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Biochemistry

Mechanism of phenolic activation of *Agrobacterium* virulence genes: Development of a specific inhibitor of bacterial sensor/response systems

(signal transduction/plasmid gene expression/crown gall/Vir A/α-dehydrodiconiferyl alcohol glycoside)

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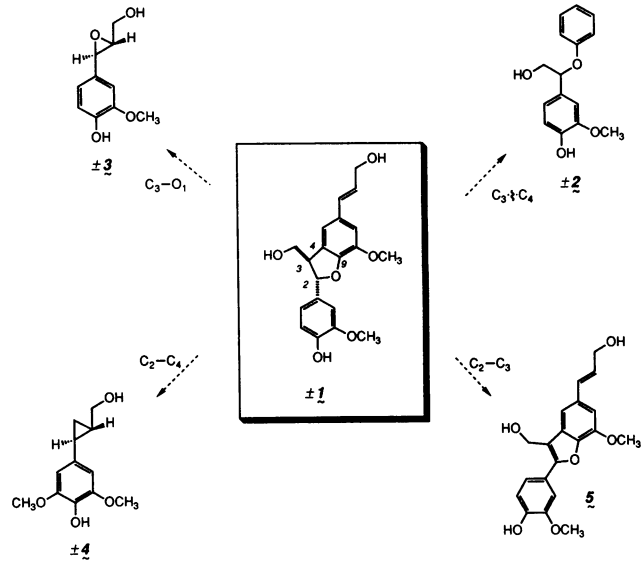
ABSTRACT **The aglycone of the dihydrodiconiferyl alcohol glycosides, a series of phenolic growth factors able to substitute for some of the hormone requirements of tobacco cell division, are also potent inducers of virulence gene expression in *Agrobacterium tumefaciens*. However, these factors do not conform to the previously established structural requirements necessary for *vir* expression. Systematic evaluation of the structural requirements of these inducers has led to a model detailing the role of the phenolics in induction. With this model, a specific inhibitor of *vir* induction has been developed. This inhibitor does not affect the induction of other genes on the Ti plasmid but irreversibly blocks *vir* expression. The inhibitor has been used to show that the inducing phenolics must be constantly present to maintain expression of the *vir* regulon.**

Agrobacterium tumefaciens can transfer genetic material (T-DNA) into cells of higher plants, where it is integrated into the nuclear genome (1, 2). This gene transfer requires both chromosomal- and Ti plasmid-encoded gene products. The virulence (*vir*) genes of the Ti plasmid encode proteins required for processing and transfer of T-DNA (3). Two of these genes, *virA* and *virG*, have been proposed to function in a manner analogous to other bacterial two-component regulatory systems (4, 5). Vir A, a periplasmic membrane-spanning protein, would be the component that senses the stimuli and autophosphorylates at a histidine residue in its cytoplasmic C-terminal domain (6, 7). The Vir A phosphohistidine is thought to transfer the phosphate to Vir G, the signal-response regulator, which then activates transcription of the *vir* genes (8, 9). The environmental signals that initiate this cascade are specific plant cell-derived phenolic compounds that are synergistically enhanced in activity by simple monosaccharides (10, 11).

Two features of this system have made it particularly attractive for further characterization of the signal-transduction pathway. (*i*) The genetics is well developed, allowing for rapid analysis of gene expression and for site-specific mutagenesis of the pathway components. (*ii*) Dehydrodiconiferyl alcohol, compound **1** (Scheme I), the aglycone of recently characterized molecules that have cell division-promoting activity in tobacco bioassays (12, 13), has now been shown to have *vir*-inducing activity. This molecule is produced rapidly after wounding of tobacco cells, concomitant with the early wound-induced cell divisions (14) critical to *Agrobacterium* transformation (15–17). Therefore, a connection may exist between signals controlling plant cell division and the signals initiating *Agrobacterium*/plant gene transfer (18).



Scheme I. Structure of dehydrodiconiferyl alcohol, compound **1**, together with a series of structural analogues designed by the formal formation or breakage of the bonds between the atoms shown by dashed arrows.

