Journal of Chromatography, 538 (1991) 203–212
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 730

Isolation of bacitracins A and F by high-speed counter-current chromatography

KEN-ICHI HARADA, YOSHITOMO IKAI, YUMIKO YAMAZAKI, HISAO OKA and MAKOTO SUZUKI*
Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468 (Japan)
HIROYUKI NAKAZAWA
The National Institute of Public Health, Minato-ku, Tokyo 108 (Japan)
and
YOICHIRO ITO
Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Building 10, Room 7N-322, National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

ABSTRACT

The major components of bacitracin were separated and purified using high-speed counter-current chromatography (HSCCC). A systematic search for optimum two-phase solvent systems resulted in two systems: chloroform–ethanol–methanol–water (5:3:3:4) and chloroform–ethanol–water (5:4:3). These were selected based on the determination of the partition coefficients of all the components and the settling time of the phases. HSCCC with these solvent systems separated two components, bacitracins A and F. Improvements in the flow-cell arrangement eliminated noise in detection, making in-line monitoring possible. A tandem mass spectrometric technique was used to characterize the isolated components.

INTRODUCTION

Bacitracins, which are peptide antibiotics produced by *Bacillus subtilis* and *Bacillus licheniformis*, exhibit an inhibitory activity against Gram-positive bacteria [1] and are among the most commonly used antibiotics as animal feed additives [2,3]. These compounds were discovered in 1943 and were originally considered to be one substance [4]. More recently, it has been demonstrated that they consist of over twenty components of differing antimicrobial activities [5,6]. The major antimicrobial components are bacitracins A and B, and bacitracin F is a degradation product [5,7] that shows nephrotoxicity [4]. Only the structures of bacitracins A and F (Fig. 1) have been determined to date [4,8–10].

The bacitracins are highly polar compounds composed of a cyclic heptapeptide and a branch containing a thiazole ring. In order to establish appropriate chromatographic methods for the separation of the components, it is necessary to consider various properties. The bacitracin complex has low solubility in benzene, chloroform, ethyl acetate and acetone and is soluble to the approximate limit of 20 mg/ml in water or methanol. When silica gel and Sephadex are used for preparative separation, the

0021-9673/91/$03.50

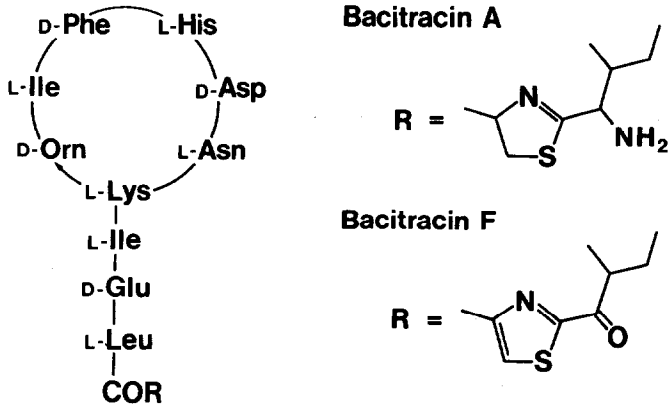

Fig. 1. Structures of bacitracins A and F.

recovery is very poor, probably because polar functional groups interact with silanol and other polar groups. Additionally, the antibiotics are thermally unstable and labile under basic conditions. We have also confirmed that bacitracin A decomposes thermally to give bacitracin F. These components have previously been separated by counter-current distribution methods [5,11–14], without complete resolution.

We have previously applied high-speed counter-current chromatography (HSCCC) to the preparative separation of six sporaviridin components, which are complicated basic glycoside antibiotics produced by *Streptosporangium viridogriseum* [15]. Here we describe the isolation of bacitracin components by HSCCC and their structural characterization by tandem mass spectrometry (MS–MS) under fast atom bombardment (FAB) conditions.

