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ACTIVITY OF BRASSINOSTEROIDS IN THE DWARF RICE LAMINA INCLINATION BIOASSAY

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Key Word Index—*Oryza sativa*; Gramineae; biological activity; rice lamina inclination bioassay; biosynthetic intermediates of brassinolide; brassinosteroids; 6-deoxobrassinosteroids; 6-oxobrassinosteroids; brassinolide; castasterone; campestanol.

Abstract—Biological activities of brassinolide, its biosynthetic intermediates and some biosynthetically related brassinosteroids were evaluated using modified dwarf rice lamina inclination bioassay. A very strong synergistic effect was observed when IAA was applied together with brassinosteroid. In particular co-application of brassinosteroid and 5 μg IAA was *ca*. 300 times more active than a single application of brassinosteroid. Under co-application of 5 μg IAA, the minimum detectable amount of brassinolide was less than 0.01 ng/rice plant. Biological activity increased according to the order of the biosynthetic pathway. Brassinosteroids in the early C-6 oxidation pathway (6-oxobrassinosteroids) were more active than corresponding brassinosteroids in the late C-6 oxidation pathway (6-deoxobrassinosteroids). Even very early biosynthetic intermediates such as campestanol showed some activity in our modified bioassay. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The rice lamina inclination bioassay was originally developed by Maeda [1]. This bioassay system has been found to be very sensitive to brassinosteroids (BRs), and has been successfully used to guide purification and isolation of BRs from various plant sources and also to evaluate biological potency of natural and synthetic BRs [2, 3]. In this bioassay, explants comprising lamina, lamina joint and leaf sheath are excised from etiolated rice seedlings and floated on test solutions. Cells aligned in the adaxial side of the lamina joint between leaf sheath and lamina are swollen by BRs, causing bending [4]. Takeno and Pharis [5] have reported that application of BR to the intact seedlings of dwarf rice *Oryza sativa* cv. Tan-ginbozu and Waito-C, induced a significant inclination between the leaf sheath and lamina. They also found that this inclination was synergized by the application of IAA and BR. Later Kim *et al*. [6] developed a sensitive and simple dwarf rice lamina inclination bioassay based on the intact seedling assay of Takeno and Pharis. Although their bioassay is simple and sensitive, the bioassay has not been used widely so far. We have slightly modified this bioassay system to improve sensitivity. Herein, we report the biological activity of various BRs, especially biosynthetic intermediates of brassinolide, by our modified rice lamina inclination bioassay using intact seedlings.

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RESULTS AND DISCUSSION

Effect of brassinosteroid with or without IAA

It has been found that pretreatment with 0.1 and 1 μg of IAA before application of brassinolide increased activity by 10- and 100-fold, respectively, in the rice lamina inclination assay, with a synergistic effect of IAA up to 1 μg [6]. We found that treatment of IAA (5 μg/plant) was more effective, and its activity increased *ca*. 300-fold, compared to single brassinolide or castasterone treatment (Fig. 1). Also with other BRs such as typhasterol and teasterone, co-application of 5 μg IAA was found to be much more effective (*ca*. 300-fold). Therefore, co-application of BR and IAA is much more effective to detect biological activities of BRs, compared to a single application of BR.

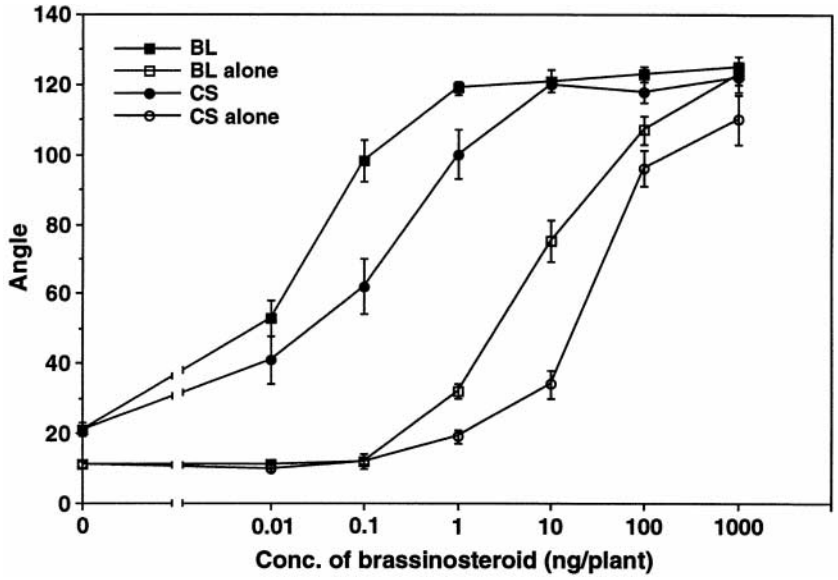


Fig. 1. Effect of various concentrations of brassinolide and castasterone with 5 μg IAA (BL, CS) or without IAA (BL alone, CS alone) in the dwarf rice lamina inclination bioassay. Each data point represents the mean of 30 replicates $\pm$ SE.

