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A concise preparation of the fluorescent amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine and extension of its utility in solid phase peptide synthesis

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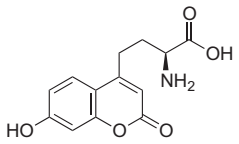
ABSTRACT

Incorporation of the unnatural amino acid L-(7-hydroxycoumarin-4-yl)ethylglycine (7-HC) is a powerful and reliable approach for the preparation of fluorescently labeled proteins. The growing popularity of this valuable amino acid prompted us to pursue an improved protocol for its synthetic preparation. The optimized procedure here described provides ready access to multi-gram quantities of 7-HC. Also reported is an extension of the utility of 7-HC in the generation of a protected building block suitable for use in solid phase peptide synthesis. The building block was successfully incorporated at various positions in a series of model peptides, including analogues of the cell penetrating HIV-Tat peptide, further illustrating the utility of this unique amino acid.

1. Introduction

The expansion of the genetic code to allow for the site-specific incorporation of unnatural amino acids (UAAs) is a hallmark of modern protein chemistry. The ability to introduce functionalities beyond those contained within the twenty regular amino acids now makes it possible to probe and tune the properties of proteins expressed in bacterial, fungal, or mammalian cells.[1–3] To date, multiple UAAs bearing structurally diverse, chemically reactive, photoreactive, and fluorescent side chains have been successfully incorporated into a variety of proteins.[4–6] Fluorescently labeled proteins are of particular value in molecular and chemical biology investigations and are often produced as fusions with other inherently fluorescent proteins. As an alternative, the use of a fluorescent UAA offers the advantage of providing greater flexibility with respect to the site of incorporation as well as a significantly smaller perturbation of the size and shape of the protein under investigation. To this end, Schultz and co-workers were the first to successfully incorporate the small coumarin-based fluorescent amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine (7-HC **1**, Fig. 1) into a model protein.[7] While other fluorescent UAAs have also been described[8–11] and in some cases successfully incorporated into proteins,[12–16] 7-HC remains a preferred choice for many applications due to its relatively large Stokes shift and the sensitivity of its fluorescent intensity to both pH and solvent polarity.[7,17–19] Of particular note is the recent application of 7-HC in what can be considered the smallest genetically encoded phosphorylation biosensor.[20,21] Specifically, Wang and co-workers showed that by incorporating 7-HC in close proximity to a known site of protein phosphorylation, it is possible to directly detect phosphorylation owing to the measurable change it induces in the 7-HC fluorescent signal.[20] This effect is ascribed to an increase in the local pH resulting from addition of a phosphate group. Increases in pH are known to red-shift the maximum excitation peak of 7-HC along with an increase in fluorescence emission intensity at 450 nm.[21]



7-HC (**1**)

Figure 1. Structure of L-(7-hydroxycoumarin-4-yl) ethylglycine (7-HC) **1**.

Despite its clear value, the methods reported to date for the preparation of 7-HC **1** in significant quantities (multi-gram) are limited. The approach most often employed, derives from a procedure originally developed by Garbay and co-workers for the synthesis of coumarin-bearing fluorescent amino acids.[7,22] While direct and straightforward, the route as described makes use of a final purification step employing preparative reverse phase high performance liquid chromatography. Recently, an alternative synthesis of **1**, avoiding HPLC purification, has also been reported, however this procedure yields **1** as a racemate.[23] To address these issues we chose to further investigate the synthesis of **1** with the aim of optimizing its preparation to allow for an efficient preparation on the multi-gram scale. Furthermore, with access to larger

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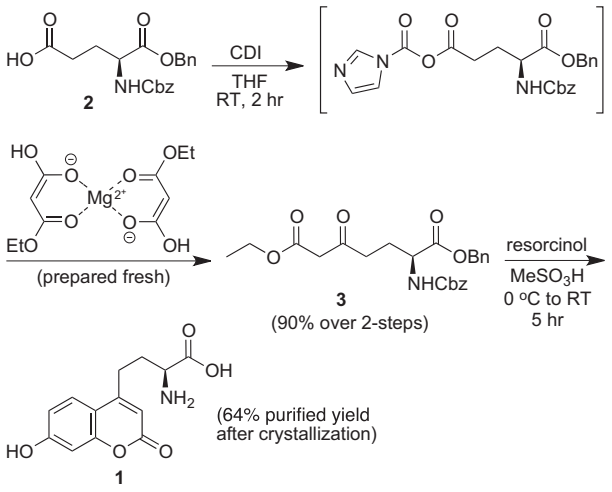
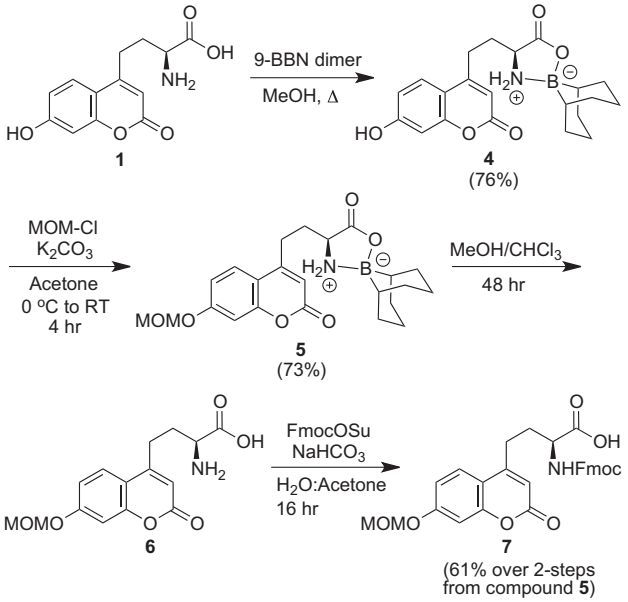


Scheme 1. Synthetic route optimized for the preparation of L-(7-hydroxycoumarin-4-yl) ethylglycine **1**.



