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Stendomycin and Pantomycin are Identical Natural Products – Preparation of a Functionalized Bioactive Analog

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Abstract - Graphic

Stendomycin = Pantomycin

Abstract

The natural products pantomycin and stendomycin were both reported as antimicrobial agents. We demonstrate by gene cluster analysis, LC-MS-analysis, and isolation that these polypeptides are identical, and we identify previously unknown congeners. We show that stendomycin can be chemically modified at its electrophilic dehydrobutyrine moiety yielding the first bioactive analog of this natural product which can undergo additional functionalization. This compound may be a valuable starting point for molecular probe development and we invite its distribution to the scientific community.

The world is standing on the verge of an antibiotics crisis with the continued rise in clinical cases of super-resistant microorganisms.¹ This most discomforting situation is the result of decades of insufficient regulation of the use of the antibiotics arsenal that has been assembled since the 1950s. Within anti-bacterials, the challenges are substantial and cases have even been reported of resistance to the polymyxins, normally considered to be anti-bacterial agents of last resort.² Multi-drug-resistant fungal infections are also increasing in prevalence, especially of the genus *Candida*, which is already a major concern in US hospitals.³ Immunocompromised patients are at particular risk of acquiring these systemic fungal infections. In recognition of the gravity of the situation, tighter regulatory measures are being passed in many countries, but it will take significant time to implement these. It is imperative that the scientific community re-focuses on this area to mitigate the duration of a future scenario where normally uncomplicated infectious diseases simply may not have an effective therapeutic option.

During the hay-days of antibiotics discovery in 1950-70s, many natural products were shelved and forgotten, as they – at the time – did not favourably compare to those that eventually became the major antibiotics.⁴ Some of these compounds could possess an untapped potential for discovery of novel therapeutics with activity towards resistant bacterial and fungal strains. Bearing in mind recent examples demonstrating how chemical diversification of well-known scaffolds can lead to novel antibiotics,⁵ it should be a high priority to systematically re-investigate all previously isolated complex natural products with antibiotic activity, in particular those compounds that appear to exploit non-standard mechanisms. Such studies can however be challenged by incomplete structural information, which will hamper attempts at re-isolation of the original natural product for evaluation. Here, we turn the attention to two lipodepsipeptide antibiotics, pantomycin and stendomycin, that have been subject to significant structural confusion.

Pantomycin was reported in 1979 as an antifungal, antibacterial, and antiviral agent.⁶ The compound was isolated from *Streptomyces hygroscopicus* NRRL 2751 and displayed activity towards a panel of plant and animal pathogens, notably clinically relevant fungi such as *Candida albicans*. The structure of pantomycin remained unclear, but the limited spectroscopic data available suggested significant structural resemblance to a known natural product, stendomycin.⁷ Stendomycin was isolated in 1963 from *Streptomyces endus* and identified as an antimicrobial agent with predominant anti-fungal activities.⁸ It is an acylated tetradecapeptide with seven D-amino acids, a dehydrobutyrine, a modified L-arginine (L-stendomycidine⁹), and a macrolactone (Figure 1a).¹⁰ The compound exists as a microheterogeneous mixture – varying by acyl chain length and at some of the hydrophobic residues. The ratio of the different congeners depends on the growth media, but the antibiotic activity remains unaltered.¹¹

Stendomycin was rediscovered in 2011; isolated from *Streptomyces himastatinicus* ATCC 53653.¹² The biosynthetic machinery was identified, and six structures were identified – stendomycin I-VI (supporting information Figure S2). In 2017, Filipuzzi and co-workers identified stendomycin as a selective inhibitor of the TIM23 complex (translocase of the inner membrane) in yeast and mammalian cells, thus inhibiting TIM23-dependent mitochondrial protein import.¹³ Stendomycin is the only known small molecule to modulate the function of TIM23, making it a valuable tool compound. Intrigued by the complex chemical scaffold of stendomycin, the unclarified relationship to pantomycin and the biological activities reported for both compounds, we set out to deconvolute their relation.

Figure 1| Stendomycin and pantomycin comparative analyses. a) Structure of stendomycin (pantomycin). **b)** *Streptomyces hygroscopicus* NRRL 2751 (pantomycin producing) and *Streptomyces himastatinicus* ATCC 53653

(stendomycin producing) on ISP-2 agar. **c)** MS spectra from bacterial extracts and MS fragmentation of 1629 m/z. **d)** Alignment of the biosynthetic gene cluster from NRRL 2751, ATCC 53653 and *Streptomyces* sp. RTd22. Red: NRPS, orange: proposed stendomycin biosynthesis,¹² purple: transport, green: sensing, regulators and unknowns.

