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Takuya Muto and Yasushi Todoroki*

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan.

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* Corresponding author. Tel./fax: +81 54 238 4871.

E-mail address: aytodor@ipc.shizuoka.ac.jp (Y. Todoroki)

ABSTRACT

A novel chemical tool compound that is an antagonist of brassinolide (BL, **1**)-induced rice lamina joint inclination was developed. Although 2-*O*-, 3-*O*-, 22-*O*-, or 23-*O*-methylation of BL causes a critical decrease in biological activity,[5] a crystal structure of the extracellular leucine-rich repeat (LRR) domain of BRASSINOSTEROID-INSENSITIVE I (BRI1) bound to BL[3,4] indicates that the loss of activity of the *O*-methylated BL may result from not only the low affinity to BRI1, but also from blocking the interaction with another BR signaling factor, a partner protein of BRI1 (e.g., BRI1-ASSOCIATED KINASE 1, BAK1). On the basis of this hypothesis we synthesized the BL 2,3-acetonide **2**, the 22,23-acetonide **3**, and the 2,3:22,23-diacetonide **4** to assess the possibility of 2-*O*- and 3-*O*- or/and 22-*O*- and 23-*O*-alkylated BL as an antagonist in BR signaling evoked by exogenously applied BL. The 2,3-acetonide **2** more strongly inhibited the lamina inclination caused by BL relative to the 22,23-acetonide **3**, whereas the diacetonide **4** had no effect most likely due to its increased hydrophobicity. This suggested that the 2,3-hydroxyl groups of BL play a more significant role in the interaction with a BRI1 partner protein rather than BRI1 itself in rice lamina joint inclination. Taken together it was demonstrated that BL, the most potent agonist of BRI1, is transformed into an antagonist by functionalization of the 2,3-dihydroxyl groups as the acetonide. This finding opens the door to the potential development of a chemical tool that modulates protein-protein interactions in the BR signaling pathway to dissect the BR-dependent processes.

1. Introduction

Brassinosteroids (BRs) are essential plant steroid hormones that play important roles in plant growth and development.[1] They are recognized by the extracellular leucine-rich repeat (LRR) domain of BRASSINOSTEROID-INSENSITIVE I (BRI1), a cell surface receptor kinase.[2] Recently, two research groups have reported the three-dimensional structure of BRI1 bound brassinolide (BL, **1**, Figure 1a), the most potent BR.[3,4] BL binds to a surface groove on BRI1 (Figure 1c) with the terminus of the BL side chain (C24–28) buried in a hydrophobic pocket. The remaining parts interact with the surface groove only on the one side (β-face) with the α-face of the ring being solvent-exposed. Although some direct, or water-mediated indirect, hydrogen bonds are observed between the hydroxyl groups and the residues of BRI1 (Figure 1b and c), all of the hydroxyl groups are solvent-accessible. As a result the solvent-exposed face might be involved in the interaction with another factor essential to BRI1 signaling. For example, BRI1-ASSOCIATED KINASE 1 (BAK1) associates with BRI1 and promotes BR-induced signaling.[6] A BL analogue with the diol moieties modified could function as a BRI1 antagonist if the solvent accessible region of the BRI1-BL complex provides a protein-protein interaction platform to induce BR signaling. The inhibition of this activation pathway could potentially shut down BR signaling. A BRI1 antagonist that causes chemical knockdown of BRI1 could function as a novel chemical tool used to dissect the BR signaling pathway. To date there have been no reports on a BRI1 antagonist, but a significant candidate may have been merely overlooked because BR analogues have never been screened from this perspective.

The 2-*O*-, 3-*O*-, 22-*O*- or 23-*O*-methylation of BL causes a critical decrease in biological activity.[6,7] These analogues could be inefficient agonists of BRI1 owing to the loss of hydrogen bonds with the BRI1 residues. It bears mentioning that the low biological activity of the methylated BL derivatives does not exclude the possibility that these compounds bind to BRI1. The *O*-alkyl functional groups should not interfere sterically with the formation of a complex with BRI1 given that the hydroxyl groups are solvent-accessible and not directly involved in

the surface binding with BRI1. Therefore, the loss of biological activity of the *O*-methylated derivatives of BL may be the result of not only the low affinity to BRI1, but also the blocked interaction with another BR signaling factor. This could be especially true considering that the C2-hydroxyl group does not hydrogen bond with the BRI1 residues. The low biological activity of 2-*O*-methyl-BL may result exclusively from the inhibitory action against the BRI1-BAK1 interaction. On the basis of this hypothesis we set out to assess the possibility that *O*-alkylated BL could antagonize BR signaling evoked by exogenously applied BL.

The direct alkylation (e.g., methylation) of BL could provide a mixture of four mono-, six di-, four tri-, and one tetra-alkylated compounds statistically speaking. Back et al. have reported a strategy toward the selective methylation by using appropriate protecting groups in a long synthetic sequence from stigmasterol.[6] It was envisioned that by taking advantage of the formation of acetonides from vicinal diols one could reduce the possibility of *O*-functionalized isomers from fifteen for alkylation to three (two mono- and one diacetonide). In addition, using this approach one can still access test substrates with either, or both, of the steroid diols functionalized allowing one to rapidly evaluate which interactions are more significant with BRI1. Using this concise and efficient approach, the direct acetonide functionalization of BL was carried out to provide the 2,3-acetonide **2**, the 22,23-acetonide **3**, and the diacetonide **4** (Figure 2a). Although **3** and **4** have been reported as synthetic intermediates[6,8–12] their biological activities have not been investigated. Interestingly, neither the synthesis nor the biological activity of the 2,3-acetonide **2** has been reported.

2. Results and discussion

2.1. Preparation of the acetonides 2–4

The acetonides **2**–**4** were prepared according to the previously reported method for the synthesis of 28-methyl-BL (28-homo-BL) acetonides.[12] Commercially available tetraol **1** was treated with boron trifluoride diethyl etherate and acetone to afford the mono- and diacetonides, which were distinguished based on ^{1}H NMR and mass spectral data. The monoacetonides **2** and **3** were identified based on NOESY data (Figure 3a). The methyl protons of the acetonide **2** were correlated to H-2 and H-3 in the BL A-ring, whereas those of **3** were correlated to H-22 and H-23.

2.2. Effect of the acetonides on rice lamina joint inclination

The acetonides **2**–**4** were subjected to the rice lamina joint inclination assay, which is a sensitive bioassay that serves as a test for BRs.[13] Accordingly BL was used as a positive standard. A 10 μg aliquot of the respective acetonide or 0.1 μg of BL dissolved in 0.5 μL ethanol was applied to the lamina joint region of the rice (Nipponbare) seedling four days after germination with the bending angle measured after an additional two days.

None of the tested acetonides displayed significant activity, and thus indicated that they did not act as BRI1 agonists (Figure 4). The lack of activity of the acetonides provided evidence that the acetonide moiety is stable to hydrolysis under the experimental conditions (i.e., BL is not released in rice seedling). Next, we examined the simultaneous application of each acetonide with BL in mixture experiments. As depicted in Fig. 4, the monoacetonides **2** and **3** dose-dependently inhibited the lamina inclination caused by BL at doses of more than 1 and 3 μg, respectively. In contrast, the diacetonide **4** had no effect on the lamina inclination at doses up to 10 μg. These two results taken together suggest that the 2,3-acetonide **2** acts as an antagonist of BRI1 as predicted. The 22,23-acetonide **3** appears to act as a weak antagonist of

BRI1, whereas the diacetonide **4** was neither an agonist nor an antagonist. The antagonistic effect of the acetonide **4** was not the summation of that of the two monoacetonides **2** and **3**. If both the 2,3- and 22,23-dihydroxyl groups of BL play a critical role in the interaction with a partner protein of BRI1, the diacetonide **4** should exhibit a more potent antagonistic effect relative to the monoacetonides **2** and **3**. One possibility is that the binding affinity for BRI1 was severely reduced as a result of losing all of the hydrogen-bond donors. Another potential explanation is that the diacetonide **4** is too hydrophobic to readily cross from the applied ethanolic solution on the epidermis through the cell walls. The calculated partition coefficient (clog*P*) of BL and the monoacetonide **2** is 3.13 and 4.83, respectively, whereas that of the diacetonide **4** is 6.53. The clog*P* of **4** appears to be too high to act as an effective drug (i.e. cannot cross cell wall) although interpreting clog*P* for plant targets may be not relatable to that of mammalian species. To test these hypotheses we synthesized compounds incorporating a hydroxyl group on one of the acetonide methyl groups (Figure 2b, **5**–**7**). The clog*P* of di-hydroxyacetonide **7** is 5.13, which is significantly lower than that of **4**.

2.3. Preparation of the hydroxyacetonides 5–7

The hydroxyacetonides **5**–**7** were prepared by a method similar to that of the acetonides **2**–**4**. BL (**1**) was treated with boron trifluoride diethyl etherate and hydroxyacetone to afford the mono- and di-hydroxyacetonides, which were distinguished based on ^{1}H NMR and mass spectral data. The mono-hydroxyacetonides **5** and **6** were identified based on NOESY data (Figure 3b). The methyl protons in the hydroxyacetonide of **5** were correlated to H-2 and H-3 in the A-ring, whereas those of **6** were correlated to H-22 and H-23. Because the hydroxyacetonide group bears a stereogenic center, all of the hydroxyacetonides were prepared as epimeric mixtures and the respective epimeric mixtures were tested in bioassays without being resolved.

