

Expanding the Genetic Code of *Caenorhabditis elegans* Using Bacterial aminoacyl-tRNA Synthetase/tRNA Pairs

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Supporting Methods

Plasmid Construction. Suppression plasmids (*unc-54p::RS*) were constructed using vector pPD30.38 from the Fire lab as a backbone. The synthetase genes (*I*) were amplified by PCR using the following primers: LeuRSfo 5'- AGCTAGCGGTAGAAAA AATGCAAGAGCAATACCGCC -3' and LeuRSrev 5'-GAGCTCTTAGCCAACGACC AGATTGAGG -3'; TyrRSfo 5'- AGCTAGCGGTAGAAAAAATGGCAAGCAGTAA CTTGATTAAACAATTGCAAG -3' and TyrRSrev 5'- AGAGCTCTTATTTCCAGC AAATCAGACAGTAATTCTTTTTACCGC -3'. The Uaa synthetase is amplified with the same primers (TyrRS set for OmeRS, LeuRS set for DanRS). The *unc-54p::RS* plasmids were produced by inserting each RS, using the restriction sites introduced in the PCR primers, in the *Nhe I/Sac I* sites between *unc-54* promoter and *unc-54* 3'UTR. To

insert the promoters and tRNAs, a linker was cloned with the primers Linkfo 5'-CAGAGATCTCCATGGGAACGCTGACGTCATCAACCCGC -3' and Linkrev 5'-GAGACCACGGATCCCCGCCCGGATGGTGGGAATCGG -3'. The suppressor tRNAs were cloned using the following primers: tRNAYfo 5'- CAGGATCCCCGGTGGGG TTCCCGAGCGGC -3' and tRNAYrev 5'- TGACTAGTATGAAAAATGCTGTAAA TTCAAAAAGATATGGTGGGGGAAGGATTCGAACC -3'; tRNALfo 5'- CCACCATCCGGGCGGGGATCCGTGGTCTCATAACAG -3' and tRNALrev 5'- TAACTAGTATGAAAAATGCTGTAAATTCAAAAAGATATACCCGGAGCGGGAC TTGAACC -3'. Both rev primers for cloning tRNA have the *C. elegans* lysyl tRNA 3' flanking sequence (2) for correct termination and processing. Overlap PCR using the linker PCR fragment and each of the tRNA fragments was performed, and this cassette was cloned into pPD30.38 using the *BamH* I/*Spe* I in the vector, and digesting the overlap PCR product with *Bgl* II/*Spe* I. This generated an *Nco* I site 5' of the linker, and a *BamH* I site in between the linker and the tRNA, and abolishing the original *BamH* I site. The tRNA promoter candidates (500 bp each) were amplified from *C. elegans* genomic DNA with the following primers: cen4fo 5'- TACCATGGCGACACGATCTTATTTT GATAAAGAACTTGG -3' and cen4rev 5'- AGGATCCGTGCAATAGGAGGCGTTT ATATAGGG -3'; cen38fo 5'- TACCATGGCCAATTCTCCATTATGAGCAATTG CTACAGTACC -3' and cen38rev 5'- ATGGATCCATTGAACTGGCGTCTCGT GGGTCAC -3'; rpr1fo 5'- TACCATGGGGCTCAGCCTCAACCAATTTTTAGTG -3' and rpr1rev 5'- AGGATCCACGCGCGCCGCGTCGTT -3'. These promoters were inserted between the *Nco* I and *BamH* I sites, taking care to retain the spacing of the +1 nucleotide and the TATA box, if present, in the primer design.

The plasmid for MosSCI integration of *unc-54p::mCherryTAG156* was generated by creating the amber mutation using the primers TAG156fo 5'- GAGCGGATGTAG CCGAGGACG -3' and TAG156rev 5'- CGTCCTCGGGCTACATCCGCTC -3' for overlapping PCR with the primers mCherryfo 5'- TAGGTACCGGTAGAAAAAAT GGTGAGCAAGGGCGAGGAGG -3' and mCherryrev 5'-AGAATTCTTACTTGTACA GCTCGTCCATGCCG -3'. This PCR product was cloned in place of the GFP coding sequence in vector pPD95.77, using the *EcoR* I/*Kpn* I sites which were included on the primers. The *unc-54* promoter was amplified from pPD30.38 using the primers unc54pfo 5'- CCAAGCTTGTCTTCTTCTAAATTCCC -3' and unc54prev 5'- ATGGTACCCAA GGGTCCTCCTGAAAATGTTC -3' and inserted using *Hind* III/*Kpn* I sites also present on these primers. This entire fragment, including the *unc-54* 3' UTR was PCR amplified with the primers unc54Xhofo 5'- AACTCGAGCCATAAAAATCCCGAAACTC CTTCCCTC -3' and uncChrev 5'- CGTCATCACCGAAACGCGCG -3' and cloned into the MosSCI vector pCFJ151 (from E. Jorgensen) using *Xho* I and *Spe* I sites found on the primers, to create *p5605-unc-54p::mCherryTAG156*.

The plasmids for MosSCI integration of *unc-54p::luciferaseTAG185* were generated by PCR amplifying the *unc-54* promoter from *unc-54p::mCherryTAG156* using the primers GWunc54fo 5'-GGGGACAACCTTTGTATAGAAAAGTTGCCCATAAAAT CCGAAACTCCTTCC -3' and GWunc54rev 5'- GGGGACTGCTTTTTTTGTACAAA CTTGCAAGGGTCCTCCTGAAAATGTTC -3' containing *attB4* and *attB1r* flanking sites. The *Photinus pyralis* luciferase gene was amplified from pECL1 (3) using the primers GWlucfo 5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTAGAAA AAATGGAAGACGCC -3' and GWlucrev 5'- GGGGACCACTTTGTACAAGAAA

GCTGGGTTTACAATTTGGACTTTCCGCCC -3' containing *attB1* and *attB2* sites. The TAG mutation at position 185 was introduced by overlapping PCR with the primers lucTAG185fo 5'- CGATTTTGTACCAGAGTAGTTTGATCGTGACAAAAC -3' and lucTAG185rev 5'- GTTTTGTCACGATCAAACACTCTGGTACAAAATCG -3' and the GWluc primers. These PCR products were recombined using BP Clonase II into the Gateway vectors pDONR P4-P1r and pDONR221, respectively (Invitrogen). These two plasmids, as well as a plasmid containing the *unc-54* 3' UTR flanked by *attR2* and *attL3* sequences (from E. Jorgensen) were recombined with LR Clonase II Plus (Invitrogen) with a MosSCI vector containing homologous regions surrounding the *ttTi4348* locus on chromosome I in *C. elegans* (from E. Jorgensen) to create *p4348-unc-54p::luciferaseTAG185*.

The plasmids for integrating 3 copies of *rpr-1p::tRNA^{Leu}_{CUA}* were made using the Gateway system. Identical copies of the gene cassette were PCR amplified for recombination from the *unc-54p::RS* plasmids with the plasmids. To amplify *rpr-1p::tRNA^{Leu}_{CUA}* the following primers were used as pairs: B4RNAsePfo 5'- GGGGACAAC TTTGTATAGAAAAGTTGGGCTCAGCCTCAACCAATTTTTAGTG -3' and B1rLtRNArev 5'- GGGGACTGCTTTTTTGTACAAACTTGATGAAAAATGCTGT AAATTCAAAAAGATATACCCGG -3'; B2RNAsePfo 5'- GGGGACAAGTTTGTAC AAAAAAGCAGGCTGGCTCAGCCTCAACCAATTTTTAGTG -3' and B1LtRNArev 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTATGAAAAATGCTGTAAATT CAAAAGATATACCCGG -3'; B2rRNAsePfo 5'- GGGGACAGCTTTCTTGTAC AAAGTGGGGCTCAGCCTCAACCAATTTTTAGTG -3' and B3LtRNArev 5'- GGGGACAACCTTTGTATAATAAAGTTGATGAAAAATGCTGTAAATTCAAAAAG

ATATACCCGG -3'. Each PCR product was recombined into the appropriate Gateway entry vector with BP Clonase II (Invitrogen) and recombined into MosSCI vector pCFJ150 (from E. Jorgensen) with LR Clonase II Plus (Invitrogen) to create *p5605-rpr-Ip:: tRNA^{Leu}_{CUA} 3x*. The single copy was cut from *unc-54p::LeuRS* using *Nco* I, blunted using Klenow extension (NEB), and then cut with *Spe* I. The *rpr-Ip:: tRNA^{Leu}_{CUA}* fragment was cloned into pCFJ151, which was cut with *Xho* I, blunted, and then cut with *Spe* I, to generate *p5605-rpr-Ip:: tRNA^{Leu}_{CUA}*. The *unc-54p::LeuRS* gene cassette was PCR amplified with the primers *unc54Xhofo* 5'- AACTCGAGCCCATAAAATCCCG AACTCCTTCCCTC -3' and *uncRSrev* 5'- GCCTAATTTCTTTGCTTATTTTT ACTAGTTTTCTTCC -3'. This fragment was digested with *Xho* I and *Spe* I and ligated into pCFJ178 (from E. Jorgensen) for MosSCI integration into chromosome IV to generate *p10882-unc-54p::LeuRS*.

The triple *rpr-Ip:: tRNA^{Tyr}_{CUA}* cassette was constructed using the same forward primers as the *rpr-Ip:: tRNA^{Leu}_{CUA}* cassette and the reverse primers *B1rYtRNArev* 5'- GGGGACTGCTTTTTTGTACAAACTTGATGAAAAATGCTGTAAATTCAAAAAG ATATGGTGGG -3'; *B2YtRNArev* GGGGACCACTTTGTACAAGAAAGCTGGGT ATGAAAAATGCTGTAAATTCAAAAAGATATGGTGGG -3'; and *B3YtRNArev* 5'- GGGGACAACCTTTGTATAATAAAGTTGATGAAAAATGCTGTAAATTCAAAAAG ATATGGTGGG -3'. This fragment was cut from the destination vector with *Avr* II/*Xho* I and ligated into pCFJ178 for MosSCI integration into Chromosome IV, to generate *p10882-rpr-Ip:: tRNA^{Tyr}_{CUA} 3x*.

