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BIOSYNTHESIS OF THE TRITERPENE HYDROCARBONS OF *POLYPODIUM VULGARE*

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Abstract—3(*RS*)-[2-^{14}C,(4*R*)-4-$^{3}H_1$]-mevalonic acid has been incorporated into diploptene, hopene-I, fernene and serratene in *Polypodium vulgare*. The retention of six tritium atoms in fernene and of only five in hopene-I indicates that no double bond intermediates are involved in the final rearrangement in the formation of fernene.

INTRODUCTION

TRITERPENE hydrocarbons are not commonly found in nature and they are found mainly in various fern species.[1] *Polypodium vulgare* L. produces at least four triterpene hydrocarbons, diploptene (I), neohop-13(18)-ene (II), fernene (III), and serratene (IV).[1] This fern also contains a number of 3β-hydroxylated-4,4-dimethyl and 4α-monomethyl triterpenes of the cycloartanol series[1-3] and β-sitosterol as the major sterol.[1,2,4,5] Three phytoecdysones, the steroidal hormones with insect moulting activity, have also been isolated[6] from *P. vulgare*.

We have recently undertaken a study of the biosynthesis of the phytosterols and phytoecdysones in *P. vulgare*.[2-4] At the same time we have taken the opportunity to examine the biosynthesis of the triterpene hydrocarbons in this fern from 4*R*-[4-$^{3}H_1$,2-^{14}C]-mevalonic acid. The results of these studies are the subject of this communication.

The co-occurrence of 3β-hydroxylated triterpenes and triterpene hydrocarbons has obvious phytochemical significance. However, this has recently acquired biosynthetic importance since it implies the ability of the plant system to cyclize squalene by both the oxidative route, via squalene epoxide, and by the proton catalysed route. Barton *et al.*[5] have recently reported that this is the case in *P. vulgare*.

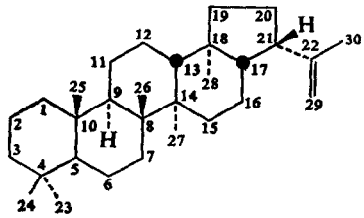
RESULTS

Characterization of Hopene-I

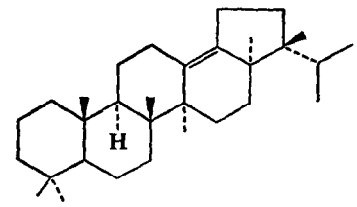
In a preliminary examination of the triterpene hydrocarbons of *Polypodium vulgare* we isolated three compounds whose physico-chemical characteristics were identical with those reported for diploptene (I), fernene (III) and serratene (IV). All three compounds had

[1] G. BERTI and F. BOTTARI, "Constituents of Ferns", in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. I, p. 589, Interscience, New York (1968).
[2] E. L. GHISALBERTI, N. J. DE SOUZA, H. H. REES, L. J. GOAD and T. W. GOODWIN, *Chem. Commun.* 1401 (1969).
[3] E. L. GHISALBERTI, N. J. DE SOUZA, H. H. REES, L. J. GOAD and T. W. GOODWIN, *Chem. Commun.* 1403 (1969).
[4] N. J. DE SOUZA, E. L. GHISALBERTI, H. H. REES and T. W. GOODWIN, *Phytochem.* 9, 1247 (1970).
[5] D. H. R. BARTON, A. F. GOSDEN, G. MELLOWS and D. A. WIDDOWSON, *Chem. Commun.* 184 (1969).
[6] G. HEINRICH and H. HOFFMEISTER, *Experientia* 23, 995 (1967); F. JIZBA, V. HEROUT and F. SORM, *Tetrahedron Letters* 1689 (1967); J. JIZBA and V. HEROUT, *Coll. Czech. Chem. Commun.* 32, 2867 (1967); G. HEINRICH and H. HOFFMEISTER, *Tetrahedron Letters* 6063 (1968); J. JIZBA, V. HEROUT and F. SORM, *Tetrahedron Letters* 5139 (1967).

