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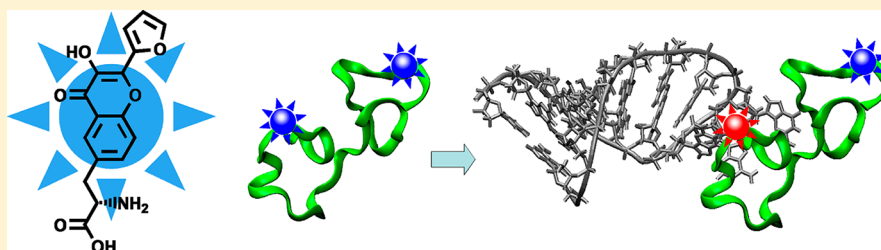
Aleksandr V. Strizhak,^{‡,§,†} Viktoriia Y. Postupalenko,^{‡,§} Volodymyr V. Shvadchak,[§] Nelly Morellet,[‡] Eric Guittet,[‡] Vasyly G. Pivovarenko,^{‡,§} Andrey S. Klymchenko,^{*,§} and Yves Mély^{*,§}

[§]Laboratoire de Biophotonique et Pharmacologie, UMR 7213 CNRS, Université de Strasbourg, Faculté de Pharmacie, 67401 Illkirch, France

[†]Department of Chemistry, National Taras Shevchenko University of Kyiv, 01601 Kyiv, Ukraine

[‡]CNRS UPR 2301, Chimie et biochimie structurales, Institut de Chimie des Substances Naturelles, Centre de recherche de Gif-sur-Yvette, 91190 Gif-sur-Yvette, France

Supporting Information



ABSTRACT: Non-natural amino acids are important tools for site-selective probing of peptide properties and interactions. Here, for the first time a fluorescent L-amino acid, exhibiting excited-state intramolecular proton transfer (ESIPT) and hydration-sensitive dual emission, was synthesized. It is an analogue of L-tryptophan bearing a slightly larger 2-(2-furyl)-3-hydroxychromone aromatic moiety instead of indole. This new amino acid was incorporated through solid-phase synthesis into NC(11–55), the zinc finger domain of the HIV-1 nucleocapsid protein, that exhibits potent nucleic acid chaperone properties. It was substituted for the Trp37 and Ala30 residues, located in the distal finger motif and the linker between the fingers of NC(11–55), respectively. Though the highly conserved Trp37 residue plays a key role in NC(11–55) structure and activity, its substitution for the new fluorescent analogue preserved the folding, the nucleic acid binding and chaperone activity of the peptide, indicating that the new amino acid can conservatively substitute Trp residues. In the presence of oligonucleotides, the Trp37-substituted peptide, but not the Ala30 variant, showed strong changes of the dual emission corresponding to local dehydration. The results are in line with NMR data, suggesting that the fluorescent amino acid interacts similarly to Trp37 with the nucleobases and is thus screened from water. Due to the exceptional sensitivity of its ESIPT fluorophore to hydration in highly polar environment, the new amino acid appears as a promising tool for substituting Trp residues and site-selectively investigating peptide–nucleic acid complexes.

■ INTRODUCTION

Non-natural amino acids are important tools to investigate peptides and proteins, since they can be incorporated at any position, and thus serve as local probes for site-selective monitoring of protein properties and functions. Probably, the most popular ones are those bearing fluorine^{1–4} and dyes^{5–7} used with NMR and fluorescence techniques, respectively. Fluorescent amino acids are particularly attractive, due to the ultimate sensitivity of fluorescence-based methods. Since the natural fluorescent amino acid tryptophan suffers from poor fluorescence properties, continuous efforts have been made to design amino acids with improved fluorophores.^{5–7} Of special interest in this respect are environmentally sensitive (or solvatochromic) fluorophores, which change their emission properties in response to changes in their environment.^{7,8} Biomolecular interactions commonly decrease the polarity at the labeling site due to screening from water and, thus, can be

readily monitored by environmentally sensitive fluorescent amino acids.⁷ For instance, a Prodan-based amino acid was used for monitoring the interaction of S-peptide with ribonuclease S⁹ and δ -opioid receptor with antagonists¹⁰ and to estimate the local dielectric constant of the B1 domain of the staphylococcal protein G.⁵ Moreover, an amino acid based on 4-dimethylaminophthalimide (4-DMAP) fluorophore was applied to sense binding of labeled octapeptides to the 14–3–3bp protein.¹¹ Finally, improved analogues of 4-DMAP, such as 6-dimethylaminonaphthalimide, were recently applied to investigate the SH2 phosphotyrosine binding domains,¹² major histocompatibility complexes (MHC) at the cell surface,⁷ and peptide–calmodulin interactions.¹³ Despite the importance of

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environmentally sensitive fluorescent amino acids in biomolecular research, their examples are limited.

Moreover, most examples are focused on protein–protein interactions,⁷ while applications of fluorescent amino acids for sensing peptide–oligonucleotide (ODNs) interactions have been poorly explored (Figure 1A). To address this problem, we

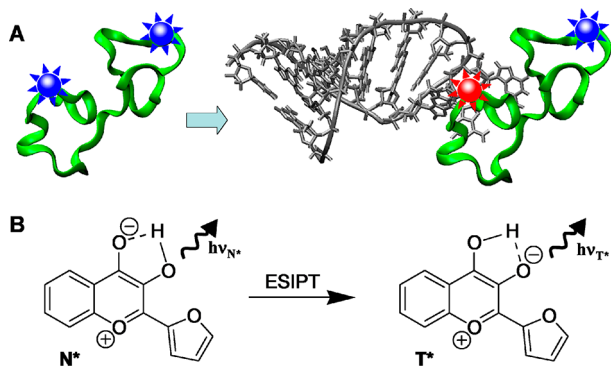


Figure 1. Monitoring of peptide/oligonucleotide interaction using solvatochromic fluorescent amino acid. (A) Principle: the interaction of the labeled peptide (in green) with oligonucleotides changes the fluorescence intensity and color of the label. (B) ESIPT reaction in 2-(2-furyl)-3-hydroxychromone dye.

selected 3-hydroxychromone (3HC) fluorophores which undergo excited-state intramolecular proton transfer (ESIPT),¹⁴ resulting in the emission of both a normal (N^*) excited state and an ESIPT product tautomer (T^*) (Figure 1B). The dual emission of 3HC dyes is highly sensitive to polarity and H-bonding interactions.^{8,15–23} The 2-(2-furyl)-3-hydroxychromone (FHC) label is particularly interesting due to the high sensitivity of its dual emission to polar environments.^{15,17,24,25} Being attached to the N-terminus of peptides and oligonucleotides, it shows strong changes in its dual emission upon interaction with ODNs,^{26,27} and allows quantification of water content in its surrounding.²⁸ This prompted us to develop an L-amino acid based on the FHC fluorophore.

