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Supporting Information

Unnatural tetradeoxy echinocandins produced by gene cluster design and heterologous expression

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Content

Strains and culture conditions

The strains used in this study are listed in Table S1. The ECB producing strain *Emericella rugulosa* NRRL 11440, formally known as *Aspergillus pachycristatus*, was obtained from American Type Culture Collection (ATCC, USA). *E. rugulosa* and *A. nidulans* were maintained as glycerol stocks at -80 °C and activated at 37 °C on solid glucose minimum medium (GMM) [1] supplemented as needed for auxotrophs. GMM was supplemented with 0.56 g/L uracil and 1.26 g/L uridine for *pyrG* auxotroph, 1mg/L pyridoxine HCl for *pyroA* auxotroph, and 2.5 mg/L riboflavin for *riboB* auxotroph. *Saccharomyces cerevisiae* BJ5464[2] was used for plasmid constructions via *in vivo* yeast recombination, and *Escherichia coli* DH5α was used for plasmid amplification.

Genetic manipulations

For genomic DNA (gDNA) extraction, *E. rugulosa* NRRL 11440 and *A. nidulans* were cultivated for 24 h at 37 °C in steady state liquid GMM, supplemented as necessary for auxotrophs. DNA extraction was carried out according to the standard protocol[3]. Echinocandin biosynthetic gene cluster (BGC) genes were cloned from *E. rugulosa* NRRL 11440 gDNA by PCR with high-fidelity DNA polymerase Primestar HS/MAX (Takara, Japan). All the primers used in this study are listed in Table S4. The lengthy NRPS gene *ecdA* was divided into four fragments *ecdA*1-4 (each 6-7kb) for PCR cloning.

As described previously, [4] plasmids (Table S2) were created to generate the expression cassettes for transformation via *in vivo* yeast recombination in *Saccharomyces cerevisiae* BJ5464. Some fragments of the plasmids were initially joined through double-joint PCR (DJ-PCR) [5] and then assembled via *in vivo* yeast recombination. The heterologous expression cassettes of *ecdA* and *ecdI* were integrated into the *yA* locus of *A. nidulans* LO8030 through three rounds of transformation. The 1st round transformation cassette from the plasmid pYX1 contained the xylose-inducible promoter *xylp* [6], the cloning fragments *ecdA*1 and *ecdA*2, the auxotrophic marker *A. fumigatus riboB* (*AfriboB*), and was flanked by 1 kb DNA fragments upstream and downstream of the gene *yA*. The cassette for the 2nd round transformation from the plasmid pYX5 consisted of the followings: the cloning fragment *ecdA*3 with a 1 kb overlap of *ecdA*2, the auxotrophic marker *A. parasiticus pyrG* (*AppyrG*), a constitutive promoter *gpdAp* [7], *ecdI*, and a 1kb DNA fragment downstream of the gene *yA*. The cassette used for the 3rd round transformation in the plasmid pYX9 had the cloning fragment *ecdA4* with 1 kb overlap of *ecdA*3, *AfriboB* and *gpdAp*. The control plasmid pYX14 was constructed to

obtain the cassette containing *xylp*, *AfriboB* and *gpdAp*. The plasmids above were digested with *Eco* RI or *Not* I to get the related cassettes for transforming *A. nidulans* LO8030. *A. nidulans* protoplast generation and transformation were carried out as previously described[8,9]. The resulted transformant with *xylp-ecdA-AfriboB-gpdAp-ecdI* in the *yA* locus was designated LO8030-4.1, whereas the control transformant with *xylp-AfriboB-gpdAp* (control cassette from pYX14) in the *yA* locus was designated LO8030-4C.1.

Via *in vivo* yeast recombination, the "*E. coli*-yeast-*Aspergillus*" shuttle plasmid pYX10 was constructed to heterologous express the oxygenase genes *ecdK* and *htyE* in the transformant LO8030-4.1. The backbone of pYX10 comprises *ori*, *2μ* and *AMA1* for self-replication in *E. coli*, yeast and *Aspergillus*, respectively. The genes *ecdK* and *htyE* of pYX10 were under the promoters *gpdAp* and *amyBp* [10], respectively. The control plasmid pYX12 was identical to pYX10 but lacked the *ecdK* and *htyE* genes. The *A. nidulans* transformant with pYX10 was designated LO8030-5.1, while the control transformant with pYX12 was designated LO8030-5C.1. Later, another *AMA1*-based fungal-replicating plasmid pJQ10 (*amyBp-htyE-AppyrG*) was constructed and transformed into LO8030-4.1, the right transformant LO8030-4.2.1 was used for producing tetradeoxy echinocandin without gene *ecdK*.

RNA extraction and semi-quantitative RT-PCR

For transcriptional-level analysis of echinocandin BGC genes in the transformant LO8030-5.1 at different time points, the mycelia were collected in duplicate from *A. nidulans* LO8030-5.1 and its control LO8030-5C.1 for the indicated time during fermentation in XSMM with appropriate supplements. Total RNA was extracted using Trizol® reagent (Ambion), and DNase I (Promaga). cDNA was synthesized using a PrimeScript RT reagent Kit (TaKaRa) with 100ng DNase I-treated RNA. Semi-quantitative RT-PCR (semi-qPCR) was performed in a 25-μL reaction mixture with a template of 1 μL diluted cDNA (diluted 10 times) and 12.5 μL 2×Es Taq MasterMix (Cwbio, China). The internal control was Histone 2B gene (*H2B*), and the primers used for each of the indicated genes are listed in Table S4.

The expression level of different biosynthetic genes was tested on 3rd (left) and 7th (right) day *via* semi-qPCR. All the four genes transformed (*ecdA*, *ecdI*, *htyE*, and *ecdK*) were transcribed with varying degrees of expression. PCR template of RTC (reverse transcription control) was total RNA without reverse transcriptase to exclude the genomic DNA contamination. Echinocandin biosynthetic

genes *ecdG*, *ecdH* and *htyF* were not transformed into LO8030 as the negative control. The expression of *H2B* was used as the internal control.

Fermentation and HPLC/MS analysis

A. nidulans transformants LO8030-4.1 and LO8030-5.1 (Fig. S1), as well as their controls, were cultivated in the fermentation medium XSMM, which was derived from the liquid GMM and replaced the carbon source - glucose (10 g/L) with xylose (20 g/L) and starch (20 g/L). Linoleic acid (300 mg/L) was added to the fermentation medium for echinocandin production. Sufficient linoleic acid could enhance echinocandin production, since the activation of linoleic acid is the first step of the ECB biosynthesis process[11]. Specifically, 1 mg/L pyridoxine HCl and 100 mg/L L-homoTyr were supplemented in the media of LO8030-4.2.1, LO8030-5.1 and its control LO8030-5C.1. Besides pyridoxine HCl and L-homoTyr, 0.56 g/L uracil, and 1.26 g/L uridine were supplemented in the media of LO8030-4.1 and its control LO8030-4C.1.

Fermentation was carried out in triplicate in a 500 mL flask with 100 mL XSMM medium for 12 days at 25 °C and 180 rpm. After the fermentation, mycelia were isolated from culture broth by filtration. Ultrasound-assisted extraction[12] was conducted in an ultrasonic bath (Shumei ultrasonic instrument, Kunshan, China) to extract metabolites from the mycelia. The mycelia were placed into a 500 mL volumetric flask with 100 mL methanol and sonicated in an ultrasonic bath at 25 °C for three 30 min cycles. The broth was extracted three times with an equal volume of ethyl acetate. The organic phases were combined and evaporated using a vacuum evaporator to remove the solvents. The extracts were dissolved in 1 mL methanol and filtered through 0.22 μm filters for HPLC and LC-MS analysis. 20 μL volumes of the filtrates were subjected to reverse-phase high-performance liquid chromatographic (RP-HPLC) on an Agilent 1260. A COSMOCIL C_{18}-MS-II column (4.6 × 250 mm, 5 μm) was used at 35 °C and a flow rate of 1 mL/min with a linear gradient of 10-100% MeOH-H_2O (*v/v*) for 60 min. Peaks of echinocandins were determined at 222 nm wavelength.

LC-MS analyses were performed on the Agilent UPLC-Triple-TOF 5600^+ LC/MS system using positive mode electrospray ionization. A Waters ACQUITY UPLC HSS SB-C_{18} column (100 ×2.1 mm, 1.7 μm) was used with 0.1% formic acid in a linear gradient of 5-100% methanol and 0.1% formic acid in water as solvents at a flow rate of 0.4 mL/min.