The pantomycin producing strain *Streptomyces hygroscopicus* NRRL 2571 and stendomycin producing strain *Streptomyces himastatinicus* ATCC 53653 were cultivated on agar and in liquid media (Figure 1b). The physical appearance of the strains clearly differed in both colony morphology and growth rates on several tested agars. However, LC-MS/MS analysis of organic extracts from both liquid cultures revealed identical collections of masses (1587, 1601, 1615, 1629) matching those reported for the known congeners (I-VI) of stendomycin (Figure S4-S11). MS fragmentation of 1629 m/z from both samples reveal an identical fragmentation pattern in agreement with stendomycin I. To further verify the LC-MS-data, the mixture of congeners was isolated from NRRL 2571 – the original pantomycin producing bacteria – and was found to match the NMR and LC-MS/MS fragmentation data previously reported for stendomycin (supporting information Table S1).¹² In addition to the known masses, we observed a series of features - most notably 1573 and 1643 – that could correspond to previously unknown congeners (Figure 1c). Extensive fragmentation analysis was confirmatory revealing a larger ensemble of stendomycin congeners than previously reported, with seven possible acyl chains and three variable amino acids. The details of stendomycin heterogeneity is discussed further in the supporting information.

Finally, we sequenced the complete genome of NRRL 2571 followed by a detection of the biosynthetic gene clusters (BGCs) using the antiSMASH genome mining tool.¹⁴ This analysis identified a BGC closely matching the known stendomycin gene cluster from ATCC 53653 with most genes showing 95% identity or more (Figure 1d). BLAST analysis of selected genes also identified *Streptomyces* sp. RTd22 – an endophyte isolated from a Mexican sunflower *Tithonia diversifolia* – as a likely producer of stendomycin.¹⁵

An interesting observation originally reported for pantomycin was the apparent presence of carbohydrate(s) due to a positive Molisch test; one of the key differences from stendomycin.⁷ We repeated this test on our isolated material but were unable to reproduce this result. We cannot conclusively account for this anomaly as our isolation protocols differ to some extent from those originally employed by Gurusiddaiah and co-workers.⁶ Interestingly, we have identified two conserved genes in the vicinity of all three biosynthetic gene clusters that are predicted glycosyl transferases, but we have not observed any compounds by MS that show microheterogeneity and which could correspond to glycosylated forms (searched mass range up to 2500 Da). Whether these putative glycosyl transferases have any functional implications therefore remains to be demonstrated. Collectively, this set of experiments conclusively determined that pantomycin and stendomycin are structurally identical natural products and further that this is the result of near identical biosynthetic gene clusters being present in the two producing microorganisms.

Stendomycin (**1**) has previously been subjected to modifications – lactone hydrolysis, methyl ester formation, alcohol per-methylation and alcohol per-acetylation – and in all cases the resulting compounds were devoid of antibiotic activity.¹¹ Dehydrobutyrine hydrogenation^{7,10} and alcohol oxidation¹⁰ was performed as part of the structure elucidation. Dihydro-stendomycin had 2.5-fold decreased activity, the oxidized analog was never evaluated. Bodanszky and co-workers reported addition of thioglycolic acid to the dehydrobutyrine of stendomycin, but the entire peptide was hydrolyzed without biological evaluation.¹⁶ Interestingly, based on that analysis, thiol-addition happens stereoselectively although the precise stereochemical outcome was not resolved. Integrating these two pieces of information regarding the dehydrobutyrine group, we hypothesized that functionalization could be tolerated at this position without compromising the biological activity. Given the recently discovered unique ability of stendomycin to inhibit the mitochondrial TIM23-complex in fungi and mammalian cells, such agents would be valuable for further investigations into the molecular details. We therefore attempted to conjugate 2-(Boc-amino)ethanethiol to stendomycin **1** under alkaline conditions to yield stendomycin thiol-conjugate **2**. Aqueous sodium bicarbonate resulted in lactone hydrolysis along with conjugation,

but we discovered that sodium phosphate buffered conditions resulted in thiol conjugation without affecting the lactone (Figure 2). Given identification of appropriate deprotection conditions, analog **2** could be a versatile compound for further installation of affinity/bioorthogonal tags or fluorophores to the stendomycin scaffold. Following some optimization, we found that the Boc-group could be removed by treatment with TFA in CH₂Cl₂ leaving the amine **3** as the TFA salt, and we demonstrated that the amine could be selectively conjugated to an NHS ester as exemplified with preparation of stendomycin-biotin **4**. We also performed a hydrolysis to stendomycin acid **5** under alkaline conditions as previously reported.¹⁰ Attempts to proteolytically cleave stendomycin **1** with subtilisin A, elastase, and pronase E were unsuccessful, which we attribute to the presence of D-amino acids in the linear portion of the molecule and the macrocycle (data not shown).

Figure 2 | Chemical functionalization of stendomycin. Thiol conjugation selectively to the dehydrobutyrine unit enables instalment of tag/handle.

