Supporting Information

Wheldone: Characterization of a unique scaffold from the co-culture of *Aspergillus fischeri* and *Xylaria flabelliformis*

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EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation data were obtained using a Rudolph Research Autopol III polarimeter, and UV spectra were measured with a Varian Cary 100 Bio UV−vis spectrophotometer. The NMR data were collected using an Agilent 700 MHz spectrometer (Agilent Technologies), equipped with a cryoprobe, operating at 700 MHz for ^{1}H and 175 MHz for ^{13}C. HRMS experiments utilized either a Thermo LTQ Orbitrap XL mass spectrometer or a Thermo Q Exactive Plus (Thermo Fisher Scientific); both were equipped with an electrospray ionization source. A Waters Acquity UPLC (Waters Corp.) was utilized for both mass spectrometers, using a BEH C_{18} column (1.7 μm; 50 mm x 2.1 mm) set to a temperature of 40ºC and a flow rate of 0.3 mL/min. The mobile phase consisted of a linear gradient of CH_3CN-H_2O (both acidified with 0.1% formic acid), starting at 15% CH_3CN and increasing linearly to 100% CH_3CN over 8 min, with a 1.5 min hold before returning to the starting condition. The HPLC separations were performed with Atlantis T3 C_{18} preparative (5 μm; 19 x 250 mm) column, with a Varian Prostar HPLC system equipped with a Prostar 210 pumps and a Prostar 335 photodiode array detector (PDA), with the collection and analysis of data using Galaxie Chromatography Workstation software. Flash chromatography was performed on a Teledyne ISCO Combiflash Rf 200 and monitored by both ELSD and PDA detectors.

Fungal Strain Isolation and Identification. *Aspergillus fischeri* strain NRRL 181 was obtained from ARS Culture Collection (NRRL).[1] *Xylaria flabelliformis* (formally *Xylaria cubensis*) strain G536 was isolated as an endophyte from surface sterilized twigs of *Asimina triloba* and identified using molecular methods as outlined previously.[2] The genome for *X. flabelliformis* was reported recently.[3]

Fermentation, Extraction, and Isolation. Storage, fermentation, and extraction conditions were reported previously.[4] Briefly, *A. fischeri* and *X. flabelliformis* were grown separately in 10 mL of YESD broth (2% soy peptone, 2% dextrose, and 1% yeast extract; 5 g of yeast extract, 10 g of soy peptone, and 10 g of D-glucose in 500 mL of deionized H_2O) and cultivated at 22˚C with agitation at 100 rpm for 3 (*A. fischeri*) and 5 days (*X. flabelliformis*). YESD seed cultures of both fungi grown individually were subsequently used to inoculate 16, 250 mL Erlenmeyer flasks that contained 10 g of autoclaved Quaker Breakfast Oatmeal each (10 g of oatmeal with 17 mL of deionized H_2O and sterilized for 15–20 mins at 121°C). Following incubation at room temperature for 3 weeks, each solid culture was extracted by adding 60 mL of 1:1 MeOH-$CHCl_3$. Subsequently, each culture was chopped with a spatula and left to shake overnight (~16 hrs) at ~100 rpm at room temperature. The cultures (16 flasks) were combined and filtered *in vacuo*, and 1500 mL $CHCl_3$ and 2400 mL H_2O were added to the filtrate. The mixture was stirred for 30 min and then transferred to a separatory funnel. The organic layer ($CHCl_3$) was drawn off and evaporated to dryness *in vacuo*. The dried organic layer was reconstituted in 300 mL of (1:1) MeOH-CH_3CN and 300 mL of hexanes, transferred to a separatory funnel, and shaken vigorously. The defatted organic layer (MeOH-CH_3CN) was evaporated to dryness *in vacuo*. The organic layer (1.28 g) was dissolved in $CHCl_3$, absorbed onto Celite 545 (Acros Organics), and fractionated by normal phase flash chromatography using a gradient of hexane-$CHCl_3$-MeOH at a 30 mL/min flow rate and 61.0 column volumes, which yielded six fractions. Fraction two (239.22 mg) was further purified via preparative HPLC using a gradient system 50:50 to 90:10 of CH_3CN-H_2O with 0.1% formic acid over 30 min at a flow rate of 16.9 mL/min to yield 14 subfractions. Subfraction four (5.48 mg), which eluted at 14.0 min, yielded wheldone (**1**). This process has been

repeated several times to isolate larger quantities of **1**, showing the reproducibility of co-culturing *A. fischeri* and *X. flabelliformis*.

