**Electronic Supplementary Information** for “Long-range PEG Stapling: Macrocyclization for Increased Protein Conformational Stability and Resistance to Proteolysis”

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Safety and Hazards:

The work described below was carried out using standard laboratory PPE, including a lab coat, nitrile gloves, and goggles. Work with trifluoroacetic acid or with thiols was conducted in a fume hood.

1. Structures and sequences of WW, SH3, and GCN4 variants

The structures and sequences of newly synthesized WW and SH3 variants are shown in Figure S1. WW variants 16/19-00, 16/19-o23, s16/19-o23, 16/32-00, 16/32-o44, s16/32-o44 and SH3 variant SH3 20/37-00 mentioned in the main text, those structures and sequences were reported before by our lab.\(^1\)\(^2\)

![Structures of WW and SH3 variants](image)

**Figure S1.** Sequences and structures of olefin-stapled WW variants s16/19-o23, s16/32-o44 and s14/30-o55; triazole-stapled WW variants s16/32-c44 and s14/30-c44; and olefin-stapled SH3 variant s20/37-o44 and their non-stapled and non-PEGylated counterparts N represents a PEG-modified Asn residue; the PEG oligomer(s) within each variant have the number of ethylene oxide units and the olefin, azide, alkyne, or triazole functional groups as indicated in the structural drawings.
Figure S2. Acidic GCN4 monomer 27A-c4, basic GCN4 monomer 29A'-c0, noncovalent GCN4 heterodimer 27/29'-c40, and its triazole-stapled counterpart 27/29'-c40. Also shown are cysteine-containing acidic GCN4 monomer 27-c4, basic GCN4 monomer 29'-c0, disulfide-bound GCN4 heterodimer d27/29'-c40, and its triazole-stapled counterpart sd27/29'-c40. X represents propargyl glycine; N represents a PEG-modified Asn residue; the PEG oligomer(s) within each variant have the number of ethylene oxide units indicated in the structural drawings.

2. Synthesis of WW, SH3 and GCN4 variants

Peptide Synthesis: WW variants 16/32-00, 16/32-o44, s16/32-o44, and SH3 variant 20/37-00 were prepared previously. WW variants 16/32-c44, 14/30-00, 14/30-o55, and 14/30-c44 were synthesized as C-terminal acids on Fmoc-Gly-Wang LL resin (EMD Biosciences) and SH3 variant 20/37-o44 and GCN4 variants 27-c4, 29'-c0, and their Cys33Ala mutants 27A-c4, 29A'-c0 were prepared as C-terminal amides on and Rink amide MBHA LL resin (EMD Biosciences), by microwave-assisted solid phase peptide synthesis using a standard Fmoc Nα protection strategy as described previously. Fmoc-protected amino acids were purchased from Advanced Chem Tech, except for the PEGylated asparagine derivatives, which were synthesized as described in section 7 below. Unstapled WW, SH3, and GCN4 variants were cleaved from resin and purified by preparative reverse-phase high-performance liquid chromatography (HPLC) on a C18 column using a linear gradient of water in acetonitrile with 0.1% v/v trifluoroacetic acid. Peptide identity was confirmed by electrospray ionization time-of-flight mass spectrometry. We used an analogous approach to prepare the stapled WW variants and SH3 variant, except we reduced the resin loading (i.e. the number of Fmoc-protected amino groups on resin) by approximately 50% using a 1:1 mixture of Fmoc- and Boc-protected amino acids during the first coupling reaction.

Stapling via olefin metathesis: We prepared olefin-stapled variants s14/30-o55 and s20/37-o44 from their resin-bound non-stapled precursors 14/30-o55 and 20/37-o44 via on-resin olefin metathesis, as we have done previously, except we heated the reaction mixture to 60 °C. We monitored reaction completeness by...
microcleavage and ESI-TOF MS. Following the reaction, we cleaved and purified s14/30-o55 and s20/37-o44 as described above.

**Disulfide formation in the GCN4 heterodimer:** We prepared the disulfide-bonded GCN4 heterodimer d27/29'-c40 (notebook number QX22031) by mixing its purified cysteine-containing precursors 27-c4 (notebook number QX21971) and 29'-c0 (notebook number QX21973) in a 1:1 ratio (total peptide concentration of 8 mg/mL) in an aqueous solution of ammonium bicarbonate with exposure to air for 3 h. Reaction completeness was monitored by analytical HPLC; GCN4 variant d27/29'-c40 was then purified by preparative HPLC and characterized by ESI-TOF MS.

**Stapling via copper-catalyzed azide/alkyne cycloaddition (CuAAC):** We prepared triazole-stapled variants s16/32-c44, s14/30-c44, sd27/29'-c40, and s27/29'-c40 (i.e., the non-disulfide-bound counterpart of sd27/29'-c40) by stirring their purified non-stapled precursors 16/32-c44, 14/30-c44, d27/29'-c40, and 27/29'-c40 (i.e., the non-covalent GCN4 heterodimer formed by a 1:1 mixture of 27A-c4 and 29A'-c0) in 2:1 (v/v) water/tert-butanol with 10 eq. copper sulfate pentahydrate and 10 eq. sodium ascorbate at room temperature for 2 hours. The reaction was monitored by analytical HPLC, where we observed small changes in retention time upon stapling. The triazole-stapled variants were purified via preparative HPLC.

The successful conversion of non-covalent heterodimer 27/29'-c40 into triazole-stapled s27/29'-c40 was readily confirmed by ESI-TOF MS; the non-covalent heterodimer 27/29'-c40 separates into its component monomers 27A-c4 (notebook number QX22091) and 29A'-c0 (notebook number QX22092) in the mass spectrometer, whereas triazole-stapled s27/29'-c40 does not. However, the CuAAC reaction does not change the mass of the monomeric triazole-stapled variants s16/32-c44, s14/30-c44, sd27/29'-c40 (notebook number QX2204) relative to their non-stapled azide/alkyne precursors 16/32-c44, 14/30-c44, and d27/29'-c40 (notebook number QX22031). To confirm the completion of the CuAAC reaction, we subjected each azide-containing variant (16/32-c44, 14/30-c44, and d27/29'-c40) and its triazole-stapled counterpart (s16/32-c44, s14/30-c44, and sd27/29'-c40) separately to a solution of dithiothreitol (DTT, 31 mg) and diisopropylethylamine (DIEA, 17 μL) in 100 μL water, followed by stirring for 1 h. Presence of the azide groups in non-stapled 16/32-c44 (notebook number QX21852), 14/30-c44, and d27/29'-c40 was confirmed by their conversion to amino groups under reducing conditions, as detected by ESI-TOF MS (i.e., a loss of N2 and the addition of two hydrogens). Presence of the triazole group in s16/32-c44 (notebook number QX2188), s14/30-c44, and sd27/29'-c40 were confirmed by the absence of this mass change under reducing conditions. The results of this analysis are shown below in Figures S3–S8.

**Choice of PEG length in variants s16/19-o23, s16/32-o44, s16/32-c44, s14/30-o55, and s14/30-c44.** We previously found that the PEG linker between positions 16 and 19 in WW variant s16/19-o23 provided optimal stabilization relative to linkers of other lengths, including one with two four-unit PEGs. In the same previous

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publication, we used two four-unit PEG linkers to connect positions 16 and 32 owing to the increased distance between positions 16 and 32 relative to that between positions 16 and 19 in the crystal structure of the parent WW domain from which these variants were derived. We did not attempt any further optimization of linker length between positions 16 and 32. Here, we have continued to use two four-unit PEG linkers to facilitate direct comparison between the impact of click-stapling on variant s16/32-c44 vs. olefin-stapling on variant s16/32-o44.

In preparing the current manuscript, we initially attempted to use two four-unit PEG linkers to staple positions 14 and 30. We successfully prepared PEGylated variant 14/30-44 and its non-PEGylated counterpart 14/30-00. However, several attempts at on-resin olefin metathesis failed to provide sufficient amounts of s14/30-o44 for variable temperature CD experiments, precluding us from assessment of the impact of this particular staple on WW conformational stability. We hypothesized that the on-resin olefin metathesis reaction might have failed because the two four-unit PEGs were not sufficiently long to bridge the distance between positions 14 and 30 in the ensemble of conformations adopted by the protected resin-bound protein. Accordingly, we prepared a new olefin-terminated Asn-PEG residue with five ethylene oxide units instead of four. We incorporated this residue at positions 14 and 30 to generate WW variant 14/30-o55. This time, on-resin olefin-metathesis provided sufficient amounts of stapled WW variant s14/30-o55 for variable temperature CD experiments.

Click-stapling efforts at these positions occurred before we knew that our efforts to obtain s14/30-o44 would be unsuccessful. Accordingly, we prepared WW variant 14/30-c44 (in which an Asn-linked propargyl-terminated four-unit PEG occupies position 14, whereas an Asn-linked azide-terminated four-unit PEG occupies position 30). The click reaction was carried out in a 2:1 solution of water/tert-butanol and successfully converted the purified non-stapled precursor 14/30-c44 into its stapled counterpart s14/30-c44. We speculate that the conformation adopted by deprotected purified 14/30-c44 in this water/tert-butanol solution allowed positions 14 and 30 to be close enough to each other that the click reaction could succeed. Unfortunately, our efforts to carry out olefin metathesis on purified deprotected 14/30-o44 in aqueous solution have not yet been successful.

