Epimerization at C-5 of brassinolide with sodium methoxide and the biological activity of 5-*epi*-brassinolide in the rice lamina inclination test

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Brassinolide **1** easily epimerized at C-5 by treatment with sodium methoxide in refluxing methanol *via* two intermediary methyl esters, and the subsequent re-lactonization with acid led to the formation of 5-*epi*-brassinolide **2** (42%) and 6(6a→3a)*abeo*-5-*epi*-brassinolide **3** (18%), along with recovery of **1** (37%); **3** was quantitatively converted to a 94:6 equilibrium mixture of **2** and **3** by prolonged treatment with acidic resin at 60 °C. The NMR experiments allowed the conformations of the A/B ring moiety of **1** and **2** and the corresponding part of **3** in solution to be elucidated. Biological activity of **2** in the rice lamina inclination test was less than 1/1000 compared with **1**, providing clear evidence that the A/B *trans* fused ring junction of brassinosteroids is an essential structural factor for high biological activity.

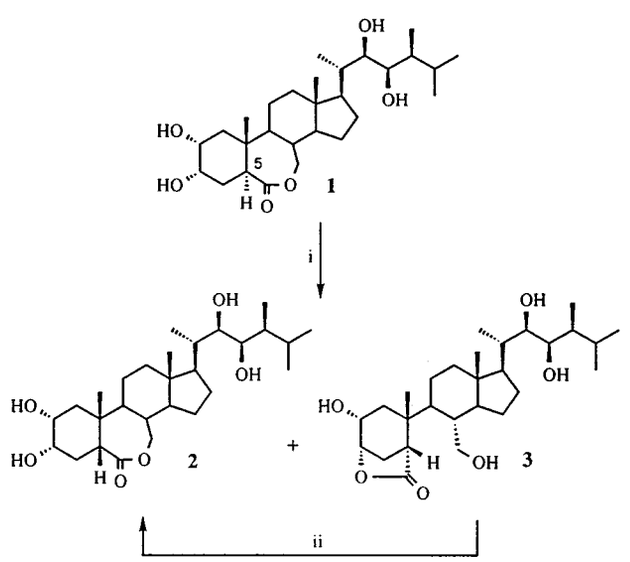
Introduction

Brassinosteroids (BRs) are considered as a new class of phytohormones due to a variety of physiological activities and their ubiquitous distribution in the plant kingdom.[1] With increasing attention to the mode of action of BRs and their receptors at physiological and molecular levels the structure-activity relationships of BRs have been widely studied and are now well documented on the basis of activity data of natural BRs and their synthetic analogs;[2,3] in general, the A/B *trans* fused ring junction common to natural BRs is postulated as an essential structural factor for high biological activity. However, this is still unclear due to the lack of any key data: 5-*epi*-brassinolide (5-*epi*-BL) **2** with an A/B *cis*-ring junction has hitherto not been available either from natural sources or organic synthesis, and thus has never been assayed and compared with BL **1**, the most active natural BR. In this paper we describe a simple method for preparation of **2** directly from **1** by epimerization with sodium methoxide and we also give the result of the rice lamina inclination test on **2**, a typical bioassay employed for testing BR activity.[4,5]

Results and discussion

In the many synthetic works on BL **1** and its congeners reported so far,[6] removal of the acetyl protecting group of their tetra-acetate derivatives at the final step appears to have been performed with no problem by treatment with 5% methanolic potassium hydroxide at refluxing temperature for a few hours. However, we found that the reported high yields were not always attainable, especially when the reaction was carried out on a milligram scale. For example, in the case of the 2,3,22,23-tetra-*O*-acetate of **1**, the yield of the desired **1** was sometimes reduced to less than 60%, and one of the major by-products was suspected to be 5-*epi*-BL **2**. This prompted us to refine the conditions for preparation of **2** from **1** by the direct epimerization of C-5 with base.

