had been obtained, the test drugs 3-5 (again in 0.2-mL injection volumes) were injected in increasing doses into the renal artery.

Agonists that exhibited vasodilation were also tested with SCH 23390 to determine the degree of DA_1 inhibition. A dose of 2.5 μg/kg of SCH 23390 was given iv and 3 min later a dose of DA was given to ascertain complete blockade, followed by a dose of the test drug. In order to determine whether the dilation was β-adrenergic in origin, propranolol (2.5-5 mg/kg) was infused intraarterially over a 20-min period. β-Adrenergic blockade was established by abolition of the vasodilator effects of isoproterenol (3 nmol).

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Chemistry and Inhibitory Activity of Long Chain Fatty Acid Oxidation of Emeriamine and Its Analogues

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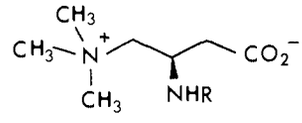
Emericedins A, B, and C, new betaines having inhibitory activity of long chain fatty acid oxidation, were isolated from the culture broth of *Emericella quadrilineata* IFO 5859. Their structures were determined by spectroscopic analyses as (*R*)-3-(acylamino)-4-(trimethylammonio)butyrate (acyl: A, acetyl; B, propionyl; C, *n*-butyryl). Structural confirmation and assignment of absolute configuration were made by chemical synthesis from L-asparagine. Deacylation of emericedin gave a potent derivative, (*R*)-3-amino-4-(trimethylammonio)butyrate, designated as emeriamine. In order to study the structure–activity relations, various analogues of emeriamine, including a stereoisomer, were prepared. Among them, *N*-palmitoyl and *N*-myristoyl derivatives showed much stronger inhibition of fatty acid oxidation than emeriamine.

In the course of searching for new biologically active compounds among microbial metabolites, we found that *Emericella quadrilineata* IFO 5859 produced novel betaines, emericedins A (1), B (2), and C (3). Emericedin



1 : R = CH_3CO
2 : R = CH_3CH_2CO
3 : R = $CH_3CH_2CH_2CO$
4 : R = H

is a new type of inhibitor of long chain fatty acid oxidation, although its activity is very low. Earlier, we reported the biochemical properties of deacylemericedin, designated as emeriamine (4), which showed potent inhibitory activity of fatty acid oxidation and hypoglycemic and antiketogenic activities in fasted rats after oral administration.[1] For the purpose of obtaining more potent analogues and also studying structure–activity relations, various analogues of 4, including a stereoisomer, were prepared. This paper deals with the isolation and structures of 1–3 and includes the synthesis and inhibitory activity of long chain fatty acid oxidation in rat liver mitochondria of 4 and its analogues.

Chemistry. Compound 1 was isolated as colorless crystals from the culture broth of *Emericella quadrilineata* IFO 5859 by column chromatography using cation-exchange resins. The molecular formula of 1 was determined to be $C_9H_{18}N_2O_3$ from elemental analysis and secondary ion mass spectrometry (SI-MS).

Structural elucidation of 1 was carried out by the following spectroscopic analyses. The IR spectrum showed characteristic bands at 1660 (amide C=O) and 1590 (CO-O^-) cm^{-1}. Its ^1H NMR spectrum (100 MHz, CD_3OD) indicated the presence of an acetyl group (δ 1.98), two methylenes (δ 2.42 and 3.56, each d, 4 H), a trimethylammonio group (δ 3.20, s, 9 H), and a methine (δ 4.7, m, 1 H). Two doublet methylene signals were each collapsed into a singlet, by the irradiation of the methine proton at 4.7 ppm, suggesting the structural unit of CH_2CHCH_2. Acid hydrolysis of 1 with 6 N HCl at 100 °C yielded a ninhydrin-positive compound, named emeriamine (4), as the dihydrochloride, $C_7H_{16}N_2O_2 \cdot 2HCl$. The ^1H NMR spectrum of the dihydrochloride of 4 showed two methylenes (δ 3.27 and 4.10, each d, 4 H), a trimethylammonio group (δ 3.50, s, 9 H), and a methine (δ 4.57, m, 1 H), but showed the absence of an acetyl methyl signal. From these results, the structures of 1 and 4 were elucidated to be 3-(acetylamino)-4-(trimethylammonio)butyrate and its deacetyl derivative, respectively. Table I shows the ^{13}C NMR spectral data of 1 and the dihydrochloride of 4. The signal at δ 69.8 (t) in the spectrum of 1 was assigned to the methylene carbon attached to a quarternary amino group on the basis of comparison with the ^{13}C NMR spectrum of L-carnitine (Table I). The minor components, emericedins B ($C_{10}H_{20}N_2O_3$) and C ($C_{11}H_{22}N_2O_3$), were also isolated and found, from their ^1H NMR spectra, to have a propionyl and a *n*-butyryl group, respectively, instead of an acetyl group.

In order to confirm the structure of 1 including the C-3 stereochemistry, synthesis of 1 from L-asparagine was carried out as illustrated in Scheme I. L-2-[(Benzyloxy-

Table I.[a] ^{13}C NMR Spectral Data of 1 and 4 (25 MHz, D_2O)

$(CH_3)_3N^+CH_2CH(R)CH_2COO^-$
4 3 2 1

carbon no.	chemical shift, ppm 1	4[b]	carnitine[c]
C-1	173.9 (s)	172.9 (s)	174.5 (s)
C-2	42.4 (t)	37.6 (t)	40.9 (t)
C-3	43.9 (d)	43.8 (d)	63.6 (d)
C-4	69.8 (t)	67.7 (t)	70.4 (t)
N^+CH_3	54.9 (q)	54.5 (q)	55.2 (q)
	54.4 (q)		55.1 (q)
	54.3 (q)		54.9 (q)
$COCH_3$	177.7 (s)		
$COCH_3$	23.1 (q)		

[a] 1: R = $NHCOCH_3$. 4: R = NH_2. Carnitine: R = OH. [b] Dihydrochloride. [c] Hydrochloride.

