Electronic Supporting Information
for
Heterologous Production of the Lantibiotic Ala(0)Actagardine in *Escherichia coli*

Yanxiang Shi, Alejandro Bueno and Wilfred A. van der Donk*

Supplementary text

Materials and Methods

Materials

All oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were purchased from New England Biolabs. Media components for culturing bacteria were purchased from Difco laboratories. Chemicals were purchased from Fisher Scientific or from Aldrich unless noted otherwise. Trypsin was purchased from Worthington Biosciences. Aminopeptidase A8200 was purchased from Sigma. *E. coli* DH5α was used as host for cloning and plasmid propagation, and *E. coli* BL21 (DE3) was used as a host for co-expression.

General methods

Positive residue numbers are used for amino acids in the core peptide counting forward starting from the N-terminal residue in the core peptide. Actagardine can be produced with an additional N-terminal alanine in *Actinoplanes garbadinensis*, and this N-terminal alanine residue is numbered Ala(0).[1, 2] We also constructed actagardine derivatives with an extra lysine residue located at the C-terminus of GarA generating GarA-19insK. Another derivative with Val15 and Ile16 substituted with Leu and Val residues and an extra lysine residue attached to the C-terminus was also constructed, and this mutation was named as GarA-V15L/I16V/19insK.

All polymerase chain reactions (PCR) were carried out on a C1000™ thermal cycler (Bio-Rad). DNA sequencing was performed by ACGT Inc. and the Biotechnology Center at the University of Illinois at Urbana-Champaign, using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on an ultrafleXtreme™ MALDI TOF/TOF system (Bruker Daltonics). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole TOF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). Fast protein liquid chromatography (FPLC) purification was carried out on an Amersham Biosciences/GE Healthcare ÄKTA system.

Genomic DNA extraction of *Actinoplanes garbadinensis* ATCC 31049

Actinoplanes garbadinensis ATCC 31049 bacteria were purchased from the American Type Culture Collection (ATCC). Lyophilized bacteria were inoculated in 50 mL of GYM or LB media. The mixtures were shaken at 30 °C and 200 r.p.m. for a week and then centrifuged. The *Actinoplanes garbadinensis* ATCC 31049 genomic DNA was extracted from the cell pellet, using Wizard® Plus SV Minipreps kit (Promega).

Construction of pRSFDuet-1 derivatives for *garM*, *garA,* and *garO*

The *garM* gene was cloned by PCR from *A. garbadinensis* ATCC 31049 genomic DNA using stepwise overlap extension PCR (for all primer sequences, see Table S1): the first round PCR generated fragment

GarM_NdeI_1456 using GarM_NdeI_FLP_Duet-1 and GarM_1456seq_RP primer pair, fragment GarM_1117_2074 using GarM_1117seq_FP and GarM_2074seq_RP primer pair, fragment GarM_576_2074 using GarM_576seq_FP and GarM_2074seq_RP primer pair, and fragment GarM_1689_KpnI using GarM_1689seq_FP and GarM_KpnI_RLP_Duet-1 primer pair. The second round PCR generated fragment GarM_NdeI_2074 using GarM_NdeI_1456 and GarM_1117_2074 megaprimer pair, and fragment GarM_576_KpnI using GarM_576_2074 and GarM_1689_KpnI megaprimer pair. The third round PCR generated the GarM_NdeI_KpnI full-length product using GarM_NdeI_2074 and GarM_576_KpnI megaprimers. The gene encoding GarM was inserted into multiple cloning site 2 (MCS2) of the pRSFDuet-1 vector between the *Nde*I and *Kpn*I restriction sites. The *garA* gene was cloned by PCR from *A. garbadinensis* ATCC 31049 genomic DNA and inserted between the *EcoR*I and *Hind*III restriction sites of MCS1 in pRSFDuet-1. The *garA* mutant gene *GarA-19insK* was generated using overlap extension PCR with the GarA_C+K_RP primer and the *garA/pRSFDuet-1* vector as the template. The *garA* mutant gene *GarA-V15L/I16V/19insK* was generated using overlap extension PCR with the GarA_LV_C+K_RP primer and the *garA/pRSFDuet-1* vector as the template. The mutant *garA* genes were individually cloned into MCS1 of the pRSFDuet-1 vector between the *EcoR*I and *Hind*III restriction sites, with the *garM* gene in MCS2. The sequences of the resulting plasmids were confirmed by DNA sequencing. One copy of *garO* was inserted into MCS1 of pCDFDuet-1 between the *Nco*I and *Hind*III restriction sites resulting in a protein without His_6-tag, and a second copy of *garO* was inserted into MCS2 of pCDFDuet-1 between the *Nde*I and *Xho*I restriction sites, also resulting in a protein without His_6-tag.

