pleted the mixture was allowed to stir for 3 hr at 60°. The AcOH was removed *in vacuo* and the residue was diluted with H_2O (300 ml). The precipitated solid was filtered, washed with hot $(CH_3)_2CO$ and recrystallized from EtOH to give 6.2 g (33%) of pure product (**2**), mp 213—215°. *Anal.* Calcd. for $C_{12}H_{11}O_3N_2Br$: C, 46.32; H, 3.56; N, 9.00. Found: C, 46.21; H, 3.40; N, 9.23. NMR (CF_3COOH) δ: 1.65 (t, 3H, J=6.7 Hz, CH_2CH_3), 4.66 (s, 2H, $-CH_2Br$), 4.68 (q, 2H, J=6.7 Hz, CH_2CH_3), 8.08 (d, 1H, J=9 Hz, H-6), 9.02 (d, 1H, J=9 Hz, H-5), 9.50 (s, 1H, H-2).

Ethyl 7-[1-Bromo-2-(5-nitro-2-furyl)vinyl]-4-hydroxy-1,8-naphthyridine-3-carboxylate (**3**)——To a solution of **2** (0.16 g, 0.0005 mole) in AcOH (5 ml) and conc. H_2SO_4 (0.5 ml), 5-nitrofurfural (0.07 g, 0.0005 mole) was added and a mixture was heated at 100° for 3 hr. After cooling, the reaction mixture was diluted with H_2O and the precipitated solid was filtered to give the product (**3**).

7-[1-Bromo-2-(5-nitro-2-furyl)vinyl]-4-hydroxy-1,8-naphthyridine-3-carboxylic Acid (**4**)——A mixture of **3** (0.1 g, 0.00025 mole), 90% AcOH (2.7 ml) and conc. HCl (0.3 ml) was heated at 130° for 1 hr. After cooling, the precipitated solid was filtered to give the product (**4**).

1-Alkyl-7-[1-bromo-2-(5-nitro-2-furyl)vinyl]-4-oxo-1,8-naphthyridine-3-carboxylic Acids (5 and 6) General Procedure——A mixture of **4** (0.01 mole), anhydrous K_2CO_3 (0.02 mole) and appropriate alkyl iodide (0.05 mole) in dimethyl formamide (DMF) (30 ml) was heated at 100° for 3 hr. The reaction mixture was evaporated *in vacuo*, and the residue was washed with H_2O, then with EtOH to give the corresponding alkylated product (**5** and **6**).

7-[1-Alkylamino-2-(5-nitro-2-furyl)vinyl]-4-hydroxy-1,8-naphthyridine-3-carboxylic Acids (**7—9**)——A mixture of **4** (0.0005 mole) and appropriate amine (0.0015 mole) in DMF (10 ml) was heated at 100° for 5 hr. DMF was removed *in vacuo* and the residue was washed with H_2O to give the corresponding product (**7—9**).

1-Alkyl-7-[1-alkylamino-2-(5-nitro-2-furyl)vinyl]-4-oxo-1,8-naphthyridine-3-carboxylic Acids (**10—14**) **General Procedure**——A mixture of **5** (or **6**) (0.0005 mole) and appropriate amine (0.0015 mole) in DMF (10 ml) was heated at 100° for 5 hr. DMF was removed *in vacuo* and the residue was washed with H_2O to give the corresponding product (**10—14**).

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Substrate Specificity of Carboxylesterase (E.C.3.1.1.1)[1] from Several Animals

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Experiments were made to see the substrate specificity of purified esterase from various origins using *p*-nitrophenyl acetate, α-naphthyl acetate and *trans*-4-aminomethylcyclohexanecarboxylic acid esters as a substrate. And attension was focused on the influence of structural properties of *trans*-4-aminomethylcyclohexanecarboxylic acid esters. The hydrolysis rate of α-naphthyl acetate of *p*-nitrophenyl acetate differed markedly according to animal species. In all the enzymes from rats, guinea pigs, rabbits, and pigs, phenyl ester was hydrolyzed more readily than benzyl ester or alkyl ester. The hydrolysis rate of phenyl esters was affected by the steric as well as electronic effect of the substituents.