Purification of novel echinocandins and NMR characterization

For purification of the novel echinocandins, the transformant LO8030-5.1 was grown in 10 L

XSMM medium for 12 days at 25 °C and 180 rpm. When the fermentation was finished, the broth was extracted three times with equal volumes of ethyl acetate and mycelium were extracted three times with 2 L methanol by ultrasonic wave. And then the organic phase was combined and distilled under reduced pressure to obtain the crude extracts (2.20 g). The extracts were separated by a silica gel column using CH_2Cl_2 /MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 1:1) in turn as the mobile phase. Preliminary identification of the echinocandins in different sub-factions was performed by TLC and HPLC. Then, further purification was carried out by the semi-preparative HPLC using MeOH/H_2O (85:15, 3.0 mL/min), with a COSMOCIL C_{18}-MS-II column (10 × 250 mm, 5 μm). Isolated compounds were dissolved in CD_3OD for NMR analysis. ^1H, ^{13}C and 2D (^1H-^{13}C HSQC, ^1H-^{13}C HMBC and ^1H-^1H COSY) NMR spectra were recorded on a JEOL 600 MHz spectrometer.

Antifungal susceptibility test

A described broth microdilution method[13,14] was used to evaluate the antifungal activity of the echinocandins against the growth of *Candida albicans* ATCC 10231. Amphotericin B was used as a positive control, while the solvent dimethyl sulfoxide (DMSO) was used as a negative control. *C. albicans* was cultured in Mueller-Hinton Broth (MHB) medium[13] in 96-well plates at a concentration of 1×10^6 CFU/mL. Final concentrations of the four tested compounds (Amphotericin B, ECB, ECE and ECF) were 0.05 to 2.0 μg/mL (μg/mL: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0). The MIC was determined after 12 h incubation at 37 °C.

Stability experiment

ECE (5.0 mg) was solved in 50% (*v/v*) methanol-water solution (2.5 mL) with the pH value of 10 and 1 mL to prepare the stock solution, which was incubated at 37 °C to test stability and measure the remaining concentration by HPLC analysis. At each stability time point (2, 4, 12, 14, 16, 24, 32, 43, 60, 84, and 166 h), 20 μL of the stock solution were injected into HPLC and separated on A COSMOCIL C_{18}-MS-II column (4.6 × 250 mm, 5 μm) at 35 °C. 85% MeOH-H_2O (*v/v*) was used to elute the samples with a flow rate of 1.0 mL/min and detective wavelength was 222 nm. The chromatographic peak area was used to present the remaining concentrations.

Supplementary Tables

Table S1. Strains used in this study.

Table S2. Plasmids constructed in this study.

Table S3. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of ECE (**1**) and ECF (**2**) in CD_3OD

Table S4. PCR primers used in this study

Table S5. Antifungal susceptibility test

Table S1. Strains used in this study.

Strain	Description	Purpose	Resource
Emericella rugulosa NRRL 11440	Wild type	To obtain *ecd/hty* BGC genes	ATCC
Escherichia coli DH5α	/	Plasmid cloning and storage	In the lab
Saccharomyces cerevisiae BJ5464	MATalpha, *ura*3-52, *trp*1, *leu*2-Δ1, *his*3-Δ200, *pep*4::*HIS*3, *prb*1-Δ1.6R, *can*1, GAL	Plasmid construction	[2]
Aspergillus nidulans LO8030 [a]	*pyr*G89, *pyro*A4, *ribo*B2,KO-DELETE :ST:::*pyr*G (Afu) integration(native); KO-DELETE :emericellamide:::*pyr*G (Afu) integration(native); KO-DELETE :asperfuranone:::*pyr*G (Afu) integration(native); KO-DELETE :monodictyphenone::*pyr*G (Afu) integration(native); KO-DELETE :terrequinone::*pyr*G (Afu) integration(native); KO-DELETE :austinol::*pyr*G (Afu) integration(native); KO-DELETE :F9775A/B::*pyr*G (Afu) integration(native); KO-DELETE :asperthecin::*pyr*G (Afu) integration(native)	Chassis strain	[15]
LO8030-1.1/1.3/1.6 [b]	*pyr*G89, *pyro*A4, *ΔyA::*(*xylp-ecdA*1-2-*AfriboB*)	1st transformants	This study
LO8030-2.2/2.4/2.5 [b]	*pyro*A4, *ribo*B2, *ΔyA::*(*xylp-ecdA*1-2-*ecdA*3-*gpdAp-ecdI-AppyrG*)	2nd transformants	This study
LO8030-4.1/4.2/4.3 [b]	*pyr*G89, *pyro*A4, *ΔyA::*(*xylp-ecdA-gpdAp-ecdI-AfriboB*)	3rd transformants	This study
LO8030-5.1/5.2/5.3/5.4/5.5/5.6 [b]	*pyro*A4, *ΔyA::*(*xylp-ecdA-gpdAp-ecdI-AfriboB*), pYX10	4th transformants	This study
LO8030-4.2.1	*pyro*A4, *ΔyA::*(*xylp-ecdA-gpdAp-ecdI-AfriboB*), pJQ10	LO8030-5.1 without *ecdK*	This study
LO8030-4C.1	*pyr*G89, *pyro*A4, *ΔyA::*(*xylp-gpdAp-AfriboB*)	Control for 3rd transformants without biosynthetic genes	This study
LO8030-5C.1	*pyro*A4, *ΔyA::*(*xylp-gpdAp-AfriboB*), pYX12	Control for 4th transformants without biosynthetic genes	This study

[a] The description of *Aspergillus nidulans* LO8030 transformants omit the same part as LO8030.

[b] 1.1/1.3/1.6, 2.2/2.4/2.5, 4.1/4.2/4.3 and 5.1/5.2/5.3/5.4/5.5/5.6 mean different transformants in each round of transformation.

Table S2. Plasmids constructed in this study.

Name	Description	Purpose
pYX1	*xylp-ecdA*1-2-*AfriboB*	Contain cassette for 1st tranformation
pYX5	*ecdA*3-*AppyrG-gpdAp-ecdI*	Contain cassette for 2nd tranformation
pYX9	*ecdA*4-*AfriboB-gpdAp*	Contain cassette for 3rd tranformation
pJQ10	*amyBp-htyE-AppyrG*, *AMA1*	Heterologous expression of *htyE*
pYX10	*amyBp-htyE-gpdAp-ecdK-AppyrG*, *AMA1*	Heterologous expression of oxygenase genes
pYX14	*xylp-AfriboB-gpdAp*, *AMA1*	Contain cassette for control transformant
pYX12	*amyBp- gpdAp-AppyrG*, *AMA1*	Control for pYX10

Table S3. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of ECE (**1**) and ECF (**2**) in CD_3OD

position	**1**	**2**	position	**1**	**2**
1st L-Orn					
C-1	175.0	175.1			
C-2	53.4	53.5	2-H	4.38	4.39
C-3	28.4	28.4	3-H_2	1.54 , 2.06	1.54 , 2.10
C-4	25.1	25.1	4-H_2	1.69 , 1.69	1.69 , 1.69
C-5	38.5	38.5	5-H_2	3.02 , 3.41	2.98 , 3.47
2nd L-Thr					
C-1	172.8	172.9			
C-2	58.9	58.9	2-H	4.81	4.83
C-3	68.5	68.7	3-H	4.41	4.42
C-4	19.9	19.9	4-H	1.26 (d, *J* = 6.3 Hz)	1.26 (d, *J* = 6.3 Hz)
3rd 4*R*-OH-L-Pro					
C-1	173.8	173.8			
C-2	62.3	62.3	2-H	4.55	4.54
C-3	38.7	38.7	3-H_2	1.82 , 2.32	1.81 , 2.32
C-4	71.4	71.4	4-H	4.49	4.48
C-5	57.2	57.2	5-H_2	3.76 , 3.76	3.75 , 3.75
4th L-homoTyr					
C-1	174.0	174.0			

