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THE ABSOLUTE CONFIGURATION OF BELACTIN A, A β-LACTONE-CONTAINING SERINE CARBOXYPEPTIDASE INHIBITOR: IMPORTANCE OF THE β-LACTONE STRUCTURE FOR SERINE CARBOXYPEPTIDASE INHIBITION

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The absolute configuration of belactin A, a β-lactone-containing serine carboxypeptidase inhibitor was studied by a crystal X-ray diffraction analysis and its absolute structure was determined to be (2*R*,3*S*)-2-{(3*S*)-3-[(2-amino-5-chlorophenyl)carboxamido]-1,1-dimethyl-2-oxobutyl}-3-methyl-4-oxooxetane. The importance of the β-lactone structure for inhibitory activity was found by preparing several derivatives of belactin A.

Keywords: Serine carboxypeptidase; Carboxypeptidase Y; Enzyme inhibitors; β-lactone; X-ray diffraction; Absolute structure

Abbreviations: BZ, benzoyl; FAB-MS, fast atom bombardment mass spectrometry; TLC, thin layer chromatography; NMR, nuclear magnetic resonance

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INTRODUCTION

Carboxypeptidase-Y[1] (CP-Y) is well known among the serine carboxypeptidases, which are widely distributed in higher organisms[2–4] and have different substrate specificities from metallocarboxypeptidases, such as CP-A,CP-B[5] or CP-N.[6] Serine carboxypeptidases have a Ser–His–Asp catalytic triad as found in the trypsin and chymotrypsin families of endopeptidase.[7] Gene product of KEX1 of yeast,[8] CP-W of wheat[9] and platelet deamidase of human[3] are also classified as serine carboxypeptidases, and it was recently reported that CP-Y-like exopeptidase, which is characterized as a serine carboxypeptidase, mainly contributed to the degradation of bradykinin in rat urine.[10,11] Irrespective of its practical usage, such as in the sequencing of carboxy-terminal amino acids or chemical reactions,[12–14] serine carboxypeptidase is one of the physiologically unknown peptidase families and detailed studies concerning serine carboxypeptidases with specific inhibitors need to be conducted.

In order to understand the physiological roles of these enzymes or to find a useful tool for their biochemical, cellular or pharmaceutical study, we searched for inhibitors in microbial products[15] and discovered belactins A and B. These are new β-lactone-containing inhibitors of serine carboxypeptidase, the structures of which were determined as 2-{3-[(2-amino-5-chlorophenyl)carboxyamido]-1,1-dimethyl-2-oxobutyl}-3-methyl-4-oxooxetane and 2-{3-[[2-(β-glucopyranosylamino)-5-chlorophenyl]carboxamido]-1,1-dimethyl-2-oxobutyl}-3-methyl-4-oxooxetane respectively, from the fermentation broth of *Saccharopolyspora* sp. MK19-42F6. These compounds have been characterized as selective inhibitors of CP-Y.[16,17]

In this communication we report a crystal X-ray diffraction analysis of belactin A, a β-lactone-containing serine carboxypeptidase inhibitor, and the inhibitory activities of several belactin A derivatives against CP-Y.

MATERIALS AND METHODS

Chemicals

Chemicals employed were as follows; carboxypeptidase Y (CP-Y, EC 3.4.16.1) from yeast was obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan), TLC-plate Silica gel F254 (0.25 mm thickness) from E. Merck (Darmstadt, FRG) and benzoyl-glycyl-L-phenylalanine (Bz-Gly-Phe)

from Sigma Chem. Ltd. (Saint Louis, USA). All other chemicals were of analytical grade.

Analytical Instruments

NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer and mass spectra were obtained using a JEOL JMS-SX102 spectrometer. The crystal X-ray diffraction analysis was performed on a Rigaku AFC5R diffractometer.

Preparation of *N*-acetylbelactin A

Belactin A (5.0 mg) was mixed with acetic anhydride (0.02 ml) in pyridine (0.5 ml) at room temperature for 2 h. The reaction was terminated by adding of H_2O (0.01 ml). The product was purified by preparative silica gel TLC (toluene : ethylacetate, 2 : 1) to give *N*-acetylbelactin A (3.1 mg), Rf value = 0.29. The molecular weight was 394 by FAB-MS (FAB positive, 395) and the chemical shifts in ^1H NMR ($CDCl_3$) were as follows.

