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A novel method for screening peptides that bind to proteins by using multiple fluorescent amino acids as fluorescent tags

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Experimental procedures for synthesis of Fmoc-Lys(Moc)-OH, Fmoc-Lys(Hoc)-OH Fmoc-Lys(Mac)-OH, and Fmoc-Orn(Cm3)-OH, and peptide oligomers modified with these Fmoc-derivatives

General: 7-Methoxycoumarin-3-carboxylic acid, SE, 7-hydroxycoumarin-3-carboxylic acid, SE and dimethylaminocoumarin-3-acetic acid, SE were purchased from Ana Spec (San Jose, CA, USA) and used without further purification. Coumarin 343 was purchased from Kanto Chemical (Tokyo, Japan) and used without further purification. 9-Fluorenylmethyl-succinimidyl carbonate (Fmoc-OSu), Boc-Orn-OtBu・HCl, Fmoc-Lys-OH・HCl, TFA, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotiazole (HOAt), diisopropylethylamine (DIPEA), triisopropylsilane (TIS), Fmoc-SH-SAL-PEG resin and other Fmoc-derivative natural amino acids were purchased from Watanabe Chemical (Hiroshima, Japan) and used without further purification. DMF, *N*-methylpyrrolidone (NMP), $NaHCO_3$ and acetic anhydride (Ac_2O) were purchased from Wako Chemicals (Osaka, Japan) and used without further purification. 2,6-Lutidine was purchased from Tokyo Kasei (Tokyo, Japan) and used without further purification. Fmoc-NH-PEG5-COOH was purchased from Merck (Darmstadt, Germany) and used without further purification. Distilled water was used throughout the synthesis. ^{1}H NMR spectra of the fluorescent amino acids were recorded on a Mercury 300 spectrometer (Varian). MALDI-TOF masses of the peptide oligomers were recorded on a Voyager DE Pro (Applied Biosystem).

Synthesis of Fmoc-Lys(Moc)-OH

Fmoc-Lys-OH・HCl (70.3 mg, 174 μmol) was mixed with 7-methoxycoumarin-3-carboxylic acid, SE (50.0 mg, 158 μmol) in acetonitrile/0.2M $NaHCO_3$ aq. (=2/1 v/v) at room temperature overnight. Then the mixture was purified with HPLC (acetonitrile/0.1% TFA aq.) and dried *in vacuo*. Fmoc-Lys(Moc)-OH was obtained as a white powder (84 mg, 93%):^{1}H NMR (300MHz, DMSO-d_6) δ 1.37 (m, 2H, Lys γ -CH_2-), 1.52 (m, 2H, Lys δ -CH_2-), 1.64 (m, 2H, Lys β -CH_2-), 3.30 (m, 2H, Lys ε -CH_2-), 3.88 (s, 3H, Coumarin (7)-C-OCH_3), 3.92 (m, 1H, Lys α -C*H*-), 4.1-4.3 (m, 3H, Fmoc -C*H*-CH_2-), 6.994, 7.003, 7.024, 7.031 (q, 1H, Coumarin (6)-C*H*), 7.064, 7.071 (d, 1H, Coumarin (8)-C*H*), 7.3 (t, 2H, Fmoc (2) -C*H*-), 7.35, 7.38, 7.40 (t, 2H, Fmoc (3) -C*H*-), 7.62, 7.64 (d, 1H, Lys α C-N*H*-), 7.68, 7.70 (d, 2H, Fmoc (1) -C*H*-), 7.84, 7.86 (d, 2H, Fmoc (4) -C*H*-), 7.84, 7.87 (d, 1H, Coumarin (5)-C*H*), 8.64 (t, 1H, Lys ε C-N*H*-), 8.78 (s, 1H, Coumarin (4)-C*H*), 12.5 (br, 1H,

-COO*H*).

