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In silico exploration for agonists/antagonists of brassinolide

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ABSTRACT

Brassinolide (BL) is a plant steroid hormone that is necessary for stem elongation and cell division. To date more than 70 steroidal BL-like compounds, which are collectively named as brassinosteroids, have been identified. However, non-steroidal compounds that mimic BL have not been reported yet, which can be used as plant growth regulators. Twenty-two non-steroidal compounds were screened from the database containing about 5 million compound structures using a pharmacophore-based in silico screening method. The crystal structure (PDB: 4LSX) of the BL receptor was used to generate a pharmacophore model required for in silico screening. Among 22 hit compounds, 15 compounds that are thought to be physicochemically acceptable were submitted to the in vivo rice lamina inclination assay. Although no compound showed BL like activity, three compounds were detected as BL antagonist. The most potent compound was an ester derivative of 1,4-diphenlenedimethanol and isoxazole-4-carboxylic acid, and the other two compounds contain 2-phenylfuran and pyrimidin-2(1*H*)-one moieties bridged by an ethenyl substructure. The 50% effective doses (ED_{50}) for the antagonistic activity were in a range of 0.6–5 nmol per plant. The inhibition of the lamina inclination by the most potent agonist was recovered by the co-application of BL in a dose-dependent manner.

In 1970, a novel plant growth-regulatory substance was isolated from the pollen of *Brassica napus* L.[1] and its chemical structure was later characterized by X-ray analysis.[2] This compound is a steroid hormone and named as brassinolide (BL) as shown in Figure 1, which has a ε-lactone (seven-membered ring) structure.[2] Three years later, castasterone (Cas) containing six-membered cyclohexanone structure was isolated from the chestnut gall as BL like compound.[3] BL and Cas were categorized into brassinosteroids (BRs) and characterized by the hormonal regulatory activity toward cell elongation/division as well as by the association with the disease resistance. To date more than 70 BRs have been identified and/or chemically synthesized. While BRs have been deemed a group of secondary metabolites for long time, the discovery and characterization of BL-deficient dwarf mutant established BL as the 6th plant hormone.[4] Nowadays, many genes encoding enzymes essential for the BL biosynthesis have been cloned from *Arabidopsis thaliana*.[5]

Steroid hormones are also important for animals including insects, and non-steroidal compounds that mimic the steroid hormones are used in pharmaceutical and agricultural markets. Diethylstilbestrol is well known as non-steroidal mimetic for female hormone, estradiol. Diacylhydrazine (DAH) type compounds that mimic the molting hormone, 20-hydroxyecdysone, are used as insecticides.[6,7] By contrast, even though the chemical structure of BL was characterized about a half century ago, no BL-like non-steroidal compound has been discovered to date, except for one paper reporting an ambiguous BL activity for non-steroids.[8] Recently, antagonistic activity of BL analogs was reported by Muto and Todoroki,[9] but non-steroidal antagonists have not been found yet.

In 2001, Wang et al. published that BR insensitive receptor kinase 1 (BRI1) is the receptor for BL,[10] and later Kinoshita and co-workers reported that BRs bind to the leucine-rich repeat (LRR) domain of the BRI1.[11] Recently, two research groups published three-dimensional (3D) structures of BRI1-BL complex.[12,13] In these crystal structures, three hydroxyl groups of BL (2-OH, 3-OH, 22-OH) do not interact with the receptor protein in the ligand–receptor binding. However, it has been reported that these OH groups are essential for BL activity by the structure–activity relationship study. Meanwhile, a family of somatic embryogenesis receptor kinases (SERKs) has been genetically implicated in mediating early brassinosteroid signaling events as co-receptor candi-

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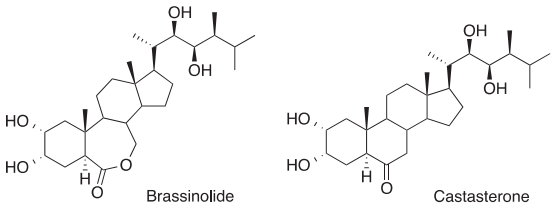

Figure 1. Structures of representative brassinosteroids.

dates.[14] Eventually, the 3D structure of the BRI1-BL-SERK1 complex was solved by X-ray crystal structure analysis,[15] which is timely for the starting of in silico screening. In this novel receptor complex, three OH groups (2-OH, 3-OH, and 22-OH) form the hydrogen bonding network.

