Bioorganic & Medicinal Chemistry Letters 21 (2011) 4905–4908

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

New branching inhibitors and their potential as strigolactone mimics in rice

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ARTICLE INFO

Article history:
Received 13 April 2011
Revised 30 May 2011
Accepted 6 June 2011
Available online 22 June 2011

Keywords:
Chemical synthesis
Drug design
Strigolactone
Mimic
Branching
Root parasitic weed
Germination
Plant hormone

ABSTRACT

Strigolactones (SLs) are rhizosphere communication chemicals. Recent studies of highly branched mutants revealed that SL or its metabolites work as a phytohormone to inhibit shoot branching. When SLs are exogenously applied to the rice *d10-1* mutant that has a highly branched phenotype caused by a defect in the SL biosynthesis gene (CCD8), they inhibit tiller bud outgrowth (branching in rice) of the mutant. We focused our attention on the SL function as a phytohormone and tried to find new chemicals mimicking the hormonal action of SL by screening chemicals that inhibit branching of rice *d10-1* mutant. Fortunately, we found 5-(4-chlorophenoxy)-3-methylfuran-2(5*H*)-one (**3a**) as a new chemical possessing SL-like activity against the rice *d10-1* mutant. Then, we prepared several derivatives of **3a** (**3b–3k**) to examine their ability to inhibit shoot branching of rice *d10-1*. These derivatives were synthesized by a one-pot coupling reaction between phenols and halo butenolide to give 5-phenoxy 3-methylfuran-2(5*H*)-one (**3**) derivatives, which possess a common substructure with SLs. Some of the derivatives showed SL-like activity more potently than GR24, a typical SL derivative, in a rice assay. As SLs also show activity by inducing seed germination of root parasitic plants, the induction activity of these derivatives was also evaluated. Here we report the structure–activity relationships of these compounds.

Strigol, a member of the strigolactones (SLs), was first discovered from cotton root exudates as a seed germination stimulant of root parasitic plants such as *Striga*.[1] This compound was identified as a member of the terpenoids whose structural core consists of tricyclic-lactone (ABC ring) and a butenolide group (D-ring) that are connected via an enolether moiety (Fig. 1 and **1**). A variety of SLs, chemically substituted at A- and/or B-rings, has been isolated from the root exudates of many different host plants as seed germination stimulants of several kinds of root parasitic plants.[2] The discovery of natural SLs led chemists to synthesize various artificial analogues based on the structure of natural products for the application of such compounds to the field of agriculture.[3] Recent studies revealed that SLs stimulate not only seed germination of root parasitic plants but also hyphal branching in *Arbuscular mycorrhizal* fungi (AM fungi), which establish a symbiotic relationship with plant roots.[4] This relationship provides the fungus with carbohydrates produced by the plant, and the plant with water and phosphate taken up by the fungus. Therefore, it was thought that SLs were originally released as symbiotic chemicals and abused by root parasitic plants. More recently, two research groups reported that these rhizosphere communication chemicals or their metabolites also work as a novel class of plant hormones inhibiting shoot branching.[5]

Until now, many kinds of artificial SL analogs were synthesized and studied as stimulants of seed germination of root parasitic plants, some of which also showed hypal branching activity.[6] Structure–activity relationship studies of SL analogs as seed germination stimulants of root parasitic plants revealed that D-ring lactone connected by an enolether-moiety is important for their activity.[7] GR24 (Fig. 1 and **2**) is the first artificial SL analog synthesized more easily than natural SLs and is therefore widely used as a mimic of natural SLs in several biological situations.[2a,3b,5,6] In the first report GR24 was synthesized in a 6-step reaction from *trans*-2-bromo-1-indanol. Later, an improved method which requires a 4-step reaction from 1-indanone, was reported.[8] This method facilitated the formation of the ABC ring part and reduced the number of steps, although GR24 diastereomer (epi-GR24) was simultaneously prepared. Although the activity of epi-GR24 is the same as that of GR24, however, in research GR24 should be evaluated separately from epi-GR24. Subsequently, asymmetric synthesis of GR24 was reported,[9] but this report did not mention the formation of the ABC ring. At present the formation of the ABC ring of GR24 still required at least a one-step reaction followed by formylation. Therefore efficient and short routes for the synthesis of potent SL mimics are still required. To date, the activity of SL analogs has been evaluated by measuring the activity of germination stimulation of root parasitic plants, although their activity as a phytohormone has not been well investigated.

