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Functional importance of the sugar moiety of jasmonic acid glucoside for bioactivity and target affinity

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12-*O*-β-D-Glucopyranosyljasmonic acid (JAG, 1) induces nyctinastic leaf-folding of *Samanea saman*. The SAR studies of 1 revealed the unique role of its glycone moiety. Biological activity and target affinity of 1 were affected by the stereochemistry of the glycone moiety. JAG belongs to a unique class of ligands in which the structure of the glycone moiety is involved in the molecular recognition by the target protein.

Jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates, play important roles in the life of higher plants.[1-4] We identified 12-*O*-β-D-glucopyranosyljasmonic acid, also referred to as jasmonic acid glucoside (JAG, **1**), as the bioactive metabolite that induces nyctinastic leaf-folding of *Samanea saman*.[5-8] Nyctinastic leaf-folding of plants has been a historic matter of interest among biologists since Darwin's era[9] and *S. saman* has been used as a standard plant material for the biology of nyctinasty.[10, 11] Thus, **1** is a key compound for the molecular understanding of this intriguing biological phenomenon.

The mode of action of JA has been verified through identification of the COI1-JAZ signalling module[12-14] in which a complex of cytosolic COI1 and JAZ proteins functions as the jasmonate receptor. However, the leaf-folding induction by **1** was revealed to be independent of the COI1-JAZ signalling pathway.[15] Instead, the membrane target protein referred to as MTJG (membrane target of jasmonic acid glucoside)[15, 16] is believed to be involved. Considering most glycosides are regarded as biologically inactive derivatives of secondary metabolites, this is a rare example in which a glucoside of a plant hormone functions as a ligand totally different from the parent ligand. Here, we report that **1** belongs to a unique class of ligands in which the D-glucopyranosyl moiety participates in the molecular recognition by the target protein.

Three possible roles of the D-glucopyranosyl moiety can be proposed for the target selectivity of **1**. First, the conjugation of the D-glucopyranosyl moiety could decrease the lipophilicity and membrane permeability of jasmonate and then a membrane permeable jasmonate targeting COI1-JAZ could be converted to a membrane-impermeable **1** targeting membrane protein MTJG. Second, the D-glucopyranosyl moiety in **1** could provide steric hindrance to inhibit binding with the COI1-JAZ receptor complex. In the crystal structure of the COI1-JAZ-jasmonoyl L-Ile (JA-Ile) complex,[14] JA-Ile is packed tightly between COI1 and JAZ, and no free space is left for accommodation of additional D-glucopyranosyl moieties. Third, the D-glucopyranosyl moiety in **1** could enhance the affinity with the target protein as shown in the case of a steroid glycoside, ouabain. Recently, Cornelius *et al.* reported an intriguing example in which the conjugation of a glycone moiety strongly enhanced the affinity of a ligand with its target protein. The affinity of ouabain with its target protein, Na,K-ATPase, is enhanced as much as 26-fold by the glycosidation of the corresponding aglycone.[17] The strong hydrogen bond network between the glycone moiety of ouabain and the ligand binding pocket of Na,K-ATPase strongly enhanced their binding. We examined this third possibility as shown below.

We first carried out structure activity relationship (SAR) studies on **1** to estimate the importance of the D-glucopyranosyl moiety. We designed *ent*-**1**,[18] diastereomeric 12-*O*-β-L-glucopyranosyljasmonic acid (**2**), diastereomeric 12-*O*-β-D-glucopyranosyl-*ent*-jasmonic acid (*ent*-**2**), epimeric 12-*O*-β-D-galactopyranosyljasmonic acid (**3**),[18] and open-chain type 12-*O*-D-sorbitoyljasmonic acid (**4**) (Figure 1). The hydrophilicity of **1** was maintained in *ent*-**1**, **2**, *ent*-**2**, **3**, and **4**, whereas the structure recognition of the glycone moiety may be affected by structural modifications in *ent*-**1**, *ent*-**2**, **3**, and **4**. We synthesized **1**, *ent*-**1**, **2** (Scheme S1), *ent*-**2** (Scheme S2), and **3** according to the procedure in reference 19. The open-chain type **4** was synthesized as shown in Scheme S3. In the previous study,[15] we demonstrated that **1** induced shrinking of extensor motor cell protoplasts isolated from *S. saman*, whereas *ent*-**1** cannot induce the shrinking. As the shrinking of motor cells directly causes leaf-

folding movement,[10] the biological evaluations of **1**-**4**, *ent*-**1**, and *ent*-**2** were carried out based on this cell-shrinking assay (Figure 1).