The *vir*-inducing activity of **1** was not anticipated based on the structures of previously identified phenolic inducers (19, 20). The specific structural features of **1** have now led to a model for phenolic interaction that incorporates all structures active in *vir* induction. Attempts to exploit this model allowed for the development of other potent agonists and to a specific *vir* induction antagonist. This antagonist has also allowed for an analysis of the time dependence of *vir* induction and provided insight into the plasticity of commitment to the gene-transfer event in *Agrobacterium*-mediated plant transformation.

MATERIALS AND METHODS

Bacterial Strains and Media. *A. tumefaciens* strains 358mx (*virE*::*lacZ*) (3) and strain A348 containing pSM102 (*occ*::*lacZ*) (21) were maintained on AB (9) minimal medium supplemented with carbenicillin at 100 μg/ml and 50 μg/ml, respectively. Induction broth at pH 5.5 was used as described (22) or modified by using 1% glucose (Fig. 1) for 3% sucrose. Induction broth was made fresh daily, and all components were sterilized by filtration.

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Abbreviations: ASBr, α-bromoacetosyringone; AS, acetosyringone; APBr, 3′,5′-dimethoxyphenacyl bromide; THF, tetrahydrofuran; DIBAL, diisobutyl aluminum hydride; β-Gal, β-galactosidase.

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Syntheses. The full experimental section for the reported compounds has been condensed here and will be published elsewhere. The methods are outlined in Scheme II, and critical data for key intermediates are presented below. Dehydrodiconiferyl alcohol (Scheme I, compound 1) was prepared enzymatically as described (14). α-Bromoacetosyringone (ASBr) (23) and α-bromoacetophenone (APBr) (24, 25) were prepared by the reported procedures.

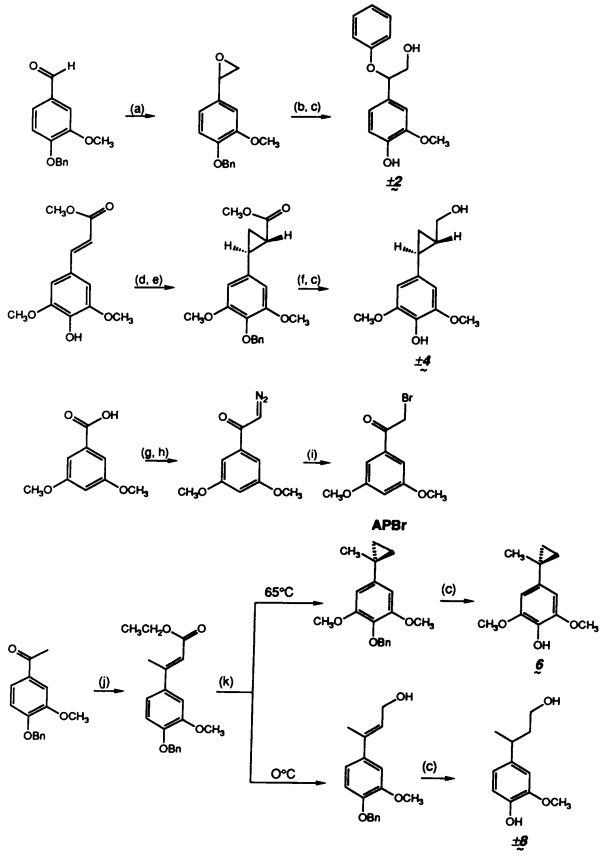
2-Phenoxy-2-(4'-hydroxy-3'-methoxyphenyl)-ethanol (Schemes I and II, compound ±2). Benzyl vanillin (4 mmol) was treated with dimethyloxosulfonium methylide in dimethyl sulfoxide to afford 4-benzyloxy-3-methoxystyrene oxide (40%) (26). This epoxide (0.4 mmol) and phenol (1 mmol) were dissolved in dry toluene, allowed to stand at room temperature overnight, and the entire mixture was chromatographed (SiO_2 ether/pentane, 1:4) (27). The benzyloxy ether (38 μmol) underwent hydrogenolysis with Pd/C to compound ±2 in quantitative yield. ^1H NMR (500 MHz, benzene-d_6) δ 7.03 (t, 2H, J = 7.6 Hz, H-3″), 6.90 (d, 2H, J = 7.6 Hz, H-2″), 6.87 (d, 1H, J = 8.5 Hz, H-5′), 6.76 (7, 1H, J = 7.3 Hz, H-4″), 6.60 (d, 1H, J = 1.6 Hz, H-2′), 6.60 (dd, 1H, J = 1.6, 8.2 Hz, H-6′), 5.34 (bs, 1H, -OH), 4.99 (dd, 1H, J = 3.8, 8.0 Hz, H-2), 3.79 (dd, 1H, 8.0, 11.7, H-1), 3.63 (dd, 1H, J = 3.8, 11.7 Hz, H-1), 3.01 (s, 3H, H-7′). ^{13}C NMR ($CDCl_3$), δ 156.6 (C-1″), 147.4 (C-4′), 146.2 (C-3′), 131.4 (C-1′), 130.3 (2C, C-3″), 121.1 (C-4′), 120.8 (C-6′), 116.1 (2C, C-2″), 115.1 (C-5′), 109.6 (C-2′), 83.2 (C-2), 68.0 (C-1), 56.6 (C-7′).

