STUDIES ON INHIBITORS OF RAT MAST CELL DEGRANULATION PRODUCED BY MICROORGANISMS

I. SCREENING OF MICROORGANISMS, AND ISOLATION AND PHYSICO-CHEMICAL PROPERTIES OF EUROCIDINS C, D AND E

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Microorganisms producing anti-inflammatory substances were screened by the inhibitory effect on mast cell degranulation. Three new compounds related to pentaene macrolide eurocidins, eurocidins C, D and E, have been isolated from the culture broth of *Streptoverticillium eurocidicum* IFO 13491 as the inhibitors. Their molecular weights and molecular formulae were estimated as 781.89 and $C_{39}H_{59}NO_{15}$ for eurocidin C, 795.92 and $C_{40}H_{61}NO_{15}$ for eurocidin D, and 779.92 and $C_{40}H_{61}NO_{14}$ for eurocidin E, respectively.

By inflammatory stimulations such as heat shock, cold shock, toxins, medicines and antigen-antibody complexes, cytoplasmic granules and various chemical mediators including histamine, serotonin and prostaglandins are released from mast cells, and these materials are known to cause various allergic and inflammatory reactions[1]. Mast cells have been recognized as one of the mediator cells of inflammatory and allergic reactions in animals[2].

YAMASAKI *et al.*[3] reported that the inhibitory activities of drugs against mast cell degranulation *in vitro* show a good correlation with their anti-inflammatory effects *in vivo*, and HIRAI *et al.*[4] reported the screening of anti-inflammatory chinese drugs based on inhibitory effect of histamine release from mast cells. We have developed a simple and fast screening method for microorganisms (culture filtrates and mycelium extracts) by the inhibitory effects on mast cell degranulation induced by compound 48/80[5], a typical inducer of rat peritoneal mast cell degranulation[6]. New anti-allergic and anti-inflammatory substances of microbial origin are expected to find out by this screening method.

In this paper, we report the screening of microorganisms producing anti-inflammatory materials, isolation and physico-chemical properties of new compounds of eurocidin family inhibiting rat mast cell degranulation.

Materials and Methods

Chemicals

Chemicals employed were as follows: Bovine serum albumin (BSA) and compound 48/80 from Sigma

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Chemical Co., St. Louis, U.S.A.; CM-Toyopearl 650M and DEAE-Toyopearl 650M from Tosoh Co., Ltd., Tokyo, Japan; MCI gel CHP20P from Mitsubishi Chemical Industries Limited, Tokyo, Japan; packed column of Lichrosorb RP-8 (5 μm, 125 × 4 mm i.d.) and Lobar column (Lichroprep RP-18, size B) from E. Merck, Darmstadt, FRG; Nucleosil $5C_8$ column (250 × 10 mm i.d.) and Nucleosil $5C_8$ column (250 × 20 mm i.d.) for preparative HPLC from Chemco Co., Osaka, Japan; YMC A-302 column (5 μm, 150 × 4.6 mm i.d.) from Yamamura Chemical Laboratories Co., Kyoto, Japan; and TMS-250 column (75 × 4.6 mm i.d.) from Tosoh Co., Ltd., Japan. All other chemicals were of analytical grade. Reference substance was a gift from Takeda Chemical Industries Ltd., Japan, designated as Eurocidin-T in this paper.

Physico-chemical Studies

The UV spectra were recorded on a Hitachi 220A or a Shimadzu UV250 spectrophotometer in MeOH solutions, and IR spectra on a Jasco A302 IR spectrophotometer with KBr method. Mass spectra were obtained with a Jeol JMS HX100 mass spectrometer.

Cultivation of Microorganisms

Actinomycetes from type cultures and microorganisms isolated from soil samples were cultured at 28~30°C in test tubes for 3~4 days with the culture medium containing glucose 1.5%, Polypeptone 0.5%, meat extract 0.5%, yeast extract 0.5% and NaCl 0.5%, and the pH was adjusted to 7.0.

