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Possible evolutionary relationships in a signal transduction system

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Abstract. Cells generally respond to external chemical stimuli by binding interactions with proteinaceous receptors. However, in many cases this process is not structurally specific and the information can be transmitted by a general class of molecules rather than one specific structure. One such case is the plant pathogen *Agrobacterium tumefaciens*. A broad spectrum of simple phenolic structures are capable of inducing oncogenetic gene transfer from pathogen to host. Consideration of the ~40 different active structures have led us to propose a mechanism whereby the receptor catalyses a *reaction* with the signal molecule to induce a stoichiometric transmembrane signaling event. Attempts to experimentally substantiate the model have led to the development of mechanism-based inactivators and the use of radiolabeled inhibitors have made it possible to identify the receptor proteins. In addition, a subset of the active structures not only activate *A. tumefaciens* gene transfer but serve as growth factors for the host plant cell. It may be that the pathogen has evolved to recognize these molecules as representative of critical chemical events that control plant cell division. Our attempts to exploit the mechanistic details of this signal transduction system are discussed.

INTRODUCTION

If the field of organic chemistry can be dated to Wöhler's synthesis of urea, then in just 164 years, a small fraction of the life of the lovely city of Strasbourg, natural products chemists have developed the methods enabling the structural characterization of all carbon compounds made by nature. The technology required for the total synthesis of this seemingly unlimited array of structural architectures has also been developed. These awesome achievements, contributing greatly to our understanding of chemical bonding and structure, have now flowed into practical improvements in agriculture, the cure of human diseases, and the design and construction of new materials. Nevertheless, our knowledge in some areas of natural products chemistry remains rudimentary; most notably in the area of function and mechanism of action of these natural products. We now face the ultimate challenge of using this understanding of molecular architecture and chemical reactivity to define the essence of living systems; the structural basis of biological recognition, chemical replication, and molecular evolution.

Excellent examples of the deficiencies in our current understanding of natural products is provided by two compounds, indole-3-acetic acid, auxin, and N^6-isopentenyl adenine, a cytokinin. These simple structures are well-known plant growth factors. Unlike animal cells where in excess of

50 growth factors have been characterized, the variation in the relative concentration of these two compounds in tissue culture is sufficient to control the growth and development of the plant; in some cases organizing the growth of single cells into the mature plant. While the structure and the biological activity of both of these compounds have been known for almost 50 years, we do not yet know how these molecules are biosynthesized in the plant, how the biosynthetic pathways are controlled, and we know virtually nothing about the mechanism by which these factors exert their control on growth and development. In this paper we will outline an approach to define the biological activities of these growth factors.

EXPLOITING A PATHOGEN

Armin Braun recognized that the tumor-inducing bacterial pathogen, *Agrobacterium tumefaciens*, might provide insight into the control of plant cell growth (1). He and Henry Wood provided evidence for and initiated the isolation of a new growth factor from crown gall tumors (2). Together with Wood, our lab was able to isolate and characterize DCG growth factors (3). These growth factors (4, 5) originate biosynthetically from coniferyl alcohol in what appears to be a branch from cell wall biosynthetic pathways (6) and the pathway is activated in cultured tobacco cells by exposure to cytokinins. These data are consistent with the action of the cytokinins on cell growth being mediated through a second metabolic signal, the DCGs, and open new approaches to the study of cytokinin action.

The transformation of the plant cell in crown gall formation is the consequence of gene transfer (T-DNA) from the bacterial tumor-inducing plasmid (Ti-plasmid) to the host (7-10). The integrated genes encode enzymes which catalyze the initial steps in the conversion of adenine to zeatin and the synthesis of auxin from tryptophan (11). More importantly, these are bacterial biosynthetic pathways not present in nor controled by the plant since the genes are driven by constitutively expressed eukaryotic promoter and activator sequences (9). Consistent with Braun's early suggestions, the inability of the plant to control these oncogenes establishes these transformed tissues as reasonable sources for the identification of growth factors whose concentrations are modulated by the tumorous phenotype.

