Supplementary Information

Insight into Bicyclic Thiopeptide Biosynthesis Benefited from Development of a Uniform Approach for Molecular Engineering and Production Improvement

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1. Supplementary Methods

1. 1. General materials and methods

Bacterial strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are summarized in Table S1. The primer sequences are listed in Table S2. The biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were purchased from standard commercial sources unless otherwise stated.

DNA isolation, manipulation, and sequencing. DNA isolation and manipulation in *Escherichia coli* and *streptomyces* were performed according to standard protocols.[1,2] PCR amplifications were carried out on an Authorized Thermal TM Cycler (AG 22331; Eppendorf, Hamburg, Germany) using either *Taq* DNA polymerase or PrimeStar HS DNA polymerase (TaKaRa). Primer synthesis and DNA sequencing were performed at Shanghai GeneCore Biotechnology Inc. .

Sequence Analysis. Protein comparison was carried out by available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequence alignment was performed by the CLUSTALW method, and the DRAWTREE and DRAWGRAM methods, respectively, from BIOLOGYWORKBENCH 3.2 software (http://workbench.sdsc.edu). DNA hairpin was determined by using the programs from Vector NTI Advance 11. 0 software (Invitrogen).

Compound Analysis. High performance liquid chromatography (HPLC) analysis was carried out on an Agilent™ 1200 HPLC system (Agilent Technologies Inc., USA). Measurement of UV-visible absorbance was carried out on a JASCO V-530 spectrophotometer (Tokyo, Japan). Electrospray ionization MS (ESI-MS) was performed on a Thermo Fisher LTQ Fleet ESI-MS S4 spectrometer (Thermo Fisher Scientific Inc., USA). NMR data were recorded on Bruker AV500 (Bruker Co. Ltd., Germany) with tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (δ); coupling constants (J) in Hz; assignments were supported by COSY, HSQC, HMBC, and NOESY experiments. Electrospray ionization MS (ESIMS) was performed on a Thermo Fisher LTQ Fleet ESIMS spectrometer (Thermo Fisher Scientific Inc. ,

USA).

1. 2. Mutant construction

Construction of SL2051 (with the mutation of GAG for Glu-7 of TsrH into TAG). The primers Spe-P1/Spe-P2 was used to introduce a SpeI site into the vector pET28a by site-specific mutation, yielding pSL2054. After digestion of the cosmid pSL2053 (carrying the partial *tsr* gene cluster)[3] with NotI, a 2.2 kb *tsrH*-containing fragment was recovered and inserted into pSL2054, giving pSL2055. Using pSL2055 as the template, PCR amplification with the primers H3-F/H3-R was carried out to mutate the codon for Glu-7 of the precursor peptide TsrH into the stop codon TAG, giving pSL2056. After sequencing to confirm the fidelity, the 2.2 kb fragment was recovered from pSL2056 by SpeI/HindIII digestion and then inserted into the XbaI/HindIII site of pOJ260, yielding the recombinant plasmid pSL2057.

Introduction of pSL2057 into the thiostrepton-producing strain *S. laurentii* was performed by *E. coli-Streptomyces* conjugation, following the procedure described previously.[3] The colonies that were resistant to apramycin were identified as the integrating mutants, in which a single-cross over homologous recombination event took place. These mutants were further cultured in liquid TSB medium for three rounds in the absence of apramycin. The genotypes of resulting strains that were apramycin-sensitive were examined by PCR amplification and sequencing, leading to identification of the recombinant strains SL2051, in which the precursor peptide-encoding gene *tsrH* was mutated by changing the codon GAG (for Glu-7 within the leader peptide part) to the stop codon TAG.

Construction of SL2052 (*in trans* expressing *tsrH* in SL2051). Cosmid pSL2053 served as the template for PCR amplification with the primers SB-F/SB-R, resulting in a 1.0 kb product that contains intact *tsrH* along with the 530 bp upstream sequence and 288 bp downstream region. This product was digested with BamHI/SpeI and then inserted into BamHI/XbaI site of pSET152, yielding the recombinant plasmid pSL2050. Site-specific integration of pSL2050 onto the chromosome of the thiostrepton non-producing strain SL2051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2052 for *in trans* expressing

tsrH.

Construction of SL2053 (producing the precursor peptide TsrH-Ile1Val). Using the recombinant plasmid pSL2050 as the template, PCR amplification with the primers I1V-F/I1F-R was carried out for site-specific mutation of ATC (coding for Ile1 of the precursor peptide TsrH) into GTC (coding for Val1), yielding the recombinant plasmid pSL2051. Site-specific integration of pSL2051 onto the chromosome of the thiostrepton non-producing strain SL2051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2053 for producing the precursor peptide TsrH-Ile1Val.

Construction of SL2054 (producing the precursor peptide TsrH-Ala2Ser). Using the recombinant plasmid pSL2050 as the template, PCR amplification with the primers A2S-F/A2S-R was carried out for site-specific mutation of GCG (coding for Ala2 of the precursor peptide TsrH) into TCG (coding for Ser2), yielding the recombinant plasmid pSL2052. Site-specific integration of pSL2052 onto the chromosome of the thiostrepton non-producing strain SL2051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2054 for producing the precursor peptide TsrH- Ala2Ser.

Construction of SL2055 (*tsrH* duplication in the wild type strain). The recombinant plasmid pSL2050, which carries *tsrH* along with along with the 530 bp upstream sequence and 288 bp downstream region, was introduced into the wild type strain by *E.coli-Streptomyces* conjugation. Integration of pSL2050 onto the chromosome occurred via pSET152-mediated, site specific recombination, yielding the recombinant strain SL2055, which contains two *tsrHs* as one *in trans* and one *in cis* (within the cluster).

Construction of SL2057 (with the mutation of GGA for Gly-30 of TsrH into TAG). Using pSL2055 as the template, PCR amplification with the primers H1-F/H1-R was carried out to mutate the codon for Gly-30 of the precursor peptide TsrH into the stop codon TGA, giving pSL2058. After sequencing to confirm the fidelity, the 2.2 kb fragment was recovered from pSL2058 by SpeI/HindIII digestion and then inserted into the XbaI/HindIII site of pOJ260,

yielding the recombinant plasmid pSL2059.

Introduction of pSL2059 into the thiostrepton-producing strain *S. laurentii* was performed by *E. coli-Streptomyces* conjugation, following the procedure described above (for SL2051) to yield the recombinant strains SL2057, in which the precursor peptide-encoding gene *tsrH* was mutated by changing the codon GGA (for Gly-30 within the leader peptide part) to the stop codon TGA.

Construction of SL2058 (*in trans* expressing *tsrH* in SL2051). Cosmid pSL2053 served as the template for PCR amplification with the primers SE/SB-R, resulting in a 0.73 kb product that contains intact *tsrH* along with the 260 bp upstream sequence and 288 bp downstream region. This product was digested with BamHI/SpeI and then inserted into BamHI/XbaI site of pSET152, yielding the recombinant plasmid pSL2060. Site-specific integration of pSL2060 onto the chromosome of the thiostrepton non-producing strain SL2051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2058 for *in trans* expressing *tsrH*.

Construction of SL2059 (*in trans* expressing *tsrH* in SL2051). Cosmid pSL2053 served as the template for PCR amplification with the primers SB-F3/SB-R3, resulting in a 0.2 kb product that contains intact *tsrH* region. This product was digested with NdeI/EcoRI and then inserted into NdeI/EcoRI site of pIB139 (a pSET152 derivative harboring the constitutive *ermE* promoter),[11] yielding the recombinant plasmid pSL2061. Site-specific integration of pSL2061 onto the chromosome of the thiostrepton non-producing strain SL2051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2059 for *in trans* expressing *tsrH*.

Construction of SL2060 (*in trans* expressing *tsrH* in SL2057). Site-specific integration of pSL2050 onto the chromosome of the thiostrepton non-producing strain SL2057 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL2060 for *in trans* expressing *tsrH*.

Construction of SL4051 (with the mutation of AAG for Lys-5 of NosM into TAG). Cosmid pSL4053[4] served as the template for PCR amplification. Amplification with the primers

KM-F/M3-R produced a 1.9 kb fragment, which overlaps a 2.0 kb fragment obtained by using the primers M3-F/KM-R. The mixture of these two products was subjected to PCR amplification for the extension, giving a 3.8 kb DNA fragment. After digestion with HindIII/EcoRI, the recovered 3.8 kb fragment was inserted into pKC1139, yielding the recombinant plasmid pSL4052. Sequencing of pSL4052 confirmed that the codon AAG, for Lys-5 of the precursor peptide NosM, was mutated into the stop codon TAG.

Introduction of pSL4052 into the nosiheptide-producing strain *S. actuosus* was carried out by *E. coli-Streptomyces* conjugation. The colonies that were apramycin-resistant at 37℃ were identified as the integrating mutants, in which a single-crosssover homologous recombination event took place. These mutants were further cultured in liquid TSB medium for several rounds in the absence of apramycin. The genotypes of resulting strains that were apramycin-sensitive were examined by PCR amplification and sequencing, leading to identification of the recombinant strains SL4051, in which the precursor peptide-encoding gene *nosM* was mutated by changing the codon AAG (for Lys-5 within the leader peptide part) to the stop codon TAG.

Construction of SL4052 (*in trans* expressing *nosM* in SL4051). Cosmid pSL4001 served as the template for PCR amplification with the primers MC-F/MC-R, resulting in a 1.3 kb product that contains intact *nosM* along with the 972 bp upstream sequence and 69 bp downstream region. This product was digested with BamHI/XbaI and then inserted into pSET152, yielding the recombinant plasmid pSL4050. Site-specific integration of pSL4050 onto the chromosome of the thiostrepton non-producing strain SL4051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4052 for *in trans* expressing *nosM*.

Construction of SL4053 (*nosM* duplication in the wild type strain). The recombinant plasmid pSL4050, which carries *nosM* along with along with the 972 bp upstream sequence and 69 bp downstream region, was introduced into the wild type strain by *E. coli-Streptomyces* conjugation. Integration of pSL4050 onto the chromosome occurred via pSET152-mediated, site specific recombination, yielding the recombinant strain SL4053, which contains two *nosMs* as one *in trans* and one *in cis* (within the cluster).

Construction of SL4054 (*nosM* triplication in the wild type strain). Cosmid pSL4001 served as the template for PCR amplification. While the primers MC-F/MC1-R were used to amplify a 1.3 kb product that contains intact *nosM* along with the 972 bp upstream sequence and 69 bp downstream region (the flanking sequence of *nosM* and the original downstream PTM-encoding gene *nosN*), the primers MC1-F/ MC-R were used to produce a 229 bp fragment that contains *nosM* only with the 69 bp downstream sequence. After digestion, the 1.3 kb BamHI/StuI fragment and the 229 bp StuI/XbaI fragment were recovered and co-ligated into the BamHI/XbaI site of pSET152, yielding the recombinant plasmid pSL4051 for sequencing to confirm the fidelity. In this construct, the second *nosM* replaced *nosN* by using its start codon. Site-specific integration of pSL4051 onto the chromosome of the wild type strain took place after the introduction via *E. coli-Streptomyces* conjugation, yielding the recombinant strain SL4054, in which the precursor peptide encoding gene *nosM* was triplicated.

Construction of SL4055 (*nosO* inactivation). Cosmid pSL4053 served as the template for PCR amplification. To inactivate *nosO*, a 1.7 kb fragment amplified by PCR using the primers O-inf-Ls/O-inf-La and a 1.8 kb fragment amplified by using the primers O-inf-Rs/O-inf-Ra were initially cloned into pMD19-T, giving pSL4055 and pSL4056, respectively. After sequencing to confirm the fidelity, the 1.7 kb HindIII/ EcoRI and 1.8 kb XbaI/EcoRI fragments were recovered and co-ligated into the HindIII/XbaI site of pKC1139, yielding the recombinant plasmid pSL4024, in which a 969 bp in-frame coding region (corresponding to AA26-AA348 of the deduced product NosO) of *nosO* was deleted.

Introduction of pSL4054 into the nosiheptide-producing strain *S. actuosus* was carried out by *E.coli-Streptomyces* conjugation. The colonies that were apramycin-resistant at 37℃ were identified as the integrating mutants, in which a single-crosssover homologous recombination event took place. These mutants were further cultured in liquid TSB medium for three rounds in the absence of apramycin. The genotypes of resulting strains that were apramycin-sensitive were examined by PCR amplification and sequencing, leading to identification of the recombinant strains SL4055, a nosiheptide non-producing strain in which *nosO* was deleted in frame.

Construction of SL4056 (*nosM* triplication in SL4055). The recombinant plasmid pSL4051, in

which *nosM* was duplicated, was introduced into SL4055 by *E.coli-Streptomyces* conjugation. Integration of pSL4051 onto the chromosome via pSET152-mediated, site specific recombination, yielding the recombinant strain SL4056, which contains three *nosMs* as two *in trans* and one *in cis* (within the cluster).

Construction of SL4057 (with the mutation of GAG for Glu-23 of NosM into TAG). Cosmid pSL4053[4] served as the template for PCR amplification. Amplification with the primers KM-F/M1-R produced a 1.9 kb fragment, which overlaps a 2.0 kb fragment obtained by using the primers M1-F/KM-R. The mixture of these two products was subjected to PCR amplification for the extension, giving a 3.8 kb DNA fragment. After digestion with HindIII/EcoRI, the recovered 3.8 kb fragment was inserted into pKC1139, yielding the recombinant plasmid pSL4058. Sequencing of pSL4058 confirmed that the codon GAG, for Glu-23 of the precursor peptide NosM, was mutated into the stop codon TAG.

Introduction of pSL4058 into the nosiheptide-producing strain *S. actuosus* was carried out by *E. coli-Streptomyces* conjugation as described above, leading to identification of the recombinant strains SL4057, in which the precursor peptide-encoding gene *nosM* was mutated by changing the codon GAG (for Glu-23 within the leader peptide part) to the stop codon TAG.

Construction of SL4058 (with the mutation of CGA for Arg-14 of NosM into TGA). Cosmid pSL4053[4] served as the template for PCR amplification. Amplification with the primers KM-F/M2-R produced a 1.9 kb fragment, which overlaps a 2.0 kb fragment obtained by using the primers M2-F/KM-R. The mixture of these two products was subjected to PCR amplification for the extension, giving a 3.8 kb DNA fragment. After digestion with HindIII/EcoRI, the recovered 3.8 kb fragment was inserted into pKC1139, yielding the recombinant plasmid pSL4059. Sequencing of pSL4059 confirmed that the codon CGA, for Arg-14 of the precursor peptide NosM, was mutated into the stop codon TGA.

Introduction of pSL4059 into the nosiheptide-producing strain *S. actuosus* was carried out by *E. coli-Streptomyces* conjugation as described above, leading to identification of the recombinant strains SL4058, in which the precursor peptide-encoding gene *nosM* was mutated by changing the codon CGA (for Arg-14 within the leader peptide part) to the stop codon TGA.

Construction of SL4059 (*in trans* expressing *nosM* in SL4057). Cosmid pSL2053 served as the template for PCR amplification with the primers Nos16com-F3/ Nos16com-R3, resulting in a 0.4 kb product. This product was digested with HindIII/EcoRI and then inserted into HindIII/EcoRI site of pSET152, yielding the recombinant plasmid pSL4060. Site-specific integration of pSL4060 onto the chromosome of the nosiheptide non-producing strain SL4057 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4059 for *in trans* expressing *nosM*.

Construction of SL4060 (*in trans* expressing *nosM* in SL4057). Cosmid pSL2053 served as the template for PCR amplification with the primers Nos16com-F3/ Nos16com-R3, resulting in a 0.4 kb product. This product was digested with HindIII/EcoRI and then inserted into HindIII/EcoRI site of pIB139,[11] yielding the recombinant plasmid pSL4061. Site-specific integration of pSL4061 onto the chromosome of the nosiheptide non-producing strain SL4057 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4060 for *in trans* expressing *nosM*.

Construction of SL4061 (*in trans* expressing *nosM* in SL4058). Site-specific integration of pSL4060 onto the chromosome of the nosiheptide non-producing strain SL4058 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4061 for *in trans* expressing *nosM*.

Construction of SL4062 (*in trans* expressing *nosM* in SL4058). Site-specific integration of pSL4061 onto the chromosome of the nosiheptide non-producing strain SL4058 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4062 for *in trans* expressing *nosM*.

Construction of SL4063 (*in trans* expressing *nosM* in SL4051). Site-specific integration of pSL4060 onto the chromosome of the nosiheptide non-producing strain SL4051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4063 for *in trans* expressing *nosM*.