Cultivations for the production of active substances were carried out in the same medium. The spores of *Streptoverticillium eurocidicum* IFO 13491 were inoculated into 40 ml of a medium in a 200-ml Sakaguchi flask and cultured at 28°C for 3 days on a reciprocal shaker. Twenty ml of the above seed culture was transferred to 1,200 ml of the same medium in a 5-liter Erlenmeyer flask and cultured at 28°C for approximately 40 hours.

Preparation of Rat Peritoneal Mast Cells

Mast cells were collected from peritoneal cavities of normal Wistar rats weighing 150~250 g as described by HIRAI *et al.*[4]. The cells were suspended in TYRODE's solution (NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 1.8 mM, NaH_2PO_4 0.4 mM, $NaHCO_3$ 11.9 mM and glucose 5.6 mM) and layered on BSA-saline solution whose density and pH were adjusted to 1.068 and 7.0, respectively. After centrifugation at 100 × *g* at 4°C for 12 minutes, the bottom layer containing mast cells was washed twice with 5 ml of 0.1% BSA in TYRODE's solution, and suspended in the same medium at about 10^6 cells/ml. The purity of mast cells was about 80~90% as observed with an Olympus phase contrast microscope.

Screening of Microorganisms

Culture filtrates were separated from microbial cells by centrifugation or filtration, added to 10 volumes of phosphate buffered saline (PBS), and then applied to the screening. Microbial cells were extracted with MeOH. The extracts were evaporated to dryness *in vacuo* and the residues were dissolved in PBS to be applied to the screening.

The assay method about screening of microorganisms for the inhibitory effect on mast cell degranulation was as follows; compound 48/80 was used as an inducer of mast cell degranulation. Twenty μl of cell suspension was added to 20 μl of a test sample, and the mixture was incubated for 10 minutes, then another 5 minutes with compound 48/80. The degree of mast cell degranulation was measured by counting the degranulated cells under an Olympus phase contrast microscope (× 400). In the case of the measurement of inhibitory effect on histamine release, those cells were cooled on ice, then centrifuged at 1,500 × *g* for 5 minutes after the incubation. Thirty μl of the supernatant was applied to HPLC as described by HIRAI *et al.*[4]. The primary screening was carried out at 25°C (room temperature) with 1 μg/ml of the inducer, and then the secondary screening for the strains picked up in the primary screening was carried out at 37°C with 2 μg/ml of the inducer.

Results and Discussions

Screening

The primary screening was carried out using the culture filtrates of 2,032 strains of microorganisms

Table 1. Primary screening for culture filtrates of microorganisms.

Microorganisms	Numbers of strains	Inhibitory effects for degranulation				
		+++	++	+	±	−
Actinomycetes (type cultures)	384	16	36	31	76	225
Actinomycetes (from soil)	638	7	15	29	79	508
Bacteria (from soil)	978	0	2	4	15	957
Fungi (from soil)	32	0	0	0	0	32
Total	2,032 (100%)	23 (1%)	53 (3%)	64 (3%)	170 (8%)	1,722 (85%)

from soil samples and our type culture collections (1,022 of actinomycetes, 978 of bacteria and 32 of fungi) as described above (Table 1). Among them, 134 strains of actinomycetes and 6 strains of bacteria showed inhibitory activity against mast cell degranulation. The inhibitory effects were found more frequently in actinomycetes than in bacteria or fungi. In the secondary screening, 8 strains (*Streptoverticillium albireticuli* IFO 12737, *Streptomyces californicus* IFO 12750, *Actinomyces streptomycini* IFO 12918, *Streptomyces ornatus* IFO 13069, *Streptomyces capoamus* IFO 13411, *S. eurocidicum* IFO 13491, *Streptomyces* sp. from soil and *Bacillus* sp. from soil) among the above 140 strains were selected. Finally we decided *S. eurocidicum* IFO 13491 as the most suitable strain for stable production of potent active substances in repetitive cultivation.

Isolation of Active Substances

The inhibitory activity was observed both in the culture filtrate and mycelial cake of *S. eurocidicum* IFO 13491. The active principles were isolated according to the procedures outlined in Fig. 1.

