Chloptosin, an Apoptosis-Inducing Dimeric Cyclohexapeptide Produced by *Streptomyces*

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In the course of screening for apoptosis-inducing agents, chloptosin (**1**) was isolated from the culture broth of *Streptomyces*. The dumbbell-type structure of the dimeric cyclohexapeptide consisting of D-valine, (3*S*)- and (3*R*)-piperazic acids, *O*-methyl-L-serine, D-threonine, and (2*S*,3a*R*,8a*R*)-6-chloro-3a-hydroxy-2,3,3a,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid was elucidated by spectroscopic and chemical degradation studies. The amino acid components in each cyclohexapeptide domain were presented in alternating *R* and *S* configurations. Chloptosin (**1**) was found to induce apoptotic activity in apoptosis-resistant human pancreatic adenocarcinoma cell line AsPC-1 and showed a strong antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*.

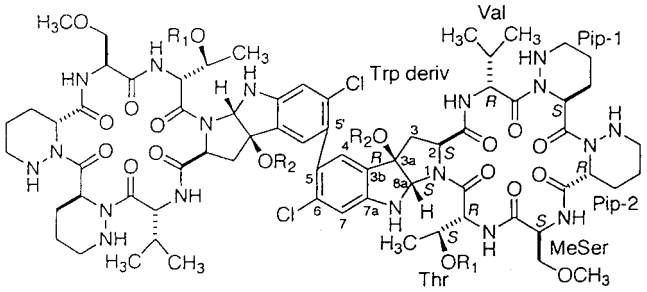
Introduction

Many anticancer agents such as adriamycin[1] and paclitaxel[2] are known to induce apoptosis in cultured neoplastic cells. However, human carcinoma cells are often resistant to apoptosis, and this phenotype may partly explain the poor therapeutic effect of present cancer chemotherapy on most solid tumors. Therefore, we selected apoptosis-resistant human carcinoma cells and began screening for apoptosis-inducing chemicals from nature that would be effective against these types of cell lines. As a result, we isolated the novel cyclic hexadepsipeptides, polyoxypeptins A and B, from *Streptomyces*.[3,4]

In the course of our screening for novel apoptosis inducers, we also isolated the dimeric cyclohexapeptide chloptosin from a polyoxypeptin-producing strain of *Streptomyces*. The structure was determined by extensive NMR spectroscopic analysis and degradation studies, and the results are reported herein. Interestingly, the structure of chloptosin supported the stereochemistry of himastatin,[5] a related cyclic depsipeptide, which was recently corrected by a synthetic method.[6,7]

Results and Discussion

Chloptosin (**1**) was isolated from the culture broth of the polyoxypeptin-producing *Streptomyces* MK498-98F14 strain, as the third metabolite of interest. As described in our previous paper,[3] the whole culture broth (5 L) of the strain was extracted with EtOAc, and the extract was evaporated to yield a residue (1.36 g) containing two cyclohexadepsipeptides, polyoxypeptins A and B, and a new compound, **1**. The residue was applied onto a silica gel column and eluted with EtOAc/hexane (5:3) followed by $CHCl_3$ and then $CHCl_3$/MeOH (150:1 and 100:1). Polyoxypeptins were isolated from the EtOAc/hexane eluate, whereas, concentration of the $CHCl_3$/MeOH eluate gave **1** (181.0 mg) as colorless crystals. However, satisfactory crystals for X-ray analysis could not be obtained.



Chloptosin (**1**) $R_1, R_2 = H$
Diacetylchloptosin (**2**) $R_1 = Ac, R_2 = H$
Tetraacetylchloptosin (**3**) $R_1, R_2 = Ac$

Figure 1. Chloptosin (**1**) and its acetates (**2** and **3**).

The IR spectrum of **1** exhibited typical absorption bands of amides at 1650 and 1529 cm^{-1} but no ester carbonyl band. The molecular formula of **1** was determined to be $C_{68}H_{94}N_{18}O_{18}Cl_2$ by HRFABMS (neg) *m*/*z* 1519.6281 $(M - H)^-$ ($C_{68}H_{93}N_{18}O_{18}Cl_2$ calculated mass 1519.6292). The presence of chlorine was suggested by the isotope abundance peaks in the MS spectrum. ^1H and ^{13}C NMR spectra (Table 1) of **1** showed duplicate signals

