Biosynthesis of ilamycins featuring unusual building blocks and engineered production of enhanced anti-tuberculosis agents

Junying Ma\(^1\), Hongbo Huang\(^1\), Yunchang Xie\(^1\), Zhiyong Liu\(^2\), Jin Zhao\(^3\), Chunyan Zhang\(^1,4\), Yanxi Jia\(^1,4\), Yun Zhang\(^1\), Hua Zhang\(^3\), Tianyu Zhang\(^2\) & Jianhua Ju\(^1,4\)

Tuberculosis remains one of the world’s deadliest communicable diseases, novel anti-tuberculosis agents are urgently needed due to severe drug resistance and the co-epidemic of tuberculosis/human immunodeficiency virus. Here, we show the isolation of six antimycobacterial ilamycin congeners (1-6) bearing rare L-3-nitro-tyrosine and L-2-amino-4-hexenoic acid structural units from the deep sea-derived Streptomyces atratus SCSIO ZH16. The biosynthesis of the rare l-3-nitrotyrosine and l-2-amino-4-hexenoic acid units as well as three pre-tailoring and two post-tailoring steps are probed in the ilamycin biosynthetic machinery through a series of gene inactivation, precursor chemical complementation, isotope-labeled precursor feeding experiments, as well as structural elucidation of three intermediates (6-8) from the respective mutants. Most impressively, ilamycins E\(_1\)/E\(_2\), which are produced in high titers by a genetically engineered mutant strain, show very potent anti-tuberculosis activity with an minimum inhibitory concentration value \(\approx 9.8 \text{ nM}\) to Mycobacterium tuberculosis H37Rv constituting extremely potent and exciting anti-tuberculosis drug leads.
uberculosis (TB) ranks as the top infectious killer in the world, with the number of TB deaths exceeding those from human immunodeficiency virus (HIV) \(^3\). It is estimated that two billion people—one-third of the world’s population—have latent TB, and about 9.6 million people fall ill; 1.5 million people die from TB annually.\(^3\, \, \, 4\). Moreover, the prevention and control of TB have become more difficult because of the co-epidemic of TB/HIV as well as the emergence and rapid dissemination of multidrug-resistant, extensively drug-resistant, and totally drug-resistant strains.\(^3\, \, \, 5\, \, \, 6\). Hence, novel anti-TB agents with increased potency and efficacy are urgently needed.

During the course of our efforts to discover and engineer anti-infective and anti-tumor agents from marine-derived actinomycetes,\(^6\, \, \, 8\) six compounds with the ultraviolet (UV) spectrum of 220, 285, and 352 nm were obtained from a deep South China Sea-derived strain *Streptomyces atratus* SCSIO ZH16. The high-resolution mass spectrometry (HRMS) profiles and nuclear magnetic resonance (NMR) spectroscopic data characterized them to be ilamycins bearing two rare units of \(\alpha\)-3-nitrotyrosine and \(\gamma\)-2-amino-4-hexenoic acid (\(\gamma\)-AHA), which was originally isolated as single, or as mixtures, of components from several *Streptomyces* in the early 1960s–1970s,\(^9\, \, \, 11\) and then re-isolated as rufomycins in another *S. macrosporeus* DSM-12818 in 2000.\(^12\, \, \, 14\) The structures, including most of the stereochemistry, of five ilamycins B\(_1\), B\(_2\), C\(_1\), C\(_2\), and D\(_2\) were characterized by HRMS, 1D and 2D NMR spectroscopic data analyses, and chemical transformations.\(^12\, \, \, 14\) Notably, the biological activities of ilamycins B\(_1\), B\(_2\), C\(_1\), C\(_2\), and D\(_2\) were not reported; only ilamycin C\(_1\) was noted to display inhibitory activity against *Mycobacterium tuberculosis* H37Rv (minimum inhibitory concentration (MIC) < 1.3 \(\mu\)g mL\(^{-1}\)).\(^13\) However, in all previous publications, the absolute configurations of the epoxy groups in ilamycins B\(_2\), C\(_1\), C\(_2\), and D\(_2\) as well as the \(\gamma\)-C (C\(_{32}\)) of the 2-amino-4-methylpentanedioic acid unit in ilamycin D\(_2\) were still unsolved.

Recently, the repurposing of old drugs and the re-evaluation of natural product leads have become new approaches to identifying anti-TB drug candidates.\(^15\, \, \, 17\) Given the intriguing anti-mycobacterial activities that had been reported for the ilamycins, the still unresolved questions about absolute stereochemistries, and the lack of systematic studies and precise knowledge about the biological activities of each of the purified compounds, a thorough and in-depth investigation of the chemistry and biology of the full set of ilamycins was clearly warranted. Furthermore, elucidation of the biosynthetic pathway to these interesting agents was deemed necessary; the ultimate goal envisioned entails ilamycin engineering to generated analogs with improved anti-mycobacterial potencies.

In this paper, we isolate six ilamycins (B\(_1\), B\(_2\), C\(_1\), C\(_2\), D\(_1\), and E\(_1\); 1–6) (Fig. 1) from a marine-derived *S. atratus* SCSIO ZH16 strain (Fig. 2, trace i) and determine the absolute stereochemistries of the epoxide groups (C\(_{13}\)) and the \(\gamma\)-C (C\(_{32}\)) of the 2-amino-4-methylpentanedioic acid residue by X-ray diffraction analysis of ilamycins B\(_3\), C\(_2\), and D. We also identify and analyze the ilamycin biosynthetic gene cluster in *S. atratus* SCSIO ZH16. In addition, the biosynthetic routes to two rare natural product building blocks, \(\alpha\)-3-nitrotyrosine and \(\gamma\)-AHA, are determined via gene inactivation, iso-tolabeled precursor feeding, and chemical complementation experiments. Moreover, we elucidate the pre-tailoring and post-tailoring steps in ilamycin biosynthesis and obtain three ilamycins analogs (E\(_1\), E\(_3\), and F; 6–8, Fig. 1). Finally, we evaluate eight ilamycins (1–8) for anti-mycobacterial activities against *M. smegmatis* MC\(_{54\, 1}\) and *M. tuberculosis* H37Rv and cytotoxic activities against a panel of human tumor and normal cell lines. These last studies revealed two engineered ilamyccins as anti-TB drug leads with potencies in the nanomolar range.

