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Supporting Information for

Characterization of a Carboxyl Methyltransferase in *Fusarium graminearum* Provides Insights into the Biosynthesis of Fusarin A

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1. Experimental Procedures

1.1 General experimental procedures

The NMR experiments were run on a Bruker Avance III 500, ^{1}H (500 MHz) and ^{13}C NMR (100 MHz), J in Hz, δ in ppm. HRESIMS data were measured on an Agilent 6200 series Q-TOF LC-MS spectrometer with C18 column (4.5×50 mm, 2.7 μm, Agilent). High performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1260 HPLC system (Agilent Technologies Inc., USA) equipped with a DAD detector. Semi-preparative HPLC was performed on an Agilent 1200 system. HPLC electrospray ionization MS (HPLC-ESI-MS) was performed on a Thermo Fisher LTQ Fleet ESI-MS spectrometer (Thermo Fisher Scientific Inc., USA), and the data were analyzed using Thermo Xcalibur software. Column chromatography was performed with silica gel (200-300 mesh, Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Biochemicals and media were purchased from Sinopharm Chemical Reagent Co., Ltd. (China), Oxoid Ltd. (U.K.) or Sigma-Aldrich Co. LLC. (USA) unless otherwise stated. Enzymes were purchased from Takara Biotechnology Co. Ltd. (China). Restriction endonucleases were purchased from Thermo Fisher Scientific Co. Ltd. (USA). Chemical reagents were purchased from standard commercial sources.

1.2 Alignment biosynthetic gene clusters *fus* and *Fgfus* and their predicted functions.

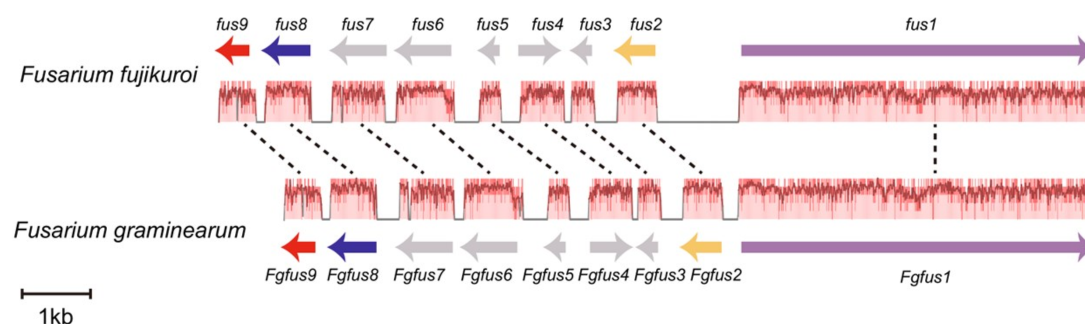

Figure S1 Alignment biosynthetic gene clusters *fus* and *Fgfus*. Biosynthetic gene cluster of *fus* from *Fusarium fujikuroi* and *Fgfus* from *F. graminearum*.

Table S1 The similarity of Fusarins biosynthetic genes between *Fusarium fujikuroi* and *F. graminearum* and the predicted functions.

Gene Name in *Fusarium fujikuroi*	Gene Name in *Fusarium graminearum*	Similarity	Predicted Function
fus1	*Fgfus1*	74.18%	Polyketide synthase/nonribosomal peptide synthetase
fus2	*Fgfus2*	87.35%	Related to a putative α/β hydrolase, probably involved in the 2-pyrrolidone ring formation
fus3	*Fgfus3*	73.18%	Subunit of elongation factor 1B
fus4	*Fgfus4*	74.53%	Peptidase
fus5	*Fgfus5*	73.28%	Glutathione *S*-transferase
fus6	*Fgfus6*	87.01%	Transporter
fus7	*Fgfus7*	70.59%	Aldehyde dehydrogenase
fus8	*Fgfus8*	82.85%	Cytochrome P450
fus9	*Fgfus9*	67.58%	Carboxyl methyltransferase

1.3 Generation and characterization of *Fgfus9* deletion mutant

To further investigate the biological function of the *Fgfus9* and its role in hyphae growth and pathogenesis, we generated three independent transgenic knockout strains (Δ*Fgfus9*-1, Δ*Fgfus9*-2, Δ*Fgfus9*-3) using homologous recombination-mediated knockout technology to replace the *Fgfus9* with neomycin resistance gene. Furthermore, we amplified a cassette consisting of the promoter and gene and terminator region of the *Fgfus9* and ligated it into a vector containing the hygromycin resistance cassette. The vectors were linearized and transferred into the Δ*Fgfus9* protoplasts and three independent transgenic complementary strains were obtained (Δ*Fgfus9C*-1, Δ*Fgfus9C*-2, Δ*Fgfus9C*-3, **Figure S2**).

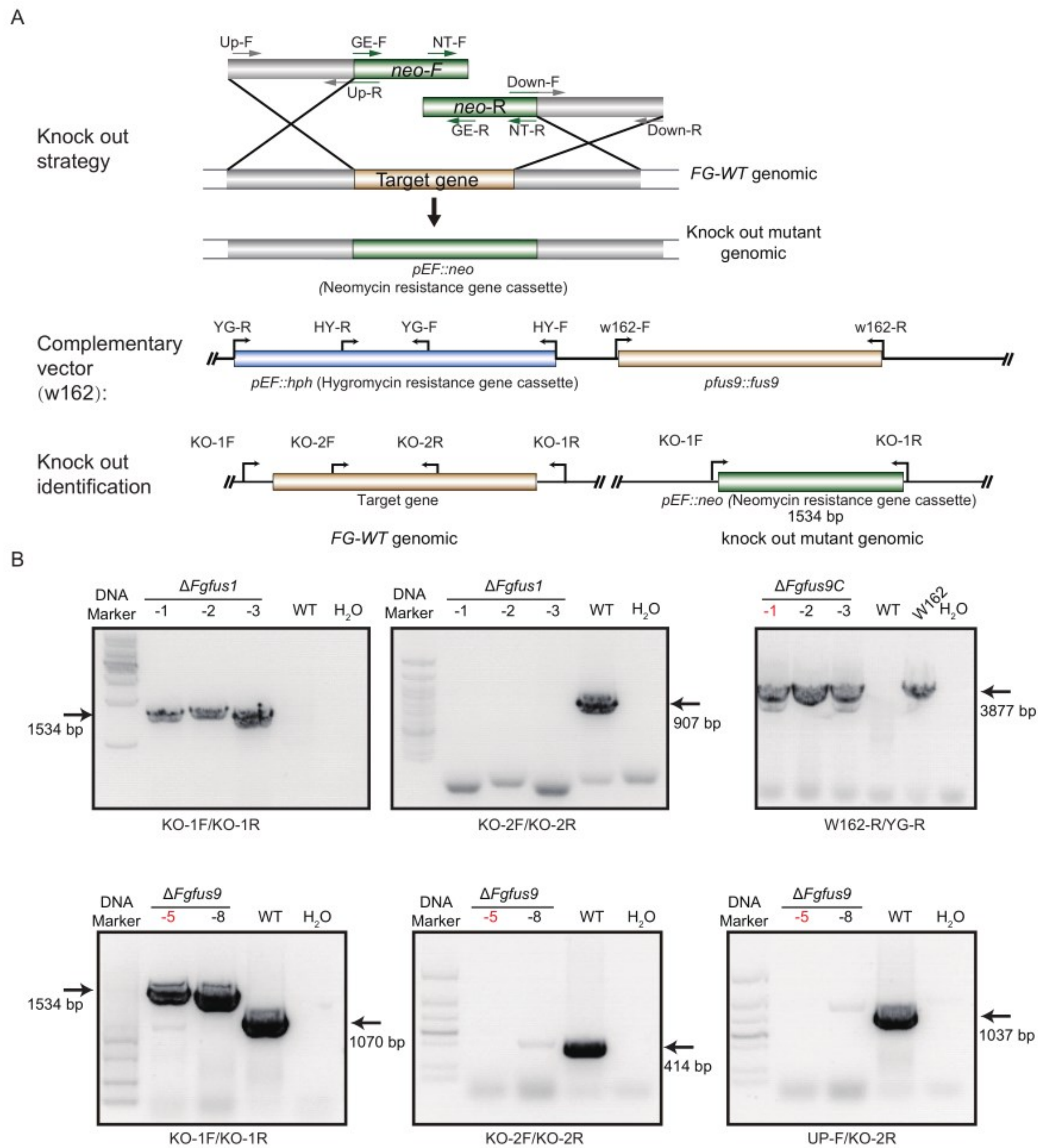

Figure S2 Construction of *F. graminearum* gene deletion mutants. **A.** Diagram of gene knock out strategy via homologous recombination. **B.** Verification of *Fgfus1* and *Fgfus9* deletion mutants by genomic DNA PCR experiment. The corresponding positions of primers are indicated in panel A. *WT*: wild-type strain. Δ*Fgfus9C*: *Fgfus9* complementary strain. The number marked with a dash represent independent transgenic lines of homologous recombination mutants. The line used for the construction of *Fgfus9* complementary strain are labeled with red.

1.4 Strains and culture conditions

The wild type strain *F. graminearum* PH-1 (NRRL 31084) was used as the parent strain for Δ*Fgfus9* transgenic construction. The gene knock out construction was mainly performed by Split-marker homologous recombination approach[1] with the following modifications. Primer UP-F/UP-R and DOWN-F/DOWN-R were used for *Fgfus9* upstream and downstream amplification, then transformed PCR products into protoplasts of wild type and screened using TB3 plate containing gromycin. Verified the positive transformants using genomic DNA PCR with primers internal (Primer IF and IR) and external (Primer U and D). All fungal mutant strains and primer sequences used in this study are listed in Supplementary Table S3. For *Fgfus9* complementation assay, a 2.3-kb region containing the gene and the promoter and the terminator region was amplified using the primer pairs (Supplementary TableS3) and then cloned into a vector with the hygromycin resistant cassette. The correct construct was transformed into protoplasts of FG-Δ*Fgfus9*. All strains of the *F. graminearum* were incubated at 25℃ in the dark. For the fungal growth assays, each construct with three independent lines were grown in a 9-cm Petri dish containing different medium. Photographed and measured the diameter of the fungal colony every day until reached the edge of the Petri dish.

1.5 Protein expression and purification

PCR amplifications were carried out on an Applied Biosystems Veriti™ Thermal Cycler using HS KOD DNA polymerase (Takara Biotechnology Co., Ltd. Japan) for high fidelity amplification. The synthesis of primers and genes were performed at Shanghai BioSune Biotech. The *c*DNA fragments containing target genes *Fgfus9* was amplified from genomic DNA of *F. graminearum*. While *Fgfus9* gene was cloned into pET28a(+) (linearized by NdeI and XhoI) for the expression of the recombinant Fgfus9 protein that is tagged by 6xHis at the *C*-terminus. The above derivatives of pET28a(+) and a pGro7 were introduced into *E. coli* BL-21(DE3). The culture of each resulting recombinant *E. coli* strain was incubated in Luria-Bertani (LB) medium (5 g of yeast extract, 10 g of tryptone and 10 g of NaCl per liter) containing 50 μg/mL kanamycin at 37°C and 220 rpm until the cell density reached 0.4-0.6 at OD600. Protein expression was induced by the addition of L-arabinose to a final concentration of 0.5 g/L. Then, the *E. coli* strain was incubated until the cell density reached 0.6-0.8 at OD600 and was cooled to 16 °C and induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG), continued to cultivate at 16°C (220 rpm) for 20 hours. *E. coli* cells were re-suspended in lysis buffer (10% 20xPBS, 10% glycerol and 10 mM imidazole, pH 7.5). After disruption by FB-110X Low Temperature Ultra-Pressure Continuous Flow Cell Disrupter (Shanghai Litu Mechanical Equipment Engineering Co., Ltd, China), soluble fractions were collected by centrifugation. The protein that contains a 6xHis-tag was purified on a HisTrap HP column (GE Healthcare, USA), which was pre-treated with 10 column volumes (CVs) of lysis buffer followed by 10 CVs of wash buffer (10% 20xPBS, 10% glycerol and 40 mM imidazole, pH 7.5), using elution buffer (10% 20xPBS, 10% glycerol and 250 mM imidazole, pH 7.5). Desired protein fractions were concentrated (to 500 μM^{-1} mM) using Amicon® Ultra-15 Centrifugal Filter Devices (MILLIPORE, USA) and desalted using a PD-10 Desalting Column (GE Healthcare, USA) according to the manufacturer's protocols, and then quantified in concentration by Bradford assay

using bovine serum albumin as the standard. Finally, the resulting Fgfus9 protein was concentrated and stored at -80 °C for *in vitro* assays.