We realize that comparing the staple-based stabilization of variant s14/30-c44 vs. s14/30-o55 conflates the impact of staple chemistry with the impact of different PEG lengths. Ideally, we would have prepared variants 14/30-c55 and s14/30-c55 to facilitate a more direct comparison. However, at this point comparison between variants s16/32-o44 and s16/32-c44 (where linker length stayed constant) had already revealed that click-stapling provide similar levels of stabilization as does olefin-stapling (see Table 1 in the main text). The comparison between variants s14/30-c44 vs. s14/30-o55 did not contradict this finding, especially in light of previous observations where changes in PEG-length beyond three or four-units has a minimal effect. We concluded that the limited insights to be gained by generating variants 14/30-c55 and s14/30-c55 did not sufficiently justify the extensive effort that would have been required to prepare the two new Asn-PEGs comprised of propargyl or azide-terminated five-unit PEGs.
**Figure S3.** Analytical HPLC retention time for WW variant 16/32-c44 (notebook number QX21852) before (A) and after (B) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h; and for its triazole-stapled counterpart s16/32-c44 (notebook number QX2188) before (C) and after (D) exposure to reducing conditions for 1 h. Peptide solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

**Figure S4.** ESI-TOF MS data for azide-containing WW variant 16/32-c44 (notebook number QX21852) before (A, C) and after (E, G) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h. Expected [(M+4H⁺)⁴]/4 = 1113.82 Da for 16/32-c44 prior to reduction; expected [(M+4H⁺)⁴]/4 = 1107.32 Da after reduction of the azide at position 32 to the corresponding amine. Also shown are ESI-TOF MS data for triazole-stapled s16/32-c44 (notebook number QX21852) before (B, D) and after (F, H) exposure to reduction conditions for 1 h. Expected [(M+4H⁺)⁴]/4 = 1113.82 Da for triazole-stapled s16/32-c44 both before and after reduction.
Figure S5. Analytical HPLC retention time for WW variant 14/30-c44 (notebook number QX22154) before (A) and after (B) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h; and for its triazole-stapled counterpart s14/30-c44 (notebook number QX22154s) before (C) and after (D) exposure to reducing conditions for 1 h. Peptide solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S6. ESI-TOF MS data for azide-containing WW variant 14/30-c44 (notebook number QX22154) before (A,C) and after (E,G) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h. Expected [M+4H]$^+$/4 = 1089.80 Da for 14/30-c44 prior to reduction; expected [M+4H]$^+$/4 = 1083.30 Da after reduction of the azide at position 30 to the corresponding amine. Also shown are ESI-TOF MS data for triazole-stapled s14/30-c44 (notebook number QX22154s) before (B,D) and after (F,H) exposure to reducing conditions for 1 h. Expected [M+4H]$^+$/4 = 1089.80 Da for triazole-stapled s14/30-c44 both before and after reduction.
Figure S7. Analytical HPLC retention time for disulfide-bound GCN4 heterodimer d27/29'-c40 (notebook number QX22031) before (A) and after (B) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h; and for its triazole-stapled counterpart sd27/29'-c40 (notebook number QX2204) before (C) and after (D) exposure to reducing conditions for 1 h. Peptide solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H2O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S8. ESI-TOF MS data for disulfide-bound GCN4 heterodimer d27/29'-c40 (notebook number QX22031) before (A,C) and after (E,G,H) exposure to reducing conditions (aqueous DTT and DIEA) for 1 h. Expected [M+6H]+/6 = 1410.59 Da for d27/29'-c40 prior to reduction. Following reduction, d27/29'-c40 splits into its component peptides: alkyne-containing 27-c4 (notebook number QX21971; expected [M+4H]+/4 = 1031.62 Da) and 29'-c0 (notebook number QX21973) in which the azide at position 29' has been reduced to an amine (expected [M+4H]+/4 = 1078.78 Da). Also shown are ESI-TOF MS data for triazole-stapled GCN4 variant sd27/29'-c40 before (B,D) and after (F) exposure to reducing conditions. Expected [M+6H]+/6 = 1410.59 Da for sd27/29'-c40 prior to reduction. After reduction, the triazole staple should be intact, but the disulfide bond should not; expected [M+6H]+/6 = 1410.92 Da.
3. Global Fitting of Variable Temperature CD Data

Following purification and characterization (as described above), the conformational stability of stapled and unstapled WW, SH3, and GCN4 variants was assessed by variable-temperature circular dichroism spectropolarimetry. Data from three replicate variable temperature CD experiments were each fit globally to a model for a two-state thermally induced unfolding transition using the program Mathematica (Wolfram Research).

We used a model based on two-state monomer folding for WW variants 16/19-00, 16/19-o23, 16/32-c44, s16/32-c44, 14/30-00, 14/30-o55, s14/30-o55, 14/30-c44 and s14/30-c44; SH3 variant s20/37-o44; and GCN4 variants d27/29'-c40, sd27/29'-c40, and s27/29'-c40. This model is described in equation S1:

\[
[\theta] = \frac{[K(a_n+b_nT)+(c_n+d_nT)]}{1+K},
\]  

where \( T \) is temperature in Kelvin, \( a_n \) is the \( y \)-intercept and \( b_n \) is the slope of the pre-transition baseline for melt \( n \) (\( a_1 \) and \( b_1 \) for replicate 1, \( a_2 \) and \( b_2 \) for replicate 2, \( a_3 \) and \( b_3 \) for replicate 3, etc.); \( c_n \) is the \( y \)-intercept and \( d_n \) is the slope of the post-transition baseline for replicate \( n \) (\( c_1 \) and \( d_1 \) for replicate 1, \( c_2 \) and \( d_2 \) for replicate 2, \( c_3 \) and \( d_3 \) for replicate 3, etc.); and \( K \) is the temperature-dependent folding equilibrium constant. \( K \) is related to the temperature-dependent free energy of folding \( \Delta G \) according to the following equation:

\[
K = \exp\left[\frac{-\Delta G}{RT}\right],
\]  

where \( R \) is the universal gas constant (0.0019872 kcal/mol/K). \( \Delta G \) is a function of temperature, as shown in the following equation:

\[
\Delta G = \frac{\Delta H_0(T_m-T)}{T_m} + \Delta C_p \cdot (T - T_m - T \cdot \ln\left[\frac{T}{T_m}\right]),
\]  

where \( T_m \) is the midpoint of the unfolding transition and the temperature at which \( \Delta G_f = 0 \); \( \Delta H_0 \) is the change in enthalpy upon folding at \( T = T_m \); and \( \Delta C_p \) is the change in heat capacity upon folding.

In contrast, we used a model based on two-state dimer folding for noncovalent GCN4 heterodimer 27/29'-c40, as described in equation S4:

\[
[\theta] = F \cdot (a_n + b_n \cdot T) + (1 - F) \cdot (c_n + d_n \cdot T),
\]  

where \( T, a_n, b_n, c_n, \) are defined as described above, and \( F \) is the temperature-dependent fraction of the total peptide concentration that is folded. \( F \) is related to the temperature-dependent equilibrium constant \( K \) and the total peptide concentration \( P \) as shown in equation S5:

\[
F = 1 + \frac{1}{4KP} - \sqrt{\frac{1}{16K^2P^2} + \frac{1}{2KP}}.
\]  

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K is related to the folding free energy $\Delta G$ as described above in equation S2. Because equation S3 is only appropriate for monomer folding equilibria, we used the following polynomial expression to describe the temperature-dependence of $\Delta G$ for noncovalent GCN4 heterodimer $27/29'\cdot c40$:

$$\Delta G = \Delta G_0 + \Delta G_1 \cdot (T - T_0) + \Delta G_2 \cdot (T - T_0)^2,$$

where $\Delta G_0$, $\Delta G_1$, and $\Delta G_2$ are parameters of the fit and $T_0$ is an arbitrary reference temperature, usually chosen as $T_m$, the temperature at which $F = 0.5$ (determined numerically for monomer-dimer equilibria).

In some cases, we found that some fit parameters had sufficiently high standard errors as to render them indistinguishable from zero and therefore not essential to the fit (as judged by their p-values). When this occurred, we repeated the fitting process without the non-essential parameters. We used the fit parameters for each variant to calculate the $\Delta G$ values given in the main text; we calculated the uncertainty for each $\Delta G$ value by propagation of error using the standard errors of the fit parameters.

CD spectra and variable temperature CD data for WW variants $16/19\cdot 00$, $16/19\cdot 023$, $s16/19\cdot 023$, $16/32\cdot c44$, $s16/32\cdot c44$, $14/30\cdot 00$, $14/30\cdot 055$, $s14/30\cdot 055$, $14/30\cdot c44$ and $s14/30\cdot c44$; SH3 variant $s20/37\cdot o44$; and GCN4 variants $d27/29'\cdot c40$, $sd27/29'\cdot c40$, $27/29'\cdot c40$, and $s27/29'\cdot c40$ are shown in Figures S9–S27, along with the fit parameters $\pm$ standard error. Standard parameter errors were used to estimate the uncertainty in the thermodynamic values given in the main text by propagation of error.
Figure S9. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 16/19-00 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔG, ΔH, and -TΔS at 332.0 K (the melting temperature of 16/19-00), with the indicated standard errors.

R^2 = 0.99989
sum residuals^2 = 0.26348

For 16/19-00, at 332.0 K,
ΔG = 0.00 ± 0.02 kcal/mol
ΔH = -31.0 ± 0.4 kcal/mol
-TΔS = 31.0 ± 0.4 kcal/mol

Figure S10. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 16/19-o23 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔG, ΔH, and -TΔS at 332.0 K (the melting temperature of 16/19-00), with the indicated standard errors.