Synthesis of Fmoc-Lys(Hoc)-OH

Fmoc-Lys-OH・HCl (73.5 mg, 182 μmol) was mixed with 7-hydroxycoumarin-3-carboxylic acid, SE (50.0 mg, 165 μmol) in acetonitrile/0.2M $NaHCO_3$ aq. (=2/1 v/v) at room temperature overnight. Then the mixture was purified with HPLC (acetonitrile/0.1% TFA aq.) and dried *in vacuo*. Fmoc-Lys(Hoc)-OH was obtained as a white powder (107 mg, quant.): ^{1}H NMR (300MHz, DMSO-d_6) δ 1.38 (m, 2H, Lys γ -CH_2-), 1.52 (m, 2H, Lys δ -CH_2-), 1.64 (m, 2H, Lys β -CH_2-), 3.30 (m, 2H, Lys ε -CH_2-), 3.92 (m, 1H, Lys α -C*H*-), 4.1-4.3 (m, 3H, Fmoc -C*H*-CH_2-), 6.768, 6.775 (d, *J*=2.1 Hz, 1H, Coumarin (8)-C*H*), 6.840, 6.847, 6.868, 6.875 (q, *J*=8.4 and 2.1 Hz, 1H, Coumarin (6) -C*H*), 7.28, 7.30, 7.32 (t, 2H, Fmoc (2) -C*H*-), 7.36, 7.39, 7.41 (t, 2H, Fmoc (3) -C*H*-), 7.62, 7.65 (d, 1H, Lys α C-N*H*-), 7.69, 7.71 (d, *J*=7.5 Hz, 2H, Fmoc (1) -C*H*-), 7.77, 7.79 (d, *J*=8.7 Hz, 1H, Coumarin (5)-C*H*), 7.85, 7.88 (d, *J*=7.5Hz, 2H, Fmoc (4) -C*H*-), 8.64 (t, 1H, Lys ε C-N*H*-), 8.75 (s, 1H, Coumarin (4)-C*H*), 11.02 (s, 1H, Coumarin (7)-C-O*H*), 12.5 (br, 1H, -COO*H*).

Synthesis of Fmoc-Lys(Mac)-OH

Fmoc-Lys-OH・HCl (64.8 mg, 160 μmol) was mixed with 7-dimethylaminocoumarin-3-carboxylic acid, SE (50.0 mg, 145 μmol) in acetonitrile/0.2M $NaHCO_3$ aq./DMF (=9/6/2 v/v) at room temperature overnight. Then the mixture was purified with HPLC (acetonitrile/0.1% TFA aq.) and dried *in vacuo*. Fmoc-Lys(Hoc)-OH was obtained as a slightly yellow powder (48 mg, 45%): ^{1}H NMR (300MHz, DMSO-d_6) δ 1.37 (m, 4H, Lys γ -CH_2- and Lys δ -CH_2-), 1.6 (m, 2H, Lys β -CH_2-), 2.99 (s, 6H, Coumarin (7)-N(CH_3)$_2$), 3.0 (m, 2H, Lys ε -CH_2-), 3.56 (s, 2H, Coumarin (4)-C-CH_2-), 3.89 (m, 1H, Lys α -C*H*-), 4.1-4.3 (m, 3H, Fmoc -C*H*-CH_2-), 5.97 (s, 1H, Coumarin (3)-C*H*), 6.52, 6.53 (d, 1H, Coumarin (8)-C*H*), 6.67, 6.68, 6.70, 6.71 (q, 1H, Coumarin (6)-C*H*), 7.28, 7.30, 7.33 (t, 2H, Fmoc (2) -C*H*-), 7.37, 7.39, 7.42 (t, 2H, Fmoc (3) -C*H*-), 7.49, 7.52 (d, 1H, Coumarin (5)-C*H*), 7.59, 7.61 (d, 1H, Lys α C-N*H*-), 7.69, 7.71 (d, 2H, Fmoc (1) -C*H*-), 7.86, 7.88 (d, 2H, Fmoc (4) -C*H*-), 8.17 (t, 1H, Lys ε C-N*H*-), 12.53 (br, 1H, -COO*H*).

