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# Natural product antibiotics: synthesis and next generation analogues

Lysenko, V.

**Citation**
Lysenko, V. (2026, May 21). *Natural product antibiotics: synthesis and next generation analogues*. Retrieved from https://hdl.handle.net/1887/4304553

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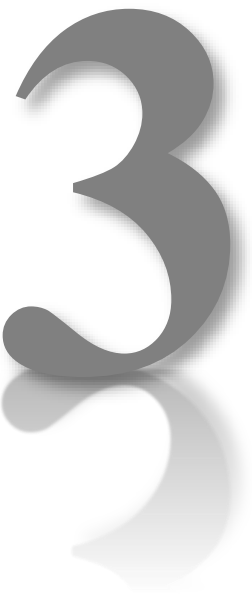


# Chapter 3

## Total Synthesis and Structural Reassignment of the Antitubercular Natural Product Evybactin


### Abstract

The escalating threat posed by antibiotic resistance is a global concern and underscores the need for new antibiotics. In this context, the recent discovery of evybactin, a nonribosomal depsipeptide antibiotic that selectively and potently inhibits the growth of *M. tuberculosis*, is particularly noteworthy. Here, we present the first total synthesis of this natural product, along with a revision of its assigned structure. Our studies revealed a disparity between the structure originally proposed for evybactin and its actual configuration. Specifically, the 3-methylhistidine residue present in the evybactin core macrocycle was found to be of the D-configuration rather than the previously assigned L-His(Me). Having addressed this, we further optimized our solid-phase synthetic route to provide access to evybactin on a multi-hundred-milligram scale. Access to such quantities will allow for more comprehensive studies with this promising antibiotic.



Parts of this chapter have been published in:

Lysenko, V.*; Son, S.*; Theriault, M. E.; Slingerland, C. J.; Hauk, G.; Cleenewerk, L.; Speer, A.; Berger, J. M.; Lewis, K.; Martin, N. I. Total Synthesis and Structural Reassignment of the Antitubercular Natural Product Evybactin. *Chem. Eur. J.* **2025**, *31* (1), e202403767.


* - these authors contributed equally.

## Introduction

Globally, tuberculosis (TB) is responsible for millions of infections each year and, with the exception of the recent COVID-19 pandemic period, has historically been the pathogen with the single highest attributable annual death toll. The standard treatment for TB infection typically involves daily administration of multidrug cocktails for many months, often resulting in poor compliance and the emergence of resistance.[1,2] The threat of extensively drug-resistant strains of TB underscores the need for innovative antibacterial therapies to counter these and other serious bacterial pathogens.[3-6] In recent years, screening uncultured bacteria[7] or targeting of microbiome symbionts such as *Photorhabdus* has led to the discovery of compounds with unique antibacterial mechanisms of action.[8] Among recent demonstrations of this strategy is the Lewis group's discovery of evybactin - a unique DNA gyrase inhibitor that selectively and potently inhibits the growth of TB.[9]

Isolated from culture extracts of the nematode microbiome symbionts *Photorhabdus*, evybactin was found to be a nonribosomal depsipeptide containing 12 amino acids. Extensive NMR and MS/MS studies combined with analysis of the biosynthetic gene cluster responsible for the biosynthesis of evybactin led to a proposed structure (**Figure 1**).[9]

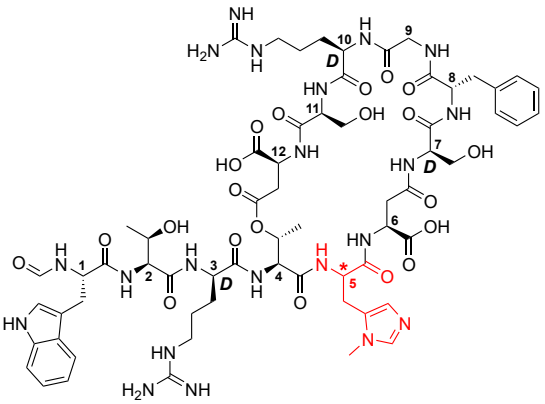



**Figure 1**. Originally proposed structure of evybactin (**1**) and revised structure (**2**) established via total synthesis.

Evybactin was found to contain a unique macrocyclic structure formed between the side chain carboxyl group of the C-terminal Asp[12] residue and the side chain hydroxyl of Thr[4]. Also of note, within the macrocycle, it is the side chain carboxyl group of Asp[6] that forms a peptide bond with neighboring D-Ser[7]. In addition to these features, evybactin was also found to contain D-Arg residues at positions 3 and 10, along with N-terminal formylation at Trp[1] and methylation at N3 in the imidazole side chain of His[5].

Evybactin's unique narrow spectrum activity against TB was shown to be due to its capacity to gain entry by hijacking the BacA transporter, used by TB as a multi-solute ABC-type transporter for hydrophilic molecules[10] such as vitamin $B_{12}$.[11] Upon cell entry, evybactin acts as a DNA gyrase poison, with structural studies subsequently showing that it binds to a recently discovered site that is also exploited by a novel class of synthetic thiophene antibiotics.[9,12] Evybactin's specific entry mechanism and strong gyrase inhibition are responsible for its potent antibacterial activity against TB, with minimum inhibitory concentration (MIC) values as low as 0.25 µg/mL while exhibiting no measurable toxicity against mammalian cells.[9]



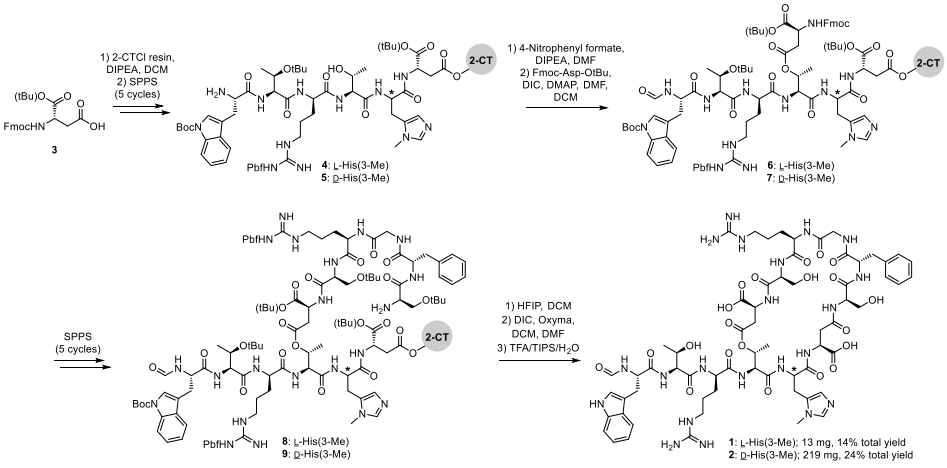
While evybactin's promising anti-TB activity merits further exploration, its isolation from natural sources presents a barrier to the investigation and exploitation of its potential. Isolation of evybactin from fermentation of *P. noenieputensis* is labor-intensive, requiring complex isolation and purification procedures and yields small quantities of material.[9] Furthermore, access to analogues to enable structure-activity relationship (SAR) studies is not readily achievable starting from the natural product. For these reasons, we were motivated to develop a synthetic route that would provide access to high-purity evybactin in larger quantities and provide a means for generating structural analogues.

