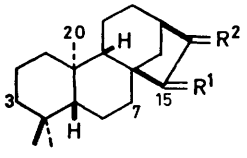
Biosynthesis of Natural Products. Part 4.[1] Biosynthesis of Enmein and Oridonin from Mono- or Di-oxygenated Kaurenoids

By **Tetsuro Fujita,*** Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770, Japan
Sachiko Takao and **Eiichi Fujita,** Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan

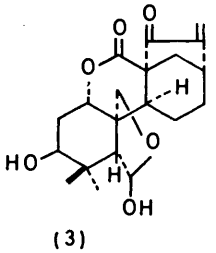
Incorporation of mono-oxygenated *ent*-kaur-16-ene derivatives (6a), (7a), and (8a) and dioxygenated derivatives (9a) and (12a) into both enmein (3) and oridonin (4) and those of C-20 or C-3 oxygenated *ent*-kaurene derivatives (13a), (14a), (15a), and (16a) into (3) by *Isodon japonicus* Hara have been demonstrated by the tracer experiments using the corresponding labelled compounds. It is probable, on the basis of the experimental results, that the positions in (3) and (4) which are similarly oxygenated are not necessarily oxygenated simultaneously at an early stage in the biosynthetic route from *ent*-kaurene (1). However, control of the biosynthesis of enmein (3) may be initiated by the introduction of the C-3 oxygen-function of *ent*-kaurene (1) at an early stage.

RECENTLY, we reported the incorporation of *ent*-kaur-16-ene (1) and *ent*-kaur-16-en-15-one (2a) into enmein (3) and oridonin (4) and the direct oxygenation at the C-15 atom in (1) by *Isodon japonicus* Hara.[2] It has not been clarified, however, whether the oxygenation of the *Isodon* diterpenes at C-15 occurs at the first step in the biosynthesis of (3) and (4) or not. All the *Isodon* diterpenoids reported so far [3-6] except for inflexin (5) [7] bear oxygen functions at C-7 and C-15,[2] hence the C-7 atom is also oxidised, presumably at an early stage.

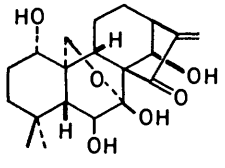
Thus, we investigated first whether 7-oxygenated *ent*-kaurene derivatives, *ent*-kaur-16-en-7α-ol (6a), *ent*-kaur-16-en-7-one (7a), *ent*-kaur-16-en-7β-ol (8a), and *ent*-kaur-16-en-15-on-7α-ol (9a) are available as precursors in the biosynthesis of enmein (3) and oridonin (4) or not.



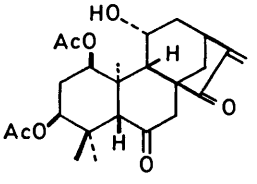
(1) $R^1 = H_2$, $R^2 = CH_2$
(2a) $R^1 = O$, $R^2 = CH_2$
(2b) $R^1 = O$, $R^2 = {}^{14}CH_2$
(2c) $R^1 = O$, $R^2 = C^3H_2$



(3)

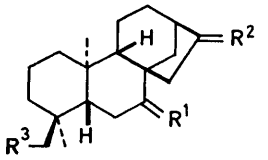


(4)

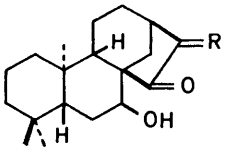


(5)

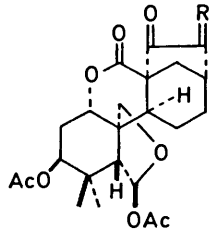
[17-^{14}C]Labelled compounds, 7β-ol (6b), 7-one (7b), 7α-ol (8b), and 15-oxo-7β-ol (9b), synthesised *via* a reported route,[8] were dissolved in acetone, and each solution was applied to the lower side of the leaves of growing *Isodon japonicus* plants. The leaves were harvested after a week, and extracted with methanol. From the methanolic extracts, the crude enmein and oridonin were isolated as described in the Experimental