Among several basic conditions tested, the following afforded the best result so far. BL **1** was refluxed in a 1 M sodium methoxide (NaOMe) solution of methanol (MeOH) for 5 h, followed by re-lactonization with Dowex-50W-2X resin (H^+ form) at pH 3–4 in MeOH–H_2O (4:1) at room temperature for 3 h, giving 5-*epi*-BL **2** (42%) and 6(6a→3a)*abeo*-5-*epi*-BL **3** (18%) along with the starting material **1** (37%) (Scheme 1).



Scheme 1 *Reagents and conditions*: i, NaOMe, MeOH, reflux, 5 h, then Dowex-50W (H^+ form), pH 3–4, MeOH–H_2O (4:1), rt, 3 h; ii, Dowex-50W (H^+ form), MeOH–H_2O (4:1), 60 °C, 4 days.

Elongation of the reaction time to 24 h was not effective in increasing the amount of **2** and **3** formed, suggesting that this epimerization had been completed at this stage. By further treatment with Dowex resin in MeOH–H_2O (4:1) at 60 °C for 4 days, γ-lactone **3** was quantitatively converted to a 94:6 mixture of **2** and **3**. Thus, the total yield of conversion of **1** to **2** was

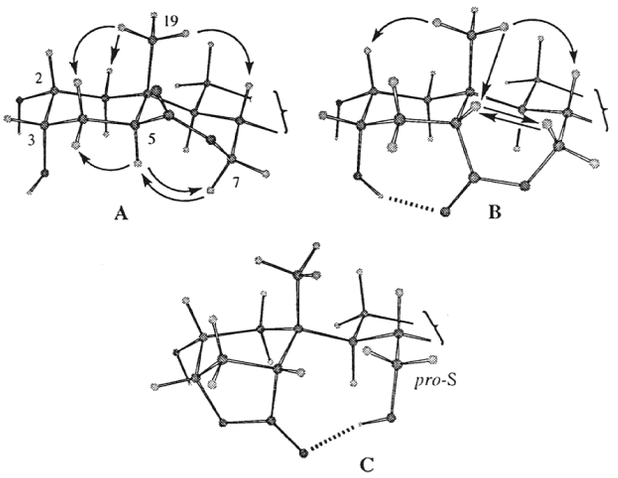


Fig. 1 Conformations of the A/B ring of BL **1**, 5-*epi*-BL **2** and the corresponding part of the γ-lactone **3**, **A**, **B** and **C**, respectively, in solution deduced by ^{1}H NMR study. Arrows on **A** and **B** show NOE effects centered on the 5-H and 19-H_3 protons, and broken lines show hydrogen bonding between the hydroxy and carbonyl groups.

59%. Under the same conditions, **2** also gave the same mixture, which indicated that this lactone rearrangement was reversible and the obtained ratio was a result of equilibrium.

The structures of the new compounds **2** and **3** were verified by MS and NMR spectroscopy. NMR experiments on both compounds, as well as **1** for a reference, were carried out at 600 MHz by PFG-DQFCOSY, PFG-HMQC and PFG-HMBC, and NOE differential experiments especially on **1** and **2**, by which all resonances were completely assigned (see Experimental section). In the ^{1}H NMR spectra, characteristic chemical shifts for both new compounds with a 5β-H proton were the downfield shifts of 19-H_3: δ 1.11 for **2** and δ 1.10 for **3**, compared with δ 0.91 for BL **1** with a 5α-H proton. In addition, the coupling patterns of 5β-H having no vicinal *anti*-proton at C-4 [δ 2.96 (d, $J_{5,4\alpha}$ 5.9) of **2** and δ 2.93 (dd, $J_{5,4\alpha}$ 5.9 and $J_{5,1\beta}$ 1.5) of **3**] were clearly distinguishable from that of **1** [δ 3.14 (dd, $J_{5,4\beta}$ 12.2 and $J_{5,4\alpha}$ 4.4)].

