OF4949, NEW INHIBITORS OF AMINOPEPTIDASE B

IV. EFFECTS OF OF4949 AND ITS DERIVATIVES ON ENZYME SYSTEMS

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OF4949-I and II inhibited aminopeptidase B from Ehrlich ascites carcinoma in a competitive way and the *Ki* value for both against L-arginine-β-naphthylamide was 8×10^{-9} M. Inhibition by I and II of various exopeptidases and endopeptidases was examined. OF4949-I and II both strongly inhibited leucine aminopeptidase and enkephalin-degrading aminopeptidase; I also inhibited enkephalinase B.

The inhibitory effects of various derivatives of I and II on aminopeptidase B activity, showed that the terminal amino and carboxamide groups are essential for activity.

We have reported on the fermentation, isolation, structure, biological activity, and biosynthesis of the cyclic peptides OF4949-I (**1**), II (**2**), III, and IV obtained from the culture broth of *Penicillium rugulosum* OF4949.[1~3] Compounds **1** and **2** strongly inhibit aminopeptidase B (AP-B) from Ehrlich ascites carcinoma (EAC) cells and enhance delayed-type hypersensitivity (DTH) to sheep red blood cells in mice.[1]

To find a derivative with yet stronger enzyme inhibition, and immunological enhancement, and also to elucidate the relationship between structure and biological activity, we prepared various derivatives of **1** and **2**.

Here, the inhibition by **1** and **2** of several exopeptidases and endopeptidases and the inhibition of AP-B by derivatives synthesized from **1** and **2** were studied.

Materials and Methods

Compounds **1** and **2** were prepared from the fermentation broth of *P. rugulosum* OF4949 as described in a previous paper.[1]

Enzymes

Aminopeptidase from rat kidney was prepared by a modification of the method of OYA *et al.*[4] The enzyme preparation was purified by fractionation with $(NH_4)_2SO_4$ [30~55% saturation for AP-B[5,6] and leucine aminopeptidase (Leu-AP), and 55~80% saturation for aminopeptidase A (AP-A)[7] and dipeptidyl aminopeptidase IV (DAP-IV)[4]]. AP-B from various tumor cell lines maintained in the peritoneal cavity of mice was prepared by the same methods for the EAC AP-B as described before.[1]

Carboxypeptidase A (CP-A) from bovine pancreas and carboxypeptidase B (CP-B) from porcine pancreas were purchased from the Sigma Chemical Co., St. Louis, U.S.A. Dipeptidyl carboxypeptidase (DCP) from *Escherichia coli* K-12 was prepared by the method of DEUTCH.[8] Carboxypeptidase P (CP-P) from *Penicillium janthinellum* was supplied by Dr. SADAJI YOKOYAMA of Takara Shuzo Co., Ltd. The enkephalinase A (EKL-A) and enkephalinase B (EKL-B) and the enkephalin-degrading aminopeptidase (EDAP) used here were partly purified from rat cerebrum by the procedure of GORENSTEIN and SNYDER.[9]

Trypsin from bovine pancreas was purchased from Difco Laboratories, Detroit, U.S.A.; α-chymotrypsin from bovine pancreas, papain from papaya, and elastase from porcine pancreas were purchased from Sigma; pepsin from porcine stomach mucosa and thermolysin from *Bacillus thermoproteolyticus* were from Nakarai Chemicals, Ltd., Kyoto, Japan.

Cells

Mouse leukemias L1210 and P388 were maintained in DBA/2 mice (females, 6 weeks old) and EAC and Sarcoma-180 (S-180) in out-bred *dd*Y (females, 6 weeks old) or ICR mice (females, 6 weeks old) by ip inoculation every 7 days.

The cultured cell line, L5178Y (mouse leukemia) was propagated in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) with 10% fetal calf serum (FCS). CHO/Pro$^-$, Chinese hamster ovary cells, were propagated in the alpha modification of EAGLE's minimal essential medium (α-MEM, GIBCO Laboratories, Grand Island, U.S.A.) with 10% FCS.

Mouse spleen lymphocytes and peritoneal macrophages were prepared from CDF_1 mice (females, 6 weeks old).