The *Luciola cruciate* (Japanese) firefly luciferase plasmid (*unc-54p::JFFluciferaseTAG158*) for MosSCI integration was amplified from pJFF12 (from J.

Noel) with the primers JFFlucfo 5'- AAGGATCCATGGAAAACATGGAAAAT GATGAAAACATTGTGG -3' and JFFlucrev 5'- AAGCGGCCGCCAGAGGAGCCCA TTTTGGCCACCGGTTTTTTCAGAA -3' and cloned into pIRES-hrGFP II (Agilent Technologies) using *BamH* I and *Not* I, in frame with the 3xFLAG tag present on the vector. The TAG mutation was made with overlapping PCR using the above primers and JFFTAG158fo 5'- CCTGGATAGCAAATAGGATTATCGTGGTTATCAG -3' and JFFTAG158rev 5'- CTGATAACCACGATAATCCTATTTGCTATCCAGG -3' and cloned into the same vector. The full gene with the 3xFLAG was amplified with the primers GWJFFlucfo 5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTAG AAAAAACCATGGAAAACATGGAAAATGATGAAAACATTGTGG -3' and GWFLAGrev 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTCGAGGAA TTGCTATTATTTGTCGTCAT -3' and recombined into pDONR221 with BP Clonase II (Invitrogen). This entry plasmid was recombined with the Gateway vector above containing the *unc-54* promoter and an entry vector containing the *tbb-2* 3'UTR followed the *gpd-2* operon linker and a histone H2B:GFP fusion (pCFJ236, from E. Jorgensen) to generate a MosSCI vector targeting Chromosome I, to generate *p4348-unc-54p::JFFluciferaseTAG158::H2B::GFP*.

All fragments were PCR amplified with phusion polymerase (NEB), and all constructs were sequenced before injection. All Gateway reactions are done according to the manufacturer's instructions (Invitrogen).

Mass Spectrometric Analysis of the JFFluc Protein and the Orthogonality of the OmeRS

To assess the orthogonality of the OmeRS in *C. elegans* and to verify the incorporation of OmeY into the JFFluc protein, we analyzed the JFFluc protein expressed in strain LWA717 with mass spectrometry.

Expression and immunoprecipitation of the JFFluc protein from LWA717 animals were described in the text and methods section. After incubating the anti-FLAG beads with the lysate from the animals, the beads were washed extensively and resuspended in 100 μ L 50 mM Hepes buffer (pH 7.2). Proteins were reduced and alkylated using 1 mM Tris(2-carboxyethyl) phosphine (Fisher, AC36383) at 94 °C for 5 minutes and 2.5 mM iodoacetamide (Fisher, AC12227) at 37 °C in the dark for 30 minutes, respectively. Proteins were digested on beads with 1 μ g Lys-C (Roche) at 37 °C overnight.

Automated 2D nanoflow LC-MS/MS analysis was performed using an LTQ tandem mass spectrometer (Thermo Electron Corporation, San Jose, CA) employing automated data-dependent acquisition. An Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) was used to deliver a flow rate of 500 nL min⁻¹ to the mass spectrometer through a splitter. Chromatographic separation was accomplished using a 3 phase capillary column. Using a pressure cell constructed in-house, 5 μ m Zorbax SB-C18 (Agilent) packing material was packed into a fused silica capillary tubing (200 μ m ID, 360 μ m OD, 10 cm long) to form the first dimension RP column (RP1). A similar column (200 μ m ID, 5 cm long) packed with 5 μ m PolySulfoethyl (PolyLC) packing material was used as the SCX column. A zero dead volume 1 μ m filter (Upchurch, M548) was attached to the exit of each column for column packing and connecting. A fused silica capillary (200 μ m ID, 360 μ m OD, 20 cm long) packed with 3.5 μ m Zorbax SB-C18 (Agilent) packing material was used as the analytical column

(RP2). One end of the fused silica tubing was pulled to a sharp tip with the ID smaller than 1 μm using a laser puller (Sutter P-2000) as the electro-spray tip. The peptide mixtures were loaded onto the RP1 column using the same in-house pressure cell. To avoid sample carry-over and maintain reproducibility, a new set of three columns with the same length was used for each sample. Peptides were first eluted from the RP1 column to the SCX column using a 0 to 80% acetonitrile gradient for 150 minutes. Then, peptides were fractionated by the SCX column using a series of 8 step salt gradients (10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 70 mM, 100 mM, and 1 M ammonium acetate for 20 minutes), followed by high resolution reverse phase separation using an acetonitrile gradient of 0 to 80% for 120 minutes.

Each sample was analyzed using mixed data-dependent and MRM (Multiple Reaction Monitoring) scan modes. The mass spectrometer was programmed to perform data-dependent MS/MS scans on the 5 most intense ions from the full MS scan (400-1600 Da), followed by 4 MRM scans of the non-substituted (Valine 158) peptide or OMe-tyrosine substituted peptide: m/z 928.4 (OMeY 158, 2+), 619.3 (OMeY 158, 3+), 889.4 (Val 158, 2+), and 593.3 (Val 158, 3+).

Raw data were extracted and searched using Spectrum Mill (Agilent, version A.04.00). MS/MS spectra with a sequence tag length of 1 or less were considered poor spectra and discarded. The filtered MS/MS spectra were searched against a customized protein database containing common protein contaminants (keratins, trypsin, etc.) and the *Luciola cruciata* luciferase. The enzyme parameter was limited to full lys-C peptides with a maximum mis-cleavage of 1. All other search parameters were set to SpectrumMill's default settings (carbamidomethylation of cysteines, +/- 2.5 Da for

precursor ions, +/- 0.7 Da for fragment ions, and a minimum matched peak intensity (SPI) of 50%). Substitution of all 20 amino acid to OMeY was defined as variable modifications. A maximum of 1 substitution per peptide was used. Search results for individual spectra were automatically validated using the filtering criteria listed in Table S1.

Table S1. Filtering criteria for autovalidation of database search results.

Mode	Protein score	1+ peptide (Score, SPI%)	2+ peptide (Score, SPI%)	3+ peptide (Score, SPI%)
Protein Details	>20	>11, >50%	>11, >50%	>13, >50%
Peptide	NA	>13, >50%	>13, >50%	>15, >50%

For the purpose of verifying the incorporation of OmeY at site 158, we unfortunately did not detect the peptide containing this position. We then repeated the experiment with wild type *Luciola cruciata* luciferase purified from strain LWA1580. Again the peptide containing Val at position 158 was not detectable, indicating that this peptide has very weak ionization ability and is unlikely to be detected by the mass spectrometer.

We did identify 11 unique peptides from the JFFluc protein purified from strain LWA717. These peptides were colored in red in Figure S8, and their MS-MS spectra were shown in Figure S9. To assess whether the OmeRS is orthogonal in *C. elegans* cells, we interrogated whether OmeY was mis-incorporated at any position in these 11 peptides. A comprehensive search for OmeY substitution of all 20 amino acids was performed, and no peptide was found to have OmeY misincorporation. All identified peptides were from the non-substituted, wild-type luciferase sequence. These results support that the OmeRS did not mischarge OmeY onto endogenous *C. elegans* tRNAs and thus is orthogonal.

References

1. Wang, Q., and Wang, L. (2008) New Methods Enabling Efficient Incorporation of Unnatural Amino Acids in Yeast, *J. Am. Chem. Soc.* *130*, 6066-6067.
2. Tranquilla, T. A., Cortese, R., Melton, D., and Smith, J. D. (1982) Sequences of four tRNA genes from *Caenorhabditis elegans* and the expression of *C. elegans* tRNA^{Leu} (anticodon IAG) in *Xenopus* oocytes, *Nucleic Acids Res* *10*, 7919-7934.
3. Lagido, C., Pettitt, J., Porter, A. J., Paton, G. I., and Glover, L. A. (2001) Development and application of bioluminescent *Caenorhabditis elegans* as multicellular eukaryotic biosensors, *FEBS Lett* *493*, 36-39.

Supporting Figures

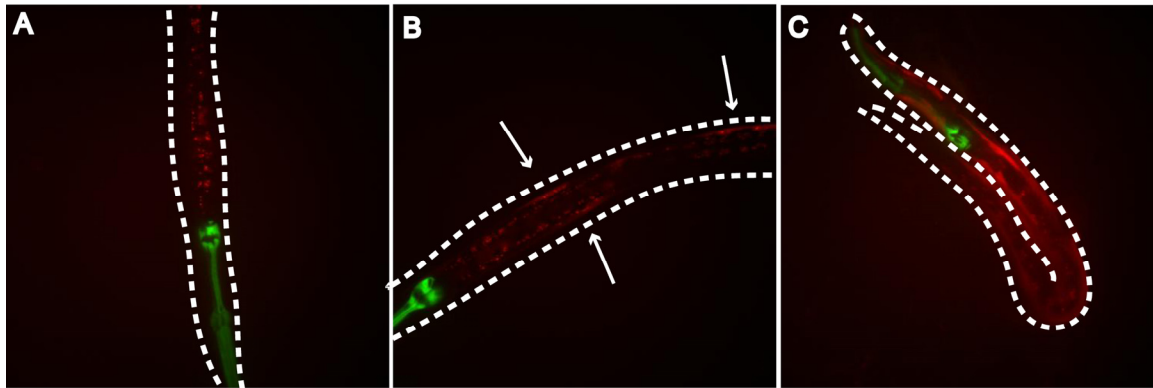


Figure S1. Integration of the orthogonal tRNA/RS array leads to uniform mCherry expression when crossed to the reporter strain. (A) LWA1565 with the $tRNA_{CUA}^{Leu}$ /DanRS array integrated. Red dots are intestinal autofluorescence. (B) mCherry reporter line LWA1560 with extrachromosomal array $wlEx1565[unc-54::DanRS_{rpr-1}::tRNA_{CUA}^{Leu} + myo-2::GFP]$ exposed to 2 mM Ala-DanAla dipeptide. Arrows point to individual red muscle cells; mCherry expression was mosaic. (C) LWA1565 exposed to 2 mM Ala-DanAla dipeptide. Note red fluorescence throughout head and body, rather than isolated muscles. All animals were raised in liquid culture for 1 generation at 15 °C. Dashed lines indicate the outline of the worm. All images are confocal Z stacks and representative of each genotype at larval stage 3-4.