In this report, we focused our attention on chemicals that inhibited shoot branching. This is because, ever since the discovery of

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doi:10.1016/j.bmcl.2011.06.019

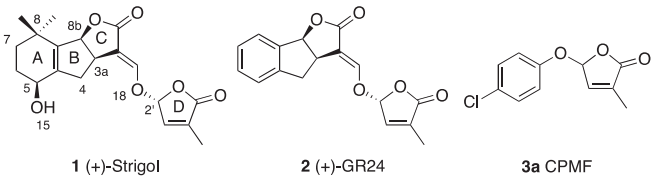
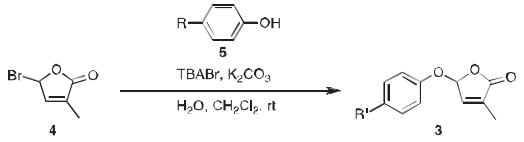


Figure 1. Structures of natural SL; (+)-strigol (**1**), synthetic SL GR24 (**2**), and a lead compound CPMF (**3a**) used in this report.

SLs as a phytohormone, easily obtainable SL mimics have been in demand by many plant biologists to investigate the functions of SLs in crop production and plant physiology. Chemicals mimicking the hormonal action of SLs can be useful for agriculture in the future. Thus, we attempted to explore the compounds that can mimic the action of SL as a phytohormone. Recently, Kondo et al. reported that the imino analog of GR24 showed germination stimulating activity in some root parasitic weeds.[3e] This finding indicates that it is not always essential to have the Michael acceptor of the C–D ring junction moiety. We expected that the π-bond conjugated ether is important for SL activity. To examine this expectation, we synthesized new chemicals lacking an enolether moiety, 5-phenoxy-3-methylfuran-2(5*H*)-one (PMF) and 5-(4-chlorophenoxy)-3-methylfuran-2(5*H*)-one (**3a**, CPMF), and estimate their activity in a shoot branching inhibition assay at 1 μM, as reported for SL biosynthesis inhibitors.[10] Fortunately, in this assay, CPMF had distinct activity but PMF, which has no substituent on the phenyl group, had almost no activity. We then initiated structure–activity relationship studies of **3a**. Here we report the chemical synthesis of **3a** and analogs of **3a** and their efficacy in inhibiting shoot branching in the rice d*10-1* mutant.

Compounds **3a**–**3k** in Table 1 were synthesized using modified procedures reported in the literature.[11] The first step was bromination at the 5-position of 3-methylfuran-2(5*H*)-one using *N*-bromo-succinimide, which was then coupled with a variety of phenols under basic conditions to give a crude mixture of phenol ethers in two steps. Purification with silica gel flash column chromatography and re-crystallization from *n*-hexane and ethyl acetate gave pure **3a**–**3k** in 13–73% yield as shown in Table 1. The compounds substituted with electron-withdrawing groups (Table 1, entries 1–6 and 10) likely gave better yield than those with electron-donating groups (Table 1, entries 7–9 and 11).

Table 1
Percent yields of phenyl ether derivatives



Entry[a]	R=	R[1]=		Yield[b] (%)
1	Cl	Cl	**3a**	29
2	F	F	**3b**	40
3	Br	Br	**3c**	53
4	I	I	**3d**	73
5	CF_3	CF_3	**3e**	59
6	CN	CN	**3f**	55
7	Me	Me	**3g**	29
8	^{t}Bu	^{t}Bu	**3h**	24
9	OMe	OMe	**3i**	38
10			**3j**	50
11	OH		**3k**	13

[a] Reaction conditions : The reaction was carried out in 4.0 ml of CH_2Cl_2 and 3.0 ml of H_2O with 1.0 mmol of TBABr, 1.2 mmol of K_2CO_3, 1.0 mmol of phenol and 1.0 mmol of substrate at room temperature for over 8 h.
[b] Isolated yield.

