New Lead Compounds for Brassinosteroid Biosynthesis Inhibitors

Yong Ki Min,[1,2] Tadao Asami,[1]* Shozo Fujioka,[1] Noboru Murofushi, [2] Isomaro Yamaguchi,[2] and
Shigeo Yoshida[1]

[1]*The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan*
[2]*Department of Applied Biological Chemistry, The University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan*

Abstract. The first brassinosteroid biosynthesis inhibitor is reported. Among newly synthesized triazole derivatives, 4-(4-chlorophenyl)-2-phenyl-3-(1,2,4-triazoyl)butan-2-ol (**6**) was found to inhibit the growth of cress seedlings, and this inhibition was recovered by the treatment of brassinolide, suggesting that compound **6** primarily inhibits brassinosteroid biosynthesis. © 1999 Elsevier Science Ltd. All rights reserved.

Brassinosteroids have recently been recognized as a new class of phytohormone[1,2] by coupling molecular genetics with studies of biosynthesis. Since the establishment of brassinosteroid chemistry, their homologues have been shown to dramatically affect the growth responses in plants, including stem elongation, pollen tube growth, leaf bending, leaf unrolling, root inhibition, proton pump activation,[3] promotion of ethylene production,[4] tracheary element differentiation,[5,6] and cell elongation.[7] In addition, extensive studies on their biosynthesis have begun to elucidate their mechanism of action.[8,9] At present, over 40 brassinosteroids have been identified, and it is thought that most of the C28-brassinosteroids are biosynthesized from campesterol, which is a common plant sterol with a side chain with the same carbon skeleton as brassinolide.
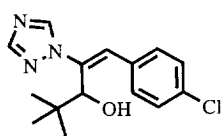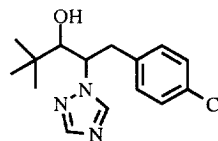
1 (uniconazole)   2 (paclobutrazol)

In general, specific inhibitors of biosynthesis are quite effective for determining the physiological functions of endogenous substances, as shown in studies of the mode of action of gibberellin.[10] Therefore, a specific inhibitor of brassinosteroids biosynthesis could provide a new approach to understand the functions of brassinosteroids. Uniconazole (**1**), a gibberellin biosynthesis inhibitor, has been reported to inhibit brassinosteroid biosynthesis,[5,6,11] even though its main target is gibberellin biosynthesis[12] rather than brassinosteroid biosynthesis. Various triazole compounds including uniconazole and paclobutrazol (**2**), another gibberellin biosynthesis inhibitor, have been shown to inhibit many types of cytochrome P-450, which are found in many oxidative processes in living systems,[13] however, the inhibition of individual enzymes is strictly controlled by the structure of the inhibitor. On the basis of these facts, we have been studying the

design and synthesis of brassinosteroid biosynthesis inhibitors among triazole compounds based on their analogy to uniconazole or paclobutrazol, since many steps in brassinosteroid biosynthesis are thought to involve cytochrome P-450; for example, the production of 6α-hydroxycampestanol from campestanol, cathasterone from 6-oxocampestanol, teasterone from cathasterone, castasterone from typhasterol, and brassinolide from castasterone.[1,14]

