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Purification and Characterization of Antistaphylococcal Substance from *Pseudomonas* sp. KUH-001

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Abstract A bacterium producing unique antistaphylococcal substance (ASS) was isolated from soil samples. The isolated strain KUH-001 was identified to belong to *Pseudomonas* species from the characteristic properties of its fluorescence and cellular 3-hydroxy fatty acid composition, etc. The ASS component was purified by procedures employing activated carbon adsorption, column chromatography with silica gel, preparative TLC and HPLC. This compound could also be purified mainly by repeating of trituration and precipitation with chilled ether. Purified ASS with a m.p. value of 140~142°C showed marked stability at high temperature (at 121°C for 10 min) and extreme pHs (in 1N HCl and 1N NaOH for 1 day) without significant loss of antibiotic activity. From spectral data of UV, IR, NMR, and FAB-MS, the compound was elucidated as 2-heptyl-4-hydroxyquinoline N-oxide (HHQO). Under the conditions employed, HHQO exhibited a narrow antimicrobial spectrum, active particularly against *Staphylococcus aureus* including the methicillin resistant strain. Moreover, it did not induce resistance, and besides, interacted synergistically with certain antibiotics such as vancomycin or erythromycin.

Key words: 2-Heptyl-4-hydroxyquinoline *N*-oxide, antistaphylococcal antibiotics, *Pseudomonas* sp.

Human diseases caused by staphylococci in particular have an explosive potential, often rapidly fatal. Therefore, their systemic infections have been regarded as a world-wide matter of concern for human health [23, 29]. Epidemiologically, these bacteria are known as endemic nosocomial pathogens [14]. The risk of incidental outbreaks to multi-drug resistance is thereby enormous. Moreover, since the resistant can be readily transmitted either by hand contact or air, special caution is required particularly at hospitals.

Within a few decades after the introduction of methicillin for the treatment of penicillinase-producing staphylococcal infections, fulminant spread of resistants against methicillin and related β-lactam antibiotics has brought on difficulties for medical treatment [1]. Methicillin resistance is usually found in *S. aureus* (MRSA) or *S. epidermidis* (MRSE), endemic nosocomial entities [20]. Unlike the streptococcal infections, staphylococci in bacteremia are known to survive for prolonged periods within polymorphonuclear leukocytes [24]. Furthermore, tissue necrosis or abscess formation may protect the pathogen from host bactericidal machinery. Staphylococcal infections often result in rapid valvular destruction, heart failure or even metastasis when host defence systems are defective [15]. Accordingly, curative therapy for serious infections with these bacteria such as bacteremia, meningitis, or endocarditis, requires administration of potential agents possessing bactericidal activities *in vivo* at low concentrations. However, because of distinct characteristics with broad antibacterial resistance patterns of MRSA and MRSE strains, only a few antibiotics are being clinically recommended [16]. Recently, a number of β-lactam antibiotics (e.g., cephamandole and carbapenems) were suggested to be useful for the clinical treatment of MRSA, having good *in vitro* activity [18]. Nevertheless, none of these can be used alone because of the high incidence of emerging resistance except vancomycin [4]. Although vancomycin has potential efficacy, its use is generally restricted per se to be an ultimate drug of choice for which life can be saved under serious infections. Because, its administration usually accompanies with marked side effects such as initial syndromes (e.g., flushing, tachycardia, or hypotention), oto- or nephro-toxicities, etc. [2, 5]. Especially, vancomycin therapy in patients with chronic renal failure, peritonitis

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or central nervous system infection needs special caution. Developing rational way to cure diseases caused by methicillin-resistant staphylococci is therefore thought to be one of the most challenging areas for human health.

In the course of screening novel antibiotics from nature, we recently found a unique microorganism producing antibiotics which were active only against *S. aureus* among those tested. This paper deals with the isolation, purification, and characterization of antistaphylococcal substance (ASS) from the isolated bacterium KUH-001. Results in taxonomic studies of the strain will also be described.