δ 10.73 (1H, br s), 8.57 (1H, d, 8.8 Hz), 7.45 (1H, d, 2.4 Hz), 7.43 (1H, dd, 8.8 and 12.4 Hz), 6.73 (1H, br d, 7.3 Hz), 5.17 (1H, dq, 7.3 and 6.8 Hz), 4.40 (1H, d, 4.4 Hz), 3.48 (1H, dq, 4.4 and 7.6 Hz), 2.18 (3H, s), 1.45 (3H, d, 7.6 Hz), 1.44 (3H, d, 6.8 Hz), 1.43 (3H, s) and 1.34 (3H, s).

Preparation of Belactinic Acid and Belactinic Acid Methylester

Belactin A (10.0 mg) was treated with 2 N HCl (1.0 ml) in MeOH (1.0 ml) at 60°C for 16 h. The product was evaporated and purified by preparative silica gel TLC (toluene : ethylacetate, 2 : 1) to give belactinic acid methylester (belactin A, ring open methylester derivative, 5.5 mg); belactinic acid (belactin A, ring open acid derivative, 1.7 mg) was given by another preparative silica gel TLC ($CHCl_3$: MeOH, 3 : 1) from the same reaction. The physicochemical properties of belactinic acid and belactinic acid methylester were as follows.

The Rf values of belactinic acid and belactinic acid methylester on silica gel TLC were 0.57 ($CHCl_3$: MeOH : H_2O, 65 : 25 : 2) and 0.32 (toluene : ethylacetate, 2 : 1), and the molecular weights were 370 (FAB negative, 369) and 384 (FAB positive, 385) respectively. The ^1H NMR of belactinic acid (DMSO-d_6) was: δ 8.37 (1H, d, 7.3 Hz), 7.60 (1H, d, 2.4 Hz), 7.15 (1H, dd,

2.4 and 8.8 Hz), 6.70 (1H, d, 8.8 Hz), 6.44 (2H, br s), 4.96 (1H, dq, 7.3 and 6.8 Hz), 3.51 (1H, br s), 2.01 (1H, m), 1.20 (3H, d, 6.8 Hz), 1.18 (3H, s), 1.07 (3H, d, 7.3 Hz), 0.99 (3H, s). The ^{1}H NMR of belactinic acid methylester (DMSO-d_6) was: δ 8.51 (1H, d, 7.3 Hz), 7.60 (1H, d, 2.4 Hz), 7.17 (1H, dd, 8.8 and 2.4 Hz), 6.70 (1H, d, 8.8), 6.43 (2H, br s), 5.13 (1H, d, 7.3 Hz), 5.02 (1H, dq, 7.3 and 6.8 Hz), 3.91 (1H, dd, 7.3 and 6.8 Hz), 3.56 (3H, s), 2.58 (1H, quintet, 6.8 Hz), 1.26 (3H, d, 6.8 Hz), 1.17 (3H, s), 1.16 (3H, s), 0.98 (3H, d, 6.8 Hz).

Assay for CP-Y Inhibitory Activities

The CP-Y carboxypeptidase activity was determined according to the method reported previously.[16,18] Briefly the reaction mixture (total 0.1 ml) for CP-Y consisted of 25 mM sodium phosphate buffer (pH 6.5), 2 μg/ml CP-Y, 1 mM Bz-Gly-Phe and water or aqueous solution containing the test compound. The reaction was started by adding the substrate solution (0.01 ml), followed by incubation at 37°C for 40 min, after which 6 μl of 1 N NaOH was added to terminate the reaction. Ten minutes later, 0.05 ml of 0.36 M sodium phosphate buffer (pH 7.2) and 0.15 ml of 2% (W/V) cyanuric chloride, freshly dissolved in 2-methoxyethanol, were added to the mixed solution. The absorbance at 405 nm was measured with a microplate reader model 3550 (BIO-RAD). The percent inhibitions were calculated by the formula $(A - B)/A \times 100$, where A is the value obtained in the enzymatic assay without an inhibitor and B is that with an inhibitor. The IC_{50} value, which is, the concentration of inhibitor at 50% inhibition, was measured graphically from two independent experiments.

