Rational Generation of Lasso Peptides Based on Biosynthetic Gene Mutations and Site-Selective Chemical Modifications

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Supplementary Information

General materials and experimental procedures .......................................................... S5
Bacterial strains, plasmids and DNA sequences .......................................................... S5
Fermentation of *Streptomyces* sp. PKU-MA01240 ......................................................... S5
Construction of the *stla* expression plasmid .............................................................. S6
Gene activation by the insertion of promoter *KasOp* at the upstream of *stlaA* .............. S6
Site-directed mutagenesis of *stlaA* gene ................................................................. S6
Heterologous expression, fermentation and HPLC analysis .......................................... S7
Large-scale fermentation and isolation of stlassins ..................................................... S8
Solution NMR structures determination ..................................................................... S9
Thermal stability assays and carboxypeptidase Y-treatment experiments .................... S9
Site-selective glycosylation of stlassin F15Y (16) and stlassin I4C (21) ......................... S10
Site-selective S-arylation of stlassin I4C (21) ................................................................. S10
Bis-allylation-elimination reaction of stlassin I4C (21) ............................................... S10
Conjugate addition of 2-mercaptoethanol and conjugate addition of N-acetylcysteamine S10
Biological activity assays of stlassins ......................................................................... S11
Table S1. The deduced functions of genes in the *stla* gene cluster ................................. S13
Table S2. Primers used in this study ........................................................................... S14
Table S3. Plasmids and strains used in this study .......................................................... S17
Table S4. The HRESIMS data of compounds 1-25 ................................................... S22
Table S5. Assignment of *1H, 13C* and *15N* signals (ppm) of stlassin (1) ................. S23
Table S6. Assignment of *1H, 13C* and *15N* signals (ppm) of stlassin V3A (3) ......... S24
Table S7. Assignment of *1H, 13C* and *15N* signals (ppm) of stlassin I4A (4) ............ S25
Table S8. Assignment of *1H signals (ppm) of stlassin W14F (14) ......................... S26
Table S9. Assignment of *1H signals (ppm) of stlassin F15Y (16) ......................... S27
Table S10. Assignment of *1H and 13C* signals (ppm) of stlassin V2C/A11C (18) ...... S28
Table S11. The Optical density (OD) values and inhibition ratios (% in parentheses) from parallel ELISA experiments for each stlassins against the binding of LPS to TLR4 S29
Table S12. Inhibition ratios from parallel ELISA experiments for each concentration in dose-response determination ................................................................. S30
Figure S1. The phylogenetic analysis of strain PKU-MA01240 ...................................... S31
Figure S2. The construction of plasmid pMM2002 for the heterologous expression .......... S32
Figure S3. The HPLC analysis of fermentations of heterologous expression strains .......... S33
Figure S4. The construction of plasmid pMM2003 and pMM2004 ................................ S34
Figure S5. HPLC analysis of stlassin (1, labeled with asterisk) in cells and supernatant of the heterologous expression strain MM20003 (*S. coelicolor* A3(2) with pMM2004 integrated) after fermentation using three different media ......................................................................... S35
Figure S6. The HPLC analysis of thermal stability and peptidase-treatment reactions ...... S36
Figure S7. The diagram for site-directed mutagenesis of *stlaA* gene ......................... S37
Figure S8. The HPLC analysis of stlassin (1) and its derivatives produced in the crude extracts of different strains ................................................................................. S38
Figure S9. The design of double mutations for introducing two cysteine residues ......... S39
The superimposition of the 20 solution structures with the lowest total energy

The TOCSY spectrum and NOESY spectrum of stlassin I4A

The TOCSY spectrum and NOESY spectrum of stlassin V3A

The HRESIMS spectrum of stlassin I4C

The HRESIMS spectrum of stlassin V3A

The HRESIMS spectrum of stlassin V2A

The HRESIMS spectrum of stlassin I4C

The HRESIMS spectrum of stlassin V2C/A11C

The antagonistic activities of stlassins at different concentrations

The OD values of four control ELISA experiments (N1–N4)

The HSQC spectrum of stlassin V3A

The HRESIMS spectra of stlassins I4C and V3A

The HRESIMS spectrum of stlassin I4C

The HRESIMS spectrum of stlassin V3A

The HRESIMS spectrum of stlassin I4C

The HRESIMS spectra of stlassins I4C and V3A

The HRESIMS spectrum of stlassin I4C

The HRESIMS spectrum of stlassin V3A

The ESIMS spectra of stlassins I4C and V3A

The TOCSY spectrum and NOESY spectrum of stlassin (I)

The 'H-13C HSQC spectrum of stlassin (1)

The 'H-15N HSQC spectrum of stlassin (1)
Figure S53. The $^1$H-$^{13}$C HSQC spectrum of stlassin I4A (4) .......................................................... S68
Figure S54. The $^1$H-$^{15}$N HSQC spectrum of stlassin I4A (4) .......................................................... S69
Figure S55. The TOCSY spectrum and NOESY spectrum of stlassin W14F (14) ............................ S70
Figure S56. The $^1$H-$^{13}$C HSQC spectrum of stlassin W14F (14) .................................................. S71
Figure S57. The TOCSY spectrum and NOESY spectrum of stlassin F15Y (16) ......................... S72
Figure S58. The $^1$H-$^{13}$C HSQC spectrum of stlassin F15Y (16) ................................................. S73
Figure S59. The TOCSY spectrum and NOESY spectrum of stlassin V2C/A11C (18) ............... S74
Figure S60. The $^1$H-$^{13}$C HSQC spectrum of stlassin V2C/A11C (18) .................................. S75
References ........................................................................................................................................ S76
General materials and experimental procedures

NMR data were collected on a Bruker Avance 600 MHz spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with an inverse triple resonance 1H-13C-15N probe with z-gradient. High-resolution mass spectra were obtained on a Shimadzu IT-TOF spectrometer (Shimadzu, Kyoto, Japan) and a Waters Alliance e2695-SQD spectrometer (Waters, Milford, MA, USA). HPLC analysis was performed on an Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) with a C18 RP-column (Extend-C18, 250 × 4.6 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA). Semi-preparative HPLC was performed on a SSI 23201 system (Scientific Systems Inc., State College, PA, USA) with a YMC-Pack ODS-A column (250 × 10 mm, 5 µm, YMC CO., LTD. Shimogyo-ku, Kyoto, Japan). Medium pressure liquid chromatography (MPLC) was performed on a LC3000 series (Beijing Tong Heng Innovation Technology, Beijing, China) with a Claricep™Flash i-series C18 cartridge (20-35 µm, 40 g, Bonna-Agela, Wilmington, DE, USA). All fermentations were carried out in MQD-B1R shakers (Minquan Instrument Co., Ltd., Shanghai, China).

Antibiotics were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and Fisher-Scientific (Waltham, MA, USA), respectively. The α-d-fluoroglucose tetraacetate was purchased from Beijing Chemsynlab Co., Ltd. (Beijing, China). The 2-nitrophenylboronic acid was purchased from Energy Chemical (ShangHai, China).

Bacterial strains, plasmids and DNA sequences

Strain Streptomyces sp. PKU-MA01240 was isolated from a sponge sample collected from Xieyang island, Guangxi Province, China.1 Genomic DNA of Streptomyces sp. PKU-MA01240 was extracted following standard protocols2 and sequenced by Majorbio Co. (Shanghai, China). The 16S rRNA gene (GeneBank accession number MT826211.1) was amplified by PCR using the forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer (5′-TACGGYTACCTTGTTACGACTT-3′).3 Common DNA sequencing and synthesis of all oligonucleotide primers used in this study (Table S2) were performed by RuiBiotech Co., Ltd. (Beijing, China). Bacterial strains and plasmids used and generated in this study are listed in Table S3. The secondary metabolite gene clusters were analyzed with antiSMASH (http://antismash.secondarymetabolites.org/).4 The gene functional annotations were performed with BLAST on the NCBI website. Multiple alignments were performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)5 and the phylogenetic tree was generated with Mega 6.0 using the Neighbor-Joining algorithm.

Small-scale fermentation of wild-type Streptomyces sp. PKU-MA01240

The S. sp. PKU-MA01240 strain was preserved as a spore solution at -80 °C. Each of 250 mL Erlenmeyer flasks, containing 50 mL of the M1 medium (yeast extract 1 g, peptone 5 g, beef extract 1 g, FePO₄ 0.01 g, and sea salt 33 g in 1 L distilled H₂O, pH 7.4) with 3% sea salt, was inoculated with 50 µL of the PKU-MA01240 spore solution and incubated in shaker at 28.0 °C, 200 rpm for three days, to afford the seed culture. The seed culture was inoculated into 50 mL of three different production media including M2 medium (yeast extract 4 g, malt extract 10 g, glucose 4 g, in 1 L distilled H₂O, pH 7.2) with 3% sea salt, medium M3 (sucrose 100 g, glucose 10 g, casamino acids 0.1 g, yeast extract 5 g, MOPS 21 g, trace elements (FeSO₄ 1 g, MnCl₂ 1 g, ZnSO₄ 1 g, in 1 L distilled H₂O) 1 mL, K₂SO₄ 0.25 g, MgCl₂·6H₂O 10 g, in 1.0 L distilled H₂O, pH 7.0) with 3% sea salt, and medium M4 (glycerol 20 g, malt extract 4 g, yeast extract 4 g, NZ Amine A 2 g, trace elements 1 mL, in 1.0 L distilled H₂O, pH 7.0) with 3% sea salt. The fermentation continued at 28 °C, 200 rpm for seven days.
The Amberlite XAD-16 (2 g/50 mL) resins were added into each of the fermentation flasks 12 h before the fermentation finished. The resins and cells were harvested by centrifugation, washed with distilled H₂O and extracted with 50 mL of MeOH at 28 °C, 200 rpm for 3 h. Then, the extract was concentrated and dissolved in 2 mL of MeOH/H₂O (1/1, v/v). Ten μL of each concentrated extract was used for HPLC analysis, using an elution program of 5% CH₃CN in H₂O (0.1% formic acid) from 0 to 5 min; 5% CH₃CN in H₂O (0.1% formic acid) to 100% CH₃CN from 5 to 23 min; 100% CH₃CN from 23 to 28 min, with a flow rate of 1 mL/min under the UV detection at 280 nm. No stlassin (I) was detected in these fermentation.

**Construction of the stla expression plasmid**

All procedures were performed according to those reported previously.⁵ ⁷ Ten micrograms of genomic DNA digested with BglII was used for the RecET-mediated direct cloning. The p15A vector used to clone the 20.3-kb fragment released with BglII digestion was amplified with pMM2001-F and pMM2001-R as the primers (Table S2) using p15A-cm-tetR-tetO-hyg-cccB as the template. Then the direct cloning procedure was conducted following previous publication,⁷ and the subsequent recombinant p15A plasmid (pMM2001) containing the stla gene cluster was extracted from colonies selected on LB plates containing chloramphenicol and verified with ApaLI restriction analysis (Figure S2). To construct the E. coli-Streptomyces shuttle vector pMM2002 for integration into heterologous hosts, 200 ng of apra-oriT-attP-phiC31 cassette was electroporated into GB05RedTrfA-containing pMM2001, which had been treated with l-rhamnose to induce expression of Redαβ.⁷ The pMM2002 plasmid was extracted from colonies selected on LB plates containing apramycin and verified with ApaLI restriction analysis (Figure S2).

**Gene activation by the insertion of promoter KasOp* at the upstream of stlaA**

The KasOp* promoter and 40-bp homology arms for Redαβ recombineering was attached to ampicillin resistance gene by overlap extension PCR using primers listed in Table S2. In the first round PCR, the ampicillin-resistance gene Amp was amplified with pET32a(+) as the template using the primers pMM2003-1-F and pMM2003-1-R (Table S2), both of which had been introduced with a AclI-recognition sequence. The PCR products was purified with the FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech Co., Ltd.) after agarose gel electrophoresis and used as the template for the second round PCR. The second round PCR products were amplified with primers pMM2003-2-F and pMM2003-2-R, and then electroporated into the GB05RedTrfA-containing pMM2002, which had been treated with l-rhamnose to induce expression of Redαβ. The correct recombinant plasmid pMM2003 was extracted from colonies selected based on ampicillin resistance and verified with ApaLI restriction analysis. The plasmid pMM2003 was further digested by AclI and self-ligated by T4 DNA ligase to generate the KasOp* insertion plasmid pMM2004 (Figure S4).

