Regular Article

Complestatin Exerts Antibacterial Activity by the Inhibition of Fatty Acid Synthesis

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Bacterial enoyl-acyl carrier protein (ACP) reductase has been confirmed as a novel target for antibacterial drug development. In the screening of inhibitors of *Staphylococcus aureus* enoyl-ACP reductase (FabI), complestatin was isolated as a potent inhibitor of *S. aureus* FabI together with neuroprotectin A and chloropeptin I from *Streptomyces chartreusis* AN1542. Complestatin and related compounds inhibited *S. aureus* FabI with IC_{50} of 0.3–0.6 μM. They also prevented the growth of *S. aureus* as well as methicillin-resistance *S. aureus* (MRSA) and quinolone-resistant *S. aureus* (QRSA), with minimum inhibitory concentrations (MICs) of 2–4 μg/mL. Consistent with its FabI-inhibition, complestatin selectively inhibited the intracellular fatty acid synthesis in *S. aureus*, whereas it did not affect the macromolecular biosynthesis of other cellular components, such as DNA, RNA, proteins, and the cell wall. Additionally, supplementation with exogenous fatty acids reversed the antibacterial effect of complestatin, demonstrating that it targets fatty acid synthesis. In this study, we reported that complestatin and related compounds showed potent antibacterial activity *via* inhibiting fatty acid synthesis.

Key words complestatin; enoyl-acyl carrier protein reductase; antibacterial; fatty acid synthesis; *Staphylococcus aureus*

Bacterial fatty acid synthesis (FAS) is an attractive antibacterial target, since FAS is organized differently in bacteria and mammals.[1,2] Fatty acid biosynthesis in bacteria is crucial for the production of a number of lipid-containing components, including the cell membrane. Bacterial enoyl-acyl carrier protein (ACP) reductase, which catalyzes the final and rate-limiting step in bacterial fatty acid synthesis, has been validated as a novel target for the development of antibacterial drugs.[3] Four isoforms, FabI, FabK, FabL, and FabV have been detected in enoyl-ACP reductase. FabI is distributed broadly throughout the majority of bacteria including *S. aureus*, while *Streptococcus pneumoniae* contains only FabK, *Enterococcus faecalis* and *Pseudomonas aeruginosa* contain both FabI and FabK, and *Bacillus subtilis* contain both FabI and FabL. Indeed, FabI has been identified as the antibacterial target of both triclosan,[4] a broad spectrum biocide used in a wide range of consumer goods, and isoniazid,[5] which has been utilized for 50 years in the treatment of tuberculosis. Therefore, inhibitors of *S. aureus* FabI may prove to be interesting lead compounds for the development of effective antibacterial drugs.

In our screening for *S. aureus* FabI inhibitors from microbial metabolites, we isolated complestatin (**1**) together with neuroprotectin A (**2**) and chloropeptin I (**3**) from the mycellium of *Streptomyces chartreusis* AN1542 as potent inhibitors of FabI (Fig. 1). Complestatin has previously been isolated from *S. lavendulae* SANK 60477 as an anti-complement substance,[6] from *Streptomyces* sp. MA7234 as human immunodeficiency virus-1 (HIV-1) integrase inhibitor,[7] and from *Streptomyces* sp. WK-3419 as an inhibitor of gp120-CD4 binding.[8] Chloropeptin I, a structural isomer of complestatin, was isolated along with complestatin (Chloropeptin II).[9] Neuroprotectins A and B, analogs with an oxindole-alanine in place of the tryptophan, have been isolated together with complestatin from *Streptomyces* sp. Q27107 as neuroprotective agents.[10–12] An antimicrobial activity of complestatin and related compounds, however, has not yet been reported. Here, we describe the isolation, FabI-inhibitory, and antibacterial activity of **1**–**3**.

MATERIALS AND METHODS

General Experimental Methods NMR spectra were recorded on a Bruker 300 and 500 spectrometer. The electrospray ionization (ESI)-MS data were recorded with a Jeol JMS-HX110/110 A mass spectrometer. Column chromatography on silica gel (Kieselgel 60, 70–230 mesh, Merck) and Sephadex LH-20 (Amersham Biosciences) were conducted. All chemicals utilized in the study, including methanol (MeOH), ethyl acetate (EtOAc), chloroform ($CHCl_3$), butanol (BuOH), acetonitrile (ACN), and hexane, were of analytical grade. Triclosan, rifampin, norfloxacin, chloramphenicol, vancomycin, trifluoroacetic acid (TFA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, U.S.A.).

Bacterial Strains The actinomycetal strain AN1542 was isolated from soil collected near Gonju city, Chungcheongnam-do, Korea. The strain was identified as *S. chartreusis* based on the 16S ribosomal RNA (rRNA) sequence. The bacterial strains used in antibacterial activity were obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea (CCARM) and the Korean Collection for Type Cultures (KCTC).