## Results

### Discovery and structural elucidation of ilamycins

The strain SCSIO ZH16 was isolated from a deep South China Sea sediment sample and identified as *Streptomyces atratus* by morphology and 16S DNA sequence analyses. Subsequent large-scale fermentation (16L), extraction, and careful isolation by silica gel column chromatography (CC) followed by preparative high performance liquid chromatography (HPLC) afforded analytically pure compounds 1–6. The structures of compounds 1–5 were identified and designated as ilamycins B\(_1\), B\(_2\), C\(_1\), C\(_2\), and D\(_2\), respectively, by HRMS (Supplementary Figs. 35–37) and 1D and 2D (correlation spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) NMR data (Supplementary Figs. 42, 43, 45–54) analyses and by comparison with previously reported data for the ilamycins (or rufomycins).\(^19\, \, \, 14\)

In this study, after careful incubation in suitable solvents, qualified single crystals of ilamycin B\(_3\) in MeOH, ilamycin C\(_2\) in MeOH-CHCl\(_3\), (9:1), and ilamycin D in MeOH-ETOH (1:1) were obtained for X-ray diffraction. We subsequently collected X-ray diffraction data for ilamycins B\(_3\), C\(_2\), and D (Supplementary Table 1; Supplementary Data 1–3, 5). Analysis of the X-ray diffraction data confirmed the previous structures that had been deduced by spectroscopic analysis and chemical derivatization (Supplementary Figs. 1–3)\(^9\, \, \, 14\). In addition, the crystal of ilamycin C\(_2\) (4) contains two CHCl\(_3\) molecules inserted into the ilamycin C\(_2\) molecule in a unit cell, enabling the stereochemistries within the six-membered ring at C\(_{32}\) and C\(_{33}\) both be established as S (absolute structure parameter = -0.012(3)). The single-crystal X-ray data of ilamycin C\(_2\) also revealed that the conformation of the hemiaminal-containing six-membered ring adopts a twist boat conformation vertical to the macro lactam ring. Analysis of the X-ray diffraction data for ilamycin B\(_2\) and ilamycin D (5), together with the \(\gamma\)-configurations established for all amino acid

---

**Fig. 1** Structures of ilamycins. Compounds 1–6 were isolated from *S. atratus* SCSIO ZH16 wild-type strain, compounds 6–8 were isolated from engineered mutant strains

---
judged by HPLC – mutant fed with AHA; (xiii) suggesting that the epoxy group of ilamycin C 1 was replaced by a corresponding to the epoxy group of ilamycin C 1 (iv) Comparison of the 1H and 13C NMR spectroscopic data 

residues in these heptapeptides 10, determined the S configurations for C13 and C32 in 2 and 5. Minor product 6 was purified as a yellow powder. The molecular formula of 6 was established by high resolution electrospray ionization massspectroscopy (HRESIMS) to be C54H75N9O11, 16 mass units less than that of ilamycin C 1. Comparison of the 1H and 13C NMR spectroscopic data (Supplementary Table 2; Supplementary Figs. 55, 56) of 6 with that of ilamycin C 1 revealed the absence of the signals corresponding to the epoxy group of ilamycin C 1 (δC 59.7, δH 3.26, CH2-13; δC 46.0, δH 2.88, 2.83, CH2-14), and the appearance of a new set of signals consistent with a terminal olefin at δC 145.5, δH 6.15 (CH-13) and δC 114.1, δH 5.22, 5.19 (CH2-14), suggesting that the epoxy group of ilamycin C 1 was replaced by a terminal olefin in 6. Further 2D NMR (Supplementary Figs. 57–59) analysis confirmed the presence of an isopentenyl group in 6, which is also present in ilamycin B1 (1). Comparison of the 1H and 13C NMR data of 6, especially the chemical shifts at C33 and C34, also suggest that 6 has the same stereochemistries as ilamycin C 1 (3);12–14 compound 6 was subsequently designated ilamycin E1.

Identification of the ilamycins gene cluster. The presence of non-proteingenic amino acids in the ilamycin cyclopeptide backbone supported our hypothesis that ilamycin biosynthesis is governed by nonribosomal peptide synthetases (NRPSs). Accordingly, we sought to identify the ila gene cluster by whole-genome sequencing of S. atratus SCSIO ZH16 using a combination of second-generation 454 and third-generation PacBio sequencing technology. The size of the entire linear genome of S. atratus SCSIO ZH16 is 9.64 Mbp. Upon data annotation and

---

**Fig. 2** HPLC analyses of fermentation broths. (i) wild-type S. atratus SCSIO ZH16; (ii) ΔilaS mutant; (iii) ΔilaO mutant; (iv) ΔilaG mutant; (v) ΔilaE mutant; (vi) ΔilaD mutant n; (vii) ΔilaF mutant; (viii) ΔilaM mutant fed with 2,4-HDA; (ix) ΔilaM mutant fed with AHA; (x) ΔilaE mutant fed with AHA; (xi) ΔilaD mutant fed with 4-HA; (xii) ΔilaE mutant fed with 4-HA; (xiii) ΔilaD mutant fed with 3-NO2-tyr; (xiv) ΔilaM mutant fed with 2,4-HDA; (xv) ΔilaM mutant; (xvi) ΔilaN mutant; (xvii) ΔilaD mutant fed with 3-NO2-tyr; (xviii) ΔilaN mutant fed with 3-NO2-tyr; (xx) ΔilaR mutant; the peaks labeled with asterisks are not ilamycin analogs judged by HPLC-DAD-UV analysis.
Fig. 3 Biosynthetic gene cluster and proposed biosynthetic pathway of ilamycins. **a** Organization of the ilamycin gene cluster. **b** Biosynthetic pathway of ilamycins. **c** The prenylation of Trp. **d** The nitration of Tyr. **e** The biosynthesis of l-AHA unit. **f** The post-tailoring biosynthetic steps en route to ilamycins. A adenylation, C condensation, T thiolation, MT methylation, TE thioesterase, KS keto synthases, ACP acyl carrier protein, AT acyl transferase, AT-L acyl transferase-like protein, DH dehydratase, KR keto reductase, ER enoyl reductase.
analysis with antiSMASH\textsuperscript{18}, a \(ca\) 57.1 kb DNA segment consisting of 20 open reading frames was identified to likely be involved in the biosynthesis of ilamycins (Supplementary Table 4). The nucleotide sequences have been deposited in GenBank with accession number KY173348, and the gene cluster is shown in Fig. 3a. A cosmid library of \textit{S. atratus} SC510 ZH16 was constructed using the SuperCos1 vector system and 12 positive clones were screened for gene inactivations.

