ORIGINAL ARTICLE

A novel isoquinoline alkaloid, DD-carboxypeptidase inhibitor, with antibacterial activity isolated from *Streptomyces* sp. 8812. Part I: Taxonomy, fermentation, isolation and biological activities

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A novel isoquinoline alkaloid of molecular formula C₁₀H₉NO₄, labeled JS-1, was isolated from the culture broth of *Streptomyces* sp. 8812. It was purified by acetone protein precipitation from the culture supernatant, followed by anion exchange and C18 RP HPLC columns. JS-1 is an inhibitor of exocellular DD-carboxypeptidases/transpeptidases (DD-peptidases) 64-575 II from *Saccharopolyspora erythraea* 64-575 II, and R39 from *Actinomadura* R39. JS-1 exhibits activity against Gram-negative bacteria, such as *Bordetella bronchiseptica*, *Stenotrophomonas maltophilia*, *Proteus vulgaris*, *P. mirabilis*, *Burkholderia cepacia* and *Acinetobacter baumannii*, with MIC values 10–160 µg ml⁻¹, and against Gram-positive bacteria, such as *Staphylococcus aureus*, with MIC values 40–206 µg ml⁻¹.

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INTRODUCTION

The increasing bacterial resistance to antibiotics is at present a serious therapeutic problem. Multiresistant pathogenic bacteria occur very frequently.¹ Hence, there is a necessity for the discovery of new classes of antibiotics for the treatment of drug-resistant bacterial infections. Apart from combinatorial chemistry, rational drug design and computer-assisted design technology, one of the solutions to bacterial resistance involves screening for microorganisms producing novel antimicrobial drugs.^{2,3} Currently, the majority of antibacterial agents in clinical use are either microbial natural products or one of their analogs.⁴ Streptomycetes are well-known producers of antibiotics and other bioactive metabolites. More than half (56%) of all bacterial metabolites consist of derivatives of amino acids, peptides, peptolides and polypeptides.⁵ The purpose of this study was to search for novel antimicrobial agents, specifically inhibitors of DD-peptidases (EC 3.4.16.4). DD-carboxypeptidases/transpeptidases are the enzymes involved in peptide cross-linking during the last stage of bacterial cell wall peptidoglycan biosynthesis.^{6,7} Enzymes participating in cell wall biosynthesis have proved to be excellent targets for antibacterial agents because the cell wall pathway is conserved among bacterial pathogens and is absent in mammalian cells. β-Lactam antibiotics are the most important class of DD-peptidases/penicillin-binding proteins inhibitors and antimicrobial agents.⁶ However, resistance mechanisms of patho-

genic bacteria, for example, changes in penicillin-binding proteins and production of β-lactamases, render the β-lactam antibiotics ineffective. In our screening program for novel inhibitors of DD-peptidases from microbial secondary metabolites, we used DD-peptidase 64-575 II^{8,9} from our collection of streptomycetes strains. A novel compound having antibacterial activity, DD-peptidase inhibitor, labeled JS-1, a member of isoquinoline alkaloids, was isolated from the culture broth of *Streptomyces* sp. 8812. In this paper, we describe the taxonomy and fermentation of *Streptomyces* sp. 8812 and the isolation procedure of JS-1. We also characterize the biological properties of this compound.¹⁰ The physico-chemical properties and structure elucidation of JS-1 are described in the following paper of this issue.¹¹ The structure of the discovered alkaloid is shown below (Figure 1).

RESULTS AND DISCUSSION

Taxonomy of the producing strain

The strain *Streptomyces* sp. 8812 forms well-developed and branching substrate mycelium and aeral mycelium. The culture characteristics and carbon utilization of sp. 8812 are shown in Table 1. Good growth was observed on inorganic salts–starch agar, on glycerol–asparagine agar and Czapek's agar. The best culture medium for this strain was yeast extract–malt extract agar and tyrosine agar on which it grew

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Figure 1 The structure of the alkaloid, JS-1.

Table 1 Cultural characteristics and carbon utilization of *Streptomyces* sp. 8812

Medium	Growth	Aerial mycelium	Reverse side color	Melanin
Yeast extract–malt extract agar	Abundant	R7a, R6a	Or3r	–
Inorganic salts–starch agar	Good	Oc7m	O4b	–
Glycerol–asparagine agar	Good	Oc7a	Oc7a	–
Tyrosine agar	Abundant	R5a	R7s	+
Czapek's agar	Good	Coo7c	Coo7c	–

Carbon utilization	
D-Glucose	++
D-Xylose	+
L-Arabinose	++
L-Rhamnose	–
D-Fructose	++
D-Mannitol	++
D-Sucrose	–
Raffinose	+
L-Inositol	–
Cellulose	–

Figure 2 Scanning electron micrograph of the spore chain of *Streptomyces* sp. 8812 grown on yeast–malt agar for 14 days. Bar represents 2.0 μm.

abundantly. Substrate and aerial mycelium color were medium dependent. Spore chains were of the open loop (*Retinaculum apertum*) type with smooth surface and spore dimensions of 0.65–0.8 μm × 1.3–1.5 μm (Figure 2).

