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•Research article•

Shunt products of aminoansamycins from *aas1* overexpressed mutant strain of *Streptomyces* sp. S35

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[ABSTRACT] Constitutively expression of the pathway-specific activators is an effective method to activate silent gene clusters and improve natural product production. In this study, nine shunt products of aminoansamycins (**1**−**9**) were identified from a recombinant mutant strain S35-LAL by overexpressed the large-ATP-binding regulator of the LuxR family (LAL) gene *aas1* in *Streptomyces* sp. S35. All the compounds showed no anti-microbial, anti-T3SS and cytotoxic activities.

[KEY WORDS] *Streptomyces* sp. S35; Aminoansamycins; Shunt products
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Introduction

Ansamycins are a family of macrolactams with diverse biological activities [1-3]. Because of their significant biological properties and structural diversities, our group had been devoted to discovery of novel ansamycins for many years. More than 60 AHBA synthase gene-positive strains have been picked by PCR screening using the primers that target AHBA synthase genes [4]. Bioinformatics analysis of natural products biosynthetic potential of these strains revealed a series of cryptic or silent ansamycin gene clusters. By constitutively overexpression of the pathway-specific activators, series of novel ansamycins, including hygrocins [5-6], ansatrienins [7], divergolides [8], juanlimycins [9], microansamycins [10] and aminoansamycins [11] had been discovered from those AHBA-positive strains.

Streptomyces sp. S35 was a soil-derived AHBA synthase gene positive bacterium. A pentaketide ansamycin biosynthetic gene cluster (*aas*, GenBank No. MH045679) was identified in genome of strain S35 using antiSMASH 4.0 software [11]. In order to awake the *aas* gene cluster, three recombinant strains, including S35-LAL, S35-SARP and S35-SARP/LAL, have been constructed by constitutively overexpression of the pathway specific LAL and SARP transcriptional regulatory genes *aas1*, *aas10* and *aas1*/*aas10* in *Streptomyces* sp. S35, respectively [11]. The HPLC profiles of the metabolites for strain S35-LAL suggested significant difference from those of S35-SARP and S35-SARP/LAL. Chemical investigations of strain S35-SARP and S35-SARP/LAL led to novel aminoansamycins (Fig. 1) [11]. And in this study, the strain *S.* sp. S35-LAL was incubated on ISP3 agar media (15 L) for 10 days at 28 ℃. After organic solvent extraction, the MeOH extract (5.0 g) was subjected to column chromatography over *Sephadex* LH-20, MPLC and followed by HPLC purification to yield compounds **1**−**9** (Fig. 1).

Results and Discussion

Compound **1** was obtained as a colorless square crystal. Its molecular formula was deduced to be $C_{15}H_{19}NO_5$ from the HR-ESI-MS peak at *m*/*z* 294.1333 $[M + H]^+$ and NMR spectroscopic data (Tables 1 and 2). The ^1H, ^{13}C NMR and HSQC spectra revealed 15 carbon signals, corresponding to two methyls, three methylenes, four methines and six quaternary C-atoms. A fragment from C-7 to C-13 was established based on the ^1H-^1H COSY correlations of H-7 to H-10 and H-12 to H-13 and the HMBC correlations from H-10 and H-12 to C-11 (δ_C 211.1). A tetra-substituted *p*-hydroquinone moiety was confirmed by the HMBC correlations of H-3 to C-1, C-2, C-4, and C-5, and H-5 to C-1, C-3, C-4 and C-7 and the down

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These authors have no conflict of interest to declare.

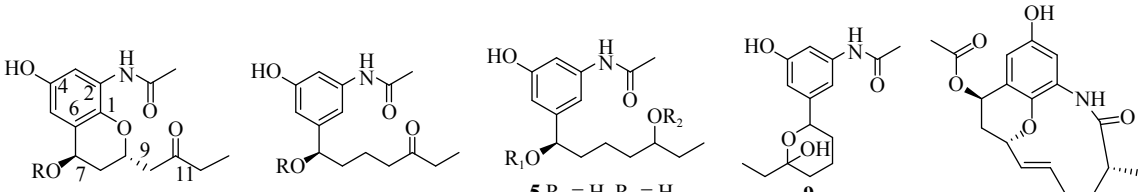

Fig. 1 Structures of compounds 1–9

Table 1 ^{1}H NMR (400 MHz) spectroscopic data of compounds 1–9 (in CD_3OD)