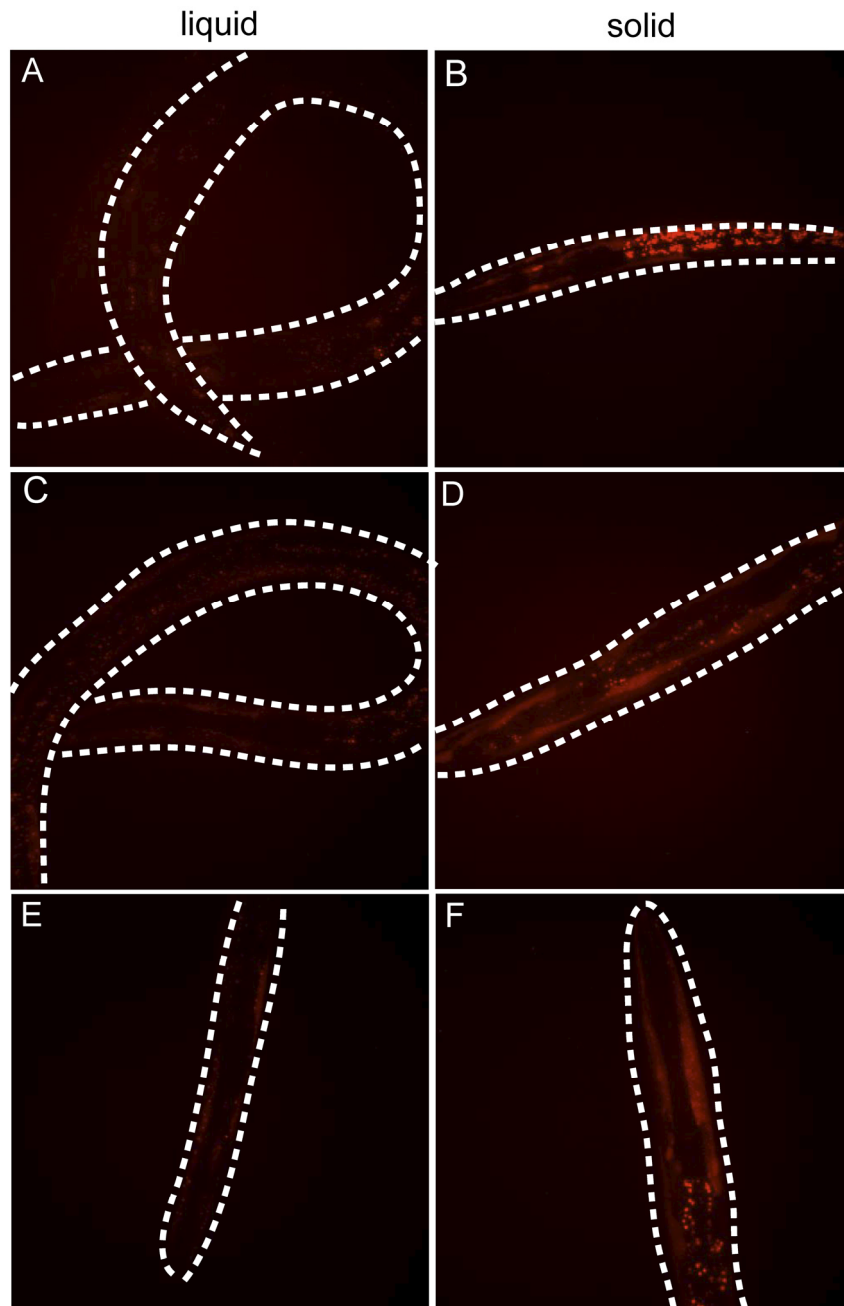


Figure S2. Addition of Uaa to solid media leads to greater Uaa incorporation than in liquid media. All animals are LWA1565. (A-B) 0.5 mM Ala-DanAla dipeptide. (C-D) 1 mM Ala-DanAla dipeptide. (E-F) 2.5 mM Ala-DanAla dipeptide. Punctate dots are intestinal autofluorescence. In each case, there was more uniform and bright mCherry expression when delivered with solid food, and animals appeared healthier on solid food.

All animals were raised for 1 generation at 15 °C. Dashed lines indicate the outline of the worm. All images are confocal Z stacks and the healthiest animals from each condition were imaged, ranging from larval stage 2-4.

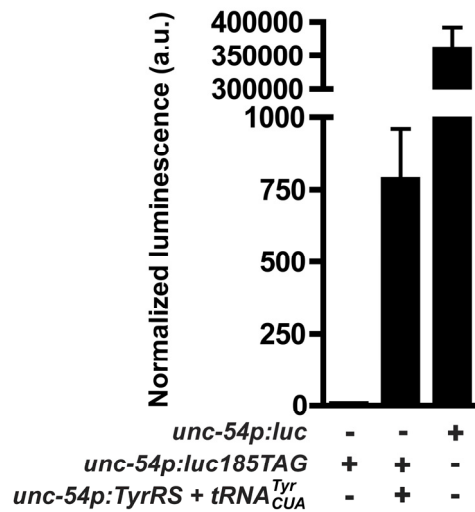


Figure S3. A singly integrated luciferase gene can be used to quantitatively report amber suppression in body wall muscle. Luciferase activity was readily detectable when the wild type luciferase gene was expressed from a single integrated copy in body wall muscle by the *unc-54* promoter. LWA1850 has a singly integrated luciferase gene with a TAG mutation at permissive site 185 driven by the *unc-54* promoter. No luciferase activity was detected from this reporter strain. Only when crossed to a strain with an integrated *E. coli* tRNA_{CUA}^{Tyr}/TyrRS array was luciferase detectable. Animals were grown in duplicate. Luminescence was normalized to total protein concentration. Error bars represent s.e.m.; n ≥ 2.

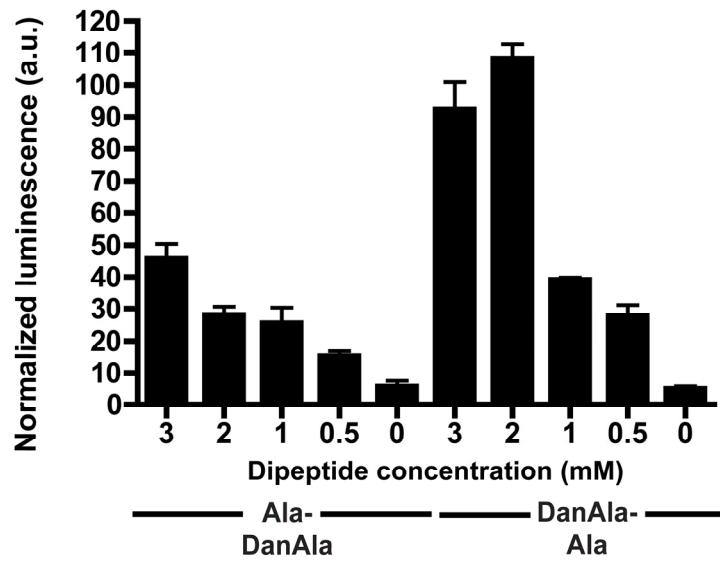


Figure S4. The orientation of Uaa dipeptide affects Uaa incorporation. LWA1031 showed differential luciferase activity depending on the Uaa position in the dipeptide. Animals were exposed to the indicated concentrations of dipeptides for 3 generations at 15 °C on solid media. Animals were grown in duplicate. Luminescence was normalized to total protein concentration. Error bars represent s.e.m.; $n \geq 2$.

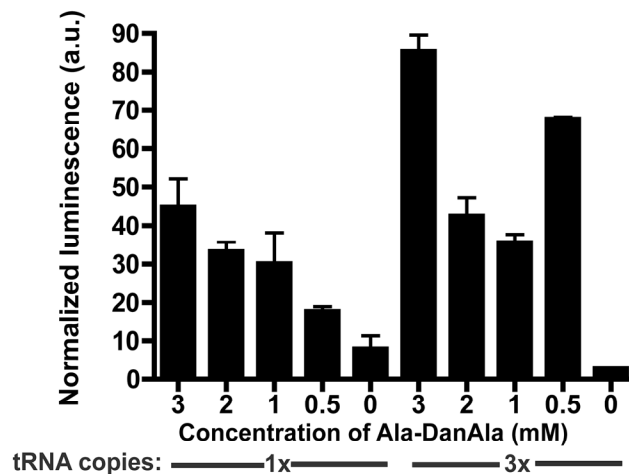


Figure S5. tRNA copy number affects Uaa incorporation with Ala-DanAla dipeptide. LWA1031 (with 1 copy of tRNA) or LWA1856 (with the 3x cassette of the tRNA) were

grown on the indicated concentrations of Ala-DanAla dipeptide. With additional tRNAs a moderate increase of luciferase activity was observed. Animals were grown in duplicate at 15 °C on solid media. Luminescence was normalized to total protein concentration. Error bars represent s.e.m.; $n \geq 2$.