The aim of this study was to find novel non-steroidal BL-like compounds or antagonists using a pharmacophore based in silico screening technique. We used the software package LigandScout developed by Inte:ligand GmbH (Austria),[16] in which the query pharmacophore was generated from the ligand–receptor complex to perform the virtual screening. The pharmacophore was constructed from the crystal structure of the BRI1-BL-SERK complex (PDB: 4LSX). Screened compounds were purchased and their BL agonist/antagonist activity was evaluated with the rice lamina inclination assay.[17,18] The bioassay method was shown in the Supplement figure.

The database containing 5,251,975 structures in sdf format was kindly supplied by Namiki Shoji Co., Ltd (2011.06; Tokyo, Japan). These structures were converted to a multi-conformational compound database in ldb format using OMEGA module of LigandScout (ver. 3.12). The conformation number per molecule was set as 200 in the conformer generation step. The crystal structure BRI1-BL-SERK1 complex (PDB: 4LSX) was used in this pharmacophore-based screening.

To construct the pharmacophore model, the excluded volume for the ligand-receptor interaction, hydrophobic interaction, and hydrogen bonding (acceptor and/or donor) features were used. In the query model generation, spheres were used to express the excluded volume and hydrophobic interactions and arrows for hydrogen bonds as shown in Figure 2a. Excluded volume spheres were derived automatically and placed on alpha carbon atom coordinates of ligand surrounding amino acids. These spheres and arrows can be removed and added manually. Hydrophobic and hydrogen bonding features of this pharmacophore are schematized in Figure 2b.

We performed the in silico screening of BR agonists using the query model generated from the BRI1-BL complex (3RGZ and 3RJ0) published in 2011,[12,13] but did not find potential BR agonists.

Table 1
Sphere positions and hydrogen-bond acceptor/donor groups interacting with amino acid residues of receptor in the pharmacophore models generated by the default setting

Interactions	Sphere position	Amino acid residues participating ligand–receptor interactions[a]	
		Model-1	Model-2
Hydrophobic	C18	Trp564, Tyr599, Phe681, Ile682	Trp564, Tyr599, Ile682, Phe681
	C19	Ile682, Ile706, Tyr729	Ile682, Ile706, Tyr729
	C20, C21	Met657, Phe658, Phe681	Met657, Phe658, Phe681
	C24, C28	Trp564, Tyr597, Leu615, Thr646, Met657	Trp564, Tyr597, Leu615, Thr646, Met657
	C25, C27, C28	Ile540, Ile563, Try564, Thr646, Met657,	Ile540, Ile563, Try564, Thr646, Met657
HB-donor	6-O		Tyr642
	22-OH	Tyr597	Tyr597
HB-acceptor	2-OH	His62 (side chain)[b], His62 (amide)[b]	—
	3-OH	His62[b]	—
	6-O		Tyr642
	22-OH	Tyr597	Tyr597
	23-OH	Tyr597, Ser647	Ser647

[a] Unless noted the numbering of amino acid residues is for BRI1.
[b] Numbering is for SERK1 protein.