C-2	55.4	55.4	2-H	4.23	4.26
C-3	34.5	34.5	3-H_2	2.14 , 2.24	2.11 , 2.24
C-4	33.0	33.0	4-H_2	2.58 , 2.58	2.58 , 2.58
C-1’	133.2	133.2			
C-2’/ C-6’	130.6	130.6	2’/ 6’- H	7.01	7.01
C-3’/ C-5’	116.4	116.4	3’/ 5’- H	6.70	6.70
C-4’	156.9	156.9			
5th L-Thr					
C-1	170.8	170.6			
C-2	56.7	56.6	2-H	4.90	4.89
C-3	69.4	69.5	3-H	4.27	4.23
C-4	20.6	20.6	4-H	1.18 (d, *J* = 6.5 Hz)	1.18 (d, *J* = 6.5 Hz)
6th 3*S*-OH-L-Pro/ 3*S*-OH-4*S*-Me-L-Pro					
C-1	172.5	172.6			
C-2	70.1	69.9	2-H	4.34	4.22
C-3	76.0	74.3	3-H	4.18	4.35
C-4	39.1	34.5	4-H_2 / H	2.45	1.97 , 2.24
C-5	52.9	46.8	5-H_2	3.39 , 3.88	3.81 , 3.81
4-CH_3	11.5		4-CH_3	1.06 (d, *J* = 6.5 Hz)	
Fatty acid side chain					
C-1	176.7	176.7			
C-2	36.9	37.0	2-H	2.23 (m, 2H)	2.24 (m, 2H)
C-3	27.2	27.2	3-H	1.60 (m, 2H)	1.61 (m, 2H)
C-8 / C-14	28.3, 28.3	28.3, 28.3	8-H / 14-H	2.06 (m, 4H)	2.06 (m, 4H)
C-9 / C-10 / C-12 / C-13	131.1, 131.1 129.2, 129.2	131.1, 131.1 129.2, 129.2	9-H / 10-H /12-H / 13-H	5.29-5.39 (m, 4H)	5.29-5.39 (m, 4H)
C-11	26.7	26.7	11-H	2.77 (t, *J* = 6.8 Hz, 2H)	2.78 (t, *J* = 6.8 Hz, 2H)
C-4 / C-5 / C-6 / C-7 / C-15 / C-16 / C-17	32.8, 30.9 30.6, 30.5 30.4, 30.4 23.8	32.8, 30.9 30.6, 30.5 30.4, 30.4 23.8	4-H / 5-H /6-H / 7-H /15-H / 16-H /17-H	1.28-1.39 (m, 14H)	1.28-1.39 (m, 14H)
C-18	14.6	14.6	18-H	0.91 (t, *J* = 7.2 Hz, 3H)	0.91 (t, *J* = 7.2 Hz, 3H)

Table S4. PCR primers used in this study.

Name	Sequence (5'-3')	Purpose
*ecdA*1-F	ATGCACAGAACCAACGAGATGG	Amplification of *ecdA*1
*ecdA*1-R	TCGCTGCAGGTGGAGTGAATG	
*ecdA*2-F	CGTTTGCGACCCTTACCTCTAC	Amplification of *ecdA*2
*ecdA*2-R	TGGCCTGCTCCTCAACCATTTC	
pYX1-*riboB*-*yA*dn-F	AACAATGTGTGCATGAAATGGTTGAGGAGCAGGCCAGCGGACTGAGTTATGGATGAC	Amplification of *AfriboB*-*yA*-dn for pYX1
pYX1-*riboB*-*yA*dn-R	AGGAACCGAAATACATACATTGTCTTCCGTAAAGCCCCAGTCACGACGTTGTAAAAC	
pYX1-AscI-F	GCTTTACGGAAGACAATG	Amplification of backbone for pYX1
pYX1-AscI-R	CAATTCCACACAACATACGAGCCGGAAGCATAAAGAATTCGGGTGCCTAATGAGTGAG	
pYX1-*yA*up-F	TTTATGCTTCCGGCTCG	Amplification of *yA*-up for pYX1
pYX1-*yA*up-R	ATAAGTATACTCTATTGACCTATAGGACCTGAGTGATGCTCGGCTTGATGTTGTTCG	
pYX1-*xylp*-F	GCATCACTCAGGTCCTATAGG	Amplification of *xylp* for pYX1
pYX1-*xylp*-R	CAGTTGGTTCTTCGAGTCGATG	
*ecdA*3-F	ACTCATTAGGCACCCAAATATGCGGCCGCTATAAAGAACAGTTCTTCGTATGGACG	Amplification of *ecdA*3
*ecdA*3-R	CTTTCGTTGGATCTGGTATTC	
pYX5-*pyrG*-F	CGAGGTCGGTGAGGTTGAATACCAGATCCAACGAAAGAGCTTATACGAACAGATGG	Amplification of *AppyrG* for pYX5
pYX5-*pyrG*-R	CAAATAGAATTAAATGGTACTCGAGTCCACTGAAGGTGCCCAACGCATTCTTCATG	
pYX5-g*pdA*-F	CACCTTCAGTGGACTCGAGTAC	Amplification of *gpdAp* for pYX5
pYX5-*gpdA*-R	CCAGTTGGCTGCACCATGCTGGGGAAGTGAAGACCATTGTGATGTCTGCTCAAGCG	
ecdI-F	ATGGTCTTCACTTCCCCAGCATG	Amplification of *ecdI*
ecdI-R	CCAAGGCGCATGTACTAGTCAAG	
pYX5-*yA*-dn-F	GTATCATAACAATCTTGACTAGTACATGCGCCTTGGGGGGTTGATGTTTGGGATTC	Amplification of *yA*-dn for pYX5
pYX5-*yA*-dn-R	ACATTGTCTTCCGTAAAGCTTTATAGCGGCCGCATATTTCCTCGGTTCAGTCTTACTAG	

pYX5-AscI-F	AAATATGCGGCCGCTATAAAGCTTTACGGAAGACAATG	Amplification of backbone for pYX5
pYX5-AscI-R	TTTATAGCGGCCGCATATTTGGGTGCCTAATGAGTGAG	
*ecdA*4-F	GACCTTGCTGCCTCATTTGC	Amplification of *ecdA*4
*ecdA*4-R	GGCTCCCTTTATGCTCTTCG	
pYX9-*riboB*-F	*AGTCAGACAATGTTCCGAAGAGCATAAAGGGAGCC*GCGGACTGAGTTATGGATGAC	Amplification of *AfriboB* for pYX9
pYX9-*riboB*-R	CAACAAGTGCCACTCAACGC	
pYX9-*gpdAp*-F	*TCACTGAGTCAATGGCGTTGAGTGGCACTTGTTG*CACCTTCAGTGGACTCGAGTAC	Amplification of *gpdAp* for pYX9
pYX9-*gpdAp*-R	*ATTGTCTTCCGTAAAGCTTTATAGCGGCCGCATATTT*TGTGATGTCTGCTCAAGCG	
pYX9-AscI-F	AAATATGCGGCCGCTATAAAGCTTTACGGAAGACAATG	Amplification of backbone for pYX9
pYX9-AscI-R	AGCAAATGAGGCAGCAAGGTCTTTATAGCGGCCGCATATTTGGGTGCCTAATGAGTGAG	
pYX14-*yA*-up -F	ACTCATTAGGCACCCAAATATGCGGCCGCTATAAATTTATGCTTCCGGCTCGTAT	Amplification of *yA*-up for pYX14
pYX14-*yA*-up -R	ATAAGTATACTCTATTGACCTATAGGACCTCGGCTTGATGTTGTTCG	
pYX14-*xylp*-F	ACGGAAGCGCGCAGTCGGCG	Amplification of *xylp* for pYX14
pYX14-*xylp*-R	GAAAGGGAGTCATCCATAACTCAGTCCGCAGTTGGTTCTTCGAGTCGATG	
pYX14-*riboB*-F	GCGGACTGAGTTATGGATGAC	Amplification of *AfriboB* for pYX14
pYX14-*riboB*-R	CAACAAGTGCCACTCAACGC	
pYX14-*gpdAp*-F	*GAGTCAATGGCGTTGAGTGGCACTTGTTG*CACCTTCAGTGGACTCGAGTAC	Amplification of *gpdAp* for pYX14
pYX14-*gpdAp*-R	TGTGATGTCTGCTCAAGCG	
pYX14-*yA*-dn-F	*ACAGCTACCCCGCTTGAGCAGACATCACA*GGGGTTGATGTTTGGGATTC	Amplification of *yA*-dn for pYX14
pYX14-*yA*-dn-R	TGTCTTCCGTAAAGCTTTATAGCGGCCGCATATTTCCTCGGTTCAGTCTTACTAG	
pYX14-AscI-F	AAATATGCGGCCGCTATAAAGCTTTACGGAAGACAATGTA	Amplification of backbone for pYX14
pYX14-AscI-R	TTTATAGCGGCCGCATATTTGGGTGCCTAATGAGTGAGCT	
pYX10-*amyBp*-F	CCATCATGGTGTTTTGATC	Amplification of *amyBp* for pYX10
pYX10-*amyBp*-R	AATTCGGAGCTTGCTGTGG	

