Total Structures and Antimicrobial Activity of Bacitracin Minor Components

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Total structures of 13 minor components of bacitracin (BC) were proposed, and their antimicrobial activities were investigated. The components of BC including bacitracins A (BC-A) and F (BC-F) were isolated by preparative HPLC and were hydrolyzed under acidic conditions. The resulting amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide and were separated by HPLC to determine their absolute configurations. It was found that the *N*-terminal amino acids of BC-A and its related components were epimerized during the hydrolysis to yield their enantiomers. The formation of these artifactual amino acids suggests that our previously proposed structures of the BC minor components are incorrect; therefore, the structures were corrected based on these results. The structures of the BC minor components were the same as that of BCs-A and -F except that one to three of the L-isoleucines, including the *N*-terminal one, were replaced by L-valines. These structures were confirmed by tandem mass spectrometry under fast atom bombardment (FAB) conditions and Frit-FAB liquid chromatography/mass spectrometry. Based on the UV spectra of the BC components determined by photodiode array detection-HPLC analysis, a new systematic nomenclature was proposed for the minor components. The isolated components were also used for the determination of their minimal inhibition concentrations and it was found that BC-A is 2~8 times more potent than the other minor components against strains of *Micrococcus luteus* and *Staphylococcus aureus*.

Bacitracin (BC) is a peptide antibiotic produced by *Bacillus subtilis* and *B. licheniformis* that exhibits activity against Gram-positive organisms[1]. BC is a complex of many similar compounds and bacitracin A (BC-A) is a main component with the most potent activity among the components[2~5]. BC-F is a degradation product of BC-A that shows no antibiotic activity and is nephrotoxic[6,7]. The structures of the above two components were elucidated[8,9] and their biological properties are well known. However, in spite of some trials for characterization[4~6], the structures and biological properties of other minor components have remained unresolved for over 40 years. To terminate these problems, we have been carrying out a series of studies concerning the separation and characterization of BC minor components[10~14].

Since the discovery of BC in 1943, many researchers have tried to isolate and characterize its components. In 1948, Barry *et al.*[2] reported that BC is separable into 3 or more components by countercurrent distribution (CCD) and the main component yields 9 amino acids by acid hydrolysis. A few years later, Craig *et al.*[4] and Newton *et al.*[5] established new CCD conditions and separated BC into 5 and 10 components, respectively, and they named the components BCs-A, -B, -C, -D, -E, -F and -G based on the separation. The two groups competitively characterized the BC components and published many papers. Craig *et al.*[15] established the absolute configurations of constituent amino acids of BC-A and proposed a cyclic structure for these peptides. Newton *et al.*[16] suggested the presence of a thiazoline ring in the structure of BC-A. Following this, many workers reported additional structural information such as sequence data by partial hydrolysis studies of BC-A[17~29]. Based on such information, several structures were proposed[18,20,22,24,28] for BC-A one after another between 1953 and 1957 but they were all unsatisfactory. The final and well accepted structure of BC-A was proposed[8] by Ressler and Kashelikar in 1966, and the structure was partially confirmed by synthesis[30~38]. The suggestion that BC-F is a degradation product of BC-A was made by Craig *et al.*[4] who explained the conversion from BC-A to -F by oxidation of the *N*-terminal amino thiazoline moiety to a keto-thiazole[6]. The structure of BC-F has been completely confirmed by synthesis[39]. A few partial structures of other minor components have also been reported. Craig *et al.*[6] suggested that the structure of

BC-B is the same as that of BC-A except that one isoleucine residue was replaced by valine, however the exact location of this substitution was unknown. They also suggested that BC-B is transformable to a BC-F type component. On the other hand, NEWTON *et al.*[5] reported that BCs-B, -D and -E yield valines by hydrolysis and these components show the same UV absorption spectra as does BC-A, suggesting structural similarity among them. In addition, they reported that two groups of components, BCs-C and -G and BCs-F_1, -F_2 and -F_3 show the same UV spectra as each other. BCs-B, -C, -D and -E were reported to be antimicrobially active but to be less potent than BC-A[4,5,40~42].

In the 1970s, HPLC was used for the separation of BC components instead of CCD and it was demonstrated that some minor components such as BC-B were still mixtures of two or three components[41,42]. The relatively low resolution of CCD may be one of the reasons that the structures of the minor components were not yet solved. We have optimized separation conditions of BC components by thin-layer chromatography (TLC), HPLC and high-speed countercurrent chromatography (HSCCC) in previous studies. Two types of conditions were developed using different types of TLC plates[10]. These TLC methods allowed us to separate the BC components into 8~10 spots on the plates. To simply and rapidly separate the BC components, isocratic HPLC conditions were established and for the first time it was possible to separate the whole components of BC[11]. Other HPLC conditions were established for liquid chromatography/mass spectrometry (LC/MS) under a gradient elution mode using a volatile mobile phase[13]. Thus, the BC components were separated into more than 30 peaks on the HPLC chromatogram as shown in Fig. 1. Preparative separation of 50 mg of a commercial BC complex gave BCs-A and -F in a pure state within a few hours by HSCCC[12]. Although other fractions containing minor components were still mixtures, they were highly enriched by this operation. These isolated and enriched components were useful for characterization of the minor components in previous and present studies. In addition, we characterized the minor components by using Frit-FAB LC/MS[13], and the structures of 13 minor components containing BC-B were proposed[14] as follows: the minor components are classified into two groups; BC-A and BC-F related components, depending on their common *N*-terminal moieties. The structures of BC-A related components are the same as that of BC-A except that one to three of the leucine or isoleucine residues have been replaced by valines. The BC-F related components are the degradation products of the BC-A related components that have gone through the same degradation process as BC-A. This is the first time the total structures of BC minor components, except for that of BC-F, have been proposed since the discovery of BC.

