Structural Requirements of Strigolactones for Shoot Branching Inhibition in Rice and Arabidopsis

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The structural requirements of strigolactones (SLs) involved in germination induction of root parasitic plants and hyphal branching in arbuscular mycorrhizal (AM) fungi have been extensively studied. However, our knowledge of the requirements of SLs involved in shoot branching inhibition in plants is still limited. To address this question, we investigated the structure–activity relationships of SLs in shoot branching inhibition in rice and Arabidopsis. SLs possess a four-ring structure, with a tricyclic lactone (ABC-rings) connected to a methylbutenolide part (D-ring) via an enol ether bridge. Here, we show that the (2R) configuration at C-2, which determines the steric position of the D-ring relative to the enol ether olefin bond, is critical for the hormonal activity in rice. Replacement of the enol ether moiety by an alkoxyl or imino ether resulted in a severe reduction in bioactivity in rice. Moreover, yeast two-hybrid experiments using a possible SL receptor, DWARF14 (D14), and a repressor in the SL signaling pathway, DWARF53 (D53), showed that D14 can interact with D53 in the presence of (2’R) stereoisomers of SLs, but not (2’S) stereoisomers, suggesting that the stereosstructure of SLs is crucial for the interaction of these proteins. When GR5, an AB-ring-truncated analog, was applied to the hydroponic culture medium, strong inhibition of shoot branching was observed both in rice and in Arabidopsis. However, GR5 was only weakly active when directly applied to the axillary buds of Arabidopsis. Our results indicate that the difference in plant species and application methods greatly influences the apparent SL biological activity.

Keywords: Arabidopsis thaliana • Oryza sativa • Stereoisomer • Strigolactone.

Abbreviations: AM fungi, arbuscular mycorrhizal fungi; Atd14, Arabidopsis DWARF14 ortholog; CCD, carotenoid cleavage dioxygenase; CD, circular dichroism; CL, carlactone; D, dwarf; DAD, decreased apical dominance; 3’,4’-dhDS5; 3’,4’-dihydroDS5, 3’, 4’-dihyGR3s, 3’, 4’-dihyGR3s, 3’, 6’-dihyGR24, 3,6’-dihydroGR24; DMF, dimethylformamide; 5DS, 5-deoxystrigol; HMB, hydroxymethylbutenolide; HTL, hypoositive to light; KAI2, karrikin insensitive 2; KAR, karrikin; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MAX, more axillary growth; NMR, nuclear magnetic resonance; RMS, ramosus; SL, strigolactone; SMXL, SMAX1-LIKE; THF, tetrahydrofuran; TLC, thin-layer chromatography; WT, wild type; Y2H, yeast two-hybrid.

Introduction

Strigolactones (SLs) are a group of terpenoid lactones that are synthesized from carotenoids. A natural SL, (+)-strigol, was initially found from cotton root exudates as a seed germination stimulant for a root parasitic plant, Striga lutea (Cook et al. 1966, Cook et al. 1972). Studies have shown that plants generally produce multiple SL species and release them from their roots and induce seed germination of Striga and Phelipanche species (for reviews, see Yoneyama et al. 2009, Xie et al. 2010). SLs also function as host recognition signals for arbuscular mycorrhizal (AM) fungi, which assist the uptake of inorganic nutrients by the host plants (Akiyama et al. 2005). Besides their roles in the rhizosphere, SLs act as plant hormones or their biosynthetic precursors that inhibit shoot branching in plants (Gomez-Roldan et al. 2008, Umehara et al. 2008).

The effect of SLs on shoot branching inhibition was discovered from studies using a series of enhanced shoot branching mutants, including ramosus (rms) of pea, more axillary growth (max) of Arabidopsis and dwarf (d) mutants of rice. Cloning of these genetic loci revealed that RMS5/MAX3/D17 and RMS1/MAX4/D10 genes encode carotenoid cleavage dioxygenase 7 (CCD7) and CCD8, respectively (Sorefan et al. 2003, Booker et al. 2004, Foo et al. 2005, Johnson et al. 2006, Arite et al. 2007, Umehara et al. 2008). SL levels in ccd7 and ccd8 mutants of pea, Arabidopsis and rice were significantly reduced, and application of natural or synthetic SLs inhibited shoot branching (Gomez-Roldan et al. 2008, Umehara et al. 2008). The D27 gene encodes an iron-containing protein (Lin et al. 2009) and was recently shown to catalyze the reversible conversion of all-trans-β-carotene into 9-cis-β-carotene, which is cleaved by...
CCD7 to produce 9-cis-\(\beta\)-apo-10'-carotene (Alder et al. 2012). CCD8 incorporates three oxygens into 9-cis-\(\beta\)-apo-10'-carotene, and produces carlactone (CL), a compound that carries the D-ring part of SL and exhibits SL-like biological activities (Alder et al. 2012). CL was identified from plant tissues, and was shown to be converted into SLs in rice plants (Seto et al. 2014). These results demonstrate that CL is an endogenous biosynthetic precursor for SLs. MAX1 was demonstrated to convert CL into a carboxylated derivative, carlactonic acid (Abe et al. 2014). In contrast to SL biosynthetic mutants, the \(rms4/mx2/d3\) mutants are defective in an F-box protein (Stirmberg et al. 2002, Ishikawa et al. 2005, Johnson et al. 2006) and are insensitive to SL treatment (Gomez-Roldan et al. 2008, Umehara et al. 2008). d14 is another SL-insensitive mutant in rice (Arite et al. 2009). The DWARF 14 (AtD14) and decreased apical dominance2 (DADD2) genes have been identified from Arabidopsis and petunia, respectively, as orthologs of D14 (Hamiaux et al. 2012, Waters et al. 2012). D14 family genes encode proteins that belong to the \(\alpha/\beta\)-fold hydrolase superfamily (Arite et al. 2009). Moreover, biochemical functional analysis of D14 family proteins suggests that they are involved in SL perception (Hamiaux et al. 2012, Kagiya et al. 2013, Nakamura et al. 2013, Zhao et al. 2013). More recently, a repressor protein in the SL signaling pathway was identified using a dominant SL-insensitive mutant of rice, d53 (Jiang et al. 2013, Zhou et al. 2013). D53 encodes a protein with weak homology to heat shock protein 101 in the Clp ATPase family. D53 protein was shown to interact with D14 protein in an SL-dependent manner. Furthermore, D53 was found to be degraded through the 26S proteasome pathway in an F-box protein (D3)-dependent manner (Jiang et al. 2013, Zhou et al. 2013). These results suggest that D14 associates with D53 and D3 proteins and mediates SL signaling for shoot branching inhibition.

