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Biosynthesis of Dehydrodiconiferyl Alcohol Glucosides: Implications for the Control of Tobacco Cell Growth[1]

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ABSTRACT

The dehydrodiconiferyl alcohol glucosides A and B are factors isolated from transformed *Vinca rosea* tumor cells that can replace the cytokinin requirement for growth of tobacco (*Nicotiana tabacum*) pith and callus cells in culture. These factors, present in tobacco pith cells, have their concentrations elevated approximately 2 orders of magnitude after cytokinin exposure. Biosynthesis experiments showed that these compounds are not cell wall fragments, as previously suggested, but are produced directly from coniferyl alcohol. Their synthesis is probably associated with the existing pathway for cell wall biosynthesis in both *Vinca* tumors and tobacco pith explants. The pathway requires only two steps, the dimerization of coniferyl alcohol by a soluble intracellular peroxidase and subsequent glycosylation. Biosynthetic experiments suggested that dehydrodiconiferyl alcohol glucoside breakdown was very slow and control of its concentration was exerted through restricted availability of coniferyl alcohol.

Over the past few years, a diverse array of signal transduction systems initiated by low mol wt phenolic compounds have been discovered. Remarkably similar compounds induce the development of the host attachment organ in the parasitic plants (22), the expression of the genes required for *Agrobacterium* T-DNA transfer to the plant cell (31), and activate the *Rhizobium* genes necessary for symbiotic integration with its plant host (19). Impressive definition of the signal transduction systems are now available (19, 20, 31), but little is known about the control of the production of these initiating compounds by the plant hosts. Another class of structurally related phenolics, the DCGs[3] A and B, are plant growth factors that were discovered by screening plant tumor lines for factors that were overproduced as a result of the oncogenetic transformation (21). These phenolics induce plant cell division (2, 28) and show activity both in the *Agrobacterium* (14) and *Striga* (T.R. Ruttledge and D.G. Lynn, unpublished) signal transduction pathways. Therefore, the cytokinin-activated pathway to the DCGs provides a system suitable to define the controls on the biosynthesis of such compounds.

The DCGs A and B were initially isolated from a rapidly growing transformed *Vinca* cell line (21). These structures differed only in the enantiomeric nature of the phenylpropanoid dimer, reminiscent of the racemic plant cell wall polymer, lignin, that is typically laid down in terminally differentiated cells (3). Phenylpropanoids are incorporated into the growing primary cell wall (8, 10), and oxidatively dimerized ferulates (8, 13) are abundant components of these structures. Therefore, it seemed likely that the DCGs could be released as preformed dimers from the growing cell wall or produced through a direct biosynthetic pathway (Scheme 1). This study was designed to test the two proposed pathways for DCG biosynthesis.

DCG concentrations are greatly elevated in proliferative tissue and very low or below detectable limits in quiescent cells (2). For example, the rapidly proliferating *Vinca* callus lines contain micromolar quantities of these compounds. Fresh tobacco pith explants contain nanomolar amounts of DCG A and B, but these concentrations are elevated by a factor of 100 when the explants are cultured in the presence of cytokinins. Evidence is presented that for both the transformed tissues and the cytokinin-activated tobacco tissue, the DCGs were made by a direct biosynthetic pathway and together the two systems have allowed for the elucidation of the individual biosynthetic steps.

MATERIALS AND METHODS

Chromatography

All solvents were HPLC grade, and H_2O was twice distilled, the second time from an all glass still. HPLC columns were analytical size, containing 5 μm particle size packing and included: Zorbax C8 and Zorbax ODS (25 × 0.45 cm, DuPont), a C18 "Short-one" (10 × 0.45 cm, Ranin), and Waters C8 and C18 Radial Pak columns (10 × 0.5 cm) and ODS cartridges (1 × 1 cm). SiO_2 chromatography included thin layer (Silica Gel 60 F_{254}, 0.2 mm, aluminum) and open column (Silica Gel 60, 230–400 mesh).

Spectroscopy

^{1}H-NMR spectra were recorded at 270 MHz on a heavily modified Bruker WM system; mass spectra were obtained with a VG 70–250 spectrometer; UV spectra were recorded on a Perkin-Elmer Lambda 5 UV-Visible spectrometer; and

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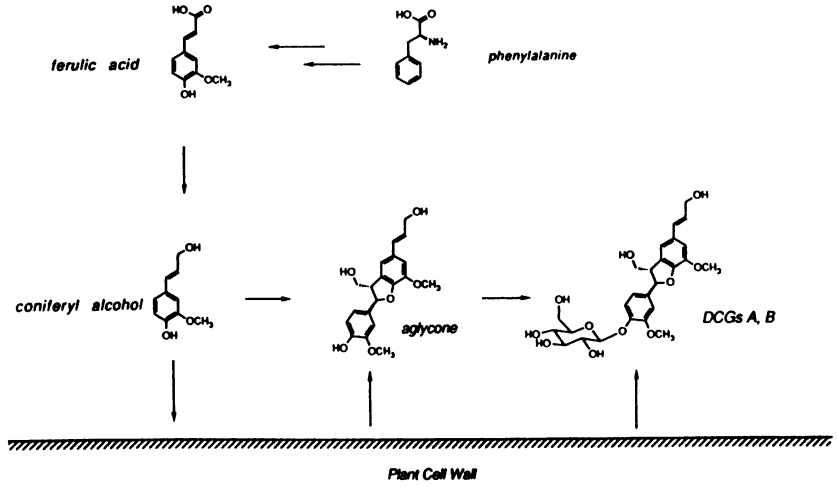
[3] Abbreviations: DCG, dehydrodiconiferyl alcohol glucoside; ODS, octadecylsilane; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide.



Scheme 1. Proposed biosynthetic pathways to DCGs. Both a direct pathway and the release of either dehydrodiconiferyl alcohol or the DCGs from the plant cell wall are represented.

IR spectra were recorded with a Nicolet 20 FT-IR system. Aquasol liquid scintillation counting cocktail (New England Nuclear) was used for scintillation counting.

