The Formation of (−)-Kaurene in a Cell-free System from *Gibberella fujikuroi*

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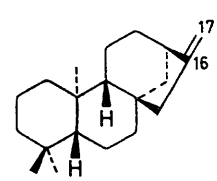
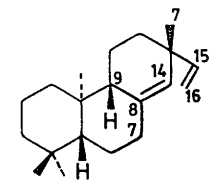
The preparation from *Gibberella fujikuroi* of a cell-free system that converts mevalonate into (−)-kaurene is described. This system is used to show that a pimaradiene intermediate is not involved in the biosynthesis of (−)-kaurene by *Gibberella fujikuroi*.

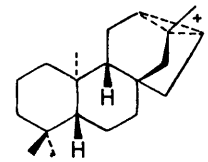
THE tetracyclic diterpene (—)-kaurene (1) occupies a central position in gibberellin biosynthesis.[1] The proposal has been made [2] that the tetracyclic diterpenes are formed by cyclization of tricyclic pimaradienes (2) through the intervention of a non-classical carbonium ion (3). We originally tested this hypothesis by use of the intact fungal system of *Gibberella fujikuroi*.[3] The intervention of a pimaradiene involves the loss of a hydrogen atom from C-7, C-9, or C-14. (—)-Kaurene was shown to incorporate four 4-*pro*-*R*-mevalonoid hydrogen atoms and eight 2-mevalonoid hydrogen atoms, thus excluding as intermediates pimaradienes containing an 8,9- or a 7,8-double bond. A [16-$^3H_2$]-labelled 8,14-unsaturated (—)-pimaradiene was then shown to be specifically incorporated into the tetracyclic diterpene gibberellic acid, but in extremely low radiochemical yield (0·024%) in comparison with the labdadienol pyrophosphate (4) (5·13%) which immediately precedes it in the biosynthesis. Consequently we were uncertain of the status of (—)-pimaradiene as an intermediate although confident of its incorporation. We now report the preparation from *Gibberella fujikuroi* of a soluble cell-free enzyme system which produces (—)-kaurene, and its use in the solution of this problem. Fall and West have recently reported [4] a partial purification of kaurene synthetase [5] and have demonstrated that there are two catalytic activities associated with this system. The first is the cyclization of geranylgeranyl pyrophosphate to copalyl pyrophosphate (labdadienol pyrophosphate) and the second is the cyclization of the copalyl pyrophosphate to (—)-kaurene.



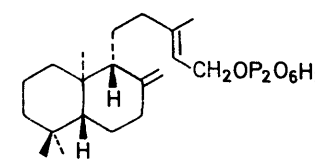
(1)



(2)



(3)



(4)

The mycelium from a young (four-day) shake culture of *Gibberella fujikuroi* was disrupted and centrifuged to remove cell debris and then the microsomes. The supernatant, when supplemented with ATP and magnesium ions, was used to prepare (—)-kaurene from

[1] B. E. Cross, R. H. B. Galt, and J. R. Hanson, *J. Chem. Soc.*, 1964, 295.
[2] E. Wenkert, *Chem. and Ind.*, 1955, 282; A. J. Birch, R. W. Rickards, H. Smith, A. Harris, and W. B. Whalley, *Tetrahedron*, 1959, **7**, 241.
[3] J. R. Hanson and A. F. White, *J. Chem. Soc.* (*C*), 1969, 981.
[4] R. R. Fall and C. A. West, *J. Biol. Chem.*, 1971, **246**, 6913.
[5] C. D. Upper and C. A. West, *J. Biol. Chem.*, 1967, **242**, 3285; *Arch. Biochem. Biophys.*, 1968, **127**, 112.

mevalonate. This preparation had a sharp activity optimum at pH 7 but lost its activity rapidly even when stored at −20°. The optimum co-factor requirements, mevalonate concentration, and time of incubation were found (see Experimental section) for the conversion of mevalonate into (−)-kaurene. Incorporations of up to 74% (based on the 3*R*-isomer of mevalonate) were obtained.

Where similar preparations have been made from *Ricinus communis*,[6] other diterpene hydrocarbons have also been formed. Consequently it was necessary to prove that our preparation was making (−)-kaurene. First, the product from a [2-^{14}C]mevalonate incubation was examined by t.l.c. and then by simultaneous g.l.c.–radiochromatography,[7] and finally by mass spectrometry. The radioactive fraction showed chromatographic behaviour identical with that of (−)-kaurene.