Antagonistic activity was not evaluated for the screened compounds. In this pharmacophore model generated from 3RGZ and 3RJ0, 2-OH and 3-OH of A-ring of BRs were not considered as HB acceptor/donor, although it is reported that these OH groups are essential for the activity.[19–21]

In 2013, the novel crystal structure BRI1-BL-SERK1 complex (PDB: 4LSX) was published as the homo-dimeric structure with quaternary structure containing two BL-binding sites.[15] Since there are two binding sites, two pharmacophore models (Model-1, Model-2) were constructed for the screening. Hydrophobic spheres were found to be similar in both models, but different hydrogen bondings (HBs) were observed between Model-1 and Model-2. In these models, hydrophobic interactions were detected surrounding the hydroxyalkyl side chain and CH_3 groups (C18, C19) locating at the A/B and C/D ring junction (Table 1). In addition, 12 excluded volume spheres were considered for screening. However, we could not obtain any compounds using these models. In these in silico screening, the number of maximum conformations generated by OMEGA was set as 25. Biological activity was evaluated with the lamina inclination assay.[9]

We further revised the pharmacophore model as shown in Figure 2. In the revised pharmacophore model, four functional

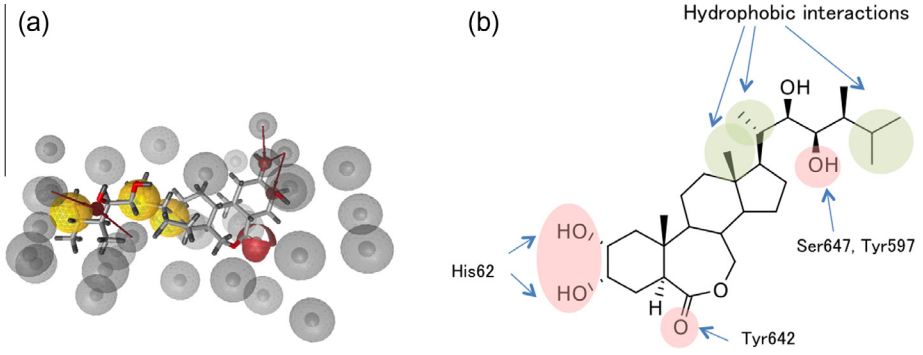

Figure 2. The pharmacophore model generated from BRI1/SERK1/BL complex used for in silico screening. (a) Pharmacophore model with exclusion volume regions (gray), hydrophobic regions (yellow), and HB-acceptor/donor (red arrow). (b) Summary of hydrogen bond interactions and hydrophobic regions.

