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ELECTRONIC SUPPLEMENTARY INFORMATION

Unique post-translational oxime formation in the biosynthesis of the azolemycin complex of novel ribosomal peptides from *Streptomyces* sp. FXJ1.264

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Strains

Bacterial strains used and constructed in this study are listed in Table S1. *Streptomyces* sp. FXJ1.264 was isolated from a red-soil sample collected from Yaoli town, Jiangxi Province, China, in 2007. The isolation medium was GTV agar[1] and the growth medium was GYM agar (0.4% glucose, 0.4% yeast extract, 1.0% malt extract, 0.2% $CaCO_3$, 2% agar, ddH_2O). The strain was preliminarily identified by morphology and 16S rRNA gene sequence analysis using standard procedures.[2] The 16S rRNA gene sequence of *Streptomyces* sp. FXJ1.264 (GenBank accession number HQ537067) showed the highest similarity (99.86%) to that of the type strain of *Streptomyces cuspidosporus* (GenBank accession number AB184090).[3]

Table S1. Strains used and constructed in this study

Strain name	Strain description	Source
E. coli DH5α	For plasmid propagation	[4]
E. coli ET12567/pUZ8002	For conjugal transfer to *Streptomyces*	[5, 6]
Streptomyces sp. FXJ1.264	Wild-type azolemycin producer	This study
Streptomyces sp. FXJ1.264DM47	*ΔazmF::neo*	This study
Streptomyces sp. FXJ1.264DM48	*ΔazmA*	This study
Streptomyces sp. FXJ1.264DM49	*ΔazmC/D::neo*	This study
Streptomyces sp. FXJ1.264DM51	*ΔazmE::neo*	This study
Staphylococcus aureus CGMCC 1.2386	For antimicrobial testing	CGMCC
Bacillus subtilis CGMCC 1.2428	For antimicrobial testing	CGMCC
Escherichia coli CGMCC 1.2385	For antimicrobial testing	CGMCC
Candidia albicans CGMCC 2.538	For antimicrobial testing	CGMCC
Candida pseudorugosa CGMCC 2.3107	For antimicrobial testing	CGMCC

Growth, extraction and LC-MS analysis of small-scale cultures of *Streptomyces* sp. FXJ1.264

Spores of *Streptomyces* sp. FXJ1.264 were spread on two GYM agar plates (the pH of the medium was adjusted to 5.0-5.1 with HCl prior to sterilization). The resulting cultures were incubated at 28°C for 8 days, then mashed and extracted three times with an equal volume of ethanol. The combined extracts were concentrated to dryness under vacuum and the residue was re-dissolved in 2 ml of MeOH.

1 μL of the methanol solution was injected onto an Waters ACQUITY UPLC BEH C18 column (2.1 mm×50 mm, 1.7 μm, 45 °C) connected to a Waters ACQUITY UPLC/Xevo G2 QTof MS system (Waters Corporation, Milford, USA), equipped with an electrospray source. The column was eluted as follows: 0 min – 95% A + 5% B, 10 min – 0% A + 100% B, where A was water containing 0.1% formic acid and B was acetonitrile. The full-scan data were acquired in the positive ion mode from 50 to 1200 Da with a 0.2 s scan time, using the following settings: capillary voltage 3.0 kV; de-solvation temperature 350 °C; sample cone voltage 35 V; extraction cone voltage 4 V; source temperature 120 °C; cone gas flow 50 L/h; and desolvation gas flow 800 L/h. The mass spectrometer was calibrated across the mass range of 50–1200 Da using a solution of sodium formate. Data were centroided and *m/z* values were corrected during acquisition using an external reference consisting of a 0.2 ng/mL solution of leucine enkephalin infused at a flow rate of 5 μL/min via a lockspray interface, generating a reference ion at 556.2771 Da ($[M+H]^+$). The lockspray scan time was set at 0.5 s, with an interval of 15

s, and data were averaged over 3 scans. MS and MS/MS data were acquired using two interleaved scan functions in the MSE mode. The first scan function was set at 6 eV in order to collect data on the intact precursor ions in the sample, and the second scan function was ramped from 15eV to 35eV to obtain fragment ion data from the ions observed in the preceding scan.

Isolation of azolemycins from large-scale cultures of *Streptomyces* sp. FXJ1.264

250 Erlenmeyer flasks (500 mL), each containing 100 mL of GYM agar (pH 5.0-5.1), were inoculated with spores of *Streptomyces* sp. FXJ1.264. After 3 days growth at 28 °C, the agar was mashed with sterilized knives and the resulting cultures were incubated for a further 8 days at the same temperature.

The cultures were combined and extracted with 3 x 25 L of ethanol. The combined extracts were concentrated under vacuum and fractionated on a silica gel column (100 to 200 mesh; Qingdao Haiyang Chemical) using a step gradient of $CHCl_3$:MeOH from 95:5 to 70:30. Fractions containing the compounds of interest were combined and passed repeatedly through a Sephadex LH-20 column eluting with MeOH to remove fatty acids and other lipophilic impurities. The resulting mixture of azolemycins was separated by HPLC on a Ascentis RP-Amide column (4.6 x 150-mm) connected to a Shimadzu SPD-M20A instrument eluted with isocratic MeCN/H_2O (3:1) at a flow rate of 1.0 ml/min, monitoring absorbance between 190 and 800 nm using a diode array detector, to afford azolemycin A (122 mg, retention time = 6.00 min), azolemycin B (44 mg, retention time = 4.51 min), azolemycin C (160 mg, retention time = 6.43 min) and azolemycin D (67 mg, retention time = 9.22 min).

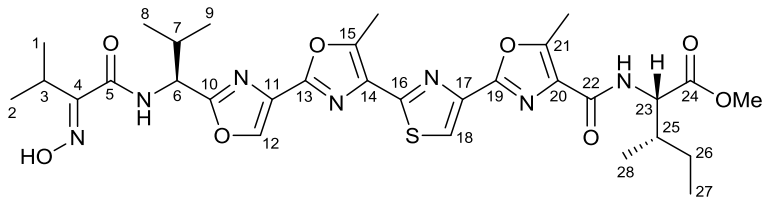
Azolemycins A, B, C, and D were further purified using a Supelco Ascentis C18 column (100 x 21.1 mm, 5 μm) connected to an Agilent 1100 HPLC at a flow rate of 5 ml/min, monitoring absorbance at 268 nm. Mobile phases consisted of A: water containing 0.1% formic acid; and B: acetonitrile containing 0.1% formic acid. The following program was used to elute the column: 0 min, 80% B; 16 min, 80% B; 18 min, 100% B; 21 min, 100% B; 23 min 80% B; 38 min 80% B. Fractions containing each azolemycin were identified using ESI-HR-Q-TOF-MS and pooled together. The organic solvent was removed from each combined fraction using a rotary evaporator and the resulting aqueous solutions were freeze dried, then immediately analyzed by NMR spectroscopy to minimize oxime isomerization.

Structure elucidation of the azolemycins

High resolution MS analyses established the molecular formula of azolemycins A and B as $C_{31}H_{39}N_7O_8S$ (*m/z* calculated for $C_{31}H_{40}N_7O_8S^+$: 670.2659; *m/z* measured for azolemycin A: 670.2672, $[M+H]^+$; *m/z* measured for azolemycin B: 670.2666, $[M+H]^+$) and the molecular formula of azolemycins C and D as $C_{32}H_{41}N_7O_8S$ (*m/z* calculated for $C_{32}H_{42}N_7O_8S^+$: 684.2816; *m/z* measured for azolemycin C: 684.2830, $[M+H]^+$; *m/z* measured for azolemycin D: 684.2815, $[M+H]^+$).

^1H, ^{13}C, COSY, HSQC and HMBC NMR spectra (Figures S1-S5) of a solution of azolemycin A in $CDCl_3$ (180 μL) were recorded in a 3 mm tube on a Bruker Avance II 700 spectrometer equipped with a TCI cryoprobe at 298K. The peak due to residual $CHCl_3$ in the solvent was used to calibrate the spectra. Assignments for the ^1H and ^{13}C resonances observed for azolemycin A are listed in Table S2.

Table S2. Assignments for signals observed in the ^{1}H and ^{13}C NMR spectra of azolemycin A ($CDCl_3$, 700 MHz)



Position	δ_H/ppm (no. of protons, multiplicity, J/Hz)	δ_C/ppm
1/2	1.28 (3H, d, 7.0)	18.8
1/2	1.25 (3H, d, 7.0)	18.2
3	3.47 (1H, m)	25.6
4		158.8
OH	10.02 (1H, broad s,)	
5		163.5
6-NH	7.65 (1H, broad s)	
6	5.25 (1H, dd, 6.5, 8.5)	52.7
7	2.30 (1H, m)	33.0
8/9	0.98 (3H, d, 6.5)	19.0
8/9	1.00 (3H, d, 6.5)	18.4
10		165.4
11		129.8
12	8.24 (1H, s)	138.7
13		153.0
14		130.9
15		148.4
15-Me	2.89 (3H, s)	12.2
16		162.2
17		143.9
18	7.99 (1H, s)	120.2
19		154.4
20		129.9
21		153.7
21-Me	2.74 (3H, s)	11.8
22		161.6
23-NH	7.52 (1H, d, 9.0)	
23	4.73 (1H, dd, 5.5, 9.0)	56.3
24		172.2
25	2.00 (1H, m)	37.9
26	1.28/1.56 (2 x 1H, 2 x m)	25.6
27	0.96 (3H, t, 7.5)	11.5
28	0.97 (3H, d, 6.5)	15.6
OMe	3.76 (3H, s)	52.6

