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THE GLUCOSIDES OF CAMBIAL SAP OF SPRUCE

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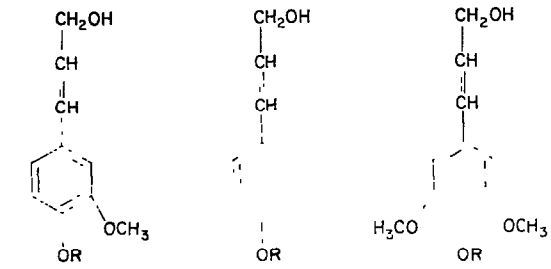
Abstract—In addition to large amounts of coniferin (4-*O*-glucosyl-coniferyl alcohol (I)) the cambial sap from spruce (*Picea excelsa* Link) was shown to contain small amounts of syringin (VI) and *p*-coumaryl alcohol 4-*O*-glucoside (IV), both of which have been isolated in a pure state. This finding supports the theory that conifer lignin is a co-polymer, obtained by enzymatic dehydrogenation of a mixture of coniferyl alcohol, sinapyl alcohol and *p*-hydroxycoumaryl alcohol, in which the coniferyl alcohol predominates. *p*-Coumaryl alcohol 4-*O*-glucoside (IV), although predicted for a long time, has not been encountered in nature before. Coniferyl alcohol and its main dehydrogenation products are also present in the cambial sap.

INTRODUCTION

IT IS well known that large amounts of coniferin (I), the 4-*O*-β-glucoside of coniferyl alcohol, occur in the sap of the cambium and surrounding cells of the spruce (*Picea abies* L. = *P. excelsa* Link), during the main period of growth in the early summer. It is also now well established that coniferyl alcohol (II) is the major precursor of conifer lignin.[1] The isolation of small amounts of trimethylgallic acid[2] from methylated spruce wood, and the discovery of *p*-hydroxybenzaldehyde among the products of degradation of conifer lignin with nitrobenzene and alkali,[3-6] as well as the isolation of anisic acid and trimethylgallic acid from a mixture of the methoxylated aromatic acids obtained after oxidative degradation of spruce milled wood lignin[7,8] indicate that small quantities of *p*-hydroxycinnamyl alcohol (*p*-coumaryl alcohol) (III) and sinapyl alcohol (V) are co-polymerized as their dehydrogenated derivatives along with coniferyl alcohol into spruce lignin. But neither of the alcohols (III) and (V) nor their glucosides (IV) and (VI) have so far been found in the cambium zone of conifers. We have now succeeded in isolating these β-glucosides (IV)[9] and (VI) in small amounts from the cambial sap of growing spruce trees.

Syringin, coniferin and *p*-coumaryl alcohol 4-*O*-glucoside have also been detected in green shoots from young spruce saplings using thin layer chromatography (K. Freudenberg and J. Torres-Serres). All three glucosides were found to be radioactive when phenylalanine-β-^{14}C had been administered to the plants. These results will be reported fully elsewhere.

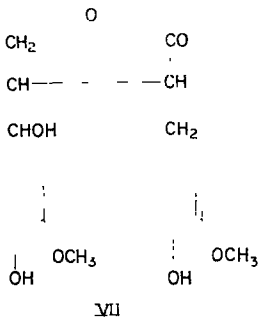
[1] See, for example, (*a*) K. FREUDENBERG in L. ZECHMEISTER's "*Fortschritte der Chemie organischer Naturstoffe*", Vol. XX, 41 (1962), Springer Verlag, Vienna; (*b*) K. FREUDENBERG, *Pure and Applied Chem.* **5**, 9 (1962).
[2] K. FREUDENBERG, K. ENGLER, E. FLICKINGER, A. SOBEK and F. KLINK, *Chem. Ber.* **71**, 1810 (1938).
[3] D. E. BLAND, G. HO and W. E. COHEN, *Australian J. Sci. Research Ser.* **A3**, 642 (1950).
[4] B. LEOPOLD, *Acta. Chem. Scand.* **6**, 38 (1952).
[5] B. LEOPOLD and J. L. MALMSTROM, *Acta. Chem. Scand.* **6**, 49 (1952).
[6] F. F. NORD and G. DE STEVENS, *Naturwiss.* **39**, 479 (1952).
[7] K. FREUDENBERG and C.-L. CHEN, *Chem. Ber.* **93**, 2533 (1960).
[8] K. FREUDENBERG, C.-L. CHEN and G. CARDINALE, *Chem. Ber.* **95**, 2814 (1962).
[9] K. FREUDENBERG in ZECHMEISTER's "*Fortschritte der Chemie Organischer Naturstoffe*", Vol. XX, 61 (1962); K. FREUDENBERG, *Pure and Applied Chem.* **5**, 11 (1962).