In order to purify the *garO* protein to perform *in vitro* assays, the *garO* gene was inserted between *Nde*I and *Xho*I into a pET28b vector. In order to construct a vector for three-gene co-expression (*garA/garM/garO*), the *garO* gene was amplified from *garO*/pET28b including the T7 promoter and lac operator and inserted into *garA/garM/pRSFDuet-1* or *garA-V15L/I16V/19insK/garM/pRSFDuet-1* vector between the *Pac*I and *Avr*II sites.

Table S1 Primer sequences for cloning and mutagenesis of *garA*, *garM* and *garO*

Primer Name	Primer Sequence (5'-3')
GarA_EcoRI_FP_Duet-1	GTA AGT ACG AAT TCG ATG TCT GCT CTC GCC ATC GAG AAG
GarA_HindIII_RP_Duet-1	ATA ATA TCA AGC TTT CAG CAG GCG CAG ATC ACG GTG
GarA_C+K_RP	ATA ATA TCA AGC TTT CAT TTG CAG GCG CAG ATC ACG GTG
GarA_LV_C+K_RP	ATA ATA TCA AGC TTT CAT TTG CAG GCG CAA ACC AGG GTG CCG CAC TCG ATC
GarM_NdeI_FLP_Duet-1	GTA AGT ACC ATA TGT CAC CGG TTC CTT CAC TCA ATT CCA CCT CGG TAC GCG
GarM_KpnI_RLP_Duet-1	ATA ATA TCG GTA CCT CAG GTC AGC GGC GGC TGC AGC CAG
GarM_576seq_FP	GCT GCT CAC CGA CCC CGC CTA C
GarM_1117seq_FP	CCG ATC GAC CTG GAG ACC GTG CTG
GarM_1689seq_FP	CGA GTC GGA GAA GCG ACA ACT GCT CG
GarM_2249seq_FP	CAT CGT CGA GAC CGT CGG CGC CTA CAG
GarM_303seq_RP	GTG ACC ACA GTT TGG TGG ACA ACT CG
GarM_867seq_RP	CAT CGT CTC GGC GTA GGC GCG TTC
GarM_1456seq_RP	GCA TGA TGT CGT AGG CGT CGG TGA ACC C
GarM_2074seq_RP	GCT TGT AGC TGT AGG TCT CCT GGG C
GarM_2616seq_RP	GAG TTC GGT GGC GAG CCG GTG CAG
GarO_NdeI_FP_Duet-1	TTA AGT CAC ATA TGG TGC TCA GCG TGC TGG ACC AG
GarO_XhoI_RP_Duet-1	ATA ATA TGC TCG AGT CAG GCC GCC GGG GAT G
GarO_EcoRI_FP	TTA AGT CAG AAT TCG ATG CTC AGC GTG CTG GAC CAG
GarO_HindIII_RP	ATA ATA TGA AGC TTT CAG GCC GCC GGG GAT G
GarO_NcoI_FP	TTA AAT CA CCATGGGC ATG CTC AGC GTG CTG GAC CAG
GarO_T7_PacI_FP	TCC AGG CA TTAATTAA CGAAATTAATACGACTCACTATAGGGG
GarO_AvrII_RP	ATA ATA TGC CTA GGT CAG GCC GCC GGG GAT G

Overexpression and purification of His_6-tagged GarM-modified GarA and GarA-V15L/I16V/19insK

E. coli BL21 (DE3) cells transformed with a pRSFDuet-1 vector carrying the genes encoding GarM and one of the GarA peptides were shaken at 225 r.p.m. in 2 L of LB medium containing 50 mg/L kanamycin at 37 °C until the $O.D._{600\ nm}$ reached 0.6 − 0.8. The culture was then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and shaken continually at 18 °C for an additional 20 h.

