MURACEINS — MURAMYL PEPTIDES PRODUCED BY *NOCARDIA ORIENTALIS* AS ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

II. ISOLATION AND STRUCTURE DETERMINATION

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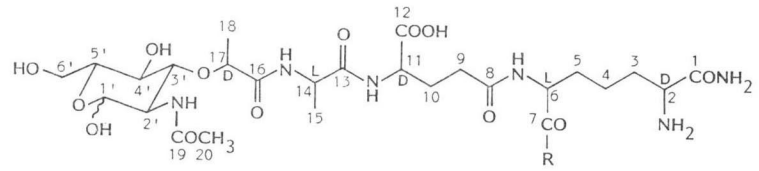
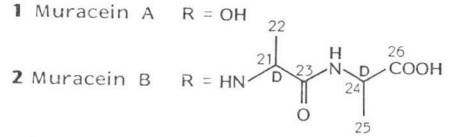
Three new muramyl peptides (muraceins A, B and C) have been isolated from fermentations of *Nocardia orientalis* as inhibitors of angiotensin-converting enzyme (ACE). Structures **1** and **2**, for muraceins A and B respectively, were deduced from their spectroscopic properties. Muracein C, **3**, was shown to be a muramyl pentapeptide containing *N*-acetylmuramic acid, alanine, glutamic acid, serine and diaminopimelic acid in a 1:2:1:1:1 ratio based on the ^{1}H NMR integration.

During the course of a screening program directed toward the isolation and evaluation of angiotensin-converting enzyme (ACE) inhibitors of microbial origin, three new *N*-acetylmuramyl peptides manifesting a specific inhibitory action against ACE were found in the culture broth of *Nocardia orientalis* SC 12,482 (ATCC 39444). Biological and taxonomic studies on the strain ATCC 39444 were presented in the preceding paper[1]. In this paper we describe the isolation and structure elucida-

Fig. 1. Structures of muraceins A, B, C and SQ 28,370.

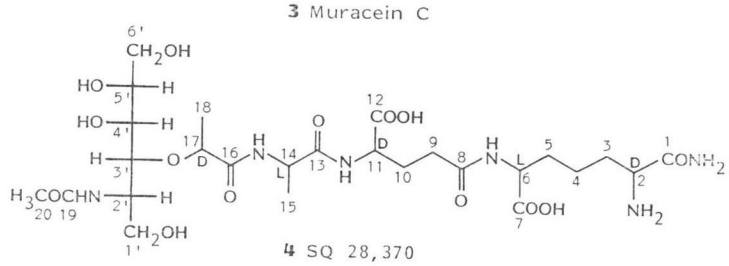


1 Muracein A R = OH



2 Muracein B R = HN

3 Muracein C



4 SQ 28,370

Fig. 2. Isolation of muraceins A, B and C.

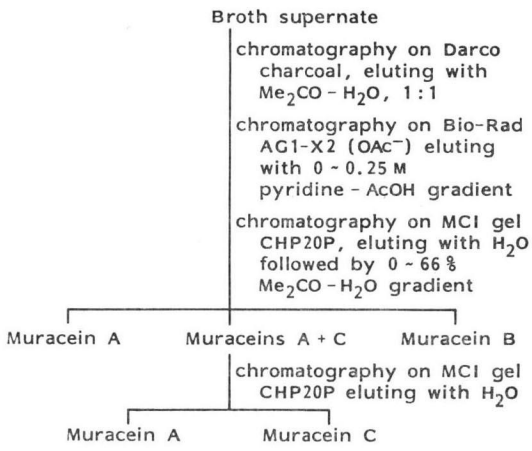


Table 1. Chromatographic behavior of muraceins A, B and C.

		Muracein A	Muracein B	Muracein C
TLC (Rf)[a]		0.19	0.19	0.15
HPLC[b]				
(retention time, minutes)	α	6.2	9.7	8.5
	β	6.9	11.9	10.0

[a] TLC on silica gel using EtOAc - BuOH - AcOH - H_2O, 1:1:1:1, Rydon-visualization.

