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Asymmetric Total Synthesis of 6-Tuliposide B and Its Biological Activities against Tulip Pathogenic Fungi

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The structure-activity relationship was investigated to evaluate the antifungal activities of tuliposides and tulipalins against tulip pathogenic fungi. 6-Tuliposide B was effectively synthesized *via* the asymmetric Baylis-Hillman reaction. Tuliposides and tulipalins showed antifungal activities against most of the strains tested at high concentrations (2.5 mM), while *Botrytis tulipae* was resistant to tuliposides. Tulipalin formation was involved in the antifungal activity, tulipalin A showed higher inhibitory activity than 6-tuliposide B and tulipalin B. Both the tuliposides and tulipalins showed pigment-inducing activity against *Gibberella zeae* and inhibitory activity against *Fusarium oxysporum* f. sp *tulipae*. These activities were induced at a much lower concentration (0.05 mM) than the antifungal MIC values.

Key words: tuliposide; tulipalin; Baylis-Hillman reaction

Tuliposides are the secondary metabolites mainly occurring in Liliaceae and Alstroemeriaceae.[1–3] Among them, 6-tuliposide A (6-*O*-(4′-hydroxy-2′-methylenebutanoyl)-D-glucose) and 6-tuliposide B (6-*O*-[(3′*S*)-3′,4′-dihydroxy-2′-methylenebutanoyl]-D-glucose) are present in tulip cultivars at high concentrations (0.2–2% w/w fresh weight in all parts of the plant).[4] These tuliposides can be respectively converted into tulipalin A (2-methylene-γ-butyrolactone) and (−)-tulipalin B ((*S*)-3-hydroxy-2-methylene-γ-butyrolactone).[5] It has remained unclear whether or not this conversion in plant tissues proceeded in an enzymatic manner, because tulipalin formation also occurs in a pH-dependent manner (above pH 7). An enzyme responsible for converting tuliposides into tulipalins has recently been purified from tulip bulbs and characterized.[6] Among tuliposides and tulipalins, tulipalin A is known to be allergic[7,8] for humans and to have insecticidal activity against *Thrips palmi*.[9]

6-Tuliposide B has been found to have potent antimicrobial activity against a broad panel of bacteria,[4] prompting us to synthesize 6-tulipoisde B.[10] We have clarified that the formation of tulipalin B from 6-tuliposide B was involved in the antibacterial activity and that its target molecule could be MurA from the results of a SAR study.[11] These results also demonstrated that 6-tuliposide B and tulipalin B would be strategic weapons against bacterial infection.[12,13]

It is also known that tulip cultivars can be damaged by pathogenic fungi; for example, *Fusarium oxysporum* f. sp *tulipae* and *Botrytis tulipae* respectively cause bulb rot disease and Botrytis blight disease. We presumed that tuliposides and tulipalins would have comparable growth-inhibiting activities against tulip pathogenic fungi. We therefore tested the biological activities of 6-tuliposide A, 6-tuliposide B, tulipalin A and tulipalin B against these natural enemies, focusing on their antifungal activities. We first explored the asymmetric synthesis of 6-tuliposide B. We had already established the total synthesis of 6-tuliposide B *via* chiral column separation of protected 6-tuliposide B and its 3′*R*-epimer,[10] and Katuski-Sharpless kinetic resolution of the (*S*)-enantiomer and (*R*)-enantiomer of methyl 3-hydroxy-4-(*p*-methoxybenzyloxy)-2-methylenebutanoate.[11] However, approximately half of the synthetic compounds would be wasted by using this separation method when the naturally occurring type of 6-tuliposide B was needed for further study. We therefore developed a superior synthetic method for preparing the naturally occurring type of 6-tuliposide *via* an asymmetric Baylis-Hillman reaction to prepare these molecular probes more efficiently. After that, we evaluated the biological activities of tuliposides and tulipalins against several tulip pathogenic fungi, and investigated SAR by using synthetic analogues of tuliposides to gain insight into the action mechanism of these compounds.

