Supplementary Information

Optimal Design of Thiostrepton-derived Thiopeptide Antibiotics and Their Potential Application against Oral Pathogens

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1. Supplementary Methods

1.1. General materials and methods

Materials, Bacterial strains, and Plasmids. Biochemicals and media were purchased from Sinopharm Chemical Reagent Co., Ltd. (China), Oxoid Ltd. (U.K.) or Sigma-Aldrich Co. LLC. (USA) unless otherwise stated. Enzymes were purchased from Takara Biotechnology Co. Ltd. (China). Restriction endonucleases were purchased from Thermo Fisher Scientific Co. Ltd. (USA). Chemical reagents were purchased from standard commercial sources. The bacterial strains and plasmids used in this study are listed in Table S1.

DNA Isolation, Manipulation, and Sequencing. DNA isolation and manipulation in *E. coli* or *streptomyces* were carried out according to standard methods[1]. PCR amplifications were carried out on an Applied Biosystems Veriti Thermal Cycler using either Taq DNA polymerase (Vazyme Biotech Co. Ltd, China) for routine genotype verification or Kod DNA polymerase (Takara Biotechnology Co., Ltd.) or PrimeSTAR HS DNA polymerase (Takara Biotechnology Co., Ltd. Japan) for high fidelity amplification. Primer synthesis was performed at Shanghai Sangon Biotech Co. Ltd. (China). DNA sequencing was performed at Shanghai Majorbio Biotech Co. Ltd. (China). Primers used for diagnostic PCR were listed in Table S2.

Sequence Analysis. TSR biosynthetic gene clusters (BGCs) were obtained from NCBI database(https://www.ncbi.nlm.nih.gov/nuccore/FJ436358.1). DNA hairpin was determined by using the programs from Vector NTI Advance 11. 0 software (Invitrogen).

General Chemical Analysis. High performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1260 HPLC system (Agilent Technologies Inc., USA) equipped with a DAD detector. Semi-preparative HPLC was performed on an Agilent 1100 system. HPLC Electrospray ionization MS (HPLC-ESI-MS) and tandem MS (MS/MS) were performed on a Thermo Fisher LTQ Fleet ESI-MS spectrometer (Thermo Fisher Scientific Inc., USA), and the data were analyzed using Thermo Xcalibur software. ESI-high resolution MS (ESI-HR-MS) analysis was carried out on an instrument consisting of a 1260 HPLC system or a 6538 UHD quadrupole time of flight (QTOF) high resolution mass spectrometry (Agilent Technologies, Santa Clara, USA).

NMR data were recorded on a Bruker AV-600 or Bruker AV-500 spectrometer (Bruker Co. Ltd, Germany).

1.2. Mutant construction

Gene Inactivation of *tsrT*. Genomic DNA of SL2051 strain served as the template for PCR amplification unless otherwise stated. To exclude polar effects on downstream gene expression, gene inactivation in SL2051 was performed by in-frame deletion. The gene *tsrT* was inactivated using a modified CRISPR-Editing method.[2] Briefly, the 20-bp *sg* sequence was generated and cloned into the plasmid pWHU2653 through homologous recombination among the two 140-bp PCR products amplified from pWHU2653 using the primer pairs sg-for/*tsrT*-sg-rev and *tsrT*-sg-for/sg-rev, respectively (Table S3), and *NheI/XbaI*-digested pWHU2653, yielding pWHU1001. 2-kb PCR products amplified from the genome of SL2051 using the primer pairs *tsrT*-L-for/ *tsrT*-L-rev and *tsrT*-R-for/ *tsrT*-R-rev were obtained as the left and right arms of gene *tsrT* (Table S3). *HindIII*-digested pWHU1001, left arm and right arm of gene *tsrT* were recombined to generate pWHU1002. To transfer pWHU1002 into the template SL2051, conjugation between *E. coli* ET 12567 (pUZ8002) and SL2051 was carried out. The colonies that growing at 5-flucytodine-resistant at 30°C were identified as integrating mutants, in which a single-crossover homologous recombination event took place. Then the resulting apramycin-sensitive isolates were subjected to PCR amplification to examine the genotype of the SL3051 mutant strain (Figure S3). PCR amplification for examination primers were *tsrT*–Y-for/ *tsrT*–Y-rev (Table S3). Previous constructed pSL3051 containing the gene that encodes TsrH-Ile1Val-Ala2Ser was then introduced into SL3051 by conjunction to acquire the targeted strain SL3052.

1.3. Chemical purification and characterization

Fermentation, Examination and Isolation of 5'-fluoro-SIO. Cultivation, production and analysis of thiostrepton variants were performed according to the methods described previously.[3,4,8] The methanol sample was subjected to HPLC and HPLC-MS analysis on an Agilent Zorbax column (SB-C18, 4.6×250 mm, 5μm, Agilent Technologies Inc., USA) by gradient elution of solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) with a flow rate of 1 mL / min over a 50 min period as follows: T = 0 min, 5% B; T = 20 min, 15% B; T = 30 min, 40% B;

