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Rational Design of an Auxin Antagonist of the SCF^{TIR1} Auxin Receptor Complex

Ken-ichiro Hayashi,*[,†] Joshua Neve,[‡] Masakazu Hirose,[†] Atsuhito Kuboki, Yukihisa Shimada,[§] Stefan Kepinski,[‡] and Hiroshi Nozaki[†]

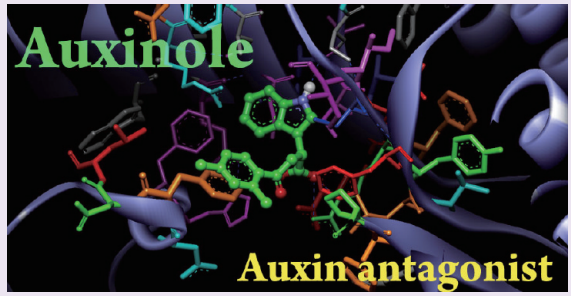
[†]Department of Biochemistry, Okayama University of Science, Okayama 700-0005, Japan

[‡]Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

[§]RIKEN Plant Science Center, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

Supporting Information

ABSTRACT: The plant hormone auxin is a master regulator of plant growth and development. By regulating rates of cell division and elongation and triggering specific patterning events, indole 3-acetic acid (IAA) regulates almost every aspect of plant development. The perception of auxin involves the formation of a ternary complex consisting of an F-box protein of the TIR1/AFB family of auxin receptors, the auxin molecule, and a member the Aux/IAA family of co-repressor proteins. In this study, we identified a potent auxin antagonist, α-(phenylethyl-2-oxo)-IAA, as a lead compound for TIR1/AFB receptors by *in silico* virtual screening. This molecule was used as the basis for the development of a more potent TIR1 antagonist, auxinole (α-[2,4-dimethylphenylethyl-2-oxo]-IAA), using a structure-based drug design approach. Auxinole binds TIR1 to block the formation of the TIR1−IAA−Aux/IAA complex and so inhibits auxin-responsive gene expression. Molecular docking analysis indicates that the phenyl ring in auxinole would strongly interact with Phe82 of TIR1, a residue that is crucial for Aux/IAA recognition. Consistent with this predicted mode of action, auxinole competitively inhibits various auxin responses in planta. Additionally, auxinole blocks auxin responses of the moss *Physcomitrella patens*, suggesting activity over a broad range of species. Our works not only substantiates the utility of chemical tools for plant biology but also demonstrates a new class of small molecule inhibitor of protein−protein interactions common to mechanisms of perception of other plant hormones, such as jasmonate, gibberellin, and abscisic acid.



The plant hormone auxin plays a central role in plant growth and development. Indole 3-acetic acid (IAA), the predominant naturally occurring auxin, controls the cell division and elongation and triggers specific differentiation events (Figure 1). As such, this small molecule regulates numerous and diverse developmental processes such as the maintenance of embryo polarity, vascular differentiation, apical dominance, and tropic responses to light and gravity.[1] Auxin is perceived by TIR1/AFB (Transport Inhibitor Response 1/Auxin signaling F-Box protein) auxin receptors, which prompts the ubiquitin-dependent degradation of a family of Aux/IAA transcriptional repressor proteins leading to the derepression of auxin-responsive genes: Aux/IAA repressors dimerize with members of the auxin response factor (ARF) family of DNA-binding transcription factors to block the transcriptional function of ARFs. The TIR1/AFB auxin receptors are F-box proteins that form part of an SCF-type E3 ubiquitin−ligase complex that catalyzes the addition of ubiquitin to target substrates. F-box proteins are responsible for the recognition of specific target proteins for ubiquitination in conjunction with the core catalytic proteins of the SCF complex, Skp1 (ASK1), Cullin (CUL1), and RBX. In the case of Aux/IAA targets, auxin enhances the interaction of Aux/IAAs with SCF$^{TIR1/AFB}$ complexes, thereby promoting the ubiquitination and consequent degradation of Aux/IAAs in the 26S proteasome.[2−4]

Structural analysis of the TIR1 receptor in complex with IAA and a short motif in domain II of the Aux/IAA protein that is required for interaction with TIR1 (known as the domain II degron) has illustrated the molecular mechanism of auxin perception (Figure 2a and b). Both IAA and the Aux/IAA bind to the same pocket of TIR1.[4] IAA nestles on the base of binding pocket, and the WPPV motif of Aux/IAA domain II degron binds on top of IAA. The tryptophan and second proline residues in WPPV are positioned close to the aromatic ring of IAA, interacting by hydrophobic bonds. In this model, Aux/IAA functions as co-receptor with TIR1, forming a small hydrophobic cavity to trap IAA. Thus auxin works as a "molecular glue" that increases the affinity of the ternary TIR1-IAA-Aux/IAA complex by increasing the extent of the hydrophobic interactions among the components.[3,4]

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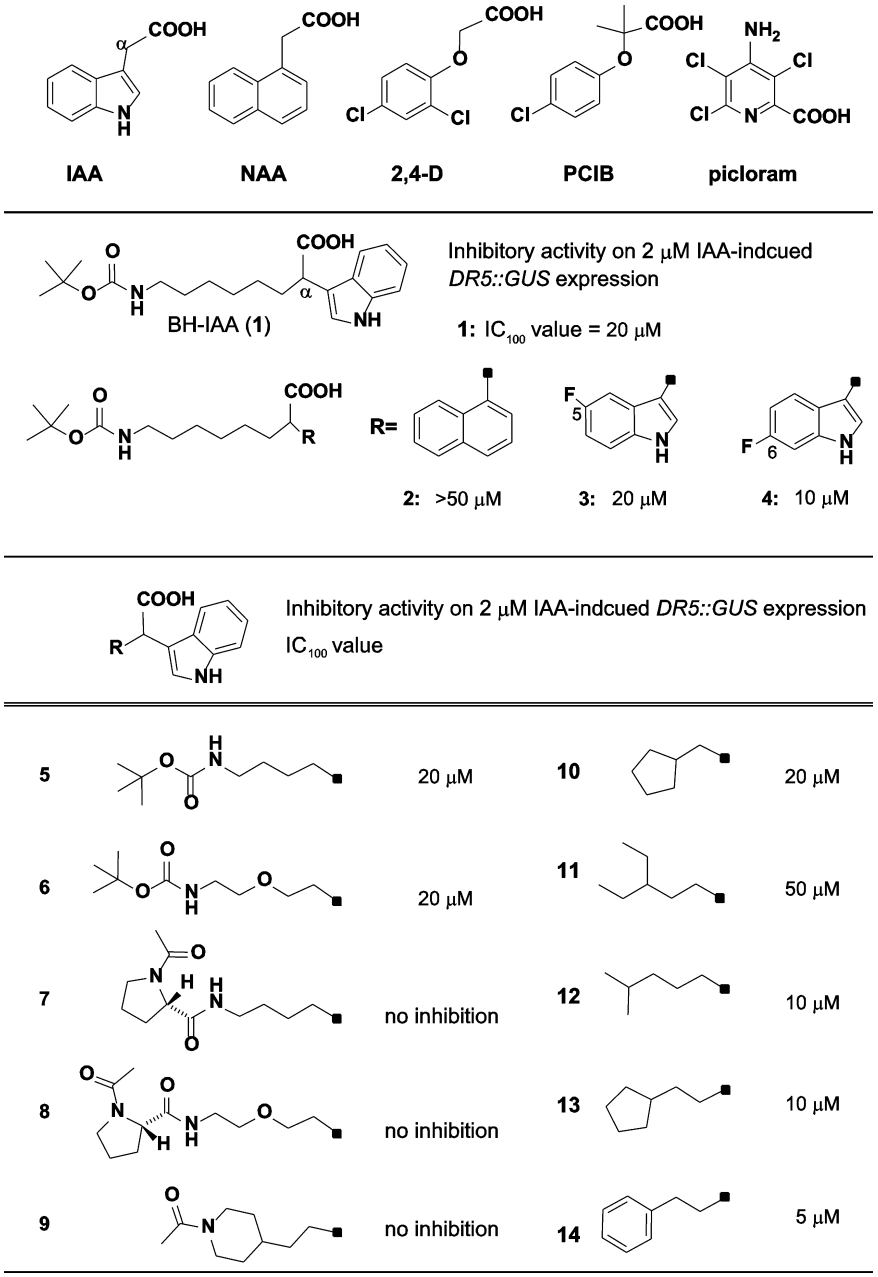

