Supporting Information

Solid-Phase Synthesis of C-Terminal Peptide Libraries for Studying the Specificity of Enzymatic Protein Prenylation

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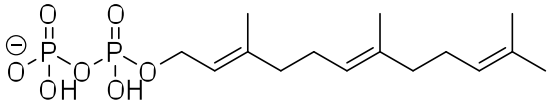
Abbreviations.

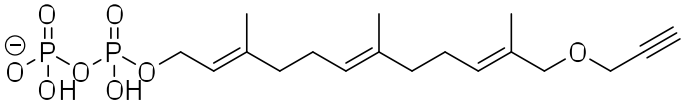
BCIP, 5-Bromo-4-chloro-3-indolyl phosphate disodium salt;
BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate;
BPB, bromophenol blue;
CHCA, α-cyano-4-hydroxycinnamic acid;
6-Cl-HOBt, 6-chloro-1-hydroxy-1H-benzotriazole;
DCC, dicyclohexylcarbodiimide;
Dde, 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl;
DIC, *N,N'*-diisopropylcarbodiimide;
DIEA, diisopropylethylamine;
Dmab,
2-{1-[4-(Hydroxymethyl)phenylamino]-3-methylbutylidene}-5,5-dimethyl-1,3-cyclohexanedione;
DMAP, dimethylamino-pyridine ;
DTT, dithiothreitol;
FPP, farnesyl pyrophosphate;
HCTU, O-(6-Chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMPA, 4-hydroxymethyl-phenoxyacetic acid;
PFTase, protein farnesyltransferase;
SP-AP, streptavidin alkaline phosphatase;
TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine;
TCEP, tris(2-carboxyethyl)phosphine hydrochloride,
TFA, trifluoroacetic acid;
Tris, tris(hydroxymethyl)aminomethane;

General:

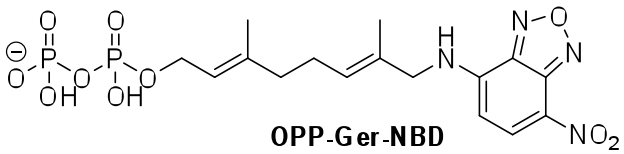
All synthetic reactions were carried out at 25° C and stirred magnetically unless otherwise noted. TLC was performed on precoated (250 mm) silica gel 60 F-254 plates (Merck). Plates were visualized by staining with $KMnO_4$ or hand-held UV lamp. Flash chromatography silica gel (60–200 mesh, 75–250 μm) was obtained from Mallinckrodt Inc. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories, Inc. Biotin-azide was purchased from ChemPep. All amino acids and peptide coupling reagents were purchased from NovaBiochem. Other general reagents, including salts and reagents, were purchased from Sigma-Aldrich. ^{1}H NMR spectra were obtained at 300 MHz; ^{31}P NMR spectra were obtained at 121 MHz. All NMR spectra were acquired on Varian instruments at 25°C. Chemical shifts are reported in ppm and *J* values are in Hz. SPOT synthesizer is ResPep SL obtained from INTAVIS Bioanalytical Instruments. Fluorescence assay data were obtained by using a Varian Cary Eclipse Fluorescence Spectrophotometer. Analytical HPLC was performed on a Beckman model 125/166 instrument, equipped with a diode array UV detector, ABI Analytical Spectroflow 980 fluorescence detector, and a Varian C_{18} column (Microsorb-MV, 5 μm, 4.6x250 mm). Preparative HPLC separations were performed by using a Beckman model 127/166 instrument, equipped with a UV detector and a Phenomenex C_{18} column (Luna, 10 μm, 10x250 mm). Fluorescence scanning was done by Storm 840 Phosphor-Chemifluorescence Workstation (excitation 450 nm & emission 540-560 nm). MS spectra for small molecules were obtained on a Bruker BioTOF II instrument. MS spectra for peptide libraries were obtained on an AB Sciex 4800 MALD TOF/TOF or Bruker Biflex III instrument. Yeast PFTase[1], Ds-GCVIA[1] and OPP-Far-alkyne[2] were prepared as previously described. OPP-Ger-NBD was prepared using a reported reaction procedure.[3]



FPP

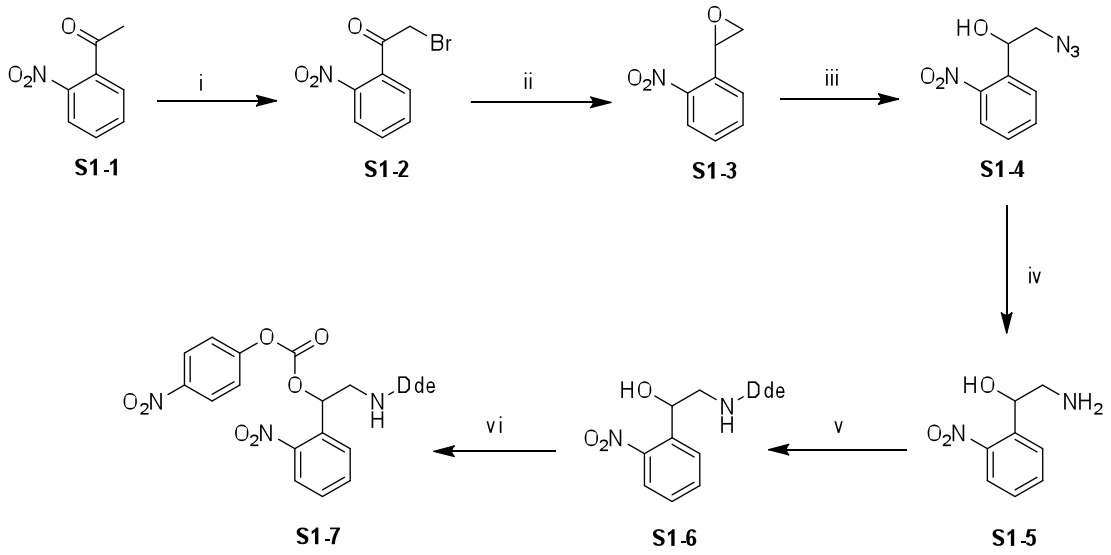


OPP-Far-alkyne



OPP-Ger-NBD

Analogues used to screen peptide libraries for PFTase activity.



Scheme S1. Synthesis of the photocleavable linker used for library synthesis. Reagents and conditions: (i) Br_2, 1,4-dioxane, room temp, 3 h; (ii) $NaBH_4$, H_2O/1,4-dioxane, 0 °C to RT, 1.5 h, then $NaOH_{(aq)}$, RT, 15 min; (iii) NaN_3, NH_4Cl, EtOH/H_2O, reflux, 12 h; (iv) PPh_3, THF, 0 °C to RT, 2 h, then H_2O, RT, 12 h; (v) Dde-OH, CH_2Cl_2, RT, 18 h; (vi) p-nitrophenyl chloroformate, pyridine, CH_2Cl_2, RT, 350 min.

O-nitrophenacyl bromide (S1-2)

Steps (i) and (ii) were performed according to a previously published procedure.[4] A solution of Br_2 (4.5 mL, 87.6 mmol) in dioxane (80 mL) was added dropwise over a 30 min period to a solution of o-nitroacetophenone **(S1-1)** (14.44 g, 87.4 mmol) in dioxane (40 mL). After the addition was completed, the mixture was stirred for 2.5 h. The solvent and HBr formed were removed by rotary evaporation followed by the addition of H_2O (60 mL). The solution was extracted with Et_2O (3×50 mL) and the combined organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation to yield **S1-2** as a brown oil. Crude compound (**2**) was used without further purification. ^1H NMR ($CDCl_3$): δ 4.30 (s, 2H), 7.51 (dd, J = 1.5, 7.2, 1H), 7.68 (ddd, J = 1.8, 7.8, 8.4, 1H), 7.80 (ddd, J = 1.2, 7.5, 7.5 1H), 8.24 (dd, J = 1.2, 8.4, 1H).

O-nitroepoxystyrene (S1-3)

A suspension composed of $NaBH_4$ (3.31 g, 87.4 mmol) and H_2O (30 mL) was added dropwise to a solution of o-nitrophenacyl bromide (**S1-2**) (prepared in previous step) in dioxane (30 mL) over a 40 min period at 0 °C with stirring. The mixture was stirred at 0 °C for 40 min and then at RT for 70 min. An aqueous solution of NaOH (4.14 g / 30 mL) was added to the mixture. The mixture was stirred at RT for 15 min. H_2O (40 mL) was then added and the solution was extracted with Et_2O (3×50 mL). The

combined organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting oil was purified by silica gel column chromatography (1:60 Et_2O/hexanes containing 2% Et_3N) to yield **S1-3** as a yellow solid (8.63 g, 59.8% yield). ^1H NMR ($CDCl_3$): δ 2.68 (dd, *J* = 2.7, 5.4, 1H), 3.30 (dd, *J* = 4.5, 5.7, 1H), 4.49 (dd, *J* = 2.7, 4.5, 1H), 7.48 (ddd, *J* = 1.8, 6.9, 6.9, 1H), 7.65 (m, 2H), 8.16 (dd, *J* = 1.2, 8.4, 1H).

2-azido-1-(2-nitrophenyl)ethanol (S1-4)

To a mixture of compound (**S1-3**) (8.63 g, 52.3 mmol) in EtOH (70 mL) was added NaN_3 (4.08 g, 62.8 mmol), NH_4Cl (2.87 g, 53.7 mmol) and then water (70 mL). The mixture was stirred at reflux for 16 h. EtOH was removed by rotary evaporation. H_2O (40 mL) was added and the solution was extracted with Et_2O (3×50 mL). The combined organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting oil was purified by silica gel column chromatography (1:3 Et_2O/hexanes) to yield **S1-4** as a yellow oil (4.41 g, 40.5% yield). ^1H NMR ($CDCl_3$): δ 3.46 (dd, *J* = 7.5, 12.3, 1H), 3.76 (dd, *J* = 3.0, 12.3, 1H), 5.51 (dd, *J* = 3.3, 7.2, 1H), 7.50 (ddd, *J* = 1.5, 8.4, 8.4, 1H), 7.71 (ddd, *J* = 1.2, 7.5, 7.5, 1H), 7.92 (dd, *J* = 1.5, 7.8, 1H), 8.02 (dd, *J* = 1.2, 8.4, 1H).

2-amino-1-(2-nitrophenyl)ethanol (S1-5)

Compound **S1-4** (4.41 g, 21.2 mmol) was dissolved in dry THF (35 mL). The mixture was placed under an N_2 (g) atmosphere and cooled to 0 ^oC. PPh_3 (5.85 g, 22.3 mmol) was added to this mixture, stirred at 0 ^oC for 10 min and then RT for 110 min. H_2O (3 mL) was added to this mixture which was then stirred at RT for 16 h. Et_2O (50 mL) was added and the solution was extracted with 1M HCl (2×35 mL). The aqueous layers were combined, adjusted to a basic pH with $NaOH_{(s)}$ and extracted with Et_2O (3×40 mL). The combined organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation to yield compound **S1-5** as a yellow solid that was used without further purification. ^1H NMR (d_6-acetone): δ3.79 (dd, *J* = 7.2, 12.3, 1H), 5.29 (dd, *J* = 5.7, 6.6, 1H), 7.54 (ddd, *J* = 1.5, 7.8, 8.7, 1H), 7.72 (ddd, *J* = 1.2, 7.2, 8.7, 1H), 7.89 (dd, *J* = 1.2, 7.8, 1H), 8.02 (dd, *J* = 1.2, 8.1, 1H).

2-(1-(2-hydroxy-2-(2-nitrophenyl)ethylamino)ethylidene)-5,5-dimethylcyclohexane-1,3-dione (S1-6)

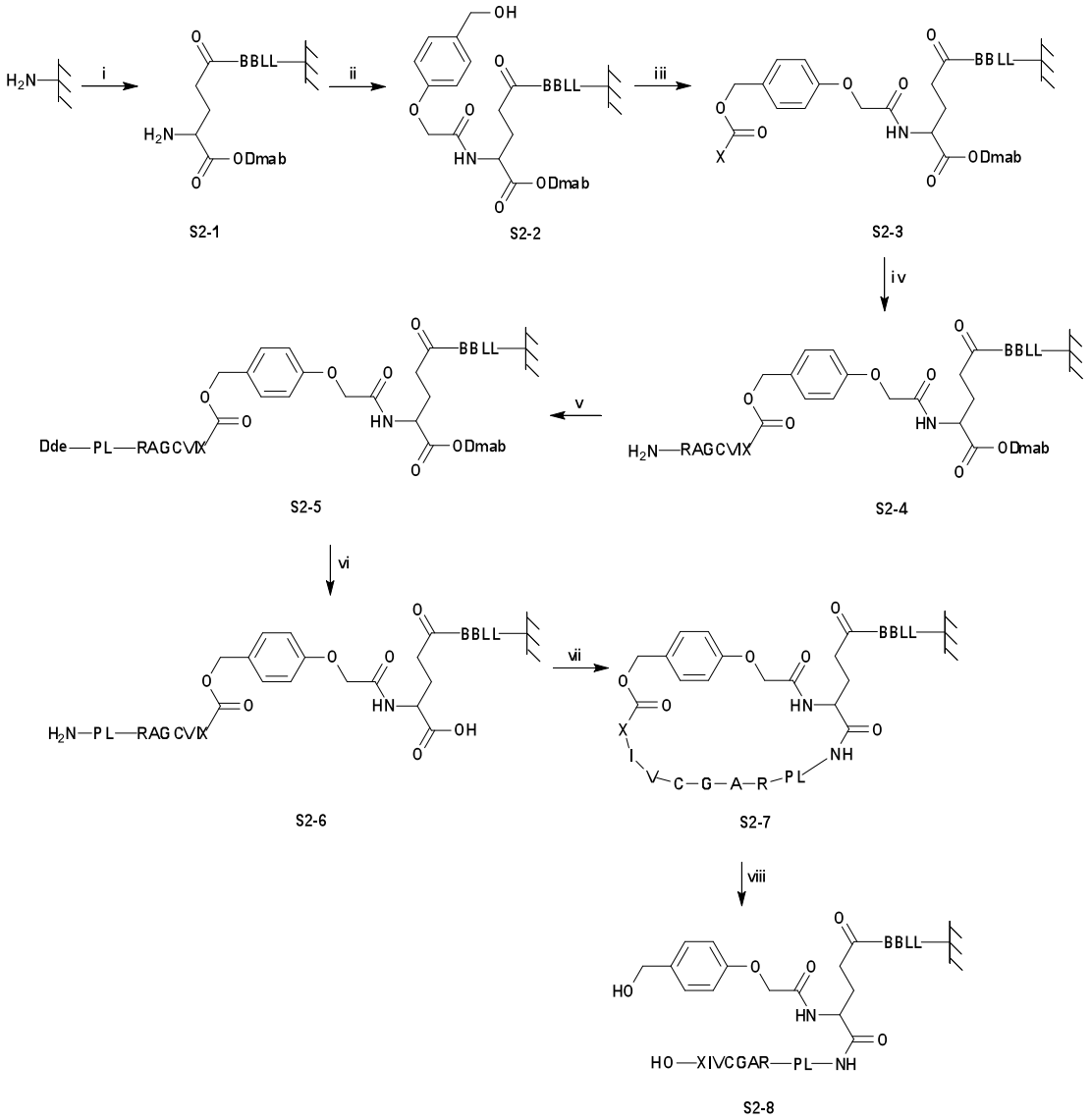
Preparation of Dde-OH: Dimedone (5.0 g, 0.0357 mol) was dissolved in DMF (75 mL) with HOAc (2.0 mL, 34.9 mmol), DCC (7.38 g, 35.8 mol), and DMAP (4.36 g, 35.7 mmol) and allowed to react for 16 h. Precipitating dicyclohexylurea (DCU) was removed by filtration, and the solvent was evaporated in vacuo. After dissolution in

EtOAc (50 mL) and washed with 1M HCl (2×50 mL) and subsequently with H_2O (2×50 mL), the organic phase was dried over $MgSO_4$, filtered and concentrated to give a yellowish liquid that slowly solidified upon standing. ^{1}H NMR ($CDCl_3$): δ 1.08 (s, 6H), 2.36 (s, 2H), 2.53 (s, 2H), 2.61 (s, 3H). ESI-MS [M-H^+]: m/z calc. for $C_{10}H_{13}O_3^-$: 181.0870; found:181.0852.