However, a reversal in the focus of this research came with the discovery that the aglycone of the DCGs, dehydrodiconiferyl alcohol, DCA, was active in inducing the virulence response in *A. tumefaciens* (12). This finding suggested that plant growth factors might provide the signal for host viablity to the pathogen (13, 14), but, more importantly, identified the bacteria as an exploitable system to study the mechanism of action of the growth factors.

MECHANISM OF PHENOL DETECTION

Phenol recognition in *A. tumefaciens* is mediated by a two-component sensory-kinase / response regulatory system (15, 16). VirA and VirG, the two protein components of the system, are encoded by constitutively expressed Ti-plasmid-localized genes. Genetic and biochemical studies have suggested that VirA serves as the phenol receptor (9). This protein is encoded by a single copy of the *virA* gene (17), suggesting that there is but a single phenol receptor.

Structure/activity studies collectively involving over 80 commercially available compounds (18-20) have documented the activity of more than 40 compounds. The phenol substituents essential for activity were (i) orthomethoxy or bis-orthomethoxy, where dimethoxyphenols were more active, and (ii) carbonyl-containing functional groups oriented para to the phenol hydroxyl group, where activity varied according to the series $-COCH_3 \geq -CHO \geq -CO_2CH_3 > -CO_2H$.

These data and crystallographic information available for proteins with phenol binding sites (21-24), a model binding site was proposed (12) and is shown in FIG 1. The proposed site contained: (i) a largely hydrophobic cleft able to accommodate the planar aromatic ring, (ii) a conformationally flexible hydrogen bond donor in a sterically unconstrained region occupied by the para substituent, (iii) a conformationally rigid hydrogen bond donor appropriately oriented for interaction with the oxygen of the ortho-methoxy groups, and (iv) a rigid hydrogen bond acceptor for interaction with the phenol hydroxyl. In this model, the interactions of the hydrogen bond pairs are additive so the highest affinity interaction would be with molecules containing all these substituents.

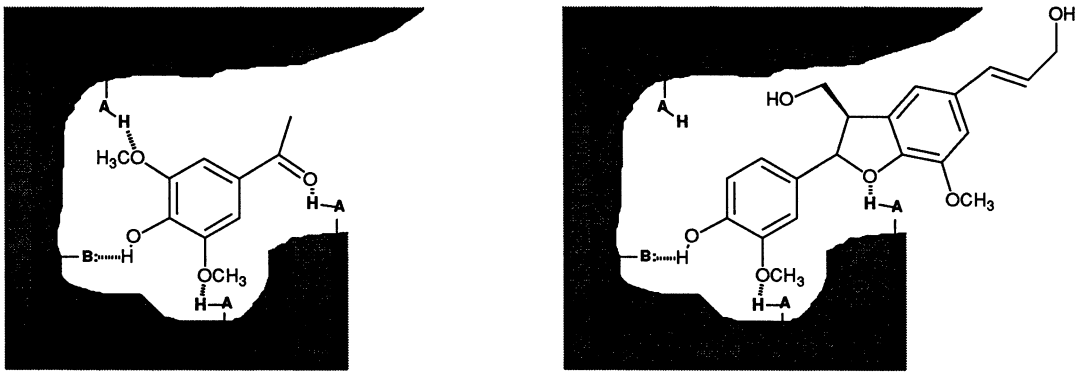


FIG 1. Binding site model.

STRUCTURAL TESTS OF THE MODEL

Models of this type can be tested by altering the structure of either the ligand or the receptor and both have been attempted (12, 25). In the initial experiments (12), advantage was taken of structural permutations that were possible in the para dihydrobenzofuran substituent of DCA, the substituent which distinguished it from other known inducers. A series of analogs were prepared which suggested that the activity was not critically dependent upon the hydrogen bond potential of the para substituent or steric factors, but rather the hydrolytic reactivity of the compound (12). These data led to the proposal of a driven proton transfer as the critical signaling event, and in the case of DCA, hydrolysis of the ligand occurs on binding to the receptor. The experimental support for this proposal has been recently reviewed (14).

