Phytochemistry, Vol. 25, No. 11, pp. 2457–2461, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

INHIBITION OF CONJUGATION OF INDOLE-3-ACETIC ACID WITH AMINO ACIDS BY 2,6-DIHYDROXYACETOPHENONE IN *TEUCRIUM CANADENSE*

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(*Received* 22 *October* 1985)

Key Word Index—*Teucrium canadense*; Labiatae; American germander; conjugation of indole-3-acetic acid; *N*-(indole-3-acetyl)-L-aspartic acid; *N*-(indole-3-acetyl)-L-glutamic acid; oxindole-3-acetic acid; 2,6-dihydroxyacetophenone.

Abstract—2,6-Dihydroxyacetophenone and five structurally related compounds were tested for their effects on metabolism of [2-^{14}C]IAA in stem segments of 3-week-old American germander (*Teucrium canadense*). Pre-treatment of the plants with 2 mM 2,6-dihydroxyacetophenone for 12 hr significantly reduced the formation of two radioactive metabolites, which were tentatively identified as *N*-(indole-3-acetyl)-L-aspartic acid and *N*-(indole-3-acetyl)-L-glutamic acid. The chemical pre-treatment also decreased the level of a less polar metabolite chromatographically indistinguishable from oxindole-3-acetic acid, an oxidative product of IAA, and other unidentified metabolites of IAA. Concomitantly, the level of free [2-^{14}C]IAA increased significantly in the treated tissue. 2,4-, 2,5- and 3,4-Dihydroxyacetophenones, as well as 3-bromo-2,6-dihydroxyacetophenone and 2-hydroxy-6-methoxyacetophenone, did not show a similar effect.

INTRODUCTION

It is well known that IAA supplied exogenously to plant tissues is metabolized at varied rates by oxidation and conjugation with amino acids and sugars. The existence of such conjugates as natural compounds is also well documented [1–7]. Although the physiological significance of conjugation of IAA has not been fully understood, evidence obtained from studies of corn seedlings [8], tomato callus [9] and bean stems [10] indicates that both sugar and amino acid conjugates of IAA are slow-release sources of free IAA. It has been suggested that formation and hydrolysis of IAA conjugates play an important role in the homeostatic control of free IAA levels in plants [8]. From the widely varying rates of IAA metabolism observed in different plant species [11], it is reasonable to believe that plants must have their own control mechanisms to regulate internal IAA concentrations.

Chemical regulation of conjugation of IAA is of interest from both academic and practical viewpoints. It has been reported that a high level of exogenous auxin induced the formation of *N*-(indole-3-acetyl)-L-aspartic acid (IAAsp) in pea seedlings [12] and in other plants, but inactive analogs did not cause the induction [13]. Kinetin suppressed the formation of IAAsp in mungbean seedlings [14], but ABA enhanced it in the same tissue [14] and in *Coleus* petiole segments [15]. There have been conflicting reports on the effect of GA [16, 17] and ethylene [18, 19] on the formation of IAAsp. More recently, it has been reported that a spray of GA_3 decreased IAAsp levels in dwarf pea seedlings [20]. The effect of phenolic compounds on conjugation of IAA has been reported in only a few instances. 4-Methylumbelliferone decreased IAAsp levels in pea stems [21] and *p*-coumaric acid inhibited the formation of alkaline-labile conjugates of IAA in peas and corn [22]. In corn stems, 4-methylumbelliferone, *p*-coumaric acid and ferulic acid were also observed to decrease the crude, polar metabolite fraction that contained conjugates of IAA [23]. We now report that 2,6-dihydroxyacetophenone inhibits the formation of two metabolites of [2-^{14}C]IAA chromatographically indistinguishable from standard IAAsp and *N*-(indole-3-acetyl)-L-glutamic acid (IAGlu) as well as oxidation of IAA in the stems of American germander, *Teucrium canadense* L.

