The hydrolysis rate of AMCHA-Phe was much lower than that of PNPA or α-NA, and the rate of α-NH, PNPA or AMCHA-Phe differed markedly with animal species.

Various esters of AMCHA were prepared, such as saturated alkyl esters, unsaturated alkyl ester, benzyl ester, substituted benzyl esters, phenyl ester, and substituted phenyl esters.

Table II shows hydrolysis rates of AMCHA esters with each enzyme expressed relative to that of AMCHA-Phe. In all enzymes from rats, guinea pigs, rabbits, and pigs, phenyl ester was hydrolyzed more rapidly than those of benzyl ester and alkyl esters. In the esterases from guinea pigs and rabbits, the following relations between the substituted groups and hydrolysis were observed. (1) Generally, the presence of substituent groups, such as halogen or alkyl, at the *p*-position, enhanced the hydrolysis. (2) The hydrolysis rate of *m*-substituted phenyl ester was less than that of the corresponding *p*-substituted compounds. (3) The hydrolysis rate of the *o,p*-disubstituted ester was lower than that of the *p*-substituted ester. (4) Introduction of substituent groups into the *o,o*-position of the phenyl moiety resulted in lowering of the hydrolysis rate. In esterases from the rat and pig, the hydrolysis rate was enhanced by the presence of an alkyl group at the *p*-position. Lowering of the hydrolysis rate was observed by introduction of a substituent group into the *o,o*-position and *o,p*-position of the phenyl moiety. These findings suggest that the hydrolysis of these phenyl esters was affected by the steric as well as electronic effect of the substituents.

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Microbiological Transformation of (—)-Kaur-16-en-19-oic Acid[1]

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(—)-Kaur-16-en-19-oic acid (I) is transfered by *Cunninghamella blakesleeana* to a series of differently hydroxylated derivatives, four compounds of which have been isolated and characterized as 7β-hydroxy-(—)-kaur-16-en-19-oic acid (II), 16-hydroxy-(—)-kauranoic acid (III), 16α,17-dihydroxy-(—)-kauran-19-oic acid (IV) and 7β,16α-dihydroxy-(—)-kauran-19-oic acid (V).

(—)-Kaur-16-en-19-oic acid (I) was isolated as a major constituent of *Cacalia bulbifera* Maxim. with (—)-kauran-16α-ol, (—)-kaur-16-en-19-ol, (—)-kaur-16-en-19-al and others.[1] Microbial transformation of this major constituent was investigated in the hope that hydroxylated kaurenoids, especially 7-hydroxy derivatives, which are important as intermediates in gibberellins biosynthesis,[3] might be obtained.

Cunninghamella blakesleeana was our first choice for this kind of microbiological transformation, because we have some experience of good results in transformation of sesquiterpenes and triterpenes.[4]

Fermentation of (—)-kaur-16-en-19-oic acid (I) at this time gave a mixture of several products, four compounds (II—V) of which were separated through SiO_2 chromatography.

1) Part III in the series "Constituents of Cacalia spp." For Part II see N.A. El-Emary, G. Kusano, and T. Takemoto, *Phytochemistry*, **1975**, 1660.
2) Location: *Aobayama, Sendai.*
3) A.F. White and J.R. Hanson, *Chem. Comm.*, **1969**, 410.
4) H. Hikino, Y. Tokuoka, Y. Hikino, and T. Takemoto, *Tetrahedron*, **24**, 3147 (1968).

The first metabolite (II), mp 238—239°, $C_{20}H_{30}O_3$, was eluted with petr. benzine–ethyl acetate (9: 1—2) from the SiO_2 column. The mass spectrum (M^+, *m/e* 318) and the infrared (IR) spectrum (3455 cm^{-1}) showed an additional hydroxy group on (—)-kaur-16-en-19-oic acid. The nuclear magnetic resonance (NMR) spectrum of the methyl ester (II m), suggested the presence of an axial hydroxy group with not more than two adjacent hydrogens (an equatorial hydrogen at 3.75 ppm with $W_1/_2$ 6 Hz in pyridine-d_5), excluding the possibility of 2, 6, 11 or 12 positions for the hydroxy group of II. Two tertiary methyl groups (two singlets at 0.85 and 1.16 ppm), a methyl ester (a singlet at 3.63 ppm) and an end methylene (a broad unresolved signal at 4.77 ppm) are recognized similarly in the NMR spectrum of methyl (—)-kaur-16-en-19-oate (I m Table I), excluding the possibility of 2α, 3β, 6α or 11α hydroxy derivative for II.