pYX10-*htyE*-F	TTCTCTGAACAATAAACCCCACAGCAAGCTCCGAATTATGGCTATCACTACGCTAG	Amplification of *htyE*
pYX10-*htyE*-R	TGTCAACTACGACTGTCATG	
pYX10-*gpdAp*-F	CTCATATTCATATTCATGACAGTCGTAGTTGACACACCTTCAGTGGACTCGAGTAC	Amplification of *gpdAp*
pYX10-*gpdAp*-R	TGTGATGTCTGCTCAAGCG	for pYX10
pYX10-*ecdK*-F	CTTGACTAACAGCTACCCCGCTTGAGCAGACATCACAATGTCTGTTCTAACTCTCG	Amplification of *ecdK*
pYX10-*ecdK*-R	GGAATAGTCCTCTCGGGCCATCTGTTCGTATAAGCTGATAAACAGCAGGTGACATG	
pYX10-*pyrG*-F	AGCTTATACGAACAGATGG	Amplification of *AppyrG*
pYX10-*pyrG*-R	ATACAAAAAATAAGCTGGCTTTCCCCGTCAAGCTCTAACCCAACGCATTCTTCATG	for pYX10
pYX10-*AMA1*-F	TTAGAGCTTGACGGGGAAAGC	Amplification of *AMM1*
pYX10-*AMA1*-R	CCAGGAACCGAAATACATACATTGTCTTCCGTAAAGCACTCTAGAGGATCCTGCAG	for pYX10
pYX10-AscI-F	GCTTTACGGAAGACAATG	Amplification of backbone
pYX10-AscI-R	CCATATAAAAATTTAAAATGATCAAAACACCATGATGGGGGTGCCTAATGAGTGAG	for pYX10
pYX12-*gpdAp*-F	TCTGAACAATAAACCCCACAGCAAGCTCCGAATTCACCTTCAGTGGACTCGAGTAC	Amplification of *gpdAp*
pYX12-*gpdAp*-R	CGGAATAGTCCTCTCGGGCCATCTGTTCGTATAAGCTTGTGATGTCTGCTCAAGCG	for pYX12
pJQ10-*pyrG/AMA1*-F	AGCTTATACGAACAGATGG	Amplification of *pyrG-AMA1* for pJQ10
pJQ10-*pyrG/AMA1*-R	CCAGGAACCGAAATACATACATTGTCTTCCGTAAAGCACTCTAGAGGATCCTGCAG	
pJQ10-AscI-F	GCTTTACGGAAGACAATG	Amplification of backbone
pJQ10-AscI-R	GGGTGCCTAATGAGTGAG	for pJQ10
pJQ10-*amyBp/htyE*-F	CGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCCATCATGGTGTTTTGATC	Amplification of *amyBp-htyE* for pJQ10
pJQ10-*amyBp/htyE*-R	CGGAATAGTCCTCTCGGGCCATCTGTTCGTATAAGCTTGTCAACTACGACTGTCATG	
RT-*ecdA*-F	TTCTCCGGGTCTTCTTGTC	Semi-quantitative RT-PCR
RT-*ecdA*-R	TTCTTCCCATTCTGGTGTC	
RT-*ecdI*-F	TCTTTGGCATTAACGACG	
RT-*ecdI*-R	CTGTGACCCTTGGGAATAC	

RT-*ecdK*-F	ATCTGCCTGTGCGGTTTG	
RT-*ecdK*-R	ATCCTGGAACGATGGACG	
RT-*htyE*-F	TTTGTTCGGCGAATCATCG	Semi-quantitative
RT-*htyE*-R	GCAACGCAGTCAAATGGTC	RT-PCR
RT-*H2B*-F	CTGCCGAGAAGAAGCCTAGCAC	
RT-*H2B*-R	GAAGAGTAGGTCTCCTTCCTGGTC	

Table S5. Antifungal susceptibility test

Compounds	**MIC** (μg/mL)
ECB	0.4
ECE	0.6
ECF	1.0
Amphotericin B	0.2

Supplementary Figures

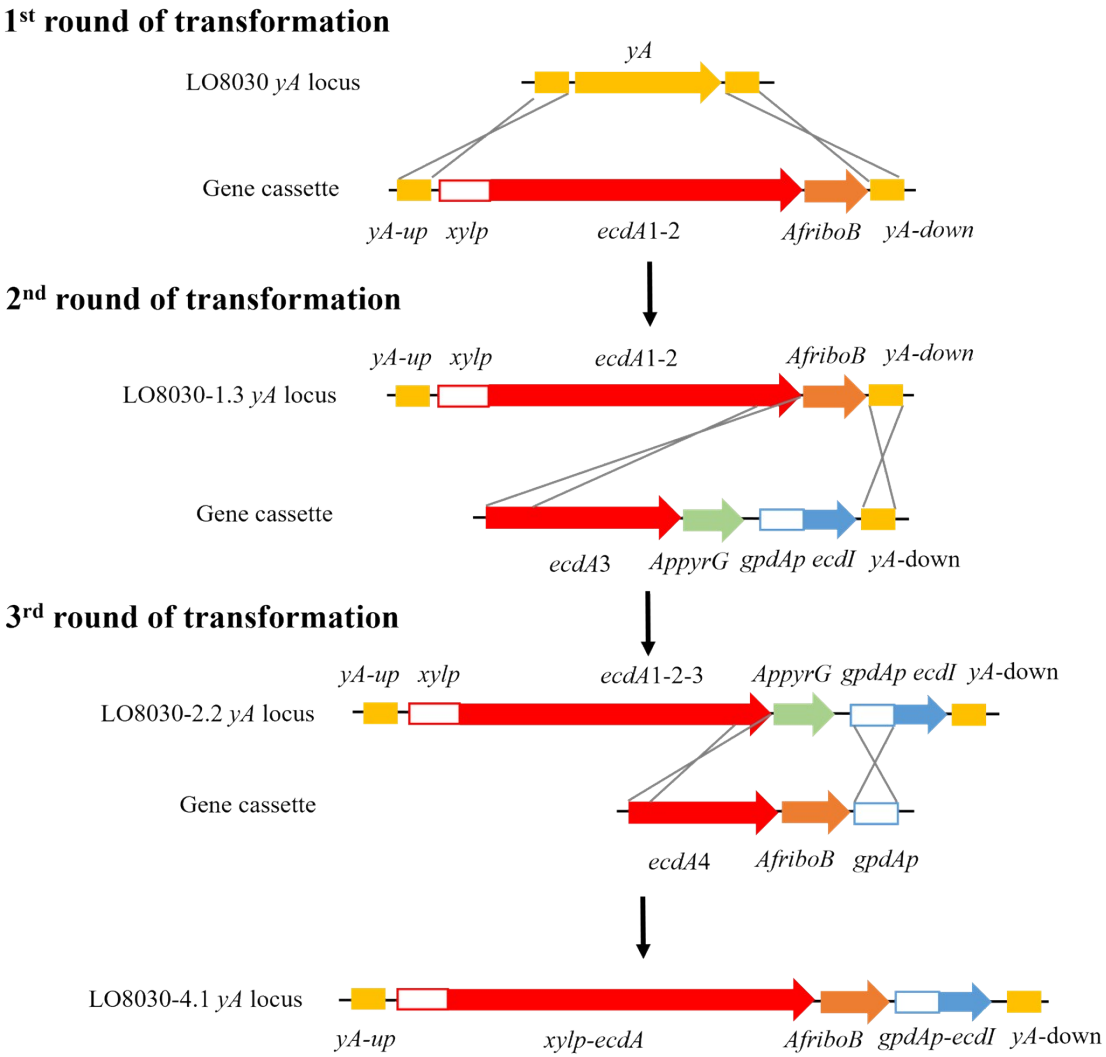

Fig. S1 The strategy for integrating large NRPS gene *ecdA* and *ecdI* into *yA* locus of *A. nidulans* LO8030