Scheme 2. Preparation of protected SPPS building block **7**.

quantities of **1**, a protecting group strategy was also devised so as to allow for its ready incorporation into synthetic peptides via standard Fmoc solid phase peptide synthesis (SPPS) approaches.

2. Results and discussion

The most direct route described in the literature for the preparation of **1** offers the advantage of starting from a commercially available protected glutamic acid building block **2** (Scheme 1).[7,22] The conversion of **2** into β-ketoester **3** has previously been achieved in moderate-to-good yield by pre-activation of the side chain carboxyl group of **2** with carbonyldiimidazole, followed by condensation with ethyl magnesium malonate. In optimizing this step we found that a large enhancement in the yield of **3** (up to 90%) could be attained by using freshly prepared magnesium monoethylmalonate. The direct conversion of β-ketoester **3** to the desired amino acid product is then accomplished by condensation with resorcinol in neat methanesulfonic acid.[7] Under these strongly acidic conditions, the von Pechmann condensation leads to formation of the coumarin moiety while the excess resorcinol employed serves as a scavenger leading to concomitant removal of both the benzyl ester and benzyl carbamate. Following optimization, purified yields of up to 64% were achieved for this transformation, providing **1** in higher yield than previously reported. The most significant improvement in this particular step relates to the purification protocol employed. Previous approaches to purifying **1** have relied on the use of extensive RP-HPLC severely limiting the practicality and ease of large-scale preparation. Thus, we were pleased to find that a straightforward acid–base titration/crystallization approach could be applied to provide compound **1** as a dark red crystalline material in high purity. In this manner multi-gram quantities of **1** can be rapidly prepared in routine fashion.

With ready access to gram quantities of **1**, we next turned our attention to developing a protecting group strategy that would allow for incorporation of the amino acid into synthetic peptides. While there are reports of using an Fmoc-protected analogue of **1**, without protection of the phenol group, the yields of peptides obtained via this approach were low.[24] Furthermore, we found that unprotected **1** decomposes under the basic conditions typically utilized for Fmoc protection. We therefore chose to pursue a fully protected variant of **1** wherein the 7-hydroxy group of the coumarin is masked with an acid labile group and the amine with the standard Fmoc carbamate. Initial attempts at directly protecting the phenolic group were unsuccessful due to the limited solubility of **1** and the cross reactivity of the amino acid functionalities. To simultaneous address both of these issues we turned to a known 9-borabicyclononane (9-BBN)-based strategy previously developed for the protection of α-amino acids.[25] Treatment of **1** with a slight excess of 9-BBN dimer led to formation of complex **4** which exhibited good solubility in organic solvent (Scheme 2). Compound **4** was next treated with chloromethyl methyl ether to provide MOM-protected intermediate **5**. Decomplexation of the 9-BBN moiety was achieved using very mild conditions whereby compound **5** was treated with a 1:6 mixture of methanol and chloroform to yield the free amino acid **6**.[26,27] Standard conditions were then employed to convert compound **6** directly into the Fmoc-protected building block **7** in good yield.

To demonstrate the utility of building block **7** in SPPS, a small model peptide based upon the Leu-enkephalin pentapeptide

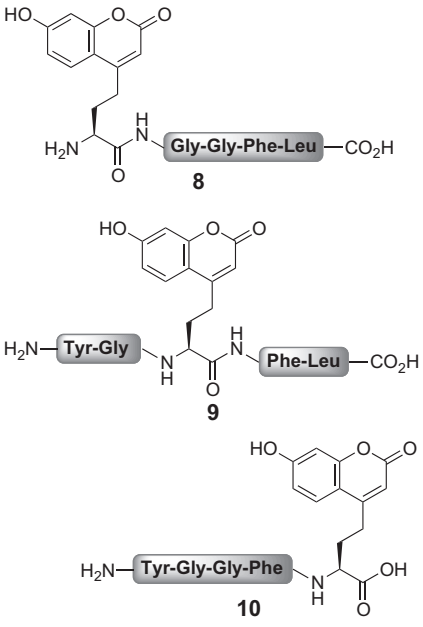


Figure 2. Leu-Enkephalin analogues **8**–**10** prepared with incorporation of 7-HC building block **7**.

