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Convergent synthesis of a helical, prehairpin HR1 trimer from HIV gp41

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A helical, prehairpin trimer covering the majority of the HR1 region of human immunodeficiency virus gp41 was achieved by chemically coupling three identical 51 amino acid peptides. A 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene pinwheel 'cap' was used to trimerize the peptides by taking advantage of the unique property of triacyl fluoride and orthogonal protection and deprotection. The resulting protein is fully helical, highly thermostable and soluble. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: convergent synthesis; protein synthesis; peptides; prehairpin gp41 HR1 trimer; oligomer

Introduction

Infection by human immunodeficiency virus type 1 (HIV-1) requires fusion of the viral and cellular membranes [1]. Binding of viral glycoproteins gp120 and gp41 to the CD4 receptor and coreceptors (such as CCR5 and CXCR4) triggers a series of conformational changes in gp120 and gp41 and ultimately leads to formation of the trimer-of-hairpins structure in gp41 [2].

gp41 contains two heptad repeat regions: a trimeric coiled-coil HR1 region which is located near the fusion peptide and the initially unstructured HR2 region which is proximal to the viral membrane [1]. After binding to CD4 and coreceptors, the fusion peptide inserts into the target cell to form the fusion intermediate state. The HR2 region then binds to hydrophobic grooves present on the HR1 to form the six-helix bundle or trimer-of-hairpins. The transition to the bundle state is thought to provide the energy required to facilitate membrane fusion [1,3,5]. Both heptad repeat regions have been proven to be attractive targets for blocking the fusion process [4–6]. Enfuvirtide (ENF), a peptide derived from the HR2 region and targeting the HR1, is the only fusion inhibitor approved for clinical use [4a,4b,7].

The success of HR1- and HR2-derived peptide fusion inhibitors has focused much attention on gp41 as a target for the development of small molecule fusion inhibitors as well as an antigen for vaccine development. Efforts in this area have been inhibited due to the difficulty in presenting the HR1 prehairpin intermediate in a stable, soluble state that accurately represents the viral HR1 trimer [8]. Several approaches have been used to avoid these issues. Several groups have attempted to stabilize the HR1 trimer, either by forming a chimera between the GCN4 trimer and a short segment of the HR1 [9] or to substantially mutate the peptide to stabilize the trimeric form [6]. Another approach has been to form a '5-helix' bundle, where one groove of the HR1 trimer remains exposed [1]. Unfortunately, each of these approaches introduces extraneous sequence that may preclude some of the applications where native sequence is required. Recently, Taylor *et al*., Bianchi *et al*. and Nakahara *et al*. have reported effective solutions to the synthesis of covalent HR1 trimers (albeit only up to 36-aa peptides) respectively [10].

We sought to stabilize a long segment of the wild type, helical trimer using a small chemical cap. Templates have been shown previously to reinforce intramolecular folding in designed proteins [11–14] and the cooperative conformational network [15] in 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene (hereafter called the pinwheel) has attracted much attention to its application for the design of receptors for assessing glycoside [16], nitrate, citrate [17], and self-assembling sieves [18] and controlling the oxygenation level of hemoglobin [19].

In our effort to design a stable HR1 trimer, we incorporated a 1,3,5-tris (aminomethyl)-2,4,6-triethylbenzene pinwheel template to the less structured, *N*-terminal end of the HR1. A Gly-Gly spacer was used between the pinwheel and the peptide to help maintain the integrity of the trimeric coiled coil. A glutarate spacer was used on the pinwheel to avoid steric congestion and allow more conformational mobility. For the first time, we were able to engineer a 53 amino acid trimer, based on the peptide T-865 [20], covering the majority of the HR1 region of gp41. This pinwheel trimer not only includes the deep pocket [4c,8a,21] but the entire HR1 groove including regions known to be important for HR2 binding and areas that mutate in response to ENF resistance [20]. The trimer is shown to be fully helical, soluble and exceptionally stable.

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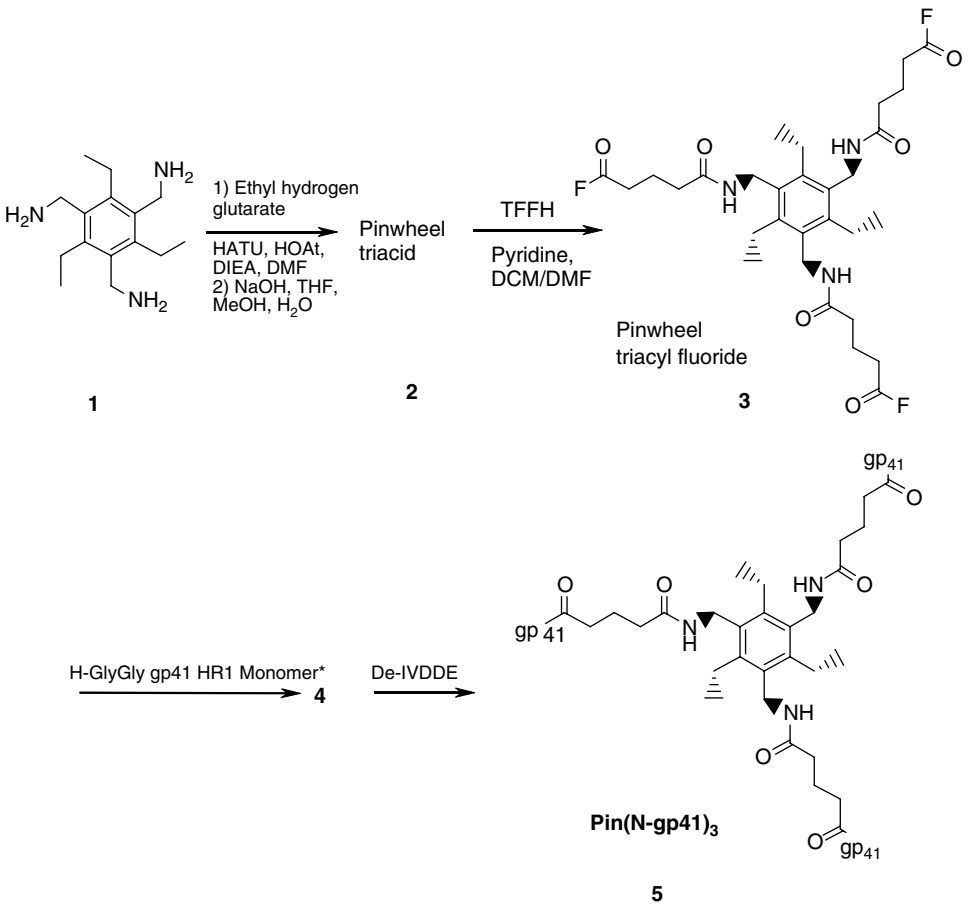

