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Paper Chromatography of the Tropolones of *Cupressaceae*

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A chromatographic procedure has been developed for separation and identification of the known tropolonic constituents of *Cupressaceae* heartwood extracts and the heartwoods of eight species of the above family have been analyzed.

In our investigation of the tropolonic fraction[1] obtained from incense-cedar heartwood, *Libocedrus decurrens* Torrey, the only compound that could be isolated in sufficient quantity for characterization purposes was γ-thujaplicin.[2] All attempts to preparatively isolate the other isomeric thujaplicins, if present, namely α- and β-thujaplicin in sufficient amount for characterization, failed. Thus, it became apparent that it would be desirable to develop a chromatography procedure capable of detecting traces of thujaplicins. Such a procedure was developed and was based on the chromatographic separation of the azo dyes obtained by coupling the thujaplicins with diazotized *p*-aminobenzoic acid.[2] When the method was applied to the mixture obtained from the crude copper chelates isolated from incense-cedar heartwood extract, β-thujaplicin was detected, together with γ-thujaplicin.

While this method proved quite satisfactory, it was not applicable to nootkatin,[3] another tropolonic constituent found in *Cupressaceae*, since this compound does not couple readily with diazotized *p*-aminobenzoic acid. This prompted us to develop a chromatography procedure which would detect all of the known naturally occurring tropolones found in *Cupressaceae*,[4,5] namely the three isomeric thujaplicins together with nootkatin. Such a method would be of value in reference to taxonomic studies of the family cited.

(1) Zavarin and Anderson, *J. Org. Chem.*, **20**, 82 (1955).
(2) Zavarin and Anderson, in press.
(3) Carlsson, Erdtman, Frank, and Harvey, *Acta Chem. Scand.*, **6**, 690 (1952).
(4) Erdtman, *Progress in Organic Chemistry*, ed. by J. W. Cook, Vol. I, Academic Press, Inc., New York, N. Y. (1952), pp. 40–53.
(5) Pauson, *Chem. Revs.*, **55**, 22–25 (1955).

The difficulties encountered in the chromatographic separation of the *Cupressaceae* tropolones, in addition to the similarity in their solubility characteristics, stem from the rather unfavorable distribution coefficient between water and organic solvents, *i.e.*, R_f values being approximately 1, and from their rather pronounced streaking tendency. A method with the novel feature of using phosphoric acid solution as the immobile phase in the presence of sodium sulfate as desiccant and with isoöctane-toluene mixtures as developers seemed to overcome the aforementioned difficulties. A 5% ferric chloride solution was used as the chromogenic spray. Table I summarizes the results of several runs using papers of different phosphoric acid contents and several developing solvents. The column under X refers to an unidentified enolic compound present in the copper complex isolated from the incense-cedar and western red cedar (*Thuja plicata*) heartwood extracts which were chromatographed along with the individual *Cupressaceae* tropolones. This unknown compound is being investigated.

As the data indicate, the effect of increasing the phosphoric acid content of the paper results in a decrease in R_f values. It may be assumed that the basic nature of tropolones is at least partly responsible for this behavior. The effect of the addition of toluene to the isoöctane at a given phosphoric acid content of the paper is, as expected, in direction of increasing the R_f values of the various tropolones. In spite of the clearly defined spots, the use of paper impregnated with 8.5% phosphoric acid does not produce satisfactory results because of the comparable R_f values of both α-thujaplicin and nootkatin spots.

From the many runs performed, it would seem

TABLE I

R_f VALUES OF THE TROPOLONES

Mobile Phase	Immobile Phase ($\%H_3PO_4$)	α-Thujaplicin	Nootkatin	X[a]	β-Thujaplicin	γ-Thujaplicin
Isoöctane	8.5	0.85	0.84	0.48	0.42	0.27
Isoöctane	12.8	.78	.84	.28	.22	.13
Isoöctane	17.0	.70	.79	.23	.15	.10
Isoöctane-toluene (3:1)	8.5	.88	.90	.60	.51	.40
Isoöctane-toluene (3:1)	12.8	.87	.92	.45	.38	.26
Isoöctane-toluene (3:1)	17.0	.86	.92	.40	.31	.23
Isoöctane-toluene (3:1)	21.2	.80	.89	.29	.21	.15
Toluene	17.0	.94	.97	.69	.66	.57

[a] Unknown enolic compound present in incense-cedar and western red cedar extracts.

