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Biological Evaluation of 5-Substituted Pyrimidine Derivatives as Inhibitors of Brassinosteroid Biosynthesis

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A series of 5-substituted pyrimidine derivatives was synthesized, and their ability to inhibit brassinosteroid biosynthesis was tested. The biological activity of these compounds was evaluated by the cress stem elongation method. Among the synthesized compounds, α-(4-chlorophenyl)-α-phenyl-5-pyrimidinemethanol (DPPM 4) exhibited potent inhibitory activity for retarding cress stem elongation in the light. This inhibition was reversed by the application of 10 nM brassinolide, but not by 1 μM GA_3. DPPM 4 also affected *Arabidopsis* growth in the dark. DPPM 4-treated *Arabidopsis* had phenotypes like those of brassinosteroid-deficient mutants, with short hypocotyls and open cotyledons, in the dark. These biological changes were restored by the co-application of 10 nM brassinolide, but not by 1 μM GA_3, suggesting that the primary site of action of DPPM 4 was the brassinosteroid biosynthetic pathway.

Key words: brassinosteroids biosynthesis; inhibitor; pyrimidine derivative

Brassinosteroids (BRs) are naturally occurring steroids that are widely distributed in the plant kingdom. Since the discovery of brassinolide (BL), which was originally isolated from the pollen of *Brassica napus* L.,[1] more than 40 BRs have been identified from various plant species.[2] Based on their diverse physiological effects and requirement for plant growth,[3-6] it is now widely accepted that BRs are important phytohormones that regulate plant growth and development.[7-9]

Recent progress in chemical studies on BRs, coupled with the application of molecular genetics to the analysis of BR biosynthesis, have given information about the biosynthesis of BRs.[7-9] In the biosynthetic pathway of BRs, many steps are thought to involve cytochrome P450s. These steps include the production of 6α-hydroxycampestanol from campestanol, cathasterone from 6-oxo-campestanol,[10] teasterone from cathasterone,[11] castasterone from typhasterol, and brassinolide from castasterone.[8] These facts suggest that the biosynthetic pathway of BRs involves potential sites for cytochrome P450 inhibitors.

The use of specific biosynthesis inhibitors is an alternative way to determine the physiological functions of endogenous substances and the mechanism for regulating their biosynthesis. As has been shown in mode-of-action studies on gibberellins (GAs), GA-deficient mutants and GA biosynthesis inhibitors were both quite effective.[12-13] Therefore, a specific inhibitor of BR biosynthesis could provide a new and complementary approach to understanding the function of BRs and the regulation mechanism for BR biosynthesis.

Azoles are compounds that inhibit many types of cytochrome P450. These compounds include uniconazole, a potent inhibitor of GA biosynthesis,[14] which reduced the concentration of endogenous castasterone[15] and inhibited brassinolide (BL)-induced tracheary element differentiation,[16] implying that uniconazole would also affect BR biosynthesis. We have recently reported that brassinazole (Brz; its structure is shown in Fig. 1), a specific inhibitor of BR biosynthesis,[17] strongly inhibited BR biosynthesis by interfering with the cytochrome P450 enzyme catalyzing the biochemical conversion of cathasterone to teasterone in the biosynthesis of BRs.[18] These observations suggest that cytochrome P450 inhibitors could possibly inhibit BR biosynthesis. We have consequently been searching for new inhibitors of BR biosynthesis by chemically modifying known cytochrome P450 inhibitors.

Pyrimidine derivatives such as fenarimol (its structure is shown in Fig. 1) are compounds that inhibit many types of cytochrome P450. These compounds inhibit the cytochrome P450 enzyme responsible for the 14α-demethylation of ergosterol biosynthesis,[19] ent-kaurene oxidation[20] and aromatase (estrogen synthetase) isolated from rats.[21]

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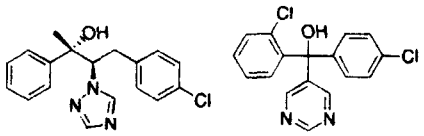
Abbreviations: BRs, brassinosteroids; BL, brassinolide; Brz, brassinazole; GA, gibberellin; **DPPM 4**, α-(4-chlorophenyl)-α-phenyl-5-pyrimidinemethanol



Brassinazole Fenarimol

Fig. 1. Structural Formulae for Brassinazole and Fenarimol.

