

Supplementary Information

Novel macrolactam compound produced by heterologous expression of a large cryptic biosynthetic gene cluster of *Streptomyces rochei* IFO12908

Takuya Hashimoto¹, Ikuko Kozono², Junko Hashimoto², Reiko Ueoka¹, Noritaka Kagaya¹,
Manabu Fujie³, Noriyuki Sato³, Haruo Ikeda⁴, Kazuo Shin-ya^{*,1,5,6}

¹National Institute of Advanced Industrial Science and Technology, 2-4-7 Aomi, Koto-ku, Tokyo, 135-0064, Japan.

²Japan Biological Informatics Consortium (JBIC), Tokyo Japan. 2-4-7 Aomi, Koto-ku, Tokyo, 135-0064, Japan.

³Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa 904-0495, Japan

⁴Kitasato Institute for Life Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagami-hara, Kanagawa 252-0373, Japan.

⁵Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, To-kyo, 113-8657, Japan.

⁶Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan.

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Experimental section

General Experimental Procedures.

Optical rotations were measured on a Horiba SEPA-500 polarimeter (Horiba, Kyoto, Japan) using a sodium lamp at a wavelength of 589 nm. UV and IR spectra were measured with a DU730 UV/Vis spectrophotometer (Beckman Coulter Japan, Tokyo, Japan) and an FT-720 spectrophotometer (Horiba) equipped with DuraSamplIR II (Horiba), respectively. ^1H , ^{13}C NMR, and 2D NMR data such as double-quantum-filtered correlation spectroscopy (DQF-COSY), gradient-enhanced heteronuclear single quantum coherence with adiabatic pulses (HSQCAD), and gradient-selected heteronuclear multiple bond correlation using adiabatic pulses (HMBCAD) were collected using a Varian NMR 600 NB CL spectrometer with DMSO- d_6 as the solvent (δ_{C} 39.7 ppm, δ_{H} 2.49 ppm). The coupling constants (J) are given in Hertz. Reversed-phase MPLC was carried out using Purif-Pack ODS-60 columns (Shoko Scientific Co., Ltd., Yokohama, Japan). Analytical RP-UPLC and HR-ESI-MS (positive mode) were performed using a Waters ACQUITY UPLC System (Waters, Taunton, MA in conjunction with a BEH ODS column (2.1 i.d. \times 100 mm, Waters), a Waters ACQUITY UPLC photodiode array λ detector (Waters), and a XevoG2 Tof system (Waters). Preparative RP-HPLC was conducted using a Develosil RPAQUEOUS-AR5 C₃₀ column (20 i.d. \times 150 mm, Nomura Chemical, Seto, Japan). Oligonucleotides for the polymerase chain reaction (PCR) were purchased from Life Technologies (Carlsbad, CA). Pulse field gel electrophoresis was performed with a CHEF Mapper XA System (Bio-Rad, Hercules, CA). DNA manipulation was carried out using standard methods.¹ Restriction enzymes and other molecular biology reagents were purchased from New England BioLabs (Ipswich, MA). PCR reactions for screening were performed with GoTaq Master Mix (Promega, Madison, WI).

Cloning and Isolation of a BAC Clone Containing Target Gene Cluster and Transformation to Host Strain.

Genome sequencing of *Streptomyces rochei* IFO12908 strain was performed using Roche 454 GS FLX Titanium chemistry (Roche, Basel, Switzerland). The sequence was assembled using the Newbler software package. After the genome, embedded in 0.6% agarose gel, was partially digested with *Bam*HI, the resulting fragments were separated by CHEF electrophoresis in 1% agarose gel, and approximately 220-240 kb fragments were harvested from the gel. The purified DNA fragments were ligated with *Bam*HI-cut pKU518 and the ligation products were introduced into *E. coli* NEB 10-beta (New England Biolabs, Inc., MA, USA) by electroporation.^{2,3} Each BAC clone was stored in four 384-well plates containing

