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Hybrid stereoisomers of a compact molecular probe based on a jasmonic acid glucoside: Syntheses and biological evaluations

Minoru Ueda [a,*], Gangqiang Yang [a], Yasuhiro Ishimaru [a], Tetsuya Itabashi [a], Satoru Tamura [a], Hiromasa Kiyota [b], Shigefumi Kuwahara [b], Sho Inomata [a], Mitsuru Shoji [c], Takeshi Sugai [c]

[a] Department of Chemistry, Tohoku University, 6-3 Aramaki-aza Aoba, Aoba-ku, Sendai 980-8578, Japan
[b] Department of Applied Bioorganic Chemistry, Division of Bioscience & Biotechnology for Future Bioindustry, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiya, Aoba-ku, Sendai 981-8555, Japan
[c] Faculty of Pharmaceutical Sciences, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

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ABSTRACT

12-*O*-β-D-glucopyranosyl jasmonic acid (JAG) shows unique biological activities, including leaf-closing of *Samanea saman*. It is expected that the mode of action for such regulation is distinct from that of other jasmonates. We developed high-performance compact molecular probes (CMPs) based on JAG that can be used for the FLAG-tagging of JAG target. We synthesized four hybrid-type JAG-CMP stereoisomers (**7**, *ent*-**7**, **8**, and *ent*-**8**), which are composed of (−)-12-OH-JA (**2**)/D-galactopyranoside, (−)-**2**/L-galactopyranoside, (+)-*ent*-**2**/D-galactopyranoside, and (+)-*ent*-**2**/L-galactopyranoside moieties, respectively, and we examined their biological features, such as the stereospecific induction of shrinkage, rate of the cellular response, and dependence on potassium channel activity. These features of the JAG-CMPs were completely consistent with those of the original JAG. These results indicate the biological equivalence of JAG and the JAG-CMPs. During the course of such biological evaluations, it was revealed that the biological activity of the CMPs is greatly dependent on the D/L-stereochemistry of a glycon moiety. To the best of our knowledge, this is the first study suggesting that the D/L-stereochemistry of the glycon moiety significantly affects the biological activity of the associated glycoside.

1. Introduction

Jasmonic acid (JA, **1**) and its derivatives, collectively referred to as jasmonates, play important roles in controlling growth and development and regulating plant responses to environmental changes in higher plants. Recently, it was revealed that (+)-7-*iso*-jasmonoyl isoleucine (JA-Ile) functions as a trigger for a JA signaling module. Accordingly, (+)-7-*iso*-JA-Ile binds to the co-receptor complex COI1-JAZ[1] and triggers the degradation of the JAZ repressor by 26S-proteasome, thereby releasing the transcription factor MYC2 and allowing the activation of gene expression.[2,3]

On the other hand, 12-*O*-β-D-glucopyranosyl jasmonic acid (JAG, **3**) shows unique biological activities, such as inducing leaf-closing of *Samanea saman*[4–6] as well as tuber induction if *Solanaceous* species,[7] but no involvement in the above-mentioned COI1-JAZ signaling module.[8] This result implies the existence of an unknown mode of action mediated by jasmonates. Therefore, studies to determine the protein target of **3** would provide important clues to help identify this new mechanism.

However, it is widely recognized that the identification of the protein target of a bioactive metabolite remains a difficult task.[9,10] We and Uesugi et al. have proposed that the stereospecific nature of ligand recognition by a target protein provides a powerful strategy for identifying the target of a bioactive metabolite. For this strategy, the stereoisomer of the bioactive metabolite can be used as the ideal control.[11,12] Differences in the affinity profile among the stereoisomers of a ligand suggest the specific binding of the ligand based on the stereospecificity of ligand recognition by the specific target. We applied an 'enantio-differential' approach (EDA) in which an enantiomer was adopted as the control for investigating the target of **3**. EDA using a pair of probes (**5** and **6**) enabled the discovery of a membrane target protein, referred to as membrane target protein of jasmonate glucoside (MTJG), in the motor cells of *S. saman*.[11] However, the trace amounts of MTJG present in the cells limit its accessibility, making identification difficult. Thus, it is essential to develop a high-performance molecular probe for the efficient tagging of target proteins having low-abundance.

Recently, we reported the advantages of a method for designing a high-performance compact molecular probe (CMP) for target identification in which the retention of the original bioactivity of the ligand was given the highest priority.[13] An azide-functionalized ligand equipped with a carbon elecrophile/photoreactive functional

* Corresponding author.
E-mail address: ueda@m.tohou.ac.jp (M. Ueda).

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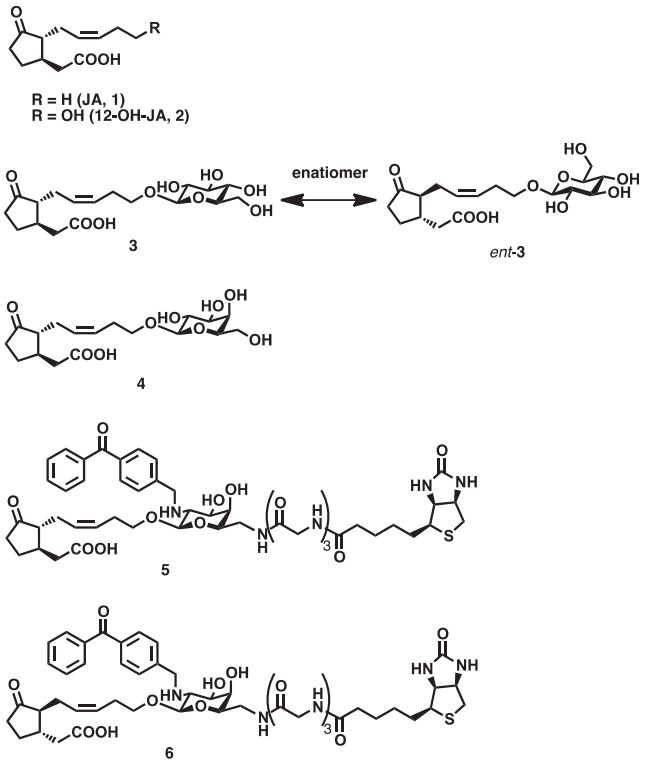
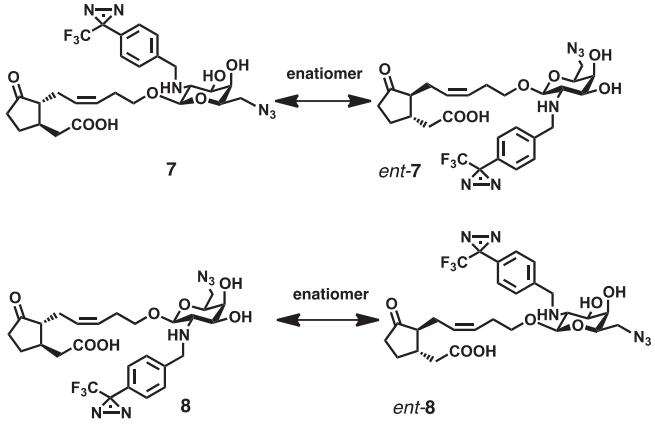
group represents a very simple design for a molecular probe to which a large tag unit, such as FITC, biotin, or FLAG epitope peptide, can be attached after crosslinking with the target protein. The use of stepwise tagging enables the incorporation of a large FLAG peptide tag, which is an octapeptide antigen with the sequence DYKDDDDK and known to give only low levels of non-specific binding.[14] The CMP method was highly successful for the detection and purification of the cytosolic target for isolespedezate, CoMetE.



On the basis of these positive results, we planned to integrate EDA with the CMP method for the chemical tagging of the target protein. The use of a JAG-CMP combined with the subsequent introduction of an alkyne-linked FLAG tag by Hüsgen [3+2] cycloaddition is expected to enable the highly efficient tagging of the low-abundant MTJG (Fig. 1). In this report, we describe the syntheses of four hybrid stereoisomeric JAG-CMPs (**7**, *ent*-**7**, **8**, and *ent*-**8**) and their biological evaluations, which substantiated their reliability as probes for tagging the target protein of JAG. The results also showed an intriguing importance of the specific glycoside moiety for the bioactivity and target affinity of **3**.



2. Results and discussion

Protein labeling using a probe with an azide handle and subsequent on-the-cell click chemistry[15–17] are employed as a powerful method for tagging a membrane protein. In this method, a target protein on living cells is labeled by a sterically inconsequential azide handle that is subsequently used to introduce a tag by Hüsgen [3+2] cycloaddition employing CuAAC[18,19] or copper-free click chemistry[20–23] (Fig. 1). The different results obtained using stereoisomeric probes, such as enantiomers and diastereomers, provide robust information concerning the stereospecific recognition of a ligand by its target protein. Furthermore, a related co-dependence of both target affinity and bioactivity on ligand stereochemistry affords strong support for target validation.

We already have probe **7** in hand,[24] then we synthesized other three hybrid-type JAG-CMP stereoisomers (*ent*-**7**, **8**, and *ent*-**8**), which are composed of (+)-*ent*-**2**/L-galactopyranoside, (−)-**2**/L-galactopyranoside, and (+)-*ent*-**2**/D-galactopyranoside moieties, respectively. Additionally, the related co-dependence of both target affinity and bioactivity on the stereochemistry of **7**, *ent*-**7**, **8**, *and ent*-**8** would afford strong support for target validation.

The synthesis of *ent*-**7** requires synthetic supply of commercially unavailable L-galactosamine derivative. We planned to synthesize L-galactosamine from a commercially available L-galactose derivative. Some methods to convert hexose into 2′-aminohexose have been reported.[25,26] Among them, Lemieux's azidonitration[27] is recognized as a very useful one and represents a unique approach involving the azidonitration via glycals. Using this method, we synthesized L-galactosamine derivative **16**. The stereoselective addition of an azido radical to the galactal[28] intermediate gave a 10:1:5 mixture of the desired 2′*S* derivative (**10**) and undesired 2′*R* derivative (**11**) as well as the 1α-acetamide analog (**12**) (Scheme 1). The mixture was then reduced by catalytic hydrogenation and protected by phthalimide using the acid chloride[29] to give **15**.

Galactosamine derivative **15** was brominated to form **16** which was used as a glycosyl donor. The glycosylation of **17**[11,30] by glycosyl donor **16** gave **18**, and subsequent modifications gave probe *ent*-**7**, an enantiomer of probe **7** (Scheme 2). Thus, we obtained an enatiomeric pair of CMPs (**7** and *ent*-**7**). Similarly, we synthesized two diastereomeric CMPs (**8** and *ent*-**8**), which were composed of (−)-**2**/L-galactosamine or (+)-*ent*-**2**/D-galactosamine moieties, respectively (Scheme 3a and b).

