterone (6)[7], brassinone (7)[8], 24-ethylbrassinone (10)[9] and dolichosterone (8)[11].

Methaneboronic acid was obtained from Alfa Products, Ventron Corporation.

Derivatization

Methaneboronic acid (10 μg) in dry pyridine (50 μl) was added to 10 μg of brassinolide (1). The mixture was heated at 70°C for 30 min. Several microlitres of the solution were injected into the gas chromatograph.

GC analysis

Packed columns of 2% OV-17 (0.5 m × 2 mm I.D.) and 2% OV-1 (2 m × 2 mm I.D.) on Chromosorb W (80–100 mesh) were used. A fused-silica WCOT capillary column (25 m × 0.2 mm I.D.) containing OV-101 was used at 280°C. A Shimadzu GC-7APrF chromatograph with a solventless inlet system (moving needle type) and flame ionization detector was used. The carrier gas (helium) flow-rate was 1.0 ml/min; solvent cut time, 3 min; purge flow-rate, 2.72 ml/min.

When a packed column with 2% OV-17 on Chromosorb W (80–100 mesh) was used at 290°C, the relative retention times of the brassinosteroid bismethaneboronates were as follows: brassinolide(1a), 1.00 (5.30 min); norbrassinolide (2a), 0.85; dolicholide (3a), 1.00; 28-homobrassinolide (5a), 1.05; castasterone (6a), 0.66; brassinone (7a), 0.56; dolichostrone (8a), 0.66; 24-ethylbrassinone (10a), 0.76.

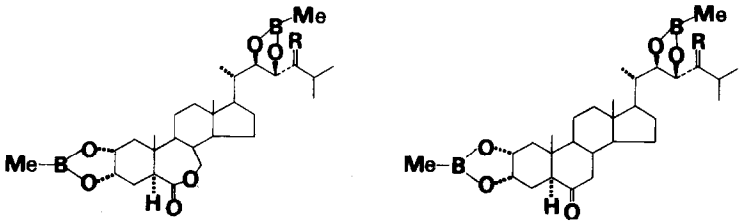


Fig. 2. Structures of bismethaneboronate derivatives of brassinosteroids.

(1a) R =  (24S)

(2a) R = H_2

(3a) R = CH_2

(4a) R = (E)-$CHCH_3$

(5a) R =  (24S)

(6a) R =  (24S)

(7a) R = H_2

(8a) R = CH_2

(9a) R = (E)-$CHCH_3$

(10a) R =  (24S)

GC–MS analysis

A Shimadzu GC–MS 6020 gas chromatograph–mass spectrometer with electron impact (EI) and chemical ionization (CI) sources and a SCAP-1123 was used. For GC–EI-MS, a column packed with 2% OV-17 on Chromosorb W (80–100 mesh) (0.5 m × 2 mm I.D.) was used at 290°C; the carrier gas (helium) flow-rate was 30

ml/min; electron energy, 20 eV; electron current, 60μA; acceleration high voltage, 3.5 kV; ion source temperature, 290°C. For GC–CI-MS, the same packed column was used; the reagent gas was isobutane; carrier gas (helium) flow-rate, 30 ml/min; electron energy, 150 eV; box current, 150 μA; acceleration high voltage, 3.5 kV; ion source temperature, 250°C.

Purification procedure

The insect galls (40 g) and the leaves (16 kg) of *Distylium racemosum* Sieb. et Zucc. and the whole plant (6.8 kg) except the root of the rice *Oryza sativa* L. cv. Arborio J1 were processed as follows to obtain the active fractions. The rice-lamina inclination test, a highly sensitive and specific bioassay for brassinosteroids, was used to guide the fractionation[12,26].

The methanol extract of the plant material was separated in the usual way to afford an ethyl acetate-soluble neutral fraction. This was then partitioned between acetonitrile and hexane (1:1), and the acetonitrile fraction was chromatographed on aluminium oxide (Activity II–III, E. Merck, 30 × 3 cm I.D.). The active fraction was eluted with 20–40% ethanol in ethyl acetate, evaporated to dryness and residue was dissolved in water. This solution was placed on an Amberlite XAD-2 column (30 × 3 cm I.D.) and eluted with aqueous ethanol. The activity appeared in fractions of 50–70% ethanol in water. These fractions were successfully purified by gel chromatography on Sephadex LH-20 (1% acetic acid in ethanol), and by silica gel thin-layer chromatography (ethyl acetate–ethanol, 22:3). The activities appeared as zones of $R_F \approx 0.36$ and ≈ 0.39. These two active fractions were further purified by high-performance liquid chromatography on Finepak SIL (25 cm × 4.6 mm I.D.) (isooctane–ethanol, 70:30) and on Finepak SIL C_{18} (25 cm × 4.6 mm I.D.) (acetonitrile–water, 75:25). The two highly active fractions thus obtained were *ca.* 100 times more active than indol-3-acetic acid (IAA) in the rice-lamina inclination test.

