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# Action mechanism of 6, 6′-dihydroxythiobinupharidine from *Nuphar japonicum*, which showed anti-MRSA and anti-VRE activities

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ABSTRACT

*Background:* Multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE), cause serious infections at clinical sites, for which the development of new drugs is necessary. We screened candidates for new antibiotics and investigated its action mechanism.
*Methods:* An antimicrobial compound was isolated from an extract of *Nuphar japonicum*. Its chemical structure was determined by NMR, MS, and optical rotation. We measured its minimum inhibitory concentration (MIC) using the microdilution method. The effects of the compound on DNA gyrase and DNA topoisomerase IV were investigated with DNA supercoiling, decatenation, and cleavage assay.
*Results:* We isolated and identified 6,6′-dihydroxythiobinupharidine as the antimicrobial compound. The MIC of this compound was 1–4 μg/mL against various MRSA and VRE strains. We also demonstrated that this compound inhibited DNA topoisomerase IV ($IC_{50}$ was 10–15 μM), but not DNA gyrase in *S. aureus*, both of which are known to be the targets of quinolone antibiotics and necessary for DNA replication. However, this compound only exhibited slight cross-resistance to norfloxacin-resistant *S. aureus*, which indicated that DTBN might inhibit other targets besides topoisomerase IV. These results suggest that 6,6′-dihydroxythiobinupharidine may be a potent candidate or seed for novel antibacterial agents.
*Conclusions:* DTBN from *N. japonicum* showed anti-MRSA and anti-VRE activities. DTBN might be involved in the inhibition of DNA topoisomerase IV.
*General significance:* DTBN might be useful as a seed compound. The information on the inhibition mechanism of DTBN will be useful for the modification of DTBN towards developing novel anti-MRSA and anti-VRE drug.


## 1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961 and has been spread worldwide [1]. MRSA has recently become one of the most important pathogenic bacteria that cause pneumonia, meningitis, and sepsis at clinical sites. MRSA infections are difficult to treat and are more severe because of their resistance to various antibiotics. Glycopeptides (vancomycin, teicoplanin), aminoglycosides (arbekacin), oxazolidinones (linezolid), and lipopeptides (daptomycin) have been developed to treat MRSA infections. These new antibiotics are suitable against modifying enzymes and have different targets from those of early antibiotics. However, vancomycin intermediate-resistant *S. aureus* (VISA), which has a thick cell wall, and vancomycin-resistant *S. aureus* (VRSA) carrying *vanA* from vancomycin-resistant enterococci (VRE) have recently been identified [2,3]. Arbekacin-resistant *S. aureus*, which acquired the arbekacin-modifying enzyme, and linezolid-resistant *S. aureus* have also been reported [4,5]. Since *S. aureus* is acquiring resistance to drugs currently used in clinical settings, the development of new drugs is needed.

*Abbreviations:* AcOEt, ethyl acetate; CFU, colony forming units; DTBN, 6, 6′-dihydroxythiobinupharidine; $Et_2NH$, diethylamine; MeOH, methanol; MIC, minimal inhibitory concentration; NOR, norfloxacin; O.D., optical density; VCM, vancomycin

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In our laboratory, we have been trying to identify candidate compounds for new drugs from crude drugs or herbs that have been used for a long time as folk medicine. We have so far discovered inhibitors for bacterial drug-resistant mechanisms, such as tellimagrandin I, baicalein, and corilagin. Tellimagrandin I and corilagin were shown to inhibit PBP2a and potentiated β-lactams [6], while baicalein inhibited TetK, a tetracycline efflux transporter [7]. Kaempferol-3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside and kaempferol-3-*O*-a-L-(2″-*E*-*p*-coumaroyl-4″-*Z*-*p*-coumaroyl)-rhamnoside are flavonoids from Laurel that exhibit strong anti-MRSA activities [8]. We also showed that these compounds inhibited both *S. aureus* DNA gyrase and topoisomerase IV, which are essential enzymes for DNA replication, transcription, and repair.

We screened candidates for new antibiotics from seven natural medicines and found that the methanol extract from *Nuphar japonicum* showed strong anti-MRSA and anti-VRE activities. In the present study, we identified an antimicrobial compound against MRSA in the methanol extract from *N. japonicum*.

*N. japonicum* is a perennial plant that grows naturally in aquatic marshes and the rhizome is called Nuphar Rhizome. It has been used as a crude drug for the treatment of edema, irregular menstruation, hysteria with diuretics, and as a blood purifier and sedative. It includes alkaloids such as nupharamine, nuphamine, and deoxynupharidine [9,10], and tannins such as nupharins [9]. *Nuphar pumilum*, which is a plant that is closely related to *N. japonicum*, includes many alkaloids such as nupharidine, deoxynupharidine, 7-epideoxynupharidine, 6-hydroxythiobinupharidine, 6,6′-dihydroxythiobinupharidine (DTBN), nupharolutin, and neothiobinupharidine [11]. The immunosuppressive effects and inhibitory actions of deoxynupharidine on the central nervous system [12], and antifungal activity and immunosuppressive effects of DTBN have been described previously [13,14]. However, to the best of our knowledge, its antimicrobial activity has not yet been demonstrated. Therefore, we isolated and identified DTBN from *N. japonicum* as a substance exhibiting strong antibacterial activity against MRSA. We also showed that DNA topoisomerase IV was one of the targets of this compound.