(6a) R^1 = α—H, β—OH, $R^2 = CH_2$, R^3 = H
(6b) R^1 = α—H, β—OH, $R^2 = {}^{14}CH_2$, R^3 = H
(7a) R^1 = O, $R^2 = CH_2$, R^3 = H
(7b) R^1 = O, $R^2 = {}^{14}CH_2$, R^3 = H
(7c) R^1 = O, $R^2 = CH_2$, $R^3 = {}^3H$
(8a) R^1 = α—OH, β—H, $R^2 = CH_2$, R^3 = H
(8b) R^1 = α—OH, β—H, $R^2 = {}^{14}CH_2$, R^3 = H



(9a) R = CH_2
(9b) R = $^{14}CH_2$



(10a) R = CH_2
(10b) R = $^{14}CH_2$

section. Enmein and oridonin were purified as the diacetate (10a) and the tetra-acetate (11a), respectively.† Ozonolysis of radioactive enmein diacetate (10b) and oridonin tetra-acetate (11b) gave formaldehyde. It was trapped as its dimedone derivative, and its radioactivity was counted. The results are summarised in Table 1, which indicates the incorporation of (6b), (7b), and (9b) into enmein (3) and oridonin (4), and of (8b) into enmein. Although these incorporation ratios are very low, the

† The same treatment and counting of the radioactivity with the purified acetates was carried out in all the following experiments.

TABLE 1

Results of the feeding experiments with the ^{14}C-labelled compounds

Expt.	Compound fed: Amount (mg)	Compound fed: Disint. min^{-1} $mmol^{-1} \times 10^{-9}$	Radioactivity fed: Disint. $min^{-1} \times 10^{-7}$	Enmein diacetate: Yield (mg)	Enmein diacetate: Disint. min^{-1} $mmol^{-1} \times 10^{-4}$	Enmein diacetate: Incorporation (%)	Enmein diacetate: Radioactivity at C-17 (%)
1	(6b) 5.1	3.70	6.56	60.0	1.04	0.0021	97.1
2	(7b) 8.2	3.67	10.5	32.5	7.91	0.0054	102.3
3	(8b) 9.6	3.69	12.3	97.0	0.872	0.0015	108.9
4	(9b) 11.0	3.71	13.5	35.0	6.22	0.0035	100.2

Oridonin tetra-acetate: Yield (mg)	Disint. min^{-1} $mmol^{-1} \times 10^{-4}$	Incorporation (%)	Radioactivity at C-17 (%)
72.5	0.785	0.0016	95.0
35.0	6.69	0.0042	90.0
3.2 [a]			
30.5	6.64	0.0028	97.9

[a] In this case, dilution with (11a) was used, but no definite conclusion was obtained.

almost complete localisation of the radioactivity in C-17 is shown. The incorporation of (8b) into oridonin remains unresolved because of the very low yield of oridonin. These results show that 7-oxygenated compounds as well as the 15-oxo-compound (2a) are available as potential precursors of the *Isodon* diterpenes.

In our tracer experiments [2] so far, we have experienced a large variation in the incorporation percentage of the same compound in individual plants. (See the Experimental results in Part 1 [2] of this series). Hence, in order to compare the relative efficiency of two mono-oxygenated compounds as precursors of enmein (3) and oridonin (4), the compounds labelled with different radioisotopes were fed simultaneously to the same plant. Thus, ^{3}H-labelled compound (7c) was synthesised,[8] and the mixtures of (7c) + (6b) and (7c) + (8b) were applied to the plants, respectively. The results of the feeding experiments are summarised in Table 2. Incorporation of the 7β-hydroxy-compound (6b) and 7-oxo-compound (7c) into enmein (3) show a close similarity (see expt. 5), but the 7α-hydroxy-compound (8b) incorporates to less than 1/5 of the extent of (7c). A comparison of the incorporation of the foregoing compounds into oridonin shows the order 7-oxo-compound (7c) > 7β-hydroxy-compound (6b) > 7α-hydroxy-compound (8b) (see expts. 5 and 6). The 7-oxo-compound (7c), which was the compound most effectively incorporated among the three, and the 15-oxo-compound (2b) [2] were then compared (see expt. 7). The result suggests that the 7-oxo-compound (7c) is a more preferable precursor of enmein (3) and oridonin (4) than the 15-oxo-compound (2b).

The possibility that a 7,15-dioxygenated compound could be a common intermediate in the biosynthesis of *Isodon* diterpenes from *ent-kaur*-16-ene is suggested, because C-7 and C-15 are oxygenated in common in all the *Isodon* diterpenes except for one, inflexin (5). This suggests that these positions may be oxidised at an early step in the biosynthesis.