The ^{1}H NMR data allowed further elucidation of the conformations of the A/B ring moiety of **1** and **2** and the corresponding part of **3** in solution as illustrated in Fig. 1. In the case of **1**, 1α-, 2-, 4β- and 5-protons respectively had large coupling constants with each vicinal *anti*-proton, $J_{1\alpha,2}$ 12.2 and $J_{4\beta,5}$ 12.2, and a NOE effect was observed between 5α-H and 7α-H, but not between 19-H_3 and 7β-H. These results verified the chair conformation of the A-ring and the specific conformation of the B-ring. The conformer **A** of **1** was accordingly assigned. The conformer **B** of **2** was deduced from NOE effects observed between 5β-H and 7β-H [δ 4.39 (dd, J_{gem} 13.7 and $J_{7\beta,8}$ 6.8)] and between 19-H_3 and 2-H [δ 3.55 (m)] and from the very low field resonance due to the 3-OH proton [δ 5.67 (d, $J_{OH,3}$ 8.3)] which suggested hydrogen bonding with the lactone carbonyl function. These conformers in solution were eventually found to be identical to the global minimum conformers of **1** and **2** obtained from molecular dynamics calculations which incorporated energy minimization. In the case of **3**, the 7α-OH proton gave a resonance at δ 3.09, further downfield than those of the 2-, 22- and 23-OH protons (δ 1.68, 1.91 and 1.96, respectively) and had sharp coupling constants. This implied the fixed conformation of **C** (Fig. 1) due to hydrogen bonding between the 7α-OH proton and the lactone carbonyl function. The large vicinal coupling, $J_{2,1\alpha}$ 10.2, of 2-H (δ 3.83) indicated a chair conformation of the A-ring and the coupling constants of $J_{OH,7pro\text{-}S}$ 10.7, $J_{OH,7pro\text{-}R}$ 1.5, $J_{7pro\text{-}S,8}$ 1.0 and $J_{7pro\text{-}R,8}$ *ca.* 1.5 were consistent with the conformer **C**.

In C-5 epimerization of **1** using methanolic NaOMe, a complex equilibration takes place as illustrated in Scheme 2. However, monitoring the reaction by TLC showed that the major

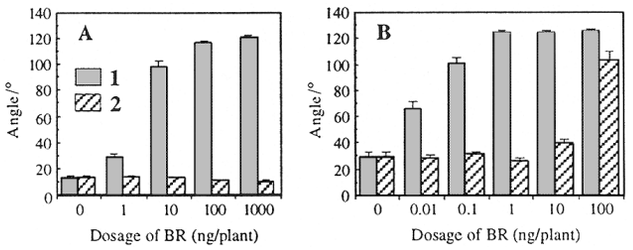
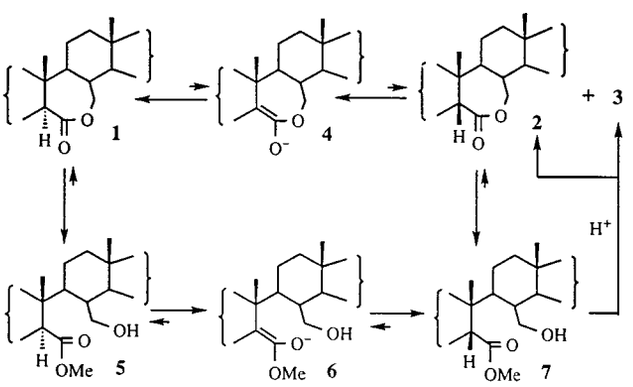


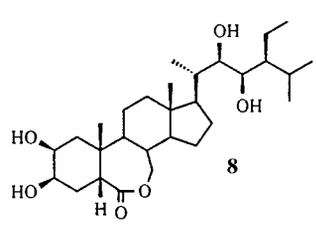
Fig. 2 Rice lamina inclination test (*Oryza sativa* cv. Tan-ginbozu) of BRs, BL **1** and 5-*epi*-BL **2**. A: application of BR alone; B: co-application with IAA (5 μg/plant). Each data point represents the mean of 30 replicates ± SE.