Carboxylesterases[1] are widely distributed in animals, plants, and bacteria. They hydrolyze a variety of esters, and are sensitive to organophosphate inhibitors.[3] Esterases have been purified from the liver of pigs,[4] oxen,[5] rats,[6] and rabbits.[7]

1) Enzyme: Carboxyl ester hydrolase (E.C. 3.1.1.1).
2) Location: *1432-1 Horinouchi, Hachioji-shi, Tokyo, 192-03, Japan.*
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Catalytic properties of esterase have been the subject of extensive research in recent years. The behavior of horse liver esterase with aliphatic esters was examined by Dixon and Webb,[8] pig liver esterase by Ocken and Levy,[9] and rat liver esterase by Arndt and Krisch.[10]

We examined the substrate specificity of esterases using *p*-nitrophenyl acetate (PNPA), α-naphthyl acetate (α-NA), and *trans*-4-aminomethylcyclohexanecarboxylic acid ester (AMCHA-ester) as substrate. The present communication deals with the substrate specificity of rat, guinea pig, rabbit, and pig microsomal esterases. In particular, attention was focused on the effect of the structural properties of AMCHA-esters on the enzyme action.

Materials and Methods

Enzyme Preparation——Carboxylesterase was prepared from rat, rabbit, and guinea pig liver microsomes according to the method of Ljungquist and Augustinsson[6b] (involving solubilization of esterase using phospholipase-A treatment, gel filtration, DEAE-cellulose chromatography, and isoelectric focusing), except that 20 mM phosphate buffer (pH 7.0) was used for the preparation. These enzymes (rat e_1, rat e_2, guinea pig e_1, guinea pig e_2, and rabbit e_2) were homogeneous in disc electrophoretic analysis at pH 9.4. Pig esterase was purchased from C.F. Boehringer and Sohne Gmbh. The protein was measured by the method of Lowry, *et al.*[11] with bovine serum albumin as a standard.

Substrate——*p*-Nitrophenyl acetate and α-naphthyl acetate were obtained from Tokyo Kasei Organic Chem. Co. AMCHA-esters were synthesized according to the method of Okano, *et al.*[12]

Assay of Esterase——1) PNPA used as a Substrate: The reaction mixture of 4.9 ml of 50 mM phosphate buffer (pH 8.0), 0.1 ml of 100 mM PNPA (in MeOH), and 0.2 ml of the enzyme solution was incubated at 37°. Absorbance at 405 nm was measured to determine the products (*p*-nitrophenol) in the reaction mixture.

2) α-NA used as a Substrate: One-half ml of enzyme solution was incubated with 5 ml of a substrate mixture conteaing α-NA (0.1 mg/ml) in 50 mM phosphate buffer (pH 8.0) at 37° for 30 min. The liberated α-naphthol was captured by the addition of 1 ml of an aqueous solution of diazonium salt Fast Blue b (4 mg/ml). Enzyme reaction was stopped by adding 1 ml of 40% trichloroacetic acid and the colored diazotate was extracted with 5 ml of AcOEt. Diazotate of extract was determined by spectrophotometric measurements at 560 nm.

3) AMCHA-ester used as a Substrate: A mixture of 0.1 ml of 1—3 μmol AMCHA-ester (in MeOH), 0.2 ml of 50 mM phosphate buffer (pH 8.0), and 0.1 ml of the enzyme solution was incubated at 37° for 30 min. The enzyme reaction was stopped by adding 0.06 ml of 12.5% trichloroacetic acid. The product formed (AMCHA) was measured by the method using gas chromatography (GLC).

Measurement of AMCHA——AMCHA was determined according to a minor modification of the procedure described by Isoda, *et al.*[13] The reaction mixture was extracted with ether, then was made alkaline by adding Na_2CO_3, and reextracted with ether. The aqueous layer was evaporated and the residue was acetylated by adding 1 N NaOH and Ac_2O. The acetylated substance was extracted with acetone at 50° and, after removal of the solvent, the residue was dissolved in MeOH, CH_2N_2 was added, and the mixture was allowed to stand for 30 min at room temperature. Methyl *trans*-4-acetamidmethylcyclohexanecarboxylic acid (Ac-AMCHA-Me) was collected, dissolved in tetrahydrofuran, and Ac-AMCHA-Me was measured by the method using GLC.