Next, we performed the biological evaluations of the resulting four stereoisomeric CMPs (**7**, *ent*-**7**, **8**, and *ent*-**8**). Nyctinastic leaf-folding of *S. saman* has been attributed to volume changes in specific motor cells located on the opposite side of the vascular bundle: the extensor cells that reside on the adaxial side of the tertiary pulvinus shrink, whereas the flexor cells that reside on the abaxial side swell during leaf-folding.[31,32] We have recently reported that JAG **3** is able to selectively induce the shrinking of protoplasts isolated from extensor motor cells, whereas flexor motor cell protoplasts prove to be insensitive to JAG treatment.[8] Thus, the leaf-folding of *S. saman* is attributed to cell shrinking in extensor motor cells. This unique feature can be employed for the biological evaluation of the four stereoisomeric CMPs (**7**, *ent*-**7**, **8**, and *ent*-**8**). Each CMP was incubated with the freshly prepared protoplasts of extensor motor cells, and then, the change in the size of the cells was measured and plotted in Figure 2a and b. The cell-shrinking activity was evaluated using the radii of the protoplasts surviving the preparation and 40-min incubation (Fig. 2d). CMP **7** as well as **3** induced the shrinking of extensor protoplasts, whereas the other stereoisomers, *ent*-**7**, **8**, and *ent*-**8**, did not induce any shrinking. Thus, the stereospecific induction of cell shrinkage was found among the JAG-based CMPs. It should also be emphasized

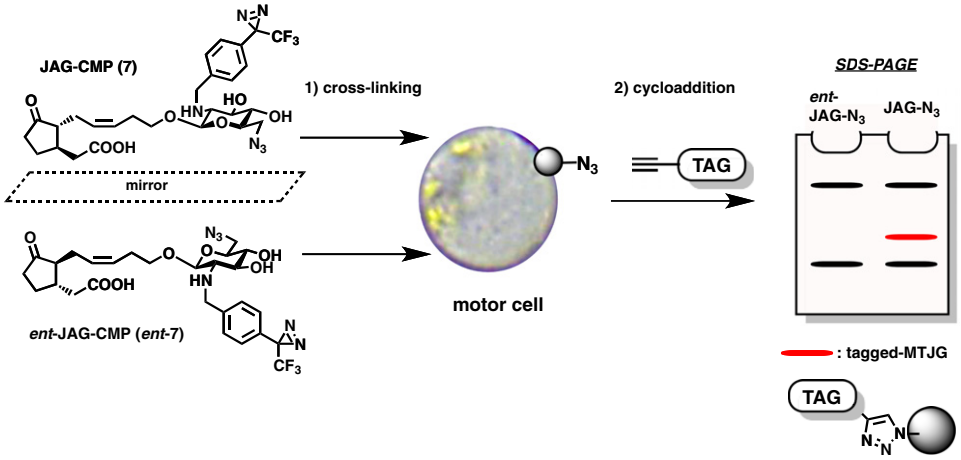
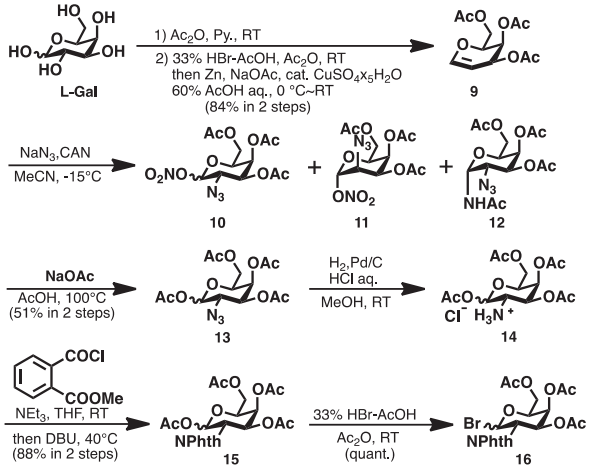


Figure 1. Overview of CMP-CuAAC strategy using stereoisomeric CMPs for the tagging of target protein.



Scheme 1. Synthesis of L-galactosamino unit (**16**).

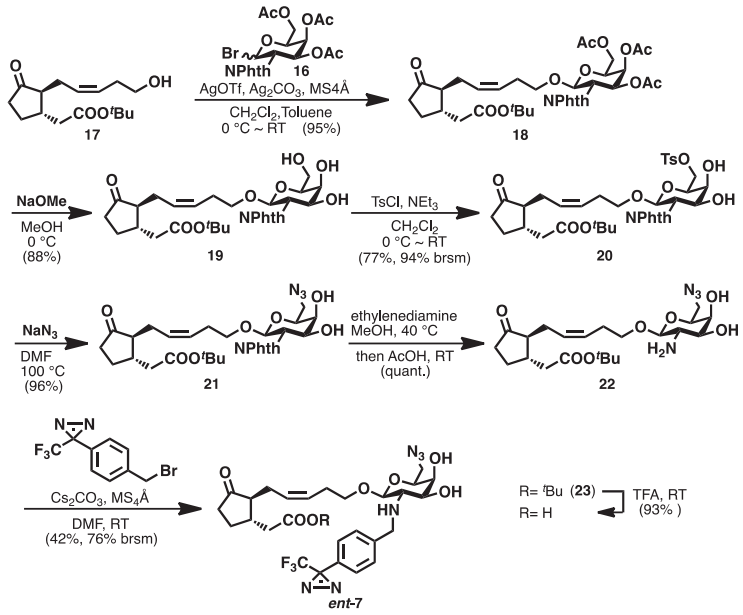
that CMP **7** was as effective as the original JAG **3** in the cell-shrinking assay (Fig. 2a), suggesting that **7** has an affinity for MTJG comparable to the original JAG **3**. In addition, we examined the time-course profiles of the cellular response to the JAG-based CMPs against those to JAG **3**. The response of protoplasts to CMP **7** was as quick as that to JAG **3**; the shrinking started within 10 min after the addition of each compound. This rapid response suggests that the shrinking of motor cell protoplasts upon CMP application is the result of an ion channel-mediated process. Regarding the cell shrinking in response to JAG **3**, the involvement of a potassium channel was confirmed by the inhibition of this response using a potassium channel blocker, namely, tetraethyl ammonium chloride (TEA).[8] This is based on Moran's experiment in which the outward-directed K^+ current from extensor motor cells was blocked by TEA.[33] We also examined the effect of TEA on the cell shrinking induced by CMP **7**. As shown in Figure 2c, the addition of TEA completely inhibited cell shrinking in response to CMP **7** as well as JAG, which suggested that regulation through potassium channels is involved in the shrinking response induced by both compounds.

Next, we examined the biological features of the JAG-CMPs, such as the stereospecific induction of shrinkage, rate of the cellular response, and dependence on potassium channel activity, using motor cells. These features were completely consistent with those of original JAG. These results support the biological equivalence of JAG **3** and probe **7**.

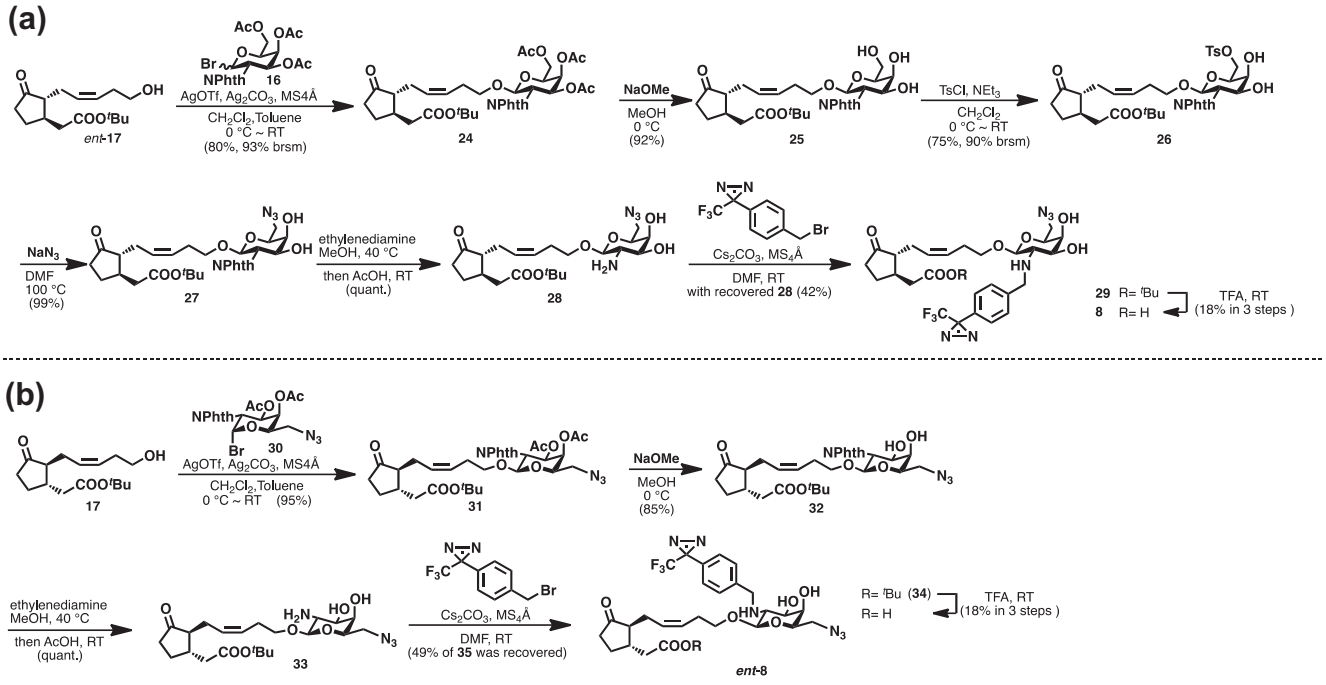
In addition, the biological evaluation also revealed an intriguing feature of JAG **3**, namely, the influence of the glycon moiety on its biological activity. Previous SAR studies[8] unequivocally demonstrated that the glycon moiety of **3** is indispensable for its bioactivity because (−)-12-OH-JA ((−)-**2**), the aglycon of JAG **3**, cannot induce leaf-folding of *S. saman* as well as shrinking of the motor cells. However, biological activity of **3** was retained when its D-glucopyranoside was replaced by the 4′-epimer, D-galactopyranoside (**4**),[34] or a D-galactosaminopyranoside derivative (in **7**).[11] This implies that structural modification of the glycon moiety of **3**, such as that exemplified in **5** and **7**, will not severely affect its biological activity. On the other hand, the importance of the stereochemistry of the 12-OH-JA moieties[11,30,34] was suggested by EDA studies using probes **5** and **6** in which probe **6** with the enantiomeric 12-OH-JA moiety cannot induce leaf-folding and bind to MTJG. Thus, it was very intriguing that diastereomeric CMP **8**, which is composed of (−)-**2**/L-galactopyranoside, cannot induce shrinking even though it has the naturally occurring stereoisomer found with 12-OH-JA. The biological activity of **3** was lost when D-glucopyranoside was replaced by an L-galactosaminopyranoside derivative (in **8**). These results suggest that the naturally occurring stereochemistry in both the aglycon and glycon moieties is indispensable for the leaf-folding/motor-cell-shrinking activity of JAG-related compounds.

Glycosylation dramatically changes the physical properties of a substrate. For example, it causes reduced lipophilicity leading to a decrease in cell permeability. Therefore, the glycosylation of JA **1** may lower its cell permeability and thereby decrease its binding to the cytosolic COI1-JAZ receptor complex. However, the above-mentioned results strongly suggested that the glycon moiety of **3** may be involved in or related to its recognition by MTJG rather than simply functioning as a hydrophilic anchor decreasing the cell permeability of **3**.

Next, we examined the viability of the motor cells during the course of each step, which include the photoaffinity-labeling (PAL) and CuAAC reaction (Fig. 3a). Cell viability under the two conditions used for PAL were compared (Fig. 3b): Freshly prepared protoplasts[11,32] were (1) incubated with probe **7** at 4 °C for 20 min and then photo-crosslinked at 4 °C for 5 min or (2) incubated with probe **7** at 4 °C for 5 min and photo-crosslinked at 4 °C for 20 min. 'Condition 1' (Fig. 3b-C) gave better viability than 'condition 2' (Fig. 3b-F). Surprisingly, longer UV-irradiation (20 min) improved the viability of the protoplasts (Fig. 3(b), C and F, D and G). Considering the fact that photo-crosslinking by trifluoromethyldiazirine (TFMD) is completed within 30 min, it was concluded that condition 1 is superior to condition 2. The longer irradiation time provides a better cross-linking result as well as increased cell viability.