Synthesis (Scheme 2)

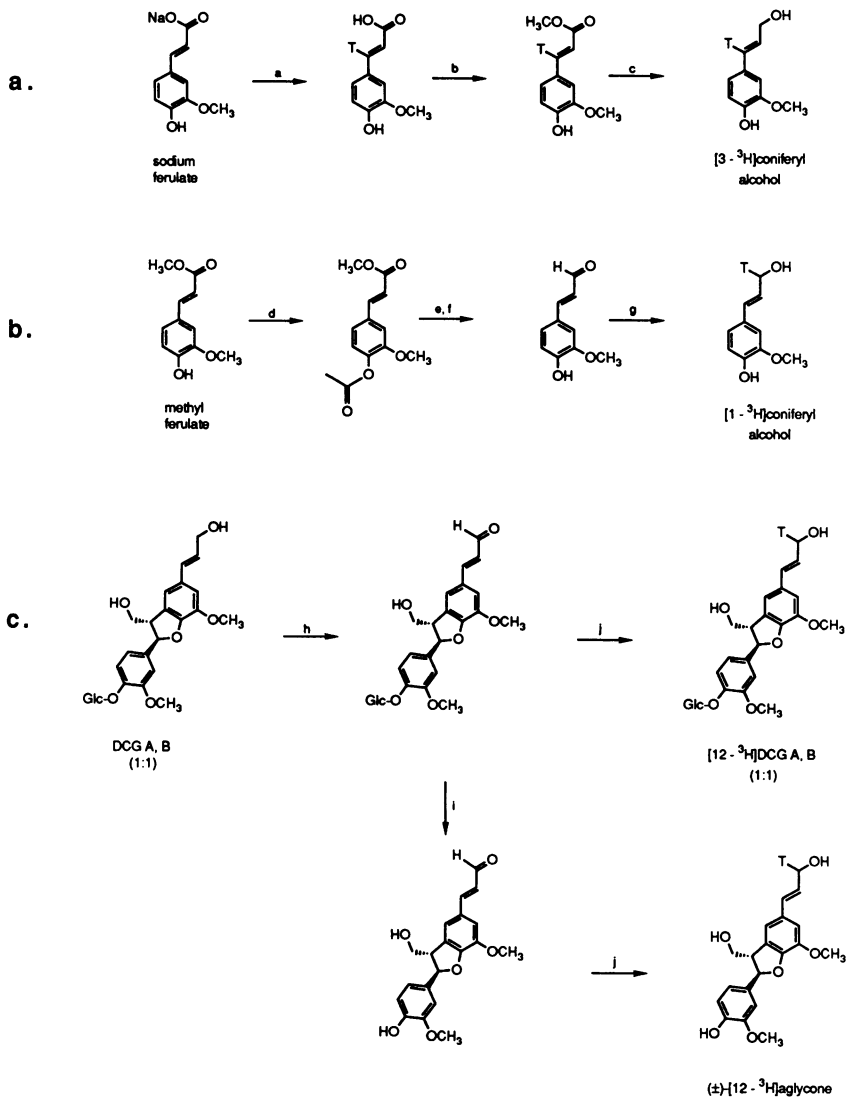
[3-^{3}H]Coniferyl Alcohol

The methods for a hydrogen exchange reaction (18) were developed with ^2H_2O in an NMR tube. Ferulic acid (42 μmol), DMF-d_7 (300 μL), ^2H_2O (300 μL), and $RhCl_3 \cdot 3\ H_2O$ (21 μmol) were dissolved in an NMR tube and the solution was frozen, evacuated, and thawed (2×) before the tube was sealed and heated to 105°C for 3 h. These conditions proved sufficiently mild such that no other products were detected by ^{1}H-NMR or by TLC and regiospecific exchange occurred at C-3. The exchange varied between 20 and 42%. With H_2O: ^{1}H-NMR (270 MHz, ^2H_2O) δ7.67 (1H, d, J = 16 Hz, H-3), 7.32 (1H, d, J = 2 Hz, H-2′), 7.23 (1H, dd, J = 2, 8 Hz, H-6′), 7.01 (1H, d, J = 8 Hz, H-5′), 6.45 (1H, d, J = 16 Hz, H-2), 3.96 (3H, s, $-OCH_3$). With ^2H_2O: ^{1}H-NMR (270 MHz, ^2H_2O) δ7.67 (<1H, d, J = 16 Hz, H-3), 7.32 (1H, d, J = 2 Hz, H-2′), 7.23 (1H, dd, J = 2, 8 Hz, H-6′), 7.01 (1H, d, J = 8 Hz, H-5′), 6.45 (<1H, d, J = 16 Hz, H-2), 6.44 (<1H, s, H-2 in 3-^{2}H), 3.96 (3H, s, $-OCH_3$). For mass spectral analysis, the reaction was repeated the same way except that the amount of ferulic acid was increased to 45 μmol and the mixture was heated in a 0.1 mL screw-capped vial. The mixture was extracted from 0.1 N HCl with $CHCl_3$ (3 × 2 mL), washed with H_2O (3 × 2 mL), and dried *in vacuo*. With H_2O: MS (electron impact (+), 70 eV): *m/z* (%), 195 (M^+ + 1)(13), 194 (100), 180 (1), 179 (17), 134 (7), 133 (19), 106 (3), 105 (11). With ^2H_2O: MS (electron impact (+), 70 eV): *m/z* (%): 195 (M^+ + 1)(42), 194 (100), 180 (5), 179 (17), 134 (14), 133 (19), 106 (8), 105 (11).

Sodium ferulate (62.0 μmol), $RhCl_3 \cdot 3\ H_2O$ (17.8 μmol), [^{3}H]H_2O (New England Nuclear; 10 μL, 50 mCi) and DMF (75 μL) were heated to 105°C for 1.5 h in a 0.1 mL capped vial as above. The reaction was quenched with 5 mL 0.1 N HCl, extracted with EtOAc (3 × 5 mL) and dried with a stream of N_2. A portion of this material (26 μmol), THF (1 mL), and excess etherial diazomethane (Diazald, Aldrich) were allowed to react at ambient temperature for 2 h. The mixture was dried with a stream of N_2, and the residue dissolved in EtOAc, filtered through a cotton plug, and dried with a stream of N_2. This material was dissolved in THF (1 mL) and allowed to react with diisobutylaluminumhydride (250 μmol) for 2 h at 0°C before the reaction was quenched with methanol (50 μL). The mixture was extracted with EtOAc (3 × 10 mL), the EtOAc layers were filtered through a cotton plug, dried *in vacuo*, and the residue was dissolved in Et_2O and eluted (Et_2O, 10 mL) through an SiO_2 column (5 × 0.5 cm). The eluate was dried with a stream of N_2 and applied to HPLC (Waters C8; iPrOH:H_2O:AcOH, 7:92.5:0.5, v/v; flow rate 2 mL/min, t_R 10 min). The appropriate peak was collected, dried, and traces of acid were removed by adsorption to Sep-Pac and elution with MeOH to give an overall yield of 24% from ferulic acid; UV (MeOH), λ_{max}, 267 nm; 304 nm shoulder. Specific activity was determined from HPLC (MeOH:H_2O:AcOH, 25:74.5:0.5, v/v, flow rate 1.5 mL/min, t_R 10 min) to be 16 mCi/mmol).