We report in this paper a series of newly synthesized 5-substituted pyrimidine derivatives (named the **DPPM** series) that inhibit BR biosynthesis.

Materials and Methods

Plant materials and growth conditions. Seeds of cress (*Lepidium sativum* L.) were purchased from Sakata Co., and *Arabidopsis* (ecotype Columbia) was purchased from Lehle Seeds (Round Rock, TX, U.S.A.). The seeds used for the assay were sterilized in 1% NaOCl for 20 min and washed with sterile distilled water. Seeds were sown on a 1% solidified- agar medium containing half Murashige and Skoog salts and 1.5% sucrose (w/v) in agripots (Kirin Brewery. Co., Tokyo, Japan) with or without added chemicals. Plants were grown in 16-hr light (240 μE/m^{2}s) and 8-hr dark conditions in a growth chamber (25°C) with or without added chemicals. To create the dark conditions, agripots were wrapped in four layers of aluminum foil. The biological activities of the test compounds were measured 7 days after sowing the seeds.

Synthesis of pyrimidinemethanols. Melting point (mp) data were determined with Yanako melting point apparatus. ^{1}H-NMR spectra were recorded with a Bruker AC-300 Plus spectrometer, chemical shifts being expressed in ppm downfield from TMS as an internal standard. 5-substituted pyrimidine derivatives were prepared by the method described previously.[21]

α,α-Bisphenyl-5-pyrimidinemethanol **(1)**. A solution of 5-bromopyrimidine (1.27 g, 8 mmol) and benzophenone (1.45 g, 8 mmol) in tetrahydrofuran (30 ml) was cooled to −80°C to produce a white slurry. A 5-ml amount of 1.6 M *n*-BuLi in hexane was added dropwise to the slurry over 10 min, and the reaction mixture formed a thick paste. After the reaction mixture had been allowed to warm slowly to ambient temperature, it was stirred for 10 min. The reaction mixture was cooled to −20°C, and water (5 ml) was added. The upper organic phase was concentrated under reduced pressure. The resulting residue was dissolved in hot toluene (50°C, 20 ml) and successively washed with water (50 ml) containing concentrated HCl (0.1 ml) and then with water (3 × 50 ml). The toluene was evaporated under reduced pressure. After being recrystallized twice from toluene and hexane, 1.26 g of the target compound (α,α-bisphenyl-5-pyrimidinemethanol; 60% yield) was obtained. Mp 168–169°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.59 (1H, br), 7.21–7.26 (4H, m), 7.31–7.37 (6H, m), 8.56 (2H, s), 9.03 (1H, s). *Anal.* Found: C, 77.87; H, 5.35; N, 10.64%. Calcd. for $C_{17}H_{14}N_2O$: C, 77.84; H, 5.38; N, 10.68%.

Pyrimidine carbinols **2–13** were prepared in a similar way by adding 5-lithiopyrimidine to the appropriate starting material (a series of ketones) instead of benzophenone. Poorly crystallized compounds were purified by column chromatography on silica gel.

α-(4-Methoxyphenyl)-α-phenyl-5-pyrimidinemethanol **(2)**. Mp 177–180°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.70 (3H, s), 5.29 (1H, s), 6.51–6.53 (1H, m), 6.85–6.89 (1H, m), 6.97–7.00 (1H, m), 7.22–7.37 (6H, m), 8.59 (2H, s), 9.13 (1H, s). *Anal.* Found: C, 73.86; H, 5.52; N, 9.48%. Calcd. for $C_{18}H_{16}N_2O_2$: C, 73.96; H, 5.52; N, 9.58%.

α-(2-Methoxyphenyl)-α-phenyl-5-pyrimidinemethanol **(3)**. Mp 93–95°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.61 (1H, s), 3.81 (3H, s), 6.83–7.14 (4H, m), 7.23–7.36 (5H, m), 8.65 (2H, s), 9.02 (1H, s). *Anal.* Found: C, 73.40; H, 5.54; N, 9.49%. Calcd. for $C_{18}H_{16}N_2O_2$: C, 73.96; H, 5.52; N, 9.58%.

α-(4-Chlorophenyl)-α-phenyl-5-pyrimidinemethanol **(4)**. Mp 161–162°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) d: 4.46 (1H, s), 6.75–6.77 (1H, m), 7.17–7.45 (8H, m), 8.62 (2H, s), 9.10 (1H, s). Anal. Found: C, 68.84; H, 4.34; Cl, 11.82; N, 9.40%. Calcd. for $C_{17}H_{13}ClN_2O$: C, 68.81; H, 4.42; Cl, 11.95; N, 9.44%.

