Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Characterisation and heterologous biosynthesis of burnettiene A, a new polyene-decalin polyketide from *Aspergillus burnettii*

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Supporting Experimental Methods

1. General experimental details

The analyses of small-scale liquid culture extracts were performed on an Agilent 1260 liquid chromatography (LC) system coupled to a diode array detector (DAD) and an Agilent 6130 single quadrupole mass spectrometer (MS) with an electrospray ionisation (ESI) source. In all cases 3 µL of the methanol dissolved crude extract (66.7 times concentrated from liquid media extract) was injected. Chromatographic separation was performed at 40 °C using a Kinetex C18 column (2.6 µm; 2.1 mm i.d. × 100 mm; Phenomenex). Chromatographic separation was achieved with a linear gradient of 5–95% MeCN/H_2O (containing 0.1% v/v formic acid) in 10 min followed by 95% MeCN for 3 min, at a flow rate of 0.70 mL/min. The MS data were collected in the *m/z* range 100–1000 in negative ion mode and UV observed at DAD λ = 330 nm. Peak areas from relevant peaks of DAD λ = 330 nm chromatogram were integrated using Masshunter Workstation Qualitative Analysis (Agilent).

Analytical HPLC was performed on a gradient Agilent 1260 Infinity quaternary HPLC system. The column was an Agilent Zorbax SB-C18 (2.1 × 50 mm, 1.8 µm) eluted with a 0.6 mL/min gradient of 10-100% MeCN/H_2O (0.01% TFA) over 8.33 min. LC-MS was performed on an Agilent 1260 Infinity series HPLC equipped with an Agilent 6130 Infinity series single quadrupole mass detector in both positive and negative ion modes.

NMR spectra were recorded in 5 mm Pyrex tubes (Wilmad, USA) on a Bruker Avance II DRX-600K 600 MHz spectrometer. All NMR spectra were acquired at 25 °C, processed using Bruker Topspin 3.5 software and referenced to residual solvent signals (DMSO-d_6 δ_H 2.49/δ_C 39.5 ppm). High resolution electrospray ionisation mass spectra (HRESIMS) were obtained on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) by direct infusion in MeCN.

Silica fractionation was performed on a silica gel column (100 g, 300 × 50 mm) (Agilent Silica Bondesil-SI 40 µm). The column was washed once with hexane, then eluted with 50% hexane/$CHCl_3$, 25% hexane/$CHCl_3$ and $CHCl_3$, followed by a stepwise gradient of 1, 2, 4, 8, 16, 32 and 100% MeCN/$CHCl_3$ (500 mL each step).

2. Comparative genomic analysis

The putative *bue* BGC genes from *Aspergillus burnettii* FRR 5400[1] (isotype from MST FP2249) were analysed by with cblaster[2] against NCBI. Identified homologous BGCs where compared with clinker.[3] PKS domain architecture was plotted with Synthaser.[4]

3. Vector construction

All vectors are listed on Table S6 and primers in Table S8, with their purposes indicated. The different combinations of *bue* genes were cloned by isothermal assembly. The fragments were PCR-amplified from gDNA extracted from *A. burnettii* MST FP2249 using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). The fragments were cloned into pYFAC episomal vectors (Addgene IDs #168978, #168979, #168980)[5] with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, MA, USA). The *gpdA* promoter sequence was amplified from pBARGPE1[6], obtained from the Fungal Genetics Stock Centre. The vectors were verified by whole plasmid next generation sequencing. Vector schematics can be found at Fig. S27.

4. *Aspergillus nidulans* transformation and small-scale culture conditions

Strains are indicated in Table S7. Protoplasts of *Aspergillus nidulans* LO8030[7] were prepared from germlings as in Lim *et al.*[8] Protoplasts were transformed with pYFAC vectors with the following procedure: in a 2 mL microcentrifuge tube, 60 µL of thawed protoplast solution was incubated with 50 µL of STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) and 3 µg of each vector contained in maximum total volume of 10 µL. After 20 min of incubation on ice, 350 µL of the calcium PEG 60% mix was added and mixed gently by inversion, followed by a 20 min incubation at room temperature. After adding 1 mL of STC buffer the mix was spread on Sorbitol Minimal Medium (SMM) (For 1 L SMM: 218.6 g sorbitol, 10 g glucose, 6 g $NaNO_3$, 1.52 g K_2HPO_4, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 11 mg H_3BO_3, 5 mg $MnCl_2 \cdot 4H_2O$, 1.6 mg $FeSO_4 \cdot 7H_2O$, 1.6 mg $CoCl_2 \cdot 5H_2O$, 1.6 mg $CuSO_4 \cdot 5H_2O$, 1.1 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 mg Na_4EDTA, adjusted to pH 6.5, 16 g agar).[5] Plates were then incubated for three days at 37 °C to generate transformant colonies.

For each transformant strain, spores from three individual colonies were picked as biological replicates for culture analysis and re-streaked individually in a solidified GMM plate (SMM without sorbitol) and cultivated for three days at 37 °C. Spores were harvested from plates in 1 mL of 0.1% Tween 80 (Sigma, MO, USA) and 2×10^8 spores were inoculated into 250-mL flasks containing 50 mL liquid GMM medium as described previously.[5] Additionally, ampicillin was added to 50 µg/mL. Cultures were grown for 2.5 days with shaking set to 200 rpm at 25 °C. At the end of the culture, 20 mL of media was collected in 50 mL conical tubes by filtration with Miracloth (Milipore, MA, USA). The metabolites were extracted from the liquid culture with 20 mL of ethyl acetate, methanol, and acetic acid (89.5 : 10 : 0.5 ratio). The crude extracts were dried down *in vacuo* and re-dissolved in 0.3 mL of methanol for LC-DAD-MS analysis.

5. Scale up and purification

To purify preburnettiene A (**3**), a preparative-scale cultivation of *A. nidulans* strain 1 (Table S7) was carried out. To prepare the jasmine rice media, 2000 g of dry rice was hydrated with 1200 mL distilled water then and autoclaved at 121 °C for 40 min. A spore suspension made using a 7-day old petri plate (GMM agar) stirred in 200 mL sterile water was used to inoculate 3000 g of hydrated jasmine rice media and incubated at 24 °C for 14 days. The rice was extracted into acetone (2 × 6000 mL), pooled and evaporated *in vacuo*, leaving an aqueous fraction (49.9 g, 500 mL). The aqueous fraction was partitioned with ethyl acetate (2 × 1000 mL), and the organic layer evaporated under vacuum to an oily residue (12.0 g) that was dissolved in methanol (400 mL), and defatted against hexane (2 × 500 mL) to give the crude extract (5.0 g). The crude extract was adsorbed onto silica gel which was loaded onto a silica gel column (100 g, 300 × 50 mm). The column was washed once with hexane, then eluted with 50% hexane/$CHCl_3$, 25% hexane/$CHCl_3$ and $CHCl_3$, followed by a stepwise gradient of 1, 2, 4, 8, 16, 32 and 100% MeCN/$CHCl_3$ (500 mL each step), to yield 11 fractions (Fr 1–11). Fraction 6 of this separation gave a pure orange residue of preburnettiene A (200 mg). For full isolation scheme of **3**, see Fig. S34.

For preburnettiene B (**2**), similar cultivation procedures were carried out with strain 2 (Table S7). The rice (3200 g) was extracted with acetone (3 × 2800 mL), pooled and evaporated *in vacuo* to an aqueous concentrate. The concentrate (63.3 g, 1250 mL) was partitioned with ethyl acetate (2 × 2500 mL) and the organic layer evaporated under vacuum to an oily residue (21.1 g) that was dissolved in methanol (400 mL) and defatted against hexane (2 × 400 mL) to give the crude extract (7.5 g). The crude extract was adsorbed onto silica gel which was loaded onto a silica gel column (100 g, 300 × 50 mm). The column was washed once with hexane, then eluted with 50% hexane/$CHCl_3$, 25% hexane/$CHCl_3$ and $CHCl_3$, followed by a stepwise gradient of 1, 2, 4, 8, 16, 32 and 100% MeCN/$CHCl_3$ (500 mL each step), to yield 11 fractions (Fr 1–11). Fraction 11 of this separation gave a pure yellow solid of preburnettiene B (1.5 g). For full isolation scheme of **2**, see Fig. S35.

Finally, for burnettiene A (**1**) isolation similar cultivation procedures were carried out with *A. nidulans* strain 3 (Table S7). The rice (2873 g) was extracted with acetone (2 × 3000 mL), pooled and evaporated *in vacuo*, to an aqueous concentrate (62.4 g, 750 mL). The concentrate was partitioned against ethyl acetate (2 × 1500 mL), and the organic layer evaporated under vacuum to produce an oil (15.6 g) that was dissolved in methanol (400 mL) and defatted with hexane (2 × 400 mL) to give the crude extract (9.1 g). The crude extract was adsorbed onto silica gel which was loaded onto a silica gel column (100 g, 300 × 50 mm). The column was washed once with hexane, then eluted with 50% hexane/$CHCl_3$, 25% hexane/$CHCl_3$ and $CHCl_3$, followed by a stepwise gradient of 1, 2, 4, 8, 16, 32 and 100%

MeCN/$CHCl_3$ (500 mL each step), to yield 11 fractions (Fr 1–11). Fraction 9 of this separation gave a pure yellow solid of burnettiene A (1.1 g). For full isolation scheme of **1**, see Fig. S33.

6. Bioactivity assays

Purified metabolites were dissolved in DMSO to provide stock solutions (10,000 µg/mL). An aliquot of each stock solution was transferred to the first lane of Rows B to G in a 96-well microtitre plate and two-fold serially diluted with DMSO across the 12 lanes of the plate to provide a 2,048-fold concentration gradient. Bioassay medium was added to an aliquot of each test solution to provide a 100-fold dilution into the final bioassay, thus yielding a test range of 100 to 0.05 µg/mL in 1% DMSO for majority of our test organisms, and 200 to 0.1 µg/mL in 2% DMSO for yeasts. Row A contained no test compound (as a reference for no inhibition) and Row H was uninoculated (as a reference for complete inhibition). The assays were tested as single replicates.

CyTOX is an indicative bioassay platform for discovery of antitumour actives. NS-1 (ATCC TIB-18) mouse myeloma was inoculated in 96-well microtitre plates (190 µL) at 50,000 cells/mL in DMEM (Dulbecco's Modified Eagle Medium + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (10,000 U/mL / 10,000 µg/mL, Life Technologies Cat. No. 15140122), together with resazurin (250 µg/mL; 10 µL) and incubated in 37 °C (5% CO_2) incubator. The plates were incubated for 96 h during which time the positive control wells change colour from a blue to pink colour. The absorbance of each well was measured at 72 h and 96 h at 605 nm using a Spectromax plate reader (Molecular Devices).

ProTOX is a bioassay platform for antibiotic discovery. In the present study *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923) were used as indicative species for Gram positive antibacterial activity, respectively. A bacterial suspension (50 mL in 250 mL flask) was prepared in nutrient media by cultivation for 24 h at 250 rpm, 28 °C. The suspension was diluted to an absorbance of 0.01 absorbance units per mL, and 10 µL aliquots were added to the wells of a 96-well microtitre plate, which contained the test compounds dispersed in 190 µL nutrient broth (Amyl) with 10 µL of resazurin (12.5 µg/mL). The plates were incubated at 28 °C for 48 h during which time the positive control wells change colour from a blue to light pink colour. At 24 h and 48 h, MIC end points were determined visually, and the absorbance was measured using Spectromax plate reader (Molecular Devices) at 605 nm.

EuTOX is a bioassay platform for antifungal discovery. In the present study, the yeasts *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) were used as indicative species for antifungal activity. A yeast suspension (50 mL in 250 mL flask) was prepared in 1% malt extract broth by cultivation for 24 h at 250 rpm, 24 °C. The suspension was diluted to an absorbance of 0.005 and 0.03 absorbance units per mL for *C. albicans* and

S. cerevisiae, respectively. Aliquots (20 µL and 30 µL) of *C. albicans* and *S. cerevisiae*, respectively, were applied to the wells of a 96-well microtitre plate, which contained the test compounds dispersed in malt extract agar containing bromocresol green (50 µg/mL). The plates were incubated at 24 °C for 48 h during which time the positive control wells change colour from a blue to yellow colour. At 24 h and 48 h, MIC end points were determined visually and the absorbance was measured using Spectromax plate reader (Molecular Devices) at 620 nm.

TriTOX is a bioassay focused on the discovery of inhibitors of the animal protozoan pathogen, *Tritrichomonas foetus*. In the present bioassay *T. foetus* (strain KV-1) were inoculated in 96-well microtitre plates (200 µL) at 4×10^4 cells/mL in *T. foetus* medium (0.2% tryptone, Oxoid; 0.1% yeast extract, Difco; 0.25% glucose; 0.1% L-cysteine; 0.1% K_2HPO_4; 0.1% KH_2PO_4; 0.1% ascorbic acid; 0.01% $FeSO_4.7H_2O$; 1% penicillin/streptomycin (10,000 U/mL / 10,000 µg/mL, Life Technologies Cat. No. 15140122), 10% new born calf serum (NBCS), Life Technologies). The plates were incubated in anaerobic jars (Oxoid AG25) containing Anaerogen sachet (Oxoid AN25) in 37 °C (5% CO_2) incubator. At 48 h and 72 h, MIC end points were determined visually and absorbance was measure using Spectromax plate reader (Molecular Devices) at 570 nm.[9]

Phytox is a bioassay platform for herbicidal discovery. In the present study, *Eragrostis tef* (teff) seed was used as indicative monocotyledon species for herbicidal discovery. 10 to 15 teff seeds were dispensed using LabTIE seed dispenser into the wells of a 96-well microtitre plate containing the test compounds dispersed in 200 µL of agar (1% w/v) per well. The plates were placed in a tray wrapped with semi-opaque bag, exposed to 1600 lux (inside the bag) using Power-Glo (20 W) and Sun-Glo (20 W) tubes, and incubated for 72 h at 24 °C.

7. Ketoreductase sequence alignment

Sequence alignment was performed using KR sequences from BueA, FSL1 (accession EYB31408), Tyl1 (accession 2Z5L_A), Ery (accession Q03131) and Hpm8 (accession B3FWT3) with Geneious 11.1. Fingerprint residues were interpreted in the context of previous analysis by Keatinge-Clay *et al*.[10,11]

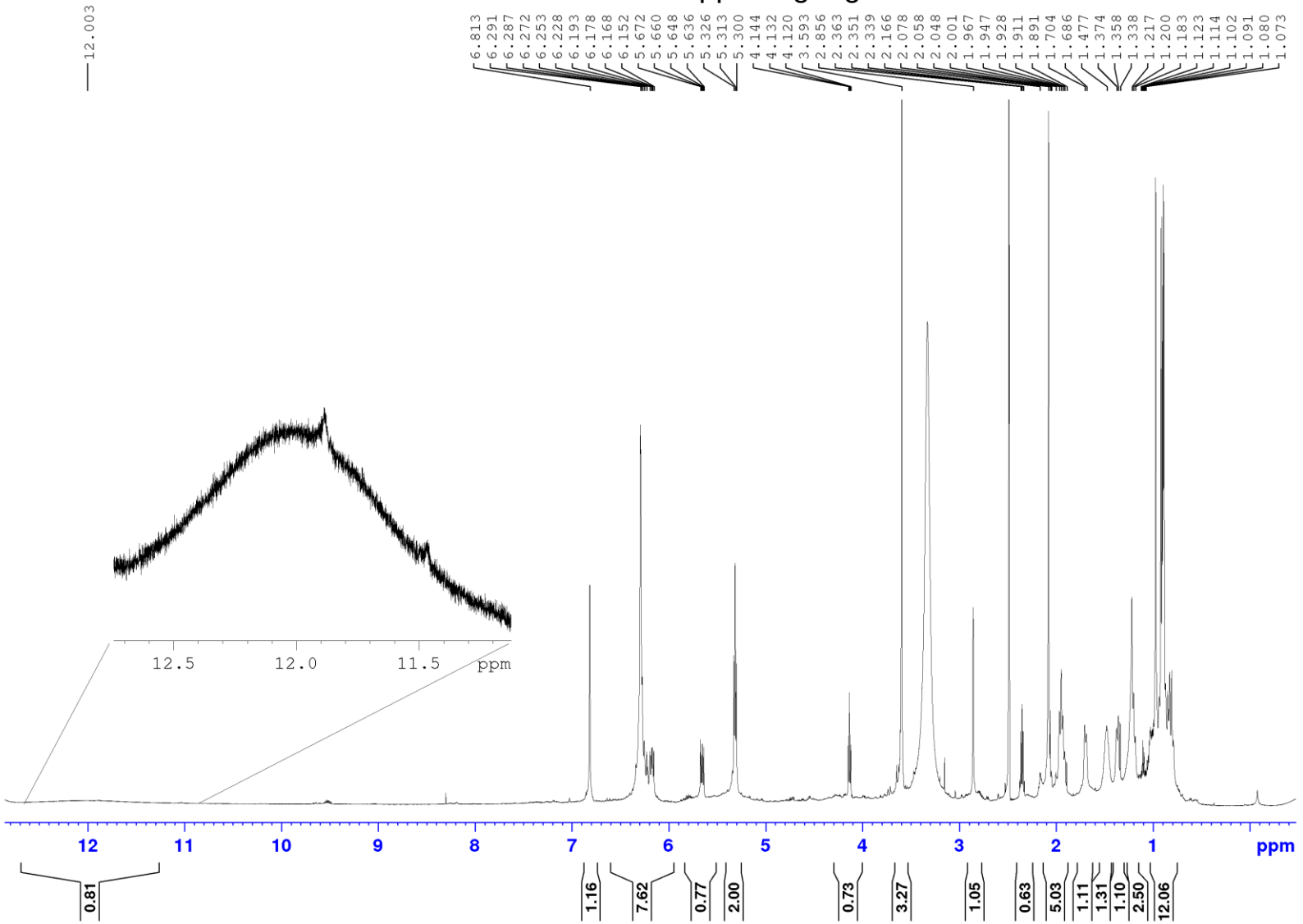


Fig. S1. ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

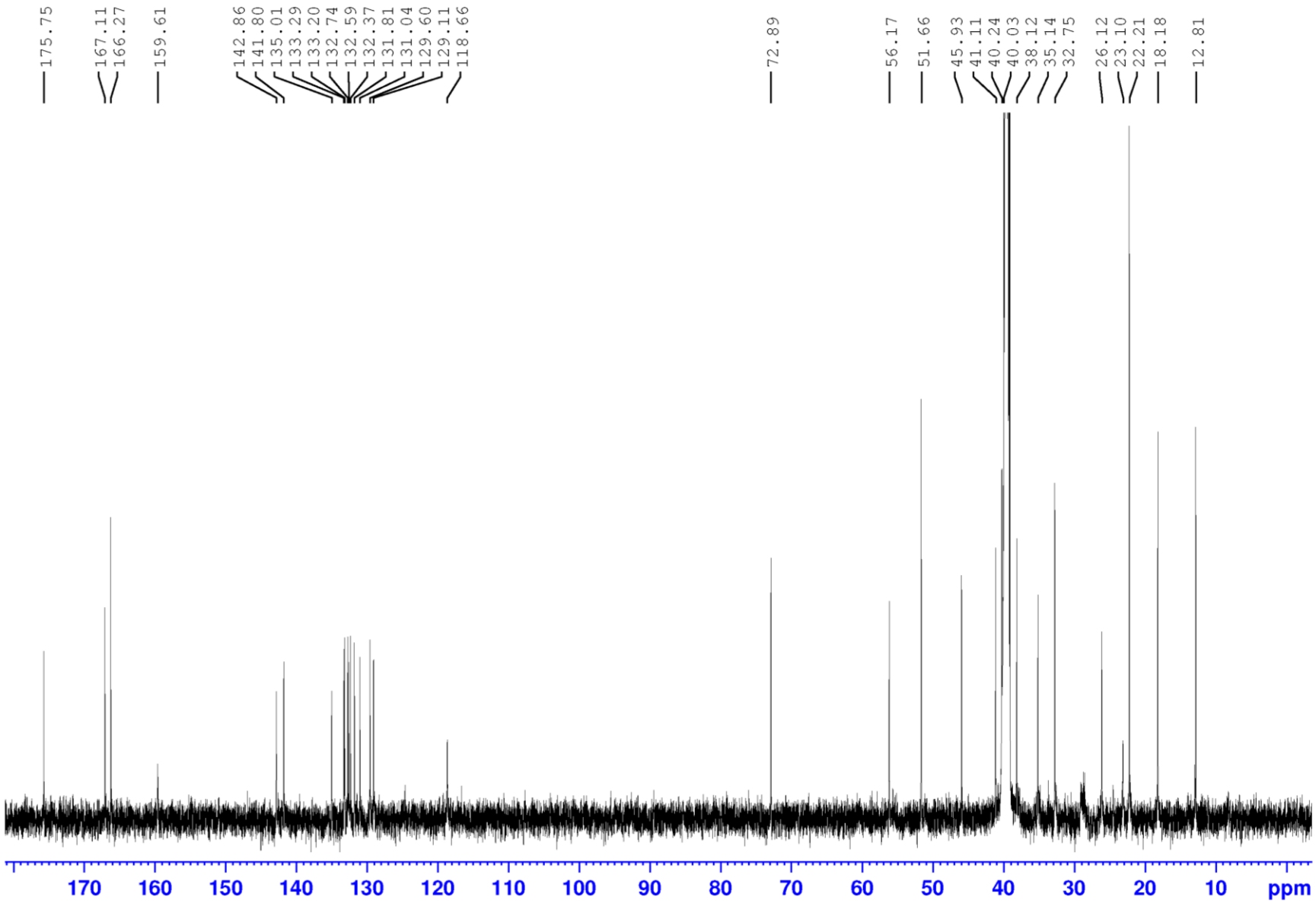


Fig. S2. ^{13}C NMR spectrum (150 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

