PERGAMON

Phytochemistry 59 (2002) 627–634

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Synthesis and bioactivity of C-2 and C-3 methyl ether derivatives of brassinolide

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Received 17 September 2001; received in revised form 4 December 2001

Abstract

The following six novel methyl ether derivatives of brassinolide were prepared and their brassinosteroid activity was measured by means of the rice leaf lamina inclination bioassay: 2-*O*-methylbrassinolide, 3-*O*-methylbrassinolide, 2,22,23-tri-*O*-methylbrassinolide, 3,22,23-tri-*O*-methylbrassinolide, 2-*O*-methyl-25-methoxybrassinolide and 3-*O*-methyl-25-methoxybrassinolide. Brassinolide was used as a standard for comparison. All six compounds were also tested in the presence of 1000 ng of indole-3-acetic acid (IAA), an auxin that synergizes the effects of brassinosteroids. The 2-*O*-methyl- and 3-*O*-methylbrassinolide derivatives showed weak activity at high doses, which was enhanced by IAA, especially in the case of the 3-*O*-methyl derivative. Similarly, the 2,22,23-tri-*O*-methyl- and 3,22,23-tri-*O*-methyl derivatives displayed weak bioactivity on their own, but significantly stronger activity when applied with IAA. The 3-*O*-methyl and 3,22,23-tri-*O*-methyl analogues plus IAA were comparable in bioacivity to brassinolide alone, but were less active than brassinolide plus IAA. In each case, *O*-methylation at C-2 resulted in a greater loss of activity than *O*-methylation at C-3 under the same conditions. The relatively strong activity of 3,22,23-tri-*O*-methylbrassinolide in the presence of IAA is especially noteworthy as it indicates that free hydroxyl groups at C-3, C-22, and C-23 are not essential for bioactivity. Finally, 2-*O*-methyl- and 3-*O*-methyl-25-methoxybrassinolide were essentially inactive alone, and showed only a modest increase in bioactivity when coapplied with IAA.

Keywords: *Oryza sativa*; Gramineae; Rice leaf lamina inclination assay; Brassinolide; 2- And 3-*O*-methyl brassinolide derivatives

1. Introduction

Brassinolide (**1**) is a potent steroidal plant growth-regulator that was first reported by Grove et al. (1979). The chemistry, biology and field applications of **1** and related brassinosteroids have since provoked considerable scrutiny (Cutler et al., 1991; Khripach et al., 1999; Sakurai et al., 1999). Recent studies of the metabolism of brassinosteroids, mainly conducted with 24-epibrassinolide, (for reviews, see: Adam et al., 1996; Adam and Schneider, 1999; Khripach et al., 1999) in various species of intact plants and cell cultures have revealed that, inter alia, glucosylation of existing hydroxyl groups at C-2, C-3 and C-23 is a common pathway. Furthermore, enzymatic hydroxylation at C-25 and C-26 affords **2** and **3**, again followed by glucosylation (Fig. 1) (for example, see: Hai et al., 1995, 1996; Kolbe et al., 1996; Schneider et al., 1994). It is generally accepted that the glucosylated conjugates are considerably less active than the parent brassinosteroids, suggesting that they are the products of metabolic deactivation. The bioactivity of the C-25 and C-26 hydroxylated metabolites has been the subject of some earlier debate (Adam et al., 1996; Hai et al., 1995; Kolbe et al., 1996; Seto et al., 1999; Voigt et al., 1996). However, very recent studies indicate that the free aglycones **2** and **3** in the brassinolide series are only weakly bioactive, suggesting that they too are deactivation products (Pharis et al., 2001).

We therefore considered it of interest to prepare brassinosteroid analogues where glucosylation is blocked by an appropriate modification of the free hydroxyl groups. Such compounds might be expected to resist metabolic deactivation in plants and, therefore, to produce more persistent activity in field applications, providing that the structural modifications did not

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0031-9422/02/$ - see front matter
PII: S0031-9422(02)00019-5

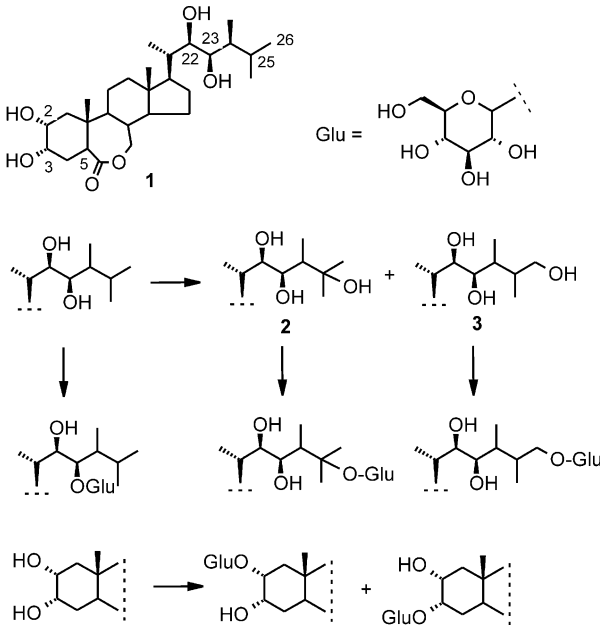

Fig. 1. Structure of brassinolide (**1**) and metabolites of brassinosteroids.

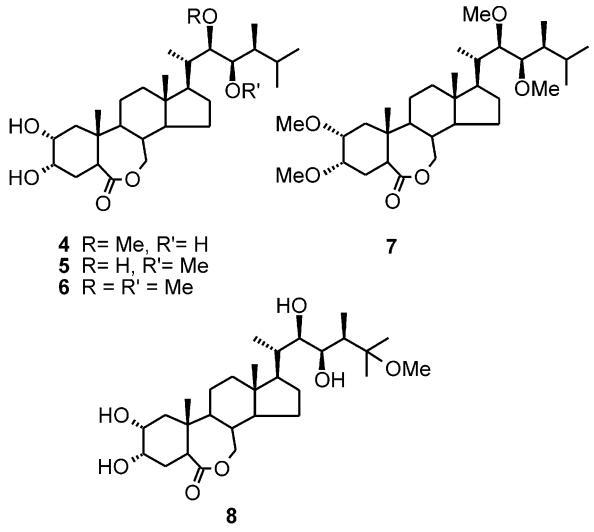

Fig. 2. Structures of known methyl ether analogues **4**–**8** of brassinolide.

