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Isolation and structure determination of a new lantibiotic cinnamycin B from Actinomadura atramentaria based on genome mining

メタデータ	言語: eng 出版者: 公開日: 2017-06-13 キーワード (Ja): キーワード (En): 作成者: Kodani, Shinya, Komaki, Hisayuki, Ishimura, Sho, Hemmi, Hikaru, Ohnishi-Kameyama, Mayumi メールアドレス: 所属:
URL	http://hdl.handle.net/10297/10284

Isolation and structure determination of a new lantibiotic cinnamycin B from *Actinomadura atramentaria*

Running headline: Structural determination of cinnamycin B

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Abstract

New lantibiotic cinnamycin B was isolated from the extract of *Actinomadura atramentaria* NBRC 14695^{T}, based on genome-mining and chemical investigation. The partial structure of cinnamycin B was established by 2D NMR experiments, which indicated that cinnamycin B had same methyl lanthionine bridging pattern with cinnamycin. The reduction with $NaBH_4$-$NiCl_2$ afforded the reduced cinnamycin B, and MS/MS experiment indicated the presence of hydroxy asparatic acid in the molecule. Cinnamycin B showed an antibacterial activity against *Streptomyces antibioticus* with dosage of 5 μg at spot-on-lawn testing method. The gene cluster of cinnamycin B on the genome of *A. atramentaria* was identified and discussed in comparison with that of cinnamycin.

Keywords: *Actinomadura atramentaria* NBRC14695, lantibiotic, NMR spectrum, MS/MS analysis

Introduction

Lantibiotics are a class of peptide antibiotics that contain thioether amino acids including lanthionine and/or methyllanthionine [26,21,5]. In addition to the thioeter amino acids, lantibiotics normally possess the unsaturated amino acids including dehydroalanine and/or 2-aminoisobutyric acid. Lantibiotics are produced by a large number of gram-positive bacteria such as *Streptococcus* and *Streptomyces*. A quadrocyclic lantibiotic cinnamycin (**2**, Fig. 1) was isolated as an antibacterial compound from *Streptomyces cinnamoneus* [3,6,1]. Cinnamycin had the structure containing unique unusual amino acids such as lysinoalanine and β-hydroxy aspartic acid (HOAsp) in addition to unusual amino acids like lanthionine and methyllanthionine to form cyclic structure. The cinnamycin-related lantibiotics including ancovenin [11] and duramycins [8,7] were isolated from actinomycetes. Among the peptides, the biosynthetic cluster of cinnamycin was cloned and engineered in heterologous expression experiments [25]. The posttranslational modification system of cinnamycin was studied [19]. The genetic studies indicated that just 4 genes including *cinA*, *cinM*, *cinX*, and *cinorf7* are required for formation of cinnamycin [19]. Especially, a small protein encoded by *cinorf7* is indicated to be critical for the formation of lysinoalanine by coexpression studies in *E. coli* [19]. Among four genes, *cinX* coding protein was

indicated to be an enzyme catalyzing the hydroxylation of Asp to give HOAsp [19].

Recently, the bioactivities of duramycin and cinnamycin have been focused since they can become possible probes for phosphatidylethanolamine (PE) [10]. Duramycin and cinnamycin were indicated to bind the head group of PE with high affinity at a molar ratio of 1:1, with the dissociation constant toward PE-containing lipid membranes in the low nanomolar range [16]. The compounds like duramycin and cinnamycin which disrupt PE association with phosphatidylserine receptors necessary for entry of many enveloped viruses are a promising broad-spectrum antiviral strategy [22]. These circumstances prompted us to find new lantibiotic related to cinnamycin. Based on the genome mining approach using cinnamycin biosynthetic gene, we found new lantibiotic named cinnamycin B from *Actinomadura atramentaria* NBRC 14695^{T}. The structure was determined by the combination of NMR and MS analyses. Here we describe the isolation and structural determination of new lantibiotic cinnamycin B (**1**, Fig. 1) and the assignment of putative biosynthetic gene cluster of cinnamycin B.

