Overexpression of a pathway-specific transcription factor CicD leads to the discovery of novel phytotoxins in *Aspergillus nidulans*

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1. Supplementary Materials and methods

1.1 General Experimental Procedures

Polymerase chain reaction (PCR) was performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, NEB, Beijing, China). DNA restriction enzymes were used as recommended by the manufacturer (New England Biolabs, NEB, Beijing, China). High performance liquid chromatography (HPLC) analysis was performed on a Shimadzu LC-20AT system (Shimadzu, Japan) using a Shimadzu ShimNex CS C18 analytical column (5 μm, 4.6 × 150 mm, 1 mL/min). NMR spectra were recorded on an Agilent 500 MHz DD2 spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). Specific rotations were obtained on a JASCO P-1020 digital polarimeter. HRESIMS were obtained on a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) or Micromass Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer (Waters Aisa Ltd. Singapore). A JASCO J-715 spectropolarimeter (JASCO, Tokyo, Japan) was used to obtain ECD spectra. Column chromatography (CC) was performed with SiliaSphere C18 (Octadecylsilyl, ODS) monomeric (SiliCycle Inc., Quebec, Canada, 50 μm, 120 A). The compounds were purified by a HPLC Hitachi 1110 system equipped with a 1430 PDA detector or HPLC system (PuriMaster-2000, Shanghai Kezhe Biochemical Technology Co., Ltd, China) and a C18 column (SilGreen, 10 mm × 250 mm, 5 μm, 3 mL/min).

1.2 Strains and media

A. nidulans A1145 was used for strain construction. *A. nidulans* strains were grown at 37 °C in CD (1% glucose, 0.5% v/v 20 × nitrate salts, 0.01% v/v trace elements, and 2% agar) medium for sporulation. CDS medium (1% glucose, 1.2 M D-sorbitol, 0.5% v/v 20 × nitrate salts, 0.01% v/v trace elements, and 2% agar) was used to screen auxotrophic transformants. CD-ST (2% starch, 2% casamino acids, 5% v/v 20 × nitrate salts, 0.1% v/v trace elements) was used for compound production. In addition, 10 mM uridine, 5 mM uracil, 1 μg/mL pyridoxine HCl and 0.25 μg/mL riboflavin were supplemented, when necessary.

Escherichia coli strain XL-1 Phage Resistant Chemically competent cells (prepared by

Supercometent Cell Preparation Kit, Beyotime, Shanghai, China) were used for plasmid preservation and amplification, following standard recombinant DNA techniques. *E. coli* cultures were grown at 37 ℃ in LB medium (Hope Biotech, Qingdao, China). *Saccharomyces cerevisiae* BJ5464-NpgA was used for in vivo yeast DNA recombination cloning and cultured on yeast medium (2% glucose, 0.5% casamino acids, and 2% agar) at 28 °C.

1.3 Plasmid construction

To construct expression plasmid for *A. nidulans*, plasmid pYTU with auxotrophic marker for uracil (*pyrG*) was digested with restriction enzymes (*Pac*I and *Not*I) and used as vector to insert gene. The final plasmid was obtained by yeast homologous recombination in *S. cerevisiae* BJ5464-NpgA. Following the selection of correct colonies through PCR validation, the yeast miniprep procedure (Zymoprep™ Yeast Plasmid Miniprep Kit Zymo Research, USA) was carried out to obtain plasmid aliquots. Plasmid constructs were then introduced into *E. coli* XL-1. After acquisition of transformants and subsequent plasmid extraction from *E. coli*, constructs were validated by sequencing.

1.4 Preparation and transformation of *A. nidulans* protoplasts.

A. nidulans was cultured in solid CD medium containing 10 mM uridine, 5 mM uracil, 0.5 μg/mL pyridoxine HCl and 2.5 μg/mL riboflavin at 37 ℃ for 3 days, and then spores were collected in 20% glycerol. The spores were inoculated in 50 mL CD medium with supplements and cultured at 37 ℃ and 220 rpm for 8 h. After germination, culture fluid was centrifuged at 4 ℃ and 4000 rpm for 15 min to harvest the mycelia. The precipitate was washed with 30 mL osmotic buffer (1.2 M $MgSO_4 \cdot 7H_2O$, 10 mM sodium phosphate, pH 5.8), centrifuged at 4 ℃ and 4000 rpm for 15 min, and then resuspended with 15 mL osmotic buffer containing 30 mg *Lysing Enzymes* from *Trichodema harzianum* (Sigma, Germany) and 20 mg *Yatalase* (Takara, Japan). The suspension was transferred to a 250 mL Erlenmeyer flask and cultured at 28 ℃ and 80 rpm for 12 h. The resulting culture fluid was transferred directly to a sterile 50 mL centrifugal tube and overlaid gently with 15 mL of trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.0), then centrifuged at 4 ℃ and 4000 rpm for 15 min. The protoplasm layer was transferred and fully scattered into STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCI, pH 7.5) with 2 × volume and centrifuged at 4 ℃ and 4000 rpm for 20 min. The supernatant was removed and STC buffer was added to resuspend the protoplasts for transformation.

For *A. nidulans* transformations, the requisite plasmids were added to 100 µL of *A. nidulans* A1145 protoplast suspension prepared as described above and the mixture was incubated on ice for 60 min. After incubation on ice, 500 µL of PEG solution (60% PEG, 50 mM calcium chloride and 50 mM Tris-HCl, pH 7.5) was added to the protoplast mixture, followed by additional incubation at room temperature for 20 min. The mixture was spread on the regeneration medium (CDS solid medium with appropriate supplements including 10 mM uridine, 5 mM uracil and/or 0.5 µg/mL pyridoxine HCl and/or 2.5 µg/mL riboflavin depending on the plasmids being transformed) and then incubated at 37 °C for 2-3 days until single colonies appeared.

