Supplemental Information for:

Title: Uncovering the biosynthetic potential of rare metagenomic DNA using co-occurrence network analysis of targeted sequences

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Supplementary Table 1: Soil samples and metagenomic libraries information

Library name	subpools	clones per subpool	library clones	avg. insert size [kbp]	total library size [Gbp]	amplicon sequencing [M reads]				
						adenylation (NRPS)	ketosynthase (PKS)	enduracididine	AHBA	capreomycidine
Arizona	2304	5000	1E+07	40	400	24	11*	0.2	0.1	15
Oregon	768	25000	2E+07	40	800	11	10	0.2	N/A	N/A
New Mexico	768	25000	2E+07	40	800	12	11	N/A	0.05	N/A
Hawaii	768	25000	2E+07	40	800	11	N/A	N/A	N/A	N/A

*Due to low sequencing coverage of one 384 plate, only 1920 subpools were processed for Arizona ketosynthase domains

Supplementary Table 2: Primers table

Target domain	Annealing T°	Forward binding sequence	Reverse binding sequence	Primer$_{len}$ /Total$_{len}$
AD1 (Arizona)	65	5′-GCSTACSYSATSTACACSTCSGG	5′-SASGTCVCCSGTSCGGTA	23 / 210
AD2 (Oregon, Hawaii, New Mexico)	56.3	5′-SATBTAYACSTCVGGHWCSAC	5′-CCANRTCNCCBGTSYKGTACA	21 / 210
KS (Arizona, Oregon, Hawaii, New Mexico)	56.3	5′-GCNATGGAYCCNCARCARMGNVT	5′-GTNCNNGTNCCRTGNSCYTCNAC	23 / 210
END. (Arizona, Oregon)	64.5	5′-CCNCCRCCSTGGCACTTCKCSG	5′-CSAACTGCTTGGGCATSCCCTG	22 / 110
AHBA (Arizona, New Mexico)	61	5′-CAGAACGGCAAGCTGATGACSG	5′-GGAMCATSGCCATGTAGYKSG	22 / 110
CAP. (Arizona)	55	5′-SGACRTSWSCWSSWSCGGYGT	5′-CRSTNGGRTTRTGSGGRAAGT	21 / 210

Supplementary Table 3: BGCs similarity analysis of 60 metagenomic clones

Recovered BGC #	Highest median identity to a reference BGC (%)	BiG-SCAPE Raw distance	Closest relative (BiG-SCAPE)	Source organism
29:Genbank MN161611	95.6	0.36	GCF_001886595_NZ_CP018074.1.cluster027	*Streptomyces venezuelae* NRRL B-65442
9:Genbank MN161603	95.6	0.36	GCF_000023245_NC_013093.1.cluster022	*Actinosynnema mirum* DSM 43827
28:Genbank MN161610	83.5	0.43	GCF_001302585_NZ_CP012752.1.cluster008	*Kibdelosporangium phytohabitans*
38:Genbank MN161613	79.7	0.43	GCF_001579845_NZ_CP007440.1.cluster002	*Rhodoplanes sp.* Z2-YC6860
25:Genbank MN161608	89.8	0.43	GCF_001443625_NZ_CP013129.1.cluster026	*Streptomyces venezuelae*
73:Genbank MN161622	89.7	0.44	GCF_002796545_NZ_CP024894.1.cluster009	*Amycolatopsis sp.* AA4
355:Genbank MN161648	94.2	0.45	GCF_000282715_NC_018266.1.cluster017	*Amycolatopsis mediterranei* S699
46:Genbank MN161620	89.8	0.45	GCF_001302585_NZ_CP012752.1.cluster017	*Kibdelosporangium phytohabitans*
900:Genbank MN161659	88.4	0.47	GCF_000282715_NC_018266.1.cluster017	*Amycolatopsis mediterranei* S699
16:Genbank MN161605	92.7	0.48	GCF_000739085_NZ_CP009110.1.cluster003	*Amycolatopsis methanolica* 239
41:Genbank MN161615	97.1	0.49	GCF_001650215_NZ_CP015726.1.cluster030	*Streptomyces sp.* RTd22
40:Genbank MN161614	85.5	>0.5	NA	NA
47:Genbank MN161621	84	>0.5	NA	NA
96:Genbank MN161625	76.8	>0.5	NA	NA
683:Genbank MN161657	75.7	>0.5	NA	NA
917:Genbank MN161660	75.3	>0.5	NA	NA
All remaining BGCs (44)	<75	>0.5	NA	NA

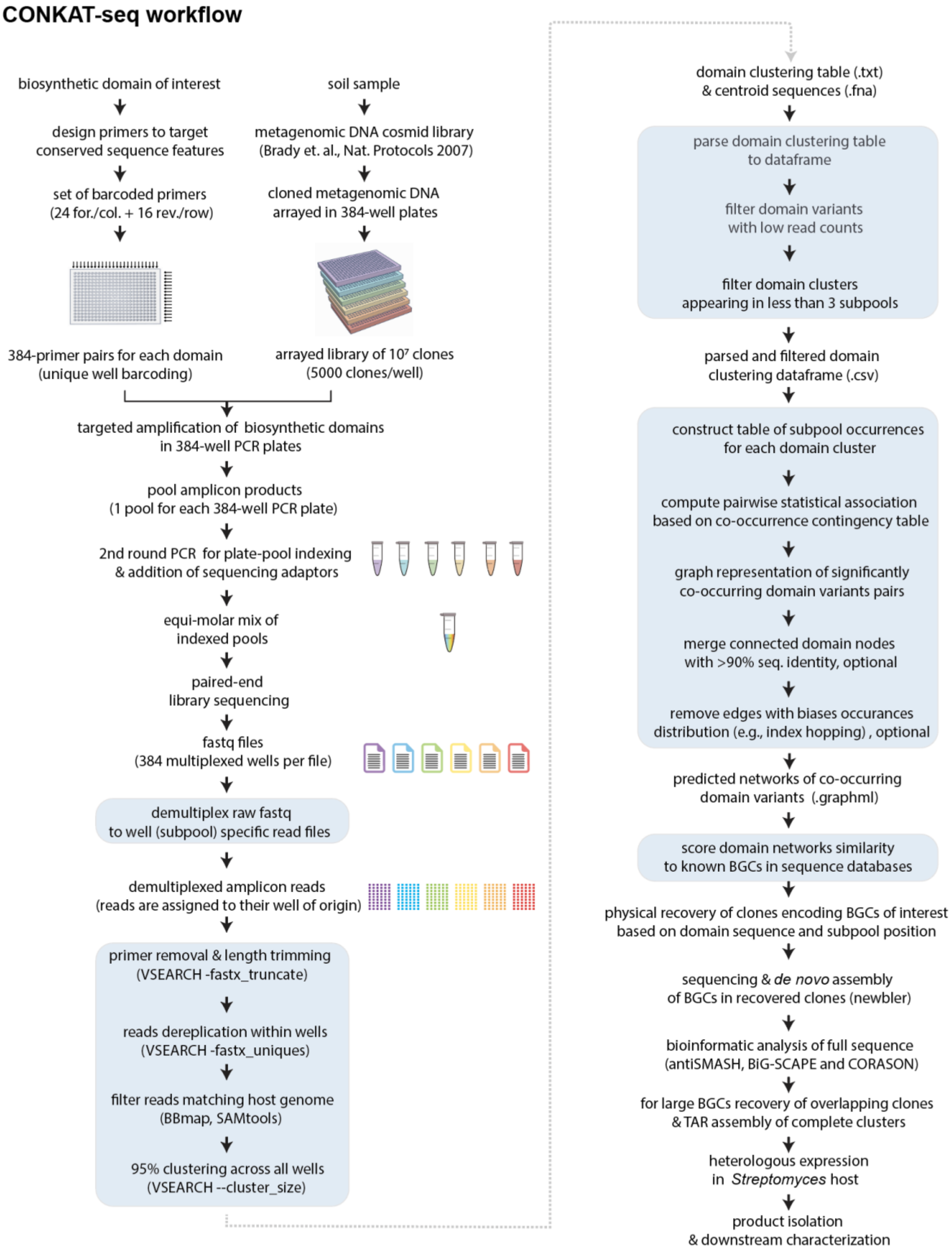
Supplementary Table 4: Omnipeptin biosynthetic gene analysis

