Biosynthesis of Natural Products. Part 1. Incorporations of *ent*-Kaur-16-ene and *ent*-Kaur-16-en-15-one into Enmein and Oridonin [1]

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Incorporations of *ent*-kaur-16-ene (7a) and *ent*-kaur-16-en-15-one (14a) into enmein (1a) and oridonin (6a) by *Isodon japonicus* Hara have been demonstrated by tracer experiments with seven labelled *ent*-kaurene derivatives. Furthermore, evidence has been obtained that functionalisation of *ent*-kaur-16-ene at the allylic position C–15 proceeds through direct oxygenation.

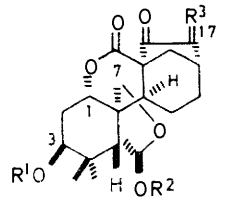
THE diterpenes enmein (1a) [2] and oridonin (6a) [3] are the major bitter principles in the leaves of *Isodon japonicus* Hara, which has been used as a folk medicine for gastro-intestinal disorders in Japan. The biosynthesis of these diterpenes has been thought to proceed through a pathway similar to that for cyclic diterpenes in general, and *ent*-kaur-16-ene (7a) has been regarded as an important precursor. Thus, oridonin (6a) is thought to be formed *via* oxygenation at C-1, -6, -7, -14, -15, and -20 of *ent*-kaur-16-ene (7a) and enmein (1a) *via* oxidative cleavage [4] of the 6,7-bond of an oridonin-like precursor.

The thirty-one diterpenoids isolated from *Isodon* species commonly bear oxygen functions at C-7 and C-15; hence these carbon atoms are thought to be oxidised at an early stage in the biosynthetic route from *ent*-kaur-16-ene (7a). On the basis of these considerations, a biogenetic pathway has been proposed.[5] It was thought that functionalisation at C-15 would occur by one of the following pathways: (i) direct oxygenation at the allylic position of *ent*-kaur-16-ene (7a), (ii) oxygenation at C-15 of *ent*-kaur-15-ene (9a) by singlet oxygen, accompanied by allylic rearrangement (ene reaction), and (iii) epoxidation of (9a) followed by rearrangement. We have now demonstrated that both *ent*-kaur-16-ene (7a) and *ent*-kaur-16-en-15-one (14a) are precursors of enmein (1a) and oridonin (6a), by feeding some [17-^{14}C]-*ent*-kaur-16-ene derivatives to growing *I. japonicus* plants, and have also obtained evidence supporting pathway (i) for the functionalisation at C-15.

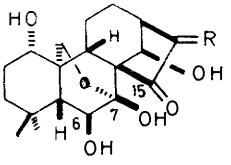
A Wittig reaction under modified conditions of *ent*-17-norkauran-16-one (8) with [^{14}C]methyltriphenylphosphonium iodide gave *ent*-[17-^{14}C]kaur-16-ene (7b) in better yield than reported.[6] Treatment of the product (7b) with iodine in benzene [7] gave the isomer, *ent*-[17-^{14}C]kaur-15-ene (9b). Epoxidation of (9b) with *m*-chloroperbenzoic acid afforded *ent*-15β,16-epoxy[17-^{14}C]kaurane (10). *ent*-Kaur-15-ene (9a) on photosensitised oxygenation yielded *ent*-kaur-16-en-15β-ol (11a),[7] which on acetylation and subsequent ozonolysis gave the 17-nor-16-ketone (13). A Wittig reaction of compound (13) with [^{14}C]methyltriphenylphosphonium iodide followed by hydrolysis yielded *ent*-[17-^{14}C]kaur-16-en-15β-ol (11b). Photosensitised oxygenation of the labelled *ent*-kaur-15-ene (9b) also gave directly the