Construction of SL4064 (*in trans* expressing *nosM* in SL4051). Site-specific integration of pSL4061 onto the chromosome of the nosiheptide non-producing strain SL4051 took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL4064 for *in trans* expressing *nosM*.

1. 3. Chemical purification and characterization

Cultivation, production and analysis of thiostrepton or nosiheptide, variants and intermediate were perform according to the methods described previously.[5,6]

Isolation and purification of the thiostrepton variants. For each variant (**1** or **2**), 25 L of the culture broth was prepared and centrifuged for 20 min at 6,000 rpm. The mycelia cake was soaked with 3 L of acetone overnight and then evaporated in vacuum. The solvent dissolved in H_2O was extracted four times by equal volume of EtOAc together with the supernatant. Upon monitoring by HPLC analysis, the organic layer was sequentially subjected to isolation and purification by chromatography on the silica chromatography (300-400 mesh, Qingdao Marine Chemical Inc., Qingdao, China) with petroleum ether/chloroform/methanol system (1:0:0, 1:1:0, 0:100:1, 0:50:1, 0:20:1, 0:10:1, 0:5:1, 0:1:1, 0:0:1). The collection was further purified by preparative HPLC that was performed on an Agilent 1100 with a Zorbax SB-C18 column (21.2 × 250 mm), yielding the product **1** (470 mg) or **2** (860 mg) subjected to spectrum analysis. For **1** purification, the column was equilibrated with 47% solvent A (H_2O) and 53% B (CH_3CN), and the program was developed as the following: 0 to 3 min, constant 47% A/53% B; 3 to 12 min, a linear gradient from 47% A/53% B to 31% A/69% B; 12 to 14 min, constant 31% A/69% B to 47% A/53% B. For **2** purification, the collumn was equilibrated with 45% solvent A (H_2O) and 55% B (CH_3CN), and the program was developed as the following: 0 to 3 min, constant 45% A/55% B; 3 to 12 min, a linear gradient from 45% A/55% B to 27.5% A/72.5% B; 12 to 14 min, constant 27.5% A/72.5% B to 45% A/55% B.

Isolation and purification of the nosiheptide shunt intermediate. 40 L of the culture broth of

SL4056 was centrifuged for 20 min at 6,000 rpm. The mycelia cake was treated as described above, yielding 43 mg of product subjected to spectrum analysis. For purification, the Zorbax SB-C18 column (21.2 × 250 mm) was equilibrated with 60% solvent A (H_2O) and 40% B (CH_3CN), and the HPLC program was developed as the following: 0 to 4 min, constant 60% A/40% B; 4 to 10 min, a linear gradient from 60% A/40% B to 4% A/60% B; 18 to 20 min, constant 40% A/60% B to 60% A/40% B.

1. 4. NMR data of new compounds

Compound 1. ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 3:1): δ9.91 (s, 1H), 8.84 (d, 1H), 8.33 (s, 1H), 8.32 (s, 1H), 8.21 (s, 1H), 8.08 (s, 1H), 7.83 (s, 1H), 7.62 (d, 1H), 7.61 (s, 1H), 7.33 (s, 1H), 7.19 (d, 1H), 7.14 (d, 1H), 6.93 (d, 1H), 6.72 (s, 1H), 6.54 (s, 1H), 6.44 (t, 1H), 6.38 (q, 1H), 6.26 (q, 1H), 5.85 (s, 1H), 5.80 (s, 1H), 5.79 (d, 1H), 5.73 (s, 1H), 5.65 (s, 1H), 5.54 (d, 1H), 5.48 (d, 1H), 5.39 (s, 1H), 5.36 (s, 2H), 5.02 (t, 1H), 4.76 (q, 1H), 4.45 (m, 1H), 4.44 (s, 2H), 4.11 (t, 3H), 4.02 (Q, 1H), 3.96 (t, 1H), 3.84 (m, 2H), 3.67 (t, 1H), 3.64 (d, 2H), 3.50 (d, 2H), 3.36 (s, 1H), 3.21 (t, 1H), 2.97 (m, 1H), 2.95 (d, 1H), 2.36 (m, 1H), 2.27 (m, 1H), 1.75 (d, 3H), 1.64 (d, 3H), 1.46 (d, 3H), 1.41 (d, 3H), 1.33 (d, 3H), 1.19 (d, 3H), 1.17 (d, 3H), 1.08 (d, 3H), 0.84 (m, 6H). **^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 3:1):** δ173.49, 173.20, 171.80, 170.02, 169.97, 169.60, 168.84, 168.21, 166.34, 165.98, 165.38, 162.61, 162.56, 162.00, 161.93, 161.84, 161.60, 160.57, 159.48, 157.04, 154.39, 153.39, 149.90, 149.69, 146.11, 143.41, 133.99, 132.86, 132.58, 131.99, 129.88, 128.27, 127.60, 127.52, 127.09, 125.51, 125.45, 124.89, 123.09, 122.20, 118.15, 107.38, 104.31, 103.20, 97.67, 78.79, 71.85, 67.58, 67.35, 67.08, 66.74, 66.23, 65.79, 64.32, 63.96, 58.81, 57.47, 55.69, 55.46, 52.81, 51.65, 49.39, 49.30, 34.57, 33.16, 32.85, 31.79, 30.95, 30.82, 29.18, 28.82, 26.00, 25.13, 24.71, 23.46, 23.05, 22.45, 22.19, 19.00, 18.77, 18.69, 18.64, 18.22, 16.19, 16.15, 15.74, 15.15, 13.50. UV/Vis λ_{max} 198 nm, 250 nm, and 294 nm. Assignment of the ^{1}H, ^{13}C NMR and ^{1}H-^{13}C COSY correlations (HSQC) of **1** was summarized in Table S3.

Compound 2. ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 3:1): δ9.83 (s, 1H), 9.42 (s, 1H), 8.74 (d, 1H), 8.56 (s, 1H), 8.33 (s, 1H), 8.31 (s, 1H), 8.19 (s, 1H), 7.61 (s, 1H), 7.58 (s, 2H), 7.33 (s, 1H), 7.10 (d, 1H), 6.95 (d, 1H), 6.73 (d, 1H), 6.55 (d, 1H), 6.44 (t, 1H), 6.41 (t, 2H), 6.25 (q, 1H), 5.80

(s, 2H), 5.78 (d, 1H), 5.71 (s, 1H), 5.64 (s, 1H), 5.41 (s, 1H), 5.35 (t, 1H), 5.33 (s, 1H), 5.17 (s, 1H), 5.01 (q, 1H), 4.81 (t, 1H), 4.45 (t, 1H), 4.44 (s, 1H), 4.18 (t, 1H), 4.04, 3.85 (q, 1H), 3.67 (q, 1H), 3.63 (d, 1H), 4.50 (q, 1H), 3.36 (t, 1H), 3.20 (t, 1H), 3.07 (d, 1H), 2.97 (m, 1H), 2.34 (m, 1H), 1.67 (d, 3H), 1.64 (d, 3H), 1, 47 (d, 3H), 1.40 (d, 3H), 1.32 (d, 3H), 1.17 (s, 3H), 1.06 (d, 3H), 0.89 (t, 3H), 0.85 (d, 3H). **^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 3:1):** δ172.09, 172.04, 172.01, 170.82, 169.02 168.91, 168.45, 167.09, 165.10, 164.84, 164.23, 161.78, 160.88, 160.84, 160.74, 160.60, 159.48, 158.38, 155.95, 153.14, 152.48, 148.86, 148.63, 145.12, 142.36, 133.04, 132.90, 131.72, 131.52, 128.39, 127.16, 126.50, 126.25, 124.47, 123.75, 122.33, 121.23, 117.07, 115.24, 103.18, 102.10, 101.23, 99.03, 77.65, 70.64, 67.27, 66.45, 65.24, 65.20, 63.17, 62.96, 58.08, 56.23, 54.58, 54.45, 54.36, 51.77, 50.64, 37.03, 33.62, 35.67, 32.08, 30.70, 29.74, 27.91, 24.92, 23.56, 22.81, 21.49, 21.43, 21.13, 18.39, 17.69, 17.63, 17.55, 17.14, 14.64, 14.13, 10.29. UV/Vis λ_{max} 198 nm, 250 nm, and 294nm. Assignment of the ^{1}H, ^{13}C NMR and ^{1}H-^{13}C COSY correlations (HSQC) of **2** was summarized in Table S3.

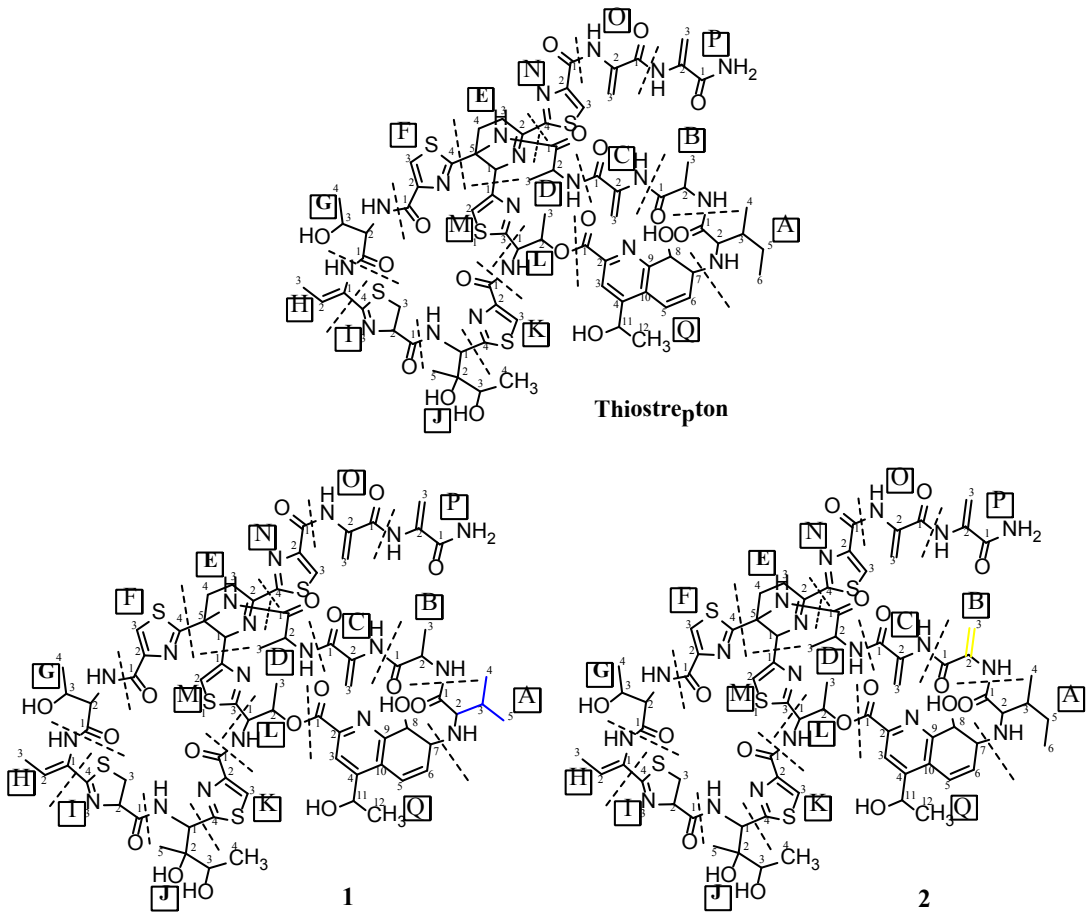
Compound 3. ^{1}H NMR (500 MHz, d6-DMSO): δ11.90 (s, 1H), 10.27 (s, 1H), 9.93 (s, 1H), 8.84 (s, 1H), 9.77 (s, 1H), 9.10 (d, 1H), 8.77 (s, 1H), 8.53 (s, 1H), 8.44 (s, 1H), 8.38 (d, 1H), 8.27 (s, 1H), 8.25 (s, 1H), 8.23 (d, 1H), 7.47 (d, 1H), 7.22 (t, 1H), 7.07 (s, 1H), 6.83 (q, 1H), 6.47 (s, 1H), 6.41 (s, 1H), 5.97 (s, 1H), 5.81 (s, 1H), 5.78 (s, 1H), 5.57 (s, 1H), 5.50 (s, 1H), 5.40 (dd, 1H), 5.29 (m, 1H), 4.59 (dd, 1H), 4.33 (m, 1H), 4.03 (m, 1H), 2.70 (s, 1H), 2.68 (s, 1H), 2.42 (m, 1H), 2.29 (m, 1H), 2.20 (m, 1H), 1.74 (d, 1H), 1.22 (d, 1H). **^{13}C NMR (125 MHz, d6-DMSO):** δ 191.23, 182.48, 173.88, 172.77, 171.09, 169.47, 167.53, 166.54, 165.42, 165.25, 160.87, 160.068, 160.09, 159.04, 158.68, 150.72, 149.03, 148.66, 148.62, 148.54, 137.73, 135.13, 133.63, 131.28, 130.99, 129.92, 128.68, 127.03, 126.44, 126.17, 125.30, 125.16, 125.02, 124.38, 123.10, 118.73, 118.05, 114.09, 105.05, 103.01, 101.01, 66.87, 65.79, 58.75, 51.13, 50.49, 48.59, 31.66, 31.10, 29.30, 25.74, 20.73, 12.59, 13.31. UV/Vis λ_{max} 198 nm, 250 nm, and 294nm. Assignment of the ^{1}H, ^{13}C NMR and ^{1}H-^{13}C COSY correlations (HSQC) of **3** was summarized in Table S4.

1. 5. Bioassay

Minimum Inhibitory Concentrations (MICs). The MICs were measured by broth dilution using a modified method described previously. [7, 8]

2. Supplementary results

2. 1. Structural elucidation of compounds 1 and 2

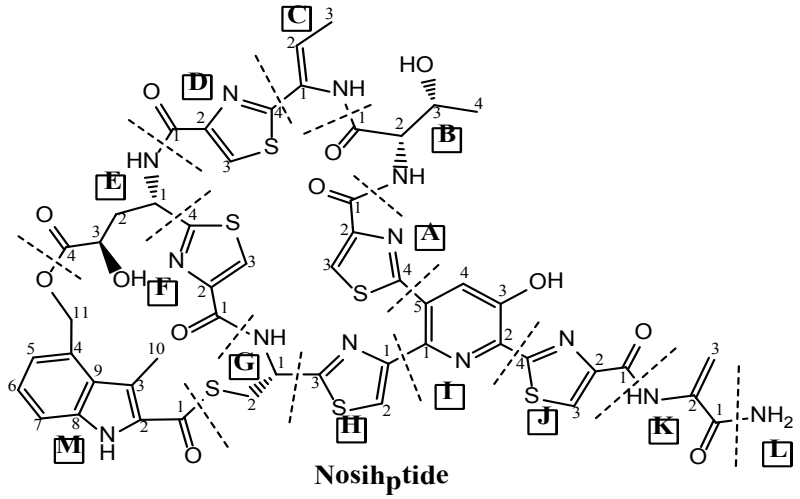


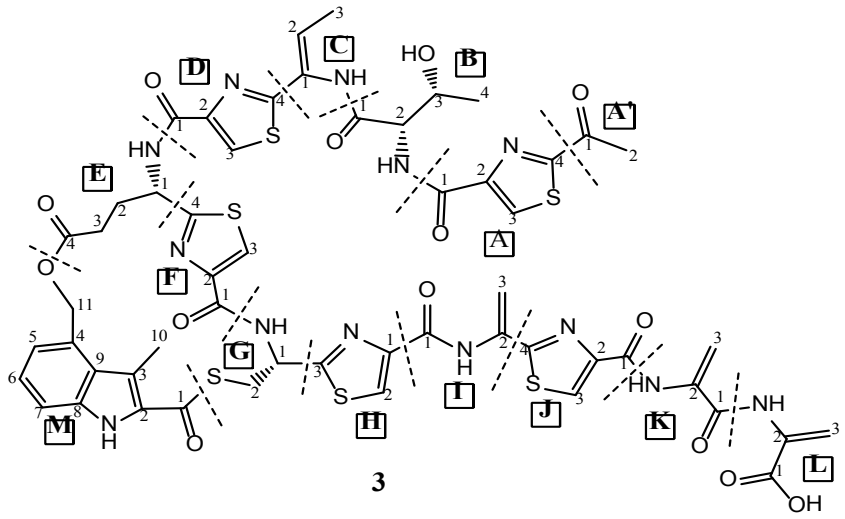
Compound **1** shares the overall similarity in spectrum patterns to the parent compound thiostrepton (Figures S1, S2, and Table S3). Analysis of their 1D and 2D NMR spectra revealed the only difference present in the unit A. **1** apparently lacks the ^{1}H NMR signal (δ_{H} 0.74 ppm) and ^{13}C NMR signal (δ_{C} 11.25 ppm) corresponding to H_3-6Me of thiostrepton. In the unit A, establishment of the COSY (C4, C5, C3 and C2) and HMBC (H_3-5CH_3 to C4 and C2; H_3-4CH_3 to C3; and H-3CH to C2) correlations further supports the substitution of an isopropyl group at C2 for compound **1**, instead of an isobutyl group for thiostrepton. This is consistent with the fact that the unit A in **1** is derived from the Val residue (rather than the Ile residue in thiostrepton).

