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# Synthesis of novel brassinosteroid biosynthesis inhibitors based on the ketoconazole scaffold

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ABSTRACT

Brassinosteroids (BRs) are steroidal plant hormones that control several important agronomic traits such as plant architecture, seed yield, and stress tolerance. Inhibitors that target BR biosynthesis are candidate plant growth regulators. We synthesized novel triazole derivatives, based on the ketoconazole scaffold, that function as inhibitors of BR biosynthesis. The biological activity of the test compounds was evaluated by determining their ability to induce dwarfism in *Arabidopsis* seedlings grown in the dark. The chemically induced dwarfism of *Arabidopsis* seedlings was further evaluated by a rescue experiment using the co-application of brassinolide and/or gibberellins (GA). The structure–activity relationship studies revealed a potent BR biosynthesis inhibitor, 2*RS*, 4*RS*-1-{2-(4-chlorophenyl)-4-[2-(2-ethoxyphenyl)-ethyl]-1, 3-dioxolan-2-ylmethyl}-1*H*-1,2,4-triazole (**7m**), with an $IC_{50}$ value of 0.10 ± 0.03 μM for retardation of *Arabidopsis* seedling stem elongation. The compound-induced hypocotyl dwarfism was counteracted by the co-application of 10 nM brassinolide, but not 1 μM $GA_3$, which produced seedlings that resembled BR-deficient mutants. This result suggests that **7m** is a potent and specific inhibitor of BR biosynthesis.


Brassinosteroids (BRs) are steroidal plant hormones that play critical roles in regulating broad aspects of plant growth and development.[1] When applied exogenously at nanomolar to micromolar levels, BRs exhibit a wide spectrum of physiological effects, including the promotion and/or enhancement of stem elongation, pollen tube growth, leaf bending, ethylene biosynthesis, and stress resistance.[2,3] The functions of endogenous BRs have been elucidated by the characterization of BR synthesis mutants of *Arabidopsis*, tomato, rice, and pea.[4–7] Mutants with impaired BR synthesis display dramatic growth defects such as decreased cell elongation, resulting in pleiotropic dwarf phenotypes.[4–7] Because BRs control several important agronomic traits such as flowering, plant architecture, seed yield, and stress tolerance,[1–3] efforts have recently been made to genetically control BR levels in plant tissues. The use of transgenic techniques to control the BR levels in plant tissues increased grain yields in rice, and available evidence indicates that mutations in BR biosynthesis may be a means to improve biomass production.[8,9] Thus, the biosynthetic pathway of BRs is a potential target site for enhancing the crop yield and/or stress tolerance of plants. The importance of BRs in plant growth and development has sparked great interest in the regulation of BR biosynthesis. An alternative method to control the BR levels in plant tissues is the use of specific inhibitors that target the enzymes responsible for BR biosynthesis; BR biosynthesis inhibitors have consequently become highly viable candidates for plant growth regulators.

With the establishment of the biosynthetic pathways of BRs[10] and functional analysis of BR biosynthesis enzymes, an educated search for specific inhibitors of BR biosynthesis can be conducted. Several steps in BR biosynthesis are carried out by P450 enzymes. CYP90s are involved in C22 and C23 side-chain hydroxylation of BRs,[11] CYP92A6 catalyzes the biochemical conversion of castasterone from typhasterol,[12] and CYP85A2 catalyzes the lactonization of castasterone to brassinolide.[13] Accordingly, strategies for designing P450 inhibitors can be applied to the identification of BR synthesis inhibitors. Cytochrome P450 inhibition mechanisms have been studied in considerable detail.[14] Triazole derivatives demonstrate widespread inhibition of P450s, due to the intrinsic affinity of the nitrogen electron pair in heterocyclic molecules for the prosthetic heme iron. The triazoles thus bind not only to lipophilic regions of the protein but also simultaneously to the prosthetic heme iron.[15] Our research interests are in developing novel plant hormone biosynthesis inhibitors and using these compounds to explore the functions of plant hormones in plant growth and development.[16–19] Toward this end, we carried out a systemic search for novel BR biosynthesis inhibitors by the optimization of known BR biosynthesis inhibitors.

Asami and Yoshida reported the discovery of brassinazole (Brz91, Chemical structure was shown in Fig. 1), the first synthetic

*Abbreviations:* BRs, brassinosteroids; Brz, brassinazole; GA, gibberellin.