RESULTS AND DISCUSSION

Effect on conjugation of [2-^{14}C]*IAA*

The stems of *Teucrium canadense* showed a high rate of IAA metabolism as indicated by the results of incubation with [2-^{14}C]IAA (Table 1). Eighty-five per cent of [2-^{14}C]IAA taken up was metabolized in 5 hr. However, the plants pre-treated with 2 mM 2,6-dihydroxyacetophenone metabolized only 25% in the same period, a decrease of 70%, whereas the total uptake of [2-^{14}C]IAA decreased only by 12%. When the radioactivity of the crude fractions after solvent partitioning was compared, it was found that there were four fractions that showed marked differences between the control and the treated (Table 2). In the control tissue, the H_2O-soluble fraction from partition of the MeOH extract between Et_2O and Pi buffer was the major metabolite fraction as it contained 47.6% of the radioactivity. HPLC indicated that this fraction had four major radioactive components (Fig. 1) and several minor metabolites (not shown) which were retained longer on the reverse-phase column. The four

Table 1. Uptake and metabolism of [2-^{14}C]IAA in the control and 2,6-dihydroxyacetophenone-treated stems of *Teucrium canadense**

Treatment†	Total uptake of [2-^{14}C]IAA‡ (dpm × 10^3/g fr wt)	Free [2-^{14}C]IAA recovered from tissue (dpm × 10^3/g fr wt)	[2-^{14}C]IAA metabolized (%)
Water (control)	67.28 ± 1.76	9.73 ± 1.68	85.5 ± 2.5
2,6-Dihydroxyacetophenone	58.74 ± 2.63	44.15 ± 2.60	24.8 ± 2.9

* The data are averages of 3 expt ± s.e.
† The plants were pre-treated with deionized water or 2 mM 2,6-dihydroxyacetophenone for 12 hr.
‡ The duration of incubation with [2-^{14}C]IAA was 5 hr.

Table 2. Distribution of radioactivity in major fractions of tissue homogenates of *Teucrium canadense* after a 5-hr incubation with [2-^{14}C]IAA*

	Radioactivity (% of total [2-^{14}C]IAA uptake)			
	MeOH-soluble‡		MeOH-insoluble‡	
Treatment†	Et_2O fraction	H_2O fraction	7 N NaOH hydrolyzable	Residue
Water (control)	30.9 ± 1.7	47.6 ± 2.0	17.9 ± 0.8	3.6 ± 0.7
2,6-Dihydroxyacetophenone	86.3 ± 2.0	9.8 ± 1.7	3.6 ± 0.3	0.3 ± 0.1

* The data are the averages of 3 expt ± s.e.
† The plants were pre-treated with deionized water or 2 mM 2,6-dihydroxyacetophenone for 12 hr.
‡ See procedures in the experimental section.

Table 3. Effect of 2,6-dihydroxyacetophenone on conjugation of [2-^{14}C]IAA with L-aspartic acid and L-glutamic acid in stems of *Teucrium canadense**

	[2-^{14}C]IAAsp		[2-^{14}C]IAGlu	
Treatment†	dpm × 10^3/g fr wt	% of total [2-^{14}C]IAA uptake	dpm × 10^3/g fr wt	% of total [2-^{14}C]IAA uptake
Water (control)	5.68 ± 1.59	8.4 ± 2.5	6.40 ± 1.50	9.5 ± 2.4
2,6-Dihydroxyacetophenone	0.56 ± 0.11	1.0 ± 0.2	1.90 ± 0.36	3.2 ± 0.7

* The data are the averages of 3 expt ± s.e.
† The plants were pre-treated with deionized water or 2 mM 2,6-dihydroxyacetophenone for 12 hr.

major peaks were isolated and further analyzed by TLC and other HPLC systems.

Peaks 1 and 2 had retention times similar to two indole-3-acetyl-*myo*-inositol isomers in the HPLC system used, but they were not such conjugates as indicated by the following evidence: (1) 1 N NaOH hydrolyzed indole-3-acetyl-*myo*-inositol but not peaks 1 and 2; (2) in silica gel TLC with EtOAc–MeCOEt–EtOH–H_2O (5:3:1:1) as solvent, authentic indole-3-acetyl-*myo*-inositol showed two spots (R_fs 0.47 and 0.45), but peaks 1 and 2 remained at the point of application. Chemical identification of peaks 1 and 2 remain to be resolved. A pre-treatment with 2,6-dihydroxyacetophenone substantially decreased radioactivity in peaks 1 and 2 (Fig. 1).