Figure S1. ^{1}H NMR spectrum of azolemycin A

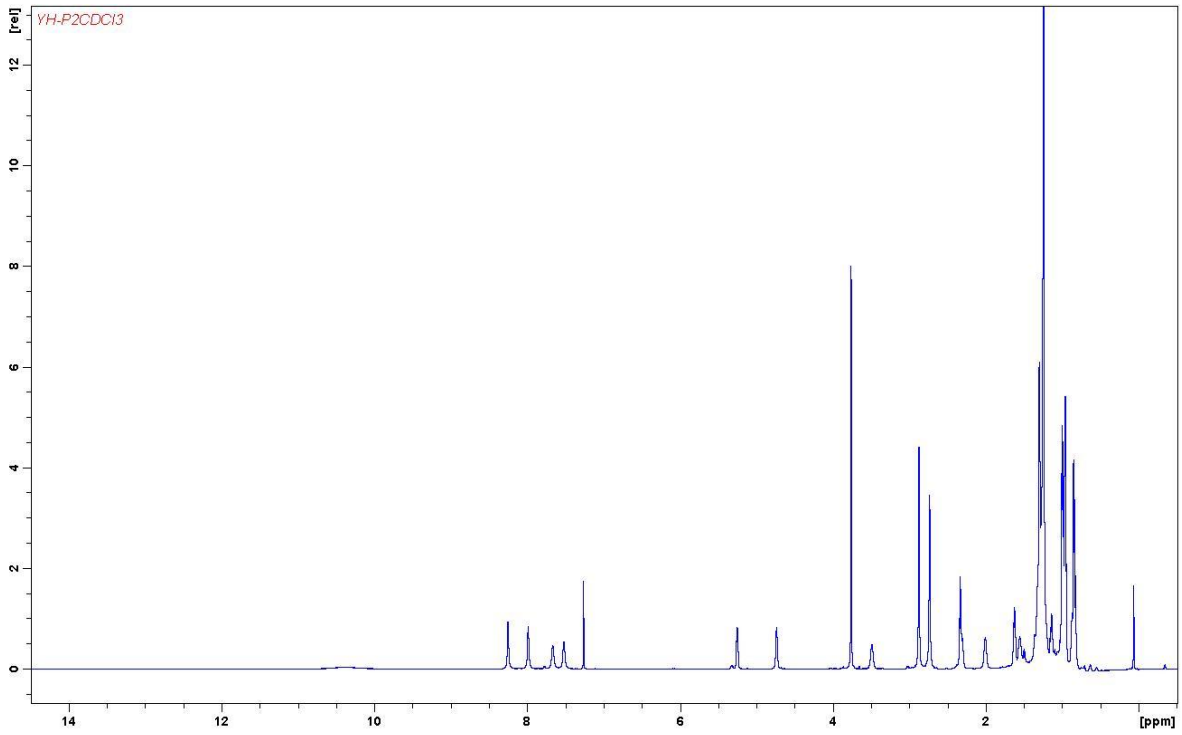


Figure S2. ^{13}C NMR spectrum of azolemycin A

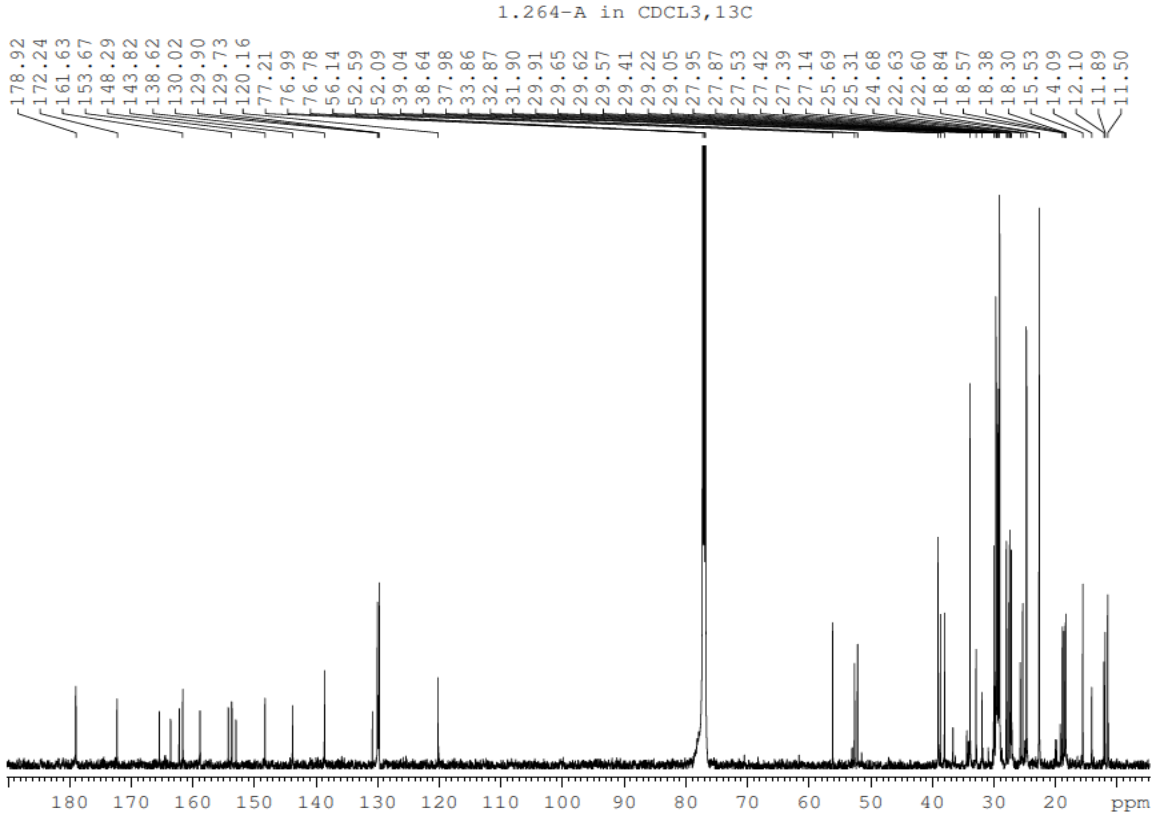


Figure S3. COSY spectrum of azolemycin A

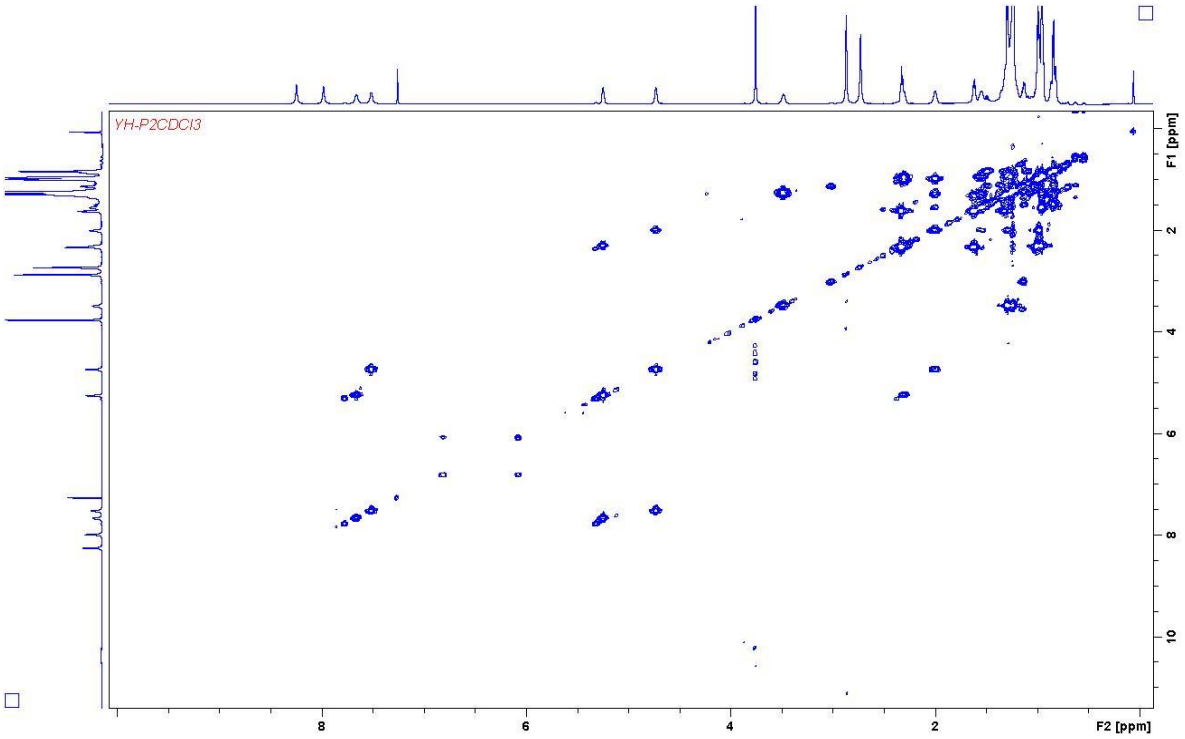


Figure S4. HSQC spectrum of azolemycin A

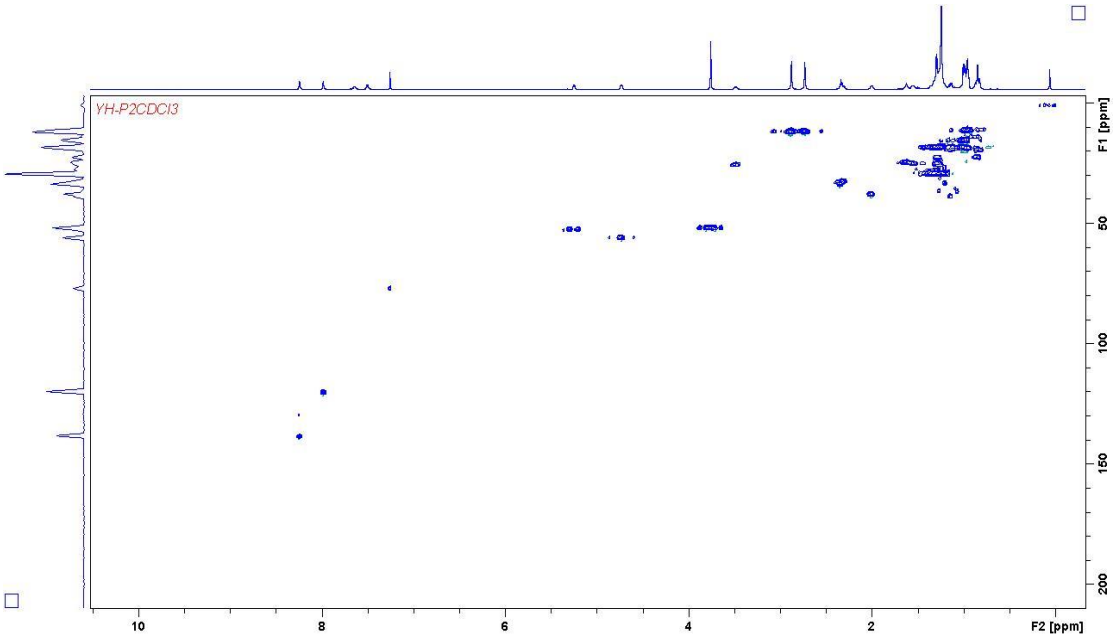
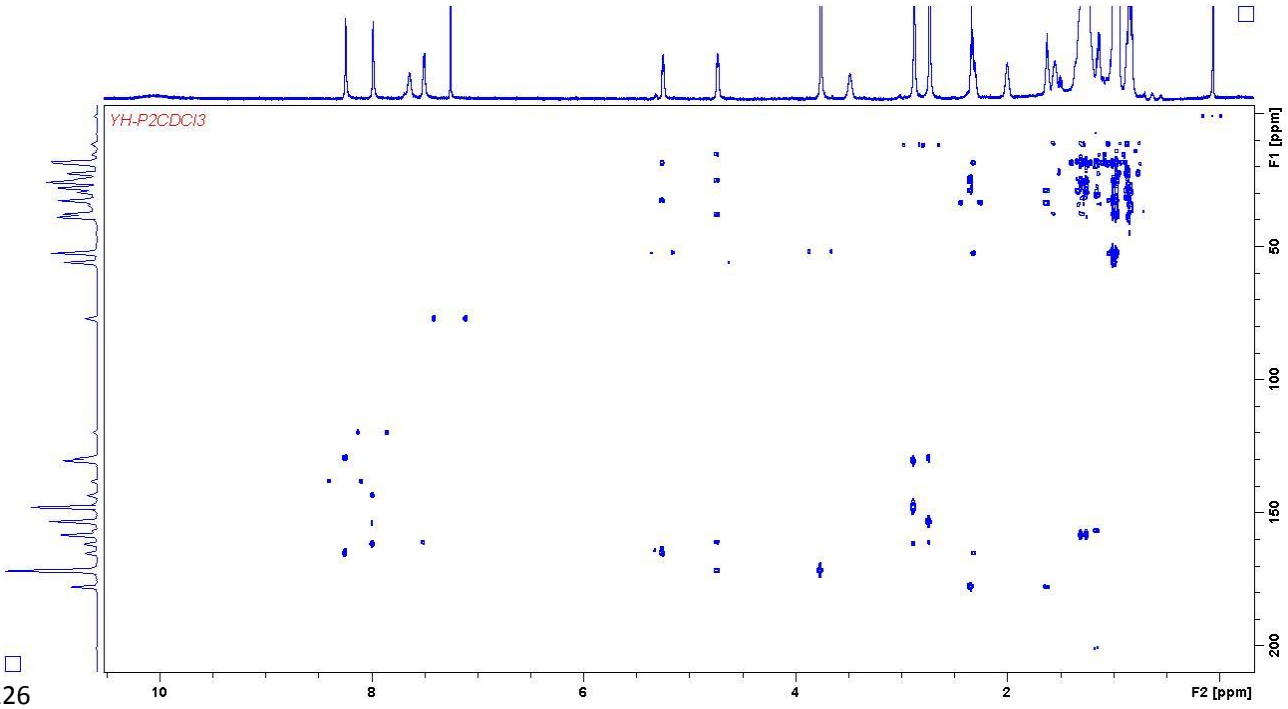


Figure S5. HMBC spectrum of azolemycin A



To determine the absolute stereochemistry of C-6 and C-23 in azolemycin A, 100 µg of pure azolemycin A was heated at 110 °C in 6 M HCl for 1 hour. The mixture was evaporated to dryness and the residue was dissolved in 100 µl of water. 50 µl of the resulting solution was added to 40 µl of 1 M sodium bicarbonate. 50 µl of 1% 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone was added and the mixture was incubated for 60 min at 37 °C. The reaction was quenched by adding 40 µl of 1 M HCl. 400 µl of acetonitrile was added and the sample was centrifuged for 10 min at 13,000 rpm. The supernatant was analyzed by LC-MS using an RP-C18 column (Agilent Zorbax, 100 x 2.1 mm, 1.8 µm), attached to a Dionex 3000 RS UHPLC connected to a Bruker MaXis mass spectrometer (Figures S6 and S7). 1 µl of sample was injected and the column was eluted at 0.2 ml/min using the following program: 0 min, 30% B; 40 min, 70% B. Mobile phases consisted of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. The reaction products were also analyzed on a homochiral stationary phase (ChiralPACK IC, 5µm), but separation of the Marfey's derivatives of L-Ile and L-*allo*-Ile could not be achieved.

Figure S6. Extracted ion chromatograms at *m/z* = 370.13 from LC-MS analyses of Marfey's derivatives of D- and L-valine (top and middle panels, respectively), and the acid hydrolysate of azolemycin A (bottom panel).