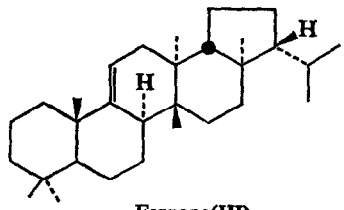
previously been isolated from this fern (see Ref. 1 for leading references). Berti and Bottari[1] reported that a fourth hydrocarbon, neohop-13(18)-ene (II), was also present in *P. vulgare*. We have also isolated a fourth triterpene hydrocarbon, but its characteristics did not correspond with those reported[7,8] for neohop-13(18)-ene (II). Reasons which lead us to suggest that it is hopene-I (hop-17(21)-ene) (V) are (i) GLC analysis (1% NGS column) showed it to have a retention time of 2·10 (relative to cholestane) in agreement with that found[9] for hopene-I (V) (2·10), but in contrast with that observed[9] for neohop-13(18)-ene (III) (2·70); (ii) the mass spectrum of our hydrocarbon showed significant peaks at *m/e* 410, 395, 367 (base peak), 231, 203, 191, 189, 175, 161, which do not differ greatly from those reported[7] for (II), but it



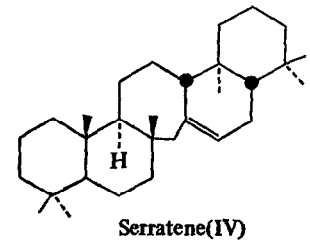
Diploptene(I)



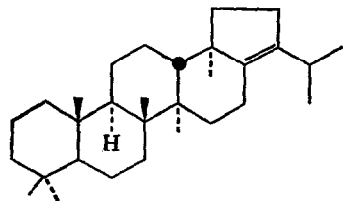
Neohop-13(18)-ene(II)



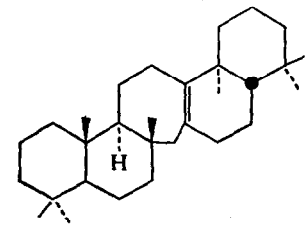
Fernene(III)



Serratene(IV)



Hopene-I(V)



Isoserratene(VI)

showed only a minimal peak at *m/e* 205. This peak is of high intensity in the mass spectrum of neohop-13(18)-ene (II),[10] and is considered characteristic for triterpenes with a 13–18 double bond;[11a] (iii) its NMR spectrum (100 Mc) included signals centred at 7·38 τ (quintet) attributable[11b] to the allylic methine proton of the isopropyl group; (iv) the m.p. and optical rotation of this compound (182–183°, $[\alpha]_D + 45°$) agree well with those recorded[8] for hopene-I

[7] G. N. PANDEY and C. R. MITRA, *Tetrahedron Letters* 4683 (1967).
[8] H. AGETA, K. IWATA and Y. OTAKE, *Chem. Pharm. Bull. Tokyo* **11**, 407 (1963).
[9] N. IKEKAWA, in *Methods in Enzymology* (edited by R. B. CLAYTON), Vol. XV, p. 200, Academic Press, New York (1969).
[10] G. BERTI, F. BOTTARI, A. MARSILI, I. MORELLI and A. MANDELBAUM, *Tetrahedron Letters* 529 (1968).
[11a] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Structure Elucidation of Natural Products by Mass Spectrometry*, Vol. II, p. 127, Holden-Day, San Francisco (1964).
[11b] E. O. BISHOP, in *Nuclear Magnetic Resonance for Organic Chemists* (edited by D. W. MATHIESON), Chapter 7, p. 121, Academic Press, (1967).

(V), (m.p. 183·5–185°; $[\alpha]_D$ + 49°) but differ significantly from those of neohop-13(18)-ene (II) (m.p. 197·0–198·5°; $[\alpha]_D \pm 0°$[7], $[\alpha]_D^{26}$ + 2°[8]); (v) the epoxyderivative (M^+ 416), obtained from the hydrocarbon by treatment with *m*-chloroperbenzoic acid, had m.p. 270–271° in agreement with that of 17,21-epoxyhopane (m.p. 272–273°)[12a] (the epoxide from neohop-13(18)-ene has m.p. 202–204°).[12b]

To our knowledge the presence of hopene-I (V) in *P. vulgare* has not previously been noted although its oxy-derivative, 17,21-epoxyhopane, has been isolated[12a] in low yields from the same plant.