Consistent with the cyclic peptide scaffold of the ilamycins, we found a giant 8022 aa protein, \textit{IlaS}, comprising seven modules that incorporate building blocks to form the full-length heptapeptide (Fig. 3b). Polyketide synthase (PKS)/NRPS analyses employing online software revealed that the predicted substrate amino acids of the A1–A7 binding pocket domain of \textit{IlaS} do not match those found in the ilamycins. To confirm that the analyzed gene cluster is responsible for ilamycin biosynthesis, we constructed a \(\Delta i\text{laS}\) mutant by using \(\lambda\)-Red recombination with an apramycin gene cassette\textsuperscript{19–21}. The mutant was identified and confirmed by its kanamycin-sensitive and apramycin-resistant phenotype and further validated by polymerase chain reaction (PCR). The \(\Delta i\text{laS}\) mutant completely lost the ability to produce ilamycins but the HPLC profile showed accumulation of compound 9 (Fig. 2, trace ii). Compound 9 was isolated upon large-scale fermentation and purification; MS and \(^{1}H\) and \(^{13}C\) NMR (Supplementary Figs. 70, 71) data analyses of 9 (Supplementary Table 2) allowed us to determine the compound to be N-(1, 1-dimethyl-1-allyl)-tryptophan (Fig. 3c). This result demonstrated the necessity of \textit{IlaS} in the construction of the ilamycin core, and also suggested that the Trp is first N-precatalyzed before being loaded onto the ilamycin biosynthetic assembly line. The \textit{ila} cluster indeed contains an aromatic prenyltransferase, \textit{IlaO}, that displays high identity to CymD in cyclomarin biosynthesis\textsuperscript{22}. We inactivated \textit{ilaO} and found that the \(\Delta i\text{laO}\) mutant completely lost the ability to generate both the ilamycins and fragment 9 (Fig. 2, trace iii). Consequently, \textit{IlaO} appears to catalyze the reverse prenylation of Trp during a pre-tailoring step during ilamycin biosynthesis (Fig. 3c). The \textit{ilaG} gene encoding a MbtH-like protein was also inactivated, the \(\Delta i\text{laG}\) mutant almost completely lost the ability to produce ilamycins (Fig. 2, trace iv), suggesting that \textit{IlaG} is necessary for ilamycin biosynthesis and might be involved in the biosynthesis of the unique \(\alpha\)-AHA structural unit.

### Biosynthesis of the \(\alpha\)-AHA unit

The ilamycins contain a \(\alpha\)-AHA building block whose biosynthetic origin is unclear; the \(\alpha\)-AHA structural element is unique and has not been observed in any other natural products. In our examination of the \textit{ila} gene cluster, we observed a 4835 aa PKS, \textit{IlaE}, consisting of three modules comprising 13 domains (Fig. 3e). In contrast to canonical type I PKSs, \textit{IlaE} possesses three features. An AT-like (AT\textsubscript{1}L) domain and a canonical AT (AT\textsubscript{2}) domain are assignable to \textit{IlaE}, but the conversed active site residues S and R in the GXXXG...R motif are mutated (Supplementary Fig. 31) and the malonyl-CoA specificity motif (HAFH) are present only in the later canonical AT (AT\textsubscript{2}) domain\textsuperscript{28}, suggesting that the later canonical AT\textsubscript{2} domain maybe used iteratively to synthesize the C6 chain. Two keto synthases (KSS), KS\textsubscript{L} and KS\textsubscript{S}, along with a small portion of the linker regions before and after KS\textsubscript{L} (ca 1500 bp in size) has completely identical DNA sequences. The giant PKS lacks a TE domain for releasing the polyketide chain. To investigate the exact role of \textit{IlaE} in ilamycin biosynthesis, a \(\Delta i\text{laE}\) mutant was constructed. The production of ilamycins was completely abolished in the \(\Delta i\text{laE}\) mutant (Fig. 2, trace v), suggesting that \textit{IlaE} is necessary for ilamycin biosynthesis and might be involved in the biosynthesis of the unique \(\alpha\)-AHA structural unit.