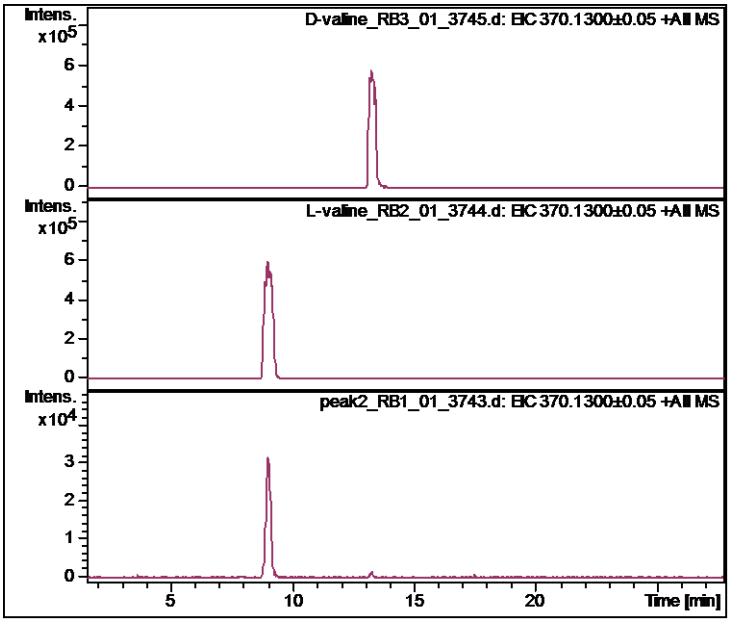
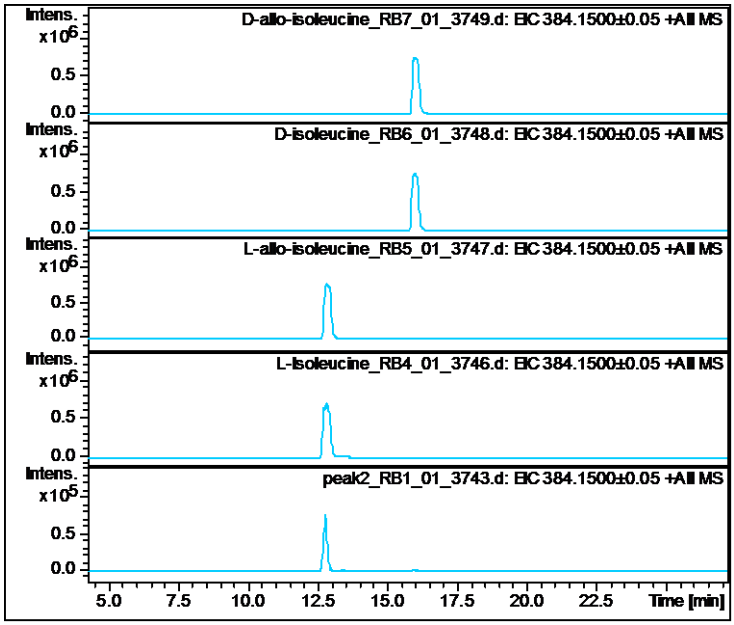


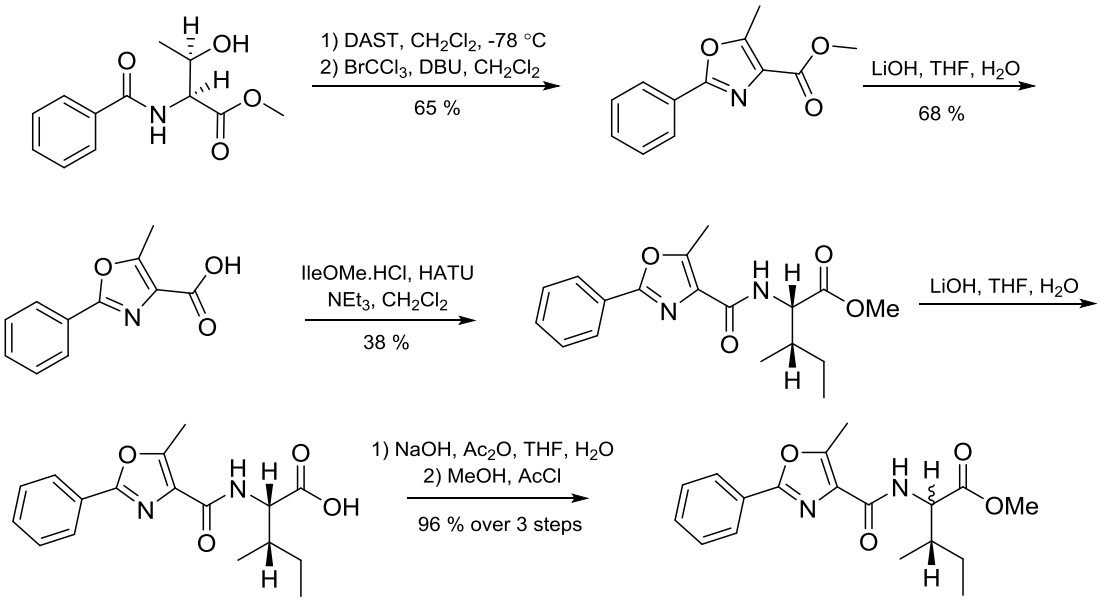
Figure S7. Extracted ion chromatograms at *m/z* = 384.15 from LC-MS analyses of Marfey's derivatives of D-*allo*-isoleucine (top panel), D-isoleucine (second from top panel), L-*allo*-isoleucine (third from top panel), L-isoleucine (second from bottom panel) and the acid hydrolysate of azolemycin A (bottom panel).



Azolemycin A was assigned the 25*S* configuration by comparison of the chemical shift for H-23 with the δ_H values reported for the corresponding protons in the methyl esters of N-benzoyl-*allo*-isoleucine and N-benzyol-isoleucine.[7] To further confirm this stereochemical assignment, the methyl esters of (2*S*, 3*S*)- and (2*R*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid were synthesized for spectroscopic comparison with the

natural product (Scheme S1).

Scheme S1. Synthesis of (2*S*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester and a 1:1 mixture of its (2*S*, 3*S*) and (2*R*, 3*S*) isomers



***N*-Benzoyl-L-threonine methyl ester.** Acetyl chloride (15.6 mL, 200 mmol) was added dropwise with stirring to MeOH (100 mL) at 0-5 °C. L-Threonine (6.54 g, 54.9 mmol) was added and the resulting solution was heated to reflux for 2 hours. The reaction mixture was concentrated *in vacuo* to give L-threonine methyl ester hydrochloride, which was used without further purification; it was dissolved in CH_2Cl_2 (160 mL) and triethylamine (17.4 mL, 125 mmol), and the resulting solution was cooled to 0 °C with stirring. Benzoyl chloride (5.8 mL, 49.9 mmol) was added dropwise and the reaction mixture was allowed to warm to room temperature over 18 hours. After quenching with saturated sodium hydrogen carbonate solution (100 mL), the mixture was separated and the aqueous phase was extracted with CH_2Cl_2 (2 x 100 mL). The combined organic extracts were concentrated *in vacuo* and the resulting yellow oil was partitioned between EtOAc (100 mL) and pH 2 buffer (100 mL). The organic phase was further washed with pH 2 buffer (100 mL), water (100 mL), saturated aqueous $NaHCO_3$ solution (2 x 100 mL) and saturated aqueous NaCl solution (100 mL), dried over Na_2SO_4 and concentrated *in vacuo* to give the crude product as a yellow oil. Recrystallisation (EtOAc:petroleum ether) afforded the product as a fluffy white solid (8.03 g, 33.8 mmol, 62 %); mp. 96 - 97 °C (lit.[8] 97 - 98 °C); $[\alpha]_D^{25}$ +18.4 (c = 1.02, $CHCl_3$), (lit.[9] $[\alpha]_D^{25}$ +22.6 (c = 1.0 in $CHCl_3$)); υ_{max}/cm^{-1} 3425 (O-H), 3348 (N-H), 1741 (ester C=O), 1640 (amide C=O), 1521 (N-H); δ_H (400 MHz, $CDCl_3$) 7.91 - 7.78 (2H, m, *ortho* C-*H*), 7.55 - 7.48 (1H, m, *para* C-*H*), 7.46 - 7.41 (2H, m, *meta* C-*H*), 7.04 (1H, d, *J* 8.5 Hz, CHN*H*), 4.82 (1H, dd, *J* 8.5, 2.5 Hz, C*H*NH), 4.50 - 4.40 (1H, m, C*H*OH), 3.78 (3H, s, OCH_3), 2.77 (1H, d, *J* 4.0 Hz, CHO*H*), 1.28 (3H, d, *J* 6.5 Hz, CHCH_3); δ_C (100 MHz, $CDCl_3$) 171.7 (*C*ONH), 168.0 (*C*O_2Me), 133.7 (*ipso C*), 132.0 (*para* C-H), 128.6 (*ortho C*-H), 127.2 (*meta C*-H), 68.2 (*C*HOH), 57.7 (*C*HNH), 52.7 (O*C*H_3), 20.0 (CH*C*H_3); *m/z* (ESI+) 260.1 ($[M+Na]^+$, 100%); HR-ES-MS, *m/z* = 260.0888, (calculated for $C_{12}H_{15}NO_4Na^+$ *m/z* = 260.0893). These data are consistent with a previous report.[10]

5-Methyl-2-phenyl-oxazole-4-carboxylic acid methyl ester. The method used was modified from a procedure reported by Deeley *et al.*[11] (Diethylamino)sulfur trifluoride (2.4 mL, 18.0 mmol) was added dropwise to a stirred solution of *N*-Benzoyl-L-threonine methyl ester (3.6 g, 15.0 mmol) in dry CH_2Cl_2 (150 mL) at -78 °C under N_2. The mixture was stirred at -78 °C for 1.5 hours, then allowed to reach room temperature and stirred for a further 15 minutes. The reaction was quenched by addition of saturated sodium bicarbonate solution (80 mL) and separated. The organic phase was dried and concentrated *in vacuo* to give the crude oxazolidine as a clear oil, which was used immediately without further purification. Bromotrichloromethane (4.4 mL, 46 mmol) was added to a stirred solution of the crude oxazolidine in dry CH_2Cl_2 (150 mL) at 0 °C under N_2 and the mixture was stirred at this temperature for 5 minutes. 1, 8-Diazabicyclo[5.4.0]undec-7-ene (6.9 mL, 46 mmol) was added dropwise and the mixture was allowed to warm to room temperature overnight. The reaction was quenched with 10% aqueous citric acid (80 mL) and the phases were separated. The aqueous phase was extracted with dichloromethane (2 x 80 mL) and the combined organic extracts were concentrated *in vacuo* to give a dark brown residue. This was partitioned between EtOAc (100 mL) and 10% aqueous citric acid (80 mL) and the separated organic extract was washed with saturated $NaHCO_3$ solution (80 mL) and saturated aqueous NaCl solution (80 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica chromatography (9:1 hexanes:EtOAc, Rf. = 0.33) to give the product as a white crystalline solid (2.10 g, 9.7 mmol, 65 % over 2 steps); m.p. 94 - 95°C (lit.[12] 93 - 94°C); υ_{max} /cm^{-1} (neat) 2960 (aromatic C-H), 1722 (C=O); δ_H (400 MHz, $CDCl_3$) 8.14 - 8.02 (2H, m, *ortho* C-*H*), 7.52 - 7.42 (3H, m, *meta, para* C-*H*), 3.96 (3H, s, OCH_3), 2.72 (3H, s, CCH_3); δ_C (100 MHz, $CDCl_3$) 162.8, 159.6, 156.4 (3 x quaternary *C*), 130.7 (*para C*-H), 128.7 (*meta C*-H), 128.5 (quaternary *C*), 126.5 (*ortho C-H*), 52.0 (OCH_3), 12.1 (CCH_3). The signal due to one of the quaternary carbon atoms was not observed due to low relative intensity; *m/z* (ESI+) 240.0 ($[M+Na]^+$, 100%), 218.0 ($[M+H]^+$, 54%); HR-ESI-MS, *m/z* = 240.0628, (calculated for $C_{12}H_{11}NO_3Na^+$ *m/z* = 240.0631). The data are consistent with those reported previously.[13]

