chloroform (3 × 10 mL). The organic layer was washed with water, dried (Na_2SO_4), and evaporated to dryness, and the residue was dissolved in 30 mL of 5% sulfuric acid in methanol. The solution was kept at 20 °C for 20 h. It was then diluted with 50 mL of water and extracted with chloroform (3 × 15 mL), and the extracts were washed with water, with a 5% sodium bicarbonate solution, and again with water. The chloroform solution was dried (Na_2SO_4) and evaporated to dryness, and the residue was recrystallized from chloroform–hexane: 17 mg (53%); mp 222 °C (lit.[10,11] mp 218–220, 222 °C[5]); NMR (0.005 M in $CDCl_3$) δ 3.31 (t, 4, CH_2CH_2COR), 3.65, 3.68, 3.77 (3 s, 6, 6, 6, C_2CH_3 and C_7CH_3, OCH_3, $C_{12}CH_3$, and $C_{18}CH_3$), 4.46 (t, 4, $CH_2CH_2CO_2R$), 9.11 (br, 2, H_3 and H_8), 10.05, 10.09, 10.14, 10.17 (4 s, 1, 1, 1, 1, H_{20}, H_{10}, H_5, and H_{15}); mass spectrum, *m/e* (relative intensity) 538 (M^+, 100). Anal. Calcd for $C_{32}H_{34}N_4O_2$: C, 71.37, H, 6.35; N, 10.40. Found: C, 71.20; H, 6.20; N, 10.31. When [5-^{14}C]-4 was used (1.2 × 10^5 dpm/mg), the [5-^{14}C]deuteroporphyrin IX dimethyl ester had 1.4 × 10^5 dpm/mg.

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Registry No. 1, 81971-96-2; 2, 81971-97-3; 3, 52091-10-8; 4, 81971-98-4; 5, 81971-99-5; 9, 68999-91-7; 10, 975-45-1; 11, 4458-65-5; 12, 81972-00-1; 13, 81972-01-2; 14, 4563-77-3; 15, 81972-02-3; 16, 81972-03-4; 17, 81972-04-5; 18, 81972-05-6; 19, 6277-13-0; 20, 81972-06-7; 21, 81972-07-8; 22, 81972-08-9; 23, 81972-09-0; 24, 81972-10-3; 25, 81972-11-4; sodium nitrite, 7632-00-0; benzyl acetoacetate, 5396-89-4; ethyl acetoacetate, 141-97-9.

Crystal and Molecular Structure of Bestatin and Its Implications Regarding Substrate Binding to the Active Site of Leucine Aminopeptidase[1,2]

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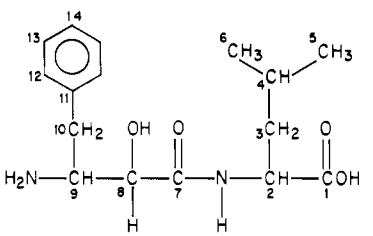
The X-ray crystal structure of bestatin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine ($C_{16}H_{24}N_2O_4$), has been determined. Four molecules of bestatin crystallize with four molecules of 2-methyl-2,4-pentanediol (MPD) and eight molecules of water in the space group $P2_12_12_1$. Unit cell dimensions are a = 6.653 (1), b = 15.150 (3), and c = 27.309 (4) Å. The final R was 8.5%, based on 2871 independent structure amplitudes. The MPD was found to be disordered. In addition to the usual functional groups needed for binding to leucine aminopeptidase, bestatin includes a tetrahedral carbon, C(8), as might be found in the putative transition-state intermediate. The structure indicates that the nonpolar side chains are oppositely disposed and separated by ~10 Å. The peptide bond is trans. There is no H bonding between OH on C(8) and the adjacent carbonyl. These data suggest possible modes of binding of this transition-state analogue to leucine aminopeptidase.

Bestatin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine[3] (1), is a tightly bound inhibitor of hog



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kidney leucine aminopeptidase (K_i = 2 × 10^{-8}) and aminopeptidase B.[4,5] This peptide analogue, originally isolated from actinomycetes, has three optical centers.[6] Kinetic tests of synthetically prepared isomers show that isomers with the 8*S*,9*S*,2*S* or 8*R*,9*R*,2*S* configurations are 7- and 750-fold less active, respectively, as inhibitors of LAP than (8*S*,9*R*,2*S*)-bestatin.