Scheme 1. Synthesis of Pin(N-gp41)$_3$.

Experimental Procedures

Section 1: Biophysical Analysis

Analytical ultracentrifugation

The sedimentation equilibrium experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge at 4 °C as described [20]. Peptide samples were diluted using 50 mM KH_2PO_4, 100 mM KCl pH 7.0 and data were collected at 18 000 and 22 000 rpm at wavelengths of 235 240 and 280 nm. Weight-averaged molecular weights were obtained by fitting each data file individually using a single ideal species model in the Beckman-Origin software (version 3.78 for Windows) (Figure 1). The solvent density of the phosphate buffer (equal to 1.00894) and partial specific volume (0.7424) were calculated using the program SEDNTERP [20].

Circular dichroism

The midpoint of the thermal unfolding transition (Tm) of the Pin(N-gp41)$_3$ and the HR1/HR2 complex was determined at 222 nm with 2 °C increments from 25 to 97 °C with 16 s averaging time on an AVIV 202-01 circular dichroism spectrophotometer. The Tm was determined to be the value that corresponded to the maximum value of the first derivative of the thermal transition. Wavelength scans were also performed (Figure 2) and ranged from 200 to 260 nm, with measurements at every 0.5 nm, a 4 s averaging time and a 1.5 nm bandwidth.

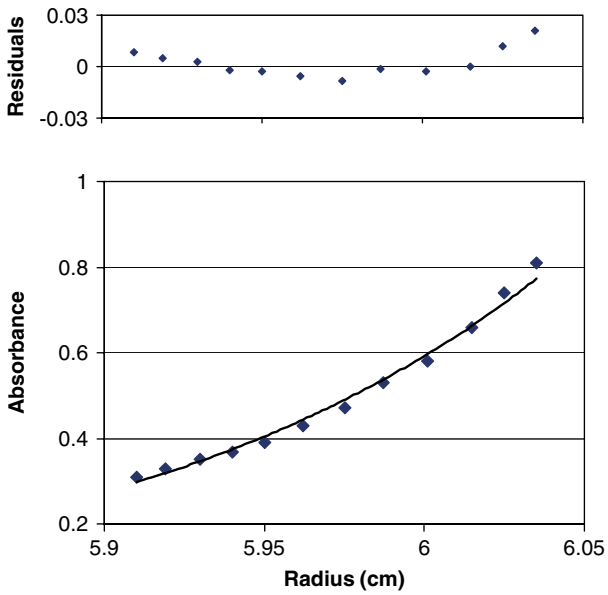

Figure 1. Sedimentation equilibrium results for a 10 μM solution of Pin(N-gp41)$_3$ at 4 °C in 50 mM KH_2PO_4, 100 mM KCl pH 7.0. The line represents the fit to the single ideal species model. For clarity, not all data points are shown.