5-Methyl-2-phenyl-oxazole-4-carboxylic acid. 5-Methyl-2-phenyl-oxazole-4-carboxylic acid methyl ester (2.04 g, 9.4 mmol) was dissolved in a mixture of 3 M aqueous NaOH solution (20 mL, 60 mmol) and MeOH (32 mL) and the resulting solution was heated to 45 °C for 2 hours. The mixture was acidified to pH 1 with 12 M aqueous hydrochloric acid and extracted with diethyl ether (3 x 100 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo* to give the product as an off white crystalline solid (1.30 g, 6.4 mmol, 68 %); m.p. 182 - 183 °C (lit.[14] 181.5 - 183 °C); υ_{max}/cm^{-1} 2970 (O-H), 1717 (C=O); δ_H (400 MHz, DMSO-d_6) 13.36 - 12.73 (1H, m, CO_2H), 8.15 - 7.89 (2H, m, *ortho* C-*H*), 7.68 - 7.25 (3H, m, *meta* and *para* C-*H*), 2.65 (3H, s, CCH_3); δ_C (100 MHz, DMSO-d_6) 163.1, 158.5, 156.2 (3 x quaternary *C*), 131.0 (*para C*-H), 129.3 (*meta C*-H), 128.8, 126.3 (2 x quaternary *C*), 126.0 (*ortho C*-H), 12.0 (CCH_3); *m/z* (ESI+) 204.0 ($[M+H]^+$, 100%), 226.0 ($[M+Na]^+$, 30%); HR-ESI-MS, *m/z* = 226.0478, (calculated for $C_{11}H_9NO_3Na^+$ *m/z* = 226.0475). The data are consistent with those reported previously.[13]

(2*S*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester. Acetyl chloride (16.3 mL, 229 mmol) was added dropwise with stirring to MeOH at 0 °C. L-Isoleucine (5.00 g, 38.1 mmol) was added and the mixture was heated to reflux overnight. The resulting solution of L-isoleucine methyl ester hydrochloride was concentrated *in vacuo* and used

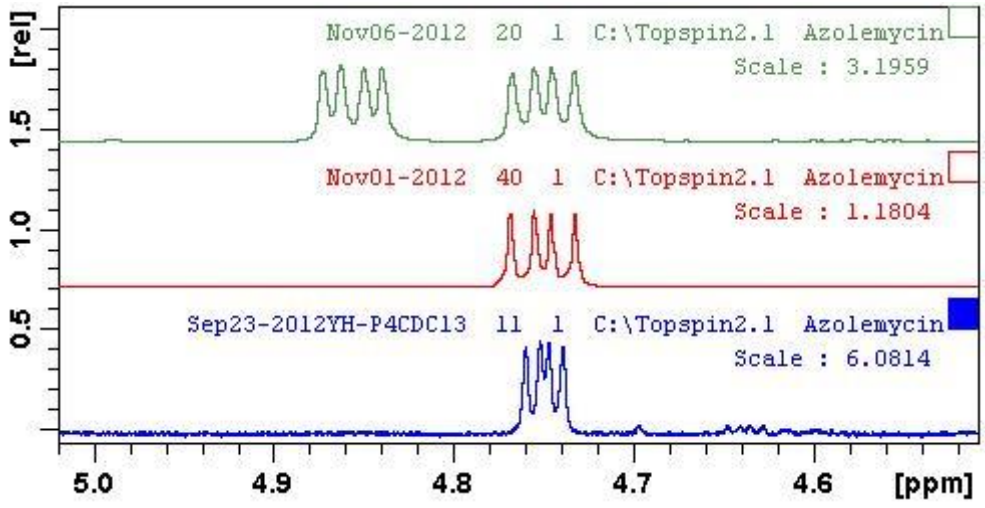
without further purification. A solution of L-isoleucine methyl ester hydrochloride (1.08 g, 6.40 mmol) and triethylamine (0.9 mL, 6.4 mmol) in CH_2Cl_2 (20 mL) was added to a stirred and cooled suspension of HATU (2.4 g, 6.4 mmol) and 5-Methyl-2-phenyl-oxazole-4-carboxylic acid (1.3 g, 6.4 mmol) in CH_2Cl_2 (20 mL) at 0-5 °C. The resulting mixture was stirred at 0-5 °C for 15 minutes, then triethylamine (1.8 mL, 12.8 mmol) was added dropwise and mixture was allowed to warm to room temperature overnight. To the resulting yellow solution was added pH 2 buffer (100 mL), and the separated aqueous phase was extracted with CH_2Cl_2 (2 x 50 mL). The combined organics were concentrated *in vacuo* and the resulting yellow residue was partitioned between pH 2 buffer (100 mL) and toluene (100 mL). The separated organic phase was washed with water (2 x 50 mL), saturated aqueous $NaHCO_3$ solution (50 mL) and saturated aqueous NaCl solution (50 mL), dried over Na_2SO_4 and concentrated *in vacuo*. Purification using silica gel chromatography (2% MeOH: CH_2Cl_2) yielded the product as a colorless oil (0.8 g, 2.4 mmol, 38 %); $[\alpha]_D^{25}$ -23.2 (c = 1.1, $CHCl_3$); υ_{max}/cm^{-1} 2964 (N-H), 1740 (ester C=O), 1670 (amide C=O), 1580 (N-H); δ_H (600 MHz, $CDCl_3$) 8.26 - 7.94 (2H, m, *ortho* C-*H*), 7.53 (1H, d, *J* 8.5 Hz, CHN*H*), 7.49 - 7.41 (3H, m, *meta* and *para* C-*H*), 4.74 (1H, dd, *J* 9.0, 5.5 Hz, NHC*H*), 3.77 (3H, s, OCH_3), 2.71 (3H, s, CCH_3), 2.03 (1H, dqt, *J* 9.5, 7.0, 5.0 Hz, C*H*CH_3), 1.56 (1H, dqd, *J* 15.0, 7.0, 4.5 Hz, CHCH_2), 1.29 (1H, ddq, *J* 15.0, 9.5, 7.5 Hz, CHCH_2), 1.00 (3H, d, *J* 7.0 Hz, CHCH_3), 0.97 (3H, t, *J* 7.5 Hz, CH_2CH_3); δ_C (125 MHz, $CDCl_3$) 172.3 (CO_2Me), 161.8, 158.6, 153.2 (3 x quaternary *C*), 130.6 (Ar *C*-H), 129.9 (quaternary *C*), 128.7 (Ar *C*-H), 126.8 (quaternary *C*), 126.4 (Ar *C*-H), 56.0 (NH*C*H), 52.1 (O*C*H_3), 38.0 (*C*HCH_3), 25.2 (CH_2CH_3), 15.6 (CH*C*H_3), 11.8 (oxazole *C*H_3), 11.5 (CH_2CH_3); *m/z* (ESI+) 331.1 ($[M+H]^+$, 100%), 353.1 ($[M+Na]^+$, 85%); HR-ESI-MS, *m/z* = 353.1479, (calculate for $C_{18}H_{22}N_2O_4Na^+$ *m/z* = 353.1472).

Mixture of (2*S*, 3*S*) and (2*R*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester. The method used was modified from a procedure reported by du Vigneaud *et al.*[15] A solution of (2*S*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester (0.10 g, 0.29 mmol) in 3 M aqueous NaOH solution (6 mL, 18.2 mmol) and MeOH (5 mL) was heated to 45 °C overnight. The reaction was acidified to pH 1 with 12 M aqueous hydrochloric acid and extracted with EtOAc (3 x 20 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo* to give the acid as a white solid, which was dissolved in 3 M aqueous NaOH solution (0.5 mL, 1.8 mmol), THF (0.5 mL) and water (0.5 mL). Acetic anhydride was added (0.05 mL, 0.5 mmol) and the resulting mixture was heated to 65 °C for 3 days. The mixture was cooled to room temperature, acidified with pH 2 buffer (5 mL) and extracted with EtOAc (5 x 10 mL). The combined organics were dried over Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in a solution of acetyl chloride (0.43 mL, 6 mmol) in MeOH (2.5 mL) and heated to reflux for 18 hours. The mixture was concentrated *in vacuo* to afford the products as a brown oil (0.096 g, 0.28 mmol, 96%, 1:1 (2*S*, 3*S*) and (2*R*, 3*S*) isomers, determined by ^{1}H NMR spectroscopy); υ_{max}/cm^{-1} 2962 (N-H), 1739 (ester C=O), 1649 (amide C=O), 1506 (N-H); δ_H (500 MHz, $CDCl_3$) 8.10 - 7.99 ((2*S*, 3*S*), 2H, m, *ortho* C-*H*; (2*R*, 3*S*), 2H, m, *ortho* C-*H*), 7.51 - 7.43 ((2*S*, 3*S*), 4H, m, *meta* and *para* C-*H*, N*H*CH; (2*R*, 3*S*), 4H, m, *meta* and *para* C-*H*, N*H*CH), 4.85 ((2*R*, 3*S*), 1H, dd, *J* 9.5, 4.5 Hz, NHC*H*), 4.73 ((2*S*, 3*S*), 1H, dd, *J* 9.0, 5.5 Hz, NHC*H*), 3.77 ((2*S*, 3*S*), 3H, s, OCH_3; (2*R*, 3*S*), 3H, s, OCH_3), 2.72 ((2*S*, 3*S*), 3H, s, CCH_3; (2*R*, 3*S*), 3H, s, CCH_3), 2.15 - 1.94 ((2*S*, 3*S*), 1H, m, C*H*CH_3; (2*R*, 3*S*), 1H, m, C*H*CH_3), 1.66 - 1.44 ((2*S*, 3*S*), 1H, m, CHCH_2; (2*R*, 3*S*), 1H, m, CHCH_2), 1.39 - 1.16 ((2*S*, 3*S*), 1H, m, CHCH_2; (2*R*, 3*S*), 1H, m, CHCH_2), 1.02 - 0.94 ((2*S*, 3*S*), 6H, m, CHCH_3, CH_2CH_3; (2*R*, 3*S*), 6H, m, CHCH_3, CH_2CH_3); δ_C (125 MHz, $CDCl_3$) 172.7, 172.3 ((2*S*, 3*S*), CO_2Me; (2*R*, 3*S*), CO_2Me), 162.0

161.8, 158.6, 153.2 ((2*S*, 3*S*), 3 x quaternary *C*; (2*R*, 3*S*), 3 x quaternary *C*), 130.6 ((2*S*, 3*S*), Ar *C*-H; (2*R*, 3*S*), Ar *C*-H), 129.9 ((2*S*, 3*S*), quaternary *C*; (2*R*, 3*S*), quaternary *C*), 128.7 ((2*S*, 3*S*), Ar *C*-H; (2*R*, 3*S*), Ar *C*-H), 126.8 ((2*S*, 3*S*), quaternary *C*; (2*R*, 3*S*), quaternary *C*), 126.4 ((2*S*, 3*S*), Ar *C*-H; (2*R*, 3*S*), Ar *C*-H), 56.0, 55.0 ((2*S*, 3*S*), NH*C*H; (2*R*, 3*S*), NH*C*H), 52.2, 52.1 ((2*S*, 3*S*), O*C*H$_3$; (2*R*, 3*S*), O*C*H$_3$), 38.0, 37.8 ((2*S*, 3*S*), *C*HCH$_3$; (2*R*, 3*S*), *C*HCH$_3$), 26.3 ((2*S*, 3*S*), *C*H$_2$CH$_3$; (2*R*, 3*S*), *C*H$_2$CH$_3$), 15.6, 14.8 ((2*S*, 3*S*), CH*C*H$_3$; (2*R*, 3*S*), CH$_2$*C*H$_3$), 11.8 ((2*S*, 3*S*), oxazole *C*H$_3$; (2*R*, 3*S*), oxazole *C*H$_3$), 11.7, 11.5 ((2*S*, 3*S*), CH$_2$*C*H$_3$; (2*R*, 3*S*), CH$_2$*C*H$_3$); *m/z* (ESI+) 353.1 ([M+Na]$^+$, 100%), 331.2 ([M+H]$^{1+}$, 82%); HR-ES-MS, *m/z* = 331.1659, (calculate for $C_{18}H_{23}N_2O_4$ *m/z* = 331.1652).

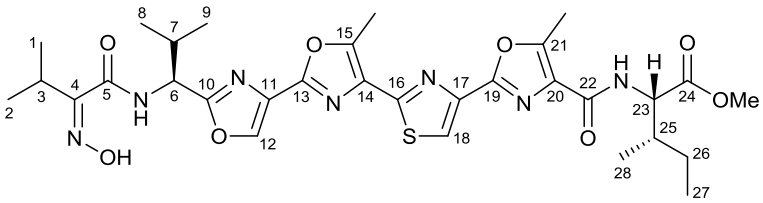
Comparison of the ^{1}H NMR spectra for (2*S*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester, the mixture of (2*S*, 3*S*)- and (2*R*, 3*S*)-2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl esters and azolemycin A confirmed the (23*S*, 25*S*) stereochemical assignment for the natural product (Figure S8).