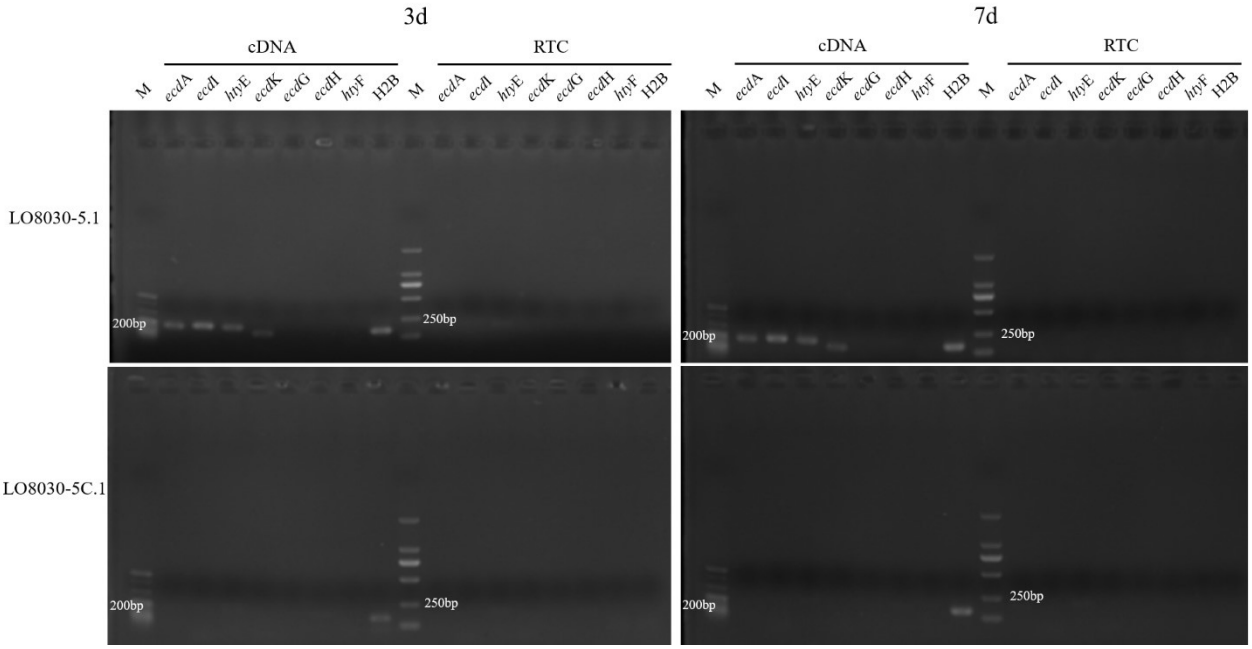

Fig. S2 Transcriptional analysis of echinocandin biosynthetic genes in LO8030-5.1

The expression level of different biosynthetic genes was tested on 3rd (left) and 7th (right) day *via* semi-qPCR. All the four genes transformed (*ecdA*, *ecdI*, *htyE*, and *ecdK*) were transcribed with varying degrees of expression. PCR template of RTC (reverse transcription control) was total RNA without reverse transcriptase to exclude the genomic DNA contamination. Echinocandin biosynthetic genes *ecdG*, *ecdH* and *htyF* were not transformed into LO8030 as the negative control. The expression of *H2B* was used as the internal control.

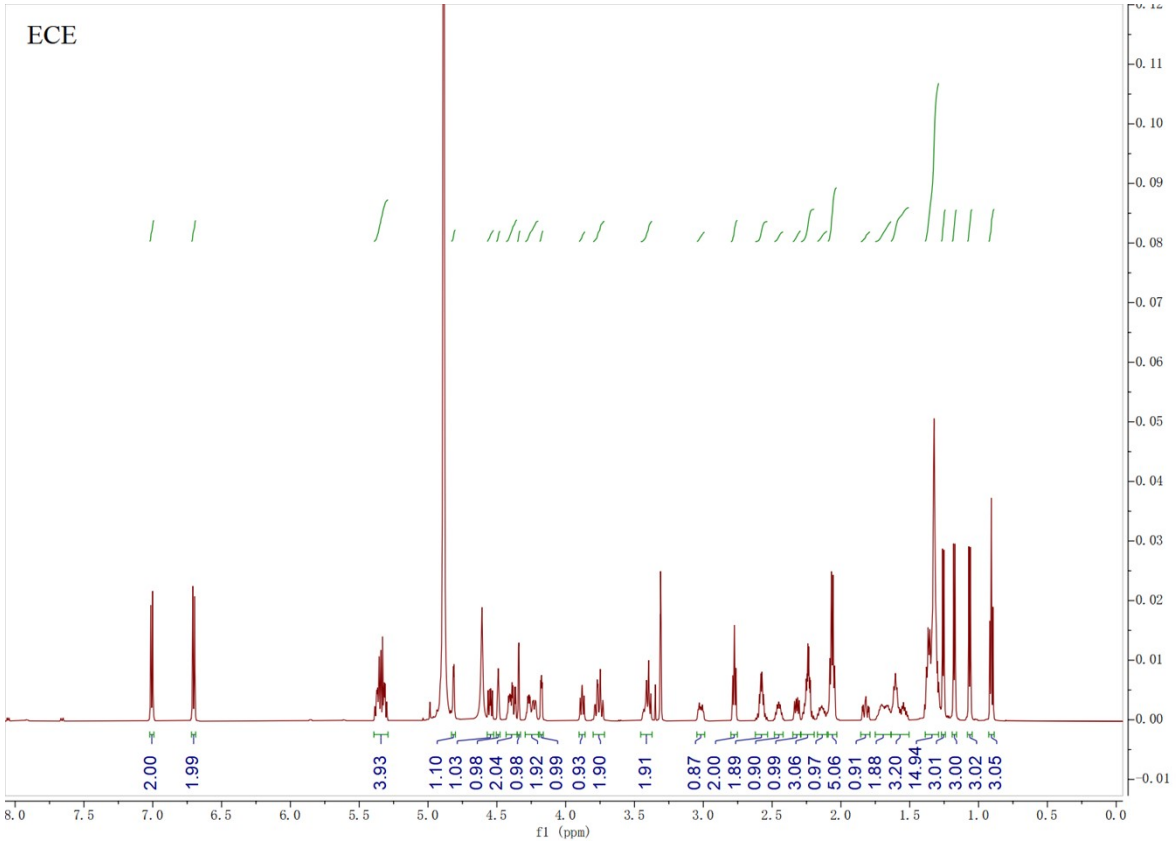

Fig. S3 ^{1}H spectrum of ECE (compound **1**) in CD_3OD (600 MHz)

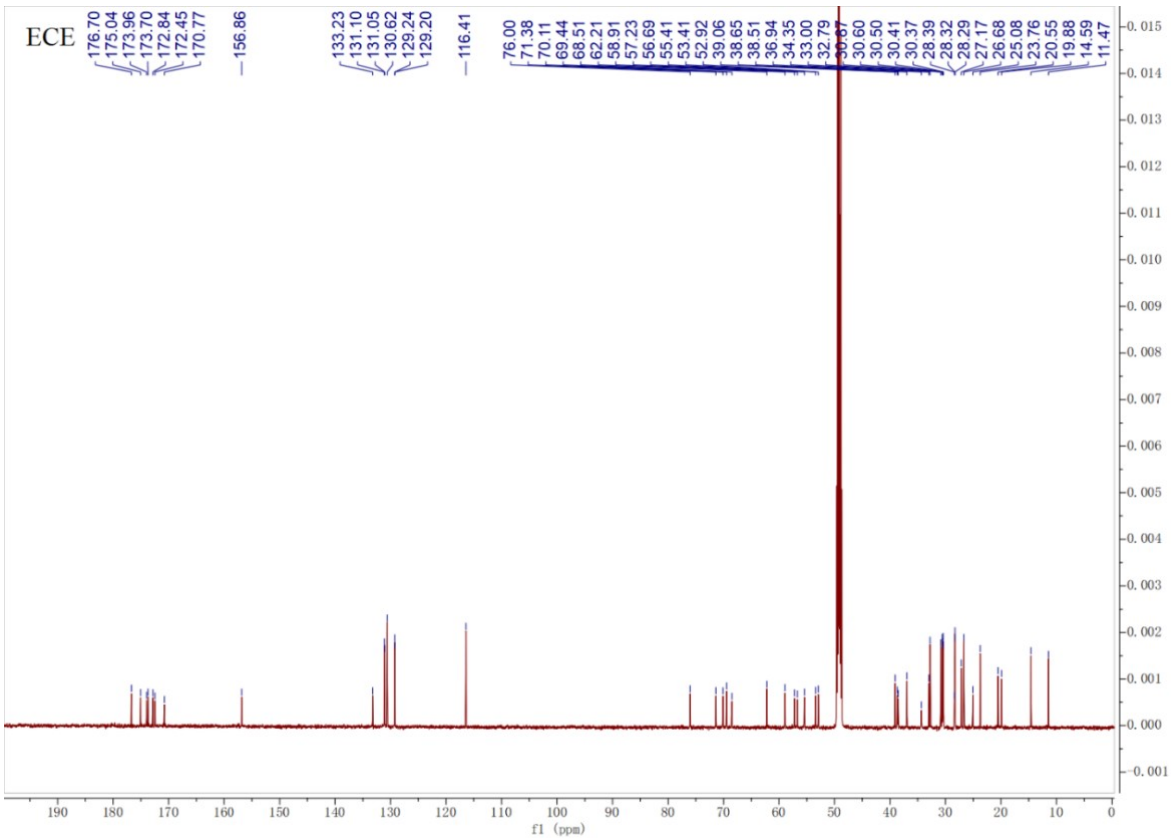

Fig. S4 ^{13}C spectrum of ECE (compound **1**) in CD_3OD (150 MHz)