To confirm that the unique \(\alpha\)-AHA unit found in the skeleton of ilamycins is indeed biosynthesized by \textit{IlaE}, and to explore the biosynthetic origin of this unit, feeding experiments using \([1\text{-}\textsuperscript{13}C]\), \([2\text{-}\textsuperscript{13}C]_{2}\), and \([1, 2\text{-}\textsuperscript{13}C]_{2}\)-labeled sodium acetate were conducted with \textit{S. atratus} SC510 ZH16, and the representative product, ilamycin B\(_{2}\), was purified. Inspection of the \(^{13}C\) NMR spectra (Supplementary Fig. 44) of the \(^{13}C\)-labeled ilamycin B\(_{2}\) revealed that: (i) feeding with \([1\text{-}\textsuperscript{13}C]\) acetate led to C\(_{17}\), C\(_{19}\), and C\(_{21}\) enrichment in ilamycin B\(_{2}\); (ii) feeding with \([2\text{-}\textsuperscript{13}C]\) acetate led to C\(_{19}\), C\(_{20}\), and C\(_{22}\) enrichment in ilamycin B\(_{2}\); and (iii) feeding with \([1, 2\text{-}\textsuperscript{13}C]\) acetate led to enrichment of C\(_{17}\)–C\(_{22}\) in ilamycin B\(_{2}\), and each of the C\(_{17}/\text{C}_{18}\), C\(_{19}/\text{C}_{20}\), and C\(_{21}/\text{C}_{22}\) pairs appeared as coupled doublets (\(J_{\text{CC}} = 25.2\) Hz). These data convincingly demonstrate that acetate is the direct precursor of this unique C6 structural element, and that the \(\alpha\)-AHA unit is assembled by \textit{IlaE}, a type II PKS with clearly unusual features. \textit{IlaE} does not contain a TE domain for the release of the C6 polyketide chain; transformation of the C6 polyketide chain to the \(\alpha\)-AHA unit requires further amination at the \(\alpha\)-position. Nearby \textit{IlaE}, we found four genes that might be involved in these \(\alpha\)-amination tailoring steps: \textit{ilaC} encoding a hydroxase, \textit{ilaD} encoding a cytochrome P450 monooxygenase, \textit{ilaF} encoding a type II thioesterase, and \textit{ilaH} encoding an aminotransferase. To probe if these four genes are involved in the \(\alpha\)-AHA biosynthesis, we individually inactivated each one to yield four mutants: \(\Delta i\text{laC}\), \(\Delta i\text{laD}\), \(\Delta i\text{laF}\), and \(\Delta i\text{laH}\). HPLC analysis of the fermentation extracts showed that the \(\Delta i\text{laD}\) strain failed to produce ilamycins (Fig. 2, trace vi); the \(\Delta i\text{laF}\) strain produced significantly lower titers of ilamycins (Fig. 2, trace vii); and the titers of ilamycins from the \(\Delta i\text{laC}\) (Fig. 2, trace viii) and \(\Delta i\text{laH}\) (Fig. 2, trace ix) mutants were only one-half and two-thirds as great as the titers from wild-type producer, respectively. To further test if \textit{IlaD} indeed takes part in the biosynthesis of the \(\alpha\)-AHA unit and to determine the biosynthetic timing of \(\alpha\)-AHA formation, as well as to exclude the polar effect of \textit{IlaE} knockout on the expression of the down stream genes, synthesized \(\alpha\)-D(A)-AHA and 4-hexenio acid (4- HA) were fed to the \(\Delta i\text{laD}\) mutant. Additionally, synthesized \(\alpha\)-D(A)-AHA, 2,4-hexenoic acid (2,4-HDA), and 4-HA were supplied to the \(\Delta i\text{laE}\) mutant strain. For each feeding experiment, synthetic precursors were added (individually) to a final reaction concentration of 0.5 mM in precursor. The fed mutant strains were all cultivated for 7 days alongside the wild-type strain as a control. HPLC analyses of the fermentation extracts revealed that ilamycins production was restored in both the \(\Delta i\text{laD}\) and \(\Delta i\text{laE}\) mutants when supplied with \(\alpha\)-D(A)-HA (Fig. 2, traces x and xii). However, neither mutant strain produced ilamycins when supplied with 4-HA to the \(\Delta i\text{laD}\) and \(\Delta i\text{laE}\), as well as feeding 2,4-HDA to the \(\Delta i\text{laE}\) mutant also failed to restore ilamycin production (Fig. 2, traces xi, xiii, xiv).

Based on the above feeding results and combined with the gene knockout results using \textit{ilaDEH}, we propose that \textit{IlaD} performs an \(\alpha\)-oxidation to form \(\alpha\)-keto intermediate 10, and that the sole aminotransferase within the cluster, \textit{IlaH}, is responsible for transamination of 10 to form the \(\alpha\)-AHA unit. The oxidation of the C6 polyketide chain by \textit{IlaD} and subsequent transamination at the \(\alpha\)-position likely occurs while the substrate is tethered to the ACP\textsubscript{3} of \textit{IlaE} (Fig. 3e). However, the chemical complementation experiment with AHA demonstrates that the \(\alpha\)-AHA unit is released from the PKS before being loaded onto the NRPS assembly line to form ilamycins. Two enzymes likely able to hydrolyze and release the \(\alpha\)-AHA unit from \textit{IlaE} are the hydrolase \textit{IlaC} and the type II TE \textit{IlaF}. Both of these kinds of enzymes have...
been shown to have similar functions in other antibiotic biosyntheses. Although, the ΔilaC and ΔilaH mutants still produced ilamycins, other similar enzymes within the SC510 ZH16 genome may complement their functions.

**Biosynthesis of the 1-3-nitrotyrosine unit**. Nitro-containing natural products possess diverse structures and remarkable biological activities. However, the biosynthesis of nitro groups in natural products is poorly elucidated, and only a limited number of the enzymes involved in the formation of aromatic nitro groups have been characterized. The mechanism for tyrosine nitration remains an unsolved mystery. Nevertheless, a growing number of studies reveal that the presence of 3′-nitro-tyrosine and 3′-nitro-tyrosine-modified proteins are closely related to or serve as a biomarker for many diseases, including atherosclerosis, Parkinson’s disease, cardiomyocyte disease, respiratory disease, Alzheimer’s disease, and various kinds of cancers and infectious diseases. The presence of an 1-3-nitrotyrosine unit in the ilamycins provides an excellent opportunity to elucidate the biosynthetic mechanism(s) leading to this rare unit. In the biosynthetic gene cluster of ilamycins, IlaM and IlaN encode a nitric oxide synthase and a cytochrome P450 oxygenase, respectively. Silico analysis revealed that IlaM shared the same active sites with other nitric oxide synthase originate from the human or murine, but clustered in different clades. Silico analysis revealed that IlaM shared the same active sites with other nitric oxide synthase originate from the human or murine, but clustered in different clades. Silico analysis revealed that IlaM shared the same active sites with other nitric oxide synthase originate from the human or murine, but clustered in different clades. Silico analysis revealed that IlaM shared the same active sites with other nitric oxide synthase originate from the human or murine, but clustered in different clades.

To validate this hypothesis, the ilaM and ilaN genes were each inactivated using the aforementioned method. HPLC analyses of the fermentation extracts of the resultant mutant strains revealed that both the ΔilaM and ΔilaN mutants failed to produce ilamycins or their analogs (Fig. 2, traces xv and xvi). These results, along with the bioinformatics analysis, suggest that IlaM and IlaN might be responsible for pre-tailoring 1-Tyr to 1-3-nitrotyrosine since no evidence of de-nitro ilamycin analogs could be found.