R^2 = 0.99990
sum residuals^2 = 0.18113

For 16/19-o23, at 332.0 K,
ΔG = -0.75 ± 0.01 kcal/mol
ΔH = -26.2 ± 0.4 kcal/mol
-TΔS = 25.5 ± 0.4 kcal/mol
Figure S11. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant *s16/19-o23* in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGi, ΔHi, and -TΔSi at 332.0 K (the melting temperature of 16/19-00), with the indicated standard errors.

| Parameters | Values (kcal/mol) | Units | P-Values |
|------------|-------------------|-------|----------|
| ΔHf        | -32.06 ± 0.36     | kcal/mol | <0.001 |
| Tm         | 71.7 ± 0.1        | °C    | ---      |
| ΔCp        | -0.613 ± 0.015   | kcal/mol/K | <0.001 |
| a1         | 15.8 ± 0.2        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b1         | -0.033 ± 0.001    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c1         | -1.47 ± 0.02      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d1         | ---               | ---   | ---      |
| a2         | 16.4 ± 0.2        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b2         | -0.033 ± 0.001    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c2         | -0.97 ± 0.02      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d2         | ---               | ---   | ---      |
| a3         | 16.1 ± 0.2        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b3         | -0.034 ± 0.001    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c3         | -1.45 ± 0.02      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d3         | ---               | ---   | ---      |

R² = 0.99994
sum residuals² = 0.18768

For *s16/19-o23*, at 332.0 K,
ΔG = -1.04 ± 0.02 kcal/mol
ΔH = -24.2 ± 0.4 kcal/mol
-TΔS = 23.2 ± 0.4 kcal/mol

Figure S12. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant *16/32-00* in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGi, ΔHi, and -TΔSi at 322.3 K (the melting temperature of 16/32-00), with the indicated standard errors.

| Parameters | Values (kcal/mol) | Units | P-Values |
|------------|-------------------|-------|----------|
| ΔHf        | -22.68 ± 0.76     | kcal/mol | <0.001 |
| Tm         | 49.2 ± 0.6        | °C    | <0.001  |
| ΔCp        | 0.369 ± 0.107     | kcal/mol/K | <0.001 |
| a1         | 15.1 ± 0.6        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b1         | -0.035 ± 0.002    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c1         | -1.80 ± 0.13      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d1         | ---               | ---   | ---      |
| a2         | 16.1 ± 0.6        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b2         | -0.036 ± 0.002    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c2         | -1.53 ± 0.13      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d2         | ---               | ---   | ---      |
| a3         | 15.9 ± 0.6        | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b3         | -0.037 ± 0.002    | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c3         | -1.61 ± 0.13      | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d3         | ---               | ---   | ---      |

R² = 0.99982
sum residuals² = 0.26677

For *16/32-00*, at 322.3 K,
ΔG = 0.00 ± 0.04 kcal/mol
ΔH = -22.7 ± 0.8 kcal/mol
-TΔS = 22.7 ± 0.8 kcal/mol
Figure S13. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 16/32-o44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGf, ΔHf, and -TΔSf at 322.3 K (the melting temperature of 16/32-00), with the indicated standard errors.

Figure S14. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant s16/32-o44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGf, ΔHf, and -TΔSf at 322.3 K (the melting temperature of 16/32-00), with the indicated standard errors.
**Figure S15.** (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 16/32-c44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGᵢ, ΔHᵢ, and -TΔSᵢ at 322.3 K (the melting temperature of 16/32-00), with the indicated standard errors.

| Parameters | Values       | Units          | P-Values |
|------------|--------------|----------------|----------|
| ΔHᵢ        | -30.33 ± 0.59 kcal/mol |                | <0.001   |
| Tₘ         | 54.2 ± 0.2 °C |                | <0.001   |
| ΔCᵥ        | -0.678 ± 0.049 kcal/mol/K |            | <0.001   |
| a₁         | 14.9 ± 0.6 deg cm⁻² dmol⁻¹ x 10⁻³ |     | <0.001   |
| b₁         | -0.032 ± 0.002 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ | | <0.001   |
| c₁         | 4.45 ± 0.66 deg cm⁻² dmol⁻¹ x 10⁻³ |        | <0.001   |
| d₁         | -0.02 ± 0.00 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| a₂         | 15.8 ± 0.5 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| b₂         | -0.034 ± 0.002 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |
| c₂         | 4.97 ± 0.64 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d₂         | -0.016 ± 0.002 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |
| a₃         | 14.9 ± 0.5 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| b₃         | -0.032 ± 0.002 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |
| c₃         | 4.30 ± 0.64 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d₃         | -0.014 ± 0.002 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |

R² = 0.99988

sum residuals² = 0.23480

For 16/32-c44, at 322.3 K,
ΔG = -0.44 ± 0.02 kcal/mol
ΔH = -26.9 ± 0.6 kcal/mol
-TΔS = 26.4 ± 0.6 kcal/mol

**Figure S16.** (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant s16/32-c44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGᵢ, ΔHᵢ, and -TΔSᵢ at 322.3 K (the melting temperature of 16/32-00), with the indicated standard errors.

| Parameters | Values       | Units          | P-Values |
|------------|--------------|----------------|----------|
| ΔHᵢ        | -33.79 ± 0.41 kcal/mol |                | <0.001   |
| Tₘ         | 71.4 ± 0.1 °C |                | 0.000    |
| ΔCᵥ        | -0.668 ± 0.014 kcal/mol/K |            | <0.001   |
| a₁         | 15.6 ± 0.3 deg cm⁻² dmol⁻¹ x 10⁻³ |     | <0.001   |
| b₁         | -0.030 ± 0.001 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ | | <0.001   |
| c₁         | -1.14 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |        | <0.001   |
| d₁         | ---          |                | ---      |
| a₂         | 16.7 ± 0.3 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| b₂         | -0.031 ± 0.001 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |
| c₂         | -0.65 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d₂         | ---          |                | ---      |
| a₃         | 16.5 ± 0.3 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| b₃         | -0.030 ± 0.001 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ |     | <0.001   |
| c₃         | -0.36 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d₃         | ---          |                | ---      |

R² = 0.99994

sum residuals² = 0.26311

For s16/32-c44, at 322.3 K,
ΔG = -1.68 ± 0.03 kcal/mol
ΔH = -18.5 ± 0.5 kcal/mol
-TΔS = 16.8 ± 0.5 kcal/mol
Figure S17. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 14/30-00 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔG, ΔH, and -TΔS, at 301.7 K (the melting temperature of 14/30-00), with the indicated standard errors.

| Parameters | Values | Units | P–Values |
|------------|--------|-------|----------|
| ΔH₀        | -20.58 ± 0.39 kcal/mol |       | <0.001   |
| Tₘ         | 28.6 ± 0.2 °C |       | <0.001   |
| ΔC_p       | --- | --- | --- |
| a₁         | 3.0 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| b₁         | --- | --- | --- |
| c₁         | 1.86 ± 0.29 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| d₁         | -0.01 ± 0.00 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| a₂         | 3.1 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| b₂         | --- | --- | --- |
| c₂         | 2.09 ± 0.29 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| d₂         | -0.008 ± 0.001 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| a₃         | 3.3 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| b₃         | --- | --- | --- |
| c₃         | 2.18 ± 0.29 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| d₃         | -0.006 ± 0.001 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |

R² = 0.99945  
sum residuals² = 0.17339

For 14/30-00, at 301.7 K,  
ΔG = 0.00 ± 0.01 kcal/mol  
ΔH = -20.6 ± 0.4 kcal/mol  
-TΔS = 20.6 ± 0.4 kcal/mol

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Figure S18. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 14/30-o55 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔG, ΔH, and -TΔS, at 301.7 K (the melting temperature of 14/30-00), with the indicated standard errors.

| Parameters | Values | Units | P–Values |
|------------|--------|-------|----------|
| ΔH₀        | -22.48 ± 0.31 kcal/mol |       | <0.001   |
| Tₘ         | 33.2 ± 0.1 °C |       | <0.001   |
| ΔC_p       | --- | --- | --- |
| a₁         | 5.5 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | --- | <0.001 |
| b₁         | --- | --- | --- |
| c₁         | 1.35 ± 0.40 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| d₁         | -0.01 ± 0.00 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| a₂         | 5.5 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| b₂         | --- | warning! | |
| c₂         | 0.50 ± 0.40 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| d₂         | -0.005 ± 0.001 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ | warning! | |
| a₃         | 5.5 ± 0.0 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| b₃         | --- | warning! | |
| c₃         | 0.94 ± 0.39 deg cm⁻² dmol⁻¹ x 10⁻³ | warning! | |
| d₃         | -0.006 ± 0.001 deg cm⁻² dmol⁻¹ K⁻¹ x 10⁻³ | warning! | |

R² = 0.99959  
sum residuals² = 0.47214

For 14/30-o55, at 301.7 K,  
ΔG = -0.34 ± 0.01 kcal/mol  
ΔH = -22.5 ± 0.3 kcal/mol  
-TΔS = 22.1 ± 0.3 kcal/mol
Figure S19. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant s14/30-o55 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGf, ΔHf, and -TΔSf at 301.7 K (the melting temperature of 14/30-00), with the indicated standard errors.

