Auxin Transport Inhibitors

IV. EVIDENCE OF A COMMON MODE OF ACTION FOR A PROPOSED CLASS OF AUXIN TRANSPORT INHIBITORS: THE PHYTOTROPINS

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GERARD F. KATEKAR AND ART E. GEISSLER
Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra 2601, Australia

ABSTRACT

The more active members of a proposed class of auxin transport inhibitors have been shown to have the ability to inhibit the active movement of auxin at concentrations where they have little effect on auxin action and no significant auxin activity. They have also been shown to give rise to characteristic biphasic dose-response curves on cress root growth. Based on these physiological similarities and other common physiological properties, it is concluded that they may achieve their effects by a common mode of action which differs from that of other known auxin transport inhibitors. It is suggested that the name "phytotropins" be given to the class of auxin transport inhibitors now defined by a similar mode of action and common chemical properties.

It has been shown that a group of chemicals which were known to affect the geotropic response can be defined by a common set of chemical requirements (16) and that these requirements are at least similar to those which can give rise to inhibition of auxin transport (18). The group can be divided into eight chemical types, and these are shown in Figure 1. Rules which define the chemical requirements so far known have been formulated (18). In summary, a 2-carboxyphenyl group separated by a conjugated system of atoms from a second aromatic ring may be necessary for a molecule to have high activity.

At least one member of each type has been established to be an auxin transport inhibitor, as well as having the additional physiological properties of being able to abolish the root and stem geotropic responses, the phototropic response, and the apical dominance effect. From the close chemical similarity between compounds within each type, it can be inferred that members within each type may act by a similar mode of action. The broader structure-activity correlation, however, implies a relationship between the types (18). It also implies that the mode of action of the group as a whole may be different from other auxin transport inhibitors which do not conform to the chemical parameters. An investigation of this relationship, therefore, would seem warranted, especially since some compounds from within the group, e.g. NPA (3), CPP IV-1, and DPX 1840 VIII-1 (Fig. 2), have found use in plant physiological research, together with other auxin transport inhibitors which do not belong to the group, e.g. TIBA IX and the morphactins X (Fig. 2).

To enable a class of compounds having similar physiological and chemical properties to be defined, chemicals of known high auxin transport inhibiting activity from within types II through VII were comparatively assessed for relevant physiological activities. In some assays, they were also compared with other known transport inhibitors which, on chemical grounds, were not regarded as members of the proposed class. Since the testing of all compounds for a range of physiological activities was impractical, representatives from types II to V were chosen mainly as model compounds. This can be justified on the grounds that types VII and VIII are themselves closely related and are known to have chemicals of type V-1 as their active form (4). Type VI is also only the lactone form of IV. Type I compounds, of their nature, do not fulfill all the chemical requirements and possess only low activity, so that direct comparison is rendered difficult. They are, therefore, not included in this correlation. The specific chemicals assessed are shown in Figure 2. Three activities which could be assessed in stem tissue were chosen, namely, auxin activity, the capacity to affect auxin action, and auxin transport activity. Two activities in roots, root elongation and the ability to affect the root geotropic response, were also assessed.

The name "phytotropins" is suggested as a descriptive term for the class defined below (see "Discussion"), as a major manifestation of their activity is their ability to interfere with the tropic responses of plants.

MATERIALS AND METHODS

Chemicals. The phytotropins CPD and VI-1 (7), CPP and VII-1 (12), NPA (25) and PBA (17) have been previously synthesized.

Fig. 1. Chemical types of phytotropins.

1 Abbreviations: CFM, methyl-2-chloro-9-hydroxyfluorene-9-carboxylate; CPD, 1-(2-carboxyphenyl)-3-phenylpropane-1,3-dione; CPP, 5-(2-carboxyphenyl)-3-phenylpyrazole; DPX 1840, 3,3a-dihydro-2-(p-methoxycarbonyl)-8H-pyrazolo[5-1a]isindol-8-one; NPA, N-(naphth-1-yl)-phthalamic acid; PBA, 2-(1-pyrenyl)benzoic acid; PCIB, p-chlorophenoxyisobutyric acid; TCBA, 2,3,6-trichlorobenzoic acid; TIBA, 2,3,5-triiodobenzoic acid.
V-2 and V-4 were synthesized by published methods (5). 2,4-D, IAA, TCBA, and TIBA were obtained from the Aldrich Chemical Company, whereas CFM was obtained from Celamerck, Germany. Lycoricidinol was obtained from Dr. J. M. Sasse of the University of Melbourne.

