Pergamon

PII: S0031-9422(98)00367-7

Phytochemistry Vol. 49, No. 7, pp. 1849–1858, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

STRUCTURE–ACTIVITY STUDIES OF BRASSINOLIDE B-RING ANALOGUES

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(*Received* 22 *January* 1998; *in revised form* 27 *April* 1998)

Key Word Index—Rice leaf lamina inclination assay; brassinosteroids; brassinolide; brassinolide B-ring analogues; indole-3-acetic acid.

Abstract—Six new analogues of brassinolide were prepared in order to investigate their structure–activity relationship: 7-azabrassinolide, 7-thiabrassinolide, 6-deoxybrassinolide, B-homocastasterone, 6-methylidenecastasterone and 6-methylidene-B-homocastasterone. These compounds were subjected to the rice leaf lamina inclination assay, in comparison with brassinolide and 24-epibrassinolide and/or castasterone to test for brassinosteroid activity. The activity of 7-azabrassinolide, 7-thiabrassinolide and 6-deoxybrassinolide was comparable to that of 24-epibrassinolide, but lower than that of brassinolide. B-Homocastasterone was less active than either brassinolide or castasterone. The B-ring carbocycles 6-methylidenecastasterone and 6-methylidene-B-homocastasterone were essentially inactive. These results indicate that neither the oxygen atom at the 7-position of brassinolide, nor its carbonyl group, is essential for activity. However, the complete absence of a polar functional group from the B-ring, as in 6-methylidenecastasterone and 6-methylidene-B-homocastasterone, results in a total loss of bioactivity. This inactivity persists even in the presence of an exocyclic methylidene group that flattens the B-ring to resemble that of brassinolide or castasterone by virtue of the sp^2-hybridized carbon atom at C-6. Finally, the bioactivity of several, but not all, of the brassinosteroids, was significantly and synergistically increased by the simultaneous application of the auxin, indole-3-acetic acid.

INTRODUCTION

Ever since the discovery of the potent plant growth-regulator brassinolide (**1**) by Grove *et al*. in 1979 [1], there has been intense interest in its chemical and biological properties and in related brassinosteroid analogues [2–7]. It has been suggested that **1** interacts very specifically with a putative receptor, ultimately leading to the expression of genes responsible for the growth induced by **1** [8–11]. Unfortunately, **1** occurs in very low concentrations in plant tissues [2, 3] and is difficult and expensive to synthesize. Structure–activity relationships of brassinosteroids have been conducted to identify key features that are required for biological activity [10, 12–16]. This information is potentially useful, both for providing insight into possible brassinosteroid–receptor interactions and in the task of developing more effective and less expensive brassinosteroids for agricultural and horticultural applications. Structure–activity studies have thus demonstrated that the (2α,3α)- and (22*R*,23*R*)-vicinal diol moieties are required for optimum bioactivity, although conversion of the side chain hydroxyl groups of **1** to the corresponding methyl ethers in **2** affords a product that retains significant biological activity [17]. The nature of the substituent and configuration at C-24 are also important, since 24-epibrassinolide (**3**), 28-homobrassinolide (**4**) and 28-norbrassinolide (**5**) show generally diminished bioactivity compared to **1**. However, it should be noted that the relative activities of brassinosteroids do vary with the type of bioassay used [12, 14]. The greater ease of synthesis of **3** and **4** compared to **1** has made them popular subjects in field trials, despite their lower bioactivity [2]. Moreover, the 5α-configuration (A/B *trans*-fused rings) is required for optimum activity, although some 5β analogues have recently been reported to show substantial activity [13].

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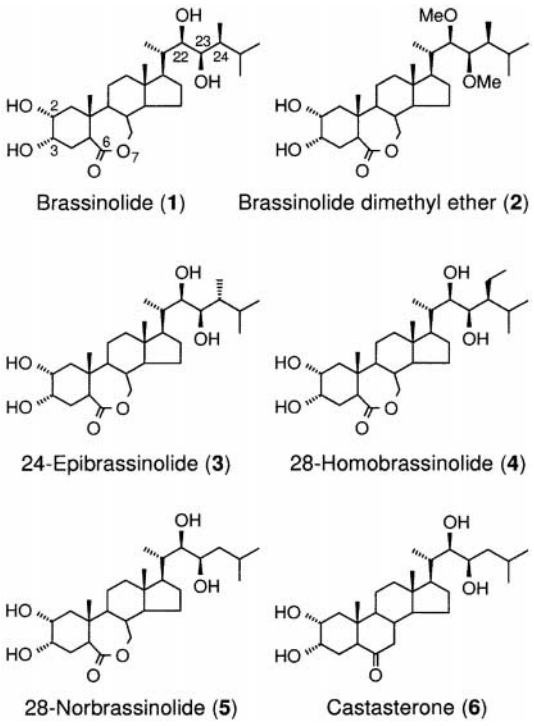

Fig. 1. Structures of brassinosteroids.

The effect of the unusual seven-membered B-ring lactone on the bioactivity of brassinosteroids has also been investigated. The 6-oxalactones are much less active than their 7-oxa regioisomers [15, 18, 19], but castasterone (**6**) (for the structures of **1–6**, see Fig. 1), where the seven-membered B-ring lactone of **1** is replaced by a six-membered ketone, shows significant per se activity in some plant species [20], although it can serve as the biosynthetic precursor of brassinolide in others [21]. Several heterocyclic B-ring analogues have been previously reported [22–25], but these have side chains that differ from **1**, thereby precluding direct comparisons of their bioactivity with that of **1**. We now report the preparation of a series of novel B-ring analogues and their bioactivity as measured by the rice leaf lamina inclination bioassay [26]. Since synergy between brassinosteroids and auxins has been previously noted [6, 26, 27], we also report the effects of applying the novel brassinosteroids together with the auxin, indole-3-acetic acid (IAA).