Materials and Methods

Bacterial strains

The microorganisms (bacterial strains including *Actinomadura atramentaria* NBRC 14695^{T}, *Escherichia coli* NBRC 102203^{T}, *Serratia marcescens* NBRC102204^{T},

Pseudomonas aeruginosa NBRC 12689^{T}, *Bacillus subtilis* subsp *subtilis* NBRC 13719^{T}, *Staphylococcus aureus* subsp. *aureus* NBRC 100910^{T}, *Micrococcus luteus* NBRC 3333^{T}, *Streptomyces antibioticus* NBRC 3117; Yeast strains including *Saccharomyces cerevisiae* NBRC 2376, *Schizosaccharomyces pombe* NBRC0340, *Kloeckera apiculata* NBRC 0154; fungi strains including *Aspergillus niger* NBRC 33023^{T}, *Aspergillus oryzae* NBRC 4290, *Mucor hiemalis* NBRC 9405^{T}) were obtained from the NBRC culture collection (Biological Resource Center, NITE, Japan).

Isolation of cinnamycin B

Actinomadura atramentaria NBRC 14695^{T} was cultured using 3L of ISP2 agar medium for 7 days at 30 °C. The aerial hyphae and spore cells on the agar surface were harvested with steel spatula. Double volume of MeOH was added to the harvested cells, followed by filtration with paper filter (Whatman No. 1, GE Healthcare Life Sciences, Little Chalfont, UK). The MeOH extract was concentrated to an aqueous residue using rotary evaporator. The aqueous residue was subjected to open column chromatography using hydrophobic resin CHP-20P (Mitsubishi Chemical, Tokyo, Japan), eluted with 10% MeOH, 60% MeOH, and 100% MeOH. The 100% MeOH fraction was subjected to HPLC purification using C8 column (4.6 × 250 mm, 5C8-MS, nacalai tesque, Kyoto, Japan) with isocratic elution from 30% MeCN

containing 0.05% trifluoroacetic acid with UV detector set at 220 nm to yield 5.3 mg of **1** (Retention time: 15.1 min).

Amino acid analysis

The amino acid content of **1** was determined by HPLC analysis of PTC-derivatized amino acids [9]. For hydrolysis, compound **1** (0.2 mg) was incubated in 100 μL of 6N HCl at 110 °C for 16 h. Aliquot of 40 μL of hydrolyzed sample, or aliquot of 10 μL of amino acid mixture standard solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was evaporated by a freeze dryer. Aliquots of 20 μL of ethanol/water/triethylamine (2/2/1, v/v/v) were added to each sample and evaporated by a freeze dryer. For PTC-derivatization of amino acids, aliquots of 50μl of ethanol/water/triethylamine/phenylisocyanate (7/1/1/1, v/v/v/v) were added to each sample, and derivatization was accomplished by incubation at room temperature for 20 min. After evaporation using a freeze dryer, 1.0 ml of PTC-derivatized amino acid mobile phase A (60mM CH_3COONa aqueous solution pH 6.0/MeCN, 6:94) was added to dissolve each sample. The PTC-derivatized samples were analyzed using an HPLC system (PU980 system, JASCO, Tokyo, Japan). The conditions for HPLC analysis were following: HPLC column, Wakopak Handy-ODS (4.6 mm × 250 mm; Wako Pure Chemical Industries, Ltd.); mobile phase A (60mM CH_3COONa aqueous solution pH

6.0/MeCN, 6:94); mobile phase B (60mM CH_3COONa aqueous solution pH 6.0/MeCN, 60:40); flow rate, 1 ml/min; gradient of mobile phase B, 5% to 65% from 0 to 30 min (linear gradient); UV detector, 254 nm.

Reduction of cinnamycin B with $NaBH_4$-$NiCl_2$

Compound **1** (1.0 mg) and $NiCl_2$ (1.0 mg) were suspended in 1.0 mL of MeOH/H_2O(1:1). $NaBH_4$ (1.0 mg) was added to the mixture, followed by stirring for 1h at 50 °C. A black precipitate (Ni_2B) formed with generation of deuterium gas. After centrifugation at 14500 rpm for 1 min, supernatant was evaporated by rotary evaporator. The dried material was re-dissolved in MeOH (0.5 mL). The solution was subjected to HPLC analysis using ODS column C8 column (4.6 × 250 mm, 5C8-MS, nacalai tesque) with isocratic elution from 30% MeCN containing 0.05% trifluoroacetic acid with UV detector set at 220 nm to yield reduced cinnamycin B (Retention time: 11.2 min).

