Small-molecule agonists and antagonists of F-box protein–substrate interactions in auxin perception and signaling

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The regulation of gene expression by the hormone auxin is a crucial mechanism in plant development. We have shown that the Arabidopsis F-box protein TIR1 is a receptor for auxin, and our recent structural work has revealed the molecular mechanism of auxin perception. TIR1 is the substrate receptor of the ubiquitin–ligase complex SCFTIR1. Auxin binding enhances the interaction between TIR1 and its substrates, the Aux/IAA repressors, thereby promoting the ubiquitination and degradation of Aux/IAA proteins, altering the expression of hundreds of genes. TIR1 is the prototype of a new class of hormone receptor and the first example of an SCF ubiquitin-ligase modulated by a small molecule. Here, we describe the design, synthesis, and characterization of a series of auxin agonists and antagonists. We show these molecules are specific to TIR1-mediated events in Arabidopsis, and their mode of action in binding to TIR1 is confirmed by x-ray crystallographic analysis. Further, we demonstrate the utility of these probes for the analysis of TIR1-mediated auxin signaling in the moss Physcomitrella patens. Our work not only provides a useful tool for plant chemical biology but also demonstrates an example of a specific small-molecule inhibitor of F-box protein–substrate recruitment. Substrate recognition and subsequent ubiquitination by SCF-type ubiquitin ligases are central to many cellular processes in eukaryotes, and ubiquitin–ligase function is affected in several human diseases. Our work supports the idea that it may be possible to design small-molecule agents to modulate ubiquitin-ligase function therapeutically.


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Chemical biology | TIR1 | ubiquitin ligase | SCF | plant hormones

The plant hormone auxin is a core regulator of plant growth and development. By controlling cell division and elongation and triggering specific differentiation events, indole-3-acetic acid (IAA), the predominant naturally occurring auxin, regulates developmental phenomena as diverse as embryo polarization, vascular differentiation, tropic response to light and gravity, and apical dominance, among many others (1). Auxin’s influence over these manifold events stems from its ability to regulate differentially the expression of hundreds of genes. Central to this transcriptional control is the auxin-enhanced and ubiquitin-mediated degradation of a family of transcriptional repressor proteins called Aux/IAAs (1). Recent work has identified TIR1 and related AFB proteins as auxin receptors for this response (2–4). TIR1 is an F-box protein and a component of the E3 ubiquitin ligase complex SCFTIR1. E3 ubiquitin ligases are responsible for catalyzing the ubiquitination of target proteins, which in most cases results in the rapid degradation of the target proteins in the 26S proteasome. SCF-type E3s consists of three components, a SKP subunit (termed ASK proteins in Arabidopsis), and CULLIN subunit and the F-box protein (5). It is the F-box protein component, of which there are >700 in Arabidopsis, that is responsible for the recruitment of specific protein substrates (6). In the case of TIR1, the Aux/IAA targets are recruited via an interaction between the leucine-rich repeat (LRR) domain of TIR1 and a short so-called “degron” motif within domain II of the Aux/IAA (7). This interaction is enhanced by the direct binding of auxin to TIR1 make the SCFTIR1–auxin–Aux/IAA complex the hub of the fundamental derepression model for auxin-regulated control of gene expression: When auxin levels are low, Aux/IAAs are relatively stable and able to exert repression on target genes. As auxin levels rise, TIR1-mediated proteolysis of Aux/IAAs relieves this repression, and genes are expressed (5).

Recent structural analysis of the receptor protein TIR1 in complex with auxin and the Aux/IAA domain II peptide has revealed the molecular mechanism of auxin perception. Both auxin and Aux/IAA bind to the same pocket formed by the LRRs on the surface of TIR1. Auxin nests on the floor of this common pocket, whereas the Aux/IAA binds on top of auxin, trapping it underneath. In this way, auxin is thought to act as a “molecular glue” by increasing the extent of hydrophobic interaction among the three components (8).

The vast majority of the molecular genetic research on which our understanding of auxin signaling is based has come from work on the model plant Arabidopsis. Beyond this dicot model, the role of auxin in the growth and development of divergent plant species is unclear. Assessing the physiological role of auxin in these species is often hampered by lack of genetic tools to dissect auxin response. To address this problem and to complement molecular genetic approaches to auxin study, we have taken a chemical biology approach, by generating specific auxin signaling probes that can be used to dissect auxin signal transduction (9, 10).

Many structural and biological investigations of auxin compounds have led to the development of several synthetic auxins, including 2,4-dichlorophenoxy acetic acid (2,4-D) and 1-naphthylacetic acid (NAA). In contrast, there are few reports on auxin-like compounds. P-Chlorophenoxyisobutyl acid (PCIB) is used as an auxin analog although it does not antagonize all auxin responses, and its mode of action is not clear (11, 12).

Here, we report a series of systematically designed small-molecule agonists and antagonists of TIR1 receptor function. By introducing different alkyl chains to the α-position of IAA, we have generated specific TIR1 agonists and antagonists that modulate all tested TIR1-mediated auxin responses in Arabidopsis, from the molecular to the whole-plant level. The mode of action of these small-molecule probes is demonstrated by x-ray crystallographic analysis among the three components (8).

Supporting Information includes 26 tables and 22 figures. This article contains supporting information online at www.pnas.org/cgi/content/full/0711146105/DCSupplemental.

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Data deposition: The structure coordinates and structural factors reported in this paper have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3C60, 3C6G, and 3C6P).

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Further, these probes inhibited NAA-induced longer chain molecules. The potency of the antiauxin activity of 4 probes and/or 0.5 μM IAA, without chemicals for 5 h. The induced GUS activity is expressed relative to 1 μM NAA treatment (100%). Error bars, mean ± SD of three independent experiments. (B) Auxin activity of 1–3 (10 μM) and 4–8 (50 μM). (C) Antiauxin activity of 4–8 (light gray, 20 μM; dark gray, 50 μM). (D) The effect of probes on the Aux/IAA–TIR1 interaction. c-myc-tagged TIR1 was pulled-down using biotinylated IAA7 domain II peptide in the presence of chemicals (100 μM probes and/or 0.5 μM IAA), and recovery of TIR1-myc was monitored by Western blot analysis with anti-c-Myc antibody. (E) The effect of probes on Aux/IAA stability. The HS::AXR3NT-GUS line was incubated with chemicals (2 μM 3, 1 μM IAA, and/or 50 μM 4–8) after heat-shock induction. GUS expression was visualized by histochemical GUS staining.

