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# IDENTIFICATION AND QUANTIFICATION OF BRASSINOLIDE-RELATED STEROIDS IN THE INSECT GALL AND HEALTHY TISSUES OF THE CHESTNUT PLANT

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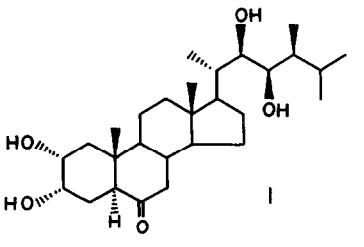
**Key Word Index**—*Castanea crenata*; Fagaceae; chestnut; insect gall; steroidal growth promotors; castasterone; 6-deoxocastasterone; brassinolide; brassinone.

**Abstract**—Castasterone and 6-deoxocastasterone were identified by GC/MS and/or selected ion monitoring not only in the insect gall of chestnut but also in the healthy tissues, including the shoot, leaf and flower bud. In addition, the gall was found to contain a small amount of brassinolide. Brassinone, which had been reported to be present in the gall, could not be detected in these tissues. The contents of castasterone, 6-deoxocastasterone and brassinolide, which were determined by using selected ion monitoring, are discussed.

## INTRODUCTION

Brassinolide (**3**) was first isolated from rape pollen as a steroidal growth promotor [1]. Since then, as many as 11 steroids with such activity have been isolated from several plant sources (for example, see refs [2–4] and refs cited therein). The insect gall of the chestnut tree (*Castanea crenata* Sieb. et Zucc.) is one of the sources in which strong brassinolide-like activity is observed. We have isolated castasterone (**1**) as the major active principle and determined its structure [5]. On the other hand, the occurrence of brassinolide (**3**) and brassinone (28-norcastasterone, **4**) in the gall has been demonstrated by Ikeda *et al.* [6] and Abe *et al.* [4], respectively, on the basis of selected ion monitoring (SIM) using the chemical ionization technique [7].

We have recently investigated the growth-promoting steroids present in the gall, larvae excised from the gall, shoot, leaf and flower bud obtained from resistant and/or susceptible (to chestnut wasp infestation) varieties of the chestnut plant. The results obtained are discussed in this paper. The isolation procedure of castasterone, which has only been preliminarily reported [5], is also described in detail.

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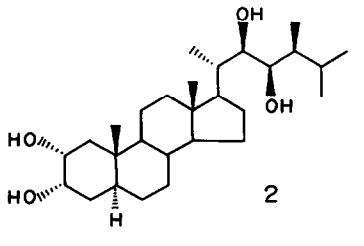


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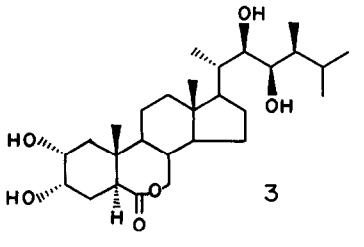


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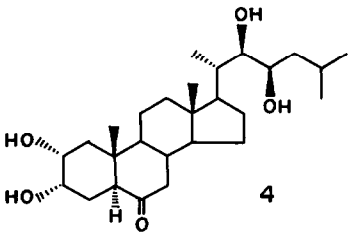


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## RESULTS

### *Isolation of castasterone from chestnut gall*

The distribution of brassinolide-like activity after each purification step was examined by the rice-lamina inclination test [8, 9]. The methanol extract obtained from the galls collected from an unidentified susceptible variety was partitioned between benzene and water. The benzene fraction, which contained *ca* 99% of the original activity, was again partitioned between hexane and 90% methanol. The latter was purified twice by silica gel chromatography with different solvent systems. Further purification by silica gel and reversed-phase HPLC followed by crystalliz-

ation led to the isolation of castasterone (**1**). Its structure was rigorously determined by spectroscopic analyses, the details of which have already been reported [5].

