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Guadinomines, Type III Secretion System Inhibitors, Produced by *Streptomyces* sp. K01-0509

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Dedicated to the late Prof. Shigeo Iwasaki

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Abstract Enteropathogenic *Escherichia coli* (EPEC) expressing the Type III secretion system (TTSS) induced hemolysis of sheep blood cells. Using this assay, six structurally related compounds designated as guadinomines were isolated as inhibitors of TTSS-induced hemolysis by ion exchange column chromatography and HPLC from the culture broth of *Streptomyces* sp. K01-0509. Guadinomines A and B showed potent inhibition with IC_{50} values of 0.02 and 0.007 μg/ml, respectively, guadinomine D showed moderate activity (IC_{50}: 8.5 μg/ml), while guadinomines C_1 and C_2 and guadinomic acid had no activity.

Keywords guadinomines, Type III secretion system (TTSS)-induced hemolysis, *Streptomyces* sp. K01-0509, EPEC

Introduction

Our research group has focused on the discovery of anti-infectives from microbial metabolites [1～3]. The Type III secretion system (TTSS) is a common virulence system present in many Gram-negative bacteria, including *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, EPEC, enterohemorragic *E. coli* (EHEC), and *Chlamydia* spp. [4]. These bacteria use TTSS to deliver effector proteins into the cytosol of the eukaryotic target cell and depend on their respective TTSS to invade the host, resist phagocytosis, grow in deep tissues, and cause disease [5]. Recent studies have revealed that TTSS is not essential for the survival of bacteria and is not found in non-pathogenic Gram-negative bacteria except for some kinds of symbiotic bacteria.

Based on the new concept of "anti-infective drugs" developed by Ōmura [6], we have focused on TTSS as a new target for anti-infective drugs. Namely, a specific inhibitor of TTSS is expected to attenuate pathogens specifically and inhibit the process of their infection without killing pathogens and affecting normal bacterial flora. And such a specific agent would be expected to target the system only involved in the virulence mechanism. Thus, there would be little or no selective pressure for viability, potentially reducing the development of resistance. A convenient assay system in screening for TTSS inhibitors from microbial metabolites was employed using TTSS-induced hemolysis [7]. As a part of this screening program, we discovered six novel compounds designated guadinomines A to D and guadinomic acid (Fig. 1), produced by actinomycete strain K01-0509. The structure elucidation of these guadinomines will be

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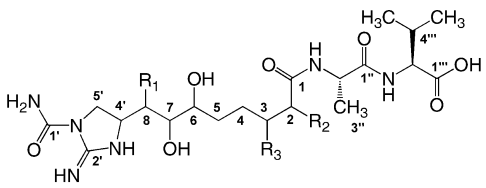
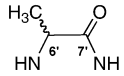
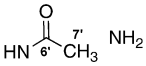
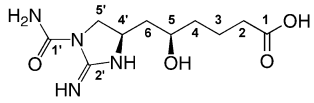


	R_1	R_2	R_3
Guadinomine A (**1**)	OH	NH_2	NH_2
Guadinomine B (**2**)	H	NH_2	NH_2
Guadinomines C_1 (**3**) and C_2 (**4**) (diastereomers at C-6')	H		
Guadinomine D (**5**)	H		NH_2



Guadinomic acid (**6**)
(K01-0509 B)

Fig. 1 Structures of guadinomines A, B, C_1, C_2 and D and guadinomic acid.

published in an accompanying study [8]. In this study, the taxonomy, fermentation, isolation, and biological properties of guadinomines are described.

Materials and Methods

Taxonomic Studies

The International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb [9] and media recommended by Waksman [10] were used to investigate the cultural and physiological characteristics. Cultures were routinely observed after incubation for two weeks at 27°C. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium containing 1.0% carbon at 27°C [11]. Morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). The type of diaminopimelic isomers present was determined by the method of Becker *et al.* [12]. Menaquinones were extracted and purified after Collins *et al.* [13], then analyzed by high performance liquid chromatography (HPLC) equipped with a CAPCELL PAK C18 column (Shiseido) [14].