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Scheme I. Synthesis of Emericedin A

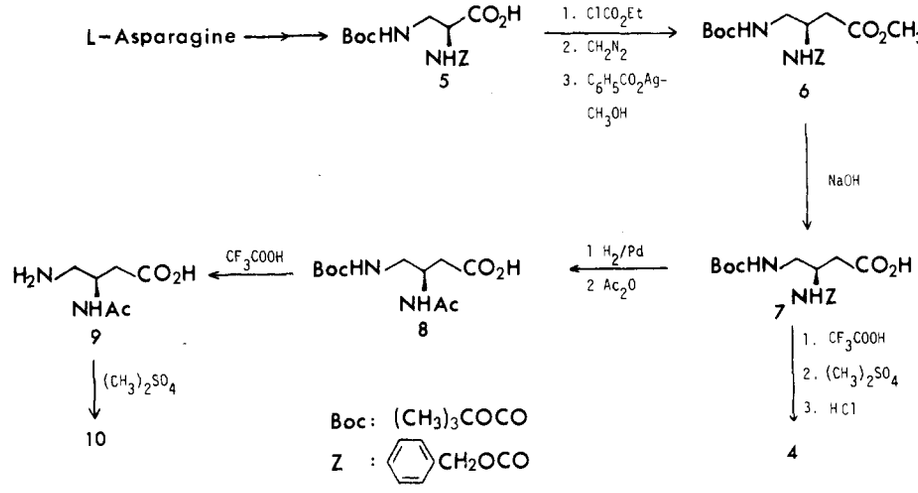

Table II. Inhibition of Fatty Acid Oxidation by Emeriamine and Its Analogues

$Me_3N^+CH_2CH(NHR_1)CH_2COR_2$

compound no.	R_1	R_2	inhibition (%)[a] 2 mM	2×10^{-1} mM	2×10^{-2} mM	2×10^{-3} mM
1	CH_3CO	O^-	38	6		
2	CH_3CH_2CO	O^-	44			
3	$CH_3CH_2CH_2CO$	O^-	50			
4	H	O^-			90	59
11	HCO	O^-	84	53		
12	NH_2CO	O^-	72	84		
13	CH_3OCO	O^-	0	0		
14	$C_6H_5CH_2OCO$	O^-	98	85		
15	$CH_3(CH_2)_{12}CO$	O^-			100	100
16	$CH_3(CH_2)_{14}CO$	O^-			100	100
17	$CH_3C_6H_4SO_2$	O^-	100	84		
18	CH_3	O^-	100	89		
19	H	OCH_3			73	26
20	H	OCH_2CH_3			85	37
21	H	NH_2			27	10
22	*S* isomer of 4		36	11		

[a] Inhibition of carnitine-dependent oxidation of [1-^{14}C]palmitate to $^{14}CO_2$ in rat liver mitochondria was measured as described in the Experimental Section.

carbonyl)amino]-3-[(*tert*-butoxycarbonyl)amino]propionic acid (**5**), which was prepared from (benzyloxycarbonyl)-L-asparagine through two reaction steps according to the method of Waki et al.,[2] was treated with ethyl chloroformate at −10 °C and then diazomethane to afford a diazo ketone. From the diazo ketone, methyl L-3-[(benzyloxycarbonyl)amino]-4-[(*tert*-butoxycarbonyl)amino]butyrate (**6**) was obtained by the Wolff rearrangement using silver benzoate in MeOH.[3,4] Saponification of **6** with aqueous NaOH produced a free carboxylic acid (**7**), which was hydrogenated over Pd-black and acetylated with acetic anhydride to give L-3-(acetylamino)-4-[(*tert*-butoxycarbonyl)amino]butyric acid (**8**). The *tert*-butoxycarbonyl group of **8** was removed by treatment with TFA, affording L-3-(acetylamino)-4-aminobutyric acid (**9**). N-Trimethylation of **9** was carried out with $(CH_3)_2SO_4$ in a manner similar to that described for the synthesis of laminine by Takemoto et al.[5] The hydrochloride of (*R*)-3-(acetylamino)-4-(trimethylammonio)butyrate (**10**) thus obtained was identical with naturally occurring emericedin A hydrochloride.

Compound **4** was synthesized alternatively from **7** in a way similar to that described for the synthesis of **1** (Scheme I). The analogues of **4** prepared are N-substituted derivatives, ester and amide derivatives, and a stereoisomer of **4**. The derivatives of **4** with a substituted amino group or blocked carboxyl group were prepared as shown in Scheme II. N-Acylation or N-tosylation of **4** with a formic acid–acetic anhydride mixture, cyanate,[6] methoxycarbonyl chloride, benzyloxycarbonyl chloride, myristic anhydride, palmitic anhydride, and *p*-toluenesulfonyl chloride gave the corresponding N-substituted derivatives **11**, **12**, **13**, **14**, **15**, **16**, and **17**, respectively. The *N*-methyl derivative of **4** (**18**) was obtained by treatment of **1** with silver oxide–methyl iodide in dimethylformamide (DMF)[7] followed by deacetylation with 6 N HCl.

Esterification of **4** with HCl–MeOH and thionyl chloride–EtOH gave the methyl ester (**19**) and the ethyl ester (**20**), respectively. Treatment of **19** with ammonia–MeOH afforded the amide derivative (**21**). For the synthesis of (*S*)-emeriamine (**22**), the stereoisomer of **4**, (*S*)-emericedin A (**23**) was prepared by starting from D-asparagine as de-

(2) Waki, M.; Kitajima, Y.; Izumiya, N. *Synthesis* **1981**, 266.
(3) Wakamiya, T.; Teshima, T.; Sakakibara, H.; Fukukawa, K.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1984.
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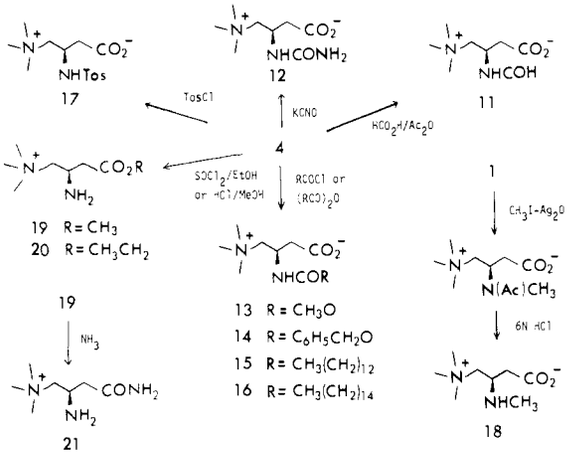
(5) Takemoto, T.; Daigo, K.; Takagi, N. *Yakugaku Zasshi* **1964**, *84*, 1180.
(6) Stark, G. R. *Biochemistry* **1965**, *4*, 1030.
(7) Olsen, R. K. *J. Org. Chem.* **1970**, *35*, 1912.