We then evaluated the inhibitory activity of the newly synthesized compounds on the shoot branching of rice grown in a hydroponic culture system as reported previously.[11] In this test, we used an SL-deficient *d10-1* mutant in which a gene encoding the SL biosynthesis enzyme (CCD8) is mutated.[12] This mutant, whose phenotype shows more tillering, reverts back to a phenotype almost identical to that of wild type after the application of SLs. Second tiller bud outgrowth was observed in 2-week-old rice *d10-1* seedlings without chemical treatment, but this characteristic phenotype in mutant rice was not observed in the SL-treated *d10-1* mutant. We examined the inhibitory effect of the compounds synthesized as described above in this assay system at 1 μM (Fig. 2). Treatment of GR24 (**2**), a synthetic SL analog, at 1 μM inhibited the second tiller outgrowth of *d10-1*. All of the compounds synthesized and listed in Table 1 are 4-substituted phenoxyfuranone derivatives that show clear biological activity depending on the substituent on the phenoxy ring. Halogenated compounds, which contain fluorine (**3b**), bromine (**3c**) or iodine (**3d**), are highly active; they also include a chlorine-substituted compound (**3a**). Compounds substituted with an electron-withdrawing group such as trifluoromethyl (**3e**) or nitrile (**3f**), are also more active than GR24, although, interestingly, the compound substituted with methoxycarbonyl (**3j**) as an electron-withdrawing group was not so active. In contrast, the compound substituted with an electron-donating group, such as methyl (**3g**), was active. On the other hand, compounds substituted with bulky groups, such as *tert*-butyl (**3h**), methoxy (**3i**) or butenolide (**3k**), were likely not so active as compounds **3a**–**3g**. When combining the results in Figure 2, compounds including a bulky substituent were likely less active than those including a smaller substituent. This result obtained by structure–activity relationship studies of the derivatives reported here could be useful for designing new SL mimics in future research.

Next, we examined the concentration dependency of the inhibitory activity of each compound substituted with an electron-withdrawing but not a bulky group (Fig. 3A). All of the compounds tested here inhibited second tiller bud outgrowth at 0.1 μM as effectively as GR24 at 1 μM. Compounds **3c**–**3f** were so potent in our assay system even at 0.1 μM that little or no second tiller bud outgrowth was observed in the *d10-1* mutant treated with **3c**–**3f** at this concentration. To further estimate the activity of **3c**–**3f** at a low concentration (Fig. 3B), we again treated the *d10-1* mutant with **3c–3f** at 0.01 μM. The result showed that **3c** is likely the most active. There were statistically unclear points in these data but every time **3c** was the most active in the same

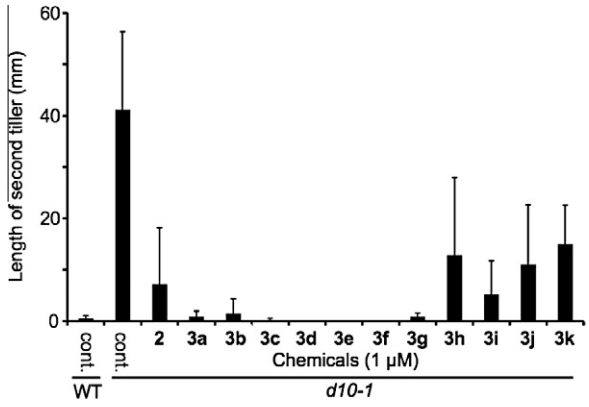


Figure 2. Effect of chemicals on 16-day-old rice seedlings. Length of second tiller of rice seedlings treated with 1 μM of chemicals. The data are means ± SE of six samples.