Results and Discussion

To establish a stereoselective synthesis of the natural type of 6-tuliposide B (3′*S*-epimer), we first tried the β-isocupreidine-catalyzed asymmetric Baylis-Hillman reaction of 1,1,1,3,3,3-hexafluoroisopropyl acrylate (HFIPA) with protected 2-hydroxyacetaldehyde.[14] We found that the Baylis-Hillman reaction of 3 eq. of HFIPA and 1 eq. of 2-(*tert*-butyldimethylsilyloxy)-acetaldehyde (**1a**) in the presence of 1 eq. of β-isocupreidine gave the corresponding adduct (63% e.e.) in a 16% yield. The reaction was slow (120 h), and using the 2-(*p*-methoxybenzyloxy)-acetaldehyde (**1b**) instead of **1a** did not give any desired product under the conditions tested (data not shown). Brzezinski *et al.* have reported that *N*-acyl camphor sultam (**2**) promoted the asymmetric Baylis-Hillman reaction. This

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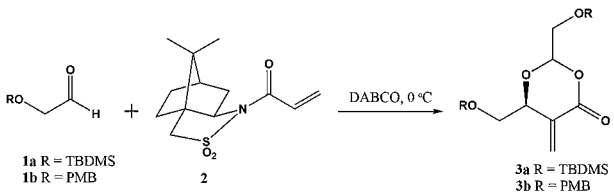
Abbreviations: SAR, structure-activity relationship; MIC, minimum inhibitory concentration; MurA, UDPGlcNAc *enol*pyruvyltransferase

Table 1. Asymmetric Synthesis of the 6-Tuliposide B Side-Chain



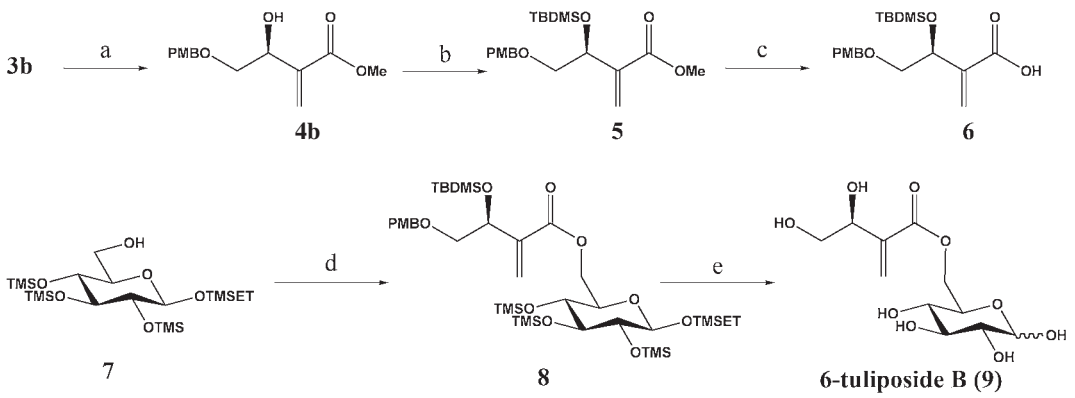
Entry	Reactant	Solvent	Time (h)	Yield (%)	e.e. (%)[a]
1	**1a**	CH_2Cl_2	24	0	—
2	**1a**	DMF	24	7	97
3	**1b**	CH_2Cl_2	24	0	—
4	**1b**	DMF	24	20	98
5	**1b**	DMF	120	90	>99

[a]The enantiomeric excesses are shown as those of butanoates (**4a** and **4b**) prepared from corresponding dioxanones (**3a** and **3b**) by triethylamine treatment.

coupling reaction has afforded 1,3-dioxan-4-ones in high selectivity from a variety of aliphatic aldehydes, and subsequent ring opening gave (3*S*)-3-hydroxy-2-methylene esters in moderate to good yields (33–98%) with high selectivity (>99% e.e.).[15] We therefore next examined whether this method could be applied for asymmetric synthesis of the naturally occurring type of 6-tuliposide B by using two types of protected 2-hydroxyacetaldehydes (**1a** and **1b**) as shown in Table 1.

