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Bacillus subtilis SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated β-amino acid

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ABSTRACT

Subtulene A, a new cyclic lipopeptide, was isolated from the culture broth of *Bacillus subtilis* SSE4. This antibiotic compound contained the seven common α-amino acids, L-Asn-1, D-Tyr-2, D-Asn-3, L-Gln-4, L-Pro-5, D-Asn-6, L-Ser-7 and the unique β-amino acid-8 present in the iturin family. 1D and 2D NMR, as well as MS analyses, identified the β-amino acid as 3-amino-13-methyltetradec-8-enoic acid, an Iso C15 long chain β-amino acid. *B. subtilis* SSE4 was also found to produce iturin A. *B. subtilis* SSE4 culture filtrate exhibited both antifungal and antibacterial activities.

1. Introduction

The iturins, produced by several strains of *Bacillus subtilis*, are cyclic lipopeptide antibiotics [1,2]. Iturins A–E [3–5], bacillomycins D, F, L and Lc [6–10], and mycosubtilin [11] have been previously described as the main variants of the iturin family. They are cyclic lipopeptides containing a heptapeptide of α-amino acids that is cyclized with a C_{13} to C_{17} β-amino fatty acid (Table 1, entries 1–8). The α-amino acids of the heptapeptide are arranged in the constant chiral sequence, LDDLLDL and the stereochemistry of the β-amino fatty acid side chain has been determined to be in the *R*-configuration at the β-carbon [1]. The length of the fatty acid side chain (R) of iturinic acid varies from 10 to 14 carbons (C_{13} to C_{17} total carbons) in the *n*-, *iso*-, or *anteiso*-configurations. These can give rise to eight distinct iturin isomers (A1–A8) based on chain length. Of these, the *n*-C_{14} isomer (iturin A2) is the predominant isomer in iturin A [12].

Previous biological activity studies have shown iturins to be effective antibiotics against bacteria and fungi [13]. The first three amino acids, L-Asx (x = p or n), D-Tyr, and D-Asn, are conserved in this family and are known to play an important role in antibiotic action. For example, the replacement of L-Asn-1 of iturin A by L-Asp-1 in iturin C abolishes antibiotic action [10]. Also, the methylation of the phenoxyl group of the D-Tyr-2 residue of iturin A dramatically decreases the activity [14,15]. Mycosubtilin differs from iturinA only by the sequence inversion of the two amino acid residues, D-Ser-6 and L-Asn-7. However, they have different conformation and mycosubtilin is more active than iturinA [2]. The side chain length of iturinic acid is also critically important. The synthetic cyclopeptide (SCP) which contains β-alanine instead of iturinic acid to give only the iturin peptide core, loses antibiotic activity (Table 1, entry 7) [10].

Herein, we report the isolation of *B. subtilis* SSE4 that extracellularly produces two antibiotic compounds: iturin A and the novel lipopeptide, subtulene A (Table 1, entry 9). The structure of the β-amino acid of subtulene A was determined

Abbreviations: SCP, synthetic cyclopeptide; DMSO, dimethyl sulfoxide

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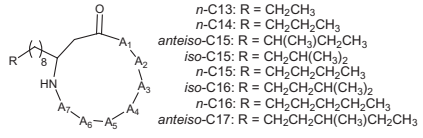
Table 1
Amino acid composition of the iturin family and compositional differences of the β-amino acid.[a]



Entry	Antibiotic	Amino acid sequences L-A1-D-A2-D-A3-L-A4-L-A5-D-A6-L-A7
1	Iturin A	Asn-Tyr-Asn-Gln-Pro-Asn-Ser
2	Iturin C	Asp-Tyr-Asn-Gln-Pro-Asn-Ser
3	Bacillomycin D	Asn-Tyr-Asn-Pro-Glu-Ser-Thr
4	Bacillomycin F	Asn-Tyr-Asn-Gln-Pro-Asn-Thr
5	Bacillomycin L	Asp-Tyr-Asn-Ser-Glu-Ser-Thr
6	Bacillomycin Lc	Asn-Tyr-Asn-Ser-Glu-Ser-Thr
7	SCP[b]	Asp-Tyr-Asn-Ser-Glu-Ser-Thr
8	Mycosubtilin	Asn-Tyr-Asn-Gln-Pro-Ser-Asn
9	Subtulene A[c]	Asn-Tyr-Asn-Gln-Pro-Asn-Ser

[a] Normal β-amino acid side chain (C13 to C17).
[b] SCP is the synthetic cyclopeptide (β-amino acid = Ala).
[c] β-Amino acid side chain is 3-amino 13-methyltetradec-8-enoic acid ($R = (CH_2)_3CH{=}CH(CH_2)_3CH(CH_3)_2$).

to be 3-amino-13-methyltetradec-8-enoic acid and represents a novel side chain structure for this class of lipopeptide. The biological activities of subtulene A are also reported.