Auxin Activity. This was assessed by means of pea stem elongation. In general, the method followed was as outlined in the Agriculture Handbook of the United States Department of Agriculture (1).

Seeds (Pisum sativum cv. Victory Freezer) were soaked in running tap water for 6 h and planted in a mixture of perlite and vermiculite (50:50). Plants were grown in total darkness at 27 C. All observations and manipulations were made under green safety light. Plants received 4 min of red light (Philips red fluorescent tube TL-40 W/15; 263 µW/cm², 600–800 nm, of which 10% was between 700 and 800 nm) 24 h before harvesting. When the fourth internode was not more than 4 mm long, 6-mm segments were cut from the apical end of the third internode. After excision, no fewer than 12 stem sections were placed in each Petri dish, containing 30 ml of the test solutions. All solutions were buffered with sodium citrate and the pH was adjusted to 6.8 with NaOH. The segments were allowed to elongate in the dark for 24 h at 27 C and were subsequently measured to the nearest 0.1 mm.

Competitive Auxin Activity. Control segments obtained as above were soaked for 3 h in buffer alone (pretreatment) and were subsequently transferred to a fresh solution of buffer. Other sections were transferred after pretreatment to solutions of IAA at various concentrations. The remainder of the sections were pretreated with the test compounds by similarly soaking for 3 h at appropriate concentrations. After pretreatment, sections were transferred to test solutions of the same strength in which IAA was also incorporated at the required concentrations.

Auxin Transport-inhibiting Activity. (a) The assay using bean petioles has been previously described (18). (b) Where peas were used, [2-14C]IAA-labeled transport was assessed in pea stem sections identical to those used above. In other respects, the assay was based on that used by Keitt and Baker (19). After 3 h pretreatment as described above, excess solution on the sections was removed by blotting paper, and 20 sections were placed in Perspex holders with the apical ends resting on donor plantchets containing 1 µg/ml [2-14C]IAA (2.5 cm diameter; 1.5 ml volume; 1.5% agar; IAA activity, 30 mCi/mmol). Receiver blocks contained 1.5 ml 1.5% agar. These latter were placed on the basal ends of the sections, which were incubated in a humid environment for 4 h under laboratory lighting conditions. The radioactivity transported to the receiver blocks was determined by a gas flow counter. At least three replicates/concentration were used.

Cress Root Growth. Seeds of cress (Lepidium sativum) were allowed to germinate on agar (0.75%) for 24 to 26 h in darkness at 20 to 22 C. Seedlings were selected for uniform length, 9.5 to 10.5 mm, and the root tips were embedded in 0.75% agar containing the required substance under test in a Petri dish. On the underside of the dish a line was drawn, and the tips of the roots were placed against the leading edge of this line. Eight seedlings were placed in each dish and there were two replicates/concentration. The dishes then were stored vertically so that the root tips pointed downwards. Increase in length to the nearest 0.5 mm then was measured after 26 to 28 h, when control roots had grown 24 to 30 mm. Roots which grew on top of the agar generally grew faster than those growing in the agar and, therefore, were discarded. The seedlings were grown in darkness at 20 to 22 C. Embedding and measuring of the roots was carried out under laboratory lighting conditions. It was found unnecessary and cumbersome for the purposes here to carry out the manipulations under a safety light.

In an experiment done under these conditions and grown in darkness with CPD IV-1, the roots grew about 10% longer after 24 h, but the overall shape of the dose-response curve was unaffected.