**Site-directed mutagenesis of stlaA gene**

The heterologous expression plasmids with site-directed mutagenesis of stlaA were constructed as the following steps:

(i) Construction of the plasmid pMM2007 with the KasOp*-stlaA cassette deletion. To delete the KasOp*-stlaA cassette within pMM2004, the Amp gene flanked by AclI sites and 40-bp homologous arms for Redαβ recombineering was amplified by PCR with primers pMM2006-F and pMM2006-R (Table S2). Then, the PCR product was purified and used to replace the KasOp*-stlaA region in pMM2004 by Redαβ
recombineering following standard protocols mentioned above, to generate the plasmid pMM2006 (Figure S7). The plasmid pMM2006 was identified with ApaLI restriction digestion analysis and further digested by AclI and self-ligated by T4 DNA ligase to generate the plasmid pMM2007.

(ii) Preparation of Amp-KasOp*-stlaA cassette bearing mutations for Redaβ recombineering. To perform the site-directed mutagenesis of stlaA gene via PCR, plasmid pMM2005 carrying Amp-KasOp*-stlaA cassette was constructed. The p15A-cm and Amp-KasOp*-stlaA cassettes were amplified by PCR from plasmids p15A-cm-tetR-tetO-hyg-ccdB and pMM2003, respectively, using primers listed in Table S2. The two fragments were ligated with the Gibson assembly method and then transformed into E. coli DH5α to yield the plasmid pMM2005. Then, site-directed mutagenesis was performed by PCR amplification using two overlapping primers (Table S2) containing the mutation sites with pMM2005 as the template. The resulting mixtures containing the mutated plasmids were digested with DpnI for 1 h at 37 °C to remove residual template DNA. After transforming into E. coli DH5α, the mutated plasmids were individually prepared from transformants and confirmed by DNA sequencing. Using these mutated plasmids as the templates, the Amp-KasOp*-stlaA cassette bearing different mutations for recombineering were individually amplified by PCR, using the same primers stla-2-F and stla-2-R.

(iii) Construction of the heterologous expression plasmids pMM2008-pMM2037 with mutated stlaA. The Amp-KasOp*-stlaA cassettes prepared from step ii were inserted into pMM2007 constructed in step i following standard protocols for Redaβ recombineering. Afterwards, to construct the heterologous expression plasmids pMM2008-pMM2037 with mutated stlaA, Amp gene was removed by AclI digestion and self-ligation by T4 DNA ligase. All the plasmids pMM2008-pMM2037 were identified with ApaLI restriction digestion and DNA sequencing.

**Heterologous expression, fermentation and HPLC analysis**

According to the standard procedure, all of the heterologous expression plasmids were first transformed into E. coli ET12567/pUZ8002 and then conjugated into heterologous hosts S. coelicolor A3(2) or S. lividans K4-114. Colonies of exconjugants (MM20001-MM20034) were selected for apramycin resistance and verified by PCR with stla-1-F and stla-1-R as the primers.

For detection of stlassin (I) and its variants, a two-stage culture procedure was used in the small-scale fermentations. The seed cultures of heterologous expression strains generated above were prepared by inoculating spores into 250 mL flasks containing 50 mL of medium M1, and incubated at 28.0 °C, 200 rpm for three days. The seed cultures were then inoculated (2% v/v) into 50 mL of medium M2, and the fermentation continued at 28 °C, 200 rpm for seven days. Another two production media, medium M3 and medium M4 were also used. The Amberlite XAD-16 (2 g/50 mL) resins were added into each of the fermentation flasks 12 h before the fermentation finished. The resins and cells were harvested by centrifugation, washed with distilled H₂O and extracted with 50 mL of methanol at 28 °C, 200 rpm for 3 h. Then, the extract was concentrated and dissolved in 2 mL of MeOH/H₂O (1/1, v/v). Ten μL of each concentrated extract was used for HPLC analysis, using an elution program of 5% CH₃CN in H₂O (0.1% formic acid) from 0 to 5 min; 5% CH₃CN in H₂O (0.1% formic acid) to 100% CH₃CN from 5 to 23 min; 100% CH₃CN from 23 to 28 min, with a flow rate of 1 mL/min under the UV detection at 280 nm. For detection of stlassin I4C (21), dithiothreitol (DTT, final concentration: 100 mM) was added to the concentrated extract samples and stirred at 25 °C overnight before HPLC analysis.

Production yields of stlassin (I) were calculated from the HPLC peak areas in the analytical HPLC based on a linear equation of peak area of purified I versus quantity. The relative production yields of derivatives from mutations were calculated based on the comparison of their peak areas to that of I. Triplicate
experiments were carried out for each production yields calculation.

**Large-scale fermentation and isolation of slassins**

To obtain enough slassin (1) for solution NMR structure determination, large-scale fermentation (5 L) of the strain MM20003 was carried out with similar procedures used in the small-scale fermentation. After the fermentation, the resins and cells were separated from supernatants by centrifugation, and extracted with 2 L of MeOH for three times at 28 °C, 200 rpm for 3 h. The MeOH extract was concentrated under reduced pressure to give a crude extract. The crude extract was resuspended in H₂O and extracted with EtOAc for three times. Since the EtOAc soluble fraction contains little amount of 1, only the water soluble fraction that contains most of 1 was subjected to MPLC eluted with a gradient of MeOH in H₂O (0% to 100% over 30 min) followed by 100% MeOH for 10 min at a flow rate of 8 mL/min under the UV detection at 210 nm, to give 22 fractions (a1-a22). The fractions a17-a20 were combined based on HPLC analysis and further purified by semi-preparative HPLC eluted with a gradient of CH₃CN in H₂O (0.1% formic acid) from 40% to 60% over 30 min with a flow rate of 2 mL/min under the UV detection at 210 nm, to afford compound 1 (51 mg).

Compounds 3, 4 and 21 were isolated using the similar procedures as that for 1, except that the purification of compound 4 by semi-preparative HPLC was carried out with an increasing gradient of CH₃CN in H₂O (0.1% formic acid) from 40% to 55% over 30 min; and for the isolation of compound 21, dithiothreitol (DTT, final concentration: 100 nM) was added to the combined MPLC fractions and stirred at 25 °C overnight. Totally 26 mg of 3, 20 mg of 4 and 30 mg of 21 were isolated from 5 L fermentation of MM20007, 4 L fermentation of MM20008 and 12 L fermentation of MM20034, respectively.

The isolation of compounds 14, 16, 18 and 19 was carried out using similar procedures as that for 1 with changes listed below. Firstly, after the crude extracts were resuspended in H₂O and extracted with EtOAc, the EtOAc extracts, instead of the water soluble fractions, were used for further purification. Secondly, the elution programs used for compound 16 purification by semi-preparative HPLC was the same as that for 4; the purification of compound 14 by semi-preparative HPLC was carried out with an increasing gradient of CH₃CN in H₂O (0.1% formic acid) from 44% to 62% over 30 min; and the purification of compounds 18 and 19 by semi-preparative HPLC was carried out with 43% CH₃CN in H₂O (0.1% formic acid) over 30 min. Finally, 18 mg of 14, 27 mg of 16, 33 mg of 18 and 15 mg of 19 were isolated from 6 L fermentation of MM20024, 16 L fermentation of MM20028, 6 L fermentation of MM20031 and 30 L fermentation of MM20032, respectively.

For biological activity assays, more derivatives were isolated from large-scale fermentations of mutants, which were carried out with similar procedures used in that of MM20003. After the fermentation, the treatments with MeOH and EtOAc were similar as those in the isolation of 1. L1A (10), V2A (2), V5A (5), Q6A (6) and A7G (11) were isolated using the similar MPLC procedures as that for 1, while N10A (7), A11G (8), P12A (9), W9F (12) and W14Y (15) were isolated using the similar MPLC procedures as that for 14. The purification of compounds 2, 5 and 10 by semi-preparative HPLC were carried out with an increasing gradient of CH₃CN in H₂O (0.1% formic acid) from 40% to 55% over 30 min. Totally 25 mg of 2, 18 mg of 5 and 1.8 mg of 10 were isolated from 5.2 L fermentation of MM20006, 5.2 L fermentation of MM2009 and 6 L fermentation of MM2005, respectively. The purification of compounds 6, 7, 9 and 11 by semi-preparative HPLC were carried out with an increasing gradient of CH₃CN in H₂O (0.1% formic acid) from 45% to 65% over 30 min. Totally 20 mg of 6, 10 mg of 7, 8.6 mg of 9 and 2.4 mg of 11 were isolated from 4.8 L fermentation of MM20010, 4.4 L fermentation of MM20017, 5.6 L fermentation of MM20019 and 5.2 L fermentation of MM20011, respectively. The purification of compounds 8, 12 and 15...
by semi-preparative HPLC were carried out with an increasing gradient of CH$_3$CN in H$_2$O (0.1% formic acid) from 40% to 60% over 30 min. Totally 21 mg of 8, 10 mg of 12 and 16 mg of 15 were isolated from 4 L fermentation of MM20018, 6 L fermentation of MM20015 and 4 L fermentation of MM20025 respectively.

F15Y-I (20) was generated by site-selective glycosylation reaction of 16. The purification of compounds 20 by semi-preparative HPLC was carried out with an increasing gradient of CH$_3$CN in H$_2$O (0.1% formic acid) from 36% to 55% over 30 min. 14C-III (24) was generated by site-selective S-arylation reaction of 21. The purification of compounds 24 by semi-preparative HPLC was carried out with an increasing gradient of CH$_3$CN in H$_2$O (0.1% formic acid) from 52% to 67% over 30 min. Finally, 5.3 mg of 20 and 6.5 mg of 24 were isolated respectively.

**Solution NMR structures determination**

Compounds 1, 3, 4, 14 and 16 (5 mg for each compound) were dissolved in 500 μL of 20% DMSO-$_d_{6}$, 80% H$_2$O, 100 mM NaCl, and compound 18 (8 mg) was dissolved in 500 μL of 100% DMSO-$_d_{6}$ for the NMR experiments. All NMR spectra were acquired at 298 K on an Agilent DD2 or a Bruker Avance-600 spectrometer equipped with cold probes. The DMSO-$_d_{6}$ in the samples served as the deuterium lock solvent. For 1, 3 and 4, TOCSY, NOESY, $^{1}$H-$^{15}$C HSQC and $^{1}$H-$^{13}$N HSQC spectra were collected. For 14, 16 and 18, TOCSY, NOESY, $^{1}$H-$^{13}$C HSQC spectra were collected. All the TOCSY experiments were carried out using a 70 ms DIPSI-2 spin-lock pulse with a field strength of 6000 Hz. The mixing time for the NOESY experiments were 300 ms. The spectra widths were 8445.9*8445.9 Hz for the $^{1}$H-$^{1}$H, 8445.9*21124.9 Hz for the $^{1}$H-$^{15}$C and 8445.9*1450 Hz for the $^{1}$H-$^{13}$N correlation spectra, and 200, 128 and 32 complex points were collected for the indirect proton, carbon and nitrogen dimension respectively, while 512 complex points were collected for the directly observing proton dimension. The chemical shifts for the samples in 20% DMSO-$_d_{6}$ were referenced to the external DSS at 0.00 ppm for the proton and indirectly for the carbon and nitrogen dimension, and those for the sample in 100% DMSO-$_d_{6}$ were referenced to the internal TMS at 0.00 ppm. All NMR data were processed using the NMRPipe and analyzed with CcpNmr program suite. Signal assignment was obtained by following the method developed by Wuthrich and inter-proton distance restraints were derived from the NOESY spectra. Initial structure calculation, NOE peak refinement and assignment were performed simultaneously by using the program CNS and ARIA2. Then the structure calculation was carried out using Xplor-NIH with distance constraints derived from ARIA2. Patched topology and parameter files were used for creating the isopeptide bond between the amino group of Leu1 and the carbonyl group of the Asp8 side chain. A total of 100 structures were calculated and the 20 structures with the lowest total energy were selected to perform a refinement procedure in water. The structures of 1, 3, 4, 14, 16 and 18 have been deposited in Protein Data Bank with the PDB IDs of 7BZA, 7BZ8, 7BZ9, 6M19, 7BZ7, and 7CU6, respectively. The chemical shift assignments of 1, 3, 4, 14, 16 and 18 have been deposited in the Biological Magnetic Resonance Data Bank with the accession numbers of 36351, 36349, 36350, 36317, 36348, and 36372 (http://www.bmrbr.wisc.edu), respectively. The structures were displayed and analyzed with PyMOL (http://www.pymol.org/).