EXPERIMENTAL

Materials

Methanol, ethanol, *n*-butanol, isopropanol, acetone, diethyl ether, ethyl acetate, disodium hydrogenphosphate, sodium chloride, potassium chloride, ammonium chloride and ammonium acetate were of analytical-reagent grade. Bacitracin was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.).

Operation of HSCCC

The apparatus used was a Shimadzu (Kyoto, Japan) prototype of the coil planet centrifuge (HSCCC-1A) for HSCCC with a 160 m- length of 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing around a 10-cm diameter column holder making multiple coiled layers. The total capacity was 325 ml and the column was revolved at 800 rpm. Solvents were delivered with a Shimadzu LC-6A pump. After filling with the stationary phase, followed by sample injection, mobile phase was applied at 3 ml/min. UV detection at 254 nm was performed using a Shimadzu SPD-6A instrument. Eluates were collected in a Pharmacia (Uppsala, Sweden) FRAC-100 fraction collector.

High-performance liquid chromatography (HPLC)

HPLC was carried out using the following conditions: pump, Shimadzu LC-6A; detector, Shimadzu SPD-2A; column, Capcell Pak C_{18} (150 × 4.6 mm I.D.) (Shiseido, Tokyo, Japan); mobile phase, methanol–0.04 *M* aqueous disodium hydrogenphosphate (6:4).

Mass spectrometry

Liquid secondary ion mass spectrometry (SIMS) was performed using a Hitachi (Tokyo, Japan) M-80B mass spectrometer under the following operating conditions: primary ion, Xe^+; accelerating voltage, 8 kV (primary) and 3 kV (secondary). A mixture of glycerol and 10% aqueous oxalic acid was used as the matrix. MS–MS was carried out with a JEOL (Tokyo, Japan) JMS-HX110/HX110 system. The fast atom beam was operated at 6 kV using xenon gas and the spectrometer was operated at 10 kV accelerating potential. A mixture of thioglycerol, glycerol and 10% aqueous oxalic acid was used as the matrix. The collisionally activated dissociation (CAD) experiment was run using helium as the collision gas.

RESULTS AND DISCUSSION

As over twenty components are contained in the bacitracin complex, it is necessary to develop the chromatographic conditions carefully. We have already established an HPLC method for the analysis of the bacitracin complex, in which we used a Capcell Pak C_{18} column and an isocratic solvent system, methanol–phosphate buffer (pH 9.4) (6:4) [16]. As shown in Fig. 2, 22 peaks are separated. The peak numbers shown also applied to later HSCCC separations.

Precise separation by HSCCC is mainly dependent on the selection of the opti-

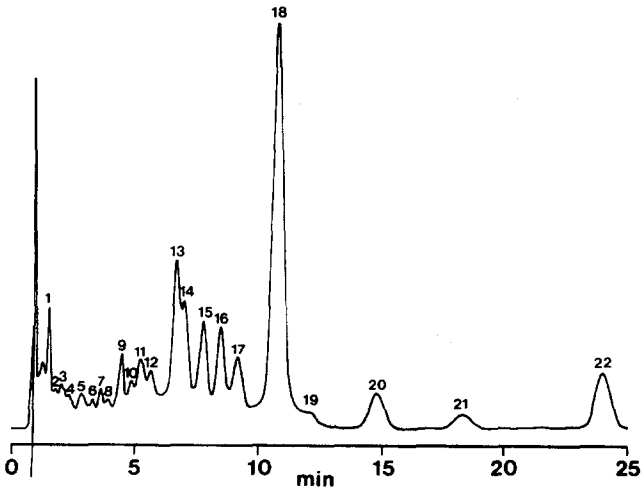

Fig. 2. Analytical HPLC of bacitracin components. Sample size, 3 μg; column, Capcell Pak C_{18} (5 μm) (150 × 4.6 mm I.D.); mobile phase, methanol–0.04 *M* disodium hydrogenphosphate (6:4); flow-rate, 1.3 ml/min; detection, UV (234 nm).

mum two-phase solvent system [17]. To select a suitable solvent system, the solubility of bacitracin components, partition coefficients (K) of the individual components and the settling time of the solvent system were carefully measured. A two-phase solvent system with a settling time of less than 30 s is desirable for high retention of the stationary phase in the HSCCC apparatus [18,19]. The partition coefficient was determined by a simple test-tube experiment using HPLC [16]. Partition coefficients were determined by dividing the corresponding peak area of components of the upper phase by that of components of the lower phase.