(E)-1-Hydroxymethyl-2-(3',5'-dimethoxy-4'-hydroxyphenyl)-cyclopropane (compound ±4). Methyl synapate (4.2 mmol) was benzylated (28), and the product (1.0 mmol) was treated with dimethyloxosulfonium methylide (26) in tetrahydrofuran (THF) to give a mixture of the cyclopropane and the starting olefin (85 mg). This mixture (85 mg) was dissolved in THF and cooled in an ice bath before DIBAL was added (0.25 mmol) over 15 min. Standard workup and chromatography (SiO_2, ether/pentane, 3:2) gave the alcohol (5 mg). The benzyloxy ether (16 μmol) underwent hydrogenolysis in 5 min to afford compound 4 in quantitative yield. ^1H NMR (500 MHz, $CDCl_3$) δ 6.25 (s, 2H, H-2′), 3.78 (s, 6H, H-5′), 3.54 (m, 2H, H-4), 1.72 (m, 1H, H-2), 1.48 (bs, 1H, -OH), 1.34 (m, 1H, H-1), 0.85–0.80 (m, 2H, H-3). EI^+, 70 eV, m/z 224 (83, M^+), 193 (78, M-CH_3O), 167 (96), 161 (100, M-$C_2H_7O_2$), 154 (35), 133 (44). The same procedures were used for preparation of compound ±7 of Fig. 2, but the yields were significantly higher for this monomethoxy derivative. This cyclopropanation procedure has been shown to give cleanly trans products. Only one product was detected in these reactions, and in the benzyl-protected alcohols the methine coupling constants of the cyclopropane confirmed that assignment ($^3J_{1-2}$ = 4.4 Hz) (29). ^1H NMR (500 MHz, $CDCl_3$) δ 6.77 (d, 1H, J = 8.1 Hz, H-5′), 6.57 (bs, 1H, H-2′), 6.54 (dd, 1H, J = 1.4, 8.1 Hz, H-6′), 5.51 (bs, 1H, -OH), 3.84 (3H, H-7′), 3.70 (m, 2H, H-4), 1.77 (m, 1H, H-2), 1.57 (bs, 1H, -OH), 1.36 (m, 1H, H-1), 0.90–0.84 (m, 2H, H-3). EI^+, 70 eV, m/z 194 (28, M^+), 163 (37, M-CH_3O), 137 (26) 131 (100, M-$C_2H_7O_2$), 103 (55).

Benzofuran (compound 5). Dehydrodiferulic acid dimethylester was isolated as a side product in the synthesis of a dehydrodiconiferyl alcohol glucoside (14). This ester (29 μmol) was dissolved in THF at −78°C before DIBAL was added in 30-μmol aliquots every 15 min until the reaction was complete as determined by TLC (SiO_2, ether). Standard workup and chromatography (SiO_2, ether/pentane/methanol, 12:6:1) yielded compound 5 (35%). ^1H NMR (500 MHz, acetone-d_6) δ 7.50 (d, 1H, J = 1.7 Hz, H-2′), 7.38 (dd, 1H, J = 1.7, 8.2 Hz, H-6′), 7.29 (bs, 1H, H-5), 6.98 (bs, 1H, H-7), 6.95 (d, 1H, J = 8.2 Hz, H-5′), 6.66 (d, 1H, J = 15.9 Hz, H-9), 6.37 (dt, 1H, J = 15.9, 5.3 Hz, H-10), 4.84 (bs, 2H, H-12), 4.23 (bs, 2H, H-11), 4.02 (s, 3H, H-13), 3.92 (s, 3H, H-7′).