Gene	Proposed function	NCBI similarity	Species	%ID
omn1		flavin reductase	*Kibdelosporangium sp. MJ126-NF4*	71
omn2		cation/H(+) antiporter	*Amycolatopsis antarctica*	45
omn3	Halogenation of tryptophan	tryptophan 7-halogenase	*Streptomyces sp. NRRL S-146*	76
omn4	fatty acyl-AMP ligase	fatty acyl-AMP ligase	*Streptomyces formicae*	65
omn5	Attachment of acyl chain	acyl carrier protein	*Streptomyces caeruleatus*	49
omn6	NRPS	non-ribosomal peptide synthetase	*Sciscionella sp. SE31*	53
omn7	NRPS	non-ribosomal peptide synthetase	*Actinoplanes friuliensis*	50
omn8	NRPS	non-ribosomal peptide synthetase	*Streptomyces sp. LUP47B*	57
omn9		protein mbtH	*Saccharomonospora viridis*	68
omn10	Deacylation	Cyclic lipopeptide acylase	*Streptomyces canus*	55
omn11		alpha/beta hydrolase	*Streptomyces sp. M1013*	67
omn12		hypothetical protein	*Streptomyces sp. LUP47B*	61
omn13		hypothetical protein	*Sciscionella sp. SE31*	38
omn14	Transport	ABC transporter permease	*Herbihabitans rhizosphaerae*	66
omn15	Transport	daunorubicin resistance protein DrrA family ABC transporter ATP-binding protein	*Saccharothrix espanaensis*	65
omn16		glutamate synthase	*Amycolatopsis mediterranei*	70
omn17		asparagine ligase	*Saccharothrix syringae*	75
omn18		methylaspartate mutase E	*Streptomyces pratensis ATCC 33331*	64
omn19		methylaspartate mutase S	*Streptomyces erythrochromogenes*	59
omn20	Regulation	ArsR family transcriptional regulator	*Actinoplanes utahensis*	43
omn21		hypothetical protein	*Streptomyces canus*	62
omn22	Transport	ABC transporter permease	*Streptomyces sp. LUP47B*	59
omn23	Transport	ABC transporter ATP-binding protein	*Streptomyces sp. LUP47B*	76
omn24	Regulation	signal transduction histidine kinase	*Actinokineospora cianjurensis*	60
omn25	Regulation	response regulator transcription factor	*Actinokineospora cianjurensis*	80
omn26	Hydroxylation of tyrosine	cytochrome P450	*Streptomyces sp. NRRL B-24051*	64
omn27	Hydroxylation of proline	proline hydroxylase	*Streptomyces malaysiensis*	53
omn28		phytanoyl-CoA dioxygenase	*Streptomyces sp. H23*	66
omn29		alcohol dehydrogenase	*Streptomyces sp. LUP47B*	73
omn30		myo-inositol-1-phosphate synthase	*Kibdelosporangium aridum*	64
omn31		4-hydroxybenzoate polyprenyltransferase	*Actinocrispum wychmicini*	57
omn32		sugar phosphate isomerase/epimerase	*Actinosynnema sp. ALI-1.44*	70
omn33		TatD family hydrolase	*Amycolatopsis nigrescens*	79
omn34		xylose isomerase	*Kibdelosporangium aridum*	67
omn35		alkaline phosphatase family protein	*Prauserella shujinwangii*	72
omn36	Regulation	AfsR/SARP family transcriptional regulator	*Kibdelosporangium phytohabitans*	60
omn37	Regulation	TetR family transcriptional regulator	*Actinophytocola oryzae*	47
omn38	Regulation	TetR/AcrR family transcriptional regulator	*Kibdelosporangium aridum*	66
omn39		gamma-butyrolactone biosynthesis protein	*Streptomyces sp. RP5T*	49
omn40		NAD-dependent epimerase/dehydratase family protein	*Herbihabitans rhizosphaerae*	56

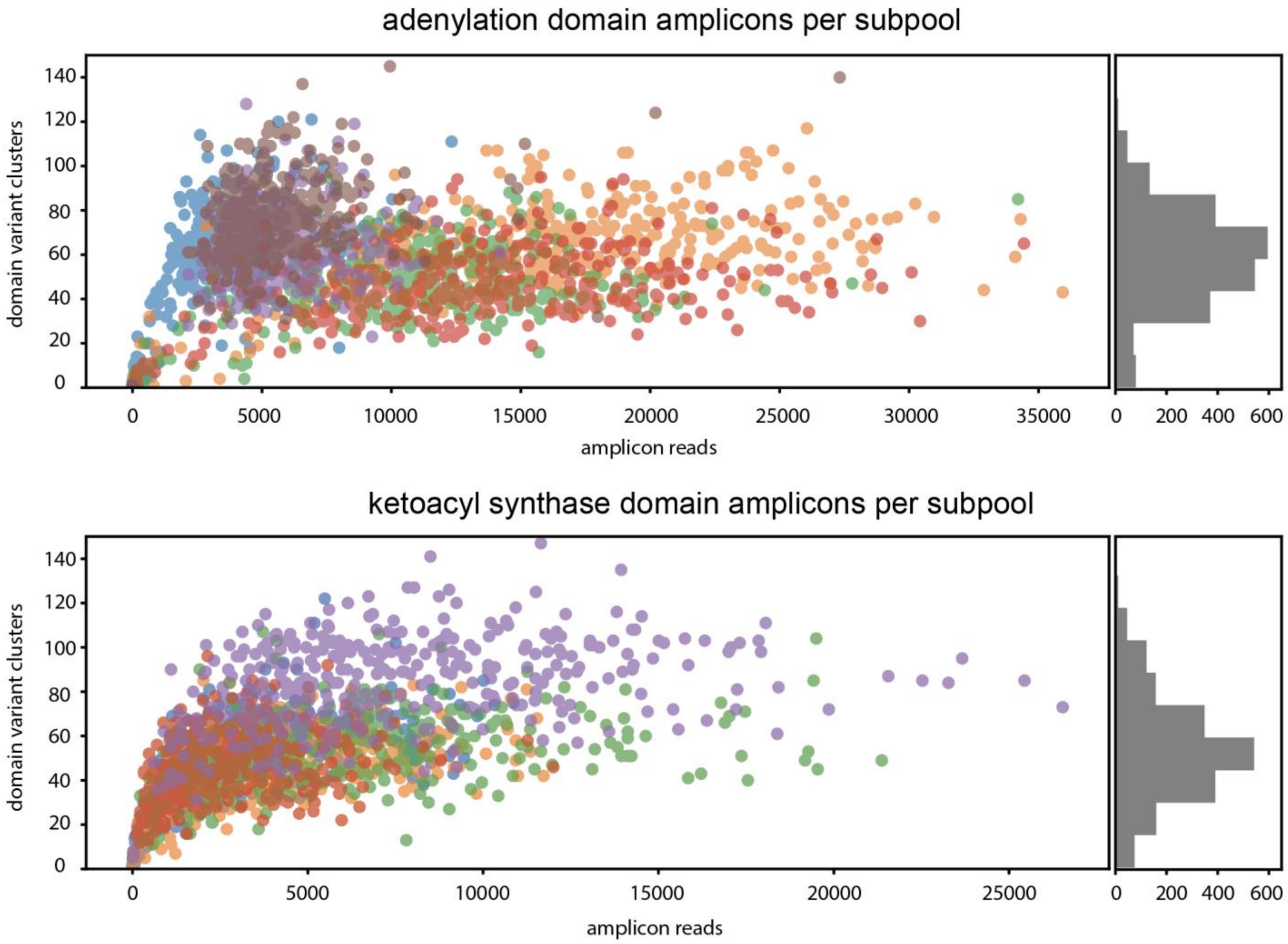
Supplementary Table 5. ^{1}H and ^{13}C NMR (600 MHz, DMSO-d_6) data for omnipeptin (**1**)

Residue	Pos.	δ_C	δ_H, mult. (*J* in Hz)*	Residue	Pos.	δ_C	δ_H, mult. (*J* in Hz)*
isoAsp-1	1	170.9, C	−		$5\text{-CO}\underline{\text{NH}_2}$	−	6.90, brs; 7.32
	2	50.9, CH	3.57		2-NH	−	7.87, br s
	3	36.4, CH$_2$	2.19, 2.75	Cl-Trp-8	1	170.7, C	−
	4-CONH	169.1, C	−		2	53.4, CH	4.55
hyTyr-2	1	170.4, C	−		3	27.7, CH$_2$	2.85, 3.13
	2	59.9, CH	4.28, d (7.4)		4	110.3, C	−
	3	72.4, CH	4.66, d (7.4)		4a	126.0, C	−
	4	132.1, C	−		5	119.8, C	7.58, d (8.4)
	5/9	128.0, CH	7.22		6	118.6, CH	6.97, d (8.4)
	6/8	114.5, CH	6.65, d (7.9)		7	125.6, C	−
	7	156.7, C	−		8	110.8, CH	7.34, s
	2-NH	−	8.52, br s		8a	136.5, C	−
Ser-3	1	169.1, C	−		9	125.0, CH	7.19
	2	52.0, CH	4.58		2-NH	−	7.64
	3	64.0, CH$_2$	4.34, d (9.9); 4.40		9-NH	−	11.0, s
	2-NH	−	8.23, d (6.6)	Val-9	1	170.2, C	−
Glu-4	1	171.5, C	−		2	57.6, CH	4.05
	2	52.5, CH	4.47		3	30.3, CH	1.66, m
	3	27.1, CH$_2$	1.81, m; 2.02, m		4	18.8, CH$_3$	0.39, d (6.5)
	4	30.6, CH$_2$	2.14, 2.23		5	17.8, CH$_3$	0.47, d (5.9)
	5-COOH	174.5, C	−		NH	−	7.70, br s
	2-NH	−	8.13, br s	Phe-10	1	170.6, C	−
Thr-5	1	170.4, C	−		2	51.1, CH	4.74, br s
	2	57.9, CH	4.43		3	36.5, CH$_2$	2.76; 2.98, d (11.8)
	3	66.7, CH	4.05		4	137.5, C	−
	4	19.6, CH$_3$	1.01, d (6.1)		5/9	129.4, CH	7.31, d (7.7)
	2-NH	−	7.67		6/8	128.0, CH	7.23
Ser-6	1	170.8, C	−		7	126.3, CH	7.16
	2	54.7, CH	4.46		NH	−	8.37
	3	61.5, CH$_2$	3.51, br s; 3.69, dd (10.1, 5.7)	MehyPro-11	1	169.5, C	−
	2-NH	−	8.36		2	62.3, CH	4.37, d (7.8)
MeAsn-7	1	169.6, C	−		3	74.9, CH	4.21, br s
	2	55.2, CH	4.47		4	37.5, CH	2.21
	3	40.1, CH	2.68, m		5	50.6, CH$_2$	3.20, 3.81, br s
	4	11.8, CH$_3$	0.66, br s		6	14.3, CH$_3$	1.04, d (6.1)
	$5\text{-}\underline{\text{CO}}\text{NH}_2$	175.0, C	−				