As a target protein for labeling, we selected the nucleocapsid protein (NC) of the human immunodeficiency virus, type 1 (HIV-1), which plays a crucial role in the viral life cycle.^{29,30} NC is a small (55 amino acids) basic protein, characterized by two rigid zinc fingers connected by a flexible basic linker and flanked by poorly folded N- and C-terminal basic domains.^{31,32} NC binds both specifically^{33–38} and nonspecifically,^{35,39–41} to a large range of nucleic acid sequences. Specific binding is mainly mediated through the folded finger motifs, with a key role being played by the hydrophobic residues forming a hydrophobic platform at the top of the folded fingers.^{34,36,38} This hydrophobic plateau also plays a key role in the nucleic acid chaperone properties of NC, which direct the structural rearrangement of ODNs into their most stable conformation and promote the annealing of complementary ODN sequences through specific pathways.^{42–44} Within this hydrophobic plateau, the Trp37 residue plays a particularly important role, since its substitution by a nonaromatic residue leads to a dramatic decrease in ODN affinity,^{35,40,45} a complete loss in the highly specific ODN destabilization component of the NC chaperone properties,^{40,42} as well as a complete loss of HIV-1 infectivity.⁴⁶ In the present work, we synthesized an L-amino acid analogue based on 2-(2-furyl)-3-hydroxychromone dye through an original route from L-tyrosine. It was then

incorporated at two different positions of the NC(11–55), inside and outside of its fingers, which allowed us to site-selectively investigate its interaction with ODNs. Remarkably, substitution of Trp residue for the new amino acid marginally alters the folding as well as the nucleic acid binding and chaperone activity of the peptide, indicating that this new amino acid could be used as a Trp mimic. The ODNs selected for binding (Figure S1, Supporting Information) to the labeled peptides were SL2 and SL3, two stem-loops of the HIV-1 RNA encapsidation sequence^{33,35,38} and $\Delta P(-)$ PBS, the cDNA copy of the primer binding site, deleted of its 5' overhang.³⁶ These three ODNs bind NC with high affinity and 1:1 stoichiometry, and their structures in complex with NC are known.^{34,36,38} The results showed that this amino acid allows probing of protein sites responsible for DNA binding.

MATERIALS AND METHODS

All the solvents and chemicals were purchased from Sigma-Aldrich Chemical Co. For absorption and fluorescence studies, the solvents were of spectroscopic grade. $\Delta P(-)$ PBS DNA, SL2, and SL3 RNAs were synthesized and HPLC-purified by IBA GmbH (Germany). Concentrations of the ODNs were calculated from their absorbance using the molar extinction coefficients at 260 nm provided by the supplier.

NMR spectra were recorded on a 400 and 500 MHz BrukerAvance III, BBFO+ at room temperature. Mass spectra were obtained on a Bruker HCT Ultra and Agilent Technologies Accurate-Mass Q-TOF LC/MS 6520 mass spectrometers.

Synthesis of Fmoc-3HCaa. The synthesis of compounds **1** and **2** (Scheme S1) was performed as described previously.^{47,48}

N-(tert-Butoxycarbonyl)-3-[(2E)-3-(2-furyl)prop-2-enoyl]-L-tyrosine (3). 6.425 g (19.89 mmol) of 3-acetyl-N-(tert-butoxycarbonyl)-L-tyrosine **2** was dissolved in 48 mL of ethanol upon stirring under Ar atmosphere. To the reaction mixture, 48 mL of degassed 25% solution of sodium hydroxide was added upon cooling in a cold water bath. Then, 2.409 g of furfural (25.09 mmol, 1.26 equiv) was added and the mixture was stirred for 12 h at RT under Ar. Additional 0.5 g of furfural was added and the mixture was stirred overnight. The reaction mixture was acidified with HCl and diluted twice with water. The formed precipitate was filtered and washed with diluted ethanol and water, dried, and used in the next step without further purification. Yield 7.62 g (95%) of chalcone **3** as a yellow powder. ¹H NMR (500 MHz, MeOD) δ 1.33 (s, 9H), 2.87–2.92 (dd, J = 13.9 Hz, J = 8.85 Hz, 1H), 3.16–3.19 (dd, J = 13.9 Hz, J = 4.43 Hz, 1H), 4.31–4.34 (dd, J = 8.85 Hz, J = 4.43 Hz, 1H), 6.61–6.62 (dd, J = 3.16 Hz, J = 1.89 Hz, 1H), 6.87–6.89 (d, J = 8.21 Hz, 1H), 6.94 (d, J = 3.8 Hz, 1H), 7.39–7.41 (dd, J = 8.22 Hz, J = 1.9 Hz, 1H), 7.64–7.71 (dd, J = 15.16 Hz, J = 4.42 Hz, 2H), 7.71 (d, J = 1.9 Hz, 1H), 7.86–7.87 (d, J = 1.9 Hz, 1H). ¹³C NMR (500 MHz, MeOD) δ 28.66, 38.11, 55.49, 80.47, 114.01, 118.33, 118.84, 119.02, 120.97, 129.45, 131.65, 132.31, 138.58, 147.17, 152.98, 157.68, 163.09, 175.69, 194.74 (two carbon signals are absent due to incidental equivalence). HRMS (EI) m/z calcd for $C_{21}H_{23}NO_7$ 401.14745, found: 401.147.

