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MICROANALYSIS OF BRASSINOLIDE AND ITS ANALOGUES BY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

SUGURU TAKATSUTO, BAIPING YING, MASUO MORISAKI and NOBUO IKEKAWA*
Department of Chemistry, Tokyo Institute of Technology, Ohokayama, Meguro-ku, Tokyo 152 (Japan)

SUMMARY

The microanalysis of brassinolide, a new plant growth promotor, was investigated using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Bismethaneboronate was found to be a suitable derivative for analysis of brassinolide and its analogues by gas-phase analysis. GC and GC–MS analysis of the derivative are useful for identification and screening of brassinolide in plants.

INTRODUCTION

Recently a new plant growth-promoting steroid, brassinolide, has been isolated from rape pollen (*Brassica napus* L.). The structure was determined by mass spectrometry (MS) and X-ray crystallography as (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-6,7-seco-5α-cholestano-6,7-lactone (1)[1]. Brassinolide is especially noteworthy because it is the first natural steroid containing a seven-membered *B*-ring lactone and an α_F configuration at the C_{22} position. The synthesis of brassinolide has been achieved by us[2] and by Siddall *et al.*[3] and its analogues have also been synthesized by three groups[4–6]. Brassinolide promotes both cell elongation and cell division, resulting in curvature, swelling and, more dramatically, splitting of the internode in the bean second-internode bioassy[1] and shows a strong activity in the lamina inclination assay at very low concentration[7]. Brassinolide also exhibits a broad spectrum of biological activities, compared with the known plant hormones[8,9]. The presence of brassinolide-like substances has been demonstrated in other pollens[10] and also in the leaves of *Distylium racemosum*[11].

The remarkable biological activities and the very small amounts contained in plants prompted us to develop a microanalysis and a screening method for brassinolide and its analogues. Thus we investigated analysis by gas chromatography (GC) and GC–MS.

EXPERIMENTAL

Samples and reagents

The following standard samples were synthesized in this laboratory: brassi-

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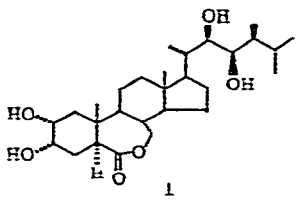

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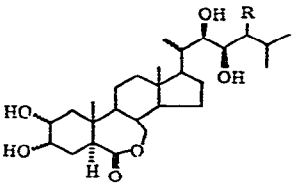
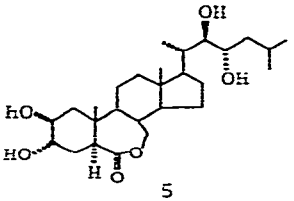


2 R = β-CH_3

3 R = α-C_2H_5

4 R = H



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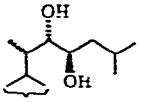

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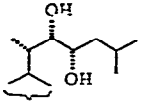

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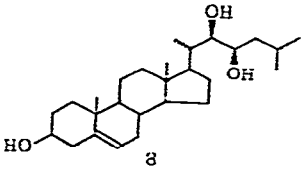

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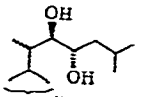

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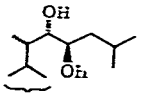

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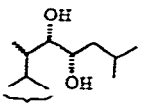

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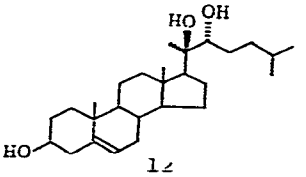

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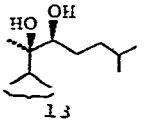

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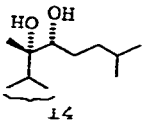

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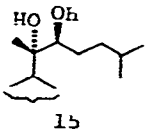

