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# Structure–Function Relationships of Four Stereoisomers of a Brassinolide Mimetic on Hypocotyl and Root Elongation of the Brassinosteroid-Deficient *det2-1* Mutant of *Arabidopsis*

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**Abstract** Brassinosteroids (BRs) are a group of plant hormones and the bioactive BR, brassinolide (BL), is causally implicated in promoting cell elongation and cell proliferation. In *Arabidopsis,* the biosynthesis of BL is essential for hypocotyl etiolation in the dark, and application of bioactive BRs can promote both hypocotyl and root elongation, although high concentrations of applied BRs result in inhibition of root elongation. A non-steroidal structure consisting of four stereoisomers was designed to contain subunits bearing key functional groups mimicking those of BL. The bioactivity of each of these individual stereoisomers was tested using the *Arabidopsis thaliana det2-1* mutant line, which is deficient in BL, and thus does not etiolate in the dark. Application of BL at each of 0.1, 1.0, and 10.0 μM promotes hypocotyl elongation in dark-grown *det2-1* plants while simultaneously inhibiting elongation of their primary root. In contrast, the mimetic structures, when applied to dark-grown *det2-1* plants, promote hypocotyl elongation without negatively affecting primary root elongation. In fact, two of the mimetic structures, applied at a 10 μM concentration, significantly promoted both hypocotyl and root elongation. Correlation of this contrasting behavior with the configurations of the hydroxylated stereocenters of the mimetics is described. This is the first example of a non-steroidal BL mimetic where the biological activities of individual stereoisomers were tested and compared.

**Keywords** Brassinosteroids · Brassinolide · Non-steroidal mimetics of brassinolide · *Arabidopsis det2-1* mutant · Hypocotyl and root elongation

## Introduction

Brassinosteroids (BRs) are a class of plant hormones that have been shown to be involved in the stem etiolation that occurs in the dark (Li and others 1996; Nagata and others 2000; Choe 2010). To date over fifty BR structures have been identified from a wide range of species, including gymnosperms, angiosperms, pteridophytes, and algae (Fujioka and others 2002). Among the BRs identified so far, brassinolide (BL) exhibits the greatest growth-promoting activity (Grove and others 1979; Takatsuto and others 1983). The BRs also participate in cell proliferation (Clouse and others 1993) and applied BL promotes cell division in cell cultures of *Arabidopsis* (*Arabidopsis thaliana*) (Hu and others 2000) and tobacco (*Nicotiana tabacum*) (Miyazawa and others 2003). Low concentrations of applied BRs can also promote root growth in several higher plants, including *Arabidopsis* (Clouse and others 1996; Müssig and others 2003; González-García and others 2011) and tomato (*Solanum lycopersicum*) (Roddick and others 1993), though higher concentrations inhibited root growth.

However, it is unclear if the promotive effects of BRs on plant growth is direct or indirect, for example, the latter being via interactions with other plant hormones, such as

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auxins, gibberellins, or abscisic acid (Cohen and Meudt 1983; Katsumi 1991; Tanaka and others 2003; Symons and Reid 2003a, b; Kurepin and others 2008; 2012a; Kurepin and Pharis 2014).