*Identification of 6-deoxocastasterone from chestnut gall*

Our initial aim in this experiment was to examine whether our gall contained brassinone (**4**). The 90% methanol fraction was obtained from the young galls collected from a resistant variety (Tsukuba) and was subjected to silica gel column chromatography. The eluate with 5% methanol in chloroform, which was expected to contain brassinone, was fractionated, then subjected to bioassay (Fig. 1). Among the fractions showing activity, fractions 3, 5 and 7 were subjected to methane boronation [7] prior to SIM, where an ion (*m/z* 498) corresponding to the $[M]^+$ of brassinone bis-methane boronate was monitored. The ion was found only in fraction 3, which had only weak activity, but the retention time did not agree with that of brassinone bis-methane boronate but exactly with that of 6-deoxocastasterone (**2**) bis-methane boronate, which happens to have the same MW as brassinone bis-methane boronate. 6-Deoxocastasterone has recently been found together with its dehydro ($\Delta^{24(28)}$) derivative, 6-deoxodolichosterone, in *Phaseolus vulgaris* seed [2]. Fraction 3 was purified by HPLC on Partisil, affording an active zone at 14–16 min (corresponding to the retention time of 6-deoxocastasterone and 6-deoxodolichosterone). This was subjected to reversed-phase HPLC. The zone between 12 and 15 min which was expected to contain 6-deoxocastasterone was subjected to methane boronation prior to GC/MS analysis. Thus 6-deoxocastasterone was detected as the bis-methane boronate with the same retention time (4.62 min) and mass spectrum as the authentic specimen [2]. Major fragment ions were observed at *m/z* (relative intensity): 498 $[M]^+$ (47), 483 (11), 343 (9), 313 (14), 288 (20), 273 (100), 205 (21) and 155 (41).

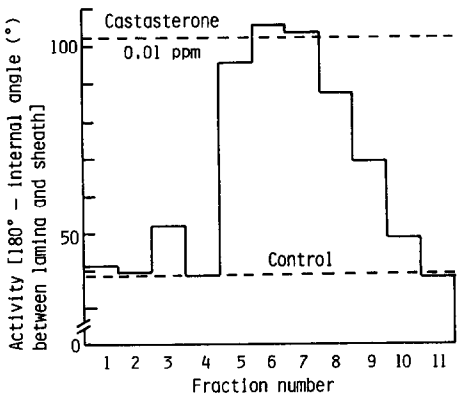


Fig. 1. Distribution of biological activity determined by the rice-lamina inclination test after silica gel chromatography of the chestnut gall extract. A portion (0.71 g, equivalent to 650 g fr. wt) of the 90% methanol extract obtained from young chestnut gall of a resistant variety was chromatographed on a silica gel column (10 g). After washing the column with chloroform (100 ml) and 2.5% methanol in chloroform (100 ml), the eluate with 5% methanol in chloroform (200 ml) was fractionated into 11 fractions, 12 g fr. wt equivalent portions of which were used for bioassay.

On the other hand, the zone (retention time, 8–12 min) corresponding to 6-deoxodolichosterone, separated in the above-mentioned reversed-phase HPLC, was not found to contain 6-deoxodolichosterone, as similarly analysed by GC/MS. Thus our attempt to search for brassinone resulted in the identification of 6-deoxocastasterone. This fact was also substantiated by the SIM data as will be described below.

*Identification and contents of castasterone, 6-deoxocastasterone and brassinolide in gall, leaf, stem and flower bud of the chestnut plant*

The 90% methanol fraction derived from each tissue was purified using a pair of Sep-Pak silica cartridges with two different solvent systems (see Experimental), by which brassinolide-related steroids ranging in polarity from 6-deoxocastasterone to brassinolide could be separated as a group. It was shown by bioassay that any serious loss was not observed in this purification step (Table 1). Materials thus obtained were subjected to methane boronation, then analysed by SIM with electron-impact ionization. The following ions were monitored: *m/z* 512 ($[M]^+$ of castasterone bis-methane boronate), 498 ($[M]^+$ of bis-methane boronates of 6-deoxocastasterone and brassinone), 528 and 374 ($[M]^+$ and $[M-154]^+$ due to C20–C22 cleavage of brassinolide bis-methane boronate, respectively) [7, 10]. As shown in Fig. 2, both castasterone (**1**) and 6-deoxocastasterone (**2**) could be detected from all the tissues examined, while brassinone (**4**) was never detected.