In 1993, TETLER *et al.*[44] used tandem mass spectrometry (MS/MS) to characterize the structures of BC minor components under FAB conditions without any chromatographic separation and proposed the structures for BC-B which are different from ours. The following year MORRIS[45] and SIEGEL *et al.*[46] proposed the structures of BC-A related components and supported the structures of BC-B[44] proposed by TETLER *et al.*, suggesting that our previously proposed structures[14] are incorrect. The newly proposed structures are different from ours only at one substitution position of valine in the *N*-terminus. The new structures are the same as ours except that the *N*-terminal isoleucine has been replaced by valine, and leucine has not been replaced. It is considered that the discrepancy between the two proposed structures can be resolved by determining whether or not leucine can be detected in all of the components of BC.

In this paper, we reported the isolation of 12 minor components using preparative HPLC and the determination of the absolute configuration of the constituent amino acids of each isolated component. Based on the results of amino acid analysis and sequence data given by previous LC/MS analysis, the total structures of the minor components have been deduced, and the structures have been confirmed by using MS/MS and LC/MS. Furthermore, a new systematic nomenclature has been proposed for the minor components based on the UV spectra given by photodiode-array detection HPLC analysis. The minimal inhibition concentrations (MICs) of BC components against several organisms has also been measured.

Experimental

Materials

Acetonitrile, acetone, glycerol, hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), methanol, and trifluoroacetic acid (TFA) were of analytical reagent grade. Bacitracin and 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA)[47] were purchased from P-L Biochemicals (Milwaukee, WI, USA) and Tokyo Chemical Industry (Tokyo, Japan), respectively. D,L-Alloisoleucine and other standard amino acids were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Muller-Hinton II agar medium was

purchased from BBL (Cockesville, MD, USA).

Isolation and Separation of Minor Components

The isolation of the minor components was carried out by preparative HPLC. The original mixtures of the minor components, enriched BC-A and BC-F related components were used[14]. The BC-A related components were separated on a Chromatorex Phenyl HPLC column ($5\,\mu$m, 250×20.0 mm i.d.) (Fuji-Silysia Ltd., Kasugai, Japan) using acetonitrile - 0.01 M TFA aqueous solution (23 : 73) as the mobile phase at a flow rate of 10.0 ml/minute. The eluent was monitored at 234 nm, and eluents of the corresponding peaks were collected and lyophilized, after removing the acetonitrile using a rotary evaporator, to yield each isolated component. The BC-F related components were isolated in the same way as described above except that an acetonitrile - 0.01 M TFA aqueous solution (38 : 62) was used as the mobile phase. To check the purity of the isolated components, the following HPLC conditions were used: column, Chromatorex Ph ($5\,\mu$m, 250×4.6 mm i.d.); mobile phase, acetonitrile - 0.01 M TFA aqueous solution; flow rate, 1.0 ml/minute; gradient rate, acetonitrile $26 \sim 44\%$ (40 minutes, linear); detection, 234 nm. For the measurement of the UV absorption spectra of each component, a photodiode-array detector, Waters 991J (Millipore Co., Milford, MA, USA) was used at the scanning range of $220 \sim 300$ nm.

Determination of the Absolute Configuration of the Constituent Amino Acids of BC Components

In sealed vials, 0.2 mg of individual isolated components were hydrolyzed at 110°C for 24 hours with $100\,\mu$l of distilled 6 N HCl aqueous solution. This solution was evaporated to dryness and the residue was dissolved in $50\,\mu$l of water. The amino acids solution was transferred to a 2-ml reaction vial, and $20\,\mu$l of 1 M $NaHCO_3$ aqueous solution and $100\,\mu$l of 1% FDAA acetone solution were added to the vial. The vials were capped and incubated at 37°C for 60 minutes in a water bath. After the addition of $20\,\mu$l of 1 N HCl aqueous solution to the vial to stop the reaction, the reaction mixture was evaporated to dryness. The residual derivatized amino acids were dissolved in 1 ml of methanol and $2\,\mu$l aliquots of this solution were injected into the HPLC system. The separation was performed on a Inertsil C8 HPLC column ($5\,\mu$m, 150×4.6 mm i.d.) (GL-Science, Tokyo, Japan) using an acetonitrile - 0.01 M TFA aqueous solution as a mobile phase under gradient elution mode (acetonitrile, $20\% \sim 40\%$, 30 minutes followed by hold at 40% for 10 minutes). The flow rate of the mobile phase was 1.0 ml/minute and the monitoring wavelength was set at 340 nm.

Measurement of MS/MS Spectra

MS/MS was carried out using a JMS-HX/HX 110A tandem mass spectrometer (JEOL, Tokyo, Japan). The samples were ionized under FAB conditions and the resulting protonated molecules $[M+H]^+$ were selected as the precursor ions at the first mass spectrometer. The precursor ions were decomposed at the collision cell under collision induced decomposition conditions and the resulting product ions were separated and detected at the second mass spectrometer. The product ion spectra were obtained by processing the mass data using a JMA-DA7000 data system (JEOL). The fast atom beam was operated at 6 kV using xenon gas and the first mass spectrometer was operated at a 10 kV accelerating potential. The floating cell was used at a voltage of 5 kV. A mixture of glycerol and 1 N HCl (1 : 1) was used as the matrix for the ionization of the samples under FAB conditions, and helium was used as the collision gas.

Determination of Minimal Inhibition Concentration

Minimal inhibition concentrations (MICs) of BC components were determined by the agar plate dilution method[48] using *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (IFO 12732), *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 6633) and *Escherichia coli* (IFO 3806) as test strains. A commercial BC complex (58.6 units/mg) was used as a reference sample. After the preparation of the Muller-Hinton II agar medium containing a series of concentrations ($25 \sim 0.05\,\mu$g/ml) of samples, the suspension of individual test strains ($10^5 \sim 10^6$ cell/ml) were inoculated by micromanipulator (MIT-P, SAKURA, Tokyo, Japan). The growth of the strains was observed after incubation at 37°C for 20 hours.