NMR experiments

An NMR sample was prepared by dissolving **1** in 500 μl of DMSO-d_6. 1D ^1H, ^{13}C, DEPT-135, and all 2D NMR spectra were obtained on Bruker Avance800 spectrometer with quadrature detection. The 1D ^1H, ^{13}C, DEPT-135 spectra were recorded at 25°C with 20 ppm for proton and 240 ppm for carbon. The following 2D ^1H-NMR spectra were

recorded at 25°C with 10 ppm or 12 ppm spectral widths in *t1* and *t2* dimensions in the phase-sensitive mode by States-TPPI method: 2D double quantum filtered correlated spectroscopy (DQF-COSY), recorded with 512 and 2048 complex points in *t1* and *t2* dimensions; 2D homonuclear total correlated spectroscopy (TOCSY) with MLEV-17 mixing sequence, recorded with mixing time of 80 ms, 256 and 2048 complex points in *t1* and *t2* dimensions; 2D nuclear Overhauser effect spectroscopy (NOESY), recorded with mixing time of 200 ms, 256 and 2048 complex points in *t1* and *t2* dimensions. Water suppression was performed using presaturation method. 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 or 1024 × 256 complex points for 12 ppm in the ^{1}H dimension and 170 ppm in the ^{13}C dimension or for 10 ppm in the ^{1}H dimension and 170 ppm in the ^{13}C dimension, respectively, at a natural isotope abundance. 2D ^{1}H-^{15}N HSQC spectrum was also recorded at 25°C with 1024 × 128 complex points for 12 ppm in the ^{1}H dimension and 50 ppm in the ^{15}N dimension in the phase-sensitive mode by States-TPPI method at a natural isotope abundance. All NMR spectra were processed using XWINNMR (Bruker). Before Fourier transformation, the shifted sinebell window function was

applied to *t1* and *t2* dimensions except for the HMBC spectrum. All ^{1}H and ^{13}C dimensions were referenced to DMSO-*d6* at 25°C.

MS experiments

Composition formulae of compounds were calculated from accurate mass values obtained from an electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (solarix XR, Bruker Daltonics) in the positive-ion mode. MS/MS spectra were recorded on a matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)/TOF mass spectrometer (4800 plus TOF/TOF analyzer, AB SCIEX, CA, USA) in the positive-ion mode with an acceleration voltage of 20 kV. α-Cyano-4-hydroxycinnamic acid (Bruker Daltonics, MA, USA) was used as the matrix.

Results

By BLAST similarity search [4] using the amino acid sequence of cinnamyin (accession number, P29827.1), we found four cinnamycin analogue prepeptide genes in four actinomycetes (*Actinomadura atramentaria, Actinomadura oligospora. Streptomyces roseoverticillatus ,and Nocardiopsis potens*, in Fig. 2). The leader peptide sequences (63 amino acids residues from N-terminus in the case of CinA, Fig. 2) that are cleaved off during modification had the conserved amino acid motif shown by underlines in Fig. 2. The structural peptide sequences were conserved and the sequence of CSXGXFTXXCDGXTK was found to be common motif for cinnamycin analogues as shown in bold letters in Fig. 2. The genetic data prompted us to perform chemical investigation on *Actinomadura atramentaria* searching for new cinnamycin analogue. As a result of preliminary culture and chemical analysis using HPLC and ESI-TOF-MS (Fig. S1), the production of new cinnamycin analogue cinnamycin B (**1**, Fig. 1) was detected in MeOH extract of culture of *A. atramentaria* NBRC 14695^T.

To obtain enough amount of **1** for structure determination, cultivation of *A. atramentaria* NBRC 14695^T was performed with 3 L of ISP2 agar media. After 7 days of cultivation, cells of spore and aerial hyphae were harvested by steel spatula. The cells were extracted with double volume of MeOH, followed by centrifugation. After

condensation using rotary evaporator, the extract was subjected to open column chromatography using hydrophobic resin CHP-20P, eluted with 10%, 60%, and 100% MeOH. The 100% MeOH fraction was repeatedly subjected to HPLC purification to afford **1**.