α-(2-Chlorophenyl)-α-phenyl-5-pyrimidinemethanol **(5)**. Glassy. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 5.14 (1H, s), 7.15–7.19 (4H, m), 7.25–7.32 (5H, m), 8.53 (2H, s), 8.84 (1H, s). *Anal.* Found: C, 68.90; H, 4.38; Cl, 11.82; N, 9.52%. Calcd. for $C_{17}H_{13}ClN_2O$: C, 68.81; H, 4.42; Cl, 11.95; N, 9.44%.

α-(2-Fluoro-4-trifluoromethylphenyl)-α-phenyl-5-pyrimidinemethanol **(6)**. Amorphous. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 7.22–7.44 (9H, m), 8.68 (2H, s), 9.16 (1H, s). Anal. Found: C, 61.82; H, 3.48; F, 21.96; N, 7.97%. Calcd. for $C_{18}H_{12}F_4N_2O$: C, 62.07; H, 3.47; F, 21.82; N, 8.04%.

α,α-Bis(4-chlorophenyl)-5-pyrimidinemethanol **(7)**. Mp 158–159°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 4.38 (1H, s), 6.96–7.17 (4H, m), 7.30–7.33 (4H, m), 8.57 (2H, s), 8.97 (1H, s). *Anal.* Found: C, 61.85; H, 3.64; Cl, 21.56; N, 8.26%. Calcd. for $C_{17}H_{12}Cl_2N_2O$: C, 61.65; H, 3.65; Cl, 21.41; N, 8.46%.

α,α-Bis(4-fluorophenyl)-5-pyrimidinemethanol (**8**). Amorphous. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.10 (1H, s), 7.05–7.10 (4H, m), 7.22–7.28 (4H, m), 8.69 (2H, s), 9.14 (1H, s). *Anal.* Found: C, 68.37; H, 4.09; F, 12.62; N, 9.37%. Calcd. for $C_{17}H_{12}F_2N_2O$: C, 68.45; H, 4.05; F, 12.74; N, 9.39%.

α-(4-Fluorophenyl)-α-phenyl-5-pyrimidinemethanol (**9**). Mp 118–119°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 2.97 (1H, s), 7.02–7.07 (2H, m), 7.20–7.26 (5H, m), 7.34–7.38 (2H, m), 8.69 (2H, s), 9.13 (1H, s). *Anal.* Found: C, 72.79; H, 4.64; F, 6.84; N, 9.90%. Calcd. for $C_{17}H_{13}FN_2O$: C, 72.85; H, 4.67; F, 6.78; N, 9.99%.

α-(4-Chlorobenzyl)-α-phenyl-5-pyrimidinemethanol (**10**). Mp 158–160°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 1.58 (2H, s), 2.51 (1H, s), 6.90–6.93 (2H, m), 7.20–7.26 (4H, m), 7.32–7.41 (3H, m), 8.74 (2H, s), 9.09 (1H, s). *Anal.* Found: C, 69.48; H, 4.81; Cl, 11.21; N, 8.84%. Calcd. for $C_{18}H_{15}ClN_2O$: C, 69.57; H, 4.86; Cl, 11.41; N, 9.01%.

α-(2,4-Difluorophenyl)-α-phenyl-5-pyrimidinemethanol (**11**). Mp 112–113°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.41 (1H, d, J=7.5 Hz), 6.87–6.99 (3H, m), 7.25–7.28 (4H, m), 7.40–7.42 (4H, m), 8.69 (2H, s), 9.19 (1H, s). *Anal.* Found: C, 68.28; H, 4.05; F, 12.72; N, 9.25%. Calcd. for $C_{17}H_{12}F_2N_2O$: C, 68.45; H, 4.05; F, 12.74; N, 9.39%.

α-(2-Fluorophenyl)-α-(4-fluorophenyl)-5-pyrimidinemethanol (**12**). Mp 121–123°C. ^{1}H-NMR ($CDCl_3$/TMS, ppm) δ: 3.60 (1H, d, J=9.5 Hz), 6.85–6.90 (1H, m), 7.06–7.18 (3H, m), 7.24–7.29 (3H, m), 7.40–7.43 (1H, m), 8.68 (2H, s), 9.19 (1H, s). *Anal.* Found: C, 68.40; H, 3.98; F, 12.71; N, 9.36%. Calcd. for $C_{17}H_{12}F_2N_2O$: C, 68.45; H, 4.05; F, 12.74; N, 9.39%.