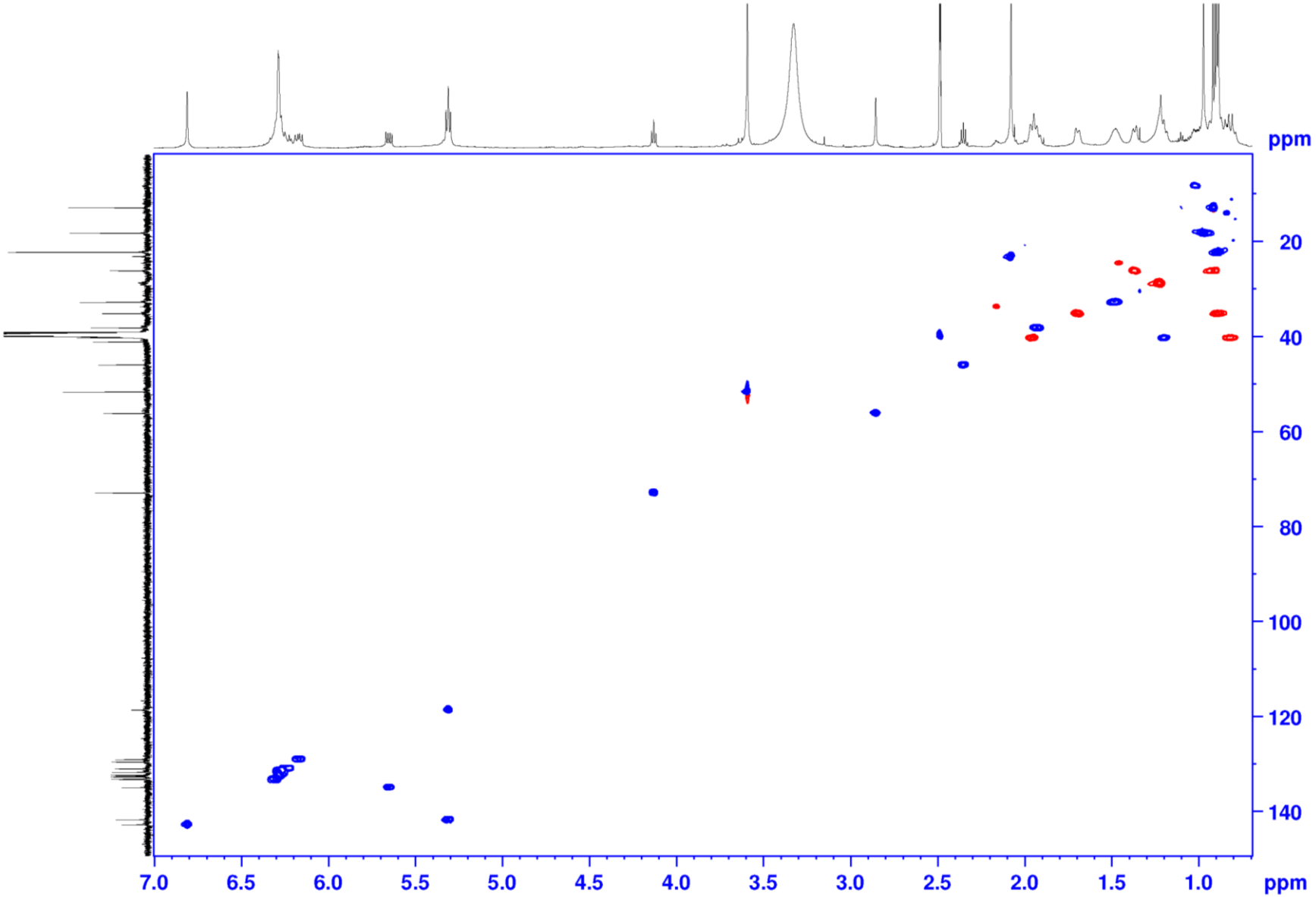


Fig. S3. ^{1}H-^{13}C HSQC NMR spectrum (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

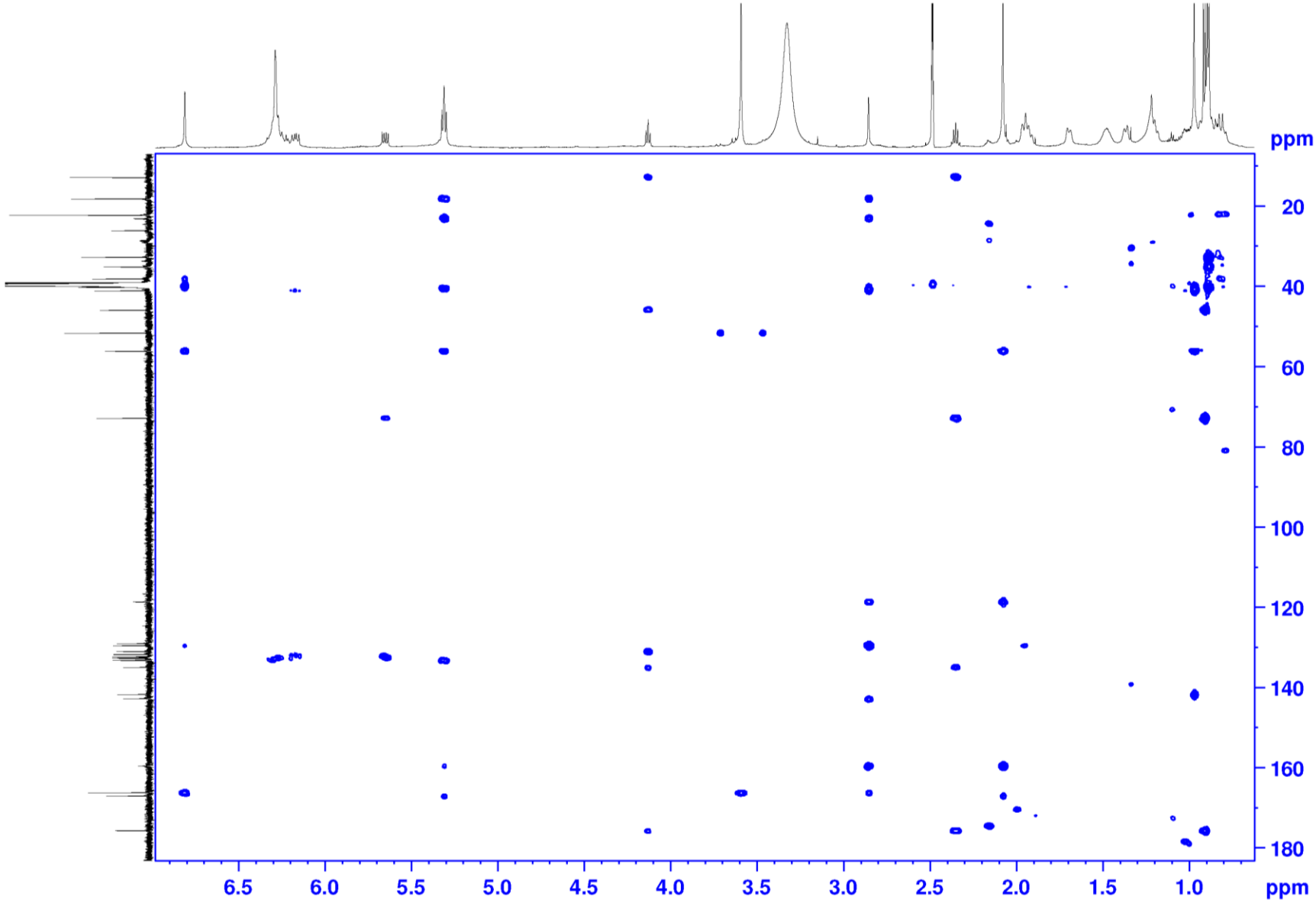


Fig. S4. ^{1}H-^{13}C HMBC NMR spectrum (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

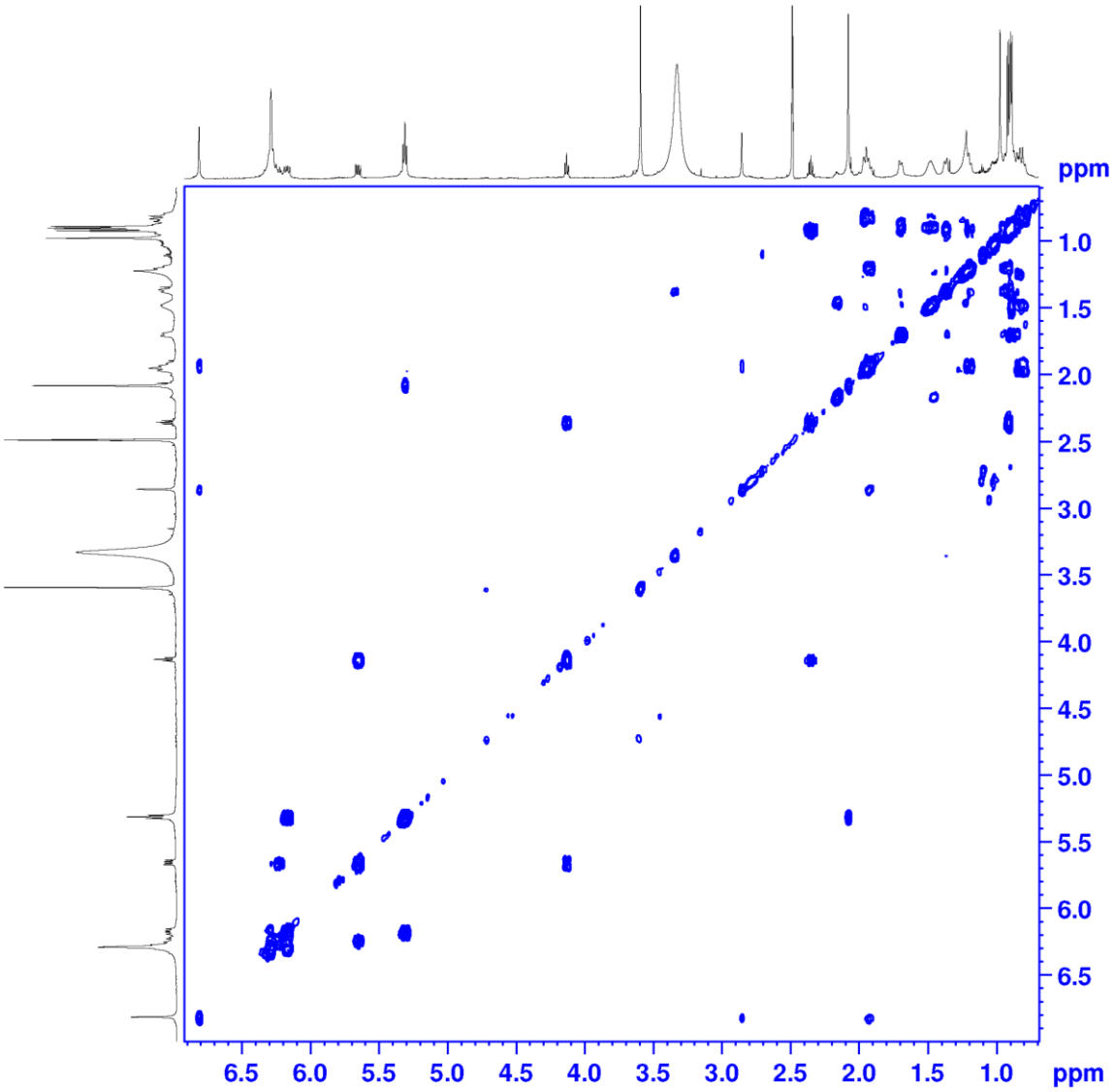


Fig. S5. COSY NMR spectrum (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

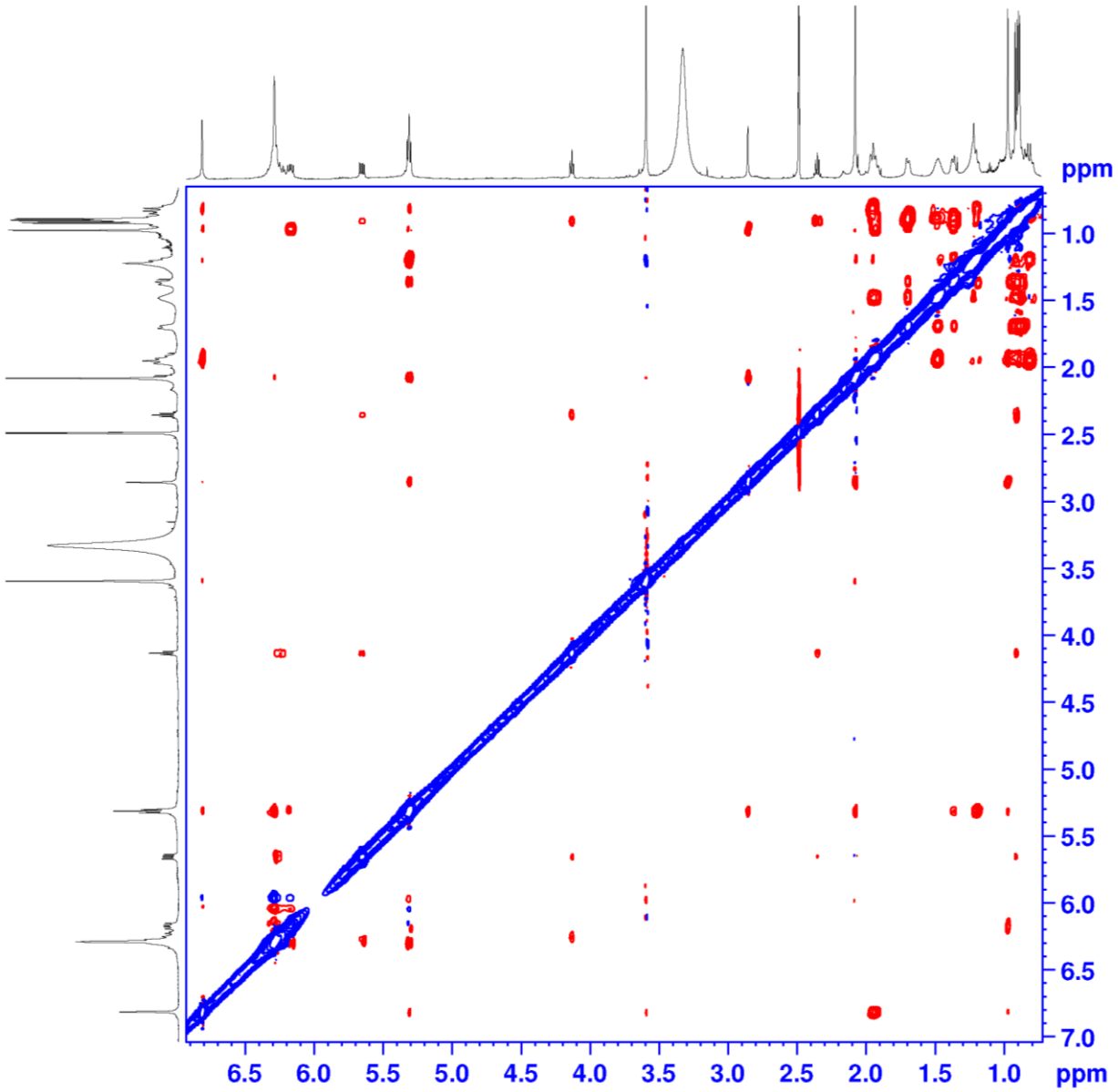


Fig. S6. ROESY NMR spectrum (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

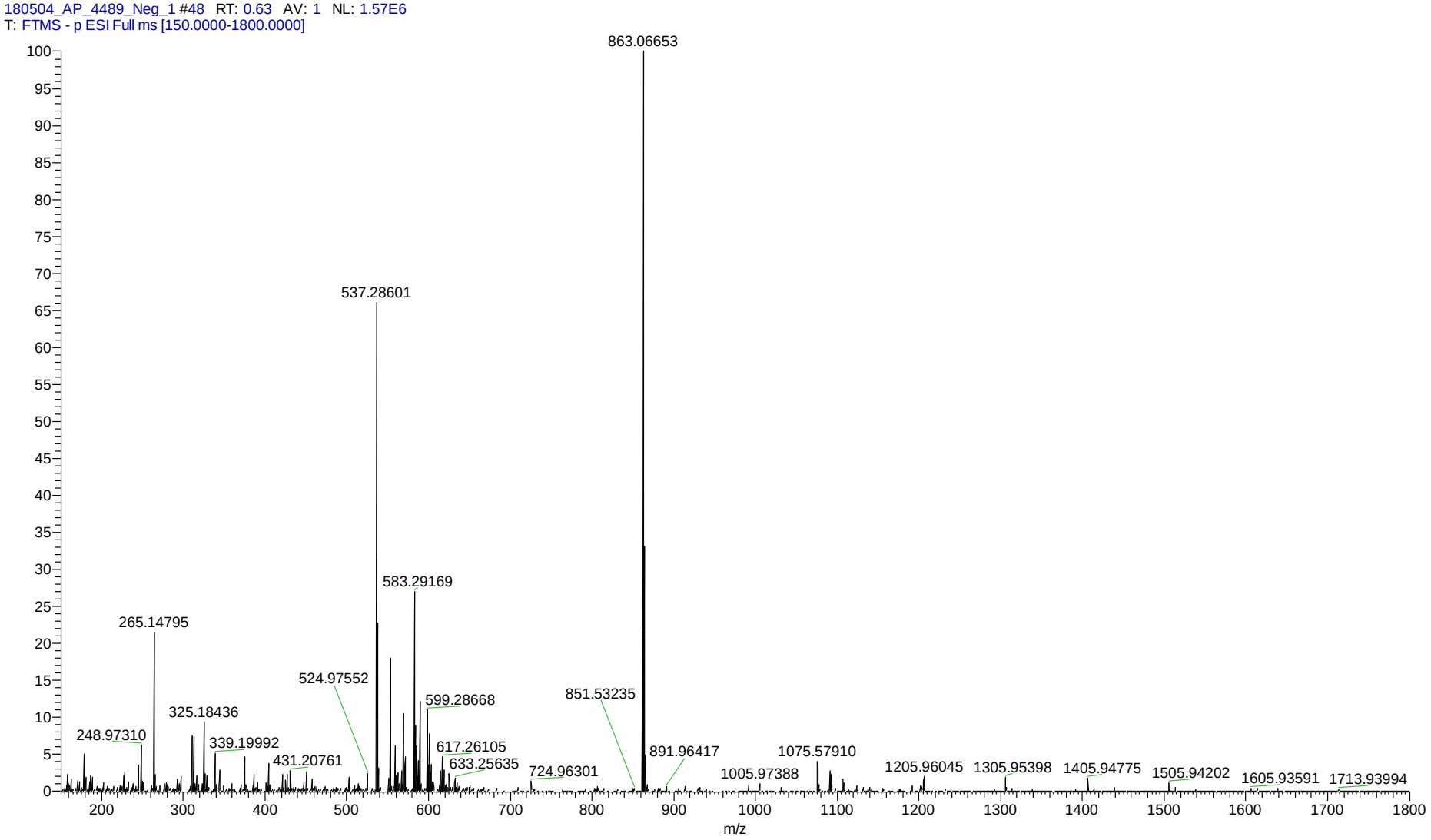


Fig. S7. HRESI(−)MS spectrum of burnettiene A (**1**) isolated from *Aspergillus burnettii*.