Pos.	1	2	3	4	5	6	7	8	9
1	/	/	6.90, s	6.49, s	6.91, s	6.94, s	6.89, s	6.92, s	6.56, s
3	7.41, d (2.8)	7.44, d (2.7)	7.07, s	7.09, s	7.08, s	7.09, s	7.08, s	7.10, s	7.09, t (1.8)
5	6.55, d (2.6)	6.52, d (2.7)	6.54, s	6.93, s	6.55, s	6.50, s	6.54, s	6.48, s	6.95, s
7	4.61, m	5.85, t (3.2)	4.50, t (5.2)	5.59, t (6.7)	4.50, t (6.4)	5.60, t (6.0)	4.48, t (7.1)	5.58, t (6.8)	4.51, dd (11.9, 1.2)
8	1.85, td (3.8, 8.0) 2.06, td (14.8, 2.5)	1.94, m; 2.15, s	1.71, m	1.73, m 1.86, m	1.73, m	1.73, m 1.85, m	1.70, m	1.88, m	1.50, m 1.80, m
9	4.66, m	4.61, m	1.55, m 1.68, m	1.59, m	1.54, m	1.45, m	1.27, m 1.41, m	1.37, m	1.70, m 1.98, m
10	2.81, dd (16.0, 5.0) 3.02, dd (16.0, 7.0)	2.85, dd (16.6, 8.8) 3.07, dd (16.6,6.7)	2.47, m	2.48, t (7.4)	1.44, m	1.47, m	1.57, m	1.58, m	1.47, m 1.79, m
11	/	/	/	/	3.44, m	3.43, m	4.80, m	4.79, m	
12	2.64, q (7.2)	2.63, q (7.4)	2.45, q (6.8)	2.44, q (6.1)	1.46, m	1.49, m	1.55, m	1.58, (f, 6.0)	1.58, m 1.78, m
13	1.08, t (7.3)	1.09, t (7.2)	1.01, t (6.6)	1.01, t (7.3)	0.93, t (7.4)	0.93, t (7.4)	0.88, t (7.4)	0.88, t (8.0)	0.93, t (7.5)
2'	2.11, s	2.12, s	2.09, s	2.09, s	2.09, s	2.09, m	1.99, s	2.06, s	2.09, s
7b	/	2.07, s	/	2.06, s	/	/	/	1.99, s	/
11b	/		/	/	/	/	2.09, s	2.09, s	/

Table 2 ^{13}C NMR (100 MHz) NMR spectroscopic data of compounds 1–9 (in CD_3OD)

Pos.	1	2	3	4	5	6	7	8	9
1	140.0	140.6	110.1	110.0	110.2	110.1	110.9	110.1	109.9
2	127.9	127.9	140.9	141.1	140.7	141.0	140.9	141.1	140.7
3	111.4	112.1	107.1	107.6	107.2	107.5	107.1	107.6	107.2
4	151.6	151.5	158.9	159.0	158.8	159.0	158.8	159.0	158.7
5	112.9	113.1	109.8	110.1	109.8	110.0	109.8	110.0	110.2
6	125.5	121.6	148.4	143.8	148.5	144. 0	148.5	143.8	146.4
7	64.7	66.9	75.1	77.1	75.0	77.4	75.1	77.1	73.7
8	37.6	34.7	39.9	36.7	40.1	37.4	39.6	37.0	34.2
9	69.7	70.1	21.1	20.8	23.1	22.9	22.7	22.3	20.3
10	48.5	48.1	43.1	42.4	37.8	37.5	34.4	34.1	32.5
11	211.7	211.3	214.4	213.9	73.8	73.7	77.2	76.5	101.8
12	37.4	37.4	36.5	36.5	31.0	31.1	28.1	28.0	30.1
13	8.0	7.9	8.2	8.1	10.4	10.4	10.1	9.9	8.1
1'	171.4	172.0	171.8	171.6	171.6	171.6	171.6	171.6	171.6
2'	23.8	21.2	24.3	21.1	23.9	21.1	23.9	21.1	23.9
7a	/	171.6	/	172.2	/	172.1	/	172.2	/
7b	/	23.8	/	23.9	/	23.9	/	21.0	/
11a	/	/	/	/	/	/	172.9	172.9	/
11b	/	/	/	/	/	/	21.0	23.9	/