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BR biosynthesis inhibitor.[20] Subsequent screening of azole derivatives revealed a series of potent inhibitors of BR synthesis (Fig 1).[21–23] The Brz series have been characterized as potent inhibitors of BR biosynthesis with respect to their high binding affinity to CYP90s.[24,25] The inhibition of other P450 enzymes, such as CYP92A6 and CYP85A2, that are also involved in BR biosynthesis remain unexplored. CYP90s catalyze the hydroxylation of BRs at their side chains, whereas CYP92A6 and CYP85A2 alter the steroid skeleton. The steroid skeleton is a multi-cyclic structure, implying that the introduction of ring structures to an analogue inhibitor may enhance the potency and/or selectivity of those inhibitors. To explore this possibility, we carried out a systemic search for novel BR inhibitors based on the chemical structure of ketoconazole (Fig. 1). Ketoconazole was selected as a molecular scaffold for the following reasons: (1) ketoconazole shares a 1,3-dioxolane moiety with Brz220 (Fig. 1), the most potent inhibitor of BR biosynthesis reported to date;[21] (2) ketoconazole is a well-known P450 inhibitor that is widely used experimentally and clinically,[27] suggesting that analogues of ketoconazole may inhibit P450 enzymes involved in BR synthesis; and (3) the target compounds (Fig. 1) would be analogues with a substituted phenoxy moiety, a ring structure, instead of the propyl moiety found in BRz220 (Fig. 1). Based on this rationale, we synthesized a series of novel triazole derivatives and evaluated their inhibitory activity on BR biosynthesis.

The development of a synthetic route for the preparation of target compound **7** is outlined in Scheme 1. The key transformation of **2** with **5** consisted of four steps: (1) formation of 1-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1yl) ethanone **2**; (2) tosylation of isopropylideneglycerol **3**; (3) deprotection of isopropylidene ketal **4**; and (4) ketal formation to generate **6**. Compound **2** was prepared by reacting *a*-bromoketone **1** with triazole in DMF using a method that we described previously.[26] The tosylation of isopropylidene glycerol **3** was achieved using a standard protocol (tosyl chloride in pyridine at 0 °C), and hydrolysis with 1 M HCl in MeOH yielded glyceryl tosylate **5**. Ketal formation to generate **6** was carried out using 3 equiv of trifluoromethanesulfonic acid (TfOH) in toluene at room temperature for 60 h, according to a method previously described.[28] The target compound **7** was prepared by reacting **6** with corresponding phenols in a basic condition, as described previously.[29] All of the compounds synthesized in this work consist of four stereoisomers, and they were subjected to biological studies without further purification.

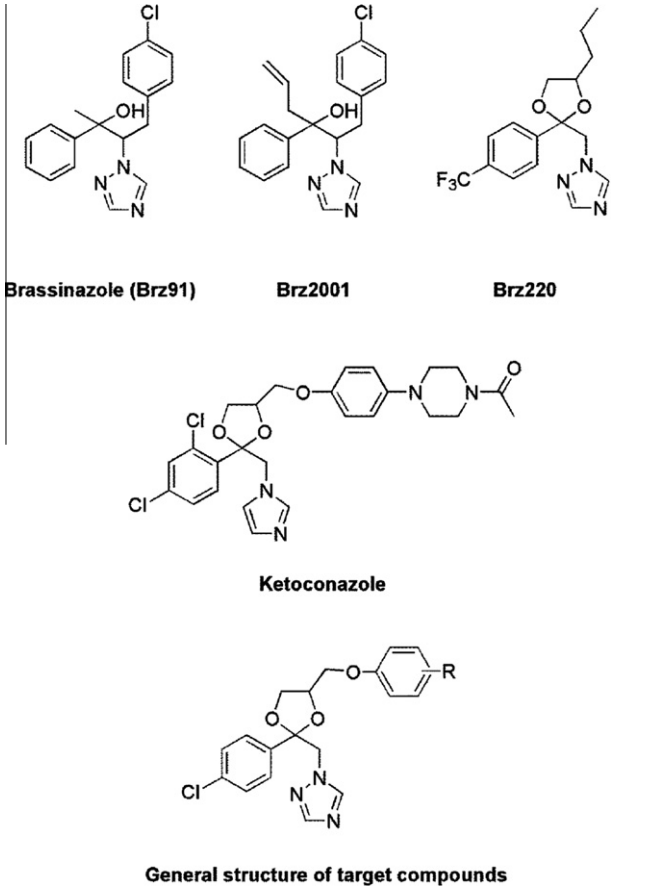
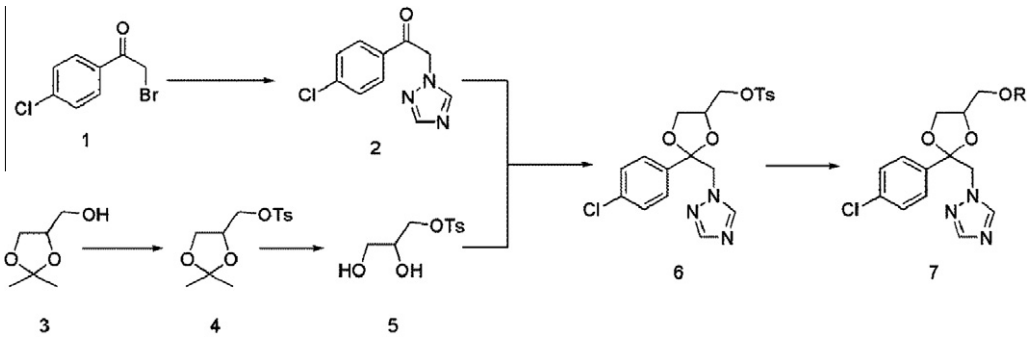