[b] HPLC using C_{18} spherical packed column (8 mm × 10 cm, 10 μm) with Waters Z-module; 10% CH_3CN, 0.1% 1-heptanesulfonic acid sodium salt in water, isocratic, pH 2.1 with HCl; 1.5 ml/minute; 210 nm.

tion of two muramyl peptides, muracein A (**1**, I_{50}=0.18 μg/ml), muracein B (**2**, I_{50}=145 μg/ml) and characterization of a third muramyl peptide, muracein C (**3**, I_{50}=9.8 μg/ml) (Fig. 1).

Isolation Procedure

The extraction and purification procedure is outlined in Fig. 2. The ACE inhibitory activity was measured spectrophotometrically using captopril as a positive standard, *p*-nitrobenzyloxycarbonyl-glycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine[2] as a chromogenic substrate and partially purified rabbit lung ACE.

The activity which was found in the broth supernate, was adsorbed on a column of Darco granular charcoal, eluted with $Me_2CO - H_2O$ (1:1), and further purified by ion-exchange chromatography on Bio-Rad AG1-X2, OAc^-. The separation of muraceins A, B and C was accomplished by reversed-phase chromatography on MCI gel CHP20P eluting with water followed by a 0~66% $Me_2CO - H_2O$ gradient. The fractions containing muraceins A and C eluted with water; these were combined on the basis of TLC (silica gel, EtOAc - BuOH - AcOH - H_2O, 1:1:1:1, Rydon visualization) and HPLC analysis (Table 1). Muracein B eluted with ~54% $Me_2CO - H_2O$.

Physico-chemical Properties of Muraceins A, B and C

The most active component of the fermentation, muracein A (**1**, monosodium salt, I_{50}=0.18 μg/ml) is a water-soluble, acidic, *N*-acetylmuramyl peptide, mp 171~174°C (dec), $[\alpha]_D^{25}$ +6.7° (*c* 0.46, H_2O). Muracein A has the molecular formula $C_{26}H_{44}N_6O_{14}$ as confirmed by microanalysis and the high resolution fast atom bombardment (FAB)[3] mass spectrum. The UV spectrum showed only end absorption and the IR spectrum exhibited bands at 3300 (OH, NH) and 1650 (CONH) cm^{-1}. Hydrolysis of muracein A in 1 N HCl at 100°C for 5 hours gave a mixture that was separated by ion-exchange chromatography into alanine, glutamic acid, muramic acid and diaminopimelic acid. Alanine and glutamic acid were assigned L- and D-configurations by peak enhancement on gas chromatography with authentic *N*-pentafluoropropionyl-L-alanine isopropyl ester and *N*-pentafluoropropionyl-D-glutamic acid diisopropyl ester, respectively[4,5]. The α,ε-diaminopimelic acid was identified as *meso* by direct comparison of its Rf value with authentic *meso*-α,ε-diaminopimelic acid on cellulose TLC with MeOH - H_2O - 6 N HCl - pyridine (80:26:4:10)[6]. No L,L- or D,D-α,ε-diaminopimelic acid could be detected in the acid hydrolysate. The identity of muramic acid was confirmed by comparison

of its optical rotation and ^{1}H NMR spectrum with those of authentic muramic acid.

The L-carbon of α,ε-diaminopimelic acid was shown to be involved in the peptide linkage by measurement of the optical rotation of the mono-DNP-diaminopimelic acid obtained by the reaction of muracein A with dinitrofluorobenzene, followed by acid hydrolysis (6 N HCl, 100°C, 15 hours) and purification by ion-exchange chromatography[7].