Five liters of the culture broth was centrifuged to give a supernatant fluid and a mycelial cake (about 200 g at wet weight). The active substances in the supernatant were adsorbed to 500 ml of Amberlite XAD-7, and then eluted with 1.5 liters of 80% MeOH after the resin was washed with water and with 20% MeOH. After the eluate was evaporated to dryness, the residue was dissolved in 1 liter of water, adjusted to pH 4.0 and applied to a CM-Toyopearl 650M cation exchange column (272 × 50 mm i.d.) which was pre-equilibrated with 50 mM $AcONH_4$ buffer (pH 4.0) before use. The column was washed with 50 mM $AcONH_4$ buffer (pH 4.0) and eluted with the same buffer (pH 6.0). Three active fractions (F-1, F-2 and F-3) were eluted at pH 4.8, 5.4 and 5.8, respectively (Fig. 2). Each fraction (F-1: 360 ml, F-2: 576 ml and F-3: 648 ml) was adjusted to pH 9.0 and applied to a DEAE-Toyopearl 650M anion exchange column (230 × 25 mm i.d.) which was pre-equilibrated with 50 mM $AcONH_4$ buffer (pH 9.0) before use. The column was washed with 50 mM $AcONH_4$ buffer (pH 9.0) and eluted with the same buffer (pH 7.5). The active fractions were applied to a MCI gel CHP20P column (100 × 12.5 mm i.d.) and the column was washed with water and with 20% acetonitrile, and then eluted with 40% acetonitrile solution. The eluates were concentrated and applied to preparative HPLC using a Nucleosil $5C_8$ column (250 × 20 mm i.d.) with acetonitrile - 10 mM $AcONH_4$ buffer (pH 5.0) (35 : 65) as a mobile phase. F-1, F-2 and F-3 were obtained 1, 7 and 13 mg, respectively, as pale yellow powders.

The mycelial cake (200 g at wet weight) was extracted with 4 liters of MeOH at the room temperature

Fig. 1. Isolation procedures for eurocidins C, D and E.

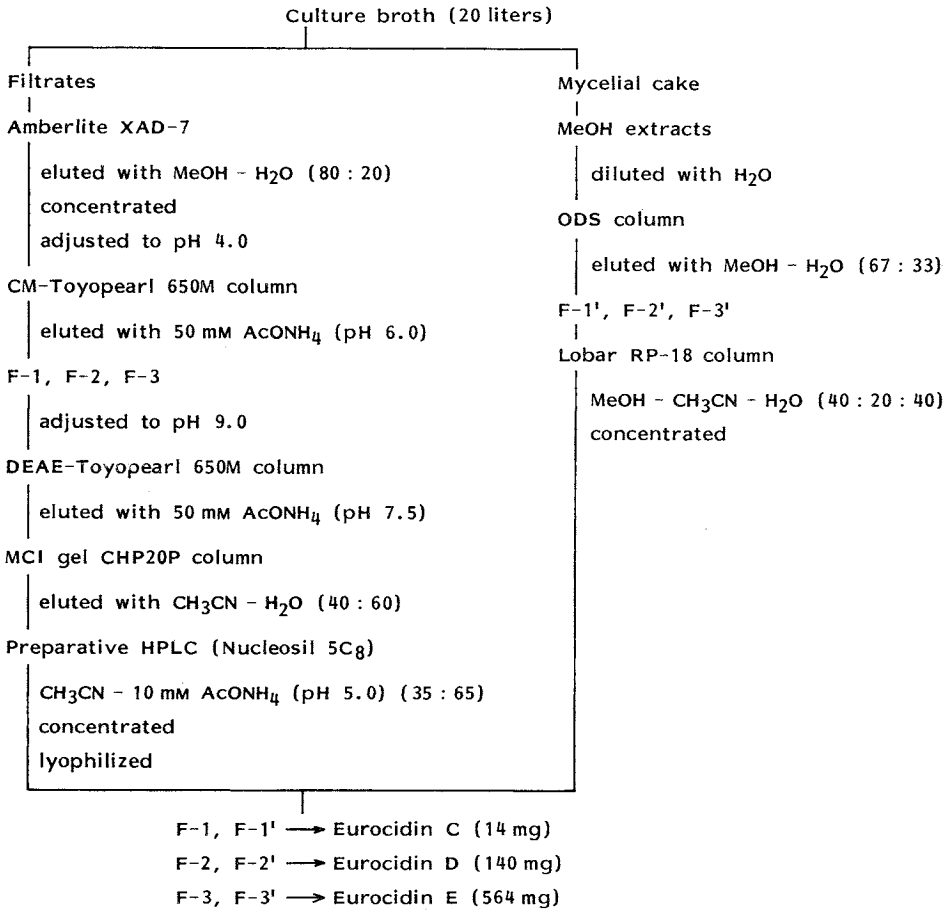
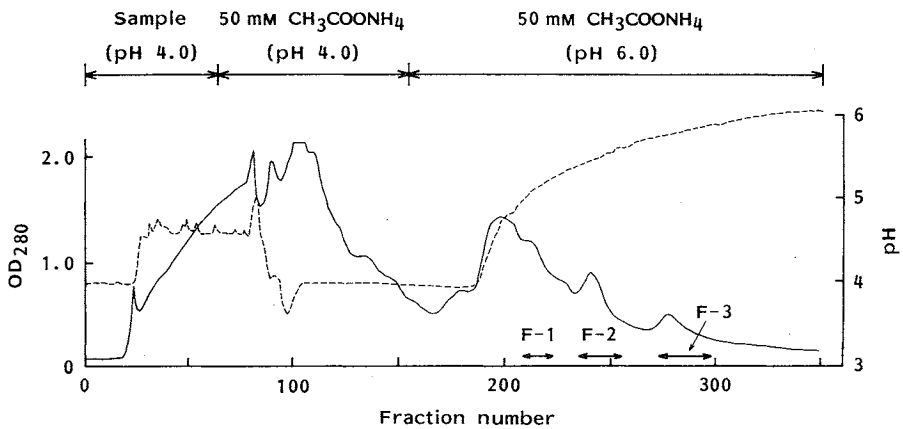


