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The Chemistry of the Natural Order Cupressales

XXV *. Heartwood Constituents of *Juniperus chinensis L.*

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From the heartwood of *Juniperus chinensis* there were isolated: thujopsene, cuparene, cedrol, widdrol, hinokiic acid, "widdringtonia acid II", carvacrol, thymohydroquinone, 3-hydroxy-thymoquinone and 3,6-dihydroxy-thymoquinone. Paper chromatographic evidence was obtained for the presence of nootkatin, α-, and β-thujaplicin and gas chromatographic evidence for the presence of α-cedrene.

Most of the genera into which the family Cupressaceae has been divided botanically are small or monotypic. The largest genus, *Juniperus*, which is found almost only in the northern hemisphere is divided into two subgenera, *Oxycedrus* and *Sabina*, with altogether some 50—60 species. It thus appears to be particularly suitable for systematic chemical investigation.

Only a few juniper woods have been investigated before and interest has been mainly directed towards the constituents of the berries, leaves and bark [1,2].

Bredenberg and Gripenberg [3–5] made the interesting discovery that the diterpene sugiol and related compounds are present in *Juniperus communis* and Nakatsuka and Hirose [6] have found tropolones (nootkatin and β-thujaplicin) in *J. chinensis*. Cedrol and "cedrene" have frequently been found in juniper woods. However, they appear to be absent from those species of the subgenus *Oxycedrus* that have been investigated and Bredenberg has suggested that cedrol and cedrene might be characteristic of the *Sabina* junipers.

Thirty years ago Ushida [7] published a note on some constituents of the wood of *J. chinensis (Sabina)* and recorded an interesting "cedrol" of abnormally high rotation $[\alpha]_D + 85.4°$. Three years ago the sesquiterpene alcohol widdrol [8] (m.p. 98°, $[\alpha]_D + 105°$) from *Widdringtonia* was isolated in this institute and one of us (C.P.), working with Professor H. Erdtman, made a preliminary study of the wood of *J. chinensis* with the intention of comparing Ushida's "cedrol" with widdrol. Cedrol of normal optical properties was

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easily obtained but some fractions had a higher rotation (up to about + 60°) and may have been similar to the preparation obtained by Ushida.

Other constituents isolated during this study included thymohydroquinone, a constituent of *Tetraclinis articulata* [9] and *Libocedrus decurrens* [10] that had not previously been found among the junipers, and a crystalline acid. Ultraviolet spectra of the ligroin-soluble part of the acid fraction indicated the presence of tropolones. A more detailed study has now been made (J.R.) of the extract from a larger sample of *Juniperus chinensis* heartwood.

J. chinensis is a native of China and Japan but the wood used in these investigations came from a tree grown in the Glasnevin National Botanic Garden, Dublin. Our thanks are due to Mr. T. Walsh, keeper, for kindly putting the wood at our disposal. It should be noted that the wood came from a tree that was probably killed by a fungal infection. It is therefore possible that some of the substances found, *e.g.* the hydroxythymoquinones (see below), do not occur in sound wood. An interesting case of changed metabolism due to infection has been described by Hasegawa and Shirato [11].

The air-dried wood was extracted with acetone and the light petroleum-soluble part of the extract was separated into neutral and acid fractions with alkali.

The tropolones were concentrated by precipitation from the ligroin soluble part of the acid fraction with cupric acetate, decomposition of the crude copper salts with hydrochloric acid and steam distillation. The fraction most volatile in steam gave 3-hydroxy-thymoquinone. According to paper chromatographic analysis the next fraction contained a mixture of nootkatin and α- and β-thujaplicins but in such small amounts that a separation was not attempted (Nakatsuka and Hirose obtained 6 g nootkatin and 0.3 g β-thujaplicin from 147 kg of wood).

Fractional distillation of the tropolone-free acid portion of the oil yielded thymohydroquinone, 3,6-dihydroxy-thymoquinone and carvacrol. The high-boiling fractions gave two sesquiterpene acids. One was identified as hinokiic acid ("widdrenic acid" [12]), recently isolated from a number of *Widdringtonia* species and also prepared by oxidation of thujopsene ("widdrene"). The other was found to be identical with the "Acid II", m.p. 192—194°, $[\alpha]_D$ −94°, from the same source [8].

The neutral portion of the oil was fractionally distilled. It contained only traces of monoterpenes or compounds of similar boiling point. The lowest boiling fraction was yellow and it was therefore expected that thymoquinone would be present but this could not be confirmed.