1-Methyl-1-(4'-hydroxy-3',5'-dimethoxyphenyl)-cyclopropane (compound 6). Benzyl acetosyringone (3.4 mmol) dissolved in 5 ml of 1,2-dimethoxyethane was added to a solution of triethylphosphonoacetate anion (3.4 mmol) in 5 ml of the same solvent , stirred for 12 hr, and flash chromatographed (SiO_2 ether/pentane, 1:4) to give the (*E*)-olefin (15% conversion, 25% yield) (30). This carboxymethyl compound (0.50 mmol) and $LiAlH_4$ (1.3 mmol) were refluxed in THF for 2 hr (31). Workup and chromatography (SiO_2, ether/pentane, 1:9) gave the cyclopropane in low yield. Hydrogenolysis and chromatography (SiO_2, ether/pentane) gave low yields of compound 6 (29%). ^1H NMR (500 MHz, $CDCl_3$) δ 6.54 (s, 2H, H-2″), 5.36 (bs, 1H, -OH), 3.90 (s, 6H, H-5″), 1.40 (s, 3H, H-1′), 0.84 (m, 2H, H-2a), 0.70 (m, 2H, H-2b). EI^+, 70 eV, m/z 208 (62, M^+), 193 (32, M-CH_3), 177 (100, M-CH_3O).

3-(4'-Hydroxy-3'-methoxyphenyl)-butan-1-ol (compound ±8). Condensation of benzyl acetovanillone (4.0 mmol) with the triethylphosphonoacetate anion (4.5 mmol) in benzene as above gave the (*E*)-unsaturated ester (30) (50% conversion, 78% yield). Reduction with $LiAlH_4$ at 0°C gave the allylic alcohol (45%), which was hydrogenated as above to give compound ±8 (100%). ^1H NMR (500 MHz, $CDCl_3$) δ 6.81 (d, 1H, J = 8.4 Hz, H-5′), 6.66 (m, 2H, H-2′, H-6′), 5.44 (s, 1H, -OH), 3.56 (m, 2H, H-1), 2.80 (m, 1H, H-3), 1.81 (m, 2H, H-2), 1.24 (d, 3H, J = 7.0 Hz, H-4). EI^+, 70 eV, m/z 196 (27, M^+), 181 (2, M-CH_3), 151 (100, M-C_2H_5O), 137 (9), 119 (12).



Scheme II. (*a*) $(Me)_3S(O)I$, NaH/dimethyl sulfoxide; (*b*) phenol/toluene; (*c*) Pd/C, H_2; (*d*) BnCl, KI, K_2CO_3; (*e*) $(Me)_3S(O)I$, NaH/THF; (*f*) diisobutyl aluminum hydride (DIBAL)/THF; (*g*) $SOCl_2$/benzene; (*h*) CH_2N_2/ether; (*i*) HBr/HOAc; (*j*) $(C_2H_5O)_2P(O)CH_2CO_2C_2H_5$, NaH; (*k*) $LiAlH_4$/THF.

***vir* and *occ* Gene Induction and Inhibition.** *A. tumefaciens* strain A348 pSM102 or 358mx was grown to OD_{600} of 0.2–0.55 in AB minimal medium supplemented with carbenicillin at 50 μg/ml or 100 μg/ml, respectively. Cells were pelleted and resuspended at 0.05 OD unit per ml or 0.1 OD unit per ml in induction broth supplemented with the various

compounds. The phenolic compounds were dissolved in 10% dimethyl sulfoxide/water mixtures at millimolar concentrations, diluted to appropriate concentrations, and filter sterilized. These solutions were diluted 10-fold with induction medium to obtain the desired concentration. When required, octopine was added at 100 μg/ml. Samples were placed in a 15-ml sterile centrifuge tube and agitated (200 rpm) at 28°C for the indicated time. At the end of the experiment, samples were either worked up directly for β-galactosidase (β-Gal) activity or frozen with liquid N_2 and stored at −20°C for later analyses. β-Gal activity was measured as described (20); background (buffer alone) was subtracted from measured value, and positive controls from different experiments were justified. Values are reported as SEM with errors propagated for each calculation.

