Heterologous production of coryneazolicin in Escherichia coli

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2 Running headline: Heterologous production of coryneazolicin

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16

Abstract

Coryneazolicin belongs to a plantazolicin type peptide which is a ribosomally synthesized and post-translationally modified peptide (RiPP) that contains multiple azole rings. Although coryneazolicin was previously synthesized by in vitro experiments, its biological activity has not been evaluated. In this report, the heterologous production of coryneazolicin was accomplished to obtain enough coryneazolicin for biological activity tests. The structure of coryneazolicin was confirmed by ESI-MS and NMR analyses. The biological activity tests indicated that coryneazolicin possessed potent antibacterial activity and cytotoxicity. Although antibacterial activity of plantazolicin was previously reported, the cytotoxicity was newly found in coryneazolicin among plantazolicin type peptides. In addition, we revealed that coryneazolicin induced apoptosis.

Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a class of naturally occurring peptides that includes more than 20 subclasses such as lanthipeptides.[1-4] Based on their structural characteristics, RiPPs are classified into several groups, such as lantibiotics,[5, 6] lasso peptides,[7-9] and linear azole-containing peptides (LAPs).[10] LAPs are defined as linear peptides containing several azole and azoline rings biosynthesized from Cys, Ser, and Thr residues in the precursor peptide.[10] LAPs, such as microcin B17,[11, 12] streptolysin S,[13-15] goadsporin, [16-20] and plantazolicin,[21-23] have been reported to have a wide variety of bioactivities. Plantazolicin was originally isolated as an anti-*Bacillus anthracis* agent[22, 24] from *Bacillus velezensis* FZB42 (formerly *Bacillus amyloliquefaciens*). Total synthesis of plantazolicin(s) was accomplished with different approaches by several groups.[25-28]

The biosynthetic gene cluster of plantazolicin was reported to include six essential genes (*bamA*, *bamB*, *bamC*, *bamD*, *bamE*, and *bamL*).[29] The azole and azoline rings in the plantazolicin precursor peptide (BamA) are enzymatically biosynthesized by a trimeric complex of a cyclodehydratase (BamC and BamD) and a FMN-dependent dehydrogenase (BamB).[29] After heterocycle formation, the *N*-terminal (leader peptide) region of the precursor peptide can be removed by the putative protease (BamE).[29] Two

methyl residues are added to the amino residue of the *N*-terminus Arg in the core of the peptide by an S-adenosylmethionine (SAM)-dependent methyltransferase (BamL) to afford the target plantazolicin.[29-31] Genome mining for gene clusters analogous to that of plantazolicin revealed the distribution of similar biosynthetic gene clusters in Gram-positive bacteria.[32] Among them, the plantazolicin analog coryneazolicin (**1**, Fig. 1a) was synthesized in an in vitro experiment based on the biosynthetic gene cluster of actinobacterium *Corynebacterium urealyticum*.[32] However, the biological activity of coryneazolicin has not yet been reported. Based on this precedent, we successfully accomplished the heterologous production of coryneazolicin using *Escherichia coli* as host cells. Here, we describe the heterologous production and biological activities of coryneazolicin (**1** in Fig 1a).

Results and Discussion

As shown in Fig. 1b, the gene cluster of coryneazolicin (length: 6052 bp) includes six essential genes (*curA*: precursor gene, *curB*: dehydrogenase, *curC*/*curD*: cyclodehydratase complex, *curE*: protease, and *curL*: methyltransferase) and two unknown protein coding genes.[32] The full length of the gene cluster was amplified by PCR and integrated into the vector pET-28a to give the coryneazolicin expression vector pET-28a-10395 (Fig. 2). The vector pET-28a-10395 was cloned in *E. coli* DH5α and transformed into *E. coli* BL21 (DE3). The bacterium *E. coli* BL21 (DE3), harboring pET-28a-10395, was cultured on modified basal agar medium[33] at 23 °C for 4 days with isopropyl-β-D-1-thiogalactopyranoside (IPTG) to express the genes. The bacterial cells were harvested with a spatula and extracted with twice the volume of MeOH. The MeOH extract of the cells was analyzed by HPLC and ESI-MS. ESI-MS analysis showed incompletely modified coryneazolicin analogs (Fig. S1a). Judging from the ESI-MS results, non specific cleavages of the leader sequence were possibly caused by endogenous proteases of *E .coli*. In addition, dimethylation of the *N*-terminus amino residue seemed to be incomplete in this expression system. The gene cluster of coryneazolicin includes genes coding for a protease and a methyltransferase (*curE* and *curL*), and we proposed that the enzymes derived from these genes would not function

