SYNTHESIS OF HISTARGIN AND RELATED COMPOUNDS AND THEIR INHIBITION OF ENZYMES

MAKOTO MORIGUCHI, YOSHIHISA UMEDA, KEIKO MIYAZAKI,
TERUYA NAKAMURA, KEIJI OGAWA,† FUKIKO KOJIMA,†
HIRONOBU IINUMA† and TAKAAKI AOYAGI†

Central Research Laboratories, Takara Shuzo Co., Ltd.,
3-4-1 Seta, Otsu-shi, Shiga-ken
†Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication July 9, 1988)

A novel method for the synthesis of histargin and its analogs is described. It includes two kinds of *N*-alkylation reactions that prevent the formation of side products. The inhibition of enzymes by these compounds was also measured. Some of the compounds strongly inhibited carboxypeptidase B, carboxypeptidase A, carboxypeptidase N (kiniase I), and angiotensin converting enzyme.

Histargin (**1**) (Fig. 1) inhibits some exopeptidases, such as carboxypeptidase B (CPase B),[1] angiotensin converting enzyme (ACE),[2] and enkephalinase.[2] It was isolated from culture filtrates of *Streptomyces roseoviridis* MF118-A5 by UMEZAWA *et al.*[1] The structure is *N*-[(*S*)-1-carboxy-4-guanidinobutyl]-*N'*-[(*S*)-1-carboxy-2-(imidazol-4-yl)ethyl]ethylenediamine;[3] that is, L-histidine and L-arginine are linked together by their α-amino groups with an ethylene group. A number of proteinase inhibitors have amino acids as their constituents, and these amino acids are usually linked by amide bonds. The unusual structure of histargin and its biological activities prompted us to investigate its structure-activity relationship. OGAWA *et al.*, and also NEAL and ROSE have synthesized such compounds by the condensation of two amino acids and 1,2-dibromoethane in one pot.[3,4] However, this method yields many side products and may give rise to difficulties in the purification of histargin. In this paper, we describe a novel method for the synthesis of histargin and

Fig. 1. Structure of histargin (**1**).

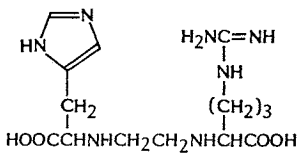
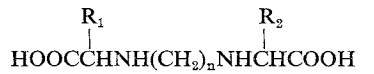


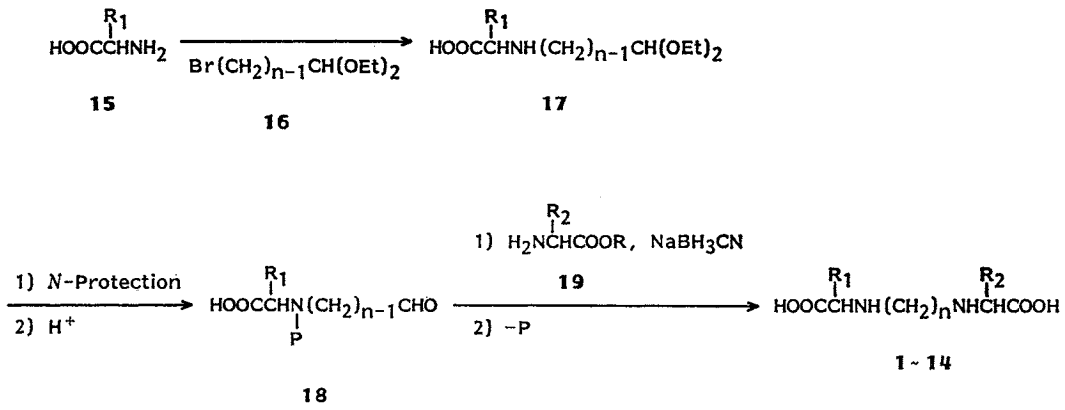
Fig. 2. Structure of histargin analogs.