Incorporation of 3(*RS*)-[2-^{14}C,(4*R*)-4-$^3H_1$]-*Mevalonic Acid into* Polypodium vulgare

Sliced rhizomes and chopped leaves of *P. vulgare* were incubated with a mixture of [2-^{14}C]-MVA and 4*R*-[4-$^3H_1$]-MVA for 24 hr at 25°. The non-saponifiable portion of the extract was separated by column chromatography and preparative TLC into fractions whose R_f corresponded to those of squalene, 4,4-dimethyl, 4-monomethyl and 4-desmethyl sterols. The fraction with R_f similar to squalene was further separated into its components by TLC on silica gel-silver nitrate. The bands taken, in order of increasing R_f, were those corresponding to squalene, diploptene (I), hopene-I (V), serratene (IV), and fernene (III). After further purification the hydrocarbons were recrystallized to constant specific activity. The levels of incorporation of MVA and the ^3H:^{14}C ratios (based on squalene) for the triterpene hydrocarbons are shown in Table 1.

TABLE 1. INCORPORATION OF 3*R*-[2-^{14}C,(4*R*),4-$^3H_1$]MVA INTO THE TRITERPENES HYDROCARBONS OF *P. vulgare*

	Observed ^3H:^{14}C ratio	Normalized ^3H:^{14}C ratio	% Incorporation
Squalene	10·32	6:6	0·35
Fernene (III)	10·31	5·99:6	0·44
12-Ketofernene	10·87	6·32:6	
Hopene-I (V)	8·82	5·13:6	0·04
Diploptene (I)	11·34	6·60:6	0·04
Serratene (IV)	10·57	6·15:6	0·01
Isoserratene (VI)	8·82	5·13:6	

Oxidation of radioactive fernene (III) with chromium trioxide yielded 12-ketofernene[13] which retained all tritium atoms (^3H:^{14}C; 6·32:6). Treatment of radioactive serratene (IV) with gaseous HCl in chloroform gave isoserratene[14] (VI) whose ^3H:^{14}C ratio showed that it had lost the tritium atom (^3H:^{14}C; 5·13:6), which was originally located at C-13.

The high ^3H:^{14}C ratio obtained for diploptene (I) indicated the presence of a radioactive impurity. Attempts to remove this involves formation of the corresponding 22,29-diol.[15] Purification of the diol failed to lower the radioactivity ratio as did periodate cleavage to 29-norketo-diploptene.[15]

The general lack of reactivity of the hydrocarbons and lack of carrier material precluded any further degradative experiments.

[12a] G. BERTI, F. BOTTARI, A. MARSILI and I. MORELLI, *Tetrahedron Letters* 979 (1966).
[12b] Y. TSUDA and K. ISOBE, *Tetrahedron Letters* 3337 (1965).
[13] H. AGETA, K. IWATA and S. NATORI, *Tetrahedron Letters* 1447 (1963).
[14] G. BERTI, F. BOTTARI, A. MARSILI, I. MORELLI and A. MANDELBAUM, *Chem. Commun.* 50 (1967).
[15] G. V. BADDELEY, T. G. HALSALL and E. R. H. JONES, *J. Chem. Soc.* 3891 (1961).

DISCUSSION

Members of three different skeletal groups can be recognized in the triterpene hydrocarbons of *Polypodium vulgare*; (i) the hopane groups represented by diploptene (I); (ii) the rearranged hopane by hopene-I (V) and fernene (III); (iii) the onocerane group represented by serratene (IV).[16]

