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(−)-KAUR-16-EN-7β-OL-19-OIC ACID, AN INTERMEDIATE IN GIBBERELLIN BIOSYNTHESIS*

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Abstract—17-^{14}C-(−)-kaur-16-en-19-oic acid is converted to at least five chromatographically-separable products in cell-free preparations of endosperm from immature *Echinocystis macrocarpa* seeds. One of these products (X_{2A}), isolated in milligram quantites from the pooled extracts of a large number of incubation mixtures, was identified as (−)-kaur-16-en-7β-ol-19-oic acid (I) from its physical and chemical characteristics. A second product (X_{2B}), which was apparently generated non-enzymically from X_{2A} during the isolation of the latter, was tentatively identified as (−)-kaur-15-en-7β-ol-19-oic acid (IV). I stimulated leaf sheath growth in seedlings of the *dwarf*-5 mutant of *Zea mays*. Also I was convertible to ^{14}C-gibberellin A_3 and probably other ^{14}C-gibberellins in *Gibberella fujikuroi* mycelial suspensions. On the basis of these properties it is suggested that I is a normal intermediate in gibberellin biosynthesis.

INTRODUCTION

PREVIOUS investigations have established that mevalonic acid is converted to *trans*-geranylgeranyl pyrophosphate[1] and this acyclic precursor is cyclized to the tetracyclic diterpene (−)-kaur-16-ene‡ (kaurene)[2,3] via the intermediate bicyclic diterpene copalyl pyrophosphate[4] in a soluble enzyme fraction prepared from the endosperm of immature *Echinocystis macrocarpa* seed. Kaurene is further converted to (−)-kaur-16-en-19-ol (kaurenol), (−)-kaur-16-en-19-al (kaurenal) and (−)-kaur-16-en-19-oic acid (kaurenoic acid) in a sequence of oxidative steps catalyzed by a particulate fraction prepared from the endosperm.[5] The general characteristics of these oxidative reactions are those expected of a mixed function oxygenase.[6] It has been concluded from studies of the conversion of kaurene and its oxidized metabolites labeled with ^{14}C to gibberellic acid-^{14}C in cultures of *Gibberella fujikuroi*[2,5,7–10] and from the activity of these compounds in stimulating the growth of the

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‡ Abbreviations used include kaurene for (−)-kaur-16-ene, kaurenol for (−)-kaur-16-en-19-ol, kaurenal for (−)-kaur-16-en-19-al and kaurenoic acid for (−)-kaur-16-en-19-oic acid. All references to kaurene and kaurene derivatives are to compounds of the (−)-enantiokaurane skeleton (as illustrated in structures I–IV).

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[2] J. E. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST. *J. Biol. Chem.* **240,** 1847 (1965).
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[4] I. SHECHTER and C. A. WEST, *J. Biol. Chem.* **244,** 3200 (1969).
[5] D. T. DENNIS and C. A. WEST, *J. Biol. Chem.* **242,** 3293 (1967).
[6] P. J. MURPHY and C. A. WEST. *Arch. Biochem. Biophys.* **133,** 395 (1969).
[7] B. F. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).
[8] T. A. GEISSMAN, A. J. VERBISCAR, B. O. PHINNEY and G. CRAGG, *Phytochem.* **5,** 933 (1966).
[9] B. E. CROSS, R. H. B. GALT and K. NORTON, *Tetrahedron* **24,** 231 (1968).
[10] T. A. GEISSMAN, A. J. VERBISCAR, B. O. PHINNEY and G. CRAGG, *Phytochem.* **6,** 807 (1967).

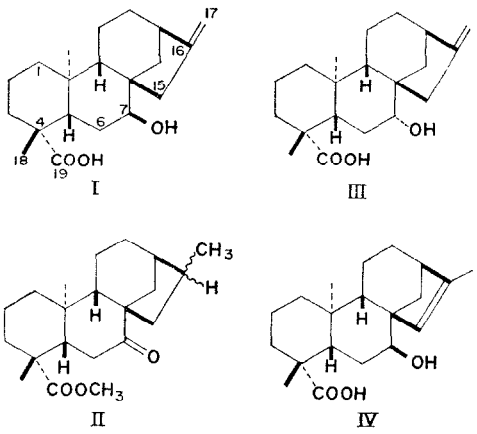

FIG. 1.

I = (−)-Kaur-16-en-7β-ol-19-oic acid; II = methyl (−)-kauran-7-on-19-oate; III = (−)-kaur-16-en-7α-ol-19-oic acid; IV = (−)-kaur-15-en-7β-ol-19-oic acid.

dwarf-5 mutant of *Zea mays*[11,12] that this sequence of oxidations is a part of a normal biosynthetic pathway leading to the gibberellins.

