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Chapter 4

Metabolomics-driven discovery of a prenylated isatin antibiotic produced by *Streptomyces* species MBT28

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ABSTRACT

Actinomycetes are a major source of antimicrobials, anti-cancer compounds and other medically natural products, and their genomes harbor extensive biosynthetic potential. Major challenges in the screening of these microorganisms are to activate the expression of cryptic biosynthetic gene clusters and the development of technologies for efficient dereplication of known molecules. Here we report the identification of a previously unidentified isatin-type antibiotic produced by *Streptomyces* sp. MBT28, following a strategy based on NMR-based metabolomics combined with the introduction of streptomycin resistance in the producer strain. NMR-guided isolation by tracking the target proton signal resulted in the characterization of 7-prenylisatin (**1**) with antimicrobial activity against *Bacillus subtilis*. The metabolite-guided genome mining of *Streptomyces* sp. MBT28 combined with proteomics identified a gene cluster with an indole prenyltransferase that catalyzes the conversion of tryptophan into 7-prenylisatin. This study underlines the applicability of NMR-based metabolomics in facilitating the discovery of novel antibiotics.

1. INTRODUCTION

The discovery and development of antibiotics to fight bacterial diseases is one of the greatest triumphs of modern medicine. However, the increase of antimicrobial resistance means that bacterial infections which once seemed conquered, now form a major threat to human health.¹ The high frequency of re-discovery of known compounds by high-throughput (HT) screening regimes necessitates new approaches to discover the antimicrobial lead compounds.²⁻⁵ As producers of approximately two thirds of all known antibiotics and many other medically relevant natural products, actinomycetes are a major source of clinical drugs.^{6,7} It is probable that the antimicrobial compounds so far been identified represent only a fraction of the total products.⁸ In support of this, sequencing the genomes of actinomycetes revealed the potential of even the best-studied model organisms as producers of natural products had been grossly underestimated.⁹⁻¹¹ The biosynthetic gene clusters are under extensive genetic control.^{12,13} Many of these gene clusters are poorly expressed under routine laboratory conditions and are therefore generally referred as cryptic or silent gene clusters (reviewed in¹⁴⁻¹⁷).

A drug-discovery pipeline that is rapidly gaining momentum combines genome sequence information with different culturing conditions to allow fluctuation of the production of (cryptic) antibiotics, followed by the metabolic profiling-based identification of the bioactivity of interest. A major challenge thereby lies in finding the appropriate chemical triggers or ecological cues to elicit the production of cryptic antibiotics (recently reviewed in^{18,19}). Strategies that have been employed include heterologous expression,²⁰ the use of chemical elicitors,^{21,22} and inducing antibiotic resistance.^{23,24} Selection of streptomycin- or rifampicin-resistant mutants, typically caused by mutations in *rpsL* (for ribosomal protein S12) or *rpoB* (for the β -subunit of RNA polymerase), respectively, enhances the production of antibiotics^{23,25,26} and may also lead to the production of novel antibiotics.²⁷

Once differential expression of a bioactivity is achieved in a producing organism, *e.g.*, by varying culturing conditions or by making mutants, the next challenge is to rapidly establish whether the natural product is sufficiently novel to warrant full structure elucidation. Robust chemical analysis is required to monitor the differences in metabolic complexity and antibiotic production when using different strains or culturing conditions. Metabolomics approaches globally identify the low molecular weight metabolites in biological samples. Chemical profiling techniques using LC-MS or NMR followed by multivariate data analysis allows scientists to compare and detect molecules that are differentially synthesized between diverse biological samples, thereby effectively narrowing down the search for the sought-after biomarker and avoiding chemical redundancy in an early stage.^{28,29}

In this study, we describe the activation of antimicrobial activity in *Streptomyces* sp. MBT28, by selecting streptomycin-resistant mutants. Multivariate data analysis correlated the bioactivity to the corresponding NMR signals, facilitating the characterization of a previously undescribed antibiotic, 7-prenylisatin (**1**). The likely genes encoding the key biosynthetic enzymes for the synthesis of 7-prenylisatin were uncovered using natural product proteomining,³⁰ a proteomics-based genome mining approach.

2. RESULTS AND DISCUSSION

2.1. Induced antibiotic production by selection for streptomycin resistance

Previous screening of over 800 actinomycetes from our in-house actinomycete collection

obtained from remote mountain soils, showed that a subset of 96 isolates was of particular relevance for drug discovery as they produced antibiotics against many ESKAPE pathogens under specific growth conditions.²² One of these isolates is *Streptomyces* sp. MBT28, which has a broad spectrum activity against multidrug-resistant (MDR) bacterial pathogens, including *Enterococcus faecium*, *Klebsiella pneumonia* and *Acinetobacter baumannii*. The strain produced the known antibiotic borrelidin as shown by bioactivity-guided separation,²² but other bioactive molecules remained uncharacterized due to their low abundance.

To enhance the expression of antimicrobials in *Streptomyces* sp. MBT28, we made use of the well-established fact that the introduction of streptomycin resistance induces antibiotic production by actinomycetes.³¹ *Streptomyces* sp. MBT28 spores were plated onto SFM agar containing 10 µg/ml streptomycin, which resulted in the formation of streptomycin resistant (Str^R) mutants after around nine days of growth. A surprisingly high number of these spontaneous Str^R mutants showed altered colony size and/or pigmentation, with many white colonies appearing. White colonies also arose in the absence of streptomycin, but at a much lower frequency (Figure S1). As a control, we also selected Str^R colonies of the phylogenetically related *Streptomyces lividans* 1326. The resulting *S. lividans* Str^R mutants did not show the same high frequency of white mutants (Figure S1), suggesting that MBT28 is genetically unstable.