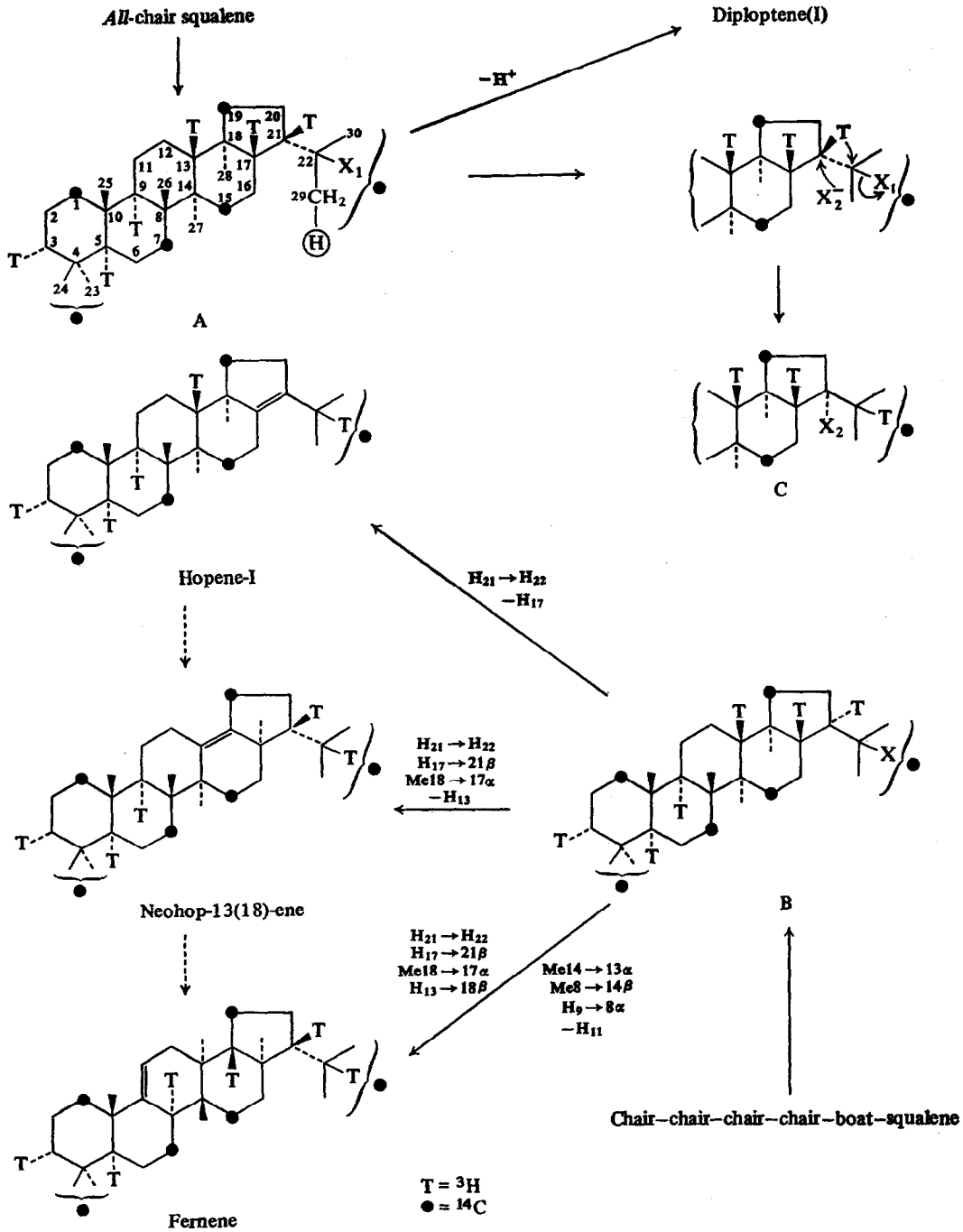
It has been suggested[16] that the hopane skeleton can arise by direct cyclization of squalene in the all-chair sequence to the ion derivable from form A (Scheme I). Loss of a proton could then generate diploptene (I). The rearranged hopane skeleton can conceivably be formed in two ways: (i) by cyclization of squalene in the chair–chair–chair–chair–boat sequence[16] and (ii) from form A. In case (i) the first-formed precursor (B) could then undergo the appropriate 1,2-shifts thus leading to hopene-I (V), neohop-13(18)-ene (II) and fernene (III). In the second case the side chain in form A must suffer inversion to give C before rearrangement can occur. Suggestions[16] for the formation of onocerane triterpenes invoke cyclization from both ends of the squalene molecule.