Fermentation

Strain K01-0509 was grown and maintained on an agar slant consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loopful of spores of *Streptomyces* sp. K01-0509 was inoculated into 100 ml of the seed medium consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The inoculated flask was incubated in a rotary shaker (210 rpm) at 27°C for 3 days.

For production of guadinomines A, B and D, a 1-ml portion of the seed culture was transferred to a 500-ml Erlenmeyer flask (total 49 flasks) containing 100 ml of production Medium A (2.0% galactose, 2.0% dextrin, 0.5% corn steep liquor, 1.0% Bacto-soytone, 0.2% $(NH_4)_2SO_4$ and 0.2% $CaCO_3$ (adjusted to pH 7.4 before sterilization)) and fermentation was carried out in a rotary shaker (210 rpm) at 27°C for 9 days.

For production of guadinomines C_1 and C_2, the seed culture (200 ml) was transferred to a 30-liter Jar fermentor (total of three fermentors) containing 20 liters of production Medium B (2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% $CaCO_3$, 5.0×10^{-4}% $FeSO_4\cdot7H_2O$, 5.0×10^{-4}% $MgCl_2\cdot4H_2O$, 5.0×10^{-4}% $CuSO_4\cdot5H_2O$ and 5.0×10^{-4}% $CoCl_2\cdot6H_2O$ (adjusted to pH 7.0 before sterilization)) and fermentation was carried out at 27°C with an air flow of 10 liters/minute for 4 days.

For production of guadinomic acid, a 1-ml portion of the seed culture was transferred to a 500-ml Erlenmeyer flask (total 42 flasks) containing 100 ml of production Medium B and fermentation was carried out in a rotary shaker (210 rpm) at 27°C for 6 days.

The time courses of inhibitory activity against TTSS by the supernatant of the culture broth (0.5 μl), the pH of the culture and packed cell volume (PCV, ml/10 ml) after centrifugation (3,000 rpm, 10 minutes) of the culture (10 ml) during fermentation were measured.

Assay of TTSS Activity

TTSS activity was measured as the hemolytic activity caused by TTSS of EPEC in a 96-well microplate as reported previously [7]. TTSS-expressing EPEC and erythrocytes were mixed and the hemolytic activity was measured spectrometrically. Namely, noninfectious strain EPEC ΔCesT, which was defective of the chaperon protein of Tir (translocated intimin receptor), was used in this assay. One loopful of this strain was inoculated in liquid medium (5.0 ml) consisting of 2.5% LB medium (Funakoshi Co. Ltd., Japan) and cultured at 37°C for 12 hours without shaking. The seed culture (1.0%) was transferred to M9 medium (0.4% glucose, 0.1% casamino

acids, 0.68% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl and 0.012% $MgSO_4$), and the main culture was incubated at 37°C for 4.5 hours without shaking. The culture was centrifuged at 3,000 rpm for 15 minutes to collect the bacterial cells, which were suspended in M9 medium (5.0 ml) and used as TTSS-expressing EPEC. In parallel, sheep blood cells (Nippon Biological Materials Center, Japan) were washed three times by suspension in saline and centrifugation at 2,500 rpm for 5 minutes. The red cells (1.0 g), suspended in M9 medium (2 ml), were used as erythrocytes. Then, a test sample dissolved in M9 medium (10 μl), TTSS-expressing EPEC (45 μl) and erythrocytes (45 μl) were added to each well of a 96-well microplate (Corning). The microplate was centrifuged at 1,500 rpm for 10 minutes to make EPEC and erythrocytes come into contact, and was incubated at 37°C for 90 minutes to promote the hemolytic reaction, which was stopped by adding cooled PBS (150 μl/well). The mixture was centrifuged at 1,500 rpm for 10 minutes, and the supernatant (100 μl) was transferred to a 96-well microplate to measure the hemolytic activity by determining the absorbance at 550 nm with a microplate reader (Bio-Instruments Inc., U.S.A.). In control experiments (no drug) an absorbance of 0.3～0.5 at 550 nm was obtained as a full hemolytic activity induced by TTSS-expressing EPEC.

