Supporting Information

Functional Characterization of *ttnI* Completing the Tailoring Steps for Tautomycetin Biosynthesis in *Streptomyces griseochromogenes*

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General

^{1}H and ^{13}C NMR data were recorded at 25°C on Bruker 700 instrument operating 700 MHz for ^{1}H and 175 MHz for ^{13}C nuclei, respectively. ^{1}H and ^{13}C NMR chemical shifts were referenced to residual solvent signals: δ_H 7.26 and δ_C 77.36 for $CDCl_3$. ^{1}H-^{1}H COSY, HMQC, and HMBC were performed using standard Bruker pulse sequences. Optical rotations were measured on AUTOPOL®IV automatic polarimeter (Rudolph Research Analytical) using a quartz cell with 1 mL capacity and a 10 cm path length. High-resolution ESI-MS analyses were acquired on a LTQ orbitrap spectrometer (Thermo). HPLC was carried out on a Varian system equipped with Prostar 210 pumps and a photodiode array detector. RP-C18 silica gel for column chromatography was purchased from Fisher Scientific. Common chemical and biochemicals and chemicals were purchased from Sigma, Fisher Scientific, or commercial sources.

Escherichia coli strains carrying plasmids were grown in Luria-Bertani (LB) medium with appropriate antibiotics selection.[S1] All media for *Streptomyces* growth were prepared according to standard protocols.[S2] ISP-4 and tryptic soy broth (TSB) were from Difco Laboratories (Detroit, MI), and modified ISP-4 is ISP-4 supplemented with 0.05% yeast extract and 0.1% tryptone.[S3] ISP-4 medium and MS medium were used for *Streptomyces griseochromogenes* sporulation at 30°C for 5-7 days. *E. coli* DH5α was used as the host for general subcloning.[S1] *E. coli* ET12567/pUZ8002[S4] was used as the cosmid donor host for *E. coli-Streptomyces* conjugation. *E. coli* BW25113/pIJ790 and *E. coli* DH5α/pIJ773 were provided by John Innes Center (Norwich, UK) as a part of the REDIRECT Technology kit.[S5]

Plasmids and DNA manipulation

Plasmid extraction and DNA purification were carried out using commercial kits (Qiagen, Santa Clarita, CA). Genomic DNAs were isolated according to literature protocol.[S2] The digoxigenin-11-dUTP labeling and detection kit (Roche Diagnostics Corp, Indianapolis, IN) was used for preparation of DNA probes, and Southern hybridization was carried out per manufacturer instructions.

Gene inactivation and complementation procedure

The *ttnC* and *ttnI* gene were inactivated by the REDIRECT Technology according to literature protocols.[S5] Briefly, the apramycin resistance gene *aac(3)IV*/*oriT* cassette was used to replace an internal region of the target gene. Mutant cosmids pBS13024 (*⊿ttnC*) and pBS13031 (*⊿ttnI*) were constructed from pBS13009[S6] and pBS13012[S6] (Table S1), respectively, and introduced into *S. griseochromogenes* by conjugation from *E. coli* ET12567/pUZ8002 according to the previously described procedures.[S6,S7] *S. griseochromogenes* spores were suspended in TSB medium and heat-shocked at 45°C for 15 min, followed by incubation at 30°C for 6 hr. Germinated spores were mixed with *E. coli* ET12567/pUZ8002 harboring pBS13024 or pBS13031 and spread onto modified ISP-4 plates freshly supplemented with 20 mM $MgCl_2$. After incubation at 28°C for 16 to 22 h, each plate was overlaid with 1 mL of sterile H_2O containing apramycin at a final concentration of 30 μg/mL and nalidixic acid at a final concentration of 50 μg/mL. Incubation continued at 28°C until exconjugants appeared. The Δ*ttnC* or Δ*ttnI* double crossover mutant, selected by the apramycin resistant and kanamycin sensitive phenotype, was isolated as SB13012 (Δ*ttnC*) and SB13017 (Δ*ttnI*), whose genotype was verified by PCR (*ttnC*FP2 5'-GCAATGCATGCGATTGATCGTTG-3'/*ttnC*RP2 5'-GCTCTAGATCATTCACCTGTTCCCTT-3' for SB13012) or Southern analysis (for SB13017 and see Table S2 and Figure S2). To construct the expression plasmid of pBS13032 for Δ*ttnI* complementation, the *ttnI* gene was amplified by PCR (Table S3), digested with *Nsi*I and *Xba*I,

and cloned into the same sites of pBS6027[S8] to yield pBS13032. Introduction of pBS13032 into SB13017 by conjugation afforded SB13018, in which the Δ*ttnI* mutation was complemented by the constitutive expression of functional copy of Δ*ttnI*, under the control of the *ErmE** promoter[S2] (Table S3).