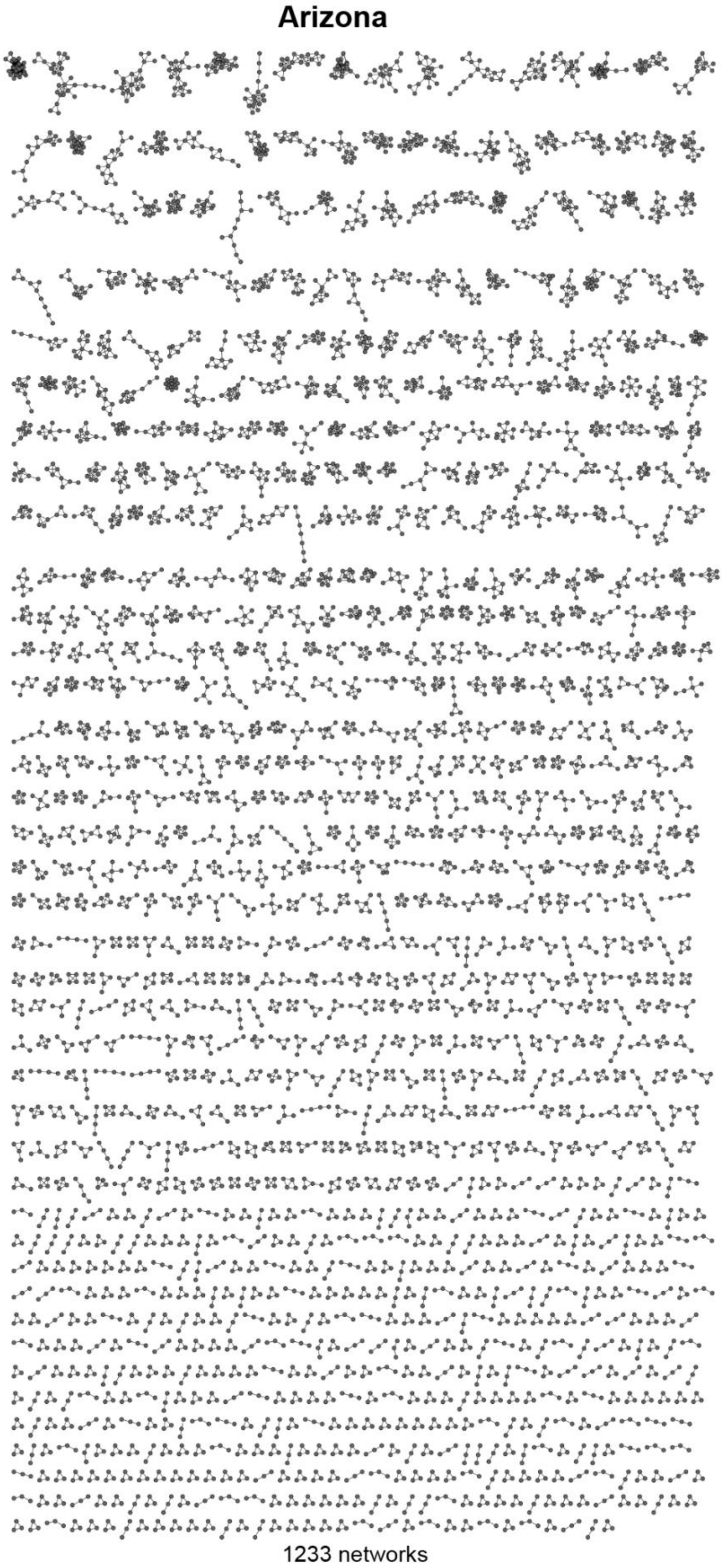
* The assignments of overlapping ^{1}H NMR signals were supported by HSQC, HMBC, TOCSY and HSQC-TOCSY. The peak multiplicity and coupling constants are presented for non-overlapping signals only.


CONKAT-seq workflow
biosynthetic domain of interest
design primers to target conserved sequence features
set of barcoded primers (24 for./col. + 16 rev./row)
384-primer pairs for each domain (unique well barcoding)
soil sample
metagenomic DNA cosmid library (Brady et. al., Nat. Protocols 2007)
cloned metagenomic DNA arrayed in 384-well plates
arrayed library of 10⁷ clones (5000 clones/well)
targeted amplification of biosynthetic domains in 384-well PCR plates
pool amplicon products (1 pool for each 384-well PCR plate)
2nd round PCR for plate-pool indexing & addition of sequencing adaptors
equi-molar mix of indexed pools
paired-end library sequencing
fastq files (384 multiplexed wells per file)
demultiplex raw fastq to well (subpool) specific read files
demultiplexed amplicon reads (reads are assigned to their well of origin)
primer removal & length trimming (VSEARCH -fastx_truncate)
reads dereplication within wells (VSEARCH -fastx_uniques)
filter reads matching host genome (BBmap, SAMtools)
95% clustering across all wells (VSEARCH --cluster_size)
domain clustering table (.txt) & centroid sequences (.fna)
parse domain clustering table to dataframe
filter domain variants with low read counts
filter domain clusters appearing in less than 3 subpools
parsed and filtered domain clustering dataframe (.csv)
construct table of subpool occurrences for each domain cluster
compute pairwise statistical association based on co-occurrence contingency table
graph representation of significantly co-occurring domain variants pairs
merge connected domain nodes with >90% seq. identity, optional
remove edges with biases occurances distribution (e.g., index hopping) , optional
predicted networks of co-occurring domain variants (.graphml)
score domain networks similarity to known BGCs in sequence databases
physical recovery of clones encoding BGCs of interest based on domain sequence and subpool position
sequencing & *de novo* assembly of BGCs in recovered clones (newbler)
bioinformatic analysis of full sequence (antiSMASH, BiG-SCAPE and CORASON)
for large BGCs recovery of overlapping clones & TAR assembly of complete clusters
heterologous expression in *Streptomyces* host
product isolation & downstream characterization

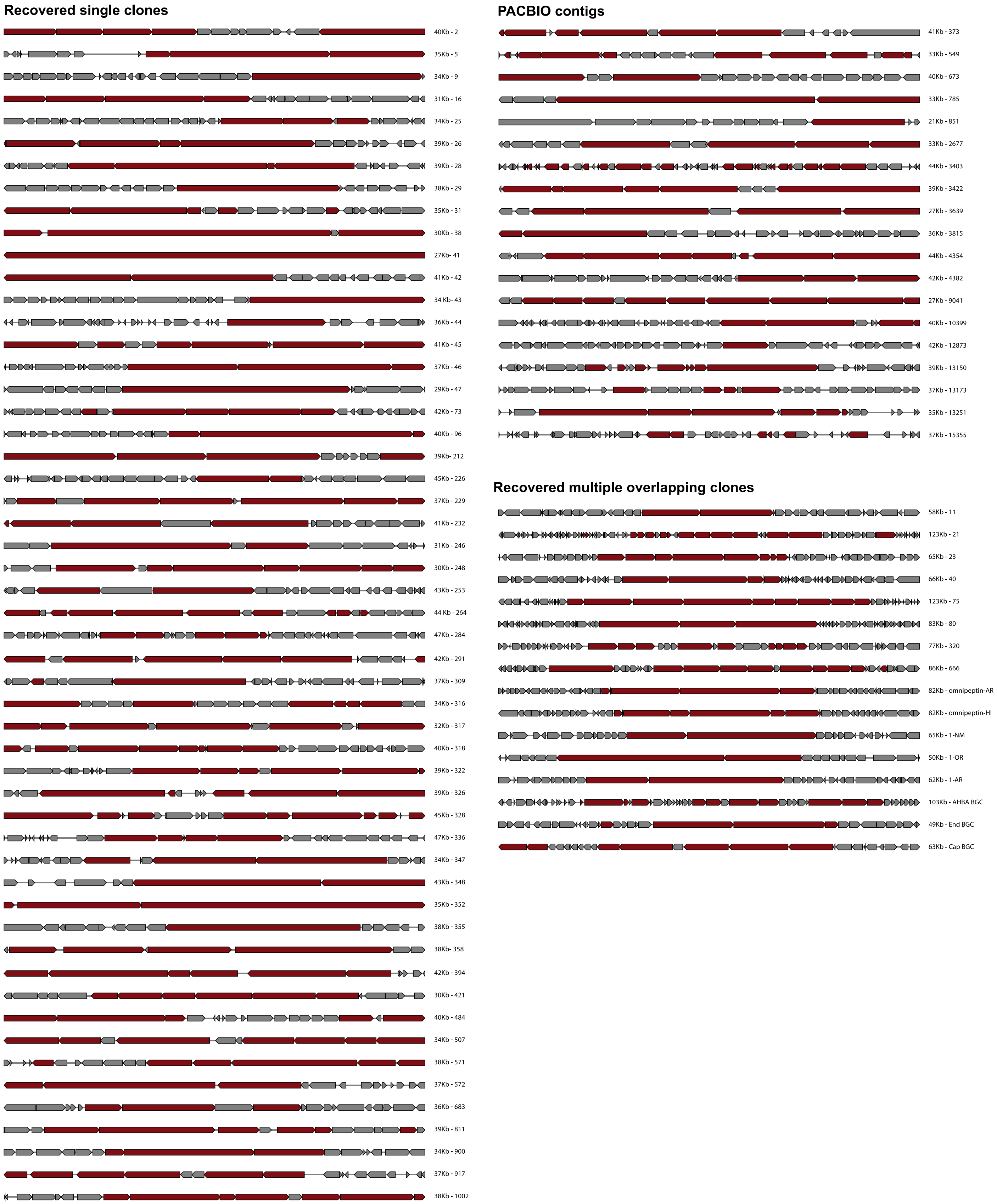
Supplementary Fig. 1 - CONKAT-seq workflow. CONKAT is based on the statistical analysis of amplicon co-occurrence in a partitioned library of large insert metagenomic clones. High molecular weight DNA from soil samples are extracted and cloned to construct large insert metagenomic library which preserves the linkage between co-clustered genes. Library clones are randomly partitioned into hundreds of wells (subpools), and DNA sequence encoding for biosynthetic domains of interest are amplified using barcoded primers. Amplicon de-barcoding identifies the positioning of each biosynthetic domain within the array of subpools and enables the statistical analysis of domain co-occurrence. Domains encoded within a BGC are expected to show high level of co-occurrence across subpools due to their physical linkage. Significantly associated domains are grouped into networks, that can be guide the recovery of novel BGCs based on their similarity scores to known BGCs. The domain variant sequences and the subpool localization information that are associated with networks of interest can guide the physical recovery of metagenomic clones encoding for novel BGCs from the library using serial dilution PCR strategy with domain variant specific primers. Isolated metagenomic clones can be sequenced and assembled to obtain the full BGC sequence encoded within the clone, or heterologously expressed in a suitable host to attempt and obtain the molecular product. Scripts for domain clustering prediction from amplicon data (highlighted in blue) are available at https://github.com/brady-lab-rockefeller/conkat_seq.