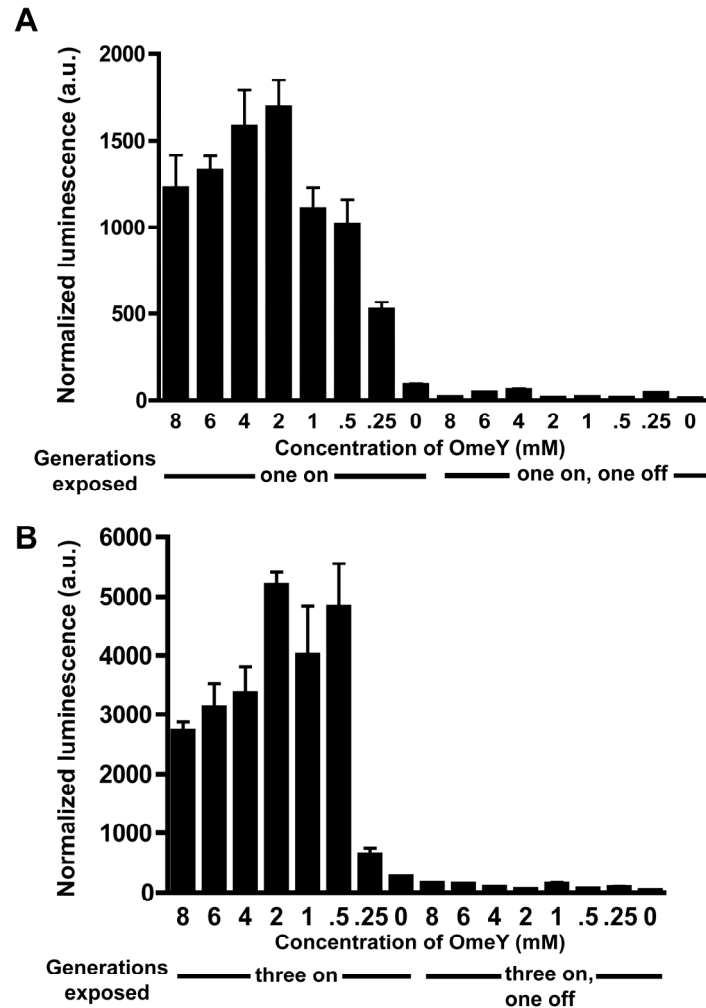


Figure S6. Withdrawal of Uaa for one generation precludes Uaa incorporation. (A) LWA1852 was grown at the indicated concentrations of OmeY for 1 generation in duplicate. Animals were removed from each plate to 0 mM plates for 1 generation and assayed. (B) LWA1852 was grown at the indicated concentrations of OmeY for 3

generations in duplicate. Animals were removed from each plate to 0 mM plates for 1 generation and assayed. All animals were grown in duplicate at 15 °C on solid media. After 1 generation in the absence of Uaa, the luciferase activity dropped back to background levels in all cases. Error bars represent s.e.m.; n ≥ 2.

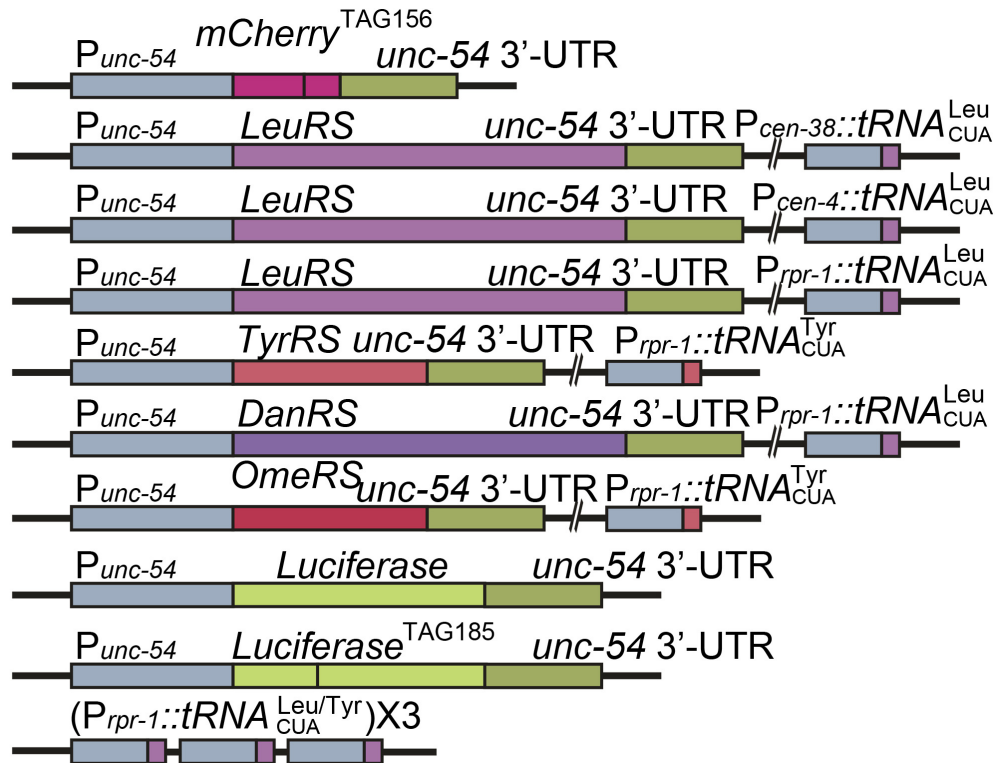
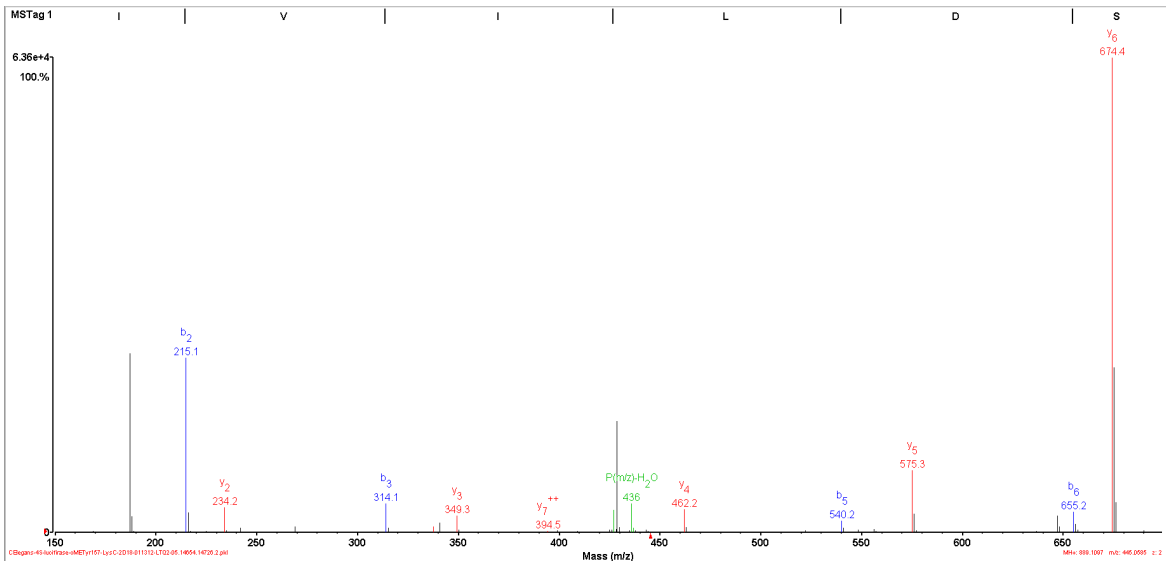


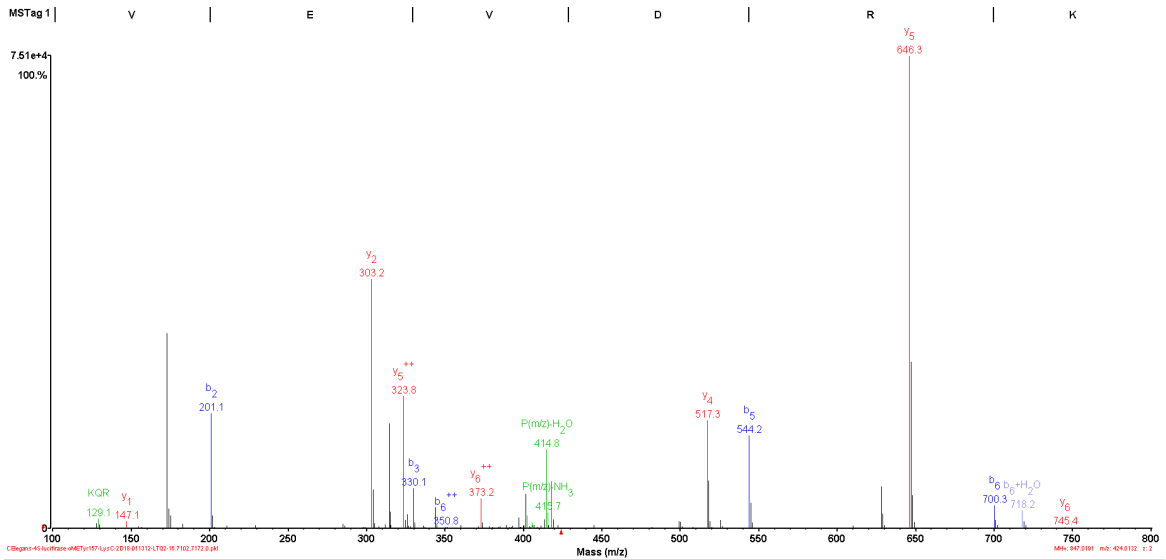
Figure S7. Constructs generated. Representation of the plasmids used to make the animals in this work. All tRNA 3' flanking sequences come from *C. elegans* tRNA^{Lys}.