The primary amide was shown to be on the diaminopimelic acid residue by a HOFMANN-type oxidative degradation using bis(1,1-diacetoxy)iodobenzene[8]. Treatment of acetylated muracein A with 1.5 equivalents of bis(1,1-diacetoxy)iodobenzene (18 hours, 25°C), followed by peptide hydrolysis (1 N HCl, 100°C, 5 hours), gave α-aminoadipic acid δ-semialdehyde, the identity of which was established by GC-MS of the trifluoroacetyl 1-butyl ester derivative.

The ^{1}H NMR spectrum of muracein A was complex due to the existence of both α- and β-anomers of the sugar. The signals at δ 4.58 and 5.05 were assigned to C-1′Hβ and C-1′Hα, respectively. A triplet at δ 3.92 (J=6.4 Hz) was attributed to the methine proton of the diaminopimelic acid residue at the position bearing both the free amine and the carboxamide functionalities. Decoupling experiments indicated that the alanyl and muramyl methyl protons (δ 1.30) were coupled to the methine protons at C-14 (δ 4.12) and C-17 (δ 4.20), the C-4 protons (δ 1.40, 1.60) were coupled to each other and to the protons at C-3 and C-5 (δ 1.70~1.90), the proton at C-2 (δ 3.92) was coupled to the C-3 protons (δ 1.70~1.90), the proton at C-11 (δ 4.12) was coupled to the C-10 protons (δ 1.70~1.90, 2.00) and the C-9 (δ 2.20) and C-10 protons (δ 1.70~1.90, 2.00) were coupled to each other.

The ^{13}C NMR spectrum of muracein A was also complex due to the existence of the α- and β-anomeric sugars.

A mass spectrum of muracein A was obtained by FAB mass spectrometry, which gave peaks at m/z 665 and 687 in the positive-ion mode and at m/z 663 and 685 in the negative-ion mode, indicating a molecular weight of 664 and 686 for the free acid and monosodium salt, respectively. The major fragment at m/z 462 (loss of 203) in the positive-ion FAB mass spectrum corresponded to the loss of the muramic acid part ($C_8H_{13}NO_5$) of the molecule. A mass-analyzed ion kinetic energy (MIKE) spectrum[9] of the $(M-H)^-$ 663 ion also indicated the loss of $C_8H_{13}NO_5$ in addition to losses of NH_3, H_2O and CO_2.

In order to simplify the ^{1}H and ^{13}C NMR spectra of muracein A, a sample of muracein A (42.2 mg) was reduced with sodium borohydride and purified by reversed-phase chromatography on MCI gel CHP20P to give SQ 28,370 (**4**, 45.2 mg, I_{50}= 0.08 μg/ml). The ^{1}H NMR data for SQ 28,370 and assignments made with the aid of decoupling experiments are given in Table 2. The decoupling experiments indicated that the alanyl and C-18 methyl protons (δ 1.30) were coupled to the methine protons at C-14 (δ 4.13) and C-17 (δ 4.20), the C-4 protons (δ 1.35, 1.65) were coupled to each other and to the protons at C-3 and C-5 (δ 1.70~1.90), the proton at C-2 (δ 3.92) was coupled to the C-3 protons (δ 1.70~1.90), the proton at C-11 (δ 4.13) was coupled to the C-10 protons (δ 1.70~1.90, 2.00) and the C-9 (δ 2.20) and C-10

Table 2. ^{1}H NMR data for SQ 28,370 in D_2O.

Position	δ (J Hz)[a]
15	1.30 (d, 6.7)
18	1.30 (d, 6.7)
4α	1.35 (m)
4β	1.65 (m)
3, 5, 10α, 20	1.70~1.90 (m, s)
10β	2.00 (m)
9	2.20 (tr, 6.4)
2′~6′	3.55~3.82 (m)
2	3.92 (tr, 6.4)
6, 11, 14	4.13 (m)
17	4.20 (q, 6.7)