From the characteristics stated above, the strain 8812 was identified as *Streptomyces* sp. This strain was deposited in the Polish Collection

Culture broth (5 L)
|
Supernatant
| Protein precipitation with acetone
BioLogic System, IRA-400 (AcO[–])
| 0–2 M AcOH
BioLogic System, Atlantis dC18
| 9–11 % phase B [phase B, CH₃CN–0.1 % TFA,
| 30 : 70 (v/v)]; phase A, 0.05 % TFA
HPLC, Atlantis dC18
| 2 % phase B [phase B, CH₃CN–0.05 % TFA,
| 20 : 80 (v/v)]; phase A, 0.05 % TFA
JS-1

Figure 3 Isolation and purification of JS-1 under guidance of DD-peptidase 64-575 II inhibition reaction.

of Microorganism, Wrocław, under the name of *Streptomyces* sp. 8812, with an accession number B/00017.

The culture broth was centrifuged (8000 g for 60 min at 4 °C; Beckman Coulter, Sykesville, MD, USA) to obtain the supernatant, followed by acetone precipitation of proteins (Figure 3). The precipitated proteins were centrifuged (8000 g for 30 min at 4 °C; Beckman) and discarded. The acetone solution was evaporated under reduced pressure to afford an aqueous solution, which was frozen and lyophilized (24 h, Beta 1-8, Christ, Osterode, Germany).

The crude material (22 g) was dissolved in deionized water (200 ml), applied to an anion exchange resin IRA-400 (AcO[–]) (Supelco, Bellefonte, PA, USA) column 5 × 25 cm and washed with deionized water (1.5 l). Chromatography was performed using the BioLogic system (Bio-Rad, Hercules, CA, USA) at flow rates of 9.9 ml min^{–1} and detection by UV absorption at 254 nm. Elution was performed with a stepwise gradient of aqueous acetic acid (0.5, 1, 2 M, 1 l each) and 6 ml fractions were collected. The active compound (fractions exhibiting an inhibitory effect on DD-peptidase activity) was eluted with ~0.5 M aqueous acetic acid and lyophilized to yield the partially purified material (140 mg). This procedure was repeated 4 times and 560 mg of the material was obtained.

Further purification of JS-1 was carried out by the BioLogic System (Bio-Rad) on a semi-preparative modified reversed-phase column (Atlantis, dC18, 10 × 250 mm, 10 μm, Waters, Milford, MA, USA); UV detection at 214 nm; flow rate of 4.7 ml min^{–1}; mobile phase: isocratic phase A: 0.05% trifluoroacetic acid (10 ml), and linear gradient from 0 to 19% of phase B (phase B was CH₃CN–0.1% trifluoroacetic acid, 30:70 (v/v)), 175 ml. The most active fractions obtained at 9–11% of phase B, exhibiting 100% inhibition of DD-peptidase activity, were collected. The fractions eluted in 22 repeated runs were pooled and lyophilized to yield 40 mg of powder.

This was then subjected to HPLC (Atlantis, dC18, 4.6 × 250 mm, 5 μm, Waters); detection by UV at 214 nm; flow rate of 1 ml min^{–1}; mobile phase: 2% phase B (B: CH₃CN–0.05% trifluoroacetic acid, 20:80 (v/v)), phase A (0.05% trifluoroacetic acid). Under these conditions, JS-1 was eluted at a retention time of 30.4 min. The fractions eluted in 80 repeated runs were pooled and lyophilized to yield 7 mg of yellow powder.

Further purification of JS-1 was carried out by the same procedure as described previously, namely, the detection by diode array detection (Figure 4). JS-1 of 5 mg was obtained.

Figure 4 HPLC profile of the purified JS-1 compound.

