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Structure and biosynthesis of scabichelin, a novel tris-hydroxamate siderophore produced by the plant pathogen *Streptomyces scabies* 87.22†

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Scabichelin and turgichelin, novel tris-hydroxamate siderophores, were isolated from *Streptomyces antibioticus* NBRC 13838/*Streptomyces scabies* JCM 7914 and *Streptomyces turgidiscabies* JCM 10429, respectively. The planar structures of scabichelin and turgichelin were elucidated by mass spectrometry, and 1- and 2-D NMR spectroscopic analyses of their gallium(III) complexes. The relative and absolute stereochemistry of the metabolites was determined by the modified Marfey's method in conjunction with computational modelling and NOESY NMR analysis of Ga-scabichelin and Ga-turgichelin. Genome sequence analysis of the plant pathogen *Streptomyces scabies* 87.22 identified a gene cluster containing a gene encoding a nonribosomal peptide synthetase (NRPS) that was predicted to direct the production of a pentapeptide with structural similarities to scabichelin and turgichelin. Comparative LC-MS/MS analyses of iron-deficient culture supernatants from wild type *S. scabies* 87.22 and a mutant in which the NRPS gene had been disrupted, and scabichelin purified from *S. antibioticus*, showed that scabichelin is the metabolic product of the cryptic gene cluster, strongly suggesting that it functions as a siderophore.

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Introduction

Iron acquisition is a vital process for bacterial survival, because more than 100 primary and secondary metabolic enzymes possess iron-containing cofactors such as iron-sulfur clusters and heme groups. Iron in Earth's crust exists predominantly in the ferric form due to the oxygen-rich atmosphere of the planet. However, the solubility of ferric ion is as low as 10^{-18} μM in water of biological pH, whereas optimum growth of bacterial cells requires an intracellular iron concentration in excess of 1 μM .¹ In order to utilize such a low concentration of

ferric ion in natural habitats, bacteria excrete low molecular weight compounds called siderophores, which have the ability to chelate ferric ion. Ferric-siderophore complexes are typically transported into bacterial cells *via* receptors associated with the extracellular membrane.²

Siderophore-dependent iron uptake systems of pathogenic bacteria have been extensively studied as potential targets for chemotherapeutic intervention, because iron acquisition is often critical for pathogen survival within hosts. Indeed, in several cases the secretion of siderophores has been reported to enhance the virulence of pathogenic bacteria.³ As a consequence, numerous siderophores including pyochelin,⁴ yersiniabactin,⁵ petrobactin,⁶ and mycobactin⁷ have been isolated from pathogenic bacteria. Two main pathways for siderophore biosynthesis in bacteria are known.⁸ One pathway utilizes non-ribosomal peptide synthetase (NRPS) multienzymes,⁹ whereas the other pathway is NRPS-independent.¹⁰

Streptomyces scabies is a plant pathogenic bacterium that causes serious damage to potatoes and other root vegetables. As a consequence, studies over several decades, culminating recently in a complete genome sequence, have focused on elucidating the molecular mechanism of plant infection by *S. scabies*.^{11,12} Production of the phytotoxin thaxtomin A, which causes apoptosis of plant cells, has been shown to be a key pathogenicity determinant of *S. scabies*.^{13,14} The

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Fig. 1 Structures of scabichelin and turgichelin.

necrogenetic factor, *nec1*, is also produced by *S. scabies* and related strains.^{15,16} However, the iron uptake systems of *S. scabies* have received relatively little attention, even though the correlation between virulence and iron uptake in pathogenic bacteria is well known.

Mining of the *S. scabies* genome has identified several gene clusters that are hypothesized to direct the production of siderophores. Among these, the *scab1371–scab1481* locus has recently been shown to encode a NRPS-dependent pathway for the biosynthesis of pyochelin,¹⁷ a siderophore produced by pathogenic *Pseudomonas* and *Burkholderia* species that is known to contribute to virulence.^{18–20} Here we report the isolation and structure elucidation of scabichelin (Fig. 1), a novel siderophore produced by *S. scabies*, along with the identification of a gene cluster that encodes the nonribosomal peptide synthetase multienzyme responsible for its biosynthesis. We also report the discovery of the structurally-related iron-chelator turgichelin (Fig. 1) from *Streptomyces turgidiscabies*, a close relative of *S. scabies*.

Materials and methods

NMR spectroscopy and mass spectrometry

One milligram of Ga-siderophore was dissolved in 0.7 mL of DMSO-d₆, and the following NMR spectra were recorded at 303 K on a Bruker AVANCE 800 MHz spectrometer equipped with a cryogenic probe (Bruker Biospin, Karlsruhe, Germany): ¹H, ¹³C, DQF-COSY, TOCSY, ROESY, NOESY, HSQC and HMBC.

An electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Apex II, Bruker Daltonics, Billerica, MA, USA) was used to record high resolution mass spectra. The spectrometer was operated in positive-ion mode, and an *m/z* range of 294.9 to 974.8 was calibrated using ESI calibration standard mixture (YOKUDELNA, JEOL, Tokyo, Japan) with an error range of 0.015 to 0.67 ppm. Optimal acquisition parameters at a flow rate of 100 µL h^{−1} for 1 : 1 methanol water solutions (containing 2% AcOH) of **1** or **2**, respectively, were: capillary voltage of −4.1 and −3.7 kV, end-plate voltage of −3.6 and −3.3 kV, capillary exit voltage of 70 and 100 V, skimmer voltage 1: 12.0 and 15.0 V, skimmer voltage 2: 8.0 and 5.5 V. The drying gas temperature was set at

120 °C and the nebulizing gas pressure was set at approximately 30 psi (N₂). MSⁿ spectra were recorded in positive-ion mode using an ESI quadrupole ion trap mass spectrometer (LCQ, ThermoFischer, MA, USA). Acquisition parameters were as follows. Spray voltage: 4.5 kV, capillary voltage: 31 V, capillary temperature: 220 °C, sheath gas and aux gas flow rates were set at 88 and 48 (arb, N₂), respectively.

Strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in the ESI Table S1.† *S. antibioticus* NBRC 13838, *S. noursei* NBRC 15452, *S. virginiae* NBRC 12827, *S. aureofaciens* NBRC12843, *S. albus* NBRC 13014, *S. scabies* JCM 7914 and *S. turgidiscabies* JCM10429 were cultured in a shaking incubator at 180 rpm and 30 °C for 4 days using polypropylene flasks containing 50 mL of an iron deficient culture medium, which was prepared as follows. 2 g of K₂SO₄, 3 g of K₂HPO₄, 1 g of NaCl and 5 g of NH₄Cl were dissolved in 1 L of deionized water. To remove ferric ions, the solution was stirred with 50 g of chelex-100 Na⁺ form (Bio-Rad) for 16 h. The solution was filtered through Whatman No. 1 filter paper and the following quantities of each stock solution were added: 100 µL of thiamine (20 mg mL^{−1}), 100 µL of ZnSO₄·7H₂O (20 mg mL^{−1}), 20 µL of CuSO₄·7H₂O (0.5 mg mL^{−1}), 20 µL of MnSO₄·4H₂O (3.5 mg mL^{−1}). The resulting solution was autoclaved. 10 µL of each of the following sterile solutions were added to the medium immediately prior to use: CaCl₂·H₂O (10 mg mL^{−1}), glucose (250 mg mL^{−1}), and 0.5% yeast extract (Difco).

S. scabies 87.22 and W1000 were grown for 7 days at 30 °C on oatmeal agar medium for spore stock preparation and for 4 days at 30 °C and 180 rpm in a previously-reported iron-deficient liquid medium²¹ for analysis of scabichelin production. *Escherichia coli* strains were grown in LB medium with appropriate antibiotics added.

Preparation and manipulation of DNA

E. coli DNA mini-preparations, *E. coli* transformations and *E. coli*/*S. scabies* conjugation were performed according to published procedures.^{22–24} Plasmids and PCR/restriction digestion products were purified from agarose gels using a Qiaquick gel extraction kit (Qiagen). The Expand high-fidelity DNA polymerase kit (Roche) was used to amplify the *oriT-apr* cassette from pIJ773. PCR reactions used to verify the replacement of the 3 kb internal fragment of the *scab85471* gene in cosmid S57 and *S. scabies* 87.22 chromosomal DNA were carried out using Taq polymerase (Fermentas). *S. scabies* 87.22 and W1000 chromosomal DNA was isolated according to a published protocol.²⁵

Disruption of *scab85471* in *S. scabies* 87.22

A 3 kb internal region of *scab85471* was replaced with the *oriT-apr* cassette from pIJ773 in cosmid S57 (a clone from an *S. scabies* supercos1 genomic library containing *scab85251* to *scab85521* kindly provided by Prof. R. Loria of Cornell University) using PCR targeting.²⁴ The sequences of the primers used (replacement_fw and replacement_rv) are given in the ESI Table S1.† The mutagenized cosmid (S57::*oriT-apr*) was

introduced into *S. scabies* 87.22 by conjugation from *E. coli* ET12567 containing pUZ8002, selecting for apramycin resistance. Transconjugants were screened for kanamycin sensitivity and apramycin resistance, resulting from double homologous recombination of the engineered cosmid with the chromosome to replace the 3 kb internal region of *scab85471* with the *oriT-apr* cassette. The integrity of one such mutant, named *S. scabies* W1000, was confirmed by PCR with primers designed to anneal up- and downstream of the region of *scab85471* that was mutagenized, and sequencing of the PCR product. The sequences of the PCR primers used (test_fw and test_rv) are given in the ESI Table S1.†