The above-mentioned procedure, however, did not allow us to detect brassinolide (**3**), because the contents of brassinolide were relatively low and electron-impact ionization does not give rise to strong fragments suitable for SIM of brassinolide methane boronate [7, 10]. Thus further purification to enrich brassinolide was attempted. The young gall tissue collected from Tsukuba was purified successively by Sephadex LH-20 chromatography, Sep-Pak treatment and finally reversed-phase HPLC. The SIM analysis of the brassinolide-enriched fraction thus obtained led to the successful identification of brassinolide (Fig. 3). Whether the other tissues contained brassinolide or not was not determined.

The quantitation data, which were calculated on the basis of the SIM data, of castasterone, 6-deoxocastasterone and brassinolide in the various tissues are shown in Table 1.

## DISCUSSION

The young galls collected from the resistant variety Tsukuba were found to contain 6-deoxocastasterone (**2**), castasterone (**1**) and brassinolide (**3**), which constitutes a series of steriods differing in their B-ring oxidation patterns. Brassinone (**4**) which had been reported present in the chestnut gall [4], however, could not be detected: this contradictory result is discussed below. The contents of castasterone and brassinolide (Table 1) are in accord with the data reported by Ikeda *et al.* [6]. A comparison of the contents of growth-promoting steroids between the young and aged galls suggests that the contents of growth-promoting steroids tend to decrease during ageing. The larvae collected from the galls contained only a trace amount of activity, as was reported by Ikeda *et al.* [6], and

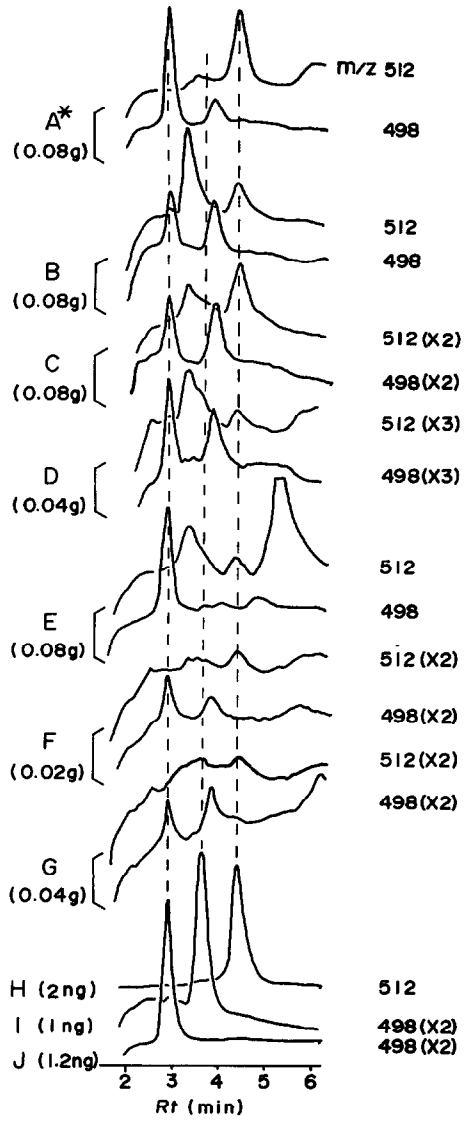


Fig. 2. SIM profiles obtained for the insect gall and healthy tissues of chestnut. (A and B) Young and aged galls collected from a resistant variety, respectively; (C, D, E, F and G) gall, current shoot, 2-year-old shoot, leaf and flower bud collected from a susceptible variety respectively; (H, I and J) bis-methane boronates of authentic castasterone, brassinone and 6-deoxocastasterone, respectively. Each tissue extract was purified as stated in the text, then subjected to methane boronation prior to monitoring of ions of $m/z$ 512 ($[M]^+$ of castasterone bis-methane-boronate) and $m/z$ 498 ($[M]^+$ of 6-deoxocastasterone and brassinone bis-methane boronate). *Figures in parentheses denote the amounts (fr. wt equivalent or wt) used per injection.