Figure S8. Comparison of the pertinent region of the ^{1}H NMR spectra in $CDCl_3$ for the mixture of (2*S*, 3*S*)- and (2*R*, 3*S*)-2-(*N*-(5-Methyl- 2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl esters (top panel, 400 MHz), (2*S*, 3*S*)- 2-(*N*-(5-Methyl-2-phenyl-oxazole-4-carboxyamino)-3-methyl-pentanoic acid methyl ester (middle panel, 400 MHz) and azolemycin A (bottom panel, 700 MHz)



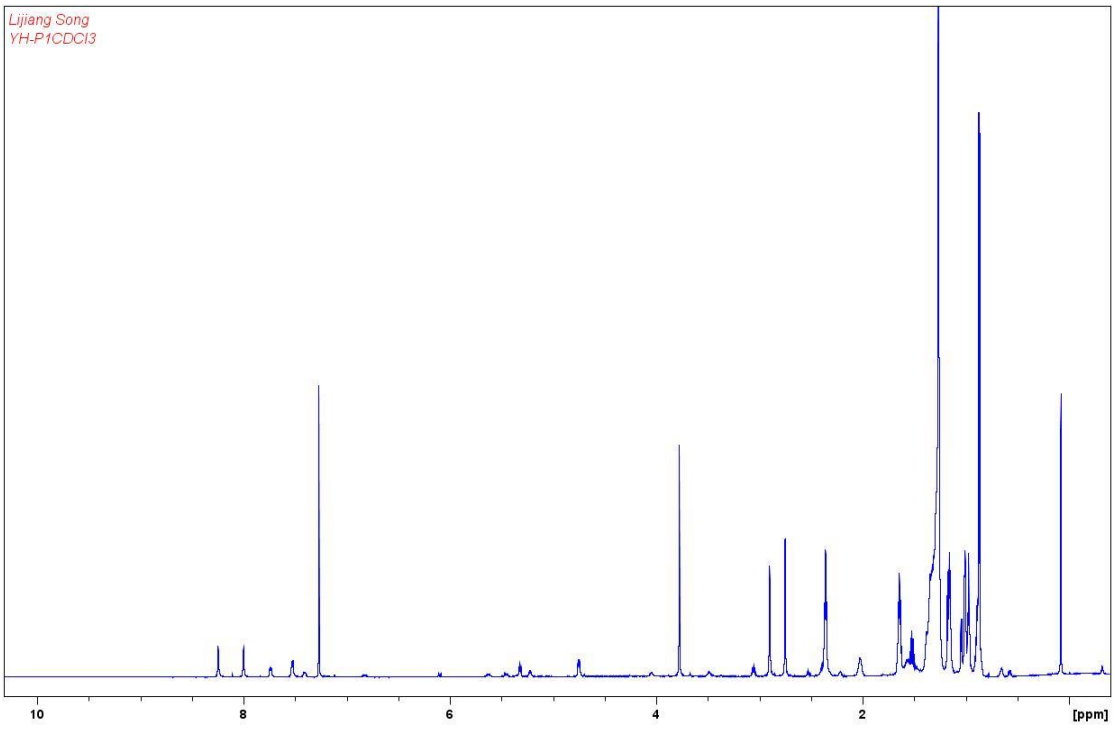
The ^{1}H NMR spectra of azolemycins A and B were found to be very similar (Figures S1 and S9, Tables S2 and S3). Only the signals due to protons in the vicinity of the oxime group differed significantly in chemical shift.

Table S3. Assignments for resonances observed in the ^{1}H NMR spectrum of azolemycin B ($CDCl_3$, 700 MHz)



Position	δ_H/ppm (no. of protons, multiplicity, J/Hz)
1/2	1.23 (3H, d, 7.0)
1/2	1.15 (3H, d, 7.0)
3	3.05 (1H, m)
OH	not observed
6-NH	7.40 (1H, d, 8.5)
6	5.21 (1H, dd, 6.5, 8.5)
7	2.31 (1H, m)
8/9	0.95 (3H, d, 6.5)
8/9	1.04 (3H, d, 6.5)
12	8.25 (1H, s)
15-Me	2.88 (3H, s)
18	8.00 (1H, s)
21-Me	2.74 (3H, s)
23-NH	7.53 (1H, d, 9.0)
23	4.73 (1H, dd, 5.5, 9.0)
25	2.00 (1H, m)
26	1.28/1.56 (2 x 1H, 2 x m)
27	0.96 (3H, t, 7.2)
28	0.99 (3H, d, 6.3)
24-OMe	3.76(3H, s)

Figure S9. ^{1}H NMR spectrum of azolemycin B



Azolemycin B was assigned as the *Z*-oxime isomer and azolemycin A was assigned as the *E*-oxime isomer on the basis of the comparison of chemical shift values observed for H-3/C-3 and calculated for the corresponding protons/carbons in the oxime derived from condensation of the N-methyl amide derivative of a-ketoisovaleric acid with hydroxylamine (Figure S10, Table S4). Structure conformational searching was done by systematic rotation of all bonds that create distinguishable conformations in Chem3D using the PM3 semi-empirical basis set. PM3 ground-state structures were energy minimized with Firefly using the B3LYP-D3(BJ) functional and the 6-31G+(d,p) basis set (including Hessian and thermochemistry calculations at 298K). DFT ground-state structures were analyzed with the GIAO method in Gaussian03 using the mPW1PW91 functional and the 6-311+G(2d,p) basis set and scrf=(solvent=chcl3,cpcm,read) radii=uaks nosymcav options. NMR shifts were calculated using parameters specific to the functional, basis set and option combination described by Lodewyk *et al.*[16]

Figure S10. Energy minimized structures of the lowest energy conformations of the *Z*- (left) and *E*-oxime (second from left) derived from the condensation of hydroxylamine with the N-methyl amide of α-ketoisovaleric acid, and the *Z*- (second from right) and *E*-O-methyloxime (right) derived from the condensation of O-methyl-hydroxylamine with the N-methyl amide of α-ketoisovaleric acid.

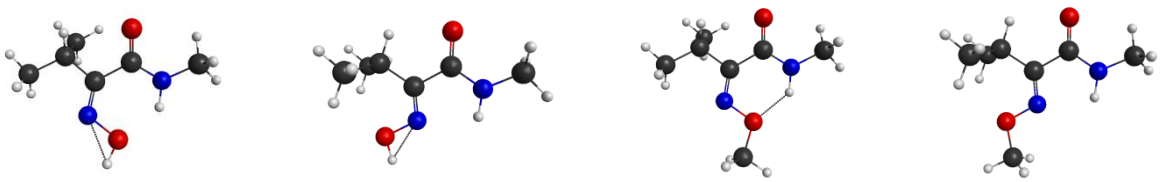
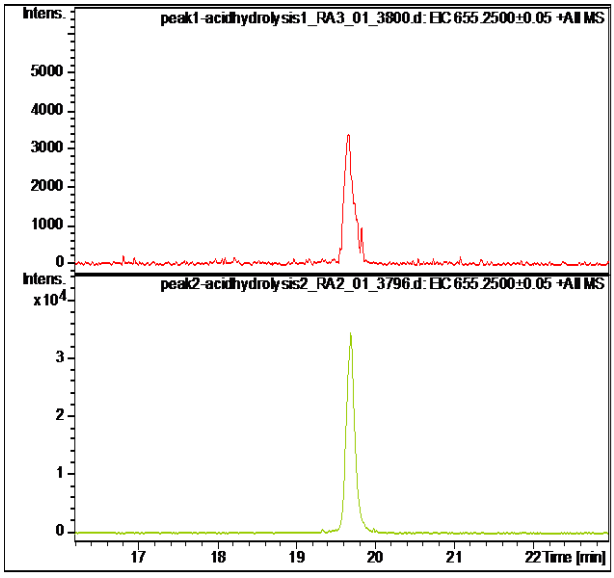


Table S4. Comparison of the observed chemical shift values for H-3 and C-3 of azolemycins A and B with the calculated chemical shift values for the methine proton and carbon of the *Z*- and *E*-oximes, respectively, derived from the condensation of hydroxylamine with the N-methyl amide of α-ketoisovaleric acid, and the observed chemical shift values for H-3 and C-3 of azolemycins C and D with the calculated chemical shift values for the methine proton and carbon of *Z*- and *E*-O-methyloximes, respectively, derived from the condensation of O-methyl-hydroxylamine with the N-methyl amide of α-ketoisovaleric acid.

Geometry	B3LYP-D3(BJ)/6-31G+(d,p)											
NMR	mPW1PW91/6-311+G(2d,p) scrf=(solvent=chcl3,cpcm,read)											
intercept 1H	31.8486		intercept 13C	186.0596								
slope 1H	1.0823		slope 13C	1.0448								
	631G+dp	G at 298	total energy	degeneracy	relative energy	population	isotropic H	Calculated		isotropic C	Calculated	
	Hartrees	kJ/mol	Hartrees		kJ/mol	inc degen.	ppm	NMR SHIFT	weighted NMR	ppm	NMR SHIFT	weighted NMR
Z-Ox111	-495.847933	387.617	-495.7002691	2	5.2378	0.24124526	29.1172	2.523699529	0.214262228	151.9146	32.680896	2.774609848
Z-Ox121	-495.846271	385.87	-495.6992733	1	7.8517	0.04197731	28.7803	2.834981059	0.0418807	148.1755	36.259667	0.535657978
Z-Ox211	-495.838508	385.702	-495.6915741	2	28.062	2.3969E-05	28.5607	3.037882288	2.56254E-05	150.4978	34.036945	0.000287112
Z-Ox212	-495.849377	386.17	-495.7022644	2	0	2	28.2442	3.33031507	2.34403717	151.3348	33.235835	23.39299137
Z-Ox221	-495.83796	385.223	-495.691208	2	29.023	1.6259E-05	28.9997	2.632264622	1.5062E-05	147.0924	37.296325	0.000213412
Z-Ox222	-495.848338	386.604	-495.7010606	2	3.1601	0.5582579	29.1341	2.508084635	0.492749555	145.823	38.511294	7.566101537
						2.8415207		**predicted shift**	**3.09297034**			**34.26986126**
								OBS SHIFT	**3.05**			**not measured**
E-Ox111	-495.842994	382.976	-495.697098	1	20.16	0.00029135	27.6491	3.880162617	0.000843619	160.2521	24.7009	0.005370429
E-Ox112	-495.851729	385.747	-495.7047781	1	0	1	27.9339	3.617019311	2.699223891	156.8654	27.942381	20.85218151
E-Ox221	-495.844046	388.891	-495.6958968	1	23.313	8.1556E-05	28.7957	2.820752102	0.000171676	159.5457	25.37701	0.001544488
E-Ox222	-495.852105	389.406	-495.7037594	1	2.6741	0.33964907	27.9837	3.571006191	0.905126147	157.6603	27.181566	6.889583676
						1.34002197		**predicted shift**	**3.605365332**			**27.74868011**
								OBS SHIFT	**3.47**			**25.6**
Z-MeOx121	-535.159045	453.29	-534.9863626	1	3.5581	0.23768837	29.1348	2.507437864	0.266341294	145.5509	38.771727	4.118352042
Z-MeOx211	-535.137579	448.756	-534.9666245	2	55.37	3.8945E-10	29.0838	2.554559734	4.44597E-10	145.4752	38.844181	6.76046E-09
Z-MeOx21211	-535.160277	452.967	-534.987718	2	0	2	28.2139	3.358311004	3.001589543	151.5176	33.060873	29.54913057
						2.23768837		**predicted shift**	**3.267930837**			**33.66748261**
								OBS SHIFT	**2.97**			**31.1**
E-MeOx111	-535.16263	447.763	-534.992054	1	0	1	27.9709	3.582832856	2.709682955	156.6724	28.127106	21.27242377
E-MeOx121	-535.162923	451.335	-534.9909857	1	2.8044	0.32223323	28.2194	3.353229234	0.817194631	156.8911	27.917783	6.803669247
						1.32223323		**predicted shift**	**3.526877586**			**28.07609302**
								OBS SHIFT	**3.39**			**25.6**

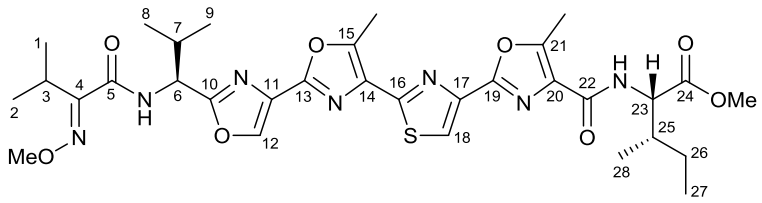
To confirm that azolemycins A and B differ only in the configuration of the oxime, a small amount of each compound was dissolved separately in chloroform, then diluted 100-fold with 5M HCl and incubated for 4 hours at 42 °C. UHPLC-HRMS analyses showed that in both cases a compound with a molecular formula corresponding to the ketone resulting from oxime hydrolysis was formed (*m/z* calculated for $C_{31}H_{39}N_6O_8S$: 655.2545; found: 655.2539) had been formed. These compounds had identical retention times (Figure S11).