The molecular formula of **1** was established to be $C_{79}H_{114}N_{20}O_{25}S_3$ by the accurate mass analysis using ESI-FTCR mass spectrometry ($[M+2H]^{2+}$ at *m/z* 920.3792 was of $C_{79}H_{116}N_{20}O_{25}S_3$ whose calculated value was 920.3786). The molecular formula indicated that same posttranslational modification (four dehydration:-H_2O × 4, one hydroxylation: +O) may occur during maturation of cinnamycin prepeptide (CASTCSAGPFTFVCDGTTK; molecular formula, $C_{79}H_{122}N_{20}O_{28}S_3$), compared to biosynthesis of cinnamycin. According to previous report [9], amino acid content analysis of cinnamycin B was performed and the analysis gave 2 moles each of Gly, Ala, and Phe, 1 mole each of Ser, Thr, Pro, Val at mole rate. To obtain further information on the chemical structure, NMR experiments including ^{1}H, ^{13}C, HMQC, HMBC, DQFCOSY, TOCSY, and NOESY analyses of **1** were performed in DMSO-d_6 (Fig. S4-S10). As a result of analysis of NMR data, four partial peptide sequences (Gly16-Thr17-S-Abu18-Lys19, S-Ala1-Ala2-Ser3-S-Abu4-S-Ala5, Phe12-Val13-S-Ala14, Phe10-S-Abu11) were established by correlations of HMBC and NOESY

experiments (Fig. 3a). The bridging pairs of methyl lanthionines were established to be S-Ala1/S-Abu18, S-Ala14/S-Abu4, and S-Ala5/S-Abu11 by correlation of NOESY between β-protons of S-Ala and S-Abu (Fig. 3a). This result indicated that cinnamycin B possessed the same lanthionine bridging pattern with cinnamycin [3]. The amino acid residues of Ala7, Gly8, and Pro9 were assigned by NMR spectra, although amide protons of Ala7 and Gly8 were not observed. The connections could not be established by HMBC or NOESY experiment regarding the amino acids of Ala7, Gly8, and Pro9. Based on the amino acid sequence of biosynthetic gene of cinnamycin B prepeptide, the sequence of the amino acids was proposed to be Ala7-Gly8-Pro9. Since the methyl lanthionine bridges hamper the fragmentation analysis by MS/MS experiment, $NaBH_4$-$NiCl_2$ reduction was performed on **1**, according to the previous reports [18,15,12]. After HPLC purification, the molecular formula of the reduced cinnamycin B was established to be $C_{79}H_{116}N_{20}O_{25}S$ by the accurate mass analysis using ESI-FTCR mass spectrometry ($[M+2H]^{2+}$ at *m/z* 889.4146 whose calculated value was 889.4143). Reduction with $NaBH_4$-$NiCl_2$ on lanthibiotic was reported to give amino butyric acid (Abu) and Ala from methyl lanthionine [18,15,12]. The discrepancy of the molecular formula between before/after the reduction indicated that two methyl lanthionine were reduced (theoretically, loss of two sulfur:-S_2 and gain

of four hydrogen: $+H_4$). However, the discrepancy was loss of two sulfur ($-S_2$) and gain of two hydrogen ($+H_2$), which indicated that an unexpected dehydrogenation or incomplete reduction could occur during the reaction. The MS/MS fragmentation analysis indicated the two amino acid sequences of Ala-Ala-Ser-dehydrobutric acid (Dhb) and Val-Ala-HOAsp-Gly (Fig. 3b), indicating the presence of HOAsp in the molecule. Although two amino acids including N-Ala6 and HOAsp15 were not detected by NMR experiments, we proposed the structure of cinnamycin B to be **1** (Fig. 1), based on the chemical analyses described above and the biosynthetic prepeptide sequence.

The anti-microbial assay using spot-on-lawn method was accomplished against microorganisms (Bacterial strains including *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Streptomyces antibioticus*; Yeast strains including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kloeckera apiculata*; fungi strains including *Aspergillus niger*, *Aspergillus oryzae*, *Mucor hiemalis*) following a previous report [24,20]. Cinnamycin B showed antibacterial activity only against *Streptomyces antibioticus* at the dosage of 5 μg (Fig. S11).