MATERIALS AND METHODS

Microorganisms, Growth, and Screening Conditions

Microorganisms used in this study were as follows; *Escherichia coli* No. 20 and *S. aureus* No. 19 were stock strains in our laboratory [12]. *Arthrobacter* sp., *Salmonella typhimurium, Micrococcus luteus, Streptococcus pneumonia,* and *Bacillus megaterium* were provided from The Kitasato Institute, Japan. *Proteus mirabilis, Klebsiella pneumonia, Providentia rettgeri,* and *Aspergillus oryzae* were obtained from KRIC, Korea. *Pseudomonas aeruginosa* ATCC 1711, *Bacillus subtilis* ATCC 2101, *Aspergillus niger* ATCC 13497, and *Candida albicans* KCTC 1940 were obtained from KCTC, Korea. Bacteria isolated from domestic soil samples and above test organisms were grown on nutrient medium (NM; 0.5% polypeptone, 0.3% yeast extract, pH 7.0) at 30°C by reciprocal shaking at 90 rpm. Culture broths of formers (20 µl each) were directly applied onto paper discs (8 mm in diameter; Toyo Roshi Kaisha, Ltd., Japan), and then placed on agar plates of NM, suspended homogeneously by viable cells (test organisms: 0.25×10^7 cells per milliliter). After overnight incubation at 30°C, antimicrobial activities were determined by measuring disc zones of growth inhibitions [10].

Taxonomic Studies of Strain KUH-001 Producing ASS

Cells of KUH-001 producing ass were seen microscopically, tested for gram staining and motility. Fluorescence of the cell colonies was observed on petri plates containing NM, King A (2% peptone, 0.14% $MgCl_2$, 1% $(NH_4)_2SO_4$, 1% glycerol and 1.5% agar, pH 7.2) and King B media (2% peptone, 0.15% K_2HPO_4, 1% $MgSO_4$, 1% glycerol and 1.5% agar, pH 7.2) [27]. Cellular fatty acid composition was determined by the following methods [22]: Method A — 10 mg of lyophilized cells and 2 ml of 5% HCl in methanol were placed on a Teflon-lined, screw-capped tube, followed by heating at 100°C for 3 h. To this was added 3 ml of solution composed of 1 ml of distilled water: 4 N HCl (1:1, v/v) and 2 ml of petroleum ether: ethylacetate (1:1, v/v), and then vigorously mixed using separatory funnel. After discarding the aqueous phase, residual water in organic phase was successively removed by extracting 3 times with the same solvent system as above (1:1, v/v). In order to eliminate the trace amounts of water 2 g of Na_2SO_4 was added. Following filtration, the resulting solvent was stripped to dryness *in vacuo.* Method B — Lyophilized cells were pretreated with 4 N HCl for 5 h at 100°C before esterification of fatty acids by 5% HCl in methanol. Above concentrates were then applied for gas chromatography (Shimadzu GC-14A, Japan) using a column (Shimadzu CBP1-M25-025, Japan) with flame ionization detector at 2 ml/min of He carriage.

Isolation and Purification of ASS from Strain KUH-001

Unless otherwise specified, the following procedures were carried out at room temperature. All materials used were of analytical grades commercially available.

Seed cultures of KUH-001 grown on 100 ml of NM using 500-ml Erlenmyer flask (at 30°C for 1 day by reciprocal shaking) were inoculated into 3 *l* NM in a jar fermentor (KFC-5 L, Korea), and then incubated for 2 days with the aid of 30% neurin (aeration: 1 vvm, 100 rpm, at 30°C). Cell growth was monitored spectrophotometrically by measuring turbidity at 660 nm (Shimadzu Model 2101PC, Japan).

Each culture broth was centrifuged at $9,000 \times g$ for 10 min, and the resulting supernatant was extracted by ethylacetate (1:1, v/v). The extract was then placed in a tightly-capped bottle and stored in the refrigerator. All of the extracts were combined and concentrated. The concentrate was subjected to a column chromatography using silica gel (70×200 mm; Kiesel gel 60, 230~400 mesh, Merk) and the activity was eluted by concentrated chloroform/methanol gradient. The active fractions were collected, concentrated by evaporation. The concentrate was dissolved in chloroform, and then preparative TLC (Kiesel gel 60 F_{254}, Merk Art. 5717) with chloroform-methanol (6:1, v/v) was performed. The activity spot ascertained by UV lamp (254 nm) and the growth inhibitory zone of the disc was carefully scraped up, extracted by chloroform-methanol (1:1, v/v) and filtered. Preparative HPLC (Hitachi, Japan) was carried out using ODS column (20×250 mm; TSK gel ODS-120T), eluted at 7 ml/min with 30% acetonitrile. The purification summary is shown in Scheme 1.

Analysis of the Chemical Structure of Purified ASS

Ultraviolet spectrum was monitored by UV-spectrophotometry (Shimadzu UV-160A, Japan). Infrared spectum was recorded on the Shimadzu IR 435 spectrophotometer, and high-resolution mass spectrum was obtained by FAB-MS (JMS-DX 300, JEOL). Nuclear magnetic resonance (^{1}H-NMR) spectrum was recorded on a Varian XL-400 spectrophotometer using tetramethylsilane (δ=0) as the internal standard.