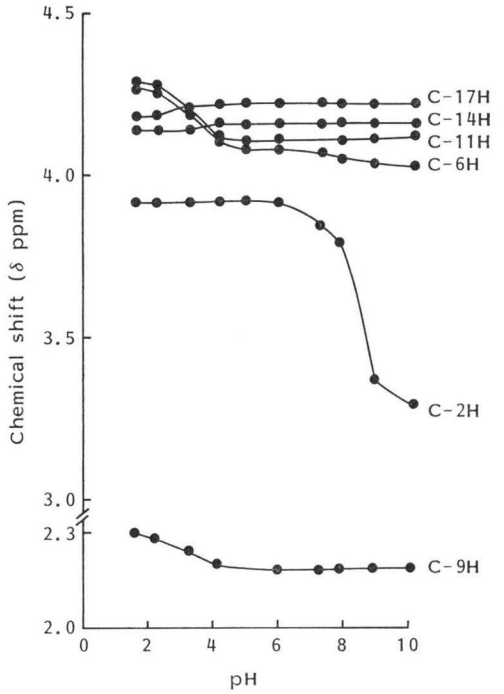
[a] ppm downfield from TMS using HDO (4.73 ppm) as internal standard.

Table 3. ^{13}C NMR data for SQ 28,370 in D_2O.

Chemical shifts (δ)[a]	Carbon type[b]	Assignment
18.0, 19.6	CH_3	C-15, C-18
22.0	CH_2	C-4
23.3	CH_3	C-20
29.1, 31.5, 32.2, 33.2	CH_2	C-3, C-5, C-9, C-10
50.7, 53.9, 55.3 (2C), 55.4	CH	C-2, C-6, C-11, C-14, C-2′
61.8, 64.1	CH_2	C-1′, C-6′
71.0, 71.5, 77.5, 78.3	CH	C-17, C-3′, C-4′, C-5′
172.5 (2C), 174.4, 175.2, 176.3, 177.7, 178.7	C=O	C-1, C-7, C-8, C-12, C-13, C-16, C-19

[a] ppm downfield from TMS, using dioxane (67.6 ppm) as internal standard.
[b] Assignments are made by the INEPT technique.

Fig. 3. pH dependent ^1H NMR of SQ 28,370.



protons (δ 1.70~1.90, 2.00) were coupled to each other.

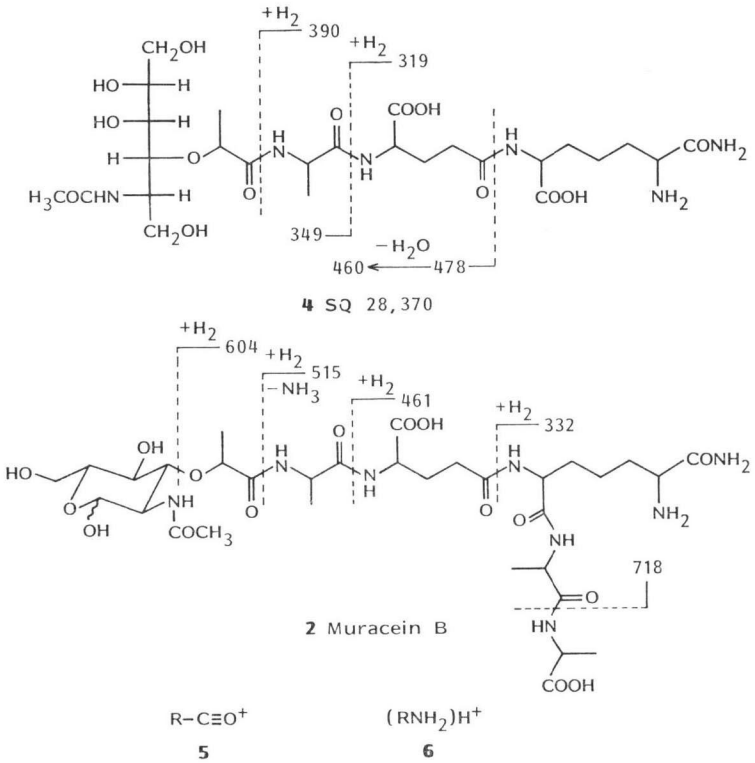
The ^{13}C NMR data is presented in Table 3. The multiplicities of the carbon were determined using the INEPT[10] technique.