Plusgrow II (100 µg/mL ampicillin and 20% glycerol) at –80 °C. Clones containing the biosynthetic gene cluster for **1** were subjected to PCR amplification using two pairs of primers corresponding to its upstream and downstream regions (F6K295_P1_1F: 5'-GTGGTCCAGACACGTCAGCTTC-3'/F6K295_P1_1R: 5'-AGGATTCGTCCGATTCTTCGACTC-3' and F6K295_P1_2F: 5'-GAGCACCAGCGTGACCAGAT-3'/F6K295_P1_2R: 5'-TACCGCTGTCACACCACAAGAGAT-3'). The amplification was performed as follows: denaturation at 95 °C for 300 s, 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s, incubation at 72 °C for 5 min then soaking at 12 °C. A clone pKU518F6K295_P1_P9-M23 was selected, and the inserted sequence was confirmed by end-sequencing. The pKU518F6K295_P1_P9-M23 was introduced into the *S. avermitilis* SUKA32 strain carrying the SAP1 vector that contains the synthetic sequence of *attB*_{ϕK38-1}, *attB*_{R4}, *attB*_{ϕBT1}, *attB*_{ϕC31}, and *attB*_{TG1} of *S. avermitilis* MA-4680, according to the reported protocol.⁴

Fermentation, Extraction, and Isolation.

The obtained clone was cultivated in 50-mL test-tubes, each containing 15 mL of a seed medium consisting of 0.5% glucose, 1.5% soybean meal and 0.5% yeast extract at pH 7.5 (adjusted before sterilization). The test tubes were shaken on a reciprocal shaker (320 rpm) at 27 °C for 2 days. Aliquots (2.5 mL) of the culture were transferred into 500-mL baffled Erlenmeyer flasks containing 100 mL of a production medium consisting of 2% glycerol, 1% molasses, 0.5% casein, 0.1% polypepton, 0.4% CaCO₂, (adjusted pH to 7.2 before sterilization), and were cultured on a rotary shaker (180 rpm) at 27 °C for 5 days.

The fermentation broth (1 L) was centrifuged, and the collected mycelial cake was extracted with acetone (200 mL × 3). After concentration in vacuo, the residual aqueous concentrate was partitioned between EtOAc and water (100 mL × 3). The resultant ethyl acetate layer was concentrated to dryness (321 mg). The sample was subjected to ODS RP-MPLC by using a H₂O–MeOH stepwise solvent system (0%, 40%, 60%, 80%, and 100% MeOH). Fractions were monitored using the UPLC-DAD-MS system. The 80% MeOH fraction was collected and lyophilized (30.7 mg). The fraction was further purified by RP HPLC employing a C30 column, in isocratic mode with 55% CH₃CN to afford 3.6 mg of **1**.

JBIR-156 (1)

[α]_D²⁴ –25 (c 0.02, MeOH); UV (MeOH) λ_{\max} (log ϵ) 264 nm (4.22), 303 nm (4.53), 313 nm (4.54), 348 nm (4.11); IR (ATR) ν_{\max} 3400 and 1646 cm^{–1}; ¹H NMR (600 MHz) and ¹³C NMR (150 MHz), see Table 1.

Cytotoxicity Assays.

The cytotoxic activities of **1** against human ovarian adenocarcinoma SKOV-3 cells, malignant pleural mesothelioma Meso-1 cells and immortalized human T lymphocyte Jurkat cells were examined. SKOV-3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 µg/mL). Meso-1 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 µg/mL). Jurkat cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin (50 U/mL), streptomycin (50 µg/mL) and glutamax. All cell lines were seeded in 384-well plates at the density of 1000 cells/well in 20 µL media and incubated at 37 °C in a humidified incubator with 5% CO₂. Samples were resolved in DMSO. After 4 h, two-fold dilution samples were added to the cell culture at the final concentration of 0.5% and incubated for 72 h. Cell viabilities were measured using CellTiter-Glo Luminescent Cell Viability Assay and EnVision Multilabel Plate Reader.

References

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4. Kim JH, Komatsu M, Shin-ya K, Omura S, Ikeda H. Distribution and functional analysis of the phosphopantetheinyl transferase superfamily in *Actinomycetales* microorganisms. Proc Natl Acad Sci U S A. 2018;115:6828-33.

Table S1. The deduced functions of the genes in the biosynthetic gene cluster.