## Results and discussion

Given our previous experience in the synthesis of macrocyclic peptide antibiotics,[13-17] we elected to pursue a microwave-assisted solid-phase peptide synthesis (SPPS) approach for the preparation of evybactin. To this end, we first explored a direct route wherein a protected linear 12-mer peptide was synthesized starting from the C-terminal $Asp^{12}$ (based on the numbering in **Figure 1**), loaded via its side chain carboxyl group onto 2-chlorotrityl chloride (2-CTCl) resin (see supplemental **Scheme S1**). In preparing the linear precursor peptide, we used 10 % piperazine in EtOH/NMP for Fmoc deprotections and HBTU and DIPEA for amino acid couplings. We also used Fmoc-Thr-OH (with the side chain hydroxyl unprotected) for the installation of $Thr^4$, which did not interfere with any of the subsequent couplings. Following the final Fmoc deprotection, the N-terminus of $Trp^1$ was cleanly formylated on resin using a mixture of 4-nitrophenylformate and DIPEA in DMF.[18] Given the limited stability of the formylation reagent, this step was performed manually at room temperature. The protected peptide was then cleaved from the resin using hexafluoroisopropanol (HFIP) to maintain all side chain protecting groups. Next, we explored a variety of conditions for closing the macrocycle via ester bond formation between the free side chain hydroxyl of $Thr^4$ and the free side chain carboxyl of $Asp^{12}$. Our initial attempt using DIC/DMAP failed to yield any of the cyclized product, however, upon addition of DBU, we observed partial conversion to a species with the expected mass. However, following global deprotection using TFA/TIPS/$H_2O$, the crude peptide contained many closely eluting impurities that precluded purification of the desired product. Switching to different cyclization conditions using HATU and DBU also

resulted in incomplete conversion and again yielded complex mixtures, preventing purification.

We next opted for a strategy wherein the ester linkage between Thr[4] and Asp[12] was formed on resin at an earlier stage of the synthesis, followed by closure of the macrocycle by subsequent amide formation between D-Ser[7] and Asp[6] (**Scheme 1**). In doing so, we started by loading Fmoc-Asp-OtBu **3** (corresponding to Asp[6]) onto 2-CT resin via its side chain carboxyl group. Five rounds of SPPS resulted in the resin-bound intermediate hexapeptide **4** terminating in Trp[1]. As in our previous route, we used Fmoc-Thr-OH (with the side chain hydroxyl unprotected) for the installation of Thr[4], which did not affect the quality of any of the following couplings. On resin formylation of the N-terminus of Trp[1] was performed manually at room temperature, after which the hydroxyl side chain of Thr[4] was esterified with Fmoc-Asp-OtBu (also under manual conditions) using DIC/DMAP activation in DMF/DCM, resulting in a reasonable 85 % conversion to intermediate **6** as assessed by LC-MS analysis. Automated SPPS was again used for the installation of the remaining amino acids, yielding the resin-bound intermediate **8** terminating at D-Ser[7]. The peptide was then cleaved from the resin using HFIP treatment to maintain all side chain protection. The crude protected peptide was then directly treated with DIC/Oxyma (6:6) in DCM/DMF (6:1), which, gratifyingly, was found to result in the clean and complete formation of the desired macrocycle by amide bond formation between the amine of D-Ser[7] and the side chain carboxyl of Asp[6]. Following this, global deprotection with TFA/TIPS/$H_2O$ and subsequent purification using reverse-phase high-performance liquid chromatography (RP-HPLC) resulted in a yield of 13 mg of compound **1** (14 % overall yield, corresponding to an average yield of 93 % per step).




**Scheme 1.** Combined solid- and solution-phase route developed for the synthesis of evybactin.

We next proceeded to characterize the antibacterial activity of compound **1** and, to our surprise, found it to be completely inactive against *M. tuberculosis* H37Rv mc[2]6020, *E. coli* ATCC 25922, and *E. coli* MG1655 while the natural product isolated from

fermentation of *P. noenieputensis*, exhibited the same activity against these strains as previously reported (**Table 1**).[9] Compound **1** was also found to be much less active against the hypersensitive *E. coli* WO153 strain with increased membrane permeability. Subsequent investigations revealed that **1** is not identical to the natural product based on LC-MS co-injection experiments (**Figure 2**) and a comparison of the NMR data acquired (see supplemental **Figures S1** and **S2**). These findings, and the lack of antibacterial activity observed for **1**, suggested that the structure originally assigned to evybactin might require revision. To this end, we first assessed the amino acid connectivity of evybactin by performing and comparing MS/MS analyses for the natural product and compound **1**. The results obtained from these studies confirmed the originally assigned connectivity, with that of natural evybactin matching that of compound **1** (see supplemental **Tables S1** and **S2**). These findings prompted us to further investigate the stereochemistry assigned for the different amino acids present in evybactin. To this end, we performed Marfey's analysis[19] on both the natural product and **1**. Notably, the data thus obtained suggested that the stereochemistry of the His(3-Me) residue at position 5 was D- rather than L- as originally assigned (see supplemental **Table S3**). This led to a reanalysis of the biosynthetic gene cluster of evybactin, which, upon closer inspection, was also found to support the presence of D-His(3-Me). Specifically, antiSMASH analysis[20] of the 12 nonribosomal peptide synthetases (NRPS) modules encoding evybactin revealed a previously unassigned epimerase domain downstream of module 5, responsible for the incorporation of Me-His (see supplemental **Figure S4**).

Equipped with these insights, we next proceeded to synthesize compound **2** (**Figure 1**) according to the route developed for **1** (**Scheme 1**) but with the notable incorporation of D-His(3-Me) at position 5. Interestingly, while Fmoc-L-His(3-Me)-OH is widely available, the corresponding D-enantiomer could not be readily sourced from commercial suppliers. The published methods for synthesizing Fmoc-D-His(3-Me)-OH were found to be complicated and low-yielding.[21,22] We, therefore, elected to develop a new protocol for the preparation of this key building block with the aim of pursuing a more convenient, chromatography-free route (see supplemental **Scheme S3**). Starting from readily available Fmoc-D-His(Trt)-OH, we first protected the carboxyl group as the Trt ester by treatment with TrtCl and DIPEA. Following a simple extractive work-up, treatment with MeI in DMF was found to cleanly alkylate position 3 of the imidazole ring. Subsequent removal of both Trt groups by treatment with TFA/TIPS/$H_2O$, followed by precipitation using MTBE/PE, provided Fmoc-D-His(3-Me)-OH in 92 % yield over 3 steps. This procedure was found to be reproducible at a gram scale, providing the building block in purity suitable for direct use in SPPS.

**Table 1.** MICs determined for evybactin isolated from fermentation of *P. noenieputensis* compared with synthetic compounds **1** and **2**.

| Strain | MIC (µg/mL) | | |
|---|---|---|---|
| | Evybactin (natural) | **1** | **2** |
| *M. tuberculosis* H37Rv mc$^2$6020[a] | 0.25 | 128 | 0.25 |
| *E. coli* ATCC 25922[b] | 8 | >128 | 8 |
| *E. coli* MG1655[b] | 16 | >128 | 16 |
| *E. coli* WO153 (AB1157; *recJ asmB1 ΔtolC*::KanR)[b] | 0.0625 | 0.8 | 0.0625 |
| *S. aureus* HG003[b] | 128 | >128 | >128 |

[a] Difco Middlebrook 7H9 medium; [b] MHIIB medium.

The synthesis of **2** was carried out as for **1**, with some minor adjustments that were found to improve the overall yield. Specifically, for the transformation of resin-bound intermediate **5** to **7**, we found that reducing the number of formylation cycles from 3 to 2 led to suppression of an unwanted side product presumably formed due to formylation of the side chain hydroxyl of Thr$^4$. We also found that for the subsequent esterification of the Thr$^4$ side chain with Fmoc-Asp-OtBu, a lower amount of DMAP (0.2 eq) improved the conversion, as higher concentrations of DMAP resulted in premature Fmoc deprotection at Asp$^{12}$, leading to unwanted side products. As for the cyclization step, we found that the original extractive work-up used resulted in a significant loss of material and negatively impacted the yield. To avoid this, we reduced the number of DIC/Oxyma equivalents to 3 and decreased the concentration of DMF in DCM to 0.5 %, which allowed for direct evaporation of the solvent after the cyclization was complete (as established by LC-MS analysis). The material thus obtained was then deprotected by direct treatment with TFA/TIPS/$H_2O$, followed by precipitation of the product from MTBE. This optimized protocol both improved the purity of the crude product (which greatly facilitated final purification by RP-HPLC) and resulted in an increased overall yield for compound **2** of 24 % (corresponding to an average yield of 94.8 % per step). This method was found to be reproducible and performed well at a 0.5 mmol scale, providing access to quantities of **2** in excess of 200 mg.