We selected *n*-butanol, ethyl acetate and chloroform as the organic components and then prepared two-phase solvent systems composed of one of these three solvents and water and/or methanol, which dissolve the bacitracin complex. Settling times and individual partition coefficients of peaks 13–22 using *n*-butanol and ethyl acetate systems are summarized in Table I. Ideally, partition coefficients of the bacitracin components should be dispersed around $K = 1$. For *n*-butanol solvent systems, peaks 13–18 have suitable partition coefficients, but those of peaks 20–22 are too high. These solvent systems also have the disadvantage of being laborious to evaporate.

TABLE I

PARTITION COEFFICIENTS OF BACITRACIN COMPONENTS WITH *n*-BUTANOL AND ETHYL ACETATE SYSTEMS

Solvent system[a]	Volume ratio	Settling time (s)	Peak No.							
			13,14	15	16	17	18	20	21	22
n-Bu–DE–W	10:0:10	15	0.63	1.52	0.63	1.48	0.75	13.9	UP[b]	23.14
n-Bu–DE–W	10:1:10	15	0.49	1.25	0.51	1.24	1.03	11.1	UP	15.47
n-Bu–DE–W	10:3:10	14	0.22	0.59	0.21	0.51	0.53	6.37	UP	9.95
EA–E–W	5:2:3	50	0.10	0.12	0.08	0.10	0.16	0.56	0.49	0.60
EA–i-P–W	4:2:3	60	0.07	0.14	0.06	0.13	0.15	0.74	0.68	0.85
EA–M–A–W	5:1:1:2	60	LP[c]	LP	LP	LP	0.01	0.08	LP	0.12
EA–E–i-P–W	4:1:1:4	60	0.06	0.13	0.05	0.10	0.12	0.57	LP	0.60
EA–n-Bu–M–W	4:1:1:4	40	LP	LP	LP	LP	0.03	0.50	LP	0.76
EA–n-Bu–M–W	5:1:1:4	30	LP	LP	LP	LP	0.02	0.29	LP	0.50
EA–E–i-P–W	4:1:1:4	60	0.06	0.13	0.05	0.10	0.12	0.57	LP	0.61
EA–E–i-P–10% aq. NaCl	4:1:1:4	30	0.07	(0.08)[d]		0.08	0.11	0.50	LP	0.67
EA–E–i-P–10% aq. KCl	4:1:1:4	30	0.21	(0.22)		0.15	0.33	0.88	0.85	1.25
EA–E–i-P–10% aq. NH_4Cl	4:1:1:4	32	LP	LP	LP	LP	0.07	0.45	0.64	0.64
EA–E–i-P–10% aq. NH_4OAc	4:1:1:4	35	–[e]	–	–	–	–	–	–	–
EA–M–A–W	5:1:1:2	60	LP	LP	LP	LP	0.01	0.08	LP	0.12
EA–M–A–10% aq. NaCl	5:1:1:2	30	LP	LP	LP	LP	LP	LP	LP	LP
EA–M–A–10% aq. KCl	5:1:1:2	30	LP	LP	LP	LP	0.01	LP	LP	0.15
EA–M–A–10% aq. NH_4Cl	5:1:1:2	30	LP	LP	LP	LP	LP	LP	LP	LP
EA–M–A–10% aq. NH_4OAc	5:1:1:1	40	LP	LP	LP	LP	LP	LP	LP	LP

[a] n-Bu = *n*-butanol; DE = diethyl ether; W = water; EA = ethyl acetate; E = ethanol; i-P = isopropanol; M = methanol; A = acetone; OAc = acetate.