Gene product	Amino acids (aa)	Proposed function	BLAST Hit protein [Origin]	Identity/ Similarity (%)	Accession No.
Orf-3	234	Hypothetical protein	Hypothetical protein SCO0976 [Streptomyces coelicolor A3(2)]	49/53	NP_625272.1
Orf-2	116	Hypothetical protein	Hypothetical protein SCO0977 [Streptomyces coelicolor A3(2)]	90/94	NP_625273.1
Orf-1	135	Putative L-aspartate 1-decarboxylase	Aspartate 1-decarboxylase [Streptomyces coelicolor A3(3)]	95/98	NP_625274.1
ORF C	253	Type II thioesterase	PimI protein [Streptomyces natalensis]	51/65	CAC20922.1
ORF A1	5629	Modular polyketide synthase	Modular polyketide synthase [Streptomyces halstedii]	49/60	BAD08373.1
ORF A5	3776	Modular polyketide synthase	Modular polyketide synthase [Streptomyces halstedii]	52/64	BAD08360.1
ORF A4	3544	Modular polyketide synthase	Modular polyketide synthase [Streptomyces halstedii]	50/62	BAD08360.1
ORF A3	1610	Modular polyketide synthase	Type I polyketide synthase [Streptomyces rochei]	100/100	WP_086876767.1
ORF A2	3586	Modular polyketide synthase	Modular polyketide synthase [Streptomyces halstedii]	49/60	BAD08373.1
ORF D	391	Aspartate aminotransferase	VinF [Streptomyces halstedii]	64/75	BAD08362.1
ORF B1	496	dTDP-4-keto-6-deoxy-L-hexose 2,3-dehydratase	VinD [Streptomyces halstedii]	53/66	BAD08363.1
ORF B2	339	dTDP-4-keto-6-deoxy-L-hexose 2,3-reductase	VinE [Streptomyces halstedii]	74/85	BAD08364.1
ORF B3	326	dTDP-glucose 4,6-dehydratase	dTDP-glucose 4,6-dehydratase [Micromonospora sp. ATCC 39149]	69/78	EEP73304.1
ORF E	518	Non-ribosomal peptide synthetase	VinM [Streptomyces halstedii]	57/67	BAD08370.1
ORF F	318	Malonyl-CoA-[acyl-carrier-protein] transacylase	VinK [Streptomyces halstedii]	53/71	BAD08368.1
ORF G	427	Glycosyltransferase	VinC [Streptomyces halstedii]	45/61	BAD08357.1
ORF B4	290	Glucose-1-phosphate thymidyltransferase	AveBIII [Streptomyces avermitilis MA-4680]	68/78	NP_822122.1
ORF T	521	Transporter	Multidrug resistance efflux protein [Streptomyces avermitilis MA-4680]	47/62	NP_827958.1
ORF R1	1067	Transcriptional regulator	AtsR-like transcriptional regulator [Streptomyces griseus subsp. griseus NBRC 13350].	37/49	YP_001821581.1
ORF H	471	Glutamate mutase E-chain	VinI [Streptomyces halstedii]	57/64	BAD08366.1
ORF I	173	Glutamate mutase S-chain	VinH [Streptomyces halstedii]	51/62	BAD08365.1
ORF J	441	Decarboxylase	VinO [Streptomyces halstedii]	57/68	BAD08372.1
ORF K	528	Non-ribosomal peptide synthetase	VinN [Streptomyces halstedii]	62/75	BAD08371.1
ORF L	82	Peptidyl carrier protein	VinL [Streptomyces halstedii]	55/68	BAD08369.1
ORF M	313	Peptidase	VinJ [Streptomyces halstedii]	67/80	BAD08367.1
ORF R2	876	Transcriptional regulator	OlmRII [Streptomyces avermitilis MA-4680]	33/46	NP_824077.1
ORF B5	240	N,N-dimethyltransferase	DesVI [Streptomyces venezuelae]	45/59	AAC68678.1
Orf+1	206	Hypothetical protein	Hypothetical protein [Streptomyces coelicolor A3(2)]	73/80	NP_627042.1
Orf+2	343	Iron transporter	Iron transporter protein [Streptomyces avermitilis MA-4680]	30/46	WP_010982040.1