Geotropic Assay. a) The dishes from the growth experiment described above were turned through 90° so that the root tips pointed horizontally. In untreated plants the root tip turned downwards and the shoot grew upwards, the changes being assessed after 24 h. When the gravitational response of roots was destroyed, they continued to grow horizontally and, when it was destroyed in shoots, they also continued to grow horizontally. b) Seedlings were placed in the agar as for the growth experiment described above but were placed vertically with the roots pointing downwards for 1 h and then were turned so that the roots were horizontal and grown in darkness. Reproducibility in both geotropic assays was high.

**RESULTS AND DISCUSSION**

Since NPA III-1 has been described as a weak auxin (19), a possible mechanism of action could be an ability to prevent auxin action, either as a competitive inhibitor or by some other means. The auxin activity, as well as the ability to prevent auxin action, was therefore assessed on pea stem sections and compared with their ability to prevent polar auxin movement in identical pea stem sections. Under the experimental conditions, the concentration of IAA which gave the optimum effect was found to be 10⁻⁵ M in either the presence or absence of 10⁻⁵ M CPD (Fig. 3). This concentration of IAA was therefore used in the competitive experiments.

If the compounds possess auxin activity or can affect auxin action, they would be expected to reduce stem growth in competitive experiments where optimal IAA is present. In the experiments without auxin present, auxin activity would be expected to result in increased elongation, whereas an inhibitory effect could be due to factors other than auxin activity. Auxin activity and the ability to prevent auxin activity are shown in Figure 4. To test for auxin transport activity, similar pea stem sections were pretreated as before and then used on the donor-receiver agar block technique (Fig. 4).

Inasmuch as there is a strong correlation between the chemical requirements for inhibiting auxin transport and the ability to affect the root geotropic response (18), dose-response curves with respect to cress root growth were obtained for the phytotropins (Fig. 5) as well as other auxin transport inhibitors (Figs. 6 and 7), together with their ability to abolish the root geotropic response. Although results have been normalized in these figures to show growth as a percentage of control, typical standard errors are...
physiological response to IAA was abolished at a concentration of 10^{-5} M CPD.

Consistently, representatives of those types where either it is known, as in VII-1 and VIII-1 (4), or can be concluded, as in VI-1 (7), that they undergo conversion to the active form, also show dose-response curves which are similar to the other compounds (Fig. 5). These observations would be consistent with at least a similar mode of action. Additionally, however, NPA is known to interact at a specific binding site in corn coleoptiles (21), and it is also known that this binding site does not interact with any known hormone (29). If the phytotropins achieve their effect by a common mode of action, it would be expected that other members may also interact with the same site. Preliminary observations indicate that this is the case for CPD (R. Hertel, personal communication). The fluoresceins also bind to the same site (M. H. Goldsmith and M. R. Sussman, personal communication). It is concluded that the similarities in the physiological responses which these compounds elicit is consistent with the suggestion that they may achieve their effects by a common mode of action.

Differences in Responses. The pyrenoyl benzoic acid PBA II-1

Fig. 3. The effect of IAA on pea stem elongation in the presence and absence of 10^{-5} M CPD.

Fig. 4. Auxin and auxin transport inhibiting activities of phytotropins in pea stem sections. Elongation in the presence (upper curves) and absence (lower curves) of 10^{-5} M IAA after 24 h. Hatched bars, polar auxin transport.

included in the curve for CFM, (Fig. 6), where actual length measurements are recorded.

Comparable Physiological Responses. The effects of the phytotropins upon pea stems show similarities at the lower concentrations. First, the representatives of types II, IV, and V have no significant effect on stem elongation at concentrations as high as 10^{-4} M, whereas the effect of the type III compound (NPA) was barely significant (Fig. 4, lower curves). Second, in combination with IAA, they also do not markedly affect auxin-induced elongation at concentrations below 10^{-3} M (Fig. 4, higher curves).