[1-^{3}H]Coniferyl Alcohol

Methyl ferulate (10 mmol), CH_2Cl_2 (10 mL), acetic anhydride (50 mmol), and pyridine (10 mL) were stirred in a closed flask at 25°C for 38 h. This mixture was diluted with diethyl ether (50 mL) and washed with saturated $NaHCO_3$ (25 mL), saturated $CuSO_4$ (2 × 25 mL), water (25 mL), and finally saturated NaCl (25 mL). The organic layer was filtered through a bed of Celite and dried to give a 58% yield of the acetate; ^{1}H-NMR (270 MHz, C^2HCl_3) δ2.32 (3H, s, $OCOCH_3$). A toluene solution (15 mL) of this material (5.8 mmol) was added as to a mixture of $LiAlH_4$ (12 mmol) and Et_2NH (23 mmol), and the entire mixture was stirred under N_2 at 25°C for 6 h (5). Ether (50 mL) and dilute HCl (60 mL) were added and the biphasic mixture was stirred for 12 h. The etherial layer was collected, washed (4 × 25 mL) with water until the pH = 7, and dried *in vacuo*. The residue was applied to an SiO_2 column and eluted with ether:pentane (1:1) to give low yields of the aldehyde; ^{1}H-NMR (270 MHz, $CDCl_3$) δ9.61 (1H, d, J = 7 Hz, CHO), UV (MeOH), λ_{max},



Scheme 2. Synthesis of labeled compounds. a, [3-^{3}H]coniferyl alcohol. (a) [^{3}H]H_2O, $RhCl_3 \cdot 3H_2O$, DMF, 105°C; (b) CH_2N_2, Et_2O; (c) diisobutylaluminumhydride, THF, 0°C. b, [1-^{3}H]coniferyl alcohol. (d) Ac_2O, pyridine, CH_2Cl_2; (e) $LiAlH_4 \cdot 2Et_2NH$; toluene; (f) 2 N HCl, Et_2O; (g) [^{3}H]$NaBH_4$, EtOH. c, [12-^{3}H]DCGs A and B, (1:1) and (±)-[12-^{3}H]dehydrodiconiferyl alcohol. (h) MnO_2, CH_3CN; (i) β-D-glucosidase, phosphate buffer, pH 5; (j) [^{3}H]$NaBH_4$, MeOH.

331 nm; 300 nm shoulder; IR (NaCl) ν1669 cm^{-1} (C = O). A portion of this material (80 μmol) was added to an ethanolic suspension (2.5 mL) of [^{3}H]$NaBH_4$ (30.4 μmol, 12 mCi) and allowed to react at 25°C for 24 h. The reaction was pushed to completion by adding a small portion of unlabeled $NaBH_4$. The mixture was filtered through a 0.45 μM nylon filter, dried *in vacuo,* and purified (Waters, C8; iPrOH:H_2O:AcOH, 7:92.5:0.5, v/v; flow rate 2 mL/min, t_R 10 min) to give a 52% yield of the alcohol with specific activity (measured as above) of 68 mCi/mmol; UV (MeOH), λ_{max}, 267 nm; 304 nm shoulder.

Dehydrodiconiferyl Alcohol Glucosides A and B and (±)-Dehydrodiconiferyl Alcohol

The syntheses of these compounds were performed in this laboratory as previously reported (28).

[12-^{3}H]Dehydrodiconiferyl Alcohol Glucosides A and B

A 1:1 mixture of DCG A and B (2.9 μmol), MnO_2 (200 mg), and CH_3CN (1 mL) were allowed to react at ambient temperature for 12 h. Direct HPLC analysis (DuPont, C8; MeOH:H_2O, 30:70, v/v; flow rate 2.0 mL/min, t_R(alcohol) = 7.5 min; t_R(aldehyde) = 11 min) showed >95% completion of reaction before the reaction was quenched with water (5 mL), the manganese salt removed by centrifugation, and the solvent removed *in vacuo.* The residue was dissolved in H_2O and loaded onto an ODS cartridge, washed with H_2O, and eluted with MeOH, to give a 57% yield of the aldehydes; UV (H_2O), λ_{max}, 332 nm. An equimolar mixture of the aldehydes (0.64 μmol), [^{3}H]$NaBH_4$ (0.19 μmol, 14.5 mCi), and methanol (500 μL) were allowed to react for 12 h at ambient temperature. The mixture was dried *in vacuo* and purified by HPLC (Waters, C18; MeOH:H_2O, 25:75, v/v; flow rate 2.0 mL/min, t_R 20 min); UV (MeOH), λ_{max}, 273 nm, 306 nm shoulder; specific activity, 1.2 Ci/mmol.

(±)-[12-^{3}H]Dehydrodiconiferyl Alcohol

Dehydroconiferolconiferal glucosides A and B from above (0.5 μmol each), β-D-glucosidase (almonds, Sigma, 2 mg, 80 units), and phosphate buffer (0.05 M, pH 5, 500 μL) were

incubated for 4 h at 37°C. The reaction mixture was loaded onto an ODS cartridge, washed with H_2O, and the product was eluted with MeOH and dried *in vacuo*. The (±)-aldehyde was purified by HPLC (DuPont, C8; MeOH:H_2O, 40:60, v/v; flow rate 2.0 mL/min t_R 7.5 min) giving a 43% yield; UV (MeOH), λ_{max}, 344 nm. This aldehyde (0.43 μmol) and [^{3}H]$NaBH_4$ (0.16 μmole, 10.5 mCi) were allowed to react in methanol (500 μL) at ambient temperature for 12 h. The mixture was dried *in vacuo* and purified by HPLC (Waters, C18; MeOH:H_2O, 30:70, v/v; flow rate 2.0 mL/min; t_R 25 min); UV (MeOH), λ_{max}, 278 nm, 306 nm shoulder, 22% yield, specific activity of 2.1 Ci/mmol.

Isolation and Analysis of Plant Materials

Tobacco (*Nicotiana tabacum* L. cv H425) pith explants and *Vinca rosea* tumors (21) were ground at 0°C in a glass Tenbroeck tissue grinder in 1% (v/v) 2-mercaptoethanol (1 mL/g tissue). Insoluble material was removed by centrifugation, and the soluble material was loaded onto an ODS cartridge. The cartridge was washed with 6 mL each of 15 and 60% (v/v) methanol in H_2O. The latter fraction was dried *in vacuo,* chromatographed on HPLC (Ranin, C18; MeOH:H_2O, 25:75, v/v, flow rate 2 mL/min), and the peak absorbance corresponding to coniferyl alcohol, dehydrodiconiferyl alcohol, or the DCGs were collected (t_R = 5.5, 23, 7.5 min, respectively) and purified on HPLC (DuPont, C18); coniferyl alcohol, (iPrOH:H_2O:AcOH, 5:94.5:0.5, v/v, flow rate 2 mL/min, t_R 13.8 min); DCGs (iPrOH:H_2O:AcOH, 7:92.5:0.5, v/v, flow rate 2 mL/min, t_R 15.0 min); dehydrodiconiferyl alcohol (THF:H_2O:AcOH, 15:84.5:0.5, v/v, flow rate 2.5 mL/min, t_R 14.8 min).

Biosynthetic Studies with *V. rosea* Tumor Cultures

Axenic cultures of *V. rosea* crown gall tumors were grown on White's basic medium solidified with 1% Bacto-Agar (Difco). Six pieces of tumor tissue (approximately 0.5 g each) were placed in a culture dish (25 × 100 mm) containing 100 mL of solid White's medium. Tumors were grown under constant cool-white fluorescent illumination. After 4 weeks of growth, a small portion was used for subculture and the remainder was harvested for isolation of the DCGs as reported earlier (21).

