J. Enzyme Inhibition, 1995, Vol. 9, pp. 277–284
Reprints available directly from the publisher
Photocopying permitted by license only

BELACTINS A AND B, NEW SERINE CARBOXYPEPTIDASE INHIBITORS PRODUCED BY ACTINOMYCETE. II. PHYSICO-CHEMICAL PROPERTIES, STRUCTURE DETERMINATIONS AND ENZYMATIC INHIBITORY ACTIVITIES COMPARED WITH OTHER ß-LACTONE CONTAINING INHIBITORS

SHOICHI MURAKAMI,[1] YOSHIKAZU TAKAHASHI,[1] HIROSHI NAGANAWA,[1] TOMIO TAKEUCHI[1] and TAKAAKI AOYAGI[1,2,*]

[1]*Institute of Microbial Chemistry, 3–14–23 Kamiosaki, Sinagawa-ku, Tokyo 141, Japan*

[2]*Showa College of Pharmaceutical Sciences, 3–3165 Higashitamagawagakuen, Machida-shi, Tokyo 194, Japan*

(Received 4 April 1995)

Belactins A and B, new inhibitors of serine carboxypeptidase were discovered in the fermentation broth of *Saccharopolyspora* sp. MK19–42F6. The structures of belactins A and B were determined to be 4-[3-[(2-amino-5-chlorobenzoyl)amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone and 4-[3-[[2-(β-glucopyranosylamino)-5-chlorobenzoyl]amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone respectively by various spectral analyses. Belactins A and B do not inhibit esterase or lipase at 100 μg/ml but have more specific inhibitory activities towards carboxypeptidase Y (CP-Y) compared with other β-lactone-containing inhibitors, such as ebelactones A, B and esterastin.

KEY WORDS: Serine carboxypeptidase, carboxypeptidase Y, enzyme inhibitors, β-lactone, structure determination, natural products

INTRODUCTION

In the preceding paper[1] we have described the taxonomy, the production, the purification and the biological properties of belactins A and B, new inhibitors of serine carboxypetidase.

*Correspondence.

ABBREVIATIONS: FAB-MS, fast atom bombardment mass spectrometry; DEPT, distortionless enhancement by polarization transfer; COSY, correlated spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; Bz, benzoyl.

In this communication we report physico-chemical properties, structure determinations and enzymatic inhibitory activities against esterase and lipase of belactins A and B.

MATERIALS AND METHODS

Chemicals

Chemicals employed were as follows: TLC-plate Silica gel F254 (0.25 mm thickness) from E. Merck, Darmstadt, FRG; benzoyl-glycyl-L-phenylalanine (Bz-Gly-Phe) from Peptide Institute Inc., Minoh-shi, Japan; *p*-nitrophenyl acetate (PNP acetate) 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. UV spectra were recorded on a Hitachi U-3210 spectrometer. IR spectra were measured on a Hitachi Model 260–10 spectrometer. Optical rotations were determined using a Perkin-Elmer 241 polarimeter. Melting points were measured on a Yanaco melting point apparatus MP500D.

Enzymes

Carboxypeptidase Y (CP-Y, EC 3.4.16.1) from yeast was obtained from Oriental Yeast Co. Ltd., Japan. Esterase from hog liver were purchased from Boehlinger Mannheim GmbH, F.R.G., Germany. Lipase from hog pancreas was obtained from Nutritional Biochem Co., USA.

Assays of Enzymes and Inhibitory Activities

CP-Y activities were measured by the method reported in the preceding paper.[1] The reaction mixture (total 0.1 ml) for CP-Y consisted of 25 mM sodium phosphate buffer (pH 6.5), 1 mM Bz-Gly-Phe, 2 μg/ml enzyme, and water or aqueous solution containing the test compound. The activities of lipase and esterase were determined from the absorbance at 405 nm of *p*-nitrophenol liberated in these enzyme reactions. In case of lipase the reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0) containing 0.125 mM PNP acetate, 0.03% triton X-100, 25 μg/ml partially purified enzyme, and water or aqueous solution containing the test compound. The enzyme reaction was started by addition of the enzyme, followed by incubation at 37°C for 60 min and the absorbance at 405 nm was then determined without terminating the reaction. Esterase inhibitory activities were measured according to the same procedure as lipase except that 0.4 μg/ml enzyme (esterase from hog liver) was used and the incubation time was 30 min. The absorbance at 405 nm was measured with a microplate reader model 3550 (BIO-RAD).