Protection with Dde-OH: Compound **S1-5** (1.48 g, 8.15 mmol) was stirred with Dde-OH (1.45 g, 7.96 mmol) in 20 mL CH_2Cl_2 at RT for 16 h. Some CH_2Cl_2 was removed by rotary evaporation and the resulting mixture was purified by silica gel column chromatography (3:1 Et_2O/hexanes) to yield **S1-6** as a yellow solid (2.06 g, 73.1 %). ^{1}H NMR ($CDCl_3$): δ 0.97 (s, 6H), 2.27 (s, 4H), 2.53 (s, 3H), 3.98 (m, 2H), 5.55 (dd, *J* = 3.3, 7.5, 1H), 7.54 (ddd, *J* = 7.5, 7.5, 1H), 7.72 (dd, *J* = 7.8, 7.8, 1H), 8.02 (m, 2H). ESI-MS [M+Na^+]: m/z calc. for $C_{18}H_{22}N_2O_5Na^+$: 369.1421; found: 369.1480.

2-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethylamino)-1-(2-nitrophenyl)ethyl 4-nitrophenyl carbonate (S1-7)

To a solution of compound **S1-6** (0.454 g, 1.31 mol) in CH_2Cl_2 (5 ml) was added 4-nitrophenyl chloroformate (0.394 g, 1.95 mmol) and pyridine (0.19 ml, 2.35 mmol). The mixture was stirred at room temperature for 350 min. Some CH_2Cl_2 was removed by rotary evaporation and the resulting mixture was purified by silica gel column chromatography (3:1 Et_2O/hexanes) to yield **S1-7** as a white solid (0.487 g, 72.7 %). ^{1}H NMR ($CDCl_3$): δ 1.047 (s, 6H), 2.36 (s, 2H), 2.43 (s, 2H) 2.57 (s, 3H), 3.97 (dd, *J* = 5.7, 14.4, 1H), 4.22 (dd, *J* = 3.3, 9.6, 1H), 6.50 (d, *J* = 4.2, 1H), 7.38 (d, *J* = 9.6, 2H), 7.61 (dd, *J* = 8.1, 8.1, 1H), 7.88 (m, 2H), 8.14 (d, *J* = 8.1, 1H), 8.25 (d, *J* = 7.8, 2H). ESI-MS [M+H^+]: m/z calc. for $C_{25}H_{26}N_3O_9^+$: 512.1664; found: .512.1591.



Scheme S2. Synthesis of inverted peptides. Reagents and conditions: (i) standard DIC coupling (2×), then, capping, then 20% piperidine, then BPB staining; (ii) standard DIC coupling (2×); (iii) 0.5 M Fmoc-Aa and 0.5 M 6-Cl-HOBt in DMF/CH_2Cl_2, 0.5 M DIC in CH_2Cl_2, 0.2 M DMAP in CH_2Cl_2, (6-8×), then, capping, then 20% piperidine, then BPB staining; (iv) standard DIC coupling (2×), then, capping, then 20% piperidine, then BPB staining; (v) 0.5 M photocleavable linker, 0.5 M Et_3N in DMF (3-4×); (vi) 2% NH_2NH_2; (vii) 0.05 M BOP, 0.05 M 6-Cl-HOBt and 0.1 M DIEA in DMF (2×); (viii) modified reagent K.

Amino acid residues used in the library synthesis

Abbreviation	Chemical name	Abbreviation	Chemical name
B	Fmoc-β-Ala-OH	K	Fmoc-Lys(Boc)-OH
L	Fmoc-Leu-OH	H	Fmoc-His(Trt)-OH
R	Fmoc-Arg(Pbf)-OH	P	Fmoc-Pro-OH
A	Fmoc-Ala-OH	F	Fmoc-Phe-OH

G	Fmoc-Gly-OH	Y	Fmoc-Tyr(tBu)-OH
C	Fmoc-Cys(Trt)-OH	W	Fmoc-Trp(Boc)-OH
V	Fmoc-Val-OH	M	Fmoc-Met-OH
I	Fmoc-Ile-OH	S	Fmoc-Ser(tBu)-OH
Q	Fmoc-Gln(Trt)-OH	T	Fmoc-Thr(tBu)-OH
D	Fmoc-Asp(OtBu)-OH	E	Fmoc-Glu(OtBu)-OH
N	Fmoc-Asn(Trt)-OH		Fmoc-Glu-ODmab (For cyclization)

Synthesis of inverted peptides

Manual synthesis:

The inverted peptides were synthesized on amino-functionalized cellulose membranes (0.40 mmol/g). All of the manipulations were performed at RT unless otherwise noted. The peptides were synthesized by spotting 1 μL of 0.5 M Fmoc-amino acid and 1 μL of 0.5 M DIC in DMF. The coupling reaction was typically allowed to proceed for 30 min twice. The membrane was incubated with 2% (v/v) Ac_2O in DMF (capping solution A) for 5 min, followed by 2% (v/v) acetic anhydride, 2% (v/v) DIEA in DMF (capping solution B) for 15 min without shaking. The membrane was then washed with DMF (3×). The Fmoc groups were removed by treatment twice with 20% piperidine in DMF (5+5 min) followed by washing of the membrane with DMF (4×) and MeOH (2×). The membrane was then stained with BPB in MeOH (40 mg/L) to visualize the spot locations. The membrane was then washed with MeOH (4×) and Et_2O (2×).

Next, HMPA was coupled to the growing peptide chain using the above procedure. The membrane was washed with MeOH (4×), diethyl ether (2×) without capping.

Next, esterification of the links which will be the C-terminal residue in the final product was accomplished by spotting sequentially 1 μL of 0.5 M Fmoc-amino acid, 6-Cl-HOBt in DMF/CH_2Cl_2 (v/v = 50:50), 1 μL of 0.5 M DIC in CH_2Cl_2 and 1 μL of 0.2 M DMAP in CH_2Cl_2. Each coupling reaction was typically allowed to proceed for 30 min. The process was repeated for six to eight times. The membrane was then incubated with capping solution A for 5 min followed by capping solution B for 15 min without shaking. The membrane was washed with DMF (3×) and the Fmoc group was removed by treatment twice with 20% piperidine in DMF (5+5 min). The membrane was washed with DMF (4×), MeOH (2×). The membrane was stained with BPB in MeOH (40 mg/L) to indicate spot locations. The membrane was washed with MeOH (4×) followed by Et_2O (2×).

To add the photocleavable linker, 1 μL of 0.5 M photocleavable linker solution and 1 μL of 0.5 M Et_3N in DMF were spotted onto the membrane. The coupling reaction

was typically repeated three to four times with each time to proceed for 30 min.
Next, the Dmab and Dde protecting groups were removed by treatment with 2% hydrazine solution in DMF (3×10 min) followed by washing with DMF and MeOH.
For peptide cyclization, a solution of (BOP)/6-Cl-HOBt/DIEA (0.05 M, 0.05 M, 0.1 M respectively) in DMF was mixed with the membrane. This reaction was performed twice, the first time for 2 h and the second time for 16 h. The membrane was washed with DMF (3×), MeOH (2×) and Et_2O (2×).
The membrane was treated with a modified reagent K (6.5% phenol, 5% H_2O, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 2 h for ester cleavage (ring opening) and side-chain deprotection.

SPOT Synthesizer:

The peptides were synthesized by ResPep SL SPOT-robot (INTAVIS Bioanalytical Instruments). The synthesis started with spot definition by a standard protocol followed by the coupling of a solution of 0.11 μL of Fmoc-amino acid (0.5 M), 0.055 μL of DIC (1.1 M) and 0.055 μL of 6-Cl-HOBt (1.2 M) in DMF (double coupling, 10 and 15 min reaction each). The membrane was acetylated with acetic anhydride in DMF (2%), washed with DMF (3×) and EtOH (3×) and finally air-dried. Fmoc was removed by 20% piperidine in DMF (3×). After HMPA was coupled to the growing peptide chain using the above procedure, the membrane was washed with DMF (3×), EtOH (3×) without capping.
For ester-bond formation (step iii), 0.22 μL of solution Fmoc-amino acid (0.4M) activated with 1,1'-carbonyldiimidazole (CDI, 3 equiv.) in DMF were spotted on the membrane (4× coupling, 15 min reaction time each).
To add the photocleavable linker, 0.22 μL of 0.5 M photocleavable linker solution mixed with Et_3N (2 equiv.) in DMF were spotted onto the membrane (4× coupling, 15 min reaction time each).
The procedure of peptide cyclization, side-chain cleavage and other steps was the same as manual synthesis.

MALDI MS analysis of peptides obtained via SPOT synthesis before and after enzymatic prenylation

Although it has been reported that treatment of prenylated peptides with CNBr, does not cause loss of the prenyl group,[8] we found substantial cleavage occurred. Thus CNBr could not be used to cleave the peptides from the resin and our strategy switched to a photocleavable linker. Accordingly, each spot membrane was incubated with 500 μL CH_3CN/H_2O (v/v = 5/95) in a microcentrifuge tube. Peptide release was accomplished by shining UV light (λ = 365 nm) on the side of the tube for 1 h. After photolysis, the solvent was transferred to a new microcentrifuge tube. The membrane

was washed with 500 µL CH_3CN and then 500 µL MeOH. The combined solution was concentrated *in vacuo*. The resulting residue was dissolved in 70 µL CH_3CN/H_2O (v/v = 5/95) with 0.1% TFA. In a typical MALDI experiment, 0.7 µL of peptide solution was mixed with 0.7 µL CHCA matrix (10 mg/mL; CH_3CN/H_2O (v/v = 50/50) with 0.1% TFA) on the MALDI target and air dried.

Screening for PFTase specificity with OPP-Far-alkyne

Spots from manual synthesis:

Spots from the membranes produced above were excised and incubated with 155 mM DTT for 1 h and then washed with H_2O (1×). Typical conditions for PFTase reactions were 5.1 mM DTT, 5 mM $MgCl_2$, 50 µM $ZnCl_2$, 200 mM Tris (pH=7.5), 1 mg/mL BSA, 260 µM OPP-Far-alkyne, 0.09 mg/mL PFTase. The membranes were incubated in the solution for 30 min before adding OPP-Far-allkyne and PFTase. The enzymatic reactions were typically allowed to proceed for 4 h followed by washing with 25 mM $NH_4HCO_{3(aq)}$ (4×), CH_3CN (4×) after which the membranes were transferred to new 1.5 mL microcentrifuge tubes.

Typical conditions for click reactions were 0.1 mM biotin-azide, 1 mM TCEP, 0.2 mM TBTA, 1 mM $CuSO_4$ in PBS buffer 1 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, pH=7.4). The reaction was typically allowed to proceed for 16 h. After the reactions, the membranes were washed with H_2O (4×), DMF (4×) and placed in new tubes.

The membranes were incubated with 5 % milk in PBS buffer 2 (100 mM KH_2PO_4, 500 mM NaCl, pH=6.5) for 1 h and then incubated with SP-AP in 5% milk PBS buffer 2 (v/v = 1/800) for 30 min. After the reactions, the membranes were washed with PBS buffer 2 (7×) and placed in new tubes. Upon addition of 750 µL of buffer E (5 mM $MgCl_2$, 20 µM $ZnCl_2$, 100 mM NaCl, 30 mM Tris, pH=8.5) and 50 µL of 5 mg/mL BCIP solution, turquoise color developed on positive spots after 1 h. The staining was terminated by adding 200 uL of 10 M HCl.

Spots from SPOT synthesizer:

The whole membrane was incubated with 155 mM DTT for 1 h and then washed with H_2O (1×). Typical conditions for PFTase reactions were 5.1 mM DTT, 5 mM $MgCl_2$, 50 µM $ZnCl_2$, 100 mM Tris (pH=7.5), 1 mg/mL BSA, ~100 µM OPP-Far-alkyne, 0.045 mg/mL PFTase. The enzymatic reactions were typically allowed to proceed for 4.5 h followed by washing with 25 mM $NH_4HCO_{3(aq)}$ (3×), CH_3CN (3×), and H_2O (3×).

Typical conditions for click reactions were 0.1 mM biotin-azide, 1 mM TCEP, 0.2 mM TBTA, 1 mM $CuSO_4$ in PBS buffer 1. The reaction was typically allowed to proceed for 16 h. After the reactions, the membranes were washed with H_2O (3×),

DMF (3×), and H_2O (3×).

The membranes were incubated with 5 % milk in PBS buffer 2 for 1 h and then incubated with SP-AP in 5% milk PBS buffer 2 (v/v = 1/800) for 30 min. After the reactions, the membranes were washed with H_2O (9×). Upon addition of BCIP solution in buffer E (0.313 mg/mL), turquoise color developed on positive spots after 1.5 h. The staining was terminated by washing with H_2O (2×) and CH_3CN (1×).

The quantification of color intensity was performed using Image J software. The membrane was scanned and saved. The file was opened by Image J. Under Image>Type, 8-bit was selected to convert the image to grayscale. Under the menu Process>Subtract Background, the rolling ball radius was set to 50. Under Analyze>Set Measurements, the Area, Mean Gray Value, and Integrated Density were selected. Under Analyze>Set Scale, "pixels" in the box next to Unit of length were selected. Under Edit>Invert, the colors on the image were inverted, a line was drawn around the image boundary and the measurement of the selected are was then performed using the "m" key.