Around 200 randomly chosen Str^R mutants were transferred to MM agar plates, allowed to grow for six days at 30 °C and then overlaid with *Bacillus subtilis* to assess their antimicrobial activity. 14% of the Str^R mutants had enhanced antimicrobial activity as compared to the parental strain, while more than half of the mutants showed reduced bioactivity as compared to the parental strain. Furthermore, no clear correlation between the phenotype (*i.e.* pigmentation) and antibiotic production was observed.

One white mutant derivative (MBT28-91) with particularly strong bioactivity was chosen for further investigation, and a second mutant (MBT28-30) with less enhanced bioactivity was selected for comparison. On SFM agar plates, *Streptomyces* sp. MBT28 and Str^R mutant MBT28-30 produced grey-pigmented spores after two days of incubation, while MBT28-91 remained white even after nine days of incubation at 30 °C (Figure 1A). After prolonged incubation (two weeks), MBT28-91 eventually turned light grey. To compare the antimicrobial activity of the strains, antibiotic production was examined at 24 h intervals during growth on MM agar plates (Figure 1B). MBT28-91 produced a much larger zone of inhibition against *B. subtilis* than the parent, indicative of enhanced antibiotic production, while MBT28-30 exhibited slightly higher antibiotic activity than the parent.

The *rpsL* gene, which encodes ribosomal protein S12, is the major hotspot for streptomycin-resistant mutations and therefore also referred to as the *str* gene.³² An alternative site for Str^R mutations, particularly associated with lower streptomycin resistance, is the *rsmG* gene for an rRNA methyltransferase.³³ In an attempt to elucidate the nature of the Str^R mutations, the *rpsL* and *rsmG* genes of the two mutants were amplified by PCR and the DNA products were sequenced. Neither of the mutants had sustained a mutation in *rpsL*. However, the *rsmG* gene of MBT28-30 carried a G to A mutation at nt position 92, which resulted in a Gly→Asp mutation at aa position 31 of RsmG. Surprisingly, Mutant MBT28-91 carried wild-type copies of both *rpsL* and *rsmG*, and the origin of the enhanced Str^R is therefore as yet unclear.

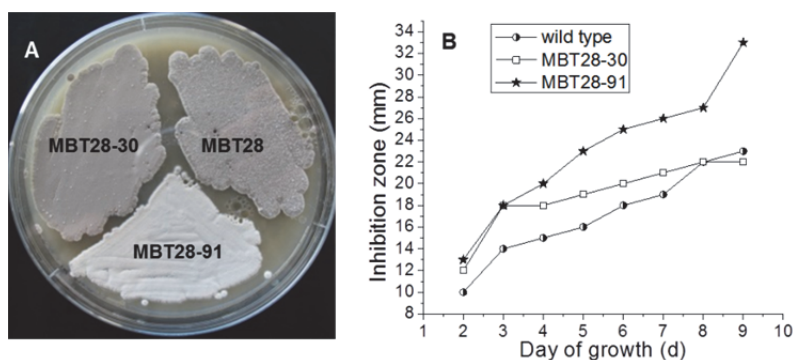


Figure 1. Antibiotic activity of *Streptomyces* sp. MBT28 and its streptomycin-resistant mutants. Morphologies of colonies of *Streptomyces* sp. MBT28 and its Str^R mutants MBT28-30 and MBT28-91 grown on SFM agar for six days at 30 °C (A), and time course of inhibition activity against *Bacillus subtilis* (B). For the activity test, the three strains were grown on MM agar supplemented with 0.5% mannitol and 1% glycerol, and samples taken at 24 h intervals (*X* axis) were overlaid with *B. subtilis* to give inhibition zone (*Y* axis). Note the strong increase in bioactivity of MBT-28-91 as compared to the parental strain MBT28 or to mutant MBT28-30.

2.2. Metabolic profiling and multivariate data analysis

¹H nuclear magnetic resonance (¹H NMR) based metabolic profiling was conducted to analyze and compare the metabolomes of wild-type *Streptomyces* sp. MBT28 and its Str^R-mutant derivatives, so as to identify the compound(s) responsible for the elevated bioactivity. NMR spectroscopy, and in particular ¹H NMR which is commonly used for natural product profiling, is well suited for such comparative metabolomics as it is fast, reproducible, and benefits from relatively straightforward sample preparation.³⁴ Multivariate data analysis (MVDA) was used to reduce the dimensionality of the multivariate dataset and to discriminate the samples. For this, the strains were grown on MM agar plates supplemented with 0.5% (w/v) mannitol and 1% (w/v) glycerol as carbon sources. For metabolomics experiments, ten biological replicates were analyzed for each sample.