Scheme II. Synthesis of Analogues of Emeriamine



scribed for the synthesis of (*R*)-emericedin A (**1**), followed by deacetylation with 6 N HCl.

Biological Results and Discussion

Emeriamine and its analogues were compared for the in vitro inhibitory activity of L-carnitine-dependent oxidation of long-chain fatty acids.[1] Emeriamine (**4**) exhibited potent inhibitory activity as shown in Table II. Esterification (**19**, **20**) or amidation (**21**) of the carboxyl group of **4** resulted in a decrease in activity, whereas N-acylation (**11**, **13**, **14**), N-carbamylation (**12**), N-tosylation (**17**), or N-methylation (**18**) markedly reduced the inhibitory activity. On the contrary, introduction of a long chain acyl group to the amino nitrogen of **4** (**15**, **16**) caused a surprising increase in activity. These results suggest the presence of two types of inhibitors, one with a free amino group and the other with a long-chain acyl group. It is noteworthy that the N-palmitoyl derivative (**16**) shows much more potent inhibitory activity than **4**, because the main inhibition site of **4** has been suggested to be carnitine palmitoyltransferase.[1] The *S* isomer of **4** (**22**) shows very weak inhibitory activity, indicating that the mode of action of **4** is stereospecific. Studies on the in vitro inhibitory mechanism of fatty acid oxidation and carnitine palmitoyltransferase by **4** and in vivo hypoglycemic and antiketogenic activities of analogues of **4** will be described elsewhere.

Recently, Jenkins and Griffith[8,9] reported the synthesis and inhibitory activity of fatty acid oxidation and carnitine acyltransferase of racemic emeriamine and racemic acyl emeriamine and described the results similar to those obtained by us as to (*R*)-emeriamine and its acyl derivatives, mentioned above. However, it would have been better if they had used optically active compounds, not racemates, when studying their biological properties. Each stereoisomer may have different biological activities.

Experimental Section

Melting points were taken on a Yamato Model MP-21 melting point apparatus, and specific rotations were determined with a JASCO DIP-181 instrument at 23–25 °C. The IR spectra were measured with a Hitachi 285 grating spectrophotometer. The NMR spectra were obtained with a Varian EM-390 or XL-100 instrument with Me_4Si as the internal standard. Each analogue of **4** had spectral data compatible with its assigned structure and was found to be a single spot on TLC or single peak in high-performance liquid chromatography (HPLC). TLC was carried out on precoated silica gel plates ($60F_{254}$, Merck). HPLC was performed on a YMC-pack A-312 column (equipment, Waters Model 6000A/660/440; detection, UV absorbance at 214 nm). The desalting procedure was as follows, unless otherwise mentioned: The solution was applied to a Dowex 50 W × 2 (H^+) column, the column was washed with H_2O, the adsorbed compound was eluted with 0.5 N NH_4OH, and the eluate was evaporated.

Fermentation and Isolation of Emericedins A (1), B (2), and C (3). *Emericella quadrilineata* IFO 5859 was cultivated with 100 L of a medium, composed of oleic acid 3%, raw soybean flour 0.5%, malt extract 0.5%, polypeptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.05%, $MnSO_4 \cdot nH_2O$ 0.05%, and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 4.5, in a 200-L fermentator at 28 °C for 138 h with aeration and agitation. The culture broth (70 L) thus obtained was filtered with Hyfro Super Cel, and the filtrate was loaded onto Amberlite IR-120 (H^+, 10 L). Emericedins were eluted with 1 N NH_4OH. The eluate was concentrated and subjected to activated carbon chromatography (15 L), eluting with 50% MeOH. The eluate was concentrated to give a syrupy residue. The residue obtained from the culture broth (135 L) was dissolved in 0.05 M acetate buffer (pH 4.0, 100 mL), and the solution was passed through a column of Dowex 50 W × 2 (0.5 L) that had been equilibrated with the same buffer. The adsorbed emericedins were eluted with 0.1 M acetate buffer (pH 5.0). The eluate was desalted with a column of Amberlite IR-120 (H^+, 0.3 L) and concentrated. The concentrate was passed through a column of Dowex 1 × 2 (OH^-, 0.2 L). The passed solution was concentrated and freeze-dried to give emericedin complex (9.2 g) as a hygroscopic solid. Crystallization of the solid (8.0 g) from EtOH–Et_2O afforded the major component **1** as colorless prisms (6.4 g): mp 174–175 °C; $[\alpha]_D$ −16.3° (c 1.0, H_2O); SI-MS, *m/z* 203 (MH^+). Anal. Calcd for $C_9H_{18}N_2O_3 \cdot H_2O$: C, 49.08; H, 9.15; N, 12.72. Found: C, 49.27; H, 9.10; N, 12.64. The emericedin complex recovered from the mother liquor was dissolved in H_2O and subjected to a Dowex 50 W × 8 (0.4 L) column chromatography eluting with 0.05 M citrate buffer (pH 3.8). The pure fractions of **2** and **3**, detected by HPLC, were individually combined, desalted, and concentrated to give syrupy residues of **2** (150 mg) and **3** (65 mg), respectively. The picrates of **2** and **3** were obtained as yellow needles from 5% picric acid–EtOH. The picrate of **2**: mp 191–193 °C; $[\alpha]_D$ −6.4° (c 0.39, MeOH); SI-MS, *m/z* 217 (MH^+); ^1H NMR (Me_2SO-d_6) δ 1.00 (t, 3 H, CH_2CH_3), 2.12 (q, 2 H, CH_2CH_3), 2.52 (m, 2 H, CH_2), 3.12 (s, 9 H, Me_3N^+), 3.50 (m, 2 H, CH_2), 4.54 (m, 1 H, CH), 8.08 (d, 1 H, NHCO). Anal. Calcd for $C_{10}H_{20}N_2O_3 \cdot C_6H_3N_3O_7$: C, 43.15; H, 5.20; N, 15.72. Found: C, 43.32; H, 5.27; N, 15.69. The picrate of **3**: mp 176–177 °C; $[\alpha]_D$ −5.1° (c 0.39, MeOH); SI-MS, *m/z* 231 (MH^+); ^1H NMR (Me_2SO-d_6) δ 0.83 (t, 3 H, $CH_2CH_2CH_3$), 1.52 (sext, 2 H, $CH_2CH_2CH_3$), 2.08 (t, 2 H, $CH_2CH_2CH_3$), 2.52 (m, 2 H, CH_2), 3.12 (s, 9 H, Me_3N^+), 3.50 (m, 2 H, CH_2), 4.54 (m, 1 H, CH), 8.12 (d, 1 H, NHCO). Anal. Calcd for $C_{11}H_{22}N_2O_3 \cdot C_6H_3N_3O_7$: C, 44.45; H, 5.48; N, 15.24. Found: C, 44.38; H, 5.43; N, 15.30.