[b] UP: bacitracins were exclusively partitioned into the upper phase.

[c] LP: bacitracins were exclusively partitioned into the lower phase.

[d] ( ): Both components were not separated.

[e] –: Decomposition.

TABLE II

PARTITION COEFFCIENTS OF BACITRACIN COMPONENTS WITH CHLOROFORM SYSTEMS

Solvent system[a]	Volume ratio	Settling time (s)	Peak No.							
			13,14	15	16	17	18	20	21	22
C–E–M–W	5:2:3:4	18	7.20	(4.92)[c]		2.46	4.17	0.64	0.65	0.48
C–E–M–W	5:2:1:4	19	UP[b]	UP	UP	UP	33.27	1.62	1.38	0.75
C–E–M–W[d]	5:3:3:4	27	3.35	1.59	4.43	1.40	2.37	0.57	0.47	0.45
C–E–W	5:3:3	15	11.14	(6.37)		3.20	5.34	0.32	0.35	0.27
C–E–W	5:4:2	20	3.19	(2.19)		1.05	2.00	0.25	0.26	0.21
C–E–W[d]	5:4:3	26	5.49	1.78	6.74	1.46	2.20	0.16	LP[e]	0.16
C–E–W	5:4:4	37	6.10	2.25	6.45	2.04	2.68	0.14	LP	0.10

[a] C = chloroform; E = ethanol; M = methanol; W = water.
[b] UP: Bacitracin components were exclusively partitioned into the upper phase.
[c] ( ): Both components were not separated.
[d] Optimum solvent systems.
[e] LP: Bacitracin components were exclusively partitioned into the lower phase.

Another disadvantage is that the ethyl acetate, alcohol and water system showed too long settling times. Addition of inorganic salts, such as sodium chloride, potassium chloride, ammonium chloride and ammonium acetate, to these systems did not improve the K values.

Chloroform, methanol and water systems have been extensively used in droplet CCC [20]. Direct application of these solvent systems to HSCCC was unsuccessful in bacitracin separation but the complete or partial replacement of methanol with etha-

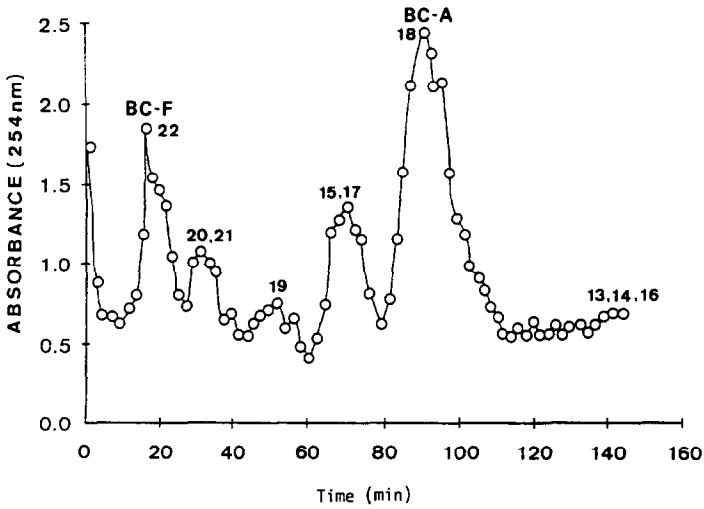

Fig. 3. Counter-current chromatogram of bacitracin components. Sample size, 50 mg; solvent system, chloroform–ethanol–water (5:4:3); mobile phase, lower phase; flow-rate, 3 ml/min; revolution speed, 800 rpm; fractionation volume, 3 ml per tube. The absorbance of fractions was determined manually.

nol gave good results. Other systems are summarized in Table II. The systems using chloroform–ethanol–methanol–water (5:3:3:4) and chloroform–ethanol–water (5:4:3) gave the best combination of partition coefficients.