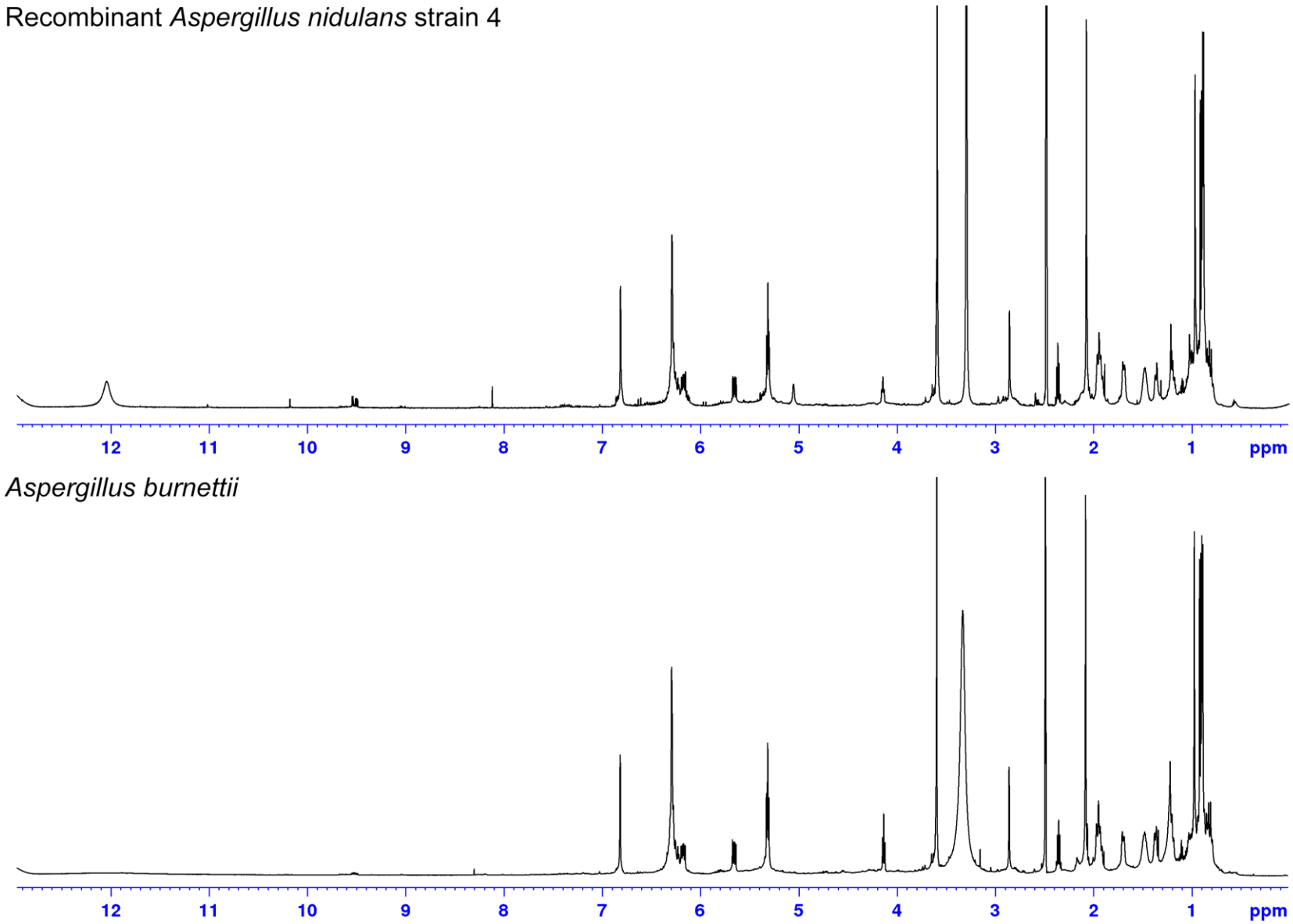


Fig. S8. Comparison of the ^{1}H NMR spectra (600 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from recombinant *Aspergillus nidulans* strain 4 (top) and from *Aspergillus burnettii* (bottom).

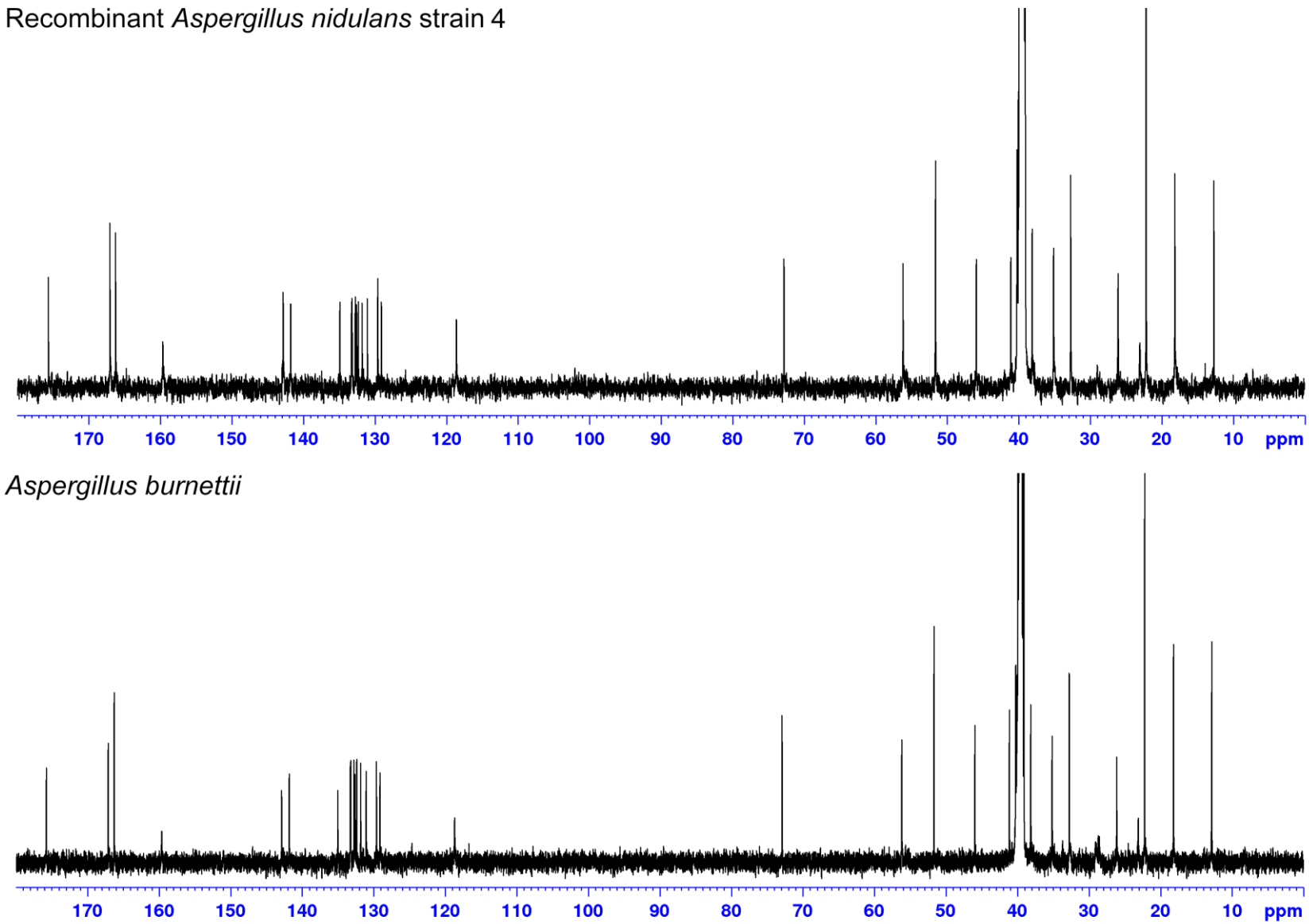


Fig. S9. Comparison of the ^{13}C NMR spectra (150 MHz, DMSO-d_6) of burnettiene A (**1**) isolated from recombinant *Aspergillus nidulans* strain 4 (top) and from *Aspergillus burnettii* (bottom).

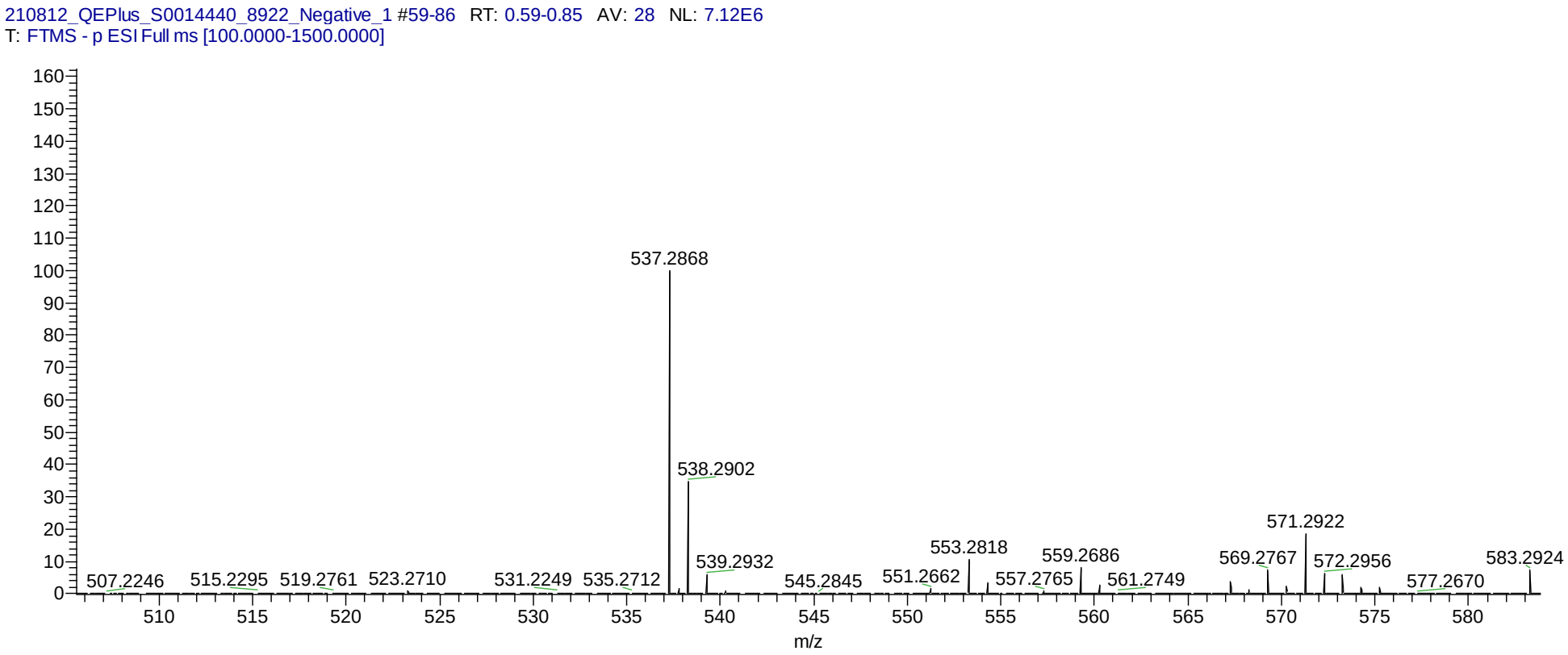


Fig. S10. HRESI(−)MS spectrum of burnettiene A (**1**) isolated from recombinant *Aspergillus nidulans* strain 4.

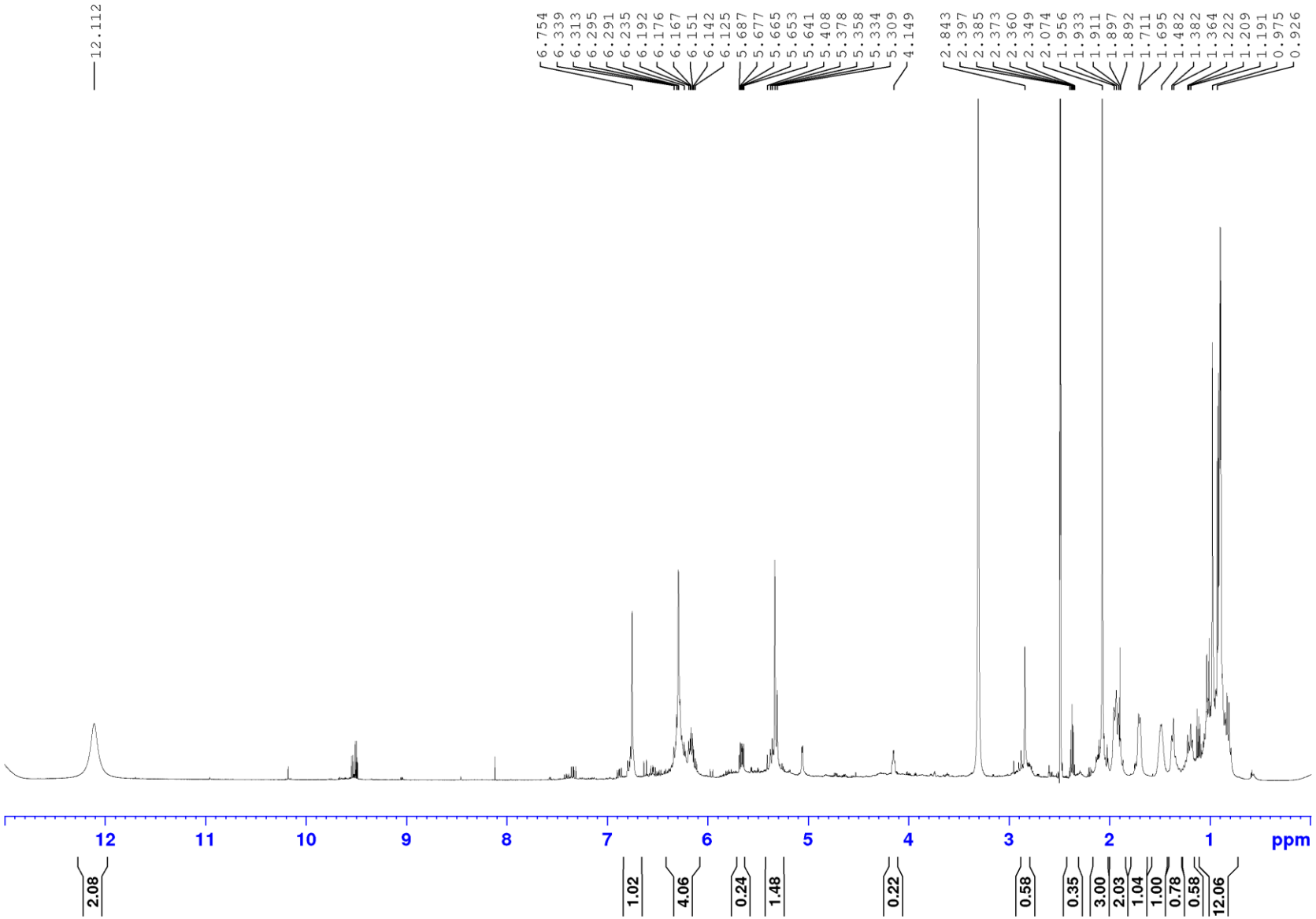


Fig. S11. ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene B (**2**).

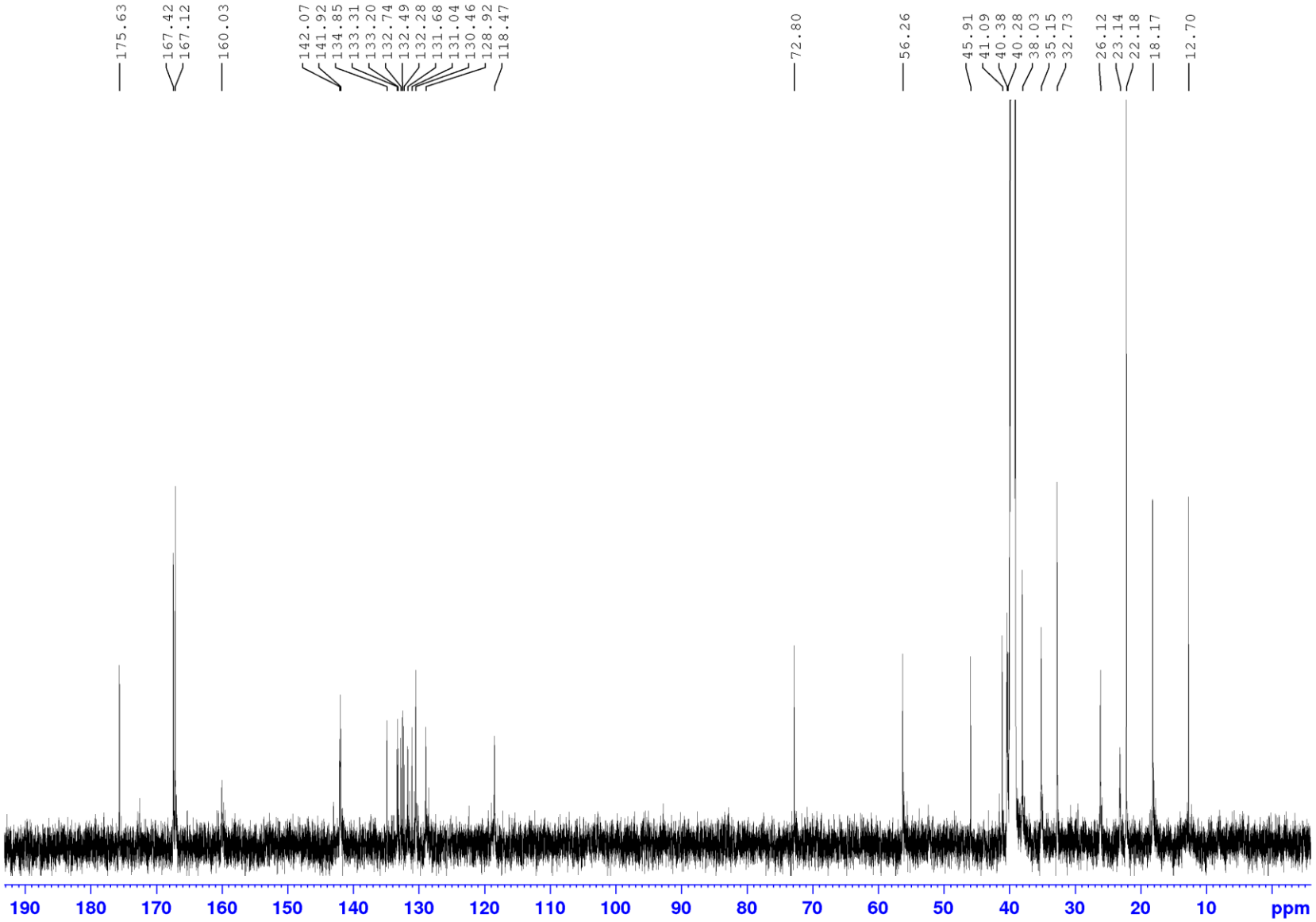


Fig. S12. ^{13}C NMR spectrum (150 MHz, DMSO-d_6) of preburnettiene B (**2**).