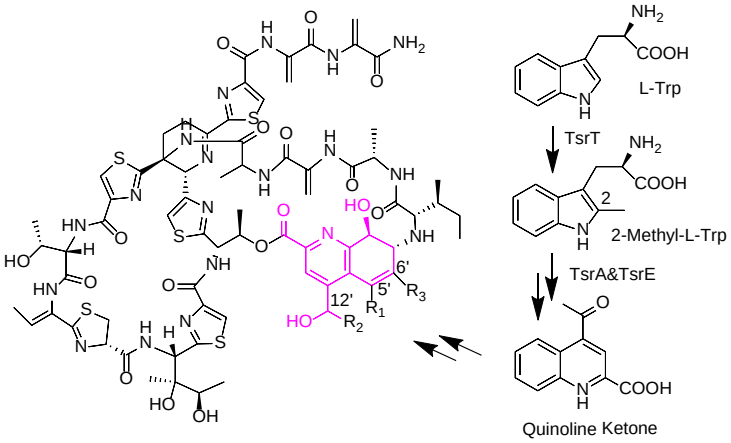
T = 37 min, 55% B; T = 40 min, 85% B; T = 47 min, 85% B; T = 50 min, 5% B. Absorbance was monitored at 254 nm. Related data were analyzed using Thermo Xcalibur software. Isolation and purification of the thiostrepton variants were performed according to the methods described previously[5].

1.4. Physical property and bioactivity of TSR and its analogs

Minimum Inhibitory Concentrations (MICs). Oral Gram-positive facultative anaerobes (*Streptococcus mutans* UA159, *Lactobacillus acidophilus* ATCC 4356 and *Actinomyces viscosus* ATCC 19246) and Gram-negative obligate anaerobes (*Fusobacterium nucleatum* ATCC 25286 and *Porphyromonas gingivalis* ATCC 33277) were used to determine antibacterial activities *in vitro*. Brain-heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) was used for culture of facultative anaerobic bacteria and incubated for 24 h at 37 °C. For the obligate anaerobic bacteria, *F. nucleatum and P. gingivalis*, BHI containing hemin (Sigma-Aldrich, St. Louis, MO, USA) and menadione(Sigma-Aldrich, St. Louis, MO, USA) (BHI-modified) was used and incubated for 48 h at 37 °C. The procedure was in accordance to standard procedure recommended by the Clinical Laboratory Standard Institute (CLSI) with a few modifications[6,7]. The broth microdilution technique was operated to determine the MIC values of the tested compounds as previously stated[4,5]. Each tested sample was dissolved in DMSO to produce stock solution (100 μg/ml), which was serially diluted into 100 μL of BHI or BHI-modified broth according to the corresponding strain in a 96-well Microtiter plate to a final concentration ranging from 10 to 0 μg/ml in two-fold serial. 100 μL of the testing strain (calculated according to 0.5 McFarland diluted 1:100 to a final concentration of 0.5-1*10^6 CFU/ml) was then added into each well of the microtiter plate, followed by 24–48 h of incubation period either in aerobic or anaerobic condition accordingly（*S. mutans*, *L. acidophils* and *A. viscosus* for 24 h, *F. nucleatum* and *P. gingivalis* for 48 h. Sodium fluoride (NaF) and chlorhexidine (CHX) were chosen as Gram-positive controls, Levofloxacin (LVX) and tinidazole (TNZ) were chosen as Gram-negative controls, respectively. The MIC was defined as the lowest concentration that inhibited visible bacterial growth. All tests were carried out in 3 times.

Water solubility. The solubility was measured according to a saturation shake-flask method described previously[4,5]. To determine the solubility of TSR, SIO, 5'-fluoro-TSR and 5'-fluoro-SIO, an excess amount of solid was added into 1 mL of water in a vial. The vial was gently stirred over a 24-hour period at the room temperature to ensure the equilibrium of the solution. A 0.22 μm centrifugal filter unit (Millipore Utrafree-MC, USA) was used for filtration to remove the excess solid. The saturated solution was then subjected to HPLC analysis to determine the concentration of the sample based on the established calibration curve. To establish the calibration curve, a set of the solutions for TSR, SIO, 5'-fluoro-TSR and 5'-fluoro-SIO varying in concentration were analyzed independently by HPLC. The peak areas correlated with the concentrations were fitted to a regression equation, resulting in a linear standard curve for estimating the concentration.

Fig. S1. Structures of TSR, 5’-fluoro-TSR, 6’-fluoro-TSR and 12’-methyl-TSR



R_1	R_2	R_3	Name
H	H	H	Thiostrepton (TSR)
F	H	H	5'-fluoro-thiostrepton (5'-fluoro-TSR)
H	H	F	6'-fluoro-thiostrepton (6'-fluoro-TSR)
H	CH_3	H	12'-methyl-thiostrepton (12'-methyl-TSR)

Fig. S2. The UV spectra of 5’-fluoro-SIO (254 nm)

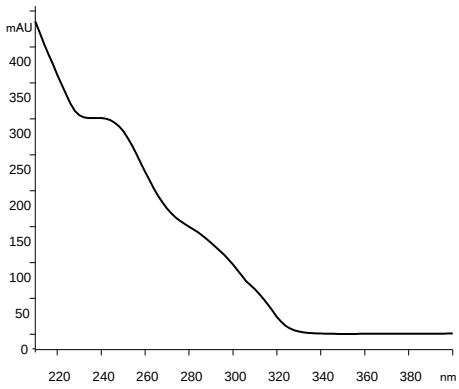


Fig. S3. DNA examination. DNA examination by agarose gel electrophoresis analysis. The gel contains the DNA ladder (left) and the PCR products of mutant SL3051 (665 bp) and SL2051 (2340 bp) (right)