1.5 Fermentation, Extraction and Compound Isolation

A. nidulans A1145 transformants were cultivated on solid CD-ST medium at 28 °C for 4 d. The cultures were extracted thrice with CH_3OH for 30 min with sonication. The organic phases were combined and solvent was removed by a vacuum evaporator. The extracts were dissolved in 1 mL HPLC-grade methanol and filtered using 0.22 µm filters for HPLC measurements.

For compound isolation, a large-scale solid fermentation (CD-ST medium, 10 L) was performed. The cultures were extracted thrice with CH_3OH for 30 min with sonication. The organic phase was evaporated under reduced pressure to afford a crude extract (6 g).

The extract was applied to C18 column using a stepped gradient elution of MeOH/H2O to yield six subfractions (Fr.1-Fr.6, 30% to 80%). Fr.1 was separated by semi-preparative HPLC to afford **3** (40% MeOH in H_2O, 0.1% THF, 13.2 mg, t_R = 16 min); Fr.2 was separated by semi-preparative HPLC with a gradient of 50% MeOH in H_2O with 0.1% THF to afford **4** (3.5 mg, t_R = 14 min) and **5** (4.8 mg, t_R = 34 min); Fr.3 was separated by semi-preparative HPLC to afford **1** (55% MeOH in H_2O, 0.1% THF, 4.0 mg, t_R = 28 min) and Fr.4 was purified by semi-preparative HPLC to obtain **2** (40% MeCN in H_2O, 0.1% THF, 4.0 mg, t_R = 15 min).

Isoleucylcichorine (**1**): colorless oil; $[\alpha]_D^{25}$ -11.34, (*c* 0.17, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 214 (3.45), 255 (2.98), 297 (2.41) nm; ECD (0.5 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 214 (+12.20), 234 (-18.9), 254 (+0.57), 267 (-1.37), 299 (+1.43) nm; ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data (DMSO-d_6), see Table 1; positive ion HRESIMS *m/z* 308.1501 $[M + H]^+$ (calcd. for $C_{16}H_{22}NO_5$, 308.1492).

Leucylcichorine (**2**): yellow oil; $[\alpha]_D^{25}$ +0.4, (*c* 0.07, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 214

(3.00), 255 (2.44), 297 (1.90) nm; ECD (0.5 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 228 (-1.96), 246 (+2.45), 264 (-0.88), 282 (+0.62) nm; ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data (DMSO-d_6), see Table 1; positive ion HRESIMS *m/z* 308.1497 $[M + H]^+$ (calcd. for $C_{16}H_{22}NO_5$, 308.1492).

1.6 ECD Calculations

Conformation searches based on molecular mechanics with MMFF (Merck molecular force field) force fields were performed for stereoisomers to get stable conformers. All the stable conformers were further optimized by the density functional theory (DFT) method at the B3LYP/6-31+G(d) level by Gaussian 16 program package[1]. The ECD were calculated using time-dependent density functional theory (TDDFT) at B3LYP/6-31+G(d) level in methanol with IEFPCM model. The calculated ECD curves were all generated using SpecDis 1.71 program package and the calculated ECD data of all conformers were Boltzmann averaged by Gibbs free energy[2].

1.7 Phytotoxic Bioassay

The phytotoxic activity of the compounds was tested on cut leaves using the wet chamber technique. The leaves were from *Medicago polymorpha L*, *Zea mays* and *Lactuca sativa L. var. ramosa Hort.* All the tested compounds were prepared with the concentration at 5×10^{-3} M. A puncture was made using the tip of a syringe and a drop (10μL) of test solution was placed at the puncture site from the upper side of the leaves. The diameter of the lesion was measured after 72 hours of incubation at 28℃ in a humid sterile room. The compounds were dissolved in 2% ethanol. 2% ethanol was chosen as a negative control.

2. Supplementary figures

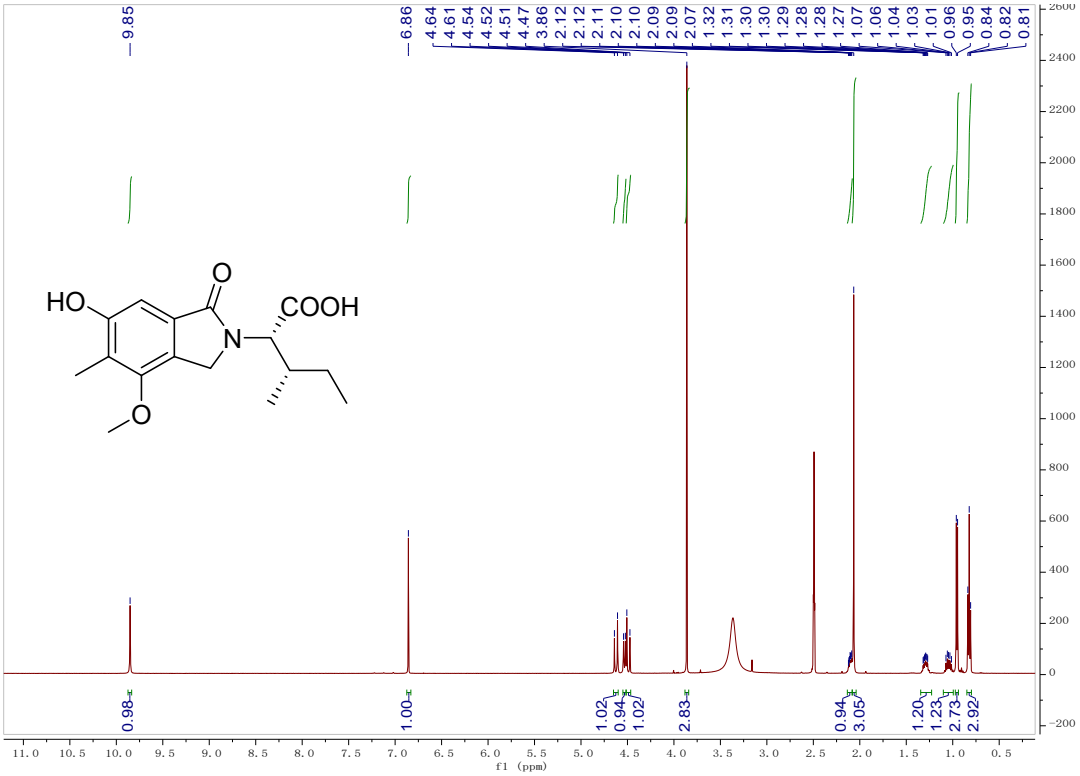

Figure S1. ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of isoleucylcichorine (**1**).