2.4. Effect of the hydroxyacetonides on rice lamina joint inclination

The mono-hydroxyacetonides **5** and **6**, although weak, caused bending at 10 μg (Figure 5) in contrast to the results obtained with the *des*-hydroxy-acetonides. Although it is sterically larger than the unsubstituted acetonide, the hydroxyacetonide has hydrogen-bonding ability and this effect in **5** and **6** may have slightly assisted the approach of the BRI1 partner protein in cooperation with the unmodified two remaining hydroxyl groups. With the simultaneous application of the hydroxyacetonides with BL in mixture experiments (Figure 5), the 2,3-hydroxyacetonide **5** dose-dependently inhibited the lamina inclination caused by BL at doses of more than 1 μg; however, it was less potent than the corresponding unsubstituted acetonide **2**. In contrast, the 22,23-hydroxyacetonide **6** slightly inhibited the BL-induced bending only at 10 μg, indicating that the hydroxyl group attached to the acetonide moiety caused a decrease in the antagonistic effect. Especially, the weak antagonistic effect of the 22,23-acetonide **3** seems to have been almost completely offset by the agonistic effect induced by the additional hydroxyl group on the acetonide. On the other hand, the di-hydroxyacetonide **7** acted not as an agonist, but an antagonist. This is most likely due to the steric effect derived from the two hydroxyacetonides, which may have been too great to have been compensated by the two hydroxyl groups on the acetonides that are located in different orientations from the original four hydroxyl groups in BL. Considering that an antagonistic effect was observed with the di-hydroxyacetonide **7**, it may be reasonable to postulate that the diacetonide **4** behaved neither as an agonist nor an antagonist because it was not absorbed into plant tissue as a result of the increased hydrophobicity.

3. Conclusions

In this study it has been demonstrated that BL, the most potent agonist of BRI1, is transformed into an antagonist by acetonide functionalization of the 2,3-dihydroxyl groups. Although this effect was also observed with the 22,23-acetonide, the antagonistic effect was weaker. This suggests that the 2,3-dihydroxyl groups of BL play a more significant role in the

interaction with a BRI1 partner protein rather than BRI1 itself in rice lamina joint inclination. The acetonide functional group was carefully selected as a substituent to introduce steric hindrance on the α-face of the BL A-ring while providing a concise and efficient synthetic route to test the posed hypothesis. By taking advantage of the formation of acetonides from vicinal diols one could reduce the possibility of *O*-functionalized isomers from fifteen for methylation to three (two mono- and one diacetonide) without the use of lengthy protecting group manipulations. In addition, using this approach one can still access test substrates with either, or both, of the steroid diols functionalized allowing one to rapidly evaluate which interactions are more significant with BRI1. At this point it remains unclear as to which of the hydroxyl groups (2- vs. 3-) is more effective in producing a potent antagonist. The length, bulkiness, and polarity of the substituent have also yet to be investigated. However, it is clear that a single hydroxyl group should not be introduced onto the modified groups. As an initial communication, our finding opens the door to the development of a chemical tool that modulates the protein-protein interactions in BR signaling to dissect the BR-dependent processes.

4. Experimental

4.1. General

Brassinolide was purchased from Brassino Co. Ltd., Japan. ^{1}H NMR, ^{13}C NMR and 2D-correlation NMR experiments were recorded with tetramethylsilane as the internal standard using JNM-LA500 (500 MHz) NMR spectrometers. High-resolution mass spectra were obtained with a JEOL JMS-T100LC AccuTOF mass spectrometer.

4.2. Synthesis of substrates 3–7

4.2.1. The monoacetonides 2 and 3 and diacetonide 4 of BL

Brassinolide (BL, **1**) (10 mg, 20 μmol) and acetone (0.2 mL, 2.7 mmol) were dissolved in THF (2 mL) at −5 °C under Ar. Borontrifluoride diethyl etherate (5 μL) was added to the stirred solution. The resulting mixture was stirred for 4 h at the same temperature and subsequently quenched with pyridine (30 μL). The solution was concentrated in vacuo and the residue was purified by silica gel column chromatography eluted with hexane-EtOAc to obtain **2** (1.6 mg), **3** (2.7 mg), and **4** (1.5 mg) as colorless oils. The same procedure was repeated several times to obtain enough material for structural analyses and bioassays. The acetonide **2**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.73 (3H, s, H_3-18), 0.85 (3H, d, *J* = 6.7 Hz, H_3-28), 0.88 (3H, s, H_3-19), 0.91 (3H, d, *J* = 6.4 Hz, H_3-21), 0.95 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.97 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 1.14 (1H, dd, *J* =15.9 and 2.4 Hz, H-1β), 1.21 (1H, m, H-24), 1.24 (1H, m, H-14), 1.31 (3H, s, methyls in the acetonide), 1.35 (1H, m, H-12α), 1.49 (1H, m, H-20), 1.52 (3H, s, methyls in the acetonide), 1.58 (1H, m, H-17), 1.62 (1H, m, H-25), 1.65 (2H, m, H_2-15), 1.73 (1H, m, H-9), 1.78 (2H, m, H_2-4), 1.80 (1H, m, H-8), 1.83 (2H, m, H_2-11), 1.97 (2H, m, H_2-16), 2.01 (1H, m, H-12β), 2.13 (1H, brs, HO-22 or -23), 2.31 (1H, dd, *J* = 15.9 and 3.7, H-1α), 3.29 (1H, dd, *J* = 10.7 and 4.3 Hz, H-5), 3.54 (1H, brd, *J* = 8.2 Hz, H-22), 3.71 (1H, brd, *J* = 8.2 Hz, H-23), 3.75 (1H, brs, HO-22 or -23), 4.10 (2H, m, H_2-7), 4.35 (1H, m, H-2), 4.38 (1H, m, H-3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 10.1 (C28), 11.8 (C21), 12.1 (C18), 19.6 (C19), 20.7 (C26 or 27), 20.8 (C26 or 27), 23.0 (C11), 23.6 (methyl carbon in the acetonide), 24.4 (C15), 26.5 (methyl carbon in the acetonide), 27.6 (C16), 27.7 (C4), 30.7 (C25), 33.5 (C1), 35.9 (C10), 36.8 (C20), 39.4 (C8), 39.8 (C12), 40.1 (C24), 40.2 (C5), 42.9 (C13), 51.9 (C14), 52.2 (C17), 54.7 (C9), 71.2 (C7), 72.4 (C3), 73.0 (C2), 73.5 (C23), 74.5 (C22), 107.6 (quaternary carbon in the acetonide), 176.6 (C6); HRMS (ESI-TOF, positive mode): calcd for $C_{31}H_{52}O_6Na$ $[M+Na]^+$ 543.3662, found 543.3657. The acetonide **3**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.70 (3H, s, H_3-18), 0.86 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.89 (3H, d, *J* = 7.0 Hz, H_3-28), 0.92 (3H, s, H_3-19), 0.94 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.98 (3H, d, *J* = 6.7 Hz, H_3-21), 1.19 (1H, m, H-11β), 1.22 (1H, m, H-14), 1.28 (1H, m, H-9), 1.33 (1H, m, H-24), 1.34 (3H, s, methyl protons in the acetonide), 1.36 (3H, s, methyl protons in the acetonide), 1.39 (1H, m, H-15α), 1.41-1.44 (1H, m, H-20), 1.52 (1H, m, H-17), 1.56 (1H, m, H-1β), 1.62 (1H, brs, HO-2 or -3), 1.64 (1H, m,

H-25), 1.71 (1H, m, H-11α), 1.75 (1H, m, H-8), 1.78 (1H, m, H-15β), 1.87 (1H, dd, *J* = 12.5 and 4.6, H-1α), 1.94 (1H, m, H-4α), 1.97 (2H, m, H_2-12), 1.96 (2H, m, H_2-16), 2.09 (1H, m, H-4β), 3.11 (1H, dd, *J* = 12.5 and 4.6 Hz, H-5), 3.71 (1H, m, H-2), 3.73 (1H, dd, *J* = 8.2 and 4.0 Hz, H-22), 3.80 (1H, brd, *J* = 8.2 Hz, H-23), 4.02 (1H, m, H-3), 4.09 (2H, m, H_2-7); ^{13}C NMR (125 MHz, $CDCl_3$): δ 9.9 (C28), 11.6 (C18), 12.6 (C21), 15.5 (C19), 18.4 (C26 or 27), 21.2 (C26 or 27), 22.2 (C15), 24.7 (C11), 27.3 (methyl carbons in the acetonide), 27.7 (C16), 30.6 (C25), 31.1 (C4), 36.2 (C20), 38.3 (C10), 39.2 (C8), 39.4 (C12), 40.6 (C24), 40.9 (C5), 41.5 (C1), 42.6 (C13), 51.1 (C14), 53.3 (C17), 58.2 (C9), 68.1 (C2), 68.1 (C3), 70.4 (C7), 79.1 (C23), 80.3 (C22), 107.6 (quaternary carbon in the acetonide), 176.2 (C6). HRMS (ESI-TOF, positive mode): calcd for $C_{31}H_{52}O_6Na$ $[M+Na]^+$ 543.3662, found 543.3655. The diacetonide **4**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.71 (3H, s, H_3-18), 0.86 (3H, d, *J* = 7.0 Hz, H_3-26 or -27), 0.88 (3H, s, H_3-19), 0.89 (3H, d, *J* = 7.0 Hz, H_3-28), 0.94 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.98 (3H, d, *J* = 6.7 Hz, H_3-21), 1.11 (1H, dd, *J* =15.9 and 2.4 Hz, H-1β), 1.23-1.25 (1H, m, H-14), 1.31 (3H, s, methyl protons in the acetonide), 1.32 (1H, m, H-12α), 1.32 (1H, m, H-24), 1.33 (3H, s, methyl protons in the acetonide), 1.34 (1H, m, H-11β), 1.36 (3H, s, methyl protons in the acetonide), 1.44 (1H, m, H-20), 1.51 (3H, s, methyl protons in the acetonide), 1.55 (1H, m, H-17), 1.63 (2H, m, H_2-15), 1.66 (1H, m, H-25), 1.74 (1H, m, H-9), 1.78 (2H, m, H_2-16), 1.80 (1H, m, H-4α), 1.82 (1H, m, H-8), 1.84 (1H, m, H-11α), 1.99 (1H, m, H-4β), 2.01 (1H, m, H-12β), 2.32 (1H, dd, *J* = 15.9 and 3.7, H-1α), 3.29 (1H, dd, *J* = 10.7 and 3.7 Hz, H-5), 3.74 (1H, dd, *J* = 8.5 and 4.6 Hz, H-23), 3.81 (1H, brd, *J* = 8.5 Hz, H-22), 4.09 (2H, m, H_2-7), 4.36 (1H, m, H-2), 4.38 (1H, m, H-3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 9.9 (C28), 11.9 (C18), 12.5 (C21), 18.4 (C26 or 27), 19.6 (C19), 21.1 (C26 or 27), 23.0 (C11), 23.6 (methyl carbon in the acetonide), 24.4 (C15), 26.4 (methyl carbon in the acetonide), 27.2 (methyl carbons in the acetonide), 27.5 (C16), 27.9 (C4), 30.6 (C25), 33.5 (C1), 35.9 (C10), 36.2 (C20), 39.4 (C8), 39.6 (C12), 40.2 (C5), 40.6 (C24), 43.1 (C13), 51.7 (C14), 53.3 (C17), 54.7 (C9), 71.2 (C7), 72.5 (C3), 73.1 (C2), 79.0 (C23), 80.3 (C22), 107.5, (quaternary carbon in the acetonide), 107.6 (quaternary carbon in the acetonide), 176.6 (C6); HRMS (ESI-TOF, positive mode): calcd for $C_{34}H_{56}O_6Na$ $[M+Na]^+$ 583.3975, found 583.3981.

