The Bacterial Transformation of Abietic Acid

BY B. E. CROSS AND P. L. MYERS

Department of Organic Chemistry, University of Leeds

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An *Alcaligenes* species, which was isolated from soil, can utilize abietic acid as its sole carbon source. During growth, the bacterium transforms abietic acid into 5 α -hydroxyabietic acid (I, R=OH), a product considered to be 7 β -hydroxy-13-isopropyl-8 ξ -podocarp-13-en-15-oic acid (II, R=H) and a compound, C₂₀H₂₈O₃, which is believed to be an epoxy- γ -lactone.

The bacterial degradation of steroids has been investigated in some detail (Pasqualini, 1963) and many of the reactions involved in the breakdown of steroids such as androst-4-ene-3,17-dione have been elucidated (Dodson & Muir, 1961*a,b*; Sih & Wang, 1963; Sih, Lee, Tsong & Wang, 1966; Gibson, Wang, Sih & Whitlock, 1966). Although the terpenes are biogenetically closely related to the steroids their bacterial transformation products have received little attention (Tamm, 1962), except for some of the monoterpenes. Thus (+)-camphor has been examined in detail (Bradshaw, Conrad, Corey, Gunsalus & Lednicer, 1959; Chapman, Kuo & Gunsalus, 1963; Conrad, DuBus, Namtvedt & Gunsalus, 1965; Chapman, Meerman, Gunsalus, Srinivasan & Rinehart, 1966) and fenchone has received less attention (Chapman, Meerman & Gunsalus, 1965). There do not appear to be any reports of bacterial transformation products of diterpenes, although two strains of bacteria, namely *Flavobacterium resinovorum* and *Pseudomonas resinovorans*, have been isolated that can utilize abietic acid (I, R=H) and a dihydro derivative as their sole carbon source (Delaporte & Daste, 1956; Daste, 1958; Raynaud & Daste, 1962). Since abietic acid is readily available and might be expected to afford bacterial degradation products that are accessible only with difficulty by chemical transformations, it was decided to investigate its bacterial transformation.

RESULTS AND DISCUSSION

An *Alcaligenes* sp. capable of utilizing abietic acid as its sole carbon source was isolated from a soil sample by elective culture on a medium containing abietic acid (I, R=H). The high concentration of cells required for the transformation of abietic acid on the preparative scale was produced by growing the organism in a large volume of succinate medium and then suspending the col-

lected cells in a small volume of phosphate buffer. When abietic acid was added to this suspension it was rapidly degraded, but, if the cells were harvested after 3 hr., two, and sometimes three, transformation products of abietic acid were obtained; none of these products was formed when a suspension of abietic acid in sterile buffer was aerated.

The minor transformation product, which was isolated in very small amounts, was shown to have the formula C₂₀H₂₈O₃ by high-resolution mass spectrometry. The infrared spectrum of the compound revealed the presence of a γ -lactone ring (C=O band at 1768 cm.⁻¹) and an ethylenic double bond (C=C band at 1620 cm.⁻¹), but did not show any hydroxyl absorption. The γ -lactone did not contain the diene system of abietic acid, since it showed no maximum in the ultraviolet above 220 m μ , and hence it is believed that the third oxygen atom is present as an epoxide ring. Thus this transformation product appears to be either a 15 $\rightarrow$ 2- or a 15 $\rightarrow$ 6-lactone of abietic acid in which one of the double bonds has been epoxidized. Further work on this compound has been prevented by lack of material.

The major transformation product was isolated as a crystalline hemihydrate, C₂₀H₃₀O₃· $\frac{1}{2}$ H₂O. It was very unstable; extensive decomposition took place during 24 hr. at room temperature, but it could be stored without appreciable decomposition at -40°. Its infrared spectrum (bands at 3390, ~2400-~2700 and 1689 cm.⁻¹) revealed the presence of hydroxyl and carboxyl functions, and in the ultraviolet, maxima at 229 and 240 m μ (ϵ 22 000 and 22 800 respectively) showed that the diene system of abietic acid had been retained. Hence the transformation product could be formulated as a hydroxyabietic acid. The nuclear-magnetic-resonance spectrum of the hydroxy acid showed peaks that were interpreted as follows: τ = 9.16 (singlet; 17-methyl group), two

