Supporting Information for

Bioactivity-HiTES Unveils Cryptic Antibiotics Encoded in Actinomycete Bacteria

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Bacterial strains

Strains *Streptomyces lavendulae* B-1230 and *Amycolatopsis keratiniphila* B-24117 were obtained from the NRRL. *Streptomyces hiroshimensis* A18, *B. subtilis* 168 and *E. coli* K12 (ZSR638) were kindly provided by the laboratory of Prof. Roberto Kolter at Harvard Medical School.

General procedures

Materials. All chemicals were purchased from Sigma-Aldrich. Bacterial media were purchased from Becton-Dickinson. Restriction enzymes, Q5 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs. Plasmid isolation, DNA gel extraction, and PCR purification kits were purchased from Qiagen.

Media. TSBY and R4 media were routinely used to culture the three actinomycete strains. TSBY (tryptic soy broth yeast extract) consisted of 3% (w/v) tryptone soy broth supplemented with 0.5% yeast extract. R4 medium consisted of 0.5% glucose, 0.1% yeast extract, 0.5% $MgCl_2$•$6H_2O$, 0.2% $CaCl_2$•$2H_2O$, 0.15% proline, 0.118% valine, 0.28% TES, 50 mg/L casamino acid, 100 mg/L K_2SO_4, and 1X trace element solution (40 mg/L $ZnCl_2$, 200 mg/L $FeCl_3$•$6H_2O$, 10 mg/L $CuCl_2$•$2H_2O$, 10 mg/L $MnCl_2$•$4H_2O$, 10 mg/L $Na_2B_4O_7$•$10H_2O$, 10 mg/L $(NH_4)_6Mo_7O_{24}$•$4H_2O$).

Routine HPLC-MS and NMR Analysis. Low resolution HPLC-MS analysis was performed on a 1260 Infinity Series HPLC system (Agilent) equipped with an automated liquid sampler, a diode array detector, and a 6120 Series ESI mass spectrometer using an analytical Luna C18 column (Phenomenex, 5 µm, 4.6 x 100 mm) operating at 0.5 mL/min with a gradient of 10% MeCN in H_2O to 100 % MeCN over 20 minutes. Both MeCN and H_2O contained 0.1% (v/v) formic acid. High-resolution (HR) HPLC-MS and HR-tandem HPLC-MS were carried out on a 6540 UHD Accurate Mass Q-tof LC-MS system (Agilent), consisting of a 1260 Infinity Series HPLC system, an automated liquid sampler, a diode array detector, a JetStream ESI source, and the 6540 Series Q-tof. HPLC purifications were carried out on an Agilent 1260 Infinity Series analytical or preparative HPLC system equipped with a photodiode array detector and an automated fraction collector. NMR spectra were collected at the Princeton Chemistry NMR Core Facility in a Bruker Avance III 500 MHz spectrometer equipped with a ^{1}H-optimized cryoprobe.

Bioactivity-coupled high-throughput-elicitor screening

High-Throughput-Elicitor Screening. The procedure is described for *S. hiroshimensis*. An identical procedure was used for *S. lavendulae* and *A. keratiniphila*. 15 µL of a spore stock suspension (~5 x 10^6 spores per mL) of *S. hiroshimensis* were used to inoculate

20 mL TSBY medium in a 125 mL Erlenmeyer flask. The culture was incubated at 30°C/250 rpm for two days. Then, the mycelia cell mass was pelleted by centrifugation, the supernatant discarded, and a stock solution of 100 mg/mL cell mass was generated in R4 medium. The stock solution was used to prepare the appropriate volume (see below) of R4 medium containing 0.5 mg/mL mycelia cell mass in a 250 mL Erlenmeyer flask. The suspension was homogenized by stirring at room temperature. Subsequently, a Biotek MultiFlow Dispenser was used to dispense 150 µL of the suspension into each well of six sterile round bottom 96 well plates (Corning). Candidate elicitors were added from a commercially-available 502-member Natural Products Library (Enzo Scientific) using a CyBi-Well automated liquid transfer robot (CyBio). A volume of 0.5 µL was transferred from the compound library into each well to give a final concentration of ~33 µM. Then each plate was covered with a Breathe-Easy sealing membrane (Sigma) and cultured at 30°C/200 rpm for 5 days. Humidity was maintained in the shaker/incubator by incubating several flasks containing sterile water.

High-throughput Liquid Bioactivity Assays. After 5 days, the plates were spun down in a 96-well plate centrifuge and the supernatants (10 µL) transferred to fresh 96-well assay plates, each well containing 100 µL LB-*E. coli* culture, with the *E. coli* cells inoculated to an initial $OD_{600\ nm}$ of 0.002. The assay plates were cultured at 37°C/200 rpm overnight (10-12 h) and $OD_{600\ nm}$ was then determined. To generate the plots in Figs. 3B, 3C, and 3D, the $OD_{600\ nm}$ from each well was divided by the average $OD_{600\ nm}$ from the entire plate and multiplied by 100 to give the percent growth. This normalization was carried out to account for plate-to-plate variation in growth rates.

Medium-throughput Disc Diffusion Bioactivity Assays. For disc diffusion assays, the elicitor plates were prepared as described above. The only difference was that a single elicitor plate was used per strain. After 5 days, each plate was spun down in a 96-well plate centrifuge and the supernatant from each well (15 µL) was manually spotted onto a sterile filter disc (Fisher Scientific). Once dry, the discs were placed onto a soft agar (~1%) plate (30 x 30 cm) inoculated with *B. subtilis* (~10^7 cells/mL). The plate was incubated for 5-10 h at 37°C and subsequently examined visually for halos. To generate Fig. 2, the halo diameter, if any, was measured and categorized as none (n), weak (w), medium (m), or strong (s) inhibition as described in the Fig. 2 caption.

Elicitor validation assays

Seed cultures of *S. hiroshimensis* A18 were prepared as described for the HiTES screen above. Briefly, 15 µL of a spore stock suspension (~5 x 10^6 spores per mL) was used to

inoculate 20 mL TSBY medium in a 125 mL Erlenmeyer flask. After two days, the pelleted mycelial cell stock was used to inoculate 20 mL of R4 medium in a 125 mL Erlenmeyer flask to a concentration of 0.5 mg/mL cell stock. Atenolol was added to from a DMSO stock to a final concentration of 33 µM. A control flask was prepared containing the same volume of DMSO, rather than atenolol. The culture was incubated at 30°C/200 rpm. After five days, the cell mass was removed by centrifugation and the resulting supernatant extracted twice with 30 mL of ethyl acetate. The organic phases were combined, divided in two, and dried in vacuo. The dried sample was dissolved in a small volume of methanol for LR-HPLC-MS analysis and DMSO for agar disc diffusion assays (see below).

Disc diffusion assay. The dried control and atenolol-treated culture extracts were dissolved in 100 µL of DMSO each. Filter paper discs were infused with 30 µL of each sample, allowed to dry, and then placed on a soft LB agar (1% agarose) plate inoculated with *E. coli* ZSR638 at a concentration of 2 x 10^6 cells/mL. The agar plate was incubated at 37°C for 10 h and then inspected visually.

Low-resolution HPLC-MS validation. Dried control culture extracts were dissolved in 200 µL of MeOH each and filtered. Subsequently, 10 µL of each sample was analyzed by HPLC-MS (see General procedures above).