For biosynthetic studies, tissues were incubated with radiolabeled precursor for 30 min, unincorporated radiolabel was washed off with approximately 100 mL H_2O in a Buchner funnel, the tissue was rehydrated with White's media and replaced onto the same solidified media. At the times indicated, tissues were removed from the media, ground, extracted, and compounds of interest were isolated as described above. Any changes in the feeding procedures or analyses of the isolated compounds were performed as described in the figure legends.

Biosynthetic Studies with *N. tabacum* cv Havana 425

Tobacco plants were grown in a greenhouse in 8-inch pots in a mixture of potting soil/sand/vermiculite (4:2:1). Eighty-five- to 100-d-old vegetative plants were used that were 35 to 45 cm tall and contained 19 to 20 leaves >25 mm long. The stems were surface sterilized by washing with Ivory soap, passing three times through 7% commercial bleach (Clorox) and 70% EtOH/H_2O (1 min each), and washing with sterile water (3×). Pith was aseptically removed from mature internode sections, cut into approximately 10 mg segments, and 16 of these explants were placed in each culture dish (9 × 2 cm), which contained 40 mL solid Linsmaiser-Skoog growth media, 1 μM 1-naphthaleneacetic acid, and 1 μM zeatin riboside. Precursors were delivered (four times, at 12-h intervals) to each explant between the 4th and 6th d on culture. Eight hours after the final pulse, the DCGs were isolated as described above.

Detection of Enzymatic Activity in Cell Free Extracts

The *V. rosea* tumor tissues were washed with water (approximately 250 mL/10 g tissue) and 5 g portions were ground in a glass Tenbroeck tissue grinder at 0 to 4°C as described in the legends to Figures 4 and 5. Insoluble material was removed by centrifugation (1600*g*, 4°C) followed by filtration (0.45 μm). The supernatant was incubated at room temperature with the appropriate labeled precursor and enzymatic conversion was quantified by HPLC and liquid scintillation counting analyses.

Cell Wall Autolysis and Enzymatic Hydrolysis

Tumor tissue (48 g) was ground in a glass Tenbroeck tissue grinder at 0 to 4°C in 250 mL of 0.05 M NaOAc buffer, pH 5.7, containing 0.5% SDS, and then sonicated in a Branson ultrasonic bath for 4 min (13). The cell wall material was pelleted by centrifugation for 6 min at 3000 rpm with a GSA preparatory rotor in a Sorvall superspeed centrifuge maintained at 4°C. The residue was washed four more times with the same buffer, the last three without the SDS. Finally, the cell wall material was washed with cold buffer on a bed of sand on top of a Buchner funnel, resuspended in 50 mL buffer, and decanted. Portions of this suspension corresponding to approximately 3 g tissue were either gently agitated at 38°C or pelleted and suspended in 1 mL of 0.1 M citrate/phosphate buffer, pH 5.6, and incubated with 10 mg of driselase (Basidomycetes), cellulase (*Aspergillus niger,* EC 3.2.1.4), or β-D-glucosidase (almonds, EC 3.2.1.21). After 0, 1, 2, 6, 12, and 24 h, aliquots were removed, partially purified by ODS cartridge chromatography, and analyzed by HPLC (DuPont, C8; MeOH:H_2O:AcOH, 32:67.5:0.5, v/v; flow rate 1.5 mL/min; t_R for coniferyl alcohol, the DCGs, and the aglycone were 7.0, 10.1, and 15.8 min, respectively).

RESULTS

Wall Release Pathway

The entire solid *Vinca* callus was harvested while the cells were still in log-phase growth. These cells were easily dispersed and washed and the amounts of the DCGs (A + B) in the cells and in the extracellular matrix were estimated by HPLC analysis. The concentrations within the cells were found to be as high as 130 μM and as low as 21 μM, but over 32 measurements the average concentration was 66 ± 4 μM. No detectable

quantities of the DCGs were found to accumulate in the growth media. The extracellular material removed from the cells by suction contained less than 0.1 μM DCG, and this concentration could have arisen from cell breakage during processing.

The low concentrations of the DCGs in the media could have resulted from an efficient cellular uptake system. A series of radiolabeled phenolic compounds were synthesized in order to test for such a transport system (Scheme 2). Tritium labels were placed at two different positions in coniferyl alcohol to control for possible oxidative loss of the tritium. Both DCG A/B and the aglycone were prepared from the synthetic DCG A/B sample (28). Each of these methods ensured very high specific activity and each product was purified by HPLC immediately before the experiment.

Incubation of 8.5 g of *Vinca* cells with [12-^{3}H]DCG A/B (2.8 nmol, 80 mCi/mmol) for 1 h resulted in the internalization of 1.7% (48 pmol) of the applied label. However, [1-^{3}H] coniferyl alcohol (68 mCi/mmol) was incorporated into the DCGs just as effectively. When 6.6 nmol was incubated with 10 g of tissue for 30 min, 1.5% (98 ± 16 pmol) of the label was converted into the internal DCGs (n = 8). [12-^{3}H]Dehydrodiconiferyl alcohol (540 mCi/mmol) was incorporated into the DCGs eightfold more effectively; 0.21 nmol given to 10 g of the tissue for 30 min gave 26 ± 1 pmol or 13% incorporation. In all cases, verification of label incorporation into the DCGs was established by the coelution of the radioactivity with the DCGs on HPLC followed by β-glucosidase-catalyzed hydrolysis to the aglycone and comigration of the radioactivity on HPLC (Fig. 1). The finding that potential biosynthetic precursors, coniferyl alcohol and the aglycone, were incorporated into the internal DCG pool at least as well as the DCGs was not consistent with an efficient DCG import system that would be required for a cell wall fragment release mechanism of DCG biosynthesis.

Finally, the presence of the DCGs in the walls was investigated by isolating the cell walls (11, 13) and attempting to release the DCGs or aglycone by enzymatic hydrolysis (8). The autolysis reaction and the reactions with driselase, β-glucosidase, and cellulase were monitored throughout a 24-h incubation period with HPLC. In each case, low mol wt, UV-absorbing material accumulated in the incubation media, but none of the desired compounds could be detected. Thermolysis in an autoclave (1), however, did release easily detectable (by HPLC) amounts of coniferyl alcohol, but neither the aglycone nor the DCGs were detected.