4.2.2. The mono-hydroxyacetonides 5 and 6 and di-hydroxyacetonide 7 of BL

BL (**1**) (10 mg, 20 μmol) and hydroxyacetone (0.1 mL, 1.5 mmol) were dissolved in THF (1 mL) at −5 °C under Ar. Sulfuric acid (20 μL) was added to the stirred solution. The resulting mixture was stirred for 3 h at the same temperature prior to quenching with sat. aq. $NaHCO_3$ (10 mL) and subsequent extraction with EtOAc (8 mL×3). The residue was purified by silica gel column chromatography eluted with hexane-EtOAc to obtain **5** (2.2 mg), **6** (1.7 mg), and **7** (2.0 mg) as colorless oils. The same procedure was repeated several times to obtain enough material for structural analyses and bioassays. The hydroxyacetonide **5**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.73 (3H, s, H_3-18), 0.85 (3H, d, *J* = 7.0 Hz, H_3-28), 0.88 (3H, s, H_3-19), 0.90 (3H, d, *J* = 6.7 Hz, H_3-21), 0.95 and 0.97 (each 3H, d, *J* = 6.7 Hz, H_3-26 or -27), 1.17 (1H, brd, *J* =16.8 Hz, H-1β), 1.21 (1H, m, H-14), 1.22 (1H, m, H-24), 1.26 (3H, s, methyl protons in the hydroxyacetonide), 1.29 (1H, m, H-4α), 1.32 (1H, m, H-11β), 1.34 (1H, m, H-12α), 1.49 (1H, m, H-20), 1.57 (1H, m, H-17), 1.60 (1H, m, H-25), 1.63-1.66 (2H, m, H_2-15), 1.79 (1H, m, H-9), 1.79 (1H, m, H-8), 1.81 (1H, m, H-11α), 1.83 (1H, m, H-4β), 2.00 (1H, m, H-12β), 2.00 (2H, m, H_2-16), 2.35 (1H, brd, *J* = 16.8 Hz, H-1α), 2.18, 2.63, and 3.49 (each 1H, s, hydroxy proton in the hydroxyacetonide, H-22 or H-23), 3.54 (1H, brd, *J* = 8.5 Hz, H-22), 3.65 (1H, dd, *J* = 14.7 and 4.3 Hz, H-5), 3.69 (2H, s, methylene protons in the hydroxyacetonide), 3.72 (1H, brd, *J* = 8.5 Hz, H-23), 4.05 (1H, m, H-7α), 4.19 (1H, m, H-7β), 4.44 (2H, brs, H-2 and 3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 10.1 (C28), 11.8 (C21), 12.1 (C18), 19.3 (C19), 19.9 (methyl carbons in the acetonide), 20.7 (C26 or 27), 20.8 (C26 or 27), 23.0 (C11), 24.4 (C15), 27.8 (C16), 27.8 (C4), 30.7 (C25), 33.5 (C1), 35.8 (C10), 36.9 (C20), 39.4 (C8), 39.6 (C5), 39.8 (C12), 40.1 (C24), 42.9 (C13), 51.8 (C14), 52.2 (C17), 54.1 (C9), 66.1 (hydroxymethyl carbons in the acetonide), 70.8 (C7), 72.6 (C2 or 3), 73.2 (C2 or 3), 73.4 (C23), 74.6 (C22), 107.7, (quaternary carbon in the acetonide), 177.1 (C6); HRMS (ESI-TOF, positive mode): calcd for $C_{31}H_{52}O_7Na$ $[M+Na]^+$ 559.3611, found 559.3604. The hydroxyacetonide **6**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.70 and 0.71 (3H, s, H_3-18), 0.87 and 0.87 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.91 (3H, d, *J* = 7.0 Hz, H_3-28), 0.92 (3H, s, H_3-19), 0.94 and 0.95 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.99 (3H, d, *J* = 6.7

Hz, H_3-21), 1.20 (1H, m, H-11β), 1.22 (1H, m, H-14), 1.24 (1H, m, H-8), 1.26 (1H, m, H-9), 1.26 (1H, m, H-16α), 1.30 and 1.33 (3H, s, methyl protons in the hydroxyacetonide), 1.38 (1H, m, H-24), 1.45 (1H, m, H-20), 1.54 (1H, m, H-1β), 1.58 (1H, m, H-17), 1.65 (1H, m, H-25), 1.70 (1H, m, H-11α),1.71 (1H, m, H-12α), 1.78 (2H, m, H_2-15), 1.87 (1H, dd, *J* = 12.5 and 4.6 Hz, H-1α), 1.94 (1H, m, H-4α), 1.97 (1H, m, H-16β), 2.00 (1H, m, H-12β), 2.15 (1H, m, H-4β), 3.12 (1H, dd, *J* = 12.5 and 4.6 Hz, H-5), 3.48 (2H, m, methylene protons in the hydroxyacetonide), 3.70 (1H, m, H-2), 3.82 (1H, m, H-23), 3.86 (1H, m, H-22), 4.01 (1H, brs, H-3), 4.08 (2H, m, H_2-7); ^{13}C NMR (125 MHz, $CDCl_3$): δ 9.8 and 9.9 (C28), 11.5 (C18), 12.6 (C21), 15.4 (C19), 18.4 and 18.6 (C26 or 27), 21.0 (C26 or C27), 22.2 (C15), 22.6 and 22.7 (methyl carbons in the acetonide), 24.7 (C11), 27.7 and 27.8 (C16), 30.8 and 3.09 (C25), 31.0 (C4), 36.0 and 36.1 (C20), 38.3 (C10), 39.2 (C8), 39.4 (C12), 40.3 (C24), 40.9 (C5), 41.5 (C1), 42.6 (C13), 51.2 (C14), 53.3 (C17), 53.3 (C9), 67.5 (hydroxymethyl carbons in the acetonide), 68.0 (C3), 68.1 (C2), 70.4 (C7), 79.0 and 79.9 (C23), 80.1 and 81.5 (C22), 107.5 and 107.7 (quaternary carbon in the acetonide), 176.3 (C6); HRMS (ESI-TOF, positive mode): calcd for $C_{31}H_{52}O_7Na$ $[M+Na]^+$ 559.3611, found 559.3604. The di-hydroxyacetonide **7**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.72 and 0.73 (3H, s, H_3-18), 0.87 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 0.89 (3H, s, H_3-19), 0.91 (3H, d, *J* = 7.0 Hz, H_3-28), 0.94 and 0.95 (3H, d, *J* = 6.7 Hz, H_3-26 or -27), 1.00 (3H, d, *J* = 6.7 Hz, H_3-21), 1.14 (1H, dd, *J* = 15.6 and 2.4 Hz, H-1β), 1.26 (1H, m, H-14), 1.30 and 1.33 (3H, s, methyl protons in the hydroxyacetonide), 1.33 (1H, m, H-16α), 1.35 (1H, m, H-12α), 1.38 (1H, m, H-24), 1.44 (1H, m, H-20), 1.51 (3H, s, methyl protons in the hydroxyacetonide), 1.59 (1H, m, H-17), 1.65 (2H, m, H_2-15), 1.69 (1H, m, H-25), 1.74 (1H, m, H-9), 1.81 (2H, m, H_2-4), 1.84 (2H, m, H_2-11), 1.87 (1H, m, H-12β), 2.00 (1H, m, H-16β), 2.01 (1H, m, H-8), 2.31 (1H, dd, *J* = 15.6 and 2.4 Hz, H-1α), 3.28 (1H, dd, *J* = 11.3 and 3.7 Hz, H-5), 3.44 (2H, m, methylene protons in the hydroxyacetonide), 3.51 (2H, m, methylene protons in the hydroxyacetonide), 3.82 (1H, m, H-23), 3.86 (1H, m, H-22), 4.10 (2H, m, H_2-7), 4.42 (1H, m, H-2), 4.44 (1H, brs, H-3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 9.9 and 9.9 (C28), 11.9 (C18), 12.6 (C21), 18.4 and 18.6 (C26 or C27), 19.5 (C19), 21.0 (C26 or C27), 21.9 (methyl carbons in the acetonide), 22.7 (methyl carbons in the acetonide), 23.0 (C11), 24.4 (C15), 27.8 (C16), 27.9