## 2. Materials and methods

### 2.1. Bacterial strains

A description of all the strains used in this study was described in Table 1. The MRSA OM series and HU series were clinical isolates. MRSA N315 and MRSA COL were standard strains for genome analysis [15,16]. *S. aureus* K1 and 3-2 (288-3) were given by Dr. T. Takenouchi of Daiichi Sankyo Co., Ltd. [17]. VISA Mu50 was a kind gift from Dr. K. Hiramatsu of Juntendo University (Japan). NF42 and NF82 were isolated as norfloxacin-resistant mutants from N315. The DTR series was isolated as DTBN-resistant mutants from N315. *Enterococcus faecalis*

**Table 1**
Bacterial strains and their description.

| Strain | Description | Mutation in the QRDR | | | | Reference |
|---|---|---|---|---|---|---|
| | | GyrA | GyrB | ParC | ParE | |
| *Staphylococcus aureus* | | | | | | |
| 209P | Laboratory strain | – | – | – | – | |
| NCTC8325 | Laboratory strain | – | – | – | – | [18] |
| RN4220 | Genomic standard | – | – | – | – | [19] |
| N315 | Genomic standard | – | – | – | – | [16] |
| NF42 | NOR$^{r}$ from N315 | – | – | E84K | – | This study |
| NF82 | NOR$^{r}$ from N315 | – | – | E84K | – | This study |
| DTR8AL1 | DTBN$^{r}$ from N315 | S84L | – | S80F | – | This study |
| DTR8BL1 | DTBN$^{r}$ from N315 | S84L | – | S80F | – | This study |
| DTR8DL1 | DTBN$^{r}$ from N315 | S84L | – | S80F | – | This study |
| DTR8EL1 | DTBN$^{r}$ from N315 | S84L | – | S80F | – | This study |
| Mu50 | VCM$^{i}$ | – | – | S80F | – | [16] |
| MRSA–COL | Genomic standard | – | – | – | – | [15] |
| 3-2(288-3) | Clinical isolate | S84L, E88G | – | S80Y, E84K | – | [17] |
| K-1 | Clinical isolate | S84L, E88K | – | S80F, E84K | – | [17] |
| OM481 | Clinical isolate | – | – | S80F | – | [20] |
| OM505 | Clinical isolate | – | – | S108N | – | [20] |
| OM584 | Clinical isolate | E88K | – | S80F | – | [20] |
| OM623 | Clinical isolate | E88K | – | S80F | – | [20] |
| HU2 | Clinical isolate | S84L | – | E84K | – | This study |
| HU3 | Clinical isolate | S84L | – | S80F | – | This study |
| HU4 | Clinical isolate | S84L | – | S80Y | – | This study |
| HU6 | Clinical isolate | S84L | – | E84K | – | This study |
| HU7 | Clinical isolate | S84L | – | S80Y | – | This study |
| HU11 | Clinical isolate | S84L | – | S80Y | – | This study |
| HU12 | Clinical isolate | S84L | – | S80Y | – | This study |
| HU13 | Clinical isolate | S84L | – | S80Y | – | This study |
| HU14 | Clinical isolate | S84L | – | E84K | – | This study |
| HU20 | Clinical isolate | S84L | – | S80Y | – | This study |
| *Enterococcus faecium* | | | | | | |
| ATCC19434 | VCM$^{s}$ | n.d. | n.d. | n.d. | n.d. | |
| FN-1 | VCM$^{r}$(*vanA*) | n.d. | n.d. | n.d. | n.d. | |
| *Enterococcus faecalis* | | | | | | |
| ATCC19433 | VCM$^{s}$ | n.d. | n.d. | n.d. | n.d. | |
| ATCC29212 | VCM$^{s}$ | n.d. | n.d. | n.d. | n.d. | |
| ATCC51299 | VCM$^{r}$(*vanB*) | n.d. | n.d. | n.d. | n.d. | |
| NCTC12201 | VCM$^{r}$(*vanA*) | n.d. | n.d. | n.d. | n.d. | |
| NCTC12203 | VCM$^{r}$(*vanA*) | n.d. | n.d. | n.d. | n.d. | |

n.d.: not determined, $^{r}$ resistant, $^{s}$ sensitive, $^{i}$ intermediate.

ATCC19433 and NCTC12203 were obtained from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Tsukuba, Japan.

### 2.2. Isolation and identification of the antimicrobial agent from N. japonicum

The isolation procedure (Fig. 1) has already been described previously [11]. In brief, dried rhizomes of *N. japonicum* (4.0 kg, Takasago Yakugyo (Osaka, Japan)) were crushed and extracted with approximately 80 L methanol (MeOH). The MeOH extract (323 g) was then vacuum filtrated and evaporated. A total of 102 g of this extract was subjected to liquid–liquid separation. We obtained a chloroform ($CHCl_3$) extract (10.1 g), ethyl acetate (AcOEt) extract (9.0 g), water extract (113 g), and insoluble matter (64.3 g). The AcOEt extract (8.17 g) was bedded to silica gel column chromatography (silica gel 60) and eluted with $CHCl_3$–AcOEt–ethylenediamine (EDA) (20:1:1) and MeOH–EDA (10:1) to obtain three fractions (fr.1 5.56 g, fr.2 1.27 g, and fr.3 1.30 g). Fr.1 (0.92 g) was bedded to silica gel column chromatography and eluted with n-Hexane–AcOEt–$NH_4OH$ (75:25:1) and MeOH–$NH_4OH$ (100:1) to obtain fractions (fr.1-I 64.9 mg, fr.1-II 51.9 mg, fr.1-III 91.8 mg, fr.1-IV 36.4 mg, fr.1-V 50.6 mg, fr.1-VI 73.7 mg, fr.1-VII 14.5 mg, fr.1-VIII 159.9 mg, fr.1-IX 167.5 mg, and fr.1-X 1276.3 mg). Silica gel (SIGMA-ALDRICH), pore size 60 Å, and a 70–230 mesh were used for column chromatography.