field shifts of C-4 (δ_C 151.6) and C-1 (δ_C 140.1). The two fragments were linked together and supported by the HMBC correlations of H-7 to C-5, C-6 and C-9, and further the existence of ether linakge between the C-1 and C-9 was confirmed by the HMBC correlation of H-9 to C-1. An acetyl moiety in **1** was assigned by the HMBC correlation of H-2' to C-1'. The absolute configurations of 7*R*, 9*S* in **1** were determined by a single-crystal X-ray diffraction analysis with Cu *Kα* radiation (CCDC 1956537, Fig. 2), the same as these in aminoansamycins A and B [11].

The NMR data of **2** (Tables 1 and 2) were highly similar to those of **1** except that C-7 was substituted by an acetoxy group deduced by the HMBC correlations of H-7b (δ_H 2.07) to C-7a (δ_C 172.6) and H-7 (δ_H 5.85) to C-7a (δ_C 172.6), which was further supported by the HR-ESI-MS peak at *m/z* 336.1441 $[M + H]^+$.

Compound **3** was obtained as a light yellow oil with the molecular formula $C_{15}H_{21}NO_4$ according to HR-ESI-MS ion peak at *m/z* 280.1538 $[M + H]^+$ and NMR data (Tables 1 and 2). The same fragment from C-7 to C-13 as that in **1** was deduced on the basis of the ^{1}H-^{1}H COSY correlations of H-7 to H-10 and H-12 to H-13 and the HMBC correlations from H-10 and H-12 to C-11 (δ_C 214.4). Further a tri-substituted benzene ring was confirmed by the presence of three aromatic protons and their HMBC correlations. The HMBC correlations from H-7 to C-1, C-8 and C-9 and from H-5 to C-7 linked the two fragments together. The marked difference between **3** and **1** is the oxygenated methine at C-9 (δ_C 69.7, δ_H 4.67) in **1** replaced by a methylene (δ_C 21.1, δ_H 1.55 /1.68) in **3**.

The difference between **4** and **3** was an additional acetoxy group (δ_C 172.2 (C-7a) and δ_C 21.0/δ_H 2.06 (C-7b)) in **4**, and it was attached at C-7 deduced by the HMBC correlations of H-7b to C-7a and H-7 to C-7a, which was further confirmed by the HR-ESI-MS peak at *m/z* 322.1644 $[M + H]^+$.

By comparing the NMR spectra of **5** and **3**, we found the ketone group in **3** was replaced by an oxygenated methine at C-11 (δ_C73.8, δ_H 3.44) in **5**, which was confirmed by HR-ESI-MS peak at *m/z* 282.1697 $[M + H]^+$.

Fig. 2 The single-crystal *X*-ray structure of 1

Both the molecular formula of **6** and **7** were established to be $C_{17}H_{25}NO_5$ based on their HR-ESI-MS with ion peaks at *m/z* 324.1805 $[M + H]^+$ and 324.1803 $[M + H]^+$, respectively. The NMR spectra of **6** and **7** were similar to those of **5**, except that an additional acetoxyl group attached at C-7 (δ_C172.2, δ_H 2.06/δ_C 23.9) in **6** and C-11 (δ_C 172.9, δ_H 2.09/δ_C 21.0) in **7** were confirmed by the HMBC correlations of H-7 to C-7a in **6** and H-11 to C-11a in **7**, respectively.

The NMR data of **8** were similar to those of **5**, except that two acetoxyl groups [C-7a (δ_C 172.2) and C-7b (δ_H 1.99/21.0) and C-11a (δ_C 172.9) and C-11b (δ_H 2.09/23.9)] attached at C-7 and C-11 were confirmed by the HMBC correlations of H-7 (δ_H 5.62) to C-7a and H-11 (δ_H 4.79) to C-11a, respectively.

