Supplementary information

Aliphatic dipeptide tags for multi 2-plex protein quantification

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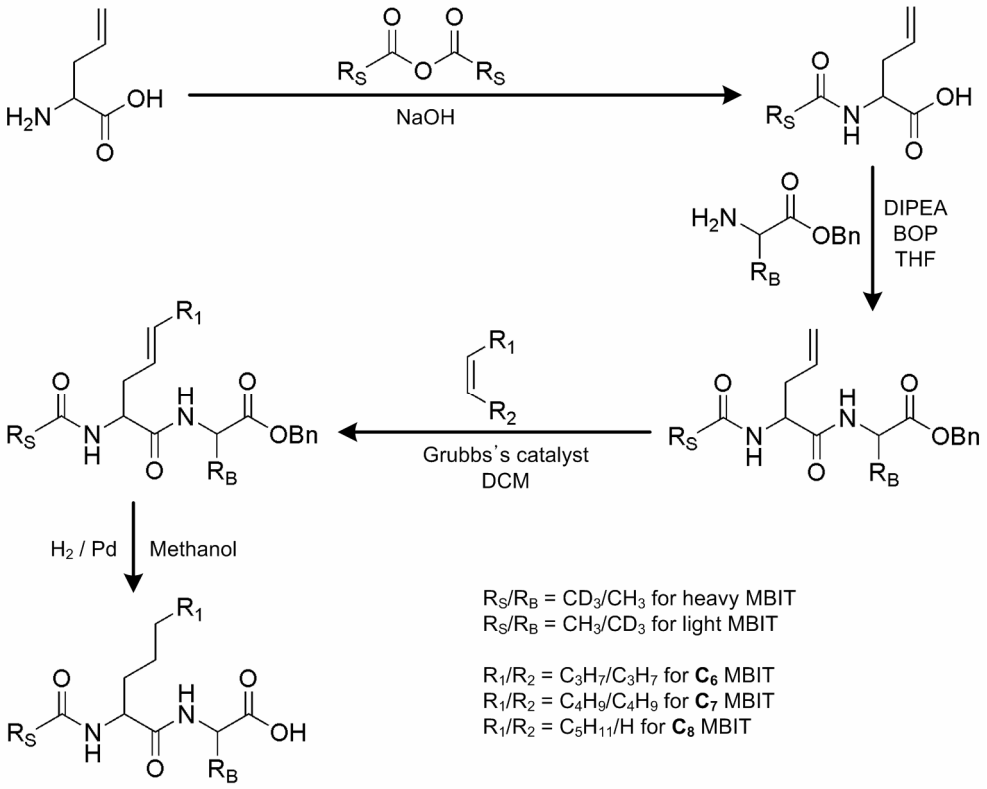
I. Solution-phase syntheses of acid forms of aliphatic MBITs (C_6–C_8 tags)



Scheme S1. Solution-phase synthesis of the acid form of C_6–C_8 tags

Materials

Acetic anhydride (Ac_2O-d_0), 2-amino-4-pentenoic acid, Boc-L-alanine, (benzotriazol-1-yloxy)tris (dimethylamino)phosphonium hexafluorophosphate (BOP), DCM, DIPEA, tetrahydrofuran (THF), methanol, TFA, 4-octene, 5-decene, 1-heptene, and Grubbs's catalyst (2nd generation) were purchased from Sigma-Aldrich. Acetic anhydride-d_6 (Ac_2O-d_6) and *N*-Fmoc-alanine-*3,3,3*-d_3 were from CDN Isotopes.

Synthesis procedure

Step 1. Acetylation of 2-amino-4-pentenoic acid: 2-Amino-4-pentenoic acid (2.0 mmol) was dissolved in water (pH 9–10, 4 mL) and Ac_2O-d_0 or -d_6 (4.0 mmol) was added at 0 °C. After adjusting pH to 10 by adding NaOH (8 M), the reaction mixture was stirred for 4 h at 0 °C. The reaction was terminated by adding concentrated hydrochloric acid to adjust pH to less than 2. The crude product was dissolved in methanol, filtered, and dried to recover solid 2-acetamido-4-pentenoic acid.