Compound **2** shares the overall similarity in spectrum patterns to the parent compound thiostrepton

(Figures S1, S3, and Table S3). Analysis of their 1D and 2D NMR spectra revealed the only difference present in the unit B. In this unit of **2**, the ^{1}H NMR signal at 1.03 ppm (d) for H_3-3Me of thiostrepton was changed to 5.16 ppm (s) and 6.40 ppm (s), while the ^{13}C NMR signal at 18.88 ppm for C3 of thiostrepton was shifted to 98.96 ppm. For **2**, the presence of a double bond between C2 and C3 was further supported by the HMBC correlations of H_2-3CH_2 to C2 and C1, respectively. This is consistent with the fact that the unit B in **2** is derived from the Ser residue (rather than the Ala residue in thiostrepton) by dehydration.

2. 2. Structural elucidation of compound 3





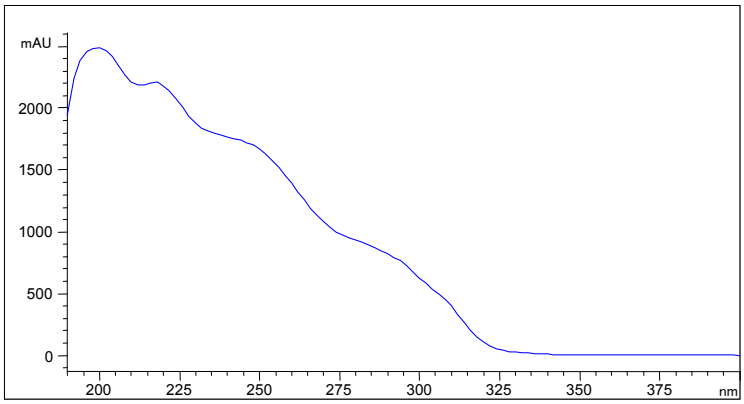
Compound **3** shares the overall similarity in spectrum patterns to the parent compound nosiheptide (Figures S1, S4, and Table S4). Comparison of their 1D and 2D NMR spectra revealed the identity in

the units A, B, C, D, F, G, H, J, K, and M, indicating that **3** is fully installed with thiazoles and Dhas, and particularly, the indole-containing side ring system. The differences are present in the units A' (for **3** only), I, E and L. For the unit I, **3** lacks the ^{13}C NMR signals corresponding to the carbons of the central pyridine domain of nosiheptide, including C1 (δ_C 142.52ppm, C), C4 (δ_C 127.12 ppm, CH), C5 (δ_C 129.90ppm, C), and in line with the assignment of **3** as a linear product. In this unit of **3**, the specific ^{1}H NMR signals, including δ_H 5.81 ppm *Z* (s) and 6.41ppm *E* (s) for H_2-3CH_2 and δ_H 10.26 ppm for H-N, and ^{13}C NMR signal δ_C 159.04 ppm for C1 were observed, suggesting that there is an enamide moiety. The HMBC corrections (e.g., H-N to C1, C2, and C4 of the neighboring unit J; H_2-3CH_2 to C2 and C4 of the neighboring unit J) further supports this assignment. The unit A' is specific to **3**, corresponding to the ^{1}H NMR signal δ_H 2.70 ppm (s) for H_3-2CH_3, the ^{13}C NMR signals δ_C 191.23 ppm for C1 and 25.74 ppm for C2, and the HMBC correlation of H_3-2CH_3 to C1 and C4 of the neighboring A unit. For the unit E, there is the ^{1}H NMR signal δ_H 2.42 ppm (m) for H_2-3CH_2 of **3** (instead of 4.09 ppm (dd) for H-3CH of nosiheptide), and COSY (C1, C2 and C3) and HMBC (e.g., H_2-2CH_2 to C3, C4 and C4 of the neighboring unit F; H_2-3CH_2 to C1, C2, and C4 of the neighboring unit F) correlations, suggesting that **3** lacks a hydroxyl group at this position. Consistently, the signals of H_2-2CH_2 are shifted, as δ_H 3.86 ppm (dd) and 3.56 ppm (dd) for nosiheptide versus 2.20 ppm (m) and 2.29 ppm (m) for **3**. In addition, the ^{1}H NMR signal δ_H 9.77 ppm for H-N, δ_H 5.50 ppm *Z* (s) and δ_H 5.97 ppm *E* (s) for H_2-3CH_2; the ^{13}C NMR signals δ_C 137.73 ppm for C2, 101.01 ppm for C3, and 165.25 ppm for C1 were found in the unit L of **3**. The HMBC correlations (e.g., H_2-3CH_2 to C1, C2 and H-N to C2 and C2 of the neighboring unit K) further supports that **3** has a *C*-terminal Dha residue.

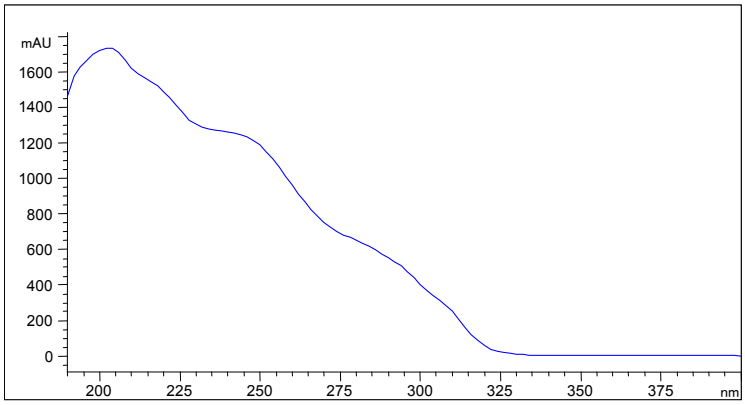
3. Supplementary Figures

Figure S1. Ultraviolet absorption patterns of compounds 1 (A), 2 (B) and 3 (C).

A



B



C

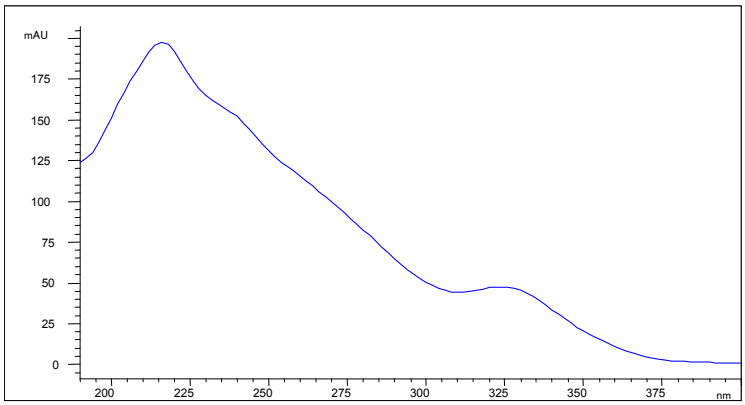
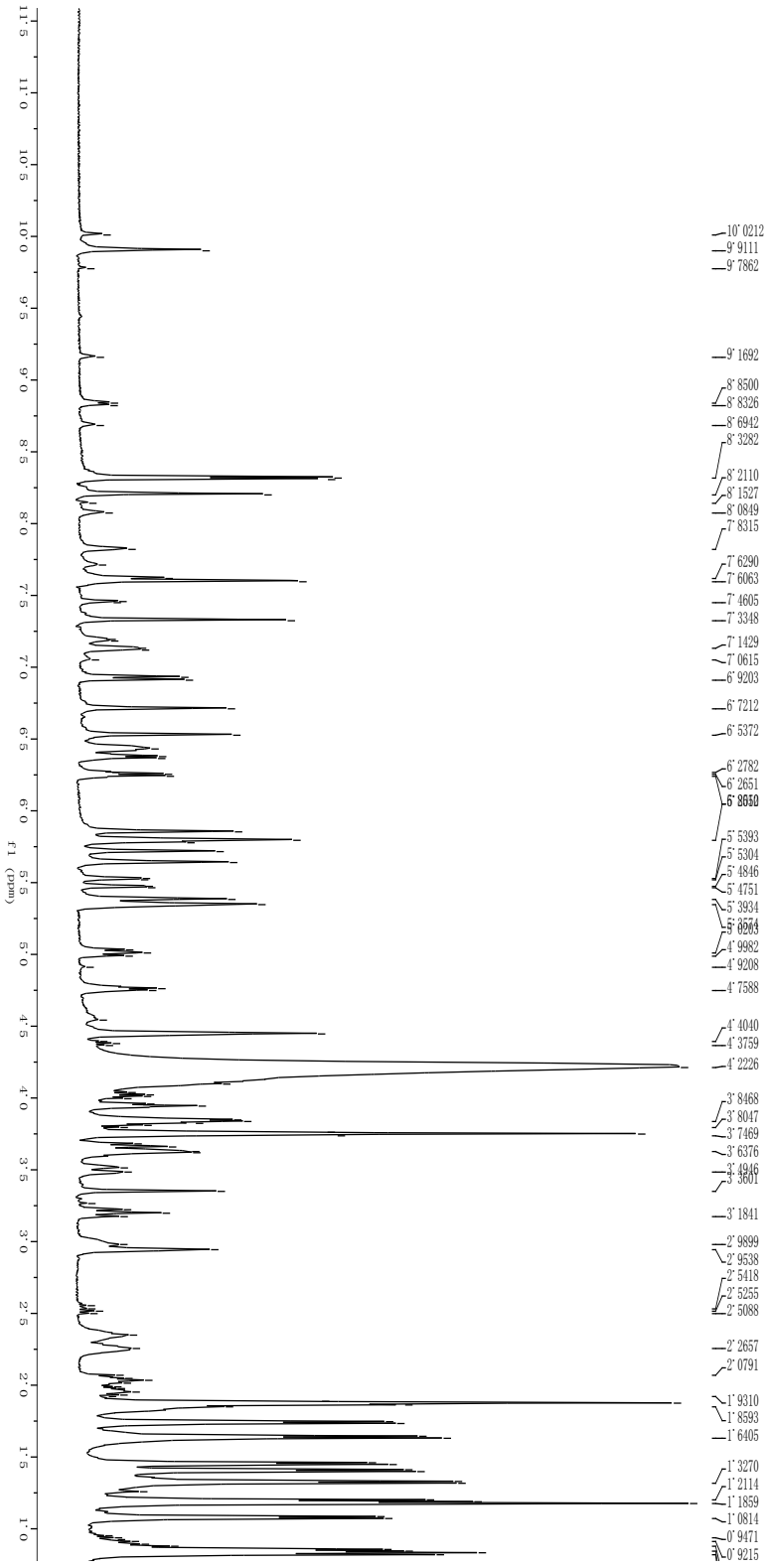
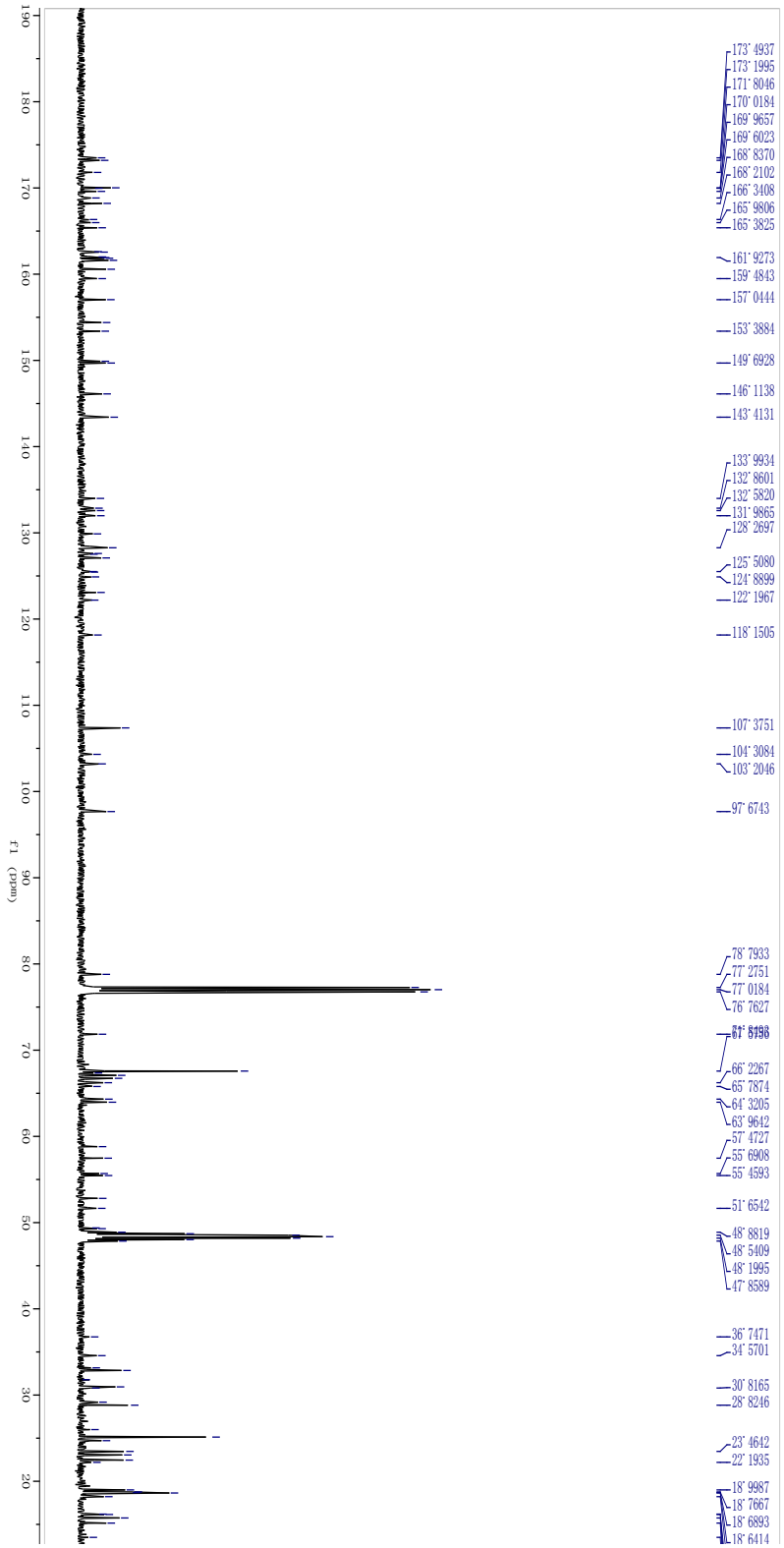


