Psammaplin A, a Natural Bromotyrosine Derivative from a Sponge, Possesses the Antibacterial Activity against Methicillin-resistant *Staphylococcus aureus* and the DNA Gyrase-inhibitory Activity

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Psammaplin A, a natural bromotyrosine derivative from an associated form of two sponges (*Poecillastra* sp. and *Jaspis* sp.) was found to possess the antimicrobial effect on the Gram-positive bacteria, especially on methicillin-resistant *Staphylococcus aureus* (MRSA). The minimal inhibitory concentration of psammaplin A against twenty one MRSAs ranged from 0.781 to 6.25 μg/ml, while that of ciprofloxacin was 0.391~3.125 μg/ml. Psammaplin A could not bind to penicillin binding protein, but inhibited the DNA synthesis and the DNA gyrase activity with the respective 50% (DNA synthesis) and 100% (DNA gyrase) inhibitory concentration 2.83 and 100 μg/ml. These results indicate that psammaplin A has a considerable antibacterial activity, although restricted to a somewhat narrow range of bacteria, probably by inhibiting DNA gyrase.

Key words : Psammaplin A, Antibacterial activity, *Staphylococcus aureus*, DNA gyrase

INTRODUCTION

Antibiotics such as β-lactams, quinolones, and macrolides have been widely used in the chemotherapeutic treatment of bacterial infections, because of their broad antimicrobial spectra and highly efficacious antimicrobial activities. However, these antibiotics have encountered some problems such as the emergence of drug-resistant bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA). Unfortunately, MRSAs are found resistant not only to methicillin but also to many other antibiotics including quinolones (Goswitz *et al.*, 1992; Hori *et al.*, 1993), and the frequency of this resistance keeps increasing (Nakanishi *et al.*, 1991; Okuda *et al.*, 1991). Thus, MRSAs have become major microorganisms causing the failure in the successful use of these antimicrobial agents, which necessitates the development of new antibiotics which have the outstanding effect on MRSA and other bacteria resistant to the preexisting antibiotics.

Here we report that psammaplin A, which was previously isolated from a sponge, *Psammaplysilla* sp. (Quinoa and Crews, 1987) has antibacterial efficacy against MRSAs. As a bromotyrosine derivative, psammaplin A is unique in that its structure is quite different from those of preexisting antibiotics (see Fig. 1). We also provide some evidence concerning its antimicrobial mechanism of action.

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MATERIALS AND METHODS

Materials

Mueller-Hinton agar, peptone, beef extract, and yeast extract were purchased from Difco (Detroit, MI, USA), ^{3}H-benzylpenicillin (TRK.779) and ^{3}H-thymidine (TRA.120) from Amersham Co (Buckinghamshire, UK). Ciprofloxacin and cefpirome were synthesized at Korea Research Institute of Chemical Technology (Taejon, Korea) and Korea Institute of Science and Technology

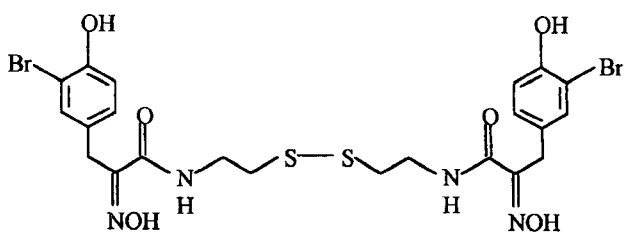

Fig. 1. Structure of psammaplin A.

(Seoul, Korea), respectively.

Psammaplin A was prepared from the associated form of the two sponges, *Poecillastra* sp. and *Jaspis* sp. collected in Komun island, Korea as described previously (Jung *et al.*, 1995).

Bacterial strains and microbial susceptibility test

A total of forty two bacteria including twenty one MRSAs from Hoechst Pharmaceutical Co. in Germany and Yonsei Medical Center in Korea were cultured in Fleisch extract broth (10 g of beef extract, 10 g of peptone, 3 g of NaCl, 2 g of Na_2HPO_4, pH 7.4 per liter of distilled water) and Mueller-Hinton agar.

Minimal inhibitory concentration (MIC) that was defined as the lowest drug concentration which prevented visible growth of bacteria was determined by agar dilution method as described by National Committee for Clinical Laboratory Standards (1990).