Preparation of the (R)- and (S)-MTPA Ester Derivatives of wheldone

To 0.25 mg of compound **1** were added 450 μL of pyridine-*d*5, and the solution was transferred into an NMR tube. To initiate the reaction, 20 μL of (*R*)-(–)- *α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA)* chloride were added with careful shaking and then monitored immediately by ^{1}H NMR at the following time points: 0, 5, 10, and 15 min. The reaction was found to be complete in 5 min, yielding the mono (*S*)-MTPA ester derivative (**1a**). ^{1}H NMR data of **1a** (500 MHz, pyridine-*d*5): δ*H* 1.55 (3H, H_3-22), 1.88 (3H, H_3-25). In an analogous manner, 0.25 mg of compound **1** dissolved in 400μL pyridine-*d*5 was reacted in a second NMR tube with 20 μL of *S*-(+)-*α*-methoxy-*α*-(trifluoromethyl)phenylacetyl (MTPA) chloride for the same time points, to afford the mono (*R*)-MTPA ester derivative (**1b**). ^{1}H NMR data of **1b** (500 MHz, pyridine-*d*5): δ*H* 1.61 (3H, H_3-22), 1.76 (3H, H_3-25).

*wheldone (**1**).*White, amorphous powder; $[\alpha]_D^{20}$ = +182 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 262 (3.74), 217 (3.70) nm; ^{1}H NMR (CD_3OD, 700 MHz) and ^{13}C (CD_3OD, 175 MHz) (See Table 1); HRESIMS *m/z* 431.2423 $[M+H]^+$ (calcd. For $C_{25}H_{35}O_6$, 431.2433).

Cytotoxicity Assay

Human melanoma cancer cells MDA-MB-435, human breast cancer cells MDA-MB-231 and human ovarian cancer cells OVCAR3 were purchased from the American Type Culture Collection (Manassas, VA). The cell line was propagated at 37°C in 5% CO_2 in RPMI 1640 medium, supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells in log phase growth were harvested by trypsinization followed by two washing to remove all traces of enzyme. A total of 5,000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96®, Falcon) and incubated overnight (37°C in 5% CO_2). Samples dissolved in DMSO were then diluted and added to the appropriate wells. The cells were incubated in the presence of test substance for 72 h at 37°C and evaluated for viability with a commercial absorbance assay (CellTiter-Blue Cell Viability Assay, Promega Corp, Madison, WI) that measured viable cells. IC_{50} values are expressed in µM relative to the solvent (DMSO) control.

