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Separation of WAP-8294A components, a novel anti-methicillin-resistant *Staphylococcus aureus* antibiotic, using high-speed counter-current chromatography

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Abstract

The WAP-8294A complex was isolated from the fermentation broth of *Lysobacter* sp. WAP-8294, whose major component, WAP-8294A2, showed a strong activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* in vitro, and also exhibited a potent activity against MRSA in vivo. The previous separation procedure using the conventional chromatographic methods was laborious and time-consuming, and the recovery of the desired compound was often unsatisfactory. In the present study, high-speed counter-current chromatography (HSCCC) was applied to the separation of the main components of the WAP-8294A complex. Due to the high polarity of the target compounds, we selected a hydrophilic two-phase solvent system composed of *n*-butanol–ethyl acetate–aqueous 0.005 *M* trifluoroacetic acid (1.25:3.75:5, v/v/v) which provided a suitable range of partition coefficient values for these compounds. Although the settling time of this solvent system was much longer than the optimum range, suggesting a low retention of the stationary phase under the standard experimental conditions, the separation was successfully performed at the low flow-rate of 0.5 ml/min. A sample size of 25 mg yielded pure fractions of three components (1–6 mg). The identification of each component was carried out by HPLC and fast atom bombardment mass spectrometry. The method will contribute to the clinical development of WAP-8294A2 as an anti-MRSA agent.

Keywords: Antibiotics; WAP-8294A; Anti-methicillin-resistant *Staphylococcus aureus* agent

1. Introduction

One of the most significant problems in clinical practice is the increase in methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA infection was first reported in Europe in 1961 and has become a serious

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clinical problem as a nosocomial infection [1,2]. Although the cyclic glycopeptide antibiotic, vancomycin, is used for the treatment of MRSA infections, vancomycin-resistant *Enterococci* (VRE) were reported in 1989 [3], and the possibility of the emergence of vancomycin-resistant MRSA (VRSA) strains was suggested by the demonstration of transferability of high-level vancomycin resistance from *E. faecalis* to *S. aureus* in vitro and in vivo [4]. In fact, the clinical isolation of the VRSA Mu50 strain was reported in Japan in 1997 [5], which indicates the urgent need for a new anti-MRSA antibiotic.

We have screened microbial metabolites for anti-MRSA antibiotics and found a novel antibiotic, WAP-8294A, that is a complex of anti-MRSA antibiotics produced by a Gram-negative bacterium *Lysobacter* sp. [6]. The WAP-8294A complex was shown to consist of at least 19 closely related components by high-performance liquid chromatography (HPLC) analysis, in which WAP-8294A2 was present as the major component and A1, A4, Ax8, Ax9 and Ax13 were minor components. The structure of WAP-8294A2 has been elucidated as a cyclic depsipeptide with the molecular mass of 1561 based on two-dimensional nuclear magnetic resonance (NMR) experiments and chemical degradations (Fig. 1) [7]. During the course of isolating each component we had to repeat the laborious chromatographic steps using ODS (octadecylsilanized) silica gel and ion-exchange columns.

In the present study, we applied high-speed counter-current chromatography (HSCCC) [8] to the preparative separation of the three main components of the WAP-8294A complex. Successful separation by HSCCC depends significantly on the selection of a suitable two-phase solvent system which requires the following considerations [9–12]: (1) for satisfactory retention of the stationary phase, the settling time [13] of the solvent systems should be considerably less than 30 s; (2) to avoid excessive solvent waste, the mixture should provide nearly equal volumes of each phase; and (3) for efficient separation, the partition coefficient (K) of the target compound should be close to 1 and the separation factor between the two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be greater than 1.5. We have successfully separated many compounds using HSCCC by optimizing the two-phase solvent system according to the above requirements [14–17]. However, there are many samples for which is difficult to find the ideal operating conditions, and WAP-8294A is a typical example whose settling time of the optimized two-solvent system was 16 min. In this study, the operating conditions of HSCCC for WAP-8294A were optimized and the preparative separation was carried out under optimized conditions.