Fig. 3 shows the counter-current chromatogram of bacitracin components using the chloroform system. The settling time was 26 s and the lower phase was delivered at 3 ml/min. A 50-mg amount of the complex was introduced and the apparatus was rotated at 800 rpm. The retention of the stationary phase was 74.1% and the experiment run was *ca.* 3 h. These components were eluted in order of their partition coefficients and peaks 18 and 22 could be almost completely separated.

Real-time UV monitoring showed a high degree of noise and an unstable baseline. These effects are due to turbidity of phase droplets in the mobile phase in the flow tube along with decavitation. It was necessary, therefore, to draw the elution curve manually after spectrometric analysis of individual fractions. To improve the in-line detection we modified the UV monitoring system by heating the effluent usually at 40°C near the inlet of the UV flow cell. A narrow-bore PTFE tube about 1 m long was attached at the outlet of the monitor [21]. The real-time UV detection in HSCCC is shown in fig. 4. From our previous work in separating the components by HPLC we have established that peaks 18 and 22 correspond to bacitracin A and F, respectively [16]. We were therefore able to compare the HSCCC and HPLC separations. Bacitracin A from HPLC was not always pure [16], whereas HSCCC gave bacitracin A in a pure state and the recovery was high. Similarly, bacitracin F was completely purified by HSCCC in the same run.

Liquid SIMS of the isolated bacitracins A and F was applied using glycerol and 10% oxalic acid as the matrix (Fig. 5). The protonated molecules $[M+H]^+$ are observed at m/z 1422 and 1419, correctly indicating their molecular weights. However,

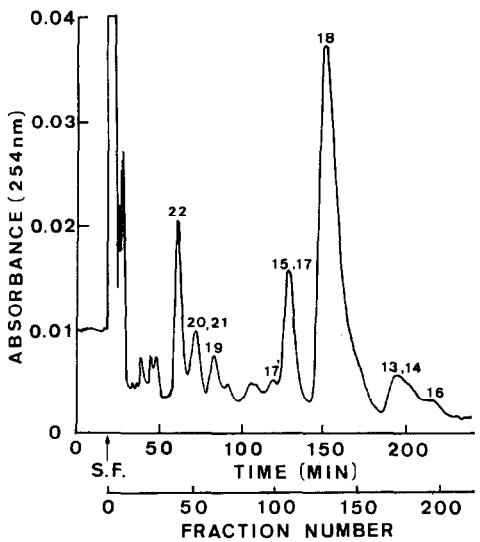

Fig. 4. Counter-current chromatogram of bacitracin components using improved in-line detection. Sample size, 50 mg; solvent system, chloroform–ethanol–methanol–water (5:3:3:4); mobile phase, lower phase; flow-rate, 3 ml/min; fractionation volume, 3 ml per tube; detection, UV (254 nm).