superimposed methyl doublets centred at 9.05 ($J = 7$ cyc./sec.; isopropyl group), 8.66 (singlet; 16-methyl group), 4.84 (broad) and 4.32 (sharp) (vinyl protons on C-7 and C-14 respectively). The vinyl proton signals were very similar to those shown by abietic acid, thus providing further evidence for the presence of the diene chromophore in the hydroxy acid. A double-irradiation experiment confirmed that the signal at 4.84 τ was due to the proton on C-7, because this peak was considerably sharpened when it was spin-spin decoupled with a proton in the methylene envelope at 7.67 τ . The lack of a resonance attributable to a $>CH\cdot OH$ function showed that the hydroxyl group must be tertiary. Of the three tertiary positions, namely 5, 9 and 18, in abietic acid, that at 18 appeared to be excluded because the isopropyl methyl groups were not deshielded relative to abietic acid. Since it is known that a hydroxyl group introduced by a micro-organism has the stereochemistry of the hydrogen atom it replaces (Talalay, 1965), the transformation product must be either 5 α - or 9 α -hydroxyabietic acid. However, 9 α -hydroxyabietic

acid is known (Fieser & Campbell, 1938; Herz & Wahlborg, 1965) and preparation of a sample showed that it is not the same as the transformation product. Hence the latter was formulated as 5 α -hydroxyabietic acid (I, $R=OH$). The α -orientation of the hydroxyl group was confirmed by the chemical shift of the 17-methyl group, which was found only 0.04 p.p.m. downfield of its position in abietic acid. This shift is of the same order as that found for the 19-methyl group in 5 α -hydroxy steroids (Bhacca & Williams, 1964a). In a 5 β -hydroxyabietic acid however, the β -hydroxyl group and the resultant *cis*-junction of rings A/B would, by analogy with the steroids (Bhacca & Williams, 1964a), be expected to shift the 17-methyl group downfield by a total of about 0.15 p.p.m. with respect to abietic acid.

The structure of 5 α -hydroxyabietic acid was rigorously established by degradation. Hydrogenation of 5 α -hydroxyabietic acid with Adams catalyst in ethanol gave the tetrahydro acid (IV, $R=H$) and two dihydro acids (see below). It was not possible to assign the 8 β -configuration to

the tetrahydro acid with certainty solely on the basis of its nuclear-magnetic-resonance spectrum. Thus in carbon tetrachloride solution it showed the 17-methyl-group resonance at $\tau = 9.04$, which, allowing for deshielding by the 5α -hydroxyl group (Bhacca & Williams, 1964a), is characteristic of *b/c trans*-fused podocarpanes (Huffman, Kamiya, Wright, Schmidt & Herz, 1966; Cross & Myers, 1968). However, in pyridine solution the 17-methyl-group peak of the tetrahydro acid appeared at $\tau = 8.94$; this value is clearly anomalous since the acid was shown (see below) to have the 8β -configuration. The tetrahydro acid was most readily purified by methylation with diazomethane, followed by chromatography of the ester and then alkaline hydrolysis to regenerate the acid.

The tertiary nature of the hydroxyl group in 5α -hydroxyabietic acid was confirmed when the methyl ester of the tetrahydro acid (IV, R=Me) was recovered unchanged from treatment with Jones reagent (Curtis, Heilbron, Jones & Woods, 1953). The position of this hydroxyl group was established by pyrolysis of the tetrahydro acid (IV, R=H) at 180 – 200° . The product, formed in 83%

yield, was a hydrocarbon, $C_{19}H_{32}$, shown to be the Δ^4 -ene (VI) by its nuclear-magnetic-resonance spectrum, which showed the 17-methyl-group peak at $\tau = 9.07$, a vinylic methyl-group resonance at $\tau = 8.43$, i.e. the 16-methyl group, and no olefinic protons. Since the 17-methyl-group resonance occurred at a chemical shift characteristic of *b/c trans*-fused podocarpanes (Huffman *et al.* 1966; Cross & Myers, 1968), the Δ^4 -ene (VI), and consequently also the tetrahydro acid (IV, R=H), must have had the 8β -configuration. Since tetrahydroabietic acid was recovered unchanged after pyrolysis at 200 – 250° , the formation of the hydrocarbon (VI) can only be explained if the hydroxyl group in the hydroxytetrahydro acid is placed at C-5. Dehydration would then give a Δ^5 -ene, which as a $\beta\gamma$ -unsaturated acid would decarboxylate with double-bond migration. The bacterial transformation product must therefore be 5α -hydroxyabietic acid (I, R=OH).