Peaks 3 and 4 (Fig. 1) had retention times similar to authentic IAAsp and IAGlu, respectively. The peaks were collected and further analyzed by HPLC using Partisil 10 SAX anion-exchange and μBondapak Phenyl reverse-phase columns and by silica gel TLC. All results indicated that each radioactive peak was homogeneous with R_T and R_f values identical with those of the corresponding standard. Both peaks were stable to 1 N NaOH, indicating that they were not esters of IAA but were hydrolyzable by 6 N HCl or 7 N NaOH. Based on this evidence, peaks 3 and 4 were tentatively identified as IAAsp and IAGlu, respectively.

In a 5-hr incubation with [2-^{14}C]IAA, 8.4% of the radioactivity taken up was found in IAAsp (peak 3) and 9.5% in IAGlu (peak 4) (Table 3). A pre-treatment with 2,6-dihydroxyacetophenone markedly decreased the levels of the two radioactive peaks. The decrease was 88% for IAAsp and 66% for IAGlu. Preliminary results from a

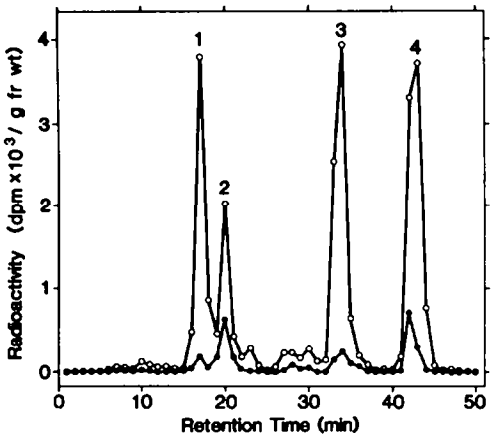

Fig. 1. HPLC analysis of [2-^{14}C]IAA metabolites contained in the 50% MeOH eluate from the Sep-Pak C_{18} separation of the H_2O-soluble fraction from the MeOH extract. Column: μBondapak C_{18}, 30 cm × 3.9 mm i.d. Solvent: (A) 1% HOAc (pH 2.9), (B) MeOH. Elution: 45 min gradient (curve 10) from 15 to 20% B followed by 20% B for 5 min. Flow rate: 1 ml/min. Based on retention times, peak 3 = IAAsp and peak 4 = IAGlu. Hollow circle: control; solid circle: 2 mM 2,6-dihydroxyacetophenone-treated.

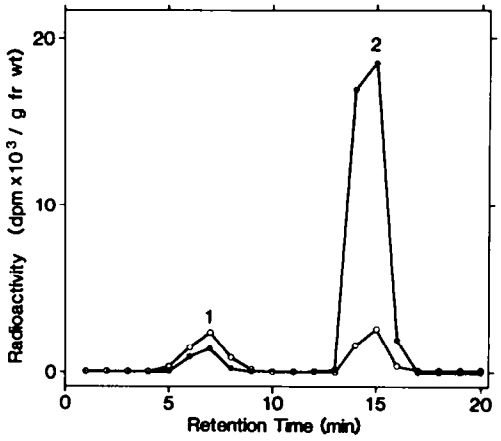

Fig. 2. HPLC analysis of radioactive substances contained in the 50% MeOH eluate from the Sep-Pak C_{18} separation of the Et_2O-soluble fraction from the MeOH extract. Column: Partisil 10 ODS, 25 cm × 4.6 mm i.d. Solvent: 20 mM ammonium acetate (pH 4.4)–MeCN (9:1). Elution: isocratic. Flow rate: 1 ml/min. Peak 2 = IAA. Open circle: control; solid circle: 2 mM 2,6-dihydroxyacetophenone-treated.

comparison study indicated that 2,4-, 2,5- and 3,4-dihydroxyacetophenones did not have a similar effect on these two peaks. Bromination at the 3 position on the ring or methylation of one hydroxyl group also diminished the inhibitory activity.