78 properly. To compensate for the incompleteness of the modification, we planned to
79 utilize the genes for protease *bamE* and methyltransferase *bamL* in the plantazolicin
80 biosynthetic gene cluster[29] of *Bacillus velezensis*. The genes of protease *bamE* and
81 methyltransferase *bamL* were amplified by PCR and integrated into the vector
82 pACYCDuet-1 to give the vector pACYC-BamLE (Fig. 2). The vector pACYC-BamLE
83 was transformed into *E. coli* BL21 (DE3) harboring pET-28a-10395. The bacterium *E.*
84 *coli* BL21 (DE3), harboring two vectors, pET-28a-10395 and pACYC-BamLE, was
85 cultured on modified basal agar medium containing IPTG at 23 °C for 4 days to express
86 the genes. The HPLC and ESI-MS analyses of the extract of the cells indicated the
87 complete modification by this system to give **1** (Fig. S1b).

88 The molecular formula of **1** was confirmed to be $C_{55}H_{56}N_{18}O_9S_7$ by accurate ESI-MS
89 (Fig. S2) since the ion corresponding to $[M+H]^+$ was observed at m/z 1337.2587 (mass
90 error: -0.972 ppm, the calculated m/z value, 1337.2600). In the ESI-MS experiment, the
91 observed fragmentation ions at m/z 693.2 and 665.2 corresponded to the fragment ion
92 diMeArg-Thz-Oxz-Thz-MeOxz-MeOxz-Ile and its decarbonylated ion, respectively
93 (Fig. 3). These data coincided with the ESI-MS data in a previous report.[32] To
94 determine the structure, the NMR spectra, including the ^1H, ^{13}C, DEPT-135,
95 DQF-COSY, TOCSY, NOESY, HMBC, and HSQC spectra, of coryneazolicin in 500

96 μL of DMSO-d_6 were analyzed. A spin system containing four normal amino acids, one
97 Ile, one Pro, and two Gly, was constructed based on the HSQC, HMBC, DQF-COSY,
98 and TOCSY spectra (Fig. 4 and Table S3). In the same manner, azole groups including
99 seven Thz and two methyl oxazole (MeOxz) units were identified from the NMR
100 experiments (Fig. 4 and Table S3). However, some of the units that were expected in
101 coryneazolicin, including N^{α},N^{α}-dimethylarginine (diMeArg) and Oxz, were not
102 detected in the NMR experiments. Two fragments (partial structures A and B) were
103 constructed from the HMBC and NOESY results (Fig. 4a). The sequence of partial
104 structure A was determined to be MeOxz1-MeOxz2-Ile-Pro. Briefly, the connection
105 between Ile and Pro was determined based on the NOESY correlation between the
106 α-proton of Ile (δH 4.76) and the δ-proton of Pro (δH 3.89). The connection between
107 MeOxz2 and Ile was confirmed based on the NOESY correlation between the amide
108 proton of Ile (δH 7.76) and the methyl protons of MeOxz2 (δH 2.77). A long-range
109 HMBC coupling was observed from the methyl proton of MeOxz1 (δH 2.67) and the
110 methyl proton of MeOxz2 (δH 2.77) to the carbon of MeOxz2 (δC 153.0). The
111 sequence of partial structure B was determined to be Thz-Gly-Gly. The connection
112 between the two Gly units was determined based on the HMBC correlations from the α
113 proton of Gly1 (δH 3.98) and the amide proton of Gly2 (δH 8.32) to the carbonyl