1	MENMENDENI	VVGPKPFYPI	EESGAGTQLR	<u>K</u> YMERYAKLG	AIAFTNAVTVG	VDYSYAEYLE	<u>K</u> SCCLGKALQ	NYGLVVDGRI	80
81	ALCSENC EE F	FIPVLIAGLFI	GVGVAPTNEI	YTLREL VH SL	GISKPTIVFS	SKKGLD K VIT	VQKT VTTIK T	IVILDSK V VDY	160
161	RGYQC LD TFI	<u>K</u> RNTPPGFQA	SSF K TVEVDR	<u>K</u> EQVALIMNS	SGSTGLF K GV	QLTHENIVTR	FSHARDPIYG	NQVSPGTAVL	240
241	TVVFFHHGFG	MFTTLGYLIC	GFRV VMLT KF	DEETFL K TLQ	DY K CTSVILV	PTLFAILN K S	ELLNKYDLSN	LVEIASGGAP	320
321	LSK EVGEAVA	RRFNLP GV RQ	GYGLTET TS A	IIITPEGDDK	PGASG K VVPL	FKAK V IDLDT	KK SLGPNRRG	EVC V KG P MLM	400
401	<u>K</u> GYVNNPEAT	<u>K</u> ELIDEEGWL	HTGDIGYDE	EKHFFIVDRL	<u>K</u> SLIKY K GYQ	VPPAELESVL	LQHPSIFDAG	VAGVPDPVAG	480
481	ELPGAVVVLE	<u>S</u> GKNMTE K EV	MDYVASQVSN	<u>A</u> KRLRG G VRF	VDEVF K G L TG	<u>K</u> IDGRAIREI	<u>L</u> K K P V A K M G S	SGGRDY K DDD	560
561	<u>D</u> KDY K DDDD K	<u>D</u> Y K DDDD K							578

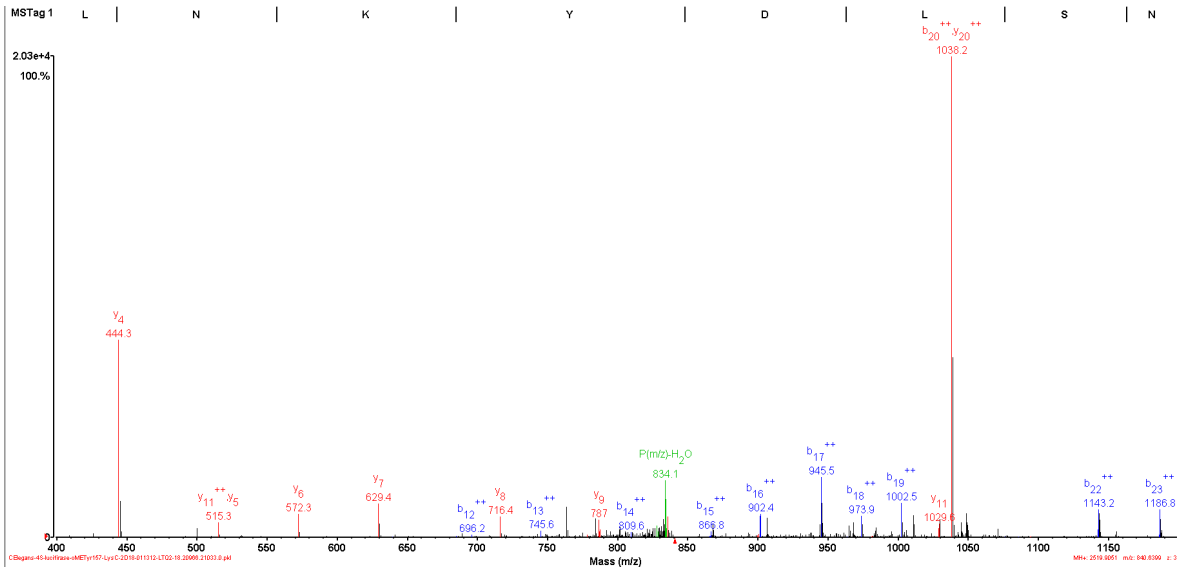
Figure S8. Mass spectrometric analysis of the JFFluc protein purified from strain LWA717. Eleven unique peptides, highlighted in red, were identified. All identified peptides were from the luciferase sequence as indicated. No peptides with OmeY misincorporated at any position in these peptides were found, suggesting that the OmeRS did not mischarge the endogenous *C. elegans* tRNA with the Uaa.



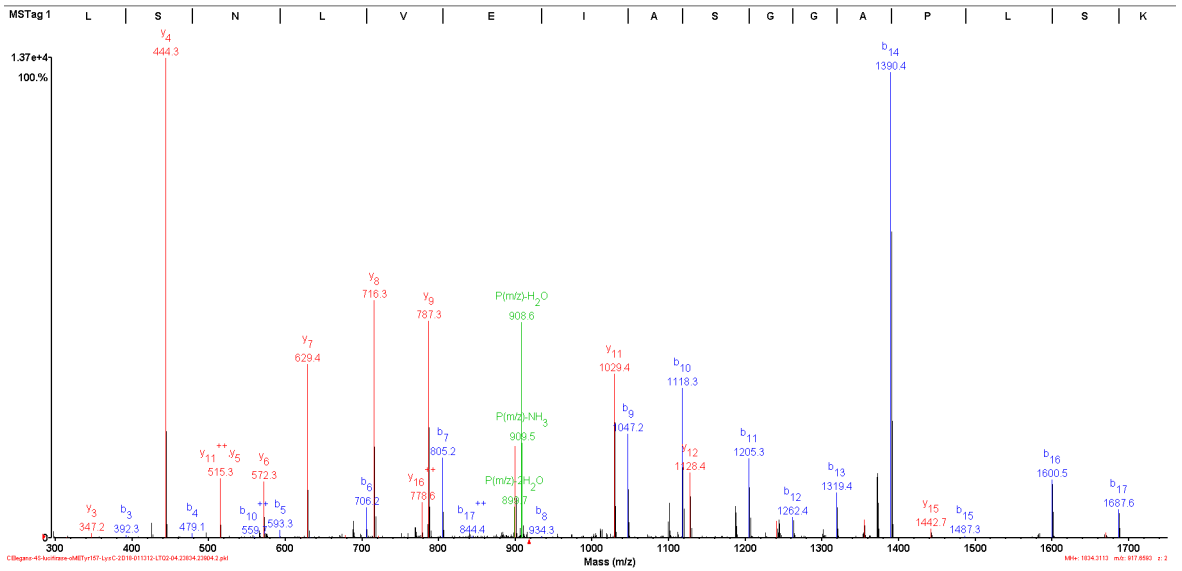
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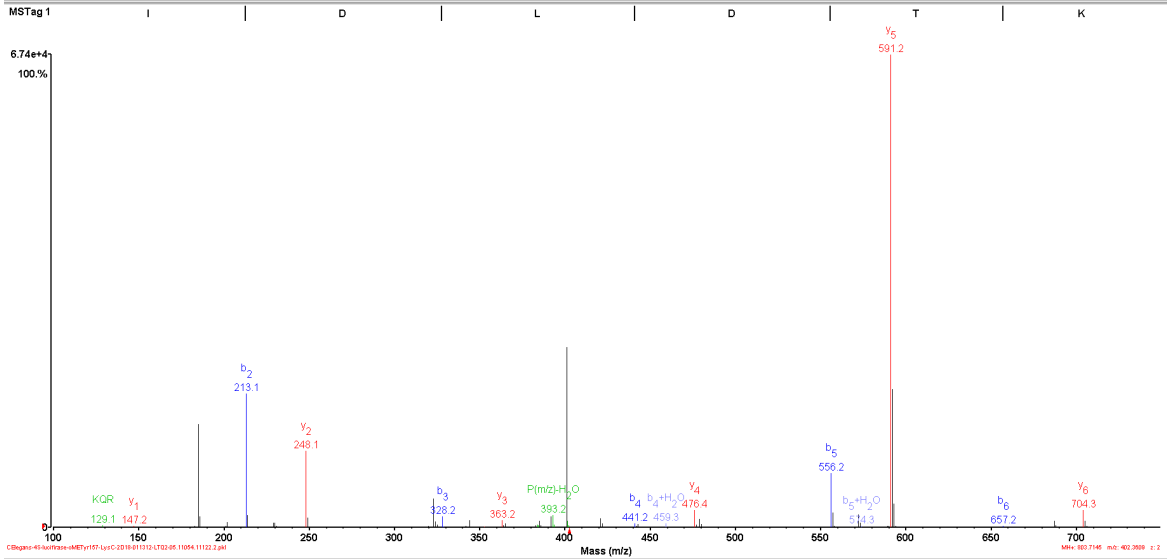
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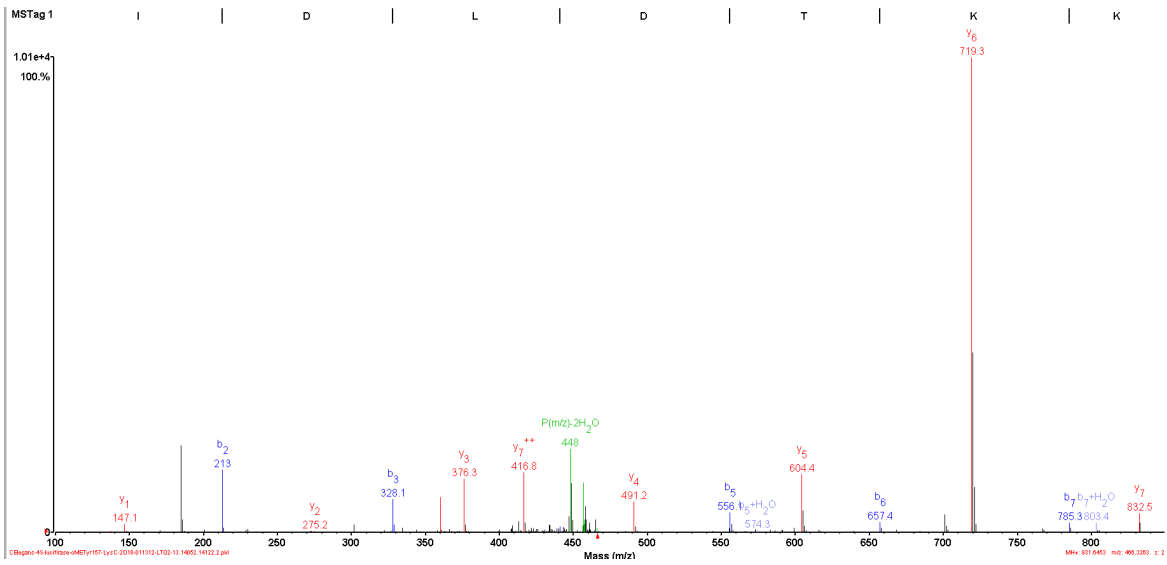
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Spectral count: 1; Start aa position: 300.