Kinetic analysis of individual peptides for substrate verification

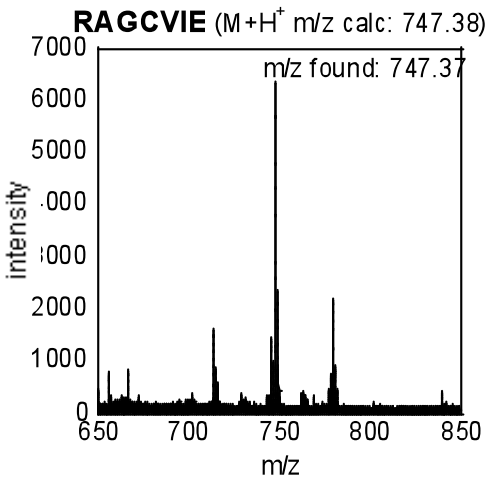
The rates of farnesylation of individual purified peptides by PFTase were determined using the time-dependent increase in fluorescence (λ_{ex} 340 nm, λ_{em} 510 nm) upon prenylation of a dansylated form of the peptide. Assays were performed with 2.4 μM dansylated peptide, 70 nM PFTase, 10 μM OPP-Far-alkyne, 200 mM Tris, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 50 μM $ZnCl_2$, and 0.040 % (*w/v*) *n*-dodecyl-*β*-D-maltoside at 25 °C. Peptides were incubated in reaction buffer for 5 min prior to initiation by the addition of PFTase. Fluorescence was measured as a function of time to define both the initial linear velocity and the reaction end point. The total fluorescence change observed upon reaction completion was divided by the initial concentration of the peptide substrate in a given reaction to yield a conversion from fluorescence units to product concentration (Amp_{conv}). The linear initial rate, V, in fluorescence intensity per minute, was then converted to a velocity (μM product produced per minute) by dividing V with Amp_{conv}.

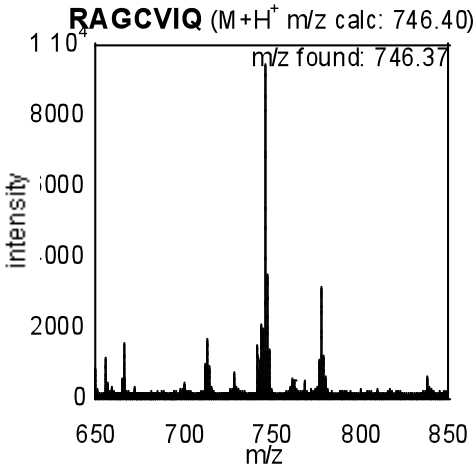
Evaluation of Rce1 activity for RAGC(farnesyl)VIA on CPG beads

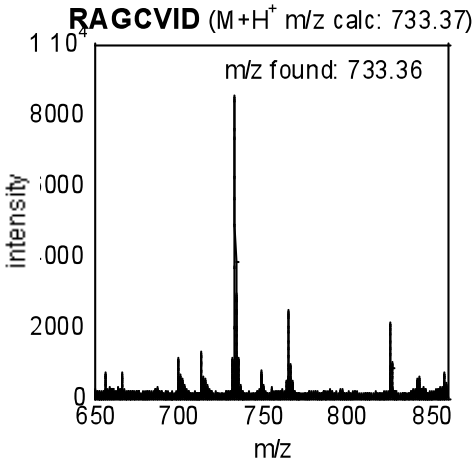
CPG beads with a large pore size (1000 Å) were functionalized with the peptide RAGCVIA as described above. 0.16 g of the resulting CPG beads (~11.7 μmol peptides) were incubated with 4 mL of DTT (155 mM) for at least 1 h. After removing the solvent, those CPG beads were washed with DMF (9×). To prepare farnesylation solution, $Zn(OAc)_2 \cdot 2H_2O$ (12.8 mg, 58.3 μmol) and farnesyl bromide

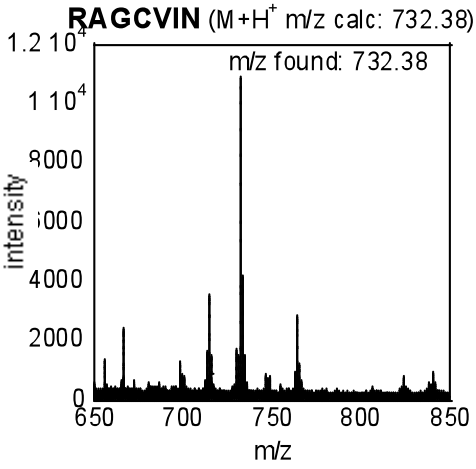
(12.7 μL, 46.8 μmol) were dissolved in a mixture of 2 mL DMF, 1 mL 1-BuOH and 1 mL 0.1% TFA. CPG beads were mixed with farnesylation solution for 3 h. After removing the solvent, the CPG beads were washed with DMF (3×) and H_2O (3×).

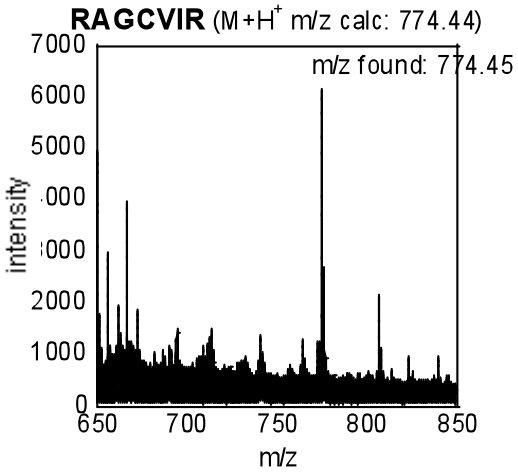
For the Rce1 proteolysis experiment, the assay involved mixing of CPG beads with yeast membranes enriched for the Rce1 protease.[5] Membranes used as the source of activity were stored as 1 mg/mL stocks. CPG beads (~ 1-2 mg) were incubated with a mixture of 500 μL assay buffer (100 mM HEPES, pH 7.5, 5 mM $MgCl_2$) and 50 μL of BSA (10 mg/mL) for at least 1 h. 50 μL of the stock Rce1 membranes were added to the solution and the reaction tube was gently shaken for 16 h. After removing the solvent, the CPG beads were washed with H_2O (2×), CH_3CN (2×) and H_2O (2×). For peptide release and MALDI analysis, the procedure described above uder **MALDI MS analysis of peptides obtained via SPOT synthesis** was employed.

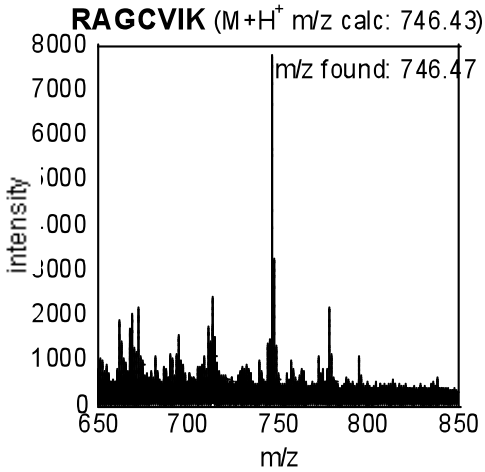

RAGCVIE (M+H+ m/z calc: 747.38)
m/z found: 747.37
intensity
m/z

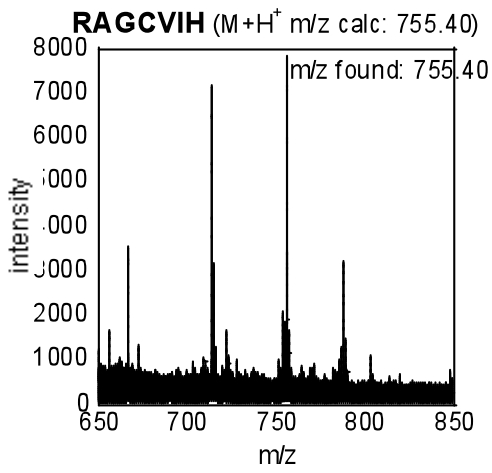

RAGCVIQ (M+H+ m/z calc: 746.40)
m/z found: 746.37
intensity
m/z

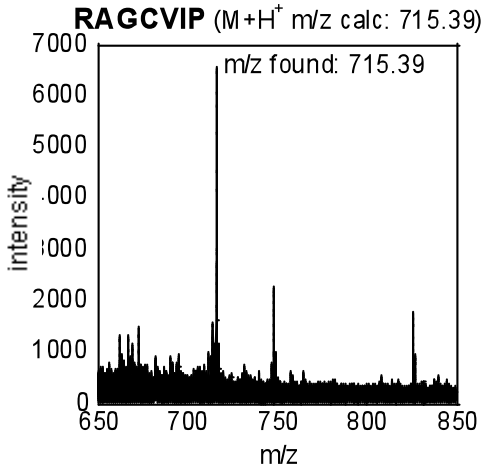

RAGCVID (M+H+ m/z calc: 733.37)
m/z found: 733.36
intensity
m/z

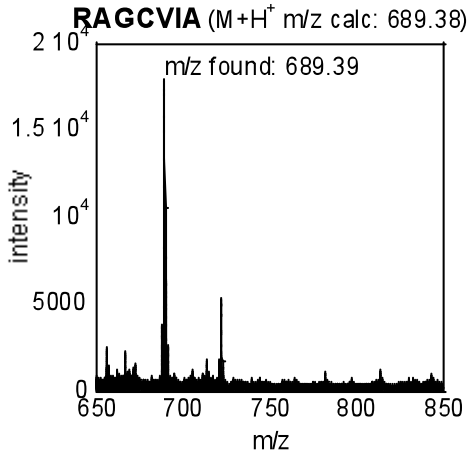

RAGCVIN (M+H+ m/z calc: 732.38)
m/z found: 732.38
intensity
m/z

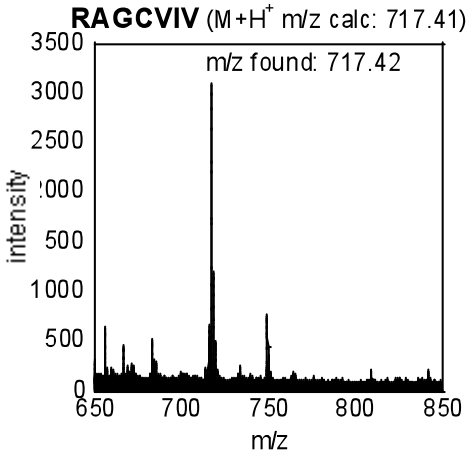

RAGCVIR (M+H+ m/z calc: 774.44)
m/z found: 774.45
intensity
m/z

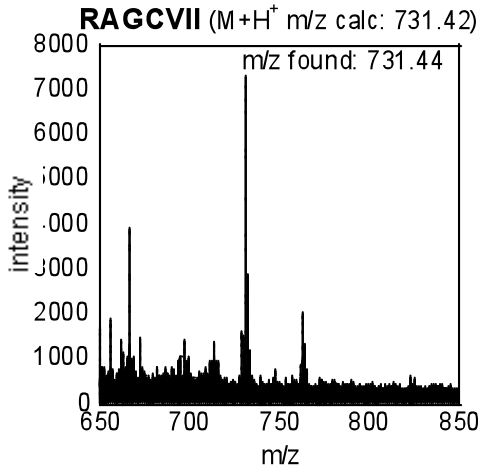

RAGCVIK (M+H+ m/z calc: 746.43)
m/z found: 746.47
intensity
m/z

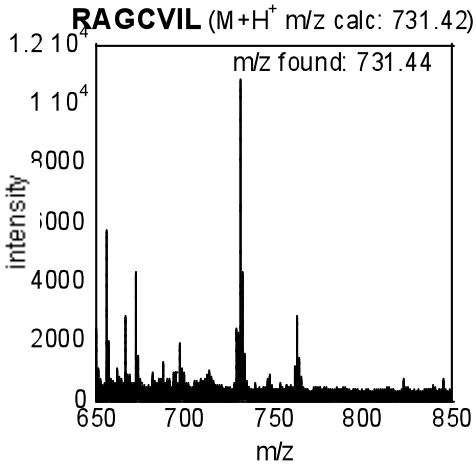

RAGCVIH (M+H+ m/z calc: 755.40)
m/z found: 755.40
intensity
m/z

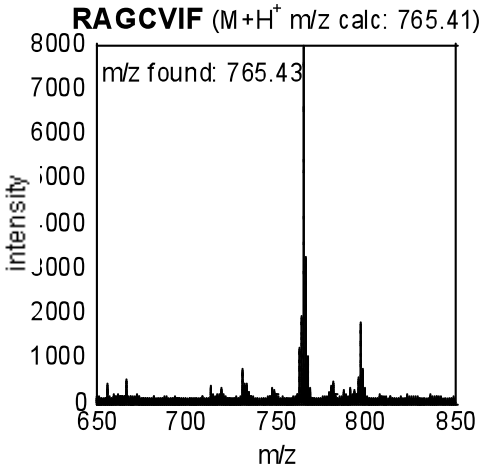

RAGCVIP (M+H+ m/z calc: 715.39)
m/z found: 715.39
intensity
m/z

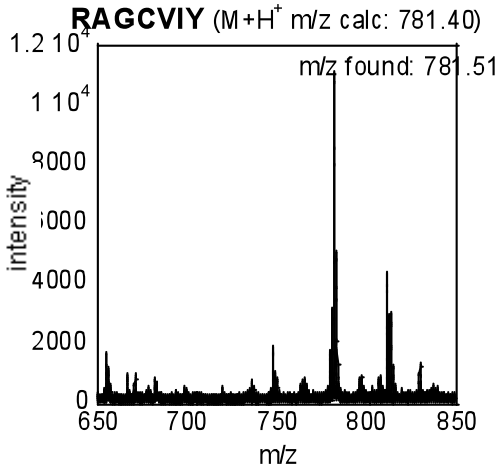

RAGCVIA (M+H+ m/z calc: 689.38)
m/z found: 689.39
intensity
m/z

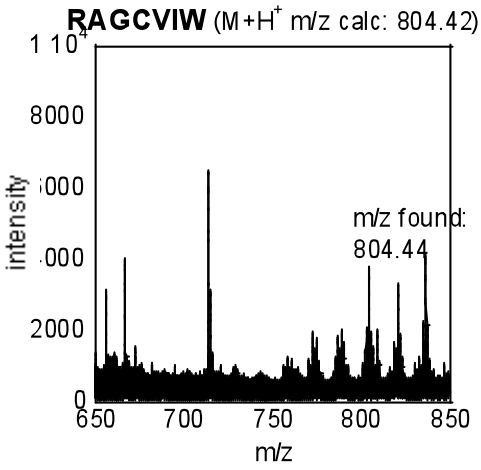

RAGCVIV (M+H+ m/z calc: 717.41)
m/z found: 717.42
intensity
m/z

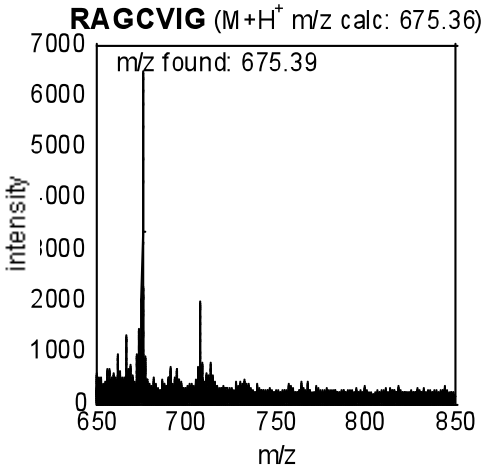

RAGCVII (M+H+ m/z calc: 731.42)
m/z found: 731.44
intensity
m/z

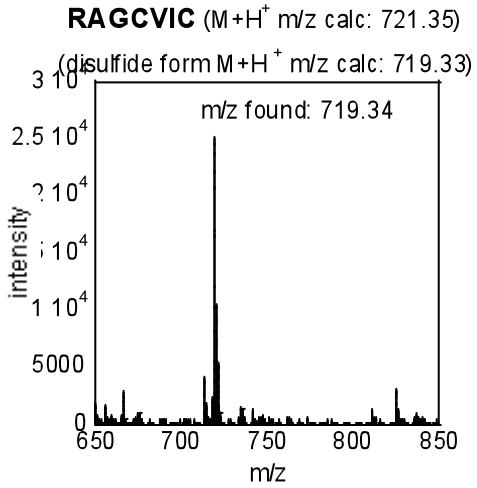

RAGCVIL (M+H+ m/z calc: 731.42)
m/z found: 731.44
intensity
m/z

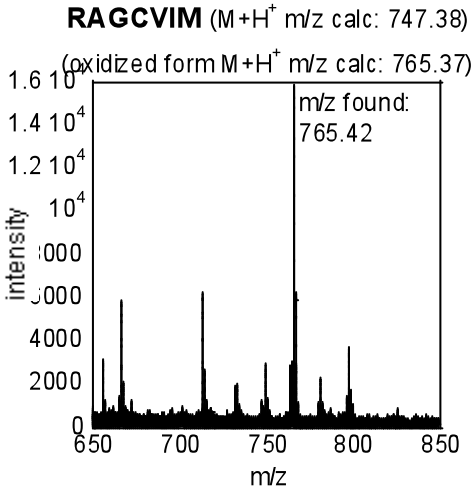

RAGCVIF (M+H+ m/z calc: 765.41)
m/z found: 765.43
intensity
m/z


RAGCVIY (M+H+ m/z calc: 781.40)
m/z found: 781.51
intensity
m/z


RAGCVIW (M+H+ m/z calc: 804.42)
m/z found: 804.44
intensity
m/z


RAGCVIG (M+H+ m/z calc: 675.36)
m/z found: 675.39
intensity
m/z


RAGCVIC (M+H+ m/z calc: 721.35)
(disulfide form M+H+ m/z calc: 719.33)
m/z found: 719.34
intensity
m/z