RESULTS AND DISCUSSION

Compounds **7** and **8** were selected for investigation because they should demonstrate the effect of replacing the ring oxygen at C-7 of **1** with the heteroatoms N and S, respectively. Compound **9** should indicate the effect of removing the carbonyl function at C-6. B-Homocastasterone (**10**) was selected for comparison with the naturally occurring six-membered B-ring analogue **6** to see if expansion to a seven-membered ring (i.e. as in **1**) would increase activity. Furthermore, comparison of **10** with **7**, **8** and **1** should show the effect of replacing the heteroatom at C-7 with carbon. Finally, the exocyclic methylidene derivatives **11** and **12** (for the structures of 7–12, see Fig. 2) were chosen for comparison with castasterone (**6**) and brassinolide (**1**), respectively. The olefinic moieties of **11** and **12** are expected to flatten the B-ring in a manner similar to that of the carbonyl groups of **6** and **1** because of the sp^2-hybridized carbon atom at C-6 that is present in all four compounds. This comparison should therefore provide insight into whether the lactone ring in brassinolide (**1**) interacts with a putative receptor through polar effects (H-bonding, dipole interactions, etc.), or whether the lactone ring merely maintains a suitable B-ring conformation for optimum interaction with the active site of the receptor.

The synthesis of compounds **7–12** was achieved as shown in Fig. 3. Brassinolide (**1**), prepared by our previously reported procedure [28, 29], was converted into its bisacetonide **13**. Transesterification of **13** with sodium methoxide provided **14**. Direct cyclization of the tosylate of **14** with ammonia, or sulfurization with potassium thioacetate followed by cyclization, produced lactam **7** and thiolactone **8**, respectively, after deprotection. Reduction of **13** to diol **15**, followed by monotosylation, intramolecular Williamson ether synthesis and deprotection, afforded the cyclic ether **9**.

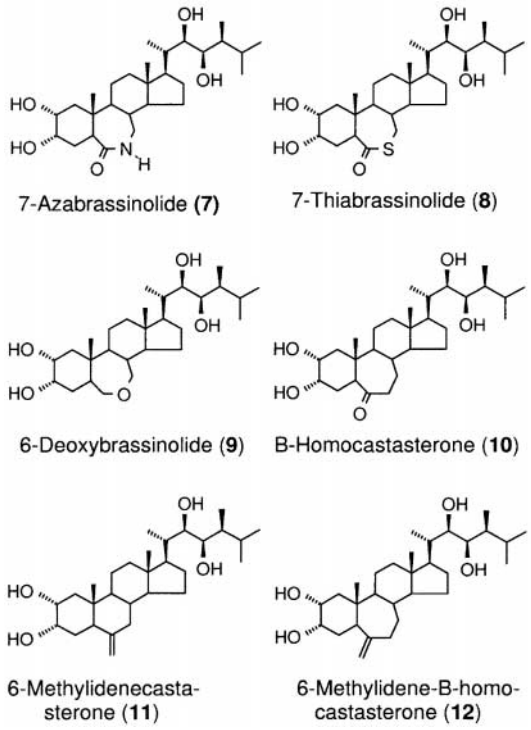

Fig. 2. Novel brassinosteroid B-ring analogues.

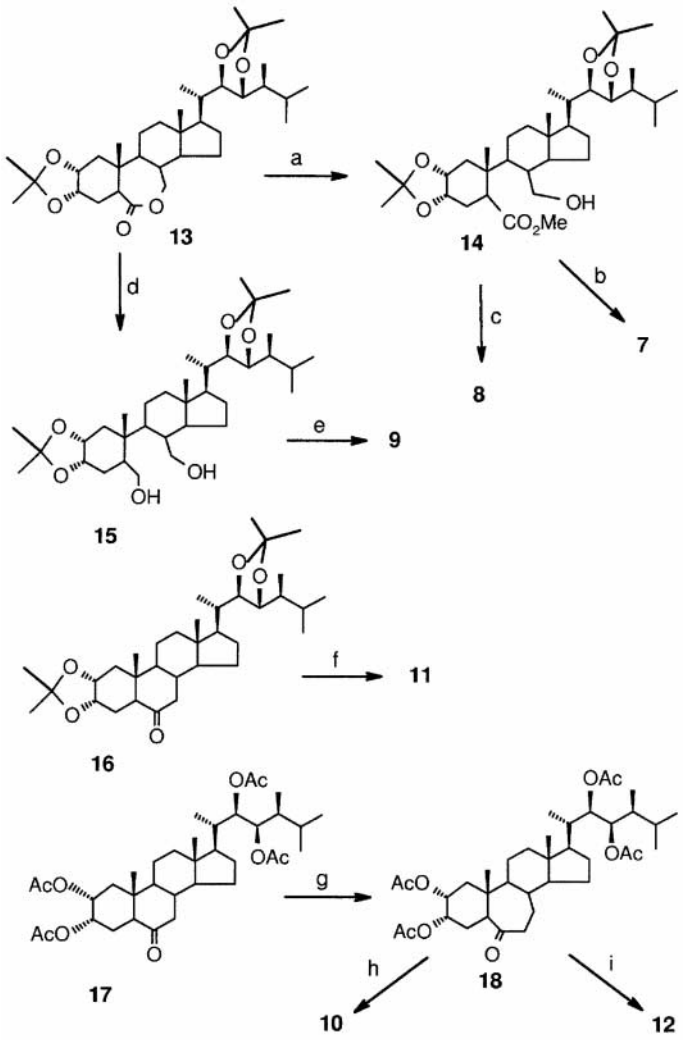

Fig. 3. Synthesis of novel brassinosteroids, **7–12**. Reagents: (a) KOH–MeOH–H_2O; (b) (i) TsCl–Py, (ii) NH_4OH, (iii) AcOH–H_2O; (c) (i) TsCl–Py, (ii) AcSH–NaH, (iii) NaOH–MeOH–H_2O, (iv) O=P(OPh)Cl_2–Py, (v) CF_3CO_2H–H_2O; (d) $LiAlH_4$; (e) (i) TsCl–Py, (ii) Py (reflux), (iii) AcOH–H_2O; (f) (i) CH_2=PPh_3, (ii) AcOH–H_2O; (g) (i) CH_2N_2–BF_3·Et_2O; (h) NaOH–MeOH–H_2O; (i) (i) Lombardo–Oshima reagent, (ii) NaOH–MeOH–H_2O.