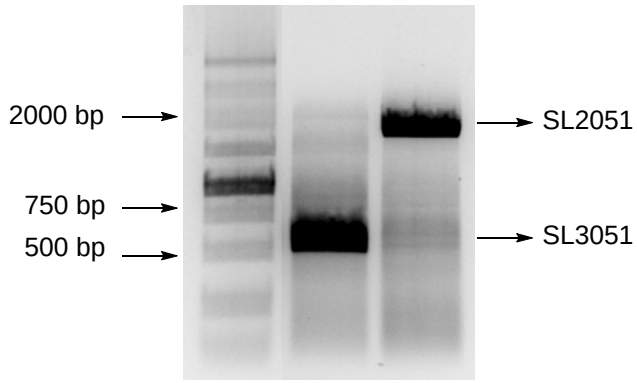


Fig. S4. Structure of 5’-fluoro-SIO

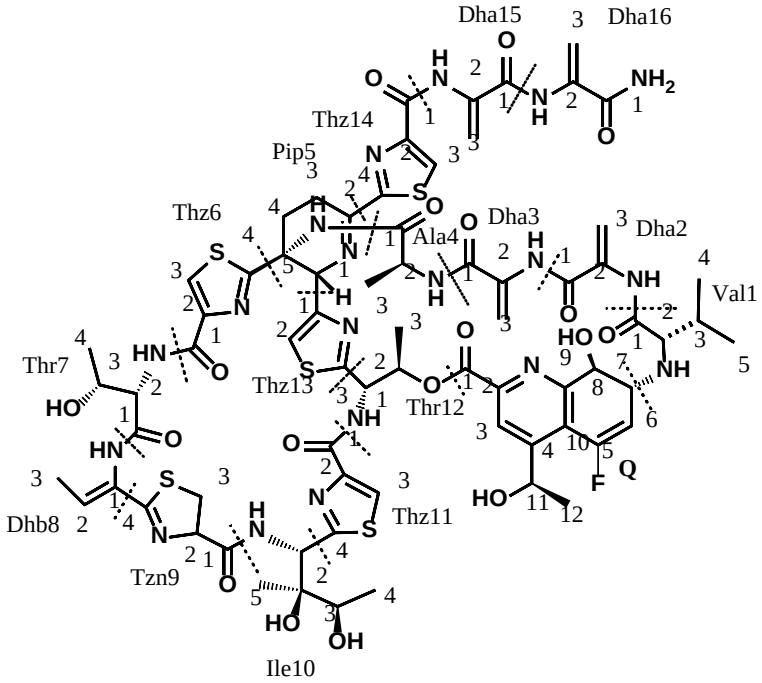
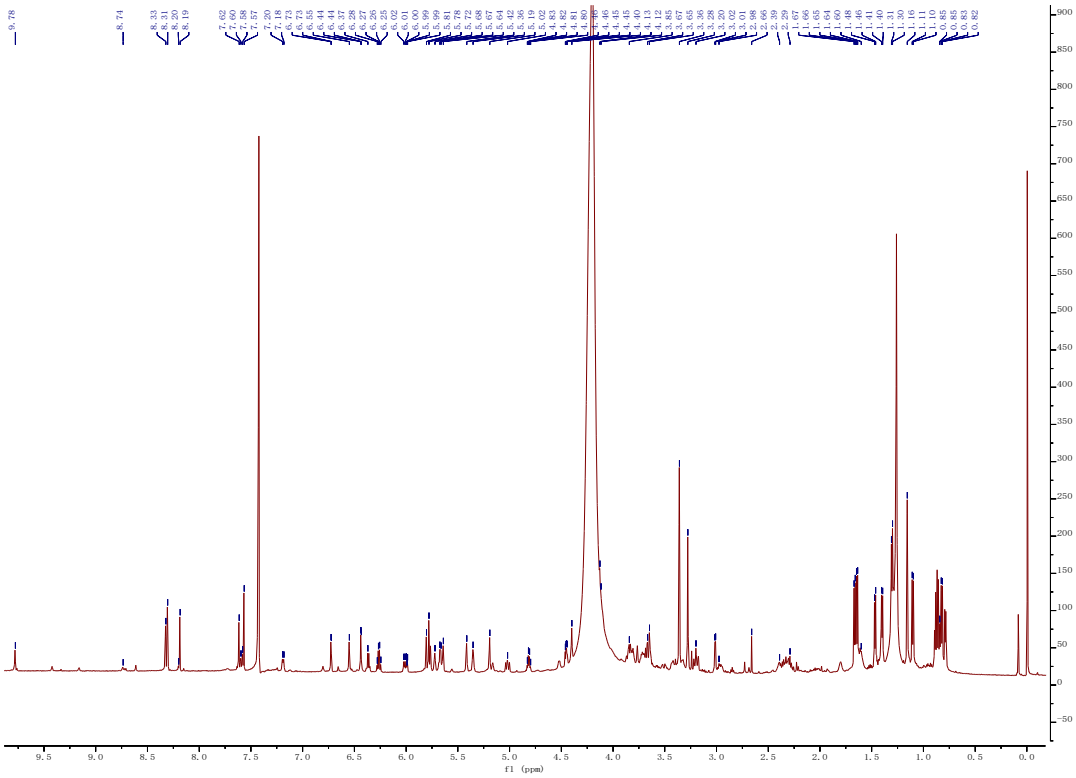
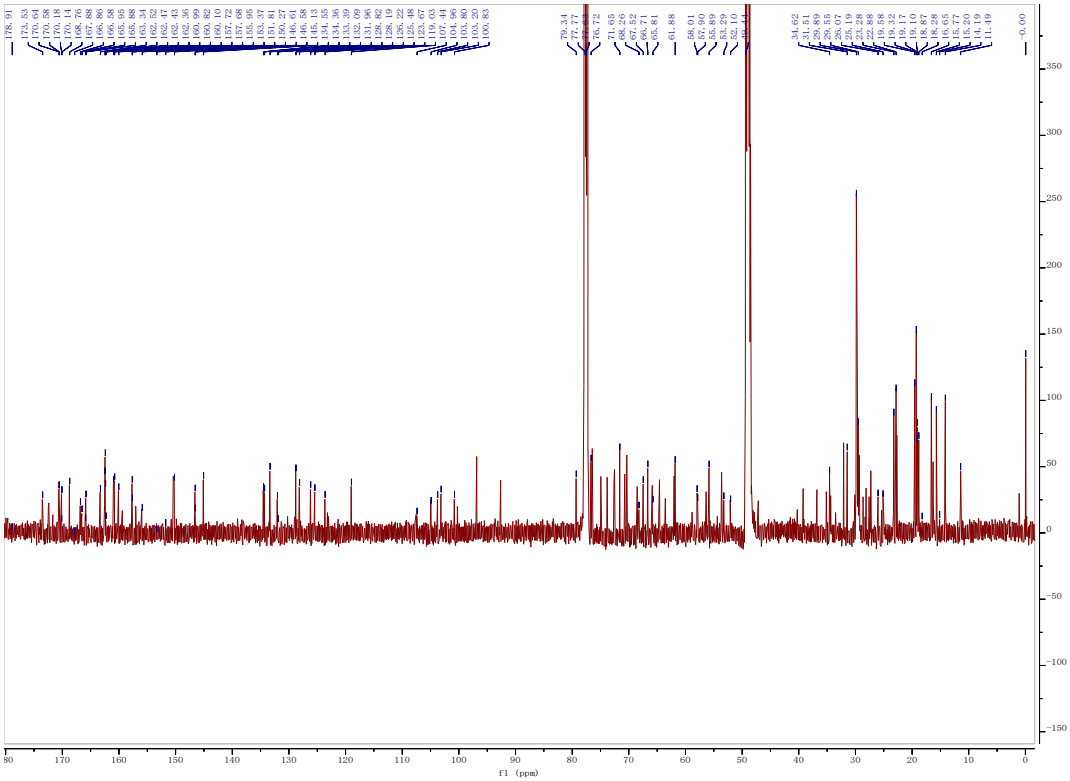


