Electronic Supplementary Information

Active site modification of the β-ketoacyl-ACP synthase FabF3 of *Streptomyces coelicolor* affects the fatty acid chain length of the CDA lipopeptides.

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Content

Materials and Methods

Bacterial strains, microbiological methods and genetic manipulations. The *Streptomyces coelicolor* A(2) strain, MT1110 which is a SCP1$^-$, SCP2$^-$ derivative of the wt strain 1147 was used [S1]. All methods used for growth and transformation of *E. coli* and *S. coelicolor* are as described previously [S1, S2]. A 2kb DNA fragment spanning *fab*F3 was amplified by PCR using *S. coelicolor* MT1110 genomic DNA as template and primers fabF3Up and fabF3Down (Table 1). The PCR product was digested with *Xba*I (Roche) and cloned into similarly cut pHSG398 [8], treated with Antarctic phosphatase (NEB), to yield pRAL75. This construct was used in the QuikChange protocol (Stratagene) using a series of mutagenic primer pairs (Table 1) designed to substitute Ile (FabF3IleUp & fabF3IleDown), Leu (FabF3LeuUp & fabF3LeuDown) and Ala (FabF3AlaUp & fabF3AlaDown) residues for Phe108.The mutagenized codons were selected according to *S. coelicolor* codon usage preference. The presence of the desired mutations was verified by DNA sequencing (MWG) using forward and reverse universal M13 primers, and the mutated inserts were each cloned into the *S. coelicolor* suicide vector pMAH as *Xba*I fragments to generate the deletion constructs pRAL7591 (F-I), pRAL7592 (F-L) and pRAL7593 (F-A) respectively. After passaging through the DNA demethylating *E. coli* strain ET12567 these constructs were used to transform *S. coelicolor* MT1110 protoplasts and transformants were selected for using hygromycin. Following two rounds of unselective growth hygromycin sensitive colonies, identified by replica plating, were screened, using DNA sequencing (MWG) of PCR products generated using primers fabf3M13foward & faBf3M13reverse (Table 1) spanning the mutated region of *fabF3*, to identify mutants. CDA plate bioassays were effected as described previously [S3] using standardized numbers of spores (5×10^7).

Cultivation of *S. coelicolor*, Isolation and Detection of CDA variants. Wild-type MT1110 and mutant strains f*abF3* F-I, F-L, F-S were grown on solid MS media, then inoculated into SV2 liquid media[S2] and incubated for a further 5 days at 28ºC and 180 rpm. Liquid media was spun down at 4500 rpm for 10 minutes; the supernatant was filtered, adjusted to pH 2.0 with 1M HCl and then re-filtered before being loaded onto a Varian C-18 Bond Elute SPE cartridge. Crude CDA was extracted using a methanol gradient (30-100%), evaporated under reduced pressure and analysed using LC-MS [4].

Purification of CDA variants. CDA extracts were purified on a Varian Prostar instrument, equipped with a Prostar 335 photodiode array detector. Purification was achieved using semipreparative reversed phase HPLC: Phenomenex C-18 5mm, 250 × 10 mm column. Solvent A was H_2O containing 0.1% HCO_2H and solvent B was acetonitrile containing 0.1% HCO_2H. At a flow rate of 3 mL min^{-1} B was increased from 5% to 100% over a 30 min linear gradient, held at 100% for 5 min before being brought back down to 5% (pre-equilibration for next run); detection by UV at 280 nm and 350 nm. Fractions were analysed by LC-MS [4].

Analysis of CDA Extracts Using LC-MS. General LC-MS analysis was carried out on a Micromass LCT orthogonal acceleration time of flight mass spectrometer, equipped with an electrospray ionisation source run in positive mode (scanning from 700 to 1700 *m/z*) combined with a Waters 2790 Separation module. A reversed phase C-18 150 x 4.6 mm 3 μm Phenomenex column was used. Solvent A was H_2O with 1 % acetonitrile and 0.1 % HCO_2H and solvent B was acetonitrile with 1 % H_2O and 0.1 % HCO_2H. At a flow rate of 1 ml min^{-1} with a gradient of 80 % A and 20 % B, increasing to 70 % B over 10 min and increased to 100 % B over the 1 min and held for a further 4 min.