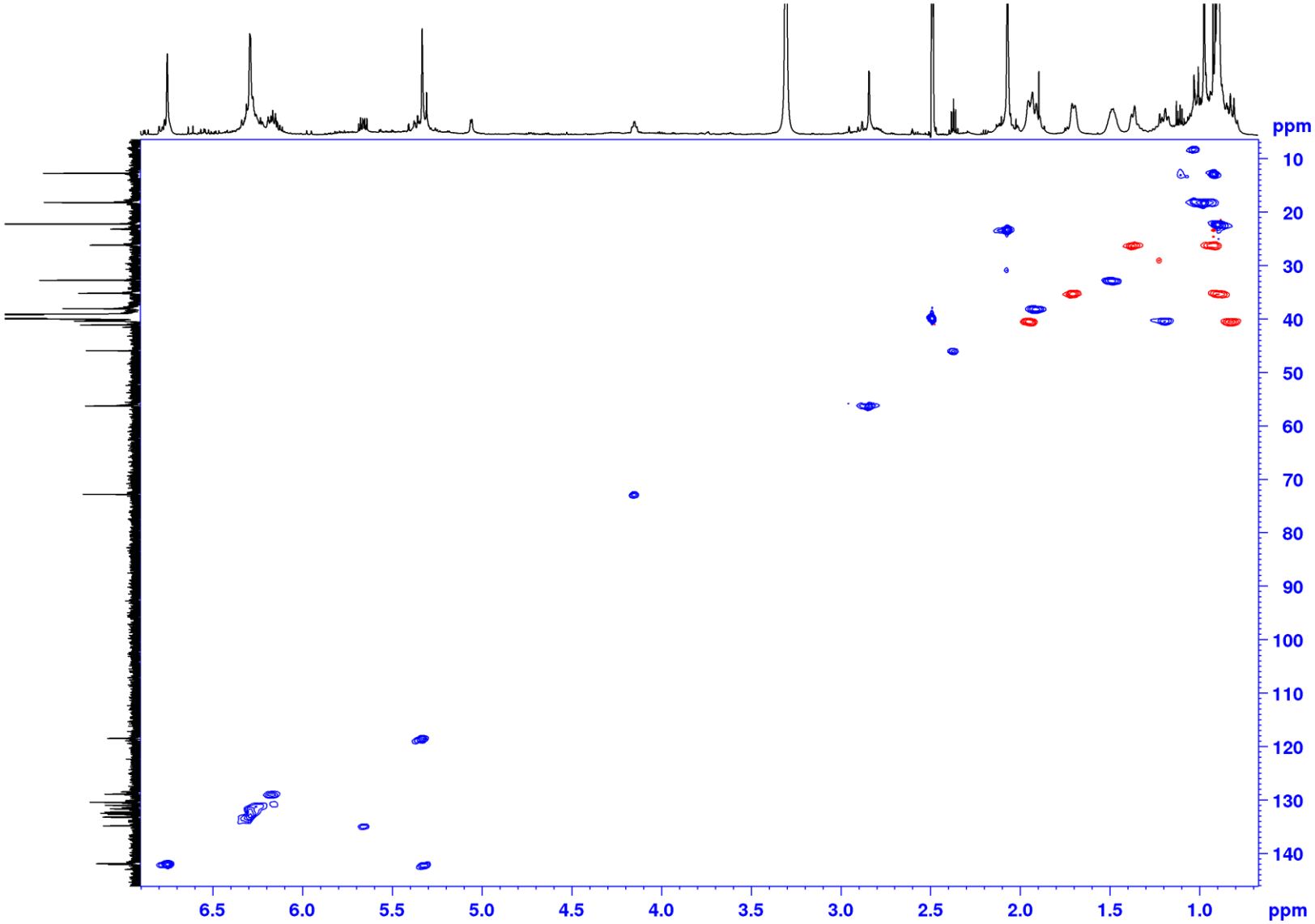


Fig. S13. ^{1}H-^{13}C HSQC NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene B (**2**).

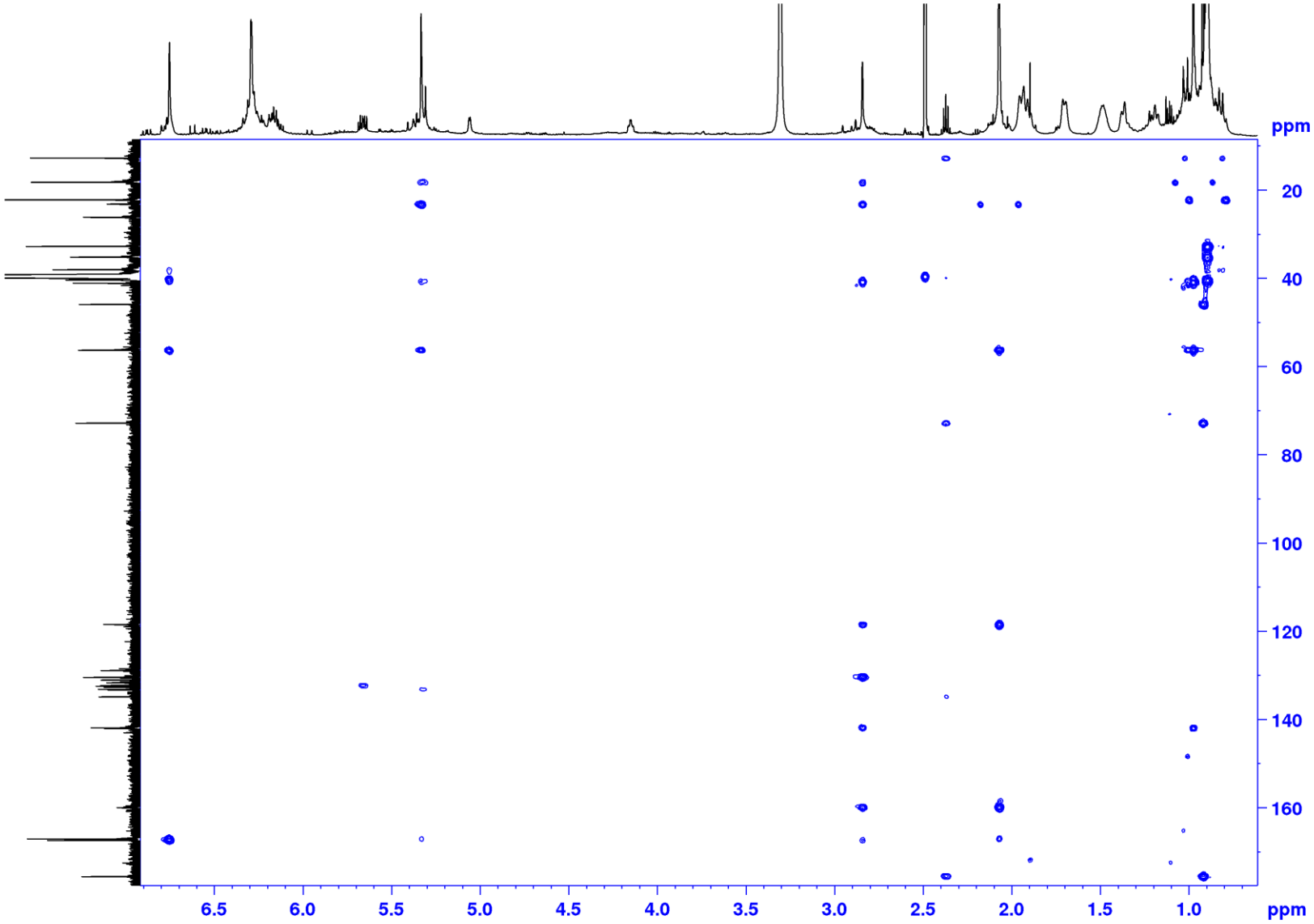


Fig. S14. ^{1}H-^{13}C HMBC NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene B (**2**).

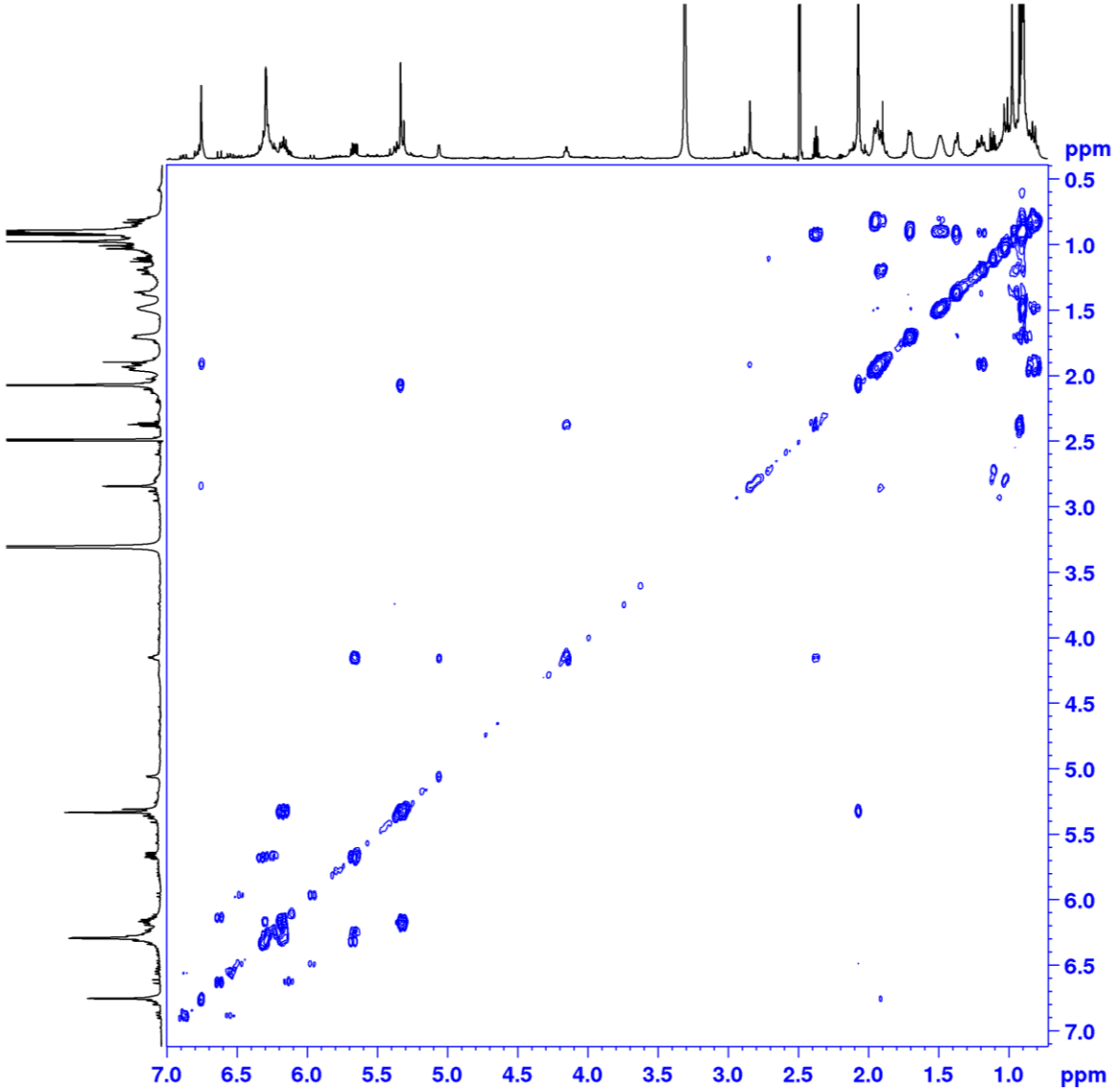


Fig. S15. COSY NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene B (**2**).

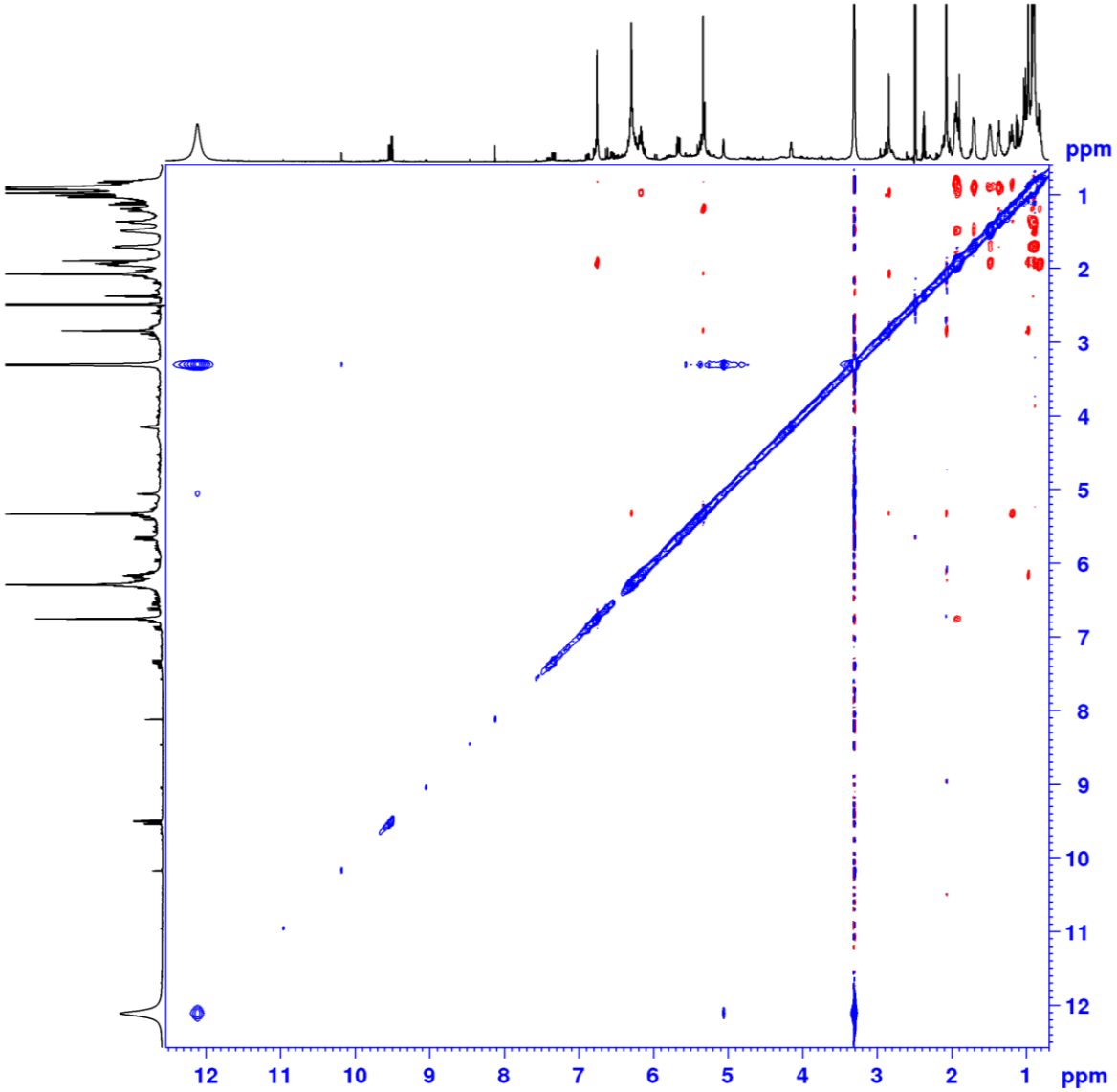


Fig. S16. ROESY NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene B (**2**).

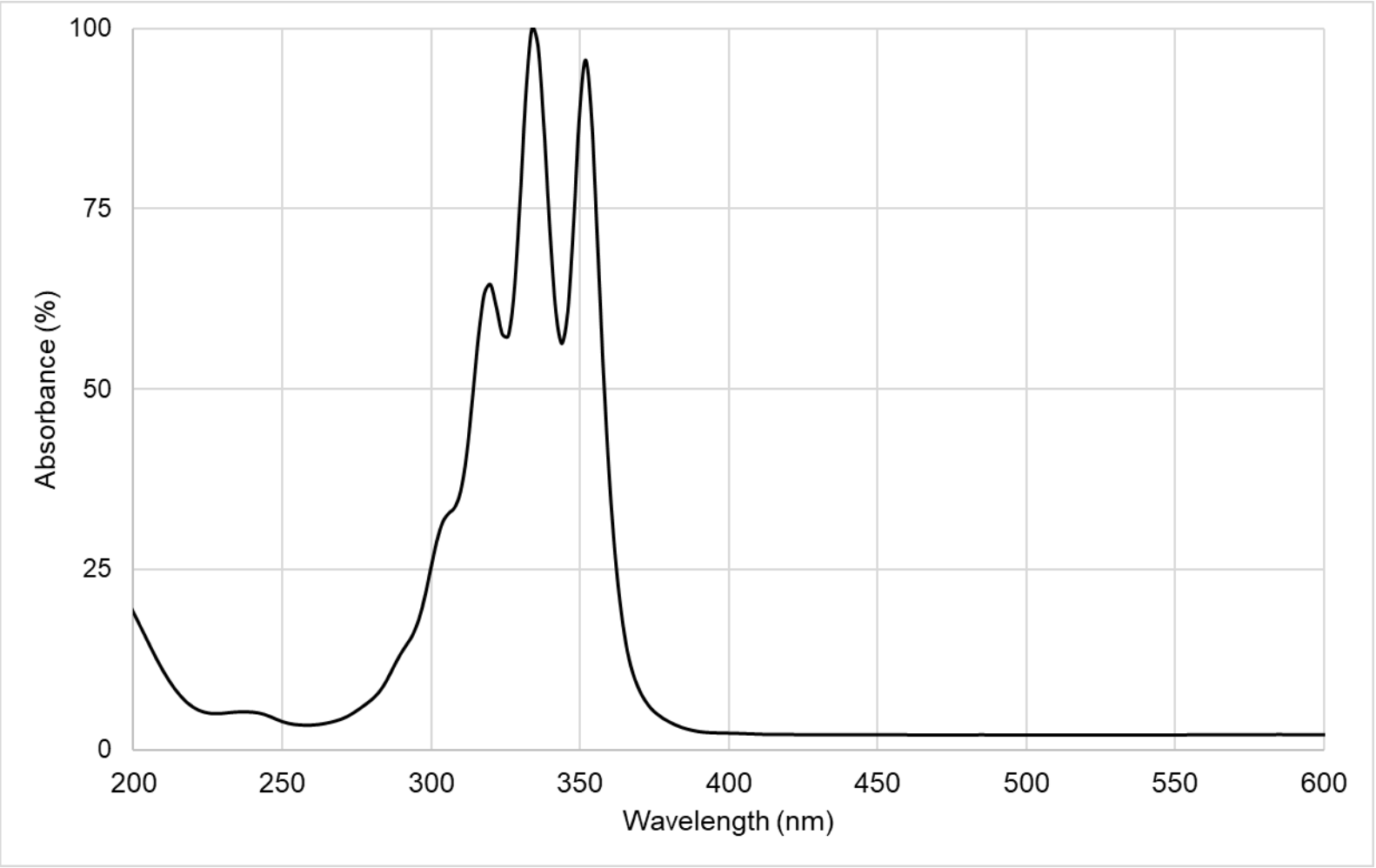


Fig. S17. UV-Vis spectrum of preburnettiene B (**2**).