N-(tert-Butoxycarbonyl)-3-[2-(2-furyl)-3-hydroxy-4-oxo-4H-chromen-6-yl]-L-alanine (4). 7.62 g (19 mmol) of chalcone **3** was dissolved in 75 mL of ethanol, cooled in an ice bath. Then, 75 mL of 1 M solution of sodium hydroxide was added with stirring. Next, 4.73 mL (41.8 mmol) of 30% hydrogen peroxide was added and the mixture was stirred in ice bath for 4

h. The reaction was monitored by TLC (silica, EtOAc/MeOH 9:1). After completing the reaction, the mixture was acidified and the formed precipitate was filtered. Yield of the crude 3-hydroxychromone **4** was 3.56 g (45%). It was purified to about 95% purity by crystallization several times from toluene and then from acetonitrile. Final yield ~1.5 g (20%) of yellow crystals. ^1H NMR (500 MHz, MeOD) δ 1.33 (s, 9H), 2.98–3.03 (dd, J = 13.9 Hz, J = 9.48 Hz, 1H), 3.26–3.30 (m, J = 5.05 Hz, 1H), 4.36–4.39 (dd, J = 9.48 Hz, J = 5.05 Hz, 1H), 6.67 (m, 1H), 7.33 (d, J = 3.16 Hz, 1H), 7.51–7.53 (d, J = 8.85 Hz, 1H), 7.60–7.62 (d, J = 8.85 Hz, 1H), 7.78 (s, 1H), 7.97 (d, J = 1.27 Hz, 1H). ^{13}C NMR (500 MHz, MeOD) δ 28.6, 38.27, 56.26, 80.52, 113.58, 116.91, 119.29, 122.83, 126.26, 135.77, 135.97, 138.44, 141.29, 145.86, 146.08, 155.09, 157.77, 173.81, 175.11 (two carbon signals are absent due to incidental equivalence). HRMS (EI) m/z calcd for $\text{C}_{21}\text{H}_{21}\text{NO}_8$ 415.12672, found: 415.12659.

3-[2-(2-Furyl)-3-hydroxy-4-oxo-4H-chromen-6-yl]-L-alanine Hydrochloride (5**)**. 944 mg of 3-hydroxychromone **4** was dissolved in 10 mL of dioxane. Then, 10 mL of conc. HCl was added to the reaction mixture in cold water bath. After 5–10 min, the bath was removed and the mixture was stirred for 2–3 h. The reaction mixture was concentrated under reduced pressure to give the desired product **5** as a dark yellow powder, yield 0.769 g (96%). ^1H NMR (500 MHz, MeOD) δ 3.28–3.34 (dd, J = 14.56 Hz, J = 7.78 Hz, 1H), 3.42–3.48 (dd, J = 14.56 Hz, J = 5.77 Hz, 1H), 4.34–4.37 (dd, J = 7.53 Hz, J = 5.77 Hz, 1H), 6.7–6.72 (dd, J = 3.52 Hz, J = 1.76 Hz, 1H), 7.37–7.38 (d, J = 3.51 Hz, 1H), 7.63–7.65 (d, J = 8.79 Hz, 1H), 7.68–7.71 (dd, J = 8.79 Hz, J = 2.26 Hz, 1H), 7.81–7.82 (d, J = 1.00 Hz, 1H), 8.05–8.06 (d, J = 1.76 Hz, 1H). ^{13}C NMR (500 MHz, MeOD) δ 36.74, 54.95, 113.70, 117.23, 120.29, 123.34, 126.81, 132.56, 135.79, 138.64, 141.67, 145.83, 146.33, 155.71, 171.00, 173.74. HRMS (EI) m/z calcd for $\text{C}_{16}\text{H}_{13}\text{NO}_6$ 315.07429, found: 315.07419.

N-[(9H-Fluoren-9-ylmethoxy)carbonyl]-3-[2-(2-furyl)-3-hydroxy-4-oxo-4H-chromen-6-yl]-L-alanine (6**)**. 614 mg (1.744 mmol) of amino acid hydrochloride **5** was dissolved in a solution of 500 mg (5.95 mmol) of sodium bicarbonate in 10 mL of water. Then, 30 mL of acetonitrile and 588 mg (1 equiv) of FmocOSu were added and the mixture was stirred for 24 h. The reaction mixture was filtered and the precipitate was dissolved in hot water (about 100 mL per 1 g) with further acidification with HCl. Yield: 900 mg (96%) of yellow powder. ^1H NMR (500 MHz, DMSO- d_6) δ 3.00–3.05 (m, 1H), 3.22–3.26 (dd, J = 14.04 Hz, J = 4.27 Hz, 1H), 4.12–4.19 (m, 3H), 4.26–4.30 (m, 1H), 6.77–6.78 (dd, J = 3.05 Hz, J = 1.83 Hz, 1H), 7.21–7.25 (m, 2H), 7.27–7.28 (d, J = 3.05 Hz, 1H), 7.34–7.37 (t, J = 7.32 Hz, 2H), 7.57–7.58 (d, J = 7.32 Hz, 2H), 7.59–7.61 (d, J = 8.55 Hz, 1H), 7.68–7.70 (d, J = 8.55 Hz, 1H), 7.75–7.76 (d, J = 8.55 Hz, 1H), 7.82–7.83 (d, J = 7.32 Hz, 2H), 8.01 (s, 1H), 8.04 (s, 1H), 9.88 (s, 1H), 12.76 (br s, 1H). ^{13}C NMR (500 MHz, DMSO- d_6) δ 35.80, 46.54, 55.39, 65.64, 112.82, 115.27, 118.03, 120.04, 121.56, 124.95, 125.11, 125.18, 126.98, 127.55, 134.68, 137.20, 139.19, 140.63, 143.67, 144.09, 145.17, 152.96, 156.01, 171.78, 173.07 (six carbon signals are absent due to incidental equivalence). HRMS (EI) m/z calcd for $\text{C}_{31}\text{H}_{23}\text{NO}_8$ 537.14237, found: 537.14187.

Proof of Optical Purity. To test the enantiomeric purity of the obtained chromone-amino acid derivative, we prepared the dibenzyl ether ester of the amino acid, which was acylated by two enantiomers of Mosher's acid chloride to give compounds **9** and **10** (Scheme S1). The NMR spectra of the diastereomeric

Mosher amides were then compared. No signal of the second diastereomer was found in each spectrum.