HPLC and LC-MS analysis of ferric-hydroxamate production

Culture supernatants from *S. antibioticus* NBRC 13838, *S. noursei* NBRC 15452, *S. virginiae* NBRC 12827, *S. aureofaciens* NBRC12843, *S. albus* NBRC 13014, *S. scabies* JCM 7914 and *S. turgidiscabies* JCM10429 were centrifuged at 3000 rpm (700 g) for 10 min and 5 mL of each clarified supernatant was transferred to a new tube. 0.05 mL of 1 M FeCl₃ was added to each tube to convert hydroxamate-containing metabolites to the corresponding ferric complexes. After centrifugation at 10 000 rpm (4500 g) for 5 min, 50 µL of the solution was analyzed by HPLC, monitoring absorbance at 435 nm, on a C18 column (4.6 × 250 mm, particle size 5 µm, Cosmosil C18-MSII, Nacalai Tesque) eluted with solvent A (MilliQ water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) using the following elution conditions: 0 min – 100% solvent A; 10 min – 100% solvent A; 60 min – 50% solvent A, 50% solvent B. The retention times of scabichelin and turgichelin were 8.2 and 11.0 min, respectively.

1 mL supernatants from separate 50 mL cultures of *S. scabies* 87.22 and W1000 grown in iron-deficient medium were analysed by ultra-high resolution LC-MS on a Sigma Ascentis Express column (C18, 150 × 2.1 mm, 2.7 µm) attached to a Dionex 3000RS UHPLC coupled to a Bruker MaXis ESI-Q-TOF mass spectrometer. The mobile phases were water containing 0.1% formic acid (eluent A) and methanol containing 0.1% formic acid (eluent B). A gradient of 5% B to 100% B in 15 minutes was employed using a flow rate of 0.2 mL min⁻¹. Absorbance at 210 nm was monitored.

The mass spectrometer was operated in positive ion mode with a scan range 50–2000 *m/z*. Source conditions were: end plate offset at –500 V; capillary at –4500 V; nebulizer gas (N₂) at 1.6 bar; dry gas (N₂) at 8 L min⁻¹; dry temperature at 180 °C. Ion transfer conditions were: ion funnel RF at 200 Vpp; multiple RF at 200 Vpp; quadrupole low mass at 55 *m/z*; collision energy at 5.0 eV; collision RF at 600 Vpp; ion cooler RF at 50–350 Vpp; transfer time at 121 µs; pre-Pulse storage time at 1 µs. For MS/MS experiments, an isolation window of ±4 Da was used and the collision energy was optimised to achieve maximum fragmentation. Calibration was done with sodium formate (10 mM) through a loop injection of 20 µL at the beginning of each run.

Isolation of ferric-hydroxamate complexes

S. antibioticus and *S. turgidiscabies* were separately cultured in a shaking incubator at 180 rpm and 30 °C for 4 days in 10 polypropylene flasks each containing 100 mL of iron deficient culture medium. The spent medium (1 L total volume in each case) was harvested by filtration through Whatman No. 1 filter paper. After addition of 1 M FeCl₃ (50 µL), the culture supernatants were concentrated to a volume of 20 mL using a rotary evaporator, centrifuged at 3000 rpm (700 g) for 10 min and filtered (Millipore, 0.45 µm pore size) to remove insoluble material. The ferric-hydroxamate complexes were purified by semi-preparative HPLC, monitoring absorbance at 435 nm, on a C18 column (10 × 250 mm, particle size 5 µm, Capcell Pak C18 UG80, Shiseido) eluted with an isocratic mobile phase of 2% MeCN–98% water containing 0.05% TFA, yielding 3.2 mg of ferri-scabichelin and 2.5 mg of ferri-turgichelin.

Conversion of Fe-hydroxamates to Ga-hydroxamates

The ferric-hydroxamate complex (ferri-scabichelin or ferri-turgichelin) was dissolved in 3 mL of water. The solution was mixed with 3 mL of 1 M 8-quinolinol and stirred at room temperature for 30 min. The resulting solution was extracted with 4 × 6 mL of CH₂Cl₂ and then lyophilized. The residual solid was dissolved in 2 mL of water and purified by semi-preparative HPLC on a C18 column eluting with 3% MeCN–97% water containing 0.05% TFA and monitoring absorbance at 215 nm. The peaks corresponding to the desferri-hydroxamates (retention times: turgichelin – 9.79 min; scabichelin – 11.79 min) were collected and evaporated to dryness. The residues were redissolved in 2 mL of distilled water and 10 mg of GaCl₃ was added to each solution. The resulting gallium-hydroxamate complexes were purified by HPLC using the method employed for purification of the desferri-hydroxamates.