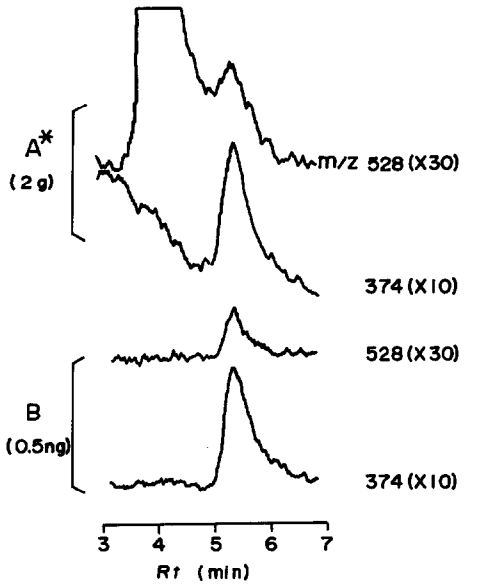


Fig. 3. SIM profile obtained for young galls collected from a resistant variety. (A) Gall; (B) brassinolide bis-methaneboronate. The tissue extract was purified as stated in the text, then subjected to methane boronation prior to monitoring of ions of $m/z$ 528 and 374 corresponding to $[M]^+$ and $[M-154]^+$ of brassinolide bis-methane boronate. *Figures in parentheses denote the amounts (fr. wt equivalent or wt) used per injection.

therefore it is evident that activity found in the gall is exclusively due to the plant tissue.

In order to know whether growth-promoting steroids are specific to the gall tissue, various tissues of a susceptible variety, such as gall, shoot (current and 2-year-old), leaf and flower bud, were examined. All were found to contain castasterone and 6-deoxocastasterone, and the contents of these steroids did not vary largely with the tissues. It is also a common feature that the content of 6-deoxocastasterone is higher than that of castasterone. Brassinolide could not be found in all the tissues of the susceptible variety, although it should have been possible to detect it if these tissues were subjected to extensive purification as in the case of the gall of the resistant variety. It is biosynthetically reasonable to assume that low levels of brassinolide would be present in the tissues. In any event, it is concluded that brassinolide-related steroids are widely distributed in the various tissues of the plant.

That brassinone could not be detected in any of the tissues examined does not agree with the findings by Abe *et al.* [4], who have identified brassinone from the gall and estimated its content to be approximately equal to that of castasterone. Their data, however, do not seem to give conclusive evidence for the presence of brassinone because an SIM peak of brassinone reported by them apparently had a shorter retention time than authentic brassinone. Taking our findings into account, there is a possibility that the peak in question is due to 6-deoxocastasterone. If this is not the case, it is possible that the tissues we examined happened to be at the stage where brassinone was lacking, since the contents of compounds in plant tissue generally fluctuate during growth.

Lastly, it must be pointed out that there was a big difference between the quantitation data obtained by bioassay and SIM, although the same sample was used in both cases (Table 1). For example, in the case of the young gall of Tsukuba, the content obtained by bioassay is about one order of magnitude larger than that obtained by SIM, considering that, in our bioassay system, biological activities of 6-deoxocastasterone and brassinolide are about

Table 1. Contents of brassinolide-related steroids in various tissues of chestnut

| Plant material (date of harvest) | Content determined by: Bioassay (μg castasterone equivalents/kg fr. wt) 90% Methanol fraction | Bioassay (μg castasterone equivalents/kg fr. wt) After Sep-Pak (×2) | SIM (μg/kg fr. wt)* Castasterone | SIM (μg/kg fr. wt)* 6-Deoxo-castasterone | SIM (μg/kg fr. wt)* Brassinolide |
|---|---|---|---|---|---|
| Resistant variety, Tsukuba | | | | | |
| Gall (May 7) | 120 | 120 | 12 | 26 | n.d. (0.25†) |
| Larvae (May 7) | 0.6‡ | — | — | — | — |
| Gall (June 17) | 25 | 65 | 5 | 13 | n.d. |
| Susceptible variety (May 18) | | | | | |
| Gall | 45 | 35 | 4 | 9 | n.d. |
| Larvae | 0.13‡ | — | — | — | — |
| Current shoot | 57 | 70 | 2 | 20 | n.d. |
| 2-Year-old shoot | 28 | 20 | 3 | 27 | n.d. |
| Leaf | 26 | 30 | 6 | 30 | n.d. |
| Flower bud | 10 | 10 | 3 | 15 | n.d. |

—, Not analysed; n.d., not detectable.

*SIM was carried out after purification with a Sep-Pak cartridge.

†SIM was carried out after successive purification with LH-20, a Sep-Pak cartridge and Develosil ODS.