Substrates

L-Arginine-β-naphthylamide hydrochloride (L-Arg-β-NA), L-Lys-β-NA, L-Leu-β-NA, L-Glu-β-NA, hippuryl-L-phenylalanine (Hip-L-Phe), Hip-L-Arg, Z-Gly-L-Tyr, casein, and elastin-Congo red were purchased from Sigma; Gly-L-Pro-β-NA from the Bachem Feinchemikalin AG, Bubendorf, Switzerland; Hip-L-His-L-Leu and Leu-enkephalin from the Peptide Institute, Osaka, Japan; [Tyr-3.5-^{3}H(N)]Leu-enkephalin (43.6 Ci/mmol) from New England Nuclear Corp., Boston, U.S.A.; L-Tyr-L-Tyr-Gly and L-Tyr-Gly from the Kokusan Chemical Co., Tokyo, Japan; and α-*N*-benzoyl-L-Arg-ethyl ester HCl (base) from Nakarai.

Assay for Enzyme Inhibition

Inhibition of AP-A and -B, Leu-AP, and DAP-IV was measured by colorimetry by the method of HOPUS *et al.*[9] Inhibition of CP-A and -B and of DCP was assayed by the method of HAYAKARI *et al.*[10] The effect of the inhibitors on the activity of CP-P was measured by the method of ICHISHIMA.[11] Inhibition of the hydrolysis of [^{3}H]Leu-enkephalin by EKL-A and -B and by EDAP was assayed by the measurement of radioactive reaction products by the method of HUDGIN *et al.*[12] Inhibition of trypsin, α-chymotrypsin, papain, pepsin, thermolysin, elastase, and kallikrein was measured by the method of AOYAGI *et al.*[13]

Preparation of OF4949 (-I, -II, -D, -F) Derivatives

I-Methyl ester (**3**), I-amide (**6**), **D** (**7**), **D**-dimethyl ester (**8**), **D**-diol (**10**), bromo-I (**14**), II-methyl ester (**17**), **F** (**18**), **F**-dimethyl ester (**19**), **F**-diol (**20**) and *N*-formyl-II (**22**) were prepared as described in another paper.[2] For purification of derivatives, preparative HPLC was done on a Nucleosil $30C_{18}$ column (4×150 mm) with 0.1 M citrate buffer, pH 5.7, containing 5 to 20% acetonitrile, and the eluate was desalted on an XAD-2 column with 50% MeOH.

I-Butyl Ester (**4**): A solution of 200 mg of **1** in a mixture of 10 ml of *N*,*N*-dimethylformamide (DMF) and 10 ml of 2 N butanolic HCl was stirred at room temp for 15 hours to give 72.9 mg of **4** as a white powder. IR ν_{max} cm^{-1} 1735 (ester carbonyl); ^{1}H NMR (CD_3OD+DMSO-d_6) δ 0.8~1.1 (3H, m, CH_3), 1.2~1.9 (4H, m), 3.86 (3H, s, OCH_3).

I-Benzyl Ester (**5**): To a solution of 200 mg of **1** in a mixture of 10 ml of benzyl alcohol and 20 ml of benzene was added 100 mg of *p*-toluenesulfonic acid, and the mixture was refluxed for 4 hours. A mixture of ether - *n*-hexane (1 : 1) was added to this solution and the precipitate was filtered to give 343.7 mg of product. The product was purified by preparative HPLC, giving 83.5 mg of **5** as a white powder. ^{1}H NMR (CD_3OD+DMSO-d_6) δ 3.87 (3H, s, OCH_3), 5.22 (2H, s, OCH_2-Ar), 7.41 (5H, s, OCH_2-*Ar*).

D-Amide (**9**): Compound **8** (59.6 mg) was treated with 28% ammonia in the same way as for **6**[2] to give 9.2 mg of **9** with 30.6 mg of **6**. ^{1}H NMR (DMSO-d_6) δ 3.79 (3H, s, OCH_3), 7.17 and 7.35 (2H, m, $CONH_2$).