**Figure 1.** Chemical structure of brassinosteroid biosynthesis inhibitors.

The bioassay for evaluating the activity of BR biosynthesis inhibitors was carried out using *Arabidopsis* seedlings grown in the dark, as previously described.[21] In the postembryonic development of higher plants, light signals activating the phytochrome or photoreceptors induce photomorphogenesis and de-etiolation, resulting in the inhibition of hypocotyl elongation, the opening of the apical hook of cotyledons, the induction of greening, and the elongation of leaf primordia. In the absence of light, the elongation of the hypocotyl and root is not inhibited, and the apical hook of cotyledons is maintained. *Arabidopsis* BR synthesis-deficient mutants such as *dwarf 1* show remarkable dwarfism and the opening of the apical hook of cotyledons in the dark.[4] This unique de-etiolation in the dark phenotype has been used for screening for BR biosynthesis inhibitors.[21] In the present study, we adapted this assay method to determine the effects of test compounds on hypocotyl elongation of *Arabisopsis* seedlings grown in the dark, and we co-applied BL and GA with the test compounds to determine the reversibility of their effects. With this assay system, we evaluated the biological activities of synthesized compounds.

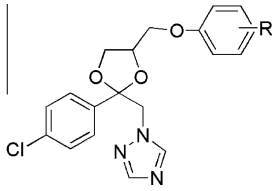
The chemical structures of compounds tested in the biological studies are shown in Table 1. To identify the chemical substituent on the phenoxy moiety that was responsible for the retardation of *Arabidopsis* stem elongation, various substituents were introduced onto the aromatic ring (compounds **7a**–**j**). Compound **7a,** which has no substituent on the phenyl ring, was used as a baseline reference, and brassinazole (Brz) was used as a positive control for structure–activity relationship discussions. The concentrations of all of the test compounds were assigned to be 0, 0.1, 0.5, and 1 μM, and the $IC_{50}$ values were calculated accordingly. As shown in Table 1, compound 7**a** exhibits inhibitory activity, retarding hypocotyl elongation of *Arabidopsis* seedling grown in the dark, with an $IC_{50}$ value of 0.41 ± 0.13 μM, whereas the $IC_{50}$ of Brz was 0.58 ± 0.38 μM in our assay system. Analogues **7b** and **7c** contain a chlorine atom at position 2 and 3 of phenyl ring and enhanced the inhibitory activity, with $IC_{50}$ values of 0.13 ± 0.02 and 0.11 ± 0.04 μM, respectively. Interestingly, moving the chlorine atom to position 4 (**7d**) reduced the inhibitory activity, with an $IC_{50}$ value of 0.32 ± 0.10 μM. The $IC_{50}$ values of analogues **7e**–**7j**, which contain two chlorines atoms with different variations on the phenyl ring, are between 0.14 and 0.87 μM. These results suggest that the introduction of two chlorines atoms on the phenyl moiety (**7e**–**7j**) did not produce a significant effect on the compound's ability to retard hypocotyl elongation of *Arabidopsis* seedlings, compared with that of mono-chloride substitute analogues (**7b**–**7d**). It is worthwhile to note that chlorine substitution at position 4 seemed to have a negative effect on the inhibitory potency. As shown in Table 1, analogue **7b** is more potent than **7f**, and analogue **7c** is more potent than **7i**. Among the compounds with chlorine atom(s) substituents in Table 1 (**7a**–**7j**), compound **7b**, **7c**, **7g**, and **7h** exhibit potent inhibitory activity.