α-(3,4-Dichlorophenyl)-α-phenyl-5-pyrimidinemethanol (**13**). Amorphous. ^{1}H-NMR ($CDCl_3$/TMS, ppm) d: 4.53 (1H, s), 7.04–7.07 (1H, m), 7.15–7.19 (2H, m), 7.32–7.39 (5H, m), 8.58 (2H, s), 9.04 (1H, s). *Anal.* Found: C, 61.85; H, 3.45; Cl, 21.52; N, 8.35%. Calcd. for $C_{17}H_{12}Cl_2N_2O$: C, 61.65; H, 3.65; Cl, 21.41; N, 8.46%.

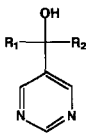
Results and Discussion

Biological activity of the DPPM series

We have previously shown cress (*Lepidium sativum* L.) to be a useful plant for screening BR biosynthesis inhibitors, because cress responded well to the inhibitors, and the inhibited plant recovered well after adding BL, a very potent brassinosteroid.[22] We therefore used this assay method to evaluate the inhibitory activity of the newly synthesized compounds.

It has been reported that chemical substitution on the aromatic ring affected the inhibitory activity against cytochrome P450-dependent aromatase.[21] Accordingly, we introduced various substituents on to the aromatic ring of α,α-bisphenyl-5-pyrimidinemethanol derivatives (Table 1), and tested their inhibitory activity for reducing the longitudinal growth of cress (Fig. 2). The test compounds inhibited the longitudinal growth of cress at a concentration of 3 μM (Fig. 2). Chemical modification of the phenyl substitution in this synthesized series did not significantly enhance the retardation of cress stem elon-

Table 1. Compounds Synthesized and Assayed in This Report



	R_1	R_2
1	phenyl	phenyl
2	phenyl	*p*-methoxyphenyl
3	phenyl	*o*-methoxyphenyl
4	phenyl	*p*-chlorophenyl
5	phenyl	*o*-chlorophenyl
6	phenyl	2-fluoro-4-trifluoromethylphenyl
7	*p*-chlorophenyl	*p*-chloromethoxyphenyl
8	*p*-fluorophenyl	*p*-fluorophenyl
9	phenyl	*p*-fluorophenyl
10	phenyl	*p*-chlorobenzyl
11	phenyl	2,4-difluorophenyl
12	*o*-fluorophenyl	*p*-fluorophenyl
13	phenyl	3,4-dichlorophenyl

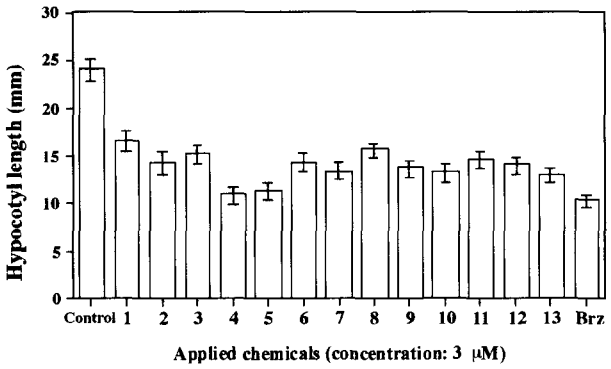


Fig. 2. Effect of **DPPM** Series and Brz on Cress Stem Elongation.

Cress seeds were sown on a 1% agar-solidified medium containing half Murashige and Skoog salts and 1.5% sucrose (w/v) in an agripot (Kirin Brewery. Co., Tokyo, Japan) with or without chemicals. The plants were grown in a growth chamber (25°C) under a 16-hr/8-hr light (240 mE/m^{2}s)/dark cycle. The hypocotyl length was measured 7 days after sowing. The average length of untreated cress hypocotyls calculated from 11 seeds was 24 mm. The compound number refers to the data in Table 1. The experiment was done three times, a vertical bar representing S.D.

gation. Of the synthesized compounds, compound 4 was the most active (named **DPPM 4**) and was used for further investigations.