Figure S2. 1D and 2D NMR spectra of 1

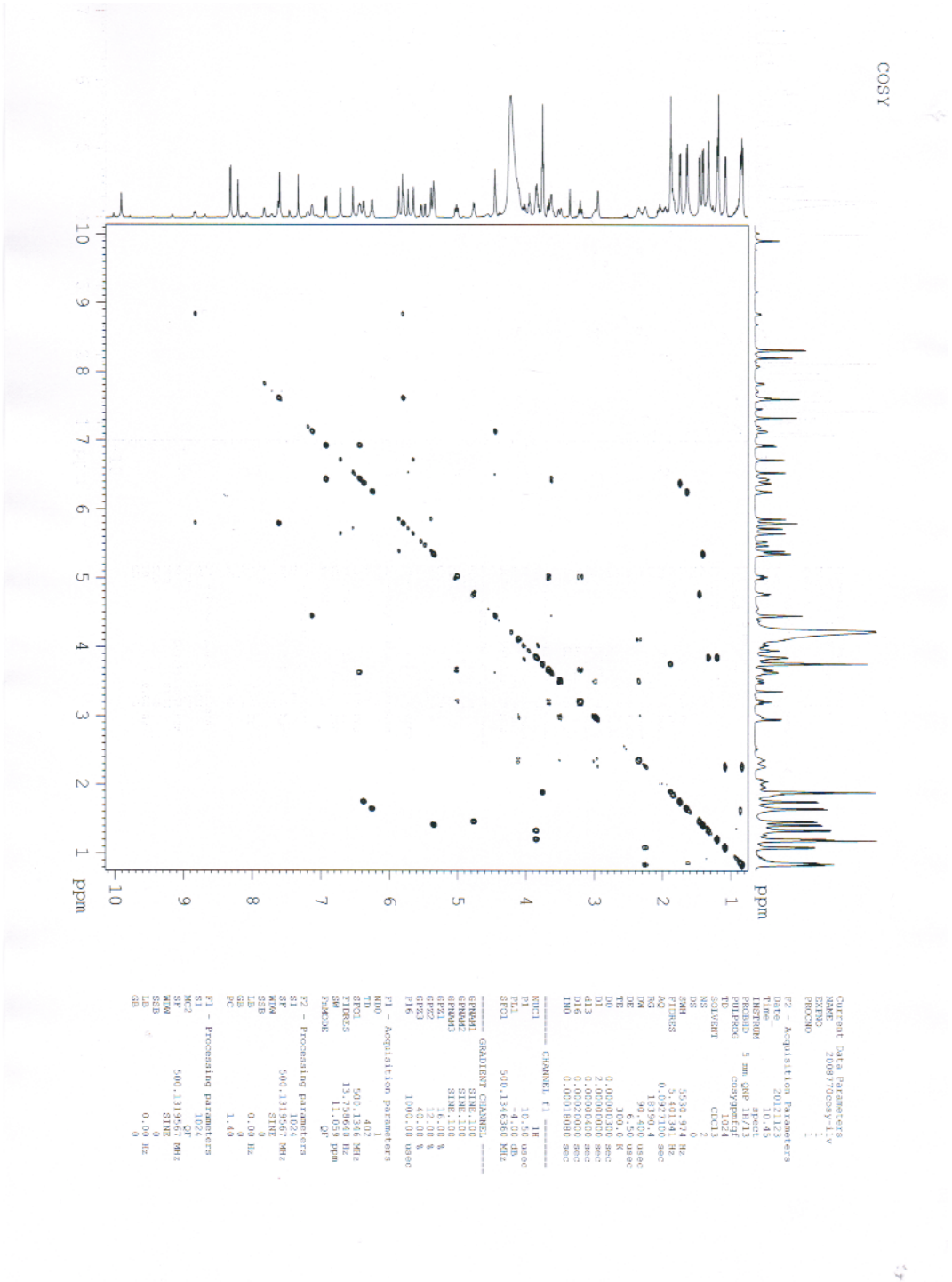
(A) ^{1}H spectrum



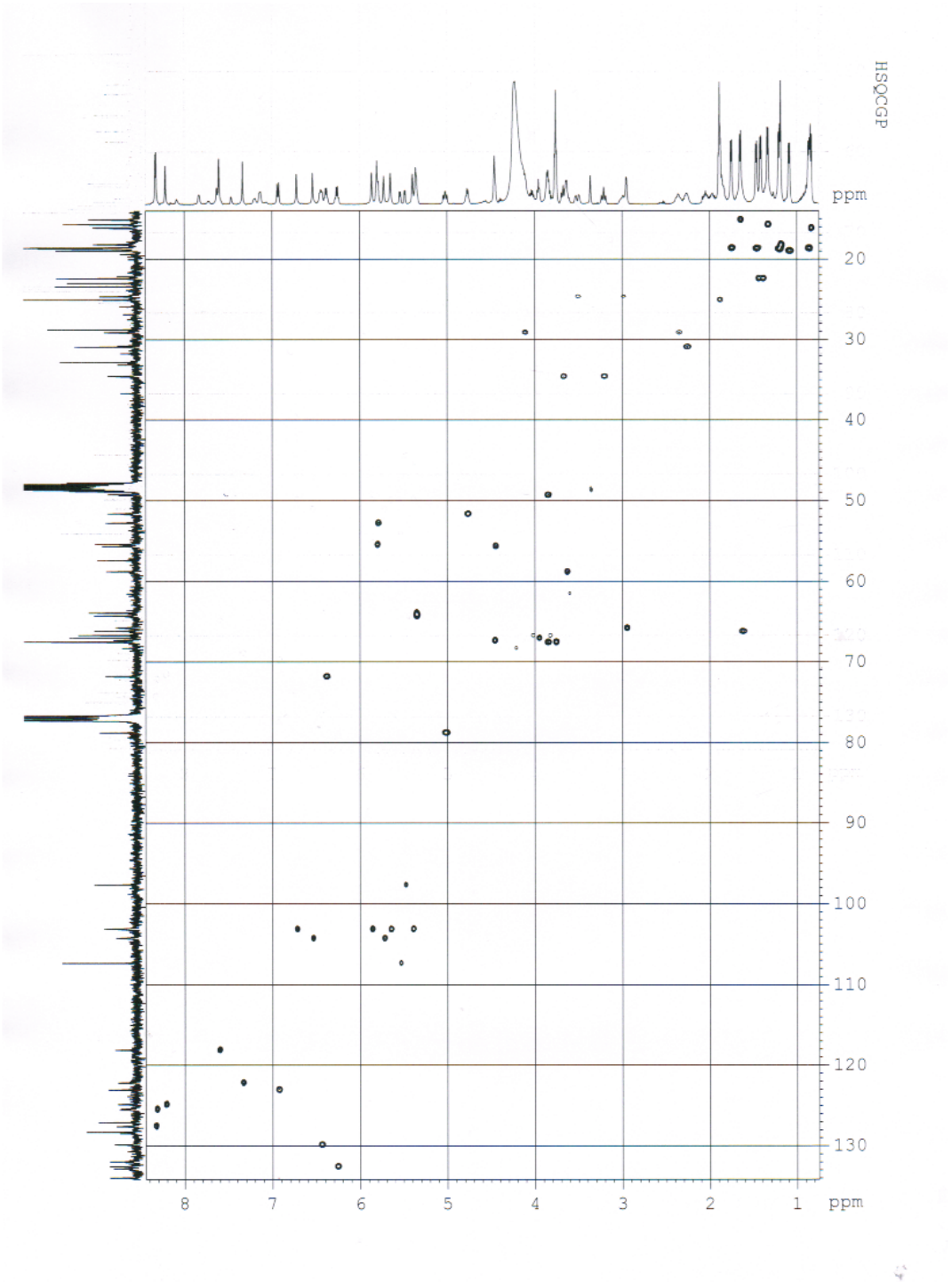
(B) ^{13}C NMR spectrum



(C) COSY spectrum



(D) HSQC spectrum



(E) HMBC spectrum

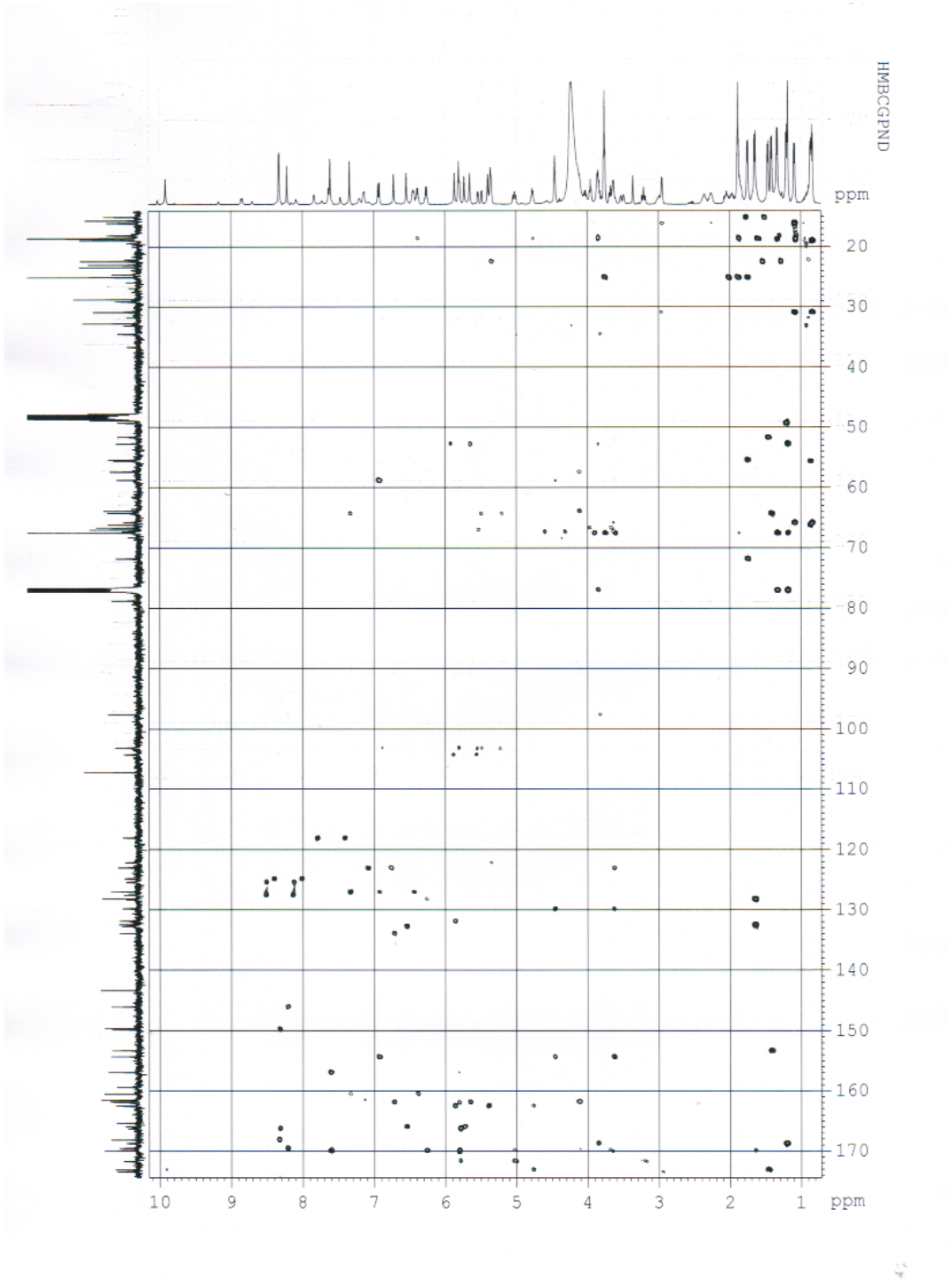
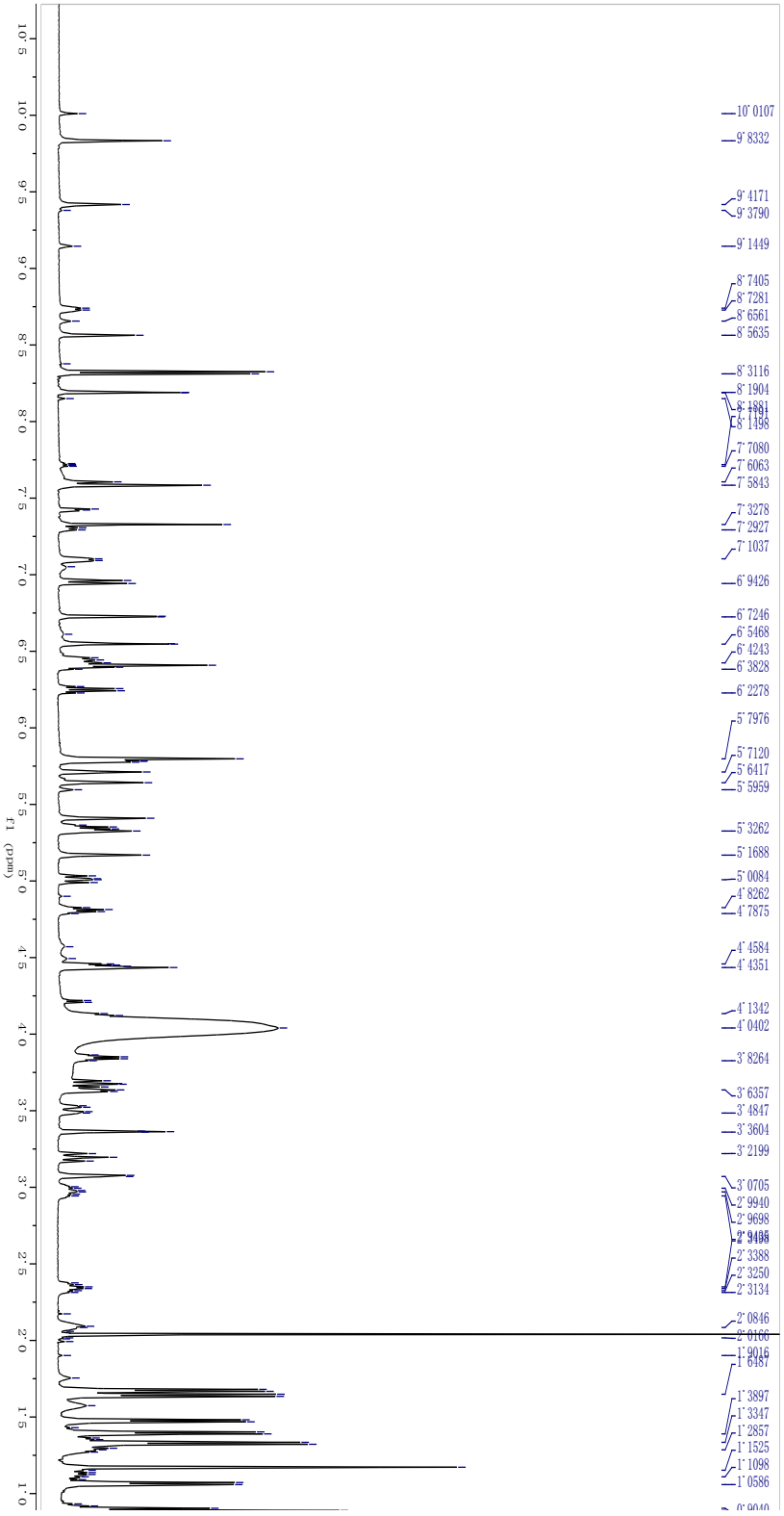
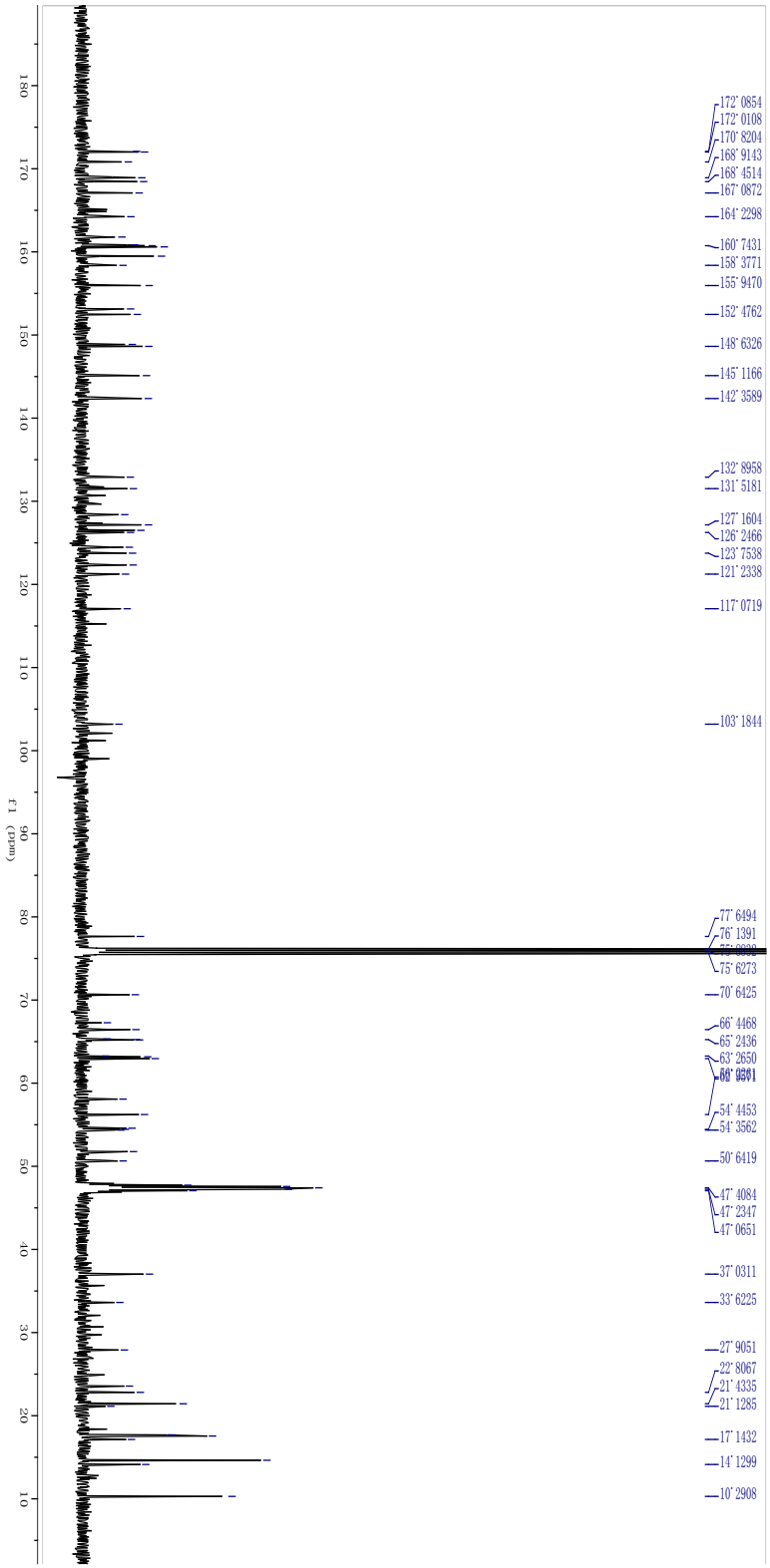