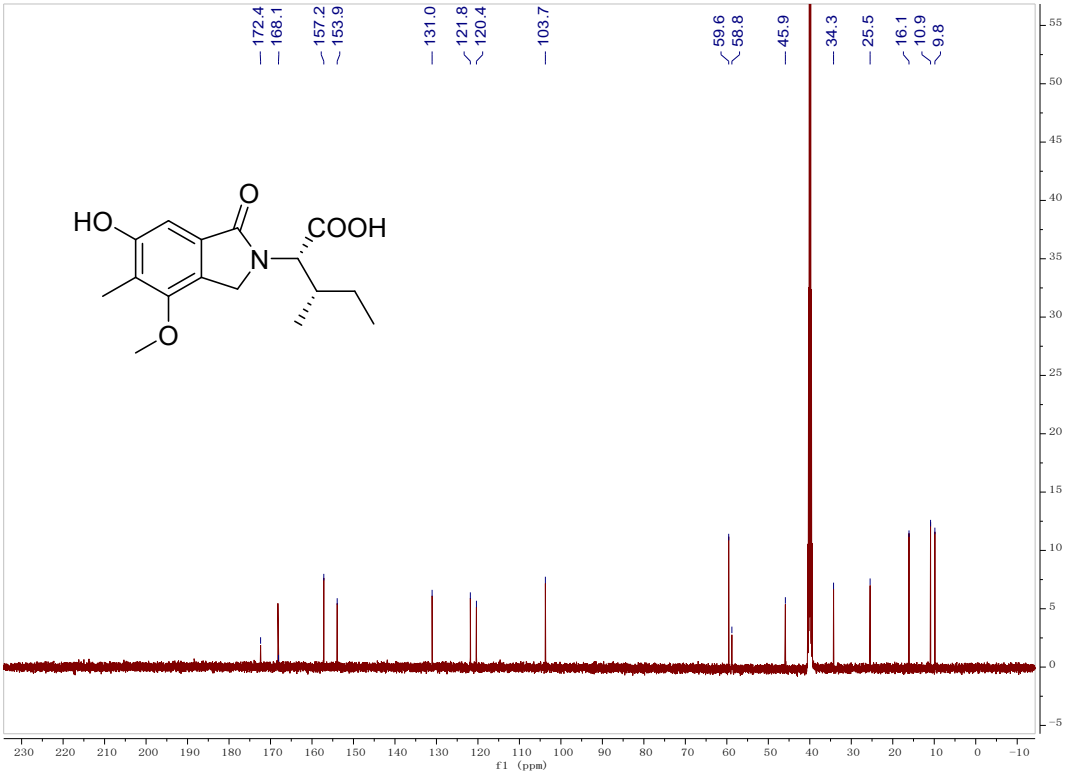

Figure S2. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of isoleucylcichorine (**1**).

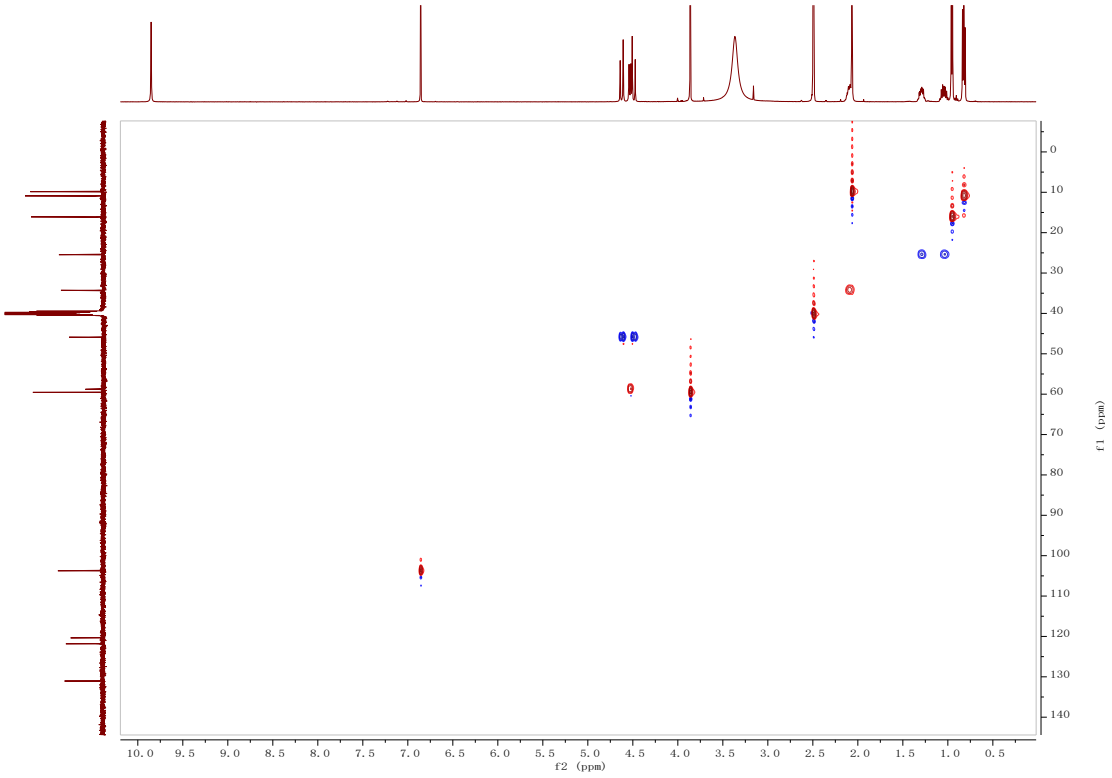

Figure S3. HSQC spectrum of isoleucylcichorine (**1**).