Accurate Mass and Tandem MS analyses of CDA using LTQ-Orbitrap XL- nUPLC

Samples were initially diluted in water, and subsequently in 95 % water/ 5 % acetonitrile supplemented with 0.1 % HCO_2H (hereafter referred to as Buffer A1). Data were acquired using a nanoacquity chromatograph (Waters MS technologies) coupled to an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific) equipped with the manufacturer's dynamic nanospray source, fitted with a PicoTip emitter (20-10 µm diameter, New Objective). The sample temperature in the autosampler was maintained at 10 ºC, and 4 µl of sample was injected initially onto a trapping column (Waters C-18 180 µm × 20 mm), with a sample loading time of 1 min, using the partial loop mode of injection, at a flow rate of 18 µl/min (Buffer A1). Following loading, the valve position was switched, such that the trapping and analytical columns were in-line with respect to each other, and on-line with the mass spectrometer. The analytical column (nanoACQUITY UPLC™ BEH C-18 75 µm × 150 mm 1.7 µm column) was maintained at a temperature of 35 ºC, and at a constant flow rate of 300 nl/min. The gradient conditions were as follows: 0.33 min – 5 % acetonitrile/0.1 % HCO_2H,

up to 50 % acetonitrile/ 0.1 % HCO_2H over a 30 min linear gradient; 31 min – 85 % acetonitrile/ 0.1 % HCO_2H, 35 min, 5 % acetonitrile/ 0.1 % HCO_2H (pre-equilibration for the next run). The total length of an analytical run was 50 min.

For high mass accuracy, all spectra were acquired in an LTQ-Orbitrap XL with the Orbitrap operating at a resolution of 30,000 (defined at m/z 400). The Orbitrap was set to acquire alternate full MS scans, and tandem MS data from the linear ion trap. Data were acquired in positive ion mode using Xcalibur version 2.0.5 and Tuneplus version 2.4 SP1, and in data-dependent mode, with the most intense peak from full MS spectra directing collision induced dissociation (CID), at normalized collision energy of 35 %, and an activation q of 0.25. Dynamic exclusion was applied for a duration of 30 sec, with a repeat count of 2, and an exclude duration width of 180 sec to avoid repeated selection of the same m/z precursors for MSMS.

Supplementary References

[S1] Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics (Norwich, UK: The John Innes Foundation).

[S2] Sambrook, J. and Russel, D.W. (2001). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory).

[S3] Chong, P.P., Podmore, S.M., Lieser, H.M., Redenbach, M., Turgay, K., Marahiel, M.A., Hopwood, D.A., and Smith, C.P. (1998). Physical identification of a chromosomal locus encoding biosynthetic genes for the lipopeptide calcium-dependant antibiotic (CDA) of *Streptomyces coelicolor* A3(2). Microbiology **144**, 193-199.

Primer name	Sequence: 5'-3'
fabF3Up	CTGGTCAGATCTAGACCTCGACAACCG
fabF3Down	CCGACCTCTAGACGGGCCAGCACC
fabF3IleUp	GGGCAGCGCC**ATC**GGGGGCGTGACC
fabF3IleDown	GGTCACGCCCCCGATGGCGCTGCCC
fabF3LeuUp	GGGCAGCGCC**CTC**GGGGGCGTGACC
fabF3LeuDown	GGTCACGCCCCCGAGGGCGCTGCCC
fabF3AlaUp	GGGCAGCGCC**GCC**GGGGGCGTGACC
fabF3AlaDown	GGTCACGCCCCCGGCGGCGCTGCCC
fabF3M13forward	CGCCAGGGTTTTCCCAGTCACGACG CGATGTGCGCAGCCCGGTCCC
fabF3M13reverse	AGCGGATAACAATTTCACACAGGAG CGTCTTGCCGAGCCCGAACGCC

Table S1 Sequences of all primers used in the present study. Non-native residues are underlined. **Bold** type indicates codon 107.