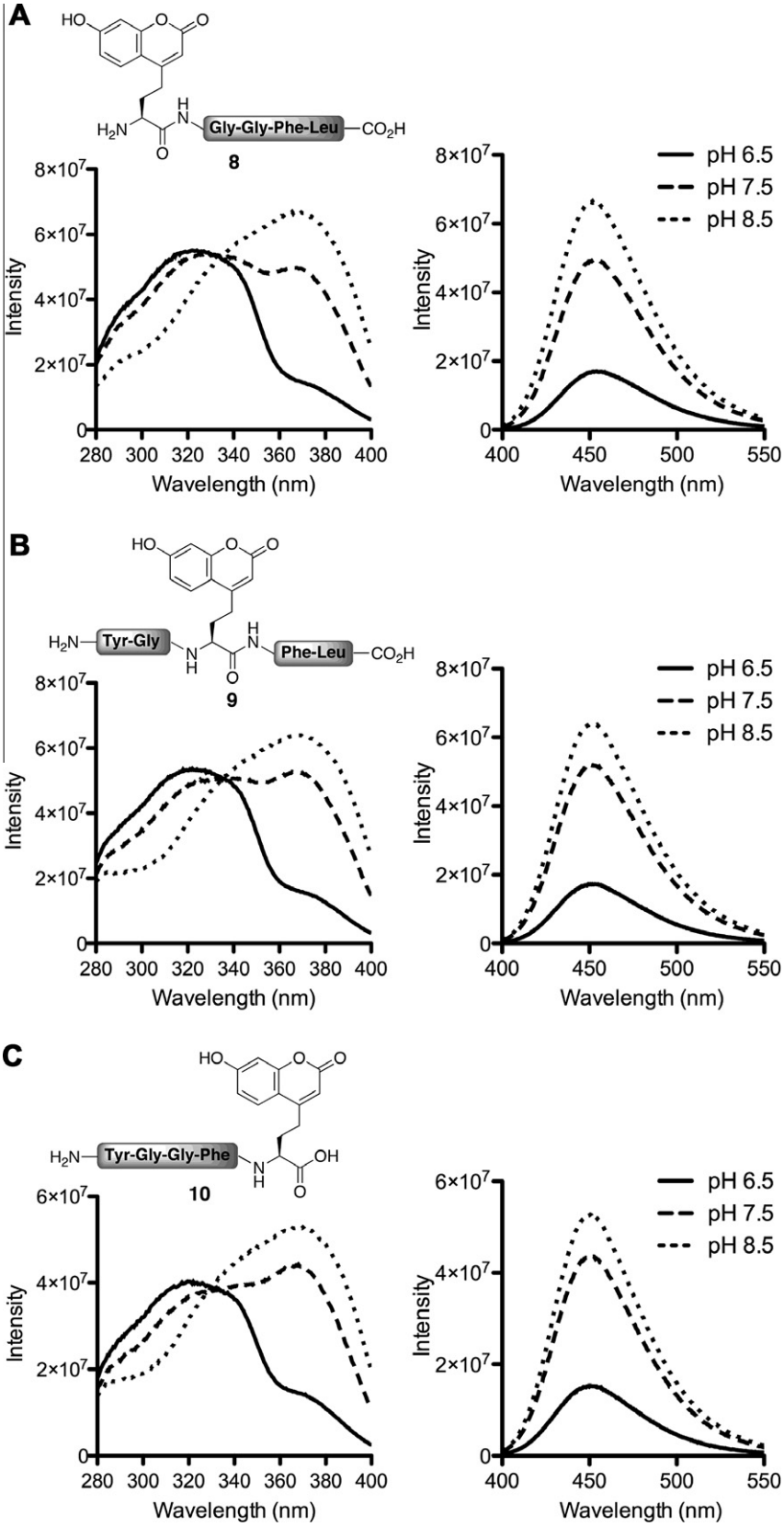


Figure 3. Fluorescence excitation spectra, emission recorded at 450 nm (left) and fluorescence emission spectra, excitation at 363 nm (right) at pH 6.5, 7.5, and 8.5 for peptides **8** (A), **9** (B), and **10** (C).

(YGGFL) was employed. Three different enkephalin analogues were prepared whereby **7** was incorporated at either the C-terminus, N-terminus or in the center of the peptide (Fig. 2). In all cases **7** was incorporated with ease using standard SPPS coupling and Fmoc deprotection conditions to provide the desired peptide as the major product after global deprotection and cleavage from the resin.

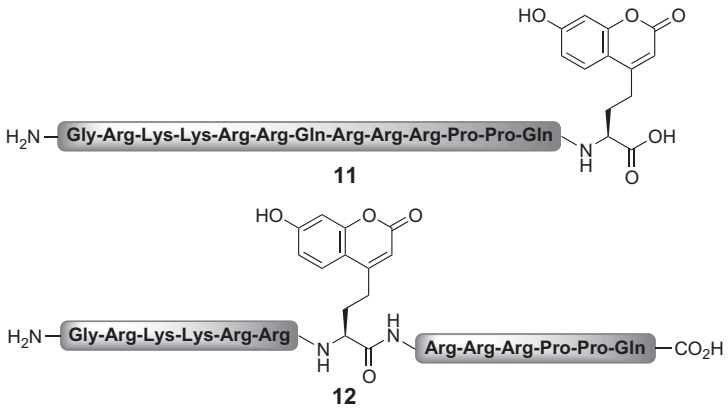


Figure 4. Analogues of the HIV-Tat$_{48–60}$ peptide prepared with addition of 7-HC at the C-terminus (compound **11**) or substitution for Gln$_{54}$ (compound **12**).