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(1) Ling, Y.-H.; Priebe, W.; Perez-Soler, R. *Cancer Res.* **1993**, *53*, 1845−1852.
(2) Haldar, S.; Chintapalli, J.; Croce, C. M. *Cancer Res.* **1996**, *56*, 1253−1255.
(3) (a) Umezawa, K.; Nakazawa, K.; Uemura, T.; Ikeda, Y.; Kondo, S.; Naganawa, H.; Kinoshita, N.; Hashizume, H.; Hamada, M.; Takeuchi, T.; Ohba, S. *Tetrahedron Lett.* **1998**, *39*, 1389−1392. (b) Umezawa, K., Nakazawa, K.; Ikeda, Y.; Naganawa, H.; Kondo, S. *J. Org. Chem.* **1999**, *64*, 3034−3038.
(4) Umezawa, K.; Nakazawa, K.; Uchihata, Y.; Otsuka, M. Screening for Inducers of Apoptosis in Apoptosis-resistant Human Carcinoma Cells. In *Advances in Enzyme Regulation*; Weber, G., Ed.; Elsevier Science: Oxford U.K., 1999; Vol. 39, pp 145−156.
(5) Leet, J. E.; Schroeder, D. R.; Golik, J.; Matson, J. A.; Doyle, T. W.; Lam, K. S.; Hill, S. E.; Lee, M. S.; Whitney, J. L.; Krishnan, B. S. *J. Antibiot.* **1996**, *49*, 299−311.
(6) Kamenecka, T. M.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **1998**, *37*, 2993−2995.
(7) Kamenecka, T. M.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **1998**, *37*, 2995−2998.

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Table 1. ^{1}H and ^{13}C NMR Data for Chloptosin (1)[a]

	position	^{13}C		^{1}H		^{1}H−^{1}H ROESY
Val	CO	173.4	173.5			
	α	55.1 or 55.2	55.0 or 54.9	5.24 dd	5.28 dd	OMe, Pip-1-NH
	β	30.6	30.6	2.11 or 2.15 m	2.11 or 2.15 m	
	γ	19.9	19.9	0.98 d	0.98 d	
	γ′	16.5	16.9	0.84 d	0.86 d	
	NH			7.76 d	7.79 d	
Pip-1	CO	176.7	176.3			
	α	45.6	46.2	6.14 dd	6.08 dd	Thr-OH, Pip-2-NH
	β	25.7	25.8	1.92 m	1.92 m	
				1.93 m	1.93 m	
	γ	20.7 or 20.8	20.7 or 20.5	1.60 m	1.60 m	
				1.79 m	1.79 m	
	δ	47.2	47.2	2.75 m	2.75 m	
				3.15 m	3.15 m	
	δ-NH			5.05 dd	5.04 dd	OMe, Valα
Pip-2	CO	170.4	170.4			
	α	50.2	50.2	4.91 dd	4.88 dd	MeSer-NH
	β	23.2	23.5	1.70 m	1.70 m	
				2.30 br d	2.27 br d	
	γ	20.7 or 20.8	20.7 or 20.5	1.56 m	1.56 m	
				1.99 m	2.03 m	
	δ	47.2	47.2	2.75 m	2.75 m	
				3.18 br d	3.18 br d	
	δ-NH			4.76 dd	4.71 dd	Thr-OH, Pip-1α
MeSer	CO	172.6 or 172.7	172.6 or 172.7			
	α	55.1 or 55.2	55.0 or 54.9	4.40 ddd	4.42 ddd	
	β	71.4	71.4	3.45 dd	3.43 dd	
				3.83 dd	3.85 dd	8a
	OMe	59.5	59.4	3.26 s	3.18 s	3a-OH, Valα, 8a, Pip-1-NH
	NH			7.54 d	7.55 d	Pip-2α
Thr	CO	170.5	170.5			
	α	55.3	55.7	4.84 d	4.82 d	8a, 8-NH
	β	66.0	66.0	4.65 dq	4.69 dq	
	γ	17.5	17.5	1.09 d	1.08 d	
	OH			4.07 br s	4.10 br s	Pip-1α, Pip-2-NH
	NH			6.49 d	6.52 d	
Trp	CO	172.6 or 172.7	172.6 or 172.7			
deriv	2	61.3	61.5	5.30 d	5.32 d	3
	3	39.1	39.0	2.13 m	2.13 m	2
				2.69 d	2.72 d	3a-OH, 2
	3a	90.6	90.4			
	3a-OH			6.06 s	6.14 s	OMe, 8a, 3(2.69, 2.72)
	3b	130.3 or 130.4	130.3			
	4	126.0	126.0	7.18 s	7.18 s	
	5	130.3 or 130.4	130.3			
	6	135.0	134.9			
	7	111.9	111.9	6.79 s	6.77 s	8-NH
	7a	148.4	148.2			
	8-NH			6.19 d	6.07 d	7, Thrα
	8a	86.1	86.4	5.36 d	5.47 d	3a-OH, OMe, MeSer*β*, Thrα

[a] NMR spectra (*δ* ppm) were measured in $CDCl_3$ with a JEOL JNM-A500 spectrometer. Duplicate signals were observed.

(ca. 3:2 ratio), suggesting its dimeric structure. ^{1}H and ^{13}C chemical shift assignments were made by standard 1D and 2D NMR techniques, such as DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and ^{1}H−^{1}H ROESY. By 2D NMR studies, the structure of **1** (Figure 1) was proposed to be a dimer of cyclohexapeptide consisting of valine, two molecules of piperazic acid (Pip-1 and Pip-2), *O*-methylserine (MeSer), threonine, and a chlorinated pyrroloindoline derivative (Trp deriv) considered to be derived from tryptophan.