Echinocystis macrocarpa endosperm preparations catalyze the conversion of ^{14}C-kaurenoic acid to a group of ^{14}C-labeled products. This paper describes the isolation of the metabolite apparently formed initially from kaurenoic acid in such incubation mixtures and its identification as (−)-kaur-16-en-7β-ol-19-oic acid (I). Evidence is also presented for the role of I as an intermediate in gibberellin biosynthesis and some of the characteristics of its enzymic formation from kaurenoic acid are described. A preliminary report of some of these findings has appeared.[13] Hanson and White have independently presented evidence for the participation of I in gibberellin biosynthesis in *G. fujikuroi*.[14]

RESULTS

Incubation of 17-^{14}C-kaurenoic acid (2 μM) under aerobic conditions with lyophilized *E. macrocarpa* endosperm in the presence of NADPH (0·5 mM) led to the formation of several ^{14}C-labeled products which were extractable by ethyl acetate from acidified solution. Five radioactive peaks at R_f values of 0·86 (X_1), 0·74 (X_2), 0·60 (X_3), 0·42 (X_4) and 0·02 (X_5) were separated on silica gel thin layer chromatograms developed with isopropyl ether–acetic acid (95:5) in addition to a small peak very close to the solvent front which co-chromatographed with kaurenoic acid and presumably represented unreacted substrate. The relative amounts of these acidic radioactive metabolites varied with different enzyme preparations and incubation conditions. In particular, it was found that substrate kaurenoic acid concentrations of 50 μM or higher led to the formation of X_2 to the virtual exclusion of other products. Furthermore, a time course of formation of the various products showed

[11] M. RUDDAT, A. LANG and E. MOSETTIG, *Naturwissenschaften*. **50**, 23 (1963).
[12] M. KATSUMI, B. O. PHINNEY, P. R. JEFFERIES and C. A. HENRICK, *Science* **144**, 849 (1964).
[13] C. A. WEST, M. OSTER, D. ROBINSON, F. LEW and P. J. MURPHY, *Biochemistry and Physiology of Plant Growth Substances* (edited by F. WIGHTMAN and G. SETTERFIELD), pp 313–332, The Runge Press, Ottawa, Canada (1969).
[14] A. F. WHITE and J. R. HANSON, *Chem Comm.* 410 (1969).

that the initial rate of formation of X_2 was more rapid than the other products. For these reasons it was considered likely that X_2 represented the first conversion product from kaurenoic acid while the other substances were derived from X_2.

The biosynthesis of X_2 on a large enough scale to permit its isolation and characterization was undertaken. The following conditions were established from preliminary experiments as best for the production of X_2: 17-^{14}C-kaurenoic acid (0·1 mM), reconstituted lyophilized whole endosperm (0·25 ml), $MgCl_2$ (5 mM) and Tris chloride, pH 7·2 (50 mM) in a total volume of 1 ml were incubated in a 25-ml flask with shaking at 30° for 2 hr. The kaurenoic acid (16×10^3 counts/min ^{14}C/0·1 m-mole) was added in 0·02 ml acetone containing 0·35 mg Tween 20. The yields of X_2 were better in 1-ml incubations carried out as described than in incubations in larger volumes even though equivalent concentrations of all components were included. Therefore, a total of 1440 separate 1-ml incubations were carried out in order to accumulate several milligrams of X_2 for characterization.

The precipitate formed from heat–denaturation of the incubation mixtures was extracted with benzene–acetone (3:1) and the aqueous phase from the incubations was acidified to pH 2 and extracted with ethyl acetate. The pooled organic extracts were chromatographed on silicic acid developed with a gradient of increasing concentrations of ethyl ether in benzene applied by means of a Hirsch–Ahrens elution apparatus.[15] A peak of radioactive material eluted first from the column contained 33% of the recovered radioactivity, most of which possessed the chromatographic properties of kaurenoic acid. The second peak contained 49% of the recovered radioactivity, all of which resembled X_2 in chromatographic behavior. Minor amounts of radioactivity (3·5%) in later fractions were not further characterized. A total of 85% of radioactivity applied to the column was recovered.

Recrystallization of the crude X_2-containing fractions (22·6 mg) from aqueous methanol yielded 9·2 mg of crystalline material (m.p. 222–238° with decomposition) containing $39{\cdot}94 \times 10^5$ counts/min. This corresponds to a specific radioactivity of $1{\cdot}59 \times 10^5$ counts/min/μmole for a substance of molecular weight 318 (see below) which is comparable to the specific radioactivity of $1{\cdot}60 \times 10^5$ counts/min/μmole of the kaurenoic acid employed as substrate. An additional 4·9 mg of material of lower specific radioactivity was recovered in subsequent crystal crops, but these less pure products were not utilized in the characterization studies described below.

The purified sample of X_2 migrated as a single, rather broad radioactive peak on silica gel thin layer plates developed with isopropyl ether–acetic acid (95:5) ($R_f = 0{\cdot}75$). However, the methyl ester prepared by treatment of X_2 with diazomethane showed three peaks of radioactivity when chromatographed on silica gel plates impreganted with 3% $AgNO_3$ developed with benzene–ethyl acetate (8:2). The methyl esters of X_{2A} ($R_f = 0{\cdot}30$) and X_{2B} ($R_f = 0{\cdot}10$) accounted for 95% of the radioactivity, while the other 5% was associated with a minor component migrating to $R_f = 0{\cdot}70$ in this system. Thus, the purified sample of X_2 was heterogeneous.

