THE STRUCTURE OF HISTARGIN

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The structure of histargin, an inhibitor of carboxypeptidase B, produced by *Streptomyces roseoviridis* MF118-A5 strain, has been defined as *N*-[(*S*)-1-carboxy-4-guanidinobutyl]-*N'*-[(*S*)-1-carboxy-2-(imidazol-4-yl)ethyl]ethylenediamine, by spectral analysis and chemical synthesis.

In a previous communication[1], we reported on the isolation and physico-chemical properties of histargin. In this communication, the structure determination and chemical synthesis of histargin are reported.

Histargin (**1**) was obtained as its monohydrate, and its molecular formula was suggested to be $C_{14}H_{25}N_7O_4 \cdot H_2O$ by elemental analysis and secondary ion mass spectrometry [m/z 356 (M + 1)] as reported. Compound **1** showed positive SAKAGUCHI and PAULI reactions.

The ^1H NMR spectrum (in D_2O) of **1** revealed the presence of two carbon chains, $-CH_2CH_2CH_2-$ CH– and $-CH_2$CH– in addition to the broad singlet-appearing four proton multiplet at δ 2.96 and one proton singlets at δ 7.75 and 7.00. The two down field singlets indicate the presence of an imidazole group (Fig. 1).

The potentiometric titration showed the presence of six dissociable groups with pKa' values of <3.5, <3.5, 4.4, 6.8, 9.0 and >11. They can be assigned to the two carboxyl functions, secondary

Fig. 1. ^1H NMR spectrum of histargin (**1**) monohydrate (D_2O).
400 MHz, internal DHO, 4.80 ppm.

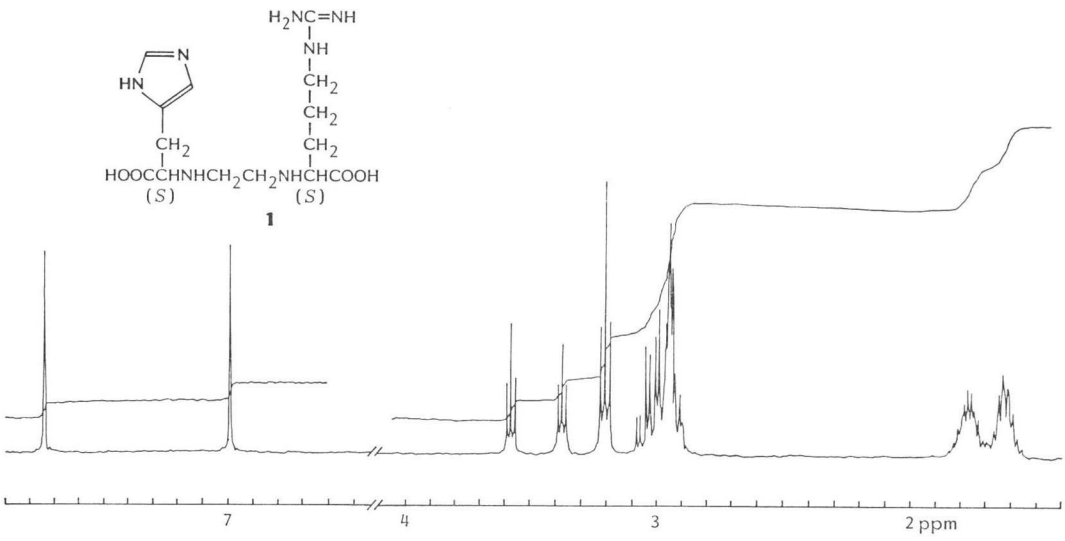
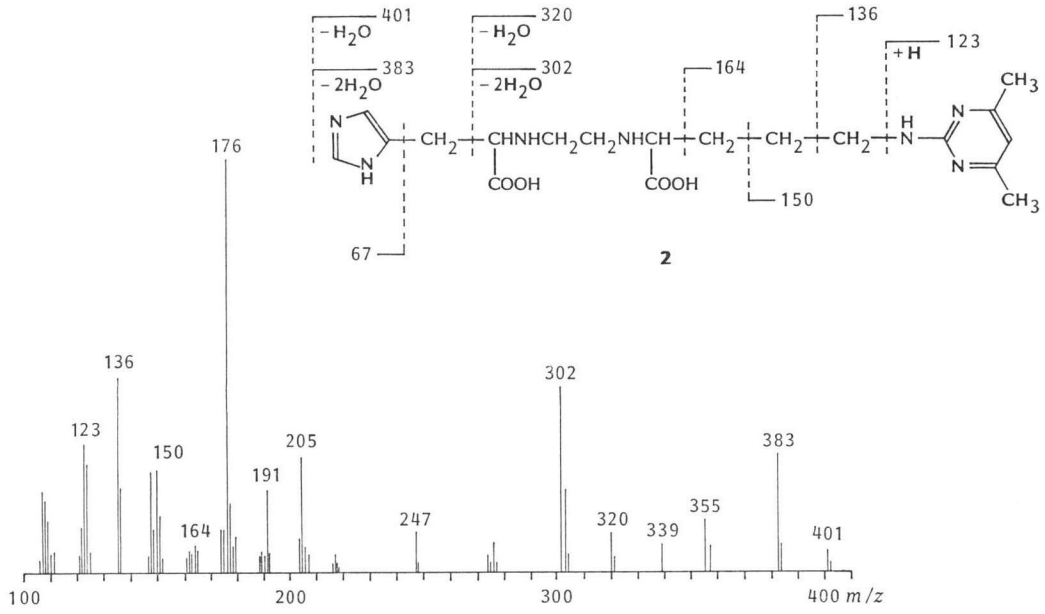


