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FULL PAPER

Cell Penetrating Peptides containing Fluorescent D-Cysteines

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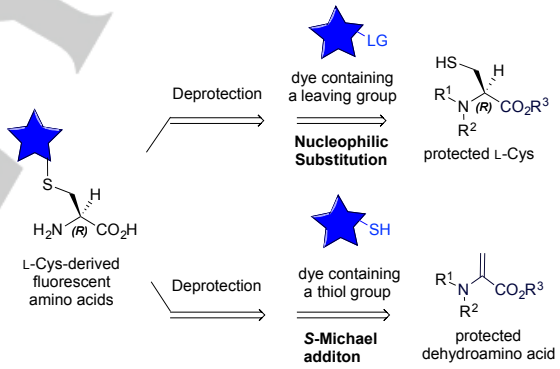
Abstract: A series of fluorescent D-cysteines (Cys) has been synthesized and their optical properties were studied. The key synthetic step is the highly diastereoselective 1,4-conjugate addition of aryl thiols to a chiral bicyclic dehydroalanine recently developed by our group. This reaction is fast at room temperature and proceeds with total chemo- and stereoselectivity. The Michael adducts were easily transformed into the corresponding amino acids to study their optical properties and, in some selected cases, into the corresponding *N*-Fmoc-D-cysteine derivatives to be used in solid phase peptide synthesis (SPPS). To further demonstrate the utility of these non-natural Cys-derived fluorescent amino acids, the coumaryl and dansyl derivatives were incorporated into cell penetrating peptide sequences through standard SPPS and their optical properties studied in different cell lines. The internalization of these fluorescent peptides was monitored by fluorescence microscopy.

Introduction

Nowadays, fluorescence spectroscopy is routinely used to investigate complex biological processes as well as a diagnostic and research tool in many fields of biomedical sciences.[1] Although natural amino acids such as tryptophan, tyrosine and phenylalanine have been used as intrinsic fluorescent probes to monitor different biological events, their use is restricted due to their poor optical properties.[2] Therefore, the design and synthesis of fluorescent non-natural amino acids and their incorporation into peptides or proteins has emerged as a powerful tool to study biological processes using fluorescence spectroscopy.[3-4] Given the continuous development of imaging techniques and synthetic biology methods, there is an urgent need for expanding the toolbox of available amino acids incorporating a variety of fluorescent moieties with different spectroscopic properties.[3-4] Although several synthetic protocols have been developed for the synthesis of fluorescent non-natural L-amino acids,[1,3-4] very few are described for D-amino acids.[5] In this context, it has been shown that fluorescent D-amino acids allow quick detection of cell wall biosynthesis in live cells and in numerous bacterial species.[6] This method has also been used to study the synthesis of peptidoglycans in some bacterial species.[7]

Functionalized amino acids such as lysine, cysteine or tyrosine have been found to be ideal candidates for the development of fluorescent labels through conjugation of the corresponding electrophilic dye. Thus, the aim of this work is to introduce different commercially available fluorophores into the D-Cys scaffold for potential diagnostic or imaging purposes when the resulting amino acids are incorporated into peptides.

L-Cys-derived fluorescent amino acids are normally accessed through nucleophilic attack of the sulfhydryl group of L-Cys to a fluorescent building block equipped with a suitable leaving group. (Scheme 1).



Scheme 1. Retrosynthetic analysis of Cys-derived fluorescent amino acids.

However, the formation of C-S bonds involving the opposite approach –the nucleophilic reaction of dyes incorporating a thiol group with electrophilic amino acid derivatives- has received less attention. In this work, we report a new methodology using thiol-containing dyes as nucleophiles which undergo stereoselective *S*-Michael addition to a chiral dehydroamino acid (Scheme 1).

Dehydroalanine (Dha)[8] is an α,β-unsaturated amino acid of biological and synthetic interest[9] which acts as a chemical precursor of a range of site-selective post-translational modifications. However, in general, these reactions are poorly stereoselective. We recently reported the synthesis of a new cyclic chiral Dha-derivative **ent-1** derived from D-serine. This compound behaves as a versatile substrate for stereoselective *S*-Michael additions, generally providing high yields and diastereoselectivity (Scheme 2).[10] More recently, we developed a rigidified variant of this Dha[11] through a lactonization reaction to

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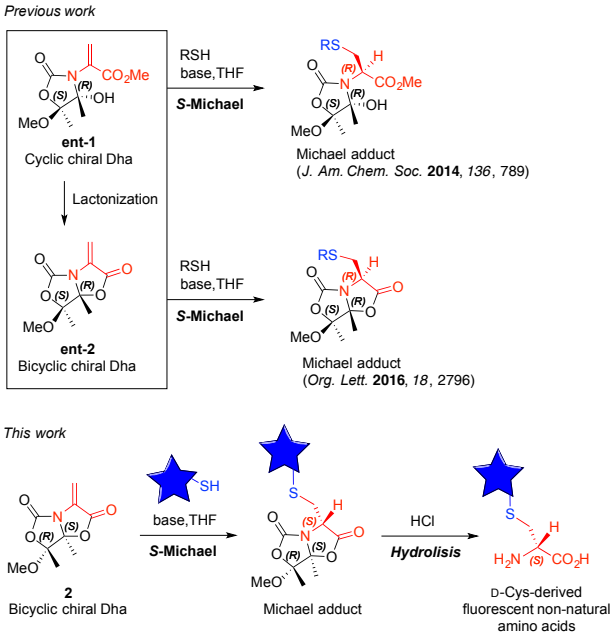
give the bicyclic chiral Dha **ent-2**. The reduced conformational flexibility of this bicyclic scaffold confers higher diastereoinducing properties even at room temperature (Scheme 2).



Scheme 2. Synthesis of fluorescent D-Cys from a bicyclic chiral dehydroalanine (Dha **2**) through *S*-Michael additions.

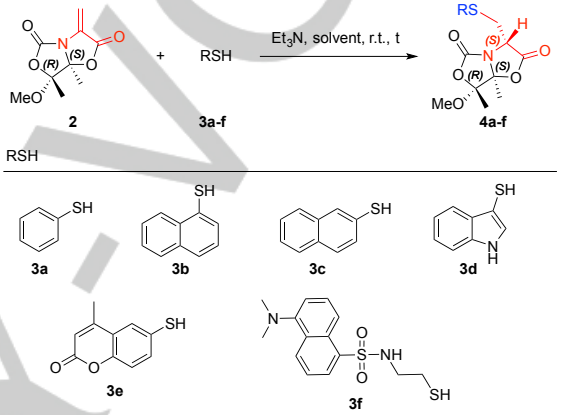
This new scaffold (**ent-2**) also behaved as an excellent *S*-Michael acceptor when several thiols, including tri-*O*-acetyl-2-acetamido-2-deoxy-1-thio-α-D-galactopyranose, were used as nucleophiles. The advantage of this synthetic approach over the previously published work[10a] is an increased yield in the 1,4-conjugate addition and the ability to hydrolize the Michael adduct in a cleaner way.[10b]

From this previous, it was apparent that D-Cys-derived amino acids with enhanced fluorescence properties could be readily accessible using the same methodology. We envisioned the *S*-Michael addition of different commercially available fluorescent aryl thiols to chiral Dha **2**, (the enantiomer of **ent-2**) as the key step to obtain a family of *S*-functionalized D-Cys-derived amino acids after hydrolysis of the corresponding Michael adducts (Scheme 2). To date, enantiopure fluorescent non-natural amino acids have been synthesized in poor global yields, using stereoselective procedures that need for multiple steps.[1] Herein, we report an efficient and versatile two-step synthesis of fluorescent non-natural amino acids. Our synthetic protocol includes general guides for researchers to synthesize their own custom fluorescent side chain-functionalized D-Cys derivatives, with different user-defined features from an appropriately SH-activated fluorescent dye. We focused on coumaryl and dansyl tags because they show strong fluorescence and comprise a wide range of commercially available fluorescent tags. Moreover, they are photostable and present good solubility in many solvents.[1]

Results and Discussion

Starting from the chiral bicyclic Dha **2**,[11] we assayed the *S*-Michael addition reaction of different SH-containing fluorophores (**3a-f**) as nucleophiles, applying the same procedure[11] developed for its enantiomer **ent-2**. Such protocol involves dropwise addition of equimolecular amounts of aromatic thiols in the presence of triethylamine as a base and THF or CH_2Cl_2 as a solvent at room temperature (Table 1).

Table 1. Stereoslective *S*-Michael addition of thiols **3a-f** to the chiral Dha **2**.



Entry	RSH	Solvent/time (min)	Product	Yield[a]
1	**3a**	THF/5	**4a**	89
2	**3b**	THF/60	**4b**	96
3	**3c**	THF/60	**4c**	95
4	**3d**	THF/60	**4d**	60
5	**3e**	THF/5	**4e**	93
6	**3f**	CH_2Cl_2/180	**4f**	92

[a] Yield after a column chromatography. Diastereoselectivity was higher than 95:5 in all cases, since only one product was detected by ^{1}H NMR spectra of the reaction mixtures before purification.