2. Materials and methods

2.1. Isolation of B. subtilis SSE4

Samples from shrimp shell waste were serially diluted and spread on tryptic soy agar (TSA) supplemented with 100 μg/ml ketoconazole to inhibit fungal growth. All plates were incubated at 28 °C in the dark for 24 h.

2.2. Antagonistic activity screening using the dual culture technique

The phytopathogenic fungi, *Colletotrichum gloeosporioides* DOAC1690 and *Sclerotium rolfsii* DOAC 1521 were maintained on potato dextrose agar (PDA) plates at 28 °C in the dark for 4 days. Mycelium tip of 4-day-old culture was used as the inoculum for this experiment. A bacterial isolate was aerobically cultivated at 28 °C with shaking at 150 rpm for 18 h. Twenty microliters of bacterial suspension (OD_{600} ~0.1) was placed onto one side of a PDA plate. The plates were incubated at 28 °C for 2 days. A 6-mm diameter plug, taken from the margin of a 4-day-old PDA plate with confluent growth of a fungal pathogen, was placed onto the center of the plate previously inoculated with the bacterial suspension. The plate was then incubated at 28 °C and the extent of fungal growth inhibition was examined after 5–7 days of incubation. The level of inhibition was calculated as previously reported [16].

2.3. Identification of bacteria by 16S ribosomal RNA gene sequencing

Strain SSE4 was gram stained and found to be a Gram positive rod. In order to precisely identify this bacterium, the sequence of part of the 16S ribosomal RNA gene was determined. PCR amplification of the 16S rRNA gene with the universal primers of forward F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse R (5′-ACCAGGGTATCTAATCCTGT-3′) was carried out and the resulting 755 bp product was sequenced [17]. The sequences were compared to those found in the Genbank database. PCR amplification conditions were as follows: preheated at 94 °C for 5 min then 35 cycles of 94 °C for 45 s, annealed at 55 °C for 45 s and extend at 72 °C for 45 s.

2.4. Preparation of cell-free culture filtrate

Bacterial cells were cultivated in TSB and incubated with continuous shaking at 28 °C. The supernatant was collected at 4 h (exponential phase) and 48 h (stationary phase) by centrifugation at 8000 rpm for 20 min at 4 °C. The supernatant was rendered cell-free by filtration through a sterile membrane with a 0.45 μm pore size and stored at 4 °C until further use in the antifungal assay.

2.5. Radial growth inhibition assay

Culture filtrate was added to warm molten PDA (at 45 °C) to a final concentration of 20% (v/v). A 6 mm-diameter mycelial plug from a 3-day-old plate culture of either *Colletotrichum gloeosporioides* or *Sclerotium rolfsii* was placed in the center of the PDA plate followed by incubation at 28 °C in the dark. Mycelial growth was measured daily until the fungal mycelia on the control plate reached the edge of the plate. Fungal growth inhibition was expressed as the percentage of inhibition of radial growth relative to the control.

2.6. Protection against anthracnose disease caused by C. gloeosporioides

Bioassays for suppression of fungal diseases caused by *C. gloeosporioides* were performed using 1-year-old *Dendrobium* orchid plants. The experiment used a Completely Randomized Design (CRD) which was divided into five treatments with five replications. Both culture filtrate and a fungal pathogen were not applied in T1 as negative control. Orchid leaves were inoculated with *C. gloeosporioides* as positive control (T2). The surface of orchid leaf was sprayed with 200 μl of 1 mg/ml of mancozeb as chemical antifungal agent for T3. Two hundred microliters of exponential (T4) or stationary (T5) culture filtrates were applied on the abaxial surface of the leaf. The abaxial surface of the orchid leaf was then scratched with a sterile needle. A piece of a 6-mm plug of *C. gloeosporioides* was then placed on the surface for all treatments, except T1. The inoculated orchid plants were covered with polyethylene bags for 24 h. The size of visible anthracnose disease lesions on the orchid leaves was measured daily for 5 days.