Step 2. Construction of intermediate: Alanine benzyl esters (alanine-d_0 benzyl ester or alanine-d_3 benzyl ester) were prepared by adding benzyl bromide to *N*-Boc-alanine and de-protecting *N*-Boc-alanine benzyl ester with TFA.[1] BOP (1.01 mmol) was added to alanine benzyl ester (0.55 mmol) in THF (5 mL) and stirred at room temperature for 30 min. DIPEA (3.36 mmol) was added at 0 °C and stirred at room temperature for 15 min. This mixture was mixed with 2-acetamido-4-pentenoic acid dissolved in anhydrous THF and stirred at room temperature overnight: Alanine-d_0 benzyl ester was mixed with 2-acet-d_3-amido-4-pentenoic acid, while alanine-d_3 benzyl ester was mixed with 2-acet-d_0-amido-4-pentenoic acid. After evaporating the solvent, the residue was dissolved in ethyl acetate and washed with water. The residual product was purified by silica gel flash chromatography to obtain benzyl 2-(2-acetamido-4-penteneamido) propanate.

Step 3. Olefin cross-metathesis[2] to vary the mass-tunable group: Benzyl 2-(2-acetamido-4-penteneamido) propanate prepared in Step 2, Grubbs's catalyst, and one of alkene (4-octene for C_6-, 5-decene for C_7-, or 1-heptene for C_8-tags) were added to DCM, and refluxed for 24 h at 40 °C. In the case of C_8 MBIT synthesis, 1-heptene was used because 6-dodecene was not commercially available. After removing the catalyst and solvent, the product was purified by silica gel chromatography.

Step 4. Recovery of MBIT: The reaction product prepared in Step 3 was mixed with $Pd(OH)_2$ (20 mol%) in anhydrous methanol and stirred overnight under 1 atm hydrogen gas at room temperature. After filtering out the catalyst, the product was concentrated under vacuum, followed by recrystallization using a methanol/ether mixture (1:1, v/v) to produce the acid form of MBIT reagent.

II. Construction of yeast strains

Construction of *HSP82*-deficient yeast strain. The haploid *hsp82Δ* null mutant [YHY240d1 {MAT*a ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 hsp82Δ::TRP1 CFIII (CEN3.L. YPH983*) *HIS3 SUP11*}] was constructed as follows. A PCR product containing the *HSP82* ORF with a *Spe*I site before the start codon and a *Sal*I site after the stop codon, was digested with these enzymes and cloned into the respective restriction sites of the yeast expression plasmid pBFG1,[3] resulting in the plasmid pBFG1-

HSP82. The 1360 bp *Bgl*II-*Eco*RI fragment of the *HSP82*-coding region was replaced with a 850 bp *Bgl*II-*Eco*RI fragment containing the yeast *TRP1* gene, the resulting plasmid pBFG1-*dHSP82* was digested with *Spe*I and *Sal*I, and transformed into the isogenic wild-type strain. Chromosomal deletion of the *HSP82* gene was verified by PCR with genomic DNA of the *TRP*+ transformants.

Construction of yeast strains expressing the hemagglutinin (HA)-tagged Hsc82 protein. To generate plasmid pYHY306-*HA-HSC82* expressing the hemagglutinin (HA)-tagged *HSC82* under control of the endogenous *HSC82* promoter, a 1370-bp *Xho*I-*Eco*RI cassette containing the *HSC82* promoter (a 650-bp PCR fragment), triple HA repeats, and a 5′-595 bp fragment of *HSC82* ORF (PCR fragment) was inserted into the *Xho*I and *Eco*RI sites of the yeast integrating plasmid pRS306.[4] Plasmid pYHY306-*HA-HSC82* was linearized within the *HSC82* gene by *Hpa*I digestion and transformed into the *hsp82Δ* deletion strain (YHY240d1) or isogenic wild-type strain by selecting colonies on uracil-minus medium. The resulting strains are YHY404 [*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 hsp82Δ::TRP1 hsc82::HA-HSC82-URA3 CFIII (CEN3.L. YPH983) HIS3 SUP11*] and YHY186HAN2 [*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 hsc82::HA-HSC82-URA3 CFIII (CEN3.L. YPH983) HIS3 SUP11*]. Correct integration of the plasmid at the *HSC82* locus was confirmed by PCR.