Scheme 2. Synthesis of enantiomeric CMP (*ent*-**7**).



Scheme 3. (a) Synthesis of diastereomeric CMP (**8**). (b) Synthesis of diastereomeric CMP (*ent*-**8**).

Subsequently, we examined the conditions for the CuAAC reaction using the FLAG-linked alkyne unit **35**.[13,32] Two conditions were examined for the CuAAC reaction (Fig. 3c): (I) one based on the reports by Finn et al[35,36] and (II) another based on our previous reports.[37] Under both conditions, a tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) ligand was added as a catalyst.[35] However, under the first condition, CuAAC was used without the addition of aminoguanidine[35] because the motor cells were treated at a pH less than 5.5, a level at which aminoguanidine cannot function efficiently to intercept the byproducts of ascorbate oxidation that can react with proteins. Better cell viability was observed using condition I (Fig. 3c-C) than using condition II (Fig. 3c-D). The increase in copper (I) ion concentration (and equivalence against THPTA) caused a severe decrease in the viability of the protoplasts. This may be because of the toxicity of copper (I) ion, which was reported in Ref. 36. This combination of mild conditions (1) and (I) was selected for the application of CMP to pull-down the purification of the target (MTJG, Fig. 1).

3. Conclusion

Jasmonate glucoside is a metabolite of an important plant hormone, jasmonic acid (JA).[38] The mode of action of JA has been verified through the identification of the COI1-JAZ signaling module[39,40] in which a complex of cytosolic COI1 and JAZ proteins directly functions as a JA receptor.[40,41] Then MTJG[8,11] became the

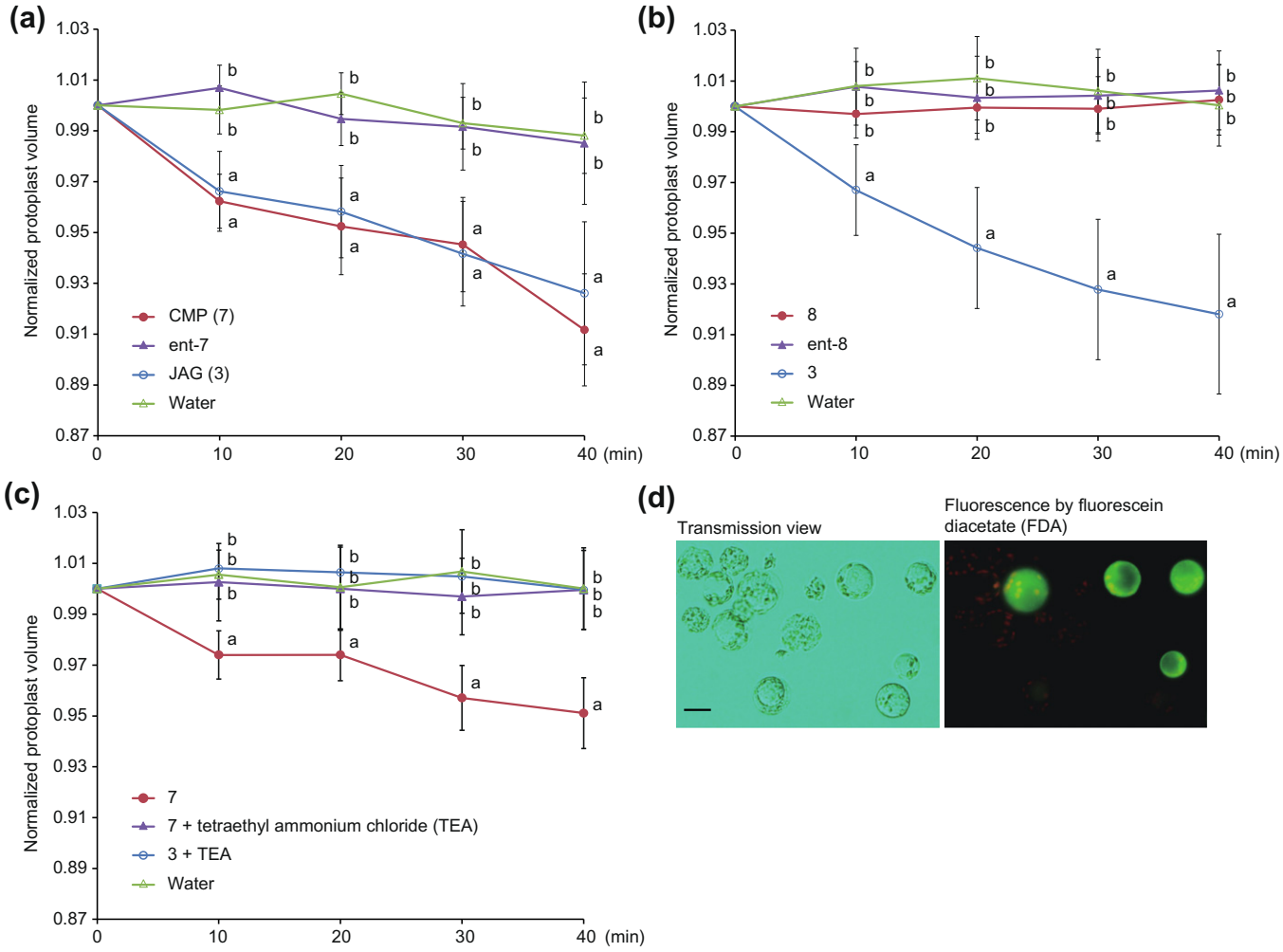


Figure 2. CMP induced shrinkage of extensor motor cell protoplast in *S. saman* (a) Extensor protoplasts were treated with 100 μM CMP (**7**) (red circles), and the response was compared to the treatments with 100 μM *ent*-**7** (purple triangles), 100 μM JAG (blue circles), or water (green triangles). (b) Extensor protoplasts were treated with 100 μM **8** (red circles) or 100 μM *ent*-**8** (purple triangles), and the responses were compared to the treatments with 100 μM JAG (blue circles), or water (green triangles). (c) The response of extensor protoplasts to treatment with **7**and TEA (purple triangles) is compared to the ones caused by **7** (red circles), JAG and TEA (blue circles), or water (green triangles). (d) Transmission view and FDA fluorescence in extensor protoplast after 40 min incubation. Scale bar = 20 μm. Error bars represent the SD. The values followed by different letters are statistically different according to analysis of variance followed by SNK test (**a**, p <0.01; **7**, n = 10; *ent*-**7**, n = 12; JAG, n = 13; Water, n = 13; **b**, p <0.01; **8**, n = 12; *ent*-**8**, n = 11; JAG, n = 12; Water, n = 12; **c**, p <0.01; **7**, n = 9; **7**+TEA, n = 11; JAG + TEA, n = 8; Water, n = 7).

first example of a membrane target protein that binds to jasmonate-related compounds. Our CMP probes were confirmed to have the same biological features as those of the original JAG **3**, that is, they can induce a rapid shrinking response on motor-cells through the activation of a potassium channel (Fig. 3). Thus, it was concluded that JAG **3** and CMP **7** were involved in the same signaling path for inducing the shrinkage of *S. samanea* motor cells, which supports the suitability of using CMP **7** for the exploration of the JAG **3** target.

It is also interesting that a unique feature of ligand recognition using JAG-related compounds was found, that is, the stereochemistry of their glycon moieties strongly influences the induced biological response. In SAR studies, attention is usually focused on the biological consequences of the stereochemistry of the aglycon, whereas there are no reports of those of the glycon moiety. In some cases, hydrogen bonds to the glycon moiety of a glycoside provide an important contribution to target affinity. For example, the rhamnose moiety of ouabain can form hydrogen bonds with its target, an Na/K-ATPase, thereby conferring a much higher affinity to ouabain than that of the corresponding aglycon, ouabagenin.[42,43] Similar involvement of the glycon moiety can be anticipated in our case.

To date, many secondary metabolites with unique bioactivities have been identified from nature. However, in many cases, their mode of action remains unknown. Thus, studies aimed at identifying these unknown targets bridge the fields of chemical biology and natural products chemistry.[44] CMPs employing stereoisomers of bioactive metabolites provide a powerful method for target identification.

4. Experimental section

Unless otherwise stated, reactions were performed in flame-dried glassware under an argon or a nitrogen atmosphere using dry solvents. Solvents were dried over activated molecular sieves under an argon atmosphere. All the starting materials were purchased from commercial sources and used as received, unless otherwise stated. Liquids and solutions were transferred via syringe or positive-pressure cannula. Brine solutions refer to saturated aqueous sodium chloride solutions. Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} precoated plates (0.25 mm) and visualized by UV fluorescence quenching, anisaldehyde, or $H_3(PMo_{12}O_{40})$ staining. Silica gel 60 N (particle size 63–210 mm) was used for column chromatography. HPLC purifications were