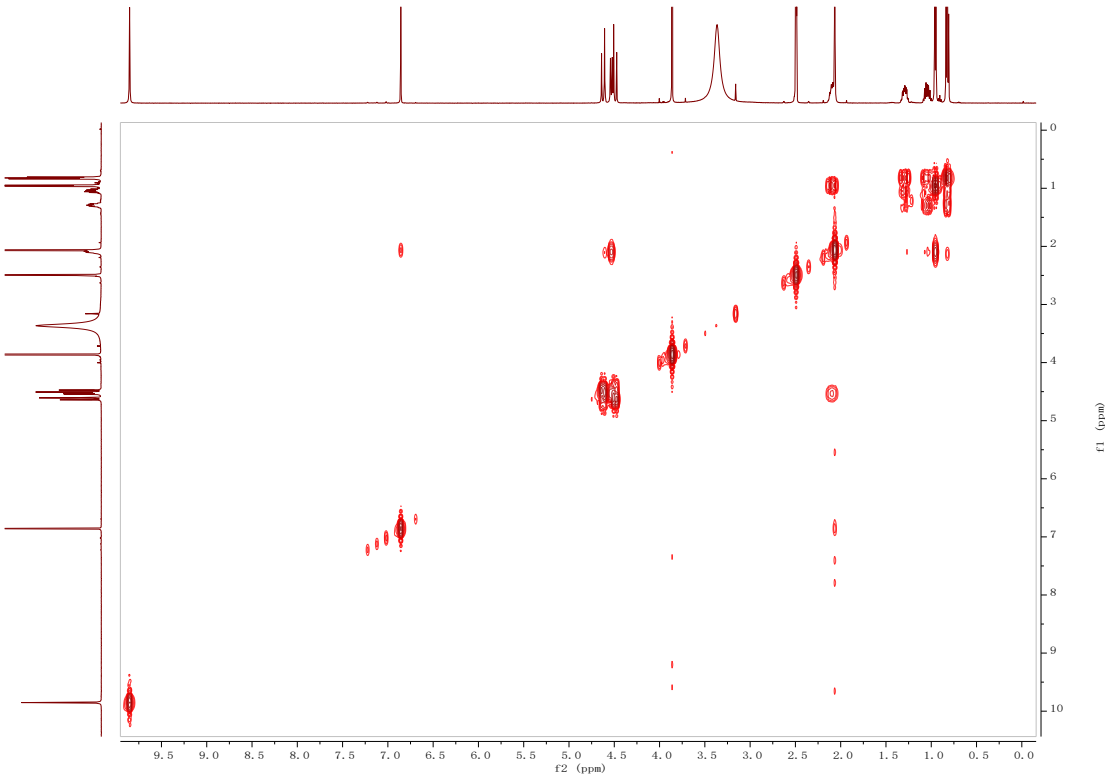

Figure S4. ^{1}**H-**^{1}**H COSY** spectrum of isoleucylcichorine (**1**).

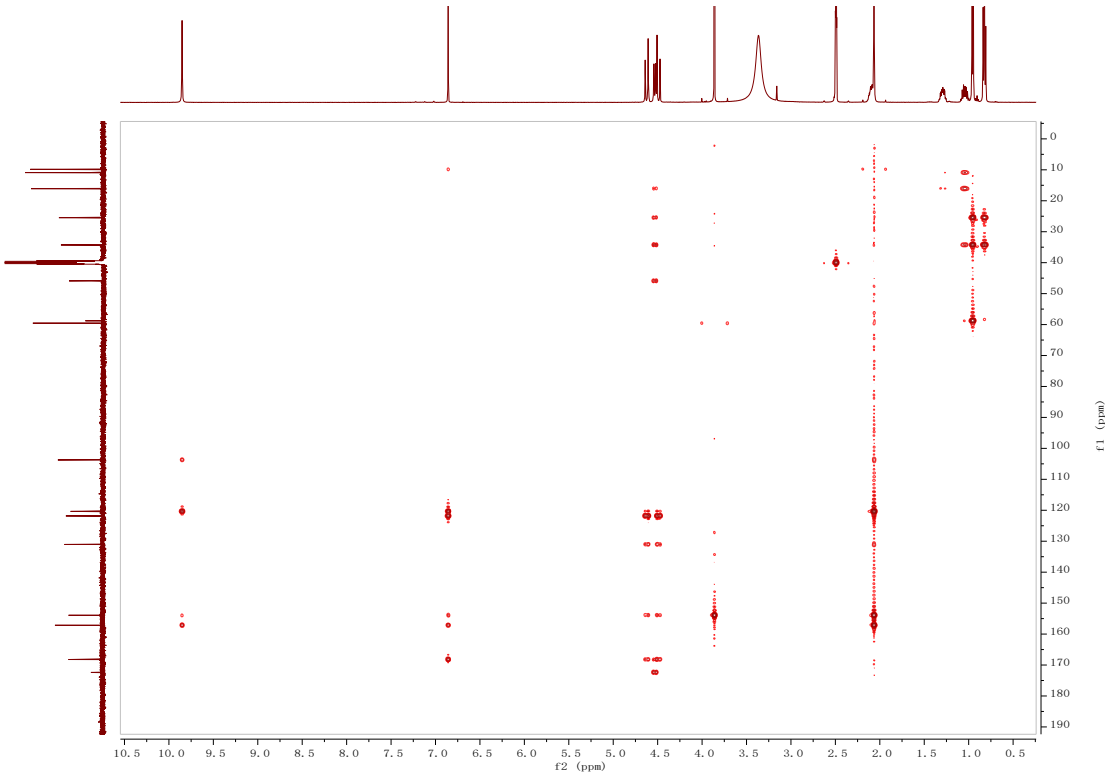

Figure S5. ^{1}H-^{13}C **HMBC** spectrum of isoleucylcichorine (**1**).