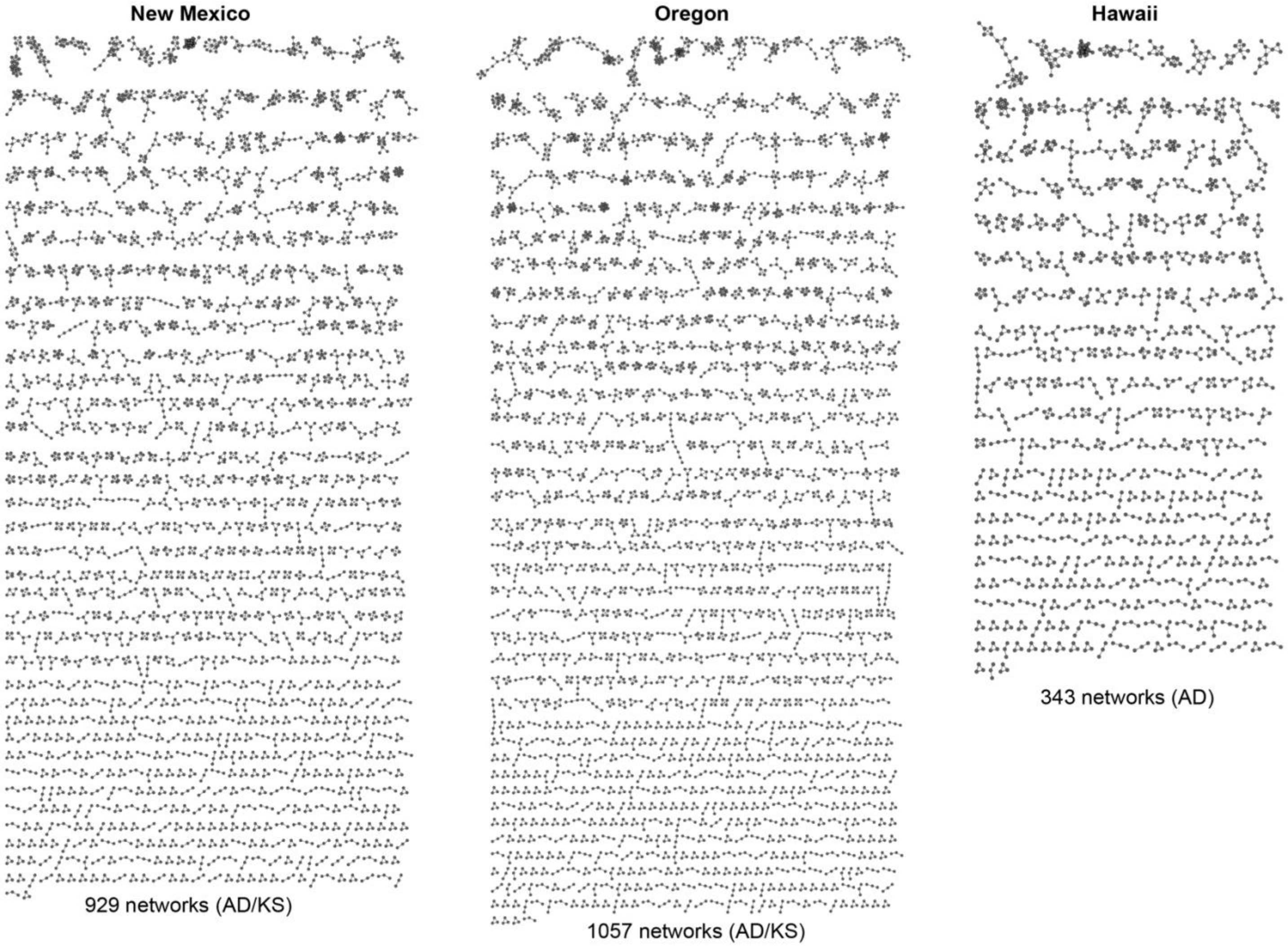
Supplementary Fig. 2 - Adenylation and ketoacyl-synthase domain variants in the Arizona library subpools. Scatter plot of amplicons reads and domain variant clusters (95% identity threshold) in subpools of the Arizona metagenomic library. Each subpool (containing ~5000 metagenomic clones) was PCR amplified using adenylation and ketoacyl-synthase subpool-barcoed domain primers. Approximately 3000 reads per subpool were required to saturate the diversity of domain variants amplified by these primers in each subpool. On average, we identified 60 adenylation domain variants and 55 ketoacyl-synthase domain variants per subpool and a total of 10^5 unique domain variants across the library. Colors represent 384-subpools PCR reaction plates that have been pooled and sequenced as an indexed sample.



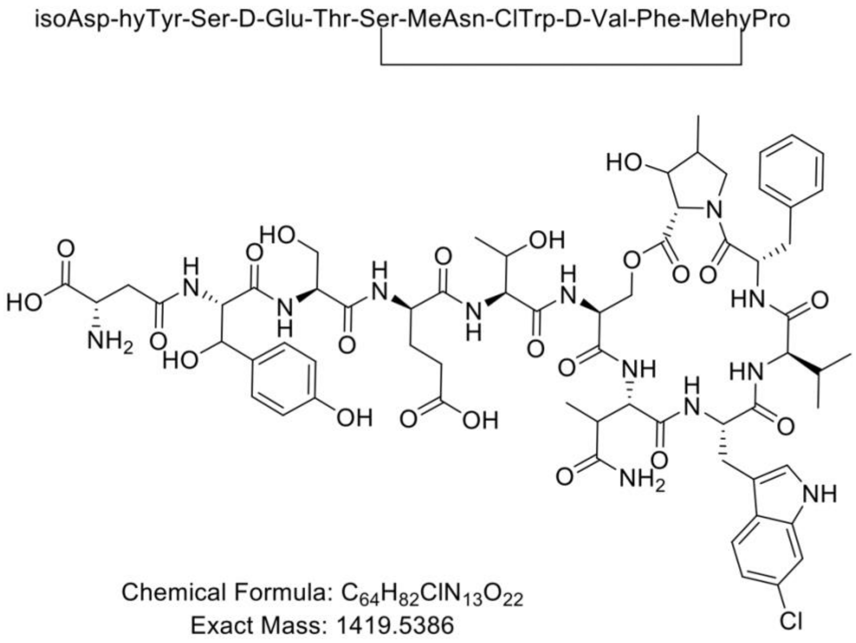
Supplementary Fig. 3 - Predicted domain networks (Arizona)



Supplementary Fig. 4 - NRPS and PKS genes locations in metagenomic insert sequences. NRPS and PKS genes are depicted in red. Recovered clones have been sequenced individually with short-read technology (Illumina) while PacBio contigs have been obtained from bulk sequencing of 2 subpools of ~5000 clones each with long-read technology.



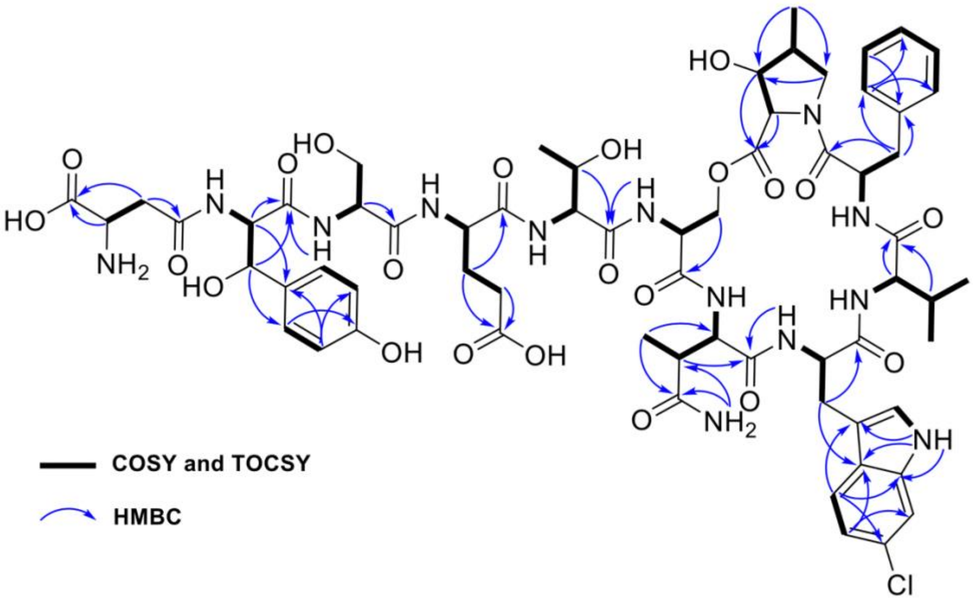
Supplementary Fig. 5 - Predicted domain networks (Oregon, New Mexico, Hawaii)


isoAsp-hyTyr-Ser-D-Glu-Thr-Ser-MeAsn-ClTrp-D-Val-Phe-MehyPro

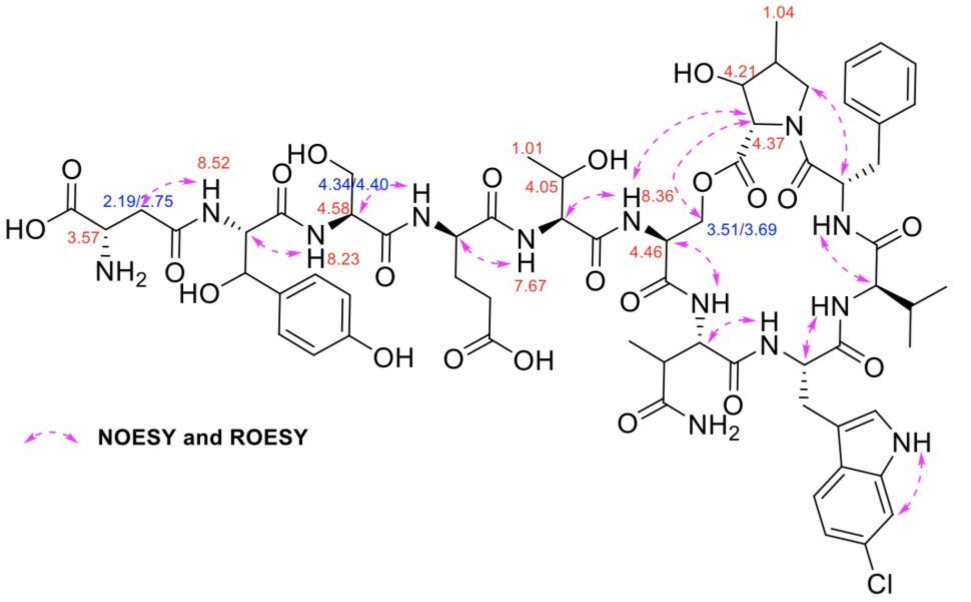
Chemical Formula: $C_{64}H_{82}ClN_{13}O_{22}$
Exact Mass: 1419.5386

*The stereochemistry of α-protons in amino acid residues were predicted by bioinformatics analysis.