‡These values represent the contents in the larvae collected from 1 kg of galls.

one-hundredth and three times that of castasterone, respectively. IAA is known to be synergistic with brassinolide in several bioassay systems [11, 12]. The samples used for bioassay and SIM, however, were not found to contain auxin activity on the basis of the *Avena* curvature test (data not shown). This suggests that a synergist(s) other than auxin might be present in the extract. This assumption, however, awaits further experimentation.

## EXPERIMENTAL

*Plant material.* Insect galls used for the isolation of castasterone were collected from an unidentified susceptible (to the infestation by cecidogenetic chestnut wasp) variety of the chestnut plants grown at Fruit Research Station, Hiratsuka, between April and May 1976. Young and aged insect galls of the resistant variety Tsukuba ('resistant' means less prone to chestnut wasp infestation) and insect galls, current shoots, 2-year-old shoots, leaves and flower buds of an unidentified susceptible variety were collected at the Ibaraki Horticultural Station in 1982. The larvae of the chestnut wasp were obtained from chestnut galls by dissecting them with knives.

*Bioassay.* The rice-lamina inclination test [8, 9] was used with some modifications. Rice seedlings (*Oryza sativa* L. cv Koshihikari), germinated in water for 2 days in the light were planted on 1% agar and grown for 7 days in the dark, where the plantlets were exposed to red light for 1–2 hr per day. From the 2nd leaves of the etiolated seedlings, explants including the lamina joint were cut out 2 cm below the joint and incubated while floating on $H_2O$ for 1 day in the dark. The explants which were bent *ca* 15° were selected, and 10 pieces of them were placed in a Petri dish (9 cm diameter) containing 20 ml aq. soln of a test sample. After 2-day incubation in the dark, the internal angles between the laminae and sheaths were measured. All operations were carried out at 30°.

*GC/MS and SIM.* Samples were heated at 70° for 30 min in pyridine containing 2 mg/ml of methane boronic acid [7] prior to GC/MS and SIM. GC/MS (EI) identification of 6-deoxocastasterone was carried out with a JEOL DX-300 (ionization voltage, 70 eV). GC conditions were as follows: column, 2% OV-1 (1 m × 3 mm); column temp., 270°; flow rate of He, 30 ml/min. SIM (EI) was done with a Hitachi 80A (ionization voltage 22 eV) under the same GC conditions as described above except the He flow rate was 50 ml/min. Quantification data were calculated from the peak areas observed in the SIM; standard calibration curves were obtained using known amounts of authentic compounds.

*HPLC.* (A) Partisil column (particule size 5 μm; 150 × 8 mm) with gradient elution. Varian Aerograph 8500 fitted with a Rheodine 7125 injector (Berkley, CA, U.S.A.). (B) Reversed-phase Develosil ODS (particle size, 3 μm; 150 × 8 mm; Nomurakagaku, Japan). NP-800 double-plunger micropump system (Nihonseimitsu, Japan) fitted with the same injector as in A.

*Isolation of castasterone.* The MeOH extract from insect galls (40 kg) was concd to give an aq. residue, which was extracted with $C_6H_6$. The extract (89 g) was partitioned (×2) between hexane (1 l.) and 90% MeOH (1 l.) which had previously been saturated with each other. The 90% MeOH fraction (45 g) was chromatographed on a silica gel column (500 g). Elution (flow rate 200 ml/hr) was carried out stepwise with $CHCl_3$ (4 l.) and MeOH–$CHCl_3$ (1.5:98.5, 3 l.; 5:95, 8 l.; 7.5:92.5, 4 l.; 10:90, 4 l.). Activity was found only in the eluate with 5% MeOH. This eluate was purified on a 2nd silica gel column (110 g). Elution (flow rate 200 ml/hr) was performed with 1 l. each of EtOAc–$C_6H_6$ (1:1, 9:1), EtOAc, EtOAc–MeOH (9:1) and MeOH. The eluate with EtOAc in which most of the activity was found was purified on a Sephadex LH-20 column (45 × 2 cm) using 70% EtOH (flow rate 30 ml/hr), yielding an active fraction (elution vol. 100–115 ml). This was dissolved in 0.2 ml $CHCl_3$ and subjected to Partisil HPLC: flow rate, 3 ml/min; mobile phase, $CHCl_3$ during 10 min, then linear gradient from 0 to 22% *iso*-PrOH in $CHCl_3$ during 33.3 min. The active fraction ($R_t$ 27–29 min) was dissolved in 60 μl