(C4), 30.8 and 3.09 (C25), 33.8 (C1), 35.9 (C10), 36.1 (C20), 39.4 (C8), 39.6 (C12), 40.3 (C24), 40.3 (C5), 43.1 (C13), 51.8 (C14), 53.3 (C17), 54.8 (C9), 64.9 (hydroxymethyl carbons in the acetonide), 67.6 (hydroxymethyl carbons in the acetonide), 71.1 (C7), 73.7 (C3), 74.0 (C2), 79.0 and 79.9 (C23), 80.1 and 81.5 (C22), 107.2 and 107.7 (quaternary carbon in the acetonide), 108.4 (quaternary carbon in the acetonide), 176.3 (C6); HRMS (ESI-TOF, positive mode): calcd for $C_{34}H_{56}O_8Na$ $[M+Na]^+$ 615.3873, found 615.3871.

4.3. Bioassays

Seeds of rice (*O. sativa* L. cv. Nipponbare) were sterilized with EtOH for 5 min and washed with running tap water. The sterilized seeds were soaked in water for 3 days at 25 °C to germinate. The compounds were dissolved in EtOH and applied as 0.5 μL microdrops to the rice plant 96 h after planting germinated seeds on 0.4% water agar under continuous light (4000 lx) at 25 °C. The resultant leaf lamina angle was measured 48 h later. An application of EtOH alone (control) was always carried out. Each data point is the mean of the leaf angles from 7 plants for all the doses. All assays were conducted at least triplicate.

Acknowledgments

We thank Prof. Akira Kitajima (Kyoto University) for kindly providing rice (Nipponbare) seeds. Part of this research was carried out using an instrument at the Center for Instrumental Analysis of Shizuoka University.

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:xx.xxxx/j.bmc.201x.xx.xxx.

References and notes

1. Yang, C. J.; Zhang, C; Lu, Y. N.; Jin, J. Q.; Wang, X. L. *Mol. Plant* **2011**, *4*, 588.

2. Li, J.; Chory, J. *Cell* **1997**, *90*, 929.

3. Hothorn, M.; Belkhadir, Y.; Dreux, M.; Dabi, T.; Noel, J. P.; Wilson, I. A.; Chory, J. *Nature* **2011**, *474*, 467.

4. She, J.; Han, Z.; Kim, T.-W.; Wang, J.; Cheng, W.; Chang, J.; Shi, S.; Wang, J.; Yang, M.; Wang, Z.-Y.; Chai, J. *Nature* **2011**, *474*, 472.

5. Li, J.; Wen, J.; Lease, K. A.; Doke, J. T.; Tax, F. E.; Walker, J. C. *Cell* **2002**, *110*, 213.

6. Back, T. G.; Janzen, L.; Pharis, R. P.; Yan, Z. *Phytochemistry* **2002**, *59*, 627.

7. Back, T. G.; Pharis, R. P. *J. Plant Growth Regul.* **2003**, *22*, 350.

8. Baron, D. L.; Luo, W.; Janzen, L.; Pharis, R. P.; Back, T. G. *Phytochemistry* **1998**, *49*, 1849.

9. Back, T. G.; Baron, D. L.; Luo, W.; Nakajima, S. K. *J. Org. Chem.* **1997**, *62*, 1179.

10. Khripach, V. A.; Khripach, N. B.; Zhabinskii, V. N.; Zhiburtovich, Y. Y.; Schneider, B.; de Groot, A. *J. Label. Compd. Radiopharm.* **2007**, *50*, 1153.

11. Watanabe, T.; Yokota, T.; Shibata, K.; Nomura, T.; Seto, H.; Takatsuto, S. *J. Chem. Res. (S)* **2000**, 18.

12. Kerb, U.; Eder, U.; Krahmer, H. *Ger. Offen.* **1984**, DE3234606 A1 19840315.

13. Takeno, K.; Pharis, R. P. *Plant Cell Physiol.* **1982**, *23*, 1275.

14. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.

Figure legends

Figure 1. (a) The molecular structure of BL (**1**); (b) the direct and water-mediated indirect hydrogen bonds between the hydroxyl groups of BL and the BRI1 residue, estimated from the three-dimensional structure (PDB code: 3RJ0[3] and 3RGZ[4]) of BRI1 bound BL; (c) the three-dimensional structure (PDB code: 3RGZ) of BRI1 bound BL in which BL is depicted as a stick model (carbons, green; oxygens, red; hydrogens, white). Hydrogens and a molecular surface (upper) of BRI1 is calculated and displayed with Chimera.[14]

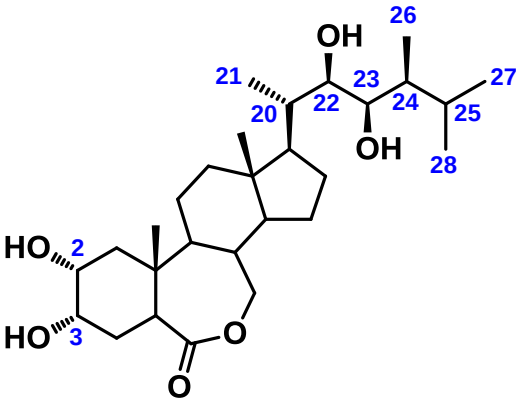
Figure 2. The molecular structure of the acetonides **2**–**4** (**a**) and hydroxyacetonides **5**–**7** (**b**).

Figure 3. Selected NOESY correlations (arrows) for the acetonides **2** and **3** (**a**) and hydroxyacetonides **5** and **6** (**b**).

Figure 4. Rice lamina joint inclination assay of the acetonides **2**–**4** in the absence and presence of BL.

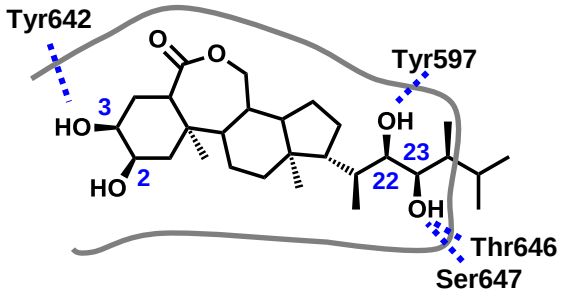
Figure 5. Rice lamina joint inclination assay of the hydroxyacetonides **5**–**7** in the absence and presence of BL.

a

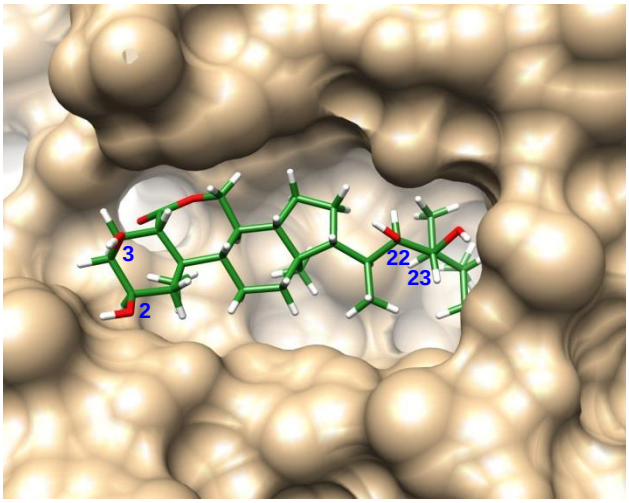


BL (**1**)

b



c



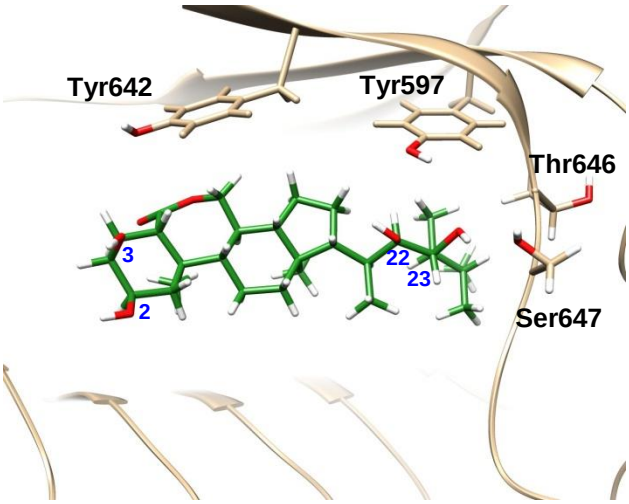
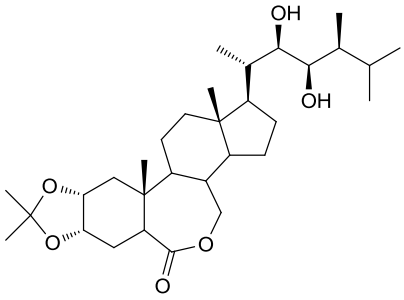