Stereochemical elucidation of scabichelin and turgichelin

Each peptide (0.5 mg) was subjected to acid hydrolysis at 100 °C for 24 h in concentrated HI (0.5 mL), and the hydrolysates were dried over a stream of N₂ gas. After re-suspending the residues in H₂O (200 µL), 10 µL of a solution of *N*_α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-DFVA, 10 mg mL⁻¹) in acetone was added to each hydrolysate. 100 µL of 1 M NaHCO₃ was added and the resulting mixtures were heated to 80 °C for 3 min. The reaction mixtures were cooled, neutralized with 2 N HCl (50 µL), and diluted with MeCN (200 µL). About 10 µL of each solution of FDVA derivatives was subjected to HPLC analysis, monitoring absorbance at 360 nm, on a C18 column (Cosmosil MSII, 4.6 × 50 mm) eluted at a flow rate of 1 mL min⁻¹ with solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) according to the following method: 0 min – 100% solvent A; 60 min – 40% solvent A, 60% solvent B. The retention times (min) of the L-FDVA-derivatized amino acids were L-Ser (40.32 min), D-Ser (41.08 min), D-Orn (33.84 min), and L-Orn (36.36 min).

Lowest energy conformers for all ten diastereomers of Ga-scabichelin and Ga-turgichelin were calculated as described previously for Ga-coelichelin.²⁶ The inter-residue α -H/NH distances for *N*-Me-fhOrn1/Ser2, *N*-Me-hOrn3/Orn4 and Orn4/hOrn5, as well as the α -H/ δ -H inter-residue distance for Ser2/hOrn3 and the α -H/ γ -H intra-residue distance for hOrn5 in the lowest energy conformers for each of the ten possible diastereomers of Ga-scabichelin and Ga-turgichelin were compared to the corresponding distances calculated from the ROESY data. In each case, good agreement between theory and experiment was observed for only one of the ten diastereomers.

Molecular modelling

The 3D structures were modelled using our in-house extension of the Molecular Operating Environment software Dommi-MOE, using essentially the same force field as in our previous study.^{26,27} The stochastic search algorithm applies random changes to all torsion angles and random displacements of the Cartesian coordinates to generate highly distorted starting geometries for subsequent optimisation. All structures were optimised to an rms gradient threshold of 0.01 which ensures energy convergence to at least 0.001 kcal mol⁻¹.

Results

Identification and isolation of tris-hydroxamate iron chelators produced by *Streptomyces* species

Seven strains of *Streptomyces* were cultured in an iron-deficient medium, a process that is known to induce the excretion of ferric iron-chelating metabolites in many bacteria. After cultivation, FeCl₃ was added to the cell-free spent medium to generate the ferric complexes of the excreted chelators. A small portion of each of the resulting solutions was filtered and subjected to HPLC analysis monitoring absorbance at 435 nm to detect ferric-tris-hydroxamate complexes. Ferrioxamines, ferric-tris-hydroxamate complexes known to be produced by several *Streptomyces* species and shown in *S. coelicolor* and *Streptomyces pilosus* to function in ferric iron uptake,^{21,28,29} were detected in the culture supernatants of all seven strains. Additional ferric-hydroxamates were detected in the culture supernatants of three of the strains (*S. antibioticus* NBRC 13838, *S. scabies* JCM 7914, and *S. turgidiscabies* JCM 10429). The additional metabolites produced by *S. antibioticus* and *S. scabies* had the same retention time, indicating that they have identical structures, whereas the additional metabolite produced by *S. turgidiscabies* had a different retention time from the metabolites produced by *S. antibioticus*/*S. scabies*. The *S. antibioticus*/*S. scabies* metabolite was named scabichelin and the *S. turgidiscabies* metabolite was named turgichelin. Large-scale cultures of *S. antibioticus* and *S. turgidiscabies* were grown in the iron deficient medium and the ferric complexes of scabichelin/turgichelin were isolated from the ferric chloride treated culture supernatants by semi-preparative HPLC to yield 3.2 mg and 2.5 mg, respectively.

Planar structure elucidation of scabichelin and turgichelin

Iron was removed from ferric-scabichelin by treatment with 8-quinolinol in water, followed by organic extraction and HPLC purification of the lyophilized aqueous phase. FT-ICR ESI-MS analysis of scabichelin showed it has the molecular formula C₂₆H₄₉N₉O₁₀ (*m/z* calculated for C₂₆H₅₀N₉O₁₀⁺: 648.3675; found: 648.3671). Because this molecular formula does not correspond to that of any known tris-hydroxamate siderophores we concluded that scabichelin is novel.