The TIM23 complex is evolutionarily conserved from yeast to mammalian cells. We evaluated stendomycin **1** for its ability to reduce viability of a panel of human cancer cell lines (breast, lung, cervix, pancreas, Figure 3) and found that although inhibitory activity was observed in all cases, a surprisingly large span (17 fold) was observed between the most sensitive (A549) and least sensitive (HCC827) cell line. Given the central function of TIM23 for mitochondrial protein import, differential sensitivity is somewhat unexpected. These results indicate that it would be important to identify molecular predictors for stendomycin sensitivity, in particular as the precise TIM23-inhibitory mechanism remains unclear and that stendomycin also displays a more abrupt effect on the mitochondrial membrane at low micromolar concentrations (in HeLa cells).¹³ We next evaluated analogs **2**, **4**, and **5** in A549 cells. As expected, opening of the lactone renders compound **5** inactive. Gratifyingly, in accord with our original hypothesis, thiol conjugate **2** turned out almost equipotent to **1** which strongly suggests that the portion around the dehydrobutyrine-motif is e.g. not involved in key target interactions and can tolerate significant steric bulk. Biotin-conjugate **4** displayed >20-fold reduced activity, which is surprising given the tolerance to the large carbamate group in **2**. This result will require further scrutiny, however biotin-conjugation has previously been observed to reduce cellular penetration.¹⁷ All in all, thiol-conjugate **2** appears to provide a flexible starting point for making conjugations to the stendomycin scaffold.

Figure 3 | Effects of stendomycin and analogs on cancer cell viability. **a)** Cytotoxicity of stendomycin in 7 cell lines after 48 h treatment. **b)** Cytotoxicity of stendomycin (1) and analogs (2,4,5) in A549 cells after 72 h treatment. All data points represent mean $\pm$ standard deviation ($n=3$) and are normalized to the two lowest dosings of compound.

In conclusion, the identity of stendomycin and pantomycin demonstrated here - by logic - fuses the biological data reported for these compounds. As already mentioned, pantomycin (stendomycin) was demonstrated to have anti-viral activity and furthermore to inhibit myogenesis – muscle cell differentiation – at non-toxic concentrations.¹⁸ While we have not addressed the potential anti-viral effects, we have so far been unable to reproduce the anti-myogenic activity using H9C2 rat myoblasts (data not shown). This cellular model differs from that originally reported, but this negative result at least indicate that the effect is not general. What stands more firm is the anti-fungal properties of stendomycin and here it would be of interest to evaluate the compound against e.g. azole-resistant *Candida* strains which is an emerging clinical issue. As further details on the TIM-23 inhibitory mechanism emerge, optimization of the structure to increase the anti-fungal selectivity may also be possible.

Experimental Section

Fermentation:

Growth media:

Pantomycin growth medium (PGM)⁶: Yeast extract 5 g/L, corn-steep liquor 10 mL/L, glucose 20 g/L, pH 6.5, then add CaCO_3 1 g/L.

Pantomycin production medium (PPM)⁶: Soy hydrolysates 30 g/L, corn-steep liquor 10 mL/L, glucose 10 g/L, yeast extract 5 g/L, pH 6.5, then add CaCO_3 1.5 g/L.

ISP-2 medium: Yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L, pH=7.3

M8 medium: Soluble starch 20 g/L, glucose 10 g/L, yeast extract 2 g/L, meat extract 2 g/L, hydrolyzed casein 4 g/L, CaCO_3 3 g/L, pH 7.

Streptomyces hygroscopicus NRRL 2571 was obtained from USDA ARS culture collection.

Streptomyces himastatinicus ATCC 53653 was obtained from ATCC.

NRRL 2571 and ATCC 53653 were both grown on ISP-2 agar (15 g agar per liter) at 28 °C. A container with water was kept in the incubator to keep the agar from drying. Sporulation was observed after 20 days. A spore stock in glycerol was created and applied for any subsequent inoculations.

Medium dependent heterogeneity:

Streptomyces hygroscopicus NRRL 2571 and *Streptomyces himastatinicus* ATCC 53653 were grown in 2 x 50 mL liquid cultures of PGM, PPM, ISP-2, and M8 at 28 °C, 160 rpm for four days. The cultures were extracted with EtOAc (1:1 relative to medium) and concentrated. Analytical

LC-MS analysis confirmed production of stendomycin with a congener mixture dependent on the media. The concentrated extracts were dissolved in MeOH/H₂O 1:1, and NaOH (0.2 M) was added. After stirring for 1.5 hours at ambient temperature, the samples were analyzed by LC-MS/MS. Lactone hydrolysis is required for sufficient peptide fragmentation. See supporting info for data.

LC-MS/MS analysis:

Extract samples in MeOH were analyzed by LC-MS; C8, 2.1 mm x 15 cm, 2.7 μ m, column temperature of 40 °C, 0.4 mL/min, water w. 0.1% formic acid (A), MeOH/MeCN 1:1 w. 0.1% formic acid (B). Gradient: 5% B (1 min), 5% to 95% B (16 min), hold 95% B (5 min). Mass range 50-2500 m/z.

