BIOSYNTHESIS OF TWO DILIGNOL RHAMNOSIDES IN LEAVES OF *THUJA PLICATA* DONN

Gary D. Manners
Faculty of Forestry, University of British Columbia, Vancouver, B.C., Canada

and

Eric P. Swan[a]
Department of Fisheries and the Environment, Canadian Forestry Service, Western Forest Products Laboratory, 6620 NW Marine Drive, Vancouver, British Columbia, Canada V6T 1X2

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ABSTRACT

One of a series of dilignol glycosides previously isolated from western red cedar leaves has now been identified as 2, 3-dihydro-7-hydroxy-2— (4′-hydroxy-3′methoxyphenyl)-3-hydroxymethyl-5-benzofuran-propan-3″-*O*-α-L-rhamnopyranoside. In an experiment to determine biosynthesis rate, cut western red cedar leaves took up 37% radioactive phenylalanine (label) after 10 h. The newly identified compound took 3 h to reach a maximum uptake of label of 0.4%. A previously identified dilignol glycoside reached a maximum uptake of 0.3% in the same period. Both glycosides in leaves were rapidly anabolized, therefore, and since their label decreased after 5 h, they were precursors to other unknown compounds. The possible role of these glycosides in wound response or leaf lignin formation is considered.

Keywords: *Thuja plicata*, biosynthesis, anabolism, dilignols, phenyl propane, dimers, lignification, glycosides, leaves, needles.

INTRODUCTION

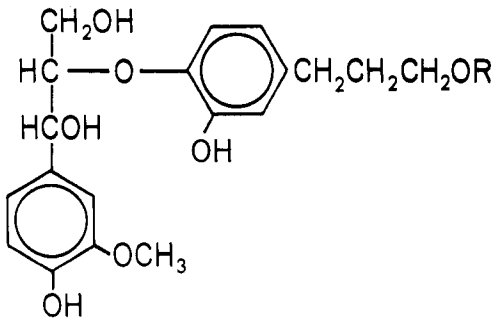
A series of dilignol glycosides were isolated previously from western red cedar (*Thuja plicata* Donn) leaves and one of them was characterized as Compound A (Manners and Swan 1971), 1-(3′methoxy-4′-hydroxyphenyl)-2-*O*-1″-[2″-hydroxy-4″-(propane-3‴-*O*-α-L-rhamnopyranoside)-phenyl]-propane-1, 3-diol. Another then uncharacterized Compound B, 2, 3-dihydro-7-hydroxy-2-(4′-hydroxy-3′-methoxyphenyl-3-hydroxymethyl-5-benzofuran-propane-3″-*O*-α-L-rhamnopyranoside, was also isolated (Manners and Swan 1971).

Figure 1 shows the structures of Compounds A and B. The occurrence of Compound B in western red cedar leaves is described here for the first time, with details in the experimental section. These compounds and related dilignol glycosides have been isolated from the needles of Scots pine by Popoff and Theander (1976), who characterized *inter alia* Compound B. Comparison of A and B from the two sources has shown their identity. We now report the results of an experiment to determine the rate of biosynthesis (anabolism) of Compounds A and B in western red cedar leaves.

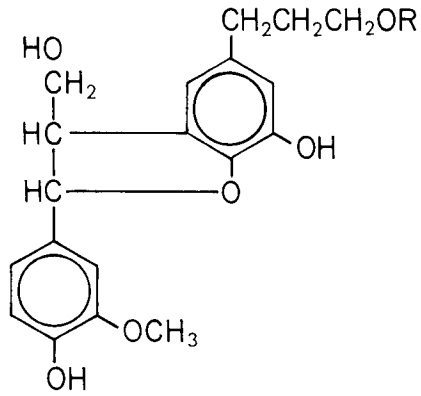
The metabolic activity of leaves makes them a very promising tissue for the study of rapid incorporation of known aromatic precursors. Phenylalanine represents a well-established aromatic precursor (Neish 1965) which, when administered to actively metabolizing tissues, will participate in the formation of aromatic compounds with a minimum reversion to the glycolytic pathway. Therefore, it was for this reason that phenylalanine was chosen for incorporation studies in the leaves of western red cedar.