Hydrochloride of 1. Compound **1** (3.1 g) was dissolved in 1 N HCl (15 mL), and the solution was concentrated. The residue was crystallized from MeOH–EtOH to give the hydrochloride of **1** as colorless prisms (2.5 g, 66%): mp 219–221 °C; $[\alpha]_D$ −20.0° (c 0.8, H_2O). Anal. Calcd for $C_9H_{18}N_2O_3 \cdot HCl$: C, 45.28; H, 8.02; N, 11.73; Cl, 14.85. Found: C, 45.39; H, 7.73; N, 11.50; Cl, 14.77.

Acid Hydrolysis of 1. Compound **1** (11.5 g) was dissolved in 6 N HCl (500 mL) and kept at 100 °C for 10 h. The mixture was evaporated, and the resulting crystals were recrystallized from H_2O–EtOH to give colorless crystals of **4** as the dihydrochloride (11.0 g): mp 217–218 °C; $[\alpha]_D$ +7.1° (c 0.95, 1 N AcOH); $[\alpha]_D$ +7.6° (c 1.0, H_2O). Anal. Calcd for $C_7H_{18}N_2O_2Cl_2$: C, 36.05; H, 7.77; N, 12.01; Cl, 30.40. Found: C, 35.98; H, 7.68; N, 11.73; Cl, 30.38.

Methyl L-3-[(Benzyloxycarbonyl)amino]-4-[(*tert*-butoxycarbonyl)amino]butyrate (6). To a solution of L-2-[(benzyloxycarbonyl)amino]-3-[(*tert*-butoxycarbonyl)amino]propionic acid[2] (**5**, 6.1 g) in 100 mL of AcOEt were added *N*-ethylmorpholine (1.8 g) and then ethyl chloroformate (1.9 g), and the mixture was stirred at 0 °C for 1 h. The mixture was filtered to remove insolubles, and a large excess of diazomethane in Et_2O was added to the filtrate. The mixture was stirred at 0 °C for 1 h and then at room temperature for 12 h. The solvent was removed by evaporation, and the resulting crystals of diazo ketone were dissolved in MeOH (50 mL). Silver benzoate (200 mg) in tri-

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(9) Jenkins, D. L.; Griffith, O. W. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 290.

ethylamine (2 mL) was added to this mixture. The mixture was stirred in a dark place at room temperature for 4 h. The insoluble material was filtered off, and the filtrate was concentrated. The residue was dissolved in AcOEt (100 mL). The AcOEt was washed with 10% citric acid, 5% $NaHCO_3$, and H_2O and dried over Na_2SO_4. The AcOEt was evaporated, and the resulting crystals of **6** were recrystallized from AcOEt–petroleum ether (4.8 g, 73%): mp 100–101 °C; $[\alpha]_D$ +6.0° (c 1, DMF). Anal. ($C_{18}H_{26}N_2O_6$) C, H, N.

L-3-[(Benzyloxycarbonyl)amino]-4-[(*tert*-butoxycarbonyl)amino]butyric Acid (7). To a solution of **6** (3.6 g) in MeOH (20 mL) was added 1 N NaOH (12 mL) dropwise at 0 °C. The mixture was stirred at room temperature for 3 h, then acidified with 10% citric acid, and extracted with AcOEt. The organic layer was washed with H_2O and dried over Na_2SO_4. The solvent was evaporated, and the resulting crystalline residue was recrystallized from AcOEt to give **7** (2.96 g, 85%): mp 136–137 °C; $[\alpha]_D$ +12.2° (c 1, DMF). Anal. ($C_{17}H_{24}N_2O_6$) C, H, N.

L-3-(Acetylamino)-4-[(*tert*-butoxycarbonyl)amino]butyric Acid (8). Compound **7** (1.40 g) was dissolved in 80% MeOH (30 mL) and hydrogenated over Pd-black. The catalyst was filtered off, and the MeOH was evaporated. The resulting residue was dissolved in a solution of $NaHCO_3$ (840 mg) in H_2O (10 mL). To this was added acetic anhydride (0.5 mL) in acetonitrile (10 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature for 12 h, and then the solvent was evaporated. The residue was washed with AcOEt, and the aqueous layer was acidified with 0.1 N HCl and extracted with AcOEt. The organic layer was washed with saturated NaCl and dried over Na_2SO_4. The AcOEt was evaporated, and the residue was crystallized from petroleum ether and recrystallized from AcOEt to afford **8** (0.88 g, 86%): mp 140–141 °C; $[\alpha]_D$ +26.0° (c 0.9, DMF). Anal. ($C_{11}H_{20}N_2O_5$) C, H, N.

L-3-(Acetylamino)-4-aminobutyric Acid (9). Compound **8** (700 mg) was dissolved in TFA (10 mL), and the solution was allowed to stand at room temperature for 30 min. The TFA was evaporated, and the residue was dried in vacuo and then dissolved in H_2O (100 mL). The solution was desalted and concentrated, and the crystalline residue was recrystallized from MeOH to give **9** (348 mg, 81%): mp 177–178 °C; $[\alpha]_D$ −15.4° (c 0.7, 0.1 N HCl). Anal. ($C_6H_{12}N_2O_3$) C, H, N.