Figure S11. Extracted ion chromatograms at *m/z* = 655.25 (corresponding to the ketone product of oxime hydrolysis) from UHPLC-HRMS analyses of the mild acid hydrolysis of azolemycin A (bottom panel) and azolemycin B (top panel).



Azolemycins C and D have very similar NMR spectra to azolemycins A (Tables S5 and S6, and Figures S12-S17). The only significant difference is an additional signal in the spectra for azolemycins C and D that is attributable to an O-methyl group. Azolemycins C and D are therefore assigned as derivatives of azolemycins A and B, respectively, in which the oxime is O-methylated.

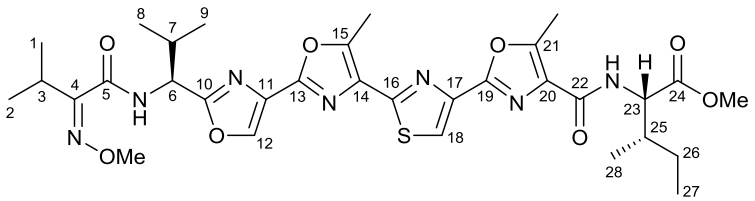
Table S5. Assignments for signals observed in the ^{1}H and HSQC/HMBC spectra of azolemycin C ($CDCl_3$, 700 MHz)



Position	δ_H/ppm (no. of protons, multiplicity, J/Hz)	δ_C/ppm*
1	1.24 (3H, d, 7.0)	18.4
2	1.20 (3H, d, 7.0)	18.3
3	3.39 (1H, m)	25.6
4		157.6
4-NOMe	3.99 (3H, s)	62.7
5		162.8
6-NH	7.29 (1H, d, 9.0)	
6	5.19 (1H, dd, 6.5, 9.0)	52.6
7	2.30 (1H, m)	32.9
8	0.98 (3H, d, 6.5)	19.0
9	1.00 (3H, d, 6.5)	18.4
10		165.3
11		129.8
12	8.23 (1H, s)	138.6
13		153.0
14		130.9
15		148.2
15-Me	2.88 (3H, s)	11.8
16		161.9
17		143.9
18	7.99 (1H, s)	120.1
19		154.3
20		129.8
21		153.7
21-Me	2.74 (3H, s)	11.4
22		161.7
23-NH	7.51(1H, d, 8.5)	
23	4.74 (1H, dd, 5.5, 8.5)	56.1
24		172.1
25	2.00 (1H, m)	37.9
26	1.28/1.56 (2 x 1H, 2 x m)	25.4
27	0.96 (3H, t, 7.5)	11.6
28	0.97 (3H, d, 6.5)	15.5
24-OMe	3.77 (3H, s)	52.6

* Based on HSQC and HMBC data

Table S6. Assignments for signals observed in the ^{1}H and HSQC/HMBC spectra of azolemycin D ($CDCl_3$, 700MHz)



Position	δ_H/ppm (no. of protons, multiplicity, J/Hz)	δ_C/ppm*
1/2	1.14 (3H, d, 7.0)	19.9
1/2	1.15 (3H, d, 7.0)	20.0
3	2.97(1H, m)	31.1
4		156.8
4-NOMe	3.95 (3H, s)	62.4
5		161.8
6-NH	7.38(1H, d, 8.9)	
6	5.28(1H, dd, 6.4, 8.9)	53.0
7	2.35(1H, m)	32.6
8	1.00(3H, d, 6.7)	18.8
9	1.03 (3H, d, 6.7)	18.4
10		164.8
11		129.9
12	8.23(1H, s)	138.3
13		153.0
14		130.7
15		148.3
15-Me	2.90(3H, s)	12.0
16		162.1
17		143.8
18	7.99(1H, s)	119.9
19		154.0
20		129.9
21		153.5
21-Me	2.74(3H, s)	11.5
22		161.6
23-NH	7.51(1H, d, 8.8)	
23	4.74 (1H, dd, 5.4, 8.8)	55.9
24		172.2
25	2.00 (1H, m)	37.8
26	1.28/1.56(2H, m)	25.3
27	0.96(3H, t, 7.4)	11.5
28	0.97(3H, d, 6.3)	15.5
24-OMe	3.77(3H, s)	52.0