Effect on oxidation of [2-^{14}C]*IAA*

The marked difference between the control and the treated (Table 2) in the amount of radioactivity in the Et_2O-soluble fraction from partition of the MeOH extract between Et_2O and Pi buffer reflects mainly the preservation of free [2-^{14}C]IAA by 2,6-dihydroxyacetophenone. When purified using a Sep-Pak C_{18} cartridge, the material in this fraction which was retained and eluted by 50% MeOH showed two major radioactive peaks (Fig. 2). Peak 1 had a retention time of 7.0 min which was identical with that of oxindole-3-acetic acid (OxIAA). On repeated HPLC, however, a minor peak was eluted before the major peak. A breakdown product with a similar retention time was also observed with authentic OxIAA after being in solution for some time. Neither peak 1 nor OxIAA was detectable by the fluorescence detector. Based on these similarities, peak 1 is likely to be OxIAA. This compound has been reported to be a metabolite of IAA in rice bran [24], corn shoots [25] and corn roots [26]. Peak 1 isolated from the control tissue had an average of 8.4% of the total radioactivity taken up, an amount similar to that of free [2-^{14}C]IAA remaining in the tissue after a 5-hr incubation with the labelled IAA (Fig. 2). A pre-treatment with 2,6-dihydroxyacetophenone resulted in a 50% decrease in peak 1. This phenol has been found previously to affect peroxidase-catalyzed decarboxylation of IAA *in vitro* [27]. The present results suggest that it may also affect oxidation of IAA that does not involve decarboxylation.

Peak 2 had a retention time similar to IAA (Fig. 2). Further analysis of peak 2 by HPLC with a μBondapak Phenyl reverse-phase column and by TLC on silica gel with two solvent systems showed a single radioactive component with R_T and R_f values identical with IAA. The effect of 2,6-dihydroxyacetophenone on the level of free [2-^{14}C]IAA is clearly shown in Fig. 2.

In addition to IAA and OxIAA, there were three groups of minor radioactive metabolites in the crude Et_2O fraction: (1) 2% of the radioactivity of this fraction was not retained on the Sep-Pak when washed with H_2O or dilute acidic buffer; (2) the 50% MeOH-eluted subfraction that contained IAA and OxIAA also included some minor metabolites which were retained on the Partisil 10 ODS and μBondapak C_{18} reverse-phase columns longer than IAA; (3) 20% of the radioactivity in the Et_2O fraction was not eluted by 15 ml of 50% MeOH but was eluted by 100% MeOH. Further analysis of this latter material by HPLC and TLC showed several radioactive compounds. In addition, hydrolysis with 7 N NaOH at 100° for 3 hr and subsequent TLC analysis of the neutralized hydrolysates also showed several radioactive spots. These metabolites were less polar than IAA and appeared to be oxidation products rather than conjugates of IAA. Obviously, the Et_2O fraction from the control tissue contains, in addition to IAA, several radioactive compounds including probably OxIAA. However, the total amount of these metabolites was less than 20% of all the [2-^{14}C]IAA metabolized in the 5-hr period.

It is noteworthy that a considerable amount of radioactivity (21.5%) from [2-^{14}C]IAA was incorporated into the MeOH-insoluble material of the control tissue in 5 hr (Table 2). A treatment with 7 N NaOH at 100° for 3 hr released 83% of the radioactivity. Among the solubilized, 22% was identified as [2-^{14}C]IAA, which represented 3.9% of the total [2-^{14}C]IAA taken up by the tissue. The

incorporation of this amount of [2-^{14}C]IAA into the insoluble material occurred probably through initial conjugation with amino acids, such as L-aspartic acid and L-glutamic acid, and subsequent formation of higher peptides. The major portion of radioactivity released by alkaline hydrolysis is likely to be oxidative metabolites. A pretreatment with 2,6-dihydroxyacetophenone reduced the incorporation of radioactivity into the MeOH-insoluble fraction by 82% (Table 2). This decrease probably was due mainly to an inhibition of IAA oxidation by this compound.