RESULTS

Absolute Structure of Belactin A

Belactin A was recrystallized from hexane–acetone solution and colourless needles were obtained. A colourless crystal having approximate dimensions of 0.3 × 0.04 × 0.03 mm was mounted on a glass fibre and the measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated CuKα radiation and a 3 kW rotating anode generator. Cell constants were $a = 9.451(5)$ Å, $b = 5.963(5)$ Å, $c = 15.550(3)$ Å, $V = 875.3(8)$ Å^3, $\beta = 92.79(2)°$, $Z = 2$, formula weight = 352.82 and calculated density was

1.34 g/cm^3. Based on the systematic absences of: $0k0$ when $k \neq 2n$, the space group was determined to be $P2_1$(#4). The data was collected at room temperature using ω–2θ scan technique to a maximum 2θ value of 120.2°. Omega scan of several intense reflections, made prior to data collection, had an average width at half-height of 0.29° with a take-off angle of 6.0°. Scans of $(1.26 + 0.30 \tan \theta)°$ were made at a speed of 4.0°/min (in ω). Of the total 1512 reflections, 1454 were unique ($R_{int} = 0.080$). The intensities of three representative reflection were measured after every 150 reflections and no decay correction was applied. The linear absorption coefficient, μ, for CuKα radiation was 21.4 cm^{-1}. Azimuthal scans of several reflections indicated no need for an absorption correction. The data were corrected for Lorentz and polarization effects.

The structure was solved by direct methods[19] and expanded using Fourier techniques.[20] All calculations were performed on a VAX II using the TEXSAN crystallographic software package of Molecular Structure Corporation (1985, 1992). The non-hydrogen atoms were refined anisotropically and the hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 486 observed reflections ($I > 3.00\ \sigma\ (I)$) and 217 variable parameters, and the parameter refinement converged at $R = \Sigma||F_o| - |F_c||/\Sigma|F_o| = 0.051$, $Rw = \{\Sigma w(|F_o| - |F_c|)^2/\Sigma w F_o^2\}^{1/2} = 0.035$, S (standard deviation of an observation of unit weight) $= \{\Sigma w(|F_o| - |F_c|)^2/(N_o - N_v)\}^{1/2} = 1.69$ where N_o = number of observations, N_v = number of variables. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.20 and $-0.21\ e^-/Å^3$ respectively. A correction for secondary extinction was applied (coefficient = $3.01578\ e^{-8}$).

Neutral atom scattering factors were from Cromer and Waber.[21] Anomalous dispersion effects were included in F[22] and the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley.[23] The values for the mass attenuation coefficients were those of Creagh and Hubbel.[24] The absolute configuration was determined by the Bijvoet's method[25] using the anomalous dispersion of Cu radiation by chroline atom. The intensities of 12 most enantiomer-sensitive Friedel pairs were measured (data not shown). From the agreement of intensity differences of Friedel pairs with those of the absolute configuration shown in Figure 1, we concluded that the absolute structure of Belactin A was (2*R*,3*S*)-2-{(3*S*)-3-[(2-amino-5-chlorophenyl)-carboxamido]-1,1-dimethyl-2-oxobutyl}-3-methyl-4-oxooxetane. The crystal structure data, atomic parameters, anisotropic displacement parameters, bond lengths, bond angles, torsion angles and other parameters will be lodged in and can be obtained from the Cambridge Crystallographic Database.