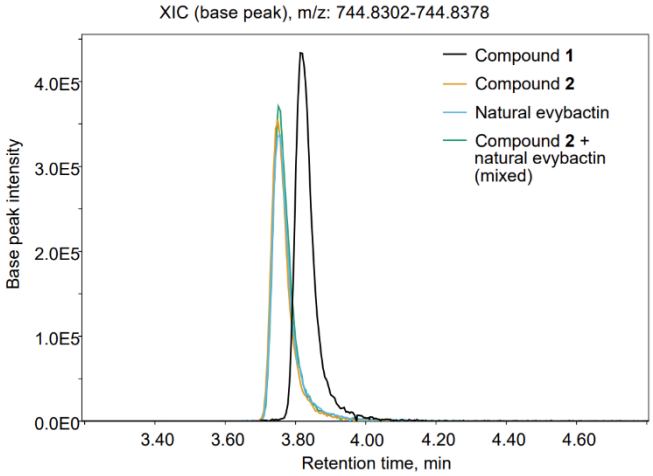



**Figure 2**. Extracted ion chromatograms of authentic evybactin (*m/z* = 744.8344 for $[M+2H]^{2+}$) isolated from fermentation of *P. noenieputensis* overlaid with traces obtained for synthetic compounds **1** and **2**. Compound **2** co-elutes with natural product. Traces for authentic evybactin in blue, compound **1** in black, compound **2** in yellow, and the combination of authentic evybactin + compound **2** in green (detection range for doubly charged species *m/z* = 744.8302–744.8378).

With compound **2** in hand, we next proceeded to assess its antibacterial activity against the same panel of strains previously used for natural evybactin and compound **1**. We were delighted to find that the activity profile of compound **2** completely matches that of authentic evybactin (**Table 1**). In addition, the NMR spectra (see supplemental **Figures S1-S3**) and MS data obtained for compound **2** match those of the natural product along with co-injection experiments (**Figure 2**), further confirming that the structure of evybactin is indeed that of compound **2**.

Having confirmed that the structure of evybactin contains D-His(3-Me) at position 5, we also revisited the previously reported crystal structure of the evybactin-DNA gyrase complex (**Figure 3**).[9] Somewhat surprisingly, the change in stereochemistry at D-His(3-Me)$^5$ was not found to have a significant impact on the modeled conformation of evybactin when bound to gyrase. As indicated by the revised model, it appears that the side chain of D-His(3-Me)$^5$ does not make key contacts with the enzyme, which in turn raises the question as to why there is such a pronounced difference in the antibacterial activity for the position 5 epimers **1** and **2**. A possible explanation may be that the BacA transporter, responsible for the active transport of evybactin into the bacterial cell, may have a strong preference for the natural product. In support of this explanation is the finding that unnatural L-His(3-Me)$^5$ containing **1** does exhibit antibacterial activity against *E. coli* WO153 (MIC = 0.8 µg/mL), a strain with a hyperpermeable outer membrane, along with knockout of the TolC porin to which MDR pumps dock (**Table 1**). While authentic evybactin is clearly more active against this strain (MIC = 0.0625 µg/mL), these findings suggest that if able to gain entry to the cell, the unnatural analogue may also be able to engage with the gyrase in a manner similar to the natural product.

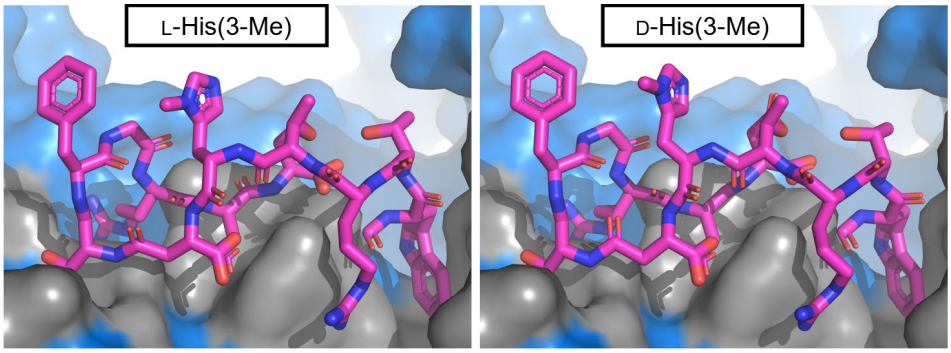



**Figure 3**. Crystal structure of evybactin bound to DNA gyrase from *M. tuberculosis*, modeled with compounds **1** and **2** containing L- vs. D-methyl histidine stereoisomer, respectively (PDBID 7UGW). Gyrase is depicted in light blue surface representation, with the contact surface (residues within 4Å) for evybactin colored in grey. Evybactin structures are illustrated as sticks with carbons in magenta.

## Conclusions

In conclusion, we here report the first total synthesis of the potent antitubercular natural product evybactin. Our synthetic studies led to the reassignment of the stereochemical configuration of the His(3-Me) residue at position 5 to the D-configuration, in contrast to the L-configuration in the originally proposed structure. Synthetic evybactin containing D-His(3-Me) at position 5 was found to have the same antibacterial activity profile as the natural product, along with matching analytical data. Notably, our synthetic route provides a convenient method for the preparation of evybactin at a scale that will enable studies aimed at establishing its therapeutic potential. Furthermore, the convenient SPPS-based route reported here can also provide access to evybactin analogues for SAR studies. The evaluation of evybactin in relevant in vivo models of disease, along with the design and synthesis of novel evybactin analogues, are ongoing in our laboratories and will be reported in due course.

## Acknowledgement

We thank Dr. Jason Guo for the assistance in acquiring and analyzing NMR spectra and Dr. Akira Iinishi for the assistance with LC-MS operations. Financial support was provided by the Dutch Research Council (Open Technology Programme grant to NIM, project no. 19384) and the National Institutes of Health (grant RO1AI170962 to KL).

## Materials and methods

### General information

Extended supporting information, which includes NMR and HPLC figures, is available free of charge at https://doi.org/10.1002/chem.202403767.

**Reagents**

All reagents employed were of American Chemical Society (ACS) grade or higher and were used without further purification unless otherwise stated.

**HRMS**

High-resolution mass spectra (HRMS) analyses were performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 10 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 4 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionization) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000. MZMine 3.2.8 was used to analyze the obtained data.[24]

**Analytical HPLC**

HPLC analyses were performed on a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring at 214 and 254 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 100:0 (A/B) for 3 min, 100:0 to 0:100 (A/B) over 47 min, 0:100 (A/B) for 4 min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 5 min.

**NMR**

$^{1}$H and $^{13}$C NMR spectra were recorded on Bruker AV 400 MHz (at 400.2 ($^{1}$H) and 100.6 ($^{13}$C) MHz), AV 600 MHz (at 600.1 ($^{1}$H) and 150.9 ($^{13}$C) MHz). The temperatures of the NMR experiments were 298 K or 320K. Chemical shifts are reported in ppm (δ) and were calibrated using residual deuterated solvent as an internal reference. (δ $^{1}$H NMR: DMSO 2.50, δ $^{13}$C NMR: DMSO 39.52). The NMR data are processed as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, tt = triplet of triplets, m = multiplet), integration, coupling constants (*J*, reported in Hz) and a number of nuclei. NMR spectra were analyzed and processed using MestreNova version 14.2.0.