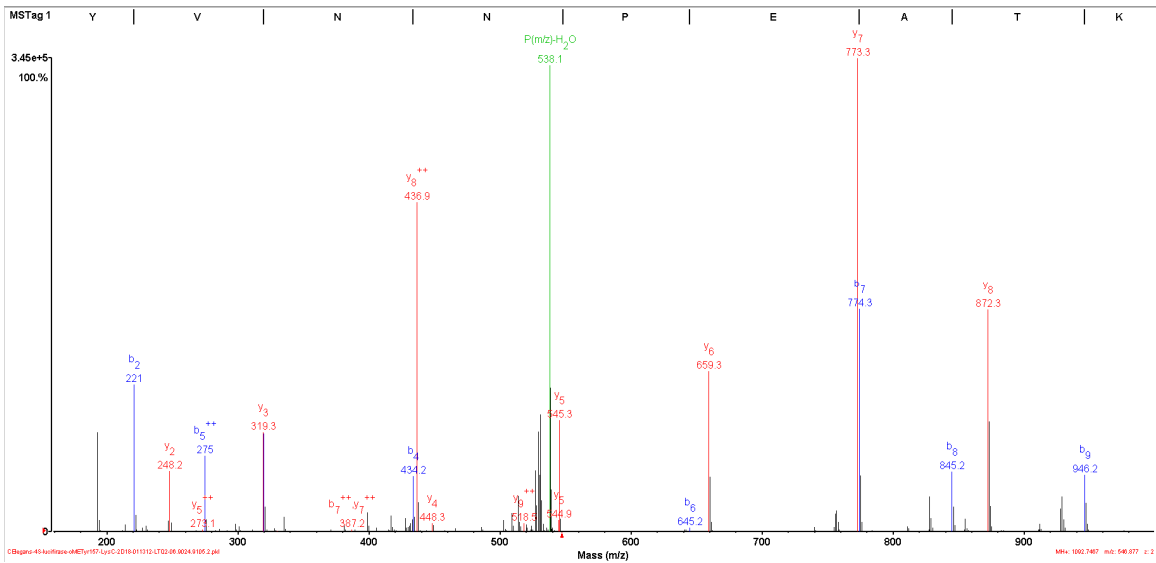
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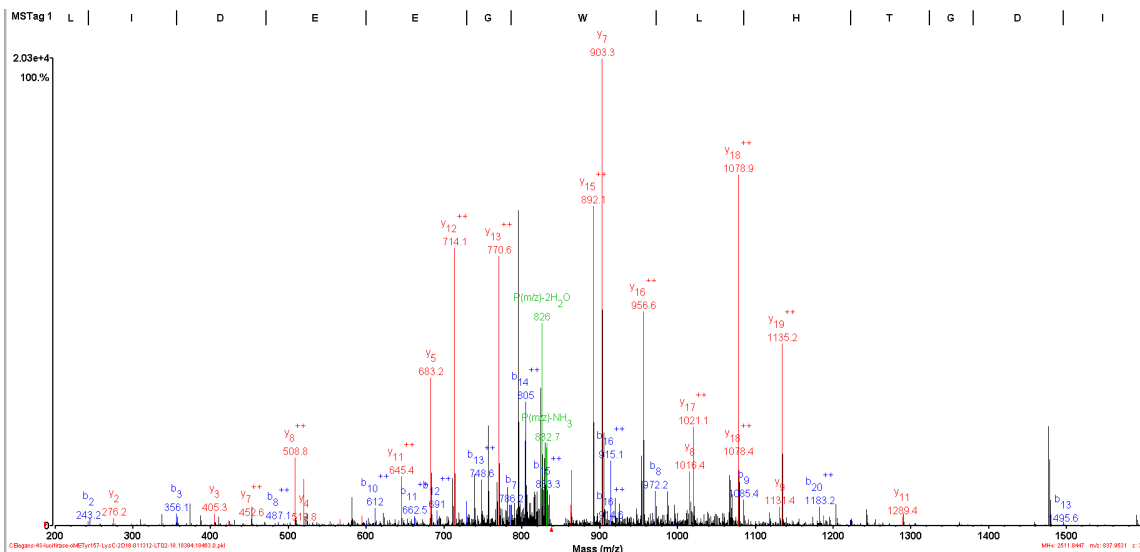
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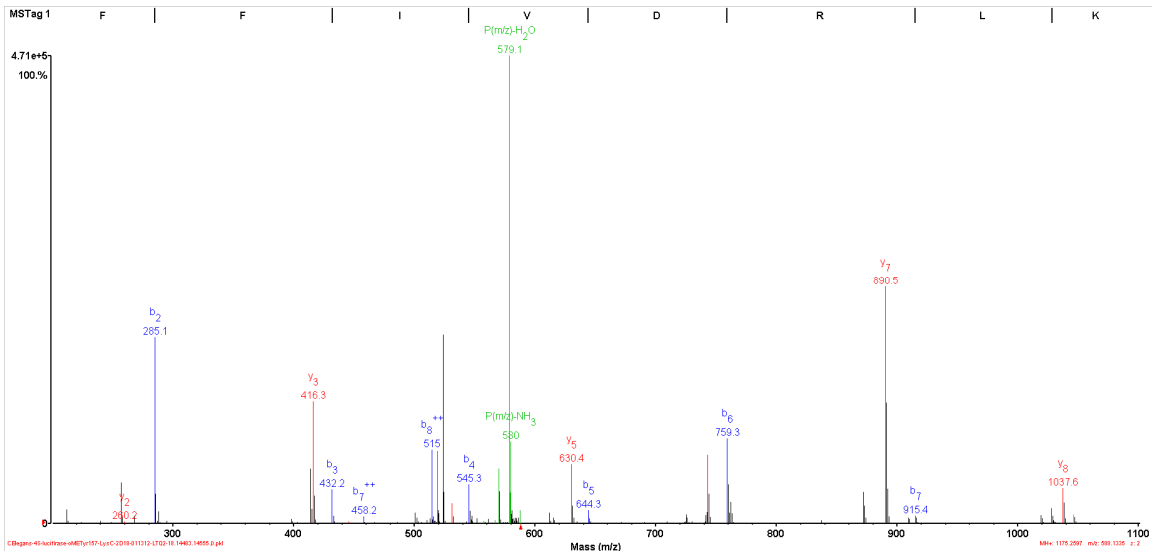
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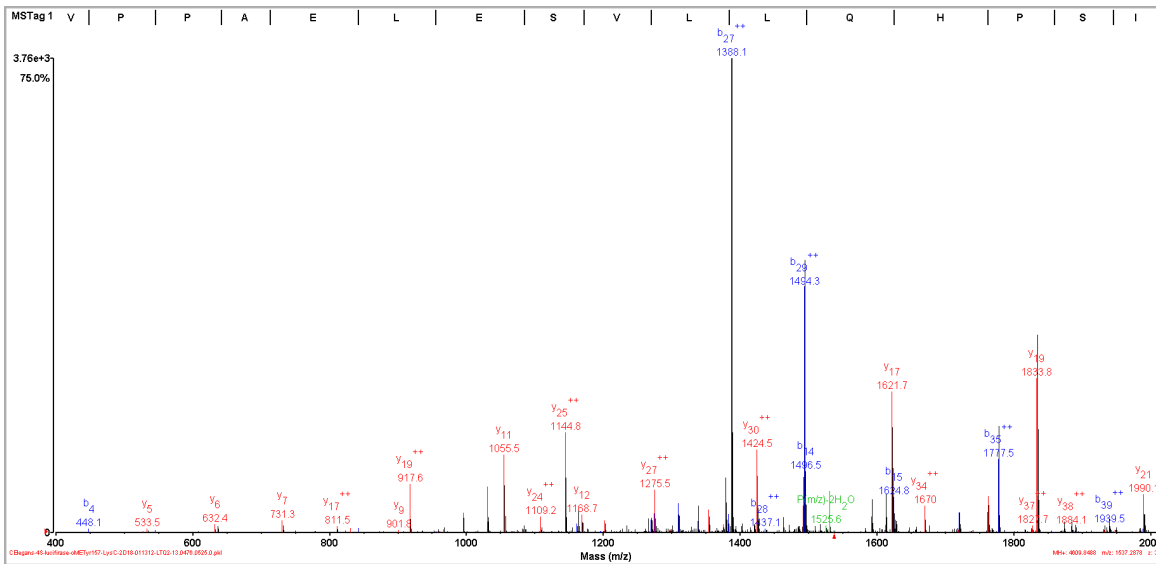
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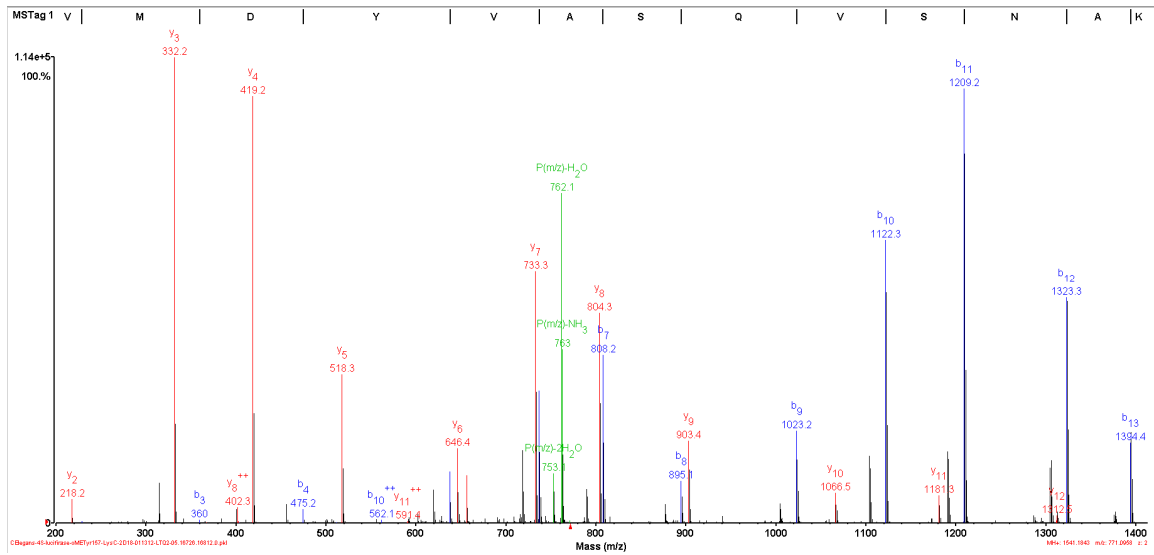
Peptide: (K)ELIDEEGLHTGDIGYYDEEK(H); Score: 21.24;
Spectral count: 28; Start aa position: 412.