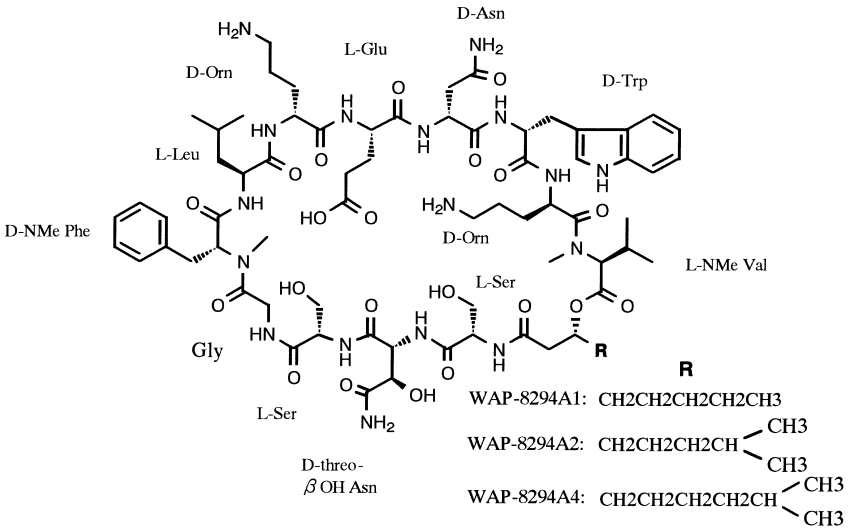

Fig. 1. Structures of WAP-8294A components.

2. Experimental

2.1. Reagents

Acetonitrile, *n*-butanol, ethyl acetate, chloroform, methanol, phosphoric acid, and glycerol were analytical grade and purchased from Nacalai Tesque (Kyoto, Japan). Trifluoroacetic acid (TFA) was obtained from Wako (Osaka, Japan). The WAP-8294A complex was kindly provided by Wakamoto (Tokyo, Japan) and treated according to a previously reported method [6].

2.2. HPLC analysis

HPLC was performed using a constant-flow pump (PU-970) and a variable-wavelength UV–Vis detector (UV-970), both from Jasco (Tokyo, Japan), and a Model C-R6A integrator from Shimadzu (Kyoto, Japan). Separations were carried out on a COSMOSIL $5C_{18}$ (150×4.6 mm I.D., Nacalai Tesque) column heated at 40°C with methanol–acetonitrile–0.01 *M* TFA (57:57:86) as the mobile phase. A flow-rate of 1 ml/min was applied with UV detection at an absorbance of 280 nm.

2.3. Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10-ml test tube to which 2 ml of each phase of the equilibrated two-phase solvent system was added. The tube was vigorously shaken for 1 min to thoroughly equilibrate the sample with the two phases. Equal volumes of each phase were then analyzed by HPLC to obtain the partition coefficient of each component. The *K* value of each component was determined from a pair of its corresponding peaks by dividing the peak height obtained from the upper phase by that from the lower phase.

2.4. HSCCC separation

The apparatus used was an HSCCC-1A prototype multi-layer coil planet centrifuge (Shimadzu) with a 10 cm orbital radius that produces a synchronous type-J planetary motion at 800 rpm. The multi-layer coil was prepared by winding a ca. 160 m length of 1.6 mm I.D. PTFE (polytetrafluoroethylene) tubing onto the column holder with a 10 cm hub diameter and a 15 cm hub length, making six coiled layers with a total capacity of about 300 ml. The two-phase solvent system composed of *n*-butanol–ethyl acetate–0.005 *M* TFA, aqueous solution (pH 2.5) (1.25:3.75:5, v/v/v) was thoroughly equilibrated at room temperature in a separatory funnel by repeating vigorous shaking three times each followed by disposal of the generated gas by inverting the vessel and manipulating its stopcock. First, the column was entirely filled with the upper non-aqueous stationary phase, then 25 mg of the sample dissolved in 1 ml of each phase was loaded. The column was rotated at 780 rpm, while the lower aqueous mobile phase was pumped into the head of the column (the head–tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects with different densities, either lighter or heavier than the surrounding medium, are driven toward the head of the coil) at a flow-rate of 0.5 ml/min with an HPLC pump (LC-6A, Shimadzu). The effluent from the outlet of the column was fractionated into test tubes at 1 ml per tube using a fraction collector. After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas under slow coil rotation in the tail-to-head elution mode.