Various natural SLs have been identified from root exudates, and a number of SL analogs have been chemically synthesized so far. In particular, the structure–activity relationships of SLs have been extensively studied for the induction of seed germination of root parasitic plants (for reviews, see Yoneyama et al. 2009, Zwanenburg et al. 2009). SLs consist of a tricyclic lactone (ABC-ring) and a hydroxymethyl butenolide (HMB, D-ring), connected through an enol ether bond (Fig. 1A). The D-ring portion is a common part in all SLs active on root parasitic plants, suggesting that this part of the SL structure may be important for biological activity. In fact, modification of the D-ring reduces SL bioactivity for the induction of root parasitic seed germination (Mangnus et al. 1992). In contrast, many natural SLs have a chemically modified ABC-ring. From their structure–activity data, the bioactiphore of SLs for the induction of germination of root parasitic plants was found to reside in the C–D-ring part of the molecule (Mangnus and Zwanenburg 1992b). The stereochemistry of SLs is also important for biological activity on root parasitic plants. For example, in the case of GR24 and sorgolactone, the stereoisomer whose configurations at position C-3a, C-8b and C-2' are (R), (S) and (R), respectively, is the most active (Thuring et al. 1997, Sugimoto et al. 1998). The structural requirements of SLs for AM fungi have also been studied in detail (Akiyama et al. 2010). The natural stereoisomer showed higher activity in hyphal branching of AM fungi. To induce hyphal branching of AM fungi, the C-ring of the tricyclic lactone and the D-ring must be connected, but the enol ether was not essential and could be replaced by an alkoxyl ether. When the A-ring or AB-rings were truncated, the resulting analogs (GR7 and GR5, respectively) exhibited drastically reduced hyphal branching-inducing activity.

Recently, SL structural requirements for shoot branching inhibition were studied in pea using a variety of natural SLs and SL analogs (Boyer et al. 2012). The results showed that the presence of both an \(\alpha/\beta\)-unsaturated system and a methylbutenolide as the D-ring in the same molecule is essential for biological activity in pea. Moreover, the stereochemistry at C-2' is not an important structural feature for pea shoot branching (Boyer et al. 2012). In contrast, the stereoisomers which have (2'R) configuration were more active in Arabidopsis shoot branching inhibition (Scaffoldi et al. 2014). In rice, (−)-ent-2'-epi-GR7 showed stronger activity for the inhibition of tiller bud outgrowth than its 2'-epimer, suggesting that the stereochemistry at C-2' is also important in rice (Nakamura et al. 2013). However, the structural requirements of SLs in rice and Arabidopsis have not been fully investigated. In the present study, we used an SL-deficient rice mutant, d10, to evaluate the effect of about 30 compounds including natural and synthetic SLs on shoot branching inhibition, and determined some structural features necessary for the hormonal activity. Some of these compounds were also applied to the Arabidopsis SL-deficient mutant \(\text{max}4\) in order to compare the results between these two plant species. Moreover, we evaluated the effects of some of these compounds on the induction of the interaction between D14 and D53, which are the possible SL receptor complex components, as examined by yeast two-hybrid (Y2H) experiments. Based on these results, we discuss the structural requirements of SLs for shoot branching inhibition in rice and Arabidopsis.

Results
Effect of SL stereoisomers on shoot branching inhibition
To examine the inhibitory effect of test compounds on tiller bud outgrowth, we pre-cultured wild-type (WT), d3 and d10 seedlings on agar media and then grew them in hydroponic culture medium containing the test compounds for 1 week. In our experimental conditions, we can observe the outgrowth of the first and second leaf tillers in the \(d\) mutants, but not in the WT. We regarded tiller buds \(>2\) mm as growing out, because dormant tiller buds are \(<2\) mm in size in our growth conditions (Umehara et al. 2008, Umehara et al. 2010). We scored the number of first and second leaf tillers that grew out in the \(d10\) mutant to evaluate the biological activity. We included WT and d3 seedlings in order to make sure that tiller bud outgrowth does not occur in the WT and that the test
Fig. 1 Effect of SL stereoisomers. (A) 5DS (1–4), (B) orobanchol (5–8), (C) GR24 (9–12), (D) GR7 (13–16) and (E) GR5 (17, 18). Each chemical was added to the hydroponic solution. The number of outgrowing tillers (>2 mm) per plant in six d10 seedlings is shown as the mean ± SD (n = 3).
compounds inhibited tiller bud outgrowth specifically in the D3-dependent SL signaling pathway, respectively (Supplementary Fig. S1).

We first examined the effect of stereochemistry using natural and synthetic SLs. As mentioned above, (2'R) isomers of GR24 and 5-deoxystrigol (SDS) showed stronger shoot branch-inhibiting activities compared with (2'S) isomers in Arabidopsis (Scaffidi et al. 2014). In rice, among four stereoisomers of SDS (compounds 1–4; Fig. 1A), (+)-SDS (1) and (-)-ent-2'-epi-SDS (4), both of which have the (2'R) configuration, showed stronger activity in inhibiting d10 tiller bud outgrowth (Fig. 1A), consistent with the result in Arabidopsis. Similar trends were observed for stereoisomers of orobanchol (compounds 5–8; Fig. 1B) and GR24 (compounds 9–12; Fig. 1C). In contrast to the effect of the C-2' configuration, the difference in the configurations at C-3a and C-8b did not affect the inhibition of tiller bud outgrowth very much.

GR7 and GR5 are A-ring- and AB-ring-truncated SL analogs, respectively. (GR7, compounds 13–16; GR5, compounds 17, 18; Fig. 1D, E), and they were reported as active chemicals for the induction of seed germination of root parasitic plants (Johnson et al. 1976, Johnson et al. 1981). For both SL analogs, (2'R) stereoisomers showed stronger activities in the inhibition of d10 tiller bud outgrowth, as was the case with other SLs having four rings (Fig. 1D, E). In particular, a clear difference in the biological activity between the (2'R) and (2'S) isomers of GR5 (Fig. 1E) suggests that the C-2' configuration is a critical structural feature in determining the shoot branching inhibition activity in rice.