Fig. S5. NMR spectra of 5'-fluoro-SIO. **A**. ^{1}H spectrum. **B.** ^{13}C spectrum. **C.** COSY spectrum. **D.** HSQC spectrum. **E.** HMBC spectrum. **F.** ^{19}F NMR. ($CDCl_3$:CD_3OD=4:1)

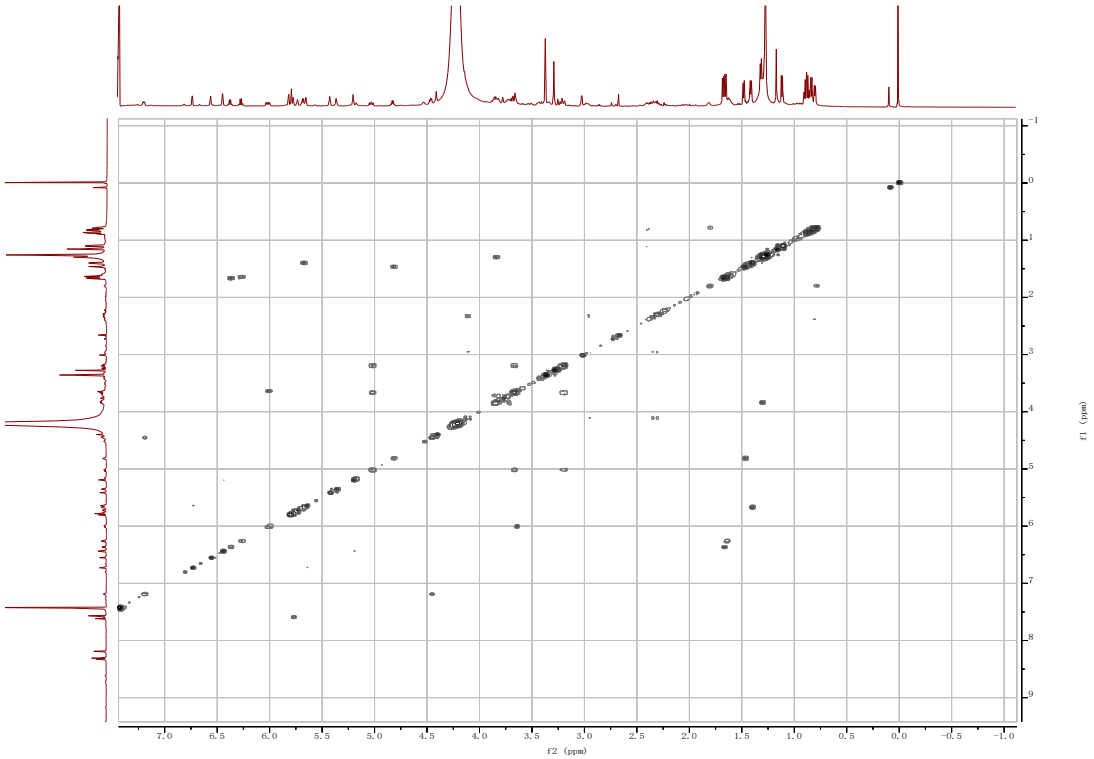
A. ^{1}H NMR



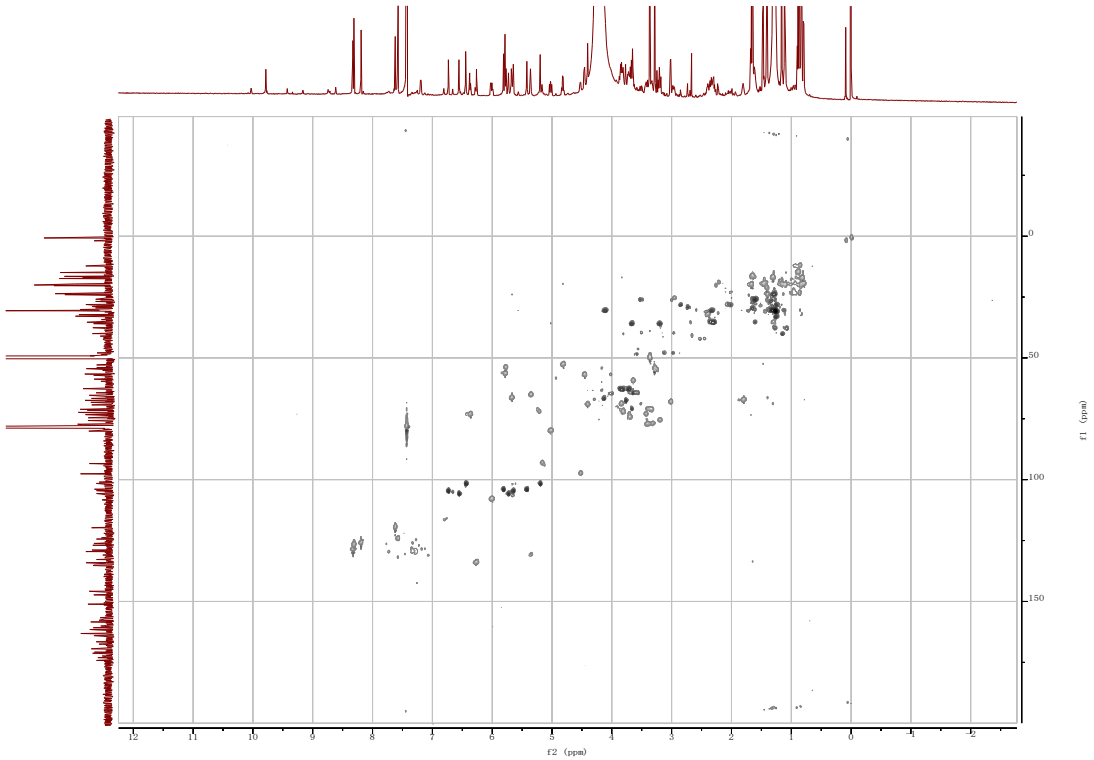
B. ^{13}C NMR



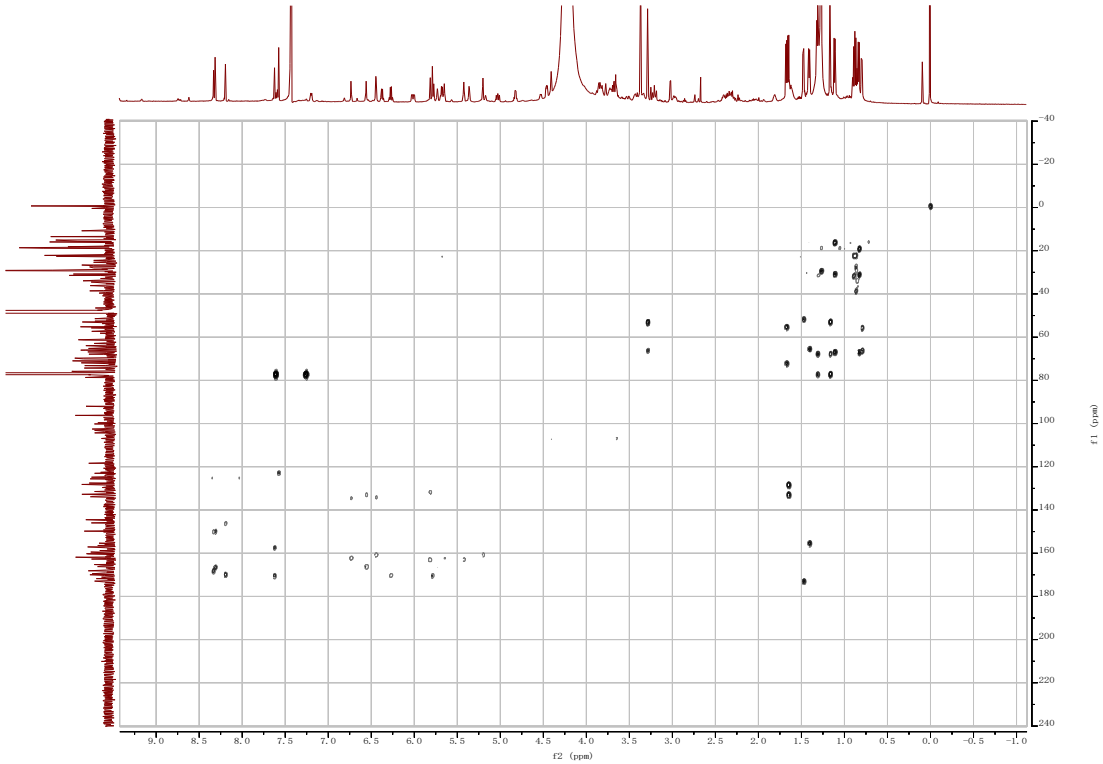
C. COSY



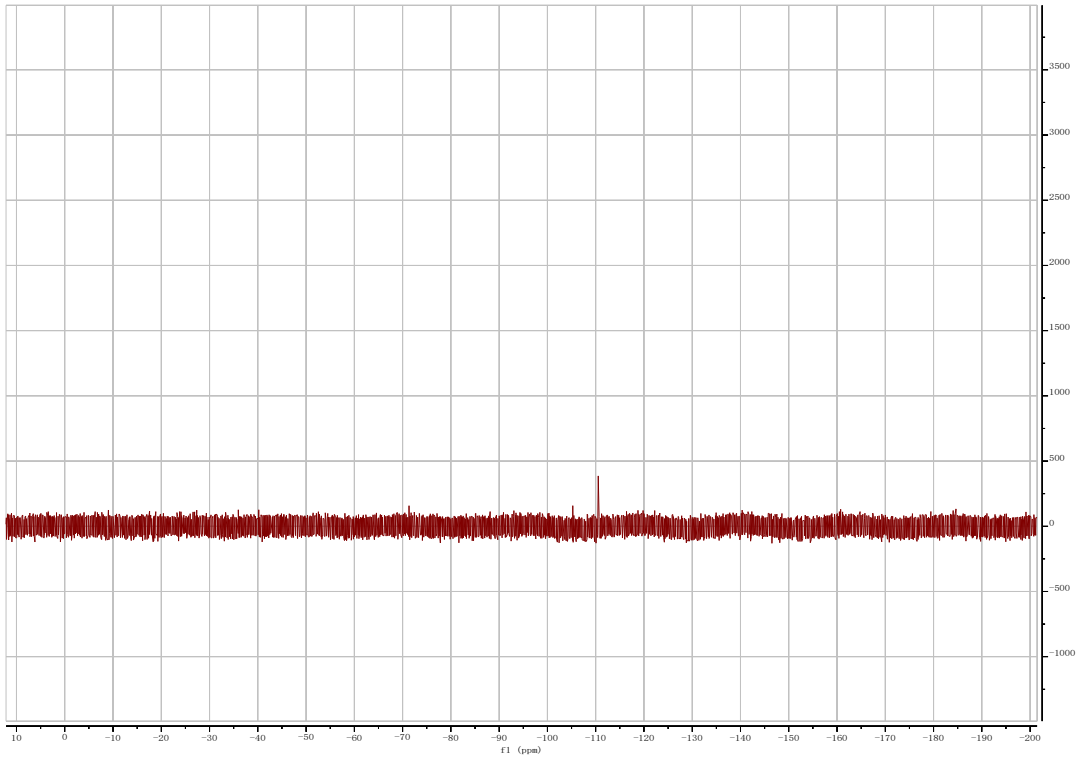
D. HSQC



E. HMBC



F. ^{19}F NMR



2. Supplementary Tables

Table S1. Strains and plasmids used in this study

Strains/Plasmids	Description	Source / Reference
Strains		
Escherichia coli		
DH5α	Host for general cloning	Transgen