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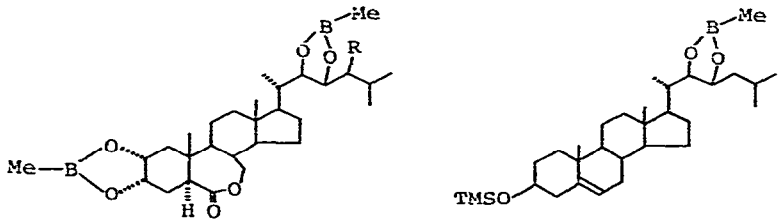
nolide (1)[2]; 24-epibrassinolide (2)[4]; (22*R*,23*R*)-(4); (22*R*,23*S*)-(5), (22*S*,23*R*)-(6) and (22*S*,23*S*)-28-norbrassinolide (7)[6]; (22*R*,23*R*)-(8), (22*R*,23*S*)-(9), (22*S*,23*R*)-(10) and (22*S*,23*S*)-dihydroxycholesterol (11)[6]; (20*R*,22*R*)-(12), (20*R*,22*S*)-(13), (20*S*,22*R*)-(14) and (20*S*,22*S*)-dihydroxycholesterol (15)[13]. (22*S*,23*S*)-28-Homobrassinolide (3)[5] was supplied by Dr. Wada, Nagoya University.

Methaneboronic acid was obtained from Alfa Products, Ventron Corporation, and trimethylsilylimidazole was from Tokyo Kasei.

Derivatization

Bismethaneboronate. Methaneboronic acid (100 μg) was dissolved in 50 μg of dry pyridine and this solution was added to 100 μg of brassinolide. The mixture was heated at 60°C for 30 min. Several microlitres of this solution were injected into the gas chromatograph.

Methaneboronate trimethylsilyl ether. The triol (100 μg) was converted into monomethaneboronate as described above. To this reaction mixture 30 μl of trimethylsilylimidazole were added and, after allowing to stand at room temperature for 30 min, several μl of this solution were injected into the gas chromatograph.



GC analysis

A Shimadzu Model GC-7A chromatograph equipped with dual hydrogen flame-ionization detector was employed. A glass capillary column coated with OV-17 (SCOT column) (40 m × 0.25 mm) was used at 270°C. The carrier gas (nitrogen) flow-rate was 0.4 ml/min, and the split ratio was 100:1. A U-shaped column packed with 2% OV-17 on Chromosorb W (80–100 mesh) (150 cm × 4 mm I.D.) was also used.

GC–MS analysis

A Shimadzu GC–MS 6020 gas chromatograph–mass spectrometer with electron impact (EI), 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, 60 μA; 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 was 30 ml/min; electron energy, 150 eV; box current, 150 μA; acceleration high voltage, 3.5 kV; ion source temperature 250°C.

RESULTS AND DISCUSSION

GC analysis

Since brassinolide has two vicinal diols in the side chain and A-ring, a methaneboronate[12] seems to be the best derivative for GC analysis. The bismethaneboronate of brassinolide and its analogues exhibited sharp peaks and the derivatives can be separated as shown in Fig. 1. Brassinolide and its 24-epimer can also be separated by glass capillary column (Fig. 2). The compounds can be analysed using a packed column, but a capillary column afforded better resolution and sharper peak. It was