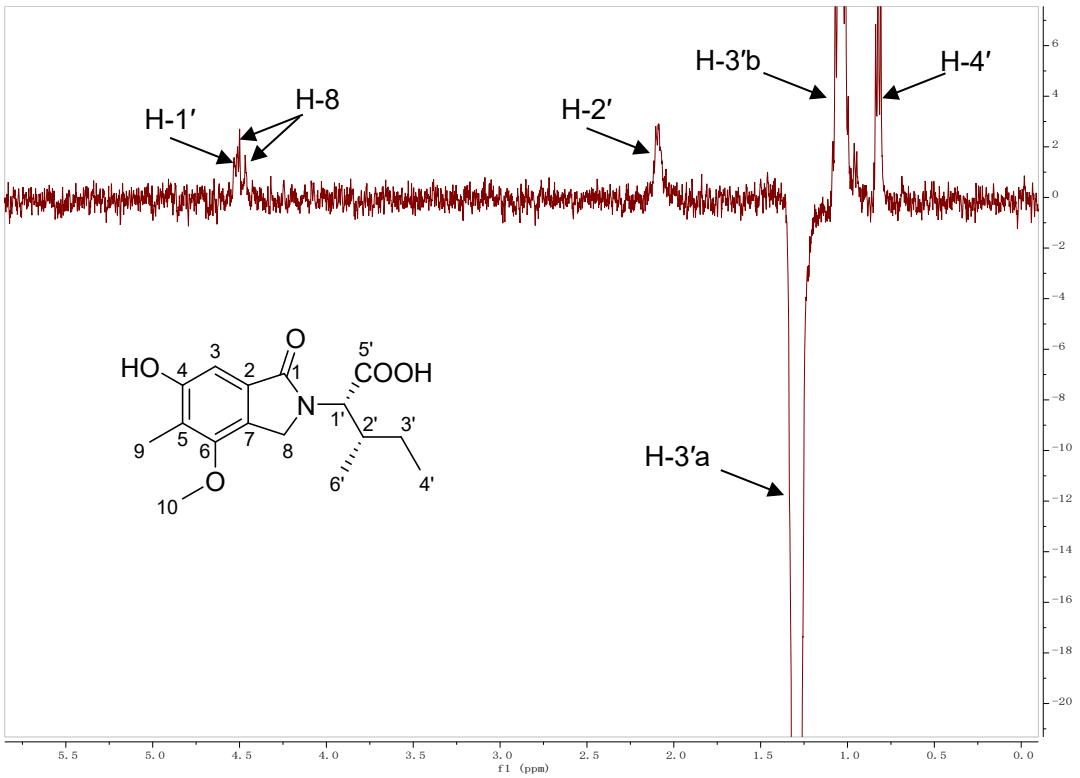

Figure S6. NOE spectrum of isoleucylcichorine (**1**).

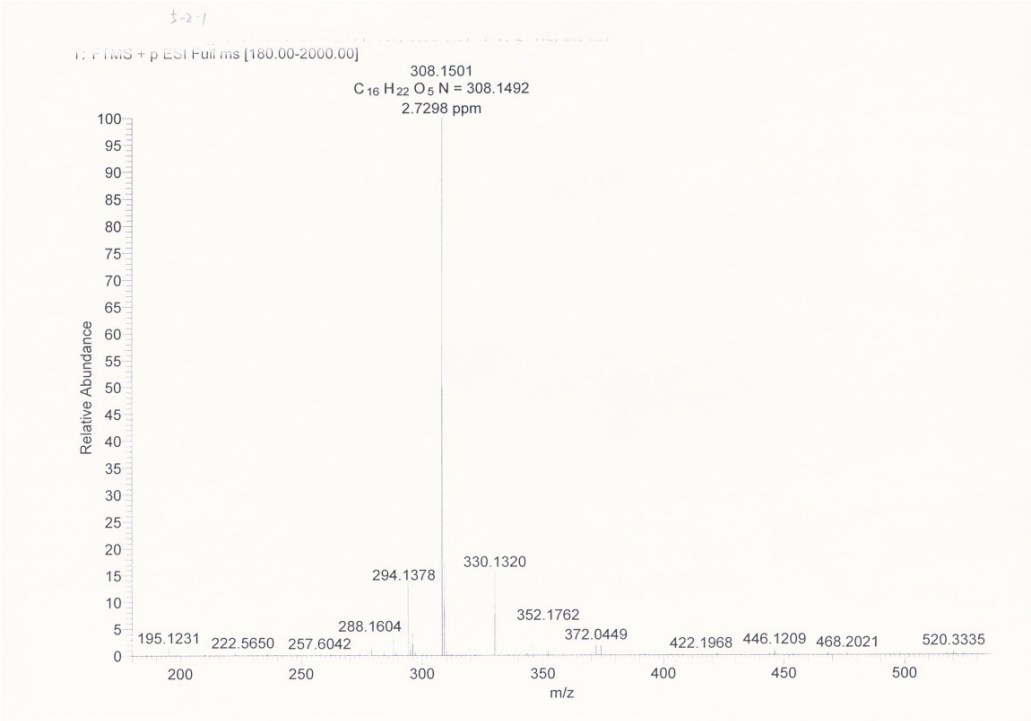

Figure S7. HRESIMS spectrum of isoleucylcichorine (**1**).