Results

α-Alkyl IAA Probes Are Specific to TIR1. Although extensive structure-activity analysis of modifications to the indole ring of IAA has failed to identify experimentally useful antiauxin and auxin derivatives, the effect of substitutions at the α-position of IAA on auxin activity has not been investigated systematically. To examine the effect of such modifications, a series of α-alkyl chains were introduced to the α-position of IAA (Fig. L4). The synthesis procedures are described in supporting information (SI) Appendix. The α-alkyl IAs were prepared as racemic mixtures at α-position, and the racemate was used for biological evaluation because of the difficulty of separating each enantiomer.

To examine the auxin and antiauxin activity of the probes, we used the Arabidopsis auxin response reporter line DR5::GUS, which has a synthetic auxin responsive promoter driving GUS expression (13). The IAs with methyl to propyl chain substitutions (probes 1–3) at the α-position induced DR5::GUS expression demonstrating that these molecules retain auxin activity in this assay (Fig. 1B). In contrast, the introduction of butyl or longer chains (probes 4–8) to IAA abolished auxin activity. Further, these probes inhibited NAA-induced DR5::GUS expression (Fig. 1C), indicating that the introduction of butyl or longer chain (4–8) confers an antiauxin activity to these new molecules. The potency of the antiauxin activity of 4–8 was approximately proportional to chain length.

To test the idea that these probes act by affecting the binding of Aux/IAAs to the receptor TIR1, we performed in vitro pulldown assays in the presence and absence of both IAA and each of the probes using a biotinylated Aux/IAA domain II degron peptide and c-myc-tagged TIR1 (14). Fig. 1D shows that probes 1–3 have auxinic activity in this assay, enhancing the interaction between TIR1 and Aux/IAA domain II peptides. In contrast, probes 4–8 (butyl or longer chains) block the IAA-enhanced interaction, indicating that these molecules are antiauxins blocking the TIR1-Aux/IAA interaction (Fig. 1D). Consistent with DR5::GUS reporter analysis, this inhibitory activity was also proportional to alkyl chain length, with probe 8 exhibiting the most potent antiauxin activity. To confirm the effects of the probes on TIR1–Aux/IAA interaction in vivo, Aux/IAA stability was monitored by using the Arabidopsis Hs::AXR3NT-GUS line, in which a translational fusion between domains I and II of the Aux/IAA AXR3 and the GUS reporter protein is expressed under the control of a heat-shock promoter (15). Hs::AXR3NT-GUS seedlings were treated with the probes in the presence or absence of IAA after heat induction. After a 20-min incubation, probe 3 enhanced the degradation of the fusion, whereas probes 4, 7, and 8 blocked IAA-enhanced degradation (Fig. 1E). Together, these data indicate that these auxin and antiauxin molecules act at the level of TIR1–Aux/IAA interaction by mimicking or interfering with auxin activity.

Antiauxin Probes Block Auxin Response via the SCFTIR1–Aux/IAA Pathway. To examine further the biological activity of the antiauxin probes, we evaluated their effects on typical auxin responses in the Arabidopsis root. Auxin inhibits primary root growth while promoting root hair and lateral root formation (1). Consistent with its activity at the molecular level, probe 8 antagonized these root responses to auxin, suppressing the inhibition of primary root growth.
The effects of probe B on the growth and development of Arabidopsis. (A–C) The effects of B on the elongation of hypocotyl and root in wild-type and auxin overproducing yucca mutants. The seedlings were grown for 6 days in the presence of chemicals. Five μM NPA and 50 nM IAA were used for assays unless otherwise stated. (A) Photos of Arabidopsis seedlings grown in the presence or absence of chemicals (a–f, wild-type; g–i, yucca mutant). Plants grown with 8 (b, c, and h), with NPA (d and i), or with IAA and/or 20 μM B (e and f). (B and C) Root and hypocotyl length was measured after cultivating with B and with and without 50 nM IAA. Values are the mean +/- SD of two independent experiments. (D) Wild-type plants treated with B phenocopy auxin-insensitive mutants, axr1–3, axr1–12, and axr2–1. Wild type were grown for 14 days with or without B. Mutants were grown for 14 days without B. (Scale bars, 5 mm in A and 10 mm in D.)

Fig. 3. The effects of probe B on the growth and development of Arabidopsis. (A–C) The effects of B on the elongation of hypocotyl and root in wild-type and auxin overproducing yucca mutants. The seedlings were grown for 6 days in the presence of chemicals. Five μM NPA and 50 nM IAA were used for assays unless otherwise stated. (A) Photos of Arabidopsis seedlings grown in the presence or absence of chemicals (a–f, wild-type; g–i, yucca mutant). Plants grown with 8 (b, c, and h), with NPA (d and i), or with IAA and/or 20 μM B (e and f). (B and C) Root and hypocotyl length was measured after cultivating with B and with and without 50 nM IAA. Values are the mean +/- SD of two independent experiments. (D) Wild-type plants treated with B phenocopy auxin-insensitive mutants, axr1–3, axr1–12, and axr2–1. Wild type were grown for 14 days with or without B. Mutants were grown for 14 days without B. (Scale bars, 5 mm in A and 10 mm in D.)

root (Fig. 3Ae and f and B) and the promotion of root hair and lateral root formation (Fig. 2A and B and Fig. S2). Probes 4 and 7 similarly antagonized these three root auxin responses (Figs. S1 and S2 in SI Appendix). For further detailed analysis of antiauxin action, probe 8 was used because of its higher potency. In the same bioassays, probe 3 continued to exhibit auxin activity (Figs. S1 and S2). Auxin is an essential regulator of the root gravitropic response and is also required for the proper development of columella cells in which the gravity is perceived (1). To test the effects of 8 on gravitropism, 5-day-old Arabidopsis seedlings were transferred onto agar plates with or without both IAA and 8. The plates were then rotated 135° and cultured for another 2 days in the dark. As shown in Fig. 2C, probe 8 perturbed gravitropic response of Arabidopsis roots although this effect could be rescued by exogenous IAA.

Although auxin transport inhibitors such as 1-N-naphthylphthalamic acid (NPA) can elicit similar gravitropic defects (16), other developmental abnormalities arising from inhibition of auxin transport are very different (Fig. 3Aa and d). For example, NPA induces abnormal columella cell positioning by perturbing auxin transport, whereas probe 8 affects columella cells by reducing their number but not their positioning (Fig. 2D), suggesting probe 8 activity is distinct from that typical of auxin transport inhibitors such as NPA. The antiauxin activity of probe 8 is not confined to root responses to auxin. The etiolation response of Arabidopsis hypocotyls is strongly inhibited by auxin but could be fully recovered by cotreatment with 8 (Fig. S3).