Similarly, the known bisacetonide **16** [30] was prepared from castasterone (**6**) and converted into the exo-methylidene derivative **11** by a Wittig reaction followed by deprotection. Alternatively, the known tetraacetate **17** [29, 31] was subjected to ring-expansion with diazomethane in the presence of boron trifluoride etherate [32] to give **18**, and B-homocastasterone (**10**) was obtained after saponification of the latter. Finally, **18** afforded **12** when treated with the Lombardo–Oshima reagent [33, 34], followed by saponification.

Biological activity of compounds **7**, **8** and **9**, as determined by the rice leaf lamina assay, is shown in Fig. 4, which depicts the leaf lamina angle vs dose in ng on a logarithmic scale. Brassinolide (**1**) and 24-epibrassinolide (**3**) are included as standards. It is evident that all three novel analogues (**7–9**) show substantial bioactivity. The activity of lactam **7** and thiolactone **8** was comparable to that of 24-epibrassinolide (**3**) at low to mid-range doses, but in the case of **7** was nearly identical to that of brassinolide (**1**) at the higher dose of 1000 ng/plant. Cyclic ether **9** resembled **1** at the two lowest doses, was intermediate in activity between **3** and **1** at doses of 10 and 100 ng/plant, and was comparable to **3** at 1000 ng/plant.

Effects of B-homocastasterone (**10**) and the exocyclic methylene derivatives **11** and **12** are shown in Fig. 5, along with effects of **1** and **6**. Analogue **10** displayed significant activity at high doses. It was, however, less active than either **1** or **6**. Finally, the carbocycles **11** and **12** were essentially devoid of activity at all dosage levels.

The synergy between IAA and the novel analogues **7–10** is shown in Figs 9–12, respectively, while that between IAA and brassinolide (**1**), 24-

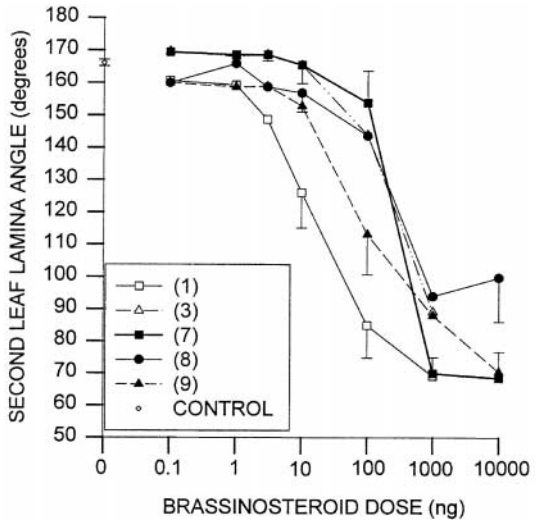

Fig. 4. Rice leaf lamina bending assay of lactam **7**, thiolactone **8** and cyclic ether **9** with brassinolide (**1**) and 24-epibrassinolide (**3**) as standards.

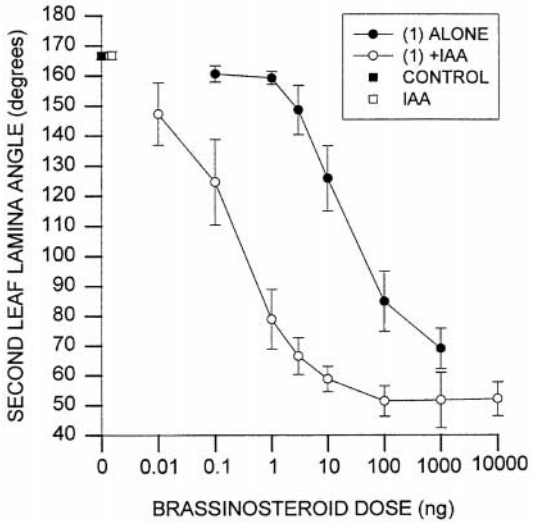

Fig. 6. Rice leaf lamina bending assay of brassinolide (**1**), alone and with IAA.

epibrassinolide (**3**) and castasterone (**6**) is shown for comparison in Figs 6, 7 and 8, respectively. The synergy of **1** with IAA (Fig. 6) is striking across a broad range of doses, with significant leaf lamina bending even at 0.01 ng. Synergy of IAA with **3** (Fig. 7) was also observed over the entire dosage range, but was less pronounced than for **1**. On the other hand, IAA synergized the activity of lactam **7** only slightly at most doses in the range of 10–1000 ng (Fig. 9), though the synergism was significant and appreciable at two doses of **7** (100 and 1000 ng). In contrast, the thiolactone **8** and cyclic ether **9** showed significant and appreciable synergism across a wide range of doses (Figs 10 and 11, respectively). A general enhancement of the bioactivity of brassinosteroid derivatives **8** and **9** with IAA gave bioactivities comparable to or better than brassinolide (**1**) alone, but much lower than for **1** plus IAA.

The synergistic effect of IAA upon the bioactivity of B-homocastasterone (**10**) is clearly demonstrated at two relatively high doses (100 and 1000 ng) (Fig. 12), whereas significant ($P \leq 0.05$) synergism for castasterone (**6**) occurred only at 10 ng (Fig. 8). Analogues **11** and **12** remained inactive when applied together with IAA, except that significant ($P \leq 0.05$), but very slight enhancement of activity was observed with a dose of 1 ng of **11** and very slight inhibition with 1 or 100 ng of **12** (data not shown).

A number of conclusions can thus be made regarding structure–activity relationships of the

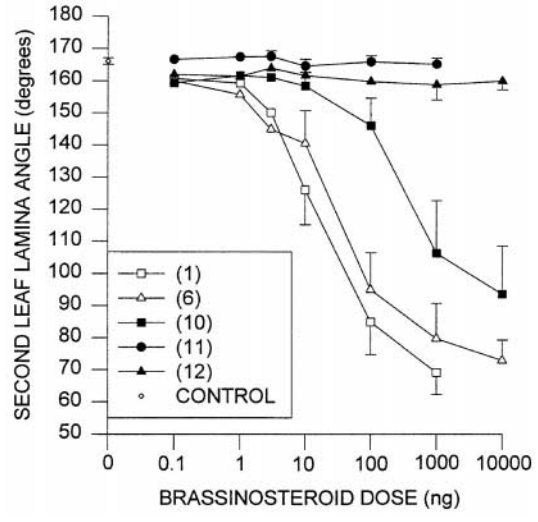

Fig. 5. Rice leaf lamina bending assay of B-homocastasterone (**10**), 6-methylidenecastasterone (**11**) and 6-methylidene-B-homocastasterone (**12**) with brassinolide (**1**) and castasterone (**6**) as standards.