Assay of Antibacterial Activity

Antimicrobial activity was measured by a paper disk method (6 mm, ADVANTEC) [15]. The culture conditions were as follows: *Bacillus subtilis* ATCC6633 [Davis synthetic medium (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.5% sodium citrate, 0.1% ammonium sulfate, 0.2% glucose, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.8% agar), 1.0% inoculation, 37°C, 24 hours], *Staphylococcus aureus* ATCC6538P [Nutrient agar (0.5% peptone, 0.5% meat extract and 0.8% agar), 0.2% inoculation, 37°C, 24 hours], *Micrococcus luteus* ATCC9341 [Nutrient agar, 0.2% inoculation, 37°C, 24 hours], *E. coli* NIHJ [Nutrient agar, 0.5% inoculation, 37°C, 24 hours], *Xanthomonas camestris* KB88 [Nutrient agar, 1.0% inoculation, 37°C, 24 hours], *Aspergillus niger* ATCC9642 [GY agar, 0.3% inoculation, 27°C, 48 hours], *Mucor racemosus* IFO4581 [GY agar, 0.3% inoculation, 27°C, 48 hours], *Candida albicans* ATCC64548 [GY agar, 0.2% inoculation, 27°C, 24 hours].

Assay of Cytotoxic Activity

Cytotoxic activity against Jurkat cells was measured using the colorimetric 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in a 96-well microplate [16]. Jurkat cells growing exponentially in RPMI-1640 (4×10^5 cells/ml; 50 μl) and drugs in RPMI-1640 containing 1.0% DMSO (50 μl) were added to each well of a 96-well microplate and were then cultured at 37°C for 48 hours in a 5.0% CO_2 atmosphere. After incubation, MTT in phosphate buffered saline (5.5 mg/ml; 10 μl) was added to each well, followed by further incubation at 37°C for 4 hours. A 90-μl aliquot of extraction solution (40% (v/v) *N*,*N*-dimethylformamide, 2.0% (v/v) CH_3COOH, 20% (w/v) SDS and 0.03 N HCl) was added and the mixtures were shaken at room temperature for 2 hours. Cytotoxic activity was determined by measuring optical density at 550 nm with a microplate reader (BIO-TEK Instruments).

Results

Taxonomy of the Producing Strain K01-0509

Strain K01-0509 was isolated from a soil sample collected at Amamioshima, Kagoshima, Japan. The vegetative mycelia grew abundantly on yeast extract - malt extract agar, oatmeal agar, inorganic salt - starch agar, glycerol-asparagine agar and glucose-asparagine agar, and did not show fragmentation into coccoid forms or bacillary elements. The color of vegetative mycelia was yellow to brown. The aerial mycelia grew abundantly on yeast extract-malt extract and glycerol - asparagine agar and the aerial mass color was white to gray. From observation of the scanning electron micrograph of the strain (Fig. 2), the spore chains were straight and with more than 20 spores per chain. The spores were cylindrical in shape, 0.6～0.8×1.0～1.8 μm in size and had a smooth surface. Whirls, sclerotic granules, sporangia and flagellate spores were not observed. The growth temperature range was 10～38°C. D-Glucose and L-arabinose were used as the sole carbon source and D-fructose, *myo*-inositol, D-mannitol, melibiose, raffinose, L-rhamnose, sucrose and xylose were not used. Melanoid pigment was produced in tryptone -

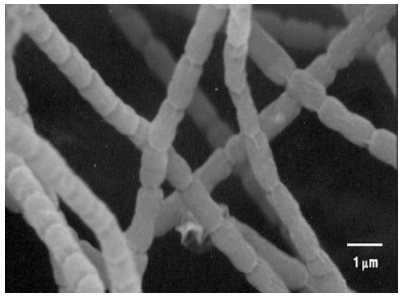


Fig. 2 Scanning electron micrograph of *Streptomyces* sp. K01-0509.