We first used CH_2Cl_2 as a solvent in the reaction of TBDMS-protected aldehyde (**1a**) and sultam (**2**) because of its suitability for **1a**, although the reaction provided no desired adduct (entry 1). However, the use of DMF, which is poor solvent for **1a**, afforded the desired adduct (**3a**) with superior selectivity in a poor yield (7%, 97% e.e., entry 2). We have previously reported that α-silyloxy aldehyde afforded a 1,2-silyl-migrated adduct in the Baylis-Hillman reaction,[16] which could lead to the formation of undesired by-products (*e.g.*, γ-lactone). 2-(*p*-Methoxybenzyloxy)-acetaldehyde (**1b**) was examined to prevent this undesired migration of the protecting group, resulting in the cyclic adduct (**3b**) being obtained in better yield (20%) with satisfactory selectivity (98% e.e., entry 4). The reaction in CH_2Cl_2 did not afford any product (entry 3). Finally, a prolonged reaction time (120 h) afforded the Baylis-Hillman adduct (**3b**) with outstanding enantiomeric excess in a good yield (90%, >99% e.e., entry 5). Acidic silica gel column chromatography (Wakogel C200, EtOAc:hexane, 1:4 to 1:2) was effective for purifying **3b** which was easily converted into a butanoate (**4b**) by treating with triethylamine.[15] Protection of the hydroxyl group of **4b** and subsequent alkaline hydrolysis of the ester gave acid **6** which was converted into **8** by condensation with 1-*O*-(2-trimethylsilylethyl)-2,3,4-tri-*O*-(trimethylsilyl)-β-D-glucopyranoside (**7**). The resulting sugar ester was deprotected by trifluoroacetic acid to give the natural type of 6-tuliposide B (**9**) as shown in Scheme 1.

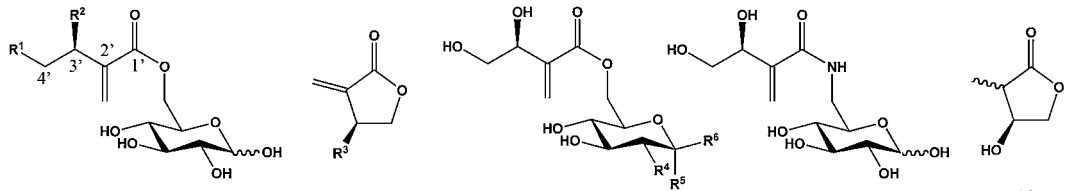
This synthesized 6-tuliposide B (**9**) was evaluated for its antifungal activity, together with its synthetic 3′*R*-epimer (***epi*-9**), (−)-tulipalin B (**10**), (+)-tulipalin B (***ent*-10**), natural 6-tuliposide A (**11**) and commercially available tulipalin A (**14**). Several tuliposide B analogues and their 3′-epimers were also synthesized as described,[11] and their antifungal activity was analyzed. The structures of all the compounds are depicted in Table 2. Four species of tulip pathogenic fungi, namely *Pythium ultimum*, *Rhizoctonia solani*, *Botrytis tulipae*, *Fusarium oxysporum* f. sp *tulipae* and non-tulip pathogenic *Gibberella zeae* were employed as test organisms. All assay results are expressed as minimum inhibitory concentration (MIC) values against each fungus in Table 2. The growth of *P. ultimum*, *R. solani* and *G. zeae* was inhibited by all naturally occurring 6-tuliposide B (**9**), 6-tuliposide A (**11**), (−)-tulipalin B (**10**) and tulipalin A (**14**) compounds. *P. ultimum* was highly sensitive to these compounds, especially so to (−)- and (+)-tulipalin B (**10** and ***ent*-10**). Synthetic (+)-tulipalin B (***ent*-10**) exhibited the same activities as natural **10** against these three strains. 6-Tuliposide A (**11**) and 6-tuliposide B (**9**) showed equal antifungal activity. In the case of the lactones, however, tulipalin A (**14**) showed higher activity than tulipalin B (**10**), except against *P. ultimum*. This characteristic was contrary to that of the antibacterial activity, for which (−)- and (+)-tulipalin B (**10** and ***ent*-10**) showed higher antibacterial activity than tulipalin A (**14**). The higher antibacterial activity of tulipalin Bs has been attributed to its putative target, MurA.[11] In contrast, the high antifungal activity of tulipalin A (**14**) may be ascribed to its cell permeability being enhanced by the absence of a β-hydroxyl