Genome sequencing:

DNA was extracted from *Streptomyces hygroscopicus* NRRL 2571 using the Qiagen Blood and Cell Culture DNA Maxi Kit following standard protocols and adding both Protease and Proteinase K as suggested. The library preparation, PacBio sequencing, assembly, and annotation was carried out by Macrogen Inc.

Stendomycin Isolation:

Starting from *Streptomyces hygroscopicus* NRRL 2571 grown on ISP-2 agar, a liquid culture was created. The bacteria grow in millimeter sized clumps at 28 °C and shaking at 160 rpm. The culture was stepwise diluted with ISP-2 media every other day until the total volume was 600 mL. From this culture was taken 100 mL into each of 6 x 5 L flasks with indents containing 2 L M8 media. The cultures were incubated at 28 °C at 120 rpm. After 11 days, the 12.6 L culture was extracted with EtOAc, and concentrated to dryness. Preparative LC-MS followed by lyophilization yielded stendomycin-TFA salt (175 mg) as a white solid in a mixture of congeners. LC-MS conditions: C18 3x25cm, 25 mL/min, UV; 254 nm and 210, H₂O/MeCN 0.1% TFA, gradient; 50% to 90% MeCN (25 min), hold (6 min), 90% to 100% MeCN (1 min), hold (4 min). Stendomycin **1** eluted from 26-31 min. NMR data and LC-MS/MS fragmentation series matched what have been reported in the literature.¹²

R_f 0.22 (CH₂Cl₂/MeOH) 9:1, UV and KMnO₄. The spot streaks.

HRMS

ESI-TOF m/z [M+H]⁺

Calcd.: C₇₅H₁₃₀N₁₇O₁₉⁺ 1572.9723; found 1572.9729.

Calcd.: C₇₆H₁₃₂N₁₇O₁₉⁺ 1586.9880; found 1586.9868.

Calcd.: C₇₇H₁₃₄N₁₇O₁₉⁺ 1601.0036; found 1601.0017.

Calcd.: C₇₈H₁₃₆N₁₇O₁₉⁺ 1615.0193; found 1615.0192.

Calcd.: C₇₉H₁₃₈N₁₇O₁₉⁺ 1629.0349; found 1629.0348.

Calcd.: C₈₀H₁₄₀N₁₇O₁₉⁺ 1643.0506; found 1643.0522.

IR (neat)

vmax / cm⁻¹: 3393, 2976, 2927, 1753, 1652, 1531, 1155.

¹H NMR

(400 MHz, CD₃OD) 9.30 (s, 1H), 8.86 (s, 1H), 8.54 – 8.46 (m, 1H), 8.17 (s, 1H), 8.11 (d, *J* = 6.5 Hz, 2H), 7.97 – 7.89 (m, 1H), 7.85 – 7.76 (m, 2H), 7.71 (s, 1H), 7.69 (s, 1H), 7.48 – 7.42 (m, 1H), 7.39 (d, *J* = 9.3 Hz, 1H), 7.37 – 7.30 (m, 1H), 6.46 (q, *J* = 6.9 Hz, 1H), 5.81 – 5.69 (m, 1H), 5.31 – 5.19 (m, 1H), 4.97 – 4.91 (m, 1H), 4.73 – 4.67 (m, 1H), 4.57 – 4.47 (m, 1H), 4.49 – 4.42 (m, 1H), 4.40 – 4.29 (m, 1H), 4.29 – 4.22 (m, 1H), 4.18 – 4.10 (m, 1H), 4.09 – 4.03 (m, 2H), 4.04 – 3.97 (m, 1H), 3.96 – 3.92 (m, 1H), 3.88 – 3.85 (m, 1H), 3.85 – 3.82 (m, 1H), 3.80 – 3.52 (m, 5H), 3.45 (s, 3H), 3.39 – 3.33 (m, 2H), 3.15 – 3.09 (m, 1H), 3.08 (s, 3H), 2.89 (s, 3H), 2.41 – 2.31 (m, 2H), 2.30 – 2.20 (m, 1H), 2.20 – 2.14 (m, 2H), 2.13 – 1.97 (m, 4H), 1.96 – 1.91 (m, 2H), 1.93 – 1.91 (m, 1H), 1.82 (d, *J* = 7.1 Hz, 3H), 1.62 (d, *J* = 7.3 Hz, 3H), 1.58 (s, 3H), 1.55 – 1.47 (m, 2H), 1.39 – 1.31 (m, 11H), 1.27 (s, 15H), 1.20 – 1.11 (m, 2H), 1.11 – 1.06 (m, 3H), 1.06 – 1.01 (m, 9H), 0.99 (s, 6H), 1.01 – 0.94 (m, 3H), 0.92 – 0.84 (m, 9H).