Interestingly, clear differences were observed among them. Only naturally occurring **1** induced cell-shrinking, whereas no cell-shrinking was observed with the enantiomeric and diastereomeric isomers (*ent*-**1**, **2** and *ent*-**2**) (Figure 1b). It should be noted that the stereochemistry of the glycone moiety was proven to be important for the bioactivity of **1**. Additionally, substitution of glucopyranosyl into galactopyranosyl (4'-epimer) in **3** did not affect the cell-shrinking activity or the previously reported leaf-folding activity (Figure 1c).[18] Subtle structural modifications, such as epimerization of one hydroxy group in the sugar moiety, did not affect the biological activities of **1**. In contrast, the glycopyranosyl structure is important because no cell-shrinking was observed in open-chain type **4**. These results strongly suggested that the structure of the D-glycopyranosyl moiety is indispensable for cell-shrinking activity.

Next, we considered that photoaffinity labeling experiments could provide direct proof that the stereostructure of the glycone moiety in **1** affected its affinity with its target protein, MTJG. We previously prepared four stereochemical hybrids of compact molecular probes[19] (CMPs: **5**, *ent*-**5**, **6,** and *ent*-**6**, Figure 2a) based on **1**.[20] The CMPs contained D-galactopyranosyl due to its stability against hydrolysis by glucosidases in living cells. Among them, only the naturally occurring form (**5**) could induce the shrinking of *Samanea* motor cells.[20] The target protein in living cells was labeled by a sterically minimal azide handle and subsequent introduction of a tag was achieved by Hüsgen [3+2] cycloaddition employing Cu-catalyzed azide alkyne cycloaddition (CuAAC)[21, 22] or copper-free click chemistry (CFCC).[23] Thus, living motor cells were photo-crosslinked using CMP **5** (1×10^{-4} M) followed by either CuAAC with 4-ethynyl-2,6-bis(trifluoromethyl)-cinnamamide (**7**; 1×10^{-4} M)[24, 25] or CFCC with strained difluorinated cyclooctyne DIFO[23] derivative (**8**; 1×10^{-4} M). In both alkyne units, the FLAG peptide was conjugated as a molecular tag.[19, 26] As shown in Figure S1, CuAAC using **7** gave the better result. CFCC using **8** gave many non-specific bindings (Figure S1). These nonspecific bindings were also observed when DIFO **8** was used alone without the addition of CMP **5** (Figure S1), and could be due to nonspecific thiol adducts because the strained DIFO unit can react with thiol groups in proteins to give nonspecific adducts.[27, 28] In contrast, no nonspecific binding was observed in CuAAC, and the result was very clear because only one target band was observed (Figure S1).

The photo-crosslinking against a living *Samanea* motor cell was examined by using these four CMPs (**5**, *ent*-**5**, **6,** and *ent*-**6**) and subsequent cycloaddition was carried out with alkyne **7**. Results of SDS-PAGE analysis and subsequent chemiluminescence detection are shown in Figures 2b and S2. The order of target affinity was **5** > *ent*-**6** > **6** = *ent*-**5**. The difference between the results by probe **5** or *ent*-**5** clearly demonstrated the stereospecific recognition of the probe by MTJG. Binding of probe **5** with this protein was competitively inhibited by the coaddition of excess **1** (50-fold), whereas it could not be inhibited by the excess coaddition of methyl-