Acetylation of **1** with acetic anhydride in pyridine gave diacetyl (**2**) and tetraacetylchloptosin (**3**) (Figure 1), the former containing acetylated hydroxyl groups of threonine moieties and the latter having all four hydroxyls acetylated. These structures were determined by FABMS and extensive NMR analyses (Table 2). Because ^{13}C chemical shifts of the biphenyl moiety in **3** were clearly distinguished, the $^nJ_{CH}$ coupling constants[8,9] were measured for determination of the biphenyl bond and the positions of chlorine atoms (Table 3). These couplings were compared with two-bond couplings ($^2J_{C5,H4}$ and $^2J_{C6,H7}$) and three-bond couplings ($^3J_{C5,H7}$ and $^3J_{C6,H4}$) of the authentic 6-chloroindole, and the structure of the dimeric chlorinated pyrroloindoline moiety (Trp deriv) was elucidated.

Acid hydrolysis of **1** with 6 N HCl at 105 °C for 16 h, followed by preparative high-voltage paper electrophoresis (HVPE), gave five known amino acids. Piperazic acid[10] (R_m 0.98) was isolated as a racemic mixture. The chiralities of valine (R_m 0.86), threonine (R_m 0.81), and serine (R_m 0.88) were determined to be D, D, and L, respectively,

(8) Nevalainen, T.; Kolehmainen, E.; Vilen, E. *Magn. Reson. Chem.* **1995**, *33*, 355−366.
(9) Ernst, L.; Wray, V. *J. Magn. Reson.* **1977**, *25*, 123−139.
(10) Bevan, K.; Davies, J. S.; Hassall, C. H.; Morton, R. B.; Phillips, D. A. S. *J. Chem. Soc. C* **1971**, 514−522.

Table 2. ^{1}H and ^{13}C NMR Data for Diacetyl (2) and Tetraacetylchloptosin (3)[a]

		2		3	
	position	^{13}C	^{1}H	^{13}C	^{1}H
Val	CO	174.3		175.3	
	α	56.0	5.15 m	55.7	5.14 dd
	β	30.3	2.41 m	31.0	2.38 m
	γ	20.0	0.97 d	20.2	0.95 d
	γ′	16.0	0.81 d	16.0	0.77 d
	NH		7.84 d		7.67 d
Pip-1	CO	177.7		177.8	
	α	46.8	5.96 dd	47.0	5.89 dd
	β	25.2	1.96 m	25.0	1.98 m
	γ	21.0	1.67 m	21.1[b]	1.65 m
			1.71 m		1.70 m
	δ	46.8	2.77 dddd	46.7	2.80 dddd
			3.13 br d		3.13 br d
	δ-NH		4.98 br d		4.89 br d
Pip-2	CO	171.4		171.3	
	α	51.0	5.00 m	50.9	5.02 dd
	β	22.5	1.68 m	22.5	1.68 m
			2.41 m		2.42 m
	γ	21.0	1.60 m	21.0[b]	1.61 m
			1.87 ddddd		1.85 ddddd
	δ	47.4	2.77 dddd	47.3	2.78 dddd
			3.21 br d		3.22 br d
	δ-NH		5.20 m		5.22 d
MeSer	CO	172.4		172.3	
	α	54.7	4.55 m	54.9	4.60 m
	β	73.0	3.26 m	72.2	3.28 m
			3.80 dd		3.80 dd
	OMe	59.5	3.27 s	59.5	3.28 s
	NH		7.17 d		7.09 d
Thr	CO	168.4		169.0	
	α	54.8	5.20 dd	55.2	5.48 dd
	β	68.8	5.71 dq	69.2	5.71 dd
	γ	18.3	1.42 d	18.4	1.42 d
	NH		6.52 d		6.72 d
	AcCO	172.4		172.4	
	AcMe	20.8	2.08 s	21.3	2.08 s
Trp	CO	172.5		169.5	
deriv	2	60.4	5.29 d	59.6	5.22 d
	3	39.1	2.14 dd	33.4	2.38 m
			2.78 d		3.68 d
	3a	90.7		96.1	
	3a-OH		5.84 s		
	3b	130.5		128.5	
	4	126.2	7.22 s	129.2	7.46 s
	5	130.8		130.4	
	6	135.2		135.9	
	7	112.3	6.83 s	112.4	6.88 s
	7a	148.7		149.9	
	8-NH		6.12 d		6.43 d
	8a	85.4	5.17 m	81.9	5.42 d
	AcCO			169.6	
	AcMe			21.9	1.97 s

[a] NMR spectra (*δ* ppm) were measured in $CDCl_3$ with a JEOL JNM-A500 spectrometer. The major peaks of duplicate signals are shown. [b] Assignments are exchangeable.

by application onto a chiral HPTLC plate. Threonine was differentiated from allothreonine by cellulose TLC. Interestingly, acid hydrolysis of authentic *O*-methyl-DL-serine was found to partially generate serine under the same condition. Therefore, it was decided that the *O*-methylserine residue in **1** was in the L configuration.