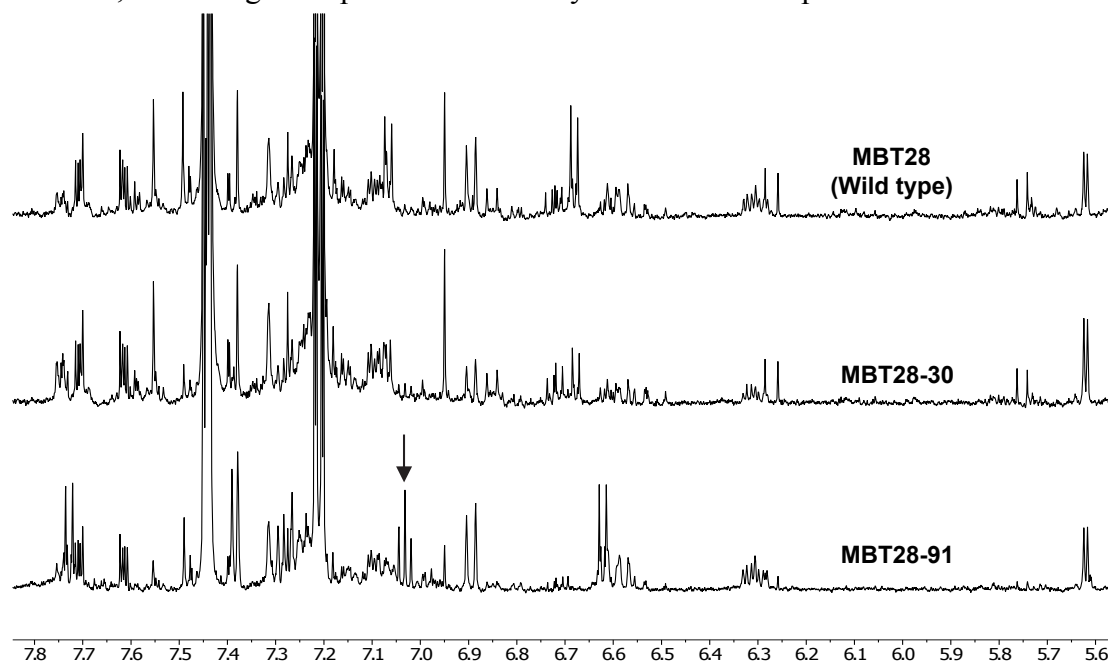


Figure 2. Comparison of ¹H NMR spectra. ¹H NMR (600 MHz) was done on samples from *Streptomyces* sp. MBT28 and its Str^R mutants MBT28-30 and MBT28-91. The solvent was CH₃OH-*d*₄, and peaks are shown for the region of δ 5.6–7.8 ppm. The signal δ 7.03 (t, *J* = 7.2 Hz) that was exclusively identified in mutant MBT28-91 is highlighted by an arrow.

While only small differences were found in the ^1H NMR spectra between the parental strain and its mutant MBT28-30, those of the antibiotic-overproducing mutant MBT28-91 displayed distinctly different NMR signals, particularly in the aromatic region (δ 5.7–7.8, Figure 2). The compounds were elucidated by 2D NMR including COSY, HSQC, and HMBC experiments (Table S1). The signals of the main aromatic compounds in the wild-type strain were attributed to caffeic acid, *p*-cresol, and syringic acid, which were not detected in MBT28-91. Conversely, ^1H NMR spectra of MBT28-91 specifically exhibited resonances for 4-hydroxybenzoic acid and an unknown compound with typical resonances at δ 7.03 (t, $J = 7.2$ Hz), which were absent in the other two strains. The known antimicrobial compound borrelidin (δ 6.60, 6.90, and 6.31), which was previously shown to be produced by MBT28,²² was detected in all samples, but its production level was 10% lower in MBT28-91 as compared to the parent or mutant MBT28-30. This strongly suggests that borrelidin was not the causative agent of the increased bioactivity.

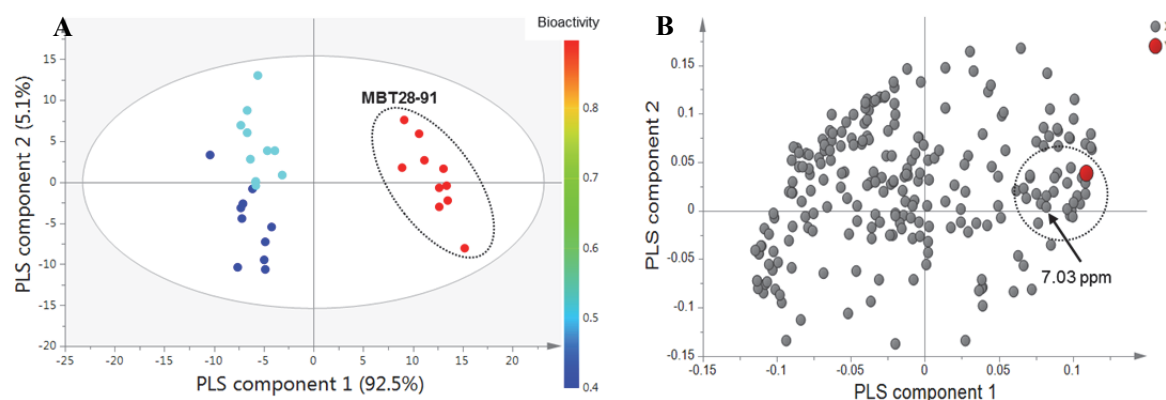


Figure 3. Multivariate data analysis. The PLS-DA score plot (A) reveals good separation between wild-type *Streptomyces* sp. MBT28 (blue) and its mutant derivatives MBT28-30 (light blue) and MBT28-91 (red) with low, medium, and high antibiotic activity against *B. subtilis*, respectively. The corresponding loading plot (B) presents the NMR signals (dashed circles) that make major contributions to the separation of the samples (and the bioactivity) of MBT28-91 from those of the other two strains. The arrow refers to the characteristic proton signal δ 7.03 (t, $J = 7.2$ Hz). X, primary variable of the chemical shift; Y, bioactivity.