The rice-lamina inclination test[12,26]

Etiolated seedlings of rice (*Oryza sativa* L. cv. Arborio J1) were cultivated in water at 28°C in the dark for 6 days. The leaf segments, each consisting of the second lamina (length 1 cm), a lamina joint and a sheath (length 1 cm), were excised from the seedlings. After the leaf segments had been floated in water at 28°C for 24 h in the dark, they were transferred into 1 ml of 2.5 m*M* potassium malate aqueous solution containing a known amount of the test sample. After incubation at 28°C for 48 h in the dark, the magnitudes of the induced angles between laminae and sheaths were measured. The activity of standard brassinolide (1) was detected at levels as low as 0.0001 μg/ml, while that of IAA was 10 μg/ml.

RESULTS AND DISCUSSION

GC analysis

In a previous paper[25] we have shown that the bismethaneboronates of brassinosteroids are useful derivatives for GC analysis. In the present study, we examined the minimum detection limit of these brassinosteroid derivatives using an OV-101 glass capillary column and the solventless inlet system. As shown in Fig. 3, the bis-

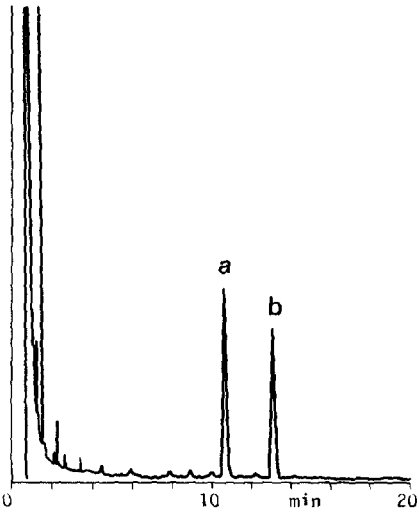


Fig. 3. Separation of castasterone bismethaneboronate (6a) (a) and brassinolide bismethaneboronate (1a) (b) on an OV-101 capillary column (25 m × 0.2 mm I.D.). Helium flow-rate: 1.0 ml/min. Splitting ratio: 1/3.

methaneboronates of brassinolide (1) and castasterone (6) afforded sharp peaks at 10.7 and 13.1 min, respectively. The amounts of these derivatives are 2.6 ng for 1a and 2.2 ng for 6a. Thus, the boronate derivatives of brassinosteroids can be analyzed at the nanogram level.

GC–MS analysis

The bismethaneboronate derivatives of brassinolide (1), 28-norbrassinolide (2), 28-homobrassinolide (5) and their corresponding 6-oxo compounds (6, 7 and 10) showed similar fragment ions in EI mass spectra as summarized in Table I. They afforded molecular ions at *m/z* 528, 514, 542, 512, 498 and 526, respectively, ions at *m/z* 457 (lactones) and *m/z* 441 (ketones) resulting from C_{23}–C_{24} fission, at *m/z* 374 (lactones) and *m/z* 358 (ketones) resulting from C_{20}–C_{22} fission and at *m/z* 345 (lactones) and *m/z* 329 (ketones) resulting from C_{17}–C_{20} fission. The 6-oxo derivatives generally showed more intense molecular ions than the 7-oxalactone derivatives as shown in Table I. The ions at *m/z* 374 (lactones) and *m/z* 358 (ketones) were accompanied by hydrogen transfer. Thus, the fragment ions at *m/z* 457, 374, 345 and 177 are common for the brassinolide skeleton, while ions at *m/z* 441, 358 and 329 are common for the castasterone skeleton. The 6-oxo derivatives also had another ion in common at *m/z* 287 resulting from C_{14}–C_{15} and C_{13}–C_{17} fissions. The 7-oxalactone derivatives afforded characteristic ions for a seven-membered B-ring lactone at *m/z* 332, 346, 318 and 177 (common), respectively. The ions at *m/z* 155, 141 and 169 corresponding to C_{20}–C_{22} side chain cleavage are base peaks in those spectra.