Mild acid hydrolysis of **1** with 6 N HCl at 105 °C for 4 h, followed by purification by reverse-phase column chromatography, gave (3*RS*)-piperazic acid, piperazic acid anhydride (4a*R*,8a*S*-perhydro-1,5,9a,10a-tetraazaanthracen-9,10-dione, **4**), and valylpiperazic acid (**5**) (Figure 2). Finally, the stereochemical structural determination of **1** was accomplished by further degradation of the isolated **5**. Acid hydrolysis of **5**, followed by preparation

Table 3. Comparison of $^nJ_{CH}$ Coupling Constants of Tetraacetylchloptosin (3) and 6-Chloroindole[a]

	Trp deriv in **3**			6-chloroindole		
position	^{13}C	^{1}H	$^nJ_{CH}$ (Hz)	^{13}C	^{1}H	$^nJ_{CH}$ (Hz)
4 or 4′	129.24	7.46		121.55	7.54	
	129.35	7.50				
5 or 5′	130.35			120.59	7.09	$^1J_{C5,H5} = 164.5$
	130.36		$^2J_{C5,H4} =$ $^2J_{C5',H4'} =$ 1.0			$^2J_{C5,H4} = 1.0$
			$^3J_{C5,H7} =$ $^3J_{C5',H7'} =$ 4.4			$^3J_{C5,H7} = 4.9$
			$^3J_{C5,H4'} =$ $^3J_{C5',H4} =$ 4.4			
6 or 6′	135.89			127.86		$^2J_{C6,H5} = 2.8$
	135.97		$^2J_{C6,H7} =$ $^2J_{C6',H7'} =$ 4.4			$^2J_{C6,H7} = 5.0$
			$^3J_{C6,H4} =$ $^3J_{C6',H4'} =$ 10.2			$^3J_{C6,H4} = 11.5$
7 or 7′	112.38	6.88		110.94	7.37	
	112.46					

[a] ^{1}H and ^{13}C NMR spectra (*δ* ppm) were measured in $CDCl_3$ (50 mM **3** and 300 mM 6-chloroindole) with a JEOL JNM-A500 spectrometer. Proton-coupled ^{13}C spectra were obtained by a gated decoupling method.

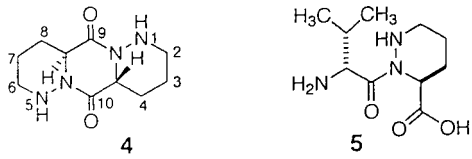

Figure 2. Piperazic acid anhydride (**4**) and valylpiperazic acid (**5**) obtained by mild acid hydrolysis of **1**.

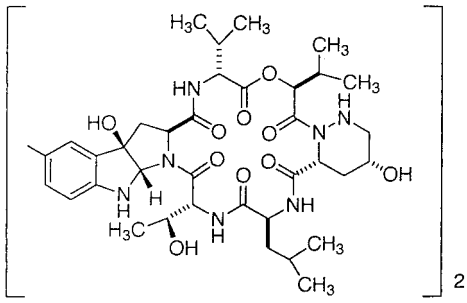

Figure 3. Himastatin.

of its 2,4-dinitrophenyl (DNP) derivative,[10] gave DNP−(3*S*)-piperazic acid, $[\alpha]^{25}_D$ −232° (*c* 0.05, MeOH). Similarly, the other piperazic acid was determined to possess the 3*R* configuration.

Compound **1** is structurally related to himastatin[5] (Figure 3), produced by *Streptomyces hygroscopicus*, but is significantly different in the amino acid content, the absence of an ester moiety, and the presence of chlorine atoms on the pyrroloindoline moieties.[11] The structure of the pyrroloindoline moiety of himastatin was determined to be (2*R*,3a*R*,8a*R*)-3a-hydroxyhexahydropyrrolo-[2,3-*b*]indole-2-carboxylic acid by the extensive NMR studies and CD measurement of the derivative by Leet et al.[5] However, Kamenecka and Danishefsky[6,7] recently

(11) Nakagawa, M.; Kato, S.; Kataoka, S.; Kodato, S.; Watanabe, H.; Okajima, H.; Hino, T.; Witkop, B. *Chem. Pharm. Bull.* **1981**, *29*, 1013−1026.

revised the stereochemistry at C-2 in the pyrrolo[2,3-*b*]-indoline system into the *S* configuration by their synthetic methods. The ^{1}H chemical shifts of the isopropyl methyl groups (δ 0.98 and 0.84) of **1** were very close to those of the synthetic *syn-cis* pyrroloindoline-D-valinol monomer with 2*S* configuration (δ 0.92 and 0.88). Another synthetic *anti-cis* monomer with 2*R* configuration showed the ^{1}H chemical shifts of the isopropyl methyls at δ 0.78 and 0.62. The *syn-cis* relationship was also confirmed by observation of NOEs between 3a-OH and 8a-H, and between 3a-OH and 3-H (δ 2.69 or 2.72) having a small coupling with 2-H (Table 1). Therefore, the pyrroloindoline moiety of **1** was suggested to be (2*S*,3a*R*,-8a*R*)-6-chloro-3a-hydroxy-2,3,3a,8a-hexahydropyrrolo-[2,3-*b*]indole-2-carboxylic acid (absolute configuration of C-8a or C-8a′ in **1** is *S*, but that in the pyrroloindoline moiety is *R* as a result of the deacylation at N-1). Thus, in the chemically elucidated structure of **1**, the amino acid components in each cyclohexapeptide domain are presented in alternating *R* and *S* configurations. The dumbbell-type dimer structure of **1**, like that of himastatin,[5] was confirmed by the fragment peak at *m*/*z* 761 $(M/2 + H)^+$ in FABMS (pos), which can be derived from the fragmentation of the 5,5′-biphenyl linkage.[12] A half of the molecular ion peak at *m*/*z* 803 $(M/2 + H)^+$ was also observed in the FABMS (pos) spectrum of **2**.