Results and Discussion

Determination of the Absolute Configuration of the Constituent Amino Acids

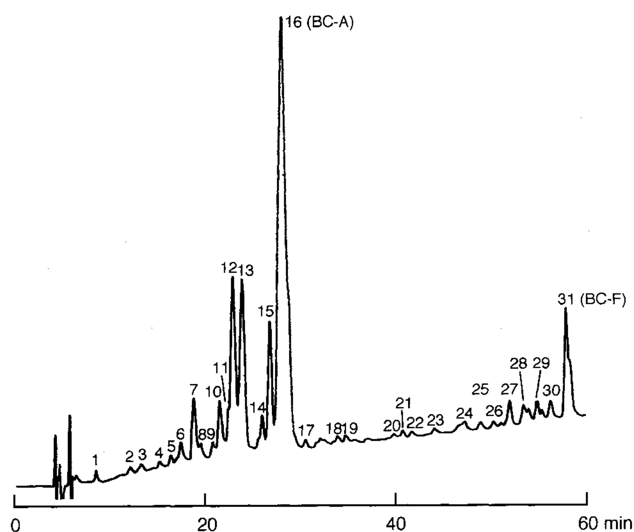
To determine the absolute configuration of the constituent amino acids of BC components, components including BCs-A and -F were isolated by preparative HPLC and the purity of the isolated components (peaks No. 7, 10, 11, 12, 13, 15, 16, 24, 25, 26, 27, 28, 29 and 31 as indicated in Fig. 1) were checked under analytical HPLC conditions. Samples of $0.5 \sim 5$ mg were obtained and the purity of each component was over 95% on the HPLC chromatogram. The isolated components were hydrolyzed under acidic conditions, and the resulting amino acids were derivatized with Marfey reagent[47]. The resulting mixture of derivatives was separated by HPLC and the absolute configurations of the amino acids were determined from the retention times of peaks on the chromatogram. Tables 1 and 2 show the constituent amino acids and their absolute configurations detected from isolated BC-A and BC-F related components, respectively. Although L-cysteine and D-asparagine are present as constituent amino acids in every component,

cysteine was not detectable by this method because of decomposition during acid hydrolysis, and D-asparagine was detected as D-aspartic acid.

Total Structures of BC Minor Components

Small amounts of D-isoleucine or D-valine were detected together with the L-enantiomers from BC-A related components, but were not detected from BC-F related components as listed in Tables 1 and 2. We consider that these D-amino acids are originated from the *N*-terminal moieties of BC-A related components and that isoleucine and valine, except for those at the *N*-terminus, have L-configuration, because the *N*-terminal moieties of BC-F related components do not undergo hydrolysis.

Fig. 1. HPLC profiles of BC components.



HPLC conditions; column, Chromatorex Ph (5μm, 250×4.6mm i.d.); mobile phase; acetonitrile-0.01 M TFA aqueous solution; flow rate, 1.0ml/minute; gradient rate, acetonitrile 26~44% (40 minutes, linear); detection, 234nm.

It has been reported that D-alloisoleucine was detected from BC-A as an artifactual amino acid formed during hydrolysis[19,22]. The formation of D-alloisoleucine was explained by involving an epimerization of the L-isoleucine moiety in the thiazoline ring at the *N*-terminus of BC-A under acidic conditions as shown in Fig. 2 (I)[49]. Because D-alloisoleucine and D-isoleucine appear at almost the same retention time on the HPLC chromatogram by this method, the D-isoleucines listed in Table 1 were considered to be all D-alloisoleucines derived from *N*-terminal L-isoleucines. If so, the D-valines in Table 1 could also be artifacts derived from *N*-terminal amino acids by way of the same mechanism as that of D-alloisoleucine as shown in Fig. 2 (II). This strongly indicates that the minor components of peaks No. 11, 13 and 15 have the same *N*-terminal moieties as that of BC-A, whereas, the *N*-terminal moieties of peaks No. 7, 10 and 12 are different from that of BC-A, consisting of L-cysteine and L-valine. Based on the results described above, four types of variations of *N*-terminal moieties among the components of BC are suggested as shown in Fig. 3.

As listed in Tables 1 and 2, L-leucine was detected in all of the components. Both this result and the above structures of *N*-terminal moieties not only support the structures of the minor components proposed by TETLER

Table 1. Absolute configurations of constituent amino acids of BC-A related components.

Peak No.	His	Asp	Glu	Orn	Lys	Val	Ile	Leu	Phe
7	L	D, L	D	D	L	D, L	L	L	D
10	L	D, L	D	D	L	D, L	L	L	D
11	L	D, L	D	D	L	L	D, L	L	D
12	L	D, L	D	D	L	D, L	L	L	D
13	L	D, L	D	D	L	L	D, L	L	D
15	L	D, L	D	D	L	L	D, L	L	D
16 (BC-A)	L	D, L	D	D	L	—	D, L	L	D

Table 2. Absolute configurations of constituent amino acids of BC-F related components.

Peak No.	His	Asp	Glu	Orn	Lys	Val	Ile	Leu	Phe
24	L	D, L	D	D	L	L	L	L	D
25	L	D, L	D	D	L	L	L	L	D
26	L	D, L	D	D	L	L	(L)	L	D
27	L	D, L	D	D	L	–	L	L	D
28	L	D, L	D	D	L	L	L	L	D
29	L	D, L	D	D	L	L	L	L	D
31 (BC-F)	L	D, L	D	D	L	—	L	L	D

( ) Trace level.

Fig. 2. Epimerization mechanism of *N*-terminal amino acids for bacitracin A related components during hydrolysis.