However, compared with those of brassinolide (1) and castasterone (6), the bismethaneboronate derivatives of dolicholide (3) and dolichosterone (8), which possess a C-24 (28) double bond, showed rather different fragment ions as shown in Figs. 4 and 5, particularly those resulting from cleavages of the cyclic boronate moiety of the side chain. EI mass spectra of the boronates of brassinolide (1) and castasterone

TABLE I

CHARACTERISTIC (EI-MS) FRAGMENT IONS OF BRASSINOSTEROID BISMETHANEBORONATE DERIVATIVES

Values given are *m/z* with relative intensities (%) in parentheses.

Bismethane-boronate	M^+	C_{23}–C_{24} *fission*	C_{20}–C_{22} *fission*	C_{17}–C_{20} *fission*	*B-ring lactone*
Brassinolide (1)	528 (1.8)	457 (4.0)	374* (20.9) 155 (100)	345 (9.6)	332 (12.4) 177 (53.0)
Norbrassinolide (2)	514 (2.5)	457 (0.83)	374* (10.4) 141 (100)	345 (5.8)	318 (27.9) 177 (57.5)
Homobrassinolide (5)	542 (0.90)	457 (6.3)	374* (22.5) 169 (100)	345 (16.2)	346 (5.9) 177 (47.3)
Dolicholide (3)	526 (17.9)	457 (–)	373 (21.1) 153 (70.5)	345 (21.1) 343** (100)	330 (2.1) 177 (8.4)
Castasterone (6)	512 (38.3)	441 (6.4)	358* (24.5) 155 (100)	329 (7.4)	
Brassinone (7)	498 (100)	441 (5.3)	358* (25.5) 141 (97.9)	329 (12.8)	
Ethylbrassinone (10)	526 (18.1)	441 (9.6)	358* (17.0) 169 (100)	329 (5.3)	
Dolichosterone (8)	510 (40.0)	441 (–)	357 (11.6) 153 (88.4)	329 (8.4) 327** (100)	

* Hydrogen-atom transfer.
** Two-hydrogen-atom transfer.

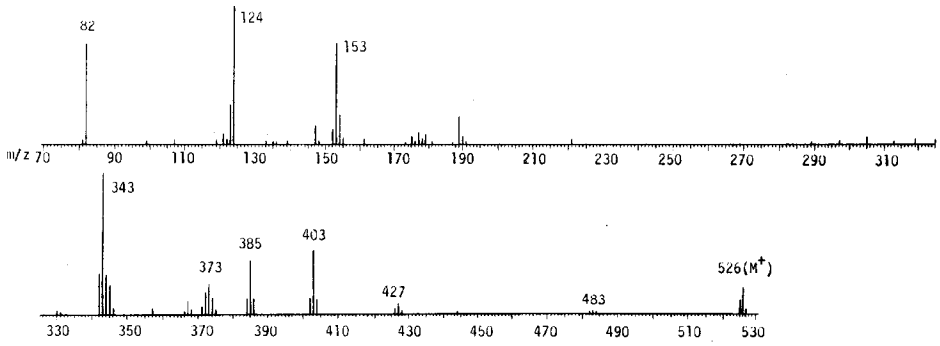


Fig. 4. EI-MS of dolicholide bismethaneboronate (3a).

(6) have already been reported[25,27]. The fragment ions occur at *m/z* 427, 403, 385, 124 and 82 for the dolicholide derivative (3a) and at *m/z* 411, 387, 369, 124 and 82 for the dolichosterone derivative (8a) as shown in Figs. 4 and 5. Another remarkable difference is that hydrogen transfer observed upon C_{20}–C_{22} fission in the saturated derivatives was not recorded in the case of the unsaturated derivatives (3a and 8a), but two-hydrogen transfer upon C_{17}–C_{20} fission was observed for the unsaturated derivatives (3a and 8a). The derivative of dolicholide (3) did not afford the characteristic ions for a B-ring lactone. The differences described above in the fragment ions of EI mass spectra are summarized in Fig. 6.