Fermentation and HPLC analyses of *S. griseochromogenes* strains

A two stage fermentation procedure was utilized to grow the *S. griseochromogenes* wild-type and recombinant strains SB13012, SB13017, and SB13018 for TTN and analogue production as previously described.[S6,S7] Thus, seed medium (50 mL in a 250-mL flask) was inoculated with spores, and the flasks were incubated on a rotary shaker at 250 rpm and 28°C for 2 days. The resulting seed culture (5 mL) was used to inoculate 50 mL production medium in a 250-mL flask and incubated on a rotary shaker at 28°C and 250 rpm for 5 days. Both seed and production media consist of glucose 2% (separately autoclaved), soluble starch 0.5%, beef extract 0.05%, yeast extract 0.3%, soybean flour 1%, NaCl 0.1%, K_2HPO_4 0.0025%, pH 7.0, and were sterilized by autoclaving at 121°C for 30 min.

The fermentation broth was adjusted to pH 4.0 with 1 N HCl and centrifuged and filtered to remove the mycelia. The resulting supernatant was extracted twice with an equal volume of EtOAc. The combined EtOAc extracts were concentrated in vacuo to afford an oily residue. The latter was dissolved in CH_3CN, filtered through a 0.2 μM filter, and subjected to HPLC analysis. The mycelia were extracted twice with acetone. The combined acetone extracts were concentrated in vacuo to afford the water phase. The resulting water phase was extracted twice with EtOAc. The combined EtOAc extracts were concentrated in vacuo to afford an oily residue. The latter was dissolved in CH_3CN, filtered through a 0.2 μM filter, and subjected to HPLC analysis. The HPLC was performed on an Alltech Alltima C18 column (5 μM, 250 x 4.6 mm) eluted with a linear gradient from 15% to 80% CH_3CN/H_2O in 20 min followed by an additional 15 min at 80% CH_3CN/H_2O at a flow rate of 1 mL/min and UV detection at 210 nm (Figures 2 and S1).

Large scale fermentation, isolation and purification for TTN I-1

The *S. griseochromogenes* Δ*ttnI* mutant strain SB13017 was fermented similarly to the procedure noted above except that the seed culture (50 mL) was used to inoculate 500 mL batches of production medium in 2-L flasks. A total of 24 flasks were used. Following fermentation, the broth was adjusted to pH 4.0 with 1 N HCl, centrifuged and filtered to obtain the mycelia. The mycelia were extracted twice with acetone. The combined acetone extracts were concentrated in vacuo to afford the water phase. The resulting water phase was extracted twice with EtOAc. The combined EtOAc extracts were concentrated in vacuo to afford an oily residue. The latter was dissolved in small amount of EtOH and mixed with 4 g silica gel. The dried silica gel containing sample was loaded onto a RP-C18 flash column developed by stepwise elution with 0-100% CH_3CN in H_2O, yielding 20 fractions, each of which was analyzed by analytical HPLC. Fractions containing TTN I-1 were combined and the solvents were removed under reduced pressure for further purification by semipreparative HPLC. Semipreparative HPLC was carried out on an Alltech Alltima C18 column (5 μM, 250 × 10.0 mm), employing a linear gradient from CH_3CN/H_2O (70:30) to 100% CH_3CN over 20 min and continued at 100% CH_3CN for an additional 10 min, at a flow rate of 3.8 mL/min and monitored by UV detection at 210 nm. Finally, 20.2 mg of TTN I-1 was isolated.

Physicochemical properties of TTN I-1

TTN I-1: $[\alpha]_D^{25}$ +27.0 (*c* 1.0, $CHCl_3$); HR-ESI-MS (positive ion) at *m*/*z* 593.3688 (calcd for $C_{33}H_{53}O_9$ $[M+H]^+$, 593.3684). ^1H and ^{13}C NMR ($CDCl_3$) data, see Table 1.