Multivariate data analysis was conducted to correlate NMR signals with the bioactivity, which does not require extensive pre-fractionation and chromatographic separation of the crude extract.³⁵ An unsupervised principal component analysis (PCA) revealed that MBT28-91 fingerprints did not overlap with those of the two other strains, but it was rather difficult to correlate the significant signals to the improved activity in MBT28-91. Projection to latent structures (PLS) is a robust multivariate linear regression technique to predict a set of dependent variables from a set of independent variables or predictors.³⁶ To investigate the detailed correlation between bioactivity and metabolites, PLS was employed as a supervised data reduction algorithm in which bioactivity values - determined as the diameter of the inhibition zone in an agar diffusion assay - were used instead of dummy Y-variables. The score plot of the PLS analysis (Figure 3A) showed that not only samples from the highly bioactive MBT28-91 were well separated, but that separation was also found between all independent samples of MBT28-30 and those of the wild-type strain. Examining the corresponding loading plot again identified characteristic signals at δ 7.03 (t, $J = 7.2$ Hz) that correlated very well to the enhanced bioactivity in MBT28-91 (Figure 3B), but full structure

elucidation could not be done on crude extracts. The supervised model was validated by a permutation test that is a calculation of quality of the fit and the predictive ability. For antimicrobial activity, the R² and Q² values for PLS analysis were 0.98 and 0.95, respectively, indicating an excellent reproducibility and predictability.

2.3. Identification of 7-prenylisatin as the elicited bioactivity

To elucidate the structure of the bioactive component produced by mutant MBT28-91, NMR-guided fractionation³⁷ tracing the characteristic signal δ 7.03 (t, $J = 7.2$ Hz) was conducted. Extracts of MBT28-91 grown on MM agar plates were sequentially defatted using *n*-hexane, followed by silica gel, and Sephadex LH-20 chromatography. After each step, the collected fractions were analysed by ¹H NMR for target signal tracking, and relevant fractions were subjected to preparative TLC chromatography. A bright orange compound (0.35 mg, R_f = 0.45 on TLC, developed in CHCl₃) was readily obtained. Its ¹H NMR spectrum (600 MHz, CDCl₃) showed an aromatic ring ABC spin system, δ_H 7.39 (brd, $J = 7.8$ Hz, H-6), 7.06 (t, $J = 7.8$ Hz, H-5), 7.49 (brd, $J = 7.8$ Hz, H-4). A set of typical signals attributable to a prenyl group, including δ_H 3.31 (brd, $J = 7.2$ Hz, H-8), 5.24 (m, H-9), 1.80 (d, $J = 1.2$ Hz, H₃-11), and 1.78 (brs, H₃-12) were also observed (Table 1). The prenyl chain was located at the C-7 position of a benzene ring, which was established from the key HMBC correlation from δ_H 3.31 (d, $J = 7.2$ Hz, H-8) to a heteroatom-bearing carbon δ_C 147.9 (C-7a). The HMBC correlation from H-4 to the carboxyl carbon δ_C 183.1 (C-3) proved its conjugation to the benzene ring. The 1*H*-indole-2,3-dione backbone (isatin) was ultimately confirmed by the chemical formula C₁₃H₁₃NO₂ established from high-resolution mass [M + H]⁺ peak at m/z 216.1018 (calculated for C₁₃H₁₄NO₂ 216.1019). Thus, compound **1** was identified as 7-prenylisatin, not previously identified in nature (Figure 4).

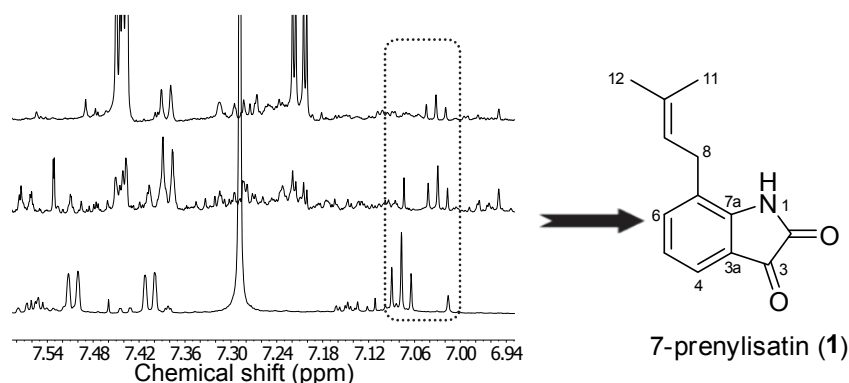


Figure 4. NMR-guided purification of 7-prenylisatin from *Streptomyces sp.* MBT28-91. Characteristic proton signals δ 7.03 (t, $J = 7.2$ Hz) that correlated to the high bioactivity are boxed (dashed box), which were used as tracking signals for chromatographic separation. Chemical shift divergence was due to the different deuterated solvents used (methanol-*d*₄ for top and middle rows, and CDCl₃ for bottom row).