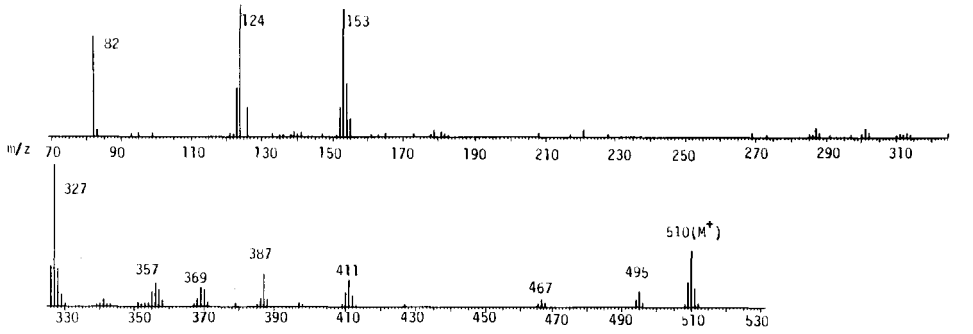


Fig. 5. EI-MS of dolichosterone bismethaneboronate (8a).

In the CI mass spectra, the bismethaneboronate derivatives of brassinosteroids gave ions corresponding to M + 1 as base peaks as shown in Table II. CI mass spectra of the boronates of brassinolide (1) and castasterone (6) have already been reported[25,27]. These derivatives afforded ions resulting from M + 1 − 60 [$MeB(OH)_2$] and also weak ions resulting from C_{17}–C_{20} and C_{20}–C_{22} fissions. There is not much difference between the saturated and unsaturated derivatives in the side chain. Since in the CI mass spectra the molecular ions of the brassinosteroid derivatives are base peaks, these ions can be used for selected ion monitoring for screening of brassinosteroids.

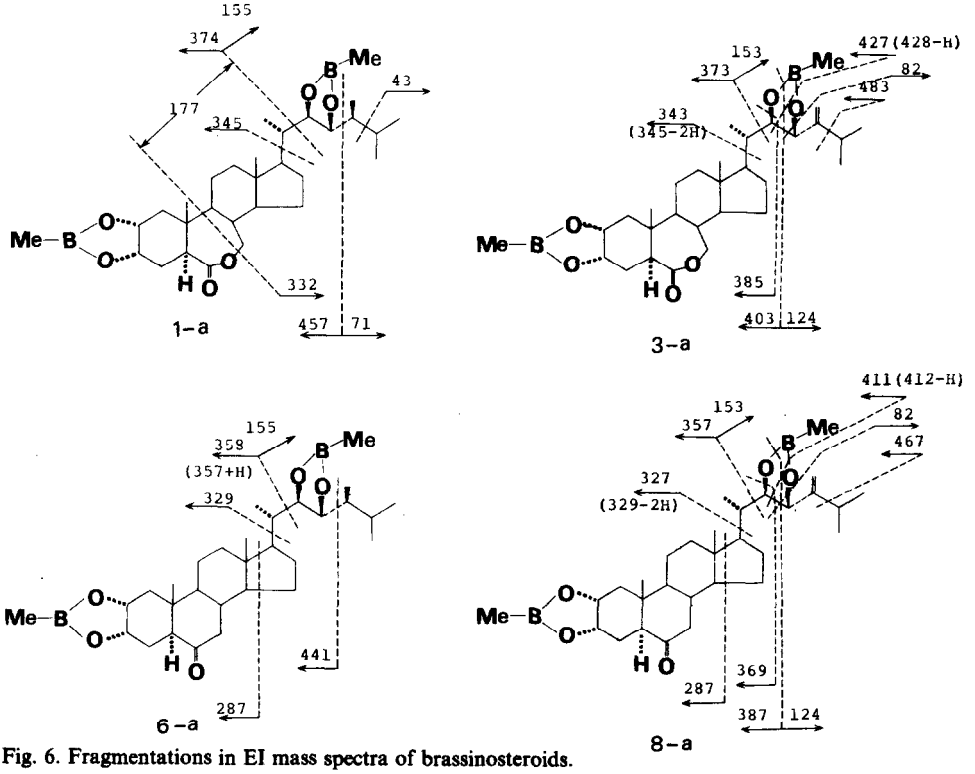


Fig. 6. Fragmentations in EI mass spectra of brassinosteroids.

TABLE II

CHARACTERISTIC (CI-MS) FRAGMENT IONS OF BRASSINOSTEROID BISMETHANEBORONATE DERIVATIVES

Values are *m/z* with relative intensities (%) in parentheses.