The plant *Teucrium canadense* L. metabolized applied [2-^{14}C]IAA rapidly yielding several MeOH-soluble compounds, including three tentatively characterized as IAAsp, IAGlu and OxIAA, as well as smaller amount of MeOH-insoluble material. A pre-treatment with 2,6-dihydroxyacetophenone substantially reduced the formation of these metabolites. This study not only confirmed the complexity of IAA metabolism in plants, but more significantly demonstrated an effective regulation of IAA levels by chemical treatment. Although the study involved only one plant species and a short experimental period, the magnitude and the persistence of the effect of 2,6-dihydroxyacetophenone suggests a potential for control of IAA levels by manipulating its metabolism in plants. As IAA is a natural hormone involved in various aspects of plant growth and development, the effectiveness of this phenolic compound reported here has practical implications.

EXPERIMENTAL

Plant material. Two-bud segments of the rhizomes of American germander (*Teucrium canadense* L.) were planted in soil in clay pots in the greenhouse. After germination, uniform plants were selected and grown in a growth chamber at 28° under 12-hr light and 12-hr dark cycles. The light with 270 $\mu E/M^2/sec$ was provided by a mixture of incandescent and fluorescent lamps. Three-week-old plants were used for the experiments. This plant species was chosen because it had been observed to have a high rate of IAA metabolism [11] and a high sensitivity to phenolic compounds (unpublished results).

Chemicals. 2,6-Dihydroxyacetophenone and 3,4-dihydroxyacetophenone were purified by chromatography over a column of silicic acid–celite (4:1) using CH_2Cl_2 as eluent. 2,4-Dihydroxyacetophenone and 2,5-dihydroxyacetophenone were used as received. 3-Bromo-2,6-dihydroxyacetophenone and 2-hydroxy-6-methoxyacetophenone were synthesized according to reported procedures [27]. [2-^{14}C]IAA (42 μCi/μmol) was purified by HPLC before use. IAAsp and IAGlu were synthesized by the method of Mollan *et al.* [28]. Indole-3-acetyl-*myo*-inositol and OxIAA were synthesized by the methods of Nowacki *et al.* [29] and Hinman and Bauman [30], respectively.

Radioactivity measurement. Aliquots of all fractions from solvent extraction and HPLC were counted for radioactivity by liquid scintillation in a Beckman LS-9000 system. The zones of TLC after development were scraped, mixed with 0.5 ml MeOH, and similarly counted.

Chemical treatment. The stems were cut near the soil surface and kept in the dark for 12 hr with the cut end dipping in a beaker containing deionized water (control) or 2 mM aq. soln of 2,6-dihydroxyacetophenone or related compounds. All plants appeared healthy at the end of the chemical treatment.

Incubation with [2-^{14}C]IAA. After the pre-treatment, the internodes were cut into 5-mm segments. The segments (5 g) were used for incubation with [2-^{14}C]IAA by the reported procedure [11] except that the duration was increased to 5 hr. Duplicate samples were used for each treatment and the experiment was repeated twice.

Extraction. At the end of incubation with [2-^{14}C]IAA, the stem segments were removed from the soln, stirred vigorously in 100 ml sterile H_2O for 1 min, washed thoroughly on a Buchner funnel with H_2O, then immediately homogenized with MeOH containing 0.01% 2,6-di-*tert*-butyl-4-methylphenol (BHT). The extract was filtered and the residue was re-extracted six times with fresh MeOH containing BHT. After adding 5 ml of KH_2PO_4–H_3PO_4 (0.05 M, pH 2.5) to the combined extract, MeOH was removed at 35° *in vacuo*, and the aq. soln was partitioned with peroxide-free Et_2O six times. Thus, the sample was separated into three fractions: Et_2O-soluble, H_2O-soluble and insoluble. These fractions were subjected to further treatment as described below.

Chromatography. (1) *Sep-Pak* C_{18}. Sep-Pak C_{18} cartridges (Waters) were used for initial purification of the two subfractions from the MeOH extract and the alkaline hydrolysates from the MeOH-insoluble material. The Sep-Pak, after being washed with MeOH and H_2O, was equilibrated with 0.05 M KH_2PO_4–H_3PO_4 (pH 2.5). The samples from which any organic solvent was removed were applied to the Sep-Pak. After washing with H_2O, the retained material was eluted with 50% MeOH containing BHT and then with 100% MeOH.