In all cases, the Michael adducts were cleanly formed with excellent yields (89 to 96%), with the exception of thiol **3d**, probably due to its bidentate (S and N nucleophile) character. Adducts **4a** and **4e** were formed in five minutes (entries 1 and 5, Table 1), while adducts **4b**, **4c** and **4d** needed one hour to achieve complete conversion, probably due to steric hindrance (entries 2-4, Table 1). When thiol **3f** were used (entry 6, Table 1), the reaction was carried out in dichloromethane as a solvent because of reduced solubility in THF, and 3 h were required to complete the reaction. This thiol is not commercially available and was prepared following a reported procedure,[12]

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Interestingly, all *S*-Michael reactions proceeded with complete diastereoselectivity, since only a unique diastereoisomer was observed in the reaction mixture. Therefore, the results reported herein indicate a robust stereoinduction mechanism for the protonation of the enolate adduct upon conjugate addition. In general, the short time needed, together with the mild conditions used (room temperature), the high yield, and total chemo- and stereoselectivity achieved, make these 1,4-conjugate additions true click reactions.

The high diastereoselectivity obtained can be explained attending to the pyramidalization of the enolate intermediate, as we previously demonstrated by theoretical calculations[11] in the case of its enantiomer Dha **ent-2**. The absolute configuration of the new stereocenter created in the Michael addition (C3) was determined by NOESY-NMR experiments (Supporting Information). For instance, the observation of a NOE cross-peak between the hydrogens of the –CH_2S– substituent at C3 and the CH_3 group at C7a of the 2,5-dioxotetrahydro-5*H*-oxazolo[4,3-b]oxazol rigid bicyclic system confirmed that C3 displays an *S*-configuration in compound **4e** (Figure 1).

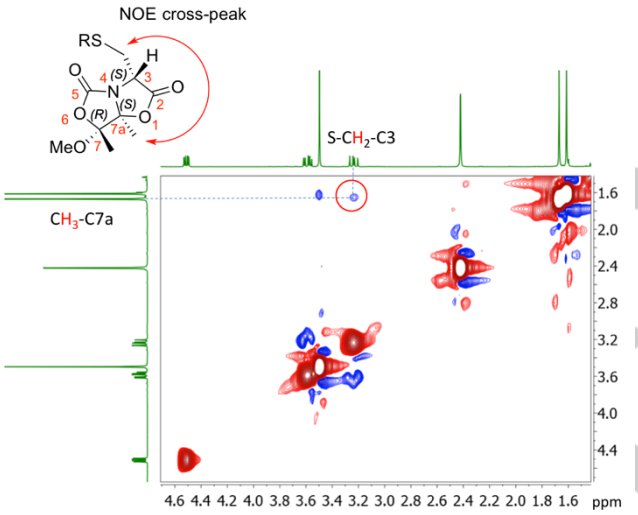
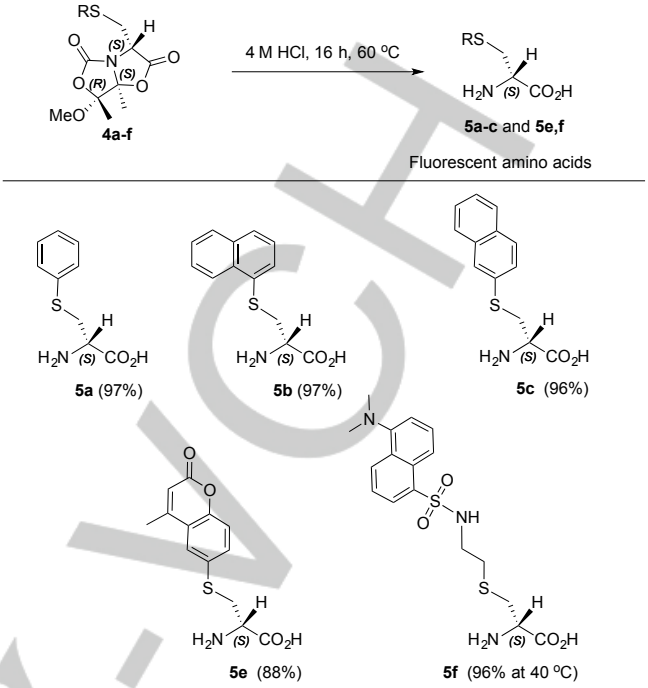


Figure 1. 2D-NOESY-NMR experiment for compound **4e** performed in a 400 MHz equipment using $CDCl_3$ as a solvent at 20 °C.

These bicyclic adducts **4a-f** can be visualized as protected Cys-derivatives, in which the amino moiety is protected as a cyclic carbamate and the carboxylic acid as a lactone. Therefore, the next step involved a simple acid hydrolysis with aqueous 4 M HCl at 60 °C for 16 h, giving the corresponding D-Cys derived fluorescent non-natural amino acids **5a-c** and **5e-f** in good yields (88-97%, Scheme 3). To avoid hydrolysis of the sulfonamide group in compound **4f**, the temperature of the reaction was fixed at 40 °C, leading to amino acid **5f** in 96% yield.



Scheme 3. Synthesis of D-Cys derived fluorescent non-natural amino acids **5a-c** and **5e,f** from acid hydrolysis of the corresponding Michael adducts **4a-c** and **5e,f**.

All these amino acids are stable solids at room temperature and can be stored without the requirement of any special precautions. Absorption and fluorescence emission were measured for each compound and are presented in Table 2. The optical properties of the aromatic natural amino acids L-tyrosine, L-tryptophan and L-phenylalanine were also included for comparison purposes.[13]

All non-natural amino acids showed absorption maxima wavelengths (λ_{max}) in the ultraviolet region ranging from 302–350 nm, being compounds **5e** and **5f** the ones with absorption peaks at lower energies (337 and 350 nm, respectively). In all cases, single absorption bands at lower energies were observed (Table 2 and Supporting Information).

Upon photoexcitation of all non-natural amino acids **5a-c** and **5e-f** at wavelengths corresponding to their maximum absorptions, all these compounds showed a strong emission in the ultraviolet (compounds **5a-c** and **5e**) or in the visible regions (compound **5f**). Spectra emission corresponding to normalized fluorescence intensity are given in Figure 2. Coumaryl and dansyl derivatives, **5e** (396 nm, blue region) and **5f** (519 nm, green region), are the more suitable candidates to be used as fluorescent dyes. These two amino acids looked more whitish when visualized under UV lamp (Figure 2).

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Table 2. Optical properties of fluorescent D-Cys-derived amino acids **5a-c,e,f** and natural amino acids L-Trp, L-Tyr and L-Phe.

Entry	Amino acid	Concentration[a] (M)	λ_{max} Abs.[b] (nm)	λ_{max} Em.[b] (nm)
1	**5a**	$1.1 \cdot 10^{-5}$	302	334
2	**5b**	$8.3 \cdot 10^{-5}$	307	370
3	**5c**	$7.3 \cdot 10^{-5}$	285	375
4	**5e**	$7.2 \cdot 10^{-5}$	337	396
5	**5f**	$5.0 \cdot 10^{-5}$	350	519
6	L-**Trp**	-	287	352
7	L-**Tyr**	-	254	303
8	L-**Phe**	-	254	282

[a] Determined in DMSO at room temperature for **5a-c** and **5e,f** and in water at pH 7 for L-Trp, L-Tyr and L-Phe.[13] [b] Maximum wavelength of the absorption UV-Vis spectra (λ_{max} Abs.) and maximum wavelength of the emission (λ_{max} Em.)

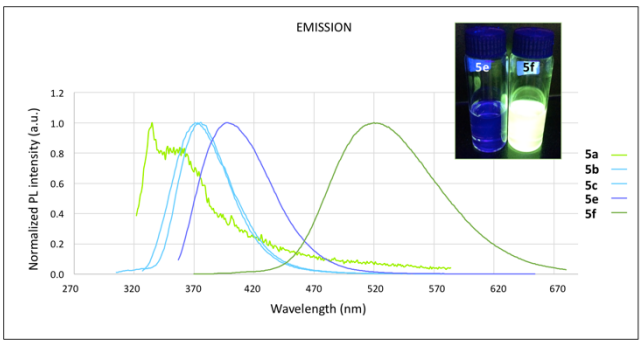

Figure 2. Fluorescence emission spectra of amino acids **5a-c,e,f** (left to right) when they were irradiated at the corresponding maximum absorption. Vials show the fluorescence of amino acids **5e** and **5f** after irradiation at 254 nm (see Supporting Information for details).

In principle, only those derivatives displaying absorbance maxima at wavelengths longer than 320 nm[1d] are adequate to be efficiently incorporated into peptides and/or proteins, thus avoiding any interference from the endogen aromatic residues. In our case, non-natural amino acids **5e,f** satisfy this requirement (entries 5 and 6, Table 2) and therefore, were subsequently used to synthesize well-known cell-penetrating peptides that can be used in cell imaging.