Furthermore, although several authors pointed out the importance of the order of treatment of IAA and BR for the synergistic activity [7–9], Kim *et al.* [6] found no significant effect on their assay, which was confirmed in our studies. So, in the present study, we chose to apply IAA (5 μg fixed) and BR (various concentration) mixture simultaneously instead of pretreatment or posttreatment with IAA.

Biological activity of brassinolide and its biosynthetic intermediates

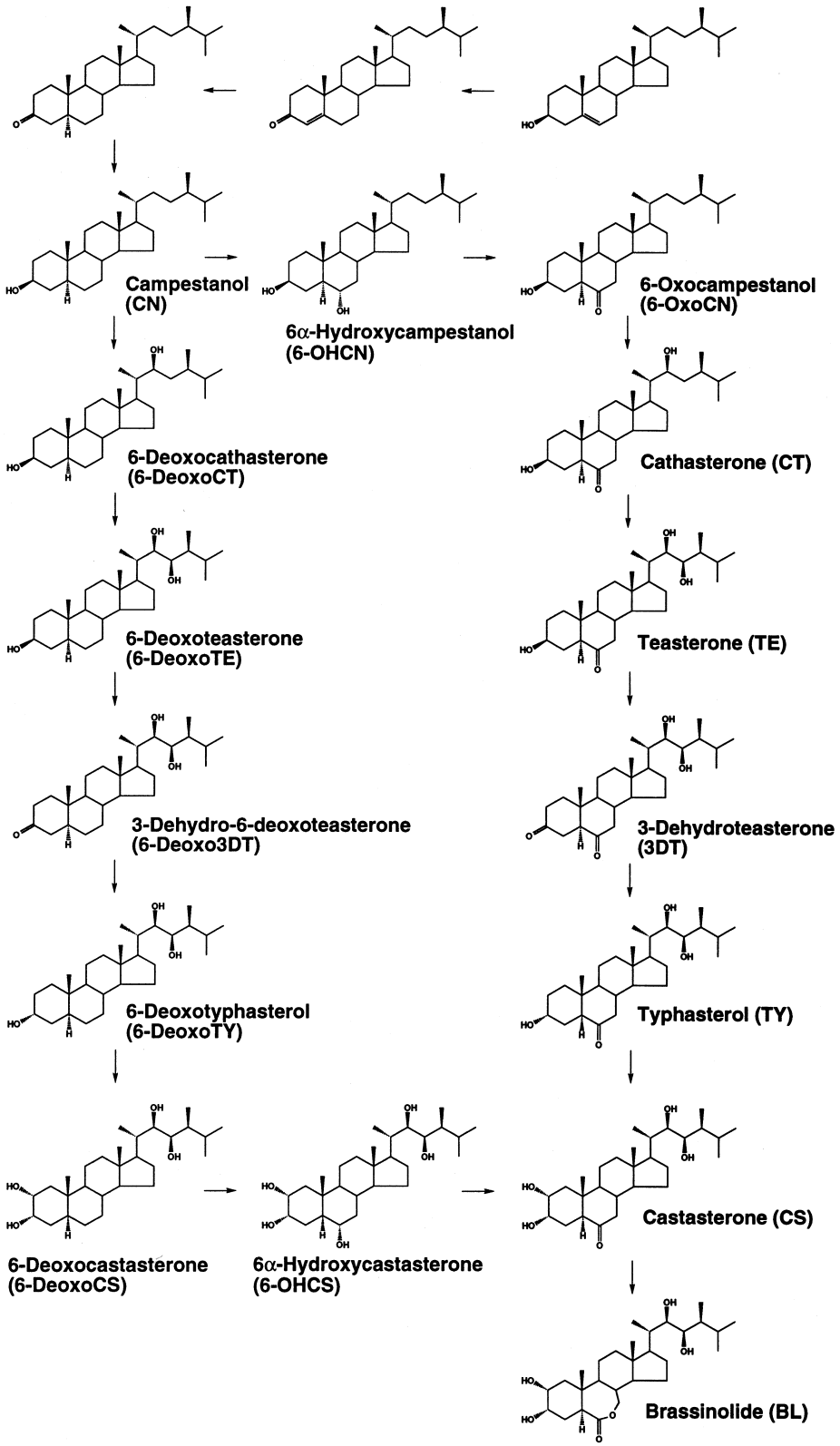
Recently we have established biosynthetic pathways of brassinolide from campesterol (Scheme 1) using a cell culture system of *Catharanthus roseus* [10, 11]. There are two pathways, named early C-6 oxidation pathway (6-oxoBRs) and late C-6 oxidation pathway (6-deoxoBRs), leading to brassinolide. Also recent studies using BR biosynthesis mutants in *Arabidopsis thaliana* provided strong genetic and biochemical evidence that brassinolide is synthesized in two parallel pathways [12]. Biological activities of biosynthetic intermediates in the pathways were examined by our modified dwarf rice lamina inclination bioassay using intact seedlings.