Scheme 2 Mechanism of formation of 5-*epi*-BL **2** and γ-lactone **3** from BL **1** with sodium methoxide.

components of the intermediates were more polar than lactones, **1**, **2** and **3**, and were assumed to be methyl esters, **5** and **7**. Thus, it is conceivable that this epimerization proceeds mainly through the pathway, **1**→**5**→**6**→**7**, and that **7** is thermodynamically more stable than **5**. Another feasible pathway, **1**→**4**→**2**, is unlikely, because the energy difference between **1** and **2**, calculated by molecular dynamics and energy minimization, is in favor of **1**, $\Delta E = 9.1$ kJ mol^{-1}. It should be noted that when **1** was treated with 5% KOH in 90% aqueous MeOH at room temperature for 1 h until the lactone ring completely opened to the carboxylate and then at refluxing temperature for 2 h, the formation of **2** and **3** was largely reduced. After re-lactonization, **1** was recovered in 84% yield along with a small amount of **2** and **3** (4% combined yield). This eliminated the possibility of enolization of the carboxylate under these conditions and provided a better procedure for removal of the tetraacetyl protecting group of BL **1** and its congeners.

Biological activity of 5-*epi*-BL **2** in the rice lamina inclination test was examined and the activity was compared with BL **1**, as shown in Fig. 2. Two assay methods were employed:[5] single application of BR, **1** or **2**, and the co-application with indole-3-acetic acid (IAA) whose synergistic effect is well known to significantly enhance the assay sensitivity.[4] By single application, **2** exhibited no activity (graph A), while by co-application with IAA considerable activity was observed at a dosage of 100 ng/plant, although this was 1/1000 lower than that of **1** (graph B). The antagonistic effect of **2** on **1** was also examined, and the result showed that **2** had no antagonistic effect (data not shown). In this connection, Brosa *et al.* recently reported the preparation of a 5-*epi*-BL analog, 28-homo-2,3,5-tri-*epi*-BL **8**,



and described **8** as having high activity comparable to that of **1** in the rice lamina inclination test.[3] However, we are sceptical about their activity evaluation in the absence of any dose dependence tests. The tested dosage of 1 μg/plant employed by Brosa *et al*. should be too high for such a comparison study, because even at 10 ng/plant the activity of **1** nearly reached the maximum (graph A, Fig. 2).

In conclusion, 5-*epi*-BL **2** was easily prepared by a simple treatment of BL **1** with sodium methoxide, in which efficient C-5 epimerization *via* two intermediary methyl esters took place. BL **2** had neither an agonistic nor an antagonistic effect on the rice lamina inclination test. This is the first experimental proof that the A/B *trans* fused ring junction of BRs is an essential structural factor not only for high BR activity, but also for interacting with the binding site of the BR receptor.

Experimental

General

Melting points (mp) were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. NMR measurements were performed on a JEOL JNM-A600 spectrometer. All spectra were recorded using standard pulse sequences. Chemical shifts were recorded as δ values in parts per million (ppm) relative to tetramethylsilane (δ 0 ppm) for ^{1}H NMR or to the solvent (δ 77.0 ppm) for ^{13}C NMR as an internal reference. All J values are given in Hz. High resolution mass spectra, HR-FAB-MS, were obtained with a JEOL-HX-110 mass spectrometer. Analytical thin layer chromatography (TLC) was conducted on micro-slides coated with Merck Kieselgel KG60F-254; the developed plates were stained with 10% (w/v) vanillin in concentrated sulfuric acid at 180 °C. All reactions were carried out under a nitrogen atmosphere. Column chromatography was conducted using silica gel FL-60D [Fuji Silysia Chemical Ltd.] as the adsorbent. The ratios of mixed solvents were v/v.

Treatment of BL 1 with methanolic sodium methoxide

A mixture of BL **1** (25.0 mg) and 1 M NaOMe solution of MeOH (2 cm^3) was stirred at refluxing temperature for 5 h, to which was added MeOH (2 cm^3) and H_2O (1 cm^3) at 0 °C. The mixture was acidified with Dowex 50W resin (H^+ form) to pH 3–4 and stirred at room temperature for 3 h, and was then filtered through a glass filter. The filtrate was evaporated and the residue was subjected to column chromatography. The first elution with CH_2Cl_2–MeOH (30 : 1) gave *epi*-BL **2** (10.4 mg, 42%), and the second gave 6(6a→3a)*abeo*-5-*epi*-BL **3** (4.4 mg, 18%). Further elution with CH_2Cl_2–MeOH (20 : 1) gave BL **1** (9.3 mg, 37%).