Culture broth (48 *l*)
↓ Centrifugation 9,000×g for 10 min
Culture filtrate
↓ Ethyl acetate (EA) extraction
↓ [EA:supernatant=1:1 (v/v)]
Silica gel column chromatography
↓ ($CHCl_3$:MeOH=100:1~1:1)
Preparative TLC ($CHCl_3$:MeOH=6:1)
↓ Extraction ($CHCl_3$:MeOH=1:1)
Preparative HPLC
↓ (ODS column, 30% CH_3CN, 210 nm)
Analytical HPLC
↓ (ODS column, 50% CH_3CN, 220 nm)
Purified ASS (16.7 mg)

Scheme 1. Purification procedure for ASS from the culture broth of strain KUH-001.

Antimicrobial Activity Assay

Antimicrobial activity of ASS was determined by measuring the growth inhibitory disc zone, formed by the procedure as described above. Minimal inhibitory concentration (MIC) was assessed by extrapolating straight lines [11], achieved by a distance plot of disc zones of growth inhibitions as a function of logarithmic concentrations of ASS. MIC values included both of cidal and stasis.

RESULTS

Production of Specific Antibiotics from Nature

From the thousands of isolates, it was found that a few bacterial broths exhibited specific antibacterial activities pertaining to the particular strains among those tested. Data in Table 1 reveals that *M. luteus* and *Arthrobacter* sp. were relatively susceptible to naturally occurring antibiotics. It was interesting that even *P. aeruginosa* and *S. aureus* could also be specifically attacked by natural antibiotics. In this paper, we selected strain Q-0, renamed as KUH-001 to study about its production of antistaphylococcal substance (ASS).

Taxonomical Studies of Strain KUH-001

The ass producer was determined to be a gram-negative bacterium, motile rod with flagellar. Since this bacterium expressed characteristic fluorescence on NM, King A and King B media, we thought it would be a species of *Pseudomonas* [27]. From the knowledge that *Pseudomonas* species per se can be grouped on the basis of their cellular fatty acid compositions [13], we analyzed the cellular fatty acid composition of this bacterium. GC-profile of liberated fatty acids from cells by methanolysis with 5% HCl-methanol (Fig. 1A) were compared with that found in the methylating method upon hydrolysis of fatty acids by 4 N HCl (Fig. 1B). GC-data illustrated that the cell's fatty acids were mainly composed of even-numbered straight chains of

Table 1. Isolated microorganisms exhibiting specific antibiotic activities.

Strain	Susceptible organisms	Disc zone diameter (mm)
E-43	*M. luteus*	32
E-151	*M. luteus*	38
E-189	*M. luteus*	(11)
E-200	*M. luteus*	(12)
8	*M. luteus*	10
D-97	*Arthrobacter* sp.	12
D-137	*Arthrobacter sp.*	12
D-171	*Arthrobacter sp.*	22
E-209	*Arthrobacter sp.*	18
E-242	*Arthrobacter sp.*	40
E-364	*P. aeruginosa*	(17)
F-207	*P. rettgeri*	12 (14)
Q-0 (KUH-001)	*S. aureus*	30

Bacterial species denoted in the middle of the table indicate the only strains susceptible to culture broths of numbered strains isolated from nature. Parentheses for disc zone diameters (mm) indicate bacteriostatic activities other than bacteriocides.

$C_{16:0}$ and $C_{18:1}$. In the case of 3-hydroxy fatty acids 3-$OH_{10:0}$ and 3-$OH_{12:0}$ were identified, ubiquitous in *Pseudomonas* species. Data in the two figures appeared to be considerably different. It seemed that this might be due to the presence of amide bonds, which were readily disrupted by treatment with 4 N HCl, resulting to yield corresponding free fatty acids. These observations are known to be common in gram-negative bacteria.