Inhibitory effect of JS-1 on DD-peptidases

JS-1 exhibited inhibitory effects on DD-peptidases 64-575 II and R39. The concentrations of the compound necessary to inhibit DD-carboxypeptidases by 50% (IC_{50}) were calculated. The IC_{50} values of JS-1 for these enzymes were 4.7 and 6.0 μM , respectively. The IC_{50} values of β -lactam antibiotics for DD-carboxypeptidase 64-575 II were compared with the IC_{50} value of JS-1 for DD-carboxypeptidase 64-575 II, for example, the IC_{50} values of carbenicillin, cephalothin and imipenem for DD-carboxypeptidase 64-575 II were 1.4, 1.1 μM and 58 nM, respectively.⁹ The IC_{50} value of 6-aminopenicillanic acid for DD-carboxypeptidase R39 was comparable with the IC_{50} value of JS-1 for this enzyme.¹² The IC_{50} values of β -lactam antibiotics such as 6-aminopenicillanic acid, carbenicillin and cephalothin for DD-carboxypeptidase R39 were 5.7, 1.95 μM and 42 nM, respectively.¹² Until today, there have not been any described inhibitors of DD-peptidases from the group of isoquinoline alkaloids. It is assumed that JS-1 may inhibit cell wall biosynthesis by disturbing the cross-linking of peptidoglycan, analogous to the mechanism of action of β -lactam antibiotics.

Inhibitory effect of JS-1 on β -lactamase

As the examined alkaloid has been shown to inhibit DD-peptidase 64-575 II activity, similar to clavulanic acid,⁹ which is a known inhibitor of β -lactamases, an inhibition test was carried out for this enzyme. JS-1 did not show inhibitory activity against β -lactamase from *Bacillus cereus*.

Inhibitory effect of JS-1 on common proteolytic enzymes

To examine the specificity of the JS-1 compound, inhibition assays were performed for a range of common proteolytic enzymes. It was determined that at a concentration of 65 μM , the JS-1 alkaloid causes a 100% inhibition of DD-peptidase 64-575 II and DD-peptidase R39, whereas the rest of the enzymes remained active even in a twofold higher concentration. Only pepsin lost 10% of its activity, whereas the activity of papain, a proteolytic enzyme with a disulfide bond in its active center, was inhibited by 7%. The obtained results on the examination of the JS-1 alkaloid implied it to be a specific inhibitor of DD-peptidases.

Hemolytic activity

No hemolytic activity was observed on human erythrocyte.

Table 2 MICs and MBCs of alkaloid JS-1 against Gram(+), Gram(–) bacteria and yeast^a

Microorganism	MIC, ($\mu g\ ml^{-1}$)	MBC, ($\mu g\ ml^{-1}$)
<i>S. aureus</i> ATCC 25923	80	80
<i>S. aureus</i> ATCC 43300	40	80
<i>E. faecalis</i> ATCC 29219	> 320	> 320
<i>B. bronchiseptica</i> ATCC 4617	10	20
<i>A. baumannii</i> ATCC 19606	160	160
<i>K. pneumoniae</i> ATCC 13882	> 320	> 320
<i>B. cepacia</i> ATCC 25416	160	160
<i>P. aeruginosa</i> ATCC 27853	> 320	> 320
<i>S. maltophilia</i> ATCC 13637	160	160
<i>P. vulgaris</i> ATCC 6896	160	160
<i>P. mirabilis</i> ATCC 12453	160	160
<i>E. coli</i> ATCC 25922	> 320	> 320
<i>E. coli</i> ATCC BAA-198	> 320	> 320
<i>B. subtilis</i> ATCC 6633	> 206	> 206
<i>B. stearothermophilus</i> ATCC 7953	> 206	> 206
<i>C. albicans</i> ATCC 90028	> 320	> 320
<i>C. parapsilosis</i> ATCC 22019	206	> 206
<i>C. krusei</i> ATCC 6258	206	> 206

^aMIC's of the reference compounds: penicillin G against *S. aureus* ATCC 43300, 5.0 $\mu g\ ml^{-1}$; polymyxin B against *P. aeruginosa* ATCC 27853, 1.2 $\mu g\ ml^{-1}$; amphotericin against *C. krusei*, 1 $\mu g\ ml^{-1}$.

Genotoxicity

Bacillus subtilis M45 (rec^{-}) is devoid of the recombinant-based DNA repair mechanism and, therefore, is much more susceptible to genotoxic damage than its rec^{+} counterpart, which is evidenced by a wider zone of growth inhibition for this strain around the reference genotoxin (4-nitroquinoline *N*-oxide)-carrying discs. The diameter of the zone of growth inhibition reached 24 mm for the rec^{-} strain and 12 mm for the rec^{+} strain. The examined JS-1 alkaloid did not inhibit the growth of the tested *B. subtilis* strains and, hence, was determined as nongenotoxic. The lack of genotoxic features of JS-1 allows the consideration of potential modifications of the naturally isolated molecule to increase its antibacterial and antifungal activity.