Fig. 2. CM-Toyopearl cation exchange column chromatogram for inhibitors in the culture filtrate.

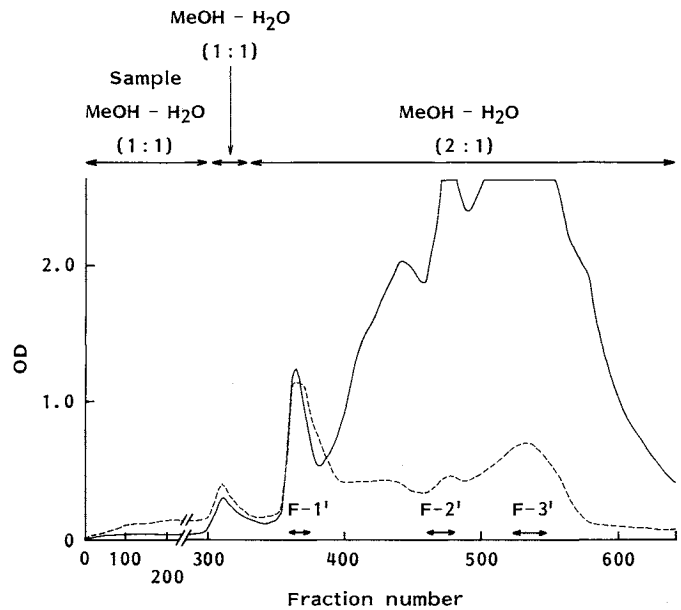
—— OD_{280}, ------ pH.



Column: CM-Toyopearl 650M (272 × 50 mm i.d.), sample: adjusted to pH 4.0, eluent: 50 mM $AcONH_4$ buffer (pH 6.0), flow rate: 96 ml/hour, fraction: 24 ml/tube.

Fig. 3. ODS column chromatogram for inhibitors in the MeOH extracts of mycelial cake.

—— OD_{350}, ------ OD_{280}.



Column: ODS (75~150 μm, 275×60 mm i.d.), sample: dissolved in MeOH-water (1:1), eluent: MeOH-water (2:1), flow rate: 150 ml/hour, fraction: 25 ml/tube.