| Parameters | Values  | Units                | P-Values |
|------------|---------|----------------------|----------|
| ΔHf        | -23.67 ± 0.80 | kcal/mol             | <0.001   |
| Tm         | 39.5 ± 0.6  | °C                   | <0.001   |
| ΔCp        | ----      | ----                 | ----     |
| a1         | 10.8 ± 1.3 | deg cm² dmol⁻¹ x 10⁻³| <0.001   |
| b1         | -0.025 ± 0.005 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |
| c1         | 3.38 ± 0.69 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d1         | -0.01 ± 0.00 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| a2         | 20.6 ± 1.4  | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| b2         | -0.054 ± 0.005 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |
| c2         | -1.21 ± 0.03 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d2         | ----      | ----                 | ----     |
| a3         | 11.1 ± 1.3  | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| b3         | -0.026 ± 0.004 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |
| c3         | 3.58 ± 0.68 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d3         | -0.014 ± 0.002 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |

R² = 0.99869
sum residuals² = 1.08171

For s14/30-o55, at 301.7 K,
ΔG = -0.83 ± 0.05 kcal/mol
ΔH = -23.7 ± 0.8 kcal/mol
-TΔS = 22.8 ± 0.8 kcal/mol

Figure S20. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant 14/30-c44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGf, ΔHf, and -TΔSf at 301.7 K (the melting temperature of 14/30-00), with the indicated standard errors.

| Parameters | Values  | Units                | P-Values |
|------------|---------|----------------------|----------|
| ΔHf        | -19.99 ± 0.33 | kcal/mol             | <0.001   |
| Tm         | 30.5 ± 0.2  | °C                   | <0.001   |
| ΔCp        | ----      | ----                 | ----     |
| a1         | 4.7 ± 0.0  | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| b1         | ----      | ----                 | ----     |
| c1         | 2.32 ± 0.38 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d1         | -0.01 ± 0.00 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| a2         | 4.5 ± 0.0  | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| b2         | ----      | ----                 | ----     |
| c2         | 2.73 ± 0.37 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d2         | -0.012 ± 0.001 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |
| a3         | 4.5 ± 0.0  | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| b3         | ----      | ----                 | ----     |
| c3         | 2.42 ± 0.37 | deg cm² dmol⁻¹ x 10⁻³ | <0.001   |
| d3         | -0.011 ± 0.001 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001   |

R² = 0.99948
sum residuals² = 0.38222

For 14/30-c44, at 301.7 K,
ΔG = -0.13 ± 0.01 kcal/mol
ΔH = -20.0 ± 0.3 kcal/mol
-TΔS = 19.9 ± 0.3 kcal/mol
Figure S21. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM WW variant s14/30-c44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGi, ΔHi, and -TΔSi at 301.7 K (the melting temperature of 14/30-00), with the indicated standard errors.

| Parameters | Values       | Units           | P-Values |
|------------|--------------|-----------------|----------|
| ΔHf        | -18.09 ± 0.21 kcal/mol |                 | <0.001   |
| Tm         | 41.3 ± 1.0   °C       |                 | <0.001   |
| ΔCp        | ---          | ---             | ---      |
| a1         | 4.8 ± 0.0    deg cm⁻² dmol⁻¹ x 10⁻³ |             | <0.001   |
| b1         | ---          | ---             | ---      |
| c1         | -1.27 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| d1         | ---          | ---             | ---      |
| a2         | 4.7 ± 0.0    deg cm⁻² dmol⁻¹ x 10⁻³ |             | <0.001   |
| b2         | ---          | ---             | ---      |
| c2         | -1.41 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| d2         | ---          | ---             | ---      |
| a3         | 4.8 ± 0.0    deg cm⁻² dmol⁻¹ x 10⁻³ |             | <0.001   |
| b3         | ---          | ---             | ---      |
| c3         | -1.28 ± 0.02 deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| d3         | ---          | ---             | ---      |

R² = 0.99937

sum residuals² = 0.62679

For s14/30–c44, at 301.7 K,
ΔG = -0.73 ± 0.01 kcal/mol
ΔH = -18.1 ± 0.2 kcal/mol
-ΔTS = 17.4 ± 0.2 kcal/mol

Figure S22. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM SH3 variant 20/37-00 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for ΔGi, ΔHi, and -TΔSi at 334.2 K (the melting temperature of 20/37-00), with the indicated standard errors.

| Parameters | Values       | Units           | P-Values |
|------------|--------------|-----------------|----------|
| ΔHf        | -31.66 ± 1.03 kcal/mol |            | <0.001   |
| Tm         | 61.1 ± 0.3   °C       |                 | <0.001   |
| ΔCp        | ---          | ---             | ---      |
| a1         | -2.0 ± 0.0   deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| b1         | ---          | ---             | ---      |
| c1         | -2.96 ± 0.01 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d1         | ---          | ---             | ---      |
| a2         | -2.1 ± 0.0   deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| b2         | ---          | ---             | ---      |
| c2         | -3.04 ± 0.01 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d2         | ---          | ---             | ---      |
| a3         | -2.0 ± 0.0   deg cm⁻² dmol⁻¹ x 10⁻³ |         | <0.001   |
| b3         | ---          | ---             | ---      |
| c3         | -2.98 ± 0.01 deg cm⁻² dmol⁻¹ x 10⁻³ |       | <0.001   |
| d3         | ---          | ---             | ---      |

R² = 0.99982

sum residuals² = 0.14525

For SH3 20/37–00, at 334.2 K,
ΔG = 0.00 ± 0.02 kcal/mol
ΔH = -31.7 ± 1.0 kcal/mol
-ΔTS = 31.7 ± 1.0 kcal/mol
**Figure S23.** (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 50 μM SH3 variant s20/37-o44 in 20 mM sodium phosphate (pH 7). Fit parameters from equations S1–S3 appear in the table, as do calculated values for $\Delta G_f$, $\Delta H_f$, and $-T\Delta S_f$ at 334.2 K (the melting temperature of 20/37-00), with the indicated standard errors.

**Figure S24.** (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 15 μM disulfide bound GCN4 heterodimer d27/29-c40 in 20 mM sodium phosphate (pH 7) with 4 M GdnHCl. Fit parameters from equations S1–S3 appear in the table, as do calculated values for $\Delta G_f$, $\Delta H_f$, and $-T\Delta S_f$ at 334.2 K (the melting temperature of d27/29-c40), with the indicated standard errors.
Figure S25. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 15 μM disulfide bound triazole-stapled GCN4 variant s\textsubscript{d27/29'}-c\textsubscript{40} in 20 mM sodium phosphate (pH 7) with 4 M GdnHCl. Fit parameters from equations S1–S3 appear in the table, as do calculated values for Δ\text{G}_f, Δ\text{H}_f, and -TΔS\text{f} at 334.2 K (the melting temperature of d\textsubscript{27/29'}-c\textsubscript{40}), with the indicated standard errors.

| Parameters | Values         | Units                | P-Values |
|------------|----------------|----------------------|----------|
| Δ\text{G}_f | -29.84 ± 0.36  | kcal/mol             | <0.001   |
| T\text{m}  | 48.2 ± 0.1     | °C                   | <0.001   |
| Δ\text{C}_p | -----         | -----                | -----    |
| a1         | -100.2 ± 2.0   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b1         | 0.218 ± 0.007  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c1         | -3.90 ± 0.07   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d1         | -----         | -----                | -----    |
| a2         | -104.5 ± 1.9   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b2         | 0.233 ± 0.007  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c2         | -3.62 ± 0.07   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d2         | -----         | -----                | -----    |
| a3         | -102.8 ± 1.9   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b3         | 0.230 ± 0.007  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c3         | -3.67 ± 0.07   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d3         | -----         | -----                | -----    |

\[R^2 = 0.99989\]

sum residuals\textsuperscript{2} = 8.86391

For s\textsubscript{d27/29'}-c\textsubscript{40}, at 314.3 K,

\[Δ\text{G} = -0.65 ± 0.01\text{ kcal/mol}\]

\[Δ\text{H} = -29.8 ± 0.4\text{ kcal/mol}\]

\[-TΔS = 29.2 ± 0.4\text{ kcal/mol}\]

Figure S26. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 15 μM non-covalent GCN4 heterodimer 27/29'-c40 in 20 mM sodium phosphate (pH 7) with 0.5 M GdnHCl. Fit parameters from equations S4–S6 appear in the table, as does the calculated value for Δ\text{G}_f at 308.0 K (the melting temperature of 27/29'-c40), with the indicated standard errors.

| Parameters | Values         | Units                | P-Values |
|------------|----------------|----------------------|----------|
| Δ\text{G}_f | -12.56 ± 0.10  | kcal/mol             | <0.001   |
| Δ\text{G}_1 | 0.077 ± 0.002  | kcal/mol/K           | <0.001   |
| Δ\text{G}_2 | -----         | -----                | -----    |
| a1         | -42.5 ± 2.1    | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b1         | 0.086 ± 0.008  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c1         | -2.90 ± 0.02   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d1         | -----         | -----                | -----    |
| a2         | -47.8 ± 2.1    | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b2         | 0.103 ± 0.008  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c2         | -2.97 ± 0.02   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d2         | -----         | -----                | -----    |
| a3         | -47.2 ± 2.0    | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| b3         | 0.102 ± 0.007  | deg cm\textsuperscript{-2} mol\textsuperscript{-1} K\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| c3         | -3.03 ± 0.02   | deg cm\textsuperscript{-2} mol\textsuperscript{-1} \times 10\textsuperscript{-3} | <0.001   |
| d3         | -----         | -----                | -----    |

\[R^2 = 0.99991\]

sum residuals\textsuperscript{2} = 1.26647

For 27/29'-c40, at 308.0 K,

\[Δ\text{G} = -9.62 ± 0.12\text{ kcal/mol}\]
Figure S27. (A) CD spectra and (B–D) variable temperature CD data (triplicate) for 15 μM triazole-stapled GCN4 s27/29'-c04 in 20 mM sodium phosphate (pH 7) with 0.5 M GdnHCl. Fit parameters from equations S1–S3 appear in the table, as does the calculated value for ∆G, ∆H, and -T∆S at 355.2 K (the melting temperature of s27/29'-c04), with the indicated standard errors.