Table S1. Targeted gene inactivation by the REDIRECT technology in *S. griseochromogenes*

Gene	Primers[a]	Cosmid[b]	Strain
ΔttnC	*ttnC*F1:5'-CATGCGATTGATCGTTGCCATCACCGGCGCCACCGGTGCCATTCCGGGGATCCGTCGACC-3' *ttnC*R1: 5'–CTTCATTCACCTGTTCCCTTCAGGAAGCCCTCGTCGGTCAGTGTAGGCTGGAGCTGCTTC-3'	pBS13024	SB13012
ΔttnI	*ttnI*F1: 5'-GGCGCGCTGGGCGCTCACCCACGGGCTGATCAGGCTGGCGATTCCGGGGATCCGTCGACC-3' *ttnI*R1: 5'–CCTCAGCGCACCGGCGTGGGGGCCGGCGTGGCCATCAACCGTGTAGGCTGGAGCTGCTTC-3'	pBS13031	SB13017

[a]Underlined letters represent the nucleotide homologous to the DNA regions internal to targeted genes.
[b]pBS13024 is based on pBS13009, and pBS13031 is based on pBS13011.[S6]

Table S2. Southern analysis confirming the genotypes of the *ΔttnI* mutant strain[a]

Strain	Gene targeted	Probe		Fragment replaced (bp)	Restriction digestion	Signal size (kb)	
		primers used to amplify the probe	Size (bp)			WT	mutant
SB13017	*ΔttnI*	*ttnI*FP2: 5'- GCCGGGCGGGACCTGTCGAAG -3' *ttnI*RP2: 5'- ATCTCCCGCCGGGCGCTG -3'	980	1272	*Sal*I	1.03	2.04

[a]See Figure S2 for details.

Table S3. Expression constructs for complementation to the *ΔttnI* mutant

Mutant strain	Gene mutated	Primers used to make the expression constructs[a]	Construct	Complemented strain
SB13017	*ΔttnI*	*ttnI*FP3: 5'- GCAATGCATGACCCACGCGGTTC -3 *ttnI*RP3: 5'- GCTCTAGATCAGCGCACCGGCGTGGG -3'	pBS13032	SB13018

[a]*Nsi*I and *Xba*I restriction sites are underlined.

Figure S1. HPLC analysis of *S. griseochromogenes* wild-type and *ΔttnC* mutant strains SB13012. ♦ TTN.

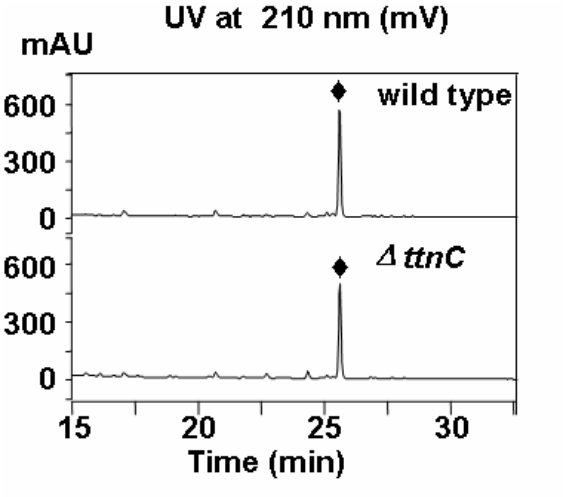
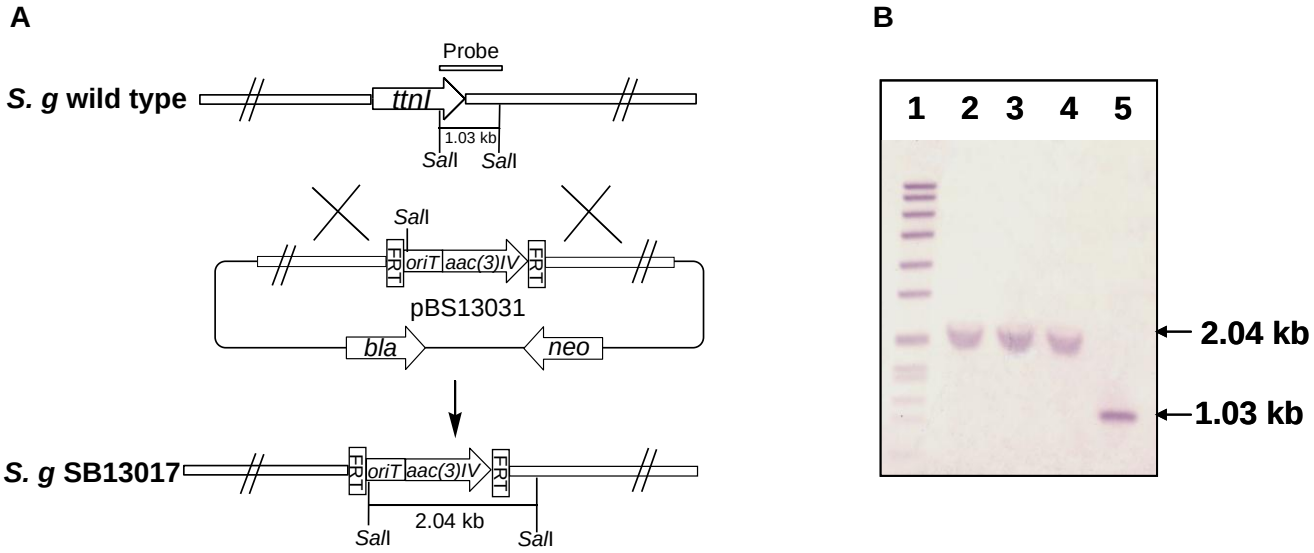