¹³C NMR (101 MHz, CD₃OD) δ 178.2, 177.9, 177.1, 176.2, 175.9, 175.5, 174.8, 174.6, 174.2, 173.9, 173.4, 172.7, 172.4, 172.1, 168.4, 157.3, 131.5, 130.8, 72.9, 70.9, 70.7, 68.8, 68.0, 66.0, 64.9, 64.0, 63.3, 63.1, 61.6, 59.2, 59.1, 59.0, 56.9, 55.0, 53.1, 49.0, 45.9, 41.3, 40.4, 40.3, 40.3, 37.7, 37.5, 36.6, 36.6, 36.3, 35.3, 35.1, 35.0, 34.9, 33.1, 31.2, 31.2, 31.1, 31.0, 30.9, 30.8, 30.8, 30.7, 30.6, 30.6, 30.5, 30.5, 30.3, 30.3, 30.2, 29.8, 29.2, 28.7, 28.7, 28.6, 28.4, 28.4, 28.3, 28.2, 27.0, 26.6, 26.2, 26.1, 26.0, 25.8, 25.7, 23.8, 23.8, 23.8, 23.2, 23.1, 23.1, 21.9, 21.7, 21.2, 20.8, 20.7, 20.1, 19.9, 19.8, 19.6, 19.4, 18.9, 17.2, 16.5, 15.1, 14.5, 14.5, 12.9, 12.1, 10.6.

¹⁹F NMR (376 MHz, CD₃OD) δ -77.53.

Synthesis:

General: Dichloromethane was dried over aluminum oxide via an MBraun SPS-800 solvent purification system. DMF was purchased anhydrous. The dryness of solvent was controlled via Karl Fischer titration. Et₃N was dried by stirring for at least 30 minutes over CaH₂ followed by distillation onto pre-activated molecular sieves (4 Å). Reagents were used as received from commercial suppliers. Concentration *in vacuo* was performed using a rotary evaporator with the water bath temperature at 40 °C, followed by further concentration using a high vacuum pump. TLC analysis was carried out on silica coated aluminum foil plates (Merck Kieselgel 60 F254). The TLC plates were visualized by UV irradiation and/or by staining with KMnO₄ (KMnO₄ (5.0 g), 5% NaOH (aq., 8.3 mL) and K₂CO₃ (33.3 g) in H₂O (500 mL)). Syringe filters: Q-Max, RR syringe filters, 0.22 µm nylon (Cat#: 25NY022-100).

Infrared spectra (IR) were acquired on a PerkinElmer Spectrum Two™ UATR. Mass spectra (HRMS) were recorded on a Bruker Daltonics MicrOTOF time-of-flight spectrometer with positive electrospray ionization. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker BioSpin GmbH 400 MHz spectrometer, running at 400, 376 and 101 MHz for ¹H, ¹⁹F, and ¹³C, respectively. Chemical shifts (δ) are reported in ppm relative to the residual solvent signals (CD₃OD @ 3.31 ppm ¹H NMR, 49.00 ppm ¹³C NMR). ¹⁹F NMR are reported without reference peak. LC-MS purification was performed using a Gilson HPLC system and a PerkinElmer Flexar SQ 300 MS detector.

Molisch carbohydrate test:

The test was performed according to Gurusiddaiah *et. al.*⁶ Sample prep: Stendomycin-TFA salt **1** (6.0 mg, 0.0034 mmol, 1 equiv.) was put in a dry sealable reaction tube under argon. H₂SO₄ (1 M, 2.0 mL) was added, the tube was sealed, and the mixture was heated to 95 °C. After 2 h, the mixture was allowed to cool to room temperature. Ba(OH)₂·8H₂O was added until the pH was >10 to neutralize the reaction. BaSO₄ precipitates as a white solid. The mixture was transferred to a falcon tube and centrifuged. The supernatant was evaluated. Molisch test: Three samples were employed; (1) glucose (positive control), (2) water (negative control), and (3) the stendomycin sample. For each reaction a 15 mL reaction tube was employed. (1) glucose (3.6 mg) and 2 mL water, (2) 2 mL water, (3) the 2 mL stendomycin sample. 1-Naphthanol (50 mg) was dissolved in EtOH (99%, 2.5 mL). From this solution was added 10 drops to each reaction tube. H₂SO₄ (conc., 2 mL) was added carefully along the side of the tubes, it runs through the water to create a biphasic system. For sample (1), a purple band was observed within the first minute at the interphase of the two phases (positive result). For sample (2) no color was observed (negative result). For sample (3) a white solid was formed due to remaining Ba(OH)₂, but no color was observed (negative result).