**Effect of ABC-ring modification**

To determine which part of the SL molecule is important for shoot branching inhibition activity in rice, we examined the bioactivity of truncated or modified SLs. As mentioned above, we found that the (2'R) isomers of the A-ring-truncated analog GR7 and the AB-ring-truncated analog GR5 were fairly active in our assay. In addition, GR7 and GR5 strongly stimulated seed germination of non-parasitic plants, such as lettuce and wild oat (Bradow et al. 1988, Bradow et al. 1990), while these truncated analogs were not as strong as GR24 in stimulating seed germination of root parasitic plants (Johnson et al. 1976, Johnson et al. 1981, Mangus and Zwanenburg 1992a). To compare the effect of deletions of the A- and AB-rings directly, we carried out a dose–response analysis of (+)-GR24, (+)-GR7 and (+)-GR5 in more detail. Among these analogs, (+)-GR5 showed the strongest inhibitory activity, while GR24, possessing a four-ring structure like natural SLs, exhibited the weakest activity (Fig. 2A). Thus, the inhibition of tiller bud outgrowth tended to be more potent for analogs with a smaller number of ABC-rings. In contrast, the opposite trend was observed for the germination stimulation of root parasitic plants, including *Striga hermonthica* and *Orobanche minor* (Fig. 2B, C) and for the induction of hyphal branching of AM fungi (Akiyama et al. 2010).

Our results showed that the AB-rings are dispensable for the inhibition of tiller bud outgrowth in rice. Both d3 and d14 mutant seedlings were insensitive to GR5 (Supplementary Figs. S1, S2), suggesting that GR5, consisting of only the C–D part, still acted in the SL signaling pathway. In an effort to determine the minimal SL structure necessary for the inhibition of tiller bud outgrowth in rice, we synthesized some GR5 analogs as racemic mixtures whose C-ring is cleaved (Fig. 3A, compounds 19–21). In all cases, they were nearly equally as active as (±)-GR5, indicating that the C-ring does not have to be intact to exhibit potent activity in rice (Fig. 3B). In contrast, rac-3',4'-dihydroGR5 (3',4'-dihydroGR5, compound 22, Fig. 3A), in which the D-ring double bond (C-3',4') is reduced to a single bond, displayed drastically reduced activity. This result suggests that the C-3',4' double bond is important for the inhibition of tiller bud outgrowth in rice (Fig. 3C). A similar analog prepared from rac-(±)-SDS, rac-(±)-3',4'-dihydroSDS, also did not show shoot branching inhibition activity (3',4'-dihydroSDS, compound 23, Supplementary Fig. S3).
outgrowing tillers (Fig. 3B–D). The number of 2nd leaf tillers per plant was added to the hydroponic solution. (B–D) The number of 2nd leaf tillers per plant was added to the hydroponic solution. (B–D) The number of 2nd leaf tillers per plant.

**Importance of the enol ether bridge**

The enol ether bridge connecting the C- and D-rings is a common part in natural SLs, and has been identified as an important structure for stimulation of seed germination of root parasitic plants (Mangnus and Zwanenburg 1992b, Zwanenburg et al. 2009), while the enol ether could be replaced by an alkoxy ether for the induction of hyphal branching of AM fungi (Akiyama et al. 2010). To explore the importance of the enol ether structure in shoot branching inhibition, we synthesized four stereoisomers of 3,6'-dihydrorGR24 (3,6'-dhGR24) (compounds 24–27, Fig. 4A). None of the 3,6'-dhGR24 isomers that we tested inhibited tiller bud outgrowth even at 1 μM (Fig. 4B). Next, we tested the effect of GR24 imino-analogs, which showed weak activity in root parasitic plants and AM fungi (Kondo et al. 2007, Akiyama et al. 2010) (Fig. 4A). (E)- or (Z)-iminoGR24 (Akiyama et al. 2010) exhibited very weak activity in the inhibition of tiller bud outgrowth in rice seedlings (compounds 28, 29, Fig. 4C).

**Effects of SLs and analogs on Arabidopsis shoot branching and hypocotyl elongation**

As described above, GR5 was more effective in inhibiting tiller bud outgrowth of rice than GR24 when applied to the hydroponic solution. We examined whether similar trends were observed in Arabidopsis. We applied racemic (±)-GR24 or (±)-GR5 to the media of hydroponically grown Col-0 (WT), max2 and max4 plants. max2 is an SL-insensitive mutant defective in F-box protein, whereas max4 is an SL-deficient mutant defective in CCD8. Treatment with 0.1 μM GR5 suppressed the outgrowth of axillary shoots nearly completely in max4, but not in max2 (Fig. 5A). GR24 was able to inhibit the outgrowth of axillary shoots of max4 at 5 μM, but not at 0.1 μM (Fig. 5A). These results indicate that GR5 is a more potent inhibitor of axillary bud outgrowth than GR24 in Arabidopsis when supplied to the root (Fig. 5A). However, when these chemicals were applied directly to the axillary buds, GR5 was not as effective as GR24 in inhibiting their outgrowth (Fig. 5B). These results illustrate that GR5 shows stronger activity than GR24 in inhibiting shoot branching when applied to the roots in hydroponic culture both in rice and in Arabidopsis, while GR24 is more active than GR5 when applied to the axillary bud directly in Arabidopsis (Fig. 5B).

The relative strength of GR5 and GR24 activity depended on the application method. This might be due to the difference in the chemical properties between GR5 and GR24, such as their solubility and stability in water, and their membrane permeability. It is also possible that these two chemicals differ in their stability in plants. As a first step to address these questions, we determined the bioactivity of stereoisomers of SDS using two different application methods, because their chemical properties are similar to each other, unlike the case of GR5 and GR24. The results showed that (+)-SDS (1) was the most potent in the inhibition of axillary bud outgrowth and that the relative activities of the four stereoisomers are similar whichever application method was used (Fig. 5C, D). Although (−)-2'-epi-SDS (4) showed relatively weak activity, the (2'R) isomers showed stronger activities than the (2'S) isomers, consistent with reported results (Scaffidi et al. 2014). Therefore, the influence of the application methods on the relative bioactivity between GR5 and GR24 might be due to the difference in their chemical properties.