### 2.3. Analysis of thin layer chromatography (TLC)

TLC Silica gel 60 $F_{254}$ Aluminum sheets (MERCK KGaA) were used for the stationary phase. The mobile phase was the same as the above silica gel chromatography. We detected spots using UV (254 nm) absorption and Dragendorff's spray reagents.

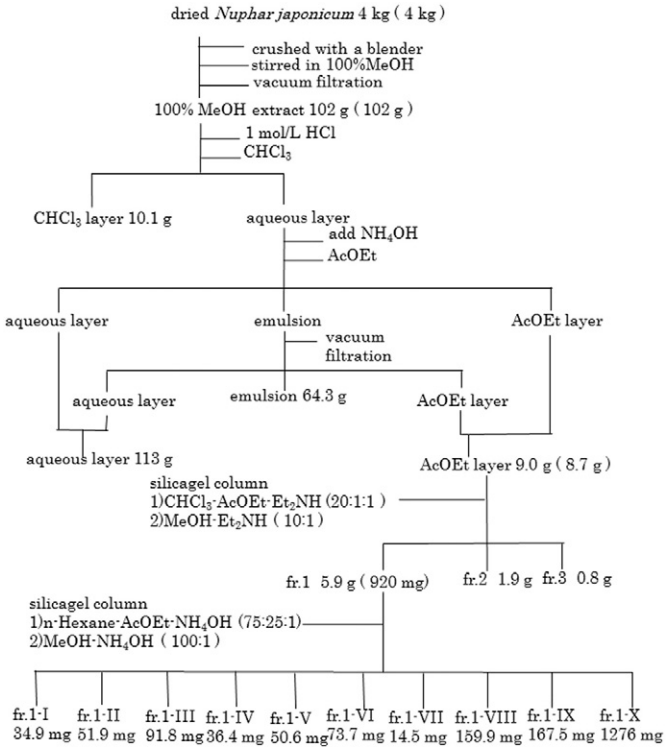


**Fig. 1.** Fractionation of the methanol extract from *Nuphar japonicum*. The isolation procedure has already been reported previously [11]. The weights beside the parentheses show the yield, while the weights in the parentheses show the quantity used for the next fractionation.

### 2.4. Measurement of nuclear magnetic resonance (NMR) spectrum, mass spectrum (MS), and optical rotation

We analyzed fr.1-VIII with $^1$H NMR analysis, MS analysis, and optical rotation measurements to identify the compounds. The NMR spectrum was measured in deuterated MeOH by Varian INOVA 600 ($^1$H NMR; 600 MHz). Chemical shifts are given in δ (ppm) values relative to those of the solvent signal [MeOH-d4 (δH 3.30)] on the tetramethylsilane scale. The MS spectrum was measured by JMS-700 MStation. Optical rotation was measured by JASCO DIP1000 in dichloromethane.

Fr.1-VIII (DTBN): Dragendorff's reagent: +, $[\alpha]_D^{24.2}$ + 74.0° (c = 1.5, dichloromethane) lit.$[\alpha]_D^{25}$ + 78° (c = 1.5, dichloromethane), $^1$H NMR (deuterated MeOH, 600 MHz, δ): given in Table 2. FAB-MS(m/*e*): 230 (characteristic fragment for most Nuphar alkaloids [21]), 262 (230 + S).

### 2.5. Investigation of antibacterial activity

MICs were determined using the broth microdilution method according to the recommendations of the Japanese Society of Chemotherapy [22]. Briefly, MICs were determined in Mueller–Hinton broth (Difco) containing each compound in a two-fold serial dilution series. Approximately $10^4$ cells were inoculated in each well and incubated at 37 °C for 24 h, and growth was examined visually. The MIC was defined as the lowest concentration that prevented visible growth.

### 2.6. Effect of the compound on the survival rate of S. aureus cells

*S. aureus* cells were cultured in N broth (Nissui) at 37 °C until $O.D._{650}$ = 0.7. Cells were then divided into four test tubes. DTBN was added to three tubes at final concentrations of 0.5 μg/mL, 2 μg/mL, and 8 μg/mL, and one tube, to which DTBN was not added, was the control.