The NMR data of compound **9** were similar to those of **5**, except that C-11 was an acetal quaternary carbon at δ_C 101.8 rather than an oxygenated methine. The ether linkage between C-7 and C-11 was determined by the HMBC correlation of H-7 (δ_H 4.51) to C-11 (δ_C 101.8). HR-ESI-MS with the ion peak at *m/z* 280.1537 $[M + H]^+$ further confirmed the structure.

The relative configurations of C-7 and C-9 in **2** were deduced to be 7*R*, 9*S* and the stereochemistry of C-7 in **3**–**8** were assumed to be the same as that of in **1** on the basis of biosynthetic logic, but the stereochemistry of C-11 in **5**–**9** could't be confirmed in this study.

All the compounds showed no antimicrobial activity against all tested strains with 40 μg/disc in the paper disc diffusion assay. In the anti-T3SS experiment, no inhibitory activities were indicated for compounds **1**–**9** on the secretion of SPI-1 effector proteins SipA/B/C/D. In the CCK-8 assay using HeLa and MDA-MB-231 cells, no cytotoxic activities were observed even at the concentration of 200 μmol·L^{-1} of compounds.

The external environments affect the production of microbial secondary metabolism. The changes of these environmental signals are ultimately acting on the secondary metabolism pathway through the regulatory factors [12-14]. Therefore, the manipulation of regulatory genes is an effective way to activate silent gene clusters. Our recent work revealed that co-expression of pathway-specific regulator genes *aas1* (LAL family) and *aas10* (SARP family) in strain S35 resulted in the identification of seven novel pentaketide ansamycin anminoansamycins A–G [11]. In this study, we performed the isolation of the extra peaks presented in strain S35-LAL, and identified nine immature anminoansamycins at the tetraketide stage, which is probably because that other essential genes are not activated in strain S35-LAL. Pathway-specific LAL and SARP family regulatory genes are conserved in almost all known benzenic ansamycin gene clusters except for the geldanamycin gene cluster, which contains two LAL family regulatory genes. Previous study for the biosynthesis of ansamitocins indicated that the LAL family regulator Asm8

was a specific positive regulator of AHBA biosynthesis [15], and the SARP family regulator Asm18 increased the transcription levels of the PKS genes, tailoring genes, and the LAL regulatory gene *asm8* in *Actinosynnema pretiosum* ssp. auranticum ATCC 31565 [16]. Therefore, overexpression of *aas1* may only upregulated partial biosynthetic genes in *aas* gene cluster, which led to the production of pre-released tetraketide products, followed by a series of oxido-reduction and acetylation to give compounds **1**–**9**.

Experimental

General experimental procedures

The optical rotations were carried out using an Anton Paar MCP200 polarrimeter. NMR spectra were recorded on Bruker DRX-400 MHz NMR spectrometer with tetramethylsilane (TMS) as an internal standard. HR-ESI-MS were carried out on an LTQ-Orbitrap XL. Sephadex LH-20 (25−100 μm; Pharmacia Biotek, Denmark) and LiChroprep RP-18 (40−63 μm; Darmstadt, Germany) were used for column chromatography (CC). Preparative HPLC were performed on a Waters 2545 equipped with a ZORBAX Eclipse XDC18 5 μm column (9.4 mm × 250 mm). All solvents used were of analytical grade.

Strain and fermentation

The strain S35-LAL was constructed by our group previously [11]. Strain S35-LAL was cultured on Petri dishes with *ca*. 20 mL ISP3 medium (1.5% agar, 2% oatmeal, 0.1% trace element solution, pH 7.2) with a total volume of 15 litres for 14 d at 28 ℃.