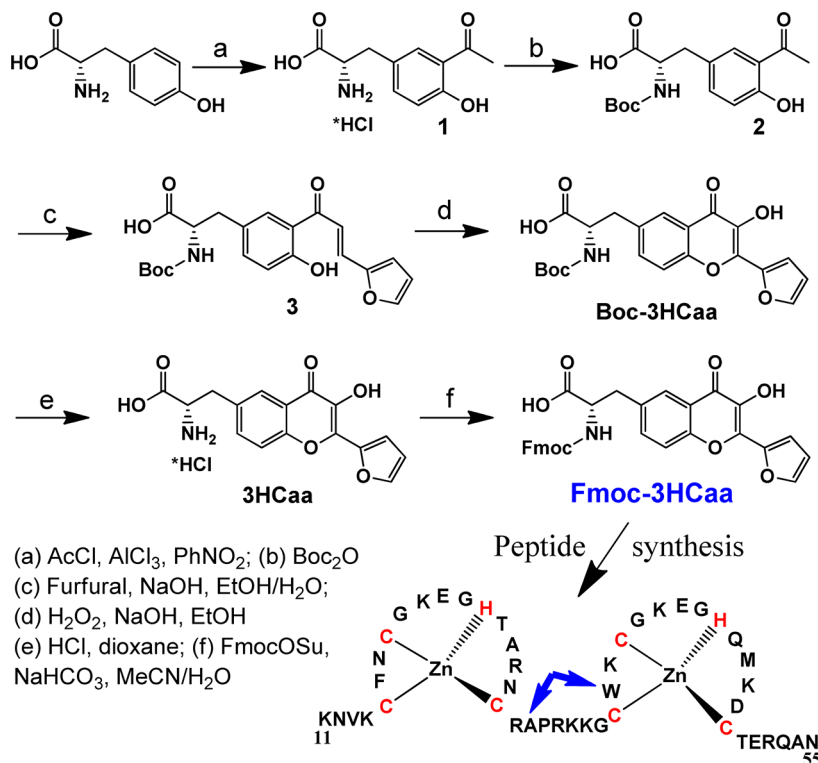
Benzyl 3-[3-(Benzyloxy)-2-(2-furyl)-4-oxo-4H-chromen-6-yl]-N-[(2S)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl]-L-alaninate (9**)**. ^1H NMR (400 MHz, CDCl_3) δ 3.14–3.19 (dd, J = 14.24 Hz, J = 7.12 Hz, 1H), 3.20 (s, 3H), 3.24–3.29 (dd, J = 14.24 Hz, J = 5.60 Hz, 1H), 4.89–4.94 (dd, J = 14.25 Hz, J = 6.62 Hz, 1H), 5.05–5.08 (d, J = 12.21 Hz, 1H), 5.11–5.14 (d, J = 12.21 Hz, 1H), 5.24 (s, 2H), 6.48–6.49 (dd, J = 3.56 Hz, J = 1.52 Hz, 1H), 7.18–7.44 (m, 17H), 7.59 (d, J = 1.01 Hz, 1H), 7.96 (d, J = 1.53 Hz, 1H). ^{19}F -NMR (376 MHz, CDCl_3) δ –69.5 (9), –72.03 (Mosher acid).

Benzyl 3-[3-(Benzyloxy)-2-(2-furyl)-4-oxo-4H-chromen-6-yl]-N-[(2R)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl]-L-alaninate (10**)**. ^1H NMR (400 MHz, CDCl_3) δ 3.02–3.07 (dd, J = 14.05 Hz, J = 7.53 Hz, 1H), 3.16–3.21 (dd, J = 14.05 Hz, J = 5.52 Hz, 1H), 3.35 (d, J = 1.50 Hz, 3H), 4.94–5.00 (m, J = 5.52 Hz, J = 7.28 Hz, 1H), 5.07–5.10 (d, J = 12.05 Hz, 1H), 5.14–5.17 (d, J = 12.05 Hz, 1H), 5.20–5.23 (d, J = 10.54 Hz, 1H), 5.23–5.26 (d, J = 10.54 Hz, 1H), 6.49–6.51 (dd, J = 3.52 Hz, J = 1.76 Hz, 1H), 7.01–7.04 (dd, J = 8.79 Hz, J = 2.26 Hz, 1H), 7.05–7.07 (d, J = 8.53 Hz, 1H), 7.17–7.32 (m, 15H), 7.42–7.45 (dd, J = 8.29 Hz, J = 1.76 Hz, 1H), 7.60 (dd, J = 1.76 Hz, J = 0.75 Hz, 1H), 7.84 (d, J = 2.26 Hz, 1H). ^{19}F -NMR (376 MHz, CDCl_3) δ –69.41 (10), –72.03 (Mosher acid).

Peptide Synthesis. The labeled NC(11–55) peptides were synthesized by solid-phase peptide synthesis on a 433A synthesizer (ABI, Foster City, CA), according to a previously established protocol.⁴⁹ The synthesis was performed at a 0.1 mmol scale using the standard fluorenylmethoxycarbonyl (Fmoc)-amino acid-coupling protocol starting from 0.44 mmol/g Wang LL resin. At selected positions of the peptide, the fluorescent amino acid analogue was incorporated by the following procedure. In a flask, 2–4 mol equiv of the Fmoc-3HCaa were mixed with 4 equiv of HBTU/HOBt coupling solution (in DMF) and 5 equiv of DIEA. This mixture was immediately added to the peptidyl resin and shaken at 37 °C for 12 h. Then, the resin was washed with 1-methyl-2-pyrrolidone (NMP) and peptide synthesis was continued on the synthesizer. At the end of the synthesis, the Fmoc-deprotected peptidyl resin was isolated and washed with NMP, methanol, and dichloromethane.

Cleavage of the peptidyl resin and deprotection were performed for 2 h using a 10 mL trifluoroacetic acid (TFA) solution containing water (5%, v/v), phenol (2%, w/v), thioanisole (5%, v/v), triisopropylsilane (2.5%, v/v), and ethanedithiol (2.5%, v/v). The peptide was precipitated using ice-cold diethyl ether and pelleted by centrifugation. The pellet was air-dried for approximately 15 min, solubilized with aqueous TFA (0.05%, v/v), and lyophilized. Purification by HPLC was carried out on a C8 column (uPtisphere 300 Å, 5 μm ; 250 \times 10, Interchim, France) in water/acetonitrile mixture containing 0.05% TFA with a linear gradient 10–35% of acetonitrile for 30 min and monitored at 210 and 360 nm (3HC dye absorption). Obtained peptides were characterized by ESI-MS analysis. NC(11–55)-W37-3HCaa peptide: calculated M = 5245.40, found $[M+7\text{H}]^{7+}$ = 750.64 corresponding to M = 5245.38 after deconvolution; NC(11–55)-A30-3HCaa peptide: calculated M = 5360.43, found $[M+7\text{H}]^{7+}$ = 767.06 corresponding to M = 5360.37 after deconvolution.