Figure S2. Inactivation of *ttnI* by gene replacement. (A) Construction of the *ΔttnI* gene replacement mutant and restriction maps of *S. griseochromogenes* wild-type and SB13017 mutant strains showing predicted fragment sizes upon *Sal*I digestion. (B) Southern analysis of the wild-type (lane 5) and SB13017 (lanes 2, 3 and 4 are three individual isolates) genomic DNAs digested with *Sal*I using the 980-bp amplified DNA fragment as a probe. Lane 1, molecular weight standards.



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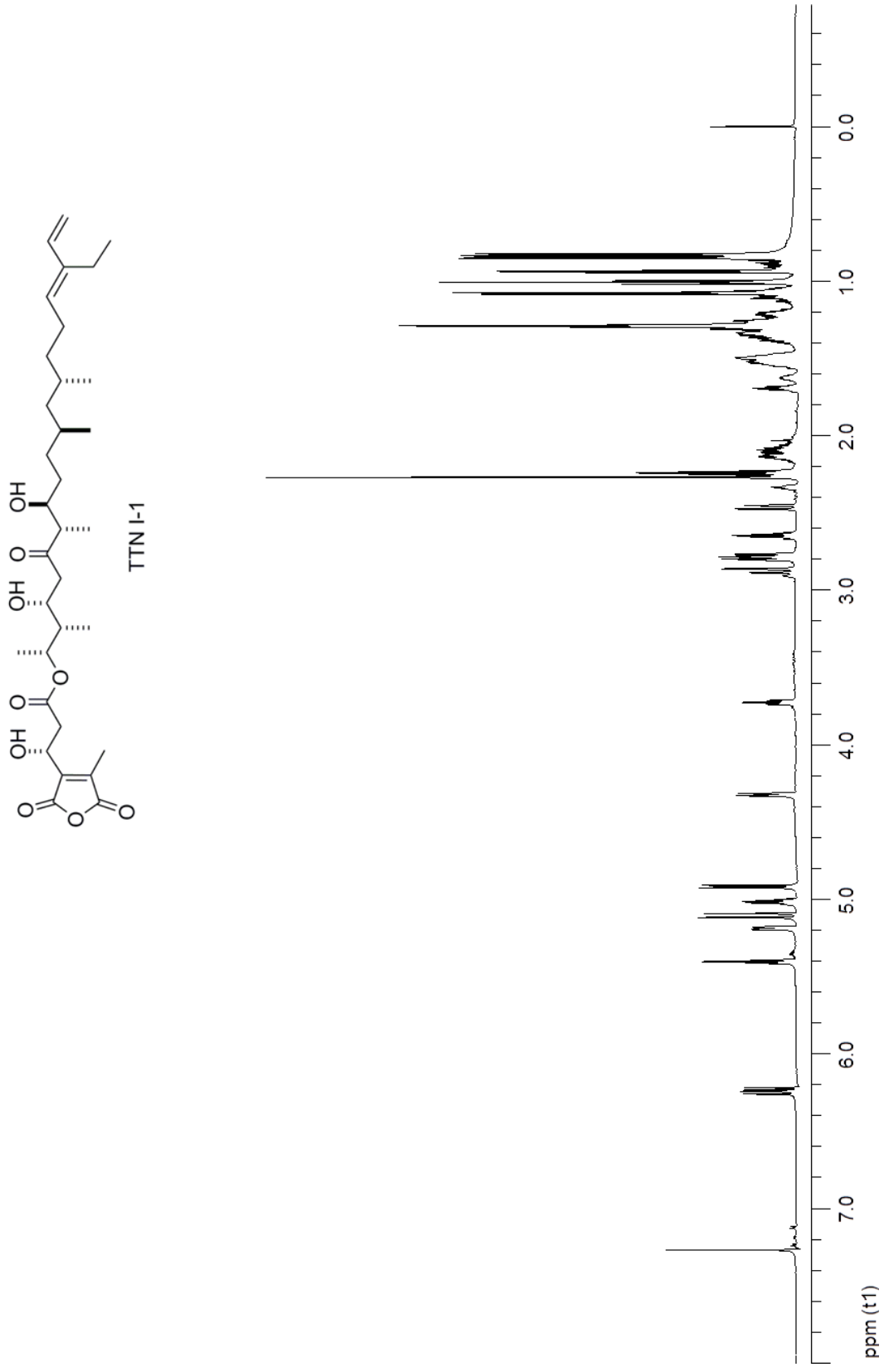