Antimicrobial activity

JS-1 was tested against three Gram-positive bacteria, reference bacteria (*Staphylococcus aureus* subsp. *aureus* ATCC 25923; *S. aureus* subsp. *aureus* ATCC 43300 resistant to methicillin; and *Enterococcus faecalis*), 12 Gram-negative bacteria (*Bordetella bronchiseptica*; *Stenotrophomonas maltophilia*; *Proteus vulgaris* and *P. mirabilis*; *Burkholderia cepacia*; *Acinetobacter baumannii*; *Klebsiella pneumoniae*; *Pseudomonas aeruginosa*; *Escherichia coli* ATCC 25922; *E. coli* ATCC BAA-198-producing extended spectrum β -lactamase ESBL TEM-26; *Bacillus subtilis*; *B. stearothermophilus*) and three yeasts (*Candida albicans*; *C. parapsilosis* and *C. krusei*) by the broth microdilution method. MIC and MBC are presented in Table 2. JS-1 exhibited the highest activity against *B. bronchiseptica* (MIC 10 $\mu g\ ml^{-1}$) and *S. aureus* strains, including methicillin-resistant *S. aureus* (MIC 40–80 $\mu g\ ml^{-1}$). Bacteria *E. faecalis*, *K. pneumoniae*, *E. coli* and *P. aeruginosa*, and the yeast *C. albicans* proved insensitive (MIC > 320 $\mu g\ ml^{-1}$).

Antibiotic resistance has reached a crisis point in many hospitals around the world. The problem is related to methicillin-resistant *S. aureus* and to many multidrug-resistant Gram-negatives.^{13–15} Therefore, new classes of potent antimicrobial agents, including natural products, are needed to resolve this problem.

Table 3 MICs of alkaloid JS-1 against clinical isolates of *S. aureus*

Strains	MIC, ($\mu\text{g ml}^{-1}$)	Number of strains
<i>S. aureus</i> (methicillin-resistant <i>S. aureus</i>)	80	4
	103	6
	206	12
	> 206	5
<i>S. aureus</i> (methicillin-sensitive <i>S. aureus</i>)	103	1
	206	2
	> 206	1

In addition, the activity of JS-1 alkaloid on 31 clinical isolates of *S. aureus*, including 27 *S. aureus* methicillin-resistant *S. aureus* strains, was examined. MIC values are presented in Table 3. Of the clinical isolates, 30% exhibited sensitivity to the new compound (MIC 80–103 $\mu\text{g ml}^{-1}$). The obtained MIC values of JS-1 alkaloid for the clinical strains were similar to the MIC values determined for the *S. aureus* ATCC 25923 reference strain (MIC 80 $\mu\text{g ml}^{-1}$). No differences between the sensitivity of clinical methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus* strains toward JS-1 alkaloid have been observed.

On the contrary, the relatively poor MIC values of JS-1 in comparison with the good inhibitory activity of DD-peptidases was most probably associated with the weak permeability of the bacterial cell envelopes to the examined alkaloid. The inhibitory activity of JS-1 on DD-peptidases is comparable with the activity of certain β -lactam antibiotics.⁹ Yet, in bacteria, DD-peptidases are known to occur in the periplasmic space. Hence, most probably the structure of JS-1 (Figure 1), which suggests low lipophilicity of the molecule, impedes its transport to the periplasmic space.

JS-1, the novel DD-carboxypeptidase inhibitor isolated from the culture broth of *Streptomyces* sp. 8812, belongs to isoquinoline alkaloids, some of which exhibit antibacterial activity (for example, plant origin products such as berberine, canadine, canadine, (+)-*N*-(methoxycarbonyl)-*N*-norbofine).^{16,17} Some of them show an activity toward *S. aureus*, which is analogous to JS-1. Iwasa *et al.*¹⁸ described antimicrobial, antimalarial, cytotoxic and anti-HIV activities of simple, natural and synthetic isoquinoline and benzylisoquinoline alkaloids. Some simple alkaloids were significantly active in each assay; especially those with a quaternary nitrogen atom of isoquinolinium or dihydroisoquinolinium type may contribute to enhanced potency in the first three types of activities.¹⁸

This study reports the discovery of a simple isoquinoline alkaloid, JS-1, and its biological activity.

Specific inhibition of DD-peptidase activity (occurring solely in *Prokaryota*) by JS-1 alkaloid implies the directed action of this compound and its derivatives on bacteria. Moreover, the demonstrated lack of both genotoxic and hemolytic activities toward human blood cells suggests the possible application of the JS-1 alkaloid as an initial compound in the search of a potentially new drug group. The active compound JS-1 must be studied further to determine its possible biological activity and toxicity thereof, and to resolve the mode of antibacterial action of isoquinoline alkaloids. Moreover, a synthetic modification of the natural molecule may generate new compounds with improved antibacterial activity.