### Parameters

| Parameter | Value | Units | P-Values |
|-----------|-------|-------|----------|
| ∆H0 | -36.09 ± 0.62 | kcal/mol | <0.001 |
| Tm | 82.0 ± 0.2 | °C | <0.001 |
| ∆Cp | ----- | ----- | ----- |
| a1 | -55.2 ± 0.3 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b1 | 0.113 ± 0.001 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c1 | -2.31 ± 0.14 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d1 | ----- | ----- | ----- |
| a2 | -56.9 ± 0.3 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b2 | 0.115 ± 0.001 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c2 | -3.01 ± 0.14 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d2 | ----- | ----- | ----- |
| a3 | -55.1 ± 0.3 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| b3 | 0.113 ± 0.001 | deg cm² dmol⁻¹ K⁻¹ x 10⁻³ | <0.001 |
| c3 | -2.36 ± 0.14 | deg cm² dmol⁻¹ x 10⁻³ | <0.001 |
| d3 | ----- | ----- | ----- |

| R² | 0.99996 |
| Sum residuals² | 1.73168 |

For s27/29'-c40, at 355.2 K,

| Parameter | Value | Units |
|-----------|-------|-------|
| ∆G | 0.00 ± 0.02 | kcal/mol |
| ∆H | -36.1 ± 0.6 | kcal/mol |
| -T∆S | 36.1 ± 0.6 | kcal/mol |

### 4. Proteolysis of WW variants

50 μM protein solutions in 20 mM sodium phosphate buffer (pH 7) were incubated at ambient temperature with 17 μg/mL proteinase K respectively for up to 1 hour. At each of the several time points, the proteolysis reaction was quenched by adding 100 μL of aqueous trifluoroacetic acid (1% v/v) to 50 μL of the reaction mixture. The quenched mixture was then analyzed in triplicate by reverse phase HPLC analytical column, monitored by a UV-Vis detector at 220 nm. The degradation of the proteins was assessed using the integrated HPLC peak area to account for how much of the full-length protein remained at each time point. The protein half-lives were calculated by fitting the integrated peak areas as a function of time to a mono exponential decay equation:

\[
\text{Area}(t) = A \cdot \exp \left[ -kt \right],
\]

where t is time in minutes, A is a constant corresponding to relative integrated peak area at t = 0, and τ is the decay time, which is related to the protein half-life \( t_{1/2} \) \( (t_{1/2} = \tau \ln 2) \). Decay traces for proteins WW variants, 16/19-00, 16/19-o23, s16/19-o23, 16/32-00, 16/32-o44, s16/32-o44, are shown in Figures S28–S33.
**Figure S28.** Proteolysis of 16/19-00 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for 16/19-00 are shown as blue circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

**Figure S29.** Proteolysis of 16/19-o23 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for 16/19-o23 are shown as orange circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

**Figure S30.** Proteolysis of 16/19-o23 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for 16/19-o23 are shown as pink circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

| 16/19-00 Parameters | Values | P-Values |
|---------------------|--------|----------|
| A                   | 1.01 ± 0.0 | <0.001 |
| k                   | 0.54 ± 0.01 | <0.001 |
| R²                  | 0.9997 |          |

| 16/19-o23 Parameters | Values | P-Values |
|---------------------|--------|----------|
| A                   | 0.93 ± 0.1 | <0.001 |
| k                   | 0.12 ± 0.02 | 0.016 |
| R²                  | 0.9888 |          |

| s16/19-o23 Parameters | Values | P-Values |
|----------------------|--------|----------|
| A                    | 0.93 ± 0.1 | <0.001 |
| k                    | 0.08 ± 0.02 | 0.013 |
| R²                   | 0.9922 |          |
Figure S31. Proteolysis of 16/32-00 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for 16/32-00 are shown as blue circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

![Graph](attachment:figure_s31.png)

| 16/32-00 Parameters | Values  | P-Values |
|---------------------|---------|----------|
| A                   | 1.01 ± 0.1 | 0.004    |
| k                   | 1.71 ± 0.23 | 0.018    |
| R²                  | 0.9938   |          |

Figure S32. Proteolysis of 16/32-044 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for 16/32-044 are shown as orange circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

![Graph](attachment:figure_s32.png)

| 16/32-044 Parameters | Values  | P-Values |
|----------------------|---------|----------|
| A                    | 1.01 ± 0.0 | 0.002    |
| k                    | 0.72 ± 0.07 | 0.008    |
| R²                   | 0.9975   |          |

Figure S33. Proteolysis of s16/32-044 (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (17 μg/mL) as monitored by HPLC. Data points for s16/32-044 are shown as orange circles, and each represents the average of three replicate experiments. Solid lines represent fits of the data to a mono exponential decay function, which was used to calculate the indicated half-lives.

![Graph](attachment:figure_s33.png)

| s16/32-044 Parameters | Values  | P-Values |
|-----------------------|---------|----------|
| A                     | 0.77 ± 0.1 | 0.002    |
| k                     | 0.12 ± 0.06 | 0.097    |
| R²                    | 0.9418   |          |
5. ESI-TOF MS data for WW, SH3, and GCN4 variants

Table S1. Summary of the mass spectrum data for the new WW, SH3, and GCN4 variants described here.

| Name | Notebook Number | Molecular Formula | $z$ | Expected $[M+z\cdot H]/z$ | Observed $[M+z\cdot H]/z$ |
|------|----------------|------------------|-----|--------------------------|-------------------------|
| 14/30-00 | DA10434 | C$_{175}$H$_{321}$N$_{52}$O$_{31}$S | 4 | 985.989 | 985.996 |
| 14/30-55 | QX2183 | C$_{201}$H$_{310}$N$_{52}$O$_{61}$S | 4 | 1116.070 | 1116.071 |
| s14/30-55 | QX2183s | C$_{196}$H$_{306}$N$_{52}$O$_{61}$S | 4 | 1109.062 | 1109.058 |
| 14/30-c44 | QX22154 | C$_{194}$H$_{294}$N$_{55}$O$_{57}$S | 4 | 1089.797 | 1089.790 |
| s14/30-c44 | QX22154s | C$_{194}$H$_{294}$N$_{55}$O$_{57}$S | 4 | 1089.797 | 1089.790 |
| 16/32-c44 | QX21852 | C$_{198}$H$_{303}$N$_{59}$O$_{55}$S | 4 | 1113.817 | 1113.806 |
| s16/32-c44 | QX2188 | C$_{198}$H$_{303}$N$_{59}$O$_{55}$S | 4 | 1113.817 | 1113.810 |
| 20/37-44 | KT1027 | C$_{216}$H$_{471}$N$_{77}$O$_{88}$ | 4 | 1711.114 | 1711.092 |
| s20/37-44 | QX2217 | C$_{306}$H$_{467}$N$_{77}$O$_{88}$ | 4 | 1704.106 | 1704.108 |
| 27-c4 | QX21971 | C$_{187}$H$_{324}$N$_{80}$O$_{46}$S | 4 | 1031.620 | 1031.618 |
| 29'-c0 | QX21973 | C$_{184}$H$_{320}$N$_{80}$O$_{46}$S | 4 | 1085.277 | 1085.271 |
| d27/29'-c40 | QX22031 | C$_{277}$H$_{611}$N$_{105}$O$_{114}$S$_2$ | 6 | 1410.592 | 1410.753 |
| sd27/29'-c40 | QX2204 | C$_{277}$H$_{611}$N$_{105}$O$_{114}$S$_2$ | 6 | 1410.592 | 1410.736 |
| 27A-c4 | QX22091 | C$_{183}$H$_{324}$N$_{56}$O$_{46}$ | 4 | 1023.627 | 1023.620 |
| 29A'-c0 | QX22092 | C$_{184}$H$_{293}$N$_{51}$O$_{68}$ | 4 | 1077.284 | 1077.276 |
| s27/29'-c40 | QX2214 | C$_{371}$H$_{613}$N$_{105}$O$_{114}$S$_2$ | 6 | 1400.271 | 1400.583 |

ESI-TOF MS data for WW variants 14/30-00, 14/30-55, s14/30-55, 14/30-c44, s14/30-c44, 16/32-c44 and s16/32-c44; SH3 variants 20/37-o44 and s20/37-o44; and GCN4 variants 27-c4, 29'-c0, d27/29'-c40, sd27/29'-c40, 27A-c4, 29A'-c0, and s27/29'-40 are shown in Figures S34–S49.

**Figure S34.** ESI-TOF spectrum for WW variant 14/30-00. Expected $[M+4H^+/4 = 985.989$ Da. Observed $[M+4H^+/4 = 985.996$ Da.
Figure S35. ESI-TOF spectrum for WW variant 14/30-o55. Expected \([\text{M}+4\text{H}^+]^4 = 1116.070 \text{ Da}\). Observed \([\text{M}+4\text{H}^+]^4 = 1116.071 \text{ Da}\).