Table 1. ^{13}C NMR of histargin (**1**) monohydrate (D_2O).

Signal No.	Chemical shift (ppm)	Multiplicity	Assignment	Signal No.	Chemical shift (ppm)	Multiplicity	Assignment
1	178.9	s	–COOH of His or Arg	8	63.2	d	$_{\alpha}$CH of Arg
2	177.9	s	〃	9	45.8	t	–N–*CH_2–*CH_2–N– H H
3	157.7	s	$_{\zeta}$C of Arg	10	45.4	t	〃
4	136.7	d	C-2 of His	11	41.6	t	$_{\delta}CH_2$ of Arg
5	133.6	s	C-5 of His	12	30.0	t	$_{\beta}CH_2$ of His
6	118.2	d	C-4 of His	13	29.0	t	$_{\beta}CH_2$ of Arg
7	63.7	d	$_{\alpha}$CH of His	14	25.0	t	$_{\gamma}CH_2$ of Arg

100 MHz, internal dioxane, 67.40 ppm.

Fig. 2. Electron impact mass spectrum of dimethylpyrimidine derivative (**2**).



amino, imidazole, secondary amino and guanidino groups, respectively in the proposed structure.

The ^{13}C NMR spectrum of **1** showed 14 signals which are summarized and assigned in Table 1. The presence of carboxyl groups was also confirmed by the appearance of the absorption band at 1735 cm^{-1} in the IR spectrum[1] of the tetrahydrochloride of **1**.

Treatment of **1** with 2,4-pentanedione in aqueous alkaline solution at 37°C, 18 hours[2] afforded the dimethylpyrimidine derivative (**2**), which showed UV absorption maxima at 235 nm and 300 nm, characteristic of dimethylpyrimidines. It also gave a reasonable fragmentation pattern in electron impact mass spectrometry [m/z 401 (M − H_2O)]. A key fragment ion at m/z 176 suggests the existence of N^{δ}-dimethylpyrimidylornithine residue[3] and fragment ions at m/z 123, 136, 150, 151 and 164 are also considered to be derived from this moiety[4] (Fig. 2). Furthermore, the fragment ion at m/z 67 suggests the presence of an imidazole group in **2**. In secondary ion mass spectrometry, **2** showed a protonated molecular ion peak at m/z 420 (M + 1).

The high resolution mass spectrum of **2** showed *m/z* 401.2182 (Calcd 401.2173 for $C_{19}H_{27}N_7O_3$), which corresponds to M − H_2O. From these results, and their elemental analyses the most probable empirical formulae of **2** and **1** were concluded to be $C_{19}H_{29}N_7O_4$ and $C_{14}H_{25}N_7O_4$, respectively.