In order to confirm that the nitration of 1-Tyr is a pre-tailoring process in the biosynthesis of ilamycins and that the nitration is catalyzed by IlaM and IlaN, 1-3-nitrotyrosine (0.5 mM) was individually fed to cultures of the resultant mutant strains and that the nitration is catalyzed by IlaM and IlaN, 1-3-nitrotyrosine (0.5 mM) was individually fed to cultures of the resultant mutant strains. HPLC analyses of the fermentation extracts of the mutant strains were analyzed by HPLC (Fig. 2, traces xvii and xix). The structural elucidation of the 1-3-nitrotyrosine might be similarly catalyzed by IlaM and the downstream IlaN.

Post-tailoring steps in ilamycin biosynthesis. We next elucidated the post-tailoring steps in the biosynthesis of ilamycins. Among all of the ilamycin analogs, Ilamycin D (5) is the most highly oxidized; a terminal methyl group in the N-methyl Leu unit is oxidized to its COOH moiety and the double bond of the isopentene is oxidized to an epoxy group (Fig. 3f). The p450 enzymes with diverse oxidation functions maybe the better candidates to fulfill these roles. In the ila gene cluster, there is a cytochrome P450 monoxygenase IlaL in the middle of the cluster and another cytochrome P450 monoxygenase IlaR adjacent to the giant NRPS protein IlaS. Both of these p450s may be responsible for these post-tailoring oxidation steps. Similarly, the ilaL and ilaR genes were each inactivated and the fermentation extracts of the mutant strains were analyzed by HPLC (Fig. 2, traces xx and xx).

HPLC analysis revealed that the ΔilaR mutant accumulated four products, 1 and 6–8 (Fig. 2, trace xx), and LC-MS analysis of the metabolites revealed that the latter two adducts might be other ilamycin analogs. To elucidate the exact structures of these metabolites, cultures of the ΔilaR mutant were scaled-up in a 16L fermentation, and the four compounds, 1 and 6–8, were subsequently isolated and purified. Compounds 1 and 6 isolated from the ΔilaR mutant were identified as ilamycin B1 and ilamycin E1, respectively, on the basis of HRESIMS and 1H and 13C NMR spectroscopic data comparisons. The molecular formula of compound 7 (designated ilamycin E2) was established to be C34H32N6O12 by HRESIMS. The planar structure of 7 coincided with that of ilamycin E1 (6), which was elucidated in the same fashion. Comparisons of NMR data (Supplementary Figs. 60–64) further showed that ilamycins E2 and E3 (6 and 7) have the same stereochemistries at the γ- and δ-carbons (C5 and C13) of the cyclic hemiaminals containing each N-methyl leucine unit as seen in ilamycins C1 and C2 (3 and 4), respectively. The molecular formula of compound 8 (designated ilamycin F) was established to be C47H56N8O12 by HRESIMS, 16 mass units less than that of ilamycin D (5). The 1H and 13C NMR spectroscopic data comparisons (Supplementary Table 2 and Supplementary Figs. 65, 66) of 8 were very similar to that of ilamycin D, except that the signals ascribed to the epoxy group in ilamycin D were missing in spectra of 8. Conversely, additional signals at δH 145.5, δH 6.18 (CH-13) and δC 114.1, δH 5.25, 5.21 (CH2-14) in the spectrum of 8 were clearly present indicating the presence of a terminal double bond. The COSY, HMOC, and HMBAC correlations (Supplementary Figs. 67–69) confirmed the planar structure of compound 8. Analysis of the X-ray diffraction data for 8 revealed that the γ-C (C15) stereochemical configuration in the 2-amino-4-methylpentan-4-enoic acid residue is S (Supplementary Table 1; Supplementary Fig. 4 and Supplementary Data 4, 5). The structural elucidation of these four products in the ilaR mutant fermentations allowed us to assign IlaR as an isopentene epoxidase.

An interesting feature we identified during HPLC studies is that compounds 7 and 6 epimerize/interconvert in aqueous solvents (e.g., MeOH or CH3CN); this interconversion also was noted under neat conditions following periods of long-term storage. The ratio of compound 7 to 6 in MeOH was found to be ca 6:4 at equilibrium and compound 6 is reasonably stable in non-aqueous solvents. At room temperature and under acidic aqueous condition (15% CH3CN in H2O containing 0.1% acetic acid) or in MeOH, purified 6 was found to slowly convert to 7, and purified 7 was found to slowly convert to 6 (Supplementary
Fig. 34A); similar chemistry was noted under neat conditions but only after prolonged periods. Similarly, such conditions were found to facilitate more rapid conversion of compound 4 into 3; at equilibrium we found the ratio of 4:3 to be ca 5:5. The possibility interchange of these two pairs of compounds was postulated (Supplementary Fig. 34B). Interestingly, a similar reaction able to take place under neat (solvent and catalyst-free) conditions to form bicyclic hemiaminals using a pair of substrates containing an aldehyde group and N-substituted amide group has been noted18. It is indeed interesting to consider that cyclic hemiaminal installation in ilamycins C1, C2, E1, and E2 is likely reflective of a transient C33 aldehyde and that the αOH orientation is preferred; presumably this is driven to some extent by the fixed stereochemical orientations at C23 and C32 in each respective case (Fig. 3B). By virtue of this interconversion chemistry it is significant to note that assays carried out with these agents likely involved the use of isomeric mixtures of 6/7 and 3/4 and no one specific isomer.

Antimycobacterial and cytotoxic activities of ilamycins. Having never been reported previously, we assessed the cytotoxicity of 1–8 using five human tumor cell lines and two normal human cell lines. The results demonstrated that compounds 3/4, 6/7 exhibited cytotoxic activities against HeLa, HepG2, and A549 cell lines with IC50 values in the range 3.2–6.2 μM (Supplementary Table 6). Additionally, the cytotoxic ilamycins generally displayed a 3–5-fold reduced activity against two normal cell lines; these agents showed a clear preference for harming cancerous cell lines.

Finally, we systematically evaluated antimicrobial activities of compounds 1–8 using a panel of six Gram-positive and Gram-negative bacteria, and two mycobacteria, including M. smegmatis MC2 155 and M. tuberculosis H37Rv. The ilamycins failed to show antibacterial activities (MICs > 121 μM) against the first six bacteria (Supplementary Table 7). However, selective activities against the mycobacteria were, in some cases, quite prominent. Most notably, ilamycins E1/E2 (6/7) showed the strongest inhibitory activity against M. tuberculosis H37Rv with an MIC value of 9.8 nM, which was 30-fold superior to that of positive control, rifampin (Table 1). More intriguingly, 6 and 7 (likely as a mixture) bear a therapeutic activity/toxicity index of 400–800 indicating they will hold great promise as the lead drugs for anti-TB agents.