Isatins exhibit antibacterial, antifungal, and cytotoxic properties.³⁸ However, only a few isatin-type compounds of microbial origin have been identified.³⁹⁻⁴¹ 5-prenylisatin from the fungus *Chaetomium globosum* was reported to have antifungal activity,³⁹ while 6-prenylisatin was the first example of an isatin derivative discovered in a streptomycete, namely *Streptomyces albus*, which showed antimicrobial activity against Gram-positive bacteria.⁴⁰ Purified 7-prenylisatin was tested for its efficacy against *B. subtilis* using a microtitre plate-based test for the minimal inhibitory concentration (MIC) (see Materials and Methods

section for details). This showed that 7-prenylisatin acts as an antibiotic with an MIC of around 25 $\mu\text{g/ml}$ against *B. subtilis*, which is similar to that of 6-prenylisatin. No antimicrobial activity was observed against the Gram-positive *Staphylococcus aureus* or the Gram-negative *Escherichia coli* K12.

Table 1. ^1H and ^{13}C NMR data assignments for 7-prenylisatin (1) ^a

NO.	δ_{C} , type	δ_{H} (J in Hz)	HMBC correlations	COSY correlations
2	159.2, C			
3	183.1, C			
3a	118.4, C			
4	123.7, CH	7.49, brd (7.8)	C-3, C-6, C-7a	H-5
5	124.0, CH	7.06, t (7.8)	C-7, C-3a	H-4, H-6
6	139.1, CH	7.39, brd (7.8)	C-8, C-7a, C-4	H-5
7	125.1, C			
7a	147.9, C			
8	29.7, CH ₂	3.31, brd (7.2)	C-7a, C-6, C-7, C-9, C-10	
9	120.0, CH	5.24, m	C-8, C-11, C-12	H-8, H-11, H-12
10	136.1, C			
11	25.9, CH ₃	1.80, d (1.2)	C-9, C-10, C-12	H-9, H-8
12	18.3, CH ₃	1.78, brs	C-9, C-10, C-11	H-9, H-8

^a **1** recorded in CDCl_3 . Proton coupling constants (J) in Hz are given in parentheses. ^1H NMR and ^{13}C APT NMR spectra were recorded at 600 MHz. All chemical shift assignments were done on the basis of 1D and 2D NMR techniques.

2.4. Identification of the biosynthetic gene cluster for 7-prenylisatin

A biosynthetic route for 7-prenylisatin was proposed based on the existing literature for related isatin compounds (Figure 5). The initial step of isatin biosynthesis is prenylation at the C-7 carbon of tryptophan, followed by cleavage of the amino acid chain to generate the intermediate 7-prenylindole.^{42, 43} This intermediate is then further oxidized at C-2 and/or C-3 into the isatin skeleton through indoxyl or oxindole.⁴⁴⁻⁴⁶

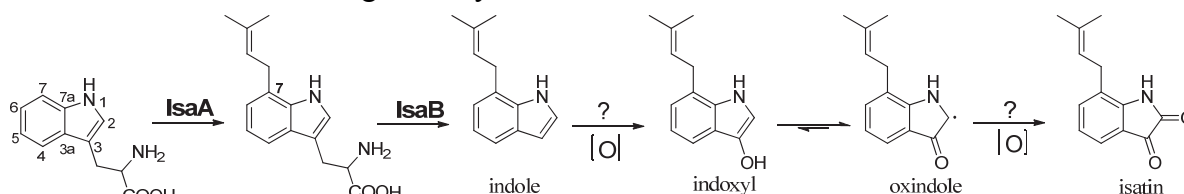


Figure 5. Proposed biosynthetic pathway of 7-prenylisatin. IsaA is the likely enzyme for prenylation at the C-7 carbon of tryptophan and IsaB for the subsequent cleavage of the amino acid chain. The intermediate 7-prenylindole is then further oxidized into 7-prenylisatin by a yet uncharacterized oxygenase.

To characterize the 7-prenylisatin biosynthetic pathway, the genome of *Streptomyces* sp. MBT28 was sequenced by Illumina paired end sequencing, and the resulting draft genome sequence was annotated as described.⁴⁷ The genes responsible for synthesis of the 1*H*-indole-2,3-dione scaffold (isatin) have not previously been identified in bacteria. However, the prenyltransferase (PTase) catalyzing the initial prenylation of the indole nucleus has been well characterized. For instance, enzymes that prenylate at all non-bridgehead atoms of the indole ring (N-1, C-2, C-3, C-4, C-5, C-6, and C-7) have been identified (Figure S2 and Table 2).^{48, 49} Therefore, the genome was scanned for genes encoding possible PTases, which might give clues as to which gene cluster is responsible for 7-prenylisatin biosynthesis. Genes for indole PTases have been found in actinomycetes in two different genomic contexts,⁴² Type A in combination with a tryptophanase, and Type B in combination with a flavin-dependent

monooxygenase (FMO). For instance, in *Streptomyces sp.* SN-593, IptA (Type A) installs a dimethylallyl group on the C-6 position of the tryptophan indole core, while in *Streptomyces coelicolor* A3(2), a Type B PTase prenylates tryptophan at C-5.⁴³ Interestingly, the *Streptomyces sp.* MBT28 genome contained both types of gene clusters (Tables S2 and S3). However, considering that a tryptophanase is required for cleavage of the side chain (Figure 5), the Type A cluster was the most likely candidate for the bioassembly of 7-prenylisatin.