To assess the effects of probe 8 on responses to endogenous auxin in Arabidopsis, wild-type and yucca mutant plants (which have an auxin overproduction phenotype) (17) were grown in the presence of 8, with or without exogenous IAA. yucca mutants have shorter root and longer hypocotyls than wild-type because of elevated endogenous auxin levels (Fig. 3Ag, B, and C). Probe 8 promoted root elongation in wild-type and yucca in a dose-dependent manner, indicating that endogenous auxin action is being antagonized (Fig. 3A b, c, and h, and B). Exogenous IAA (50 nM) counteracted this effect of probe 8 on root elongation in both WT and yucca (Fig. 3B). In contrast to root response, probe 8 blocked endogenous auxin action to inhibit WT and yucca hypocotyl elongation in a dose-dependent manner (Fig. 3A b, c, and h, and C). The control of hypocotyl growth with respect to auxin is complex (18). Although yucca mutants have longer hypocytol than WT, both genotypes exhibit reduced hypocotyl elongation in response to exogenous auxin (Fig. 3C), suggesting that in each background, there is an optimal level of auxin with compensatory changes in response in the yucca mutant. This is supported by the observation that the dose–response curve of hypocotyls treated with 8 and exogenous IAA displayed a bell-shaped response in both WT and yucca mutant, but higher concentrations of 8 (40 μM) inhibited hypocotyl growth to the same extent in all conditions (Fig. 3C). Probe 8 (20 μM) was sufficient to almost fully rescue the yucca phenotypes under our conditions (Fig. 3A). Together, these experiments support the idea that the antiauxin 8 antagonizes responses to endogenous auxin.

Several Arabidopsis mutants with specific defects in the TIR1-Aux/IAA pathway have been identified (1, 19). These range from the strongly auxin-resistant mutants axr1–3, axr1–12, and axr2–1 to the more moderately resistant mutants tir1–1 and str–1. The strong auxin resistance phenotypes of axr1–3, axr1–12, and axr2–1 mutants were phenocopied by the treatment of WT plants with 20–50 μM probe 8 (Fig. 3D) consistent with the idea that probe 8 blocks TIR1 function in vivo. This is further supported by the finding that strongly auxin resistant mutants such as

Fig. 4. Effects of 8 on the growth of Arabidopsis auxin mutants. Arabidopsis wild type and mutants were grown for 6 days. Upper shows the effect of increasing concentrations of 8 on root length relative to untreated control (100%). (Lower) Hypocotyl length relative to untreated control (100%). Values are the mean +/- SD of two independent experiments.
axr1–12 and axr2–1 were also highly insensitive to 8, whereas tir1–1 and slr1 were more moderately insensitive (Fig. 4) presumably because the effect of 8 on the growth of wild-type hypocotyls and roots depends on the response to endogenous IAA, and this response is attenuated in each mutant. In contrast, auxin transport mutations affecting either auxin influx (aux1–7) or auxin efflux (eir1) displayed only weak or no resistance to 8, suggesting that probe 8 acts to disrupt auxin signaling principally by affecting the initial auxin perception event.

**Molecular Mechanism of Auxin Probe Action in Auxin Perception.** To understand the molecular mechanism of the α-alkyl probes, we solved the crystal structures of TIR1 (and ASK1) in complex with probes 3, 4, and 8 (Table S1 in SI Appendix) and also carried out molecular docking calculations using the program AutoDock (20). In the crystal structures of the TIR1 complexed with 3 (auxin), 4, or 8 (antiauxin), the indole 3-α-acetic acid moiety of three probes sits in the auxin-binding pocket of TIR1 in the same way as IAA (8), whereas the alkyl chain of the probes is oriented to the Aux/IAA-binding cavity (Fig. 5A and B and Fig. S4). We could not determine the exact position of alkyl chains beyond β-carbon in alkyl chain (the one next to α-position) because of weak electron density of alkyl carbons (Fig. S4). The coordinates of alkyl chain are represented as one of the most likely chain conformations (Fig. 5A and B). IAA and IAA7 molecules were superimposed into experimentally determined coordinates (8). Interestingly, all bound probes had S-form stereochemistry even though racemic probes were used for crystallization, indicating that it is the S-form, which binds, and is thus more potent than the R-form. These structures indicate that the alkyl chains is significantly disordered and oriented into Aux/IAA binding cavity. Consistent with crystal structure of auxin-bound TIR1, no conformational change in TIR1 was observed among three complexes (with auxin 3 or antiauxin 4 and 8) supporting the molecular glue hypothesis of Tan et al. (8).

Molecular docking calculation analysis complemented the crystallographic analysis of the TIR1–probe complexes. Docking analysis predicted the possible conformers of the three probes in the TIR1 auxin/Aux/IAA-binding site. The predicted binding conformers of 3 and 4 are indicated as blue and yellow colored molecules, and red- and green-colored molecules represent IAA and IAA7 superimposed into the coordinates in crystal (Fig. 5C). In the predicted binding position of 3 and 4, the IAA moiety of both probes sits in the TIR1 groove in a manner similar to IAA. Consistent with crystal structures, alkyl chains are disordered (Fig. 5C). The butyl chains of 4 (antiauxin) were directed to the Aux/IAA-binding cavity, where they would prevent access of the domain II Aux/IAA degron in the binding cavity. The butyl chain conflicts with the second proline in the IAA7 core degron sequence GWPPV, which is critical for Aux/IAA recognition by TIR1. In contrast, the propyl chain of 3 (auxin) fits into a small vacant cavity without hindering subsequent binding of the Aux/IAA (Fig. 5C). Thus, the addition of a methyl group in the butyl chain of 4 was all that is required to convert an auxin to an antiauxin. This was further confirmed by auxinic and anti- auxinio activity of another two types of α-alkyl auxins, α-alkyl NAA, and 2,4-D. Previous structural work has demonstrated that NAA and 2,4-D bind to TIR1 with a binding conformation similar to IAA (8). Therefore, it is predicted that the introduction of α-alkyl chain to two different types of auxins would confer similar auxin/antiauxin properties to the α-alkyl IAA derivatives. Consistent with our structural analysis, α-methyl to ethyl NAA and α-methyl to propyl 2,4-D retained auxin activity in DR5::GUS expression assays, whereas the introduction of over butyl chain showed antiauxin activity as observed with probes 4–8 (Figs. S5 and S6). The activity of propyl NAA was more complex showing both weak auxin activity in the absence of NAA and weak antiauxin activity in the presence of NAA. This reflects the fact that some conformers of propyl NAA with the disordered chain are predicted to allow the molecule to occupy the auxin-binding site without blocking the Aux/IAA pocket, whereas other conformers do not.