Large-scale fermentation, isolation, and structure elucidation

Large-scale fermentation and isolation. Large-scale fermentation of *S. hiroshimensis* was carried out as described above. *S. hiroshimensis* spore stocks were used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of TSBY seed medium. After growth for 2-3 days, the mycelial cell mass was isolated, and a 100 mg/mL mycelia cell stock was generated in R4 medium. This stock was used to inoculate 4 x 1 L R4 media (in 3.8 L Fernbach flasks) to an initial concentration of 0.5 mg/mL mycelium. The suspension was supplemented with a final concentration of 33 µM atenolol (from a DMSO stock). The four Fernbach flasks were cultured for five days at 30°C/200 rpm. After five days, the cells mass was removed by centrifugation and the supernatant applied directly to a manually-prepared C_{18} open-column (Phenomenex, 5 µm, 10 g). After application of the supernatant, the column was washed with 150 mL each of 20%, 40%, 60%, 80%, and 100% MeOH in water. Each fraction was dried in vacuo. Taylorflavin A (**7**) and B (**8**) were detected in the 20% fraction, pyridindolol (**9**) and hiroshidine (**12**) were found in the 40% and 60% fractions, and 6,7,8-trimethoxy-3-methylisocoumarin (**10**) and 6,8-dihydroxy-3-methylisocoumarin (**11**) were identified in the 80% fraction. Taylorflavin A (**7**) and B (**8**) were purified at a retention time of 10 and 25 min, respectively, by HPLC on a semi-preparative

Phenyl-Hexyl column (Phenomenex, 5 µm, 10 x 250 mm) operating at flow late 2 mL/min with a 10% aqueous acetonitrile isocratic step (0.1% formic acid). Hiroshidine (**12**) was obtained at 30 min by HPLC with a semi-preparative Luna C_{18} column (Phenomenex, 5 µm, 10 x 250 mm), operating at flow rate 2 mL/min and a gradient 10–40% aqueous acetonitrile (0.1% TFA) over 30 min. 6,7,8-Trimethoxy-3-methylisocoumarin (**10**) was purified by HPLC with the same Luna C_{18} column using 55% aqueous acetonitrile isocratic solvent (0.1% formic acid), where it eluted at 23 min. Pure taylorflavin A (1.2 mg), taylorflavin B (3.0 mg), 6,7,8-trimethoxy-3-methyl-isocoumarin (3.2 mg), and hiroshidine (2.4 mg) were obtained using these procedures.

Structure elucidation. The structures of compounds **7**–**12** were determined by analysis of 1D (^{1}H and ^{13}C) and 2D (gCOSY, NOESY, HSQC and HMBC) NMR spectra (**7**, **8**, **10**, **12**) and/or HPLC-MS data (**9**, **11**). Molecular formulae and HR-MS data are listed in Table S1.

MIC determination

Antibacterial assays with taylorflavin B (**8**) were carried out by Micromyx, LLC in accordance with methods from the Clinical and Laboratory Standards Institute. Minimal inhibitory concentrations were determined with the following strains (listed in Table 1): *E. coli* ATCC 25922, *A. baumannii* ATCC 19606, *V. cholerae* BAA-2163, *N. gonorrhoeae* ATCC 49226, *P. aeruginosa* ATCC 27853, *K. pneumoniae* MMX 214. *S. aureus* ATCC 29213, *S. aureus* MMX 2011, *S. pneumoniae* ATCC 49619, *S. pyogenes* MMX 6253, *S. agalactiae* MMX 6189, *E. faecalis* ATCC 29212, *E. faecalis* MMX 486, *B. subtilis* ATCC 6633, *C. difficile* ATCC 700057, *B. fragilis* ATCC 25285. Assays with taylorflavin A (**7**) were carried in-house according to the same protocols.

Sequencing of *S. hiroshimensis*

S. hiroshimensis A18 was cultured in 50 mL TSBY medium for two days, as described above. Then, the mycelium was collected by centrifugation and genomic DNA isolated using the Promega Wizard Genomic DNA Purification Kit as per manufacturer's instructions. Genomic DNA of high quality was obtained at a concentration of 1.1 µg/µL and a $UV_{260/280}$ value of 1.9. The genomic DNA of *S. hiroshimensis* A18 was sent to the Lewis Sigler Institute Sequencing Core Facility, where short DNA fragment libraries were prepared via an Illumina MiSeq Reagent Kit, and the fragments then sequenced on an Illumina MiSeq System. The raw sequence data were assembled with Unicycler software. A total of 86 contigs covering 8.4 Mbp sequence were obtained. Genome annotation analysis was conducted via the RAST server 2.0. The obtained data were then searched using the reported toxoflavin (*toxB* from *P. protegens*) and qinimycin

(*qin19* from *Streptomyces* sp. MBT76) biosynthetic gene clusters as queries. Each search delivered a single high-scoring hit, which allowed us to identify the clusters and determine cluster boundaries. In each cluster, the predicted protein functions were assigned using the IMG and antiSMASH databases (Tables S4 and S5). The sequence for the whole *tfl*/*tox* and *hrs* gene clusters were uploaded to NCBI (accession number MH743142 and MH743143, respectively).

DNA manipulation and construction of Mutants

Generating the tfl/tox gene deletion plasmid. To generate a *tfl*/*tox* gene deletion, ~2 kb regions upstream of *toxB* and ~2 kb downstream of *tflA* were amplified using appropriate primers (Table S6) and digested with BamHI/EcoRI and HindIII/KpnI respectively. The apramycin resistance gene (*apr*) fused to *oriT* was amplified by PCR with appropriate primers from vector pIJ773. This was then digested with EcoRI/HindIII to give a 1.6 kb insert. Then each of the three fragments obtained was in turn cloned into pJTU1289 (which carries the thiostrepton marker, Tsr^R) to create a plasmid construct containing a fragment that comprises 2-kb-up-*toxB*_oriT-*apr*_2-kb-dn-*tflA*. This plasmid (*pΔtoxB–tflA*) was amplified in *E. coli* DH5α cells and introduced into *E. coli* ET12567 by heat shock transformation. Conjugation of this transformed *E. coli* strain with *S. hiroshimensis* was carried out as described below.

Generating the hrs gene deletion plasmid. To create the plasmid for large-fragment deletion within the *hrs* cluster, a similar strategy as above was employed: 2 kb regions upstream of *hrsC* and downstream of *hrsJ* were amplified using the appropriate primers (Table S6), and subsequently digested with BamHI/EcoRI and HindIII/KpnI, respectively. The apramycin resistance gene (*apr*) fused to *oriT* was amplified by PCR with the appropriate primers from vector pIJ773. This was then digested with EcoRI/HindIII to give a 1.6 kb insert. Then each of the three fragments obtained was in turn cloned into pJTU1289 to create a plasmid construct containing a fragment that comprises 2-kb-up-*hrsC*_oriT-*apr*_2-kb-dn-*hrsJ*. This plasmid (*pΔhrsC–hrsJ*) was amplified in *E. coli* DH5α cells and introduced into *E. coli* ET12567 by heat shock transformation.

Conjugation. Conjugation of both gene deletion plasmids into *S. hiroshimensis* used the following protocol: Each plasmid (Tsr^R, Apr^R) was transformed into *E. coli* ET12567 (Cm^R), which also carries the mobilizing vector pUZ8002 (Kan^R),[1-3] as mentioned above. Then, *E. coli* ET12567 containing the desired plasmids was cultured in LB containing Apr (50 μg/mL), Kan (50 μg/mL), and Cm (25 μg/mL) to an $OD_{600\ nm}$ of ~0.4. The cells were collected by centrifugation and washed with LB to remove the antibiotics. These were resuspended in ~0.5 mL LB to be used later in the protocol (see below).