To check the possibility that the 3- and 3,6-hydroxylated thymoquinones might have been formed from thymoquinone during isolation, a solution of thymoquinone in light petroleum was shaken with either aqueous or ethanolic alkali in the presence of air. The product was chromatographed on paper by the method developed for tropolones by Wachtmeister and Wickberg [13]; this indicated that both the hydroxylated thymoquinones were present. Even though attempts to isolate thymoquinone from the wood failed, it is thus possible that the two hydroxythymoquinones are artefacts formed during the alkali extraction of the oil or as a result of *post mortem* enzymatic oxidation in the wood.

Since the presence of carvacrol methyl ether in the neutral oil was to be expected, a sample of the lowest boiling fraction was demethylated and chromatographed on paper. No carvacrol was detected but spots corresponding to two unidentified phenols were found.

The infrared spectrum of the rather large sesquiterpene fraction that followed resembled those of corresponding fractions isolated from *Widdringtonia* [8]. According to gas chromatographic analysis, fraction 2 (Table 1, see experimental part) contained four components. The substances were present approximately in the ratio 300:90:10:1, assuming complete resolution. The principal constituent was identified as thujopsene by conversion into the corresponding aldehyde by oxidation with selenium dioxide. The next largest constituent, according to gas chromatographic evidence, was α-cedrene. Fraction 3 (Table 1) contained the two main components in a ratio of 1:4, but the minor constituents could not be detected in this fraction.

The next higher boiling fraction was redistilled and treated with ozone at low temperature. From the material unaffected by ozone cuparene [14] was isolated.

According to the infrared spectrum the highest boiling fraction of the neutral material appeared to contain alcohols and carbonyl compounds.

The compounds isolated and a very approximate estimate of the amounts present are given below. The figures are subject to the uncertainties of the estimation and to the wide variations possible in biological material. The amounts are given as a percentage of the air-dried wood. Total acetone extract 8.37, ether-soluble acetone extract 7.48, light petroleum-soluble acetone extract 5.92, sodium bicarbonate-soluble 0.058, sodium hydroxide-soluble 0.83, soluble in ethanolic potassium hydroxide 0.43, neutral 4.6. Tropolones (nootkatin, α- and β-thujaplicin) 0.007, thymohydroquinone 0.018, 3-hydroxythymoquinone 0.0009, 3,6-dihydroxy-thymoquinone 0.0003, carvacrol 0.11, hinokiic acid 0.001, "Widdringtonia acid II" 0.061, thujopsene 0.27, cuparene 0.10, cedrol 1.7, widdrol 0.14, high-boiling carbonyl compounds 0.043.

EXPERIMENTAL

Rotations were measured in chloroform. Melting points, taken on a hot stage, are uncorrected. Light petroleum refers to the fraction b. p. 40—60°.

Isolation of the oils. Ground, air-dried heartwood (6.0 kg) was continuously extracted with acetone for 48 h. The acetone was evaporated and the remaining oil was poured with stirring into ten volumes of ether. The ether solution was filtered (precipitate 54 g), evaporated, and the rest similarly poured into ten volumes of light petroleum. After filtration (precipitate 94 g) the solution was evaporated to about three l and extracted successively with a saturated solution of sodium bicarbonate, with 2 N sodium hydroxide and with 2 N ethanolic potassium hydroxide. On acidification and extraction with ether the sodium bicarbonate solution yielded a viscous oil (3.5 g) which was not further investigated. The sodium hydroxide-soluble material (50.6 g) (A) was isolated in the same way by acidification of the alkaline solution and extraction with ether. The ethanolic potassium hydroxide solution was shaken several times with light petroleum, acidified and extracted with ether. The ether extract was washed with water and gave on evaporation a reddish brown oil (39.4 g) (B). The remaining petroleum ether solution was washed with water and evaporated yielding neutral material (260.8 g) (C).

I. *Alkali-soluble fraction.* The viscous product A (19.8 g) was dissolved in acetone, poured onto cotton wool and the acetone was evaporated. This material was then

extracted with hot ligroin (b. p. 100—125°) and the extract was shaken with a saturated solution of cupric acetate; the sticky copper salts were filtered off, washed with ligroin and water and decomposed with 2 N hydrochloric acid. The product was extracted with ether and the ether solution was washed with water, dried, and evaporated affording a resin (6.8 g) (A_1) which was steam distilled.

The first 100 ml of distillate deposited a small amount (20 mg) of orange red needles which gave a strong violet colour with sodium hydroxide. This substance was recrystallised from ethanol-water and sublimed, m. p. 168—170°, undepressed when mixed with an authentic sample of *3-hydroxy-thymoquinone* prepared from 2,4-dinitrothymol *via* 2,4-diaminothymol [15]. The infrared spectra of the two samples were identical.

The next 200 ml of steam distillate was extracted with ether. The ether extract on evaporation gave a syrupy material (314 mg) which according to paper chromatographic [13] evidence contained nootkatin and *α*- and *β*-thujaplicins in amounts decreasing in this order.