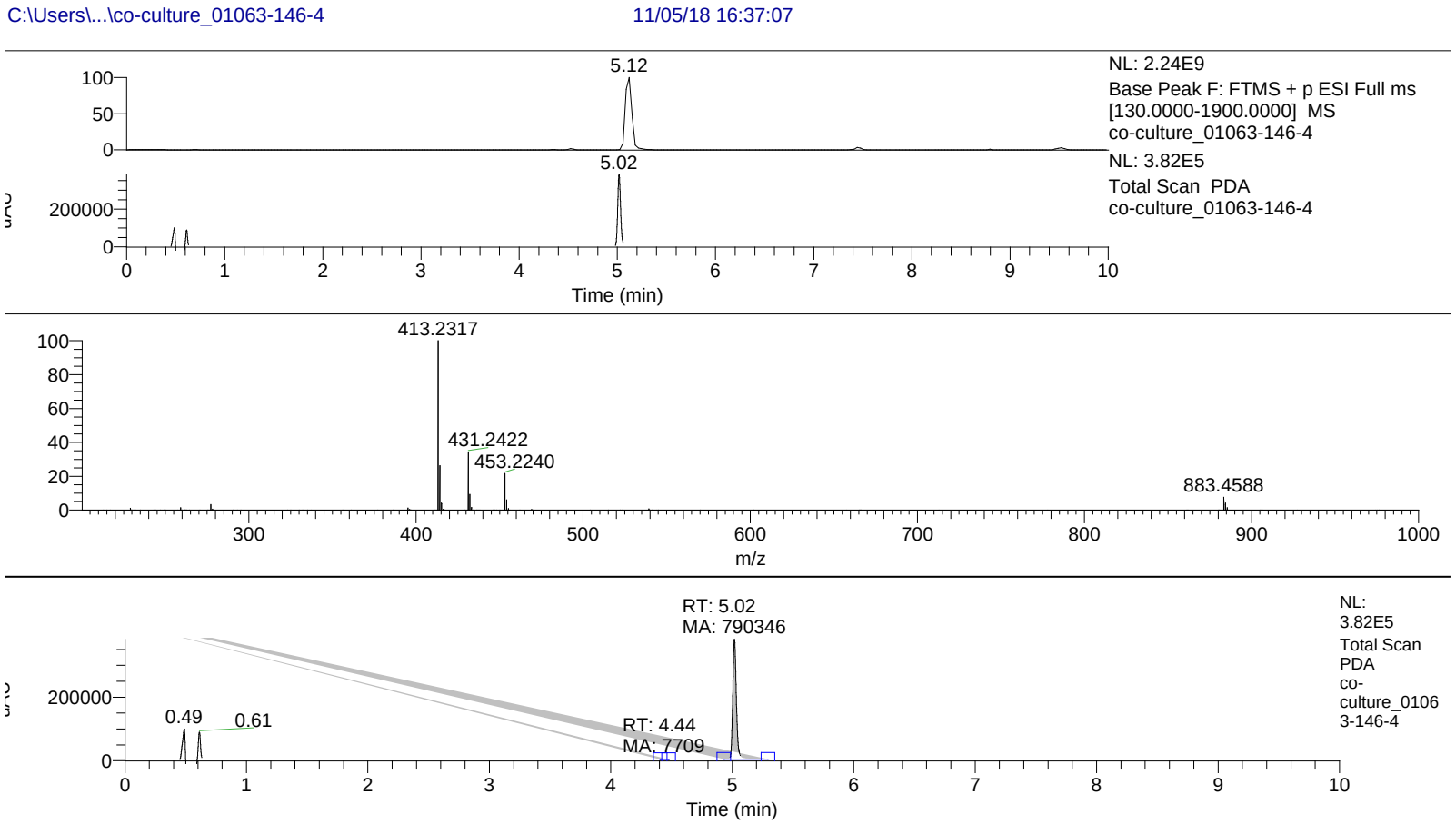

Figure S1. UPLC-MS chromatogram (base peak and PDA data; top), (+)-HRESIMS spectrum (middle), purity (bottom) of compound **1**.

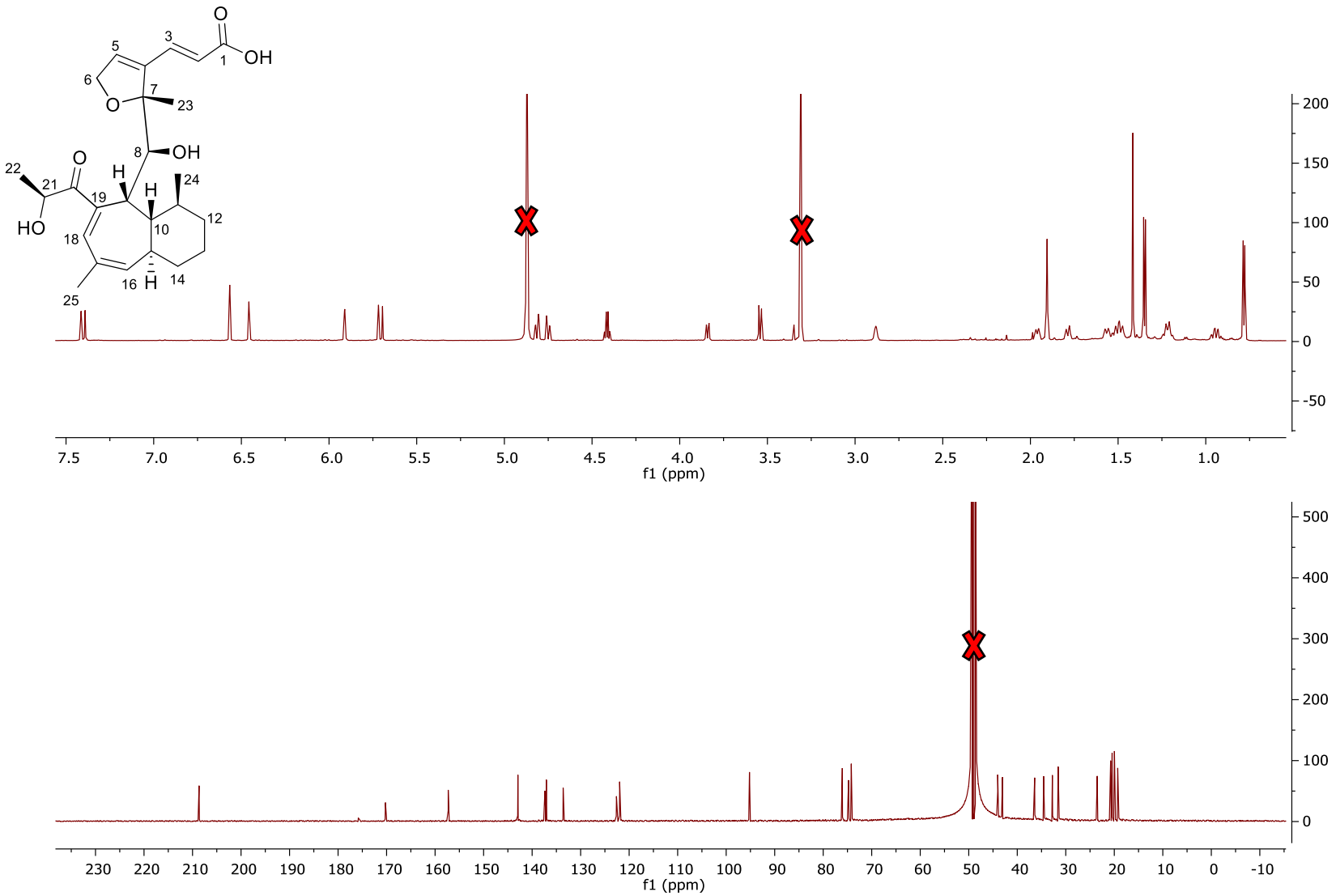

Figure S2. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data for wheldone (**1**) in CD_3OD.