Spores of *S. hiroshimensis* were grown on ISP-2 medium (yeast extract: 0.4% (w/v), malt extract: 1%, dextrose: 0.4%, Agar: 2%, pH=7.2) and isolated using standard methods.[3] *S. hiroshimensis* spores were washed with TES buffer (50 mM, pH 8.0), collected, resuspended in 500 µL TES buffer, and heat-shocked at 50°C for 10 min. The spores were then supplemented with 500 µL of 2x spore-activating media (yeast extract: 1% (w/v), casein hydrolysate: 1%, $CaCl_2$: 5 mM), and then cultured at 37°C for 3 h. The spore stocks were then collected by centrifugation, resuspended in ~0.5 mL of LB and mixed with the *E. coli* cells that were also resuspended in 0.5 mL of LB (see above). The suspension was then plated on SFM agar plates (which consists of 2%(w/v) soybean meal prepared in tap water, which is sterilized by autoclaving, filtered, and then mixed with 2% mannitol and 2% agar, and then autoclaved again). The plates were grown at 30°C for 16-20 h. The single-site integration mutants were selected with 25 µg/mL thiostrepton, 35 µg/mL Apr, and 50 µg/mL trimethoprim (to kill the *E. coli* donor strain). For selection of double-crossover mutants, the exconjugants were picked and cultured in YEME medium without any antibiotics. Surviving cells were subcultured again in YEME medium. They were then diluted to obtain single colonies on SFM agar plates. The colonies were tested individually and separately for Apr resistance and Tsr-susceptibility. The mutants that satisfied both criteria were tested by PCR for the absence of the *toxB* and *hrsD* gene, thus delivering the *toxB-tflA::apr* and *hrsC-hrsJ::apr* strains. These were then tested in small scale fermentations as described below.

Metabolic profiling of wild-type and mutant *S. hiroshimensis*

Frozen spore stocks of wild type *S. hiroshimensis* A18 as well as mutant strains *toxB-tflA::apr* and *hrsC-hrsJ::apr* were used to inoculate into 3 mL of TSBY medium in separate 14 mL bacterial culture tubes, which were then incubated at 30°C/200rpm for two days. The isolated mycelial cell mass was then diluted into 20 mL of R4 medium (in a 125 mL Erlenmeyer flask) to a final concentration of 0.5 mg/mL. The cultures were grown at 30°C/200 rpm for 5 days in the presence of 33 µM atenolol, and then cells were removed by centrifugation. The resulting supernatant was extracted twice with 30 mL of ethyl acetate. The organic phases were combined for each strain, dried in vacuo, dissolved in 400 µL MeOH, and analyzed by LR-HPLC-ESI-MS using an analytical Luna C_{18} column (Phenomenex, 5 µm, 4.6 x 100 mm) operating at 0.5 mL/min with a gradient of 10% MeCN in H_2O to 100 % MeCN over 20 minutes (mobile phase of water and MeCN contain 0.1% formic acid).

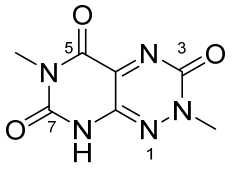
Table S1. Observed and calculated HR-MS data for compounds **7**–**12**.

	$[M+H]^+_{calc}$	$[M+H]^+_{obs}$	Δppm
Taylorflavin A (**7**)	210.0627	210.0620	3.3
Taylorflavin B (**8**)	224.0784	224.0778	2.7
Pyridindolol (**9**)	259.1083	259.1080	1.2
6,7,8-Trimethoxy-3-methylisocoumarin (**10**)	251.0919	251.0922	1.2
6,8-Dihydroxy-3-methylisocoumarin (**11**)	193.0501	193.0496	2.6
Hiroshidine (**12**)	478.2077	478.2089	2.5
3-^{13}C-taylorflavin A	211.0661	211.0667	2.8
3-^{13}C-taylorflavin B	225.0818	225.0815	1.3

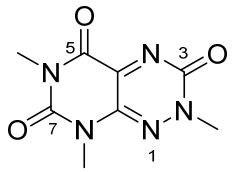
Table S2. NMR assignments of taylorflavin A (**7**) and B (**8**) in DMSO-d_6. The structures and number schemes for the two compounds are shown below the table.

Taylorflavin A			**Taylorflavin B**		
No.	δ_C[a]	δ_H[b]	No.	δ_C[a]	δ_H[b]
1			1		
2-Me	41.5	3.67	2-Me	41.8	3.75
3	153.7		3	153.2	
4			4		
4a	145.6		4a	145.6	
5	159.3		5	158.4	
6-Me	28.4	3.22	6-Me	29.3	3.29
7	150.0		7	149.8	
8a	138.3		8-Me	29.5	3.35
			8a	138.3	

[a]125 MHz, [b]500 MHz



Taylorflavin A

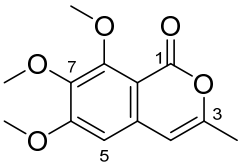


Taylorflavin B

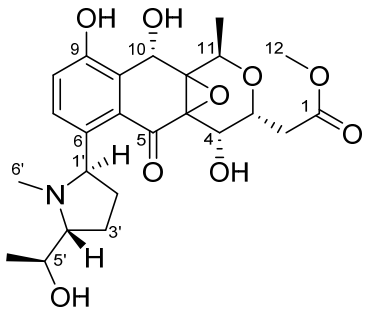
Table S3. NMR assignments of 6,7,8-trimethoxy-3-methylisocoumarin (**10**) and hiroshidine (**12**). The structures and number schemes for the two compounds are shown below the table.

6,7,8-Trimethoxy-3-methylisocoumarin[a]			**Hiroshidine**[b]		
No.	δ_C[c]	δ_H[d]	No.	δ_C[c]	δ_H[d]
1	-		1	171.3	
2			2	35.8	2.54, 2.45
3	156.4		3	66.0	3.97
3-Me	19.8	2.18	4	62.2	4.15
4	105.4	6.32	4a	61.9	
4a	139.3		5	195.1	
5	104.8	6.77	5a	130.6	
6	161.8		6	130.0	
6-OMe	57.6	3.92	7	129.0	7.55
7	144.2		8	120.3	7.16
7-OMe	62.4	3.8	9	155.0	
8	157.6		9a	127.5	
8-OMe	62.9	3.87	10	59.8	5.22
8a	108.6		10a	66.8	
			11	64.0	4.75
			11-Me	14.7	1.24
			12-Me	51.5	3.66
			1’	63.5	4.90
			2’	32.6	2.41, 1.76
			3’	22.1	2.03, 1.94
			4’	68.2	3.32
			5’	63.8	4.10
			5’-Me	20.4	1.07
			6’-Me	34.2	2.22

[a]CD_3OD, [b]DMSO-d_6, [c]125 MHz, [d]500 MHz



6,7,8-Trimethoxy-3-methylisocoumarin



Hiroshidine

Table S4. Annotation of the *S. hiroshimensis tfl*/*tox* biosynthetic gene cluster.

Gene	Length (AA)	Putative Function
toxB	202	GTP cyclohydrolase II
toxC	571	WD-40 repeat-containing protein
toxA	252	methyltransferase
toxD	331	serine/threonine kinase
toxE	377	riboflavin biosynthesis protein, RibD
tflA	418	quinone oxidoreductase
tflB	247	methyltransferase
tflC	201	hypothetical protein
tflD	494	MFS transporter
tflE	216	TetR family transcriptional regulator

Table S5. Annotation of the *S. hiroshimensis hrs* biosynthetic gene cluster.

Gene	Length (AA)	Putative Function
hrsA$_1$	323	dTDP-glucose 4,6-dehydratase
hrsB	360	glucose-1-phosphate thymidylyltransferase
hrsC	285	C5-O-methyltransferase
hrsD	366	glycosyltransferase IroB
hrsE	386	DegT/DnrJ/EryC1/StrS family aminotransferase
hrsF$_1$	422	hypothetical protein
hrsA$_2$	437	NDP-hexose 2,3-dehydratase
hrsA$_3$	352	NDP-hexose 3-ketoreductase
hrsA$_4$	433	NDP-hexose 3,4-dehydratase
hrsG	381	hydrolase
hrsH	268	SARP family transcriptional regulator
hrsI$_1$	307	putative polyketide cyclase
hrsJ	292	4-phosphopantetheinyl transferase
hrsK$_1$	591	acyl-CoA dehydrogenase
hrsK$_2$	143	hydroxyacyl-CoA dehydrogenase
hrsI$_2$	335	cyclase
hrsL	88	acyl carrier protein
hrsM$_2$	412	chain length factor, beta-ketoacyl synthase beta subunit
hrsM$_1$	423	polyketide beta-ketoacyl synthase, alpha subunit
hrsN	410	transposase
hrsO	167	flavin reductase
hrsK$_3$	262	acetoacetyl-CoA reductase
hrsK$_4$	328	3-hydroxyacyl-CoA dehydrogenase
hrsP$_1$	304	quinone oxidoreductase
hrsQ	218	DsbA family oxidoreductase
hrsF$_2$	214	hypothetical protein
hrsK$_5$	603	3-hydroxyacyl-CoA dehydrogenase
hrsP$_2$	329	quinone oxidoreductase
hrsR	202	NADPH-dependent FMN reductase

Table S6. Primers used for creating *S. hiroshimensis* gene deletion mutants.

