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The Chemistry of the Natural Order Cupressales 39 *

Heartwood Constituents of *Cupressus torulosa* Don.

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The heartwood of *Cupressus torulosa*, Don contains carvacrol, carvacrol methyl ether, α-thujaplicin, β-thujaplicin, β-thujaplicinol, nootkatin, ferruginol, hinokiol, hinokione, manool and the new diterpenes torulosal and torulosol. Paper and gas chromatographic evidence was obtained for the presence of cuparene, humulene and thujopsene and paper chromatographic and spectroscopic evidence for the presence of β-dolabrin.

Cupressus torulosa, the Bhutan or Nepal cypress, belongs to the comparatively large genus *Cupressus*, which is being subjected to a systematic chemotaxonomic study [1,2] in this laboratory. This species grows wild in the outer ranges of the western Himalayas and in western Szechwan, China, but the material used in this investigation was obtained from a tree grown in Switzerland and was kindly made available to us by Professor A. U. Däniker, Zürich. The first preliminary investigation of this material was carried out by one of us (H.B.) in 1958. Ahluwalia and Seshadri [3] have already shown that the heartwood contains nootkatin.

Cupressus torulosa heartwood was extracted with acetone and the light petroleum-soluble part of this extract was separated into acidic and neutral fractions. The neutral oil was chromatographed on alumina giving nine main fractions.

The first fraction was very small and its infrared spectrum and the results of gas-liquid and liquid-liquid partition chromatography showed that it was a mixture of at least nine hydrocarbons. The main component of this fraction was identified as humulene [4] and two other components as thujopsene [5] and cuparene [6]. The liquid-liquid partition chromatography was carried out with a silver fluoborate-hexadecane system [7].

The very small second fraction was partly crystalline. The crystalline material, according to its infrared spectrum, was a mixture of straight chain hydro-

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carbons; the non-crystalline part was mainly carvacrol methyl ether, which was identified by conversion to carvacroxyacetic acid.

Fraction three, by far the largest fraction, was practically pure manool [8].

Fraction four also contained manool and was shown by paper chromatography to be a mixture of ferruginol and manool.

Fraction five was a mixture of ferruginol and a second component. It was separated on a light petroleum-impregnated polyvinyl chloride column, using aqueous methanol as the mobile phase [7], into ferruginol and its main component, a new hydroxyaldehyde which was named torulosal [9]. Ferruginol was identified by conversion to the crystalline acetate. The ultra-violet spectrum and the optical rotation of the acetate showed that, within the limits of error, it was free from the Δ^6-dehydrocompound (steroid numbering) which apparently [10] often accompanies ferruginol.

Fractions six and seven, in addition to ferruginol, contained a new diol which was named torulosol. The structures of torulosol and torulosal have been discussed in a preceding paper [9].

Fraction eight was largely crystalline and contained torulosol as the main component. On fractional crystallisation it gave hinokione and a mixture of hinokione and hinokiol, which was separated by preparative paper chromatography on dimethyl sulphoxide-impregnated paper. The structures of these two substances have recently been discussed by Chow and Erdtman [11].

Fraction nine, the end fraction, was subjected to a quick distillation at low pressure. The distillate contained, according to paper chromatography, the same components as the previous fraction.

The acidic fraction from *Cupressus torulosa* contained nootkatin as the main component. The remaining material was separated into eight fractions by preparative chromatography on paper impregnated with dimethyl sulphoxide and ethylenediaminetetraacetic acid [12]. The compounds obtained on extraction of the strips were purified by sublimation and, where possible, by crystallisation. It was possible to identify carvacrol (after conversion to carvacroxyacetic acid), hinokiol, hinokione, β-thujaplicinol [13], nootkatin, α-thujaplicin and β-thujaplicin by mixed melting points and infra-red spectra. One of the zones, in addition to hinokione, contained a more volatile material, which, according to paper chromatographic results obtained by two methods and in agreement with the spectroscopic data, contained β-dolabrin [14] as the main component.

Though wide variations are possible in biological material approximate estimates of the amount of each compound are given in the annexed table as percentages of air-dry heartwood.

Total acetone extract 5.7 %

Light petroleum-soluble acetone extract 3.1 % (acidic 0.3 %, neutral 2.8 %)

Carvacrol	0.003 %	Cuparene	0.002 %	Ferruginol	0.2 %
Carvacrol methyl ether	0.007 %	Humulene	0.02 %	Hinokiol	0.03 %
β-Dolabrin	0.0004 %	Nootkatin	0.2 %	Hinokione	0.09 %
α-Thujaplicin	0.008 %	Thujopsene	0.003 %	Manool	1.4 %
β-Thujaplicin	0.004 %			Torulosal	0.4 %
β-Thujaplicinol	0.004 %			Torulosol	0.4 %

Simonsen [15] in 1923 investigated the leaf oil of *Cupressus torulosa* and found that this, unlike the heartwood we investigated here, was very rich in monoterpenoids.