1.6 *In vitro* assay of FgFus9 activity.

The conversion was conducted at 30°C for 2 hr in 50 μL of the reaction mixture that contained 200 μM **2**, 30 μM FgFus9, 200 μM SAM, 50 μM Hepes (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), add dd H_2O to 50 μL. Conversions were quenched as described above, and after centrifugation, reaction mixtures were subjected to HPLC-HR-MS and HR-MS/MS analyses. The HPLC was conducted on a reversed-phase Agilent ZORBAX column (300SB-C18, 4.6 mm × 100 mm, Agilent Technologies Inc., USA) by gradient elution of solvent A (ddH_2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) with a flow rate of 1 mL/min over a 40 min period as follows: T = 0 min, 20% B; T = 25 min, 80% B; T = 30 min, 100% B; T = 35 min, 100% B; T = 37 min, 20% B; T = 40 min, 20% B.

1.7 Wheat coleoptiles infection assay.

For pathogenicity evaluation, wheat (*Triticum aestivum*) cultivars Zhongyuan 98–68 was used for coleoptile infection. The wheat coleoptiles infection was mainly performed according the assay we reported previously. Briefly, picked plump seeds and rinsed the wheat seeds under running water overnight. Transferred the imbibed wheat seeds into a plate and cultivated at 25℃. Cut off the top of the wheat seedlings and drop 2 μL spore suspension (concentration: 10^6 per mL) on the wound at the 3th day. Incubated the inoculated seedlings in growth chamber at 25 °C with 95% relative humidity. Lesion sizes were photographed and measured at 7 dpi.

1.8 Phylogenetic analysis of protein sequences.

The processes of phylogenetic analysis of Fgfus9 and other well-documented methyltransferases was run on MEGA7,[2] and the results indicated that FgFus9 probably is a new carboxyl methyltransferase.

Table S2 The protein sequences well documented methyltransferase for phylogenetic analysis.

Category/Name	protein sequences
	***N*-methyltransferase**
EryCVI	MYEGAFAELYDRFYRGRGKDYASEAAQVARLVRDRRPSASSLLDVACGT
	GAHLRRFADVFDDVTGLELSAAMIELARPQLDGIPVLQGDMRGFALDREF
	DAVTCMFSSIGHMRDSAELERALASFAHHLAPGGVVVVEPWWFPEDFLD
	GYVAGDVVRDGDLTISRVSHSVRAGDTTRMEIHWVVADPVNGPRHQVE
	HYEITLFERQQYEKAFIAAGCSVEYLADGPSGRGLFIGVRR
RavNMT	MSTLSVSQPGTPPTPADVEQAYGAEHADVYDAIYRGRGRDYAAEADAFL
	AVLRTRRPEITSLLDVACGTGAHLAALRGSVDHVEGVELSPWMHTLATR
	QLPGVAVHQDDMRSFNLSRRFSAVTCLFSSVGYLKSVEDLRRTLRTFRAH
	VAPGGVALVEPWWFPETFLDGYVGSSLVEVDGRTIARVSHTVRAGSTSR
	MTVEYTVAEPVNGVRRFSDLHVLSLFTREQYEDAFERAGFAVEFLKDGPS
	GRGLFLGVAPETTDGGPR
Veg24	MPARILACMTDQMARAAVRTTRADVLLASAGERGVLCDFYSETAADIYQ
	DIMGDGDKDGSPEAGEYATHIRPESGPVLELAAGTGRLTFPLLDLGLEVT
	ALELSTAMLGALQKRLAEAPVEIQDRCTVVHGDMSAFALDTRFGTVMISS
	GSINVLDDADRPGVYASVREHLAPGGRFLLGVAVSEAVGPDEVERRQELP
	GRSGRRYVLHARRSPAEEIHEITIYPADEAADPFLVCTNRVRLLKEDQVVR
	ELEQAGFDVIARTPFASQGAEGVLLLEAVVRG
Veg25	MSDQLAQDAVRTTRADVLLASAGERGILCDYYSETAAEIYRDIMDDEGE
	DPAASSEAEVFASHIPAGSAPVLELAAGAGRLTFPLLDLGLEVTALELSAK
	MVATLRTRLAEAPAEVQARCTAVVGDMADFAFDEQFRAVIISPGTIAVLD
	DAGRAGLYASVRKHLAPGGQFLVGVGQQNPSKAEPLERFQDLTGESGRK
	YMLHHERWFPGDEEVHDITIYPADEAADPFLICTHRMRIPNMDRIVRECEA
	AGFDVTVQPFAGKLPEDVLLLKASLRGDS
MtfA	XGSSHHHHHHSGGLVPRGSXSNQLERGPVRTPHADVLLASVGERGVLCD
	FYDEGAADTYRDLIQDADGTSEAREFATRTGPVSGPVLELAAGXGRLTFP
	FLDLGWEVTALELSTSVLAAFRKRLAEAPADVRDRCTLVQGDXSAFALD
	KRFGTVVISSGSINELDEADRRGLYASVREHLEPGGKFLLSLAXSEAAESE
	PLERKQELPGRSGRRYVLHVRHLPAEEIQEITIHPADETTDPFVVCTHRRRL
	LAPDQVVRELVRSGFDVIAQTPFASGGAGRKDXVLVEAVXPGATADAR
H074_31692	MTNVDLEVVLMTDQLERGPARTAHAGVVLASAGDRGVLCDFYDEAGSD
	TYRDLIQDADGTLETREFAARVPQDPGPVLELAAGMGRLTFPFLELGWEM
	TALELSATVVTAFRKRLAESPPELRERCTVVQGDMSAFALGRRFGAVVISS
	GSINELGEADRKGLYAAVLEHLRPGGKFLLSLELTKPVEAEPVERSQELSG
	RSGRRHVLHVKILPSEELQEITIYPADETADPFVVCTHRRRLFTVDRIAREL
	GQAGFDVVAREPFASTGAGRDDMVLVEAIPREVTADAK
Bn4651_P2188	MEADAGGTEEAHGFAALVRPESGAILELGAGTGRLTIPLLELGWEVTALE
	LSTAMLTTLRTRLADAPADLRDRCTLVHADMTAFKLGERFGTAILSPSTID
	LLDDADRPGLYSSVREHLRPGGRFLLGMANPDASGRQEPLERTQEFTGRS
	GRRYVLHAKVYPSEEIRDVTIHPADESADPFVICVNRFRVITPDQIARELEQ
	AGFDVVARTPLPGVRNHELVLEAQWGSVEDAH
	***C*-methyltransferase**

ChryCMT	MSDLPNCRICGGALREFLDLGEQPMGNAFVTPGRQDDEAGAFRFRLTVA
	VCEVCTMVQLTDYVRHDRMFHAAYPYRSSQSSVMSAHFENLARRFLQDE
	LNGTDPFIVELGSNDGIMLRTVAKAGVRHLGVEPCGDVAEVGRANGVRV
	VSEFFDEDLAARIRAADGTADVVFAANTFSHIDFLDSILRGVKNLLAPGGV
	FVFEDPYFGDIVQRASFDQIYDEHIFFFTARSVQDMAARHGLELVDVELLP
	VHGGELRCTLAHTGARPVSPAVPRLLRQEEEQGLTETATLTRFADGVREL
	GETLVTRLRELRDQGKRVVGYGATAKSTTLLNYCGIGPDLVSFVCDSTAE
	KQGRLTPGTHIPVRSSEAFADPYPDYALLLAWNHKDEILDKESAFREAGG
	RWIHYVPDVHID
AviG1	MSTTGHSTVIDRCRICDNTELLPVLDLGPQALTGVFPRTRGEDVPYVPLEL
	VRCSPGGCELVQLRHTADFGLMYGEGYGYRSSLNRSMADHLRGKVAAIT
	GLVDLGPGDLVVDIGSNDGTLLAAYPADGPRLVGVDPAATVFAASYPPG
	VELIPDFFAYDLLGGRRAKVVTSIAMFYDLPRPMEFMREVGRLLTDDGIW
	VTEQSYLPAMLHACAYDVVCHEHLDYYGLRQIEWMAERTGLKVVDAEL
	TPVYGGSLSLVLARRGSSRQVNEPALARIRAGETDLPYAEFARRTEESRDR
	LLEFLTASRDKGLHTLGYGASTKGNVILQYCGLDETLLPCIAEVNEDKFGC
	YTPGTNIPIVSEEEARALEPDQFLVLPWIYRDAMVARERDFLASGGSLVFP
	LPTLEVV
Sam34	MSNNSRCRVCGGTVREFFDFGRQPLSDAFAPPDADFTQEFFFRLATGVCE
	GCTMVQLMEEVPRDRMFHEDYPYHSSGSSVMREHFEQLAKRFLATELTG
	DDPFIVEIGCNDGIMLKAVAEAGVRHLGVEPSGGVAALAATKGIQVRNAF
	FEQGTAGEIVTEQGRADVIYAANTLCHIPYMESILTGVTALLKPTGVFVFE
	DPYLGDIVERTSFDQIYDEHFFLFSAQSVQAMASRFGLELVDVERLAVHG
	GEVRYTLAPAGRRTPTPEVAALVAQERERGIADPATLEAFGARVLKIRDD
	LVALLEKLRAEGRRVVAYGATAKSATVTNLCGIGPELVEFVCDSTPAKQG
	RVTPGTHIPVRESAAFAQPYPDYALLFAWNHAEEIMAKEQEFRDRGGKWI
	LYVPDVRIV
VEG17	MSTTTQCRICDGTVREFIDFGRQPLSDAFVVPGDTREEFFYHLATGICDTC
	TMVQLMEEIPRDRMFHEEYPYLSSGSAYMRTHFEELAKRFLATELTGEDA
	FIVELGSNDGIMLKAVAEAGVRHLGVDPSGVADLAAAKGIRVRKDFFEEA
	TAADIRANERPADVIYAANTLCHIPYMDSILKGVNALLGPNGVFVFEDPYL
	GDIVERTSFDQIYDEHFFFFTARSVQEMARRHGLELVDVERIPVHGGEVRY
	TLALAGARTPTNAVAELLAWEAERQLSEYATLERFAVKVKKNKEDLIAL
	LTKLRDEGKRVVGYGATAKGATVNNFCGVTPDLVEFISDTTPAKQGKLSP
	GQHIPVHPHKKFADNYPDYALLFAWNHADEIMNAEQAFRDAGGKWILY
	VPDVHVI
KijD1	GHMTGPTDATAPARCRVCGDTVDEFLDLGRQPLSDRFLTPADTDGEFFYR
	LAVGRCHACGMVQLTEEVPRHLMFHEEYPYHSSGSSVMREHFAKVAQRL
	LATELTGADPFVVEIGCNDGIMLRAVHEAGVRHLGFEPSAGVAEVARSRG
	VRVRTEFFEKATATAVRESEGPADVIYAANTMCHIPYLESVFQGADALLG
	PDGVVVFEDPYLGDIVAKTSFDQIYDEHFYLFSAGSVAAMAERFGFELVD
	VERLPVHGGEVRYTLARRGARTPTEAVGRLLAEEREQGLDDLATLRTFA
	ANVHTVRDELVALLTRLRAEGHRVVGYGATAKSATVTNFCGIGPDLVSF
	VCDTTPGKQHRLTPGKHLPVRPAEAFADPYPDYALLFAWNHADEIMAKE