Scheme 1.

a) Et_3N, MeOH, 90%; b) TBDMSOTf, Et_3N, CH_2Cl_2, quant.; c) LiOH (1.1 eq), MeCN:H_2O = 1:1, 60 °C, 92%; d) **6**, diisopropyl carbodiimide, DMAP, CH_2Cl_2, 0 °C, 61%; e) TFA:CH_2Cl_2 = 2:1, 83%.

Table 2. Structures of the 6-Tuliposide B and Tulipalin B Analogues and Their Antifungal Activities



11 $R^1 = OH$, $R^2 = H$
12 $R^1 = H$, $R^2 = OH$
13 $R^1 = CH_2CH_2OH$, $R^2 = OH$

10 $R^3 = OH$
14 $R^3 = H$

15 $R^4 = OH$, $R^5 = OMe$, $R^6 = H$
16 $R^4 = OH$, $R^5 = H$, $R^6 = OMe$
17 $R^4 = H$, $R^5 = H$, $R^6 = H$

18

19

	MIC (mM)				
	Pythium ultimum	*Rhizoctonia solani*	*Botrytis tulipae*	*Fusarium oxysporum* t.	*Gibberella zeae*
6-tuliposide B (**9**/***epi*-9**)	0.25/0.25	2.5/2.5	n.d./n.d.	2.5[a]/2.5[a]	2.5[b]/2.5[b]
tulipalin B (**10**/***ent*-10**)	0.05/0.05	2.5/2.5	n.d./n.d.	n.d.[a]/n.d.[a]	0.25[b]/0.25[b]
6-tuliposide A (**11**)	0.25	2.5	n.d.	2.5[a]	2.5[b]
tulipalin A (**14**)	0.25	0.5	2.5	0.5[a]	0.25[b]
α-Me glucoside (**15**/***epi*-15**)	0.25/0.25	2.5/2.5	n.d./n.d.	n.d.[a]/n.d.[a]	2.5[b]/2.5[b]
β-Me glucoside (**16**/***epi*-16**)	0.25/0.25	2.5/2.5	n.d./n.d.	n.d.[a]/n.d.[a]	2.5[b]/2.5[b]
1,2-dideoxy type (**17**/***epi*-17**)	0.25/0.25	2.5/2.5	n.d./n.d.	n.d.[a]/n.d.[a]	2.5[b]/2.5[b]

12, **13**, **18**, **19**, ***epi*-12**, ***epi*-13**, ***epi*-18** and ***ent*-19** did not show any activities at 2.5 mM.
n.d., No antifungal activity was detected at 2.5 mM.
[a]The color of culture medium did not change to pink at 0.05 mM.
[b]The color of culture medium changed to pale yellow at 0.05 mM.

group. *P. ultimum* has recently been classified in the chromalveolata kingdom, rather than fungi kingdom, so this strain might have shown different behavior toward the tulipalins. Tulipalin A (**14**) showed higher activity than 6-tuliposide A (**11**) in most cases and was the only growth inhibitor against *B. tulipae*. It is interesting to note that *B. tulipae* had enzyme systems capable of converting 6-tuliposide A (**11**) into the hydroxyacid form of tulipalin A that had no antifungal activity (data not shown). Further studies are in progress to clarify its mode of resistance against tulip secondary metabolites from enzymatic and genetic points of view. Tuliposides and tulipalins exhibited growth inhibitory activities against fungi, but higher MICs (2.5 mM) than those against bacteria (0.1–0.3 mM[11]) were needed. We would be interested to learn about the detailed mechanism for their antifungal activities.