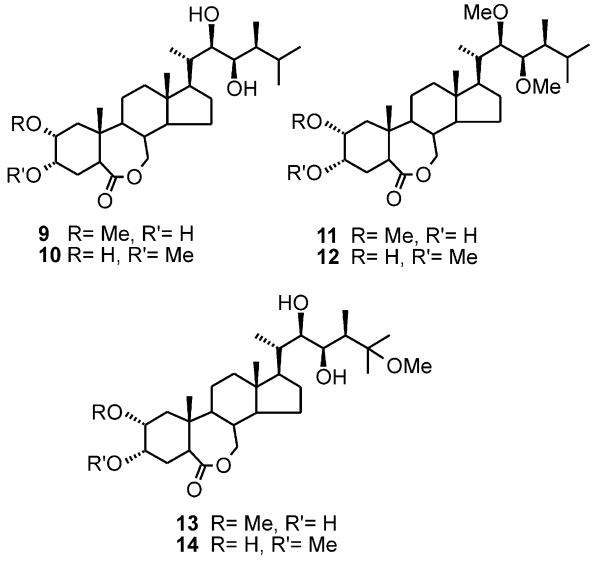

Fig. 3. Structures of novel methyl ether analogues **9**–**14** of brassinolide.

themselves adversely affect bioactivity. Moreover, the effects of systematic modifications of the hydroxyl groups of brassinolide upon its bioactivity could lead to new insight into the nature of the interactions of these functionalities with a putative brassinosteroid receptor(s) (for the molecular mechanism of action of brassinosteroids, see: Clouse and Feldmann, 1999; Clouse and Sasse, 1998; Fujioka and Sakurai, 1997; Li and Chory, 1997). We recently reported the preparation and bioactivity of the brassinolide methyl ethers **4**–**7** (Luo et al., 1998) and **8** (Back et al., 1999) (see Fig. 2 for structures **4**–**8**). Interestingly, the dimethyl derivative **6** proved strongly bioactive (surprisingly, even more so than the monomethyl compounds **4** and **5**), whereas the tetramethyl analogue **7** was completely inactive. Furthermore, 25-methoxybrassinolide (**8**) was strongly active, in contrast to its 25-hydroxy analogue **2**. These observations suggested that free hydroxyl groups on the side chain are not essential for strong bioactivity, whereas the A-ring diol moiety is less tolerant of modification through methyl ether formation.

In order to investigate more closely the role of the 2- and 3-hydroxyl groups in determining brassinosteroid bioactivity, we decided to prepare analogues where the A-ring hydroxyl groups were individually blocked as methyl ethers. Thus, we now report the synthesis and bioactivity of the novel analogues **9**–**14** (see Fig. 3), where first only the 2-hydroxyl and 3-hydroxyl moieties were individually *O*-methylated (**9** and **10**, respectively). Second, the *O*-methylation at C-2 or C-3 was accompanied by *O*-methylation at both C-22 and C-23 (**11** and **12**, respectively) and third, a 25-methoxy substituent was included along with *O*-methylation at C-2 or C-3 (**13** and **14**, respectively).

2. Results and discussion

The synthesis of the novel compounds **9**–**14** is shown in Fig. 4. The preparation of **9** and **10** was achieved by starting with **15**, an intermediate used in our earlier synthesis of brassinolide and castasterone (Back et al., 1997). The side-chain diol moiety was acetylated and the product **16** was subjected to one-pot deketalization and Baeyer–Villiger oxidation to produce **17**. This was treated with diazomethane in the presence of a Lewis acid catalyst ($SnCl_2$) to afford a mixture of 2- and 3-*O*-