Penicillin binding assay

Penicillin binding protein (PBP) binding was assayed following the method of Preston *et al.* (1990). The outer membrane preparation (70 μg protein) from *S. aureus* SG511 was incubated with varying concentrations of each agent at 37°C for 15 min, and then with 10 μCi of ^{3}H-benzylpenicillin for 15 min. The final concentrations of each agent were 5~100 μg/ml. After the reaction was terminated by adding sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenolblue, and 10% glycerol), samples were analyzed by SDS-polyacrylamide gel electrophoresis using 8% acrylamide, followed by fluorography.

In vitro DNA synthesis assay

DNA synthesis assay was performed by the modified method of Chow *et al.* (1988). A 0.9 ml volume of *S. aureus* SG511 culture in logarithmic phase (0.5 OD at 595 nm) was incubated with 0.1 ml of varying concentrations (0.1~10.0 μg/ml) of psammaplin A, ciprofloxacin, or vehicle at 37°C for 15 min and then with 10 μCi of thymidine at 37°C for 5 min. The reaction was terminated by adding 1 ml of 20% ice-cold trichloroacetic acid (TCA). After incubation in an ice-water bath for 30 min, TCA-precipitable materials were collected using GF/C glass fiber filters and cell harvester (Brandel Co., M-12R). Filters were washed four times with 5 ml of 2.5% TCA, dried in the air, and the radioactivities on the filters were measured with a scintillation counter (Beckman LS 6000TA).

DNA gyrase assay

DNA gyrase was partially purified from *Citrobacter freundii* ATCC 8090 with the slight modifications of the published procedures (Aoyama *et al.*, 1988; Takahata and Nishino, 1988), and one unit of gyrase was defined as the amount of activity that converts relaxed DNA (0.15 μg) into supercoiled form in an hour at 30°C. Reaction mixtures containing 0.15 μg of relaxed pBR322 DNA and 1 unit of gyrase in the supercoiling buffer (40 mM Tris-HCl, pH 7.5, 20 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, 2 mM spermidine hydrochloride, and 2 mM dithiothreitol) were incubated with varying concentrations of psammaplin A or other control agent at 37°C. After an hour, the reaction mixtures were analyzed by 0.8% agarose gel electrophoresis. The 100% inhibitory concentration (IC_{100}) of each agent was estimated by determining the concentration at which the conversion of relaxed form into supercoiled form of DNA was completely blocked (Barrett *et al.*, 1990).

RESULTS

Microbial susceptibility to psammaplin A

As shown in Table I, psammaplin A had some antibacterial activity. Quite interestingly, this antibacterial activity of psammaplin A appeared to be restricted to the Gram-positive bacteria: the MIC values against Gram-positive bacteria ranged between 0.78 and 12.5 μg/ml, while those against Gram-negative were between 25 and something greater than 100 μg/ml. Among the Gram-positive bacteria, *S. aureus* species were the most susceptible with the MICs being 0.78 μg/ml, while the most susceptible Gram-negative bacteria among tested were *Citrobacter freundii* (ATCC 8090) and *E. coli* DC2 with the MICs being 25 μg/ml. Psammaplin A also showed considerable antibacterial effects against MRSAs, which were almost comparable to those of ciprofloxacin (Table II).

Penicillin binding

To elucidate the antibacterial mechanism of psammaplin A, it was first determined whether psammaplin A could bind to PBPs like β-lactams. This was tested by examining whether binding of radiolabeled benzylpenicillin (^{3}H-benzylpenicillin) to PBPs could be competitively inhibited by psammaplin A. To this end, *S. aureus* SG511 was chosen as a source of PBP, since this microorganism was shown to be susceptible to psammaplin A as well as cefpirome, a β-lactam and ciprofloxacin, a quinolone antibiotic. As shown in Fig. 2, cefpirome at all of the concentrations tested (5~100 μg/ml) was indeed capable of inhibiting the binding of ^{3}H-benzylpenicillin to PBPs. However, psammaplin A and ciprofloxacin could not compete with ^{3}H-benzylpenicillin at even 100 μg/ml. These data indicate that