	QEFRQAGGRWILYYVPEVRVL
TcaB9	GHMSHLADVSPPTACRVCGGGVQEFLDLGRQPLSDRFRKPDELDDEFTYR LAVGRCDSCEMVQLTEEVPRDLMFHEVYPYHSSGSSVMREHFAMLARDF LATELTGPDPFIVEIGCNDGIMLRTIQEAGVRHLGFEPSSGVAAKAREKGIR VRTDFFEKATADDVRRTEGPANVIYAANTLCHIPYVQSVLEGVDALLAPD GVFVFEDPYLGDIVAKTSFDQIYDQHFFLFSATSVQGMAQRCGFELVDVQ RLPVHGGEVRYTLARQGSRTPSAAVAQLLAAEREQELSDMATLRAFAGN VVKIRDELTALLHRLRAEGRSVVGYGATAKSATVTNFCGIGPDLVHSVYD TTPDKQNRLTPGAHIPVRPASAFSDPYPDYALLFAWNHAEEIMAKEQEFH QAGGRWILYVPEVHIR
	***O*-methyltransferase**
BmbA	MKISLTGAAETLLAPLCARALDARSAHPLLGDHIAAELIDRIDYDFGRLGM GEATAVGVALRARYFDRRVRAFLQAHPECTVVHLGCGLDSRFERLAPGA GVRWFDLDQPDVIELRKRLYPARPGHETLAASVTEPDWPAQVPTDRPVL VVAEGLSMYLSAEEGPRMLRTLVARFPHGELLFDTYSRFAVRSTRSLSLFR RTGARLAWGVDDPRELEREIPGLRLIEADSAYATAAGVDVRHLPRRLRLR MRIDTLLLSRLPVLRGIGHLSRFAFGGSG
BstB	MKVPLTGAAATLLAPLYARALDASSPHPMLADSTAPELVQKIEYDFGALG MRPSNAVGVALRARFFDRRTREFLTAHPNSTVLHLGCGLDNRVDRVERG PGVRWFDVDQPEVIEVREQLFSPRSDHRTIAASVTETDWLAEVPTDLPVL VIAEGLTMYLAADEGPRMLRALVEHFSEGEAVFDTYARYAVRSTRSMAL FRKTGARLAWGVDDPRELEREVPGLRLVKSVKAYHTADPADFRRLPFAL RTRMRVDAEVLARLPVLGRVGHLSRYAFG
BotOMT	MKISLTGAAETLLAPLYARALDARSAHPLLGDHIAAELLDRIDYDFDRLG MGEASAVGVALRARYFDRRVRAFLDAHPESTVVHLGCGLDSRFERLAPG PGVRWFDLDQPDVIELRKQLYPARPGHETLATSVTEPDWPAQVPTDRPVL VVAEGLSMYLSADEGPRMLRTLVAHFPQGELLFDTYSRFAVRSTRSLALF RKTGARLAWGVNDPRELERGIPGLRLIETDSAYATAAGVDVSHLPRTLRR RMRIDTHILARLPVLRHIGHISRYAFGPPA
bsmH	MMSRALENAVYGLISTPVLHTAVKHGVFASLIEDGPDTAEGLAGRLGTDQ DTLERMLLVLTSLGVVERAAGGEYSPAPDAVPFLDRRNSRYLGGFVEHLT LETQGRLGRLDTYLAEGKPDTEASPFDDVYRDADSLRAFMRAMWDLSFG MSQELAALAGMAGTKRLVDVGGATGPFAVAALLTEPTLSATVFDMPAV GHLVGEDADARPVADRLDFVGGDFFADELPHGDCLAFGYILSDWDDDTC VTLLEKAYRACEAPGRVLIMDRLFDDDRSGPLATAAMNLVMHIEMAGRH RTAAEFVALLERAGFGGCEVRRGSGEKHLVIGHKK
HrbU	MHPGPIMGLMAGYWQVRMLLAALEHDIFTELSKGPATSGEVADRMGLV ELGTHDLLTGLGHLGLLQVADGRFANSPLADRFLVRGGPEFLGGYLRFCE QELNPAWDGLATSLRTGRPTNRAAVVGNPYDTLYSDPDATDGFLDSMDL LSTPIGLAISRYDWSQYSSFIDVGGARGNFAHQVVTENPHLTAGVFDLPPL EGTFKRHIERLGATASGISFHGGDFFKDKLPKADVIVLGHVLHNWGVEDR VQLLKNVYDAVRPGGAVFVYDPMAGGEQPSLHAVLAGLAMLVWSRGG HEYSVEELHGWLREAGFWPETAEVPGLHEDVLVIGHKDA
	Carboxyl-methyltransferase

FgFus9	MIKALPLFQKAAKVVADHDGDHTAIIEYGSAHGNNSLEPIQAILKATPSRQ VELLFSDRPENDFSTLSTTITSWADTLDKTEFPHALFLSMIPRNFYQKVVPL KSAHLGFSLAALHHLDHIPPPTGVQSEDDQLLKKQAHLDLSTFLELRAQEI ISGGSLVLSFVGQASAGYENYSGPVDACRNAMIEMVQQGIIPVSVAAAFR VPTYNRTLDDVRKILGEMSNLWNVHDLFENDITHPAIHDLKKKQVEGED ASQEYANVVIDWMMAVCSGYFLKALKVGNGGRYSDEEEERLLGDWVA KTKALFIRDHKDEEVVCSFLYLHLGRV
LCM	MKNLTTIKQTNKNVKQERRKKYADLAIQGTNNSSIASKRSVELLYLPKLS SANNFQMDKNNKLLEYFKFFVPKKIKRSPCINRGYWLRLFAIRSRLNSIIEQ TPQDKKIVVVNLGCGYDPLPFQLLDTNNIQSQQYHDRVSFIDIDYSDLLKI KIELIKTIPELSKIIGLSEDKDYVDDSNVDFLTTPKYLARPCDLNDSKMFST LLNECQLYDPNVVKVFVAEVSLAYMKPERSDSIIEATSKMENSHFIILEQLI PKGPFEPFSKQMLAHFKRNDSPLQSVLKYNTIESQVQRFNKLGFAYVNVG DMFQLWESADEATKKELLKVEPFDELEEFHLFCHHYVLCHATNYKEFAF TQGFLFDRSISEINLTVDEDYQLLECECPINRKFGDVDVAGNDVFYMGGS NPYRVNEILQLSIHYDKIDMKNIEVSSSEVPVARMCHTFTTISRNNQLLLIG GRKAPHQGLSDNWIFDMKTREWSMIKSLSHTR FRHSACSLPDGNVLILGGVTEGPAMLLYNVTEEIFKDVTPKDEFFQNSLVS AGLEFDPVSKQGIILGGGFMDQTTVSDKAIIFKYDAENATEPITVIKKLQHP LFQRYGSQIKYITPRKLLIVGGTSPSGLFDRTNSIISLDPLSETLTSIPISRRIW EDHSLMLAGFSLVSTSMGTIHIIGGGATCYGFGSVTNVGLKLIAIAK
Q63KM8	MSNQSIGAPDSTAVRVALWRALHARIDAPPHVLDDEIGLALAAPDDDWR SRPDMDPQASRGYRASIVGRARFVEDLVDEQADRGVAQYVVLGAGLDTF AQRRAKLASHLRVFEIDQPGTQAWKRQRLIALGYGVPQWLRLVPVDFET SGAWRAQLSAAGFDDSRPAVVSSTGVSMYLTREAIAGTLSQIATLAPGST LAMTFLLPLELIDDPAERAQHAAVYERARAAGTPFVSFFSPSDMLALARE AGFREARHVSTDDLVRRYFESRPDGLRPASGEAFLVAST
1RJE	MERIIQQTDYDALSCKLAAISVGYLPSSGLQRLSVDLSKKYTEWHRSYLIT LKKFSRRAFGKVDKAMRSSFPVMNYGTYLRTVGIDAAILEFLVANEKVQ VVNLGCGSDLRMLPLLQMFPHLAYVDIDYNESVELKNSILRESEILRISLGL SKEDTAKSPFLIDQGRYKLAACDLNDITETTRLLDVCTKREIPTIVISECLLC YMHNNESQLLINTIMSKFSHGLWISYDPIGGSQPNDRFGAIMQSNLKESRN LEMPTLMTYNSKEKYASRWSAAPNVIVNDMWEIFNAQIPESERKRLRSLQ FLDELEELKVMQTHYILMKAQWHHHHHH
3IEI	MATRQRESSITSCCSTSSMDENDEGVRGTCEDASLCKRFAVSIGYWHDPYI QHFVRLSKERKAPEINRGYFARVHGVSQLIKAFLRKTECHCQIVNLGAGM DTTFWRLKDEDLLSSKYFEVDFPMIVTRKLHSIKCKPPLSSPILELHSEDTL QMDGHILDSKRYAVIGADLRDLSELEEKLKKCNMNTQLPTLLIAECVLVY MTPEQSANLLKWAANSFERAMFINYEQVNMGDRFGQIMIENLRRRQCDL AGVETCKSLESQKERLLSNGWETASAVDMMELYNRLPRAEVSRIESLEFL DEMELLEQLMRHYCLCWATKGGNELGLKEITY

Table S3. Primers used in this study. Small letters indicate the homologous sequences.

Gene ID	Primer number	Primer type	Primer sequence	Relevant characteristics
pks10 (Fgfus1)	P1	UP-F	GCAACACTGGCTTTTGACGA	mutant contstruction
	P2	UP-R	TAGCCACGATTCGAAGCCGCGGGAAAGATTGACTCGACAT	
	P3	DOWN-F	GAAATGATAGCATTGAAGGATGAGACTAATCCATTCGAACGGGTAGTTCATAG	
	P4	DOWN-R	TTGCAACAGAAAGAACAGGA	
	P5	KO-1F	AGTGTGGACTATCTTCAACATCTTC	
	P6	KO-1R	GGGAGGCTGAAGTGGACAAG	
	P7	KO-2F	CTATGCGCGAGGAGAGGGTGTAGCA	
	P8	KO-2R	TAACTACATCCTCAACTATGGCACT	
Fgfus9	P9	UP-F	GAAGCTGGTTGAAGAAGTTG	mutant contstruction
	P10	UP-R	TAGCCACGATTCGAAGCCGCTCATCGCTTCGTGCTGTAGT	
	P11	DOWN-F	GAAATGATAGCATTGAAGGATGAGACTAATCCAAGTTGGCGGTGTTTTAGTGA	
	P12	DOWN-R	TTTGAAACCAGTGTCCCGGA	
	P13	KO-1F	CACTACAGCACGAAGCGATG	
	P14	KO-1R	GACGTCACTAAAACACCGCC	
	P15	KO-2F	CCTGAAAGCAACTCCTTCCCG	
	P16	KO-2R	GCGTCGACAGGTCCACTGTAG	
hph	P17	HY-F	GCGGCTTCGAATCGTGGCTA	mutant contstruction
	P18	HY-R	GTATTGACCGATTCCTTGCGGTCCGAA	
	P19	YG-F	GATGTAGGAGGGCGTGGATATGTCCT	
	P20	YG-R	TCATACTCTTCCTTTTTCAATTCAATGCG	
neo	P21	GE-F	GCGGCTTCGAATCGTGGCTA	
	P22	GE-R	CACGACGAGATCCTCGCCGTCG	
	P23	NT-F	TGCTCGACGTTGTCACTGAAGCGG	
	P24	NT-R	TGGATTAGTCTCATCCTTCAATGCTATCATTTC	
w162	P25	F	GCCACGATTCGAAGCCGCGAAGCTTAGAAGCTGGTTGAAGAAGTT	plasmid construction
	P26	R	CAGCTATGACCATGATTACGAATTCTGTTGGTTTGTTTGTGAAAG	

Table S4 Strains, plasmids, and primers used in protein express assay.

