LEUHISTIN, A NEW INHIBITOR OF AMINOPEPTIDASE M, PRODUCED BY *Bacillus laterosporus* BMI156-14F1

II. STRUCTURE DETERMINATION OF LEUHISTIN

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Leuhistin, a new inhibitor of aminopeptidase M, has been isolated from the culture broth of *Bacillus laterosporus* BMI156-14F1. The structure of leuhistin was determined by NMR studies. X-Ray and chemical analysis confirmed the absolute structure to be (2*R*,3*S*)-3-amino-2-hydroxy-2-(1*H*-imidazol-4-ylmethyl)-5-methylhexanoic acid.

In the preceeding paper[1], we have described the taxonomy, isolation, physico-chemical properties and biological properties of leuhistin, a novel inhibitor of aminopeptidase M (AP-M). In this paper, we describe the structure determination of leuhistin. The molecular formula of leuhistin was elucidated as $C_{11}H_{19}N_3O_3$ from the mass spectrum and the elemental analysis. The IR spectrum (KBr) of leuhistin showed absorption bands at 3400, 3130, 2970, 1625, 1470, 1395, 1270, 1220, 1150, 1090, 835 cm^{-1}.

Table 1 shows ^1H and ^{13}C NMR data for leuhistin monohydrochloride. A positive color reaction with ninhydrin reagent suggested the presence of an amino group in the molecule. The chemical shifts of 3-H (δ 3.57) and C-3 (δ 55.8) indicated the amino group was attached to C-3. ^1H-^1H COSY spectrum of leuhistin revealed the partial structure (Fig. 1A). A positive color reaction with Pauly reagent and NMR data suggested the presence of an imidazole moiety (Fig. 1B). The other three carbons are shown as C,

Table 1. ^1H and ^{13}C NMR spectral data for leuhistin monohydrochloride in D_2O.

Assignment	^1H[a]	^{13}C[b]
1-CO	—	176.9 (s)
2-C	—	78.2 (s)
3-CH	3.57 dd (J=2.5, 10.8)	55.8 (d)
4-CH_2	1.58 ddd (J=3.2, 10.8, 13.2), 1.70 ddd (J=2.5, 10.2, 13.2)	37.1 (t)
5-CH	1.75 m	24.7 (d)
6-CH_3	0.96 d (J=5.6)	20.8 (q)
7-CH_3	1.02 d (J=6.4)	23.5 (q)
8-CH_2	3.05 d (J=15.0), 3.25 d (J=15.0)	31.2 (t)
2'-CH	8.61 d (J=1.6)	133.9 (d)
4'-C	—	128.7 (s)
5'-CH	7.28 br s	118.0 (d)

[a] 400 MHz, δ in ppm, J in Hz.
[b] 100 MHz, δ in ppm.

Fig. 1. Partial structures of leuhistin.

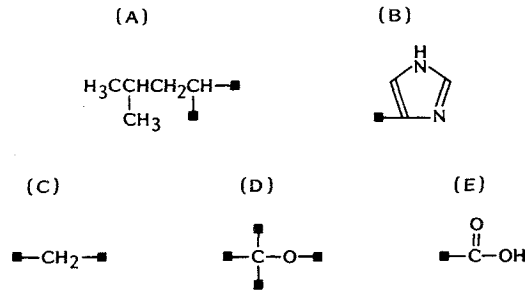


Fig. 2. Long range ^{1}H-^{13}C coupling pattern of leuhistin.

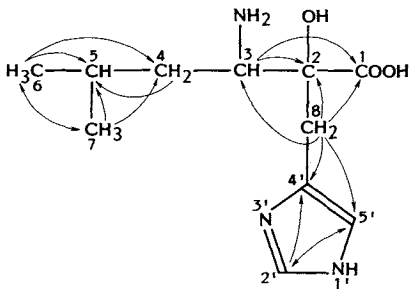


Fig. 3. Structure of leuhistin.

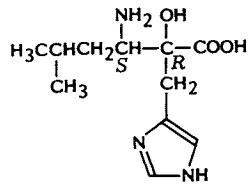
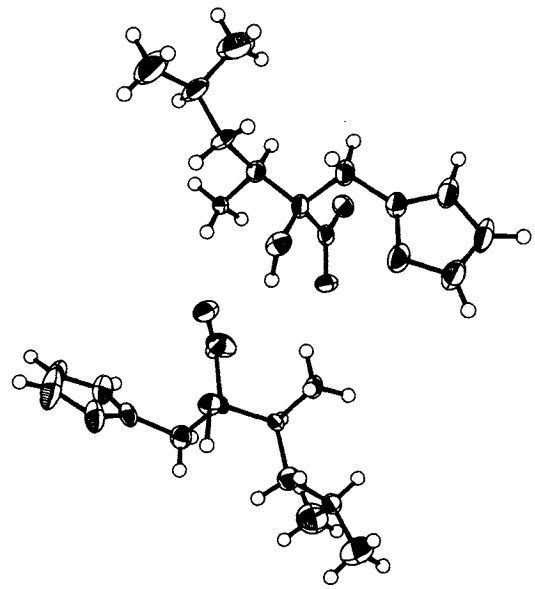


Fig. 4. An ORTEP drawing of leuhistin.