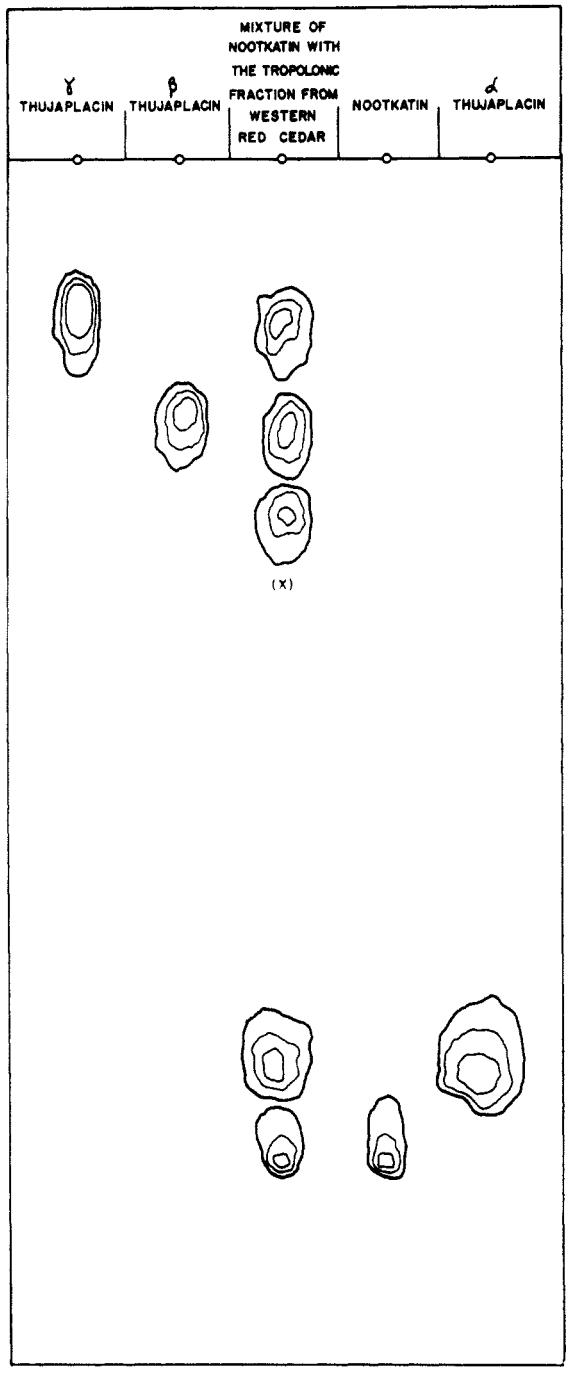

FIG. 1. A TYPICAL CHROMATOGRAM OF *Cupressaceae* TROPOLONES. Thinner lines denote the approximate intensity distribution of the various spots. The spot marked with (X) corresponds to the unknown enolic constituent present in western red cedar and incense-cedar tropolonic fractions.

that the most promising combination consists of the use of 17% phosphoric acid impregnated paper in conjunction with the isoöctane-toluene (3:1) as developer. The time for the chromatograms to develop varied somewhat with the solvent and impregnated paper employed. In general, isoöctane required 2.5 to 3.0 hours and isoöctane-toluene (3:1) about 2.0 to 2.5 hours. The color of the spots, although dependent upon the amount of chromogenic spray used and concentration of the tropolone, can also be utilized for identification purposes. The thujaplicin spots are either green or brown with a greenish border, the unidentified enolic compound is either violet or brown with a violet border, while the nootkatin spot is light brown. In general, as little as 4–1 $\times$ 10^{-6} g. of the tropolone could be detected by this procedure. Figure 1 represents a typical chromatogram, using 17% phosphoric acid impregnated paper and isoöctane-toluene (3:1) as the developing solvent.

The heartwoods of eight species of the Cupressaceae family available to us were tested for tropolonic compounds using the presently developed chromatographic method. The results are summarized in Table II. It appears that this is the first report on the analysis for tropolones in three of the species, namely Port Orford Cedar (*Ch. lawsoniana*), Atlantic white cedar (*Ch. thyoides*), and western juniper (*Juniperus occidentalis*). Interestingly enough, while Port Orford cedar and western juniper gave copper chelates, no tropolones identifiable by our method appeared to be present in the wood samples analyzed. On the other hand, Atlantic white cedar was found to contain the three isomeric thujaplicins. Carlsson, *et al.*, reported upon the isolation of nootkatin from Alaska cedar (*Ch. nootkatinsis.*)[3] When we submitted the tropolone fraction of the same species to chromatographic analysis, no tropolones, other than nootkatin could be detected. Corbett and Wright have isolated nootkatin from Monterey cypress (*Cupressus macrocarpa*).[6] In addition to nootkatin, we found that β-thujaplicin was also present in the heartwood of the above species. No copper complex could be isolated from the extract of eastern red cedar (*Juniperus virginiana*) and the extract upon being chromatographed indicated the absence of tropolones. We had previously reported upon the presence of β- and γ-thujaplicin in incense-cedar heartwood extract using another chromatographic procedure.[2] While the three isomeric thujaplicins have been isolated from western red cedar heartwoods, the American tree was reported to contain β- and γ-thujaplicin[7,8,9,10] while the Swedish grown tree yielded α- and γ-thujaplicin, but no β-thujaplicin.[11] When the present chromatographic procedure was applied to the tropolone mixture isolated from the American grown western red cedar heartwood,