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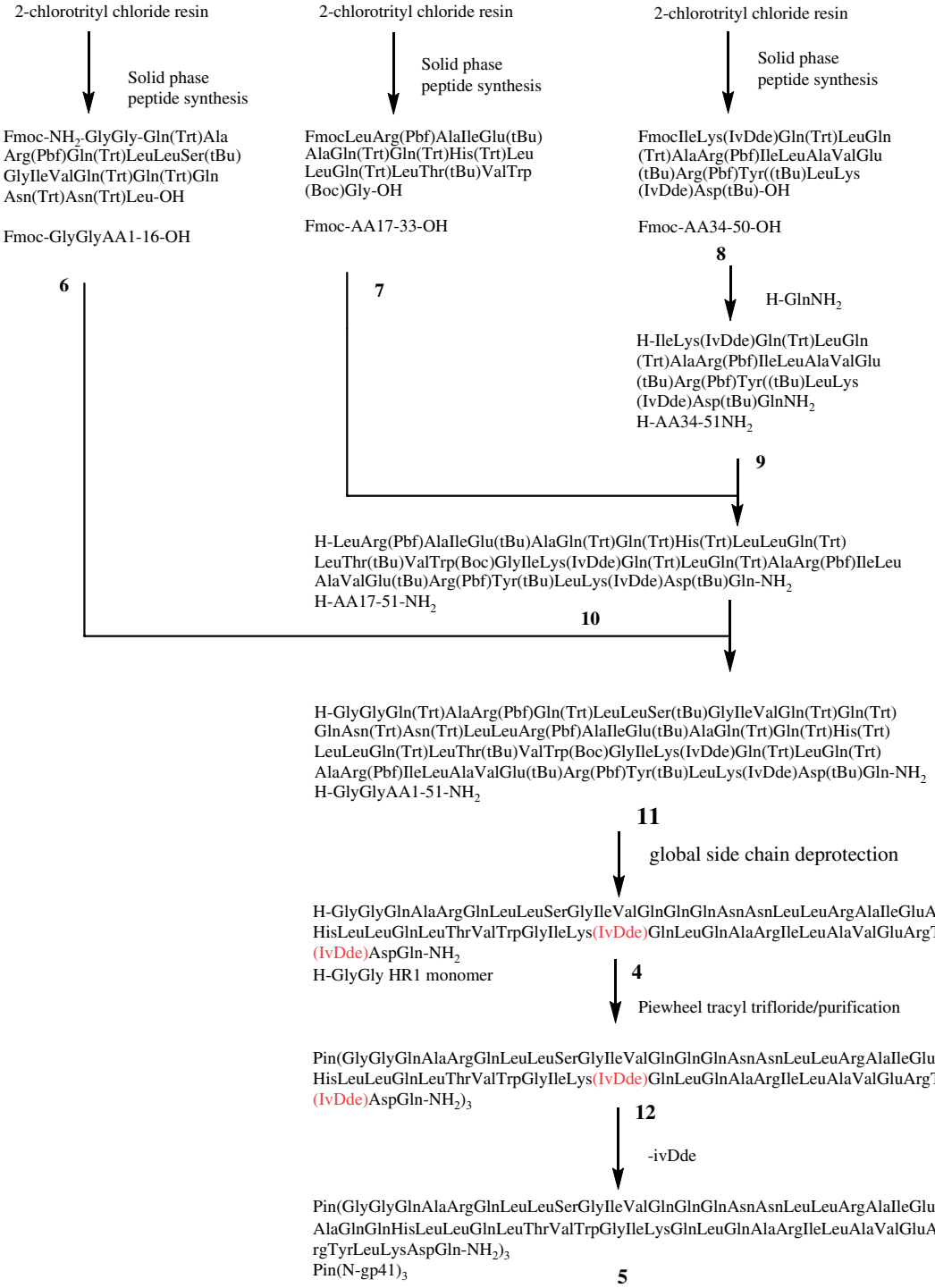

Scheme 2. Synthesis of H GlyGly HR monomer (**11**).

Section 2: Synthesis

*Synthesis of Pin(N-gp41)$_3$ (**5**)*

The Pin-F$_3$ (**3**, 5.9 mg, 0.009 mmol) and H-GlyGly-HR1 monomer (ivdde) (**12**, 532 mg, 0.082 mmol) were dissolved in dimethyl formamide (DMF) (2 ml) at 25 °C (Scheme 1). Then diisopropyl ethyl amine (DIEA) (120 µl, 0.689 mmol) was added and the solution was stirred at 25 °C for 21 h. The reaction mixture was cooled in an ice bath and then the pH was adjusted to *ca* 6.5 with HOAc. The product was precipitated with cold methyl t-butyl ether (MTBE) (100 ml), filtered off, washed with MTBE (3 × 25 ml) and dried on the funnel for 1 h. The crude product was purified using reverse phase high-performance liquid chromatography (RP-HPLC) (Column: Phenomenex Jupitor C4, 300 Å, 10 µm, 250 × 21.4 mm; mobile phase: A: 0.1% trifluoroacetic acid (TFA) in H_2O; B: 0.1% TFA in acetonitrile; gradient: 55–72% B in 63 min; flow rate: 20.0 ml min^{-1}; detection: UV at 220 nm) to afford pure ivDde protected product Pin(GlyGly HR1-ivdde)$_3$ (**4**)

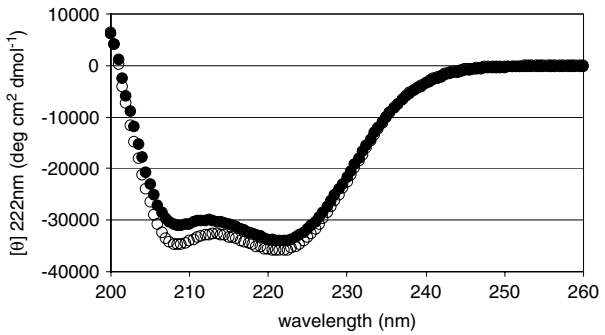

Figure 2. Wavelength scan of 6.6 μM Pin(N-gp41)$_3$ (●) and 20 μM T865 (○) in phosphate buffered saline (PBS) pH 6.0 at 25 °C as determined by circular dichroism. The helicity of Pin(N-gp41)$_3$ and T865 was found to be 90% and 97%, respectively.

as a fluffy white solid in 25% yield (45 mg). MS(Da) calculated for $C_{912}H_{1497}N_{255}O_{243}$ 19910.24, found 19909.65. To a 100 ml round bottom flask were charged Pin(GlyGly HR1-ivdde)$_3$ (**4**, 45 mg, 0.00226 mmol) and 5% hydrazine in DMF (4 ml). The solution was stirred for 2 h, then cooled to 0–5 °C and HOAc (5–6 drops) was added bringing the pH to 6.5. To the cooled solution was added MTBE (50 ml) resulting in precipitation of the trimer. The solid was collected by filtration, washed with MTBE (3 × 15 ml) and dissolved in 20% acetonitrile in water (10 ml, 0.1% TFA). The solution was frozen and lyophilized to give Pin(N-gp41)$_3$ (**5**, 37 mg) in 88% yield and as a single peak in the HPLC chromatogram purity 100% by LC-MS. MS(Da): calculated monoisotope for $C_{834}H_{1389}N_{255}O_{231}$ 18673.48, found 18674.84 (Figures 3 and 4).