To examine the effect of chemical modification of the ABC-ring part on shoot branching inhibition in rice, we compared the bioactivity of a racemic mixture of SDS, orobanchol, orobanchyl acetate and their 2'-epimers in our assay. There was no great difference in bioactivity among these compounds (Supplementary Fig. S4). These results indicate that introduction of a hydroxyl group at C-4 and its acetylation do not greatly affect the biological activity of SDS in rice seedlings.
properties, as discussed above. When the biological activity of 

\(+\)-GR5 (17) was compared with that of \(-\)-GR5 (18) by adding 

them to Arabidopsis hydroponic culture media, only \(+\)-GR5, 

but not \(-\)-GR5, was effective in inhibiting the out-
growth of axillary shoots (Fig. 5E). These results further support 
the idea that the stereochemistry at C-2\(_0\) is crucial for the hor-
monal activity in Arabidopsis, as was the case in rice.

In addition to shoot branching inhibition, SLs have been 
shown to inhibit hypocotyl elongation in Arabidopsis (Nelson 
et al. 2011, Waters et al. 2012). SL-dependent regulation of 
hypocotyl elongation is mediated by two \(\alpha/\beta\)-hydrolase 
family proteins, AtD14 and hyposensitive to light (HTL)/karri-
kin insensitive 2 (KAII2) (Sun and Ni 2011, Waters et al. 2012). 
AtD14 is a possible SL receptor in Arabidopsis, while HTL was 
initially characterized by a long hypocotyl phenotype of the 
htl mutant and then by an mutant insensitive to the smoke-
derived germination stimulant, karrikin (KAR). Moreover, HTL 
was predicted to function as a KAR receptor due to the insensi-
tivity of the \(htl\) mutant to KAR (Waters et al. 2012) and 
the in vitro binding activity of recombinant HTL with KAR (Waters 
mediates KAR and some SL signal, AtD14 mediates only SL 
signal (Waters et al. 2012). MAX2, an F-box protein, was re-
ported to be involved in the signaling pathway of both AtD14 
and HTL (Nelson et al. 2011, Waters et al. 2012). In order to 
evaluate the contribution of SLs to the control of hypocotyl 
length through these two pathways, we evaluated the 
stereo-specificity of SLs on hypocotyl elongation using 
Arabidopsis WT (Col-0) and some mutants (\(atd14\), \(htl\), \(atd14 \htl\) and \(max2\)). In the \(atd14\) mutant, HTL can function, while in 
the \(htl\) mutant AtD14 can function; thus, we can evaluate the 
stereo-specificity of SL isomers for each pathway. Very recently, 
Scaffidi et al. (2014) carried out the same experiments using 
four stereoisomers of GR24 and 5DS under different light con-
ditions from ours. They found that \((2\_R)\) isomers are more 
active than \((2\_S)\) isomers for AtD14-dependent control of hypo-
cotyl elongation, whereas only \((\sim)\)-5DS and its GR24 counter-
part were active for HTL-dependent regulation. In agreement 
with this report, \((\sim)\)-5DS and \((\sim)\)-\(2\_epi\)-5DS, both of which 
have the \((2\_R)\) configuration, strongly suppressed hypocotyl 
elongation of the \(htl\) mutant (Supplementary Fig. S5). 
Moreover, \((\sim)\)-GR5 showed much stronger activity than 
\((\sim)\)-GR5, and the contrast between the two isomers was 
clear, as was the case with shoot branching inhibition 
(Supplementary Fig. S5). On the other hand, for the \(atd14\) mutant, all four of the stereoisomers of 5DS showed equally 
weak activities in our bioassay conditions (white light, short 
days), in contrast to the reported results (Supplementary Fig. S5). 
In addition, both \((\sim)\)- and \((\sim)\)-GR5 inhibited hypocotyl 
elongation of the \(atd14\) mutant to the same degree. The \(atd14 \htl\) double mutant was insensitive to all of the tested SLs, sup-
porting the involvement of both AtD14 and HTL in the control 
of SL-dependent hypocotyl elongation. The \(max2\) mutant was 
also insensitive to the tested SLs, supporting the idea that MAX2

Fig. 4 Effect of SL analogs with a modified enol ether bridge. (A) Chemical structures. (B) Effect of stereoisomers of 3,6\(_0\)-dhGR24 (24–27). (C) 
Effect of iminoGR24 regioisomers (28, 29). Each chemical was added to hydroponic solution at 1 \(\mu\)M. Data shows the mean ± SD (\(n=3\)). The numbers of outgrowing tillers (>2 mm) in six \(d10\) seedlings are shown.
is involved in both the AtD14 and HTL pathways. Our results strongly suggest that SLs are stereo-specifically recognized in the AtD14 pathway, whereas there is no stereo-specificity for SLs in the HTL-dependent control of hypocotyl elongation.

Effects of SLs and analogues on D14-D53 interaction

As mentioned above, our SAR studies demonstrated that the (2'R) configuration is important for SL biological activity for shoot branching inhibition in rice and Arabidopsis. One possible interpretation of these results is that (2'R) isomers are preferentially recognized in the SL perception step. D14 is a possible SL receptor protein. It is reported that D14 interacts with D53, a repressor protein in the SL signaling pathway, in an SL-dependent manner, and that the degradation of D53 through the 26S proteasome pathway transduces the SL signal for shoot branching inhibition (Jiang et al. 2013, Zhou et al. 2013). The SL-dependent interaction between D14 and D53 was examined by the Y2H system and pull-down assays in previous reports. To address whether only biologically active isomers of SLs can induce the interaction between D14 and D53, we performed Y2H experiments using D14 and D53 according to the reported method (Zhou et al. 2013). We tested the stereoisomers of GR24 (9–12), GR7 (13–16) and GR5 (17, 18). As a result, D14–D53 interaction was observed only in the presence of (2'R) isomers of these SLs (Fig. 6A). These data conclusively demonstrate that (2'R) isomers of SLs inhibit shoot branching due to their ability to induce receptor complex formation between D14 and D53. We also tested the effects of 3,4′-dhGR5 (22) and 3,6′-dhGR24 (24–27), which are inactive analogs in shoot branching inhibition assays, on the D14–D53 interaction. The results showed that neither of these biologically inactive analogs induced the D14–D53 interaction in Y2H experiments (Fig. 6B).