Scabichelin was converted to its gallium(III) complex prior to NMR analysis, to aid signal dispersion and facilitate stereochemical analysis, as reported previously for coelichelin.²⁶ The ¹H NMR spectrum of Ga-scabichelin showed several amide protons between 8.5 and 9 ppm and α -protons around 4–5 ppm. Interpretation of TOCSY (bold lines in Fig. 2) and DQF-COSY spectra allowed the proton spin systems for 5 amino acids, including a proteinogenic amino acid (Ser) and four non-proteinogenic amino acids [Orn, *N*2-methyl-*N*5-formyl-*N*5-hydroxyornithine (*N*-Me-fhOrn), *N*2-methyl-*N*5-hydroxyornithine (*N*-Me-hOrn), and cyclic *N*5-hydroxyornithine (cyclo-hOrn)] to be assigned, as shown in the ESI Table S2.† The formyl group attached to N-5 of the *N*-Me-fhOrn residue was indicated by a ROESY correlation between the δ -protons of Orn and the proton of the formyl group and an HMBC correlation between the δ -protons of Orn and the carbonyl carbon (δ_c = 152 ppm) of the formyl group. The cyclic nature of the cyclo-hOrn residue was revealed by HMBC correlations between the δ -protons and the carbonyl carbon (δ_c = 159 ppm).

Fig. 2 Key correlations observed in 2-D NMR spectra of scabichelin and turgichelin.

The sequence of residues within the peptide was deduced from the HMBC and ROESY spectra as shown in Fig. 2.

ESI-MS/MS analysis of scabichelin yielded a daughter ion with $m/z = 518$ ($[M + H - 130]^+$), and this ion fragmented to produce ions with $m/z = 346$ ($[M + H - 130 - 172]^+$) and $m/z = 259$ ($[M + H - 130 - 172 - 87]^+$). The neutral losses of 130, 172, and 87 corresponded to the masses of the cyclo-hOrn, *N*-Me-fhOrn, and serine, respectively, in agreement with the results of the NMR analyses.

The molecular formula $C_{24}H_{44}N_8O_{11}$ determined for turgichelin by FT-ICR ESI-MS analysis (m/z calculated for $C_{24}H_{45}N_8O_{11}^+$: 621.3202 found: 621.3199 for $[M + H]^+$ indicated that, like scabichelin, it is a novel metabolite. Analogous NMR methods to those employed for Ga-scabichelin were used to elucidate the structure of the gallium complex of turgichelin (Fig. 2 and ESI Table S3†). Daughter ions with $m/z = 449$ ($[M + H - 172]^+$), 362 ($[M + H - 172 - 87]^+$), 235 ($[M + H - 172 - 87 - 127]^+$), and 218 ($[M + H - 172 - 87 - 144]^+$) were observed in ESI-MS/MS analyses of turgichelin. The observed neutral losses of 172, 87, 127, and 144 correspond to those expected for *N*-Me-fhOrn, serine, cyclo-hOrn, and *N*-Me-hOrn, respectively, further corroborating the results of the NMR spectroscopic analyses. Turgichelin has a very similar planar structure to scabichelin. The only difference is the substitution of the ornithine residue in scabichelin with a serine residue in turgichelin.

Stereochemical analysis

The stereochemistry of the residues in scabichelin and turgichelin were determined as *L*-*N*-Me-fhOrn-*L*-Ser-*L*-*N*-Me-hOrn-*L*-Orn-*D*-cyclo-hOrn and *L*-*N*-Me-fhOrn-*L*-Ser-*L*-*N*-Me-hOrn-*L*-Ser-*D*-cyclo-hOrn, respectively, by a combination of chemical analyses and comparisons of internuclear distances derived from the NMR analyses with those derived from computer-generated molecular models of the gallium complexes.

For the chemical analyses, the modified Marfey's method³⁰ was employed. Aqueous HI was used for peptide hydrolysis, resulting in reduction of hOrn residues to Orn as described previously.³¹ The hydrolysate of each compound was derivatized with *N*_α-(2,4-dinitro-5-fluorophenyl)-*L*-valinamide (*L*-DFVA), and the derivatised hydrolysates were subjected to HPLC comparisons with *L*-DFVA derivatives of amino acid standards. The results of these analyses showed that scabichelin contains *L*-Ser, *L*-Orn and *D*-Orn in a 1:1:1 molar ratio, whereas turgichelin contains *L*-Ser and *D*-Orn in a 2:1 molar ratio.