I R = Glucosyl
II R = H

III R = H
IV R = Glucosyl

V R = H
VI R = Glucosyl



VII

EXPERIMENTAL AND RESULTS

At about the end of May, three spruce trees (each about 20 yr old) were felled, their branches removed, and the trunks cut into sections each about 3 ft long. Each section was then worked up as quickly as possible. A number of longitudinal incisions were made in the bark, and the latter stripped off. The peeled trunk was stood on its end on a galvanized iron grating over a large polythene dish and washed down with boiling water containing 2% formalin, in order to inactivate the enzymes. This arrangement must be used to prevent the sap from being sucked up by capillarity by the vessels at the ends of the trunk. The cambial tissue around the surface of the trunk was then scraped off and the sap and tissues washed into the dish with more boiling formaldehyde solution. The inside of the strips of bark were worked up separately as the sap from this contained greater quantities of hydroxymatairesinol (VII).[10]

It is noteworthy that the sap from the cambial cells obtained *immediately* after peeling off the bark couples with diazotized *p*-sulfanilic acid to give the same orange-red colour as is observed with the mixture of main oligomeric intermediates obtained by enzymatic dehydrogenation of coniferyl alcohol during the preparation of biosynthetic lignin (DHP).[1]

The sticky solution of cambial sap was separated from tissue material by filtration through cloth, and the residue extracted with warm dilute formaldehyde solution. The combined filtrates were evaporated to dryness at 40–45° *in vacuo* in a rotary evaporator, the receiver being cooled in a freezing mixture. The residue obtained was a grey–green amorphous solid, which was pulverized and extracted at room temperature successively with petroleum ether, benzene and *n*-butanol. The benzene extract was shown to contain a small amount of coniferyl alcohol and the main phenolic substances which are

[10] K. FREUDENBERG and L. KNOF, *Chem. Ber.* **90**, 2857 (1957).

formed on dehydrogenation of the latter compound with laccase *in vitro*.[1] The butanol extract (1·2 g) contained larger amounts of the same materials, some of which were identified by paper chromatography using the solvent mixtures described by Freudenberg and Lehmann.[11] These included coniferyl alcohol, coniferyl aldehyde, dehydrodiconiferyl alcohol, pinoresinol, guaiacylglycerol-β-coniferyl ether, and surprisingly, relatively large amounts of guaiacylglycerol-β-pinoresinol ether, a substance only recently recognized as a trimeric intermediate of lignification.[12]

Undoubtedly these substances must be regarded as occurring naturally and are probably accompanied by smaller amounts of the other intermediates observed during the dehydrogenation of coniferyl alcohol *in vitro*.[11] Hydroxymatairesinol (VII)[10] was also present, probably extracted from the resin ducts or from knots or the stumps of the branches in the wood.

Part of the residue insoluble in butanol (1270 g) was extracted with several portions of boiling water. The combined extracts were filtered hot through a sintered glass filter, and the dark coloured filtrate concentrated *in vacuo* at 55° to a thin syrup, which was seeded with a few crystals of pure coniferin. On standing, 80 g of grey crystals of crude coniferin separated out, which, after repeated recrystallization from water (with addition of activated carbon) gave the pure compound m.p. 185°. It was later discovered, using thin layer chromatography, described below, that the crude coniferin was mixed with a little *p*-coumaryl alcohol 4-*O*-glucoside.

The crystallization of the crude coniferin from the aqueous extract was facilitated when the sugars in the extract were removed by fermenting with yeast before concentration.

Paper chromatography of the mother liquor remaining after removal of most of the coniferin revealed that it contained at least sixteen substances other than phenolic glucosides. Judged by chromatography, these included glucose, mannose, xylose, galactose, arabinose, chlorogenic acid, and caffeic acid. It was impossible to tell at this stage whether the solution contained syringin or *p*-coumaryl alcohol glucoside, because of the amount of coniferin still in solution. This was far more than the amount normally required to produce a saturated solution in pure water (0·5%); obviously the other polyhydroxy compounds in solution prevented its complete precipitation.

The mixture of substances was therefore partitioned in small portions by countercurrent distribution in a 200 tube apparatus, each tube containing 25 ml of both upper and lower phases, using as solvent system sec-butanol/water, containing 2% pyridine to prevent emulsification. During the separation, pure coniferin crystallized out in several of the tubes and was removed.

After 660 transfers, the *p*-coumaryl alcohol 4-*O*-glucoside was found mainly in tubes 180–200, the coniferin in tubes 140–180, and the syringin in tubes 110–140. The sugars remained in the tubes at the start, and the phenolic impurities passed through into the leading tubes as the first fraction. Each glucoside fraction was examined by paper chromatography as shown in Table 1.