Chloptosin **1** induced the loss of viability in apoptosis-resistant AsPC-1 cells[13] with an EC_{50} of 2.5 μg/mL after 24 h. It also induced loss of viability in apoptosis-sensitive human pancreatic adenocarcinoma BxPC-3, human fibrosarcoma HT1080, and human T cell leukemia Jurkat cells with EC_{50} values of 0.49, 0.07, and 0.20 μg/mL, respectively. Furthermore, it inhibited the growth of AsPC-1, BxPC-3, HT1080, and Jurkat cells with IC_{50} values of 50, 12, 5.4, and 6.4 ng/mL, respectively, assessed after 48 h of treatment. Compound **1** induced nuclear fragmentation after 24 h and internucleosomal DNA fragmentation after 18 h at 3−10 μg/mL in AsPC-1 cells, showing the induction of apoptosis. Chloptosin-induced apoptosis was not suppressed by the addition of 100 μM *N*-(benzyloxycarbonyl)-L-aspartyl-(2,6-dichlorobenzoyl)oxymethane (Z-Asp-CH_2-DCB), an inhibitor of caspases,[14] in AsPC-1 cells. However, Z-Asp-CH_2-DCB partially inhibited chloptosin-induced apoptosis in human T cell leukemia Jurkat cells, indicating that caspases would be involved in the mechanism of apoptosis in this cell line. The effects on macromolecular synthesis were studied in terms of cellular incorporation of radioactively labeled thymidine, uridine, and leucine in 1 h. Compound **1** selectively inhibited RNA synthesis at 0.1−1.0 μg/mL in AsPC-1 cells. Chloptosin **1** also strongly inhibited the growth of Gram-positive bacteria, such as *Staphylococcus*, *Micrococcus*, and *Bacillus* strains (MIC range 0.20−0.78 μg/mL), but not that of Gram-negative bacteria and was very effective against MRSA (MIC range 0.025−1.56 μg/mL, MIC_{50} 0.78 μg/mL, MIC_{90} 0.78 μg/mL). When a solution of **1** was administered intraperitoneally into 4-week old ICR male mice, the LD_{50} value was 50−100 mg/kg.

Experimental Section

General Procedures. TLC was performed on two types of silica gel plates: TLC-a, Merck Art.5715 plate developed with $CHCl_3$/MeOH, 20:1, and stained with phosphomolybdic acid/H_2SO_4 reagent composed of $Na_2MoO_4 \cdot 2H_2O$ (12 g), 85% H_3PO_4 (7.5 mL), concd H_2SO_4 (25 mL), and H_2O (500 mL); or TLC-b, Merck Art.5626 plate developed with PrOH/H_2O, 7:3, and stained with ninhydrin spray (Wako Pure Chemical). Chirality of amino acids was determined with a chiral HPTLC plate (Merck, HPTLC plate CHIR Art.14101) developed with MeCN/MeOH/H_2O, 4:1:1, and the R_f value was compared with that of the authentic D- or L-amino acid. High-voltage paper electrophoresis[15] (HVPE) was carried out with a CAMAG HVE system at 3300 V for 15 min, with HCOOH/CH_3COOH/H_2O (25:75:900, pH 1.8) used as an electrolyte solution; the paper was illuminated by a UV lamp (254 nm) or was stained with ninhydrin and Rydon−Smith[16] reagents. The relative mobility (*Rm*) to alanine was calculated.

Producing Strain and Fermentation. *Streptomyces* strain MK498-98F14[3] was isolated from a soil sample collected in Aomori Prefecture, Japan, in 1995 and deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan, under the accession numbers FERM P-16200 and FERM BP-6874. Mycelia of strain MK498-98F14 were inoculated into a 500-mL Erlenmeyer flask containing 110 mL of a medium composed of 2.0% galactose, 2.0% dextrin (Wako Pure Chemical), 1.0% Bacto-Soytone (Difco), 0.5% corn steep liquor (Ajinomoto), 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ (adjusted to pH 7.4) and cultured at 27 °C for 3 d on a rotatory shaker at 180 rpm. The seed culture (2.2 mL) was transferred to each of 46 Erlenmeyer flasks, each containing 110 mL of a medium composed of 1.0% potato starch (Yoshida Seiyaku), 1.0% glucose, 1.0% glycerol, 0.5% peptone (Polypepton, Wako Pure Chemical), 0.5% meat extract (Kyokuto), 0.5% NaCl, and 0.32% $CaCO_3$ (adjusted to pH 7.4). The culture was incubated at 27 °C for 6 d on a rotatory shaker at 180 rpm.