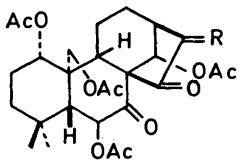
Since, of the 7-oxygenated compounds, the 7-oxo-derivative (7a) was shown to be the most effective precursor for enmein (3) and oridonin (4) and, further, that only the 15-oxo-derivative (2a) of the 15-oxygenated compounds was effective as a precursor,[2] *ent*-kaur-16-ene-7,15-dione (12a) was thought to be a potential pre-

TABLE 2

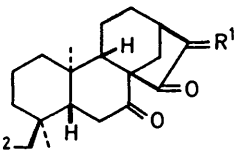
Results of the feeding experiments for comparison of the relative incorporations of the compounds mono-oxygenated at C-7 or C-15 into enmein and oridonin

	Expt. no.	5	6	7
Compound fed		(7c) + (6b)	(7c) + (8b)	(7c) + (2b)
	mg	5.8 6.6	5.7 9.2	5.6 9.7
Radioactivity fed Disint. $min^{-1} \times 10^{-8}$	^{3}H	5.70	5.60	5.48
	^{14}C	0.92	1.27	2.07
$^{3}H/^{14}C$ Ratio fed	(R_F)	6.20	4.41	2.65
$^{3}H/^{14}C$ Ratio in enmein diacetate	(R_E)	6.66	23.0	4.64
R_E/R_F		1.07	5.20	1.76
$^{3}H/^{14}C$ Ratio in oridonin tetra-acetate	(R_O)	8.99	7.72	4.26
R_O/R_F		1.45	1.75	1.61
Enmein diacetate: Yield	mg	347.5	23.5	54.6
Enmein diacetate: Radioactivity disint. min^{-1} $mmol^{-1} \times 10^{-4}$	^{3}H	11.2	7.85	14.1
	^{14}C	1.68	0.342	3.04
Enmein diacetate: Incorporation (%)	^{3}H	0.0150	0.000 723	0.003 08
	^{14}C	0.0139	0.000 139	0.001 75
Oridonin tetra-acetate: Yield	mg	10.2	40.0	34.6
Oridonin tetra-acetate: Radioactivity disint. min^{-1} $mmol^{-1} \times 10^{-4}$	^{3}H	54.1	11.2	31.3
	^{14}C	6.02	1.45	7.35
Oridonin tetra-acetate: Incorporation (%)	^{3}H	0.001 81	0.001 50	0.003 71
	^{14}C	0.001 25	0.000 858	0.002 30

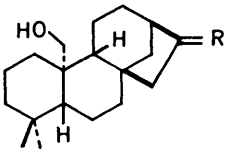
cursor. If then the latter is the real intermediate, the following two biogenetic pathways may be possible, although route (i) seems preferable to (ii) in the light of the result of expt. (7). (i) *ent*-Kaur-16-ene (1) ⟶ 7-



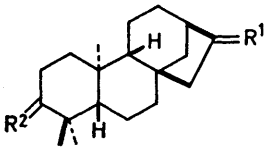
(11a) R = CH_2
(11b) R = $^{14}CH_2$



(12a) R^1 = CH_2, R^2 = H
(12b) R^1 = $^{14}CH_2$, R^2 = H
(12c) R^1 = CH_2, R^2 = ^3H



(13a) R = CH_2
(13b) R = $^{14}CH_2$



(14a) R^1 = CH_2, R^2 = α–OH, β–H
(14b) R^1 = $^{14}CH_2$, R^2 = α–OH, β–H
(15a) R^1 = CH_2, R^2 = α–H, β–OH
(15b) R^1 = $^{14}CH_2$, R^2 = α–H, β–OH
(16a) R^1 = CH_2, R^2 = O
(16b) R^1 = $^{14}CH_2$, R^2 = O