Thus, 6-oxoBR belonging to the early C-6 oxidation pathway was applied together with 5 μg IAA. As shown in Fig. 2, biological activity increased according to the order of the biosynthetic pathway. Relative activity was roughly estimated by the concentration at the midpoint of the maximum response as follows: cathasterone (1), teasterone (5), 3-dehydroteasterone (50), typhasterol (50), castasterone (300), and brassinolide (2000). This is in good accordance with the results obtained with the standard rice lamina inclination bioassay using explants of etiolated seedlings of rice (*Oryza sativa* cv. Koshihikari) [13]. Therefore, our modified bioassay can evaluate the biological activity in the same way as the standard bioassay. More advantageously, minimum detectable levels of brassinolide and castasterone were less than 0.01 ng/plant. Furthermore, our system needs only one week for the entire bioassay, while standard bioassay needs two weeks.

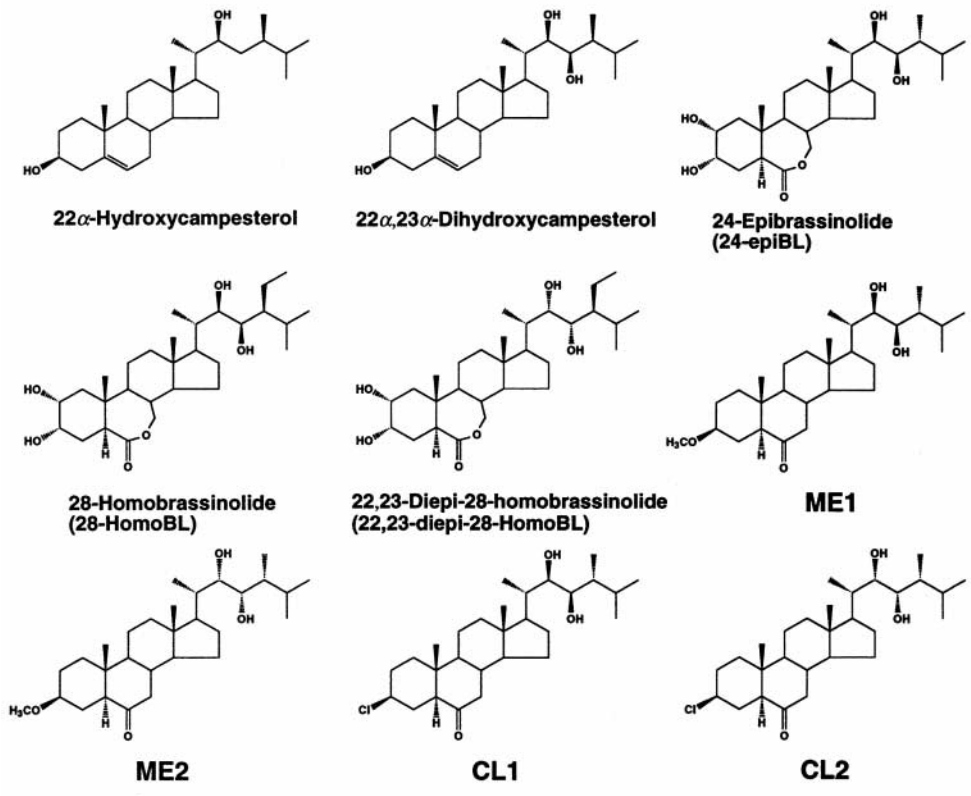
Biological activity of 6-deoxoBRs belonging to the late C-6 oxidation pathway was also investigated. The results were shown in Fig. 3. Biological activity tended to increase according to the order of the biosynthetic pathway. However, the activities of 6-deoxocastasterone, 6-deoxotyphasterol and 3-dehydro-6-deoxoteasterone was almost similar. The relative activity was as follows: 6-deoxocathasterone (1), 6-deoxoteasterone (10), 3-dehydro-6-deoxoteasterone (50), 6-deoxotyphasterol (50), 6-deoxocastasterone (50), 6α-hydroxycastasterone (500), and castasterone (10000). Although 6α-hydroxycastasterone has not been found in plants yet, we have synthesized this compound as a possible candidate of intermediate between 6-deoxocastasterone and castasterone. Interestingly the activity of 6α-hydroxycastasterone was in the middle of that of 6-deoxocastasterone and castasterone, supporting that 6α-hydroxycastasterone is a possible intermediate between 6-deoxocastasterone and castasterone.