(6) Corbett and Wright, *Chemistry & Industry*, 1258 (1953).

(7) Anderson and Gripenberg, *Acta Chem. Scand.*, **2**, 644 (1948).

(8) Anderson and Sherrard, *J. Am. Chem. Soc.*, **55**, 3813 (1933).

(9) Erdtman and Gripenberg, *Acta Chem. Scand.*, **2**, 625 (1948).

(10) Barton and Gardner, *Pulp & Paper Mag. Can.*, **55**, No. 10, 132–137 (1954).

(11) Gripenberg, *Acta Chem. Scand.*, **2**, 639 (1948).

TABLE II

CHROMATOGRAPHY OF *Cupressaceae* TROPOLONES

Species	Copper Acetate Test	Thujaplicins			Nootkatin
		α-	β-	γ-	
Port Orford cedar (*Chamaecyparis lawsoniana*)	+	−	−	−	−
Alaska cedar (*Chamaecyparis nootkatensis*)	+	−	−	−	+
Atlantic white cedar (*Chamaecyparis thyoides*)	+	+[a]	+	+	−
Monterey cypress (*Cupressus macrocarpa*)	+	−	+	−	+
Western Juniper (*Juniperus occidentalis*)	+	−	−	−	−
Eastern red cedar (*Juniperus virginiana*)	−	−	−	−	−
Incense-cedar (*Libocedrus decurrens*)	+	−	+	+	−
Western red cedar (*Thuja plicata*)	+	+	+	+	−

[a] This compound could only be detected by diazotization chromatographic procedure because of the presence of apparently mere traces of it in the sample analyzed.[2] The latter method is generally more sensitive in the case of α- and β-thujaplicins.

each of the three isomeric thujaplicins was found to be present.[12]

EXPERIMENTAL

PAPER CHROMATOGRAPHY OF TROPOLONES

Paper preparation. The appropriate amount of the 85% phosphoric acid was diluted with distilled water to make-up a solution of the desired strength and transferred to a 2-liter beaker. A 46 × 57 cm. sheet of Whatman No. 1 paper was cut into 3 to 4 strips 57 cm. long. The strips were submerged in about 150 ml. of the acid solution to thoroughly saturate the paper. The wet paper strips then were removed and the suspended strips were allowed to air-dry. If some solution accumulated on the lower edge of the paper strips during the drying period, it was removed by touching it slightly with a piece of dry filter paper. After the moisture content of the strips came to equilibrium, the strips were stored in a desiccator over anhydrous sodium sulfate until just prior to use. The paper strips, so prepared, are rather stable. In experiments performed with paper impregnated with 17% phosphoric acid and sodium sulfate as desiccant, it was found that there was no marked difference in R_f values and reproducibility when using freshly prepared paper strips or those that were stored over a three weeks period.

Development of chromatograms. About 300 g. of anhydrous sodium sulfate was placed in a tray on the bottom of the chromatographic chamber, followed by a beaker containing the developer to be used, and the whole was allowed to stand overnight. Prior to development using the descending procedure, the impregnated paper strips were suspended in the chamber overnight to permit the system to come to equilibrium. Authentic samples of each of the three thujaplicins, *i.e.*, α-, β-, and γ- together with nootkatin prepared from Alaska cedar heartwood (*Chamaecyparis nootkatinsis*)[3] (1 mg. of each) was dissolved in 1 ml. of acetone. Each of these solutions was used as a reference sample for chromatographic purposes. An appropriate amount of each of the reference materials together with unknowns then was brought to the paper and the trays were filled with dry solvent. The developer was permitted to migrate about 40 cm. from the point of reference sample application, the strips were removed and the position of the solvent front was marked. The strips then were air-dried and sprayed with 5% aqueous ferric chloride solution until the first distinctive spot appearance. When our interest was in the spots of relatively high R_f values, it was advantageous to reduce the migration distance at the solvent front to about 20 cm. The spots tend to fade, thus it is imperative that their respective positions be marked immediately after spraying. The yellow color of the ferric chloride does not interfere because of the formation on the paper of the colorless ferric phosphate complexes.