**Table 2**
Peak and coupling constant of NMR spectrum.

| Position | Fr.1-VIII | DTBN[a] |
|---|---|---|
| 1 | 1.24[b] | 1.26[b] |
| 2a | 1.63[b] | 1.62[b] |
| 2b | 1.11[b] | 1.10[b] |
| 3 | 1.65[b] | 1.65[b] |
| 4 | 3.70 (d.d., J = 7.8, 9.6 Hz) | 3.70 (d.d., J = 5.0, 8.0 Hz) |
| 6 | 3.77 (s) | 3.77 (s) |
| 8a | 1.40[b] | 1.40[b] |
| 8b | 1.86[b] | 1.86[b] |
| 9a | 1.20[b] | 1.21[b] |
| 9b | 1.83[b] | 1.82[b] |
| 10 | 2.31[b] | 2.32[b] |
| 11 | 0.92 (d, J = 6.6 Hz) | 0.92 (d, J = 6.5 Hz) |
| 13 | 6.42 (d, J = 0.6 Hz) | 6.43 (d, J = 1.0 Hz) |
| 14 | 7.45 (t, J = 1.8 Hz) | 7.45 (t, J = 1.0 Hz) |
| 16 | 7.43 (s) | 7.43 (s) |
| 17a | 1.79 (d, J = 14.4 Hz) | 1.79 (d, J = 13.0 Hz) |
| 17b | 1.85 (d, J = 14.4 Hz) | 1.84 (d, J = 13.0 Hz) |
| 1′ | 1.28[b] | 1.28[b] |
| 2′a | 1.62[b] | 1.62[b] |
| 2′b | 1.10[b] | 1.10[b] |
| 3′ | 1.65[b] | 1.65[b] |
| 4′ | 3.65 (d.d., J = 8.4, 10.2 Hz) | 3.65 (d.d., J = 5.0, 8.0 Hz) |
| 6′a | 3.93 (s) | 3.93 (s) |
| 8′a | 1.60[b] | 1.60[b] |
| 8′b | 1.27[b] | 1.27[b] |
| 9′a | 1.20[b] | 1.21[b] |
| 9′b | 1.80[b] | 1.79[b] |
| 10′ | 2.34[b] | 2.35[b] |
| 11′ | 0.93 (d, J = 6.6 Hz) | 0.93 (d, J = 6.5 Hz) |
| 13′ | 6.43 (d, J = 1.2 Hz) | 6.42 (d, J = 1.0 Hz) |
| 14′ | 7.45 (t, J = 1.8 Hz) | 7.45 (t, J = 1.0 Hz) |
| 16′ | 7.43 (s) | 7.43 (s) |
| 17′a | 2.19 (d, J = 12.0 Hz) | 2.19 (d, J = 12.0 Hz) |
| 17′b | 2.64 (d, J = 12.0 Hz) | 2.64 (d, J = 12.0 Hz) |

[a] Peak and coupling constant of DTBN was referred to previous report [11].
[b] Signals were overlapped, d: doublet, d.d.: double doublet, s: singlet.

These tubes were incubated at 37 °C with shaking. Small aliquots were obtained at 3 h, 6 h, 12 h, and 24 h, diluted, and spread on N plates including 1.5% agar. Plates were incubated at 37 °C for 16–24 h and the number of colonies was then counted to calculate CFU/mL.

### 2.7. Effect of the compound on the growth rate of S. aureus cells

*S. aureus* cells were incubated in N broth at 37 °C for 16–24 h. Cells were $10^4$ diluted in N broth, which included no or 4 μg/mL DTBN. All samples were incubated at 37 °C for 48 h. Small aliquots were obtained at 6 h, 12 h, 24 h, and 48 h. They were diluted and spread on N plates including 1.5% agar. Plates were incubated at 37 °C for 16–24 h and the number of colonies was counted to calculate CFU/mL.

### 2.8. DNA gyrase supercoiling assay and topoisomerase IV decatenation assay

The *S. aureus* gyrase supercoiling assay kit (Inspiralis Co., Ltd.) was used to investigate the effects of DNA gyrase. One unit of DNA gyrase converted 500 ng relaxed pBR322 to the supercoiled form in 30 min. The enzyme and substrate DNA were incubated at 37 °C in reaction mixture (40 mM HEPES-KOH (pH 7.6), 10 mM magnesium acetate, 500 mM potassium glutamate, 10 mM DTT, 2 mM ATP, and 50 μg/mL albumin) for 30 min.

The *S. aureus* topoisomerase IV decatenation assay kit (Inspiralis Co., Ltd.) was used for DNA topoisomerase IV. One unit of DNA topoisomerase IV decatenated 200 ng of kinetoplast DNA to mini-circle DNA in 30 min. The enzyme and substrate DNA were incubated at 37 °C in reaction mixture (50 mM Tris–HCl (pH 7.5), 5 mM magnesium chloride, 5 mM DTT, 1.5 mM ATP, 350 mM potassium glutamate, and 50 μg/mL albumin) for 30 min.

The reaction mixture following incubation was electrophoresed in 1% agarose gels at 50 V for 1.5 h for decatenation assay and 3 h for supercoiling assay, respectively. Gels were stained with 1 μg/mL ethidium bromide for 20 min and washed with distilled water for 15 min.

### 2.9. Isolation of resistant mutants from S. aureus N315

Regarding norfloxacin-resistant mutants, *S. aureus* N315 was cultured in Tryptic soy broth medium and spread on Mueller–Hinton agar plates including norfloxacin (16 μg/mL or 32 μg/mL). Plates were incubated at 37 °C and several colonies were obtained. After single colony isolation, the norfloxacin resistance of these mutants was confirmed again. Two mutants were named NF42 and NF82.

We could not isolate any mutants for DTBN using the method described above. Therefore, DTBN-resistant mutants were isolated by a subculture in liquid medium. *S. aureus* N315 was cultured in N broth. The cell solution was diluted in N broth including DTBN 1 μg/mL and incubated at 37 °C overnight. Small aliquots of cultured cells were inoculated in N broth including 2 μg/mL DTBN. This procedure was repeated and provided cells that could grow in the presence of 8 μg/mL DTBN. These cells were spread on N plates without DTBN and incubated at 37 °C. After single colony isolation, the DTBN resistance of these mutants was confirmed. Operations were carried independently four times, and we obtained four resistant mutants, named as DTR8AL1, DTR8BL1, DTR8DL1 and DTR8EL1.