(*R*)-3-(Acetylamino)-4-(trimethylammonio)butyrate Hydrochloride (10). Compound **9** (288 mg) was dissolved in 10% NaOH (8 mL). To this solution was added dimethyl sulfate (0.8 mL) at 0 °C, and the mixture was stirred at 0 °C for 30 min and then at room temperature for 30 min. The solution was desalted and concentrated. The residue was dissolved in 1 N HCl (1.5 mL), and the solution was concentrated to dryness. The resulting residue was crystallized from MeOH–Et_2O to give **10** (220 mg, 51%): mp 217–218 °C; $[\alpha]_D$ −20.2° (c 0.96, H_2O). Anal. ($C_9H_{19}N_2O_3Cl$) C, H, N, Cl. The compound **10** was identical with the hydrochloride of **1** from the fermentation broth by HPLC, IR, and ^1H NMR.

Synthesis of 4 from 7. Compound **7** (0.75 g) was dissolved in TFA (10 mL) and allowed to stand at room temperature for 10 min. The TFA was removed by evaporation, and the residue was washed with Et_2O and dried in vacuo. The resulting material was dissolved in 10% NaOH (7 mL), and dimethyl sulfate (0.65 mL) was added to the solution at 0 °C. After being stirred at room temperature for 1 h, the solution was desalted with a column of Amberlite IR-120 (H^+, 50 mL), concentrated, and lyophilized to give (*R*)-3-[(benzyloxycarbonyl)amino]-4-(trimethylammonio)butyrate (0.44 g) as a syrup. This was identical with *N*-(benzyloxycaronyl)emeriamine (**14**), mentioned afterwards, by TLC and HPLC. The compound (0.39 g) thus obtained was dissolved in 5.7 N HCl (5 mL) and kept at 90 °C for 30 min. The mixture was evaporated, H_2O (5 mL) was added to the residue, and the mixture was evaporated again. The residue was crystallized from MeOH–Et_2O to give **4** as the dihydrochloride (228 mg, total 53%): mp 219–220 °C dec; $[\alpha]_D$ +6.7° (c 1.0, 1 N AcOH). Anal. ($C_7H_{18}N_2O_2Cl_2$) C, H, N, Cl.

***N*-Formylemeriamine (11).** The dihydrochloride of **4** (930 mg) was dissolved in formic acid (10 mL). Acetic anhydride (2.8 mL) and $NaHCO_3$ (336 mg) were added to this solution at 0 °C. After being stirred at room temperature for 4 h, the mixture was diluted with H_2O (50 mL) and evaporated. The residue was dissolved in H_2O (100 mL), and the solution was desalted and evaporated. The oily residue (880 mg) was dissolved in MeOH (10 mL). A solution of picric acid (1.10 g) in EtOH (30 mL) was added to the solution. The resulting crystals were collected and recrystallized from MeOH to give the picrate of **11** (1.25 g, 75%): mp 174–176 °C; $[\alpha]_D$ −3.3° (c 1.0, DMF); ^1H NMR (90 MHz, Me_2SO-d_6) δ 2.54 (m, 2 H, CH_2), 3.14 (s, 9 H, Me_3N^+), 3.56 (m, 2 H, CH_2), 4.58 (m, 1 H, CH), 8.07 (s, 1 H, HCO), 8.38 (d, 1 H, CONH). Anal. ($C_{14}H_{19}N_5O_{10}$) C, H, N.

***N*-Carbamoylemeriamine (12).** The dihydrochloride of **4** (700 mg) was dissolved in H_2O (20 mL), and potassium cyanate (364 mg) was added to this solution at room temperature. After being stirred at room temperature for 12 h, the mixture was desalted and evaporated. The residue was dissolved in 1 N HCl (3 mL). The solution was evaporated to dryness, and the syrupy residue was crystallized from MeOH–Et_2O and recrystallized from MeOH–EtOH to give colorless crystals of **12** as the hydrochloride (0.53 g, 74%): mp 215–217 °C dec; $[\alpha]_D$ −24.1° (c 1.0, H_2O); ^1H NMR (90 MHz, CD_3OD) δ 2.66 (d, 2 H, CH_2), 3.24 (s, 9 H, Me_3N^+), 3.65 (m, 2 H, CH_2), 4.60 (m, 1 H, CH). Anal. ($C_8H_{18}N_3O_3Cl$) C, H, N, Cl.

***N*-(Methoxycarbonyl)emeriamine (13).** The dihydrochloride of **4** (816 mg) was dissolved in a solution of $NaHCO_3$ (1.29 g) in H_2O (20 mL). Methoxycarbonyl chloride (395 mg) was added to the solution. After being stirred at 0 °C for 30 min and at room temperature overnight, the solution was neutralized with 1 N HCl and washed with AcOEt (50 mL). The aqueous layer was desalted and evaporated. The residue was dissolved in H_2O (30 mL). The solution was then applied to a column (10 mL) of Amberlite IRC-50 (H^+), and the column was washed with H_2O (200 mL). The passed solution and washings were combined and evaporated to give a syrupy residue (0.65 g), which was dissolved in 1 N HCl (3 mL). The solution was evaporated to dryness, and the residue was crystallized from Et_2O to give **13** as the hydrochloride (0.65 g, 73%): mp 201–205 °C dec; $[\alpha]_D$ −22.3° (c 0.75, H_2O); ^1H NMR (90 MHz, D_2O) δ 2.96 (d, 2 H, CH_2), 3.43 (s, 9 H, Me_3N^+), 3.82 (m, 2 H, CH_2), 3.92 (s, 3 H, OCH_3), 4.75 (m, 1 H, CH). Anal. ($C_9H_{19}N_2O_4Cl$) C, H, N, Cl.

***N*-(Benzyloxycarbonyl)emeriamine (14).** The dihydrochloride of **4** (1.63 g) was dissolved in 1 N NaOH (28 mL). A solution of benzyloxycarbonyl chloride (1.70 g) in acetonitrile (5 mL) was added to the solution at 0 °C. After being stirred at 0 °C for 2 h and at room temperature overnight, the reaction mixture was evaporated to remove acetonitrile and washed with Et_2O. The aqueous layer was desalted and evaporated to dryness. The residue was dissolved in H_2O (30 mL) and extracted with 1-butanol (100 mL × 3). The 1-butanol was evaporated, and the resulting crystals were collected and recrystallized from 1-propanol–Et_2O to give colorless crystals of **14** (0.90 g, 44%): mp 196–197 °C; $[\alpha]_D$ −19.8° (c 0.51, H_2O); ^1H NMR (90 MHz, Me_2SO-d_6) δ 2.10 (d, 2 H, CH_2), 3.26 (s, 9 H, Me_3N^+), 3.50 (d, 2 H, CH_2), 4.2 (m, 1 H, CH), 5.04 (s, 2 H, OCH_2), 7.34 (s, 5 H, C_6H_5). Anal. ($C_{15}H_{22}N_2O_4$) C, H, N.