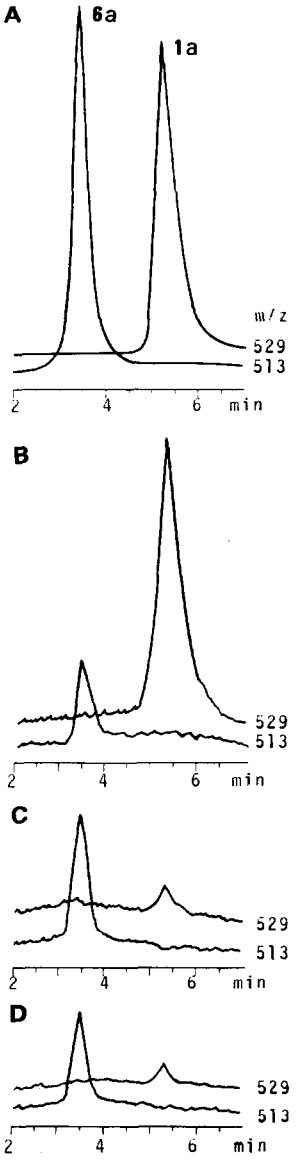
Bismethane-boronate	*M + 1*	*M + 1 − 18*	*M + 1 − 60*	C_{20}–C_{22} *fission*	C_{17}–C_{20} *fission*
Brassinolide (1)	529 (100)	511 (15.7)	469 (21.4)	373 (5.7) 155 (10.0)	345 (14.3)
Norbrassinolide (2)	515 (100)	497 (17.1)	455 (32.8)	373 (5.7) 141 (7.1)	345 (11.4)
Homobrassinolide (5)	543 (100)	525 (120)	483 (28.6)	373 (8.6) 169 (21.4)	345 (21.4)
Dolicholide (3)	527 (100)	509 (6.3)	467 (8.4)	373 (33.7) 153 (8.4)	345 (24.2) 183 (4.2)
Castasterone (6)	513 (100)	495 (1.0)	453 (20.2)	358 (2.1) 155 (2.1)	329 (8.5)
Brassinone (7)	499 (100)	481 (1.2)	439 (18.9)	358 (2.9) 141 (2.7)	329 (9.1)
Ethylbrassinone (10)	527 (100	509 (0.8)	467 (21.6)	358 (3.8) 169 (3.5)	329 (7.8)
Dolichosterone (8)	511 (100)	493 (2.1)	451 (20.0)	358 (4.2) 153 (4.2)	329 (8.4) 183 (23.2)

Application

The application of our microanalytical technique has already led to the identification of brassinolide (1) and castasterone (6) in three different kinds of plants, the immature seeds and sheaths of Chinese cabbage, *Brassica campestris* L. var. pekinensis, the leaves of green tea, *Thea sinensis*, and the insect galls of the chestnut tree, *Castanea crenata* L. Sieb. et Zucc., as shown in Fig. 7[22,27]. As reported previously[17,27], our further investigations into the identification of new brassinolide analogues also led us to detect brassinone (7), 28-norbrassinolide (2) and 24-ethylbrassinone (10) in the three plants, as shown in Fig. 8. The amounts of these brassinosteroids are summarized in Table III. The utility of the bismethaneboronates of brassinosteroids in structure determination and identification has also been verified by Takahashi and Yokota[19–21,24,28].

In the present study, we investigated brassinosteroids contained in both the insect galls and the leaves of *Distylium racemosum* Sieb. et Zucc., and the rice plant, *Oryza sativa* L. cv. Arborio J1.

The molecular ion at *m/z* 499 of the bismethaneboronate derivative of brassinone (7) can be effectively used for selected ion monitoring (SIM) using GC–CI-MS as shown in Fig. 9. The peak of the standard sample in Fig. 9A corresponds to 1.96 ng of the brassinone derivative (7a). About one-fifth of the less polar and more polar, active fractions obtained as described in the Experimental section were derivatized, respectively, as bismethaneboronate. Fig. 9B shows the presence of *ca.* 4 ng