GA biosynthesis inhibitors such as paclobutrazol retard the stem elongation of many plant species by blocking *ent*-kaurene oxidation and also mildly affect other cytochrome P450 monooxygenases.[22] This retardation can be rescued by the application of GA.



**Scheme 1.** Reagents and conditions: (a) 1,2,4-triazole, triethylamine, DMF, −10 °C, 1 h, rt, 3 h; (b) TsCl, pyridine, 0 °C, acetone; (c) HCl, Reflux, 6 h; (d) 3 equiv TfOH, toluene, rt, 60 h; and (e) phenol, KOH, DMF, 50 °C, 12 h.

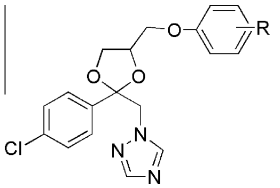
**Table 1**
Inhibitory activity of triazole derivatives on *Arabidopsis* seedling growth



| No. | R | Inhibition of *Arabidopsis* stem elongation ($IC_{50}$) (μM)* |
|---|---|---|
| **7a** | H | 0.41 ± 0.13 |
| **7b** | 2-Cl | 0.13 ± 0.02 |
| **7c** | 3-Cl | 0.11 ± 0.04 |
| **7d** | 4-Cl | 0.32 ± 0.10 |
| **7e** | 2,3-$Cl_2$ | 0.51 ± 0.08 |
| **7f** | 2,4-$Cl_2$ | 0.36 ± 0.10 |
| **7g** | 2,5-$Cl_2$ | 0.14 ± 0.05 |
| **7h** | 2,6-$Cl_2$ | 0.17 ± 0.09 |
| **7i** | 3,4-$Cl_2$ | 0.39 ± 0.05 |
| **7j** | 3,5-$Cl_2$ | 0.87 ± 0.04 |
| **7k** | 2-F | 0.14 ± 0.06 |
| **7l** | 2-$OCF_3$ | 0.17 ± 0.03 |
| **7m** | 2-OEt | 0.10 ± 0.03 |
| **7n** | 2-OMe | 0.14 ± 0.05 |
| **7o** | 2-Me | 0.17 ± 0.04 |
| Brz | — | 0.58 ± 0.38 |

* The $IC_{50}$ values of the test compounds for the inhibition of *Arabidopsis* stem elongation were calculated by determining the hypocotyl length of untreated *Arabidopsis* seedlings as 0% inhibition and using hypocotyl length of 0 mm as 100% inhibition. Data were collected from 15 to 20 seedlings. All of the experiments were performed at least in duplicate to establish their replicability.

**Table 2**
Retardation of *Arabidopsis* seedling growth by triazole derivatives and rescue of growth by BL and GA



| No. | R | Hypocotyl length, % relative to untreated *Arabidopsis* seedlings (%) | | |
|---|---|---|---|---|
| | | Chem. | Chem. + BL (10 nM) | Chem. + GA (1 μM) |
| Control | — | **100** | 117 ± 6 | 112 ± 8 |
| **7a** | H | 50 ± 3 | 111 ± 7 | 53 ± 5 |
| **7b** | 2-Cl | 30 ± 4 | 104 ± 9 | 33 ± 3 |
| **7c** | 3-Cl | 36 ± 7 | 72 ± 8 | 46 ± 5 |
| **7d** | 4-Cl | 53 ± 3 | 90 ± 9 | 58 ± 4 |
| **7e** | 2,3-$Cl_2$ | 44 ± 5 | 72 ± 9 | 57 ± 4 |
| **7f** | 2,4-$Cl_2$ | 58 ± 6 | 87 ± 11 | 62 ± 5 |
| **7g** | 2,5-$Cl_2$ | 32 ± 5 | 102 ± 12 | 38 ±4 |
| **7h** | 2,6-$Cl_2$ | 43 ± 5 | 100 ± 8 | 56 ± 4 |
| **7i** | 3,4-$Cl_2$ | 58 ± 6 | 81 ± 13 | 63 ± 6 |
| **7j** | 3,5-$Cl_2$ | 78 ± 5 | 96 ± 6 | 85 ± 4 |
| **7k** | 2-F | 29 ± 4 | 96 ± 6 | 34 ± 5 |
| **7l** | 2-$OCF_3$ | 20 ± 3 | 83 ± 5 | 26 ± 4 |
| **7m** | 2-OEt | 22 ± 4 | 93 ±4 | 22± 3 |
| **7n** | 2-OMe | 25 ± 5 | 92 ± 4 | 34 ± 5 |
| **7o** | 2-Me | 28 ± 4 | 95 ± 5 | 32 ± 4 |
| Brz | — | 34 ± 4 | 63 ± 6 | n.d.* |

* n.d.: not determined.