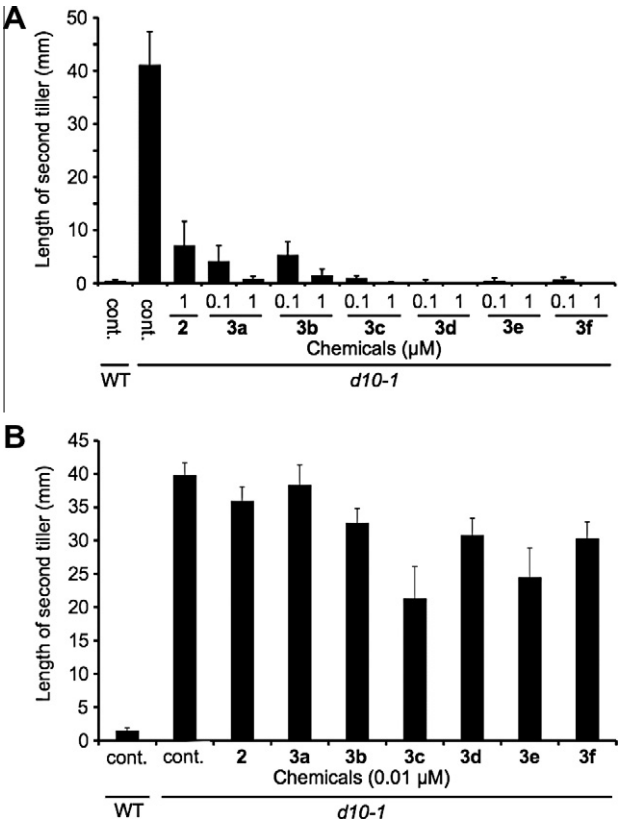


Figure 3. Effect of chemical concentration on 16-day-old rice seedlings. (A) Length of second tiller of rice seedlings treated with chemicals at the described concentration. The data are means ± SE of six samples. (B) Length of second tiller of rice seedlings treated with 0.01 μM of chemicals. The data are means ± SE of six samples.

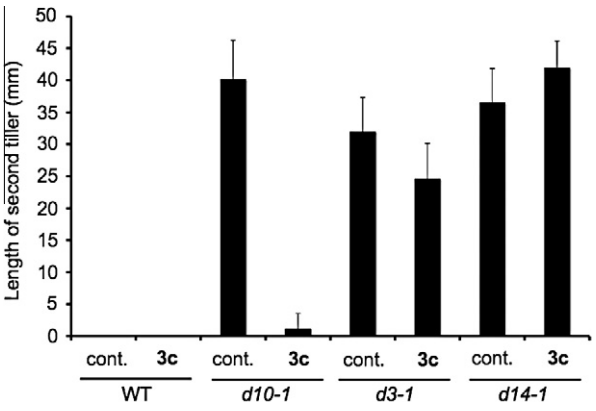


Figure 4. Effect of **3c** on rice tillering. A comparison of second tiller length in 16-day-old wild type (WT) and *d* mutants. Concentration of **3c** was 1 μM in hydroponic culture.

Table 2
Effect of chemicals on inducing seed germination of *Striga hermonthica*

No.	Germination rate (%)				
	0.001 μM	0.1 μM	1 μM	10 μM	100 μM
2	13.8 ± 8.4	29.5 ± 5.2	33.8 ± 0.8	24.0 ± 11.1	—
3a	—	—	1.0 ± 1.7	3.3 ± 3.8	2.1 ± 1.8
3b	—	—	1.1 ± 2.0	1.4 ± 2.5	4.8 ± 4.4
3c	—	—	0.0	5.4 ± 4.8	19.9 ± 1.6
3d	—	—	0.9 ± 1.6	5.7 ± 5.2	8.3 ± 14.4
3e	—	—	1.4 ± 2.5	3.4 ± 3.1	11.1 ± 2.9
3f	—	—	4.5 ± 7.9	22.2 ± 6.8	21.7 ± 2.9
3g	—	—	0.0	0.0	0.0
3h	—	—	0.0	1.1 ± 1.9	2.1 ± 3.6
3i	—	—	1.1 ± 1.9	3.1 ± 3.2	4.5 ± 4.8
3j	—	—	3.7 ± 3.5	14.0 ± 7.2	11.9 ± 7.7
3k	—	—	0.6 ± 1.0	0.7 ± 1.2	4.4 ± 5.3

Each data value is mean germination percentage ± standard deviation from three replicates.

assay. In this assay, our compounds showed a clear inhibitory effect on rice second tiller bud outgrowth in a dose-dependent manner; however, even at a high concentration (10 μM, data not shown) no morphological change was observed such as dwarfing or bleaching in rice treated with these chemicals.