[1] Preliminary communications, T. Fujita, S. Takao, and E. Fujita, *J.C.S. Chem. Comm.*, 1973, 434; T. Fujita, S. Takao, Y. Nagao, and E. Fujita, *ibid.*, 1974, 666.
[2] E. Fujita, T. Fujita, and M. Shibuya, *Chem. Comm.*, 1966, 297; *Yakugaku Zasshi*, 1967, **87**, 1076.
[3] E. Fujita, T. Fujita, H. Katayama, M. Shibuya, and T. Shingu, *J. Chem. Soc.* (*C*), 1970, 1674.
[4] T. Kubota, T. Matsuura, T. Tsutsui, S. Uyeo, H. Irie, A. Numata, T. Fujita, and T. Suzuki, *Tetrahedron*, 1966, **22**, 1659.
[5] E. Fujita, M. Node, Y. Nagao, and T. Fujita, *Yakugaku Zasshi*, 1974, **94**, 788.
[6] R. A. Bell, R. E. Ireland, and R. A. Partyka, *J. Org. Chem.*, 1966, **31**, 2530; B. E. Cross, R. H. B. Galt, and J. R. Hanson, *J. Chem. Soc.* (*C*), 1964, 295.
[7] M. F. Barnes and J. MacMillan, *J. Chem. Soc.* (*C*), 1967, 361.

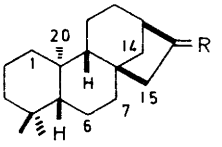
radioactive alcohol (11b) and *ent*-[17-^{14}C]kaur-16-en-15-one (14b). The former on oxidation with chromic



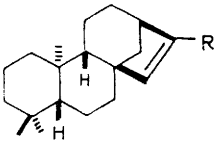
(1a) $R^1 = R^2 = H$, $R^3 = CH_2$
(1b) $R^1 = R^2 = H$, $R^3 = {}^{14}CH_2$
(2a) $R^1 = R^2 = Ac$, $R^3 = CH_2$
(2b) $R^1 = R^2 = Ac$, $R^3 = {}^{14}CH_2$
(3) $R^1 = Ac$, $R^2 = H$, $R^3 = {}^{14}CH_2$
(4a) $R^1 = R^2 = H$, $R^3 = \alpha\text{-}CH_3\ \beta\text{-}H$
(4b) $R^1 = R^2 = H$, $R^3 = \alpha\text{-}{}^{14}CH_3\ \beta\text{-}H$
(5) $R^1 = R^2 = Ac$, $R^3 = \alpha\text{-}CH_3\ \beta\text{-}H$



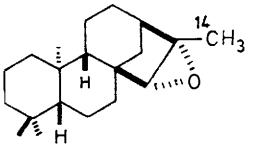
(6) a; $R = CH_2$
b; $R = {}^{14}CH_2$



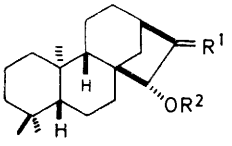
(7a) $R = CH_2$
(7b) $R = {}^{14}CH_2$
(8) $R = O$



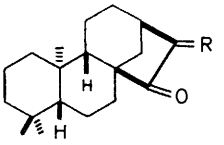
(9) a; $R = CH_3$
b; $R = {}^{14}CH_3$



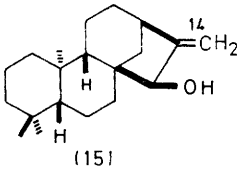
(10)



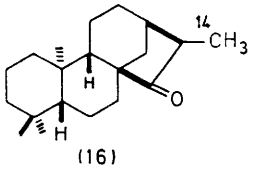
(11a) $R^1 = CH_2, R^2 = H$
(11b) $R^1 = {}^{14}CH_2, R^2 = H$
(12) $R^1 = CH_2, R^2 = Ac$
(13) $R^1 = O$, $R^2 = Ac$



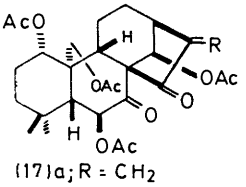
(14) a; $R = CH_2$
b; $R = {}^{14}CH_2$



(15)



(16)



(17) a; $R = CH_2$
b; $R = {}^{14}CH_2$

anhydride–pyridine gave the latter,[7] which on reduction with sodium borohydride yielded *ent*-[17-^{14}C]kaur-16-en-15α-ol (15),[7] the 15-epimer of (11b). Treatment with concentrated hydrochloric acid converted (15) into *ent*-[17-^{14}C]kauran-15-one (16).[7]

Three methods for feeding, *i.e.*, inoculation, hydroponics, and application to the leaves, were examined with *ent*-[17-^{14}C]kaur-16-ene (7b). Application to the leaves proved to be the most suitable.