In addition to their antifungal activities, tuliposides and tulipalins also exhibited "unexpected" acceleration or inhibition of fungal pigment production at a low concentration (0.05 mM). The culture medium for *G. zeae* (a teleomorph of *Fusarium graminearum*) turned pale yellow in an early phase (12 h) of the culture period after adding tuliposides and tulipalins, whereas the pigment was only induced after a week without adding these compounds. In contrast, the culture medium of *F. oxysporum*, which normally turns pink during the culture, did not show any particular color by adding these compounds. Moreover, the non-natural type of (−)-tulipalin B (***ent*-10**) and 6-tuliposide B (***epi*-9**) that did not show any antifungal activity against *F. oxysporum* also inhibited production of the pigment. The *F. oxysporum* species are known to produce such naphthoquinone-type pigments as fusarubins[17] and javanicins,[18] and *G. zeae* secretes such pigments as aurofusarin,[19] zearalenone[20] and deoxynivalenol.[21] It is very interesting that these tulip metabolites affected the secondary metabolism of pathogenic *F. oxysporum* and non-pathogenic *G. zeae*. Although tuliposides and tulipalins actually showed antifungal properties as we speculated, they were unexpectedly efficient as pigment-inducers or -inhibitors rather than as phytoanticipins against some fungi.

Antifungal SAR was subsequently examined by using the synthetic analogues,[11] 4′-deoxy type (**12**), hexanoyl type (**13**), α-methyl glucoside (**15**), β-methyl glucoside (**16**), 1,2-dideoxy type (**17**), amide-type (**18**), and their 3′-epimers (***epi*-12**, ***epi*-13**, ***epi*-15**, ***epi*-16**, ***epi*-17** and ***epi*-18**). Hydrogenated tulipalin Bs (**19** and ***ent*-19**) were also tested as shown in Table 2. Among the compounds tested, **15**, **16**, **17** and their epimers in which the sugar part of 6-tuliposide B was modified exhibited antifungal activities. All the active analogues gave the same MIC values against each fungus irrespective of the sugar modification. Meanwhile, **12**, **13**, **18** and their epimers in which the side-chain moiety of 6-tuliposide B was modified were all inactive. The 4′-deoxy-type (**12** and ***epi*-12**) and hexanoyl-type (**13** and ***epi*-13**) analogues are unable to form tulipalin B, and the amide-type analogues (**18** and ***epi*-18**) are difficult to lactonize because of robust amide bonds. On the other hand, 6-tuliposide A (**11**), which can be regarded as a side-chain analogue of 6-tuliposide B (**9**), retained its antifungal activity. These results indicate that tulipalin formation played a key role in the mechanism for antifungal action, like it did in the antibacterial activities. Tuliposides can spontaneously or enzymatically lactonize to release their sugar moieties, and resulting tulipalin A (**14**) or tulipalin B (**10**) would behave as an active principle. In most cases, the configuration of the β-hydroxyl group did not affect the antifungal activity, and this characteristic was analogous to that for the antibacterial activity. Hydrogenated tulipalin Bs (**19** and ***ent*-19**) did not show any activity, indicating that the unsaturated α-methylene moiety was necessary for antifungal activity. The results of the SAR study also demonstrate that tulipalin formation was involved in the pigment-inducing or -inhibiting activity. Side-chain-modified **12**, **13** and **18**

and hydrogenated **19** did not show these activities, while the analogues which could form tulipalins showed the same activities irrespective of their configuration at the 3′-position. Tulipalins would have been the active principles, and tuliposides their precursors for exhibiting both antifungal and pigment-inducing or -inhibiting activities.