ET12567 (pUZ8002)	Donor strain for conjugation between *E.coli* and *Streptomyces*	1
Streptococcus mutans UA159	Oral caries pathogenic bacteria, Gram-positive	ATCC
Lactobacillus acidophilus ATCC 4356	Oral caries pathogenic bacteria, Gram-positive	ATCC
Actinomyces viscosus ATCC 19246	Oral caries pathogenic bacteria, Gram-positive	ATCC
Fusobacterium nucleatum ATCC 25286	Oral periodontopathic bacteria, Gram-negative	ATCC
Porphyromonas gingivalis ATCC 33277	Oral periodontopathic bacteria, Gram-negative	ATCC
Streptomyces		
laurentii	Wild type strain, thiostrepton(TSR)-producing strain	ATCC
SL2051	S. laurentii derivative, in which the codon GAG for Glu-7 of TsrH was mutated into the stop codon TAG	4
SL3051	The *tsrT* in-frame deletion mutant of SL2051	This study
SL3052	SL3051 derivative, containing pSL-I1V-A2S	This study
Plasmids		
pWHU2653	*E. coli-Streptomyces* shuttle vector for gene inactivation	2
pSET152	*E. coli-Streptomyces* shuttle vector containing the aac(3)IV gene and the C31 attP site and integrase gene	1
pSL2050	pSET152 derivative for in trans expressing tsrH with the 530 bp upstream sequence and 288 bp downstream region	8

pSL3051	pSL2050 derivative for in trans expressing the gene that encodes TsrH- Ile1Val-Ala2Ser	4
pWHU1001	pWHU2653 derivative containing *sg* sequence of *tsrT*	This study
pWHU1002	pWHU2653 derivative for *tsrT* in-frame deletion	This study