60% MeCN and subjected to reversed-phase HPLC: flow rate, 2 ml/min; mobile phase, 50% MeCN (isocratic). The active eluate ($R_t$ 10–11 min) was collected in a test-tube (10 × 1.2 cm). The test-tube was capped loosely with an Al foil to permit gentle evapn of the solvent, then allowed to stand at 4° for a week, yielding crystals of the active principle castasterone. The mother liquor was removed by means of a pipette having a capillary end so that crystals were left in the test-tube. After the crystals in the test-tube were dried *in vacuo*, a trace amount was removed with a sewing needle and sandwiched between a pair of cover glasses, which were then placed in a micro mp apparatus equipped with a microscope (Yanagimoto, Japan) to determine the mp (259–261°). The wt (95 $\mu$g) of the crystals was determined after the crystals left in the test-tube were dissolved in $CHCl_3$, transferred to a small test-tube (5 × 0.5 cm) and the solvent was removed in a stream of $N_2$ followed by drying *in vacuo*.

The eluate with EtOAc–MeOH (9:1) from the 2nd silica gel column in which minor activity was found was similarly treated, affording a very small amount of castasterone crystals.

*Purification of 6-deoxocastasterone.* The young galls (1 kg) were soaked in MeOH (1 l.), homogenized with a blendor and filtered by suction. The residue was further extracted with MeOH (1.5 l. × 2). After evapn of the MeOH *in vacuo*, the aq. residue was extracted with $CHCl_3$ (350 ml × 3). After evapn of the $CHCl_3$, the residue was dissolved in EtOAc (200 ml). This was washed with 0.5 M Pi buffer, pH 8.8 (100 ml × 2) to give a neutral fraction, which was partitioned between hexane and 90% MeOH (200 ml each). The hexane phase was again partitioned with 90% MeOH (100 ml). The 90% MeOH fractions were combined, and evapn of the solvent gave an oil in which most of the biological activity was recovered. This was purified on a silica gel column (for details, see Fig. 1), and the 3rd fraction (40 mg) was purified by HPLC on Partisil: flow rate, 3 ml/min; mobile phase, linear gradient from 0 to 15% *iso*-PrOH in $CHCl_3$ during 33.3 min. Prior to GC/MS analysis, further purification was done by HPLC on Develosil ODS: flow rate 2.8 ml/min; mobile phase, 70% MeCN (isocratic).

*Purification procedures for SIM analysis.* (A) *Sep-Pak silica gel treatment (group separation of brassinolide-related steroids)*: The 90% MeOH fractions (42–95 mg) prepared, from the tissues, as described in the preceding section were dissolved in $CHCl_3$, then passed through a Sep-Pak silica gel cartridge (Waters). After successive washing with $CHCl_3$ (20 ml) and 1% MeOH in $CHCl_3$ (20 ml), brassinolide-related steroids were eluted with 5% MeOH in $CHCl_3$ (20 ml). Note that the Sep-Pak silica gel cartridge was weak in its adsorbing power so that even brassinolide was eluted with 5% MeOH vs 10% MeOH in the case of ordinary silica gel. The eluate was dissolved in 30% EtOAc in $C_6H_6$, then passed through another cartridge. After washing with the same solvent (20 ml), elution with 2% MeOH in EtOAc (30 ml) gave a sample for SIM.

(B) *Procedure to enrich brassinolide*: The 90% MeOH fraction (53 mg) obtained from the young galls (50 g fr. wt) was chromatographed on a LH-20 column (35 × 2.2 cm) using $CHCl_3$–MeOH (1:4) as solvent, affording an active fraction (elution vol. 95–115 ml). This was purified with a Sep-Pak cartridge. After washing with 2% MeOH in $CHCl_3$ (20 ml), the eluate with 5% MeOH in $CHCl_3$ (30 ml) was collected. Further purification was carried out by Develosil ODS HPLC: flow rate, 2 ml/min; 45% MeCN (isocratic); and a fraction ($R_t$ 8–12 min) corresponding to the $R_t$ of brassinolide was collected.

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