Conclusion

We established the asymmetric total synthesis of 6-tuliposide B, using *N*-acyl camphor sultam as a chiral template, to selectively prepare the natural type of 6-tuliposide B without monotonous HPLC separation to remove the unwanted 3′*R*-epimer. We also clarified its biological activities against tulip pathogenic fungi. Tuliposides and tulipalins had the potential to inhibit both bacterial and fungal growth. Tulipalins showed stronger antifungal activities than tuliposides, with tulipalin A being more effective than tulipalin B. However, those activities were lower than the antibacterial activities. Tuliposides and tulipalins perturbed the production of fungal pigments at a lower concentration than MIC in their antifungal activities against certain pathogenic fungi. The results of the SAR study proved tulipalins to have been the active principles in all these activities. Further molecular biological studies are needed to clarify details of the mechanism of action for these activities.

Experimental

Chemicals of the highest commercial purity were used without further purification unless otherwise stated. Thin-layer chromatography was performed with Merck 60 F_{254} silica gel. Silica gel column chromatography was performed with Kanto Chemicals 60 N silica gel (spherical, neutral), except for purifying dioxanone **3b** which used C200 Wakogel (Wako Co.). Chiral HPLC was performed with a Daicel Chiralpak® IA column (ϕ 4.6 mm × 250 mm) and a Hitachi L-7455 photodiode array detector at 30 °C. ^{1}H- and ^{13}C-NMR spectra were measured in $CDCl_3$ with a Jeol JNM-EX270 instrument and in D_2O with a Jeol JNM-LA400. Chemical shifts are reported in δ ppm, using tetramethylsilane as the internal standard, and coupling constants (J) are given in Hertz. All the FD-mass spectra were measured with a JMS-SX102A instrument by the GC-MS and NMR Laboratory of Faculty of Agriculture at Hokkaido University. Optical rotation data were determined with a Jasco P-2200 polarimeter in ϕ 3.4 mm × 50 mm cells at 24 °C. Dichloromethane was distilled from phosphorous oxide. All of the compounds having an α,β-unsaturated carbonyl structure were supplemented with 200 ppm of hydroquinone to prevent polymerization after being purified. Tulipalin A (**14**) was purchased from Aldrich Chemical Company. The synthetic procedures and spectral data for compounds **5**, **6**, **7**, **9**, ***epi*-9**, **10**, ***ent*-10**, **12**, ***epi*-12**, **13**, ***epi*-13**, **15**, ***epi*-15**, **16**, ***epi*-16**, **17**, ***epi*-17**, **18**, ***epi*-18**, **19** and ***ent*-19** have been presented in our previous reports.[10,11]

*Synthesis of (6S)-2,6-Di-(*p*-methoxybenzyloxy)methyl-5-methylene-1,3-dioxan-4-one (**3b**).* Acylated camphor sultam **2** (47.8 mg, 0.178 mmol) and DABCO (1.98 mg, 0.018 mmol) were dissolved in 0.2 mL of DMF at room temperature. The mixture was cooled at 0 °C for 1 h, and PMB-protected aldehyde **1b** (467 mg, 2.59 mmol) was added. After 120 h, the reaction mixture was diluted with Et_2O and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to remove the solvent. The obtained crude product was purified by silica gel (Wakogel C200) column chromatography (EtOAc:hexane, 1:4 to 1:2). Evaporation of the corresponding fractions gave 66.1 mg of **3b** (90%), $[\alpha]_D^{24} = +25.4$ (c 2.15, $CHCl_3$). HR-FD-MS m/z $[M]^+$: calcd. for $C_{23}H_{26}O_7$, 414.1679; found, 414.1655. ^{1}H-NMR (270 MHz, $CDCl_3$): 3.65–3.69 (4H, m, C$\underline{H}_2$), 3.80 (6H, s, OMe), 4.52 (2H, s, benzyl C$\underline{H}_2$), 4.56 (2H, s, benzyl C$\underline{H}_2$), 4.79 (1H, m, β-C$\underline{H}$), 5.45 (1H, t, J = 4.6 Hz, acetalic C$\underline{H}$), 5.73 (1H, d, J = 2.3 Hz, =C$\underline{H}_2$), 6.51 (1H, d, J = 2.3 Hz, =C$\underline{H}_2$), 6.87 (4H, d, J = 8.6 Hz, aromatic H), 7.25 (2H, d, J = 8.6 Hz, aromatic H), 7.27 (2H, d, J = 8.6 Hz, aromatic H). ^{13}C-NMR (67.5 MHz, $CDCl_3$): 55.2 (PhO$\underline{Me}$), 69.8 ($\underline{C}H_2$), 71.4 (γ-$\underline{C}H_2$), 73.2 (benzyl $\underline{C}H_2$), 73.5 (benzyl $\underline{C}H_2$), 99.5 (acetalic $\underline{C}H$), 113.8 (aromatic C), 127.3 (=$\underline{C}H_2$), 129.3 (aromatic C), 129.39 (aromatic C), 129.44 (aromatic C), 129.6 (aromatic C), 133.8 (β-$\underline{C}$), 159.3 (aromatic C), 159.4 (aromatic C), 163.0 (carbonyl C).