BL **1**: δ_H (600 MHz; $CDCl_3$–CD_3OD, 8 : 1) 0.72 (3H, s, 18-H_3), 0.84 (3H, d, J 6.8, 28-H_3), 0.89 (3H, d, J 6.8, 21-H_3), 0.91 (3H, s, 19-H_3), 0.94 and 0.96 (each 3H, each d, J 6.8, 26-H_3 and 27-H_3), 1.18 (1H, m, 24-H), 1.22 (1H, m, 14-H), 1.24 (1H, m, 12α-H), 1.26 and 1.70 (each 1H, each m, 15-H_2), 1.29 (1H, m, 9-H), 1.30 and 1.99 (each 1H, each m, 16-H_2), 1.41 (1H, m, 11β-H), 1.48 (1H, m, 20-H), 1.57 (1H, m, 17-H), 1.57 (1H, dd, J 12.7 and 12.2, 1α-H), 1.63 (1H, m, 25-H), 1.72 (1H, m, 8-H), 1.79 (1H, m, 11α-H), 1.85 (1H, dd, J 12.7 and 4.4, 1β-H), 1.89 (1H, ddd, J 15.1, 4.4 and 3.9, 4α-H), 1.99 (1H, m, 12β-H), 2.09 (1H, ddd, J 15.1, 12.2 and 2.0, 4β-H), 3.14 (1H, dd, J 12.2 and 4.4, 5-H), 3.51 (1H, br d, J 8.8, 22-H), 3.62 (1H, ddd, J 12.2, 4.4 and 2.4, 2-H), 3.68 (1H, br d, J 8.8, 23-H), 3.95 (1H, ddd, J 3.9, 2.4 and 2.0, 3-H), 4.11 (2H, d-like, J 5.4, 7-H_2); δ_C (150 MHz; $CDCl_3$) 9.87 (C-28), 11.47 (C-18), 11.55 (C-21), 15.17 (C-19), 20.46 and 20.59 (C-26 and C-27), 22.02 (C-11), 24.49 (C-15), 27.32 (C-16), 30.41 (C-25), 30.97 (C-4), 36.72 (C-20), 38.04 (C-10), 38.96 (C-8), 39.45 (C-12), 40.03 (C-24), 40.78 (C-5), 40.98 (C-1), 42.23 (C-13), 51.07 (C-14), 52.06 (C-17), 57.91 (C-9), 67.63 (C-2), 67.68 (C-3), 70.46 (C-7), 72.96 (C-23), 74.12 (C-22), 177.22 (C-6).

5-*epi*-BL **2**: an amorphous powder; δ_H (600 MHz; $CDCl_3$) 0.70 (3H, s, 18-H_3), 0.85 (3H, d, J 6.8, 28-H_3), 0.90 (3H, d, J 6.8, 21-H_3), 0.95 (3H, d, J 6.4, 26-H_3 or 27-H_3), 0.97 (3H, d, 6.8, 26-H_3 or 27-H_3), 1.11 (3H, s, 19-H_3), 1.13 and 1.83 (each 1H, each m, 15-H_2), 1.20 (2 × 1H, each m, 9-H and 24-H), 1.24 and 1.98 (each 1H, each m, 12-H_2), 1.28 and 1.83 (each 1H, each m, 1-H_2), 1.30 and 2.00 (each 1H, each m, 16-H_2), 1.45 and 1.79 (each 1H, each m, 11-H_2), 1.49 (1H, m, 20-H), 1.58 (1H, m, 14-H), 1.61 (1H, m, 17-H), 1.64 (1H, m, 25-H), 1.79 (1H, m, 8-H), 2.02 (1H, m, 4-H), 2.02, 2.20 and 2.44 (each 1H, br s, 2-OH, 22-OH and 23-OH), 2.22 (1H, d, J 16.1, 4-H), 2.96 (1H, d, J 5.9, 5-H), 3.54 (1H, br d, J 8.3, 22-H), 3.55 (1H, m, 2-H), 3.71 (1H, dd, J 8.3 and 1.5, 23-H), 3.91 (1H, m, 3-H), 4.23 (1H, d, J 13.7, 7α-H), 4.39 (1H, dd, J 13.7 and 6.8, 7β-H), 5.67 (1H, d, J 8.3, 3-OH); δ_C (150 MHz; $CDCl_3$) 10.11 (C-28), 11.44 (C-18), 11.88 (C-21), 18.92 (C-19), 20.72 and 20.87 (C-26 and C-27), 21.84 (C-11), 24.86 (C-15), 27.19 (C-16), 28.35 (C-4), 30.74 (C-25), 35.65 (C-8), 36.71 (C-1), 36.80 (C-20), 38.92 (C-10), 39.12 (C-12), 40.08 (C-24), 42.63 (C-13), 43.72 (C-5), 48.39 (C-9), 52.25 (C-17), 53.69 (C-14), 66.07 (C-3), 67.75 (C-2), 69.52 (C-7), 73.37 (C-23), 74.54 (C-22), 179.31 (C-6); HR-FAB-MS *m*/*z* ([M + 1]$^+$: positive ion, glycerol): Found, 481.3522. Calc. for $C_{28}H_{49}O_6$, 481.3529.