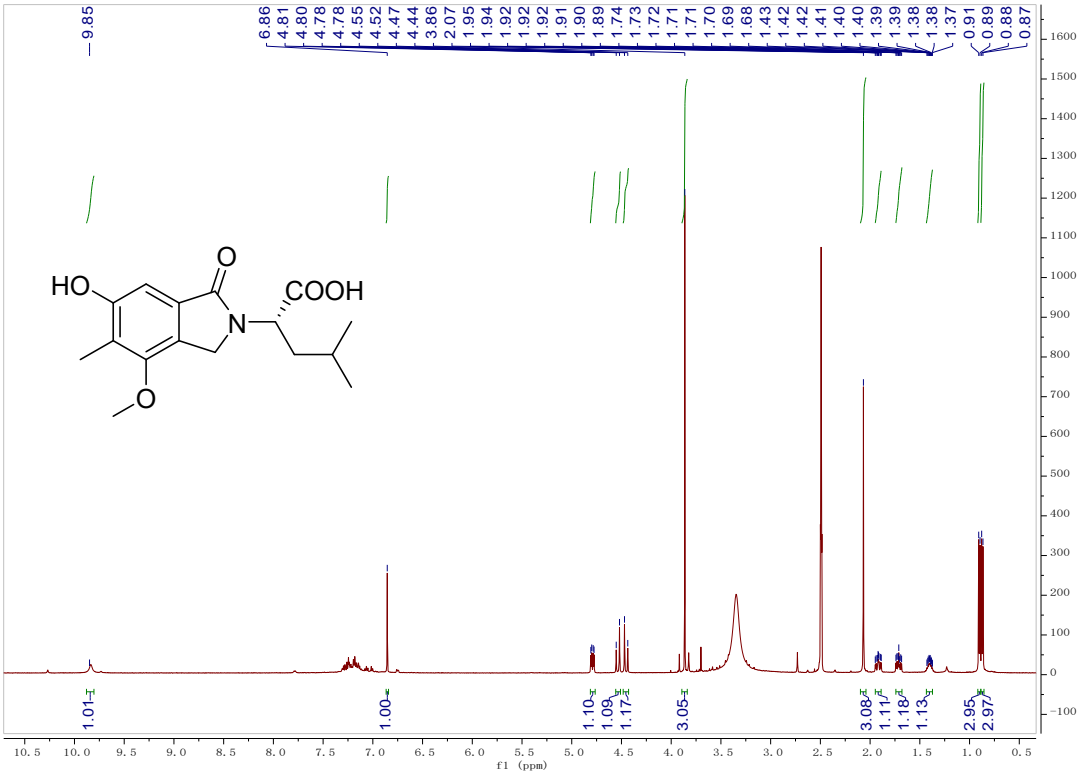

Figure S8. ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of leucylcichorine (**2**).

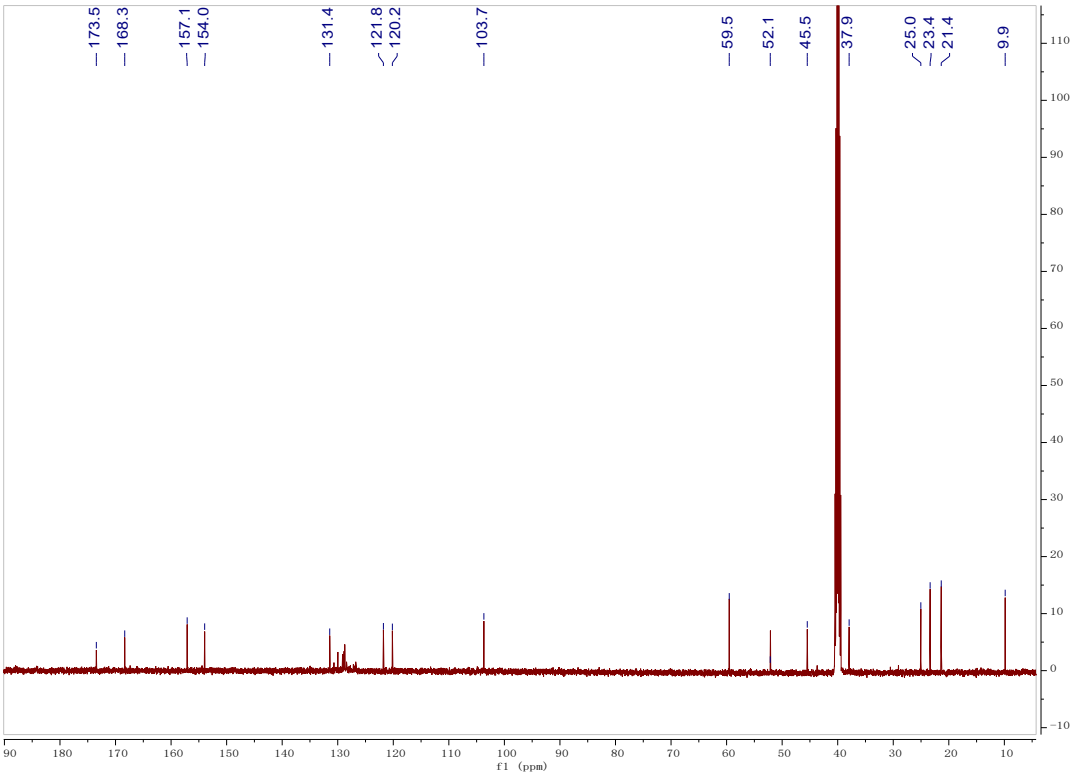

Figure S9. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of leucylcichorine (**2**).