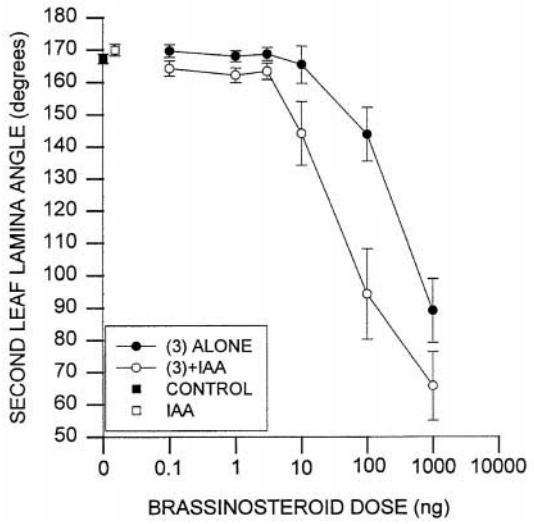

Fig. 7. Rice leaf lamina bending assay of 24-epibrassinolide (**3**), alone and with IAA.

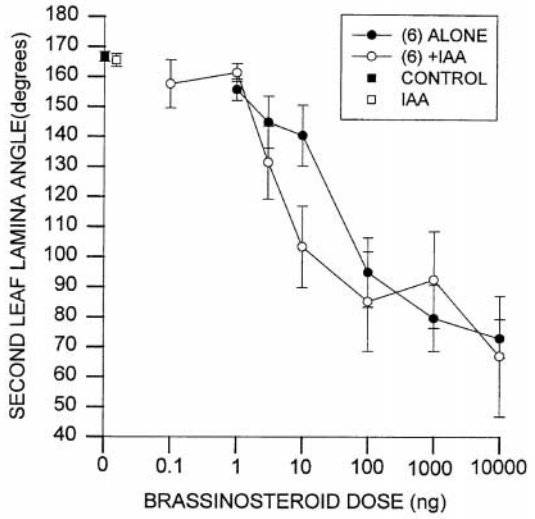

Fig. 8. Rice leaf lamina bending assay of castasterone (**6**), alone and with IAA.

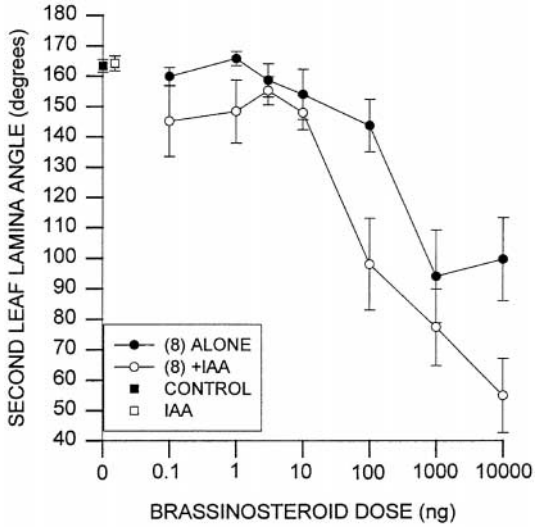

Fig. 10. Rice leaf lamina bending assay of thiolactone **8**, alone and with IAA.

B-ring of brassinosteroids. First, replacement of the lactone oxygen atom at C-7 with nitrogen, sulfur or carbon (i.e. **7**, **8** and **10**, respectively) produces compounds that retain quite significant bioactivity, roughly comparable to 24-epibrassinolide (**3**) in the case of **7** and **8**. Second, the carbonyl group at C-6 is not essential, providing that the oxygen atom at C-7 remains in place, as evidenced by the relatively high activity of cyclic ether **9**. Third, B-homocastasterone (**10**) is significantly active, but less so than either brassinolide (**1**) or castasterone (**6**). The comparison with castasterone is particularly interesting, since it shows that ring-expansion to a seven-membered ring in the 6-keto series does not produce the high level of activity associated with brassinolide (**1**), which is the analogous seven-membered lactone. It is thus possible, by analogy to the biosynthetic transformation of **6** to **1** in some species of plants [21], that **10** is oxidized to the possibly less active corresponding eight-membered lactone. A direct comparison of the latter with **1** may thus be warranted. Furthermore, bioassay activities of **11** and **12** indicate that a flattened B-ring conformation caused by an sp^2-hybridized center at C-6 is insufficient for activity in either the six- or seven-membered series. A polar functional group associated with a heteroatom at C-7, or a carbonyl group at C-6 is clearly required for activity. This in turn suggests that the B-ring interacts with a putative receptor by either acting as a hydrogen-bond acceptor, or through other types of polar interactions.

The synergy in bioactivity between brassinosteroids and IAA is not well understood, although it

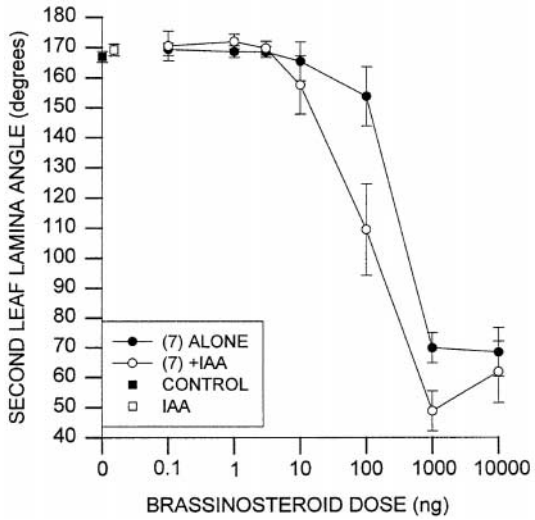

Fig. 9. Rice leaf lamina bending assay of lactam **7**, alone and with IAA.