The structure:function relationship between BRs and their biological activities have been studied by several groups (Yokota and Mori 1992; Adam and others 1996; Brosa 1997; 1999; Khripach and others 1999; Andersen and others 2001; Back and Pharis 2003, Back and others 2004). In view of the low natural abundance and lengthy synthetic protocols that make BL expensive and difficult to prepare, a series of twelve non-steroidal structures (mimetics) was designed to mimic the structure and activity of BL. These compounds contained hydroxylated subunits connected by rigid linkers that could be superimposed upon the 2,3-dihydroxylated A-ring and the 22,23-diol side-chain moiety of BL. In addition, most of the mimetics also contained polar phenolic or ketone substituents to emulate the lactone-containing B-ring of BL (Andersen and others 2001). Several of the mimetic structures exhibited BL-like bioactivity in the rice (*Oryza sativa* L.) leaf lamina bending bioassay (Andersen and others 2001). However, these compounds, including a highly active pentahydroxylated bis(tetralin)acetylene, were initially prepared and assayed as mixtures of stereoisomers. To gain further insight into the effects of stereochemistry upon the biological activity of the pentahydroxylated bis(tetralin)acetylene mimetics, the four possible stereoisomers (two diastereomeric pairs of enantiomers: M21, M56, and M44, M59; Fig. 1b) were synthesized individually (Back and others 2004). The four stereoisomers were then assessed independently using the *det2-1 Arabidopsis* mutant, which has a known defect in an early stage of BR biosynthesis, for example, it is blocked in the conversion of campesterol to campestanol (Li and others 1996). Plants of the *det2-1* mutant line thus exhibit a dwarf shoot phenotype, especially in the dark where they show no hypocotyl etiolation, but do exhibit good growth of the primary root (Li and others 1996). The absence of hypocotyl elongation in *det2-1* is the result of reduced cell size and also reduced cell numbers (Szekeres and others 1996). However, application of BL to *det2-1* seedlings germinated in the dark can restore the wild type tall phenotype, and this BL-induced growth restoration was attributed both to increased cell elongation and cell division (Nakaya and others 2002).

We have thus utilized dark-grown *det2-1 Arabidopsis* plants to assess the bioactivity of each of the four mimetic stereoisomers on hypocotyl growth. The four mimetics and BL were applied via an agar medium, and elongation growth of each of the hypocotyl and primary root was measured 7 days after germination and growth in the dark for the *det2-1* seedlings.

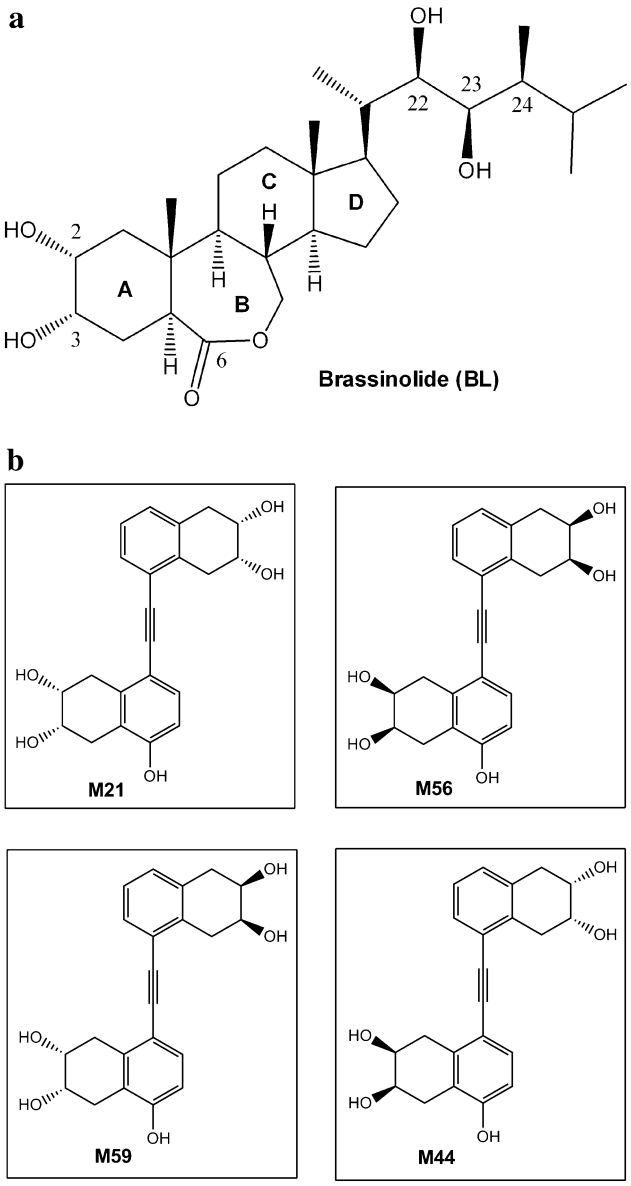


**Fig. 1** **a** Structure of brassinolide (BL); **b** structures of four non-steroidal BL mimetics (M21, M44, M56, and M59)