Figure 3 shows the dose-response effects of **DPPM 4**, Brz and uniconazole (a GA biosynthesis inhibitor) on cress seedling growth. The length of cress treated with uniconazole and Brz decreased in a dose-dependent manner, the concentration of these compounds for 50% inhibition being 0.05 μM and 0.8 μM, respectively (Fig. 3). This result confirmed the early observations that the cress assay method was effective for screening BR biosynthesis inhibitors.[22] **DPPM 4** also showed dose-dependent inhibition of the longitudinal growth of cress. At a concentration of 3 μM or more, the length was less than half of the control value, the I_{50} value for **DPPM 4** to retard cress stem elongation in the light being 2.5 μM by the cress assay (Fig. 3). This result indicates that **DPPM 4** exhibited potent inhibitory activity by retarding cress stem elongation.

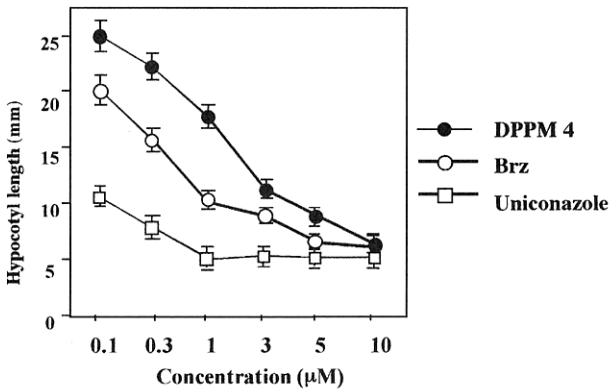


Fig. 3. Inhibitory Activity of **DPPM 4**, Brz and Uniconazole Toward Cress Growth.

The experimental procedure was the same as that described in Fig. 2. The average length of untreated cress hypocotyls calculated from 11 seeds was 24 mm. The experiment was done three times, a vertical bar representing S.D.

Mode of action of **DPPM 4**

Effects of GA and BL on **DPPM 4***-treated cress.* GA biosynthesis inhibitors are known to retard the stem elongation of many plant species. This retardation can be rescued by the application of GA. Similarly, a common characteristic of brassinosteroid-deficient mutants and Brz-treated cress or *Arabidopsis* is reduced longitudinal growth.[17-18] To investigate the mode of action of the **DPPM** series, we examined the effects of GA and BL on **DPPM 4**-treated cress seedlings. While **DPPM 4** retarded the stem elongation of cress seedlings, this biological activity could be reversed by the co-application of 10 nM BL, but not of 1 μM GA_3 (Fig. 4). This result indicates that **DPPM 4** may have affected BR biosynthesis.

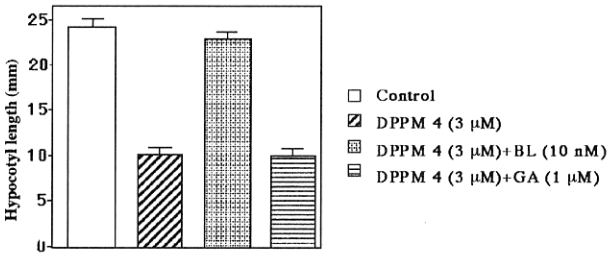


Fig. 4. Effects of BL and GA3 on **DPPM 4**-Treated Cress (7-day-old).

The experimental procedure was the same as that described in Fig. 2. The experiment was done three times, a vertical bar representing S.D.

Effect of **DPPM 4** *on the morphological changes to Arabidopsis seedlings germinated in the dark.* *Arabidopsis* mutants such as *det2* and *cpd* have a deetiolated shape with short hypocotyls and open cotyledons in the dark. These transformations can be rescued by the application of BL, although other plant hormones have no effect.[8] We therefore examined the effects of **DPPM 4** on *Arabidopsis* growth in the dark.