For the co-expression of GarA, GarM and GarO, *E. coli* BL21 (DE3) cells transformed with a pRSFDuet-1 vector carrying *garA/garM/garO*, or transformed with two vectors (one was *garA/garM/pRSFDuet-1* or *garA-V15L/I16V/19insK/garM/pRSFDuet-1*, while another was *garO/pCDFDuet-1* or *garO/garO/pCDFDuet-1*) were grown in 2 L of LB medium containing 50 mg/L kanamycin and 25 mg/L spectinomycin (spectinomycin was not needed for *garA/garM/garO*/*pRSFDuet-1* overexpression) at 37 °C until the $O.D._{600\ nm}$ reached 0.6 − 0.8. The culture was then induced with 0.2 mM IPTG and shaken continually at 18 °C for an additional 30 h with *garO/pCDFDuet-1* or 20 h with *garO/garO/pCDFDuet-1*.

The induced cells were harvested by centrifugation (11,900 × g for 20 min at 4 °C). The cell pellet was resuspended in 20 mL of LanA Start Buffer (20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 pause, 15 min). The sample was centrifuged (23,700 × g for 40 min at 4 °C) and the supernatant was kept for further purification. The pellet was resuspended in another 20 mL of LanA Start Buffer to obtain any soluble proteins remaining in the pellet. Insoluble materials were removed from the combined soluble fractions by centrifugation (11,900 × g for 20 min at 4 °C), and the resulting sample was clarified using a 0.45 μm syringe filter (Corning). The His_6-tagged peptides were then purified by immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare Life Sciences). The filtered sample was applied to the column and the column was washed with 2 column volumes of LanA Buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole), followed by 2 ~ 3 column volumes of LanA Buffer 2 (4 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 300 mM NaCl, 30 mM imidazole), and then eluted with 3 column volumes of LanA Elution Buffer (4 M guanidine hydrochloride, 20 mM Tris, pH 7.5 at 25 °C, 100 mM NaCl, 1 M imidazole).

The elution fractions were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) using a Waters Delta-pak™ C4 15 μm 300 Å 25 × 100 mm PrepPac® Cartridge. Solvents for the RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile / 20% water). A gradient of 2-100% of solvent B was executed over 45 min at a flow rate of 8 mL/min, and peptides were detected by absorbance at 220 nm. The fractions were analyzed by MALDI-TOF-MS. All the fractions containing the desired product were combined and the organic solvents were removed by rotary evaporation, followed by lyophilization overnight. The yields of modified GarA and GarA-V15L/I16V/19insK from *garA/garM/pRSFDuet-1* were 4.2 mg and 2.1 mg per liter respectively, while the yields of modified GarA and GarA-V15L/I16V/19insK from *garA/garM/pRSFDuet-1* and *garA-V15L/I16V/19insK/garM/pRSFDuet-1* with *2garO/pCDFDuet-1* were 0.17 mg and 0.74 mg per liter, respectively. The products were kept at −20 °C for short-term storage and −80 °C for long-term storage.

Overexpression and purification of His_6-tagged GarO

E. coli BL21 (DE3) cells transformed with *garO/pET28b* vector were shaken at 225 r.p.m. in 2 L of LB medium containing 50 mg/L kanamycin at 37 °C until the $O.D._{600\ nm}$ reached 0.6 − 0.8. The culture was then induced with 0.33 mM IPTG and shaken at 18 °C for an additional 20 h.