Primers	Sequences
toxB-KO-L-For	5'-ATGGATCCAACTCCGACGTCGCCTTCCACAGC-3'
toxB-KO-L-Rev	5'-TAGAATTCTGTCAGCCGGAACGGTTCCTTACC-3'
tflA-KO-R-For	5'-ATAAGCTTACTCCGTGGTCTTCGGCACC-3'
tflA-KO-R-Rev	5'-TAGGTACCTGGAGAACGTACTGCTGCTGG-3'
hrsC-KO-L-For	5'-ATGGATCCTGGTGAACTTCCACAAGAAGTTCG-3'
hrsC-KO-L-Rev	5'-TAGAATTCAGCAGCACGTCGTACAGGTCGAGG-3'
hrsJ-KO-R-For	5'-ATAAGCTTTTGCGAGAATTTCTGGGGAACC-3'
hrsJ-KO-R-Rev	5'-TAGGTACCTTGTCGTCGTTGATCGCGTAGC-3'

Figure S1. Bioactivity-HiTES with *S. lavendulae* B-1230. Cultures of *S. lavendulae* B-1230 were challenged with 96 different small molecules and the resulting supernatants were used directly in disc diffusion assays against *B. subtilis*. The best elicitors of antibiotic activity, 7,2'-dimethoxyflavone ('D'), isobutylmethylxanthine ('I'), and methoxyvone ('M') are marked, as are the negative (water, 'W') and positive (tetracycline, 'T') controls.

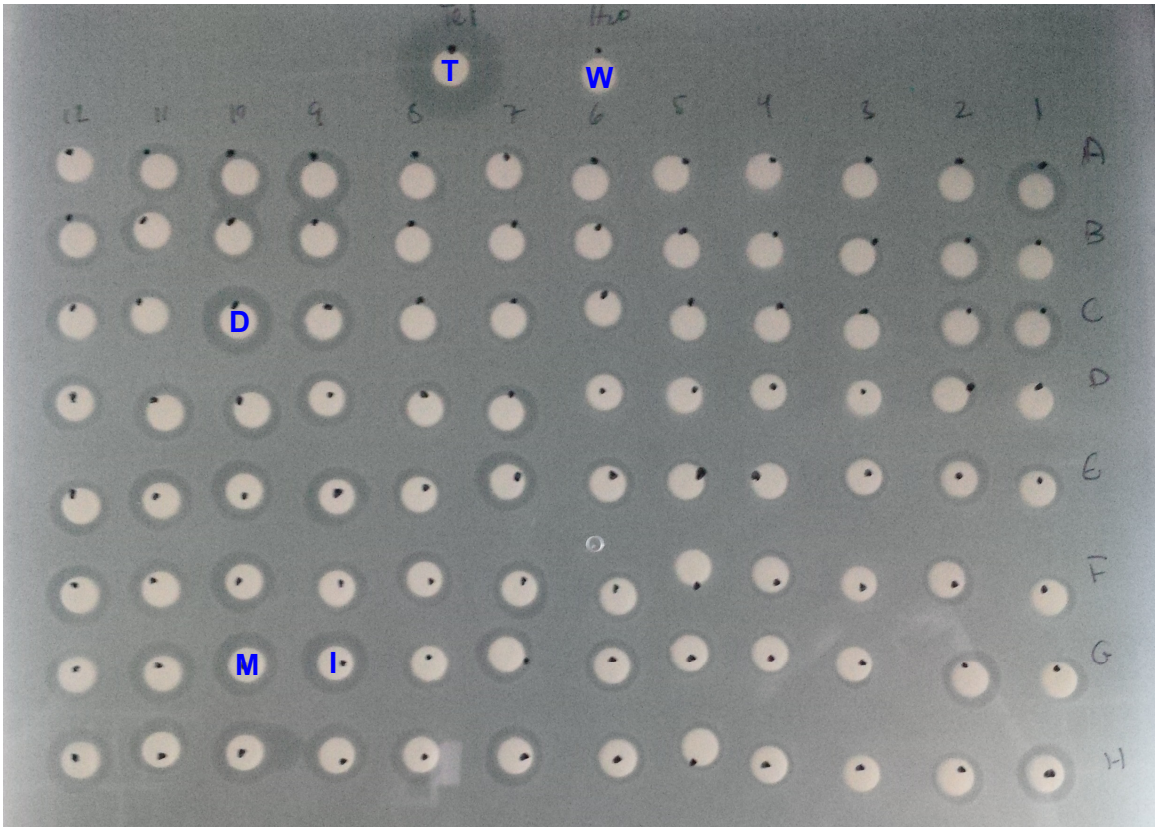


Figure S2. Bioactivity-HiTES with *A. keratiniphila* B-24117. Cultures of *A. keratiniphila* B-24117 were challenged with 96 different small molecules and the resulting supernatants were used directly in disc diffusion assays against *B. subtilis*. The best elicitors of antibiotic activity, baccatin III ('B') and isoscopoletin ('I') are marked, as are the negative (water, 'W'), and positive (tetracycline, 'T') controls.