Figure 1

a



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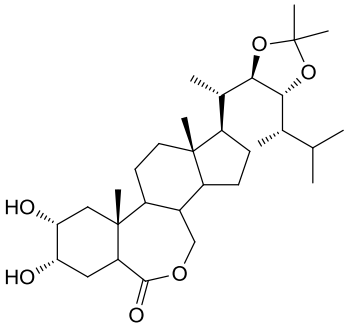
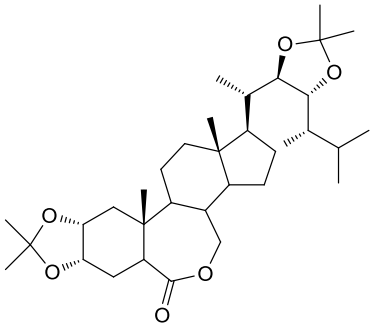
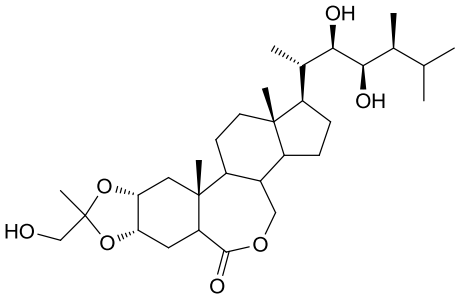


3



4

b



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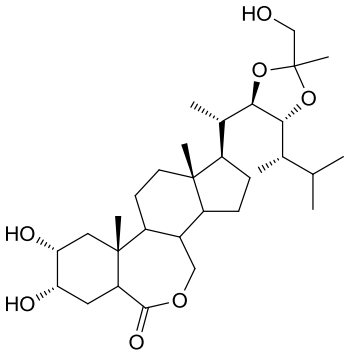

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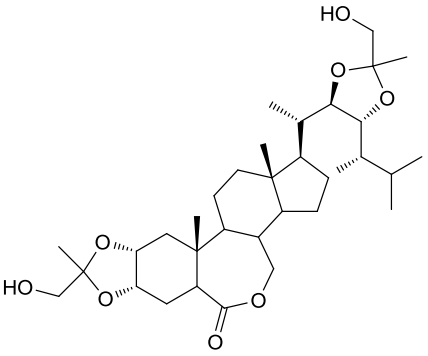
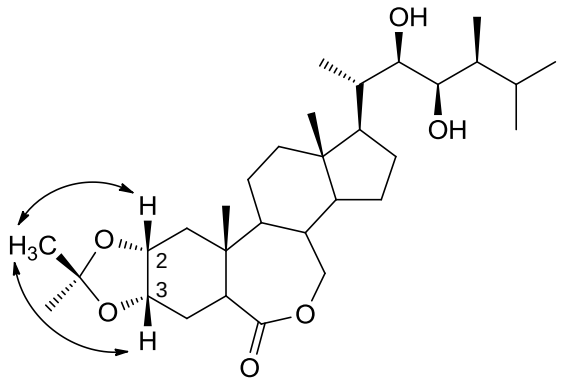


7

Figure 2

a



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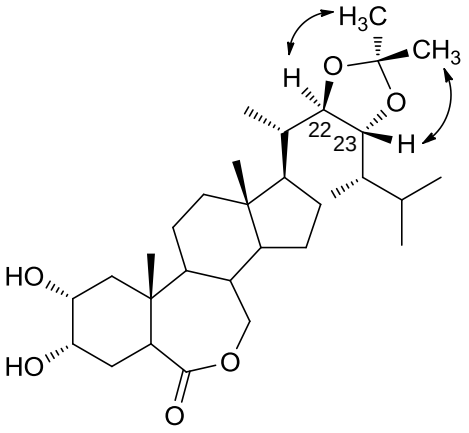
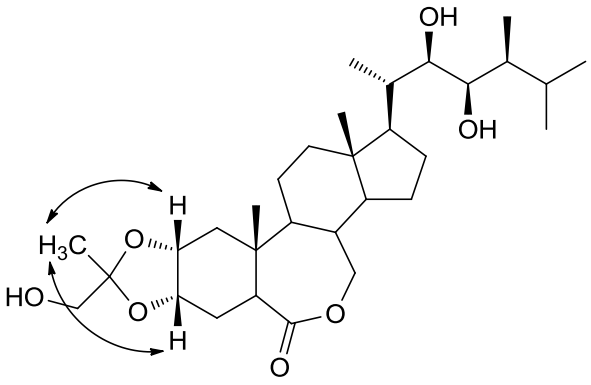


3

b



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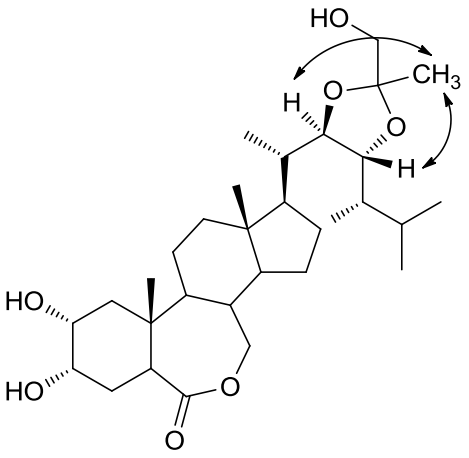


6

Figure 3

	BL (0.1 μg)	dose (μg)	angle (degree)
none	−		0±0
	+		115±12
2	−	10	0±0
	+	0.3	112±28
	+	1	37±6
	+	3	28±6
	+	10	17±9
3	−	10	3±5
	+	0.3	117±12
	+	1	83±32
	+	3	36±7
	+	10	24±4
4	−	10	0±0
	+	0.3	100±0
	+	1	98±8
	+	3	106±11
	+	10	106±9

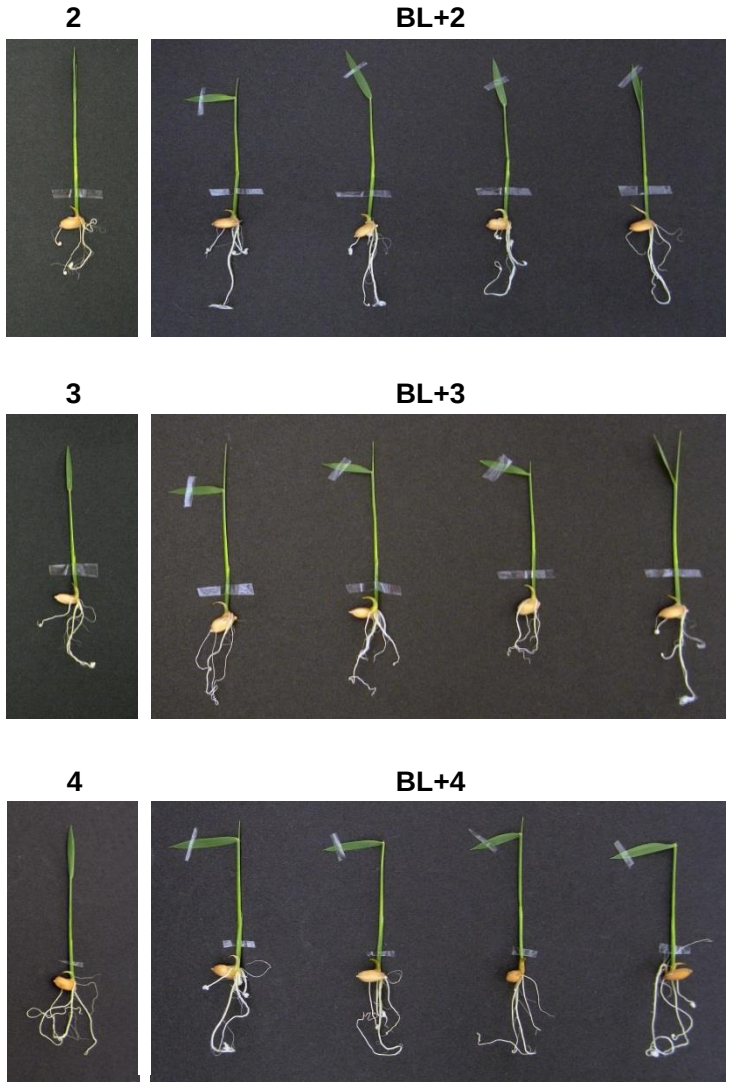


Figure 4

	BL (0.1 μg)	dose (μg)	angle (degree)
none	−		0±0
	+		115±12
5	−	10	15±5
	+	0.3	93±5
	+	1	68±15
	+	3	40±22
	+	10	25±11
6	−	10	27±10
	+	0.3	117±10
	+	1	94±15
	+	3	97±7
	+	10	73±16
7	−	10	0±0
	+	0.3	101±10
	+	1	85±23
	+	3	48±11
	+	10	39±13