METHODS

Taxonomy

The JS-1-producing actinomycetes strain, *Streptomyces* sp. 8812, was isolated from soil in Brasil. The strain, *Streptomyces* sp. 8812, was grown at 28 °C for 14

days on inorganic salts–starch agar, glycerol–asparagine agar, yeast extract–malt extract agar, tyrosine agar and on Czapek's medium and was examined visually to determine substrate mycelium pigmentation and spore color. Colors were determined by using the H. Prauser Code.¹⁹ Morphological characteristics were observed with a scanning electron microscope (Jeol JSM-35, Tokyo, Japan). Standard biochemical properties of this strain were tested according to the methods of Williams *et al.*²⁰ All carbon sources for carbon utilization tests were tested at the concentrations recommended by Shirling and Gottlieb²¹ and Williams *et al.*²⁰

Fermentation

A slant culture of strain 8812 was inoculated into a 500-ml Erlenmeyer flask containing 40 ml of liquid medium consisting of tryptone (Difco, Sparks, MD, USA) 17 g, peptone (Difco) 4 g, yeast extract (Difco) 5 g, corn steep liquor (Cerestar, Milan, Italy) 10 g, lactose 10 g, CaCO_3 3 g, K_2HPO_4 4 g, KH_2PO_4 2 g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g in 1.0 l of tap water, pH 6.7. The inoculated flasks were incubated for 24 h on a rotary shaker (220 r.p.m.) at 28 °C. A volume of 4 ml of the seed culture was transferred into 500 ml flasks containing 36 ml of the same medium, and incubated on a rotary shaker (220 r.p.m.) at 28 °C. Fermentation was carried out for 22 h. The production of JS-1 was analyzed by testing for DD-carboxypeptidase 64-575 II inhibition. After 22 h of fermentation, the amount of JS-1 in the broth filtrate reached a maximum (100% inhibition of DD-peptidase 64-575 II activity).

Assay of DD-carboxypeptidase activity

Enzyme activity was measured as described previously.²² Samples for assay of DD-carboxypeptidase activity consisted of 10 μl of exocellular DD-carboxypeptidase 64-575 II (50 μM) from *Saccharopolyspora erythraea* 64-575 II (Polish Collection of Microorganism, Wroclaw, accession number B/00018),^{9,23} 10 μl of the substrate solution containing 2.98 mg ml^{-1} $\text{N}^{\alpha},\text{N}^{\beta}$ -diacetyl-L-lysyl-D-alanyl-D-alanine in 0.1 M phosphate buffer, pH 8.0, and 20 μl of 0.1 M phosphate buffer, pH 8.0. Standard sample contained 20 μl of D-alanine in distilled water. Samples were incubated for 30 min at 37 °C and then boiled for 2 min. After cooling, 77 μl of the reaction mixture was added, and all samples were incubated for 10 min at 37 °C. Reaction mixture for assay of DD-carboxypeptidase activity consisted of 60 μl of 0.05 mg ml^{-1} flavin adenine dinucleotide in 0.1 M phosphate buffer, pH 8.0, 10 μl of 0.2 mg ml^{-1} horseradish peroxidase (250–330 U mg^{-1}) in distilled water, 5 μl of 5 mg ml^{-1} of *o*-dianisidine-HCl in distilled water and 2 μl of 11.9 mg ml^{-1} of D-amino acid oxidase from hog kidney (20.7 U mg^{-1}) in 0.1 M phosphate buffer, pH 8.0. Finally, 350 μl of a mixture consisting of methanol, distilled water and sulfuric acid (5:5:6 by volume) was added to each sample. Absorbance of the resulting solution was measured spectrophotometrically at 540 nm (Jasco V-630, Tokyo, Japan).

Assay of JS-1 inhibitory activity on DD-peptidases

Inhibition of DD-peptidase 64-575 II by the JS-1 compound was evaluated as follows. To detect the active compound during cultivation of strain 8812 and in subsequent stages of JS-1 purification, the DD-peptidase 64-575 II inhibition test was carried out. Mixtures of 10 μl of DD-peptidase 64-575 II (50 μM), 10 μl of the supernatant (or aliquots of concentrated fractions during purification steps) and 10 μl of 0.1 M phosphate buffer pH 8.0 were incubated for 30 min at 37 °C. After incubation, 10 μl of the substrate solution was added to 30 μl of each sample and the resulting mixtures were incubated again. The rest of the procedure was performed as mentioned above. To determine the inhibitory activity of the purified JS-1 alkaloid on DD-peptidase 64-575 II, the assay was performed as described above with the only exception being that the enzyme was incubated with JS-1 at different concentrations ranging from 0.1 mM to 0.1 μM (instead of the supernatant). The inhibition of DD-peptidase was expressed as IC_{50} values, molar concentrations of JS-1 necessary to inhibit the DD-peptidase activity by 50%.

The inhibition of exocellular DD-peptidase R39 from *Actinomadura* R39²⁴ by JS-1 was performed as described previously. DD-peptidase R39 of 10 μl (Penzym test, UCB Bioproducts, Braine-l'Alleud, Belgium)²⁵ was used in the reaction mixtures.