Isolation and Purification of ASS from *Pseudomonas* sp. KUH-001

Cell growth and production of the antibiotic by jar fermentor were monitored, and the resultant data are shown in Fig. 2. Cell growth was accompanied by somewhat delayed production of ASS with increasing pH of broth. Its maximal activity was observed after 1 day of cultivation. Culture broth (48 *l*) at this period was centrifuged (10,000 rpm for 10 min). The resulting culture filtrate was extracted by ethylacetate and concentrated to give 2.9 g of crude ASS. Since crude ASS was able to be dissolved in a large volume of distilled water, it could be adsorbed to activated carbon for which excess pigments were removed. In the adsorbed active pool eluted by 80% methanol, there were at least two kinds of ASS, found from TLC slice test (silica gel; ethylacetate-hexane=9:1, $R_{f0.2}$ and $R_{f0.6}$, data not shown). Of these, the major active fraction corresponding to the R_f value of 0.2 was used for further purification. The purification procedure, presented in Scheme 1, yielded 16.7 mg of leaflet powder from 48 *l* of culture broth. Purification degree could be ascertained from its m.p. (140~142°C), analytical HPLC (ODS column, 50% CH_3CN: r.t. 30 min; Fig. 3) and TLC (chloroform-methanol=9:1, $R_{f0.44}$).

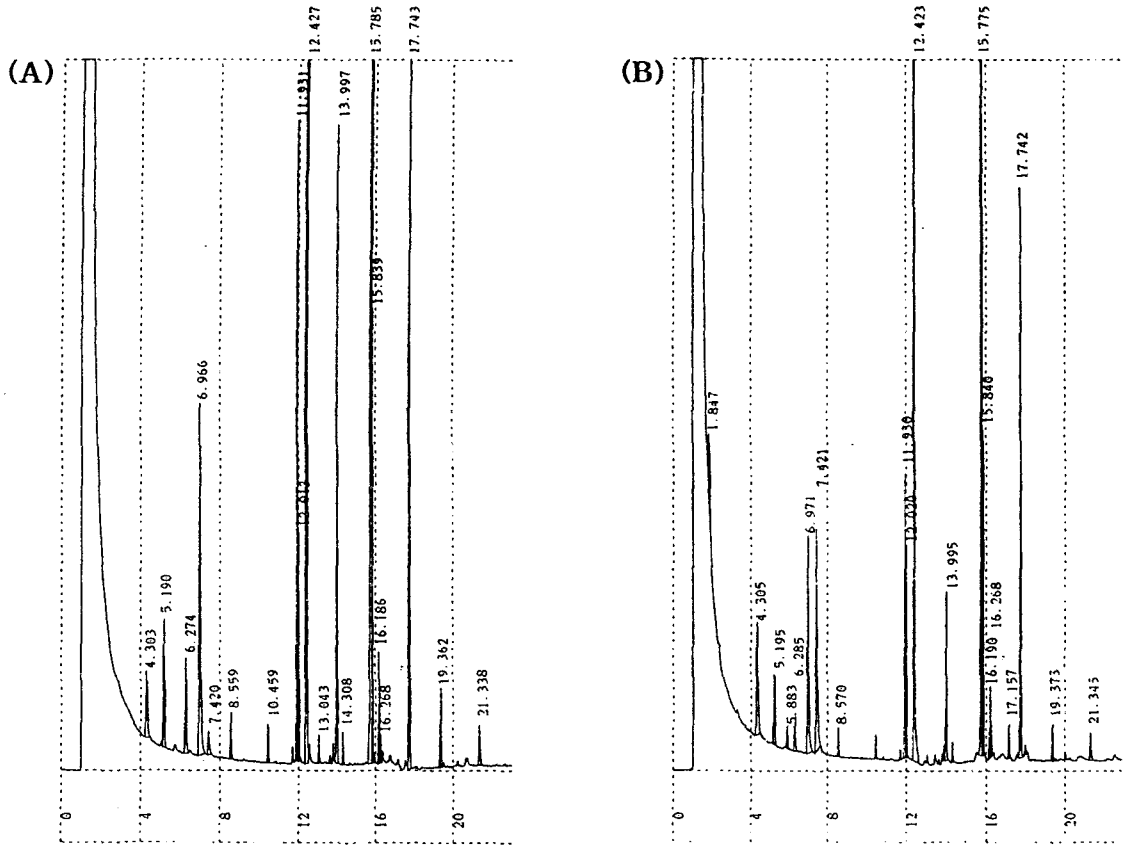

Fig. 1. Gas chromatographic profiles of cellular fatty acids from strain KUH-001.
(A) Fatty acids were directly extracted after treatment with 5% HCl in methanol. (B) Cells were treated by 4 N HCl prior to the esterification of fatty acids.

