Phytochemistry, 1970, Vol. 9, pp. 1589 to 1594. Pergamon Press. Printed in England.

THE REACTION OF THIOGLYCOLIC ACID WITH POLYFLAVANOID BARK FRACTIONS OF *TSUGA HETEROPHYLLA*

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(*Received* 21 *October* 1969)

Abstract—Methylated western hemlock tannin (*Tsuga heterophylla*) was cleaved with thioglycolic acid to give approximately equal amounts of the methyl 2,3-*cis* and 2,3-*trans*-(3-hydroxy-3′,4′,5,7-tetramethoxyflavan-4-ylthio) acetates (I and VI) after esterification. These results indicate that the tannin is comprised internally of equivalent amounts of 2,3-*cis* and *trans* leucocyanidin units and further substantiates the presence of carbon–carbon linkages between them. Reaction of extractive free bark with this reagent also gave I and VI thus verifying existing evidence that the *in situ* bark phenolic acid fraction of extractive-free bark is similar in structure to the tannin.

INTRODUCTION

THE REACTION of thioglycolic acid with a polymeric leucoanthocyanidin was first utilized by Brown *et al.*[1] in an attempt to determine the mode of linkage in *Calluna vulgaris* tannin. Degradation of this tannin with subsequent permethylation gave the S-benzylthioglycolate (I), which led them to conclude that the condensed tannin was comprised of flavanoid units attached by C-4 benzylic ether linkages (II) since thioglycolic acid was known to cleave such bonds.[2] However, the isolation of numerous, dimeric proanthocyanidins[3,4] supports the suggestion that the most commonly occurring condensed tannins are comprised of flavan-3-ol nuclei in which the acid-labile carbon–carbon bond from the 4-position of the one is linked to the 6- or 8-position of the others (e.g. III).[3,5] Although thioglycolic acid had not been reported to cleave carbon–carbon bonds, we thought that such suitably activated carbon–carbon linkages should be readily capable of rupture with this reagent. This cleavage was demonstrated with the synthetic proanthocyanidins IV and V which resulted in the expected S-benzylthioglycolate VI[6] after esterification.

We report here the application of this technique as a structural tool in the degradation of tannin from western hemlock (*Tsuga heterophylla*), a condensed tannin believed to have structural units linked by carbon–carbon bonds[7] as represented in III. Additionally, we have

* Contribution No. 113 from ITT Rayonier Inc. Research Laboratories.

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[7] H. L. HERGERT, Abstracts of Papers, 155th ACS Mtg. 21D (1968).

used this reagent to degrade extractive free bark which contains the *in situ* bark phenolic-acid fraction. There is a substantial amount of evidence,[8-12] which will be briefly discussed, indicating that this fraction is similar in structure to the bark tannin, but is not removed by solvent extraction because of either polymeric size or a three-dimensional polymeric network. To remove this fraction it is necessary to employ dilute basic extraction (1 per cent NaOH at 100°) of bark previously treated with neutral solvents to remove wax, tannins, etc. Alkaline treatment alone is sufficient to alter the *in situ* polymer, to generate carboxyl groups,[12] and solubilize the bark phenolic-acid fraction which coexists with lignin and holocellulose in extractive-free bark.

RESULTS AND DISCUSSION

Reaction with Tannin

The degradation reaction of the tannin substrate was performed on methylated tannin; this circumvented separation problems resulting from the co-solubility of reaction products from thioglycolic acid and unreacted tannin in water. The cleavage reaction conditions were patterned from those given by Lindgren,[2] since the specific experimental details employed to degrade *Calluna vulgaris* tannin have not yet been published. The reaction conditions which were utilized to cleave proanthocyanidins[6] (108°, 30 per cent w/w, $HSCH_2CO_2H/H_2O$) were found to be too mild for cleavage of methylated tannin. Higher temperatures (135–145°) and concentrations of thioglycolic acid (56–63 per cent w/w, $HSCH_2CO_2H/H_2O$) were found to give the best results.

After degradation of methylated tannin with thioglycolic acid and subsequent permethylation of the degradation products with dimethyl sulfate and potassium carbonate in refluxing acetone, TLC analysis showed predominantly two cerise-colored spots of approximately equal intensity upon detection with corrosive acid. Isolation and purification of these two compounds was accomplished by column chromatography followed by multiple-elution preparative TLC. The material corresponding to the foremost spot was found to be identical (i.r., u.v., NMR) to the 2,3-*trans*-S-benzylthioglycolate (VI) which was synthesized by an independent route and characterized in our earlier study.[6] We were able to obtain this material in crystalline form. Analysis of the other compound (i.r., u.v., NMR, m.s.) shows it to be identical to the 2,3-*cis* compound (I) obtained as a degradation product from *C. vulgaris* tannin and characterized by Brown *et al.*[1] They have recently determined the relative stereochemistry of this product to be 3,4-*trans* and have also found that the reaction of flavan model compounds with thioglycolic acid gives predominantly the 3,4-*trans* compound irrespective of the stereochemistry of the starting material, thus constituting evidence for an S_n1 mechanism.[13] This suggests that the stereochemistry of VI is 3,4-*cis*, although this cannot be determined with certainty from its NMR spectrum.