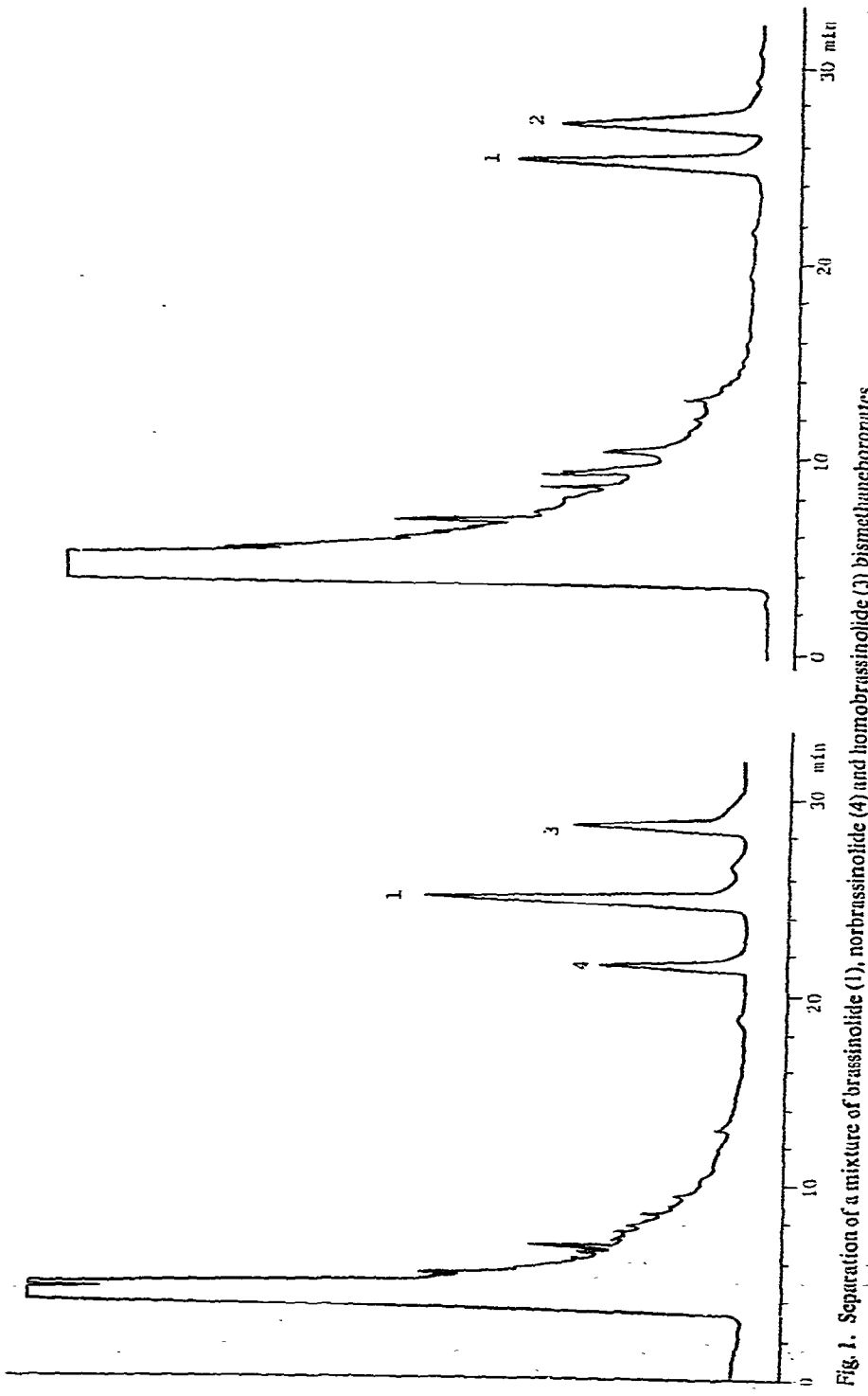


Fig. 1. Separation of a mixture of brassinolide (1), norbrassinolide (4) and homobrassinolide (3) bismethaneboronates.

Fig. 2. Separation of a mixture of brassinolide (1) and 24-epibrassinolide (2) bismethaneboronates.