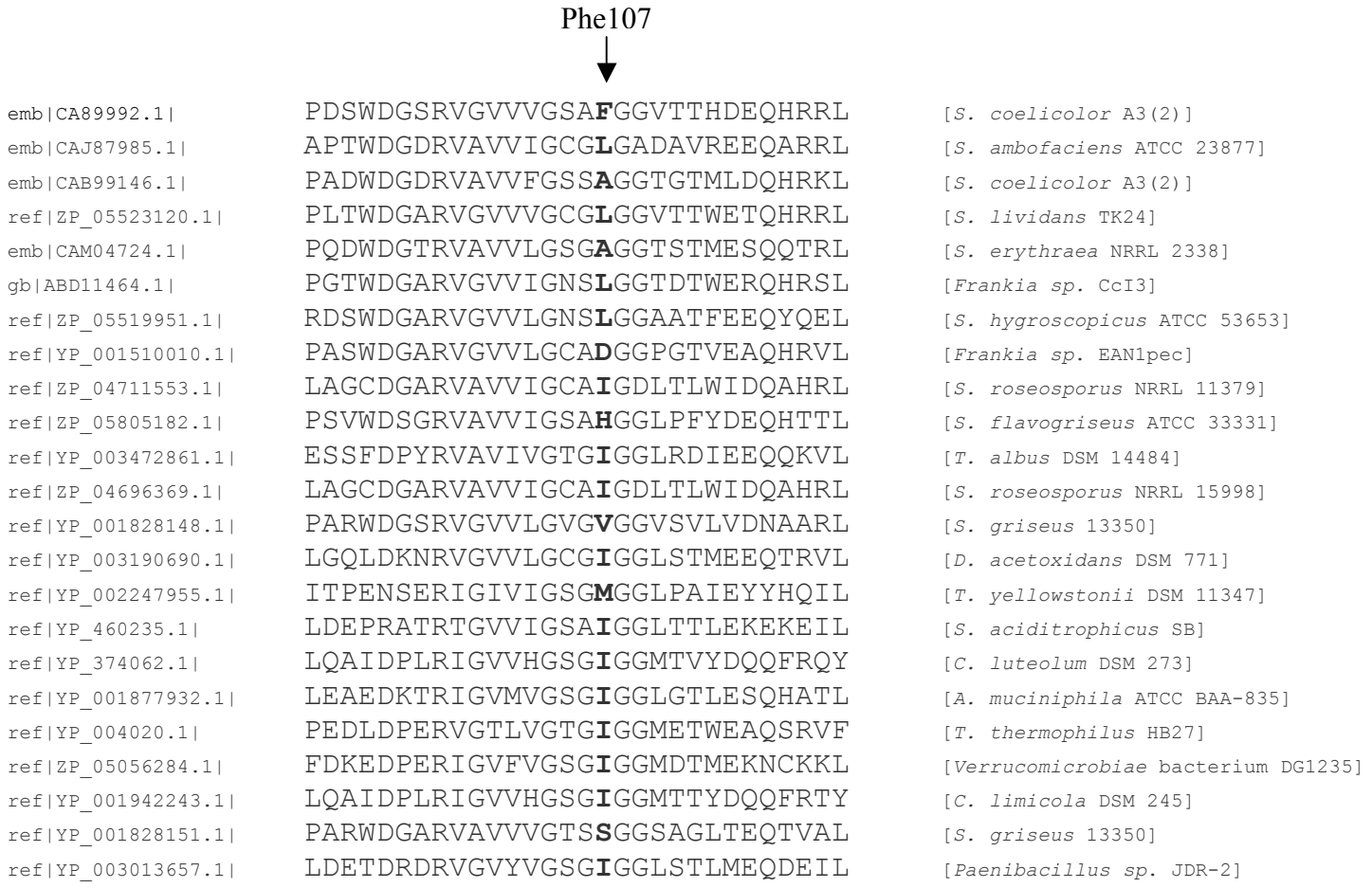

Figure S1. More extensive multiple sequence alignments of KAS-II enzymes, highlighting the other amino acid residues that are found at position 107.