Fermentation and Isolation Fermentation was carried out in a liquid culture medium containing soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization). A sample of the strain from a mature plate culture was inoculated into a 500-mL Erlenmeyer flask containing 80 mL of the above sterile seed

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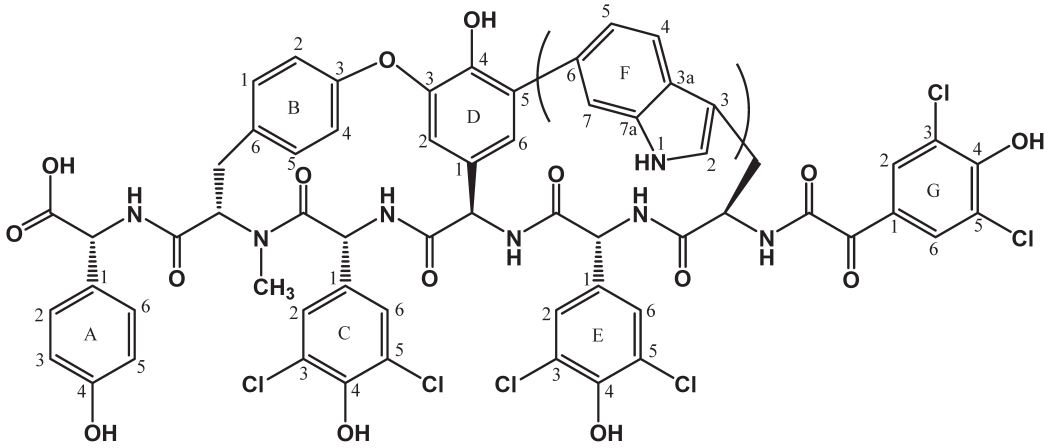

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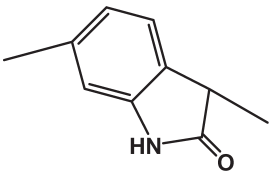

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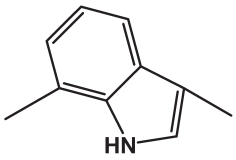

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Fig. 1. Chemical Structures of Complestatin (**1**), Neuroprotectin A (**2**) and Chloropeptin I (**3**)

liquid medium and cultured on a rotary shaker (150 rpm) at 28°C for 3 d. For the production of the active compounds, 5 mL of the seed culture was transferred into 500-mL Erlenmeyer flasks 60 flasks containing 100 mL of the same medium, then cultivated for 7 d at 28°C. The fermented whole medium (6 L) was centrifuged at 6000 rpm for 10 min and then the resultant mycelium was extracted twice with 80% acetone. The extract was concentrated *in vacuo* to an aqueous solution, which was adjusted to pH 3.0 with 1 N HCl and then extracted with an equal volume of ethyl acetate (EtOAc) twice. The EtOAc extract was concentrated *in vacuo* to dryness. The crude extract was subjected to SiO_2 (Merck Art No. 7734.9025) column chromatography followed by stepwise elution with $CHCl_3$–methanol (MeOH) (20 : 1–1 : 1) to give two active fractions (I and II). The active fractions (I) eluted with $CHCl_3$–MeOH (2 : 1) were pooled and concentrated *in vacuo*. The residue (70 mg) was applied again to a Sephadex LH-20 and then eluted with MeOH. The active fractions were pooled and concentrated *in vacuo*. The residue (24 mg) dissolved in MeOH was further purified by HPLC column (10×250 mm, YMC C_{18}) chromatography. The column was eluted with ACN–water (45 : 55) containing 0.025% TFA at a flow rate of 2 mL/min to afford **1** (2.2 mg), **2** (1.9 mg), and **3** (1.8 mg) with retention times of 34.8, 15.8, and 50.3 min, respectively, as yellow powders.

The second active fraction (II) eluted with $CHCl_3$–MeOH (1 : 1) were pooled and concentrated *in vacuo*. The residue (80 mg) was applied to the Sephadex LH-20 column chromatography and eluted with MeOH to give **1** (5 mg) as a yellow powder.

Compound **1**: $C_{61}H_{45}N_7O_{15}Cl_6$; a yellow powder: $[\alpha]_D^{25}$ 22.6° (c=0.13, dimethyl sulfoxide (DMSO)); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.86 (2H, d, J=8.0 Hz, A-2, 6), 6.63 (2H, d, J=8.0 Hz, A-3, 5), 4.67 (1H, brs, A-αCH), 7.88 (1H, d, J=9.0 Hz, A-NH), 7.17 (1H, dd, J=2.0, 8.5 Hz, B-2), 7.08 (1H, dd, J=2.0, 8.5 Hz, B-3), 6.84 (1H, dd, J=2.0, 9.0 Hz, B-5), 7.86 (1H, dd, J=2.0, 9.0 Hz, B-6), 5.01 (1H, d, J=10.6 Hz, B-αCH), 2.98 (1H, t, J=12.5 Hz, B-βCH$_a$), 3.24 (1H, d, J=11.9 Hz, B-βCH$_b$), 2.90 (3H, s, B-NCH$_3$), 7.33 (2H, s, C-2, 6), 5.06 (1H, d, J=5.4 Hz, C-αCH), 8.62 (1H, d, J=5.4 Hz, C-NH), 5.49 (1H, d, J=2.5 Hz, D-2), 5.07 (1H, d, J=2.5 Hz, D6), 5.53 (1H, d, J=9.0 Hz, D-αCH), 8.21 (1H, d, J=9.0 Hz, D-NH), 7.26 (2H, s, E-2, 6), 5.55 (1H, d, J=8.5 Hz, E-αCH), 7.74 (1H, d, J=8.5, E-NH), 10.88 (1H, s, F-1), 7.27 (1H, s, F-2), 7.45 (1H, d, J=8.5 Hz, F-4), 6.81 (1H, d, J=8.5 Hz, F-5), 7.24 (1H, s, F-7), 4.11 (1H, m, F-αCH), 2.86 (1H, d, J=11.7 Hz, F-βCH$_a$), 3.39 (1H, t, J=12.5 Hz, F-βCH$_b$), 8.27 (1H, d, J=7.0 Hz, F-NH), 7.78 (2H, s, G-2, 6), ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 131.4 (A-1), 127.5 (A-2, 6), 114.5 (A-3, 5), 155.6 (A-4), 171.2 (A-CO), 57.6 (A-αCH), 134.6 (B-1), 130.4 (B-2), 121.7 (B-3), 155.1 (B-4), 123.1 (B-5), 131.6 (B-6), 168.5 (B-CO), 61.9 (B-αCH), 34.3 (B-βCH$_2$), 31.0 (B-NCH$_3$), 131.1 (C-1), 127.2 (C-2, 6), 122.1 (C-3, 5), 149.8 (C-4), 169.2 (C-CO), 51.9 (C-αCH), 126.3 (D-1), 110.7 (D-2), 149.0 (D-3), 139.0 (D-4), 131.0 (D-5), 129.4 (D-6), 167.5 (D-CO), 54.9 (D-αCH), 131.9 (E-1), 126.7 (E-2, 6), 121.7 (E-3, 5), 149.0 (E-4), 169.5 (E-CO), 54.7 (E-αCH), 123.5 (F-2), 111.7 (F-3), 126.0 (F-3$_a$), 118.4 (F-4), 123.6 (F-5), 134.6 (F-6), 114.4 (F-7), 136.1 (F-7$_a$), 170.4 (F-CO), 56.9 (F-αCH), 28.4 (F-βCH$_2$), 127.2 (G-1), 130.6 (G-2, 6), 122.5 (G-3, 5), 166.7 (G-4), 181.6 (G-αCO), 164.5 (G-βCO); high resolution-electrospray ionization-mass spectrometry (HR-ESI-MS): m/z 661.5490 $[M-2H]^{2-}$, $C_{61}H_{45}N_7O_{15}Cl_6$ requires 661.5480.