Assay of the universal protease activity

Pancreatic elastase (type IV) from porcine pancreas (0.017 U ml⁻¹) was assayed by monitoring the release of p-nitroanilide at 410 nm from 0.3 mM *N*-succinyl-Ala-Ala-Val-Ala p-nitroanilide in 0.1 M Tris buffer, pH 8.0, at 25 °C.²⁶

Carboxypeptidase A from bovine pancreas (0.4 U ml⁻¹) was assayed by examining hydrolysis at 254 nm of 1 mM Hippuryl-L-Phe in 0.025 M Tris buffer with 0.5 M NaCl, pH 7.5, at 25 °C.²⁷

Thrombin from bovine plasma (3.7 U ml⁻¹) was assayed by monitoring the release of p-nitroanilide at 410 nm from 1 mM *N*-Benzoyl-L-Arg p-nitroanilide in 10 mM HEPES, 10 mM Tris buffer, with 0.1 M NaCl, 0.1% PEG₆₀₀₀ and 1% dimethylsulfoxide, pH 7.8, at 25 °C.²⁸

Trypsin from porcine pancreas (73 U ml⁻¹) was assayed by monitoring the release of p-nitroanilide at 410 nm from 1 mM *N*-Benzoyl-D,L-Arg p-nitroanilide in 0.1 M Tris buffer with 20 mM CaCl₂, 1% dimethylsulfoxide, pH 8.0, at 25 °C.^{29,30}

Papain from Papaya latex (2.5 U ml⁻¹) was assayed by examining the release at 410 nm of p-nitroanilide from 1 mM *N*-Benzoyl-L-Arg p-nitroanilide in 0.1 M phosphate buffer with 2 mM Cys, 2 mM EDTA, 1% dimethylsulfoxide, pH 7.0, at 25 °C.³¹ Earlier, papain was activated by incubation at room temperature in the same buffer without dimethylsulfoxide for 1.5 h.

Endoproteinase Glu-C from *S. aureus* V8 (6.7 U ml⁻¹) was assayed by monitoring the release of p-nitroanilide from 1 mM Ac-Glu-p-nitroanilide in 0.1 M Tris buffer, pH 8.0, containing 2% dimethyl formamide and 2 mM CaCl₂, at 37 °C.³²

The activity of pepsin from porcine gastric mucosa (30.3 U ml⁻¹) was assayed using 2.5% bovine hemoglobin as substrate, at acidic pH and at 37 °C. After 10 min of incubation, the reaction was stopped by the addition of 5% trichloroacetic acid. The samples were centrifuged and the resulting supernatant was analyzed at 280 nm.³³

All enzyme assays were performed using a spectrophotometer (Jasco V-630).

Assessment of the inhibition of the above-listed proteolytic enzymes was carried out by preincubating the enzymes with JS-1 at a concentration of 65 µM, which causes 100% inhibition of DD-peptidase 64-575 II activity and, additionally, at a concentration twofold greater.

Assay of β-lactamase activity

Inhibition of penicillinase (Penase, 5 × 10⁶ IU ml⁻¹) by the JS-1 compound was evaluated on the basis of the method described in literature.³⁴ A mixture of β-lactamase and JS-1 in 0.1 M phosphate buffer, pH 7.0, was incubated for 30 min at 37 °C; thereafter, nitrocephin was added and the mixture was incubated for an additional 10 min at 37 °C. Subsequently, absorbance was measured at 482 nm.

Antimicrobial activity

The antimicrobial spectrum of JS-1 was evaluated by the MIC method using the serial twofold dilution method under standard conditions as described in the Committee Laboratory Standards (CLSI) reference method M7-A7.³⁵ Test reference bacterial strains are presented in Table 2. The organisms were cultivated according to ATCC recommendation. Thirty-one clinical isolates of *S. aureus*, including 27 methicillin-resistant *S. aureus* strains, were cultivated on tryptic soy agar. All strains were incubated for 24 h at 37 °C.

The reference method (broth microdilution susceptibility test) was as follows: The compound JS-1 was dissolved in double-distilled water. A series of the twofold JS-1 alkaloid dilutions were diluted with cation-adjusted Mueller–Hinton broth. Aliquots of 95 µl were dispensed into microdilution sterile plates (Mar-Four). Then, 5 µl of bacteria inoculum, containing 5 × 10⁴ CFU ml⁻¹, was added. The final concentration of the JS-1 compound ranged from 320 to 5 µg ml⁻¹ (or 206–12.9 µg ml⁻¹), all in twofold dilution steps. The experiments for each sample were conducted in triplicate. Polymyxin B and penicillin G were used as controls (from 8 to 0.15 µg ml⁻¹). The plates were incubated at 37 °C (35 °C) for 18–24 h depending on the bacterial strain. MIC was defined as the lowest drug concentration that reduced growth by 100%. For the MBC assay, aliquots (10 µl) of each culture without visible growth were transferred onto tryptic soy agar and incubated during 24 h at 37 °C.