Here, sections are floating on auxin solutions at 10^{-5} M and thus receive an optimum concentration of auxin from the medium. Cell to cell transport is not limiting for growth in this experimental situation and, hence, inhibiting it does not affect growth. Third, in contrast to their weak effects on elongation, they strongly reduce the polar movement of auxin in the same stem tissue (Fig. 4, hatched bars). The dose-response curves of the phytotropins with respect to cress root growth can also be seen to have basic similarities (Fig. 5). The highly active representatives show biphasic curves in which there are two concentration ranges where root growth is inhibited by the compound, and these two ranges are separated by a plateau in which root growth inhibition is insensitive to concentration over ranges of 2 to 3 orders of magnitude. Further, in all cases, the root geotropic response is lost at the beginning of the plateau (Fig. 5, arrows).

Consistently, representatives of those types where either it is known, as in VII-1 and VIII-1 (4), or can be concluded, as in VI-1 (7), that they undergo conversion to the active form, also show dose-response curves which are similar to the other compounds (Fig. 5). These observations would be consistent with at least a similar mode of action. Additionally, however, NPA is known to interact at a specific binding site in corn coleoptiles (21), and it is also known that this binding site does not interact with any known hormone (29). If the phytotropins achieve their effect by a common mode of action, it would be expected that other members may also interact with the same site. Preliminary observations indicate that this is the case for CPD (R. Hertel, personal communication). The fluoresceins also bind to the same site (M. H. Goldsmith and M. R. Sussman, personal communication). It is concluded that the similarities in the physiological responses which these compounds elicit is consistent with the suggestion that they may achieve their effects by a common mode of action.

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FIG. 6. Effect of other compounds on the roots of L. sativum. Curves and arrows have the same meaning as in Figure 6.

FIG. 7. Effect of lycoricidinol and PCIB on the roots of L. sativum. Curves and arrows have the same meaning as in Figure 7.

at $10^{-3} \text{ M}$ substantially prevents elongation of the stem sections, both on its own and in the presence of IAA. This property is not shared by the other compounds tested. The degenerated appearance of the stem sections would indicate that this may be a phytotoxic reaction. The naphthylphthalamic acid III-1 NPA, on the other hand, has a weak ability to increase stem growth at concentrations above $10^{-6} \text{ M}$, which is also not shared by the other chemicals. Such weak auxin activity of NPA has previously been observed (19). It was considered possible that this activity could be due to in vivo hydrolysis to phthalic acid and $\alpha$-napthylamine since NPA is an amide. Testing of both these compounds showed that neither has significant auxin activity so that the auxin activity of NPA may be intrinsic to the molecule itself. It is suggested that nonspecific phytotoxicity at high concentrations and very weak auxin activity may be due to minor chemical variations between the types and do not affect significantly the conclusions drawn above with respect to the much stronger physiological effects achieved at lower concentrations.

Nature of Mode of Action. The very weak or nonexistent auxin activity shown by the compounds tested indicates that their effectiveness in preventing polar transport is unrelated to auxin activity. All except PBA II-1 at $10^{-3} \text{ M}$ do not appear to be toxic, even at the highest concentrations, because the treated pea stem sections show at least control level expansion. At high concentrations, however, PBA, CPD, and CPP can inhibit to some extent the auxin-induced elongation of the stems. This could be due to either competitive inhibition or to some other type of inhibition of auxin
action. The root growth-inhibition assays show two ranges where the relationship between the response and the logarithm of the chemical's concentration approximates a linear one. They thus resemble a hormone-induced dose-response curve (20, 23). It is possible to speculate that the activity shown at low concentrations is related to the effect which gives rise to inhibition of auxin transport in stems, whereas that shown at high concentrations may be due to their effect on auxin action, although toxic effects at high concentrations cannot be excluded. The physiological properties examined here may not be the only properties possessed by the class. For example, ABA is considered to be involved in the root georesponse (33) so that it may be that the compounds can affect the movement of this hormone as well. Since all the known natural hormones are capable of affecting IAA transport (2, 22), presumably by an indirect mechanism (see below), it is at least possible that the effect of the phytotropins is similarly indirect, and that the ability to inhibit auxin transport is not their primary mode of action.