Compound **2**: $C_{61}H_{45}N_7O_{16}Cl_6$; a yellow powder: $[\alpha]_D^{25}$ 11.6° (c=0.14, MeOH); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.10 (2H, d, J=8.5 Hz, A-2, 6), 6.77 (2H, d, J=8.5 Hz, A-3, 5), 5.06 (1H, d, J=6.5 Hz, A-αCH), 8.44 (1H, d, J=6.5 Hz, A-NH), 7.19 (1H,

dd, J=2.0, 8.0 Hz, B-2), 7.15 (1H, dd, J=2.5, 8.0 Hz, B-3), 6.78 (1H, dd, J=2.0, 9.0 Hz, B-5), 7.79 (1H, dd, J=2.0, 9.0 Hz, B-6), 5.08 (1H, m, B-αCH), 3.05 (2H, m, B-βCH_2), 2.99 (3H, s, B-NCH_3), 7.37 (2H, s, C-2, 6), 5.18 (1H, d, J=6.0 Hz, C-αCH), 8.91 (1H, d, J=6.0 Hz, C-NH), 5.77 (H, d, J=2.5 Hz, D-2), 5.69 (1H, d, J=2.5 Hz, D-6), 5.71 (1H, d, J=9.5 Hz, D-αCH), 8.56 (1H, d, J=9.5 Hz, D-NH), 7.02 (2H, s, E-2, 6), 5.56 (1H, d, J=8.5 Hz, E-αCH), 7.10 (1H, d, J=8.5 Hz, E-NH), 10.61 (1H, s, F-1), 3.70 (1H, t, J=3.7 Hz, F-3), 7.11 (1H, d, J=8.0 Hz, F-4), 6.74 (1H, d, J=8.0 Hz, F-5), 6.75 (1H, s, F-7), 3.47 (1H, m, F-αCH), 3.11 (1H, d, J=5.0, 13.5 Hz, F-βCH_a), 1.98 (1H, br d, J=13.5 Hz, F-βCH_b), 9.63 (1H, d, J=7.5 Hz, F-NH), 7.94 (2H, s, G-2, 6); HR-ESI-MS: m/z 669.5454 $[M-2H]^{2-}$, $C_{61}H_{45}N_7O_{16}Cl_6$ requires 669.5454.

Compound **3**: $C_{61}H_{45}N_7O_{15}Cl_6$; a yellow powder: $[\alpha]_D^{25}$ −16.4° (c=0.17, DMSO); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.808 (2H, d, J=8.5 Hz, A-2, 6), 6.75 (2H, d, J=8.5 Hz, A-3, 5), 5.04 (1H, d, J=6.5 Hz, A-αCH), 8.40 (1H, d, J=6.5 Hz, A-NH), 7.19 (1H, d, J=8.0 Hz, B-2), 7.15 (1H, dd, J=2.5, 8.0 Hz, B-3), 6.78 (1H, dd, J=2.5, 8.5 Hz, B-5), 7.81 (1H, d, J=8.5 Hz, B-6), 5.06 (1H, m, B-αCH), 3.05 (2H, m, B-βCH_2), 2.99 (3H, s, B-NCH_3), 7.39 (2H, s, C-2, 6), 5.16 (1H, d, J=6.5 Hz, C-αCH), 8.79 (1H, d, J=6.5 Hz, C-NH), 5.71 (H, d, J=2.0 Hz, D-2), 5.94 (1H, d, J=2.0 Hz, D-6), 5.62 (1H, d, J=8.5 Hz, D-αCH), 8.22 (1H, d, J=8.5 Hz, D-NH), 7.29 (2H, s, E-2, 6), 5.42 (1H, d, J=9.0 Hz, E-αCH), 8.20 (1H, d, J=9.0 Hz, E-NH), 10.58 (1H, s, F-1), 7.65 (1H, s, F-2), 7.23 (1H, d, J=8.0 Hz, F-4), 6.92 (1H, t, J=7.5 Hz, F-5), 7.07 (1H, d, J=8.0 Hz, F-6), 5.08 (1H, m, F-αCH), 3.02 (2H, m, F-CH_2), 9.05 (1H, d, J=16.0 Hz, F-NH), 7.86 (2H, s, G-2, 6); HR-ESI-MS: m/z 1326.1196 $[M+H]^+$, $C_{61}H_{46}N_7O_{15}Cl_6$ requires 1326.1183.