Preparation of Zn-Bound Peptides. Lyophilized labeled peptides were dissolved in water (~0.5 mg in 500 μL). Then, about 10 μL of this solution was used to determine the peptide

Scheme 1. Synthesis of the Fluorescent Amino Acid and the Labeled Peptides^a


^aThe substituted positions of the NC(11–55) peptide are shown by blue arrows.

concentration using an extinction coefficient of $15\,000\text{ M}^{-1} \times \text{cm}^{-1}$ at 350 nm. Next, 2 mol equiv of ZnSO_4 was added to the peptide and pH was raised to its final value, by adding buffer. This last step was done only at the end to prevent peptide oxidation. Noticeably, a large excess of Zn^{2+} ions should be avoided since this ion could affect the 3HC fluorescence.

NMR Structural Studies. The two NC(11–55) conjugates at 0.5 mM concentration in neat water, pH 6.5 at 293 K, were studied on an Avance Bruker spectrometer operating at 600.13 MHz equipped with a cryoprobe. A mixing time of 200 ms and a water gate pulse program for water signal suppression in NOESY experiments were used.

Spectroscopic Measurements. Unless otherwise indicated, the experiments were performed in 10 mM phosphate buffer, pH 6.5, 30 mM NaCl, at 20 °C. This low pH was used to prevent deprotonation of the 3-hydroxy group of the 3HC amino acid. Absorption spectra were recorded with a Cary 4000 UV–visible spectrophotometer (Varian). Fluorescence spectra were recorded on FluoroMax3 and FluoroLog spectrofluorimeters (Jobin Yvon) equipped with thermostatted cell compartments. Fluorescence spectra were corrected for Raman scattering. Quantum yields were calculated using quinine sulfate in 0.5 M sulfuric acid (quantum yield, $\phi = 0.577$) as a reference.⁵⁰ Excitation wavelength was 350 nm for 3HCaa.

To determine the affinity of the labeled peptides for the ODNs, fixed amounts of the ODN were titrated with peptides by monitoring the two-band fluorescence of the labeled peptides. For each data point, the emission of the same concentration of labeled peptide in buffer was subtracted from the signal measured in the presence of the ODN. Affinity constants were determined from direct fitting of the corrected signal to the rewritten Scatchard equation

$$I = I_0 - \frac{(I_0 - I_t)}{N_t} \times \left\{ [1 + (P_t + nN_t)K_a] - \sqrt{[1 + (P_t + nN_t)K_a]^2 - 4P_tN_tK_a^2} \right\} / 2K_a \quad (1)$$

where I and I_t are the signal at a given and a saturating peptide concentration, respectively, I_0 is the signal in the absence of peptide, N_t is the total ODN concentration, P_t is the total concentration of peptide, K_a is the apparent affinity constant, and n is the number of binding sites. The parameters were recovered from nonlinear fits of eq 1 to experimental data sets using the *MicrocalOrigin 6.0* software.

Peptide Activity Tests. The ability of the labeled peptides to destabilize the secondary structure of ODNs was tested using cTAR DNA, the complementary sequence of the trans-activation response element, involved in the minus strand DNA transfer during reverse transcription. To this end, we used a cTAR sequence labeled at its 3' and 5' ends by 5(6)-carboxytetramethylrhodamine (TMR) and 4-(4'-methylaminophenylazo) benzoic acid (Dabcyl), respectively. In the absence of NC, cTAR is mainly in a nonfluorescent closed form where the TMR and Dabcyl labels are close together, giving excitonic coupling.⁵¹ The destabilization was measured through the fluorescence intensity increase of TMR in the labeled cTAR as a function of NC(11–55) concentration.^{43,52}

The ability of the labeled peptides to promote the annealing of cTAR (55 base hairpin DNA) with its complementary dTAR sequence was compared to that of the unlabeled NC peptide.⁵³ The kinetic measurements were performed under pseudo-first-order conditions by using unlabeled dTAR at a concentration which was 30-fold higher than the concentration of cTAR labeled with carboxytetramethylrhodamine (TMR) at the 5'

end and with 5/6-carboxyfluorescein (Fl) at the 3' end.⁵³ Excitation and emission wavelengths were 480 and 520 nm, respectively, for monitoring the Fl fluorescence. All reported concentrations correspond to those after mixing. To avoid high local concentrations during mixing, both reactants were mixed at the same volume. Peptides were added to each reactant separately at a peptide/ODN ratio of 3:1, and then, the reaction was initiated by mixing the peptide-coated ODNs together. Experiments were performed in 25 mM Tris, 30 mM NaCl, 0.2 mM MgCl₂, pH 7.5 at 20 °C.

RESULTS AND DISCUSSION

To obtain the L-amino acid bearing the 3HC fluorophore, we started with L-tyrosine that was converted into corresponding acetophenone **1** using a described procedure^{47,48} based on Fries reaction (Scheme 1). Then, the amino group was protected with the Boc group, and the product **2** was condensed with furaldehyde in the presence of a base. The obtained chalcone **3** was further converted into a 3HC derivative (Boc-3HCaa) using the Algar-Flynn-Oyamada reaction. Then, the Boc group was removed to obtain the desired amino acid (3HCaa). Finally, it was protected with the Fmoc group, which is required for solid-phase peptide synthesis. We checked that the basic conditions used in the synthesis do not alter the optical purity of the obtained amino acid. For this purpose, the obtained Boc-protected amino acid was first benzylated, and then the amino group was deprotected and finally modified with (R)- and (S)-Mosher agents (Scheme S2, Supporting Information). The ¹⁹F-NMR spectra of the two products **9** and **10** showed only one fluorine peak corresponding to pure diastereomers, indicating that the synthesis did not produce any racemization. This conclusion was confirmed by ¹H NMR spectra of these two diastereomers.