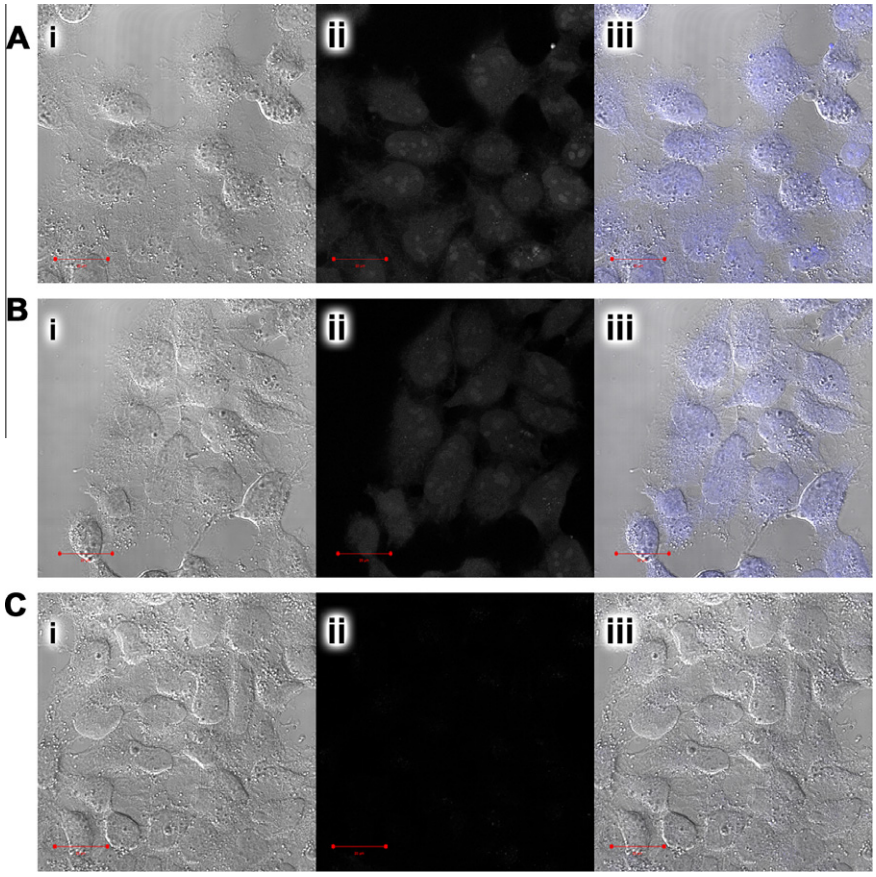


Figure 5. HeLa cell internalization of fluorescent HIV-Tat$_{48–60}$ analogues visualized by confocal microscopy: (A) Analogue **11**, (B) Analogue **12**, and (C) Negative control (no peptide added). For each panel: (i) differential interference contrast image, (ii) confocal fluorescence image (grayscaled for clarity) and (iii) overlay (native colors).

Peptides **8–10** were further characterized by fluorescence spectroscopy over a range of pH values to provide the excitation and emission spectra illustrated in Figure 3. Of particular note in Figure 3 is the clear pH dependence of the fluorescence exhibited by each peptide. While the fluorescence excitation spectrum of each peptide is essentially identical at both pH 6.5 and pH 8.5, at pH 7.5 there are differences. In the case of peptide **10**, the spectrum obtained at pH 7.5 indicates a more red-shifted excitation maximum which may suggest an equilibrium laying further towards the deprotonated coumarin species. By comparison, the spectral shift observed for peptide **8** at pH 7.5 is significantly less while the spectrum obtained for peptide **9** at pH 7.5 appears to fall in between that obtained for **8** and **10** (for comparative purposes, Supplementary Fig. S1 provides an overlay of the fluorescence excitation spectra obtained for peptides **8–10** at pH 7.5). These observations can be rationalized based upon the proximity of the 7-HC residue to the C-terminus and the impact this likely has on the effective pK_a of the 7-hydroxy coumarin moiety (the approximate pK_a of 7-hydroxy coumarin is reported to be 7.8).[28]

To further establish the utility of building block **7** in the preparation of more challenging and biologically relevant constructs, analogues of the HIV-Tat peptide were next investigated. It is well established that truncated peptides comprising the arginine rich motif of the HIV-Tat protein are able to cross membranes and as

such can also be used to deliver various molecular cargos to the interior of cells.[29,30] In this regard, one of the more commonly employed HIV-Tat peptide derived sequences, GRKKRRQRRRPPQ, is a 13-mer spanning residues 48–60 of the full-length Tat protein.[31,32] To generate two fluorescent analogues of HIV-Tat$_{48-60}$, building block **7** was incorporated, again using standard Fmoc-SPPS approaches. In analogue **11** the fluorescent amino acid was included at the C-terminus of the peptide while in analogue **12** the central glutamine residue was replaced with 7-HC (Fig. 4).

To assess both the cell permeability and fluorescent properties of Tat analogues **11** and **12**, each peptide was incubated with HeLa cells after which internalization was visualized using confocal microscopy. As is seen in Figure 5, those cells treated with either **11** or **12** exhibit a clear and evenly distributed fluorescence indicative of cellular uptake of the peptides. While internalization of analogue **11** was expected, given that it retains the native HIV-Tat$_{48-60}$ sequence, the observation that analogue **12** is also able to effectively enter cells is of note. In analogue **12** the glutamine residue at position 54 is replaced by 7-HC to generate a fluorescent mutant of HIV-Tat$_{48-60}$. While it is has been reported that the substitution of alanine for glutamine at position 54 is tolerated,[33] the inclusion of larger or unnatural amino acids such as 7-HC at this position, and the impact this might have on cell permeability, has not been described.