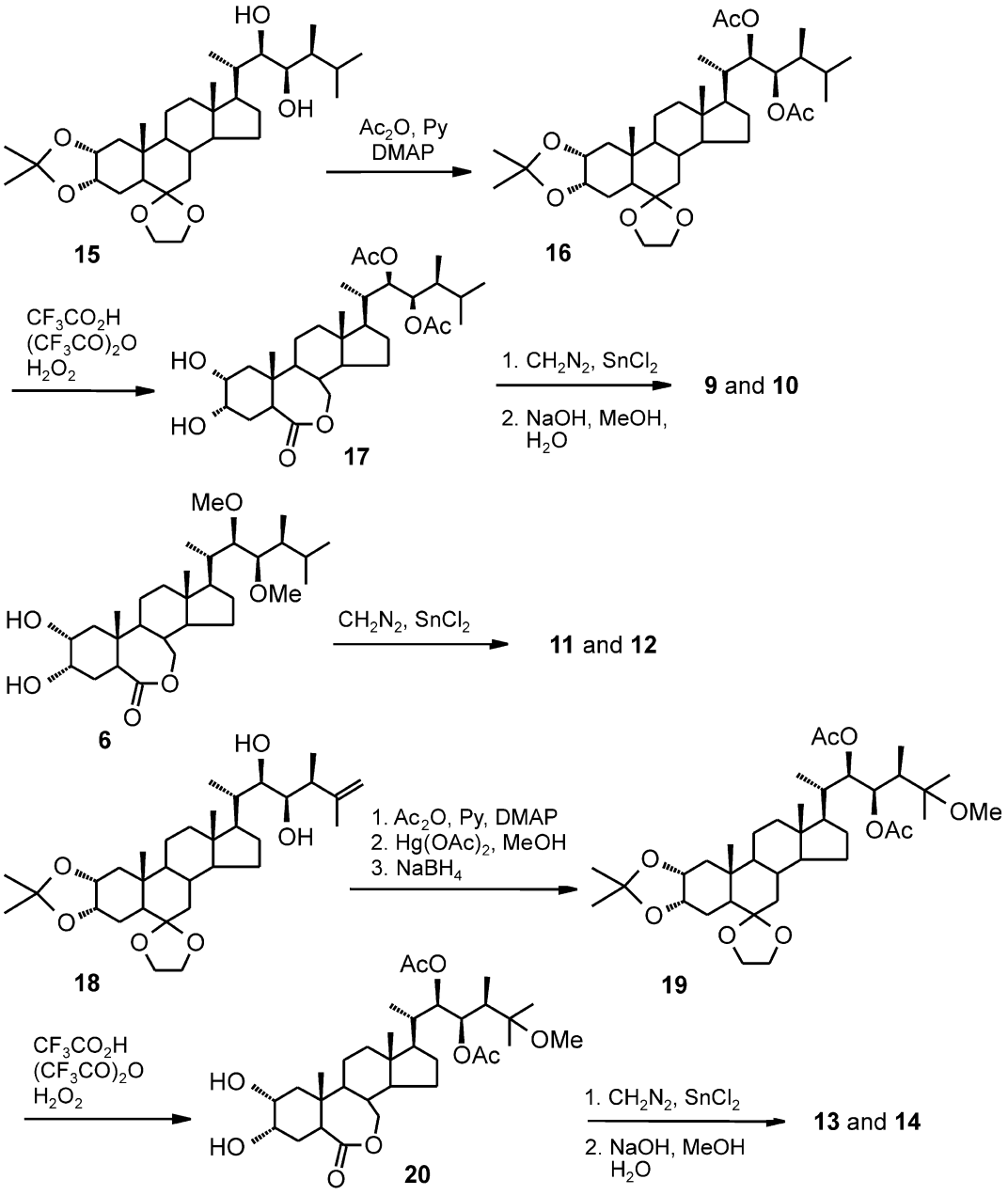

Fig. 4. Preparation of methyl ether analogues **9**–**14**.

methylated products, along with recovered starting material **17**. Saponification of the side-chain acetate groups then completed the preparation of **9** and **10**. Compounds **11** and **12** were similarly prepared by treating **6** (Luo et al., 1998) with diazomethane. Finally, the unsaturated brassinosteroid intermediate **18** (Back et al., 1999) was acetylated and subjected to oxymercuration in methanol to produce the 25-methoxy derivative **19**. The latter compound was then subjected to deketalization and Baeyer–Villiger oxidation, followed by the usual *O*-methylation of **20** with diazomethane and saponification of the acetate groups to afford **13** and **14**.

In each case, the *O*-methylation step was cleaner when brief exposure to diazomethane was used and the reaction was stopped at an early stage, instead of allowing it to go to completion. This proved more expedient, despite the need to recover and recycle the starting material. Several other methylation procedures, such as the use of sodium hydride and methyl iodide, were also attempted but gave inferior results. The pairs of 2- and 3-*O*-methylated isomers (**9** and **10**, **11** and **12**, and **13** and **14**) were separated by flash chromatography and were distinguished from each other on the basis of their ^{1}H NMR spectra, in which the C-5α proton acted as a convenient reporter signal for identifying the C-3α sub-

stituent. Thus, in the 2-*O*-methylated series (**9**, **11** and **13**), containing an axial hydroxyl group at C-3α, the proton at C-5α consistently displayed a signal at ca. δ 3.1 ppm, as in brassinolide (**1**). On the other hand, in the 3-*O*-methylated series (**10**, **12** and **14**), where the C-5α proton has a 1,3-diaxial relationship with the methoxy group at C-3α, this signal was consistently shifted upfield to ca. δ 2.9 ppm. Moreover, NOE's were observed between the C-5α protons and the 3α-methoxy groups in the latter series.

We then subjected the products **9**–**14** to the rice leaf lamina inclination bioassay, a rapid, convenient and highly sensitive method for measuring brassinosteroid activity (Takeno and Pharis, 1982). Since the effects of brassinosteroids are synergized by auxins such as indole-3-acetic acid (IAA) (Takeno and Pharis, 1982; Mandava, 1988; Sasse, 1991), the bioassays were also repeated in the presence of 1000 ng of IAA. The coapplication of IAA has been observed to enhance the activity of **1** by a factor of 10–100, thus dropping the threshold activity from ca. 1 to 0.1–0.01 ng (e.g. see Baron et al., 1998). The results of the bioassays of **9**–**14** are shown in Figs. 5–7, in which brassinolide (**1**) is included as a standard for comparison. In each of these Figures, the leaf lamina angle is plotted against the logarithm of the brassinosteroid dose in ng. Untreated control plants typically display a leaf lamina angle of ca. 160–170° (i.e. nearly upright), while ones showing a strong brassinosteroid response may reach 50° or even less. Error bars in Figs. 5–7 indicate 95% confidence levels.