Figure 1. Structures of auxins and α-alkyl-indole 3-acetic acids. The IC_{100} value represents the concentration required for the complete inhibition for auxin-responsive *DR5::GUS* expression induced by 2 μM IAA (Supplementary Figure 1).

Forward and reverse genetic analysis in the model plant *Arabidopsis thaliana* has identified a fundamental signaling mechanism that can account for how changes in auxin concentration can be translated into transcriptional change. The *Arabidopsis* genome encodes 6 TIR1/AFB receptors, 29 Aux/IAA repressors, and 23 ARF transcription factors,[2] and these families of auxin signaling components regulate complex and cell-type-specific responses to auxin. Functional redundancy between members of these multigene families has often hindered the genetic analysis of the contribution of individual genes and proteins in auxin response at specific developmental stages.[5] Conversely, the disruption of multiple auxin receptors can result in embryo lethality because of the pivotal role of auxin in embryogenesis.[6] Furthermore, although a role for auxin in the development of diverse land plants (liverworts, moss, ferns, gymnosperms, and angiosperms) is suggested by the conservation of TIR1/AFB-Aux/IAA-ARF signaling components across these species, a lack of genetic, molecular genetic, and genomic resources has made it difficult to assess the physiological role of auxin among species with such disparate morphologies and life cycles.[5,7]

To overcome some of these difficulties and as a complement to genetic analysis, the use of small molecules to specifically modulate aspects of auxin signal transduction represents an attractive tool for auxin biology. Inhibitors of hormone biosynthesis, for example, would be useful chemical tools to reversibly block hormonal action in the plant, but this is often only achievable where the hormone is produced by a single linear pathway, as in the case of gibberellic acid and brassinosteroids. Inhibitors of the biosynthesis of these plant hormones have been developed and are widely used as chemical tools to study their action in a number of species.[7]

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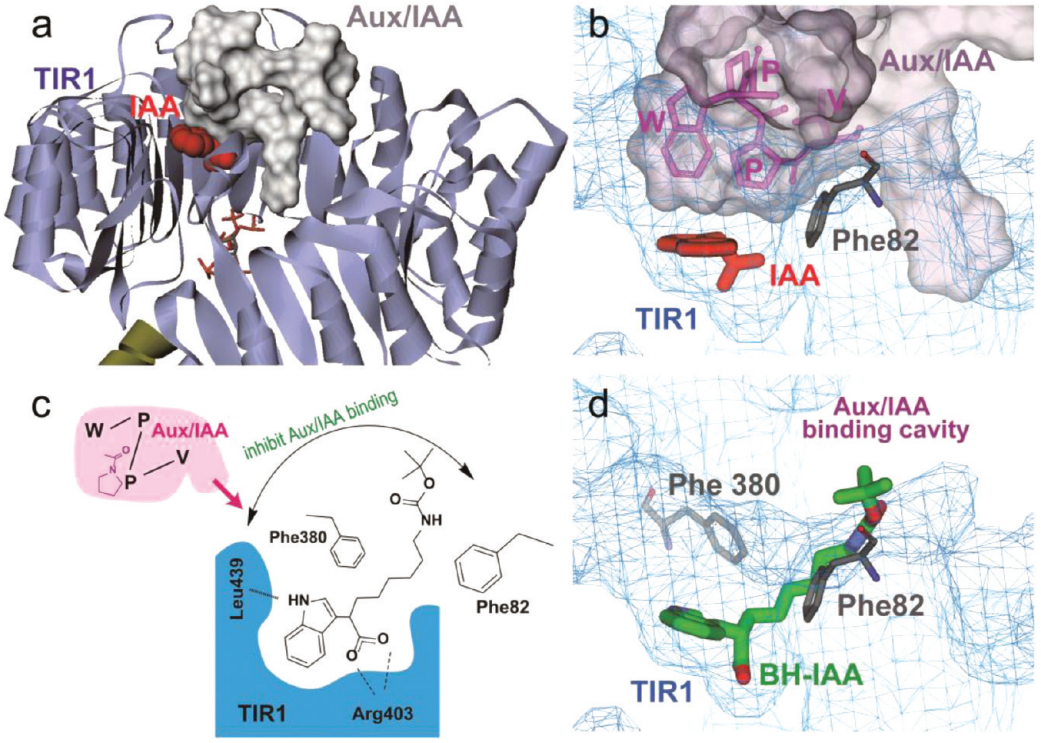

Figure 2. Molecular structure of auxin perception of TIR1 auxin receptor and Aux/IAA. (a, b) Crystal structure of TIR1−ASK1−IAA−Aux/IAA (PDB ID 2P1Q). IAA is captured in small hydrophobic cavity formed by TIR1 and WPPV motif of the domain II degron peptide of Aux/IAA protein. (c, d) Molecular mechanism of auxin antagonist BH-IAA action; BH-IAA blocks the interaction between TIR1 and Aux/IAA. BH-IAA binds to same binding pocket as IAA, and the long alkyl chain is oriented to the Aux/IAA binding site. The structure of BH-IAA−TIR1−ASK1 was based on PDB ID 3C6N.