Table 2. Examples of prenyltransferases (PTs) for prenylation of indoles. All possible positions in the indole core can be prenylated, depending on the enzyme. See also Figure S2.

Enzyme	Function	PT site	Enzyme origin	Homology	Accession number
IsaA	regular	C-7	<i>Streptomyces sp.</i> MBT28	100%	PRJNA279348
LtxC	reverse	C-7	<i>Lyngbya majuscula</i>	48%	NP_631515
EAL92290	regular	C-7	<i>Aspergillus fumigatus</i>	23%	ABS89001.1
IptA	regular	C-6	<i>Streptomyces sp.</i> SN-593	46%	BAJ07990.1
EAW08391	regular	C-5	<i>Aspergillus clavatus</i> NRRL 1	29%	EAW08391
SCO7467	regular	C-5	<i>Streptomyces coelicolor</i> A3(2)	48%	NP_631515
FgaPT2	regular	C-4	<i>Aspergillus fumigatus</i>	33%	AAX08549.1
MaPT	regular	C-4	<i>Malbranchea aurantiaca</i>	36%	ABZ80611.1
AnaPT	reverse	C-3	<i>Neosartorya fischeri</i>	23%	EAW16181.1
CdpNPT	reverse	C-3	<i>Aspergillus fumigatus</i>	45%	ABR14712.1
FgaPT1	reverse	C-2	<i>Aspergillus fumigatus</i>	22%	EAL94098
FtmPT1	regular	C-2	<i>Aspergillus fumigatus</i>	35%	AAX56314.1
FtmPT2	regular	N-1	<i>Aspergillus fumigatus</i>	35%	AAX56314.1
CymD	reverse	N-1	<i>Salinispora arenicola</i>	31%	ABW00334

To validate that 7-prenylisatin is synthesized by a Type A indole PTase, we made use of the fact that the expression of biosynthetic genes correlates closely with the bioactivity of interest, so that fluctuations in the proteome (as assessed by quantitative proteomics) correlate directly to the level of the bioactivity.³⁰ Protein extracts of MBT28 and its mutants MBT28-30 and MBT28-91 had been obtained from the same cultures as used for metabolomics, and stable isotope dimethyl labeling (see Materials and Methods section for details) and subsequent LC-MS/MS analysis were used for relative quantification of protein levels between the three strains. 1,382 proteins were positively identified and the changes in expression levels of 1,035 proteins could be quantified. Based on the intensity-dependent significance-*B* value,⁵⁰ 177 of the quantified proteins were significantly enhanced or reduced ($p < 0.05$) in at least one comparison (Table S4 and Figure S3). Among the proteins that were significantly enhanced in strain MBT28-91 in comparison to the wild-type strain, we found the two key enzymes of the Type A indole PTase gene cluster, namely the aromatic prenyltransferase (IsaA) and the tryptophanase (IsaB) (Table 3 and Table S2). The two enzymes were also upregulated in strain MBT28-30 compared to the wild-type strain, but much less than for strain MBT28-91. Thus, the expression level of Type A indole PTase gene cluster showed excellent correlation to the amount of 7-prenylisatin produced. In contrast, none of the proteins encoded by the Type B cluster were detected, which may therefore not be expressed under these conditions (Table S3).

Tryptophan is a known key precursor for the biosynthesis of the prenylated indole alkaloids and acts as the biogenetic origin of indole rings.^{51, 52} Indole alkaloids that contain prenylated tryptophan moieties at their core represent an important class of natural products for which considerable synthetic and biosynthetic interest exists. Gene clusters containing genes for indole prenyltransferases are widely distributed in actinomycetes. Although some

prenylated indole derivatives have been isolated from *Streptomyces* species,^{42, 43, 53, 54} only two studies on the biosynthesis of prenylated indoles have been reported.^{41, 42} IsaA is the third actinomycete prenyltransferase known to catalyze the addition of a dimethylallyl group to the C-7 of tryptophan. It is yet unclear which gene(s) are responsible for the oxidation of indole into isatin backbone, as no such genes were found within or in close proximity of the *isa* gene cluster. Three proteins with an oxidation-related PQQ domain were all found significantly up-regulated ($p < 0.05$) in the overproducing strain as compared to the parental strain (Table S4). Further analysis is required to establish whether one of these enzymes may execute the oxidation reaction to generate the final isatin moiety.

Table 3. Differential expression (in fold change mutant/wt) of the proteins of the Type A indole PTase gene cluster as determined by quantitative proteomics. See also Table S4. The sequences have been deposited as Genbank Bioproject Accession PRJNA279348.