Figure S3. 1D and 2D NMR spectra of 2

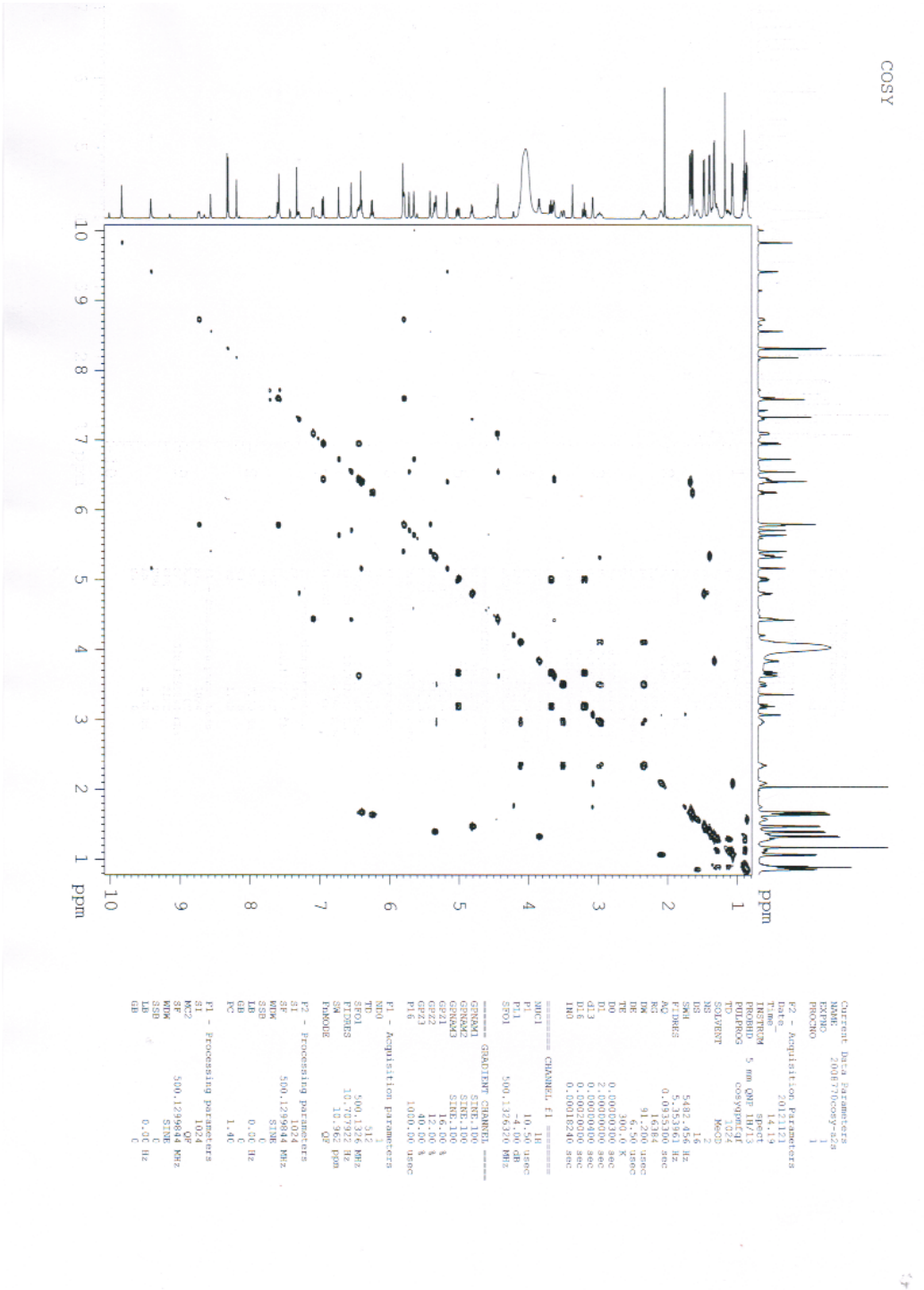
(A) ^{1}H NMR spectrum



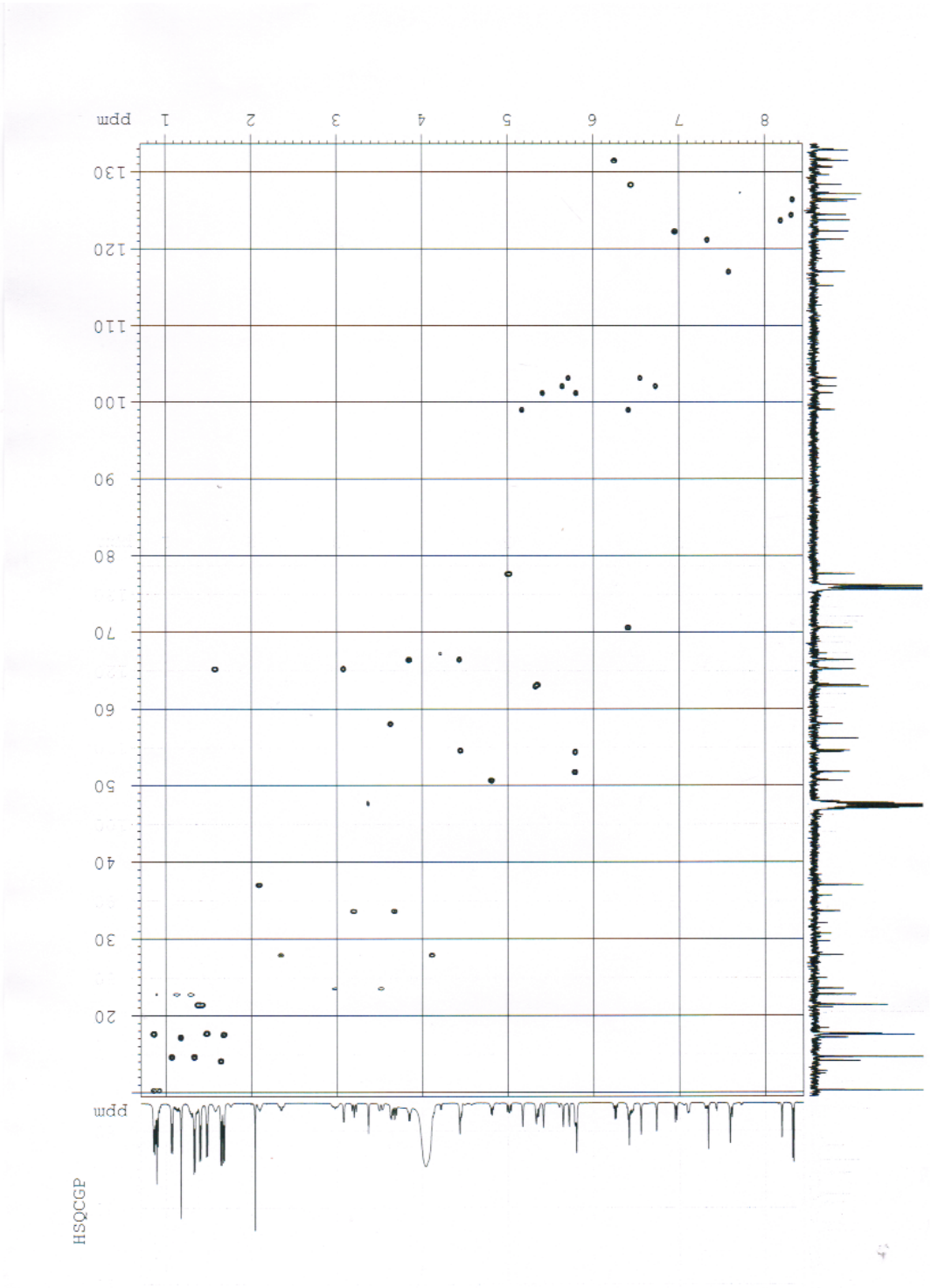
(B) ^{13}C NMR spectrum



(C) COSY spectrum



(D) HSQC spectrum



(E) HMBC spectrum

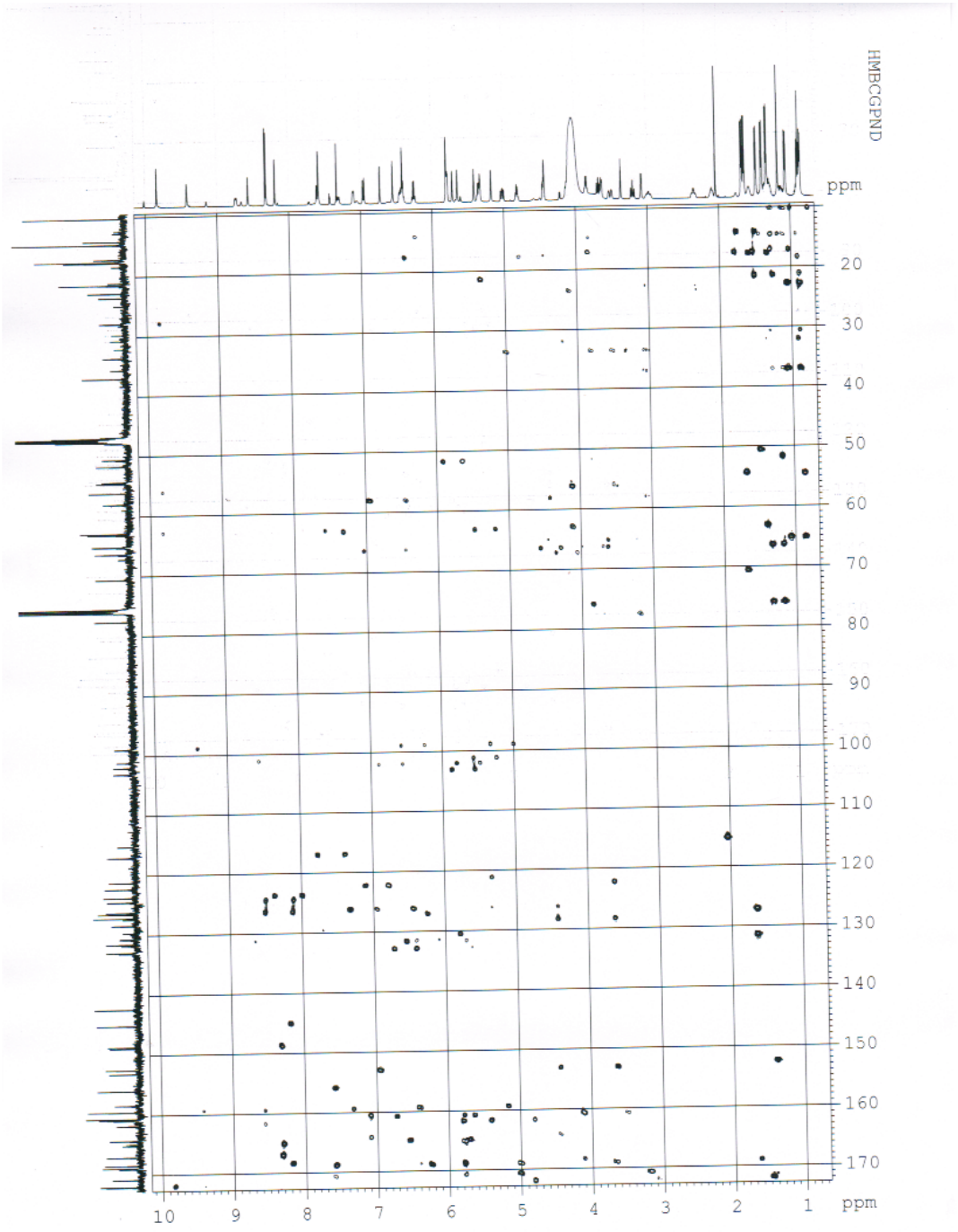
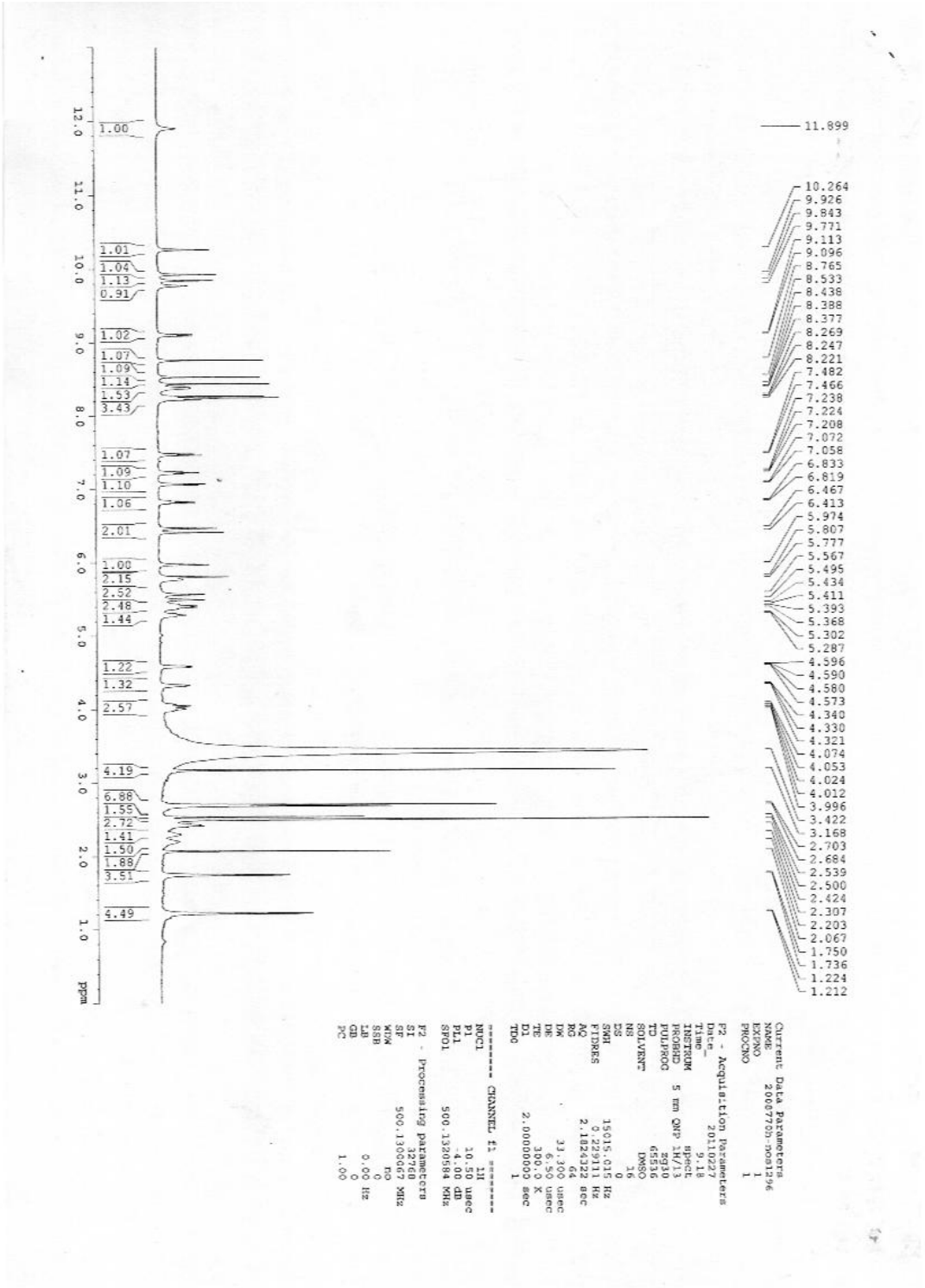
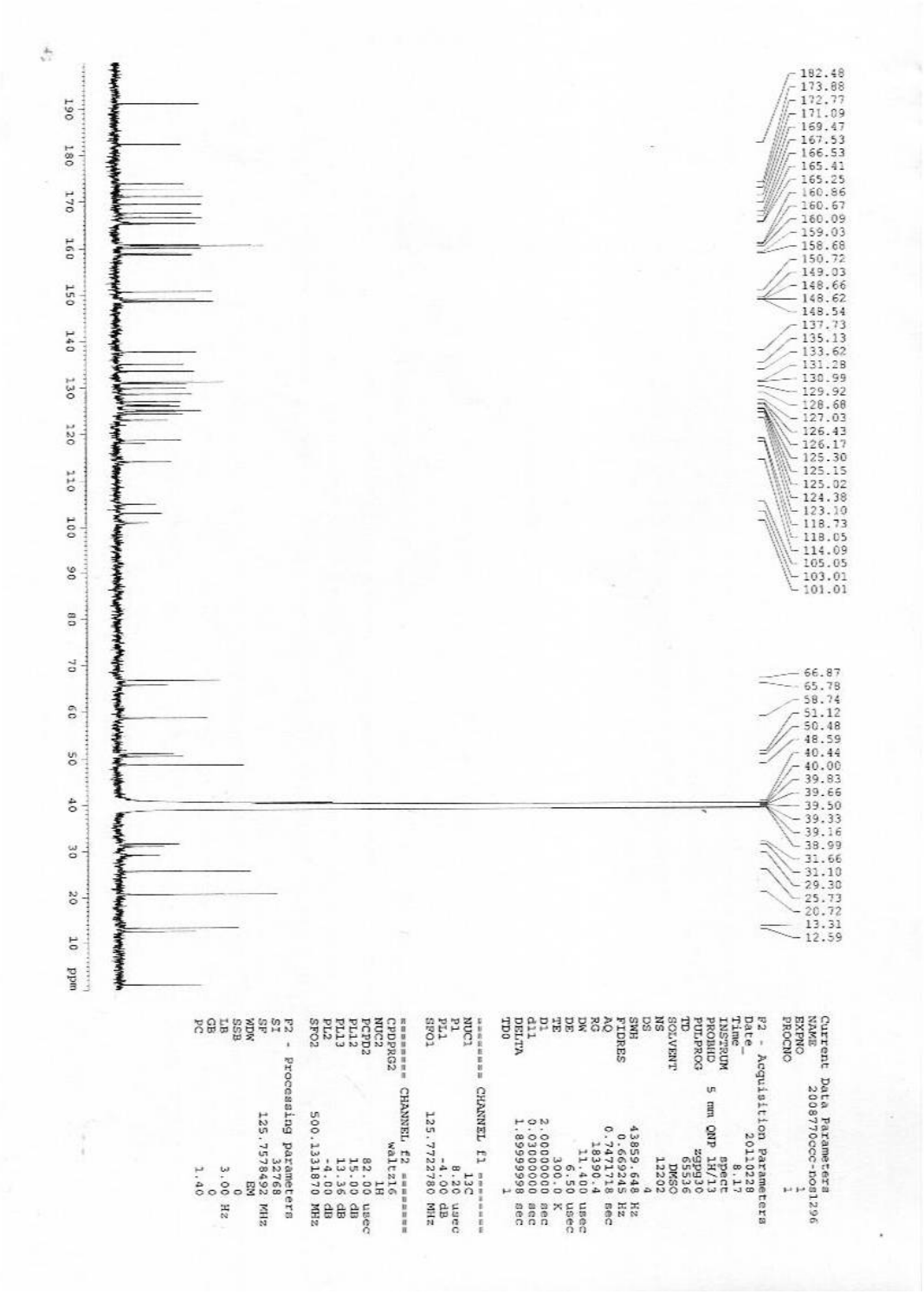