The purification steps of GarO were all performed at 4 °C. Induced cells were first harvested by centrifugation (11,900 × g for 20 min at 4 °C). The cell pellet was resuspended in 20 mL of Start Buffer (20 mM Tris, pH 8.0 at 4 °C, 500 mM NaCl, 10% glycerol) and lysed by a high pressure homogenizer (Avestin, Inc.). After centrifugation (23,700 × g for 40 min at 4 °C), the supernatant was clarified using a 0.45 μm syringe filter (Corning), followed by injection onto a fast protein liquid chromatography (FPLC) system

(ÄKTA, GE Heathcare Life Sciences), equipped with a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare Life Sciences) pre-charged with Ni^{2+} and pre-equilibrated with Start Buffer. After loading, the column was washed with Buffer A (20 mM Tris, pH 8.0 at 4 °C, 1 M NaCl, 30 mM imidazole). GarO protein was eluted using a linear gradient of 0-100% of Buffer B (20 mM Tris, pH 8.0 at 4 °C, 1 M NaCl, 200 mM imidazole), over 45 min at a flow rate of 2 mL/min and detection by UV absorbance at 280 nm. The fractions were analyzed by SDS-PAGE (4 – 20% tris-glycine gel, Bio-Rad). All the fractions containing the desired product were combined and concentrated to 2.5 mL using an Amicon Ultra-15 Centrifuge Filter Unit (10 kDa MWCO, Millipore). Then the buffer of the concentrated protein was exchanged to Start Buffer using a PD-10 desalting column (GE Healthcare Life Sciences). The concentration of the protein was determined by absorbance at 280 nm, and the resulting protein sample was stored at −80 °C.

To evaluate the oligomerization state of GarO, purified GarO (Calc. M.W.: 38.3 kDa) was injected in to the FPLC system equipped with a Superdex 200 (GE Healthcare) column pre-equilibrated with Column Buffer (20 mM NaH_2PO_4, 500 mM NaCl, 10% glycerol, pH 8.0 at 4 °C). The protein was eluted at a flow rate of 1 mL/min and proteins were detected by UV absorbance at 280 nm. The major peak was eluted with a retention time of 78 min, indicating a monomer oligomerization state when compared with molecular weight standard (Fig. S6).

Proteolytic removal of GarA leader peptide

Modified GarA and its mutants were dissolved in water generating 6 mg/mL peptide solutions. An aliquot of 10 μL GarA stock solution was incubated with 1 μL of trypsin solution (0.6 mg/mL) in 50 mM Tris buffer (pH = 8.3) for at least 1 h to remove the major N-terminal portion of the leader peptide. An aliquot of 4 μL of aminopeptidase A8200 (0.1 Unit/ mL) was then added to the mixture and incubated for at least three hours to remove the residual C-terminal portion of the leader peptide.

Product evaluation for *in vivo* coexpression of GarA, GarM and GarO

For the co-expression of GarA, GarM and GarO in *E. coli*, the same expression procedure was used as described above under "Overexpression and purification of His_6-tagged GarM-modified GarA and GarA-V15L/I16V/19insK". GarA with four dehydrations and one oxygen addition was observed after proteolytic cleavage, regardless of whether one-vector (*garA/garM/garO/pRSFDuet-1*) or two vectors (*garA/garM/pRSFDuet-1* together with *2garO/pCDFDuet-1*) were used (Fig. S4 and Fig. 4 in the main text). Two-vector co-expression using *garA-V15L/I16V/19insK/garM/pRSFDuet-1* together with *2garO/pCDFDuet-1* generated garA-V15L/I16V/19insK with four dehydrations but incomplete addition of one oxygen (Fig. S5).