***N*-Myristoylemeriamine (15).** The dihydrochloride of **4** (700 mg) was dissolved in a mixture of H_2O (5 mL) and MeOH (50 mL). Myristic anhydride (1.70 g) and $NaHCO_3$ (720 mg) were added to the mixture at room temperature. After being stirred overnight, the mixture was evaporated and acidified with 1 N HCl. The solution was washed with Et_2O and extracted with 1-butanol (50 mL). The organic layer was washed with H_2O and evaporated. The residue was dissolved in H_2O (20 mL) and applied to a column (5 mL) of Amberlite IRA-402 (OH^-). The passed solution and washings were combined and lyophilized to give a colorless powder of **15** (690 mg, 63%): $[\alpha]_D$ −11.9° (c 0.56, MeOH); ^1H NMR (90 MHz, CD_3OD) δ 0.87 (t, 3 H, CH_3), 1.27 (s, 20 H, $(CH_2)_{10}$), 1.60 (m, 2 H, CH_2), 2.22 (t, 2 H, CH_2CO), 2.40 (d, 2 H, CH_2), 3.16 (s, 9 H, Me_3N^+), 3.52 (d, 2 H, CH_2), 4.64 (m, 1 H, CH). Anal. ($C_{21}H_{42}N_2O_3 \cdot 1.2H_2O$) C, H, N.

***N*-Palmitoylemeriamine (16).** From the dihydrochloride of **4** (700 mg) and palmitic anhydride (1.90 g) was obtained the desired compound (**16**) as colorless powder in a manner similar to that described for the synthesis of **15** (910 mg, 82%): $[\alpha]_D$ −11.2° (c 0.5, MeOH); ^1H NMR (90 MHz, CD_3OD) δ 0.87 (t, 3 H, CH_3), 1.27 (s, 24 H, $(CH_2)_{12}$), 1.60 (m, 2 H, CH_2), 2.20 (t, 2 H, CH_2CO), 2.40 (d, 2 H, CH_2), 3.17 (s, 9 H, Me_3N^+), 3.53 (d, 2 H, CH_2), 4.64 (m, 1 H, CH). Anal. ($C_{23}H_{46}N_2O_3 \cdot 2H_2O$) C, N; H: calcd,

11.59; found, 11.10.

***N*-Tosylemeriamine (17).** The dihydrochloride of 4 (1.39 g) was dissolved in H_2O (20 mL). Triethylamine (3.0 mL) and a solution of tosyl chloride (1.74 g) in THF (10 mL) were added to the mixture at 0 °C. The mixture was stirred at room temperature for 5 h, evaporated to remove THF, and washed with AcOEt. The aqueous layer was acidified with 1 N HCl and extracted with 1-butanol (50 mL × 2). The 1-butanol was evaporated. The residue was dissolved in H_2O (300 mL), and the solution was desalted and concentrated to a small volume, which was then applied to a column (150 mL) of CM-Sephadex C-25 (H^+). The column was developed with 0.05 M acetate buffer (pH 4.5). The fractions from 260 to 370 mL were combined, desalted, and lyophilized to give a colorless powder of 17 (790 mg, 42%): $[\alpha]_D$ +43.4° (*c* 0.41, H_2O); ^1H NMR (90 MHz, D_2O) δ 2.12 (ddd, 2 H, CH_2), 2.64 (s, 3 H, CH_3), 3.46 (s, 9 H, Me_3N^+), 3.67 (m, 2 H, CH_2), 4.34 (m, 1 H, CH), 7.70 and 8.04 (each d, 4 H, C_6H_4). Anal. ($C_{14}H_{22}N_2O_4S\cdot H_2O$) C, H, N, S.

***N*-Methylemeriamine (18).** The hydrochloride of 1 (1.90 g) and silver oxide (7.0 g) was suspended in anhydrous DMF (40 mL). Methyl iodide (4.0 mL) was added to the mixture. The reaction mixture was stirred at 45 °C for 5 h and filtered to remove insolubles. The filtrate was evaporated, and MeOH (20 mL) and 1 N NaOH (30 mL) were added to the residue. The mixture was stirred at room temperature for 2 h and filtered to remove insolubles. The filtrate was evaporated, and the residue was dissolved in H_2O (100 mL). The aqueous solution was desalted, concentrated, and applied to a column (1000 mL) of CM-Sephadex C-25, which was pretreated with 0.05 M acetate buffer (pH 4.4). The column was developed with the same buffer. The fractions from 1400 mL to 1600 mL were combined, desalted, concentrated, and lyophilized to give an oil (0.80 g) of *N*-methylemericedin A: ^1H NMR (90 MHz, D_2O) δ 2.35 (s, 3 H, CH_3CO), 2.68 (d, 2 H, CH_2), 3.22 (s, 3 H, CH_3N), 3.36 (s, 9 H, Me_3N^+), 3.8 (m, 2 H, CH_2), 5.66 (m, 1 H, CH). The oil (0.50 g) was dissolved in 6 N HCl (30 mL) and kept at 100 °C for 24 h. The mixture was evaporated to dryness. The residue was dissolved in H_2O (30 mL) and again evaporated to dryness. The residue was dissolved in a small amount of H_2O and lyophilized to give the dihydrochloride of 18 as a syrup (0.45 g, total 36%): $[\alpha]_D$ +7.4° (c 0.64, 1 N AcOH); ^1H NMR (90 MHz, D_2O) δ 3.12 (s, 3 H, CH_3N), 3.39 (d, 2 H, CH_2), 3.52 (s, 9 H, Me_3N^+), 4.18 (d, 2 H, CH_2), 4.45 (m, 1 H, CH). Anal. ($C_8H_{20}N_2O_2Cl_2\cdot H_2O$) C, H, N; Cl: calcd, 26.51; found, 25.31.