(A-$(CH_2)_n$-B A, B=amino acids)

1	A=His,	B=Arg,	n=2		
2	A=His,	B=His,	n=2		
3	A=His,	B=Phe,	n=2		
4	A=His,	B=Lys,	n=2		
5	A=His,	B=Pro,	n=2		
6	A=His,	B=Asp,	n=2		
7	A=His,	B=Orn,	n=2		
8	A=His,	B=Phe-OEt,	n=2		
9	A=His-OCH_3,	B=His-OCH_3,	n=2		
10	A=His,	B=Z-Arg,	n=2		
11	A=Arg,	B=Arg,	n=2		
12	A=Arg,	B=Phe,	n=2		
13	A=Arg,	B=Lys,	n=2		
14	A=His,	B=Arg,	n=3		

Scheme 1. Synthesis of histargin analogs.



related compounds (**1**~**14**) (Fig. 2). We also report the inhibitory activities of these compounds against CPase B, CPase A, CPase N, and ACE.

Chemistry

The general method we used for histargin analogs is shown in Scheme 1. Our method involves two kinds of *N*-alkylation reactions: Alkylation of amines with alkyl halides and reductive *N*-alkylation. This stepwise procedure eliminates the formation of side products that include undesired sets of amino acids. One amino acid (**15**), L-histidine, for example, and an equimolar amount of bromoacetoaldehyde diethylacetal (**16**, n=2) were refluxed with potassium carbonate to give N^{α}-(diethoxyethyl)-L-histidine (**17**). The amino group was protected by benzyloxycarbonyl group (Z) and then the diethylacetal was hydrolyzed by treatment with 1 N HCl to give N^{α}-formylmethyl-N^{α}-Z-L-histidine (**18**). Reductive *N*-alkylation of the other amino acid (**19**), L-arginine, with compound **18** by the use of sodium cyanoborohydride followed by *N*-deprotection gave histargin (**1**).

Using this method, we replaced L-histidine and L-arginine with various amino acids to synthesize compounds **1** to **14**.

Table 1. Inhibition of enzymes by synthesized compounds.

Compound	IC_{50} (μg/ml)			
	CPase B	CPase A	CPase N	ACE
1	17.6	>200	6	1.7
2	60	>200	48	0.7
3	64	8.4	100	1.2
4	>200	>200	80	4.0
5	>200	>200	134	1.0
6	>200	>200	—	0.5
7	112	>200	22	1.7
8	>200	146	>200	24
9	>200	>200	—	>200
10	240	>200	26	>200
11	360	>200	1.4	>200
12	130	49.6	10	260
13	>200	>200	16	>200
14	360	>200	150	4.0

Inhibition of CPase B, CPase A, CPase N, and ACE

The inhibition of CPase B, CPase A, CPase N, and ACE by these synthesized compounds was tested with a modification of the assay method for ACE activity described by HAYAKARI *et al.*[5]

CPase B was inhibited by compounds **1**, **2**, **3**, and **7** with the IC_{50} of 17.6, 60, 64, and 112 μg/ml, respectively. Although histargin (**1**) was inactive against CPase A, compound **3** at the concentration of 8.4 μg/ml inhibited the activity of this enzyme by 50%. Compounds **1** to **7** inhibited ACE strongly

with low concentrations. Of these seven, the strongest inhibitors of ACE were **2** and **6**, for which the IC_{50} were 0.7 and 0.5 μg/ml, respectively (Table 1). It seemed that the chelating activity of histargin with the metal atom of the proteinases was the primary mode of action. The functional groups for the chelation are the two amino groups and the carbonyl groups, but side chains of the constituent amino acids are also important to the inhibition. Our results showed that the histidyl residue was necessary to inhibit ACE. On the other hand, CPase N was inhibited by histargin and other compounds that have arginine residue(s), even if they did not inhibit ACE, such as compounds **10** to **13**.

Experimental

Optical rotations were measured with a Jasco DIP-181 polarimeter. MP's were found with a Yanagimoto melting point apparatus, and were uncorrected. IR spectra were taken on a Hitachi EPI-G2 or 273-30 spectrophotometer, and NMR spectra on a Hitachi R-24B (60 MHz) spectrometer. Fast atom bombardment mass spectra (FAB-MS) were recorded on a Jeol JMS-DX302 mass spectrometer.