These results suggested the presence of histidine and arginine residues in **1**. These two amino acids could not be combined by a peptide linkage, because they could not be liberated from **1** by acid hydrolysis (6 N HCl, at 110°C for 18 hours).

Taking the chelating ability of **1**[1] into account, and considering our inability to recover the postulated two amino acids from **1** by acid hydrolysis, the singlet-appearing four proton band in the ^1H NMR of **1** most probably arises from an ethylenediamine moiety. This moiety is considered to be the connecting group between the two amino acids, sharing nitrogen atoms with them and showing the typical two-step protonation of the nitrogens[5] in *pKa* analysis.

From the above-mentioned results, the structure of histargin was postulated to be *N*-(1-carboxy-4-guanidinobutyl)-*N'*-[1-carboxy-2-(imidazol-4-yl)ethyl]ethylenediamine.

In order to determine the structure and the absolute configuration of **1**, the chemical synthesis of **1** was undertaken. NISHIKIORI *et al.*[6] has reported the synthesis of ethylenediaminedisuccinic acid from L-aspartic acid and 1,2-dibromoethane in alkaline aqueous ethanolic solution. Its optical rotation was compared with that of natural ethylenediaminedisuccinic acid which they obtained from culture filtrate of a *Streptomyces*. Histargin was synthesized by a modification of this method using L-histidine and L-arginine as amino acid components as described below. Synthetic histargin was purified using methods we described previously.[1] The product was followed by the thin-layer chromatograms (visualized using the SAKAGUCHI and PAULI reactions) and by the carboxylpeptidase B inhibitory activity, with total yield of 6.7%.

All the physico-chemical and biochemical properties of the synthetic histargin thus obtained were identical with that of the naturally occurring histargin. Thus the absolute chemical structure of histargin was established as *N*-[(*S*)-1-carboxy-4-guanidinobutyl]-*N'*-[(*S*)-1-carboxy-2-(imidazol-4-yl)-ethyl]ethylenediamine.

Experimental

Melting points were uncorrected. UV spectra were recorded on a Beckman DU-8 spectrophotometer, IR spectra on a Hitachi 260-10 infrared spectrophotometer, ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra on a Jeol JNM-GX400 spectrometer, mass spectra on a Hitachi RMU-6M or Hitachi M-80H spectrometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. High performance liquid chromatography was performed using Partisil-10-ODS-3 (Magnum 20, Whatman, USA) or Nucleosil 5 C_{18} (20 × 300 mm, Macherey-Nagel, Germany) with solvent systems of 10% aqueous ammonium acetate or acetonitrile - 5% potassium acetate containing 1% citric acid (pH 5.2), 12: 88.

Histargin (1) Monohydrate

The inhibitor (**1**) was purified from the culture filtrate of *Streptomyces roseoviridis* strain MF118-A5 by the method described in a previous paper[1]. Physico-chemical properties: mp 161 ~ 165°C (dec); $[\alpha]_D^{25}$ +36.3° (*c* 1, constant boiling HCl); IR (KBr) 3375, 1670, 1630, 1580, 1450, 1400, 1320, 1180, 1100, 980, 940, 760 cm^{-1}. The ^1H NMR spectral data (400 MHz, D_2O, internal DHO, 4.80 ppm) is shown in Fig. 1. *Anal* Found: C 45.53, H 7.53, N 26.28, O 20.80. Calcd for $C_{14}H_{25}N_7O_4 \cdot H_2O$: C 45.03, H 7.29, N 26.26, O 21.42. SIMS *m/z* 356 (M + H). IC_{50} 17 μg/ml against carboxypeptidase B.