TABLE 1

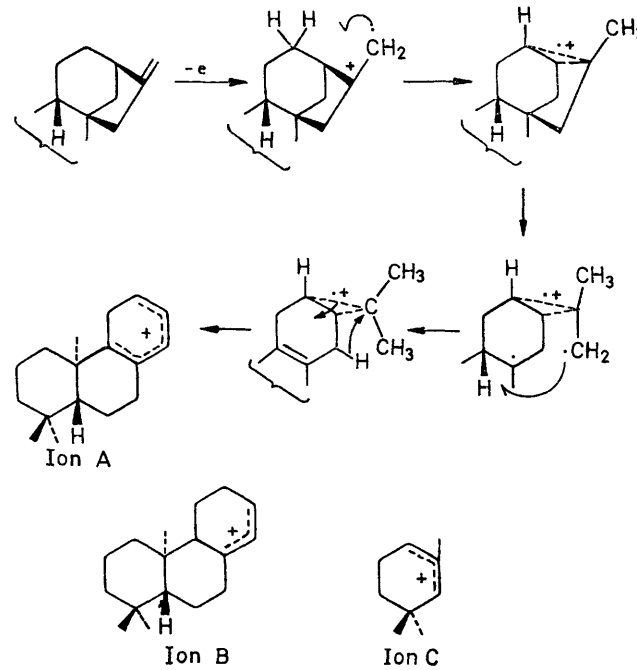
Mass spectra of (−)-kaurene and related compounds

	M^+	$M^+ - CH_3$	Ion A	Ion B	Ion C
Kaurene	272	257	229		123
Biosynthetic kaurene	282	264	235		129
Norkauran-16-one	274	259		231	123
[$^2H_2$]-17-Norkauran-16-one	276	261		231	123
Biosynthetic 17-norkauran-16-one	282	264		237	129
Biosynthetic 17-norkauran-16-one after exchange	280	262		237	129

Second, the biosynthetic material was diluted with inactive (−)-kaurene and converted into the 16,17-diol and thence into (−)-17-norkauran-16-one. There was no change in the specific activity at each step.

[*methyl*-$^2H_3$,2-^{14}C]Mevalonic acid (37·59 μmol [*methyl*-$^2H_3$]mevalonic acid and 77·96 nmol [2-^{14}C]mevalonic acid) [8] was incubated with the enzyme preparation and the (−)-kaurene was isolated. The formation of the 8-double bond of the labdadienol pyrophosphate and the 16,17-double bond of (−)-kaurene would result in the loss of two deuterium atoms. If an 8,14-unsaturated pimaradiene was involved, then a third deuterium atom would be lost. The mass spectrum of the (−)-kaurene produced biosynthetically from [$^2H_3$]mevalonic acid had a molecular ion at 282 showing the presence of ten deuterium atoms. Hence only two deuterium atoms are lost and the (−)-pimaradiene can be excluded as a discrete intermediate. The (−)-kaurene was converted into the 16,17-diol with osmium tetroxide and thence with sodium periodate into (−)-17-norkauran-16-one. The molecular ion of the nor-ketone showed the presence of eight deuterium atoms. After exchange with 2N-sodium hydroxide for 24 h the nor-ketone showed 100% loss of one deuterium atom and a 30% loss of a second. Further exchange in sodium hydroxide for 48 h resulted in a 70% [$^2H_6$]-(−)-17-norkauran-16-one. The ready loss of one deuterium atom and the far more difficult exchange of the second were paralleled by the deuteriation of 17-norkauran-16-one in sodium [^{2}H]hydroxide.

The fragmentation patterns of the hydrocarbon and the nor-ketone (see Table 1) permit the location of six deuterium atoms in two of the methyl groups of ring A, two deuterium atoms at C-15 and two at C-17. The formation of ion A (*m/e* 229) from (−)-kaurene requires the loss of a fragment C_3H_7 ($C_3D_4H_3$ in the deuteriated material). This may be visualized as in Scheme 1.



SCHEME 1

The fragmentation of 17-norkauran-16-one to form ion B (*m/e* 231) requires the loss of a fragment C_2H_3O (C_2D_2HO in the deuteriated material); this again represents the loss of ring D. The fragment *m/e* 123 (ion C) clearly represents ring A; this ion appears at an *m/e* value six mass units higher in the deuteriated product.