Assays of Enzymatic Activity

The activities of the enzymes were measured by the assay for ACE activity described by HAYAKARI *et al.*[5] with a slight modification. The assay was carried out in 0.5 ml of incubation mixture containing the enzyme (a), the substrate (b), Tris-HCl buffer (c), 0.1 mg/ml bovine serum albumin (d), and the test material. The reaction was started by the addition of the enzyme, and the reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1 N NaOH (0.3 ml). The activity of the enzyme was found from the absorbance at 382 nm after the addition of 2 ml of 0.06 M phosphate buffer (pH 7.2) and 2 ml of 1% cyanuric chloride dissolved in 2-methoxyethanol.

(a): CPase B purified from porcine pancreas (Boehringer-Mannheim, FRG), CPase A (type I) from bovine pancreas (Sigma Chem. Co., U.S.A.), CPase N partly purified from human serum, and ACE partly purified from bovine lungs were used. (b): 0.005 M Hippuryl-L-lysine for CPase B and CPase N, 0.005 M hippuryl-L-phenylalanine for CPase A, and 0.01 M hippuryl-L-histidyl-L-leucine for ACE were used. (c): 0.025 M Tris-HCl buffer (pH 8.0) for CPase B, 0.025 M Tris-HCl buffer (pH 7.5) containing 0.45 M NaCl for CPase A, and 0.05 M Tris-HCl buffer (pH 8.0) containing 0.03 M NaCl for ACE were used. (d): It was not added for CPase N and ACE.

Preparation of Compounds 1 to 14

Compounds **1** to **14** were prepared by almost the same method. The procedure for the synthesis of histargin (**1**) is described below as a typical example.

N-(2,2-Diethoxyethyl)-L-histidine (17)

To a solution of L-histidine (**15**) (50.0 g, 0.32 mol) and anhydrous potassium carbonate (44.5 g) in EtOH - H_2O (1 : 1) (1 liter), bromoacetoaldehyde diethylacetal (**16**) (63.5 g, 0.32 mol) was added. The mixture was refluxed for 24 hours. Then it was diluted with water (1 liter) and adjusted to pH 7.0. After EtOH was evaporated under reduced pressure, the aqueous layer was washed with EtOAc. The crude material was chromatographed on Sepabeads SP-207 (3 liters, Mitsubishi Chemical Industries Limited) with water (5 liters) and MeOH - H_2O (1 : 2) as eluents. The fractions that were eluted with MeOH - H_2O (1 : 2) were combined and evaporated to give N^{α}-(diethoxyethyl)-L-histidine (**17**) (31.8 g, yield 36.3%): MP 160~162°C; $[\alpha]_D$ −27.4° (*c* 1, MeOH); IR (KBr) cm^{-1} 2970, 2870, 1645, 1580, 1570, 1450, 1380, 1300, 1125, 1090, 1065, 845, 800, 630, 550, 480; ^1H NMR (CD_3OD) δ 1.20 (6H, t, J=7 Hz), 3.0~3.5 (4H, m), 3.5~4.0 (5H, m), 4.79 (1H, d, J=5 Hz), 6.98 (1H, s), 7.62 (1H, s).

N^{α}-Formylmethyl-N^{α}-Z-L-histidine (18)

To an ice-cooled solution of compound **17** (19.5 g, 0.072 mol) in 1 N NaOH (72 ml), a solution of benzyloxycarbonyl chloride (22.6 ml) was added dropwise. During the addition, the pH was adjusted

from 9 to 10 with 2 N NaOH. After the addition, the mixture was stirred for 2 hours at 5°C. The reaction mixture was extracted with EtOAc (150 ml) three times and the combined EtOAc layer was washed with 1 N HCl and the saturated NaCl solution, and dried over anhydrous Na_2SO_4. The evaporation of the solvent gave N^{α}-(2,2-diethoxyethyl)-N^{α},N^{im}-Z_2-histidine (40.1 g), which was then dissolved in anhydrous MeOH (250 ml). To this solution was added 28% sodium methoxide in MeOH (27.7 ml), and the mixture was stirred for 2.5 hours at room temperature. After the reaction, the mixture was diluted with water (259 ml) and adjusted to pH 7.0, and MeOH was removed under reduced pressure. The aqueous layer was washed twice with EtOAc, and concentrated to dryness. Desalting with MeOH gave N^{α}-(2,2-diethoxyethyl)-N^{α}-Z-L-histidine (26.0 g, yield 89.5%).