The partial sequence of amino acids in SQ 28,370 was determined by pH-dependent NMR spectroscopy. A graph showing the dependence of the chemical shifts[11] of the protons of interest *versus* the pH of the solution is shown in Fig. 3. Analysis of Fig. 3 shows that the C-6 and C-11 protons are adjacent to a free CO_2H (titrate in the pH range 2~4), the C-2 proton is adjacent to a free NH_2 (titrates in the pH range 7~9) and the C-9, C-14 and C-17 protons form the internal peptide backbone (unaffected by the change in pH).

The sequence of amino acids in SQ 28,370 was determined by FAB mass spectrometry. A MIKES[9] scan of peak *m/z* 667 $(M+H)^+$ resulted in two kinds of sequence ions, acylium ions of type **5** and protonated amines of type **6** (Fig. 4). The sequence ions of type **5** occur at *m/z* 349, 478 (460) and of type **6** at *m/z* 319, 390 establishing the sequence shown (Fig. 4). Fragments resulting from the loss of H_2O and the loss of 92 mass units (unexplained) were also observed.

The least active component of the fermentation, muracein B (**2**, monosodium salt, I_{50}=145 μg/ml), is a water-soluble, acidic, *N*-acetylmuramyl peptide, mp 159~165°C (dec), $[\alpha]_D^{25}$ +6.7° (*c* 0.46, H_2O). Muracein B has the molecular formula $C_{32}H_{54}N_8O_{16}$ established by microanalysis and the high resolution FAB mass spectrum. The UV spectrum showed only end absorption and the IR spectrum exhibited bands at 3300 (OH, NH) and 1650 (CONH) cm^{-1}. Hydrolysis of muracein B in 1 N HCl at 100°C for 5 hours gave a mixture that was separated by ion-exchange chromatography into alanine, glutamic acid, muramic acid and diaminopimelic acid. Alanine, as the 2,3,4,6-tetra-*O*-acetyl-β-D-

Fig. 4. Mass spectrometric fragmentations in SQ 28,370 and muracein B.



glucopyranosyl isothiocyanate (GITC)[12] derivative, was shown to be a mixture of the L- and D-isomers in a 1:2 ratio by HPLC. By peak enhancement with authentic glutamic acid GITC derivative, the configuration of glutamic acid in muracein B was shown to be D. The identities of *meso*-α,ε-diaminopimelic acid and muramic acid, the involvement of the L-carbon of α,ε-diaminopimelic acid in the peptide linkage and the position of the primary amide on diaminopimelic acid were determined as described for muracein A.

The ^{1}H NMR spectrum of muracein B was complex due to the existence of the α- and β-anomers of the sugar. The signals at δ 4.58 and 5.05 were assigned to C-1'Hβ and C-1'Hα, respectively. A triplet at δ 3.92 (J=6.4 Hz) was attributed to the methine proton of the diaminopimelic acid residue bearing both the free amine and the carboxamide functionalities. Decoupling experiments indicated that the alanyl and muramyl methyl protons (δ 1.30) were coupled to methines at C-14 (δ 4.12), C-21 (δ 4.20), C-24 (δ 4.20) and C-17 (δ 4.20), the C-4 protons (δ 1.40, 1.60) were coupled to each other and to the protons at C-3 and C-5 (δ 1.70~1.90), the proton at C-2 (δ 3.92) was coupled to the C-3 protons (δ 1.70~1.90), the proton at C-11 (δ 4.12) was coupled to the C-10 protons (δ 1.70~1.90, 2.00) and the protons at C-9 (δ 2.20) and C-10 (δ 1.70~1.90, 2.00) were coupled to each other.

The ^{13}C NMR spectrum of muracein B was also complex due to the existence of α- and β-anomeric sugars.