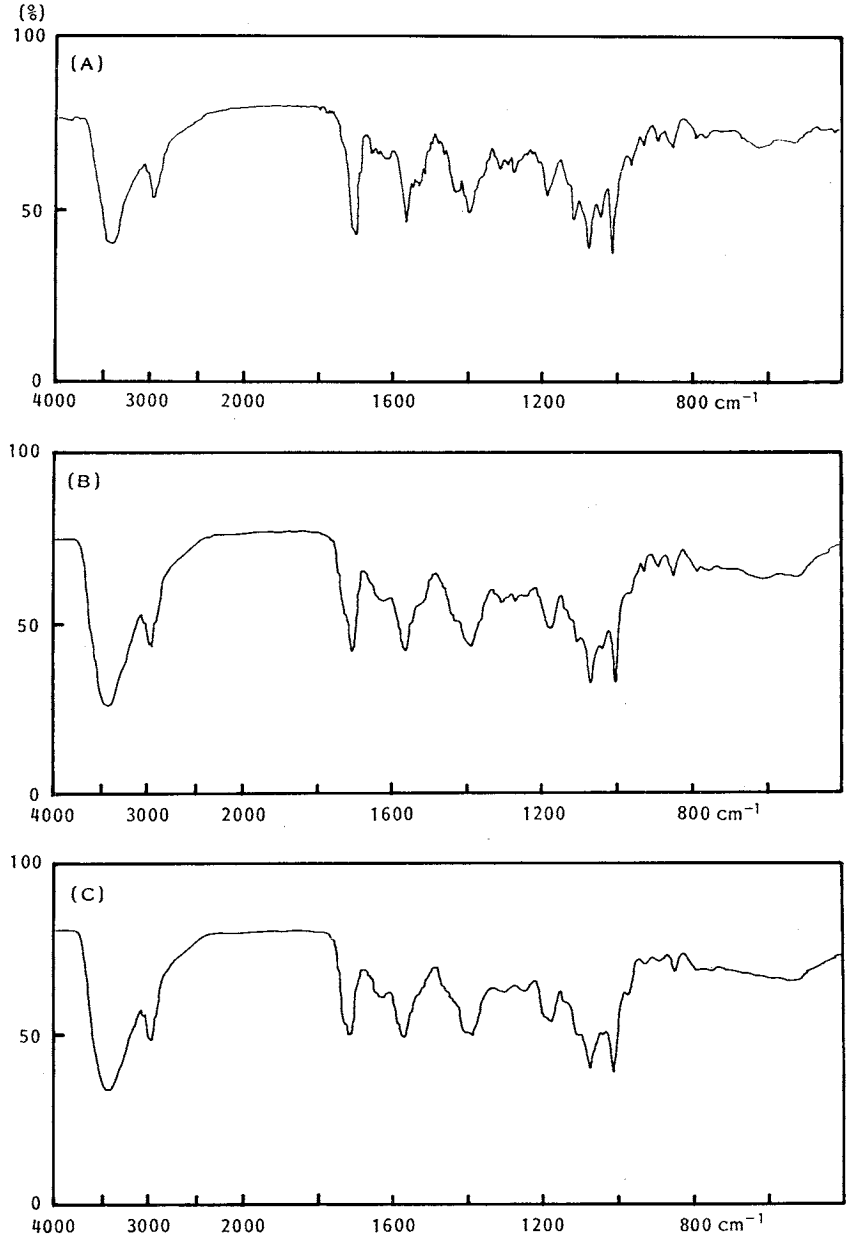
Table 2. Physico-chemical properties of eurocidins C, D and E.