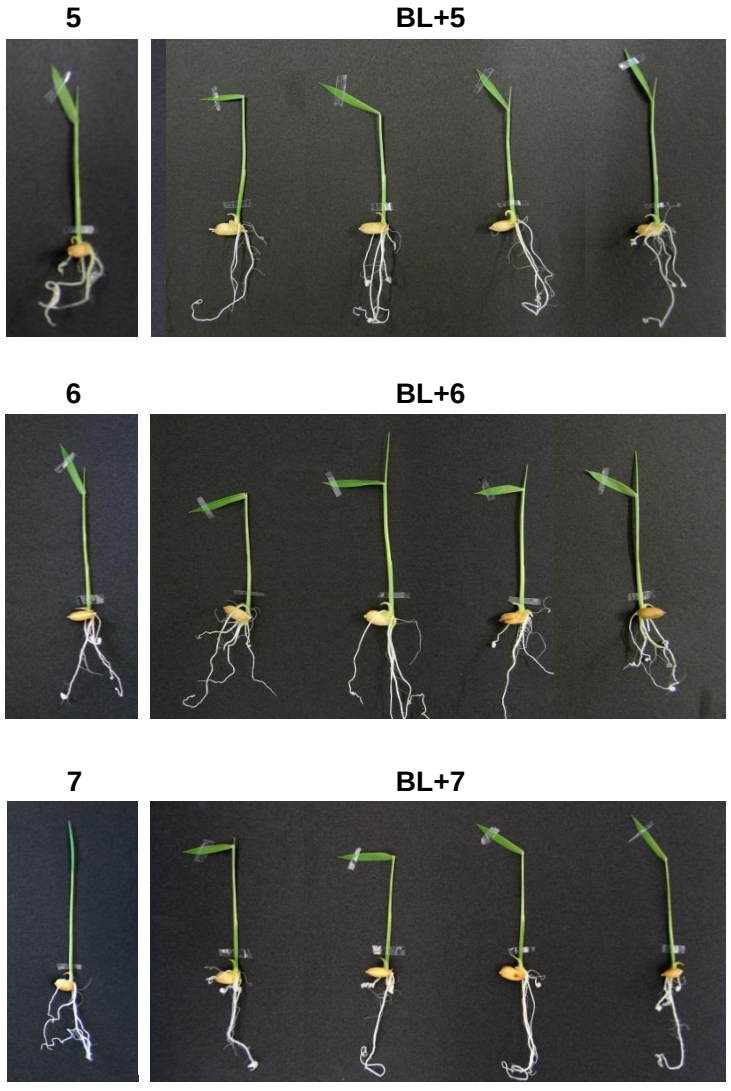


Figure 5

Brassinolide-2,3-acetonide: a brassinolide-induced rice lamina joint inclination antagonist

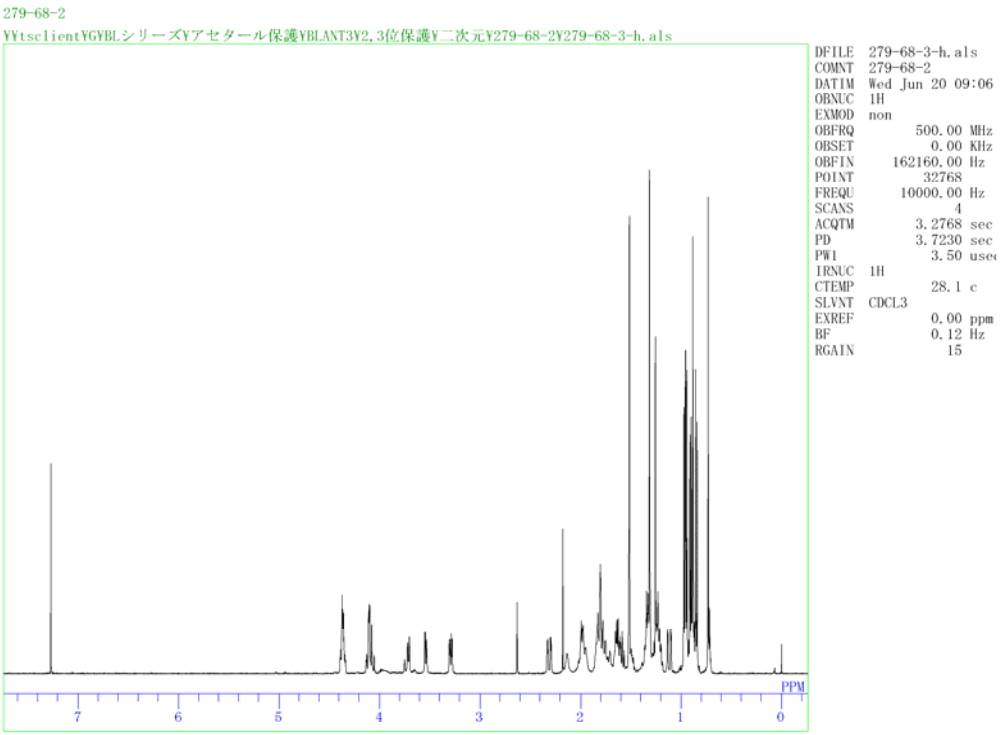
Takuya Muto and Yasushi Todoroki*

Supplementary data

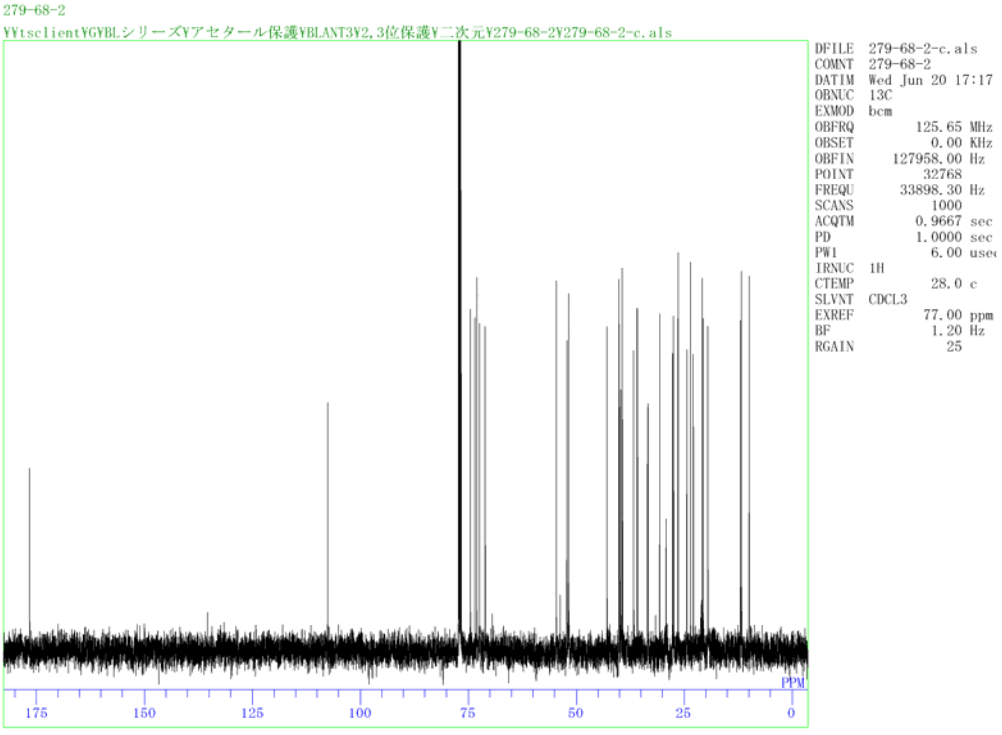
^{1}H and ^{13}C NMR spectra of compounds **2-7**

