Stereospecificity of the Oxidation of *ent*-Kauren-19-ol to *ent*-Kaurenal by a Microsomal Enzyme Preparation from *Marah Macrocarpus*

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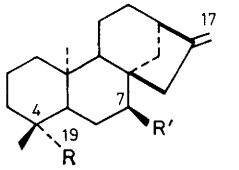
The oxidation of *ent*-kaur-16-en-19-ol (**2**) by a microsomal enzyme preparation from *M. macrocarpus* seeds proceeds with stereospecific loss of the C-19 hydrogen atom in (**2**) which appears at higher field in the n.m.r. spectrum and is assigned the *pro-R* configuration.

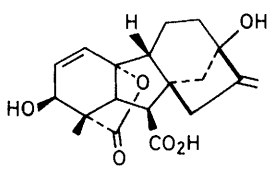
West and co-workers have described the preparation of a microsomal enzyme extract from immature seeds of *Marah macrocarpus* (wild cucumber) which catalyses the oxidation of *ent*-kaurene (**1**) to *ent*-7β-hydroxykaurenoic acid (**5**) *via* the isolable intermediates *ent*-kauren-19-ol (**2**), *ent*-kauren-19-al (**3**), and *ent*-kauren-19-oic acid (**4**).[1] This oxidation sequence is believed to be one of the preliminary stages in the biosynthesis of gibberellic acid (**6**) from kaurene.[2] The enzyme system has been characterized as a cytochrome P-450-dependent mixed function oxidase, and is highly specific for kauranoid substrates.[1] The stereochemistry and isotope effects of these oxidations are of interest as probes of mixed function oxidase action in higher plants, and as a basis for comparison with similar oxidations in mammalian and bacterial systems.[1] We have determined the stereospecificity of the kaurenol to kaurenal oxidation (**2**) $\rightarrow$ (**3**) with the microsomal enzyme preparation from *M. macrocarpus*.

Methyl *ent*-kauren-19-oate[3] was converted in three steps into the *ent*-[19-$^2H_1$]kaurenol diastereomers (**2b**) [$LiAlH_4$, pyridinium chlorochromate (PCC),[4] NaB^2H_4] and (**2c**) ($LiAl^2H_4$, PCC, $NaBH_4$). The 360 MHz ^1H n.m.r. spectra ($CDCl_3$) of (**2b**) and (**2c**) exhibit singlets for the CH^2HOH group at δ 3.42 and 3.72, respectively, in place of the AB quartet for the CH_2OH group of (**2a**) [δ 3.75 (H_A) and 3.44 (H_B); J_{AB} 10.9 Hz]. Careful integration established the stereoselectivity of the aldehyde reductions to be 95 $\pm$ 2%. The C-19 isotopic configurations of (**2b**) and (**2c**) are assigned as *S* ($H_A = H_S = {}^2H$) and *R* ($H_B = H_R = {}^2H$) respectively, based on the assignments for the analogous podocarpane and beyerane derivatives.[5,6]

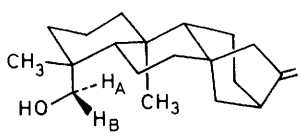
The *ent*-[19-$^2H_1$,19-$^3H_1$]kaurenol diastereomers (**2d**) (19-*S*; 13 mCi mmol^{-1}) and (**2e**) (19-*R*; 30 mCi mmol^{-1}) were prepared by a similar method. Each was mixed with an internal standard of *ent*-[17-^{14}C]kaurenol [(**7**) 12 mCi mmol^{-1}]† to give the respective *ent*-kaurenol substrates **A** [10.6:1 molar ratio of (**2d**):(**7**); ^3H:^{14}C ratio 11.19 $\pm$ 0.25:1], and **B** [4.3:1 molar ratio of (**2e**):(**7**); ^3H:^{14}C ratio 10.97 $\pm$ 0.66:1].