Protein	MBT28-91/WT (² log)	Significance -B	MBT28-30/WT (² log)	Significance -B	Description*
Isa Regulator	-	-	-	-	Transcriptional regulator
IsaA	3.0	0.023	0.9	0.116	Aromatic prenyltransferase
IsaB	2.8	0.031	1.6	0.040	Tryptophanase

* based on blast homology searches

3. CONCLUSION

The increasingly high frequency of re-discovery of known antibiotics using routine screening methods poses the challenge of prioritizing the bioactive molecules of interest.^{2, 5, 55, 56} Starting from a ‘novel’ biosynthetic cluster identified by genome sequencing is tricky, because the compound may already have been seen. Our work followed a strategy based on (i) fluctuations in the production level of the compound of interest; (ii) metabolomics combined with multivariate data analysis to correlate bioactive molecules to the observed bioactivity, followed by NMR-guided purification; and (iii) correlating the changes in gene expression profiles to the bioactivity to identify candidate gene clusters. This efficiently identified 7-prenylisatin, an antibiotic with activity against the Gram-positive bacterium *B. subtilis*, and also partially elucidated the biosynthetic pathway and the responsible biosynthetic genes. Further experiments are required to elucidate the mode of action and the activity spectrum of 7-prenylisatin and related compounds of the (prenyl)isatin family. The integrated approach of metabolomics as an efficient way to elucidate the nature of any bioactivity of interest, even in complex crude extracts, combined with eliciting strategies for the activation of novel bioactive molecules, forms a promising pipeline in the search for novel antibiotics and other medically relevant natural products.

4. EXPERIMENTAL SECTION

4.1. General Experimental Procedures

FT-IR spectra were recorded on a Shimadzu FT-IR 83000 spectrometer. UV measurements were performed using a Shimadzu UV mini-1240. NMR spectra were recorded in MeOD on a

Bruker 600 MHz calibrated to a residual $\text{CH}_3\text{OH-}d_4$ (3.30 ppm). The UHPLC-TOF-MS analyses were performed on an Ultimate 3000 UHPLC system (ThermoScientific, Pittsburgh, PA, USA) coupled to a micro-ToF-2Q mass spectrometer from Bruker Daltonics (Bremen, Germany) with an electrospray (ESI) interface. Silica gel (pore size 60 Å, 70–230 mesh) for open column chromatography was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sephadex LH-20 (GE Healthcare Life Sciences, Eindhoven, The Netherlands) was used for size-exclusion chromatography. Silica gel 60 F254, 1 mm (Merck, Darmstadt, Germany) was used for preparative TLC separation. All organic solvents and chemicals were of analytical or HPLC grade, depending on the experiment.

4.2. Strains and culturing conditions

Streptomyces sp. MBT28 (Biosample Accession 3444561) was obtained from the Leiden University strain collection.²² Spores were stored in 20% glycerol and maintained in -20°C . Soy flower agar medium (SFM⁵⁷) was used to generate streptomycin-resistant (Str^{R}) mutants and for preparing spore suspensions. Minimal medium (MM) agar plates⁵⁷ supplemented with 0.5% (w/v) mannitol and 1% (w/v) glycerol as carbon sources were used for activity tests and to prepare samples for metabolomics and proteomics experiments. *Bacillus subtilis* 168 was grown in Luria-Bertani broth (LB) at 37°C . Imaging was done using a Zeiss standard 25 phase-contrast microscope and a Zeiss Lumar V12 stereo microscope as described.⁵⁸

4.3. Selection of streptomycin-resistant mutants and antibiotic activity assays

Str^{R} mutants were selected by plating 10 μl of spores (1×10^7 cfu/ml) onto SFM agar plates containing streptomycin (10 $\mu\text{g/ml}$) at 30°C . Colonies typically developed after nine days. For bioactivity screening, colonies were grown for six days on MM agar plates and overlaid with LB agar containing 100 μl of exponentially *B. subtilis* cells (OD_{600} of 0.4). The activity was initially assessed as the zone of growth inhibition after overnight incubation at 37°C . MIC values against *B. subtilis* were carried out as described.²² All MIC determinations were performed in triplicate. Ampicillin was used as positive control, and the solvent chloroform as the negative control.

4.4. PCR amplification

PCRs were performed in a minicycler (MJ Research, Watertown, MA, USA), using Phusion polymerase (Stratagene, La Jolla, CA) as described.⁵⁹ *rpsL* was amplified from genomic DNA using oligonucleotides *rpsL*_For (5'-CGGCACACAGAAACCGGAGAAG) and *rpsL*_Rev (5'-GTCGATGATGACCGGGCGCTTCG), and *rsmG* was amplified using oligonucleotides *rsmG*_For (5'-TGACGAATTCGTCAGCCTGATAGTTCTGGTGG) and *rsmG*_Rev (5'-TCAGTAAGCTTGCCGTGCAGCAGTGAGCGACG).

4.5. Metabolomics sampling and NMR-guided separation

Streptomyces were grown on MM agar plates supplemented with 0.5% mannitol and 1% glycerol. The agar with mycelia was cut into small pieces and extracted with ethyl acetate by soaking in the solvent overnight at room temperature. Samples were dried under reduced pressure at 40°C and redissolved in methanol. The solvent was then evaporated at room temperature under nitrogen gas flow, and subsequently dipped into liquid nitrogen and

lyophilized using a freeze dryer (Edwards Ltd., Crawley, UK). Ten replicates were performed for each sample.