Then, the spectroscopic properties of the new amino acid (Boc-3HCaa) were characterized in different solvents (Figure 2, Table S1, Supporting Information). The absorption and emission maxima of Boc-3HCaa were remarkably close to those reported previously for the basic fluorophore FHC (Table S1),²⁴ indicating that 6-substitution in this fluorophore did not change its spectroscopic properties. The dual emission

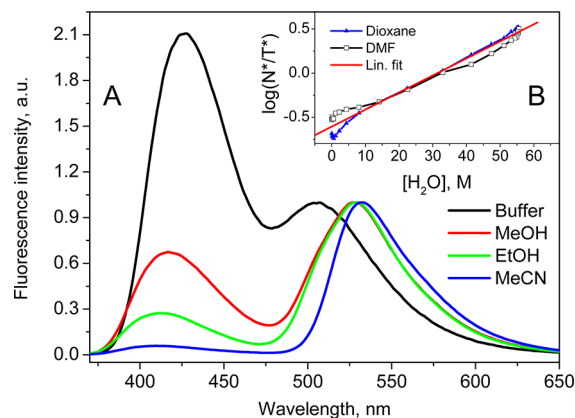


Figure 2. Fluorescence spectra of the Boc-3HCaa derivative in different solvents (A) and dependence of $\log(N^*/T^*)$ of 2-(2-furyl)-3HC (FHC) on water concentration in organic solvents based on previous data²⁸ (B). In (A), 10 mM phosphate buffer, 30 mM NaCl, pH 6.5 was used. All the spectra were normalized at the T^* band. Excitation wavelength was 350 nm. Concentration of Boc-3HCaa was 1 μ M.

of Boc-3HCaa was found to depend strongly on solvent polarity, showing an increase of the relative intensity of the N^* band in more polar solvents (Figure 2A) due to an inhibition of the ESIPT reaction.⁸ However, it should be noted that the ratio of the two emission bands of Boc-3HCaa in polar protic solvents is considerably lower than that of the parent fluorophore, indicating that the Boc-protected amino acid group partially screens the fluorophore from polar solvents. Therefore, to estimate the local water concentration, we used the calibration curve $\log(N^*/T^*)$ vs water concentration previously reported for the parent FHC dye (Figure 2B).²⁸

Using the Fmoc-protected 3HCaa, we then synthesized two labeled NC(11–55) peptides: NC(11–55)-A30-3HCaa and NC(11–55)-W37-3HCaa, where the fluorescent amino acid substituted Ala30 and Trp37 residues, located in the linker and the distal zinc finger, respectively (Scheme 1). Importantly, the 3HCaa was compatible with the standard protocols of peptide synthesis and purification. The NOESY spectra show that the folding of the zinc fingers is maintained in the two labeled NC(11–55) peptides (Figure S2, Supporting Information), since similar medium- and long-distance NOEs were found as for the native peptide. Thus, the incorporated amino acid analogue 3HCaa does not significantly affect the native folding in the two labeled peptides.

Next, we compared the chaperone activity of the labeled peptides with that of the native peptide. First, we checked the ability of the peptides to promote destabilization of DNA stem-loops, such as, for instance, the cTAR sequence involved in the first strand transfer of reverse transcription.⁴⁰ This test is particularly important, because substitution of Trp37 in NC(11–55) for a nonaromatic amino acid results in the loss of this component of NC chaperone activity.⁴⁰ Remarkably, NC(11–55)-W37-3HCaa showed high destabilization activity, nearly identical to that of the native peptide, while NC(11–55)-A30-3HCaa was less active (Figure 3). Second, we tested the ability of the labeled peptides to promote the annealing of two complementary sequences. Both labeled peptides exhibited similar activities comparable to that of the native peptide (Figure S3, Supporting Information), indicating that this

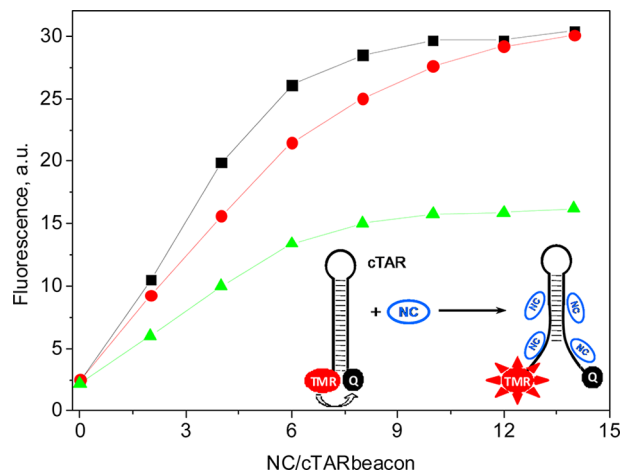


Figure 3. Destabilization of doubly labeled cTAR DNA by NC(11–55) (squares), NC(11–55)-W37-3HCaa (disks), and NC(11–55)-A30-3HCaa (triangles). Titration of 100 nM Dabcyl-5'-cTAR-3'-TMR (cTAR beacon) by NC peptides was performed in 25 mM TRIS (pH 7.5), 30 mM NaCl, and 0.2 mM MgCl₂. To monitor TMR fluorescence, excitation wavelength was set at 520 nm.

component of NC chaperone activity is also preserved. The preserved chaperone activity of NC(11–55)-W37-3HCaa shows that the new amino acid does not affect the peptide folding and can functionally substitute the Trp37 residue. We could explain this outstanding feature of the new amino acid by two factors. First, similarly to Trp, 3HCaa bears a flat and relatively small aromatic moiety. Second, the 3HC moiety, due to its compact structure, can easily intercalate within the nucleobases, as was shown in different recent reports.^{26,27,54} This property allows the 3HC moiety to mimic the interaction of the Trp37 indole moiety with nucleobases in the complexes of the peptide with nucleic acid sequences.^{34–36,38}

Labeled peptides in aqueous solutions (Figure 4) showed dual emission, characteristic of 3HC dyes, where the short- and