The crystal structure of TIR1 complexed with 8 shows clearly that the probe would block Aux/IAA access (Fig. 5B). The predicted binding conformers of 8 are shown in Fig. S7. The IAA moiety of the conformers is consistent with experimental coordinates of 8. The alkyl chain is more disordered than probe 4 because of a longer chain length and would hinder the access of Aux/IAA more effectively (Fig. S7). These data explain, at molecular level, why 3 and 4 have opposite activities (agonist/antagonist), and why probe 8 is more potent than 4.

Auxin-binding protein 1 (ABP1) is a candidate auxin receptor of unknown function, distinct from the TIR1/AFB family. The crystal structure of ABP1–auxin complex (21) has demonstrated that auxin binds to small pocket in ABP1 (Fig. S8A). Molecular modeling analysis revealed that probe 8 could not bind to ABP1 because of the bulky alkyl chain of the probe (Fig. S8B), suggesting that probe 8 could not block ABP1 function and that probe 8 is specific to TIR1 and TIR1-related AFB receptors.

**SCF^TIR1 Pathway Is Conserved in Lower Land Plants.** To confirm the utility of the probes for studying auxin response in other plant species, we examined the effect of 8 on auxin responses in the monocot grass *Oryza sativa* (rice) and the moss *P. patens*. As expected from the high homology of the rice TIR1 orthologue to Arabidopsis TIR1, probe 8 blocked typical auxin responses in rice, such as lateral root and root hair formation (Fig. S9). The moss *P. patens* is a lower-land plant, and available sequence data suggests that a functional TIR1-Aux/IAA pathway could be conserved in moss. Moss is therefore an attractive model in which to assess the early evolution of auxin signaling mechanisms. *P. patens* shows two distinct developmental stages, the protonema, a filamentous network of the chloronemata and caulonemata; and the gametophore, a leafy shoot. The gametophore is developed from a subapical cell of the caulonema (22). In *P. patens*, exogenous auxin is known to elongate the gameto-
and the formation of gametophore by blocking the transition also antagonized the enhancement by NAA of this development.

FIG. 6. The TIR1/AFB specific probe 8 blocks auxin responses of moss P. patens. (A) Effects of 8 on NAA-induced elongation of P. patens gametophores. The juvenile gametophore was incubated for 60 h with chemicals (2 μM NAA and/or 20 μM 8). Arrows indicate the elongation zone in response to NAA. (Scale bar, 10 mm.) (B) Effects of 8 and NAA on the development of chloronema. Chloronema cells were cultured on a BCDATG medium for 10 days in the presence of 0.5 μM NAA and/or 10 μM 8. Arrows indicate caulonema. [Scale bars, 2.5 mm (Upper) and 1 mm (Lower).]

phore and promote the formation of rhizoids and caulonemata (23). The P. patens genome contains a TIR1 orthologue (PpTIR1) with high homology to TIR1. Homology modeling indicates auxin-binding site of TIR1 is conserved in PpTIR1 (Fig. 10A). Molecular docking calculation between PpTIR1 and 8 indicated that 8 could bind to PpTIR1 in similar way to TIR1 (Fig. S10B).

We investigated the effects of 8 on P. patens auxin responses as a potential means of probing the existence of functional PpTIR1-mediated auxin signaling pathway in moss. Probe 8 antagonized NAA-induced gametophore elongation (Fig. 6A). Additionally, probe 8 inhibited the elongation of chloronema cell and the formation of gametophore by blocking the transition from chloronema to caulonema (Fig. 6B and Fig. S11). Probe 8 also antagonized the enhancement by NAA of this developmental transition from chloronema to caulonema cells (Fig. 6B and Fig. S11). Auxin application promotes the transition gametophore bud cells to rhizoid-like bud structure and probe 8 also blocked this process (Fig. S11). Taken together with homology modeling and molecular docking calculation, these findings support the idea thus that auxin response mediated by TIR1 and Aux/IAA orthologues is an ancient mechanism. Interestingly, this contrasts with the lack of a functional gibberellin receptor DELLA GA response pathway in moss, despite the existence of apparent homologs for both components (24). The developmental role of auxin and its means of perception and signal transduction in other lower land plants such as liverworts and ferns is unknown, and our probes offer useful tools for the study of auxin-regulated development across diverse plant species.

Discussion

We have designed a set of molecules that modulate, either positively or negatively, the interaction between TIR1 (and AFBs) and Aux/IAAs and hence transcriptional responses to auxin. These auxin agonists and antagonists affect all assayed TIR1-mediated responses to auxin. By x-ray crystallography of TIR1 in complex with the probes, we show how binding events at the receptor relate to these in vitro and in vivo responses. Further, we demonstrate the utility of these molecules for probing auxin response in species in which the role of auxin and the mechanism of its perception are unclear.

The probe concentrations required to block IAA activity are relatively high (10–50 μM probe 8 against 0.1–2 μM IAA). There are three possible reasons for this. First, the long and disordered alkyl-chain of probes such as 8 would probably decrease the affinity of the probe to TIR1 compared with IAA. Second, the structural analysis of TIR1–probe complex revealed that the S-form of the probes are the predominant binding enantiomer, suggesting that pure S-form probes would show higher activity than the racemates assayed here. Finally, there are no affinity data available for the binding of IAA to TIR1 in the absence of Aux/IAA protein. A prediction of the molecular glue hypothesis would be that without Aux/IAA binding on top of the TIR1-bound IAA, the TIR1-IAA affinity would be relatively low (8). Because the alkyl chains of the antiauxins such as 8 preclude Aux/IAA binding, much higher concentrations of antiauxin would be required to compete effectively with IAA, which is retained more readily in the tight complex of Aux/IAA–auxin–TIR1.