Administration of lignin precursors into actively metabolizing tissues generally falls into two separate classes, (1) infusion and

[a] To whom enquiries should be addressed.



COMPOUND A



COMPOUND B

R = α - L - Rhamnopyranoside

FIG. 1. Formulae for Compounds A and B.

(2) implantation. Kratzl (1965) has shown that the infusion of radioactive lignin precursors into the stem produced higher radioactive incorporation into lignin than the implantation of the same precursors into the cambial area. Implantation techniques initiated secondary wound reactions, which subsequently altered the pathway to aromatic compounds. Freudenberg (1962) noted the formation of radioactive lignin using the technique of infusion of known lignin precursors through the leaves of coniferous species. Therefore, infusion feeding methods allow the efficient uptake of lignin precursors that may be altered in the leaf tissue prior to the ultimate formation of lignin. Thus the close examination of western red cedar leaves for lignin precursors should show active metabolism of radioactivity labeled aromatic precursors during infusion feeding.

EXPERIMENTAL

Leaves were taken in the summer from a mature western red cedar tree growing outside the Western Forest Products Laboratory in Vancouver, B.C. *U*-^{14}C-L-Phenylalanine (350 mc/mM) was purchased from New England Nuclear Corp. Samples were counted in a Packard 1200 scintillation counter with a calibrated external standard at the Vancouver Laboratory of Canada Fisheries and Marine Service.

The identity of Compound B was determined as follows. The ultraviolet, visible, infrared and proton magnetic resonance spectra found (Manners 1970) are in agreement with those published by Popoff and Theander (1976). The proton magnetic resonance and mass spectra of the hexaacetate derivative (Manners 1970) agreed with the spectra shown by Popoff and Theander using the same techniques, but on the hexamethyl ether derivative (1976). Finally, thin-layer chromatographic comparison of Compound B and an authentic sample from Prof. Theander, using the technique described below, showed them to have identical R_f values.

The collected leaves were cut with scissors into pieces averaging about 2 mm in length; all woody material was eliminated.

A portion of the leaves was immediately weighed and placed in a 105 C oven for 18 h to determine oven-dry weight. The remaining leaves were weighed into four 2-g portions and placed in four petri dishes containing approximately 2 μc/ml of *U*-^{14}C-L-phenylalanine in 6 ml of sterilized water.

Three 10-μl samples of the feeding solution were withdrawn from each petri dish immediately after introduction of the samples. These samples were placed in

TABLE 1. *Uptake of U-^{14}C-L-phenylalanine and its incorporation into Compounds A and B in the leaves of western red cedar*

Feeding time hr.	Leaves		Compound A		Compound B	
	Activity dpm	Percent of Activity*	Activity dpm	Percent of Activity*	Activity dpm	Percent of Activity*
1	1.22×10^6	16.1	1865	0.15	3063	0.25
3	1.34×10^6	20.3	3970	0.30	5370	0.40
5	1.78×10^6	23.5	5340	0.30	7040	0.39
10	2.82×10^6	37.1	5035	0.18	6600	0.24

* By difference

counting vials containing Liquifluor (New England Nuclear Corp.) and counted to determine the amount of labeled phenylalanine available to the leaves at the beginning of the feeding period. The petri dishes were covered and illuminated by two 250-watt incandescent bulbs at a distance of approximately 1 m. The four dishes represented feeding times of 1, 3, 5 and 10 h. At the end of each time period, three 10-μl samples were withdrawn from the individual feeding solutions and counted to determine percent uptake of the labelled solution with time. Upon completion of each feeding, the leaves were filtered from the radioactive solution and washed with distilled water. The washed leaves were then placed without delay in individual micro-soxhlets for extraction with methyl alcohol. Extraction and workup, according to the method of Manners and Swan (1971), yielded gross ethyl acetate solubles. Based upon earlier chromatographic evidence, the compounds of interest were known to be present in the ethyl acetate extract. The ethyl acetate extract was taken to dryness under vacuum and transferred in ethyl alcohol to four 2-ml volumetric flasks. Three 60-μl replicates of each solution (representing the four time periods) were applied to separate thin-layer cellulose plates and developed two-dimensionally with *n*-butanol, chloroform, acetic acid, water (4:1:1:1 v/v) in the first direction, followed by air drying and development in the second direction with chloroform, acetic acid, water (2:3:1.5 v/v lower layer). The plates were dried and sprayed with diazotized sulfanilic acid. The compounds were then stripped from the plate and counted, using the method of Manners (1974).