N-Formyl-I (**11**): Compound **1** (100 mg) was treated with formic acid and acetic anhydride in

the same way as for **22**[2] to give 42.2 mg of **11**.

Anal Calcd for $C_{24}H_{26}N_4O_9$: C 56.03, H 5.09, N 10.89.
Found: C 55.87, H 5.08, N 10.94.

^{1}H NMR (D_2O) δ 3.84 (3H, s, OC*H*$_3$), 8.07 (1H, s, C*H*O).

N-Acetyl-I (**12**) and *N*-Acetyl-II (**23**): We added 5 ml of acetic anhydride to 200 mg of **1** in 20 ml of H_2O being stirred over an ice bath. Stirring was continued in the cold for 15 minutes and then at room temp for 15 hours, giving 157 mg of **12** as a colorless powder. Compound **23** (45 mg) was obtained from 50 mg of **2** in the same way.

12: ^{1}H NMR (CD_3OD) δ 1.99 (3H, s, CH_3CO), 3.87 (3H, s, OCH_3).

23: ^{1}H NMR (CD_3OD) δ 1.97 (3H, s, CH_3CO).

N-Ethyl-I (**13**) and *N*-Ethyl-II (**25**): To a solution of **1** (200 mg) dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.0, was added 40 ml of acetoaldehyde. The solution was stirred at room temp for 10 minutes; then 100 mg of sodium cyanoborohydride ($NaBH_3CN$) was added, and stirring was continued for 20 minutes, to give 129 mg of **13** as a colorless powder. Compound **25** (28.6 mg) was obtained from 50 mg of **2** in the same way.

13: ^{1}H NMR (DMSO-d_6) δ 1.04 (3H, t, CH_3), 3.78 (3H, s, CH_3), 4.70 (1H, d, CH).

25: ^{1}H NMR (DMSO-d_6) δ 1.04 (3H, t, CH_3).

Bromo-**D** (**15**): Compound **15** (38.6 mg) was obtained from 41.0 mg of **7** in the same way as for **14**.[2] ^{1}H NMR (0.5 N ND_4OD) δ 3.98 (3H, s, OCH_3), 7.34 (1H, s, Ar).

O-Butyl-II (**16**): A solution of 279 mg of **22** in a mixture of 20 ml of DMF, 200 mg of K_2CO_3, and 0.5 ml of *n*-butyl bromide was stirred at room temp for 15 hours. The solution was neutralized with 2 N HCl and chromatographed on a Nucleosil $30C_{18}$ column. The precipitate (52 mg) from the eluate was hydrolyzed with 4 N NaOH at room temp for 2 hours, giving 25.5 mg of **16** as colorless needles.

Anal Calcd for $C_{26}H_{32}N_4O_6$: C 59.08, H 6.10, N 10.60.
Found: C 58.55, H 6.10, N 10.49.

^{1}H NMR (DMSO-d_6) δ 1.96 (3H, s, CH_3), 1.4~1.8 (4H, m, $CH_2\times2$), 4.04 (2H, t, CH_2).

II-Ol (**20**): First, 37 mg of **17** was refluxed in 40 ml of tetrahydrofuran with 500 mg of lithium borohydride for 6 hours, giving 6.1 mg of **20** as a colorless powder. ^{1}H NMR (D_2O) δ 3.72 (2H, m, C*H*$_2$OH).

N-Dimethyl-II (**24**): To a suspension of 50 mg of **2** in 5 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 ml of formaldehyde was added and the whole was stirred at room temp for 10 minutes. Then 30 mg of $NaBH_3CN$ was added to the solution and the mixture stirred for another 60 minutes, giving 24.6 mg of **24** as a colorless powder. ^{1}H NMR (CD_3OD+D_2O) δ 2.52 (6H, s, $CH_3\times2$).

N-Diethyl-II (**26**): Compound **2** (50 mg) was dissolved in a mixture of 5 ml of 0.1 M phosphate buffer, pH 7.0, and 0.05 ml of acetoaldehyde and stirred for 10 minutes at room temp; then 30 mg of $NaBH_3CN$ was added and the whole was stirred for another 60 minutes, to give 31.4 mg of **26** as a colorless powder. ^{1}H NMR (CD_3OD) δ 1.06 (6H, t, $CH_3\times2$).