Washing Experiments. Cells were prepared for assay as described above and resuspended for 2 hr in medium containing 10 μM ASBr and either 100 μM acetosyringone (AS) or octopine at 100 μg/ml. Cells were then microcentrifuged for 4 min, supernatant was removed, and cells were resuspended in medium with only inducer for 6 more hr.

Timing Assay. Cells were induced with either 100 or 10 μM AS for 8 hr. At 2-hr intervals, a 1-ml aliquot was removed and analyzed for β-Gal activity. ASBr was added at a final concentration of 10 or 1 μM, and the incubation was continued. β-Gal activity was measured at 8 hr.

RESULTS

Structural Requirements for *vir* Induction. The ability of **1** to induce *vir* expression was not anticipated because the molecule was neither a simple monocyclic phenol nor did it contain the sp^2 hybridized benzylic carbon para to the phenolic OH (19, 20, 32). Because **1** did induce, the structure could be systematically modified to better characterize the mechanistic aspects of *vir* induction (Scheme I). Attention was focused on the benzofuran ring as the site of greatest structural difference from previous inducers. Formal breakage of the C_3—C_4 bond led to structure-type **2.** This benzyl phenyl ether function proved more sensitive to acidic and basic conditions than the cyclized dihydrobenzofuran but sufficiently stable for analysis. However, the epoxide **3,** which resulted from a formal removal of the upper aromatic ring and bonding C_3 to the oxygen of the dihydrofuran, gave an unstable structure under the assay conditions. Removal of the aromatic ring and construction of a bond between C_2 and C_4 gave the cyclopropane **4.**

Both compounds **2** and **4** consistently showed comparable, if not better, biological activity than did AS (Fig. 1*A*). It was difficult to rationalize the activity of compounds **1, 2,** and **4** with the other active compounds in terms of overall binding energetics. Two explanations seemed reasonable: (*i*) These materials were oxidatively converted into the phenyl ketones. To test this possibility, compound **6,** which contained a quaternary carbon preventing benzylic hydroxylation, was prepared. This compound was found as active as AS (Fig. 1*B*). (*ii*) An explanation centered on the acidity of the phenol. Electron-withdrawing substituents para to the OH dramatically lower its pK_a; the measured (33) value for AS is 7.4 (data not shown). This correlation between inducing activity and reduced phenolic pK_a held for the previously identified inducers (19, 20, 32), the exceptions being **1** and **2.** The benzyl phenyl ether functionality in these structures, however, provided a good benzylic leaving group. A reaction that would drive the elimination of such a leaving group would be assisted by phenol ionization. The importance of such a leaving group was tested with **5.** Aromatization of the dihydrobenzofuran ring of **1** reduces the leaving group potential of the benzyl phenyl ether. Benzofuran **5** was inactive.

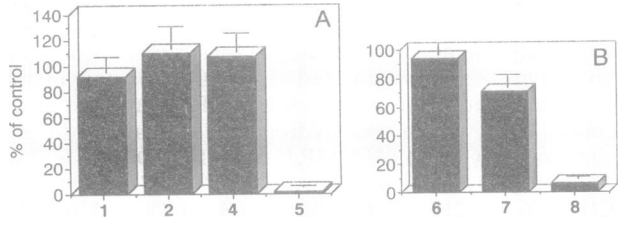
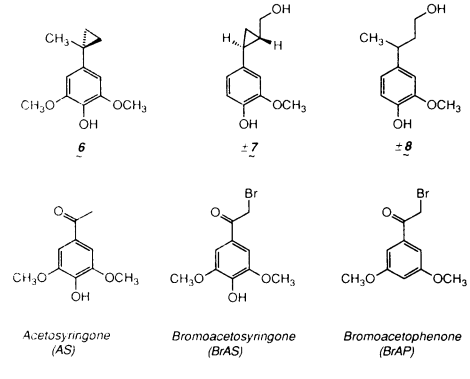