TABLE 1
Physico-chemical properties of belactin A.

Appearance	Slightly yellowish solid
Melting point	42.5~44.5°C
$[\alpha]_D^{25}$ (c 0.5, $CHCl_3$)	–30.2°
FAB-MS (positive, m/z)	$353(M+H)^+$
FAB-MS (negative, m/z)	$351(M-H)^-$
Molecular weight	352
Elemental analysis	Found: C, 57.62; H, 6.03; Cl, 9.79; N, 7.96%
	Calcd for $C_{17}H_{21}ClN_2O_4$: C, 57.87; H, 6.00; Cl, 10.05; N, 7.94%
Molecular formula	$C_{17}H_{21}ClN_2O_4$
UV λ max nm (ε) in MeOH	213(22,000) 256(9,300) 340(3,500)
IR ν max cm^{-1}	3480, 3360, 2990, 2950, 2890, 1820, 1710, 1650, 1620, 1580, 1480, 1460, 1420, 1380, 1320, 1260, 1160, 1140, 1060, 1010, 990, 940, 910, 880, 830, 790, 720, 690
Rf value on TLC	0.45 (Toluene-EtOAc, 2/1, Silica gel)
Color reaction	Mo-H_2SO_4, Greig-Leaback, 2,4-D
Solubility	Soluble: EtOAc, $CHCl_3$, MeOH, benzene

TABLE 2
Physico-chemical properties of belactin B.

Appearance	White powder
Melting point	150.0~153.0°C.
$[\alpha]_D^{23}$ (c 0.5, $CHCl_3$)	–87.0°
FAB-MS (positive, m/z)	$515(M+H)^+$
FAB-MS (negative, m/z)	$513(M-H)^-$
Molecular weight	514
Molecular formula	$C_{23}H_{31}ClN_2O_9$
UV λ max nm (ε) in MeOH	215(22,300) 257(13,300) 337(4,050)
IR ν max cm^{-1}	3400, 2990, 2950, 2890, 1820, 1710, 1640, 1580, 1520, 1480, 1460, 1420, 1390, 1380, 1360, 1320, 1270, 1180, 1130, 1080, 1060, 1020, 940, 910, 880, 820, 740
Rf value on TLC	0.42 ($CHCl_3$-MeOH-H_2O, 100/20/1, Silicagel)
Color reaction	Mo-H_2SO_4, Greig-Leaback, 2,4-D
Solubility	Soluble: EtOAc, acetone, CH_3CN, MeOH

RESULTS AND DISCUSSION

Physico-chemical Properties of Belactins

The physico-chemical properties of belactins are summarized in Tables 1 and 2. The molecular formula of belactins were determined by FAB-MS, ^{13}C NMR spectra and elemental analysis. The IR spectra of both compounds indicated that the presence

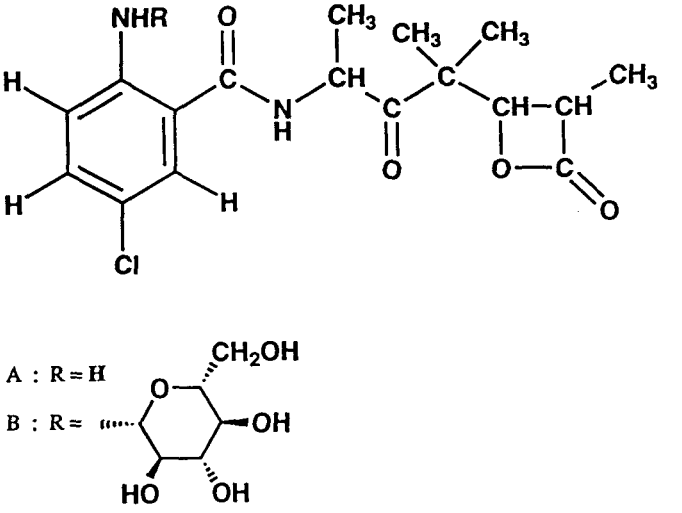


FIGURE 1 Structures of belactins A and B.

of an hydroxyl or amino group (~3400 cm^{-1}), β-lactone group (~1820 cm^{-1}), ketone group (~1710 cm^{-1}) and amide group (~1650 and ~1580 cm^{-1}). Belactins gave positive color reactions to Greig-Leaback, Phosphomolybdate-H_2SO_4 (Mo-H_2SO_4) and 2,4-dinitrophenylhydrazine (2,4-D).