Bioassay results of compounds **9** and **10** are shown in Fig. 5, which indicate that neither compound **9** (*O*-methylated at C-2) nor **10** (*O*-methylated at C-3) is as potent as **1**. However, **10** has stronger activity than **9** at high dosage levels, suggesting that the presence of a free hydroxyl group at the 2-position is more critical than at C-3. When IAA was coapplied with the brassinosteroids, the activity of both compounds **9** and **10** increased considerably and that of **10** with IAA actually exceeded

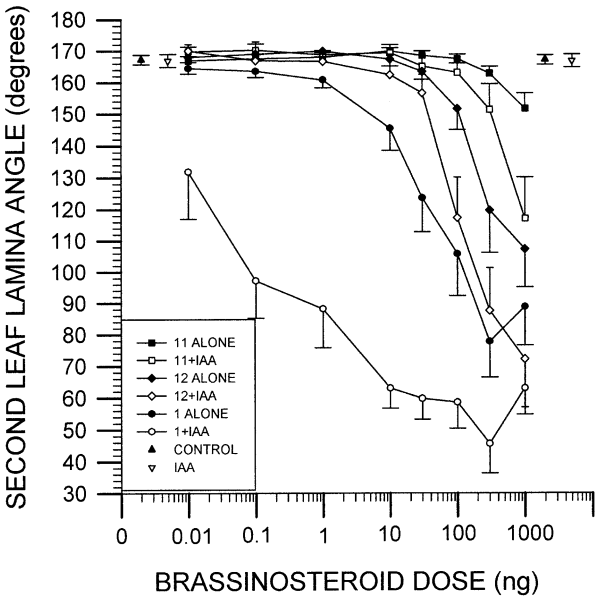

Fig. 6. Rice leaf lamina inclination bioassay of **11** and **12**, compared to brassinolide (**1**), with and without coapplication of 1000 ng of IAA. Error bars represent 95% confidence limits.

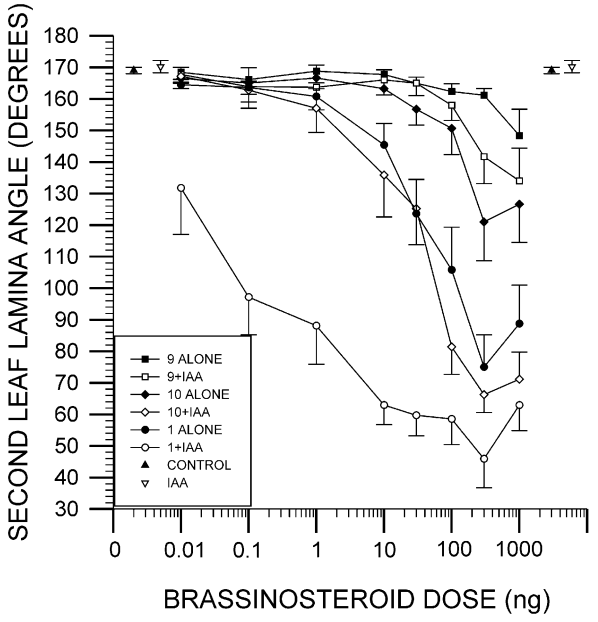

Fig. 5. Rice leaf lamina inclination bioassay of **9** and **10**, compared to brassinolide (**1**), with and without coapplication of 1000 ng of IAA. Error bars represent 95% confidence limits.

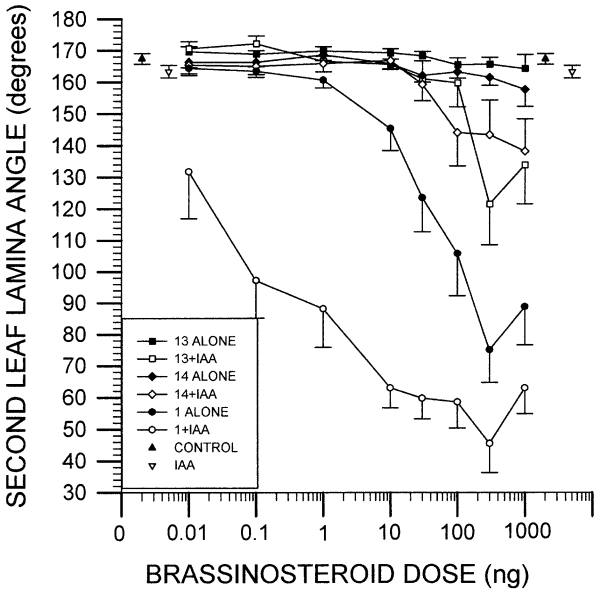

Fig. 7. Rice leaf lamina inclination bioassay of **13** and **14**, compared to brassinolide (**1**), with and without coapplication of 1000 ng of IAA. Error bars represent 95% confidence limits.

that of **1** alone, but not of **1** with IAA. A reversal in the leaf lamina angle at the highest dose of 1000 ng of **1** and **10** was observed both with and without IAA. This effect has been noted previously for brassinolide and many other active brassinosteroids that we have tested (Pharis et al., 2001) and is probably due to saturation and/or toxicity effects at very high doses.

A similar trend is seen in Fig. 6, where **12** (*O*-methylated at C-3, as well as at both C-22 and C-23), displayed stronger activity than **11** (O-methylated at C-2, as well as at C-22 and C-23). The activity of **12** was only ca. 1 order of magnitude lower than that of brassinolide (**1**) (i.e. it takes ca. 10 times the dose of **12** to elicit the same response as a given dose of **1**). Again, the activity of both compounds **11** and **12** was significantly enhanced by the coapplication of 1000 ng of IAA, and the activity of **12** with IAA approached that of **1** alone, except at the highest dose of 1000 ng, where **12** with IAA exceeded **1** alone. However, when **1** was applied with IAA, it gave a stronger response than either **11** or **12** plus IAA. It is highly noteworthy that compound **12**, where three of the hydroxyl groups have been blocked as methyl ethers, displays such strong bioactivity. The results in Fig. 6 again indicate that the presence of a free hydroxyl group at C-2 is more critical than at C-3. Clearly, free hydroxyl groups on the side chain (C-22, C-23) are not essential for strong bioactivity, confirming similar conclusions based on previous studies of the corresponding 22,23-bis(methyl ether) **6** (Luo et al., 1998).

The analogues **13** (*O*-methylated at C-2 and C-25) and **14** (*O*-methylated at C-3 and C-25) showed no significant activity on their own and only slight activity at the highest doses of 100–1000 ng when synergized with 1000 ng of IAA (Fig. 7). This is somewhat surprising in view of the relatively high activity of 25-methoxybrassinolide (**8**) found previously (Back et al., 1999) and suggests that it may be difficult to prevent glucosylation at C-25 and, simultaneously, at the A-ring hydroxyl groups.