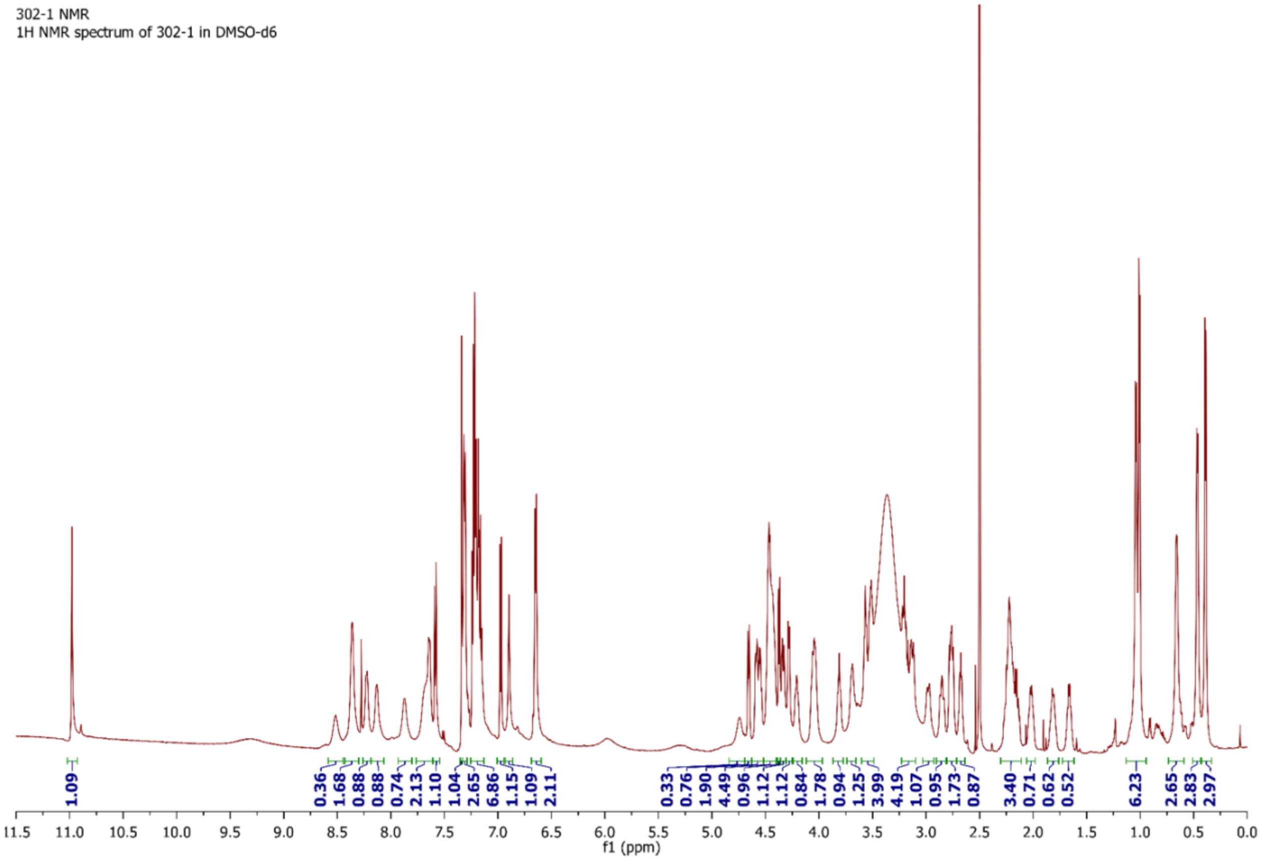
Supplementary Fig. 6 - Chemical structure of omnipeptin **(1)**



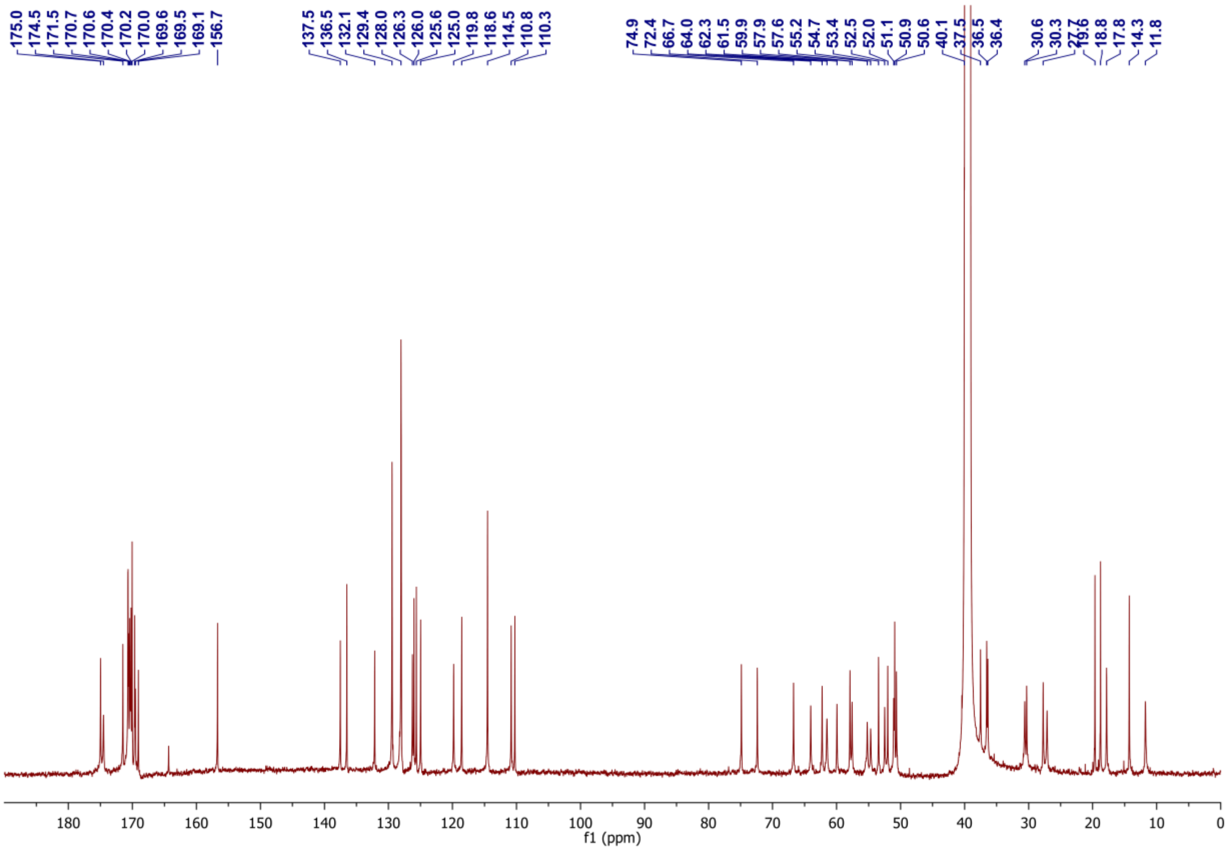
Supplementary Fig. 7 - 2D NMR correlations of omnipeptin (**1**)



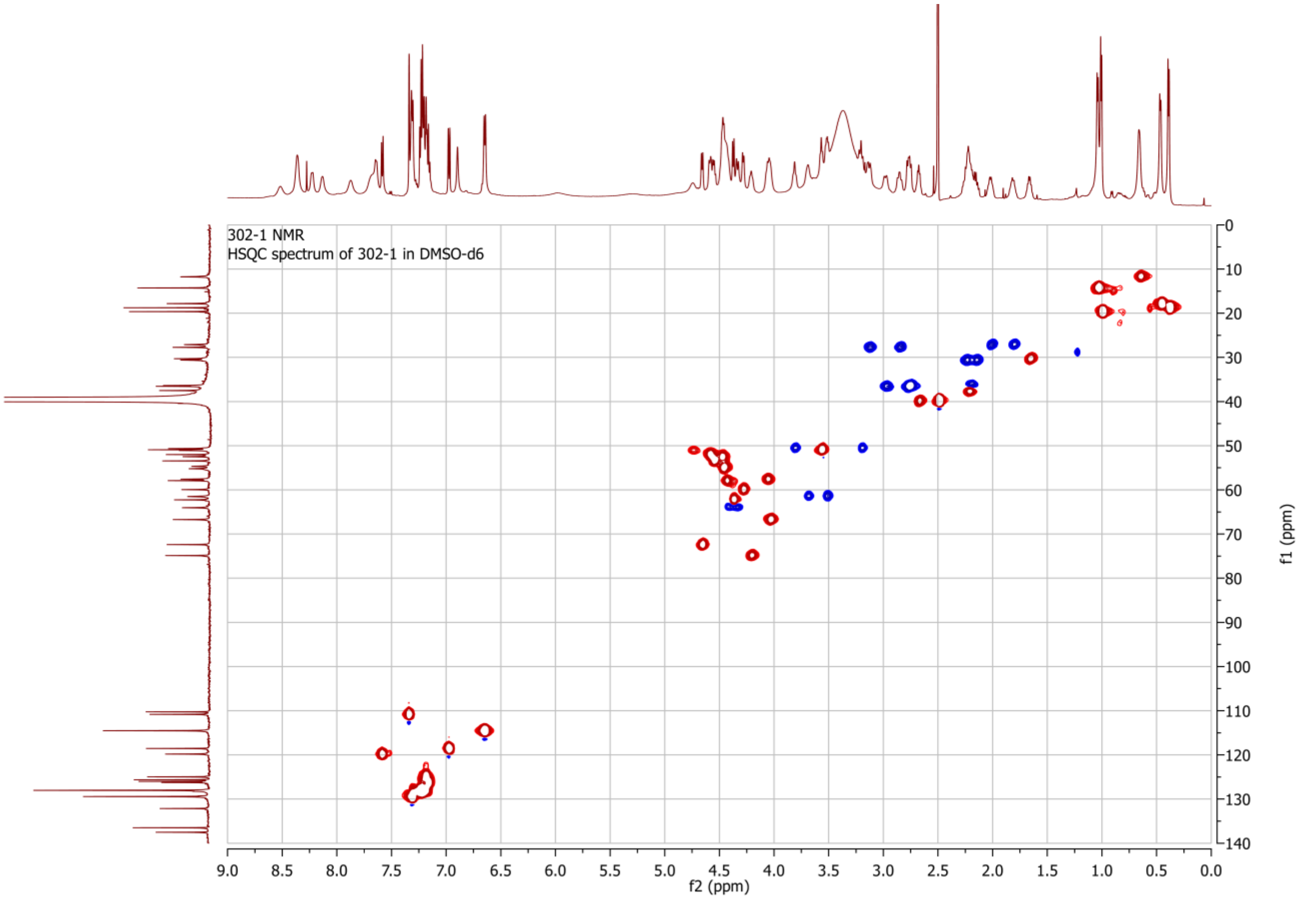
Supplementary Fig. 8 - NOE/ROE correlations of omnipeptin (**1**) establishing the connectivity of amino acid residues