Structure Determination of Belactin A (Figure 1)

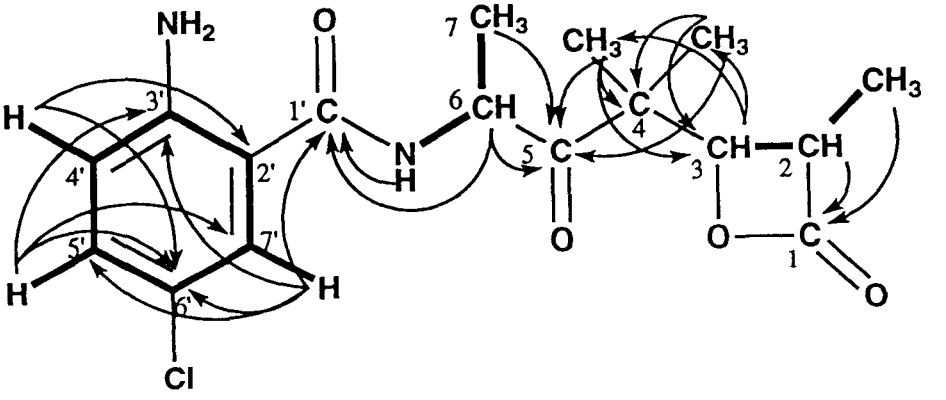
The molecular formula of belactin A was established as $C_{17}H_{21}ClN_2O_4$ by FAB-MS and elemental analysis. All seventeen carbons were detected in the ^{13}C NMR spectrum of this compound in $CDCl_3$ (Table 3). The DEPT spectra established the presence of four methyls and six methines, all of which were confirmed by HSQC spectrum. The ^1H NMR spectrum showed three exchangeable proton signals. The partial structures were deduced through ^1H-^1H COSY spectrum as shown in Figure 2.

The coupling constants of 8.8 Hz and 2.4 Hz among the three aromatic protons (δ6.61, d, 4′-H; δ7.16, dd, 5′-H; δ7.32, d, 7′-H) show *ortho* and *meta* coupling of benzene derivatives respectively. The connection of the partial structures and the carbons bearing no protons; quarternary (C-4), carbonyl carbons (C-1, C-5 and C-1′) and substituted aromatic carbons (C-2′ C-3′ and C-6′) were determined by the HMBC spectrum as shown below. The methine proton at δ3.48 (2-H) and the methyl protons at δ1.44 (2-CH_3) were coupled to the carbonyl carbon at δ171.3 (C-1), which forms a β-lactone with the oxygen attached to the carbon at δ81.9 (C-3). The presence of β-lactone was supported by the IR spectrum of belactin A. The methyl

TABLE 3
^{13}C and ^{1}H NMR data of belactin A in $CDCl_3$.

Position	δ_c ppm	M	δ_H ppm (J=Hz)
1	171.3	s	—
2	47.1	d	3.48 dq(4.4, 7.3)
2-CH_3	12.9	q	1.44 d(7.3)
3	81.9	d	4.40 d(4.4)
4	49.1	s	—
4-CH_3(a)	18.9	q	1.34 s
4-CH_3(b)	19.9	q	1.43 s
5	231.1	s	—
6	49.1	d	5.19 dq(7.3, 6.8)
6-NH			6.50 br d(7.3)
7	18.4	q	1.40 d(6.8)
1′	167.2	s	
2′	115.2	s	
3′	147.4	s	
3′-NH_2			5.48 br s
4′	118.6	d	6.61 d(8.8)
5′	132.6	d	7.16 dd(8.8, 2.4)
6′	121.0	s	
7′	126.9	d	7.32 d(2.4)

M = multiplicity on the basis of DEPT spectrum.



