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2-DEOXYBRASSINOLIDE, A BRASSINOSTEROID FROM *PISUM SATIVUM* SEED

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Key Word Index—*Pisum sativum*; Leguminosae; garden pea; seed; brassinosteroids; 2-deoxybrassinolide; mass spectrum.

Abstract—GC-MS analysis of fully grown seed of *Pisum sativum* revealed the occurrence of a new brassinosteroid, 2-deoxybrassinolide, (22*R*, 23*R*, 24*S*)-3α, 22,23-trihydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one, together with known brassinosteroids, brassinolide, castasterone, typhasterol and 6-deoxocastasterone. In addition, the presence of a brassinolide with an extra hydroxyl group and an epimer of 6-deoxocastasterone was indicated although their structures could not be clarified.

INTRODUCTION

In *Pisum sativum* (garden pea), it was revealed by using a variety of gibberellin biosynthesis and/or response mutants that gibberellins play important roles in the growth of peas [1, 2]. It has been reported that exogenously applied brassinoide elongates pea tissue [3], indicating that endogenous brassinosteroids (BRs) should be also involved in the growth of pea. We have already reported that BRs are endogenous in pea shoots and their synthesis was suppressed by uniconazol, a growth retardant [4]. In the present work, we have further investigated on the endogenous BRs which are considered to be important in the fruit growth.

RESULTS

Figure 1 shows the separation of endogenous BRs in fully grown pea seed by reverse-phase HPLC as monitored by the rice lamina inclination bioassay. The HPLC fractions were analysed by GC–mass spectrometry after conversion into methaneboronates or methaneboronate-trimethylsilyl (TMSi) ethers resulted in the identification of various BRs (Table 1). Among them, three BRs, namely brassinolide (fractions 14/15), castasterone (fractions 20/21) and 6-deoxocastasterone (fractions 36/37 and 38/40) were identified by full-scan mass spectra and Kovats retention indices. Typhasterol was identified by GC–selected ion monitoring (SIM) where ions characteristic of typhasterol methaneboronate-TMSi ether were observed at *m/z* 544 $[M]^+$, 529, 515, 454 and 439 with a relative intensity ratio of 10:5:9:7:3. In addition, an epimer of 6-deoxocastasterone, which showed a mass spectrum similar to that of 6-deoxocastasterone, but had a different GC retention time, was found in the combined fractions 38–40. The position of the epimerization remained undetermined.

A BR in fraction 32 was a new compound. This was converted into a methaneboronate-TMSi ether, which exhibited the molecular ion at *m/z* 560.4088 (calc. for $C_{32}H_{57}O_5BSi$: 560.4107). A *m/z* 177 ion strongly indicated the presence of a 7-oxa-6-one (lactone) structure in the B-ring. In addition, a peak at *m/z* 155 was suggestive of the presence of 22, 23-vicinal hydroxyls [5]. The above spectral evidence suggests that this compound may be 2-deoxybrassinolide (**1**) or its 3-epimer. By direct GC-mass spectral comparison with the synthetic standards [6] supplied from Dr H. Abe, the new BR was rigorously identified with 2-deoxybrassinolide, (22*R*, 23*R*, 24*S*)-3α, 22, 23-trihy-

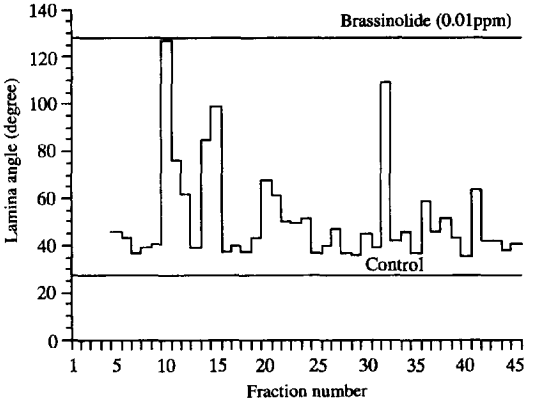


Fig. 1. Separation of endogenous brassinosteroids in pea seed by reverse-phase HPLC. Extracts equivalent to 30 g fr. wt tissue were bioassayed by rice lamina inclination test.