FIG. 1. (*A*) Effect of various compounds on induction of *vir E. A. tumefaciens* 358mx (*virE*::*lacZ*) was incubated with compounds **1** (200 μM), **2** (100 μM), **4** (100 μM), and **5** (100 μM) and monitored for β-Gal activity. AS (100 μM) induced β-Gal activity provided the 100% control; for **1** and **5,** 2545 ± 206.5 (no. of replicates, $n = 16$) and for **2** and **4,** 2912 ± 165.1 ($n = 7$). (*B*) Compounds **6, 7,** and **8** were monitored at 100 μM. AS control for **6** was 1810 ± 453 ($n = 3$) and for **7** and **8** was 5051 ± 327 ($n = 6$).

These data suggested that either some electron-withdrawing group and/or a reaction at the benzylic carbon stabilized by phenol ionization could be involved in induction of *vir* expression. The cyclopropane inducers offered the most dramatic extremes between a structure susceptible to the reaction suggested above and an isosteric structure completely resistent to such a reaction. Compounds **7** and **8** represent such a comparison and (see Fig. 1*B*) the presence of the cyclopropane makes **7** 10–30 times more biologically active. As in **1** and **2,** the cyclopropane ring would not be expected to alter the pK_a of the phenol unless a proximal acid induced a ring opening of the cyclopropane. The presence of a carboxylic acid residue at the phenol receptor site could then explain the biological activity of these dehydrodiconiferyl alcohol analogues!

Active-site carboxylates have been shown to be present in many enzymes. A notable example is triose phosphate isomerase, where Glu-165 of the chicken muscle enzyme was identified by the covalent esterification of dihydroxyacetone phos-



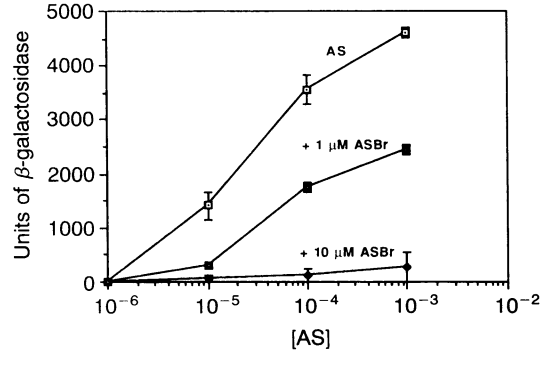


FIG. 2. Dose-dependent inhibition of *vir* induction with ASBr. Strain 358mx (*virE*::*lacZ*) was induced with increased concentrations of AS with AS alone or with 1 and 10 μM ASBr for 8 hr in induction medium at pH 5.5.

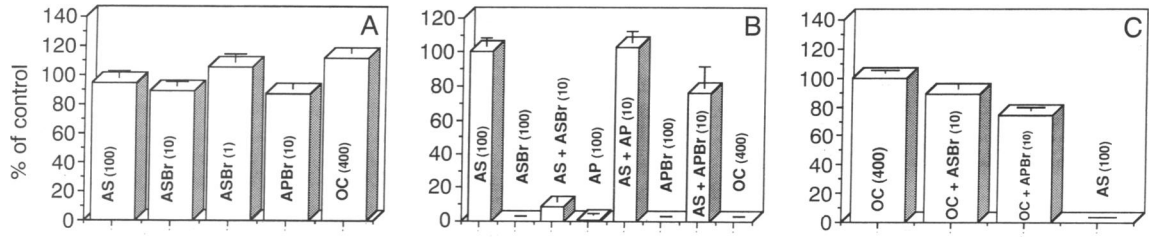


FIG. 3. Specificity of ASBr inhibition. *A. tumefaciens* strain 358mx (*virE::lacZ*) (*A* and *B*) or A348pSM102 (*occ::lacZ*) (*C*) was incubated in induction broth at pH 5.5 with indicated compounds for 8 hr. Values represent % control with *A* (growth), $OD_{(600)}$ = 0.198 ± 0.004 (n = 3) for induction broth alone; *B* (*vir*), β-Gal = 3,090 ± 156.4 (n = 12) for 100 μM AS; and *C* (*occ*), β-Gal = 311 ± 4.4 (n = 3) for octopine (OC) + APBr and 775 ± 9.06 (n = 3) for OC + ASBr. Concentrations in μM are indicated in parentheses.

phate (34). We reasoned that if a carboxylic acid was involved in the protonation of the carbonyl of AS, thereby generating an active site carboxylate, a similar α-haloketone might provide a further test for its presence.