2.5. Fast atom bombardment mass spectrometry (FAB-MS) analysis

The FAB mass spectra were obtained using a double-focusing mass spectrometer (JMS-HX 110, JEOL, Tokyo, Japan). A xenon ion gun was operated at 10 kV. The matrix used was glycerol.

3. Results and discussion

3.1. Selection of two-phase solvent system

Prior to the HSCCC separation, the HPLC system was established for the WAP-8294A components as shown in Fig. 2, which was effectively used to determine the partition coefficient of the target compounds for selection of the two-phase solvent

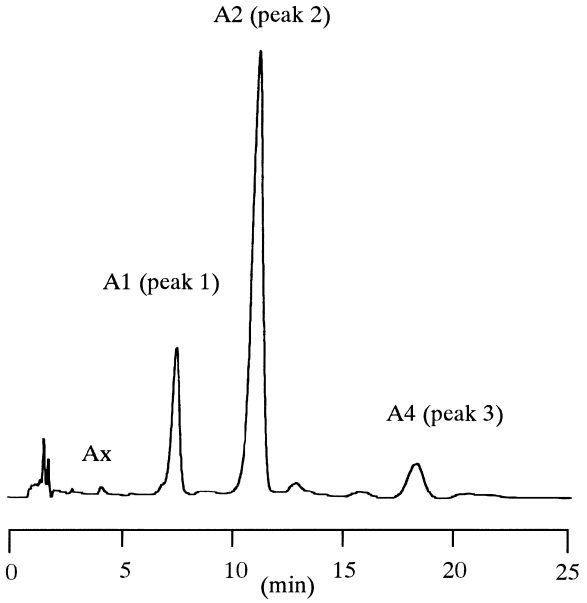

Fig. 2. High-performance liquid chromatogram of WAP-8294A complex. HPLC conditions: column, Cosomosil 5C-18AR (150× 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.01 aq. *M* TFA (57:57:86); detection, UV 280 nm; flow-rate, 1.0 ml/min; column temperature, 40°C.

system and also to analyze the HSCCC fractions in order to evaluate the separation. Peak 2 corresponds to the main component, WAP-8294A2, and two other components, peak 1 (WAP-8294A1) and peak 3 (WAP-8294A4), are also observed (Fig. 2). The component Ax contains at least 13 components, which will be discussed elsewhere. The successful separation by HSCCC largely relies on the selection of a two-phase solvent system that provides good solubility and a proper range of partition coefficients for the target compounds. WAP-8294A is soluble in water, but is not readily soluble in organic solvents such as ethanol, chloroform, ethyl acetate, and *n*-butanol. Therefore, most components of the WAP-8294A were distributed in the aqueous layer of the *n*-butanol or chloroform and water binary system. However, they moved to the organic layer when TFA was added to this solvent system at or above 0.005 *M*, apparently due to protonation of their carboxylic group. This result suggested that one can obtain desirable *K* values by adding the appropriate amount of a hydrophobic modifier to the two-phase solvent system to increase the hydrophobicity of the organic phase. Ethyl acetate was selected as the modifier in this study and a two-phase solvent system composed of *n*-butanol, ethyl acetate and 0.005 *M* TFA were examined at various mutual volume ratios (Table 1).

As mentioned earlier, the *K* value was estimated by a simple test tube experiment followed by HPLC analysis. At a volume ratio of 1:4:5, three components (peaks 1, 2 and 3) were all unilaterally partitioned into the lower phase, but they were mostly distributed in the upper phase at the volume ratio of 2:3:5. These results indicated that the proper *K* values of the target compounds may be obtained by varying the volume ratio of these three solvents between these two extremes. Table 1 summarizes the results. Although the volume ratio at 1.3:3.7:5 gave suitable *K* values around 1, the separation factor (α) between these components was less than 1.5. Finally, it was found that the volume ratio at 1.25:3.75:5

Table 1
Partition coefficients and separation factors (α) of WAP-8294A components

Solvent system (*n*-butanol–ethyl acetate–0.005 *M* TFA)	Peak No.				
	1		2		3
		($\alpha_{1,2}$)		($\alpha_{2,3}$)	
(1:4:5)	0.057	(1.34)	0.076	(1.23)	0.094
(2:3:5)	6.48	(1.70)	11.04	(1.61)	17.8
(1.5:3.5:5)	1.58	(1.61)	2.55	(1.53)	3.91
(1.3:3.7:5)	0.57	(1.29)	0.74	(1.23)	1.0
(1.25:3.75:5)	0.53	(1.68)	0.89	(1.67)	1.5

gave the more evenly dispersed K values around 1 and desirable α values between these three components (Table 1).