HPLC purification of Ala(0)-DAB-Lys

The Ala(0)-DAB-Lys generated by trypsin and aminopeptidase A8200 cleavage reaction was purified by analytical RP-HPLC using a Vydac™ C18 5 μm 300 Å 4.6 i.d. × 250 mm column. Solvents for RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile / 20% water). A gradient of 0.8-100% of solvent B was executed over 50 min at a flow rate of 1 mL/min, and peptides were detected by absorbance at 220 nm. The fractions were tested by MALDI-ToF-MS for the desired peptides. All the fractions containing the desired fully modified product were combined and the solvents were removed by lyophilization overnight. The product was kept at −20 °C for short-term storage and −80 °C for long-term storage.

Iodoacetamide treatment of modified His_6-GarA and its derivatives to detect free cysteines

An aliquot of 20 μL of the trypsin cleavage reaction of GarM-modified GarA or its derivatives was mixed with 50 mM Tris-HCl (pH 8.3), 50 mM IAA, and 0.5 mM TECP. The resulting solution was then

incubated at room temperature overnight, followed by desalting using $ZipTip_{C18}$ (Millipore) and MALDI-TOF-MS analysis. No carboxyamidomethyl (CAM) thiol modification was observed after IAA treatment (Fig. S1), suggesting the cyclization was complete without any free cysteine residues.

GarO enzymatic assays

Activity assays for GarO with dehydrated and cyclized GarA, GarA-V15L/I16V/19insK, and purified Ala(0)-DAB-Lys were carried out in an assay mix containing 50 mM Na_2HPO_4 (pH 8.0), 0.2 mM FMN, and 0.5 mM NADH. The final concentrations of GarA (or its derivatives) and GarO were 75 μM and 10 μM, respectively. The reaction mixtures were incubated at room temperature overnight. An aliquot of 20 μL reaction was treated with 1 μL trypsin (0.6 mg/mL) before analysis by MALDI-ToF mass spectrometry. When NADH was substituted with NADPH, no product was observed after overnight incubation.

To obtain insights into the site of oxidation, an aliquot of 5 μL GarO-modified Ala(0)-DAB-Lys was analyzed by LC/ESI-MS/MS. The trypsin cleavage reaction mixture was injected to a BEH C8 column (1.7 μm, 1.0 × 100 mm), and the fully modified product was purified by UPLC using a gradient of 3% mobile phase B (0.1% formic acid in acetonitrile) to 97% mobile phase B in mobile phase A (0.1% formic acid in water) over 12 min. Mass spectra were acquired in ESI positive mode in the range of 50-2000 m/z. The capillary voltage was 3200 V, and the cone voltage was 40 V. The other parameters used were as follows: 120 °C source temperature, 300 °C desolvation temperature, 150 L/h cone gas flow, and 600 L/h desolvation gas flow. A transfer collision energy of 4 V was used for both MS and tandem MS, while the trap collision energy was set to 6 V for MS and 35 V for MSMS. Glu-fibrinopeptide B (Sigma) was directly infused as the lock mass. The tandem mass spectra were processed with MaxEnt3 and analyzed by Protein/Peptide Editor in BioLynx 4.1. The software for analyzing both precursor-ions and fragment-ions was set to report any mass within 0.3 amu of the calculated values. Fragments corresponding to b7 and y”14 were observed (Fig. S2), confirming the sulfoxide group was added to the C-terminal intertwined rings. The loss of one molecule of H_2O was observed during MSMS when collision energy was applied on the parent ion.

Bioactivity assay for deoxyactagardine derivatives

Bacillus subtilis W23 was inoculated from frozen stock into 5 mL DSMZ Medium 1 Nutrient Broth and grown overnight at 37 °C and 250 r.p.m. An aliquot of 100 μl overnight culture was inoculated into 20 mL molten DSMZ Medium 1 Nutrient Agar kept at 55 °C, followed by dispensing into a round petri dish. While the bacteria-containing agar was cooled at room temperature for 0.5 h, 20 μL of nisin (100 μM), trypsin digested followed by aminopeptidase A8200 cleaved GarM-modified GarA-V15L/I16V/19insK (peptide final concentrations: 100 μM; other components included 50 mM Tris buffer, 0.03 mg/mL trypsin, and 0.1 U/mL aminopeptidase A8200, pH = 8.3), or negative control with buffer and proteinases only, was spotted onto the agar (Fig. 3).