ASBr as a Specific Inhibitor of *vir* Expression. The covalent binding of an AS derivative to the phenol receptor could lead to either of two extremes, permanent activation or permanent inactivation of *vir* expression. ASBr was found not to be active in *vir* induction (at ≤100 μM), as measured by expression of *virE::lacZ* and *virB::lacZ* gene fusions. However, with AS, micromolar concentrations of ASBr significantly reduced β-Gal synthesis (Fig. 2). The inhibitory effects of 1 μM ASBr were partially overcome by coincubation with higher concentrations of AS. In contrast, such inhibition could not be relieved by structurally similar, noninducing compounds, such as the acetophenones (see below). This protection was consistent with the antagonistic ASBr competing for the AS-receptor site.

Specificity of inhibition by ASBr was tested in three ways. (*i*) ASBr at concentrations that completely blocked *vir* induction did not affect *Agrobacterium* growth (Fig. 3*A*). (*ii*) A structurally similar but noninducing acetophenone lacking the phenolic hydroxyl group (AP) was converted into the brominated derivative APBr. This compound was not an active inducer (≤100 μM) and at 10 μM had only marginal effects on *vir* induction (Fig. 3*B*), not unlike its effects on growth (Fig. 3*A*). Therefore, the inhibitory activity of ASBr required the essential structural features of the inducer and was not simply due to the presence of the α-bromoacetophenones. (*iii*) The octopine-mediated induction of the Ti plasmid-borne octopine catabolism locus, *occ*, was monitored by using strains carrying a Tn*3*Hoho*lacZ* fusion in this operon (21). Octopine-inducible β-Gal was seen with ASBr concentrations that completely inhibited *vir* induction (Fig. 3*C*). AS did not induce the *occ* locus, nor did APBr show inhibition any greater than its general inhibition of *vir* expression or growth. Further, direct ASBr addition to the β-Gal assays (100 μM for 30 min) did not alter enzymatic activity (data not shown), consistent with the inability of this compound to inhibit β-Gal placed under *occ* control. Therefore, ASBr does not affect β-Gal synthesis, its activity, or the expression of different operons within the same plasmid.

Inability of AS to overcome completely the effect of ASBr (Fig. 2) supported an irreversible inhibition. This irreversibility was confirmed by washing experiments in which the inhibitor was removed and replaced with AS (Table 1). Over the subsequent 6-hr period, *vir* induction, as measured by β-Gal activity, was minimal. These same washing experiments had no effect on *occ* expression. In these experiments, the overall level of expression was reduced, due to stress of the washing treatment. However, under the same conditions, *occ* expression was not inhibited by 2-hr treatment with ASBr.

Table 1. β-Gal activity after washing at 2 hr in 8-hr induction

Strain	Inducer (control)	Inducer + 10 μM ASBr	% control
virE::lacZ	668 ± 31.5	21.8 ± 2.72	3.3 ± 0.57
occ::lacZ	123 ± 9.62	121 ± 7.94	100 ± 14.4

Inhibitor was present only during first 2 hr.

Time Dependence of *vir* Induction. In these experiments, ASBr was added to induction medium at various times after AS exposure. Data show that addition of the inhibitor effectively blocks further induction of the *vir* genes (Table 2). This result suggests that the *vir* activation system needs to be continuously stimulated for *vir* expression to proceed. The differential between activity at time of inhibitor addition and final activity probably reflects the time dependence of the inhibition including both inhibitor–receptor interaction as well as the stability of downstream components in the signal cascade.

DISCUSSION

Discovery of the dehydrodiconiferyl alcohol glycoside aglycone as an inducer of *vir* expression has dramatically altered our perception of the structural requirements of these phenolic compounds necessary for biological activity. The possible involvement of a structural change occurring at the benzylic carbon as a mechanism for gene activation has led to the construction of a series of unexpected *vir* expression agonists. In this communication we have also extended this structural insight into the development of a specific inhibitor of *vir* gene expression.