Because of the presence of 3β-hydroxylated triterpenes and sterols in *P. vulgare* it can be argued that the precursor A (X = OH) could also arise by a "reverse cyclization" of squalene epoxide.[5] This has been excluded by the experiments of Barton *et al.*[5] on fernene biosynthesis in *P. vulgare*. It seems logical to assume that the same is true for the other triterpene hydrocarbons in *P. vulgare*.

Although the formation of diploptene appears straightforward, that of hopene-I and fernene involves a series of 1,2-shifts. Indeed, for fernene, seven such shifts must occur if form B is a precursor (Scheme I). A question which arises is whether these shifts are concerted or whether stable intermediates are involved in the biosynthesis of fernene. The presence of hopene-I (V) and, apparently, neohop-13(18)-ene (II),[1] in *P. vulgare* suggests that these could be intermediates in the formation of fernene (III). However, our results show that fernene retains all six tritium atoms, whereas hopene-I retains only five. If the reasonable assumption is made that the hydrogen atoms arising from the 4-*pro*-R position of MVA retain their identity from squalene through to the first completely cyclized product, as in the case of lanosterol[17] and β-amyrin,[18] then the hydrogen atoms involved in the 1,2-shifts in fernene formation are those arising from the same position of MVA. Therefore, the retention of all of these hydrogens in fernene, biosynthesized from the specifically labelled MVA, precludes the formation of the intermediates just mentioned because of the position of the double bonds present in these compounds. Diploptene and fernene can be chemically converted into neohop-13(18)-ene; however, the isolation of other intermediate olefins formed during these rearrangements indicates that a series of discrete steps are involved.[1]

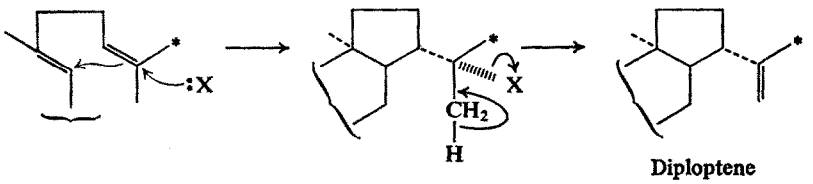
Although the results obtained for diploptene must be viewed with caution, they point to a stereospecific formation of the 22(29) double bond. Thus no change in the ^3H:^{14}C ratio is observed in going from diploptene (I) to the corresponding 29-norketone (see Experimental), suggesting that C-29 arises from the C-3′ of MVA and C-30 from C-2 of MVA. More accurate observations of this type have been reported[19] for lupeol, betulin and betulinic acid and for cyclolaudenol.[2] In the cases so far reported, the introduction of the double bond

[16] See leading references in R. MCCRINDLE and K. H. OVERTON, in *Rodd's Chemistry of Carbon Compounds* (edited by S. COFFEY), Second edition, Vol. IIc, Chapter 14, p. 369, Elsevier (1969).
[17] J. W. CORNFORTH, R. H. CORNFORTH, C. DONNINGER, G. POPJÁK, Y. SHIMIZU, S. ICHII, E. FORCHIELLI and E. CASPI, *J. Am. Chem. Soc.* **87**, 3224 (1965).
[18] H. H. REES, G. BRITTON and T. W. GOODWIN, *Biochem. J.* **106**, 659 (1968).
[19] D. ARIGONI, *Biogenesi delle Sostanze Naturali*, p.1 ff., Accademia Nazionale dei Licei, Rome (1964).



SCHEME I. POSSIBLE BIOSYNTHETIC PATHWAYS FOR FORMATION OF THE TRITERPENE HYDROCARBONS FROM 3*R*-[2-^{14}C,(4*R*),4-$^3H_1$]MEVALONATE [X, X_1 AND X_2 COULD BE NUCLEOPHILIC GROUPS ON AN ENZYME].

follows closely a major event in the biosynthesis of the compounds. Thus in the case of cyclolaudenol the formation of the double bond is linked with the alkylation of the C-24,25 double bond. In diploptene the double bond is introduced at the centre where a nucleophilic group is expected to attack, thus inducing or facilitating the cyclization of squalene. In lupeol the introduction of the double bond is expected to conclude the sequence which results in the formation of ring E. The stereospecificity observed for double bond formation in all these cases is best explained by considering that the rotation of the isopropyl group is severely restricted,[19] as would be the case if the double bond begins to form while the nucleophilic group is not yet completely dissociated from the central carbon atom, as shown below for diploptene (Scheme II).