**Marfey's analysis**

The stereochemistries of chiral centers present at α carbons were assigned by applying derivatization methods coupled with chromatographic analysis. The advanced Marfey's

method using L-FDAA (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide) established the absolute configurations of amino acids.[19]

The general method for Marfey's analysis (as described):[25]

A sample of authentic evybactin (30 µg) in 6 M HCl (100 µL) was heated to 100 °C in a sealed vial for 15 h, after which the hydrolysate was concentrated to dryness at 40 °C under a stream of dry $N_2$. The hydrolysate was then treated with 1 M $NaHCO_3$ (20 µL) and L-FDAA (1% solution in acetone, 40 µL) at 40 °C for 1 h, after which the reaction was neutralized with 1 M HCl (20 µL). An aliquot of the analyte was diluted 50 times with $H_2O$/MeCN (1:1) and injected (2 µL) into an HRMS instrument following the standard protocol of the analysis (see general methods). The analyte amino acid content was assessed by comparison to authentic standards.

**MIC of synthetic evybactins**

*M. tuberculosis* H37Rv mc$^2$6020 (ΔlysA ΔpanCD) expressing mCherry was grown in complete 7H9 OADC media to mid-log. Serial 2-fold dilutions of evybactins in water were made the day of assay, accounting for a final 10% of total assay volume. Mtb was added to a final OD600 of 0.01 in fresh 7H9 OADC media. Plates were incubated for 7 days at 37 °C prior to analyses via fluorescent plate reader of mCherry fluorescence. MICs were assigned as the concentration where there was at least >90% inhibition relative to negative (water) control values. For MICs against *E. coli* and *S. aureus*, overnight cultures of *E. coli* MG1655, *E. coli* 25922, *E. coli* WO153, and *S. aureus* HG003 in Mueller-Hinton II broth (MHIIB) were diluted 1:100 and cultured at 37 °C with aeration at 220 rpm. After 2 h of incubation, the exponential phase cultures were further diluted to an $OD_{600}$ of 0.001. Then, 98 µl of this diluted culture was added to 96-well plates containing 2 µl of serial 2-fold dilutions of evybactins in water. The activity was evaluated after 20 h of incubation at 37 °C. MICs were defined as the lowest concentration at which no visible cell growth was observed.

**LC-MS/MS**

For LC-MS analyses, compounds were dissolved at a concentration of 15 ug/mL in either $H_2O$/MeCN (1:1) or 0.01 M NaOH (followed by 3h incubation), and 1 µL was injected into Shimadzu Nexera X2 UHPLC system coupled to a Shimadzu 9030 QTOF mass spectrometer, and data acquisition was performed as previously described.[23] All the samples were analyzed in positive polarity, using data-dependent acquisition mode. In this regard, full scan MS spectra (*m/z* 100–1700, scan rate 10 Hz, ID enabled) were followed by two data-dependent MS/MS spectra (*m/z* 100–1700, scan rate 10 Hz, ID disabled) for the two most intense ions per scan. The ions were fragmented using collision-induced dissociation with fixed collision energy (20 eV) and excluded for 1 s before being re-selected for fragmentation. The parameters used for the ESI source were: interface voltage 4 kV, interface temperature 300 °C, nebulizing gas flow 3 L/min, and drying gas flow 10 L/min. MZMine 3.2.8 was used to analyze the obtained data.[24]

**Conversion to the formic acid salt**

To allow for direct NMR comparison with natural product synthetic peptides **1** and **2** were converted from the TFA salt into the formic acid salt using the method below:

7 mg of the TFA salt of the previously purified peptide and 10 mg of the $Na_2CO_3$ were weighed out in the vial and diluted with 4 mL of water and 0.5 mL of the MeCN. The mixture was purified using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm) and equipped with an ECOM Flash UV detector monitoring at 214 nm and 254 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % formic acid in water/acetonitrile 95/5; solvent B, 0.1 % formic acid in water/acetonitrile 5/95. Gradient elution was as follows: 100:0 (A/B) for 3 min, 100:0 to 50:50 (A/B) over 48 min, 50:50 to 0:100 (A/B) over 0.5 min, 0:100 (A/B) for 4 min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 4 min. The fractions containing the product were combined and lyophilized to obtain compounds **1** or **2** as a white fluffy powder in quantitative yield with >95% purity as determined by HPLC.

**Analysis of the biosynthetic gene cluster**

The genome sequence of *Photorhabdus noenieputensis* DSM 25462 (GenBank accession number: GCA_023108895.1) was submitted to antiSMASH 6.0 software.[20] The biosynthetic gene cluster was identified as non-ribosomal peptide synthetases harboring a total of 12 modules by prediction of the adenylation domain specificity. Analysis of module 5, incorporating the Me-His residue, was further analyzed by BlastP to confirm the presence of the methyltransferase domain.

**X-ray crystallography**

Refinement restraints for evybactin containing D-methyl histidine were generated using the ELBOW PHENIX software package. D-methyl histidine was then manually re-fit into electron density using the previous L-methyl histidine evybactin structure as a guide.[9] Refinement was conducted using PHENIX refine, restricted to atoms within evybactin.

**Detailed procedures for synthesis**

**Resin loading**

2-CTCl resin (4 g, 1.51 mmol/g) was loaded by coupling via the free sidechain carboxyl group of Fmoc-Asp-OtBu (4.97 g, 12.08 mmol, 2 eq.) with DIPEA (5.26 mL, 30.2 mmol, 5 eq.) in 70 mL of $CH_2Cl_2$. After 3 h at RT, an extra amount of DIPEA (5.26 mL, 30.2 mmol, 5 eq.) and 24 mol of MeOH were added, and the reaction was left shaking for another 15 minutes. The resin was then filtered, washed with DMF, EtOH, DCM, $Et_2O$, and dried overnight under a stream of $N_2$. The resin loading was then determined to be 0.558 mmol/g.

**Automated solid-phase peptide synthesis**

A CEM Liberty Blue automated peptide synthesizer with microwave irradiation was used to perform SPPS. The following system was used for couplings: 0.05 mmol scale – 5 eq. HBTU (0.25 M in DMF), 10 eq. DIPEA (0.5 M in DMF), 5 eq. of amino acid (0.2 M in DMF); 0.5 mmol scale – 4 eq. HBTU (0.25 M in DMF), 8 eq. DIPEA (0.5 M in DMF), 4 eq. of amino acid (0.2 M in DMF). Fmoc group removal was performed using Piperazine/EtOH/NMP (1:1:9, m/v/v). A detailed overview of the automated protocols can be found below.

**Resin swelling**

The resin was swollen in 10 mL of DMF for 300 s prior to the first coupling.