Figure S36. ESI-TOF spectrum for WW variant s14/30-o55. Expected \([\text{M}+4\text{H}^+]^4 = 1109.062 \text{ Da}\). Obs. \([\text{M}+4\text{H}^+]^4 = 1109.058 \text{ Da}\).

Figure S37. ESI-TOF spectrum for WW variant 14/30-c44. Expected \([\text{M}+4\text{H}^+]^4 = 1089.797 \text{ Da}\). Obs. \([\text{M}+4\text{H}^+]^4 = 1089.790 \text{ Da}\).
Figure S38. ESI-TOF spectrum for WW variant s14/30-c44. Expected [M+4H\(^+\)]/4 = 1089.797 Da. Observed [M+4H\(^+\)]/4 = 1089.790 Da.

Figure S39. ESI-TOF spectrum for WW variant 16/32-c44. Expected [M+4H\(^+\)]/4 = 1113.817 Da. Observed [M+4H\(^+\)]/4 = 1113.806 Da.

Figure S40. ESI-TOF spectrum for WW variant s16/32-c44. Expected [M+4H\(^+\)]/4 = 1113.817 Da. Observed [M+4H\(^+\)]/4 = 1113.809 Da.
Figure S41. ESI-TOF spectrum for SH3 variant 20/37-o44. Expected \([M+4H^+] / 4 = 1711.114 \text{ Da}\). Observed \([M+4H^+] / 4 = 1711.092 \text{ Da}\).

Figure S42. ESI-TOF spectrum for SH3 variant s20/37-o44. Expected \([M+4H^+] / 4 = 1704.106 \text{ Da}\). Observed \([M+4H^+] / 4 = 1704.108 \text{ Da}\).

Figure S43. ESI-TOF spectrum for GCN4 monomer 27-c4. Expected \([M+4H^+] / 4 = 1031.620 \text{ Da}\). Observed \([M+4H^+] / 4 = 1031.618 \text{ Da}\).
Figure S44. ESI-TOF spectrum for GCN4 monomer 29'-c0. Expected [M+4H⁺]/4 = 1085.277 Da. Obs. [M+4H⁺]/4 = 1085.271 Da.

Figure S45. ESI-TOF spectrum for disulfide-bound GCN4 heterodimer d27/29'-c40. Expected [M+6H⁺]/6 = 1410.592 Da. Observed [M+6H⁺]/6 = 1410.753 Da.

Figure S46. ESI-TOF spectrum for triazole-stapled disulfide-bound GCN4 heterodimer sd27/29'-c40. Expected [M+6H⁺]/6 = 1410.592 Da. Observed [M+6H⁺]/6 = 1410.736 Da.
Figure S47. ESI-TOF spectrum for GCN4 monomer 27A-c4. Expected [M+4H\(^+\)]/4 = 1023.627 Da. Obs. [M+4H\(^+\)]/4 = 1023.620 Da.

Figure S48. ESI-TOF spectrum for GCN4 monomer 29A-c0. Expected [M+4H\(^+\)]/4 = 1077.284 Da. Obs. [M+4H\(^+\)]/4 = 1077.276 Da.

Figure S49. ESI-TOF spectrum for triazole-stapled GCN4 heterodimer s27/29*-c40. Expected [M+6H\(^+\)]/6 = 1400.271 Da. Observed [M+6H\(^+\)]/6 = 1400.583 Da.
6. Analytical HPLC data

Analytical HPLC data WW variants 14/30-00, 14/30-o55, s14/30-o55, 14/30-c44, s14/30-c44, 16/32-c44 and s16/32-c44; SH3 variants 20/37-o44 and s20/37-o44; and GCN4 variants 27-c4, 29'-c0, d27/29'-c40, sd27/29'-c40, 27A-c4, 29A'-c0, and s27/29'-40 are shown in Figures S50–S65.

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**Figure S50.** Analytical HPLC Data for WW variant 14/30-00 (notebook number DA10434). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H2O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

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**Figure S51.** Analytical HPLC Data for WW variant 14/30-o55 (notebook number QX2183). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H2O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

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**Figure S52.** Analytical HPLC Data for WW variant s14/30-o55 (notebook number QX2183S). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H2O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
Figure S53. Analytical HPLC Data for WW variant 14/30-c44 (notebook number QX22154). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S54. Analytical HPLC Data for WW variant s14/30-c44 (notebook number QX22154s). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S55. Analytical HPLC Data for WW variant 16/32-c44 (notebook number QX21852). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
Figure S56. Analytical HPLC Data for WW variant s16/32-c44 (notebook number QX2188). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S57. Analytical HPLC Data for SH3 variant 20/37-o44 (notebook number KT1027). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S58. Analytical HPLC Data for SH3 variant s20/37-44 (notebook number QX2117). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
Figure S59. Analytical HPLC Data for GCN4 monomer 27-c4 (notebook number QX21971). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S60. Analytical HPLC Data for GCN4 monomer 29'-c0 (notebook number QX21972). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S61. Analytical HPLC Data for disulfide-bound GCN4 heterodimer d27/29'-c40 (notebook number QX22031). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H₂O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
Figure S62. Analytical HPLC Data for triazole-stapled disulfide-bound GCN4 variant sd27/29-c40 (notebook number QX2204). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S63. Analytical HPLC Data for GCN4 monomer 27A-c4 (notebook number QX22091). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.

Figure S64. Analytical HPLC Data for GCN4 monomer 29A’-c0 (notebook number QX22092). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H$_2$O, 0.1% TFA; B = MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
Figure S65. Analytical HPLC Data for triazole-stapled GCN4 heterodimer s27/29'-c40 (notebook number QX2214). Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A = H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10-minute rinse (95% B), and a 10-minute column re-equilibration.
7. Synthesis and characterization of PEGylated Asn derivatives

**((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) dimethanesulfonate (QX2157)**

Methanesulfonyl chloride (23 g, 200 mmol) was added dropwise to a stirred solution of tetra ethylene glycol (10 g, 50 mmol) and TEA (20 g, 200 mmol) in dichloromethane (250 mL) at 0 °C. After the addition was complete, the resulting mixture was stirred at r.t. for 15 h. Water was added to quench the reaction. The organic phase was separated, and the aqueous phase was extracted with dichloromethane (2 × 100 mL). The combined organic layers were washed with brine (3 × 100 mL), dried with anhydrous sodium sulfate, filtered, and the solvent was removed by rotary evaporation to afford 17.5 g of colorless oil, which was used in the next step without purification. Yield quantitative. MS(ESI-TOF) m/z calc. for C_{10}H_{23}O_{9}S_{2}^{+} 351.08, found 351.08 [M+H^{+}]; calc. for C_{10}H_{26}NO_{9}S_{2}^{+} 368.10, found 368.11 [M+NH_{4}^{+}].

1-Azido-2-(2-(2-azidoethoxy)ethoxy)ethoxyethane (QX2158)

To a solution of NaN_{3} (13 g, 200 mmol) in DMF (200 mL) was added QX2157 (17.5 g, 50 mmol) at room temperature. The reaction mixture was heated to 70 °C and stirred for 12 hours. The crude mixture was extracted with DCM for 3 times after the reaction was completed. The combined organic phases were washed with saturated brine and dried over anhydrous sodium sulfate, evaporated and purified by flash column (Hexane/EA 1:1 to 0:1) to obtain a colorless oil. (10 g, 82% yield). MS(ESI-TOF) m/z calc. for C_{8}H_{17}N_{6}O_{5}^{+} 245.14, found 245.13 [M+H^{+}]. ^1H NMR (300 MHz, Chloroform-d) δ 3.70 – 3.66 (m, 12H), 3.39 (t, J = 5.5 Hz, 4H). ^13C NMR (126 MHz, Chloroform-d) δ 70.74, 70.73, 70.71, 70.70, 70.66, 70.04, 50.67.
Triphenylphosphine (4 g, 15 mmol, 0.9 eq.) dissolved in ether (75 mL) was added to a solution of QX2158 (4 g, 16.5 mmol) in 5% aqueous HCl (50 mL). Addition was performed in 30 minutes at room temperature and the reaction was stirred for additional 2.5 hours. Phases were separated by a separation funnel and the aqueous layer was washed with DCM (3 × 50 mL). The aqueous layer was adjusted to pH 10 using sodium hydroxide powder. Product was then extracted with DCM (3 × 75 mL). Combined organic layer was dried over anhydrous sodium sulfate and filtered. After removal of the solvent under reduced pressure, a yellow oil was afforded (1.5 g, yield 46%) MS(ESI-TOF) m/z calc. for C₈H₁₉N₄O₃+ 219.15, found 219.15 [M+H⁺]. ¹H NMR (500 MHz, Chloroform-d) δ 3.65 – 3.62 (m, 10H), 3.60 – 3.58 (m, 2H), 3.47 (t, J = 5.5 Hz, 2H), 3.35 (t, J = 5.5 Hz, 2H), 2.8 (t, J = 5.5 Hz, 2H). ¹³C NMR (500 MHz, Chloroform-d) δ 73.49, 70.69, 70.63, 70.61, 70.26, 70.02, 50.64, 41.7.
tert-Butyl (S)-15-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-1-azido-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate (QX2161)