The initial experiments on ASBr have shown that inhibition of *vir* induction is irreversible. The inhibitor is specific for the *vir* operon in that (*i*) expression of other inducible genes (e.g., *occ*) was not affected and (*ii*) inhibition depended on structural requirements of the inhibitor that were in common with the active inducer molecules. The inhibition can be partially overcome with high concentrations of AS. As bacteria continue to grow (≥12 hr after ASBr removal) *vir* inducibility gradually returns (data not shown), probably due to continued expression of the signal-transduction components.

The *vir*-inducing activity of compounds **1**, **2**, **4**, **6**, and **7** together with the ability of ASBr to inhibit *vir* induction has provided support for a very simple and testable molecular mechanism regulating virulence in *Agrobacterium* (Fig. 4).

Table 2. Effect of variation in exposure time on β-Gal levels both at time of inhibitor addition and at 8-hr time point with *virE::lacZ* strain

Incubation time before ASBr (10 μM) addition, hr	β-Gal activity at ASBr addition, % control	β-Gal activity after 8-hr incubation, % control
0	0	8 ± 2
2	1 ± 0.1	6 ± 2
4	14 ± 1.4	21 ± 5.3
6	63 ± 6.7	75 ± 13
8	100 ± 5.4	100 ± 22

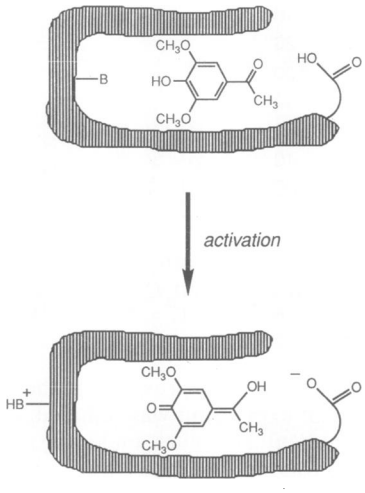


FIG. 4. Proposed mechanism for phenolic induction of *vir* expression. Protonation at a basic site (B) on the phenolic receptor, presumably VirA, induces indicated conformational change for VirG activation.

Inducing phenolics such as AS can be viewed as vinylogous carboxylic acids where ionization of the phenol is stabilized by resonance delocalization through the carbonyl oxygen. Under assay conditions, the phenol of AS is protonated, but in the binding site, an acidic residue protonates the carbonyl oxygen, greatly increasing acidity of the phenol. This activated phenol, probably bound in a relatively hydrophobic pocket, could protonate a basic site on the receptor surface and induce a conformational change (Fig. 4) that would set up the proposed phosphorylation cascade involved in Vir G activation (35). Because *o*-methoxy groups are not predicted to alter phenol acidity (36), we suggest that they are involved primarily in receptor binding. The mechanism of Fig. 4 suggests that activation is controlled by a chemical reaction, the transfer of a proton across ≈10 Å on the phenol-receptor surface. Further evaluation of this model is now possible both through structural manipulation of the signal phenolic and by mutagenesis of the presumed phenol-binding protein Vir A.

Our data showing that addition of the *vir* antagonist ASBr blocks further *vir* induction by AS (Table 2) suggest that the phenol receptor must be bound to the phenol throughout the induction period. Consistent with this requirement for receptor occupancy are experiments in which removal of AS also results in termination of induction (37). From the bacterial sensor/response models (38), Vir A phosphorylation of Vir G would constitute the "on" switch. This rapid "off" switch must be due either to dephosphorylation or to some other active form of deactivation (38) because the phosphorylated form of Vir G has been shown to be surprisingly stable *in vitro* (35). This ability to terminate *vir* expression immediately after signal removal may be very important to the pathogen. As in the parasitic plants (18, 39), commitment to the parasitic phase involves a significant redistribution of the resources of the organism. If the host is not a viable, transformable organism, the commitment can be rapidly aborted.

The availability of this specific and irreversible inhibitor should continue to be useful in defining the signal-transduction system of *Agrobacterium* and, most importantly, can now be used to characterize the phenol-binding protein and the mechanism for the activation event. The technology developed may be particularly valuable because similar, and in some cases the same, compounds are involved both in activation of cell growth in higher plants (12) and in activation of *nod* gene expression in *Rhizobium* spp. (40).

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