Assay of FabI and FabK *S. aureus* FabI and *S. pneumoniae* FabK enzymes were cloned, overexpressed and purified as described previously.[13,14] Assays were carried out in half-area, 96-well microtiter plates. Compounds were evaluated in 100 μL assay mixtures containing components specific for each enzyme (see below). Reduction of the *trans*-2-octenoyl *N*-acetylcysteamine (*t*-o-NAC thioester) substrate analog was measured spectrophotometrically by following the utilization of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm at 30°C for the linear period of the assay. *S. aureus* FabI assays contained 50 mM sodium acetate, pH 6.5, 400 μM *t*-o-NAC thioester, 200 μM NADPH, and 150 nM *S. aureus* FabI. The rate of decrease in the amount of NADPH in each reaction well was measured by a microtiter enzyme-linked immunosorbent assay (ELISA) reader using SOFTmax PRO software (Molecular Devices, CA, U.S.A.). The inhibitory activity was calculated by the following formula: % of inhibition=100×[1−(rate in the presence of compound/rate in the untreated control)]. IC_{50} values were calculated by fitting the data to a sigmoid equation. An equal volume of dimethyl sulfoxide solvent was used for the untreated control. FabK assays contained 100 mM sodium acetate, pH 6.5, 2% glycerol, 200 mM NH_4Cl, 50 μM *t*-o-NAC thioester, 200 μM NADH, and 150 nM *S. pneumoniae* FabK.

Determination of Antibacterial Susceptibility The whole-cell antimicrobial activity was determined using broth microdilution as described previously.[13] Most of the test strains were grown to mid-log phase in Mueller–Hinton broth and diluted 1000-fold in the same medium. Cells (10^5/mL) were inoculated into Mueller–Hinton broth and dispensed at 0.2 mL/well in 96-well microtiter plates. *Enterococcus* strains and *Streptococcus pneumonia* were grown in Tryptic Soy Broth and Todd–Hewitt medium, respectively, instead of Mueller–Hinton broth. The minimum inhibitory concentrations (MICs) were determined in triplicate by serial two-fold dilutions of the test compounds. The MIC was defined as the concentration of a test compound that completely inhibited cell growth during a 24 h incubation at 37°C. Bacterial growth was determined by measuring the absorption at 650 nm using a microtiter ELISA reader.

Measurement of Inhibition of Macromolecular Biosynthesis To monitor the effects of **1** on lipid, DNA, RNA, protein, and cell wall biosynthesis, its effects on the incorporation of [1-^{14}C]acetate (50 mCi/mmol), [2-^{14}C]thymidine (59.8 mCi/mmol), [U-^{14}C]uridine (539 mCi/mmol), L-[U-^{14}C]isoleucine (329 mCi/mmol), and *N*-acetyl-D-[1-^{14}C]glucosamine (58.1 mCi/mmol) into *S. aureus* RN4220 were measured as described previously.[13] *S. aureus* was exponentially grown to an A_{650} of 0.2 in Mueller–Hinton broth. Test compounds were added to the 1-mL culture at concentrations of 0.25, 0.5, 1, 2, and 4 times the MIC for 10 min. An equal volume of DMSO solvent was added to the untreated control. After incubation with the radiolabeled precursors at 37°C for 1 h, followed by centrifugation, the cell pellets were washed twice with phosphate-buffered saline (PBS) buffer. After acetate incorporation, the total cellular lipids were extracted with chloroform–methanol–water. The incorporated radioactivity in the chloroform phase was measured using scintillation counting. For the other precursors, incorporation was terminated by adding 10% (w/v) trichloroacetic acid (TCA) and cooling on ice for 20 min. The precipitated material was collected on Whatman GF/C glass microfiber filters, washed with TCA and ethanol, dried, and counted using a scintillation counter. The inhibition of radiolabeled precursor incorporation was calculated using the following formula: % inhibition=100×[1−(radioactivity values of the treated samples/control (no antibacterial) values)]. In all experiments, known antibacterial agents were included as positive controls.

Supplementation of Exogenous Fatty Acids The effects of supplementation with exogenous fatty acids on the antibacterial activity of **1** were assessed as described previously.[14] *S. aureus* was grown to mid-log phase in Luria broth (LB) medium and diluted 1000-fold in the same medium. A 100-μL aliquot of the diluted cell suspension (2×10^5 cells) was used to inoculate each well of a 96-microtiter plate containing 95 μL of LB medium with the test compound at the MICs. Subsequently, 5 μL of the serially diluted fatty acid solution was added, and the cell suspension was incubated at 37°C for 18 h. The bacterial growth was measured at 650 nm using a microtiter ELISA reader.