DEVELOPMENT OF MECHANISM-BASED INACTIVATORS

The model predicts changes in ionization of two amino acids residues, a proton donor and a proton acceptor. The possible generation of the more strongly nucleophilic residue following ionization of the proton donor, e.g., a carboxylate, was tested by the preparation of α-bromoacetosyringone

(ASBr) (12). This analog attempted to build on the chemistry of a known class of mechanism-based irreversible inhibitors of general acid-mediated enzymic transformations (26) and required nucleophilic displacement of the bromide. Consistent with this requirement, the α-bromo and α-iodo-derivatives were comparable inhibitors (85% and 90% inhibition respectively at 10 μM) whereas the chloride analog was not inhibitory (13% inhibition at 10 μM). The bromide proved to be specific and irreversible; the inhibition of *vir* expression was seen without deleterious effects on the expression of other genes or on bacterial growth in general and on pre-incubation with this compound, *A. tumefaciens* was insensitive to subsequent induction (12).

The most critical feature of this model however involves the proposed proton acceptor. Our initial investigations attempted to determine the pKa dependence of the phenol inducers. However, attempts to correlate pKa data with activity in these in vivo assays was not successful. Nevertheless several nitrophenols were found to be active inducers. Both the nitro analog of acetosyringone, **2**, and the *o*-nitrophenol, **3**, were active inducers (FIG 2A). Dinitrophenol (DNP), **4**, was not active,

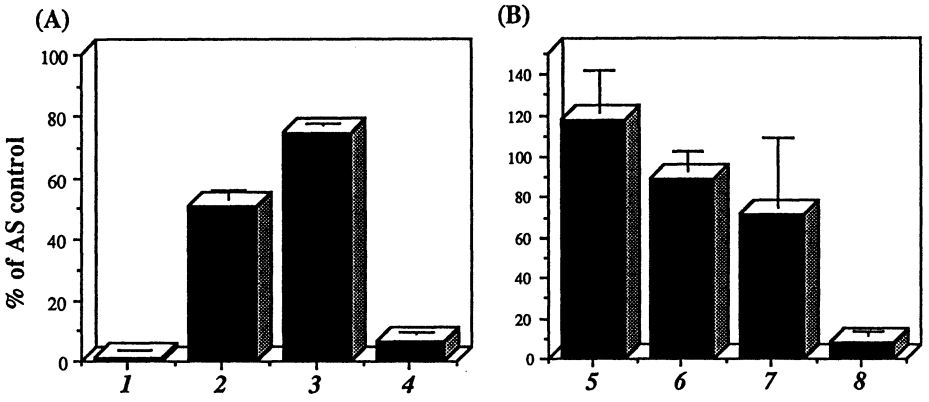
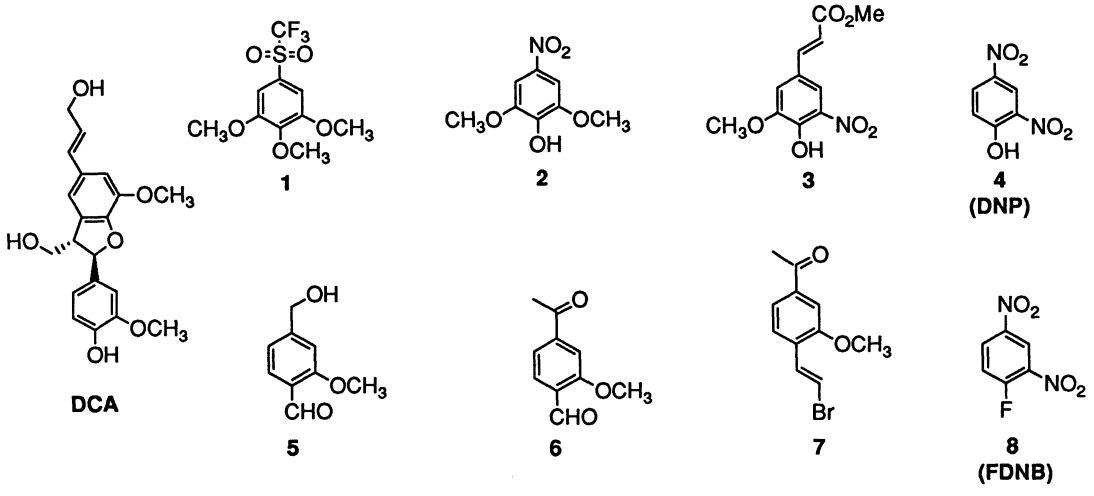