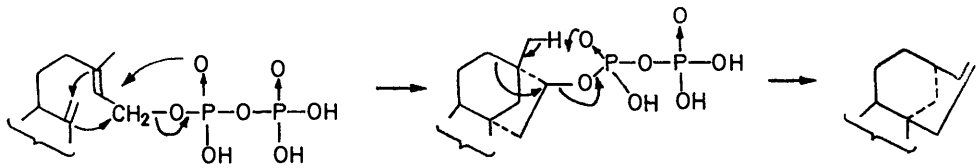
Although these results exclude a discrete pimaradiene intermediate, they do not eliminate an enzyme-bound pimarane with, for example, a stabilized C-8 carbonium ion system. They are also in accord with the illustrated direct cyclization scheme (Scheme 2). The incorporation described in our earlier work [3] was shown to be specific and the degradation revealed the label at a centre to be expected from a (−)-pimaradiene. Consequently this must be a case of a microbiological conversion, by, for example, protonation of the 8,14-double bond. *Gibberella fujikuroi* has been shown to be capable of transforming diterpenoid substances.[9]

[6] D. R. Robinson and C. A. West, *Biochemistry*, 1970, **9**, 80.
[7] G. Popjak, A. E. Lowe, and D. Moore, *J. Lipid Res.*, 1962, **3**, 364.
[8] M. Fetizon, M. Golfier, and J.-M. Louis, *Chem. Comm.*, 1969, 1118.
[9] J. R. Hanson and A. F. White, *Tetrahedron*, 1968, **24**, 6291.

EXPERIMENTAL

General experimental details have been described previously.[3,10]

Radioactive samples were counted in a Packard Tri-Carb Liquid Scintillation spectrometer, model 3375, and t.l.c. plates were scanned for radioactivity with a Packard Radiochromatogram scanner, model T201. G.l.c. was carried out on a Varian 1400 instrument (5 ft × ⅛ in stainless steel column packed with 3% SE 30 on 80—100 mesh Chromosorb W; carrier gas nitrogen at 60 ml min^{-1}; column temperature programmed to rise from 100—220° at 10° min^{-1}). G.l.c.–mass spectra were carried out with an A.E.I. MS 30 spectrometer.



SCHEME 2

Incubation Procedure.—Gibberella fujikuroi (in 1 l of culture medium) was grown as described.[10] After 4 days the mycelium was filtered off, washed with distilled water, and resuspended in an ice-cold 0·2M-potassium phosphate buffer (30 ml) at pH 7·0. This cell suspension was then passed through a French press under 5—6 tons pressure. The resultant broken cell mass was centrifuged at 10,000*g* for 15 min. The resulting supernatant was then centrifuged at 100,000*g* for 1 h. The clear yellow supernatant from this (protein concentration of 7 mg ml^{-1}) was used for the incubations. This preparation was supplemented with ATP and magnesium chloride (see later), the mevalonate was added, and the incubation was carried out in a stoppered flask at 30° for 5 h. At the end of the incubation period, acetone (20 ml) was added and the mixture was extracted with light petroleum; the extract was concentrated and chromatographed on 10% silver nitrate–silica gel which was eluted with 5% benzene in n-hexane. The fractions were assayed for radioactivity and where necessary for the amount of (−)-kaurene present by g.l.c.–radiochromatography on 3% SE 30.

Identification of (−)-*Kaurene.*—Biosynthesized (−)-kaurene (53,200 disint. min^{-1}) was mixed with authentic (−)-kaurene (12·3 mg) and crystallized to a constant activity [m.p. 50° (lit.,[11] 50°)] of 4324 disint. min^{-1} mg^{-1} (1176 disint. min^{-1} μmol^{-1}). The (−)-kaurene (5 mg) in dioxan (3 ml) was treated with osmium tetroxide (5 mg) for 24 h. The solution was treated with hydrogen sulphide and filtered, and the 16,17-dihydroxykaurane was purified by preparative layer chromatography and crystallized from acetone to give needles, m.p. 187—188° (lit.,[11] 189—190°), activity 3899 disint. min^{-1} mg^{-1} (1193 disint. min^{-1} μmol^{-1}). The diol (3 mg) in dioxan (3 ml) was treated with aqueous sodium periodate (8 mg) for 18 h. 17-Norkauran-16-one was recovered in ether and crystallized from ethyl acetate as needles, m.p. 114—116° (lit.,[11] 114—116°), activity 4209 disint. min^{-1} mg^{-1} (1153 disint. min^{-1} μmol^{-1}).