carbon (δC 169.3). The connection between Thz and Gly1 was determined based on the HMBC correlations from the methine proton of Thz (δH 8.58) and the amide proton of Gly1 (δH 8.61) to the carbonyl carbon (δC 160.8).In addition to the Thz unit in fragment B, presence of six other Thz units were indicated in the molecule based on the NMR spectra (Fig. 4b). It was not possible to determine the connections of these Thz units due to their lack of long-range couplings in the HMBC spectrum. Over all, we proposed the structure of coryneazolicin to be **1**, which was identical to the previously reported structure.[32]

The antibacterial activity of **1** was tested against several bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*, to determine the minimum inhibitory concentrations (MIC values). Coryneazolicin (**1**) displayed antibacterial activity against Gram-positive bacteria, including *B. subtilis* (MIC: 0.5 μg/mL), *S. aureus* (MIC: 4 μg/mL), and *M. luteus* (MIC: 2 μg/mL), but no antibacterial activity against the tested Gram-negative bacteria at a concentration of 32 μg/mL. Previously one of LAPs, microcin B17, was reported to have potent inhibitory activity on DNA gyrase, an essential topoisomerase of bacteria.[34, 35] The antibacterial activity of **1** might be due to similar inhibition on DNA gyrase.

To investigate whether treatment with **1** alters the proliferation of cancer cells, we incubated HCT116 and HOS with various concentrations of **1** for 72 h and assessed cell viability using the CellTiter-Glo luminescent cell viability assay. Coryneazolicin (**1**) showed dose-dependent cytotoxicity against these cell lines and showed an IC_{50} values of 6.5 nM (HCT116) and 3.2 nM (HOS) against these cell lines, respectively. These data indicate that **1** is highly toxic to the HCT116 and HOS cancer cell lines. To investigate further mechanism of cytotoxicity, the apoptosis inducing assay using **1** was performed. As a result, apoptosis was observed after 48h from inoculation of **1** with dosage of 20 nM on HCT116 and HOS cancer cell lines. A macrocyclic peptide with eight azole rings, telomestatin, was reported to inhibit the telomerase activity of in vitro cancer cells.[36] The mechanism of apoptosis induction of **1** was also thought to be inhibition of telomerase or topoisomerase.

Materials and methods

Bacterial Strains

The bacterium *Corynebacterium urealyticum* JCM 10395^T was obtained from JCM (Japan Collection of Microorganisms, RIKEN BioResource Research Center, Japan). The bacterial strains *Escherichia coli* NBRC 102203^T, *Pseudomonas aeruginosa* NBRC 12689^T, *Bacillus subtilis* NBRC 13719^T, *Staphylococcus aureus* NBRC 100910^T and *Micrococcus luteus* NBRC 3333^T were obtained from the NBRC culture collection (NITE Biological Resource Center, Japan). The bacterium *Bacillus velezensis* DSM 23117 (formerly *Bacillus amyloliquefaciens* strain FZB42) was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

Molecular cloning of the biosynthetic gene cluster of coryneazolicin.

The gene cluster of coryneazolicin was integrated into the expression vector pET-28a (Merck Millipore, USA) by performing amplification and integration of the partial sequences twice. The crude genome DNA was extracted from *Corynebacterium urealyticum* JCM 10395 using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). The partial sequence (2050 bp) of the gene cluster was amplified by PCR with the primer pair 10395-F2 and 10395-R1 (Table S1) using template DNA of *C.*