***Protocol 1: Standard Coupling protocol***

| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |
| **5** | Coupling amino acid | 150 s at 25°C then 750 s at 50°C |

***Protocol 2: 3-Me Histidine coupling protocol***

| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |
| **5** | Coupling amino acid | 1800 s at 25°C then 300 s at 50°C |

***Protocol 3: Arg coupling protocol***

| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |
| **5** | Coupling amino acid | 2400 s at 25°C |
| **6** | Coupling amino acid | 1800 s at 25°C then 300 s at 50°C |

***Protocol 4: L-Ser coupling protocol***

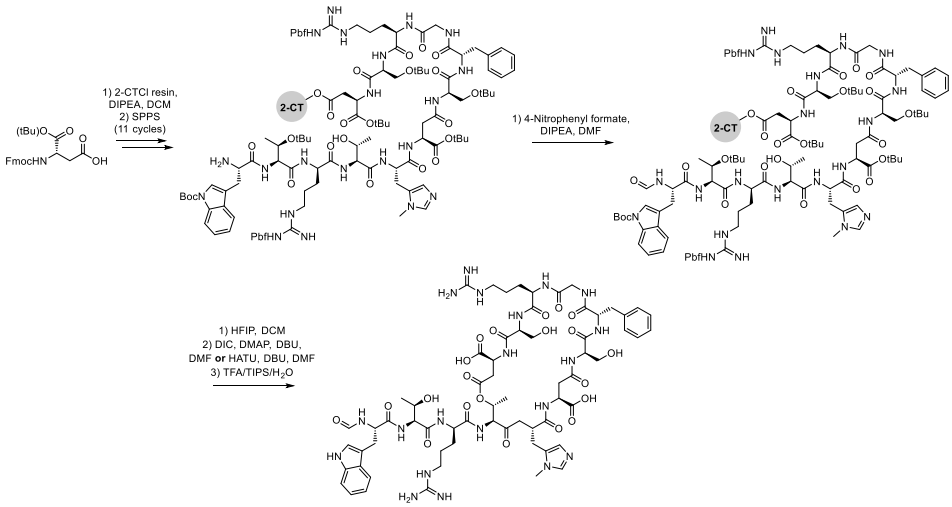
| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 400 s at 25°C |
| **2** | Deprotection N-terminus | 1200 s at 25°C |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |
| **5** | Wash (DMF) | RT |
| **6** | Coupling amino acid | 3600 s at 25°C |



***Protocol 5: Final deprotection N-terminus***

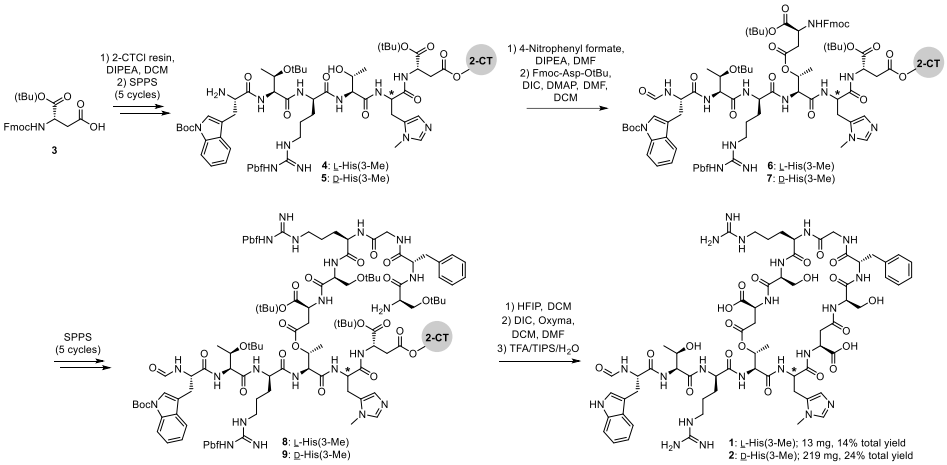
| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |

**Attempted synthesis of compound 1 via linear SPPS strategy**




**Scheme S1.** Initially attempted synthesis of compound **1** that resulted in incomplete conversion and yielded complex mixtures preventing proper purification

## Synthesis of the compounds 1 and 2 – proposed and revised structures of evybactin




**Scheme S2.** SPPS route developed for the synthesis of compounds **1** and **2**

## Procedure for the synthesis of the compound 1 – proposed structure of evybactin

2-CT resin loaded with Fmoc-Asp-OtBu was transferred into a CEM Liberty Blue μwave peptide synthesizer at a 0.05 mmol scale. The amino acids were coupled in the following order: 1) Fmoc-His(3-Me)-OH using protocol 2; 2) Fmoc-Thr-OH using protocol 1; 3) Fmoc-D-Arg(Pbf)-OH using protocol 3; 4) Fmoc-Thr-OH using protocol 1; 5) Fmoc-Trp(Boc)-OH using protocol 1 followed by a final deprotection using protocol 5. The resin was then transferred to a manual reactor for the SPPS connected to the nitrogen flow to perform formylation. After the resin was swollen in DMF (5 mL) for 1 min, 4-nitrophenyl formate (42 mg, 0.25 mmol) and DIPEA (88 mkl, 0.5 mmol) were added. After bubbling with $N_2$ for 30 min, the resin was filtered, washed with DMF (2 × 5 mL), and the formylation procedure was repeated 2 more times. After that, the resin was washed with DMF (2 × 5 mL), DCM (2 × 5 mL) and dried using $N_2$ flow. Fmoc-Asp-OtBu (206 mg, 0.5 mmol), was weighed out in the round-bottom flask and diluted with 4 mL of dry DCM:DMF mixture (1:1), followed by the addition of DIC (79 mkl, 0.5 mmol). The mixture was left shaking for 1 h under an Ar atmosphere. After that, DMAP (2.44 mg, 0.02 mmol) was added to the flask, followed by the addition of the dried resin after formylation. The reaction was left shaking overnight at RT under an Ar atmosphere. After the reaction was finished the resin was filtered, washed with DMF (2 × 5 mL), DCM (2 × 5 mL) and transferred to the CEM Liberty Blue μwave peptide synthesizer to perform the next couplings in the following order: 1) Fmoc-Ser(tBu)-OH using protocol 4; 2) Fmoc-D-Arg(Pbf)-OH using protocol 3; 3) Fmoc-Gly-OH using protocol 1; 4) Fmoc-Phe-OH using protocol 1; 5) Fmoc-D-Ser(tBu)-OH using protocol 1 followed by final deprotection using protocol 5. The peptide was then detached from the resin with 6 mL of the 20% HFIP in DCM. The solvents were removed by rotary evaporation, yielding the protected peptide, which was used directly in the next step.

Dry DMF (10 mL) was poured into the round-bottom flask containing a stirring bar, protected peptide (0.05 mmol), and Oxyma (42.6 mg, 0.3 mmol), followed by the addition of dry DCM (60 mL). The DIC (47 mkl, 0.3 mmol) was added to the resulting mixture, and the reaction was left stirring overnight. The next day, the solvents were evaporated under reduced pressure, and the residue was diluted with water (20 mL) and extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with 1 M $NaHCO_3$ (25 mL), water (25 mL), brine (20 mL), dried under $Na_2SO_4$, and concentrated using rotary evaporation. Final sidechain deprotection was carried out by treating the obtained powder with 3 mL of the TFA/TIPS/$H_2O$ (95:2.5:2.5) mixture for 2.5 h. The reaction mixture was precipitated in MTBE/PE (1:1) and centrifuged (4500 rpm, 5 min). The pellet was then resuspended in MTBE/PE (1:1) and centrifuged again (4500 rpm, 5 min). Finally, the pellet containing the crude peptide was dissolved in $H_2O$/MeCN (95:5) and lyophilized overnight.

The peptide was purified using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm) and equipped with an ECOM Flash UV detector monitoring at 214 nm and 254 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 100:0 (A/B) for 3 min, 100:0 to 50:50 (A/B) over 48 min, 50:50 to 0:100 (A/B) over 0.5 min, 0:100 (A/B) for 4 min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 4 min. The fractions containing the product were combined and lyophilized to obtain compound **1** as a white fluffy powder (13 mg, 14% yield, calcd for 3×TFA salt) with >95% purity as determined by HPLC.

