Unlike II in methanol, IV in *n*-butyl alcohol did not undergo alcoholysis under acid catalysis in the temperature range 25–60°.

Equilibrium Measurements. 1.—A reaction solution 0.080 *M* in I, 0.100 *M* in II, and 0.001 *M* in trifluoroacetic acid in dry 1,2-dimethoxyethane was prepared from stock solutions. Aliquots of 3 ml. of this solution were sealed in 5-ml. glass ampoules and the ampoules were thermostated at 40°. From time to time, ampoules were removed from the bath and the contents were quantitatively analyzed for methanol by g.l.p.c.[23] From the equilibrium plot of the data, the equilibrium constant was calculated at 45.1.

2.—Using the same technique, a reaction solution 0.085 *M* in III, 0.095 *M* in methanol, and 0.001 *M* in trifluoroacetic acid in dry 1,2-dimethoxyethane gave an equilibrium constant of 0.0198.

3.—In a similar experiment, a reaction solution 0.205 *M* in III, 0.125 *M* in methanol, and 0.001 *M* in trifluoroacetic acid in dry 1,2-dimethoxyethane gave an equilibrium constant of 0.0219.

Reactions at Low Conversions and Different Reactant Concentrations.—Reaction solutions of I, II, and trifluoroacetic acid were prepared in dry 1,2-dimethoxyethane. The solutions were thermostated at 25° and from time to time samples were withdrawn and analyzed for methanol.[23] The experiments were carried out with the following reactant concentrations at a constant concentration of 0.001 *M* trifluoroacetic acid. The results are plotted in Figure 1.

Expt.	Concn. of I, *M*	Concn. of II, *M*
1	0.0803	0.0802
2	0.1606	0.0802
3	0.0803	0.1604

Acknowledgment.—The authors wish to express their appreciation to Professor Nathan Kornblum of Purdue University for many stimulating discussions during the course of this work.

(23) G.l.p.c. analyses were carried out on an Aerograph A-600 Hy Fy using a 5 ft. × 1/8 in. column packed with G. E. Silicone SF-96 on 60–80 mesh Chromosorb W (regular). The injector and oven temperature were maintained at the reaction temperature. Methanol concentrations were computed from an area *vs.* concentration calibration curve at constant sample volume.

Constituents of Hiba Wood Oil. The Isolation and Synthesis of Two Isomeric Cuprenenes[1]

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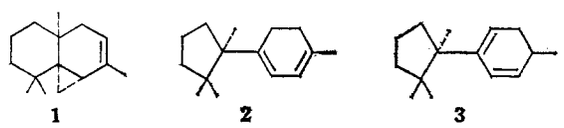
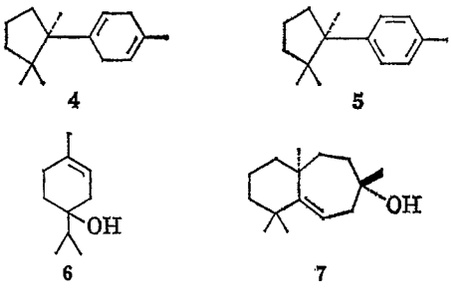
In 1960, Nozoe and Takeshita[3] reported that the sesquiterpene thujopsene (**1**) was the major constituent of the essential oil of the Japanese Hiba tree (*Thujopsis dolabrata*). In addition, they found that a late fraction in the sesquiterpene distillation range was a mixture of other sesquiterpenes. From chemical and spectral studies of this fraction it was suggested that, among other things, the two isomeric cuprenenes **2** and **3** were present, but the materials were never isolated in pure form. This oil has been re-examined and two isomeric cuprenenes (**2** and **4**) as well as cuparene (**5**), terpinen-4-ol (**6**), and widdrol (**7**) have been isolated in pure form.



1 **2** **3**



4 **5** **6** **7**

Hiba wood oil[4] was chromatographed on neutral alumina. The petroleum ether eluate (~90%) consisted mainly of hydrocarbons (plus a small amount of alcohols) and the methanol eluate (~5%) consisted of alcohols. The remainder of the oil (~5%) remained on the column and presumably contained acids; this material was not investigated.