oxo-derivative (7a) ⟶ 7,15-dioxo-derivative (12a) ⟶ enmein (3) or oridonin (4); (ii) (1) ⟶ 15-oxo-derivative (2a) ⟶ (12a) ⟶ (3) or (4). Mixtures of [18-$^3H_1$]dione (12c) [8] and [17-^{14}C]7-one (7b), of [17-^{14}C]dione (12b) [8] and [18-$^3H_1$]7-one (7c), and of (12c) and [17-^{14}C]15-one (2b) were applied to plants. The results are summarised in Table 3, which shows that all the mono-oxo-compounds, (7b), (7c), and (2b), are more effectively incorporated into enmein and oridonin than the dioxo-compounds (12b) and (12c). The relative incorporation of 7-oxo- and 15-oxo-compounds into both diterpenes are similar, which contrasts with the result of expt. (7). Accidental error because of low incorporations cannot, however, be ruled out. On the basis of the foregoing experimental results, therefore, the biogenetic pathways described above cannot be accepted; moreover, it is not possible to say that the 7 and 15 positions are oxygenated in preference to the other positions.

All the diterpenes isolated from *Isodon japonicus* so far bear the oxygen functions at the 1-, 6-, and 20-positions, in addition to the 7- and 15-positions. The compounds oxygenated at the 20 position were subsequently investigated because their synthesis was easier than that of the C-1- or C-6-oxygenated compounds. Thus, *ent*-[17-^{14}C]kaur-16-en-20-ol (13b) [1] synthesised from enmein (3) was used for the tracer experiment. However, enmein has an additional oxygen function at C-3 and the possibility that this position might be oxygenated in preference to the other positions in the biosynthesis of enmein cannot be excluded. Work on the enzymatic biosynthesis of *ent*-kaur-16-en-3-ols published recently by Coates *et al.*[9] is of interest in this connection. Thus, *ent*-[17-^{14}C]kaur-16-en-3β-ol (14b), *ent*-[17-^{14}C]kaur-16-en-3α-ol (15b), and *ent*-[17-^{14}C]kaur-16-en-3-one (16b) were used for the feeding experiments. As a reference for comparing the relative incorporation, *ent*-[17-$^3H_2$]kaur-16-en-15-one (2c) was used.* Thus, mixtures of (13b) + (2c), (14b) + (2c), (15b) + (2c), and (16b) + (2c) were applied to the *Isodon* plants. The experimental results are summarised in Table 4. In order to see the fates of ^{3}H and ^{14}C of the same compound in the plant, a mixture of (2c) and (2b) was fed. Since enmein was not identified in this case, checking was not possible for enmein, but there was no major difference between the retentions of ^{3}H and ^{14}C

TABLE 3

Results of the feeding experiments for comparison of the relative incorporations of the mono-oxo-compounds and the dioxo-compound into enmein and oridonin

		Expt. no.	8	9	10
Compound fed			(12c) + (7b)	(7c) + (12b)	(12c) + (2b)
		mg	5.5 8.2	5.7 4.0	5.2 9.1
Radioactivity fed disint. $min^{-1} \times 10^{-8}$		^{3}H	6.91	5.60	6.52
		^{14}C	1.12	0.591	1.94
$^3H/^{14}C$ Ratio fed		(RF)	6.17	9.48	3.36
$^3H/^{14}C$ Ratio in enmein diacetate		(RE)	4.07	11.1	2.13
RE/RF			0.660	1.17	0.633
$^3H/^{14}C$ Ratio in oridonin tetra-acetate		(RO)	3.45	12.6	2.13
RO/RF			0.559	1.33	0.633
Enmein diacetate	Yield	mg	348.0	99.2	38.2
	Radioactivity disint.min^{-1} $mmol^{-1} \times 10^{-4}$	^{3}H	10.1	9.39	74.4
		^{14}C	2.48	0.845	34.9
	Incorporation (%)	^{3}H	0.0111	0.003 65	0.009 57
		^{14}C	0.0168	0.00311	0.0151
Oridonin tetra-acetate	Yield	mg	23.0	39.7	24.3
	Radioactivity disint. min^{-1} $mmol^{-1} \times 10^{-4}$	^{3}H	11.1	12.7	6.21
		^{14}C	3.22	1.01	2.91
	Incorporation (%)	^{3}H	0.000 694	0.001 69	0.000 434
		^{14}C	0.001 24	0.001 27	0.000 685

* Although the 7-oxo-derivative (7c) seemed most suitable for a reference compound, it was unfortunately not available.