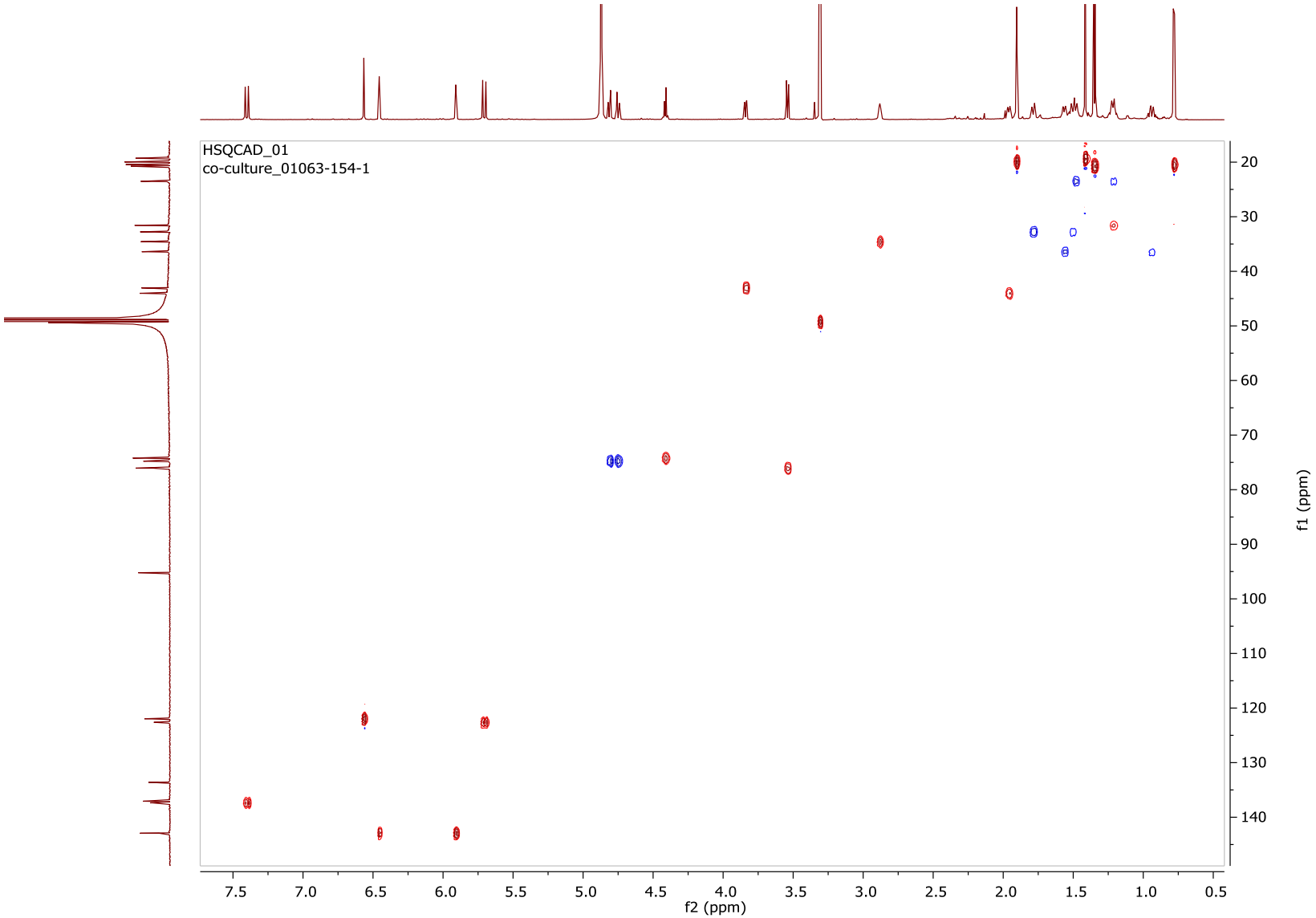

Figure S3. DEPT-edited HSQC NMR spectrum of compound **1** (700 MHz, CD_3OD).