Optimization of Co-factor and Mevalonate Concentrations.—(a) *Variation of ATP and magnesium chloride concentrations.* The incubations (2 ml) contained the 100,000*g* supernatant (1·7 ml), the mevalonic acid [in water (0·1 ml)], and equimolar amounts of the co-factors. The incubations were carried out at 30° for 5 h.

(b) *Variation of* (−)-*kaurene formation with time.* The incubations were carried out at 30° in a final volume of 2 ml comprising the 100,000*g* supernatant (1·7 ml), the mevalonic acid solution (0·1 ml), and the co-factors (20 μmol).

Incorporation of [methyl-$^2H_3$,2-^{14}C]*Mevalonic Acid.*—The incubation contained, in a final volume of 43 ml, the 100,000*g* supernatant (38 ml), ATP (400 μmol), magnesium chloride (400 μmol) and [*methyl*-$^2H_3$,2-^{14}C]mevalonic acid

TABLE 2

Variation of ATP and magnesium chloride concentration

(A) Mevalonic acid 50 nmol, 15,762 disint. min^{-1} (3*R*-isomer)						
ATP and $MgCl_2$ (μmol ml^{-1})	2·5	5·0	10·0	15·0	20·0	25·0
Kaurene (disint. min^{-1})	1361	4113	8314	10,234	10,412	10,448
Kaurene (nmol)	1·08	3·26	6·59	8·12	8·26	8·28
(B) Mevalonic acid 200 nmol, 67,202 disint. min^{-1} (3*R*-isomer)						
Kaurene (disint. min^{-1})	14,243	16,995	33,183	25,811	20,629	6838
Kaurene (nmol)	10·6	12·6	24·68	19·2	15·36	5·08
(C) Mevalonic acid 500 nmol, 173,204 disint. min^{-1} (3*R*-isomer)						
Kaurene (disint. min^{-1})	16,849	34,978	58,643	55,643	55,621	19,727
Kaurene (nmol)	12·15	25·2	42·3	40·14	33·4	14·2

TABLE 3

Variation of kaurene formation with respect to time of incubation

Time (h)	Kaurene (disint. min^{-1})	% Mevalonic acid incorporated	Kaurene (nmol)
(A) Mevalonic acid 50 nmol, 15,762 disint. min^{-1} (3*R*-isomer)			
1	6710	42·6	5·32
2	8196	52·0	6·5
3	9086	57·7	7·21
4	10,088	64·0	8·0
5	11,693	74·2	9·27
6	11,681	74·1	9·26
(B) Mevalonic acid 500 nmol, 173,204 disint. min^{-1} (3*R*-isomer)			
1	28,845	16·7	20·8
2	32,972	19·0	23·8
3	37,652	21·7	27·2
4	44,195	25·5	31·89
5	44,340	25·6	32·0
6	45,864	26·6	33·1

[10] J. R. Hanson and J. Hawker, *J.C.S. Perkin I*, 1972, 1892.
[11] J. R. Hanson, *J. Chem. Soc.*, 1963, 5061.

(37·59 μmol [*methyl*-$^2H_3$]mevalonate; 77·96 nmol [2-^{14}C]-mevalonate). The incubation was carried out anaerobically at 30° for 5 h. Acetone (20 ml) was added and the solution was extracted with light petroleum. The extract was dried and evaporated. The residue was chromatographed on 10% silver nitrate–silica gel and eluted with 5% benzene in n-hexane. The (—)-kaurene had 203,570 disint. min^{-1} activity (23% incorporation) (300 μg). One fifth of this sample was used for mass spectrometry (see Table 1). The remainder was converted into 17-norkauran-16-one (122 μg) as already described. This was divided into two portions. One was submitted to mass spectrometry (see Table 1). The remainder in acetone (1 ml) containing 5N-sodium hydroxide (0·6 ml) was heated under reflux for 24 h and the product was analysed by mass spectrometry. The experiment was then continued for a further 48 h. The deuteriation of 17-norkauran-16-one was followed under identical conditions in [$^2H_6$]acetone and sodium [^{2}H]-hydroxide.

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