The common method to obtain fluorescently labeled peptides involves the bioconjugation of a fluorescent dye to the corresponding synthesized peptide.[1d,13] Because of this, commercial fluorescent dyes often contain leaving groups to react with the amine or thiol nucleophilic groups of the peptides. This strategy can be challenging with peptides bearing more than one amine or thiol group, since an non-homogenous mixture of labeled species can be generated. An alternative strategy to circumvent this issue involves incorporation in the peptidic sequence of commercially available amino acids labelled at the N terminus with a fluorescent dye. However, this methodology presents the handicap that the fluorescent amino acid only can be incorporated at the N-terminus of the sequence. Therefore, the use of adequately protected (Fmoc) fluorescent non-natural amino acids, such as **5e** and **5f**, incorporating a dye in the side chain, allows the direct and site-specific incorporation of the fluorescent dye as an integrated part of the SPPS methodology (Scheme 4).

Initially, we protected amino acids **5e,f** as Fmoc derivatives **6e,f**, which are ready-to-use in solid phase peptide synthesis (SPPS, Scheme 4 and Supporting Information). To evaluate the utility of these amino acids in cell imaging, they were incorporated into the well-known cell penetrating peptides[14] Val-Pro-Ala-Leu-Lys (VPALK) and Val-Pro-Ala-Leu-Arg (VPALR). We selected these pentapeptides, also named Bax-Inhibiting Peptides (BIP), because they belong to the subclass of linear hydrophobic peptides based on natural amino acids containing the minimal cell-penetrating sequence able to be internalized. Additionally, these short sequences composed of five natural amino acids are non-cytotoxic and it has been demonstrated that they could be potentially utilized as tools to deliver therapeutic molecules into cells.[14a,15]

Using a standard SPPS protocol involving the use of a 2-chlorotrityl chloride resin and 1-(bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), as a coupling agent, along with the Hünig's base (*N,N*-diisopropylethylamine, DIEA) and DMF as a solvent, we manually synthesized the following peptides: Ac-D-Cys(Coum)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-OH, **7e**, (where D-Cys(Coum) = **5e**) and Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-OH, **7f**, (where D-Cys(Dans) = **5f**).

Furthermore, and using an automatic microwave assisted solid phase peptide synthesizer (MW-SPPS) and, a Rink amide MBHA resin and Oxyma pure/DIC (*N,N'*-diisopropylcarbodiimide) as a coupling cocktail, we promptly synthesized with higher yields the following peptides: Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-NH_2, **8f**, and Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Arg-NH_2, **9f** (Scheme 4).

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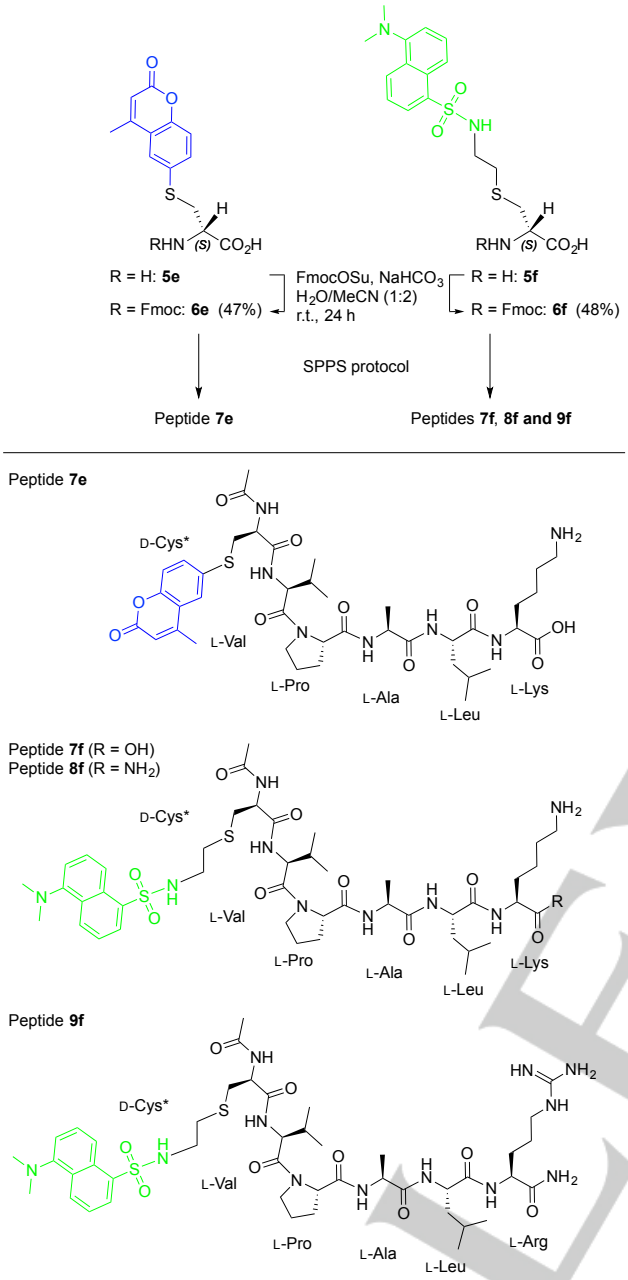

Scheme 4. SPPS protocol to achieve peptides **7e** and **7-9f**.

After purification by semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) and lyophilization, these peptides **7e,f, 8f** and **9f** were further characterized by UV-Vis and fluorescence spectroscopy, the excitation and emission spectra being shown in figure 3. To evaluate both their cell permeability and fluorescent properties, all peptides were incubated for 3 h with a human epithelial cell line (HeLa) and internalization was visualized using fluorescence microscopy (Figure 4 and Supporting Information).

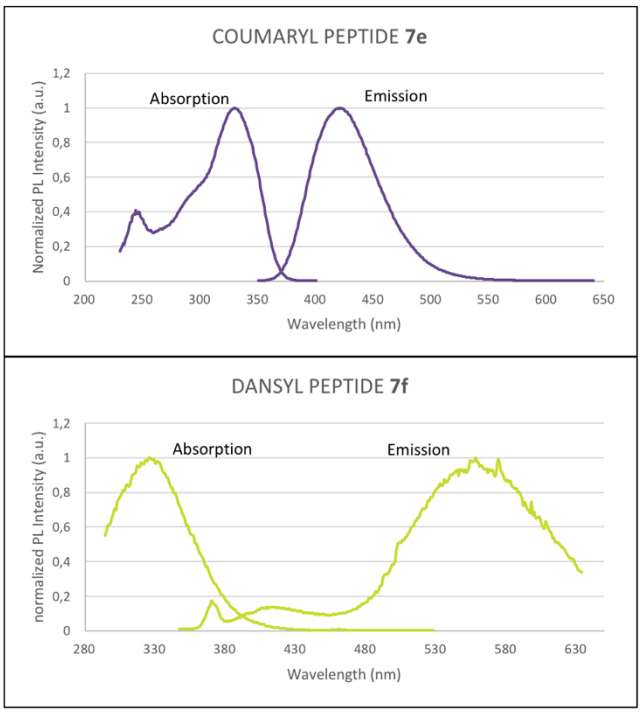

Figure 3. Absorption and emission spectra of coumaryl (up) and dansyl (down) peptides **7e** and **7f** in DMSO.

As a result, the fluorescence observed with peptide **7e** was similar to that obtained in the control experiment. This fact is probably due to the fact that the intrinsic fluorescence of cells is comparable to those emitted by this peptide. Thus, we decided to focus on the peptides incorporating the dansyl Cys (**7f, 8f** and **9f**). Fluorescence observed with peptides **7f** and **8f** was very similar and different from the control experiment, both molecules being adequate candidates for this type of assay. However, and in agreement with the above-described difference of internalization capacity of sequences VPALK and VPALR,[14a] the best result was obtained for peptide **9f**, thus validating the use of these amino acids as intrinsic fluorescent labels.

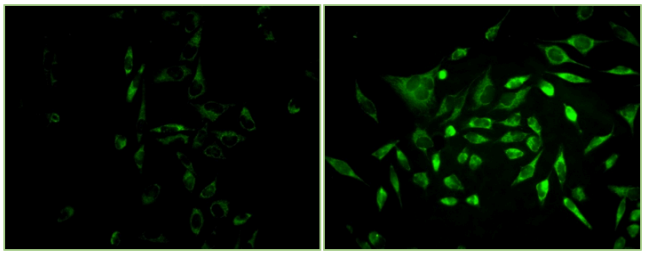

Figure 4. Control (left) an internalization of peptide **9f** by HeLa (right) cells visualized by a fluorescence microscope. Differences between control cells and any of the treated cells are significant at $p<0.01$ (Mann-Whitney U Test, see Supporting Information).