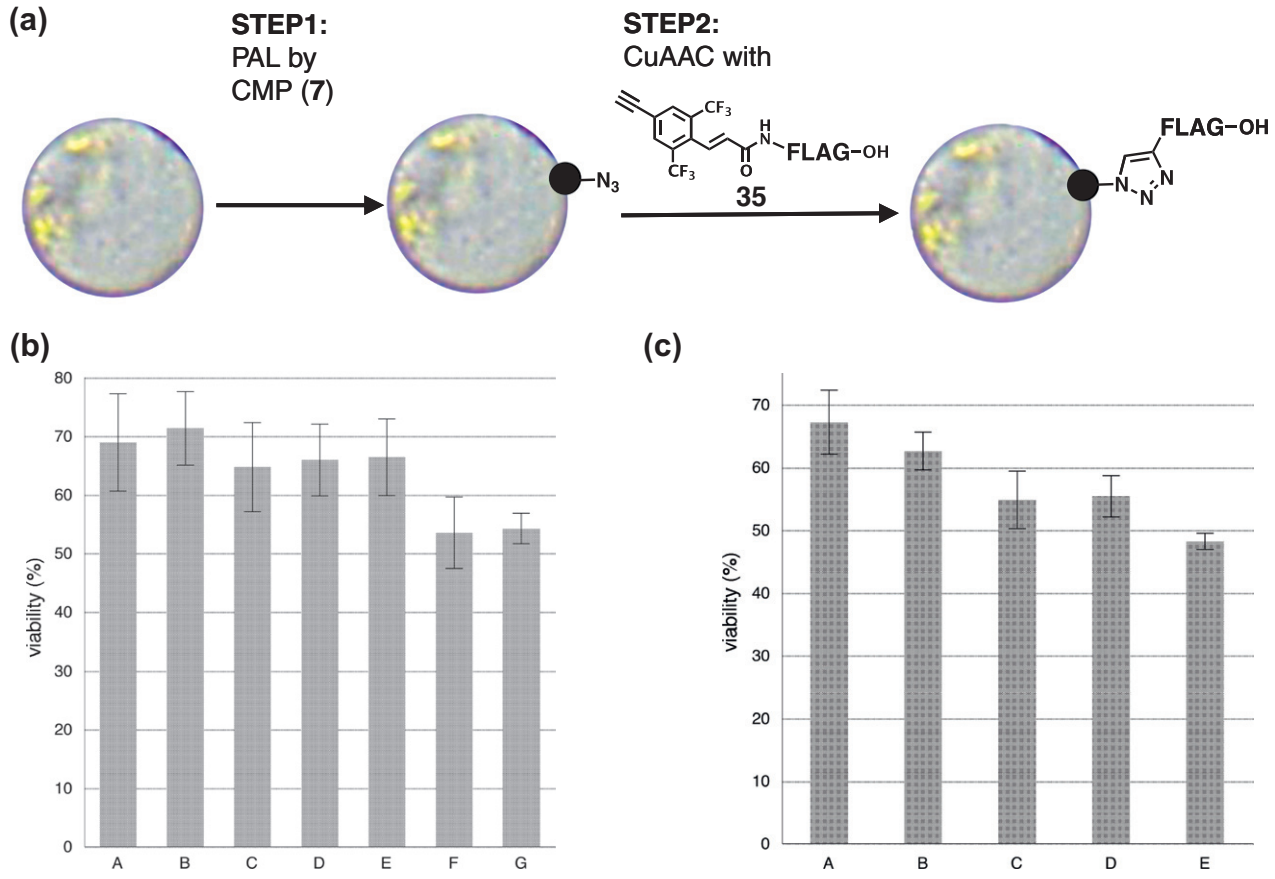


Figure 3. (a) FLAG-tagging by PAL-CuAAC (STEP1 and STEP2) and subsequent fluorescence-labeling by suing anti-FLAG-antibody. Cell viabilities during STEP1 and STEP2 were observed. (b) Viability of protoplasts in STEP1: The viability of prepared protoplasts (= basal) was evaluated by the ratio of alive cells in total cells, and that of each condition was calculated on the basis of the number of alive protoplasts in basal. A: basal, B–D: 'condition 1' (incubation at 4 °C for 5 min, then irradiated with UV light at 4 °C for 20 min), E–G: 'condition 2' (incubation at 4 °C for 20 min, then irradiated with UV light at 4 °C for 5 min). B and E: intact (protoplast with buffer), C and F: control (protoplast in 5% DMSOaq), D and G (protoplast with 500 μM **7** in 5% DMSOaq). The data shows average and SD of quadruplicate individual experiments. (c)Viability of protoplasts in STEP 2: The viability of prepared protoplasts (= basal) was evaluated by the ratio of alive cells in total cells, and that of each condition was calculated on the basis of the number of alive protoplasts in basal. A: basal, B: intact (protoplast with buffer), C: control (protoplast with DMSO), D: condition I (50 μM CuSO4, 250 μM THPTA ligand, 2.5 mM sodium ascorbate, 0.5 mM alkyne-FLAG **35**, 7.5% DMSO), E: condition II (500 μM $CuSO_4$, 500 μM THPTA ligand, 0.5 mM sodium ascorbate, 0.5 mM alkyne-FLAG **35**, 7.5% DMSO). The data shows average and SD of triplicate individual experiments.

carried out using PU-2089 with UV-2075 detector (Jasco Ltd.) equipped with Cosmosil 5C18AR (φ 20 × 250 mm, Nakalai. Tesque Ltd.) at a flow rate of 4.0 mL/min. ^{1}H and ^{13}C NMR spectra were recorded on ECA400 and Alpha 500 spectrometers (Jeol Ltd.). Chemical shifts are given in parts per million (ppm) relative to Me_4Si (0.0 ppm). Data for ^{1}H NMR spectra are reported as follows: chemical shift in ppm (integration, multiplicity, coupling constant in Hz). Multiplicity and qualifier abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. IR spectra were recorded on FT/IR-410 spectrometer (Jasco Co., Ltd.) and are reported in frequency of absorption (cm $^{-1}$). LR and HR MS were recorded on an ESI-mode by using MicrOTOF-II and APEX-III spectrometers (Bruker Daltonics Ltd.). Optical rotation was recorded on a DIP-360 spectrometer using 100-mm cell.

4.1. Compound 10, 11 and 12

Compound **9**[27] (2.12 g, 7.79 mmol) in dehydrated MeCN (39 mL) was added to a mixture of CAN (15.84 g, 28.9 mmol) and NaN_3 (763 mg, 11.7 mmol) at −15 °C under N_2. After being stirred for 7 h at −15 °C, the reaction mixture was diluted with cold Et_2O and H_2O, extracted with Et_2O. The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The obtained crude mixture (2.81 g, containing **10**, **11** and **12** was subjected to the next reaction without further purification.

4.2. Compound 13

The crude mixture of **10**, **11** and **12** (2.76 g) in AcOH (18 mL) was added NaOAc (1.20 g, 14.6 mmol) at RT under N_2. After being stirred for 3 h at 100 °C, the reaction mixture was cooled to 0 °C, and diluted with $CHCl_3$ and sat. $NaHCO_3$ aq, extracted with $CHCl_3$. The combined organic layer was washed with sat. $NaHCO_3$ aq and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography (*n*-hexane/EtOAc = 4/1–3/1) gave **13** (α: β = 7: 4, 1.46 g, 3.91 mmol, 51% in 2 steps) as a colorless viscous oil.

13-α, ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.33 (d, *J* = 3.6 Hz, 1 H, 1-H), 5.48 (dd, *J* = 3.2 Hz, 1.2 Hz, 1 H, 4-H), 5.32 (dd, *J* = 11.2 Hz, 3.2 Hz, 1 H, 3-H), 4.29 (td, *J* = 6.8 Hz, 1.2 Hz, 1 H, 5-H), 4.07 (dd, *J* = 11.2 Hz, 6.8 Hz, 1 H, 6-H_a), 4.03 (dd, *J* = 11.2 Hz, 6.8 Hz, 1 H, 6-H_b), 3.94 (dd, *J* = 11.2 Hz, 3.6 Hz, 1 H, 2-H), 2.18 (s, 3 H, 1-OAc), 2.17 (s, 3 H, 4-OAc), 2.08 (s, 3 H, 3-OAc), 2.04 (s, 3 H, 6-OAc); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (1 C, 6-$OCOCH_3$), 169.9 (1 C, 4-$OCOCH_3$), 169.7 (1 C, 3-$OCOCH_3$), 168.6 (1 C, 1-$OCOCH_3$), 90.4 (1 C, 1-C), 68.7 (1 C, 3-C), 68.7 (1 C, 5-C), 66.9 (1 C, 4-C), 61.0 (1 C, 6-C), 56.9 (1 C,

2-C), 20.8 (1 C, 1-OCOCH$_3$), 20.5(3 C, 3, 4, 6-OCOCH$_3$); IR (film) 2964, 2938, 2116, 1754, 1434, 1372, 1217, 1133, 1110, 1077, 1045, 1013, 938, 899, 762; HRMS (ESI, positive) *m/z* [M+Na]$^+$ calcd for $C_{14}H_{19}N_3O_9Na$ 396.1019, found 396.1017. HR MS was observed as a mixture of α- and β- isomers.

13-β, ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.55 (d, J = 8.4 Hz, 1 H, 1-H), 5.38 (dd, J = 3.2 Hz, 0.8 Hz, 1 H, 4-H), 4.90 (dd, J = 10.4 Hz, 3.2 Hz, 1 H, 3-H), 4.12 (dd, J = 11.2 Hz, 6.8 Hz, 1 H, 6-H$_a$), 4.07 (dd, J = 11.2 Hz, 6.8 Hz, 1 H, 6-H$_b$), 4.02 (td, J = 6.8 Hz, 0.8 Hz, 1 H, 5-H), 3.84 (dd, J = 10.4 Hz, 8.4 Hz, 1 H, 2-H), 2.21 (s, 3 H, 1-OAc), 2.17 (s, 3 H, 4-OAc), 2.07 (s, 3 H, 3-OAc), 2.04 (s, 3 H, 6-OAc); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (1 C, 6-OCOCH$_3$), 169.8 (1 C, 4-OCOCH$_3$), 169.5 (1 C, 3-OCOCH$_3$), 168.4 (1 C, 1-OCOCH$_3$), 92.8 (1 C, 1-C), 71.7 (1 C, 5-C), 71.3 (1 C, 3-C), 66.2 (1 C, 4-C), 60.9 (1 C, 6-C), 59.7 (1 C, 2-C), 20.8(1 C, 1-OCOCH$_3$), 20.5(3 C, 3, 4, 6-OCOCH$_3$); IR (film) 2964, 2938, 2116, 1754, 1434, 1372, 1217, 1133, 1110, 1077, 1045, 1013, 938, 899, 762.

4.3. Compound 14

To a solution of **13** (1.46 g, 3.91 mmol) in MeOH (39 mL) were added 10% w/w Pd/C (416.8 mg, 0.392 mmol) and 10 M HCl aq (0.43 mL, 4.3 mmol) at 0 °C. After being stirred for 1 h at RT under H_2, the reaction mixture was filtered through a pad of Celite and concentrated in vacuo. Resultant crude amine **14** (1.37 g) was subjected to the next reaction without further purification.

4.4. Compound 15

To a suspension of **14** (1.37 g) and acid chloride (2.41 g, 12.1 mmol) in dehydrated THF (71.5 mL) was added NEt_3 (3.9 mL, 28.0 mmol) at 0 °C under N_2. After the reaction mixture was stirred for 1 h at RT, DBU (1.17 mL, 7.84 mmol) was added to the reaction mixture at 0 °C. After being stirred for 8 h at 40 °C, the reaction mixture was cooled to 0 °C, diluted with $CHCl_3$, quenched with sat. $NaHCO_3$ aq and extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography (*n*-hexane/EtOAc = 3/1–2/1–1/1) gave **15** (α:β = 2:1, 1.64 g, 3.44 mmol, 88% in 2 steps) as a colorless viscous oil.

15-α ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88–7.83 (m, 2 H, 2-NPhth), 7.78–7.74 (m, 2 H, 2-NPhth), 6.53 (dd, J = 12.0 Hz, 3.2 Hz, 1 H, 3-H), 6.34 (d, J = 3.2 Hz, 1 H, 1-H), 5.67 (dd, J = 3.2 Hz, 1.2 Hz, 1 H, 4-H), 4.91 (dd, J = 12.4 Hz, 3.2 Hz, 1 H, 2-H), 4.50 (td, J = 6.8 Hz, 1.2 Hz, 1 H, 5-H), 4.17 (dd, J = 11.2 Hz, 6.8 Hz, 1 H, 6-H$_a$), 4.13 (dd, J = 11.2 Hz, 6.8 Hz, 1 H, 6-H$_b$), 2.19 (s, 3 H, 4-OAc), 2. 06 (s, 6 H, 1, 6-OAc), 1.89 (s, 3 H, 3-OAc); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3 (1 C, 6-OCOCH$_3$), 170.1 (1 C, 4-OCOCH$_3$), 169.4 (2 C, 1, 3-OCOCH$_3$), 167.7 (1 C, 2-N (CO)$_2$C$_6$H$_4$), 167.5 (1 C, 2-N(CO)$_2$C$_6$H$_4$), 134.4 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 131.3 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 123.5 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 91.2 (1 C, 1-C), 69.2 (1 C, 5-C), 67.0 (1 C, 4-C), 64.6 (1 C, 3-C), 61.3 (1 C, 6-C), 49.5 (1 C, 2-C), 20.9 (1 C, 1 or 6-OCOCH$_3$), 20.6 (2 C, 1 or 6, 4-OCOCH$_3$), 20.5 (1 C, 3-OCOCH$_3$); IR (film) 3026, 1752, 1721, 1636, 1614, 1540, 1469, 1434, 1387, 1220, 1173, 1134, 1117, 1074, 1044, 1013, 948, 756, 723; HRMS (ESI, positive) *m/z* [M+Na]$^+$ calcd for $C_{22}H_{23}N_1O_{11}Na$ 500.1169, found 500.1155. HR MS was observed as a mixture of α- and β- isomers.