	Eurocidin C (F-1)	Eurocidin D (F-2)	Eurocidin E (F-3)
MP (°C, dec)	135~139	135~139	135~139
Elemental analysis			
Calcd (+$2H_2O$):	C 57.27, H 7.76, N 1.71	C 57.75, H 7.87, N 1.68	C 58.88, H 8.03, N 1.72
Found:	C 57.59, H 7.61, N 1.76	C 57.70, H 7.57, N 1.65	C 58.86, H 8.10, N 1.86
FAB-MS $(M+H)^+$ m/z	782	796	780
Calcd $(M+H)^+$	(782.4)	(796.5)	(780.4)
Molecular formula	$C_{39}H_{59}NO_{15}$	$C_{40}H_{61}NO_{15}$	$C_{40}H_{61}NO_{14}$
MW	781.89	795.92	779.92
IR ν_{max} (KBr) cm^{-1}	3380, 2940, 1700, 1560, 1390, 1180, 1070, 1005	3380, 2940, 1700, 1560, 1380, 1170, 1070, 1000	3380, 2940, 1710, 1560, 1380, 1170, 1070, 1005
UV λ_{max}^{MeOH} nm (ε)	302 (32,600), 316 (69,200), 331 (113,000), 349 (114,000)	302 (31,200), 316 (66,500), 331 (109,000), 349 (111,000)	302 (31,400), 316 (67,000), 331 (110,000), 349 (112,000)

soaking overnight. The extract was diluted with an equal volume of water, and 8 liters of sample solution was applied to a preparative ODS column (275×60 mm i.d.) (Fig. 3). The column was eluted with MeOH-water (2:1) to provide three active fractions (F-1′, F-2′ and F-3′), which were concentrated and purified by a Lobar column with the eluent of MeOH-acetonitrile-water (4:2:4). Each active fraction from F-1′, F-2′ and F-3′ was evaporated to dryness *in vacuo* and was obtained 2.5, 30 and 128 mg, respectively, as pale yellow powders as well as those from the culture filtrate.

Active substances (F-1′, F-2′ and F-3′) isolated from the mycelial cake were combined to F-1, F-2 and F-3, respectively, because their physico-chemical data were identical with each other. F-1 (14 mg), F-2 (140 mg) and F-3 (564 mg) were obtained from 20 liters of a culture broth, as pale yellow powders. F-1,

Fig. 4. IR spectra of eurocidins C (A), D (B) and E (C) (KBr tablet).



F-2 and F-3 showed an inhibitory effect on histamine release from rat mast cells induced by 1 μg/ml of compound 48/80, and IC_{50} values were found to be about 5.0 μg/ml for F-1, 2.2 μg/ml for F-2 and 1.1 μg/ml for F-3, as well as inhibitory activity against mast cell degranulation.

Physico-chemical Properties

Physico-chemical properties of F-1, F-2 and F-3 are summarized in Table 2 and their IR spectra are shown in Fig. 4. From the mass spectral data and elemental analysis, the molecular weights and molecular formulae were estimated 781.89 and $C_{39}H_{59}NO_{15}$ for F-1, 795.92 and $C_{40}H_{61}NO_{15}$ for F-2 and 779.92

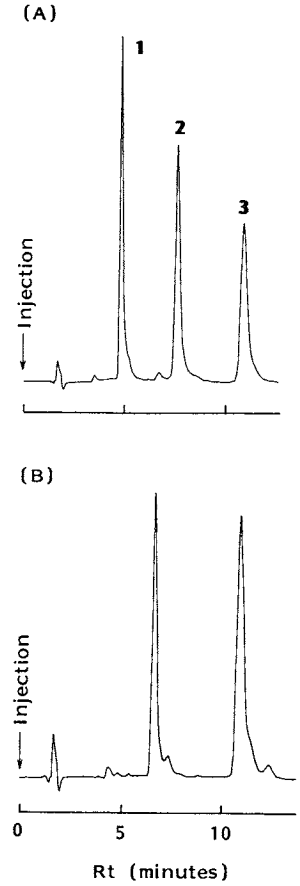
and $C_{40}H_{61}NO_{14}$ for F-3, respectively.

From the UV spectra, F-1, F-2 and F-3 were defined as the typical pentaene of polyene macrolide antibiotics. They were estimated as eurocidin-type compounds from producing microorganisms. Eurocidin was found by NAKAZAWA[7] as an anti-fungal antibiotic produced by *S. albireticuli*, and was designated as Eurocidin-T in this paper. Eurocidin-T was a mixture of several related compounds. HORII *et al.*[8] reported the partial structure of Eurocidin-T as a pentaene containing eurocidins A and B, however the whole structure of any of eurocidins was still unknown.