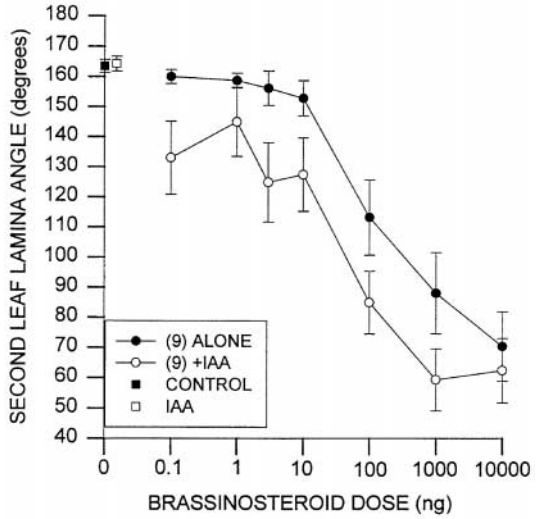

Fig. 11. Rice leaf lamina bending assay of cyclic ether **9**, alone and with IAA.

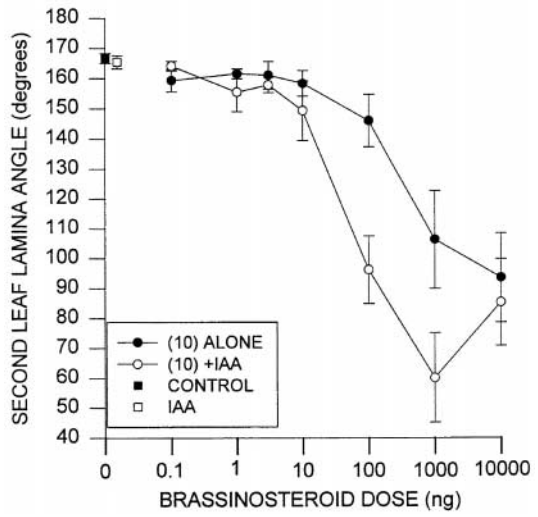

Fig. 12. Rice leaf lamina bending assay of B-homocastasterone (**10**), alone and with IAA.

presumably involves interaction effects on cell expansion. Interestingly, there are significant synergistic interactions between IAA and several of the more active novel brassinosteroid analogues (i.e. **7**–**10**). However, with the analogues, the synergy is less consistent across the dosage range studied and less pronounced than occurs between IAA and brassinolide (**1**). An interesting comparison of differential synergism is seen for the effect of IAA upon the bioactivity of B-homocastasterone (**10**) relative to castasterone (**6**). The parent compound **6** shows appreciable activity alone (without IAA), relative to the *ca*. 10-fold reduced activity shown by compound **10** alone (Fig. 5). In this situation both compounds can be assumed to be interacting with a relatively low level of endogenous IAA in the untreated rice plant. Furthermore, as mentioned earlier, a significant synergism with applied IAA was seen only at the low dose of 10 ng for castasterone, whereas compound **10** showed an appreciable and significant synergism with IAA only at the two higher doses of 100 and 1000 ng. Thus, castasterone appears to be quite efficient at interacting with low levels of IAA (endogenous or applied), whereas ring-expansion to a seven-membered ketone (i.e. **10**) results in a very reduced ability to interact synergistically with IAA at low (endogenous) levels, or low applied doses. It is only at the higher dose range (33 to 1000 ng) that B-homocastasterone can interact synergistically with applied IAA, dose ranges where castasterone no longer responds synergistically with applied IAA. As a final comparison, the analogous seven-membered lactone, brassinolide (**1**), possesses very high activity without applied IAA, and yet retains the ability to interact synergistically with IAA across a wide range of brassinosteroid doses (Fig. 6).

EXPERIMENTAL

Brassinolide (**1**) [28] and castasterone (**6**) [29] were prepared by methods reported previously. Castasterone was converted into the known bisacetonide **16** [30] and tetraacetate **17** [29, 31] by standard procedures. 24-Epibrassinolide (**3**) was obtained from the Sigma Chemical Co. ^{1}H- and ^{13}C-NMR spectra were recorded using $CDCl_3$ as the solvent and residual chloroform as the internal standard unless otherwise indicated. Mass spectra were obtained by electron impact (direct probe) at 70 eV.

Preparation of 7-*azabrassinolide* (**7**)

A mixture of brassinolide (518 mg, 1.08 mmol), 2,2-dimethoxypropane (3.0 ml), *p*-toluenesulfonic acid (30 mg) and dichloromethane (30 ml) was stirred at room temperature overnight. The mixture was poured into 50 ml of 5% aqueous sodium bicarbonate solution and extracted with 3×30 ml of dichloromethane. The extracts were washed with brine, dried (Na_2SO_4), concentrated and subjected to flash chromatography on silica-gel (elution with 20% ethyl acetate–hexane), to afford 494 mg (82%) of bisacetal **13** as a solid foam.

Compound **13** (494 mg, 0.884 mmol) and potassium hydroxide (1.0 g) were refluxed for 6 h in 15 ml of methanol and 1.5 ml of water. The mixture was poured into saturated ammonium chloride solution and extracted with 4×20 ml of ether. The extracts were dried (Na_2SO_4), concentrated and the residue was dissolved in 10 ml of ether. Ethereal diazomethane solution [35] was added dropwise until the yellow colour persisted. Volatile material was removed under reduced pressure and the residue was chromatographed on silica-gel (elution with 15–20% ethyl acetate–hexane) to afford 502 mg (96%) of the methyl ester **14** as a solid foam.