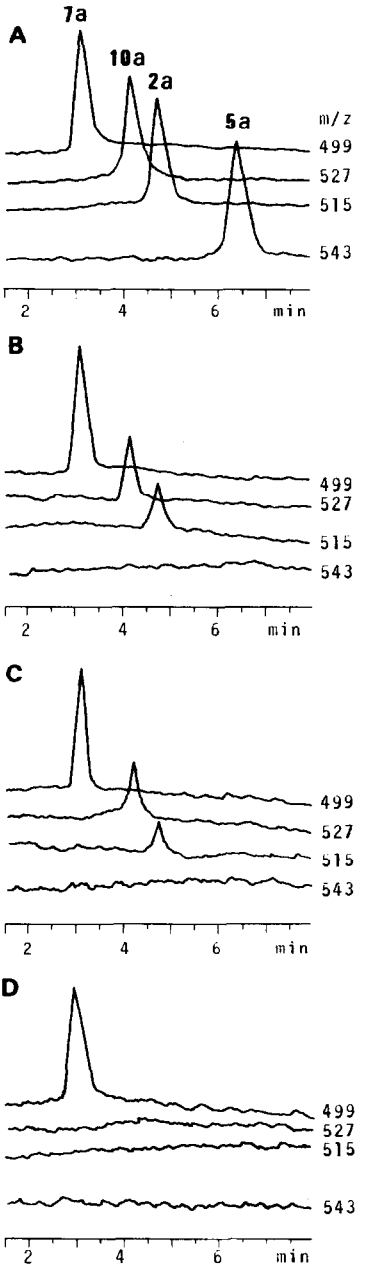


Fig. 7. Selected ion monitoring of brassinolide bismethaneboronate (1a) and castasterone bismethaneboronate (6a): A, standard mixture; B, Chinese cabbage; C, green tea; D, insect galls of chestnut tree.

Fig. 8. Selected ion monitoring of the bismethaneboronates of brassinone (7a), 24-ethylbrassinone (10a), 28-norbrassinolide (2a) and 28-homobrassinolide (5a). Key as in Fig. 7.

TABLE III

AMOUNTS OF BRASSINOSTEROIDS IN CHINESE CABBAGE, GREEN TEA AND THE INSECT GALLS OF *CASTANEA*

N.D. = Not detected.

Brassinosteroid	*Chinese cabbage (ng/kg)*	*Green tea (ng/kg)*	*Gall (Castanea) (ng/g)*
Brassinolide (1)	9.4	4.6	1.0
Norbrassinolide (2)	1.3	N.D.	N.D.
Homobrassinolide (5)	Trace	N.D.	N.D.
Castasterone (6)	1.6	110.0	11.0
Brassinone (7)	0.78	2.0	11.0
Ethylbrassinone (10)	0.13	Trace	N.D.

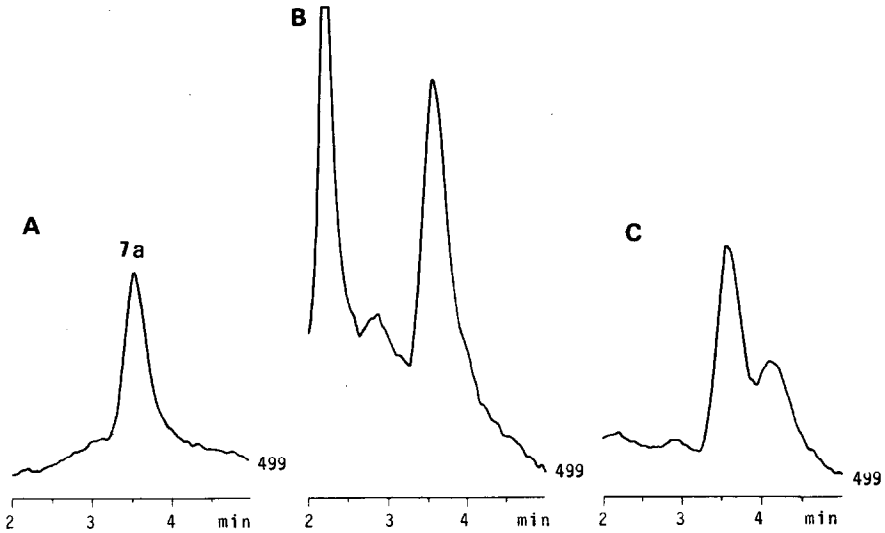


Fig. 9. Selected ion monitoring of the bismethaneboronate of brassinone (7a) in *Distylium racemosum*: A, standard; B, insect galls; C, leaves.

of 7a from a 2-μl injection of the derivatized fraction (50 μg) obtained from the insect galls of *Distylium racemosum*. As shown in Fig. 9C, *ca.* 2 ng of 7a were also detected from a 2-μl injection of the derivatized less polar fraction obtained from the leaves of *Distylium racemosum*. The retention times of those peaks coincided with that of standard 7a. These data clearly indicate that brassinone (7) is present in both the insect galls and the leaves of *Distylium racemosum*.