Peptide: (K)HFFIVDRLK(S); Score: 17.08;
Spectral count: 2; Start aa position: 433.



Peptide: (K)GYQVPPAELESVLLQHPISIFDAGVAGVDPDPVAGELPGAVVVLES GK(N);
Score: 23.46; Spectral count: 9; Start aa position: 448.



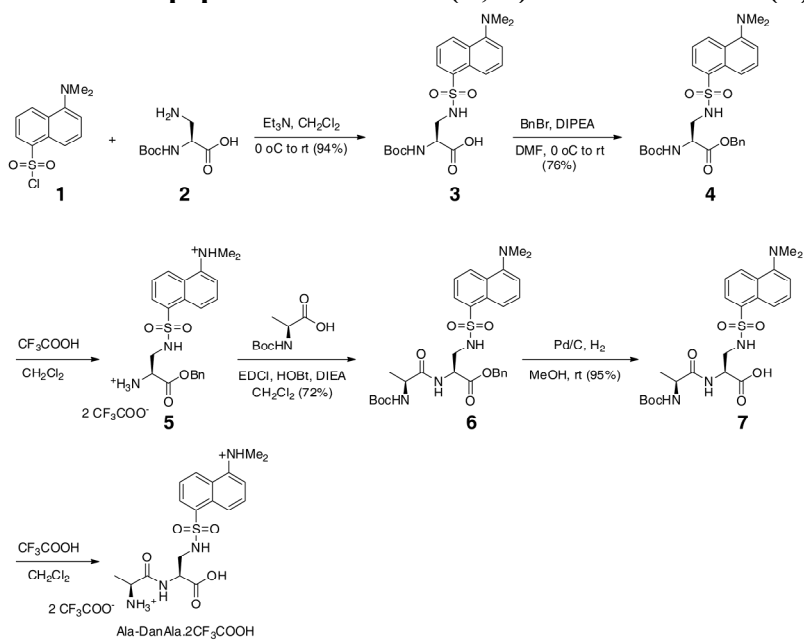
Peptide: (K)EVMDYVASQVSN(A)K; Score: 22.00;
Spectral count: 6; Start aa position: 499.

Figure S9. MS-MS spectra of peptides from JFFluc expressed in strain LWA717.

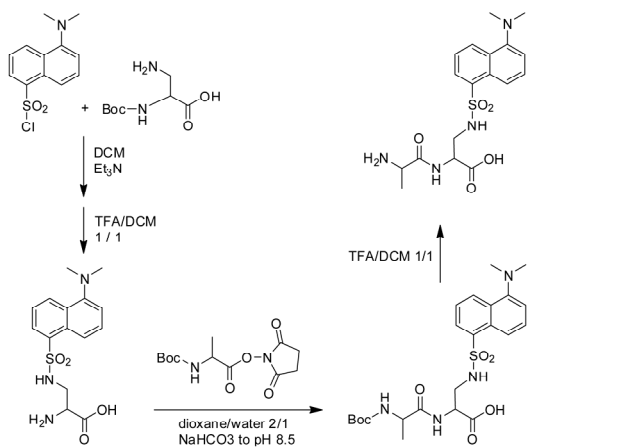
The fragment-ion masses were unambiguously identified, confirming that the sequences of the peptides are from the *L. cruciata* luciferase. No peptide with any of the natural amino acids substituted by the Uaa OmeY was identified.

Scheme S1. Synthesis of dipeptide Ala-DanAla (A, B) and DanAla-Ala (C).

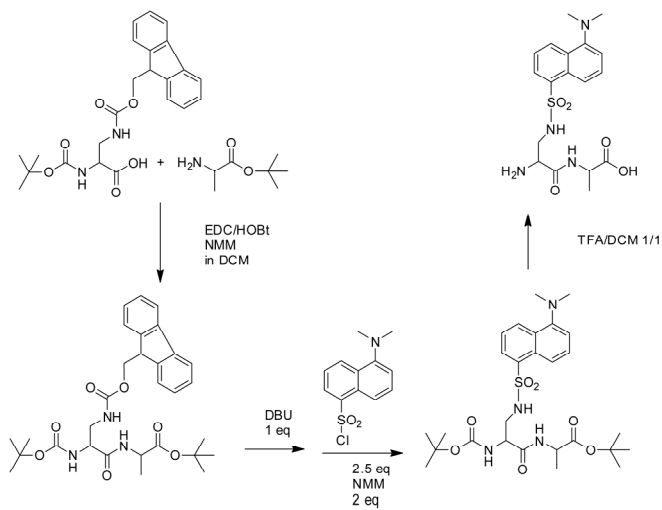
A



B



C



H-Ala-DanAla-OH

¹H NMR (500 MHz, DMSO-*d*6): δ = 8.63 (d, J = 8.0 Hz, 1H), 8.48 (d, J = 8.5 Hz, 1H), 8.28 (d, J = 9.0 Hz, 1H), 8.11-8.09 (m, 4H), 7.64 (dd, J = 7.0, 2.0 Hz, 1H), 7.60 (t, J = 8.0 Hz, 1H), 7.28 (d, J = 7.5 Hz, 1H), 4.40 (dt, J = 7.0, 5.5 Hz, 1H), 3.83 (t, J = 6.0 Hz, 1H), 3.14 (t, J = 5.5 Hz, 1H), 3.08 (dd, J = 13.5, 7.0 Hz, 1H), 2.84 (s, 6H), 1.35 (d, J = 7.0 Hz, 3H); ¹³C NMR (125MHz, DMSO-*d*6): δ = 170.7, 169.5, 151.3, 135.5, 129.6, 129.1, 129.0, 128.2, 128.0, 123.6, 119.1, 115.3, 64.9, 52.6, 48.1, 45.1, 43.2, 25.2, 16.9.

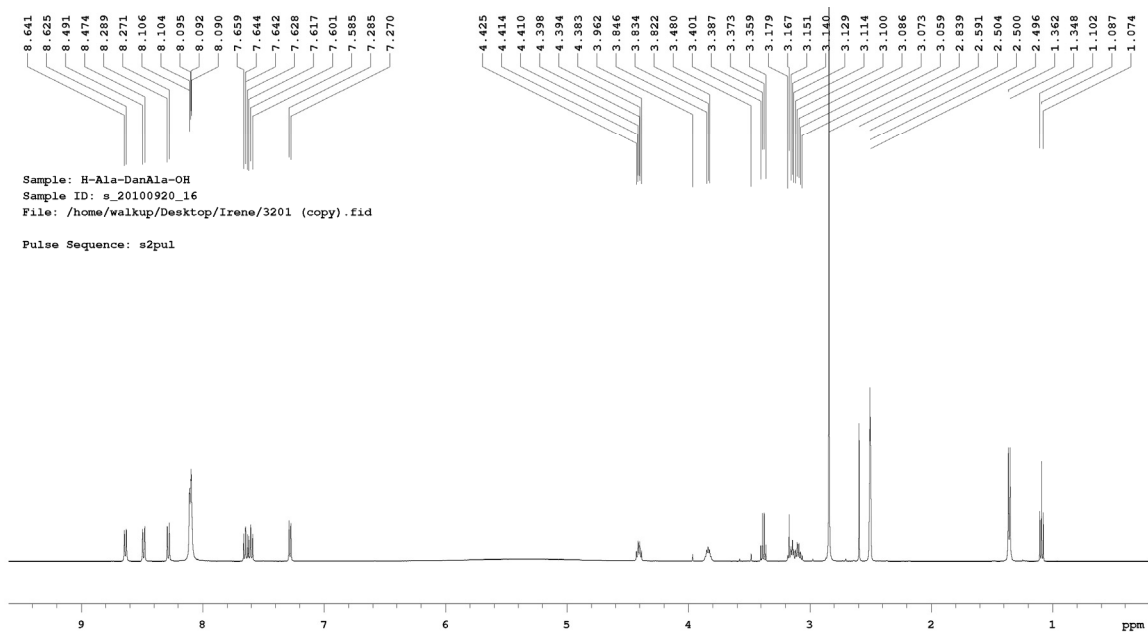
LC-MS (245 nm, single peak), ESI-ion trap, [M+H]⁺_{expected}: 409.15, [M+H]⁺_{found}: 408.5, [2M+H]⁺_{found}: 816.3