Differences from Other Auxin Transport Inhibitors. TIBA appears to act as a weak antagonist in stem tissue, being able to suppress auxin activity at high concentrations while having no significant auxin activity itself (Fig. 4e). The results for TIBA are consistent with those of other workers who have shown that it can interfere with both IAA (14) and 2,4-D activity (32). Also, it has been shown consistently that TIBA competes for the same binding sites as IAA (14, 29) and moves in the same channels as IAA, being capable of polar transport (29), whereas the phytotropins NPA and DPX 1840 are not (3, 29).

Since TIBA is a weak auxin transport inhibitor (Fig. 4e, hatched bars), its ability in this regard may be primarily due to its properties as an auxin competitor. Both TIBA and the other synthetic auxins TCBA XI and 2,4-D XII have dose-response curves on cress root growth which are similar to that of IAA (Fig. 6). None of these curves are biphasic, as with the phytotropins. These compounds also have the ability to abolish the root geotropic response, but only at concentrations where root growth is highly inhibited. This is also shown by the arrows in Figure 6. The dose-response curve of the auxin antagonist PCIB XIV (Fig. 7) also differs significantly from those of the phytotropins and is not biphasic. Its ability to abolish the geotropic response also occurs only at concentrations where root growth is highly inhibited (Fig. 7). It is concluded that these compounds, all of which are known to be auxin transport inhibitors (19, 24), achieve their effects by a different mode of action from the phytotropins.

The plant growth inhibitor lycoricidinol XIII has been shown to inhibit auxin transport by a mechanism different from that of the morphactins (15). It also differs from the phytotropins because it has the ability to prevent auxin-induced elongation (15). Its dose-response curve on cress root growth is also significantly different from those of the phytotropins, and it can only affect the root geotropic response at concentrations where very little root growth is observed (Fig. 7).

The morphactins are a further class of auxin transport inhibitors which have some physiological properties in common with NPA and TIBA (26). Their mode of action with respect to cress root growth and geotropic response may not be different from the phytotropins, despite obvious chemical differences between the two classes. Further, it has also been shown that NPA and the free acid of CFM (X; CH₃ = H) compete for a common binding site in homogenates from corn coleoptiles (30), which would also indicate a common mode of action. The morphactins, however, are known to induce a variety of characteristic morphological effects (26) not shared by the phytotropins. These include structural changes to the root cap (26). Such changes have not been reported for NPA or DPX 1840, which have been shown to inhibit cell elongation in the roots of P. sativum (11). The morphactins therefore, can be distinguished from the phytotropins on both chemical (18) and biological grounds. A relationship between the two classes thus exists, but the nature of the relationship is as yet unclear.

There are many other chemicals known to be able to inhibit auxin transport, but they are not classed as such because the property is regarded as an indirect effect, rather than a cause, of their known biological activities. Some compounds include the natural hormones C₃H₄ (2) and the gibberellins (22). The cytokinins however, are known to promote auxin transport (22). Similarly, enzyme or metabolic inhibitors may have this property. Compounds in the latter category include coumaric and caffeic acids, organomercury compounds, iodoacetate, N-ethylmaleimide (19), and sodium azide (9). The phytotropins can thus be distinguished from the other known auxin transport inhibitors by differences in both chemistry and mode of action.

Other Possible Phytotropins. Recently, large numbers of chemicals have been described in the literature, which conform to the suggested chemical rules. Some were specially synthesized with the appropriate characteristics (12, 13, 17), and all have been claimed to have either plant growth regulatory (6) or herbicidal (5) properties. These newer types are summarized in Figure 8. Chemically, compounds V-2 to V-15 can be classed as falling within type V of the phytotropins where the central portion of the molecule is an aromatic ring. Although not all have as yet been assessed for auxin transport activity, it can be seen (Fig. 9) that V-2 and V-4 are powerful inhibitors of auxin transport in bean petioles and that their dose-response curves on cress roots are consistent with a mode of action similar to the other members of the class (Fig. 4). Such remaining compounds of this type as have been tested have been shown to be able to abolish the root geotropic response (13).