*Synthesis of pinwheel triacid (**2**): 1,2,3-tri(N-methylpentanoic acidamide)-2,4,6-triethylbenzene (pinwheel triacid)*

A 100 ml round bottom flask was charged with 20 ml of anhydrous DMF under an inert atmosphere, and ethyl hydrogen glutarate (761 mg, 4.752 mmol) was added. The resulting solution was cooled to 0 °C in an ice-water bath, and *N*-[(dimethylamino)-^{1}H-1,2,3-triazolo[4,5-*b*]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (1.807 g, 4.752 mmol), 1-hydroxy-7-azabenzotriazole (HOAt) (646 mg, 4.751 mmol) and DIEA (2.55 ml, 17.28 mmol) were added and stirred at 0 °C for 10 min. Then 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene trihydrochloride (517 mg, 1.44 mmol) [22] was added. The reaction was stirred at 0 °C for 1 h and then at 25 °C for 24 h. Half of the solvent was removed under reduced pressure and the remaining solids were dissolved in 70 ml of CH_2Cl_2 and washed with cold 0.5 N HCl (2 × 20 ml), H_2O (2 × 20 ml), cold 0.5 N NaOH (2 × 20 ml) and brine (2 × 20 ml). The organic layer was collected and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the resulting crude product was dried under high vacuum overnight. The crude product was dissolved in 9 ml of tetrahydrofuran (THF) and 3 ml of MeOH