Table I. MIC of psammaplin A and other antibiotics against twenty-one primary organisms

Organisms*	MIC (μg/ml) Compound[b]			
	PSMP	CPFX	CFP	MTCLN
Streptococcus pyogenes 308A	6.25	3.125	0.004	0.01
Streptococcus pyogenes 77A	6.25	0.391	<0.002	0.01
Streptococcus faecium MD8b	12.5	0.391	1.563	25
Staphylococcus aureus SG511	0.781	0.195	0.013	1.56
Staphylococcus aureus 285	0.781	0.391	0.013	1.56
Staphylococcus aureus 503	0.781	0.391	0.007	0.78
Escherichia coli O78	>100	<0.002	0.098	>100
Escherichia coli DC 0	>100	0.195	0.195	>100
Escherichia coli DC 2	25	0.049	0.391	6.25
Escherichia coli TEM	>100	0.013	0.195	>100
Escherichia coli 1507E	>100	0.013	0.098	>100
Pseudomonas aeruginosa 9027	>100	0.195	0.391	>100
Pseudomonas aeruginosa 1592E	>100	0.098	0.391	>100
Pseudomonas aeruginosa 1771	50	0.195	0.781	>100
Pseudomonas aeruginosa 1771M	50	0.098	0.195	>100
Salmonella typhimurium	>100	0.007	0.391	>100
Klebsiella oxytoca 1082E	>100	<0.002	0.195	>100
Klebsiella oxytoca 1522E	>100	0.013	0.195	>100
Enterobacter cloacae P99	>100	0.007	0.049	>100
Enterobacter cloacae 1321E	>100	0.004	0.098	>100
Citrobacter freundii ATCC8090[a]	25	0.004	0.098	>100

*All except a were obtained from Hoechst Pharmaceutical company, Germany.

[a]American Type Culture Collection (Rockville, MD, USA)

[b]PSMP, psammaplin A; CPFX, ciprofloxacin; CFP, cefpirome; MTCLN, methicillin

psammaplin A did not bind to PBPs and thus that the antibacterial activity of psammaplin A is not due to the binding to PBP.

Inhibition of DNA synthesis

Next, it was investigated whether psammaplin A could affect the bacterial DNA replication as quinolone antibiotics do. As shown in Fig. 3, both psammaplin A and ciprofloxacin inhibited DNA synthesis of *S. aureus* SG511 in a dose-dependent manner. The IC_{50} (the concentration at which DNA synthesis is inhibited by 50%) of psammaplin A and ciprofloxacin were estimated to be 2.83 and 0.12 μg/ml, respectively. This result suggests that the antibacterial activity of psammaplin A probably originates from its inhibitory effect on DNA synthesis.

Inhibition of the supercoiling activity of DNA gyrase

Bacterial DNA synthesis or replication is, in part, controlled by DNA gyrase, an enzyme that participates in the regulation of DNA topology and serves as a target for quinolone antibiotics. However, it has been shown by others and our own experiments (data not shown) that DNA gyrases from most of *S. aureus* are relatively refractory to the inhibitory action of quinolones, requiring extraordinarily high concentration of a quinolone, while DNA gyrase from *Citrobacter freundii* can be inhibited by manageably lower concentrations of quinolones (see a review by Zimmer *et al.*, 1990). Our preliminary experiments also revealed that a further higher amount of psammaplin A would be required to inhibit DNA gyrase from *S. aureus* SG511 (data not shown). Therefore, in order to investigate whether psammaplin A has the ability to inhibit DNA gyrase *Citrobacter freundii* instead of *S. aureus* SG511 was used as a source of partially purified DNA gyrase (Fig. 4). Without any agent added, DNA gyrase was capable of converting the relaxed DNA into the supercoiled one (lane 12 vs. 11). But, when psammaplin A (lanes 1~5) or ciprofloxacin (lanes 6~10) was added in the reaction mixture, the activity of DNA gyrase became decreased and complete loss of DNA gyrase activity was observed with 100 and 2.5 μg/ml of psammaplin A and ciprofloxacin, respectively. These data indicate that psammaplin A is capable of inhibiting DNA gyrase like quinolones, although somewhat less efficiently than ciprofloxacin.

