短　報

Isolation and identification of a compound showing anti-Staphylococcal activity

Norihiro Ohtaguro[2)], Hirotaka Matsuo[2)], Kosuke Iwasaki[2)], Shohei Ueda[2)], Kazuya Nakagawa[2)], Tomofusa Tsuchiya[2)], Nobutaka Imamura[2)], Toshifumi Dohi[2)], Yoichi Yamada[1)] * and Sumiko Shiota[1)]

[1)] *School of Pharmacy, Shujitsu University,*

[2)] *College of Pharmaceutical Sciences, Ritsumeikan University*

(Received 4 November 2020; accepted 26 December 2020)

Abstract

Staphylococci are one of the major causes of bloodstream infections via medical devices. The development of new anti-Staphylococcal drugs is urgently needed. We isolated tryptanthrin from a traditional Japanese dye *sukumo* made from fermented *Polygonum tinctorium*. The minimum inhibitory concentration against gram-positive and gram-negative bacteria were measured. It showed anti-Staphylococcal activity against *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus* and *S. warneri.*

Keywords: tryptanthrin, anti-staphylococcal, *sukumo*

Introduction

Coagulase-negative staphylococci (CNS) including *S. epidermidis*, *S. saprophyticus*, *S. warneri* and coagulase-positive staphylococci, *S. aureus*, are among the normal flora on human skin and easily attach to medical devices such as catheters. These are therefore one of the most common pathogenic bacteria in bloodstream infections and are serious problem worldwide. Furthermore, drug-resistant bacteria like methicillin-resistant *S. aureus* (MRSA) and drug resistant CNS are frequently isolated at clinical sites.

Methicillin was released in 1959. Only two years later, the first MRSA isolate was reported in England. MRSA has been isolated in many hospitals all over the world. Furthermore, MRSA was also detected in the community, and it has become a bacterium indigenous to humans and the environment. Only a few drugs, such as vancomycin marketed since 1956, have been used for the treatment of MRSA infection for many years. The vancomycin-resistant *S. aureus* (VRSA) was reported in 2002. Although a few drugs such as linezolid and daptomycin were developed recently and used as anti-MRSA drugs, *S. aureus* strains showing resistance to these drugs soon emerged. Drug resistant CNS have

also been isolated. In this situation, the development of new anti-staphylococcal agents has become an urgent issue.

Humans have long been making use of plants and their products because of their useful medical effects. Dye made from fermented *Polygonum tinctorium*, which is traditionally used for the dyeing of clothes, is known as “Japan Blue”. Because the hands of dyers working with Japan Blue were considered to be very clean, it was suggested that this dye may have antibacterial and anti-inflammatory activities. In fact, tryptanthrin isolated from *P. tinctorium* was identified as having anti-*Helicobacter pylori* and anti-inflammatory effects[1, 2]. It was believed that dye made from fermented *P. tinctorium* is useful to relieve symptoms like rough skin, heat rash and athlete’s foot.

In this study, we found that a methanol extract of the traditional Japanese dye *sukumo*, made of fermented *P. tinctorium* leaves, showed anti-Staphylococcal activity. We purified and identified tryptanthrin as the effective compound. Tryptanthrin was synthesized and applied to measure its antimicrobial activity.

Experimental procedures

Bacterial strain and Growth. Bacterial strains used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) broth (Nacalai tesque Inc., Japan) or Mueller–Hinton Broth (Difco, Sparks, USA) supplemented with Mg^{2+} and Ca^{2+}.

Drug susceptibility test. The minimum inhibitory concentration (MIC) was determined in cation adjusted Muller-Hinton broth utilizing a liquid two-fold dilution method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI)[4]. Cells in the medium (5 x 10^5 cells/mL) were incubated at 37°C for 20 hours, and the growth was judged by visual inspection.

General experimental procedures. Nuclear Magnetic Resonance (NMR) spectra were measured using a JNM-ECA-600 NMR spectrometer (JEOL, Japan) with tetramethylsilane as an internal standard in $CDCl_3$. High Performance Liquid Chromatography (HPLC) analysis was conducted using a HPLC pump GL-7410 and a Photodiode Array detector GL- fractionations, a HPLC pump L - 6200 (Hitachi, Japan) and a ultraviolet (UV)