## Materials and Methods

Brassinolide (BL; Fig. 1a) was synthesized using the method of Back and others (1997). Brassinolide mimetics (Fig. 1b) were synthesized as described in Back and others (2004). *Arabidopsis thaliana det2-1* seeds (obtained from the Arabidopsis Biological Research Center at Columbus OH, USA) were prepared for germination in the dark as described in Kurepin and others (2012b). Brassinolide and the four mimetics were initially dissolved in DMSO and then diluted with double distilled water to achieve a concentration of 10 μM. Further dilution using a 0.1 % DMSO solution was used to achieve lower concentrations of the

mimetics and of BL. The *Arabidopsis det2-1* plants were grown in Petri dishes in a dark room at a constant temperature of 20 °C. Seven days after planting the seeds, the hypocotyl and root lengths were measured. Each treatment (each compound) was tested at 0.1, 1.0, and 10.0 μM concentrations. There were three separate repeat trials, each with at least four replicate Petri dishes, and ten plants were used for each concentration in each trial. The statistical significance of growth differences across the treatments was assessed using Tukey's ANOVA test.

## Results and Discussion

Application of BL at concentrations of 0.1, 1, and 10 μM in the growth medium on which the BR-deficient *Arabidopsis det-2* seeds were planted resulted, as expected (Li and others 1996), in about 2.5 to 2.6-fold increases in hypocotyl elongation (Fig. 2a). All three concentrations of BL were highly effective in promoting hypocotyl growth, relative to the two controls. The higher 10 μM concentration was slightly, but significantly more effective than the two lower BL concentrations (Fig. 2a). Application of DMSO, the solvent used to dissolve BL and various mimetics and had no significant effect on hypocotyl elongation in any of the trials (Figs. 2, 3, 4).

Among the four individual mimetic stereoisomers applied to the *det2-1* seedlings (Fig. 2c, d), M44 exhibited the highest efficacy for promoting hypocotyl elongation, though it was, at 10 μM, less active than BL when applied at the two lower doses (0.1 and 1.0 μM) (Fig. 2a). The second best mimetic structure for hypocotyl elongation of the *det2-1* plants was M21, which caused modest (but significant) hypocotyl elongation at all three concentrations (Fig. 2c). Finally, mimetic structures M56 and M59 showed the lowest activity in promoting hypocotyl elongation, with only the two highest concentrations (1.0 and 10.0 μM) giving a small, but significant increase in hypocotyl elongation (Fig. 2b, c).

Application of BL at all concentrations (0.1, 1, and 10 μM) resulted in appreciable and highly significant inhibition of primary root elongation for the *det2-1* plants (Fig. 3a). The endogenous levels of bioactive BRs are significantly lower in wild type *Arabidopsis* roots relative to shoots (Shimada and others 2003). Thus, the three BL concentrations which we tested here are likely well above the physiologically active levels of endogenous BR that are required for normal root elongation. For example, application of 24-epibrassinolide (EBL) at a concentration as low as 0.01 μM resulted in a greater than 30 % inhibition of root elongation in wild type *Arabidopsis* plants grown in hydroponics solution (Müssig and others 2003). In contrast,