2.7. Fractionation and purification of the bioactive compounds

The supernatant from 1 l of stationary phase *B. subtilis* SSE4 culture was extracted once with hexane, (ratio 1:1). After extraction with hexane, the supernatant was extracted twice with ethyl acetate, (ratio 1:1). The ethyl acetate fractions were combined and concentrated by evaporation at temperature <40 °C to give a dried powder residue. The dried residue was then dissolved in 4 ml methanol (100%) and subjected to preparative HPLC (Waters Delta 600, Massachusetts, USA). The preparative HPLC was performed using a Sunfire Prep™ C18, 5 μm, OBD 19 × 150 mm column (Waters, Massachusetts, USA). The mobile phase was water (A) and methanol (B) at a flow rate of 20 ml/min. The detection wavelength was set at 230 nm. The gradient system started from 0 min (5% B) to 5 min (30% B), 10 min (55% B), 13 min (70% B), 17 min (70% B), 22 min (90% B), 24 min (5% B) and maintained at this ratio until 27 min. The temperature of the column was 25 °C.

2.8. Microbial sensitivity assays

In order to test the susceptibility of various microorganisms, disk inhibition assay was performed as previously described [18].

Organic solvent extracted preparations were prepared by hexane and ethyl acetate extraction as mentioned above. Thus, 10 μl of 250 μg/μl dried organic extract residue was applied to the filter paper disk and the zone of growth inhibition around the 7-mm disk was measured after 24 h of incubation.

2.9. Cell lines

Human Cholangiocarcinoma (HuCCA-1) (Immunology Laboratory, Siriraj Hospital, Thailand) and human lung cancer (A549) (ATCC; CCL-185) were grown in Ham S/F12 medium (Hyclone Laboratories, Logan, UT, USA) containing 2 mM L-glutamine (Sigma, USA) supplemented with 100 U/ml penicillin–streptomycin (Sigma) and 10% fetal bovine serum (Hyclone Laboratories). Hepatocarcinoma cells (HepG2) (ATCC; HB-8065), hormone-independent breast cancer cells (MDA-MB-231) (M. D. Anderson Cancer Center, USA) and human cervical carcinoma cells (HeLA), (Environmental Toxicology Laboratory, Chulabhorn Research Institute, Thailand) were grown in DMEM medium (Hyclone Laboratories) with the same supplement. Hormone-dependent breast cancer cells (T47D ATCC HTB-133) were grown in RPMI-1640 medium (Hyclone Laboratories) containing 2 mM L-glutamine and supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), insulin (0.2 U/ml) (Sigma), 4.5 g/l glucose and 10% fetal bovine serum.

2.10. Cytotoxic activity testing

Cell lines were plated in 96-well microplates (Costar No. 3599, USA) at 100 μl/well at a density of 5×10^3–2×10^4 cells/well. Background control well contained the same volume of complete culture medium. Microplates were incubated for 24 h at 37 °C, 5% CO_2 and 95% humidity (Shellab, USA), anticancer drugs at various concentrations were added and further incubated for 48 h. Cell viability was determined by MTT assay [19,20], which entails staining with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma). The reagent was dissolved in phosphate buffered saline at 5 mg/ml and filtered. The MTT solution at the final concentration of 500 μg/ml was added to microplates and incubated for 2–4 h. Subsequently, 100 μl of dimethyl sulfoxide (DMSO) (Merck, Germany) was added to dissolve the formed formazan. The plates were read on a microplate reader (Molecular Devices, USA), using test and reference wavelengths of 550 and 650 nm, respectively. IC_{50} values were determined as the drug concentration that resulted in a 50% inhibition of cell growth.

2.11. Structure elucidation

All NMR spectra were recorded on a Bruker 600 Avance II spectrometer using CD_3OD, and $H_2O + D_2O$ as the solvent. Two-dimensional spectra, DQF-COSY, ed-HSQC, TOCSY, ROESY, NOESY and HMBC were processed using Bruker XWINNMR software. Infrared spectra were recorded on a Perkin–Elmer spectrum I spectrometer and samples were analyzed in the solid phase (UATR) (data in Supplementary data). Mass spectra (HRMS) were obtained on a Bruker microTOF using atmospheric pressure chemical ionization (APCI) in positive mode.