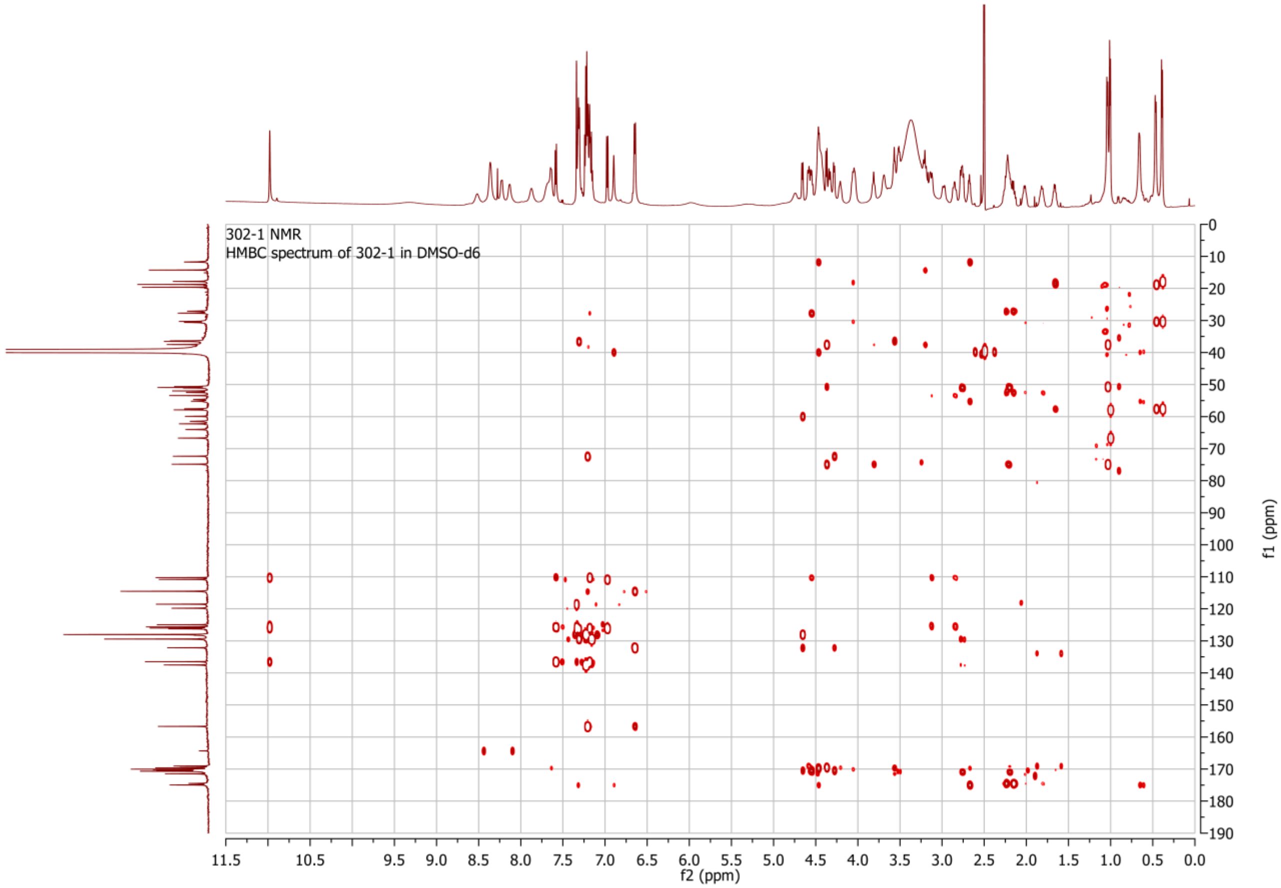
Supplementary Fig. 9 - ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



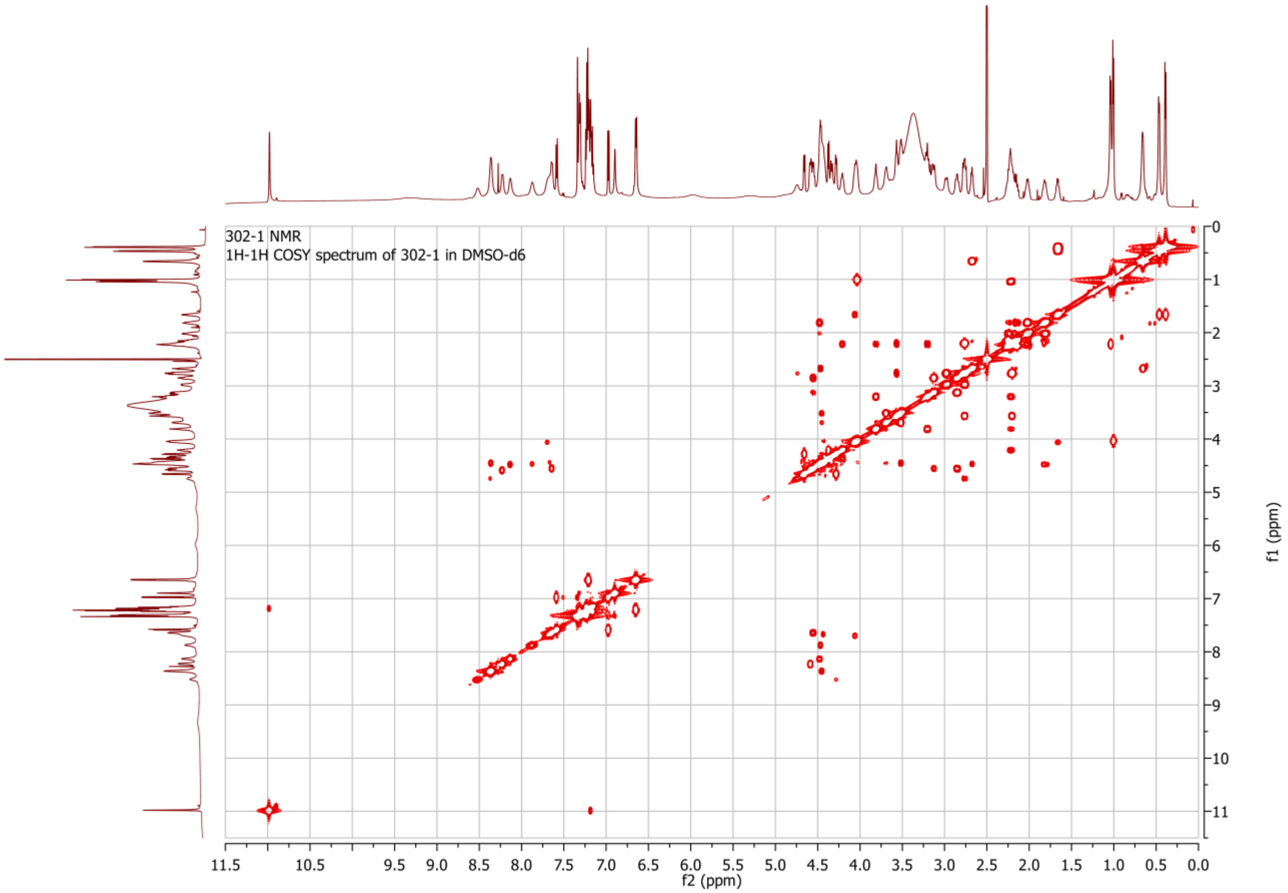
Supplementary Fig. 10 - ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