Biological activity of earlier intermediates

We have examined the activities of earlier intermediates such as campestanol and 6-oxocampestanol. Co-application of 100 ng of each steroid and 5 μg of IAA did not cause significant bending.



Scheme 1. Proposed biosynthetic pathways of brassinolide.



However, co-application of 1 μg of each steroid and 5 μg of IAA caused some response (cf. angle [degrees] $\pm$ standard error, control: 21 ± 2, campestanol: 27 ± 3, 6α-hydroxycampestanol: 31 ± 4, 6-oxocampestanol: 39 ± 5). Dosage of 5 μg of each steroid increased inclination (cf. campestanol: 41 ± 8, 6α-hydroxycampestanol: 49 ± 9, 6-oxocampestanol: 54 ± 7). As the biological activity of campestanol has not been detected in standard bioassay, our modified bioassay was shown to be very useful.

Although 22α-hydroxycampesterol and 22α,23α-dihydroxycampesterol have not been found in plants, we have synthesized both compounds as

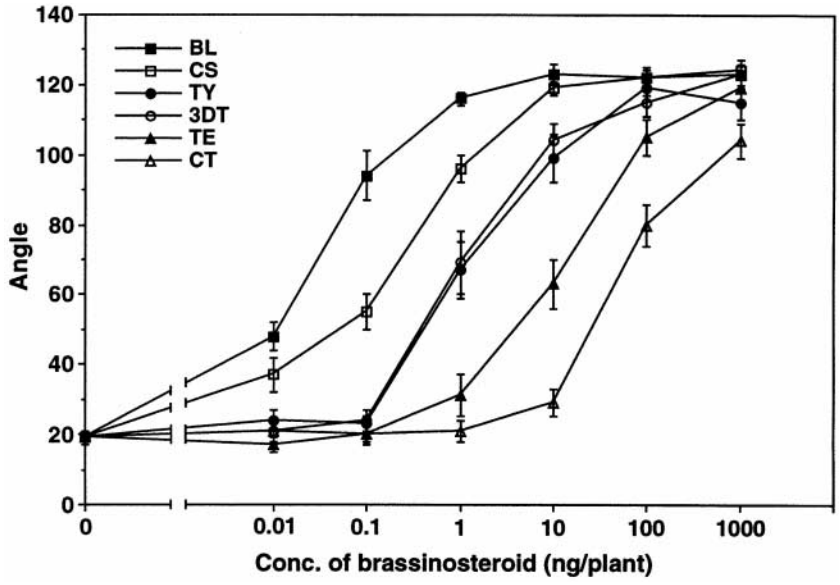


Fig. 2. Biological activity of brassinosteroids belonging to the early C-6 oxidation pathway in the dwarf rice lamina inclination bioassay. Each data point represents the mean of 30 replicates $\pm$ SE.