A v.p.c. analysis of the total hydrocarbon fraction (DEGS column, 130°) indicated the presence of approximately 60% thujopsene (**1**), 7% of cuprenene **2**, 3% of cuprenene **4**, 2.5% of cuparene (**5**), and 1.5% of monoterpenes [including terpinen-4-ol (**6**)]. The remainder of the fraction consisted of a large number of minor constituents. In order to isolate the five major components, the hydrocarbon fraction was fractionated by distillation using a spinning-band column followed by preparative v.p.c. The details of the complicated fractionation are given in the Experimental Section.

Cuparene could not be obtained completely free of olefinic impurities by the above separation scheme, and so the mixture was subjected to oxidation with performic acid and the oxidation products were separated from the unchanged cuparene by alumina chromatography. The infrared spectrum of the purified material was identical with the published spectrum of cuparene.[5]

The gross carbon skeleton of the two cuprenenes **2** and **4** was established by their conversion to cuparene upon prolonged standing in air. The presence of a conjugated diene in cuprenene **2** was indicated by its ultraviolet spectrum [λ_{max}^{EtOH} 271 mμ (ε 6300)]. The n.m.r. spectrum showed absorption for two vinyl hydrogens (τ 4.49), a methyl group on an olefinic double bond (τ 8.29), and three methyl groups on saturated centers (τ 9.01 and 9.20). These spectral data permit the specific placement of the diene system as shown in **2**. Cuprenene **4** was isomeric with cuprenene **2**, but the two double bonds were not conjugated. Again the placement of the unsaturated centers was possible on the basis of the n.m.r. spectrum which showed the presence of two vinyl hydrogens (τ 4.56 and 4.68), four diallylic hydrogens (τ 7.40), one methyl group on an olefinic linkage (τ 8.37), and three methyl groups on saturated centers (τ 8.98, 9.00, and 9.20).

These structures of the compounds were proved by synthesis from cuparene. Reduction of the aromatic

(1) This work was supported in part by Grant GP-3890, National Science Foundation.

(2) Roche Anniversary Foundation Postdoctoral Fellow, 1963–1965.

(3) T. Nozoe and H. Takeshita, *Tetrahedron Letters*, **No. 23**, 14 (1960).

(4) Kindly supplied by Fritzsche Brothers, New York, N. Y., and the Saisei Camphor Co., Ltd., Japan.

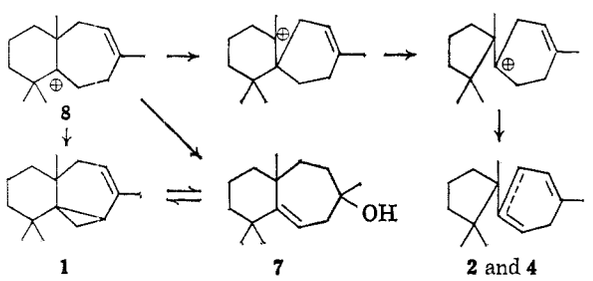
(5) C. Enzell and H. Erdtman, *Tetrahedron*, **4**, 361 (1958).

precursor **5** with lithium in ammonia under the forcing conditions of Johnson[6] (the compound resisted reduction under the normal Birch conditions) gave cuprenene **4** as the sole product. It is interesting to note that the location of the double bonds arrived at on the basis of spectral evidence is, indeed, that expected from such a reduction.[7] This nonconjugated diene was equilibrated in the presence of potassium *t*-butoxide in dimethyl sulfoxide–benzene to form a 9:1 mixture of cuprenene **2** and **4**, respectively.

The compound cuprenene **3** suggested by the earlier workers, if indeed present in the essential oil, must be a very minor constituent. On the basis of the ultraviolet spectrum of the fraction studied by them, less than 35% of the conjugated cuprenene **2** was present.

The v.p.c. analysis of the neutral alcohol fraction indicated the presence of at least ten components, four of which were in major amounts. No clean separation of the materials could be achieved except for one fraction which crystallized, and this material was shown to be widdrol (**7**). The concentration of this alcohol in Hiba wood oil is estimated to be less than 1%. Either this material or cuparene could be shown not to be an artifact arising out of the isolation procedure since these compounds are known to be formed from thujopsene and cuprenene, respectively.

Previously, Enzell and Erdtman[8] have suggested that the cuprenenes might have a biogenetic relationship to γ-bisabolene. The presence of the cuprenenes along with thujopsene calls attention to an alternate biogenetic scheme which allows for all materials to arise from a common precursor **8**. This intermediate could arise from farnesol by any one of a variety of



pathways. It is to be noted that this same precursor could give rise to both thujopsene and widdrol. Biosynthetic studies can evaluate this latter suggestion since the distribution of labels should be different if the two materials arise from a common precursor than if one is the precursor of the other, since it has previously been shown[8] that in the interconversion of thujopsene and widdrol a skeletal rearrangement occurs.