RAGCVIM (M+H+ m/z calc: 747.38)
(oxidized form M+H+ m/z calc: 765.37)
m/z found: 765.42
intensity
m/z

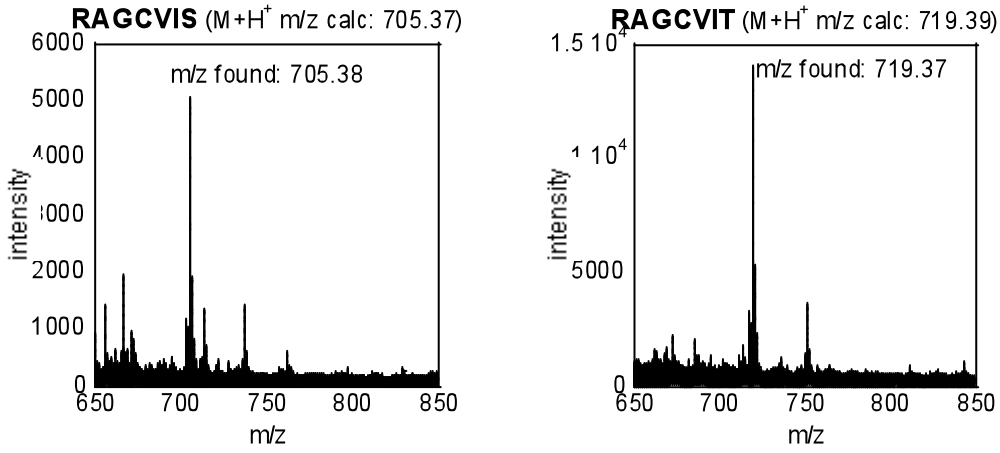
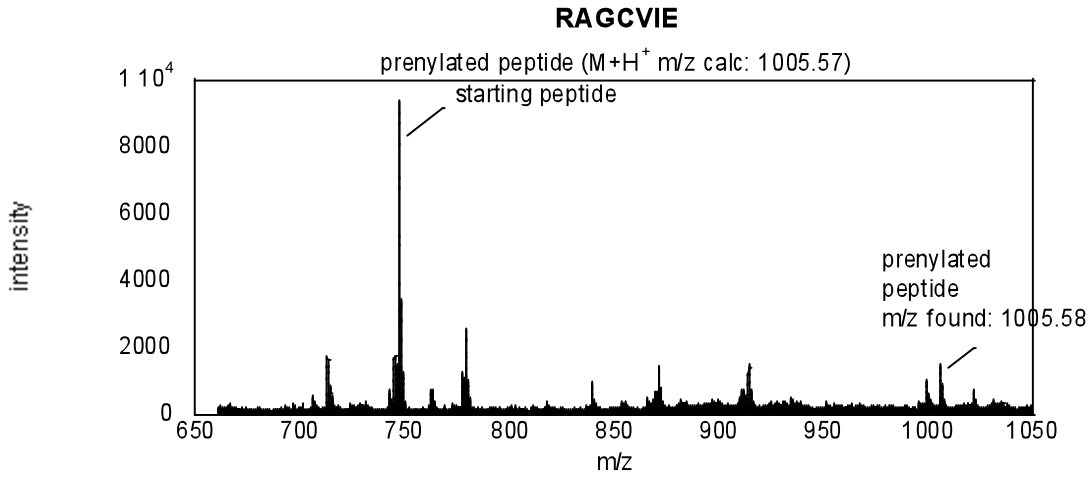
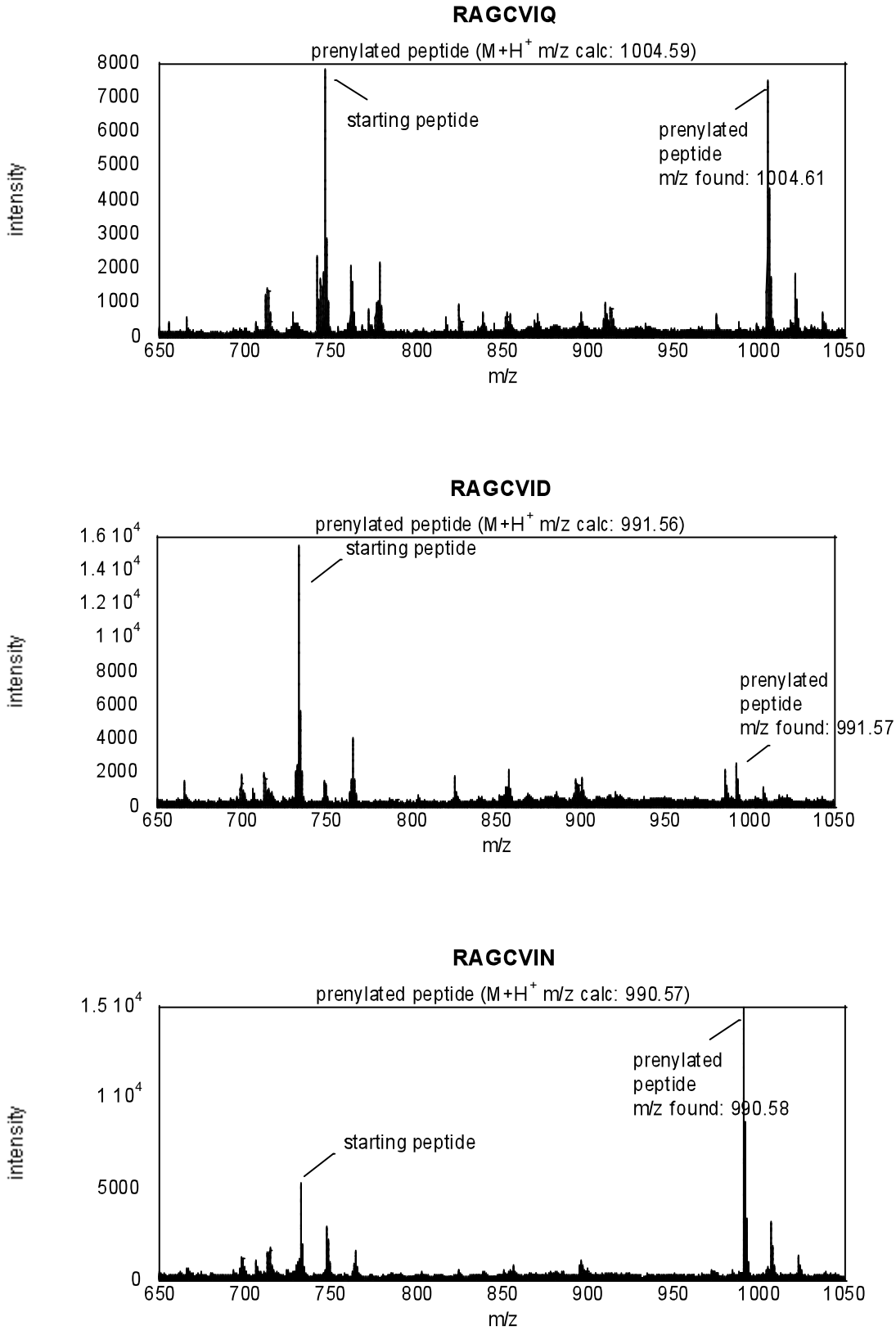
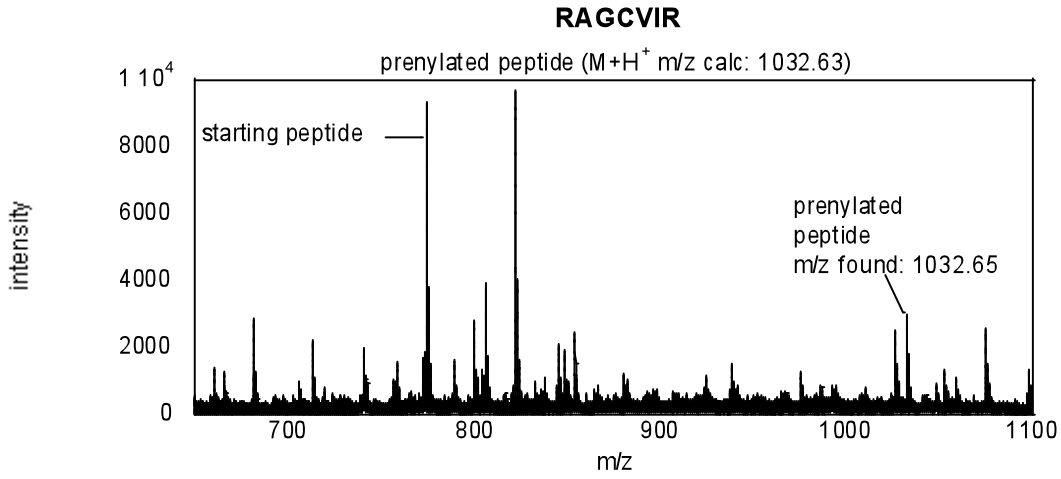
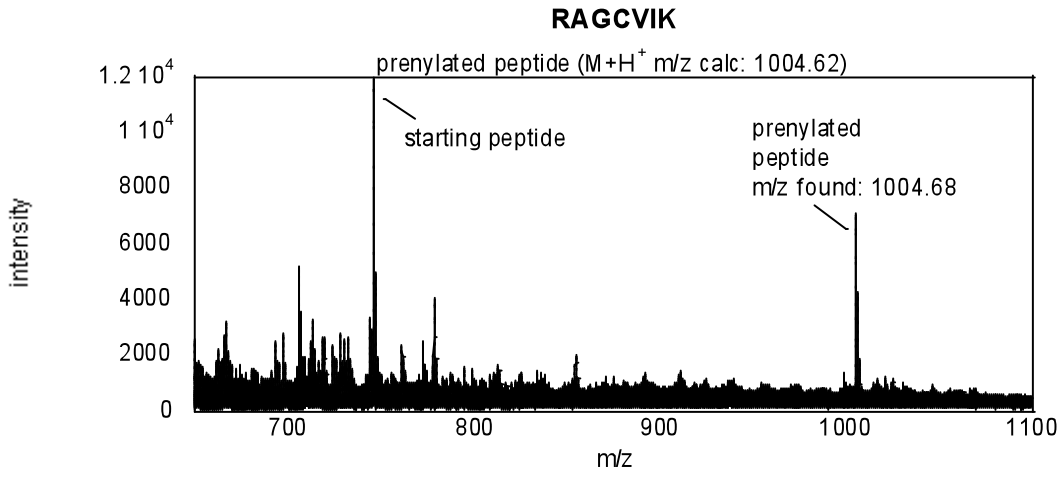


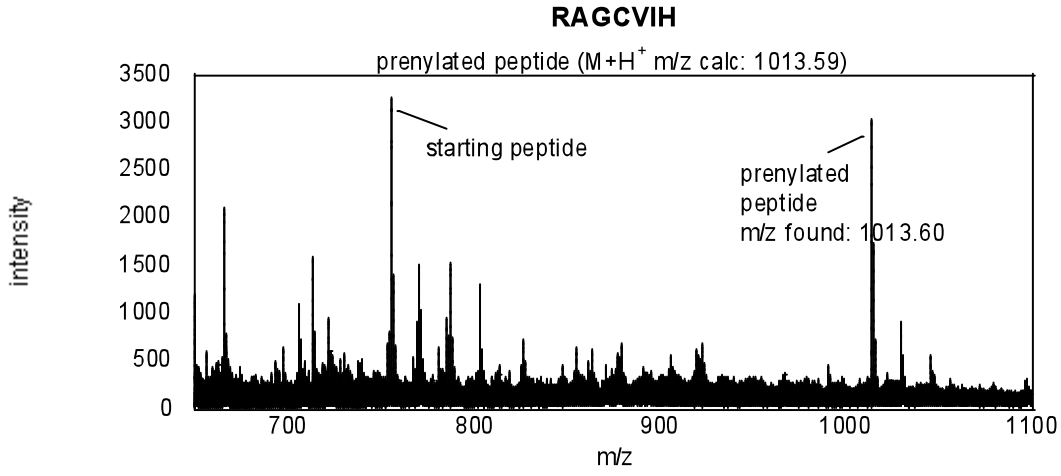
Figure S1. MALDI analysis of peptides produced by SPOT synthesis performed to verify the production of the desired product. The photocleavable moiety shown in Fig. 3B (main text) was used as the linker. The membranes were treated with UV light (365 nm) in CH_3CN/H_2O (v/v = 5/95). The resins were washed with CH_3CN and MeOH. The combined solvents were removed *in vacuo*. The peptides (RAGCVIX) were re-dissolved in CH_3CN/H_2O (v/v = 5/95) with 0.1% TFA.