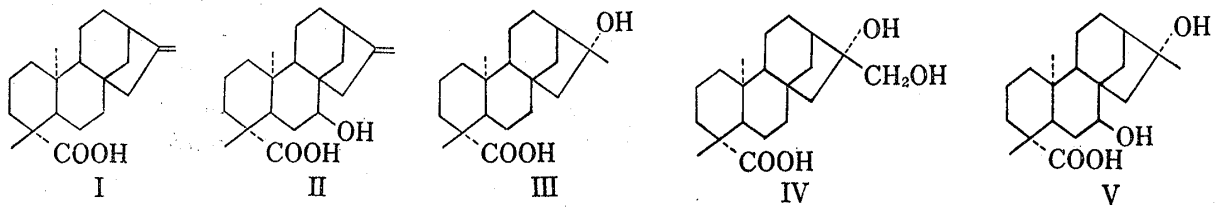

Fig. 1

TABLE I. Comparative NMR Data of (—)-Kaur-16-en-19-oic Acid Ester (IM) and Metabolite Esters (IIM-VM) in $CDCl_3$

Compounds		Im (ppm)	IIm (ppm)	IIIm (ppm)	IVm (ppm)	Vm (ppm)
Assignment	C_{18}	1.17	1.17	1.17	1.17	1.17
	C_{20}	0.83	0.83	0.83	0.83	0.83
	C_{17}	4.8 (2H)	4.77 (2H)	1.35 (3H)	3.7 (2H)	1.38 (3H)
	$OC\underline{H}_3$	3.62	3.62	3.62	3.64	3.62

The keto derivative (II k), mp 193—196°, prepared by Jones oxidation of II, showed a six membered keto band in the IR spectrum (1695 cm^{-1} in $CHCl_3$), a negative Zimmermann test,[5] a positive Cotton effect (Fig. 2), and a stability against heat, excluding the positions 1, 2 or 3 for the keto group of II k.

The authentic specimen of 7β-hydroxy-(—)-kaur-16-en-19-oic acid[6] for direct comparison was not available, but the above described properties lead us to 7β-hydroxy-(—)-kaur-16-en-19-oic acid for the first metabolite (II).

The second metabolite (III), mp 275—280°, M^+, *m/e* 320, which was eluted next to II and recrystallized from acetone, showed a hydroxyl band in the IR spectrum (3300—2900 cm^{-1}) and an addition of one molecule of water to (—)-kaur-16-en-19-oic acid in the mass spectrum. The NMR spectrum of the methyl ester (III m), mp 153—156°, showed three tertiary methyl groups at 0.78, 1.12 and 1.31 ppm, while the signal of the end methylene of I disappeared. From the above properties 16α-hydroxy-(—)-kauran-19-oic acid was proposed for III.

The third metabolite (IV), mp 260—263°, M^+, *m/e* 336, eluted with petr. benzine–ethyl acetate (5—4: 1) and recrystallized from ethyl acetate, showed hydroxyl bands at 3450, 3250 cm^{-1} and a carboxyl carbonyl band at 1710. The methyl ester of IV (IV m), mp 145—147°, gave the acetate (IV ma), mp 105—109°, which had a hydroxyl band at 3455 cm^{-1},

5) F. Piozzi, P. Venturella, A. Bellino, and R. Mondelli, *Tetrahedron*, **24**, 4073 (1968).
6) F T. Lew and Charles A. West, *Phytochemistry*, **10**, 2065 (1971).

Table II. Comparative Data of Kaurenoic Acid and Its Metabolites

Compounds	I	II	III	IV	V
Formula	$C_{20}H_{30}O_2$	$C_{20}H_{30}O_3$	$C_{20}H_{32}O_3$	$C_{20}H_{32}O_4$	$C_{20}H_{32}O_4$
Melting points	174—177	238—238	275	260—263	286
Mass spectra	M^+302	M^+318	M^+320	M^+336	M^+336
IR-spectra (cm^{-1})	3100～2300, 1690	3475, ～2300, 1700	3450, ～2300, 1700	3455, 3250, 1700	3500, 3355, 1700
Methyl ester mp.	81— 84	142—146	153—156	145—147	163—166

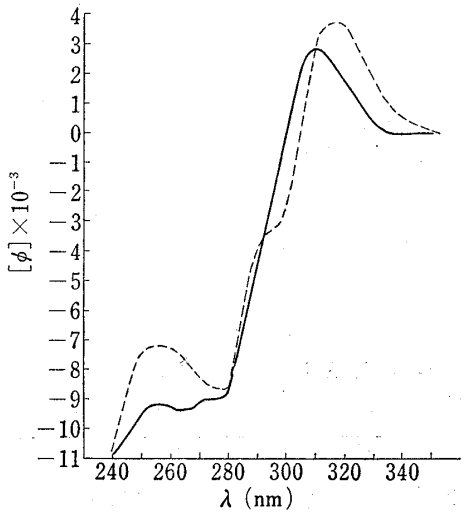

Fig. 2. ORD curves of IImk and Vk
——: II mk ------: V k

ester bands at 1710 and 1735 cm^{-1} in the IR spectrum. The NMR spectrum of IV suggested two tertiary methyl groups at 0.82 and 1.18 ppm, an acetyl at 2.12 ppm and an ester methyl at 3.66 ppm. A signal of 2H at 4.24 ppm is assigned to $-C\underline{H}_2-$OAc. A comparison with the reported data of 16α, 17-dihydroxy-(−)-kauran-19-oic acid[7] lead us to an identification of IV to 16α,17-dihydroxy-(−)-kauran-19-oic acid.