Compound **2**

^{1}H NMR

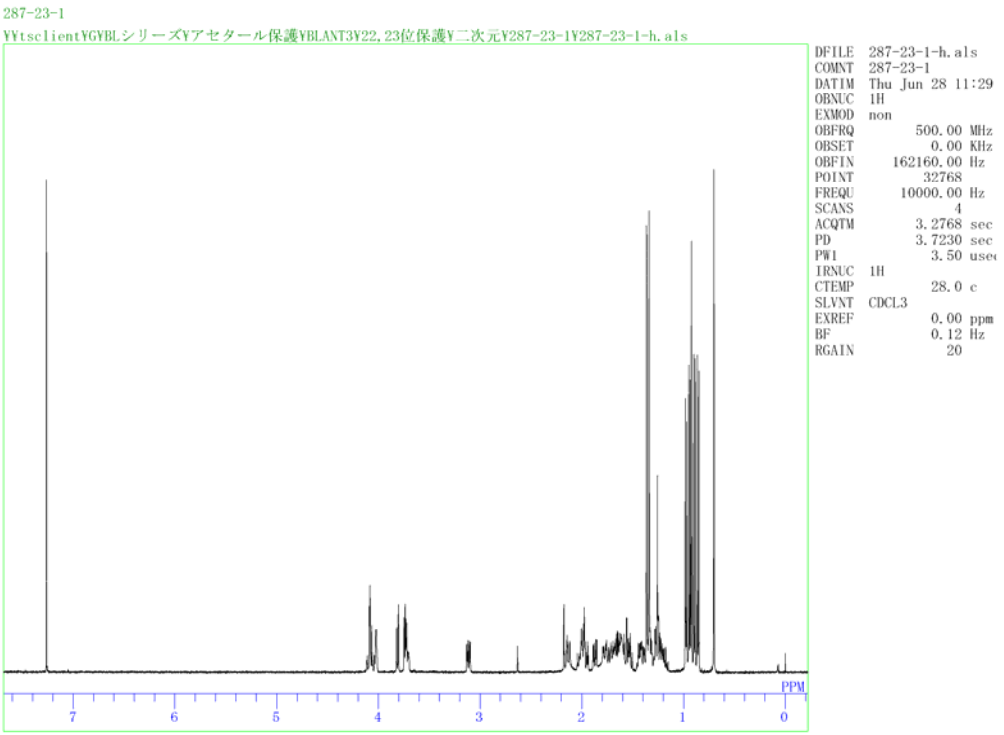


^{13}C NMR

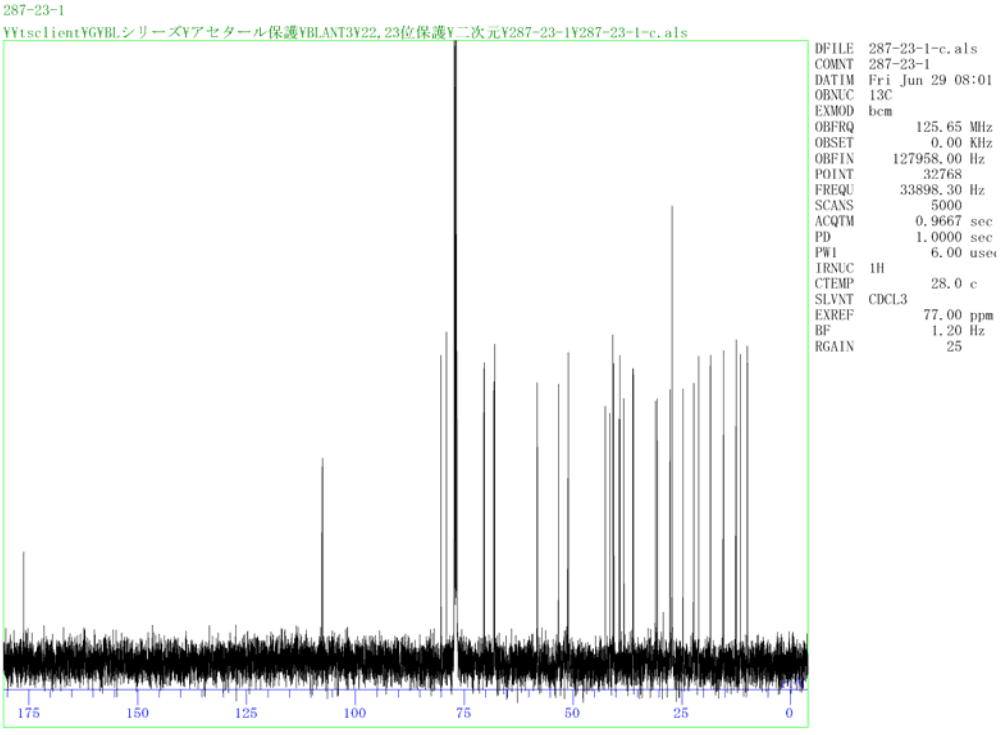


Compound **3**

^{1}H NMR

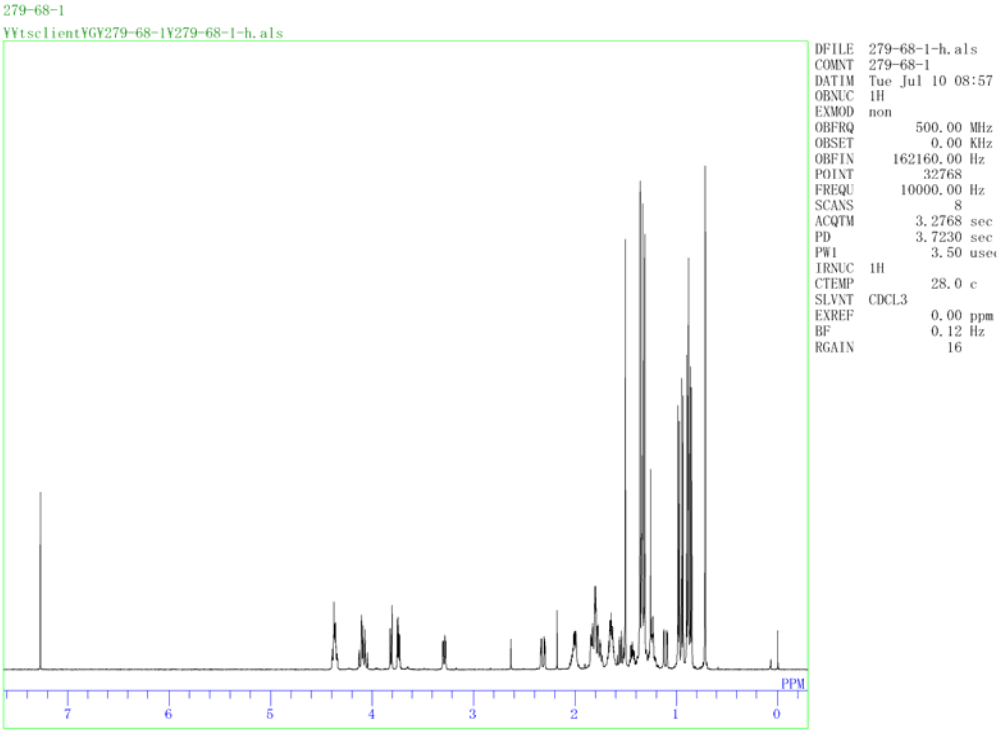


^{13}C NMR

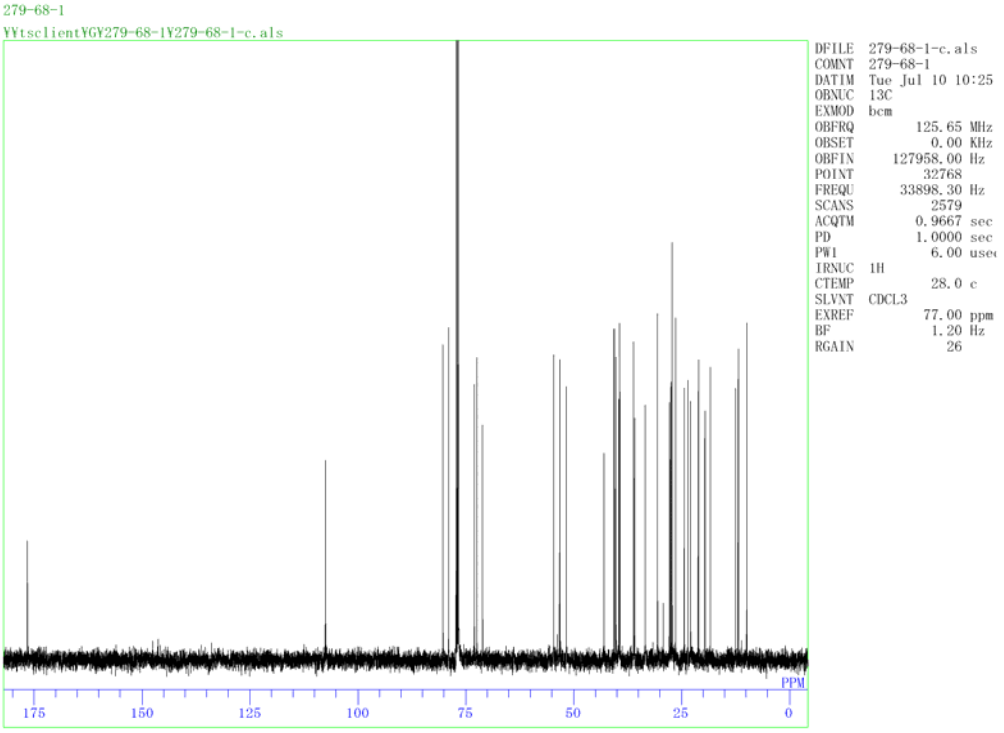


Compound **4**

^{1}H NMR

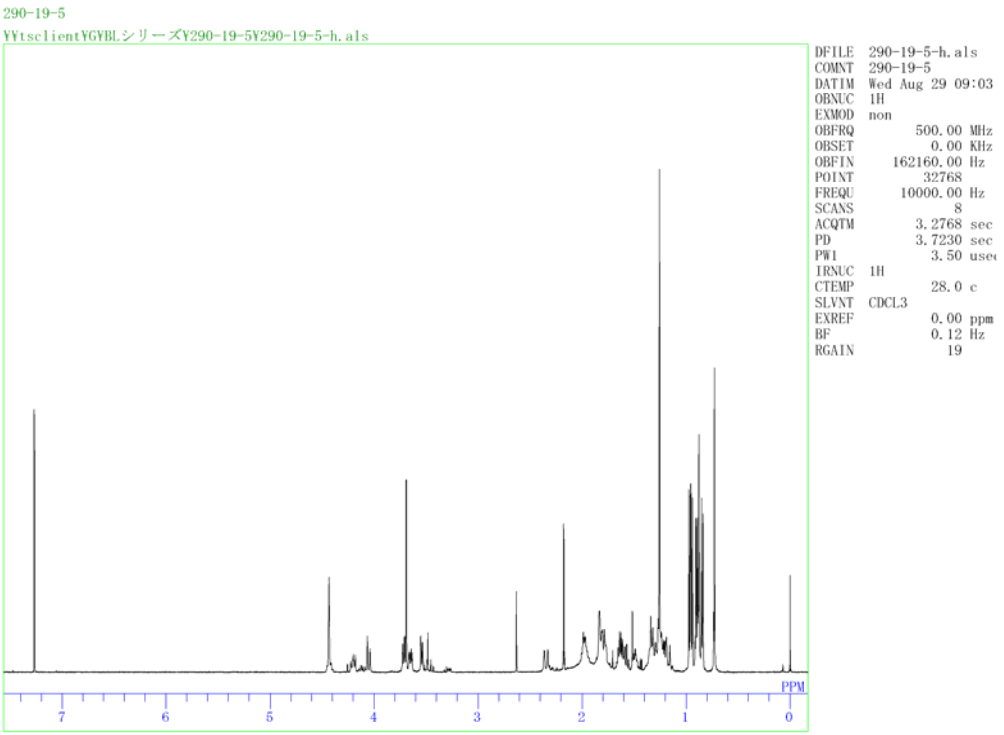