Immobile phase experiments. The paper strips impregnated with 0.0, 0.85, 4.25, 8.5, 12.8, 17.0, and 21.2% phosphoric acid solutions were used in conjunction with isoöctane and isoöctane-toluene (3:1) as the developing solvents. In all cases where the phosphoric acid solutions were less than 8.5%, heavy streaking prevented the separation of the various components. With 21.2% phosphoric acid impregnated paper and isoöctane as the solvent, the progress of the solvent front was very slow and satisfactory separations were not obtained. In the remaining runs rather clearly defined spots were attained. These results are summarized in Table I.

Mobile phase experiments. The effect of toluene addition to the isoöctane on the R_f values of the various tropolones using paper with various phosphoric acid content was determined and the results are tabulated in Table I. The addition of carbon tetrachloride in place of toluene produced a similar effect to toluene addition. When *n*-butanol was substituted for toluene or carbon tetrachloride heavy streaking resulted.

Experiments with desiccants. When calcium chloride was used in place of anhydrous sodium sulfate, a marked decrease in R_f values was observed. Furthermore, streaking was so extensive that very little separation took place. The thujaplicin spots extended over an area of 0.00 to 0.10 R_f and nootkatin gave a streak from R_f 0.12 to 0.25. Use of papers impregnated with 8.5% phosphoric acid in conjunction with isoöctane as the developer and calcium chloride or potassium carbonate as desiccants resulted in similar behavior.

Chromatography employing paper impregnated with 8.5% phosphoric acid and with isoöctane as developer but with the atmosphere saturated with water, in place of dry conditions, resulted in a very uneven solvent front advancement and unsatisfactory separation. The R_f values of all spots were shifted toward R_f 1.

Effect of temperature. The temperature throughout most of the experiments was kept at 21° ± 0.5°. To determine the effect of the temperature increase on the chromatographic behavior of the various tropolones, experiments were performed using paper impregnated with 17% phosphoric acid, isoöctane and isoöctane-toluene (3:1) as developers and sodium sulfate as desiccant at a temperature of 29.5° ± 0.5°. Practically no change in R_f values was observed. Nootkatin, however, showed a marked tendency to streak when isoöctane was used as the developer. This tendency was not present with isoöctane-toluene (3:1).

(12) Note: Professor H. Erdtman indicated in a private communication that he also found that both the American and Swedish grown western red cedar varieties contained the three isomeric thujaplicins.

IDENTIFICATION OF TROPOLONES IN *cupressaceae* HEARTWOODS

Isolation of tropolone fraction. The aforementioned tropolone chromatography procedure was applied to the extractive components which were isolated from the heartwood of 8 species of *Cupressaceae* available to us. For this purpose, 450 to 500 g. of the heartwood sawdust was extracted with acetone for 8 hours in a Soxhlet extractor. The acetone extract was evaporated to dryness and the residue was taken up in hot chloroform. The decanted chloroform mixture was evaporated to 10 ml. and 9 volumes of petroleum ether were added. Filtration removed tarry materials that separated at this point. The filtrate was evaporated to dryness and the residue again was dissolved in chloroform. The chloroform extract was shaken with a 5% solution of aqueous copper acetate, the organic phase was evaporated to dryness, and the residue was extracted with isoöctane which removed a considerable amount of contaminants from the insoluble copper chelates. The copper complex was redissolved in chloroform and hydrogen sulfide was led through the solution until the green color had disappeared. The resulting tropolone-containing mixture was evaporated to dryness on a steam-bath and taken up in acetone or alcohol at a ratio of approximately 10 mg. to 1 ml. of solvent.

In some cases the expected green-colored chloroform solution of the copper complex was not noticeable after the copper acetate treatment. In such instances, the chloroform solution was evaporated to 5 ml. and this solution was diluted with 5 to 10 volumes of methanol. Filtration gave a green filtrate, which was subsequently processed as indicated above.

Chromatography of Cupressaceae tropolones. The solutions of tropolones so prepared from heartwood of eight species of *Cupressaceae* were chromatographed according to the aforementioned procedure, and the results are summarized in Table II. The α-thujaplicin present in Atlantic white cedar was present in such small amounts that its presence could not be detected by this chromatographic procedure. However, when using the diazotization procedure which is particularly sensitive in cases of α- and β-thujaplicins, the presence of α-thujaplicin was readily ascertained.[2] In order to be assured that small amounts of thujaplicins did not escape detection in other cases, each of the eight tropolone mixtures was also run by the diazotization method. No additional thujaplicins could be detected.

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