### 2.10. Determining mutations in the quinolone-resistant determining regions (QRDRs) of gyrA, gyrB, parC, and parE

The QRDRs of these genes were amplified by PCR. The primers used in this study were described in Table 3.

**Table 3**
Primers used in this study.

| Primer | Sequence | Reference |
|---|---|---|
| *gyrA*_QRDR_F | TGCTCGTGCATTGCCAGATG | This study |
| *gyrA*_QRDR_R | AGACTGACGGCTCTCTTTCA | This study |
| *gyrB*_QRDR_F | CAGCGTTAGATGTAGCAAGT | [23] |
| *gyrB*_QRDR_R | CCGATTCCTGTACCAAATGC | [23] |
| *parC*_QRDR_F | GATGAGGAGGAAATCTAG | [23] |
| *parC*_QRDR_R | GTTGGAAAATCAGGACCTT | [23] |
| *parE*_QRDR_F | GACAATTGTCTAAATCACTTGTG | [23] |
| *parE*_QRDR_R | CACCATCAGTATCAGCATCA | This study |

### 2.11. Cleavage assay

The cleavage assay was performed as described previously [24]. Plasmid pBR322 was extracted with alkaline-SDS method and supercoiled pBR322 was separated by agarose gel electrophoresis and purified by Wizard® SV Gel and PCR Clean-Up System (Promega). One unit of topoisomerase IV (Inspiralis Co., Ltd.) and 240 ng supercoiled pBR322 were incubated at 25 °C for 1 h in reaction mixture (10 mM NaCl, 10 mM DTT, 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 200 mM potassium glutamate and 50 μg/mL BSA). SDS (0.23%) and proteinase K (115 μg/mL) were added and the sample incubated at 37 °C for 30 min. The reaction mixture following incubation was electrophoresed in 1% agarose gels at 50 V for 2 h. Gels were stained with 1 μg/mL ethidium bromide for 20 min and washed with distilled water for 20 min.

## 3. Results

### 3.1. Isolation of compounds with antimicrobial activities

We screened candidates for new antimicrobial agents from the MeOH extract of *N. japonicum*. Alkaloids and tannins were previously shown to be the main components of *N. japonicum* [9,10]. If the active compound(s) had been tannins, we could have isolated them with this procedure. Furthermore, if they had been alkaloids, pH control may have been useful for fractionation. We then partitioned the MeOH extract with liquid–liquid fractionation in 1 M HCl followed by liquid–liquid fractionation at pH 10 with ammonia water. We obtained a $CHCl_3$ layer, AcOEt layer, and emulsion (Fig. 1). The MIC at each step was shown in Table 4. The MIC of the AcOEt layer was 8-fold higher than that of the MeOH extract. The $CHCl_3$ layer and emulsion had weak or no anti-MRSA activity. The AcOEt layer was partitioned with

**Table 4**
Antimicrobial activities of the different fractions against MRSA.

| Fraction | MIC (μg/mL) | |
|---|---|---|
| | OM481 | OM584 |
| Methanol extract | 32 | 32 |
| Chloroform layer | 128 | 128 |
| Emulsion | 1024 | >1024 |
| Ethyl acetate layer | 4 | 4 |
| fr.1 | 4 | 4 |
| fr.2 | 64 | 64 |
| fr.3 | >256 | >256 |
| fr.1-I | >16 | >16 |
| fr.1-II | >16 | >16 |
| fr.1-III | 4 | 4 |
| fr.1-IV | 4 | 4 |
| fr.1-V | 4 | 4 |
| fr.1-VI | 4 | 4 |
| fr.1-VII | 4 | 4 |
| fr.1-VIII | 4 | 2 |
| fr.1-IX | 8 | 8 |
| fr.1-X | >16 | >16 |

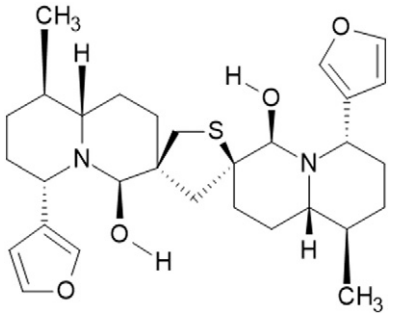

**Fig. 2.** The structure of DTBN.

silica gel column chromatography and three fractions were obtained. We further partitioned fr.1 with silica gel column chromatography (Fig. 1), and fractions were then analyzed by TLC. We finally isolated fr.1-VIII, which exhibited strong antimicrobial activity, and was expected to include only one compound because only one spot was detected by TLC. The compound was identified as 6,6′-dihydroxythiobinupharidine (DTBN) (Fig. 2) from NMR data, the MS spectrum, and optical rotation data (Table 2).

We examined its antibacterial activity against *S. aureus*, *Enterococcus faecium* and *E. faecalis* strains (Tables 5, 6). The MIC of DTBN was 1–2 μg/mL for the four methicillin-sensitive *S. aureus* (MSSA) strains, and 2–4 μg/mL for the twenty MRSA strains including VISA (Mu50). In addition, the MIC of DTBN for vancomycin-sensitive enterococci (VSE) and VRE strains was 2–4 μg/mL. These results indicated that DTBN was effective against *S. aureus* irrespective of sensitivity to oxacillin and enterococci irrespective of sensitivity to vancomycin, and this compound may be a potential candidate for new antimicrobial agents against MRSA and VRE.