15-β ^{1}H NMR (400 MHz, $CDCl_3$) δ7.88–7.83 (m, 2 H, 2-NPhth), 7.78–7.74 (m, 2 H, 2-NPhth), 6.45 (d, J = 8.8 Hz, 1 H, 1-H), 5.94 (dd, J = 11.6 Hz, 3.2 Hz, 1 H, 3-H), 5.52 (d, J = 3.6 Hz, 1 H, 4-H), 4.67 (dd, J = 11.6 Hz, 8.8 Hz, 1 H, 2-H), 4.26-4.17 (m,3 H, 5, 6-H), 2.22 (s, 3 H, 4-OAc), 2.07 (s, 3 H, 6-OAc), 2.01 (s, 3 H, 1-OAc), 1.86 (s, 3 H, 3-OAc); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3 (1 C, 6-OCOCH$_3$), 170.1 (1 C, 4-OCOCH$_3$), 169.5 (1 C, 3-OCOCH$_3$), 168.6 (1 C, 1-OCOCH$_3$), 167.7 (1 C, 2-N (CO)$_2$C$_6$H$_4$), 167.5 (1 C, 2-N(CO)$_2$C$_6$H$_4$), 134.4 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 131.3 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 123.7 (2 C, 2-N(CO)$_2$C$_6$H$_4$), 90.3 (1 C, 1-C), 71.8 (1 C, 5-C), 67.7 (1 C, 3-C), 66.5 (1 C, 4-C), 61.2 (1 C, 6-C), 50.3 (1 C, 2-C), 20.7 (2 C, 1, 4-C), 20.6 (1 C, 6-C), 20.4 (1 C, 3-C); IR (film) 3026, 1752, 1721, 1636, 1614, 1540, 1469, 1434, 1387, 1220, 1173, 1134, 1117, 1074, 1044, 1013, 948, 756, 723.

4.5. Compound 16

To a solution of **15** (675.8 mg, 1.42 mmol) in Ac_2O (266 μL) was added 33% HBr-AcOH (7.1 mL) at 0 °C. The reaction mixture was diluted with $CHCl_3$, quenched with sat. $NaHCO_3$ aq and extracted with $CHCl_3$. The combined organic layer was washed with sat. $NaHCO_3$ aq and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Resultant bromide **16** (695.7 mg) was subjected to the next reaction without further purification.

4.6. Compound 18

To a suspension of crude **16** (695.7 mg), **17**[11] (203.0 mg, 0.72 mmol), Ag_2CO_3 (476.5 mg, 1.73 mmol) and MS4Å (2.03 g) in dehydrated CH_2Cl_2 (7.2 mL) was slowly added AgOTf (221.7 mg, 0.86 mmol) in dehydrated toluene (7.2 mL) at 0 °C under N_2. After being stirred for 1 h at RT in the dark, the reaction mixture was filtered through a pad of Celite. The filtrate was washed with sat. $NaHCO_3$ aq and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography (toluene/acetone = 50/1–40/1–30/1–20/1–15/1–10/1) gave **18** (478.7 mg, 0.68 mmol, 95%) as a pale yellow viscous oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (br, 2 H), 7.76 (dd, J = 6.0 Hz, 2.8 Hz, 2 H), 5.79 (dd, J = 11.6 Hz, 3.2 Hz, 1 H), 5.48 (dd, J = 3.2 Hz, 0.4 Hz, 1 H), 5.32 (d, J = 8.8 Hz, 1 H), 5.19-5.13 (m, 1 H), 5.06–5.00 (m, 1 H), 4.53 (dd, J = 11.6 Hz, 8.4 Hz, 1 H), 4.23 (dd, J = 11.2 Hz, 6.8 Hz, 1 H), 4.19 (dd, J = 11.2 Hz, 6.8 Hz, 1 H), 4.09 (td, J = 6.8 Hz, 0.8 Hz, 1 H), 3.89 (dt, J = 9.6 Hz, 6.4 Hz, 1 H), 3.43 (ddd, J = 9.6 Hz, 7.6 Hz, 6.8 Hz, 1 H), 2.50 (dd, J = 20.0 Hz, 8.0 Hz, 1 H), 2.35–1.98 (m, 15 H), 1.86 (s, 3 H), 1.79–1.74 (m, 1 H), 1.48–1.43 (m, 1 H), 1.45 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 218.8, 171.3, 170.3, 170.3, 169.8, 168.2, 167.4, 134.2 (2 C), 131.4 (2 C), 127.8, 127.2, 123.6, 123.4, 98.6, 80.6, 70.7, 69.5, 68.0, 66.7, 61.3, 53.7, 51.3, 40.1, 38.0, 37.6, 28.0 (3 C), 27.5, 26.9, 25.2, 20.7, 20.6, 20.5; IR (film) 2977, 2933, 1776, 1750, 1718, 1389, 1369, 1239, 1154, 1131, 1107, 1076, 1047, 1017, 952, 761, 723; $[\alpha]_D^{26}$ + 38.4 (*c* 1, MeOH); HRMS (ESI, positive) *m/z* [M+Na]$^+$ calcd for $C_{36}H_{45}N_1O_{13}Na$ 722.2789, found 722.2770.

4.7. Compound 19

To a solution of **18** (457.8 mg, 0.65 mmol) in MeOH (13 mL) was added NaOMe (38.9 mg, 0.72 mmol) at 0 °C under N_2. After being stirred for 1 h at 0 °C, the reaction mixture was neutralized with Amberlite IR120B, filtered and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 50/1–25/1–10/1) gave **19** (330.2 mg, 0.58 mmol, 88%) as a colorless viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.88–7.83 (m, 2 H), 7.81 (dd, J = 5.6 Hz, 3.2 Hz, 2 H), 5.19–4.95 (m, 2 H), 5.12 (d, J = 8.0 Hz, 1 H), 4.42 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 4.33 (dd, J = 11.2 Hz, 8.4 Hz, 1 H), 3.95 (dd, J = 2.8 Hz, 0.8 Hz, 1 H), 3.86 (dt, J = 10.0 Hz, 6.0 Hz, 1 H), 3.82 (dd, J = 11.2 Hz, 6.8 Hz, 1 H), 3.78 (dd, J = 11.2 Hz, 5.6 Hz, 1 H), 3.65 (ddd, J = 6.8 Hz, 5.6 Hz, 0.8 Hz, 1 H), 3.42 (ddd, J = 9.6 Hz, 7.6 Hz, 6.0 Hz, 1 H), 2.50 (dd, J = 14.4 Hz, 3.2 Hz, 1 H), 2.31–1.96 (m, 9 H), 1.82–1.77 (m, 1 H), 1.50–1.44 (m, 1 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.3, 169.8, 135.5 (2 C), 133.2, 133.2, 128.9, 128.8, 124.4, 124.0, 100.4, 81.8, 77.0, 70.2, 70.0, 69.6, 62.5, 55.5, 55.0, 41.1, 39.3, 38.5, 28.7, 28.4 (3 C), 28.0, 26.2; IR (film) 3484, 2955, 1714, 1391, 1365, 1177,

1153, 1078, 722; $[\alpha]_D^{26}$ + 39.5 (*c* 0.5, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{30}H_{39}N_1O_{10}Na$ 596.2472, found 596.2466.

4.8. Compound 20

To a solution of **19** (303.8 mg, 0.53 mmol) in dehydrated CH_2Cl_2 (10.6 mL) were added NEt_3 (177 μL, 1.27 mmol) and TsCl (121.0 mg, 0.63 mmol) at 0 °C under N_2. After being slowly warmed from 0 °C to RT, the reaction mixture was quenched with MeOH and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 100/1–50/1–20/1–10/1) gave **20** (297.5 mg, 0.41 mmol, 77%, 94% brsm) as a colorless viscous oil. The start material **19** (53.2 mg, 0.093 mmol, 17%) was recovered.

^{1}H NMR (400 MHz, CD_3OD) δ 7.83–7.79 (m, 6 H), 7.46 (d, *J* = 8.0 Hz, 2 H), 5.18–4.98 (m, 2 H), 5.05 (d, *J* = 8.4 Hz, 1 H), 4.38 (dd, *J* = 11.2 Hz, 3.2 Hz, 1 H), 4.27 (dd, *J* = 11.2 Hz, 8.4 Hz, 1 H), 4.27 (dd, *J* = 10.8 Hz, 4.8 Hz, 1 H), 4.23 (dd, *J* = 10.8 Hz, 6.8 Hz, 1 H), 3.87–3.84 (m, 2 H), 3.72 (dt, *J* = 9.6 Hz, 6.0 Hz, 1 H), 3.36 (ddd, *J* = 9.6 Hz, 7.2 Hz, 6.4 Hz, 1 H), 2.51 (dd, *J* = 15.2 Hz, 3.2 Hz, 1 H), 2.46 (s, 3 H), 2.31–1.97 (m, 9 H), 1.84–1.79 (m, 1 H), 1.53–1.46 (m, 1 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.1, 169.7, 146.6, 135.5 (2 C), 134.3, 133.1 (2 C), 131.2 (2 C), 129.1 (2 C), 128.9, 128.8, 124.4, 124.0, 100.2, 81.8, 74.1, 70.8, 70.3, 69.7, 69.1, 55.2, 54.9, 41.1, 39.3, 38.5, 28.7, 28.4 (3 C), 28.0, 26.2, 21.7; IR (film) 3484, 2955, 1714, 1391, 1365, 1177, 1153, 976, 722; $[\alpha]_D^{25}$ + 39.4 (*c* 0.5, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{37}H_{45}N_1O_{12}SNa$ 750.2560, found 750.2551.

4.9. Compound 21

To a solution of **20** (12.2 mg, 0.017 mmol) in DMF (0.34 mL) was added NaN_3 (10.8 mg, 0.17 mmol) at RT under N_2. After being stirred for 6 h at 100 °C, the reaction mixture was cooled to RT, diluted with H_2O and extracted with EtOAc. The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 300/1–100/1–50/1) gave **21** (9.6 mg, 0.016 mmol, 96%) as a pale yellow viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.89–7.83 (m, 2 H), 7.81 (dd, *J* = 5.2 Hz, 3.2 Hz, 2 H), 5.20–4.98 (m, 2 H), 5.16 (d, *J* = 8.4 Hz, 1 H), 4.44 (dd, *J* = 11.2 Hz, 3.2 Hz, 1 H), 4.34 (dd, *J* = 10.8 Hz, 8.4 Hz, 1 H), 3.89–3.82 (m, 3 H), 3.72 (dd, *J* = 12.8 Hz, 8.4 Hz, 1 H), 3.44 (ddd, *J* = 9.6 Hz, 7.6 Hz, 6.0 Hz, 1 H), 3.29 (dd, *J* = 12.8 Hz, 3.6 Hz, 1 H), 2.51 (dd, *J* = 14.4 Hz, 3.2 Hz, 1 H), 2.32–1.99 (m, 9 H), 1.84–1.78 (m, 1 H), 1.48–1.44 (m, 1 H), 1.44 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.5, 173.4, 170.2, 169.8, 135.5 (2 C), 133.2, 133.1, 128.9, 128.8, 124.4, 124.0, 100.3, 81.8, 76.2, 70.5, 70.2, 69.3, 55.3, 55.0, 52.5, 41.1, 39.3, 38.5, 28.7, 28.4 (3 C), 28.0, 26.2; IR (film) 3465, 3006, 2977, 2933, 2101, 1774, 1714, 1391, 1368, 1335, 1280, 1257, 1154, 1116, 1072, 883, 793, 757, 722; $[\alpha]_D^{27}$ + 51.8 (*c* 0.5, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{30}H_{38}N_4O_9Na$ 621.2536, found 621.2530.