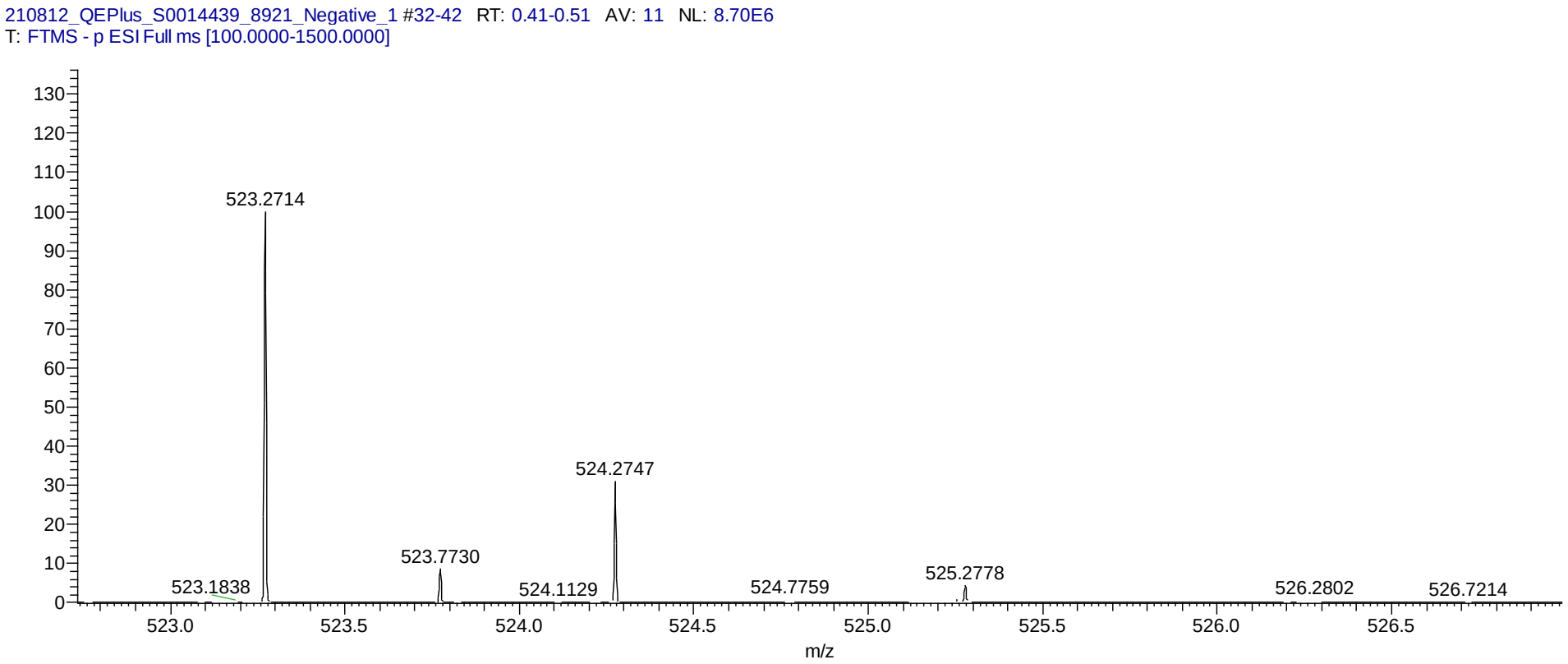


Fig. S18. HRESI(−)MS spectrum of preburnettiene B (**2**).

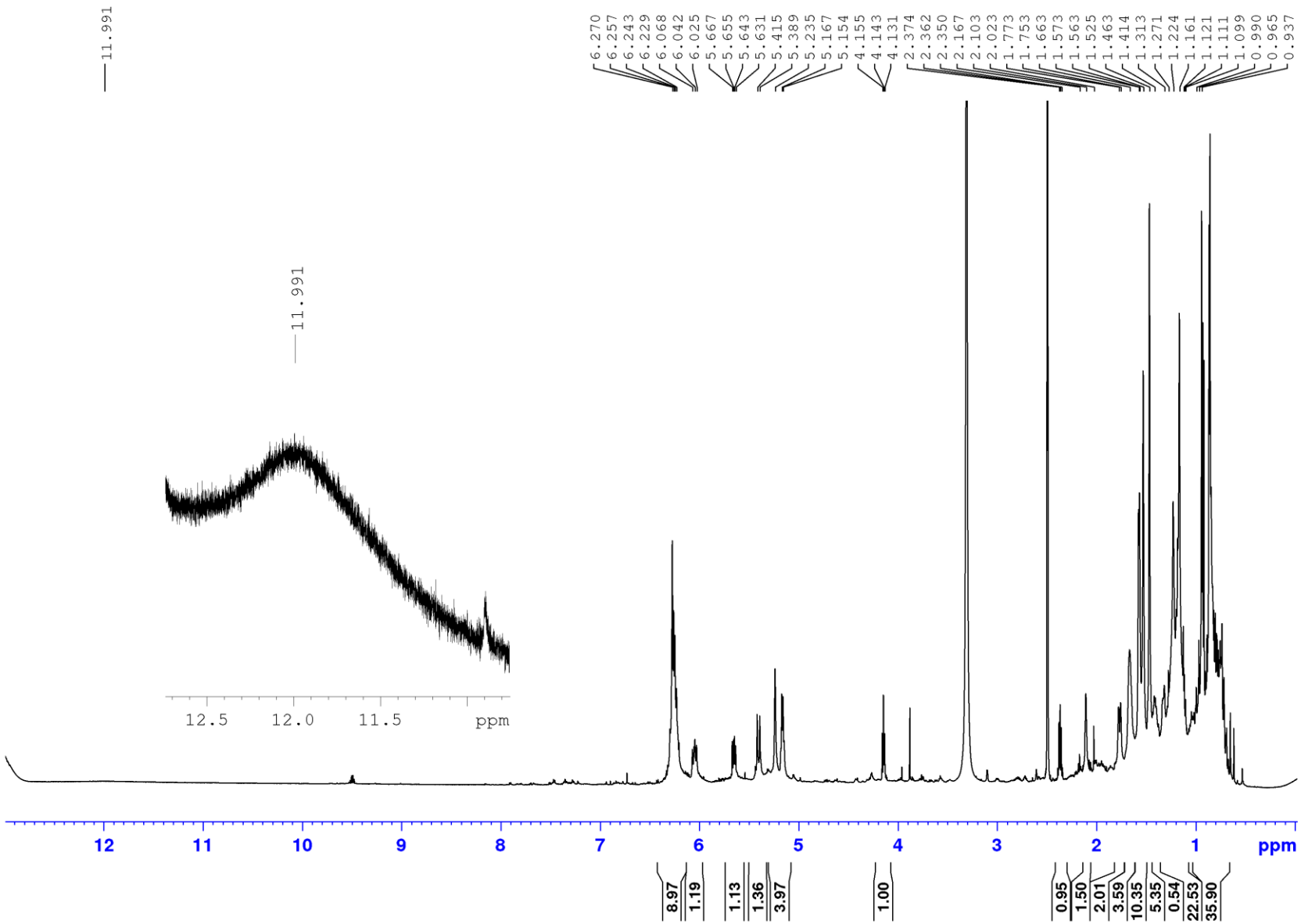


Fig. S19. ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene A (**3**).

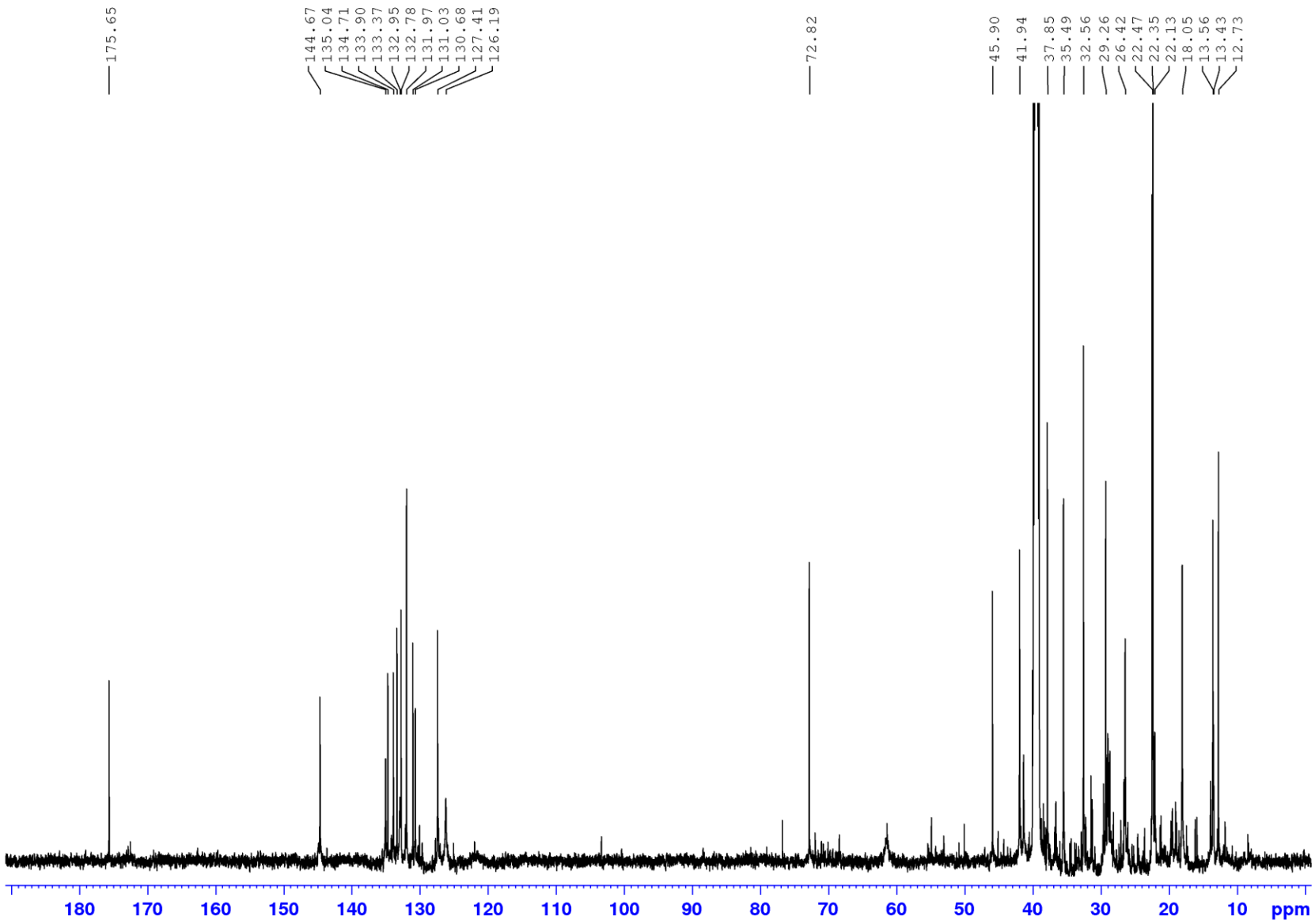


Fig. S20. ^{13}C NMR spectrum (150 MHz, DMSO-d_6) of preburnettiene A (**3**).

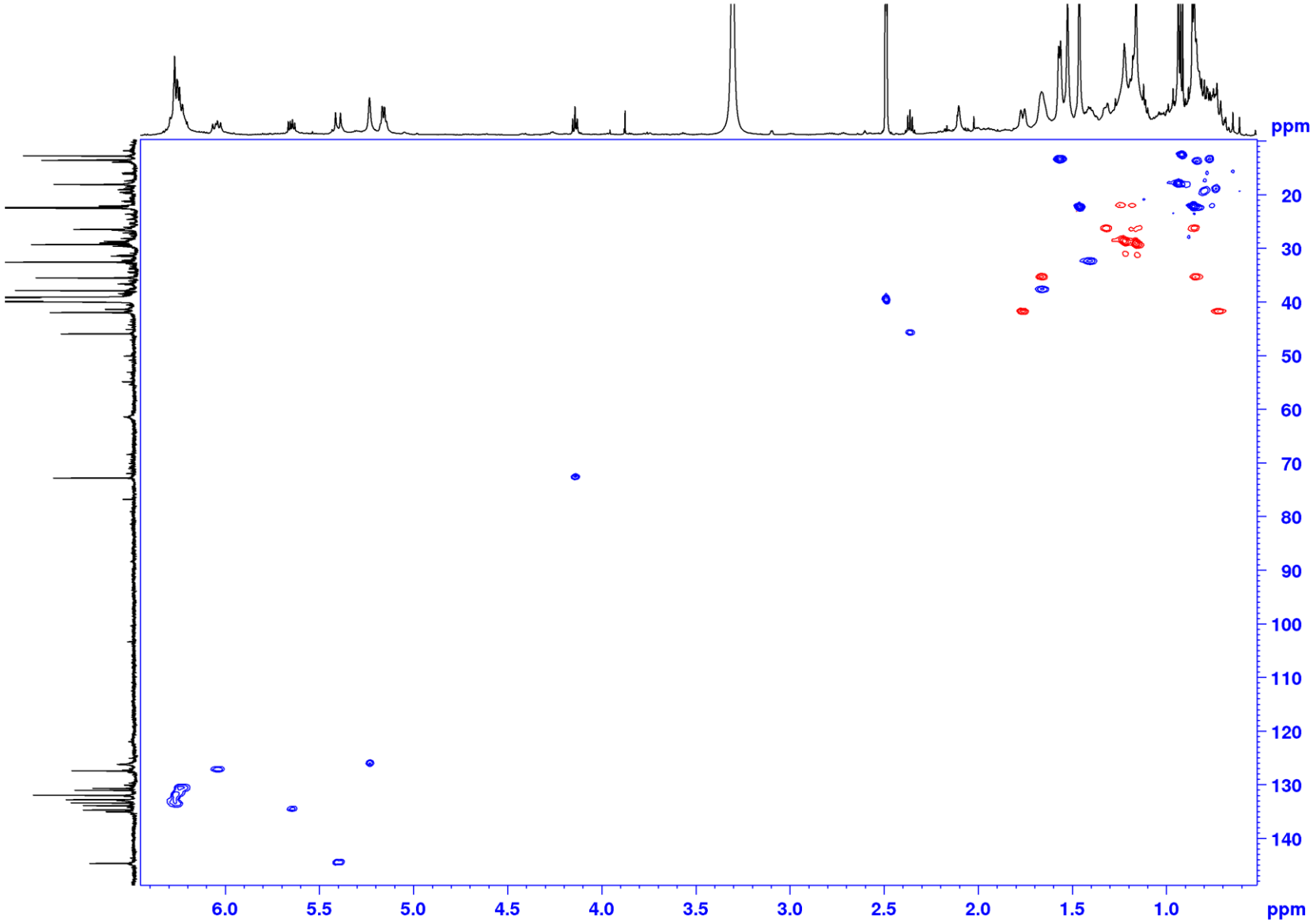


Fig. S21. ^{1}H-^{13}C HSQC NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene A (**3**).

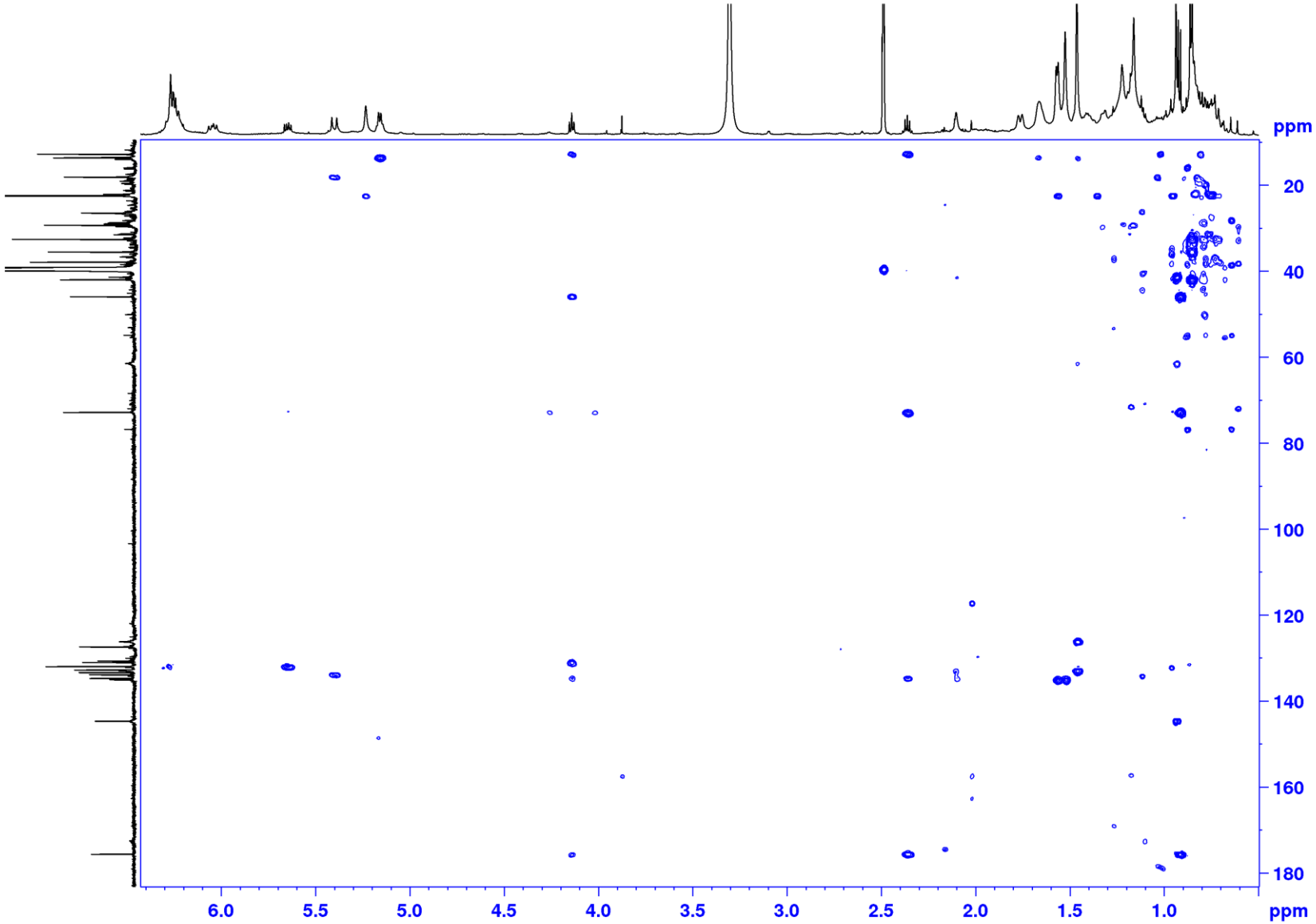


Fig. S22. ^{1}H-^{13}C HMBC NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene A (**3**).