Compound **14** (140 mg, 0.236 mmol) and *p*-toluenesulfonyl chloride (132 mg, 0.696 mmol) were stirred in 2 ml of pyridine at room temperature for 24 h. The solvent was removed under vacuum, the residue was dissolved in 20 ml of dichloromethane, washed with 40 ml of 5% aqueous sodium bicarbonate solution and 40 ml of brine. The aqueous layers were extracted with 2×20 ml of dichloromethane and the combined organic layers were dried (Na_2SO_4), concentrated and chromatographed on silica-gel (elution with 10–30% ethyl acetate–hexane) to produce 137 mg (78%) of the corresponding tosylate. The tosylate (18 mg, 0.024 mmol) was dissolved in 2 ml of ethanol and 0.8 mL of concentrated ammonium hydroxide solution in a sealed vial, which was heated in an oil bath at 80–120°C for 20 h. The mixture was cooled to room temperature, poured into 10 ml of brine and extracted with 3×15 ml of chloroform. The combined organic layers were dried (Na_2SO_4), concentrated and chro-

matographed on silica-gel (elution with 10–20% ethyl acetate–hexane) to afford 10 mg (74%) of the bisacetal of **7** as an oil. This compound (16 mg, 0.029 mmol) in 1.5 ml of 80% acetic acid was heated at 65°C for 3 h and then the solvent was removed under vacuum. The residue was recrystallized (chloroform–methanol) to give 10.3 mg (74%) of lactam **7** as a white solid, m.p. 173–176°C; ^{1}H-NMR (200 MHz, $CDCl_3$–CD_3OD) δ 3.88 (*m*, 1 H), 3.60 (*m*, 1 H), 3.55 (*m*, 1 H), 3.43 (*d*, J = 8.3 Hz, 1 H), 3.05–2.85 (*m*, 2 H), 0.89 (*d*, J = 6.6 Hz, 3 H), 0.87 (*d*, J = 6.7 Hz, 3 H), 0.83 (*s*, 3 H), 0.81 (*d*, J = 7.3 Hz, 3 H), 0.76 (*d*, J = 6.8 Hz, 3 H), 0.3 (*s*, 3 H); ^{13}C-NMR (50 MHz, $CDCl_3$–CD_3OD) δ 178.2, 73.9, 72.7, 67.7 (2 C), 58.9, 52.4, 52.0, 44.4, 41.8, 41.0, 40.8, 39.9, 39.7, 39.5, 37.1, 36.6, 30.2, 29.7, 29.2, 27.0, 24.4, 22.1, 20.3, 20.1, 14.5, 11.3, 9.6. MS *m*/*z* (relative intensity, %) 479 (0.8, M^+), 380 (20), 379 (19), 361 (41), 331 (13), 43 (100). HRMS, *m*/*z*: 479.3651; calcd. for $C_{28}H_{49}NO_5$: 479.3611.

Preparation of 7-*thiabrassinolide* (**8**)

A solution of 273 mg (3.60 mmol) of thioacetic acid and 173 mg of a 50% sodium hydride dispersion in oil (3.60 mmol) was stirred in 5 ml of THF for 10 min. The tosylate of compound **14** (269 mg, 0.36 mmol), obtained as in the preparation of lactam **7**, in 5 ml of THF was added and the mixture was refluxed for 5 h. It was then poured into 25 ml of brine and extracted with 3 × 25 ml of ether. The combined extracts were dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with 5–15% ethyl acetate–hexane) to afford 190 mg (81%) of the corresponding thioacetate as a yellow oil. The thioacetate (180 mg, 0.277 mmol) and 300 mg of sodium hydroxide were refluxed for 3 h in 18 ml of methanol and 0.8 ml of water under argon. The solution was then added to 20 ml of saturated aqueous ammonium chloride solution and the mixture was extracted with 3 × 15 ml of ether. The combined extracts were washed with brine, dried (Na_2SO_4) and concentrated. The residue was dissolved in 10 ml of THF and 0.11 ml of pyridine (1.4 mmol), followed by 0.083 ml (0.554 mmol) of phenyl dichlorophosphate were added. The mixture was stirred at room temperature under argon for 18 h and then poured into 1 M sodium hydroxide solution and extracted with 3 × 20 ml of chloroform. The combined extracts were washed with brine, dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with chloroform) to afford 159 mg (100%) of the bisacetal of **8** as a solid foam. The bisacetal (123 mg, 0.213 mmol) was stirred overnight in 7 ml of chloroform containing 1.4 ml of trifluoroacetic acid and 0.15 ml of water. The mixture was then poured into 10% aqueous potassium carbonate solution and extracted with 4 × 20 ml of chloroform. The combined organic layers were washed with brine, dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with 2.5–10% methanol–chloroform) to afford 27 mg (25%) of thiolactone **8** as a light yellow solid, m.p. 160–165°C; ^{1}H-NMR (200 MHz) δ 4.03 (*m*, 1 H), 3.73 (*m*, 1 H), 3.63 (*m*, 1 H), 3.55 (*m*, 1 H), 3.07 (*dd*, J = 16.1, 9.3 Hz, 1 H), 2.75 (*d*, J = 16.1 Hz, 1 H), 0.96 (*d*, J = 6.6 Hz, 3 H), 0.94 (*d*, J = 6.7 Hz, 3 H), 0.91 (*s*, 3 H) 0.89 (*d*, J = 8.0 Hz, 3 H), 0.84 (*d*, J = 6.8 Hz, 3 H), 0.70 (*s*, 3 H); ^{13}C-NMR (50 MHz, $CDCl_3$–CD_3OD) δ 207.4, 74.2, 73.1, 67.7 (2 C), 59.1, 55.5, 52.1, 49.9, 44.4, 42.0, 39.9, 39.5, 39.1, 36.6, 32.0, 30.6, 30.2, 29.3, 27.5, 26.9, 24.9, 23.0, 20.4, 20.3, 15.5, 11.4, 9.7. MS *m*/*z* (relative intensity, %) 478 (0.5, M^+–H_2O), 396 (14), 395 (14), 378 (11), 377 (18), 266 (11), 265 (12), 264 (11), 137 (22), 121 (21), 107 (36), 95 (47), 81 (60), 69 (62), 55 (55), 43 (100). HRMS, *m*/*z*: 478.3156; calcd for $C_{28}H_{46}O_4S$: 478.3117.

Preparation of 6-*deoxybrassinolide* (**9**)

Bisacetal **13** (171 mg, 0.305 mmol) in 5 ml of ether was added to 58 mg (1.5 mmol) of lithium aluminum hydride in 5 ml of ether. The mixture was stirred at room temperature for 15 h and then 10 ml of saturated ammonium chloride solution was added cautiously. The mixture was extracted with 4 × 20 ml of ethyl acetate, the combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated to give 172 mg (100%) of diol **15** as a white solid.