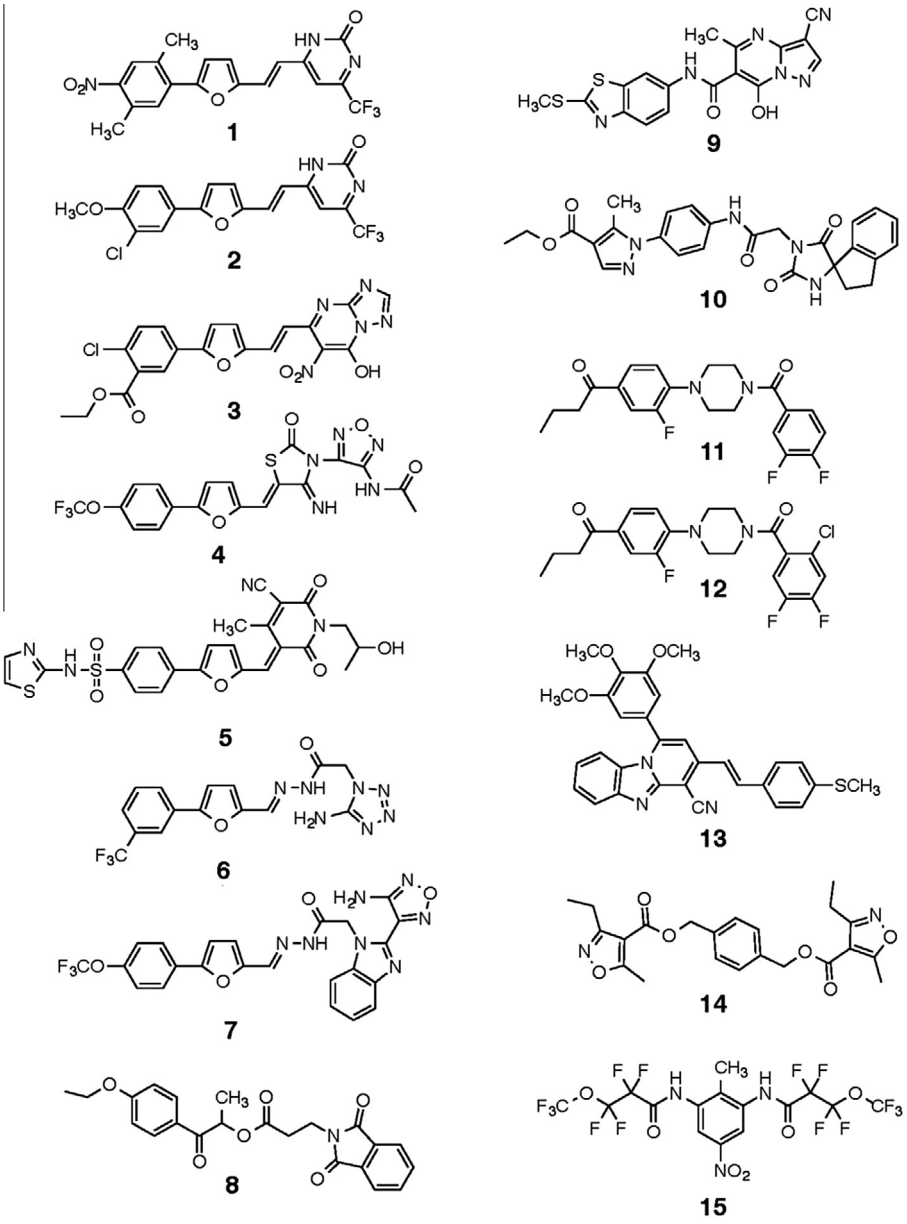

Figure 3. Structures of screened compounds that were submitted to the lamina inclination assay.

groups [2-OH, 3-OH, =O (C6), and 23-OH] were adopted as the HB acceptor, which are thought to be important for the BL activity from SAR study. HBs toward 2-OH and 3-OH groups from His62 residue of SERK1 were detected only in the Model-1, which are essential for the activity according to the SAR study. In addition to these HBs detected in the interaction of BL to SERK1, one HB donor (22-OH) pointed toward Tyr597 and two HB acceptors (22-, and 23-OH) pointed toward Tyr597 and Ser647 were found in BRI1. The carbonyl oxygen at C6 was considered to be HB acceptor, although it was depicted as hydroxyl oxygen in the PDB data. Thus, the donor sphere that was generated in the default model was removed. In fact, HBs between carbonyl oxygen at C6 and amino acid residue (Tyr642) are detected in the BRI1 crystal structure (3RJ0). In another crystal structure (3RGZ), H_2O molecule intervenes between ligand and amino acid residue. HBs of 23-OH to Ser647 and Tyr597 were considered for the screening, but the HB of 22-OH to Tyr597 was eliminated in this new model, because the HB ability of 22-OH is thought to be weaker than that of 23-OH. In fact, HB of 22-OH to receptor was not observed in the crystal structures of BRI1-BL complex without SERK1 (3RGZ and 3RJ0).

In order to judge adequacy of this pharmacophore model, BL and Cas were included in the structure database and screened. To construct the structure database, chemical structures of BL and Cas were drawn by smile expression and converted to lbd file using OMEGA. In this conformer generation step, the maximum conformation was set to 200. Under this screening condition, both BL and Cas were retrieved, but not for the database generated by setting maximum conformation number to 25.