A comparison of the different types of compounds isolated in the present investigation with those previously isolated from other conifers is of some interest. Tropolones are present in many conifers of the *Cupressaceae* and, in a recent paper-chromatographic survey [16] on their occurrence, they have been used to illuminate certain taxonomic relationships in this family. The sesquiterpenes and the tricarbocyclic diterpenes isolated during this investigation occur in several genera of the *Cupressaceae* and some of the diterpenes have also been found in a number of genera of the *Podocarpaceae;* however the chemotaxonomic value of these compounds is at present less clear. The dicarbocyclic diterpenes seem to offer an interesting chemotaxonomic peculiarity in that they have so far only been found [1] within the genus *Cupressus* of the family Cupressaceae, but whether this difference will survive more careful examination of other genera remains to be seen. General relationships among these conifers have been fully discussed by Erdtman [17] in the light of recent results.

EXPERIMENTAL

Melting points were taken on a Kofler micro hot stage, unless otherwise stated. Infrared spectra were recorded on a Perkin-Elmer No. 21 instrument (NaCl prism). Ultra-violet spectra were measured in ethanol on a Beckman recording spectrophotometer, model DK-2. Light petroleum refers to a fraction b.p. 35—60°, unless stated to the contrary. Micro-analyses by Dr. A. Bernhardt, Mülheim.

Extraction. Finely milled air-dry heartwood of *Cupressus torulosa* (5.7 kg) was extracted with acetone for 24 h. The extract was filtered, the acetone was evaporated and the residue was poured with vigorous stirring into a tenfold volume of light petroleum. The precipitate obtained was filtered off, dissolved in a small volume of acetone and the solution was poured into a fivefold volume of light petroleum and filtered. The precipitation was repeated. The combined light petroleum solutions were concentrated to a small volume, poured with vigorous stirring into a tenfold volume of light petroleum and filtered. The light petroleum insoluble precipitates (160 g) were not further investigated. The clear light-petroleum solution was concentrated to 1 litre, extracted repeatedly with aqueous potassium hydroxide (10 %) until it gave no colour reaction with bis-diazotized benzidine and then washed with water. The alkaline solutions were combined, washed with light petroleum, acidified with dilute sulphuric acid (10 %) and extracted with light petroleum. The combined neutral solutions, after drying (Na_2SO_4) and evaporation of the solvent, gave a yellow-brown oil (160 g, 2.2 % of the wood). The acidic solution, on drying (Na_2SO_4) and evaporation of the solvent, gave a solid residue (16 g, 0.5 % of the wood).

The neutral oil. Part (77 g) of the neutral oil, diluted with light petroleum (100 ml), was adsorbed on a column of alumina (2.2 kg, Merck, activity II). It was eluted with solvents of increasing polarity and collected in 1 litre fractions. These were combined according to the results of paper chromatography on hexadecane-impregnated (10 % hexadecane in light petroleum, b.p. 60—71°) glass fibre paper (Schleicher and Schüll, 0.17—0.23 mm) using methanol-water (7:3—9:1) as mobile phase and dipping the dried paper (100°, 2—3 min) in antimony pentachloride-chloroform (1:9) solution for detection of the spots [7]. The results are summarised in Table 1.

Fraction 1, according to its infra-red absorption and the results of gas-liquid and liquid-liquid partition chromatography, was a mixture of at least 9 different hydrocarbons. The gas-liquid chromatographic examination was made with a Pye instrument (one metre column; 2,4-dinitrophenyl-2-naphthyl ether (20 %) on Celite [18]; column temp. 150°; flow rate 25.5 ml/min of argon; charge 0.025 μl). Nine peaks were found, with the following elution times and areas (as percentages of the total area): A 4.2 min (2 %), B 8.6 (1 %), C 15.0 (2 %), D 17.6 (9 %), E 19.9 (3 %), F 25.2 (50 %), G 26.2 (25 %), H 30.4

Table 1.