3. Results and discussion

3.1. Isolation and identification of B. subtilis SSE4

A collection of 143 bacterial strains isolated from shrimp shell waste were screened for potential antagonistic activity toward the phytopathogenic fungi, *C. gloeosporioides* and *S. rolfsii*, using a dual culture technique. *C. gloeosporioides* and *S. rolfsii* are common causal agents of anthracnose and collar rot diseases in tropical fruits and infection by these fungal pathogens results in severe post-harvest losses [21,22]. One strain, SSE4, was found to express the highest antagonistic activity against both pathogens. The Gram positive, rod shaped bacilli was identified as *B. subtilis* according to the sequence of its 16S rRNA gene.

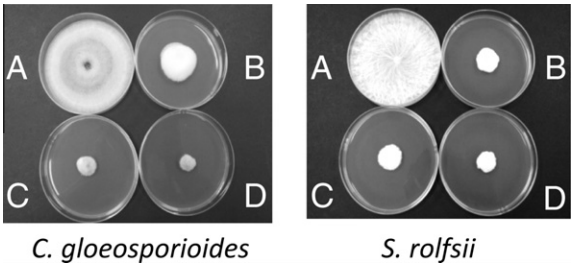


Fig. 1. The radial growth inhibition of *C. gloeosporioides* and *S. rolfsii* on PDA plates containing stationary phase culture filtrate of *B. subtilis* SSE4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) of culture filtrate.

3.2. Radial growth inhibition by extracellular antifungal metabolites

In order to evaluate the antifungal effect of extracellular metabolites of *B. subtilis* SSE4 on the radial growth of *C. gloeosporioides* and *S. rolfsii*. the fungal strains were cultivated on PDA plates containing 10%, 20%, and 30% (v/v) of *B. subtilis* SSE4 culture filtrate. When increasing amounts of stationary culture filtrate were added to the PDA plates, growth of both fungal pathogens were obviously inhibited (Fig. 1). The fungal growth on the agar plates was measured daily. The degree of *C. gloeosporioides* growth inhibition was proportional to the concentration of culture filtrate in the media, whereas *S. rolfsii* was equally inhibited by 10% and 20% culture filtrate and most inhibited by 30% filtrate concentration. The filtrates of stationary phase cultures of *B. subtilis* SSE4 exhibited higher antifungal activity than those in exponential phase suggesting that the active compound(s) or secondary metabolite(s) may be preferentially released during the stationary phase of growth (Table 2).

3.3. Protection of anthracnose disease caused by C. gloeosporioides on plant leaves

Most Thai orchid exports are of the *Dendrobium* species. To test if the active metabolites can protect *Dendrobium* orchid leaves from *C. gloeosporioides* infection, bioassays were performed to measure the ability *B. subtilis* SSE4 culture filtrate to suppress fungal infection. Development of anthracnose disease was observed 5 days post-infection in the positive control leaves while treatment with *B. subtilis* SSE4 culture filtrate arrested the development of anthracnose disease lesions to the same extent as the mancozeb treated leaves (Fig. 2, compare T2, T3, T4 and T5). This is the first experimental demonstration of the use of bioactive compound(s) to protect orchids from anthracnose disease.

Table 2
The percentage of radial growth inhibition of *C. gloeosporioides* and *S. rolfsii* by culture filtrates of *B. subtilis* SSE4 after 3 days of incubation.

B. subtilis SSE4 culture filtrate concentration (%)	% Radial growth inhibition			
	C. gloeosporioides		*S. rolfsii*	
	Exponential	Stationary	Exponential	Stationary
10	16.7	88.9	29.4	83.8
20	22.2	94.4	47.1	88.2
30	36.1	100	64.7	92.6

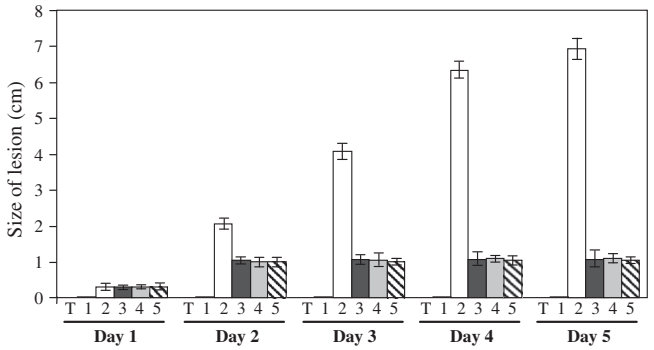


Fig. 2. The size of lesions on *Dendrobium* leaves after infection with *C. gloeosporioides*. T1 neither *C. gloeosporioides* nor culture filtrates; T2 only *C. gloeosporioides*; T3 100 μl of 1 mg/ml of mancozeb and *C. gloeosporioides*; T4 100 μl of 20% (v/v) exponential culture filtrate and *C. gloeosporioides*; T5 100 μl of 20% (v/v) stationary culture filtrate and *C. gloeosporioides*.