The molecular structures of the gallium complexes of scabichelin and turgichelin were modeled theoretically using an extended molecular mechanics method with previously-developed parameters.³² Compared to our previous study on coelichelin,²⁶ where all possible stereoisomers were built and the chiralities fixed prior to separate stochastic searches, in this work we took a single starting structure for each of scabichelin and turgichelin and carried out a series of constraint-free stochastic searches. The results from an initial 22 000 step search followed up by eight additional 8000 step searches were

combined and ordered according to their potential energies. This led to a series of 'steps' where the first conformer of each step corresponds to the lowest energy structure for a given combination of stereocentres. The structural parameters derived from the 'best' conformers were then compared to those derived from the experimental NMR data. Only four of the five distance constraints could be fitted adequately. The discrepancy with the fifth constraint was traced to an unfavourable conformation of the six-membered ring. Apparently, the stochastic search algorithm was unable to locate the most favourable ring conformation for cyclic-hOrn, presumably because it is also coordinates to the metal centre. A better conformation was located some 4.2 kcal mol⁻¹ lower in energy. A comparison of the key inter-proton distances in each of the calculated models with the corresponding inter-proton distances derived from the ROESY data for Ga-scabichelin and Ga-turgichelin leads to reasonable agreement between experiment and theory (Fig. 3 and Table 1).

Fig. 3 Relative stereochemistry elucidation of scabichelin and turgichelin by molecular modelling and ROESY NMR experiments (note that the molecular models are antipodes of the natural products). (A) Calculated lowest-energy conformation of Ga-scabichelin. (B) Calculated lowest-energy conformation of Ga-turgichelin. (C) Key inter-proton distances (d_1 – d_5) for Ga-scabichelin and Ga-turgichelin estimated from ROESY spectra.

Table 1 Comparison of key inter-proton distances (in Å – see Fig. 3) derived from ROESY data (exp) and molecular modelling (calc)

Distance	Scabichelin		Turgichelin	
	Exp	Calc	Exp	Calc
d_1	2.23	2.28	2.25	2.19
d_2	1.92	2.27	1.80	2.39
d_3	2.15	2.36	2.16	2.47
d_4	2.21	2.59	2.04	2.59
d_5	2.58	2.33	2.47	2.34

Scabichelin is the metabolic product of a cryptic nonribosomal peptide biosynthetic gene cluster in *Streptomyces scabies* 87.22

Analysis of the recently-deposited *S. scabies* 87.22 genome sequence identified a cryptic nonribosomal peptide biosynthetic gene cluster spanning the protein coding sequences (CDSs) *scab85431–scab85521* (Fig. 4). All of the proteins encoded by these CDSs are homologous to proteins encoded

by genes within the coelichelin biosynthetic gene cluster of *S. coelicolor* M145 (Table 2), which directs the biosynthesis of a tris-hydroxamate siderophore.²⁶ It was therefore tempting to speculate that the *S. scabies* biosynthetic gene cluster may be responsible for the production of a hydroxamate-containing metabolite.

Sequence analysis of the protein encoded by *scab85471* showed that it is a nonribosomal peptide synthetase (NRPS) containing 17 enzymatic domains organized into 5 modules (Fig. 4). Assuming each domain is used only once in peptide chain assembly, the metabolic product of the NRPS would be a pentapeptide. Using our previously-developed model for NRPS adenylation (A) domain substrate selectivity,³³ we predicted that modules 1, 2 and 3 of the NRPS likely recognize an *L*-N5-acyl-N5-hydroxyornithine, *L*-Ser and *L*-N5-hydroxyornithine, respectively. We were unable to make unambiguous predictions for the likely substrates of the A domains within modules 4 and 5 of the NRPS. Modules 1 and 3 of the NRPS contain *N*-methyltransferase (MT) domains, suggesting that residues 1 and 3 within the pentapeptide product are

Fig. 4 The scabichelin biosynthetic gene cluster and proposed pathway for scabichelin biosynthesis. (A) Organization of the scabichelin biosynthetic gene cluster in *S. scabies* 87.22. The proposed functions of the proteins encoded by the gene cluster are summarised in Table 2. The sequences of the putative intergenic iron-dependent repressor binding sites are shown in the boxes below the vertical arrows. (B) Proposed role of the enzymes encoded by *scab85521* and *scab85511* in the biosynthesis of the non-proteinogenic amino acids *L*-N5-hydroxyornithine (L-hOrn) and *L*-N5-formyl-N5-hydroxyornithine (L-fhOrn). (C) Module and domain organization of the NRPS encoded by *scab85471* and proposed mechanism for scabichelin assembly. The species attached to the thiolation domains (black circles) are the proposed intermediates after the adenylation (A), epimerization (E) and methyltransferase (MT) domains in each module have performed their functions.