The labelled compounds (7b), (9b), (10), (11b), (14b), (15), and (16), dissolved in acetone, respectively, were applied to the reverse sides of leaves of growing *I. japonicus* plants, and the leaves were harvested after a week. The methanolic extracts of the leaves were fractionated as described in the Experimental section, and the crude enmein and oridonin were isolated from the neutral fraction. Enmein was converted into the diacetate (2a or b), which was purified by preparative t.l.c. and recrystallisation. Oridonin was also acetylated to give the tetra-acetate (17a or b) and purified. The results of incorporation experiments are summarised in the Table.

Both *ent*-kaur-16-ene (7b) and *ent*-kaur-16-en-15-one (14b) were incorporated into enmein and oridonin, although the incorporation ratios were different in two experiments. *ent*-Kaur-15-ene (9b), the 15,16-epoxide (10), the alcohol (11b), and *ent*-kauran-15-one (16) were not incorporated into either diterpene. *ent*-Kaur-15-en-15α-ol (15) was incorporated into enmein (experiment no. 10) but not into oridonin (experiment no. 11). These results exclude pathways (ii) and (iii) for functionalisation at C-15 of the kaurene nucleus, but support pathway (i), that is, direct oxygenation at the allylic position of *ent*-kaur-16-ene. Similar direct oxygenation at an allylic position has been observed during a study of peroxidation of cholesterol with microsomes from rat liver.[8] The ketone (14a) may be formed *via* hydroperoxidation from the less hindered α-side of ring D in *ent*-kaur-16-ene (7a) followed by dehydration.

In order to demonstrate the specific incorporation of ^{14}C at C-17 of the diterpenes, ozonolyses of labelled enmein diacetate (2b) and oridonin tetra-acetate (17b) [9] were carried out. Formaldehyde produced was trapped as its dimedone derivative and its radioactivity was counted. Enmein diacetate (2b) and oridonin tetra-acetate (17b) obtained from the plant to which compound (7b) or (14b) had been fed showed almost complete localisation of radioactivity at C-17. In contrast, recovery of the radioactivity from enmein diacetate (2b) obtained from the plant to which compound (15) had been fed, was 58.1%. This enmein diacetate (3.87×10^4 disint. min^{-1} mmol^{-1}) was partially hydrolysed to the 3-monoacetate (3),[2] which showed the same radioactivity (3.57×10^4 disint. min^{-1} mmol^{-1}) (57.4% radioactivity at C-17). Generally, isolation of pure dihydroenmein and enmein from a mixture of the two is very difficult, and it was thought that the presence of dihydroenmein might be the cause of the low recovery observed. However, enmein obtained from the *Isodon japonicus* plant

[8] L. L. Smith and J. I. Teng, *J. Amer. Chem. Soc.*, 1974, **96**, 2640.
[9] E. Fujita, M. Taoka, M. Shibuya, and T. Fujita, *J.C.S. Perkin I*, 1973, 2277.

has been shown not to be contaminated by dihydroenmein (4a),[2] and the n.m.r. spectrum of the radioactive enmein diacetate obtained from the plant to which compound (15) had been fed gave no evidence of contamination by dihydroenmein diacetate. The low localisation of the radioactivity at C-17 in this case may however be explicable as follows: (i) the 15β-ol (15) is (enzymically or non-enzymically) converted into *ent*-[17-^{14}C]kauran-15-one (16) by a garryfoline–cuauchichicine-type rearrangement,[7] and then into dihydroenmein (4b); (ii) the content of dihydroenmein is so low that it was not detected by the n.m.r. spectrometry; and (iii) the dihydroenmein (4b) has a higher specific radioactivity than the enmein. We investigated whether *ent*-kauranone (16) was converted into dihydroenmein (4b), as described in the Experimental section, but no evidence was obtained for the route from (15) to (4b) *via* (16) in the plant. A further detailed investigation will be required.