One of the dihydro acids obtained by hydrogenation of 5α -hydroxyabietic acid (see above) was crystalline, and was assigned the Δ^7 -ene structure (III, R=H) since its nuclear-magnetic-resonance

(VII)

(VIII)

(IX)

(X)

(XI)

(XII)

(XIII)

spectrum showed a broad vinylic proton signal at $\tau = 4.9$, similar in shape to that of the proton attached to C-7 in abietic acid. This structure has been confirmed because dehydration of the methyl ester (III, R=Me) of this acid with phosphoryl chloride in pyridine gave the conjugated diene (VII), which showed $\lambda_{\max}$. 276m μ (ϵ 7100). It is unlikely that this diene was formed by double-bond migration, since dehydration of the isomeric dihydro ester (V) (see below) did not give a conjugated diene. The second dihydro derivative of 5 α -hydroxyabietic acid was characterized as its methyl ester, whose nuclear-magnetic-resonance spectrum showed a broad vinylic proton peak at $\tau = 4.53$. It has been tentatively assigned structure (V) by analogy with the $\Delta^8(14)$ -ene (X) formed as one of the products on hydrogenation of 12 α -hydroxyabietic acid under similar conditions (Herz, Wahlborg, Lloyd, Schuller & Hedrick, 1965). In agreement with structure (V) for the ester, dehydration with phosphoryl chloride in pyridine proceeded less readily than for the isomer (III, R=Me), and the product showed no conjugated-diene absorption in the ultraviolet.

The third transformation product was isolated as its gummy methyl ester and characterized as the 3,5-dinitrobenzoate of this ester. Since analyses of the dinitrobenzoate showed it to have the formula $C_{28}H_{36}N_2O_8$ the methyl ester can be assigned the formula $C_{21}H_{34}O_3$. The ultraviolet-absorption spectrum of the methyl ester showed that the diene chromophore was no longer present, and, in the infrared, bands due to hydroxyl (3400cm.⁻¹) and ester (1720cm.⁻¹) groups were observed. The nuclear-magnetic-resonance spectrum of the methyl ester contained signals at $\tau = 9.12$ (singlet; 17-methyl group), superimposed doublets centred at $\tau = 9.0$ ($J = 6.5$ cyc./sec.; isopropyl group), 8.84 (singlet; 16-methyl group), 6.33 (CO₂·Me) and 4.27 (vinyl proton). In addition it showed a multiplet at $\tau = 6.9$ (half band width about 20cyc./sec.), and hence the hydroxyl group was secondary and equatorial (Bhacca & Williams, 1964b). The other functional groups were presumably a trisubstituted double bond and an ester group. Recovery of the methyl ester from attempted oxidation with active manganese dioxide strongly suggested that the hydroxyl group was not allylic to the double bond. On the other hand, oxidation of the methyl ester with Jones reagent gave a product shown to be a mixture of an $\alpha\beta$ -unsaturated ketone and an unconjugated ketone. Thus the mixture showed infrared-absorption bands at 1715cm.⁻¹ (ester), 1705cm.⁻¹ (ketone), 1680cm.⁻¹ ($\alpha\beta$ -unsaturated ketone) and 1605cm.⁻¹ (C=C conjugated with C=O). In the ultraviolet the mixture had a maximum at 253m μ , which increased in intensity from ϵ 4400 to ϵ 6200 after the addition of a trace of mineral acid and

standing for 24hr. These results may be interpreted if the methyl ester $C_{21}H_{34}O_3$ contained a $\beta\gamma$ -unsaturated alcohol function. With Jones reagent, the $\beta\gamma$ -unsaturated ketone which was formed at first, underwent partial isomerization to an $\alpha\beta$ -unsaturated ketone, and further isomerization took place in ethanol solution when mineral acid was added. From the wavelength of the ultraviolet-absorption maximum (Fieser & Fieser, 1959), the double bond in the $\alpha\beta$ -unsaturated ketone was seen to be tetra-substituted, and hence it was probably between C-8 and C-9. If it is assumed that the bacterial transformation takes place by the addition of water to one of the double bonds in abietic acid, and bearing in mind that the hydroxyl group is equatorial (see above), then the most likely structure for the transformation product is (II) (R=H). The ketone (XI), formed by oxidation of the ester (II, R=Me) would, on acid-catalysed rearrangement, give the $\alpha\beta$ -unsaturated ketone (IX, R=O). Some evidence in support of structure (II, R=H) is provided by the nuclear-magnetic-resonance spectrum of its methyl ester, which showed a vinylic proton signal at $\tau = 4.27$, very similar in shape to that given by the proton attached to C-14 in abietic acid.