Given that auxin-induced interactions between the TIR1-related AFB proteins and Aux/IAAs have also been demonstrated, we predict that our probes will also act on these related receptor proteins (2, 5). In addition to binding to the TIR1/AFB receptors, there is a possibility that our probes could act in other ways, for example, by interacting with other receptors or affecting auxin transport. The evidence presented here suggests that this is not the case: structural modeling of the only other candidate auxin receptor, ABP1, clearly shows that our antiauxin probes cannot bind to its auxin-binding site. Also, although some of the effects of probe 8 are similar to those of auxin transport inhibitors such as NPA, most effects are different. For example, NPA enhances DR5 response in the presence of auxin, whereas probe 8 has the opposite effect. Together, the molecular and structural data, these observations suggest that these α-alkyl IAA molecules are effective TIR1/AFB-specific probes.

Our work also has implications beyond plant biology. The F-box protein TIR1 is the first example of an entirely new class of receptor in which the activity of an otherwise generic mechanism for protein ubiquitination, common throughout eukaryotes, is regulated by the binding of a small molecule (in this case auxin). The vast majority of the hundreds of F-box proteins identified remain uncharacterized and their target proteins unknown (6). A second example of an F-box protein acting as a small-molecule receptor has already been identified (25), suggesting it is likely that this mechanism will be more widespread. Our results thus provide not only a useful tool for plant chemical biology but also demonstrate an example of a specific small-molecule inhibitor of F-box protein-substrate recruitment. Substrate recognition and subsequent ubiquitination by SCF-type ubiquitin ligases is central to many cellular processes in eukaryotes and ubiquitin-ligase function is affected in several human diseases including Parkinson’s disease and certain cancers (26, 27). Our work substantiates the idea that it may be possible to design small-molecule agents to ameliorate ubiquitin ligase function in human diseases. Such drugs could be formulated for maximal specificity and offer an exciting opportunity for pharmaceutical research.

Materials and Methods

Materials and Plant Growth Conditions. Full details of synthetic procedures and the spectroscopic data of probes are described in SI Appendix. Arabidopsis thaliana Columbia and Physcomitrella patens were cultured at 24°C under continuous light in this study. Arabidopsis plants were grown on a dish containing germination (GM) medium (9) with 0.1% gellan gum (Sigma) containing the indicated hormone and/or chemicals for the indicated time. P. patens was maintained as protonema on a plate containing BCDATG medium (28). To observe the auxin response of gametophores, the gametophores were cultured on a plate with BCD medium (28) with 0.1 mM CaCl2 and 0.1% gellan gum for 60 h.
Probes were soaked into the crystals by placing the crystals in the crystallization and purification and crystallization were performed as described (8). Different Crystallographic Study and Molecular Docking Analysis. TIR1–ASK1 complex purification and crystallization were performed as described (8). Different assays were conducted in liquid GM medium for 5 h with or without chemicals. Induced GUS activity was determined fluorometrically. For pulldown assays with Aux/IAA and cmyc tagged TIR1, pulldown assays with the biotinylated AuxII/IAA degron peptide were performed as described (14). The AuxII/IAA protein degradation assay was assayed as described in ref. 15. Briefly, 8-day-old H5:AXR3NT–GUS transgenic seedlings after heat shock (2 h, 37°C and then 10 min, 23°C) were treated with chemicals for another 20 min. GUS activity was histochemically stained. Root gravitropic response assay were performed as described (10). For hypocotyl and root assays (n = 25–40), the length of hypocotyl and root was recorded by digital camera and analyzed by the program NIH Image (National Institutes of Health) after 6-day cultivation.

Crystallographic Study and Molecular Docking Analysis. TIR1–ASK1 complex purification and crystallization were performed as described (8). Different assays were conducted in liquid GM medium for 5 h with or without chemicals. Induced GUS activity was determined fluorometrically. For pulldown assays with Aux/IAA and cmyc tagged TIR1, pulldown assays with the biotinylated AuxII/IAA degron peptide were performed as described (14). The AuxII/IAA protein degradation assay was assayed as described in ref. 15. Briefly, 8-day-old H5:AXR3NT–GUS transgenic seedlings after heat shock (2 h, 37°C and then 10 min, 23°C) were treated with chemicals for another 20 min. GUS activity was histochemically stained. Root gravitropic response assay were performed as described (10). For hypocotyl and root assays (n = 25–40), the length of hypocotyl and root was recorded by digital camera and analyzed by the program NIH Image (National Institutes of Health) after 6-day cultivation.

Supporting Information

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Supporting Appendix: Synthesis of Chemicals

1. General Experimental Conditions. 1H and 13C NMR spectra were recorded on a Bruker ARX 400 NMR spectrometer (Bruker Japan). Chemical shifts are shown as δ values from TMS as the internal reference. Peak multiplicities are quoted in Hz. Mass spectra were measured on a JMS-700 spectrometer (JEOL). Column chromatography was carried out on columns of silica gel 60 (230–400 mesh, Merck). All chemicals were purchased from Tokyo Chemical Industry Japan and Sigma-Aldrich, unless otherwise stated.

2. Synthesis of α-Alkyl IAAs, Auxin Probes (1–8)

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\text{Scheme 1: Reagents and conditions.} \quad (a) \text{AcCl, MeOH, rt, 3h, 96%,} \quad (b) \text{Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH}_2\text{Cl}_2:30\% \text{NaOH aqueous solution = 1:1, 0°C, 2h, 87%}
\]

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

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Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

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Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

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Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

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Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

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Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.

Methyl chlorofolmatate, tetrabutyl ammonium iodide, CH2Cl2: 30% NaOH aqueous solution = 1:1, 0°C, 2h, 87%.
122.9, 119.5, 119.4, 115.2, 53.7, 52.0, 42.6, 32.2, 31.6, 29.0, 27.6, 22.6, 14.0; EI-MS: m/z 331 [M]+.

3. **Synthesis of α-alkyl NAA s, auxin probes (1a-1g)**

Scheme 2. Reagents and conditions. (a) LDA, HMPA, THF, -78°C, 1 h. (b) NaOH in H2O/MeOH, 70°C, 0.5 h.

3.1. **General synthetic procedure of α-alkyl NAA s**. To the solution of naphthalene-1-acetic acid methyl ester (13) in THF was added lithium disopropylamide (1.5 equiv.) and hexamethyldisilazane (5 equiv.), and then stirred for 30 min at -78°C. The corresponding alkyl iodide, R-I (1.2 equiv.) in THF was added dropwise to the solution, and then stirred for 1 h at -78°C. After warming to 0°C for 15 min, the resulting solution was added to water (100 mL), and extracted with EtOAc (100 mL × 3). The
organic layer was washed with saturated NH₄Cl solution and brine, and then dried over Na₂SO₄. The residue was purified by silica gel column chromatography to give the corresponding α-alkyl-naphthalene-1-acetic acid methyl ester (14a-g).