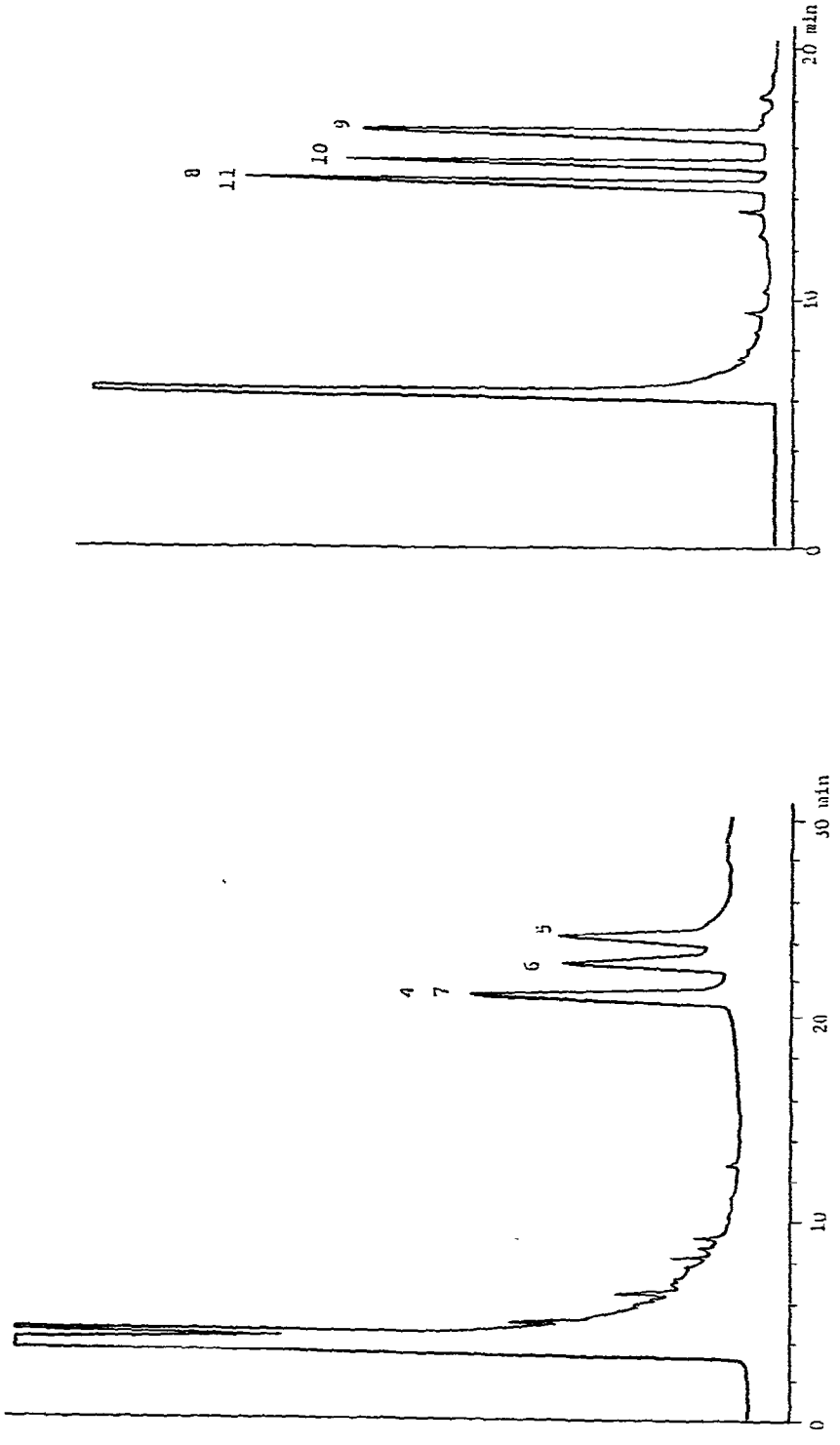


Fig. 3. Separation of four isomers of norbrassinolide bismethaneboronate.

Fig. 4. Separation of four isomers of 22,23-dihydroxycholesterol methaneboronate trimethylsilyl ether.

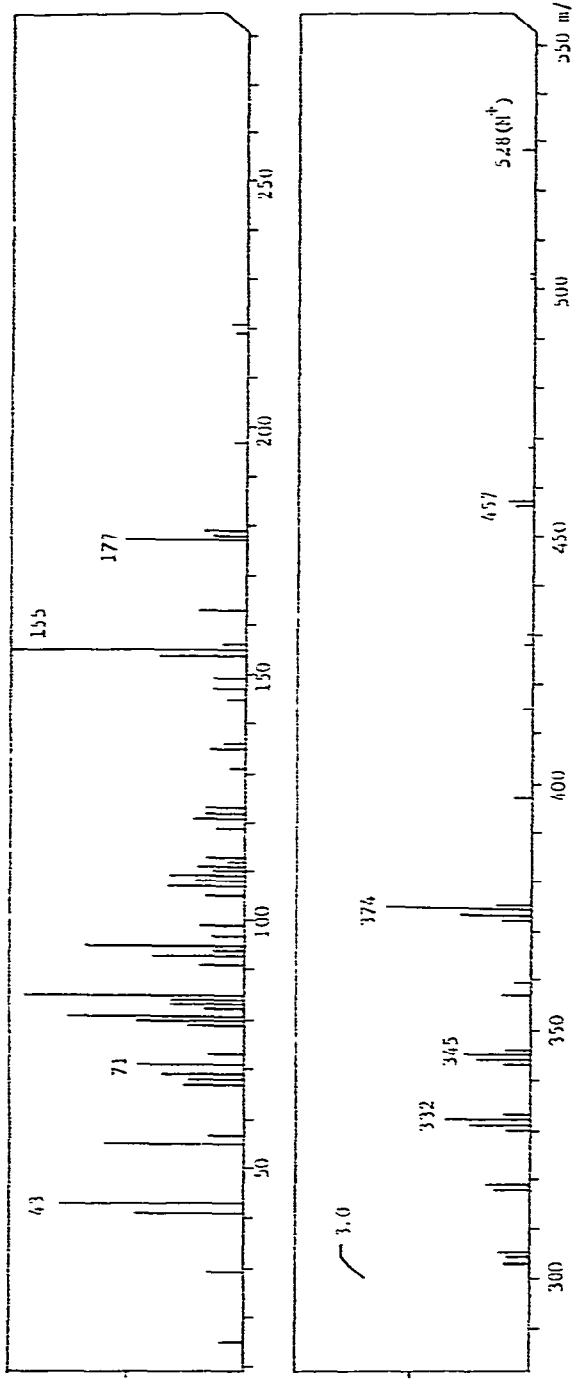


Fig. 5. EI Mass spectrum of brassinolide bismethaneboronate.