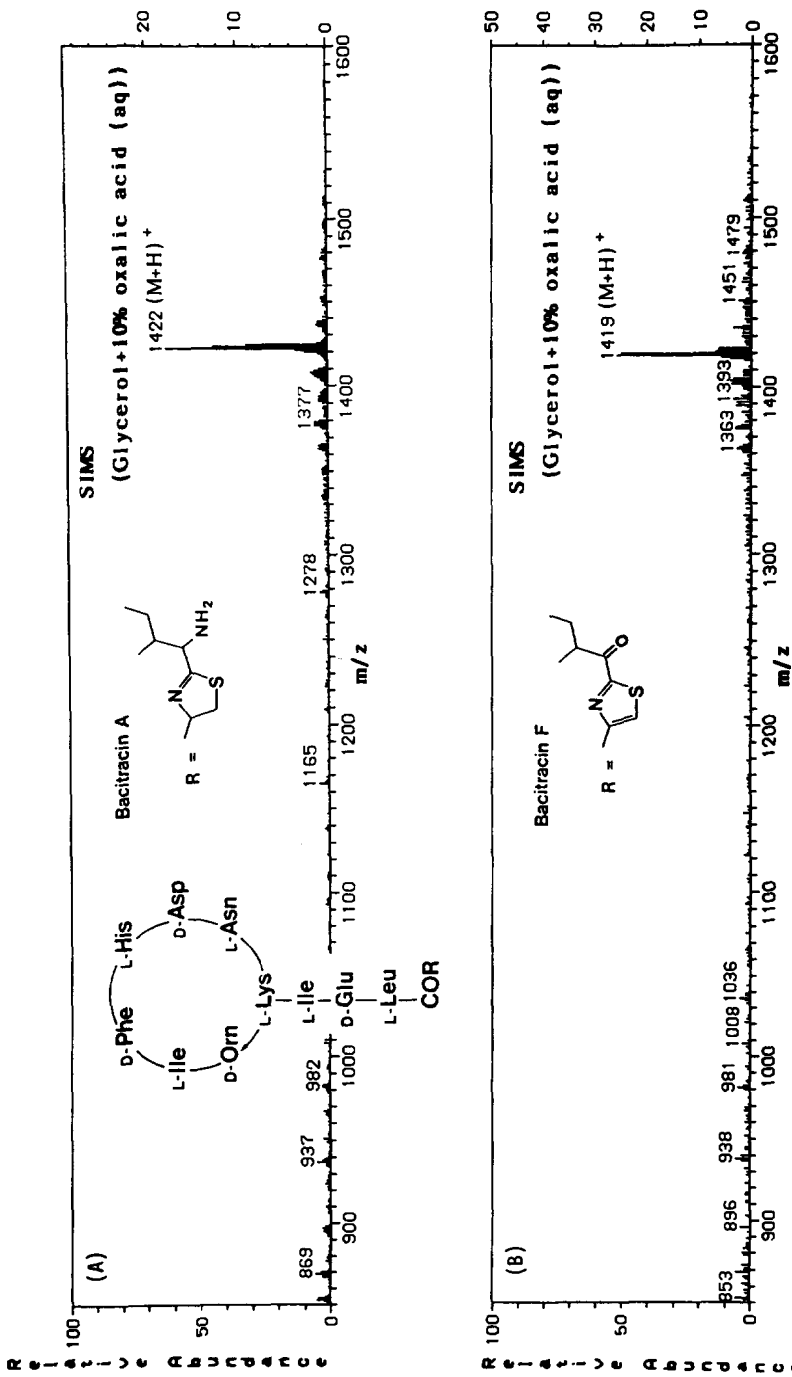

Fig. 5. Liquid SIMS of (A) bacitracin A and (B) bacitracin F using glycerol–10% oxalic acid as the matrix.