Bar represents 1.0 μm.

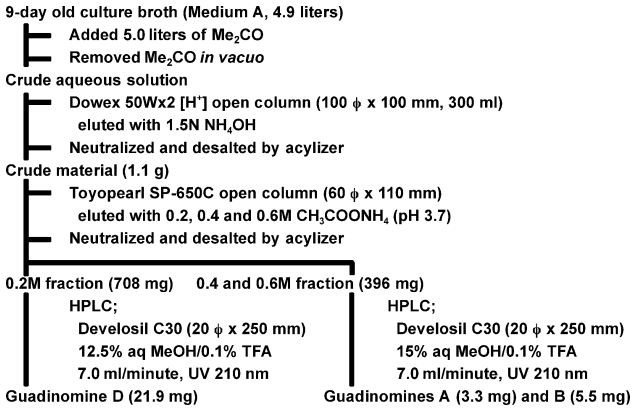
yeast extract broth. No soluble pigment was found. The isomer of DAP in whole-cell hydrolysates of strain K01-0509 was determined to be an LL-form. Major menaquinones were MK-9 (H_6) and MK-9 (H_8). Based on the taxonomic properties described above, strain K01-0509 is considered to belong to the genus *Streptomyces* [17] and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM BP-08504.

Fermentation

Typical time courses of the fermentation for production of guadinomines A, B and D in Medium A and for production of guadinomines C_1 and C_2 and guadinomic acid in Medium B in a 500-ml Erlenmeyer flask are shown in Fig. 3a and 3b, respectively. In Medium A the inhibitory activity of the culture broth against TTSS reached maximum levels on day 2~3 after inoculation and then slowly decreased.

Isolation

The procedure for the isolation of guadinomines A, B and D is summarized in Scheme 1. The 9-day old culture broth (Medium A, 4.9 liters) was extracted with 5.0 liters of Me_2CO, followed by centrifugation at 3,000 rpm to separate the mycelium and the supernatant. The supernatant was concentrated under reduced pressure to remove Me_2CO and then passed through a Dowex 50W x2 [H^+] column (100 i.d.×100 mm, Muromachi Technos Co.) previously activated. After washing with water (900 ml), the active materials were eluted with 1.5 N NH_4OH (900 ml), followed by neutralization by HCl and desalting by a micro acylizer (Asahi Chemical Co.). The whole eluate was concentrated *in vacuo* and lyophilized to yield a brown material (1.1 g). The material was dissolved in a small amount of 0.05 M CH_3COONH_4 buffer (pH 3.7) and applied to a Toyopearl SP650-C column (60 i.d.×110 mm, Tosoh Co.) previously equilibrated with 0.05 M CH_3COONH_4 buffer (pH 3.7). After washing with 0.05, 0.1 and 0.2 M CH_3COONH_4 buffer (pH 3.7, 900 ml each), the active materials were eluted with 0.2, 0.4 and 0.6 M CH_3COONH_4 buffer (pH 3.7, 900 ml each). The 0.4 and 0.6 M CH_3COONH_4 fractions were neutralized by NH_4OH. desalted by a micro acylizer and concentrated *in vacuo* to dryness to yield a brown material (396 mg). The material was dissolved in a small amount of water and purified by HPLC on a Develosil C_{30} column (20 i.d.×250 mm, Nomura Chemical Co.) with 15% aq MeOH/0.1% TFA at 7.0 ml/minute detected at UV 210 nm. The retention times of guadinomines A and B were 27 and 30 minutes, respectively (Fig. 4a). Each active fraction was concentrated *in vacuo* to dryness to afford guadinomine A (3.3 mg) and guadinomine B (5.5 mg) as pale brown powders. In contrast, the 0.2 M CH_3COONH_4 fraction was neutralized by NH_4OH, desalted by a micro acylizer and concentrated *in vacuo* to dryness to yield a brown material (708 mg). This material was dissolved in a small amount