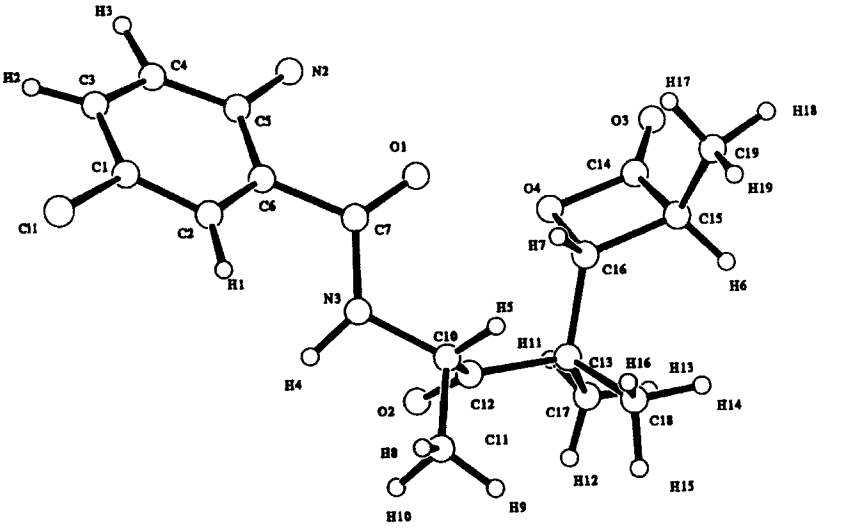


FIGURE 1 A PLUTO plot of the molecular structure of belactin A determined by X-ray diffraction analysis. ^{1}H NMR data[17] ($CDCl_3$) for the labelled protons of the belactin A structure are as follows; H1 (δ 7.32, d, 2.4), H2 (δ 7.16, dd, 8.8 and 2.4), H3 (δ 6.61, d, 8.8), H4 (δ 6.50, br d, 7.3), H5 (δ 5.19, dq, 7.3 and 6.8), H6 (δ 3.48, dq, 4.4 and 7.3), H7 (δ 4.40, d, 4.4), H8, 9, 10 (δ 1.40, d, 6.8), H11, 12, 13 or H14, 15, 16 (δ 1.34 or 1.43, s), H17, 18, 19 (δ 1.44, d, 7.3).

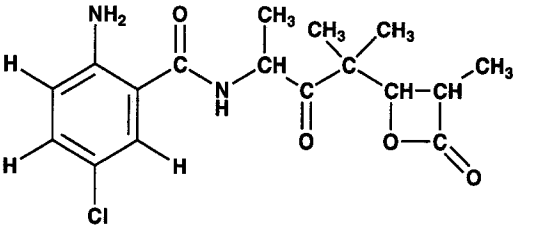
Inhibitory Activities of Belactin A Derivatives Against CP-Y

Belactinic acid and belactinic acid methylester, ring opened-derivatives of belactin A (see Figure 2), were obtained by treatment with HCl containing methanol, followed by preparative silica gel TLC using the solvent systems of $CHCl_3$–MeOH (3 : 1) and toluene–ethylacetate (2 : 1) respectively. These ring opened-derivatives were found to lose most of the inhibitory activities of the parent compound against CP-Y. On the other hand, *N*-acetyl belactin A, a derivative which has β-lactone moiety, retained its inhibitory activity as shown in Table I. The above results show that the β-lactone moiety of belactin A is essential for CP-Y inhibitory activity.

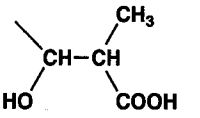
DISCUSSION

We discovered new serine carboxypeptidase inhibitors, belactins A and B in the fermentation broth of *Saccharopolyspora* sp. MK19-42F6 and determined their structures by various spectral methods and also characterized them as selective inhibitors of serine carboxypeptidase, as reported in previous papers.[17,16] Here, we performed a crystal X-ray diffraction analysis of

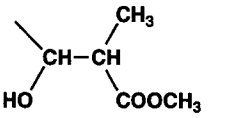
Belactin A



Belactinic acid



Belactinic acid methylester



N-acetyl belactin A

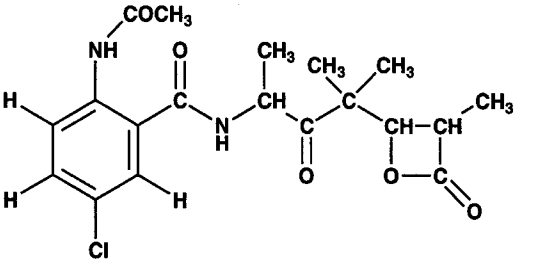


FIGURE 2 Belactin A and its derivatives.