This compound was also able to be purified in an alternative way: Ethylacetate extract of culture filtrate was directly applied to batch chromatography using silica gel, eluted by concentrated methanol. The active fraction eluted was concentrated and dissolved in a large volume of distilled water. After removing precipitate by filter funnel, pigments in the filtrate were removed by charcoal with batch method. The active pool was then eluted by chloroform-methanol (1:1), and evaporated. The resulting pellet was loaded onto a column of cellulose, eluted by toluene-ethylacetate (3:1). Following trituration of active fraction by chilled ether, a column chromatography using silica gel was undertaken, eluted by ethylacetate-chloroform-acetic acid (3:1:1). The active fractions were collected and then its trituration/precipitation process was successively carried out using chilled ether until not exceeding 2°C of the melting point. This method was far more tedious and time-consuming compared with the above method. However, for large scale preparation of the antibiotic, this latter procedure seemed to be more appropriate.

Physicochemical Properties of Purified ASS

The purified compound exhibited extraordinarily stable characteristics against change in pH or temperature. Under a dissolved state in 1 N HCl or 1 N NaOH, its antibiotic activity was maintained for 1 day at room temperature without significant change. Over 95% of the activity was retained after autoclaving (121°C, 10 min). Although the purified ASS was practically insoluble in water, it could be dissolved in water to give more than 25 mm of growth inhibitory zone of disc per 0.01 ml against *S. aureus* (data not shown). Comparative data for the relationship between activities and solubilities of related compounds are shown in Table 2.

Structural Elucidation of ASS

The ultraviolet absorption spectrum of purified ASS is shown in Fig. 4. This compound was shown to possess major absorbing peaks at wavelengths of 215 nm and 240 nm along with a broad peak at about 325 nm, suggesting that this compound consists of a quinoline skeleton [3, 28]. IR-spectral data (Fig. 5) revealed that its structure is constructed by aliphatic, aromatic C-H bonds (2940 cm^{-1}, 1250 cm^{-1}) and aromatic C=C, C-O bonds (1600 cm^{-1}, 1125 cm^{-1}). Data of FAB-MS are presented in Fig. 6. As presented, the m/z of the ass indicated to be 260 for protonated $(M+H)^+$ species and 282 for sodium salt (M+Na) species, respectively. Also, from ^{1}H-

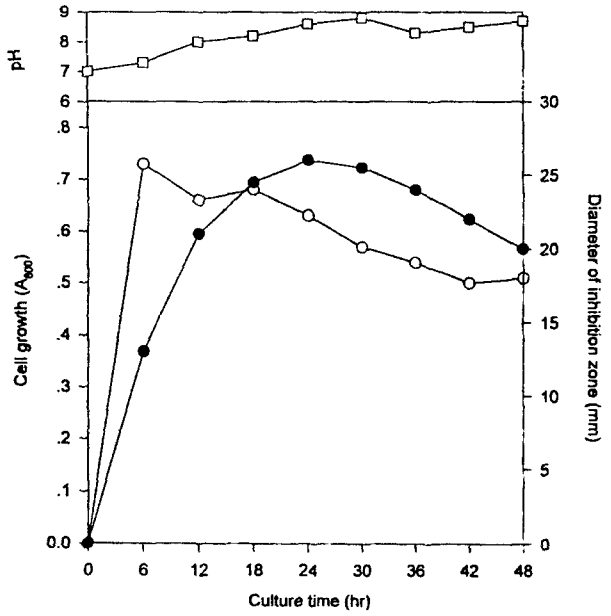

Fig. 2. Time course of cell growth and ASS production from strain KUH-001.

Cells of strain KUH-001 were grown using jar fermentor containing 3 *l* of NM (see text for detailed conditions) and time course production of ASS was monitored as follows; 0.01 ml of culture broth at each time was applied onto paper discs, placed on seeded NM agar plate with *S. aureus*, and incubated overnight at 30°C. Activity was expressed by measuring the disc zone diameter (mm) as described in 'Materials and Methods'. Symbols used: (○), cell growth; (●), ASS production; (□), pH of cell broth.