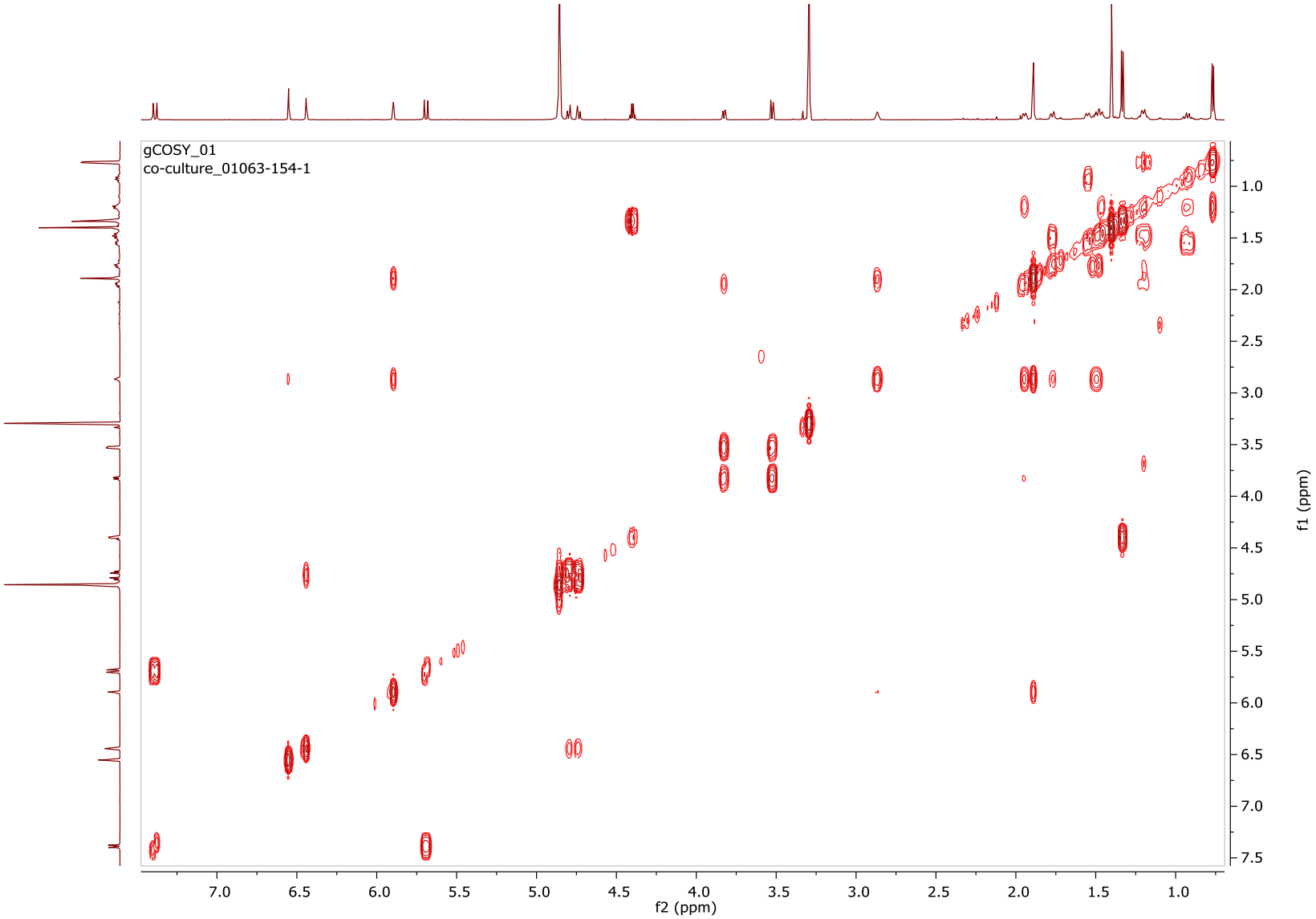

Figure S4. COSY NMR spectrum of compound **1** (700 MHz, CD_3OD).