Next, we investigated the mechanism by which **3c** exerts its hormonal activity in rice. To examine the possibility that **3c** inhibits tiller bud outgrowth through the SL signaling pathway, we treated **3c** in SL-insensitive mutants, *d3-1* and *d14-1*,[5b,13] in which SL signal transduction is blocked. Tiller bud outgrowth of *d3-1* and *d14-1* is not affected by the application of GR24, while GR24 inhibited tiller bud outgrowth of *d10-1*. As a result, a similar trend was observed for the application of 1 μM of **3c** to these three mutants and wild type rice (Fig. 4). That is, **3c** inhibited tiller bud outgrowth of *d10-1*, but by contrast, did not affect tiller bud outgrowth of both *d3-1* and *d14-1*. These results suggest that our compounds should inhibit tiller bud outgrowth by activating the SL signal transduction pathway, not by a side effect, and be good lead compounds for branching inhibitors as SL mimics. Here we propose the name of the chemicals possessing a 5-phenoxy-3-methylfuran-2(5*H*)-one skeleton as debranones (furanones showing de-branching activity).

SLs are not only shoot branching inhibitors but also seed germination stimulants of root parasitic plants *Striga* and *Orobanche*. To confirm whether debranones also act as SL mimics in the induction of seed germination of parasitic plants, we used a highly sensitive germination assay using *Striga hermonthica* seeds. The assay method was the same as that used in a former report by Ito et al.[10] We compared the activity of synthesized debranones with that of GR24 (Table 2). As a result, some of the derivatives (**3c**, **3e**, **3f**, and **3j**) clearly stimulated the germination of *Striga* seeds at 100 μM, but all of the debranones barely induced germination at 1 μM. On the other hand, GR24 showed germination stimulant activity even at 0.001 μM. That is, GR24 was 10,000-times more active than debranones in this biological test although GR24 was 10-times less active than **3c** in the rice branching test.

The important structural requirements of SLs and their synthetic derivatives for hyphal branching in AM fungi and seed germination of root parasitic plants have been studied, revealing the existence of several similarities in the structural requirements for both activities.[6a,8] Specifically, the existence of proper substituents on the A- and/or B-ring and a bond between the C-ring and the D-ring are important for exerting both activities. However, there is a distinct difference in the structural requirements for AM fungi and for parasitic plants. For example, carbon–carbon or carbon–nitrogen double bonds next to an ether bond are required for the induction activity of seed germination of root parasitic plants but not for stimulating hyphal branching. In contrast, there has been no structure–activity relationship study on the functions of SL derivatives as a phytohormone because both SL-deficient mutants and a relatively large amount of SLs are necessary for estimating the hormonal activity of SLs making it difficult to evaluate the hormonal activity of SLs and their derivatives. Therefore, whether structural requirements of SLs as a phytohormone were different from those for stimulating root parasitic seed germination or inducing hyphal branching or not is still unknown. In this report, we demonstrate that our chemicals inhibit shoot branching in rice maybe through the SL signaling pathway and therefore our

chemicals can be said to be SL mimics. However, the activity of debranones in the branching inhibition test differed greatly from that in the germination induction test. Therefore, it is possible to say that debranones are the first selective SL mimics specialized for plant hormone action and that **3c** is the most potent and selective compound inducing the de-branching phenotype in rice. At present, whether debranones function as agonists of SLs or not is unclear, but further investigations on SL-related gene expression and identification of SL receptor(s) will reveal the action mechanism(s) of debranones.

In this report, we demonstrate that our compounds could be used as selective SL mimics to inhibit the branching of plants. Further structure–activity relationship studies on debranone derivatives can provide selective SL mimics active only for the induction of root parasitic seed germination. Such types of SL mimics would be useful for reducing damage to crops by a parasitic plant attack by stimulating suicidal germination of seeds of parasitic plants. At present the synthesis of 3-methyl-2(5*H*)-furanone on a large-scale is not easy,[14] but further exploration of SL mimics may enable to find chemicals that exert SL-like activity without the butenolide moiety in our compounds.

Acknowledgements

We thank Professor A. G. T. Babiker for supplying *Striga* seeds. This research was supported by PROBRAIN (grant to T.A.) and the Japan Society for the Promotion of Science Fellows to S.I.

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