Emeriamine Methyl Ester (19). To a suspension of the dihydrochloride of 4 (1.0 g) in MeOH (20 mL) was added 6 N HCl–MeOH (5 mL). The mixture was kept at room temperature for 2 days and evaporated. The residue was dissolved in MeOH (50 mL). A solution of picric acid (2.0 g) in EtOH (50 mL) was added to the mixture. The resulting crystals were collected and recrystallized from MeOH to give yellow needles of 19 as the dipicrate (1.60 g, 59%): mp 139–140 °C; $[\alpha]_D$ −0.7° (*c* 1.0, DMF); ^1H NMR (90 MHz, Me_2SO-d_6) δ 2.92 (d, 2 H, CH_2), 3.22 (s, 9 H, Me_3N^+), 3.70 (s, 3 H, CH_3O), 3.76 (d, 2 H, CH_2), 4.27 (m, 1 H, CH). Anal. ($C_{20}H_{24}N_8O_{16}$) C, H, N.

Emeriamine Ethyl Ester (20). Thionyl chloride (0.4 mL) was added dropwise to EtOH (10 mL) at −20 °C. The dihydrochloride of 4 (0.92 g) was added to the solution. After being stirred at 0 °C for 1 h and at 60 °C for 8 h, the mixture was evaporated to dryness. The residue was dissolved in EtOH (10 mL). A solution of picric acid (2.0 g) in EtOH (50 mL) was added to the solution. The resulting crystals were collected and recrystallized from MeOH–EtOH to give yellow needles of 20 as the monohydrochloride monopicrate (1.20 g, 67%): mp 204–208 °C; $[\alpha]_D$ −2.8° (c 1.1, DMF); ^1H NMR (90 MHz, CD_3OD) δ 1.28 (t, 3 H, OCH_2CH_3), 3.05 (d, 2 H, CH_2), 3.34 (s, 9 H, Me_3N^+), 4.00 (m, 2 H, CH_2), 4.23 (q, 2 H, OCH_2). Anal. ($C_{15}H_{24}N_5O_9Cl$) C, H, N; Cl: calcd, 7.82; found, 7.09.

Emeriamine Amide (21). The dipicrate of 19 (1.0 g) was dissolved in MeOH (20 mL). To the solution was added 15 N NH_4OH (10 mL). After being kept at room temperature for 2 days, the solution was evaporated to dryness. The residue was dissolved in EtOH. The resulting crystals were collected and recrystallized from aqueous MeOH to give yellow needles of 21 as the dipicrate (0.65 g, 67%): mp 226–227 °C; $[\alpha]_D$ +2.9° (c 1.0, DMF); ^1H NMR (90 MHz, Me_2SO-d_6) δ 2.66 (d, 2 H, CH_2), 3.18 (s, 9 H, Me_3N^+), 3.67 (d, 2 H, CH_2), 4.16 (m, 1 H, CH), 7.33 (m, 1 H, $CONH_2$), 7.75 (m, 1 H, $CONH_2$), 8.22 (m, 2 H, $CHNH_2$). Anal. ($C_{19}H_{23}N_9O_{15}$) C, H, N.

D-2-[(Benzyloxycarbonyl)amino]-3-aminopropionic Acid (24). The desired compound (24) was obtained from (benzyloxycarbonyl)-D-asparagine (5.3 g) in a manner similar to that described for the synthesis of the corresponding L isomer[2] (3.5 g, 74%): mp 227–228 °C; $[\alpha]_D$ +8.8° (c 0.40, 1 N NaOH). Anal. ($C_{11}H_{14}N_2O_4\cdot 0.2H_2O$) C, H, N.

D-2-[(Benzyloxycarbonyl)amino]-3-[(*tert*-butoxycarbonyl)amino]propionic Acid (25). The desired compound (25) was obtained from 24 (3.30 g) in a manner similar to that described for the synthesis of the corresponding L isomer[2] (2.90 g, 62%): mp 142–143 °C; $[\alpha]_D$ +11.0° (c 0.5, MeOH). Anal. ($C_{16}H_{22}N_2O_6$) C, H, N.

Methyl D-3-[(Benzyloxycarbonyl)amino]-4-[(*tert*-butoxycarbonyl)amino]butyrate (26). The desired compound (26) was obtained from 25 (2.80 g) in a manner similar to that described for the synthesis of 6 (1.50 g, 50%): mp 101–103 °C; $[\alpha]_D$ −5.6° (*c* 0.9, DMF). Anal. ($C_{18}H_{26}N_2O_6$) C, H, N.

D-3-[(Benzyloxycarbonyl)amino]-4-[(*tert*-butoxycarbonyl)amino]butyric Acid (27). The desired compound (27) was obtained from 26 (1.45 g) in a manner similar to that described for the synthesis of 7 (1.15 g, 83%): mp 136–137 °C; $[\alpha]_D$ −12.0° (*c* 1.0, DMF). Anal. ($C_{17}H_{24}N_2O_6$) C, H, N.

D-3-(Acetylamino)-4-[(*tert*-butoxycarbonyl)amino]butyric Acid (28). Compound 27 (1.10 g) was suspended in a mixture of MeOH (5 mL) and H_2O (45 mL) and hydrogenated over Pd-black. The catalyst was removed by filtration, and the filtrate was evaporated. The aqueous residue was dissolved in a mixture of H_2O (20 mL) and dioxane (20 mL). To this solution were added triethylamine (0.80 mL) and *N*-acetoxy-5-norbornene-2,3-dicarboximide[10] (0.80 g) at 0 °C. After being stirred at room temperature for 5 h, the mixture was evaporated to remove the dioxane, acidified with 10% citric acid, and extracted with AcOEt (100 mL × 2). The AcOEt solution was washed with saturated NaCl solution, dried over Na_2SO_4, and evaporated. The residue was crystallized from petroleum ether and recrystallized from AcOEt (0.57 g, 70%): mp 139–140 °C; $[\alpha]_D$ −25.4° (c 0.8, DMF). Anal. ($C_{11}H_{20}N_2O_5$) C, H, N.