Experimental Section[9]

Fractionation of Hiba Wood Oil.—A 750-g. portion of Hiba wood oil[4] was dissolved in 750 ml. of petroleum ether (b.p. 30–60°) and the solution was passed through a column containing 750 g. of neutral Woelm alumina (activity I). The column was first eluted with a total of 3 l. of petroleum ether and then with 3 l. of methanol. Both fractions were concentrated to give the "hydrocarbon fraction," yield 683 g. (91% of Hiba wood oil), and the "alcohol fraction," yield 37.5 g. (5% of Hiba wood oil), respectively. The material (presumably acidic) which remained on the column was not investigated.

Fractionation of the Hydrocarbon Fraction.—The hydrocarbon fraction was distilled through a 2-ft. spinning-band column at a pressure of 8 mm. The fractions described in Table I were collected.

TABLE I

Fraction	Weight, g.	B.p., °C.	Remarks
1	10.6	55–90	Monoterpenes, including terpinen-4-ol
2	25.0	90–123	Intermediate fraction, with sesquiterpenes (mainly thujopsene) building up
3	361.0	124–128	Impure thujopsene
4	76.3	129–140	Intermediate fraction, with **2**, **4**, and **5** building up
5	42.4	140–141	Mixture of (mainly) **2**, **4**, and **5**.
6	43.0	141–145	Intermediate fraction, contains some alcohols
7			Distillation stopped since cracking began.

Isolation of Cuparene (5).—Portions (250 μl.) of fraction 5 were injected into a 10 ft. × 3/8 in. column containing a packing of 30% Cyanowax on Chromosorb W (60–80 mesh) kept at 148°, and a flow rate of 150 cc./min. of helium was used. The last peak of the chromatogram was collected and this peak was purified by passing it once again through the same column. A total of 33 mg. was collected and this material was added to 0.8 ml. of formic acid and 0.1 ml. of 30% hydrogen peroxide in 0.9 ml. of tetrahydrofuran. The mixture was allowed to stand at room temperature for 24 hr. and then diluted with ether. The ethereal layer was separated, washed with sodium carbonate solution and water, and dried over anhydrous magnesium sulfate. The solvent was removed, the residue was dissolved in petroleum ether, and the solution was passed through a short column of neutral Woelm alumina (activity I). The solvent was removed, and the 25 mg. of remaining colorless oil possessed spectral features which were identical with those published for cuparene.[5]

Isolation and Identification of Cuprenene 4.—Portions (150 μl.) of fraction 5 were injected into a 20 ft. × 3/8 in. column containing a packing of 20% neopentyl glycol succinate on Chromosorb P (HMDS, 60–80 mesh) kept at 168°, and a flow rate of 125 cc./min. of helium was used. The next to last peak was collected. The process was repeated several times, the collected product was dissolved in petroleum ether, and the solution was filtered through a short column containing alumina (activity I) in order to remove traces of neopentyl glycol succinate. The solvent was evaporated, and the 80 mg. of residue was cuprenene **4** of greater than 95% purity, $\nu_{max}^{CCl_4}$ 780 cm.$^{-1}$, $\epsilon_{218}^{Pentane}$ 1000 (no maximum at long wave length), and $[\alpha]D$ +50 ± 2° (*c* 1.1, $CHCl_3$).

Anal. Calcd. for $C_{15}H_{24}$: C, 88.16; H, 11.84. Found: C, 88.23; H, 12.06.

The n.m.r. spectrum had bands at τ 4.56 (1H, vinyl, multiplet), 4.68 (1H, vinyl, multiplet), 7.40 (4H diallylic, singlet), 8.37 (3H, vinyl methyl, singlet), 8.98 (3H, methyl, singlet), 9.00 (3H, methyl, singlet), and 9.20 (3H, methyl, singlet). The mass spectrum, if run immediately after the insertion of the sample, indicated a molecular weight of 204. Rerunning the mass spectrum after a few minutes showed a spectrum identical with that of authentic cuparene, mol. wt. 202, obviously obtained by pyrolytic aromatization of cuprenene **4**.