Scheme 1 Isolation procedure of guadinomines A, B and D

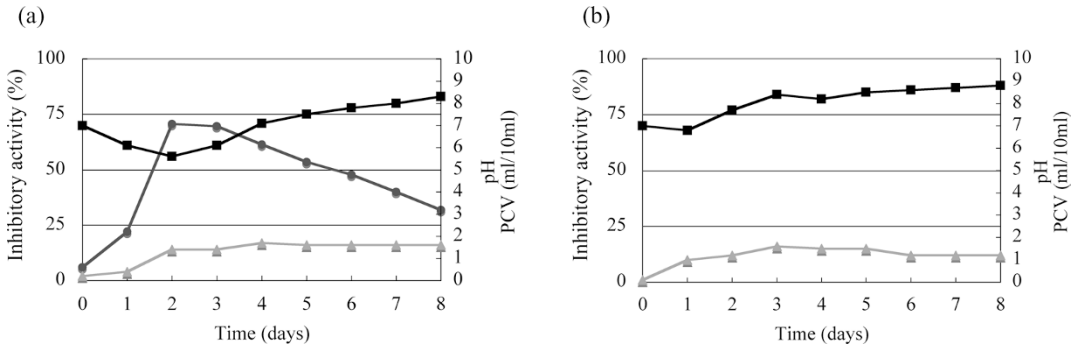


Fig. 3 Typical time course of fermentation by *Streptomyces* sp. K01-0509.

Strain K01-0509 was cultured in Medium A (a) or Medium B (b). The inhibitory activity against TTSS of EPEC by supernatant (●) of the culture broth (5.0 μl), pH (■) of the culture and packed cell volume (PCV, ▲) were measured.

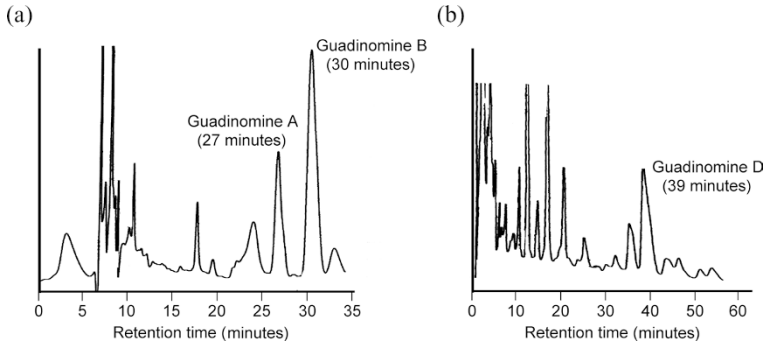


Fig. 4 Purification of (a) guadinomines A and B and (b) guadinomine D by HPLC.
The detailed conditions of HPLC are described in the "Results".

of water and purified by HPLC on a Develosil C_{30} column (20 i.d.×250 mm) with 12.5% MeOH/0.1% TFA at 7.0 ml/minute detected at UV 210 nm. The retention time of guadinomine D was 39 minutes (Fig. 4b). The active fraction was concentrated *in vacuo* to dryness to afford guadinomine D (21.9 mg) as a pale brown powder.