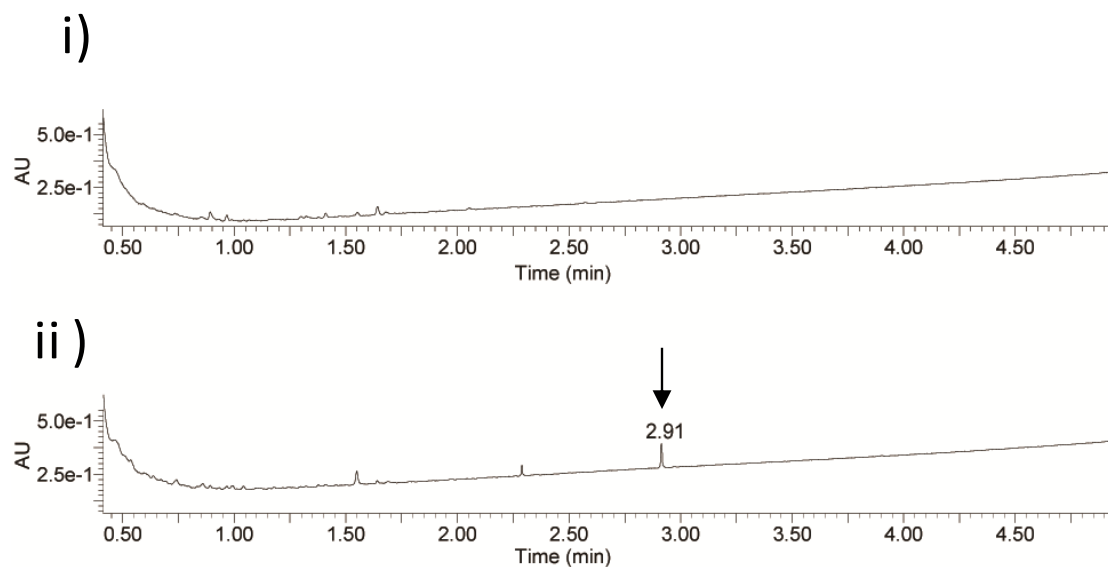


Figure S1. Detection of JBIR-156 (**1**). UPLC analysis of culture extracts from (i) *Streptomyces avermitilis* SUKA32 host strain as a negative control, and (ii) SUKA32 strain::pKU518F6K295_P1_P9-M23. The chromatogram was monitored at 220 nm. The peak of JBIR-156 (**1**) is indicated with a black arrow.

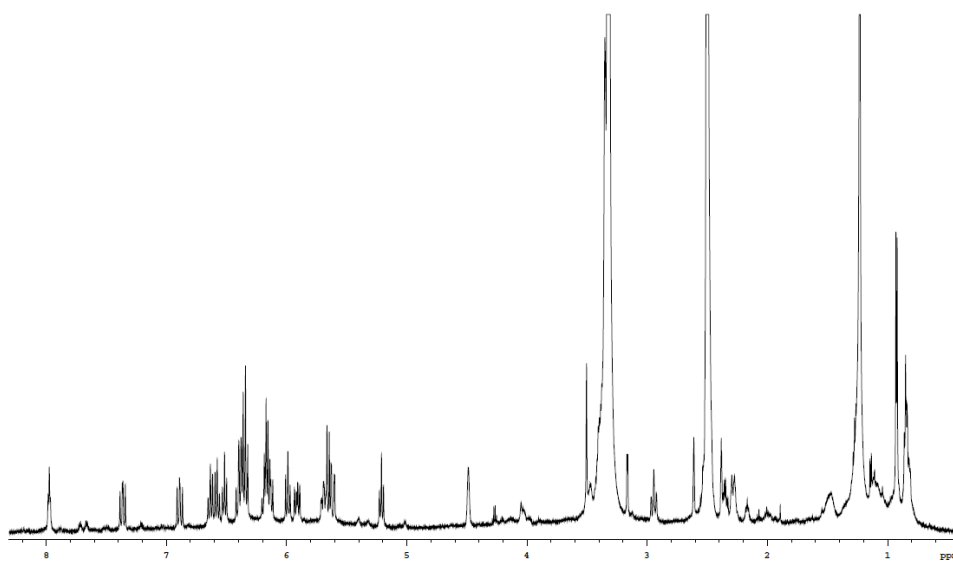


Figure S3. ^1H NMR spectrum of **1**.