6(6a→3a)*abeo*-5-*epi*-BL **3**: mp 232–236 °C (colorless granules from MeOH); δ_H (600 MHz; $CDCl_3$) 0.70 (3H, s, 18-H_3), 0.85 (3H, d, J 6.8, 28-H_3), 0.89 (3H, d, J 6.3, 21-H_3), 0.94 and 0.97 (each 3H, each d, J 6.8, 26-H_3 and 27-H_3), 1.10 (3H, s, 19-H_3), 1.14 and 1.87 (each 1H, each m, 15-H_2), 1.20 (1H, m, 24-H), 1.25 (1H, m, 1α-H), 1.25 and 1.98 (each 1H, each m, 16-H_2), 1.26 and 1.93 (each 1H, each m, 12-H_2), 1.36 and 1.65 (each 1H, each m, 11-H_2), 1.46 (1H, m, 8-H), 1.48 (1H, m, 20-H), 1.59 (1H, m, 17-H), 1.65 (1H, m, 25-H), 1.67 (1H, m, 14-H), 1.68 (1H, d, 2-OH), 1.73 (1H, td, J 10.7 and 3.4, 9-H), 1.91 (1H, d, J 3.9, 22-OH), 1.96 (1H, d, J 3.9, 23-OH), 2.05 (1H, ddd, J 13.7, 6.8 and 1.5, 1β-H), 2.08 (1H, d, J 12.7, 4β-H), 2.34 (1H, ddd, J 12.7, 6.4 and 5.9, 4α-H), 2.93 (1H, dd, J 5.9 and 1.5, 5-H), 3.09 (1H, dd, J 10.7 and 1.5, 7-OH), 3.56 (1H, br dd, J 8.3 and 3.9, 22-H), 3.64 (1H, ddd, J 12.7, 10.7 and 1.0, 7*pro*-*S*-H), 3.71 (1H, ddd, J 8.3, 3.9 and 2.0, 23-H), 3.83 (1H, m, 2-H; after D_2O displacement, br dd, J 10.2 and 6.8), 3.94 (1H, br d, J 12.7, 7*pro*-*R*-H), 4.73 (1H, d, J 6.4, 3-H); δ_C (150 MHz; $CDCl_3$) 10.10 (C-28), 11.75 (C-18), 11.80 (C-21), 17.35 (C-19), 20.74 and 20.89 (C-26 and C-27), 22.71 (C-11), 24.55 (C-15), 27.47 (C-16), 30.65 (C-4), 30.78 (C-25), 37.07 (C-20), 37.42 (C-10), 39.69 (C-12), 40.07 (C-24), 40.32 (C-8), 41.71 (C-9), 41.78 (C-13), 42.92 (C-1), 46.18 (C-5), 51.18 (C-14), 52.50 (C-17), 62.50 (C-7), 68.44 (C-2), 73.33 (C-23), 74.68 (C-22), 82.54 (C-3), 179.32 (C-6); HR-FAB-MS *m*/*z* ([M + 1]$^+$: positive ion, glycerol): Found, 481.3519. Calc. for $C_{28}H_{49}O_6$, 481.3529.