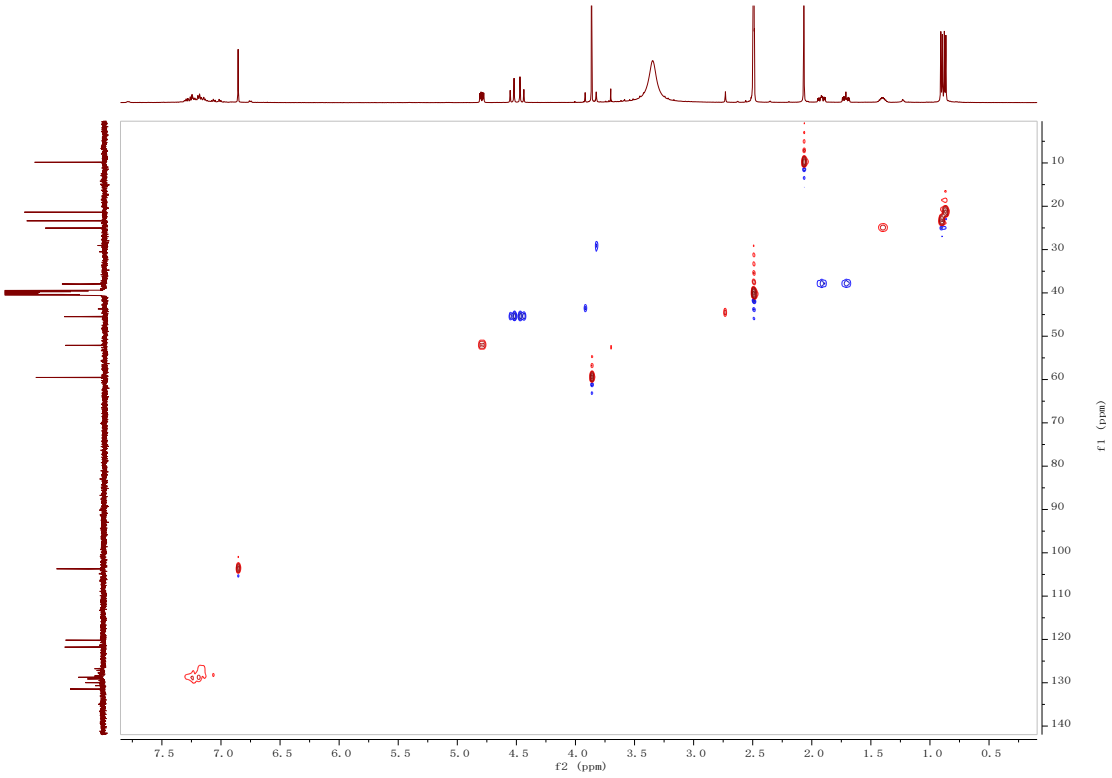

Figure S10. HSQC spectrum of leucylcichorine (**2**).

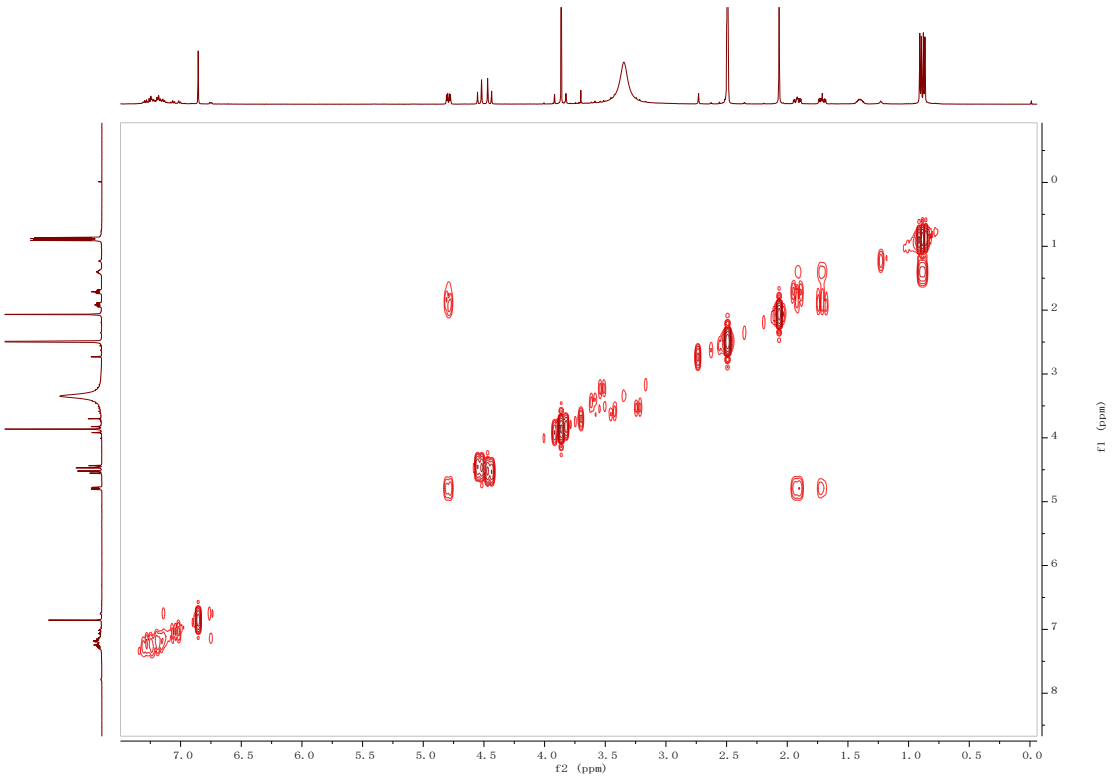

Figure S11. ^{1}**H-**^{1}**H COSY** spectrum of leucylcichorine (**2**).