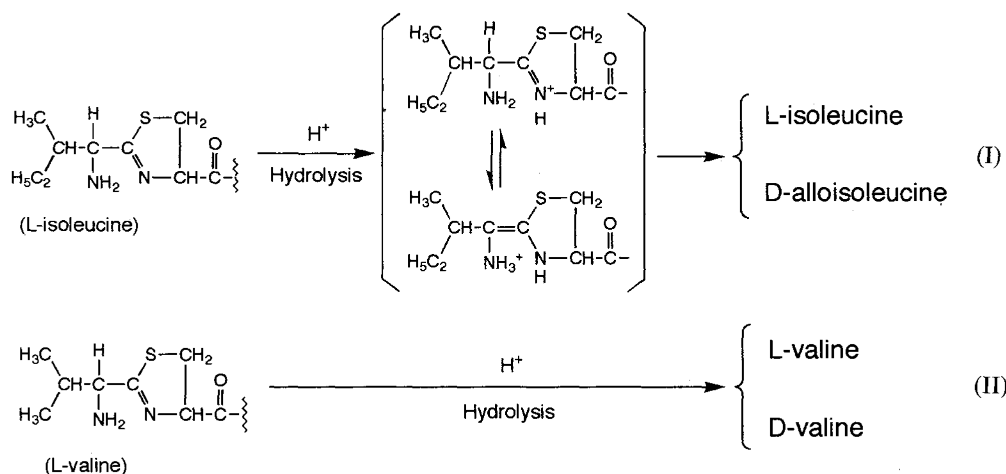


Fig. 3. Variations of *N*-terminal moieties and their fragmentation patterns.

Thiazoline ring composed of L-isoleucine (A), L-valine (B) for BC-A related components; thiazole ring composed of L-isoleucine (C), L-valine (D) for BC-F related components.

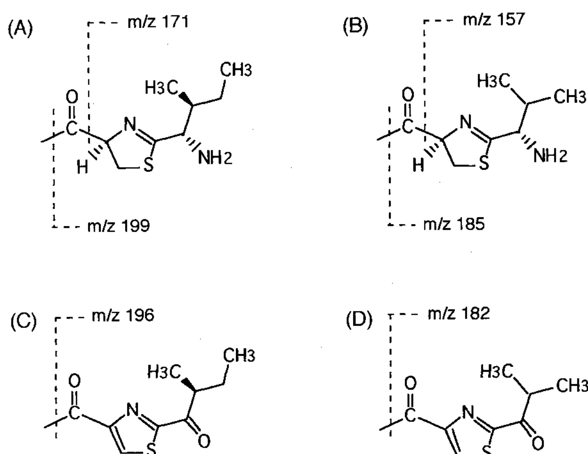


Fig. 4. General structure of BC components.

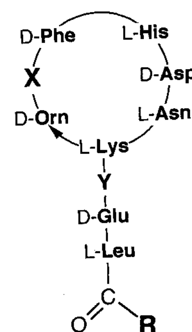


Table 3. Proposed structures and names for BC components.

Peak No.	Name	M.W.	X	Y	R*
6	BC-E	1379	L-Val	L-Val	(B)
7	BC-D_1	1393	L-Val	L-Ile	(B)
10	BC-D_2	1393	L-Ile	L-Val	(B)
11	BC-D_3	1393	L-Val	L-Val	(A)
12	BC-B_1	1407	L-Ile	L-Ile	(B)
13	BC-B_2	1407	L-Val	L-Ile	(A)
15	BC-B_3	1407	L-Ile	L-Val	(A)
16	BC-A	1421	L-Ile	L-Ile	(A)
24	BC-I_1	1390	L-Val	L-Ile	(D)
25	BC-I_2	1390	L-Ile	L-Val	(D)
26	BC-I_3	1390	L-Val	L-Val	(C)
27	BC-H_1	1404	L-Ile	L-Ile	(D)
28	BC-H_2	1404	L-Val	L-Ile	(C)
29	BC-H_3	1404	L-Ile	L-Val	(C)
31	BC-F	1418	L-Ile	L-Ile	(C)

* See Fig. 3.

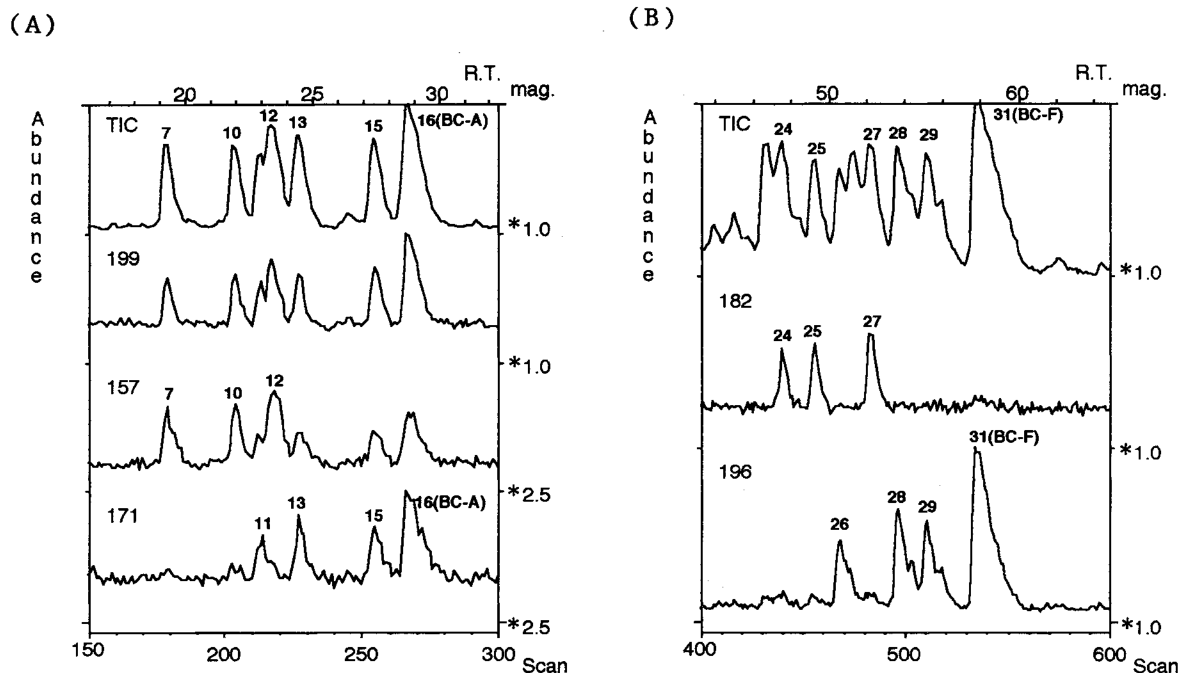
et al.[44], MORRIS[45] and SIEGEL *et al.*[46] but also point out that our previously proposed structures of BC minor components[14] are incorrect with respect to one substitution position of valine at the *N*-terminus. In addition, the following suggestion was also given: all components have the common structure shown in Fig. 4 and are different from one another only in the structures of the *N*-terminal moieties and that the constituent amino acids at the "X" and "Y" positions are L-isoleucine or L-valine. The variations of the constituent amino acids and *N*-terminal moieties among the components are summarized in Table 3.