GLC——A Shimadzu Model GC-6A gas chromatograph equipped with a hydrogen flame ionization detector was used for GLC, and the conditions used were: column of 2% Polyethylenglycol 20 M on Chromosorb Q (60—80 mesh, 3 mm × 100 cm); column temperature 180°: nitrogen as carrier gas at 60 ml/min for Ac-AMCHA-Me. N-Cyclohexylbenzamide was used as an internal standard.

Results and Discussion

Table I shows the esterase activities of purified enzymes obtained from hepatic microsomes of rats, guinea pigs, rabbits, and pigs, using PNPA, α-NA and phenyl *trans*-4-aminomethylcyclohexanecarboxylate (AMCHA-Phe) as substrate.

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Table I. Substrate Specificity of Carboxylesterase

Enzyme	Esterase activity[a] PNPA[b] (μmole/min/ml)	α-NA[c] (μmole/min/ml)	AMCHA-Phe[c] 10^{-1} μmole/min/ml
Rat e_1	65±7	44±3	0.14±0.02
e_2	87±9	274±8	0.21±0.02
Guinea pig e_1	378±11	146±14	0.17±0.03
e_2	234±19	302±19	0.18±0.02
Rabbit e_1	475±17	1482±34	0.38±0.09
e_2	87±10	625±19	0.08±0.03
Pig	435±38	1199±107	1.16±0.22

The values represent mean ± standard error.
a) Esterase activity was measured with purified carboxylesterase (30 μg of protein) in 50 mM phosphate buffer pH 8.0.
b) at 2 mM substrate
c) at 1 mM substrate

Table II. Enzymatic Hydrolysis Rate of Various AMCHA-esters by Purified Microsomal Esterase

Substrate[a] R	HX	Relative rate of hydrolysis[b] Rat (e_1,e_2)	Guinea pig (e_1,e_2)	Rabbit (e_1,e_2)	Pig
H_2NCH_2-⟨H⟩···COOR·HX					
$-C_4H_9$(*tert*)	HCl	0[c]	0[c]	0[c]	0[c]
$-C_6H_{13}$(*n*)	HCl	0.38±0.15[c]	0[c]	0[c]	0.59±0.09[c]
$-CH_2CH{=}CH_2$	HCl	0.62±0.18[c]	0.34±0.29	0.75±0.50	0.58±0.18[c]
$-CH_2-C_6H_5$	HCl	0.50±0.30[c]	0[c]	0[c]	0.56±0.13[c]
$-CH_2-C_6H_3(Cl)-CH_3$	HCl	0.54±0.19[c]	0[c]	0[c]	0.53±0.14[c]
$-CH_2-C_6H_3(CH_3)-CH_3$	HCl	0.38±0.08[c]	0[c]	0[c]	0.38±0.06[c]
$-C_6H_4-H$	HCl	1.00±0.19	1.00±0.27	1.00±0.25	1.00±0.24
$-C_6H_4-CH_3$	HCl	1.19±0.23	0.39±0.19[c]	0.25±0.10[c]	0.75±0.21
$-C_6H_4-CH_3$	HCl	1.54±0.23[c]	0.87±0.11	0.90±0.10	1.15±0.24
$-C_6H_4-CH_3$	HCl	1.73±0.21[c]	1.34±0.18[c]	1.75±0.05[c]	1.44±0.24[c]
$-C_6H_4-Cl$	HCl	0.92±0.12	1.51±0.18[c]	1.70±0.10[c]	0.97±0.25
$-C_6H_4-CH_3$	HCl	1.76±0.24[c]	1.21±0.13	1.00±0.15	1.34±0.25
$-C_6H_3(CH_3)-CH_3$	HCl	0.54±0.08[c]	0.31±0.18[c]	0.15±0.05[c]	0.78±0.09[c]
$-C_6H_3(CH_3)-CH_3$	HCl	1.23±0.15	1.18±0.09	1.20±0.10	1.15±0.06
$-C_6H_3(CH_3)_2$	HCl	0.38±0.04[c]	0[c]	0[c]	0.31±0.06[c]
$-C_6H_4-SO_2NH_2$	HBr	0.53±0.22[c]	0[c]	0[c]	0[c]

a) at 1 mM substrate
b) The hydrolysis rates of AMCHA-esters with each enzymes were represented relative to that of AMCHA-Phe. The values represent mean ± standard error.
c) significantly different from AMCHA-Phe group, $p < 0.05$

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.
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