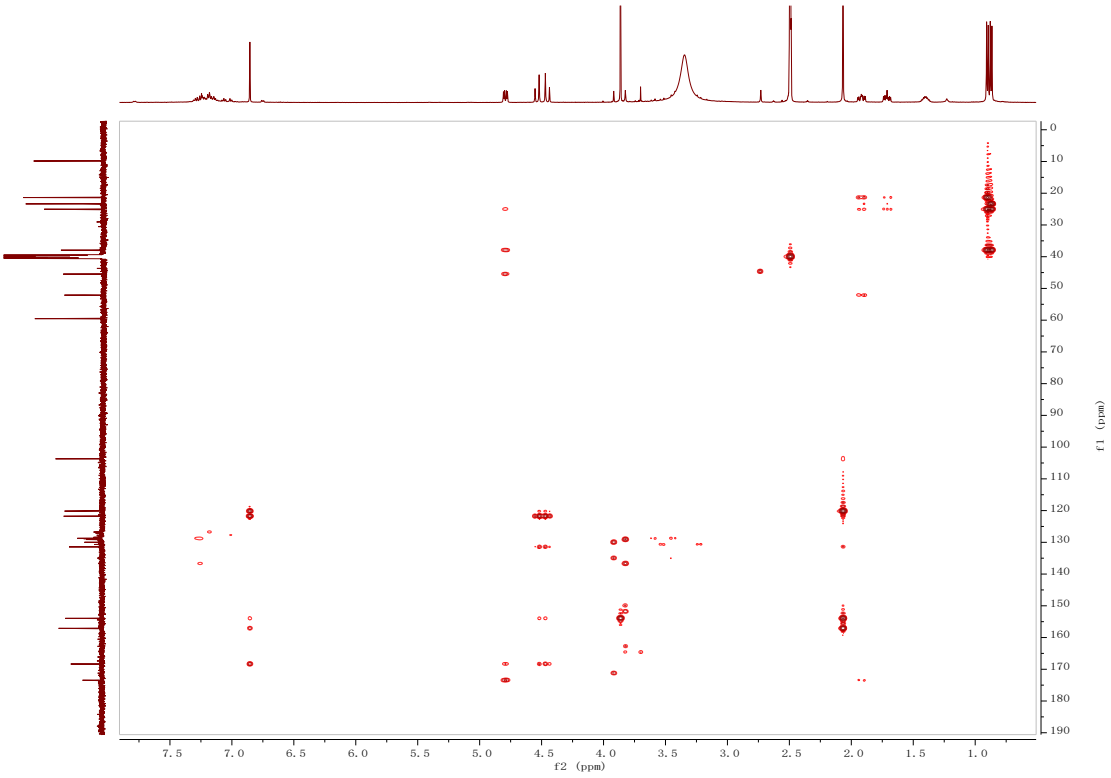

Figure S12. ^{1}H-^{13}C **HMBC** spectrum of leucylcichorine (**2**).

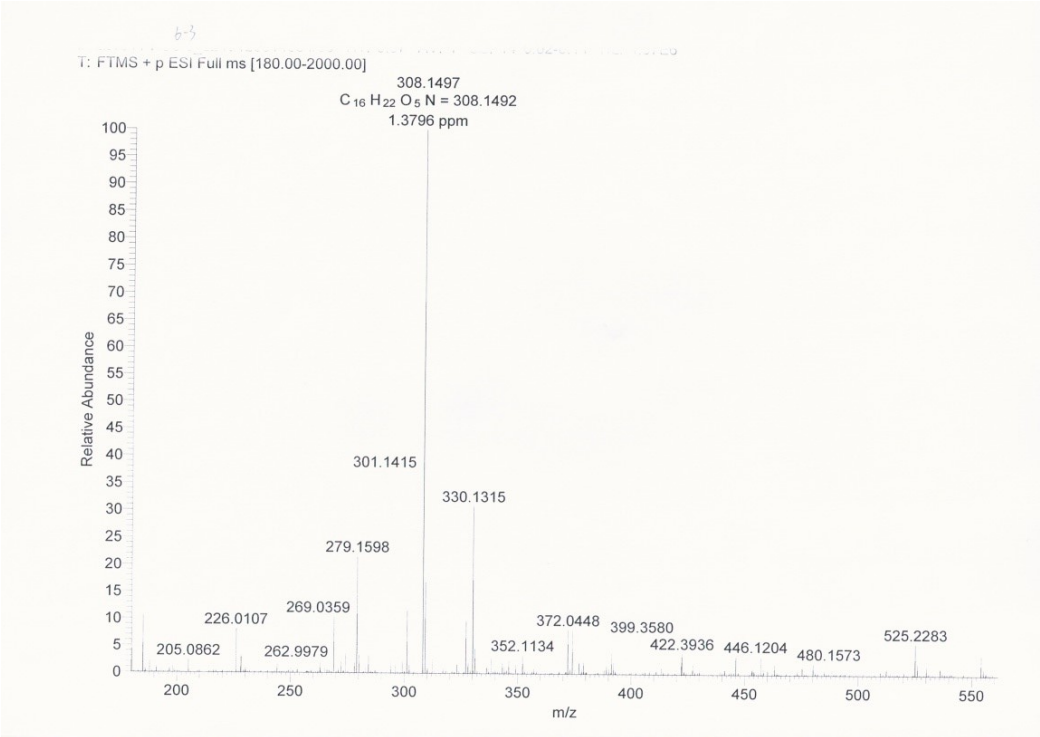

Figure S13. HRESIMS spectrum of leucylcichorine (**2**).

3. Supplementary references

1. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. *Gaussian 16 Rev. C.01*, Wallingford, CT, 2016.

2. Bruhn, T.; Schaumlöffel, A.; Hemberger, Y.; Bringmann, G., SpecDis: quantifying the comparison of calculated and experimental electronic circular dichroism spectra. *Chirality* 2013**,** *25* (4), 243-249.