Synthesis: Compounds **1-10** in Table 1 were synthesized in good yield according to the procedures in Scheme 1. (A) is for paclobutrazol-type compounds and (B) is for uniconazole-type compounds. In procedure (A), bromine is introduced to the α-position of a ketone (**a**) to give α-bromoketone. The resulting α-bromoketone (**b**) is coupled with triazole under basic conditions to give **c**, which in turn gives **d** following alkylation in aprotic and basic conditions. Subsequent reduction with sodium borohydride or alkylation with methyl·magnesium bromide gives the target compounds, **e** or **f**, respectively. In procedure (B), triazole derivative **c** is condensed with benzaldehyde under basic conditions to give α,β-unsaturated ketone derivative **h**, which is then reduced with sodium borohydride to give the target compound **i**.
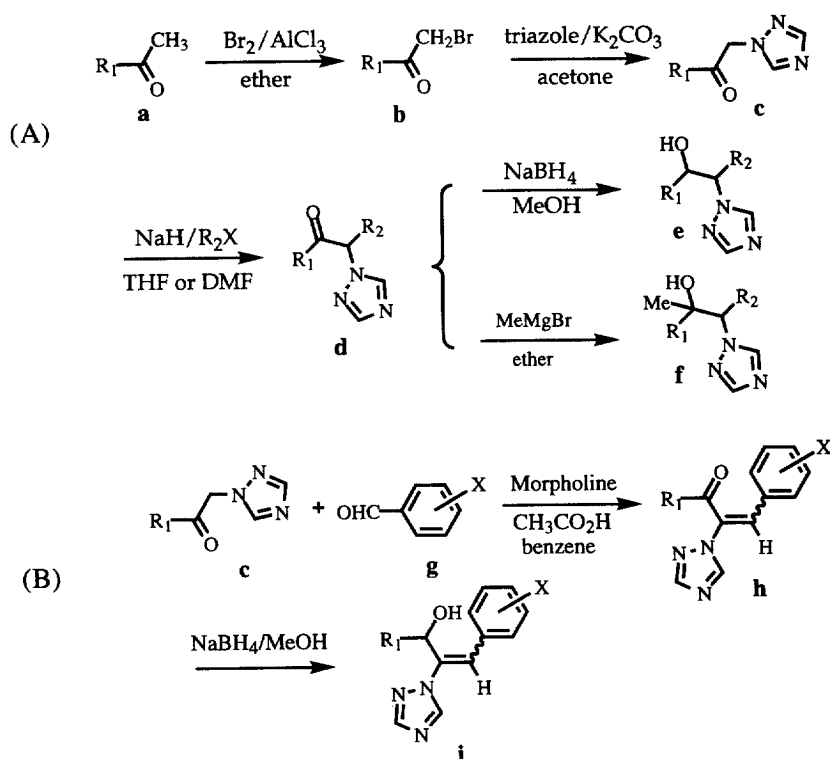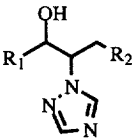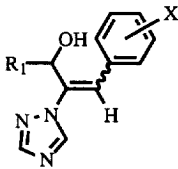
Scheme 1
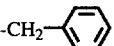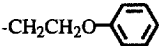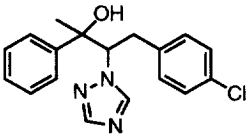
Table 1 Compounds synthesized and assayed in this report.

	R$_1$	R$_2$		R$_1$	X
2	t-Bu	⬡-Cl	**1**	t-Bu	4-Cl
3	t-Bu	-CH$_2$-⬡	**7**	t-Bu	2,4-diCl
4	t-Bu	-CH$_2$CH$_2$O-⬡	**8**	t-Bu	4-O-n-C$_4$H$_9$
5	⬡-	⬡-Cl	**9**	t-Bu	3-OCH$_3$
6			**10**	⬡-	4-Cl

Biological activities: Although the final products of both in procedure (A) and (B) consisted of 4 isomers, these compounds were subjected to biological tests without further purification. To find brassinosteroid biosynthesis inhibitors, we combined some biological assays. First, compounds were assayed using a rice stem elongation test to eliminate gibberellin biosynthesis inhibitors. It is well known that gibberellin biosynthesis inhibitors retard rice stem elongation, and such retardation is rescued by treatment with gibberellin. Therefore, we thought that this test would be suitable for identifying gibberellin biosynthesis inhibitors.
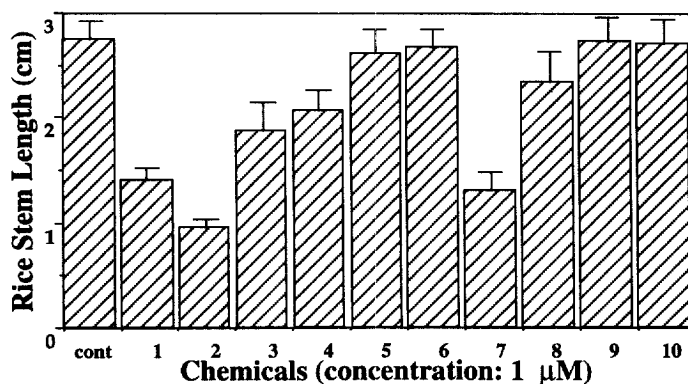

Fig. 1. Rice stem elongation test. Grains of rice (*Oryza sativa* L. cv. Koshihikari) were germinated in an incubator at 25 °C for 24 h and then transplanted into water including chemicals in a glass jar. Stem length was measured 7 days after transplantation.