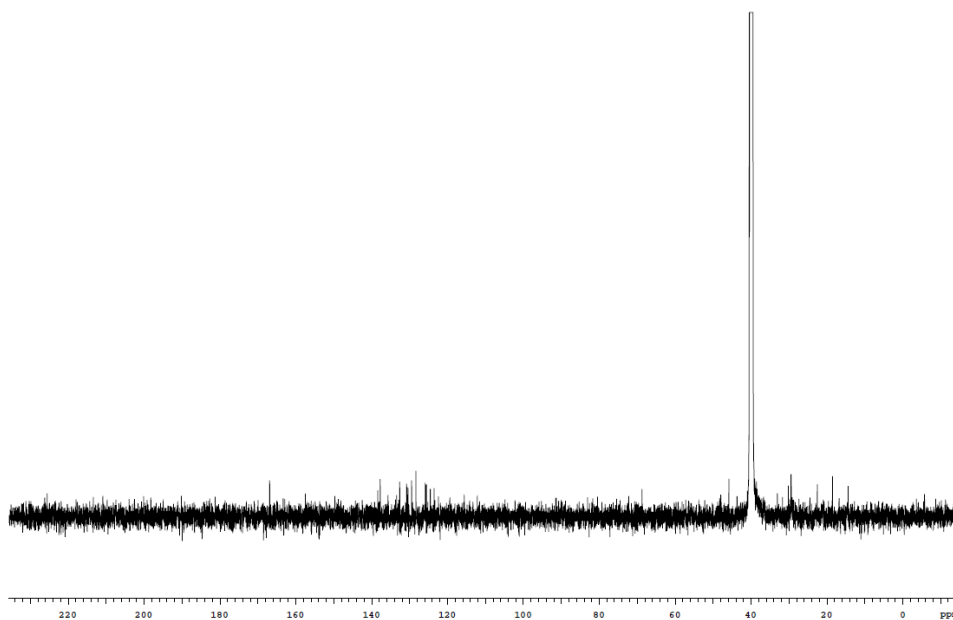


Figure S4. ^{13}C NMR spectrum of **1**.

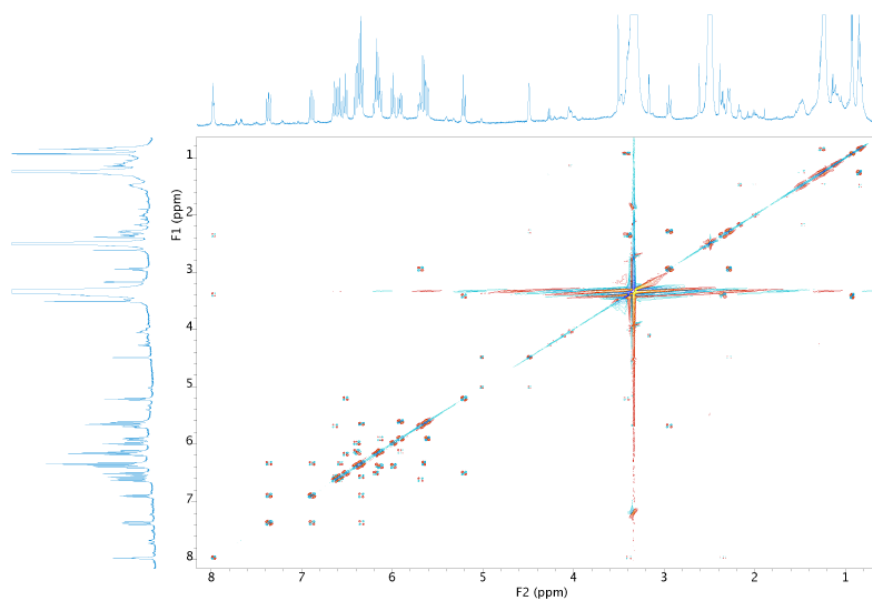


Figure S5. DQF-COSY spectrum of **1**.