urealyticum and EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The insert DNA fragment and the pET-28a vector (Merck Millipore, USA) were double-digested with BamHI-HF and NotI-HF (NEB) according to the manufacturer's instructions. The DNA products were ligated using a DNA Ligation Kit Mighty Mix (Takara Bio Inc.) to afford the plasmid pET-28a-10395A. *E. coli* DH5α cells were transformed with 2.5 μL of the ligation mixture by a chemocompetent transformation, and the cells were plated on LB agar plates containing kanamycin (final concentration of 30 μg/mL). The remaining partial sequence (4070 bp) of the gene cluster was amplified by PCR with the primer pair 10395-F1 and 10395-R2 (Table S1) using EmeraldAmp PCR Master Mix (Takara Bio Inc.). The inserted DNA fragment and the plasmid pET-28a-10395A were double-digested with NcoI-HF and BamHI-HF (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The DNA products were ligated using the DNA Ligation Kit Mighty Mix (Takara Bio Inc.) to afford the plasmid pET-28a-10395, which contained the whole gene cluster of coryneazolicin. *E. coli* DH5α cells were transformed with 2.5 μL of the ligation mixture by a chemocompetent transformation, and the cells were plated on LB agar plates containing kanamycin (final concentration of 30 μg/mL). The plasmid was extracted and isolated using the FastGene

Plasmid Mini Kit (Nippon gene Co., Ltd., Tokyo, Japan) following the manufacturer's instructions. Finally, the plasmid pET-28a-10395 was transformed into the expression host, *E. coli* BL21 (DE3), for the heterologous expression of coryneazolicin.

Construction of the vector pACYC-BamLE

The crude genome DNA was extracted from *Bacillus velezensis* DSM 23117 using a DNeasy Blood & Tissue Kit (Qiagen). The sequence of gene *bamL* was amplified by PCR with the primer pair BamL-F and BamL-R (Table S1) using template DNA of *B. velezensis* and EmeraldAmp PCR Master Mix (Takara Bio Inc.) following the manufacturer's instructions. The insert DNA fragment and the pACYCDuet-1 vector (Merck Millipore, USA) were double-digested with NcoI-HF and KpnI-HF (New England Biolabs) according to the manufacturer's instructions. The DNA products were ligated using the DNA Ligation Kit Mighty Mix (Takara Bio Inc.) to obtain the plasmid pACYC-BamL. *E. coli* DH5α cells were transformed with 5 μL of the ligation mixture by a chemocompetent transformation, and the cells were plated on LB agar plates containing chloramphenicol (final concentration of 20 μg/mL). The sequence of gene *bamE* was amplified by PCR with the primer pair BamE-F and BamE-R (Table S1) using template DNA of *B. velezensis* and EmeraldAmp PCR Master Mix (Takara Bio Inc.) according to the manufacturer's instructions. The insert DNA fragment and the

199 vector pACYC-BamL were digested with KpnI-HF (New England Biolabs) according
200 to the manufacturer's instructions. The DNA products were ligated using the DNA
201 Ligation Kit Mighty Mix (Takara Bio Inc.) to obtain the plasmid pACYC-BamLE. The
202 insertion was confirmed by PCR, and the clone possessing BamL and BamE oriented in
203 the same direction was chosen to obtain *E. coli* DH5α harboring pACYC-BamLE. The
204 plasmid pACYC-BamLE was extracted and isolated using the FastGene Plasmid Mini
205 Kit (Nippon Genetics) following the manufacturer's instructions. The plasmid
206 pACYC-BamLE was transformed into the expression host *E. coli* BL21(DE3) harboring
207 pET-28a-10395 to obtain *E. coli* BL21(DE3) harboring two vectors, pET-28a-10395
208 and pACYC-BamLE. The bacterium *E. coli* BL21(DE3) harboring pET-28a-10395 and
209 pACYC-BamLE was selected and maintained on LB agar medium containing
210 kanamycin (final concentration of 30 μg/mL) and chloramphenicol (final concentration
211 of 20 μg/mL).