- **α-Methyl-naphthalene-1-acetic acid methyl ester (14a).** Starting material 13 (100 mg, 0.50 mmol). Reaction mixture was purified with silica gel column eluted with hexane-EtOAc (9:1) to yield 14a (70 mg, yield 65%) as a white powder: IR ν max (neat): 1737, 1434, 1198, 777 cm⁻¹; 1H NMR (400 MHz, CDCl₃); δ 8.13 (d, J = 8.4 Hz, 1H), 7.85 (d, J = 8.2 Hz, 1H), 7.42 – 7.56 (m, 4H), 3.64 (s, CH₃), 2.08 (m, 2H), 1.32 – 1.42 (m, 2H), 0.94 (t, J = 7.3 Hz, CH₃); 13C NMR (100 MHz, CDCl₃); δ 174.9, 135.6, 133.9, 131.6, 128.9, 127.6, 126.3, 125.5, 125.4, 124.8, 52.0, 46.5, 35.3, 31.2, 13.9; EI-MS: m/z 242 [M⁺].

- **α-Hexyl-naphthalene-1-acetic acid methyl ester (14f).** Starting material 13 (100 mg, 0.50 mmol). Reaction mixture was purified with silica gel column eluted with hexane-EtOAc (9:1) to yield 14f (57 mg, Yield 89%) as a white powder: IR ν max (neat): 1740, 1416, 1272, 1222, 925, 778 cm⁻¹; 1H NMR (400 MHz, CDCl₃); δ 8.12 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 8.2 Hz, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.43 – 7.56 (m, 4H), 4.39 (t, J = 7.4 Hz, 1H), 3.64 (s, CH₃), 2.08 (m, 2H), 1.32 – 1.42 (m, 2H), 1.94 (t, J = 7.3 Hz, CH₃); 13C NMR (100 MHz, CDCl₃); δ 174.9, 135.6, 134.0, 134.0, 131.6, 129.0, 127.7, 126.3, 125.6, 125.5, 124.9, 52.0, 46.5, 35.3, 31.2, 13.9; EI-MS: m/z 256 [M⁺].
Scheme 3. 4.1. General synthetic procedure of α-alkyl-2,4-dichlorophenoxy acetic acid (1h-k). To the solution of 2,4-dichlorophenol (15) in CH₂CN was added cesium bicarbonate (0.5 equiv.) and α-bromo alkyl carboxylic acids (1 equiv.). The solution was refluxed at 80°C for 4h. The reaction mixture was added to water (100 ml), and extracted with EtOAc (100 ml × 3). The organic layer was washed with saturated NH₄Cl solution and brine, and then dried over Na₂SO₄. The residue was purified by silica gel column chromatography to give the corresponding α-alkyl-2,4-dichlorophenoxy acetic acid methyl esters (14h-k).

The methyl ester (14h-k) was hydrolyzed in aqueous methanolic LiOH solution (2 equiv. of LiOH in THF:H₂O = 3:1). The solution was stirred at room temperature for 1h. The resulting solution was cooled and acidified to pH 3 with 6N HCl. After removal of MeOH in vacuo, the resulting suspension was extracted with EtOAc (50 ml × 3). The organic layer was washed successively with saturated NH₄Cl solution and brine, and then dried over Na₂SO₄. The solvent was removed in vacuo. The residue was purified by silica gel column chromatography to give the respective α-alkyl-NAAs (1h-k).

α-Ethyl-2,4-dichlorophenoxy acetic acid methyl ester (14h). Starting material 13 (200 mg, 1.23 mmol). Reaction mixture was purified with silica gel column eluted with hexane-acetone (95:5) to yield 14h (256 mg, Yield 79%) as an oil: IR ν max (neat): 1757, 1478, 1287, 1206, 1106, 1063, 1006, 804 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.38 (s, 1H), 7.13 (d, J = 8.7 Hz, 1H), 6.73 (d, J = 8.8 Hz, 1H), 4.57 (t, J = 6.1 Hz, 1H), 3.75 (s, CH₃), 2.05 (m, 2H), 1.11 (t, J = 7.5 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 171.2, 152.4, 130.2, 127.5, 128.6, 124.6, 125.5, 125.0, 123.1, 46.5, 32.8, 31.8, 29.4, 29.0, 27.8, 22.6, 14.1; FAB-MS: m/z 307 [M+Na⁺].

α-Butyl-2,4-dichlorophenoxy acetic acid methyl ester (14i). Starting material 13 (200 mg, 1.18 mmol). Reaction mixture was purified with silica gel column eluted with hexane-acetone (95:5) to yield 14i (207 mg, Yield 78%) as an oil: IR ν max (neat): 1758, 1478, 1287, 1206, 1106, 1009, 804 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.38 (s, 1H), 7.12 (d, J = 8.8 Hz, 1H), 6.72 (d, J = 8.9 Hz, 1H), 4.62 (t, J = 6.2 Hz, 1H), 3.75 (s, CH₃), 1.93 – 2.04 (m, 2H), 1.56 – 1.60 (m, 2H), 0.98 (t, J = 7.3 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 171.3, 152.3, 130.1, 127.4, 126.6, 124.3, 115.0, 77.6, 52.2, 34.6, 18.3, 13.6; EI-MS: m/z 276 [M⁺].

α-Propyl-2,4-dichlorophenoxy acetic acid methyl ester (14j). Starting material 13 (300 mg, 1.84 mmol). Reaction mixture was purified with silica gel column eluted with hexane-EtOAc (9:1) to yield 14j (294 mg, Yield 86%) as an oil: IR ν max (neat): 1758, 1478, 1287, 1106, 1009, 804 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.36 (s, 1H), 7.12 (d, J = 8.8 Hz, 1H), 6.72 (d, J = 8.9 Hz, 1H), 4.62 (t, J = 6.2 Hz, 1H), 3.75 (s, CH₃), 1.93 – 2.04 (m, 2H), 1.56 – 1.60 (m, 2H), 0.98 (t, J = 7.3 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 171.3, 152.3, 130.1, 127.4, 126.6, 124.3, 115.0, 77.6, 52.2, 34.6, 18.3, 13.6; EI-MS: m/z 276 [M⁺].