Strains/Plasmids	**Description**	**Source /Reference**
Strains		
Escherichia coli		
DH5*α*	Host for general cloning	Transgen[3]
BL21(DE3)	Host for protein expression	Novagen
Plasmids		
pET-28a(+)	Protein expression vector used in *E. coli*, encoding *N*-terminal 6xHis-tag, kanamycin resistance	Novagen
pGro7	*E. coli* vector; chaperone expression plasmid	Novagen
primer	**Sequence (The sequences of restriction enzymes are underlined)**	**Enzyme sites**
Fgfus9-For	tgccgcgcggcagccatatgatcaaggccctacctct	NedI
Fgfus9-Rev	tggtggtggtggtgctcgagctaaaccctacccaaatgaa	XhoI

1.9 Bioassays for antibacterial activities and cytotoxic

To explore whether fusarins exhibit anti-infective activity, fusarin A (**1**) and neofusarin (**2**) were evaluated for their activities against a panel of oral bacterial pathogens, including Gram-positive cariogenic bacteria (*Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Enterococcus faecalis*) and Gram-negative periodontal bacteria (*Fusobacterium nucleatum*, *Porphyromonas gingivalis*). Consequently, none of them were found to be potent.

The double dilution method[4] was used to determine the minimum inhibitory concentration (MIC) of fusarin A (**1**) and neofusarin (**2**). Brain heart infusion broth (100 μL) was added to each well in a 96-well plate and 100 μL of sample was added into the first column of the plate. Stepwise double diluted sample from the first column to the penultimate column served as the experimental group, whereas the last column served as the blank control. 10 μL of bacteria (concentration: ~3 x 10^6 CFU/mL) was added into each well of the plate. The gram-positive bacteria were incubated at 37 °C for 24 h and the gram-negative bacteria for 48 h in an anaerobic incubator. The MICs were recorded as the minimal concentration of the drug that cannot cause turbidity of the bacterial suspensions using a microplate reader (Winooski, VT 05404-0998) at 600 nm wavelength.

Table S5 Anti-Oral-Bacteria Activity.

Oral Bacteria Strains	Minimum Inhibitory Concentration (MIC) (μg/mL)	
	Fusarin A (**1**)	Neofusarin (**2**)
Streptococcus mutans UA159	>256	>256
Lactobacillus acidophilus ATCC 4356	>256	>256
Actinomyces viscosus ATCC 19246	256	>256
Enterococcus faecalis ATCC 29212	>256	>256
Fusobacterium nucleatum ATCC 25286	>256	>256
Porphyromonas gingivalis ATCC 33277	128	>256

In addition, PANC-1, A375, U87, and MHCC-97H human tumor cell lines were used in the cytotoxic assay. cells were seeded into each well of a 96-well cell culture plate. After 12 h of incubation at 37°C, the test compound (0-100 μM) was added. After incubating for 72 h, cells were subjected to the Cell Counting Kit-8 (CCK-8).[5, 6] The IC_{50} value was calculated with Reed and Muench’s method.

1.10 Extraction and purification of compounds 1-2.

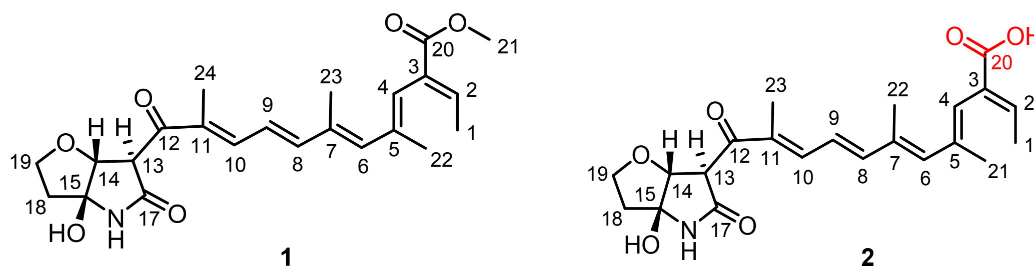

Figure S3 Chemical structures of **1-2**.

A total of 10 L *F. graminearum* fermentation broth was subjected to compound isolation according to the method described. The culture medium was extracted with EtOAc, after each extraction the solvent was evaporated. The EtOAc extraction (20.0 g) was eluted by using the column chromatography on silica gel with a CH_2Cl_2 /Me_2CO gradient system (1:0, 9:1, 8:2, 7:3, 6:4) to A-E fractions. Among them, B fraction was chromatographed on a silica gel column with a petroleum ether/Me_2CO gradient system (from 100:1 to 0:1) to afford fractions B1-B5. Fraction B4 was purified by semipreparative HPLC (3 mL/min, detector UV λ_{max} = 330 nm, $CNCH_3/H_2O$ 40:60) to yield **1** (3.0 mg, retention time = 38.2 min). Next, a total of 20 L Δ*Fgfus9* strain was subjected to YEPD cultures, 7d, 25 ℃, 180 r/min, and avoid lights. The fermentation broth was extracted with EtOAc, then the solvent was evaporated in vacuo. The extraction was also subjected to column chromatography on silica gel with a CH_2Cl_2/Me_2CO gradient system (1:0, 9:1, 8:2, 7:3, 6:4) to A-E fractions. The C fraction was purified by repeats semipreparative HPLC (3 mL/min, detector UV λ_{max} = 330 nm, $CNCH_3/H_2O$ 40:60) to yield **2** (1.0 mg, retention time = 29.5 min).

Fusarin A (**1**), was obtained as a as a yellow gum. It had the molecular formula $C_{23}H_{29}NO_6$ as deduced by the sodium (+)-HRESIMS ion at *m/z* 416.2068 $[M + H]^+$ (calcd 416.2068), indicating ten degrees of unsaturation. According to the literature reported,[7] compound **1** was determined as fusarin A.

Neofusarin (**2**), was obtained as a yellow gum. It had the molecular formula $C_{22}H_{27}NO_6$ as deduced by the sodium (+)-HRESIMS ion at *m/z* 402.1915 $[M + H]^+$ (calcd 402.1911), indicating ten degrees of unsaturation. The ^1H NMR exhibited characteristic signals for six olefinic protons at δ_H 6.94 (m, H-2), 5.97 (s, H-4), 6.24 (s, H-6), 7.51 (d, 15.2, H-8), 6.78 (dd, 15.2, 11.1, H-9), and 7.47 (d, 11.1, H-10), four methyl groups at δ_H 1.80 (d, 7.5, H_3-1), 1.70 (s, H_3-21), 2.40 (s, H_3-22), and 1.97 (s, H_3-23). Its ^{13}C NMR spectra disclosed 22 carbon resonances, including obviously one carbonyl, two ester carbonyl, and four methyls. Elaborate analysis of the 2D NMR data of neofusarin suggested its structure to be highly similar to **1**, except that the C-20 ester group in **1** was replaced with a carboxyl in **2**. In other words, the C-21 methyl was disappeared. Thus, the structure of **2** was unambiguously determined. The neofusarin (**2**) is the first intermediate that possessing furan conjugated pyrrolidone ring in *N*-terminal and carboxyl at C-20.

2. Supplementary Figures

2.1 UV absorption of compounds 1-2.

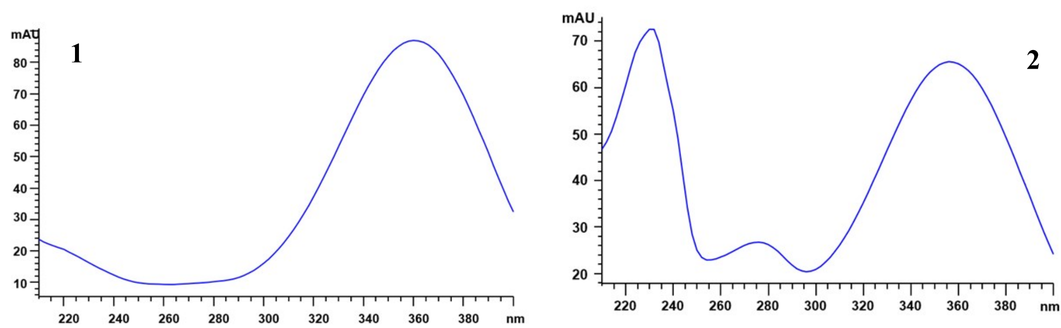

Figure S4 The UV absorption of compounds **1-2**.

2.2 HRESIMS and NMR spectra of 1-2.

Qualitative Analysis Report

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Sample Type	Sample	**Position**	Vial 25
Instrument Name	Instrument 1	**User Name**	
Acq Method	yqDD-65.m	**Acquired Time**	2021/3/24 1:51:26 PM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group **Info.**
Acquisition SW 6200 series TOF/6500 series
Version Q-TOF B.05.01 (B5125.3)

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
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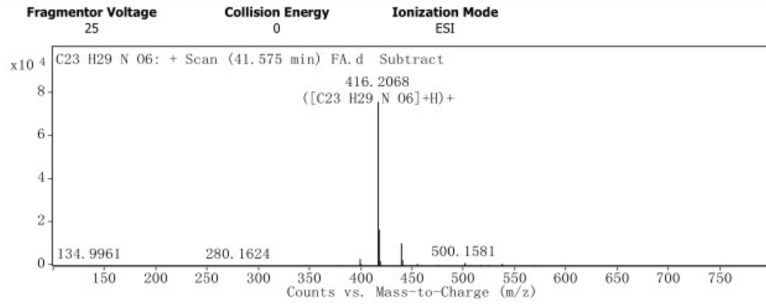


Peak List

m/z	z	Abund	Formula	Ion
416.2068	1	75852.67	C23 H29 N O6	(M+H)+
417.2098	1	16921.44	C23 H29 N O6	(M+H)+
418.2124	1	2464.99	C23 H29 N O6	(M+H)+
419.2157	1	287.76	C23 H29 N O6	(M+H)+
438.1884	1	10285.58	C23 H29 N O6	(M+Na)+
439.1927	1	2715.83	C23 H29 N O6	(M+Na)+
440.1944	1	551.44	C23 H29 N O6	(M+Na)+

Formula Calculator Element Limits

Element	Min	Max
C	3	60
H	0	120
O	1	10
N	1	5

Formula Calculator Results

Ion Formula	m/z	m/z (Calc)	DBE	Diff (ppm)	Score (MFG)
C23 H30 N O6	416.2068	416.2068	10	-0.09	100
Ion Formula	m/z	m/z (Calc)	DBE	Diff (ppm)	Score (MFG)
C23 H29 N Na O6	438.1884	438.1887	10	0.74	99.76
C21 H27 N4 Na O5	438.1884	438.1874	10.5	-2.49	97.36

--- End Of Report ---

Figure S5 HRESIMS analysis of fusarin A (**1**).