H-DanAla-Ala-OH

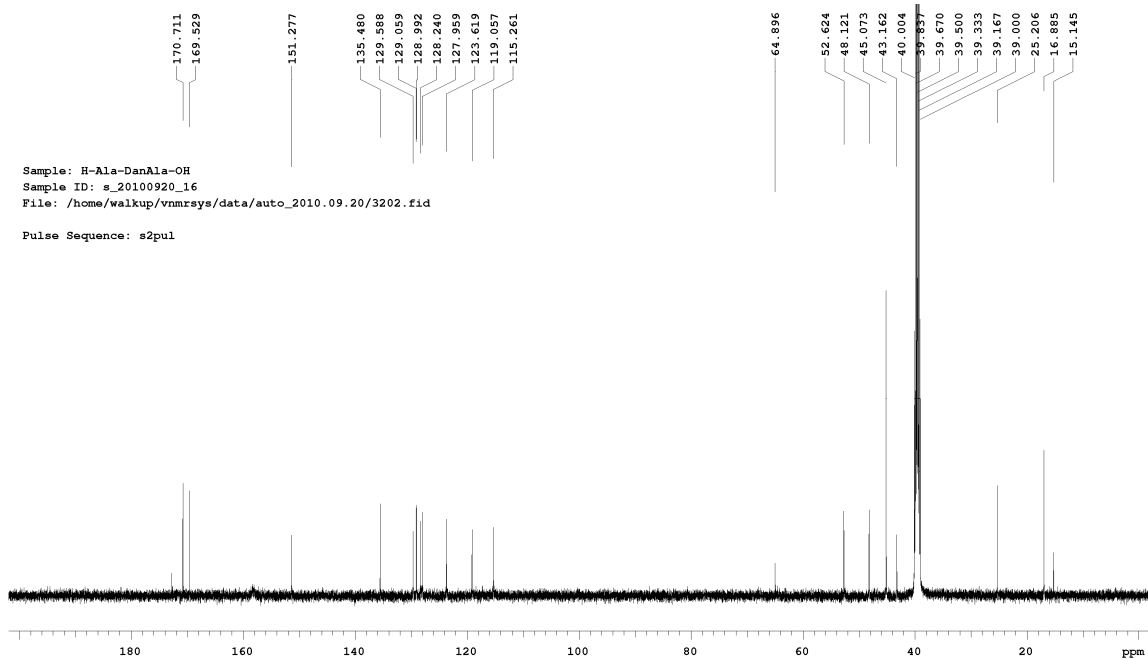
¹H NMR (500 MHz, DMSO-*d*6): δ = 8.49 (d, J = 8.5 Hz, 1 H), 8.28 (d, J = 8.5 Hz, 1H), 8.11 (d, J = 6.5 Hz, 1H), 7.64 (t, J = 8.0 Hz, 1H), 7.60 (t, J = 8.0 Hz, 1H), 7.27 (d, J = 7.0 Hz, 1H), 4.18 (t, J = 6.0 Hz, 1H), 3.66 (s, 1H), 3.07 (dd, J = 13.0, 4.0 Hz, 1H), 2.98 (dd, J = 13.0, 7.5 Hz, 1H), 2.83 (s, 6H), 1.26 (d, J = 7.0 Hz, 3H); ¹³C NMR (125MHz, DMSO-*d*6): δ = 173.8, 168.2, 151.4, 135.1, 129.7, 129.1, 129.0, 128.4, 128.1, 123.6, 118.9, 115.3,

64.9, 52.9, 47.8, 45.1, 44.6, 17.2. LC-MS (245 nm, single peak), ESI-ion trap, [M+H]⁺_{expected}: 409.15, [M+H]⁺_{found}: 408.5, [2M+H]⁺_{found}: 816.4

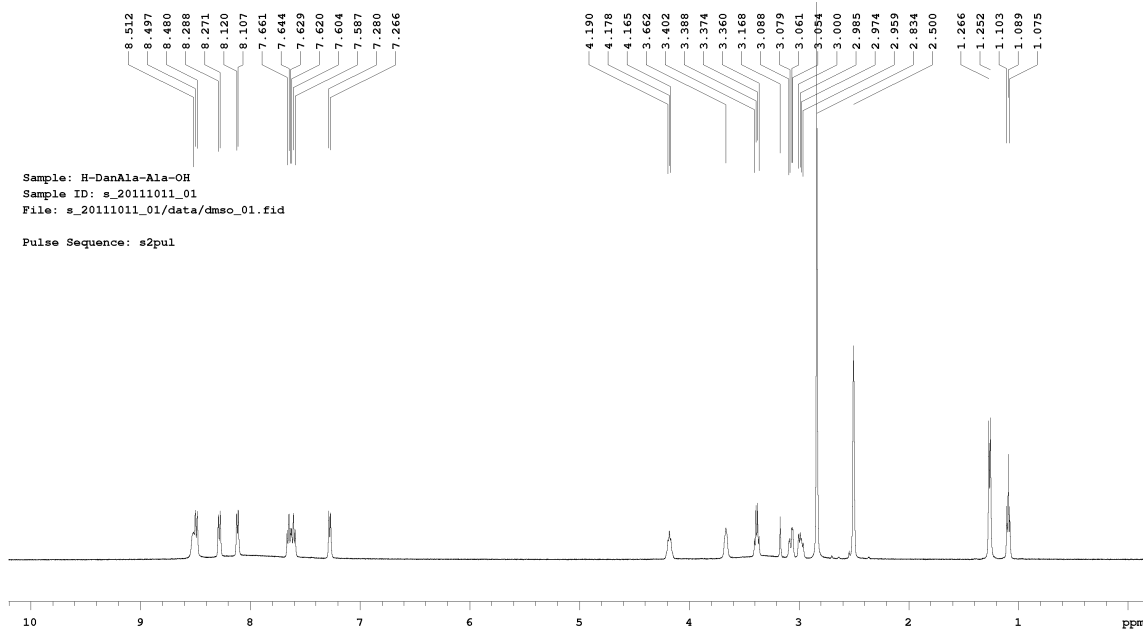
¹H NMR (500 MHz, DMSO-d₆) of H-Ala-DanAla-OH



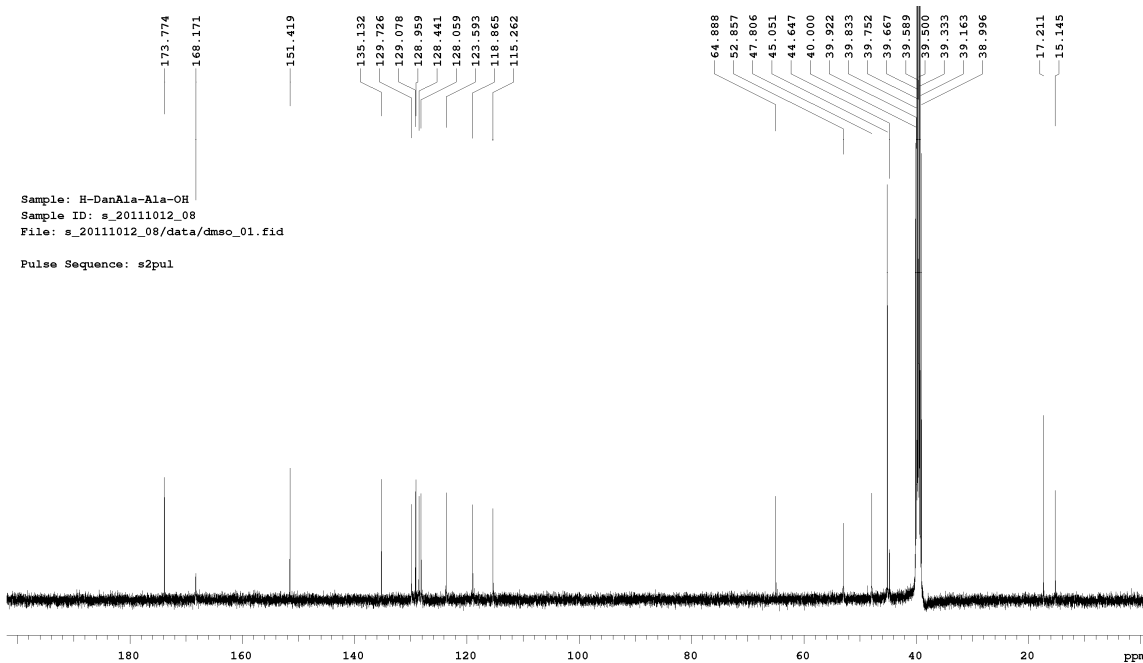
¹³C NMR (125MHz, DMSO-d₆) of H-Ala-DanAla-OH



¹H NMR (500 MHz, DMSO-*d*₆) of H-DanAla-Ala-OH



¹³C NMR (125MHz, DMSO-*d*₆) of H-DanAla-Ala-OH



Plasmid Sequences

unc-54p::LeuRS

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unc-54p::TyrRS

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