In contrast, auxin is synthesized by several redundant pathways, and most of the enzymes in the pathway have not been identified.[8] Inhibitors of auxin signaling from both natural and synthetic sources have been investigated more extensively, but in most cases, the target proteins of the inhibitor has not been determined.[5,9−11] For example (Figure 1), in early studies of synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene 1-acetic acid (1-NAA), and picloram, some auxin analogues that show auxin antagonist activity were discovered, and these analogues were termed anti-auxins.[5,12] *p*-Chlorophenoxy isobutylic acid (PCIB) is an inactive analogue of *p*-chlorophenoxyacetic acid, a synthetic auxin that has been used as an anti-auxin and is believed to bind the auxin receptor in competition with auxin. However, PCIB cannot fully antagonize auxin responses, acting only as partial agonist of auxin in some instances.[13] The target and mode of action of PCIB are yet to be determined.

We have previously demonstrated that *tert*-butoxycarbonylaminohexyl-IAA (BH-IAA, **1**), α-alkyl-IAA, specifically binds to the auxin binding site of TIR1 receptor to act as an auxin antagonist.[14] BH-IAA perturbs the interaction of Aux/IAAs and TIR1 thereby blocking the degradation of Aux/IAA repressors and so responses to auxin at the cellular and whole-plant level. The molecular mechanism of action was illustrated by the crystallographic analysis of the TIR1−BH-IAA complex (Figure 2c and d).[14] BH-IAA is the first specific auxin antagonist of TIR1/AFB auxin receptors. BH-IAA is an effective chemical tool for dissecting auxin action beyond plant species. However, while BH-IAA is a specific inhibitor of TIR1/AFB receptors, it is not a potent antagonist because of the relatively low affinity of BH-IAA for TIR1. Although BH-IAA shows the same binding affinity to the auxin recognition site of TIR1 as IAA, because it acts by blocking the binding of the Aux/IAA protein, the binding of BH-IAA to TIR1 is not stabilized by the formation of the ternary complex with the Aux/IAA.[14]

Here we present a potent auxin antagonist of TIR1/AFB receptors designated as auxinole (Figure 4). The antagonist was designed on the basis of the crystal structure of the TIR1−BH-IAA complex and by *in silico* screening of TIR1 ligands from chemical libraries. After the lead compound optimization, auxinole was found to show a high affinity for TIR1/AFB receptors and displayed potent antagonistic activity on TIR1/AFB-mediated auxin responses in planta. Auxinole, represents the next generation of auxin antagonist and offers new and powerful tool for auxin biology.

RESULTS AND DISCUSSION

Ligand Based Design of Auxin Antagonist on TIR1 Auxin Receptor. Structural analysis of auxin receptor TIR1 demonstrated that auxin binds to the surface of TIR1 receptor, and then auxin is captured within the small hydrophobic cavity formed by TIR1 and WPPV motif in the Aux/IAA degron (Figure 2a and b).[4] This suggests that Aux/IAAs function as co-receptors, enhancing the binding of auxin to the receptor complex. The structure of the TIR1−BH-IAA complex illustrated the molecular mechanism of BH-IAA, anti-auxin activity. The IAA moiety of BH-IAA binds to the auxin binding site of TIR1 in a similar way to IAA, but the alkyl chain is oriented into the Aux/IAA binding site.[14] The coordinates of the long alkyl chain are disordered thereby preventing the docking of the Aux/IAA to TIR1, resulting in the inhibition of SCF^{TIR1} mediated ubiquitination of Aux/IAA (Figure 2c and

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d). Thus, BH-IAA blocks the degradation of Aux/IAA repressors and the proper regulation of downstream auxin-regulated processes. BH-IAA interacts with TIR1 receptor only at the site of IAA moiety, but unlike IAA, Aux/IAAs cannot bind to the TIR1−ligand complex to stabilize the interactions, suggesting that BH-IAA would show affinity considerably lower than that of IAA in terms of overall TIR1 binding. To design highly active antagonists, we modified IAA moiety of BH-IAA to increase the affinity of IAA moiety to the TIR1 binding site. The auxin antagonistic activity was initially evaluated by the *Arabidopsis* transgenic plant line *DR5::GUS*.[15] This reporter line harbors β-glucuronidase (GUS) reporter gene under the control of the synthetic auxin-inducible *DR5* promoter containing a composite auxin responsive *cis* elements that are regulated by ARF transcription factors. The regulation of GUS expression in this line is therefore directly related to the capacity of auxin to bring about the proteolysis of Aux/IAA proteins via the $SCF^{TIR1/AFB}$ signaling mechanism. GUS protein expression was monitored histochemically using the chromogenic GUS substrate X-Glc or by quantitative fluorometric measurement using the fluorogenic substrate 4-MUG (Figure 3 and Supplementary Figure 1). The natural auxin IAA and the potent synthetic auxin 1-NAA strongly induce GUS expression, while BH-IAA inhibits auxin-induced *DR5::GUS* expression in a dose-dependent manner (Figure 3 and Supplementary Figure 1). 1-NAA has a naphthalene ring, rather than the indole ring of IAA, and the replacement of indole ring of BH-IAA to naphthalene ring (**2**) reduced the inhibitory activity on *DR5::GUS* expression (Supplementary Figure 1). This is consistent with data that show that IAA binds more strongly to TIR1 than 1-NAA due to the hydrogen bond between indole amino group and Leu439 amide group (Figures 2c and 5a).[4] Halogenated IAA derivatives such as 4-chloro-IAA and 6-fluoro-IAA have been reported to show potent auxin activity.[16] The introduction of fluoride into C-5 of the indole ring of BH-IAA (**3**) reduced its anti-auxin activity in *DR5::GUS* assays, and 6-fluoro-BH-IAA (**4**) displayed anti-auxin activity slightly higher than that of BH-IAA (Supplementary Figure 1), although both 5- and 6-fluoro-IAA have previously been reported to show potent auxin activity.[16] These results suggest that the modification of IAA moiety in BH-IAA would not improve significantly the affinity of the molecule for the TIR1/AFB receptors.