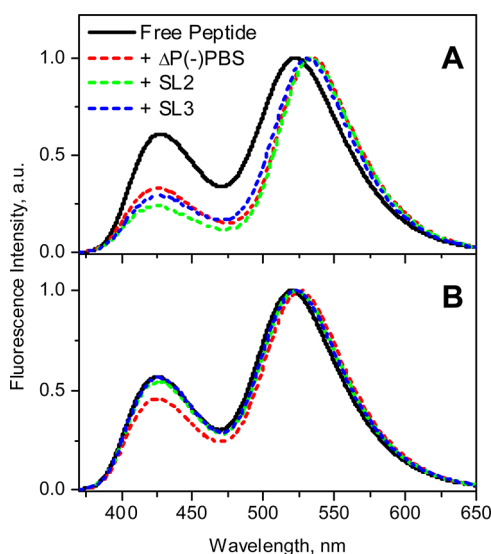


Figure 4. Effect of ODN interaction on the fluorescence spectra of the 3HCaa-labeled NC(11–55) peptides. Fluorescence spectra of NC(11–55)-W37-3HCaa (A) and NC(11–55)-A30-3HCaa (B) were normalized at the T* band. Peptide and ODN concentrations were 1 μ M. Measurements were done in 10 mM phosphate buffer, 30 mM NaCl, pH 6.5. Excitation wavelength was 350 nm.

long-wavelength bands can be assigned to N* and T* forms, respectively. The observed intensity ratio of the two emission bands, N*/T*, is close to that for the FHC fluorophore in ethanol (Tables 1 and S1, Supporting Information), indicating that the 3HCaa fluorophore is partially screened from water by the peptide in its folded form. Using a recently developed

method,²⁸ the local water concentration $[H_2O]_L$ of the 3HCaa solvation shell in both peptides was estimated around 31–32 M (Table 1), which corresponds to a water access coefficient W_A for the probe of 0.57 ($W_A = [H_2O]_L/[H_2O]$, where $[H_2O] = 55.56$ M is the concentration of neat water). This estimation indicates that almost half of the space around the 3HC fluorophore is screened from the bulk water by the peptide backbone.

On interaction with ODNs SL2, SL3, and $\Delta P(-)$ PBS, a strong change in the NC(11–55)-W37-3HCaa fluorescence spectrum was observed, with notably a large drop in the N*/T* ratio, a shift of the T* emission maximum to the red (Figures 4 and 5) and a decrease in the fluorescence quantum yield (Table

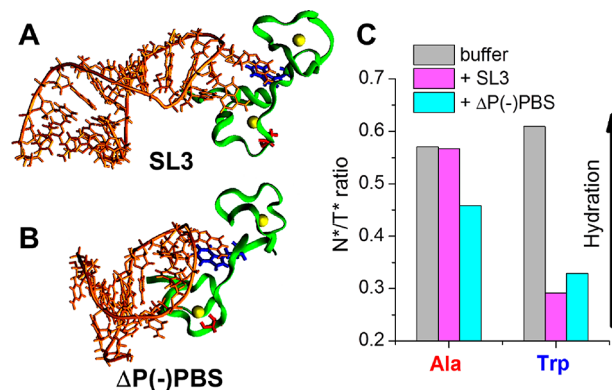


Figure 5. Correlation between the 3D structures of NC-ODN complexes and the response of the 3HCaa-labeled peptides on ODN binding. 3D structures of NC complexes with SL3 (A) and $\Delta P(-)$ PBS (B) drawn based on NMR data.^{34,36} Only the NC(11–55) part is shown. Zn atoms are represented as yellow spheres; Ala30 and Trp37 residues are in red, and blue, respectively. Fluorescence intensity ratio N*/T* for NC(11–55)-A30-3HCaa (Ala) and NC(11–55)-W37-3HCaa (Trp) peptides in buffer and bound to SL3 or $\Delta P(-)$ PBS (C).

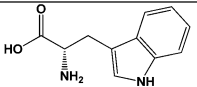
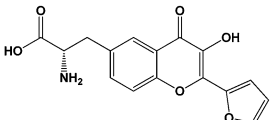
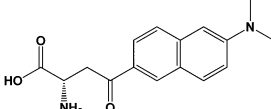
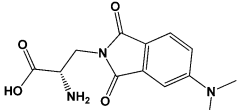
1). The drop in the N*/T* ratio can be assigned to a decrease in the local hydration of the 3HCaa environment, with a water access coefficient W_A of the probe dropping to 0.32–0.41 for the three ODNs (Table 1). The observed dehydration is likely connected to the interaction of the 3HC fluorophore with the nucleobases, which leads to an efficient screening of this fluorophore from water.^{26,27} Moreover, this conclusion is substantiated by the observed red shift of the T* band^{26,27} and is in line with the NMR-derived 3D structures of the NC-ODN complexes (Figure 5), showing that the Trp37 residue strongly

Table 1. Spectroscopic Properties of the 3HCaa-Labeled NC(11–55) Peptides in Complexes with ODNs^a

peptide	oligo-nucleotide	λ_{ABS} nm	λ_{N^*} nm	λ_{T^*} nm	N*/T*	$[H_2O]$, M	W_A	QY, %
NC(11–55)-A30–3HCaa	-	364	426	520	0.57	31	0.56	3.7
	$\Delta P(-)$ PBS	364	425	526	0.46	27	0.49	2.5
	SL3	363	426	523	0.57	30	0.54	2.5
	SL2	363	426	523	0.54	30	0.54	2.5
NC(11–55)-W37–3HCaa	-	366	427	522	0.61	32	0.58	9.0
	$\Delta P(-)$ PBS	367	425	534	0.33	23	0.41	5.1
	SL3	368	427	531	0.29	21	0.38	4.0
	SL2	368	425	533	0.24	18	0.32	4.8

^a λ_{ABS} , λ_{N^*} , and λ_{T^*} are the maxima of absorption, N* and T* emission bands, respectively; N*/T* is the intensity ratio of the two emission bands measured at the peak maxima (error $\pm 5\%$); $[H_2O]$ is the water concentration estimated using a method reported elsewhere (error ± 1 M);²⁸ W_A is water access coefficient;²⁸ QY is the fluorescence quantum yield. Excitation wavelength was 350 nm. Measurements were done in 10 mM phosphate buffer, 30 mM NaCl, pH 6.5. Peptide and ODN concentrations were 1 μ M.