3.2. Separation of WAP-8294A complex by HSCCC

As mentioned above, the two-phase solvent system, *n*-butanol–ethyl acetate–0.005 *M* TFA (1.25:3.75:5), was optimized for the separation of the WAP-8294A complex. However, its settling time was 16 min that is much longer than the optimal range (less than 30 s for conventional HSCCC), indicating that the retention of the stationary phase would be insufficient. This problem may be overcome by application of a low flow-rate. Previously, we demonstrated that the slower flow-rate increases the retention of the stationary phase and improved the peak resolution between the target compounds [18]. A 25 mg sample of the WAP-8294A complex was separated using the solvent system composed of *n*-butanol–ethyl acetate–0.005 *M* TFA (1.25:3.75:5) using the lower aqueous phase as the mobile phase. The separation required 13.3 h due to the low flow-rate at 0.5 ml/min. However, the retention of the stationary phase was within an acceptable range of 45.2%. The separated HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 280 nm to draw the elution curve (Fig. 3). As expected, these components were eluted according to the decreasing order of their polarity, i.e., A1> A2 . . . >A4>. Based on the HPLC analysis and the elution curve, all collected fractions were combined into seven pooled fractions. Fig. 4 shows the HPLC analysis of these combined fractions and Table 2 summarizes the amounts of the isolated components in each fraction. As shown in Fig. 4, pure WAP-8294A1, A2 and A4 were eluted in the fractions 2, 4 and 6, respectively. The total recovery was 85%.

The FAB mass spectra of the isolated components (fractions 2, 4 and 6) were measured using glycerol as the matrix. The molecular ion species of these components are summarized together with their composition [9] in Table 3. Finally, they were identified to be WAP-8294A1, A2 and A4 by comparison of their standard samples using HPLC.

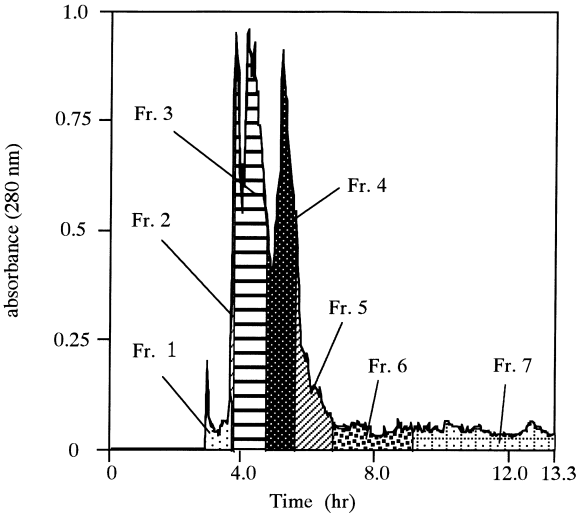

Fig. 3. Separation of WAP-8294A complex by HSCCC. HSCCC conditions: apparatus: type-J multilayer coil planet centrifuge with 10 cm orbital radius; column: multilayer coil consisting of 160 m long, 1.6 mm I.D. PTFE tubing with a total capacity of 300 ml; solvent system, *n*-butanol–ethyl acetate–0.005 *M* aq. TFA (1.25:3.75:5); sample solution, 25 mg in the upper phase (2 ml) and lower phase (2 ml); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 0.5 ml/min; revolution, 780 rpm; retention of the stationary phase measured after separation, 136 ml (45.2%).