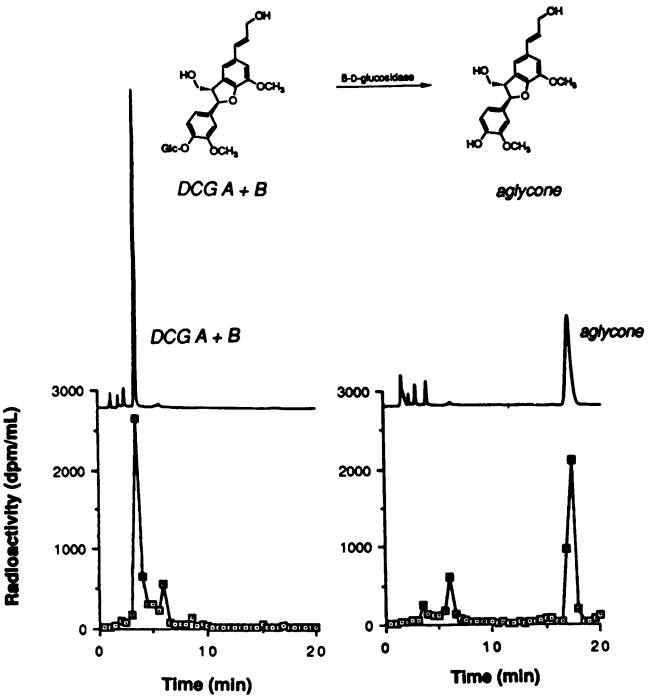


Figure 1. Analysis of dehydrodiconiferyl alcohol glucosides isolated from *Vinca* cells incubated with [3-^{3}H]coniferyl alcohol. Tumor tissue (10 g) was incubated with [3-^{3}H]coniferyl alcohol (26 mCi/mmol, 1.1 μCi, 42 nmol) for 30 min and immediately ground and extracted, and the DCG fraction was isolated. This fraction was dried *in vacuo*, dissolved in NaOAc (pH 5, 0.1 M, 600 μL), and two aliquots (150 μL each) were removed and incubated at 37°C for 45 min in the presence or absence of β-D-glucosidase (8 units). Both reactions were chromatographed on HPLC (DuPont, C_{18}; THF:H_2O: AcOH, 15:84.5:0.5, flow rate 2 mL/min, t_R (DCGs, 3.5 and aglycone 17.5 min). The upper trace is detected at 254 nm and the lower trace is the radioactivity detected in fractions collected every 30 s.

Direct Biosynthetic Pathway

The above experiments established coniferyl alcohol and dehydrodiconiferyl alcohol as suitable precursors for direct feeding experiments in *Vinca*. The average concentrations of these compounds in the cells were measured by HPLC to be 0.40 ± 0.04 (n = 10) and 0.39 ± 0.05 μM (n = 20), respectively. Although these concentrations were low, more than 10^2 lower than the sum of the DCGs A and B, they were sufficient for specific activity measurements.

Kinetic analyses of the radioactivity distribution after a 30-min exposure of 10 g of tissue to [12-^{3}H]aglycone (540 mCi/mmol, 2 × 10^{-8} M) is shown in Figure 2a. Thirteen percent of the administered radioactivity and 44% of the absorbed label was rapidly glucosylated into DCGs A and B and very little remained as the aglycone. As a result of the large intracellular concentrations of the DCGs, the specific activity was diluted by greater than 2 × 10^3. Unexpectedly, both the amount of radioactivity and the pool size remained virtually constant for the first 6 to 10 h before the specific activity was gradually depleted.

Coniferyl alcohol was a less suitable precursor probably because of competing incorporation into the cell wall and polymerization by extracellular phenol oxidases. Sixty-eight percent of the label was found in insoluble material, with less than 1% incorporation into the DCGs after a 30-min exposure of 10 g of *Vinca* tissue to 4 × 10^{-7} M [1-^{3}H]coniferyl alcohol (68 mCi/mmol). Figure 2b shows a plot of the specific activity in coniferyl alcohol and dehydrodiconiferyl alcohol as a function of time after the feeding. Immediately after removing the labeled compound, the specific activity of coniferyl alcohol dropped rapidly. The specific activity in the aglycone dropped at approximately the same rate, but persisted for a longer period of time. The specific activity of the DCGs was very low and, as before, the activity that developed early after the

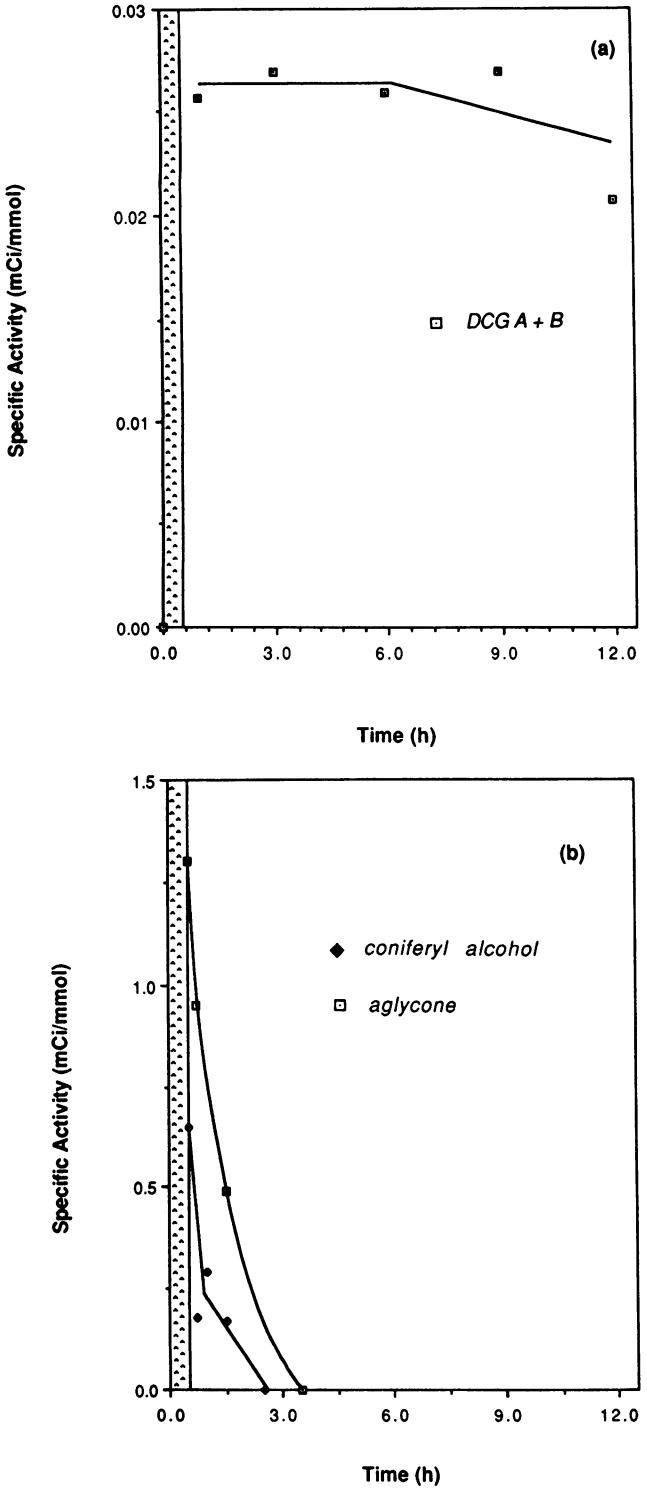


Figure 2. Kinetic changes in the specific activity of pathway intermediates after precursor feedings. a, *Vinca* cells (10 g per time point) were incubated with (±)-[12-^{3}H]aglycone (540 mCi/mmol, 110 nCi, 0.21 nmol) as described, the tissues were harvested at the times indicated, and the DCGs were isolated and their specific activity quantified. b, *Vinca* cells (10 g per time point) were incubated with [1-^{3}H]coniferyl alcohol (68 mCi/mmol, 450 nCi, 6.6 nmol) and analyzed as in panel a. The shaded region indicates the period of incubation with the radioactive precursor in both experiments.

feeding remained constant over the course of the experiment (data not shown). The flow of radioactivity from coniferyl alcohol to dehydrodiconiferyl alcohol and ultimately to the DCGs was consistent with a direct product/precursor relationship (32). Pulse/chase experiments further supported the direct biosynthetic pathway in that the slower turnover of label from the artificially enlarged precursor pools delayed the attainment of maximal specific activity in the DCGs (data not shown).