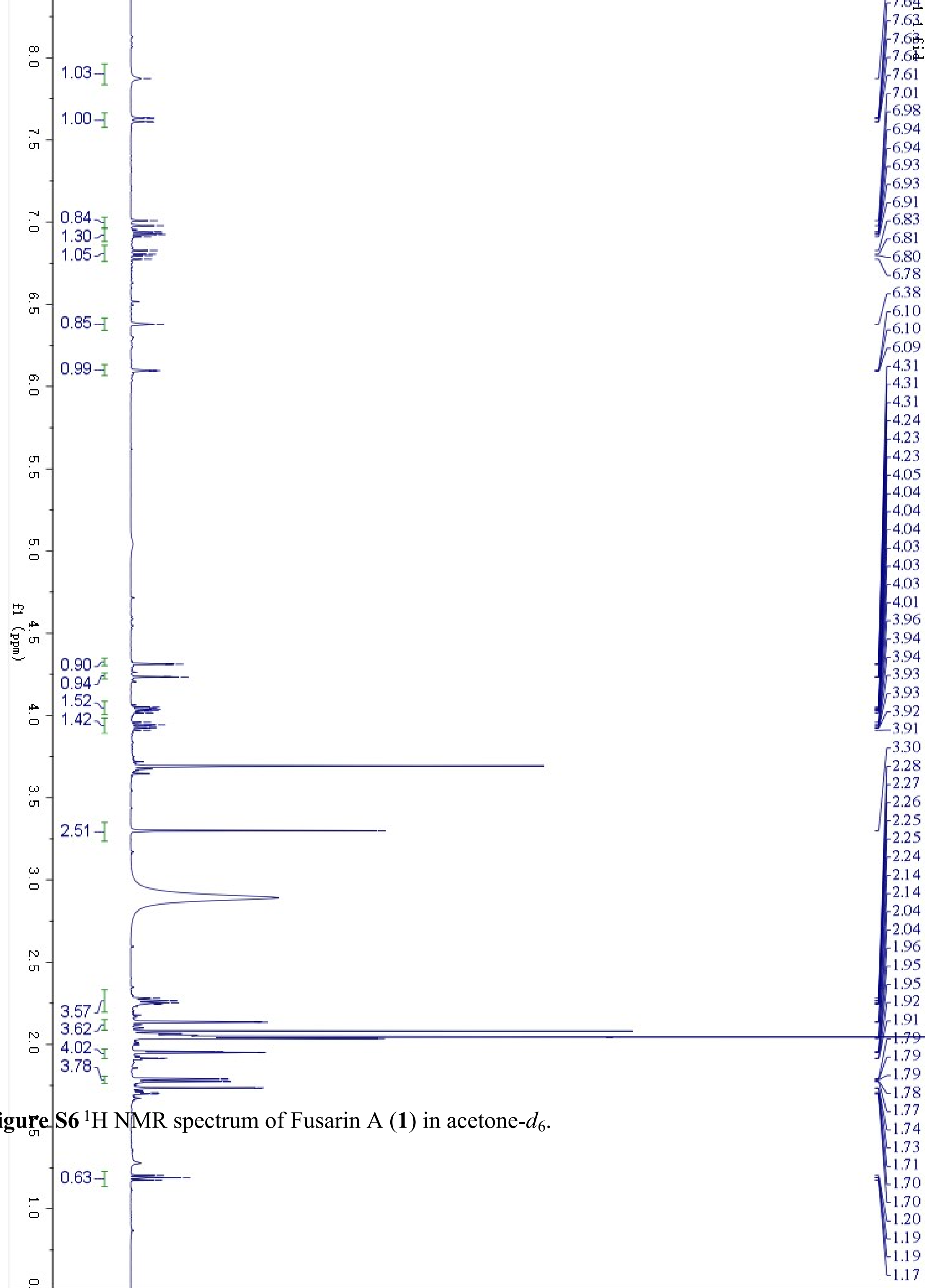


Figure S6 ^{1}H NMR spectrum of Fusarin A (**1**) in acetone-d_6.

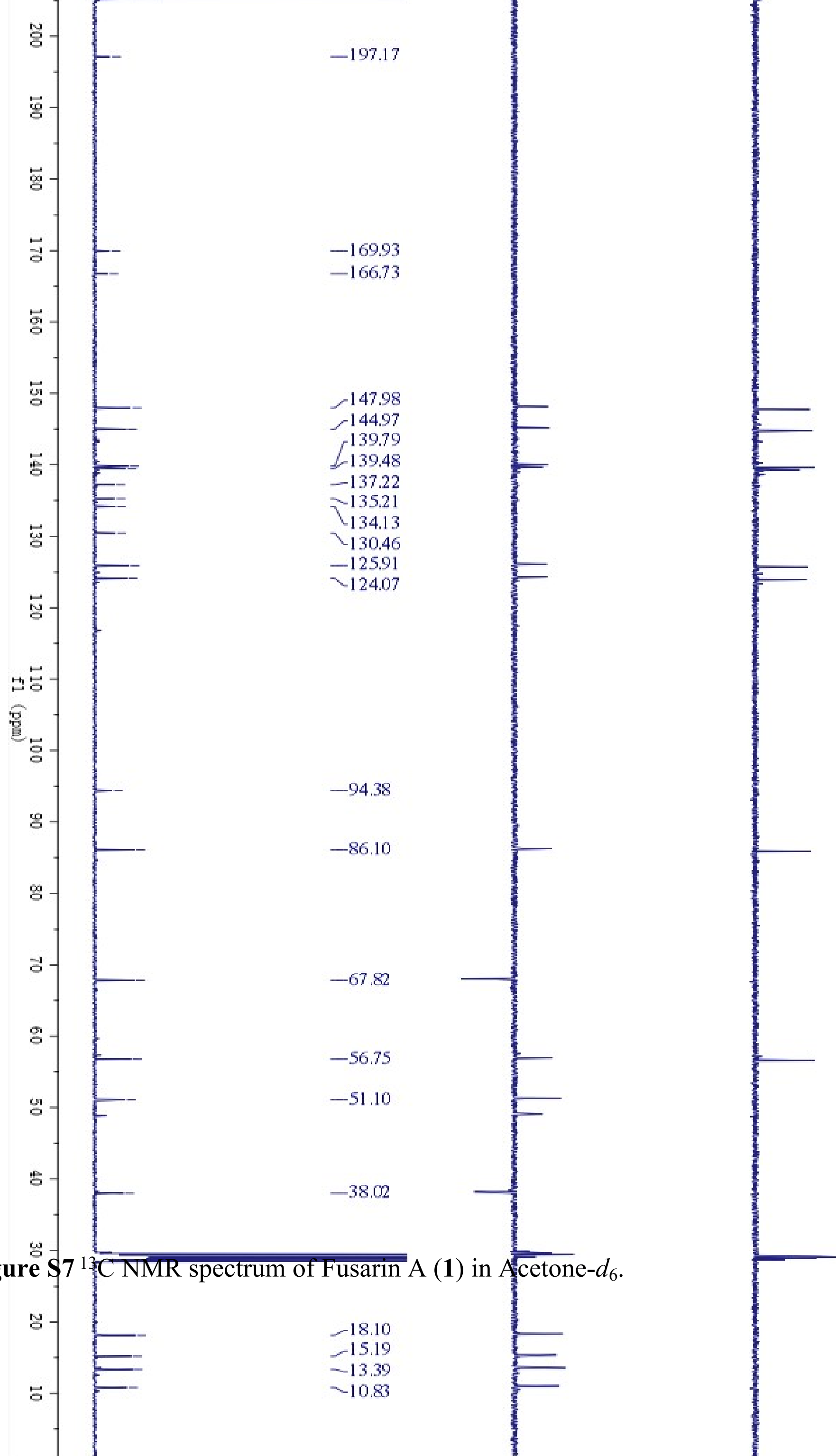


Figure S7 ^{13}C NMR spectrum of Fusarin A (**1**) in Acetone-d_6.

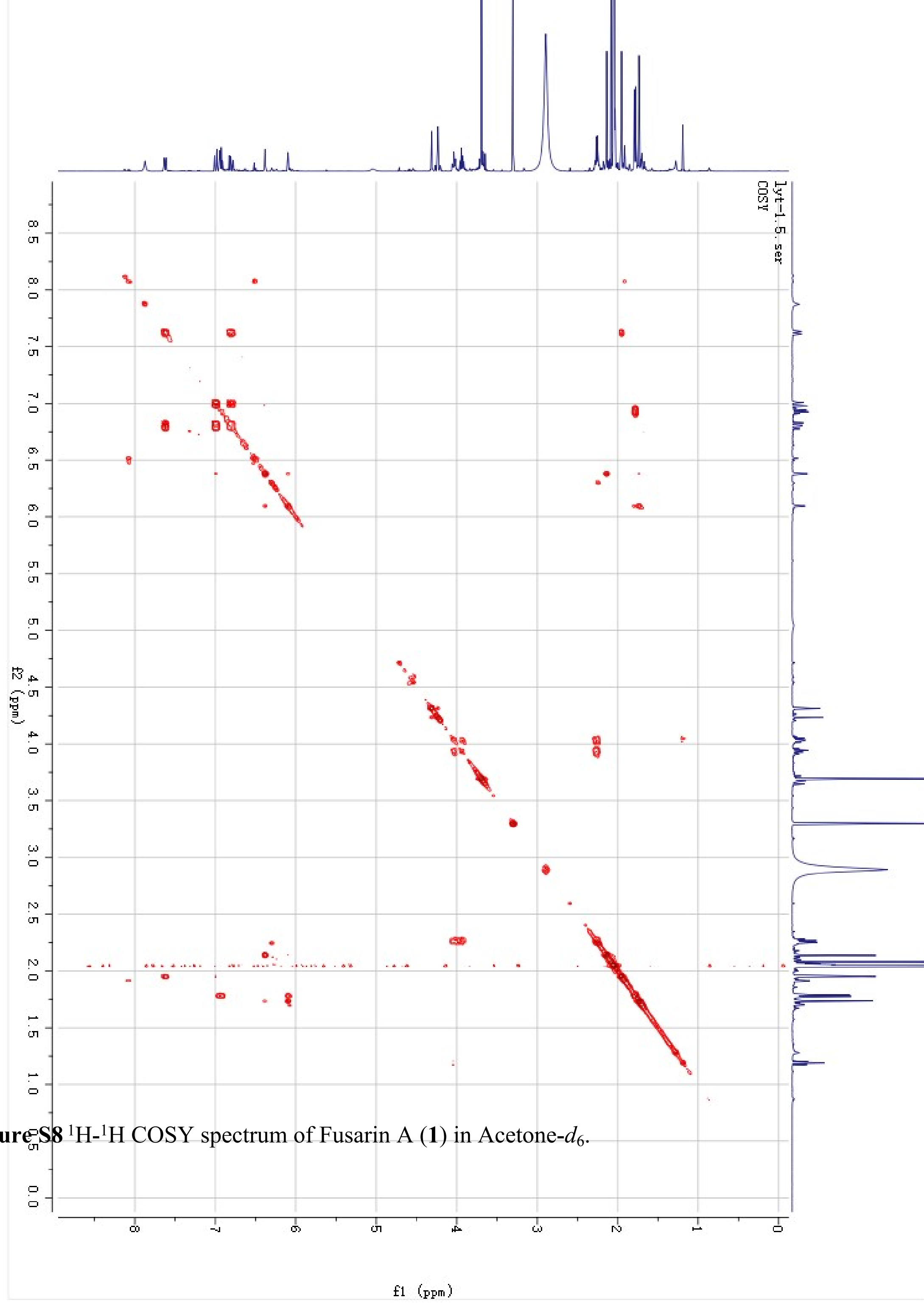


Figure S8 ^{1}H-^{1}H COSY spectrum of Fusarin A (**1**) in Acetone-d_6.