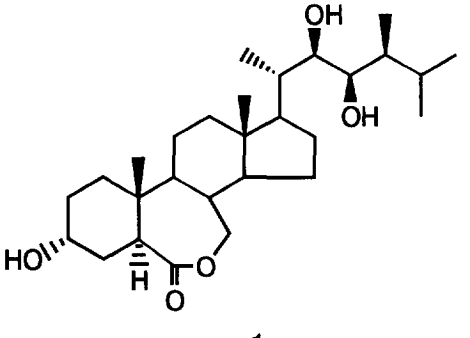
Table 1. GC-MS data for endogenous brassinosteroids in *P. sativum*

HPLC fractions	Brassinosteroid present	Derivative	R_t (min)*	KRI*	Fragment ions (rel. int.)†
10	ξ-Hydroxybrassinolide (tentative)	MB-TMSi	14.27	3783	616(M^+, 46), 601(44), 559(100), 556(56), 526(29), 500(29), 375(33), 212(50), 194(65), 155(73), 145(90)
14/15	Brassinolide	MB	13.32	3723 (3724)	528(M^+, 11), 457(13), 397(14), 374(47), 345(36), 332(34), 319(20), 177(88), 163(36), 155(100)
20/21	Castasterone	MB	11.87	3618 (3618)	512(M^+, 81), 399(15), 358(38), 287(33), 155(100)
32	2-Deoxybrassinolide (**1**)	MB-TMSi	13.83	3756 (3757)	560(M^+, 9), 545(30), 531(17), 490(16), 470(8), 440(5), 375(5), 332(8), 195(49), 177(19), 156(100), 155(17)
36/37	Typhasterol	MB-TMSi	11.12 (11.08)	n.d.	Determined by SIM (see text)
	6-Deoxocastasterone	MB	9.77	3433 (3429)	498(M^+, 58), 483(20), 455(3), 288(18), 273(100), 205(19), 155(27)
38–40	6-Deoxocastasterone	MB	9.72	3430 (3429)	Same as the above
	6-Deoxocastasterone epimer	MB	10.02	3460	498(M^+, 100), 483(20), 455(6), 273(98), 205(17), 155(30), 288(21),
Authentic	3-epi-2-deoxybrassinolide	MB-TMSi	16.30‡	3915	559($[M-1]^+$, 1), 545(100), 531(5), 169(19), 156(22)

MB = methaneboronate; KRI = Kovats retention index; n.d. = not determined.
*Data for the authentic sample are shown in parentheses.
†The fragment ions of the authentic sample identical with the endogenous BR are not shown.
‡This R_t was recorded in a separate experiment.

droxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (Table 1). The possible fragmentations of **1** methaneboronate-TMSi ether are illustrated in Fig. 2. Unexpectedly, its 3-epimer gave a very different mass spectrum in which a $[M-1]^+$ ion (m/z 559) was observed instead of $[M]^+$, and a $[M-15]^+$ ion (m/z 545) was the base peak (Table 1).

The HPLC fraction 10 was biologically very active (Fig. 1) and, on the basis of the retention time, expected to contain a BR more polar than brassinolide. In GC-mass spectrometry analysis, a compound with a large retention time was detected (Table 1). The molecular ion (m/z 616) allowed us to speculate that it was a bismethaneboronate-TMSi ether of brassinolide with an extra hydroxyl group. The presence of a m/z 155 ion indicates that this compound has the same side chain as brassinolide [5], and, therefore, the hydroxyl may be present in the steroidal ring structure. For further structural analysis of this compound, we are now trying to isolate it in the pure state, using a large amount of the pea seeds.



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DISCUSSION

Recently, it was demonstrated that a biosynthetic sequence of teasterone → typhasterol → castasterone → brassinolide is present in normal and crown gall cells of *Catharanthus roseus* [7] and also in intact seedlings of tobacco, rice and *C. roseus* [8]. Thus, it is considered that the lactonization of the B-ring occurs after the introduction of 2α-hydroxyl. However, the co-occurrence of **1** and brassinolide in pea seed shows that the lactonization of the B-ring can occur both before and after the introduction of the 2α-hydroxyl. Nonetheless, **1** has not been found in a number of other plants which were examined for the endogenous BRs, indicating that

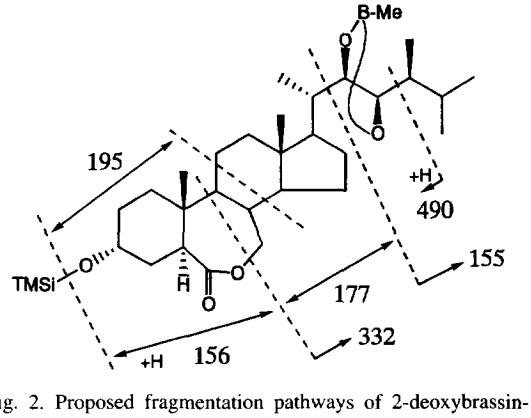

Fig. 2. Proposed fragmentation pathways of 2-deoxybrassinolide (**1**) bismethaneboronate-TMSi ether.

the lactonization before the introduction of 2α-hydroxyl may not be a major pathway in most of the plants.