Table 1. Bacterial strains

Bacteria	References
S. aureus 209P	Clinical isolate
S. aureus RN4220	Standard strain
S. aureus N315	Standard strain, MRSA
S. aureus COL	Standard strain, MRSA
S. aureus OM481	Clinical isolate, MRSA
S. aureus OM584	Clinical isolate, MRSA
S. aureus OM623	Clinical isolate, MRSA
S. epidermidis NBRC12993	National Institute of Technology and Evaluation (NITE)
S. saprophyticus NBRC102446	NITE
S. warneri NBRC109769	NITE
Bacillus subtilis ATCC9372	American Type Culture Collection (ATCC)
Enterococcus faecalis AT29212	ATCC
E. faecium DO	Ref. 3
Escherichia coli K-12	Wild type
Pseudomonas aeruginosa PAO1	Standard strain
Serratia marcescens NUSM8903	Clinical isolate

detector L-4200 (Hitachi) were using an API-3200 triple quadrupole mass spectrometer (Applied Biosystems, Japan). UV-visible (VIS) light spectra were measured using a V-630 spectrophotometer (JASCO).

Plant material, extraction, and fractionation. The traditional Japanese dye *sukumo* made from fermented *P. tinctorium* was provided by Awa-Yuzen Kojo Inc. *Sukumo* (4.1 kg) was minced and extracted once with 25 L of absolute methanol under reflux for 1 hour at 80°C. The solvent was evaporated under reduced pressure, yielding a methanol extract (109 g). The resultant methanol extract was resuspended in water. After filtration of the suspension, it was successively partitioned with *n*-hexane and ethylacetate. These organic layers were concentrated to give an *n*-hexane fraction (fraction A, 849 mg) and an ethylacetate-soluble fraction (fraction B, 7.38 g).

Isolation of effective compound. Fraction B (7.38 g) was dissolved in ethylacetate and the solution was applied to a silica gel column (Silica gel 60, i.d. 70×220 mm). It was eluted with a stepwise gradient of *n*-hexane–ethyl acetate (10:1, 5:1, 2:1, 1:1 and 0:1) and fractionated. The active fraction (188 mg) was dissolved in methanol (MeOH) and the solution was applied to an ODS column (Wakogel 50C18, i.d. 35×140 mm). It was eluted with a stepwise gradient of H_2O–MeOH (10, 40, 70 and 100%) and fractionated. Again, the active fraction of 70% MeOH was concentrated in vacuo to yield a crude material (17.1 mg). Further purification was carried out under the following conditions: HPLC ODS column; Cosmosil 5C18-AR-II (i.d. 10×250 mm, detection 230 nm, flow rate 2 mL/min), gradient elution, solvent A (98.95% H_2O + 1% acetonitrile + 0.05% trifluoroacetic acid) and solvent B (1% H_2O + 98.95% acetonitrile + 0.05% trifluoroacetic acid), gradient rate; 0 to 5 minutes (100% A), 5 to 55 minutes (0%→100% B), 55 to 60 minutes (100% B). The eluent was divided into several fractions and each fraction was bioassayed. The active compound was obtained as a yellow powder. It was soluble in MeOH and dimethylsulfoxide but insoluble in water.

Synthetic tryptanthrin samples. Tryptanthrin was prepared from the corresponding isatin by reactions with isatoic anhydride in accordance with previous reports[5, 6]. Thus, the mixture of isatin (5 mmol) and isatoic anhydride (816 mg, 5 mmol) in toluene (15 mL) including trimethylamine (3.5 mL, 25 mmol) was heated at refluxing temperature (around 110°C) for 4 hours in a nitrogen atmosphere. Completion of the reaction was checked by TLC. In our modified purification procedure, the resulting reaction mixture was washed with water, and the aqueous phase was extracted with ethyl acetate. Almost pure tryptanthrin was obtained as a light-yellow-colored solid from the combined organic phase after treatment with anhydrous sodium sulfate and evaporation. The small amount floating on the aqueous phase was collected by filtration, providing an additional amount of the product. Thus, a pure sample of tryptanthrin was obtained with a 92% yield by washing these solids with ethanol. All chemicals for the preparation were obtained from commercial suppliers and used as received without purification.

Results and discussion

Sukumo (4.1 kg) was extracted with methanol. The extract was filtered and dried, and it was then dissolved in water and extracted with ethyl acetate. The evaporated organic layer was applied to a silica gel column, and fractions were eluted with stepwise gradient of *n*-hexane-ethyl acetate.

The fraction with 50% ethyl acetate was evaporated and applied to an ODS column. A stepwise gradient of H_2O-methanol was adapted, and the 70% methanol fraction was concentrated in vacuo to yield a crude material (17.1 mg). Further fractionation was performed by HPLC with an ODS column, and an active compound was obtained (1.2 mg).