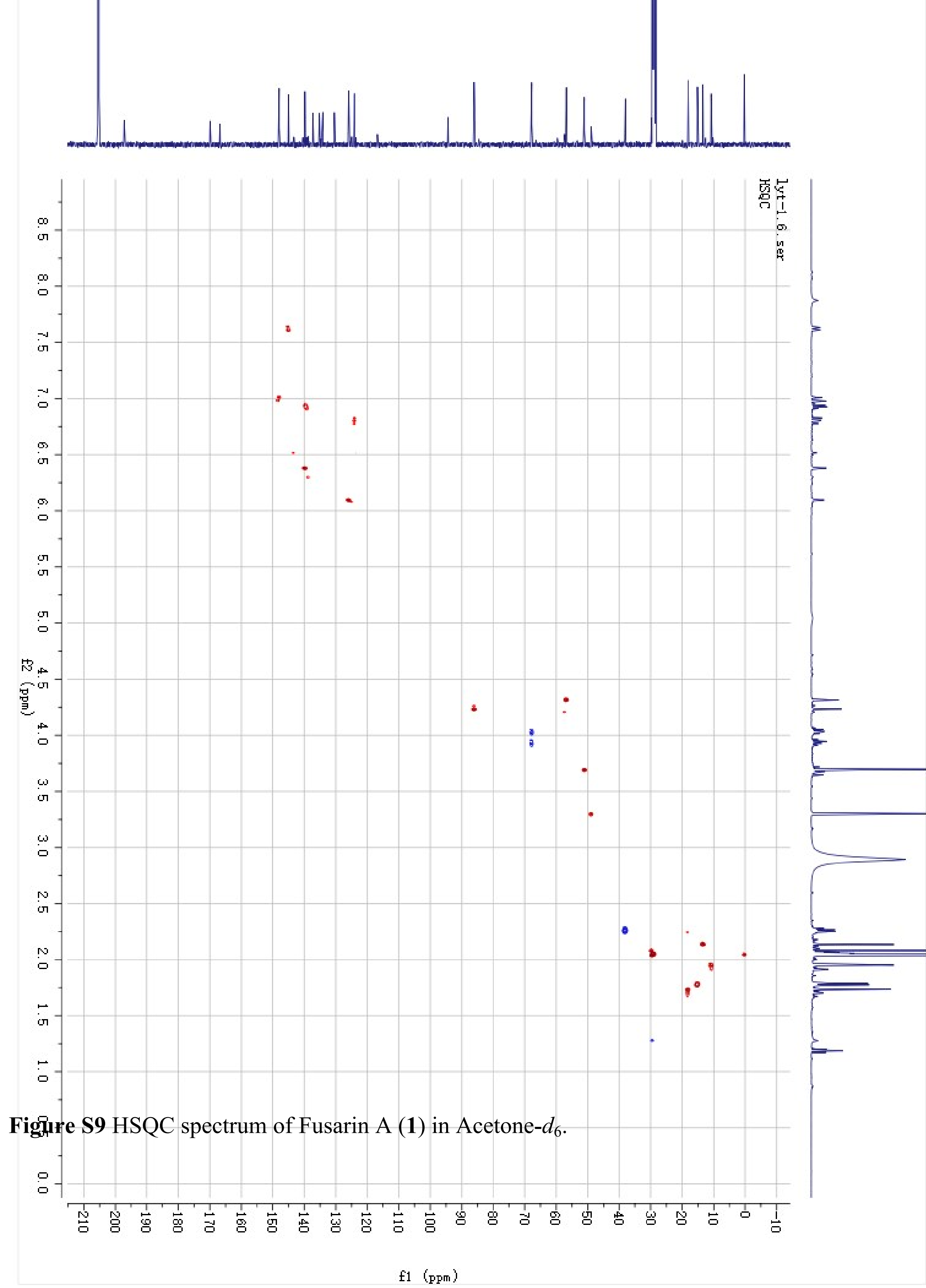


Figure S9 HSQC spectrum of Fusarin A (**1**) in Acetone-d_6.

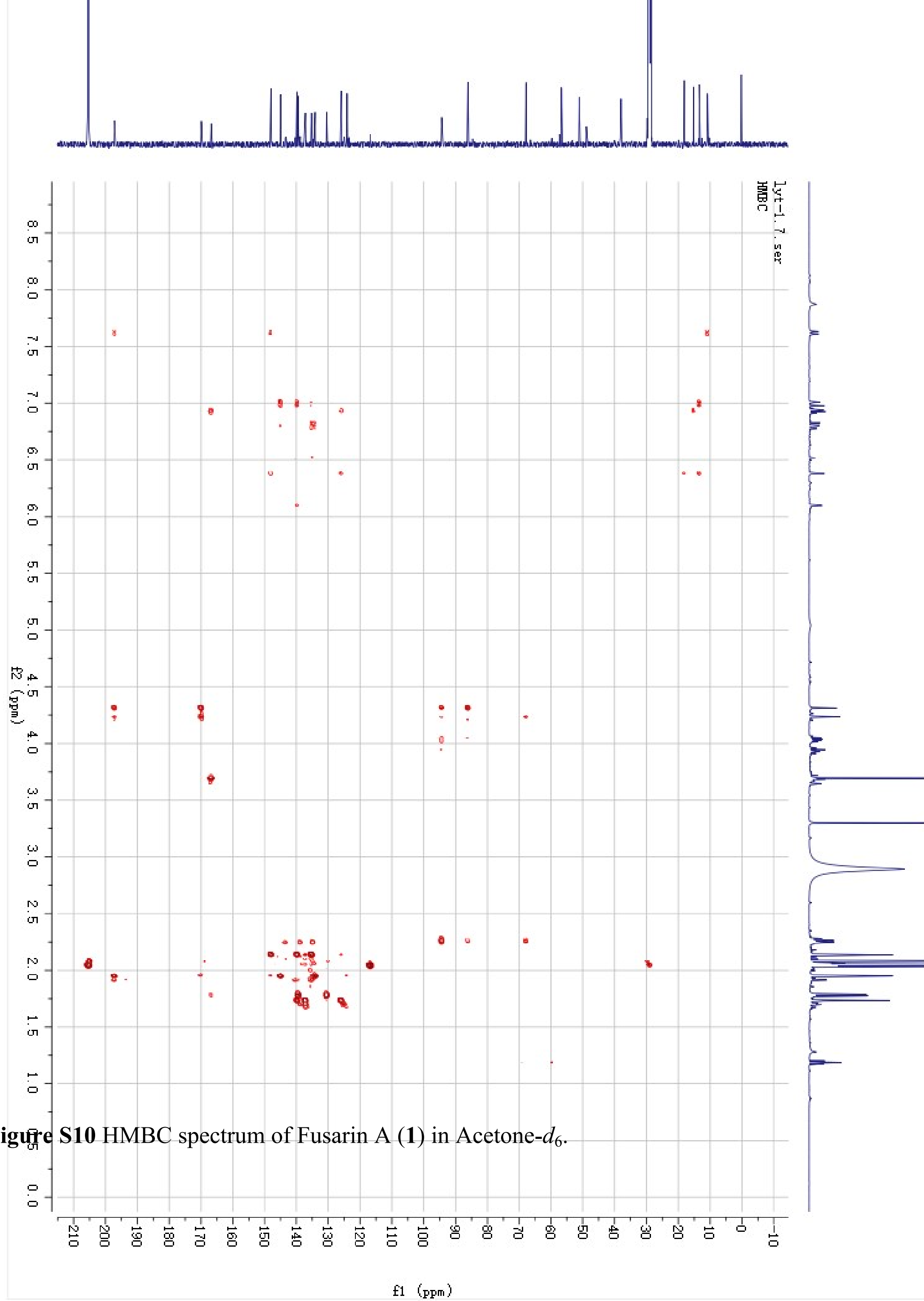


Figure S10 HMBC spectrum of Fusarin A (**1**) in Acetone-d_6.

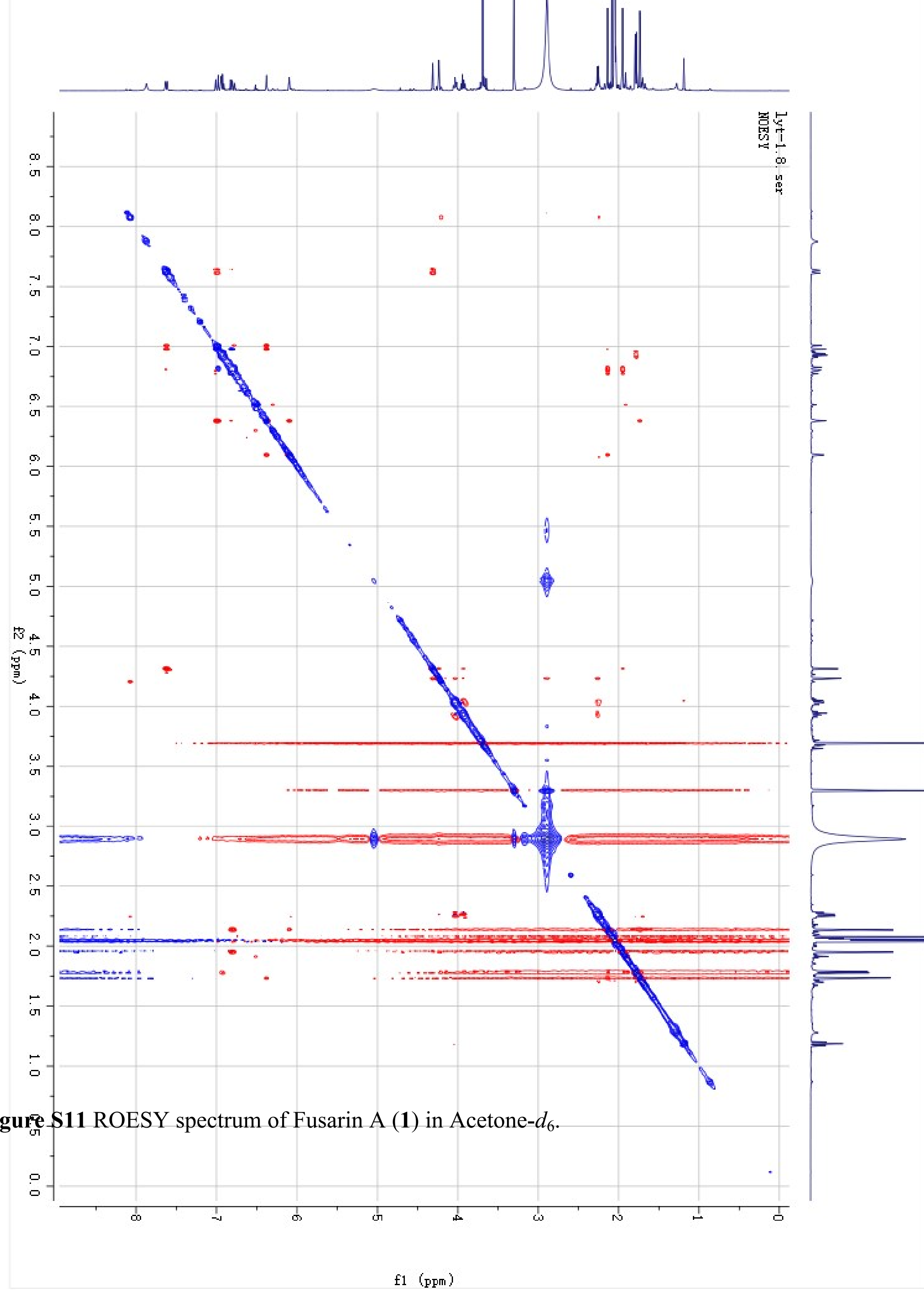


Figure S11 ROESY spectrum of Fusarin A (**1**) in Acetone-d_6.