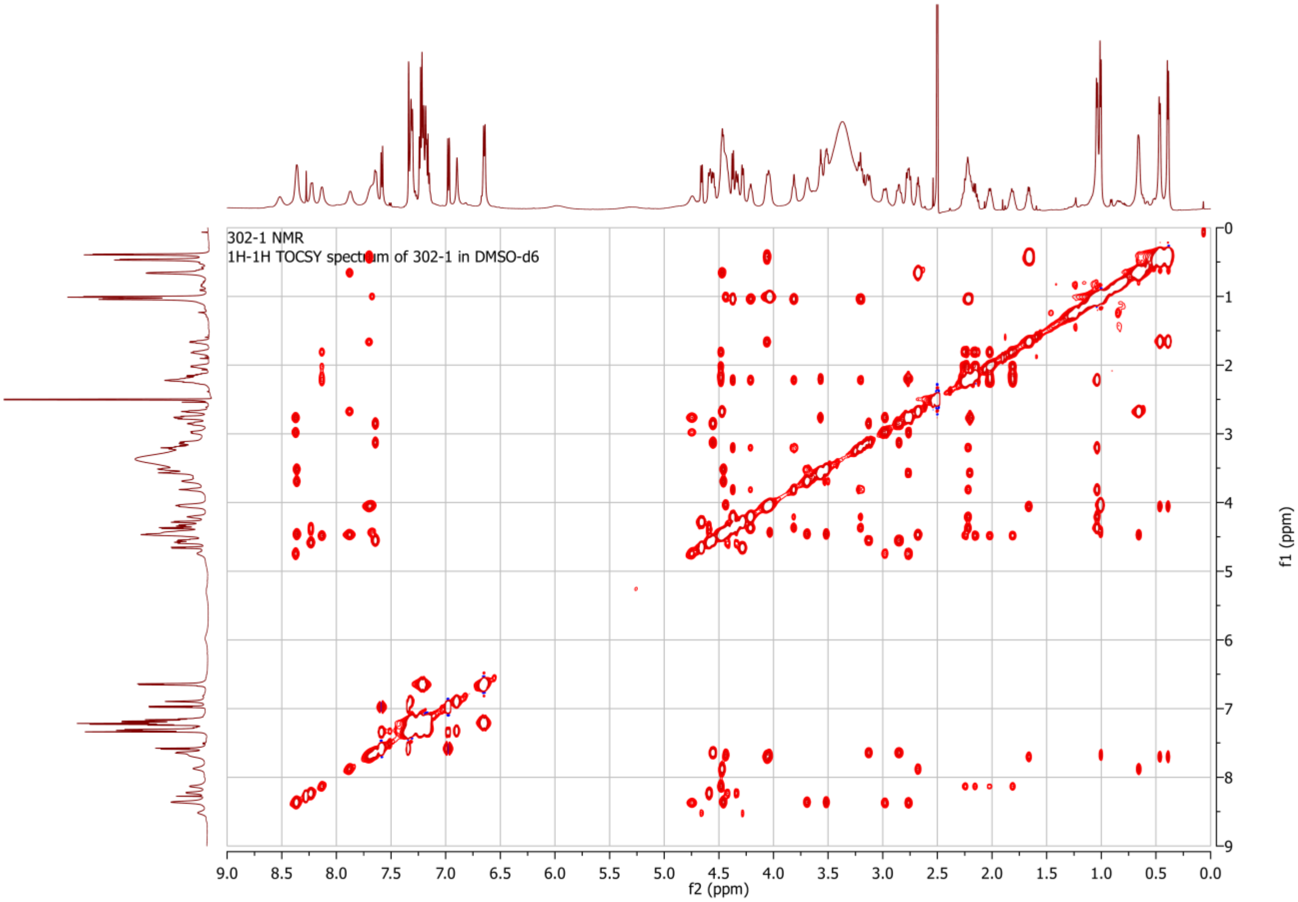
Supplementary Fig. 11 - ^{1}H-^{13}C HSQC NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



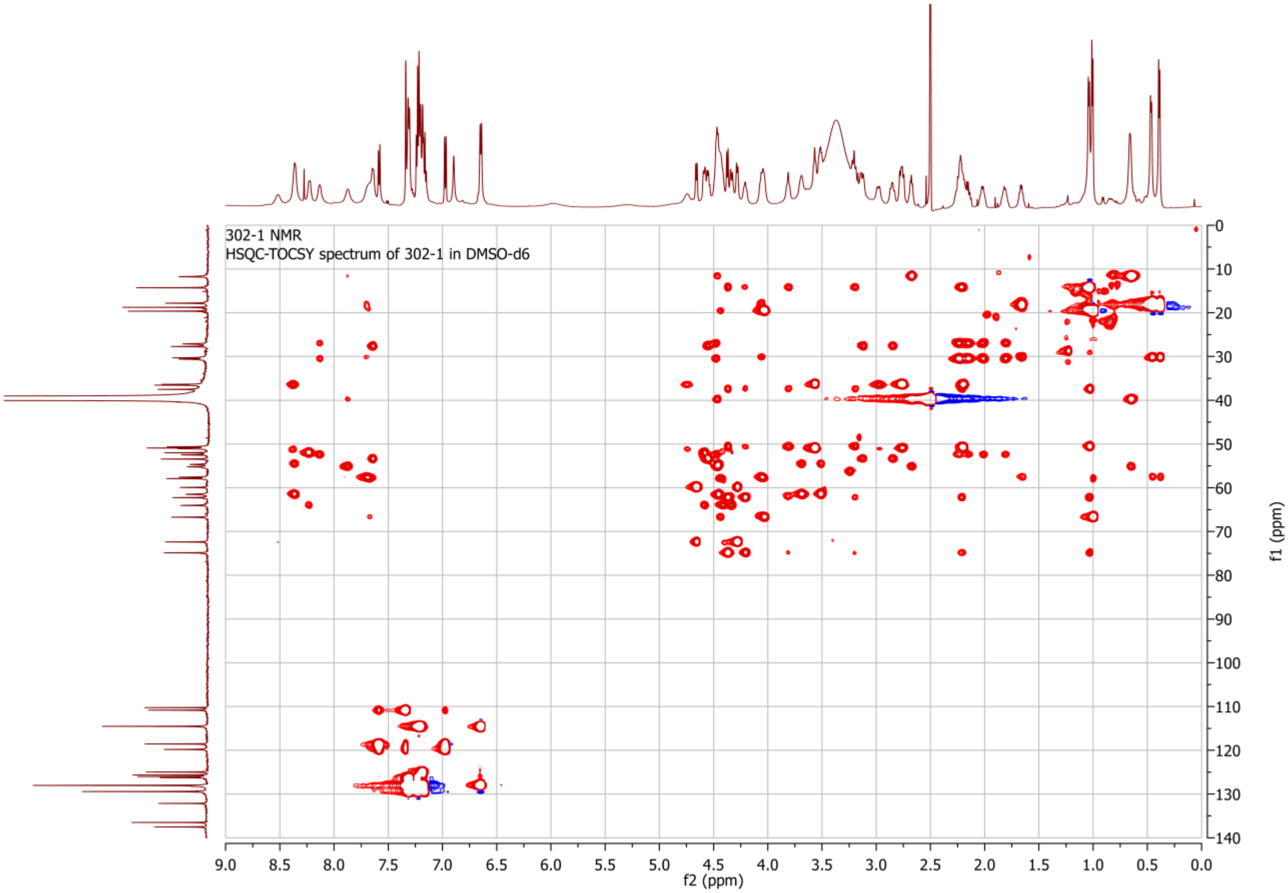
Supplementary Fig. 12 - ^{1}H-^{13}C HMBC NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



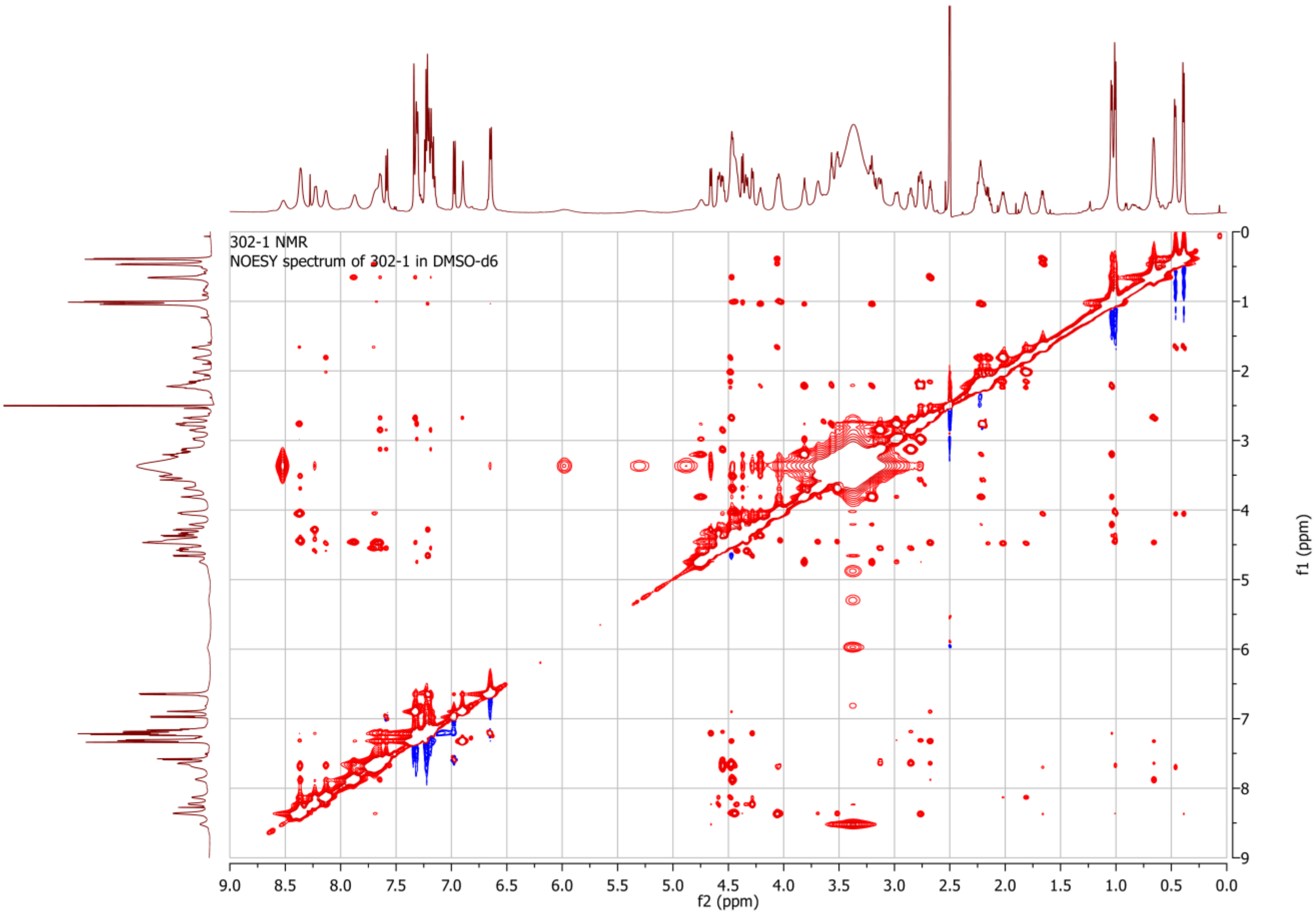
Supplementary Fig. 13 - ^{1}H-^{1}H COSY NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