Synthesis of Fmoc-Orn(Cm3)-OH

First, Boc-Orn-OtBu・HCl (104 mg, 318 μmol), Coumarin 343 (100 mg, 351 μmol), HATU (133 mg, 350 μmol) and DIPEA (123 mg, 953 μmol) were mixed in DMF at 60 ˚C overnight. Then the mixture was purified with HPLC (acetonitrile/0.1% TFA aq.) and dried *in vacuo*. Fmoc-Lys(Hoc)-OH was obtained as a white powder: ^{1}H NMR (300MHz, DMSO-d_6) δ 1.37 (s, 18H, Boc and *tert*-Butyl), 1.50-1.64 (m, 4H, Lys β -CH_2- and γ -CH_2-), 1.839, 1.859, 1.878, 1.897 (q, 4H, Coumarin N-CH$_2$-CH_2-), 2.681, 2.709, 2.731, 2.751 (q, 4H, Coumarin N-CH$_2$-CH$_2$-CH_2), 3.26 (m, 2H, Lys δ -CH_2-), 3.32 (m, 2H, Coumarin N-CH_2-), 3.79 (m, 1H, Lys α -C*H*-), 7.13, 7.16 (d, 1H, Lys

α C-N*H*-), 7.26 (s, 1H, Coumarin (5)-C*H*), 8.50 (s, 1H, Coumarin (4)-C*H*), 8.64 (t, 1H, Lys ε C-N*H*-).

Next, the obtained compound was treated with TFA for 1.5 h at room temperature. The resultant mixture was evaporated to dryness. The residue was dissolved in 5% aq $NaHCO_3$ to bring the pH to 8. A solution of Fmoc-OSu (113 g, 335 mmol) in acetonitrile was added to the aqueous solution. The reaction mixture was stirred at room temperature overnight. Then the mixture was purified with HPLC (acetonitrile/0.1% TFA aq.) and dried *in vacuo*. Fmoc-Lys(Cm3)-OH was obtained as a white powder (164 mg, 83%): ^{1}H NMR (300MHz, DMSO-d_6) δ 1.6-1.8 (m, 4H, Lys β -CH_2- and γ -CH_2-), 1.88 (q, 2H, N-CH_2-), 2.677, 2.699, 2.720, 2.742 (q, 4H, Coumarin N-CH_2-CH_2-CH_2), 3.2 (m, 4H, Lys δ -CH_2- and Coumarin N-CH_2-), 3.95 (m, 1H, Lys α -C*H*-), 4.1-4.3 (m, 3H, Fmoc -C*H*-CH_2-), 7.23 (s, 1H, Coumarin (5)-C*H*), 7.29, 7.31, 7.34 (t, 2H, Fmoc (2) -C*H*-), 7.37, 7.40, 7.42 (t, 2H, Fmoc (3) -C*H*-), 7.7 (m, 1H, Lys C-α-N*H*-), 7.70, 7.72 (d, 2H, Fmoc (1) -C*H*-), 7.86, 7.89 (d, 2H, Fmoc (4) -C*H*-), 8.50 (s, 1H, Coumarin (4)-C*H*), 8.67 (t, 1H, Lys ε C-N*H*-), 12.6 (br, 1H, -COO*H*).