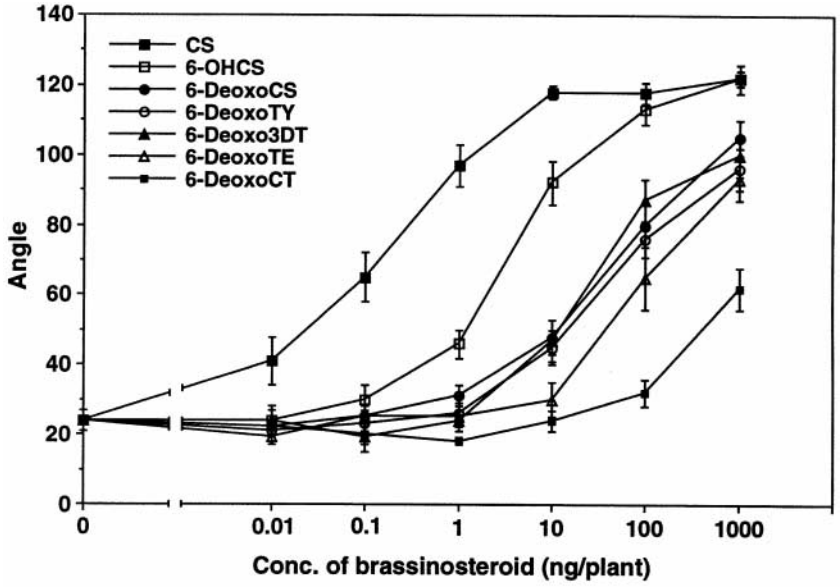


Fig. 3. Biological activity of brassinosteroids belonging to the late C-6 oxidation pathway in the dwarf rice lamina inclination bioassay. Each data point represents the mean of 30 replicates ± SE.

possible biosynthetic intermediates and examined their activities. Interestingly both compounds showed significant activities (control: 23 ± 3, 22α-hydroxycampesterol: 10 ng; 31 ± 3, 100 ng; 54 ± 5, 1000 ng; 86 ± 4, 22α,23α-dihydroxycampesterol: 10 ng; 36 ± 5, 100 ng; 70 ± 7, 1000 ng; 99 ± 4). These activities were almost equivalent to those of 6-deoxocathasterone and 6-deoxoteasterone, respectively.

Biological activity of some other brassinosteroids

Some other BRs were also tested. The results were shown in Fig. 4. 24-Epibrassinolide was 10 times less active than brassinolide. 28-

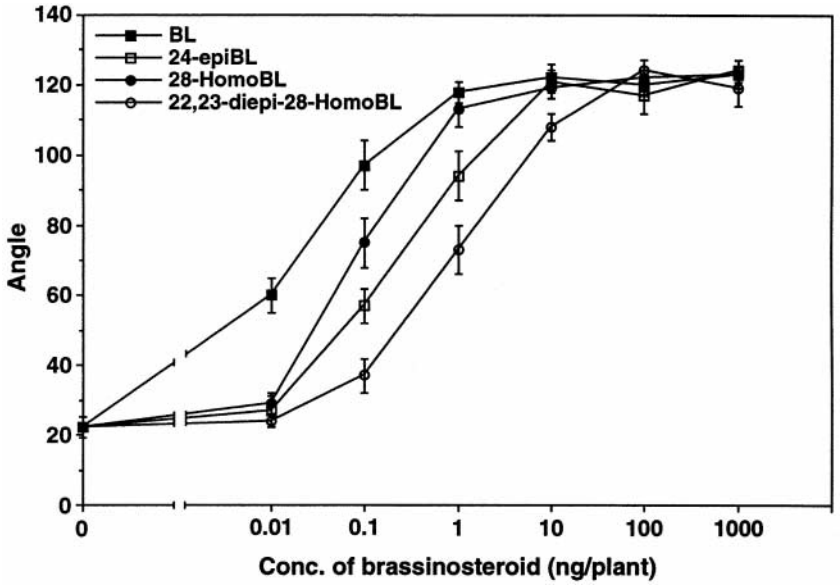


Fig. 4. Biological activity of four brassinosteroids (brassinolide, 24-epibrassinolide, 28-homobrassinolide and 22,23-diepi-28-homobrassinolide) in the dwarf rice lamina inclination bioassay. Each data point represents the mean of 30 replicates ± SE.