Samples of the methyl esters X_{2A} and X_{2B} were prepared free from the other by preparative chromatography on thin layers of silica gel impregnated with silver nitrate and developed with benzene–ethyl acetate (65:35). The minor radioactive component was not recovered in sufficient quantity for further characterization. Samples of X_{2A} and X_{2B} as the free acids were also prepared by chromatography on thin layers of silica gel impregnated with silver nitrate and developed with isopropyl ether–acetic acid (95:5) (R_f of $X_{2A} = 0{\cdot}70$ and $X_{2B} = 0{\cdot}30$).

[15] J. Hirsch and E. H. Ahrens, Jr., *J. Biol. Chem.* **233**, 311 (1958).

An experiment was performed to determine whether both X_{2A} and X_{2B} were generated from ^{14}C-kaurenoic acid in the initial incubation mixture. 17-^{14}C-Kaurenoic acid and endosperm were incubated under the same conditions described above for the large scale preparation of X_2. The ethyl acetate–extractable acidic components from 12 1-ml incubation mixtures were treated with an excess of diazomethane and the resulting methyl esters were chromatographed on a silver nitrate-impregnated silica gel plate developed with benzene–ethyl acetate. 27% of the recovered radioactivity was associated with the methyl X_{2A} region of the plate, 31% was associated with methyl kaurenoate and less than 2% was found in the region where methyl X_{2B} should be present. From this experiment it was concluded that X_{2A} was the initial metabolite formed from kaurenoic acid in incubation mixtures; most or all of the X_{2B} in the purified X_2 sample presumably arose from X_{2A} during the purification steps employed.

Properties and Structure of X_{2A}

The general chromatographic and extraction properties were consistent with the presence of a carboxylic acid group in X_{2A}. Treatment of X_{2A} with diazomethane converted it to a compound with the chromatographic properties of a neutral methyl ester. The mass spectrum of the methyl ester showed an apparent molecular ion at $m/e = 332$ ($C_{21}H_{32}O_3$) which corresponds to the monomethyl ester of an acid $C_{20}H_{30}O_3$. The proton magnetic resonance spectrum of the methyl ester was characterized by the following features (assignments are made by reference to the proton magnetic resonance spectrum of methyl kaurenoate); two unsplit three proton peaks at $\tau = 9{\cdot}15$ and $\tau = 8{\cdot}84$ (C-20 and C-18 quaternary methyl groups), an unsplit three proton peak at $\tau = 6{\cdot}37$ (methoxyl methyl group), and an unsplit two proton peak at $\tau = 5{\cdot}23$ (C-17 methylene group).

The methyl ester of X_{2A} was reduced by hydrogen over a Pd/charcoal catalyst to a saturated product whose mobility on silica gel thin layers was not influenced by the presence of silver nitrate. The mass spectrum of this product showed a molecular ion at $m/e = 334$. The proton magnetic resonance spectrum of the hydrogenated product revealed a three proton doublet at $\tau = 9{\cdot}01$ and $8{\cdot}91$ ($J = 6$–7 c/s) which is assigned to a C-17 methyl group by comparison with the reported values for kauran-2,15-diol-19-oic acid;[16] as expected, the hydrogenated product lacked the signal assigned to two vinyl protons of the C-17 methylene group in the parent compound. Thus, these data are consistent with the reduction by hydrogen in the presence of Pd/charcoal catalyst of the Δ-16,17 double bond to a dihydro derivative. It is known that hydrogenation of kaurenoic acid and its derivatives yields a mixture of α and β epimers at C-16 which are difficultly separable;[17] however, no effort was made to resolve the epimers produced on the hydrogenation of methyl X_{2A} in the present work.

The above data indicates that X_{2A} is a carboxylic acid of molecular formula $C_{20}H_{30}O_3$. Several lines of evidence were consistent with the idea that the third oxygen was present as a primary or secondary alcoholic hydroxyl group. Treatment of the methyl ester of X_{2A} with acetic anhydride in pyridine converted it to a derivative of $R_f = 0{\cdot}75$ on a silica gel thin later plate developed with benzene–ethyl acetate (9:1) as compared with an $R_f = 0{\cdot}45$ for the methyl ester of X_{2A} itself in the same system. Oxidation of X_{2A} as the free acid with Jones reagent led to the formation of a derivative which stained on thin layer chromatograms

[16] F. PIOZZI, A. QUILICO, T. AJILLO and V. SPRIO, *Tetrahedron Letters* **23**, 1829 (1965).
[17] J. R. CANNON, P. N. CHOW, P. R. JEFFERIES and G. V. MEEHAN, *Australian J. Chem.* **19**, 861 (1966).

with acidic 2,4-dinitrophenylhydrazine spray reagent[18] in a manner characteristic for aldehydes or ketones. The oxidized derivative of X_{2A} had an R_f value of 0·60 on silica gel developed with benzene–ethyl acetate–acetic acid (80:20:1), whereas X_{2A} itself had an R_f value of 0·45 in this system. The fact that the quaternary methyl groups at C-18 and C-20 appeared to be present in the methyl ester of X_{2A} from proton magnetic resonance measurements led to the conclusion that the hydroxyl group must be secondary.