Reference yeasts were presented in Table 2. *Candida* sp. was cultivated according to ATCC references. The broth microdilution susceptibility test was

performed as described in Clinical and Laboratory Standards Institute, reference method M27-A2 (1).³⁶

Hemolysis test

The hemolysis test was evaluated following the literature method.³⁷ Red blood cells prepared in phosphate-buffered saline were suspended to correspond to a hematocrit of 1% in JS-1 alkaloid at final concentrations of 0.6 and 2.4 mM and were incubated for 30, 60, 90, 120 and 180 min at 23 °C.

Genotoxicity

For genotoxicity research, two genetically modified *B. subtilis* strains, M45 rec⁻ and H17 rec⁺, obtained from Dr Sadaie were studied.^{38–40} The JS-1 alkaloid was examined by the disc-diffusion method according to Kada's procedure.^{38,39} Sterile filter paper discs (9 mm diameter, Whatman no. 3, Maidstone, UK) were dripped with tested JS-1 solution to load 206 µg of the compound per disc. As a standard, 4-nitroquinoline *N*-oxide (2 µg per disc) was used. Results of the genotoxicity test were read after 18 h of incubation of the discs at 35 °C.

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- Walsh, C. T. *Antibiotics: Actions, Origin, Resistance* (ASM Press, Washington, DC, 2003).
- Baker, D. D., Chu, M., Oza, U. & Rajgarhia, V. The value of natural products to future pharmaceutical discovery. *Nat. Prod. Rep.* **24**, 1225–1244 (2007).
- Imanaka, H. Screening microbial products for medicine. *Actinomycetologica* **14**, 22–26 (2001).
- Singh, M. P. & Greenstein, M. Antibacterial leads from microbial natural products discovery. *Curr. Opin. Drug Disc. Develop.* **3**, 167–176 (2000).
- Berdy, J. Bioactive microbial metabolites. *J. Antibiot.* **58**, 1–26 (2005).
- Waxman, D. J. & Strominger, J. L. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. *Ann. Rev. Biochem.* **52**, 825–969 (1983).
- Ghuysen, J.-M. Bacterial active-site serine penicillin-interactive proteins and domains: mechanism, structure, and evolution. *Rev. Infect. Dis.* **10**, 726–732 (1988).
- Kurzatowski, W., Solecka, J., Filipek, J., Kurzatowski, J. D. & Kurylowicz, W. *Streptomyces* excreting DD-carboxypeptidases. *Appl. Microbiol. Biotechnol.* **33**, 452–454 (1990).
- Solecka, J. & Kurzatowski, W. Affinity of exocellular DD-carboxypeptidase/transpeptidase from *Saccharopolyspora erythraea* PZH 64-575 to beta-lactam compounds. *Med. Dosw. Mikrobiol.* **51**, 151–165 (1999).
- Solecka, J. & Kozerski, L. A new inhibitor of DD-peptidase and its use as antibiotic or anticancer drug. PCT/WO 2009/028972 A1, 5 March (2009).
- Solecka, J. et al. A novel isoquinoline alkaloid, DD-carboxypeptidase inhibitor, with antimicrobial activity isolated from *Streptomyces* sp. 8812. Part II: Physico-chemical properties and structure elucidation. *J. Antibiot.* (e-pub ahead of print 28 August 2009; doi:10.1038/ja.2009.86).
- Dusart, J. et al. DD-Carboxypeptidase-transpeptidase and killing site of β-lactam antibiotics in *Streptomyces* strains R39, R61, and K11. *Antimicrob. Ag. Chemother.* **3**, 181–187 (1973).
- Gould, I. M. The epidemiology of antibiotic resistance. *Int. J. Antimicrob. Agents* **32S**, S2–S9 (2008).
- Casey, A. L., Lambert, P. A. & Elliott, T. S. J. *Staphylococci*. *Int. J. Antimicrob. Agents* **29** (Suppl. 3), S23–S32 (2007).
- Denton, M. Enterobacteriaceae. *Int. J. Antimicrob. Agents* **29** (Suppl. 3), S9–S22 (2007).
- Scazzocchio, F., Cometa, M. F., Tomassini, L. & Palmery, M. Antibacterial activity of *Hydrastis canadensis* extract and its major isolated alkaloids. *Planta Med.* **67**, 561–564 (2001).
- Feng, T., Xu, Y., Cai, X.-H., Du, Z.-Z. & Luo, X.-D. Antimicrobially active isoquinoline alkaloids from *Litsea cubeba*. *Planta Med.* **75**, 76–79 (2009).
- Iwasa, K. et al. Simple isoquinoline and benzylisoquinoline alkaloids as potential antimicrobial, antimalarial, cytotoxic, and anti-HIV agents. *Bioorg. Med. Chem.* **9**, 2871–2884 (2001).
- Prauser, H. Aptness and application of colour codes for exact description of colours of streptomycetes. *Z. Allg. Mikrobiologie* **4**, 95–98 (1964).
- Williams, S. T., Goodfellow, M. & Alderson, G. Genus *Streptomyces* Waksman and Henrici 1943. in *Bergey's Manual of Systematic Bacteriology*, Vol. 4 (ed. Williams, S. T.) 2452–2492 (Williams & Wilkins, Baltimore, 1989).
- Shirling, E. B. & Gottlieb, D. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16**, 313–340 (1966).