A mass spectrum of muracein B obtained by FAB mass spectrometry gave peaks at m/z 807 in the positive-ion mode and at m/z 805 in the negative-ion mode, indicating a molecular weight of 806 for the compound. The major fragment at m/z 604 (loss of 203) in the mass spectrum corresponded to the

loss of the muramic acid part ($C_8H_{13}NO_5$) of the molecule.

The sequence of amino acids in muracein B was determined by FAB mass spectrometry. The MIKES[9] spectrum was reminiscent of the SQ 28,370 spectrum. For muracein B, the sequence ions of type **5** occur at *m/z* 718 and of type **6** occur at *m/z* 332, 461 and 515 (loss of NH_3 from 532) establishing the sequence shown in Fig. 4. Fragments resulting from the loss of H_2O and $C_8H_{13}NO_5$ were also observed.

The third component of the fermentation, muracein C (**3**, monosodium salt, I_{50}=9.8 μg/ml), is a water-soluble, acidic, *N*-acetylmuramyl peptide, mp >200°C (dec), $[\alpha]_D^{25}$ +1.1° (*c* 0.34, H_2O). Muracein C has the molecular formula $C_{32}H_{54}N_8O_{17}$ determined by high resolution FAB mass spectrometry. Analysis of the acid hydrolysate (1 N HCl, 100°C, 5 hours) by dansylation[13] followed by TLC analysis on polyamide plates indicated the presence of alanine, glutamic acid, serine, diaminopimelic acid and muramic acid. The identities of alanine, glutamic acid, diaminopimelic acid and serine, as their trifluoroacetyl 1-butyl ester derivatives, were confirmed by GC-MS. Amino acid analysis and a ^1H NMR spectrum indicated that the ratio of alanine, glutamic acid, serine, diaminopimelic acid and muramic acid was 2: 1: 1: 1: 1.

In conclusion, muraceins, a family of closely related muramyl peptides, have been isolated from fermentations of *N. orientalis* as inhibitors of ACE. In contrast to other biologically active muramyl peptides described in the literature[14~17], the position of the primary amide in muraceins is on the diaminopimelic acid residue rather than the glutamic acid residue. The ACE inhibition of muraceins is discussed in the preceding paper[1].

Experimental

NMR spectra were determined on Varian Associates model XL-100-15 and Joel Ltd. GX 400 spectrometers. Infrared spectra were recorded on a Perkin-Elmer model 621 spectrometer. Rotations were measured on a Perkin-Elmer model 141 polarimeter. Mass spectra were determined on a VG Analytical Ltd. model ZAB 1F spectrometer. GC-MS measurements were done on an Extranuclear Laboratories modified Simulscan instrument. The configurations of amino acids were determined either by GC using a chiral column (Altech Associates 25 m glass WCOT column with RSL-007 stationary phase) or by HPLC using a Waters C_{18}-μBondapak column in a Waters Z-module.

Isolation of Muraceins A, B and C

At harvest, the cells from a 20-liter fermentation of *N. orientalis* ATCC 39444 were separated by centrifugation. The broth supernate (19 liters, pH 3.0), was passed through a column of Darco granular charcoal (5×45 cm). The column was washed with water (3 liters) and the active principle eluted with 50% aqueous acetone (4 liters). The active fractions were combined and concentrated *in vacuo* to give a yellow solid (9.97 g). A solution of the solid in water (10 ml) was adjusted to pH 9.0 with 5 M NaOH and then purified by anion-exchange chromatography on Bio-Rad AG1-X2, OAc^-, 200~400 mesh (5×30 cm). The column was washed with 600 ml of water and then eluted with a linear gradient of 0~0.25 M pyridine - AcOH, pH 5.1 (6 liters). The active fractions were combined and concentrated *in vacuo* to give a mixture of muraceins A, B and C as a yellow solid (1.2 g).