Table II. MIC of psammaplin A and other antibiotics against MRSAs obtained from Hoechst[a] and Yonsei Medical Center[b]

Bacterial strains	MIC (μg/ml)		
	Compound[c]		
	PSMP	CPFX	MTCLN
S. aureus 88E	1.563	0.781	6.25
S. aureus 690E	1.563	0.391	12.5
S. aureus 692E	1.563	0.391	6.25
S. aureus 693E	1.563	0.781	6.25
S. aureus 697E	1.563	0.391	6.25
S. aureus 701E	0.781	0.781	6.25
S. aureus 703E	0.781	0.781	25
S. aureus 707E	1.563	0.781	6.25
S. aureus 708E	1.563	0.391	12.5
S. aureus 725E	1.563	0.781	6.25
S. aureus Y-80-11-1129	3.125	0.391	25
S. aureus Y-80-12-250	3.125	0.391	12.5
S. aureus Y-80-12-1109	6.25	0.391	>100
S. aureus Y-80-12-1999	6.25	0.391	>100
S. aureus Y-80-12-598	1.563	0.391	12.5
S. aureus Y-80-12-844	3.125	3.125	>100
S. aureus Y-80-12-1891	25	0.781	50
S. aureus Y-80-12-2086	0.781	0.195	6.25
S. aureus Y-81-1-354	1.563	0.391	6.25
S. aureus Y-88-12-3791	0.781	0.391	50
S. aureus Y-88-12-3777	0.781	0.195	100
S. aureus Y-80-12-2610	0.781	0.391	50

[a]Strains designated with E at the end
[b]Strains of Y-series
[c]PSMP, psammaplin A; CPFX, ciprofloxacin; MTCLN, methicillin

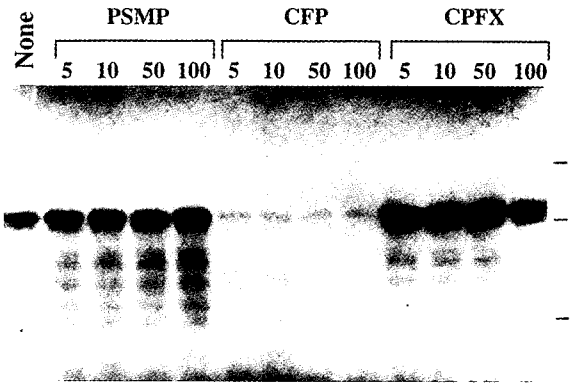

Fig. 2. Negative PBP binding of psammaplin A. After preincubation with varying concentrations of each agent, the outer membrane prepared from *S. aureus* SG511 was incubated in the presence of 10 μCi of ^{3}H-benzylpenicillin for 15 min. Reactions were terminated by adding SDS sample buffer and the samples were analyzed by SDS-PAGE of 8% acrylamide, followed by a fluorography. The final concentrations of each agent in the mixtures were 5~100 μg/ml.

DISCUSSION

Psammaplin A is derived from a sponge. Its structure is unique in that it is mainly composed of two monobromotyrosine moieties lacking the pharmacophores present in the other known antibiotics (Fig. 1). In this report, psammaplin A was shown to possess the antibacterial activity especially against MRSAs. Unlike β-lactams, psammaplin A did not bind to PBPs, indicating that psammaplin A has antibacterial mechanism(s) other than the binding to and the inhibition of PBPs. Supporting this, DNA synthesis in *S. aureus* SG511 and DNA gyrase activity from *C. freundii* were inhibited