*Synthesis of 6-*O*-[(3′*S*)-3′-(*tert*-butyldimethylsilyloxy)-4′-(*p*-methoxybenzyloxy)-2′-methylenebutanoyl]-2,3,4-tri-*O*-(trimethylsilyl)-1-*O*-(2-trimethylsilylethyl)-β-*D*-glucopyranoside (**8**).* To a solution of carboxylic acid **6** (140 mg, 0.38 mmol) in 4.0 mL of CH_2Cl_2, *N*,*N*-dimethylaminopyridine (23.2 mg, 0.19 mmol) and diisopropylcabodiimide (75 µL, 0.46 mmol) were added at −20 °C. Protected sugar **7** (150 mg, 0.30 mmol) was then added, and the mixture stirred overnight at 0 °C. The reaction mixture was diluted with Et_2O and was washed with a saturated NH_4Cl aqueous solution. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. Removal of the solvent by evaporation and subsequent purification by silica gel column chromatography (EtOAc:hexane, 1:19) afforded 154 mg of sugar ester **8** (0.18 mmol, 61%) as a syrup, $[\alpha]_D^{24} = +9.2$ (c 3.01, $CHCl_3$). HR-FD-MS m/z $[M]^+$: calcd. for $C_{39}H_{76}O_{16}Si_5$, 844.4285; found, 844.4280. ^{1}H-NMR (270 MHz, $CDCl_3$): 0.02–0.18 (42H, m, SiMe), 0.91 (9H, s, *t*-Bu), 0.95–1.04 (2H, m, $CH_2C\underline{H}_2SiMe_3$), 3.30–3.50 (6H, m, H-2, H-3, H-4, H-5, H-4′a and $C\underline{H}_2CH_2SiMe_3$), 3.56 (1H, dd, J = 10.2, 3.0 Hz, H-4′b), 3.84 (3H, s, OMe), 3.84–3.97 (1H, m, $C\underline{H}_2CH_2SiMe_3$), 4.06 (1H, dd, J = 11.7, 5.2 Hz, H-6a), 4.18 (1H, d, J = 7.6 Hz, H-1), 4.42–4.60 (3H, m, H-6b and benzyl C$\underline{H}_2$), 4.83 (1H, br dd, H-3′), 6.06 (1H, s, =C$\underline{H}_2$), 6.37 (1H, s, =C$\underline{H}_2$), 6.86 (2H, d, J = 8.2 Hz, aromatic H), 7.25 (2H, d, J = 8.2 Hz, aromatic H). ^{13}C-NMR (67.5 MHz, $CDCl_3$): −5.0 and −4.8 (Me_2Si), −1.5 ($CH_2CH_2Si\underline{Me}_3$), 0.9–1.3 ($OSiMe_3$), 18.1 and 18.2 ($CH_2\underline{C}H_2SiMe_3$ and $C\underline{Me}_3$), 25.8 ($\underline{C}Me_3$), 55.2 (PhO$\underline{Me}$), 63.9 (C-6), 66.7 ($\underline{C}H_2CH_2SiMe_3$), 70.4 (C-4′), 72.2 (C-4), 72.7 (benzyl $\underline{C}H_2$), 73.7 (C-3′), 74.8 (C-5), 76.0 (C-2), 78.5 (C-3), 102.7 (C-1), 113.5 (aromatic C), 126.5 (=$\underline{C}H_2$), 129.1 (aromatic C), 130.6 (aromatic C), 140.8 (C-2′), 158.9 (aromatic C), 165.7 (C-1′).