Table 2 Putative functions of proteins encoded by genes within the scabichelin biosynthetic gene cluster

Protein	Putative function	Homolog; origin	Similarity/identity (%)
<i>Scab85431</i>	ABC transporter permease-ATPase component	CchI; <i>S. coelicolor</i>	53/38
<i>Scab85441</i>	ABC transporter permease-ATPase component	CchG; <i>S. coelicolor</i>	58/46
<i>Scab85451</i>	Ferric-siderophore lipoprotein receptor	CchF; <i>S. coelicolor</i>	53/38
<i>Scab85461</i>	MbtH-like protein	CchK; <i>S. coelicolor</i>	72/57
<i>Scab85471</i>	Nonribosomal peptide synthetase	CchH; <i>S. coelicolor</i>	60/48
<i>Scab85481</i>	ABC transporter permease component	CchC; <i>S. coelicolor</i>	65/47
<i>Scab85491</i>	ABC transporter permease component	CchD; <i>S. coelicolor</i>	60/45
<i>Scab85501</i>	ABC transporter ATPase component	CchE; <i>S. coelicolor</i>	74/60
<i>Scab85511</i>	Formyl-tetrahydrofolate-dependent formyl transferase	CchA; <i>S. coelicolor</i>	85/76
<i>Scab85521</i>	Flavin-dependent monooxygenase	CchB; <i>S. coelicolor</i>	73/60

N-methylated. Moreover, module 5 of the NRPS, contains an epimerization (E) domain, suggesting that the absolute stereochemistry of the product is L-L-L-L-D. Taken together, these observations led us to speculate that scabichelin, or a metabolite of similar structure, is the likely product of the NRPS. This hypothesis is corroborated by the fact that *scab85511* and *scab85521* encode homologs of *S. coelicolor* CchA and CchB, respectively. CchA and CchB have been shown to be responsible for the biosynthesis of hOrn and fhOrn, the non-proteinogenic amino acid residues incorporated into coelichelein.^{34,35}

To investigate the role of the cryptic nonribosomal peptide biosynthetic gene cluster in scabichelin biosynthesis, we used PCR targeting-based methodology³⁶ to construct a mutant of *S. scabies* 87.22 in which an internal fragment of the *scab85471* gene is replaced with an *oriT-apr* cassette. This mutant was named *S. scabies* W1000. Comparative LC-MS/MS analyses of supernatants from cultures of the 87.22 and W1000 strains grown in an iron-deficient medium, to which ferric iron was added prior to analysis, identified a ferric complex that is produced by *S. scabies* 87.22, but not the W1000 mutant, with identical retention time and MS/MS fragmentation pattern to scabichelin isolated from *S. antibioticus* (Fig. 5). These data confirm that the NRPS encoded by

scab85471 is responsible for the assembly of scabichelin in *S. scabies* 87.22.

Discussion

The ability of scabichelin to complex iron, coupled with the fact that the genes for scabichelin biosynthesis in *S. scabies* 87.22 are clustered with genes encoding a putative ABC transporter (*scab85431/scab85441*: permease-ATPase; *scab85451*: ferric-siderophore lipoprotein receptor) likely involved in ferric-scabichelin uptake (Fig. 4, Table 2), suggests that scabichelin functions as a siderophore in *S. scabies*. This hypothesis is reinforced by the presence of conserved palindromic sequences, with a high degree of similarity to binding sites for iron-dependent repressor proteins that control the expression of siderophore biosynthetic genes in *S. coelicolor*,⁴⁰ in the intergenic regions between *scab85421* and *scab85431*, *scab85471* and *scab85481*, and *scab85511* and *scab85521* (Fig. 4). It is tempting to speculate that turgichelin also functions as a siderophore in *S. turgidiscabies*. A role for scabichelin in iron uptake is of significant interest because these metabolites may be *S. scabies* virulence determinants, as is the case for several siderophores

Fig. 5 LC-MS/MS analyses of scabichelin production by the *S. scabies* 87.22 and W1000 strains. (A) Extracted ion chromatograms for $m/z = 351.1400$ (corresponding to the $[M + 2H]^{2+}$ ion for the ferric-scabichelin complex) from analyses of Fe-scabichelin purified from *S. antibioticus* (top panel), ferrated culture supernatant of *S. scabies* 87.22 (middle panel), and ferrated culture supernatant of *S. scabies* W1000 (bottom panel). (B) MS/MS spectra of Fe-scabichelin purified from *S. antibioticus* (top panel) and in ferrated supernatants from iron-deficient cultures of *S. scabies* 87.22 (bottom panel).

produced by other pathogenic bacteria.³⁷ The results described herein provide a basis for future experiments aimed at further elucidating the biological function of scabichelin and investigating its role in *Streptomyces* plant pathogenicity. However, such experiments will be complicated by the fact that *S. scabies* produces pyochelin¹⁷ and desferrioxamines (L. Song, J. Bicz and G. L. Challis, unpublished), both of which are known to function as siderophores in other bacteria.