3. Conclusions

Our earlier work demonstrated that blocking both hydroxyl groups at C-22 and C-23 (i.e. **6**) results in strong bioactivity, whereas blocking all four hydroxyls of brassinolide (i.e. **7**) gives an inactive product (Luo et al., 1998). These earlier results, in conjunction with the present ones, point to a critical need for a free C-2 hydroxyl group in brassinolide analogues, perhaps because it is required as a hydrogen bond donor in binding to a putative receptor. On the other hand, *O*-methylation of the hydroxyl groups at C-3, C-22 and C-23 of brassinolide does not appear to severely diminish its bioactivity, as evidenced by the relatively strong activity of **12**. The latter compound, where glucosylation at three of the four hydroxyl groups is blocked, may prove more resistant to metabolic deactivation than brassinosteroids such as **1**, and is, therefore, a promising candidate for field applications. Moreover, *O*-methylation of the C-25 hydroxyl, which is introduced into brassinosteroids by enzymatic hydroxylation in plants, results in the active product **8** (Back et al., 1999), although *O*-methylation at this position, together with methylation of either the C-2 or C-3 alcohol moiety (**13** and **14**, respectively) causes a nearly complete loss of activity. Overall, it appears that *O*-methylation is an effective means for preventing undesired in vivo glucosylation at several of the key sites in brassinosteroids, but not for blocking all of them simultaneously, and not for blocking the C-2 hydroxyl group under any circumstance.

4. Experimental

^{1}H and 13C NMR spectra were recorded using residual chloroform as the internal standard. Mass spectra were obtained by electron impact (direct probe) at 70 eV. Chromatography refers to flash chromatography on silica-gel (230–400 mesh).

*4.1. Preparation of 2-O-methylbrassinolide (**9**) and 3-O-methylbrassinolide (**10**)*

A mixture of 300 mg (0.547 mmol) of diol **15** (Back et al. 1997), 13 mg (0.11 mmol) of 4-(dimethylamino)pyridine, 4 ml of pyridine and 1.5 ml of acetic anhydride was stirred for 20 h at room temperature. It was then diluted with 100 ml of dichloromethane and washed successively with water, 10% aqueous HCl solution, saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was subjected to chromatography (hexanes:ethyl acetate, 8:1 to 5:1) to afford 350 mg (100%) of **16**, obtained as a white solid, mp 98–100 °C; IR (KBr) 1734, 1648, 1556, 1455, 1234 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.32 (*d*, $J=8.8$ Hz, 1 H), 5.14 (*d*, $J=8.8$ Hz, 1 H), 4.26 (*m*, 1 H), 4.08 (*m*, 1 H), 3.91 (*m*, 3 H), 3.75 (*m*, 1 H), 2.00 (*s*, 3 H), 1.97 (*s*, 3 H), 1.46 (*s*, 3 H), 1.31 (*s*, 3 H), 0.99 (*d*, $J=6.8$ Hz, 3 H), 0.95 (*d*, $J=6.7$ Hz, 3 H), 0.92 (*d*, $J=6.6$ Hz, 3 H), 0.89 (*d*, $J=6.8$ Hz, 3 H), 0.82 (*s*, 3 H), 0.68 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.5, 170.4, 109.7, 107.6, 77.2, 75.8, 74.2, 72.9, 72.8, 65.6, 64.2, 55.9, 53.2, 52.4, 45.6, 42.8, 42.4, 41.0, 39.8, 39.7, 38.0, 37.0, 33.0, 30.5, 29.2, 28.7, 26.6, 24.2, 22.0, 21.0, 20.9, 20.8, 20.3, 13.5, 12.9, 11.8, 11.1; MS (*m*/*z*, relative intensity, %) 575 (3), 557 (7), 421 (100), 254 (33), 99 (39).

Aqueous hydrogen peroxide (0.3 ml of a 30% solution, ca. 3 mmol) was slowly added to 2.0 ml of

trifluoroacetic anhydride over a period of 10 min at 0 °C. After 30 min, the mixture was diluted with 20 ml of chloroform. In a separate vessel, trifluoroacetic acid (0.5 ml) was added to a solution of **16** (68 mg, 0.11 mmol) in 2 mL of chloroform. The latter solution was stirred at room temperature for 40 min and then was added dropwise to the pregenerated trifluoroperoxyacetic acid solution at 0 °C over a period of 20 min. The resulting mixture was warmed to room temperature and stirred for an additional 3 h. It was diluted with water and extracted five times with chloroform. The organic extracts were washed with 10% aqueous sodium bisulfite solution, dried over anhydrous sodium sulfate and concentrated in vacuum. The residue was purified by chromatography (hexanes:ethyl acetate, 20:1) to afford 51 mg (84%) of **17** as a white solid, mp 130–132 °C; IR (KBr) 3448, 1726, 1698, 1651, 1241 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.33 (*m*, 1 H), 5.15 (*m*, 1 H), 4.10 (*m*, 3 H), 3.72 (*m*, 1 H), 3.11 (*dd*, $J=13.5$, 5.0 Hz, 1 H), 2.03 (*s*, 3 H), 1.99 (*s*, 3 H), 1.01 (*d*, $J=6.6$ Hz, 3 H), 0.96 (*d*, $J=6.5$ Hz, 3 H), 0.94 (*d*, $J=6.5$ Hz, 3 H), 0.92 (*s*, 3 H), 0.91 (*d*, $J=6.6$ Hz, 3 H), 0.73 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 170.5, 170.4, 77.2, 75.6, 74.1, 70.3, 68.2, 68.1, 58.2, 52.3, 51.4, 42.5, 41.6, 40.9, 39.8, 39.6, 39.2, 38.3, 37.0, 31.1, 30.5, 28.0, 24.7, 22.2, 20.9, 20.8, 20.2, 15.5, 12.7, 11.6, 11.0; MS (*m*/*z*, relative intensity, %) 475 (2), 462 (2), 379 (23), 343 (27), 106 (100).