The physicochemical properties were UV λ_{max} (MeOH) nm (log ε): 251(4.77), 311(4.09), 328(4.05), 387(3.91), Mass Spectrum *m/z*: 249(M^+), ^{1}H-NMR ($CDCl_3$)δ: 8.44 (1H, d, *J*=8.9 Hz), 7.67 (1H, t, *J*=7.6 Hz), 7.85 (1H, t, *J*=7.6 Hz), 8.03 (1H, d, *J*=8.3 Hz), 7.91 (1H, d, *J*=7.6 Hz), 7.42 (1H, t, *J*=7.6 Hz), 7.79 (1H, t, *J*=7.6 Hz), 8.63 (d, *J*=7.6 Hz). All properties were completely identical to tryptanthrin (Fig. 1)[7]. The MIC of isolated tryptanthrin against MRSA N315 was 16 μg/mL.

The yield (1.2 mg) was not enough to measure MICs for many bacteria. Therefore, we synthesized tryptanthrin. MICs of synthesized tryptanthrin against various bacteria are shown in Table 2. The MIC values against methicillin-sensitive *S. aureus* (MSSA) 209P and RN4220 were 11 and 21 μg/mL respectively, and those against MRSA N315, COL, OM481, OM584, andOM623 ranged from 4 to 43 μg/mL. The MIC of streptomycin against MRSA N315 was 4 μg/mL. The antimicrobial activity of tryptanthrin against *S. aureus* was close to that of streptomycin. The MIC of synthesized tryptanthrin against *S. epidermidis* NBRC12993 was 1.3 μg/mL. These results suggest that tryptanthrin has anti - *S. aureus* and anti - *S. epidermidis* activity are consistent with a previous study[8]. MICs against CNS, *S. saprophyticus* NBRC102446 and *S. warneri* NBRC109769 were 16 and 4 μg/mL respectively. These data showed tryptanthrin also had antimicrobial activity against *S. saprophyticus* and *S. warneri*. *S. saprophyticus* has become problematic as a cause of opportunistic infections. Tryptanthrin did not have the antimicrobial effect against other gram-positive bacteria such as *B. subtilis* ATCC9372, *E. faecalis* ATCC29212, and *E. faecium* DO, or gram-negative bacteria such as *E. coli* K-12, *P. aeruginosa* PAO1, and *S. marcescens* NUSM8903. Some compounds have been reported to show anti-staphylococcal activity[9]. However, only *S. aureus* and *S. epidermidis* are used in those reports. In this study, ten kinds of gram-positive and gram-negative bacteria, including four kinds of staphylococci, were used. According to these results, tryptanthrin might have antimicrobial activity specific towards Staphylococci.

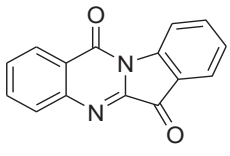

Fig. 1. The structure of tryptanthrin.

Staphylococci are indigenous bacteria of the

Table 2. Antibiotic susceptibility

bacteria	MIC(μg/mL)[a]
Gram-positive bacteria	
Methicillin-sensitive *S. aureus*	
209P	11
RN4220	21
Methicillin-resistant *S. aureus*	
N315	21
COL	11
OM481	43
OM584	16
OM623	4
S. epidermidis NBRC12993	1.3
S. saprophyticus NBRC102446	16
S. warneri NBRC109769	4
B. subtilis ATCC9372	>85
E. faecalis AT29212	>85
E. faecium DO	>85
Gram-negative bacteria	
E. coli K-12	>85
P. aeruginosa PAO1	>85
S. marcescens NUSM8903	>85

[a] All MIC measurements were done in triplicates.

skin and sometimes cause nosocomial infections. There is a possibility that synthesized tryptanthrin could be used as a disinfectant or antimicrobial agents. Tryptanthrin was isolated from the indigo plant family as an anti-*H. pylori*, anti-MRSA, anti-cancer, and anti-inflammatory compound, so fermentation might not be necessarily [1-2), 8), 10)]. Tryptanthrin - derivative compounds might be useful not only as antimicrobial agents, but also in many other cases. To overcome infectious diseases by drug resistant bacteria, new antimicrobial agents like avermectin, the treatment for parasitic worms discovered by Dr. Ōmura, are needed.

Acknowledgments

We thank to Awa-Yuzen Kojo Inc. for supplying *sukumo* and Benjamin W. Listen for critical reading of the manuscript.

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