Fifteen out of 22 compounds (Fig. 3) screened from about 5 million compounds were purchased for bioassay. The other seven compounds (**16**–**22**; Fig. 4) were not purchased. Compounds **19**–**22** were thought to be unsuitable as leads for agrochemicals due to their unfavorable substructures containing polyfluoronated alkyl or sugar moiety. In particular, the hydrophobicity of compounds **19** and **22** was estimated to be very high (11.31 and 8.38

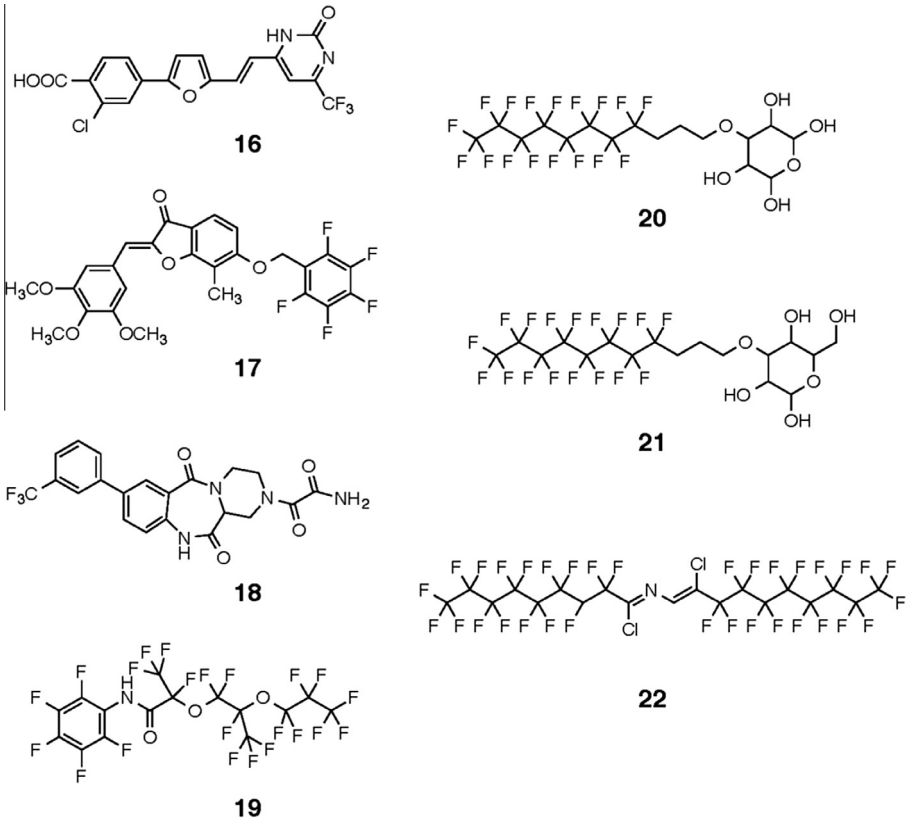

Figure 4. Structures of screened compounds that were not used in this study.

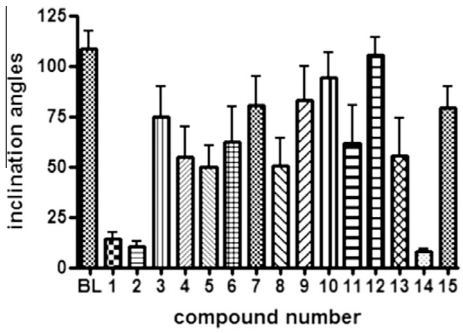

Figure 5. Inhibition of lamina inclination induced by BL treatment.

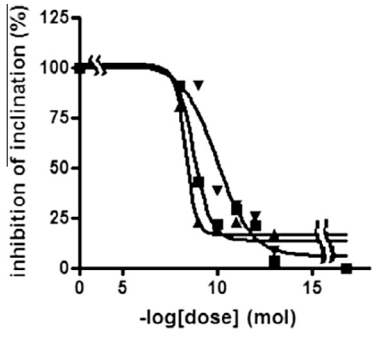

Figure 6. Dose–response relationship for BL-antagonistic activity of three compounds, **1** (■), **2** (▲), and **14** (▼).