Confirmation of Proposed Structures by LC/MS and MS/MS

To confirm the structures proposed above, previous Frit-FAB LC/MS data[14] were reexamined. Because BC components yield characteristic fragment ions at *m/z* 157, 171, 182, 196, 185 and 199 from their *N*-terminal moieties under FAB conditions, as shown in Fig. 3, these ions were used for mass chromatography. The fragment ion at *m/z* 185 could not be successfully monitored because it overlapped with a very intensive background ion from the glycerol used as a matrix. Because the mass

Fig. 5. Expanded total ion current and mass chromatograms of enriched samples monitored at *m/z* 199, 196, 182, 171, 157 by LC/MS.

(A) BC-A related components, (B) BC-F related components.



chromatogram at *m/z* 199 showed almost the same chromatographic profile as that of the total ion current chromatogram (TIC) as shown in Fig. 5 (A), the fragment ion was not usable either. However, the *N*-terminal amino acids were easily distinguished as either valine or isoleucine by comparing the mass chromatograms at *m/z* 157 and 171 for BC-A related components and *m/z* 182 and 196 for BC-F related components as shown in Fig. 5 (A) and (B), respectively.

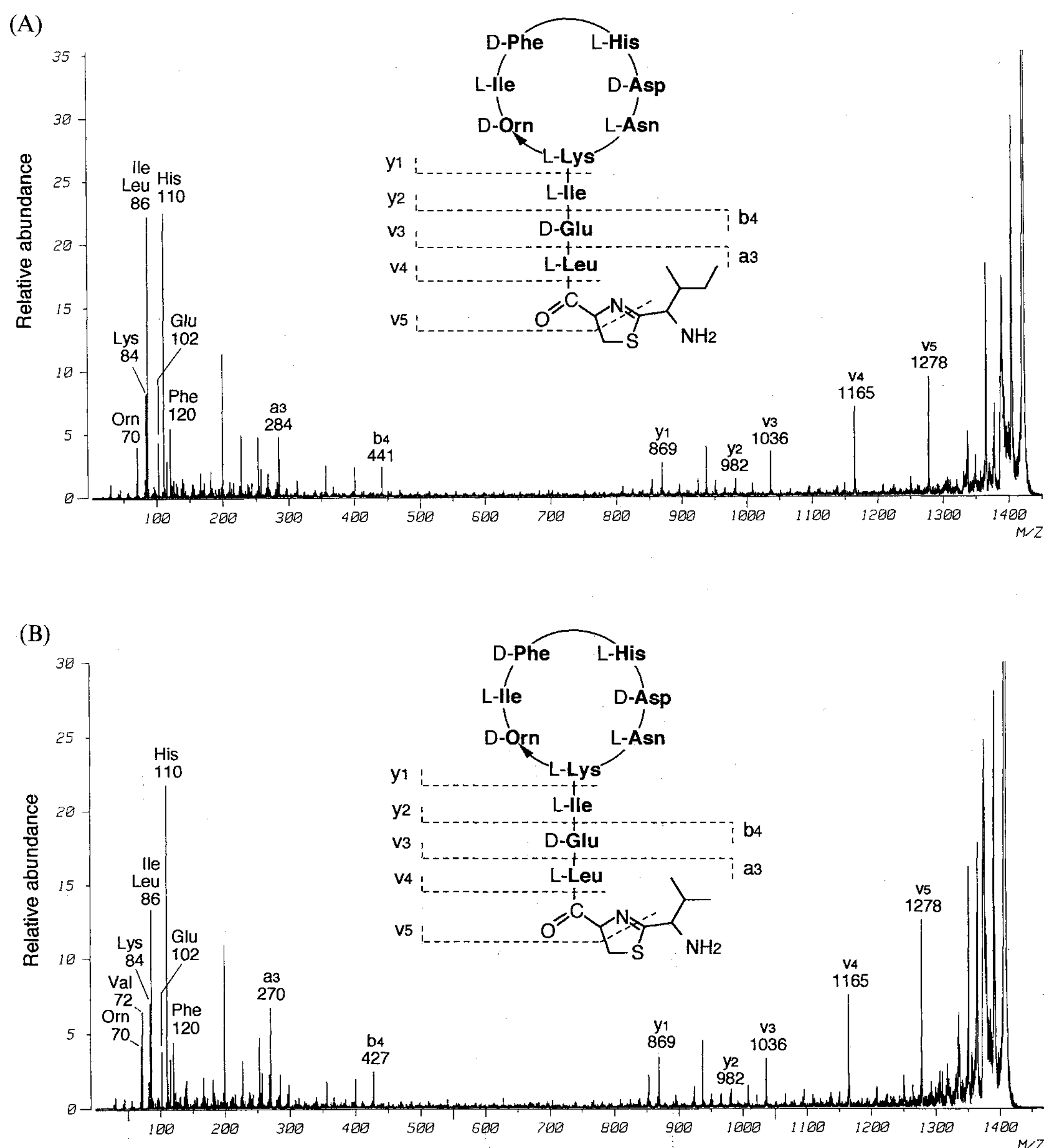
We previously applied MS/MS to structural characterization of isolated BC-A and -F under FAB conditions, and satisfactory product ion spectra containing many sequence ions were obtained[12]. In order to confirm the structures of BC-A related components proposed above, the isolated components were analyzed by MS/MS and the fragment ions in the product ion spectra were examined in detail. In the product ion spectra of BC-A many structurally informative fragment ions such as immonium ions originating from each constituent amino acid (ornithine, lysine, leucine/isoleucine, glutamic acid, histidine and phenylalanine at *m/z* 70, 84, 86, 102, 110 and 120, respectively), *C*-terminal ions (a_3 and b_4) and *N*-terminal ions (y_1, y_2, v_3, v_4 and v_5)[50] were observed as shown in Fig. 6 (A). Because the BC-A related components give the same fragmentation pattern as that of BC-A, their product ion spectra were examined in detail for immonium ions and sequence ions as shown in Table 4. As shown in Fig. 6 (B), all of the expected *C*-terminal and *N*-terminal sequence ions and immonium ions containing valine at *m/z* 72 were observed in each spectrum of BC-A related components. Thus, based on the LC/MS and MS/MS results, the proposed structures of BC minor components were supported.