212 **Production of coryneazolicin**

213 The bacterium *E. coli* BL21 (DE3) harboring pET-28a-10395 and pACYC-BamLE was
214 cultured using modified basal agar medium (8 L) containing
215 isopropyl-β-D-1-thiogalactopyranoside (final concentration of 0.1 mM) and antibiotics
216 including kanamycin (final concentration of 30 μg/mL) and chloramphenicol (final

concentration of 20 μg/mL). The modified basal agar medium[33] was prepared by combining the inorganic compounds (K_2SO_4, 2 g; K_2HPO_4, 3 g; NaCl, 1 g; NH_4Cl, 5 g; $MgSO_4 \cdot 7H_2O$, 80 mg; $CuCl_2$, 0.5 mg; $MnSO_4 \cdot H_2O$, 2.5 mg; $FeCl_3$, 0.5 mg; and $CaCl_2 \cdot 2H_2O$, 0.5 mg) and 20 g of agar in 1 L of distilled water and adjusting the pH to 7.3. After autoclaving, the medium was supplemented with sterilized glucose and yeast extracts to final concentrations of 0.25% and 0.4%, respectively. The bacterial cells were cultured at 23 °C for 4 days. The bacterial cells were harvested using a spatula and extracted with twice the volume of MeOH. After centrifugation, the MeOH extract was purified by HPLC (column: handy-ODS, 4.6 × 250 mm, FUJIFILM Wako Pure Chemical Co. Osaka, Japan, elution: isocratic mode with 40% MeCN containing 0.05% TFA, UV detector set at 220 nm). HPLC purification was performed repeatedly to obtain coryneazolicin (3.2 mg).

ESI-MS analysis

ESI-MS data were obtained in positive ion mode using a JMS-T100LP mass spectrometer (JEOL Ltd., Tokyo, Japan). For accurate ESI-MS, reserpine was used as an internal standard.

NMR experiments

An NMR sample was prepared by dissolving **1** in 500 μl of DMSO-d_6. All NMR spectra

were obtained on Bruker Avance III HD 800 spectrometers with quadrature detection in the phase-sensitive mode by States-TPPI (time-proportional phase incrementation) and in the echo-antiecho mode. One-dimensional (1D) ^{1}H, ^{13}C, and DEPT-135 spectra were recorded at 25 °C with a 12 ppm window for proton and 239 ppm or 222 ppm windows for carbon. The following 2D ^{1}H-NMR spectra were recorded at 25 °C with 10 ppm spectral widths in the t1 and t2 dimensions: 2D double-quantum filtered correlated spectroscopy (DQF-COSY), recorded with 512 and 1024 complex points in the t1 and t2 dimensions; 2D homonuclear total correlated spectroscopy (TOCSY) with DIPSI-2 mixing sequence, recorded with mixing time of 80 ms, 512 and 1024 complex points in t1 and t2 dimensions; 2D nuclear Overhauser effect spectroscopy (NOESY), recorded with mixing time of 200 ms. 256 and 1024 complex points in the t1 and t2 dimensions. 2D ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond connectivity (HMBC) spectra were acquired at 25 °C in the echo-antiecho mode or in the absolute mode, respectively. The ^{1}H-^{13}C HSQC and HMBC spectra were recorded with 1024 × 512 complex points for 12 ppm or 10 ppm in the ^{1}H dimension and 160 ppm or 222 ppm in the ^{13}C dimension, respectively, at a natural isotope abundance. 2D ^{1}H-^{15}N HSQC spectrum was recorded with 1024 × 128 complex points for 15 ppm in the ^{1}H dimension and 99 ppm in the ^{15}N dimension at a natural isotope

abundance. All NMR spectra were processed using TOPSPIN 3.5 (Bruker). Before Fourier transformation, the shifted sinebell window function was applied to the t1 and t2 dimensions. All ^{1}H and ^{13}C dimensions were referenced to DMSO-d_6 at 25 °C.

Antimicrobial activity

The antibacterial activity of **1** was established by minimum inhibitory concentration (MIC) tests in 96-well microplates following a previous report.[37, 38] The MICs of **1** against Gram-positive and Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *M. luteus*, were determined. The bacterial strains were cultured in nutrient agar media for 24 h. The testing system contained bacterial cells (approximately 10^5 CFU/mL) and various concentrations of the test compounds in 100 μL of Muller-Hinton liquid medium. The microplates were incubated at 30 °C for 24 h. Tetracycline was used as a positive control reagent to evaluate the antibacterial activity of **1** (Table S2).