Stendomycin thiol conjugate 2:

Stendomycin TFA salt **1** (43.1 mg, 0.025 mmol, 1 equiv.) was dissolved in MeOH (2 mL) in a reaction tube containing a magnet. H₂O (2 mL) was added followed by Na₂HPO₄ (100 mg, 0.70 mmol, 28.5 equiv.) yielding a white suspension. 2-(Boc-amino)ethanethiol (0.2 mL, 0.70 mmol, 48 equiv.) was added. The heterogenous mixture was stirred at ambient temperature. After 17 h, the mixture was diluted with MeOH and filtered through a nylon syringe filter. The product TFA salt **2** was obtained as the white solid (48 mg, 0.025 mmol, quant.) after purification by

preparative LC-MS and lyophilization. LC-MS conditions: C18 3x25cm, 25 mL/min, UV; 254 nm and 210, H₂O/MeCN 0.1% TFA, gradient; 50% to 90% MeCN (25 min), hold (6 min), 90% to 100% MeCN (1 min), hold (4 min).

R_f 0.6 (CH₂Cl₂/MeOH) 9:1, KMnO₄.

HRMS ESI-TOF m/z [M+H]⁺
 Calcd.: C₈₂H₁₄₅N₁₈O₂₁S⁺ 1750.0547; found 1750.0589.
 Calcd.: C₈₃H₁₄₇N₁₈O₂₁S⁺ 1764.0703; found 1764.0722.
 Calcd.: C₈₄H₁₄₉N₁₈O₂₁S⁺ 1778.0860; found 1778.0873.
 Calcd.: C₈₅H₁₅₁N₁₈O₂₁S⁺ 1792.1016; found 1791.1036.
 Calcd.: C₈₆H₁₅₃N₁₈O₂₁S⁺ 1806.1173; found 1806.1201.

IR (neat) ν_{max} / cm⁻¹: 3289, 2958, 2924, 2855, 1652, 1536, 1201, 1173, 1064.

NMR: (400 MHz, CD₃OD) δ 9.11 – 8.45 (m, 3H), 8.47 – 8.30 (m, 1H), 8.32 – 8.18 (m, 1H), 8.10 (s, 1H), 7.97 (s, 1H), 7.88 – 7.62 (m, 2H), 7.65 – 7.42 (m, 1H), 7.43 – 7.17 (m, 1H), 5.71 (s, 1H), 5.34 – 5.16 (m, 1H), 4.73 – 4.62 (m, 1H), 4.56 – 4.40 (m, 2H), 4.41 – 4.17 (m, 2H), 4.18 – 4.11 (m, 2H), 4.10 – 3.94 (m, 4H), 3.92 – 3.81 (m, 5H), 3.83 – 3.66 (m, 1H), 3.68 – 3.52 (m, 1H), 3.43 (d, *J* = 2.7 Hz, 3H), 3.35 (d, *J* = 7.9 Hz, 2H), 3.27 – 3.10 (m, 2H), 3.08 (s, 3H), 2.88 (s, 3H), 2.72 – 2.59 (m, 3H), 2.56 – 2.40 (m, 1H), 2.40 – 2.31 (m, 1H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.24 – 1.77 (m, 8H), 1.75 – 1.47 (m, 8H), 1.47 – 1.40 (m, 9H), 1.41 – 1.22 (m, 36H), 1.17 (q, *J* = 6.8 Hz, 2H), 1.11 – 1.01 (m, 12H), 1.01 – 0.96 (m, 4H), 0.97 – 0.84 (m, 18H).

Stendomycin amine 3:

The Boc-protected amine **2** (5.0 mg, 0.0028 mmol, 1 equiv.) was dissolved in anhydrous CH₂Cl₂ (0.35 mL) under argon and stirred. The mixture was cooled to 0 °C and TFA (0.35 mL) was added. After 1.5 h, toluene was added and the mixture was concentrated to dryness to yield the product. Analytical LC-MS confirm formation of the product **3**. The concentrate was utilized in the next reaction without further purification.

Stendomycin biotin 4:

The crude concentrate of stendomycin amine **3** (0.0028 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (0.2 mL) in a dry reaction tube under argon while stirred at ambient temperature. Anhydrous Et₃N (5 μL, 0.036 mmol, 13 equiv.) was added followed by Biotin-NHS ester (2 mg, 0.0059 mmol, 2.1 mmol). After 20 h, the reaction was subjected directly to purification by preparative LC-MS to yield the product **4** (3.0 mg, 0.002 mmol, 56% over two steps) as a white solid. LC-MS method: C18, 1x25 cm, 7 mL/min, UV; 254 nm and 210, H₂O/MeCN 0.1% TFA, gradient; 50% to 90% MeCN (25 min), hold (6 min), 90% to 100% MeCN (1 min), hold (4 min)

HRMS ESI-TOF m/z [M+H]⁺
 Calcd.: C₈₈H₁₅₃N₂₀O₂₁S₂⁺ 1890.0955; found 1890.0946
 Calcd.: C₈₉H₁₅₅N₂₀O₂₁S₂⁺ 1904.1112; found 1904.1108
 Calcd.: C₉₀H₁₅₇N₂₀O₂₁S₂⁺ 1918.1268; found 1918.1231
 Calcd.: C₉₁H₁₅₉N₂₀O₂₁S₂⁺ 1932.1425; found 1932.1429