The procedure for the isolation of guadinomines C_1 and C_2 guided by detection of Fearon reagent (positive for guanidine moieties) [18] is summarized in Scheme 2. The 4-day old culture broth (Medium B, 90 liters) was centrifuged at 3,000 rpm to separate the mycelium and the supernatant. The supernatant (54 liters) was passed through a column of active charcoal (100 i.d.×80 mm) previously activated. After washing with water (1,500 ml), the active materials were eluted with 20 and 40% aq Me_2CO (1,500 ml each). The whole eluate was concentrated *in vacuo* and lyophilized to yield a brown material (21.5 g). The active material was dissolved in a small amount of water and applied on a IRC-50 [H^+] column (80 i.d.×60 mm, Organo Co.) previously activated. After washing with water (900 ml), the active materials were eluted with 1.0 N HCl (900 ml) followed by neutralization by NaOH and desalting by a micro acylizer. The whole eluate was concentrated *in vacuo* and lyophilized to yield a brown material (823 mg). The material was dissolved in a small amount of water and applied to an ODS column (10 i.d.×40 mm, Senshu Scientific Co.) previously equilibrated with water. After washing with water (10 ml), the active materials were eluted with 10 and 20% MeOH/0.1% TFA (10 ml each). The whole eluate was concentrated *in vacuo* to dryness to yield a brown material (103 mg). The material was dissolved in a small amount of water and purified by HPLC on a Develosil C_{30} column (20 i.d.×250 mm) with 8.0% MeOH/0.1% TFA at 5.0 ml/minute detected at UV 210 nm. The active fraction with a retention time of 45 minutes was concentrated *in vacuo* to dryness to yield a pale brown material (3.6 mg). The material was dissolved in a small amount of water and finally purified by HPLC on a Develosil C_{30} column (20 i.d.×250 mm) with 3.0% CH_3CN/0.05% H_3PO_4 at 5.0 ml/minute detected at UV 210 nm. The active fractions with retention times of 27 and 30 minutes (Fig. 5) were concentrated *in vacuo* to dryness to afford guadinomine C_1 (1.1 mg) and guadinomine C_2 (1.5 mg) as white powders, respectively.

4-day old culture broth B (Medium B, 60 liters)
- Centrifuged at 3,000 rpm

Supernatant (54 liters)
- Active charcoal open column (100 ϕ x 80 mm, 300 ml)
  eluted with 20 and 40% aq Me_2CO

Crude material (21.5 g)
- IRC-50 [H^+] open column (80 ϕ x 60 mm)
  eluted with 1.0N HCl
- Neutralized and desalted by acylizer

Crude material (823 mg)
- ODS open column (10 ϕ x 40 mm)
  eluted with 10 and 20% aq MeOH/0.1% TFA

Crude material (103 mg)
- HPLC;
  Develosil C30 (20 ϕ x 250 mm)
  8% aq MeOH/0.1% TFA
  5.0 ml/minute, UV 210 nm

Crude material (3.6 mg)
- HPLC;
  Develosil C30 (20 ϕ x 250 mm)
  3% aq MeCN/0.05% H_3PO_4
  5.0 ml/minute, UV 210 nm

Guadinomines C_1 (1.1 mg) and C_2 (1.5 mg)

Scheme 2 Isolation procedure of guadinomines C_1 and C_2

The procedure for the isolation of guadinomic acid guided by detection of Fearon reagent (positive for guanidine moieties) is summarized in Scheme 3. The 6-day old culture broth (Medium B, 4.2 liters) was centrifuged at 3,000 rpm to separate the mycelium and the supernatant. The supernatant (3.5 liters) was passed through a cation exchange Dowex 50W x2 [H^+] column (60 i.d.×40 mm) previously activated. After washing with water (500 ml), the materials were eluted with 1.5 N NH_4OH (300 ml) followed by neutralization by NaOH and desaltation by

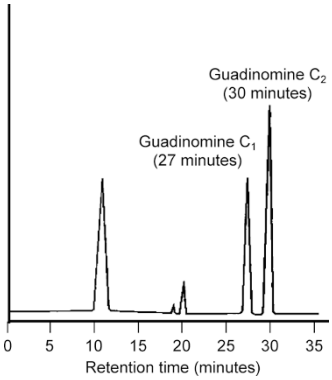


Fig. 5 Purification of guadinomines C_1 and C_2 by HPLC.

The detailed conditions of HPLC are described in the "Results".