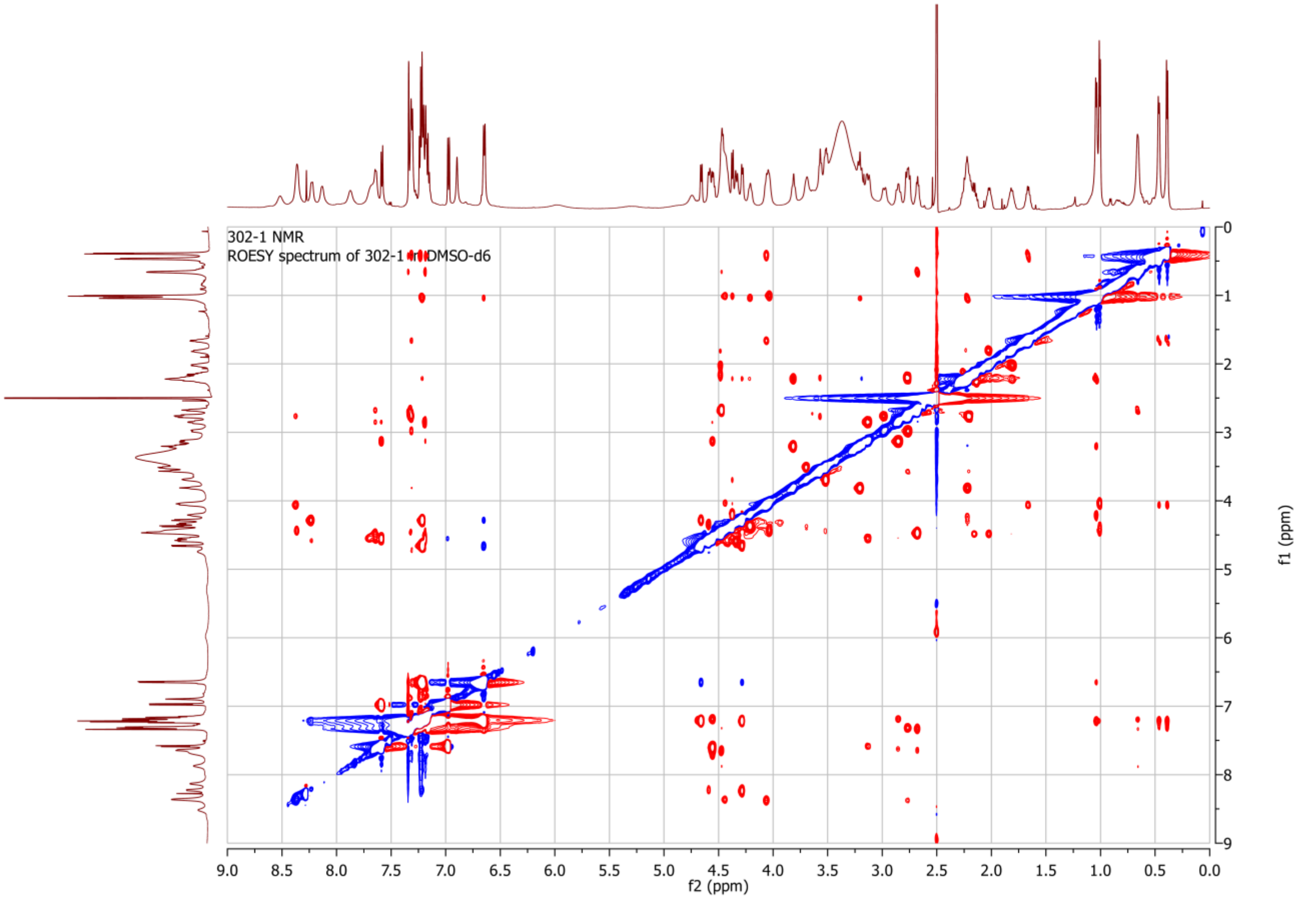
Supplementary Fig. 14 - ^{1}H-^{1}H TOCSY NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



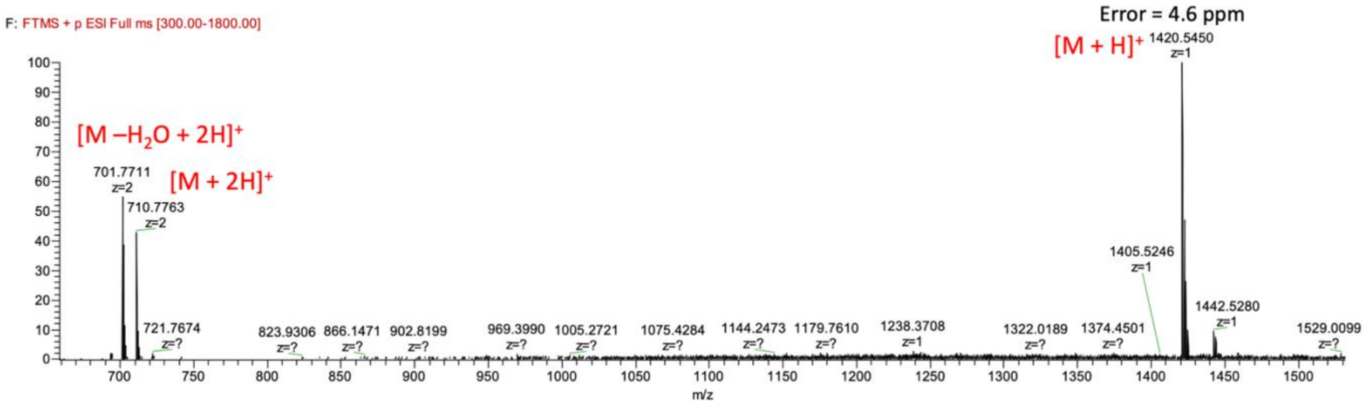
Supplementary Fig. 15 - ^{1}H-^{13}C HSQC-TOCSY NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



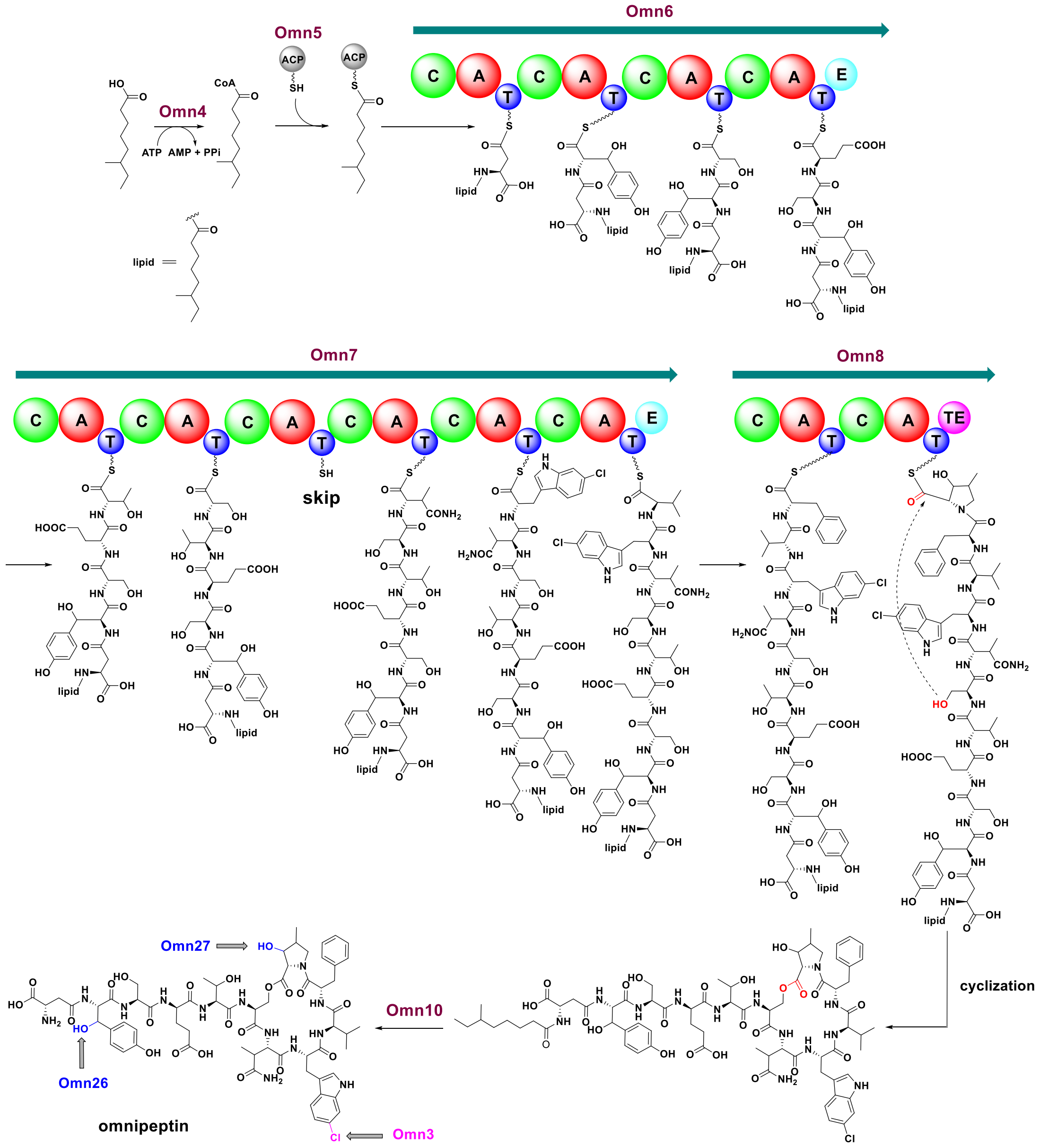
Supplementary Fig. 16 - ^{1}H-^{1}H NOESY NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



Supplementary Fig. 17 - ^{1}H-^{1}H ROESY NMR (600 MHz, DMSO-d_6) spectrum of omnipeptin (**1**)



Supplementary Fig. 18 - High-resolution mass spectrometry spectrum of omnipeptin



Supplementary Fig. 19 - Proposed biosynthesis of omnipeptin