The molecular ion at *m/z* 513 of castasterone bismethaneboronate (6a) was also selected for the identification of castasterone (6) in both the insect galls and the leaves of *Distylium racemosum*. Fig. 10A shows the peak corresponding to *ca.* 20 ng of standard 6a. As shown in Fig. 10B and 10C, castasterone (6) was detected in the above derivatized less polar fractions. The amounts of 6a were *ca.* 2 and *ca.* 17 ng,

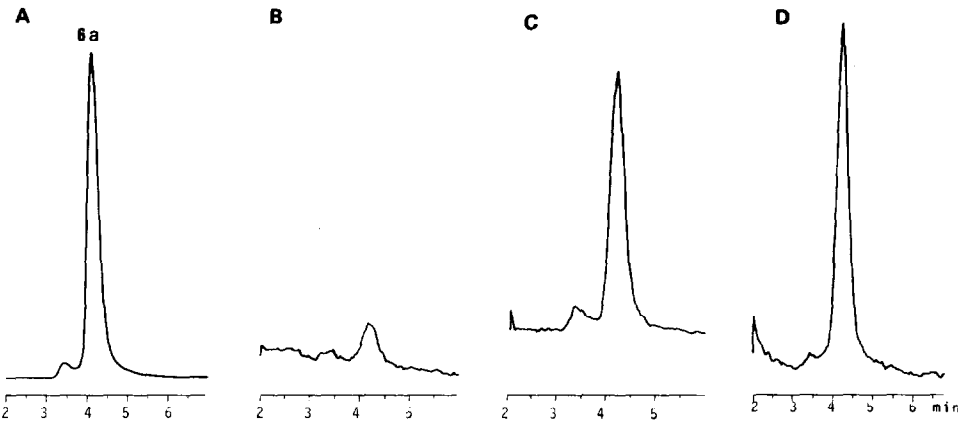


Fig. 10. Selected ion monitoring of the bismethaneboronate of castasterone (6a) in *Distylium racemosum* and green tea: A, stasndard; B, insect galls; C, leaves; D, green tea.

respectively, from 2-μl injections of the derivatized fraction (50 μl). Fig. 10D shows that castasterone (6) is present in the fraction from the leaves of green tea, *Thea sinensis*, obtained in our previous study[22]. The retention times of these peaks were completely identical with that of the standard peak.

One-half of the more polar, active fraction obtained from the leaves of *Distylium racemosum* was derivatized as bismethaneboronates. The molecular ions at *m/z* 515 of 28-norbrassinolide bismethaneboronate (2a) and at *m/z* 529 of brassinolide bismethaneboronate (1a) were selected for the identification of these steroids in the leaves of *Distylium racemosum*. Fig. 11A shows the separation of the derivatives (*ca.* 50 ng each). The natural 28-norbrassinolide (2) and brassinolide (1) in the derivatized fraction were detected as shown in Fig. 11B in amounts of *ca.* 20 and *ca.*

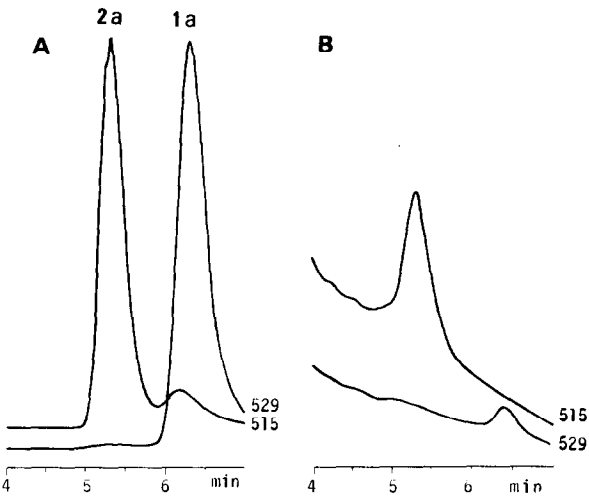


Fig. 11. Selected ion monitoring of the bismethaneboronates of 28-norbrassinolide (2a) and brassinolide (1a) in *Distylium racemosum*: A, standard; B, leaves.