In Arabidopsis, there are three D53 homologs called SMAX1-LIKE6 (SMXL6), SMXL7 and SMXL8, of which SMXL7 is the most highly expressed (Jiang et al. 2013, Stanga et al. 2013, Zhou et al. 2013). We tested the interaction of AtD14 with these three SMXLs by Y2H experiments and found that SMXL7 can interact with AtD14 strongly in the presence of rac-GR24 compared with SMXL6 and SMXL8, suggesting that SMXL7 is a D53 orthologous gene in Arabidopsis (Supplementary Fig. S6A).
Next, we examined the effects of stereoisomers of some SLs on the AtD14–SMXL7 interaction. In agreement with our bioassay results, (2′R) isomers of SLs could strongly induce the interaction between AtD14 and SMXL7 (Supplementary Fig. S6B).

Stability of SLs in rice hydroponic culture media
To determine whether the stability of the compounds tested affected the apparent bioactivity, we determined how much SLs spontaneously degrade during rice hydroponics using liquid chromatography–tandem mass spectrometry (LC-MS/MS). (+)-5DS, (−)-5DS, (−)-GR24, (+)-epi-3,6′-dhGR24, (+)-GR7 and (+)-GR5 were dissolved in hydroponic culture media and the level of each chemical remained was determined 0, 1, 3 and 7 d after the start of incubation (Supplementary Fig. S7). The levels of (−)-strigol, (+)-GR7 and (+)-GR5 did not change during the incubation. (+)-epi-3,6′-dhGR24, which was inactive in tillering inhibition, and (+)-GR24 decreased approximately 20% in 7 d. Approximately 40% of (−)-5DS and (−)-5DS was degraded during the incubation for 7 d. These results indicated that although some SLs and SL analogs partly degraded during incubation, the major portion of the compounds remained in the media in our growth conditions and there is no drastic difference in the stability of test compounds during the incubation. Therefore, it is likely that the stability of compounds in the media did not greatly affect the apparent biological activity in our current study.

Effects of SLs and analogs on parasitic seeds germination
We evaluated the germination-stimulating activity of some of the tested chemicals using S. hermonthica and O. minor seeds (Supplementary Fig. S8). With regard to the effect of stereoisomers, (2′R) stereoisomers of SLs (5DS, GR24 and GR7) showed stronger activities for O. minor seeds, similarly to shoot branching inhibition in rice (Supplementary Fig. S8B). In contrast, (−)-5DS and its counterpart of GR24 and GR7 showed strong germination-stimulating activity for S. hermonthica (Supplementary Fig. S8A).

Discussion
Structure requirements of SLs in rice and Arabidopsis
Our structure–activity relationship studies using the rice d10 mutants demonstrated that stereoisomers with a (2′R) configuration showed stronger bioactivity than those with a (2′S) configuration in the inhibition of tiller bud outgrowth. In comparison, the stereochemistry at C-3a and C-8b did not affect the hormonal activity very much. This trend was observed for all natural and synthetic SLs that we tested in this study (Fig. 1). The importance of the C-2′ configuration was particularly clear for GR5 both in rice and in Arabidopsis (Figs. 1E, 5E). In Arabidopsis, although the activity of (−)-2′-epi-5DS was weaker compared with the case in rice, (2′R) isomers generally showed stronger activity than (2′S) isomers, consistent with the reported data (Scaffidi et al. 2014). These results indicate that the stereochemistry at C-2′ is crucial for shoot branching inhibition in rice and Arabidopsis, and are in contrast to the data from garden pea, in which the C-2′ stereochemistry had a low effect on shoot branching inhibition activity (Boyer et al. 2012). It is possible that the structural requirements of SLs differ in different plant species. All of the natural SLs reported so far have the (2′R) configuration (Ueno et al. 2011, Xie et al. 2013),
thus it is reasonable that (2'R) isomers show stronger activity than non-natural (2'S) isomers. Among two stereoisomers of CN-debranone, a non-enol ether-type SL analog (Fukui et al. 2011, Fukui et al. 2013), (S)-CN-debranone showed stronger activity than (R)-CN-debranone for shoot branching inhibition in Arabidopsis (Scaffidi et al. 2014). GR5 has a similar molecular size to debranone-type analogs. However, our results clearly show that not (S)-GR5 [(−)-GR5], but rather (R)-GR5 [(+)-GR5] showed stronger activity, as did other SLs both in rice and in Arabidopsis. These results suggest that the (2'R) configuration is critical at least for SLs which have an enol ether-linked D-ring part. In a previous report, qualitative analysis of the hydrolysis reaction by D14 indicated that, when rac-GR24 was used as a substrate, a decrease in the amount of only the (2'R)-isomer, (+)-GR24, was observed, implying the possibility that D14 can recognize only the bioactive stereoisomers (Nakamura et al. 2013).

Furthermore, our Y2H experiments using D14 and D53, a possible SL receptor and its interacting protein, demonstrated that (2'R) stereoisomers of SLs (GR24, GR7 and GR5) can induce their strong interactions. In contrast, 3',6'-dhGR24 and 3',4'- dhGR5 did not induce the D14–D53 interactions. These results are consistent with our bioassay results. We also found by Y2H experiments that SMXL7 is an SL-dependent interacting protein of AtD14, and that the (2'R) isomers of SLs strongly induce their interaction. These results indicate that the (2'R) isomers of SLs show strong shoot branching inhibition activity by inducing formation of the receptor complex.

Truncation of the A- and AB-rings in the tricyclic lactone part increased the biological activity in rice (Fig. 3). The analog GR5, truncated in the AB-ring part, was also highly active in Arabidopsis when supplied to the hydroponic culture media (Fig. 3). These results demonstrate that the AB-rings are dispensable for shoot branching inhibition activity in rice and Arabidopsis. Hydroxylation at C-4 and its acetylation did not drastically influence the inhibition of tiller bud outgrowth (Supplementary Fig. S3). Also, (+)-stiglo, a natural SL with a hydroxy group at C-5, was as active as GR24 in inhibiting tiller bud outgrowth in rice in our previous study (Umehara et al. 2008). Interestingly, C-ring-cleaved GR5 still had strong biological activity (Fig. 4). Together, these results indicate that modification of ABC-rings, as far as we have examined here, does not strongly affect the biological activity in rice.