It is therefore suggested that at least some of the biological properties of these compounds are due to their activity as phytotropins. Similarly, the herbicidal benzopyran phthalides XVIII (27), o-cinnamyl benzoates XV (28), phenylbenzoxazines (XVI) (10), and the benzimidazo isoquinolines XVII (8), although not fitting within the chemical types I to VIII described above, nevertheless conform to the rules suggested for the chemical requirements for auxin transport activity (18) and may therefore have phytotropin activity. In the case of the isoquinoline XVII, it has been found here to abolish the geotropic response in cress roots at concentrations of 10⁻⁶ M or greater. This compound also possesses the apparently unrelated property of being able to inhibit photosynthesis (8).

Usefulness. Since the phytotropins have found application in plant physiological investigations, the question arises as to which are the better ones to use. Desirable criteria would be high activity, chemical stability, and absence of unrelated physiological and chemical properties which would cause difficulties of interpretation. Of the compounds currently available, it is suggested that CPD and CPP best fulfil these criteria. Although PBA is the most active of all the compounds, it would appear to be phytotoxic at high concentrations and, therefore, may be unsuitable. The type III compounds, including NPA are less active. NPA is also a weak auxin, whereas CPP and CPD are not. The lower activity of type III compounds may be at least partly due to the fact that all are amides and would be susceptible to hydrolysis within the plant. In contrast, types VII and VIII require transformation to their active form V-I in the plant (4). The reaction which inactivates NPA (amide hydrolysis) is the same one which is required to convert VII and VIII to their active forms. Type VI compounds also require a hydrolytic step to convert them to their active form IV. DPX 1840 VIII-I has been found to be less active than its active form V-1 (4), presumably because both dehydrogenation and hydrolysis are required. If the morphactins do in fact possess phytotropin activity, then they also possess other physiological
properties not shown by the phytotropins and may be polyvalent (26). It is suggested that they are unsatisfactory for this reason. Although it is possible that other type V compounds, where the central portion of the molecule is a heterocyclic ring, may prove to be suitable, it is also possible that some of these compounds may possess secondary properties. For example, some of the heterocyclic rings may themselves possess biological activity, and this may be reflected in the activity of the molecule as a whole.

If the binding site with which NPA is known to interact (discussed above) is a receptor site through which the phytotropins achieve their effect, then the implication is that it is the receptor site for an endogenous ligand or ligands within the plant. Such a compound, karotoffelfaktor, has in fact been isolated (R. Hertel, personal communication), and it is known to be a potent inhibitor of both auxin transport and geotropic curvature (31). It has been suggested that this compound may be a natural analogue of NPA (31). Since NPA is a phytoptrin, it follows that karotoffelfaktor may also be an analog of the phytotropins.

If a naturally occurring compound acts at a specific binding site and affects given physiological processes, it may be involved in the control of the processes which it is known to affect. In the case here, synthetic compounds are known, NPA and CPD, which not only compete for the same binding site and affect the same processes of auxin transport and geotropic curvature, but which also affect other processes, including phototropism and apical dominance. This spectrum of activity appears to be an integral part of the structure-activity correlation of these compounds. The natural compound, perhaps together with other endogenous analogues that are yet unknown, may be involved in the control mechanisms of plant growth and development which the phytoptins are known to affect. Current hypotheses with respect to such mechanisms may therefore be inadequate in this regard, but the class of compound defined here may be of use in the elucidation of these mechanisms.

Conclusions. It is argued that a class of auxin transport inhibitors can be defined by a common set of chemical and biological properties and that the class is potentially a large one. The name phytotropins is suggested for this class. To the extent that compounds within the class have been tested, the results are consistent with the proposition that they achieve their effects by a common mode of action. They are distinguishable from other auxin transport inhibitor classes, such as auxin competitors (including TIBA), the morphactins, and the lycoicidins. Because the chemical definition of the class remains tentative (18), it may be that the more active class members may yet be made. The development of competitive inhibitors of the phytotropins is also not excluded. Because members of the class interact at a specific binding site within the plant, it is speculated that the more active members may be either agonists or antagonists of a naturally occurring plant growth substance or substances.

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