Figure S4. 1D and 2D NMR spectra of 3

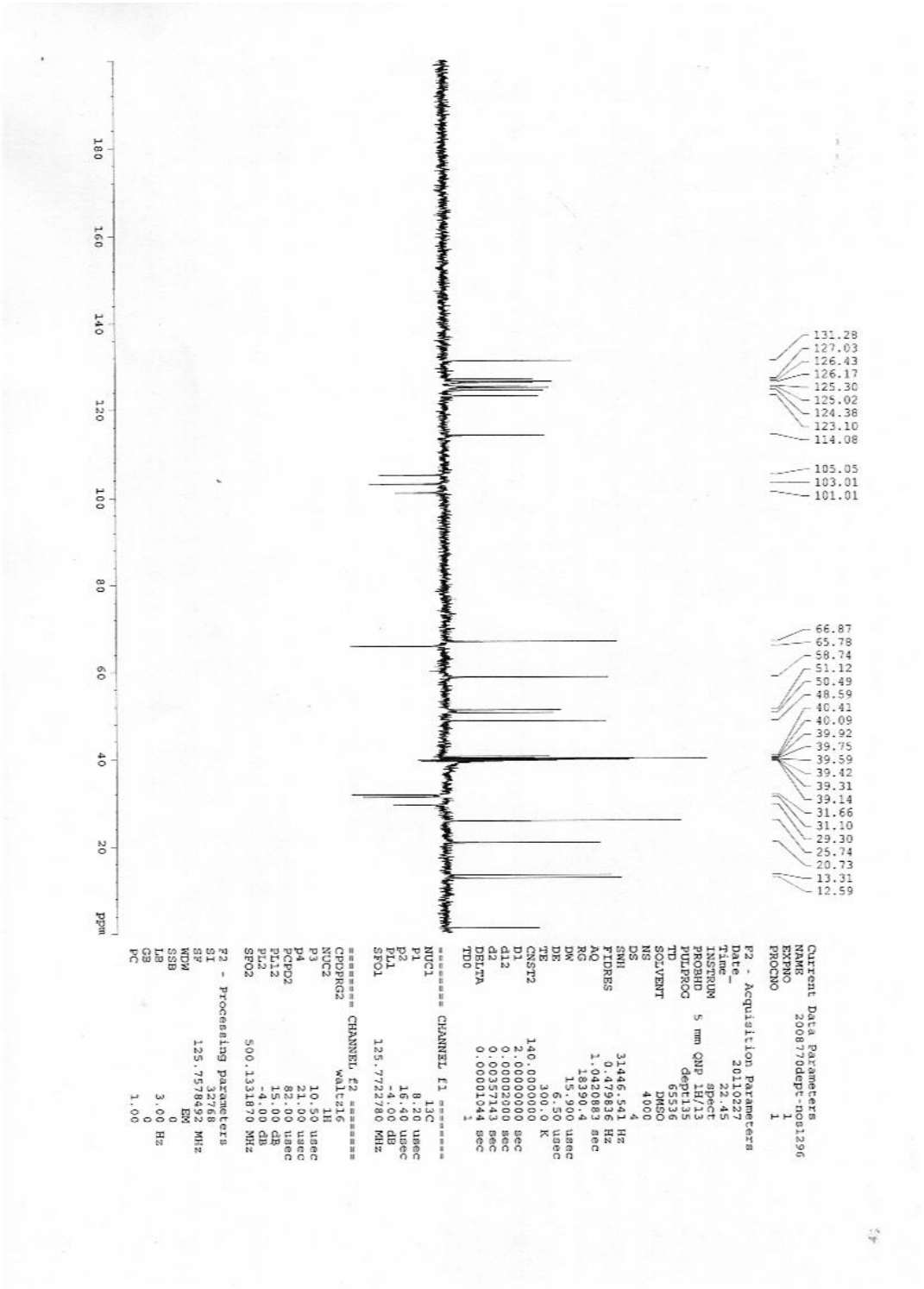
(A) ^{1}H NMR spectrum



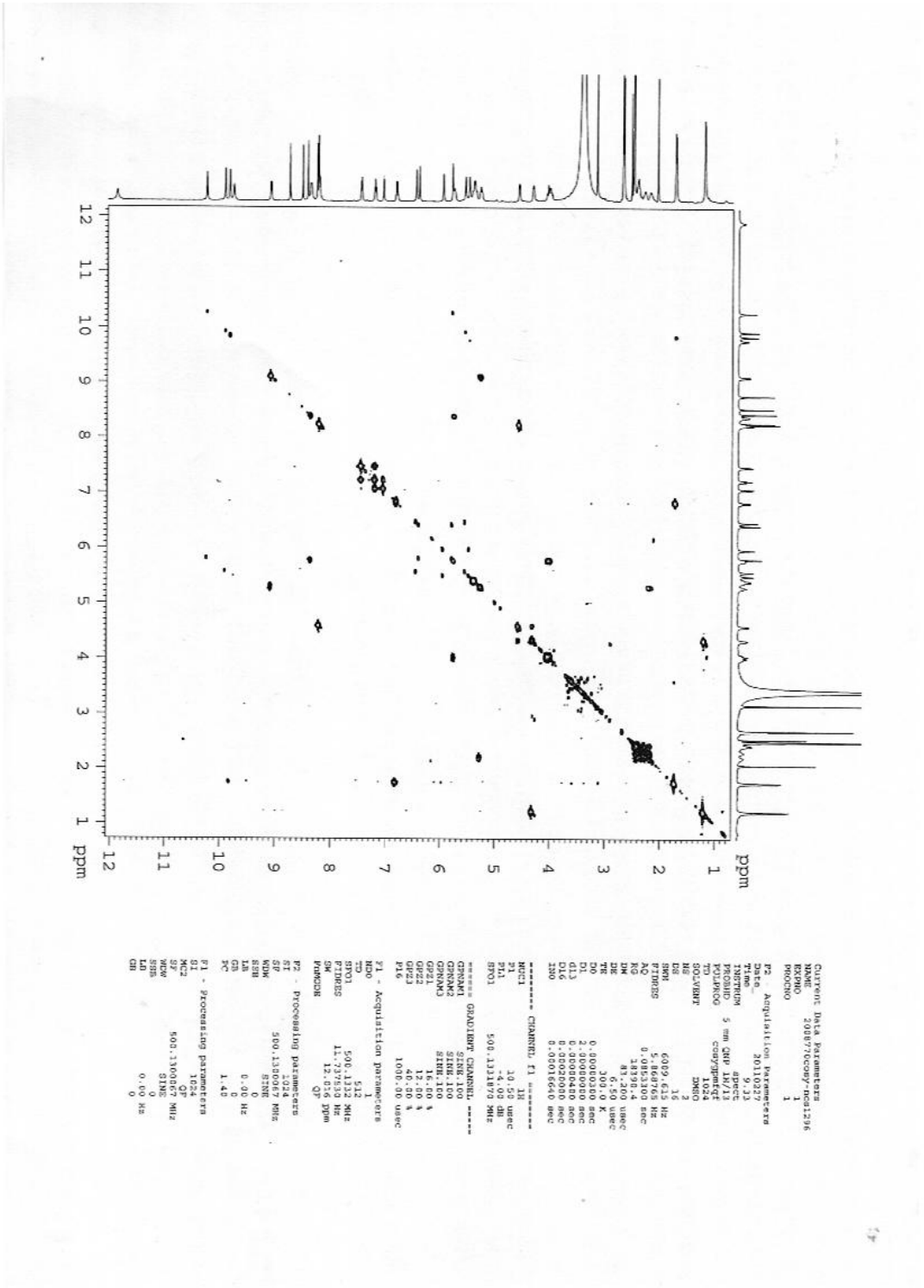
(B) ^{13}C NMR spectrum



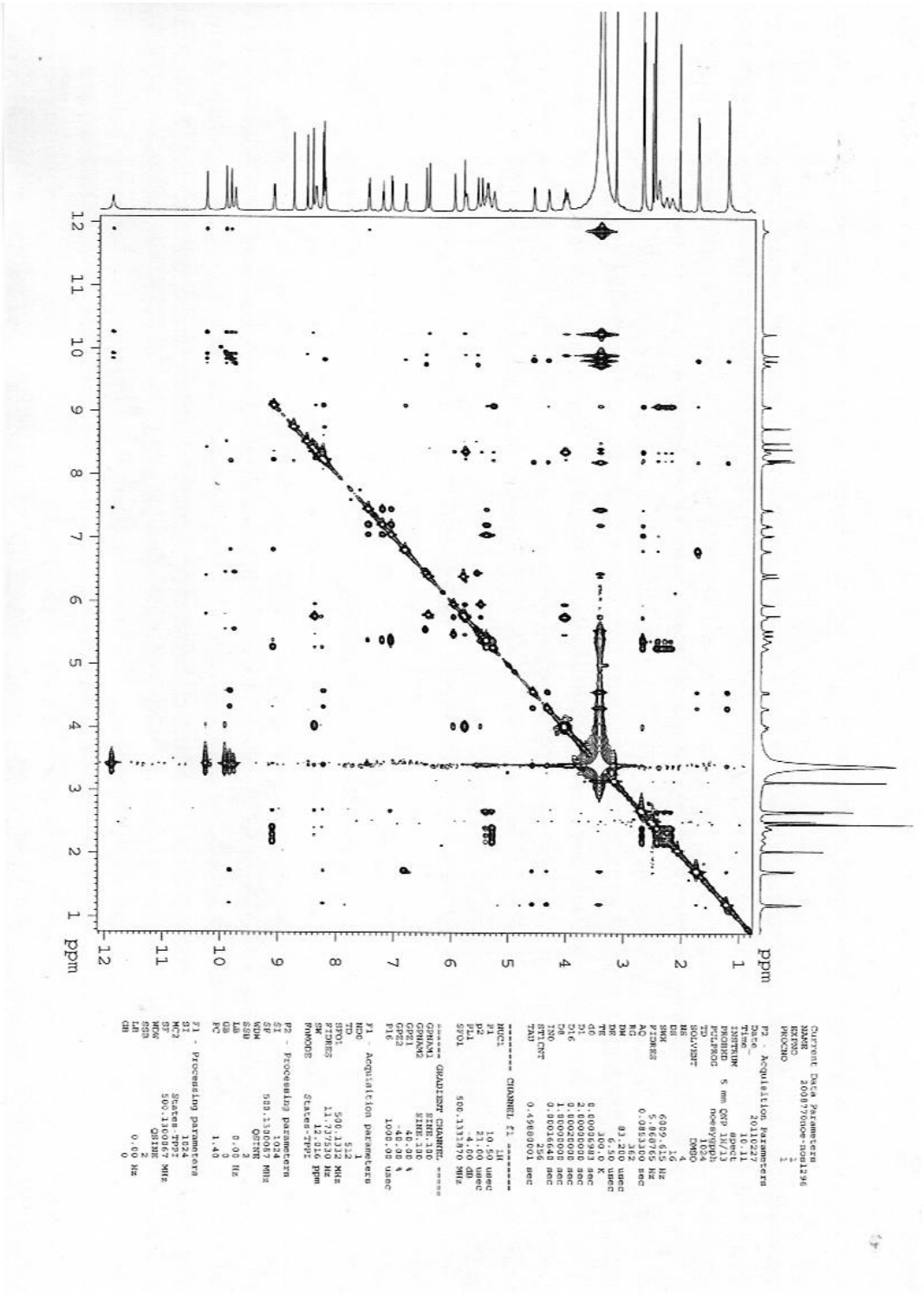
(C) DEPT 135 spectrum



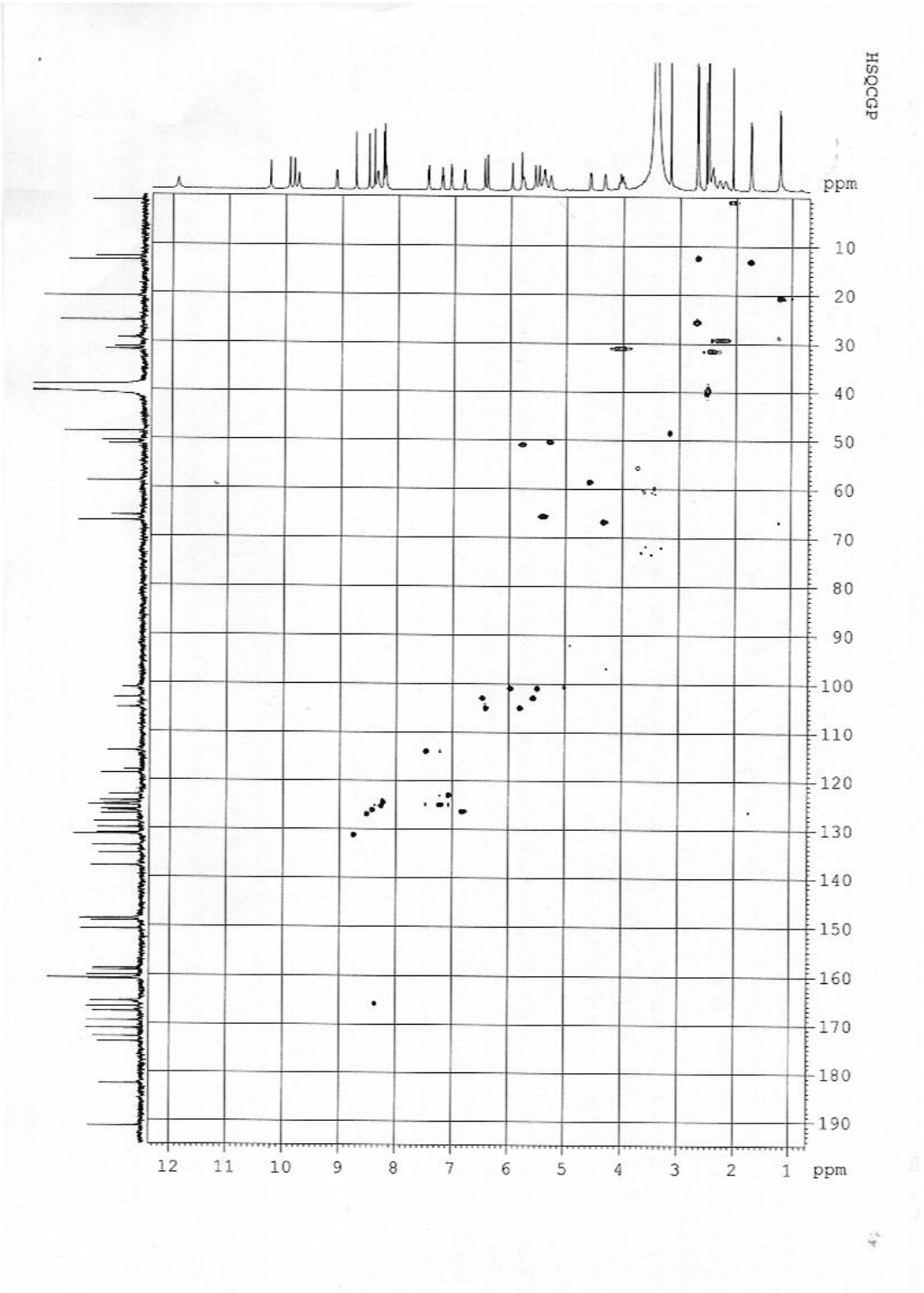
(D) COSY spectrum



(E) NOESY spectrum



(F) HSQC spectrum



(G) HMBC spectrum

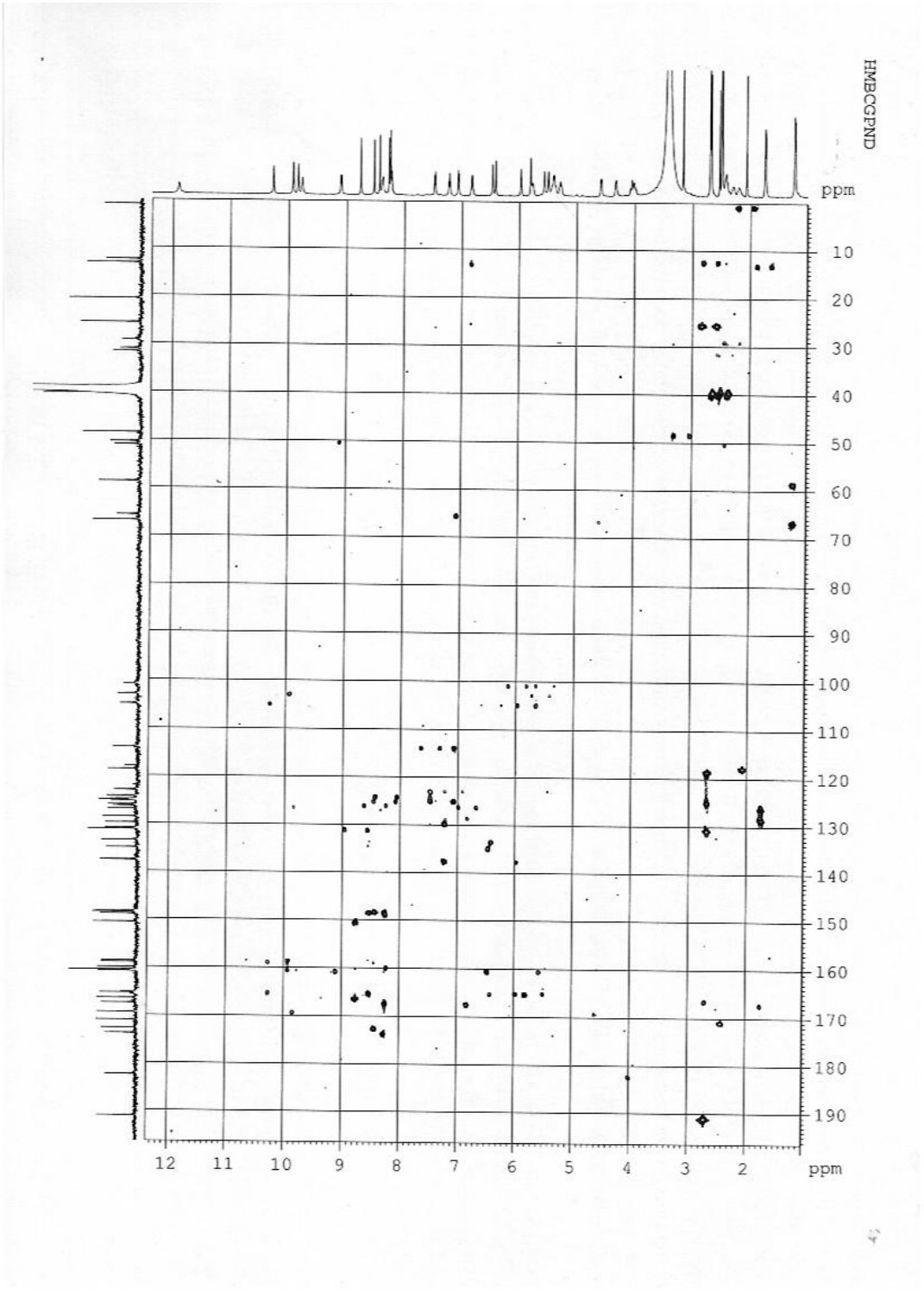
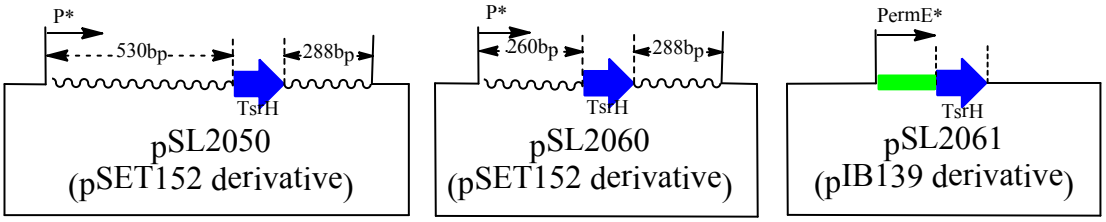
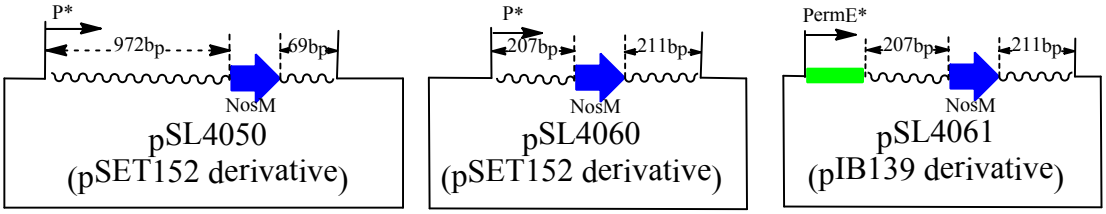


Figure S5. Recombinant plasmids for *trans* expression of *prep*

Recombinant plasmids for *trans* expression of *TsrH*



Recombinant plasmids for *trans* expression of *NosM*



4. Supplementary Tables

Table S1. Bacterial strains and plasmids

Strain/Plasmid	Characteristic(s)	Source / Reference
Strains		
Escherichia coli		
DH5□	Host for general cloning	Invitrogen
ET12567 (pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	1
S17-1	Donor strain for conjugation between *E. coli* and *Streptomyces*	ATCC
Streptomyces		
laurentii	Thiostrepton-producing	ATCC
actuosus	Nosiheptide-producing	ATCC
SL2051	*S. laurentii* derivative, in which the codon GAG for Glu-7 of TsrH was mutated into the stop codon TAG	This study
SL2052	SL2051 derivative, containing pSL2050 for producing the precursor peptide TsrH	This study
SL2053	SL2051 derivative, containing pSL2051 for producing TsrH-Ile1Val	This study
SL2054	SL2051 derivative, containing pSL2052 for producing TsrH-Ala2Ser	This study
SL2055	*S. laurentiiwas* derivative, containing pSL2050 for *tsrH* duplication	This study
SL2057	*S. laurentii* derivative, in which the codon GGA for Gly-30 of TsrH was mutated into the stop codon TGA	This study
SL2058	SL2051 derivative, containing pSL2060 for producing the	This study

	precursor peptide TsrH	
SL2059	SL2051 derivative, containing pSL2061 for producing the precursor peptide TsrH	This study
SL2060	SL2057 derivative, containing pSL2050 for producing the precursor peptide TsrH	This study
SL4051	*S. actuosus* derivative, in which the codon AAG for Lys-5 of NosM was mutated into the stop codon TAG	This study
SL4052	SL4051 derivative, containing pSL4050 for producing the precursor peptide NosM	This study
SL4053	*S. actuosus* derivative, containing pSL4050 for *nosM* duplication	This study
SL4054	*S. actuosus* derivative, containing pSL4051 for *nosM* triplication	This study
SL4055	*S. actuosus* derivative, in which *nosO* was inactivated	This study
SL4056	SL4055 derivative, containing pSL4051	This study
SL4057	*S. actuosus* derivative, in which the codon GAG for Glu-23 of NosM was mutated into the stop codon TAG	This study
SL4058	*S. actuosus* derivative, in which the codon CGA for Arg-14 of NosM was mutated into the stop codon TGA	This study
SL4059	SL4057 derivative, containing pSL4060 for producing the precursor peptide NosM	This study
SL4060	SL4057 derivative, containing pSL4061 for producing the precursor peptide NosM	This study
SL4061	SL4058 derivative, containing pSL4060 for producing the precursor peptide NosM	This study
SL4062	SL4058 derivative, containing pSL4061 for producing the precursor peptide NosM	This study

SL4063	SL4051 derivative, containing pSL4060 for producing the precursor peptide NosM	This study
SL4064	SL4051 derivative, containing pSL4061 for producing the precursor peptide NosM	This study
Plasmids		
pMD19-T simple	*E. coli* subcloning vector	Takara
pKC1139	Apramycin resistant *E. coli Streptomyces* shuttle vector for gene inactivation, temperature sensitive replication in *Streptomyces*	9
pSET152	*E. coli-Streptomyces* shuttle vector containing the *aac(3)IV* gene and the C31 *attP* site and integrase gene	10
pOJ260	Apramycin resistant *E. coli Streptomyces* suicide vector for gene inactivation	10
pIB139	pSET152 derivative with the *ermE** promoter	11
pSL2050	pSET152 derivative for *in trans* expressing *tsrH* with the 580 bp upstream sequence and 288 bp downstream region	This study
pSL2051	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH-Ile1Val	This study
pSL2052	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH-Ala2Ser	This study
pSL2053	pOJ446-based *S.laurentii* genomic library cosmid, containing the partial thiostrepton biosynthetic gene cluster	3
pSL2054	pET28a derivative containing a SpeI site	This study
pSL2055	pSL2053 derivative carrying a 2.2 kb fragment that harbors *tsrH*	This study