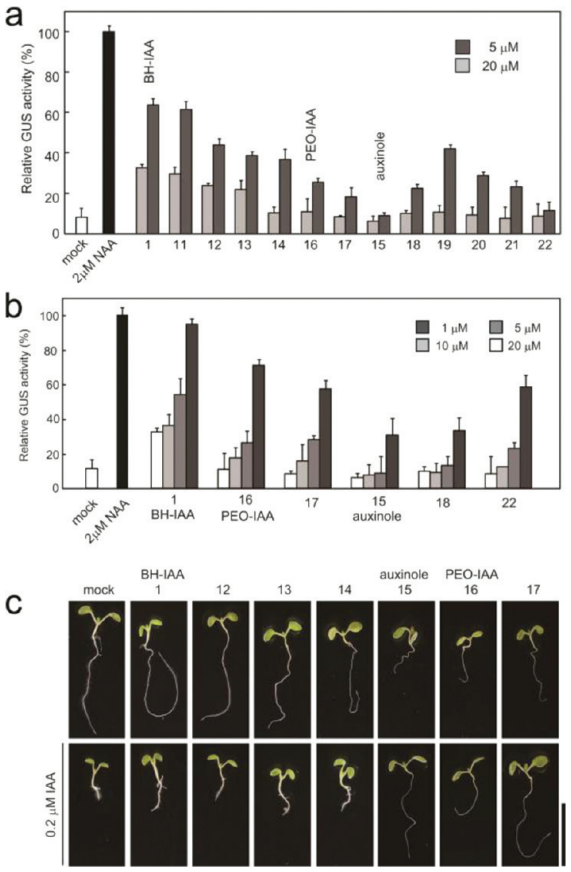

Figure 3. Effects of auxin antagonists (**11**−**22**) on auxin-responsive *DR5::GUS* reporter gene expression and auxin-inhibited root growth of *Arabidopsis* seedling. (a, b) Auxin antagonistic activity of ligands **11**−**22**. Five-day-old *Arabidopsis* auxin-responsive *DR5::GUS* reporter line was incubated in a medium containing ligands with or without 2 μM 1-NAA for 5 h. GUS enzyme activity was determined fluorometrically. Relative GUS activity induced by 2 μM 1-NAA is shown as 100% value. Error bars, SD (n = 10−15). (c) *Arabidopsis* seedlings were grown with 20 μM ligands in the absence [upper panel] or presence [lower panel] of 0.2 μM IAA on GM plates solidified with 0.1% gellan gum for 6 days under continuous light. Scale bar, 10 mm.

In the structure of the TIR1−IAA−Aux/IAA complex, the second proline residue in WPPV motif of Aux/IAA is crucial for the binding to TIR1 by hydrophobic bonding with a phenylalanine (Phe82 and Phe380) in the TIR1 auxin binding cavity (Figure 2).[4] Phe82 especially plays a pivotal role in the binding of both IAA and Aux/IAA.[17] Therefore, the introduction of a hydrophobic group at the α-position of IAA would be predicted to enhance the hydrophobic interaction of the IAA ligand with Phe82 of TIR1, and such a ligand would be expected to show higher binding affinity to TIR1. To test this idea we initially converted the aminohexyl chain of BH-IAA to a short aminobutyl chain (**5**) or the glycol chain (**6**). Ligands **5** and **6** exhibited the same activity as original BH-IAA (Supplementary Figure 1). We next attempted to design ligands that mimic the proline residues in WPPV of Aux/IAA by linking L-proline with the alkyl chain at the α-position of IAA (**7** and **8**), and a proline analogue chain was introduced to the α-position of IAA (**9**). However, ligands **7**−**9** did not show any inhibitory activity on *DR5::GUS* expression (Supplementary Figure 1), despite having high affinity values that in terms of docking score were equivalent to that of BH-IAA (**1**). We next introduced the simple alkyl ring and branched alkyl chain at the α-position of IAA. The ligands **10** and **11** inhibited *DR5::GUS* expression to the same extent as **1** (Figure 3a and Supplementary Figure 1) and ligands **12**−**14** showed more potent inhibition than **1** (Figure 3a). Especially, the phenylethyl IAA ligand (**14**) displayed most potent inhibition among examined ligands. The predicted binding orientation of ligand **14** in the TIR1 auxin receptor by Surflex molecular docking software[18] suggested the phenylethyl group was positioned close enough to Phe82 to form hydrophobic bonds (Supplementary Figure 2B).

Receptor-Based Design of Auxin Antagonist by Mean of *in Silico* Screening. To design ligands with high affinity to the TIR1 auxin binding site effectively, we performed *in silico* structural screening using Surflex molecular docking software[18] to find a lead structure for further development. The structural study for the TIR1−BH-IAA complex demonstrated that binding of BH-IAA did not elicit any conformation change of TIR1, as with the TIR1−IAA complex.[14] Therefore, the TIR1 structure would be a suitable target for the protein−ligand

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docking calculation because the conformational change induced by ligand binding would not seriously affect the docking accuracy. The chemical structure of the small molecule was obtained from the ZINC structure database,[19] and the structures were filtered by the indole substructure and drug-likeness structure to build a indole focused library (7176 chemicals with indole moiety). This library was docked against the auxin binding site of TIR1 receptor (PDB ID 2P1P) by using Surflex 2.3 docking software. Two hundred high-scored compounds were further evaluated by scoring software, X-Score[20] and DrugScore X.[21] The highest-scored chemicals (representing 1.5% of the total compound docked) were visually inspected and selected on the basis of their binding pose and interactions within the receptor. The entire docking analysis is described in Supporting Information.

Following the *in silico* screening, top-scored ligands were examined by *DR5::GUS* reporter assay. Among the high scoring ligands, α-(phenylethyl-2-oxo)-IAA (PEO-IAA, **16**, ZINC ID 350707) and its derivative (**17**, ZINC ID 517812 and **21**, ZINC ID 110177) showed very potent inhibitory activity on *DR5::GUS* expression (Figures 3a,b and 4). To optimize the

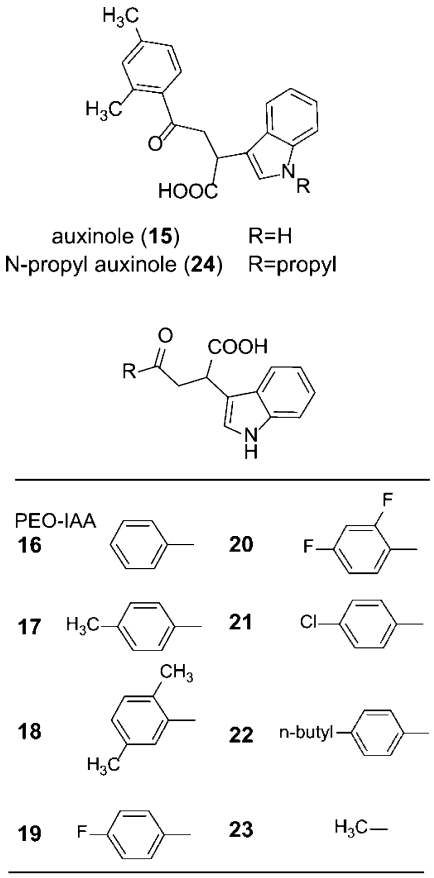

Figure 4. Chemical structures of auxinole (**15**) and derivatives (**16**–**23**).

affinity of PEO-IAA lead compound to TIR1, PEO-IAA derivatives were designed according to predicted binding position by molecular docking calculation (Figure 4). Ligand **22** is designed to show the efficient inhibition of Aux/IAA docking because of the *n*-propyl chain at the *para* position of PEO-IAA. In the *DR5::GUS* assay, ligand **22** slightly enhanced the inhibitory activity (Figure 3b). The halogenation of the phenyl ring (**19**–**21**) of PEO-IAA did not improve the inhibitory activity (Figure 3a). The methylation of the phenyl ring (**15**, **17**, **18**) of PEO-IAA greatly increased the inhibitory activity (Figure 3b). Among them, ligand **15** displayed most potent inhibition on *DR5::GUS* expression, and we designated **15** as auxinole (Figure 4).