Cytotoxic assay

HCT116 (3.0×10^3) and HOS (3.0×10^3) cells were aliquoted in 96-well plates and treated with coryneazolicin (1–20 nM) in D-MEM (HCT116) or E-MEM (HOS) containing FBS (10%), respectively. Cell viability was assayed after 72 h by using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, USA) with a JNR

271 Luminescencer (ATTO, Tokyo, Japan) according to the manufacturer's protocol.

272

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384 Figure legends

385

386 Fig. 1. a) Chemical structure of coryneazolicin (**1**), and b) biosynthetic gene cluster of

387 coryneazolicin (*curA*: precursor gene, *curB*: dehydrogenase, *curC*/*curD*:

388 cyclodehydratase complex, *curE*: protease, and *curL*: methyltransferase)

389 Fig. 2. Vector maps of the constructed vectors for the production of coryneazolicin (**1**)

390 Fig. 3. Fragmentation ions of coryneazolicin (**1**) obtained by ESI-MS in positive ion

391 mode

392 Fig. 4. a) Key NMR correlations for construction of partial structures A and B. b) Key

393 NMR correlations for detection of six thiazoles. Plain letters indicate proton chemical

394 shifts and bold letters indicate carbon chemical shifts.

395

396 Fig.1

397

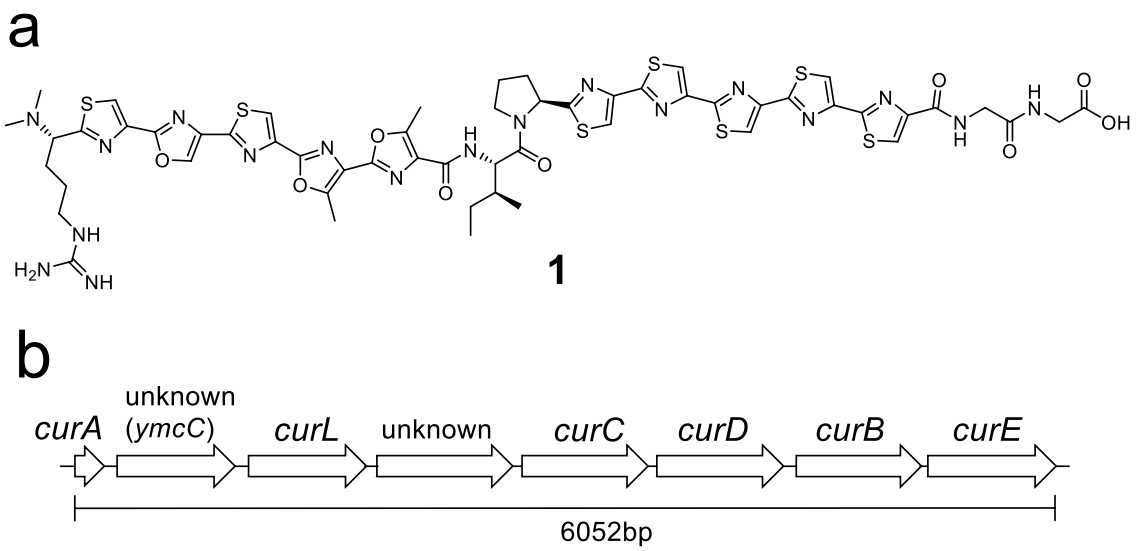
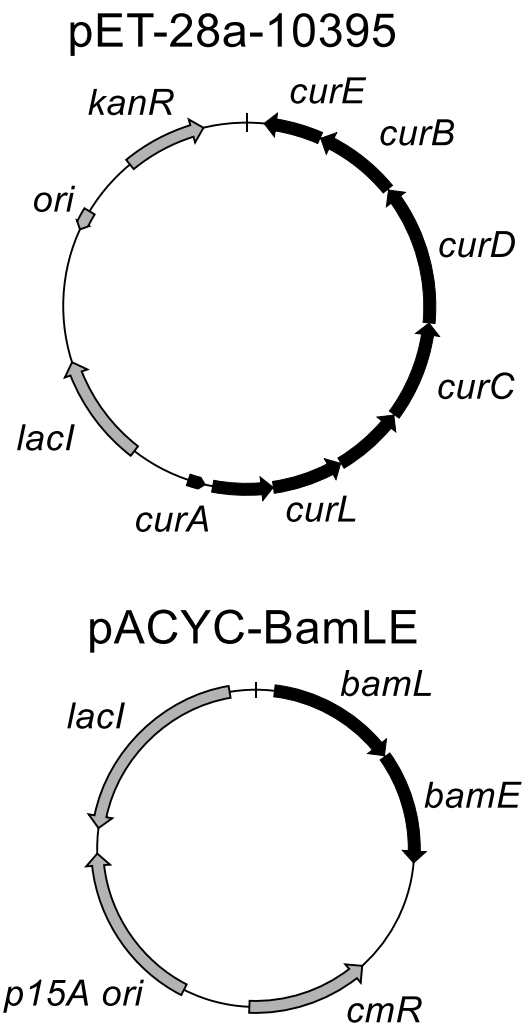