N^{α}-(2,2-Diethoxyethyl)-N^{α}-Z-L-histidine was dissolved in EtOH - H_2O (1 : 2) (200 ml) and treated with 1 N HCl (147 ml) for 3.5 hours at room temperature. The reaction solution was adjusted to pH 6.8 and the EtOH was removed under reduced pressure. The aqueous layer was purified on Diaion HP-20 (4 liters) with water and 25% MeOH as eluents. The fractions containing the desired product were combined and concentrated to give N^{α}-formylmethyl-N^{α}-Z-L-histidine (**18**) as a pale yellow powder (15.1 g, yield 71.5%); $[\alpha]_D$ −13.1° (*c* 1, MeOH); IR (KBr) cm^{-1} 3450, 2950, 1700, 1600, 1420, 1370, 1310, 1260, 1220, 1190, 1130, 1110, 990, 825, 785, 740, 705, 670; ^1H NMR (CD_3OD) δ 3.1~3.4 (2H, m), 3.9~4.2 (2H, m), 4.3~4.7 (1H, m), 5.09 (2H, s), 5.6~5.9 (1H, m), 6.70 (1H, s), 7.28 (5H, s), 7.32 (5H, s), 7.65 (1H, s).

Histargin (**1**)

To a solution of compound **18** (15.1 g, 0.046 mol) in MeOH (150 ml) was added 100 ml of an aqueous solution of L-arginine methyl ester HCl salt (0.092 mol), and the mixture was cooled in an ice bath. To this ice-cooled solution was added portionwise sodium cyanoborohydride (2.90 g, 0.046 mol). The mixture was stirred for 4 hours at room temperature, and the solvent was evaporated. Column chromatography of the residue with Sepabeads SP-207 (3 liters) gave protected histargin (N^{α}-Z-L-His-C_2-L-Arg-OCH_3) (18.9 g, yield 70.6%).

The protected histargin (18 g, 0.031 mol) was dissolved in EtOH (90 ml), and treated with 1 N NaOH (90 ml) for 1.5 hours at room temperature to hydrolyze the ester. After the solution was neutralized, 1 N HCl (63 ml) and 10% palladium on carbon (200 mg) were added. The mixture was treated by catalytic hydrogenation for 8 hours with H_2 gas bubbling. The catalyst was filtered and EtOH was evaporated under reduced pressure. The resulting aqueous solution was adjusted to pH 6.5 and then put on a Sepabeads SP-207 column (500 ml). The fractions of H_2O elution were combined and purified on a Dowex 50W (H^+ type) column (500 ml) by elution with 2 N NH_4OH. After the fractions containing the desired product were combined and concentrated under reduced pressure, the resulting residue was dissolved in distilled water and freeze-dried to give histargin (**1**) (9.76 g, yield 79.2%): $[\alpha]_D$ +30.7° (*c* 1, 6 N HCl); IR (KBr) cm^{-1} 3350, 1655, 1630, 1580, 1450, 1400, 1320, 1185, 1110, 985, 940, 770; ^1H NMR (D_2O) δ 1.3~1.8 (4H, m), 2.8 (4H, s), 2.85 (2H, d, *J*=7 Hz), 3.02 (2H, t, *J*=6 Hz), 3.3 (1H, t, *J*=6 Hz), 3.41 (1H, t, *J*=6 Hz), 6.85 (1H, s), 7.6 (1H, s); FAB-MS *m/z* 356 (M+1).

Spectral data of compounds **2** to **14** were as follows:

2: $[\alpha]_D$ +17.1° (*c* 1, 6 N HCl); mp 227~232°C (dec); IR (KBr) cm^{-1} 3400, 3100, 2840, 1580, 1470, 1390, 1340, 1320, 1290, 1160, 1100, 1080, 1060, 920, 840, 805, 660, 620, 530, 420; ^1H NMR (D_2O) δ 2.84 (4H, s), 2.9 (4H, d, *J*=7 Hz), 3.46 (2H, t, *J*=6 Hz), 6.92 (1H, s), 6.95 (1H, s), 7.84 (1H, s), 7.87 (1H, s); FAB-MS *m/z* 337 (M+1).