Eurocidin-T showed two major peaks and some minor peaks when it was subjected to HPLC (Fig. 5B). F-1, F-2 and F-3 isolated in this experiment were detected as almost a single peak (Fig. 5A shows the additive mixture of F-1, F-2 and F-3). The retention time of F-1 or F-2 was different from any component of Eurocidin-T, and only F-3 was eluted at similar retention time to that of one of the two major peaks of Eurocidin-T. The similar result was obtained by further HPLC analyses under different conditions using several different columns and mobile phases (Table 3). Consequently, F-1 and F-2 were decided as the new compounds. F-3 was closely related to a major component of Eurocidin-T, and cannot be distinguished from that of Eurocidin-T chromatographically, which will be clarified by structure elucidation of these compounds. These three compounds belonged to eurocidin family, therefore F-1 and F-2 were named eurocidins C, D, respectively. F-3 was named eurocidin E tentatively, until the identification is performed among F-3 and purified eurocidin A or B from Eurocidin-T.

Fig. 5. HPLC chromatograms of eurocidins C, D and E and Eurocidin-T.

(A)Mixture of eurocidins C (**1**), D (**2**) and E (**3**), (B) Eurocidin-T.



HPLC condition was described in Table 3 (Expt 1).

We isolated eurocidins C, D and E by the inhibitory action of mast cell degranulation induced by compound 48/80, while Eurocidin-T was screened as an antifungal antibiotic. Analogy of physico-chemical properties among eurocidins C, D, E and Eurocidin-T suggests that these compounds possess similar biological activities. Indeed, Eurocidin-T was found to have an inhibitory activity for rat mast cell degranulation. In our screening work for microorganisms, *S. albireticuli* IFO 12737 was found to possess an inhibitory activity against mast cell degranulation, which was estimated eurocidin related compounds (data not shown).

Further details on the structure elucidation of eurocidins D and E and biological activities of these compounds will be described in the succeeding papers[9,10].

Table 3. HPLC retention times of eurocidins C, D and E and Eurocidin-T.

Expt	Column	Mobile phase	Retention time (minutes)				
			Eurocidin			Eurocidin-T	
			C (F-1)	D (F-2)	E (F-3)	1st peak	2nd peak
1	C_8 (analytical)	CH_3CN - $AcONH_4$ (pH5.0) 33 : 67	4.8	7.6	11.0	6.7	11.0
2	C_8 (analytical)	CH_3CN - NaH_2PO_4 (pH 2.9) 35 : 65	8.6	14.3	23.4	13.6	23.4
3	C_8 (analytical)	MeOH - $AcONH_4$ (pH 5.0) 63 : 37	6.4	9.8	12.5	8.3	12.8
4	C_8 (preparative)	CH_3CN - $AcONH_4$ (pH 5.0) 31 : 69	3.0	21.3	31.0	19.0	31.0
5	C_{18}	CH_3CN - $AcONH_4$(pH 5.0) 35 : 65	5.4	9.4	14.3	7.7	14.2
6	C_2	CH_3CN - $AcONH_4$ (pH 5.0) 25 : 75	6.2	9.7	13.1	8.2	13.1

Column: Expt 1~3, Lichrosorb RP-8 (5 μm, 125 × 4 mm i.d.); Expt 4, Nucleosil 5C_8 (5 μm, 250 × 10 mm i.d.); Expt 5, YMC A-302 (5 μm, 150 × 4.6 mm i.d.); and Expt 6, TMS-250 (75 × 4.6 mm i.d.). Mobile phase: Expt 1, 4~6, acetonitrile - 10 mM AcOH - $AcONH_4$ buffer (pH 5.0); Expt 2, acetonitrile - 10 mM H_3PO_4 - NaH_2PO_4 buffer (pH 2.9); and Expt 3, MeOH - 10 mM AcOH - $AcONH_4$ buffer (pH 5.0). Flow rate: Expt 1~3, 5, 1 ml/minute; Expt 4, 4 ml/minute; and Expt 6, 0.8 ml/minute. Detector: UV 350 nm.

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