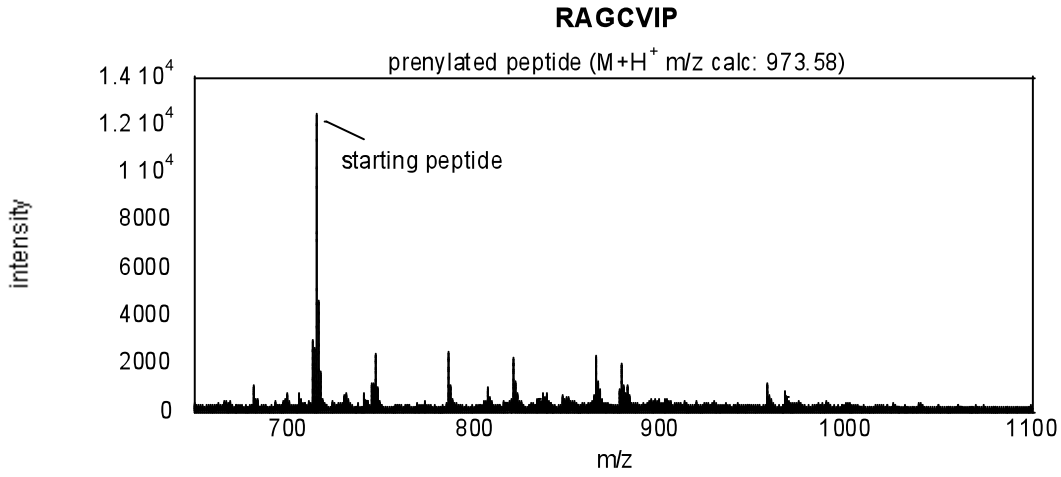


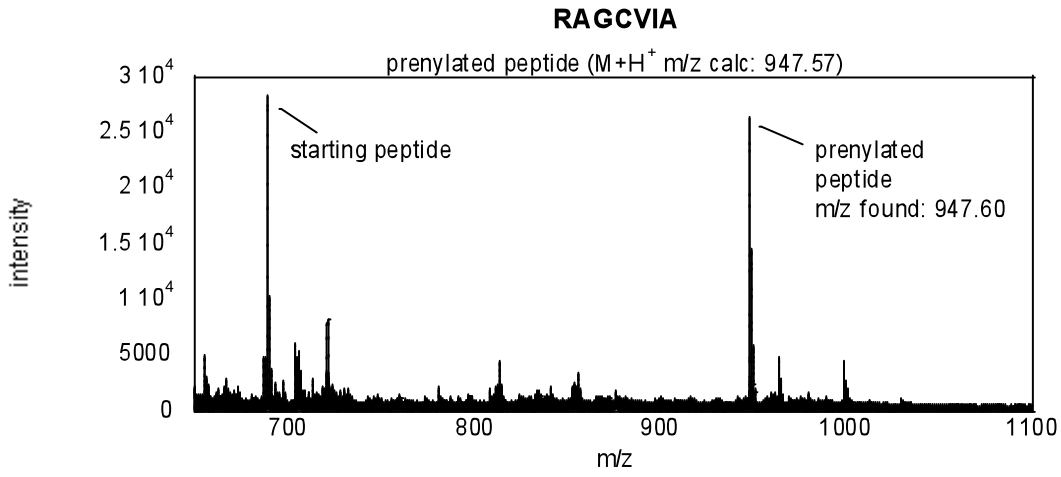

RAGCVIQ
prenylated peptide (M+H+ m/z calc: 1004.59)
starting peptide
prenylated peptide m/z found: 1004.61
intensity
m/z
RAGCVID
prenylated peptide (M+H+ m/z calc: 991.56)
starting peptide
prenylated peptide m/z found: 991.57
intensity
m/z
RAGCVIN
prenylated peptide (M+H+ m/z calc: 990.57)
prenylated peptide m/z found: 990.58
starting peptide
intensity
m/z

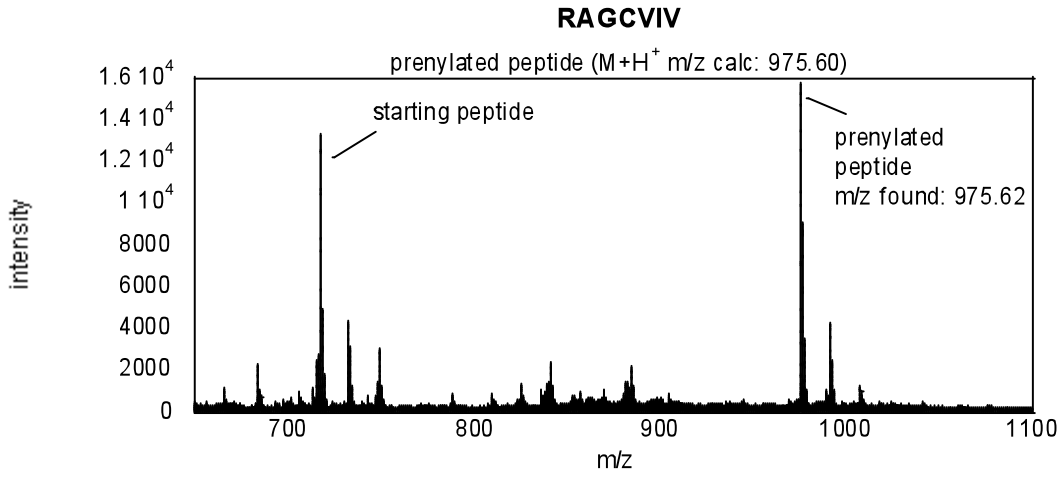

RAGCVIR
prenylated peptide (M+H+ m/z calc: 1032.63)
1 10^4
8000
6000
4000
2000
0
intensity
starting peptide
prenylated
peptide
m/z found: 1032.65
700
800
900
1000
1100
m/z

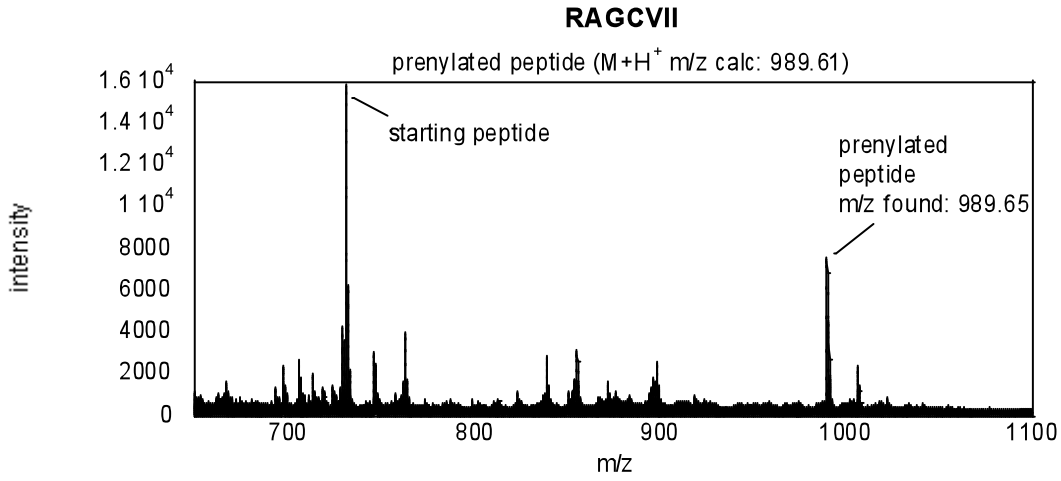

RAGCVIK
prenylated peptide (M+H+ m/z calc: 1004.62)
1.2 10^4
1 10^4
8000
6000
4000
2000
0
intensity
starting peptide
prenylated
peptide
m/z found: 1004.68
700
800
900
1000
1100
m/z

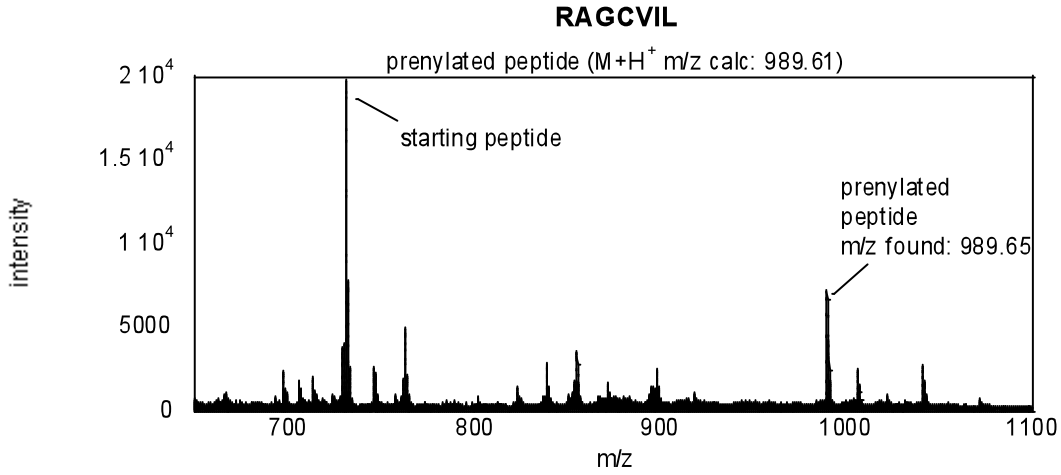

RAGCVIH
prenylated peptide (M+H+ m/z calc: 1013.59)
3500
3000
2500
2000
1500
1000
500
0
intensity
starting peptide
prenylated
peptide
m/z found: 1013.60
700
800
900
1000
1100
m/z

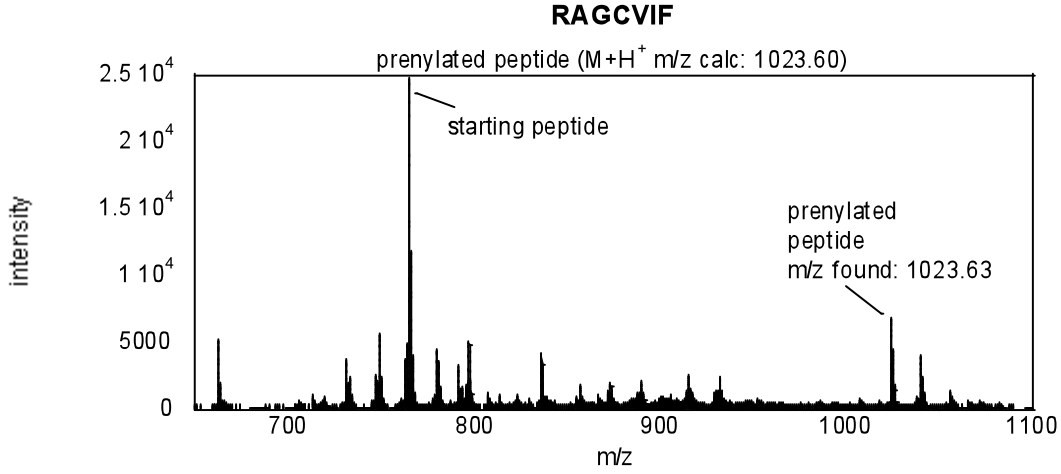

RAGCVIP
prenylated peptide (M+H+ m/z calc: 973.58)
starting peptide
intensity
m/z
1.4 10^4
1.2 10^4
1 10^4
8000
6000
4000
2000
0
700
800
900
1000
1100

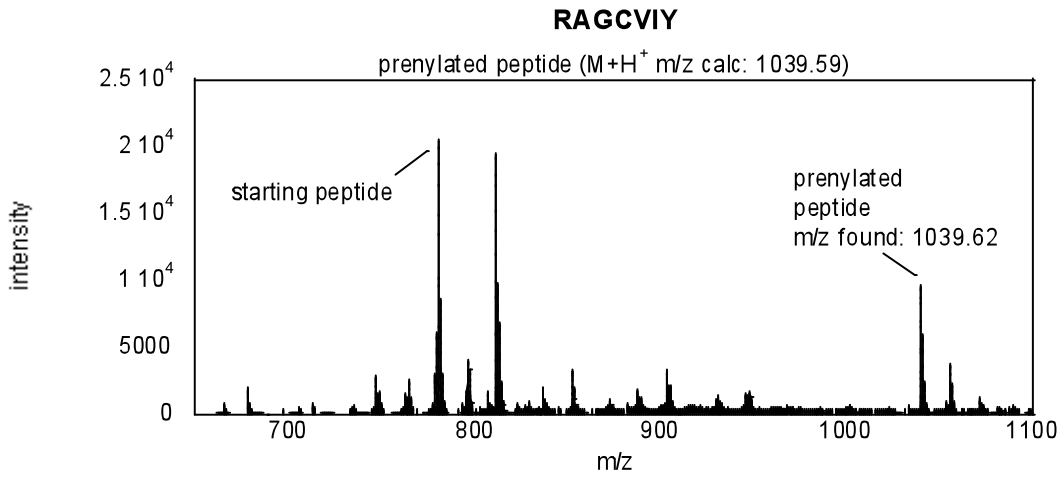

RAGCVIA
prenylated peptide (M+H+ m/z calc: 947.57)
starting peptide
prenylated peptide m/z found: 947.60
intensity
m/z
3 10^4
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
700
800
900
1000
1100

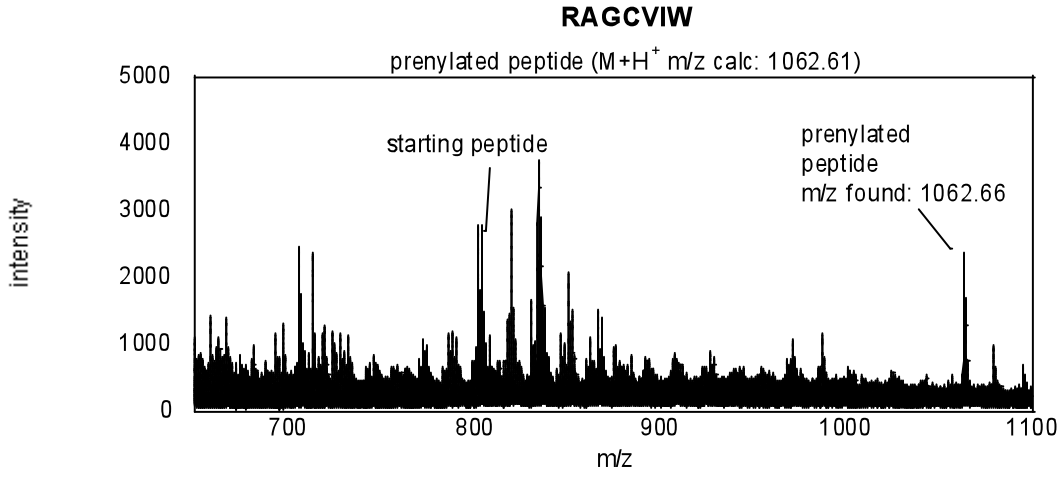

RAGCVIV
prenylated peptide (M+H+ m/z calc: 975.60)
starting peptide
prenylated peptide m/z found: 975.62
intensity
m/z
1.6 10^4
1.4 10^4
1.2 10^4
1 10^4
8000
6000
4000
2000
0
700
800
900
1000
1100