3.4. Organic solvent extracted compounds and antimicrobial activities

Next we examined the antimicrobicidial activity of organic solvent extracts of *B. subtilis* SSE4 culture filtrates against several bacterial strains as well as the yeast *Saccharomyces cerevisiae* BY 4741 using a disk diffusion inhibition assay. Interestingly, an organic solvent extract of *B. subtilis* SSE4 culture filtrate was toxic to Gram negative and Gram positive bacterial strains including human pathogens such as: *Stenotrophomonas maltophilia*, which has emerged as an antimicrobial resistant causative agent of serious nosocomial infections [23] and AIDS-related disorders [24], *Enterobacter cloacae*, which causes nosocomial bacteremia [25], and the plant pathogen, *Xanthomonas campestris* [26] (Table 3). This result is somewhat unusual since iturins have generally been shown to display strong antifungal toxicity against a variety of yeast and fungi but only limited antibacterial activity [27,28]. For example, bacilopeptins, iturin-group antifungal antibiotics, isolated from *B. subtilis* FR-2 were reported to have no antibacterial activity and only bacilopeptin C exhibited toxicity to several fungi [29]. Also, neither iturin D nor iturin E have been shown to possess antibacterial activity [4].

3.5. Purification of bioactive compounds

The HPLC fingerprint of the residue from ethyl acetate extracts of extract of *B. subtilis* SSE4 culture filtrate is shown in Fig. 3. Three fractions of A (7.20 min), B (9.75 min) and C (16.25–20.75 min), were collected for antibacterial activity assays. The results showed that fractions A and B did not have any antibacterial activity while fraction C was found to inhibit bacterial growth (data not shown). Therefore, we separated this fraction C into four pure fractions (1, 2, 3, and 4) using HPLC and examined these fractions for antibacterial activity. The results indicated that peaks 3 (19.00 min) and 4 (20.75 min) had the highest antibacterial activity (data not shown) and were therefore collected for further structural analysis (Fig. 3, Fr. C). Since only peaks 3 and 4 of fraction C have antimicrobial activity so they were assigned as compounds **1** and **2**, respectively.

Table 3
Antimicrobial activities of organic solvent extracted compounds.

Microorganisms	Diameter (mm)
Acinetobacter calcoaceticus ADP1	20.6
Agrobacterium tumefaciens NTL4	16.0
Enterobacter cloacae	25.6
Escherichia coli TISTR 887	21.6
Klebsiella pneumoniae	11.2
Proteus mirabilis	17.0
Pseudomonas aeruginosa TISTR 1467	12.2
Pseudomonas maltophilia	18.0
Pseudomonas putida	12.0
Rhodobacter capsulatus	15.3
Saccharomyces cerevisiae BY 4741	15.0
Salmonella enteritidis	16.6
Salmonella typhi	14.30
Salmonella typhimurium TISTR 292	22.25
Sinorhizobium meliloti SM1021	17.6
Stenotrophomonas maltophilia K279a	20.6
Xanthomonas campestris	30.0

3.6. Structure elucidation

Both compounds **1** and **2** (Fig. 4) gave a negative reaction with ninhydrin suggesting that they are cyclic lipopeptides. The microTOF HRMS of compound **1** displayed two major $[M+H]^+$ peaks at *m/z* 1043.5497, and 1057.5655, the difference of 14 mass units being due to the presence of the homologous C14, and C15 β-amino acids. Thus compound **1** was identified as a mixture of iturin A2, $C_{48}H_{74}N_{12}O_{14}$, and iturin A5, $C_{49}H_{76}N_{12}O_{14}$.