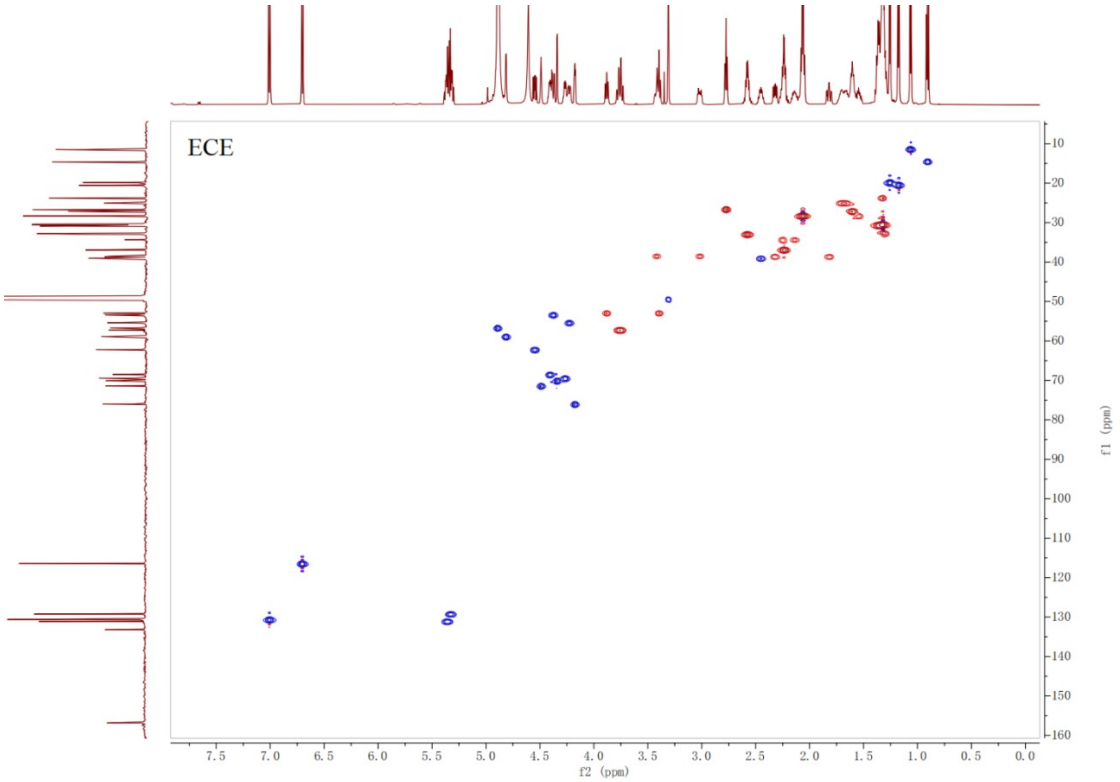

Fig. S5 ^{1}H-^{13}C HSQC spectrum of ECE (compound **1**) in CD_3OD

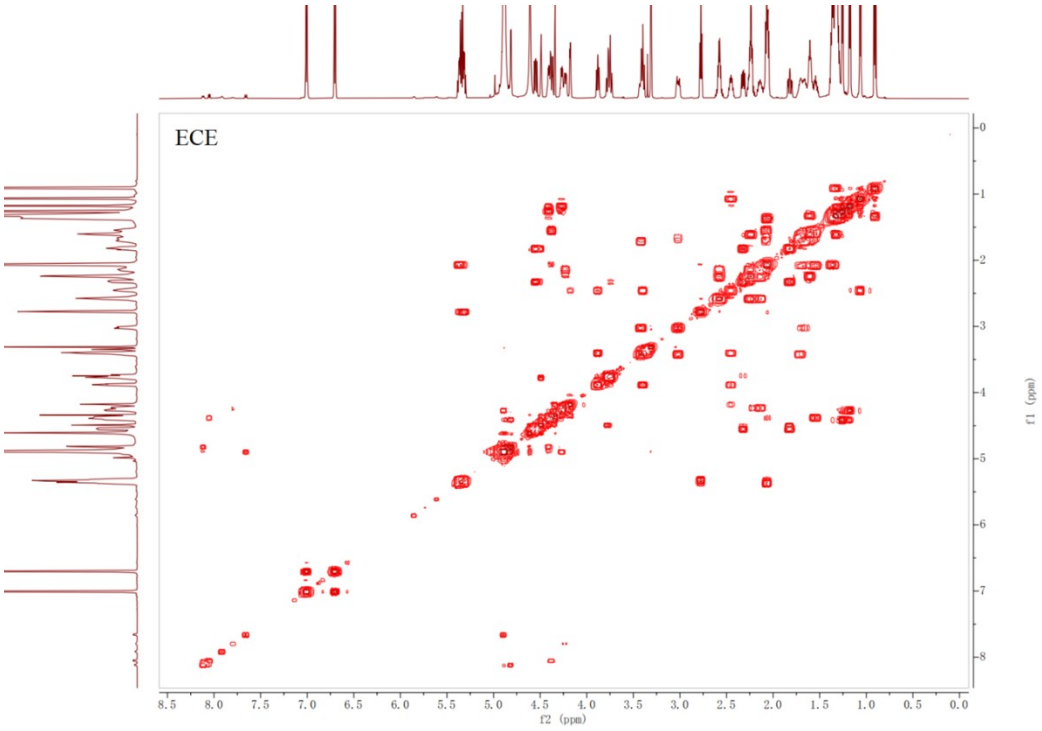

Fig. S6 ^{1}H-^{1}H COSY spectrum of ECE (compound **1**) in CD_3OD

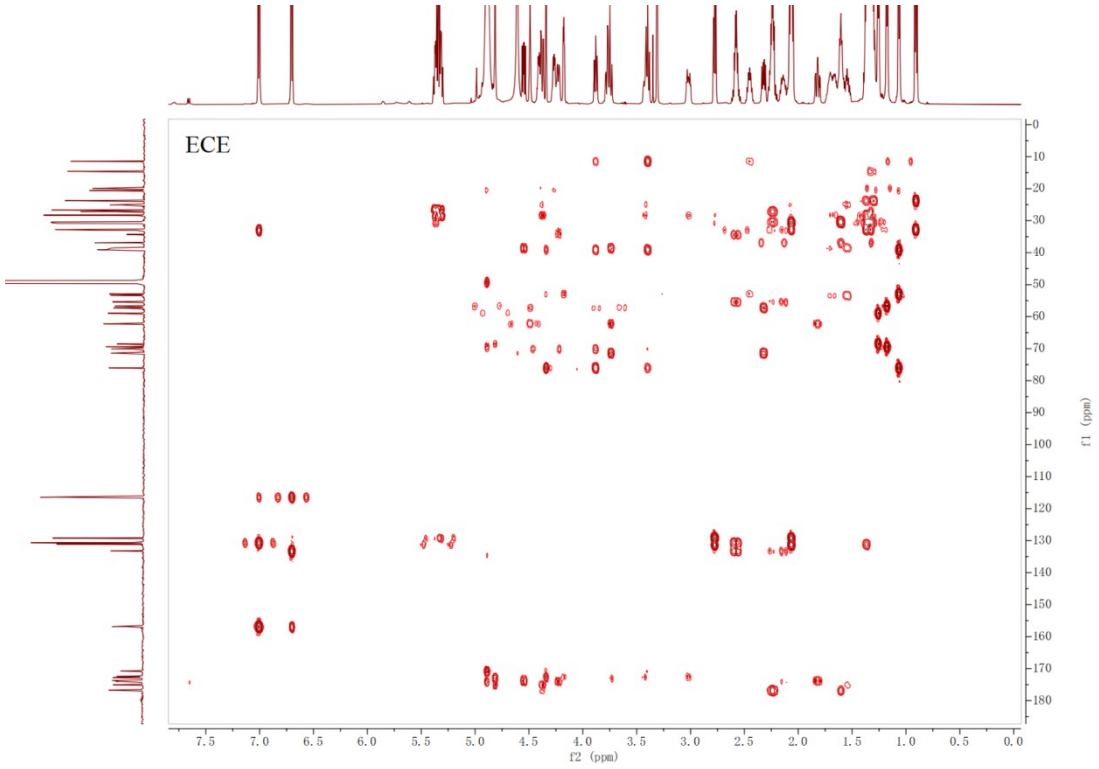