The ligroin solution remaining after the removal of the copper salts was evaporated and the residue was separated by distillation into lower boiling (b. p. below 180°/2 mm, 5.7 g) (A_2) and higher boiling (b. p. 180—184°/2 mm, 4.8 g) (A_3) fractions.

The material soluble in ethanolic potassium hydroxide (B) was subjected to a quick preliminary distillation. The fraction boiling below 160°/1.5 mm (B_1) was added to fraction A_2 and the combined material (27 g) was distilled through a spinning band column. On standing the fraction boiling at 117—152°/20 mm (2.4 g) deposited a small amount (6 mg) of bright red needles, which gave a strong violet colour with sodium hydroxide. This compound was recrystallised from 95 % ethanol and sublimed, m. p. 223—224°, undepressed when mixed with synthetic *3,6-dihydroxy-thymoquinone* prepared from thymoquinone *via* 3,6-bismethylamino-thymoquinone [16].

The fraction boiling at 117—152°/20 mm was found to contain mainly *carvacrol*. After 3,6-dihydroxy-thymoquinone had been removed by filtration a sample was converted into carvacroxyacetic acid, m. p. 150.0—151.5°, undepressed by an authentic sample.

The fraction b. p. 152—165°/20 mm crystallised on standing. This was a mixture of cedrol and widdrol (see below) indicating that the separation of neutral material from weak phenols had been unsatisfactory. (It has later been found that extraction with ethanolic potassium hydroxide should be avoided.)

The fraction b. p. 166°/20 mm (3.0 g) was dissolved in ether and extracted with 2 N sodium hydroxide. Acidification of the alkaline solution furnished a product (330 mg), which was recrystallised from ether-light petroleum and sublimed, m. p. 145.0—145.5°, undepressed by an authentic sample of *thymohydroquinone*.

A part of product A (22.4 g) was fractionally distilled in a vacuum. The fraction boiling between 145—175°/0.8 mm (8.1 g) crystallised partly on standing. It was dissolved in ether and the solution was extracted with 2 N sodium carbonate. On acidification the alkaline solution afforded a substance (1.4 g) which was recrystallised from ether, m. p. 192—194°, $[\alpha]_D$ −94° (*c*, 1.3). It was identified (mixed m. p., I.R.) as *Acid II* from *Widdringtonia* [8].

Table 1. Distillation of fraction C_1. Total distillate 107 g or 79 %.

Fraction	Weight (g)	B.p./21 mm (°C)	Rotation $[\alpha]_D$	Refractive index (n_D^{21})
1	5.3	124—136	−48.7	1.4990
2	14.5	136—137	−70.5	1.5025
3	7.5	137—141	−70.8	1.5041
4	18.1	141—165	+ 8.8	1.5088
5	27.1	165—166	+13.9	—
6	28.1	166	+14.7	—
7	6.6	166	+66.7	—

In another experiment a similar fraction of the sodium hydroxide soluble oil on treatment with light petroleum partly crystallised. Recrystallisation from methanol-water afforded thymohydroquinone. The methanol-water mother liquor was evaporated and the residue combined with the material soluble in light petroleum. On standing for several months the combined oils deposited another compound (0.2 g), which was recrystallised from methanol-water, m. p. 169.5—170.5°, $[\alpha]_D$ −86° (*c*, 1.3). It was identified as *hinokiic acid* [12] (mixed m. p., I.R.).

II. *Neutral fraction:* A quick preliminary distillation of the neutral oil C afforded a product boiling below 160°/3 mm (C_1) and a higher boiling residue (C_2). C_1 on fractional distillation through a vacuum-jacketed, packed column, gave the fractions listed in Table 1.

Attempts to demonstrate the presence of thymoquinone in fraction 1 (Table 1): Fraction 1 (0.5 g) was suspended in water (20 ml) saturated with sulphur dioxide and allowed to stand for a week. The solution was extracted with ether and the ether extract was washed with water and evaporated. The residue, like the starting material, was yellow and afforded no acidic material on extraction of a solution in light petroleum with 2 N potassium hydroxide.

No phenolic material was obtained on reduction with zinc and acetic acid.

Demethylation of Fraction 1 (Table 1): Fraction 1 (1.5 g) in glacial acetic acid (6 ml) was refluxed with hydrobromic acid (48 %, 8 ml) for 1 h. The reaction mixture was extracted with light petroleum, the organic phase was extracted with 2 N ethanolic potassium hydroxide and the alkaline extract was acidified, extracted with light petroleum, washed with water dried and evaporated affording an oil (0.48 g). This was examined by paper chromatography [13]. No spot corresponding to carvacrol was found but two spots of higher R_F value were observed.