pSL2056	pSL2054 derivative, in which the codon GAG for Glu-7 of	This study

	TsrH was mutated into the stop codon TAG	
pSL2057	pOJ260 derivative, in which the codon GAG for Glu-7 of TsrH was mutated into the stop codon TAG	This study
pSL2058	pSL2054 derivative, in which the codon GGA for Gly-30 of TsrH was mutated into the stop codon TGA	This study
pSL2059	pOJ260 derivative, in which the codon GGA for Gly-30 of TsrH was mutated into the stop codon TGA	This study
pSL2060	pSET152 derivative for *in trans* expressing *tsrH* with the 260 bp upstream sequence and 288 bp downstream region	This study
pSL2061	pIB139 derivative for *in trans* expressing *tsrH*	This study
pSL4050	pSET152 derivative for *in trans* expressing *nosM* with the 972 bp upstream sequence and 69 bp downstream region	This study
pSL4051	pSET152 derivative, in which *nosM* was duplicated	This study
pSL4052	pKC1139 derivative, in which the codon AAG for Lys-5 of NosM was mutated into the stop codon TAG	This study
pSL4053	pOJ446-based *S.actuosus* genomic library cosmid, containing the nosiheptide biosynthetic gene cluster	4
pSL4054	pKC1139 derivative for deleting *nosO* in frame	This study
pSL4055	pMD19-T derivative containing a 1.7 kb PCR product from pSL4001	This study
pSL4056	pMD19-T derivative containing a 1.8 kb PCR product from pSL4001	This study
pSL4058	pKC1139 derivative, in which the codon GAG for Glu-23 of NosM was mutated into the stop codon TAG	This study
pSL4059	pKC1139 derivative, in which the codon CGA for Arg-14 of NosM was mutated into the stop codon TGA	This study
pSL4060	pSET152 derivative for *in trans* expressing *nosM* with the 207 bp upstream sequence and 211 bp downstream region	This study

pSL4061	pIB139 derivative for *in trans* expressing *nosM* with the 207 bp upstream sequence and 211 bp downstream region	This study

Table S2. Primers used in this study

Name	Sequences
Spe-P1	CCGGAACTAGTTCCTCCTTTCAG (SpeI site underlined)
Spe-P2	ATTGGCGAATGGGACGCGC
H3-F	GCTCGACGGTTAGGACCTGACCGT (used for replacement of the base "G")
H3-R	ACGGTCAGGTCCTAACCGTCGAGC (used for replacement of the base "G")
SB-F	CCCGGATCCACAGGTCGACCAGCCAGAA (BamHI site underlined)
SB-R	GGAACTAGTTGTCCACGCCGTCATCGG (SpeI site underlined)
I1V-F	GACCTGACCGTCACGATGGTCGCGTCCGCCTCCTGCA (used for replacement of the base "A")
I1V-R	TGCAGGAGGCGGACGCGACCATCGTGACGGTCAGGTC (used for replacement of the base "A")
A2S-F	CTGACCGTCACGATGATCTCGTCCGCCTCCTGCACCAC (used for replacement of the base "G")
A2S-R	GTGGTGCAGGAGGCGGACGAGATCATCGTGACGGTCAG (used for replacement of the base "G")
KM-F	GCATAAGCTTCCTGGAACACGCACATGGTG (HindIII site underlined)
M3-F	GCGAGGTCGTGGCCTAGGTCATGTCGGCCTCG (used for replacement of the base "A")
M3-R	CGAGGCCGACATGACCTAGGCCACGACCTCGC (used for replacement of the base "A")
KM-R	GCTATCTAGAGGCCCTTCTGGTGGGACTTG (XbaI site underlined)
MC-F	GACGGATCCTCCAGCCAGCGGTCGAAGG (BamHI site underlined)
MC1-R	CGGAGGCCTTTCGGGTCGAGAAAAGCGG (StuI site underlined)
MC1-F	GAAAGGCCTCATGACGCTGCACACCTG (StuI site underlined)
MC-R	CAGTCTAGAGAGATGTACTTCTGGCGCAC (XbaI site underlined)
O-inf-Ls	AAGCTTTCTCCGTTACCCTCCCCACC (HindIII site underlined)
O-inf-La	GAATTCGAGCCCGATCTCCAGCCAG (XbaI site underlined)
O-inf-Rs	GAATTCGAGTCGATCTTCGACACCG (XbaI site underlined)
O-inf-Ra	TCTAGACGAGAACCGTCGTGTGGTCCAG (EcoRI site underlined)
H1-F	ATCGGTGTCGAGTGACTCACGGGT (used for replacement of the base "G")
H1-R	ACCCGTGAGTCACTCGACACCGAT (used for replacement of the base "G")
SE	CCAGAATTCGACGGCCGAAGTGGCGCAT (EcoRI site underlined)
SB-R	GGAACTAGTTGTCCACGCCGTCATCGG (SpeI site underlined)

SB-F3	GTGCATATGATGAGCAATGCCGCCCTGG	(NdeI site underlined)
SB-R3	GGTGAATTCTCAGGAGCTGCAGCTGCAG	(EcoRI site underlined)
Nos16com-F3	GCATAAGCTTAGGGCCGGAATCCTTGAC	(HindIII site underlined)
Nos16com-R3	GCTAGAATTCGGACCCAGTCGCAGAACG	(EcoRI site underlined)
M1-R	GACATCGACGCTCTCTAGATCTCCGAGTTCCTG	(used for replacement of the base “G”)
M1-F	CAGGAACTCGGAGATCTAGAGAGCGTCGATGTC	(used for replacement of the base “G”)
M1-R	CCTGGACGAGAGCTGACTGGAGGACAGCGAG	(used for replacement of the base “C”)
M1-F	CTCGCTGTCCTCCAGTCAGCTCTCGTCCAGG	(used for replacement of the base “C”)

Table S3. ^{1}H and ^{13}C NMR assignments of t compounds 1, 2 in $CDCl_3$:CD_3OD 3:1 (δ in ppm, J in Hz). The data for thiostrepton[12] serve as the reference. The blue box show the differences between compound **1** and thiostrepton and the yellow box show the differences between compound **1** and thiostrepton.