A comparison of the thin layer chromatographic properties of X_{2A} and an alcohol derived from it with a sample of kauren-7α-ol-19-oic acid (III) kindly supplied by Dr. J. R. Hanson were instrumental in suggesting a structure for X_{2A}. Co-chromatography of X_{2A} and III on $AgNO_3$-impregnated silica gel plates developed with isopropyl ether–acetic acid demonstrated that X_{2A} moved slightly, but reproducibly, ahead of the reference compound. However, the alcohol prepared by oxidation of X_{2A} to the keto derivative with Jones reagent followed by re-reduction of the purified keto derivative with sodium borohydride now co-chromatographed with III in the same system. This strongly suggested that X_{2A} was the 7β-hydroxy derivative of kaurenoic acid (I) since it had already been established that reduction of the kauren-7-on-19-oic acid with sodium borohydride led to the predominant formation of kauren-7α-ol-19-oic acid.[19]

Confirmation of the correctness of the proposed structure of X_{2A} came from the following observations. The dihydroderivative of the methyl ester of X_{2A} (6·0 mg; 2×10^6 counts/min) was oxidized with Jones reagent to a keto derivative which was purified on a small silicic acid column. The proton magnetic resonance spectrum of the major product ($1{\cdot}3 \times 10^6$ counts/min) measured at 60 mc/cycle showed the presence of three protons with complex, non-symmetrical splitting patterns at 7·25 τ and 7·01 τ. A scan of this region at 100 mc/sec likewise indicated the complexity of the splitting pattern. From their chemical shifts, these protons are believed to be influenced by the presence of the keto group in the molecule, but the complex nature of the splitting pattern can not be explained. A reference sample of methyl kauran-7-on-19-oate (II) was prepared from the authentic sample of kauren-7α-ol-19-oic acid supplied by Dr. Hanson using the same procedures described above. The mass spectrum of this authentic specimen was identical within experimental variation to the mass spectrum of the keto derivative prepared from methyl dihydro-X_{2A}. This would appear to conclusively establish that X_{2A} is (—)-kaur-16-en-7β-ol-19-oic acid (I).

Properties and Proposed Structure of X_{2B}

The evidence cited earlier suggested that X_{2B} may have arisen nonenzymically from X_{2A} during purification of the X_2 fractions. The relative thin layer chromatographic properties of X_{2A} and X_{2B} on plain and silica gel impregnated plates were reminiscent of those of (—)-kaurene and (—)-isokaurene ((—)-kaur-15-ene). Table 1 summarizes these properties. It can be seen that X_{2B} and isokaurene were greatly retarded on silver nitrate relative to X_{2A} and kaurene, respectively; whereas, the mobilities of X_{2A} and X_{2B} in the one case, and kaurene and isokaurene in the other, were quite similar on plain plates. This led to the suggestion that X_{2B} might bear the same structural relationship to X_{2A} that isokaurene does to kaurene, that is, that X_{2B} is a positional isomer of X_{2A} with a 15,16 instead of a 16,17 double bond.

[18] D. WALDI, in *Thin-layer Chromatography: A Laboratory Handbook* (edited by E. STAHL), p. 490, Springer-Verlag and Academic Press, New York (1965).
[19] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 2944 (1963).

TABLE 1. THIN LAYER MOBILITIES OF KAURENE, ISOKAURENE, METHYL X_{2A} AND METHYL X_{2B}

Compound	R_f	
	Silica gel plate	3% $AgNO_3$-silica gel
Kaurene	0·89*	0·59‡
Isokaurene	0·89*	0·23‡
Methyl X_{2A}	0·55†	0 30†
Methyl X_{2B}	0·63†	0 10†

* Developed with hexane.
† Developed with benzene–ethyl acetate (8:2).
‡ Developed with hexane–benzene (17:3).

The following evidence appears to be consistent with this proposal. X_{2B} behaved as an acid and reacted with diazomethane to produce a methyl ester whose mass spectrum showed a molecular ion at $m/e = 332$ ($C_{21}H_{34}O_3$). The proton magnetic resonance spectrum of methyl X_{2B} seemed to show a three proton signal at $\tau = 8{\cdot}26$ (tentatively assigned to a vinyl methyl group at C-17) and a single proton signal at $\tau = 5{\cdot}53$ (tentatively assigned to a single vinyl proton at C-15); however, the spectrum was not well-defined and appeared to change in time due to isomerization under the conditions of measurement in $CDCl_3$ and thus must be considered somewhat tentative. Methyl X_{2B} could be acetylated in acetic anhydride–pyridine mixtures. And finally, hydrogenation of methyl X_{2B} yielded a product which could not be distinguished chromatographically from methyl kauran-7β-ol-19-oate produced from methyl X_{2A} and was not retarded by silver nitrate. These findings are consistent with the tentative formulation of the structure of X_{2B} as (—)-kaur-15-en-7β-ol-19-oic acid (IV).

TABLE 2. BIOASSAY OF KAURENE DERIVATIVES ON THE *dwarf*-5 MUTANT OF *Zea mays*

Substance tested	Average length*		
	μg/plant	(cm)	% of control
Control	—	3·42	100
Gibberellin A_3	1·5	5·90	173
Kaurenoic acid	15·0	4·72	138
Kaur-16-en-7β-ol-19-oic acid (X_{2A})	15·0	5·20	152
Kaur-15-en-7β-ol-19-oic acid (X_{2B})	15·0	3·24	95
Control	—	4·38	100
Kaurenoic acid	15·0	6·96	160
Kauren-7α-ol-19-oic acid	15·0	4·84	112
Control	—	3 6	100
Gibberellin A_3	1·5	7·2	200
Kaurenoic acid	15·0	4 8	138
Kauren-7-on-19-oic acid	15·0	3·7	103

* Average length of the sums of the second and third leaf sheaths of five treated plants.