RESULTS AND DISCUSSION

Our continued screening of microbial extracts with the use of a combination of whole-cell and enzyme assays resulted in the identification of three FabI inhibitors (**1**–**3**) with potent antibacterial activity from *S. chartreusis* AN1542. Compound **1** was isolated together with **2** and **3** by activity-guided fractionation using EtOAc extraction, SiO_2 column chromatography, Sephadex LH-20 chromatography, and HPLC from the

Table 1. Inhibitory Activity of Complestatin (**1**), Neuroprotectin A (**2**), and Chloropeptin I (**3**) on *S. aureus* FabI, *S. pneumoniae* FabK, and Growth of *S. aureus* RN4220 and *S. pneumonia* KCTC 5412

Compounds	IC_{50} (μM)		MIC (μg/mL)	
	S. aureus FabI	*S. pneumoniae* FabK	*S. aureus*	*S. pneumoniae*
1	0.5	10	2	16
2	0.3	N.T.	4	N.T.
3	0.6	N.T.	4	N.T.
Triclosan	0.6	>100	0.01	64

N.T.: not tested.

Table 2. Minimum Inhibitory Concentrations (MICs) of Complestatin (**1**)

Test organisms	MIC (mg/L)			
	Complestatin	Vancomycin	Oxacillin	Norfloxacin
Staphylococcus aureus KCTC 1916	4	0.5	0.25	0.25
S. aureus RN 4220	2	1	0.25	1
MRSA CCARM 3167	2	2	500	8
MRSA CCARM 3506	2	0.5	500	1
QRSA CCARM 3505	2	1	0.5	250
QRSA CCARM 3519	4	1	0.5	125
Bacillus subtilis KCTC 1021	0.5	0.1	N.T.	N.T.
Bacillus cereus KCTC 1661	2	1	N.T.	N.T.
Streptococcus pneumoniae KCTC 5412	16	N.T.	N.T.	4
Entercoccus faecalis KCTC 5191	2	1	4	4
E. faecalis KCTC 3511	1	2	8	4
Staphylococcus epidermidis KCTC 3958	2	2	>128	0.5
Salmonella typhimurium KCTC 1926	>128	>128	128	2
Escherichia coli CCARM 1356	>128	>128	>128	>128
E. coli KCTC 1682	>128	>128	>128	0.06
Pseudomonas aeruginosa KCTC 2004	>128	>128	>128	1
P. aeruginosa KCTC 2742	>128	>128	>128	0.5
Klebsiella aerogenes KCTC 2619	>128	>128	>128	0.25
Candida albicans KCTC 7535	>128	64	16	8

mycellium of *S. chartreusis* AN1542. The ^{1}H- and ^{13}C-NMR data of **1** indicated a peptidic structure with aromatic rings. The ^{1}H- and ^{13}C-NMR assignments of **1** were independently completed by correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) spectra. The molecular formula of **1** was also confirmed by HR-ESI spectrum. The ^{1}H- and ^{13}C-NMR data of **1** were almost the same as those of complestatin in the same solvent in the literature[15] except the little differences in chemical shifts of carbons at A-1, G-CO, and G-4. The relative stereochemistry of **1** was also in agreement with that of complestatin based on nuclear Overhauser effect spectroscopy (NOESY) spectrum.[16] Additionally, the $[\alpha]_D$ value [+22.6 (c=0.13, DMSO)] of **1** was similar to the literature value [+16.3 (c=1.6, DMSO)] for complestatin.[9] Chloropeptin I and neuroprotectins have been coisolated in the complestatin-producing *Streptomyces*,[9,10] suggesting that **2** and **3** may be chloropeptin I or neuroprotectins. Indeed, the ^{1}H-NMR data of **2** and **3** were almost the same as those of neuroprotectin A[11] and chloropeptin I,[17] respectively, in the literatures. The ^{13}C-NMR data of **2** and **3** were also similar with those of neuroprotectin A and chloropeptin I although the complete ^{13}C-NMR data of **2** and **3** were not obtained due to their tiny amount. The molecular formulas of **2** and **3** were confirmed by HR-ESI spectrum.

Table 3. The Cross Resistance between Triclosan and Complestatin (**1**)

	S. aureus RN4220	Triclosan-resistant *S. aureus* RN4220
1	2	16
Triclosan	0.01	>1
Norfloxacin	1	1

Especially, the $[\alpha]_D$ values [+11.6 (c=0.14, MeOH) and −16.4 (c=0.17, DMSO), respectively] of **2** and **3** were also similar to the literature values [+18.0 (c=0.024, MeOH) and −18.8 (c=1.6, DMSO), respectively] for these compounds.[9,10] Thus, compounds **1**, **2**, and **3** were identified as complestatin, neuroprotectin A, and chloropeptin I, respectively (Fig. 1).

Compound **1** potently inhibited *S. aureus* FabI in a dose-dependent fashion with an IC_{50} of 0.5 μM (Table 1), while showed twenty-times weaker inhibition on another reductase, *S. pneumonia* FabK, with an IC_{50} of 10 μM. Also compounds **2** and **3** showed the similar inhibitory activity against *S. aureus* FabI with IC_{50} of 0.3 and 0.6 μM, respectively. In order to determine whether **1** inhibit the bacterial growth, the antibacterial activity against the Gram-positive and Gram-negative pathogen was evaluated (Table 2). Compound **1** showed potent antibacterial activity against Gram-positive bacteria, such as Staphylococci, Enterococci, and Bacilli with MICs of 2–4 μg/mL, which are

comparable to those of vancomycin. No activity, however, was observed for **1** against the Gram-negative pathogens *Escherichia coli* or *Pseudomonas aeruginosa*. Consistent with its weaker inhibition on FabK, **1** showed weaker antibacterial activity on *S. pneumonia* with an MIC of 16 μg/mL (Table 1). The cross resistance between triclosan, a FabI inhibitor, and **1** was evaluated using triclosan-resistant *S. aureus*[13] (Table 3). Indeed, triclosan-resistant *S. aureus* were resistant to **1**, while were not resistant to norfloxacin, a DNA gyrase inhibitor, as a negative control. It indicates that **1** inhibits FabI in *S. aureus*. To investigate the frequency of mutation to complestatin resistance, the isolation of resistant mutants was carried out. *S. aureus* RN4220 (1.59×10^9 cells) was plated onto LB plates containing complestatin at 4 times the MIC.[13] No resistant mutants, however, were detected.