To Fmoc-Asp-tBu (1.15 g, 2.8 mmol) dissolved in dry DMF (20 ml) was added HATU (1.6 g, 4.2 mmol), HOBT (567.5 mg, 4.2 mmol), DIPEA (1.46 ml, 8.4 mmol). Then the mixture was stirred for 15 minutes at room temperature. Then compound QX2160 (730 mg, 3.25 mmol, dissolved in 5 ml DMF) was added to the mixture and the system was stirred for another 2 hours at room temperature. Upon completion of the reaction monitored by TLC, 30 ml water was added to the flask and extracted 3 times with ethyl acetate. The organic phases were combined, washed with saturated brine, dried over anhydrous sodium sulfate and evaporated to dryness. The crude was used in the next step without purification; MS(ESI-TOF) m/z calc. for C$_3$H$_{41}$N$_5$O$_8$H$^+$ 612.30, found 612.30 [M+H$^+$];
(S)-15-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-1-azido-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oic acid (QX2162)

Compound **QX2161** (800 mg, 1.31 mmol) was dissolved in a mixture of TFA (3 ml) and water (150 uL). Then the reaction mixture was stirred at room temperature for 2 hours. After completion of the reaction, TFA and water were removed by rotary evaporation. Yield quantitative; MS(ESI-TOF) m/z calc. for C_{27}H_{34}N_{5}O_{8}H^{+} 556.24, found 556.24 [M+H]\(^{+}\); \(^{1}\)H NMR (300 MHz, Chloroform-\(d\)) \(\delta\) 7.78 (d, \(J = 6.5\) Hz, 2H), 7.63 (t, \(J = 6.5\) Hz, 2H), 7.42 (t, \(J = 6.5\) Hz, 2H), 7.34 (t, \(J = 6.5\) Hz, 2H), 6.85 (br s., 1H), 6.25 (d, \(J = 6.9\) Hz, 1H), 4.60 (m, 1H), 4.48 – 4.33 (m, 2H), 4.25 (t, \(J = 6.9\) Hz, 1H), 3.68 – 3.63 (m, 12H), 3.57 – 3.55 (m, 2H), 3.42 – 3.37 (m, 2H), 2.97 (dd, \(J = 15, 7.2\) Hz, 1H), 2.76 (dd, \(J = 15, 7.2\) Hz, 1H). \(^{13}\)C NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 172.22, 171.34, 156.03, 143.88, 141.29, 127.76, 127.14, 125.25, 125.19, 120.00, 70.75, 70.44, 70.40, 70.02, 69.94, 50.85, 50.63, 47.07, 39.71, 38.69, 37.98.
To a solution of tetraethylene glycol (3.88 g, 20 mmol) in 80 mL of freshly distilled THF was added t-BuOK (1.12 g, 10 mmol) at 0 °C, the mixture was stirred at r.t. for 30 mins, then cooled to 0 °C, and propargyl bromide (1.2 g, 10 mmol) dissolved in 5 mL of THF was added dropwise. After that the reaction mixture was stirred at 0 °C for 10 mins then warmed to r.t. and stirred at r.t. overnight. Then the reaction was filtered, washed with THF, and the filtrate was concentrated and purified by flash chromatography (Hexane/EA : 1/2 to 0/1), the desired intermediate was obtained as a yellow oil. (2.0 g Yield 86%) MS(ESI-TOF) m/z calc. for C_{11}H_{21}O_{5}^{+} 233.14, found 233.14 [M+H^{+}]; calc. for C_{11}H_{20}NaO_{5}^{+} 255.12 found 255.12 [M+Na^{+}]. \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}_{6}) \delta 4.58 (t, J = 5.5 Hz, 1H), 4.13 (d, J = 2.5 Hz, 2H), 3.55 – 3.52 (m, 4H), 3.52 – 3.50 (m, 8H), 3.47 (t, J = 5.5 Hz, 2H), 3.42 (t, J = 2.5 Hz, 1H), 3.40 (t, J = 5.5 Hz, 2H). \textsuperscript{13}C NMR (500 MHz, DMSO-\textit{d}_{6}) \delta 80.80, 77.57, 72.80, 70.69, 70.27, 70.23, 69.50, 68.97, 60.66, 57.94.
3,6,9,12-Tetraoxapentadec-14-yn-1-yl methanesulfonate (QX2167)

Procedure was performed as described above for compound QX2157 but using QX2163 as starting material. Product was obtained as yellow oil (2.08 g Yield: 78%) MS(ESI-TOF) m/z calc. for C_{12}H_{23}O_{7}S^{+} 311.11, found 311.11 [M+H^{+}]; calc. for C_{12}H_{26}NO_{7}S^{+} 328.14, found 328.15 [M+NH_{4}^{+}]. ^{1}H NMR (500 MHz, DMSO-d_{6}) δ 4.31 – 4.29 (m, 2H), 4.13 (d, J = 2.0 Hz, 2H), 3.66 (t, J = 4.0 Hz, 2H), 3.55 – 3.51 (m, 12H), 3.41 (t, J = 2.5 Hz, 1H), 3.17 (s, 3H). ^{13}C NMR (500 MHz, DMSO-d_{6}) δ 80.80, 77.56, 70.20, 70.19, 70.16, 70.14, 69.94, 68.97, 68.73, 57.94, 37.27.
1-Azido-3,6,9,12-tetraoxapentadec-14-yne (QX2168)

Procedure was performed as described above for compound QX2158 but using QX2167 as starting material. Product was obtained as yellow oil (1.68 g Yield: 97%) MS(ESI-TOF) m/z calc. for C_{11}H_{20}N_{3}O_{4}+ 258.14, found 258.15 [M+H]. ^1H NMR (300 MHz, DMSO-d6) 4.16 (d, J = 2.4 Hz, 2H), 3.64 – 3.59 (m, 2H), 3.58 – 3.54 (m, 12H), 3.43 – 3.39 (m, 3H). ^13C NMR (300 MHz, DMSO-d6) δ 80.80, 77.54, 70.29, 70.24, 70.21, 70.17, 69.99, 69.74, 69.00 57.97, 50.48.
**3,6,9,12-Tetraoxapentadec-14-yn-1-amine (QX2169)**

![Chemical Structure](image)

Compound **QX2168** (1.68 g, 6.5 mmol) and PPh\(_3\) (3.4 g, 13.0 mmol) in a mixture of THF (30 mL) and water (1.6 mL) were stirred at room temperature overnight. 1M HCl (60 mL) was added and extracted 3 times with diethyl ether. The aqueous phase was lyophilized to obtain the product as colorless oil without further purification. Yield quantitative; MS(ESI-TOF) m/z calc. for C\(_{11}\)H\(_{22}\)NO\(_4\)H\(^+\) 232.15, found 232.15 [M+H\(^+\)]. \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \(\delta\) 4.13 (d, \(J = 2.0\) Hz, 2H), 3.60 (t, \(J = 5.5\) Hz, 2H), 3.54 – 3.50 (m, 12H), 3.43 (t, \(J = 2.5\) Hz, 1H), 2.93 – 2.90 (m, 2H). \(^{13}\)C NMR (500 MHz, DMSO-d\(_6\)) \(\delta\) 80.78, 77.64, 70.17, 70.09, 70.06, 69.91, 68.95, 67.02, 57.94, 38.87.

![Mass Spectrum](image)
**tert-Butyl (S)-19-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-17-oxo-4,7,10,13-tetraoxa-16-azaicos-1-yn-20-oate (QX2170)**

Procedure was performed as described above for compound QX2161 but using QX2169 as starting material. Product was obtained as colorless oil (2.4 g Yield: 59%) MS(ESI-TOF) m/z calc. for C_{34}H_{45}N_{2}O_{9} + 625.31, found 625.31 [M+H^+]. \(^1\)H NMR (500 MHz, Chloroform-d) δ 7.78 (d, J = 7.5 Hz, 2H), 7.60 (dd, J = 7.5, 5.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 6.38 (br.s., 1H), 6.07 (d, J = 8.5 Hz, 1H), 4.54 (dt, J = 8.5, 5.0 Hz, 1H, αH), 4.45 (d, J = 7.5 Hz, 2H), 4.24 – 4.21 (m, 3H, 1H of Fmoc, 2H of propargyl group), 3.71 – 3.37 (m, 16H), 2.80 (dd, J = 15.5, 5.0 Hz, 1H), 2.66 (dd, J = 15.5, 5.0 Hz, 1H), 1.49 (s, 9H). \(^{13}\)C NMR (500 MHz, Chloroform-d) δ 171.17, 170.30, 156.31, 143.87, 143.68, 141.31, 141.23, 127.76, 127.20, 127.11, 125.08, 125.02, 119.99, 82.53, 79.38, 74.89, 69.76, 69.67, 68.17, 66.69, 58.33, 51.49, 47.11, 39.27, 38.53, 36.50, 31.43, 27.91.
(S)-19-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-17-oxo-4,7,10,13-tetraoxa-16-azaicos-1-yn-20-oic acid (QX2170A)