Structure analysis by MS-fragmentation pattern:

Stendomycin **1** and stendomycin thiol-conjugate **2** required lactone hydrolysis in order to achieve identifiable MS-fragmentation patterns. General method: 1 mg of starting material was dissolved in MeOH (0.15 mL) in a reaction tube by stirring. H₂O (0.15 mL) was added followed by NaOH (aq., 0.2 M, 50 μL). After stirring at ambient temperature for 2 h, an aliquot was taken out, diluted with MeOH and analyzed by LC-MS/MS. Full lactone hydrolysis was achieved. See supporting information for MS-fragmentation data.

Mammalian Cell Culture

Pancreatic cancer cell line PANC-1 (Cell Line Services, Eppelheim, Germany, Cat#: 300228) was cultured in high-glucose DMEM (Gibco, Cat#: 31966-021). Pancreatic cancer cell line BxPC3 (Sigma, Cat#: 93120816) was cultured in RPMI 1640 medium (Gibco, Cat#: 61870010). Lung cancer cell lines HCC827 (ATCC CRL-2868) and A549 (Sigma, Cat#: 86012804) were cultured in RPMI 1640 medium and Ham's F-12K (Kaighn's) medium (Gibco, Cat#: 21127022), respectively. Breast cancer cell line MCF7 (ATCC HTB-22) were cultured in DMEM (Gibco, Cat#: 21969035) supplemented with GlutaMAX™ supplement (Gibco, Cat#: 35050061) and 10 µg/ml human recombinant insulin (Sigma, I9278). Breast cancer cell line MDA-MB-231 (Sigma, Cat#: 92020424) was cultured in DMEM/F12 medium (Gibco, Cat#: 31331028) supplemented with 5 µg/ml human recombinant insulin. Cervical cancer cell line SiHa (gift from Dr. Morten Busk, Aarhus University Hospital) was cultured in MEM, GlutaMax supplement (Gibco, Cat#: 41090028) supplemented with 1% MEM NEAA (Gibco, Cat#: 11140035) and 1 mM sodium pyruvate (Gibco, Cat#: 11360039).

All cell media were supplemented with fetal bovine serum (FBS, 10%) and Penicillin/Streptomycin (1%) except MDA-MB-231 cells where only 5% FBS was added. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Every third day the cells were washed in PBS (2x5 mL, Sigma, Cat#: D8537) and dissociated from the culture flask by trypsin-EDTA (Sigma, T4049) and one fifth of the cells were re-seeded in fresh complete medium in a T75 flask (Thermo Scientific, Cat#: 130190).

Cell Viability Assay

Stendomycin and analogs were dissolved in DMSO and the concentrations were determined using Pierce bicinchoninic acid (BCA) Protein Assay kit according to the manufacturer's protocol (Thermo Scientific). Cells were seeded in black 96-well plates (Thermo Scientific, Cat# 137101) at a density of 2000 cells/well in complete medium (75 µL/well) and were allowed to adhere to the substratum overnight. Compounds were dosed in the designated culture plates in triplicates as 4X solutions in 25 µL medium with a normalized DMSO concentration (0.5%) in all treatments and the plates were placed at 37 °C in a humidified atmosphere containing 5% CO₂. After 46.5 or 70.5 h, plates were added CellTiter-Blue (20 µL/well) and incubated for 90 min after which the plates were analyzed in a Tecan Spark 10M multi-mode plate reader for fluorescence (552±10 nm excitation; 598±10 nm emission). Average growth of treated cells was calculated by correcting fluorescence values for background fluorescence and subsequently normalize to the average of the two lowest treatments of the compound in question. Data was plotted and fitted to a four-parameter dose-response curve using Prism 7 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

- NMR data comparison table
- Data from LC-MS analysis of bacterial extracts in different growth media.
- Analysis of the heterogeneity of stendomycin and identification of new congeners by MS/MS fragmentation.
- Additional data for structure verification including NMR and IR spectra.

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Notes

The authors declare no competing financial interests.

Requests for compounds **1** or **2** should be directed to T.B.P.