Demonstration of the Enzymatic Activity in Cell Free Extracts

Cell free extracts were prepared from *Vinca* callus by methods previously shown to preserve glucosyl transferase activity (15). The tissues were ground, extracted, and the supernatant resulting from the removal of the insoluble wall debris was used immediately. When incubated with synthetic [12-^{3}H] aglycone (530 mCi/mmol) and a 10^3 molar excess of UDP-glucose for 1 h, this extract catalyzed a 14% conversion into the DCGs (Fig. 3).

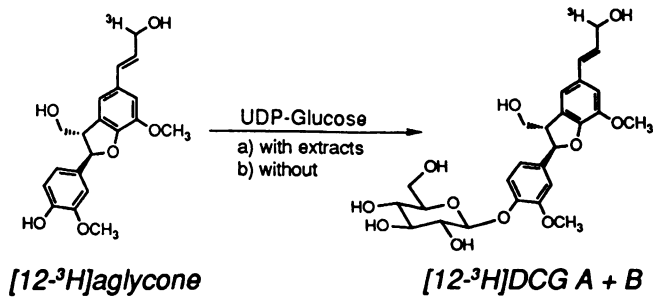
An extraction protocol (9) designed for the solubilization of intracellular peroxidases without liberating ionically bound cell wall peroxidases (6) was found to provide the desired dimerization activity. The incubation of the extracts of *Vinca* tissue with coniferyl alcohol (5 μmol) gave no reaction in the absence of H_2O_2. However, upon the addition of 0.6 equivalents of H_2O_2, one equivalent of tritiated coniferyl alcohol was converted into the aglycone in 96% yield in $\leq$1 min (Fig. 4). Therefore, both of the required enzymatic activities for the pathway were demonstrated *in vitro*, and it remained to be established that these enzymes accounted exclusively for the biosynthetic production of the DCGs.

Regulation of the Ratio of DCG A to DCG B

The ratio of DCG A to DCG B varied from batch to batch of *V. rosea* cells and varied greatly in the tobacco explants (28) as a function of time on culture. It became clear that this variability could not be due to the differences in isolation, as was previously suggested (2). Because the enzymes for DCG biosynthesis were present in the *Vinca* cells, as demonstrated by the feeding and cell free extract experiments, the DCG A/B ratio should be under their control. If not, this pathway could not be the sole biosynthetic source of the DCGs.

To evaluate this hypothesis, the same experiment as described in Figure 4 was performed except [1-^{3}H]coniferyl alcohol was added as a tracer. The ratio between the newly formed radiolabeled aglycone enantiomers was determined by conversion to DCGs A and B with intact *V. rosea* cells and analysis on HPLC (Fig. 5b). As a control, racemic radiolabeled aglycone (28) was fed to the *Vinca* cells, DCGs A and B were resolved, and their relative radioactivities were determined from HPLC (Fig. 5a).

Although different amounts of the synthetic and *Vinca*-produced aglycones were administered to the tissues, the incorporations into the DCGs were the same (6.0 and 7.6% respectively). The synthetic feeding experiment gave a [^{3}H]A/B ratio of 1.1 (Fig. 5a, lower trace), confirming that racemic aglycone was converted into DCG A and B in equal



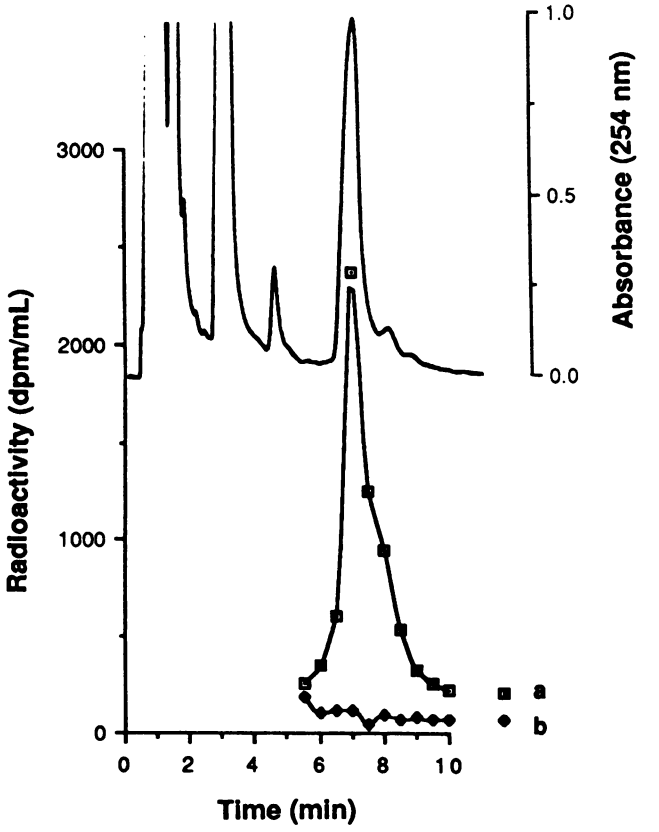


Figure 3. Assay for UDP-glucose:dehydrodiconiferyl alcohol glucosyl transferase in *V. rosea* tumor cell free extracts. *V. rosea* tumor tissue (5 g) was ground in 5 mL of Tris-HCl buffer (100 mM, pH 7.0) that contained 2-mercaptoethanol (25 mM) and 2-methoxyethanol (10%, v/v) according to the methods of Ibrahim and Grisebach (15). Both this cell free extract (a) and buffer alone (b) (288 μL) were incubated with (±)-[12-^{3}H]aglycone (110 μL, 112 nCi, 0.21 nmol), UDP-glucose (200 μL, 200 nmol) for 1 h at 37°C. One hundred microliter portions of each were chromatographed directly on HPLC (Ranin, C18; MeOH:H_2O:AcOH, 25:74.5:0.5, v/v; flow rate 2.0 mL/min, t_R [DCGs] = 7.5 min) and radioactivity was measured in fractions collected every 30 s from 5.5 to 10 min. The chromatogram detected at 254 nm is shown above the coeluting radioactivity.

amounts by these cells. In contrast, the *Vinca*-dimerized [^{3}H] aglycone feeding gave a [^{3}H]A/B ratio of 0.52, a ratio identical to the relative endogenous pool sizes of A/B (Fig. 5a and b, upper traces).