In bestatin the C(9) is derived from D-phenylalanine. The configuration about this carbon seems to be a less important determinant of the binding constant than is the configuration about C(8). C(8) is an analogue of the putative tetrahedral intermediate which is formed during the enzyme-catalyzed hydrolysis of L-aminoacyl amides. C(8) bears the hydroxyl which is thought to be ligated to the activation-site metal ion of leucine aminopeptidase.[4,7-9] The bestatin analogue derived from D-leucine is 340-fold less active as an inhibitor than bestatin.

Our previous work shows that K_m is a close approximation of K_I for peptide substrates.[10] Comparison of K_m values of peptide substrates and K_I of bestatin shows that K_m values of peptidyl substrates are 200–65 000-fold higher

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(2) Presented at the 12th Congress of the International Union of Crystallography, Aug 1981, Ottawa, Canada.

(3) In this paper the optical center in the leucyl moiety is C(2). The 2*S* and 3*R* centers of the phenylalanyl moiety are C(8) and C(9), respectively. 2-Methyl-2,4-pentanediol = MPD, and leucine aminopeptidase = LAP.

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than K_I of bestatin; thus bestatin is more tightly bound to leucine aminopeptidase than any other compound studied to date.[10,11] Because the function of enzymes is to stabilize the transition state, it is plausible that the structure of this transition-state analogue is a reflection of the architecture of the active site of leucine aminopeptidase, and information regarding the structure of bestatin should be useful in our studies of the mechanism of action of this metalloprotease.[9,10,12,13]

Experimental Section

Synthesis and Crystallization. Bestatin was synthesized according to the method of Nishizawa et al.,[4] except that the hydrolysis of the cyanohydrin proceeded for 2–4 h. Samples of bestatin also were kindly provided by Dr. Umezawa. 2-Methyl-2,4-pentanediol (MPD)[3] was from Aldrich. Crystals of bestatin were obtained as follows. An aqueous solution of bestatin at 5 mg/mL was brought to 10% v/v with MPD. Large droplets of this solution were placed on siliconized slides and the slides were suspended above a bath of 20% v/v MPD. The complete setup was contained in a sealed crystallizing dish. Hexagonal plate crystals appeared after 2–5 days at room temperature.

Crystal data: $C_{16}H_{24}N_2O_4 \cdot C_6H_{14}O_2 \cdot 2H_2O$; orthorhombic, $P2_12_12_1$; a = 6.6530 (9), b = 15.1504 (30), c = 27.3088 (35) Å; V = 2752.61 Å^3 ($\lambda_{CuK\alpha}$ = 1.5418 Å at 18 °C); Z = 4 (one formula unit per asymmetric unit); d_{calcd} = 1.12 g cm^{-3}, d_{measd} (by flotation in cyclohexane) = 1.18 g cm^{-3}; absorption coefficient μ (Cu Kα) = 7.030 cm^{-1}.

Data Collection and Refinement. Zero and first level precession photographs showed systematic extinctions consistent with space group $P2_12_12_1 = D_2^4$ with Z = 4. The cell dimensions were determined by a least-squares fit of the observed 2θ angles for 22 reflections centered automatically.

Three-dimensional intensity data were measured on an Enraf-Nonius CAD-4 diffractometer using Cu Kα radiation monochromatized by reflection from a highly oriented graphite monochromator. The data crystal was a transparent, diamond-shaped plate of approximate dimensions 0.70 × 0.60 × 0.20 mm. It was mounted inside a glass capillary along its longest dimension $\{1\bar{1}0\}$. Data were collected by θ–2θ scans to 2θ(Cu Kα) = 156°. Two complete equivalent forms of data (hkl and $hk\bar{l}$) were measured, and absorption corrections were applied to 6925 observations by using BNLABS, a local version of ORABS.[14] The minimum and maximum corrections to F_o^2 were 0.5894 and 0.8574, respectively, with an agreement between symmetry equivalent intensities of R = 0.029, where $R = \sum_{hkl}|\sum_{i=1}^{n}|F_{o_i}^2 - \langle F_o^2\rangle|| / \sum_{hkl} n|\langle F_o^2\rangle|$. These intensities were averaged to give 3333 independent structure amplitudes with $F_o^2 > 3\sigma_{count}(F_o^2)$, where $\sigma(F_o^2)$ is based on Poisson counting statistics. The intensities of three monitor reflections were measured periodically; total decay during exposure was approximately 13%. Data were scaled to these monitors. Background was measured on one-fifth of the total scan width, and normal scans which did not result in sufficiently high precision on net intensity measurements were repeated at a slower scan speed. The takeoff angle was 3.2°, and the diffracted beam was automatically corrected for coincidence losses.