— Connectivities observed in the ^{1}H-^{1}H COSY spectrum

↷ ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum

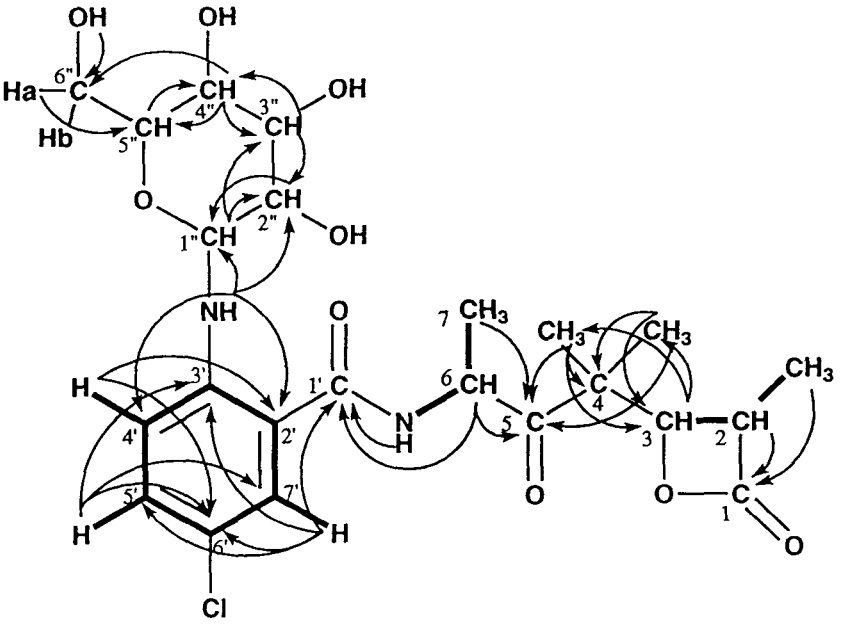
FIGURE 2 Structure of belactin A elucidated by ^{1}H-^{1}H COSY and HMBC.

protons at δ1.34 (4-CH_3(a)) and 1.43 (4-CH_3(b)) were coupled to the quatenary carbon at δ49.1 (C-4). Both the methyl protons were also coupled to C-3 and the carbonyl carbon at δ213.1 (C-5), and the methine proton at δ4.40 (3-H) were coupled to the methyl carbons at δ18.9 (C-4–CH_3(a)) and δ19.9 (C-4–CH_3(b)). The methine proton at δ5.16 (6-H) and the methyl protons at δ1.40 (7-CH_3) were coupled to C-5. The long range corrrelations from 6-H, the amino proton at δ6.50 (6-NH) and the aromatic proton at δ7.32 (7′-H) to the carbonyl carbon at δ167.2 (C-1′) revealed that the substituted benzoic acid formed an amide bond.

TABLE 4
^{13}C and ^{1}H NMR data of belactin B in CD_3CN.

Position	δ_c ppm	M	δ_H ppm (J=Hz)	δ_H ppm (+D_2O, J=Hz)
1	172.9	s	—	
2	47.8	d	3.59 dq(4.2, 7.3)	3.60 dq(4.3, 7.6)
2-CH_3	13.3	q	1.35 d(7.3)	1.35 d(7.6)
3	83.2	d	4.48 d(4.2)	4.50 d(4.3)
4	49.9	s	—	
4-CH_3(a)	19.6	q	1.32 s	1.32 s
4-CH_3(b)	20.1	q	1.35 s	1.36 s
5	213.8	s	—	
6	50.9	d	5.02 quintet(7.0)	5.02 q(7.1)
6-NH			7.22 br d(7.0)	
7	17.5	q	1.33 d(7.0)	1.34 d(7.1)
1′	168.6	s		
2′	118.0	s		
3′	147.6	s		
3′-NH			8.02 br d	
4′	116.4	d	6.90 d(9.3)	6.91 d(9.0)
5′	133.4	d	7.31 dd(9.3, 2.6)	7.32 dd(9.0, 2.5)
6′	122.1	s		
7′	128.9	d	7.55 d(2.6)	7.56 d(2.5)
1″	85.0	d	4.52 dd(7.0, 8.6)	4.52 d(8.6)
2″	74.5	d	3.22 m	3.24 dd(8.6, 9.1)
3″	78.8	d	3.37 m	3.39 t(9.1)
4″	71.7	d	3.28 m	3.28 t(9.1)
5″	77.9	d	3.37 m	3.37 m
6″	62.9	t	3.56 m, 3.73 m	3.57 dd(6.3, 12.1) 3.73 dd(2.8, 12.1)
6″-OH			2.75 br t(6.2)	

M = multiplicity on the basis of DEPT spectrum.



— Connectivities observed in the ^{1}H-^{1}H COSY spectrum

↷ ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum

FIGURE 3 Structure of belactin B elucidated by ^{1}H-^{1}H COSY and HMBC.