(A) **(B)**

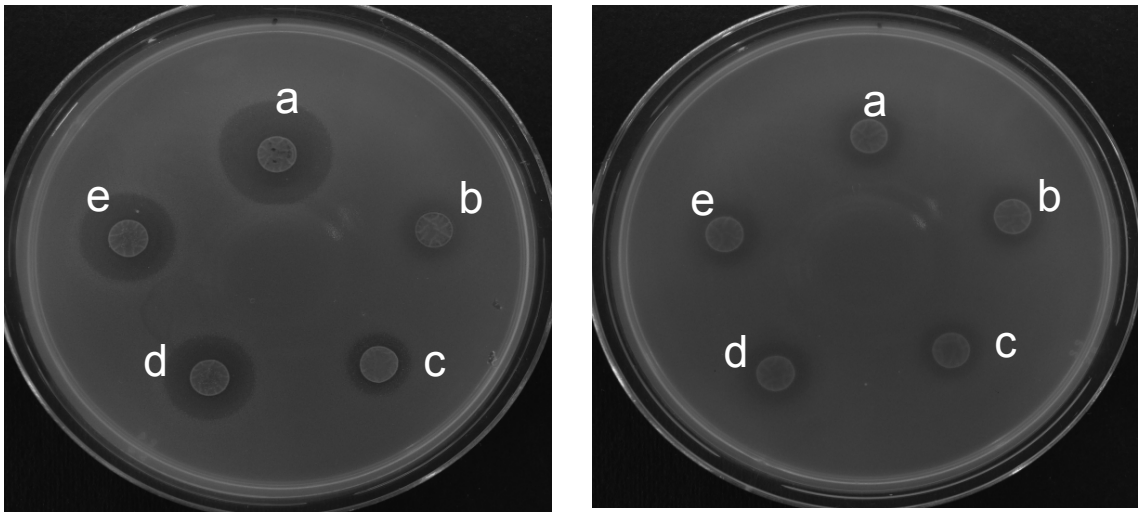

Figure S2. CDA plate bioassays of MT1110 wild-typr and *fabF3* mutant strains using *Bacillus mycoides* as an indicator strain. **(A)** Bioassay in the presence of Ca^{2+}; **(B)** bioassay in the absence of Ca^{2+}; (a) MT1110; (b) MT1110 *cdaPSI*-IIIΔ (a deletion mutant lacking the CDA NRPS encoding genes); (c) MT1110 *fabF3* Phe108Ser; (d) MT1110 *fabF3* Phe108Leu; (e) MT1110 *fabF3* Phe108Ile.