Auxin inhibits primary root growth and promotes the formation of root hairs and lateral roots. To examine the effects of TIR1 ligands (BH-IAA **1** and ligands **12**–**17**) on physiological and morphological auxin responses, *Arabidopsis* seedlings were grown for 6 days in the presence of IAA and ligands. IAA at 0.2 μM inhibited primary root elongation (Figure 3c). The α-alkyl-IAA ligands (BH-IAA, **12**–**14**) at 20 μM did not fully recover root inhibition by IAA. PEO-IAA (**16**) at 20 μM partially restored IAA-inhibited root growth, and auxinole (**15**) and *p*-methyl PEO-IAA (**17**) fully recovered the IAA-induced root phenotype. The antagonistic activity of TIR1 ligands (**12**–**17**) in root auxin response was consistent with the inhibitory activity on *DR5::GUS* assay. Additionally, TIR1 ligands (**15**–**17**) alone inhibited the growth of seedlings, and these effects could be reversed by IAA application (Figure 3c). In summary, we found auxinole (**15**) to be the most potent inhibitor of endogenous auxin action (Figure 3c).

Molecular Model for the Mechanism of Auxin Antagonists on TIR1/AFB Receptor. Molecular docking analysis of auxinole (**15**) and PEO-IAA (**16**) reveals the mechanism of TIR1 ligand action (Figure 5a, Supplementary Figure 2B and C). The IAA moiety of ligands (**15** and **16**) was positioned with the same coordination as IAA. The phenyl ring of both ligands (**15** and **16**) was stacked with Phe82 of TIR1 to form π–π hydrophobic interaction and prevent the access of the second proline residue of WPPV motif in the Aux/IAA protein (Figure 5a, Supplementary Figure 2B and 2C). Unlike auxinole, the long alkyl chain of BH-IAA (**1**) was directed to Aux/IAA binding cavity, but the disordered alkyl chain did not contribute to the affinity for TIR1 (Figure 2).[14] Therefore, in comparison with BH-IAA, the tight π–π stacking between the phenyl ring of ligands **15** and **16** and Phe82 of TIR1 would contribute significantly to the high affinity of ligands **15** and **16** binding to TIR1. Additionally, the molecular dynamics simulations of the TIR1–auxin complex has highlighted that the conformational change of the side chain of Phe82 after auxin binding is important for the subsequent Aux/IAA binding.[17] The PEO-IAA derivatives (**15**–**22**) might perturb the function of Phe82 for Aux/IAA binding. The introduction of two methyl groups to the phenyl ring (**15**) significantly enhanced the antagonistic activity in *DR5::GUS* assays. Docking analysis implies that the dimethyl groups would limit the rotation of the phenyl ring of auxinole (**15**) and consequently increase the affinity derived from π–π stacking with Phe82 (Supplementary Figure 2C). α-Phenylethyl-IAA (**14**) is an analogue of PEO-IAA (**16**) and lacks the 2-ketocarbonyl group. Docking calculations show that the phenyl ring of **14** was near Phe82, but unlike **16**, the conformation of phenyl rings (**14**) were not convergent (Supplementary Figure 2B). This suggests that π–π stacking between **14** and Phe82 of TIR1 would be less than with PEO-IAA (**16**). Consistent with this, the estimated affinity value of **16** ($-\log K_d$ = 10.20) to TIR1 was higher than that of **14** ($-\log K_d$ = 9.34). The affinity of IAA was estimated to be 7.63 ($-\log K_d$) in same calculation conditions (Supplementary Figure 2A).

To confirm our model for the mechanism of auxinole action based on docking study, we designed auxin agonist α-(2-