**HRMS** (ESI) *m/z* calcd for $C_{64}H_{89}N_{21}O_{21}+2H^+$: 744.8344 ($[M+2H]^{2+}/2$); found: 744.8344.

**Procedure for the synthesis of the compound 2 – revised structure of evybactin**

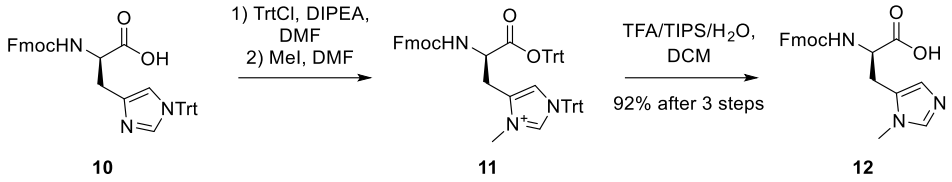
2-CT resin loaded with Fmoc-Asp-OtBu was transferred into a CEM Liberty Blue μwave peptide synthesizer at a 0.5 mmol scale. The amino acids were coupled in the following order: 1) Fmoc-D-His(3-Me)-OH using protocol 2; 2) Fmoc-Thr-OH using protocol 1; 3) Fmoc-D-Arg(Pbf)-OH using protocol 3; 4) Fmoc-Thr-OH using protocol 1; 5) Fmoc-Trp(Boc)-OH using protocol 1 followed by a final deprotection using protocol 5. The resin was then transferred to a manual reactor for the SPPS, connected to the nitrogen flow to perform formylation. After the resin was swollen in DMF (20 mL) for 1 min, 4-nitrophenyl formate (420 mg, 2.5 mmol) and DIPEA (880 mkl, 5 mmol) were added. After bubbling with $N_2$ for 20 min, the resin was filtered, washed with DMF (2 × 20 mL), and the formylation procedure was repeated 1 more time. After that, the resin was washed with DMF (2 × 20 mL), DCM (2 × 20 mL) and dried using $N_2$ flow. Fmoc-Asp-OtBu (2.06 g, 5 mmol) was weighed out in the round-bottom flask and diluted with 30 mL of dry DCM/DMF mixture (1:1), followed by the addition of DIC (790 mkl, 5 mmol). The mixture was left shaking for 20 min under an Ar atmosphere. After that, DMAP (12.2 mg,

0.1 mmol) was added to the flask, followed by the addition of the dried resin after formylation. The reaction was left shaking overnight at RT under an Ar atmosphere. After the reaction was finished the resin was filtered, washed with DMF (2 × 20 mL), DCM (2 × 20 mL) and transferred to the CEM Liberty Blue μwave peptide synthesizer to perform the next coupling in the following order: 1) Fmoc-Ser(tBu)-OH using protocol 4; 2) Fmoc-D-Arg(Pbf)-OH using protocol 3; 3) Fmoc-Gly-OH using protocol 1; 4) Fmoc-Phe-OH using protocol 1; 5) Fmoc-D-Ser(tBu)-OH using protocol 1 followed by final deprotection using protocol 5. The peptide was then detached from the resin with 25 mL of the 20% HFIP in DCM. The solvents were removed by rotary evaporation, yielding the protected peptide, which was used directly in the next step.

Dry DMF (2.5 mL) was poured into the round-bottom flask containing a stirring bar, protected peptide (0.5 mmol), and Oxyma (214 mg, 1.5 mmol), followed by the addition of dry DCM (500 mL). The DIC (235 mkl, 1.5 mmol) was added to the resulting mixture, and the reaction was left stirring overnight under an Ar atmosphere. The next day, the solvents were evaporated under reduced pressure, and the residue was treated with 20 mL of the TFA/TIPS/$H_2O$ (95:2.5:2.5) mixture for 2.5 h. The reaction mixture was precipitated in MTBE/PE (1:1) and centrifuged (4500 rpm, 5 min). The pellet was then resuspended in MTBE/PE (1:1) and centrifuged again (4500 rpm, 5 min). Finally, the pellet containing the crude peptide was dried with $N_2$ flow and then redissolved in water and purified using a BUCHI Pure C-815 Flash system with a BUCHI EcoFlex C18 50μm spherical 80g column and equipped with UV detector monitoring at 214 nm and 254 nm. The following solvent system, at a flow rate of 60 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 100:0 (A/B) for 3 min, 100:0 to 50:50 (A/B) over 40 min, 50:50 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 4 min, then reversion back to 100:0 (A/B) over 1.5 min, 100:0 (A/B) for 7 min. The fractions containing the product were combined and lyophilized to obtain compound **2** as a white fluffy powder (219 mg, 24% yield, calcd for 3×TFA salt) with >95% purity as determined by HPLC.

**HRMS** (ESI) *m/z* calcd for $C_{64}H_{89}N_{21}O_{21}+2H^+$: 744.8344 ($[M+2H]^{2+}/2$); found: 744.8350.

**Procedure for the synthesis of the Fmoc-D-His(3-Me)-OH (12)**




**Scheme S3:** Route used for the synthesis of compound **12** (Fmoc-D-His(3-Me)-OH)



Fmoc-D-His(Trt)-OH (10 g, 16.1 mmol) was weighed out in the 100 mL round-bottom flask. The compound was dissolved with 50 mL of DMF, and then DIPEA (8.44 mL, 48.4 mmol) and TrtCl (9.9 g, 35.5 mmol) were added at RT. The mixture was left stirring for 3h, and after that, MeI (5.02 mL, 80.7 mmol) was added. The reaction was left stirring overnight. The next day, the mixture was poured into 300 mL of water, and the formed precipitate was filtered. The precipitate was then dissolved in DCM (200 mL) and brine (50 mL) and extracted. The organic layer was collected, dried under $Na_2SO_4$, and concentrated at reduced pressure to afford compound **11,** which was used directly in the next step without further purification.

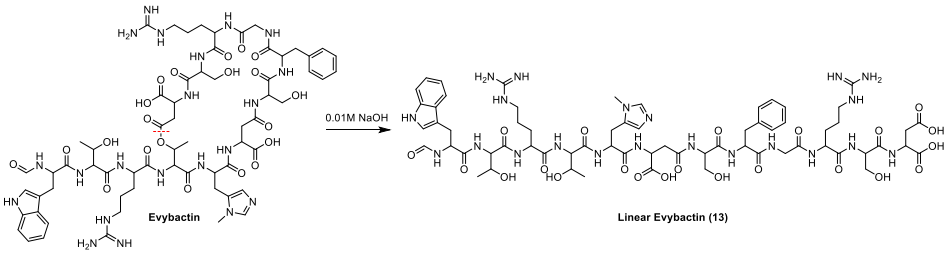
The material from the previous step was dissolved in 100 mL of DCM, and then 100 mL of cleavage cocktail (TFA/TIPS/$H_2O$ = 95:2.5:2.5) was added. The reaction was left stirring for 3h. After that, the volume was reduced to 20 mL via evaporation, and the resulting mixture was poured into 300 mL of cold MTBE/PE (1:1) mixture. The formed precipitate was filtered, washed with MTBE (3 × 100 mL), and dried to obtain TFA salt of Fmoc-D-His(3-Me)-OH (**12**) (7.5 g, 92% overall yield, calcd for 1×TFA salt) as a brown powder.

**$^1$H NMR** (400 MHz, DMSO-$d_6$, 298K): δ 14.49 (s, 1H), 13.34 (s, 1H), 9.01 (s, 1H), 7.89 (d, J = 7.6 Hz, 2H), 7.86 (d, J = 8.4 Hz, 1H), 7.69 – 7.62 (m, 2H), 7.41 (tt, J = 7.5, 1.7 Hz, 2H), 7.38 (d, J = 1.5 Hz, 1H), 7.32 (tt, J = 7.5, 1.4 Hz, 2H), 4.31 – 4.38 (m, 1H), 4.31 (d, J = 1.3 Hz, 1H), 4.29 (s, 1H), 4.20 (t, J = 6.9 Hz, 1H), 3.78 (s, 3H), 3.19 (dd, J = 15.9, 4.6 Hz, 1H), 3.02 (dd, J = 15.8, 10.1 Hz, 1H).