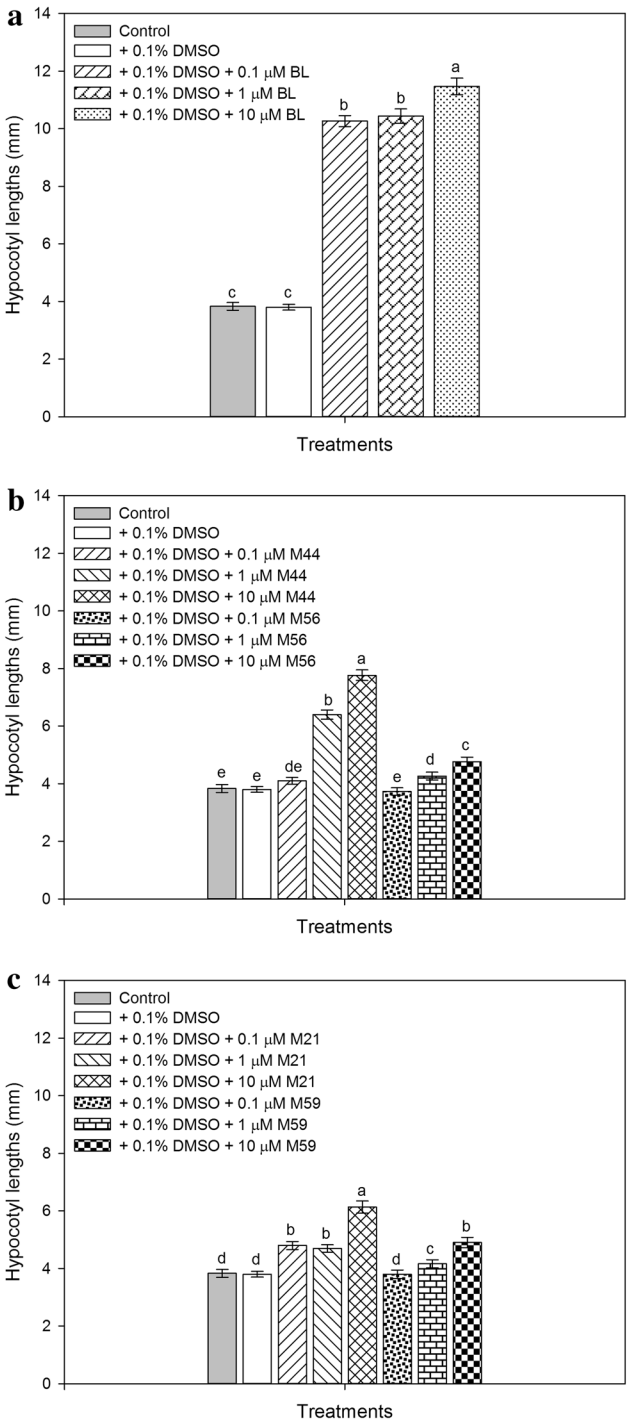


**Fig. 2** Hypocotyl lengths of 7-day old *Arabidopsis thaliana*, *det2-1* mutant seedlings treated with BL (**a**), mimetics M44 and M56 (**b**), or mimetics M21 and M59 (**c**). Seedlings were germinated and grown in continuous darkness, and controls were either untreated or treated with 0.1 % v/v DMSO, the solvent used to initially dissolve BL and the four mimetics. Treatment solutions were diluted with 0.1 % DMSO to $10^{-7}$, $10^{-6}$, or $10^{-5}$ M concentrations for brassinolide (BL) and the various mimetics. The *error bars* represent one SE of the mean. Mean values with the *same letter* do not differ significantly at $P \leq 0.05$ based on one-way ANOVA (Tukey's) test