Product **17** (260 mg, 0.461 mmol) and anhydrous $SnCl_2$ (25 mg, 0.13 mmol) were dissolved in 3 ml of dichloromethane and an ether solution of diazomethane was added in portions over 2 h until TLC analysis showed the appearance of two less polar products in addition to **17**. The reaction was then quenched with acetic acid, concentrated in vacuum and separated by chromatography (dichloromethane:methanol, 100:1 to 10:1) to afford 15 mg (30%, based on consumed starting material) of the 3-*O*-methyl diacetate derivative, mp 100–102 °C, followed by 11 mg (22%, based on consumed starting material) of the 2-*O*-methyl diacetate derivative, mp 47–49 °C, and recovered **17** (212 mg, 82%). The recovered starting material was then recycled in a similar fashion.

The above 2-*O*-methyl diacetate (30 mg, 0.052 mmol) was dissolved in 4 ml of methanol and 2 mL of 5% aqueous sodium hydroxide solution. The mixture was heated at 65 °C for 1 h, followed by concentration in vacuum and chromatography (dichloromethane: methanol, 50:1 to 20:1) to afford 17 mg (66%) of **9** as a white solid, mp 265–267 °C; IR (KBr) 3507, 1727, 1645, 1553 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$–CD_3OD) δ 4.12 (*m*, 1 H), 4.05 (*m*, 2 H), 3.64 (*m*, 1 H), 3.47 (*m*, 1 H), 3.34 (*s*, 3 H), 3.10 (*m*, 2 H), 0.91 (*d*, $J=6.6$ Hz, 3 H), 0.89 (*d*, $J=7.4$ Hz, 3 H), 0.86 (*s*, 3 H), 0.84 (*d*, $J=6.6$ Hz, 3 H), 0.79 (*d*, $J=7.0$ Hz, 3 H), 0.67 (*s*, 3 H); ^{13}C-NMR (50 MHz, $CDCl_3$–CD_3OD) δ 176.8, 77.2, 74.1, 73.0, 70.4, 64.1, 58.0, 55.7, 52.1, 51.1, 42.3, 41.0, 40.1, 39.5, 39.1, 38.4, 37.9, 36.8, 30.6, 30.5, 27.4, 24.5, 22.1, 20.7, 20.5, 15.3, 11.6, 11.5, 9.9; MS (*m*/*z*, relative intensity, %) 393 (<1), 375 (9), 128 (38), 91 (100); HRMS cal. for $C_{23}H_{37}O_5$ (M^+–$C_6H_{13}O$; from cleavage between C-22 and C-23): 393.2641; found: 393.2640.

The corresponding 3-methyl diacetate derivative was treated similarly to afford **10** in 66% yield, obtained as a white solid, mp 269–272 °C; IR (KBr) 3440, 1729, 1654, 1556 cm^{-1}; ^1H-NMR (200 MHz, $CDCl_3$–CD_3OD) δ 4.10 (*m*, 2 H), 3.72 (*m*, 1 H), 3.64 (*m*, 1 H), 3.55 (*m*, 2 H), 3.39 (*s*, 3 H), 2.92 (*dd*, $J=13.1$, 4.9 Hz, 1 H), 0.97 (*d*, $J=6.6$ Hz, 3 H), 0.95 (*d*, $J=6.6$ Hz, 3 H), 0.92 (*s*, 3 H), 0.90 (*d*, $J=8.0$ Hz, 3 H), 0.84 (*d*, $J=6.8$ Hz, 3 H), 0.71 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$–CD_3OD) δ 176.0, 77.2, 74.5, 73.5, 70.5, 67.7, 58.1, 56.9, 52.3, 51.2, 43.1, 42.4, 41.3, 40.1, 39.6, 39.2, 38.3, 36.9, 30.8, 27.6, 27.5, 24.7, 22.2, 20.8, 20.6, 15.3, 11.8, 11.7, 10.0; MS (*m*/*z*, relative intensity, %) 477 (0.5), 475 (1), 375 (42), 349 (61), 107 (64), 71 (100); HRMS calc. for $C_{29}H_{49}O_5$ (M^+–OH): 477.3580; found: 477.3568.

*4.2. Preparation of 2,22,23-tri-O-methylbrassinolide (**11**) and 3,22,23-tri-O-methylbrassinolide (**12**)*

Dimethyl ether **6** (Luo et al., 1998) (149 mg, 0.293 mmol) was treated with diazomethane and $SnCl_2$ as in the preparation of **9** and **10**. Chromatography of the product (dichloromethane:methanol, 100:1 to 10:1) afforded 6 mg (23% based on consumed starting material) of **12**, followed by 12 mg (46% based on consumed starting material) of **11** and 124 mg (83%) of recovered **6**. The recovered starting material was then recycled in a similar fashion.

Product **11**: white solid, mp 57–59 °C; IR (KBr) 3500, 1730, 1655, 1556 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.17 (*m*, 1 H), 4.09 (*m*, 2 H), 3.56 (*s*, 3 H), 3.52 (*s*, 3 H), 3.40 (*m*, 1 H), 3.39 (*s*, 3 H), 3.22 (*m*, 1 H), 3.19 (*m*, 1 H), 3.14 (*m*, 1 H), 0.97 (*d*, $J=6.6$ Hz, 3 H), 0.93 (*s*, 3 H), 0.92 (*d*, $J=6.6$ Hz, 3 H), 0.88 (*d*, $J=6.8$ Hz, 3 H), 0.83 (*d*, $J=6.8$ Hz, 3 H), 0.70 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 86.0, 84.8, 77.1, 70.4, 64.4, 61.1, 60.6, 58.3, 55.9, 52.1, 51.4, 42.5, 41.2, 41.1, 39.6, 39.3, 38.6, 38.1, 38.0, 30.4, 30.3, 28.5, 24.9, 22.3, 21.3, 20.5, 15.6, 12.4, 11.6, 10.6; MS (*m*/*z*, relative intensity, %) 507 (1), 407 (1), 375 (55), 335 (58), 83 (100); HRMS calc. for $C_{24}H_{39}O_5$ ($M^+-C_7H_{15}O$; from cleavage between C-22 and C-23): 407.2798; found: 407.2785.