**Thermal stability assays and carboxypeptidase Y-treatment experiments**

The thermal stability of 1, 3, 4, 14, 16 and 18 was tested by heating the compound in a sealed tube containing 50 μL of 50% CH$_3$CN in H$_2$O for 3 h at 55 °C, 75 °C and 95 °C. Each compound was separately tested at the three temperatures followed by immediate cooling to room temperature. After the heating experiments, the samples were subjected to HPLC analysis with the program of 5% CH$_3$CN in H$_2$O (0.1%
formic acid) from 0 to 5 min; 5% CH$_3$CN in H$_2$O (0.1% formic acid) to 100% CH$_3$CN from 5 to 23 min; 100% CH$_3$CN from 23 to 28 min, with a flow rate of 1 mL/min under the UV detection at 280 nm. The incubation of stlassin V3A (3) at 95 °C for 3 h generated compounds A and B with the same molecular weight as that of 3 based on HPLC and HRESIMS analysis (Figure S6B and S6C). Compounds A and B were purified by semi-preparative HPLC from repeated heating experiments at 95 °C, and treated with carboxypeptidase Y individually. Compound B cannot be hydrolyzed by carboxypeptidase Y, suggesting it is the threaded 3. Compound A was hydrolyzed to compound C, which was identified as stlassin V3A-ΔC$_6$ that lost the C-terminal six residues Asn10-Phe15 based on HRESIMS analysis. Thus, compound A was identified as the unthreaded 3.

For carboxypeptidase Y-treatment experiments, the tested compound was dissolved in 50 μL of 50 mM sodium acetate buffer (pH 6.0), and 0.05 U carboxypeptidase Y (0.05 U/μL) was added to the solution. The reaction was carried out at 28 °C for 12 h and analyzed by HPLC with the same program as that for the thermal stability assays.

Site-selective glycosylation of stlassin F15Y (16) and stlassin I4C (21).

To prepare the glycosyl donor α-D-fluoroglucose, deacetylation of α-D-Fluoroglucose tetraacetate was performed with NaOMe as previously described. For the glycosylation of lasso peptides 16 and 21, a 300 μL of reaction system containing 16 or 21 (final concentration: 1 mM, 1.0 equiv.), α-D-Fluoroglucose (final concentration: 1 M, 1000.0 equiv.) and Ca(OH)$_2$ (final concentration: 1 M, 1000.0 equiv.) in H$_2$O was prepared. The mixture was stirred vigorously at 25 °C for 1 h, and the reaction was quenched with 900 μL of EDTA solution (0.5 M, pH 8.0), and centrifuged at 13,000 rpm for 10 min. The supernatant was analyzed by HPLC with the program of 5% CH$_3$CN in H$_2$O (0.1% formic acid) from 0 to 5 min; 5% CH$_3$CN in H$_2$O (0.1% formic acid) to 100% CH$_3$CN from 5 to 23 min; 100% CH$_3$CN from 23 to 28 min, with a flow rate of 1 mL/min under the UV detection at 280 nm.

Site-selective S-arylation of 21

The site-selective S-arylation of 21 was carried out according to the published procedures. A 1 mL of reaction system containing 21 (0.3 mM, 1.0 equiv.), 2-nitrophenylboronic acid (3 mM, 10.0 equiv.), and Ni(OAc)$_2$·4H$_2$O (1.5 mM, 5.0 equiv.) in 10 mM N-methylmorpholine (NMM) buffer (pH 7.5) was prepared. The mixture was stirred vigorously at 25 °C for 30 min, quenched with 120 μL of EDTA solution (0.5 M, pH 8.0), and centrifuged at 13,000 rpm for 10 min. The supernatant was analyzed by HPLC with the same program as that for glycosylation products.

Bis-alkylation–elimination reaction of 21

The bis-alkylation-elimination reaction of 21 was performed with α,α'-dibromo-adipyl(bis)amide as previously described. A 100 μL of reaction system containing 21 (2.0 mM, 1.0 equiv.), K$_2$CO$_3$ (10.0 mM, 5.0 equiv.) and α,α'-dibromo-adipyl(bis)amide (10.0 mM, 5.0 equiv.) in DMF was prepared. The mixture was stirred vigorously at room temperature for 30 min and then stirred for 4 h at 37 °C. After the reaction, the mixture was concentrated and dissolved in 1 mL of MeOH/H$_2$O (1/1, v/v). After centrifuged at 13,000 rpm for 10 min, the supernatant was analyzed by HPLC with the same program as that for glycosylation products.

Conjugate addition of 2-mercaptoethanol and N-acetylcysteamine

For conjugate addition of 2-mercaptoethanol, a 100 μL system containing 25 (0.12 μM, 1.0 equiv.),
sodium methoxide (12 μM, 100 equiv.) and 2-mercaptoethanol (0.6 mM, 5000 equiv.) in MeOH was prepared. The mixture were stirred at 37 °C for 2 h, and 21 was used instead of 25 for a negative control. Conjugate addition of N-acetylcysteamine was performed with the same method as that for 2-mercaptoethanol. The HPLC analysis was performed with the same program as that for glycosylation products.

**Biological activity assays of stlassins**

To evaluate the biological activity of 1, we first carried out the antibacterial and cytotoxicity assays. However, compound 1 showed no antibacterial activities (MIC > 32 μg/mL) against Gram-positive *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6051, vancomycin resistant *Enterococcus faecalis* 1010798, Gram-negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* 14, and *Klebsiella pneumoniae* WNX-1. This compound also showed no cytotoxicity (10 μM) against human breast adenocarcinoma MCF-7, human ileocecal colorectal adenocarcinoma HCT-8, non-small cell lung carcinoma A549 and human hepatocellular carcinoma SMMC-7721 cell lines.

Then, the antagonistic activities of stlassins against the binding of LPS to TLR4 were assayed by ELISA. NCM460 (human colonic epithelial cell line) were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (fetal bovine serum, Gibco, Grand Island, NY, USA) and penicillin-streptomycin (final concentration: 100 U/mL) (Gibco, Grand Island, NY, USA,) at 37 °C in a 5% CO2 incubator, and were allowed to reach approximately 90% confluence. The cells were washed with PBS (phosphate buffered saline) and lysed with RIPA (radio immunoprecipitation assay, Sangon Biotech Co., Ltd, Shanghai, China) kit for 30 min at 4 °C. After that, the lysates were centrifuged to obtain the supernatant, which contains the NCM460 proteins including TLR4.

Mouse anti-TLR4 antibody (Sangon Biotech Co., Ltd, Shanghai, China) (2.5 μg/well) was coated onto PVC (polyvinyl chloride) plates at 4 °C overnight and washed with TBST (Tris buffered saline containing 0.05% Tween-20, Solarbio Science & Technology Co., Ltd., Beijing, China). After blocking with TBST containing 5% powdered milk (200 μL/well) for 1 h at room temperature, the plates were washed with TBST. Then NCM460 proteins (final concentration: 100 μg/well) were added to the plates and allowed to incubate for 2 h at 37 °C. The plates were washed with TBST for three times. Another round of blocking with TBST containing 5% powdered milk was carried out. To test how much amounts of TLR4 proteins have been absorbed to the PVC plates, the HRP (horseradish peroxidase)-labeled rabbit anti-mouse TLR4 antibody (Sangon Biotech Co., Ltd, Shanghai, China) solution was added (200 μL/well) and incubated for 1 h at 37 °C. Then the plates were washed with TBST for three times, and reacted with TMB (3,3′,5,5′-tetramethylbenzidine, Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature in the dark for 30 min. The reaction was quenched with the addition of 10% sulfuric acid. Optical density (OD) values were measured at 450 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA). Determining the amount of TLR4 by such ELISA method has been commonly used in literature, and similar ELISA kit and manual are commercial available (such as Elabscience online manual at https://sceti.co.jp/images/psearch/pdf/WEB_E-EL-H1539_p.pdf). The amount of TLR4 proteins that have been absorbed to the PVC plates was calculated as 0.266 ± 0.013 ng/well, based on the linear equation (Y = 0.2496X + 0.0882) of OD values versus standard TLR4 concentrations (Shanghai FANKEWEI biotechnology) (Note: the linear equation was generated by adding standard TLR4 proteins with different concentrations including 0.16, 0.32, 0.63, 1.25, 2.5, 5.0, and 10 ng/mL to the mouse anti-human TLR4 antibody-coated PVC plates).

To test the antagonistic activities of stlassins against the binding of LPS to TLR4, the NCM460 proteins
(final concentration: 100 μg/well) were added to the PVC plates as described above. Then biotin-labeled LPS (InvivoGen, CA, USA) with DMSO (negative control), TAK-242 (positive control, final concentration: 1 μg/mL, MedChemexpress, NJ, USA) or stlassins (final concentration: 1 μg/mL) were added to the plates. The plates were incubated for 2 h at 37 °C, washed with TBST and incubated with HRP-labeled streptavidin for another 1 h at 37 °C. The plates were washed with TBST for three times and reacted with TMB at room temperature in the dark for 30 min. The reaction was quenched with the addition of 10% sulfuric acid. Optical density (OD) values were measured at 450 nm with four parallel experiments (Table S11) for each sample. The statistical significances (versus DMSO treatments) were generated by SPSS Statistics 25.0 (IBM, NY, USA) and GraphPad Prism 7.0 (GraphPad Software, CA, USA) using one-way ANOVA with Dunnett's test.

In above tests, we also set several control experiments N1–N4: compared to the complete ELISA procedures described above, N1 only omits the mouse anti-human TLR4 antibody in the coating step; N2 only omits the addition of the NCM460 proteins; N3 uses biotin instead of biotin-labeled LPS; and N4 uses LPS instead of biotin-labeled LPS. All N1–N4 gave only background OD values compared to the large OD values in the DMSO control experiments (Figure S14), supporting the reasonable procedures for determining TLR4-LPS binding in our ELISA experiments.