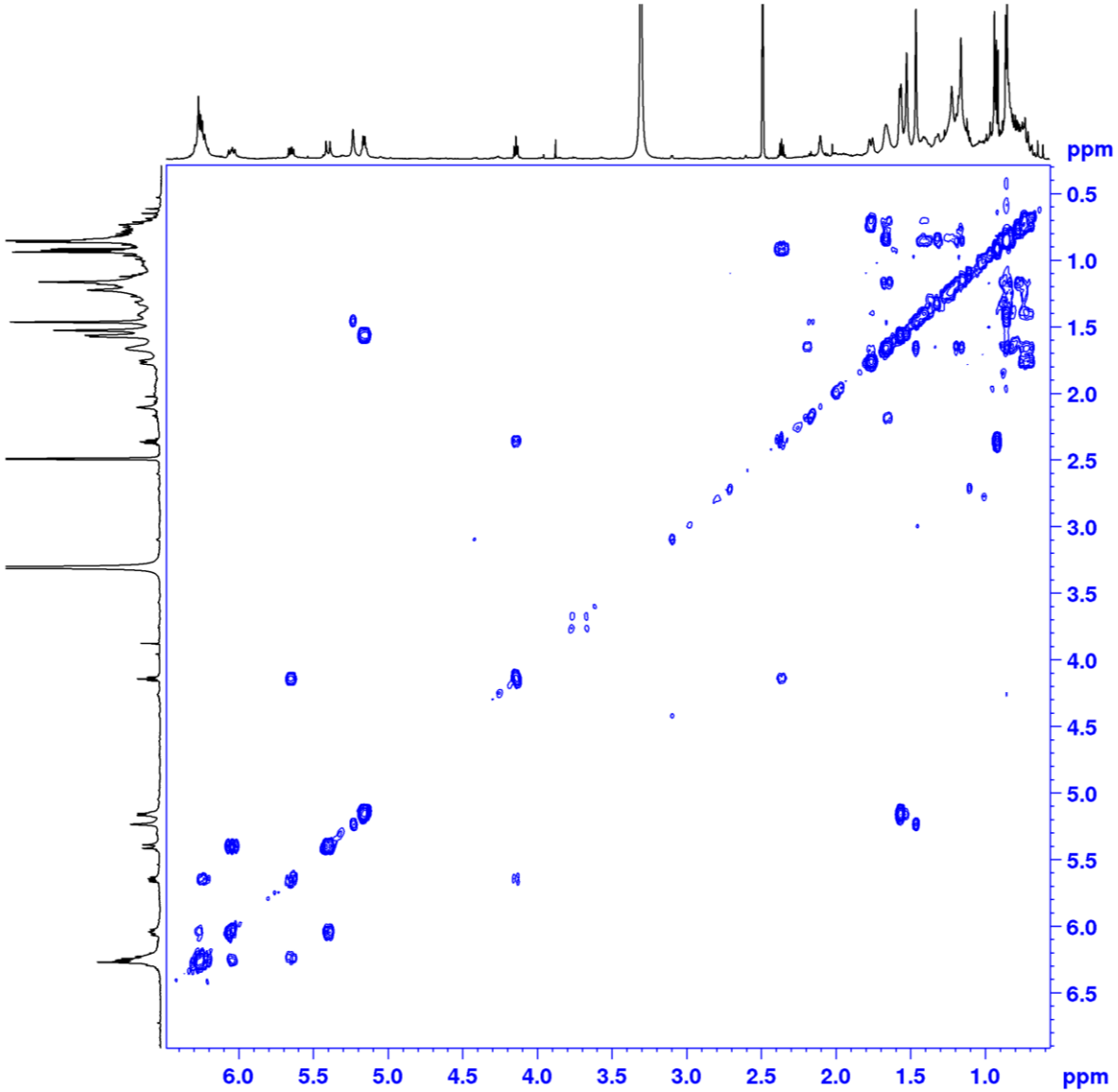


Fig. S23. COSY NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene A (**3**).

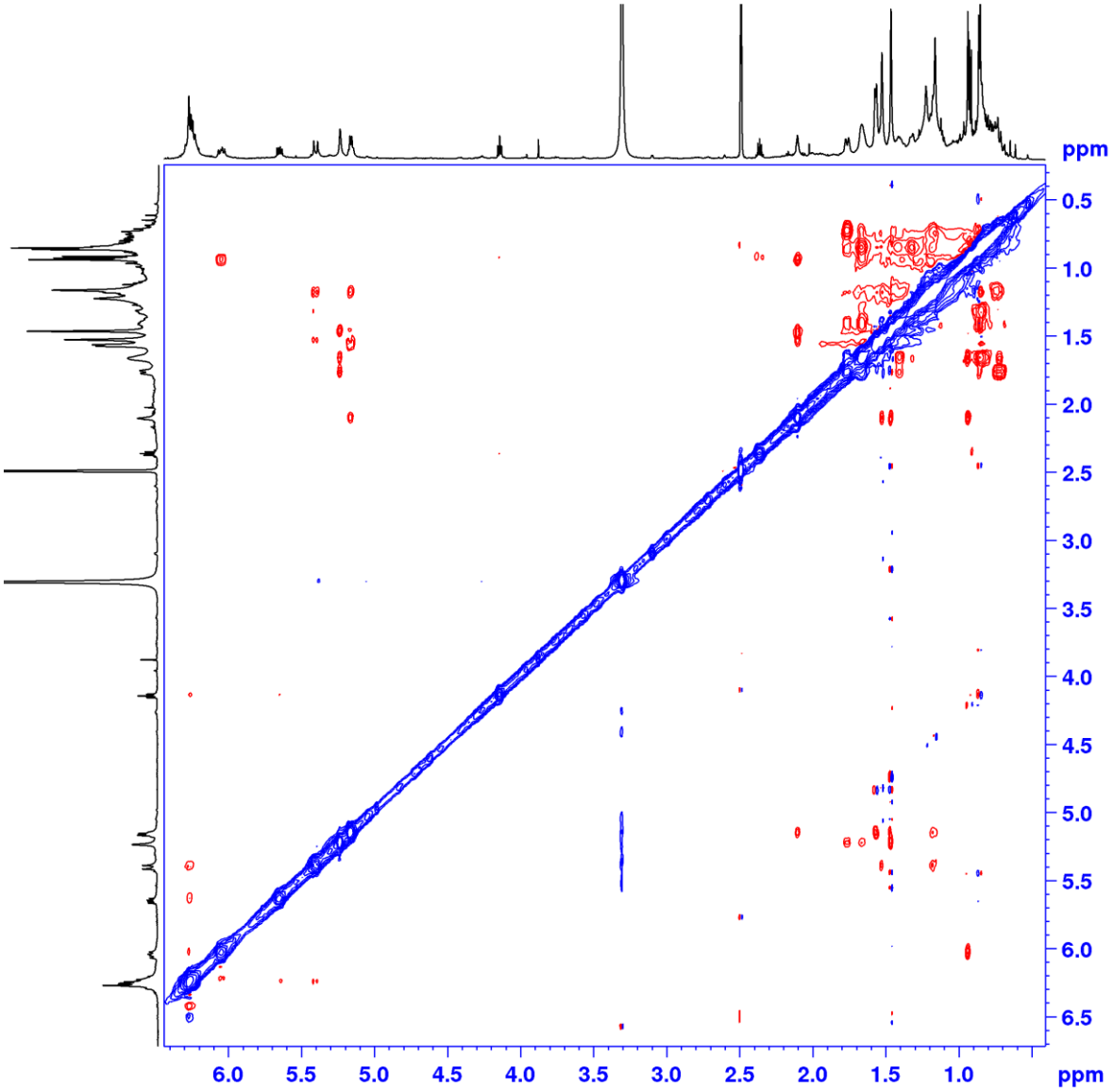


Fig. S24. ROESY NMR spectrum (600 MHz, DMSO-d_6) of preburnettiene A (**3**).

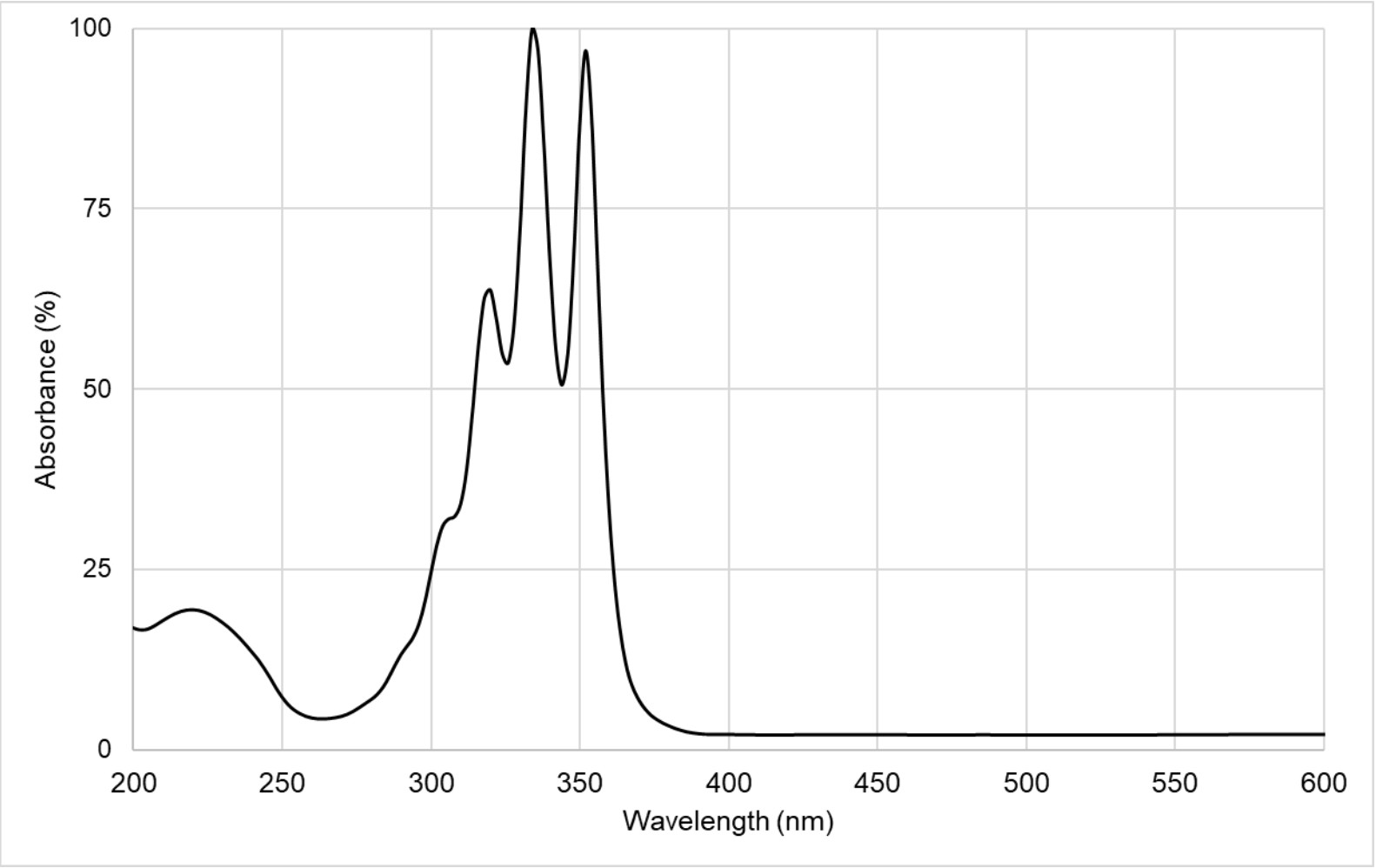


Fig. S25. UV-Vis spectrum of preburnettiene A (**3**).

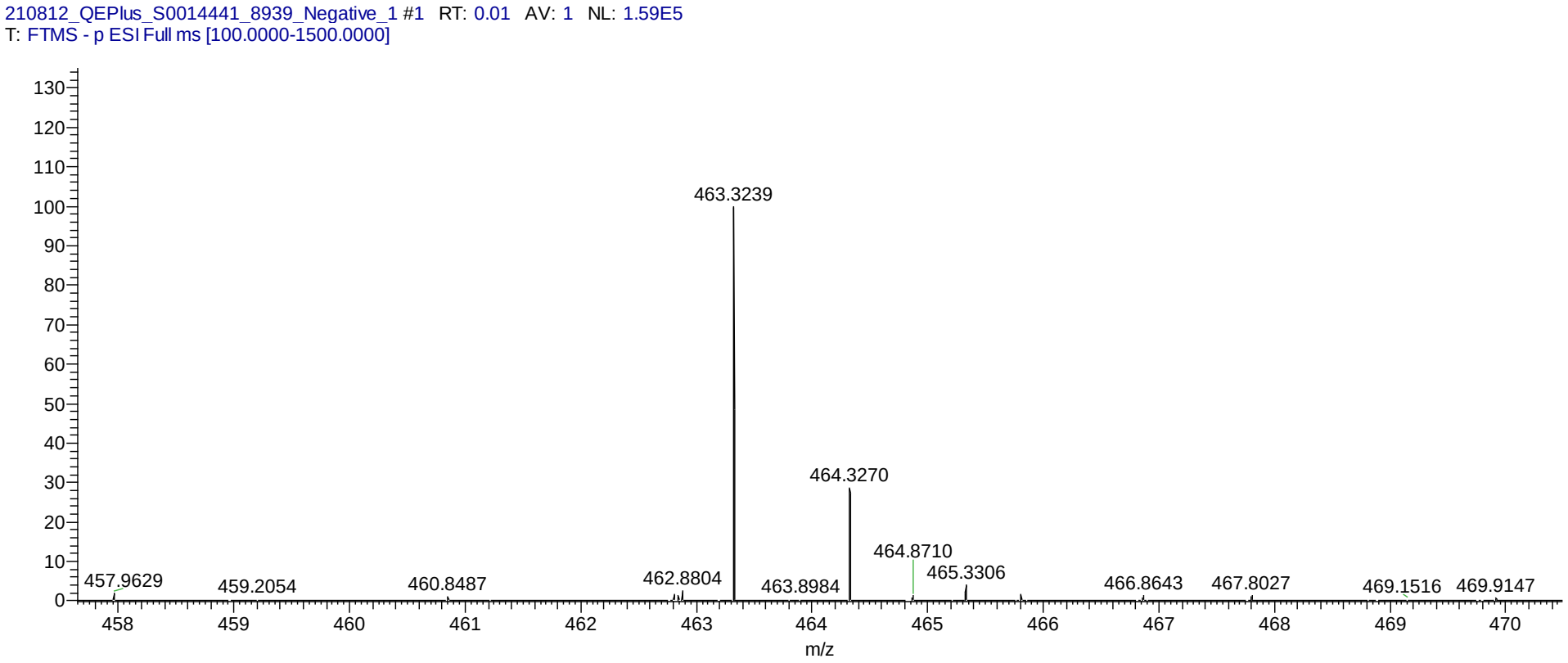


Fig. S26. HRESI(−)MS spectrum of preburnettiene A (**3**).

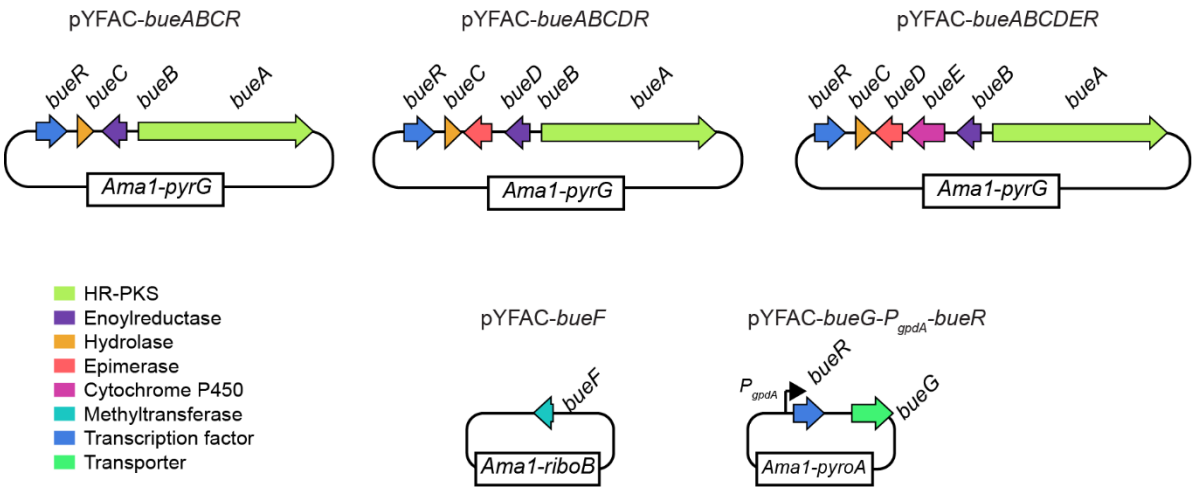


Fig. S27. Schematic of the vectors built in this work to evaluate burnettiene pathway intermediates.

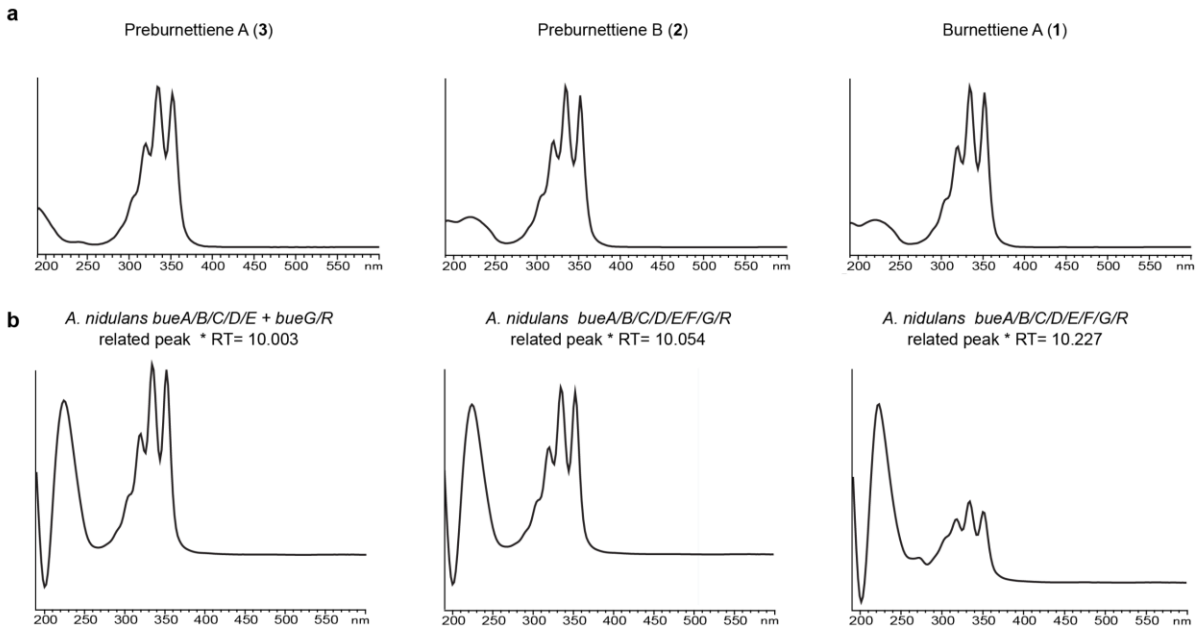


Fig. S28. UV-Vis spectra (200–600 nm) from peaks in small scale liquid cultures (LC-DAD-MS) **a.** UV-vis spectra of peaks **1**–**3** from small scale culture. **b.** UV-vis spectra of small peaks present at retention times (RT) between **1** and **2**, whose mass was not detectable.

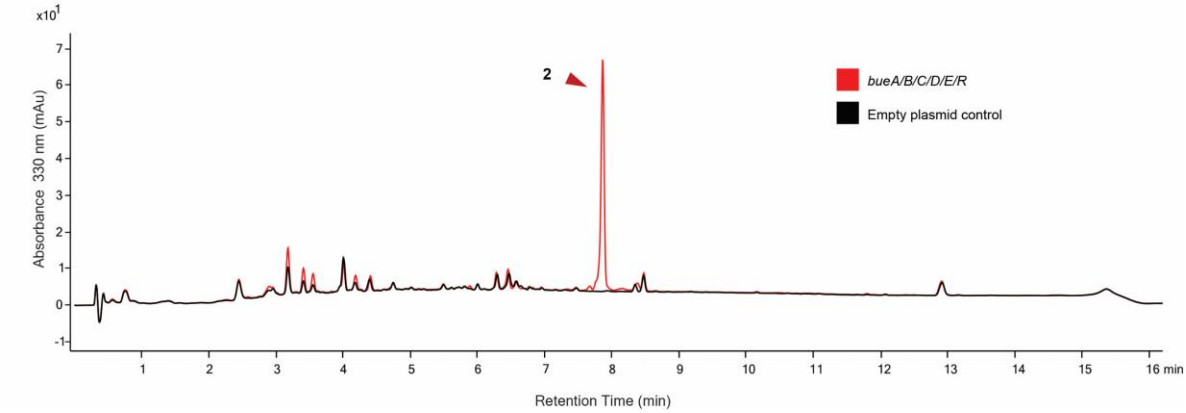


Fig. S29 HPLC 330 nm traces of media extracts from an *A. nidulans* strain expressing *bueA/B/C/D/E/R* under their native promoters (red) resulting in the production preburnettiene B (**2**), compared to a control strain carrying an empty plasmid (black).