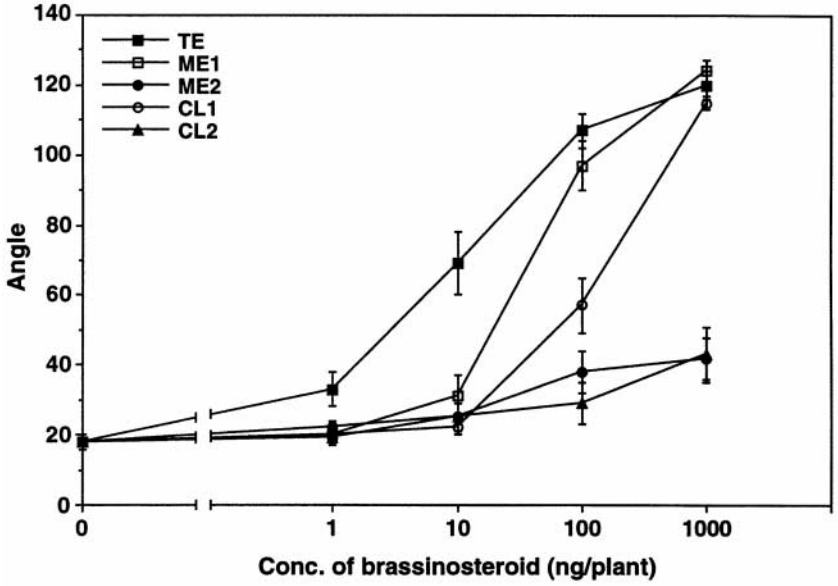


Fig. 5. Biological activity of five brassinosteroids (teasterone, ME1, ME2, CL1 and CL2) in the dwarf rice lamina inclination bioassay. Each data point represents the mean of 30 replicates ± SE.

Homobrassinolide was 4 times less active than brassinolide. 22,23-Diepi-28-homobrassinolide was 10 times less active than 28-homobrassinolide. These results are in good accordance with reported data using standard rice lamina inclination bioassay [14, 15].

We have synthesized some BR analogues containing methoxy or chlorine instead of 3-hydroxyl as possible biosynthetic inhibitors, and examined their activities. ME1 and CL1 are 3β-methoxy-24-epiteasterone and 3β-chloro-24-epiteasterone, respectively. Interestingly these two BR analogues showed activities (Fig. 5). On the contrary, their 22,23-diepimers showed only slight activities.

EXPERIMENTAL

Bioassay

Dwarf rice (*Oryza sativa* cv. Tan-ginbozu, provided by Dr. M. Koshioka, National Research Institute of Vegetable, Ornamental Plants and Tea, Mie, Japan) was used in this experiment. Seeds were soaked in water at 28° for 2 days. Germinated seeds were selected for uniformity of coleoptile length (*ca.* 1–2 mm), and five germinated seeds were planted on 30 ml of 1% agar medium in a glass jar (26 mm i.d.×60 mm), then incubated under the above conditions for three days. Samples for bioassay were dissolved in EtOH and applied by microsyringe in 0.5 μl of the samples to the top portion of lamina. After incubation of 2 days, under the same growth conditions, the external angle between the lamina and its leaf sheath was measured using a circular protractor. Totally thirty plants (six jars) were used for each treatment.

Brassinosteroids

Brassinolide and castasterone were synthesized from (20*S*)-6,6-ethylenedioxy-20-formyl-3α,5-cyclo-5α-pregnane [16]. Typhasterol and teasterone were synthesized as described previously [17]. 3-Dehydroteasterone was synthesized from teasterone [18]. 6-Deoxocastasterone, 6-deoxotyphasterol, 3-dehydro-6-deoxoteasterone, 6-deoxoteasterone and 22α,23α-dihydroxycampesterol were synthesized from (20*S*)-20-formyl-6β-methoxy-3α,5-cyclo-5α-pregnane [19]. 22α-Hydroxycampesterol, 6-deoxocathasterone, 6α-hydroxycathasterone and cathasterone were synthesized from (22*E*,24*S*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-ene [20]. 6-Oxocampestanol and 6α-hydroxycampestanol were synthesized according to the method of Ref. [21]. Campestanol was obtained by catalytic hydrogenation (palladium-charcoal) of campesterol. 28-Homobrassinolide and 22,23-diepi-28-homobrassinolide were synthesized from stigmasterol [22]. 24-Epibrassinolide was purchased from Sigma.

Synthesis of 6α-hydroxycastasterone

To a cooled (−78°) sol of castasterone [16] (51 mg) in THF-EtOH (4 ml, 3:1) was added liquid NH_3 (7 ml) and then Li (50 mg) and the mixture was stirred at −78° under Ar for 2 h. Work-up (EtOAc) and chromatography on silica gel (1.0 cm i.d. × 30 cm) with CH_2Cl_2–EtOH (20:1) provided 6α-hydroxycastasterone (39 mg), mp 253–254° (EtOAc), ^{1}H NMR (400 MHz, pyridine-d_5); δ 0.79 (3H, *s*), 0.94 (3H, *s*), 1.03 (3H, *d*, J = 6.8 Hz), 1.10 (3H, *d*, J = 6.8 Hz), 1.15 (3H, *d*, J = 6.8 Hz), 1.24 (3H, *d*, J = 6.8 Hz), 2.97 (1H, *dt*, J = 14.0 and 2.8 Hz), 3.70 (1H, *m*), 3.98 (1H, *br d*, J = 8.0 Hz),

4.14 (2H, *br d*, $J = 8.4$ Hz), 4.48 (1H, *d*, $J = 2.4$ Hz), 3.72 (1H, *m*). HR-FAB-MS *m/z* ($M^+ + H$): calcd. for $C_{28}H_{49}O_5$, 465.3580; found, 465.3573.