To test the antagonistic activities of stlassin and variants I4A, I4C, I4C-III, F15Y, and F15Y-I at different concentrations, we carried out more ELISA experiments by adding these compounds in different final concentrations (0.01, 0.05, 0.5, 2, 10, and 100 μM). The OD values, inhibition ratio values (inhibition ratio = (OD_{DMSO} - OD_{compound})/OD_{DMSO} × 100%) and error bars were calculated as mean ± standard deviation from three parallel experiments, showing increased antagonistic activities along with increased concentrations (Figure S15). The IC_{50} values were determined based on the dose-response curves using GraphPad Prism 7.0.
Table S1. The deduced functions of genes in the *stla* gene cluster.

| Genes | Numbers of amino acids | Proposed functions | Accession numbers of closest homologues | Identities with homologues |
|-------|------------------------|--------------------|----------------------------------------|---------------------------|
| Orf-13 | 278                    | hypothetical protein | ACU71420.1                             | 28.89%                    |
| Orf-12 | 172                    | hypothetical protein | KPL33761.1                             | 97.06%                    |
| Orf-11 | 252                    | SDR family oxidoreductase | WP_098891688.1 | 99.60%                    |
| Orf-10 | 357                    | ABC transporter ATP-binding protein | WP_073746171.1 | 98.88%                    |
| Orf-9  | 280                    | ABC transporter permease | KPL33696.1                             | 99.64%                    |
| Orf-8  | 330                    | ABC transporter permease | WP_073960779.1 | 97.06%                    |
| Orf-7  | 382                    | extracellular solute-binding protein | WP_057663678.1 | 99.48%                    |
| Orf-6  | 246                    | GntR family transcriptional regulator | WP_073746179.1 | 99.19%                    |
| Orf-5  | 215                    | HAD family phosphatase | WP_073746181.1 | 99.07%                    |
| Orf-4  | 203                    | dihydrofolate reductase family protein | WP_073805183.1 | 98.52%                    |
| Orf-3  | 397                    | RtcB family protein | WP_073746185.1 | 99.24%                    |
| Orf-2  | 290                    | hypothetical protein | WP_073960781.1 | 96.92%                    |
| Orf-1  | 286                    | hypothetical protein | WP_050358740.1 | 98.26%                    |
| stlaA  | 37                     | lasso RiPP family leader peptide-containing protein | WP_157420307.1 | 73.68%                    |
| stlaC  | 602                    | asparagine synthetase | EHM23871.1                             | 98.84%                    |
| stlaB1 | 85                     | lasso peptide biosynthesis PqqD family chaperone | WP_069813828.1 | 67.06%                    |
| stlaB2 | 191                    | lasso peptide biosynthesis B2 protein | WP_007460371.1 | 93.19%                    |
| Orf1   | 228                    | hypothetical protein | WP_097967018.1 | 98.25%                    |
| Orf2   | 495                    | membrane protein | KND34369.1                             | 97.05%                    |
| Orf3   | 603                    | lysine-tRNA ligase | WP_179890838.1 | 98.98%                    |
| Orf4   | 590                    | arginine--tRNA ligase | WP_098893103.1 | 98.31%                    |
Table S2. Primers used in this study.

| Primer | Sequence (5'→3') |
|--------|------------------|
| **For direct cloning (sequences underlined indicate the upstream and downstream homologic sequences of the *stla* gene cluster)** |
| pMM2001-F | GGGACCGGGTGTTGCGGCCCTCCAGGCAAGCCCCAGACGCTGAGG<br>CTGGGCACCCCTTGCTGACGAGATCTCAGATCCGAAAACCC<br>AAAGTGACGATCCTGAG<br> |
| pMM2001-R | GGGCCCGAGAGCAGACGCTGACGAGATCTCAGTCCTTGGGG<br>CGGTGCTGCTAGATCTACGCCGCCCAGATCTCCTTCTCTTT<br>TAGATCTCTTGAATTC |
| **For PCR confirmation of conjugation** |
| stla-1-F | GAACGCTCCGGCTGTTATAGTAAC<br> |
| stla-1-R | GTTGACGCGCCAGACTCTCCGGCTCTTG |
| **For promoter *KasO* insertion (sequences underlined indicate the upstream and downstream homologic sequences for Redαβ recombineering, and the bold sequences indicate the AclI recognition sites)** |
| pMM2003-1-F | CCCCCAAGTCGACGACGACGCAAGCAGCCGAAAGCAAGCTCAGCTGAAACGGTTTC<br>GCGGAACCCCTATTGTGTTTATTTTTC<br> |
| pMM2003-1-R | CACCAGCTTACACACCGACACGATCTGTCGCTCAAACGAGACGCGGTTTC<br>GAAATGACAAAGAGGGTTTTTCAATCTTACAGTGAGCACC<br> |
| pMM2003-2-F | CCCCCAAGTCGACGACGACGCAAGCAGCAGACG<br> |
| pMM2003-2-R | CAGCGGTGAACTCAGGGGTCGTAAGAAAGGCTTCTTCTACATGGACACT<br>CCTTACTTACGCTGCATTTCTCTGCCGACTTTACACAACACCGAC<br>AG |
| **For PCR confirmation of *KasO* insertion and Amp-*KasO*-*stla*A cassette preparation for Redαβ recombineering** |
| stla-2-F | GTCGACGACGACGACGACG<br> |
| stla-2-R | GAAGGATGCTGACGACTGCCTACAGAC |
| **For construction of plasmid pMM2005 (sequences underlined indicate the homologic sequences for Gibson assembly)** |
| pMM2005-1-F | GATATCGACGTCGACGACGACGACGACGACGACGACG<br> |
| pMM2005-1-R | CCGATCTGAAAGGATTGACGACGACGACGACGACG<br> |
| pMM2005-2-F | GCACATCCTTACGACGACGACGACGACGACG<br> |
| pMM2005-2-R | CGTCGACGACGTCGACGACGACGACGACGACG<br> |
| **For *KasO*-*stla*A cassette deletion (sequences underlined indicate the upstream and downstream homologic sequences of the *KasO*-*stla*A cassette, and the bold sequences indicate the AclI recognition sites)** |
| pMM2006-F | GTCGACGACGACGACGACGACGACGACGACGACG<br>CCCCCTATTTGTTTATTTTTC<br> |
| pMM2006-R | GAAAGGATGTTGACTCAGTCGACGACGACGACGACG<br>ACCAATGCTTAACTCAGTGACGAGC |
| **For site mutation (the bold codes indicate the mutation sites)** |
| L1A-F | AAGACCGGCACGTCGACGTCG |

S14
G13A-R  ACTGAACCAAGCGGGAGCGTTCC
G13S-R  CTCCCAAGCTGGTTCTAGTAACTCGATC
G13S-R  ACTGAACCAAGCTGGGAGCGTTCC
W14A-F  CCGGCGCCTTCTAGTAACTCGATCAG
W14A-R  GTTACTAGAAGCAGCCGGGAGCGTTCC
W14F-F  CCGGCTTTCTTCTAGTAACTCGATCAGCAGAG
W14F-R  GTTACTAGAAGCAGCCGGGAGCGTTCCAGTC
W14Y-F  CCGGCTACTTCTAGTAACTCGATCAGCGAG
W14Y-R  GTTACTAGAAGTACCCGGGAGCGTTCCAGTC
W14R-F  CCGGCCTCCCTTAGTAACTCGATCAGCGGAG
W14R-R  GTTACTAGAAGCAGCCGGGAGCGTTCCAGGAG
F15A-F  CCGCTGGGCTAGTAACTCGATCAG
F15A-R  AGTTACTAGGGCCAGCCGGGAGCG
F15Y-F  CCGCTGGTACTAGTAACTCGATCAGCAGGAGGAG
F15Y-R  GAGTTACTAGTACCAGCCGGGAGCGTTCCAG
F15W-F  CCGCTGGTTACTAGTAACTCGATCAGCAGGAG
F15W-R  GAGTTACTAGCACCAGCCGGGAGCGTTCC
F15R-F  CCGCTGGGCTAGTAACTCGATCAGCAGGAG
F15R-R  GAGTTACTAGGCCAGCCGGGAGCGTTCC
| Name | Description | References |
|------|-------------|------------|
| **Plasmids** | | |
| p15A-cm-tet<sup>8</sup>-tet<sup>0</sup>-hyg-ccdB | PCR template to generate a linear vector for direct cloning | 7 |
| pSC101-BAD-ETgA-tet | recET expression plasmid for linear plus linear homologous recombination | 22 |
| pR6K-oriT-phiC31 | plasmid containing the apra-oriT-attP-int cassette | 7 |
| pMM2001 | p15A-cm-tet<sup>8</sup>-tet<sup>0</sup>-hyg-ccdB-derived plasmid carrying the stla gene cluster by direct cloning | This study |
| pMM2002 | pMM2001-derived plasmid with chloramphenicol-resistance gene replaced by apra-oriT-attP-int cassette | This study |
| pMM2003 | pMM2002-derived plasmid with Amp-KasOp<sup>*</sup> cassette cloned into the upstream of the stlaA | This study |
| pMM2004 | pMM2003-derived plasmid with the ampicillin-resistance gene removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2005 | Plasmid used as a template for site-directed mutagenesis and constructed by Gibson assembly with p15A-cm cassette derived from p15A-cm-tet<sup>8</sup>-tet<sup>0</sup>-hyg-ccdB and Amp-KasOp<sup>*</sup>-stlaA cassette derived from pMM2003 | This study |
| pMM2006 | pMM2004-derived plasmid with KasOp<sup>*</sup>-stlaA cassette replaced by ampicillin-resistance gene flanked with two AciI recognition sites | This study |
| pMM2007 | pMM2006-derived plasmid with the ampicillin-resistance gene removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2008 | pMM2007-derived plasmid with Amp-KasOp<sup>*</sup>-stlaA cassette bearing mutation of L1A inserted upstream of stlaC and then the Amp removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2009 | pMM2007-derived plasmid with Amp-KasOp<sup>*</sup>-stlaA cassette bearing mutation of V2A inserted upstream of stlaC and then the Amp removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2010 | pMM2007-derived plasmid with Amp-KasOp<sup>*</sup>-stlaA cassette bearing mutation of V3A inserted upstream of stlaC and then the Amp removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2011 | pMM2007-derived plasmid with Amp-KasOp<sup>*</sup>-stlaA cassette bearing mutation of I4A inserted upstream of stlaC and then the Amp removed by AciI digestion and self-ligated by T4 ligase | This study |
| pMM2012 | pMM2007-derived plasmid with Amp-KasOp<sup>*</sup>-stlaA cassette bearing mutation of V5A inserted upstream of stlaC and then the Amp removed by AciI digestion and self-ligated by T4 ligase | This study |
This study

pMM2013

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of Q6A inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2014

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of A7G inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2015

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of D8E inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2016

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W9A inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2017

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W9R inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2018

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W9F inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2019

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W9Y inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2020

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of N10A inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase

pMM2021

pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of A11G inserted upstream of stlaC and then the Amp removed by AclI digestion and self-ligated by T4 ligase
pMM2024  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of G13S inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2025  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W14A inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2026  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W14R inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2027  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W14F inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2028  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of W14Y inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2029  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of F15A inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2030  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of F15R inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2031  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of F15Y inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2032  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of F15W inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2033  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing double mutation of L1C/A11C inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study

pMM2034  pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing double mutation of V2C/A11C inserted upstream of stlaC and then the Amp removed by AcII digestion and self-ligated by T4 ligase  This study
pMM2035: pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing double mutation of V3C/P12C inserted upstream of stlaC and then the Amp removed by AcI digestion and self-ligated by T4 ligase. This study.

pMM2036: pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing double mutation of A7C/P12C inserted upstream of stlaC and then the Amp removed by AcI digestion and self-ligated by T4 ligase. This study.

pMM2037: pMM2007-derived plasmid with Amp-KasOp*-stlaA cassette bearing mutation of I4C inserted upstream of stlaC and then the Amp removed by AcI digestion and self-ligated by T4 ligase. This study.

**E. coli strains**

GB2005: Host strain of general clone. 7

GB05RedTrfA: Host strain containing the Redαβ system for linear plus circular homologous recombination. 7

ET12567/pUZ8002: Donor strain for conjugation. 23

**Streptomyces strains**

S. sp. PKU-MA01240: Wild-type strain bearing gene cluster stla. This study.

S. coelicolor A3(2): Recipient strain for heterologous expression. 24

S. lividans K4-114: Recipient strain for heterologous expression. 25

MM20001: *S. coelicolor* A3(2) with the plasmid pMM2002 integrated. This study.

MM20002: *S. lividans* K4-114 with the plasmid pMM2002 integrated. This study.

MM20003: *S. coelicolor* A3(2) with the plasmid pMM2004 integrated. This study.

MM20004: *S. lividans* K4-114 with the plasmid pMM2004 integrated. This study.

MM20005: *S. coelicolor* A3(2) with the plasmid pMM2008 integrated. This study.

MM20006: *S. coelicolor* A3(2) with the plasmid pMM2009 integrated. This study.

MM20007: *S. coelicolor* A3(2) with the plasmid pMM2010 integrated. This study.

MM20008: *S. coelicolor* A3(2) with the plasmid pMM2011 integrated. This study.

MM20009: *S. coelicolor* A3(2) with the plasmid pMM2012 integrated. This study.

MM20010: *S. coelicolor* A3(2) with the plasmid pMM2013 integrated. This study.

MM20011: *S. coelicolor* A3(2) with the plasmid pMM2014 integrated. This study.

MM20012: *S. coelicolor* A3(2) with the plasmid pMM2015 integrated. This study.