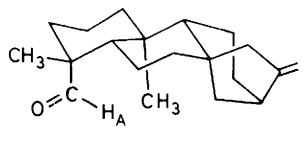
(**1**) R = Me, R′ = H
(**2**) R = CH_2OH, R′ = H
(**3**) R = CHO, R′ = H
(**4**) R = CO_2H, R′ = H
(**5**) R = CO_2H, R′ = OH
(**7**) = [17-^{14}C](**2**)



(**6**)



(**2**) **a**; $H_A = H_B = {}^1H$
b; $H_A = {}^2H$, $H_B = {}^1H$
c; $H_A = {}^1H$, $H_B = {}^2H$
d; $H_A = {}^3H$, $H_B = {}^2H$
e; $H_A = {}^2H$, $H_B = {}^3H$



(**3**) **a**; $H_A = {}^1H$
b; $H_A = {}^3H$
c; $H_A = {}^2H$

i

Reagents: i, *M. macrocarpus* enzymes, O_2, NADPH, FAD.

† ^{14}C-Labelled *ent*-kaurenol was prepared from methyl *ent*-17-nor-16-oxokauranoate by Wittig methylenation with $^{14}CH_2PPh_3$ and reduction with $LiAlH_4$.

Table 1. Radioactivity data for kaurenal produced in incubations with *ent*-kaurenol substrates **A** [(**2d**) + (**7**)] and **B** [(**2e**) + (**7**)].

Substrate	Conversion (%)	^3H d.p.m.	^{14}C d.p.m.	d.p.m. ratio	Apparent ^3H retention (%)
A	8	13009	2026	6.42	57
	15	21910	3368	6.51	58
	39	55045	6975	7.89	71
	64	75973	8240	9.22	82
B	10	2856	3634	0.786	7.2
	24	5818	7848	0.741	6.8
	27	7580	11634	0.652	5.9
	48	12185	14264	0.854	7.8

Enzyme extracts from the endosperm of immature *M. macrocarpus* seeds were prepared by resuspending the microsomal pellet obtained by differential centrifugation, as described by Hirano,[7] and had an apparent protein content of 2.9 mg ml^{-1} (Bradford assay[8]). Incubations with *ent*-[17-^{14}C]-kaurene and *ent*-[17-^{14}C]kaurenol gave satisfactory assays.

Incubations with substrates **A** and **B** were carried out in triplicate at 30 °C with gyratory shaking under a normal atmosphere, as described by Hirano,[7] and were allowed to proceed for 10, 20, 30, and 60 min intervals. Each incubation medium was 1.05 ml in volume, and contained the substrate (**A**, 14.5 ± 0.6 nmol total kaurenol; or **B**, 11.6 ± 0.8 nmol total kaurenol) in 50 μl of 0.1% Tween 20 in acetone, 4.7×10^{-4} M NADPH, 5.3×10^{-5} M FAD, 0.1 M Tris-HCl buffer (pH 7.5), and 140 μl (0.4 mg of microsomal protein) of enzyme preparation. Incubations were initiated by addition of enzyme and terminated by rapid addition of ethanol. The products and unchanged substrate were isolated by t.l.c., and analysed for radioactivity by liquid scintillation counting.

The ^3H:^{14}C d.p.m. ratios of the kaurenal‡ produced in the incubations (Table 1) show an apparent retention of ^3H label (57—82%) in the enzymic oxidation of substrate **A**, and an apparent loss of ^3H label (92—94%) from **B**.§ Since diastereomer (**2e**) has the same C-19 configuration as (**2c**), the CH_2OH hydrogen atom which is removed in the enzymic oxidation of (**2a**) is that which exhibits the higher field doublet (H_B) of the AB quartet, and is assigned as the *pro-R* hydrogen atom.

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‡ The identity and purity of the kaurenal from a series of 20 min incubations were confirmed by reduction with $NaBH_4$, dilution with cold *ent*-kaurenol, and crystallization to constant specific activity. The ^3H:^{14}C d.p.m. ratios obtained were similar to those shown in Table 1.

§ The incomplete retention and increasing ^3H:^{14}C d.p.m. ratios observed in the *ent*-kaurenal from substrate **A** are satisfactorily accounted for by an intermolecular kinetic isotope of 1.61 ± 0.09. About half of the small amount of tritium radioactivity retained in *ent*-kaurenal from substrate **B** is attributable to the presence of *ca.* 5% of (**2d**).