Isolation of Chloptosin (1). The whole cultured broth (pH 7.1, 5 L) was extracted with an equal volume of EtOAc, and the extract was evaporated to a syrup (1.36 g). The syrup was purified by column chromatography on silica gel (Wako Pure Chemical, C-200, 100 g), which was eluted stepwise with EtOAc/hexane (5:3, 1050 mL, fractions 1−78), $CHCl_3$ (250 mL, fractions 79−99), $CHCl_3$/MeOH (150:1, 1085 mL, fractions 100−200), and then $CHCl_3$/MeOH (100:1, 1034 mL, fractions 201−279). The eluate fractions 17−26 were combined and concentrated to give a crude solid of polyoxypeptin B (84.9 mg), which showed an R_f of 0.65 (TLC-a). Concentration of the next eluate fractions, 46−85, gave colorless crystals of polyoxypeptin A (443.2 mg) having an R_f of 0.59 (TLC-a). Concentration of the third eluate fractions, 214−265 (TLC-a, R_f 0.39), gave colorless crystals of **1** (181 mg), which were recrystallized from EtOAc/Me_2CO (7:3): mp >260 °C; $[\alpha]^{26}_D$ −16° (*c* 0.25, $CHCl_3$); UV λ_{max}^{MeOH} (ϵ) 213 nm (98570), 256 nm (23710), 299 nm (sh, 7900), $\lambda_{max}^{0.1\ N\ HCl-MeOH}$ (ϵ) 214 nm (105790), 256 nm (26140), 299 nm (sh, 9120), $\lambda_{max}^{0.1\ N\ NaOH-MeOH}$ (ϵ) 258 nm (23560), 299 nm (sh, 8390); IR ν_{max} (KBr) 3406, 2933, 1650 (amide), 1529 (amide), 1477, 1442, 1408, 1346, 1296, 1245, 1155, 1115, 920, 573 cm^{-1}; FABMS (pos) *m*/*z* 1543 $(M + Na)^+$, 1521 $(M + H)^+$, 761 $(M/2 + H)^+$; FABMS (neg) *m*/*z* 1519 $(M - H)^-$; HRFABMS (neg) *m*/*z* 1519.6281 $(M - H)^-$ ($C_{68}H_{93}N_{18}O_{18}Cl_2$ requires 1519.6292); ^{1}H and ^{13}C NMR data, see Table 1.

Acetylation of 1. To a solution of **1** (310 mg) in dry pyridine (10 mL) was added acetic anhydride (10 mL). After the solution had been kept for 3 d at room temperature, H_2O (100 mL) and $CHCl_3$ (100 mL) were added to the reaction mixture. The organic layer was separated and concentrated to yield a residue (352.3 mg), which was then purified by column chromatography on silica gel (Wakogel C-200, 35 g). After the column had been washed with $CHCl_3$/MeOH (150 mL of each

(12) Jossang, A.; Leboeuf, M.; Cave, A. *J. Nat. Prod.* **1986**, *49*, 1028−1035.
(13) Chen, W. H.; Horoszewicz, J. S.; Leong, S. S.; Shimano, T.; Penetrante, R.; Sanders, W. H.; Berjiann, R.; Douglass, H. O.; Martin, E. W.; Chu, T. M. *In Vitro* **1982**, *18*, 24−34.
(14) Dolle, R. E.; Hoyer, D.; Parasad, C. V. C.; Schmidt, S. J.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. *J. Med. Chem.* **1994**, *37*, 563−564.
(15) Umezawa, H.; Kondo, S. In *Methods in Enzymology. Vol. 43. Antibiotics*; Hash, J. H., Ed.; Academic Press: New York, 1975, pp 279−290.
(16) Rydon, H. N.; Smith, P. W. G. *Nature* **1952**, *169*, 922.

of 200:1 and 150:1), two acetylated compounds showing R_f 0.64 and 0.50 (TLC-a) were eluted with $CHCl_3$/MeOH (100:1). Concentration of the former eluate gave diacetylchloptosin (**2**, 55.7 mg) as colorless microcrystals: mp > 260 °C; $[\alpha]^{23}_D$ −81° (*c* 0.4, $CHCl_3$); UV λ_{max}^{MeOH} (ϵ) 213 nm (65660), 255 nm (16130), 298 nm (sh, 4810), $\lambda_{max}^{0.1\ N\ HCl-MeOH}$ (ϵ) 213 nm (63360), 256 nm (16840), 298 nm (sh, 4810), $\lambda_{max}^{0.1\ N\ NaOH-MeOH}$ (ϵ) 257 nm (17010), 298 nm (sh, 6420); FABMS (pos) *m*/*z* 1627 $(M + Na)^+$, 1605 $(M + H)^+$, 803 $(M/2 + H)^+$; ^1H and ^{13}C NMR data, see Table 2. From the latter eluate, tetraacetylchloptosin (**3**, 153.8 mg) was obtained as colorless microcrystals: mp > 260 °C; $[\alpha]^{23}_D$ −75° (*c* 1.0, $CHCl_3$); UV λ_{max}^{MeOH} (ϵ) 225 nm (29980), 257 nm (17160), 305 nm (sh, 6080), $\lambda_{max}^{0.1N\ HCl-MeOH}$ (ϵ) 218 nm (55070), 255 nm (20260), 300 nm (sh, 8020), $\lambda_{max}^{0.1\ N\ NaOH-MeOH}$ (ϵ) 257 nm (21660), 300 nm (sh, 9710); FABMS (pos) *m*/*z* 1711 $(M + Na)^+$, 1689 $(M + H)^+$; ^1H and ^{13}C NMR data, see Table 2; $^nJ_{CH}$ values, see Table 3.