*Preparation of 6-tuliposide A (**11**).* To cut petals (30 g fresh weight) of the *murasaizuishou* tulip was added 100 mL of cold 50% MeOH, and the mixture was vigorously stirred at 4 °C for 1 h. The extract was filtered through cheesecloth and centrifuged (2,000 × g, 4 °C, 5 min). The resulting supernatant was defatted by washing with $CHCl_3$, and the aqueous layer was lyophilized. The extract was dissolved in a minimum amount of water, applied to an ODS-column (ϕ 4.5 mm × 150 mm) and eluted with water. The fractions containing tuliposide A were combined, concentrated *in vacuo*, and lyophilized. Crude tuliposide A was further purified by preparative HPLC at 225 nm in an RP-18GPAqua column (ϕ 10 mm × 250 mm), using a linear gradient of MeOH (0 to 75% for 17 min) at a flow rate of 3 mL/min. Pure tuliposide A (13.7 mg) was obtained as an amorphous powder after lyophilization. ^{1}H-NMR (400 MHz, D_2O): 2.58 (2H, t, J = 6.3 Hz, H-3′), 3.74 (2H, t, J = 6.3 Hz, H-4′), 3.2–4.0 (4H, m, H-2, H-3, H-4 and H-5), 4.3–4.5 (2H, m, H-6), 4.68 (0.5H, d, J = 8.0 Hz, H-1β), 5.23 (0.5H, d, J = 3.9 Hz, H-1α), 5.81 (1H, s, =C$\underline{H}_2$), 6.32 (1H, d, J = 3.4 Hz, =C$\underline{H}_2$). ^{13}C-NMR (100 MHz, D_2O): 34.9 (C-3′), 60.7 (C-4′), 129.7 (=$\underline{C}H_2$), 137.0 (C-2′), 169.4 (C-1′); α-D-glucosyl: 64.3 (C-6), 70.4 (C-4), 70.5 (C-5), 72.2 (C-2), 73.3 (C-3), 92.4 (C-1); β-D-glucosyl: 64.4 (C-6), 74.2 (C-5), 70.4 (C-4), 74.9 (C-2), 76.3 (C-3), 96.8 (C-1).

Antifungal assay. The antifungal activities were evaluated by a microscopic analysis during the growth of each fungus. The fungal strains were maintained on potato dextrose agar (PD agar; Difco, Detroit, WI, USA). The tested compounds were each diluted with Milli Q-water or DMSO as described in our previous report.[11] The minimum inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was apparent. Each fungus was precultured on a PD agar plate for several days until the hyphae had grown well at 25 °C. The mycelia were scratched off from the surface of the

plate and suspended in sterilized saline. To a 24-well tissue culture plate (Corning, NY, USA) containing 200 μL of the PD medium supplemented with 0.05–2.5 mM of each compound was inoculated 25 μL of the mycelial suspension of a fungal strain. The morphological changes of the fungal hyphae were observed under a CK2 optical microscope (Olympus, Japan) during incubation at 20 °C for several days. The fungal strains used were *Fusarium oxysporum* f. sp. *tulipae* MAFF 235110 (tulip bulb rot disease), *Pythium ultimum* MAFF 235799 (tulip root rot disease), *Rhizoctonia solani* MAFF 235845 (tulip leaf rot disease), *Botrytis tulipae* MAFF 237887 (tulip botrytis blight disease), and *Gibberella zeae* MAFF 305135 (non-pathogenic against tulip).

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