In our earlier work on pea shoot, castasterone was identified as the major BR, being responsible for most of the biological activity in the tissue [4]. The present work revealed that pea seed contains a variety of BRs, indicating that the biosynthesis and metabolism of BRs in seed are more complicated than those in shoot. The same conclusion has been drawn from the comparative analysis of seed and shoot of *Phaseolus vulgaris* [9].

EXPERIMENTAL

Plant materials. Fully expanded green seeds of *P. sativum* cv. Kurumeyutaka grown in Chiba Prefecture were purchased in Tokyo. The average wt of one seed was 0.83 g.

Bioassay. Seed of *Oryza sativa* L. Koshihikari were germinated by immersing in H_2O for 2 days at 30° under continuous light. The germinated seeds were placed on a plastic net, which was floated on tap-water in a plastic container with a lid and incubated for 6 or 7 days in the dark with 1 hr red light illumination per day. Rice leaf explants obtained by cutting about 1 cm below the lamina joint were preincubated in a Petri dish (20 cm i.d.) containing H_2O. After 1 day, explants which bent *ca* 30° were selected and 10 of them were placed in a Petri dish (5 cm i.d.) containing a test soln (5 ml). After incubation in the dark for 2 days at 30°, bending angles were measured.

Extraction and partitioning. The seeds (1 kg) were extracted (2×) with MeOH by using a Polytron homogenizer. The extracts were combined and reduced to an aq. residue *in vacuo* prior to partitioning (3×) between $CHCl_3$ and H_2O. The $CHCl_3$ phases were combined and, after evapn of the solvent, partitioned between 80% MeOH and hexane. The 80% MeOH phase was concd to dryness (4.2 g).

Chromatographic purification. The above extract was purified on a column of silica gel (20 g, Wako gel C300), which was eluted with $CHCl_3$, $CHCl_3$ containing 1, 3, 5, 7, 10, 20 and 50% MeOH and then pure MeOH (each fr., 300 ml). The eluates with $CHCl_3$ containing 3, 5 and 7% MeOH, which were biologically active, were purified on a column of charcoal (4 g) packed with 60% aq. MeOH. The charcoal was beforehand successively washed with 80% aq. MeOH, MeOH and MeOH–$CHCl_3$ (1:1) and air-dried. The column was eluted with MeOH–H_2O (3:2, 4:1), MeOH, MeOH–$CHCl_3$ (9:1, 7:3, 1:1, 3:7) (each fr., 60 ml). The biologically active last 3 frs were combined and purified on a column of Sephadex LH-20 (bed vol., 500 ml) eluted with MeOH–$CHCl_3$ at 30 ml min^{-1} (each fr. 10 ml). The biologically active frs 32–38 were combined (16 mg) and purified on a Senshu Pak ODS-3251 D column (250 × 8 mm) at 40°. Elution solvent (flow rate, 1.5 ml min^{-1}) was: 0–20 min, CH_3CN–H_2O (11:9); 20–40 min, gradient to CH_3CN; 40–45 min, CH_3CN.

GC-MS analysis. A JEOL GC-MS model JMS-AX505W (ionization voltage, 70 eV) equipped with a J & W DB-1 capillary column (15 m × 0.25 mm; 0.25 mm film thickness). Samples were introduced by a splitless method (sampling time, 1.5 min). Injection port temp. was 270° and the carrier He flow rate was 1 ml min^{-1}. The column oven temp. was maintained at 170° for the first 1.5 min, elevated to 280° at 37° min^{-1} and then to 300° at 1.5° min^{-1}. Samples were converted into methaneboronates and analysed by GC-MS. The methaneboronates were further trimethylsilylated with bis(trimethylsilyl)acetamide [5] and again analysed by GC-MS. Kovats retention indices [10] were obtained using retention times measured by co-injecting a derivatized sample soln and a paraffin (mp 68–70°, Kanto Chemical Co.) soln in toluene.

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