Acid Hydrolysis of 1. Crystalline **1** (10.1 mg) was hydrolyzed with 6 N HCl (1 mL) in a sealed tube at 105 °C for 16 h. The hydrolysate was diluted with H_2O (5 mL) and washed with diethyl ether (5 mL twice). Evaporation of the aqueous layer gave a residue (13.3 mg) containing several ninhydrin-positive compounds (HVPE R_m 0.96, 0.87, 0.82, 0.76, and 0.45), and the five known amino acids were isolated by preparative HVPE and TLC-b. Concentration of the R_m 0.9 band yielded the racemic piperazic acid (2.1 mg): R_m 0.98 (HVPE); R_f 0.37 (TLC-b); $[\alpha]^{23}_D$ −5° (*c* 0.5, H_2O). Evaporation of the R_m 0.7−0.8 band gave a residue (7.2 mg) showing several ninhydrin-positive spots by TLC-b. Further purification by preparative TLC-b gave the following four amino acids:

D-Valine (1.2 mg) showing R_m 0.86 and R_f 0.46 was obtained, and its chirality was determined by application onto a chiral HPTLC plate (D R_f 0.40, L R_f 0.50).

D-Threonine (0.7 mg) showing R_m 0.81 and R_f 0.33 was identified by Avicel TLC (Funacel SF Cellulose, Funakoshi) developed by an upper layer of BuOH/Me_2CO/28% NH_4OH/H_2O (8:1:1:6) (threonine R_f 0.43, allothreonine R_f 0.35) and by chiral HPTLC (D-threonine R_f 0.44, L-threonine R_f 0.49).

L-Serine (0.6 mg) showing R_m 0.88 and R_f 0.25 was isolated, and its chirality was determined by chiral HPTLC (L R_f 0.41, D R_f 0.38). *O*-Methylserine (1 mg) showing R_m 0.77 and R_f 0.34 was identical with authentic *O*-methyl-DL-serine (Sigma). Acid hydrolysis of the authentic sample generated serine under the hydrolysis conditions. Therefore, the chirality of *O*-methylserine was deduced to be L.

Mild Acid Hydrolysis of 1. Crystalline **1** (799 mg) was hydrolyzed with 6 N HCl (10 mL) in a sealed tube at 105 °C for 4 h and concentrated to give a hydrolysate (1.01 g) that was then purified by column chromatography on ODS silica gel RP-18 (SSC-ODS-7515-12A, Senshu Scientific, 200 mL). The column was developed with 10% MeCN (500 mL, fractions 1−45) and then with 20% MeCN in H_2O (500 mL, fractions 46−86).

A solid (248 mg) obtained from fractions 13 and 14 was further purified by column chromatography on Dowex 50W-X2 (200−400 mesh, pyridine form 300 mL) eluted with 0.1 M pyridine−formate buffer (pH 2.7, 850 mL). The eluate of fractions 56−64 (each 10 mL) was concentrated to give a colorless hygroscopic solid (99.7 mg) of piperazic acid (racemic): HVPE R_m 0.98 (brownish purple by ninhydrin). TLC-b: R_f 0.37; $[\alpha]^{23}_D$ +2° (*c* 0.1, H_2O). Piperazic acid (46.8 mg) was dissolved in a mixture of H_2O (1.8 mL) and EtOH (2 mL) containing $NaHCO_3$ (148 mg). The mixture was added to 2,4-dinitrofluorobenzene (185 mg, Wako Pure Chemical) in EtOH (5 mL) and kept overnight at room temperature. The DNP derivative was purified by diethyl ether extraction, Sephadex LH-20 column chromatography (Pharmacia, MeOH), and then preparative TLC ($CHCl_3$/MeOH, 5:2, R_f 0.17) to give a yellowish solid (10.9 mg): FABMS (pos) *m*/*z* 319 $(M + Na)^+$, 297 $(M + H)^+$; FABMS (neg) *m*/*z* 295 $(M - H)^-$; $[\alpha]^{23}_D$ +30° (*c* 1.0, MeOH) (a racemic mixture). DNP-(3*R*)-piperazic acid obtained from polyoxypeptin A by a similar procedure showed $[\alpha]^{25}_D$ +211° (*c* 1.0, MeOH) (lit.[10] +307°).