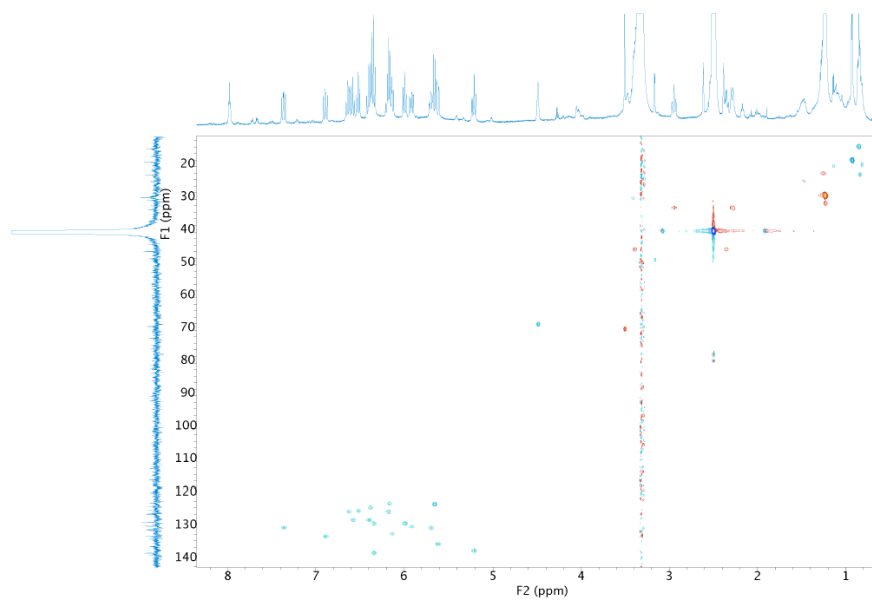


Figure S6. HSQCAD spectrum of **1**.

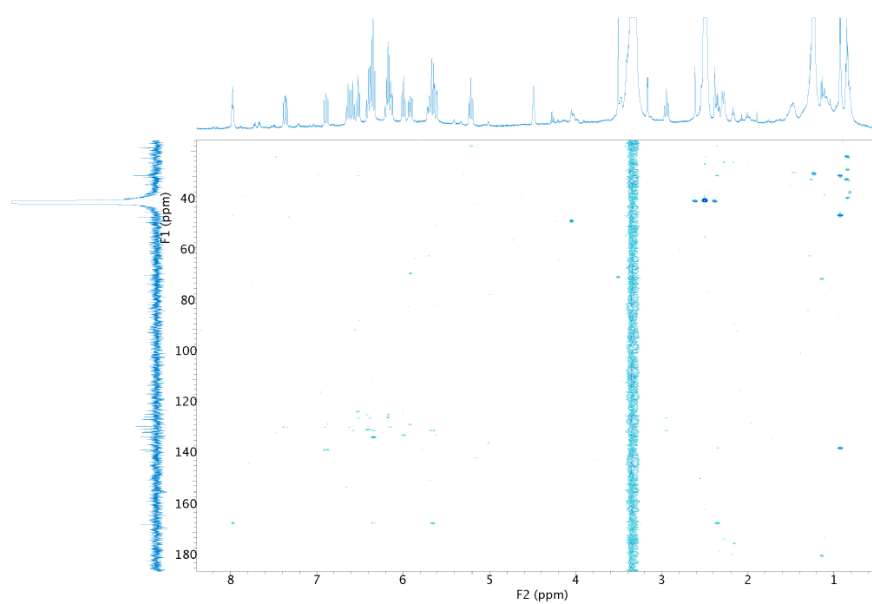


Figure S7. HMBCAD spectrum of **1**.

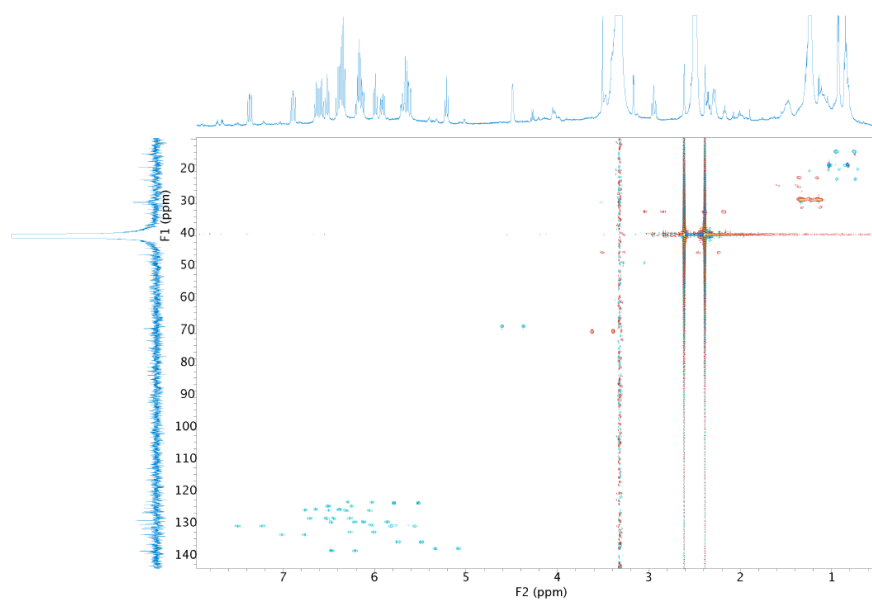


Figure S8. Coupled HSQCAD spectrum of **1**.