Treatment of BL 1 with 5% potassium hydroxide in 90% aqueous methanol

A solution of BL **1** (10.6 mg) in 90% aqueous MeOH (1 cm^3) containing 5% KOH was stirred at room temperature until the lactone **1** completely opened to the carboxylate (*ca*. 1 h), which was monitored by TLC ($CHCl_3$–MeOH = 10 : 1, R_f values: 0.21 for **1**, 0.0 for the carboxylate). The mixture was then stirred at reflux temperature for 2 h. After the same acidic work-up as described above, and column chromatography, a 72 : 28 mixture of **2** and **3** (0.42 mg, 4.0%; the ratio was estimated by the ^{1}H NMR spectrum) and **1** (8.9 mg, 84%) was obtained.

Treatment of 6(6a→3a)*abeo*-5-*epi*-BL 3 or 5-*epi*-BL 2 with acidic resin

A heterogeneous mixture of **3** (3.2 mg) and Dowex 50W resin

(H^+ form, 30 mg) in MeOH (0.8 cm^3) and H_2O (0.2 cm^3) was stirred at 60 °C for 4 days. The resin was filtered off through a glass-filter and washed with MeOH. The filtrate was concentrated *in vacuo*. After a short-pad column chromatography with CH_2Cl_2–MeOH (10:1), a 94:6 mixture of **2** and **3** (3.2 mg, 100%; the ratio was estimated by the ^1H NMR spectrum) was obtained. By the same treatment, **2** gave the same mixture of **2** and **3**.

Molecular modeling

The conformational search for each isomer, **1** or **2**, was carried out by a Low-Mode conformational search[7] with 30 000 initial structures using the MM2* force field in the Macromodel 6.0 program.[8]

Bioassay

The rice lamina inclination test was carried out as described previously.[5]

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References

1 S. Fujioka and A. Sakurai, *Nat. Prod. Rep.*, 1997, **14**, 1.
2 S. Takasuto, N. Yazawa, N. Ikekawa, Y. Takematsu and M. Koguchi, *Phytochemistry*, 1983, **22**, 2437; M. J. Thompson, W. J. Meudt, N. B. Mandava, S. R. Dutky, W. R. Lusby and D. W. Spaulding, *Steroids*, 1992, **39**, 89; V. Marquardt and G. Adam, in *Chemistry of Plant Protection*, ed. W. Ebing, Springer-Verlag, New York, 1991, vol. 7, p. 103.
3 G. Brosa, J. M. Capdevila and I. Zamara, *Tetrahedron*, 1996, **52**, 2435; G. Brosa, in *Biochemistry and Function of Sterols*, eds. E. J. Parish and W. D. Nes, CRC Press Inc., New York, 1997, p. 201.
4 K. Takeno and R. P. Pharis, *Plant Cell Physiol.*, 1982, **23**, 1275; S.-K. Kim, H. Abe, C. H. A. Little and R. P. Pharis, *Plant Physiol.*, 1990, **94**, 1709.
5 S. Fujioka, T. Noguchi, S. Takatsuto and Y. Yoshida, *Phytochemistry*, in the press.
6 S. Takatsuto, N. Yazawa, M. Ishiguro, M. Morisaki and N. Ikekawa, *J. Chem. Soc., Perkin Trans. 1*, 1984, 139; S. Takatsuto and N. Ikekawa, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2133; T. Kametani, T. Katoh, J. Fujio, I. Nogiwa, M. Tsubuki and T. Honda, *J. Org. Chem.*, 1988, **53**, 1982.
7 I. Kolossváry and W. C. Guida, *J. Am. Chem. Soc.*, 1996, **118**, 5011.
8 F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson and W. C. Still, *J. Comput. Chem.*, 1990, **11**, 440.

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