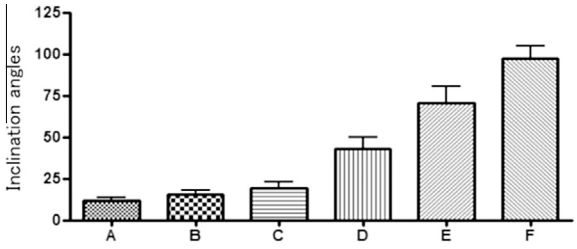

Figure 7. Lamina inclination recovery by BL treatment against the rice shoot treated with compound **14**. Rice plants for (A–E) were treated with 0.1 nmol of compound **14**. Treatment doses of BL were 0 (A), 0.001 nmol (B), 0.01 nmol (C), 0.1 nmol (D), and 1 nmol (E and F).

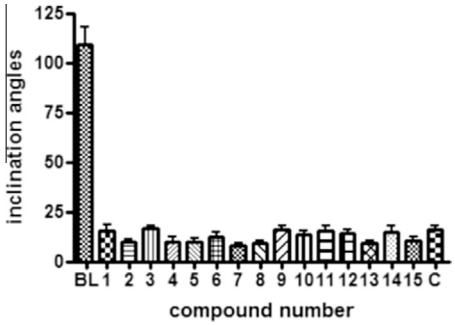

Figure 8. Brassinolide-like agonistic activity of compounds **1**–**15**. BL: brassinolide treatment (10 nmol/plant); C: control (ethanol).

by MacLogP; BioByte, Claremont, CA). We did not purchase compounds **16**–**18** due to the high cost and the delay of delivery.

Among 15 tested compounds (Fig. 3), three compounds (**1**, **2**, **14**) significantly suppressed the lamina inclination induced by

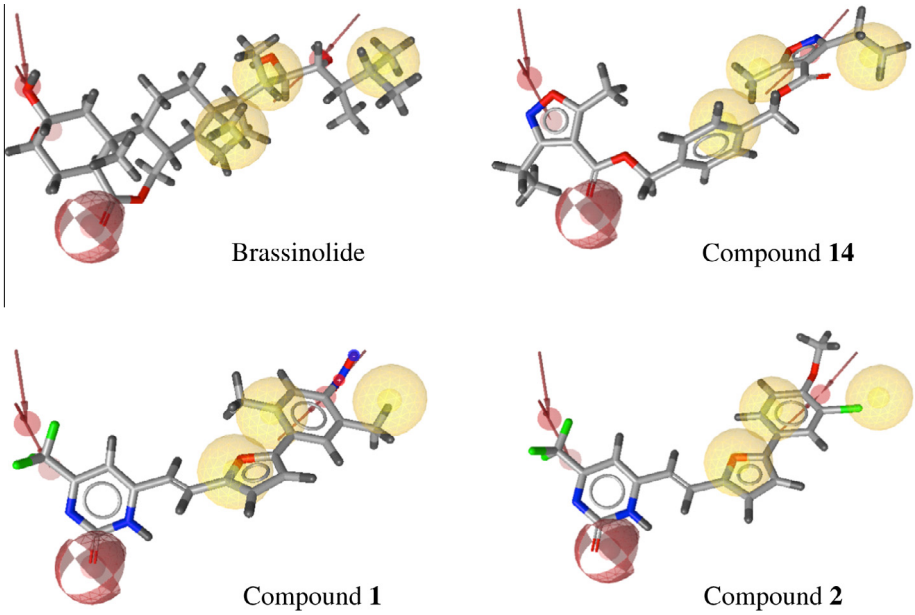

Figure 9. Superposition of spheres of pharmacophore on the structures of hit compounds.