α-Hexyl-2,4-dichlorophenoxy acetic acid methyl ester (14k). Starting material 13 (150 mg, 0.92 mmol). Reaction mixture was purified with silica gel column eluted with hexane-acetone (95:5) to yield 14k (248 mg, Yield 84%) as an oil: IR ν max (neat): 1759, 1479, 1287, 1204, 1105, 1063, 804 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.36 (s, 1H), 7.12 (d, J = 8.7 Hz, 1H), 6.72 (d, J = 8.8 Hz, 1H), 4.60 (t, J = 6.0 Hz, 1H), 3.74 (s, CH₃), 1.95 – 2.04 (m, 2H), 1.49 – 1.55 (m, 2H), 1.30 – 1.37 (m, 6H), 0.88 (t, J = 5.8 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 176.3, 152.2, 130.4, 127.6, 124.7, 124.5, 115.2, 78.0, 52.2, 32.6, 31.5, 28.8, 24.9, 22.4, 13.9; EI-MS: m/z 290 [M⁺].

α-Ethyl-2,4-dichlorophenoxy acetic acid (1h). Starting material 14h (150 mg, 0.57 mmol). Reaction mixture was purified with silica gel column eluted with hexane-acetone (95:5) to yield 14h (141 mg, Yield 75%) as a white powder: IR ν max (neat): 1714, 1479, 1244, 1106, 1065, 877, 800 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.39 (s, 1H), 7.15 (d, J = 8.8 Hz, 1H), 6.77 (d, J = 8.8 Hz, 1H), 4.60 (t, J = 5.8 Hz, 1H), 2.04 – 2.12 (m, 2H), 1.13 (t, J = 7.5 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 176.3, 152.1, 130.4, 127.6, 124.7, 124.5, 115.2, 77.1, 34.5, 18.3, 13.6; FAB-MS: m/z 285 [M+Na⁺].

α-Butyl-2,4-dichlorophenoxy acetic acid (1j). Starting material 14i (200 mg, 0.72 mmol). Reaction mixture was purified with silica gel column eluted with CHCl₃-MeOH (95:5) to yield 1j (186 mg, Yield 78%) as a white powder: IR ν max (neat): 1731, 1479, 1285, 1242, 1106, 1079, 910, 814 cm⁻¹; ¹H NMR (400 MHz, CDCl₃); δ 7.38 (s, 1H), 7.14 (d, J = 8.8 Hz, 1H), 6.76 (d, J = 8.8 Hz, 1H), 4.64 (t, J = 6.1 Hz, 1H), 2.01 – 2.11 (m, 2H), 1.52 – 1.58 (m, 2H), 1.36 – 1.43 (m, 2H), 0.93 (t, J = 7.3 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃); δ 177.2, 152.1, 130.4, 127.5, 127.1, 124.5, 115.3, 77.3, 32.2, 27.1, 22.2, 13.8; FAB-MS: m/z 299 [M+Na⁺].

α-Hexyl-2,4-dichlorophenoxy acetic acid (1k). Starting material 14k (150 mg, 0.47 mmol). Reaction mixture was purified with silica gel column eluted with CHCl₃-MeOH (98:2) to yield 1k (120 mg, Yield 84%) as a white powder: IR ν max (neat): 1706, 1479, 1287.
1242, 1105, 1089, 917, 800 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$); $\delta$ 7.39 (s, 1H), 7.15 (d, $J = 8.9$ Hz, 1H), 6.76 (d, $J = 8.8$ Hz, 1H), 4.64 (t, $J = 5.8$ Hz, 1H), 2.00 – 2.07 (m, 2H), 1.53 – 1.60 (m, 2H), 1.26 – 1.39 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$); $\delta$ 176.7, 152.1, 130.4, 127.6, 127.1, 124.6, 115.4, 77.5, 32.5, 31.5, 28.8, 24.9, 22.5, 14.0; FAB-MS: $m/z$ 327 [M+Na]$^+$. 