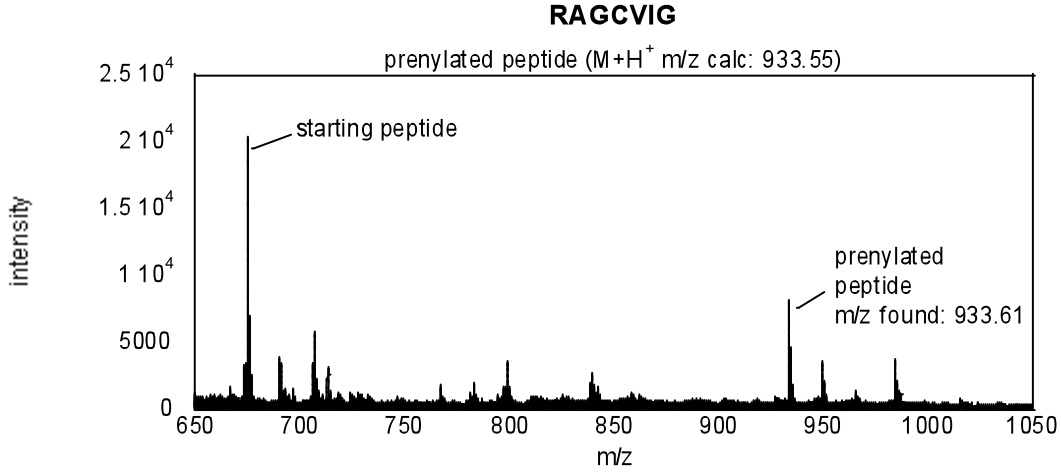

RAGCVII
prenylated peptide (M+H+ m/z calc: 989.61)
1.6 10^4
1.4 10^4
1.2 10^4
1 10^4
8000
6000
4000
2000
0
intensity
starting peptide
prenylated
peptide
m/z found: 989.65
700
800
900
1000
1100
m/z

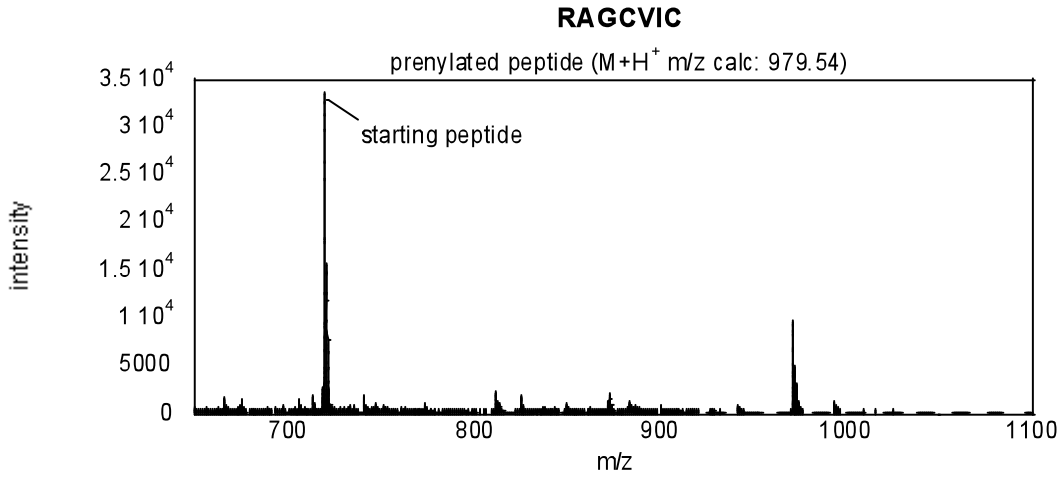

RAGCVIL
prenylated peptide (M+H+ m/z calc: 989.61)
2 10^4
1.5 10^4
1 10^4
5000
0
intensity
starting peptide
prenylated
peptide
m/z found: 989.65
700
800
900
1000
1100
m/z

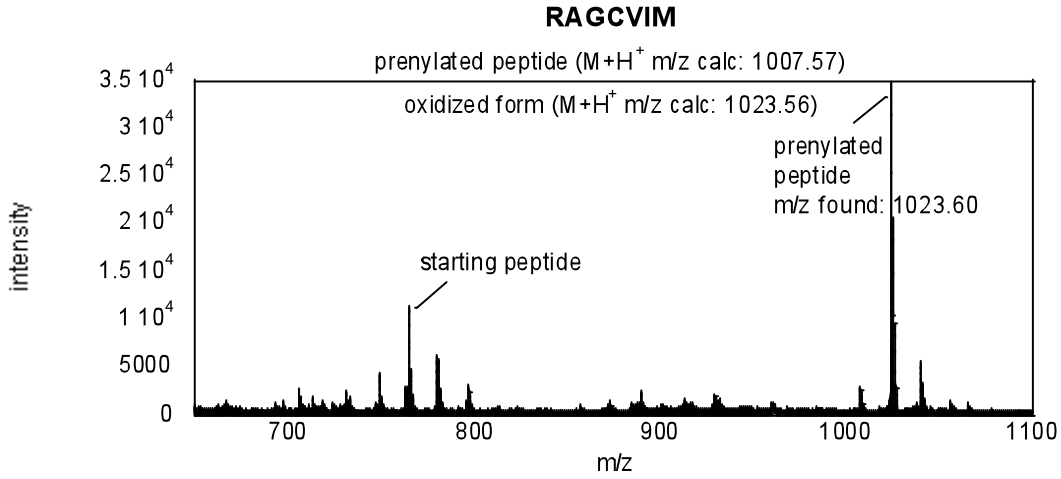

RAGCVIF
prenylated peptide (M+H+ m/z calc: 1023.60)
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
intensity
starting peptide
prenylated
peptide
m/z found: 1023.63
700
800
900
1000
1100
m/z

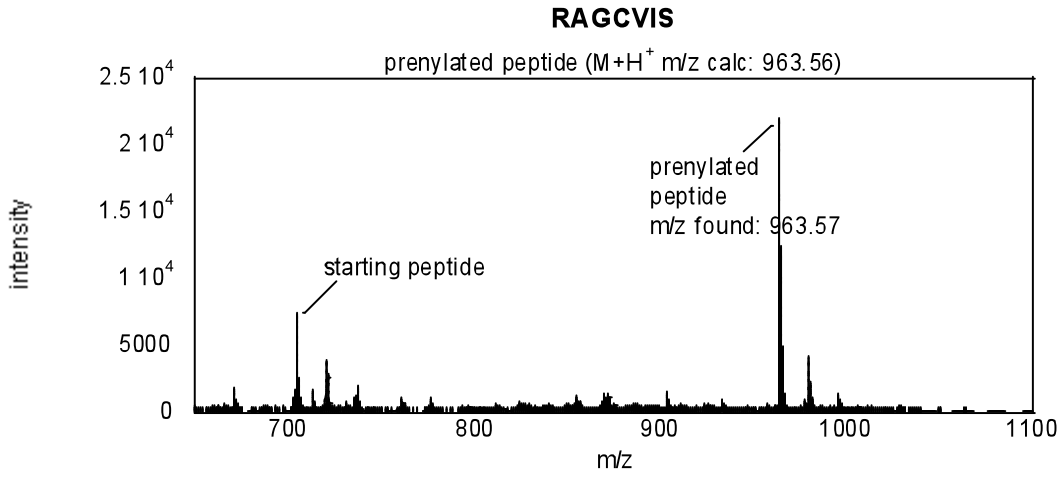

RAGCVIY
prenylated peptide (M+H+ m/z calc: 1039.59)
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
intensity
starting peptide
prenylated
peptide
m/z found: 1039.62
700
800
900
1000
1100
m/z


RAGCVIW
prenylated peptide (M+H+ m/z calc: 1062.61)
5000
4000
3000
2000
1000
0
intensity
starting peptide
prenylated
peptide
m/z found: 1062.66
700
800
900
1000
1100
m/z


RAGCVIG
prenylated peptide (M+H+ m/z calc: 933.55)
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
intensity
starting peptide
prenylated
peptide
m/z found: 933.61
650
700
750
800
850
900
950
1000
1050
m/z


RAGCVIC
prenylated peptide (M+H+ m/z calc: 979.54)
starting peptide
intensity
3.5 10^4
3 10^4
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
700
800
900
1000
1100
m/z


RAGCVIM
prenylated peptide (M+H+ m/z calc: 1007.57)
oxidized form (M+H+ m/z calc: 1023.56)
prenylated peptide m/z found: 1023.60
starting peptide
intensity
3.5 10^4
3 10^4
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
700
800
900
1000
1100
m/z


RAGCVIS
prenylated peptide (M+H+ m/z calc: 963.56)
prenylated peptide m/z found: 963.57
starting peptide
intensity
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
700
800
900
1000
1100
m/z

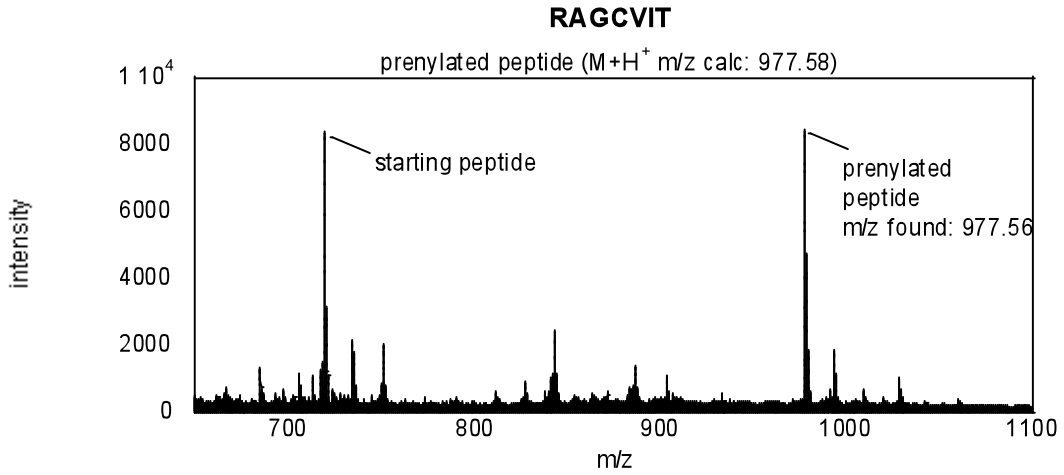


Figure S2. MALDI analysis of peptides produced by SPOT synthesis after enzymatic farnesylation performed to confirm successful enzymatic processing. The photocleavable moiety shown in Fig. 3B (main text) was used as the linker. After enzymatic reaction, the membranes were treated with UV light (365 nm) in CH_3CN/H_2O (v/v = 5/95) to release the peptides. The membranes were washed with CH_3CN and MeOH and the combined washes were evaporated *in vacuo*. The peptides (RAGCVIX) were re-dissolved in CH_3CN/H_2O (v/v = 5/95) with 0.1% TFA.

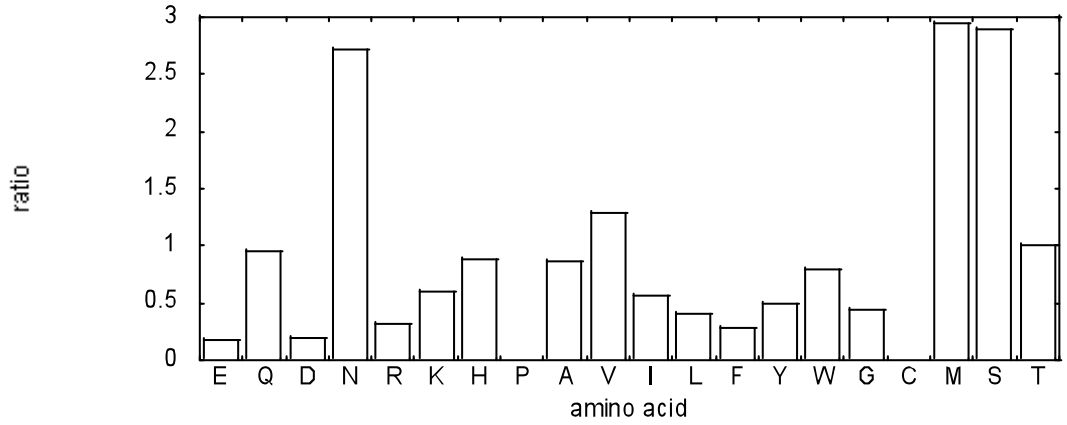


Figure S3. MS intensity ratio of prenylated peptides versus unprenylated peptides from Figure S4 performed to determine the extent of enzymatic prenylation of peptides prepared via SPOT synthesis.

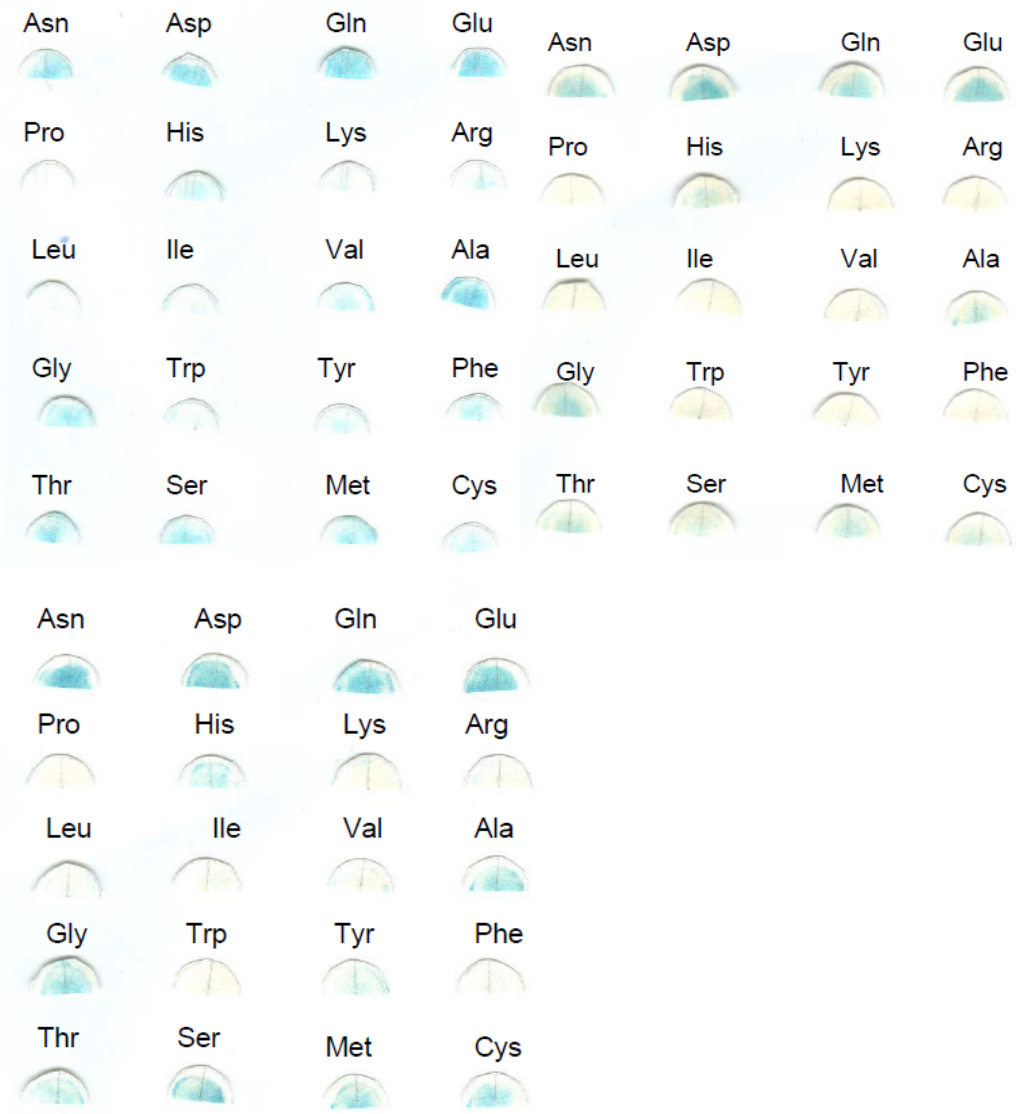


Figure S4. Screening of enzymatic prenylation of an RAGCVIX library. Each SPOT membrane was prenylated with OPP-Far-alkyne by PFTase, clicked with biotin-azide and then visualized by SP-AP in BCIP solution. Membranes from three separate library syntheses and screenings are shown.