Fraction No.	Weight of fraction in g	Volume of fraction in l	Eluent
1	0.9	1	Light petroleum
2	0.2	1	» »
3	37.0	14	Benzene and then ether-light petroleum (1:99—1:9)
4	3.0	1	Ether-light petroleum (1:1)
5	16.8	4	Ether-light petroleum (1:1—1:0)
6	1.4	2	Ether
7	1.7	1	Ether-ethanol (99:1)
8	13.2	2	Ether-ethanol (97:3)
9	2.3	6	Ether-ethanol (95:5—0:1)

(3 %), I 43.1 (5 %). The elution times of the peaks D, F and I are identical with those found for thujopsene, humulene and cuparene, respectively, under identical conditions. Paper chromatography on hexadecane-impregnated glass fibre paper (Schleicher and Schüll, 0.17—0.23 mm) with a solution of silver fluoborate (30 % w/v) in aqueous methanol (1:9) as mobile phase gave spots with R_F-values 0.04, 0.08, 0.17, 0.24, 0.42, 0.54 and 0.80 after drying and treatment with an antimony pentachloride-chloroform (1:9) solution. R_F-values 0.08, 0.17, and 0.80 are the same as those found for cuparene, thujopsene and humulene under the conditions mentioned. A further indication of the presence of cuparene is found in the infrared spectrum of the fraction, which showed a band at 1 518 cm^{-1}.

Fraction 2 was partly crystalline. The solid material was filtered off and washed with a small amount of acetone giving a white crystalline material (0.02 g) which after repeated recrystallisation from acetone had m.p. 58—61°, $[\alpha]_D$ 0° ($CHCl_3$, *c* 0.7). The infra-red spectrum of this substance was extremely simple ν_{max}^{KBr} 2 935 (s), 2 865 (s), 1 477 (m), 1 462 (m), 1 386 (w), 731 (w), 716 (m), indicating a mixture of saturated straight chain hydrocarbons. The non-crystalline part, according to its infrared spectrum, was largely carvacrol methyl ether. Demethylation with hydrogen bromide (30 %, 1.5 h) followed by etherfication with ethyl chloracetate and saponification of the ester gave carvacroxyacetic acid, m.p. and, mixed m.p. 151—152°.

Fraction 3, according to its infra-red spectrum, was manool which started to crystallise immediately on seeding. Recrystallisation from light petroleum gave pure manool, m.p. and mixed m.p. 52—53°, $[\alpha]_D$ + 33° ($CHCl_3$, *c* 2.0), with an infrared spectrum identical with that of authentic material.

Fraction 4, on recrystallisation from light petroleum, gave manool (1.1 g), which was identified as above. The mother liquors, according to paper chromatographic results, contained a mixture of manool and ferruginol in about a 3:1 ratio.

Fraction 5 was a mixture of two components. These were separated on an impregnated polyvinyl chloride (PVC) column [7]. The column was prepared as follows: PVC-powder (Wackerchemie, Germany, purified, 350 g) was washed with aqueous ethanol (2 l, 2:1) and ethanol (2 l), sucked dry on a Büchner funnel and finally air-dried at room temperature. The dry PVC-powder was suspended in aqueous methanol (2.4 l, 3:7), saturated with light petroleum (b.p. 60—71°) and then further light petroleum (245 ml, b.p. 60—71°) was added with stirring. The suspension was added, in portions, to a tube (5 × 100 cm) partly filled with the same solvent mixture. After the first addition the bottom tap was opened and the PVC-powder packed with a stopper as it settled from the flowing suspension. As the flow became slower with increasing length of the column it was speeded up by applying pressure. The last few centimetres of the column of adsorbent (5 × 50 cm) were packed by placing a cottonwool disc followed by a porcelain filter disc on the top of the column and applying pressure with the stopper. The material to be chromatographed was extracted with 5 portions (200 ml each) of aqueous methanol (1:3), which were added successively to the top of the column. The fractions (150 ml) obtained on elution with

aqueous methanol (3 l of 1:3 and thereafter 3:17) saturated with light petroleum were combined according to paper chromatographic results into two fractions. These were diluted with water (twice the amount of the eluate) and extracted with ether. The ether extracts were dried (Na_2SO_4) and the solvent was evaporated leaving torulosal (11 *g*, fract. 2—9) and ferruginol (2.9 g, fract. 16—25, identified by I.R.).

Torulosal, n_D^{25} 1.521, $[a]_D$ + 29° ($CHCl_3$, *c* 1.8) was paper chromatographically pure. It was characterised as the carbon tetrachloride "inclusion compound", m.p. 80—85° (Pyrex tube), $[a]_D$ + 24° ($CHCl_3$, *c* 2.0), and the semicarbazone, m.p. 195—197° (decomp.), $[a]_D$ −9° (pyridine, *c* 1.0) and by conversion into torulosol [9], m.p. and mixed m.p. 110—111°, $[a]_D$ + 31°.