D and E in Fig. 1. The heteronuclear multiple-bond correlation (HMBC) experiment (Fig. 2) indicated the linkage of these partial structures. Cross peaks between 8-H (δ 3.05 and 3.25) and imidazole carbons (C-4′ δ 128.7 and C-5′ δ 118.0) indicated that the methylene group is linked to the imidazole moiety. Long range coupling between 3-H and C-2 (δ 78.2), 8-H and C-3, and 8-H and C-2 suggested the quaternary carbon (C-2) is located between C-3 and C-8. Long range coupling between 3-H and C-1 (δ 176.9), and between 8-H and C-1 indicated that C-2 is connected to C-1. The C-1 carbon was identified as the carbon of carboxyl group from its chemical shift (δ 176.9). This was supported by the p*K*a values (2.3, 7.5 and 9.6) of leuhistin. Another oxygen atom, which is linked to the quaternary oxycarbon (C-2), was identified as a hydroxyl group from consideration of the molecular formula. Thus the structure of leuhistin was determined to be 3-amino-2-hydroxy-2-(1*H*-imidazol-4-ylmethyl)-5-methylhexanoic acid (Fig. 3). The absolute stereochemistry at C-3 was determined by oxidation of leuhistin. Treatment with potassium permanganate gave leucine that was identified as the L-enantiomer by TLC on a chiral precoated HPTLC plate. Thus the configuration at C-3 was found to be *S*.

The relative stereochemistry of C-2 and C-3 of leuhistin was determined by a crystal X-ray diffraction analysis using leuhistin free base. An ORTEP[2] drawing of leuhistin is shown in Fig. 4. The X-ray analysis showed that the relative configuration of C-2 and C-3 is *threo*.

Therefore, the structure of leuhistin was determined to be (2*R*,3*S*)-3-amino-2-hydroxy-2-(1*H*-imidazol-4-ylmethyl)-5-methylhexanoic acid (Fig. 3).

Experimental

The mass spectrum was obtained on a Hitachi M-80H mass spectrometer, the IR spectrum on a Hitachi 260-10 spectrophotometer and the NMR spectra on a Jeol JNM-GX400 NMR spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

Chemicals

Chemicals employed were as follows: PK208 (cation exchange resin) from Nippon Rensui Co., Tokyo, Japan; HPTLC pre-coated CHIR plates from E. Merck, Darmstadt, FRG. All other chemicals were of analytical grade.

Oxidation of Leuhistin

Potassium permanganate (450 mg) was added to a solution of 100 mg of leuhistin in 5 ml of water. After 5 hours at room temperature, the reaction mixture was filtered, and the filtrate was applied to a column of PK208 (free acid form). Elution with 0.5 N ammonium hydroxide and concentration of the ninhydrin positive fractions gave a brownish powder. The powder was subjected to a silica gel TLC with BuOH - AcOH - H_2O (2 : 1 : 1). The extract from the Rf 0.72 fraction was concentrated to give leucine as a colorless powder.

Determination of Configuration of Leucine

The solution of leucine obtained from the oxidation of leuhistin was examined by HPTLC on a Merck CHIR pre-coated plate eluting with MeOH - H_2O - MeCN (1 : 1 : 4). The configuration of the leucine, Rf 0.69, was determined by comparison with authentic D- and L-leucine which gave Rf values of 0.59 and 0.69, respectively.

X-Ray Diffraction Analysis

A colorless prism crystal of leuhistin having approximate dimensions $0.2 \times 0.1 \times 0.02$ mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated CuKα radiation and a 3KW rotating anode generator. The lattice constants were derived from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $31.0° < 2\theta < 51.6°$. Crystal data: $C_{11}H_{19}N_3O_3$, monoclinic, $P2_1$, $a=5.6163(4)$, $b=23.640(2)$, $c=9.639(1)$Å, $\beta=96.020(8)°$, $U=6473(2)$Å^3, $Z=4$, $D_{calc}=1.259$ g/cm^3, μ for CuKα radiation $=7.74$ cm^{-1}.

Intensities were measured by a $2\theta-\omega$ scan method with a scan speed 8°/minute in ω. Backgrounds were measured at each end of the scan for half the total scan time. The weak reflections ($I < 10.0\sigma$ (I)) were rescaned (maximum of 2 rescans) and the counts were accumulated to assure good counting statistics. A total of 2,087 reflections in the 2θ range $6° - 120°$ was measured.

The crystal structure was determined by direct method using MAGEX[3] program in MITHRIL package[4]. In the final refinement, the non-hydrogen atoms were refined anisotropically by full-matrix least-squares. The hydrogen atoms were eventually included in calculated positions but were not refined. The final R value was 0.045 for 1,022 observed reflections ($I > 3\sigma(I)$). The atomic parameters, bond lengths and angles have been sent to the Cambridge Crystallographic Data Centre.

Acknowledgment

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