Table S2. Primers used in this study. Small letters indicate the vector sequences. Underscored letters indicate the recognition sites of restriction endonucleases.

Primer	**Sequence**
sg-for	accaccaccaccacCACTGAGCTAGCTTCAGACGTG
sg-rev	gactagaggatccccgggtaTCTAGAAAAAAAACCCCGCC
tsrT-sg-for	GCCACCACGAGAGCCGGGccgttttagagctagaaatagc
tsrT-sg-rev	GGCCCGGCTCTCGTGGTGGCgctggatcctaccaaccggc
tsrT-L-fo*r*	gcttgcggcagcgtgaagcttGCGATCTGCTGCGGGTACG
tsrT-L-rev	ccgtgtatccgggtggatctGGTTGGGG
tsrT-R-for	agatccacccggatacacggGCGCTTTCTG
tsrT-R-rev	gacctgcaggcatgcaagcttACGGCGAACTGACCCTCTG
tsrT-Y-for	CGGGAGGCCCTGGCCTGGTACCGCTG
tsrT-Y-rev	TGACGTCGAGGAGCCGCCGGTTCCG

Table S3. ^{1}H and ^{13}C NMR spectroscopic data for 5'-fluoro-SIO.

Assignment	H	C	HMBC	COSY
Val1-1		173.5		
Val1-2	3.01 (d, 3.96）	67.5	Val1-1, Val 1-3, Val 1-4, Val 1-5, Q-7	Val1-3
Val1-3	2.39 (m)	31.5	Val1-2, Val 1-4, Val 1-5	Val1-2, Val 1-4, Val 1-5
Val1-4	0.82 (d, 6.96)	16.7	Val1-2, Val 1-3, Val 1-5	Val1-3
Val1-5	1.11 (d, 6.9)	19.6	Val1-2, Val 1-3, Val 1-4	Val1-3
Dha2-1		162.4		
Dha2-2		134.4		
Dha2-3	Ha, 6.44 (d, 1.86)	100.8	Dha2-1, Dha2-2;	Dha2-3-Hb
	Hb, 5.19 (s)		Dha2-1, Dha2-2	Dha2-3-Ha
Dha3-1		163.3		
Dha3-2		132.0		
Dha3-3	Ha, 5.81 (s)	103.2	Dha3-1, Dha3-2	Dha3-3-Hb
	Hb, 5.42 (s)		Dha3-1, Dha3-2	Dha3-3-Ha
Dha3-NH	8.33 (bs)		Dha2-1,Dha3-1,Dha3-3	
Ala4-1		173.5		
Ala4-2	4.80 (q, 6.3)	52.1	Dha3-1, Ala4-1, Ala4-3	Ala4-3
Ala4-3	1.46 (d, 6.48)	19.1	Ala4-1, Ala4-2	Ala4-2
Ala4-NH	7.12 (d, 6.9)		Dha3-1, Ala4-1	
Pip5-1	5.36 (s)	64.6	Pip5-2, Pip5-4, Pip5-5, Ala4-1, Ala4-2, Thz6-4, Thz13-1, Thz13-2,	
Pip5-2		162.4		
Pip5-3	Ha, 2.98 (m)	26.1		Pip5-3-Hb, Pip5-4-Ha, Pip5-4-Hb
	Hb, 3.36 (m)			Pip5-3-Ha, Pip5-4-Ha
Pip5-4	Ha, 4.12 (m)	29.9	Pip5-1, Pip5-3, Pip5-5	Pip5-3-Ha, Pip5-3-Hb, Pip5-4-Hb
	Hb, 2.32 (m)		Pip5-1, Pip5-2, Pip5-3, Pip5-5, Thz13-1	Pip5-3-Ha, Pip5-4-Ha
Pip5-5		57.9		
Pip5-NH	9.78 (s)		Ala4-1	
Thz6-1		161.0		
Thz6-2		146.6		
Thz6-3	8.20 (s)	126.2	Thz6-2, Thz6-4	
Thz6-4		170.2		
Thr7-1		166.8		