A part (0.50 g) of the ferruginol was dissolved in pyridine (6 ml) and acetic anhydride (2 ml) and kept for two days at room temperature and then for one hour on the water bath. The product was diluted with water, extracted with ether and after extraction with aq. NaOH (10 %) the ether solution was taken to dryness. Recrystallisation from ethanol gave pure ferruginol acetate (0.39 g), m.p. and mixed m.p. 81—83°, $[a]_D$ + 59° (EtOH, *c* 2.0), λ_{max}^{EtOH} 268 mμ (ε 1 240), 278 mμ (ε 1 330). The infra-red spectrum was identical with that of authentic material.

Fraction 6 was shown by paper chromatography to be a mixture of about equal amounts of ferruginol and torulosol.

Fraction 7 on repeated recrystallisation from *iso*propyl ether gave pure torulosol (0.7 g), m.p. 110—111°, $[a]_D$ + 31° ($CHCl_3$, *c* 2.0). The combined mother liquors, according to paper chromatographic results, were a mixture of ferruginol and torulosol in about a 3:1 ratio.

Fraction 8 was partly crystalline and on repeated recrystallisation from *iso*propyl ether gave pure torulosol (8.7 g). Concentration of the mother liquors gave two fractions with melting points higher than that of torulosol. One fraction, (m.p. 110- 170°, 2.5 g), on repeated recrystallisation from methanol, gave pure hinokione, m.p. and mixed m.p. 186—188° (slight decomp. from just above 180°), $[a]_D$ + 113° ($CHCl_3$, *c* 2.0). The infrared spectrum was identical with that of authentic material.

A part (0.090 g) of the second fraction (m.p. 186—202°, 0.13 g) was separated by chromatography on two sheets of paper (24 × 56 cm, Whatman 3MM) impregnated with dimethyl sulphoxide using *iso*propyl ether as the mobile phase. The two zones were detected by spraying strips with bisdiazotised benzidine and had R_F-values corresponding to those of hinokiol and hinokione. The slow-moving zone was cut out and extracted with ether. The ether solution, after washing with water, drying (Na_2SO_4) and removal of the solvent, gave a crystalline residue (42 mg), which was sublimed along a temperature gradient under reduced pressure (0.2 mm). This crystalline sublimate (10 mg) was resublimed twice and then showed a m.p. of 232—235° which was raised to 236—239° on admixture with hinokiol (m.p. 236—239°). The infrared spectra of the two compounds were superimposable.

A part (0.3 g) of *fraction 9* was distilled under reduced pressure (0.5 mm) and the distillate (0.2 g) was shown by paper chromatography to contain the same components as fraction 8.

The acidic fraction. A part (11 g) of the acidic fraction was recrystallised from light petroleum to give pure nootkatin (6 g), m.p. and mixed m.p. 95—96°, infrared spectrum identical with that of authentic material. The mother liquors were combined and the solvent evaporated giving a residue (5 g), a part (1.5 g) of which was separated by preparative paper chromatography. A concentrated solution in light petroleum (b.p. 60—71°, 6 ml) was applied to ten sheets of paper (Whatman 3MM, 24 × 56 cm), which had previously been impregnated with ethylenediaminetetraacetic acid and dimethyl sulphoxide [12]. The chromatograms were run in light petroleum (b.p. 60—71°) and the different zones (8), detected by ultraviolet light, were cut out and extracted with ether. These ether solutions were shaken with water and dried (Na_2SO_4). The residues, obtained on removal of the ether, were purified by sublimation or distillation along a temperature gradient and where possible by crystallisation. The results obtained are given in Table 2. The compounds identified (carvacrol after conversion to carvacroxyacetic acid) all showed undepressed mixed melting points and correct infrared spectra.

Table 2.

Fraction	R_F-value	Weight of extracted material in mg	Compound isolated
1	0.69	66	unidentified
2	0.66	500	nootkatin
3	0.55	100	α-thujaplicin
4	0.37	12	unidentified
5	0.29	46	β-thujaplicin
6	0.22	88	hinokione + β-dolabrin
7	0.14	88	β-thujaplicinol + carvacrol
8	0.02	360	hinokiol

Fraction 1, on sublimation and repeated crystallisation from methanol, gave a small amount (3 mg) of a compound, m.p. 60—63° which, according to infrared and ultraviolet spectra, seemed to be an oxoalcohol. Fraction 4 on distillation gave an easily volatile oil which according to its infrared and U.V. spectra was an α,β-unsaturated acid. Fraction 6 on sublimation gave, together with hinokione, a small amount (5 mg) of a much more volatile oil. This, according to further paper chromatographic results, using the method of Zavarin and Anderson [19], and according to its infrared and ultraviolet absorption was largely β-dolabrin [16,14,13].

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