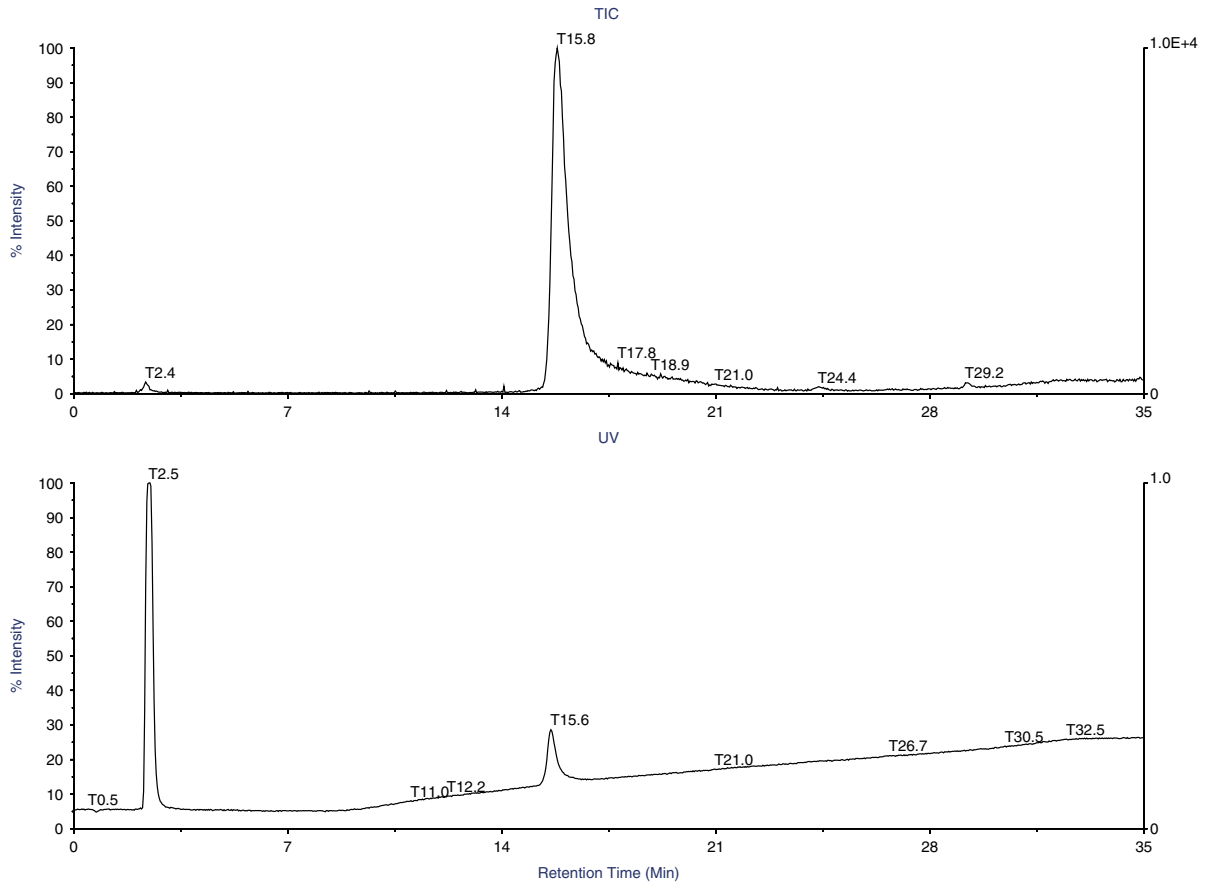

Figure 3. HPLC-MS spectrum of Pin(N-gp41)$_3$. LC column: keystone BetBasic C18, 2.0 × 150 mm (3 μm/150 Å). Mobile phase: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile. MS: positive ion mode (+); scanning 450–2500 *m/z* every 2 s; Sciex heater at 400 °C. LC: UV detector at 220 nM with flow 0.20 ml min^{-1}. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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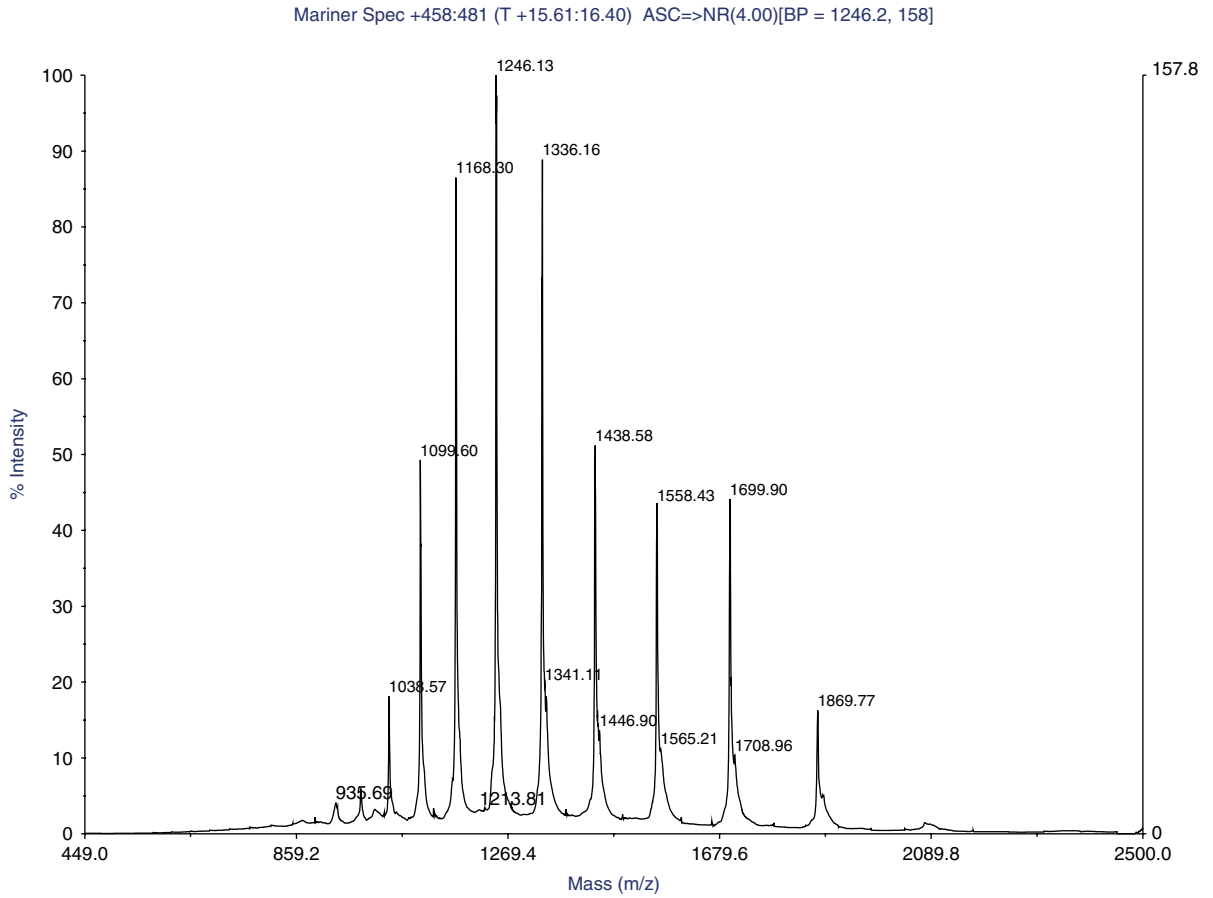

Figure 4. MS spectrum of Pin(GlyGlyHR1)$_3$ from 15.61 to 16.40 min for the peak 2 RT15.8 min in the total ion current (TIC). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

and cooled in an ice-water bath. Sodium hydroxide (218 mg, 5.46 mmol) in 3 ml of water was added dropwise and the solution was stirred at 25 °C for 5 h. The THF and methanol were removed under reduced pressure and the aqueous layer was cooled in an ice bath and the pH adjusted to 1–2 by slow addition of 1 N HCl. The crude product was purified by RP-HPLC (Phenomenex Jupiter C18, 300 Å, 10 µ, 250 × 21.4 mm, A: 0.1% TFA in H_2O; B: 0.1% TFA in acetonitrile; gradient 20–50% B in 40 min) to afford pure product as a white solid in 49% yield (414 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.01 (br s, 3H), 7.78 (t, 3H, J = 4.5 Hz), 4.26 (d, 6H, J = 4.0 Hz), 2.64–2.62 (q, 6H, J = 7.5 Hz), 2.20–2.16 (t, 6H, J = 7.3 Hz), 2.12–2.08 (t, 6H, J = 7.4 Hz), 1.71–1.67 (p, 6H, J = 7.4 Hz), 1.05 (t, 9H, J = 7.4 Hz). $[MH]^+$ calculated for $C_{30}H_{45}N_3O_9$ 592.31, found 592.13.