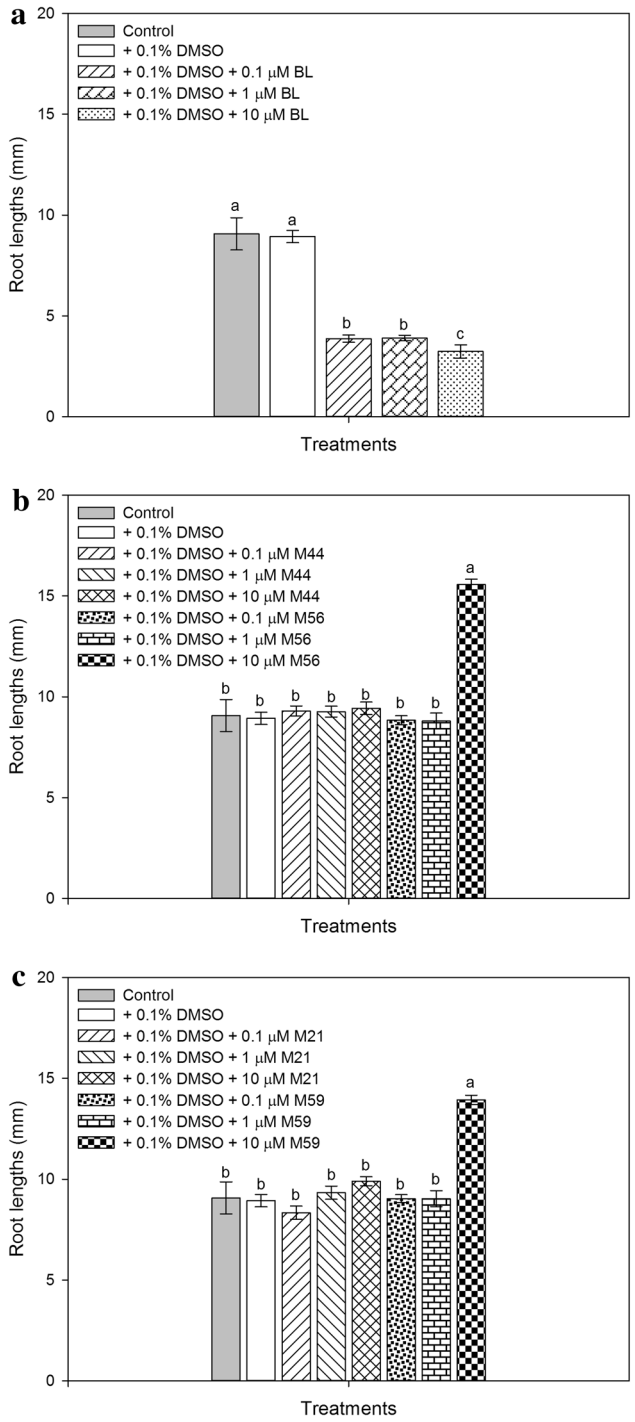


**Fig. 3** Root lengths of 7-day old *Arabidopsis thaliana, det2-1* mutant seedlings treated with BL (**a**), mimetics M44 and M56 (**b**), or mimetics M21 and M59 (**c**). Seedlings were germinated and grown in continuous darkness. Treatments with BL or the non-steroidal BL mimetics were accomplished as described above in the legend for Fig. 2. The *error bars* represent one SE of the mean. Mean values with the *same letter* do not differ significantly at $P \leq 0.05$ based on one-way ANOVA (Tukey's) test

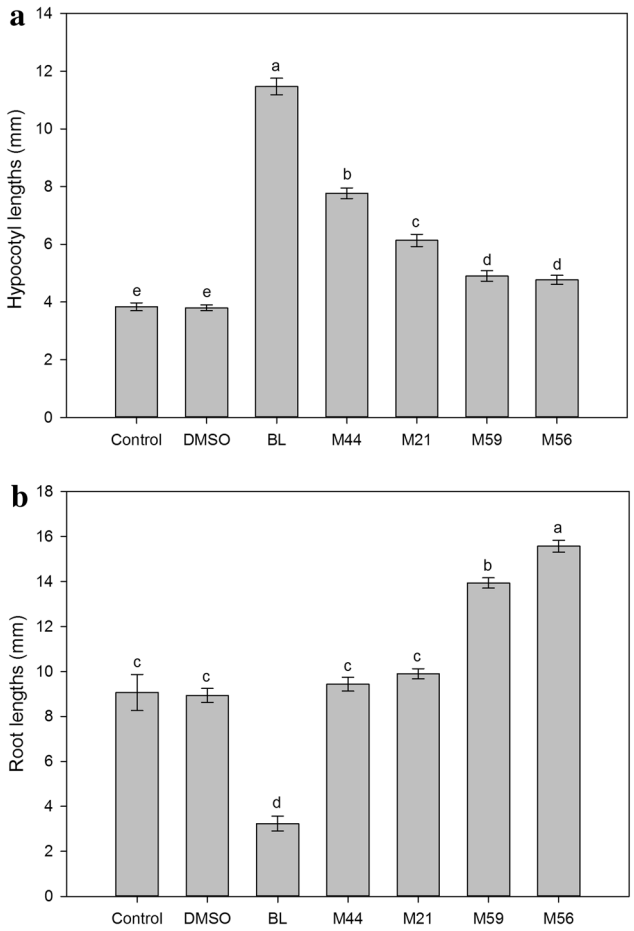


**Fig. 4** Hypocotyl (**a**) and root (**b**) lengths of 7-day old *Arabidopsis thaliana, det2-1* mutant seedlings were germinated and grown in continuous darkness. Treatments with $10^{-5}$ M concentrations of BL or of the non-steroidal BL mimetics were accomplished as described above in the legend for Fig. 2. The *error bars* represent one SE of the mean. Mean values with the *same letter* do not differ significantly at $P \leq 0.05$ based on one-way ANOVA (Tukey's) test

application of a 0.1 nM concentration of BL increased root elongation of agar-grown wild type *Arabidopsis* plants by about 20 % (Kim and others 2007).