Preparation of the Dimethylpyrimidine Derivative (2)

Inhibitor **1** (250 mg) was treated with 2,4-pentanedione (0.6 ml) in H_2O (4 ml, containing 0.2 g

of sodium carbonate) for 18 hours at 37°C. The mixture was concentrated to dryness under reduced pressure and applied on a Sephadex LH-20 column which was developed with H_2O. Fractions having the correct UV absorption and giving a PAULI-positive reaction were pooled and further purified by use of Partisil-10-ODS-3 (Magnum 20), a preparative HPLC column, developed with the mobile phase of acetonitrile - 5% potassium acetate containing 1% citric acid (pH 5.2), 12: 88 at a flow rate of 6 ml /minute. The fractions which contained PAULI reaction positive material that also had UV absorption were pooled and concentrated under reduced pressure to an aqueous solution. The resulting solution was applied to an HP-20 column. The resin bed was washed with H_2O and the adsorbed material **2** was eluted with 50% aq MeOH. The eluate was concentrated under reduced pressure to give 58 mg of crystalline powder of **2**. Physico-chemical properties: mp 243~246°C (dec); $[\alpha]_D^{22}$ +34.0° (*c* 1, constant boiling HCl); UV λ_{max} in H_2O 235 nm (ε 1.7×10^4), 300 nm (ε 4.2×10^3); IR (KBr) 3400, 2850, 2325, 1585, 1460, 1395, 1300, 1170, 1120, 1065, 980, 935, 830, 795, 670 cm^{-1}. ^1H NMR spectrum (400 MHz, D_2O+DCl, pD<2, internal DHO, 4.80 ppm): 1.96~2.15 (CH_2, m), 2.6~2.85 (CH_2, m), 2.4 ($CH_3\times2$, s), 3.4~3.65 ($CH_2\times4$, m), 4.15 (CH, dd, J=5.4, 6 Hz), 4.39 (CH, dd, J=6, 7.6 Hz), 6.71 (⌬C–H, s), 7.44 (⌬C–H, br s), 8.66 (⌬C–H, d, J=2 Hz). *Anal* Found: C 52.08, H 6.72, N 22.40. Calcd for $C_{19}H_{29}N_7O_4\cdot H_2O$: C 52.16, H 7.14, N 22.41. SIMS *m/z* 420 (M + H).

Synthesis of (*S,S*)-Histargin (**1'**)

1 g (5.21 mmol) of L-histidine monohydrate monohydrochloride and 1.06 g of L-arginine monohydrate (5.21 mmol) and 0.55 g of sodium carbonate were dissolved in 20 ml of 1 N NaOH and 20 ml of EtOH. To the resulting mixture was added 2 ml of dibromoethane and the solution was refluxed for 8 hours at oil bath temperature of 85°C. The reaction mixture was then concentrated to dryness and the residue was dissolved with 100 ml of H_2O and the new solution was adjusted to pH 7.0 with 6 N HCl. This solution was adsorbed on a column of Dowex 50WX4 (Na^+, 100~200 mesh, 200 ml) and the resin bed was washed with H_2O. The column was then developed with 1.5 N NH_4OH. The fractions that contained PAULI and SAKAGUCHI reactions-positive material and that also showed the same Rf value on TLC as histargin (**1**) were pooled and concentrated to yield 360 mg of crude material of **1'**. The material thus obtained was dissolved with 5 ml of 10% aq ammonium acetate and the solution was adjusted to pH 7.0 with 6 N HCl and divided into two equal portions. Each portion was applied to a Nucleosil $5C_{18}$ (20 × 300 mm) column that had been equilibrated with 10% aq ammonium acetate. The column was developed with the same solution at a flow rate of 6 ml/minute. The (*S,S*)-histargin containing fractions were pooled, diluted five fold with H_2O and applied to a column of Dowex 50WX4 (H^+, 100~200 mesh, 110 ml). The resin bed was washed with H_2O and the adsorbed (*S,S*)-histargin (**1'**) was eluted with 1.5 N NH_4OH. The eluate was concentrated under reduced pressure yielding 131 mg of (*S,S*)-histargin monohydrate. $[\alpha]_D^{25}$ +35.5° (*c* 1, constant boiling HCl). *Anal* Found: C 45.58, H 7.60, N 26.58. Calcd for $C_{14}H_{25}N_7O_4\cdot H_2O$: C 45.03, H 7.29, N 26.26. ^1H NMR, ^{13}C NMR, IR: identical with those of natural histargin monohydrate. SIMS *m/z* 356 (M + H). IC_{50} 17 μg/ml against carboxypeptidase B.

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