Figure S3. ^{1}H NMR spectrum of TTN I-1 in $CDCl_3$

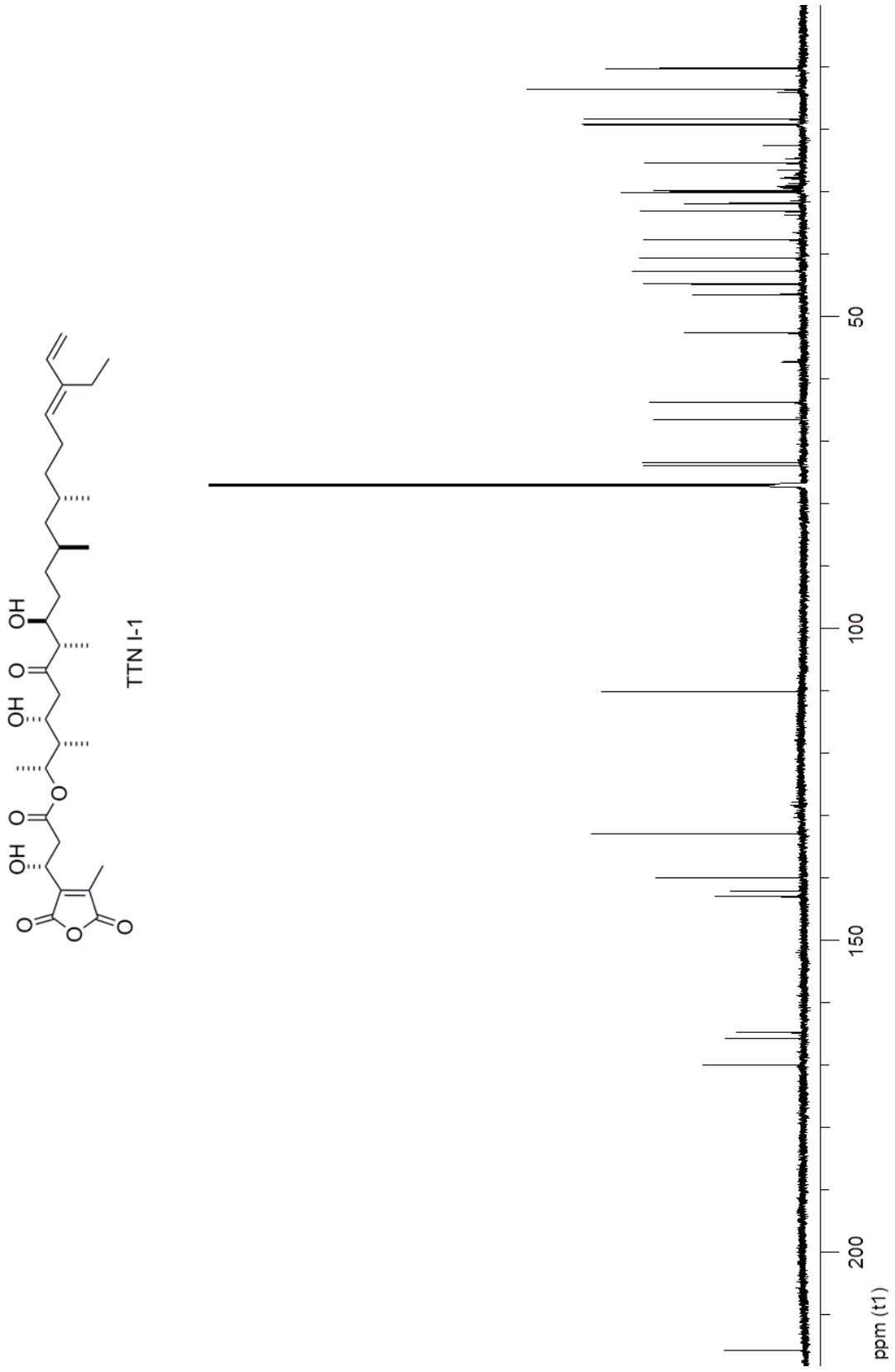