III. LC running conditions

For the separation of aliphatic MBIT-linked bradykinin, LC was run for 60 min with a flow rate of 0.3 μL min^{-1} (solvent A, 95/5/0.1 H_2O/ACN/formic acid and solvent B, 20/80/0.1 H_2O/ACN/formic acid). The solvent gradient [A]/[B] was slowly varied from 80/20 to 30/70 between 0 and 45 min, changed to 0/100 for 5 min and maintained at 0/100 for another 5 min, and then immediately dropped to 100/0 and held at 100/0 between 55 and 60 min.

For the separation of aliphatic MBIT-linked tryptic peptides of Hsc82p, LC was run at the flow rate of 0.3 μL min^{-1} (solvent A, 95/5/0.1 H_2O/ACN/TFA and solvent B, 5/95/0.1 H_2O/ACN/TFA). The

gradient [A]/[B] was varied from 100/0 to 60/40 for 9 min, changed from 60/40 to 0/100 for 31 min, held at 0/100 for 5 min, and immediately dropped to 100/0 and held at 100/0 for 15 min.

References

1. J. Seo, M.-S. Suh, T. D. Thangadurai, J. Kim, Y. H. Rhee, H.-J. Yoon, and S. K. Shin, *Anal. Chem.*, 2008, **80**, 6145–6153.
2. A. K. Chatterjee and R. H. Grubbs, *Org. Lett.*, 1999, **1**, 1751–1753.
3. R. Yelin, D. Rotem, and S. Schuldiner, *J. Bacteriol.*, 1999, **181**, 949–956.
4. R. S. Sikorski and P. Hieter, *Genetics*, 1989, **122**, 19–27.

IV. Mass spectrometric analysis of MBIT-linked bradykinin

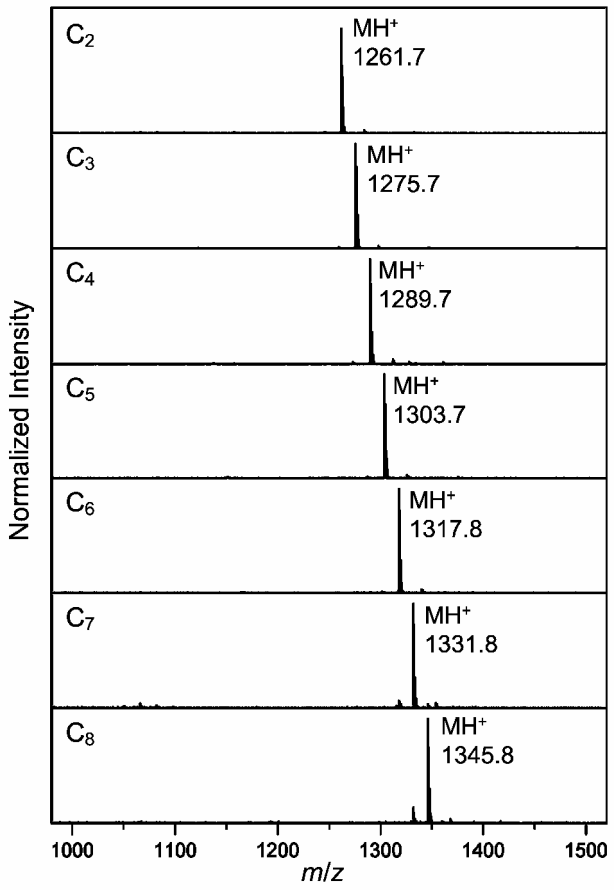


Fig. S1 MALDI mass spectra of aliphatic (C_2–C_8) MBIT-linked bradykinin (*N*-Ac-**X**A-RPPGFSPFR). Neither unmodified bradykinin peptides (*m*/*z* 1060.5) nor side products were detected.