For enrichment of isatin compounds, crude extracts were partitioned between methanol and *n*-hexane to remove the lipids. The resolved methanol fraction was subsequently separated by silica gel (pore size 60 Å, 70–230 mesh) column chromatography employing step-wise gradient elution by *n*-hexane, *n*-hexane-CHCl₃ (1:1), CHCl₃, CHCl₃-acetone (1:1), and acetone. All fractions were subsequently dissolved in CH₃OH-*d*₄ and subjected to NMR measurement. The *n*-hexane-CHCl₃ (1:1) elution fraction containing the target proton signals was firstly separated by Sephadex LH-20 (GE Healthcare Life Sciences, Eindhoven, The Netherlands) eluting with methanol, and further purified by preparative TLC (Merck, Darmstadt, Germany), migrated with solvent system CHCl₃-acetone (20:1) and detected under UV light at 254 nm wavelength.

7-prenylisatin (I): orange, amorphous powder, UV (MeOH) λ_{\max} (log ϵ) 244 (4.26), 314 (3.32) nm; IR ν_{\max} 2922, 1732, 1716, 1558, 1456 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRMS (positive mode) *m/z* 216.1018 [M + H]⁺ (calculated for C₁₃H₁₄NO₂ 216.1019), 238.0816 [M + Na]⁺ (calculated for C₁₃H₁₃NO₂Na 238.0844).

4.6. NMR measurement and multivariate data analysis

NMR sample preparation and NMR measurement was essentially done as described previously.^{60,61} 500 μ l of CH₃OH-*d*₄ was added to the freeze-dried sample. Subsequently, the mixture was vortexed for 10 s and sonicated for 20 min at the frequency of 42 kHz using an Ultrasonicator 5510E-MT (Branson, Danbury, CT, USA), followed by centrifugation at 13,000 *g* at room temperature for 5 min. The supernatant (300 μ l) was transferred to a 3 mm micro NMR tube and analyzed. ¹H NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. Deuterated methanol was used as the internal lock. Each ¹H NMR spectrum consisted of 128 scans using the following parameters: 0.16 Hz/point, pulse width (PW) = 30 (11.3 s) and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to methanol at 3.30 ppm, using XWIN NMR (version 3.5, Bruker). The ¹H NMR data files were processed as described.⁶² The AMIX software (Bruker Biospin GmbH) was used to convert the ¹H NMR spectra to an ASCII file, with total intensity scaling and calibration to methanol signal (δ 3.30 ppm). Bucketing or binning was performed and the spectral data were reduced to included regions of equal width (0.04 ppm) equivalent to the region of δ 0.30–10.02. The regions of δ 4.85 – 4.95 and δ 3.25 – 3.35 were removed in the analysis because of the remaining signal of residual solvents of HDO and CD₃OD, respectively. Multivariate data analysis was performed with the SIMCA-P+ software (Version 13.0, Umetrics, Umeå, Sweden).

4.7. Genome sequencing, assembly, and annotation

Genome sequencing and annotation was done essentially as described.⁴⁷ Illumina/Solexa sequencing on Genome Analyzer Iix and sequencing on PacBio RS were outsourced at BaseClear BV (Leiden, Netherlands). 100-nt paired-end reads were obtained and the quality of the short reads verified using FastQC

(<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). Depending on quality, reads were trimmed to various lengths at both ends. Processed raw reads were subsequently used as input for the Velvet assembly algorithm. The resulting contigs were analyzed using the GeneMark.hmm algorithm with the *Streptomyces coelicolor* genome as the reference for identification and annotation of coding sequences (CDSs).⁶³ The genome has been deposited under Bioproject Accession PRJNA279348.

4.8. Quantitative proteomics analysis

To prepare samples for proteomics, strains were grown on MM agar plates overlaid with 0.1 µm polycarbonate filter papers prior to inoculation. After six days of incubation at 30 °C, mycelia were scraped from the filters. Protein extraction, in-solution digestion, C₁₈ column dimethyl labeling, strong cation-exchange chromatography (SCX) fractionation, and LC-MS/MS analysis were performed as previously described.^{30, 64} Generally, mycelia were washed and then sonicated for disruption of the cell wall. After removal of cell debris, protein concentration was measured using a Bradford assay and 0.167 mg of protein was precipitated for each sample. Proteins were dissolved using RapiGest SF Surfactant (Waters) and treated with iodoacetamide before trypsin digestion. The primary amines of the peptides were dimethyl labeled using three combinations of isotopomers of formaldehyde and cyanoborohydride on Ultra-Clean SPE C₁₈ columns (Altech, 200 mg, Thermo Fisher Scientific, USA) as described, via CH₂O + NaBH₃CN, CD₂O + NaBH₃CN, and ¹³CD₂O + NaBD₃CN.⁶⁵ Light, medium, and heavy-labelled samples with 4 Da mass differences were mixed to obtain 0.5 mg of peptides for fractionation by SCX. 24 fractions were collected and subjected to LC-MS/MS analysis on an LTQ-orbitrap MS (ThermoScientific, Pittsburgh, Pennsylvania, USA).⁶⁴ Data analysis was performed using MaxQuant 1.4.1.2.⁵⁰ Dimethyl labeling was selected as the quantification method, carbamidomethylation of cysteine was selected as fixed modification, and oxidation of methionine was set as variable modification.⁶⁶ MS/MS spectra were searched against a database of translated coding sequences. Peptide and protein identification FDR were set to 0.01, and a minimum of two quantification events was specified to obtain a protein quantification. To determine the significance of the found expression ratios, intensity-dependent B-significance values⁵⁰ were calculated.

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