* Based on HSQC and HMBC data

Figure S12. ^{1}H NMR spectrum of azolemycin C

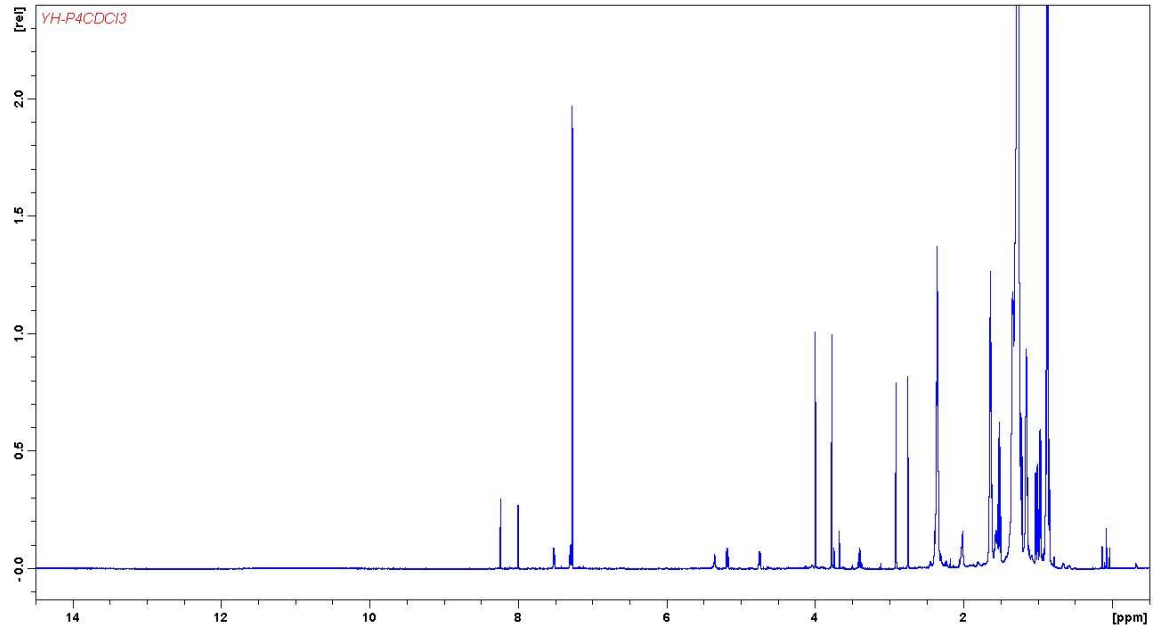


Figure S13. HSQC NMR spectrum of azolemycin C

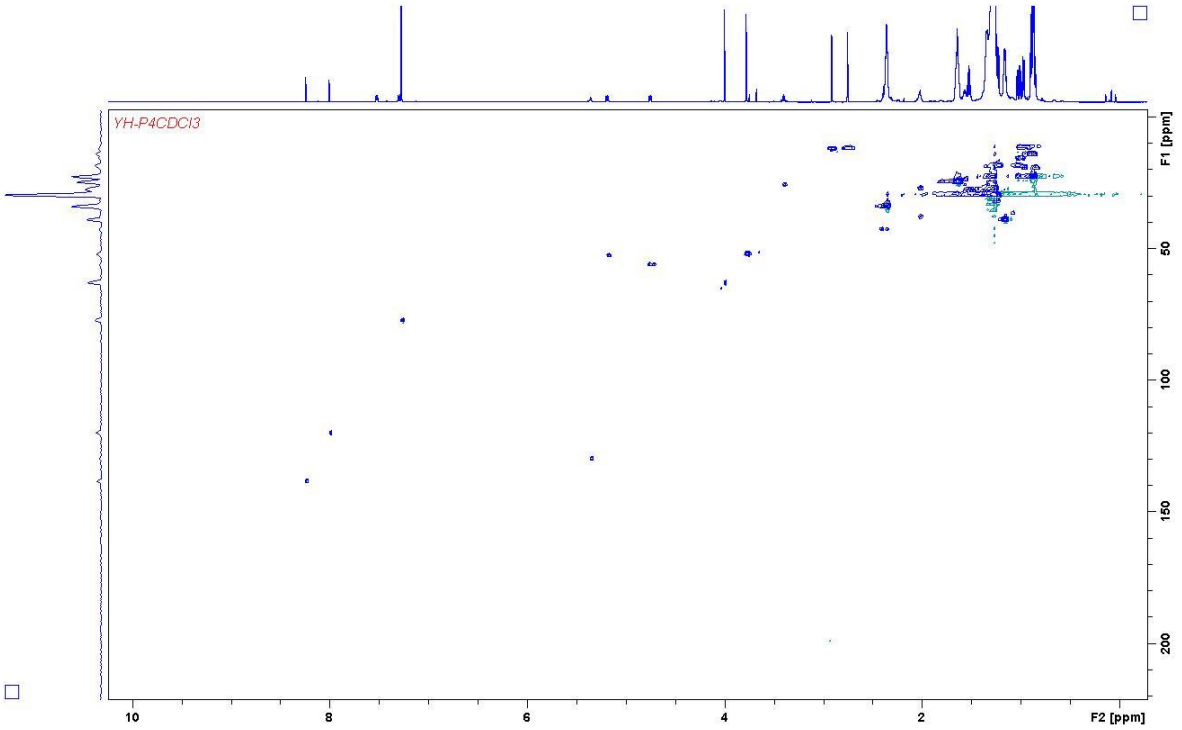


Figure S14. HMBC NMR spectrum of azolemycin C

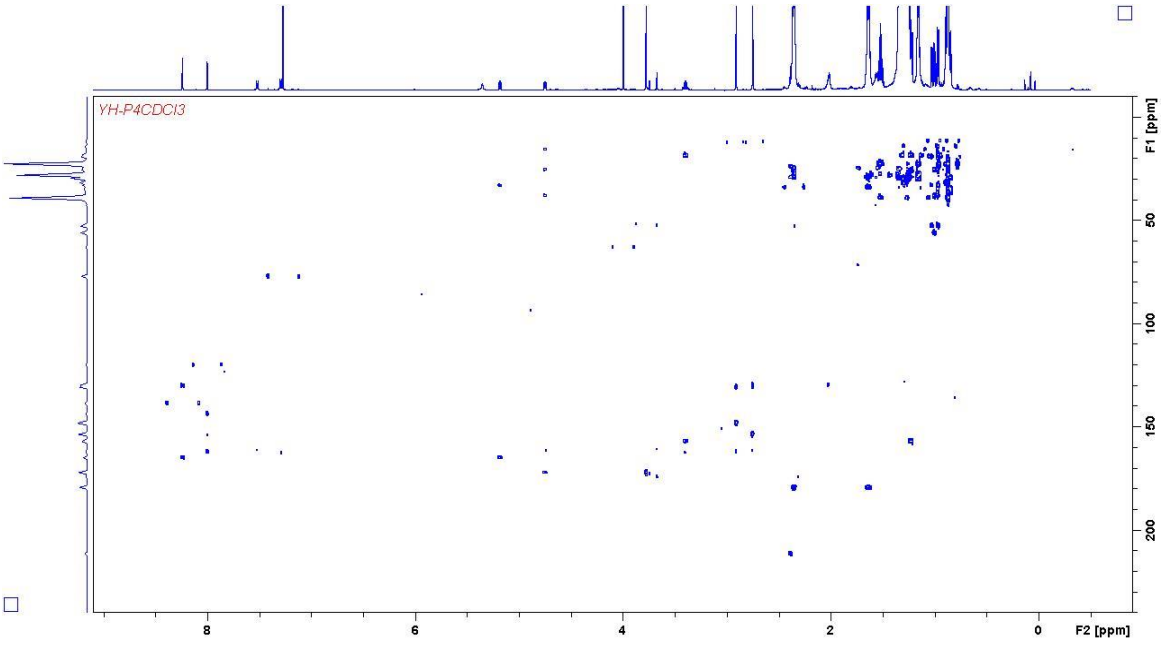


Figure S15. ^{1}H NMR spectrum of azolemycin D

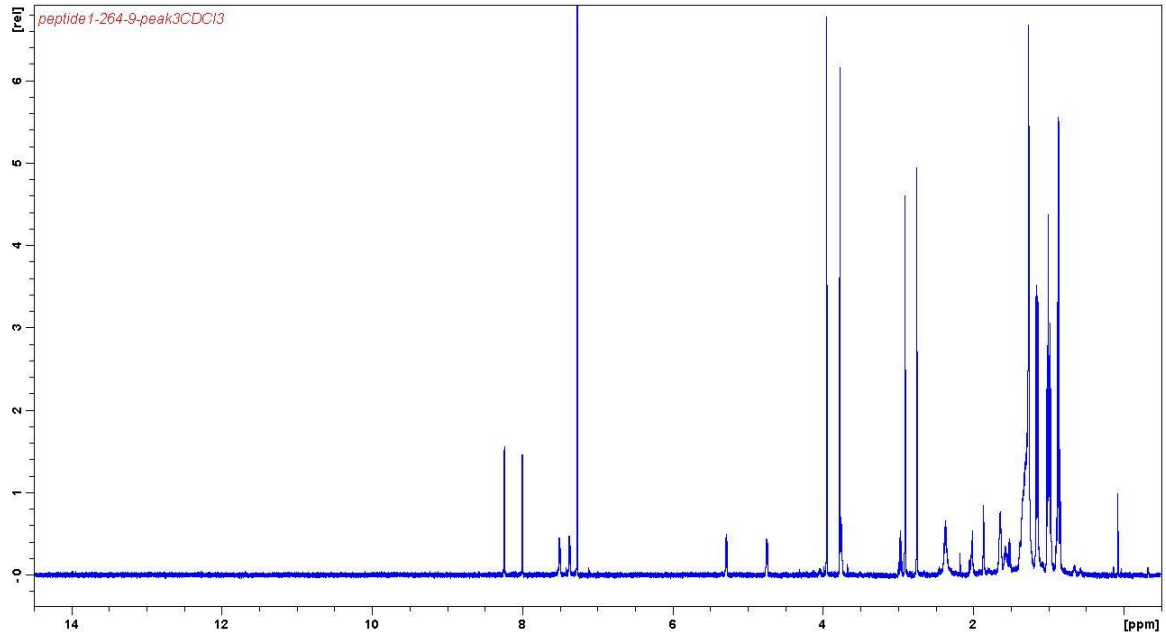


Figure S16. HSQC NMR spectrum of azolemycin D

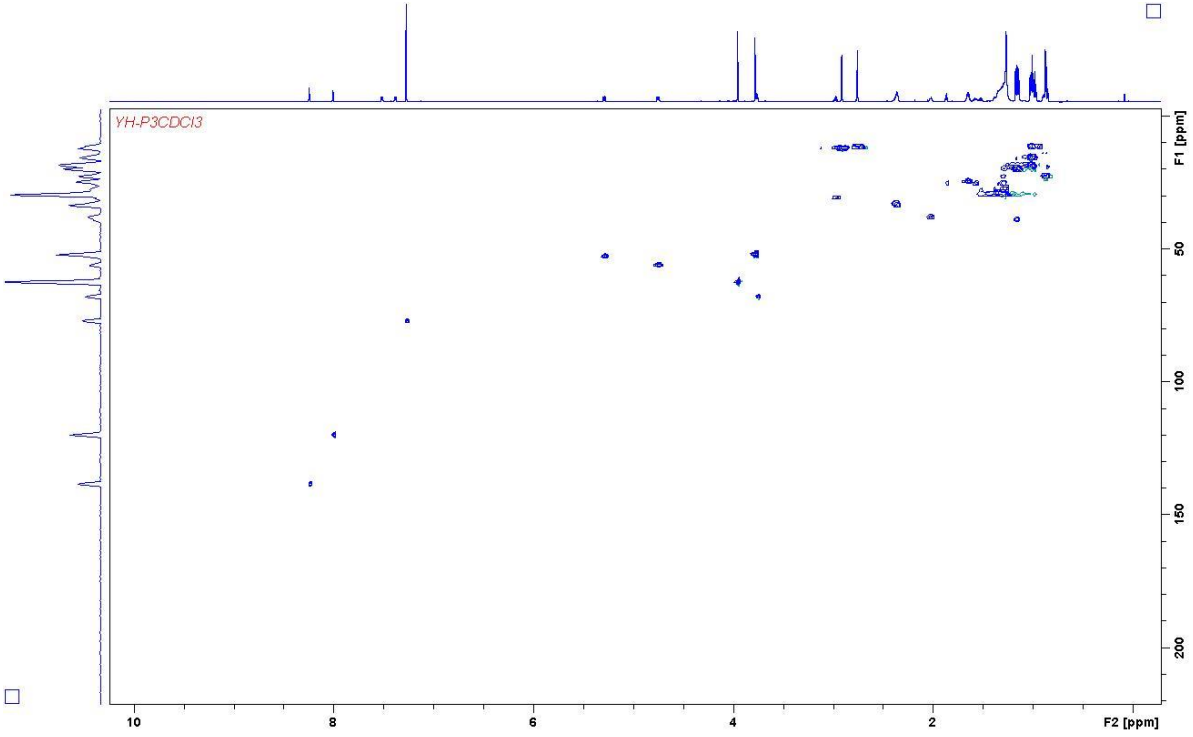
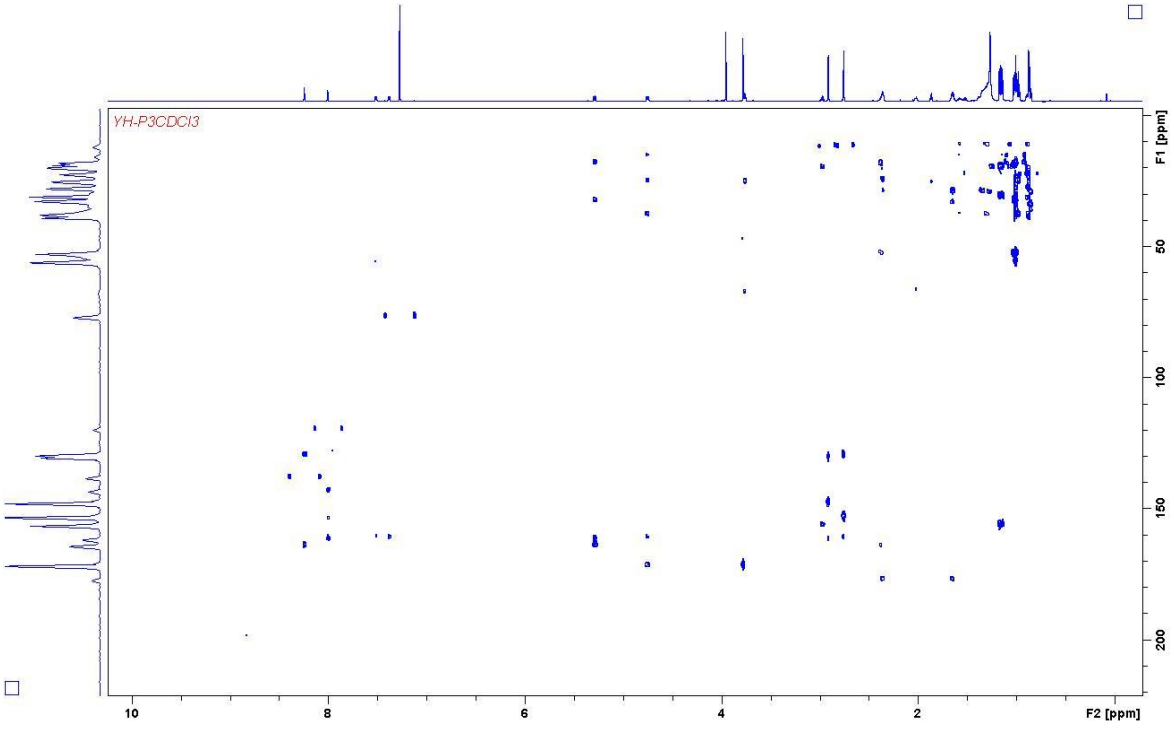


Figure S17. HMBC NMR spectrum of azolemycin D



Nucleic acid manipulations, genome sequencing and annotation

Nucleic acid manipulations were performed using standard procedures for *E. coli*[17] and *Streptomyces*[18], or according to the instructions from manufacturers (for restriction enzymes and kits). Roche 454 FLX sequencing performed by the Chinese National Human Genome Center (Shanghai, China) resulted in a total of 9,436,877 bp of genome sequence distributed across 254 contigs of ≥ 500 bp with an average size of 37,153 bp. The sequences obtained were analyzed using a combination of Glimmer 3.02,[19] Genemark[20] and BLASTP[21] to identify and annotate CDSs, respectively. The proposed functions of proteins encoded by genes in the azolemycin biosynthetic gene cluster are listed in Table S7. The sequence data of the *azm* gene cluster have been deposited in the GenBank/EMBL/DDBJ database under the accession no. KT336319.

Table S7. Proposed functions of proteins encoded by genes in the azolemycin biosynthetic gene clsuter

Gene name	Size (aa)	Closest homologue (identity/similarity %)	Source of homologue	Proposed function
cds1	159	Hypothetical protein (76/85)	*Streptomyces himastatinicus*	Not involved
cds2	1448	Haloacid dehalogenase (89/92)	*Streptomyces bingchenggensis* BCW-1	Not involved
azmF	389	FAD-binding monooxygenase protein (51/61)	*Stackebrandtia nassauensis* DSM 44728	Oxime formation
azmA	72	-	-	Precursor peptide
azmB	400	SagB-type dehydrogenase (56/65)	*Streptomyces sulphureus*	Azoline oxidation
azmC/D	697	YcaO-domain containing protein (60/68)	*Streptomyces* sp. MspMP-M5	Azoline biosynthesis
azmE	290	Isoleucine-carboxyl methyl transferase (45/56)	*Streptomyces violaceusniger* Tu 4113	Methylation of carboxylic acid and oxime groups
azmR	339	LysR family transcriptional regulator (75/83)	*Streptomyces violaceusniger* Tu 4113	Pathway-specific regulator
cds3	345	Histidinol-phosphate aminotransferase (80/87)	*Streptomyces violaceusniger* Tu 4113	Not involved

Identification of a putative LAP biosynthetic gene clusters containing an *azmF* homologue

The sequence of AzmF was used to search the all genomes database of the Joint Genome Institute (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=FindGenes&page=geneSearch). The proteins encoded by the genes surrounding the top 20 BLAST hits were manually examined to determine whether they are homologues of enzymes known to be involved in LAP biosynthesis. This resulted in the identification of a single putative LAP biosynthetic gene cluster in the genome of *Actinomadura oligospora* ATCC 43269. The proposed functions of the proteins encoded by the genes in this gene cluster are summarised in Table S8.

Table S8. Organisation and proposed functions of the encoded proteins of the putative azolemycin-like biosynthetic gene cluster in *Actinomadura oligospora* ATCC 43269

Gene ID	Size (aa)	Predicted protein families	Homologue in the *azm* pathway (identity/similarity %)	Proposed function
2540954195	627	YcaO-domain containing protein	AzmC/D (27/35)	Azoline biosynthesis
2540954196	485	SagB-type dehydrogenase	AzmB (19/26)	Azoline oxidation
2540954197	62	-		Precursor peptide
2540954198	45	-		Precursor peptide
2540954199	45	-		Precursor peptide
2540954200	46	-		Precursor peptide
2540954201	386	FAD-binding monooxygenase protein	AzmF (52/61)	Oxidation of one or more amino group(s) in the LAPs resulting from maturation of the precursor peptides
2540954202	157	Possible lysine decarboxylase		Unknown
2540954203	162	Hypothetical protein		Unknown
2540954204	321	Transcriptional regulator YafY		Pathway-specific regulator

Mutant construction and confirmation

The *azmC/D*, *azmE* and *azmF* mutants were constructed as follows. The *neo* gene, which confers kanamycin resistance, was amplified from pUC119::*neo* using primers *neo-Bgl*II-Forward and *neo-Bgl*II-Reverse (Tables S9 and S10).[22] Upstream and downstream regions of approximately 2 kb flanking the gene to be deleted were amplified from *S.* sp. FXJ1.264 genomic DNA. The resulting fragments overlapped by approximately 50 bp with the target gene. For example, for Δ*azmC/D*, the primers used were *azmC/D*-L-Forward with *azmC/D*-L-Reverse and *azmC/D*-R-Forward with *azmC/D*-R-Reverse (Table S10). These amplimers were digested with *Hin*dIII/*Bgl*II or *Xba*I/*Bgl*II, then separated by agarose gel electrophoresis and purified from the gel. The resulting DNA fragments were ligated with the *Bgl*II-digested *neo* amplimer and *Xba*I- and *Hin*dIII-digested pKC1139 to give pKC1139::*azmC/D*::*neo* (Table S9), which was subsequently introduced into *S.* sp. FXJ1.264 via conjugation.[18,23] Spores of exconjugants were harvested and spread on ISP 2 agar (yeast extract/malt extract agar) containing kanamycin. After incubation at 42 °C for 4 days, apramycin-sensitive (Apr^S) and kanamycin-resistant (Kan^R) colonies were identified, and further confirmed as *azmC/D* disruption mutants (Δ*azmC/D*) by PCR using primers Δ*azmC/D*-Foward and Δ*azmC/D*-Reverse (Table S10) and sequencing of the resulting amplimers. The *azmE* and *azmF* mutants were constructed and verified in an analogous manner.