Designation of BC Components

The names of BC components such as BCs-D and -E were originally proposed by NEWTON *et al.*[3,5] and the naming has been well accepted until the present time. They also reported that BCs-A, -B, -D and -E show the same broad absorption maximum at 253 nm due to their thiazoline ring moiety, and that BCs-F_1, -F_2 and -F_3 absorb at 288 nm due to their thiazole ring, though BCs-C and -G show a sharp maximum at 250 nm[5]. Because the nomenclature was based on the CCD separation, it is not adaptable to the study of components separated by HPLC. Therefore, we would like to propose a new systematic nomenclature in line with the original one. Comparing the HPLC profile shown in Fig. 1 with previous CCD elution curves[5], it was found that the minor components with molecular weights (M.W.) of 1407 (peaks No. 12, 13 and 15), 1393 (No. 7, 10 and 11), 1379 (No. 6), 1404 (No. 27, 28 and 29) and 1390 (No. 24, 25 and 26) correspond to BCs-B, -D, -E, -F_2 and -F_3, respectively. To confirm these correspondences, UV absorption spectra of BC components were measured by photodiode-array detection HPLC. The components of

Fig. 6. Product ion spectra of $[M+H]^+$ from isolated BC components and their fragmentation patterns.

(A) BC-A and (B) minor components of peak No. 12.



Mass spectrometer, JMS-HX/HX 110A; data system, JMA-DA7000; ion source, FAB; primary beam, Xe^0; acceleration voltage, 6 kV (primary), 10 kV (secondary); floating cell voltage, 5 kV; matrix, glycerol - 1 N HCl aq. soln. (1 : 1); collision gas, helium.

Table 4. Fragment ions in the product ion spectra of BC-A related components.

Peak No.	*C*-Terminal ions (*m*/*z*)		*N*-Terminal ions (*m*/*z*)				
	a_3	b_4	y_1	y_2	v_3	v_4	v_5
7	270	427	855	968	1022	1151	1264
10	270	427	869	968	1022	1151	1264
11	284	441	855	954	1008	1137	1250
12	270	427	869	982	1036	1165	1278
13	284	441	855	968	1022	1151	1264
15	284	441	869	968	1022	1151	1264
16 (BC-A)	284	441	869	982	1036	1165	1278

Table 5. Minimal inhibition concentrations of BC components.

	Minimal inhibition concentration (μg/ml)								
	BC-D_1	BC-D_2	BC-D_3	BC-B_1	BC-B_2	BC-B_3	BC-A	BC*	BC-F
Micrococcus luteus ATCC 9341	3.13	3.13	1.56	0.78	0.78	0.78	0.39	0.78	>25
Staphylococcus aureus IFO 12732	25	25	12.5	12.5	12.5	6.25	3.13	6.25	>25
Bacillus cereus var. mycoides ATCC 11778	>25	>25	>25	>25	25	25	6.25	25	>25

* Commercial BC complex (58.6 units/mg).

peaks No. 6, 7, 10, 11, 12, 13, 15 and 16, and peaks No. 24, 25, 26, 27, 28, 29 and 31 showed the same spectral patterns including the absorption maxima at 254 and 288 nm, respectively. Based on these results, we propose a new nomenclature, BCs-B_1, -B_2 and -B_3 for the components of peaks No. 12, 13 and 15, BCs-D_1, -D_2 and -D_3 for peaks No. 7, 10 and 11, respectively. Because BC-E is a single component, the original name was applied to the component of peak No. 6. However, the original names, BCs-F_2 and -F_3 are not suitable for the components of MW 1404 and 1390, so a new classification and nomenclature, BCs-H_1, -H_2 and -H_3 for peaks No. 27, 28 and 29, and BCs-I_1, -I_2 and -I_3 for peaks No. 24, 25 and 26 was proposed. These names are shown in Table 3 together with their structural information. The degradation product of BC-E was not found on the HPLC chromatogram probably because of low content in the sample complex. We assumed that the component can also be contained in the BC complex and named it BC-J.