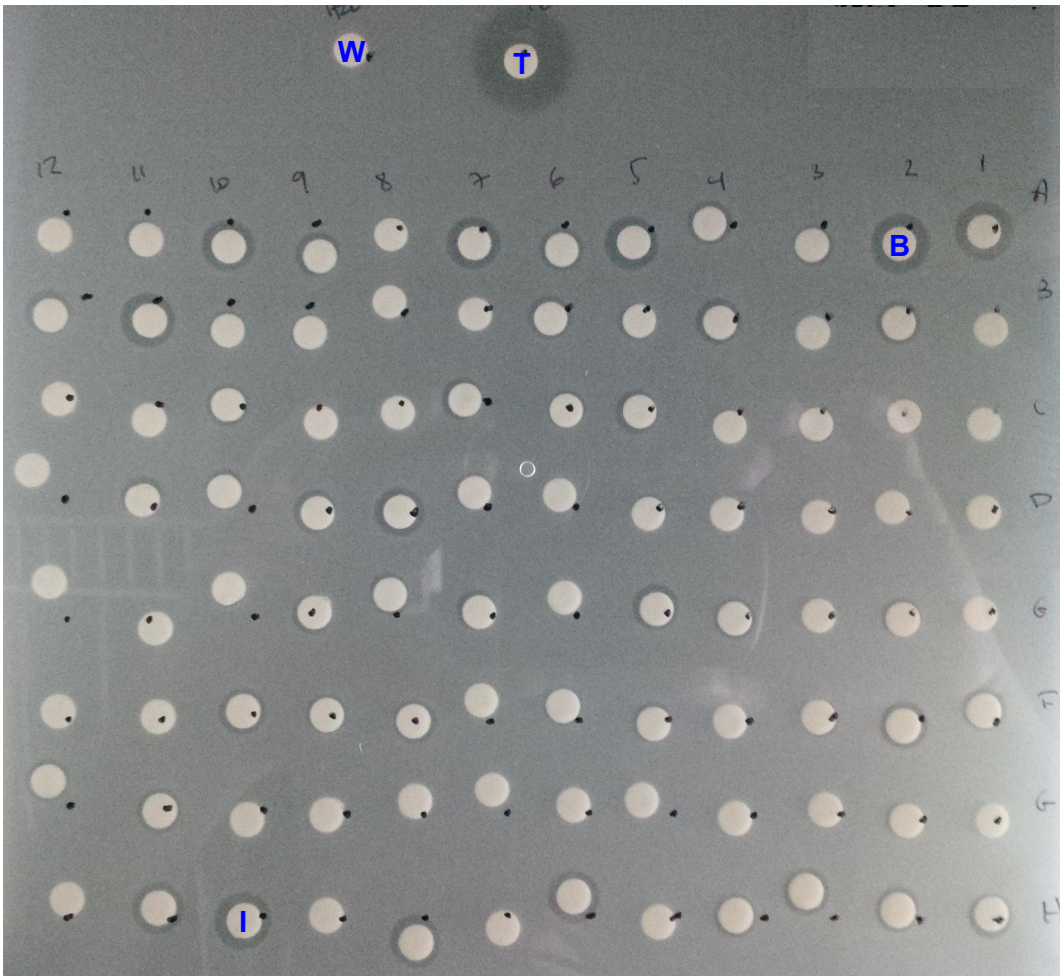


Figure S3. Bioactivity-HiTES with *S. hiroshimensis* A18. Cultures of *S. hiroshimensis* A18 were challenged with 80 different small molecules and the resulting supernatants were used directly in disc diffusion assays against *B. subtilis*. The best elicitors of antibiotic activity, primaquine ('Q'), probenecid ('P'), procainamide ('R'), and procaine ('O'), are marked, as is the positive control (gentamicin, 'G'). The large halos are possibly due to residual antibioitc elicitors: moxolactam ('M'), norfloxacin ('N'), and oxytetracycline ('T').

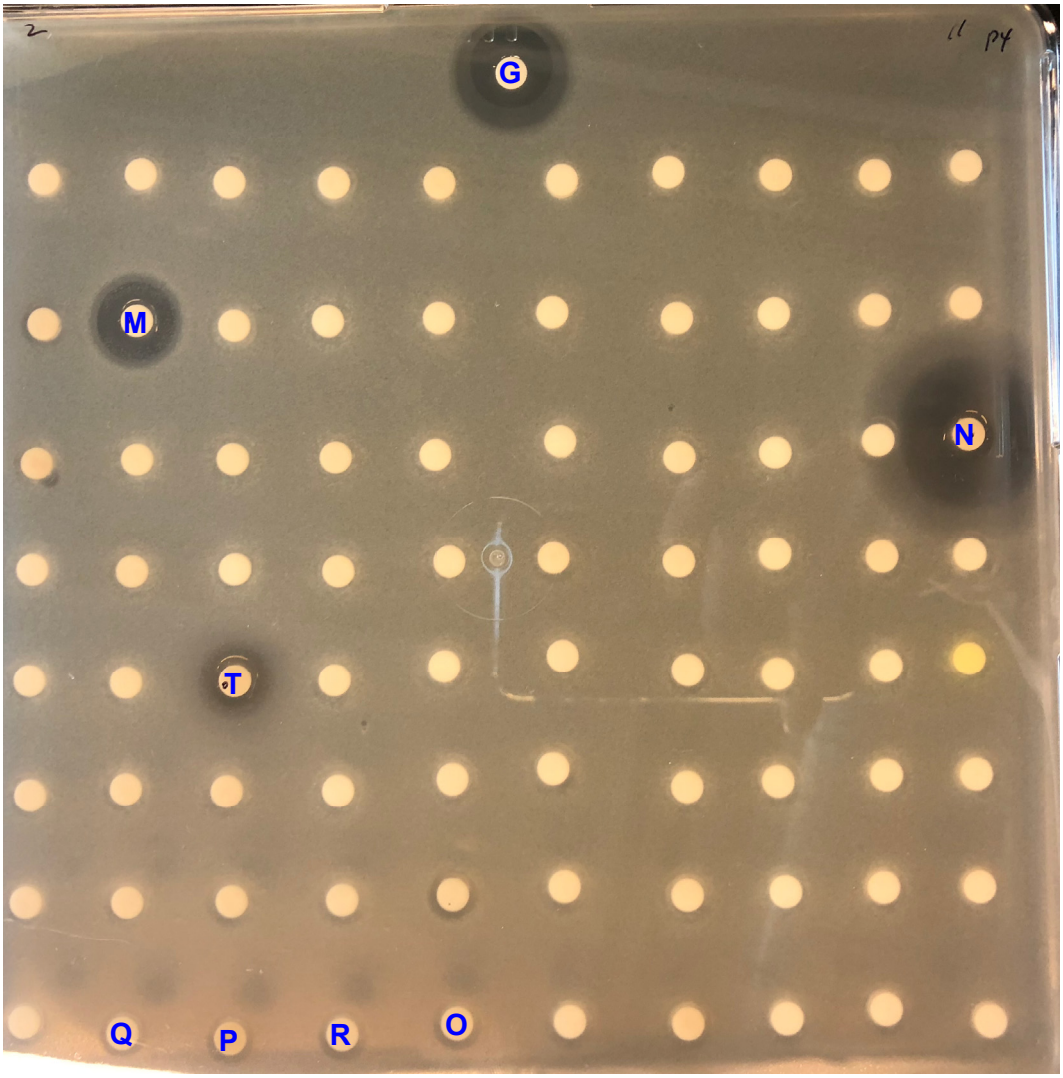


Figure S4. NMR analysis of taylorflavin A (7). ^{1}H (top), ^{13}C (middle), HSQC (bottom left) and HMBC (bottom right) spectra acquired at 500 MHz in DMSO-d_6 (See Table S2).