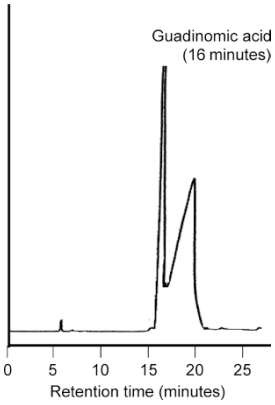


Fig. 6 Purification of guadinomic acid by HPLC.

The detailed conditions of HPLC are described in the "Results".

6-day old culture broth (Medium B, 4.2 liters)
- Centrifuged at 3,000 rpm

Supernatant (3.5 liters)
- Dowex 50W x2 [H^+] open column (60 ϕ x 40 mm, 100 ml)
  - eluted with 1.5N NH_4OH
- Washed by MeOH (10 ml)

MeOH-soluble fraction (413 mg)
- ODS open column (10 ϕ x 40 mm)
  - eluted with H_2O

Crude material (38.2 mg)
- HPLC;
  - PEGASIL ODS (20 ϕ x 250 mm)
  - 2.0% aq MeOH
  - 8.0 ml/minutes, UV 210 nm

Guadinomic acid (4.3 mg)

Scheme 3 Isolation procedure of guadinomic acid

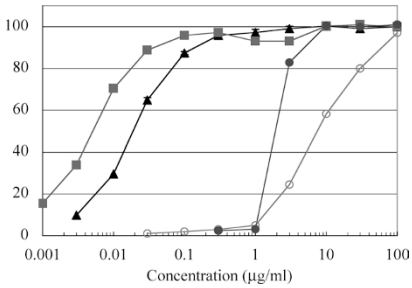


Fig. 7 Inhibitory activity of gudinomines against Type III secretion system of EPEC.

Guadinomine A (▲), B (■) and D (○) and tetracycline (●).

micro acylizer. The whole eluate was concentrated *in vacuo* and lyophilized to yield a brown material (800 mg). The material was dissolved in 10 ml of methanol and centrifuged at 3,000 rpm. The supernatant (413 mg) was applied to an ODS column (25 i.d.×165 mm) previously equilibrated by water. The materials were eluted with water (200 ml). The whole eluate was concentrated *in vacuo* and lyophilized to yield a brown material (38.2 mg). The material was dissolved in a small amount of water and purified by HPLC on a PEGASIL ODS column (20 i.d.×250 mm, Senshu Scientific Co.) with 2.0% aq MeOH at 8.0 ml/minute detected at UV 210 nm. The fraction with a retention time of 16 minute (Fig. 6) was collected and concentrated *in vacuo* to dryness to afford guadinomic acid (4.3 mg) as a white powder.

Biological Activities

As shown in Fig. 7, guadinomines A, B and D showed dose-dependent inhibition of EPEC-induced hemolysis, suggesting that these compounds inhibited TTSS. Among them, guadinomine B was the most potent with an IC_{50} value of 0.007 μg/ml, followed by guadinomine A (IC_{50}: 0.02 μg/ml). Guadinomine D was a moderate inhibitor (IC_{50}: 8.5 μg/ml). However, guadinomines C_1 and C_2 and guadinomic acid showed no activity at 100 μg/ml in this assay. All guadinomines and guadinomic acid showed no antimicrobial activity against yeasts, fungi and Gram-positive and Gram-negative bacteria at 10 μg/6 mm disk by the paper disk method. Guadinomine B showed cytotoxic activity against Jurkat cells with IC_{50} of 6.1 μg/ml. This is 100 times higher than the inhibitory activity against EPEC-induced hemolysis. Under the same condition, tetracycline inhibited EPEC-induced hemolysis with IC_{50} of 2.0 μg/ml and also showed antimicrobial activity against EPEC (inhibition zone, 19 mm at 10 μg/6 mm disk).