Intermediary Role of Kauren-7β-ol-19-oic Acid in Gibberellin Biosynthesis

It has not yet been possible to test the role of kauren-7β-ol-19-oic acid (I) as an intermediate in gibberellin biosynthesis directly in *E. macrocarpa*; however, the properties of this metabolite in two other biological systems implicate its participation in this process. Seedlings of the *dwarf*-5 mutant of *Zea mays* respond to treatment with (—)-kaurene and various of its oxidized derivatives as well as to gibberellins with increased growth of the leaf sheaths. The data in Table 2 show that I is as effective as kaurenoic acid in stimulating the growth of the second and third leaf sheaths of treated plants. It can also be seen that X_{2B} (tentatively identified as IV) and kauren-7-on-19-oic acid were inactive and kauren-7α-ol-19-oic acid was marginally active at identical levels of application in this test system.

The precursor activity of ^{14}C-labeled kauren-7β-ol-19-oic acid for the biosynthesis of gibberellins was tested in *G. fujikuroi* cultures. The ^{14}C-labeled substrate was incubated for 48 hr with a washed mycelial suspension after which the ethyl acetate–extractable acidic components were recovered from the culture filtrate and chromatographed on a silicic acid-celite column. TLC comparisons, which will not be detailed in this report, strongly suggested that the radioactivity in the three acidic fractions was associated with gibberellins A_3, A_4, A_7 and A_{14} in addition to other unidentified substances.

In order to obtain more conclusive evidence, the radioactive material from the third major fraction, 75% of which co-chromatographed with gibberellin A_3, was mixed with unlabeled gibberellin A_3 and crystallized several times from ethyl acetate–hexane mixtures until constant specific radioactivity was achieved. About 41% of the total radioactivity of this fraction cocrystallized with gibberellin A_3. These data indicate that 4% of the radioactivity added as ^{14}C-kauren-7β-ol-19-oic acid was converted to gibberellin A_3 in this experiment. This is probably a minimal figure since there could easily have been losses in ^{14}C-gibberellin A_3 during the extraction and purification steps prior to the addition of unlabelled gibberellin A_3.

In a parallel experiment for comparison purposes ^{14}C-kaurenoic acid was incubated with *G. fujikuroi* mycelial suspensions and the ethyl acetate-extractable acids were processed as described above. In this case 48% of the radioactivity present in the gibberellin A_3 fraction from silicic acid chromatography cocrystallized with gibberellin A_3. The minimal conversion of ^{14}C-kaurenoic acid to gibberellin A_3 was calculated to be 12%.

Characteristics of the Enzymic Conversion

The enzymic activity for the conversion of kaurenoic acid to kauren-7β-ol-19-oic acid resided in the pellet formed by centrifugation of the endosperm homogenate at 105,000 ***g*** for 2 hr as seen in Table 3.

Reduced pyridine nucleotide and oxygen are other requirements for the reaction which have been established. Table 4 summarizes the activities seen on the addition of reduced pyridine nucleotides to a microsomal pellet. NADPH was more effective than NADH. For unknown reasons an NADPH generating system was less effective than NADPH itself. From separate experiments also summarized in Table 4 it was shown that the combination of 0·5 mM $NADP^+$, 0·5 mM NADH and 0·5 mM ATP was much more effective than comparable concentrations of NADH and $NADP^+$ together or NADH alone. The addition of 5 mM $MgCl_2$ along with NADPH gave a further two-fold stimulation of the reaction rate over NADPH alone, although an absolute dependence on $MgCl_2$ addition was not established. Replacement of the air atmosphere with nitrogen in the presence of an alkaline pyrogallol wick to remove the last traces of O_2 gave an almost complete inhibition of the

TABLE 3. METABOLISM OF KAURENOIC ACID IN THE ENDOSPERM HOMOGENATE, 105,000 *g* PELLET AND 105,000 *g* SUPERNATANT FRACTION

Enzyme fractions	% Conversion to kauren-7β-ol-19-oic acid
Heated endosperm homogenate	1·1
Endosperm homogenate	67·0
105,000 *g* supernatant	4 7
105,000 *g* pellet	72·0

The incubation mixtures consisted in a total volume of 1·0 ml: 0 5 mM NADPH, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7 2, ^{14}C-kaurenoic acid (about 14×10^3 counts/min) in 0·02 ml acetone and 0 25 ml of enzyme preparation as indicated. The incubations were carried out at 31° for 1 hr with moderate shaking.

conversion in the presence of NADPH. This established the requirement for O_2 in the reaction.

The requirement for oxygen and reduced pyridine nucleotide in a hydroxylation reaction catalyzed by a microsome-like particulate fraction strongly suggested the involvement of a mixed function oxygenase as has been proposed for other steps in this sequence of kaurene oxidations.[6] As further evidence on this point, a number of inhibitors found to be effective for other microsomal mixed function oxygenases by Ernster and Orrenius[20] were tested.