Results

Compounds **1** and **2** strongly inhibit EAC AP-B from use of L-Arg-β-NA as a substrate.[1] The effect of these two inhibitors on AP-B from various other mammalian cells or tissues was examined. Both **1** and **2** considerably inhibited enzymes that hydrolyze L-Arg-β-NA from a wide range of sources (Table 1). Compounds **1** and **2** inhibited enzymes that originated from S-180, CHO/Pro$^-$, and L5178Y about as strongly as for the enzyme from EAC, but were weaker against enzymes from L1210, P388 lymphoma, mouse spleen lymphocytes, mouse peritoneal macrophages, rat liver homogenate, and rat kidney. Neither **1** nor **2** inhibited the hydrolysis of L-Lys-β-NA by EAC AP-B.

The kinetics of the hydrolysis of L-Arg-β-NA by EAC AP-B in the presence of **1** and of **2** were investigated as a function of the concentration of the substrate. Lineweaver-Burk plots of the data

Table 1. Inhibition by **1** and **2** of aminopeptidase B.

Enzyme source	Substrate	IC_{50} (μg/ml)	
		1	**2**
EAC	Arg-β-NA	0.0054	0.0048
EAC	Lys-β-NA	84.0	208.0
S-180	Arg-β-NA	0.0070	0.0069
L1210	Arg-β-NA	0.143	0.125
P388	Arg-β-NA	0.053	0.031
CHO/Pro$^-$	Arg-β-NA	0.0045	0.0034
L5178Y	Arg-β-NA	0.0030	0.0020
Lymphocytes	Arg-β-NA	0.025	0.020
Macrophages	Arg-β-NA	0.15	0.98
Rat liver homogenate	Arg-β-NA	0.043	0.031
Rat kidney (partially purified)	Arg-β-NA	0.0125	0.029

Table 2. Effects of **1** and **2** on enzyme systems.

Enzyme	Origin	Substrate	IC_{50} (μg/ml)	
			1	**2**
Leucine aminopeptidase	Rat kidney	Leu-β-NA	0.0041	0.018
Aminopeptidase A	Rat kidney	Glu-β-NA	>300	>300
Dipeptidyl aminopeptidase IV	Rat kidney	Gly-Pro-β-NA	>300	>300
Carboxypeptidase A	Bovine pancreas	Hip-Phe	>300	>300
Carboxypeptidase B	Porcine pancreas	Hip-Arg	>300	>300
Carboxypeptidase P	*Penicillium janthinellum*	Z-Gly-Tyr	>300	>300
Dipeptidyl carboxypeptidase	*Escherichia coli*	Hip-His-Leu	>300	>300
Enkephalinase A	Rat cerebrum	[^{3}H]Leu-Enk	>300	>300
Enkephalinase B	Rat cerebrum	[^{3}H]Leu-Enk	9.8	154
Enkephalin-degrading aminopeptidase	Rat cerebrum	[^{3}H]Leu-Enk	0.0018	0.0174
Trypsin	Bovine pancreas	Casein	>300	>300
α-Chymotrypsin	Bovine pancreas	Casein	>300	>300
Elastase	Porcine pancreas	Elastin-Congo red	>300	>300
Kallikrein	Porcine pancreas	BAEE	>300	>300
Papain	*Carica papaya*	Casein	>300	>300
Pepsin	Porcine stomach mucosa	Casein	>300	>300
Thermolysin	*Bacillus thermoproteolyticus*	Casein	>300	>300

NA: Naphthylamide.
Hip: Hippuric acid.
Leu-Enk: Leucine-enkephalin.
BAEE: α-*N*-Benzoyl-L-arginine ethyl ester.

indicated that both **1** and **2** were competitive inhibitors. The Michaelis-Menten constant, *Km*, for L-Arg-β-NA was 7.0×10^{-5} M. The inhibition constant, *Ki*, for both **1** and **2** against L-Arg-β-NA was 8×10^{-9} M.