The microTOF HRMS of compound **2** found *m/z* 1079.5535 $[M+Na]^+$ of the formula, $C_{49}H_{76}N_{12}NaO_{14}$, which is a new member of the lipopeptide family and named as subtulene A. The ^1H and ^{13}C NMR data of subtulene A are nearly identical with that of iturin A. Twelve carbonyl carbons in the characteristic range δ_C 177.7–172.8 were observed in the ^{13}C NMR spectrum. Of the twelve carbonyl carbon signals, eight carbons at δ_C 175.4 (2C), 174.2, 173.9, 173.1 (2C), 172.9, and 172.8 could be assigned to the carbonyl of α- or β-amide carbons of the eight amino acid residues. Based on the molecular formula and the 2D NMR spectra, the four remaining carbons at δ_C 177.7 (2C), 175.2, and 174.7 were assigned as the terminal amide carbons of the three asparagine residues and the glutamine residue. Seven α-amino acid protons occurred in the ^1H NMR spectrum in the characteristic range δ_H 4.00–4.85, while the α-amino acid carbons showed characteristic peaks in the range δ_C 51.6–63.3 in the ^{13}C NMR spectrum. Seven NH_α protons, one NH_β proton, eight NH_{amide} protons, and two hydroxy protons occurred at low filed in the characteristic range δ_H 6.81–9.15 in the ^1H NMR spectrum corresponding to the assignment (see Table 2 in Supplementary data).

In the ^1H NMR spectrum, two pairs of doublets at δ_H 7.01 (2H, J = 8.3 Hz) and δ_H 6.64 (2H, J = 8.3 Hz) could be attributed to the protons corresponding to the aromatic carbons of the tyrosine residue as shown in the ^{13}C NMR spectra at δ_C 157.4 (C), 131.2 (2CH), 129.0 (C), and 116.5 (2CH), respectively. The carbon signals at δ_C 62.9 (methylene, C-33) and δ_C 57.4 (methine, C-32) are very characteristic of a serine residue and showed a strong correlation in the COSY and TOCSY spectra. The proline residue protons and carbons were assigned based on the TOCSY and HSQC spectra. The set of proline protons showed the α-amino proton at δ_H 4.23 (1H, J = 7.3 Hz) and three methylene protons as multiplet at δ_H 1.95 (2H), 2.14, 2.17, 3.80, and 3.95, which corresponding to signals in the ^{13}C NMR spectrum at δ_C 63.3 (C-23), 30.5 (C-24), 26.2 (C-25), and 49.0 (C-26), respectively.

The carbon signals at δ_C 43.7 and 48.1 are very characteristic of a β-amino acid and could be assigned to the α-CH_2 (C-36) and β-CH (C-35, adjacent to NH) of the long chain β-amino acid. The doublet signal at δ_H 0.85 (6H, 2 × CH_3) in the ^1H NMR spectrum and at δ_C 23.0 in the ^{13}C NMR spectrum was assigned to the isopropyl group of the long chain. The olefinic protons and carbons at δ_H 5.23 (2H) in the ^1H NMR spectrum and at δ_C 129.1 and 130.9 in the ^{13}C NMR spectrum suggested that the long chain β-amino acid contained a 1,2-disubstituted double bond. Based on the HMBC correlation, the position of the double bond was assigned at C42–C43 position [11]. The presence of an unsaturated double bond is unique among the members of the iturin family. The absolute stereochemistry

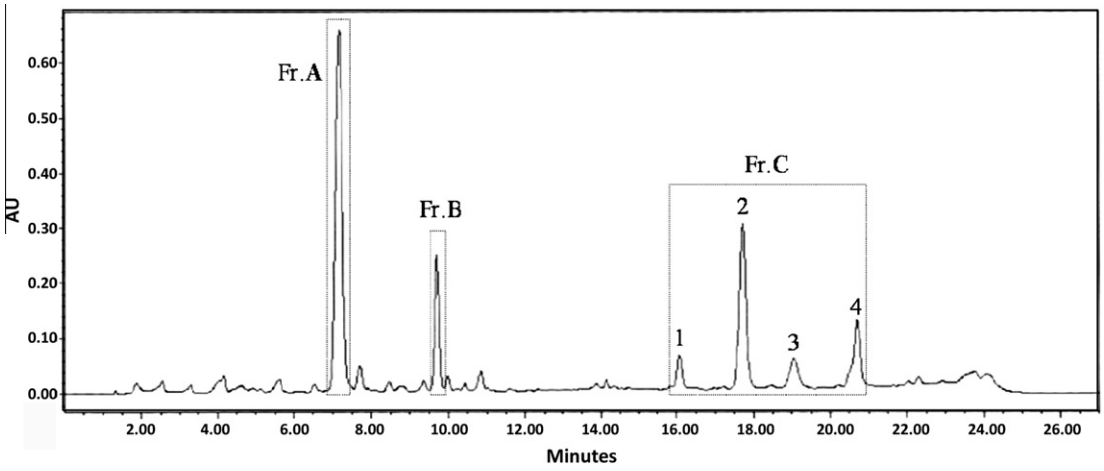


Fig. 3. Purification of bioactive compounds using HPLC. The HPLC fingerprint of the residue from an ethyl acetate extract of the culture filtrate of *B. subtilis* SSE4.