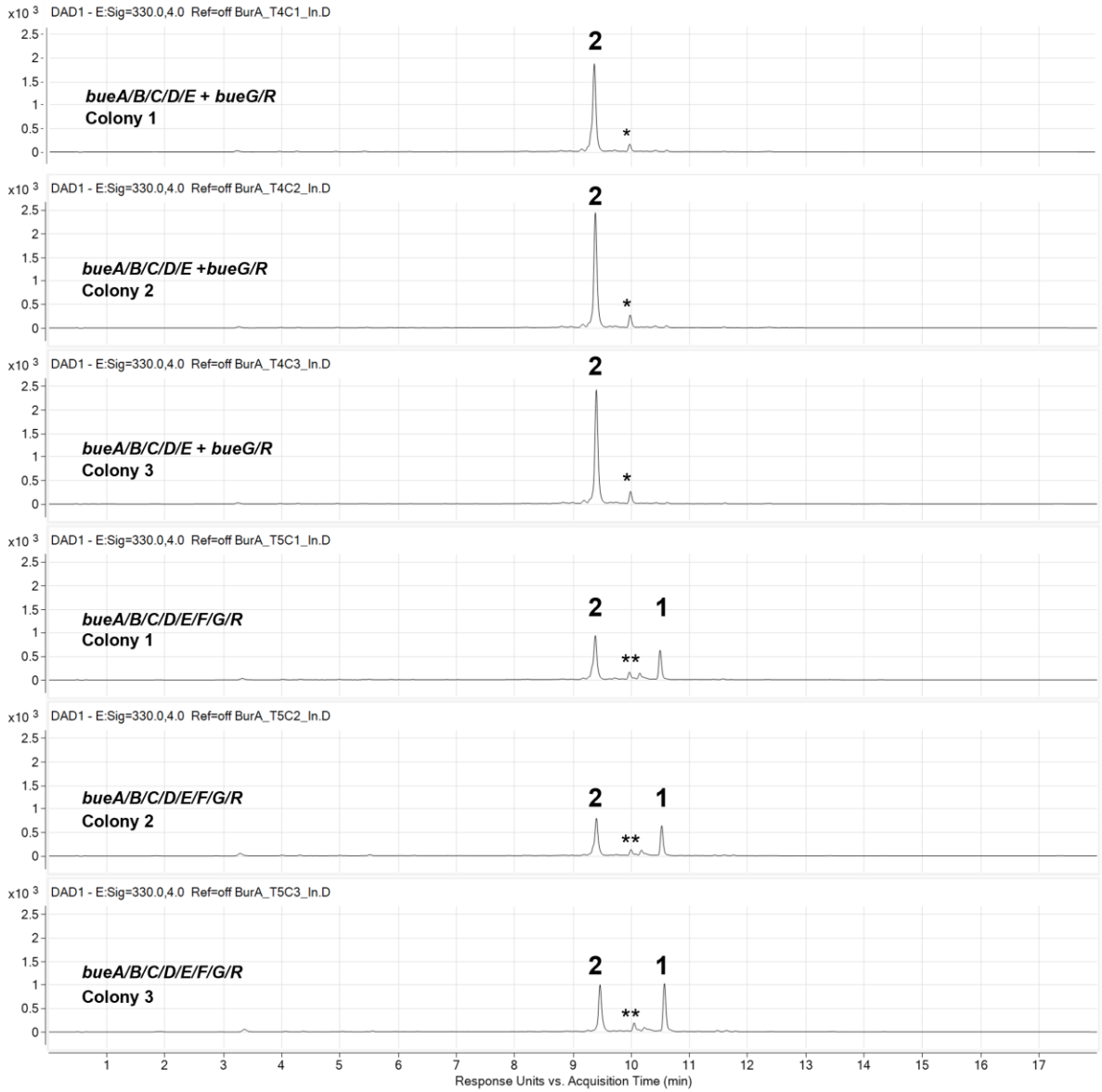


Fig. S30. HPLC traces (330 nm) of culture media extracts from strains expressing *bue* biosynthetic genes from biological triplicates showing the consistent production of **1** and **2** with minor variations on compound titre. Minor peaks with UV-vis spectra related to burnettienes are indicated with an asterisk (*).

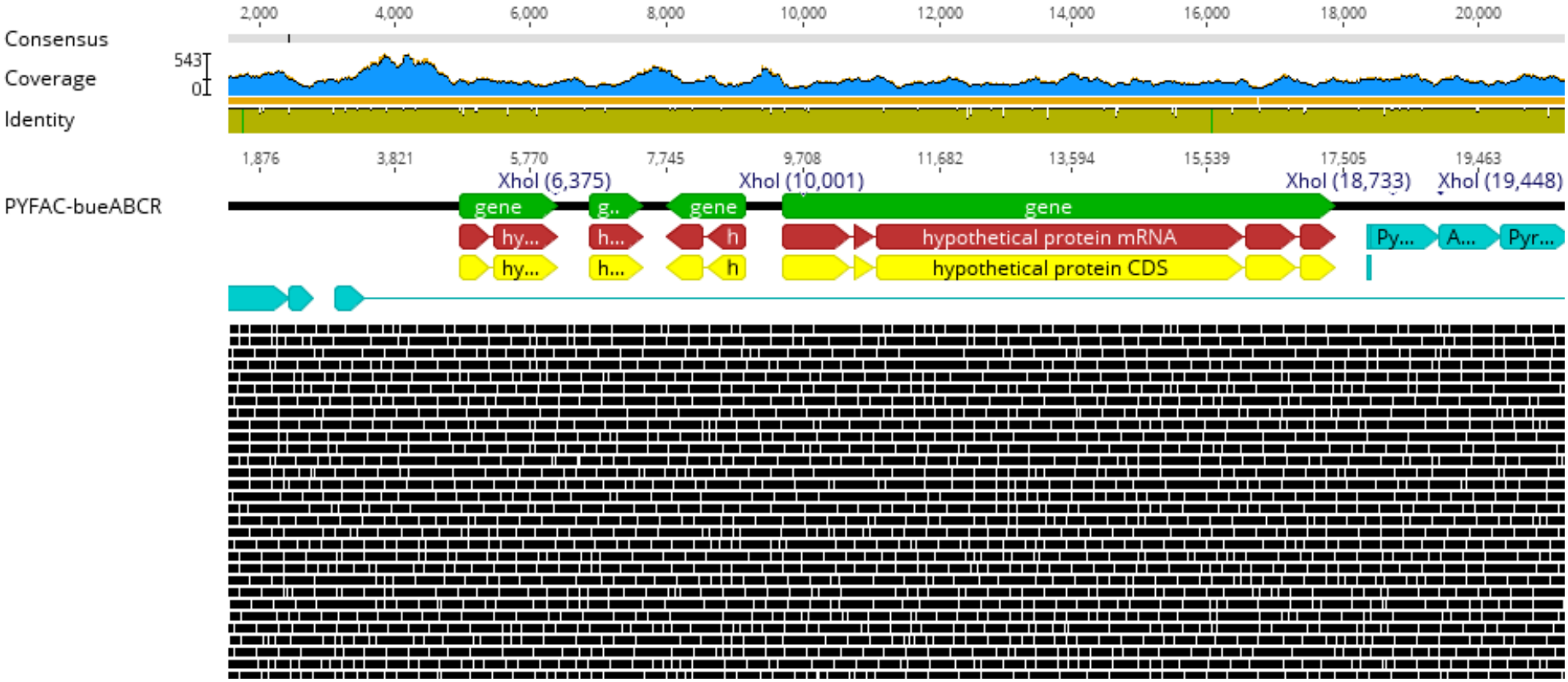


Fig. S31. Verification of correct assembly of pYFAC-bueA/B/C/R by next generation sequencing (plasmid seq). In close examination no discrepancies were found in the region containing the *bue* genes. Reads per base ≥ 100.

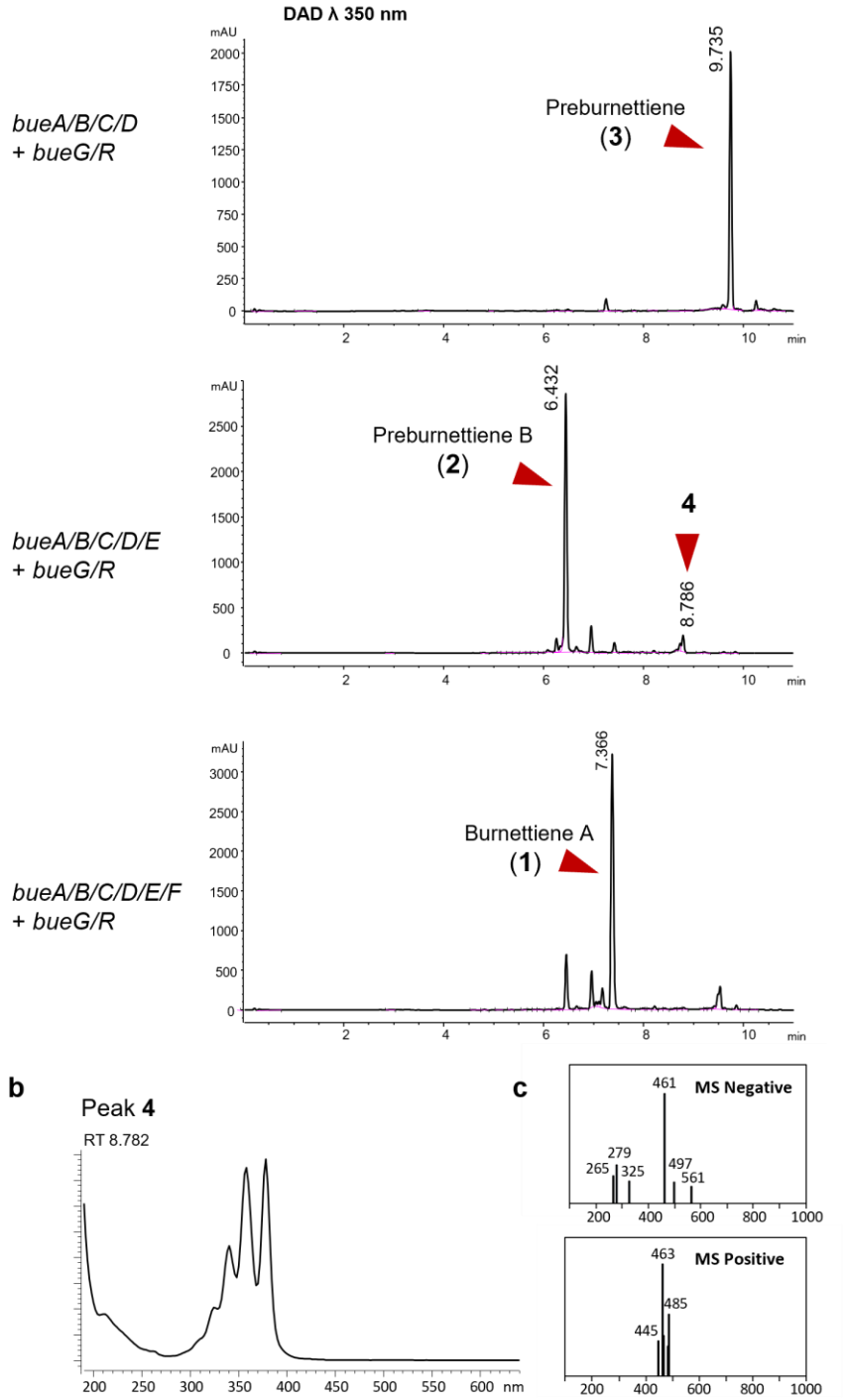


Fig. S32. Scaled up fermentations for the purification of burnettiene A (**1**) and pathway intermediates **a.** DAD (λ = 350 nm) chromatograms show production of metabolites from burnettiene cluster by heterologous expression in *Aspergillus nidulans* after 14 days of growth in jasmine rice media. **b.** UV-vis spectrum of non-polar burnettiene analogue in peak 4. **c.** Main ions detected in positive and negative mass spectrometry analysis of compound **4** indicate a molecular weight of 462.

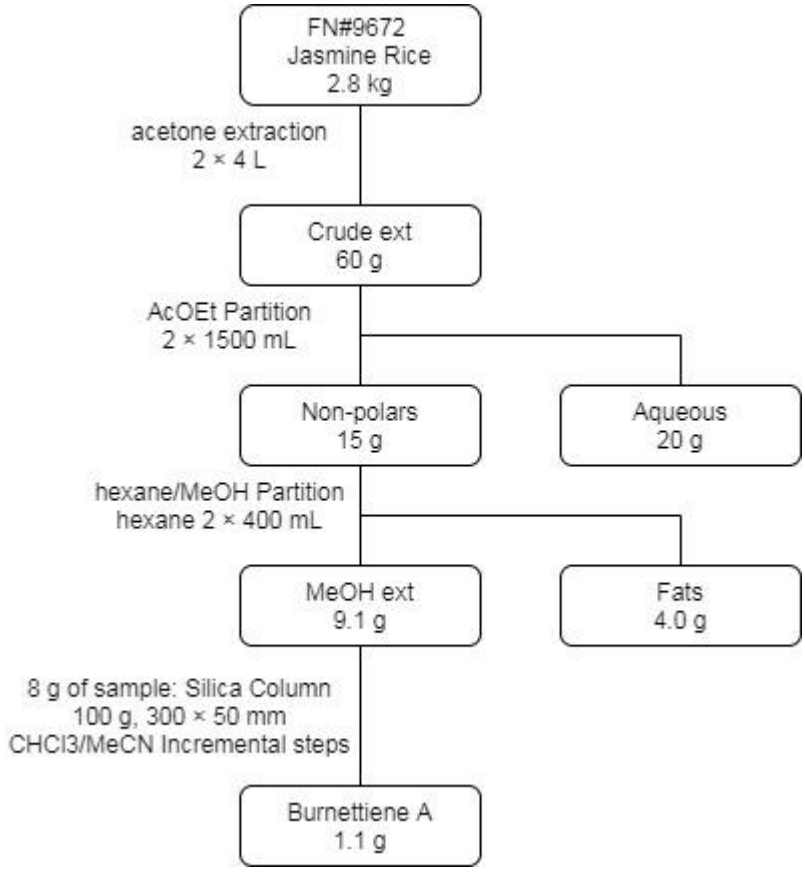


Fig. S33. Purification process for burnettiene A (**1**).

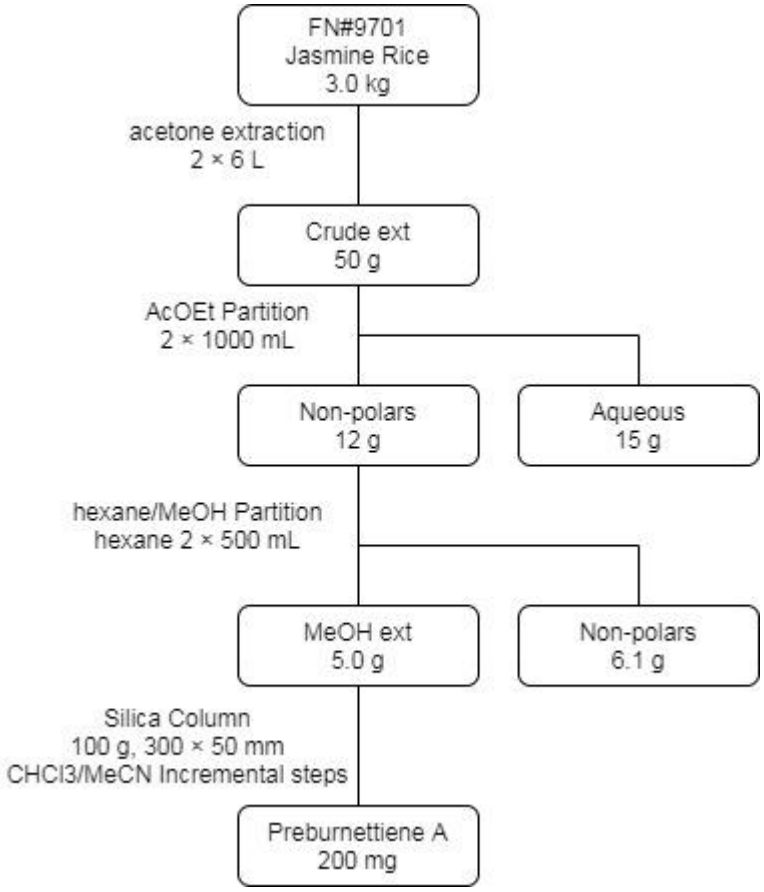


Fig. S34. Purification process for preburnettiene A (**3**).

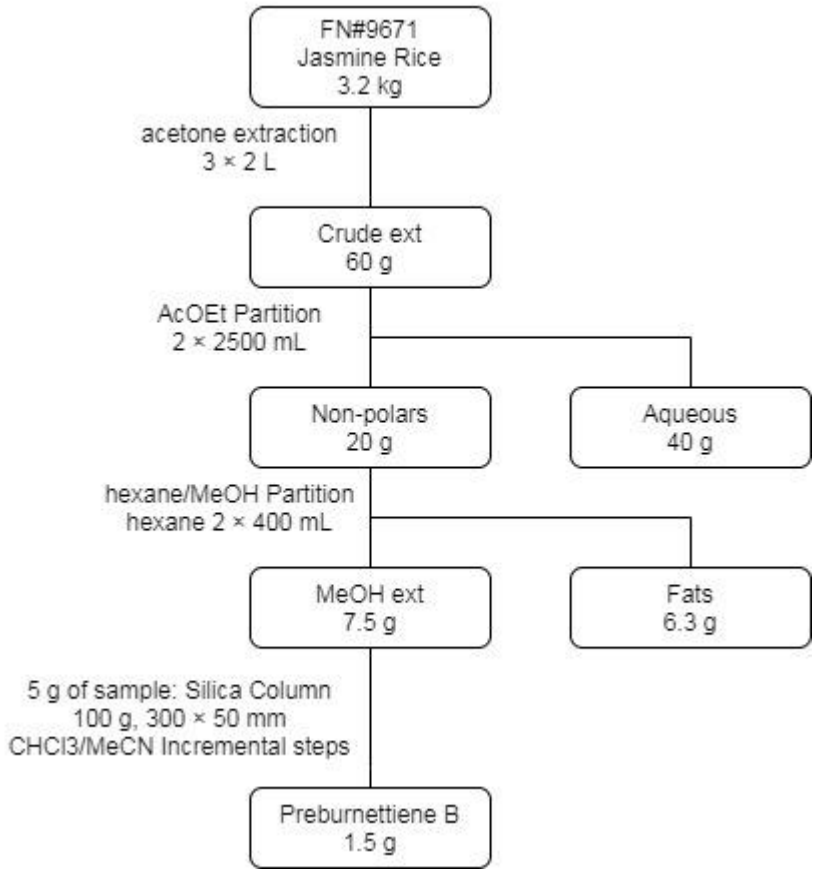


Fig. S35. Purification process for preburnettiene B (**2**).