The mixture was dissolved in water (1 ml, pH 3.5) and applied to a column of MCI gel CHP20P (2.5×54 cm). The column was eluted with water (600 ml) followed by a 0~66% Me_2CO - H_2O gradient (600 ml, collecting 8 ml fractions). The most active component eluted with water and the fractions were combined on the basis of TLC and HPLC analysis (Table 1). Fractions 44~74, which contained muracein A by TLC and HPLC analysis, were combined and concentrated *in vacuo* to give 121 mg of muracein A (monosodium salt, I_{50}=0.18 μg/ml) as a white powder. ^{13}C NMR (D_2O) δ 18~23 (C-15, C-18, C-20), 22, 29~30 (C-3, C-4, C-5, C-9, C-10), 50~57 (C-2, C-6, C-11, C-14, C-2′),

61.0 (C-6′), 70~83 (C-3′, C-4′, C-5′, C-17), 95.8 (C-1′α), 91.8 (C-1′β) and 172~176 (C-1, C-7, C-8, C-12, C-13, C-16, C-19).

Fractions 75~113, which contained a mixture of muraceins A and C by TLC and HPLC analysis were combined and concentrated *in vacuo* to give 145 mg of a white powder.

A less active band, which eluted with ~54% Me_2CO - H_2O (fractions 162~165), contained muracein B by TLC and HPLC analysis. Fractions 162~165 were combined and concentrated *in vacuo* to give 410 mg of muracein B (monosodium salt, I_{50}=145 μg/ml) as a white powder. ^{13}C NMR (D_2O) δ 18~23 (C-15, C-18, C-20, C-22, C-25), 22, 29~30 (C-3, C-4, C-5, C-9, C-10), 50~57 (C-2, C-6, C-11, C-14, C-21, C-24, C-2′), 61.0 (C-6′), 70~83 (C-3′, C-4′, C-5′, C-17), 95.8 (C-1′α), 91.8 (C-1′β) and 172~176 (C-1, C-7, C-8, C-12, C-13, C-16, C-19, C-23, C-26).

The residue from fractions 75~113 was dissolved in water (0.7 ml, pH 3.5) and applied to another column of MCI gel CHP20P (2.5×50 cm). The column was eluted with 1.2 liters of water collecting 8 ml fractions. Active fractions were combined on the basis of TLC and HPLC analysis. Fractions 30~84 were combined and concentrated *in vacuo* to give 42.2 mg of muracein A (monosodium salt, I_{50}=0.17 μg/ml). Fractions 130~145 were combined and concentrated *in vacuo* to give 5.3 mg of muracein C (monosodium salt, I_{50}=9.8 μg/ml) as a white powder.

Hydrolysis of Muraceins A, B and C

A solution of 10 mg of muracein A in 1.5 ml of 1 N HCl was heated at 110°C for 5 hours and then concentrated *in vacuo*. The residue was chromatographed on a 1.1×24 cm column of Dowex 50W-X2 resin (200~400 mesh, pyridinium form) eluting with a linear gradient prepared from 5% AcOH and 0.75 M pyridinium acetate (pH 5.1), to give glutamic acid (1.5 mg), alanine (0.9 mg), muramic acid (1.5 mg), *meso*-diaminopimelic acid (3.7 mg) and several minor peptide fragments. The ^1H NMR spectra of the hydrolysis products were identical to those of authentic material.

A solution of 8 mg of muracein B in 0.8 ml of 1 N HCl was heated at 110°C for 5 hours and then concentrated *in vacuo*. The residue was chromatographed as previously noted to give glutamic acid (0.7 mg), alanine (0.7 mg), muramic acid (1.5 mg), *meso*-diaminopimelic acid (0.9 mg) and several peptide fragments.

A solution of 1 mg of muracein C in 0.1 ml of 1 N HCl was heated at 110°C for 5 hours and then concentrated *in vacuo*. The residue was converted to the trifluoroacetyl 1-butyl ester derivative and analyzed by GC-MS.

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