Micrococcus luteus ATCC 4698 was inoculated from frozen stock into 5 mL of Mueller-Hinton Broth (MHB) and grown overnight at 37 °C and 250 r.p.m. An aliquot of 100 μL of overnight culture was inoculated into 20 mL of molten Mueller-Hinton Agar (MHA) kept at 42 °C, followed by dispensing into a Nunc OmniTray. While the bacteria-containing MHA agar was cooled at room temperature for 1 h, another aliquot of 200 μL of overnight culture was inoculated into 40 mL of molten MHA kept at 42 °C. The mixture was then dispensed onto the solidified layer of MHA. Wells were made into the top layer of seeded agar and subsequently loaded with 60 μL samples once the agar was solidified. Then the plate was transferred to a 30 °C incubator and kept in the incubator for 36 h. Both Ala(0)-deoxyactagardine and Ala(0)-DAB-Lys displayed antimicrobial activity against *M. luteus* ATCC4698. It was noted that the addition of one lysine residue to the C-terminus of deoxyactagardine increased the core peptide solubility, as precipitate was observed in the well bottom from Ala(0)-deoxyactagardine, but not from the Ala(0)-deoxyactagardine-Lys which was generated for comparison (Fig. S3).

To further determine the minimum inhibitory concentration (MIC) of Ala(0)-DAB-Lys, overnight cultures of *Bacillus subtilis* W23 in DSMZ Medium 1 Nutrient Broth or *Enterococcus faecalis* 29212 in Brain Heart Infusion Broth (BHI) were inoculated into fresh DSMZ Medium 1 Nutrient Broth or BHI, respectively, to generate cell cultures with an $O.D._{600\ nm}$ of 0.01. An aliquot of 30 μL of purified Ala(0)-DAB-Lys aqueous solution was added into 250 μL of the cell cultures in a 48-well plate (at final peptide concentrations of 60 μM, 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM, 625 nM, 312 nM, 156 nM, 78 nM, 39 nM, 20 nM, 10 nM, and 0 nM). Every concentration was tested in triplicate. The plate was shaken at 30 °C at 250 r.p.m. for 6 h, and the cell density was monitored by the plate reader at 600 nm. The MIC of Ala(0)-DAB-Lys against *Bacillus subtilis* W23 in DSMZ Medium 1 Nutrient Broth was 1.20 μM. The MIC of actagardine and deoxyactagardine against *Enterococcus faecalis* 29212 were reported to be 4.24 μM and 4.27 μM, respectively.[1] In comparison, the MIC of Ala(0)-DAB-Lys generated in this work against *Enterococcus faecalis* 29212 in BHI was 7.28 μM.

Supplementary figures

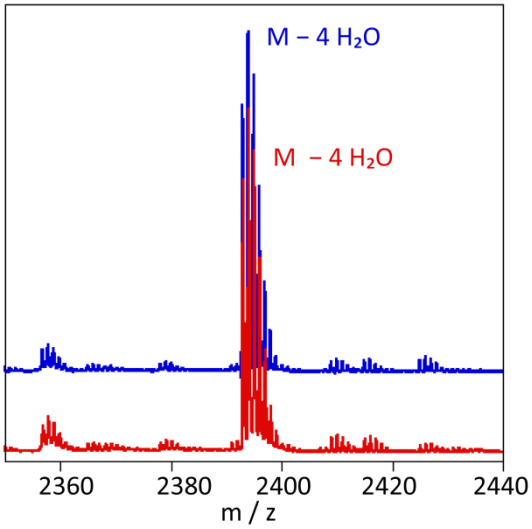

Fig. S1 MALDI-MS spectra of GarM-modified GarA incubated with IAA (blue) and without IAA (red) and treated with trypsin. M indicates the fragment of GarM-modified GarA after trypsin cleavage (sequence: TIYA-DAB, $[M — 4\ H_2O + H]^+$ Calc. m/z: 2393.08, Obs. m/z: 2393.00)