4. Conclusion

A new antibiotic agent, the WAP-8294A complex, was separated by HSCCC using a polar two-phase solvent system. The successful separation of its three main components was accomplished in a single run using the following protocol: (1) select a polar binary solvent system such as *n*-butanol–water which provides good solubility to the target compounds; (2) add an organic acid such as TFA to the solvent system to protonate the molecule so that the target compounds are mostly partitioned into the organic phase; (3) add an appropriate amount of a relatively hydrophobic organic solvent such as ethyl acetate to the solvent system to adjust the partition coefficient of the target compounds close to unity; and (4) apply a low flow-rate to the mobile phase to ensure satisfactory retention of the stationary phase, e.g., 0.5 ml/min instead of 3.0 ml/min used in standard HSCCC separations. The above method

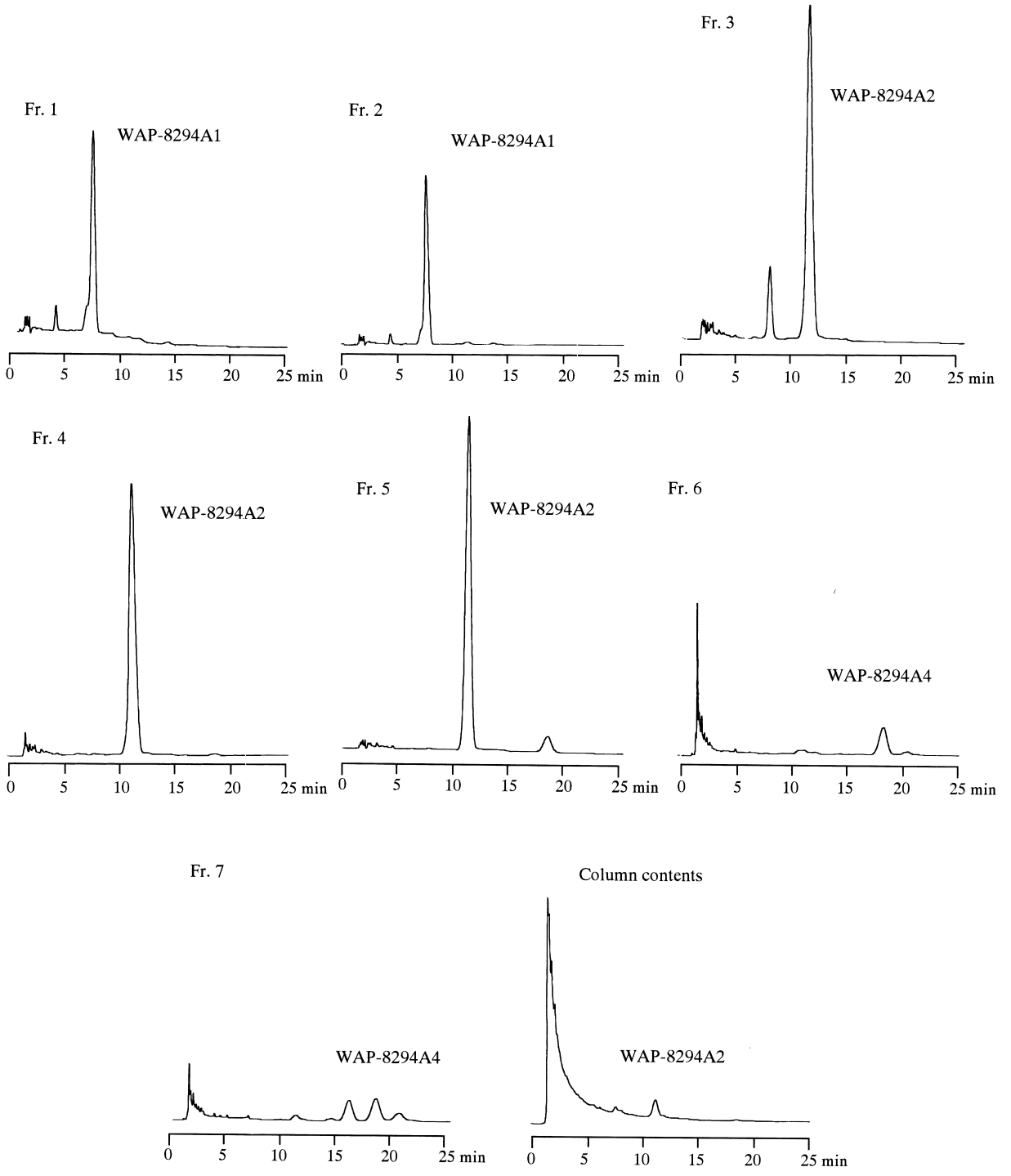

Fig. 4. High-performance liquid chromatograms of the fractions obtained from HSCCC separation of WAP-8294A components. HPLC conditions: see Fig. 2.