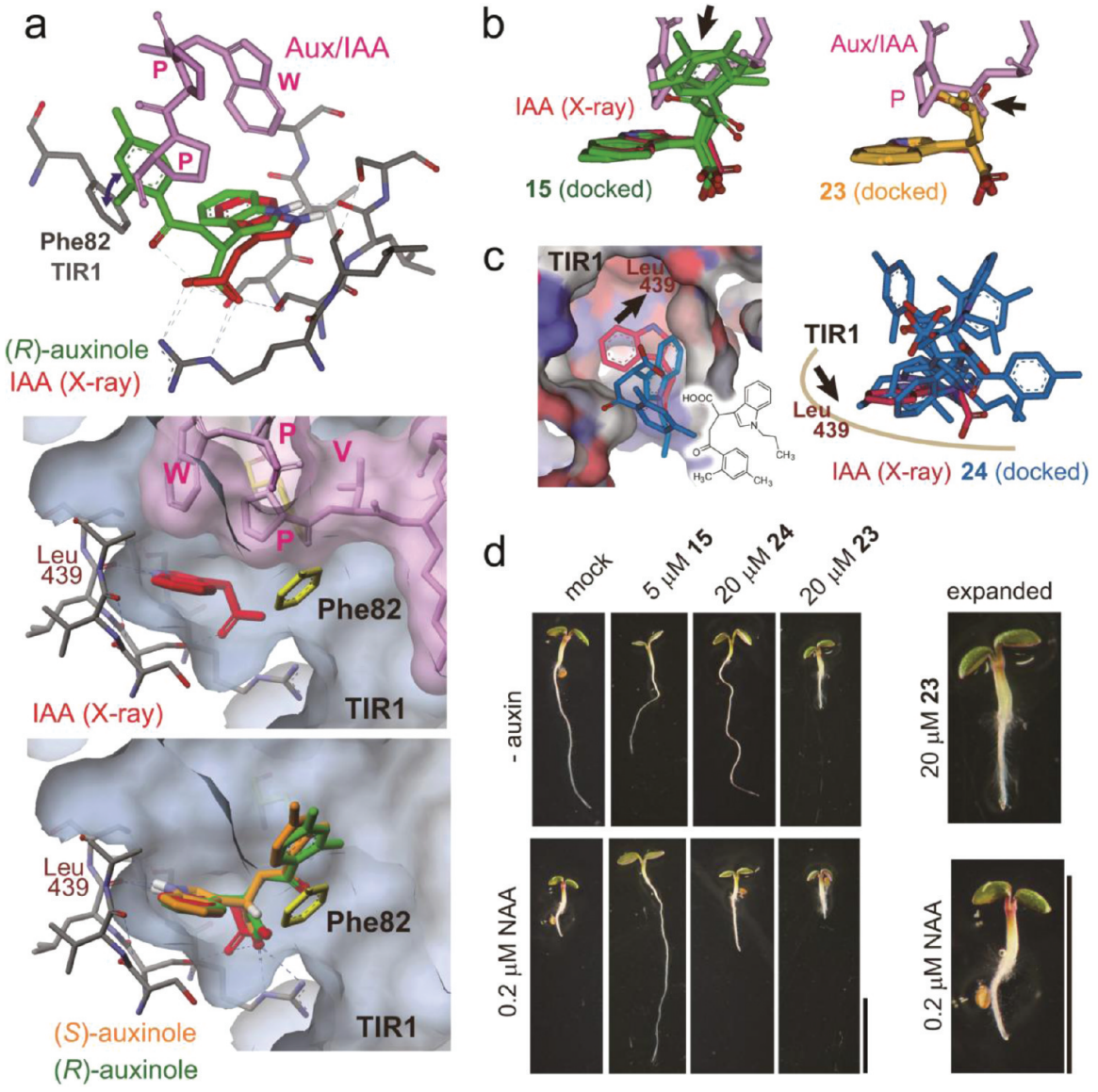

Figure 5. Molecular model for the action of auxinole (**15**) and its analogues (**23** and **24**) on the TIR1 receptor. (a) The predicted binding pose of (*R*)- and (*S*)-auxinole in TIR1 by docking calculation. Aux/IAA, IAA7 (pink colored) and IAA (red colored) were imposed on the coordinates in the crystal structure (PDB ID 2P1Q). (b) The predicted binding pose of auxinole (**15**) and α-(2-oxopropy)-IAA (**23**) in the TIR1 binding site. Aux/IAA, IAA7 (pink colored) was superimposed. (c) The predicted binding pose of *N*-propyl auxinole (**24**) in the TIR1 binding site. The top 3 conformers scored by docking calculation were represented for panels b and c. (d) *Arabidopsis* seedlings grown for 5 days on GM media containing ligands **15**, **23**, and **24** with or without 1-NAA under continuous light. Scale bar, 5 mm.

oxopropyl)-IAA (**23**) and an inactive antagonist, *N*-propyl-auxinole (**24**). We previously demonstrated that α-propyl-IAA bind to TIR1 and works as a weak auxin.[14] The molecular structure of α-propyl-IAA and TIR1 complex was resolved by X-ray crystal analysis. The propyl chain of α-propyl-IAA was fit within the small cavity formed by Aux/IAA and does not block Aux/IAA binding. The α-(2-oxopropyl)-IAA (**23**) lacks the phenyl ring of auxinole and could not interact with Phe82 of TIR1 (Figures 4 and 5b). Docking study predicts that **23** would be a weak auxin. The 2-oxopropyl chain of **23** would fit within the small vacant space under the Aux/IAA (Figure 5b). As expected, ligand **23** displayed no auxin antagonistic activity on *DR5::GUS* and root growth assay but instead showed weak auxin activity at 20 μM (Supplementary Figure 3 and Figure 5d).

The NH group of indole in IAA would interact with the amide group of Leu439 (Figure 5a).[4] The N-alkylation of IAA moiety of auxinole dramatically reduces the anti-auxin activity, because the N-alkyl chain prevents the proper binding orientation in TIR1 active site (Figure 5c). As expected from the inappropriate docking pose of *N*-propyl auxinole (**24**) in TIR1, ligand **24** was inactive as an antagonist in *DR5::GUS* and root growth inhibition assays (Supplementary Figure 3 and Figure 5d). This evidence from auxinole analogues (**23** and **24**) suggests that auxinole binds to the TIR1 auxin binding site in manner similar to that of IAA, and the phenyl ring of auxinole would play a critical role for the high affinity to TIR1, probably through the π−π stacking with Phe82 of TIR1. The synthesized compounds are all in a racemic form. The docking study was performed for each enantiomer of ligands (Figure 5a), but the biological activity of ligand was assayed against racemic form.

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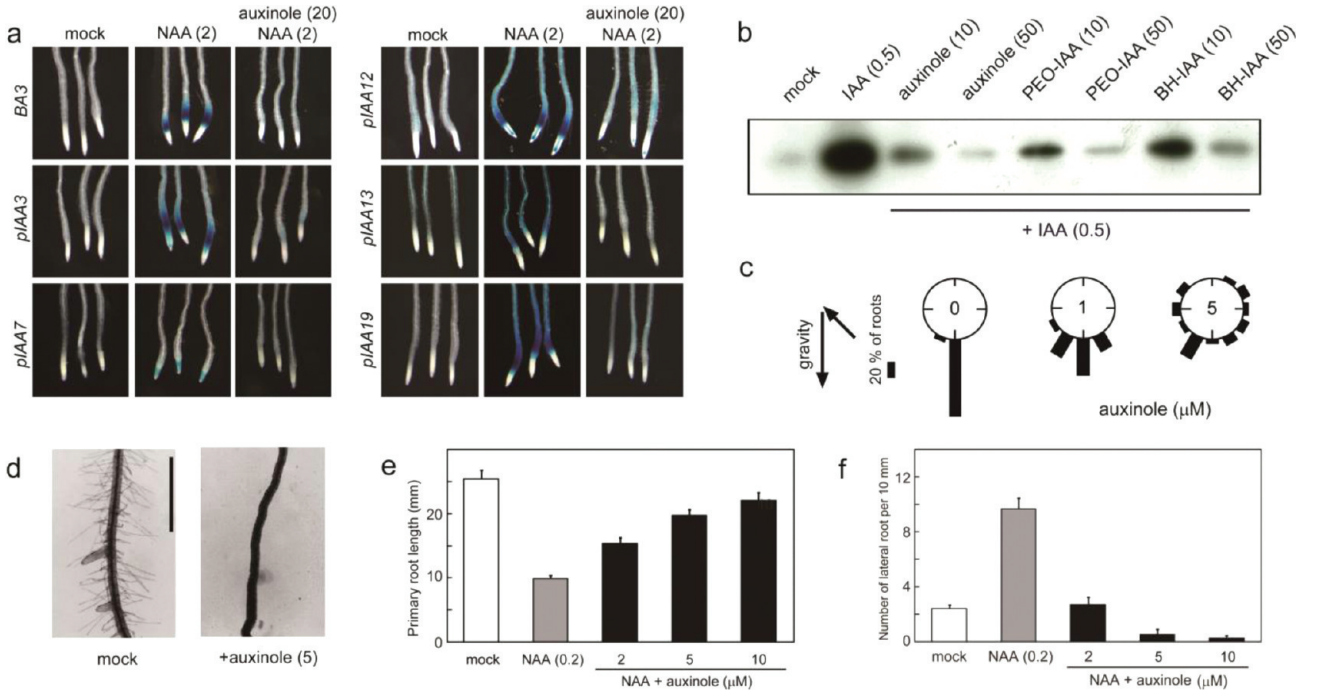