Two of the mimetic structures thus exhibited relatively high bioactivity in promoting hypocotyl elongation, that is, M44 and M21 (Fig. 2b, c). However, neither of M44 or M21 had any effect on root elongation (Fig. 3b, c). Interestingly, the two mimetic structures which exhibited only low (but significant) bioactivity in the promotion of hypocotyl elongation, M56 and M59 (Fig. 2b, c), caused appreciable and significant increases in root elongation, but only when applied at the highest (10 μM) concentration tested. Therefore, M56 and M59 possess even lower BR-like bioactivity than M44 or M21. Application of EBL to

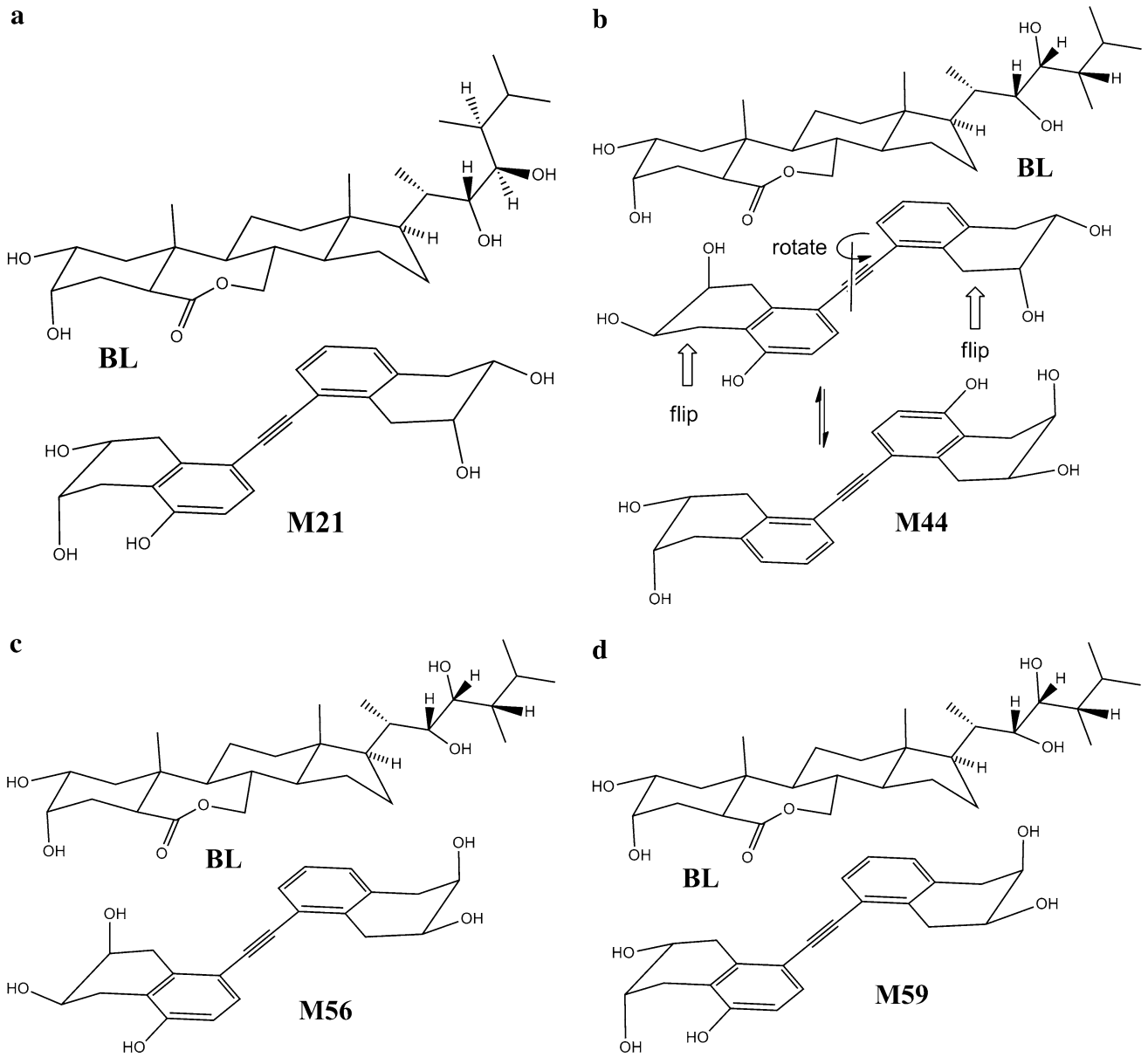


**Fig. 5** Comparison of BL conformations with non-steroidal mimetics M21, M44, M56, and M59. **a** BL and M21; **b** BL and M44; **c** BL and M56; **d** BL and M59. Only the side-chain conformation of BL that most closely matches that of the mimetic is shown in each case

another BR-deficient *Arabidopsis* mutant, *cbb3* (Szekeres and others 1996), at concentrations of 0.1 and 1 nM, caused approximately 1.5-fold increases in root elongation for hydroponically grown *cbb3* plants (Müssig and others 2003). In the present work, application of each of M56 or M59 at a concentration of 10 μM to our agar-grown *det2-1* plants also caused significant 1.5-fold increases in root elongation (Figs. 3b and c).

Figure 4 summarizes the bioactivity (on each of hypocotyl and root growth) of BL and each of the four mimetics when all compounds were applied at the highest (10 μM) concentration. Hypocotyl (a) and root (b) elongation responses are each shown relative to the untreated control and the 0.1 % DMSO-treated *det2-1* seedlings. Although all four mimetic structures caused significant increases in hypocotyl elongation, the two mimetics that yielded the highest hypocotyl elongation (M44 and M21) showed absolutely no effect on root elongation. Also, the other two mimetics, M56 and M59, which significantly promoted both hypocotyl and root elongation, increased root elongation about two- to three-fold more than the root elongation seen for control *det2-1* seedlings.

The difference in behavior between M21 and M44, relative to M56 and M59, suggests that the configurations