BL treatment (10 nmol/plant) as shown in Figure 5. Compounds **1** and **2** have the same basic structure, phenyl-furanyl-vinyl-dihydropyrimidinone, with different substitutions at benzene ring. The compound **14** is 1,4-phenylenebis(methylene)bis(3-ethyl-5-methylisoxazole-4-carboxylate), which is able to be synthesized easily from isoxazole-4-carboxylic acids and 1,4-dibenzylalcohol. Therefore, these three structures are thought to be good leads for the development of BR antagonists as well as agonist.

Since these three antagonistic compounds (**1**, **2**, and **14**) strongly inhibited the hormonal effect of BL (1 nmol/plant) at the dose of 10 nmol/plant, the dose-response relationship was examined for these three compound (Fig. 6). The half maximum inhibition dose (ID_{50}) values of compounds **1**, **2**, and **14** were 5.0, 3.2, and 0.63 nmol/plant, respectively.

These antagonists have no phytotoxic effect against rice shoot at the dose less than 10 nmol/plant. In the further study, we examined the recovery action of BL against the antagonistic effect of compound **14**. The antagonistic effect caused by 0.1 nmol of compound **14** was released by the co-application of BL in a dose-dependent manner as shown in Figure 7.

Among 22 hit compounds, 10 compounds contain 2-phenyl-5-vinyl-furane moiety (**1**–**5**, **16**) or a similar furanyl moiety (**6**, **7**), and three compounds contain a piperazine ring moiety (**11**, **12**, and **18**). Unfortunately, none of the tested compounds exhibited agonistic activity in the lamina inclination assay as shown in Figure 8.

In order to understand the potential binding mode of these hits, the hydrogen bonds and hydrophobicity pharmacophore features were matched on their chemical structures. As shown in Figure 9, the N and O atoms of the isoxazole ring of compound **14** correspond to the oxygen atoms of 2-OH and 3-OH groups of BL, and the ester carbonyl oxygen atom of ester linkage of compound **14** corresponds to the carbonyl oxygen at C6 of BL. The carbonyl oxygen atom of the other ester group corresponds to the oxygen atom of 22-OH group. The benzene ring and the alkyl substituents (CH_3 and CH_2CH_3) of the isoxazole ring correspond to the hydrophobic features. With respect to compounds **1** and **2**, the carbonyl group of dihydropyrimidinone ring matches to the carbonyl group of A-ring of BL, and the CF_3 group corresponds to 2-OH group of A-ring of BL. Phenylfuranyl moiety of compounds **1** and **2** is creating the hydrophobic features in the pharmacophore.

In conclusion, we screened 22 compounds from the database containing 5 million structures database via in silico screening using LigandScout. Three compounds were active as BR antagonists against the lamina inclination assay. Among the three antagonistic compounds, the most potent compound **14** is the ester compound derived from 1,4-phenylenedimethanol and isoxazole-4-carboxylic acid. Even though no agonistic compound was found in this study, we started the bioassay for the screened compounds using other plant materials and the BL specific gene expression. Even though it is not easy to find active compounds using in silico screening, we could get three potent antagonists using in vivo test. Even though we could not get the BL agonists, the conversion of antagonists to agonists may be possible. There are many examples for the interconversion between agonists and antagonist for G-protein coupled receptor (GPCR).[22]

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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.bmcl.2016.02.054.

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18. Rice seeds were sterilized with 2% aqueous NaClO solution and germinated by soaking in the sterilized water for three days under light at 30 °C. The sprouted rice seeds were transplanted into the 1% agar medium and cultured for 3 days under light at 30 °C. The ethanol solution of test compound was applied to the second lamina part of the rice shoot after the application of 0.5 μL of indole-3-acetic acid (50 mM in ethanol). Treatments with 1 μL of EtOH and BL solution (1×10^{-9} M) were used as negative and positive controls. To evaluate the antagonistic activity, all shoots were treated with brassinolide (1 nmol/plant) before the application of each test compound.
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