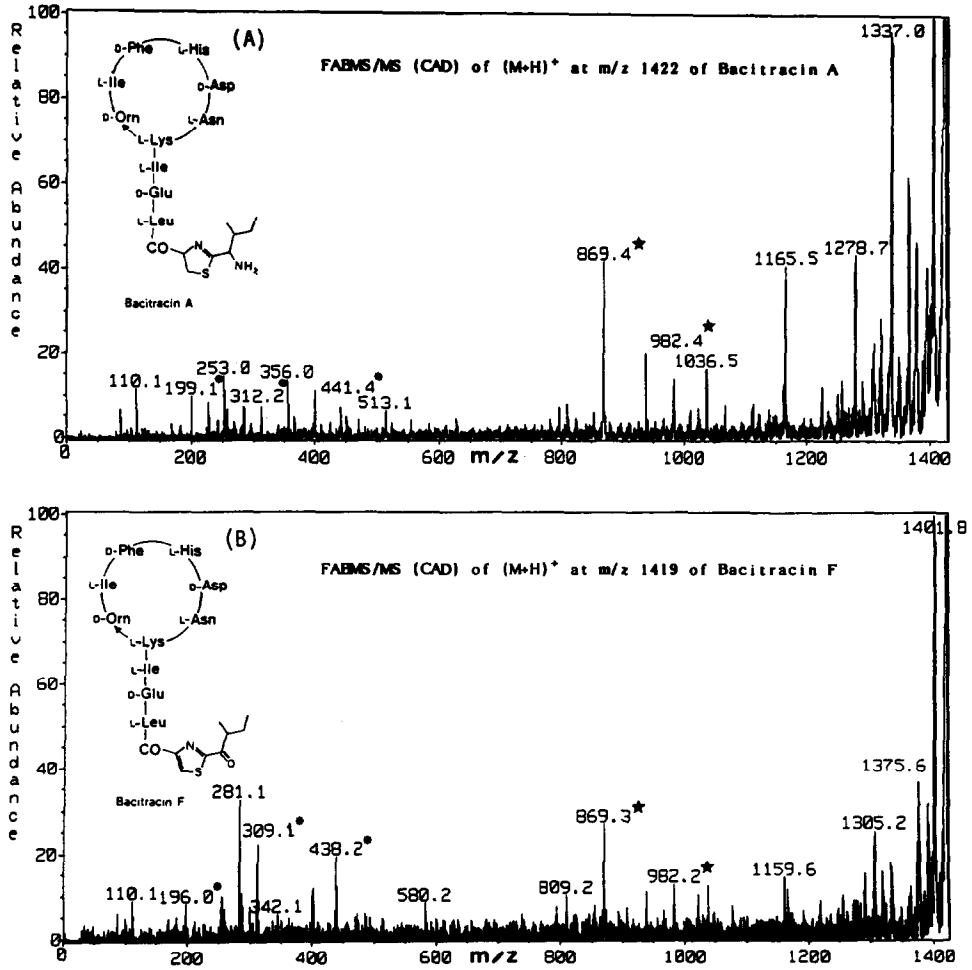

Fig. 6. Tandem mass spectra of protonated molecules of (A) bacitracin A and (B) bacitracin F.

little information concerning the peptide sequence was available from the data. MS–MS is frequently used for mixture analysis, identification of ion structure and elucidation of fragmentation. Usually CAD is applied to obtain fragment ions. Initially, the protonated molecules were formed under FAB conditions and selected as the precursor ions. Generally, peptides are cleaved at C–N bonds to yield C- and N-terminal fragment (daughter) ions which are available for sequence determination of constituent amino acids [22]. Fig. 6 shows the tandem mass spectra from the protonated molecules of bacitracins A and F. The fragmentation schemes of both components in the tandem mass spectra under CAD conditions are shown in Fig. 7. These daughter ions are classified into two groups of ions. The common fragment ions at *m/z* 982 and 869 are formed by cleavage at peptide bonds between Ile and Glu and between Lys and Ile, respectively, and are informative sequence ions including the cyclic peptide

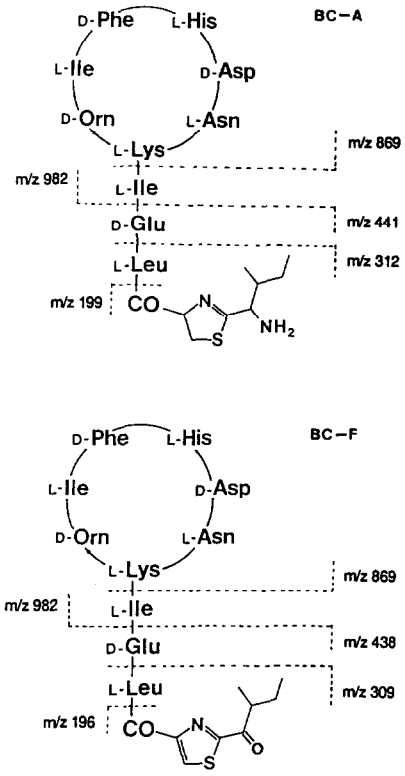

Fig. 7. Sequence ions from the tandem mass spectra of bacitracin A and B under CAD conditions.