Western hemlock tannin,[14] which had been methylated using either dimethyl sulfate in refluxing acetone/methanol solution (4/1, v/v) containing anhydrous potassium carbonate,

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(III)
(II)
(I)
(VI)
(V)
(IV)
(VII)
etc.
R =
R =

or dimethyl sulfate and sodium hydroxide in aqueous methanol solution, was found acceptable as a substrate for degradation. However, methylated tannin prepared by the latter technique appeared to give products in a purer state upon TLC analysis, since some lignin impurities were presumably removed. Precipitation of freshly methylated material with excess water on the basic side (pH 9) and subsequent redissolution in acetone resulted in a small portion of insoluble, lignin-like material. Additional lignin contaminants were presumably removed from this methylated tannin in acetone by addition of cyclohexylamine to form a small volume of precipitate.[7] This fraction was removed by filtration and the methylated tannin recovered by precipitation with a large excess of ether.

The identification of these two predominant thioglycolate products (I and VI) from the degradation of methylated hemlock tannin thus further substantiates the presence of carbon–carbon linkages between the flavanoid units of condensed hemlock tannin as advanced by Hergert;[7] his proposal is based on NMR analysis of the purified tannin polymer and upon isolation from the bark of a carbon–carbon linked dimeric proanthocyanidin. The internal leucoanthocyanidin units appear to be comprised of approximately equivalent amounts of 2,3-*cis* and *trans* leucocyanidin units (VII) since TLC showed approximately the same amount of I and VI. We have demonstrated that no equilibration takes place at C-2, C-3 in VI under the conditions of the degradation reaction with thioglycolic acid. The use of dilute mineral acid hydrolysis of western hemlock tannin by Hergert[7] gave a trace of catechin and epicatechin; therefore, the fact that the internal units are composed of units which are both *cis* and *trans* at C-2, C-3 is not unexpected. The relevant structural information obtained from dilute mineral acid hydrolysis is limited since only the terminal units of a polymeric chain are liberated.[7, 15] However, use of the thioglycolic acid offers the advantage of cleaving the internal units in the condensed methylated tannin, maintaining their structural integrity, and giving higher yields.

It has recently been demonstrated that certain appropriately substituted flavanoids can be selectively attacked with thioglycolic acid at either C-2 or C-4 depending on the pH of the reaction medium.[13] In our work with this reagent we have neither detected nor isolated any compounds that have been cleaved at C-2 (i.e. the pyran ring remains intact).

Thioglycolic acid degradation should also liberate the terminal end unit (e.g. phloroglucinol was isolated after degradation of IV[6]). Attempts to detect tetramethylcatechin by two-dimensional TLC of the neutral fraction after degradation of methylated tannin were unsuccessful. A positive identification was probably complicated by the large amounts of other material on the plate. It was found that tetramethylcatechin itself does not react under the experimental conditions used in the degradation; predominantly unreacted starting material was recovered.

Reaction with Extractive Free Bark

Sogo and Hata[8] subjected the ethylated bark lignins isolated by Bjorkman's method and the ethylated bark phenolic acids of several conifers and broad-leafed trees to permanganate oxidation. They found that bark lignins gave products identical to those from wood lignins, whereas bark phenolic acids gave a large amount of 3,4-diethoxybenzoic acid along with a small amount of the products obtained by oxidation of bark lignin. From these results Sogo and Hata concluded that the chemical properties of bark lignins are identical to those from wood lignins and that bark phenolic acids are copolymers of tannin-like substances and of a small amount of lignin. A report on the methanolysis of extractive-free Douglas fir bark

[15] V. C. QUESNEL, *Tetrahedron Letters* 3699 (1964).