Evaporation of the eluate in fractions 21−25 (ODS silica gel column) gave a solid (76 mg) that was separated into MeOH-soluble and MeOH-insoluble fractions by treatment with MeOH (3 mL). From the MeOH-soluble fraction, valylpiperazic acid (**5**) (7 mg) was isolated by preparative TLC and Sephadex LH-20 column chromatography as a colorless solid: TLC-b R_f 0.34 (ninhydrin, Rydon−Smith pos); HVPE R_m 0.80; mp 202−211 °C; $[\alpha]^{24}_D$ −6° (*c* 0.46, DMSO); EIMS *m*/*z* 229 (M^+); ^1H NMR (400 MHz, DMSO-d_6) δ 8.0 (d, *J* = 9 Hz, $ValNH_2$), 4.67, 4.59 (each dd, PipNH), 4.17 (dd, *J* = 9, 5 Hz, Valβ), 3.48 (dd, *J* = 8.4, 3.4 Hz, Pipα), 2.85, 2.74 (m, Pipδ), 2.13 (dqq, *J* = 6.5, 6.5, 5 Hz, Valβ), 1.83, 1.66 (m, Pipβ), 1.66, 1.61 (m, Pipγ), 0.91 (d, *J* = 6.5 Hz, Valγ'), 0.89 (d, *J* = 6.5 Hz, Valγ); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3 (ValCO), 171.8 (PipCO), 57.9 (Pipα), 57.1(Valα), 46.3 (Pipδ), 30.2 (Valβ), 27.2 (Pipβ), 22.7 (Pipγ), 19.3 (Valγ), 17.8 (Valγ'). Acid hydrolysis of **5** (5.5 mg) with 6 N HCl (2 mL) at 105 °C for 19 h gave (3*S*)-piperazic acid, which was changed to its DNP derivative, DNP-(3*S*)-piperazic acid (1.1 mg), by the above-mentioned procedure: TLC ($CHCl_3$/MeOH, 5:2) R_f 0.17; FABMS (pos) *m*/*z* 319 $(M + Na)^+$, 297 $(M + H)^+$; FABMS (neg) *m*/*z* 295 $(M - H)^-$; $[\alpha]^{25}_D$ −232° (*c* 0.05, MeOH).

From the MeOH-insoluble residue, piperazic acid anhydride (**4**) was obtained as colorless microcrystals (44 mg): TLC-b R_f 0.48 (ninhydrin neg, Mo-H_2SO_4 pos); mp 186−189 °C; $[\alpha]^{25}_D$ < −1° (*c* 0.25, MeCN−H_2O, 1:1); EIMS *m*/*z* 224 (M+); FABMS (pos) *m*/*z* 225 $(M + H)^+$ ($C_{10}H_{16}N_4O_2$ requires 224). ^1H NMR (400 MHz, D_2O/CD_3CN, 1:1, 50 °C, TSP δ = 0) δ 4.18 (dd, *J* = 12.4, 2.4 Hz, H4a, H8a), 3.06 (ddd, *J* = 13.0, 3.6, 3.6 Hz, H2eq, H6eq), 2.72 (ddd, *J* = 13.0, 13.0, 3.6 Hz, H2ax, H6ax), 2.28 (m, H4eq, H8eq), 1.90 (ddddd, *J* = 14.0, 4.4, 4.4, 3.6, 3.6 Hz, H3eq, H7eq), 1.81 (ddddd, *J* = 14.0, 13.0, 12.4, 4.4, 3.6 Hz, H3ax, H7ax); ^{13}C NMR (100 MHz, D_2O/CD_3CN, 1:1, 50 °C) δ 164.7 (C9, C10), 60.6 (C4a, C8a), 49.0 (C2, C6), 31.9 (C4, C8), 26.3 (C3, C7). Chemical shifts were assigned by $^1H-^1H$ COSY and HMQC experiments.

Cell Viability. AsPC-1 cells were cultured in Roswel Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 100 μg/mL kanamycin, 100 units/mL penicillin G, and 30 μg/mL glutamine in a 5% CO_2/95% air atmosphere. Cells (1×10^5) were seeded in 24-well culture plates. After 24 h, chemicals or microbial culture broths were added to the cells, and then the cells were incubated for another 24 h. Cell viability was determined by cell counting with a hemocytometer after staining with trypan blue. Surviving cells were expressed as a percentage of the total cell population. The EC_{50} values are means of triplicate determinations.

Nuclear Fragmentation. Cells (2×10^5) were plated on coverslips in 12-well culture plates and treated with **1** for 24 h. Then, the cells were fixed for 15 min with 3% paraformaldehyde solution, and the nuclei were stained for 5 min with the dye Hoechst 33258. The coverslips were examined under a fluorescence microscope.

Antimicrobial activity. In vitro antibacterial activity (minimum inhibitory concentrations, MICs) were determined by the 2-fold agar dilution method with Bacto Mueller−Hinton Medium (Difco) at 37 °C for 18 h, according to the standard method of Japan Society of Chemotherapy. The standard bacterial strains and clinical isolates (60 strains) of methicillin-resistant *Staphylococcus aureus* (MRSA) were used from the stock cultures of Institute of Microbial Chemistry.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of **1**−**3** and selected 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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