3: $[\alpha]_D$ +38.1° (*c* 1, 6 N HCl); mp 248~250°C (dec); IR (KBr) cm^{-1} 3410, 3050, 2840, 1575, 1485, 1440, 1380, 1340, 1280, 1175, 1100, 1070, 840, 820, 790, 770, 730, 690, 660, 620, 515, 410; ^1H NMR (D_2O+CF_3COOH) δ 3.16 (2H, d, *J*=6 Hz), 3.27 (2H, d, *J*=6 Hz), 3.4 (4H, s), 4.1 (1H, t, *J*=6 Hz), 4.19 (1H, t, *J*=6 Hz), 7.18 (6H, s), 8.48 (1H, s); FAB-MS *m/z* 347 (M+1).

4: $[\alpha]_D$ −32.0° (*c* 1, 6 N HCl); IR (KBr) cm^{-1} 3400, 2920, 2830, 1720, 1580, 1460, 1380, 1340, 1320, 1200, 1150, 1090, 970, 920, 800, 655, 610; ^1H NMR (D_2O) δ 1.0~1.8 (6H, m), 2.5~3.3 (4H, m), 3.05 (4H, s), 3.58 (2H, t, *J*=6 Hz), 6.74 (1H, s), 7.5 (1H, s); FAB-MS *m/z* 328 (M+1).

5: $[\alpha]_D$ −24.2° (*c* 1, 6 N HCl); IR (KBr) cm^{-1} 3420, 2870, 1630, 1460, 1400, 1340, 1240, 1170,

1095, 940, 845, 790, 670, 630; ^{1}H NMR (D_2O) δ 1.6~2.3 (4H, m), 2.8 (2H, d, J=6.5 Hz), 2.85 (4H, s), 3.15 (2H, t, J=6 Hz), 3.45 (1H, t, J=6 Hz), 3.75 (1H, t, J=6 Hz), 6.9 (1H, s), 7.9 (1H, s); FAB-MS m/z 297 (M+1).

6: $[\alpha]_D$ −28.8° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3450, 3025, 2960, 1600, 1400, 1300, 1190, 1070, 860, 810, 680, 555, 460; ^{1}H NMR (D_2O+CF_3COOH) δ 2.85 (2H, d, J=6 Hz), 3.19 (2H, d, J=6 Hz), 3.35 (4H, s), 3.88 (2H, t, J=5 Hz), 7.17 (1H, s), 8.41 (1H, s); FAB-MS m/z 315 (M+1).

7: $[\alpha]_D$ +30.8° (c 1, 6 N HCl); mp 230~235°C; IR (KBr) cm^{-1} 3425, 3260, 2840, 1635, 1585, 1480, 1400, 1310, 1295, 1280, 1175, 1110, 1060, 1030, 970, 940, 835, 800, 760, 740, 675; ^{1}H NMR (D_2O) δ 1.5~2.0 (4H, m), 2.7~3.2 (8H, m), 3.31 (1H, t), 3.63 (1H, t), 7.03 (1H, s), 7.78 (1H, s); FAB-MS m/z 314 (M+1).

8: $[\alpha]_D$ +33.4° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3460, 2955, 1750, 1650, 1510, 1460, 1420, 1210, 1100, 1045, 765, 720, 680, 640; ^{1}H NMR (CD_3OD) δ 1.1 (3H, t, J=7 Hz), 2.66 (4H, s), 2.95 (4H, d, J=7 Hz), 3.32 (1H, t, J=5 Hz), 3.52 (1H, t, J=7 Hz), 4.6 (2H, q, J=6 Hz), 6.88 (1H, s), 7.55 (1H, s); FAB-MS m/z 375 (M+1).