MM20013: *S. coelicolor* A3(2) with the plasmid pMM2016 integrated. This study.

MM20014: *S. coelicolor* A3(2) with the plasmid pMM2017 integrated. This study.

MM20015: *S. coelicolor* A3(2) with the plasmid pMM2018 integrated. This study.

MM20016: *S. coelicolor* A3(2) with the plasmid pMM2019 integrated. This study.

MM20017: *S. coelicolor* A3(2) with the plasmid pMM2020 integrated. This study.

MM20018: *S. coelicolor* A3(2) with the plasmid pMM2021 integrated. This study.

MM20019: *S. coelicolor* A3(2) with the plasmid pMM2022 integrated. This study.

MM20020: *S. coelicolor* A3(2) with the plasmid pMM2023 integrated. This study.

MM20021: *S. coelicolor* A3(2) with the plasmid pMM2024 integrated. This study.

MM20022: *S. coelicolor* A3(2) with the plasmid pMM2025 integrated. This study.

MM20023: *S. coelicolor* A3(2) with the plasmid pMM2026 integrated. This study.
| MM20024 | *S. coelicolor* A3(2) with the plasmid pMM2027 integrated | This study |
| MM20025 | *S. coelicolor* A3(2) with the plasmid pMM2028 integrated | This study |
| MM20026 | *S. coelicolor* A3(2) with the plasmid pMM2029 integrated | This study |
| MM20027 | *S. coelicolor* A3(2) with the plasmid pMM2030 integrated | This study |
| MM20028 | *S. coelicolor* A3(2) with the plasmid pMM2031 integrated | This study |
| MM20029 | *S. coelicolor* A3(2) with the plasmid pMM2032 integrated | This study |
| MM20030 | *S. coelicolor* A3(2) with the plasmid pMM2033 integrated | This study |
| MM20031 | *S. coelicolor* A3(2) with the plasmid pMM2034 integrated | This study |
| MM20032 | *S. coelicolor* A3(2) with the plasmid pMM2035 integrated | This study |
| MM20033 | *S. coelicolor* A3(2) with the plasmid pMM2036 integrated | This study |
| MM20034 | *S. coelicolor* A3(2) with the plasmid pMM2037 integrated | This study |
**Table S4.** The HRESIMS data of compounds 1-25. For most compounds, the negative ion mode gave more clearer signals than the positive mode.

| Compounds       | Molecular formula | Calculated m/z for [M-H]^- | Determined m/z for [M-H]^- |
|-----------------|-------------------|-----------------------------|-----------------------------|
| stlassin (1)    | C_{62}H_{11}N_{19}O_{19} | 1694.8694                  | 1694.8662                   |
| stlassin V2A (2)| C_{62}H_{11}N_{19}O_{19} | 1666.8381                  | 1666.8405                   |
| stlassin V3A (3)| C_{62}H_{11}N_{19}O_{19} | 1666.8381                  | 1666.8388                   |
| stlassin I4A (4)| C_{32}H_{11}N_{19}O_{19} | 1652.8225                  | 1652.8242                   |
| stlassin V5A (5)| C_{62}H_{11}N_{19}O_{19} | 1666.8381                  | 1666.8333                   |
| stlassin Q6A (6)| C_{62}H_{11}N_{19}O_{19} | 1637.8480                  | 1637.8478                   |
| stlassin N10A (7)| C_{62}H_{11}N_{19}O_{19} | 1651.8636                  | 1651.8573                   |
| stlassin A11G (8)| C_{62}H_{11}N_{19}O_{19} | 1680.8538                  | 1680.8490                   |
| stlassin P12A (9)| C_{62}H_{11}N_{19}O_{19} | 1668.8538                  | 1668.8528                   |
| stlassin L1A (10)| C_{62}H_{11}N_{19}O_{19} | 1652.8225                  | 1652.8276                   |
| stlassin A7G (11)| C_{62}H_{11}N_{19}O_{19} | 1680.8538                  | 1680.8539                   |
| stlassin W9F (12)| C_{62}H_{11}N_{19}O_{19} | 1655.8585                  | 1655.8507                   |
| stlassin W9Y (13)| C_{62}H_{11}N_{19}O_{19} | 1671.8534                  | 1671.8504                   |
| stlassin W14F (14)| C_{62}H_{11}N_{19}O_{19} | 1655.8585                  | 1655.8571                   |
| stlassin W14Y (15)| C_{62}H_{11}N_{19}O_{19} | 1671.8534                  | 1671.8534                   |
| stlassin F15Y (16)| C_{62}H_{11}N_{19}O_{19} | 1710.8643                  | 1710.8608                   |
| stlassin F15W (17)| C_{62}H_{11}N_{19}O_{19} | 1733.8803                  | 1733.8746                   |
| stlassin I4C (21)| C_{32}H_{11}N_{19}O_{19} | 1684.7945                  | 1684.7978                   |
| stlassin I4C-I (22)| C_{62}H_{11}N_{19}O_{20} | 1846.8474                  | 1846.8480                   |
| stlassin I4C-III (24)| C_{62}H_{11}N_{19}O_{20} | 1805.8109                  | 1805.8148                   |
| stlassin I4C-IV (25)| C_{62}H_{11}N_{19}O_{19} | 1652.8225                  | 1652.8258                   |
| stlassin V2C/A11C (18)| C_{62}H_{11}N_{19}O_{19} | 863.8794                   | 863.8757                    |
| stlassin V3C/P12C (19)| C_{62}H_{11}N_{19}O_{19} | 850.8716                   | 850.8723                    |
| stlassin F15Y-I (20)| C_{62}H_{11}N_{19}O_{19} | 935.9547                   | 935.9551                    |
| stlassin I4C-II (23)| C_{62}H_{11}N_{19}O_{19} | 1683.7868                  | 1683.7888                   |
| stlassin D8E (18)| C_{62}H_{11}N_{19}O_{19} | 853.9386                   | 853.9380                    |
**Table S5.** Assignment of $^1$H, $^{13}$C and $^{15}$N signals (ppm) of stlassin (1). The signals were assigned based on TOCSY, NOESY, $^1$H-$^{13}$C HSQC and $^1$H-$^{15}$N HSQC spectra.

| Residue | Atom | $\delta$<sub>H</sub> | $\delta$<sub>C</sub>/N<sub>H</sub> | Residue | Atom | $\delta$<sub>H</sub> | $\delta$<sub>C</sub>/N<sub>H</sub> |
|---------|------|----------------|-----------------|---------|------|----------------|-----------------|
| Leu1    | NH   | 8.48           | 119.33          | Trp9    | NH   | 6.40           | 110.55          |
|         | α    | 4.45           | 55.15           | Nε      |       | 4.16           | 131.14          |
|         | β    | 1.62, 1.72     | 44.18           |        | α    | 6.40           | 129.89          |
|         | γ    | 1.45           | 27.39           | β       | 3.13, 3.22 | 29.00          |
|         | δ    | 0.76           | 23.53           | δ       | 7.28          |
|         |      | 0.98           | e               | ε       | 7.57           | 120.66          |
| Val2    | NH   | 8.55           | 125.88          |        |       | 10.55          |
|         | α    | 4.20           | 61.57           |         |        | 122.65         |
|         | β    | 1.78           | 29.76           |         |        | 115.34         |
|         | γ    | 0.77           | 22.13           |         |        | 125.24         |
|         |      | 1.12           | 25.73           | Asn10   | NH   | 7.43           | 113.38          |
| Val3    | NH   | 8.10           | 118.92          |         | α    | 4.83           |
|         | α    | 4.34           | 66.58           |         | β    | 2.32, 2.34    | 40.79          |
|         | β    | 2.12           | 36.23           |         | γ    | 7.63           | 27.58          |
|         | γ    | 0.93           | 22.21           |         |         | 125.73         |
|         |      | 0.93           | 22.21           |         |        | 20.78          |
|         |      | 1.12           | 25.73           |         |        | 1.38           |
| Ile4    | NH   | 9.71           | 123.36          | Pro12   | α    | 4.23           | 64.63          |
|         | α    | 4.44           | 61.42           |         | β    | 1.92, 2.37    | 32.78          |
|         | β    | 1.89           | 40.55           |         | γ    | 1.90, 2.03    | 27.58          |
|         | γ    | 1.10           | 18.35           |         | δ    | 3.65, 3.75    | 51.16          |
|         |      | 1.25, 1.63     | 27.59           | Gly13   | NH   | 6.81           | 101.05         |
|         |      | 0.91           | 13.26           |         | α    | 2.92, 4.82    | 44.33          |
| Val5    | NH   | 8.60           | 119.92          | Trp14   | NH   | 8.46           | 120.65         |
|         | α    | 3.42           | 66.88           |         | Nε   | 4.03           | 130.24         |
|         | β    | 3.48           | 29.28           |         | α    | 4.29           | 59.07          |
|         | γ    | 1.08           | 22.20           |         | β    | 2.17, 2.30    | 31.84          |
|         |      | 1.25           | 22.33           |         | δ    | 6.77           | 127.49         |
| Gln6    | NH   | 8.64           | 126.87          | ε       | 7.55           | 121.11         |
|         | Nε   | 112.03         |                 |         |       | 9.93           |
|         | α    | 4.69           | ζ                |         |       | 6.95           | 121.50         |
|         | β    | 2.04, 2.10     | 29.67           |         |       | 7.30           | 115.07         |
|         | γ    | 2.58, 2.61     | 34.20           |         | η    | 6.87           | 123.73         |
|         | ε    | 6.92, 7.69     |                 |         |       | 9.20           | 123.03         |
| Ala7    | NH   | 7.13           | 124.76          | Phe15   | NH   | 9.20           | 123.03         |
|         | α    | 3.86           | 53.81           |         | α    | 4.85           |
|         | β    | 1.34           | 24.13           |         | β    | 2.73, 3.46    | 42.35          |
| Asp8    | NH   | 5.63           |                 |         | β    | 7.36           |
|         | α    | 3.00           | 50.29           |         |       | 16.0, 2.18    | 38.12          |

**S23**
Table S6. Assignment of $^1$H, $^{13}$C and $^{15}$N signals (ppm) of stlassin V3A (3). The signals were assigned based on TOCSY, NOESY, $^1$H-$^{13}$C HSQC and $^1$H-$^{15}$N HSQC spectra.