The chemical shifts of the substituted aromatic carbons (C-2′, C-3′ and C-6′) were identified as δ115.2, 147.5 and 121.0 respectively by the HMBC spectrum as shown in Figure 2. The positions of the amino group and the chlorine atom in the benzoic acid moiety were determined by the chemical shifts of aromatic carbons at δ147.4 (C-3′) and δ121.0 (C-6′) respectively. From the above results the structure of belactin A was determined to be 4-[3-[(2-amino-5-chlorobenzoyl)amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone.

Structure Determination of Belactin B

The molecular formula of belactin B was deduced as $C_{23}H_{31}ClN_2O_9$ by FAB-MS and ^{13}C NMR spectrum. The assignments of ^{1}H and ^{13}C NMR of belactin B are shown in Table 4. The various NMR experiments in CD_3CN revealed that the aglycone of belactin B was the same as that of belactin A and that the amino group at 3′ was substituted by a carbohydrate (Figure 3). The coupling constants between the vicinal protons ($J_{1''2''}$, $J_{2''3''}$, $J_{3''4''}$ and $J_{4''5''}$) were about 9 Hz, which revealed that the carbohydrate had a six membered ring structure and all these protons were situated at the axial positions. From these results we determined the structure of

TABLE 5
Inhibitory activities of β-lactone compounds against CP-Y, esterase and lipase.

β-lactone Compounds	IC_{50} (μg/ml) value		
	Esterase	Lipase	CP-Y
Belactin A	>100	>100	0.18
Belactin B	>100	>100	0.65
Esterastin	5.0	0.2×10^{-3}	70×10^{-3}
Ebelactone A	56×10^{-3}	3.0×10^{-3}	80×10^{-3}
Ebelactone B	0.35×10^{-3}	0.8×10^{-3}	12×10^{-3}

belactin B to be 3′-N-β-glucopyranosyl-belactin A; 4-[3-[[2-(β-glucopyranosylamino)-5-chlorobenzoyl]amino]-1,1-dimethyl-2-oxobutyl]-3-methyl-2-oxetanone. The stereochemistries of the belactins remain to be elucidated.

Enzymatic Inhibitory Properties of Belactins Compared with Other β-lactone Compounds

The inhibitory activities (IC_{50}) of belactins A and B against CP-Y were determined to be 0.18 and 0.65 μg/ml respectively as reported in the preceding paper.[1] During the characterization of various enzymatic inhibitors against CP-Y, it was found that β-lactone lipase and esterase inhibitors, such as esterastin,[2,3] ebelactones A and B[4-6] inhibited CP-Y. New inhibitors belactins A and B, which were also β-lactone compounds, did not inhibit lipase from hog pancreas or esterase from hog liver but inhibited CP-Y potently (Table 5). It was considered that belactins A and B have high specificities against CP-Y because they did not inhibit other various peptidases at 100 μg/ml except that belactins inhibited prolylendopeptidase (PEP) and dipeptidylaminopeptidase I (DPP-I) weakly, as reported in the preceding paper.[1]

In conclusion belactins A and B are potent serine carboxypeptidase inhibitors, which have high specificities, and are considered to be a useful tool for biochemical, cell biological or pharmaceutical studies where carboxypeptidases are concerned.

References

1. Murakami, S., Harada, S., Kojima, F., Kinoshita, N., Takahashi, Y., Hamada, M., Takeuchi, T. and Aoyagi, T. (1995) *J. Enz. Inhib.*, in press.
2. Kondo, S., Uotani, K., Miyamoto, M., Hazato, T., Naganawa, H. and Aoyagi, T. (1978) *J. Antibiot.*, **31**, 797.
3. Umezawa, H., Aoyagi, T., Hazato, T., Uotani, K., Kojima, F., Hamada, M. and Takeuchi, T. (1978) *J. Antibiot.*, **31**, 639.
4. Majima, M., Kuribayashi, Y., Ikeda, Y., Adachi, K., Kato, H., Katori, M. and Aoyagi, T. (1994) *Jpn. J. Pharmacol.*, **65**, 79.
5. Umezawa, H., Aoyagi, T., Uotani, K., Hamada, M., Takeuchi, T. and Takahashi, S. (1980) *J. Antibiot.*, **33**, 1594.
6. Uotani, K., Naganawa, H., Kondo, S., Aoyagi, T. and Umezawa, H. (1982) *J. Antibiot.*, **35**, 1495.