Fig. S7 ^{1}H-^{13}C HMBC spectrum of ECE (compound **1**) in CD_3OD

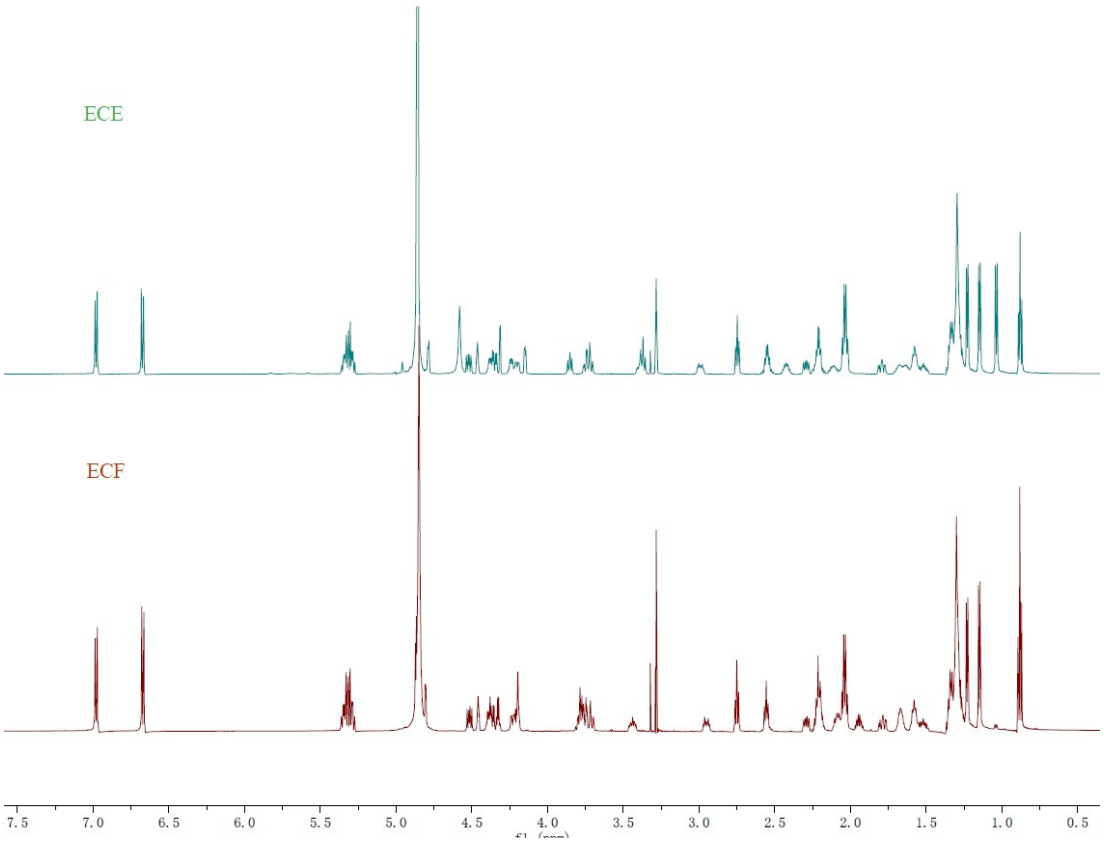

Fig. S8 Comparison of ^{1}H spectra of ECE (**1**) and ECF (**2**) in CD_3OD (600MHz)

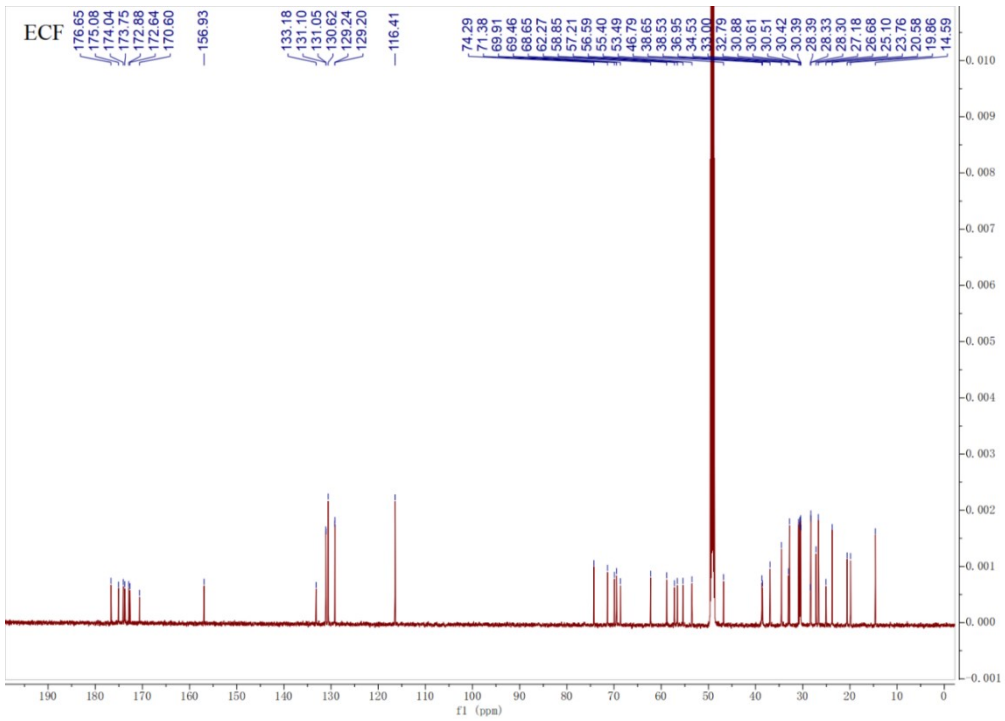

Fig. S9 ^{13}C spectrum of ECF (compound **2**) in CD_3OD (150MHz)