Figure 6. Effect of auxinole on auxin-regulated process in *Arabidopsis* plant. (a) Effects of auxinole on GUS expression in the root of the native early auxin-responsive reporter lines. Five-day-old seedlings were treated with 20 μM auxinole (**15**) and 2 μM 1-NAA for 10 h. (b) Effects of auxinole on auxin-enhanced interaction between TIR1 and Aux/IAA. Pull-down assays using a FLAG-tagged TIR1 and synthetic Aux/IAA domain II peptide in the presence of 0.5 μM IAA and antagonists (auxinole **15**, PEO-IAA **16**, and BH-IAA **1**) at indicated concentration (in μM) in parentheses. (c) Effects of auxinole on *Arabidopsis* root gravitropism. Five-day-old *Arabidopsis* seedling were vertically grown in the first gravity direction (first g) with 1 and 5 μM of auxinole and then rotated at an angle of 135° (second g) and cultured for a additional 24 h. The angles were grouped into 30° classes and plotted as circular histograms (n = 40). (d) *Arabidopsis* root hair growth with 5 μM of auxinole for 5 days. Scale bar, 1 mm. (e, f) Effect of auxinole on auxin-induced primary root growth inhibition and lateral root formation. Four-day-old vertically grown seedlings (8.7 ± 0.8 mm of primary root length) were transferred to GM plates containing auxinole and/or 0.2 μM 1-NAA and incubated for additional 3 days. The primary root length (e) and number of lateral roots (f) were measured. Error bar and values, SD (n = 15–20).

With auxinole, a similar binding orientation in TIR1 is predicted for both enantiomers (Figure 5a, Supplementary Figures 2C and 6B). The binding of optically active auxin antagonists to TIR1 will be the subject of future work.

Auxinole Blocks the Binding of Auxin to TIR1/AFB Receptor and Subsequent Auxin Responses in Planta. To examine the effects of auxinole on the expression of native $SCF^{TIR1/AFB}$-regulated auxin responsive genes, six early auxin-responsive Aux/IAA promoter::GUS transcriptional reporter lines for the *Arabidopsis Aux/IAAs IAA3*, *IAA7*, *IAA12*, *IAA13*, and *IAA19* [22−24] and composite auxin-responsive *BA3* synthetic promoter[25] (derived from a pea Aux/IAA promoter) were subjected to histochemical analysis (Figure 6a). 1-NAA at 2 μM induced GUS expression in the roots of seedlings, and this induction was blocked by co-incubation with auxinole at 20 μM. Both auxinole (**15**) and PEO-IAA (**16**) blocked auxin-responsive gene expression induced by exogenous auxin (Figures 3a,b and 6a). This suggests the direct inhibition of auxin biosynthesis is unlikely to be the basis of auxinole and PEO-IAA action. To exclude this possibility, we measured the endogenous IAA level after the incubation of auxinole and PEO-IAA. Both antagonists at 100 μM slightly reduced endogenous IAA level (>20% inhibition) but had no inhibition at an effective dose at 10 μM (Supplementary Figure 4).

To confirm the competitive binding of auxinole (**15**) and PEO-IAA (**16**) to the TIR1 active site, we performed pull-down assays with FLAG-tagged TIR1 and biotin-tagged Aux/IAA degron peptide (IAA7 peptide).[26] In the presence of IAA, the FLAG-TIR1 signal was detected after the pull-down with biotin-tagged IAA7 from the extract of plants expressing FLAG-TIR1 transgene (Figure 6b). As previously reported, BH-IAA (**1**) reduced the IAA-dependent TIR1 signal indicating BH-IAA binds to TIR1 to block the formation of the TIR1−IAA−Aux/IAA complex. The inhibitory effect of PEO-IAA (**16**) at 10 μM was more potent than that of BH-IAA, and auxinole (**15**) showed the most potent inhibition by this assay (Figure 6b). The rank of inhibitory activities of the ligands in pull-down assays was consistent with the results of *DR5::GUS* reporter and root growth assays. These results indicate that auxinole and PEO-IAA bind to auxin binding site of TIR1 and show higher affinity binding to TIR1 than BH-IAA.

We next examined the biological effects of auxinole on the typical auxin response in the *Arabidopsis* plant. Auxin inhibits primary root growth but promotes the root hair and lateral root formation. Auxin also regulates the responses to gravity.[1] All of these auxin-related root responses are mediated by the SCF^{TIR1} pathway.[1−3] Consistent with the potent anti-auxin activity of auxinole on the SCF^{TIR1} pathway, auxinole at 5 μM completely inhibited root gravitropism and root hair formation (Figure 6c and d). Auxinole also antagonized with auxin to block typical root auxin responses, primary root inhibition (Figure 6d) and lateral root promotion (Figure 6f).

Auxinole inhibited the growth of *Arabidopsis* seedlings in a dose-dependent manner by blocking endogenous auxin action. *Arabidopsis* seedlings grown with auxinole at 10 μM for 7 days showed severe phenotypes (Figure 7a and Supplementary

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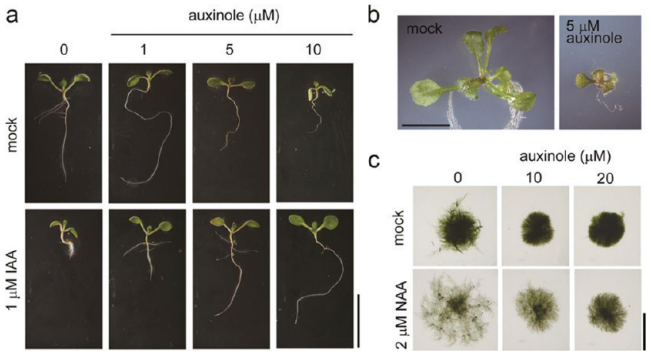

Figure 7. Antagonistic effects of auxinole on phenotypes of *Arabidopsis* plant and moss *P. patens*. (a) *Arabidopsis* seedlings were grown with auxinole in the absence [upper panel] or presence [lower panel] of 0.2 μM IAA on GM media for 7 days under continuous light. (b) Fourteen-day-old *Arabidopsis* plants grown on GM media with/without 5 μM auxinole. (c) Effects of auxinole on development of chloronemata in *P. patens*. The chloronema cells were cultured for 17 days with/without auxinole and 1-NAA. (Scale bars = 10 mm in panels a, b and 5 mm in panel c).