*Synthesis of pinwheel triacyl fluoride (**3**): 1,2,3-tri(N-methyl(pentanyl fluoride)amide)-2,4,6-triethylbenzene (Pin-F_3)*

To a solution of pinwheel triacid (0.426 g, 0.72 mmol), pyridine (0.175 ml, 2.16 mmol), anhydrous CH_2Cl_2 (20 ml), and DMF (4 ml) the fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate (TFFH) (0.856 g, 3.24 mmol) was added under an inert atmosphere. The reaction was stirred at 25 °C for 20 h and then diluted with 250 ml of CH_2Cl_2 and washed with ice water (3 × 50 ml). The organic layer was collected and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was re-crystallized with CH_2Cl_2 affording pure product as a white solid in 59.1% yield (253 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.85 (br, 3H), 4.26 (b, 6H), 2.68–2.61 (m, 12H), 2.30–2.27 (m, 6H), 1.77 (t, 6H, J = 7.2 Hz), 1.06 (t, 9H, J = 7.3 Hz). IR: 1850 cm^{-1}.

*Synthesis of H-GlyGly HR1 (ivdde) monomer (**12**)*

A 500 ml round bottom flask containing trifluoroacetic acid (85.5 ml), dithiothreitol (8.55 g), 8.55g phenol and water (4.28 ml) was purged with nitrogen for 5 min. The solution was cooled to 0–5 °C for 10 min and H-GlyGlyGln(Trt)AlaArg(Pbf)Gln(Trt)LeuLeuSer(tBu)GlyIleValGln(Trt)Gln(Trt)GlnAsn(Trt)Asn(Trt)LeuLeuArg(Pbf)AlaIleGlu(tBu)AlaGln(Trt)Gln(Trt)His(Trt)LeuILeuGln(Trt)LeuThr(tBu)ValTrp(Boc)GlyIleLys(ivDde)Gln(Trt)LeuGln(Trt)AlaArg(Pbf)IleLeuAlaValGlu(tBu)Arg(Pbf)Tyr(tBu)LeuLys(ivDde)Asp(tBu)GlnNH_2 (2.1 g, 0.19 mmol) was added. The reaction was removed from the ice bath and stirred at 25 °C for 4 h. The reaction mixture was then cooled to 0–5 °C and cold MTBE (400 ml) was added over 20 min with rapid stirring. The resulting slurry was stirred for an additional 30 min, the solids filtered off, washed with MTBE (3 × 50 ml), and dried on the funnel for 1 h. The solids were suspended in 60 ml of 50 : 50 H_2O : acetonitrile and the pH adjusted to *ca* 7.5 with 0.35 N $NaHCO_3$. When all the solids had dissolved, the pH was lowered back to *ca* 4 with acetic acid (HOAc), and the resulting cloudy suspension was stirred at 25 °C overnight to effect decarboxylation of the tryptophans. The solvent was lyophilized off to afford a fluffy ivory colored crude product that was then purified by RP-HPLC (Column: Phenomenex Jupiter C18,

300 Å, 10 μ, 250 × 21.4 mm; mobile phase: A: 0.1% TFA in H_2O; B: 0.1% TFA in acetonitrile; gradient: 40–60% B in 45 min; flow rate: 20.0 ml min^{-1}; detection: UV at 220 nm) to afford 0.62 g of H-GlyGlyGlnAlaArgGlnLeuLeuSerGlyIleValGlnGlnGlnAsnAsnLeuLeu ArgAlaIleGluAlaGlnGlnHisLeuILeuGlnLeuThrValTrpGlyIleLys(**iv Dde**)GlnLeuGlnAlaArgIleLeuAlaValGluArgTyrLeuLys(**ivDde**)Asp $GlnNH_2$ (**12**, H-GlyGly HR1 monomer) as a fluffy white solid in 49% yield and a single peak in the HPLC (Method D). MS(Da): calculated monoisotope for $C_{294}H_{486}N_{84}O_{79}$ 6457.65, found 6457.79 (Scheme 2).

*Synthesis of H-GlyGlyAA1-51 NH_2 (**11**, fully protected)*

A 500 ml round bottom flask was charged with Fmoc GlyGlyGln(Trt)AlaArg(Pbf)Gln(Trt)LeuLeuSer(tBu)GlyIleValGln(Trt) Gln(Trt)GlnAsn(Trt)Asn(Trt)LeuLeuArg(Pbf)AlaIleGlu(tBu)AlaGln (Trt)Gln(Trt)His(Trt)LeuILeuGln(Trt)LeuThr(tBu)ValTrp(Boc)GlyIle Lys(ivDde)Gln(Trt)LeuGln(Trt)AlaArg(Pbf)IleLeuAlaValGlu(tBu)Arg (Pbf)Tyr(tBu)LeuLys(ivDde)Asp(tBu)$GlnNH_2$ (10.5g, 0.95 mmol), DMF (60 ml) and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) (0.10 ml, 0.67 mmol). The solution was stirred at ambient temperature for 90 min and DCM was added (100 ml). The solution was transferred to a separator funnel and 0.1 N HCl (100 ml) was added. The aqueous layer was discarded and the process repeated. The process was repeated two more times with water (2 × 100 ml). The organic solution was dried over $MgSO_4$, then filter and concentrated using a rotary evaporator. The resulting residue was cooled to 0–5 °C and MTBE (300 ml) was added to precipitate the protected peptide. The suspension was stirred for 2 h, collected by filtration, washed with MTBE (2 × 100 ml) and dried affording H-GlyGlyGln(Trt)AlaArg(Pbf)Gln(Trt)LeuLeuSer(tBu)GlyIleValGln(Trt) Gln(Trt)GlnAsn(Trt)Asn(Trt)LeuLeuArg(Pbf)AlaIleGlu(tBu)AlaGln (Trt)Gln(Trt)His(Trt)LeuILeuGln(Trt)LeuThr(tBu)ValTrp(Boc)GlyIle Lys(ivDde)Gln(Trt)LeuGln(Trt)AlaArg(Pbf)IleLeuAlaValGlu(tBu)Arg (Pbf)Tyr(tBu)LeuLys(ivDde)Asp(tBu)$GlnNH_2$ (9.75 g) in 94.8% yield. Method C HPLC purity, 69.8A%. MS(Da): calculated monoisotope for $C_{603}H_{774}N_{84}O_{93}S_4$, 10807.73, found 10807.88.