**$^{13}$C{1H} NMR** (101 MHz, DMSO-$d_6$, 298K) δ 172.2, 156.0, 143.8, 143.7, 140.8, 135.6, 131.2, 127.7, 127.1, 125.2, 120.2, 117.7, 65.7, 52.3, 46.6, 33.1, 24.8.

**HRMS** (ESI) *m/z* calcd for $C_{22}H_{21}N_3O_4+H^+$: 392.1605 $[M+H]^+$; found: 392.1617.

# Supplementary information




**Scheme S4.** Hydrolysis reaction of evybactin in a prepared HRMS sample, 0.01 M solution of NaOH.

**Table S1.** Comparison of the synthetic compound **1** to an authentic evybactin sequence before and after hydrolysis with NaOH, using the combination of nearest-neighbor relationships (di-, tripeptide fragments) obtained. CO = Formyl group; H* = His(3-Me).

| Dipeptides | | | | | | | | | | | | Calc. | Found | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | **1** | **1** (NaOH) | Authentic evybactin | Authentic evybactin (NaOH) |
| CO | W | | | | | | | | | | | 215.0815 | 215.8013 | 215.0816 | 215.0817 | 215.0816 |
| | W | T | | | | | | | | | | 288.1343 | 288.1333 | 288.1355 | 288.1333 | 288.1339 |
| | | T | R | | | | | | | | | 258.1561 | 258.1536 | 258.1574 | 258.1562 | 258.1562 |
| | | | R | T | | | | | | | | 258.1561 | 258.1536 | 258.1574 | 258.1562 | 258.1562 |
| | | | | T | H* | | | | | | | 253.1295 | 253.1303 | 253.1283 | 253.1299 | 253.1301 |
| | | | | | H* | D | | | | | | 267.1088 | 267.1088 | 267.1087 | 267.1090 | 267.1090 |
| | | | | | | D | S | | | | | 203.0662 | 203.0661 | 203.0657 | 203.0668 | 203.0667 |
| | | | | | | | S | F | | | | 235.1077 | 235.1078 | 235.1076 | 235.1091 | 235.1085 |
| | | | | | | | | F | G | | | 205.0972 | 205.0999 | 205.0982 | 205.0975 | 205.0955 |
| | | | | | | | | | G | R | | 214.1299 | 214.1285 | 214.1315 | 214.1291 | 214.1278 |
| | | | | | | | | | | R | S | 244.1404 | 244.1416 | 244.1421 | 244.1405 | 244.1418 |
| | | | | | | | | | | | S | 203.0662 | 203.0661 | 203.0657 | 203.0668 | 203.0667 |

**Table S2.** Comparison of the synthetic compound **1** to an authentic evybactin sequence before and after hydrolysis with NaOH, using the combination of nearest-neighbor relationships (di-, tripeptide fragments) obtained. CO = Formyl group; $H^*$ = His(3-Me).

| Tripeptides | | | | | | | | | | | | Calc. | Found | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | **1** | **1** (NaOH) | Authentic evybactin | Authentic evybactin (NaOH) |
| CO | W | T | | | | | | | | | | 316.1292 | 316.1298 | 316.1290 | 316.1307 | 316.1293 |
| | W | T | R | | | | | | | | | 444.2354 | 444.2349 | 444.2332 | 444.2336 | 444.2352 |
| | | T | R | T | | | | | | | | 359.2037 | NF | NF | NF | NF |
| | | | R | T | $H^*$ | | | | | | | 409.2306 | 409.2327 | 409.2325 | 409.2321 | 409.2303 |
| | | | | T | $H^*$ | D | | | | | | 368.1565 | 368.1599 | 368.1570 | 368.1562 | 368.1570 |
| | | | | | $H^*$ | D | S | | | | | 354.1408 | 354.1432 | 354.1381 | 354.1396 | 354.1441 |
| | | | | | | D | S | F | | | | 350.1347 | NF | NF | NF | NF |
| | | | | | | | S | F | G | | | 292.1292 | 292.1292 | 292.1290 | 292.1297 | 292.1292 |
| | | | | | | | | F | G | R | | 361.1983 | 361.1976 | 361.1979 | 361.1982 | 361.1976 |
| | | | | | | | | | G | R | D | 301.1619 | 301.1621 | 301.1652 | 301.1629 | 301.1635 |
| | | | | | | | | | | R | S | D | 359.1674 | 359.1681 | 359.1686 | 359.1671 | 359.1689 |

NF – not found

**Table S3.** Retention times ($t_R$, min) of the FDAA derivatives for authentic evybactin and standard amino acids.

| **Amino acid** | $[M+H]^+$ | $t_R$, min | | | |
|---|---|---|---|---|---|
| | | **L-AA** (standard) | **D-AA** (standard) | Authentic evybactin | Stereochemical assignment |
| **Trp** | 457.1466 | 6.37 | 6.63 | NF | **NF** |
| **Thr** | 372.1150 | 4.8 | 5.18 | 4.8 | **L** |
| **Arg** | 427.1684 | 4.27 | 4.23 | 4.23 | **D** |
| **His(3-Me)** | 422.1419 | 4.05 | 3.96 | 3.96 | **D** |
| **Asp** | 386.0943 | 4.81 | 4.91 | 4.81 | **L** |
| **Ser** | 358.0994 | 4.66 | 4.72 | 4.66/4.72 | **L and D** |
| **Phe** | 418.1357 | 6.51 | 6.9 | 6.51 | **L** |
| **Gly** | 328.0888 | 5.08 | 5.08 | 5.08 | **NR** |

NF – not found

NR – not relevant

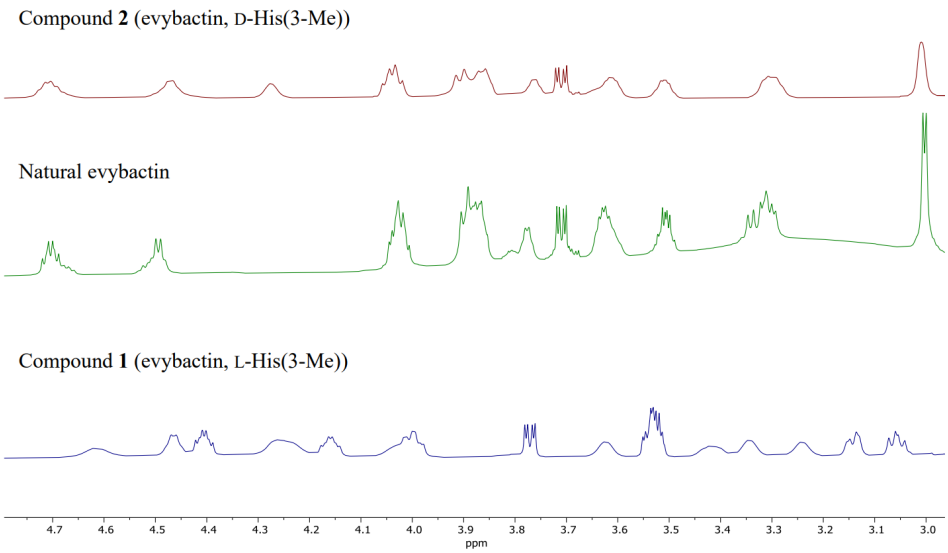



**Figure S1.** Alpha proton region (4.8 – 3.0 ppm) of the previously published $^{1}$H-NMR (700 MHz, DMSO-$d_6$, 320K) spectrum of natural evybactin (as formic acid salt) isolated after fermentation of the producing organism overlaid with $^{1}$H-NMR (600 MHz, DMSO-$d_6$, 320K) spectra of synthetic compounds **1** and **2** (as formic acid salts).