On prolonged contact with air or when oxygen was bubbled through the oil for a few hours, cuprenene **4** was converted to cuparene.

Isolation and Identification of Cuprenene 2.—A total of 0.5 ml. of fraction 5 was injected into a 10 ft. × 3/8 in. column containing a packing of 30% Cyanosilicon on Chromosorb P (HMDS,

(6) W. S. Johnson, B. Bannister, and R. Pappo, *J. Am. Chem. Soc.*, **78**, 6312 (1956).
(7) A. J. Birch, *J. Chem. Soc.*, 430 (1944).
(8) W. G. Dauben and L. E. Friedrich, *Tetrahedron Letters*, 2675 (1964).
(9) Analyses were by the Microanalytical Laboratory, College of Chemistry, University of California, Berkeley, Calif. The n.m.r. spectra were taken in carbon tetrachloride on a Varian A-60 spectrometer, using tetramethylsilane as an internal standard. The mass spectra were taken with a Consolidated 21-103-C spectrometer.

60–80 mesh) kept at a 150°, and a flow rate of 150 cc./min. of helium was used. The first (and largest) peak was collected. The material was reinjected in 10-μl. fractions into a 5 ft. × 0.25 in. column containing 20% Cyanowax on Chromosorb P (60–80 mesh) at 150°, and a flow rate of 80 cc./min. of helium was used. The center portion of the peak was collected and a total of 25 mg. of 90% pure cuprenene **2** was obtained: $\nu_{max}^{CCl_4}$ 822 cm.$^{-1}$; λ_{max}^{EtOH} 271 mμ (ε 6350); n.m.r., τ 4.49 (2H, vinyl, broad singlet), 8.29 (3H, allylic methyl, singlet), 9.01 (6H, 2 methyl, singlet), and 9.20 (3H, methyl, singlet).

Anal. Calcd. for $C_{15}H_{24}$: C, 88.16; H, 11.84. Found: C, 88.29; H, 11.71.

On prolonged contact with air or when oxygen was bubbled through the oil for 3 hr., cuparene was formed.

Synthesis of Cuprenene 4 from Cuparene (5).—Following the general procedure of Johnson,[6] a solution of 338 mg. of cuparene in 65 ml. of absolute ethanol was added cautiously to 75 ml. of liquid ammonia. Lithium metal (5 g.) was added in portions with vigorous stirring along with 50 ml. of ethanol and 75 ml. of ammonia; the reaction mixture contained a bronze-colored phase. The addition required about 20 min. The stirring was continued until all the lithium had dissolved, and then the ammonia was allowed to evaporate. The solid residue was diluted with water and the mixture was extracted with ether. The ethereal layer was washed with water, dried over magnesium sulfate, and percolated through a short column containing 5 g. of Woelm alumina. The solvent was evaporated, and the 347 mg. of remaining colorless oil possessed infrared, ultraviolet, and n.m.r. spectra identical with cuprenene **4** isolated from Hiba wood oil. On standing in air the product was converted back to cuparene.

Conversion of Cuprenene 4 to Cuprenene 2.—A mixture of 43 mg. of cuprenene **4**, 321 mg. of potassium *t*-butoxide, 5 ml. of dimethyl sulfoxide, and 5 ml. of benzene was heated under a nitrogen atmosphere at 67° for 3 hr. The reaction mixture was cooled to room temperature, diluted with water, and extracted with petroleum ether. The organic phase was washed with water and dried over magnesium sulfate. The solvent was evaporated to yield a yellow oil containing a 9:1 mixture of cuprenenes **2** and **4**, respectively, and sulfur compounds. To obtain pure cuprenene **2**, the oil was injected in 10-μl. portions into a 5 ft. × 0.25 in. column containing 20% DEGS on Chromosorb W, and the peak corresponding to cuprenene **2** was collected. The material was dissolved in a small volume of pentane, and the solution was filtered through a short column of neutral Woelm alumina. The ether was evaporated, and 31 mg. of cuprenene **2** in 95% purity was obtained as a colorless oil: $\nu_{max}^{CCl_4}$ 823 cm.$^{-1}$; λ_{max}^{EtOH} 271 mμ (ε 6500); n.m.r. identical with natural material reported earlier; $[\alpha]_D$ +99° (*c* 0.82, $CHCl_3$).

Anal. Found: C, 88.18; H, 11.82.