Although the use of D-amino acids is a common strategy used for developing therapeutic peptides with improving properties, control experiments with peptides containing L-cysteine are always convenient. Hence, we also synthesized the peptide Ac-L-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Arg-NH_2, **9f'**, which incorporates the fluorescent L-Cys **ent-5f**. This amino acid **ent-5f** is the enantiomer of amino acid **5f** and was obtained through acidic hydrolysis from the Michael adduct **ent-4f**. This intermediate was synthesized starting from the enantiomer of chiral bicyclic Dha **2** (**ent-2**) and dansyl fluorescent thiol **3f,** in the same conditions described for the *S*-Michael addition reaction between Dha **2** and thiol **3f**. The cell permeability and fluorescent properties of this new peptide **9f'** were also evaluated and resulted to be very similar to those of peptide **9f** (Supporting Information).

The stability of both fluorescent peptides **9f** and **9f'** was studied in human plasma. Both peptides are stable for at least 4 h, which is the standard time required for the cell permeability assays with HeLa cells (Supporting Information).

Cell toxicity assays were carried out with fluorescent peptides **9f** and **9f'** using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) hydrolysis method to determine the cell viability as an indicator of HeLa cells sensitivity to the peptides. As a result, none of the studied peptides resulted to be toxic in the range of 7.8 μM to 200 μM (Supporting Information).

Conclusions

There is a need for developing non-natural, stereochemically defined fluorescent amino acids labeled at their side chains to expand the toolkit of commercially available N-tagged amino acids. We have developed a direct, simple, cost effective and efficient synthesis of enantiopure, fluorescent non-natural amino acids, derived from D-cysteine. The key synthetic step is a *S*-Michael addition of thiol-containing dyes, as nucleophiles, on a chiral bicyclic dehydroalanine, as a Michael acceptor, which can be easily obtained from the inexpensive natural amino acid L-serine. This 1,4-conjugate addition proceeds with total chemo- and stereoselectivity, fast and at room temperature, making it a true click reaction. The Michael adducts were easily transformed into the corresponding amino acids to study their optical properties. In view of the optical properties of the coumaryl and dansyl D-cysteine derivatives, these two free amino acids were N-Fmoc-protected to be used in solid phase peptide synthesis (SPPS) and incorporated into cell penetrating pentapeptides through standard SPPS. The resulting fluorescent peptides were treated with different HeLa cell lines, monitoring their internalization by fluorescence microscopy.

Experimental Section

Reagents and general procedures. Commercial reagents were used without further purification. Solvents were dried and redistilled prior to use in the usual way. All reactions were performed in oven dried glassware with magnetic stirring under inert atmosphere unless noted otherwise. Analytical thin layer chromatography (TLC) was performed on glass plates precoated with a 0.25 mm thickness of silica gel. TLC plates were visualized with UV light and by staining with phosphomolibdic acid (4.5 g) in ethanol (100 mL) or potassium permanganate (1.5 g $KMnO_4$, 10 g K_2CO_3 and 1.25 mL 10% NaOH in 100 mL H_2O) solutions Column chromatography was performed on silicagel (230-400 mesh). Optical rotations (OR) were measured with a Perkin-Elmer 341 polarimeter at a concentration (c) expressed in g/100 mL in 1.0 dm cells of capacity 1.0 or 0.35 mL. Melting points were determined on a Büchi B-545 melting-point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were measured with a Bruker Avance-400 and Bruker ARX-300 spectrometer with TMS as the internal standard. Multiplicities are quoted as singlet (s), broad singlet (br s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), or multiplet (m). Spectra were assigned using COSY and HSQC. All NMR chemical shifts (δ) were recorded in ppm, and coupling constants (J) were reported in Hz. The results of these experiments were processed with MestreNova software. High-resolution electrospray mass (ESI) spectra were recorded on a microTOF-Q-BRUKER spectrometer; accurate masseasurements were achieved by using sodium formate as an external reference. UV-Vis spectra were recorded in approximately 10^{-5} M solutions in water (or DMSO) using an OceanOptics USB4000UV-Vis spectrometer and quartz cells (1 cm path length). Luminescence measurements were recorded with same solutions using a Jobin-Yvon Horiba Fluorolog 3-22 Tau-3 spectrofluorimeter.

NMR experiments. NMR experiments were performed on a 400 MHz spectrometer at 298 K. Magnitude-mode ge-2D COSY spectra were acquired with gradients by using the cosygpqf pulse program with a pulse width of 90º. Phase-sensitive ge-2D HSQC spectra were acquired by using z-filter and selection before t1 removing the decoupling during acquisition by use of the invigpndph pulse program with CNST2 (JHC)=145. Phase-sensitive ge-2D NOESY experiments were performed. NOE intensities were normalized with respect to the diagonal peak at zero mixing time.

General procedure for the *S*-Michael addition reaction. To a solution of chiral α,ß-dehydroamino acid (Dha) derivative **2** (or **ent-2**) as a Michel acceptor and triethylamine as a base in dry THF, a solution of the corresponding SH-containing dye (**3a-f**) as a nucleophile in dry THF was added dropwise (0.2 mL/min) at room temperature. After the completed addition, the reaction was followed by TLC chromatography until total conversion of the Dha derivative **2** and an aqueous saturated NH_4Cl solution (same volume as THF) was added. The aqueous phase was extracted twice with ethyl ether and the combined organic phases were dried with anhydrous Na_2SO_4, filtered and concentrated. The conversion and the diastereomeric excess were determined by ^{1}H-NMR experiments. In order to optimize the Michael additions different conditions were tested. In the case of compound **3f**, dichloromethane was used instead THF due to problems of solubility in THF.

(3*S*,7*R*,7a*S*)-7-Methoxy-7,7a-dimethyl-3-((phenylthio)methyl)dihydro-5*H*-oxazolo[4,3-b]oxazole-2,5(3*H*)-dione (4a). Following the general procedure for the Michael additions, Dha derivative **2** (20.0 mg, 0.094 mmol), triethylamine (14.4 μL, 0.103 mmol), and thiophenol **3a** (11.4 mg, 0.103 mmol) were reacted affording compound **4a** as a colourless oil (27 mg, 0.084 mmol, 89%) after purification by silica gel column chromatography (hexane/ethyl acetate 8:2). $[\alpha]_D^{25}$ (1.00, $CHCl_3$) = -4.2. HRMS (ESI) m/z: 346.0725 $[M+Na]^+$; calculated $C_{15}H_{17}NO_5SNa^+$ 346.0720. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.62 (s, 3H, Me^{7a}), 1.63 (s, 3H, Me^7), 3.14 (dd, 1H, *J* = 14.2, 10.5 Hz, CH_2S), 3.49 (m, 4H, CH_2S, OMe), 4.49 (dd, 1H, *J* = 10.5, 4.3 Hz, H^3), 7.35 (m, 3H, arom), 7.57 (m, 2H, arom). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 16.6 (Me^{7a}), 22.1 (Me^7), 36.6 (CH_2S), 51.6 (OMe), 60.4 (C^3), 101.6 (C^{7a}),

108.2 (C^7), 127.9, 129.3, 132.2, 133.4 (5xC_{arom}), 158.8 (C^5=O), 170.4 (C^2=O).

(3*S*,7*R*,7a*S*)-7-Methoxy-7,7a-dimethyl-3-((naphthalen-1-ylthio)methyl)dihydro-5*H*-oxazolo[4,3-b]oxazole-2,5(3*H*)-dione (4b). Following the general procedure for the Michael additions, Dha derivative **2** (25.0 mg, 0.117 mmol), triethylamine (18.0 µL, 0.129 mmol) and 1-naphtalenethiol **3b** (16.0 µL, 0,129 mmol) were reacted affording compound **4b** as a yellow foam (42 mg, 0.113 mmol, 96%) after purification by silica gel column chromatography (CH_2Cl_2/MeOH 95:5). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): -3.3. HRMS (ESI+) (m/z): 396.0879 [M+Na]$^+$; calculated $C_{19}H_{19}NO_5SNa^+$: 396.0876. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.48 (s, 3H, Me^{7a}), 1.57 (s, 3H, Me^7), 3.15 (dd, 1H, *J* = 14.2, 10.5 Hz, H_β), 3.11-4.46 (m, 4H, H_β, OMe), 4.50 (dd, 1H, *J* = 10.9, 4.2 Hz, H_α), 7.42-7.61 (m, 3H, H^{arom}), 7.83-7.94 (m, 3H, H^{arom}), 8.50 (d, 1H, *J* = 8.5 Hz, H^{arom}). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 16.7 (Me^{7a}), 21.9 (Me^7), 36.4 (C_β), 51.5 (OMe), 60.6 (C_α), 101.6 (C^{7a}), 107.8 (C^7), 125.3, 125.6, 126.4, 127.1, 128.8, 129.6, 130.2, 133.2, 133.6, 134.3 (10xC^{arom}), 158.8 (C^5=O), 170.3 (C^2=O).