Structure factors were derived in the usual way, and the program MULTAN[15] was used to determine phases from which an E map revealed the coordinates of nearly three-fourths of the bestatin molecule. The remaining nonhydrogen atoms, including those of solvent molecules, were readily located by a combination of differential synthesis refinement[16] and Fourier methods. This procedure revealed the presence of two water molecules and a solvent molecule, MPD, in addition to bestatin. The methyl groups and the methylene carbon, C(19), of the MPD solvent were found to be disordered. However, only C(19) could be accommodated by assigning the populations of the two positions in the ratio 0.67:0.33. The three methyl groups were simply allowed large isotropic thermal parameters. All 24 hydrogens of the bestatin, three of the water hydrogens, and one hydroxy hydrogen of MPD were located by difference maps and finally placed at idealized positions. The 28 hydrogens were given isotropic thermal parameters of 5.5 Å^2 and were added in a fixed manner to subsequent structure factor calculations. Atomic scattering factors were taken from a standard source,[17] while that for hydrogen was the best spherically averaged value of Stewart et al.[18] In the final least-squares calculations the function minimized was $\sum w\Delta^2$, where $\Delta = |F_o| - |F_c|$, $w = 4F_o^2/\sigma^2(F_o^2)$, and $\sigma^2(F_o^2) = \sigma_{count}^2(I) + (0.03F^2)^2$. $\sigma_{count}^2(I)$ is the variance based on counting statistics. Because of the large number of variables involved, each complete cycle of refinement consisted of two parts: the first included refinement of the water molecules only and the second included the remainder of the structure. The final least-squares calculations gave values for eq 1 and 2 of 0.084 and 0.112, respectively, and

$$R_1 = \sum||F_o| - |F_c|| / \sum|F_o| \quad (1)$$

$$R_2 = [[\sum w||F_o| - |F_c||^2] / \sum w|F_o^2|]^{1/2} \quad (2)$$

the error in an observation of unit weight was 4.13. The extinction coefficient refined to 0.73 with the (002) reflection requiring the maximum correction (0.34). The maximum density in a final difference electron density synthesis was 0.44 electron Å^{-3}. Final parameters are presented in Tables I and II (see paragraph concerning supplementary material at the end of the paper). The structure amplitudes are available from the authors.

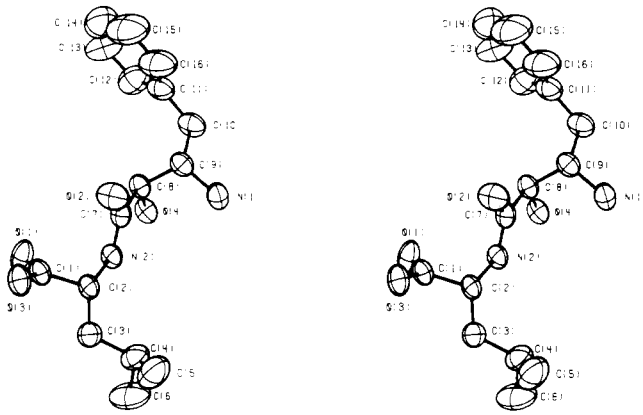

Figure 1. Stereodrawing of the bestatin molecule shown with 50% probability ellipsoids.

Discussion

The formal structure and numbering system for the bestatin molecule are given in Figure 1. The vibrational thermal ellipsoids are shown at 50% probability and appear reasonable on the basis of the data in Table I. A stereodrawing of the packing is given in Figure 2 (see paragraph concerning supplementary material at the end of the paper). In this figure is shown the hydrogen-bonding network among water molecules, bestatin, and the MPD solvent. The intermolecular hydrogen bond (2.67 Å) between O(3) and O(4) of adjacent molecules is not shown. The MPD is drawn with two positions for the (disordered) methylene C(19) atom. From the positional parameters

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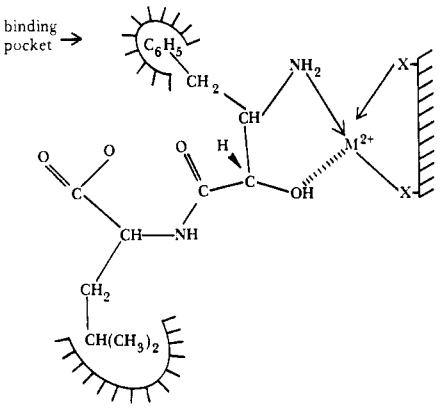

Figure 3. Proposed mode of binding of bestatin to LAP, adapted from Nishizawa et al.[4]

and correlation matrix, the relevant interatomic distances and bond angles and their standard deviations were calculated and are presented in Table III (see paragraph concerning supplementary material at the end of the paper).