Methods

General materials and experimental procedures. NMR spectra were obtained with an AVANCE-500 spectrometer (Bruker). CC was performed using silica gel (100–200 mesh; Jiangyao). Medium-pressure liquid chromatography was performed using a CHEETAH 100 automatic flash chromatography system (Bonna-Agela) with an ODS-A flash column (50 μm, 12 nm; 100 × 20 mm, YMC). Semi-preparative HPLC was performed with two 210 solvent delivery modules equipped with a diode array detector (Hidra, Uvikon 930, Rudy, Switzerland). The samples were detected at 254 nm or 280 nm. Low-resolution mass spectra were monitored on an Agilent 6120B triple quadrupole mass spectrometer (Agilent, Santa Clara, CA). The MS/MS spectra were obtained from an API 2000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA). An electrospray ionization source was used. ESI mass spectrometry was used for structure elucidation. ESI mass spectrometry was also used to support the chemical structures of compounds presented in this manuscript. The structures were confirmed by high-resolution mass spectrometry (HRMS). HRMS spectra were obtained from a Waters Micromass GCT Premier mass spectrometer equipped with a water microsource source. Mass spectra were collected in the mass range m/z 100–1000.

Bacterial strains and plasmids. Strain SCSIO ZH16 was isolated from a sediment sample collected from the South China Sea (120°0.250′E, 20°22.971′N) at a depth of 3538 m. Phylogenetic analysis based on the nearly complete 16S rRNA gene sequence indicated that strain SCSIO ZH16 belongs to the genus Streptomyces. The highest 16S rRNA gene sequence similarity value was 100% between strain SCSIO ZH16 and Streptomyces atratus atratus PY-1 (KJ627770). The 16S rRNA gene sequence has been deposited in GenBank under accession number KX9708. This strain was deposited in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) as Streptomycetes atratus SCSIO ZH16. Strains and plasmids used and generated in this study are listed in Supplementary Table 3.

Cultural conditions and DNA manipulations. S. atratus SCSIO ZH16 was cultured on modified ISP (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, and 3.0% crude sea salt) plates with additional 20 mM MgSO4 at 30 °C. For the isolation of ilamycins, their analogous cultures of S. atratus SCSIO ZH16 were grown in Am2ab medium for 60 h and then inoculated into Am3b production medium (0.5% soybean meal, 1.5% bacterial peptone, 1.5% soluble starch, 1.5% glycerol, 0.2% CaCO3, and 3% sea salt, pH 7.2–7.4) at a ratio of 1:10 before being cultured for another 7 days at 30°C and 200 rpm. All DNA isolation and manipulation procedures in Escherichia coli and Streptomyces were performed according to standard procedures or the manufacturer’s protocol. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). DNA sequencing was performed at IGE Biotech Co., Ltd. (Guangzhou, China). Restriction enzymes and DNA ligase were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Plasmid, gel extraction, and cycle-pure kits were purchased from Omega Bio-Tek Inc. (GA, USA). PCR amplifications were carried out using either EasyTaq or high-fidelity polymerase purchased from Transgene Biotech Co., Ltd. (Beijing, China).

Purification of compounds 1–9. For the isolation of compounds 1–6, the strain S. atratus SCSIO ZH16 was cultivated on an 18 L of Am3 medium using the methods mentioned above. After 8 days cultivation, the whole culture medium was centrifuged to separate the liquid broth from the solid cell mass. The liquid broth and the solid cell mass were then extracted with an equal volume of butanone and a double volume of acetone, respectively. The organic layer was dried under vacuum, and the solid cell mass were then extracted with an equal volume of butanone and a double volume of acetone, respectively. The organic layer was dried under vacuum, and the two parts of the extracts were combined.

The extract was applied to silica gel CC using a gradient elution of CHCl3/MeOH (1000, 98.2, 96.4, 94.6, 92.8, 91.2, 82, and 5) to give eight fractions (Fr. A1–A8). Fr. A2 and A3 were combined and purified by silica gel CC, eluting with gradient ratios of petroleum ether/ethyl acetate (1000:0, 82:6, 64:4, 28:2, and 0:100), ethyl acetate/MeOH (95:5), and CHCl3/MeOH (9:1) to give eight fractions (Fr.
B1–B8). Fr. B5–B8 were each separated using semi-preparative HPLC with an ODS column, eluting with CH₃CN/H₂O (40:60–100:0 over 30 min, 10 mL/min) to yield ilamycin B1 (1.5 mg), ilamycin E6 (1.8 mg), and ilamycin B2 (2.2 mg); Fr. A4 and Fr. A5 were purified by silica gel CC, eluting with gradient ratios of petroleum ether/ethyl acetate (100:0, 8:2, 6:4, 4:6, 2:8, and 0:100), ethyl acetate/MeOH (95:5), and CHCl₃/MeOH (9:1) to give eight fractions (Fr. C1–C8). Fr. C1–C8 were each separated using semi-preparative HPLC with an ODS column, eluting with CH₃CN/H₂O (30:70–100:0 over 30 min, 10 mL/min) to yield ilamycin C3 (3.28 mg), ilamycin C4 (3.3 mg), and ilamycin D5 (7.58 mg).

Based on HPLC analysis results of the mutant cultures, we selected ΔilaL, ΔilaR, and Δala mutants for large scale fermentation using the aforementioned methods. Finally, 88 mg of 1, and 152 mg of 2 were isolated from a 13.5 L fermentation of the ΔilaL mutant, 130.5 mg of 6, 85.6 mg of 8, and 150.5 mg of 1 were isolated from a 16 L fermentation of the ΔilaR mutant, and 5.6 mg of 9 was isolated from a 13.5 L fermentation of the Δala mutant using isolation procedures similar to those described above.