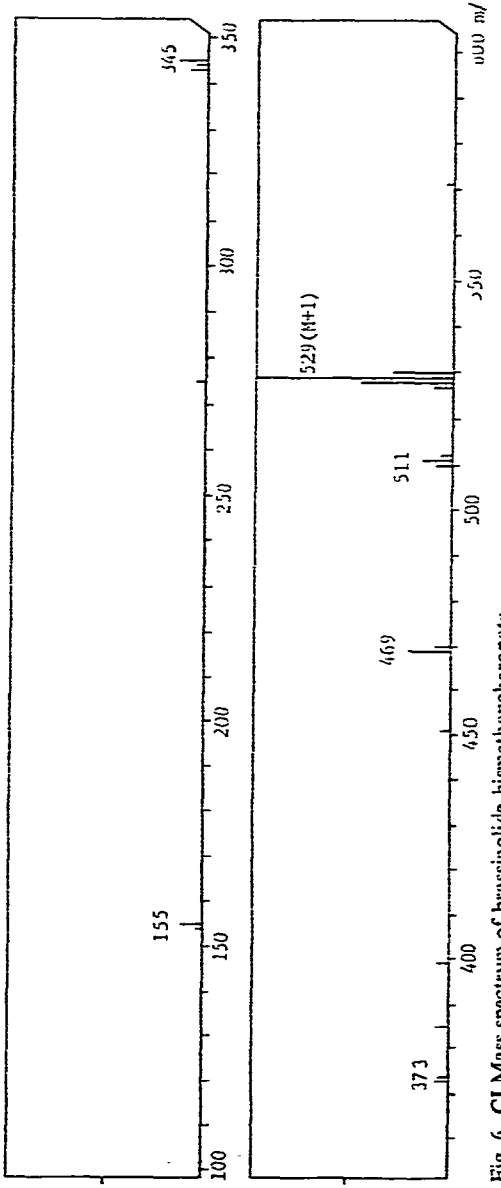


Fig. 6. CI Mass spectrum of brassinolide bismethaneboronate.

TABLE I

RETENTION TIMES OF DERIVATIVES OF BRASSINOLIDE AND ITS ANALOGUES

Compound	*Packed column (min)*	*Capillary column (min)*
Bismethaneboronate		
Brassinolide (1)	24.5	24.67
24-Epibrassinolide (2)	27.0	26.50
Homobrassinolide (3)	29.0	28.08
(22*R*,23*R*)-Norbrassinolide (4)	20.5	20.90
(22*R*,23*S*)-Norbrassinolide (5)	24.0	23.84
(22*S*,23*R*)-Norbrassinolide (6)	22.5	22.48
(22*S*,23*S*)-Norbrassinolide (7)	20.5	20.90
Methaneboronate trimethylsilyl ether		
(22*R*,23*R*)-Dihydroxycholesterol (8)		14.41
(22*R*,23*S*)-Dihydroxycholesterol (9)		16.46
(22*S*,23*R*)-Dihydroxycholesterol (10)		15.17
(22*S*,23*S*)-Dihydroxycholesterol (11)		14.41
(20*R*,22*R*)-Dihydroxycholesterol (12)		15.94
(20*R*,22*S*)-Dihydroxycholesterol (13)		15.49
(20*S*,22*R*)-Dihydroxycholesterol (14)		15.07
(20*S*,22*S*)-Dihydroxycholesterol (15)		15.94
Column	1.5% OV-17	OV-17
Column temperature (°C)	290	270
Nitrogen flow-rate (ml/min)	40	0.4

clear that the methaneboronate was a better derivative than the trimethylsilyl ether for GC analysis.