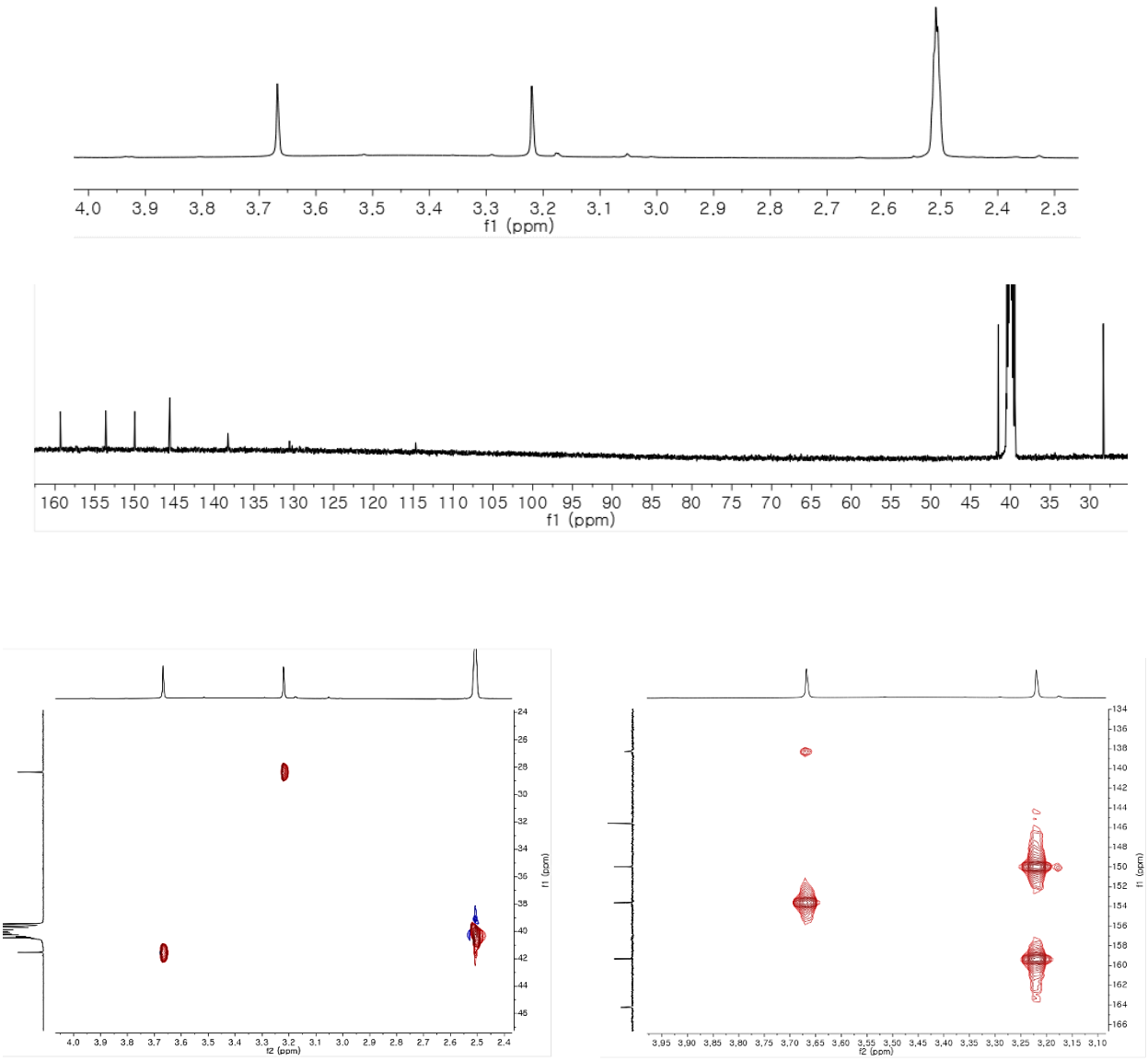


Figure S5. NMR analysis of taylorflavin B (8). ^{1}H (top), ^{13}C (middle), HSQC (bottom left) and HMBC (bottom right) spectra acquired at 500 MHz in DMSO-d_6 (see Table S2).

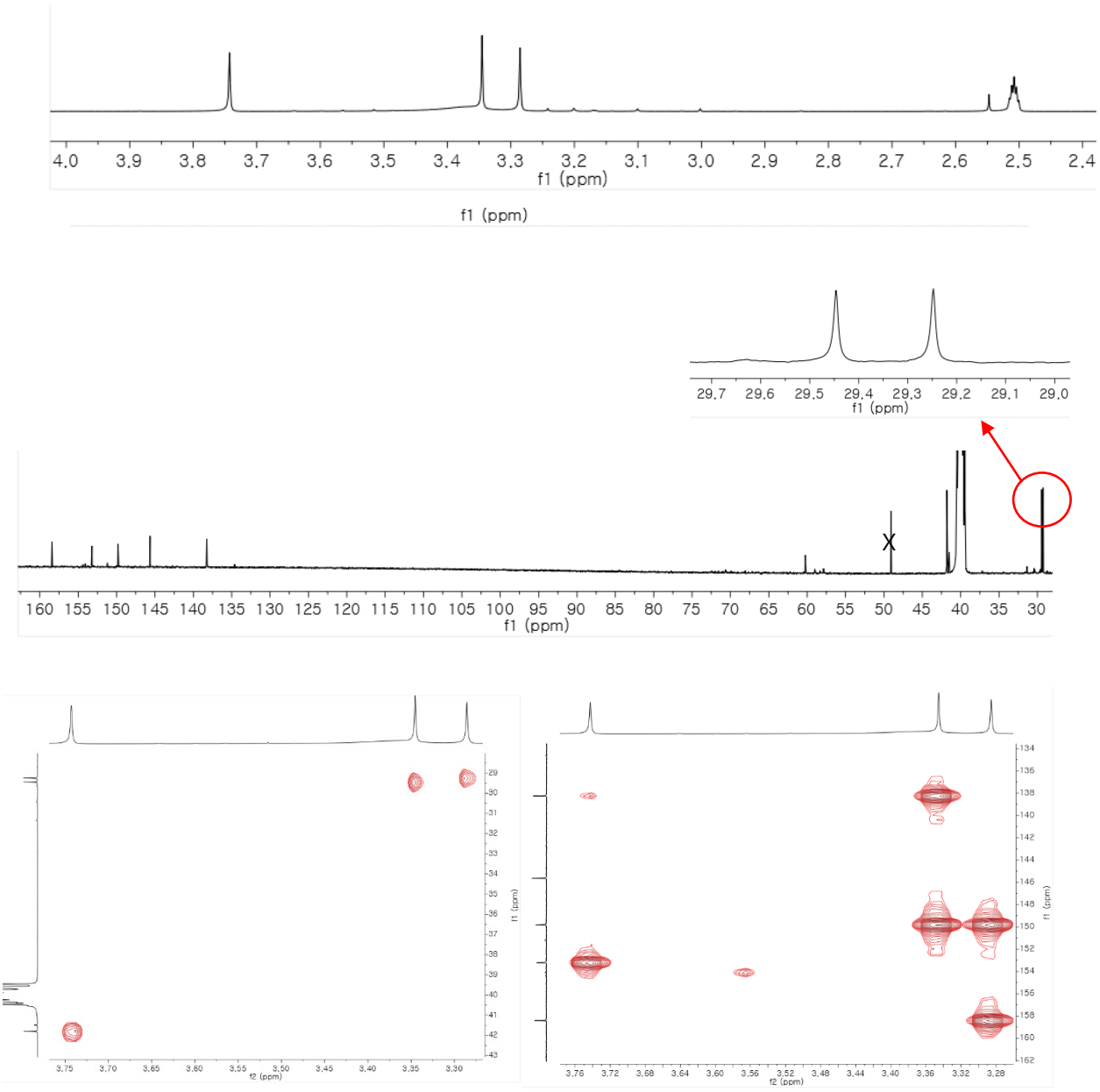
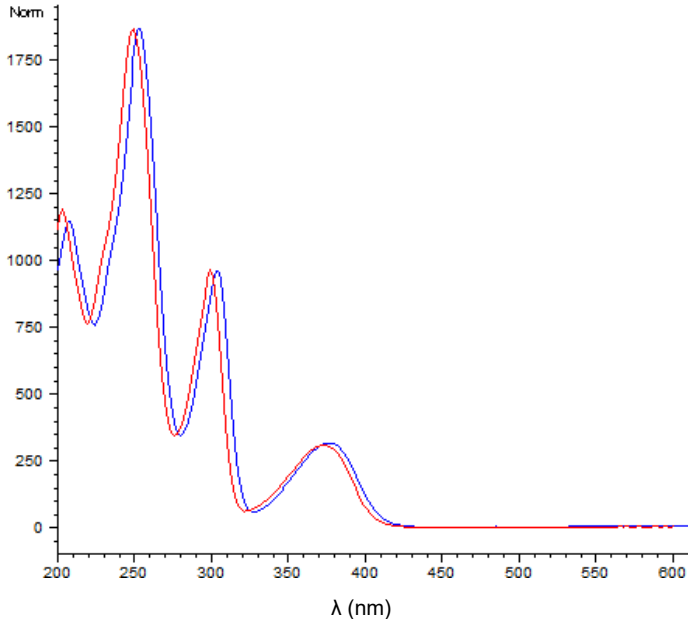


Figure S6. Identification of pyridindolol (9) and 6,8-dihydroxy-3-methylisocoumarin (11). (Top) UV-vis spectral comparison of authentic (red) and atenolol-induced (blue) pyridindolol (**9**) in *S. hiroshimensis* cultures. (Bottom) UV-vis spectral comparison of authentic (red) and atenolol-induced (blue) 6,8-dihydroxy-3-methylisocoumarin (**11**) in *S. hiroshimensis* cultures. Normalized absorbance units are plotted on the y-axes in both panels.