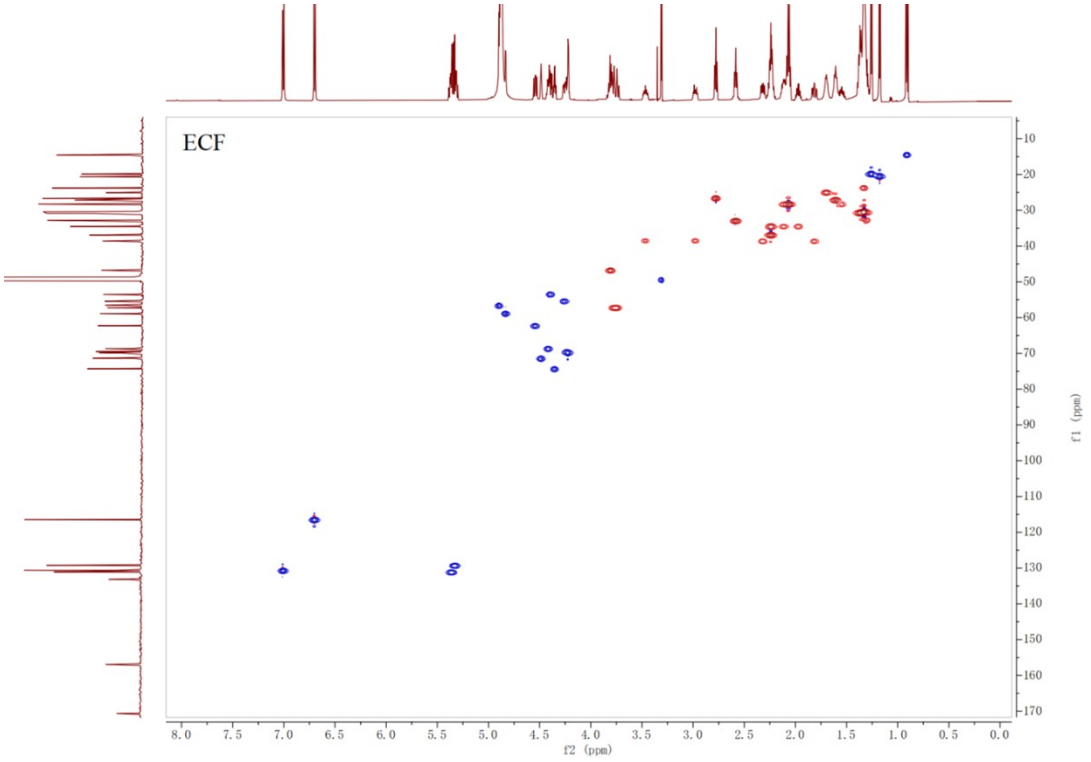

Fig. S10 ^{1}H-^{13}C HSQC spectrum of ECF (compound **2**) in CD_3OD

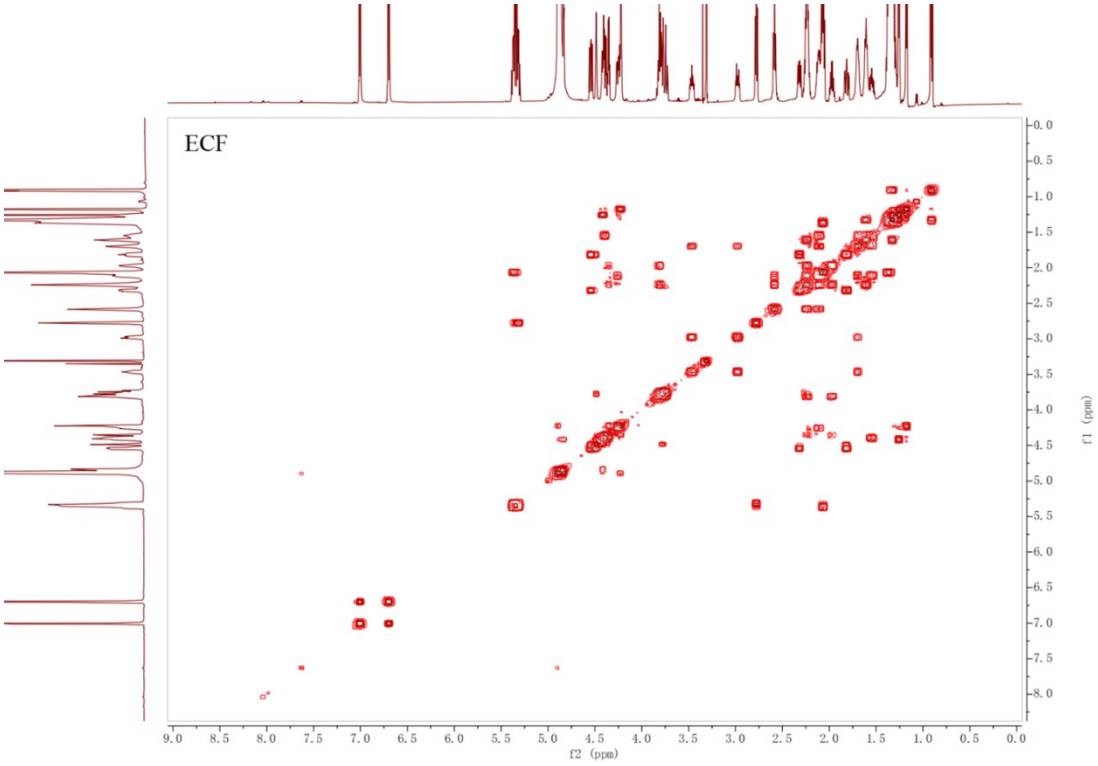

Fig. S11 ^{1}H-^{1}H COSY spectrum of ECF (compound **2**) in CD_3OD

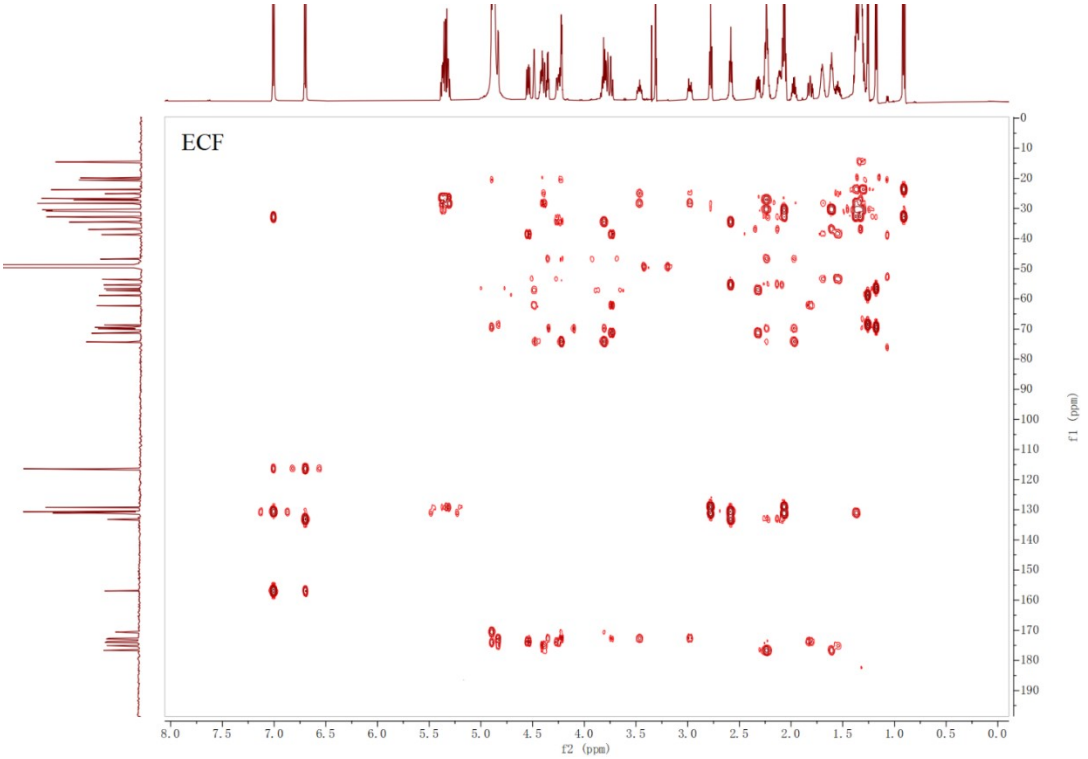

Fig. S12 ^{1}H-^{13}C HMBC spectrum of ECF (compound **2**) in CD_3OD

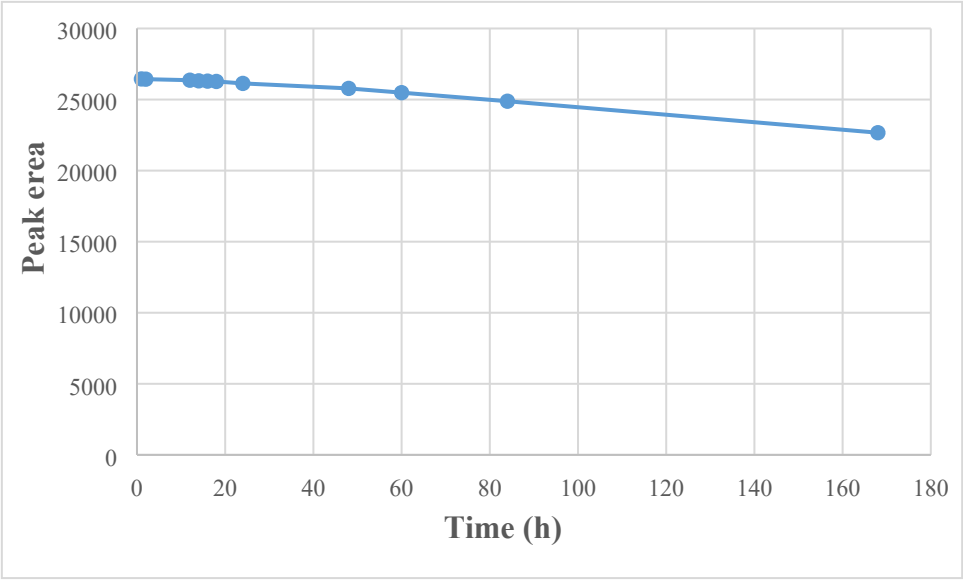

Fig. S13 The stability of ECE (**1**) in methanol-water solution (pH 10)

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