4.10. Compound 22

0.8 M Ethylenediamine in MeOH (1.1 mL) was added to **21** (30.3 mg, 0.51 mmol) at RT under N_2. After being stirred for 26 h at 40 °C, the reaction mixture was cooled to 0 °C, quenched with AcOH (0.1 mL), diluted with EtOAc and sat. $NaHCO_3$ aq, extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Crude amine **22** (24.0 mg) was obtained as a pale yellow viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 5.54–5.39 (m, 2 H), 4.22 (d, *J* = 8.0 Hz, 1 H), 3.90 (dt, *J* = 9.6 Hz, 6.8 Hz, 1 H), 3.69–3.62 (m, 3 H), 3.55 (dt, *J* = 9.6 Hz, 6.8 Hz, 1 H), 3.40 (dd, *J* = 10.4 Hz, 3.2 Hz, 1 H), 3.21 (dd, *J* = 10.4 Hz, 1.2 Hz, 1 H), 2.90 (dd, *J* = 10.4 Hz, 8.0 Hz, 1 H), 2.65–2.58 (m, 1 H), 2.46–2.04 (m, 9 H), 1.99–1.94 (m, 1 H), 1.58–1.48 (m, 1 H), 1.46 (s, 9 H).

4.11. Compound 23

To a suspension of **22** (24 mg) and MS4Å (130.0 mg) in dehydrated DMF (0.4 mL) was added Cs_2CO_3 (25.2 mg, 0.077 mmol) at RT under an Ar atmosphere. After being stirred for 1.5 h at RT, 4-(bromomethyl)trifluoromethyldiazirine[24] (11.8 mg, 0.042 mmol) was added at 0 °C in dark. After being stirred for further 3 h at RT in dark, the reaction mixture was cooled to 0 °C, quenched with AcOH (3 d), filtered through a pad of Celite and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 1/0–100/1–80/1–60/1–40/1–20/1–10/1–5/1) gave **23** (14.2 mg, 0.021 mmol, 42% in two steps, 76% brsm) as a pale yellow viscous oil. The start material **22** (10.7 mg, 0.023 mmol, 45%) was recovered.

^{1}H NMR (500 MHz, CD_3OD) δ 7.47 (d, *J* = 8.5 Hz, 2 H), 7.21 (d, *J* = 8.5 Hz, 2 H), 5.54–5.39 (m, 2 H), 4.33 (d, *J* = 8.0 Hz, 1 H), 4.10 (d, *J* = 13.5 Hz, 1 H), 3.98–3.93 (m, 2 H), 3.67–3.60 (m, 3 H), 3.55 (dt, *J* = 9.5 Hz, 7.0 Hz, 1 H), 3.43 (dd, *J* = 10.5 Hz, 3.0 Hz, 1 H), 3.19 (dd, *J* = 11.5 Hz, 2.5 Hz, 1 H), 2.73 (dd, *J* = 10.5 Hz, 8.0 Hz, 1 H), 2.59 (dd, *J* = 13.5 Hz, 3.0 Hz, 1 H), 2.47–2.14 (m, 8 H), 2.09–2.02 (m, 1 H), 1.97–1.93 (m, 1 H), 1.55–1.46 (m, 1 H), 1.44 (s, 9 H); ^{13}C NMR (125 MHz, CD_3OD) δ 221.4, 173.4, 143.8, 130.4 (2 C), 129.4, 129.1, 128.6, 127.7 (2 C), 123.7 (q, *J* = 274 Hz), 105.9, 81.8, 75.9, 73.5, 70.1, 70.0, 59.9, 55.1, 53.4, 52.6, 41.2, 39.5, 38.5, 29.5 (q, *J* = 40 Hz), 29.1, 28.4 (3 C), 28.0, 26.5; IR (film) 3433, 2972, 2931, 2878, 2100, 1730, 1613, 1519, 1457, 1368, 1343, 1233, 1155, 1080, 1054, 938, 881, 844, 812, 739; $[\alpha]_D^{24}$ + 32.4 (*c* 0.3, MeOH); HRMS (ESI, positive) *m/z* $[M+H]^+$ calcd for $C_{31}H_{42}F_3N_6O_7$ 667.3067, found 667.3061.

4.12. Compound *ent*-7

TFA (0.5 mL) was added to **23** (14.2 mg, 0.021 mmol) at 0 °C in dark. After being stirring for 1 h at RT in dark, the reaction mixture was concentrated in vacuo. Purification by HPLC (38% MeCN aq containing 0.1% TFA, UV 227 nm) gave *ent*-**7** (12.1 mg, 0.020 mmol, 93%) as a pale yellow viscous oil.

^{1}H NMR (500 MHz, CD_3OD) δ 7.61 (d, *J* = 8.5 Hz, 2 H), 7.36 (d, *J* = 8.0 Hz, 2 H), 5.53–5.45 (m, 2 H), 4.73 (d, *J* = 8.5 Hz, 1 H), 4.47 (d, *J* = 13.5 Hz, 1 H), 4.43 (d, *J* = 13.5 Hz, 1 H), 3.97 (dt, *J* = 9.5 Hz, 6.7 Hz, 1 H), 3.83 (dd, *J* = 11.0 Hz, 3.0 Hz, 1 H), 3.78 (dd, *J* = 3.0 Hz, 1.0 Hz, 1 H), 3.74 (ddd, *J* = 8.5 Hz, 4.0 Hz, 1.0 Hz, 1 H), 3.65 (dt, *J* = 9.5 Hz, 6.7 Hz, 1 H), 3.64 (dd, *J* = 13.0 Hz, 8.5 Hz, 1 H), 3.24 (dd, *J* = 13.0 Hz, 4.0 Hz, 1 H), 3.16 (dd, *J* = 11.0 Hz, 8.5 Hz, 1 H), 2.65 (dd, *J* = 14.5 Hz, 3.5 Hz, 1 H), 2.56–2.19 (m, 8 H), 2.09–1.99 (m, 2 H), 1.57–1.49 (m, 1 H); ^{13}C NMR (125 MHz, CD_3OD) δ 221.7, 176.0, 134.3, 132.3 (2 C), 131.4, 129.6, 128.8, 128.4 (2 C), 123.5 (q, *J* = 274 Hz), 100.3, 76.1, 70.3, 70.3, 69.8, 59.6, 55.1, 52.2, 51.1, 39.7, 39.2, 38.6, 29.4 (q, *J* = 40 Hz), 28.7, 28.2, 26.4; IR (film) 3390, 3018, 2935, 2894, 2103, 1733, 1677, 1523, 1429, 1411, 1347, 1232, 1189, 1145, 1058, 940, 884, 840, 801, 759, 723; $[\alpha]_D^{22}$ + 17.0 (*c* 0.2, MeOH); HRMS (ESI, positive) *m/z* $[M+H]^+$ calcd for $C_{27}H_{34}F_3N_6O_7$ 611.2441, found 611.2442.

4.13. Compound 24

To a suspension of *ent*-**17**[11] (43.6 mg, 0.15 mmol), **16** (142.5 mg, 0.29 mmol), Ag_2CO_3 (105.2 mg, 0.38 mmol) and MS4Å (440.0 mg) in dehydrated CH_2Cl_2 (1.55 mL) was slowly added AgOTf (47.6 mg, 0.19 mmol) in dehydrated toluene (7.2 mL) at 0 °C under N_2. After being stirred for 1 h at RT in the dark, the reaction mixture was filtered through a pad of Celite. The filtrate was washed with sat. $NaHCO_3$ aq and brine, dried over Na_2SO_4, filtered and concentrated in

vacuo. Purification by silica gel column chromatography (toluene/acetone = 50/1–40/1–30/1–20/1–15/1–10/1) gave **24** (86.5 mg, 0.12 mmol, 80%, 93% brsm) as a pale yellow viscous oil. The starting material *ent*-**17** (6 mg, 0.02 mmol, 14%) was recovered.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (br, 2 H), 7.76 (dd, J = 5.6 Hz, 3.2 Hz, 2 H), 5.78 (dd, J = 11.4 Hz, 3.0 Hz, 1 H), 5.48 (dd, J = 3.4 Hz, 0.6 Hz, 1 H), 5.32 (d, J = 8.8 Hz, 1 H), 5.20–5.14 (m, 1 H), 5.08–5.02 (m, 1 H), 4.53 (dd, J = 11.2 Hz, 8.4 Hz, 1 H), 4.24 (dd, J = 11.2 Hz, 6.4 Hz, 1 H), 4.19 (dd, J = 11.2 Hz, 6.8 Hz, 1 H), 4.09 (ddd, J = 6.8 Hz, 6.4 Hz, 0.8 Hz, 1 H), 3.89 (dt, J = 9.6 Hz, 6.4 Hz, 1 H), 3.43 (ddd, J = 9.6 Hz, 7.6 Hz, 6.8 Hz, 1 H), 2.50 (dd, J = 18.8 Hz, 8.0 Hz, 1 H), 2.35–1.99 (m, 15 H), 1.86 (s, 3 H), 1.79–1.74 (m, 1 H), 1.47–1.40 (m, 1 H), 1.45 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 218.8, 171.3, 170.4, 170.3, 169.8, 168.2, 167.4, 134.3, 131.5, 129.0, 128.2, 127.9, 127.2, 123.6, 123.5, 98.6, 80.7, 70.8, 69.6, 68.1, 66.7, 61.4, 53.8, 51.4, 40.1, 38.1, 37.6, 28.1 (3 C), 27.5, 26.9, 25.3, 20.7, 20.7, 20.5; IR (film) 3478, 3016, 2975, 2933, 2895, 1776, 1750, 1723, 1469, 1431, 1389, 1370, 1338, 1239, 1216, 1154, 1131, 1107, 1073, 1047, 1016, 952, 915, 876, 844, 760, 722, 633, 531; $[\alpha]_D^{20}$ − 14.3 (c 1, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{36}H_{45}N_1O_{13}Na$ 722.2789, found 722.2788.