Results of the feeding experiments

Expt. no.	Compound fed	mg	disint. min^{-1} mmol^{-1} × 10^{-9}	Radioactivity fed (disint. min^{-1} × 10^{-7})	Enmein diacetate: Yield (mg)	disint. min^{-1} mmol^{-1} × 10^{-4}	Incorporation (%)	Oridonin tetra-acetate: Yield (mg)	disint. min^{-1} mmol^{-1} × 10^{-4}	Incorporation (%)
1	(7b)	9.4	0.399	1.38	40	0.642	0.004	40	0.544	0.003
2	(7b)	7.6	6.91	19.2	83.6	1.29	0.001	35	1.04	0.0004
3	(9b)	10.4	6.62	25.4	143	0	0	Trace [a]		
4	(9b)	6.9	7.76	19.7	47.9	0	0	82.6	0	0
5	(10)	10.2	6.79	24.1	49	0	0	56.3	0	0
6	(11b)	4.3	6.48	9.67	2.3 [a]			33.3	0	0
7	(11b)	5.4	3.92	7.35	53.7	0	0	36.2	0	0
8	(14b)	3.5	5.89	7.27	185	4.12	0.02	Trace [a]		
9	(14b)	4.6	3.99	6.42	19	1.58	0.001	7.5	1.69	0.0004
10	(15)	4.9	6.03	10.3	140	3.87	0.01	Trace [a]		
11	(15)	4.5	4.06	6.35	64.3			71.0	0	0
12	(16)	2.9	4.02	4.05	125	0	0	20.5	0	0

[a] In these cases, dilution with the corresponding non-labelled compounds was used, but no definite conclusion was obtained.

The recovery of the radioactivity from the leaves to which *ent*-[17-^{14}C]kaur-16-ene (7b) had been fed was very low (10.6—10.9%). A similar observation has been reported by Bennett *et al.*,[10,11] in feeding experiments with (7b). They assumed that oxidative cleavage of the exocyclic methylene group was occurring, to give carbon dioxide. We tried to trap the carbon dioxide with 2-aminoethanol during 3 h after the administration of (7b), but only obtained a trace of radioactive material corresponding to only 0.04% recovery of the total activity, contrary to the previous assumption. From a sublimate collected as a solution in hexane from the inside of the polyethylene bag covering the plant, however, 11.6% of the radioactivity was recovered. T.l.c. suggested the presence of a polar product, presumably an oxidised kaurene derivative. From the methanolic extract of the leaves, 56.9% of the radioactivity was recovered in this case.

The results of the present investigations have shown that *ent*-kaur-16-ene (7a) is an important precursor of the diterpenes of *Isodon japonicus*, and that triplet oxygen species is involved in oxygenation at the allylic position of (7a). However, it remains uncertain whether oxygenation of the *Isodon* diterpenes at C-15 is the first biosynthetic step.

EXPERIMENTAL

M.p.s were taken with a micro hot-stage apparatus. I.r. spectra were recorded for KBr discs with a Hitachi EPI-S2 spectrometer, and n.m.r. spectra with a Varian T-60 spectrometer for solutions in deuteriochloroform (tetramethylsilane as internal standard). Mass spectra were determined with a JEOL JMS-01SG double-focusing spectrometer. Kieselgel G (0.05—0.2 mm; Merck) was used for column chromatography, and Kieselgel GF_{254} plates (Merck) for t.l.c. Radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer model 3320; samples were dissolved in a scintillation mixture consisting of toluene (10 ml), 2,5-diphenyloxazole (PPO) (50 mg), and 2,2′-*p*-phenylenebis-(5-phenyloxazole) (POPOP) (1 mg), or dioxan (10 ml), naphthalene (1 g), PPO (70 mg), and POPOP (5 mg). The radioactive diterpenes (2b), (17b), and (3) were recrystallised to constant radioactivity and counted for 100 min, because of the low activity level. Radioscanning of chromatographic plates was carried out with an Aloka JTC-201 chromatogram scanner. Specific activities of samples diluted with non-radioactive material are expressed as values before dilution.