Product **12**: white solid, mp 84–86 °C; IR (KBr) 3412, 1726, 1652, 1554 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.12 (*m*, 2 H), 3.56 (*s*, 3 H), 3.53 (*s*, 3 H), 3.42 (*m*, 2 H), 3.39 (*s*, 3 H), 3.23 (*m*, 2 H), 2.93 (*dd*, $J=12.0$, 6.0 Hz, 1 H), 0.97 (*d*, $J=6.9$ Hz, 3 H), 0.93 (*d*, $J=6.4$ Hz, 3 H), 0.92 (*s*, 3 H), 0.90 (*d*, $J=7.2$ Hz, 3 H), 0.82 (*d*, $J=6.8$ Hz, 3 H), 0.70 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.0, 86.0, 84.8, 77.2, 70.5, 67.8, 61.1, 60.6, 58.2, 56.9,

52.1, 51.4, 43.2, 42.5, 41.3, 41.2, 39.6, 39.2, 38.3, 37.9, 30.4, 28.5, 27.5, 24.9, 22.2, 21.3, 20.5, 15.3, 12.3, 11.6, 10.6; MS (*m*/*z*, relative intensity, %) 407 (2), 375 (90), 335 (92), 83 (91), 79 (96), 59 (100); HRMS calc. for $C_{24}H_{39}O_5$ (M^+–$C_7H_{15}O$; from cleavage between C-22 and C-23): 407.2798; found: 407.2801.

4.3. Preparation of 25-methoxy-2-O-methylbrassinolide (*13*) and 25-methoxy-3-O-methylbrassinolide (*14*)

Unsaturated diol **18** (Back et al., 1999) (1.13 g, 2.07 mmol) was acetylated as in the case of diol **15** to afford 1.26 g (97%) of the corresponding diacetate as a white solid, mp 94–95 °C; IR (KBr) 1746, 1647, 1364, 1247, 1058 cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 5.25 (*dd*, J = 8.0, 3.0 Hz, 1 H), 5.13 (*d*, J = 8.0 Hz, 1 H), 4.83 (*m*, 1 H), 4.72 (*m*, 1 H), 4.26 (*m*, 1 H), 4.10 (*m*, 1 H), 3.92 (*m*, 3 H), 3.74 (*m*, 1 H), 2.02 (*s*, 3 H), 1.97 (*s*, 3 H), 1.77 (*s*, 3 H), 1.47 (*s*, 3 H), 1.33 (*s*, 3 H), 1.08 (*d*, J = 6.8 Hz, 3 H), 1.03 (*d*, J = 7.0 Hz, 3 H), 0.83 (*s*, 3 H), 0.69 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.4, 170.3, 146.0, 111.9, 111.1, 100.8, 77.2, 75.2, 73.7, 72.9, 72.8, 55.9, 53.0, 52.6, 48.8, 45.6, 42.8, 42.5, 40.9, 40.7, 39.6, 37.9, 37.2, 33.0, 28.6, 28.2, 26.7, 24.2, 22.0, 21.8, 20.8, 20.7, 20.6, 13.4, 13.1, 12.5, 11.8; MS (*m*/*z*, relative intensity, %) 630 (17, M^+), 615 (52), 419 (61), 238 (55), 99 (93), 42 (100).

The above diacetate (1.22 g, 1.94 mmol) was added at room temperature to a vigorously stirred suspension of 2.46 g (7.72 mmol) of mercury(II) acetate in 10 ml of methanol. After 20 h, 10 ml of 10% sodium hydroxide solution was added, followed after 5 min by a solution of 380 mg (10.0 mmol) of sodium borohydride in 10 ml of 10% sodium hydroxide solution. The resulting mixture was stirred for 1 h, filtered through Celite and extracted three times with 100 ml of ether. The combined organic extracts were washed with saturated sodium bicarbonate solution and brine and dried over anhydrous sodium sulfate. The solution was concentrated in vacuum and the residue was chromatographed (hexanes:ethyl acetate, 4:1) to afford 840 mg (65%) of **19** as a white solid, mp 114–115 °C; IR (KBr) 1742, 1650, 1236, 1053 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.47 (*d*, J = 9.3 Hz, 1 H), 5.08 (*d*, J = 9.3 Hz, 1 H), 4.27 (*m*, 1 H), 4.10 (*m*, 1 H), 3.92 (*m*, 3 H), 3.75 (*m*, 1 H), 3.17 (*s*, 3 H), 2.02 (*s*, 3 H), 1.96 (*s*, 3 H), 1.47 (*s*, 3 H), 1.32 (*s*, 3 H), 1.08 (*s*, 3 H), 1.07 (*s*, 3 H), 1.04 (*d*, J = 7.2 Hz, 3 H), 1.02 (*d*, J = 7.0 Hz, 3 H), 0.82 (*s*, 3 H), 0.67 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.5, 170.2, 109.6, 107.5, 76.6, 75.7, 72.9, 72.8, 71.8, 65.6, 64.3, 56.0, 53.0, 52.6, 48.9, 45.6, 42.8, 42.6, 42.0, 41.0, 39.6, 38.2, 37.0, 33.0, 28.6, 28.3, 26.6, 24.2, 22.2, 22.0, 21.2, 21.1, 20.9, 20.7, 13.4, 12.8, 11.9, 8.7; MS (*m*/*z*, relative intensity, %) 662 (6, M^+), 647 (17), 451 (45), 98 (71), 73 (100).