Procedure was performed as described above for compound QX2162 but using QX2170 as starting material. Product was obtained as yellow oil. Yield quantitative. MS(ESI-TOF) m/z calc. for C\textsubscript{30}H\textsubscript{37}N\textsubscript{2}O\textsubscript{9}\textsuperscript{+} 569.25, found 569.25 [M+H\textsuperscript{+}]. \textsuperscript{1}H NMR (300 MHz, Chloroform-d) \(\delta\) 7.79 (d, \(J = 7.5\) Hz, 2H), 7.61 (d, \(J = 7.2\) Hz, 2H), 7.43 (t, \(J = 7.2\) Hz, 2H), 7.34 (t, \(J = 7.5\) Hz, 2H), 6.12 (d, \(J = 6\) Hz, 1H), 5.98 (br.s., 1H), 4.58 – 4.50 (m, 1H), 4.48 (d, \(J = 6\) Hz, 2H), 4.26 (s, 1H), 3.70 (s, 2H), 3.58 (s, 2H), 3.44 (s, 2H), 3.24 (s, 2H), 2.46 (s, 2H), 2.30 (s, 2H), 1.92 (s, 2H), 1.02 (s, 9H), 0.92 (s, 9H).
= 6.6 Hz, 2H), 4.24 (t, J = 6.6 Hz, 1H, from Fmoc), 4.16 (d, J = 1.8 Hz, 2H), 3.79 – 3.61 (m, 16H), 3.11 (dd, J = 17.7, 9 Hz, 1H), 2.82 (dd, J = 17.4, 5.1 Hz, 1H). $^{13}$C NMR (300 MHz, Chloroform-$d$) δ 143.61, 143.35, 141.33, 127.81, 127.15, 125.04, 120.03, 79.43, 74.82, 70.37, 70.26, 70.21, 70.14, 69.88, 69.85, 67.03, 58.30, 50.13, 47.10, 38.35, 35.70.
3,6,9,12,15-Pentaoxaoctadec-17-en-1-ol (QX2165)

To NaH (60 % in mineral oil, 360 mg, 9 mmol, washed with dry hexane) in THF (30 mL) was added pentaethylene glycol (4.76 g, 20 mmol) at 0°C under argon. The reaction mixture was stirred at 0°C for 1 hour, allyl bromide (846 uL, 10 mmol) in THF (10 mL) was added slowly at 0°C and stirring was continued for another 2 hours at room temperature. The reaction mixture was poured into cold saturated ammonium chloride solution (50 mL) and extracted with ethyl acetate. The combined organic phases were washed with saturated brine and dried over anhydrous sodium sulfate, evaporated to dryness. The products were separated by flash column chromatography to obtain the desired product QX2165 as yellow oil. (1.2 g, Yield 43%); MS(ESI-TOF) m/z calc. for C_{13}H_{27}O_{6}^+
279.18, found 279.18 [M+Na$^+$]; calc. for C$_{13}$H$_{26}$NaO$_6$ $^+$ 301.16, found 301.16 [M+Na$^+$]. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 5.91 – 5.83 (m, 1H), 5.24 (dd, $J = 17.5$, 2.0 Hz, 1H), 5.13 (dd, $J = 10.5$, 1.5 Hz, 1H), 3.94 (dt, $J = 5.5$, 1.5 Hz, 2H), 3.53 – 3.46 (m, 18H), 3.40 (t, $J = 5.0$ Hz, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 135.74, 116.74, 72.80, 71.50, 70.28, 70.25, 70.23, 70.22, 69.46, 60.66.
**3,6,9,12,15-Pentaoxaoc-tadee-17-en-l-yl methanesulfonate (QX2166)**

Procedure was performed as described above for compound QX2157 but using QX2165 as starting material. Product was obtained as yellow oil (1.45 g Yield: 95%) MS(ESI-TOF) m/z calc. for C_{14}H_{32}NO_{8}S^+ 374.18, found 374.19 [M+NH_4^+]. ¹H NMR (500 MHz, Chloroform-d) δ 5.90 (ddt, J = 17.5, 10.5, 5.5 Hz, 1H), 5.27 (dd, J =
17.5, 1.5 Hz, 1H), 5.18 (d, \( J = 10.5 \) Hz, 1H), 4.39 – 4.37 (m, 2H), 4.02 (d, \( J = 6.0 \) Hz, 2H), 3.77 – 3.75 (m, 2H), 3.65 – 3.64 (m, 14H), 3.61 – 3.59 (m, 2H), 3.08 (s, 3H). \(^{13}\)C NMR (500 MHz, Chloroform-\( d \)) \( \delta \) 134.75, 117.10, 72.23, 70.63, 70.58, 70.53, 70.52, 69.89, 69.87, 69.41, 69.34, 69.01, 37.74.
1-Azido-3,6,9,12,15-pentaaoctadec-17-ene(QX2178)

Procedure was performed as described above for compound QX2158 but using QX2166 as starting material. Product was obtained as colorless oil. Yield quantitative. (The mass spectrum information was not detected since it is hard to be charged.) $^1$H NMR (300 MHz, Chloroform-$d$) δ 5.92 (ddt, $J = 17.5, 10.5, 5.5$ Hz, 1H), 5.27 (dd, $J = 17.4, 1.8$ Hz, 1H), 5.18 (dd, $J = 10.5, 1.5$ Hz, 1H), 4.03 (d, $J = 5.4$ Hz, 2H), 3.70 – 3.65 (m, 16H), 3.62 – 3.60 (m, 2H), 3.40 (t, $J = 5.1$ Hz, 2H). $^{13}$C NMR (300 MHz, Chloroform-$d$) δ 134.78, 117.10, 72.24, 70.70, 70.68, 70.63, 70.64, 70.61, 70.04, 69.43, 50.69.
3,6,9,12,15-Pentaoxaoctadec-17-en-1-amine (QX2179)

Procedure was performed as described above for compound QX2168 but using QX2178 as starting material. Product was obtained as yellow oil (1.0 g Yield: 88%) MS(ESI-TOF) m/z calc. for C_{13}H_{28}NO_{5}^{+} 278.20, found 278.20 [M+H^{+}]. \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 5.91 (ddt, \(J = 17.5, 10.5, 5.5\) Hz, 1H), 5.29 (dd, \(J = 17.5, 1.5\) Hz, 1H), 5.21 (d, \(J = 10.0, 1\)H), 4.05 (d, \(J = 6.0\) Hz, 2H), 3.91 – 3.89 (m, 2H), 3.76 – 3.74 (m, 2H), 3.70 – 3.62 (m, 14H), 3.16 – 3.11 (m, 2H). \(^1^3\)C NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 134.16, 118.24, 72.26, 70.45, 70.18, 69.99, 69.91, 69.88, 69.86, 69.63, 68.90, 66.65, 40.41.
**tert-Butyl (S)-22-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-20-oxo-4,7,10,13,16-pentaoxa-19-azatricos-1-en-23-oate (QX2180)**

Procedure was performed as described above for compound **QX2161** but using **QX2179** as starting material. Product was obtained as colorless oil (0.94 g Yield: 41%) MS(ESI-TOF) m/z calc. for C_{36}H_{51}N_{2}O_{10} 671.35, found 671.35 [M+H^+]; calc. for C_{36}H_{54}N_{3}O_{10}^+ 688.38, found 688.38 [M+NH4^+]. ^1H NMR (500 MHz, Chloroform-d) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (dd, J = 7.5, 5.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 6.43 (br.s., 1H), 6.14 (d, J = 8.5 Hz, 1H), 5.89 (ddt, J = 17.5, 10.5, 5.5 Hz, 1H), 5.27 (dd, J = 17.5, 1.5 Hz, 1H), 5.18 (d, J = 10.5 Hz, 1H), 4.49 (dt, J = 8.5, 4.5 Hz, 1H), 4.44 (dd, J = 10.5, 7.5 Hz, 1H), 4.34 (dd, J = 10.5, 7.5 Hz, 1H), 4.23 (t, J = 7.5 Hz, 1H), 4.02 (d, J = 5.5 Hz, 2H), 3.68 – 3.51 (m, 18H), 3.44 (dt, J = 4.5, 5.0 Hz, 2H), 2.90 (dd, J = 15.5, 5.0 Hz, 1H), 2.71 (dd, J = 15.5, 5.0 Hz, 1H), 1.47 (s, 9H). ^13C NMR (500 MHz, Chloroform-d) δ 170.19, 156.24, 143.98, 143.83, 141.27, 134.62, 127.68, 127.10, 127.08, 125.25, 125.18, 119.94, 117.28, 110.00, 82.16, 72.21, 70.38, 70.21, 69.86, 69.27, 66.96, 51.42, 47.13, 39.32, 38.62, 37.82, 27.93.
(S)-22-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-20-oxo-4,7,10,13,16-pentaoxa-19-azatricos-1-en-23-oic acid (QX2181)

Procedure was performed as described above for compound QX2162 but using QX2180 as starting material. Product was obtained as yellow oil. Yield quantitative. MS(ESI-TOF) m/z calc. for C_{32}H_{43}N_{2}O_{10} [M+H]^+ 615.29, found 615.29. ^1H NMR (300 MHz, Chloroform-d) δ 7.78 (d, J = 7.5 Hz, 2H), 7.63 (dd, J = 7.5, 5.5 Hz, 2H), 7.42 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 6.30 (d, J = 7.2 Hz, 1H), 5.90 (ddt, J = 17.5, 10.5, 5.5 Hz, 1H), 5.27 (dd, J = 17.5, 1.5 Hz, 1H), 5.18 (d, J = 10.5 Hz, 1H), 4.60 (dt, J = 8.5, 4.5 Hz, 1H), 4.47 – 4.42 (m, 1H), 4.39 (dd, J = 9.9, 7.5 Hz, 1H), 4.24 (t, J = 8.1 Hz, 1H), 4.01 (d, J = 5.7 Hz, 2H), 3.66 – 3.38 (m, 20H), 2.96 (dd, J = 15.0, 2.7 Hz, 1H), 2.76 (dd, J = 15.0, 7.2 Hz, 1H). ^13C NMR (300 MHz, Chloroform-d) δ 171.49, 156.00, 143.93, 143.78, 141.28, 134.54, 127.73, 127.14, 125.25, 125.18, 119.97, 117.40, 72.18, 70.51, 70.34, 70.30, 70.18, 69.95, 69.37, 69.17, 67.14, 50.87, 47.09, 39.63, 37.91.
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