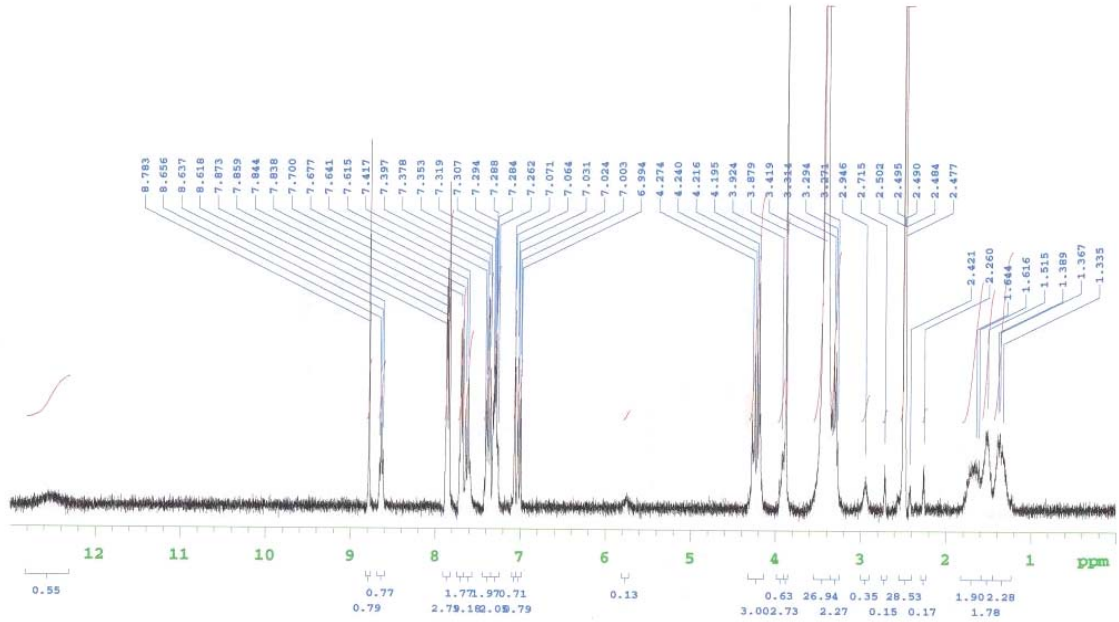

Fig. S1. ^{1}H NMR spectrum of **Fmoc-Lys(Moc)-OH** in DMSO-d_6 at room temperature.

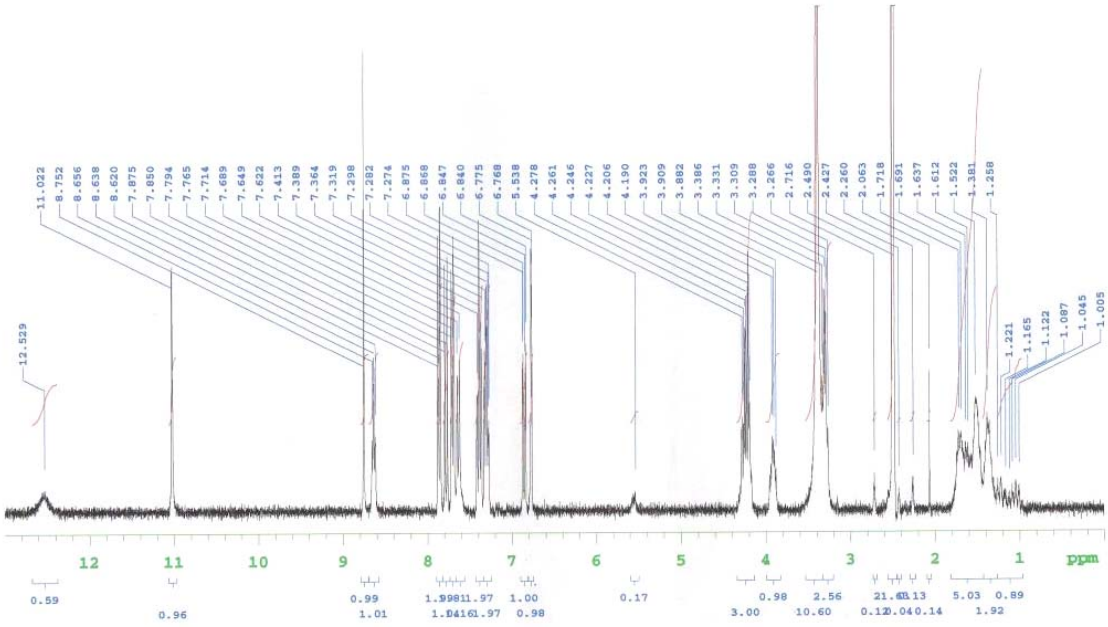

Fig. S2. ^{1}H NMR spectrum of **Fmoc-Lys(Hoc)-OH** in DMSO-d_6 at room temperature.