- 22 Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. & Perkins, H. R. Exocellular D-D-carboxypeptidases/transpeptidases from *Streptomyces*. *Methods Enzymol.* **45**, 610–636 (1976).
- 23 Solecka, J., Łysek, R., Furman, B., Chmielewski, M. & Kurzatkowski, W. Practical use of D-D-peptidase 64-575 for assay inhibition activity of natural and synthetic β -lactam compounds. *Acta. Poloniae Pharmaceutica* **60**, 115–118 (2003).
- 24 Frère, J.-M. *et al.* Molecular weight, amino acid composition and physicochemical properties of the exocellular D-D-carboxypeptidase-transpeptidase of *Streptomyces* R39. *Biochem. J.* **143**, 233–240 (1974).
- 25 Sternesjö, A. & Gustavsson, E. Biosensor analysis of [beta]-lactams I milk using the carboxypeptidase activity of a bacterial penicillin binding protein. *J. AOAC Int.* **89**, 832–837 (2006).
- 26 Bieth, J., Spiess, B. & Wermuth, C. G. Synthesis and analytical use of highly sensitive an convenient substrate of elastase. *Biochem. Medicine* **11**, 350–357 (1974).
- 27 Hass, G. M. & Ryan, C. A. Carboxypeptidase inhibitor from potatoes. *Methods Enzymol.* **80**, 778–791 (1981).
- 28 Bezeaud, A. & Guillin, M.-C. Enzymatic and nonenzymic properties of human β -thrombin. *J. Biol. Chem.* **263**, 3576–3581 (1988).
- 29 Erlanger, B. F., Edel, F. & Cooper, A. G. The action of chymotrypsin on two new chromogenic substrates. *Arch. Biochem. Biophys.* **210**, 206–210 (1966).
- 30 Kassell, B. Bovine trypsin-kallikrein inhibitor (kunits inhibitor, basic pancreatic inhibitor, polyvalent inhibitor from bovine organs). *Methods Enzymol.* **19**, 844–852 (1970).
- 31 Mole, J. & Horton, H. R. Kinetics of papain-catalyzed hydrolysis of α -N-benzoyl-L-arginine-p-nitroanilide. *Biochemistry* **12**, 816–822 (1973).
- 32 Kakudo, S. *et al.* Purification, characterization, cloning, and expressin of a glutamic acid-specific protease from *Bacillus licheniformis* ATCC 14580. *J. Biol. Chem.* **267**, 23782–23788 (1992).
- 33 Anson, M. L. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**, 79–89 (1938).
- 34 O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. Novel method for detection of β -lactamases by using a chromogenic cephalosporin substrate. *Antimicrob. Ag. Chemother.* **1**, 283–288 (1972).
- 35 CLSI. Method for Dilution Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition. M7-A7 [ISBN 1-56238-587-9]. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA (2006).
- 36 CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard—Second Edition. M27-A2, CLSI, Wayne, PA, USA (2002).
- 37 Dehghan-Noudeh, G., Housaindokht, M. & Bazzaz, B. S. F. Isolation, characterization, and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633. *J. Microbiol.* **43**, 272–276 (2005).
- 38 Kada, T., Moriya, M. & Shirasu, Y. Screening of pesticides for DNA interactions by 'rec-assay' and mutagenesis testing, and frameshift mutagens detected. *Mutat. Res.* **26**, 243–248 (1974).
- 39 Kada, T., Hirano, K. & Shirasu, Y. *Bacillus subtilis* rec-assay test. in *Chemical Mutagenesis* Vol. 6 (eds de Severs, F. E. & Hollaender, A.) 149 (Plenum Press, New York, 1980).
- 40 Sadaie, T. & Kada, T. Recombination-deficient mutants of *Bacillus subtilis*. *J. Bacteriol.* **125**, 489–500 (1976).