The crystal structure consists of individual bestatin molecules, two water molecules, and an MPD solvent molecule. The MPD molecule appeared disordered with two positions for C(19) and very large isotropic thermal parameters for the three methyl groups. The populations of C(19) and C(19′) were found to be in the ratio of (0.67:0.33), but no alternate positions were found for the methyl groups, and these were refined as occupying single positions and were allowed large isotropic motion.

The structure indicates that the nonpolar phenylalanyl and leucyl side chains of bestatin are oppositely disposed in space and separated by ~10 Å. The peptide bond is trans. It is unlikely that there is hydrogen bonding between the OH on C(8) and the adjacent carbonyl. On the assumption that the conformation of crystalline bestatin is similar to that which is found when it is at the active site, these data suggest possible modes of binding of this transition-state analogue to leucine aminopeptidase (Figure 3); they permit further description of the substrate binding site on the enzyme and should be useful in our ongoing crystallographic studies of the enzyme.[9,10,12,13]

In order to be productively bound at the active site of leucine aminopeptidase, a substrate must have an unprotonated α-amino group and an amide or ester linkage between the residue bearing the α-amino group and the penultimate residue.[19,20] For peptide substrates K_m is a reasonable approximation of the dissociation constant, K_I.[10] In general, K_m values of dipeptide substrates are lower with increasing bulk of both the amino and carbonyl terminal residues. Rates of peptide hydrolysis are most rapid if the amino terminal residue is bulky and nonpolar; however, maximal rates of hydrolysis are not necessarily linked to low K_m values.[10,11,19,20]

In addition to bulky nonpolar side chains, an amino terminus, and a peptide bond, bestatin has a hydroxyl group on a tetrahedral carbon, C(8). In peptide substrates this position is usually occupied by the scissile carbonyl which in the normal proteolytic process presumably passes through the putative tetrahedral transition-state intermediate. Since bestatin contains all the elements necessary for substrate binding as well as elements of the tetrahedral transition state, tight binding of bestatin at the active site of leucine aminopeptidase is expected. This is supported by the data of Suda et al.,[5] which demonstrate that bestatin is a competitive inhibitor, $K_I = 2 \times 10^{-8}$, of hydrolysis of leucyl-β-naphthylamide by hog kidney leucine aminopeptidase.

The crystallographic data indicate that there is close to maximal separation between the bestatin side chains. Kinetic tests indicate that the third residue of tripeptide substrates also affects hydrolytic rates significantly.[10,11,20] Taken together, these data indicate that the region of the enzyme which is involved in substrate binding is quite extended, and it is probable that many bonds between the substrate residues and the enzyme are involved in inducing the enzyme configuration necessary for maximal rates of hydrolysis of physiological substrates.

In the LAP–bestatin binding scheme it is proposed that the C(8) hydroxyl of bestatin takes the place on the enzyme surface of the carbonyl oxygen of usual peptide substrates and that this hydroxyl oxygen is liganded to the metal ion at the activation site.[4,7,21] Our recent NMR study of the mode of substrate binding indicates that direct liganding of the activation site metal ion to the scissle carbonyl is possible; therefore, the direct interaction of the bestatin C(8) hydroxyl with the Zn^{2+} is plausible.[4] Thus, it is suggested that bestatin is more tightly bound to LAP than dipeptides because the bestatin tetrahedral C(8) is a closer reflection of the active site than the analogous planar carbon found in dipeptide substrates and because, as compared with dipeptides, bestatin can ligate the activation site Zn^{2+} with some combination of the C(8) hydroxyl as well as the carbonyl oxygen and the α-amino nitrogen. Enhanced substrate–metal ion ligation is particularly intriguing if "zinc hydroxide" mechanisms or mechanisms which invoke simultaneous ligation of substrate and water are considered.[22,23]

Given that dipeptides with N-terminal D amino acids appear to be poorly bound to LAP[11] and are not cleaved[19] by the enzyme, the effect on binding of configuration about C(9) on bestatin remains to be explained.

Registry No. Bestatin MPD dihydrate, 81790-56-9; leucine aminopeptidase, 9001-61-0.

Supplementary Material Available: Atomic coordinates, bond distances, and angles (Tables I–III) and a stereoview of the packing in a unit cell (8 pages). Ordering information is given on any current masthead page.

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