The crude diol **15** (133 mg, 0.236 mmol) and *p*-toluenesulfonyl chloride (54 mg, 0.283 mmol) were stirred for 15 h at room temperature in 2 ml of pyridine. The mixture was then poured into 100 ml of 5% aqueous sodium bicarbonate solution and extracted with 4 × 20 ml of dichloromethane. The combined extracts were washed with brine, dried (Na_2SO_4), concentrated and chromatographed on silica-gel (elution with 20–50% ethyl acetate–hexane) to afford 130 mg (78%) of a mixture of monotosyl derivatives, as well as traces of the ditosyl analogue and diol **15**. The mixture of monotosylates (78 mg, 0.108 mmol) was refluxed in 3 ml of pyridine for 4 h. The solvent was then removed *in vacuo*. The residue was taken up in 20 ml of ether and washed with 30 ml of 5% aqueous sodium bicarbonate solution and 30 ml of brine. The aqueous layers were extracted with 2 × 20 ml of ether and the combined organic layers were dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with 10–20% ethyl acetate–hexane) to afford 49 mg (82%) of the bisacetal of **9** as an oil. This product was dissolved in 3 ml of 80% acetic acid and heated at 50–70°C for 3 h. The solvent was then removed under vacuum and the residue was chromatographed on silica-gel (elution with 0–10% methanol–chloroform) and recrystallized (chloroform–hexane) to afford 34 mg (81%)

of cyclic ether **9** as a white solid, m.p. 221–223°C; ^{1}H-NMR (200 MHz) δ 3.94 (*m*, 1 H), 3.68 (*m*, 4 H), 3.54 (*d*, J = 8.3 Hz, 1 H), 3.28 (*m*, 2 H), 0.98 (*s*, 3 H), 0.96 (*d*, J = 6.5 Hz, 3 H), 0.94 (*d*, J = 6.7 Hz 3 H), 0.89 (*d*, J = 6.2 Hz, 3 H), 0.84 (*d*, J = 6.9 Hz, 3 H), 0.72 (*s*, 3 H); ^{13}C-NMR (50 MHz) δ 74.4, 73.2, 72.5, 70.1, 68.9, 67.8, 58.0, 52.1, 51.9, 42.4, 41.9, 41.5, 40.1, 39.8, 36.7, 36.5, 31.1, 30.5, 27.9, 27.7, 24.3, 22.6, 20.6, 20.4, 12.8, 11.7, 11.5, 9.7. MS *m*/*z* (relative intensity, %) 466 (1.5, M^+), 365 (67), 347 (40), 208 (52), 107 (52), 95 (76), 93 (58), 83 (63), 55 (85), 43 (100). HRMS, *m*/*z*: 466.3681; calcd. for $C_{28}H_{50}O_5$: 466.3658.

Preparation of 6*-methylidenecastasterone (***11***)*

Under an atmosphere of argon, *n*-butyllithium in ether (2.5 M, 0.079 ml, 0.20 mmol) was added by syringe to a suspension of 81 mg (0.21 mmol) of methyltriphenylphosphonium iodide in 2 ml of THF. The mixture was stirred at room temperature for 15 min and a solution of castasterone bisacetal **16** (89 mg, 0.16 mmol; obtained from castasterone by the same procedure as used in the preparation of **13**) in 1 ml of THF was added by syringe. The mixture was stirred for an additional 2 h and was then poured into 20 ml of brine. It was extracted with 3×20 ml of dichloromethane, the combined extracts were dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with 2–5% ethyl acetate–hexane) to afford 70 mg (79%) of the bisacetal of **11**, obtained as a solid foam. The latter product was heated in 5 ml of 80% acetic acid and 5 ml of THF at 70°C for 5 h. It was then poured into 60 ml of 20% aqueous potassium carbonate solution and extracted with 4×20 ml of chloroform. The combined extracts were washed with brine, dried (Na_2SO_4), concentrated and flash chromatographed on silica-gel (elution with 0–8% methanol–chloroform) to give 38 mg (62%) of **11**, which was recrystallized from methanol to afford white crystals, m.p. 271–273°C; ^{1}H-NMR (200 MHz) δ 4.73 (*s*, 1 H), 4.43 (*s*, 1 H), 4.10 (*m*, 1 H), 3.72 (*m*, 2 H), 3.57 (*d*, J = 8.0 Hz, 1 H), 0.96 (*d*, J = 6.5 Hz, 3 H), 0.93 (*d*, J = 6.6 Hz, 3 H), 0.89 (*d*, J = 6.2 Hz, 3 H), 0.85 (*d*, J = 6.5 Hz, 3 H), 0.69 (*s*, 3 H), 0.67 (*s*, 3 H); ^{13}C-NMR (50 MHz, $CDCl_3$–CD_3OD) δ 149.5, 105.5, 74.6, 73.3, 69.0, 68.7, 56.4, 54.6, 52.5, 42.7, 42.6, 42.0, 40.3, 40.1, 40.0, 39.1, 37.1, 37.0, 30.7, 29.9, 27.8, 24.0, 21.3, 20.8, 20.7, 12.7, 12.0, 11.9, 10.1. MS *m*/*z* (relative intensity, %) 462 (4, M^+), 444 (16), 361 (19), 344 (74), 343 (60), 325 (47), 314 (26), 187 (35), 119 (39), 105 (43), 95 (60), 81 (64), 55 (78), 43 (100). HRMS, *m*/*z*: 462.3686; calcd for $C_{29}H_{50}O_4$: 462.3711.