Antimicrobial Activity of BC Minor Components

In order to investigate the antimicrobial activity of BC components, their MICs were determined using *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis* and *Escherichia coli* as test strains. None of samples exhibited activity against *B. subtilis* and *E. coli* at any concentrations examined. As shown in Table 5, the commercial BC complex and the BC components, except for BC-F, showed activity against the same strains. BC-A was 2~4 times more potent than the commercial BC complex and the BC-B series components and was 4~8 times more potent than the BC-D series components against the strains of *M. luteus* and *S. aureus*. Comparison of the MICs and the proposed structures of the minor components suggest that the location of the valine affects the activity of each component in the following decreasing order: *N*-terminus, the seven membered peptide ring, and the side chain peptide moiety. Because this order is related to the elution order of the minor components on the HPLC, the activity may depend on the hydrophobicity of the respective component.

Conclusion

The total structures of 13 minor components of bacitracin, including the absolute configuration of the constituent amino acids, were proposed following the results of structural characterization of the isolated components using techniques of amino acid analysis, LC/MS and MS/MS. The structures of the BC-A related components were the same as that of BC-A except that one to three of the L-isoleucines, including the *N*-terminal one, were replaced by L-valines. The BC-F related components were the degradation products of BC-A related components that went through the same degradation process as BC-A. These results agree with the proposals of TETLER *et al.*[44], MORRIS[45] and SIEGEL *et al.*[46], but are in contrast with our earlier suggestions[14].

Among the minor components described above, the three isobaric (MW 1407) components are named as BCs-B_1, -B_2 and -B_3, those of MW 1393, 1404 and 1390 are named as BCs-D_1, -D_2 and -D_3, BCs-H_1, -H_2 and -H_3, and BCs-I_1, -I_2 and -I_3, respectively, in line with their original names as much as possible. For the component of MW 1379 its original name, BC-E, is still used.

The antimicrobial activity of isolated components against some strains was determined and the results suggest that the location of valine affects the activity of the component in the following order: *N*-terminus, the seven membered peptide ring and the side chain peptide moiety.

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References

1) BREWER, G. A.: Bacitracin. Analytical profiles of drug substance 9: 1~69, 1980
2) BARRY, G. T.; J. D. GREGOLY & L. C. CRAIG: The nature