In order to determine whether the antibacterial effect of **1** is attributable to the inhibition of fatty acid synthesis, its effects on the biosynthesis of lipids, DNA, RNA, proteins, and the cell wall were examined in *S. aureus*. Compound **1** showed an MIC of 12 μg/mL in the macromolecular biosynthesis assay condition of an 1-mL shaking culture. Consistent with its FabI-inhibition, **1** blocked the incorporation of [1-^{14}C]acetate into the membrane fatty acids in a dose-dependent fashion with inhibition of 25.1% and 86.6% at 0.5 and 1 times the MIC, respectively (Fig. 2). In contrast, the incorporation of labeled thymidine, uridine, isoleucine, and *N*-acetylglucosamine into DNA, RNA, proteins, and the cell wall, respectively, was almost not inhibited at the MIC (Fig. 2, Table 3). As the positive controls, antibacterials such as triclosan, norfloxacin, rifampin, chloramphenicol, and vancomycin as inhibitors of fatty acid, DNA, RNA, protein, and cell wall, respectively, selectively inhibited their corresponding macromolecular synthesis pathway (Table 4). These data clearly indicated that **1** selectively inhibited the fatty acid synthesis in *S. aureus*.

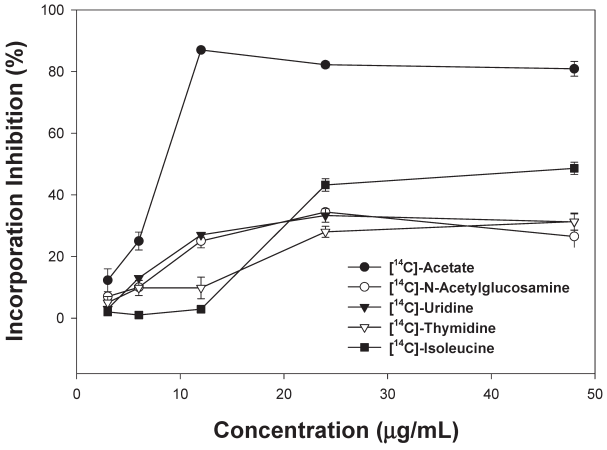

Fig. 2. Dose-Dependent Effects of **1** on the Biosynthesis of DNA, RNA, Proteins, Lipids, and the Cell Wall in *S. aureus* RN4220

To confirm whether the antibacterial effect of **1** is due to the inhibition of fatty acid synthesis, we examined whether *S. aureus* in medium containing **1** could grow with supplementation of exogenous fatty acids. Compared to untreated control cells, *S. aureus* RN4220 in medium containing **1** at the MIC showed no growth. However, when either saturated fatty acids (stearic acid and palmitic acid) or unsaturated fatty acid (oleic acid) at sub-antibacterial concentrations were supplemented to a final concentration of 50, 100, and 200 μM, the *S. aureus* cells in medium containing **1** grew well in a dose-dependent manner (Fig. 3A). Similarly, *S. aureus* cells were rescued from the growth-inhibitory effect of triclosan by the addition of exogenous fatty acids (Fig. 3B). As a negative control, *S. aureus* in medium containing chloramphenicol, a protein synthesis inhibitor, showed no growth with supplementation of the same fatty acids (Fig. 3C). This result indicates that **1** targets fatty acid synthesis.

Complestatin was first isolated from the mycelium of *Streptomyces lavendulae* SANK 60477 as an anticomplement agent in 1980.[18] It was reisolated from the *Streptomyces* sp. as an inhibitor of the binding of HIV gp120 to the CD4 protein and HIV replication.[8] Complestatin is a bicyclic chlorinated hexapeptide[15] belonging to the glycopeptide class. Glycopeptide antibiotics, such as vancomycin and teicoplanin, have unique tricyclic or tetracyclic heptapeptide aglycones, which are usually glycosylated and sometimes additionally acylated. Glycopeptide antibiotics are divided into four structural subclasses (I–IV) according to the substituents and type of residue at positions 1 and 3 of the heptapeptide backbone. Nicolaou *et al.* designated complestatin as a type V class of glycopeptide aglycone, which have a tryptophan in place of a phenyl group in the heptapeptide core.[19] Glycopeptide antibiotics showed antibacterial activity by inhibiting the transglycosylation and/or transpeptidation steps associated with cell wall biosynthesis by binding of the heptapeptide backbone to the C-terminal L-Lys-D-Ala-D-Ala subunit of the peptidoglycan Lipid II *via* five hydrogen bonds.[20] Aglycones of vancomycin and teicoplanin are known to retain antibacterial activity.[21,22] An antimicrobial activity of complestatin, however, has not yet been reported.