Since the methyl ester $C_{21}H_{34}O_3$ was obtainable only in very small amounts, it was decided to confirm the position of the hydroxyl group by relating the methyl ester to abietic acid derivatives, which were prepared for this purpose (Cross & Myers, 1968). Hydrogenation of the methyl ester $C_{21}H_{34}O_3$ gave, after preparative layer chromatography, a gum that was shown to be a mixture of methyl 7 β -hydroxy-13 α -isopropyl-8 β -podocarp-15-oate (VIII, R= $\begin{smallmatrix} \text{OH} \\ \diagup \text{H} \end{smallmatrix}$) and methyl 7 β -hydroxy-13 ξ -isopropylpodocarp-8-en-15-oate (IX, R= $\begin{smallmatrix} \text{OH} \\ \diagup \text{H} \end{smallmatrix}$) by its subsequent transformations. The nuclear-magnetic-resonance spectrum of the mixture showed no vinylic proton signal and confirmed the presence of the equatorial hydroxyl group [multiplet at $\tau = 6.95$ (half band width about 20cyc./sec.)]. The product from the hydrogenation was oxidized with Jones reagent, giving a mixture of about 85% of methyl 13 α -isopropyl-7-oxo-8 β -podocarp-15-oate (VIII, R=O) and 15% of methyl 13 ξ -isopropyl-7-oxopodocarp-8-en-15-oate (IX, R=O). The presence of the latter was demonstrated by an ultraviolet absorption maximum at 253m μ (ϵ 1600), and by $\alpha\beta$ -unsaturated ketone and C=C stretching bands at 1690 and 1610cm.⁻¹ respectively in the infrared. Finally, reaction of the mixture of keto esters with DNP-hydrazine gave a derivative identical in all respects with the DNP-hydrazone of an authentic specimen of the keto ester (VIII, R=O) in which the iso-

propyl group is known to have the α -configuration (Cross & Myers, 1968). The stereochemistry assigned above to the transformation products of the methyl ester (II, R=Me) follows from this interrelationship. That the ester $C_{21}H_{34}O_3$ was not a $\Delta^{8(14)}$ -ene was confirmed when it was shown to be different from an authentic sample of the ester (XII) (Cross & Myers, 1968). These results establish the position of the hydroxyl group in the methyl ester (II, R=Me), but do not exclude the possibility that the double bond is in the 9(11)-position, i.e. structure (XIII) for the acid. However, since bacterial attack on abietic acid is more like to give the Δ^{13} -ene (II, R=H) than the isomer (XIII), the former structure is provisionally assigned to the transformation product.

MATERIALS AND METHODS

Isolation and maintenance of organisms. The organism was isolated from a soil sample by elective culture in a medium adjusted to pH 7-7.5 with 5N-NaOH and containing (g./l.): KH_2PO_4 , 9; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.32; abietic acid, 0.5g. in 0.33N-NaOH (50ml.). Miss E. Chamberlain kindly examined the organism and identified it as a species of *Alcaligenes*; it forms motile Gram-negative rods. Stock cultures were maintained on nutrient-agar slopes sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and subcultured periodically.

Chromatography. Column chromatography was carried out on silica gel [Whatman Chromedia SG 31 (W. and R. Balston Ltd., Maidstone, Kent)] and alumina [Woelm neutral alumina, grade II (M. Woelm, Eschwege, Germany)]. The percentage composition of solvent mixtures used as eluent refers to the composition by volume. Light petroleum refers to the fraction of b.p. 60-80°.

Thin-layer chromatography was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany). Spots were rendered visible by spraying with 5% (v/v) H_2SO_4 in ethanol followed by heating at 150°. For preparative layer chromatography Kieselgel G, washed with ethyl acetate, was used in layers 0.5 mm. thick.

Physical measurements. Melting points were determined on a Kofler block apparatus. Ultraviolet-absorption spectra and optical rotations were measured in ethanol solution on a Unicam SP.800 spectrometer and a Perkin-Elmer model 141 polarimeter respectively. Unless otherwise stated, infrared-absorption spectra were determined as Nujol mulls on a Unicam SP.200 spectrometer, and nuclear-magnetic-resonance spectra were measured in CCl_4 on a Varian Associates A60 spectrometer with tetramethylsilane as internal standard ($\tau=10.00$). The mass spectrum was determined on an Associated Electrical Industries MS902 instrument.

Abietic acid. Abietic acid was isolated from resin (colophony) and was purified by crystallization of the di-n-pentylamine salt to constant optical activity (Harris & Sanderson, 1948). The free acid had m.p. 172-174°, $[\alpha]_D^{25} -95 \pm 1^\circ$ (c 2.0), λ_{max} . at 234, 241 and 250 m μ (ϵ 13 800, 14 700 and 9200 respectively), nuclear-magnetic-resonance absorptions at $\tau=9.20$ (singlet; 17-methyl group), 9.02

(doublet; $J=6.5$ cyc./sec.; isopropyl group), 8.78 (singlet; 16-methyl group), 4.88 (broad; C-7 proton) and 4.39 (C-14 proton).