Table 2
Amounts of isolated components by HSCCC separation of WAP-8294A complex (25 mg)

Fraction No.	Amount (mg)
1	1.0
2 (WAP-8294A1)	1.8
3	4.2
4 (WAP-8294A2)	6.0
5	2.5
6 (WAP-8294A4)	1.0
7	2.0
Column contents	2.6
Total	21.1

Table 3
Molecular ion species in the FAB mass spectra and composition of the three isolated components

Fraction	$[M+H]^+$ (m/z)	Composition
2 (WAP-8294A1)	1548	$C_{72}H_{109}N_{17}O_{21}$
4 (WAP-8294A2)	1562	$C_{73}H_{111}N_{17}O_{21}$
6 (WAP-8294A4)	1576	$C_{74}H_{113}N_{17}O_{21}$

may work well for other peptide antibiotics that produce problems when using conventional liquid chromatographic methods.

In our study, we confirmed that this HSCCC strategy provides an effective separation of the WAP-8294A components, which will contribute to the clinical development of WAP-8294A2 as an anti-MRSA agent.

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References

[1] M.P. Jevons, Br. Med. J. 1 (1961) 124.
[2] M.E. Mulligan, K.A. Murray-Leisure, B.S. Ribner, H.C. Standiford, J.F. John, J.A. Korvick, C.A. Kauffman, V.L. Yu, Am. J. Med. 94 (1993) 313.
[3] A.A.C. Uttlly, C.R. George, J. Naidoo, N. Woodford, P.A. Johnson, H.C. Collins, D. Morrison, J. Gilfillana, B.L. Fitch, J. Heptonstal, Epidemiol. Infect. 103 (1989) 173.
[4] W.C. Noble, Z. Virani, R.G.R. Gee, FEMS Microbiol. Lett. 72 (1992) 195.
[5] K. Hiramatsu, H. Hanaki, T. Ino, K. Yabuta, T. Oguri, F.C. Tenover, J. Antimicrob. Chemother. 40 (1997) 135.
[6] A. Kato, S. Nakaya, N. Kokubo, Y. Aiba, H. Hirata, K. Fujii, K.-I. Harada, J. Antibiot. 51 (1998) 929.
[7] A. Kato, S. Nakaya, Y. Ohashi, H. Hirata, K. Fujii, K.-I. Harada, J. Am. Chem. Soc. 119 (1997) 6680.
[8] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High Speed Countercurrent Chromatography. John Wiley & Sons, New York, 1996, p. 3, chapter 1.
[9] H. Oka, K.-I. Harada, Y. Ito, Y. Ito, J. Chromatogr. A 812 (1998) 32.
[10] F. Oka, H. Oka, Y. Ito, J. Chromatogr. 538 (1991) 99.
[11] R. Roscher, P. Winterhalter, J. Agric. Food Chem. 41 (1993) 1452.
[12] Y. Ito, in: N.B. Mandava, Y. Ito (Eds.), Countercurrent Chromatography. Theory and Practice, Marcel Dekker, New York, 1988, p. 443, Chapter 4.
[13] Y. Ito, W.D. Conway, J. Chromatogr. 301 (1984) 405.
[14] K.-I. Harada, I. Kimura, A. Yoshikawa, M. Suzuki, H. Nakazawa, S. Hattori, K. Komori, Y. Ito, J. Liq. Chromatogr. 13 (1990) 2373.
[15] K.-I. Harada, Y. Ikai, Y. Yamazaki, H. Oka, M. Suzuki, H. Nakazawa, Y. Ito, J. Chromatogr. 538 (1991) 203.
[16] H. Oka, Y. Ikai, J. Hayakawa, K.-I. Harada, M. Suzuki, A. Shimizu, T. Hayashi, K. Takeba, H. Nakazawa, Y. Ito, J. Chromatogr. A 723 (1996) 61.
[17] H. Oka, K.-I. Harada, M. Suzuki, Y. Ito, J. Chromatogr. A 903 (2000) 93.
[18] H. Oka, Y. Ikai, J. Hayakawa, K.-I. Harada, K. Nagase, M. Suzuki, H. Nakazawa, Y. Ito, J. Liq. Chromatogr. 15 (1992) 2707.