Synthesis of Fmoc-GlyGlyAA1-51 NH_2 (fully protected)

A 500 ml round bottom flask was charged with FmocGlyG-lyGln(Trt)AlaArg(Pbf)Gln(Trt)LeuLeuSer(tBu)GlyIleValGln(Trt)Gln (Trt)GlnAsn(Trt)Asn(Trt)LeuOH (**6,** 3.79 g, 0.97 mmol), H-LeuArg (Pbf)AlaIleGlu(tBu)AlaGln(Trt)Gln(Trt)His(Trt)LeuILeuGln(Trt)Leu Thr(tBu)ValTrp(Boc)GlyIleLys(ivDde)Gln(Trt)LeuGln(Trt)AlaArg (Pbf)IleLeuAlaValGlu(tBu)Arg(Pbf)Tyr(tBu)LeuLys(ivDde)Asp(tBu) $GlnNH_2$(**10**, 7.14 g, 1.00 mmol), HOAt (0.359 g, 2.91 mmol), DMF (130 ml) and DIEA (0.675 ml, 3.88 mmol). The solution was cooled to 0–5 °C and HATU (0.405 g, 1.07 mmol) was added. The solution was stirred at 0–5 °C for 10 min then warmed to ambient temperature and stirred an additional 1 h. The solution was cooled to <10 °C and water (200 ml) was added to precipitate the product. The resulting suspension was stirred at <10 °C for 30 min. The solid was collected by filtration, washed with water (3 × 75 ml) and dried to give FmocGlyG-lyGln(Trt)AlaArg(Pbf)Gln(Trt)LeuLeuSer(tBu)GlyIleValGln(Trt)Gln (Trt)GlnAsn(Trt)Asn(Trt)LeuLeuArg(Pbf)AlaIleGlu(tBu)AlaGln(Trt) Gln(Trt)His(Trt)LeuILeuGln(Trt)LeuThr(tBu)ValTrp(Boc)GlyIleLys(iv Dde)Gln(Trt)LeuGln(Trt)AlaArg(Pbf)IleLeuAlaValGlu(tBu)Arg(Pbf) Tyr(tBu)LeuLys(ivDde)Asp(tBu)$GlnNH_2$ (10.6 g) in 99% yield. MS(Da): calculated monoisotope for $C_{618}H_{784}N_{84}O_{95}S_4$, 11029.80, found 11029.87. Method C HPLC purity, 58A%. The experimental procedure for the synthesis of intermediates 6–10 is available in supporting information.

Results and Discussion

We envisioned synthesis of the Pin(N-gp41)$_3$ (**5**) from the pinwheel triacid (**2**) and the H-GlyGly HR1 monomer, where the internal lysines at positions 37 and 51 were protected. The triaminomethyl pinwheel (**1**) was coupled to the activated ester of ethyl hydrogen glutarate through standard peptide coupling conditions (HATU, HOAt, DIEA, DMF) [23]. Following saponification in methanol/water and purification by RP-HPLC, the pinwheel triacid (**2**) was isolated as a white solid in 49% yield.

Attempts to synthesize the H-GlyGly HR1 peptide by linear solid phase peptide synthesis (SPPS) using Fmoc protected amino acids proved to be difficult and failed to produce the desired quantities of material. A three fragment approach to building the H-GlyGly-HR1 peptide, similar to that used for the synthesis of ENF, was developed [24]. The three side chain protected fragments, FmocGlyGly-AA1-16OH, FmocAA17-33OH and FmocAA34-50OH, were built by SPPS on the super-acid labile 2-chlorotrityl chloride resin. The ε-amines of the two lysines in FmocAA34-50OH were protected with (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), a functionality that is stable toward the basic conditions used to remove Fmoc in peptide synthesis as well as the acidic conditions used to remove the side chain protecting groups on the other amino acids [25]. The protected fragments were assembled in solution phase condensations to provide side chain protected H-GlyGly-AA1-51 NH_2. Global side chain deprotection of H-GlyGly-AA1-51 NH2 with a cocktail composed of trifluoroacetic acid : water : dithiothreitol : phenol (80 : 4 : 8 : 8 vol, vol, wt, wt) followed by chromatographic purification produced the desired H-GlyGly HR1 monomer with the internal lysines protected. The inclusion of phenol in the cocktail was essential to avoid extensive trifluoroacetylation of the peptide during side chain deprotection.

Initial attempts at formation of the pinwheel HR1 trimer from H-GlyGly HR1 and pinwheel triacid activated as the *N*-hydroxysuccinimide, paranitrophenyl, pentafluorophenyl, *N*-hydroxybenzotriazole or *N*-hydroxy-7-azabenzotriazole esters in DMF produced pin(GlyGly HR1)$_1$ as the main product and <1% of the desired trimer. DMF competes with the peptide in reacting with the activated esters, presumably forming a Villsmeyer type reagent, which in turn formylates (after exposure to water) the *N*-terminus of the peptide.