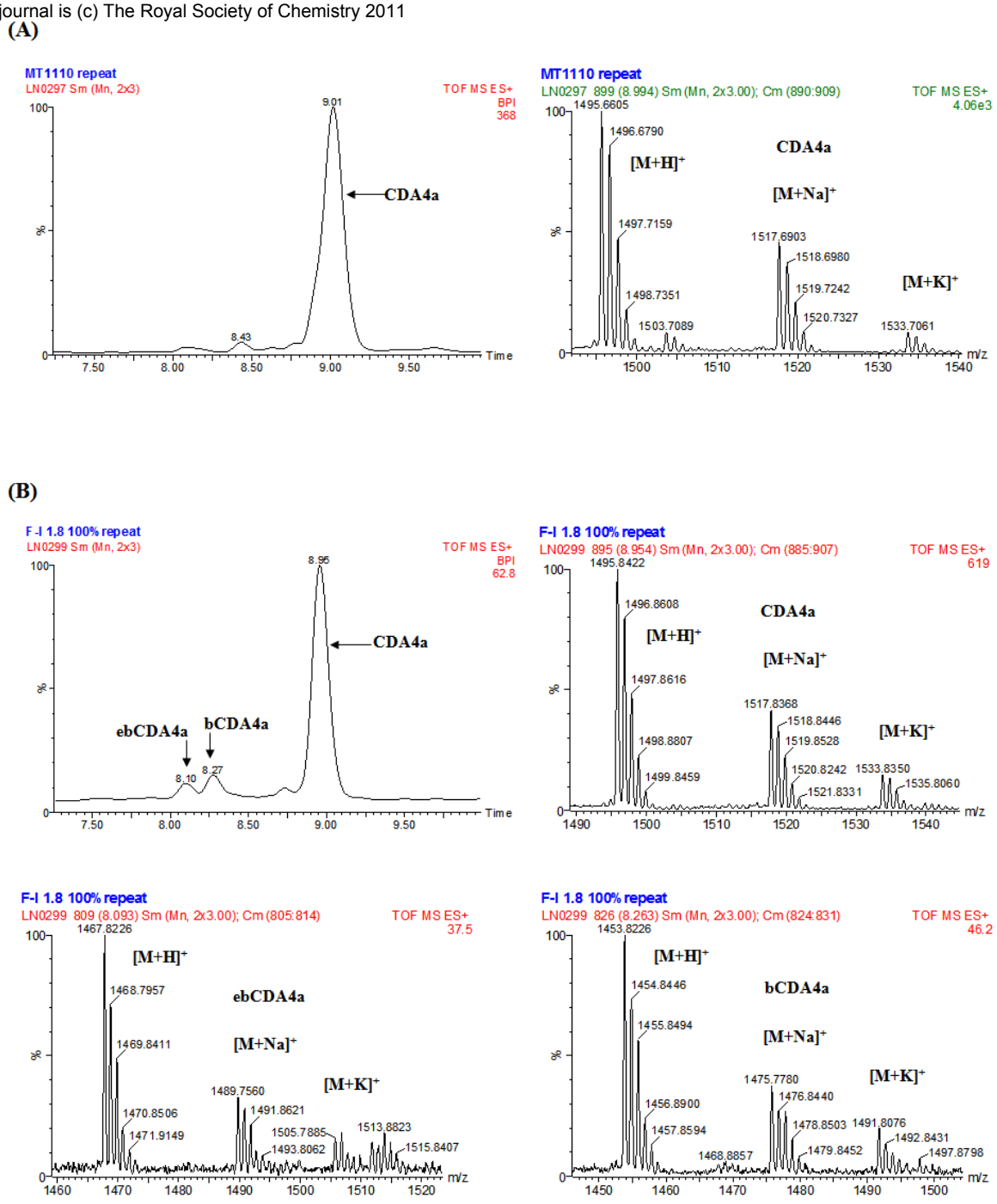

Figure S3. LC-MS analysis of CDAs from MT1110 and the mutant strain (Phe107Ile) was carried out on a Micromass LCT orthogonal acceleration time of flight mass spectrometer, equipped with an electrospray ionisation source run in positive mode (scanning from 700 to 1700 *m/z*) combined with a Waters 2790 Separation module. **(A)** The LC-MS chromatogram from MT1110 shows a product with retention time 9.01 minutes, which exhibits protonated, sodiated and potassiated ions in the mass spectrum consistent with CDA4a. **(B)** The LC-MS chromatogram of mutant strain Phe107Ile shows peaks with a retention time of 8.10 minutes, 8.27 minutes and 8.95 minutes that exhibit protonated, sodiated and potassiated ions, which correlate to ebCDA4a, bCDA4a and CDA4a respectively.