Build blocks	Thiostrepton		Compound **1**		Compound **2**	
	^{13}C chemistry	^{1}H chemistry	^{13}C chemistry	^{1}H chemistry	^{13}C chemistry	^{1}H chemistry
A-1	173.71		169.79		172.01	
A-2	65.60	2.81 (d, 4.58)	65.72	2.95 (d, 3.46)	64.96	3.06 (d, 3.61)
A-3	38.49	1.69 (m)	31.80	2.27 (m)	57.74	
A-4	15.52	0.82 (d, 6.91)	18.66	1.09 (d, 6.72)	14.30	1.05 (d, 6.81)
A-5	24.57	1.20 (m), 0.95 (m)	15.20	0.84 (d, 6.88)	22.50	1.26 (m) ; 1.10 (m)
A-6	11.25	0.74 (t, 7.13)			10.00	0.89 (t, 7.36)
A-NH						
B-1	168.84		168.77		159.37	
B-2	49.35	3.67 (dd, 5.40, 6.81)	49.07	3.84 (q, 6.29)	132.84	
B-3	18.88	1.03 (d, 6.80)	18.36	1.20 (d, 12.76)	98.86	6.40 (s) ; 5.16 (s)
B-NH		7.62 (d, 5.33)		7.62 (d, 11.39)		9.42 (s)
C-1	162.92		162.52		161.60	
C-2	132.28		131.87		130.54	
C-3	102.79	5.66 *E* (d, 1.92); 5.19*Z* (s, br)	103.20	5.39 (s); 5.86 (s)	101.06	5.79 *E* (s) ; 5.40 *Z* (s)
C-NH		7.82 (s, br)		7.83 (s, br)		8.56(s)
D-1	173.31		172.20		172.03	
D-2	51.95	4.59 (dd, 6.48, 7.77)	51.41	4.76 (q, 6.19)	50.40	4.79 (m)

D-3	18.81	1.28 (d, 6.62)	18.38	1.46 (d, 6.34)	17.37	1.47 (d, 6.53)
D-NH		6.99 (d, 7.78)		7.14 (d, 6.50)		7.09 (d, 5.60)
E-1	64.21	5.16 (s, br)	61.50	3.60 (s)	62.81	5.32 (m)
E-2	161.94		161.83		160.72	
E-3	24.57	3.32 (m); 2.76 (m)	24.45	2.97 (m); 3.45 (m)	23.34	3.50 (m) ; 2.96 (m)
E-4	29.16	3.92 (m); 2.16 (m)	28.91	2.31 (m); 4.09 (m)	27.61	4.10 (m) ; 2.33 (m)
E-5	57.57		57.28		55.96	
E-5-NH		9.72 (s, br)		9.79 (s, br)		9.83 (s, br)
F-1	161.67		168.05		160.32	
F-2	146.40		146.00		145.32	
F-3	124.88	8.01 (s)	124.79	8.20 (s)	123.65	8.18 (s)
F-4	169.73		169.94		168.43	
G-1	165.47		169.97		164.37	
G-2	55.63	4.27 (dd, 3.25, 7.60)	55.43	4.44 (m)	54.36	4.44 (m)
G-3	66.46	1.35 (m)	65.91	1.60 (m)	64.95	1.55 (m)
G-4	18.92	0.70 (d, 6.50)	18.34	0.86 (d, 5.97)	17.30	0.84 (d, 6.18)
G-NH		6.91 (d, 7.66)		6.93 (d, 9.97)		6.95 (d, 9.81)
H-1	128.45		128.27		127.07	
H-2	132.53	6.07 (q, 7.10)	132.58	6.26 (q, 6.78)	131.42	6.25 (q, 7.00)
H-3	15.20	1.46 (d, 7.05)	13.32	1.65 (d, 6.87)	13.82	1.64 (d, 7.04)
H-NH		8.47 (s, br)		8.69 (s, br)		8.66 (s, br)
I-1	171.97		171.80		170.71	
I-2	78.98	4.83 (dd, 9.62, 12.60)	78.79	5.02 (t, 10.62)	77.42	5.02 (dd, 9.3, 12.55)

I-3	34.78	3.51 (dd, 9.00, 11.50)	34.57	3.19 (t, 12.04); 3.66 (t, 12.04)	33.24	3.20 (m); 3.66 (m)
I-4	170.16		170.02		168.86	
J-1	53.06	5.62 (d, 9.90)	52.48	5.80 (s)	54.12	5.78 (m)
J-2	77.19		76.76		75.78	
J-3	67.73	3.67 (d)	67.37	3.84 (m)	66.20	3.84 (q, 6.26)
J-4	15.88	1.15 (d, 6.63)	14.80	1.33 (d, 6.26)	14.31	1.33 (d, 6.39)
J-5	18.34	0.99 (s)	17.20	1.19 (s)	16.87	1.17 (s)
J-NH		7.43 (d, 9.92)		7.46 (d, 3.86)		7.42 (d, 2.66)
K-1	162.10		165.40		167.04	
K-2	150.09		149.74		148.63	
K-3	125.36	8.13 (s)	125.39	8.31 (s)	124.37	8.31 (s)
K-4	166.38		166.26		165.16	
L-1	55.83	5.62 (d, 7.62)	55.23	5.78 (d, 8.54)	54.11	5.46 (m)
L-2	72.00	6.19 (m)	71.64	6.38 (m)	70.43	6.39 (m)
L-3	18.68	1.56 (d, 6.47)	18.32	1.75 (d, 6.38)	17.24	1.67 (d, 6.51)
L-NH		8.62 (d, 8.80)		8.84 (d, 8.72)		8.73 (d, 6.21)
M-1	157.17		156.99		153.10	
M-2	118.16	7.40 (s)	118.05	7.60 (s)	116.97	7.58 (s)
M-3	168.35		170.01		168.87	
N-1	159.56		167.20		158.20	
N-2	149.92		149.60		145.80	
N-3	127.55	8.14 (s)	127.46	8.33 (s)	126.41	8.32 (s)
N-4	165.40		168.20		172.02	

O-1	162.00		161.90		160.81	
O-2	134.20		133.90		132.80	
O-3	103.28	6.54 *E* (d, 1.93); 5.48 *Z* (d, 1.93)	103.30	6.71 *E* (s) ; 5.64 *Z* (s)	101.97	6.72 *E* (s); 5.63 *Z* (s)
O-NH		9.82 (s, br)		9.91 (s, br)		9.83 (s, br)
P-1	165.99		165.94		164.83	
P-2	133.02		132.77		131.63	
P-3	104.27	6.37*E* (d, 1.17); 5.52*Z* (d, 1.17)	104.31	5.72 (s); 6.53 (s)	103.04	5.70 (s); 6.54 (s)
P-NH		8.96 (s, br)		9.17 (s, br)		9.15 (s, br)
Q 1	160.80		160.47		159.43	
Q 2	143.56		142.40		152.38	
Q 3	122.26	7.13 (s)	122.12	7.33 (s)	121.11	7.33 (s)
Q 4	153.41		152.20		152.42	
Q 5	123.15	6.73 (d, 10.08)	122.90	6.92 (d, 9.97)	122.22	6.94 (d, 9.80)
Q 6	129.99	6.23 (dd, 5.62, 9.90)	129.80	6.43 (m)	128.34	6.43 (m)
Q 7	59.02	3.46 (dd, 1.70, 5.50)	58.59	3.62 (d, 4.17)	57.86	3.63 (d, 5.00)
Q 8	67.34	4.32 (d, 1.75)	67.13	4.45 (m)	66.21	4.42 (m)
Q 9	154.55		154.31		153.10	
Q 10	127.20		126.98		126.13	
Q 11	64.35	5.16 (d, 6.43)	63.00	5.35 (m)	62.94	5.32 (m)
Q 12	22.53	1.21 (d, 6.61)	22.14	1.41 (d, 6.42)	21.18	1.40 (d, 6.51)

Table S4. ^{1}H and ^{13}C NMR assignments of compounds 3 in d_6-DMSO (δ in ppm, J in Hz). The data for nosiheptide[13] serve as the reference. The red box show the differences between compound **3** and nosiheptide.

Build	Nosiheptide		Compound **3**	
blocks	^{13}C chemistry	^{1}H chemistry	^{13}C chemistry	^{1}H chemistry
A'-1			191.23	
A'-2			25.74	2.70 (s)
A-1	159.45		160.09	
A-2	149.83		150.72	
A-3	125.98	8.65 (s)	131.28	8.77 (s)
A-4	163.85		166.54	
B-1	167.69		169.47	
B-2	56.57	4.57 (dd, 3.75, 7.55)	58.75	4.59 (dd, 3.30, 7.95)
B-3	66.50	4.00 (m)	66.87	4.33 (m)
B-4	18.25	0.95 (d, 6.43)	13.31	1.22 (d, 6.00)
B-NH		7.64 (d, 8.01)		8.23 (d, 8.39)
B-OH		7.58 (s, br)		
C-1	129.27		128.68	
C-2	128.85	6.46 (q, 6.95)	126.44	6.83 (q, 7.00)
C-3	13.50	1.72 (d, 6.95)	20.73	1.74 (d, 7.05)
C-NH		9.32 (s)		9.84 (s)
D-1	159.60		160.87	
D-2	147.62		149.03	
D-3	124.45	8.16 (s)	124.38	8.25 (s)
D-4	166.32		167.53	
E-1	45.15	5.63 (dd, 2.37, 11.86)	50.49	5.29 (m)
E-2	37.60	3.86 (dd, 4.89, 13.86), 3.56 (dd, 5.49, 13.95)	29.30	2.20 (m); 2.29 (m)
E-3	66.40	4.09 (dd, 2.24, 11.89)	31.66	2.42 (m)
E-4	172.62		171.09	
E-NH		8.35 (d, 8.42)		9.10 (d, 8.34)
E-OH		8.00 (s, br)		
F-1	159.80		160.48	
F-2	148.70		148.54	
F-3	125.25	8.30 (s)	125.30	8.27 (s)
F-4	170.04		173.88	

G-1	49.05	5.88 (ddd, 4.89, 5.21, 9.67)	51.13	5.78 (m)
G-2	29.49	1.80 (m), 2.44 (m)	31.10	4.03 (m)
G-NH		7.72 (d, 9.67)		8.38 (d, 6.02)
H-1	153.10		148.62	
H-2	119.98		126.17	8.44 (s)
H-3	166.32		172.77	
I-1	142.52		159.04	
I-2	135.00		133.63	
I-3	150.80		105.05	6.41 *E* (s) ; 5.81 *Z* (s)
I-4	127.12	7.82 (s)		
I-5	129.90			
I-NH				10.26 (s, br)
J-1	158.20		158.68	
J-2	149.57		148.66	
J-3	126.80	8.55 (s)	127.03	8.53 (s)
J-4	167.10		165.42	
K-1	165.00		160.68	
K-2	134.26		135.13	
K-3	103.60	6.37 *E* (s); 5.76 *Z* (s)	103.01	6.47 *E* (s) ; 5.57 *Z* (s)
K-NH		10.04 (s)		9.93 (s)
L-1			165.25	
L-2			137.73	
L-3			101.01	5.97 *E* (s) ; 5.50 *Z* (s)
L-NH				9.77 (s)
M-1	181.80		182.48	
M-2	130.40		130.99	
M-3	118.35		118.05	
M-4	129.20		129.92	
M-5	123.23	7.12 (d, 7.02)	123.10	7.07 (d, 6.90)
M-6	124.91	7.28 (dd, 7.01, 8.32)	125.16	7.22 (dd, 7.31, 7.69)
M-7	114.40	7.60 (d, 8.35)	114.09	7.47 (d, 8.10)
M-8	137.60		137.73	
M-9	124.70		125.02	
M-10	12.23	2.63 (s)	12.59	2.68 (s)
M-11	65.90	5.59 (d, 11.75), 5.40 (d, 11.71)	65.79	5.40 (dd, 11.55, 20.80)
M-NH		11.19 (s, br)		11.89 (s, br)

Table S5. Comparison of Minimum Inhibitory Concentrations (MICs)

Compound	**MIC for *Bacillus subtilis* SIPI-JD1001[a]**	**MIC for *Staphylococcus aureus* SIPI-DJ1002[a]**
Thiostrepton	0. 02 μg/mL	0. 02 μg/mL
1	0. 04 μg/mL	0. 04 μg/mL
2	0. 005 μg/mL	0. 01 μg/mL
Nosiheptide	0.008 μg/mL	0.06 μg/mL
3	> 4 μg/mL	> 4 μg/mL

[a] The test organisms were deposited at the Shanghai Institute of Pharmaceutical Industry (SIPI).

Table S6. Production of thiopeptides by the strains used in this study

Strain	Characteristic(s)	Production (mg/L)	%
S. laurentii		74.0 ± 3.4 (Thiostrepton)	100
SL2051	Production of a truncated precursor peptide TsrH (MSNAALEIGVEGLTGLDVDTLEISDYMDETLLDG*)	0.0	0
SL2052	SL2051/pSL2050	41.0 ± 2.4 (Thiostrepton)	54.2
SL2058	SL2051/pSL2060	12.3 ± 0.6 (Thiostrepton)	16.6
SL2059	SL2051/pSL2061	9.2 ± 0.4 (Thiostrepton)	12.4
SL2057	Production of a truncated precursor peptide TsrH (MSNAALEIGVE*)	0.0	0
SL2060	SL2057/pSL2050	22.9±1.0 (Thiostrepton)	30.9
SL2053	SL2051/pSL2051	36.4 ± 1.7 (Compound **1**)	
SL2054	SL2051/pSL2052	52.3 ± 2.2 (Compound **2**)	
SL2055	*S. laurentii* /pSL2050	97.5 ± 3.6 (Thiostrepton)	131.7
S. actuosus		7.4 ± 0.6 (nosiheptide)	100
SL4051	Production of a truncated precursor peptide NosM (MDAAHLSDLDIDALEISEFLDESRLEDSEVVA*)	0.0	0
SL4052	SL4051/pSL4050	6.6 ± 0.5 (nosiheptide)	89.2
SL4063	SL4051/pSL4060	~0.9 (nosiheptide)	12.3
SL4064	SL4051/pSL4061	3.6 ± 0.2 (nosiheptide)	47.9
SL4057	Production of a truncated precursor peptide NosM (MDAAHLSDLDIDALEISEFLDES*)	0.0	0
SL4059	SL4057/pSL4060	~0.4 (nosiheptide)	5.0
SL4060	SL4057/pSL4061	1.9 ± 0.2 (nosiheptide)	25.8
SL4058	Production of a truncated precursor peptide NosM (MDAAHLSDLDIDAL*)	0.0	0
SL4061	SL4058/pSL4060	~0.6 (nosiheptide)	8.3
SL4062	SL4058/pSL4061	2.4 ± 0.2 (nosiheptide)	31.6
SL4053	*S. actuosus*/ pSL4050	12.5 ± 0.9 (nosiheptide)	168.9

SL4054	*S. actuosus*/ pSL4051	17.6 ±1.2 (nosiheptide)	237.8
			0
SL4055	*S. actuosus* derivative, in which *nosO* was inactivated	Trace (Compound **3**)	
SL4056	SL4055/ pSL4051	3.4 ± 0.2 (Compound **3**)	

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