Discussion

The cinnamycin biosynthetic gene (*cin*) cluster of *Streptomyces cinnamoneus* DSM 40005^{T} contains 21 open reading frames (ORFs), among which c*inA*, *cinM*, *cinX* and *cinorf7* are essential for cinnamycin synthesis. *CinT*, *cinH* and *cinY* are responsible for self-resistance, and c*inR* and *cinK* are for regulation, but the functions of the remaining twelve ORFs are unclear [25,19]. Since the draft genome sequence of *Actinomadura atramentaria* DSM 43919^{T} (=NBRC 14695^{T}) is available in public databases, we searched the biosynthetic gene cluster for cinnamycin B in the genome (Table S2, Figure 4a) and compared it with the *cin* cluster of *S. cinnamoneus* DSM 40005^{T} (Fig. 4b). G339_RS0112030 (WP_019631332) encodes preprocinnamycin B homologous to CinA. An AYA motif, similar to AFA in CinA, is present as cleavage site between the leader sequence and the core region for cinnamycin B (Fig. 2). In cinnamycin production, the AFA sequence is predicted to be recognizes by type I signal peptidase of the general secretory pathway for the explanation of the absence of cinnamycin-specific protease in the cluster [25]. In contrast, we found that a protein encoded by G339_RS0112005 has trypsin-like serine protease region, which may be responsible for the cleavage. Just next to preprocinnamycin B gene, genes homologous to *cinorf7*, *cinM* and *cinX* are present and the gene organization is the same as that of the *cin* cluster (Figure 4). Therefore, we assumed that these three genes are responsible for

posttranslational modification to the procurer peptide to yield cinnamycin B in the same manner as cinammycin synthetic pathway[19]. CinM homolog (G339_RS0112025), a member of the LanM family[2], catalyzes the dehydration Thr4, Ser6, Thr11 and Thr18 in the precursor peptide and the subsequent cyclization of three Cys residues to form three methyllanthionine bridges. CinX homolog (G339_RS0112020), an α-ketoglutarate/iron(II)-dependent hydroxylase, hydroxylates Asp15 of the precursor peptide. Cinorf7 homolog (G339_RS0112035) is involved in the formation of cross-link between Lys19 and dehydroalanine6 to make a lysinoalanine bridge. Downstream of *cinX* homolog, G339_RS33385 is present and has been annotated to encode a hypothetical protein (319 aa) by RefSeq. However, we found it to encode an ABC transporter (666 aa) through the manual annotation in this study. In the *cin* cluster of *S. cinnamoneus* DSM 40005^{T}, the ABC transporter corresponding to this composed of two proteins of CinT (309 aa) and CinH (290 aa), which encode ATP-binding subunit and membrane permease subunit, respectively, and suggested to be involved in drug efflux and resistant. The *cin* cluster of *S. cinnamoneus* DSM 40005^{T} harbors *cinY* in addition to *cinT* and *cinH* for self-resistance. In contrast, cinnamycin B gene cluster does not encode *cinY* homolog, suggesting the ABC transporter encoded by correctly annotated G339_RS33385 may entirely fulfill the immunity role for lantibiotics without

CinY-like proteins. The cinnamycin B gene cluster contains two transcriptional regulatory genes, but they are not homologs of *cinR* and *cinK*, suggesting the regulatory systems of are distinct between productions of cinnamycin by *S. cinnamoneus* DSM 40005 ^{T} and cinnamycin B by *A. atramentaria* DSM 43919^{T}. ORFs other than the homologs of *cinA*, *cinM*, *cinX cinorf7* and *cinTH* in the cinnamycin B gene cluster did not show similarities to any genes in the *cin* cluster of *S. cinnamoneus* DSM 40005 ^{T}. Thus, except for these five genes, transcriptional regulatory genes and a protease gene, the others may not be essential for the production of cinnamycins.

Normally, few secondary metabolites are produced in laboratory condition compared with the numbers of biosynthetic gene clusters especially in actinomycetes. In the same manner with *A. atramentaria*, we performed chemical investigation on the other actinomycetes (*Streptomyces roseoverticillatus*, *Nocardiopsis potens*, *Actinomadura oligospora*) which had possible cinnamycin biosynthetic genes. As a result, no production of cinnamycin analogue was observed on the three actinomycetes in the condition (Data not shown).

Genome-mining approach is powerful method to find new secondary metabolites [14], especially new ribosomally synthesized peptides including lantibiotics [23], thiopeptides [13], and lasso-peptides [17]. Without usual chemical screening of many

numbers of actinomycetes (bacterial culture, extraction, and antibacterial assay), in silico screening from massive genome data can be possible to find the new peptides. In this study, just four strains were chemically screened to find a new cinnamycin analogue, indicating there was the possibility that more peptides could be found from larger numbers of bacterial genome data. We believed that this study indicated one example which proved the efficiency of genome-mining approach.