Thr7-2	4.46 (m)	55.9	Thr7-1, Thr7-3, Thr7-4, Thz6-1	Thr7-3
Thr7-3	1.60 (m)	66.7		Thr7-2, Thr7-4
Thr7-4	0.85 (d, 6)	19.2	Thr7-2, Thr7-3	Thr7-3
Thr7-NH	7.19 (d, 6.9)		Thz6-1, Thr7-1	Thr7-2
Dhb8-1		128.8		
Dhb8-2	6.26 (q, 6.78)	132.1	Dhb8-1, Dhb8-3, Tzn9-1	Dhb8-3
Dhb8-3	1.64 (d, 7.02)	15.2	Dhb8-1, Dhb8-2, Tzn9-4	Dhb8-2
Dhb8-NH	8.74 (bs)		Dhb8-2	
Tzn9-1		170.6		
Tzn9-2	5.02 (dd, 9.78,11.94)	79.3	Tzn9-1, Tzn9-3, Tzn9-4	Tzn9-3-Ha, Tzn9-3-Hb
Tzn9-3	Ha,3.20 (m)	34.6	Tzn9-1, Tzn9-2	Tzn9-2, Tzn9-3-Hb
	Hb,3.66 (m)		Tzn9-1, Tzn9-4	Tzn9-2, Tzn9-Ha
Tzn9-4		170.6		
Ile10-1	5.78 (s)	53.3	Tzn9-1, Ile10-2, Thz11-4	
Ile10-2		76.7		
Ile10-3	3.84 (m)	67.5	Ile10-1, Ile10-2, Ile10-4, Ile10-5	Ile10-4
Ile10-4	1.31 (d,6.3)	15.8	Ile10-2, Ile10-3	Ile10-3
Ile10-5	1.16 (s)	19.1	le10-1, le10-2, le10-3, Tzn9-1	
Ile10-NH	7.60 (s)		Tzn9-1	Ile10-1
Thz11-1		166.9		
Thz11-2		146.6		
Thz11-3	8.19 (s)	125.5	Thz11-1, Thz11-2	
Thz11-4		165.9		
Thr12-1	5.81 (s)	55.9	Thz11-1, Thz13-3, Thz12-2, Thz12-3	
Thr12-2	6.37 (m)	71.6	Thz12-3, Thz13-3, Q-1	Thz12-3
Thr12-3	1.67 (d, 6.48)	18.9	Thz12-1, Thz12-2	Thz12-2
Thz13-1		157.7		
Thz13-2	7.62 (s)	119.0	Thz13-1, Thz13-3,Pip5-1	
Thz13-3		170.1		
Thz14-1		160.1		
Thz14-2		150.3		
Thz14-3	8.31 (s)	128.2	Thz14-1, Thz14-2, Thz14-4	
Thz14-4		166.0		
Dha15-1		162.4		
Dha15-2		134.4		

Dha15-3	Ha, 5.64 (s) Hb, 6.73 (s)	105.0	Dha15-1, Dha15-2 Dha15-1, Dha15-2	Dha15-3-Hb Dha15-3-Ha
Dha16-1		166.6		
Dha16-2		134.6		
Dha16-3	Ha, 6.55 (bs) Hb, 5.72 (bs)	103.8	Dha16-1, Dha16-2 Dha16-1, Dha16-2	Dha16-3-Hb Dha16-3-Ha
Q-1		160.8		
Q-2		145.1		
Q-3	7.57 (s)	123.7		
Q-4		155.9		
Q-5		170.1		
Q-6	6.0 (dd, 6.78, 6.84)	107	Q-7, Q-8, Q-9, Q-10	Q-7
Q-7	3.65 (m)	58.0	Q-5, Q-6, Q-8, Q-9,Val-2	Q-6, Q-8
Q-8	4.40 (s)	68.2	Q-6, Q-7, Q-9, Q-10	Q-7
Q-9		157.7		
Q-10		123.7		
Q-11	5.67 (q, 6.48)	65.8	Q-3, Q-4, Q-12	Q-12
Q-12	1.40 (d, 6.54)	22.9	Q-4, Q-11	Q-11

Table S4. MALDI High Resolution MS DATA of 5’-fluoro-SIO.

Compound	5’-fluoro-SIO[a]
Found	1666.4574
Calculated	1666.4589
Formula	$C_{71}H_{81}N_{19}O_{18}S_5F$

a: $[M+H]^+$

3. Supplementary references

1 T. Kieser et al., *Practical Streptomyces Genetics* (John Innes Foundation, **2000**).

2 H. Zeng, S. Wen, W. Xu, Z. He, G. Zhai, Y. Liu, Z. Deng and Y. Sun, *Appl. Microbiol. Biotechnol.*, 2015, **99**, 10575-10585.

3 R. Liao, L. Duan, C. Lei, H. Pan, Y. Ding, Q. Zhang, D. Chen, B. Shen, Y. Yu and W. Liu, *Chem. Biol.*, 2009, **16**, 141-147.

4 P. Duan, Q. Zheng, Z. Lin, S. Wang, D. Chen and W. Liu, *Org. Chem. Front.*, 2016, **3**, 1254-1258.

5 S. F. Wang, Q. F. Zheng, J. F. Wang, Z. X. Zhao, Q. Y. Li, Y. S. Yu, R. X. Wang and W. Liu, *Org. Chem. Front.*, 2015, **2**, 106-109.

6 N. Azizan, S. Mohd Said, Z. Zainal Abidin and I. Jantan, *Molecules*, 2017, **22**.

7 K. Y. Lee, S. M. Cha, S. M. Choi and J. D. Cha, *J. Oral. Sci.*, 2017, **59**, 77-86.

8 H. Guo, J. Wang, Y. Li, Y. Yu, Q. Zheng, J. Wu and W. Liu, *Chem. Sci.*, 2014, **5**, 240-246.