Bacterial transformation of abietic acid. The *Alcaligenes* sp. was grown with forced aeration at 30° in a phosphate buffer (48l.) adjusted with 5N-NaOH to pH 7-7.5 and containing (g./l.): KH_2PO_4 , 9; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.4. Succinic acid (2g./l.) was added as the carbon source. After growth for 24 hr. the cells were harvested in a De Laval continuous-flow centrifuge and resuspended in fresh phosphate buffer (3l.) to which abietic acid (6g.) in 0.13N-NaOH (150ml.) was added. The suspension was divided between 30 500ml. Erlenmeyer flasks and incubated at 30° on a shaker for 24 hr. to induce enzymes capable of degrading abietic acid. The bacteria were reharvested and growth on the abietic acid medium was repeated as above except that shaking was stopped after 3 hr. The cells were again collected in the centrifuge and the cell-free filtrate was acidified with dil. H_2SO_4 and extracted three times with ethyl acetate. The extract was washed with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo* at room temperature to give a mixture of products (100-150mg.). The cells were used to transform up to eight more batches of abietic acid by repetition of this procedure.

Isolation of the transformation products. The combined products from eight batches of the above transformations were chromatographed on silica gel (35 cm. $\times$ 3 cm.). Ether (10% in light petroleum) eluted abietic acid (360mg.). Elution with 17.5-20% ether in light petroleum gave a lactone (3-5mg.), which crystallized from ethyl acetate-light petroleum in prisms, m.p. 234-236° (Found: molecular ion, m/e 316.2056. $C_{20}H_{28}O_3$ requires M 316.2038), infrared maxima 1768 (γ -lactone) and 1620 ($C=C$) cm^{-1} , no λ_{max} . above 220 m μ .

Elution with 20-27.5% ether in light petroleum gave 5 α -hydroxyabietic acid (I, R=OH), which crystallized from ether-light petroleum at room temperature as needles (160mg.), m.p. 76-78° (Found: C, 73.2, 73.4; H, 9.2, 9.2. $C_{20}H_{30}O_3 \cdot \frac{1}{2}H_2O$ requires C, 73.35; H, 9.5%), λ_{max} . at 229, 240 and 250 (shoulder) m μ (ϵ 22 000, 22 800 and 17 300 respectively), infrared maxima (measured on a Perkin-Elmer model 125 spectrometer) 3390 (OH), ~ 2400 - ~ 2700 (OH of CO_2H) and 1689 ($C=O$ of CO_2H) cm^{-1} , nuclear-magnetic-resonance absorptions (determined on a Varian Associates HA100 spectrometer in $CDCl_3$ solution) at $\tau=9.16$ (singlet; 17-methyl group), 9.05 (doublet; $J=7$ cyc./sec., isopropyl group), 8.66 (singlet; 16-methyl group), 5.07 (CO_2H and OH), and 4.84 (broad) and 4.32 (olefinic protons attached to C-7 and C-14 respectively).

Ether (40-45%) in light petroleum eluted a gum (120mg.), which was purified by methylation with ethereal diazomethane and subsequent chromatography on alumina. Elution with 40% ether in light petroleum gave an intractable gum (55mg.), which is believed to be methyl 7 β -hydroxy-13-isopropyl-8 ξ -podocarp-13-en-15-oate (II, R=Me) and which ran as one spot on thin-layer chromatography in ethanol-benzene (1:19, v/v). It showed infrared maxima (film) at 3400 (OH) and 1720 (ester) cm^{-1} , and nuclear-magnetic-resonance absorptions at $\tau=9.12$ (singlet; 17-methyl group), 9.0 (doublet; $J=6.5$ cyc./sec.; isopropyl group), 8.84 (singlet; 16-methyl group), 8.12 (allylic protons), 7.83 (OH), 6.9 (multiplet; CH $\cdot$ OH), 6.33

(singlet; $\text{CO}_2\text{-Me}$) and 4.27 (sharp; vinylic proton), no λ_{max} above 220 μ .

Its 3,5-dinitrobenzoate was prepared by treating the hydroxy ester (35 mg.) in dry pyridine (1 ml.) with 3,5-dinitrobenzoyl chloride (48 mg.) for 15 hr. at room temperature. Water (0.25 ml.) was added and after 30 min. the solution was taken up in ether and washed with dil. H_2SO_4 and water and dried. The solution was passed through a column of alumina and the filtrate evaporated *in vacuo*. The residue crystallized from ethanol in long needles, m.p. 151–153° (Found: C, 63.6; H, 6.35. $\text{C}_{28}\text{H}_{36}\text{N}_2\text{O}_8$ requires C, 63.6; H, 6.85%). It showed an infrared maximum at 1724 (ester) cm^{-1} .