Conflict of interest

The authors disclose no conflict of interest.

Acknowledgments

This study was supported by the Japan Society for the Promotion of Science by Grants-in-aids (grant number 25350964).

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Figure legends

Figure 1. Structures of cinnamycin B (**1**) and cinnamycin (**2**).

Figure 2. Alignments of amino acid sequences of cinnmycin prepeptide gene analogues.

Arrow indicates cleavage site. Abbreviations, *S. cin*: *Streptomyces cinnamoneous; A. art:*

Actinomadura atramentaria; S.ros: Streptomyces roseoverticillatus;

N. pot: Nocardiopsis potens; A. oli: Actinomadura oligospora.

Figure 3. a) Key 2D NMR correlations of partial structure of **1**, b) MS/MS

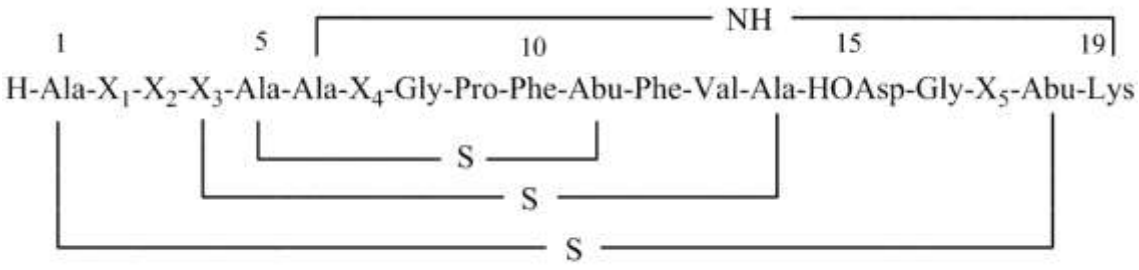
fragmentation analysis on reduced **1**

Figure 4. Gere organizations of cinnamycin B biosynthetic gene cluster in

Actinomadura atramentaria (a) and cinnamycin biosynthetic gene (*cin*) cluster in

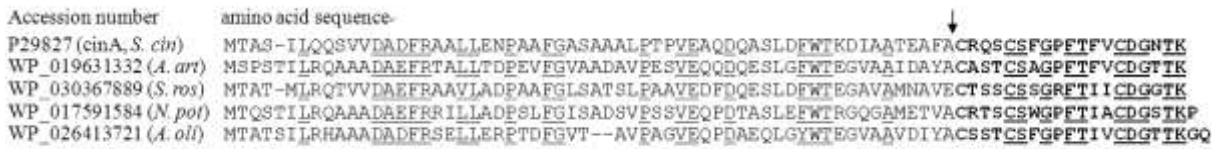
Streptomyces cinnamoneous (b).

The homologous gene pairs are shown in the same colors or patterns.



Cinnamycin B (**1**) X_1=Ala, X_2=Ser, X_3=Abu, X_4=Ala, X_5=Thr
Cinnamycin (**2**) X_1=Arg, X_2=Gln, X_3=Ala, X_4=Phe, X_5=Asn

Figure 1. Kodani et al.



Arrow indicates cleavage site.
S.cin: Streptomyces cinnamoneous; A. art: Actinomadura atramentaria; S.ros: Streptomyces roseoverticillatus
N. pot: Nocardiopsis potens; A. oli: Actinomadura oligospora

Figure 2. Kodani et al.