(2) *HPLC.* The H_2O-soluble fraction of the MeOH extract which was eluted from the Sep-Pak by 50% MeOH was first analyzed by gradient chromatography on a 30 cm × 3.9 mm i.d. μBondapak C_{18} reverse-phase column (Waters) as previously reported [31]. Preliminary results indicated that IAAsp and IAGlu were probably the major metabolites of this fraction. Modification of the method to optimize the separation of the two conjugates (Fig. 1) provided further support for this conclusion. The material contained in the radioactive peaks was isolated and further analyzed by a second HPLC system using a 25 cm × 4.6 mm i.d. Partisil 10 SAX anion-exchange column (Whatman) with a gradient of 10–100 mM KH_2PO_4 at pH 3.0 (10 mM isocratic for 3 min, 10–55 mM using curve 2 for 9 min, and 55–100 mM using curve 8 for 12 min). The material contained in the radioactive peaks was again analyzed by a third HPLC system using a 30 cm × 3.9 mm i.d. μBondapak Phenyl reverse-phase column (Waters) and an isocratic mobile phase of 1% HOAc–MeOH (75:25). In all cases, the flow-rate was 1 ml/min. Authentic IAAsp and IAGlu were used as standards and were monitored by a UV detector at 280 nm and by a fluorescence detector at 280 nm (excitation) and 338 nm (emission). The retention time (min) was 34.0 for IAAsp and 42.5 for IAGlu in system 1 (Fig. 1), 14.6 for IAGlu and 18.7 for IAAsp in system 2, and 13.6 for IAAsp and 16.7 for IAGlu in system 3.

The material from the Et_2O-soluble fraction which was retained on the Sep-Pak and eluted by 50% MeOH was analyzed by HPLC using a 25 cm × 4.6 mm i.d. Partisil 10 ODS column (Whatman) and the solvent system described in the caption of Fig. 2. The identity of the radioactive peak corresponding to IAA was confirmed by HPLC on the μBondapak C_{18} and μBondapak Phenyl columns. The same procedure was used for analysis of [2-^{14}C]IAA from the hydrolysates of the MeOH-insoluble material. The loss of IAA in the procedure was estimated by including a control with a known amount of [2-^{14}C]IAA at the beginning of homogenization and by counting the radioactivity of [2-^{14}C]IAA at the end of isolation by HPLC. The recovery was from 68 to 73%.

(3) *TLC.* The material in the four isolated radioactive HPLC peaks from the H_2O-soluble fraction (Fig. 1) and their hydrolysates were analyzed by silica gel TLC (Whatman PLK) using $CHCl_3$–EtOAc–HCO_2H (5:4:1) or EtOAc–MeCOEt–EtOH

$-H_2O$ (5:3:1:1) for development and the Van Urk-Salkowski reagent [32] for visualization. Authentic IAA and its amino acid conjugates were used for comparison.

Hydrolysis. The material in the radioactive peaks from the H_2O-soluble fraction from the MeOH extract were hydrolyzed by three methods: (1) 1 N NaOH at 21° for 1 hr under N_2; (2) 7 N NaOH at 100° for 3 hr under N_2; (3) 6 N HCl at 110° for 16 hr in a sealed tube. Method 1 hydrolyzes the ester bond of IAA conjugated with sugars and methods 2 and 3 hydrolyze the amide bond of IAA conjugated with amino acids. The alkaline hydrolysates after neutralization and the acidic hydrolysates after removal of HCl *in vacuo* were used for analyses of free [2-^{14}C]IAA, IAAsp and IAGlu. Corrections were made for losses of IAA in the procedure by using appropriate controls with a known amount of [2-^{14}C]IAA. Method 2 was also used for hydrolysis of the MeOH-insoluble residue. The material which remained insoluble after hydrolysis was solubilized by NCS tissue solubilizer (Amersham) at 45° for 16 hr and analyzed for radioactivity.

Acknowledgements—We thank J. J. Jevnikar and G. R. Lambert for technical assistance.

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