Ilamycins E5 (6): Yellow powder; H and 13C NMR data were summarized in Supplementary Table 2, 2D (COSY, HSQC, and HMBC) NMR spectra, see Supplementary Figs. 57–59; HRESIMS m/z 1026.5669 [M+H]⁺ (calculated for C₅₄H₇₆N₉O₁₂, 1042.5608), see Supplementary Fig. 40.

Metabolite analysis of wild-type and mutant strains. To analyze the metabolites of each mutant, the mutant strains were inoculated in a 250-mL flask filled with 50-mL Am2ab medium and grown at 30°C on a rotary shaker at 200 rpm for 7 days with the wild-type as a control. The fermentation was extracted with an equal volume of butanol and processed using the aforementioned method. The dried extracts of the fermentation products were re-suspended in 1-mL methanol and were centrifuged for 10 min at 14000 rpm before HPLC analysis. HPLC analysis was carried out using a reversed phase column SB-C18, 5 μm, 4.6 x 150 mm (Agilent) with UV detection at 210, 285, and 352 nm under the following program: solvent system (solvent A, 15% acetic acid in water supplemented with 0.1% acetic acid; solvent B, 85% acetic acid in water supplemented with 0.1% acetic acid; 20% B at 80% B (linear gradient, 0–20 min), 80% B to 100% B (linear gradient, 20–21.5 min), 100% B (21.5–27.0 min), 100 B to 0% B (27.0–27.1 min), 0% B at 27.1–3.0 min); flow rate was set at 1 mL/min.

Isotopic labeling experiments. To elucidate the biosynthetic origin of the 1-AHA unit, isotopic labeling experiments were carried out with cultures of the S. atratus SCSSIO ZH16. Three 13C-labeled compounds, [1-13C] sodium acetate, [2-13C] sodium acetate, and [1,2-13C] sodium acetate, were used for the feeding experiments. The 13C-labeled agent was dissolved in ddH₂O at a concentration of 500 mM and supplied to the culture of the wild-type strain at 48 h. The solution was sterilized by filtration, and then individually supplied into 1 L production cultures (Am3 medium) at 60 h, 72, and 84 h with the volume of 0.5, 0.1 mL, respectively. After cultivation at 220 rpm and 30°C for 7 days, the cultures were harvested and extracted. The representative product, 13C-labeled ilamycin B₂, was purified from the organic extract.

Precursor feeding experiments. A portion of mycelium and spores (1 cm³) of the wild-type strain was inoculated into a 250-mL flask with 50 mL of Am3 medium as described above at 30°C and 200 rpm for 60 h. Synthesized Δ(l)-AHA, 2,4-HDA, and 4-HA were dissolved in acidic water, neutralized with 1 N NaOH to pH 7.0, and sterilized via filtration. The sterilized (l)-AHA and 4-HA were supplied into the culture of the ΔilaD mutant, and the (d)-AHA, 2,4-HDA, and 4-HA were individually supplied to cultures of ΔilaLMNO mutant to achieve a final concentration of 0.5 mM. Following incubation at 30°C and 200 rpm for 7 days, the cultures were harvested and extracted with butane, evaporated to dryness, and dissolved in MeOH for HPLC analysis (Fig. 2, x–xi). The feeding procedures of 3-nitro-L-tyrosine to ΔilaM or ΔilaN mutants were similarly conducted. The 3-nitro-L-tyrosine precursor was dissolved in water, neutralized with 1 N NaOH to pH 7.0 and sterilized via filtration, and was then individually supplied into the cultures of ΔilaM or ΔilaN mutants (Fig. 2, xvii and xix).

Cytotoxicity assays. Compounds 1–8 were evaluated for cytotoxicity using HeLa, HepG2, A549, CNE2, and MCF7 cell lines using a previously reported MTT method. The L02 and Huvec-12 cell lines were used as normal cell line controls. All these cell lines were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Cell lines were checked for mycoplasma and profiled via short tandem repeat profiling to confirm their purity.
identity by the supplier. All experiments were performed in triplicate with doxorubicin and cis-platinum as control agents.

Antibacterial assays. To determine the antibacterial activities of these eight compounds (1–8), a preliminary screening of their antibacterial activities against a panel of Gram-positive and Gram-negative bacteria20–22, S. muencheni MC2 155 and M. tuberculosis H37Rv, was conducted using a broth dilution method20, 23. Compounds 1–8 were dissolved in dimethylsulfoxide (DMSO) to give 3200 μg·mL−1 stock solutions. The stock solutions were then serially diluted to concentrations of 0.0625–128 μg·mL−1. All experiments were performed in triplicate with doxorubicin and cis-platinum as control agents.

Data availability. Sequence data that support the findings of this study has been deposited in GenBank with accession codes KY173348 for ilamycin gene cluster and KT97098 for 16s rDNA gene sequence of S. atratus SCCOS ZH16. Deposition number of crystallographic data for 2, 4, 5, and 8 are CDCIC1524774, CDCIC1524775, CDCIC1524776, and CDCIC1524777, respectively. The authors declare that all other relevant data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author upon reasonable request.