Fig. S1. Effects of auxin and anti-auxin probes on auxin-induced lateral root formation in Arabidopsis. (A) Five-day-old seedlings were cultured with chemicals for another 4 days. 1 µM Probe 3 promoted lateral root formation similar to 2,4-D treatment. In contrast, probes 4, 7, and 8 blocked 2,4-D induced lateral root promotion. Scale bar represents 1 mm. (B) Black bar indicates the lateral root number treated with 0.4 µM IAA; Gray bar, the number treated with 8. Error bars show mean ± SD of two independent experiments.
Fig. S2. Effects of auxin and anti-auxin probes on Arabidopsis primary root elongation. 4-day-old Arabidopsis seedlings were grown for further 2 days in the presence of 20 μM probes with or without 100 nM auxins (IAA and 2,4-D). (A) Probe 3 inhibited root elongation similar to the test auxins (100 nM IAA (B) and 2,4-D (C)). In contrast, probe 4 had no effect and probes 7 and 8 promoted root elongation. (B and C) Probes 4, 7, and 8 were antagonistic to auxin in root elongation (scale bars, 5 mm).
Fig. S3. Effects of 8 on the hypocotyl elongation in etiolated seedlings. *Arabidopsis* etiolated seedlings were grown for 3 days in dark with probe 8 in the presence or absence of 2,4-D at indicated concentration. Bar represents 5 mm.
Fig. S4. The electron density of three probes. The electron densities are shown in red meshes (fofc map, 3α, calculated without the probes) superimposed on the crystal structures of the TIR1 auxin binding site in which the probes are shown in green. The TIR1 residues in contact the probes are shown in yellow sticks. Hydrogen bonds between the probes and TIR1 are shown as orange dashed lines.
Fig. S5. Effects α-alkyl NAA on auxin-responsive gene expression. (A) Chemical structures of NAA and seven α-alkyl NAA derivatives (C1-NAA to C7-NAA) are shown. (B and C) Arabidopsis auxin-responsive DR5::GUS reporter line was incubated for 5h with α-alkyl NAA at the indicated concentrations and/or 1 μM NAA. GUS expression was assayed histochemically by X-gluc staining. (B) α-Methyl NAA (C1-NAA) and C2-NAA induced GUS reporter gene the same as 1 μM NAA. Treatment with C3-NAA showed weak blue GUS staining at 20 μM, suggesting C3-NAA is weak auxin. On the other hand, C4-NAA to C7-NAA did not induce GUS expression at 20 μM. (C) C3-NAA and C4-NAA inhibited NAA-induced GUS expression at 50 μM. C5-NAA to C7-NAA completely suppressed NAA-induced GUS expression at 20 μM. These results suggest C1 to C3-NAAs display auxin activity, but C4 to C7-NAAs show anti-auxin activity.
Fig. S6. Effects α-alkyl 2,4-D on auxin-responsive gene expression. Chemical structures of four α-alkyl 2,4-D derivatives are shown. *Arabidopsis* auxin-responsive DR5::GUS reporter line was incubated for 5h with α-alkyl 2,4D at the concentrations indicated and/or 1 μM IAA. GUS expression was assayed histochemically by X-gluc staining. α-Ethyl 2,4-D (C2–2,4D) and C3–2,4D induced GUS expression similar to 1 μM IAA. C4–2,4D and C6–2,4D did not induce additional GUS expression (panel a). In panel b, C4–2,4D and C6–2,4D (10–20 μM) inhibited IAA-induced reporter gene expression, indicating anti-auxin activity of C4–2,4D and C6–2,4D against IAA.
Fig. S7. Predicted binding conformers of 8 to TIR1 auxin binding site. IAA7 degron peptide was shown as surface-filled model (gray) and IAA (red) and probe 8 (green) are superimposed on the coordinates determined by crystal analysis. Fifty possible binding conformers (yellow) were predicted by the program AutoDock using the Lamarckian genetic algorithm. Five representative predicted conformers of 8 (yellow) were selected and are shown by AutoDock Tools, based on the RMSD value to crystal coordinates of IAA moiety in 8 and estimated binding energy.
Fig. S8. Auxin binding pocket of *Arabidopsis* Auxin Binding Protein 1 (ABP1). The crystal data of ABP1-NAA complex was obtained from protein data bank (PDB ID, 1LRH). (A) Ribbon model (left) and surface-filled model (right) of ABP1 are shown. NAA bound to ABP1 is shown as green-colored molecule. Arrow indicates auxin binding pocket. In right-side model, the surface covering the binding pocket was removed to show the inside of ABP1 (the binding cavity is CPK-colored). The stable conformational form of 8 (yellow colored molecule) was superimposed on NAA in ABP1 (left). (B) Close-up of auxin binding pocket of ABP1. The pocket is so small that any of the conformers of 8 cannot bind to auxin binding pocket. The arrow indicates the alkyl carbon contacting with a peptide chain of binding site when 8 is superimposed.
Fig. S9. Probe 8 blocks of SCTR1-Aux/IAA pathway of rice. *Oryza sativa* CV Koshihikari was grown in 50 ml tube containing 15 ml of MS medium with 3% sucrose and 1% gellan gum for 10 days with/without chemicals in continuous light. (A) Probe 8, BH-IAA inhibited root hair formation. Plants were grown with 0.2 μM NAA and/or 8. (B) Probe 8 inhibited seminal root elongation and the formation of crown and lateral roots. Plant was grown with the indicated concentration of 8. Bar represents 10 mm.
Homology modeling of moss *Physcomitrella patens* orthologue (PpTIR1) and molecular docking calculation of 8 to PpTIR1. *P. patens* PpTIR1 with high homology (58%) to *Arabidopsis* TIR1/AFB was searched and subjected to homology modeling (A) *P. patens* genome database (PhyscoBase) using TIR1 as query (1). The homology modeling was predicted by SWISS-MODEL and the Swiss-pdbViewer (1–3) using 2p1mB as template. The PpTIR1 is shown as green colored, TIR1 as blue. IAA bound to TIR1 is shown in red. Amino acid residues in auxin binding site of TIR1 are conserved in PpTIR1 (lower panel). (B) Molecular docking calculation of the complex of PpTIR1 and 8. Fifty possible binding conformers were predicted by the program AutoDock using the Lamarckian genetic algorithm. Five representative conformers (light blue) were selected and are shown based on the RMSD value to crystal coordinates of IAA moiety of 8 and estimated binding energy. The crystal coordinates of IAA (red) and probe 8 (yellow) bound to TIR1 were superimposed on PpTIR1.

Fig. S11. Effects of 8 on the moss *P. patens* phenotype. (A, B) Probe 8 antagonized with NAA in the protonema development of *P. patens*. NAA enhanced the transition of chloronema to caulonema. Probe 8 inhibited the elongation of chloronema cell and the transition to caulonema. The chloronema were cultured in the presence of 8 and/or NAA for 10 days on BCDATG medium. (A) The photographs show the representative moss colony. Bar represents 5 mm. (B) The graph shows the average diameter of moss colony grown in the presence of 8 and/or NAA. Values are the mean ± SD of two independent experiments (*n* = 8). (C) Probe 8 inhibited gametophore formation. Gametophores were developed from protonema in the presence of 8 for 15 days on BCDATG medium. Arrow indicates gametophore. Bar represents 5 mm. (D) The phenotype of gametophore bud in *P. patens*. The gametophore bud was developed from the protonema treated with 8 and/or NAA for 10 days. Probe 8 antagonized NAA to restore the bud phenotype. Bar represents 0.5 mm.
Table S1. Summary of crystallographic data analyses

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<tr>
<td>Reflections (work/test)</td>
<td>4,753/1,324</td>
<td>23,841/1,232</td>
<td>26,541/1,440</td>
</tr>
<tr>
<td>R factor (work/test)</td>
<td>0.18/0.26</td>
<td>0.18/0.27</td>
<td>0.18/0.28</td>
</tr>
<tr>
<td>rmsd (bonds/angles)</td>
<td>0.025 Å/2.5°</td>
<td>0.024 Å/2.8°</td>
<td>0.035 Å/3.4</td>
</tr>
<tr>
<td>Ramachandran plot statistics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues in most favored regions</td>
<td>80.2%</td>
<td>80.0%</td>
<td>78.9%</td>
</tr>
<tr>
<td>Residues in additional allowed regions</td>
<td>18.6%</td>
<td>18.6%</td>
<td>19.3%</td>
</tr>
<tr>
<td>Residues in generously allowed regions</td>
<td>1.2%</td>
<td>1.4%</td>
<td>1.8%</td>
</tr>
<tr>
<td>Residues in disallowed regions</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>PDB ID code</td>
<td>3C6P</td>
<td>3C6O</td>
<td>3C6N</td>
</tr>
</tbody>
</table>

R<sub>sym</sub> = Σ h - <h<sub>h</sub>> / Σ h<sub>h</sub>, where <h<sub>h</sub>> is the average intensity of symmetry-equivalent measurements. Values in parentheses indicate outermost resolution shells.