(3*S*,7*R*,7a*S*)-7-Methoxy-7,7a-dimethyl-3-((naphthalen-2-ylthio)methyl)dihydro-5*H*-oxazolo[4,3-b]oxazole-2,5(3*H*)-dione (4c). Following the general procedure for the Michael additions, Dha derivate **2** (25.0 mg, 0.117 mmol), triethylamine (18.0 µL, 0.129 mmol) and 2-naphtalenethiol **3c** (18.8 mg, 0,129 mmol) were reacted affording compound **4c** as a yellow foam (42 mg, 0.113 mmol, 95%) after purification by silica gel column chromatography (CH_2Cl_2/MeOH 95:5). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): -53.7. HRMS (ESI+) (m/z): 396.0872 [M+Na]$^+$; calculated $C_{19}H_{19}NO_5SNa^+$: 396.0876. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.58 (s, 3H, Me^{7a}), 1.61 (s, 3H, Me^7), 3.20 (dd, 1H, *J* = 14.2, 10.4 Hz, H_β), 3,43 (s, 3H, OMe), 3.53-4.53 (m, 1H, H_β), 4.51 (dd, 1H, *J* = 10.4, 4.2 Hz, H_α), 7.47-7.50 (m, 2H, H^{arom}), 7.57 (dd, 1H, *J* = 8.6, 1.8 Hz, H^{arom}), 7.80-7.82 (m, 3H, H^{arom}), 8.04 (d, 1H, *J* = 1.5 Hz, H^{arom}). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 16.7 (Me^{7a}), 22.1 (Me^7), 36.3 (C_β), 51.6 (OMe), 60.3 (C_α), 101.6 (C^{7a}), 108.0 (C^7), 126.5, 126.8, 127.6, 127.7, 129.0, 130.6, 131.1, 132.6, 133.7 (10xC^{arom}), 158.8 (C^5=O), 170.4 (C^2=O).

(3*S*,7*R*,7a*S*)-3-(((*1H*-Indol-3-yl)thio)methyl)-7-methoxy-7,7a-dimethyldihydro-5*H*-oxazolo[4,3-b]oxazole-2,5(3*H*)-dione (4d). Following general procedure for the Michael additions, Dha derivate **2** (25.0 mg, 0.117 mmol), triethylamine (18.0 µL, 0.129 mmol) and 1*H*-indole-3-thiol **3d** (17.50 mg, 0.129 mmol) were reacted affording compound **4d** as a yellow foam (25 mg, 0.069 mmol, 60%) after purification by silica gel column chromatography (CH_2Cl_2/MeOH 95:5). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): +174.3. HRMS (ESI+) (m/z): 385.0831 [M+Na]$^+$; calculated $C_{17}H_{18}N_2O_5SNa^+$: 385.0829. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.63 (s, 3H, Me^{7a}), 1.63 (s, 3H, Me^7), 2.82 (dd, 1H, *J* = 14.1, 11.6 Hz, H_β), 3.26 (dd, 1H, *J* = 14.2, 3.8 Hz, H_β), 3,51 (s, 3H, OMe), 4.43 (dd, 1H, *J* = 11.5, 3.8 Hz, H_α), 7.25-7.29 (m, 2H, H^{arom}), 7.42-7.45 (m, 1H, H^{arom}), 7.73-7.74 (m, 1H, H^{arom}), 7.82 (dd, 1H, *J* = 7.5, 1.5 Hz, H^{arom}), 8.51 (m, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 16.7 (Me^{7a}), 22.0 (Me^7), 36.7 (C_β), 51.7 (OMe), 60.2 (C_α), 101.7 (C^{7a}), 108.0 (C^7), 111.9, 119.0, 121.0, 123.0, 129.0, 132.5, 136.6 (7xC^{arom}), 159.5 (C^5=O), 171.0 (C^2=O).

(3*S*,7*R*,7a*S*)-7-Methoxy-7,7a-dimethyl-3-(((4-methyl-2-oxo-2*H*-chromen-7-yl)thio)methyl)dihydro-2*H*-oxazolo[4,3-b]oxazole-2,5(3*H*)-dione (4e). Following the general procedure for the Michael additions, Dha derivative **2** (53.0 mg, 0.249 mmol), triethylamine (38.2 µL, 0.274 mmol) and 7-mercapto-4-methylcoumarin **3e** (52 mg, 0,270) were reacted affording compound **4e** as a yellow solid (96 mg, 0.237 mmol, 93%) after purification by silica gel column chromatography (CH_2Cl_2/MeOH 95:5). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): -51.9. Mp: 128-130 °C. HRMS (ESI+) (m/z): 428.0763 [M+Na]$^+$; calculated $C_{19}H_{19}NO_7SNa^+$: 428.0774. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.60 (s, 3H, Me^{7a}), 1.66 (s, 3H, Me^7), 2.41 (d, 3H, *J* = 1.2 Hz, Me^{4c}), 3.17-3.27 (m, 1H, H_β), 3.48 (s, 3H, OMe^7), 3.54-3.62 (m, 1H, H_β), 4.50 (dd, 1H, *J* = 10.0, 4.4 Hz, H_α), 6.25 (d, 1H, *J* = 1.2 Hz, H^{3c}), 7.30-7.36 (m, 2H, H^{6c}, H^{8c}), 7.50-7.56 (m, 1H, H^{5c}). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 16.8 (Me^{7a}), 18.7 (Me^{4c}), 22.4 (Me^7), 34.9 (CH_2S), 51.9 (OMe^7), 60.1 (C^3), 101.7 (C^{7a}), 108.4 (C^7), 115.0 (C^{3c}), 117.2 (C^{6c}), 118.8 (C_q^c), 125.3, 125.2 (C^{8c}, C^{5c}), 139.4, 152.0, 153.9 (3xC_q^c), 158.8 (C^5=O), 160.4 (C^c=O), 170.1 (C^2=O).

5-(Dimethylamino)-*N*-(2-((((3*S*,7*R*,7a*S*)-7-methoxy-7,7a-dimethyl-2,5-dioxotetrahydro-2*H*-oxazolo[4,3-b]oxazol-3-yl)methyl)thio)ethyl)naphthalene-1-sulfonamide (4f). Following the general procedure for the Michael additions, acrylate **2** (103.0 mg, 0.484 mmol), triethylamine (67 µL, 0.491 mmol) and compound **3f** (149 mg, 0,481) were reacted for 3 hours affording compound **4f** as a light green-yellowish viscous solid (231 mg, 0.441 mmol, 92%). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): -36.2. HRMS (ESI+) (m/z): 524.1515 [M+H]+; calculated $C_{23}H_{30}N_3O_7S_2^+$: 524.1520. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.58 (s, 3H, Me^{7a}), 1.63 (s, 3H, Me^7), 2.75 (td, 2H, *J* = 6.0, 1.9 Hz, CH_2-S), 2.85 (dd, 2H, *J* = 14.6, 4.4 Hz, H_β), 3.09-3.17 (m, 2H, C*H*$_2$-NH), 2.91 (s, 6H, Me), 3.53 (s, 3H, OMe^7), 4.39 (dd, 1H, *J* = 10.2, 4.4 Hz, H_α), 7.22 (d, 1H, *J* = 7.6 Hz, H^{6d}), 7.52-7.65 (m, 2H, H^{7d}, H^{3d}), 8.3- 8.57 (H^{2d}, H^{4d}, H^{8d}). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 16.7 (Me^7), 22.3 (Me^{7a}), 32.3 (*C*H_2-S), 32.6 (C_β), 42.0 (*C*H_2-NH), 45.5 (Me^d), 51.8 (OMe^7), 61.3 (C_α), 101.5 (C^{7a}), 108.5 (C^7), 115.4 (C^{6d}), 118.8, 130.0, 130.7 (C^{2d}, C^{4d}, C^{8d}). 123.3, 128.6 (C^{7d}, C^{3d}), 129.6, 129.7, 134.7, 152.1 (C^{1d}, C^{4ad}, C^{5d}, C^{8ad}), 159.0 (C^2=O), 170.2 (*C*^{5}=O).

5-(Dimethylamino)-*N*-(2-((((3*R*,7*S*,7a*R*)-7-methoxy-7,7a-dimethyl-2,5-dioxotetrahydro-2*H*-oxazolo[4,3-b]oxazol-3-yl)methyl)thio)ethyl)naphthalene-1-sulfonamide (ent-4f). Following the general procedure for the *S*-Michael addition reactions, acrylate **ent-2** (290.0 mg, 1.36 mmol), triethylamine (190 µL, 1.36 mmol) and compound **3f** (425 mg, 1.36 mmol) were reacted for 1.5 hours affording compound **ent-f** as a light green-yellowish syrup (665 mg, 1.27 mmol, 93%). $[\alpha]_D^{25}$ (1.00, $CHCl_3$): +40.6. HRMS (ESI+) (m/z): 524.1518 [M+H]+; calculated $C_{23}H_{30}N_3O_7S_2^+$: 524.1520. NMR data agree with those above reported for its enantiomer **4f**.