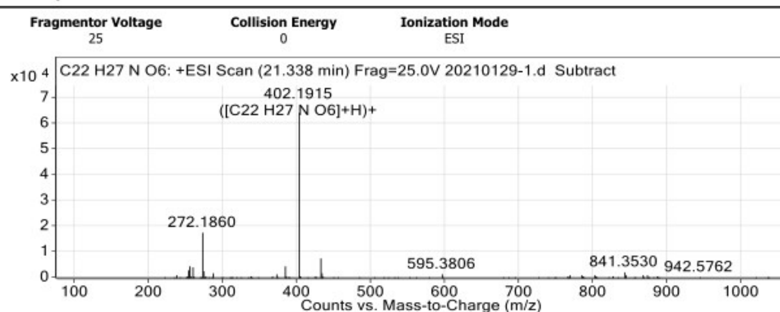
Qualitative Analysis Report

Data Filename	20210129-1.d	**Sample Name**	20210129-1
Sample Type	Sample	**Position**	Vial 41
Instrument Name	Instrument 1	**User Name**	hp-PC\hp
Acq Method	yqtd_new.m	**Acquired Time**	1/29/2021 12:10:17 PM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group **Info.**
Acquisition SW 6200 series TOF/6500 series
Version Q-TOF B.05.01 (B5125.3)

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
25	0	ESI



Peak List

m/z	z	Abund	Formula	Ion
402.1915	1	64187.27	C22 H27 N O6	(M+H)+
403.1942	1	12703.12	C22 H27 N O6	(M+H)+

Formula Calculator Element Limits

Element	Min	Max
C	3	80
H	3	120
O	1	10
N	1	5

Formula Calculator Results

Ion Formula	m/z	m/z (Calc)	DBE	Diff (ppm)	Score (MFG)
C22 H28 N O6	402.1915	402.1911	10	-0.96	99.61
C23 H24 N5 O2	402.1915	402.1925	15	2.37	97.67

--- End Of Report ---

Figure S12 HRESIMS analysis of neofusarin (**2**).

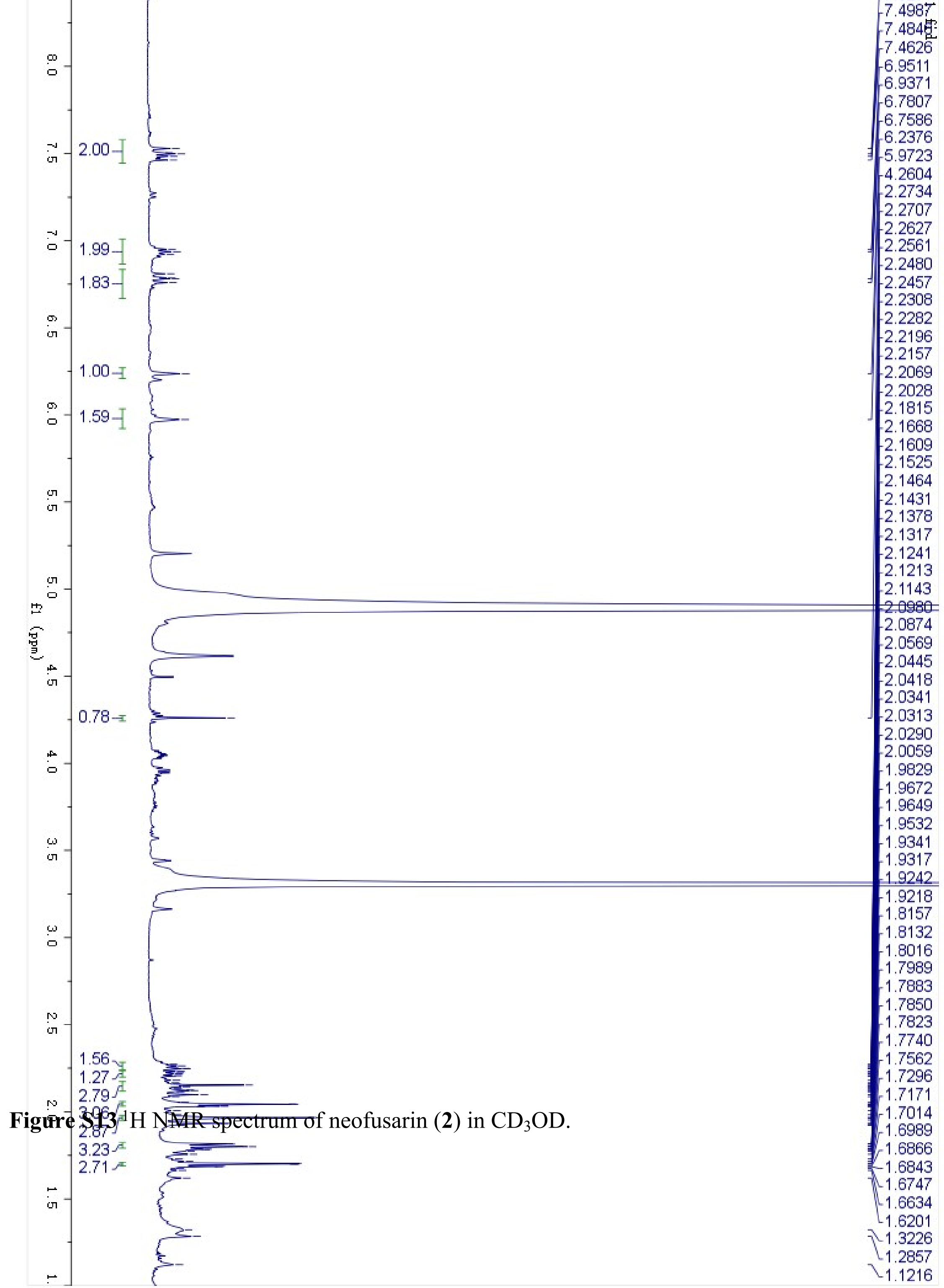


Figure S13 ^{1}H NMR spectrum of neofusarin (**2**) in CD_3OD.

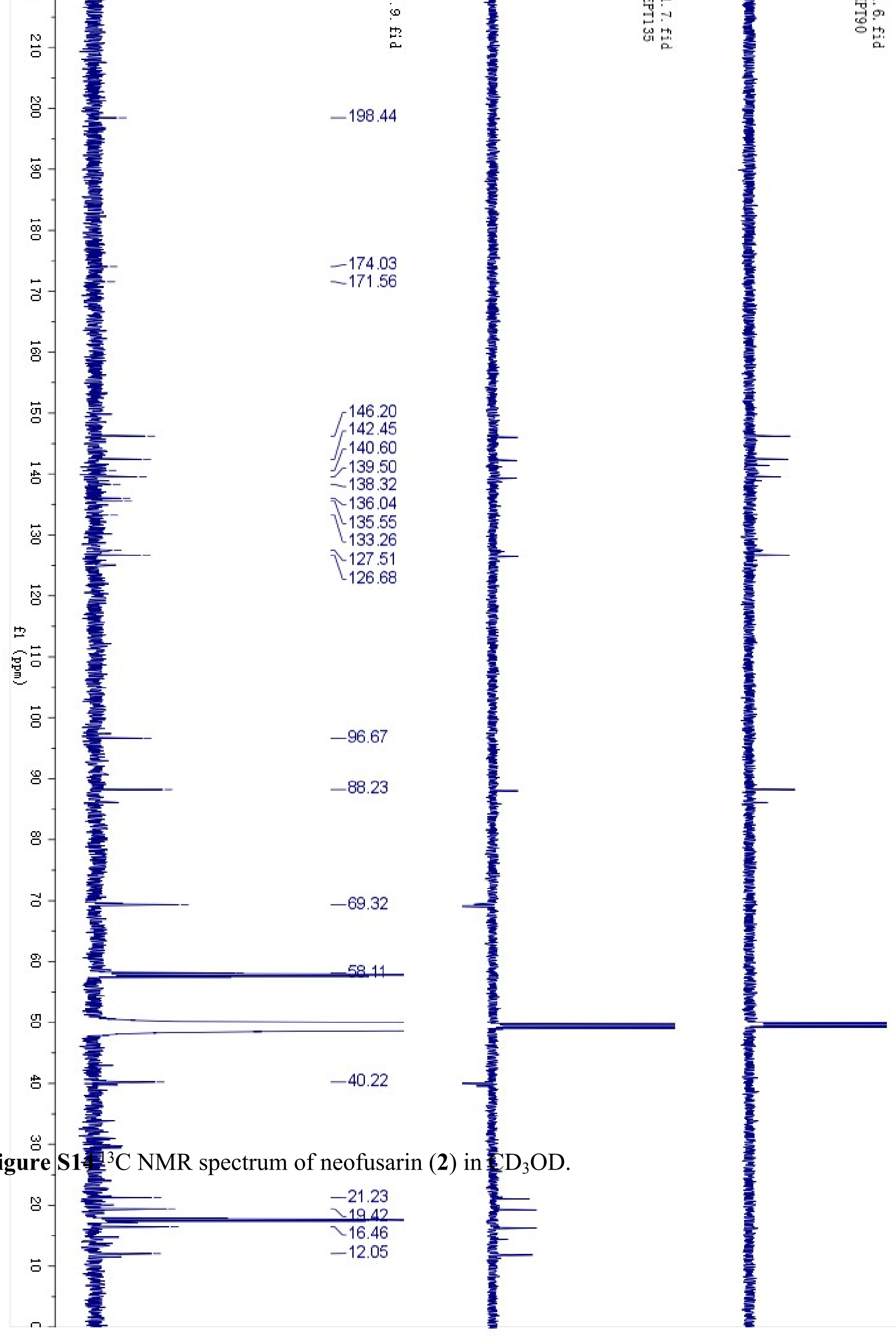


Figure S14 ^{13}C NMR spectrum of neofusarin (**2**) in CD_3OD.