a
1
b
curA
unknown
(ymcC)
curL
unknown
curC
curD
curB
curE
6052bp

Fig. 2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kanR
curE
curB
ori
curD
curC
lacI
curA
curL
pACYC-BamLE
lacI
bamL
bamE
p15A ori
cmR

404 Fig. 3

405

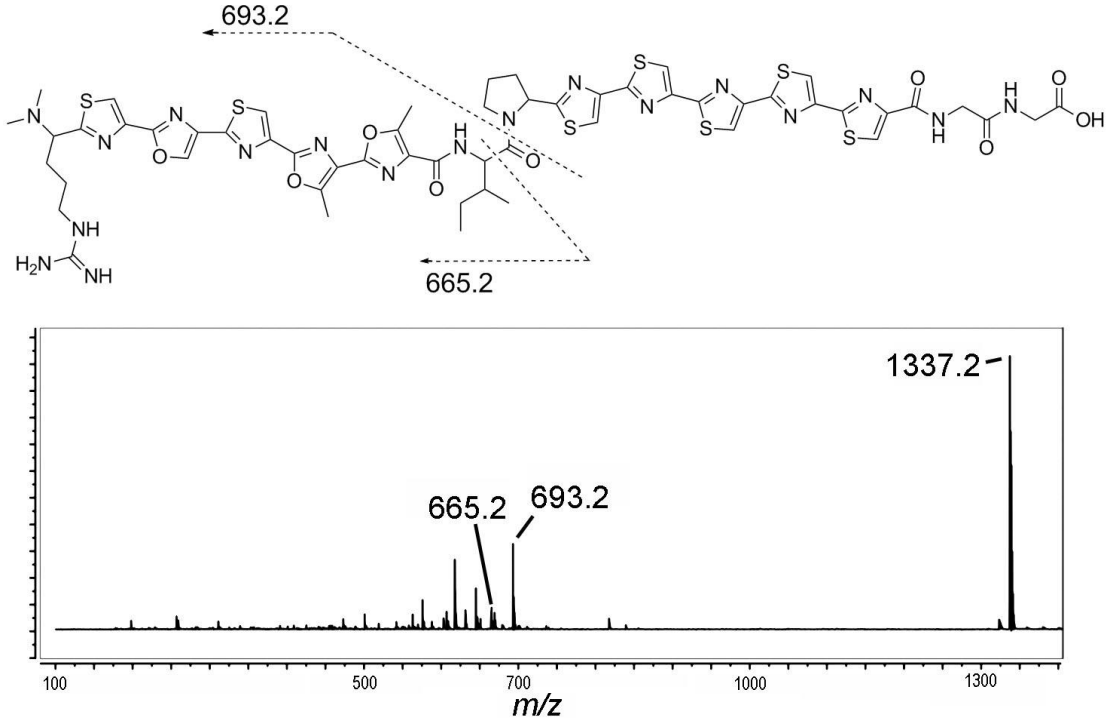
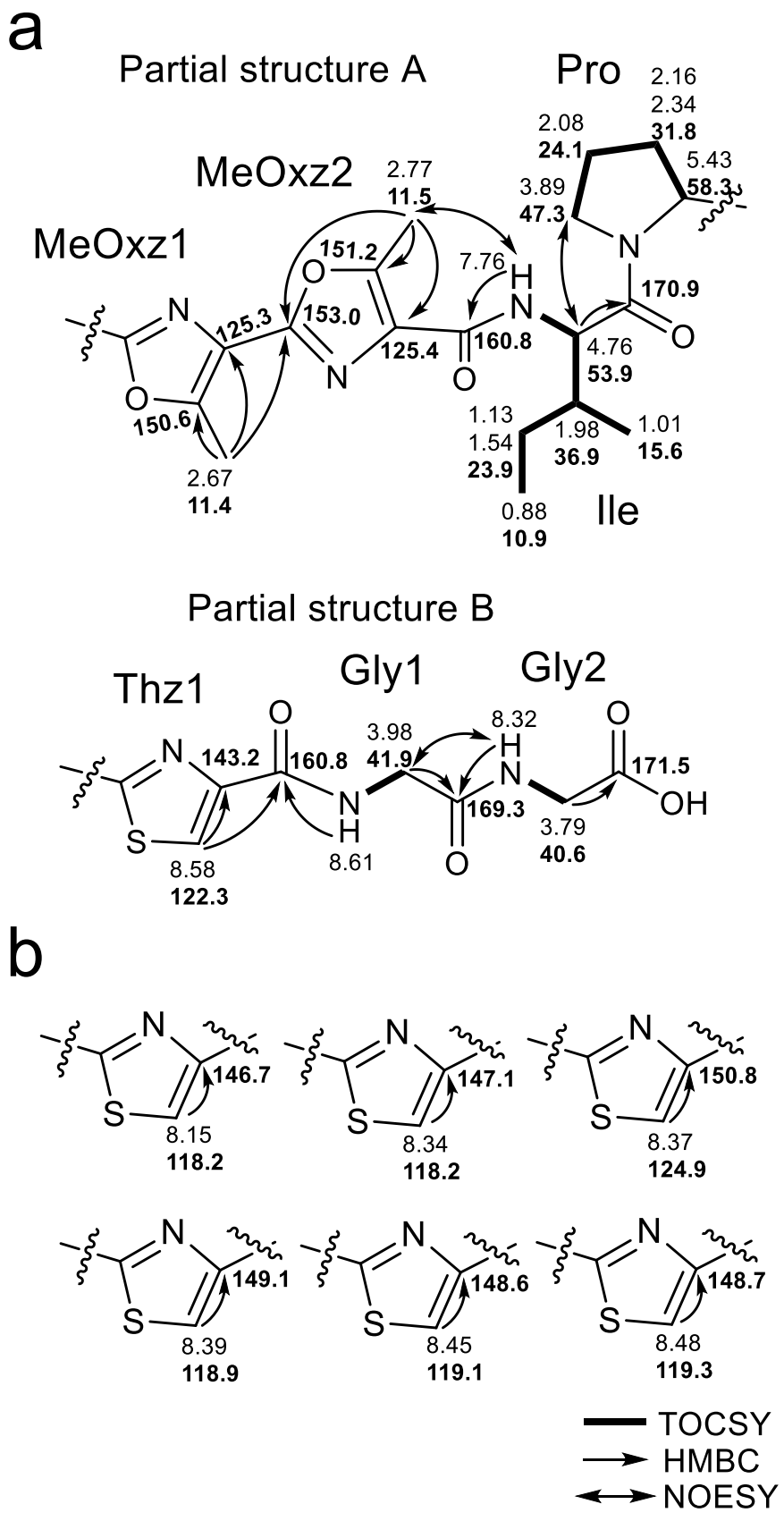


Fig. 4


a
Partial structure A
Pro
MeOxz2
MeOxz1
Ile
Partial structure B
Thz1
Gly1
Gly2
b
TOCSY
HMBC
NOESY