FIG. 2. (A) *Vir* induction. *A. tumefaciens* strain 358mx (*virE::lacZ*) was induced with each compounds at 100μM in the induction broth for 8 hr at pH 5.5 (12). β-Galactosidase activity was compared to the 100μM AS control. (B) *Vir* inhibition. *A. tumefaciens* strain 358mx (*virE::lacZ*) was incubated with each compounds at 10μM except **8** (1.5μM) in the presene of 100μM AS. After 8hr incubation, β-galactosidase activity was measured and reported as % of AS control. The indicated structures are shown below.



possibly because it is very acidic (pKa 4.07, (27)) and would be ionized under the assay conditions. These data did establish that nitro substituents could serve as H-bond acceptors on active inducers.

Attempts to exploit the nucleophilicity of the proton acceptor led to the construction of several potential methylating agents, such as the sulfone, **1**, but these were not active inducers (FIG 2A) or inhibitors. Attempts to induce the residue to react with benzylic aldehydes, **5** and **6**, or a vinyl bromide, **7**, were not successful as judged by the inhibition data (FIG 2B). The observation that nitro groups could be accommodated on active inducers led to the evaluation of Sanger's reagent (28), 1-fluoro-2,4-dinitrobenzene (FDNP). This compound proved to be one of the most active inhibitors that we have identified.

As shown in FIG 3A, FDNB proved to be a more effective inhibitor than ASBr. As with ASBr, this inhibition was partially protected by incubation with high concentrations of AS. FDNB readily alkylates amines and has been used for the determination of N-terminal amino acid residues, but at 10^{-6} M, neither the growth of the bacteria nor the expression or activity of β-galactosidase driven by the *occ* regulon of the Ti-plasmid were inhibited (FIG 3B). At this stage of analysis, FDNB appears to be as selective an inhibitor as ASBr and its reaction with the putative binding site is shown in FIG 4. The inhibition by FDNB does not prove the presence of a base in the proposed model, but it should be useful in the further definition of the receptor.

CONCLUSION

The model that we have been testing for this phenol binding site is just that, a model, and is at this point not a representation of a real protein. Nevertheless, it has proven to be a valuable device for the design of molecules and the development of inhibitors. The use of these inhibitors have recently led