**Table 6**
Antimicrobial activities of several compounds for *Enterococcus* strains.

| Strain | MIC (μg/mL) | | |
|---|---|---|---|
| | DTBN | Vancomycin | Norfloxacin |
| *Enterococcus faecium* | | | |
| ATCC19434 | 4 | 0.5 | 8 |
| FN-1 | 2 | 256 | >256 |
| *Enterococcus faecalis* | | | |
| ATCC19433 | 2–4 | 0.5 | 4 |
| ATCC29212 | 4 | 2 | 4 |
| ATCC51299 | 4 | 128 | 4 |
| NCTC12201 | 2 | >256 | 4 |
| NCTC12203 | 4 | >256 | 16 |
| FA2-2 | 2 | 1 | 8 |

–: not determined.

### 3.2. Effect of DTBN on the survival of S. aureus N315

To examine whether the antimicrobial activity of DTBN was bactericidal or bacteriostatic, the survival rate of *S. aureus* N315 was measured after exposure to DTBN (Fig. 3). When the compound was not added, the number of cells increased from $10^8$ CFU/mL to $10^{9.5}$ CFU/mL in 12 h. The compound at 0.5 μg/mL (one fourth the concentration of the

**Table 5**
Antimicrobial activities of several compounds for *S. aureus* strains.

| Strain | MIC (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | DTBN | Vancomycin | Arbekacin | Linezolid | Oxacillin | Norfloxacin |
| Methicillin-sensitive *S. aureus* | | | | | | |
| 209P | 1 | 0.5 | 1 | 1 | 0.06 | 1 |
| 8851 | 2 | 1 | – | – | 0.25 | 64 |
| NCTC8325 | 2 | 0.5 | – | – | 0.13 | 0.5 |
| RN4220 | 2 | 1 | – | – | 0.06 | 2 |
| Methicillin-resistant *S. aureus* | | | | | | |
| N315 | 2 | 0.5 | 1 | 1 | 16 | 2 |
| 9191 | 2 | 1 | – | – | 128 | 256 |
| K-1 | 2 | 1 | 1 | 1 | 64 | >256 |
| 3-2(288-3) | 2 | 1 | – | – | 128 | >256 |
| MRSA-COL | 2 | 2 | – | – | 512 | 2 |
| OM481 | 4 | 2 | 0.5 | 1 | 256 | 64 |
| OM505 | 2 | 1 | 0.5 | 1 | 128 | 8 |
| OM584 | 4 | 1 | 1 | 1 | 128 | 64 |
| OM623 | 2 | 2 | 2 | 1 | 512 | 64 |
| HU2 | 2 | 1 | – | – | 32 | 32 |
| HU3 | 2 | 1 | 1 | 1 | 8 | 64 |
| HU4 | 2 | 2 | – | – | 512 | 64 |
| HU6 | 2 | 1 | 2 | 1 | 64 | 64 |
| HU7 | 2 | 1 | – | – | 512 | 64 |
| HU11 | 2 | 2 | – | – | 512 | 64 |
| HU12 | 2 | 2 | – | – | 512 | 64 |
| HU13 | 2 | 2 | – | – | 512 | 64 |
| HU14 | 2 | 1 | 1 | 1 | 32 | 64 |
| HU20 | 4 | 2 | 2 | 1 | 512 | 64 |
| Mu50 | 4 | 8 | 1 | 1 | 512 | >256 |
| Norfloxacin-resistant mutants from N315 | | | | | | |
| NF42 | 4 | 0.5 | 1 | 1 | 8 | 16 |
| NF82 | 4 | 0.5 | 1 | 1 | 8 | 16 |
| DTBN-resistant mutants from N315 | | | | | | |
| DTR8AL1 | 4 | – | – | – | – | 64 |
| DTR8BL1 | 4 | – | – | – | – | 64 |
| DTR8DL1 | 4 | – | – | – | – | 128 |
| DTR8EL1 | 4 | – | – | – | – | 128 |

–: not determined.

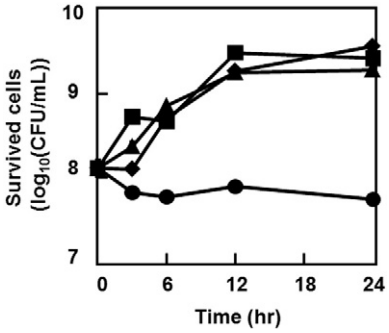


**Fig. 3.** The effects of DTBN on the survival of *S. aureus*. *S. aureus* N315 cells were incubated in the absence (square) or presence of DTBN at 0.5 μg/mL (triangles), 2 μg/mL (diamonds), and 8 μg/mL (circles). Samples were taken at the indicated times, and viable cells were investigated as CFU/ml.

MIC) or 2 μg/mL (equal concentration to the MIC) had no inhibitory effect on the survival rate or growth. On the other hand, when the compound was added at 8 μg/mL (4 times the concentration of the MIC), the number of cells did not increase and remained constant. Thus, the compound exhibited bacteriostatic activity at 8 μg/mL under this assay condition.