Synthesis of (22*R*,23*R*,24*R*)-22,23-*dihydroxy*-3β-*methoxy*-5α-*ergostan*-6-*one* (*ME*1) *and* (22*S*,23*S*,24*R*)-22,23-*dihydroxy*-3β-*methoxy*-5α-*ergostan*-6-*one* (*ME*2)

(22*E*,24*R*)-3α,5-Cyclo-5α-ergost-22-en-6-one [23] (600 mg) in MeOH (40 ml) was treated with conc. H_2SO_4 (5 drops) at 70° for 2 h. Work-up (EtOAc) and recrystallization from MeOH gave (22*E*,24*R*)-3β-methoxy-5α-ergost-22-en-6-one (410 mg), mp 133–134°. This (200 mg) in THF–H_2O (10 ml, 9:1) was treated with *N*-methylmorpholine *N*-oxide (400 mg) and OsO_4 (30 mg) at room temp. for 2 weeks. Work-up ($CHCl_3$) gave two products, which were separated by chromatography on silica gel (1.5 cm i.d. × 15 cm). From the fraction eluted with toluene–EtOAc (5:1), ME2 (98 mg) was obtained, mp 158–159° (hexane–EtOAc), ^{1}H NMR (400 MHz, $CDCl_3$); δ 0.70 (3H, *s*), 0.75 (3H, *s*), 0.89 (3H, *d*, $J = 6.8$ Hz), 0.91 (3H, *d*, $J = 6.8$ Hz), 0.97 (3H, *d*, $J = 6.8$ Hz), 1.03 (3H, *d*, $J = 6.8$ Hz), 2.33 (1H, *dd*, $J = 13.2$ and 4.4 Hz), 3.11 (1H, *m*), 3.35 (3H, *s*), 3.60 (1H, *m*), 3.72 (1H, *m*). HR-FAB-MS *m/z* ($M^+ + H$): calcd. for $C_{29}H_{51}O_4$, 463.3787; found, 463.3786.

From the fraction eluted with toluene–EtOAc (4:1), ME1 (30 mg) was obtained, mp 167–168° (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$); δ 0.68 (3H, *s*), 0.75 (3H, *s*), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.87 (3H, *d*, $J = 6.8$ Hz), 0.92 (3H, *d*, $J = 6.8$ Hz), 0.98 (3H, *d*, $J = 6.4$ Hz), 2.33 (1H, *dd*, $J = 13.2$ and 4.4 Hz), 3.11 (1H, *m*), 3.35 (3H, *s*), 3.41 (1H, *dd*, $J = 10.7$ and 5.4 Hz), 3.70 (1H, *m*). HR-FAB-MS *m/z* ($M^+ + H$): calcd. for $C_{29}H_{51}O_4$, 463.3787; found, 463.3774.

Synthesis of (22*R*,23*R*,24*R*)-3β-*chloro*-22,23-*dihydroxy*-5α-*ergostan*-6-*one* (*CL*1) *and* (22*S*,23*S*,24*R*)-3β-*chloro*-22,23-*dihydroxy*-5α-*ergostan*-6-*one* (*CL*2)

In the same manner, (22*E*,24*R*)-3β-chloro-5α-ergostan-6-one [24] (190 mg) was hydroxylated with OsO_4 to give CL2 (86 mg), mp 155–157° (hexane–EtOAc), ^{1}H NMR (400 MHz, $CDCl_3$); δ 0.70 (3H, *s*), 0.79 (3H, *s*), 0.88 (3H, *d*, $J = 6.8$ Hz), 0.91 (3H, *d*, $J = 7.1$ Hz), 0.97 (3H, *d*, $J = 6.8$ Hz), 1.02 (3H, *d*, $J = 6.8$ Hz), 2.33 (1H, *dd*, $J = 13.2$ and 4.4 Hz), 3.59 (1H, *m*), 3.72 (1H, *m*), 3.80 (1H, *m*). HR-FAB-MS *m/z* ($M^+ + H$): calcd. for $C_{28}H_{48}O_3Cl$, 467.3292; found, 467.3279, and CL1 (32 mg), mp 209–210° (EtOAc), ^{1}H NMR (400 MHz, $CDCl_3$); δ 0.68 (3H, *s*), 0.79 (3H, *s*), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.87 (3H, *d*, $J = 6.8$ Hz), 0.92 (3H, *d*, $J = 6.8$ Hz), 0.98 (3H, *d*, $J = 6.8$ Hz), 2.33 (1H, *dd*, $J = 13.2$ and 4.4 Hz), 3.41 (1H, *dd*, $J = 10.7$ and 5.4 Hz), 3.70 (1H, *m*), 3.80 (1H, *m*). HR-FAB-MS *m/z* ($M^+ + H$): calcd. for $C_{28}H_{48}O_3Cl$, 467.3292; found, 467.3289.