TABLE 4. COFACTOR REQUIREMENTS FOR THE OXIDATION OF KAURENOIC ACID TO KAUREN-7β-OL-19-OIC ACID BY MICROSOMES

Coenzyme added	% Conversion to kauren-7β-ol-19-oic acid
None	1 0
NADPH	16·0
NADPH, $MgCl_2$	34 0
NADPH generating system*	7 2
NADH	3·1
$NADP^+$, NADH and ATP	28·0
NADPH	34·0
NADH	3·1
NADH, $NADP^+$	1 4

* The NADPH generating system consisted of the following in a total of 0·5 ml: 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7 2, 0 002 mM $MnCl_2$, 1·29 mg D,L-isocitrate, 6·1 mg nicotinamide, 0·5 mM $NADP^+$ and 0·36 EU of isocitrate.

The incubation mixtures were as described in Table 3 except that 0 25 ml of microsome suspension (105,000 *g* pellet) was employed in all cases and NADPH was replaced with the coenzyme or coenzyme mixture indicated. All coenzymes where indicated were present at 0 5 mM; $MgCl_2$ where indicated was present at 5 mM. Incubations were carried out for 30 min.

[20] L. ERNSTER and S. ORRENIUS, *Federation Proc.* **24**, 1190 (1965).

TABLE 5. EFFECT OF INHIBITORS ON THE OXIDATION OF KAURENOIC ACID TO KAUREN-7β-OL-19-OIC ACID BY MICROSOMES

Inhibitor added	% Conversion to kauren-7β-ol-19-oic acid	% of control
None (—NADPH)	0·6	—
None	35·0	100
$NADP^+$, 1 mM	24·0	68
$NADP^+$, 1 mM and nicotinamide, 50 mM	8·0	23
Nicotinamide, 50 mM	14·0	40
p-Chloromercuribenzoate, 0·5 mM	0·8	2
SKF-525-A, 0·5 mM	6·0	17

The incubation mixtures were as described in Table 3 except that 0·25 ml of microsome suspension (105,000 *g* pellet) was employed in all cases. Incubations were at 29° for 40 min.

The results are shown in Table 5. $NADP^+$ alone at 1 mM or in the presence of 50 mM nicotinamide inhibited the conversion by 30% and 77%, respectively. Nicotinamide alone at 50 mM gave 60% inhibition while *p*-chloromercuribenzoate at 0·5 mM gave almost 100% inhibition. The steroid synthesis inhibitor SKF 525-A at 0·5 mM was also highly inhibitory.

DISCUSSION

The evidence clearly indicates that kauren-7β-ol-19-oic acid is a direct product of hydroxylation of kaurenoic acid in the endosperm tissue. Not only is it formed rapidly and in larger quantities than other metabolites, but when the concentration of substrate kaurenoic acid is 50 μM or higher it is the only metabolite detected in significant quantities. It was also found that reincubation of the isolated ^{14}C-kauren-7β-ol-19-oic acid with endosperm preparations under conditions equivalent to those used for its biosynthesis led to the formation of ^{14}C-metabolites which appear to be chromatographically indistinguishable from X_1, X_3, X_4 and X_5, suggesting that kauren-7β-ol-19-oic acid is the precursor of all of these substances. However, sufficient quantities of these other metabolites have not been obtained for structural investigations.

It has not been possible to obtain direct evidence for the conversion of kaurene or any of its oxidized derivatives to gibberellins in the endosperm preparations. Nonetheless, the behavior of kauren-7β-ol-19-oic acid in two other biological test systems is most readily interpreted as indicating that it, like kaurene, kaurenol, kaurenal and kaurenoic acid, serves as a normal intermediate for gibberellin biosynthesis. The growth response of the *dwarf*-5 mutant of *Zea mays* to kauren-7β-ol-19-oic acid (Table 2) was qualitatively indistinguishable from that induced by exogeneous gibberellic acid or kaurenoic acid and was quantitatively similar to that induced by the latter. Whereas it is possible that substances might be active in this bioassay by virtue of their structural features without undergoing change to normal *Zea mays* gibberellins, this seems less likely in view of the results obtained in *G. fujikuroi*. The incubation of mycelial suspensions of this organism with ^{14}C-kauren-7β-ol-19-oic acid resulted in the formation of ^{14}C-labeled substances which had the chromatographic properties of gibberellins A_4, A_7, A_{14} and A_3 (gibberellic acid). The latter substance was recrystallized to constant specific radioactivity in the presence of unlabelled,

authentic gibberellin A_3. Even though degradations to show the position of labeling in gibberellin A_3 were not done, the results of other studies of this type with kaurene[7] and kaurenol[9] make it seem likely that this conversion was accomplished directly without randomization of the label.