General procedure for the hydrolysis reaction. A 4 M HCl aqueous solution was added to the corresponding Michael adduct **4a-c** or **4e-f** or **ent-4f** and the mixture was stirred at 60 °C for 16 h. The solvent was removed under vacuum and the crude was redissolved in water and washed with ethyl acetate. The aqueous phase was evaporated affording the corresponding free amino acid **5a-c**, **5e-f** and **ent-5f**. In the case of Michael adduct **4f**, the mixture was stirred at 40 °C.

***S*-Phenyl-D-cysteine (5a).** Following the general methodology for acid hydrolysis, starting from **4a** (27 mg, 0.084 mmol) and adding a 4 M HCl aqueous solution (5 mL), compound **5a** was obtained as a yellow foam after purification (16 mg, 0.081 mmol, 97%). $[\alpha]_D^{25}$ (1.00, DMSO): -2.5. HRMS (ESI+) (m/z): 198.0588 [M+H]$^+$; calculated $C_9H_{12}NO_2S^+$: 198.0583. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 3.36-3.41 (m, 1H, H_β), 3.48-3.53 (m, 1H, H_β), 4.09 (dd, 1H, *J* = 7.1, 4.4 Hz, H_α), 7.27-7.29 (m, 3H, H^{arom}), 7.42-7.44 (m, 2H, H^{arom}). ^{13}C NMR (100 MHz, D_2O) δ (ppm): 34.2 (C_β), 52.1 (C_α),128.1, 129.5, 131.4, 132.1 (5xC^{arom}), 170.2 (COOH).

***S*-(Naphthalen-1-yl)-D-cysteine (5b).** Following the general methodology for acid hydrolysis, starting from Michael adduct **4b** (42 mg, 0.112 mmol) and adding a 4 M HCl aqueous solution (5 mL), compound **5b** was obtained as a yellow foam after purification (27 mg, 0.109 mmol, 97%). $[\alpha]_D^{25}$ (1.00, DMSO): +25.1. HRMS (ESI+) (m/z): 248.0738 [M+H]$^+$; calculated $C_{13}H_{14}NO_2S^+$: 248.0740. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 3.36-3.41 (m, 1H, H_β), 3.48-3.53 (m, 1H, H_β), 4.09 (dd, 1H, *J* = 7.1, 4.4 Hz, H_α), 7.27-7.29 (m, 3H, H^{arom}), 7.42-7.44 (m, 2H, H^{arom}). ^{13}C NMR (100 MHz,

DMSO) δ (ppm): 42.1 (C_β), 54.2 (C_α), 124.3, 125.8, 126.7, 127.2, 128.9, 129,7, 130.1, 131.9, 132.1, 133.8 (10xC^{arom}), 169.1 (COOH).

***S*-(Naphthalen-2-yl)-D-cysteine (5c).** Following the general methodology for acid hydrolysis, starting from Michael adduct **4c** (51 mg, 0.133 mmol) and adding a 4 M HCl aqueous solution (5 mL), compound **5c** was obtained as a yellow solid after purification (31 mg, 0.127 mmol, 96%). $[\alpha]_D^{25}$ (1.00, DMSO): -5.7. Mp: 128-130 °C. HRMS (ESI+) (m/z): 248.0744 $[M+H]^+$; calculated $C_{13}H_{14}NO_2S^+$: 248.0740. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 3.48-3.64 (m, 2H, H_β), 4.03 (t, 1H, *J* = 5.8 Hz, H_α), 7.46-7.58 (m, 3H, H^{arom}), 7.84-7.93 (m, 3H, H^{arom}), 7.97-8.02 (m, 1H, H^{arom}). ^{13}C NMR (75 MHz, DMSO) δ (ppm): 33.4 (C_β), 51.6 (C_α),126.1, 126.8, 127.1, 127.2, 127.2, 127.7, 128.7, 131.6 131.7, 133.3 (10xC^{arom}), 169.2 (COOH).

***S*-(4-Methyl-2-oxo-2*H*-chromen-7-yl)-D-cysteine hydrochloride (5e).** Following the general methodology for acid hydrolysis, starting from Michael adduct **4e** (176 mg, 0.435 mmol) and adding a 6 M HCl aqueous solution (20 mL), compound **5e** was obtained as yellow oil (120 mg, 0.381 mmol, 88%). $[\alpha]_D^{25}$ (1.00, H_2O): -27.7. HRMS (ESI+) (m/z): 302.0455 $[M+Na]^+$; calculated $C_{13}H_{13}NO_4SNa^+$: 302.0457. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.20 (d, *J* = 1.1 Hz, 3H, Me^{4c}), 3.44-3.64 (m, 2H, H_β), 4.23 (dd, 1H, *J* = 7.2, 4.4 Hz, H_α), 6.05 (d, 1H, *J* = 1.2 Hz, H^{3c}), 7.04 (d, 1H, *J* = 1.8 Hz, H^{9c}), 7.17 (dd, 1H, *J* = 8.4, 1.9 Hz, H^{7c}), 7.41 (d, 1H, *J* = 8.4 Hz, H^{6c}). ^{13}C NMR (100 MHz, D_2O) δ (ppm):17.8 (Me^{4c}), 32.3 (CH_2S), 51.8 ($CHNH_2$), 112.9 (C^{3c}), 115.7 (C^{6c}), 118.1 (C_q^c), 125.0, 125.6 (C^{8c} C^{5c}), 138.8, 152.3, 155.5 (3xC_q^c), 163.3 (C^c=O), 169.7 (COOH).

***S*-(2-((5-(Dimethylamino)naphthalene)-1-sulfonamido)ethyl)-D-cysteine hydrochloride (5f).** Following the general methodology for acid hydrolysis, starting from Michael adduct **4f** (231mg, 0.441 mmol) and adding a 4 M HCl aqueous solution (20 mL), compound **5e** was obtained as green-yellowish foamy oil (168 mg, 0.422 mmol, 96%). $[\alpha]_D^{25}$ (1.00, D_2O): +4.8. HRMS (ESI+) (m/z): 398.1218 $[M+H]^+$; calculated $C_{17}H_{23}N_3O_4S_2NH^+$: 398.1203. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.41-2.49 (m, 2H, CH_2-S), 2.60-2.81 (m, 2H, H_β), 3.01-3.08 (m, 2H, CH_2-NH), 3.42 (s, 6H, Me), 3.99 (dd, 1H, *J* = 7.3, 4.4 Hz, H_α), 7.70-7.84 (m, 2H, H^{7d}, H^{3d}), 7.98 (t, 1H, *J* = 8.6 Hz, H^{6d}), 8.21-8.61 (m, 3H, H^{2d}, H^{4d}, H^{8d}). ^{13}C NMR (100 MHz, D_2O) δ (ppm): 30.9 (C_β), 31.3 (CH_2-S), 41.7 (CH_2-NH), 46.7 (Me), 46.8 (Me) 52.3 (C_α), 119.6, 125.7, 126.6, 130.5 (C^2, C^4, C^6, C^8), 125.5, 127.0 (C^{4a}, C^{8a}) 128.1, 128.5 (C^7, C^3), 135.1 (C^1), 138.3 (C^5), 170.6 (COOH).

***S*-(2-((5-(Dimethylamino)naphthalene)-1-sulfonamido)ethyl)-L-cysteine hydrochloride (ent-5f).** Following the general methodology for acid hydrolysis, starting from Michael adduct **ent-4f** (665 mg. 1.27 mmol) and adding a 4 M HCl aqueous solution (20 mL), compound **ent-5f** was obtained as green-yellowish foamy oil (540 mg, 1.24 mmol, 98%). $[\alpha]_D^{25}$ (1.00, D_2O): -3.2. HRMS (ESI+) (m/z): 398.1209 $[M+H]^+$; calculated $C_{17}H_{23}N_3O_4S_2NH^+$: 398.1203. NMR data agree with those above reported for its enantiomer **5f**.