To rule out the possibility of GA biosynthesis inhibition by our analogues, we tested the effects of brassinolide, the most biologically active BR, and GA on the recovery of chemically induced dwarfism of *Arabidopsis* seedlings grown in the dark. Compounds **7a** to **7j** were subjected to the bioassay at a concentration of 0.5 μM, and *Arabidopsis* seedlings were grown in the presence of BL (10 nM) or GA (1 μM) for 5 days in the dark. Data shown in Table 2 are expressed in percentage relative to the untreated control. As shown in Table 2, in the presence of BL (10 nM) or GA (1 μM), the average hypocotyl length of *Arabidopsis* seedlings was approximately 117 ± 6 and 112 ± 8%, respectively. This result indicates that BL and GA stimulate hypocotyl elongation of *Arabidopsis* seedlings. We found that compound **7b**, **7c**, **7g**, and **7h** exhibited high inhibitory activity on *Arabidopsis* seedling elongation. The hypocotyl length of chemically treated *Arabidopsis* seedlings was approximately 30 ± 4, 36 ± 7, 32 ± 5, and 43 ± 5% of the untreated seedlings, respectively, whereas the positive control Brz (1 μM) was approximately 34 ± 4%. Co-application of BL (10 nM) showed different recoveries for different test compounds. Among the compounds **7a**–**7j** listed in Table 2, compound **7b**, **7g**, and **7h** showed good recovery with BL application; the hypocotyl lengths were 104 ± 9, 102 ± 12, and 100 ± 8% of the untreated control, respectively. Although compound **7c** exhibited potent inhibitory activity on *Arabidopsis* seedling growth, the recovery with BL was relatively low (72 ± 8%) and was partially recovered by the application of GA (from 36 ± 7% to 46 ± 5%). This result suggests that **7c** may partially inhibit GA biosynthesis.

The data obtained here clearly indicate that compound **7b** is the most potent compound among the chlorine substituent analogues, showing strong inhibition of hypocotyl elongation in *Arabidopsis* seedlings. This activity can be reversed to control levels (30 ± 4% to 104 ± 9%) by the co-application of BL (10 nM), whereas the co-application of GA (1 μM) did not show significant recovery (from 30 ± 4% to 33 ± 3%). Thus, we conclude that compound **7b** is the best compound among the chlorine substituted analogues.

To further study the structure–activity relationship of this synthetic series, we carried out the chemical modification of **7b** by introducing different substituents at position 2 of the phenyl

moiety (**7k**–**7o**). As shown in Table 1, all of the compounds (**7k**–**7o**) exhibited potent inhibitory activity on the elongation of *Arabidopsis* seedlings. We next investigated the primary site of action of these compounds by the co-application BL and GA, as described above. As shown in Table 1, all of the compounds exhibited high reversibility to BL but not GA. We found that compound **7m** was the most potent BR biosynthesis inhibitor among this synthetic series.

Thus, we have discovered and tested a novel series of specific inhibitors of BR biosynthesis. The compounds used in the biological assays consisted of four stereoisomers, but the asymmetric synthesis of these stereoisomers has been well established.[28] We expect that further studies on the stereochemical structure–activity relationship of this synthetic series may provide insights regarding the binding site of this synthetic series. Work on BR biosynthesis inhibitors such as brassinazole (Brz) has demonstrated the usefulness of inhibitors in BR research. The application of Brz to a standard genetic mutant screen that confers resistance to inhibitors allowed the identification of novel components such as *bzr1* that affect brassinosteroid signal transduction.[30] The development of chemicals that induce phenotypes of interest would provide a useful method to study biological pathways in plants, serving as a complement to classical biochemical and genetic methods.

In conclusion, we have discovered and evaluated the activity of a novel series of BR biosynthesis inhibitors. Structure–activity relationship studies revealed that 2*RS*, 4*RS*-1-{2-(4-chlorophenyl)-4-[2-(2-ethoxyphenyl)-ethyl]-1,3-dioxolan-2-ylmethyl}-1*H*-1,2,4-triazole (**7m**) is a highly selective inhibitor of BR biosynthesis. Work on the identification of the target enzyme(s) of these synthetic BR biosynthesis inhibitors is currently in progress.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.12.120.

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