Hanson and White[14] have also demonstrated the role of kauren-7β-ol-19-oic acid in gibberellin biosynthesis through experiments in which ^{14}C-labeled substrates were fed to *Gibberella fujikuroi* cultures. Small amounts of ^{14}C were detected in kauren-7β-ol-19-oic acid by dilution analyses after feeding 17-^{14}C-kaurene. They also showed that ^{14}C from 17-^{14}C-kauren-7β-ol-19-oic acid was incorporated in short term (4–24 hr) incubations into gibberellin A_{12} and the corresponding aldehyde (in which the carboxylic acid group at the 6-position of gibberellin A_{12} is replaced with a formyl group). In 5-day incubations large amounts of ^{14}C-label from this substrate were found in gibberellin A_3 and smaller amounts in 7β-hydroxykaurenolide (kauren-6α, 7β-diol-19-oic acid 19 → 6α lactone) and 7β, 18-dihydroxykaurenolide. In all of these products the label was found to be present specifically in the 17-position, indicating direct transformations. These 7β-hydroxykaurenolides are produced in *G. fujikuroi* cultures along with other diterpenes. However, it was shown earlier that these kaurenolides are not intermediates in gibberellin synthesis.[9] Thus, it seems probable that there are at least two alternate fates of kauren-7β-ol-19-oic acid in *G. fujikuroi*. In one case 6α-hydroxylation is followed by lactonization and further hydroxylation to the kaurenolides. The second fate involves oxidation and ring contraction to produce compounds of the *enantio*-gibberellane skeleton. Kauren-6β,7β-diol-19-oic acid or the 6β,7β-epoxide of kaurenoic acid would seem to be logical candidates as substrates for the ring contraction step; however, at the present time there is no evidence to support either of these substances as intermediates.

It is possible that the X_1, X_3, X_4 and X_5 fractions which appear to be derived enzymically from kauren-7β-ol-19-oic acid in *E. macrocarpa* endosperm may contain additional intermediates of gibberellin biosynthesis; however, there is no evidence to support this.

Some kaurenoic acid derivatives which became available in the course of the structural investigations were tested in the *dwarf-5 Zea mays* mutant assay. Kauren-7-on-19-oic acid was inactive and kauren-7α-ol-19-oic acid was either inactive or only slightly active. X_{2B} (tentatively identified as IV) was likewise inactive in this assay. This limited information suggests that none of these derivatives are normal intermediates of gibberellin biosynthesis.

The evidence summarized in the Results section led to the conclusion that X_{2B} was not a metabolic product and somehow had arisen from kauren-7β-ol-19-oic acid during its isolation from the incubation mixtures. The properties observed for X_{2B}, although incomplete, were consistent with the tentative structural assignment of kaur-15-en-7β-ol-19-oic acid (IV). The acid-promoted isomerization of an exocyclic to an endocyclic double bond is well known in this series, as for example in the formation of kaur-15-ene (isokaurene) from kaur-16-ene[21] kaur-15-en-19-ol from kaur-16-en-19-ol,[22] and kaur-15-en-6α, 7β-diol-19-oic acid 19 → 6α lactone from kaur-16-en-6α, 7β-diol-19-oic acid 19 → 6α lactone.[19] However, the factors which led to isomerization in the present case, if this is indeed what has occurred, have not been determined. It was ascertained that the unreacted kaurenoic acid recovered from the incubation mixture by the same procedures had not been isomerized. For this reason, it is considered possible that the presence of the 7β-hydroxyl group may in some way facilitate the double-bond migration. However, experiments to see if methyl kaur-16-en-7β-

[21] L. H. BRIGGS, R. W. CAWLEY, J. A. LOE and W. I. TAYLOR, *J. Chem. Soc.* 955 (1950).
[22] C. A. HENRICK and P. R. JEFFERIES, *Australian J. Chem.* **17**, 915 (1964).

ol-19-oate were readily isomerized in glacial acetic acid (either at room temp. for 6 hr or at 100° for 15 min) or on silicic acid columns were negative. Thus, the cause of the formation of X_{2B} under these circumstances remains obscure.

No extensive efforts were made to determine the characteristics of the enzymic system which catalyzes the conversion of kaurenoic acid to kauren-7β-ol-19-oic acid. The general properties of the system—namely, the localization of the activity in the microsome-like particulate fraction, the requirements for reduced pyridine nucleotides and oxygen and the action of selected inhibitors—suggest that this may be a microsomal mixed function oxygenase of the type which has been established for all the other oxidation steps in kaurene metabolism previously investigated in this tissue.[6]

EXPERIMENTAL

Preparation of Enzyme Fractions

The preparation of lyophilized endosperm from immature seeds of *E. macrocarpa* was essentially as described previously.[2] Before use, the lyophilized endosperm was dissolved in 0·01 M Tris-HCl, pH 7·2, at a concentration of 1·2 g dry wt./11-ml buffer. This solution was then centrifuged at low speed in a clinical centrifuge for a few minutes, gently homogenized in a glass homogenizer and filtered through glass wool for removal of cell debris. This filtrate is referred to as whole endosperm extract.

The 105,000 *g* pellet (microsomal pellet) was prepared by centrifugation of the whole endosperm extract at 10,000 *g* for 15–20 min followed by centrifugation of this supernatant fraction at 105,000 *g* for 2 hr. The resulting pellet was washed three times with 1 ml 0·01 M Tris-HCl, pH 7·2, and the pellet from the final wash was homogenized in a volume of the same buffer equivalent to half of the original volume of whole endosperm extract taken. Only freshly prepared enzyme fractions were utilized.

Substrate

A highly purified sample of 17-^{14}C-kaurenoic acid,[6] which had a specific radioactivity of $6·5 \times 10^9$ counts/min/m-mole was kindly supplied by P. J. Murphy.