Our experiments showed that modifications of the enol ether bridge resulted in a severe reduction in biological activity in rice (Fig. 4). Before the discovery of SLs as plant hormones, the enol ether part was predicted to be a possible nucleophile attack site in the receptor protein of root parasitic plants (Humphrey and Beale 2006). Because D14, a possible SL receptor for shoot branching inhibition, is a hydrolase family protein, which in fact has hydrolytic activity with GR24, one possible site of attack of D14 is the enol ether part (C-6') of SL. However, the structure of D14/AtD14/DAD2 proteins demonstrated the presence of an active site serine in the bottom of the pocket, suggesting that the site of attack is not the enol ether bridge, but the D-ring part (Hamiaux et al. 2012, Kagiya et al. 2013, Zhao et al. 2013). Furthermore, some non-enol ether-type analogs were found to have shoot branch-inhibiting activities (Fukui et al. 2011, Fukui et al. 2013, Boyer et al. 2014), suggesting that the enol ether part itself is not necessary for shoot branching-inhibiting activity. Even though the enol ether part is not essential in synthetic chemicals which can mimic SL activities, in the natural type SL structures this part might have an important role, possibly for the interaction with D14, because 3,6'-dhGR24 could not induce the interaction between D14 and D53 in our Y2H experiments (Fig. 6B).

We prepared two α,β-saturated D-ring type analogs, 3',4'- dhGR5 and 3',4'-dhDS, both of which showed drastically reduced activity for shoot branching inhibition in rice (Fig. 3; Supplementary Fig. S3). The same type of analogs prepared from GR24 and GR7 also showed very weak activity in pea and rice, respectively (Boyer et al. 2012, Nakamura et al. 2013). We also found that 3',4'-dhGR5 could not induce the interaction between D14 and D53 (Fig. 6), implying that this double bond might be important for the interaction with D14 protein. Moreover, the importance of the methyl group at the C-4' position was demonstrated for shoot branching inhibition in both rice and pea (Boyer et al. 2012, Fukui et al. 2013). On the other hand, the insertion of an additional methyl group at the C-3' position increased the activity in pea (Boyer et al. 2012). Taken together, these findings show that the D-ring structure, in particular the α,β-unaturated system, is critical for shoot branching inhibition activity. After the hydrolysis reaction by D14 with rac-GR24, HM8 was obtained as a product (Zhao et al. 2013), and the complex structure of D14 with cleaved HMB was obtained by soaking crystallized D14 with (−)-2'-epi-GR7 (Nakamura et al. 2013). It was proposed that HMB produced by D14 acts as an inactive form for shoot branching inhibition. However, there is no evidence to demonstrate that the D14–HMB complex is involved in the shoot branching inhibition pathway. More detailed analysis would be needed to determine the active form structure for shoot branching inhibition.

To summarize the essential requirements of SLs in rice, the (2'R) configuration and the presence of an intact D-ring connected with an enol ether unit are required for the inhibition of tiller bud outgrowth (Fig. 7).

We investigated the biological activity of only some SL analogs added to the hydroponic culture media in Arabidopsis because a much larger amount of SLs is required for the assay. We confirmed that GR5 strongly inhibits shoot branching compared with GR24 in Arabidopsis when they were added to hydroponic culture media, while the effect of GR5 was weaker than that of GR24 when they were directly applied to axillary buds. However, the biological activity of GR5 is stronger than that of GR24 in both assays in pea (Boyer et al. 2012). These observations suggest that the uptake of GR5 by the plant was not so efficient in comparison with that of GR24 when directly applied to the Arabidopsis axillary buds, possibly because GR5 is more hydrophilic than GR24.

SL-dependent control of Arabidopsis hypocotyl elongation

As mentioned above, SL inhibits the hypocotyl elongation of Arabidopsis through both the AtD14 and HTL pathways.
The (2'R) isomers of SDS and GR5 were highly active in inhibiting the hypocotyl elongation of the htl mutants, in which AtD14 can function (Supplementary Fig. S5), consistent with the reported data (Scaffidi et al. 2014). On the other hand, in our bioassay conditions, all stereoisomers showed equal activities for the atd14 mutant, in which HTL can function (Supplementary Fig. S5). These results were different from those of the recent report by Scaffidi et al. (2014), in which (−)-SDS and its counterpart of GR24 showed stronger activities than the other three isomers in the atd14 mutant. This difference might be due to the different bioassay conditions; we used white light under short-day conditions, whereas they used continuous red light (Scaffidi et al. 2014). It is difficult to reach a conclusion at this time, but our data along with their report both suggest that the stereo-specificity of the HTL pathway for SLs is different from that of the AtD14 pathway. HTL is thought to be a receptor protein of KAR, a smoke-derived germination stimulant, although the endogenous ligand of HTL is still unknown. Because SL can inhibit hypocotyl elongation through the HTL pathway, it is predicted that some specific SLs act in the HTL pathway to control hypocotyl elongation. However, our results showing that SLs regulate hypocotyl length in a non-stereospecific manner through the HTL pathway provide the possibility that not SL itself but its metabolites or degradation products act in the HTL pathway to control hypocotyl elongation. Therefore, we assume that some specific SLs act in the HTL pathway to control hypocotyl elongation. However, our results showing that SLs regulate hypocotyl length in a non-stereospecific manner through the HTL pathway provide the possibility that not SL itself but its metabolites or degradation products act in the HTL pathway to control hypocotyl elongation. Therefore, we assume that some specific SLs act in the HTL pathway to control hypocotyl elongation.

**Comparison of the structure requirements of SLs in root parasitic plants and AM fungi**

As shown in Supplementary Fig. S8, there was a difference in the stereo-specificity of SLs between \textit{S. hermonthica} and \textit{O. minor} for seed germination. In previous studies, similar results were obtained for strigol (Reizelman et al. 2000), sorgo-lactone (Sugimoto et al. 1998), GR24 (Thuring et al. 1997) and GR7 (Mangnus and Zwanenburg 1992a). Thus, the effects of the C-2' configuration are different in each parasitic plant. In AM fungi, (2'R) isomers of SDS were more active than their respective (2'S) isomers, but for other SLs there was no clear trend between different stereoisomers (Akiyama et al. 2010).