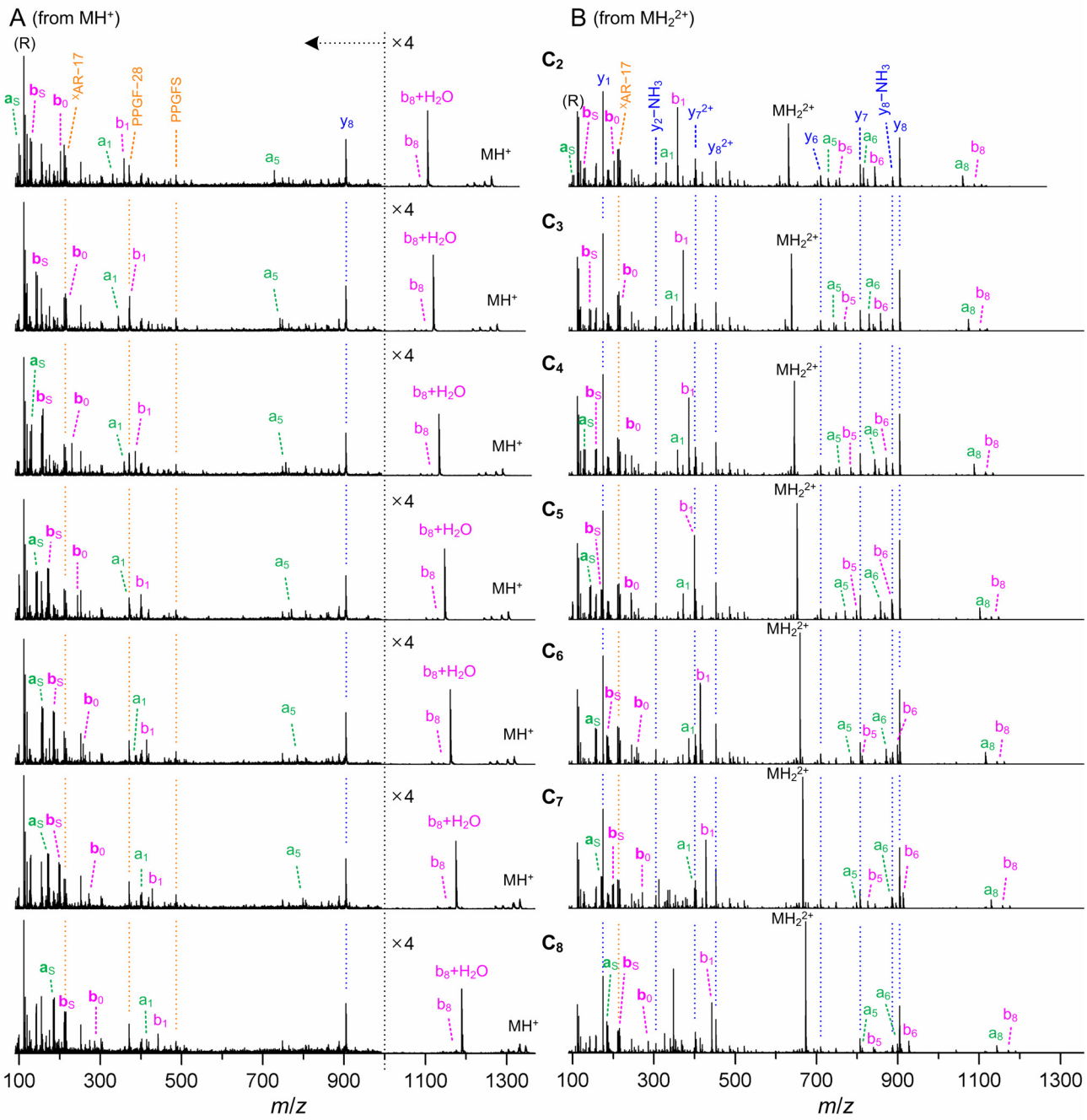


Fig. S2 (A) MALDI-MS/MS spectra of singly-protonated MBIT-linked bradykinin (*N*-Ac-XA-RPPGFSPFR) obtained using TOF/TOF (Applied Biosystems 4700 Proteomics Analyzer, Foster City, CA) and (B) ESI-MS/MS spectra of doubly-protonated MBIT-linked bradykinin obtained using Q-TOF (Waters Q-TOF Premiere, Manchester, UK). The a, b, and y-type ions are colored in green, magenta, and blue, respectively.