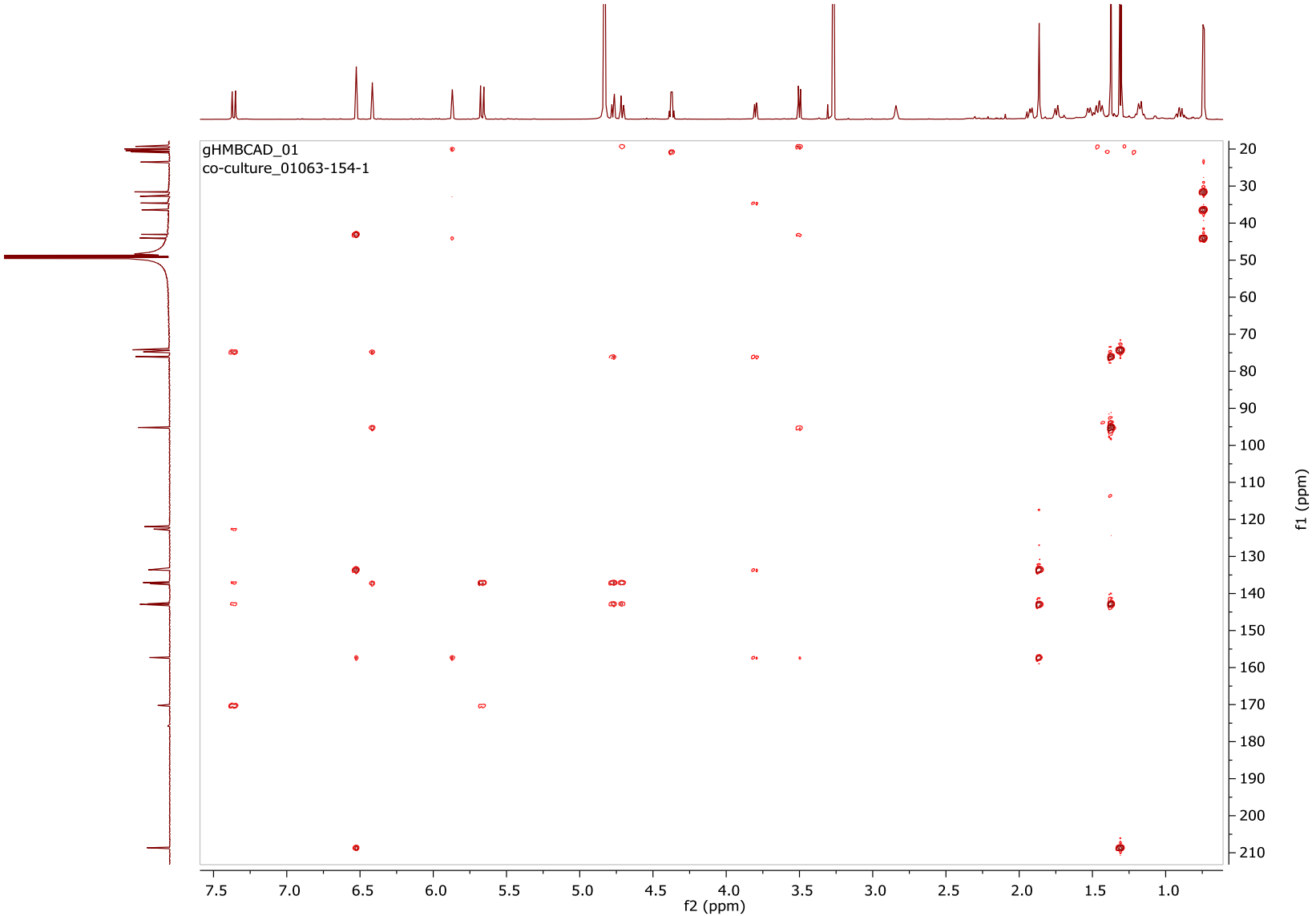

Figure S5. HMBC NMR spectrum of compound **1** (700 MHz, CD_3OD).

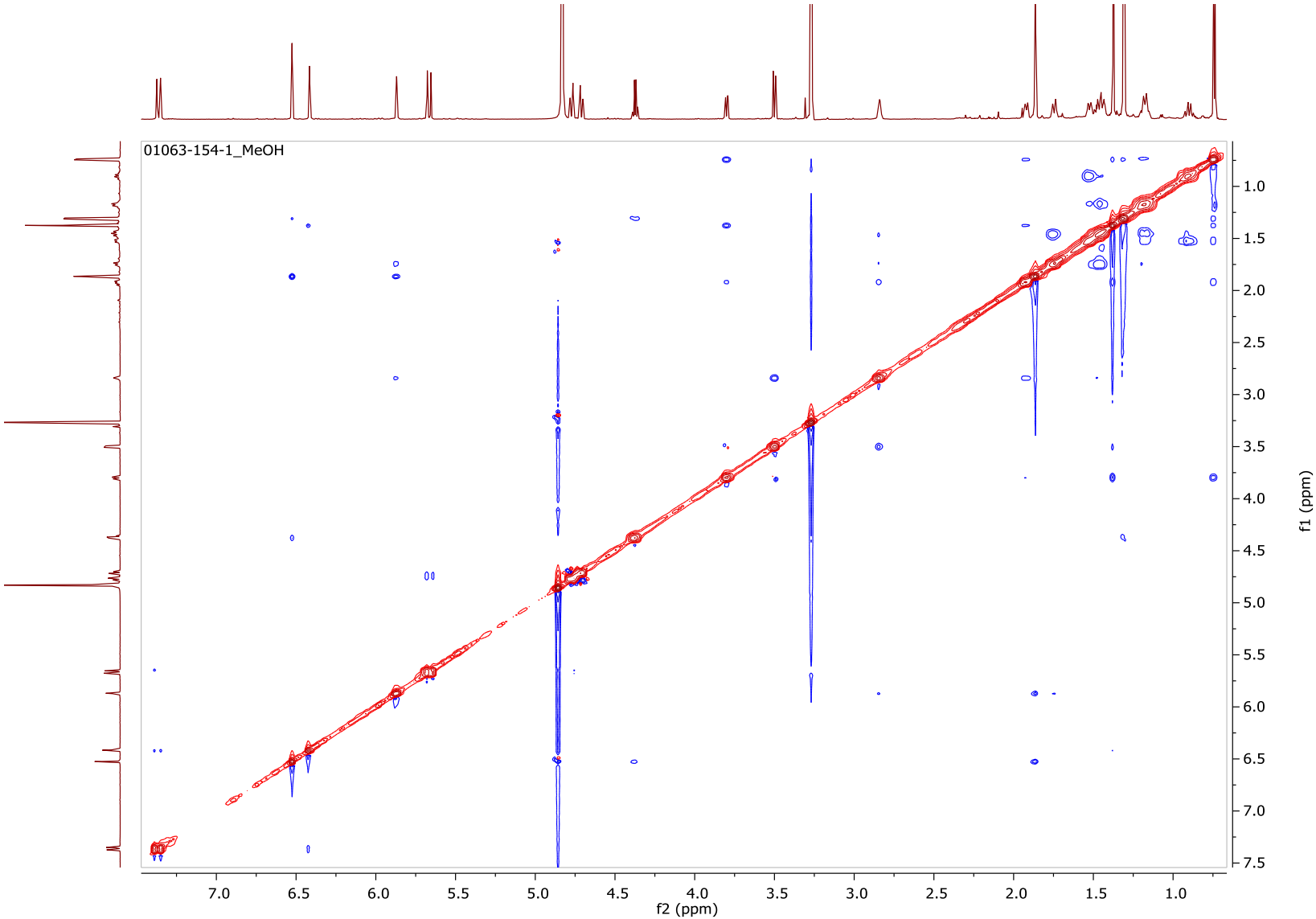

Figure S6. NOESY NMR spectrum of compound **1** (400 MHz, CD_3OD).