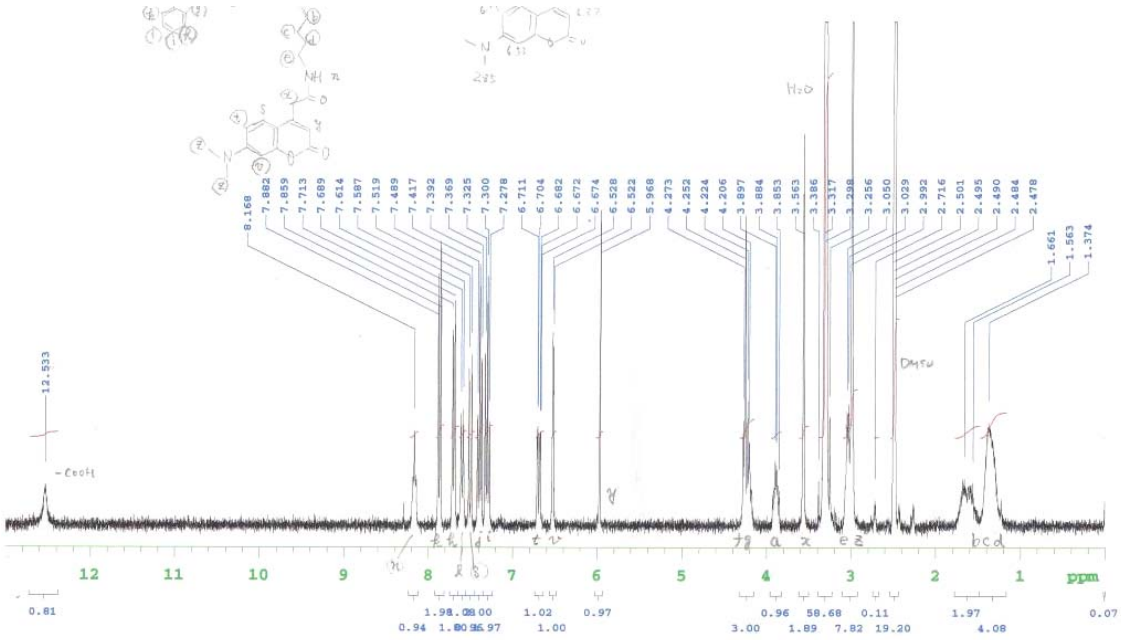

Fig. S3. ^{1}H NMR spectrum of **Fmoc-Lys(Hoc)-OH** in DMSO-d_6 at room temperature.