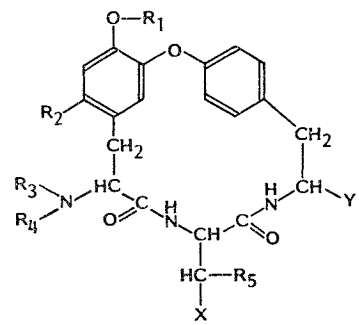
To elucidate the participation of chelation in the inhibitory reaction, zinc ion was added to the reaction mixture of EAC AP-B and inhibitors. The inhibition of 0.25 mM of 1,10-phenanthroline or

Table 3. Inhibition by OF4949 derivatives of aminopeptidase B from EAC.

Compound No.	R_1	R_2	R_3	R_4	R_5	X	Y	IC_{50} (μg/ml)
OF4949-I (1)	CH_3	H	H	H	OH	$CONH_2$	COOH	0.0054
OF4949-II (2)	H	H	H	H	OH	$CONH_2$	COOH	0.0048
OF4949-III	CH_3	H	H	H	H	$CONH_2$	COOH	3.4
OF4949-IV	H	H	H	H	H	$CONH_2$	COOH	1.7
3	CH_3	H	H	H	OH	$CONH_2$	$COOCH_3$	0.0052
4	CH_3	H	H	H	OH	$CONH_2$	$COOC_4H_9$	0.0117
5	CH_3	H	H	H	OH	$CONH_2$	$COOCH_2Ph$	0.0155
6	CH_3	H	H	H	OH	$CONH_2$	$CONH_2$	0.016
7	CH_3	H	H	H	OH	COOH	COOH	0.25
8	CH_3	H	H	H	OH	$COOCH_3$	$COOCH_3$	0.04
9	CH_3	H	H	H	OH	COOH	$CONH_2$	63
10	CH_3	H	H	H	OH	CH_2OH	CH_2OH	0.3
11	CH_3	H	CHO	H	OH	$CONH_2$	COOH	2.2
12	CH_3	H	$COCH_3$	H	OH	$CONH_2$	COOH	>100
13	CH_3	H	C_2H_5	H	OH	$CONH_2$	COOH	10.6
14	CH_3	Br	H	H	OH	$CONH_2$	COOH	0.86
15	CH_3	Br	H	H	OH	COOH	COOH	14.0
16	C_4H_9	H	H	H	OH	$CONH_2$	COOH	0.025
17	H	H	H	H	OH	$CONH_2$	$COOCH_3$	0.0056
18	H	H	H	H	OH	COOH	COOH	1.35
19	H	H	H	H	OH	$COOCH_3$	$COOCH_3$	0.095
20	H	H	H	H	OH	$CONH_2$	CH_2OH	0.025
21	H	H	H	H	OH	CH_2OH	CH_2OH	0.54
22	H	H	CHO	H	OH	$CONH_2$	COOH	3.6
23	H	H	$COCH_3$	H	OH	$CONH_2$	COOH	64
24	H	H	CH_3	CH_3	OH	$CONH_2$	COOH	>100
25	H	H	C_2H_5	H	OH	$CONH_2$	COOH	12.5
26	H	H	C_2H_5	C_2H_5	OH	$CONH_2$	COOH	>100

ethylenediaminetetraacetic acid (EDTA) was almost reversed by addition of 0.1 mM of zinc ion, but the inhibition of **1** and **2** was not, when the amounts in moles of zinc ion and inhibitors were the same as in the previous experiments.

The effects of **1** and **2** on other exopeptidases and endopeptidases were also tested. The concentrations required for 50% inhibition, that is, IC_{50}, for each enzyme are shown in Table 2. Compound **1** inhibited Leu-AP from rat kidney and EDAP from rat cerebrum, and inhibited EKL-B somewhat less. Compound **2** also inhibited Leu-AP and EDAP, but had almost no effect on EKL-B. Compounds **1** and **2** did not inhibit the activity of AP-A, DAP-IV, CP-A, -B, or -P, DCP, EKL-A, or endopeptidases such as trypsin, α-chymotrypsin, elastase, kallikrein, papain, pepsin, or thermolysin, even at the concentration of 300 μg/ml. These results indicated that **1** and **2** were selective inhibitors of AP-B, Leu-AP, and EDAP.