9: $[\alpha]_D$ +15.3° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3450, 2850, 1750, 1600, 1465, 1415, 1230, 1100, 840, 670, 630, 530; ^{1}H NMR (CD_3OD) δ 2.64 (4H, s), 2.94 (4H, d), 3.53 (2H, t), 3.7 (6H, s), 6.87 (2H, s), 7.57 (2H, s); FAB-MS m/z 365 (M+1).

10: IR (KBr) cm^{-1} 3370, 3180, 1675, 1635, 1580, 1470, 1450, 1420, 1385, 1330, 1275, 1140, 1085, 1040, 1025, 980, 775, 755, 700, 620; ^{1}H NMR (CD_3OD) δ 1.1~2.0 (2H, m), 2.6~4.1 (10H, m), 4.85 (2H, s), 6.7 (1H, s), 7.08 (5H, s), 7.32 (1H, s); FAB-MS m/z 490 (M+1).

11: $[\alpha]_D$ +27.9° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3390, 3220, 2970, 1680, 1650, 1575, 1460, 1405, 1335, 1200, 1135, 765, 685; ^{1}H NMR (CD_3OD) δ 1.3~1.7 (8H, m), 2.53 (4H, s), 2.8~3.2 (6H, m); FAB-MS m/z 375 (M+1).

12: $[\alpha]_D$ +32.2° (c 1, 6 N HCl); mp 211~216°C; IR (KBr) cm^{-1} 3300, 2925, 2840, 1655, 1615, 1480, 1440, 1380, 1310, 1175, 1120, 1070, 1045, 1020, 820, 800, 780, 740, 695, 665, 520; ^{1}H NMR (D_2O) δ 1.35~1.75 (4H, m), 2.8 (4H, s), 3.0 (2H, d, J=5 Hz), 3.05 (2H, t, J=6 Hz), 3.2 (1H, t, J=7 Hz), 3.48 (1H, t, J=7 Hz), 7.2 (5H, s); FAB-MS m/z 366 (M+1).

13: $[\alpha]_D$ +29.6° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3320, 2900, 2830, 1645, 1620, 1555, 1445, 1385, 1310, 1165, 1120, 935, 765, 660; ^{1}H NMR (D_2O) δ 1.25~1.65 (10H, m), 2.55 (4H, s), 2.7~3.15 (6H, m); FAB-MS m/z 347 (M+1).

14: $[\alpha]_D$ +35.6° (c 1, 6 N HCl); IR (KBr) cm^{-1} 3380, 1655, 1625, 1575, 1435, 1400, 1320, 1180, 1105, 980, 935, 760; ^{1}H NMR (D_2O) δ 1.4~1.9 (6H, m), 2.7 (2H, d, J=7 Hz), 2.77 (2H, t, J=6.5 Hz), 2.94 (4H, t, J=6 Hz), 3.12 (1H, t, J=5 Hz), 3.45 (1H, t, J=5 Hz), 6.72 (1H, s), 7.46 (1H, s); FAB-MS m/z 370 (M+1).

References

1) UMEZAWA, H.; T. AOYAGI, K. OGAWA, H. IINUMA, H. NAGANAWA, M. HAMADA & T. TAKEUCHI: Histargin, a new inhibitor of carboxypeptidase B, produced by actinomycetes. J. Antibiotics 37: 1088~1090, 1984
2) UMEZAWA, H.; T. TAKEUCHI, T. AOYAGI, M. HAMADA & K. OGAWA (Microbial Chemistry Research Foundation): A novel bioactive substance, histargin. Jpn. Kokai 216597 ('84), Dec. 6, 1984
3) OGAWA, K.; H. NAGANAWA, H. IINUMA, T. AOYAGI & H. UMEZAWA: The structure of histargin. J. Antibiotics 37: 984~987, 1984
4) NEAL, J. A. & N. J. ROSE: Stereospecific ligands and their complexes. I. A cobalt (III) complex of ethylenediaminedisuccinic acid. Inorg. Chem. 7: 2405, 1968
5) HAYAKARI, M.; Y. KONDO & H. IZUMI: A rapid and simple spectrophotometric assay of angiotensin converting enzyme. Anal. Biochem. 84: 361~369, 1978