| Residue | Atom | $\delta$$_H$ | $\delta$C/$\delta$N | Residue | Atom | $\delta$$_H$ | $\delta$C/$\delta$N |
|---------|------|--------------|-------------------|---------|------|--------------|-------------------|
| Leu1    | NH   | 8.48        |                   | Asp8    | NH   | 5.81        | 114.99            |
|         | $\alpha$ | 4.39        |                   |         | $\alpha$ | 3.08        | 50.23             |
|         | $\beta$ | 1.67        |                   |         | $\beta$ | 1.57, 2.10 |                   |
|         | 1.57  |             |                   | Trp9    | NH   | 6.51        | 111.54            |
|         | $\gamma$ | 1.43        |                   |         | $\alpha$ | 4.15        |                   |
|         | $\delta$ | 0.74        |                   |         | $\beta$ | 3.12, 3.18 |                   |
|         | 0.95  |             |                   |         | $\delta$ | 7.25        |                   |
| Val2    | NH   | 8.41        | 124.85            |         | $\epsilon$ | 10.48        |                   |
|         | $\alpha$ | 4.10        | 61.82             |         | $\zeta$ | 7.59        | 115.13            |
|         | $\beta$ | 1.67        | 29.80             | Asn10   | NH   | 7.45        | 113.89            |
|         | $\gamma$ | 0.76        |                   |         | $\alpha$ | 4.75        |                   |
|         | 1.01  |             |                   |         | $\beta$ | 2.30, 2.30 | 40.52             |
| Ala3    | NH   | 8.17        | 124.37            |         | $\delta$ | 6.52, 7.17 |                   |
|         | $\alpha$ | 4.60        |                   | Ala11   | NH   | 7.90        | 125.29            |
|         | $\beta$ | 1.45        | 23.10             |         | $\alpha$ | 4.64        |                   |
| Ile4    | NH   | 9.73        | 121.94            |         | $\beta$ | 1.32        |                   |
|         | $\alpha$ | 4.39        |                   | Pro12   | $\alpha$ | 4.21        |                   |
|         | $\beta$ | 1.89        |                   |         | $\beta$ | 1.88, 2.34 | 32.75             |
|         | 1.06  |             |                   |         | $\gamma$ | 1.90, 2.01 | 27.62             |
|         | 1.21, 1.55 |             |                   |         | $\delta$ | 3.64, 3.73 |                   |
|         | 1.06  |             |                   | Gly13   | NH   | 6.67        | 100.55            |
| Val5    | NH   | 8.63        | 120.53            |         | $\alpha$ | 2.93, 4.71 |                   |
|         | $\alpha$ | 3.42        | 66.93             | Trp14   | NH   | 8.45        |                   |
|         | $\beta$ | 3.46        |                   |         | $\alpha$ | 4.23        |                   |
|         | $\gamma$ | 1.24        |                   |         | $\beta$ | 2.16, 2.28 |                   |
|         | 1.07  |             |                   |         | $\delta$ | 6.69        | 127.39            |
| Gln6    | NH   | 8.66        | 126.69            |         | $\epsilon$ | 9.89        |                   |
|         | $\alpha$ | 4.62        |                   |         | $\zeta$ | 7.15        |                   |
|         | $\beta$ | 2.02, 2.05  | 29.62             | Phe15   | NH   | 9.24        | 123.01            |
|         | $\gamma$ | 2.57        | 34.11             |         | $\alpha$ | 4.83        |                   |
| Ala7    | NH   | 7.15        | 124.68            |         | $\beta$ | 2.75, 3.45 | 42.25             |
|         | $\alpha$ | 3.85        | 53.82             |         | $\delta$ | 7.36        | 132.54            |
|         | $\beta$ | 1.32        |                   |         |          |             |                   |
Table S7. Assignment of $^1$H, $^{13}$C and $^{15}$N signals (ppm) of stlassin I4A (4). The signals were assigned based on TOCSY, NOESY, $^1$H-$^{13}$C HSQC and $^1$H-$^{15}$N HSQC spectra.

| Residue | Atom | $\delta_H$ | $\delta_C/\delta_N$ | Residue | Atom | $\delta_H$ | $\delta_C/\delta_N$ |
|---------|------|-----------|-----------------|---------|------|-----------|-----------------|
| Leu1    | NH   | 8.12      | 120.61          | Trp9    | NH   | 7.70      |                  |
|         | $\alpha$ | 4.54   |                 |         | Ne   | 130.02    |                 |
|         | $\beta$ | 1.50, 1.66 | 44.81          |         | $\alpha$ | 4.39 | 59.72 |
|         | $\gamma$ | 1.44   | 27.35           | $\beta$ | 3.14, 3.31 | 29.03 |
|         | $\delta$ | 0.77   | 23.69           | $\delta$ | 7.25 |
|         |          | 0.87   | 25.27           | $\epsilon$ | 7.56 |
| Val2    | NH   | 7.91      | 120.04          |         |      |          | 10.24          |
|         | $\alpha$ | 4.31   | 60.95           | $\zeta$ | 7.12 | 122.16 |
|         | $\beta$ | 1.83   | 31.98           |         | 7.48 |
|         | $\gamma$ | 0.63   | 20.47           | $\eta$ | 7.22 |
|         |          | 0.93   | 23.15           | Asn10   | NH   | 7.88 |
| Val3    | NH   | 7.75      | 119.01          | Ala11   | NH   | 8.08      | 124.25          |
|         | $\alpha$ | 3.99   | 64.35           | $\beta$ | 2.23, 2.30 | 40.29 |
|         | $\beta$ | 2.05   | 33.44           | $\delta$ | 6.55, 7.12 |
|         | $\gamma$ | 0.89   | 21.42           | Ala11   | NH   | 8.08      | 124.25          |
|         |          | 0.90   | 21.37           | $\alpha$ | 4.68 |
| Ala4    | NH   | 7.09      |                  | Pro12   | $\alpha$ | 4.53 |
|         | $\alpha$ | 3.86   |                 |         |      |          | 4.53           |
|         | $\beta$ | 1.33   |                 |         | $\beta$ | 2.09, 2.20 | 31.24 |
| Val5    | $\alpha$ | 3.93   | 31.74           | $\gamma$ | 1.99, 1.99 | 27.76 |
|         | $\beta$ | 2.51   | 31.74           | $\delta$ | 3.59, 3.64 | 50.50 |
|         | $\gamma$ | 0.96   | 21.62           | Gly13   | NH   | 7.39 |
|         |          | 0.96   | 21.62           | $\alpha$ | 4.07, 3.31 | 46.04 |
| Gln6    | NH   | 8.13      | 119.29          | Trp14   | NH   | 8.32      | 119.65          |
|         | $\alpha$ | 4.30   | 56.19           | $\alpha$ | 4.68 |
|         | $\beta$ | 2.09, 2.13 | 28.97          | $\alpha$ | 4.68 |
|         | $\gamma$ | 2.21, 2.21 | 33.94          | $\beta$ | 2.99, 3.44 | 31.35 |
|         | $\epsilon$ | 6.65, 7.16 | 6.57           | $\delta$ | 6.57 | 126.92 |
| Ala7    | NH   | 7.83      | 122.75          | $\epsilon$ | 7.54 |
|         | $\alpha$ | 4.23   |                 |         |      |          | 10.12           |
|         | $\beta$ | 1.38   | 20.49           | $\zeta$ | 7.13 | 122.06 |
| Asp8    | NH   | 7.94      | 7.48            | Phe15   | NH   | 7.71      |                |
|         | $\alpha$ | 4.81   | 39.46           | $\eta$ | 7.22 |
|         | $\beta$ | 2.75, 2.89 | 7.48           | $\alpha$ | 4.45 | 58.57 |
|         |          |        |                  | $\beta$ | 2.83, 3.08 | 41.06 |
|         |          |        |                  | $\delta$ | 7.10 |
|         |          |        |                  | $\epsilon$ | 7.22 |
Table S8. Assignment of $^1$H signals (ppm) of stlassin W14F (14). The signals were assigned based on TOCSY, NOESY and $^1$H-$^1$C HSQC spectra

| Residue | Atom | $\delta$ | Residue | Atom | $\delta$ |
|---------|------|---------|---------|------|---------|
| Leu1    | NH   | 8.34    | Asp8   | NH   | 6.00    |
|         | $\alpha$ | 4.47   |         | $\alpha$ | 4.30    |
|         | $\beta$ | 1.56, 1.71 | $\beta$ | 2.13, 3.20 |
|         | $\gamma$ | 1.49  | Trp9   | NH   | 7.50    |
|         | $\delta$ | 0.68   |         | $\alpha$ | 4.28    |
|         |         | 0.93   |         | $\beta$ | 3.06, 3.41 |
| Val2    | NH   | 8.55    |         | $\delta$ | 7.30    |
|         | $\alpha$ | 4.17   |         | $\epsilon$ | 7.55    |
|         | $\beta$ | 1.72    |         |         | 10.26   |
|         | $\gamma$ | 0.71   |         | $\zeta$ | 7.13    |
|         |         | 1.07    |         |         | 7.47    |
| Val3    | NH   | 8.09    | Asn10  | NH   | 7.33    |
|         | $\alpha$ | 4.30   |         | $\alpha$ | 4.72    |
|         | $\beta$ | 2.10    |         | $\beta$ | 1.87, 1.95 |
|         | $\gamma$ | 0.90   |         | $\delta$ | 6.43, 6.94 |
| Ile4    | NH   | 9.66    | Ala11  | NH   | 7.96    |
|         | $\alpha$ | 4.40   |         | $\alpha$ | 4.63    |
|         | $\beta$ | 1.86    |         | $\beta$ | 1.37    |
|         | $\gamma$ | 1.07   | Pro    | $\alpha$ | 4.24    |
|         |         | 1.22, 1.61 | $\beta$ | 1.93, 2.35 |
|         | $\delta$ | 0.87   |         | $\gamma$ | 1.96, 2.01 |
| Val5    | NH   | 8.57    | Gly13  | NH   | 6.82    |
|         | $\alpha$ | 3.39   |         | $\alpha$ | 2.89, 4.82 |
|         | $\beta$ | 3.45    |         | $\gamma$ | 1.06    |
|         | $\gamma$ | 1.23   | Phe14  | NH   | 8.47    |
| Gln6    | NH   | 8.54    |         | $\beta$ | 2.03, 2.11 |
|         | $\alpha$ | 4.62   |         | $\delta$ | 6.92    |
|         | $\beta$ | 2.02, 2.07 | $\epsilon$ | 7.21    |
|         | $\gamma$ | 2.57, 2.57 | $\beta$ | 9.21    |
| Ala7    | NH   | 6.79    |         | $\alpha$ | 4.79    |
|         | $\alpha$ | 3.87   |         | $\beta$ | 2.72, 3.42 |
|         | $\beta$ | 1.36    |         | $\delta$ | 7.32    |
|         |         | $\epsilon$ | 7.17    |         |         |
Table S9. Assignment of $^1$H signals (ppm) of stlassin F15Y (16). The signals were assigned based on TOCSY, NOESY and $^1$H-$^1$C HSQC spectra.

| Residue | Atom | $\delta _H$ | Residue | Atom | $\delta _H$ |
|---------|------|-------------|---------|------|-------------|
| Leu1    | NH   | 8.46        | Trp9    | NH   | 6.43        |
|         | $\alpha$ | 4.44    |         | $\alpha$ | 4.15    |
|         | $\beta$ | 1.60, 1.70 |         | $\beta$ | 3.12, 3.20 |
|         | $\gamma$ | 1.45 |         | $\delta$ | 7.27    |
|         | $\delta$ | 0.75     |         | $\epsilon$ | 7.55 |
|         |         | 0.97      |         |         | 10.54     |
| Val2    | NH   | 8.54        |         | $\zeta$ | 7.27    |
|         | $\alpha$ | 4.19    |         |         | 7.61    |
|         | $\beta$ | 1.76      |         | $\eta$ | 7.34    |
|         | $\gamma$ | 0.75 |         |         | 1.08 |
|         |         | 1.08      | Asn10   | NH   | 7.42        |
| Val3    | NH   | 7.95        |         | $\beta$ | 2.30, 2.30 |
|         | $\alpha$ | 4.33    |         | $\alpha$ | 4.63    |
|         | $\beta$ | 2.10      | Ala11   | NH   | 7.92        |
|         | $\gamma$ | 0.91 |         | $\beta$ | 1.37    |
|         |         | 0.92      | Pro12   | $\alpha$ | 4.21 |
| Ile4    | NH   | 9.50        |         | $\beta$ | 1.90, 2.35 |
|         | $\alpha$ | 4.42    |         | $\gamma$ | 1.91, 2.00 |
|         | $\beta$ | 1.83      |         | $\delta$ | 3.64, 3.73 |
|         | $\gamma$ | 1.05 |         | Gly13 | NH   | 6.81        |
|         |         | 1.20, 1.59 |         | $\alpha$ | 2.89, 4.80 |
| Val5    | NH   | 8.57        | Trp14   | NH   | 8.46        |
|         | $\alpha$ | 3.39    |         | $\alpha$ | 4.33    |
|         | $\beta$ | 3.39      |         | $\beta$ | 2.32, 2.40 |
|         | $\gamma$ | 1.05 |         | $\delta$ | 6.77    |
|         |         | 1.20      | Gly13   | NH   | 6.81        |
|         |         | 2.58      | Tyr15   | NH   | 9.11        |
| Gln6    | NH   | 8.61        |         | $\zeta$ | 6.94    |
|         | $\alpha$ | 4.67    |         |         | 7.30    |
|         | $\beta$ | 2.03, 2.07 |         | $\eta$ | 6.86    |
|         | $\gamma$ | 2.56 |         |         | 2.58 |
| Ala7    | NH   | 7.11        |         | $\alpha$ | 4.79 |
|         | $\alpha$ | 3.86    |         | $\beta$ | 2.63, 3.32 |
|         | $\beta$ | 1.33      |         | $\delta$ | 7.18    |
| Asp8    | NH   | 5.70        |         | $\epsilon$ | 6.75 |
|         | $\alpha$ | 3.08     |         |         | 1.65, 2.23 |
Table S10. Assignment of $^1$H signals and $^{13}$C signals (ppm) of stlassin V2C/A11C (18). The signals were assigned based on TOCSY, NOESY and $^1$H-$^{13}$C HSQC spectra.