3,6'-dhGR24, which is an inactive analog for shoot branching inhibition in rice, was also inactive for \textit{S. hermonthica} germination (Supplementary Fig. S8A) (Mangnus and Zwanenburg 1992b). However, one stereoisomer of 3,6'-dhGR24, (+)-2'-epi-3,6'-dhGR24, was as effective as (+)-GR24 in inducing hyphal branching of AM fungi (Akiyama et al. 2010), suggesting that the enol ether group, a critical part for shoot branching inhibition and seed germination, is not necessary for AM fungi. Taken together, our results imply that the SL perception mechanism might be different in each organism. In particular, the SL recognition mechanism by AM fungi seems to be different from that by plants, including parasitic and non-parasitic plants. For shoot branching inhibition in plants, D14 is a possible SL receptor; however, there have been no reports on SL receptors of root parasitic plants and AM fungi. Identification of an SL receptor for each function (shoot branching inhibition, stimulation of seed germination, root parasitic plants and induction of hyphal branching of AM fungi) would be needed for the total understanding of the SL structural requirements for different organisms.

**Conclusions**

Our structure–activity relationship studies demonstrated that the presence of an intact D-ring with an enol ether bridge is essential for the inhibition of tiller bud outgrowth, and the (R) configuration at C-2' has a significant influence on the hormonal activity in rice and Arabidopsis. In addition, our results indicate that species differences and application methods of SLs have a large amount of influence on the biological activity. Furthermore, our results from Y2H experiments provide evidence to explain the different biological activity of the tested chemicals. Because D14/AtD14/DAD2 is predicted to be an SL receptor protein, it might be necessary to evaluate the direct interaction between D14/AtD14/DAD2 and these tested SLs. Although the structure of D14/AtD14/DAD2 protein has been clarified, the structures of complexes with SL or partner proteins such as D53 and D3 have not been reported yet. It is necessary to know the molecular recognition mechanism of SL by D14 family proteins for total understanding of structure–activity relationships of SLs for shoot branching inhibition.

**Materials and Methods**

**Plant materials**

We used a rice cultivar (\textit{Oryza sativa} L. cv. Shiokari) as the WT, and tillering \textit{dwarf} mutants, \textit{d3}-1, \textit{d10}-1 and \textit{d14}-1 in the Shiokari background (Ishikawa et al. 2005), and \textit{Arabidopsis thaliana} ecotype Col-0 as the WT, and \textit{max2}-3, \textit{max4}-7, \textit{max4}-8 (Umehara et al. 2008), \textit{atd14}-2 (Seto et al. 2014) and \textit{htl/kai2}-4
Rice hydroponic culture for evaluation of branching inhibitory activity

Rice hydroponic culture was performed as described previously (Umehara et al. 2008). Rice seeds were sterilized and incubated in sterile water at 28 °C for 2 d. Germinated seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d. Germinated seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d. Germinated seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d.

Arabidopsis hydroponic culture for evaluation of branching inhibitory activity

Arabidopsis hydroponic culture was performed as described previously (Umehara et al. 2008). Sterilized seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d. Germinated seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d. Germinated seeds were put on hydroponic culture media (Kamachi et al. 1991) solidified with 0.6% agar (pH 5.7) and cultured at 25 °C under dark for 2 d.

Seed germination assay of root parasitic plants

Germination assays using S. hermonthica and O. minor were performed as described previously with minor modifications (Sugimoto and Ueyama 2008). Striga hermonthica seeds were surface sterilized, and were conditioned at 30 °C for 12 d on 6 mm glass fiber filter paper disks (~50 seeds each) placed on de-ionized water-saturated filter paper. For the germination bioassay, each glass fiber filter disk with Striga seeds was transferred to each well in a 96-well plate (Falcon). A 20 μl aliquot of de-ionized water or test chemical solution was added to the well, incubated at 30 °C for 24 h and microscopically examined for germination. Orobanche minor seeds were conditioned at 25 °C for 6 d, treated with test solutions, incubated for 5 d at 25 °C, and then examined for germination.

Hypocotyl elongation assay of Arabidopsis

Sterilized seeds were sown on solidified 1/2 MS medium (pH 5.7) containing test chemical with a 1:1,000 dilution from acetone-dissolved stock solution (0.1% acetone was used as control). The plates were kept for 5 d at 30 °C.

Chemicals

SDS, GR24, GR24 imino-analogs and 3β-dRGR24 stereoisomers were prepared as reported previously (Akiyama et al. 2010). (+)-GR7, (-)-GR7, (+)-2’-epi-GR7 and (-)-2’-epi-GR7 were prepared according to a previous report (Mangnus and Zwanenburg 1992). Details of preparation of other chemicals are as follows. Mass spectra were recorded on a JEOL JMS-700 instrument, Q-Tof Premier (Waters) and TripleTOF 6600 (AB SCIEX). 1H- and 13C-nuclear magnetic resonance (NMR) spectra were obtained with a JEOL JNM-AI-600 NMR spectrometer and JEOL JNM-ECA600. Chemical shifts were referenced to tetramethylsilane as an internal standard. Optical rotations were recorded on a JASCO P-2100 polarimeter. Circular dichroism (CD) spectra were measured with a JASCO J-820 spectropolarimeter. (-)-GR5 was prepared by the one-pots synthetic procedures as described previously (Akiyama et al. 2010, Mangnus and Zwanenburg 1992a), and chromatographed on a semi-preparative Chiralpak AD-H HPLC column (φ10×250 mm; Daicel) employing isocratic elution with 40% isopropanol-n-hexane at a flow rate of 2 ml min⁻¹. Compounds eluted from the column were monitored with a photodiode array detector. (-)-GR7 and (+)-GR7 eluting at a single peak at 13.3 and 15.6 min, respectively, were collected. The enantiomeric purities of the four stereoisomers were estimated to be >99% e.e., respectively, by analytical HPLC using a Chiralpak AD-H column (φ4.6×250 mm, 5 μm; Daicel) employing isocratic elution with 40% isopropanol-n-hexane at a flow rate of 0.5 ml min⁻¹ monitored with a photo-diode array detector. (+)-GR7: [α]D₂⁰ +11 (+0.13, acetoni-trole); CD (acetoni-trole) λmax (Δε) 262 (+1), 225 (12.5) nm. (-)-GR7: [α]D₂⁰ −12 (−0.096, acetoni-trole); CD (acetoni-trole) λmax (Δε) 262 (−2, 224 (−16.4) nm. C-ring-cleaved GR5s and saturated D-ring analogs were prepared as described below. 