ent-[17-^{14}C]*Kaur*-16-*ene* (7b).—A solution (0.45 ml) of potassium t-butoxide [from potassium (0.2 g) and t-butyl alcohol (5 ml)] was added to a stirred suspension of [^{14}C]-methyltriphenylphosphonium iodide (221 mg) in dry tetrahydrofuran (THF) (3.5 ml) under nitrogen, and the mixture was stirred for 15 min. Then, a solution of *ent*-17-norkauran-16-one (8) (74 mg) in dry THF (4 ml) was added, and after 20 min a further solution of (8) (25 mg) in dry THF (1.5 ml) was added. The mixture was stirred for a further 20 min. The solvent was evaporated off *in vacuo*, and the residue was extracted with 4 : 1 n-hexane–methanol. The organic layer was washed with brine, dried, and evaporated, and the residue was subjected to column chromatography (elution with light petroleum) to give needles (85 mg) of *ent*-[17-^{14}C]kaur-16-ene (7b), radioactivity 3.99 × 10^{8} disint. min^{-1} mmol^{-1}, having the same R_F value as authentic *ent*-kaur-16-ene (7a) on t.l.c. and giving a single radioactive peak.

[10] R. D. Bennett, S.-T. Ko, and E. Heftmann, *Plant Physiol.*, 1966, **41**, 1360.

[11] R. D. Bennett, E. R. Lieber, and E. Heftmann, *Phytochemistry*, 1967, **6**, 1107.

ent-[17-^{14}C]*Kaur*-15-*ene* (9b).—To a solution of *ent*-[17-^{14}C]kaur-16-ene (7b) (6.91×10^9 disint. min^{-1} mmol^{-1}; 38 mg) in dry benzene (5.5 ml) was added iodine (3 mg), and the mixture was refluxed for 7 h. The solution was washed with 5% sodium thiosulphate and water, dried (Na_2SO_4), and evaporated to give a crystalline residue. Preparative t.l.c. (petroleum–chloroform, 3 : 1) gave *ent*-[17-^{14}C]kaur-15-ene (9b) (22.4 mg) as needles (6.62×10^9 disint. min^{-1} mmol^{-1}) and the starting material (7b) (8.5 mg). Each compound showed a single radioactive peak on t.l.c. and was identical with an authentic sample.

ent-15β,16-*Epoxy*[17-^{14}C]*kaurane* (10).—To a solution of *ent*-[17-^{14}C]kaur-15-ene (9b) (6.62×10^9 disint. min^{-1} mmol^{-1}; 11.2 mg) in benzene (1.5 ml) was added a solution of *m*-chloroperbenzoic acid (60 mg) in benzene (0.5 ml), and the mixture was left at room temperature overnight. It was washed with 5% sodium thiosulphate, 1% sodium hydrogen carbonate, and water, dried (Na_2SO_4), and evaporated, and the crystalline residue was purified by preparative t.l.c. to yield *ent*-15β,16-epoxy[17-^{14}C]kaurane (10) (11 mg) (6.79×10^9 disint. min^{-1} mmol^{-1}), identical with authentic epoxide, and showing a single radioactive peak and the same R_F value on t.l.c.

Acetylation of ent-*Kaur*-16-*en*-15β-*ol* (11a).—The alcohol (11a) [7] (105 mg) was treated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) at room temperature overnight. Methanol was added and the mixture was set aside for several hours. Evaporation *in vacuo* left a residue which was dissolved in benzene; the solution was washed with 1% hydrochloric acid and brine, dried (Na_2SO_4), and evaporated to give an amorphous acetate (12) (105 mg), δ 2.07 (3 H, s, OAc).

Ozonolysis of ent-*Kaur*-16-*en*-15β-*yl Acetate* (12).—The foregoing crude acetate (12) (100 mg) was dissolved in absolute methanol (3 ml) and chloroform (1 ml), and ozonised at −60 to −70 °C for 7 min. After removal of ozone by passing nitrogen a few drops of dimethyl sulphide were added and the mixture was stirred for 3 h at room temperature. Evaporation *in vacuo* left a residue which was dissolved in chloroform; the solution was washed with water, dried, and evaporated to give a crystalline substance, which was recrystallised from methanol to yield ent-16-*oxo*-17-*norkauran*-15β-*yl acetate* (13) (70 mg) as needles, $\nu_{max.}$ 1 770 and 1 757 cm^{-1}, δ 0.83, 0.87, and 1.12 (each 3 H, s, OAc) (Found: M^+, 332.234. $C_{21}H_{32}O_3$ requires *M*, 332.235).