Table 4. Comparative Effects of Complestatin (**1**) and Standard Antibacterials on Incorporation of Radiolabeled Precursors into *S. aureus* RN4220 at the MIC

Compounds	Inhibition of precursor incorporation (%)				
	[1-^{14}C]Acetate	[2-^{14}C]Thymidine	[U-^{14}C]Uridine	L-[U-^{14}C]Isoleucine	*N*-Acetyl-D-[1-^{14}C]-glucosamine
1	87.1	9.8	27.2	2.9	25.4
Triclosan	88.1	N.T.	9.4	8.6	1.2
Norfloxacin	30.1	74.1	N.T.	9.3	0.7
Rifampin	35.4	3.5	65.4	N.T.	2.6
Chloramphenicol	7.9	4.5	3.1	70.9	18.2
Vancomycin	19.7	1.3	7.0	15.9	78.8

N.T.: not tested.

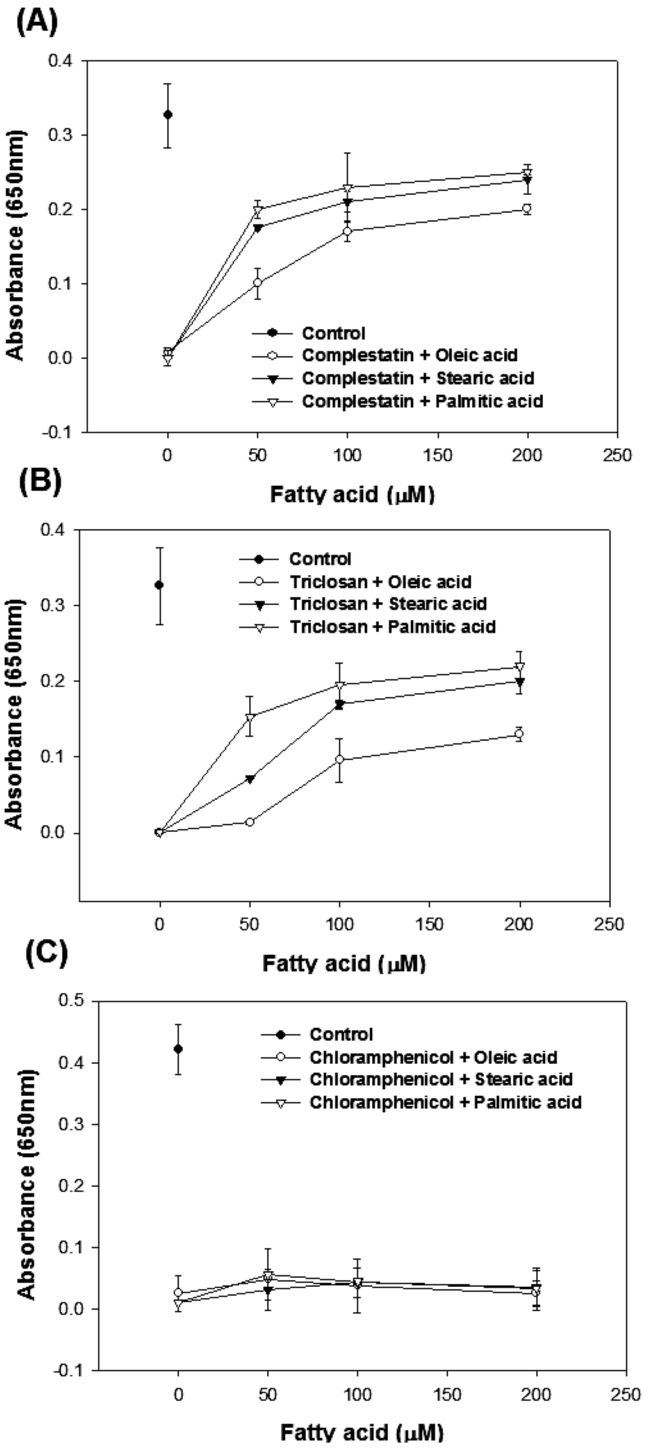

Fig. 3. Growth of *S. aureus* RN4220 in **1**-Containing Medium by Supplementation with Exogenous Fatty Acids (A)

Triclosan (B) and chloramphenicol (C) were used as positive and negative controls, respectively.

Unlike other glycopeptide antibiotics such as vancomycin inhibiting the transglycosylation and/or transpeptidation steps involved in cell wall synthesis,[20] complestatin is found to exhibit antibacterial activity by inhibiting fatty acid synthesis without affecting cell wall synthesis in this study. Interestingly, the difference in the antibacterial mechanism of complestatin from vancomycin is supported by the difference in their biosynthesis gene clusters. The biosynthesis gene clusters of vancomycin-type antibiotics contain their resistance genes (VanX, VanA, and VanH) for self-protection.[20,23] However, there is no resistance gene as such in the biosynthesis gene cluster of complestatin.[24]