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References

- (1) Brown, E. D.; Wright, G. D. Antibacterial Drug Discovery in the Resistance Era. *Nature* **2016**, 529, 336–343.
- (2) Liu, Y.-Y.; Wang, Y.; Walsh, T. R.; Yi, L.-X.; Zhang, R.; Spencer, J.; Doi, Y.; Tian, G.; Dong, B.; Huang, X.; et al. Emergence of Plasmid-Mediated Colistin Resistance Mechanism MCR-1 in Animals and Human Beings in China: A Microbiological and Molecular Biological Study. *Lancet Infect. Dis.* **2016**, 16, 161–168.
- (3) Magill, S. S.; Edwards, J. R.; Bamberg, W.; Beldavs, Z. G.; Dumyati, G.; Kainer, M. A.; Lynfield, R.; Maloney, M.; McAllister-Hollod, L.; Nadle, J.; et al. Multistate Point-Prevalence Survey of Health Care–Associated Infections. *N. Engl. J. Med.* **2014**, 370, 1198–1208.
- (4) Velkov, T.; Gallardo-Godoy, A.; Swarbrick, J. D.; Blaskovich, M. A. T.; Elliott, A. G.; Han, M.; Thompson, P. E.; Roberts, K. D.; Huang, J. X.; Becker, B.; et al. Structure, Function, and Biosynthetic Origin of Octapeptin Antibiotics Active against Extensively Drug-Resistant Gram-Negative Bacteria. *Cell Chem. Biol.* **2018**, 25, 380–391.
- (5) Seiple, I. B.; Zhang, Z.; Jakubec, P.; Langlois-Mercier, A.; Wright, P. M.; Hog, D. T.; Yabu, K.; Allu, S. R.; Fukuzaki, T.; Carlsen, P. N.; et al. A Platform for the Discovery of New Macrolide Antibiotics. *Nature* **2016**, 533, 338–345.
- (6) Gurusiddaiah, S.; Winward, L. D.; Burger, D.; Graham, S. O. Pantomycin: A New Antimicrobial Antibiotic. *Mycologia* **1979**, 71, 103–118.
- (7) Gurusiddaiah, S.; Graham, S. O. Some Chemical and Physical Characteristics of Pantomycin, and Antibiotic Isolated from *Streptomyces Hygroscopicus*. *Antimicrob. Agents Chemother.* **1980**, 17, 980–987.
- (8) Thompson, R. Q.; Hughes, M. S. Stendomycin: A New Antifungal Antibiotic. *J. Antibiot. (Tokyo)*. **1963**, 16, 187–194.
- (9) Bodanszky, M.; Marconi, G. G.; Bodanszky, A. The Structure of Stendomycin. *J. Antibiot. (Tokyo)*. **1969**, 22, 40–41.
- (10) Bodanszky, M.; Izdebski, J.; Muramatsu, I. Structure of the Peptide Antibiotic Stendomycin. *J. Am. Chem. Soc.* **1969**, 91, 2351–2358.
- (11) Losilla, B.; Pommier, M.; Bonnavero, N.; Cremieux, A.; Michel, G. Structure Activity Relationships of Stendomycin, a Lipopeptide Antibiotic from *Streptomyces*. *Microbios* **1992**, 71, 75–80.
- (12) Kersten, R. D.; Yang, Y.-L.; Xu, Y.; Cimerancic, P.; Nam, S.-J.; Fenical, W.; Fischbach, M. A.; Moore, B. S.; Dorrestein, P. C. A Mass Spectrometry-guided Genome Mining Approach for Natural Product Peptidogenomics. *Nat. Chem. Biol.* **2011**, 7, 794–802.
- (13) Filipuzzi, I.; Steffen, J.; Germain, M.; Goepfert, L.; Conti, M. A.; Potting, C.; Cerino, R.; Pfeifer,

- M.; Krastel, P.; Hoepfner, D.; et al. Stendomycin Selectively Inhibits TIM23-Dependent Mitochondrial Protein Import. *Nat. Chem. Biol.* **2017**, *13*, 1239–1244.
- (14) Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H. U.; Brucoleri, R.; Lee, S. Y.; Fischbach, M. A.; Müller, R.; Wohlleben, W.; et al. antiSMASH 3.0—a Comprehensive Resource for the Genome Mining of Biosynthetic Gene Clusters. *Nucleic Acids Res.* **2015**, *43*, W237–W243.
- (15) Chagas, F. O.; Ruzzini, A. C.; Bacha, L. V.; Samborsky, M.; Conti, R.; Pessotti, R. C.; de Oliveira, L. G.; Clardy, J.; Pupo, M. T. Genome Sequence of *Streptomyces* Sp. Strain RTd22, an Endophyte of the Mexican Sunflower. *Genome Announc.* **2016**, *4*, e00693-16.
- (16) Bodanszky, M.; Bodanszky, A. Addition of Thioglycolic Acid to Stendomycin. *J. Antibiot. (Tokyo)*. **1974**, *27*, 312–315.
- (17) Ziegler, S.; Pries, V.; Hedberg, C.; Waldmann, H. Target Identification for Small Bioactive Molecules: Finding the Needle in the Haystack. *Angew. Chemie Int. Ed.* **2013**, *52*, 2744–2792.
- (18) Gilfix, B. M.; Sanwal, B. D. Inhibition of Myoblast Fusion by Tunicamycin and Pantomycin. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1184–1191.