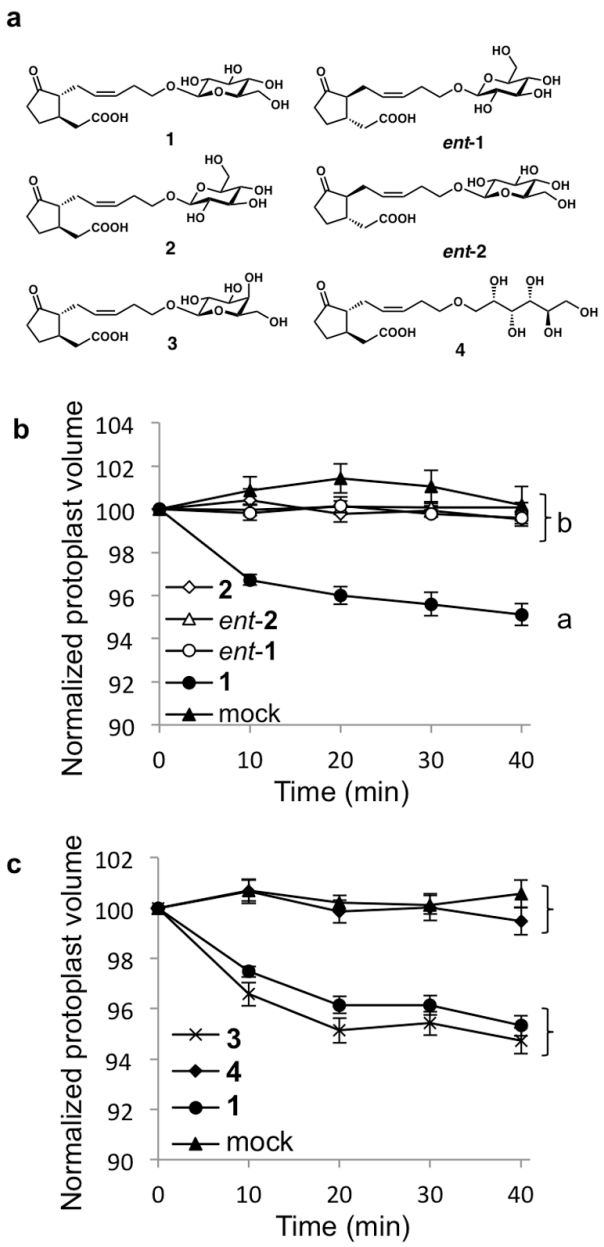

Figure 1. (a) Structures of JAG (**1**) and its derivatives; **(b)** and **(c)** Cell shrinking assays using living *Samanea* extensor motor cells. **(b)** Extensor motor cell protoplasts were treated with 100 μM **2** (white diamonds) or 100 μM *ent*-**2** (white triangles), and the response was compared to the treatments with 100 μM JAG (**1**) (black circles), 100 μM *ent*-**1** (white circles), or mock (black triangles). **(c)** Extensor protoplasts were treated with 100 μM **3** (cross marks) or 100 μM **4** (black diamonds), and the responses were compared to the treatments with 100 μM **1** (black circles) or mock (black triangles). Error bars represent the ±SE. The a and b labeled values are statistically different according to analysis of variance followed by SNK post-hoc test (**a**, $P < 0.05$, n = 8–11, i.e., $t = 40$ min, $F_{4, 41} = 20.19$, $P < 0.001$; **b**, $P < 0.05$, n = 9, 10, i.e., $t = 40$ min, $F_{3, 33} = 33.51$, $P < 0.001$).

D-galactopyranoside (**9**) or 12-hydroxy jasmonic acid (**10**) (Figures 2c and S3). Interestingly, CMP *ent*-**6** consisting of the enantiomeric aglycone and the D-galactosaminopyranosyl moiety showed weak affinity with MTJG. The band was weakly observed using *ent*-**6**, whereas it could not be seen using **6**, suggesting the importance of the D-glycopyranosyl moiety for binding with MTJG. Considering that no cell-shrinking activity was observed in *ent*-**6**,[20] the affinity of *ent*-**6** with MTJG may not be strong enough to induce cell-shrinking. However, these results provided direct proof that MTJG recognizes the glycone as well as the aglycone.