at the hydroxylated stereocenters play an important role in determining biological activity. Thus, M21 and M44 stereoisomers resemble BL, in strongly promoting hypocotyl elongation. In contrast, stereoisomers M56 and M59 are more akin to very low doses of BL, which can significantly promote root elongation (Kim and others 2007). This suggests that the two sets of stereoisomers interact differently with the BR receptors. For example, Fig. 5a shows that M21 superimposes reasonably well on BL, which is in accord with their similar promotive bioactivities on hypocotyl elongation. The gauche *cis*-diol moieties of M21 correspond closely with the 2,3-*cis* and 22(*R*),23(*R*)-diol functions of BL, whereas the phenolic hydroxyl group of M21 juxtaposes with the polar lactone carbonyl group of BL. However, the even stronger BL-like activity of M44 is somewhat surprising, as its attempted superimposition on BL reveals that the overlay of one diol unit and the phenolic hydroxyl group of M44 on its counterpart components in BL (the A-ring diol and B-ring lactone), results in poor overlap of the other mimetic diol group with that of the 22,23-diol group of BL, regardless of the side-chain conformation of the latter (Fig. 5b). Even so, it can be seen that better superimposition of the key diols can be achieved at the expense of a complete mismatch by rotation of the former structure and ring-flipping of the two tetralin half-chairs (Fig. 5b). Stereoisomer M56 (the enantiomer of M21) displays the poorest match with BL (Fig. 5c) and its low BL-like activity is therefore not surprising. On the other hand, M59 (Fig. 5d) resembles the rotated and ring-flipped form of M44 shown in Fig. 5b, but even so, M59 shows poor BL-like activity. It is also not clear why M56 and M59 are the only stereoisomers that promote root elongation, in a manner similar to a very low concentration of BL (Kim and others 2007).

Our results indicate that non-steroidal mimetics of BL can be designed and constructed, that their stereochemistry plays a crucial role in their bioactivity and that, depending on the configurations of their hydroxylated stereocenters, the mimetics are capable of promoting hypocotyl and/or primary root growth.

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