fines, which contain 45–80 per cent phenolic acids, by Kurth *et al.*,[9] has indicated that the major products are anthocyanidins accompanied by products such as methyl protocatechuate, ferulic acid, protocatechuic acid and dihydroquercetin; the dihydroquercetin is believed to arise from hydrolysis of a dihydroquercetin unit in the phenolic acid *in situ* polymer. They concluded that bark phenolic acids are polymers of catechin and related flavanoids. Higuchi and co-workers[10] submitted bark phenolic acid and bark lignin fractions to alkaline nitrobenzene oxidation. Under these conditions the phenolic acid fraction gave rise to protocatechualdehyde, whereas bark lignin yielded none of this compound. Thus, they concluded that the formation of protocatechualdehyde in alkaline nitrobenzene oxidation of phenolic acids could be ascribed to tannin-like substances. Catechin has been detected in trace amounts as a product of very mild acidolysis of extractive-free hemlock, pine, spruce and amabilis fir bark by Hergert;[7] treatment with hot isopropanol–hydrochloric acid resulted in detection of cyanidin and delphinidin as major products[14] from extractive-free inner bark. These same products are obtained from coniferous tannins. Similar treatment of coniferous bark after alkaline extraction did not yield any cyanidin or cyanidin-like products.

It was believed that the successful degradation of *in situ* bark phenolic acids with thioglycolic acid would verify these conclusions. This reagent should react with any tannin-like structure by cleaving internal flavanoid units intact as their thioglycolic acid adducts rather than fragmenting the leucoanthocyanidin structure to smaller molecules such as occurs in the methods described above. Furthermore, this technique should lend support to the existence of carbon–carbon bond linkages between the internal units. Extractive-free bark was therefore prepared by initial exhaustive extraction with hot water to remove the tannins and other soluble material. The number of hot-water extractions (7) was more that adequate to insure removal; tannins are usually removed by a series of about four 1-hr extractions.[16] Subsequent exhaustive extraction with acetone:methanol (2:1, v/v) removed other extractives such as phlobaphenes and waxes.

Percolation of extractive-free bark with boiling aqueous thioglycolic acid, followed by permethylation of the filtrate and removal of liquid impurities by centrifuging and distillation (experimental), gave material which TLC analysis showed to contain predominantly I and VI. These compounds were isolated and purified by chromatographic techniques; comparative spectra showed them to be identical with the authentic materials. The amount of I did predominate fairly heavily over VI (3:1). However, the stereochemical and structural implications of these data on the phenolic acid fraction would be difficult to assess since the extractive-free bark was unmodified prior to degradation. Hence, equilibration could be expected to occur under the acidic conditions between the 2,3-*cis* and *trans* form.

A similar reaction of extractive-free wood with thioglycolic acid did not show any I or VI. Since it is well known that extractive-free wood does not contain the phenolic-acid fraction *in situ* accompanying lignin, these data clearly demonstrate that such compounds could arise only from the phenolic-acid fraction of bark. These results, which validate the conclusions of other workers,[7–12, 14] also constitute evidence for carbon–carbon bond linkages between the internal units as depicted in III.

In addition to reacting thioglycolic acid with methylated tannin and extractive-free bark, a reaction with the phlobaphene fraction of western hemlock bark was carried out under varying conditions. The S-benzylthioglycolates I and VI were not detected, thus further substantiating that this polymer is comprised of cyanidin rather than leucoanthocyanidin units.[14]

[16] H. L. HERGERT, *Forest Prod. J.* **10**, 610 (1960).

EXPERIMENTAL

Column chromatography used Mallinckrodt SilicAR CC-7 Silica gel and TLC silica gel G. The combustion analysis was performed by Dr. A. Bernhardt, Elbach über Engelskirchen, West Germany.

Diagnostic Microscale Degradation of Methylated Tannin

Methylated tannin (0·2 g), water (3 ml) and thioglycolic acid (4 ml) were vigorously refluxed (4 hr) in N_2 at 135°. After cooling, the reaction mixture was poured into water and the aqueous solution was extracted with EtOAc (× 2). This extract was slowly added to an equivalent volume of 10% $NaHCO_3$; solid $NaHCO_3$ was added until the aqueous layer was basic; the layers were separated. The aqueous layer was acidified with HCl, and extracted with ether which was dried and evaporated to give 0·758 g of red-colored liquid.

This material was refluxed (4 hr) with acetone (25 ml), Me_2SO_4 (1·2 ml) and anhydrous K_2CO_3. The solution was filtered, evaporated to a small volume and centrifuged (0·25 hr, 10,000 r.p.m.). After decanting off the water, the viscous oil was dissolved in EtOAc which was extracted once with water, dried, and evaporated to give products (19·8 mg). TLC in isopropyl ether/methanol (9:1); ($SOCl_2$, $SnCl_4$-development) showed two predominant cerise-colored spots. The material constituting these spots was isolated and characterized as described below.