The methoxy derivative **19** (700 mg, 1.06 mmol) was subjected to the Baeyer–Villiger oxidation using the same conditions as in the preparation of **17**. Lactone **20** (440 mg, 70%) was obtained as a white solid, mp 112–114 °C; IR (KBr) 3452, 1733, 1651, 1558 cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 5.47 (*d*, J = 9.3 Hz, 1 H), 5.06 (*d*, J = 9.3 Hz, 1 H), 4.07 (*m*, 3 H), 3.72 (*m*, 1 H), 3.17 (*s*, 3 H), 3.10 (*dd*, J = 12.2, 5.0 Hz, 1 H), 2.01 (*s*, 3 H), 1.97 (*s*, 3 H), 1.06 (*s*, 3 H), 1.05 (*s*, 3 H), 1.03 (*d*, J = 7.0 Hz, 3 H), 1.02 (*d*, J = 7.1 Hz, 3 H), 0.90 (*s*, 3 H), 0.70 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.2, 170.6, 170.2, 76.1, 75.5, 71.5, 70.3, 68.1, 67.9, 57.2, 52.2, 51.8, 48.5, 42.5, 42.0, 41.4, 40.9, 39.4, 39.2, 38.3, 37.0, 31.0, 28.1, 24.6, 22.3, 22.2, 21.0, 20.9, 20.8, 15.5, 12.6, 11.5, 8.8; MS (*m*/*z*, relative intensity, %) 522 (5), 321 (28), 91 (77), 67 (100).

Lactone **20** (380 mg, 0.640 mmol) was then treated with diazomethane and $SnCl_2$ as in the case of **17** to produce 65 mg (38% based on consumed starting material) of the corresponding 2-*O*-methylated diacetate as a white solid, mp 108–110°C, 35 mg (21% based on consumed starting material) of the 3-*O*-methylated diacetate as a white solid, mp 168–170 °C, along with 215 mg (57%) of recovered starting material, which was recycled in the usual manner.

Saponification of the above two diacetates was carried out by the same procedure as used in the preparation of **9** and **10**. Thus, the 2-*O*-methyl diacetate (45 mg, 0.074 mmol) afforded 23 mg (60%) of **13** and the 3-*O*-methyl diacetate (29 mg, 0.048 mmol) produced 17 mg (68%) of **14**.

Product **13**: white solid, mp 243–245 °C; IR (KBr) 3440, 1728, 1649, 1556, 1454 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.17 (*m*, 1 H), 4.08 (*m*, 2 H), 3.98 (*m*, 1 H), 3.55 (*m*, 1 H), 3.38 (*s*, 3 H), 3.22 (*s*, 3 H), 3.18 (*m*, 1 H), 3.14 (*m*, 1 H), 1.31 (*s*, 3 H), 1.21 (*s*, 3 H), 0.93 (*d*, J = 7.0 Hz, 3 H), 0.92 (*d*, J = 6.0 Hz, 3 H), 0.91 (*s*, 3 H), 0.70 (*s*, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 78.6, 77.1, 73.9, 72.2, 70.4, 64.4, 58.2, 55.9, 52.1, 51.2, 49.1, 43.1, 42.4, 41.0, 39.6, 39.3, 38.6, 38.0, 37.1, 30.3, 27.6, 24.7, 23.6, 23.0, 22.3, 15.5, 12.0, 11.7, 7.3; MS (*m*/*z*, relatively intensity, %) 477 (5), 393 (5), 335 (44), 169 (85), 129 (83), 79 (96), 67 (100); HRMS calcd for $C_{23}H_{37}O_5$ (M^+ $-C_7H_{15}O_2$; from cleavage between C-22 and C-23): 393.2641; found: 393.2671.

Product **14**: white solid, mp 293–295 °C; IR (KBr) 3462, 1726, 1651, 1557, 1453 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.12–3.98 (*m*, 3 H), 3.63 (*m*, 1 H), 3.58–3.50 (*m*, 2 H), 3.40 (*s*, 3 H), 3.23 (*s*, 3 H), 2.93 (*dd*, J = 12.0, 6.0 Hz, 1 H), 1.32 (*s*, 3 H), 1.22 (*s*, 3 H), 0.95 (*d*, J = 7.0 Hz, 3 H), 0.92 (*s*, 3 H), 0.91 (*d*, J = 6.8 Hz, 3 H), 0.70 (*s*, 3 H); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 176.1, 78.7, 77.2, 73.9, 72.2, 70.5, 67.8, 58.1, 56.9, 52.1, 51.2, 49.1, 43.2, 43.1, 42.4, 41.3, 39.6, 39.2, 38.3, 37.1, 27.6, 27.5, 24.7, 23.6, 23.0, 22.2, 15.3, 12.0, 11.7, 7.3; MS (*m*/*z*, relative intensity, %) 393 (13), 375 (25), 79 (98), 67 (100); HRMS calc. for $C_{23}H_{37}O_5$ (M^+ $-C_7H_{15}O_2$; from cleavage between C-22 and C-23): 393.2641; found: 393.2637.

4.4. Bioassays

Compounds **9**–**14** were tested for biological activity by means of the rice leaf lamina assay, using a dwarf cv., Tan-ginbozu, as described by Takeno and Pharis (1982). Brassinolide (**1**) was included in the bioassays as a standard. The compounds were dissolved in 95% ethanol and applied as 0.5 μl drops to the rice plants 48 h after planting the germinated seeds on 0.8% water agar. At high doses, several rounds of application of the 0.5 μl drops were required to attain the desired dose per plant. 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 brassinosteroids. The resultant leaf lamina angle was measured 60–65 h later. For an individual bioassay, 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 ng doses. Parallel applications of ethanol alone (control) and IAA alone (1000 ng) were carried out for each bioassay (see Figs. 5–7).

Acknowledgements

We thank CIDtech Research Inc. and the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support. 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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