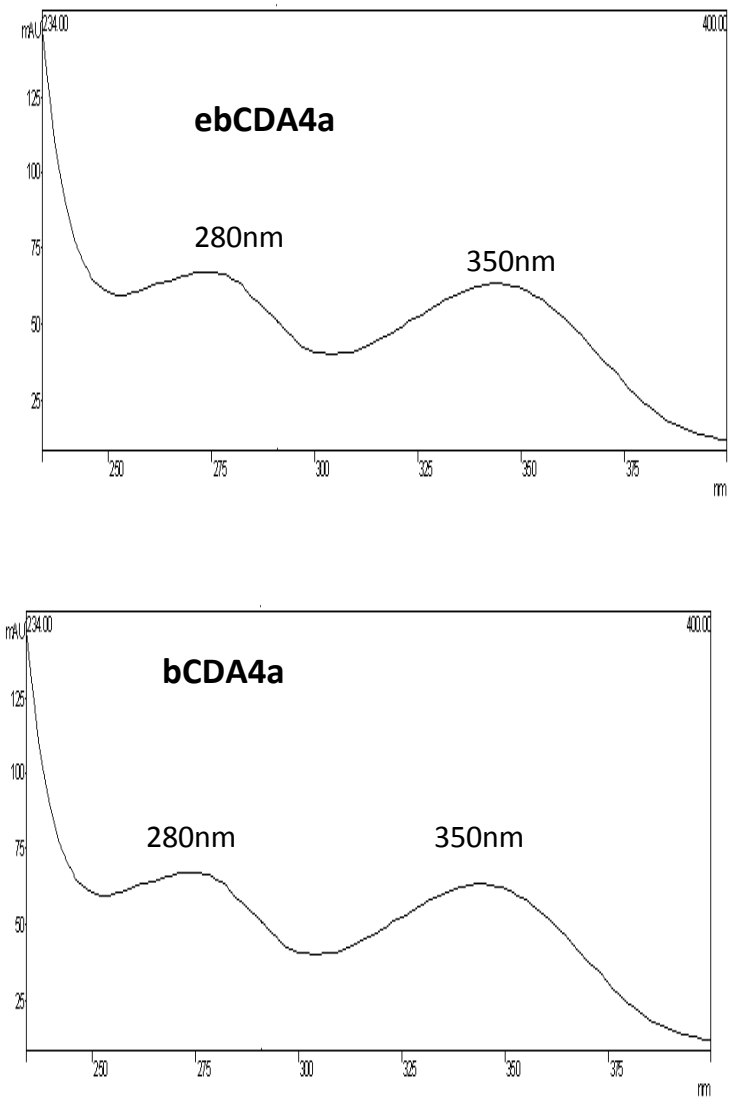

Figure S4. UV spectra of ebCDA4a and bCDA4a

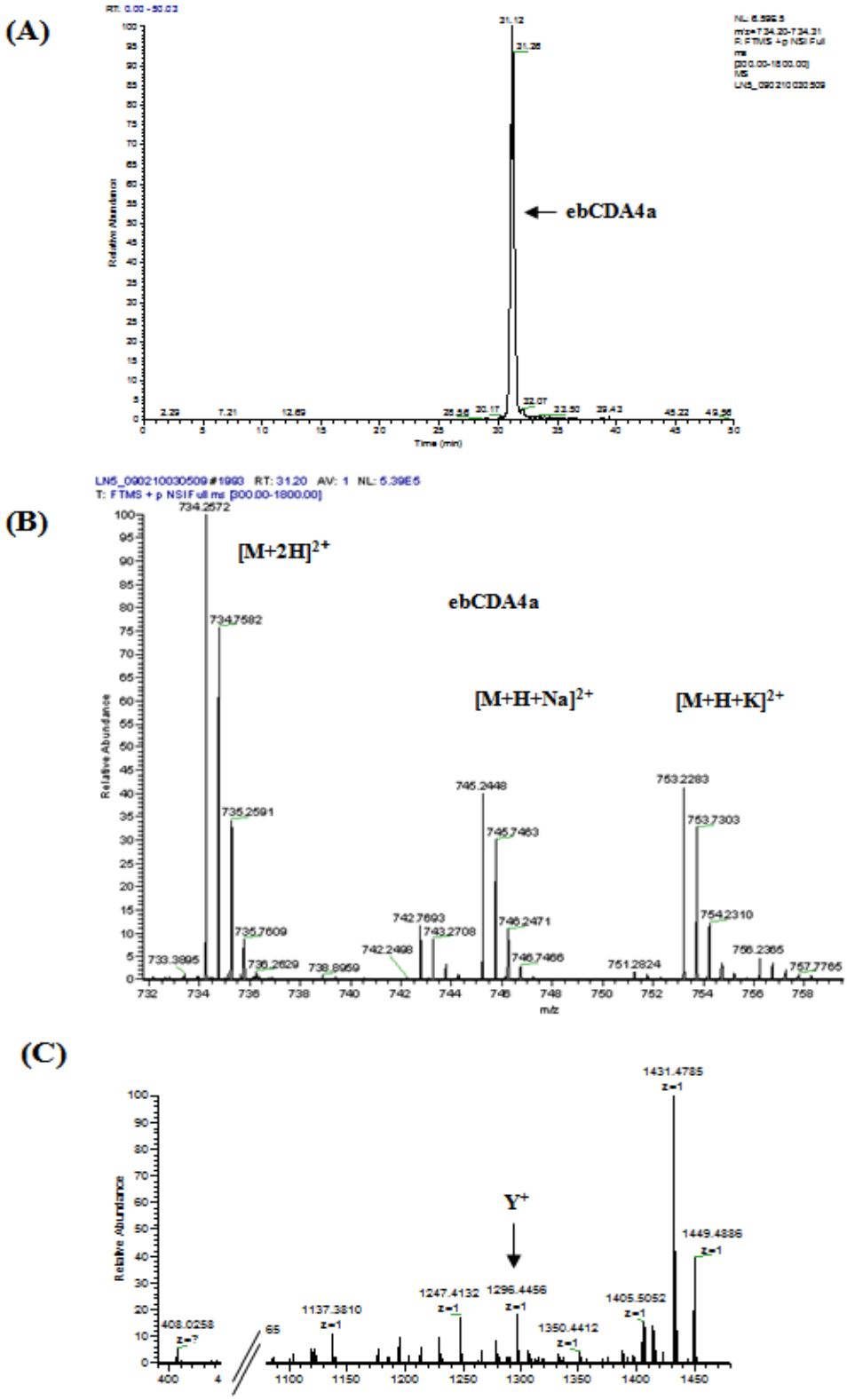

Figure S5. High resolution LC-MS and MS-MS analysis was also carried out using LTQ-Orbitrap XL-Nuplc. **(A)** An extracted ion chromatogram of ebCDA4a. **(B)** Mass spectrum for ebCDA4a with accurate masses that are consistent with the proposed formula for protonated, sodiated, and potassiated ions of ebCDA4a. **(C)** MS-MS for ebCDA4a shows the Y^+ ion consistent with formula for the decapeptide core of CDA4a.