In general, the preparation of fluorescently labeled peptides often requires the initial synthesis of the peptide of interest by SPPS after which the fluorophore is introduced, typically as a conjugate containing either an amino- or thiol-reactive moiety. Aside from requiring an additional reaction step, this approach can be problematic in peptides bearing more than one amino or thiol group, presenting the possibility of generating multiply labeled species or mixtures bearing different labeling patterns. By comparison, the use building block **7** allows for the direct and site-specific introduction of the fluorescent moiety as an integrated part of the SPPS process. These advantages are clearly illustrated in the preparation of the fluorescent Leu-enkephalin analogues **8–10** and HIV-Tat analogues **11** and **12** which were synthesized in direct fashion using conventional SPPS.

3. Conclusions

In summary, an optimized protocol for the concise and efficient preparation of the fluorescent amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine **1** on the multi-gram scale has been developed. This amino acid itself is of great value, having gained popularity as one of the smallest fluorescent labels that can be selectively incorporated into proteins via 'expanded genetic code' technologies. Furthermore, amino acid **1** was successfully converted into building block **7**, suitably protected for use in SPPS. Using standard Fmoc SPPS approaches, building block **7** was incorporated into a series of model peptides based upon the Leu-enkephalin (5-mer) and the HIV-Tat (13-mer) peptides. When used as an Fmoc SPPS building block, compound **7** requires no special handling and in all cases examined was readily incorporated into the synthetic peptides prepared. Access to such building blocks expands the repertoire of available tools for use in future explorations requiring access to proteins and peptides bearing small fluorescent labels.

4. Experimental

4.1. General

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Flash chromatography was performed using Merck type 60, 230–400 mesh silica gel. High resolution mass spectrometry (HRMS) analysis was performed using an ESI-TOF LC/MS instrument. ^{1}H NMR spectra were recorded at 300 MHz with chemical shifts reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS). ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; qn, quintet and m, multiplet), number of protons, coupling constant (J) in Hertz (Hz) and assignment. When appropriate, the multiplicity is preceded by br, indicating that the signal was broad. ^{13}C NMR spectra were recorded at 75 MHz with chemical shifts reported relative to the residual carbon resonance of the solvent used. ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. All literature compounds had ^{1}H NMR, and mass spectra consistent with the assigned structures.

4.2. Preparative details and analytical data for compounds synthesized

4.2.1. (*S*)-1-Benzyl-7-ethyl-2-(benzyloxycarbonylamino)-5-oxoheptanedioate (3)

N-Cbz-L-Glu-*O*-Bn (10.0 g, 27 mmol, 1.0 equiv) was dissolved in dry THF (100 mL) and treated with 1,1′-carbonyldiimidazole (5.69 g, 35 mmol, 1.3 equiv) and the mixture was stirred at room temperature for 2 h. Separately, potassium monoethylmalonate (6.8 g, 40 mmol) was dissolved in water (25 mL) and treated with concentrated hydrochloric acid (6 mL) and NaCl (11 g). The mixture was extracted with EtOAc (5 × 25 mL) and the combined EtOAc layers dried over Na_2SO_4, filtered and evaporated. The free acid of monoethylmalonate thus obtained was then directly dissolved in dry THF (80 mL), treated with magnesium ethoxide (2.9 g, 25 mmol), and stirred at room temperature for 1 h to generate magnesium monoethylmalonate. The fresh solution of magnesium monoethylmalonate in THF was then directly added to CDI-activated glutamatic acid species and the mixture stirred at room temperature for 16 h. The volume of the reaction mixture was next reduced to ca. 50 mL after which Et_2O (300 mL) and 1 M HCl (100 mL) were slowly added. The layers were separated and the Et_2O layer washed with 1 M HCl (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), dried over Na_2SO_4, and evaporated to yield a yellow oil. This material was applied to a silica column and eluted using a gradient of 1:3 → 2:3 EtOAc/hexane to yield compound **3** as a light yellow oil that solidified upon storage (10.76 g, 24.4 mmol, 90%). *Analytical data:* R_f 0.15 (1:3 EtOAc/hexane); mp: 55–57 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.33 (m, 10H), 5.48 (br d, 1H, J = 7.9 Hz), 5.15 (s, 2H), 5.09 (s, 2H), 4.43–4.36 (m, 1H), 4.15 (q, 2H, J = 7.1 Hz), 3.35 (s, 1H), 2.63–2.50 (m, 1H), 2.22–2.15 (m, 1H), 2.00–1.88 (m, 1H), 1.24 (t, 3H, J = 7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.5, 171.7, 167.0, 156.0, 136.2, 135.2, 128.7, 128.5, 128.4, 128.2, 128.1, 67.4, 67.1, 61.4, 53.2, 49.2, 38.6, 26.2, 14.1. HRMS (ESI) Calcd for $C_{24}H_{27}NO_7Na$ [M+Na]$^+$, 464.1685. Found 464.1673.