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REFERENCES

1. Maeda, E., *Physiologia Plantarum*, 1965, **18,** 813.
2. Wada, K., Marumo, S., Ikekawa, N., Morisaki, M. and Mori, K., *Plant and Cell Physiology*, 1981, **22,** 323.
3. Takatsuto, S., *Journal of Chromatography A*, 1994, **658,** 3.
4. Yokota, T. and Mori, K., in *Molecular Structure and Biological Activity of Steroids*, ed. M. Bohl and W. L. Duax. CRC Press, Boca Raton Florida, 1992, p. 317.
5. Takeno, K. and Pharis, R. P., *Plant and Cell Physiology*, 1982, **23,** 1275.
6. Kim, S.-K., Abe, H., Little, C. H. A. and Pharis, R. P., *Plant Physiology*, 1990, **94,** 1709.
7. Cohen, J. D. and Meudt, W. T., *Plant Physiology*, 1983, **72,** 691.
8. Katsumi, M., *Plant and Cell Physiology*, 1985, **26,** 615.
9. Yopp, J. H., Mandava, N. B. and Sasse, J. M., *Plant Physiology*, 1981, **53,** 445.
10. Sakurai, A. and Fujioka, S., *Bioscience, Biotechnology and Biochemistry*, 1997, **61,** 757.
11. Fujioka, S. and Sakurai, A., *Physiologia Plantarum*, 1997, **100,** 710.
12. Fujioka, S., Li, J., Choi, Y.-H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J. and Sakurai, A., *The Plant Cell*, 1997, **9,** 1951.
13. Fujioka, S., Inoue, T., Takatsuto, S., Yanagisawa, T., Yokota, T. and Sakurai, A., *Bioscience, Biotechnology and Biochemistry*, 1995, **59,** 1973.
14. Wada, K., Marumo, S., Mori, K., Takatsuto, S., Morisaki, M. and Ikekawa, N., *Agricultural and Biological Chemistry*, 1983, **47,** 1139.
15. Takatsuto, S., Muramatsu, M., Ohya, K., Hayashi, S. and Shida, A., *Agricultural and Biological Chemistry*, 1988, **52,** 2059.
16. Watanabe, T., Takatsuto, S., Fujioka, S. and Sakurai, A., *Journal of Chemical Research (S)*, 1997, 360.
17. Takatsuto, S., *Journal of The Chemical Society, Perkin Transactions 1,* 1986, 1833.
18. Suzuki, H., Inoue, T., Fujioka, S., Takatsuto, Yanagisawa, T., Yokota, T., Murofushi, N. and Sakurai, A., *Bioscience, Biotechnology and Biochemistry*, 1994, **58,** 1186.

19. Takatsuto, S., Watanabe, T., Fujioka, S. and Sakurai, A., *Journal of Chemical Research (S)*, 1997, 134.
20. Takatsuto, S., Kuriyama, H., Noguchi, T., Suganuma, H., Fujioka, S. and Sakurai, A., *Journal of Chemical Research (S)*, 1997, 418.
21. Suzuki, H., Inoue, T., Fujioka, S., Saito, T., Takatsuto, S., Yokota, T., Murofushi, N., Yanagisawa, T. and Sakurai, A., *Phytochemistry*, 1995, **40,** 1391.
22. Takatsuto, S. and Ikekawa, N., *Chemical and Pharmaceutical Bulletin Tokyo*, 1982, **30,** 4181.
23. Takatsuto, S. and Ikekawa, N., *Chemical and Pharmaceutical Bulletin Tokyo*, 1984, **32,** 2001.
24. Anastasia, M., Ciuffreda, P. and Fiecchi, A., *Journal of The Chemical Society, Perkin Transactions 1*, 1983, 379.