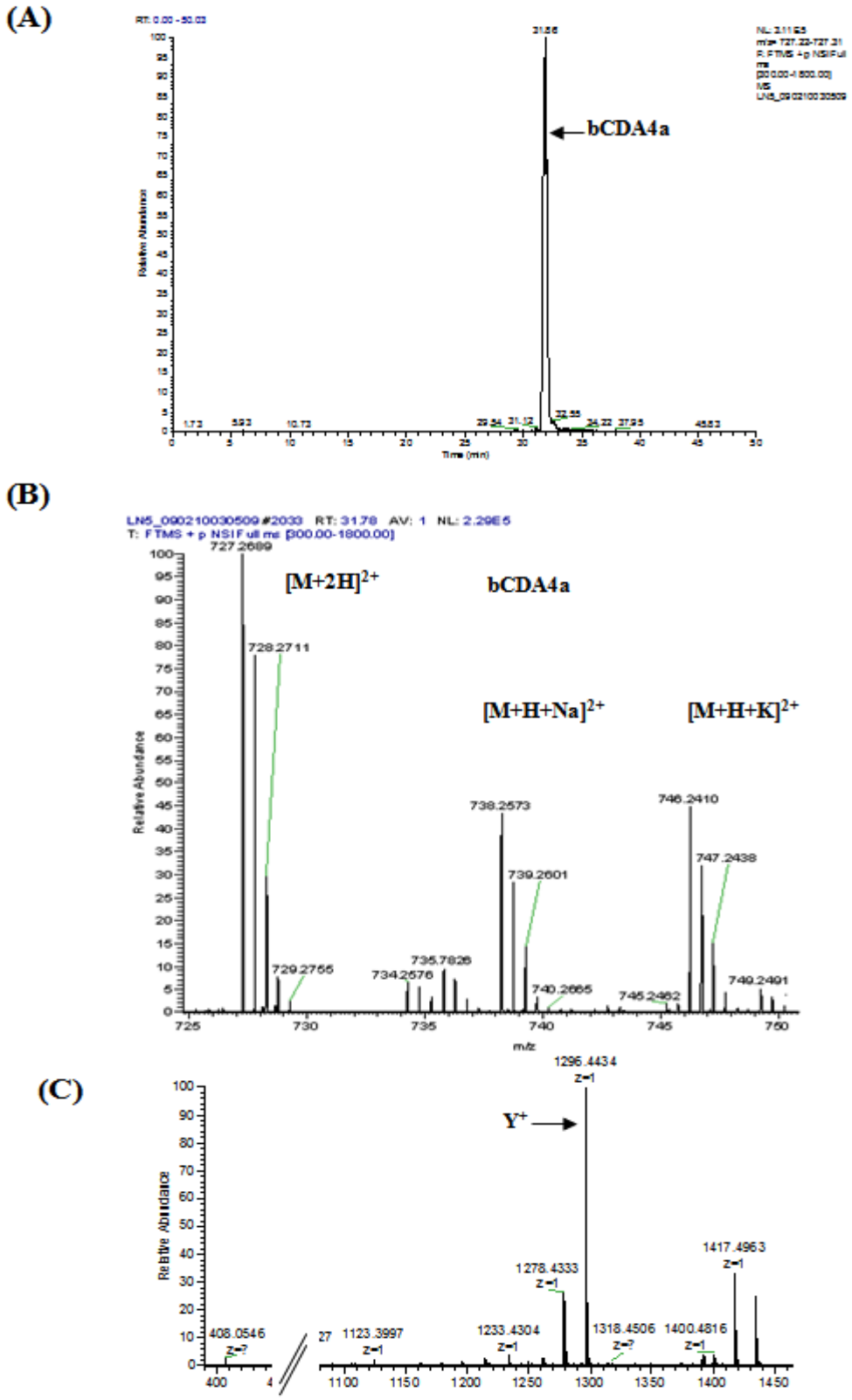

Figure S6. High resolution LC-MS and MS-MS analysis was also carried out using LTQ-Orbitrap XL-Nuplc. **(A)** An extracted ion chromatogram of bCDA4a. **(B)** Mass spectrum for ebCDA4a with accurate masses that are consistent with the proposed formula for protonated, sodiated, and potassiated ions of bCDA4a. **(C)** MS-MS for bCDA4a shows the Y^+ ion consistent with formula for the decapeptide core of CDA4a.

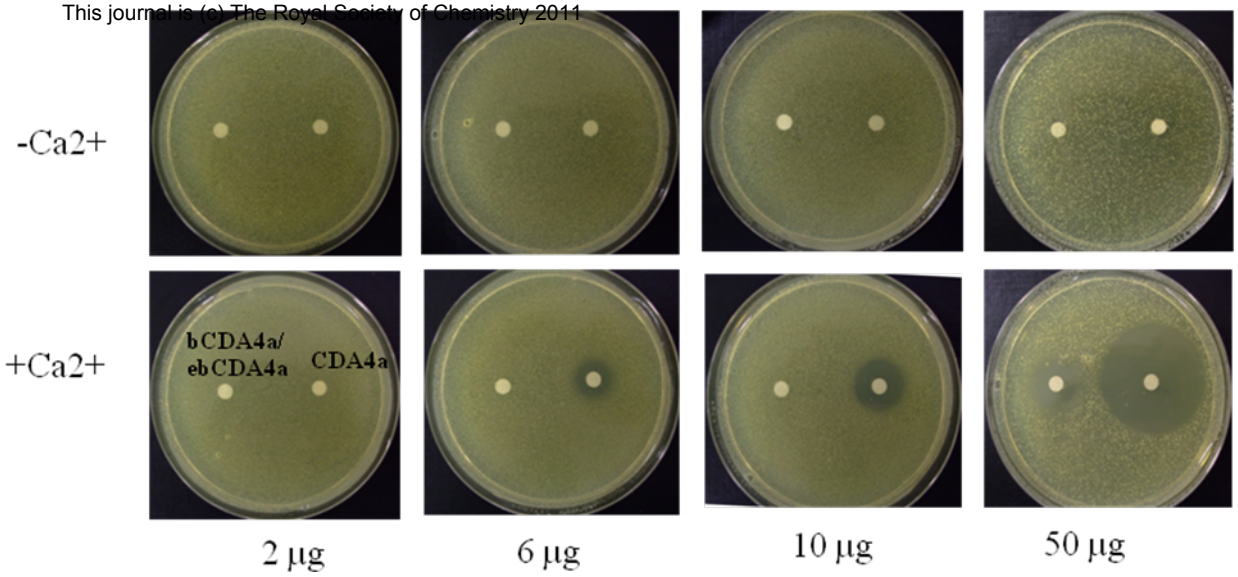

Figure S7. Bioassays comparing the antibiotic activity of purified bCDA4a/ebCDA4a and CDA4a. Increasing amounts of lipopeptides (from 2 to 50 µg) were adsorbed onto filter paper discs (*ca*. 5 mm diameter) on agar plates, which were overlaid with the indicator strain *Micrococcus Luteus* and incubated for 16 hours, with and without Ca^{2+}. Note due very similar retentions times separation of bCDA4a/ebCDA4a was not possible and these were assayed together.