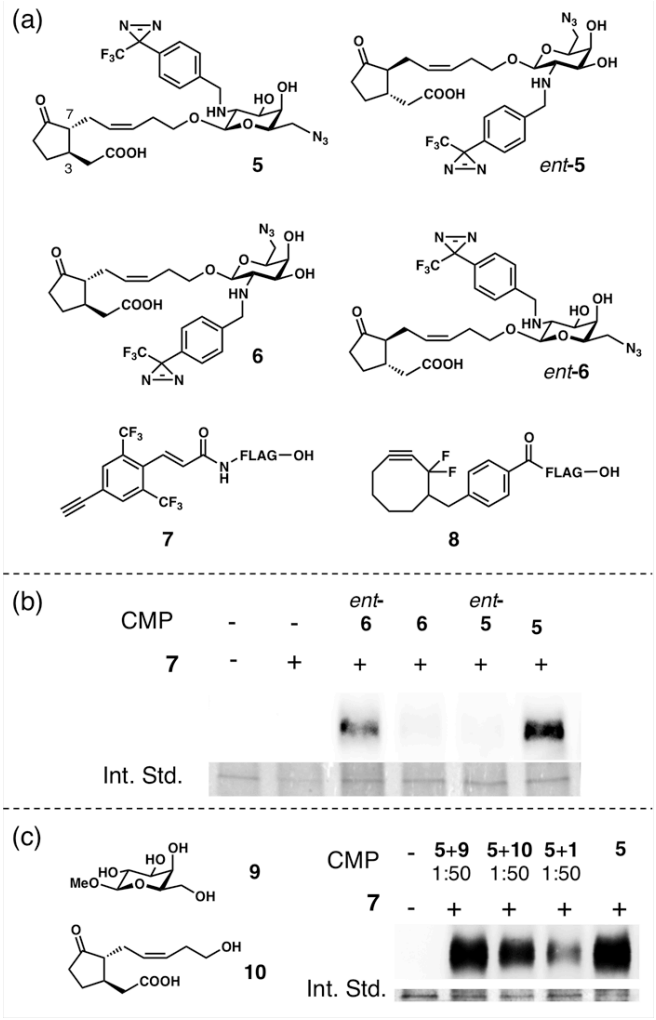

Figure 2. (a) Structures of CMPs (**5**, *ent*-**5**, **6,** and *ent*-**6**) and alkyne tag units (**7** and **8**, FLAG=DYKDDDDK); (b) Photoaffinity labeling of MTJG using live *Samanea* motor cells with CMP (**5**, *ent*-**5**, **6,** and *ent*-**6**; 4×10^{-4} M) and competitive inhibition using **5** (4×10^{-4} M) and **1** (2×10^{-2} M). Int. Std: 52 kDa in Colloidal Gold Total Protein Stain detection; (c) Competitive inhibition of binding between **5** (4×10^{-4} M) and MTJG by using **1**, **9,** or **10** (2×10^{-2} M). Int. Std: 52 kDa in Colloidal Gold Total Protein Stain detection.

The weak affinity of *ent*-**6** with MTJG can be attributed to thermodynamic equilibrium. JAG **1** is known to exist as an equilibrium mixture of two isomers at the α-position of the ketone (7*R*:7*S* = 95:5),[18] and the bioactive form was revealed to be 7*R*-**1**.[18] UPLC-MS analysis revealed that CMP *ent*-**6** also exists as an equilibrium mixture of 7*S*- and 7*R*-forms (Figures 3a,b and S4, 7*S*:7*R* = 95:5). The isomer of *ent*-**6** predominantly observed in solution was confirmed to be the 7*S*-form from NOE correlations[29] in the NMR spectrum (Figures 3c and S5). The 7*R*-*ent*-**6** is an epimer of **5** at the 3-position, and is expected to be biologically active and have some affinity with MTJG. Thus, the presence of 7*R*-