4.14. Compound 25

To a solution of **24** (83.1 mg, 0.12 mmol) in MeOH (2.4 mL) was added NaOMe (7.1 mg, 0.13 mmol) at 0 °C under N_2. After being stirred for 1.9 h at 0 °C, the reaction mixture was neutralized with Amberlite IR120B, filtered and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 50/1–20/1–10/1) gave **25** (62.4 mg, 0.11 mmol, 92%) as a colorless viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.88–7.83 (m, 2 H), 7.81 (dd, J = 5.6 Hz, 3.2 Hz, 2 H), 5.19–5.14 (m, 1 H), 5.11 (d, J = 8.0 Hz, 1 H), 5.01–4.95 (m, 1 H), 4.41 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 4.35 (dd, J = 11.2 Hz, 8.0 Hz, 1 H), 3.95 (dd, J = 2.8 Hz, 0.8 Hz, 1 H), 3.87 (dt, J = 10.0 Hz, 6.0 Hz, 1 H), 3.82 (dd, J = 11.2 Hz, 6.8 Hz, 1 H), 3.78 (dd, J = 11.2 Hz, 5.6 Hz, 1 H), 3.65 (ddd, J = 6.8 Hz, 5.6 Hz, 0.8 Hz, 1 H), 3.42 (ddd, J = 9.6 Hz, 7.2 Hz, 6.4 Hz, 1 H), 2.49 (dd, J = 14.0 Hz, 3.2 Hz, 1 H), 2.30–1.97 (m, 9 H), 1.82–1.77 (m, 1 H), 1.50–1.40 (m, 1 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.2, 169.8, 135.5, 135.4, 133.2, 133.1, 128.8 (2 C), 124.3, 124.0, 100.4, 81.8, 77.0, 70.2, 70.0, 69.6, 62.5, 55.5, 54.9, 41.1, 39.3, 38.5, 28.7, 28.4 (3 C), 27.9, 26.2; IR (film) 3473, 3006, 2972, 2939, 2900, 1773, 1713, 1391, 1368, 1337, 1256, 1154, 1115, 1073, 757, 723; $[\alpha]_D^{22}$–25.3 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{30}H_{39}N_1O_{10}Na$ 596.2472, found 596.2463.

4.15. Compound 26

To a solution of **25** (62.4 mg, 0.11 mmol) in dehydrated CH_2Cl_2 (2.2 mL) were added NEt_3 (36.4 μL, 0.26 mmol) and TsCl (24.9 mg, 0.13 mmol) at 0 °C under an Ar atmosphere. After being stirred for 11 h when it was slowly warmed from 0 °C to RT, the reaction mixture was quenched with MeOH and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 100/1–50/1–20/1–10/1) gave **26** (59.4 mg, 0.082 mmol, 75%, 90% brsm) as a colorless viscous oil. The starting material **25** (10.3 mg, 0.018 mmol 17%) was recovered.

^{1}H NMR (400 MHz, CD_3OD) δ 7.86–7.79 (m, 6 H), 7.45 (d, J = 8.0 Hz, 2 H), 5.18–5.10 (m, 1 H), 5.05 (d, J = 8.4 Hz 1 H), 5.05–4.98 (m, 1 H), 4.37 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 4.28 (dd, J = 11.2 Hz, 8.4 Hz, 1 H), 4.28 (dd, J = 10.4 Hz, 4.8 Hz, 1 H), 4.24 (dd, J = 10.4 Hz, 7.6 Hz, 1 H), 3.87–3.84 (m, 2 H), 3.73 (dt, J = 10.0 Hz, 6.0 Hz, 1 H), 3.36 (ddd, J = 9.6 Hz, 7.2 Hz, 6.4 Hz, 1 H), 2.50 (dd, J = 13.6 Hz, 2.8 Hz, 1 H), 2.46 (s, 3 H), 2.30–1.98 (m, 9 H), 1.83–1.78 (m, 1 H), 1.50–1.45 (m, 1 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.1, 169.7, 146.6, 135.5 (2 C), 134.3, 133.1 (2 C), 131.2 (2 C), 129.1 (2 C), 128.9, 128.7, 124.3, 124.0, 100.2, 81.8, 74.1, 70.8, 70.3, 69.7, 69.1, 55.2, 54.9, 41.1, 39.3, 38.5, 28.7, 28.4 (3 C), 27.9, 26.3, 21.6; IR (film) 3478, 3009, 2970, 2929, 1774, 1714, 1391, 1366, 1177, 1152, 1087, 979, 836, 816, 754, 722, 663, 554, 532; $[\alpha]_D^{24}$ − 11.7 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{37}H_{45}N_1O_{12}SNa$ 750.2560, found 750.2564.

4.16. Compound 27

To a solution of **26** (61.5 mg, 0.084 mmol) in DMF (1.7 mL) was added NaN_3 (55.1 mg, 0.85 mmol) at RT under N_2. After being stirred for 7 h at 100 °C, the reaction mixture was cooled to RT, diluted with H_2O and extracted with EtOAc. The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 100/0–100/1–50/1–20/1) gave **27** (50.0 mg, 0.84 mmol, 99%) as a colorless viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.88–7.84 (m, 2 H), 7.81 (dd, J = 5.2 Hz, 3.2 Hz, 2 H), 5.20–5.14 (m, 1 H), 5.15 (d, J = 8.4 Hz, 1 H), 5.04–4.97 (m, 1 H), 4.43 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 4.34 (dd, J = 10.8 Hz, 8.4 Hz, 1 H), 3.89–3.82 (m, 3 H), 3.71 (dd, J = 12.8 Hz, 8.4 Hz, 1 H), 3.44 (ddd, J = 10.0 Hz, 7.2 Hz, 6.0 Hz, 1 H), 3.29 (dd, J = 12.8 Hz, 4.0 Hz, 1 H), 2.50 (dd, J = 14.0 Hz, 3.2 Hz, 1 H), 2.30–1.98 (m, 9 H), 1.83–1.78 (m, 1 H), 1.51–1.42 (m, 1 H), 1.45 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.2, 169.7, 135.5, 135.5, 133.2, 133.1, 128.9, 128.8, 124.3, 124.0, 100.3, 81.8, 76.2, 70.5, 70.2, 69.3, 55.3, 54.9, 52.5, 41.1, 39.4, 38.5, 28.6, 28.4 (3 C), 27.9, 26.3; IR (film) 3461, 3011, 2977, 2933, 2889, 2101, 1774, 1714, 1391, 1368, 1335, 1280, 1257, 1153, 1115, 1072, 883, 793, 756, 722; $[\alpha]_D^{21}$ − 0.2 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{30}H_{38}N_4O_9Na$ 621.2536, found 621.2521.

4.17. Compound 28

0.8 M Ethylenediamine in MeOH (2 mL) was added to **27** (50.0 mg, 0.84 mmol) at RT under N_2. After being stirred for 40 h at 40 °C, the reaction mixture was cooled to 0 °C, quenched with AcOH (0.2 mL), diluted with EtOAc and sat. $NaHCO_3$ aq extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Crude amine **28** (36.3 mg) was obtained as an yellow viscous oil.

4.18. Compound 29

To a suspension of **28** (36.3 mg) and MS4Å (200.0 mg) in dehydrated DMF (0.7 mL) was added Cs_2CO_3 (30 mg, 0.092 mmol) at RT under an Ar atmosphere. Then, 4-(bromomethyl)trifluoromethyldiazirine (22.0 mg, 0.079 mmol) in dehydrated DMF (0.7 mL) was added at 0 °C in dark. After being stirred for 10 h at RT in dark, the reaction mixture was cooled to 0 °C, quenched with AcOH (0.2 mL), filtered through a pad of Celite and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 1/0–100/1–80/1–60/1–40/1–20/1–10/1–5/1) gave **29** (25.0 mg, mixture) as a brown viscous oil. The starting material **28** (15.3 mg, 0.033 mmol, 42%) was recovered.

4.19. Compound 8

TFA (1.5 mL) was added to **29** (25.0 mg, mixture) at 0 °C in dark. After being stirring for 2 h at RT in dark, the reaction mixture was concentrated in vacuo. Purification by HPLC (38% MeCN aq

containing 0.1% TFA, UV 227 nm) gave **8** (9.0 mg, 0.015 mmol, 18% in 3 steps, 29% brsm) as a pale yellow viscous oil.

^{1}H NMR (500 MHz, CD_3OD) δ 7.61 (d, J = 8.5 Hz, 2 H), 7.35 (d, J = 8.0 Hz, 2 H), 5.53–5.45 (m, 2 H), 4.74 (d, J = 8.5 Hz, 1 H), 4.48 (d, J = 13.0 Hz, 1 H), 4.43 (d, J = 13.0 Hz, 1 H), 3.97 (dt, J = 9.0 Hz, 7.0 Hz, 1 H), 3.84 (dd, J = 11.0 Hz, 3.0 Hz, 1 H), 3.77 (dd, J = 3 Hz, 0.5 Hz, 1 H), 3.75 (ddd, J = 8.5 Hz, 4.0 Hz, 1.0 Hz, 1 H), 3.67 (dt, J = 9.0 Hz, 7.0 Hz, 1 H), 3.64 (dd, J = 13.0 Hz, 8.5 Hz, 1 H), 3.24 (dd, J = 13.0 Hz, 4.0 Hz, 1 H), 3.16 (dd, J = 11.0 Hz, 8.5 Hz, 1 H), 2.65 (dd, J = 14.0 Hz, 3.5 Hz, 1 H), 2.55–2.27 (m, 7 H), 2.24–2.18 (m, 1 H), 2.08–1.97 (m, 2 H), 1.57–1.48 (m, 1 H); ^{13}C NMR (125 MHz, CD_3OD) δ 221.6, 176.0, 134.3, 132.3 (2 C), 131.4, 129.6, 128.9, 128.4 (2 C), 123.5 (q, J = 274 Hz), 100.3, 76.2, 70.3, 70.3, 69.8, 59.6, 55.1, 52.2, 51.1, 39.7, 39.2, 38.6, 29.4 (q, J = 40 Hz), 28.9, 28.2, 26.5; IR (film) 3356, 3019, 2960, 2938, 2895, 2103, 1732, 1671, 1436, 1347, 1232, 1190, 1153, 1058, 940, 839, 799, 759, 722; $[\alpha]_D^{23}$ − 16.9 (c 0.5, MeOH); HRMS (ESI, positive) m/z [M+H]$^+$ calcd for $C_{27}H_{34}F_3N_6O_7$ 611.2441, found 611.2415.

4.20. Compound 31

To a suspension of **17** (92.2 mg, 0.33 mmol), **30**[24] (339.8 mg, 0.71 mmol), Ag_2CO_3 (260.0 mg, 0.94 mmol) and MS4Å (1.2 g) in dehydrated CH_2Cl_2 (3.5 mL) was slowly added AgOTf (114 mg, 0.44 mmol) in dehydrated toluene (3.5 mL) at 0 °C under N_2. After being stirred for 0.5 h at RT in the dark, the reaction mixture was filtered through a pad of Celite. The filtrate was washed with sat. $NaHCO_3$ aq and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel column chromatography (toluene/acetone = 50/1–40/1–30/1–20/1–15/1–10/1) gave **31** (212.6 mg, 0.31 mmol, 95%) as a pale colorless viscous oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (br, 2 H), 7.76 (dd, J = 5.2 Hz, 3.2 Hz, 2 H), 5.77 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 5.43 (dd, J = 3.2 Hz, 0.4 Hz, 1 H), 5.34 (d, J = 8.8 Hz, 1 H), 5.17 (dt, J = 10.8, 7.2 Hz, 1 H), 5.06 (dt, J = 10.4, 7.6 Hz, 1 H), 4.54 (dd, J = 11.2, 8.8 Hz, 1 H), 4.03 (ddd, J = 8.4, 4.0, 0.4 Hz, 1 H), 3.93 (dt, J = 9.6, 6.4 Hz, 1 H), 3.61 (dd, J = 12.8, 8.0 Hz, 1 H), 3.46 (dt, J = 9.6, 7.2 Hz, 1 H), 3.17 (dd, J = 12.8, 4.0 Hz, 1 H), 2.50 (dd, J = 18.8 Hz, 8.4 Hz, 1 H), 2.35–1.99 (m, 12 H), 1.85 (s, 3 H), 1.79–1.74 (m, 1 H), 1.49–1.41 (m, 1 H), 1.45 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 218.8, 171.3, 170.2, 169.7, 168.2, 167.4, 134.2 (2 C), 131.4 (2 C), 127.9, 127.1, 123.5, 123.4, 98.5, 80.6, 72.9, 69.5, 68.0, 67.6, 53.7, 51.2, 50.6, 40.0, 38.0, 37.5, 28.0 (3 C), 27.4, 26.8, 25.3, 20.7, 20.4; IR (film) 3484, 3013, 2977, 2935, 2891, 2013, 1777, 1752, 1711, 1388, 1368, 1237, 1154, 1123, 1107, 1069, 1052, 1011, 952, 760, 722; $[\alpha]_D^{25}$ + 8.4 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{34}H_{42}N_4O_{11}Na$ 705.2748, found 705.2722.