Wittig Reaction with ent-16-*Oxo*-17-*norkauran*-15β-*yl Acetate* (13).—To a stirred suspension of [^{14}C]methyltriphenylphosphonium iodide (6.41×10^9 disint. min^{-1} mmol^{-1}; 80.8 mg) in dry THF (1 ml) in a stream of nitrogen was added a solution (0.2 ml) prepared from potassium (0.2 g) and t-butyl alcohol (5 ml) and the mixture was stirred for 30 min. Then, a solution of the ketone (13) (50 mg) in dry THF (1.5 ml) was added and the mixture was stirred for 2 h. A few drops of methanol were added to dissolve the precipitate, and the solution was stirred for a further 2 h after additions of the foregoing solution (1 ml) of potassium t-butoxide and water (1 ml). Evaporation *in vacuo* left a residue, which was extracted with 1 : 4 n-hexane–methanol. The n-hexane layer was washed with brine, dried (Na_2SO_4), and evaporated to dryness. The residue was purified by preparative t.l.c. [chloroform–acetone (10 : 1)] to give a crystalline substance. Recrystallisation from methanol gave *ent*-[17-^{14}C]kaur-16-en-15β-ol (11b) (9.2 mg) as needles (6.48×10^9 disint. min^{-1} mmol^{-1}), identical with the authentic alcohol (11a) on t.l.c. and showing a single radioactive peak.

Photosensitised Oxygenation of ent-[17-^{14}C]*Kaur*-15-*ene* (9b).—Through a solution of *ent*-[17-^{14}C]kaur-15-ene (9b) (42.8 mg) and haematoporphyrin (7 mg) in dry pyridine (3.5 ml), oxygen was passed during irradiation with fluorescent lamps (20 W × 4) for 96 h. The mixture was evaporated *in vacuo* below 40 °C to leave a residue, to which ethanol (1.7 ml), acetic acid (0.025 ml), and sodium iodide (80 mg) were added, and the mixture was set aside overnight. Evaporation *in vacuo* left a residue which was extracted with ether after addition of 5% sodium thiosulphate. Work-up of the ethereal extract gave a crude mixture which was separated by preparative t.l.c. to yield *ent*-[17-^{14}C]kaur-16-en-15β-ol (11b) (18 mg) (3.92×10^9 disint. min^{-1} mmol^{-1}) as needles, and *ent*-[17-^{14}C]kaur-16-en-15-one (14b) (7.8 mg) (3.99×10^9 disint. min^{-1} mmol^{-1}) as crystals. Each compound was identical with an authentic sample, and showed a single radioactive peak on t.l.c.

Oxidation of ent-[17-^{14}C]*Kaur*-16-*en*-15β-*ol* (11b).—To stirred dry pyridine (0.5 ml) were added chromic oxide (24 mg) at 0 °C and, after 10 min, a solution of the labelled alcohol (11b) (14 mg) in dry pyridine (0.75 ml). The mixture was stirred for 4 h at room temperature, and extracted with ether (20 ml). The extract was washed with 1% sodium hydrogen carbonate, 10% hydrochloric acid, and brine, dried (Na_2SO_4), and evaporated to dryness. The crystalline residue was purified by preparative t.l.c. to give the ketone (14b) (10 mg) (5.89×10^9 disint. min^{-1} mmol^{-1}), identical with an authentic sample (14a) and showing a single radioactive peak.

ent-[17-^{14}C]*Kaur*-16-*en*-15α-*ol* (15).—A solution of the ketone (14b) (6 mg) in dry methanol (3 ml) was treated with sodium borohydride (5 mg) at 0 °C for 1.5 h, then evaporated *in vacuo*. The residue was extracted with benzene and the extract was washed with brine, dried (Na_2SO_4), and evaporated below 30 °C to dryness. The crude crystals were purified by preparative t.l.c. [benzene–ether (9 : 1)] to afford *ent*-[17-^{14}C]kaur-16-en-15α-ol (15) (5.3 mg) (6.03×10^9 disint. min^{-1} mmol^{-1}), identical with an authentic sample, and showing a single radioactive peak on t.l.c.

ent-[17-^{14}C]*Kauran*-15-*one* (16).—To a solution of the ketone (15) (4.06×10^9 disint. min^{-1} mmol^{-1}; 4.5 mg) in methanol (1 ml) and ether (0.5 ml) was added concentrated hydrochloric acid (0.2 ml), and the mixture was stirred at room temperature for 4 h. Evaporation left a residue which was dissolved in ether; the solution was washed with brine, dried ($MgSO_4$), and evaporated. The crystalline residue was purified by preparative t.l.c. [benzene–ether (9 : 1)] to give *ent*-[17-^{14}C]kauran-15-one (16) (2.9 mg) (4.02×10^9 disint. min^{-1} mmol^{-1}), identical with an authentic sample and showing a single radioactive peak on t.l.c.