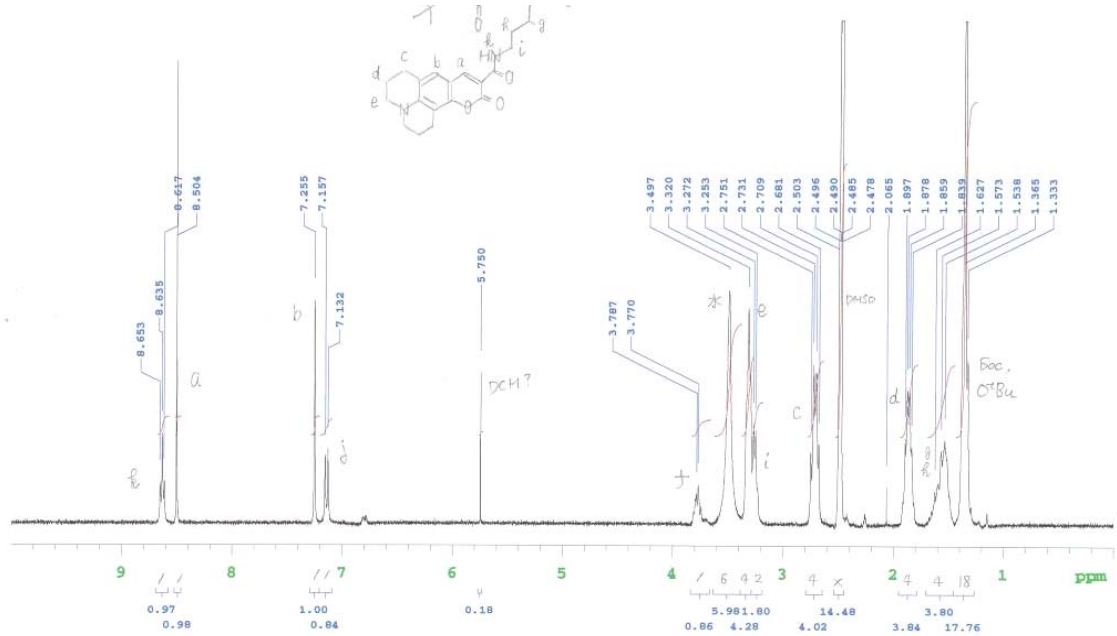

Fig. S4. ^{1}H NMR spectrum of **Boc-Orn(Mac)-OtBu** in DMSO-d_6 at room temperature.

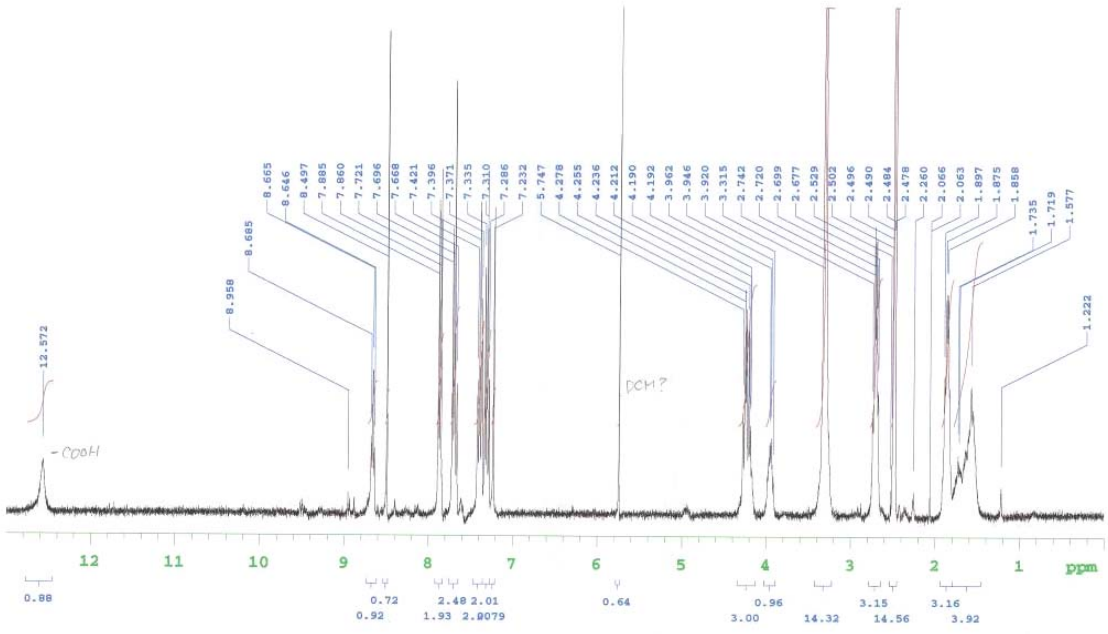

Fig. S5. ^{1}H NMR spectrum of **Fmoc-Orn(Mac)-OH** in DMSO-d_6 at room temperature.