4.2.2. L-(7-Hydroxycoumarin-4-yl) ethylglycine (1)

Resorcinol (6.98 g, 63.4 mmol, 5.0 equiv) was dissolved in methanesulfonic acid (50 mL) with vigorous stirring. Once homogenous, the clear solution was cooled on ice and compound **3** (5.6 g, 12.7 mmol, 1.0 equiv) added. After 20 min, the ice batch was removed and the mixture stirred vigorously under N_2 for 4 h at room temperature. The mixture was then poured into a 500 mL volume of Et_2O and placed on ice for 30 min. The Et_2O was decanted and the remaining precipitate dissolved in 2 M HCl (200 mL). The HCl layer was washed with EtOAc (2 × 100 mL) after which the pH of the aqueous layer was adjusted to 3.5 by slow addition of 6 M NH_4OH. Upon overnight storage at 4 °C the desired product crystallized from solution. The crystals were collected by filtration, washed with Et_2O, and dried under vacuum to yield compound **1**

as a dark red solid (2.13 g, 8.1 mmol, 64%). *Analytical data:* Mp: 251 °C (decomp.); ^{1}H NMR (300 MHz, 0.1 M KOD/D_2O) δ 7.37 (d, 1H, J = 9.0 Hz), 6.52–6.48 (m, 1H), 6.50 (ddd, 1H, J_1 = 0.6 Hz, J_2 = 2.4 Hz, J_3 = 8.9 Hz), 6.31 (dd, 1H, J_1 = 0.7 Hz, J_2 = 2.4 Hz), 5.79 (s, 1H), 3.22 (t, 1H, J = 6.2 Hz), 2.62 (t, 2H, J = 8.3 Hz), 1.87–1.69 (m, 2H); ^{13}C NMR (75 MHz, 0.1 M KOD/D_2O) δ 177.3, 171.6, 165.9, 159.0, 156.0, 125.5, 118.0, 107.9, 104.5, 104.3, 54.9, 31.1, 27.3. HRMS (ESI) Calcd for $C_{13}H_{14}NO_5$ = [M+H]$^+$, 264.0872. Found 264.0880.

4.2.3. (1*R*,4′*S*,5*S*)-4′-(2-(7-(methoxymethoxy)-2-oxo-2*H*-chromen-4-yl)ethyl)-5′-oxospiro[bicyclo[3.3.1]-nonane-9,2′-[1,3,2]oxazaborolidin]-3′-ium-11-uide (5)

9-BBN-H dimer (2.39 g, 9.78 mmol, 1.1 equiv) was dissolved in methanol (100 mL) while stirring vigorously under N_2 at reflux temperature for 30 min. Amino acid **1** (2.25 g, 8.55 mmol, 1.0 equiv) was added to the yellow solution and the mixture stirred under reflux conditions for 2 h during which time the mixture was homogenous. The mixture was then concentrated under vacuum and re-dissolved in $CHCl_3$. Silica gel was added directly to the $CHCl_3$ solution after which the solvent was evaporated leaving a mixture of the crude intermediate **4** adsorbed on silica. This silica mixture was applied to the top of a silica column and rapidly eluted using a gradient of 4:1 EtOAc/hexane → 4:1 acetone/hexane to yield compound **4** in a semi-pure form (2.5 g, 6.5 mmol, 76%). This material was then directly dissolved in acetone (130 mL) and treated with K_2CO_3 (1.8 g, 13 mmol, 2.0 equiv) while stirring under N_2. This suspension was vigorously stirred for 15 min, cooled on ice and then chloromethyl methyl ether (743 μL, 9.8 mmol, 1.5 equiv) was added. The ice bath was removed and the mixture was stirred at room temperature for 4 h. The volume was then reduced to ca. 25 mL and EtOAc (250 mL) added. The EtOAc layer was washed with brine (1 × 250 mL), dried over Na_2SO_4, filtered, and concentrated. The residue was applied to a silica column and eluting with a gradient of 1:1 → 3:2 EtOAc/hexane to yield compound **5** in pure form as an off-white solid (2.0 g, 4.7 mmol, 73%). *Analytical data:* Mp: 129 °C (decomp.) ^{1}H NMR (300 MHz, CD_3OD) δ 7.75 (d, 1H, J = 8.9 Hz), 7.00 (dd, 1H, J_1 = 2.6 Hz, J_2 = 8.9 Hz), 6.94 (d, 1H, J = 2.4 Hz), 6.54–6.48 (m, 1H), 6.19 (s, 1H), 5.99–5.93 (m, 1H), 5.25 (s, 2H), 3.88–3.83 (m, 1H), 3.46 (s, 3H), 3.12–2.97 (m, 2H), 2.35–2.26 (m, 1H), 2.15–2.06 (m, 1H), 1.94–1.46 (m, 12H), 0.61 (s, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 176.7, 163.3, 161.8, 157.6, 156.3, 127.0, 114.9, 114.5, 111.8, 104.5, 95.5, 56.6, 55.6, 32.6, 32.5, 32.4, 32.2, 30.6, 29.2, 25.6, 25.2. HRMS (ESI) Calcd for $C_{23}H_{31}BNO_6$ [M+H]$^+$, 428.2244. Found 428.2234.