The fourth metabolite (V), mp 286°, M^+, *m/e* 336, had hydroxyl bands at 3450, 3250 cm^{-1} and a carboxyl carbonyl band at 1700 cm^{-1} in the IR spectrum. The methyl ester (V m), mp 163—166°, showed three tertiary methyl signals at 0.83, 1.17 and 1.38 ppm, methyl ester at 3.62 ppm and a carbinyl hydrogen at 4.02 ppm. The last signal is an unresolved singlet with $W_1/_2$ 6 Hz. The acetate (V ma), mp 161—165°, M^+, *m/e* 392 showed a hydroxyl band at 3500 cm^{-1} and ester bands at 1710 and 1730 cm^{-1} in the IR spectrum. The NMR spectrum had three tertiary methyl groups at 0.83, 1.07 and 1.34.

The keto derivative (V k), mp 221—225°, prepared by Jones oxidation of V, had a hydroxyl band at 3500 cm^{-1} and keto carbonyl and carboxyl carbonyl bands at 1720 and 1700 cm^{-1} in the IR spectrum, and showed a positive Cotton effect in the optical rotatory dispersion (ORD) curve (Fig. 2). ORD (*c*=0.1, MeOH) $[\phi]_{312}$ +3600, $[\phi]_{278}$ −8800. These properties suggest 7β,16α-dihydroxy-(−)-kauran-19-oic acid.

Experimental[8]

Fermentation and Extraction——*Cunninghamella blakesleeana* was cultivated in an aqueous medium containing $NaNO_3$ 0.3 g, K_2HPO_4 0.1 g, KCl 0.05 g, $FeCl_2 \cdot 4H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, soluble starch 3 g, vitamine solution 1 ml and water 100 ml. The medium was adjusted to pH 6.8 using 1 N NaOH. Forty five fermentation flasks (500 ml capacity) were prepared, in each with 100 ml of the medium, then sterilized in high pressure sterilizer at 120° for 20 minutes and cooled to room temperature and inoculatedwith the mycelia. The flasks were placed in a reciprocal shaker and shaking was continued at 27° for three days, then kaurenoic acid 50 mg/1 ml EtOH was added to each flask and shaking was continued for further 3 days at 27°.

The mycelium was separated from the culture filtrate and washed with water, the filtrate and washings were acidified with HCl, saturated with NaCl and extracted with EtOAc. For better separation of emulsions EtOAc/acetone (3: 1) mixture was used for extraction of the culture filtrate. The organic layer was collected, washed with water, dried over anhydrous Na_2SO_4 and condensed to give dark orange gum.

7) P.R. Jefferies and T.G. Payne, *Aust. J. Chem.*, **18**, 1441 (1965).
8) Melting points were determined on a hot stage of Yazawa Apparatus and uncorrected. NMR spectra were determined on Hitachi Perkin-Elmer R-20 and JEOL-PS-100 spectrometers using tetramethylsilane as internal standard. Mass spectra were measured with a Hitachi RUM-7 spectrometer. ORD curves were measured with a JASCO DIP-SL polarimeter.

Chromatography and Isolation of Metabolites——The total acid gum (14 g) was mixed with 30 g SiO_2 and placed on the top of a column packed with 220 g SiO_2. Elution with petr. benzine–EtOAc (9: 1), gave kaurenoic acid (I, 560 mg) after recrystallization from aqueous MeOH.

Elution with petr. benzine–EtOAc (9: 2—1), gave II as crystalline prisms (180 mg), after recrystallization from EtOAc–petr. benzine, mp 238—239° (sublimable at 190° to very long needles or cluster crystals). IR ν_{max}^{KBr} cm^{-1}: 3455 (OH), 1700 (acid carbonyl), 880 (end methylene). Mass Spectrum: M^+, *m/e* 318, suggesting the molecular formula $C_{20}H_{30}O_3$. The methyl ester prepared with diazomethane in ether, mp 142—146°, NMR ($CDCl_3$) ppm: 0.85 and 1.16 (3H, each), 3.63 (–O–CH_3), and an unsplit 2 protons peak at 4.77. The 7-keto derivative (II mk) was prepared by stirring 5.0 mg of the methyl ester (II m) in 1 ml of pyridine and 10 mg of chromium trioxide at room temperature for 3 hr. AcOEt was added and the mixture was passed through an Al_2O_3 column. The passed fraction was condensed *in vacuo* and the residue was recrystallized from AcOEt to give colorless flakes, 4.7 mg, mp 104—106°. ORD (MeOH, $c=0.1$): $[\phi]_{310}+2800$, $[\phi]_{268}$ −9280.