^{13}C NMR

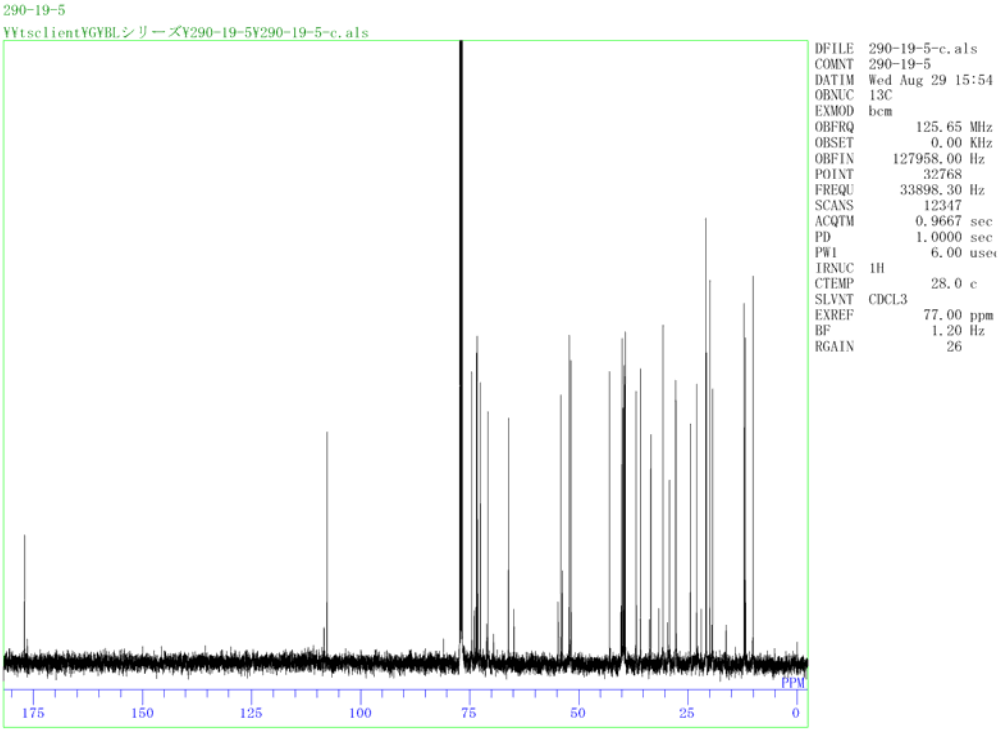


Compound **5**

^{1}H NMR

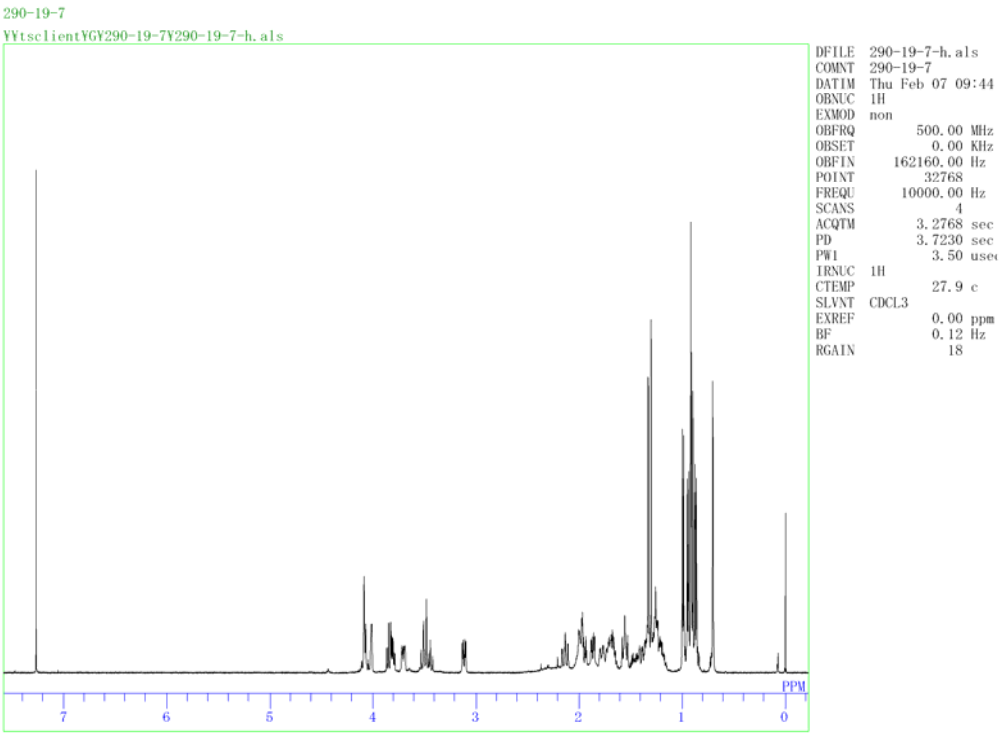


^{13}C NMR

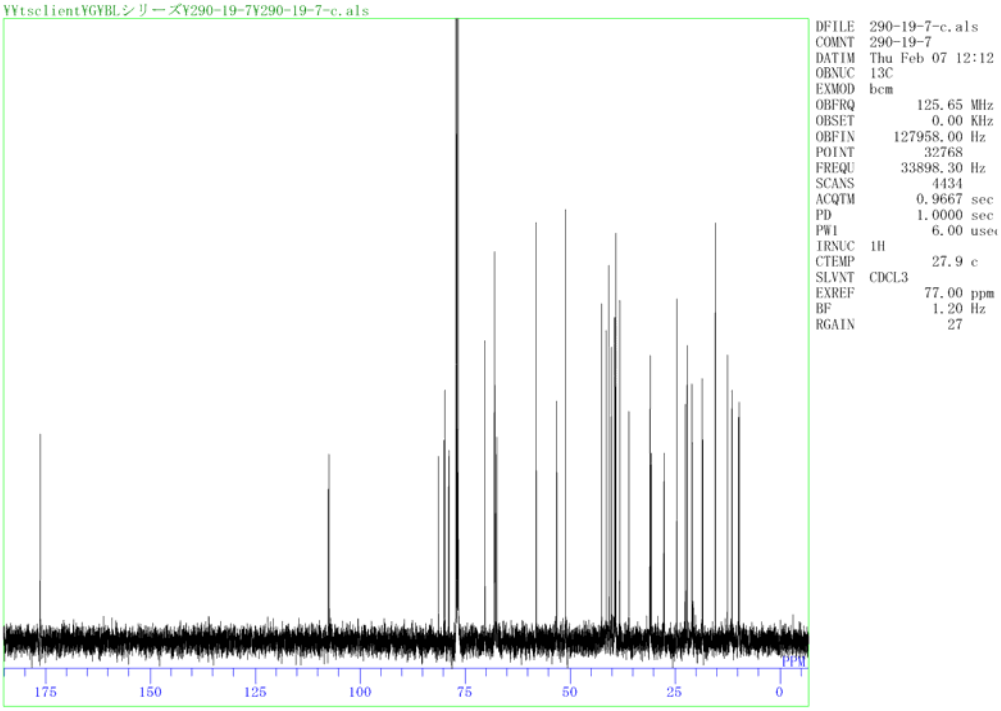


Compound **6**

^{1}H NMR

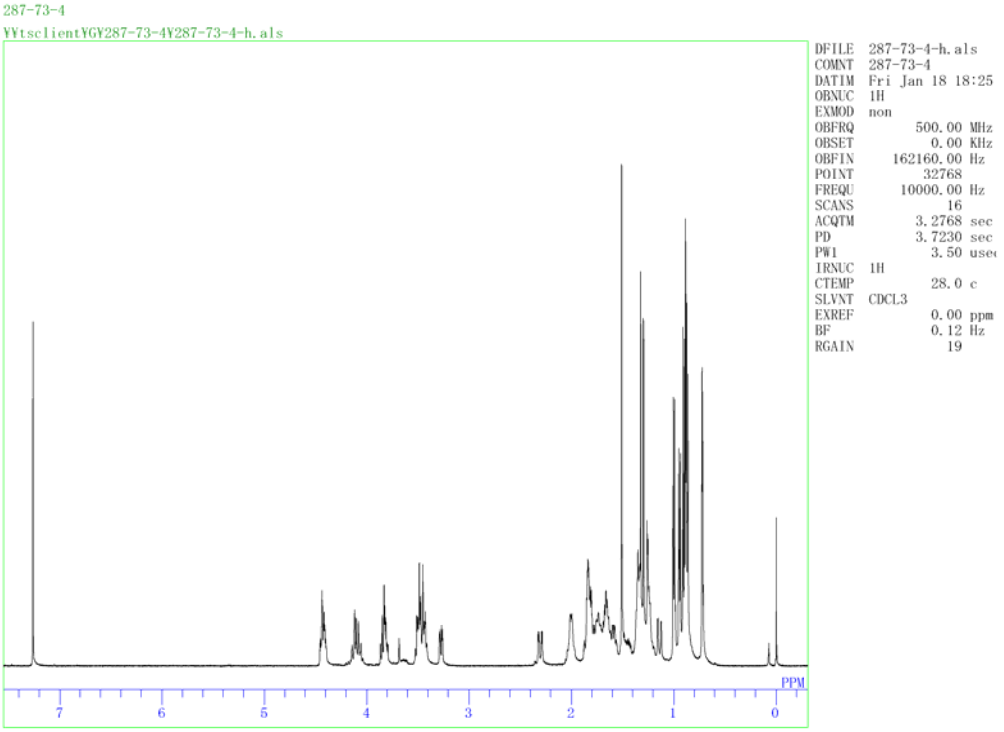


^{13}C NMR



Compound **7**

^{1}H NMR



^{13}C NMR