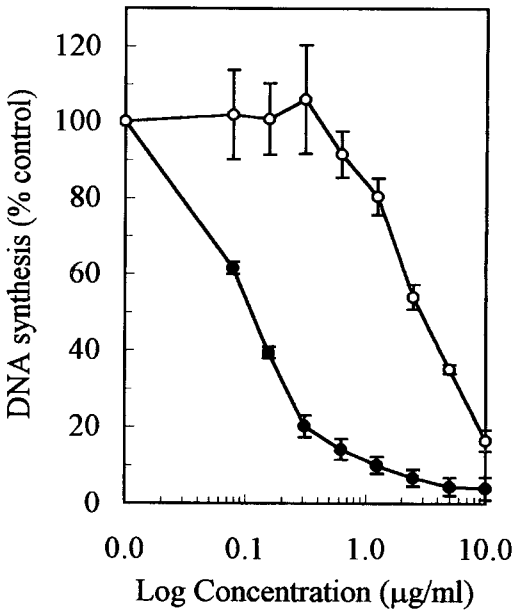

Fig. 3. Dose-dependent inhibition of DNA synthesis of *S. aureus* by psammaplin A. A logarithmic culture of *S. aureus* was preincubated with varying concentrations of psammaplin A (○) or ciprofloxacin (●), and then 10 μCi of thymidine was added. Five minutes later, the reactions were terminated with ice-cold TCA, and the radioactivities on the TCA-precipitable materials were measured as described in Materials and Methods. The final concentrations of each agent in the reaction mixtures were 0.078~10 μg/ml.

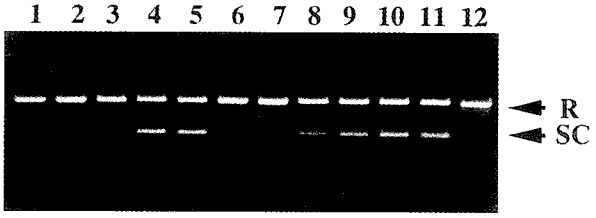

Fig. 4. Inhibition of the supercoiling action of DNA gyrase from *C. freundii* by psammaplin A. The supercoiling activity of DNA gyrase purified partially from *C. freundii* was measured in the presence of psammaplin A or ciprofloxacin using a relaxed form of pBR322 as a substrate, as described in Materials and Methods: Lanes 1-5, decreasing concentrations of psammaplin A at 200, 100, 50, 25, 12.5 μg/ml; Lanes 6-10, decreasing concentrations of ciprofloxacin at 5, 2.5, 1.25, 0.625, 0.313 μg/ml; Lane 11, no inhibitor; Lane 12, relaxed DNA. The positions of relaxed (R), and supercoiled (SC) forms of plasmid DNA are shown.

by psammaplin A. It would have been nicer if DNA gyrase from *S. aureus* was used instead, since psammaplin A had more excellent antibacterial effect on *S. aureus* than on *C. freundii.* As stated above, however, the fact that inhibition of DNA gyrase from *S. aureus* requires unmanageably high amounts of psammaplin A as well as ciprofloxacin in our and others' hands did not allow to use DNA gyrase from *S. aureus* in this assay. Nonetheless, the present study is thought to have convincingly shown that psammaplin A possesses the ability to inhibit the supercoiling activity of DNA gyrase as quinolone antibiotics do.

Consistent with studies by others, a lesser amount of ciprofloxacin was needed in inhibiting bacterial growth than in inhibiting DNA gyrase (i.e, in *C. freundii*). It has been suggested that antibacterial action might be sufficiently achieved by only a small extent of inhibition of DNA gyrase. Alternatively, DNA topoisomerase IV rather than DNA gyrase has been proposed to be critical in determining the bacterial susceptibility to quinolones (Tanaka *et al.*, 1997). Similarly, a lower concentration of psammaplin A, although less lower than ciprofloxacin, was necessary in inhibiting bacterial growth than in inhibiting DNA gyrase, which thus opens a possibility that psammaplin A might also act on DNA topoisomerase IV.

As stated above, a less lower amount was necessary in case of psammaplin A: that is, the ratio of the amount for inhibiting bacterial growth to the amount for inhibiting DNA gyrase was higher with psammaplin A than with ciprofloxacin. This can be explained as follows. Psammaplin A might have a structure less favorable for penetration into *C. freundii* than ciprofloxacin and thus not be able to inhibit bacterial growth as effectively as ciprofloxacin does. At this time, it is not known what moiety of the molecule determines the antibacterial activity and the uptake of psammaplin A.

In conclusion, this report shows that psammaplin A which has a structure distinct from that of other known antibiotics has inhibitory effects on bacterial growth (primarily of MRSAs), DNA synthesis, and DNA gyrase activity. Consequently, it raises a possibility of psammaplin A being utilized as a lead structure in the synthesis of novel agents with excellent antibacterial activities against *S. aureus* including the ones resistant to multiple antibiotics.

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