*Preparation of B-homocastasterone (***10***)*

A solution of excess alcohol-free diazomethane [35] in ether and boron trifluoride etherate (1.30 ml, 10.6 mmol) were added to a solution of castasterone tetraacetate **17** (399 mg, 0.630 mmol) in 30 ml of dry ether and the reaction mixture was stirred at room temperature for 24 h. The mixture was diluted with ether, washed twice with water, dried ($MgSO_4$), concentrated and flash chromatographed on silica-gel (elution with 10–20% ethyl acetate–hexanes), which gave 292 mg of a mixture of **18** and the starting material **17** in the ratio of 2:3. The two compounds were separated by reverse phase preparative HPLC (elution with 25% water–acetonitrile at a flow rate of 9.0 ml/min) to afford 132 mg (33%) of **18** as a white solid foam. The product (35 mg, 0.055 mmol) was dissolved in 3 ml of methanol, 0.20 ml of 1 M sodium hydroxide was added and the reaction mixture was refluxed for 1 h. The solution was neutralized with 10% aqueous HCl and the methanol was removed *in vacuo*. The residue was diluted with water and extracted with 3×20 ml of ethyl acetate. The combined organic layers were dried (Na_2SO_4) and evaporated to dryness to give a white solid, which was recrystallized from ethyl acetate to afford 15 mg (74%) of **10** as white needles, m.p. 128–130°C; ^{1}H NMR (400 MHz) δ 4.01 (*m*, 1 H), 3.71 (*m*, 2 H), 3.56 (*m*, 1 H), 3.25 (*dd*, J = 12.5, 3.8 Hz, 1 H), 2.45 (*m*, 1 H), 2.31 (*ddd*, J = 17.9, 12.7, 5.3 Hz, 1 H), 0.97 (*d*, J = 7.5 Hz, 3 H), 0.95 (*d*, J = 7.0 Hz, 3 H), 0.90 (*d*, J = 6.4 Hz, 3 H), 0.85 (*d*, J = 6.9 Hz, 3 H), 0.79 (*s*, 3 H), 0.65 (*s*, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 216.4, 74.2, 72.9, 67.9, 58.1, 55.7, 52.2, 46.9, 43.6, 41.7, 41.5, 40.5, 40.0, 39.5, 38.9, 36.7, 30.3, 29.5, 27.5, 26.9, 25.4, 22.5, 20.5, 20.4, 15.3, 11.5, 11.2, 9.8. MS *m*/*z* (relative intensity, %) 460 (0.6, M^+–H_2O), 378 (8), 377 (8), 360 (21), 359 (27), 341 (35), 95 (60), 81 (65), 55 (79), 43 (100). Anal. calcd. for $C_{29}H_{50}O_5$: C, 72.76; H, 10.53. Found: C, 72.37; H, 10.53.

Preparation of 6*-methylidene-B-homocastasterone (***12***)*

The Lombardo–Oshima reagent [33] was added to a solution of **18** (61 mg, 0.094 mmol) in 5 ml of methylene chloride. After 4 h, the mixture was poured into a 1:1 mixture of ether and 10% aqueous sodium bicarbonate solution in a separatory funnel and shaken until the ether layer was clear. The layers were separated and the ether layer was dried (Na_2SO_4) and evaporated. The residue was refluxed in a mixture of 1.5 ml of methanol and 0.1 ml of 1 M sodium hydroxide solution for 1 h. After neutralization with 10% aqueous HCl solution, the mixture was extracted with 3×20 ml of ethyl acetate. The organic layers were combined, dried (Na_2SO_4), evaporated and flash chromatographed (elution with 3% methanol–chloroform) to give 8 mg (18%) of recovered starting material and 13 mg (29%) of **12**, obtained as an off-white solid that was recrystallized from methanol–water, m.p. 238–240°C; ^{1}H NMR (400 MHz) δ 4.89 (s, 1 H),

4.66 (*s*, 1 H), 4.00 (*m*, 1 H), 3.71 (*m*, 2 H), 3.56 (*m*, 1 H), 2.56 (*m*, 1 H), 2.40 (*m*, 1 H), 0.97 (*d*, $J = 6.9$ Hz, 3 H), 0.95 (*d*, $J = 6.9$ Hz, 3 H), 0.90 (*d*, $J = 5.9$ Hz, 3 H), 0.86 (*d*, $J = 6.9$ Hz, 3 H), 0.81 (*s*, 3 H), 0.64 (*s*, 3 H); ^{13}C NMR (100 MHz) δ 150.2, 111.0, 74.8, 73.5, 69.2, 68.7, 58.3, 56.0, 52.5, 42.7, 41.7, 40.4, 40.1, 39.9, 38.4, 37.0, 36.9, 36.8, 34.1, 31.0, 30.8, 27.3, 25.7, 23.0, 20.9, 20.7, 15.7, 11.9, 11.5, 10.1. MS *m*/*z* (relative intensity, %) 476 (2, M^+), 461 (4), 444 (8), 376 (9), 357 (23), 344 (20), 339 (25), 107 (72), 95 (84), 93 (72), 81 (87), 55 (100). HRMS, *m*/*z*: 476.3848; calcd for $C_{30}H_{52}O_4$: 476.3866.

Bioassays

The novel B-ring analogues and brassinolide (standard) were tested by means of the rice leaf lamina assay, using a dwarf cv., Tan-ginbozu, as described by Takeno and Pharis [26]. The compounds were dissolved in 95% ethanol and applied as 0.5 μl microdrops to the rice plant 48 h after planting germinated seeds on 0.8% water agar. Where IAA was a co-treatment, 1,000 ng of IAA was similarly applied per plant *ca*. 2 h prior to the application of the various brassinosteroids. Thus, **1** or its derivatives were applied at 0.01 or 0.1 to 1000 or 10,000 ng/plant in microdrops and the resultant leaf lamina angle was measured 60–65 h later. Each data point is the mean of the leaf angles from *ca*. 36 plants for doses up to 100 ng and from ca. 24 plants for the 1000 and 10,000 ng doses. Parallel applications of ethanol alone (control), IAA alone (1000 ng), **1** alone and **1** plus IAA were always carried out. In all of Figs 4–12, error bars represent 95% confidence limits.

Acknowledgements—This research was supported by a Natural Sciences and Engineering Research Council of Canada Strategic Grant (No. STR0167111). D. L. B. acknowledges support from an NSERC Postgraduate Fellowship, a Ralph Steinhauer Award and an Honorary Killam Fellowship. We thank Mrs S. Horácek for assistance with the rice leaf lamina assay and Dr R. Yamdagni, Ms D. Fox and Ms Q. Wu for mass spectra.

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