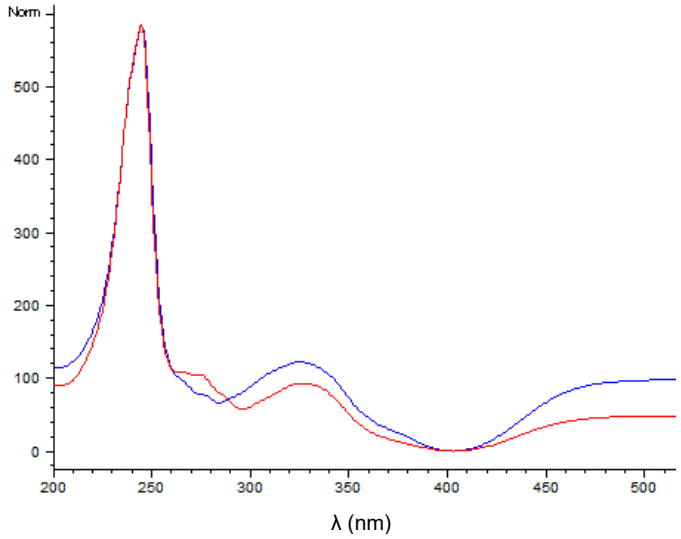


Figure S7. NMR analysis of 6,7,8-trimethoxy-3-methylisocoumarin (10). ^{1}H (top), HSQC (middle), and HMBC (bottom) spectra acquired at 500 MHz in MeOH-d_4 (see Table S3).

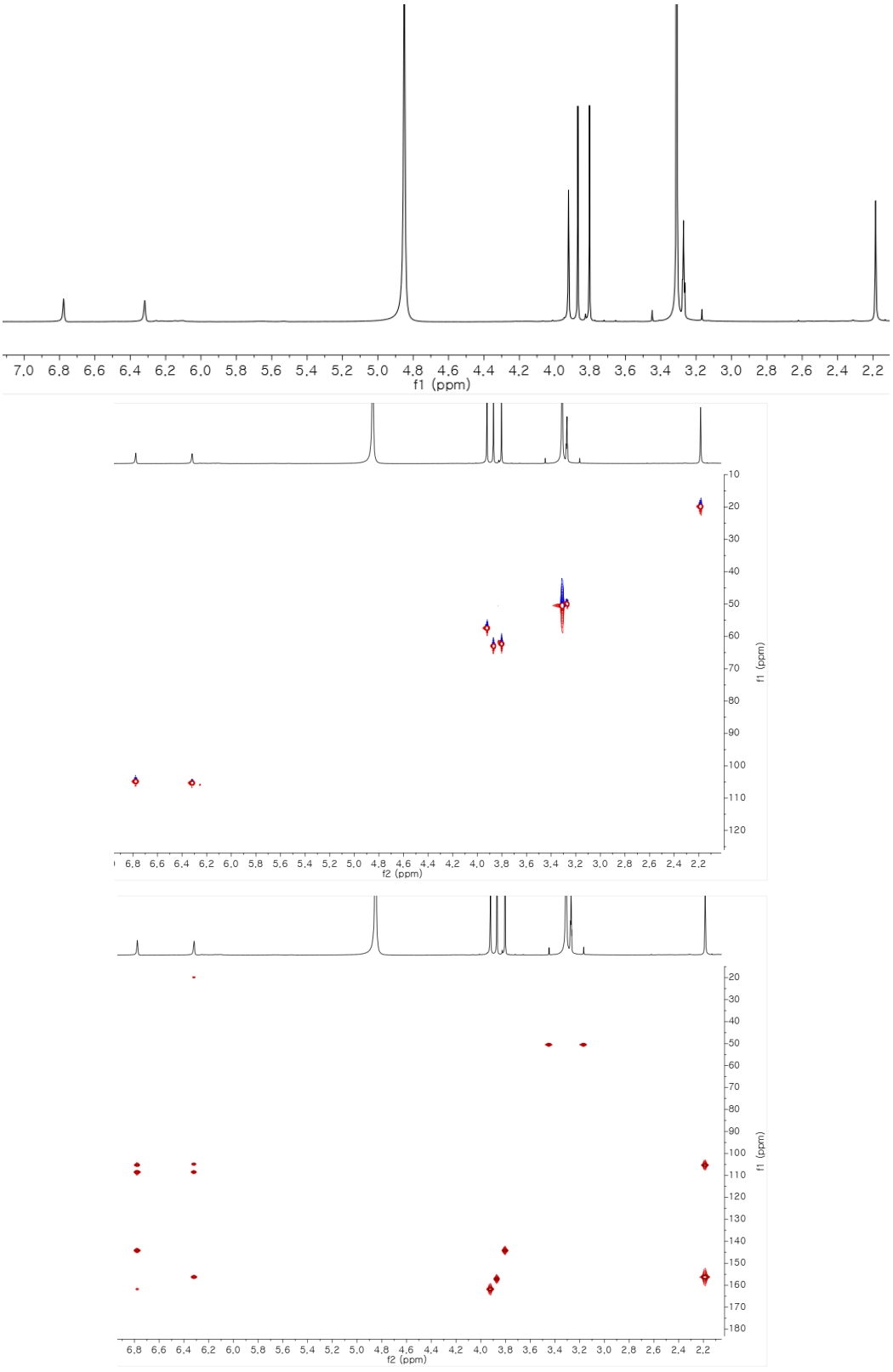


Figure S8. NMR analysis of hiroshidine (12). ^{1}H (top), ^{13}C (middle), and gCOSY (bottom) spectra acquired at 500 MHz in DMSO-d_6 (see Table S3).