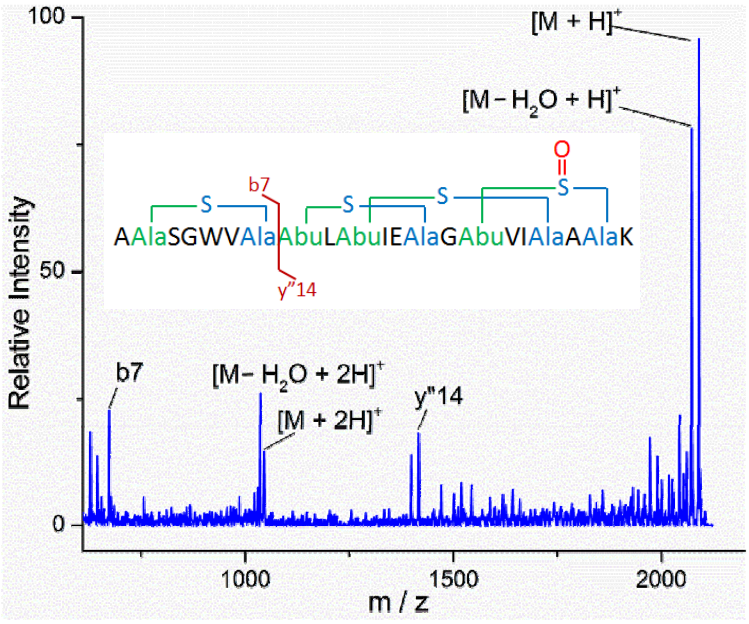

Fig. S2 ESI-MS/MS of GarA-V15L/I16V/19insK modified in *E. coli* by GarM and GarO and treated *in vitro* with trypsin and aminopeptidase A8200.

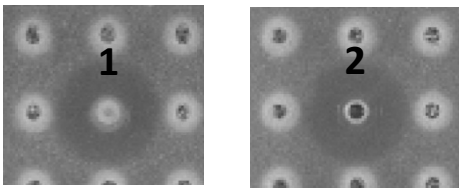

Fig. S3 Antimicrobial activity of Ala(0)-deoxyactagardine and Ala(0)-deoxyactagardine-Lys generated in *E. coli* against *M. luteus* ATCC 4698. Compounds were produced by incubating His_6-GarA and His_6-GarA-19insK with trypsin and aminopeptidase A8200 to remove the leader peptide. Compounds spotted in the wells were: (1) Ala(0)-deoxyactagardine from GarA, final concentration (f.c.) 1.87 mg/ml, (2) Ala(0)-deoxyactagardine-Lys from GarA-19insK, f.c. 1.96 mg/ml. (40 µL was spotted on each well)

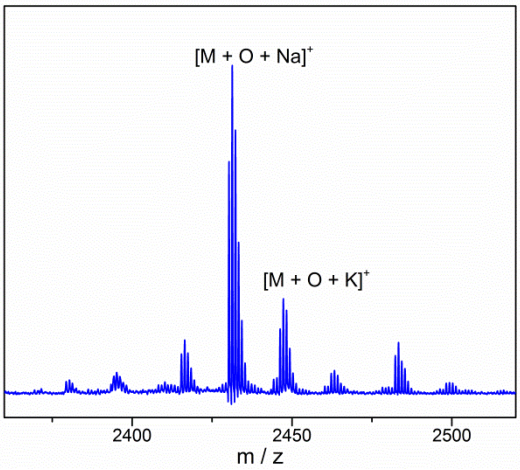

Fig. S4 MALDI-MS spectrum of GarA modified by GarO from co-expression of *garA/garM/garO/pRSFDuet-1*. M indicates GarM-modified GarA with most of the leader peptide removed by trypsin (sequence: TIYA-deoxyactagardine, $[M+O+Na]^+$ calculated (Calc.) m/z: 2431.06, observed (Obs.) m/z: 2431.34; $[M+O+K]^+$ Calc. m/z: 2447.17, Obs. m/z: 2447.38)