Discussion

Several compounds have been reported as TTSS inhibitors

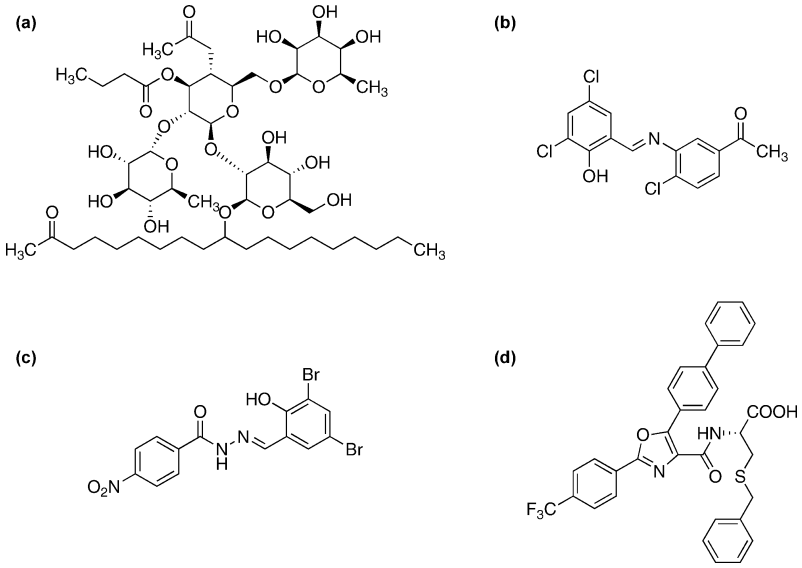


Fig. 8 Structures of (a) caminoside A, (b) synthetic TTSS inhibitor having a salicylideneaniline substructure, (c) synthetic TTSS inhibitor having an amide bond and a Shiff-base substructure and (d) synthetic TTSS inhibitor having an azole substructure.

(Fig. 8). The natural products caminosides were isolated from the marine sponge *Caminus sphaeroconia* in a screen system in which Esp proteins secreted from EPEC were detected by SDS-PAGE [19]. Caminoside A showed TTSS inhibition with IC_{50} of 38.5 μg/ml. Lactoferrin, a high-molecular weight glycoprotein, was reported to inhibit EPEC-induced hemolysis with an IC_{50} value of 3.0 mg/ml because of hydrolysis of TTSS proteins [20]. Three synthetic compounds are also known to be TTSS inhibitors. The first compound having a salicylideneaniline substructure was reported to reduce the amount of Type III secretion proteins, Tir and EspB, with IC_{50} of 5.1 μg/ml [21]. The second compound having an amide bond and a Shiff-base substructure inhibited the transcription of the TTSS-related proteins in *Yersinia* spp. with an IC_{50} of 2.2 μg/ml [22]. The third azole compound inhibited the transcription of the TTSS-related proteins, SopE, SipB and ExoU, in *Pseudomonas murine* with IC_{50} values of 1.9~80 μg/ml [23]. Thus, guadinomines are the first microbial metabolites inhibiting TTSS. Among the inhibitors listed, guadinomines A and B appear to be the most potent TTSS inhibitors reported to date with IC_{50} values of 0.007~0.01 μg/ml although the other inhibitors could not be evaluated in our assay system. Therefore, it would be useful to examine the mode of action of guadinomines in the inhibition of EPEC-induced hemolysis. TTSS inhibitors are expected to treat not only diarrhea caused by EPEC but various diseases caused by Gram-negative pathogenic bacteria.

In our assay system, guadinomines A and B showed much more potent inhibitory activity against TTSS than guadinomine D, while guadinomines C_1 and C_2 showed no activity, indicating that diamino groups are important for the activity. Furthermore, guadinomic acid had no activity, indicating that the cyclic guanidine moiety alone is not sufficient for activity.

As shown in Fig. 7, tetracycline inhibited EPEC-induced hemolysis by prevention of TTSS protein synthesis and also showed anti-EPEC activity. In contrast, guadinomines markedly inhibit EPEC-induced hemolysis with no anti-EPEC activity.

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