Methods of Feeding.—(i) A solution of [17-^{14}C]material in acetone [(7b) (9.4 mg) in acetone (10 ml)] was applied to the reverse sides of the leaves of a growing *I. japonicus* plant, and the leaves were harvested after 7 days (experiment 1).

(ii) A solution of the labelled compound in acetone (*ca.* 25 ml) was divided in two parts. Half was fed to a plant, and after 3 days the other half was fed to the same plant. The leaves were harvested after 7 days from the first feeding (experiments 2, 3, 5, 6, 8, and 10).

(iii) A solution of the labelled compound in acetone was

fed to two *I. japonicus* plants, and the leaves were harvested after 7 days (experiments 4, 7, 9, 11, and 12).

Isolations of Enmein and Oridonin.—The leaves were extracted twice with methanol (600 ml) under reflux for 2 h, and the extract was concentrated *in vacuo* (to *ca.* 300 ml). Then water (30 ml) was added and the mixture was extracted with hexane (250 ml × 3). The methanolic layer was evaporated *in vacuo* to leave a residue, which was fractionated with ethyl acetate (250 ml) and water (150 ml). The neutral substance isolated from the ethyl acetate fraction in the usual way was subjected to chromatography on a silicic acid column and subsequent preparative t.l.c. (chloroform–acetone, 7 : 3) to give crude enmein and oridonin.

Enmein Diacetate.—Experiment 1. A solution of enmein (1b) (52 mg) in dry pyridine (0.5 ml) and acetic anhydride (0.5 ml) was kept at room temperature overnight, and treated as usual to give a crude diacetone. It was purified by the preparative t.l.c. (chloroform–acetate, 8 : 2) followed by recrystallisation from methanol to yield enmein diacetate (2b) (40 mg), m.p. 165—167° (6.42×10^3 disint. min^{-1} $mmol^{-1}$) (Found: M^+, 446.193. Calc. for $C_{24}H_{30}O_8$: M, 446.194), identical with authentic enmein diacetate (2a) hemihydrate,[12] m.p. and mixed m.p. 165—167°. A similar procedure was used for the rest of the series.

Oridonin Tetra-acetate.—Experiment 1. To the crude oridonin (6b) (47 mg) were added acetic anhydride (0.5 ml) and boron trifluoride–ether (1 drop), and the mixture was kept at room temperature for 1 h. Addition of methanol and subsequent evaporation *in vacuo* left a residue, which was subjected to preparative t.l.c. (chloroform–acetone, 8 : 2) to give a crystalline product. Recrystallisation from methanol gave prisms of [17-^{14}C]oridonin tetra-acetate (17b) (40 mg), m.p. 240—242° (5.44×10^3 disint. min^{-1} $mmol^{-1}$) (Found: M^+, 532.226. Calc. for $C_{28}H_{36}O_{10}$: M, 532.231), identical with an authentic sample (17a) [9] (mixed m.p.). The same procedure was used for the rest of the series.

Ozonolysis of Labelled Enmein Diacetate (2b).—(i) *Experiment 1.* A solution of the labelled enmein diacetate (2b) (10.344 mg) diluted with (2a) (34.449 mg) in chloroform (2 ml) was ozonised at −60 °C in the usual way. After removing the ozone gas in the vessel with nitrogen, the solvent was evaporated off *in vacuo* at room temperature to leave a residue, which, after addition of water, was distilled. The distillate (*ca.* 14 ml) was led into a solution of dimedone (100 mg) in acetate buffer (20 ml; pH 4.50). The crystals (6.6 mg) of formaldehyde dimedone derivative which precipitated during 2 days at 0 °C were recrystallised from ethanol to give needles, m.p. 189—190° (7.44×10^3 disint. min^{-1} $mmol^{-1}$; recovery of radioactivity from C-17 115%; 5.4 mg).