(-)-Methyl 2-methyl-3-(1S,4S)-4-hydroxy-2,3-dihydrofuran-2-yloxyacrylate ([(-)-MePrGR5, 19]. To a stirred suspension of NaH (66 mg, 60% in oil, 2.8 mmol, washed with dry hexane before use) in dry dimethylformamide (DMF, 1 ml) at room temperature under nitrogen was added dropwise a solution of methyl propionate (176 mg, 2 mmol) in dry DMF (2 ml) followed by ethyl formate (0.97 ml, 9.8 mmol). After 17.5 h, the mixture was cooled to 0 °C, and a solution of 5-bromo-3-methyl-2(5H)-furanone (389 mg, 2.2 mmol), which was prepared according to the reported method (MacAlpine et al. 1976), in dry DMF (1 ml) was added dropwise. After stirring for 7 h at room temperature, the reaction mixture was poured into 0.5 N HCl (150 ml), and extracted three times with ether (75 ml). The organic extract was washed with water, dried over sodium sulfate, and concentrated in vacuo. The residue was chromatographed on silica gel (Kieselgel 60, Merck) employing 3% stepwise elution with 50% hexane and acetone. The 15% and 1% acetone eluates were subjected to semi-preparative Inertsil SIL-100A HPLC (φ10×250 mm, 5 μm; GL Sciences), employing isocratic elution with 15% ethanol–n-hexane at a flow rate of 3.8 ml min⁻¹ to give (-)-MePrGR5 (9.5, 14.0 min, 11.8 mg, 2.8%). H-NMR (400 MHz, CDCl₃) δ 1.75 (3H, brs, H-3), 2.00 (3H, brs, H-7), 3.71 (3H, s, OCH₃), 6.08 (3H, m, H-1), 6.91 (1H, m, H-3), 7.47 (1H, m, H-6); EIMS 70 eV, m/z (rel. int): 212 [M⁺]+ (1). 181 (2), 116 (1), 97 (100); HRESIMS m/z: 213.0761 [M+H⁺]+ (calculated for C₁₀H₁₄O₂, m/z 213.0763). (E)- and (Z)-2-methyl-3-(1S,4S)-4-hydroxy-2,3-dihydrofuran-2-yloxyacrylate [(E)- and (Z)-MeMeACGR5, 20, 21]. A solution of methyl acetate (74 mg, 1 mmol) in dry tetrahydrofuran (TFH; 5 ml) was added dropwise to a stirred solution of lithium disopropylamide (2 mmol) in dry THF (1 ml) at –78 °C under nitrogen. After stirring for 19 h at 0 °C, the mixture was poured into 0.5 N HCl (100 ml), and extracted with ether (100 ml). The organic extract was washed with water, dried over sodium sulfate and concentrated in vacuo. The residue was chromatographed on silica gel (Kieselgel 60, Merck) employing 3% stepwise elution with n-hexane and acetone. 15% and 19% acetone eluates were subjected to semi-preparative Inertsil SIL-100A HPLC (φ10×250 mm, 5 μm; GL Sciences), employing isocratic elution with 15% ethanol–n-hexane at a flow rate of 2 ml min⁻¹ to give (E)-[E]-MeMeACGR5 (20, tR 10.3 min, 2.0 mg, 1.6%). The 18% and 21% acetone eluates

Yeast two-hybrid experiments

The coding regions of rice D14 and D53 were amplified by PCR using the primer set described in Supplementary Table S1, and cloned into pGBK77 and pGAD77 (Clontech), respectively. The coding regions of Arabidopsis AD14, SMX6, SMX7 and SMX8 were amplified by PCR using the primer set described in Supplementary Table 1, and cloned into pGBKT7 respectively. The constructs were co-transformed into yeast strain Y2HGold (Clontech) and the transformants were grown on SD–Leu/Trp plates for 3 d at 30 °C. The interactions between two proteins were examined on the selective SD–Leu/Trp/His/Adr media containing different SL stereoisomers with a 1:1000 dilution from acetone-dissolved stock solution (0.1% acetone was used as control). The plates were kept for 5 d at 30 °C.

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were chromatographed using semi-preparative Inertial SIL-100A HPLC as above to give (Z)-(a)-MeAcGR5 (21, tf 13.1 min, 3.3 mg, 1.7%).

(E)-(a)-Methyl 3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yl)acrylate. 1H-NMR (400 MHz, CDCl3) δ 2.00 (3H, t, J = 15.7 Hz, H-7), 3.73 (3H, s, OCH3), 5.57 (1H, d, J = 12.4 Hz, H-2), 6.10 (1H, m, H-3), 7.56 (1H, d, J = 12.4 Hz, H-6). 13C-NMR (100 MHz, CDCl3) δ 10.7, 51.5, 99.2, 102.2, 135.4, 141.0, 157.7, 167.0, 170.4 EIMS 70 eV, m/z (rel. int): 198 [M]+ (1), 170 (5), 167 (53), 137 (11), 97 (100). HRESIMS m/z 199.0605 [M]+H+ (calculated for C9H10O5, m/z 199.0607).

Time course monitoring of SL stability in hydropnic culture media

(+)-SDS, (-)-SDS, (+)-GR24, (-)-2’,3’-epi-(+)-GR24, (+)-GR7 and (-)-GR5 were separately added to vials containing rice hydropnic culture medium at 0.1 μM. These solutions were incubated under the same condition as rice hydropnic culture. At the start of incubation (0 d) and 1, 3 and 7 d after incubation, 200 μl of hydropnic culture media containing (+)-strigol, (+)-SDS, (+)-2’,3’-epi-(+)-GR24, (+)-GR7 or (+)-GR5 was collected, and 300 pg of racemic GR24 as an internal standard was added. The solution was extracted with ethyl acetate twice. To determine the stability of (+)-GR24, 200 pg of racemic 2’,3’-epi-SDS was added to the solution as an internal standard. The combined ethyl acetate phase was dried up under nitrogen gas, and stored at 4°C in a container containing dried silica gel till LC/MS-MS analysis. Each sample was dissolved in 50% acetonitrile, and subjected to LC/MS-MS analysis equipped with a reverse-phase column (Acquity UPLC BEH-C18, 2.1 x 50 mm, 17 μm; Waters). The mobile phase was changed from 30% acetonitrile containing 0.05% acetic acid to 40% and 70% in 5 and 10 min after injection, respectively, at a flow rate of 0.2 ml min−1. Each peak area of test chemical and internal standard was monitored, and the ratio at the start of incubation was set as 100%.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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