The results are shown in Fig. 1. Except for **5, 6, 9**, and **10**, other chemicals tested here retarded rice stem elongation, and such retardation was recovered by the addition of gibberellin (GA$_3$) (data not shown), suggesting that such retardation was due to the inhibition of gibberellin biosynthesis. Therefore, **5, 6, 9**, and **10** were considered possible as brassinosteroid biosynthesis inhibitors and subjected to the next test. A good biological system for identifying brassinosteroid biosynthesis inhibitors has not yet been found. Therefore, we tested the ability of some plant systems to evaluate the potency of chemicals. As a result, we selected cress (*Lepidium sativum* L.) as a test plant because it responded well to the inhibitors, and the inhibited plant recovered well following the addition of brassinolide, a most potent brassinosteroid. In addition, cress has been used previously to investigate the effects of brassinolide[15,16] and therefore it could be useful to compare the present results with those obtained previously. The present results are shown in Fig. 2. Compound **6** and **10** inhibited the growth of cress hypocotyl, while **5** and **9** did not. An important observation is the recovery of cress growth after **6** or **10**-induced hypocotyl dwarfism by the co-application of brassinolide with **6** or **10**. On the other hand, GA$_3$ had less of an effect on **6** or **10**-induced dwarfism than brassinolide. This implies that the morphological changes in cress treated with **6** or **10** are mainly due to a deficiency of brassinosteroids and partly due to a deficiency of gibberellin in cress seedlings. Thus, the main target of **6** and **10** appears to be brassinosteroid biosynthesis .
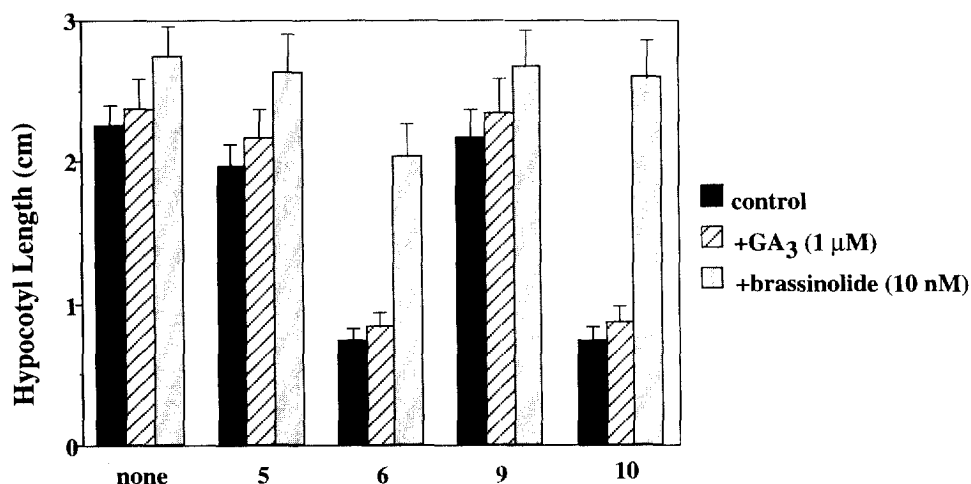

Fig. 2 Cress seeds were sown on 1% agar-solidified medium containing 0.5 x Murashige and Skoog salts and 1.5 % sucrose (w/v) in Agripot (Kirin Brewing. Co., Tokyo, Japan) with or without chemicals. Plants were grown in a growth chamber (25°C) under a 16-hr/8-hr light (240 μE/m^{2}s)/dark cycle. The hypocotyl length was measured 7 days after sowing.

Figure 3 shows the dose response of **6, 10** and uniconazole in the cress test. The length of cress treated with **6** or **10** decreased in a dose-dependent manner. At a concentration of 1 μM or higher, the length is less than half of that in the control, while **6** exhibited no activity at 0.1 μM. Uniconazole showed good potency even at 0.05 μM. Considering that **6** and **10** is the first compound targeting brassinosteroid biosynthesis as a primary site, **6** and **10** may be good lead compounds for optimizing the inhibition of brassinosteroid

biosynthesis, and may play an important role in designing new inhibitors. For example, the structural difference between paclobutrazol (2) and 6 is only the existence of a methyl group and a phenyl group attached to the carbinol carbon. These groups drastically changes the character of triazole derivatives from gibberellin biosynthesis inhibitors to brassinosteroid biosynthesis inhibitors. Therefore, it would be very important to clarify the function of these groups in this inhibition by chemical modification, such as by extension of the methyl group to an ethyl or propyl group, or by substitution with some other functional group. In addition, 6 consists of four stereoisomers: two diasteromers and two enantiomers. It would also be helpful to know the active form for designing new inhibitors.[17]