Biosynthesis in Tobacco

The Havana 425 tobacco pith explants were more compact tissues and incorporated [1-^{3}H]coniferyl alcohol less effectively than did the *Vinca* cells. The concentrations of the DCGs were shown to be low in freshly isolated tobacco pith tissue (cv H 425); however, this level markedly increased

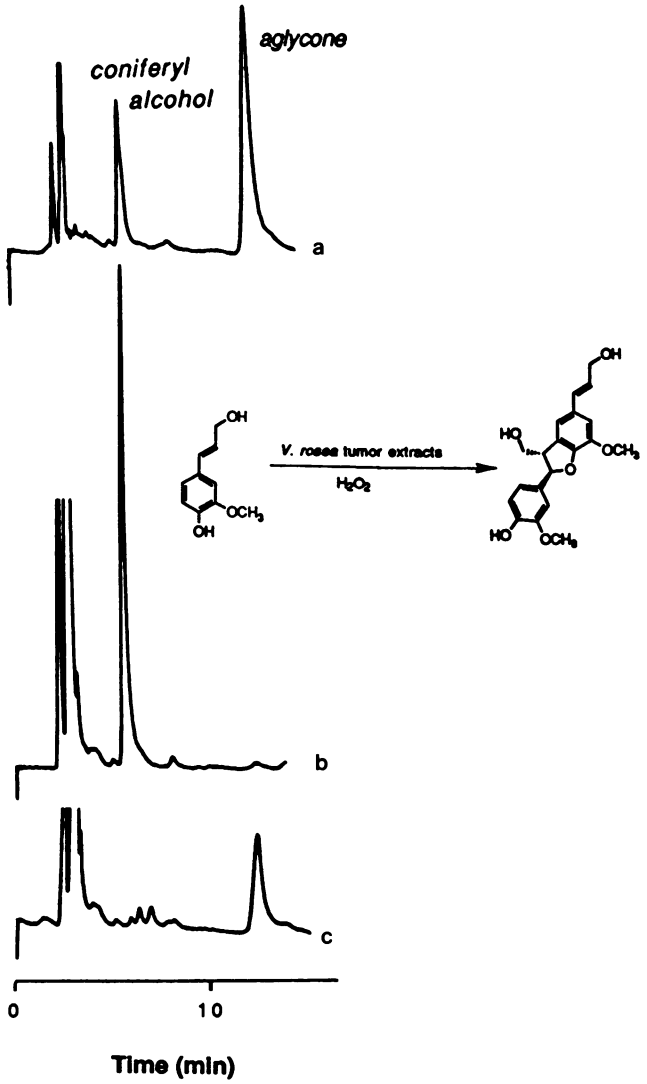


Figure 4. Assay for coniferyl alcohol dimerizing activity in *Vinca* cell free extracts. *Vinca* cells (5 g) were ground in Tris-malate buffer (50 mM, pH 7, 5 mL) and 1 mL of the supernatant was combined with (b) coniferyl alcohol (5 μmol) and Tris-malate buffer (50 mM pH 7, 4 mL). The addition of (c) H_2O_2 (3.1 μmol) gave immediate production of the aglycone. Trace (a) contains the coniferyl alcohol and aglycone standards on HPLC (DuPont, C_8; MeOH:H_2O:AcOH, 35:64.5:0.5, v/v; flow rate 1 mL/min, t_R coniferyl alcohol, 5.2 and aglycone, 12.6 min, as detected at 254 nm).

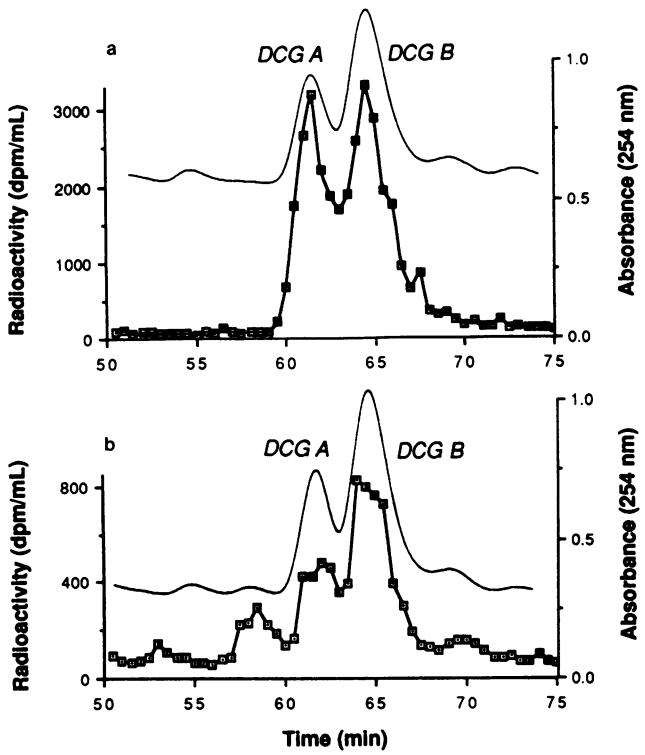


Figure 5. Enantioselectivity of the coniferyl alcohol dimerization. *Vinca* cells (5 g each) were incubated for 2.5 h with (a) (±)-[12-^{3}H] aglycone (2.1 Ci/mmol, 0.11 nmol) or (b) [12,13-^{3}H]aglycone (0.044 mCi/mmol, 390 nmol, *in vitro* dimerized material, see text, and purified by HPLC [Ranin, C18; MeOH:H_2O:AcOH, 30:79.5:0.5, v/v; flow rate 2 mL/min, t_R 6.2 min] before use). The DCG fraction was isolated from each feeding and A and B were resolved on HPLC (DuPont, C18; MeOH:H_2O, 20:80, v/v; flow rate 2.0 mL/min, t_R 62.0 and 65.0 min, respectively). The radioactivity was measured in fractions collected every 30 s from 50 to 75 min. Integration of the 254 nm detected chromatograms provided a relative measure of the concentrations of DCG A and B in these tissues.

sometime after the 4th d on cytokinin-supplemented media (28). Therefore, the feeding experiments were designed to ensure the presence of labeled precursor during this time period. Eleven nanomoles of [12-^{3}H]aglycone (2.1 Ci/mmol) were added to 282 explants in 10 μL/explant aliquots every 12 h over a 2-day period starting on the 4th d of culture. The tissue was harvested 8 h after the final pulse. This extended feeding gave a specific activity in the DCG pool of 10 mCi/mmol, an order of magnitude higher than that obtained by single feedings to the *Vinca* tumors, and this radioactivity accounted for 19% of the total soluble intracellular label. This incorporation of label into a relatively stable DCG pool was consistent with the direct pathway that had been characterized in the *Vinca* tumors.

Figure 6 contains a detail of the HPLC trace of the DCGs derived by total synthesis (trace a) and of the material derived from the [^{3}H]aglycone-administered tobacco pith explants (trace b). The radioactivity in this fraction (trace c) established that only one of the aglycone enantiomers was incorporated into the DCGs. Because it was not possible to show that the other enantiomer remained in the cells, the data were consistent with either the presence of a single enantiospecific glucosyltransferase (24) for the production of DCG A or a step that specifically removed the DCG B aglycone. This selective production of only DCG A, however, was consistent with the finding of only DCG A in H 425 explants (n = 5) during the first several days on culture (28).