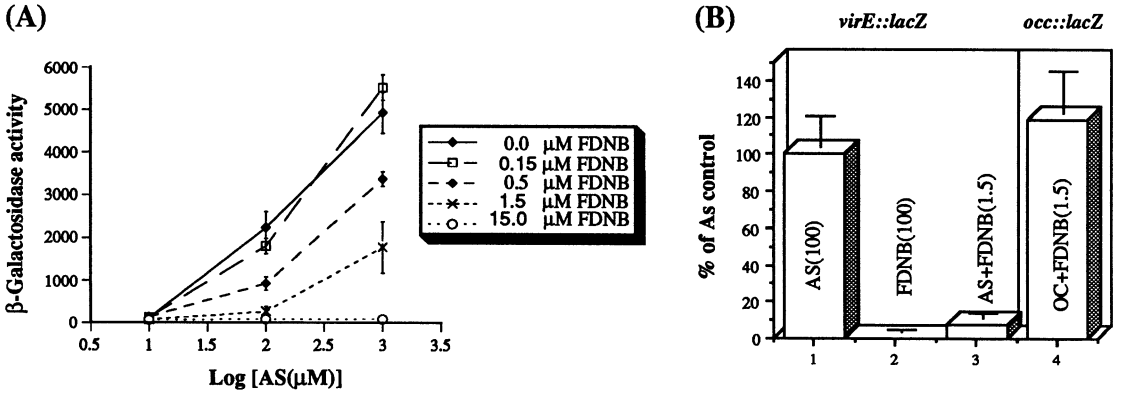


FIG. 3. (A) Dose-dependent *vir* inhibition by FDNB. *A. tumefaciens* strain 358mx (*virE::lacZ*) was induced with different concentrations of FDNB in the presence of increasing concentrations of AS for 8 hr in the induction broth at pH 5.5. β-Galactosidase activity was measured and reported as Miller unit.
(B) Specificity of FDNB inhibition. *A. tumefaciens* strain 358mx (*virE::lacZ*) or A348pSM102 (*occ::lacZ*) was incubated with FDNB in the presence of 100 μM AS or 400 μM octopine (OC), respectively. The concentrations (μM) of FDNB tested were shown above in parentheses. Values represent % control relative to 100 μM AS (in 358mx) or 400 μM OC (in A348pSM102). Under all conditions, FDNB did not affect bacterial growth according to $O.D._{600}$ measurements.

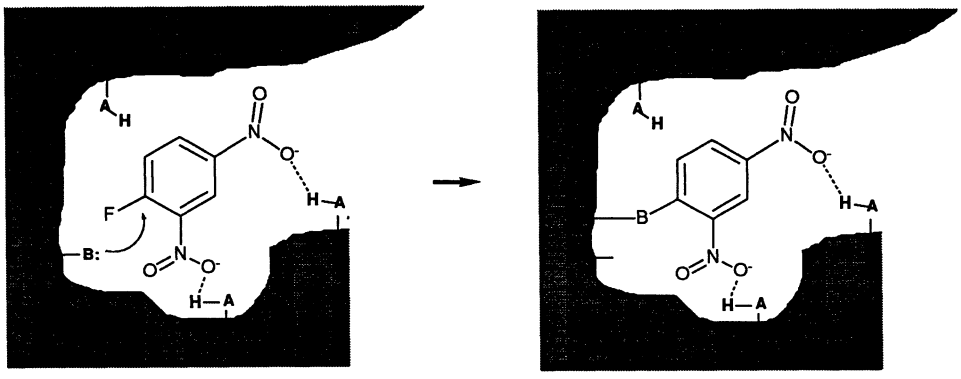


FIG. 4. Model for FDNB inhibition

to a reevaluation of the accepted role of VirA as the phenol receptor (29). The α-brominated derivative of the active inducer 5'-iodoacetovanillone was prepared in radiolabeled form (I = ^{125}I) and no evidence was found for incorporation of the radiolabel into VirA; rather, two low molecular weight proteins, p10 and p21, reacted specifically with the labeled inhibitor both in vivo and in vitro. These proteins failed to react with other compounds designed to test nonspecific reactivity. Since a plant growth factor, DCA, provided the initial motivation for these studies, we have used the inhibitors to screen for similar protein in plant tissues. Proteins of the same M_r and with similar reactivity have now been identified suggesting that it may be possible to exploit this technology for the dissection of the far more complex eukaryotic organism.

More relevant for the discussion at this meeting is the fact that these studies were initiated because of our poor understanding of the function of two very simple natural products. By exploiting spectroscopic structural assignment methods, biosynthetic feeding experiments, and organic synthesis, technology for which we owe a great deal to the people who are in attendance at this meeting, we have been able to provide insight into the control of cell growth. The process of replicating everything present within a eukaryotic cell is a monumental chemical feat. The possibility that the control of this process could be understood by defining the natural product chemistry of a simple compound is indeed appealing. In another sense, if our understanding of these simple molecules is yet so redimentary, the future is indeed exciting given the intricately complex molecular architectures discussed at this meeting.

so much depends
upon

a red wheel
barrow

glazed with rain
water

beside the white
chickens

"The Red Wheelbarrow"
William Carlos Williams

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