SCHEME II.

Our results demonstrated that all six tritium atoms which arise from the 4*R*-position of MVA are retained in serratene (IV), and that one of these tritiums is located at C-13, as indicated by the loss of one tritium atom on chemical conversion of serratene into isoserratene. These observations are in agreement with a postulated[1] pathway for the biosynthesis of serratene.

EXPERIMENTAL

General

M.ps were taken on a Kofler block and are uncorrected. U.v. spectra were recorded on a Unicam SP 800 spectrophotometer and i.r. spectra with a Perkin–Elmer Infracord 137. For preparative and analytical TLC silica gel G (Merck) plates (0·25 mm) were used.

Incorporation of 3(*RS*) [2-^{14}C, (4*R*)-4-$^{3}H_1$]-*Mevalonic Acid into the Triterpene Hydrocarbons of* Polypodium vulgare

Sliced rhizomes (6·0 g) and chopped leaves (1·0 g) of *P. vulgare* (collected in February) were incubated with the potassium salt of 3(*RS*)-[2-^{14}C, (4*R*)-4-$^{3}H_1$]-MVA (10 μc of ^{14}C) in water (1 ml) for 24 hr at 25°. Water (1 ml) was added after 2 hr and again after 17 hr. At the end of the incubation, the rhizomes and leaves were allowed to dry before being ground up in ethanol. The crushed material in ethanol (70 ml) was heated under reflux overnight. After filtration the ethanolic extract was extracted with hexane (3 × 30 ml) and the hexane layer washed with water (10 ml) and then evaporated to give a residue (9·6 mg; 3×10^7 counts/min). After addition of carrier squalene and phytosterols, the mixture was saponified by heating under reflux with ethanolic KOH (11 ml; 6%) for 1·5 hr. The residue recovered (139·5 mg) was adsorbed on a column of alumina (neutral; act. III; 15 g). Elution with light petroleum (30 ml) gave a fraction (49·6 mg) which showed the same R_f as squalene on TLC (silica gel; eluent, $CHCl_3$). This fraction was separated into its components by preparative TLC on silica gel–silver nitrate (20%) plates (eluent: light petroleum). The bands were those whose R_f corresponded with those of authentic squalene, diploptene (I), hopene-I (V), serratene (IV) and fernene (III).

The radioactive squalene was purified by preparative TLC (silica gel–silver nitrate; eluent: light petroleum) and then via the thiourea adduct,[20] to give a sample (9·5 mg), specific activity, 4042 dis/min of ^{14}C/mg.

Radioactive fernene was diluted with carrier material (20 mg) and rechromatographed on silica gel–silver nitrate plates. The fraction thus obtained was recrystallized to constant specific activity from ether–methanol to give fernene (III) as needles, m.ps 169–171° (lit.[21] 170–171°). Specific activity for the last two recrystallizations: 2281 and 2225 dis/min of ^{14}C/mg respectively.

[20] L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **99**, 735 (1966).
[21] H. AGETA, K. IWATA and K. YONEZAURA, *Chem. Pharm. Bull. Japan* **11**, 408 (1963).

A similar sequence was employed to obtain radioactively pure serratene (IV), m.p. 234–235° (lit.[14] 237–239°). Specific activity, 136 dis/min of ^{14}C/mg. The fractions containing hopene-I (V) and diploptene (I) were recombined, diluted with carrier material and separated by preparative TLC on silica gel–silver nitrate plates developed with light petroleum–5% benzene. Each fraction was then recrystallized to constant specific activity. (*a*) diploptene (I), m.p. 209–210° (lit.[19] 211–212°), needles from ether–methanol. Specific activity: 231 dis/min of ^{14}C/mg. (*b*) hopene-I (V), m.p. 179–181·5° (lit.[8] 183·5–185°), prisms from ether methanol. Sp. act.: 1400 dis/min of ^{14}C/mg. The mass spectrum showed prominent peaks at *m/e* 410(M^+), 395(M-15), 367(M-43), 231, 203, 191, 189, 175, 161. (Mass spectrum of neohop-13(18)-ene (II): 410(M^+), 395, 367, 218, 205, 191, 189, 175.)[7]