DPPM 4 caused marked a malformation of seedlings which were morphologically similar to the brassinosteroid-deficient mutants. 10 μM **DPPM 4**-treated seedlings (Fig. 5B) had short hypocotyls and open cotyledons, whereas the hypocotyls were elongated and the hook of the cotyledons was closed in the non-treated seedlings (Fig. 5A). The longitudinal growth of 10 μM **DPPM 4**-treated seedlings recovered slightly with the co-application of 1 μM GA_3, although the hook of the cotyledons remained open in the dark (Fig. 5C). Co-application of 10 nM BL to 10 μM **DPPM 4**-treated seedlings significantly restored the hypocotyl elongation, the hook of the cotyledons also being restored to the condition of non-treated seedling (Fig. 5D). This result clearly indicates that **DPPM 4** exhibited inhibitory activity toward BR biosynthesis. Comparing the results of the cress and *Arabidopsis* tests, while co-application of 1 μM GA_3 to 3 μM **DPPM 4**-treated cress did not have any recovery effect on stem elongation (Fig. 4), co-application of 1 μM GA_3 to 10 μM **DPPM 4**-treated *Arabidopsis* showed slight recovery of stem elongation (Fig. 5C). This result suggests that, at a concentration of 10 μM, **DPPM 4** may have partially inhibited GA biosynthesis as a side effect.

The inhibition mechanism for pyrimidine derivatives is believed to operate by binding heterocyclic nitrogen atoms to protoheme iron of cytochrome P450 enzyme.[19] Since many steps in BR biosynthesis involve cytochrome P450,[8] **DPPM 4** may possibly inhibit the biochemical conversion involving cytochrome P450 on the biosynthetic pathway of BRs. Although our data could not identify the target site of the pyrimidine derivatives, further investigation on the target site could be carried out with feeding experiments, as has been done by Asami *et al.*[18]

The **DPPM** series are structurally related to the po-

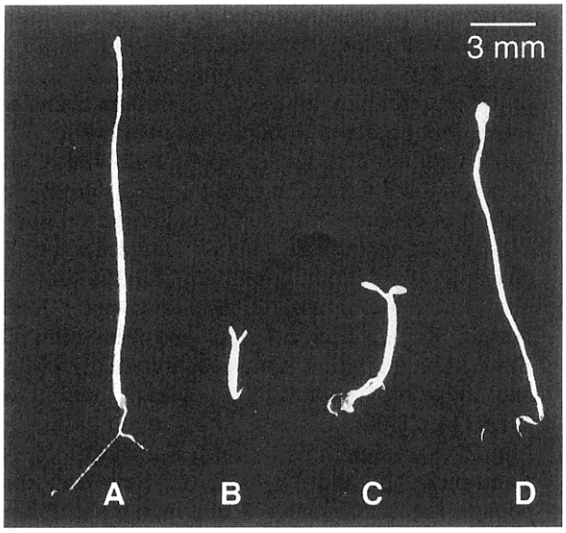


Fig. 5. Effect of **DPPM 4** on *Arabidopsis* Growth (12-day-old) in the Dark.

10 μM **DPPM 4**-treated seedlings (B) had short hypocotyls and open cotyledons, whereas the hypocotyls were elongated and the hook of the cotyledons was closed in the non-treated seedlings (A). Co-application of 1 μM GA3 slightly recovered the longitudinal growth of the 10 μM **DPPM 4**-treated *Arabidopsis* seedlings, but the hook of the cotyledons remained open in the dark (C). Co-application of 10 nM BL to the 10 μM **DPPM 4**-treated *Arabidopsis* seedlings significantly restored the hypocotyl elongation, and the hook of the cotyledons was also restored to the condition of non-treated seedlings (D). The experimental procedure was the same as that described in Fig. 2, the agripots being wrapped in four layers of aluminum foil. The experiment was done three times.

tent fungicide, fenarimol. A comparison of the action of these fungicides on fungi shows that their effects on plant growth and development were limited. These fungicides are known to have a plant growth-retarding (PGR) side effect, although the mechanism for this is far from being understood.[19] Since the retardation of hypocotyl elongation can be restored by BL, but not by GA, our results suggest that the side effect of pyrimidine fungicides like fenarimol on plant growth retardation was due to the inhibition of BR biosynthesis.

Present knowledge about the role of BR biosynthesis in plant growth and development comes mainly from studying brassinosteroid-deficient mutants and the physiological effects of brassinosteroids. Recently discovered Brz, a specific inhibitor of BR biosynthesis, has shown that BR biosynthesis inhibitors were useful to determine the functions of BRs and their biosynthesis.[18] We have demonstrated in this report that the newly synthesized pyrimidine compounds (**DPPM** series) exhibited inhibitory activity toward BR biosynthesis. Further studies on the structure-activity relationship and identification the target site of these newly synthesized compounds should provide new information about BR biosynthesis.

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