Hydrogenation of 5 α -hydroxyabiatic acid. The acid (316 mg.), in ethanol (100 ml.), was added to Adams platinum oxide (150 mg.) that had been pre-reduced in ethanol; it took up 1.25 moles of hydrogen in 6 hr. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The product (308 mg.) was chromatographed on silica gel. Elution with 12.5–15% ether in light petroleum gave fraction A (185 mg.), and further elution with 20% ether in light petroleum yielded 5 α -hydroxy-13 ξ -isopropylpodocarp-7-en-15-oic acid (III, R=H), which after sublimation at 160° (bath temperature) and 10–5 mm. Hg formed needles (98 mg.), m.p. 176–178° (Found: C, 74.7; H, 9.7. $\text{C}_{20}\text{H}_{32}\text{O}_3$ requires C, 74.95; H, 10.1%), infrared maxima at 3450 (OH) and 1690 ($\text{C}=\text{O}$ of CO_2H) cm^{-1} , nuclear-magnetic-resonance peaks at $\tau = 9.12$ (broad; 17-methyl and isopropyl groups), 8.63 (singlet; 16-methyl group) and 4.9 (broad; vinylic proton).

The methyl ester (III, R=Me), prepared with ethereal diazomethane, crystallized from ether–light petroleum in rods, m.p. 147–148° (Found: C, 74.95; H, 10.0. $\text{C}_{21}\text{H}_{34}\text{O}_3$ requires C, 75.4; H, 10.25%), infrared maxima at 3640 (OH) and 1715 (ester) cm^{-1} and nuclear-magnetic-resonance absorptions at $\tau = 9.15$ (doublet; $J = 6$ cyc./sec.; isopropyl methyl group), 9.11 (doublet; $J = 6$ cyc./sec.; isopropyl methyl group), 9.02 (singlet; 17-methyl group), 8.57 (singlet; 16-methyl group), 6.49 (singlet; $\text{CO}_2\text{-Me}$), 4.74 (broad; vinylic proton).

Fraction A was methylated with ethereal diazomethane and the product was chromatographed on an alumina column (12 cm. $\times$ 1.5 cm.). Elution with 7.5% and 10% ether in light petroleum gave methyl 5 α -hydroxy-13 ξ -isopropyl-8 β -podocarp-15-oate (IV, R=Me) as an oil (115 mg.), which was distilled at 160° (bath temperature) and 10–3 mm. Hg (Found: C, 75.1; H, 10.6. $\text{C}_{21}\text{H}_{36}\text{O}_3$ requires C, 74.95; H, 10.8%), infrared maxima (film) at 3500 (OH) and 1700 (ester) cm^{-1} and nuclear-magnetic-resonance absorptions at $\tau = 9.16$ (doublet; $J = 5.5$ cyc./sec.; isopropyl group), 9.04 (singlet; 17-methyl group), 8.68 (singlet; 16-methyl group) and 6.35 (singlet; $\text{CO}_2\text{-Me}$).

Elution with 15% ether in light petroleum gave methyl 5 α -hydroxy-13 ξ -isopropylpodocarp-8(14)-en-15-oate (V), which sublimed at 170° (bath temperature) and 2×10^{-3} mm. Hg as crystals (59 mg.), m.p. 68–69° (Found: C, 75.35; H, 10.25. $\text{C}_{21}\text{H}_{34}\text{O}_3$ requires C, 75.4; H, 10.25%), infrared maxima at 3550 (OH) and 1707 (ester) cm^{-1} and nuclear-magnetic-resonance absorptions at $\tau = 9.10$ (17-methyl and isopropyl groups), 8.67 (singlet; 16-methyl group), 6.34 (singlet; $\text{CO}_2\text{-Me}$) and 4.56 (broad; vinylic proton).

Attempted oxidation of the 5 α -hydroxy ester (IV, R=Me) with chromium trioxide. The methyl ester (15 mg.) in

acetone (25 ml.) was treated at 0° with the 8N- CrO_3 reagent (0.2 ml.) (Curtis *et al.* 1953) for 30 min. Methanol was added to destroy the excess of reagent, the solvent was removed *in vacuo*, and the residue was taken up in ether, washed with water and dried. Evaporation of the ether gave a gum (14 mg.) whose infrared spectrum was identical with that of the starting material.

Hydrolysis of the 5 α -hydroxy ester (IV, R=Me). The ester (90 mg.) was treated with NaOH (30 mg.) in 95% ethanol (10 ml.) at 30° for 3 days. The solvent was removed *in vacuo*, the residue was dissolved in water and the solution was acidified with dil. H_2SO_4 . Recovery in ether gave 5 α -hydroxy-13 ξ -isopropyl-8 β -podocarp-15-oic acid (IV, R=H) which crystallized from ether–light petroleum in prisms (72 mg.), m.p. 170–173° (decomp.). The decomposition point varied with the rate of heating (Found: C, 74.4; H, 10.8. $\text{C}_{20}\text{H}_{34}\text{O}_3$ requires C, 74.5; H, 10.6%), infrared maxima (measured on a Perkin–Elmer model 125 spectrometer) at 3330 (OH), ~ 2400 – ~ 2800 (OH of CO_2H) and 1689 ($\text{C}=\text{O}$ of CO_2H) cm^{-1} and nuclear-magnetic-resonance absorptions at $\tau = 9.16$ (doublet; $J = 5.5$ cyc./sec.; isopropyl group), 9.04 (singlet; 17-methyl group), 8.68 (singlet; 16-methyl group) and 6.34 (singlet; $\text{CO}_2\text{-Me}$), τ (in pyridine) = 9.17 (doublet; $J = 5.5$ cyc./sec.; isopropyl group), 8.94 (singlet; 17-methyl group), and 8.42 (singlet; 16-methyl group).