On standing in air, the compound was converted into cuparene.

Isolation of Widdrol.—Portions (3 μl.) of the "alcohol fraction" were injected into a 5 ft. × 3/8 in. column containing 20% DEGS on Chromosorb W at 145°, and a flow rate of 80 cc./min. of helium was used. The material from the largest peak was collected, the liquid was cooled in a refrigerator, and the solid which formed was removed. This solid possessed an infrared spectrum identical with that of authentic widdrol.

Isolation of Terpinen-4-ol.—Portions (300 μl.) of distillation fraction 1 were injected into a 10 ft. × 3/8 in. column containing a packing of 20% Carbowax-20 M on Chromosorb P (HMDS, 60–80 mesh) kept at 200°, and a flow rate of 150 cc./min. of helium was used. The main peak was collected, and the collected material was a solid at 4° but melted by 10°. The infrared spectrum fits well that of (+)-terpinene-4-ol.[10] The material distilled at 60° (0.5 mm.), $n^{20}D$ 1.4792, $[\alpha]^{20}D$ +39.8° (*c* 2.90, $CHCl_3$) [lit.[11] $n^{20}D$ 1.4778, $[\alpha]^{20}D$ +41.9° (*c* 20, $CHCl_3$)].

Anal. Calcd. for $C_{10}H_{18}O$ (154.24): C, 77.86; H, 11.76. Found: C, 77.88; H, 11.84.

The n.m.r. spectrum had the following bands: τ 4.84 (1H, broad, vinyl H), 8.11 (4H, multiplet, allylic methylenes), 8.43 (3H, singlet, vinyl methyl), 9.15 (3H, doublet, J = 2 c.p.s., *sec*-methyl), and 9.25 (3H, doublet, J = 2 c.p.s., *sec*-methyl).

(10) J. Plíva, M. Horák, V. Herout, and F. Sörm, "Collection of Spectra and Physical Properties of Terpenes," Academie-Verlag, Berlin, 1960, Spectrum M-22.

(11) Y. R. Naves and P. Tullen, *Bull. soc. chim. France*, 2123 (1960).

Anisomycin. II.[1] Biosynthesis of Anisomycin

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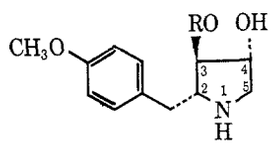
Anisomycin is a basic antibiotic which shows good biological activity against *Trichomonas vaginalis* and *Entamoeba histolytica.*[2] It is obtained by fermentation of various *Streptomyces* species,[3] and its structure has been shown to be 2-*p*-methoxybenzyl-3-*trans*-acetoxy-4-hydroxypyrrolidine[1] (I).



I, R = CH_3CO
II, R = H

The antibiotic may be extracted with chloroform from filtered broth at pH 9.0. It is recovered as white crystalline solid, and is usually accompanied by some deacetylanisomycin (II). Since the acetoxy function of anisomycin is readily hydrolyzed, it was not immediately clear whether the deacetylanisomycin (II) which accompanied the antibiotic was a product of hydrolysis during the isolation procedure or a prior fermentation product. However, a study of the fermentation showed the deacetylanisomycin (II) accumulated in the broth during the logarithmic phase of growth (Figure 1) and was converted into anisomycin during the phase of assimilation. Most of the anisomycin was produced during the third and fourth days of the fermentation when acetic acid had begun to accumulate in large quantities in the medium.

The structure of anisomycin (I) suggests that it might be derived from either tyrosine, phenylalanine, or proline. Labeled amino acids and aliphatic acids were added to the fermentation medium and specific activity of the isolated antibiotic was determined. Usually, the crude antibiotics isolated from the culture medium were hydrolyzed to deacetylanisomycin (II) which was then purified by partition chromatography. Occasionally it was advantageous to isolate anisomycin (I).

The results of these fermentations are shown in Table I. We conclude that the pyrrolidine ring of anisomycin is not derived from proline, but that the major precursor is tyrosine. Analysis of the soy meal, which was the only source of amino acids for the fermentation, showed that it contained 3.1% by weight of tyrosine, whereas phenylalanine was present in 4.8%. Thus, although the quantities of these amino acids in the fermentation medium are approximately the same, the tyrosine was incorporated 40 times more efficiently

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(3) B. A. Sobin and F. W. Tanner, *J. Am. Chem. Soc.*, **76**, 4053 (1954).