TABLE 4

Results of the feeding experiments for comparison of the relative incorporation of the compounds mono-oxygenated at C-3, C-15, and C-20 into enmein and oridonin

	Expt. no.	11	12	13	14	15
Compound fed		(2c) + (2b)	(2c) + (13b)	(2c) + (14b)	(2c) + (15b)	(2c + (16b)
	mg	12.0 12.0	10.3 10.3	8.3 8.3	14.0 14.0	9.6 9.6
Radioactivity fed disint. $min^{-1} \times 10^{-8}$	^3H	1.59	1.36	1.09	1.82	1.25
	^{14}C	1.42	1.14	0.0858	1.51	0.95
$^3H/^{14}C$ Ratio fed	(R_F)	1.12	1.19	1.27	1.21	1.31
$^3H/^{14}C$ Ratio in enmein diacetate	(R_E)		1.35	0.578	0.939	0.709
R_E/R_F			1.13	0.456	0.777	0.540
$^3H/^{14}C$ Ratio in oridonin tetra-acetate	(R_O)	0.931				
R_O/R_F		0.831				
Enmein diacetate: Yield	mg	0	144.2	72.7	304.5	65.4
Enmein diacetate: Radioactivity disint. min^{-1}	^3H		1.71	1.26	5.61	2.31
	^{14}C		1.27	2.18	5.97	3.26
Enmein diacetate: Incorporation (%) mmol^{-1} $\times 10^{-4}$	^3H		0.003 98	0.001 85	0.0206	0.002 66
	^{14}C		0.003 53	0.004 06	0.0265	0.004 93
Oridonin tetra-acetate: Yield	mg	85.2	34.9	98.0	12.0	146.0
Oridonin tetra-acetate: Radioactivity disint. min^{-1}	^3H	3.66	1.34	0.814	4.71	2.62
	^{14}C	3.93	$\geqslant 0$	0	0	0
Oridonin tetra-acetate: Incorporation (%) mmol^{-1} $\times 10^{-4}$	^3H	0.003 68	0.000 646	0.001 37	0.000 584	0.005 75
	^{14}C	0.004 43	$\geqslant 0$	0	0	0

in oridonin [see expt. (11)]. The 20-hydroxy-compound (13b) was incorporated into enmein to almost the same degree as the 15-oxo-compound (2c) although very little was incorporated into oridonin [see expt. (12)]. Such a result suggests that the oxygenation stage at the C-20 atom may be different between the biosynthetic routes of *ent*-kaurene- and *ent*-6,7-secokaurene-type diterpenes. Diterpenes not bearing an oxygen function at C-20 have been isolated from *Isodon* plants [4,5,7,10,11] other than *I. japonicus* and *I. trichocarpus*. All of them are of the *ent*-kaurene-type rather than *ent*-secokaurene-type diterpenes. This fact provides support for the foregoing suggestion. Incorporation of the 3α-hydroxy-derivative (14b) into enmein was *ca.* double that of the 15-oxo-derivative [expt. (13)], and incorporations of the 3β-hydroxy-derivatives (15b) and (2c) were almost the same [expt. (14)]. Incorporation of the 3-oxo-derivative (16b) into enmein was 1.5- to 2-fold greater than that of (2c). None of these 3-oxygenated compounds were incorporated into oridonin. A combination of the results of expts. (13)—(15) and expt. (7) indicate that the 3β-hydroxy-compound (15a) having the same configuration as that of enmein at C-3 is less available than the 7-oxo-compound (7a) as a precursor for enmein, while the 3α-epimer (14a) and the 3-oxo-compound (16a) are somewhat more favourable precursors than (7a). However, this order should be considered as only approximate since, as Bale *et al.*[12] reported, a double-isotope technique does not eliminate uncertainties due to the differences in the modes of absorption and transportation between different precursors and, except for expts. (8) and (9), only one duplicate experiment was carried out. On the basis of the above results the following conclusions may be drawn. (i) The 7-oxygenated kaurenes, (6a), (7a), and (8a), the 7,15-dioxygenated kaurenes (9a) and (12a), as well as 15-oxokaurene (2a) are possible precursors of both enmein (3) and oridonin (4). The 20-ol (13a), the 3-oxygenated kaurenes, (14a), (15a), and (16a) are shown to be effective as the precursors of enmein (3) but not for oridonin (4). (ii) The positions oxygenated in common between enmein (3) and oridonin (4) are not oxygenated at a common early stage in the biosynthetic route. Although control of the biosynthesis of enmein (3) may be achieved by the introduction at an early stage of an oxygen function at the C-3 carbon of *ent*-kaur-16-ene, the stage and the mechanism have not yet been clarified.