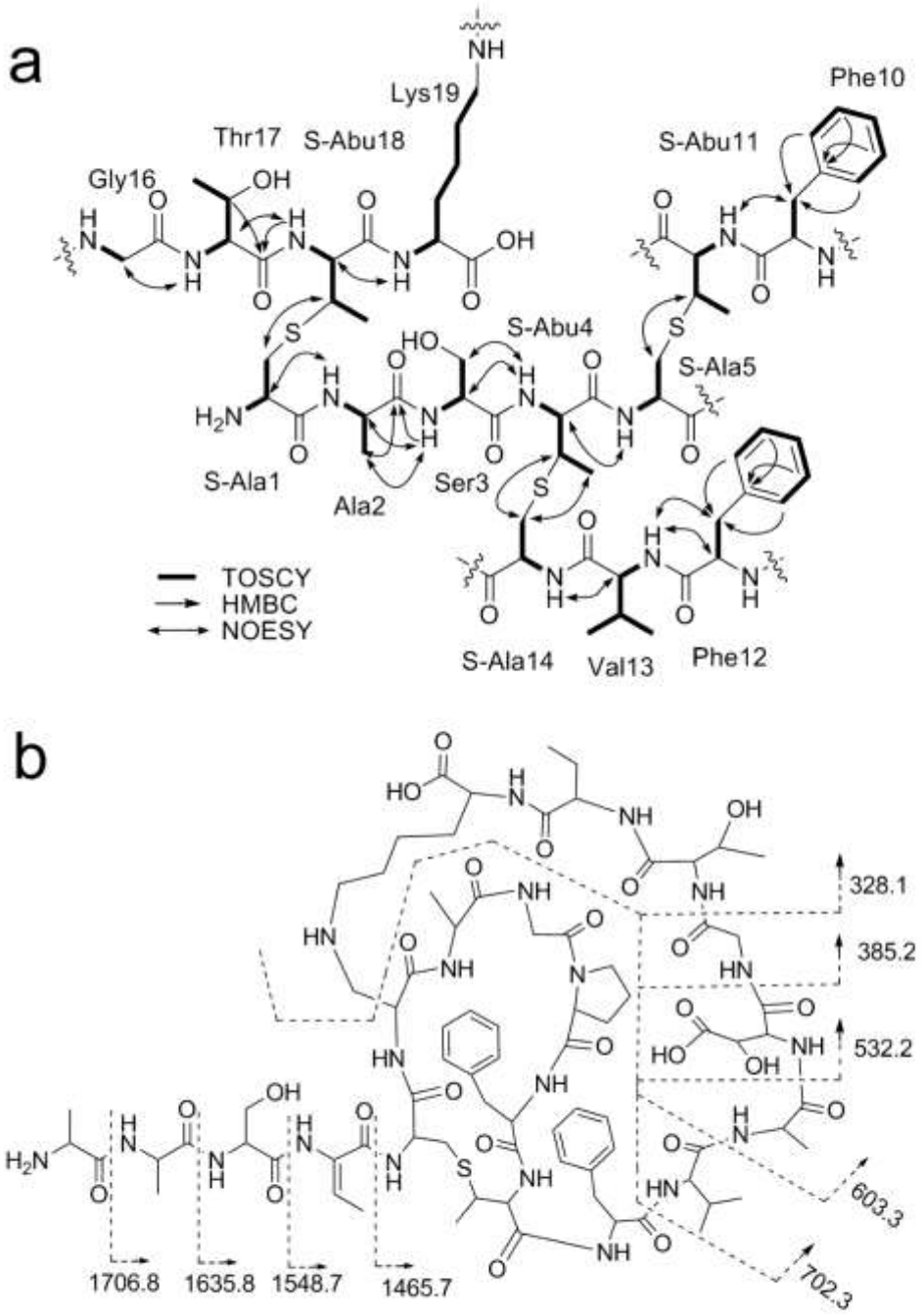


Figure 3. Kodani et al.

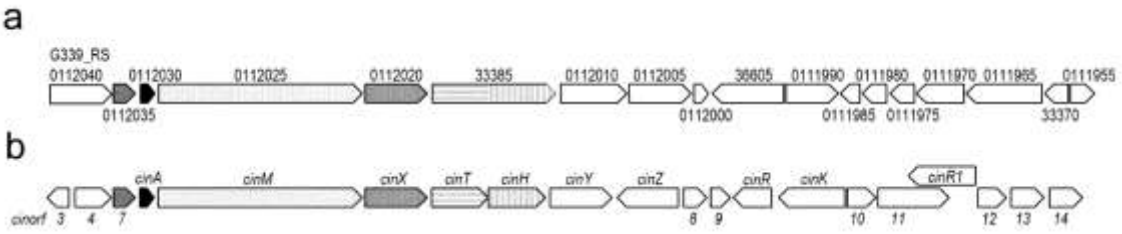


Figure 4. Kodani et al.