In summary, complestatin and related compounds exhibited a potent antibacterial activity against Gram-positive bacteria including methicillin-resistance *S. aureus* (MRSA) with the similar potency as vancomycin. Importantly, they showed antibacterial activity by inhibiting fatty acid synthesis which is distinct from that of vancomycin. Thus, complestatin could have potential as a useful lead compound for tackling existing drug resistance pathogens including MRSA.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Silver LL. Challenges of antibacterial discovery. *Clin. Microbiol. Rev.*, **24**, 71–109 (2011).
2) Heath RJ, Rock CO. Fatty acid biosynthesis as a target for novel antibacterials. *Curr. Opin. Investig. Drugs*, **5**, 146–153 (2004).
3) Zhang YM, White SW, Rock CO. Inhibiting bacterial fatty acid synthesis. *J. Biol. Chem.*, **281**, 17541–17544 (2006).
4) McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature*, **394**, 531–532 (1998).
5) Rozwarski DA, Grant GA, Barton DH, Jacobs WR Jr, Sacchettini JC. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. *Science*, **279**, 98–102 (1998).
6) Kaneko I, Kamoshida K, Takahashi S. Complestatin, a potent anti-complement substance produced by *Streptomyces lavendulae*. I. Fermentation, isolation and biological characterization. *J. Antibiot.* (Tokyo), **42**, 236–241 (1989).
7) Singh SB, Jayasuriya H, Salituro GM, Zink DL, Shafiee A, Heimbuch B, Silverman KC, Lingham RB, Genilloud O, Teran A, Vilella D, Felock P, Hazuda D. The complestatins as HIV-1 integrase inhibitors. Efficient isolation, structure elucidation, and inhibitory activities of isocomplestatin, chloropeptin I, new complestatins, A and B, and acid-hydrolysis products of chloropeptin I. *J. Nat. Prod.*, **64**, 874–882 (2001).
8) Matsuzaki K, Ikeda H, Ogino T, Matsumoto A, Woodruff HB, Tanaka H, Omura S. Chloropeptins I and II, novel inhibitors against gp120-CD4 binding from *Streptomyces* sp. *J. Antibiot.* (Tokyo), **47**, 1173–1174 (1994).
9) Tanaka H, Matsuzaki K, Nakashima H, Ogino T, Matsumoto A, Ikeda H, Woodruff HB, Omura S. Chloropeptins, new anti-HIV antibiotics inhibiting gp120-CD4 binding from *Streptomyces* sp. I. Taxonomy, fermentation, isolation, and physico-chemical properties and biological activities. *J. Antibiot.* (Tokyo), **50**, 58–65 (1997).
10) Kobayashi H, Shin-Ya K, Nagai K, Suzuki K, Hayakawa Y, Seto H, Yun BS, Ryoo IJ, Kim JS, Kim CJ, Yoo ID. Neuroprotectins A and B, bicyclohexapeptides protecting chick telencephalic neuronal cells from excitotoxicity. I. Fermentation, isolation, physico-chemical properties and biological activity. *J. Antibiot.* (Tokyo), **54**, 1013–1018 (2001).
11) Kobayashi H, Shin-Ya K, Furihata K, Nagai K, Suzuki K, Haya-

kawa Y, Seto H, Yun BS, Ryoo IJ, Kim JS, Kim CJ, Yoo ID. Neuroprotectins A and B, bicyclohexapeptides protecting chick telencephalic neurons from excitotoxicity. II. Structure determination. *J. Antibiot.* (Tokyo), **54**, 1019–1024 (2001).

12) Breazzano SP, Boger DL. Synthesis and stereochemical determination of complestatins A and B (neuroprotectins A and B). *J. Am. Chem. Soc.*, **133**, 18495–18502 (2011).

13) Zheng CJ, Sohn MJ, Lee S, Kim WG. Meleagrin, a New FabI Inhibitor from *Penicillium chryosogenum* with at least one additional mode of action. *PLoS ONE*, **8**, e78922 (2013).

14) Zheng CJ, Yoo JS, Lee TG, Cho HY, Kim YH, Kim WG. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Lett.*, **579**, 5157–5162 (2005).

15) Seto H, Fujioka T, Furihata K, Kaneko I, Takahashi S. Structure of complestatin, a very strong inhibitor of protease activity of complement in the human complement system. *Tetrahedron Lett.*, **30**, 4987–4990 (1989).

16) Shinohara T, Deng H, Snapper ML, Hoveyda AH. Isocomplestatin: total synthesis and stereochemical revision. *J. Am. Chem. Soc.*, **127**, 7334–7336 (2005).

17) Matsuzaki K, Ogino T, Sunazuka T, Tanaka H, Omura S. Chloropeptins, new anti-HIV antibiotics inhibiting gp120-CD4 binding from *Streptomyces* sp. II. Structure elucidation of chloropeptin I. *J. Antibiot.* (Tokyo), **50**, 66–69 (1997).

18) Kaneko I, Fearon DT, Austen KF. Inhibition of the alternative pathway of human complement *in vitro* by a natural microbial product, complestatin. *J. Immunol.*, **124**, 1194–1198 (1980).

19) Nicolaou KC, Boddy CN, Brase S, Winssinger N. Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew. Chem. Int. Ed. Engl.*, **38**, 2096–2152 (1999).

20) Hubbard BK, Walsh CT. Vancomycin assembly: nature's way. *Angew. Chem. Int. Ed. Engl.*, **42**, 730–765 (2003).

21) Nagarajan R. Structure–activity relationships of vancomycin-type glycopeptide antibiotics. *J. Antibiot.* (Tokyo), **46**, 1181–1195 (1993).

22) Malabarba A, Strazzolini P, Depaoli A, Landi M, Berti M, Cavalleri B. Teicoplanin, antibiotics from *Actinoplanes teichomyceticus* nov. sp. VI. Chemical degradation: physico-chemical and biological properties of acid hydrolysis products. *J. Antibiot.* (Tokyo), **37**, 988–999 (1984).

23) Pootoolal J, Thomas MG, Marshall CG, Neu JM, Hubbard BK, Walsh CT, Wright GD. Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 8962–8967 (2002).

24) Chiu HT, Hubbard BK, Shah AN, Eide J, Fredenburg RA, Walsh CT, Khosla C. Molecular cloning and sequence analysis of the complestatin biosynthetic gene cluster. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 8548–8553 (2001).