Figure S4. ^{13}C NMR spectrum of TTN I-1 in $CDCl_3$

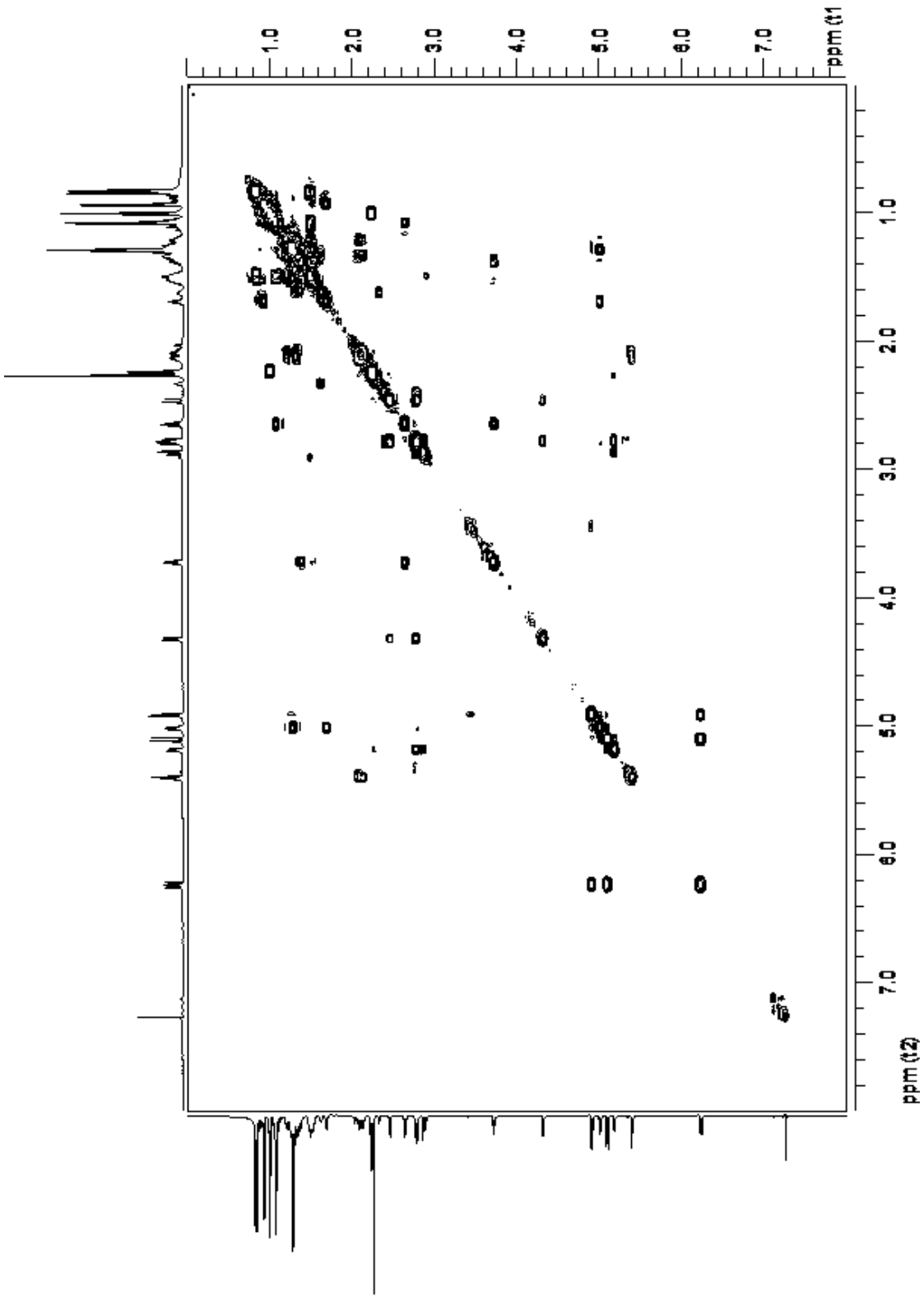

Figure S5. ^{1}H-^{1}H COSY