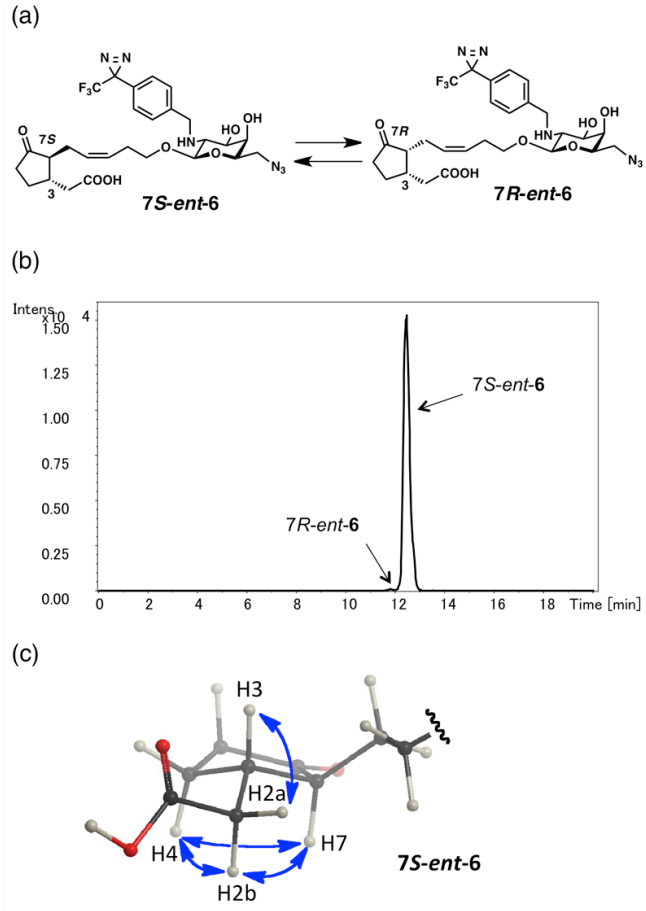

Figure 3. (a) Thermodynamic equilibrium of 7*S*-*ent*-**6** and 7*R*-*ent*-**6**; (b) UPLC-MS analysis of equilibrium mixture of 7*S*-*ent*-**6** (R_t = 14.1 min) and 7*R*-*ent*-**6** (R_t = 13.2 min) [detection: ESI (-) *m/z* = 609.300; column: Agilent Eclipse Plus C18 (1.8 m, ∅ 2.1×50 mm, Agilent Technologies); flow rate: 0.2 µL/min; mobile phase: 27% CH_3CN (0-12 min), 27-90% CH_3CN (12-14 min), 90% CH_3CN (14-18 min) each containing 0.1% TFA]; (c) NOE correlations (H7-H2, H7-H4, H3-H2a, H7-H2b, and H4-H2b) on the predominant isomer of 7*S*-*ent*-**6** in NMR spectrum.

ent-**6** (5%) can explain the weak band of MTJG in Figure 2b observed when using *ent*-**6**.

The results of photoaffinity labeling experiments demonstrated that affinity between a CMP with MTJG can be affected by structural modification in the D-galactopyranosyl moiety. Both the aglycone moiety and the D-galactopyranosyl moiety participate in the molecular recognition of **5** by MTJG.

Conclusions

Our previous SAR studies suggested that leaf-folding activity of **1** is strongly affected by the stereochemistry of the aglycone moiety.[18] In this study it was also revealed that the structure and stereochemistry of the D-glucopyranosyl moiety of **1** also severely affected the cell-shrinking activity as well as its target affinity. Our results of the SAR studies of **1** revealed the unique role of its glycone moiety. Cell-shrinking activity and target affinity of **1** were severely affected by the stereochemistry of both the aglycone and glycone moieties. The stereochemical hybrid **6** with naturally occurring aglycone and the L-galactopyranosyl moiety showed no target affinity in photoaffinity experiments. In contrast, hybrid *ent*-**6** with enantiomeric aglycone and the D-galactopyranosyl moiety containing 5% of 7*R*-*ent*-**6** isomer, a 3-epimer of **5**, showed weaker target affinity than **5** with the naturally occurring stereochemistry. These results suggested that stereochemistries of both the aglycone and glycone in **1** contribute to the target affinity.

JAG belongs to a unique class of ligands in which the glycone moiety plays an important role in the biological activity as well as the affinity with target protein.[30]

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Notes and references

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