Extraction and isolation

The culture (15 L) was extracted three times with EtOAc−MeOH−AcOH (80 : 15 : 5, *V*/*V*/*V*) to obtain the crude extract. Then the extract was partitioned between H_2O and ethyl acetate (EtOAc), and the EtOAc extract was further partitioned with petroleum ether (PE) and MeOH. The MeOH extract (5 g) was subjected to CC over Sephadex LH-20 (120 g) eluted with MeOH to obtain Frs. A−E. Fr. B (2.5 g) was subjected to Sephadex LH-20 (140 g) eluted with MeOH to obtain Frs. B1−B6. Fr. B2 (100 mg) was purified by HPLC (eluted with 18% CH_3CN, 4 mL·min^{-1}, UV 254 nm) to yield **1** (t_R 8.4 min, 12.5 mg) and **9** (t_R 11.2 min, 2.6 mg). Fr. B3 (330 mg) was purified by semi-preparative HPLC (eluted with 18% CH_3CN, 4 mL·min^{-1}, UV 254 nm) to yield **5** (t_R 8.5 min, 25.3 mg) and **3** (t_R 11.0 min, 9.8 mg). The rest of Fr. B3 (150 mg) was further purified by HPLC (eluted with 30% CH_3CN, 4 mL·min^{-1}, UV 254 nm) to yield **2** (t_R 8.0 min, 6.4 mg). Fr. B4 (250 mg) was subjected to CC over Sephadex LH-20 (60 g) eluted with MeOH to obtain Fr. B41−Fr. B43. Fr. B42 (150 mg) were purified by HPLC (eluted with 25% CH_3CN, 4 mL· min^{-1}, UV 254 nm) to yield **6** (t_R 11.6 min, 18.2 mg), **7** (t_R 16.0 min, 5.8 mg) and **4** (t_R 16.8 min, 5.0 mg). Fr. B5 (200 mg) was subjected to CC over Sephadex LH-20 (60 g) eluted with MeOH to obtain Fr.B51−Fr.B53. Fr. B52 (150 mg) were purified by HPLC (eluted with 25% CH_3CN, 4 mL·min^{-1}, UV 254 nm) to yield **8** (t_R 12.0 min, 4.6 mg).

Identification of new compounds

Compound ***1***: Colorless crystal; $[\alpha]_D^{20}$ −24.76 (*c* 0.185, MeOH); UV (MeOH) λ_{max}: 220, 254, 285 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 294.1333 $[M + H]^+$ (Calcd. for $C_{15}H_{20}NO_5^+$, *m*/*z* 294.1336), and 316.1152 $[M + Na]^+$ (Calcd. for $C_{15}H_{19}NO_5Na^+$, *m*/*z* 316.1155). The single-crystal *X*-ray diffraction intensity data of **1** was collected with a Bruker APEXII CCD diffractometer using graphite-monochromated Cu-Kα radiation (λ = 1.541 78 Å) by the ω-scan technique [scan width 0−180°, 2θ ≤ 50°] at 296 (2) K. Crystal data of **1**. $C_{15}H_{18}NO_5$; Mr = 297.80, orthorhombic, a = 8.4103(4) Å, b =11.1019(4) Å, c = 15.7742(7) Å, a = 90°, b = 90°, g = 90°, V = 1472.84(11) Å3, space group P2(1)2(1)2(1), Z = 4, *Dx* = 1.343 g·cm^{-3}, *Mu* = 0.877 mm^{-1}, F(000) = 631; Crystal dimensions: 0.37 × 0.25 × 0.31 mm^3; Rint = 0.1202. Theta range for data collection: 4.871° to 66.531°. The final R1 = 0.0723, wR2 (reflections) = 0.1995 (I > 2 s(I)). CCDC number: 1956537.

Compound ***2***: Light yellow oil; $[\alpha]_D^{20}$ +26.86 (*c* 0.325, MeOH); UV (MeOH) λ_{max}: 220, 254, 310 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 336.1441 $[M + H]^+$ (Calcd. for $C_{17}H_{22}NO_6^+$, *m*/*z* 336.1442), and 358.1259 $[M + Na]^+$ (Calcd. for $C_{17}H_{21}NO_6Na^+$, *m*/*z* 358.1261).

Compound ***3***: Light yellow oil; $[\alpha]_D^{20}$ −7.64 (*c* 0.245, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 280.1538 $[M + H]^+$ (Calcd. for $C_{15}H_{22}NO_4^+$, *m*/*z* 280.1543), and 302.1359 $[M + Na]^+$ (Calcd. for $C_{15}H_{21}NO_4Na^+$, *m*/*z* 302.1363).