TLC

Precoated silica-gel G (Brinkman), 0·25 mm, and precoated silica gel G plates impregnated with 3% $AgNO_3$ in EtOH were used for TLC. Gibberellins and kaurene derivatives showed characteristic fluorescent spots after spraying the plate with 5% conc. H_2SO_4 in 95% EtOH and heating at 120° for a few minutes.[23]

Radioassay Techniques

Gel fractions were suspended in 10 ml of toluene scintillation solution containing 4 g of 2,3-diphenyloxazole and 50 mg *p*-bis-[2′-5′-phenyloxazolyl] benzene/l. toluene for radioassay by liquid scintillation spectrhometry. Compounds in aqueous solution were counted in a scintillation solution containing 120 g naphthalene, 7 g of 2,5-diphenyloxazole and 50 mg of p-bis-[2′-(5′phenyloxazolyl)]-benzene made up to 1 l. with *p*-dioxane.

Procedures for Analysis of Incubation Mixtures

The pellet of denatured protein formed on heating the incubation mixture to terminate the reaction was washed with H_2O and then extracted with a small volume of acetone. The extract was concentrated and treated with a small amount of benzene to remove water; the acetone–benzene phase so obtained contained most of the unreacted kaurenoic acid. The other products, X_2 and other polar acids, as well as any remaining kaurenoic acid, were extracted with EtOAc from the aqueous supernatant fraction resulting from the heat precipitation after it had been acidified. The EtOAc and acetone–benzene extracts were combined and concentrated under N_2 for TLC on silica gel with isopropyl ether–HOAc (95:5) as the developing solvent. The products formed were estimated by radioassay of the appropriate gel fractions.

Bioassay Techniques

Bioassays on the *dwarf-5* mutant of *Zea mays* were performed essentially as described by Neely.[24] Seeds of the mutant were germinated under continuous exposure to light at 32–34°. On day 6 after germination test samples in 0·01 ml redistilled acetone were applied to the first unfolding leaf sheath of each plant. Control plants were treated with an equivalent amount of solvent. Plants were allowed to grow under the same

[23] G. W. Elson, D. F. Jones, J. MacMillan and P. J. Suter, *Phytochem.* **3**, 93 (1964).
[24] P. M. Neely. Doctoral Dissertation, University of California, Los Angeles (1959).

conditions for another 7 days after treatment before measurements of the length of leaf sheaths of each plant were made.

Feeding ^{14}C-labeled substrates to cell cultures of G. fujikuroi

The mycelia of a 5-day-old culture of *G. fujikuroi* grown in 1 l. of a defined medium (glucose-ammonium tartrate-minerals medium of Borrow *et al.*[25]) were harvested by centrifugation. The mycelial mass was washed twice with 0·02 M $NaHPO_4$, pH 6·55, and then suspended in 250 ml of the same buffer. The substrate to be incubated, ^{14}C-kaurenoic acid (3·75 × 10^5 counts/min, about 0·05 μmole) or X_{2A} (6·19 × 10^4 counts/min, about 0·14 μmole) was added in 0·1–0·2 ml acetone to 10 ml of this mycelial suspension in a sterile 125 ml flask. The incubations were carried out at 25° on a rotary shaker for 48 hr. As a control for each substrate, 10 ml of cell suspension was heated at 100° in a bath for a few minutes, cooled, and then supplemented with the substrate in acetone as above and incubated under identical conditions.

At the end of the incubation period, the reaction mixtures were heated to 100° and centrifuged. The acidic compounds, including gibberellins, were extracted from the supernatant solution into EtOAc. The extract was concentrated and applied to a silicic acid-celite (1:1) column (1·7 × 25 cm), which was eluted with increasing concentrations of EtOAc in $CHCl_3$ according to West and Phinney.[26] Fractions of the 3 main radioactive peaks were pooled and characterized as described in the Results sections.

Chemical Methods for the Characterization of X_{2A}

CH_2N_2 for methylation reactions was generated from 'diazald' according to the method of de Boer.[27] Acetylation reactions were carried out in a mixture of equal volumes of acetic anhydride and pyridine. Oxidation of X_{2A} to the keto derivative was carried out with 8 N chromic oxide reagent (Jones reagent[28]) in acetone solution. The keto derivative of the methyl ester of X_{2A} was prepared by adding a large excess of CrO_3 in pyridine to a solution of methyl X_{2A} in the same solvent.[29] Reductions with $NaBH_4$ were carried out in MeOH. Hydrogenations were carried out in MeOH at room temperature under H_2 at atmospheric pressure with 5% Pd/C as catalyst.

Physical Constants

NMR spectra were determined with a Varian A-60 Analytical NMR Spectrometer. Samples were dissolved in a minimal volume of $CDCl_3$ containing 20% tetramethylsilane as an internal standard. Spectra were scanned in sealed capillaries containing the samples to be measured, and if necessary, a time-averaging computer was used to give a better resolution of the spectra. Mass spectra were kindly determined on an MS-9 mass spectrometer by Mrs. K. Jones of the Chemistry Department, University of California, Los Angeles, Calif., U.S.A.

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[26] C. A. WEST and B. O. PHINNEY, *J. Am. Chem. Soc.* **81**, 2424 (1959).
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