Table 2. Spectroscopic Properties and Response to Environment of the New 3HC Amino Acid in Comparison to Other Amino Acid Analogues^a

Amino acid	Fluorophore	λ_{abs} (nm) EtOH	ϵ (M ⁻¹ cm ⁻¹)	λ_{em} (nm) EtOH	λ_{em} (nm) Water	$\Delta\lambda$ (nm)	Refs
	Indole	280	5500	342	355	13	(56)
	FHC	360	15000	531	427	-104	-
	Prodan	360	18400	496	531	35	(5, 9, 57)
	4-DMAP	408	6500	534 ^b	562	28	(11, 58)

^a λ_{abs} and λ_{em} – absorption and emission maxima of the fluorophores in the corresponding solvent; ϵ – absorption coefficient of the fluorophore in ethanol; $\Delta\lambda$ – shift of the emission maximum in response to changes in the solvent polarity (water vs ethanol). ^bData in methanol.

interacts with the nucleobases.^{34–36,38} Therefore, this observation further confirms that the 3HCaa can mimic the Trp residue at position 37. In contrast, binding of NC(11–55)-A30-3HCaa to the three ODNs led to either no change (with SL2 and SL3) or a limited decrease (with $\Delta P(-)$ PBS) in the N^{*}/T^{*} ratio with a slight decrease in its quantum yield (Table 1) and almost negligible spectral shifts of the absorption and emission maxima (Table 1). The absence of changes in the N^{*}/T^{*} ratio observed with the SL2 and SL3 sequences can be rationalized by the large distance between the A30 residue and the nucleobases in the NC-ODN complexes (Figure 5). As a consequence, the 3HCaa at position 30 in the complexes with SL2 and SL3 cannot interact with the ODN and remains highly exposed to water, as in the free peptide. Finally, the moderate change in the N^{*}/T^{*} ratio observed when NC(11–55)-A30-3HCaa was complexed with $\Delta P(-)$ PBS is fully consistent with the closer proximity of the Ala30 residue to the $\Delta P(-)$ PBS nucleobases, as compared to that in the complexes with SL2 and SL3.^{34,36,38}

Finally, using the changes in the N^{*}/T^{*} ratio or the quantum yield of 3HCaa as an analytical signal of interaction, we further measured the binding constant of the three labeled peptides to SL3. The obtained binding constants matched closely that obtained with the native peptide (Table S2, Supporting Information),^{35,37} indicating that the incorporated 3HCaa probe did not change the binding affinity of the peptides and could thus be also used for quantification of the peptide–ODN interaction. Since Trp37 was shown to be critical for the high affinity of NC to its target ODNs,^{40,42,45} this indicates that the substitution of Trp37 by 3HCaa does not alter the ability of NC to recognize its ODN targets. The results are in line with the peptide activity tests and NMR structural data, providing a clear background for considering 3HCaa as a new amino acid analogue that can readily substitute Trp even at the places where it plays a crucial role in the peptide.

Though the N^{*}/T^{*} ratio of 3HCaa is mostly controlled by the environment polarity and hydration, we could not exclude

possible quenching effects, which are frequently observed for dyes interacting with nucleobases.^{35,41} Indeed, the N^{*}/T^{*} ratio could decrease, for instance, due to a more efficient quenching of the N^{*} state compared to the T^{*} state, as a consequence of static or dynamic quenching by the neighbor nucleobases. However, we expect this effect to be minor in the probe response, because the red shift of the T^{*} band produced by the peptide–DNA interaction correlates well with the changes in the N^{*}/T^{*} ratio (Table 1). As this red shift is directly linked to the dehydration of the dye environment^{26,27,55} and not to quenching, the ratio changes appear mainly driven by the dehydration within the peptide–DNA complex. Moreover, as the N^{*} and T^{*} states are coupled by the ESIPT reaction, the quenching of the N^{*} state should influence the N^{*}/T^{*} ratio to a less significant extent, compared to the case where the two states are independent.

Compared to Trp, the new fluorophore features strongly red-shifted absorption and emission (Table 2), allowing more convenient spectroscopic studies in biological samples with less background noise and photodamage. Moreover, it preserves its fluorescence on peptide binding to nucleic acids, while Trp is frequently quenched.^{35,41} Moreover, due to ESIPT, it presents much higher sensitivity to the environment in high-polarity range, so that it almost switches its fluorescence from dominant T^{*} emission in ethanol (at 531 nm) to dominant N^{*} emission in water (at 427 nm), while Trp in these conditions changes its emission maximum only by a few nm.⁵⁶ This exclusive environmental sensitivity places it in front of all other existing fluorescent amino acid analogues of similar small size, based on Prodan and 4-DMAP (Table 2). Indeed, for highly polar environments (alcohols and water), the fluorophore of the new amino acid (FHC) is about 3-fold more sensitive to solvent polarity (hydration) as compared to Prodan and 4-DMAP. Moreover, the FHC provides an opportunity to quantitatively evaluate the hydration in the site of interaction between the two biomolecules. These exclusive properties originate from the strong effect of H-bonding solvents on the ESIPT reaction in

these derivatives.¹⁵ As peptides and nucleic acids present rather highly polar microenvironments, the new amino acid constitutes a unique tool for probing interactions of these biomolecules in a site-specific manner.

CONCLUSION

For the first time, a fluorescent L-amino acid, exhibiting ESIPT and hydration-sensitive dual emission, was synthesized. This amino acid bears, as a side chain, the 2-(2-furyl)-3-hydroxychromone fluorophore, which is flat and close in size to the indole moiety of L-tryptophan. It was incorporated at two different positions of the NC(11–55) peptide. Remarkably, substitution of the highly conserved Trp37 residue by this fluorescent amino acid was found to preserve both the native folding and the chaperone activity of the peptide. Interaction of the labeled peptides with target ODNs dramatically changed the dual emission of the incorporated amino acid, and these changes depended on its position in the peptide and on the ODN sequence. The obtained results established a correlation between the fluorescence response of the amino acid probe and its proximity to the interacting ODN bases. This amino acid appears as an attractive tool for probing the binding sites in peptide–nucleic acid complexes, being particularly suitable for substitution of tryptophan residues. Compared to tryptophan and its close fluorescent analogues, this new amino acid presents superior environment sensitivity.

ASSOCIATED CONTENT

Supporting Information

Additional characterization data on the fluorescent amino acid and its peptide conjugates and the studied ODN sequences. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail addresses: andrey.klymchenko@unistra.fr; yves.mely@unistra.fr. Tel: +33 368 854255, +33 368 854263. Fax: +33 368 854313.

Author Contributions

[‡]A.V.S. and V.Y.P. equally contributed to this work.

Notes

The authors declare no competing financial interest.

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