Compound ***4:*** Light yellow oil; $[\alpha]_D^{20}$ +14.86 (*c* 0.250, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 322.1644 $[M + H]^+$ (Calcd. for $C_{17}H_{24}NO_5^+$, *m*/*z* 322.1649), and 344.1458 $[M + Na]^+$ (Calcd. for $C_{17}H_{23}NO_5Na^+$, *m*/*z* 344.1468).

Compound ***5***: Light yellow oil; $[\alpha]_D^{20}$ +75.8 (*c* 0.1255, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2. HR-ESI-MS *m*/*z* 282.1697 $[M + H]^+$ (Calcd. for $C_{15}H_{24}NO_4^+$, 282.1700), and 304.1518 $[M + Na]^+$ (Calcd. for $C_{15}H_{23}NO_5Na^+$, *m*/*z* 304.1519).

Compound ***6***: Light yellow oil; $[\alpha]_D^{20}$ +180.5 (*c* 0.091, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 324.1805 $[M + H]^+$ (calcd. for $C_{17}H_{26}NO_5^+$, *m*/*z* 324.1805), and 346.1625 $[M + Na]^+$ (Calcd. for $C_{17}H_{25}NO_5Na^+$, *m*/*z* 346.1625).

Compound ***7***: Light yellow oil; $[\alpha]_D^{20}$ +15.21 (*c* 0.290, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m*/*z* 324.1803 $[M + H]^+$ (Calcd. for $C_{17}H_{26}NO_5^+$, *m*/*z* 324.1805), and 346.1621 $[M + Na]^+$ (Calcd. for $C_{17}H_{25}NO_5Na^+$, *m*/*z* 346.1625).

Compound ***8***: Light yellow oil; $[\alpha]_D^{20}$ +25.70 (*c* 0.230, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^{1}H and ^{13}C

NMR data, see Tables 1 and 2; HR-ESI-MS *m/z* 366.1913 $[M + H]^+$ (Calcd. for $C_{19}H_{28}NO_6^+$, *m/z* 366.1911), and *m/z* 388.1730 $[M + Na]^+$ (Calcd. for $C_{19}H_{27}NO_6Na^+$, *m/z* 388.1713).

Compound **9**: Light yellow oil; $[\alpha]_D^{20}$ +14.62 (*c* 0.080, MeOH); UV (MeOH) λ_{max}: 220, 254, 289 nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HR-ESI-MS *m/z* 280.1537 $[M + H]^+$ (Calcd. for $C_{15}H_{22}NO_4^+$, *m/z* 280.1543), and 302.1358 $[M + Na]^+$ (Calcd. for $C_{15}H_{21}NO_4Na^+$, *m/z* 346.1625).

Antimicrobial assay

The antimicrobial activities of compounds **1**−**9** against *Staphylococcus aureus* ATCC 25923, *Mycobacterium smegmatis* mc^2 155, *Pseudomonas aeruginosa* PA01 and *Bactllus subtilis* 86315 were measured with a paper disc diffusion assay [17] and Kanamycin was used as a positive control.

Effects of compounds 1−9 on the secretion of SPI-1 effector proteins SipA/B/C/D

Salmonella enterica serovar Typhimurium UK-1χ8956 (ΔP *rpoS*183: TT *ara*C PBAD *rpo*S) was used in this study [18]. It was grown in Luria-Bertani (LB) broth or on LB agar plates supplemented with 0.2% L-arabinose at 37 ºC or 25 ºC. Effector proteins SipA/B/C/D of Salmonella Pathogenicity Island-1 (SPI-1) were isolated and detected as previously described by our lab [19].

CCK-8 assay

Approximately cancer cells 4000 per well were seeded in a 96-well cell culture plate. After 24 h incubation at 37 ℃, 5% CO_2, the culture medium was replaced by a series of concentrations of drugs diluted with the corresponding culture fluid and then incubated at 37 ℃, 5% CO_2 for 48 h. Three replicates were made for each measurement. 10 μL of the CCK-8 reagent (Selleck.) was added into each well, and OD_{450} was measured using a multifunction microplate reader (Bio-Rad 680, USA) after incubation for 2−4 h at 37 ℃. The percentage each concentration accounted for the control was presented as cell viability [20].

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