The 5 α -hydroxy acid (IV, R=H) was also isolated by crystallization of fraction A from the hydrogenation of 5 α -hydroxyabiatic acid (see above) from light petroleum.

Pyrolysis of the 5 α -hydroxy acid (IV, R=H). The acid (40 mg.) was heated in a sublimation apparatus at 180–200° (bath temperature) and 14 mm. Hg for 30 min. The sublimate was chromatographed on an alumina column (20 cm. $\times$ 1.5 cm.). Elution with light petroleum yielded 13 ξ -isopropyl-8 β -podocarp-4-ene (VI) as an oil (28 mg.), which was distilled at 120° (bath temperature) and 0.1 mm. Hg (Found: C, 87.15; H, 12.8. $\text{C}_{19}\text{H}_{32}$ requires C, 87.6; H, 12.4%). It showed nuclear-magnetic-resonance peaks at $\tau = 9.16$ (doublet; $J = 6$ cyc./sec.; isopropyl group), 9.07 (singlet; 17-methyl group) and 8.43 (singlet; 16-methyl group).

Pyrolysis of tetrahydroabiatic acid. When tetrahydroabiatic acid (m.p. 161–163°, prepared by hydrogenation of abiatic acid with Adams catalyst in acetic acid; cf. Huffman *et al.* 1966) was heated at 200–250° for 2 hr. in a sealed tube it was recovered unchanged.

Dehydration of methyl 5 α -hydroxy-13 ξ -isopropylpodocarp-7-en-15-oate (III, R=Me). The ester (75 mg.) in dry pyridine (20 ml.) was treated with phosphoryl chloride (0.05 ml.) and heated under reflux in an atmosphere of N_2 for 3 hr. The solution was diluted with water, and the product was recovered in ether and chromatographed on alumina (grade V; 10 cm. $\times$ 1.0 cm.). Elution with 5% ether in light petroleum gave methyl 13 ξ -isopropylpodocarp-5,7-dien-15-oate (VII) as a gum (68 mg.), infrared maxima (film) at 1725 (ester) and 1650 ($\text{C}=\text{C}$) cm^{-1} , λ_{max} 276 $\text{m}\mu$ and 286 $\text{m}\mu$ (shoulder) (ϵ 7100 and 6300 respectively) and nuclear-magnetic-resonance absorptions at $\tau = 9.13$ (singlet; 17-methyl group), 9.12 (doublet; $J = 5$ cyc./sec.; isopropyl group), 8.66 (singlet; 16-methyl group), 6.38 (singlet; $\text{CO}_2\text{-Me}$) and 4.52 (broad; vinylic protons). The diene was not analysed because it decomposed on standing at room temperature.

Dehydration of methyl 5 α -hydroxy-13 ξ -isopropylpodocarp-

8(14)-en-15-oate (V). Treatment of the hydroxy ester (45mg.) as in the preceding experiment resulted in only about 10% dehydration as determined by thin-layer chromatography in 2.5% acetic acid in di-isopropyl ether. The reaction was therefore repeated for a further 12hr. The crude product, isolated as in the preceding experiment, was chromatographed on alumina (grade V; 10 cm. $\times$ 1 cm.). Elution with light petroleum gave a gum (28mg.), which showed no $\lambda_{\max}$. above 220m μ and infrared maximum at 1727 (ester) cm.⁻¹, but no hydroxyl absorption.

9 α -Hydroxyabietic acid. 9 α -Hydroxyabietic acid was prepared by the method of Fieser & Campbell (1938) (cf. Herz & Wahlborg, 1965), except that the compound was isolated as the free acid and purified by chromatography on silica gel. It was eluted with 30–35% ether in light petroleum and crystallized from ether–light petroleum, when it formed either prisms, m.p. 146–150°, or clusters of needles, m.p. 82–85°.

Oxidation of methyl 7 β -hydroxy-13-isopropyl-8 ξ -podocarp-13-en-15-oate (II, R=Me). (a) With MnO₂. A solution of the hydroxy ester (10mg.) in chloroform (1ml.) was shaken with active MnO₂ (Mancera, Rosenkranz & Sondheimer, 1953) (50mg.) for 24hr. Filtration followed by evaporation of the chloroform gave a gum (10mg.) identified as starting material by its infrared spectrum.