TABLE IV

AMOUNTS OF BRASSINOSTEROIDS IN *DISTYLIUM* AND *ORYZA*

Brassinosteroid	*Distylium racemosum (ng/kg)*		*Oryza sativa (ng/kg)*
	Leaves	*Insect galls*	
Brassinolide (1)	23	–	–
Norbrassinolide (2)	156	–	–
Castastrone (6)	133	2500	13.6
Brassinone (7)	16	5000	–
Dolichosterone (8)	–	–	8.4

2 ng from a 2-μl injection of the 50-μl fraction. The retention times of 1a and 2a were identical with those of standard samples.

It should be noted that the amounts of castasterone (6) and brassinone (7) contained in the insect galls of *Distylium racemosum* are higher (*ca.* 20–300 times) than those in the leaves of the same plant, as shown in Table IV, and the lactone type brassinosteroids were not found in the insect galls but were found in the leaves.

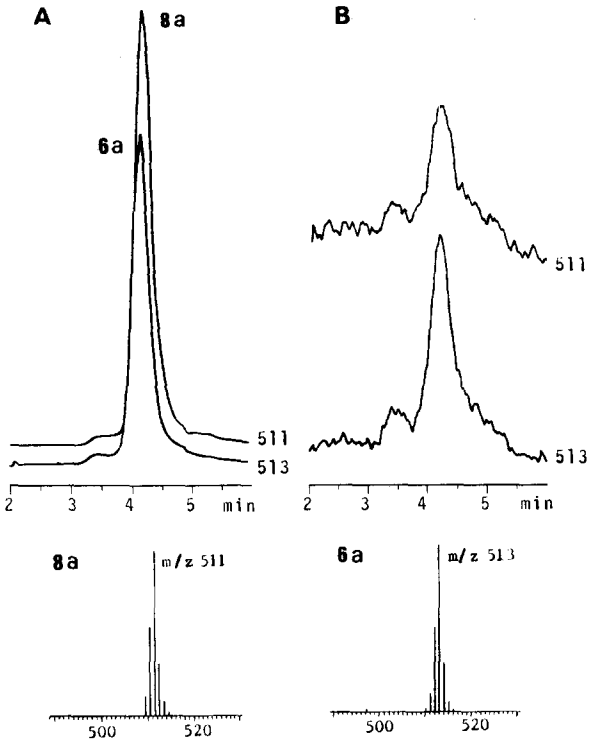


Fig. 12. Selected ion monitoring of the bismethaneboronates of dolichosterone (8a) and castasterone (6a), and the corresponding CI-mass spectra: A, standard mixture; B, rice plant.

These differences are quite interesting in connection with the chemical elucidation of the mechanism of gall formation in *Distylium racemosum*.

The brassinosteroids contained in the rice plant were analyzed by the same method. The active fraction obtained from the rice plant, *Oryza sativa*, was derivatized as bismethaneboronates. The molecular ions at *m/z* 511 of the derivative of dolichosterone (8) and at *m/z* 513 of that of castasterone (6) were selected for GC–CI-SIM to identify these steroids. The derivatives of synthetic 8 and 6 showed very similar retention times; 6a has a slightly shorter retention time than that of 8a as shown in Fig. 12A. GC–CI-SIM of these ions from the derivatized fraction showed the presence of dolichosterone (8) and castasterone (6) in amounts of *ca.* 2.3 and *ca.* 3.7 ng, respectively, for a 2-μl injection of the 50-μl fraction. Since the intensities of the isotopic ions at *m/z* 513 of dolichosterone bismethaneboronate (8a) and at *m/z* 511 of castasterone bismethaneboronate (6a) were extremely low, as shown in Fig. 12, the peaks at *m/z* 511 and *m/z* 513 in Fig. 11B could be assigned to the bismethaneboronates of dolichosterone (8) and castasterone (6), respectively. The results clearly indicate that both dolichosterone (8) and castasterone (6) are present in the rice plant in a ratio of *ca.* 2:3.

The amounts of these brassinosteroids in the plants were roughly estimated from the peak area, and are summarized in Table IV. The plant growth substances found in *Distylium racemosum* will be reported in detail elsewhere[29].

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