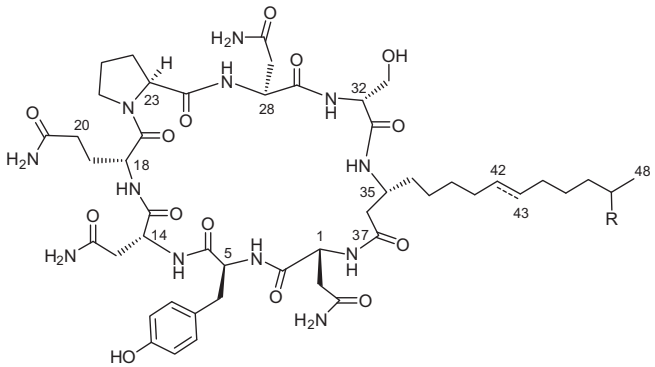


Fig. 4. Structure of iturin A (**1**) and subtulene A (**2**).

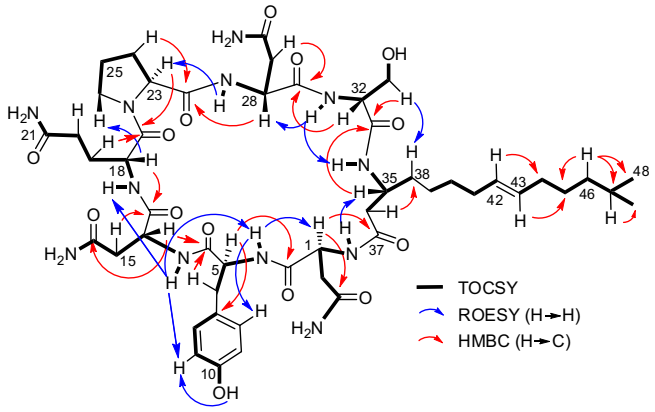


Fig. 5. Selected TOCSY, HMBC and NOESY spectrum of subtulene A (**2**).

was determined to be in the *R*-configuration at the β-carbon (C35) same as the previous reports [5,6].

The sequence of the eight amino acid residues was determined by analyzing the ROESY and HMBC spectrum as shown in Fig. 5. Six NH_α protons and one NH_β proton of the eight amino acid residues showed sequently cross correlations altogether with the seven CH_α protons and one CH_β proton in ROESY spectrum. Seven CH_α protons and one CH_β proton correlated to the carbonyl of α- and β-amide carbons of the eight amino acid residues in HMBC spectrum. The results of TOCSY, ROESY and HMBC correlations concluded the sequence of eight amino acids of subtulene A as L-Asn-1, D-Tyr-2, D-Asn-3, L-Gln-4, L-Pro-5, D-Asn-6, L-Asn-7, and β-amino acid-8, respectively.

3.7. Cytotoxic activities of subtulene A

In order to test if our new compound, subtulene A, posses any antitumor activity, the IC_{50} values for subtulene A were determined in five cancer cell lines with the anticancer drug doxorubicin serving as a positive control. Subtulene A did not exhibit significant cytotoxic effects toward the cell lines tested (data not shown). To date, there is only one report of iturins having antitumor activity. Mixirins A, B and C, which were isolated from a marine *Bacillus* sp., were reported to inhibit the human colon tumor cell line, HCT-116 [30]. However, no experimental details were given.

4. Conclusion

We isolated *B. subtilis* SSE4 that produces the extracellular antimicrobial compounds iturin A (**1**) and a new member of the lipopeptide family, subtulene A (**2**). Subtulene A has a unique long iturinic acid side chain of 3-amino-13-methyltetradec-8-enoic acid. This is the first report of an antibiotic belonging to the iturin family in which the lipid side chain contains a double bond.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.005.

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