Syntheses of fluorescent tag-modified peptide oligomers

Syntheses of fluorescent tag-modified peptide oligomers were carried out on a solid phase. The Fmoc-amino acid, Fmoc-NH-PEG5-COOH and Fmoc-fluorescent amino acids were used as monomers. The solid-phase synthesis was performed on an Fmoc-SH-SAL-PEG resin (super acid-labile polyethyleneglycol resin). Chain elongation was achieved by using HATU/HOAt as the coupling agent in the presence of DIPEA in NMP. The coupling time was set to 1 h at 45 ˚C. Then the peptide resin was treated with 5% Ac_2O containing 6% lutidine in DMF for 3 min at room temperature to prevent the formation of deletion sequences. In general, coupling efficiency at each elongation step was >95%, as estimated from UV absorption of the fulvene-adduct formed upon removal of the N_{δ}-Fmoc protecting group with 20% piperidine/DMF for 10 min at room temperature (ε_{290}= 4950 M^{-1} cm^{-1}). The resin-bound peptide was cleaved off from the resin with TFA/TIS/H_2O (= 95/2.5/2.5 v/v/v) for 90 min at room temperature. Finally, final products were identified by MALDI-TOF mass spectroscopy.

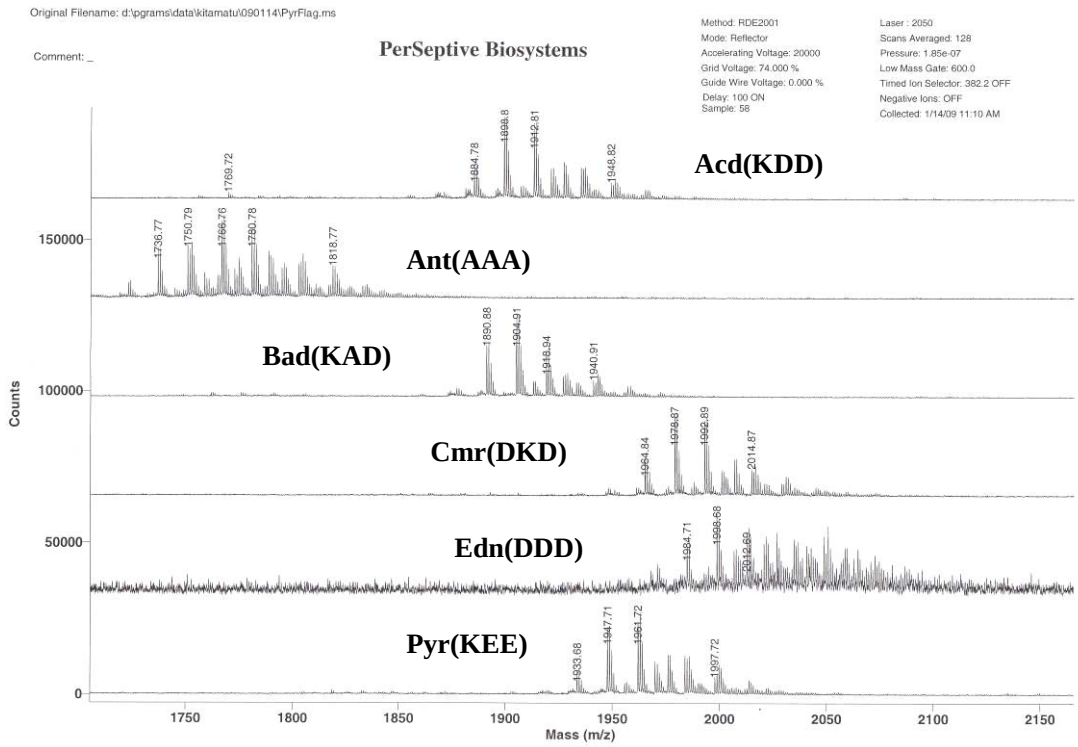

Fig. S6. MALDI-TOF mass spectra of **Acd(KDD)**, **Ant(AAA)**, **Bad(KAD)**, **Cmr(DKD)**, **Edn(DDD)** and **Pyr(KEE)**.