(*S*)-Emericedin A (23). Compound 28 (0.53 g) was dissolved in TFA (20 mL) and allowed to stand at room temperature for 15 min. The TFA was removed by evaporation, and the residue was treated with Et_2O to give a powder. To a solution of the powder in 2 N NaOH (8 mL) was added dimethyl sulfate (0.5 mL) at 0 °C. After being stirred at 0 °C for 30 min and at room temperature for 3 h, the mixture was diluted with H_2O (100 mL), desalted, and concentrated. The residue was dissolved in 1 N HCl (3 mL) and concentrated to a syrup, which was crystallized from MeOH–Et_2O to give colorless crystals of 23 as the hydrochloride (415 mg, 86%): mp 218–220 °C; $[\alpha]_D$ +20.5° (*c* 0.92, H_2O). Anal. ($C_9H_{19}N_2O_3Cl$) C, H, N, Cl. The IR and ^1H NMR spectra were identical with those of the hydrochloride of 1, respectively.

(*S*)-Emeriamine (22). Compound 23 (205 mg) was dissolved in 5.5 N HCl (10 mL) and kept at 100 °C for 10 h. The mixture was concentrated. The residue was dissolved in H_2O (10 mL) and again concentrated to dryness. The residue was crystallized from MeOH–Et_2O to give colorless crystals of 22 as the dihydrochloride (173 mg, 87%): mp 214–216 °C dec; $[\alpha]_D$ −8.2° (c 0.58, H_2O). Anal. ($C_7H_{18}N_2O_2Cl_2$) C, H, N; Cl: calcd, 30.40; found, 28.92. The IR and ^1H NMR spectra were identical with those of (*R*)-emeriamine dihydrochloride, respectively.

Biological Assays. The activity of L-carnitine-dependent oxidation of long-chain fatty acids was assayed by measuring $^{14}CO_2$ released from [1-^{14}C]palmitate in the presence or absence of L-carnitine, with rat liver mitochondria prepared from a male Sprague–Dawley rat (6 weeks old).[1] Inhibition percent of L-carnitine-dependent oxidation activity was calculated as follows.

$$\text{inhibition (\%)} = [1 - (A_i - B_i)/(A_0 - B_0)] \times 100$$

Without inhibitor: A_0, $^{14}CO_2$ released (dpm) with L-carnitine; B_0, $^{14}CO_2$ released (dpm) without L-carnitine. With inhibitor: A_i,

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$^{14}CO_2$ released (dpm) with L-carnitine; B_i, $^{14}CO_2$ released (dpm) without L-carnitine.

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Methotrexate Analogues. 30. Dihydrofolate Reductase Inhibition and in Vitro Tumor Cell Growth Inhibition by N^{ϵ}-(Haloacetyl)-L-lysine and N^{δ}-(Haloacetyl)-L-ornithine Analogues and an Acivicin Analogue of Methotrexate[1]

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Analogues of methotrexate (MTX) with strong alkylating activity were prepared by replacing the L-glutamate side chain with N^{ω}-haloacetyl derivatives of L-lysine and L-ornithine. Haloacetylation was accomplished in 30–40% yield by reaction of the preformed L-lysine and L-ornithine analogues of MTX with *p*-nitrophenyl bromoacetate or chloroacetate in aqueous sodium bicarbonate at room temperature. All four haloacetamides were potent inhibitors in spectrophotometric assays measuring noncovalent binding to purified dihydrofolate reductase (DHFR) from L1210 cells. In experiments designed to measure time-dependent inactivation of DHFR from L1210 cells and *Candida albicans*, the N^{ϵ}-(bromoacetyl)-L-lysine and N^{δ}-(bromoacetyl)-L-ornithine analogues gave results consistent with covalent binding, whereas N^{ϵ}- and N^{δ}-chloroacetyl analogues did not. The N^{δ}-(bromoacetyl)-L-ornithine analogue appeared to be the more reactive one toward both enzymes. Amino acid analysis of acid hydrolysates of the L1210 enzyme following incubation with the bromoacetamides failed to demonstrate the presence of a carboxymethylated residue, suggesting that alkylation had perhaps formed an acid-labile bond. In growth inhibition assays with L1210 cultured murine leukemia cells, the four haloacetamides were all more potent than their nonacylated precursors but less potent than MTX. The >40 000-fold MTX-resistant mutant cell line L1210/R81 was only partly cross-resistant to the haloacetamides. An analogue of MTX with acivicin replacing glutamate was a potent inhibitor of DHFR from chicken liver and L1210 cells but was 200 times less potent than MTX against L1210 cells in culture.

We have reported previously[2] that N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (**1**) is an active-site-directed irreversible inhibitor of dihydrofolate reductase (DHFR), the primary enzyme target for methotrexate (MTX) and other antifolates of the 2,4-diaminopyrimidine type.[3] In spectrophotometric and competitive ligand binding assays of reversible binding to bacterial (*Lactobacillus casei*) and mammalian (murine L1210 leukemia) DHFR, **1** showed inhibition comparable to that of MTX. Moreover, when **1** was incubated with DHFR from *L. casei* for several hours, there was a progressive loss of ability to bind MTX, which was consistent with covalent reaction at the active site. From a consideration of the known three-dimensional structure for the *L. casei* ternary DHFR–MTX–NADPH complex[4] and from the finding that the pH profile for inactivation by **1** had an inflection between 7.0 and 7.4, it was surmised that covalent reaction was occurring at His-28.[2] Subsequent work confirmed this and revealed that **1** can react with either of the imidazole nitrogens of His-28, suggesting that the imidazole ring may be capable of free rotation when the ternary enzyme complex is in solution.[5] It was also noted that **1** can bind covalently to DHFR from chicken liver and from MTX-resistant human lymphoblasts (WI-L2/M4 cells), apparently by alkylation of cysteine. This was most surprising, since the single cysteine (Cys) residue in these enzymes is believed to lie outside the active site, where it cannot easily be reached without a major conformational change in protein secondary structure. These novel findings prompted us to undertake a study of analogues of **1** with bromoacetyl or chloroacetyl groups in place of iodoacetyl and with the L-lysine moiety replaced by α,ω-diaminoalkanoic acids containing fewer CH_2 groups. This paper describes the hitherto unknown L-lysine analogues **2** and **3** and the L-ornithine analogues **4** and **5**. In addition we report the preparation and biological activity of the analogue **6**, in which the glutamate moiety is replaced by L-3-chloro-4,5-dihydro-5-isoxazoleacetate (acivicin). Interest in **6** stemmed from a desire to introduce into the molecule other amino acids with a demonstrated ability to react covalently with enzymes. Irreversible inactivation of L-glutamine synthetase by acivicin is well-known and has been ascribed to electrophilic attack of a thiol group.[6] Because of the chemical

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