NMR spectrum of TTN I-1 in $CDCl_3$

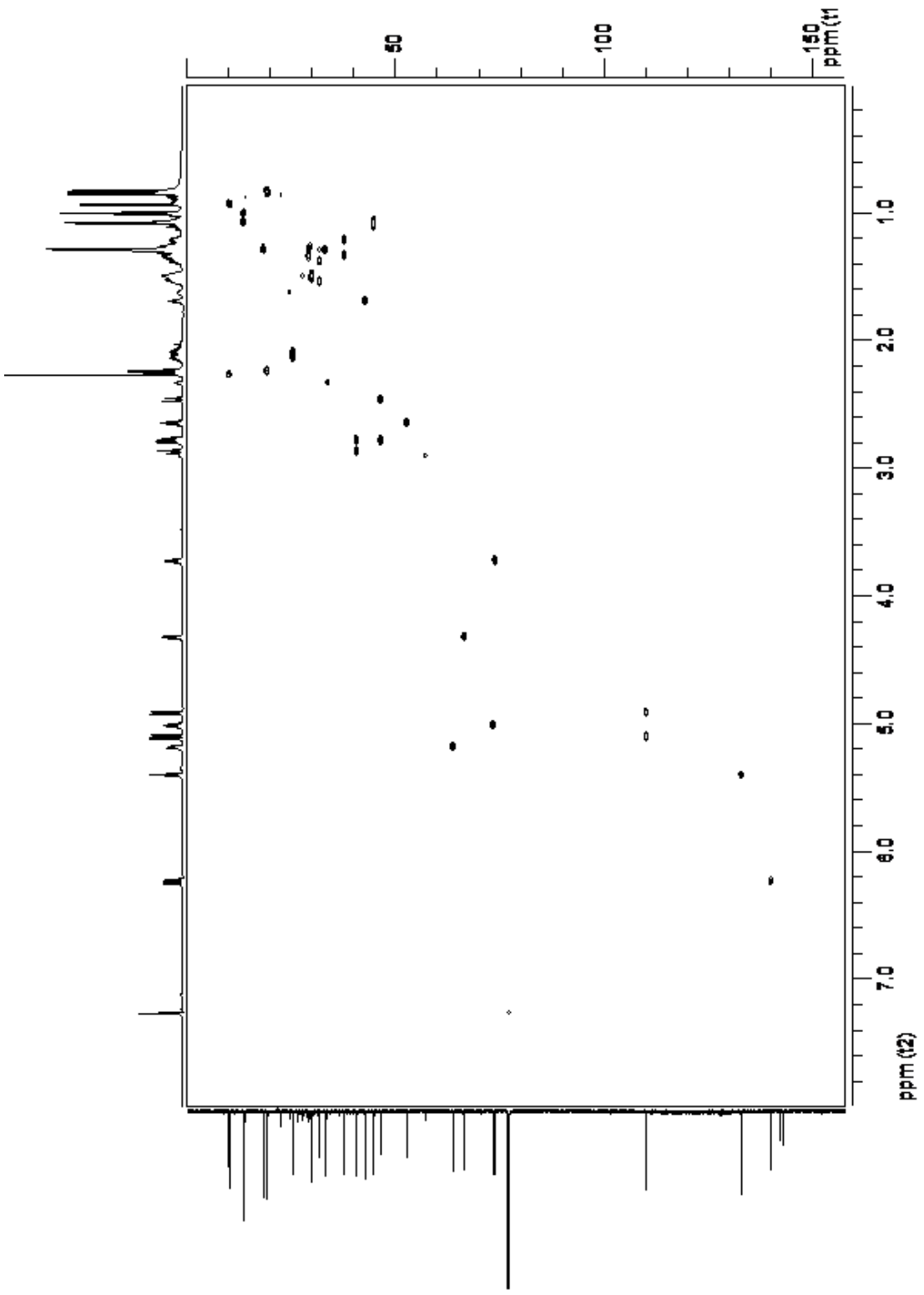

Figure S6. HSQC NMR spectrum of TTN