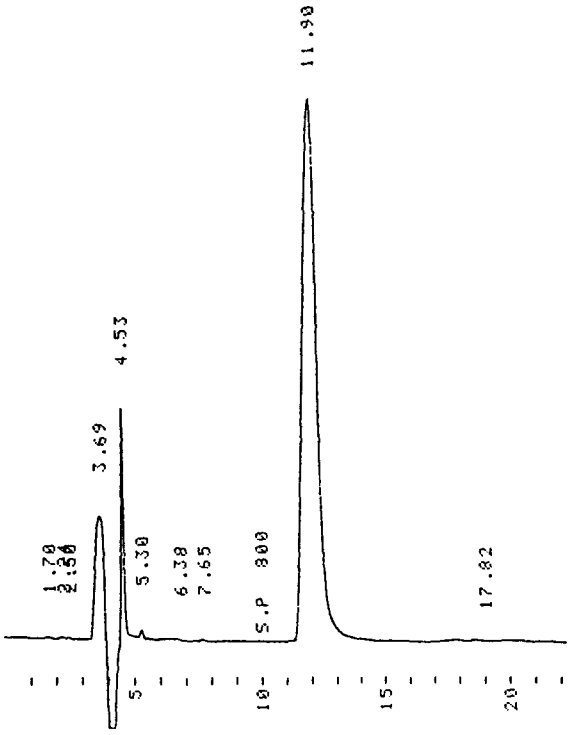

Fig. 3. HPLC profile of purified ASS.

Peaks were monitored at 220 nm. The major peak eluted at 11.90 min of retention time was used in this study.

Table 2. Comparison of some 2-alkyl-4-hydroxyquinolone N-oxides in relation to their solubilities and activities.

Compound	Solubility	Relative activity
Natural crystalline antagonist	ND	100
2-Methyl-4-hydroxyquinoline *N*-oxide	ND	< 0.1
2-n-Heptyl-4-hydroxyquinoline *N*-oxide	3.5	33
2-n-Nonyl-4-hydroxyquinoline *N*-oxide	0.3	206
2-n-Undecyl-4-hydroxyquinoline *N*-oxide	ND	66

[a]Maximal solubilities (μg/ml) of compounds in 0.35 M phosphate buffer, pH 7.0 at 22°C.

[b]Dihydrostreptomycin antagonizing activities of compounds for *B. pumilus* relative to that found in the natural crystalline antagonist, determined by a modified cup-plate assay (data from ref. 17). ND, not determined.

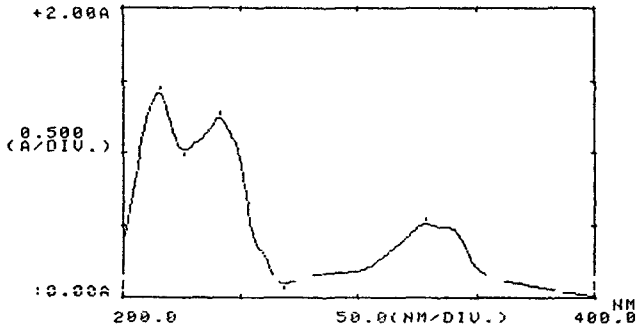

Fig. 4. Ultraviolet spectrum of purified ASS in methanol.

The spectral curve shows two narrow peaks (at 215 nm and 240 nm) and a broad peak at around 328 nm. Minimal absorption was observed at 270 nm.

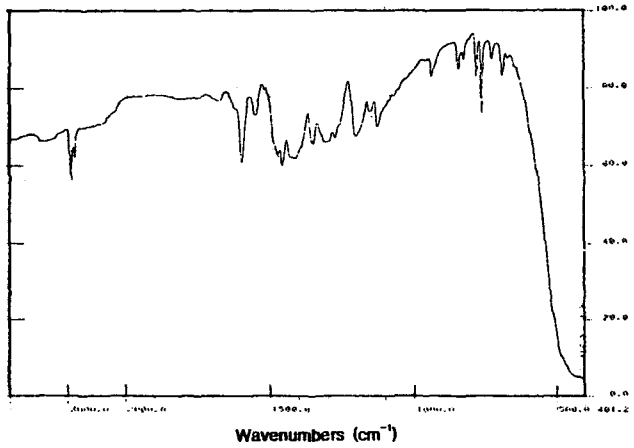

Fig. 5. Infrared absorption spectrum of purified ASS.

The spectrum was obtained using KBr plates, showing characteristic absorption bands with ranges (cm^{-1}) and intensities of 762 (m), 1125 (w), 1270 (w), 1350 (w), 1425 (w), 1600 (m), 2940 (m). Intensity: w= weak, m=medium.