The inhibition by various derivatives synthesized from compounds **1** and **2** of EAC AP-B was examined. IC_{50} by partially modified derivatives are shown in Table 3. Inhibition by derivative **16** with a butyl group substituted for

the R_1 was less than that by **1** with a methyl group or **2** with a proton. From comparison of **1** with **14** and **7** with **15**, we found that substitution of bromide for the benzene-ring R_2 proton markedly lowered the activity.

The effects of substituting something for the terminal amino, carboxamide, or terminal carboxyl groups of **1** and **2** were then examined. Replacement of the R_3 proton, the R_4 proton, or both with a methyl, ethyl, formyl, or acetyl group produced *N*-alkyl or *N*-acyl derivatives (**11**~**13** and **22**~**26**) with little or no activity. We found that the inhibitory effect of derivative **7** (**18**) with a carboxyl group substituted for the carboxamide group (X) of **1** (**2**) was much less than the effect of the parent compound. When we compared **1** with **3**, **7** with **8**, **2** with **17**, and **18** with **19**, the derivatives with carboxyl methyl ester substituted for the carboxyl group had equal or stronger activity than the parent compound. However, the substituted compounds with a carboxybutyl ester, carboxybenzyl ester, alcohol, or carboxamide group (**4**~**6** and **20**) had somewhat less activity than **1** or **2**.

Discussion

HOPUS *et al.*[5,6] found that AP-B purified from rat liver can liberate only the *N*-terminal basic amino acids, arginine and lysine, from peptide substrates. ELLIS and PERRY[14] reported the presence of two different forms of AP-B, arginyl arylamidase and lysyl arylamidase, in bovine anterior pituitary glands; the forms can be distinguished by their requirement for thiol compounds for activation and different sensitivities to puromycin. AP-B is distributed in various organs of animals, not only in the cells but also on cell surfaces, as are alkaline phosphatase, esterase, glucosidase, and other aminopeptidases.[15,16] In our experiment, **1** and **2** strongly inhibited AP-B from various mammalian cells or tissues that hydrolyzed L-Arg-β-NA, but had almost no effect on the hydrolysis of L-Lys-β-NA. This result suggests that **1** and **2** may be a kind of AP-B inhibitor unlike puromycin; puromycin is more sensitive to lysyl arylamidase than to arginyl arylamidase.[14]

Kinetic studies of both **1** and **2** with EAC AP-B and L-Arg-β-NA showed that the inhibition was competitive with the substrate. The inhibition by **1** and **2** was not reversed when zinc ion was added. The results indicated that **1** and **2** may act by a mechanism different from the chelating reaction of 1,10-phenanthroline or EDTA.[17]

Enkephalins are degraded by aminopeptidases[18~20] as well as by EKL-A and -B and angiotensin-converting enzyme.[21~23] Compound **1** strongly inhibited EDAP and EKL-B. From this result, we think that **1** might enhance morphine analgesia, as do bestatin and *p*-hydroxy-2*S*,3*R*-3-amino-2-hydroxyphenyl-4-phenylbutanoyl-L-leucine.[24] The difference in the inhibition by **1** and **2** of EKL-B may be due to the difference between the methoxyl and hydroxyl groups.[2] Neither **1** nor **2** inhibited hydrolysis by AP-A, DAP-IV, CP-A, -B, or -P, DCP, EKL-A, or any endopeptidase tested. The inhibitory effect on peptidase by **1** and **2** of these enzymes resembled that of bestatin.[25]

From our examination of various synthetic derivatives of **1** and **2**, it seems that the terminal amino and carboxamide groups are essential for activity. As reported in the first and second papers,[1,2] the difference in the activity of **1** (**2**) and the deoxy derivatives, OF4949-III (IV), of alcoholic hydroxyl group, suggested that the alcoholic hydroxyl group is also important in inhibition.

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