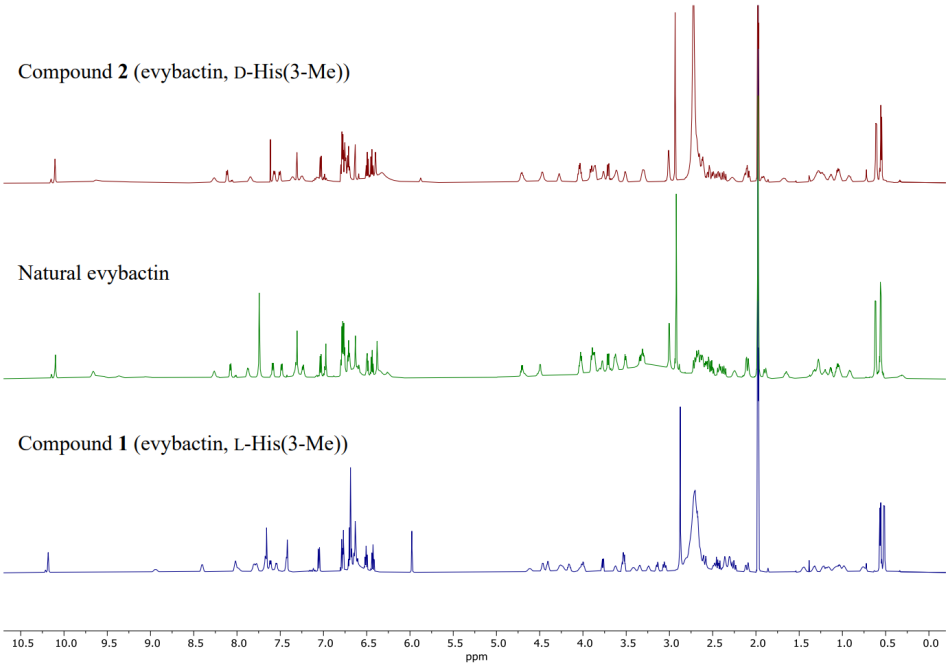



**Figure S2.** Full comparison (10.5 – 0 ppm) of the previously published $^{1}$H-NMR (700 MHz, DMSO-$d_6$, 320K) spectrum of natural evybactin (as formic acid salt) isolated after fermentation of the producing organism overlaid with $^{1}$H-NMR (600 MHz, DMSO-$d_6$, 320K) spectra of synthetic compounds **1** and **2** (as formic acid salts).

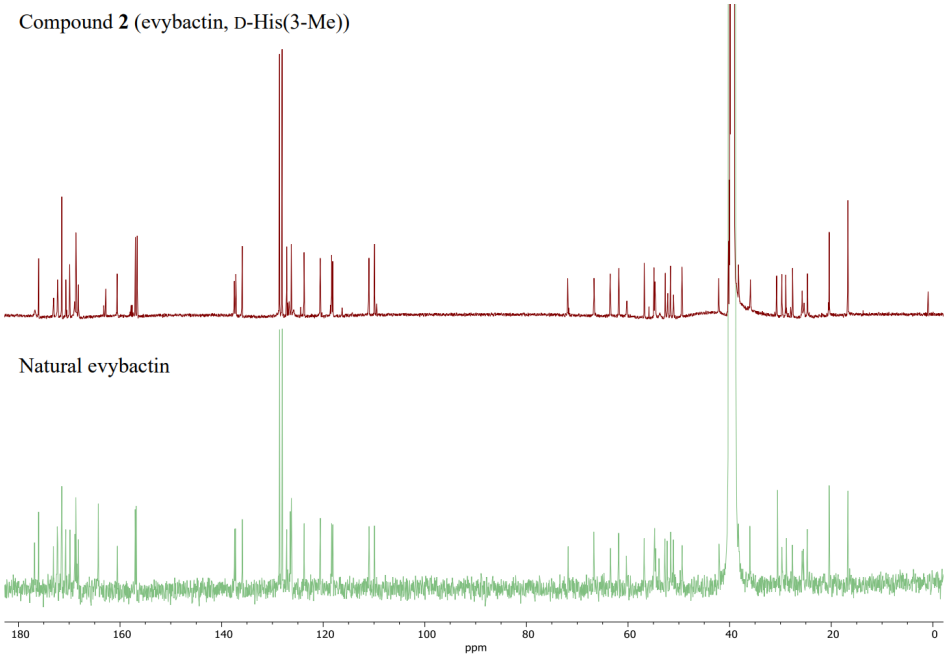



**Figure S3.** Full comparison (180 – 0 ppm) of the previously published $^{13}$C-NMR (101 MHz, DMSO-$d_6$, 320K) spectrum of natural evybactin (as formic acid salt) isolated after fermentation of the producing organism overlaid with $^{1}$H-NMR (151 MHz, DMSO-$d_6$, 320K) spectra of synthetic compound **2** (as a formic acid salt).

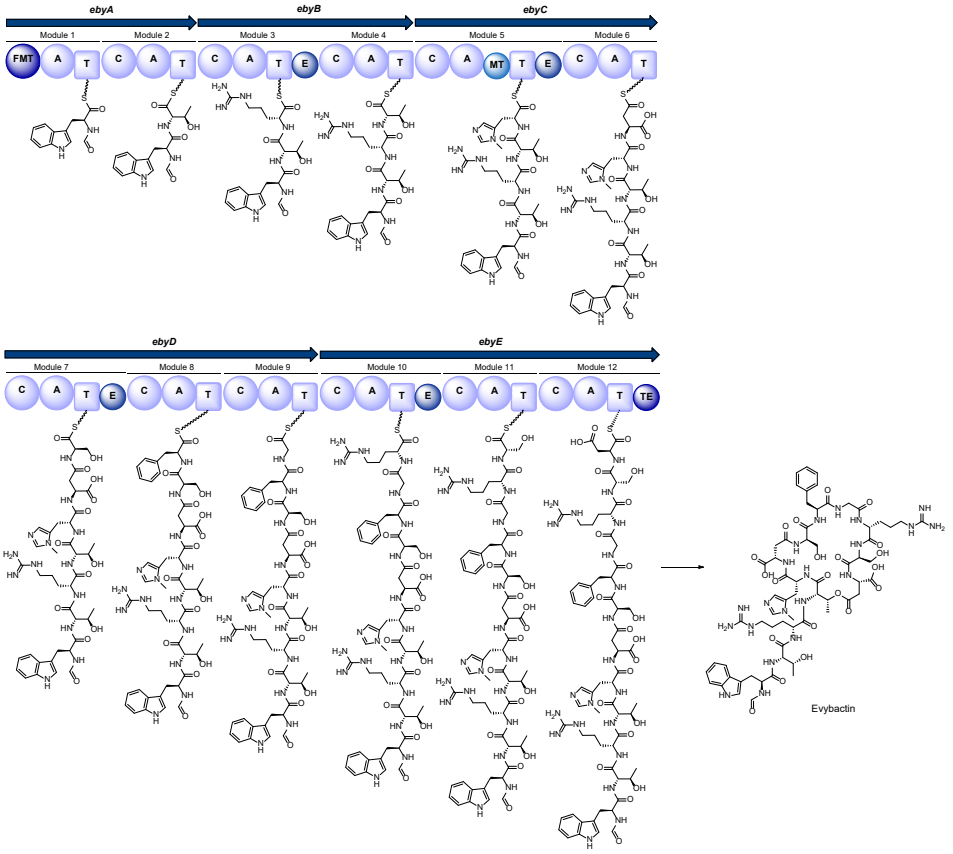



**Figure S4.** Schematic representation of evybactin biosynthesis (C, condensation; A, adenylation; T, thiolation; E, epimerization; MT, methyltransferase; TE, thioesterase; FMT, formyltransferase domain).

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