Received: 5 March 2017 Accepted: 28 June 2017

Published online: 30 August 2017

References

1. Glazou, P., Floyd, K., Weil, D. & Bavigione, M. TB deaths rank alongside HIV deaths as top infectious killer. Int. J. Tuberc. Lung Dis. 20, 143–144 (2016).
2. World Health Organization. Global tuberculosis report, http://www.who.int/tb/publications/global_report/en/ (2015).
3. Lee, B.-Y. et al. Drug regimens identified and optimized by output-driven platform markedly reduce tuberculosis treatment time. Nat. Commun. 8, 14183 (2017).
4. Hoffner, S. Unexpected high levels of multidrug-resistant tuberculosis present in the USA. Lancet 380, 1367–1369 (2012).
5. Loewenberg, S. India reports cases of totally drug-resistant tuberculosis. Lancet 379, 205 (2012).
6. Song, Y. et al. Cytotoxic and antibacterial angucyline- and prodigiosin-analogous from the deep-sea-derived Streptomyces sp. SCOSO 11594. Mar. Drugs 13, 1304–1316 (2015).
7. Song, Y. et al. Cyclic hexapeptides from the deep sea China South Sea-derived Streptomyces scopoliridus SCCOS ZJ46 active against pathogenic Gram-positive bacteria. J. Nat. Prod. 77, 1937–1941 (2014).
8. Zhou, X. et al. Marthiapenide A, an anti-infective and cytotoxic polythiazole cyclopeptide from a 60 L scale fermentation of the deep sea-derived Marinactinospora thermotolerans. J. Antibiot. 64, 750–754 (1996).
9. Rudi, R. Protein tyrosine nitration: biochemical mechanisms and structural basis for functional effects. Acc. Chem. Res. 46, 550–559 (2013).
10. Kers, J. A. et al. Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. Nature 429, 79–82 (2004).
11. Podust, L. M. & Sherman, D. H. Diversity of P450 enzymes in the biosynthesis of unsymmetrical 7-oxoaromatics. J. Biol. Chem. 282, 1405–1412 (2007).
12. Drake, E. J. et al. The 1.8 Å crystal structure of PA2412, a MbtH-like protein from the pyoverdine cluster of Pseudomonas aeruginosa. J. Biol. Chem. 282, 40245–40244 (2007).
13. Petkovšek, H. et al. Substrate specificity of the acyl transferase domains of EpoC from the epothilone polyketide synthase. Org. Biomol. Chem. 6, 500–506 (2008).
14. Xu, W. et al. LovG: the thioesterase required for dihydroxymalonoil L release and lovastatin nonaketide synthase turnover in lovastatin biosynthesis. Angew. Chem. Int. Ed. 52, 6472–6475 (2013).
15. Liu, T. et al. Identification of NanE as the thioesterase for polymer chain release in nanchangycin biosynthesis. Chem. Biol. 13, 945–955 (2006).
16. Giorgio, S. et al. Formation of nitrosyl hemoglobin and nitrotyrosine during marine leishmaniasis. Photochem. Photobiol. 63, 750–754 (1996).
17. Kulanthaivel, P. & Vasudevan, V. Rufomycin and derivatives thereof useful as antibiotic medicated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in Streptomyces coelicolor M145. Microbiology 153, 1405–1412 (2007).
18. Wolpert, M., Gust, B., Kammerer, B. & Heide, L. Effects of deletions of mbtH-like genes on chlorobiocin biosynthesis in Streptomyces coelicolor. Microbiology 153, 1413–1423 (2007).
19. Al-Mestarihi, A. H. et al. Adenylation and S-methylation of cysteine by the MbtH-like protein MtbH from M. tuberculosis. FEBS Lett. 577, 194–198 (2003).
20. Sheldrick, G. M. Crystal structure refinement and analysis and Sheldrick, G. M. A short history of SHEXL. Acta Cryst. C71, 3–8 (2015).
21. Sheldrick, G. M. A short history of SHEXL. Acta Cryst. A64, 112–122 (2008).
22. Oikawa, M. et al. Regioselective domino metathesis of unsymmetrical 7-oxoaromatics. J. Biol. Chem. 280, 339–341 (2005).
23. Bisang, C. et al. A chain initiation factor common to both modular and aromatic polyketide syntheses. Nature 401, 502–505 (1999).
50. Xue, Y., Zhao, L., Liu, H. W. & Sherman, D. H. A gene cluster for macrolide antibiotic biosynthesis in Streptomyces venezuelae: architecture of metabolic diversity. *Proc. Natl. Acad. Sci. USA* 9, 12111–12116 (1998).

51. Takahashi, S. et al. Reveromycin A biosynthesis uses RevG and RevJ for stereospecific spiroacetal formation. *Nat. Chem. Biol.* 7, 461–468 (2011).

52. Siskos, A. P. et al. Molecular basis of Celmer’s rules: stereochemistry of catalysis by isolated ketoreductase domains from modular polyketide synthases. *Chem. Biol.* 12, 1145–1153 (2005).

53. Maier, T., Jenni, S. & Ban, N. Architecture of mammalian fatty acid synthase at 4.5 Å resolution. *Science* 311, 1258–1262 (2006).

54. Maier, T., Leibundgut, M. & Ban, N. The crystal structure of a mammalian fatty acid synthase. *Science* 321, 1315–1322 (2008).

55. Knoll, M. & Pleiss, J. The crystal structure of a mammalian fatty acid synthase. *Protein Sci.* 17, 1689–1697 (2008).

56. Kwan, D. H. & Leadlay, P. F. Mutagenesis of a modular polyketide synthase enoylreductase domain reveals insights into catalysis and stereospecificity. *ACS Chem. Biol.* 5, 829–838 (2010).

57. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63 (1983).

58. CLSI (Clinical and Laboratory Standards Institute). *Performance Standards for Antimicrobial Susceptibility Testing; nineteenth Informational Supplement*. M100-S19 (Wayne, 2009).

59. Zhang, T., Li, S. Y. & Nuernberger, E. L. Autoluminescent *Mycobacterium tuberculosis* for rapid, real-time, non-invasive assessment of drug and vaccine efficacy. *PLoS ONE* 7, e29774 (2012).

**Acknowledgements**

This work was supported in part by NSFC (31270134, 81425022, U1501223, 81572037), CAS (XDA11030403, YIPA-2013226, 154144KYSB20150045), Guangdong NSF (2016A030312014, 2016A030310123), the Pearl River S&T Nova Program of Guangzhou, China (2014J200089) and Guangzhou Healthcare and Cooperative Innovation Major Project (201508020248, 201604020019). Additionally, we thank the analytical facility center (Ms. A. Sun, Dr. Z. Xiao, and Mr. C. Li) of the South China Sea Institute of Oceanology for recording MS and NMR data.

**Author contributions**

J.J. and J.M. designed the research and wrote the paper. J.M. and Y.X. did the bioinformatics analysis. J.M., C.Z., and Y.Z. constructed all the engineered strains and mutants, as well as HPLC analysis. J.M. and Y.J. carried out the isotopic labeling and precursors feeding experiments. Y.J. and J.M. isolated all the compounds. J.J. and H.H. analyzed the NMR and X-ray diffraction data, and performed the structure determination of isolated compounds. Z.L. and T.Z. carried out antibacterial assays; J.Z. and H.Z. did cytotoxicity assays.

**Additional information**

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00419-5.

**Competing interests:** The authors declare no competing financial interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017