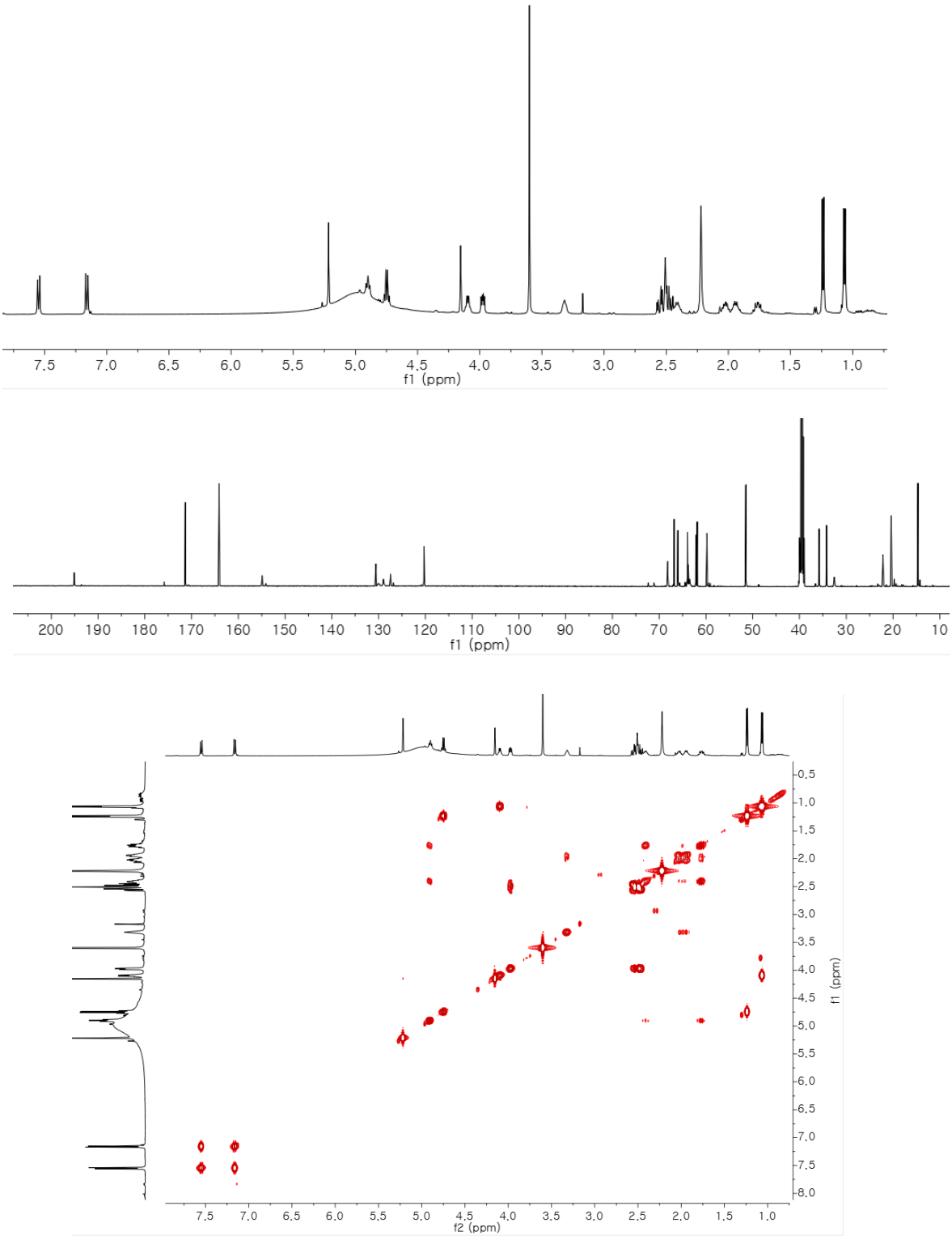


Figure S9. NMR analysis of hiroshidine (12). HSQC (top), HMBC (middle), and NOESY (bottom) spectra acquired at 500 MHz in DMSO-d_6 (see Table S3).

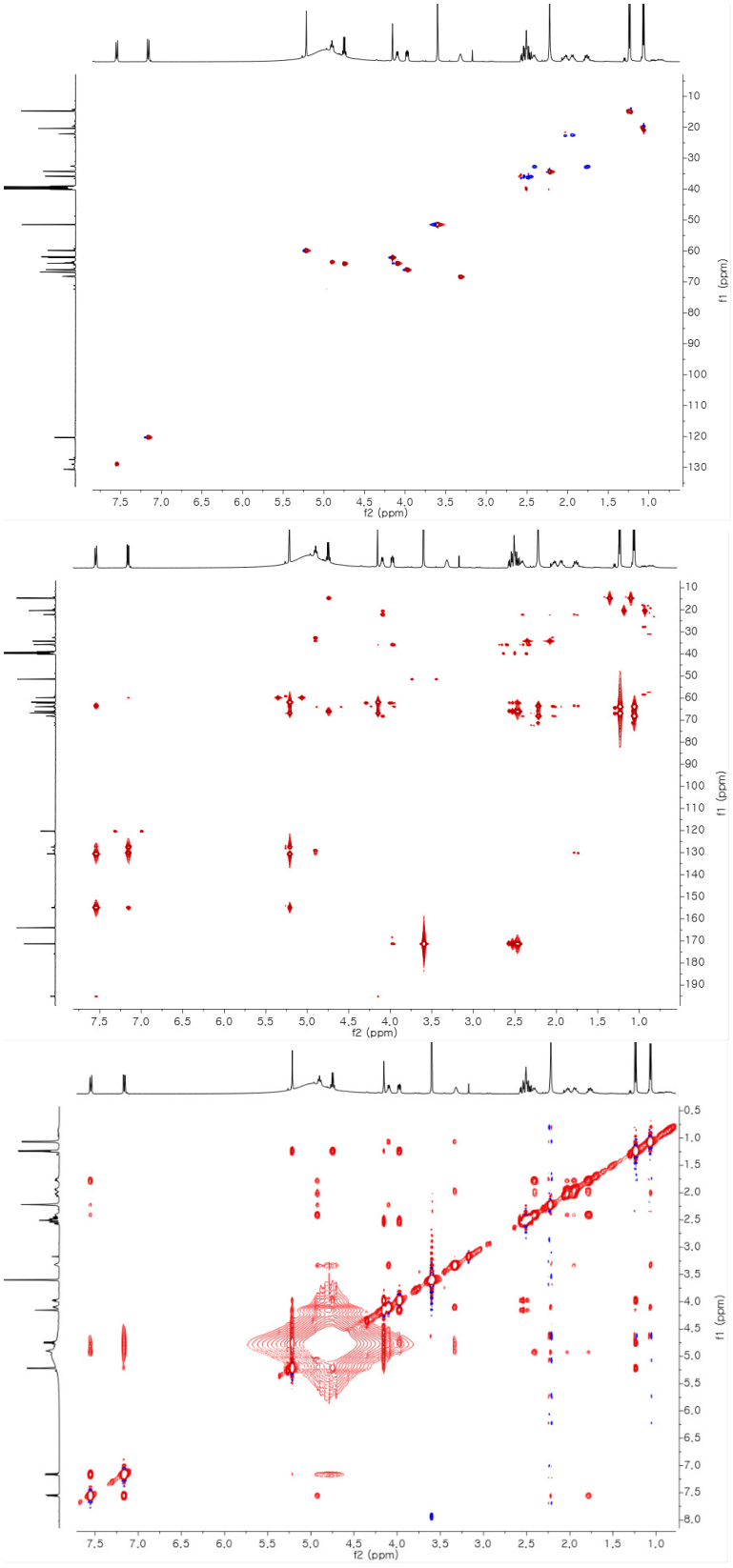
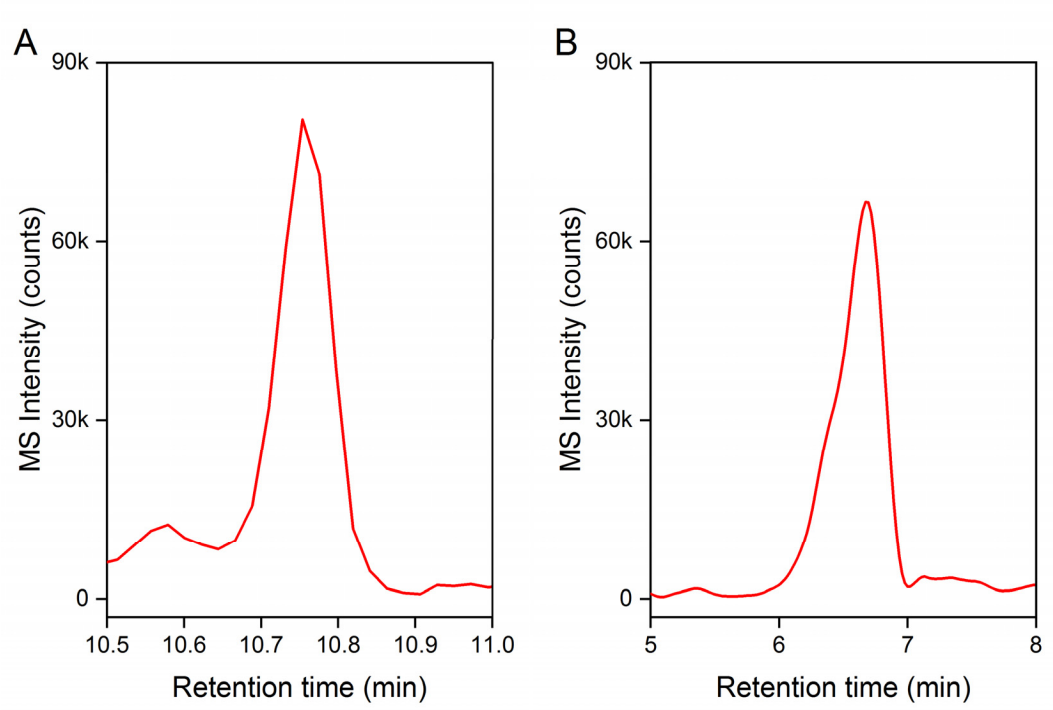


Figure S10. Production of taylorflavin B and hiroshidine by *S. hiroshimensis* mutants. (A) Extracted ion chromatogram of hiroshidine (*m/z* 478.2) in *toxB–tflA*::*apr S. hiroshimensis* cultures treated with atenolol. (B) Extracted ion chromatogram of taylorflavin B (*m/z* 224.1) in *hrsC–hrsJ*::*apr S. hiroshimensis* cultures treated with atenolol.



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