Figure 5A) that resemble the quadruple *tir1/afb* auxin receptor mutants (*tir1 afb1 afb2 afb3*).[6] IAA at 1 μM completely counteracted this effect of low concentration of auxinole on root elongation (Figure 7a). The synthetic auxins 1-NAA and 2,4-D also showed antagonistic effects on auxinole action in planta. (Supplementary Figure 5A). Long-term cultivation in the presence of auxin antagonists would be predicted to result in severe developmental defects because of changes in auxin homeostasis and response.[14] Indeed, 2-week-old *Arabidopsis* plants grown in the presence of 5 μM auxinole showed such severe defects that phenocopy SCF^{TIR1} signaling mutants, *axr1* and *axr2* (Figure 7b).[14]

Auxinole Can Overcome the Redundant Function of TIR1/AFB Receptors and Ancient TIR1 in Lower Plants. The SCF^{TIR1} pathway functions as primary auxin signaling pathway in various land plants from the moss to flowering plants.[27] The amino acid residues of TIR1/AFB receptor for the recognition of auxin and Aux/IAA are conserved in various land plants including dicots, monocots, ferns, and moss (Supplementary Figure 6A). Indeed, auxinole retarded the growth of tomato plants in the same way as in *Arabidopsis* (Supplementary Figure 6C). In order to test the efficiency of auxinole for studies of auxin response in lower plants, we examined auxinole action in the moss *Physcomitrella patens*. *P. patens* shows two distinct developmental stages: the protonema, a filamentous network of the chloronemata and caulonemata, and the gametophore, a leafy shoot-like structure. In *P. patens*, auxin dramatically promotes the transition from chloronemata to caulonemata cells, and the elongated caulonema filaments are observed in colonies grown with auxin.[27] The *P. patens* genome contains four TIR1 orthologues, and IAA-recognition resides are conserved (Supplementary Fgure 6A). Auxinole repressed the formation of caulonema and further showed potent inhibition of 1-NAA-induced promotion of caulonemata cells. This finding is consistent with the idea that the recognition mechanism of auxin by TIR1 orthologues in *P. patens* is an ancient mechanism that is essentially the same as in angiosperms. Recently, the picolinate-type auxin picloram has been shown to act mainly via the *Arabidopsis* AFB4 and AFB5 receptors.[28,29] AFB4 and 5 belong to a distinct subclade of the TIR1/AFB family and have a distinct cavity of auxin binding site from TIR1/AFB1−3.[29] Picloram promotes the elongation of hypocotyls and inhibits the root growth. Auxinole suppressed picloram-induced phenotypes in *Arabidopsis* seedling (Supplementary Figure 6B), suggesting that auxinole can overcome the redundant function of TIR1/AFB receptors.

In conclusion, we have designed a potent auxin antagonist, auxinole, that binds to TIR1/AFB receptors to block their function. Auxinole shows very potent anti-auxin activity in *Arabidopsis* and moss plants. Our work not only substantiates the value of chemical tools for plant biology but also demonstrates the development of a new class of inhibitor for the modulation of small-molecule-regulated protein−protein interactions that are also important signaling events in the perception of other plant hormones such as jasmonate, gibberellin, and abscisic acid. This approach of rational structure-based design of molecules to modulate specifically the activity of SCF-based hormone receptor complexes is significant because it represents an opportunity that may be extended to achieve greater specificity to individual F-box proteins to target distinct hormone-regulated processes either within or between plant species.

METHODS

Synthesis of Compounds. α-Alkyl-1-NAA (**2**) and α-alkyl-IAA (**3**−**14**) were synthesized essentially as described in ref 14. The PEO-IAA derivatives **15**−**24** were synthesized by the nucleophilic addition of indole to arylkotoacrylic acid, essentially as described in ref 30. The details of the synthesis of other molecules reported here, synthetic procedures, and spectroscopic data are described in Supporting Information.

***In Silico* Screening and Docking Study.** The structural data of Ask1-TIR1-IAA complex (PBD IDs 2P1M, 2P1Q, and 2P1P) were edited by Discovery Studio visualizer 3.0 (Accelrys). The chemical structure for screening was obtained from the ZINC database and filtered with indole substructure and drug likeness properties. The filtered ligand was docked into TIR1 binding site, and binding affinity was evaluated by Surflex 2.3[18] at a Linux workstation. The details of docking procedures are described in Supporting Information.

Plant Growth Condition and Plant Growth Assay. *Arabidopsis thaliana* seedlings of ecotype Columbia (Col-0) were used for all experiments unless otherwise stated, and the plants were grown on germination (GM) medium[25] containing 1.5% sucrose and 0.1% Phytagel (Sigma) under continuous white light at 24 °C. For *Arabidopsis* root growth assay, *Arabidopsis* seeds were surface-sterilized and cultured on GM agar media containing ligands and/or auxins at indicated time. The assay procedures for the lateral root growth and gravitropic response were essentially same as reported previously[10] and are described in Supporting Information.

GUS Reporter Assay. For quantitative measurement of GUS enzyme activity,[25] *Arabidposis* GUS reporter (n = 10−15) was cultured in GM liquid medium containing ligand and auxin at 24 °C for the indicated time. After the GUS induction, the seedlings were homogenized, and GUS activity was measured by a fluorophotometer (Ex 365 nm and Em 455 nm) with 1 mM 4-methyl umbelliferyl β-D-glucuronide (4-MUG) as a fluorogenic substrate. Protein concentration was determined by Bradford protein assay (Bio-Rad). For histochemical GUS enzyme staining, the transgenic reporter seedlings were washed with a GUS staining buffer[25] after hormonal induction and transferred to a GUS staining buffer containing 1 mM X-gluc. The seedlings were then incubated at 37 °C until sufficient staining developed.

Pull-Down Assay with Aux/IAA Domain II Peptide and FLAG-Tagged TIR1. Extracts of 10-day-old *35S::FLAG-TIR1* seedlings (the generation of these transgenics is described in Supporting Information) were made as described previously[31] and used in pull-down assays by combining 2.5 mg of crude extract with 5 μg of biotinylated IAA7 domain II peptide (biotinyl-NH-

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AKAQVVGWPPVRNYRKN-COOH, synthesized by Thermo Scientific) and 65 μL 50% streptavidin-agarose suspension. The assays were incubated for 1 h at 4 °C with mixing and then washed three times for 5 min in extraction buffer (0.15 M NaCl, 0.5% Nonidet P40, 0.1 M Tris-HCl pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, 1 μM dithiothreitol, 10 μM MG132) containing the appropriate auxin treatment. The final processing of the pull-down assays including electrophoresis and Western transfer were performed as described previously.[31] The immunodetection of TIR1/AFB-FLAG was performed with a 1:5000 dilution of anti-FLAG 2-Peroxidase (HRP) antibody (Sigma) followed by chemiluminescent detection with ECL plus reagents (Amersham).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: hayashi@dbc.ous.ac.jp.

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