I-1 in $CDCl_3$

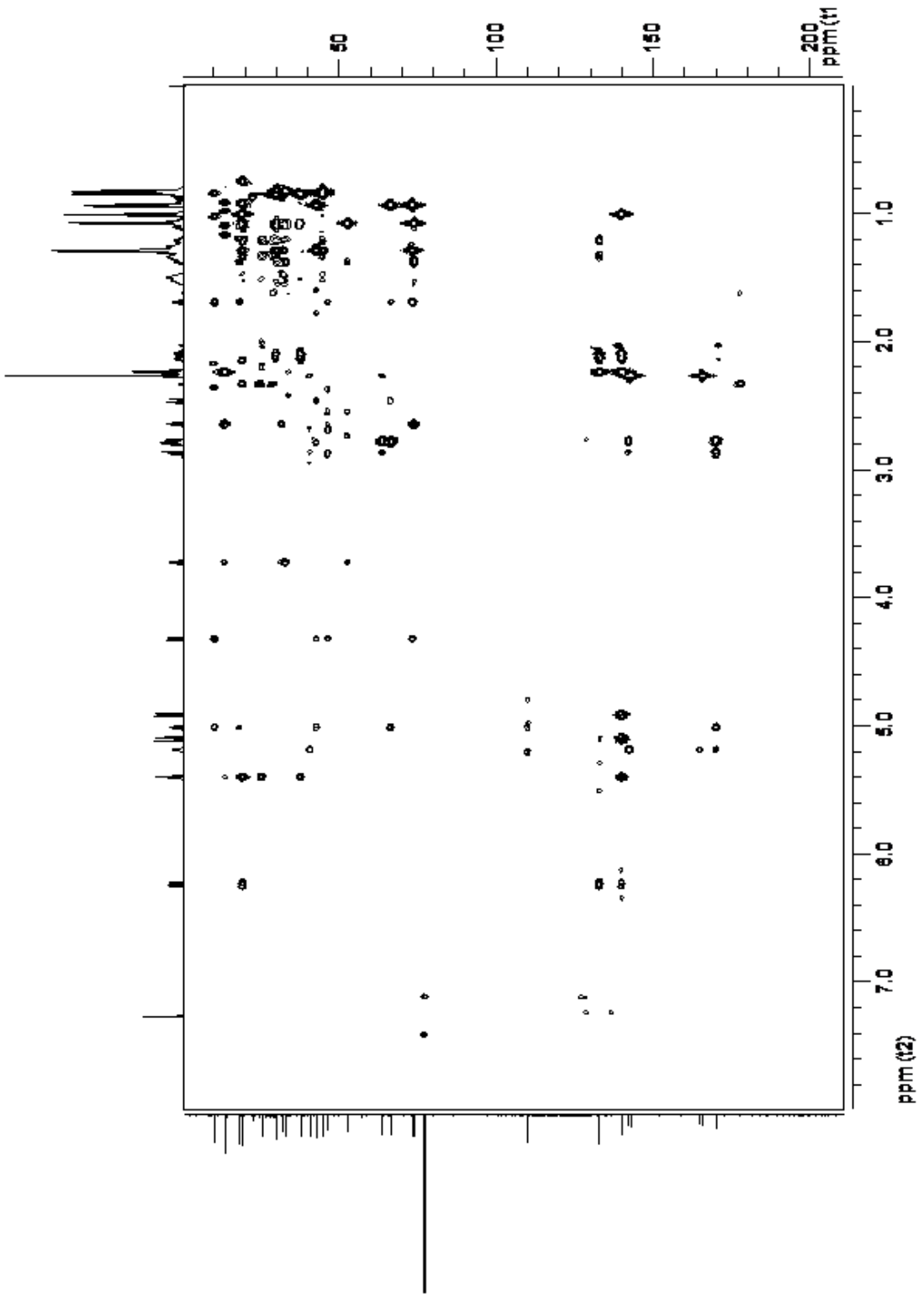

Figure S7. HMBC NMR spectrum of TTN I-1 in $CDCl_3$