Each of the four triterpenes was examined on GLC (1% neopentylglycol succinate [NGS] on chromosorb W at 240°). The retention times (in brackets those reported in the literature) relative to cholestane are as follows: fernene, 2·96 (2·96;[1] 2·96[9]), serratene, 3·91 (4·00;[1] 4·00[9]), hopene-I, 2·10 (2·10[9]), diploptene, 5·02 (5·00;[1] 5·00[9]). Ikekawa[9] quotes a retention time of 2·70 for neohop-13(18)-ene.

Oxidation of Fernene (III)

Radioactive fernene (III; 25 mg, after dilution with carrier material) was suspended in glacial HOAc (0·3 ml) and stirred vigorously at 55°. CrO_3 (15 mg) was added over a period of 2 hr after which the solution was maintained at 55° for a further 2 hr. The solution was then diluted with $NaHCO_3$ (8%) and the product recovered in the usual way. Purification was effected by preparative TLC (silica gel; eluent $CHCl_3$) and the compound obtained was recrystallized twice from ether to give needles of 12-ketofernene, m.p. 215–218°, ν_{max}^{Nujol} 1674, 1611 cm^{-1}, λ_{max}^{MeOH} 246 nm ($\epsilon = 11{,}000$). (Lit.[13] m.p. 221·5–223°, ν_{max} 1672, 1611 cm^{-1} λ_{max}^{EtOH} 246 nm ($\epsilon = 9000$). Sp. act. 161 dis/min of ^{14}C/mg. The mass spectrum of 12-ketofernene showed peaks at *m/e* 424(M^+), 409, 339, 271 and 135 (cf. with those for ferenene, *m/e* 410, 395, 257, 243, 205 and 149).

Acid Isomerization of Serratene (IV)

Radioactive serratene was dissolved in dry $CHCl_3$ and treated with dry gaseous HCl for 2 hr at room temp. TLC examination of the product isolated showed that reaction was 80% complete. Separation of the product by preparative TLC (silica gel–silver nitrate; eluent: light petroleum) and recrystallization from ether–methanol gave isoserratene (VI), m.p. 179–184° (lit.[14] 184–188°). The purity of isoserratene was confirmed by GLC analysis (NGS–1% at 240°) (RRT relative to cholestane: isoserratene 2·80 (2·78)[9] serratene; 3·91). The mass spectrum of isoserratene showed prominent peaks at *m/e* 410(M^+), 395, 257, 231, 218, 206, 205, 191 (base peak) (cf. serratene 410, 395, 286, 218, 204, 191).

Osmium Tetroxide Oxidation of Diploptene (I)

Radioactive diploptene (I, 8·0 mg) in pyridine (5 ml) was treated overnight with OsO_4 (15 mg). The osmate was decomposed by the method of Baran[22] and the product was purified by preparative TLC (silica gel, eluents: $CHCl_3$–MeOH, 19:1) and recrystallized from ether–methanol to give needles of 22,29-dihydroxy-diploptene, m.p. 249–251° (lit.[15] 239–245°) ^3H:^{14}C; 6·67:6.

The radioactive diol was treated with excess $NaIO_4$ in aqueous dioxan for 2 hr. The reaction product was recrystallized from ether–methanol to give 29-norketodiploptene. The mass spectrum of this compound showed prominent peaks at *m/e* 412(M^+), 397 (M–15), 369 (M–43, loss of CH_3—CO—) and at 191 (base peak) ^3H:^{14}C; 6·56:6.

Acknowledgements—We thank Mrs. A. Ball for the determination of mass spectra, and the SRC and Hoffmann-La Roche for financial support. E. L. G. was the holder of a Wellcome Research Fellowship.

[22] J. S. Baran, *J. Org. Chem.* **25**, 257 (1960).