### 3.3. DTBN inhibited topoisomerase IV but not DNA gyrase of S. aureus

The inhibition of DNA gyrase or topoisomerase IV activity is known to be one of the mechanisms of action of antimicrobials. We examined the inhibitory activity of DTBN on DNA gyrase and topoisomerase IV (Figs. 4, 5). Novobiocin, which is a currently available DNA gyrase inhibitor, inhibited supercoiling activity in a concentration-dependent manner and completely inhibited it at 2 μM (Fig. 4). On the other hand, DTBN had no inhibitory activity against DNA gyrase at 6 μM and faint inhibitory activity at 30 μM (Fig. 4). In the decatenation assay of topoisomerase IV, DTBN slightly inhibited topoisomerase IV at 4 μM and completely inhibited it at 30 μM. Ciprofloxacin, which was a well-known quinolone antibiotic and topoisomerase IV inhibitor, was used as the positive control. Ciprofloxacin inhibited the decatenation activity of topoisomerase IV slightly more than DTBN. DTBN inhibited 49% of the activity of topoisomerase IV, whereas ciprofloxacin inhibited 75% of its activity at 10 μM (Fig. 5). The $IC_{50}$ of DTBN was 10–15 μM while that of ciprofloxacin was 4–10 μM. These results indicated that the one of the targets of this compound was topoisomerase IV, not DNA gyrase.

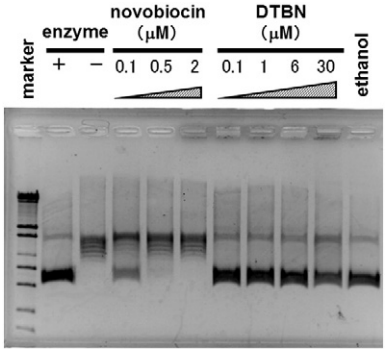


**Fig. 4.** The inhibitory activity of DTBN against *S. aureus* DNA gyrase. DNA gyrase and relaxed plasmid DNA were added to the reaction mixture and incubated at 37 °C for 30 min. The mixture was electrophoresed on a 1% agarose gel and stained by ethidium bromide.

### 3.4. Cross-resistance between the compound and quinolones

We showed that DTBN inhibited *S. aureus* topoisomerase IV, but not DNA gyrase. Both DNA gyrase and topoisomerase IV are known to be the targets of quinolone antibiotics. Fluoroquinolones were previously shown to target topoisomerase IV better than DNA gyrase in *S. aureus* [25]. If topoisomerase IV was indeed the target of DTBN, cross-resistance should have been observed between quinolone antibiotics and DTBN. We isolated the norfloxacin-resistant mutants NF42 and NF82 and DTBN-resistant mutants DTR series from *S. aureus* N315.

The MICs of norfloxacin for NF42 and NF82 were 16-fold higher than that for parental N315, whereas the MICs of DTBN were only 2-fold higher. On the other hand, the MICs of DTBN for the DTR series were 2-fold higher than for parental N315, whereas the MICs of norfloxacin were 64-fold higher (Table 5). Several point mutations were identified in the QRDR of these strains (Table 1). NF42 and NF82 had the E88K mutation in ParC. DTR8AL1 had the S80F mutation in ParC and S84L mutation in GyrA. This result also supported that topoisomerase IV is the target of DTBN.

The two-fold difference in the MIC was not significant to argue whether this difference was meaningful or not. We next evaluated the growth of N315, NF42, DTR8AL1, and OM481 in the presence of DTBN (Fig. 6). N315 and NF42 increased slowly until 48 h in the presence of 4 μg/mL DTBN. DTR8AL1 and OM481 increased quickly from $10^5$ to $10^9$ until 24 h at the same concentration. DTR8AL1 and OM481 had the S80F mutation in ParC and high resistance for quinolones, whereas NF42 had the E88K mutation in ParC and low resistance. These results showed that the S80F mutation in ParC caused high resistance to quinolones and a certain degree of resistance for quinolones to DTBN. Thus, the cross-resistance observed between DTBN and quinolones was slight, but reliable.

### 3.5. The effect of DTBN for formation of cleaved complex

QRDR in topoisomerase IV was reported as key amino acid residues for the formation of cleaved complex [26,27]. DTR strains and OM481 which were resistant to DTBN had point mutation in QRDR. We hypothesized that DTBN formed cleaved complex as well as quinolone antibiotics. We investigated the cleavage activity of DTBN for topoisomerase IV (Fig. 7). Linear DNA was increased to approximately 40% with ciprofloxacin (100 μM). However, DTBN did not increase linear DNA at 30 μM, a higher concentration of $IC_{50}$ of decatenation activity. This result indicated that DTBN did not have significant cleavage activity and formation of cleaved complex was not involved in inhibition of DTBN for topoisomerase IV.

## 4. Discussion

In the present study, we isolated and identified 6,6′-dihydroxythiobinupharidine (DTBN) from the dried rhizomes of *N. japonicum*, and showed that DTBN had anti-MRSA and anti-VRE activities. Previous studies demonstrated that DTBN exhibited antifungal activity against six human pathogenic fungi [13], immunosuppressive activity [14], and cytotoxic activity [14,28]. The principal dimeric sesquiterpene thioalkaloid 6-hydroxythiobinupharidine categorized has been reported to cause DNA fragmentation and induce apoptosis [28]. This is the first study to show that DTBN had antimicrobial activity at a lower concentration than that for antifungal activity, and also that one of the targets was DNA topoisomerase IV in *S. aureus*.

The compound examined in the present study was bacteriostatic against N315 at 4 times the concentration of the MIC, whereas growth was retarded at a lower concentration. Quinolone antibiotics have been shown to cleave DNA and exhibit bactericidal activity [29]. On the other hand, novobiocin competed with ATP against the DNA gyrase GyrB subunit and exhibited bacteriostatic activity [30,31]. From these

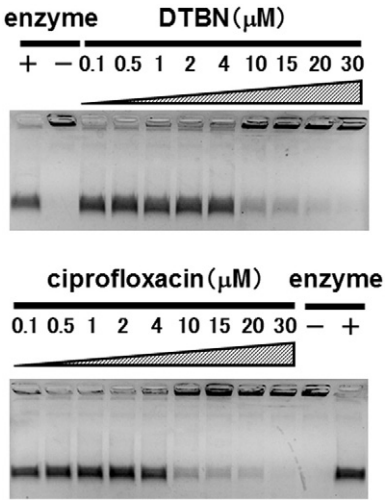


**Fig. 5.** The inhibitory activity of DTBN against *S. aureus* topoisomerase IV. Topoisomerase IV and kinetoplast DNA were added to the reaction mixture. The mixture was electrophoresed on a 1% agarose gel and stained by ethidium bromide.