Incorporation of *U*-^{14}C-L-phenylalanine into A and B was examined in a 10-h feeding experiment utilizing a thin-layer chromatographic combustion technique. Diazotized sulfanilic acid (DSA) was used as a detecting reagent, since it gave more distinct color reactions and best counting efficiency after combustion (Manners 1974).

RESULTS AND DISCUSSION

A preliminary autoradiographic verification of *U*-^{14}C-L-phenylalanine incorporation into Compounds A and B in western red cedar leaves had been performed (Manners 1970). Preliminary infusion feeding followed by autoradiography of the leaf ethyl acetate extract showed incorporation of the label in a 4-h feeding time. The subsequent analyses of scintillation results obtained in the leaf-feeding experiment are summarized in Table 1, which gives the radioactivity uptake by the leaves during infusion. The relative incorporation of radioactive phenylalanine into Compounds A and B is depicted graphically in Fig. 2. The results represent average values of three runs. Table 1 reveals that floating cut western red cedar leaves in a solution of radioactive phenylalanine is an efficient method of administering this aromatic precursor. Table 1 shows the percent uptake of the

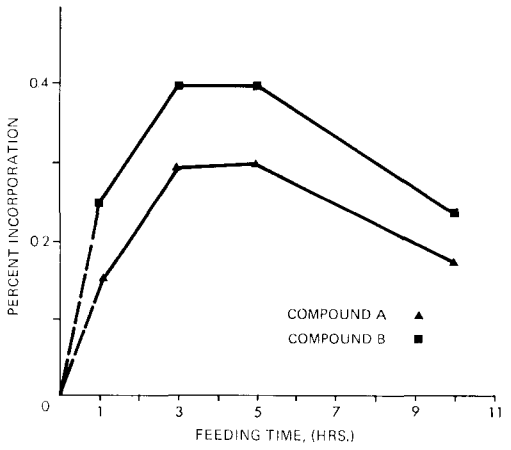


FIG. 2. Incorporation of U-^{14}C-L-phenylalanine into Compounds A and B in the leaves of western red cedar.

labeled precursor with time, to a level of 37% after 10 h. This rate of uptake effectively minimized destruction of the aromatic precursor by micro-organisms that might have been present in the leaves. This rapid rate also optimized introduction of the aromatic precursor into the metabolizing areas during a period of anabolism.

Figure 2 and Table 1 depict the level of activity incorporation into Compounds A and B during 1, 3, 5, and 10-h feeding periods. Incorporation levels of 0.15% to 0.40% were observed for radioactive phenylalanine into Compounds A and B. Compound B incorporated a larger portion of the available radioactivity than Compound A. Both compounds showed a reduction in activity after 5 h feeding, presumably associated with further metabolism of the compounds.

We offer the following hypothesis to account for the shape of the curve in Fig. 2. Compounds A and B, after rapid biosynthesis, are used in wound healing or leaf lignin formation via polymerization or oxidation. Excess L-phenylalanine present could easily give rise to more of the phenolic portions of Compounds A and B. The limiting factor in further biosynthesis of A and B is the supply of L-rhamnose; once the pool of this carbohydrate is used up, then no more can form (CO_2 not available). Thus the amounts of Compounds A and B in the experimental leaves decrease after passing through a maximum. Subsequent experiments would clarify this hypothesis.

CONCLUSIONS

A specific radioactive-infusion study revealed that the dilignol rhamnosides, Compounds A and B, incorporated 0.3% and 0.4% U-^{14}C-L-phenylalanine within a 3-h feeding period. The amount of label incorporated into these compounds was obtained by separating them chromatographically on thin-layer cellulose plates and then efficiently counting the low radioactivity in the spots. The dilignol glycosides of the leaves may be important leaf lignin or wound-healing precursors, based upon their structures. The relatively high yield of these compounds in the actively metabolizing leaf tissue make them promising candidates for future biosynthesis research.

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