Separation of the four possible isomers at the C_{22} and C_{23} positions of norbrassinolide was investigated. Although (22*R*,23*S*)- (5) and its (22*S*,23*R*)-isomer (6) can be separated, (22*R*,23*R*)- (4) and its (22*S*,23*S*)-isomer (7) have very close retention times, as shown in Fig. 3. Similar behaviour was observed in the analysis of the four isomers of 22,23-dihydroxycholesterol (8–11) as their methaneboronate trimethylsilyl derivatives (Fig. 4). In the case of 20,22-dihydroxycholesterols (12–15), the (20*R*,22*S*)- (13), (20*R*,22*R*)- (12) and (20*S*,22*R*)-isomers (14) could be separated, but the (20*S*,22*S*)-isomer (15) had a retention time close to that of the (20*R*,22*R*)-isomer (12) (all as their boronate trimethylsilyl derivatives). The retention times of these derivatives on packed and capillary columns are listed in Table I.

GC–MS analysis

The EI mass spectrum of bismethaneboronate of brassinolide is shown in Fig. 5. The derivatives of brassinolide (1), homobrassinolide (3) and norbrassinolide (4) afforded molecular ions at *m/z* 528, 542 and 514, respectively, an ion at *m/z* 457 resulting from C_{23}–C_{24} fission and a strong ion at *m/z* 345 resulting from C_{17}–C_{20} fission. Thus, the fragment ions *m/z* 457, 374, 345 and 177 are common peaks for brassinolide skeleton. The peak at *m/z* 374 was accompanied by hydrogen transfer. The ions at *m/z* 155, 169 and 141 corresponding to the side-chain cleavage are base peaks in those spectra. The derivatives afforded characteristic ions for a *B*-ring lac-

TABLE II

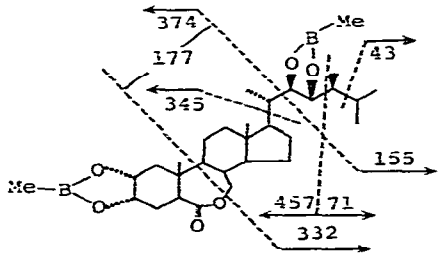
CHARACTERISTIC FRAGMENT IONS OF BRASSINOLIDE BISMETHANEBORONATE (EI-MS)

Compound (bismethaneboronate)	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 (4)	514 (2.5)	457 (0.83)	374* (10.4) 141 (100)	345 (5.8)	318 (27.9) 177 (57.5)
Homobrassinolide (3)	542 (0.90)	457 (6.3)	374* (22.5) 169 (100)	345 (16.2)	346 (5.9) 177 (47.3)

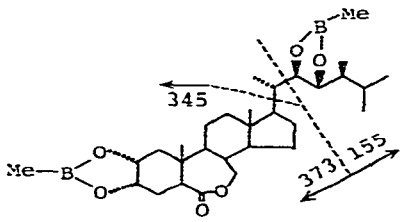
* H-Transfer.

tone at *m/z* 332, 346 and 318, respectively. These characteristic ions may be useful for structural determination of brassinolide analogues.

The CI mass spectrum of the derivative of brassinolide is shown in Fig. 6. The ions corresponding to $M + 1$ at 529, 543 and 515 are base peaks for brassinolide (1), homobrassinolide (3) and norbrassinolide (4), respectively. These derivatives also gave side-chain cleavage ions at *m/z* 345 (C_{17}–C_{20} fission), and *m/z* 155, 169 and 141, together with ions at *m/z* 373 (C_{20}–C_{22} fission). The ions at *m/z* 345 and 373 are common for the brassinolide skeleton. These ions are useful for selected ion monitor-



EI-MS



CI-MS

TABLE III

CHARACTERISTIC FRAGMENT IONS OF BRASSINOLIDE BISMETHANEBORONATE (CI-MS)

Compound (bismethaneboronate)	$M + 1$	$M + 1 - 18$	$M + 1 - 60$	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 (4)	515 (100)	497 (17.1)	455 (32.8)	373 (5.7) 141 (7.1)	345 (11.4)
Homobrassinolide (3)	543 (100)	525 (12.0)	483 (28.6)	373 (8.6) 169 (21.4)	345 (21.4)

ing for screening of brassinolide analogues. Thus, picogram amounts of brassinolide can be detected in plants by means of GC–CI-MS. The characteristic fragment ions of brassinolide derivatives are listed in Tables II and III. The mass spectra of free brassinolide and its analogues will be discussed in a future paper.

ACKNOWLEDGEMENTS

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