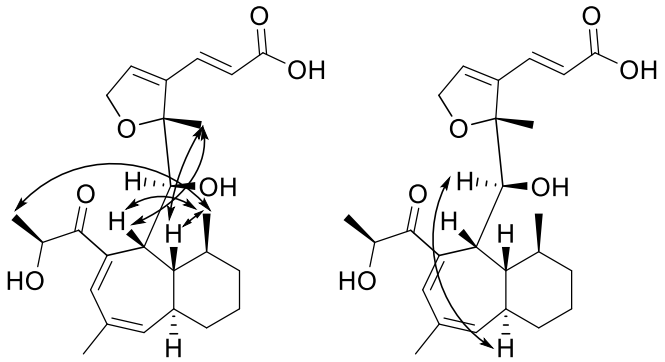

Figure S7. Key NOESY correlations for compound **1**. The structure is presented twice, so that it is easier to see the key correlations for each face of the molecule.

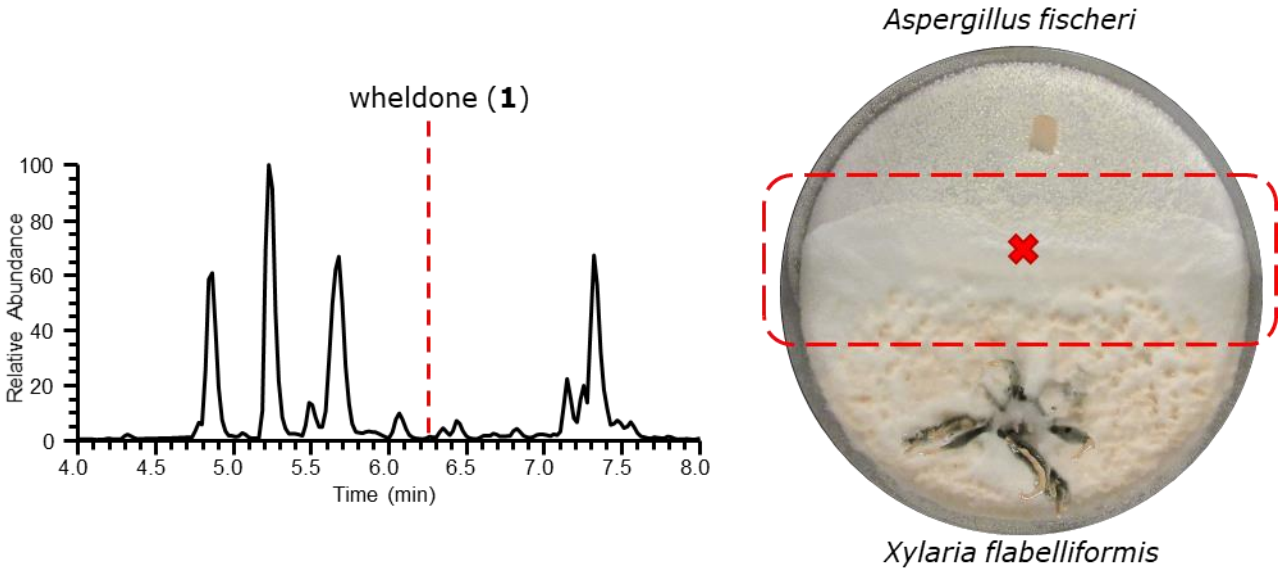

Figure S8. Wheldone (**1**) was first noted as a minor component in the base peak chromatogram during *in situ* analysis[4] of the junction that developed between *A. fischeri* and *X. flabelliformis* (shown in the box in the co-culture Petri dish at the right).

