Identification of 24-Epibrassinolide in Bee Pollen of the Broad Bean, *Vicia faba* L.

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24-Epibrassinolide (II), a C-24 epimer of naturally occurring brassinolide, was identified, together with brassinolide (I), brassinone (III) and castasterone (IV), from bee pollen of the broad bean, *Vicia faba* L. by means of the selected ion monitoring technique in gas chromatography-mass spectrometry and the rice-lamina inclination assay.

Since the first isolation of a steroidal plant-growth promoter, brassinolide (I)²⁾ from rape pollen, a number of brassinolide analogues (brassinosteroids) have been synthesized³⁾ in order to obtain information on the structure–activity relationships⁴⁾ and to search for more active analogues for application on agriculture.⁵⁾ Extensive investigations have also been carried out to search for new brassinosteroids and more than 20 brassinosteroids have been characterized in the plant kingdom.⁶⁾

Our attention has been focussed on developing a useful micro-analytical method for the screening of brassinosteroids. We were able to establish a combined system consisting of the selected ion monitoring technique in gas chromatography–mass spectrometry (GC-MS) applied to the methaneboronate derivatives (for example I_{MB}) of brassinosteroids⁷⁾ and a highly sensitive and specific rice-lamina inclination assay. By the use of these techniques we previously identified a number of new brassinosteroids.⁸⁾

As a continuation of our program for the identification and characterization of new brassinosteroids in plant sources we have screened brassinosteroids of some bee pollens. In this paper, we describe the identification of 24-epibrassinolide (II) from bee pollen of the broad bean, *Vicia faba* L. (soromame in Japanese). This brassinosteroid has not yet been encountered in plants, although it has been synthesized.^{9,10)}

A crude brassinosteroid fraction was obtained from a methanol extract of the bee pollen by a slight modification of the published procedure.¹¹⁾ Thus, the methanol extract was

partitioned between benzene and water. The water layer was then extracted with chloroform. The combined organic extracts were again partitioned between hexane and methanol. The methanol fraction was chromatographed on a silica gel column with chloroform-methanol (10:1) as an eluting solvent. Final separation by silica gel preparative thin layer chromatography with chloroform-methanol (7:1) as the developing solvent afforded the crude brassinosteroid fraction. The fraction was derivatized into bismethaneboronate and analyzed by GC-MS using a high-resolution selected ion monitoring technique.

Under our gas chromatographic conditions, authentic bismethaneboronate samples (I_{MB} and II_{MB}) of brassinolide and 24-epibrassinolide⁹⁾ were eluted at 10 min 52 s and 11 min 6 s, respectively. The selected ion monitoring of the sample from the pollen is illustrated in Fig. 1. Monitoring of an ion at m/z 374.2628 is shown in Fig. 1A and 1B. This ion is due to the C_{20} - C_{22} cleavage and loss of a hydrogen, and is specific to a type of brassinosteroids having a B-ring lactone moiety.⁷⁾ Monitoring of an ion at m/z 457.2933 (C_{23} - C_{24} fission) showed parallel results (data not shown). Further, the relative intensity (6.0) of the m/z 374/457 ions for the peak at 11 min 6 s was in good agreement with that of authentic 24-epibrassinolide. Thus, it is evident that both brassinolide (I) and 24-epibrassinolide (II) are present in the pollen. The amounts of the two compounds were calculated to be at least 190 μ g (I) and 5 μ g (II)/kg of the original pollen by the use of authentic samples for calibration.⁷⁾ In addition to the above two brassinosteroids, the occurrence of brassinone (III) and castasterone (IV) was indicated, as can be seen in Fig. 1C, by monitoring an ion at m/z 358.2679 (C_{20} - C_{22} fission-H) which is characteristic of brassinosteroids with a 6-ketone moiety such as castasterone.⁷⁾

So far, 24-epibrassinosteroids have been characterized only in two instances; 24-

Fig. 1. Selected Ion Monitoring of Bismethaneboronate Derivatives of Brassinosteroid Fraction

A: Detection of brassinosteroids with a B-ring lactone moiety by monitoring at m/z 374.2628.

B: Expansion of the pertinent peaks corresponding to brassinolide and 24-epibrassinolide in A.

C: Detection of brassinosteroids with a 6-ketone moiety by monitoring at m/z 358.2679.

Peaks I_{MB} , II_{MB} , III_{MB} , and IV_{MB} refer to bismethaneboronate derivatives of brassinolide, 24-epibrassinolide, brassinone, and castasterone, respectively.

epicastasterone from a fresh water green alga, *Hydrodictyon reticulatum*¹²⁾ and 3,24-diepicasterone from immature seeds of *Phaseolus vulgaris*.¹³⁾ Identification of 24-epibrassinolide by the present study is most remarkable in the following respect. This steroidal hormone was found to display a high activity comparable to that of natural brassinolide in several bioassays.¹⁴⁾ Further, it has recently been shown in certain cases that the 24-epibrassinolide increases crop production more effectively than natural brassinolide.¹⁵⁾ Furthermore, this (24*R*)-steroid can be conveniently prepared from easily available brassicasterol⁹⁾ or ergosterol.¹⁰⁾ Thus, 24-epibrassinolide seems to be one of the most promising brassinosteroids for agricultural use. In conclusion, the present work demonstrates for the first time the occurrence of 24-epibrassinolide in the plant kingdom.

Experimental

One kilogram of the pollen, obtained from China, was extracted with methanol (4 l × 3 times). The concentrated methanol extract (500 g) was partitioned between benzene (2 l × 2 times) and water (2 l). The water layer was extracted with chloroform (2 l). The organic layers were combined and concentrated. The residue was partitioned between methanol and hexane. The methanol layer was concentrated (9.2 g) and applied on a silica gel (120 g) column. Elution with chloroform-methanol (10:1) afforded the active fraction (144 mg). Half of the fraction was then separated by preparative thin layer chromatography (Merck Silica gel 60 F₂₅₄ pre-coated plate 20 × 20 cm², 0.5 mm thickness, developed with chloroform-methanol (7:1)). The band with *R_f* 0.47–0.59 (authentic brassinolide and castasterone were contained in this region) afforded the crude brassinosteroid fraction (8.8 mg). Half of the fraction was mixed with 50 μl of methaneboronic acid-pyridine solution [methaneboronic acid (10 mg) was dissolved in 1.0 ml of dry pyridine]. The micro test tube was capped and heated at 70 °C for 30 min. This sample was, after cooling, injected into GC-MS system (EI-MS mode) through a split/splitless injector [VG A 70-S gas chromatograph-mass spectrometer (VG Analytical Ltd.), OV-1 capillary column 12.5 m × 0.2 mm i.d., programmed oven temperature 110→320 °C (initial 1 min hold and then increased at 25 °C/min), injector temperature 285 °C].

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