From the fraction next to 7β-hydroxy-(−)-kaurenoic acid (II), we obtained the second metabolite (III) (295 mg), as needles after recrystallization from acetone mp 275—280°, IR ν_{max}^{KBr} cm^{-1}: 3300—2900 (OH), 1710 (acid carbonyl); M^+, *m/e* 320, suggesting $C_{20}H_{30}O_3$. The methyl ester (III m), mp 153—156°, NMR ($CDCl_3$) ppm: 0.78 (3H, s), 1.12 (3H, s), 1.31 (3H, s), and 3.68 (3H, s, –O·CH_3). These properties are consistent with the data reported for 16α-hydroxy-(−)kauran-19-oic acid and its methyl ester.

Elution with petr. benzine–EtOAc (5—4: 1), gave the third metabolite (IV) (650 mg), as needles after recrystallization from EtOAc, mp 260—263°. IR ν_{max}^{KBr} cm^{-1}: 3450, 3250 (OH), 1710 (acid carbonyl); Mass Spectrum: M^+, *m/e* 336, suggesting $C_{20}H_{32}O_4$. The methyl ester (IV m), mp 145—147°. NMR ($CDCl_3$) ppm: 0.84 and 1.18 (3H, each, s), 3.66 (ester methyl), and 3.67 (2H, s, –CH_2·OH).

IV m (20 mg) was dissolved in 0.2 ml dry pyridine and 4 ml Ac_2O to keep at room temperature over night. Water was added to the reaction solution and the mixture was extracted with EtOAc. The acetate extract was washed with water, dried over anhydrous Na_2SO_4, and the residue was chromatographed on SiO_2, to give the mono-acetate-methyl ester (IV ma), mp 105—109°. IR ν_{max}^{KBr} cm^{-1}: 3455 (OH), 1710 and 1735 (ester carbonyl and acetyl). NMR (100 MHz, $CDCl_3$) ppm: 0.82 and 1.18 (3H each, s), 2.12 (3H, s, acetoxyl), 3.66 (3H, s, ester methyl), 4.24 (2H, s, –CH_2 OAc). Mass Spectrum: M^+, *m/e* 392, M^+-18 *m/e* 374. This compound was identified as 16α,17-dihydroxy-(−)-kauran-19-oic acid by comparison of mp, IR and NMR spectrum of the authentic specimen.

Elution with petr. benzine–EtOAc (6—7: 10), gave the fourth metabolite (V) (60 mg) as needles after recrystallization from EtOAc, mp 286°. IR ν_{max}^{KBr} cm^{-1}: 3450, 3250 (OH), 1700 (acid carbonyl), Mass Spectrum: M^+, *m/e* 336. The methyl ester: NMR ($CDCl_3$) pm: 0.83 (3H, s), 1.17 (3H, s), 1.38 (3H, s) and 3.62 (3H, s, –O·CH_3), NMR (100 MHz, pyridine-d_5) ppm: 4.02 ppm (1H, unresolved singlet $W_{1/2}$ 6 Hz, 7α–H).

V m (15 mg), was acetylated in pyridine/acetic anhydride as mentioned above, and the product was chromatographed on SiO_2 (1 g), to give the corresponding acetate (V ma), as needles after recrystallization from EtOAc/petr. benzine, mp 161—165°, IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 and 1236 (acetoxyl), and 1710 (ester carbonyl); Mass Spectrum: M^+, *m/e* 392; NMR ($CDCl_3$) ppm; 0.83 (3H, s), 1.07 (3H, s), 1.34 (3H, s), 2.02 (3H, s, –CO·CH_3) and 3.62 (3H, s, –O·CH_3) and 4.9 (1H, unresolved singlet, $W_{1/2}$ 6 Hz).

V (10 mg) was dissolved in 4 ml acetone and treated with Jones reagent as mentioned above. The product was taken in EtOAc to chromatograph on SiO_2 (1 g), to recrystallize from EtOAc as fine needles (V k, 8 mg), mp 221—225°. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1720, 1700 (ketone and acid carbonyl). ORD (MeOH, $c=0.1$): $[\phi]_{312}$ +3600, $[\phi]_{278}$ −8800. From these properties V was thought as 7β,16α-dihydroxy-(−)-kauran-19-oic acid.