| Residue | Atom | $\delta_H$ | $\delta_C/\delta_N$ | Residue | Atom | $\delta_H$ | $\delta_C/\delta_N$ |
|---------|------|------------|---------------------|---------|------|------------|---------------------|
| Leu1    | NH   | 7.98       |                     | Trp9    | NH   | 9.09       |                     |
|         | $\alpha$ | 4.30       | 49.93               | $\alpha$ | 4.10       | 55.91               |
|         | $\beta$ | 1.60, 1.22 | 40.95               | $\beta$ | 2.97, 3.22 | 26.19               |
|         | $\gamma$ | 1.22       | 23.57               | $\delta$ | 7.13       |                     |
|         | $\delta$ | 0.71, 0.32 | 21.32               | $\varepsilon$ | 7.51       | 117.89               |
| Cys2    | NH   | 8.24       |                     |         |      | 10.81     |                     |
|         | $\alpha$ | 4.24       | 50.65               | $\zeta$ | 6.96       | 118.22               |
|         | $\beta$ | 3.04, 3.71 | 32.51               |         |      | 7.31       | 111.23               |
| Val3    | NH   | 8.55       |                     | Asn10   | NH   | 8.03       |                     |
|         | $\alpha$ | 3.95       | 58.91               | $\eta$ | 7.03       | 120.86               |
|         | $\beta$ | 1.57       | 25.48               |         |      | 51.85       |                     |
|         | $\gamma$ | 0.43       | 19.90               | $\beta$ | 2.53, 2.58 | 36.89               |
|         | $\delta$ | 0.83       | 23.01               | $\delta$ | 7.25, 6.85 |                     |
| Ile4    | NH   | 8.38       |                     | Cys11   | NH   | 6.85       |                     |
|         | $\alpha$ | 4.33       | 60.06               | $\alpha$ | 4.43       | 49.76               |
|         | $\beta$ | 2.22       | 36.57               | $\beta$ | 2.57, 3.38 | 34.81               |
|         | $\gamma$ | 0.81       | 15.72               | $\beta$ | 2.92, 2.87 | 38.02               |
|         | $\delta$ | 0.97, 1.39 | 25.13               | $\varepsilon$ | 7.12       | 127.57               |
| Val5    | NH   | 9.62       |                     | Gly13   | NH   | 6.55       |                     |
|         | $\alpha$ | 4.57       | 57.38               | $\alpha$ | 3.99, 3.22 | 42.00               |
|         | $\beta$ | 1.67       | 32.47               | $\beta$ | 2.98, 3.41 | 27.51               |
|         | $\gamma$ | 0.82       |                     | $\varepsilon$ | 6.99, 7.45 | 10.42               |
| Gln6    | NH   | 8.59       |                     |         |      | 4.12       | 56.64               |
|         | $\alpha$ | 3.69       | 55.91               | $\beta$ | 2.39, 2.86 | 24.01               |
|         | $\beta$ | 2.39, 2.86 | 24.01               | $\delta$ | 6.21       | 123.15               |
|         | $\gamma$ | 2.22, 2.39 | 32.74               | $\varepsilon$ | 7.71       | 119.06               |
| Ala7    | NH   | 8.19       |                     |         |      | 6.96       | 118.22               |
|         | $\alpha$ | 4.23       | 49.10               |         |      | 7.22       | 110.36               |
|         | $\beta$ | 1.11       | 17.73               | $\eta$ | 6.98       | 120.01               |
| Asp8    | NH   | 7.77       |                     | Phe15   | NH   | 8.01       |                     |
|         | $\alpha$ | 4.46       | 48.92               | $\alpha$ | 4.20       | 54.38               |
|         | $\beta$ | 3.61, 2.41 | 37.85               | $\beta$ | 2.92, 2.87 | 38.02               |
|         | $\delta$ | 7.12       |                     | $\varepsilon$ | 7.12       | 129.69               |
|         | $\zeta$ | 7.04       |                     | $\zeta$ | 7.04       | 125.31               |
Table S11. The Optical density (OD) values and inhibition ratios (% in parentheses) from parallel ELISA experiments for each stlassins against the binding of LPS to TLR4. N1–N4 represent four control experiments.

| Compounds or controls | OD\textsubscript{450} values (inhibition ratios (%)) from three or four parallel experiments | SD |
|-----------------------|------------------------------------------------------------------------------------------|----|
| N1                    | 0.039, 0.034, 0.045, 0.0056                                                             |    |
| N2                    | 0.042, 0.053, 0.066, 0.0120                                                             |    |
| N3                    | 0.041, 0.035, 0.036, 0.0034                                                             |    |
| N4                    | 0.042, 0.036, 0.035, 0.0036                                                             |    |
| DMSO                  | 0.837, 0.807, 0.792, 0.822, 0.0643                                                      |    |
| TAK-242               | 0.464 (44.56), 0.453 (43.87), 0.441 (44.32), 0.430 (47.69), 0.0251                     |    |
| stlassin (1)          | 0.563 (32.74), 0.549 (31.97), 0.535 (32.45), 0.521 (36.62), 0.0184                     |    |
| L1A (10)              | 0.839 (-0.24), 0.821 (-1.74), 0.803 (-1.39), 0.785 (4.50), 0.0232                      |    |
| V2A (2)               | 0.840 (-0.36), 0.804 (0.37), 0.786 (0.76), 0.822 (0.00), 0.0232                        |    |
| V3A (3)               | 0.828 (1.08), 0.824 (2.11), 0.820 (-3.54), 0.816 (0.73), 0.0052                        |    |
| I4A (4)               | 0.508 (39.31), 0.489 (39.41), 0.469 (40.78), 0.450 (45.26), 0.0246                     |    |
| I4C (21)              | 0.507 (39.43), 0.496 (38.54), 0.484 (38.89), 0.473 (42.46), 0.0146                     |    |
| I4C-III (24)          | 0.489 (41.58), 0.483 (40.15), 0.477 (39.77), 0.471 (42.70), 0.0073                     |    |
| V5A (5)               | 0.823 (1.67), 0.811 (-0.50), 0.805 (-1.64), 0.817 (0.61), 0.0077                      |    |
| Q6A (6)               | 0.710 (15.17), 0.699 (13.38), 0.687 (13.26), 0.676 (17.76), 0.0150                     |    |
| A7G (11)              | 0.687 (17.92), 0.673 (16.61), 0.659 (16.69), 0.645 (21.53), 0.0184                     |    |
| N10A (7)              | 0.840 (-0.36), 0.839 (-3.97), 0.839 (-5.93), 0.838 (-1.95), 0.0012                     |    |
| A11G (8)              | 0.709 (15.29), 0.691 (14.35), 0.673 (15.03), 0.655 (20.33), 0.0232                     |    |
| P12A (9)              | 0.690 (17.56), 0.672 (16.73), 0.654 (17.42), 0.636 (22.63), 0.0234                     |    |
| W9F (12)              | 0.838 (-0.12), 0.834 (-3.36), 0.830 (-4.80), 0.826 (-0.49), 0.0052                     |    |
| W14F (14)             | 0.884 (-5.62), 0.865 (-7.19), 0.845 (-6.69), 0.826 (-0.49), 0.0246                     |    |
| W14Y (15)             | 0.842 (-0.60), 0.831 (-2.97), 0.819 (-3.41), 0.808 (1.70), 0.0146                     |    |
| F15Y (16)             | 0.423 (49.46), 0.417 (48.33), 0.411 (48.11), 0.405 (50.73), 0.0073                     |    |
| F15Y-I (20)           | 0.419 (49.94), 0.420 (47.96), 0.400 (49.49), 0.404 (50.85), 0.0073                     |    |
| V2C/A11C (18)         | 0.669 (20.07), 0.664 (17.72), 0.658 (16.92), 0.653 (20.56), 0.0065                     |    |
| V3C/P12C (19)         | 0.662 (20.91), 0.661 (18.09), 0.661 (16.54), 0.660 (19.71), 0.0012                     |    |
Table S12. Inhibition ratios from parallel ELISA experiments for each concentration in dose-response determination.