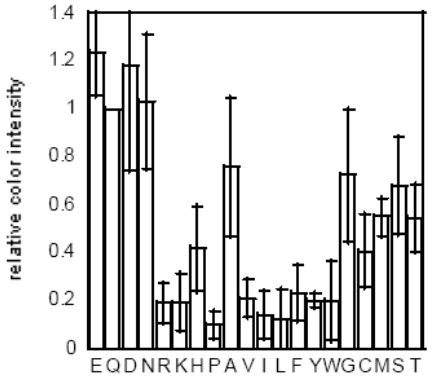


Fig. S5. Evaluation of the extent of farnesylation of a RAGCVIX library of peptides. After synthesis, the peptides were subjected to enzymatic prenylation with an alkyne-containing analogue of FPP followed by click reaction with biotin-azide and subsequent reaction with streptavidin-AP using a chromogenic substrate. Color intensity was quantified by Image J software. The library was synthesized and screened three times and the average color intensities from the three trials are plotted here along with the SD depicted by the error bars. For comparison, the intensity was normalized relative to that observed with Gln at the X position.

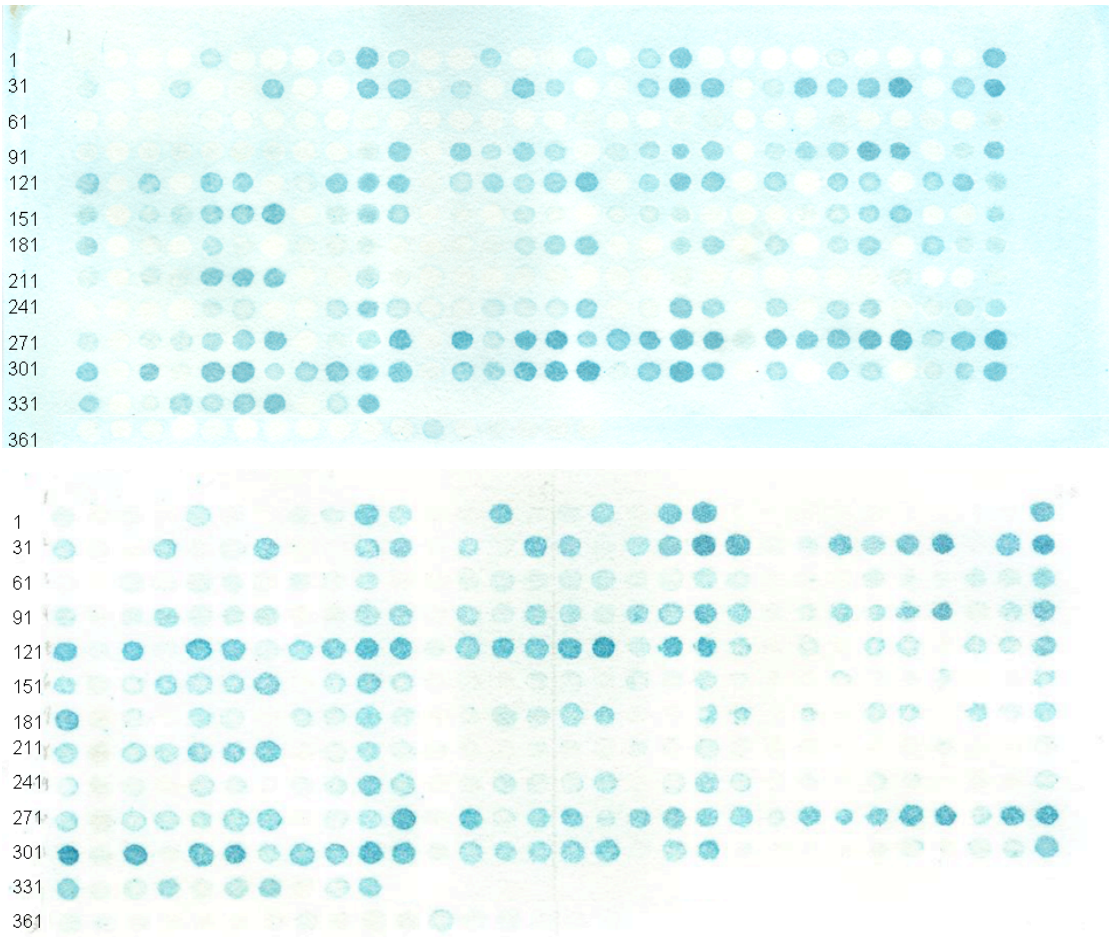


Figure S6. Screening of enzymatic prenylation of an RAGCVa_2X library. Each membrane was prenylated with OPP-Far-alkyne by PFTase, clicked with biotin-azide and then visualized by SP-AP in BCIP solution. Screenings from two separate membranes are shown.

Spot identity:

1-20: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)E

21-40: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)D

41-60: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)N

61-80: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)R

81-100: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)K

101-120: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)H

121-140: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)A

141-160: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)V

161-180: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)I

181-200: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)L

201-220: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)F

221-240: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)W

241-260: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)G

261-280: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)C
281-300: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)M
301-320: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)S
321-340: CV(A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V)T
361-377: SVI(E,D,N,R,K,H,A,V,I,L,F,W,G,C,M,S,T)

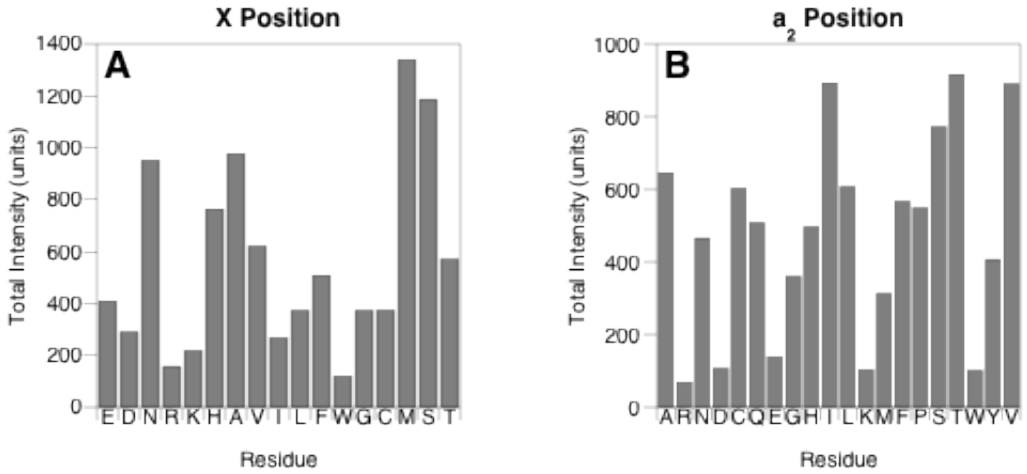
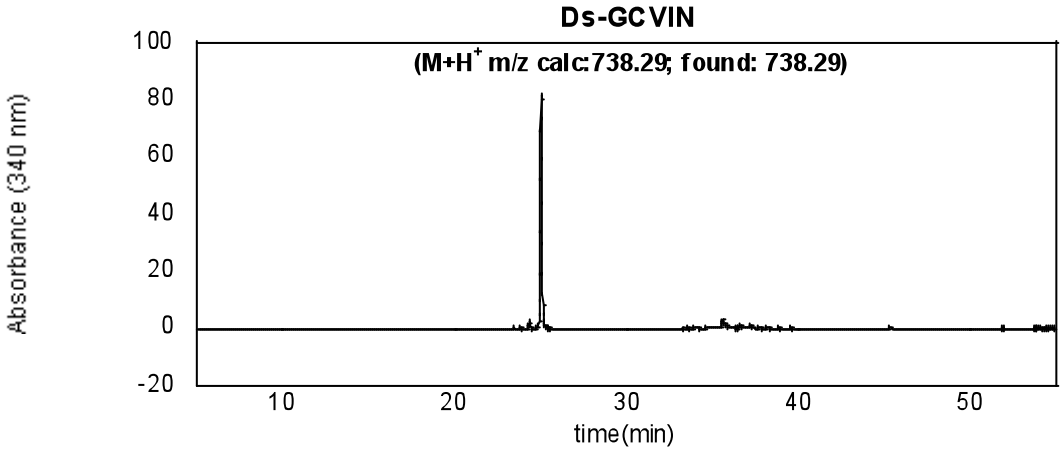


Figure S7. Analysis of preferred residues at the X position and a_2 position for a Ca_1a_2X-box obtained from screening of a 340-member library of peptides. Left (A): Preferences for the X position. Right (B): Preferences for the a_2 position. These preferences were determined by summing the intensities obtained for a single residue at the X or a2 position across all possible combinations at the adjacent position. For example, to calculate the preference for E at the X position, all intensity values present in the "E" row in Fig. 3 were summed (7+2+3+2+22 etc). To calculate the preference for A at the a_2 position, all intensity values present in the "A" column in Fig. 3 were summed (7+3+57+3+11 etc).

Sequence	Screening value	PrePS[7]		Kinetic assay[6]	Sequence	Screening value	PrePS[7]		Kinetic assay[6]
CVAM	95	-3.2	(-)	NA	CVFA	66	-3.6	(-)	NA
CVAS	79	-3.6	(-)	NA	CVPM	67	-0.4	(+)	NA
CVNM	95	-3.3	(-)	NA	CVPS	76	-0.7	(+)	NA
CVNS	80	-3.6	(-)	NA	CVSN	70	-4.0	(-)	NA
CVCN	80	-3.6	(-)	NA	CVSH	70	-2.0	(+)	NA
CVCA	74	-0.9	(+)	Sub	CVSA	77	-1.3	(+)	NA
CVCM	77	-0.5	(+)	NA	CVSF	70	-1.6	(+)	NA
CVCS	83	-0.9	(+)	NA	CVSM	94	-0.9	(+)	NA
CVQM	91	0.4	(++)	MTO	CVSS	87	-1.3	(+)	STO
CVQS	89	0.2	(++)	NA	CVTN	104	-2.7	(-)	NA
CVGM	68	-3.2	(-)	NA	CVTH	76	-0.7	(+)	NA
CVHA	68	-3.7	(-)	NA	CVTA	104	-0.0	(+)	Sub
CVHM	78	-3.7	(-)	NA	CVTV	79	-0.7	(+)	NA
CVHS	73	-3.7	(-)	MTO	CVTF	79	-0.4	(+)	NA
CVIE	82	-1.3	(+)	Sub	CVTC	70	0.2	(++)	NA
CVID	77	-1.9	(+)	NA	CVTM	103	0.4	(++)	NA
CVIN	100	-1.6	(+)	Sub	CVTS	99	0.0	(++)	NA
CVIA	81	1.1	(++)	Sub	CVTT	70	-0.7	(+)	MTO
CVIM	81	1.4	(++)	Sub	CVYM	81	-4.1	(-)	NA
CVIS	84	1.1	(++)	Sub	CVVE	74	-1.7	(+)	NA
CVIT	73	0.5	(++)	Sub	CVVN	73	-2.1	(-)	MTO
CVLN	84	-2.4	(-)	NA	CVVH	68	-0.2	(+)	NA
CVLA	78	0.3	(++)	Sub, MTO	CVVA	78	0.6	(++)	Sub
CVLM	78	0.6	(++)	NA	CVVM	99	0.9	(++)	Sub
CVLS	79	0.3	(++)	Sub	CVVS	89	0.6	(++)	NA
CVMM	71	-0.3	(+)	NA	CVVT	68	-0.1	(+)	MTO
CVFN	70	-6.5	(--)	NA					

Fig. S8. Comparison of screening results described here with Prenylation Prediction Suite PrenPS (http://mendel.imp.ac.at/PrePS/index.html)[7] and previously reported kintetic data obtained via in vitro enzyme assays.[6] Only peptides sequences giving high intensity signals (intensities above 66% in Fig. 3, highlighted in red) from the screening are included in this table. For PrenPS predictions, an upstream sequence of GGLLBBQFRAGCa_1a_2X was employed. For the previously reported kinetic assay data, NA indicates sequences not measured, Sub indicates Ca_1a_2X sequences from known farnesylated proteins, MTO indicates sequences that exhibit multiple-turnover activity and STO indicates sequences that exhibit single-turnover activity.


Ds-GCVIN
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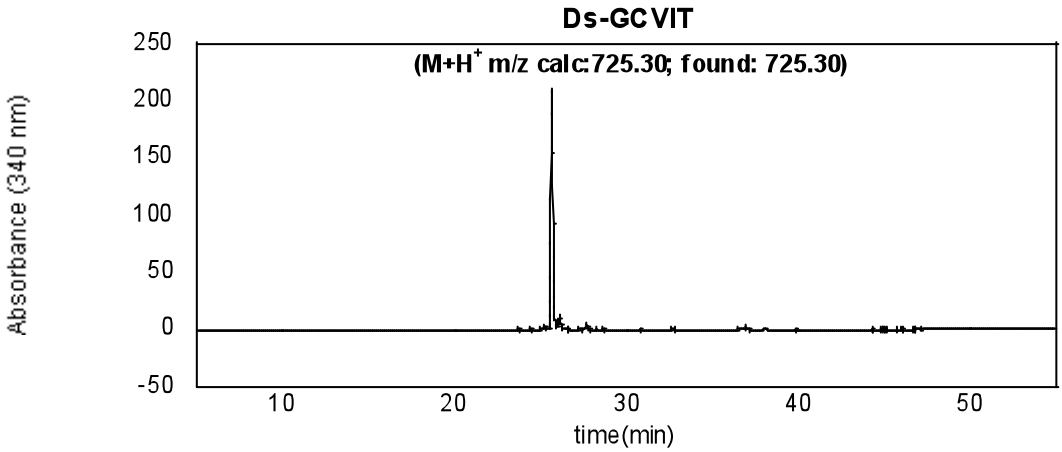
Absorbance (340 nm)
100
80
60
40
20
0
-20
10
20
30
40
50
time(min)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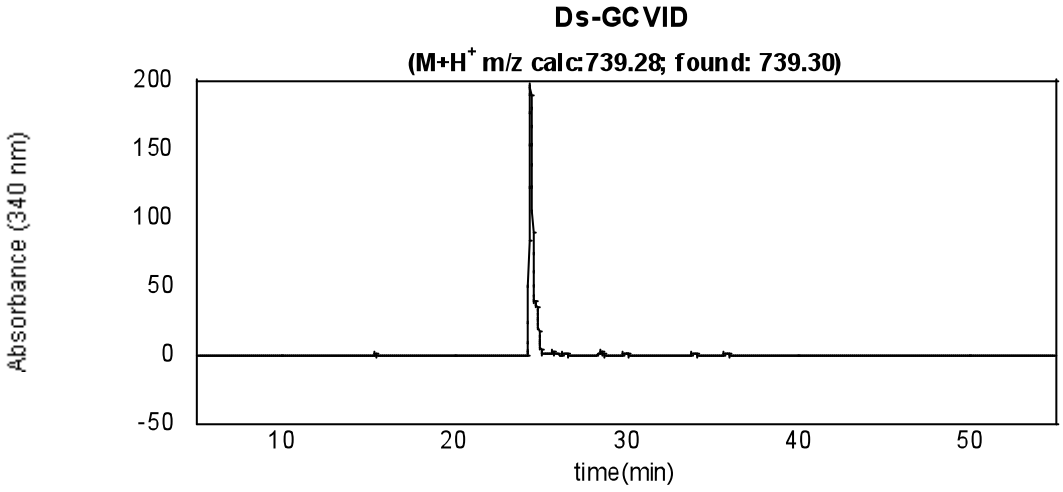
(M+H+ m/z calc:725.30; found: 725.30)
Absorbance (340 nm)
250
200
150
100
50
0
-50
10
20
30
40
50
time(min)