NMR data, shown in Fig. 7, it was found that the purified compound in colourless leaflets (m.p. 140~142°C) consisted of a typical quinolinal skeleton [3-H (6.11 ppm; s, 1H), 7.34 ppm (t, 1H), 7.61 ppm (t, 1H), 8.14 ppm (d, 1H), 8.21 ppm (d, 1H) and Ar-CH_2-CH_2 (2.55 ppm; t, 2H)], a

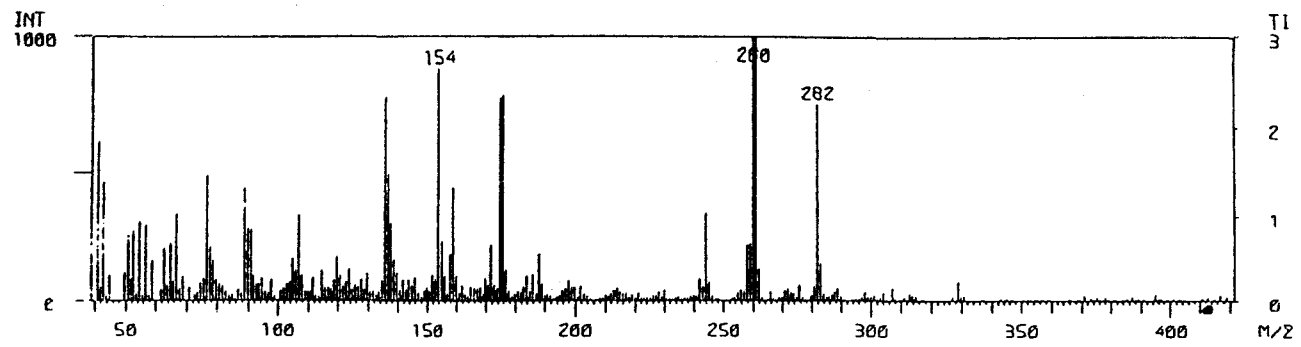

Fig. 6. Fast atom bombardment mass spectrometry of purified ASS.
From the mass spectrum of ASS the parent species of $(M+H)^+$ and (M+Na) were determined at 260 and 282 of m/z, respectively.

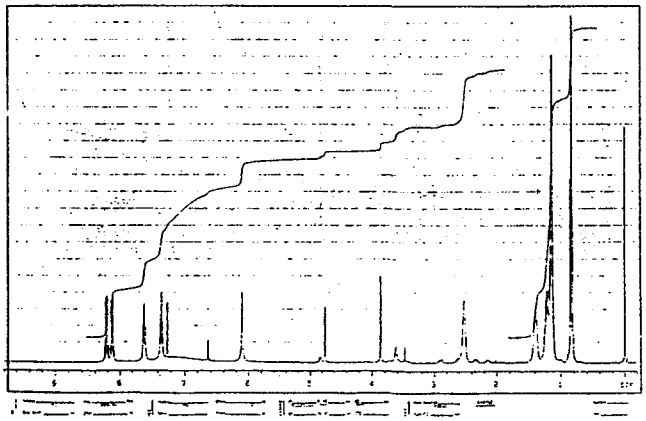

Fig. 7. The 400 MHz proton-NMR spectrum ($CDCl_3$) of purified ASS.

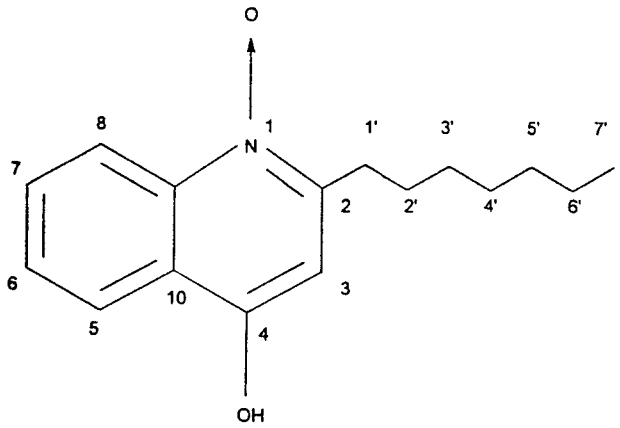

Fig. 8. Proposed structure of purified ASS.
This structure indicates the purified ass may belong to the alkyl-quinolone family.

methyl group (0.85 ppm) and a six numbered-carbohydrate chain (1.1~1.5 ppm). With extra data of its elemental composition (C:H:N, 74.1-8.4-5.4, %), the structure of this compound was clarified as 2-heptyl-4-hydroxy-quinoline N-oxide (HHQO) with molecular formula of $C_{16}H_{21}NO_2$, indicating a derivative of alkyl-hydroxyquinoline N-oxide (Fig. 8) [17].

Table 3. Antimicrobial spectrum of purified ASS from strain KUH-001.