Fig. S36. Sequence alignment of keto reductase (KR) catalytic domains indicating conserved fingerprint residues. Red asterisk (*) indicates the catalytic residues K, S, Y and N. The LDD motif signature of B-type PKS is found as LRD in the fungal PKSs Hpm8 and BueA or LKD in FSL1, and is indicated in blue.[11] BueA and FSL1 contain a glutamine (Q, yellow) signature of B1-type KR and lack a conserved proline (P, green) signature from B2-type KR. Ery KR (B2-type KR) and Tyl KR (B1-type KR) are included as examples.[11] Overall, the expected chirality of the product of BueA B1-type KR is “R,R”. However, exceptions to the rule have been observed, as is the case of Hpm8.[12]

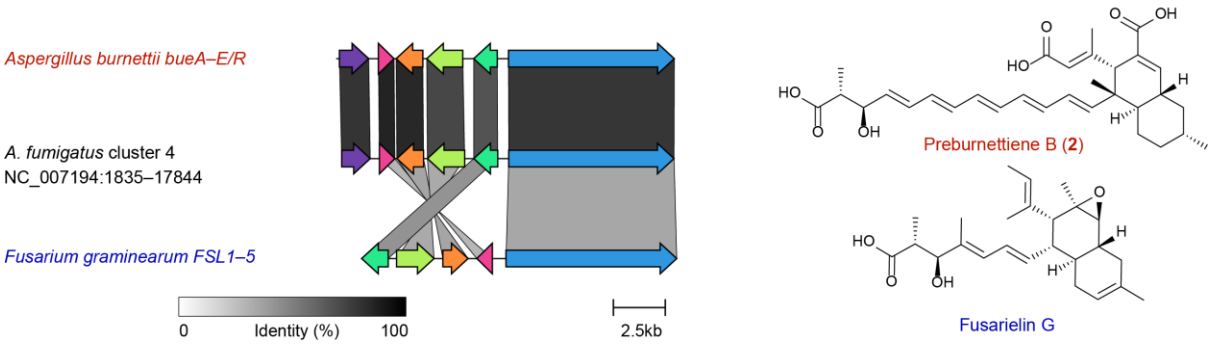


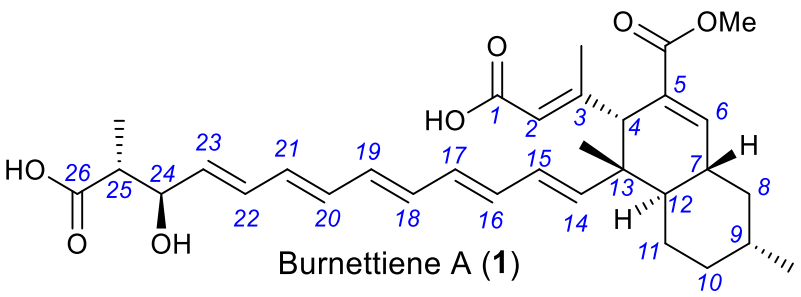
Fig. S37. Comparison of *Aspergillus fumigatus* cluster 4[13] with *bue* and *FSL* gene clusters. *Aspergillus fumigatus* cluster 4 likely encodes a compound more similar to preburnettiene B (**2**) than fusarielin G.[14] Greyscale bars linking genes indicate amino acid identity of the encoded protein (0% fully transparent, 100% black).

Supporting Tables

Table S1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for burnettiene A (**1**) in DMSO-d_6

Pos.	δ_H, mult (*J* in Hz)	δ_C	HMBC	COSY	ROESY
1		167.1			
1-OH	12.00[c], br s				
2	5.31[d], s	118.7	1, 3, 3-Me, 4	3-Me	
3		159.6			
3-Me	2.08, s	23.1	2, 3, 4	2	4
4	2.86, s	56.2	2, 3, 3-Me, 5, 5-$\underline{C}O_2$Me, 6, 12, 13, 13-Me	6	3-Me, 13-Me
5		129.6			
5-$\underline{C}O_2$Me		166.3			
5-$CO_2\underline{Me}$	3.59, s	51.7	5-$\underline{C}O_2$Me		
6	6.81, s	142.9	4, 5, 5-$\underline{C}O_2$Me, 7, 8, 12	4, 7	8a
7	1.93, m	38.1	5, 6, 8, 12	6, 8b, 12	9, 11b, 13-Me
8a	1.96, m	40.2		8b, 9	6
8b	0.81, m		6, 7, 9-Me	7, 8a, 9	
9	1.48, br m	32.8		8a/b, 9-Me, 10a	7, 10a
9-Me	0.89, d (6.5)	22.2	8, 9, 10	9	
10a	1.70, m	35.1	8, 9, 12	9, 10b, 11a	9, 11b
10b	0.89, m			10a	12
11a	1.37, m	26.1		10a, 11b, 12	
11b	0.92, m			11a, 12	7, 10a
12	1.20, m	40.3	7, 11, 13, 13-Me	7, 11a/b	10b, 14
13		41.1			
13-Me	0.97, s	18.2	4, 12, 13, 14		4, 7, 15
14	5.31[d], d (15.5)	141.8	4, 12, 13, 13-Me, 16	15	12
15	6.17, dd (15.5, 9.1)	129.1	13, 14, 16	14	13-Me
16	6.20-6.36[e], m	133.3			
17	6.20-6.36[e], m	131.8[b]			
18	6.20-6.36[e], m	132.4[b]			
19	6.20-6.36[e], m	132.6[b]			
20	6.20-6.36[e], m	132.8[b]			
21	6.20-6.36[e], m	133.2[b]			
22	6.24[e], m	131.0	24	23	
23	5.65, dd (14.2, 7.0)	135.0	21, 22, 24, 25	22, 24	
24	4.13, dd (7.2, 7.0)	72.9	22, 23, 25, 25-Me, 26	23, 25	25-Me
24-OH	[a]				
25	2.35, dq (7.2, 7.2)	46.0	23, 24, 25-Me, 26	24, 25-Me	
25-Me	0.91, d (7.2)	12.8	24, 25, 26	25	24
26		175.8			
26-OH	12.00[c], br s				

[a] Not observed; [b] Assignments interchangeable; [c-e] Overlapping signals

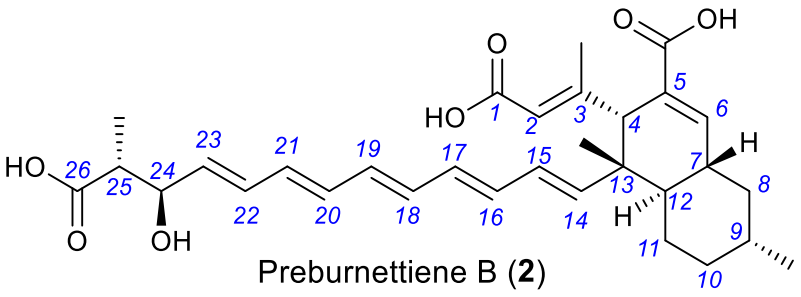


Burnettiene A (**1**)

Table S2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for preburnettiene B (**2**) in DMSO-d_6

Pos.	δ_H, mult (*J* in Hz)	δ_C	HMBC	COSY	ROESY
1		167.1			
1-OH	12.12, br s				
2	5.33, s	118.5	1, 3, 3-Me, 4	3-Me	12
3		160.0			
3-Me	2.07, s	23.1	2, 3, 4	2	4
4	2.84, s	56.3	2, 3, 3-Me, 5, 12, 13-Me	6	3-Me, 13-Me
5		130.5			
5-$\underline{C}O_2H$		167.4			
5-$CO_2\underline{H}$	12.12, br s				
6	6.78, s	141.9	4, 5-$\underline{C}$OOH, 7, 12	4, 7	8a
7	1.89, m	38.0		6, 12	
8a	1.93, m	40.4		8b	6, 9, 13-Me
8b	0.83, m			8a, 9	
9	1.48, br m	32.7		8b, 9-Me	8a, 10a
9-Me	0.90, d (6.3)	22.2	8, 9, 10	9, 10a	
10a	1.70, m	35.2	12	9-Me	9, 11b
10b	0.89, m			11a	12
11a	1.37, m	26.1	9	10b	
11b	0.93, m			12	10a, 11
12	1.19, m	40.3		7, 11b	2, 10b, 14
13		41.1			
13-Me	0.98, s	18.2	4, 12, 13, 14		4, 8a, 15
14	5.32, d (14.8)	142.1	12, 13-Me, 16	15	12
15	6.16, m	128.9	13, 17	14	13-Me
16	6.22-6.36[a], m	133.3			
17	6.22-6.36[a], m	131.7[b]			
18	6.22-6.36[a], m	132.3[b]			
19	6.22-6.36[a], m	132.5[b]			
20	6.22-6.36[a], m	132.7[b]			
21	6.22-6.36[a], m	133.2[b]		23	
22	6.23[a], m	131.0		23	
23	5.66, dd (13.9, 6.9)	134.9	21, 24	21, 22, 24	
24	4.15, dd (6.9, 6.9)	72.8	22, 25	23, 24-OH, 25	
24-OH	5.06,			24	
25	2.37, dq (7.2, 7.2)	45.9	24, 25-Me, 26	24, 25-Me	
25-Me	0.92, d (7.2)	12.7	24, 25, 26	25	
26		175.6			
26-OH	12.12, br s				

[a] Overlapping signals [b] Assignments interchangeable

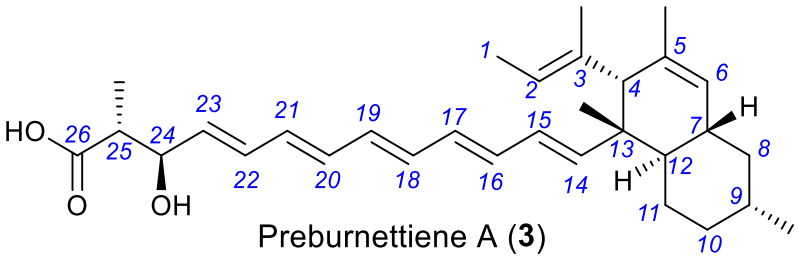


Preburnettiene B (**2**)

Table S3. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for preburnettiene A (**3**) in DMSO-d_6

Pos.	δ_H, mult (*J* in Hz)	δ_C	HMBC	COSY	ROESY
1	1.57, d (6.2)	13.6	3	2	2
2	5.16, q (6.2)	[a]	1, 4	3-Me	1
3		134.8			
3-Me	1.52, s	18.1[d]	3, 4		4
4	[a],	61.6			
5		132.8			
5-Me	1.46, s	22.5	4, 5, 6	6	6
6	5.24, s	126.2	5-Me, 8, 12	5-Me	5-Me
7	[a],	[a]			
8a	1.76, m	41.9		8b,	8b
8b	0.72, m			8a, 9	8a
9	1.40, m	32.6		8b, 9-Me	
9-Me	0.86, d (6.3)	22.4	8, 9, 10	9	
10a	1.67, m	35.5		10,	10b
10b	0.85, m			10a, 11b	10a
11a	1.22, m	29.3			
11b	1.16, m			10b, 12	14
12	1.66, m	37.9		11b	
13		41.4			
13-Me	0.94, s	18.1[d]	4, 13, 14		15
14	5.40, d (15.5)	144.7	13, 13-Me, 16	15	11b
15	6.05, dd (15.5, 10.3)	127.4	13, 16, 17	14, 16	13-Me
16	6.21-6.31[b,c], m	133.9		15	
17	6.21-6.31[b,c], m	131.0[b]			
18	6.21-6.31[b,c], m	132.0[b,e]			
19	6.21-6.31[b,c], m	132.0[b,e]			
20	6.21-6.31[b,c], m	133.0			
21	6.21-6.31[b,c], m	133.4[b]			
22	6.24[c], m	130.7	24	23	23
23	5.65, dd (14.3, 7.0)	135.0	21, 24,	22, 24	22
24	4.13, dd (7.5, 7.0)	72.9	22, 23, 25, 25-Me, 26	23, 25	
24-OH	[a]				
25	2.36, dq (7.0, 7.2)	45.9	23, 24, 25-Me, 26	24, 25-Me	
25-Me	0.91, d (7.0)	12.7	24, 25, 26	25	
26		175.7			
26-OH	12.12, br s				

[a] Not observed; [b] Assignments interchangeable; [c-e] Overlapping signals



Preburnettiene A (**3**)

Table S4. Conservation between *bue* cluster gene products and *FLS* cluster gene products.Identity and similarity values are taken from pairwise amino acid alignments realised with Clinker. N/a means no corresponding homologues.

***A. burnettii* FRR 5400 gene product name (accession number)**	**Predicted function**	***F. graminearum* PH-1 gene product name**	**Identity**	**Similarity**
BueA (ETB97_004590)	HR-PKS	FL1	0.50	0.64
BueB (ETB97_004591)	Enoyl reductase	FL5	0.60	0.70
BueC (ETB97_004594)	α/β hydrolase	FL2	0.39	0.55
BueD (ETB97_004593)	Epimerase	FL3	0.48	0.60
BueE (ETB97_004592)	Cytochrome P450	FL4	0.41	0.56
BueF (ETB97_004596)	Methyl transferase	n/a	n/a	n/a
BueR (ETB97_004595)	Transcription factor	FL7	0.25	0.35
BueG (ETB97_004597)	MFS transporter	n/a	n/a	n/a

Table S5. Additional bioactivity assays reporting minimum inhibitory concentrations (MICs).

Compound	**MIC (µg/mL)** [a]						
	Sa		*Sc*		Teff	*Tf*	
	24 h	*48 h*	*24 h*	*48 h*	72 h	*48 h*	*72 h*
Burnettiene A (**1**)	50	100	> 200	> 200	> 100	> 100	> 100
Preburnettiene B (**2**)	> 100	> 100	> 200	> 200	> 100	> 100	> 100
Preburnettiene A (**3**)	> 100	> 100	> 200	> 200	> 100	> 100	> 100
Burnettiene analogue 4 (**4**)	25	50	> 200	> 200	> 100	> 100	> 100

[a]Note: *Sa*, *Staphylococcus aureus* (ATCC 25923); *Sc*, *Saccharomyces cerevisiae* (ATCC 9763); *Teff*, *Eragrostis tef; Tf*, *Tritrichomonas foetus* KV-1.

Table S6. Vectors used in this study.

Vector name	Size (kb)	Backbone	Purpose	Origin
pYFAC-bueABCR	28.75	pKW20088 (pyrG)	Fungal expression vector containing the genes *bueA, bueB*, *bueC* and *bueR* under their native regulatory sequences.	This work
pYFAC-bueABCDR	30.24	pKW20088 (pyrG)	Fungal expression vector containing the genes *bueA, bueB*, *bueC, bueD* and *bueR* under their native regulatory sequences.	This work
pYFAC-bueABCDER	32.31	pKW20088 (pyrG)	Fungal expression vector containing the genes *bueA, bueB*, *bueC, bueD, bueE* and *bueR* under their native regulatory sequences.	This work
pYFAC-bueF	17.60	pYFAC-riboB	Fungal expression vector containing *bueF* under its native regulatory sequences.	This work
pYFAC-bueG-P_{gpdA}-bueR	19.90	pYFAC-pyroA	Fungal expression vector containing *bueG* under its native regulatory sequences, and *bueR* under the promoter P_{gpdA}.	This work
pKW20088	14.25		Empty backbone control	Tsunematsu *et al* [15]
pYFAC-riboB	14.78		Empty backbone control	Addgene ID# 168975 [5]
pYFAC-pyroA	14.58		Empty backbone control	Addgene ID# 168976 [5]

Table S7. Strains built in this study for heterologous expression. The different vectors are indicated per auxotrophic marker they complement.

Strain number	Strain name	*pyrG* complementation	*riboB* complementation	*pyroA* complementation
1	*Aspergillus nidulans bueA/B/C+bueG/R*	pYFAC-BueABCR	pYFAC-riboB	pYFAC-BueG-PgpdA-bueR
2	*A. nidulans* bueA/B/C/D +bueG/R	pYFAC-BueABCDR	pYFAC-riboB	pYFAC-BueG-PgpdA-bueR
3	*A. nidulans bueA/B/C/D/E +bueG/R*	pYFAC-BueABCDER	pYFAC-riboB	pYFAC-BueG-PgpdA-bueR
4	*A. nidulans bueA/B/C/D/E/F +bueG/R*	pYFAC-BueABCDER	pYFAC-BueF	pYFAC-BueG-PgpdA-bueR
5	*A. nidulans bueA/B/C/D/E/F*	pYFAC-BueABCDER	pYFAC-BueF	pYFAC-pyroA
6	*A. nidulans bueGR*	pKW20088	pYFAC-riboB	pYFAC-BueG-PgpdA-bueR
7	*A. nidulans bueA/B/C/D/E/R*	pYFAC-BueABCDER	pYFAC-riboB	pYFAC-pyroA
8	*A. nidulans empty plasmid control*	pKW20088	pYFAC-riboB	pYFAC-pyroA

Table S8. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′ to 3′)	Purpose
PKW-Fw1	CCTCGCGGGTGTTCTTGACGATGGCATCCTCGTGCGACCCTGAAACTACA	To build pYFAC-BueABCDER, pYFAC-BueABCDR and pYFAC-BueABCR.
Bur_Rv1	AACCGTTGGGCGACAAGG	To build pYFAC-BueABCDER and pYFAC-BueABCDR.
Bur_Fw2	AGGCTCATCTTCCTTGTCGC	To build pYFAC-BueABCDER, pYFAC-BueABCDR.
Bur_Rv2	GTCGCTCTCGATGATGGTACAC	To build pYFAC-BueABCDER, pYFAC-BueABCR and pYFAC-BueABCDR.
Bur_Fw3	CGTAGGCCGAGTGTACCATC	To build pYFAC-BueABCDER and pYFAC-BueABCR.
Bur_Rv3	GGGTTGCCTGTCTGTCCC	To build pYFAC-BueABCDER and pYFAC-BueABCR.
Bur_Fw4	GCGGACGTTATCTTTGGGGAC	To build pYFAC-BueABCDER and pYFAC-BueABCR.
Pyrg-Rv4	ACAGTGGAGGACATACCCGTAATTTTCTGGATGCTTGCGAATGGTGCACC	To build pYFAC-BueABCDER and pYFAC-BueABCR.
Fw_i143	AATTACTAGGCCAATGATACTTCAACCACCCTGCTCG	To build pYFAC-BueABCR.
Rv_i143	GGTGGTTGAAGTATCATTGGCCTAGTAATTGCTGCAG	To build pYFAC-BueABCR.
Fw_i144	CAAACAATCTTCCAGTCCTTATACTTCAACCACCCTGCTCG	To build pYFAC-BueABCDR.
Rv_i144	GTTGAAGTATAAGGACTGGAAGATTGTTTGGTATAACC	To build pYFAC-BueABCDR.
Fw_i145	TCGCGGGTGTTCTTGACGATGGCATCCTGCGGCCGCAACGATTAGCAGCTGACGGAG	To build pYFAC-BueF.
Rv_i145	TAAAGGGTATCATCGAAAGGGAGTCATCCATGTCAATTCTGTTCTACTGGGTTGTG	To build pYFAC-BueF.
Fw_i146_PCR1	ATTCATCTTCCCATCCAAGAACCTTTAATCATGATGAACGGTGAACGAAAGCTC	To build pYFAC-BueH-PgpdA-bueR.
Rv_i146_PCR1	TTTGAACAGCTGGTTACTCTGTTGGTTTCTCCACGG	To build pYFAC-BueH-PgpdA-bueR.
Fw_i146_PCR2	GAAACCAACAGAGTAACCAGCTGTTCAAAGGGGTACTG	To build pYFAC-BueH-PgpdA-bueR.
Rv_i146_PCR2	GATGAGACCCAACAACCATGATACCAGGGGGCAAGTTGTACCCGTGGACAGTAAAG	To build pYFAC-BueH-PgpdA-bueR.
Seq_i146	GGTACGGCTATTTATAGCCAAAGTAG	Sequencing or PCR screening.
i143_SEQ	GGTGAAGACGGGCGATGTG	Sequencing or PCR screening.
i144_SEQ	TCCTCAGCGGAAATGCATAAC	Sequencing or PCR screening.
PgpdA-Fw	CATGCGGAGAGACGGACGG	To build pYFAC-BueH-PgpdA-bueR.
Gpe1-Rv	GATTAAAGGTTCTTGGATGGGAAGATG	To build pYFAC-BueH-PgpdA-bueR.

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