| compounds | Concentration (μM) | Inhibition ratio (%) from three parallel experiments | SD   |
|-----------|--------------------|-----------------------------------------------------|------|
| stlassin (1) | 0.01 | 11.45 | 8.58 | 8.87 | 1.580 |
|           | 0.05 | 9.79  | 6.72 | 9.70 | 1.747 |
|           | 0.50 | 41.95 | 38.28| 40.41| 1.843 |
|           | 2.00 | 67.07 | 67.06| 68.07| 0.580 |
|           | 10.00| 85.64 | 82.04| 85.87| 2.148 |
|           | 100.00| 89.26 | 87.87| 91.15| 1.646 |
| I4A (4)   | 0.01 | 10.15 | 10.92| 11.57| 0.711 |
|           | 0.05 | 31.21 | 33.02| 34.80| 1.795 |
|           | 0.50 | 38.92 | 38.08| 40.56| 1.259 |
|           | 2.00 | 51.00 | 50.18| 52.61| 1.236 |
|           | 10.00| 59.08 | 57.91| 60.39| 1.241 |
|           | 100.00| 71.29 | 67.93| 71.53| 2.013 |
| I4C (21)  | 0.01 | 3.44  | 3.77 | 4.37 | 0.471 |
|           | 0.05 | 11.15 | 11.82| 13.93| 1.451 |
|           | 0.50 | 31.95 | 29.84| 29.86| 1.212 |
|           | 2.00 | 46.00 | 47.91| 49.93| 1.965 |
|           | 10.00| 59.85 | 58.70| 59.24| 0.575 |
|           | 100.00| 74.57 | 74.21| 75.30| 0.555 |
| I4C-III (24) | 0.01 | 5.65  | 4.47 | 5.11 | 0.591 |
|           | 0.05 | 25.36 | 26.91| 26.72| 0.845 |
|           | 0.50 | 33.91 | 33.07| 35.48| 1.223 |
|           | 2.00 | 46.12 | 47.22| 48.87| 1.384 |
|           | 10.00| 59.61 | 59.92| 61.41| 0.962 |
|           | 100.00| 66.54 | 65.76| 68.08| 1.181 |
| F15Y (16) | 0.01 | 11.27 | 12.24| 10.16| 1.041 |
|           | 0.05 | 27.74 | 28.84| 29.43| 0.858 |
|           | 0.50 | 52.53 | 52.09| 52.13| 0.243 |
|           | 2.00 | 61.60 | 59.39| 61.67| 1.297 |
|           | 10.00| 70.39 | 68.23| 70.16| 1.186 |
|           | 100.00| 79.60 | 79.72| 79.28| 0.227 |
| F15Y-I (20) | 0.01 | 16.14 | 12.69| 10.86| 2.681 |
|           | 0.05 | 32.91 | 29.76| 28.57| 2.243 |
|           | 0.50 | 44.53 | 44.33| 45.54| 0.649 |
|           | 2.00 | 64.37 | 62.86| 62.28| 1.079 |
|           | 10.00| 73.052| 72.71| 73.87| 0.596 |
|           | 100.00| 75.40 | 74.57| 74.97| 0.415 |
**Figure S1.** The phylogenetic analysis of strain PKU-MA01240 (labeled in red) based on comparison of 16S rRNA sequences. The sequence of 16S rRNA of *Kitasatospora azatica* strain OS-3256 was used as an outgroup. The GenBank accession numbers are shown in parentheses.
**Figure S2.** The construction of plasmid pMM2002 for the heterologous expression. (A) The diagram for the construction of pMM2002 using RecET-mediated direct cloning and Redαβ-mediated recombineering. The black bars labeled with “BglII” indicate the BglII restriction sites. (B) The verification of the plasmid pMM2001 construction by ApaLI digestion followed by agarose gel electrophoresis analysis. Lane 1-8, the digestion of plasmids extracted from eight *E. coli* single colonies after the RecET-mediated direct cloning; lane M, DNA ladder; lane 9 and lane M′, the predicted digestion of pMM2001 by ApaLI and predicted DNA ladder, respectively, generated by SnapGene software. (C) The verification of the plasmid pMM2002 construction by ApaLI digestion followed by agarose gel electrophoresis analysis. Lane 1, the digestion of pMM2001 as the negative control; lane 2-9, the digestion of plasmids extracted from eight *E. coli* single colonies after the Redαβ-mediated recombineering; lane M, DNA ladder; lane 10 and lane M′, the predicted digestion of pMM2002 by ApaLI and predicted DNA ladder, respectively, generated by SnapGene software. The lanes labeled with asterisks represent the digestion of correct plasmids.
**Figure S3.** Comparative HPLC analysis of fermentations of heterologous expression strains using three different media. Lane I, fermentation of strain MM20003 using media M2; lane II, fermentation of strain MM20001 using media M2; lane III, fermentation of heterologous host *S. coelicolor* A3(2) using media M2; lane IV, fermentation of strain MM20003 using media M3; lane V, fermentation of strain MM20001 using media M3; lane VI, fermentation of heterologous host *S. coelicolor* A3(2) using media M3; lane VII, fermentation of strain MM20003 using media M4; lane VIII, fermentation of strain MM20001 using media M4; lane IX, fermentation of heterologous host *S. coelicolor* A3(2) using media M4; lane X, fermentation of strain MM20004 using media M2; lane XI, fermentation of strain MM20002 using media M2; lane XII, fermentation of heterologous host *S. lividans* K4-114 using media M2; lane XIII, fermentation of strain MM20004 using media M3; lane XIV, fermentation of strain MM20002 using media M3; lane XV, fermentation of heterologous host *S. lividans* K4-114 using media M3; lane XVI, fermentation of strain MM20004 using media M4; lane XVII, fermentation of strain MM20002 using media M4; lane XVIII, fermentation of heterologous host *S. lividans* K4-114 using media M4. The asterisks indicate the peaks of stlassin (I).
Figure S4. The construction of plasmid pMM2003 and pMM2004. (A) The diagram for the construction of pMM2003 and pMM2004. (B) The verification of the plasmid pMM2003 construction by ApaLI digestion followed by agarose gel electrophoresis analysis. Lane 1, the digestion of pMM2002 as the negative control; lane 2-9, the digestion of plasmids extracted from eight *E. coli* single colonies after the Redβ-α-mediated recombineering; lane M, DNA ladder; lane 10 and lane M’, the predicted digestion of pMM2003 by ApaLI and predicted DNA ladder, respectively, generated by SnapGene software. The lanes labeled with asterisks represent the digestion of correct plasmids. (C) The PCR verification of the construction of pMM2004 with stla-2-F and stla-2-R as the primers. Lane M, DNA ladder; lane 1, the PCR product using plasmid pMM2002 as the template (negative control); lane 2, the PCR product using plasmid pMM2003 as the template; lane 3, the PCR product using pMM2004 as the template. The amplified regions corresponding to the PCR products in lane 1-3 were labeled in (A).
Figure S5. HPLC analysis of stlassin (I, labeled with asterisk) in cells and supernatant of the heterologous expression strain MM20003 (S. coelicolor A3(2) with pMM2004 integrated) after fermentation using three different media. Lane I, the extract of supernatant of MM20003 using media M2; lane II, the extract of cells of MM20003 using media M2; lane III, the extract of supernatant of MM20003 using media M3; lane IV, the extract of cells of MM20003 using media M3; lane V, the extract of supernatant of strain MM20003 using media M4; lane VI, the extract of cells of MM20003 using media M4.
Figure S6. The HPLC analysis of thermal stability and carboxypeptidase-treatment reactions. (A) The heating experiments and carboxypeptidase Y-treatment of stlassin (1). (B) The heating experiments and carboxypeptidase Y-treatment of stlassin V3A (3). (C) The HRESIMS spectra of compound A (unthreaded 3) and compound C (stlassin V3A-ΔC6) purified from the heating experiments of 3. (D) The heating experiments and carboxypeptidase Y-treatment of stlassin I4A (4). (E) The heating experiments and carboxypeptidase Y-treatment of stlassin W14F (14). (F) The heating experiments and carboxypeptidase Y-treatment of stlassin F15Y (16). (G) The heating experiments and carboxypeptidase Y-treatment of stlassin V2C/A11C (18). The “cY” represents carboxypeptidase Y.
Figure S7. The diagram for site-directed mutagenesis of stlaA gene based on the Redαβ recombineering system. (A) The diagram for construction of the plasmid pMM2007 with the KasOp*-stlaA cassette deletion. (B) The diagram for preparation of Amp-KasOp*-stlaA cassette bearing mutations for Redαβ recombineering. (C) The diagram for the construction of the heterologous expression plasmids pMM2008-pMM2037 with different mutated stlaA.
Figure S8. The HPLC analysis of stlassin (1) and its derivatives produced in the crude extracts of different strains. The specific mutations are shown in parentheses under the strain names.
**Figure S9.** The design of double mutations for introducing two cysteine residues. The four pairs of Leu1/Ala11, Val2/Ala11, Val3/Pro12 and Ala7/Pro12 that were selected for disulfide bond formation based on the superimposition of solution NMR structures of 1, 3, 4, 14 and 16. The distances between the β-carbons, which are within 5.71 Å, are indicated in yellow dashed lines.
Figure S10. The lasso presentation of the structures of compounds 1-27. The amino acid residues were shown in spheres with different colors. For all structure, the N-terminal macrolactam residues were labeled in grey except Leu1 was labeled in cyan and Asp8 was labeled in yellow, the C-terminal Trp9-Phe15 were labeled in magenta, and the residues from mutations were labeled in green.
**Figure S11.** The superimposition of the 20 solution structures with the lowest total energy of stlassin (1), stlassin V3A (3), stlassin I4A (4), stlassin W14F (14), stlassin F15Y (16) and stlassin V2C/A11C (18). For all structures, the N-terminal macrolactam residues were labeled in grey except Leu1 was labeled in cyan and Asp8 was labeled in yellow, the C-terminal Trp9-Phe15 were labeled in magenta, and the residues from mutations were labeled in green.
Figure S12. The superimposition of solution NMR structures. (A) The main chains superimposition of solution NMR structures of 1, 3, 4, 14 and 16. (B) The superimposition of solution NMR structures of 1, 3, 4, 14 and 16. (C) The superimposition of 1 (blue, partial) and 18, with the residues' names labeled in black for 18 and red for 1. For all structures except 1 in (C), the N-terminal macrolactam residues are shown in gray except Leu1 in cyan and Asp8 in yellow, the C-terminal residues are shown in magenta and the residues from mutation are shown in green.
Figure S13. Amplified region of NOESY spectra of 1, 3, 4, 14 and 16 showing the correlations (indicated with circles) between Val3 (or Ala3) and Pro12. More NOE correlations can be observed between V3 and P12 in 1, 4, 14, and 16, whereas only one can be observed between A3 and P12 in 3.
Figure S14. The OD values of four control ELISA experiments (N1–N4) compared to that of DMSO control. Compared to the complete ELISA procedures for antagonistic activities assays, N1 only omits the mouse anti-human TLR4 antibody in the coating step; N2 only omits the addition of the NCM460 proteins; N3 uses biotin instead of biotin-labeled LPS; N4 uses LPS instead of biotin-labeled LPS; DMSO control uses DMSO instead of compounds.
Figure S15. The antagonistic activities of stlassins against the binding of LPS to TLR4 at different concentrations. (A) The inhibition ratios of stlassin and variants against the binding of LPS to TLR4 with concentration of 1 μg/mL using TAK-242 as the positive control. These inhibition ratios were generated from the same sets of data used for the OD value determinations in Figure 6 in the main text. The inhibition ratios and error bars were generated based on the calculation of mean ± standard deviation from four parallel experiments. (B) The inhibition ratios of stlassin (1) and variants I4A (4), I4C (21), I4C-III (24), F15Y (16), and F15Y-I (20) at different concentrations. The inhibition ratios and error bars were generated based on the calculation of mean ± standard deviation from three parallel experiments. The IC\textsubscript{50} values were determined based on the dose-response curves using GraphPad Prism 7.0.
**Figure S16.** The HRESIMS spectrum of stlassin (1).

![HRESIMS spectrum of stlassin (1)](image1)

**Figure S17.** The HRESIMS spectrum of stlassin V2A (2).

![HRESIMS spectrum of stlassin V2A (2)](image2)
Figure S18. The HRESIMS spectrum of stlassin V3A (3).

Figure S19. The HRESIMS spectrum of stlassin I4A (4).
Figure S20. The HRESIMS spectrum of stlassin V5A (5).

Figure S21. The HRESIMS spectrum of stlassin Q6A (6).
Figure S22. The HRESIMS spectrum of stlassin N10A (7).

Figure S23. The HRESIMS spectrum of stlassin A11G (8).
Figure S24. The HRESIMS spectrum of stlassin P12A (9).

Figure S25. The HRESIMS spectrum of stlassin L1A (10).
Figure S26. The HRESIMS spectrum of stlassin A7G (11).

Figure S27. The HRESIMS spectrum of stlassin D8E.
Figure S28. The HRESIMS spectrum of stlassin W9F (12).

Figure S29. The HRESIMS spectrum of stlassin W9Y (13).
**Figure S30.** The HRESIMS spectrum of stlassin W14F (14).

**Figure S31.** The HRESIMS spectrum of stlassin W14Y (15).
Figure S32. The HRESIMS spectrum of stlassin F15Y (16).

Figure S33. The HRESIMS spectra of stlassin F15W (17).
Figure S34. The HRESIMS spectra of stillassin V2C/A11C (18).

Figure S35. The HRESIMS spectra of stillassin V3C/ P12C (19).
Figure S36. The HRESIMS spectra of stilassin F15Y-I (20).

Figure S37. The HRESIMS spectrum of stilassin I4C (21).
**Figure S38.** The HRESIMS spectrum of stlassin IαC-I (22).

**Figure S39.** The HRESIMS spectra of stlassin IαC-II (23).
Figure S40. The HRESIMS spectrum of stlassin I4C-III (24).

Figure S41. The HRESIMS spectrum of stlassin I4C-IV (25).
Figure S42. The HRESIMS spectrum of stlassin I4C-V (26a).

Figure S43. The HRESIMS spectrum of stlassin I4C-VI (26b)
Figure S44. The ESIMS spectrum of stlassin I4C-VII (27a).

Figure S45. The ESIMS spectrum of stlassin I4C-VIII (27b).
Figure S46. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin (1) in H$_2$O/DMSO-$_d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S47. The $^1$H-$^{13}$C HSQC spectrum of stlassin (I) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S48. The $^1$H-$^15$N HSQC spectrum of stlassin (1) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S49. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin V3A (3) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S50. The $^1$H-$^{13}$C HSQC spectrum of stlassin V3A (3) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S51. The $^1$H-$^{15}$N HSQC spectrum of stlassin V3A (3) in H$_2$O/DMSO-d$_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S52. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin I4A (4) in H₂O/DMSO-d₆ (8:2) with 100 mM NaCl at 298 K.
Figure S53. The $^1$H-$^1$C HSQC spectrum of stlassin I4A (4) in H$_2$O/DMSO-d$_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S54. The $^1$H-$^{15}$N HSQC spectrum of stlassin I4A (4) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S55. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin W14F (14) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S56. The $^1$H-$^{13}$C HSQC spectrum of stlassin W14F (14) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S57. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin F15Y (16) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S58. The $^1$H-$^{13}$C HSQC spectrum of sllassin F15Y (16) in H$_2$O/DMSO-$d_6$ (8:2) with 100 mM NaCl at 298 K.
Figure S59. The TOCSY spectrum (blue, mixing time: 70 ms) and NOESY spectrum (red, mixing time: 300 ms) of stlassin V2C/A11C (18) in DMSO-d6 at 298 K.
Figure S60. The $^1$H-$^{13}$C HSQC spectrum of stlassin V2C/A11C (18) in DMSO-$d_6$ at 298 K.
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