TABLE I Inhibitory activity of belactin A derivatives against CP-Y

Compounds	IC_{50} (μg/ml)*
Belactin A	0.18
N-acetylbelactin A	0.05
Belactinic acid	100
Belactinic acid methylester	> 100

*Bz-Gly-Phe, 1 mM.

belactin A and determined its absolute configuration. The results from the inhibitory characterizations of belactin A derivatives showed that its β-lactone moiety was essential for activity. In the previous experiments, the coupling constant of 4.4 Hz between the vicinal methine protons at δ 3.48 (H6) and δ 4.40 (H7) revealed belactin A should have the 2*R*,3*S*- or 2*S*,3*R*-β-lactone moiety configuration, as discussed by Kondo *et al.*[26] From the crystal X-ray diffraction analysis of belactin A, it was clarified that belactin A had the 3*S*, 2*R*, 3*S* absolute configuration and the 2*R*,3*S*-β-lactone moiety of belactin A resulted in the same configuration as the ebelactones and esterastin (2*S*, 3*S*, Figure 3), which are non-selective β-lactone-containing

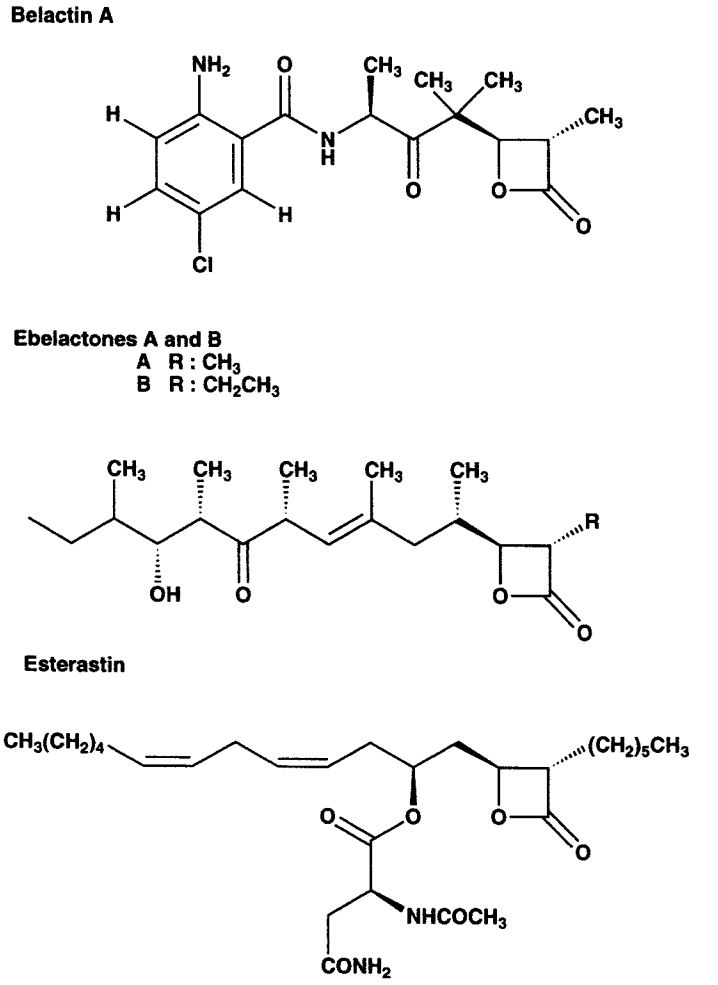


FIGURE 3 Belactin A and other β-lactone-containing inhibitors.

inhibitors of CP-Y and lipase.[27,28] In the case of lipase, the β-lactone moiety including ebelactone-type-configuration was reported to be important for the inhibitory activity, whereas it remains uncertain whether the activity of β-lactone inhibitors against CP-Y needs the ebelactone-type-configuration in the β-lactone moiety and it is also uncertain what difference selective and non-selective compounds bring about in the specificity of belactins against CP-Y and lipase. Therefore, a more detailed structure–activity relationship of belactins against CP-Y remains to be performed.

During the detailed characterization of CP-Y inhibition by belactins A and B, these compounds were found to be non-competitive and slow-binding type inhibitors which interact with CP-Y according to the one-complex model and consequently give a conformational change of the enzyme, such as in the tryptophane or tyrosine residue in the CP-Y molecule (unpublished data). In order to clarify the molecular mechanism of CP-Y inhibition by belactins, X-ray diffraction analysis of the crystalline complex of CP-Y and belactins is thought to be an important approach which will provide significant information for the design of more specific and potent inhibitors of serine carboxypeptidase.

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