Ds-GCVID
(M+H+ m/z calc:739.28; found: 739.30)
Absorbance (340 nm)
200
150
100
50
0
-50
10
20
30
40
50
time(min)

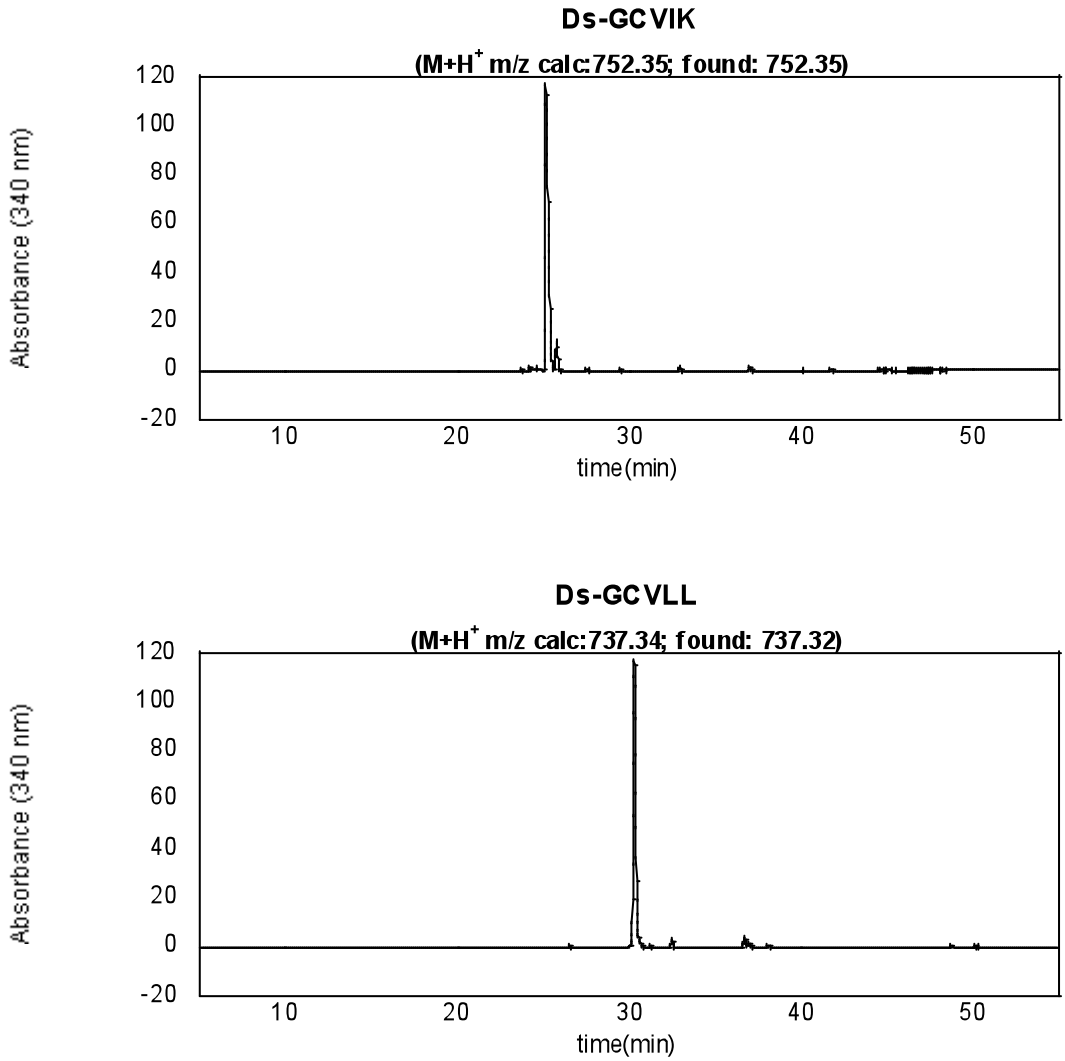


Figure S9. HPLC analysis of individually synthesized dansylated peptide used for kinetic experiments. Solvent A was H_2O with 0.1% TFA. Solvent B was CH_3CN with 0.1% TFA. The gradient was from 0% to 100% Solvent B from 5 min to 55 min. Absorbance wavelength was 340 nm. The purifity of all peptides was above 90 % as determined from peak integration.

	Ds-GCVIN	DsGCVIA	Ds-GCVIT
v (uM/min)	3.60 ± 0.25	2.39 ± 0.25	2.28 ± 0.10
	Ds-GCVID	Ds-GCVIK	Ds-GCVLL
	0.033 ± 0.003	0.048 ± 0.009	0.032 ± 0.004

Table S1 Evaluation of Ds-GCaaX peptides versus yFTase. Assays were performed using 2.4 μM dansylated peptide, 70 nM FTase, 10 μM OPP-Far-alkyne, in 200 mM Tris, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 50 μM $ZnCl_2$, and 0.040 % (*w/v*) *n*-dodecyl-β-D-maltoside.

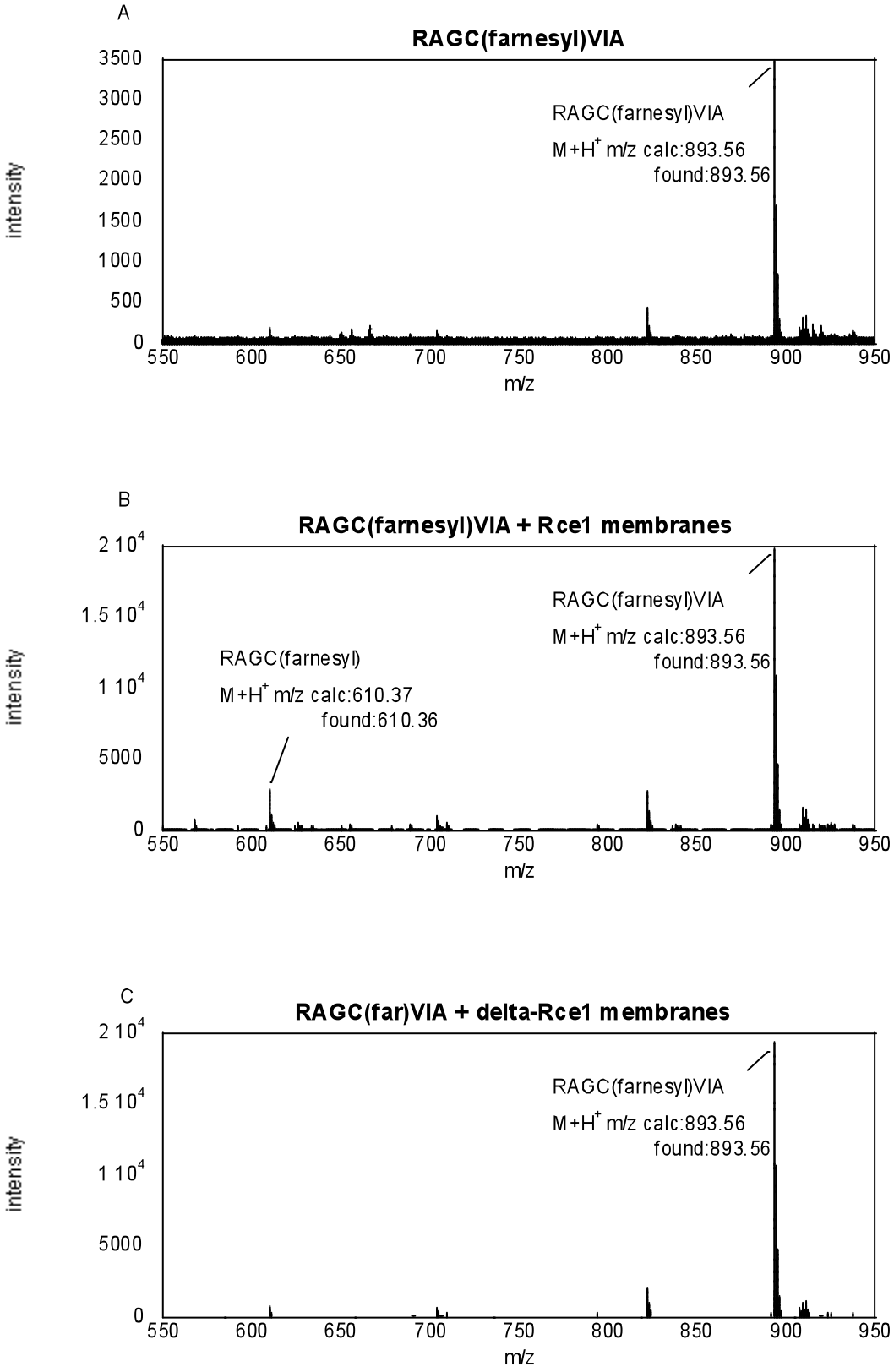


Figure S10. MALDI analysis of RAGC(farnesyl)VIA with Rce1 treatment to confirm successful enzymatic proteolysis on solid surface. (A) RAGC(farnesyl)VIA released

from CPG before Rce1 reaction. (B) Peptides released from CPG after Rce1 reaction showing the presence of both full length RAGC(farnesyl)VIA and the cleavage product RAGC(farnesyl). (C) Analysis of RAGC(farnesyl)VIA after exposure to yeast membranes produced from a deletion strain lacking Rce1 showing very low intensity of cleaved product.

Acknowledgement

This work was supported by the National Institutes of Health (GM058842 and GM084152). MALDI-MS data were obtained at the Center for Mass Spectrometry and Proteomics at the University of Minnesota. We would also like to thank Dr. Tom Krick and Dr. LeeAnn Higgins for helping with the MALDI analysis.

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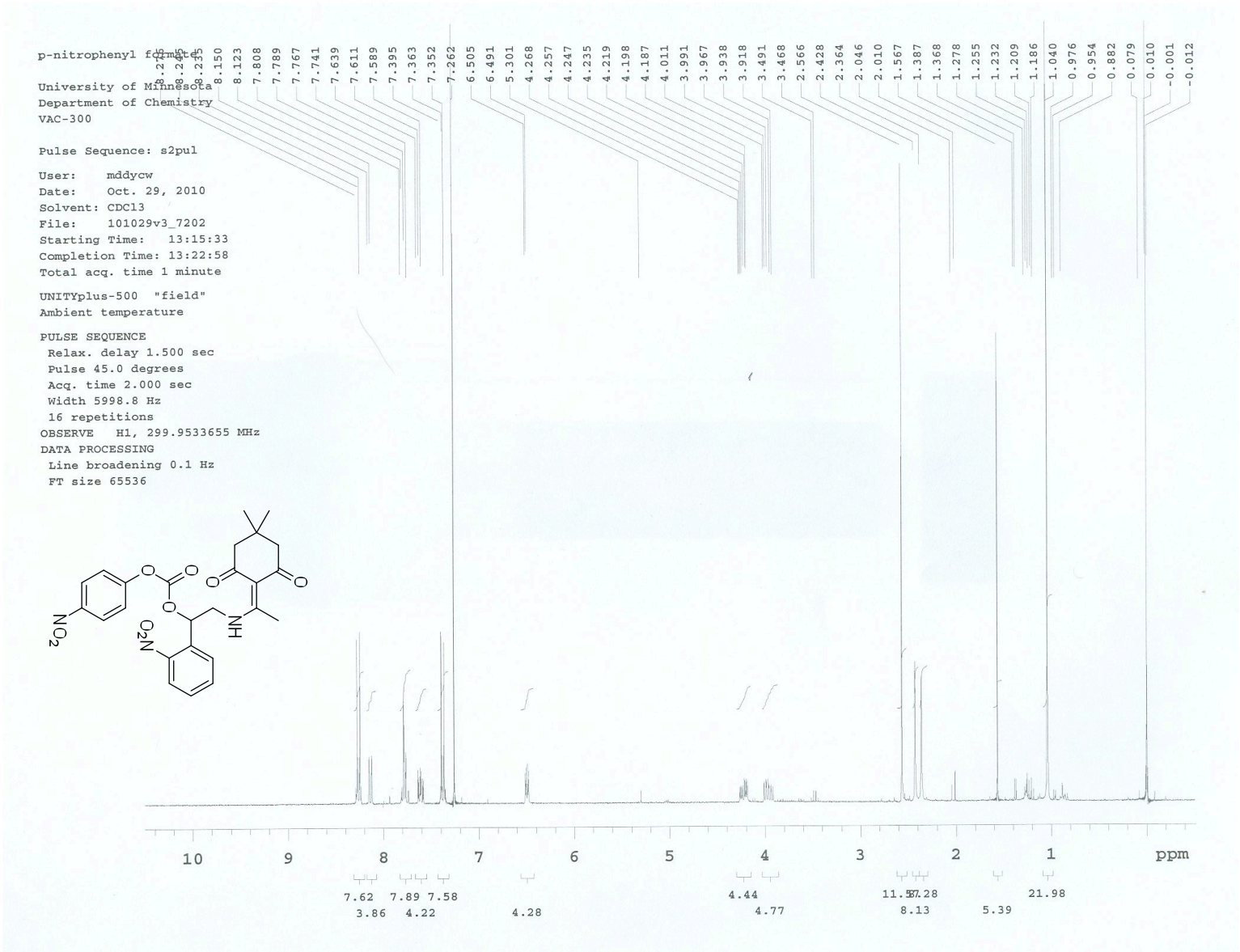
p-nitrophenyl formate
University of Minnesota
Department of Chemistry
VAC-300
Pulse Sequence: s2pul
User: mddycw
Date: Oct. 29, 2010
Solvent: CDCl3
File: 101029v3_7202
Starting Time: 13:15:33
Completion Time: 13:22:58
Total acq. time 1 minute
UNITYplus-500 "field"
Ambient temperature
PULSE SEQUENCE
Relax. delay 1.500 sec
Pulse 45.0 degrees
Acq. time 2.000 sec
Width 5998.8 Hz
16 repetitions
OBSERVE H1, 299.9533655 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 65536
8.246
8.235
8.150
8.123
7.808
7.789
7.767
7.741
7.639
7.611
7.589
7.395
7.363
7.352
7.262
6.505
6.491
5.301
4.268
4.257
4.247
4.235
4.219
4.198
4.187
4.011
3.991
3.967
3.938
3.918
3.491
3.468
2.566
2.428
2.364
2.046
2.010
1.567
1.387
1.368
1.278
1.255
1.232
1.209
1.186
1.040
0.976
0.954
0.882
0.079
0.010
-0.001
-0.012
10
9
8
7
6
5
4
3
2
1
ppm
7.62
3.86
7.89
4.22
7.58
4.28
4.44
4.77
11.57
8.13
7.28
5.39
21.98