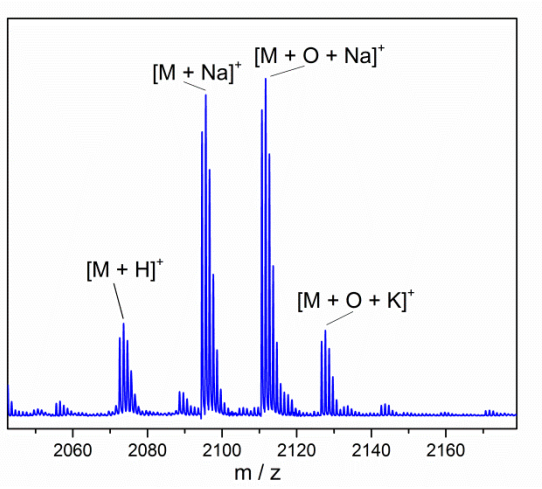

Fig. S5 MALDI-MS spectrum of modified GarA-V15L/I16V/19insK from co-expression of *garA-V15L/I16V/19insK/garM/pRSFDuet-1* and *2garO/pCDFDuet-1*. M indicates GarM-modified GarA-V15L/I16V/19insK with the leader peptide removed by trypsin and aminopeptidase A8200 (sequence: Ala(0)-DAB-Lys, $[M+O+Na]^+$ calculated (Calc.) m/z: 2095.49, observed (Obs.) m/z: 2095.64; $[M+O+Na]^+$ Calc. m/z: 2111.49, Obs. m/z: 2111.68)

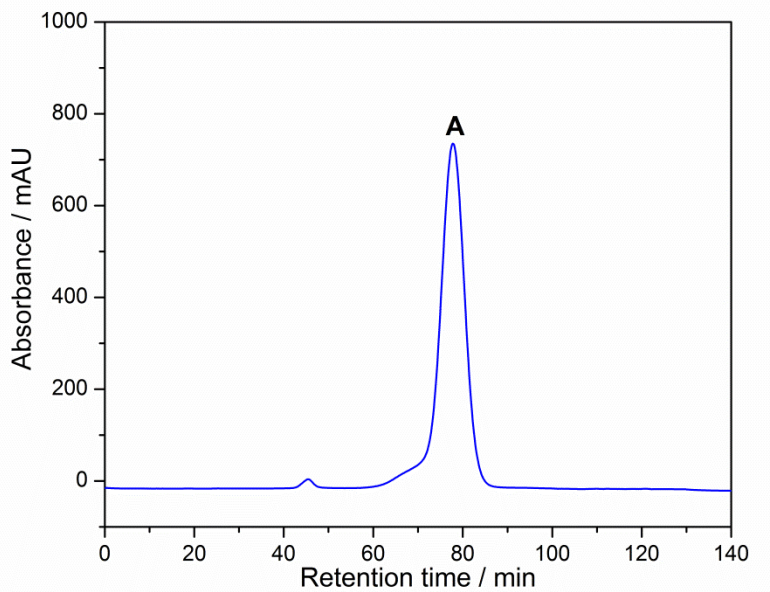

Fig. S6 Analysis of His_6-GarO by gel filtration chromatography. Peak A corresponds to the monomer of His_6-tagged GarO.

Notes and references

* Howard Hughes Medical Institute and Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. E-mail: vddonk@illinois.edu

1. S. Boakes, J. Cortes, A. N. Appleyard, B. A. M. Rudd and M. J. Dawson, *Mol. Microbiol.*, 2009, **72**, 1126-1136.
2. L. Vertesy, W. Aretz, A. Bonnefoy, E. Ehlers, M. Kurz, A. Markus, M. Schiell, M. Vogel, J. Wink and H. Kogler, *J. Antibiot.*, 1999, **52**, 730-741.