Test organisms	MIC (μg/ml)
S. aureus No. 19	1
*S. aureus*KCTC 1916	5
*S. aureus*KCTC 1621	10
B. subtilis ATCC 2101	50
B. megaterium	> 100
E. coli No. 20	> 100
P. aeruginosa ATCC 1711	> 100
A. niger ATCC 13497	> 100
A. oryzae	> 100
C. albicans KCTC 1940	

See Materials and Methods for estimation for MIC values.

Antibiotic Activity of Purified ASS

According to the screening strategy employed in this study, this compound must be active especially against *S. aureus*. As presented in Table 3, its narrow antimicrobial spectrum was already anticipated. Despite exhibition of anti *-Bacillus* property, its principal targets were thought to be staphylococci including MRSA. Interestingly, ASS showed unidirectional mode of synergy toward vancomycin. Pertaining to the synergistic effect, erythromycin was most effective among drugs examined. Detailed data will be described in a separate paper. In addition, we have not detected any HHQO resistance yet, and found that HHQO made no cross resistance with other antibiotics such as penicillin, rifampin, or chloramphenicol (personal observation).

DISCUSSION

We investigated in this paper antistaphylococcal substance (ASS) from nature, enabling to combat MRSA, specifically because methicillin resistant strains in general have characteristics of broad resistance against chemotherapy except in the case of vancomycin [6]. Furthermore, since its emerging rate is often occasioned by the presence of either impaired host factors such as abscess or slightly more resistance to β-lactam therapy

[15], methicillin resistance is now a task of great difficulty in medication [4, 7, 18]. Therefore, possible provision against this target was our initial purpose. To do this, developing new lead compounds as alternatives to vancomycin was thought to be of great significance.

A bacterial strain KUH-001, producing ASS, was isolated from nature. According to its characteristic fluorescence and cellular fatty acid composition, this bacterium was identified to belong to *Pseudomonas* species, and the purified ass was elucidated to be 2-heptyl-4-hydroxyquinoline-N-oxide (HHQO). This compound is one of the 2-alkyl-4-quinolones (pseudans) [26] which occur in certain microorganisms and plants [8]. Alkylquinolones can be synthesized [26], and are now available commercially. Surprisingly, 4-hydroxy or 4-methoxy derivatives have not yet been found in nature, but instead, isolated exclusively from *Pseudomonas aeruginosa* present in biopsy specimens of patients [9, 21]. Recently, pyocyanin and 1-hydroxyphenazine were also isolated from *Pseudomonas aeruginosa*, showing distinct properties for human respiratory ciliostasis or dyskinesia [30]. Although these compounds are structurally different from HHQO, their physiological roles seem to be related to each other as potential cytotoxins. Besides, HHQO is also of interest as a powerful inhibitor for lipoxygenase. On the other hand, quinolone alkaloids isolated from *Evodia rutaecarpa* were reported to inhibit growth of the silkworm larva, *Brombyx mori L.* [26]. These unique properties of pseudans are of great interest in the presumed natural homeostasis of the ecosystem. Alterations in the population between *P. aeruginosa* and *S. aureus* in patients with cystic fibrosis is regarded as a result of the production of akyl-substituted quinolone-N-oxides, particularly HHQO, by *P. aeruginosa* [17, 19]. Because their producers are supposed to be opportunistic, de novo production of these antibiotics would give rise to derange staphylococcal growth, followed by overwhelming colonization.

As was stressed in the text, tolerability of these compounds by gram-negative bacteria is suggested to be due to either the incompatibility of these against cytochrome components or the difficulty in their cellular transports [17]. However, this is still controversial. Meanwhile, our preliminary data indicate that HHQO could be uptaken with similar velocities towards both gram species, but then resulted in altered phenotypes of membrane proteins (data not shown). More information is needed in order to understand their mechanism of antibiosis.

We also found that the isolated HHQO exhibited synergy with certain antibiotics (e.g., vancomycin or erythromycin), although streptomycin antagonizes it with unknown reason. Although HHQO resistance has not yet been found *in vitro*, therapeutic application of this compound would be promising in the future. Its combined therapy with synergistic agents, for instance, may contribute to the current medical treatment, despite its conceivable side effects such as imperfect respiration. Although we found only one strain through the screening, it seems very unusual that the HHQO producer can occur naturally. In fact, our finding with respect to the natural occurrence of such a producer may be of great importance, definitely not to be ignored. Taking account of its significance in nature, further studies on the taxonomic status of strain KUH-001 should be undertaken. Studies on synergism of HHQO with other antibiotics also need to be pursued. These subjects are being investigated in our laboratory.

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