Degradation (Macroscale) of Methylated Tannin

Methylated tannin (5 g), thioglycolic acid (60 ml) and water (45 ml) were vigorously refluxed (2 hr) under N_2. The cooled reaction mixture was poured into water (150 ml) and extracted with EtOAc (2 × 150 ml). The EtOAc extract was rinsed with water (2 × 150 ml) and extracted with $NaHCO_3$ in the manner described above. The bicarbonate solution was acidified with HCl and then extracted with ether (3 × 300 ml) which was washed with water, dried, and evaporated to give 6·79 g of material.

The bicarbonate extractables (8·71 g prepared in this manner), acetone (165 ml), Me_2SO_4 (12·2 ml), and anhydrous K_2CO_3 (26·8 g) were refluxed (4 hr). The solution was filtered and the acetone solution evaporated to a small volume and twice centrifuged (0·25 hr; 10,000 r.p.m.). The organic phase was taken up in EtOAc which was washed twice with water, dried, and evaporated to give a liquid (2·37 g).

Isolation of Methyl 2,3-Trans-(3-*Hydroxy*-3′,4′,5,7-*tetramethoxyflavan*-4-*ylthio*) *Acetate* (*VI*) *and Methyl* 2,3-Cis-(3-*Hydroxy*-3′,4′,5,7-*tetramethoxyflavan*-4-*yl thio*) *Acetate* (*I*)

Methylated bicarbonate extractables were vacuum distilled (25°, 0·7 min) to remove methylthioglycolate (e.g. after distillation of 10·5 g, 4·6 g remained). The material was then chromatographed on a column using isopropyl ether/methanol (15:1) to remove further impurities; the desired fractions were then further purified by multiple elution preparative TLC using isopropyl ether/methanol (15:1) and benzene–EtOH–H_2O–HOAc (200:47:15:1 upper phase) to give pure products.

2,3-*Trans*-S-Benzylthioglycolate (VI): i.r. ($CHCl_3$) 3440 (w) 1735 (s), 1620 (s), 1597 cm^{-1} (s); u.v. (EtOH) 277 mμ (ε 4000); NMR ($CDCl_3$) τ 3·00 (m, 3), 3·90 (q, 2), 5·10 (d, 1), 5·50–6·00 (m, 2), 6·10 (s, 3), 6·12 (s, 6), 6·25 (s, 3), 6·27 (s, 3), 6·39 (s, 2). Crystallization from hexane–CH_2Cl_2 gave colorless needles, m.p. 122·5–123·5°. (Found: C, 58·65; H, 5·81; S, 7·11. $C_{22}H_{26}O_6S$ required: C, 58·48; H, 5·77; S, 7·00.)

2,3-*Cis*-Benzylthioglycolate (I): i.r. ($CHCl_3$) 3590 (w), 1735 (s), 1595 (s) cm^{-1}; u.v. (EtOH) 276 mμ; NMR ($CDCl_3$) τ 2·91 (m, 3), 3·80 (q, 2), 4·50 (m, 1), 5·72 (m, 2), 6·03 (s, 3), 6·08 (s, 3), 6·12 (s, 3), 6·22 (s, 3) 6·20 (s, 3), 6·50 (s, 3). Calc. mol. wt. found by m.s.: 450·1344; required: 450·1348. Base peak is at 345 (loss of $SCH_2CO_2CH_3$).

TLC of I and VI on Baker-flex, Silica Gel 1B precoated plates using isopropyl ether/methanol (9:1) (dev. with $SnCl_4$, $SOCl_2$ vapors) gave spots with R_f 0·39 and 0·43, respectively; R_fs in the second solvent were 0·66 and 0·73 respectively.

Degradation of Extractive-Free Bark

Extractive-free bark (1·50 g, 1·38 g o.d.), water (18 ml) and thioglycolic acid (18 ml) were vigorously refluxed (4 hr) under N_2 at 130°. The solution was filtered; the flask was rinsed several times with water and then dried (1·03 g). The filtrate was evaporated to give 22·7 g of product. This material was refluxed (4 hr) with acetone (350 ml), Me_2SO_4 (45 ml) and anhydrous K_2CO_3 (90 g). The filtered solution was evaporated and diluted with water. Extraction with EtOAc yielded a red-colored, rancid-smelling liquid (4·31 g). Combined material from several reactions processed in this way were centrifuged twice with water, taken up in EtOAc which was dried and evaporated. The solution was distilled (25°, 0·7 mm), to remove methylthioglycolate (residual material, 3·63 g). TLC of the remaining material showed two intense cerise-colored spots identical with those observed for I and VI above.

Column chromatography and preparative TLC gave two compounds chromatographically and spectrally identical to I and VI.

Acknowledgements—We thank Dr. H. L. Hergert for helpful discussions and for supplying us with tannin and a methylated tannin sample.