Gas chromatographic examination of the sesquiterpene fraction: A Perkin-Elmer Vapor Fractometer Model 154 was used together with a Speedomax Type G (Leeds & Northrup Co.) recorder. (Column length 2 m, internal column diameter 5 mm, stationary phase 60—80 mesh celite, impregnated with 2,4-dinitrophenyl-2-naphthyl ether (m. p. 95°C) in the proportion of 25 % by weight [17], charge 1 μl, temperature 145°, flow of helium 64 ml per minute.)

The following peaks were obtained (Recorder deflection (mV) given as a function of time (min)). Fraction 2 (Table 1): 1.1 (15.4), 4.2 (16.9). Fraction 3 (Table 1): 0.4 (11.9), 1.6 (15.4), 4.7 (16.9). Pure α-cedrene, prepared from cedrol [18] gave a peak at 15.4 min and pure thujopsene [12] gave a peak at 16.9 min.

Thujopsene: Fraction 2 (Table 1) (1.0 g) was dissolved in 95 % ethanol (20 ml) and selenium dioxide (0.6 g) was added. The solution was refluxed for 4 h, filtered and evaporated to dryness. The product on sublimation yielded a substance (160 mg) which was recrystallised from light petroleum, m. p. 74.5—75.5°. It was identified as widdrenal (mixed m. p., I.R.).

The infrared spectrum of fraction 4 (Table 1) showed medium height aromatic absorption at 1 527 cm^{-1}. This fraction (17.3 g) was redistilled through a spinning band column yielding the fractions listed in Table 2.

Table 2. Redistillation of fraction 4 (Table 1.) (17.3 g.) Total distillate 14.8 g, 86 %.

Fraction	Weight (g)	B.p./20 mm (°C)	Rotation $[\alpha]_D$	Refractive index (n_D^{21})
1	1.06	141—146	−59.8	1.5033
2	1.04	146	−60.8	1.5040
3	2.18	146—149	−26.7	1.5062
4	3.14	149—151	+18.7	1.5104
5	1.23	151	+67.5	1.5127
6	1.81	151—155	+62.0	1.5133
7	0.77	155	+27.1	1.5121
8	1.00	155—168	+13.6	1.5077
9	2.58	168	+11.4	—

Cuparene. Fractions 5 and 6 (Table 2) were combined and a sample (2.2 g) in methylene chloride (15 ml) was ozonised at −70°C until an excess of ozone was indicated by the appearance of a blue colour. The reaction mixture was then treated with hydrogen peroxide (30 %, 4 ml) in potassium hydroxide (10 %, 1.5 ml) added dropwise at room temperature. Water (30 ml) was added, the organic phase was separated and the water phase extracted with methylene chloride. The methylene chloride solution was evaporated and the residue was chromatographed on basic aluminium oxide (50 g). Light petroleum (100 ml) eluted a colourless oil (760 mg) which was fractionally distilled. The main fraction b.p. 117°/7 mm, $[\alpha]_D$ +65°, n_D^{21} 1.5230, was identified as cuparene [14] by comparison of the infrared spectrum with that of an authentic sample.

Cedrol: Fraction 6 (Table 1) was recrystallised four times from 95 % ethanol, m. p. 88.0−88.5°, $[\alpha]_D$ +10.4° (*c*, 3.2). The compound was identified as cedrol (mixed m. p., I.R.).

Widdrol: The mother liquor from the first recrystallisation of cedrol was concentrated and two further crops of crystalline material collected. The non-crystalline material (2.5 g) was chromatographed on basic aluminium oxide (200 g). Benzene eluted an oily material which was not investigated; benzene-ether (1:1) eluted mainly cedrol and ether eluted a substance, $[\alpha]_D$ +95° (*c*, 2.5), which was recrystallised from light petroleum and sublimed twice along a temperature gradient, m. p. 98.0−99.0°, $[\alpha]_D$ +105° (*c*, 1.0) identical with widdrol [8] (mixed m. p., I.R.).

The high-boiling neutral fraction: The residue from the distillation described in Table 1 was combined with the distillation residue C_2. A sample of the combined material (C_3) was fractionally distilled through a spinning band column. 65 % of the oil distilled between 143°/10 mm and 183°/5 mm. A main fraction (43 %) was obtained at 160−165°/10 mm ($[\alpha]_D$ +1.1°). According to infrared spectra all the fractions seemed to be rather complex mixtures showing strong hydroxyl and carbonyl bands.

On separation using Girard D reagent the oil C_3 gave 12 % of a mixture of carbonyl compounds. This material showed no hydroxyl absorption and on chromatography gave only liquid fractions.

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