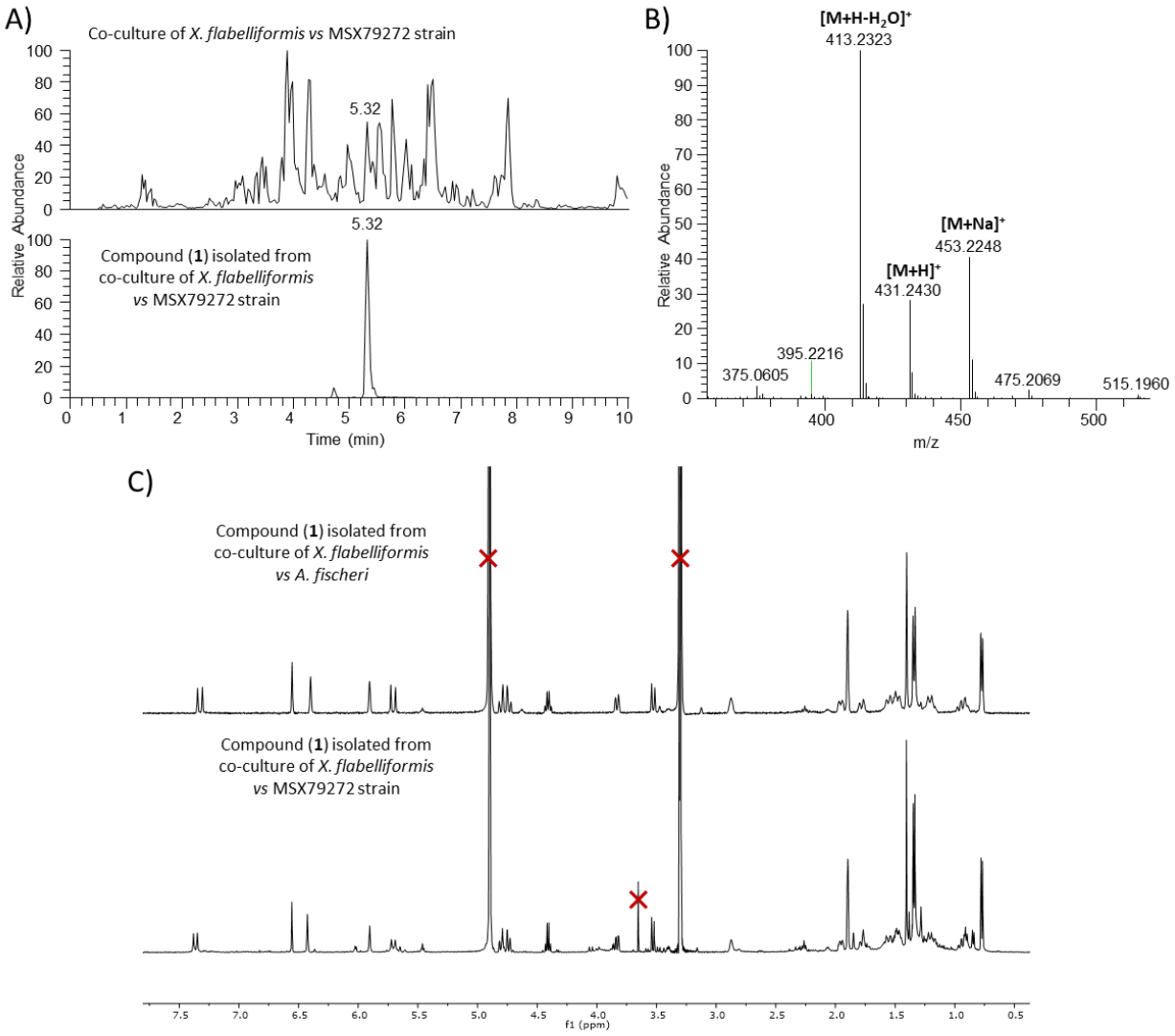

Figure S9. Data confirming the biosynthesis of **1** from a co-culture of *X. flabelliformis* vs. fungal strain MSX79272. A) UPLC-MS chromatogram (base peak of extract; top and **1**; bottom) for the co-culture of *X. flabelliformis* vs strain MSX79272. B) Mass spectrum data of **1** isolated from *X. flabelliformis* vs. MSX79272. C) ^{1}H NMR data comparison between **1** isolated from the two separate co-culture experiments.

(1) Mead, M. E.; Knowles, S. L.; Raja, H. A.; Beattie, S. R.; Kowalski, C. H.; Steenwyk, J. L.; Silva, L. P.; Chiaratto, J.; Ries, L. N.; Goldman, G. H.; Oberlies, N. H.; Rokas, A. Characterizing the pathogenic, genomic, and chemical traits of *Aspergillus fischeri*, a close relative of the major human fungal pathogen *Aspergillus fumigatus*. *mSphere* **2019**, *4*, e00018-19.
(2) Sica, V. P.; Rees, E. R.; Tchegnon, E.; Bardsley, R. H.; Raja, H. A.; Oberlies, N. H. Spatial and temporal profiling of griseofulvin production in *Xylaria cubensis* using mass spectrometry mapping. *Front. Microbiol.* **2016**, *7*, 544.
(3) Mead, M. E.; Raja, H. A.; Steenwyk, J. L.; Knowles, S. L.; Oberlies, N. H.; Rokas, A. Draft Genome Sequence of the Griseofulvin-Producing Fungus *Xylaria flabelliformis* Strain G536. *Microbiol. Resour. Announc.* **2019**, *8*, e00890-19.
(4) Knowles, S. L.; Raja, H. A.; Wright, A. J.; Lee, A. M. L.; Caesar, L. K.; Cech, N. B.; Mead, M. E.; Steenwyk, J. L.; Ries, L.; Goldman, G. H.; Rokas, A.; Oberlies, N. H. Mapping the Fungal Battlefield: Using in situ Chemistry and Deletion Mutants to Monitor Interspecific Chemical Interactions Between Fungi. *Front. Microbiol.* **2019**, *10*, 285.