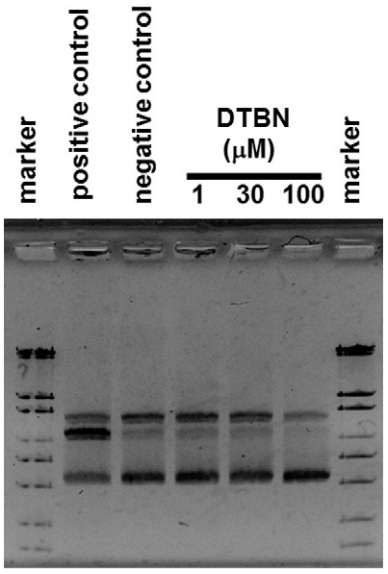


**Fig. 7.** The effect of DTBN on producing cleaved DNA. Topoisomerase IV and supercoiled pBR322 were incubated in reaction mixture at 25 °C for 1 h. SDS and proteinase K were added for disassembling complex. The mixtures were incubated at 37 °C for 30 min. Samples were electrophoresed by 1% agarose gel and gels were stained by ethidium bromide. Positive control; ciprofloxacin (100 μM), Negative control; ethanol (10%). DTBN was dissolved in ethanol.

results, DTBN is unlikely to be a cleavage complex stabilizing agent and inhibition of ATPase is one of a number of other possibilities.

However, topoisomerase IV may not be the sole target of DTBN. We isolated DTBN-resistant mutants, and found that their MICs were low, only two times that of the parental strain. If DTBN had two or more targets, the other targets may have been active against the quinolone-resistant strain. We exposed $10^{10}$ CFU cells to DTBN on plates, but did not isolate any resistant mutants. The mutation frequency has generally been reported as $10^{-5}$–$10^{-8}$ [32]. We isolated norfloxacin-resistant mutants from N315 at a frequency of $10^{-5}$–$10^{-8}$. This low frequency implied that DTBN may have had dual or more targets. The inhibition of protein and cell wall synthesis as well as membrane injury are expected if other targets exist. Of these, we showed that DTBN exhibited no inhibitory activity against protein synthesis using a cell-free protein synthesis kit. No morphological changes were observed with electron microscopy. The leakage of a small molecule such as ethidium bromide was not detected by the ethidium bromide accumulation assay (data not shown). Therefore, DTBN may exhibit inhibitory activity against cell wall synthesis or other unknown targets.

The MIC of this compound was 1 to 4 μg/mL against not only the laboratory MRSA strain, but also clinical isolates. MICs were equal or one fourth that of the antibiotics used at clinical sites, such as arbekacin, vancomycin, and linezolid. Sensitivity to DTBN was also independent of sensitivity to vancomycin. Mu50 was previously shown to have a thick cell wall and decreased permeability to vancomycin [2], while the FN-1 and NCTC12201 strains had *vanA* and high resistance to vancomycin. The *vanA* gene was on a plasmid and spread to other bacterial species [33]. Antimicrobial activity against Mu50, FN-1, and NCTC12001 suggested that this compound may be useful against various strains with different resistant mechanisms.

DTBN was previously reported to have cytotoxic effects on mouse spleen cells at a concentration lower than the MIC [14]; therefore, it may be difficult to utilize DTBN as an anti-MRSA antibiotic without any chemical modifications. However, the inhibition mechanism of DTBN may differ from that of quinolones. Furthermore, this compound exhibited strong antimicrobial activity and the emergence of spontaneously resistant mutants may be low. More information on the inhibition mechanism of DTNB will be useful for the development of novel anti-MRSA and anti-VRE antibiotics. The process of synthesizing this compound analog has recently been established [34]. If this compound is to be used as an antibiotic, total synthesis will be a major benefit as a supply method and chemical modifications can be easily achieved.

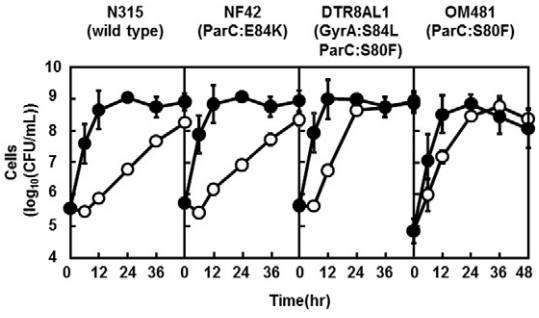


**Fig. 6.** The effects of DTBN on the growth of resistant strains. *S. aureus* N315 (wild type strain), NF42 (norfloxacin resistant mutant, ParC E84K), DTR8AL1 (DTBN resistant mutant, GyrA S84L, ParC S80F), and OM481 (clinical isolate, ParC S80F) cells were incubated in the absence (closed circle) or presence of DTBN at 4 μg/mL (open circle). Samples were taken at the indicated times, and cells that survived were investigated as CFU/ml. Error bars indicated standard error (n = 3).

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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