4.2.4. (*S*)-2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-4-(7-(methoxymethoxy)-2-oxo-2*H*-chromen-4-yl)butanoic acid (7)

Compound **5** (2.8 g, 6.6 mmol) was dissolved in a mixture of methanol (8 mL) and chloroform (40 mL) and stirred at 40 °C (with a condenser attached) for 48 h. The mixture was concentrated and the residue triturated first with hot hexanes (2 × 50 mL) and then with diethyl ether (2 × 50 mL). The residue was then dried under vacuum to yield intermediate **6** as an off-white solid (2.03 g, 6.6 mmol) that was used directly in the following step. To a mixture of compound **6** (2.03 g, 6.6 mmol, 1.0 equiv) and $NaHCO_3$ (0.61 g, 7.3 mmol, 1.1 equiv) in water (42 mL), was added a solution of FmocOSu (2.67 g, 7.9 mmol, 1.2 equiv) in acetone (34 mL). The reaction mixture was stirred overnight at room temperature after which it was concentrated under vacuum to remove the acetone. A solution of 0.2 M HCl (100 mL) was then added and the aqueous layer was extracted with EtOAc (3 × 100 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated. The residue was applied to a silica column and eluted using a gradient of 1:1 → 4:1 EtOAc/hexane to yield compound **7** as a white foam (2.14 g, 4.0 mmol, 61%). *Analytical data:* Mp: 80 °C (decomp.) ^{1}H NMR (300 MHz, CD_3OD) δ 7.75–7.71 (m, 2H), 7.67–7.58 (m, 2H), 7.37–7.23 (m, 4H), 6.97–6.93 (m, 2H), 6.14 (s, 1H), 5.24–5.21 (m, 2H), 4.38–4.35 (m, 2H), 4.30–4.25 (m, 1H), 4.19–4.14 (m, 1H), 3.43 (s, 3H), 2.83–2.78 (m, 2H), 2.58–2.15 (m, 1H), 2.08–1.98 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 173.7, 161.9, 160.4, 157.2, 156.4, 155.0, 143.9, 143.7, 141.2, 127.4, 126.8, 125.6, 124.8, 119.5, 113.4, 113.1, 110.7, 103.2, 94.1, 66.4, 55.2, 53.4, 30.2, 27.7. HRMS (ESI) Calcd for $C_{30}H_{28}NO_8$ [M+H]$^+$, 530.1815. Found 530.1802.

4.3. Peptide synthesis

Enkephalin analogues **8**, **9** and **10** were prepared using an automated peptide synthesizer. Peptides were assembled on 2-chlorotrityl resin, on a 0.25 mmol scale. Peptide couplings were performed using 4.0 equiv of Fmoc-protected amino acid, 4.0 equiv of HBTU, 4.0 equiv of HOBt and 8.0 equiv of DIPEA in an approximate total volume of 10 mL NMP at room temperature for 1 h. In the case of the 7-hydroxycoumarin building block (**7**), 2.0 equiv of the 7-hydroxycoumarin building block (**7**) were used, keeping all other reagents and reaction times equal. Upon completion of SPPS, peptides were cleaved from the resin and the side chains deprotected using TFA/TIS/H_2O (95:2.5:2.5), followed by precipitation in MTBE/hexanes (1:1) and washing twice with MTBE, to yield the crude peptides. Each peptide was purified to homogeneity using RP-HPLC, employing a C_{18} column (250 × 22 mm, 300 Å, 10 μm) with a gradient of 5–95% acetonitrile (0.1% TFA) in 90 min at a flow rate of 12.0 mL min^{-1}. Peptide purity was confirmed by analytical HPLC (see Supplementary data sections for HPLC traces) *Analytical data peptide **8**:* ^{1}H NMR (300 MHz, CD_3OD) δ 7.66 (d, 1H, J = 8.8 Hz), 7.23–7.13 (m, 5H) 6.81 (d, 1H, J = 8.8 Hz), 6.71 (s, 1H), 6.14 (s, 1H), 4.72–4.67 (m, 1H), 4.42–4.37 (m, 1H), 4.15–4.11 (m, 1H), 4.05–3.72 (m, 4H), 3.18–3.12 (m, 2H), 2.99–2.88 (m, 4H), 2.27–2.23 (m, 1H), 1.67–1.59 (m, 2H), 0.90 (dd, 6H, J_1 = 5.8 Hz, J_2 = 12.9 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 178.2, 176.2, 173.9, 173.7, 173.2, 166.2, 165.6, 159.8, 159.4, 140.8, 132.9, 131.9, 130.2, 129.7, 117.1, 115.1, 113.1, 106.3, 58.2, 56.7, 54.7, 46.3, 45.8, 44.0, 41.5, 33.7, 30.4, 25.9, 24.4; HRMS (ESI) Calcd for $C_{32}H_{40}N_5O_9$ [M+H]$^+$, 638.2826. Found 638.2825. *Analytical data peptide **9**:* ^{1}H NMR (300 MHz, CD_3OD) δ 7.58 (d, 1H, J = 8.8 Hz), 7.30–7.20 (m, 4H), 7.15–7.10 (m, 3H), 6.83–6.76 (m, 3H), 6.72–6.71 (m, 1H), 6.06 (s, 1H), 4.75–4.70 (m, 1H), 4.40 (q, 2H, J = 6.3 Hz), 4.09–4.04 (m, 1H), 4.00–3.95 (m, 1H), 3.81–3.76 (m, 1H), 3.21–3.14 (m, 2H), 3.01–2.91 (m, 2H), 2.71–2.66 (m, 2H), 2.03–1.86 (m, 2H), 1.71–1.59 (m, 3H), 0.87 (dd, 6H, J_1 = 6.1 Hz, J_2 = 17.2 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 178.2, 176.1, 175.5, 173.7, 173.3, 166.4, 165.5, 160.9, 159.4, 140.9, 134.1, 132.9, 132.0, 130.3, 130.0, 128.5, 119.4, 117.0, 115.3, 113.2, 106.2, 58.6, 58.2, 56.8, 54.7, 46.2, 44.1, 41.2, 40.3, 34.5, 31.3, 25.9, 24.4; HRMS (ESI) Calcd for $C_{39}H_{46}N_5O_{10}$ [M+H]$^+$, 744.3245, Found 744.3231. *Analytical data peptide **10**:* ^{1}H NMR (300 MHz, CD_3OD) δ 7.61 (d, 1H, J = 8.6 Hz), 7.28–7.24 (m, 3H), 7.20–7.18 (m, 2H), 7.13–7.10 (m, 2H), 6.84–6.77 (m, 3H), 6.71 (s, 1H), 6.07 (s, 1H), 4.73–4.68 (m, 1H), 4.46–4.42 (m, 1H), 4.19–4.14 (m, 1H), 4.00–3.94 (m, 2H), 3.82–3.74 (m, 2H), 3.21–3.14 (m, 2H), 3.05–2.95 (m, 2H), 2.87–2.77 (m, 2H), 2.26–2.23 (m, 1H), 2.09–2.04 (m, 1H); ^{13}C (75 MHz, CD_3OD) δ 173.0, 172.4, 170.2, 169.9, 169.8, 162.6, 161.5, 156.9, 156.6, 155.4, 136.8, 130.2, 129.0, 128.1, 126.4, 125.9, 124.6, 115.5, 113.2, 109.8, 102.4, 54.8, 54.7, 51.5, 42.7, 41.9, 37.2, 36.3, 30.0, 27.7; HRMS (ESI) Calcd for $C_{35}H_{38}N_5O_{10}$ [M+H]$^+$, 688.2619. Found 688.2622.