4.21. Compound 32

To a solution of **31** (201.8 mg, 0.30 mmol) in MeOH (6.0 mL) was added NaOMe (16.0 mg, 0.30 mmol) at 0 °C under N_2. After being stirred for 1.8 h at 0 °C, the reaction mixture was neutralized with Amberlite IR120B, filtered and concentrated in vacuo. Purification by silica gel column chromatography (n-Hexane/EtOAc = 5/1–4/1–3/1–2/1–1/1) gave **32** (149.6 mg, 0.25 mmol, 85%) as a colorless viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.88–7.84 (m, 2 H), 7.81 (dd, J = 5.6 Hz, 3.2 Hz, 2 H), 5.20–5.13 (m, 1 H), 5.15 (d, J = 8.4 Hz, 1 H), 5.04–4.97 (m, 1 H), 4.43 (dd, J = 11.2 Hz, 3.2 Hz, 1 H), 4.34 (dd, J = 11.2 Hz, 8.4 Hz, 1 H), 3.89–3.82 (m, 3 H), 3.71 (dd, J = 12.8 Hz, 8.0 Hz, 1 H), 3.42 (td, J = 10.0 Hz, 6.8 Hz, 1 H), 3.29 (dd, J = 12.8 Hz, 3.6 Hz, 1 H), 2.50 (dd, J = 13.6 Hz, 3.2 Hz, 1 H), 2.30–1.98 (m, 9 H), 1.83–1.78 (m, 1 H), 1.51–1.40 (m, 1 H), 1.44 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.4, 173.4, 170.2, 169.7, 135.5, 135.5, 133.2, 133.1, 128.9, 128.8, 124.3, 124.0, 100.2, 81.8, 76.2, 70.5, 70.2, 69.3, 55.3, 54.9, 52.5, 41.1, 39.3, 38.5, 28.6, 28.4, 27.9, 26.3; IR (film) 3464, 3011, 2977, 2933, 2889, 2101, 1774, 1714, 1391, 1368, 1335, 1280, 1257, 1153, 1115, 1073, 883, 793, 758, 722; $[\alpha]_D^{23}$ + 2.0 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+Na]$^+$ calcd for $C_{30}H_{38}N_4O_9Na$ 621.2536, found 621.2523.

4.22. Compound 33

0.8 M Ethylenediamine in MeOH (3.2 mL) was added to **32** (96.4 mg, 0.16 mmol) at RT under N_2. After being stirred for 40 h at 40 °C, the reaction mixture was cooled to 0 °C, quenched with AcOH (0.3 mL), diluted with EtOAc and sat. $NaHCO_3$ aq, extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. Crude amine **33** (71.0 mg) was obtained as a yellow viscous oil.

4.23. Compound 34

To a suspension of **33** (57.7 mg) and MS4Å (620.0 mg) in dehydrated DMF (1.2 mL) was added Cs_2CO_3 (61.0 mg, 0.18 mmol) at RT under an Ar atmosphere. Then, 4-(bromomethyl)trifluoromethyldiazirine (33.0 mg, 0.12 mmol) was added at 0 °C in dark. After being stirred for 25 h at RT in dark, the reaction mixture was cooled to 0 °C, quenched with AcOH (4 d), filtered through a pad of Celite and concentrated in vacuo. Purification by silica gel column chromatography ($CHCl_3$/MeOH = 1/0–100/1–80/1–60/1–40/1–20/1–10/1–5/1) gave **34** (28.2 mg, mixture) as a yellow viscous oil. The starting material **33** (28.3 mg, 0.060 mmol, 49%) was recovered.

4.24. Compound *ent*-8

TFA (1.0 mL) was added to **35** (28.2 mg, mixture) at 0 °C in dark. After being stirring for 2 h at RT in dark, the reaction mixture was concentrated in vacuo. Purification by HPLC (35% MeCN aq containing 0.1% TFA, UV 227 nm) gave *ent*-**8** (14.1 mg, 0.023 mmol, 18% in 3 steps) as a pale yellow viscous oil.

^{1}H NMR (400 MHz, CD_3OD) δ 7.61 (d, J = 8.0 Hz, 2 H), 7.35 (d, J = 8.4 Hz, 2 H), 5.54–5.44 (m, 2 H), 4.74 (d, J = 8.0 Hz, 1 H), 4.48 (d, J = 13.2 Hz, 1 H), 4.43 (d, J = 13.2 Hz, 1 H), 3.98 (dt, J = 9.2 Hz, 6.8 Hz, 1 H), 3.84 (dd, J = 11.2, 3.2 Hz, 1 H), 3.78 (dd, J = 3.2, 0.8 Hz, 1 H), 3.75 (ddd, J = 8.8, 3.6 Hz, 0.8 Hz, 1 H), 3.66 (dt, J = 9.2, 6.8 Hz, 1 H), 3.64 (dd, J = 12.8, 8.8 Hz, 1 H), 3.24 (dd, J = 12.8, 4.0 Hz, 1 H), 3.15 (dd, J = 11.2, 8.0 Hz, 1 H), 2.65 (dd, J = 14.0 Hz, 3.2 Hz, 1 H), 2.54–2.26 (m, 7 H), 2.24–2.18 (m, 1 H), 2.08–1.96 (m, 2 H), 1.57–1.47 (m, 1 H); ^{13}C NMR (100 MHz, CD_3OD) δ 221.6, 175.9, 134.4, 132.3, 131.4, 129.6, 128.9, 128.4, 123.5 (q, J = 273 Hz), 100.3, 76.1, 70.3, 70.3, 69.8, 59.6, 55.1, 52.3, 51.1, 39.8, 39.2, 38.6, 29.4 (q, J = 40 Hz), 28.9, 28.2, 26.5; IR (film) 3383, 3017, 2933, 2895, 2103, 1732, 1673, 1408, 1347, 1232, 1188, 1154, 1058, 940, 845, 800, 759, 721; $[\alpha]_D^{22}$ + 17.0 (c 1.0, MeOH); HRMS (ESI, positive) m/z [M+H]$^+$ calcd for $C_{27}H_{34}F_3N_6O_7$ 611.2441, found 611.2431.

4.25. Cell-shrinking assay using motor cell protoplasts of *S. saman*

The protoplasts were prepared from *S. saman* using the extensor (adaxial) part of the tertiary pulvini on the 2nd or 3rd branch from the short apex according to the previously reported method (Gorton and Satter, 1984; Moran et al., 1990; Nakamura et al., 2008). They were suspended in a wash solution (0.57 M sorbitol, 10 mM KCl, 1 mM $CaCl_2$, 20 mM MES-Tris, pH 5.5), loaded on a histopaque solution (0.5 M sorbitol, 2 mM $CaCl_2$, 90 v/v%), and centrifuged at 1500×g for 5 min. The purified protoplasts found in the middle

green layer were collected, suspended in wash buffer, centrifuged at 110×*g* for 5 min,and the washing step was then repeated. The freshly prepared protoplasts were suspended in 1.5 mL wash buffer and maintained on ice in the dark. The protoplasts were centrifuged at 110×*g* for 5 min and resuspended in a small volume of wash buffer before use.

The prepared protoplasts in 50 μL wash solution were sealed in a glass-bottom petri dish (φ 35 mm × 12 mm) coated with 300 μL of Milli Q, placed under an inverted microscope (IX-71, Olympus, Tokyo, Japan), and monitored at 24 ± 1 °C under continuous irradiation with light (50 μmol m^{-2} s^{-1} PAR) passed through a green filter (43IF550-W45, Olympus, Tokyo, Japan), as previously reported.[8]

Following incubation on the microscope for 6 min, 50 μL of a solution of the different compounds (each at 1×10^{-8} mol in wash solution, azide compounds include an additional 2% DMSO) was added to the protoplast suspension. In the experiment using a K^+ channel blocker, the protoplasts were incubated with 1 mM tetraethyleneammonium chloride (TEA) for 30 min before exposure to **7** or JAG. The status of the protoplasts was recorded with time-lapse photography (10 min intervals) for 40 min using a digital camera (DP 72, Olympus, Tokyo, Japan) and DP2-BSW analysis software (DP 72, Olympus, Tokyo, Japan). Finally, 100 μL of 0.02% fluorescein diacetate (FDA) was added to enable the selection of living protoplasts. The semidiameter for each magnified image of living protoplasts was measured by DP2-BSW analysis software (DP 72, Olympus, Tokyo, Japan).

4.26. Viability of protoplasts under the UV irradiation condition

A suspension of protoplasts was prepared at the concentration of 5×10^5 cells/mL in buffer A (20 mM MES-Tris (pH 5.5), 0.57 M D-sorbitol, 10 mM KCl, and 1 mM $CaCl_2$). A solution of probe **8** in DMSO (10 mM, 1 μL) was added to 19 μL of the protoplast suspension. The mixture was incubated under different conditions, as follows. Condition 1: the mixture was incubated at 4 °C for 5 min, and then, irradiated with black light at 4 °C for 20 min. Condition 2: the mixture was incubated at 4 °C for 20 min, and then, irradiated with black light at 4 °C for 5 min. After irradiation under each condition, living protoplasts were counted under a microscope after staining with 20 μL of 0.4% (w/v) Trypan Blue solution (Wako Pure Chemical Industries, Ltd.). The probe solution was replaced with DMSO or buffer A for the control or intact condition, respectively. The basal viability of the prepared protoplasts was calculated from the ratio of living cells to total cells. Then, the viability for each condition was calculated from the ratio of the number of living protoplasts under a given condition to that under basal conditions. Fig. 3 shows the average and SD of Individual experiments performed in quadruplicate.

4.27. Viability of protoplasts under the CuAAC condition

A suspension of protoplasts was prepared at the concentration of 5×10^5 cells/mL in buffer A (20 mM MES-Tris (pH 5.5), 0.57 M D-sorbitol, 10 mM KCl, 1 mM $CaCl_2$). The suspension was incubated at 4 °C for 30 min under different conditions, as follows. Condition 1 was based on the reports by Finn et al.[35,36] and included the following reagents: 50 μM $CuSO_4$, 250 μM THPTA ligand, 2.5 mM sodium ascorbate, 0.5 mM alkyne-FLAG **35**, 7.5% DMSO. Condition II was based on our previous report[13] and included the following reagents: 500 μM $CuSO_4$, 500 μM THPTA ligand, 0.5 mM sodium ascorbate, 0.5 mM alkyne-FLAG **35**, 7.5% DMSO. The effectiveness of each condition was calculated from the number of living protoplasts in the same manner as described in 'viability of protoplasts under the UV irradiation condition.' Fig. 3 shows the average and SD of individual experiments performed in triplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.003.

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