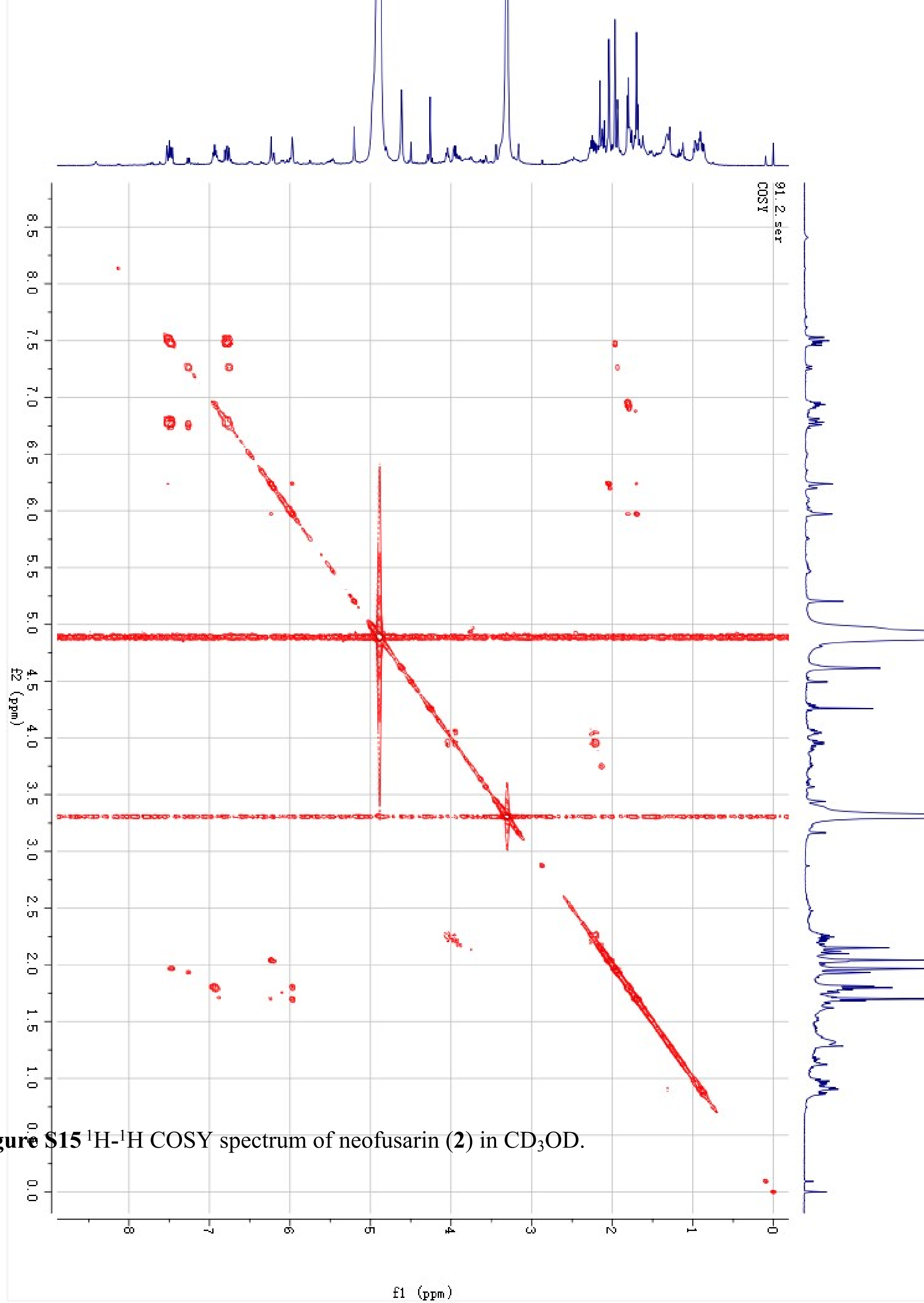


Figure S15 ^{1}H-^{1}H COSY spectrum of neofusarin (**2**) in CD_3OD.

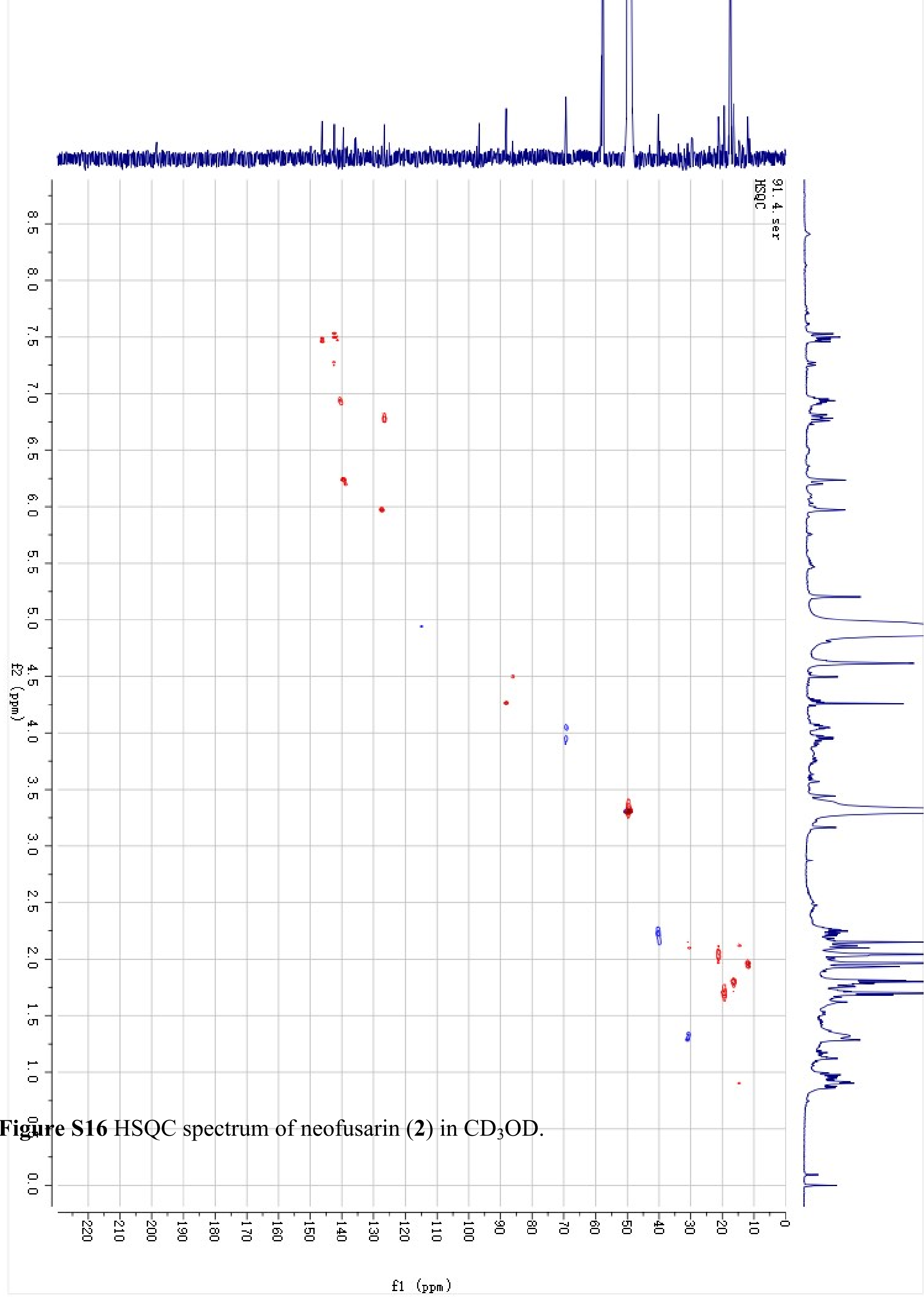


Figure S16 HSQC spectrum of neofusarin (**2**) in CD_3OD.

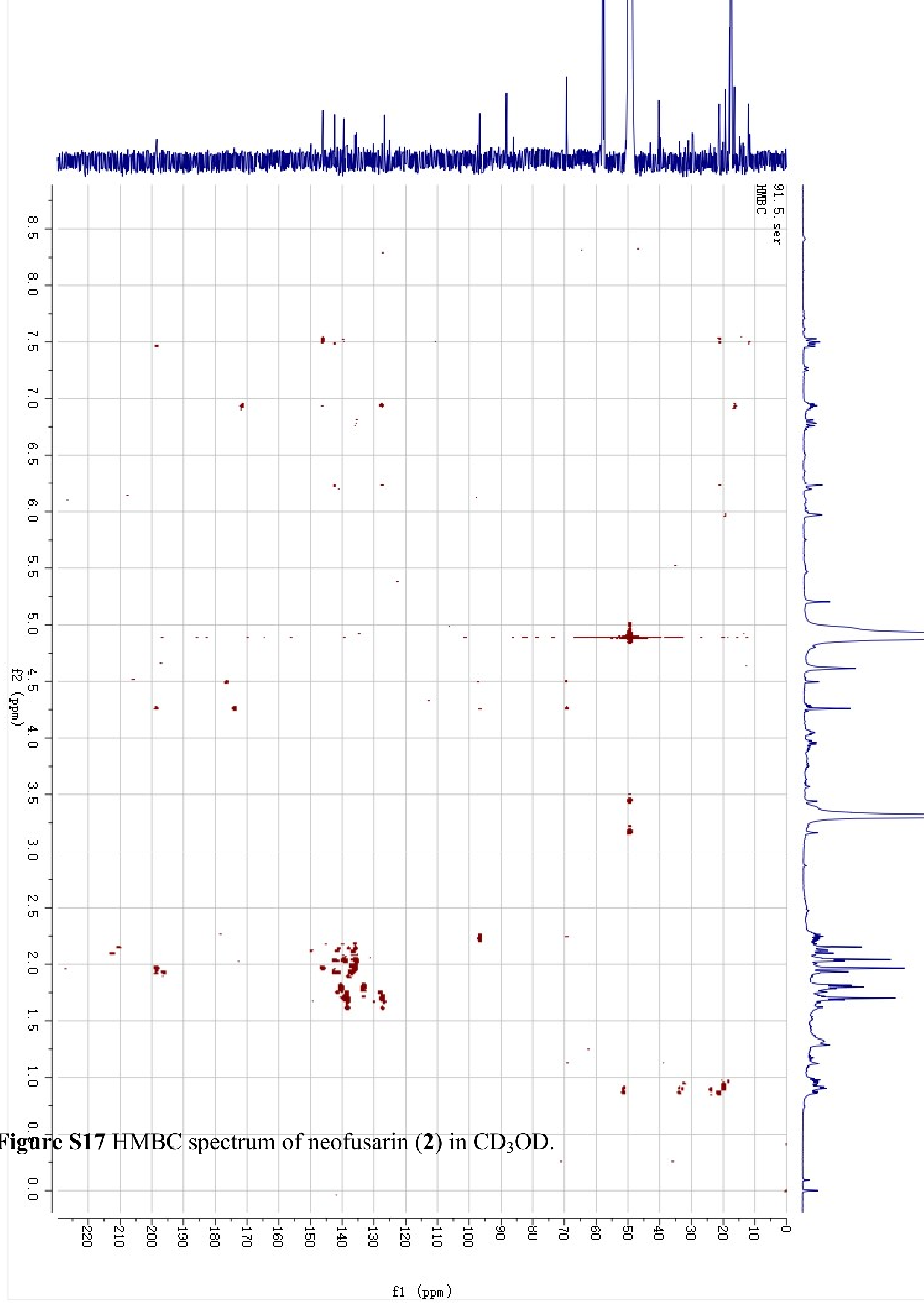


Figure S17 HMBC spectrum of neofusarin (**2**) in CD_3OD.

Figure S18 NOESY spectrum of neofusarin (**2**) in CD_3OD.

2.3 SDS-PAGE analysis for the purified protein Fgfus9.

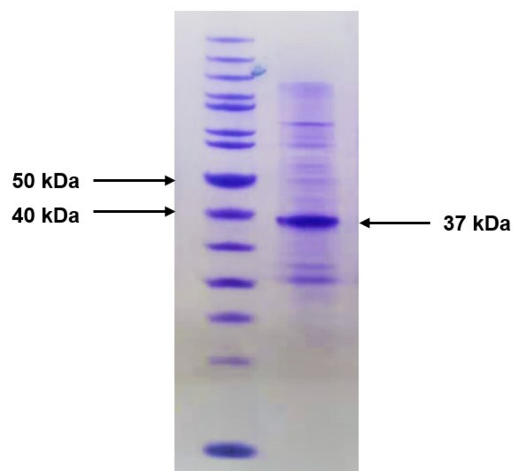


Figure S19 SDS-PAGE of purified recombinant Fgfus9. Lane 1: protein marker, Lane 2: protein

Fgfus9.

3 Supplementary references

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