Treatment of the pinwheel triacid (**2**) with Carpino's reagent [26], TFFH yielded the pinwheel triacyl fluoride (**3**). Condensation of the more stable pinwheel triacyl fluoride with an excess of H-GlyGly HR1 in DMF afforded a mixture of the desired pinwheel trimer as well as dimers and monomers. The trimer was isolated by preparative HPLC and treated with hydrazine to remove the ivDde protecting groups on the internal lysines to give Pin(N-gp41)$_3$ in 22% yield.

Circular dichroism was used to characterize the trimeric pinwheel compound. As shown in Figure 2, the wavelength scan of Pin(N-gp41)$_3$ at 6.6 μM in PBS pH 6.0 is indicative of a helical species and we determined that the construct is approximately 90% helical. Figure 5 shows that the thermal stability of Pin(N-gp41)$_3$ is substantially higher than the peptide T865. At 6.6 and 0.5 μM, helical structure is maintained to 95 °C, as compared to T865 which cooperatively unfolds at temperatures well below 95 °C.

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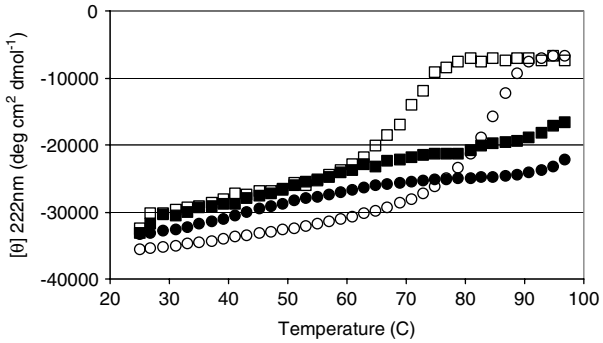

Figure 5. Thermal unfolding transitions of Pin(N-gp41)$_3$ at 6.6 μM (●), Pin(N-gp41)$_3$ at 0.5 μM (■), T865 at 20 μM (○) and T865 at 1 μM (□) as determined by circular dichroism. Thermal melts were done in PBS pH 6.0 (higher concentrations) or pH 7.0 (lower concentrations) and at 25 °C.

Therefore, Pin(N-gp41)$_3$ is almost fully helical and substantially more thermostable than the self-associated peptide.

To further investigate the enhancement in HR1 stability, we repeated the thermal unfolding experiment in the presence of 5 M urea. The observed Tm for T-865 in 5 M urea was found to be 55 °C (not shown). In contrast, Pin(N-gp41)$_3$ has a Tm of 86 °C in 5 M urea, further demonstrating that the covalent trimer has significantly enhanced thermal stability.

Although it is assumed from the crystallographic structures that the HR1 is trimeric [1,6], it has been demonstrated in solution that the peptide T-865 self-associates into a tetrameric species [20]. We analyzed Pin(N-gp41)$_3$ by analytical ultracentrifugation and found that the weight-averaged molecular weight at 10 μM to be 17 525 +/− 440 Da. This molecular weight is in good agreement with the predicted mass of a trimer (18673.48), demonstrating that the covalent construct is a trimeric species, as designed.

To investigate how well HR2 peptides could bind to the engineered trimer, we performed peptide mixing experiments using circular dichroism. Although Pin(N-gp41)$_3$ is highly helical, HR2 peptides are unstructured in solution and adopt a helical conformation upon binding. We used the HR2 peptide T-649, a peptide closely related to C34 [20], and the observed helicity of the mixture demonstrates a significant increase in helical structure, which is indicative of the formation of the six-helix bundle. The data for the interaction between Pin(N-gp41)$_3$ and T649 is shown in Figure 6. A loss of structure is observed at high Tm for T-649, which is likely due to the loss of structure of the HR2 as it dissociates from the HR1 trimer, consistent with observations from our previous work [20].

Conclusion

In summary, we have designed and engineered a helical, prehairpin HR1 trimer from HIV gp41, covering the majority of the HR1 region of gp41, that is fully helical and highly thermostable. The high affinity interaction with HR2 peptides suggests that the trimer can adopt a conformation in the six-helix bundle that is similar to that found in isolated peptides. These features make Pin(N-gp41)$_3$ attractive for screening of novel fusion inhibitors and for the development of neutralizing antibodies. The helical, prehairpin HR1 trimer described here is, to the best of our knowledge, the largest oligomer prepared by chemical synthesis via a convergent synthetic approach, taking advantage of the unique property of triacyl fluoride and orthogonal protection and deprotection. This will enable the design and preparation of a wide range of template-assembled oligomeric protein analogs for functional, immunogenic and therapeutic research.

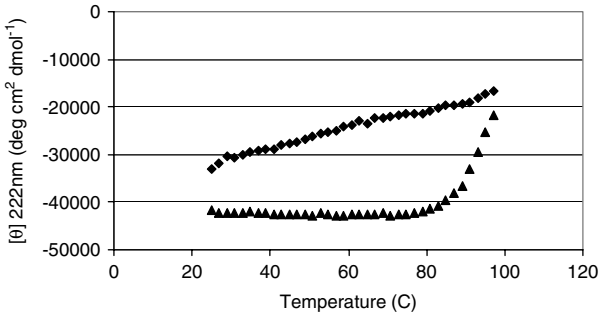

Figure 6. Thermal stability of Pin(N-gp41)$_3$ trimer alone (■) and Pin(N-gp41)$_3$/T649 bundle (▲). Data was obtained at 1 μM concentrations in PBS pH 7.0.

Supporting information

Supporting information may be found in the online version of this article.

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