EXPERIMENTAL

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*-phenylene-bis-(5-phenyloxazole) (POPOP) (1 mg). The radioactive enmein diacetate and oridonin tetra-acetate were recrystallised to constant radioactivity and, because of the low activity level, counted for 100 min. Radioscanning of chromatographic plates [Kieselgel 60 F-254 (Merck)] 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. M.p.s were taken on a micro hot-stage.

Feeding of Labelled Compounds.—A solution of [^{14}C]-labelled compound in acetone (*ca.* 10 ml) (in expts. 1—4) or a solution of the mixture of [^{14}C]labelled- and [^{3}H]-labelled compounds in acetone (*ca.* 20 ml) (in expts. 5—15) was applied to the lower sides of the leaves of two growing *I. japonicus* plants in late June. The leaves were harvested after 7 days.

Isolation of Enmein and Oridonin, and Conversion into Enmein Diacetate and Oridonin Tetra-acetate.—The leaves were extracted twice with methanol (each 1.5—2 l) under reflux each for 2 h, and the extract was concentrated *in vacuo* (to *ca.* 300 ml). Water (30 ml) was then added and the unwanted compounds were extracted with hexane (250 ml × 3). The methanolic layer was evaporated *in vacuo* to leave a residue, which was fractionated with ethyl acetate (450 ml) and water (200 ml). The neutral substance was isolated from the ethyl acetate fraction in the usual way and subjected to chromatography on a silica-gel column; subsequent preparative t.l.c. (chloroform–acetone 7 : 3) gave crude enmein and oridonin. Enmein diacetate,

prepared by treatment with acetic anhydride and pyridine at room temperature overnight, and purified by preparative t.l.c. (chloroform–acetone 8 : 2) followed by recrystallisation from methanol, had m.p. 165—167° as the hemihydrate.[2]

Oridonin tetra-acetate prepared by treatment with acetic anhydride and boron trifluoride–ether at room temperature for 1 h, and purified similarly to enmein diacetate, had m.p. 240—242° as prisms (from methanol).[2]

Ozonolysis of Labelled Enmein Diacetate (10b).—(i) *Experiment* 1. Ozone was introduced into a solution of (10b) (20.5 mg) in ethyl acetate (4 ml) at room temperature. After 5 min (10b) was consumed (t.l.c.), and the reaction was stopped. Zinc powder (20 mg) and water (3 ml) were added and the mixture was stirred overnight at room temperature. To the separated water layer was added a solution of dimedone (50 mg) in water (10 ml), and the mixture was set aside overnight. Methylenebisdimedone (1.01×10^4 disint. min^{-1} $mmol^{-1}$; 2 mg) was obtained as needles, m.p. 190—191° (from methanol). Ozonolysis was similarly carried out in expt. 3. The specific activity of the methylenebisdimedone was 9.50×10^3 disint. min^{-1} $mmol^{-1}$.

(ii) In expts. 2 and 4, (10b) diluted with (10a) was subjected to ozonolysis to give methylenebisdimedones (8.09×10^4 and 6.23×10^4 disint. min^{-1} $mmol^{-1}$, respectively).

Ozonolysis of Labelled Oridonin Tetra-acetate (11b).—The procedure for enmein diacetate was applied to ozonolysis of labelled oridonin tetra-acetate (11b) which, diluted with (11a), was used for the reaction. The specific activities of methylenebisdimedones obtained in expts. 1, 2, and 4 were 7.46×10^3, 6.02×10^4, and 6.50×10^4 disint. min^{-1} $mmol^{-1}$, respectively.

ent-[17-$^3H_2$]*Kaur*-16-*en*-15-*one* (2c).—The procedure [2] for the synthesis of (2b) from *ent*-[17-^{14}C]kaur-16-ene was applied to the preparation of (2c) from *ent*-[17-$^3H_2$]kaur-16-ene, which was obtained by Wittig reaction of *ent*-17-norkauran-16-one with [$^3H_3$]methyltriphenylphosphonium iodide. The α,β-unsaturated ketone (2c) (3.80×10^9 disint. min^{-1} $mmol^{-1}$) showed a single radioactive peak on t.l.c. and was identical with an authentic sample of (2a).

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