A 180 bp in-frame deletion from nucleotide base 1 to 180 of *azmA* was created as described above with some modifications. Upstream and downstream regions each of approximately 2 kb accurately flanking the *azmA* reading frame were amplified from *S.* sp. FXJ1.264 genomic DNA using primer

pairs *azmA*-L-Forward/Reverse and *azmA*-R-Forward/Reverse (Table S10). These amplimers were digested with *Hin*dIII/*Bgl*II or *Xba*I/*Bgl*II, then separated by agarose gel electrophoresis and purified from the gel. The resulting DNA fragments were ligated with *Xba*I- and *Hin*dIII-digested pKC1139 to give pKC1139::*azmA* (Table S9), which was subsequently introduced into *S.* sp. FXJ1.264 via conjugation.[18,23] Spores of exconjugants were harvested and spread on ISP 2 agar without antibiotics. After incubation at 42 °C for 4 days, apramycin-sensitive (Apr^S) colonies were identified, and further screened and verified as *azmA* in-frame deletion mutants (Δ*azmA*) by PCR using primers Δ*azmA*-Foward and Δ*azmA*-Reverse (Table S10) and sequencing of the resulting amplimers. In the resulting mutant, the 180 bp *azmA* coding sequence was replaced by a 6 bp *Bgl*II restriction site without alteration of the reading frame.[17]

Table S9. Plasmids used and constructed in this study

Plasmid name	Plasmid description	Reference
pKC1139	*Streptomyces* suicide vector; capable of replicating in *E.coli*	[18]
pUZ8002	Enables conjugal transfer of plasmids from *E. coli* to *Streptomyces* spp.	[23]
pKC1139::*azmA*	*azmA* deletion construct	This study
pKC1139::*azmC/D*::*neo*	*azmC* deletion construct	This study
pKC1139::*azmE*::*neo*	*azmD* deletion construct	This study
pKC1139::*azmF*::*neo*	*azmE* deletion construct	This study
pUC119::*neo*	Source of *neo* gene conferring kan^R	[22]

447 **Table S10.** Primers used in this study (restriction sites used are underlined; protective nucleotides are in
448 italics)

Primer name	Primer sequence (5'-3')
azmA-L-Forward	*CCC*AAGCTTCGGACGACGAGGAACTCACCGACGA
azmA-L-Reverse	*GC*TCTAGAAGATCTGAGGTTCCGGATCAGGGAAATCCCAAAG
azmA-R-Forward	*GA*AGATCTTCCACCTGCACCATCAAGATG
azmA-R-Reverse	*GC*TCTAGACGGATAGGGGTTCAGTTCGTC
Δ*azmA*- Forward	CTGGTGGATGTAGAGCGTGGAGAGG
Δ*azmA*- Reverse	CACACGGATCCATTTCTCAAGTGAGAG
azmC/D-L-Forward	*CCC*AAGCTTGCAACCTGGGAGAGGTCACCTTCAG
azmC/D-L-Reverse	*GC*TCTAGAAGATCTCGGTGCTGGAGCAGATATAGACATGG
azmC/D-R-Forward	*GA*AGATCTGGACGAACTGAACCCCTATCCGCTG
azmC/D-R-Reverse	*GC*TCTAGACGACCGCCGCTATGTGCTGCTG
Δ*azmC/D*-Forward	ACTTTCCCTTGGGTATGGGTTCACT
Δ*azmC/D*-Reverse	GCAGTCCACCAGCACCAGTTTG
azmE-L-Forward	*CCC*AAGCTTACACCCCACGACGACCTGGTTCTCCC
azmE-L-Reverse	*GC*TCTAGAAGATCTGCAAGCGTTTGGCACACGGCTCTCG
azmE-R-Forward	*GA*AGATCT GCTCCTGCCATGACGCCCTGT
azmE-R-Reverse	*GC*TCTAGAGTTACCTCGACTCCCGGTATTTCGC
Δ*azmE*-Forward	CGGCTGATCCATGTGGGCAACATG
Δ*azmE*-Reverse	ACACCCAACCCGCCGTCAGCTATC
azmF-L-Forward	*CCC*AAGCTTCATCGCCTTCTGCATGGGCAACTGG
azmF-L-Reverse	*GC*TCTAGAAGATCTGATGGAGCCCAGCGTGATGCCCC
azmF-R-Forward	*GA*AGATCTTGGTGGATGTAGAGCGTGGAGAGGG
azmF-R-Reverse	*GC*TCTAGATCGTCCCAGAACGGCACCAGG
Δ*azmF*-Forward	CGGCAGATCATGCTGGTCAACCTGT
Δ*azmF*-Reverse	CGGCCCTGTGCCATGAAAATGTG
*neo-Bgl*II-Forward	*GA*AGATCTATCCCCTGGATACCGCTCGCCGCAG
*neo-Bgl*II-Reverse	*GA*AGATCTTACCCGAACCCCAGAGTCCCG

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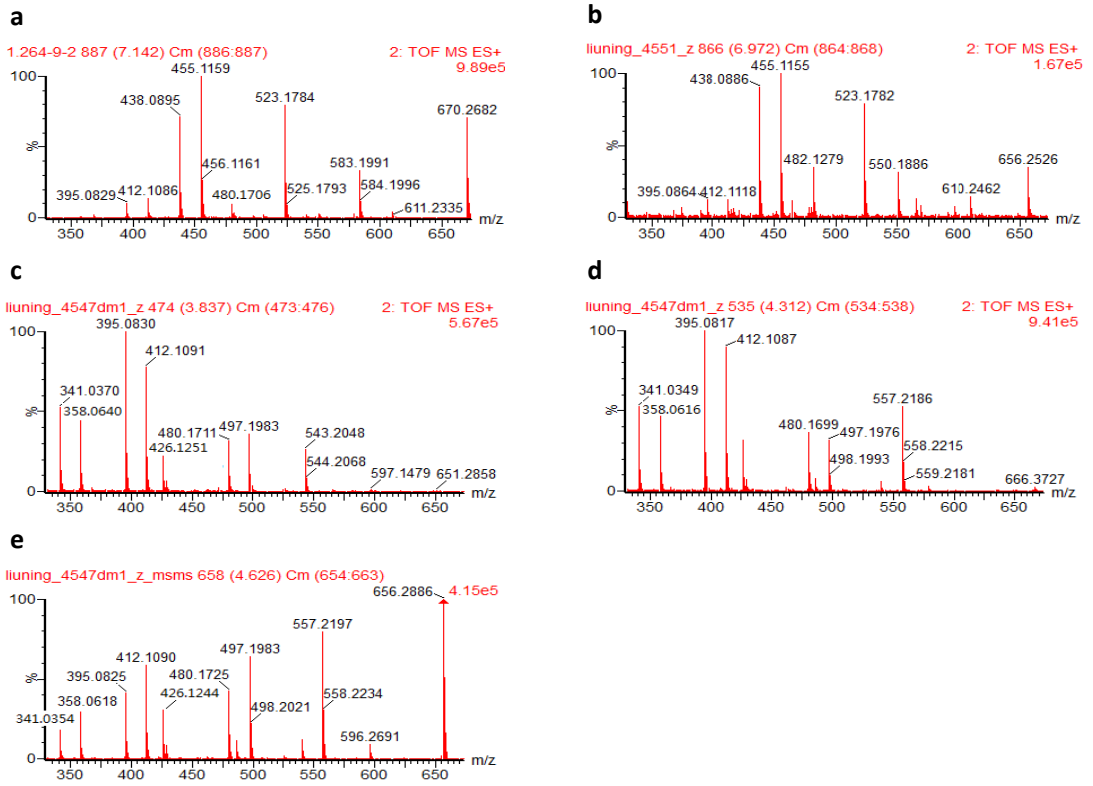
UHPLC-HRMS analyses of azolemycin production by wild type and mutant strains

The strains were cultured and extracted as described above for growth, extraction and analysis of small scale cultures. The dried ethanol extracts were re-dissolved separately in 1 ml of methanol and passed through a 0.4 µm membrane, then analyzed by LC-MS using an RP-C18 column (Agilent Zorbax, 100 x 2.1mm, 1.8 µm) connected to a Dionex 3000 RS UHPLC coupled with a Bruker MaXis mass spectrometer. 2 µl of sample was injected and the column was eluted at 0.2 ml/min using the following program: 0 min, 10% B; 5 min, 10 % B 17 min, 100% B; 22 min, 100% B; 25 min, 10% B. Mobile phases consisted of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. The mass spectrometer was calibrated using 20 µL of 10 mM sodium formate solution through loop injection prior to each run.

MS/MS analyses of azolemycin-related metabolites accumulated in the mutants

Spectra were recorded on a Waters ACQUITY UPLC/Xevo G2 QTof MS system, as described above for growth, extraction and analysis of small scale cultures (Figure S18).

Figure S18. MS/MS spectra of azolemycin-related metabolites produced by wild type *Streptomyces* sp. FXJ1.264 and the *azmE* and *azmF* mutants. (**a)** azolemycin A **1**; (**b**) intermediate **9**; (**c**) shunt metabolite **10**; (**d**) shunt metabolite **11**; **(e)** shunt metabolite **8**.



Antiproliferative and antimicrobial assays

The antiproliferative activity of the compounds was assayed using the MTT method in triplicate (Table S11).[24] Each well of a 96-well plate was filled with 10^4 cells. After cell attachment overnight, the medium was removed, and each well was treated with 50 μL of medium containing 0.2% DMSO, or an appropriate concentration of one of the test compounds, or the positive control cisplatin (DDP) (10 mg/ml as a stock solution in DMSO and serial dilutions; the test compounds showed good solubility in DMSO and did not precipitate when added to the cells). Cells were incubated at 37 °C for 4 h in a humidified incubator at 5% CO_2 and grown for another 48 h after the medium was changed to fresh Dulbecco's modified Eagle medium. The medium was removed from the wells and 50 μL of a solution containing 0.5 mg/mL MTT (Sigma) dissolved in serum-free medium or phosphate-buffered saline (PBS) was added to each well. The plate was incubated in the dark at 37 ℃ for 3 h. Upon removal of MTT/medium, 100 μL of DMSO was added to each well and the plate was agitated at 60 rpm for 5 min to dissolve the precipitate. The absorbance at 540 nm in each well was measured using a microplate reader (BioTek Synerge H4).

The antimicrobial activity of the compounds was tested using the paper disc diffusion method. 100 μL of the indicator strain at exponential phase were dispersed uniformly on LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) in a 9 cm petri-dish. Each compound was dissolved in MeOH at a concentration of 2 mg/mL, and 5 μL of compound solution were added to a 5 mm paper disc that was placed centrally on the agar. After evaporation of MeOH, the plates were incubated for 8-16 hours (37°C for bacteria and 30°C for yeasts), and the diameters of the growth inhibition zones were measured.

Table S11. IC_{50}/μM values for the azolemycins against various mammalian cell lines

	Cell line					
Compound	HCT116	Hela	T24	A549	SW480	MCF7
DDP	22.7±1.8	3.65±0.2	11.5±0.2	21.6±1.0	19.5±1.6	11.1±1.3
Azolemycin A	>200	>200	>200	>200	>200	>200
Azolemycin B	69.2±3.3	>200	>200	>200	>200	>200
Azolemycin C	>200	96.0±3.5	88.4±8.5	>200	>200	>200
Azolemycin D	>200	93.8±3.4	>200	>200	>200	>200

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