HIV-Tat$_{48–60}$ analogues **11** and **12** were prepared via standard Fmoc SPPS on 2-chlorotrityl resin, on a 0.25 mmol scale. Peptide couplings were performed using 4.0 equiv of Fmoc-protected amino acid, 4.0 equiv of HBTU, 4.0 equiv of HOBt and 8.0 equiv of DIPEA in an approximate total volume of 10 mL NMP at room

temperature for 1 h. In the case of the 7-hydroxycoumarin building block (**7**), 2.0 equiv of the 7-hydroxycoumarin building block (**7**) were used, keeping all other reagents and reaction times equal. Upon completion of SPPS, peptides were cleaved from the resin and the side chains deprotected using TFA/TIS/H_2O (95:2.5:2.5) for 4 h, followed by precipitation in MTBE/hexanes (1:1) and washing twice with MTBE, to yield the crude peptides. Each peptide was purified to homogeneity using RP-HPLC, employing a semi-preparative C_{18} column (250 × 10 mm, 300 Å, 10 μm) with a gradient of 5–33% acetonitrile (0.1% TFA) in 90 min at a flow rate of 6.0 mL min^{-1}. Peptide purity was confirmed by analytical HPLC (see Supplementary data sections for HPLC traces) and identity confirmed by MALDI-MS: Peptide **11** LRMS (MALDI) Calcd for $C_{83}H_{143}N_{36}O_{20}$ $[M+H]^+$, 1964.1. Found 1964.5. Peptide **12** LRMS (MALDI) Calcd for $C_{78}H_{135}N_{34}O_{18}$ $[M+H]^+$, 1836.1. Found 1836.5.

4.4. Fluorescence spectroscopy

60 μM solutions of peptides **8**, **9**, and **10** were prepared in Britton–Robinson buffer at pH 6.5, 7.5 and 8.5. Excitation and emission spectra were recorded on a Horiba Fluorolog-3 spectrofluorometer. Emission spectra were excited at 363 nm and the emission recorded from 400 to 550 nm. Excitation spectra were recorded from 280 to 400 nm with the emission set at 450 nm. The slit width was set at 1 nm for both excitation and emission measurements.

4.5. Cell-peptide incubations and peptide visualization

2×10^4 HeLa cells were plated onto each well of an eight-chamber culture slide and grown overnight in RPMI media complemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin in a 5% CO_2 atmosphere at 37 °C. Cells were treated with 10 μM peptide **11** or **12** for 2 h, washed twice with phosphate-buffered saline, fixed in 3.7% paraformaldehyde for 30 min at room temperature, and washed twice with phosphate-buffered saline. Culture slides were mounted using FluorSave (EMD Millipore) fluorescent mounting medium.

4.6. Confocal microscopy

Images were obtained using a Zeiss LSM710 confocal laser scanning microscope. Excitation was provided with Diode 405-30 laser at 405 nm, and the emitted light detected between 410 and 590 nm. Data were processed using Zen image analysis software.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.10.055.

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