V. Identification and quantification using multiple MBITs

Table S1. Quantification of Hsc82p expressed under four different states using aliphatic C_6–C_8 tags and LC-MALDI MS/MS

peptide sequence	*m*/*z* of unmodified peptide	number of modification[c]	L/H ratio[a] (LC elution time)[b]		
			C_6-tag Hsp82p +, 39 °C	C_7-tag Hsp82p –, 30 °C	C_8-tag Hsp82p –, 39 °C
VLEIR	629.40	1/1	1.58 ± 0.24 (44.6)	0.87 ± 0.18 (49.2)	1.36 ± 0.18 (49.7)
EIFLR	677.40	1/1	1.54 ± 0.33 (50.0)	0.89 ± 0.30 (50.1)	1.25 ± 0.20 (50.0)
LLDAPAAIR	939.56	1/1	1.57 ± 0.23 (45.2)	0.90 ± 0.15 (48.3)	1.45 ± 0.18 (49.4)
LGVHEDTQNR	1168.57	1/1	1.50 ± 0.19 (34.3)	0.82 ± 0.06 (37.1)	1.46 ± 0.12 (39.6)
QLETEPDLFIR	1360.71	1/1	1.71 ± 0.28 (42.5)	0.86 ± 0.28 (44.6)	1.50 ± 0.17 (46.3)
average			1.57 ± 0.11	0.84 ± 0.05	1.42 ± 0.07
optical imaging			1.60	0.85	1.38

[a]The ratios of the amount of the Hsc82p expressed under various conditions to that obtained from normal condition (Hsp82p +, 30 °C) are presented. The average and standard deviation of the ratio of each LC fraction are shown.

[b]The number in parenthesis represents the average LC-elution time.

[c](The number of tag)/(the total number of possible modification) is given. The total number of possible modification equals to the number of primary amines in a given peptide sequence.

Table S2. De novo sequencing of MBIT-linked tryptic peptides of Hsc82p

peptide sequence (*m*/*z*)	de novo sequencing results[a]		
	type of MBIT	identified sequence[b]	confidence (%)
VLEIR (629.40)	C_6	*V*LEIR*	99
	C_7	*V*LEIR*	62
	C_8	*V*LEIR*	82
EIFLR (677.40)	C_6	*E*IFLR*	98
	C_7	*E*IFLR*	40
	C_8	*E*IFLR*	91
LLDAPAAIR (939.56)	C_6	*L*LDAPAAIR*	87
	C_7	*L*LDAPAAIR*	97
	C_8	*L*LDAPAAIR*	99
LGVHEDTQNR (1168.57)	C_6	*L*GVHEDTAGNR*	99
	C_7	*L*GVHEDTAGNR*	99
	C_8	*L*GVHEDTAGNR*	99
QLETEPDLFIR (1360.71)	C_6	*Q*LETEPDLFIR*	99
	C_7	Q**LETEPDLFIR*	50
	C_8	*Q*LETEPDLFIR*	81

[a]De novo sequencing was performed using PEAKS 4.5 (Bioinformatics Solutions Inc., Ontario, Canada) with a mass tolerance of 0.1 Da for both the precursor and fragment ions. Each MBIT conjugation was considered as a variable modification at the N-terminal amine and/or lysine.

[b]Asterisk denotes the site of MBIT labeling. The amino acid assigned with greater than 90% confidence is marked italic and the correct sequence is underlined.

Table S3. Mascot search results from multi 2-plex quantification using aliphatic MBITs[a]

MOWSE score[b]	protein ID	description
144	HSC82	ATP-dependent molecular chaperone HSC82
140	HSP82	ATP-dependent molecular chaperone HSP82
28	HSM3	DNA mismatch repair protein HSM3

[a]Each MBIT conjugation was considered as an optional modification at the N-terminal amine and/or lysine. The database search was performed in SWISS-PROT with the taxonomy of *Saccharomyces cerevisiae* (bakers' yeast). The error tolerance was set to 50 ppm and 0.1 Da for the precursor and product ions, respectively.

[b]Scores greater than 51 are considered to be significant in the given search conditions.