moiety. Other ions are derived from the side-chain and there is a difference of 3 mass units in these ions between the components. These results confirm that bacitracins A and F [10] have the structures proposed by Ressler and Kashelikar [9] as shown in Fig. 1 and the data may be useful in the analysis of other unknown components of the bacitracins.

In conclusion, preparative separation and structural determination of the individual components of bacitracin were achieved. First we established HPLC for analysis and next optimized the operating conditions for HSCCC, so that bacitracins A and F were effectively isolated. These results indicate that HSCCC is useful for preparative separations. Other solvent systems are currently being examined to separate other components.

ACKNOWLEDGEMENTS

We are grateful to Dr. H. M. Fales, National Institutes of Health, for critically reading the manuscript, to Mr. K. Komori, Shimadzu, for technical assistance and to Mr. K. Nojima, JEOL, for the measurement of tandem mass spectra.

REFERENCES

1 G. T. Barry, J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, 175 (1948) 485.
2 D. J. Hanson, *Chem. Eng. News*, 63 (1985) 7.
3 T. Yagasaki, *J. Food Hyg. Soc. Jpn.*, 27 (1986) 451.
4 G. A. Brewer, *Anal. Profiles Drug Subst.*, 9 (1980) 1.
5 G. G. F. Newton and E. P. Abraham, *Biochemistry*, 53 (1953) 597.
6 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 112 (1975) 663.
7 L. C. Craig, W. Hausman and J. R. Weisiger, *J. Biol. Chem.*, 199 (1952) 865.
8 W. Stoffel and L. C. Craig, *J. Am. Chem. Soc.*, 83 (1961) 145.
9 C. Ressler and D. V. Kashelikar, *J. Am. Chem. Soc.*, 88 (1966) 2025.
10 Y. Hirotsu, Y. Nishiuchi and T. Shiba, in N. Izumiya (Editor) *Peptide Chemistry* 1978, Protein Research Foundation, Osaka, 1978, p. 171.
11 W. H. Hausmann, J. R. Weisiger and L. C. Craig, *J. Am. Chem. Soc.*, 77 (1955) 723.
12 L. C. Craig and W. Konigsberg, *J. Org. Chem.*, 22 (1957) 1345.
13 W. Konigsberg and L. C. Craig, *J. Org. Chem.*, 27 (1962) 934.
14 L. C. Craig, W. F. Phillips and M. Burachik, *Biochemistry*, 8 (1969) 2348.
15 K.-I. Harada, I. Kimura, A. Yoshikawa, M. Suzuki, H. Nakazawa, S. Hattori, K. Komori and Y. Ito, *J. Liq. Chromatogr.*, in press.
16 H. Oka, Y. Ikai, N. Kawamura, M. Yamada, K.-I. Harada, Y. Yamazaki and M. Suzuki, *J. Chromatogr.*, 462 (1989) 315.
17 Y. Ito in N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography*, Marcel Dekker, New York, 1988, pp. 79–442.
18 Y. Ito and W. D. Conway, *J. Chromatogr.*, 301 (1984) 405.
19 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
20 Y. Ito, in N. B. Mandava and Y. Ito (Editor) *Countercurrent Chromatography*, Marcel Dekker, New York, 1988, p. 781.
21 H. Oka and Y. Ito, *J. Chromatogr.*, 475 (1989) 229.
22 S. A. Martin, R. S. Johnson, C. E. Costello and K. Biemann, in C. J. McNeal (Editor) *The Analysis of Peptides and Proteins by Mass Spectrometry*, Wiley, New York, 1988, p. 135.