of bacitracin. J. Biol. Chem. 175: 485~486, 1948

3) NEWTON, G. G. F. & E. P. ABRAHAM: Ayfivin and bacitracin: Resolution of crude products in similar series of peptides. Biochem. J. 47: 257~267, 1950
4) CRAIG, L. C.; J. R. WEISIGER, W. HAUSMANN & E. J. HARFENIST: The separation and characterization of bacitracin polypeptides. J. Biol. Chem. 199: 259~266, 1952
5) NEWTON, G. G. F. & E. P. ABRAHAM: Some peptides of bacitracin polypeptides. Biochem. J. 53: 597~604, 1953
6) CRAIG, L. C. & W. KONIGSBERG: Further studies with the bacitracin polypeptides. J. Org. Chem. 22: 1345~1353, 1957
7) KONINGSBERG, W. & L. C. CRAIG: On bacitracin F. J. Org. Chem. 27: 934~938, 1962
8) RESSLER, C. & D. K. KASHELIKAR: Identification of asparaginyl and glutaminyl residues in endo protein in peptides by dehydration-reduction. J. Am. Chem. Soc. 88: 2025~2035, 1966
9) RATTI, E.; C. LAUINGER & C. RESSLER: Configuration of the asparaginyl and aspartyl residues of bacitracin. J. Org. Chem. 33: 1309~1310, 1968
10) OKA, H.; Y. IKAI, N. KAWAMURA, M. YAMADA, K.-I. HARADA, Y. YAMAZAKI & M. SUZUKI: Improvement of chemical analysis of antibiotics XIV. Identification of the components of bacitracin using normal- and reversed-phase thin-layer chromatography. J. Chromatogr. 449: 448~454, 1988
11) OKA, H.; Y. IKAI, N. KAWAMURA, M. YAMADA, K.-I. HARADA, Y. YAMAZAKI & M. SUZUKI: Improvement of chemical analysis of antibiotics XV. Isocratic high-performance liquid chromatographic methods for the analysis and preparative separation of the components of bacitracin. J. Chromatogr. 462: 315~322, 1989
12) HARADA, K.-I.; Y. IKAI, Y. YAMAZAKI, H. OKA, M. SUZUKI, H. NAKAZAWA & Y. ITO: Isolation of bacitracins A and F by high-speed countercurrent chromatography. J. Chromatogr. 538: 203~212, 1991
13) IKAI, Y.; H. OKA, J. HAYAKAWA, K.-I. HARADA & M. SUZUKI: Evaluation of applicability of the flow splitter to Frit-FAB LC/MS system. Mass Spectrosc. 39: 199~204, 1991
14) IKAI, Y.; H. OKA, J. HAYAKAWA, K.-I. HARADA & M. SUZUKI: Structural characterization of bacitracin components by Frit-fast atom bombardment (FAB) liquid chromatography/mass spectrometry (LC/MS). J. Antibiotics 45: 1325~1334, 1992
15) CRAIG, L. C.; W. HAUSMANN & J. R. WEISIGER: The qualitative and quantitative amino acid content of bacitracin A. J. Biol. Chem. 199: 865~871, 1952
16) NEWTON, G. G. F. & E. P. ABRAHAM: Observation on the nature of bacitracin A. Biochem. J. 53: 604~613, 1953
17) INGRAM, V. M.; The identification of peptide end-groups as dimethylamino acids. J. Biol. Chem. 202: 193~201, 1953
18) PROATH, J.: Structure of bacitracin A. Nature 172: 871~872, 1953
19) LOCKHART, I. M.; G. G. F. NEWTON & E. P. ABRAHAM: Structure of Bacitracin A. Nature 173: 536~537, 1954
20) LOCKHART, I. M. & E. P. ABRAHAM: The amino acid sequence in bacitracin A. Biochem. J. 58: 633~647, 1954
21) HAUSMANN, W; J. R. WEISIGER & L. C. CRAIG: Partial hydrolysis studies with bacitracin A. J. Am. Chem. Soc. 77: 723~731, 1955
22) CRAIG, L. C.; W. HAUSMANN & J. R. WEISIGER: Structural studies with bacitracin A. J. Am. Chem. Soc. 76: 2839~2841, 1954
23) HAUSMANN, W; J. R. WEISIGER & L. C. CRAIG: Bacitracin A. Further studies on the composition. J. Am. Chem. Soc. 77: 721~722, 1955
24) WEISIGER, J. R.; W. HAUSMANN & L. C. CRAIG: Bacitracin A. The nature of the linkages surrounding the sulfur. J. Am. Chem. Soc. 77: 3123~3127, 1955
25) WEISIGER, J. R.; W. HAUSMANN & L. C. CRAIG: On the partial hydrolysis of DNP-bacitracin A. J. Am. Chem. Soc. 77: 731~736, 1955
26) LOCKHART, I. M.; E. P. ABRAHAM & G. G. F. NEWTON: *N*-Terminal and sulfur containing residues of bacitracin A. Biochem. J. 61: 534~544, 1955
27) LOCKHART, I. M. & E. P. ABRAHAM: Lysine and aspartic residues in bacitracin A. Biochem. J. 62: 645~651, 1956
28) WRINCH, D.: Structure of bacitracin A. Nature 179: 536~537, 1957
29) SWALLOW, D. L. & E. P. ABRAHAM: The amido and carboxyl groups of bacitracin A. Biochem. J. 72: 326~332, 1959
30) STOFFEL, W. & L. C. CRAIG: Synthesis of structures related to bacitracin A. J. Am. Chem. Soc. 83: 145~149, 1961
31) ARIYOSHI, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. I. Synthesis of protected intermediate heptapeptide (5—11). Bull. Chem. Soc. Jpn. 40: 1709~1715, 1967
32) ARIYOSHI, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. II. Synthesis of a cyclic dodecapeptide, an analogue of bacitracin A. Bull. Chem. Soc. Jpn. 40: 2648~2654, 1967
33) ARIYOSHI, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. III. Synthesis of (*S*)-2-(2-methylbutyryl)-thiazole-4-carboxylic acid and its peptide derivative. Bull. Chem. Soc. Jpn. 40: 2654~2658, 1967
34) HIROTSU, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. IV. Synthesis of thiazoline peptides by iminoether coupling method. Bull. Chem. Soc. Jpn. 40: 2945~2949, 1967
35) HIROTSU, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. V. Synthesis of thiazoline peptides from cysteine peptides by dehydration procedure. Bull. Chem. Soc. Jpn. 40: 2950~2954, 1967
36) HIROTSU, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. VI. Synthesis of unprotected thiazoline peptides. Bull. Chem. Soc. Jpn. 43: 1564~1567, 1970
37) HIROTSU, Y.; T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. VII. Isomerization of amino acid components of thiazoline peptides. Bull. Chem. Soc. Jpn. 43: 1870~1873, 1970
38) MUNEKATA, E.; Y. MASUI, T. SHIBA & T. KANEKO: Synthetic studies of bacitracin. VIII. Synthesis of cycloheptapeptide moiety. Bull. Chem. Soc. Jpn. 46: 3187~3193, 1973
39) HIROTSU, Y.; Y. NISHIUCHI & T. SHIBA: Total synthesis of bacitracin F. *In* Peptide Chemistry 1978, *Ed.*, N. IZUMIYA. Protein Research Foundation, Osaka, pp. 171~176, 1979
40) TSUJI, K.; J. H. ROBERTSON & J. A. BACH: Quantitative

high-performance liquid chromatographic analysis of bacitracin, a polypeptide antibiotic. J. Chromatogr. 99: 597~608, 1974

41) Bell, R. G.: Preparative high-performance liquid chromatographic separation and isolation of bacitracin components and their relationship to microbiological activity. J. Chromatogr. 590: 163~168, 1992

42) Tsuji, K. & J. H. Robertson: Improved high-performance liquid chromatographic method for polypeptide antibiotics and its application to study the effects of treatments to reduce microbial levels in bacitracin powder. J. Chromatogr. 112: 663~672, 1975

43) Newton, G. G. F.; E. P. Abraham, H. W. Florey, N. Smith & J. Ross: Some observations on the biological properties of bacitracins A, B, and C. Brit. J. Pharmacol. 6: 417~429, 1951

44) Tetler, L. W.; S. N. Davey & M. Morris: Analysis of bacitracin B using fast atom bombardment and tandem mass spectrometry. Biol. Mass Spectrom. 22: 712~720, 1993

45) Morris, M.: Primary structural confirmation of components of the bacitracin complex. Biol. Mass Spectrom. 23: 61~70, 1994

46) Siegel, M. M.; J. Huang, B. Lin, R. Tsao & C. G. Edmonds: Structures of bacitracin A and isolated congeners: Sequence of cyclic peptide with blocked linear side chains by electrospray ionization mass spectrometry. Biol. Mass Spectrom. 23: 186~204, 1994

47) Marfey, P.: Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlesberg Res. Commun. 49: 591~596, 1984

48) Japan Society of Chemotheraphy: Rerevised assay method of MIC. Chemotheraphy: 29: 76~79, 1981

49) Koningsberg, W.; R. J. Hill & L. C. Craig: The oxidation and acid isomerization of bacitracin A. J. Org. Chem. 26: 3867~3871, 1961

50) Biemann, K.: Contributions of mass spectrometry to peptide and protein structure. Biomed. Environ. Mass Spectrom 16: 99~111, 1988