(b) With 8N-CrO₃. The hydroxy ester (70mg.) in acetone (50ml.) was treated with the 8N-CrO₃ reagent (0.15ml.) at 0° for 10min. Methanol was added and the solution was concentrated *in vacuo*, diluted with water and extracted with ether. The ether extract was washed with water, dried and evaporated to give a gum (66mg.), which was chromatographed on silica gel (30cm. $\times$ 1.5cm.). Elution with 12.5% ether in light petroleum gave a mixture of the isomeric keto esters (IX, R=O) and (XI) as a gum (42mg.), which showed infrared bands (film) at 1718 (CO₂·Me), 1705, 1685 (C=O) and 1610 (C=C) cm.⁻¹, nuclear-magnetic-resonance peaks at τ = 9.07, 8.94, 8.82, 8.75, 8.69 (singlets; methyl groups), 6.37 (singlet; CO₂·Me) and 4.27 (broad; vinylic proton). A freshly prepared solution had $\lambda_{\max}$. at 253 and 301m μ (ϵ 4400 and 1600 respectively), but after the addition of 1 drop of conc. HCl and standing for 24hr. it showed $\lambda_{\max}$. 253 and 310m μ (ϵ 6200 and 3800 respectively).

Hydrogenation of methyl 7 β -hydroxy-13-isopropyl-8 ξ -podocarp-13-en-15-oate (II, R=Me). The hydroxy ester (55mg.) was hydrogenated in acetic acid (25ml.) in the presence of Adams catalyst (25mg.) until uptake of hydrogen ceased (2hr.). The filtrate from the catalyst was diluted with water and extracted with ether. The extract was washed with dil. NaOH solution and water and dried. Evaporation of the ether gave a gum (53mg.), which was purified by preparative layer chromatography, with di-isopropyl ether–acetic acid (39:1, v/v) for development. The product was a gum (35mg.), which was a mixture of about 5 parts of methyl 7 β -hydroxy-13 α -isopropyl-8 β -podocarp-15-oate (VIII, R= $\begin{smallmatrix} \text{OH} \\ \diagup \end{smallmatrix}$) and 1 part of methyl 7 β -hydroxy-13 ξ -isopropylpodocarp-8-en-15-oate (IX, R= $\begin{smallmatrix} \text{OH} \\ \diagdown \end{smallmatrix}$). These two esters had the same R_F in 5% ethanol in benzene, and distilled at 160° (bath temperature) and 10⁻³mm. Hg (Found: C, 75.05; H, 10.3%). The mixture showed infrared bands (film) at 3550 (OH) and 1720 (ester) cm.⁻¹ and nuclear-magnetic-resonance peaks at τ = 9.12 (isopropyl group and 17-methyl group), 8.85

(singlet; 16-methyl group), 7.93 (OH), 6.95 (very broad; CH·OH) and 6.40 (singlet; CO₂·Me).

Oxidation of the mixture of esters (VIII, R= $\begin{smallmatrix} \text{OH} \\ \diagup \end{smallmatrix}$, and IX, R= $\begin{smallmatrix} \text{OH} \\ \diagdown \end{smallmatrix}$) with 8N-chromium trioxide. The above

mixture of esters (28mg.), in acetone (25ml.), was treated at 0° with the 8N-CrO₃ reagent (0.1ml.) for 10min. The product was isolated as described above and chromatographed on an alumina column (10cm. $\times$ 1cm.). Elution with 10% ether in light petroleum yielded methyl 13 α -isopropyl-7-oxo-8 β -podocarp-15-oate (VIII, R=O) containing about 15% of methyl 13 ξ -isopropyl-7-oxopodocarp-8-en-15-oate (IX, R=O) as a gum (22mg.). It showed $\lambda_{\max}$. at 253 and 301m μ (ϵ 1600 and 250 respectively) and infrared bands (film) at 1724 (ester), 1708 (cyclohexanone), 1690 ($\alpha\beta$ -unsaturated C=O) and 1610 (C=C) cm.⁻¹.

The above mixture of keto esters (20mg.) in methanol (5ml.) was treated with a solution (0.8ml.) of DNP-hydrazine (0.7g.) in methanol (30ml.) and conc. H₂SO₄ (2ml.). After 5 min. the methanol was removed *in vacuo*, the residue was diluted with water and the product was taken up in ether. The extract was washed with dil. H₂SO₄ and water and dried. Evaporation gave the DNP-hydrazone of the keto ester (VIII, R=O), which crystallized from methanol as yellow needles (15mg.), m.p. 178–179°. It was identical (mixed m.p. and infrared spectrum) with an authentic sample of the DNP-hydrazone of methyl 13 α -isopropyl-7-oxo-8 β -podocarp-15-oate (Cross & Myers, 1968).

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