(ii) *Experiment 2.* A solution of (2b) (18.8 mg) in ethyl acetate (3 ml) was ozonised at room temperature in the usual way. To the mixture were added zinc powder (20 mg), water (2 ml), and acetic acid (1 drop), and after stirring for 5 h at room temperature the mixture was left overnight. To the separated water layer was added a solution of dimedone (50 mg) in water (10 ml), and the mixture was set aside overnight. The crystals which precipitated were recrystallised from methanol to give formaldehyde dimedone derivative, m.p. 189—190°, as needles (1.21×10^4 disint. min^{-1} $mmol^{-1}$; recovery of radioactivity from C-17 93.8%; 2 mg). Ozonolyses were similarly carried out in experiments 8—10; recoveries of radioactivity from C-17 were 98.5, 89.9, and 58.1%, respectively.

Ozonolysis of Labelled Oridonin Tetra-acetate (17b).—The procedure for enmein diacetate in ethyl acetate was applied to the ozonolysis of oridonin tetra-acetate in experiments 1 and 9; recoveries of radioactivity from C-17 were 89 and 103%, respectively.

[17-^{14}C]-*Enmein 3-Acetate* (3).—*Experiment 10.* To a suspension of (2b) (30 mg) in water (4 ml) was added oxalic acid (10 mg), and the mixture was refluxed for 1 h. The usual work-up gave a crystalline product, which was purified by preparative t.l.c. (chloroform–acetone, 8 : 2) and recrystallised from methanol to yield enmein 3-acetate (3) (11.4 mg), m.p. 286—290° (decomp.) (3.57×10^4 disint. min^{-1} $mmol^{-1}$), identical with an authentic sample [2] (mixed m.p.).

Ozonolysis of [17-^{14}C]*Enmein 3-Acetate* (3).—Labelled enmein 3-acetate (3) (3.428 mg) was mixed with non-labelled material (28.527 mg) and dissolved in ethyl acetate (10 ml). Ozonolysis afforded formaldehyde, which was trapped as needles of its dimedone derivative, m.p. 189—190° (2.05×10^4 disint. min^{-1} $mmol^{-1}$; 6.4 mg).

Search for Radioactive Dihydroenmein.—Experiment 12. The mother liquors from enmein diacetate (2a) were investigated for the presence of dihydroenmein diacetate. They were combined and evaporated to leave a residue (62 mg), which, dissolved in chloroform (2 ml) and absolute methanol (2.5 ml), was subjected to ozonisation at −30 to −40 °C. After decomposition of the ozonide with dimethyl sulphide, the solvent was evaporated off and the residue was subjected to preparative t.l.c. (chloroform–acetone, 8 : 2). Material having the same R_F value as dihydroenmein diacetate (5) was extracted with chloroform–methanol (1 : 1) (50 ml), mixed with a sample of (5) (50 mg) and recrystallised from methanol. Dihydroenmein diacetate thus obtained was radioinactive.

Trapping of Carbon Dioxide from Feeding Experiments.—A solution of *ent*-[17-^{14}C]kaur-16-ene (7b) (1.29×10^6 disint. min^{-1} ; 0.88 mg)in acetone was applied to the reverse sides of the leaves of an *I. japonicus* plant, covered with a polyethylene bag having two holes. Air was passed from one hole into the bag, and the gas in the bag was passed into toluene (10 ml) and then into a solution of 2-aminoethanol (500 mg) in dioxan (10 ml). The radioactivity trapped in toluene was 2.4×10^3 disint. min^{-1} (0.2%); that in the 2-aminoethanol was only 4.75×10^2 disint. min^{-1} (0.04%). The inside of the bag was washed with hexane (50 ml); the radioactivity recovered was 1.5×10^5 disint. min^{-1} (11.6%). The extract of the leaves with 90% methanol showed radioactivity of 7.35×10^5 disint. min^{-1} (56.9% of the total radioactivity fed).

[12] E. Fujita, T. Fujita, K. Fuji, and N. Ito, *Tetrahedron*, 1966, **22**, 3423; *cf.* m.p. 223—225° for anhydrous enmein diacetate.

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