However, separation of the glucosides was best effected on thin layer chromatograms using Merck Silica Gel G and a mixture of acetone/ethyl acetate/water 10 : 10 : 1 v. as developing solution. Antimony pentachloride in carbon tetrachloride, or a mixture of concentrated sulfuric acid and formaldehyde, were used as spray reagents; the glucosides can be differentiated by the colours formed. The developed plates were heated to 50°

[11] K. Freudenberg and B. Lehmann, *Chem. Ber.* **93**, 1354 (1960).
[12] K. Freudenberg and H. Nimz, *Chem. Ber.* **95**, 2057 (1962).

TABLE 1. R_f VALUES* OF THE GLYCOSIDES FROM SPRUCE IN VARIOUS SOLVENT SYSTEMS

Solvent †‡	Syringin	Coniferin	*p*-Coumaryl alcohol glucoside
in (*a*)	0·49	0·54	0·59
in (*b*)	0·32	0·36	0·42
in (*c*)	0·27	0·33	0·40

* After 30 hr using Schleicher and Schüll paper No. 2043a.

† (*a*) s-Butanol/acetic acid/water 14 : 1 : 5
(*b*) n-Butanol/acetic acid/water 4 : 1 : 5
(*c*) n-Butanol/ethanol/water/conc. ammonia 45 : 5 : 49 : 1

‡ To show up the spots, the following reagents were used:
(1) H_5IO_6 in acetone, followed by benzidine acetate in acetic acid;
(2) Silver nitrate/conc. ammonia;
(3) Conc. nitric acid—the spots fluoresce under ultraviolet light;
(4) Antimony pentachloride in carbon tetrachloride;
(5) Aniline phthalate in acetic acid.

Silver nitrate was the most sensitive reagent, antimony pentachloride was the most suitable for distinguishing the three glucosides—syringin gives red, coniferin brown, and *p*-coumaryl alcohol glucoside greenish spots.

before spraying with the H_2SO_4/HCHO reagent; syringin then gives blue–purple spots, coniferin green–blue spots, and *p*-coumaryl alcohol glucoside light brown–red spots. The R_F values varied considerably with temperature, but the sequence is unaltered. At room temperature the R_F values were: syringin 0·24, coniferin 0·34 and *p*-coumaryl alcohol glucoside 0·42. The chromatograms were always run using samples of pure coniferin from spruce, pure syringin from lilac (*Syringa vulgaris*), and pure *p*-coumaryl alcohol glucoside (prepared synthetically)[13] as markers.

The fractions from the countercurrent separation containing the glucosides were combined and concentrated at 55° *in vacuo*. The coniferin fraction crystallized readily. Although crystals separated from the other glucoside concentrates on standing, the materials were impure and difficult to separate from the sirupy mother liquors. Hence, each of these fractions was purified by chromatography on columns of silicic acid mixed with 20% celite 35 × 2 cm, using acetone/ethyl acetate/water mixture (10 : 10 : 1 v.) as eluant.

The syringin obtained from the column was recrystallized from water containing a little sodium bicarbonate yielding white crystals (1·82 g, m.p. 191–2°) which did not depress the melting point of the pure syringin sample from lilac. Its rotation ($[\alpha]_D^{25} = -17{\cdot}1°$ (water, 0·7%)) was in accordance with the literature value. (Found: OCH_3, 16·74. Calc. for $C_{15}H_{17}O_2(OCH_3)_2$: 16·66%.)

The purified *p*-coumaryl alcohol glucoside proved more difficult to crystallize, hence a portion of the eluate from the column was evaporated to dryness, and acetylated at 50° with acetic anhydride and pyridine to give *p*-coumaryl alcohol glucoside pentacetate (*m.p.* 110–112°) from aqueous ethanol. When mixed with a synthetic sample[13] no depression of the melting point was observed. Its rotation was $[\alpha]_D^{25} = -14{\cdot}2°$ (Chloroform, 1·2%; synthetic material gives $[\alpha]_D^{20} = -15°$).

[13] K. FREUDENBERG and G. GEHRKE, *Chem. Ber.* **84**, 443 (1951).

On seeding with a trace of synthetic material, the *p*-coumaryl alcohol glucoside eventually crystallized (0·35 g). The solid was filtered off, and recrystallized from dilute aqueous sodium bicarbonate (m.p. 183°) and gave no depression of the melting point on admixture with a synthetic sample. (Found: C, 57·39; H, 6·41. Calc. for $C_{15}H_{20}O_7$: C, 57·68; H, 6·41%.)

Although the amount of syringin actually isolated was greater than that of *p*-coumaryl alcohol glucoside, this is due to the greater difficulty involved in separating the latter from coniferin, and to its greater solubility in water, and does not reflect the ratio of the sinapyl to coumaryl alcohol present either in the cambium or in lignin.