DISCUSSION

This study was designed to distinguish between two possible biosynthetic routes to the DCGs, a cell wall fragment-release mechanism and a direct biosynthesis. Very low concentrations of the DCGs were detected in the extracellular media of the *Vinca* cultures, whereas high concentrations were detected inside the cells. Administered DCGs were not absorbed effectively, and the DCGs could not be liberated from isolated cell walls. These data suggested that DCGs were not formed via cell wall autolysis followed by absorption into the cells.

Feeding experiments with coniferyl alcohol and dehydrodiconiferyl alcohol successfully demonstrated that the transformed *Vinca* tissue was capable of directly incorporating these precursors into the DCGs. The 10-fold more efficient incorporation of the aglycone relative to coniferyl alcohol in the *Vinca* cells was consistent with the sequential order of these metabolites in the biosynthesis. Kinetic analyses of the radioactivity after the pulse of [1-^{3}H]coniferyl alcohol were also consistent with a direct pathway not involving any inter-

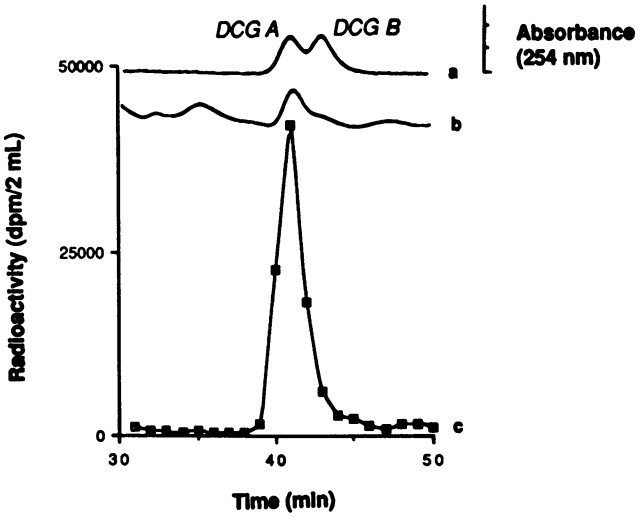


Figure 6. Chromatogram and coeluting radioactivity of resolved DCGs A and B isolated from cultured tobacco pith. *N. tabacum* cv H 425 explants (282) were administered four portions of (±)-[12-^{3}H] aglycone (2.1 Ci/mmol, 11 nmol), one every 12 h over a 2-d period starting on the 4th d of culture. The DCG fraction was isolated and A and B were resolved on HPLC (DuPont, C18; MeOH:H_2O, 21.5:78.5, v/v; flow rate 2.0 mL/min, t_R 40.5 and 41.5 min, respectively). The radioactivity was measured in fractions collected every 30 s from 30 to 50 min. Trace (a) contains the synthetic A and B (1:1) standard, 254 nm detection; trace (b) is the fraction isolated from the explants; and trace (c) shows the radioactivity coeluting with the explant isolate.

mediate association with a separate diluting metabolic pool such as the cell wall. Moreover, the ratio of aglycone enantiomers in *Vinca* was identical to the DCG A/B ratio, again consistent with this being the sole pathway for DCG biosynthesis. Recent models suggest that hydroxycinnamyl alcohols are produced on the endoplasmic reticulum and transported in vesicles to the cell surface where wall biosynthesis occurs (3, 4, 29). This pathway would provide an internal source of coniferyl alcohol that would subsequently be converted to the DCGs through dimerization and glucosylation steps in both *Vinca* and tobacco.

Peroxidases, probably the best studied family of plant enzymes, are heme-containing monomeric glycoproteins. They are best known for their role in catalyzing the racemic dimerization (26, 27) and polymerization (3) of phenylpropanoids like coniferyl alcohol in wall biosynthesis. However, the evidence presented here suggests that the *Vinca*-catalyzed dimerization requires H_2O_2 but does not produce racemic material. Likewise, fresh pith explants of H 425 converted racemic aglycone only to the DCG A isomer. At later times in this tissue (28), as well as other tobacco cultivars (2), carrot cultures and coconut milk (K. M. Hess and D. G. Lynn, unpublished), and in the *Vinca* cultures, both DCG A and B were produced.

Racemic monoterpenes isolated from sage have been shown to be derived from different enantiospecific enzymes (7). The presence of separate enantiospecific pathways would explain the presence of one enantiomer of the aglycone (30) and one diastereomer of the glycoside (25) in different plants, but further characterization of the enzymes in these tissues are required before the controls on the A/B ratio can be defined. Given the variation in the time dependence of the production of the DCGs in tobacco, the concentrations of these isomers and the enzymes responsible for their production require further characterization because they could prove to have important biological consequences.

These biosynthetic experiments have provided a preliminary look at the controls on these pathways. Probably the most important finding was that the high concentrations of the DCGs present in rapidly growing tobacco and *Vinca* tissues were not maintained by a balance between rapid synthesis and biodegradation pathways. Rather, the kinetic studies revealed the DCG pool to be large and slowly turned over. Likewise, even though the DCG biosynthetic enzymes were present, as demonstrated by the feeding experiments, the endogenous DCG concentrations did not increase, suggesting that the pathway was controlled by substrate availability.

A similar substrate-limited reaction has been found for tracheary element differentiation in *Zinnia* (6). An isoperoxidase required for lignification appeared in cultured cells more than a day before tracheary elements were observed or 4-coumarate:Co-A ligase activity was detected. In this system, the expression of the peroxidase was inhibited by anticytokinin and antiauxin treatments, treatments that delayed or inhibited the differentiation.

Peroxidases have a tissue-specific pattern of expression that is both developmentally and environmentally regulated (4). Recent experiments with transgenic tobacco plants overexpressing the anionic peroxidase resulted in phenotypic alterations occurring at specific developmental times (16). Phenylalanine ammonia-lyase genes are expressed in rapidly growing meristematic regions (17), thus providing the source of phenylpropanoid precursors in these rapidly growing tissues. One implication of this study is that these enzymes may be important not only for the biosynthesis of cell wall precursors but also for the production of DCG-like growth factors.

DCG biosynthesis is activated coincident with mitosis in tobacco (28) and exogenously applied DCGs stimulate cell division in this tissue (2). Although the original screen that led to the identification of the DCGs was established to identify growth factors, their precise role in the control of tobacco growth is still not clear. The DCGs could serve as a limiting substrate for the manufacture of a relevant structure such as the cell wall; however, these labeling experiments showing that the DCGs are not rapidly converted into other products do not support that possibility.

DCG biosynthesis then follows a pathway typical of a secondary plant metabolite. However, in this case the pathway is activated with the rise in mitotic figures (28), and the final product activates tobacco cell division when added exogenously with auxin to these cells (2, 28). An analogy with the phenolic neurotransmitter norepinephrine may be relevant. Norepinephrine must be maintained at high levels as a permissive factor required for the growth of several mammalian cells (12, 23). By this analogy, high concentrations of the DCGs may be required to allow the cell to enter the cell cycle.

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