The identification of scabichelin as the metabolic product of the *scab85431–scab85521* locus in *S. scabies* 87.22 allows a plausible pathway for scabichelin biosynthesis to be proposed. N5 hydroxylation of L-Orn catalyzed by the flavin-dependent monooxygenase encoded by *scab85521* gives L-N5-hydroxyornithine (L-hOrn), which undergoes N5-formylation catalyzed by the formyl-tetrahydrofolate-dependent formyl transferase encoded by *scab85511* yielding L-N5-formyl-N5-hydroxyornithine (L-fhOrn) (Fig. 4). Analogous reactions for the production of L-hOrn and L-fhOrn are involved in the biosynthesis of coelichelin and rhodochelin in *S. coelicolor* and *Rhodococcus jostii*, respectively.^{34,35} The A domains of the pentamodular NRPS encoded by *scab85471* catalyse ATP-dependent loading of the requisite amino acids onto the adjacent thiolation (T) domains within each module, and the aminoacyl thioesters attached to the T domains within modules 1 and 3 undergo N-methylation, catalysed by the MT domains (Fig. 4). The L-hOrn residue attached to the T-domain of module 5 undergoes α -carbon epimerization catalysed by the E domain within the same module and the condensation (C) domains of modules 2, 3, 4 and 5 then catalyse four successive N-acylation reactions to yield a L, L, L, L, D-pentapeptidyl thioester attached to the T domain of module 5 (Fig. 4). Finally, scabichelin is released from the NRPS via intramolecular addition of the δ -hydroxylamino group of the D-hOrn residue to the carbonyl group of the thioester, followed by deprotonation and collapse of the resulting tetrahedral intermediate (Fig. 4). A similar mechanism for peptide chain release has recently been suggested in the biosynthesis of amychelin, a putative siderophore produced by *Amycolatopsis* sp. AA4, which like scabichelin and turgichelin contains a C-terminal D-cyclo-hOrn residue.³⁸ This reaction was proposed to be catalysed by Acmb, a putative standalone α,β -hydrolase, because the final module of the amychelin NRPS lacks the C-terminal thioesterase (TE) domain usually required for chain release in nonribosomal peptide biosynthesis. However, a C-terminal TE domain is also missing from the last module of the scabichelin NRPS and none of the other genes within the scabichelin biosynthetic gene cluster encode a protein with sequence similarity to an α,β -hydrolase. It is possible that an α,β -hydrolase encoded by a gene elsewhere on the *S. scabies* chromosome catalyses peptide chain release. On the other hand, it is tempting to speculate that the E domain in module 5 of the scabichelin NRPS catalyzes both the epimerization of the L-hOrn thioester attached to module 5 and the subsequent release of the fully-assembled pentapeptide chain, since bi-functional epimerization-condensation domains are known in other NRPS assembly lines.³⁹ Indeed, an analogous chain release mechanism may also be involved in amychelin biosynthesis.

Experiments with purified recombinant proteins and chemically-synthesized substrate analogues will likely be required to distinguish between these different mechanistic possibilities.

Conclusions

Streptomyces scabies and closely related species cause the potato disease common scab all over the world. Siderophore-mediated iron uptake has been reported to be critical for survival of several pathogenic bacteria in their host cells. Thus, it is anticipated that siderophores also play an important role in the virulence of pathogenic streptomycetes. We isolated and elucidated the structures of two new tris-hydroxamate iron chelators, scabichelin and turgichelin, from the potato pathogens *S. scabies* and *S. turgidiscabies*, respectively. These metabolites are structurally similar to the tsukubachelins, tris-hydroxamate iron chelators that were recently isolated from *Streptomyces* sp. TM-34 and TM-74.^{41,42} Scabichelin was shown to be the metabolic product of a cryptic nonribosomal peptide biosynthetic gene cluster identified within the *S. scabies* 87.22 genome. Sequence analysis of this gene cluster indicated that scabichelin functions as a siderophore and prompted us to hypothesize that an unusual bi-functional epimerization-condensation domain catalyses release of scabichelin from the NRPS responsible for its biosynthesis, resulting in the formation of its cyclic D-hydroxyornithine residue. The identification of the scabichelin biosynthetic gene cluster will facilitate future investigations of the role played by this metabolite in *S. scabies* plant pathogenicity.

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