The step that 6 or 10 block in brassinosteroid biosynthesis is not yet known, but a feeding experiment similar to that done by Fujioka et al. with a brassinosteroid-deficient mutant[18] should reveal the target site of 6 and/or 10.
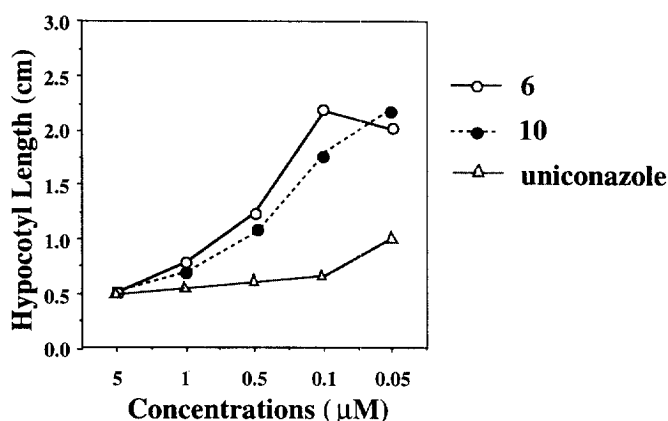

Fig. 3 Inhibitory activity of **6, 10** and uniconazole on cress growth. The experimental procedure was the same as in Fig. 2. The length of a cress hypocotyl without chemical treatment was 2.3 cm.

References and notes:

1. Yokota, T. *Trends in Plant Sci.* **1997**, *2*, 137.

2. Clouse, S. D.; Sasse, J. M. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 427.

3. Mandava, N. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *39*, 23.

4. Schlagnhaufer, C. D.; Arteca, R. N. *J. Plant Physiol.* **1991**, *138*, 191.

5. Iwasaki, T.; Shibaoka, H. *Plant Cell Physiol.* **1991**, *32*, 1007.

6. Yamamoto, R.; Demura, T.; Fukuda, H. *Plant Cell Phsyiol.* **1997**, *38*, 980.

7. Azpiroz, R.; Wu, Y.; LoCascio, J. C.; Feldman, K. A. *Plant Cell* **1998**, *10*, 219.

8. Clouse, S. D. *Plant J.* **1996**, *10*, 1.

9. Fujioka, S.; Sakurai, A. *Physiol. Plant.* **1997**, *100*, 710.

10. Rademacher, W. *Target Site for Herbicide Action;* Böger, P.; Sandmann, G. Eds.; CRC Press: Boca Raton, Florida 1989; pp. 127-145.

11. Yokota, T.; Nakamura, Y.; Takahashi, N.; Nonaka, M.; Sekimoto, H.; Oshio, H.; Takatsuto, S. *Gibberellin*; Takahashi, N.; Phinney, B. O.; MacMillan, J. Eds.; Springer-Verlag: New York 1991; pp. 339-349.

12. Izumi, K.; Yamaguchi, I.; Wada, A.; Ohshio, H.; Takahashi, N. *Plant Cell Physiol.* **1984**, *25*, 611.

13. Rademacher, W. *Gibberellin*; Takahashi, N.; Phinney, B. O.; MacMillan, J. Eds.; Springer-Verlag: New York 1991; pp. 296-310.

14. Sakurai, A.; Fujioka, S. *Biosci. Biotech. Biochem.* **1997**, *61*, 757.

15. Yopp, J.; Mandava, N.; Sasse, J. M. *Physiol. Plant.* **1981**, *53*, 445.

16. Jones-Held, S., VanDoren, M. and Lockwood, T. J. *Plant Growth Regul.* **1996**, *15*, 63.

17. The ^{1}H-NMR (CDCl3, 300 MHz) data for the diastereomers (A and B) of **6** are as follows. (A): 0.898 (s, 9H), 1.352 (s, 3H), 2.133(s, 1H, OH), 3.249 (dd, 1H, J = 11.5, 13.6Hz), 3.454 (dd, 1H, J = 2.5, 13.6Hz), 4.374 (dd, 1H, J = 2.5, 11.5Hz), 6.713 (d, 2H, J = 8.3Hz), 7.115 (d, 2H, J = 8.3Hz), 7.612 (s, 1H), 7.950 (s, 1H). (B): 0.833 (s, 9H), 1.456 (s, 3H), 3.228(dd, 1H, J = 3.6, 13.8Hz), 3.332 (dd, 1H, J = 11.3, 13.8Hz), 3.437 (s, 1H, OH), 4.466 (dd, 1H, J = 3.6, 11.3Hz), 6.747 (d, 2H, J = 8.3Hz), 7.138 (d, 2H, J = 8.3Hz), 7.529 (s, 1H), 7.935 (s, 1H).

18. Fujioka, S.; Li, J. M.; Choi, Y.-H.; Seto, H.; Takatsuto, S.; Noguchi, T.; Watanabe, T.; Kuriyama, H.; Yokota, T.; Chory, J.; Sakurai, A. *Plant Cell* **1997**, *9*, 1951.