***N*-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*S*-(4-methyl-2-oxo-2*H*-chromen-7-yl)-D-cysteine (6e).** Derivative **5e** (207 mg, 0.521 mmol), FmocOSu (263 mg, 0.780 mmol) and $NaHCO_3$ (196 mg, 2.33 mmol) were dissolved in a mixture of H_2O/CH_3CN (1:2) (15 mL). The solution was stirred at room temperature for 24 h. CH_3CN was removed *in vacuo* and the aqueous phase was acidified with 2 M HCl (pH 2-3). The solvent was then removed and the crude was purified by silica gel column chromatography (ethyl acetate/MeOH 95:5) affording compound **6e** as yellow foamy oil (145 mg, 0.234 mmol, 47%). $[\alpha]_D^{25}$ (1.00, CH_3OH): 13.1. HRMS (ESI+) (m/z): 524.1094 $[M+Na]^+$; calculated $C_{28}H_{23}NO_6SNa^+$: 524.1100. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 2.33 (s, Hz, 3H, Me^{4c}), 3.37-3.47 (m, 1H, H_β), 3.67-3.75 (m, 1H, H_β), 4.04-4.10 (m, 1H, CH^{Fmoc}), 4.19-4.37 (m, 2H, CH_2^{Fmoc}), 4.43-4.50 (m, 1H, H_α), 6.17 (s, 1H, H^{3c}), 7.23-7.82 (m, 11H, H_{arom}). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 18.4 (Me^{4c}), 35.1 (H_β), 48.4 (CH^{Fmoc}), 54.8 (H_α), 68.1 (CH_2^{Fmoc}), 114.5 (C^{3c}), 116.2 (C^{6c}), 120.8, 120.9, 125.2, 126.2, 126.3, 126.5, 128.2, 128.8, 128.8, 129.2 (CH_{arom}), 119.0, 142.5, 142.5, 143.6, 145.1, 145.2, 154.9, 155.0 (C_q^c, C_q^{Fmoc}), 158.3 (C^c=O), 162.7 (CO^{Fmoc}), 174.9 (COOH).

***N*-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*S*-(2-((5-(dimethylamino)naphthalene)-1-sulfonamido)ethyl)-D-cysteine (6f).** Derivative **5f** (207 mg, 0.521 mmol), FmocOSu (263 mg, 0.133 mmol) and $NaHCO_3$ (196 mg, 2.33 mmol) were dissolved in a mixture of H_2O/CH_3CN (1:2) (15 mL). The solution was stirred at room temperature for 24 h. CH_3CN was removed *in vacuo* and the aqueous phase was acidified with 2 M HCl (pH 2-3). The solvent was then removed and the crude was purified by silica gel column chromatography (ethyl acetate/MeOH 95:5) affording compound **6f** as a brownish viscous solid (145 mg, 0.234 mmol, 48%). $[\alpha]_D^{25}$ (1.00, DMSO): 3.7. HRMS (ESI+) (m/z): 620.1884 $[M+H]^+$; calculated $C_{32}H_{33}N_3O_6S_2H^+$: 620.1884. ^{1}H NMR (400 MHz, DMSO) δ (ppm): 2.58-2.60 (m, 1H, CH_2-S), 2.64 (dd, 1H, *J* = 13.6, 9.8 Hz, H_β), 2.73-2.76 (m, 1H, CH_2-S), 2.77-2.80 (m, 1H, H_β), 2.81 (s, 6H, Me), 2.92-2.99 (m, 2H, CH_2-NH), 3.99-4.06 (m, 1H, H_α), 4.17-4.33 (m, 3H, CH^{Fmoc}, CH_2^{Fmoc}), 7.231-8.46 (m, 16H, H_{arom}, N*H*Fmoc, $NHSO_2$).^{13}C NMR (100 MHz, DMSO) δ (ppm): 25.2 (CH_2-S), 32.6 (C_β), 42.5 (CH_2-NH), 45.1 (Me), 46.6 (CH^{Fmoc}) 53.9 (C_α), 65.7 (CH_2^{Fmoc}), 115.1, 119.1, 119.9, 120.0, 121.4, 123.6, 125.3, 127.1, 127.3, 127.6, 127.9, 128.2, 128.9, 129.5, 136.0, 137.4, 139.4, 140.7, 142.6, 143.8, 151.4, 156.0 (C_{arom}), 172.2, 173.8 (COOH, CO^{Fmoc}).

***N*-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*S*-(2-((5-(dimethylamino)naphthalene)-1-sulfonamido)ethyl)-L-cysteine (ent-6f).** Derivative **ent-5f** (500 mg, 1.15 mmol), FmocOSu (583 mg, 1.73 mmol) and $NaHCO_3$ (437 mg, 5.18 mmol) were dissolved in a mixture of H_2O/CH_3CN (1:2) (45 mL). The solution was stirred at room temperature for 24 h. CH_3CN was removed *in vacuo* and the aqueous phase was acidified with 2 M HCl (pH 2-3). The solvent was then removed and the crude purified by silica gel column chromatography (ethyl acetate/MeOH 95:5) affording compound **ent-6f** as a brownish syrup (365 mg, 0.590 mmol, 51%). $[\alpha]_D^{25}$ (1.00, DMSO): -4.8. HRMS (ESI+) (m/z): 620.1887 $[M+H]^+$; calculated $C_{32}H_{33}N_3O_6S_2H^+$: 620.1884. NMR data agree with those above reported for its enantiomer **6f**.

General methodology for C-terminal acid peptides. 2-Chlorotrityl chloride-PS resin (0.05 mmol) was swelled in CH_2Cl_2 for 15 min and reacted with the first Fmoc-protected amino acid (0.1 mmol) and diisopropylethylamine (DIPEA) (0.25 mmol) in CH_2Cl_2 (3 mL) for 2 h at room temperature. A mixture of CH_2Cl_2/MeOH/DIPEA (80:15:5, 5 mL) was added to the resin and the mixture was shaken for 10 min at room temperature and repeated once with fresh solution. Solid phase peptide synthesis (SPPS) was performed manually on the resulting resin using 1-(bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate) (HATU) and DIPEA for coupling and 20% (v/v) solution of piperidine in DMF for Fmoc deprotection. Fmoc-protected fluorescent cysteine (0.15 mmol) was coupled using HATU (0.145 mmol) and DIPEA (1.8 mmol) for 1 hour, as judged by Keiser test.[16] Acetic anhydride and pyridine (1:2) was used for N-terminus acetylation and trifluoroacetic acid (TFA), thioanisole, anisole and 1,2-ethanedithiol (90:5:2:3) for the final cleavage and removal of the side chain protecting groups. The peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in acetonitrile/water containing 0.1% TFA (1:1, v/v), and lyophilized to afford the crude peptide. Purification by reverse phase high-performance liquid chromatography (RP-HPLC) afforded the corresponding peptide.

General methodology for C-terminal amide peptides. Microwave assisted solid phase peptide synthesis (MW-SPPS) was performed automatically on Rink Amide MBHA resin (0.05 mmol) using an automated

synthesizer Liberty Blue (CEM). DIC/Oxyma pure were used for coupling and 20% (v/v) solution of piperidine in DMF for Fmoc deprotection. Fmoc-protected fluorescent cysteine (0.15 mmol) was coupled manually using HATU (0.145 mmol) and DIPEA (1.8 mmol) for 1 hour, as judged by Keiser test.[16] Acetic anhydride and pyridine (1:2) was used for N-terminus acetylation and TFA, thioanisole, anisole and 1,2-ethanedithiol (90:5:2:3) for the final cleavage and removal of the side chain protecting groups. The peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in acetonitrile/water containing 0.1% TFA (1:1, v/v), and lyophilized to afford the crude peptide. Purification by RP-HPLC afforded the corresponding peptide.

Ac-D-Cys(Coum)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-OH (7e). Peptide **7e** was synthesized following the general methodology for C-terminal acid peptides using **6e** as fluorescent cysteine. HRMS (ESI+) (m/z): 830.4041 $[M+H]^+$; calculated $C_{40}H_{59}N_7O_{10}SH^+$: 830.4044. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 0.81 (dd, 12 H, *J* = 22.6, 6.3 Hz, Me^V, Me^L), 1.27 (d, 3 H, *J* = 7.2 Hz, Me^A), 1.31-1.41 (m, 2H, CH_2-CH_2-CH_2-NH_2^K), 1.45-1.62 (m, 5H, CH_2-CH_2-NH_2^K, CH^L, $H_\beta{}^L$), 1.65-1.75 (m, 2H, $CH_2{}^P$), 1.75-1.88 (m, 4H, $H_\beta{}^K$, $CH_2{}^P$), 1.89 (s, 3H, NHAc), 1.92 (d, 1H, *J* = 7 Hz, $H_\beta{}^V$), 2.10-2.21 (m, 2H, $CH_2{}^P$), 2.32 (s, 3H, Me^{4c}), 2.90 (t, 2H, *J* = 7.5 Hz, CH_2-NH_2^K), 3.46-3.62 (m, 2H, $H_\beta{}^{C*}$), 4.00 (d, 1H, *J* = 7.7 Hz, $H_\alpha{}^V$), 4.14-4.31 (m, 4H, $H_\alpha{}^A$, $H_\alpha{}^L$, $H_\alpha{}^P$, $H_\alpha{}^K$), 4.53 (t, 1H, *J* = 6.3 Hz, $H_\alpha{}^{C*}$), 6.20 (s, 1H, H^{3c}), 7.13 (s, 1H, H^{9c}), 7.22 (d, 1H, *J* = 8.2 Hz, H^{7c}), 7.53 (d, 1H, *J* = 8.4 Hz, H^{6c}).

Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-OH (7f). Peptide **7f** was synthesized following the general methodology for C-terminal acid peptides using **6f** as fluorescent cysteine. HRMS (ESI+) (m/z): 948.4673 $[M+H]^+$; calculated $C_{44}H_{69}N_9O_{10}S_2H^+$: 948.4682. ^{1}H NMR (400 MHz, H_2O/D_2O) δ (ppm): 0.75-0.99 (m, 12 H, Me^V, Me^L), 1.39 (d, 3H, *J* = 7.2 Hz, Me^A), 1.54-1.76 (m, 7H, CH^L, $H_\beta{}^L$, CH_2-CH_2-NH_2^K, CH_2-CH_2-CH_2-NH_2^K), 1.99 (s, 3H, NHAc), 2.03-2.06 (m, 1 H, $H_\beta{}^V$), 2.22-2.37 (m, 2H, $CH_2{}^P$), 2.41-2.70 (m, 6H , $H_\beta{}^{C*}$, $CH_2{}^P$, CH_2-S^{linker}), 2.91 (s, 6H, Me^{C*}), 2.96-3.20 (m, 4H, CH_2-NH^{linker}, CH_2-NH_2^K), 3.63-3.86 (m, 4H, $H_\beta{}^K$, $CH_2{}^P$), 4.14-4.21 (m, 1H, $H_\alpha{}^{C*}$), 4.26-4.40 (m, 5H, $H_\alpha{}^A$, $H_\alpha{}^L$, $H_\alpha{}^P$, $H_\alpha{}^K$, $H_\alpha{}^V$), 7.34-8.87 (m, 12H , H_{arom}, CONH).

Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Lys-NH_2 (8f). Peptide **8f** was synthesized following the general methodology for C-terminal amide peptides using **6f** as fluorescent cysteine. HRMS (ESI+) (m/z): 970.4836 $[M+H]^+$; calculated $C_{44}H_{70}N_{10}O_9S_2H^+$: 947.4841. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 0.86-0.98 (m, 12 H, Me^V, Me^L), 1.38 (d, 3H, *J* = 7.2 Hz, Me^A), 1.51-1.97 (m, 11H, CH^L, $H_\beta{}^L$, CH_2-CH_2-NH_2^K, CH_2-CH_2-CH_2-NH_2^K, $CH_2{}^P$, $H_\beta{}^K$), 2.01 (s, 3H, NHAc), 2.02-2.11 (m, 1H, $H_\beta{}^V$), 2.22-2.37 (m, 2H, $CH_2{}^P$), 2.49-2.77 (m, 4H, $H_\beta{}^{C*}$, CH_2-S^{linker}), 3.01 (t, 2H, *J* = 7.5 Hz, CH_2-NH_2^K), 3.20 (t, 2H, *J* = 6.4 Hz, CH_2-NH^{linker}), 3.45 (s, 6H, Me^{C*}), 3.65-3.74 (m, 1H, $CH_2{}^P$), 3.83-3.91 (m, 1H, $CH_2{}^P$), 4.26-4.41 (m, 6H, $H_\alpha{}^A$, $H_\alpha{}^L$, $H_\alpha{}^P$, $H_\alpha{}^K$, $H_\alpha{}^V$ $H_\alpha{}^{C*}$), 7.91 (t, 2H, *J*=8.2 Hz, H^{3d}, H^{7d}), 8.00-8.06 (m, 1H, H^{6d}), 8.40-8.79 (m, 3H , H^{2d}, H^{4d}, H^{8d}).

Ac-D-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Arg-NH_2 (9f). Peptide **9f** was synthesized following the general methodology for C-terminal amide peptides using **6f** as fluorescent cysteine. Acetylation reaction was performed by dissolving the resulting peptide in Ac_2O and pyridine (1:2). After stirring for 2 h at room temperature, the solvent was removed *in vacuo* and the crude was purified by RP-HPLC. HRMS (ESI+) (m/z): 975.4941 $[M+H]^+$; calculated $C_{44}H_{70}N_{12}O_9S_2H^+$: 975.4903. ^{1}H NMR (300 MHz, D_2O) δ (ppm): 0.81-1.00 (m, 12H, Me^V, Me^L), 1.38 (d, 3H, *J* = 7.2 Hz, Me^A), 1.53-1.72 (m, 5H, $H_\beta{}^L$, CH^L, CH_2-CH_2-NH_2^R), 1.71-1.96 (m, 4H, $H_\beta{}^R$, $CH_2{}^P$), 1.99 (s, 3H, NHAc), 2.03 (s, 1 H, $H_\beta{}^V$), 2.04-2.08 (m, 1H, $CH_2{}^P$), 2.22-2.33 (m, 1H, $CH_2{}^P$), 2.48-2.56 (m, 2H , CH_2-S^{linker}), 2.57-2.75 (m, 2H , $H_\beta{}^{C*}$), 3.11-3.22 (m, 4 H, CH_2-NH^{linker}, CH_2-NH_2^R), 3.24 (s, 6H, Me^{C*}), 3.63-3.73 (m, 1H, $CH_2{}^P$), 3.80-3.91 (m, 1H, $CH_2{}^P$), 4.24-4.39 (m, 6H, $H_\alpha{}^{C*}$ $H_\alpha{}^A$, $H_\alpha{}^L$, $H_\alpha{}^P$, $H_\alpha{}^R$, $H_\alpha{}^V$), 7.79-7.87 (m, 3H, H^{3d}, H^{6d}, H^{7d}), 8.31-8.62 (m, 3H , H^{2d}, H^{4d}, H^{8d}).

Ac-L-Cys(Dans)-L-Val-L-Pro-L-Ala-L-Leu-L-Arg-NH_2 (9f'). Peptide **9f'** was synthesized following the general methodology for C-terminal amide peptides using **ent-6f** as fluorescent cysteine. Acetylation reaction was performed by dissolving the resulting peptide in Ac_2O and pyridine (1:2). After stirring for 2 h at room temperature, the solvent was removed *in vacuo* and the crude purified by RP-HPLC. HRMS (ESI+) (m/z): 975.4889 $[M+H]^+$; calculated $C_{44}H_{70}N_{12}O_9S_2H^+$: 975.4903. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 0.83-0.97 (m, 12H, Me^V, Me^L), 1.38 (d, 3 H, *J* = 7.2 Hz, Me^A), 1.55-1.71 (m, 5H, $H_\beta{}^L$, CH^L, CH_2-CH_2-NH_2^R), 1.73-1.93 (m, 4H, $H_\beta{}^R$, $CH_2{}^P$), 2.00 (s, 3H, NHAc), 2.02-2.08 (m, 2 H, $H_\beta{}^V$, $CH_2{}^P$), 2.23-2.32 (m, 1H, $CH_2{}^P$), 2.46-2.69 (m, 4H , CH_2-S^{linker}, $H_\beta{}^{C*}$), 3.00 (s, 6H, Me^{C*}), 3.13-3.24 (m, 4 H, CH_2-NH^{linker}, CH_2-NH_2^R), 3.63-3.73 (m, 1H, $CH_2{}^P$), 3.80-3.89 (m, 1H, $CH_2{}^P$), 4.24-4.37 (m, 6H, $H_\alpha{}^{C*}$ $H_\alpha{}^A$, $H_\alpha{}^L$, $H_\alpha{}^P$, $H_\alpha{}^R$, $H_\alpha{}^V$), 7.56 (d, 1H, H^{6d}), 7.71-7.80 (m, 2H, H^{3d}, H^{7d}), 8.29-8.55 (m, 3H , H^{2d}, H^{4d}, H^{8d}).

Cell line stocks. Cell lines were obtained from European Collection of Animal Cell Cultures (ECACC, United Kingdom) and they were maintained in liquid nitrogen for long-term storage. The freezing process was done slowly to avoid cell death due to possible crystal formation. Cells were trypsinized and spun down (1000 rpm, 5 min) at room temperature. Afterwards, cells were resuspended in culture medium with 10% FBS. In addition, dimethyl sulfoxide (DMSO, Sigma), a cryoprotectant, was added to a 10% final concentration. Cells were frozen in cryotubes at -80 ºC in isopropanol containers for a week. Afterwards, they were preserved permanently in liquid nitrogen. In contrast, thawing and recovery of cells from liquid nitrogen was done quickly. Cryotubes were removed from liquid nitrogen and maintained in dry ice until they thawed, at which point they were immediately placed in a 37 ºC water bath. Cells were washed with culture medium Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal calf serum to eliminate DMSO. Finally, cells were resuspended in fresh medium, transferred to a flask for cell culture.

Cell culture. Briefly, Hela cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal calf serum, 2 mM L-glutamine and penicillin–streptomycin (100 units/mL and 100 mg/mL, respectively). Cells were maintained in an incubator at 37 °C in an atmosphere of 5% CO_2. Hela cells were seeded in 24-well plates at a density of 50000 cells/well and were cultured overnight. Prior to starting the experiment, medium was removed from the plates, and fresh medium containing 10 µM from the stock solution (10 mM in DMSO) of the required peptide was added to the cells. Cells were cultured for 2 h before microscopy visualization. After that, peptide media was removed and cells were rinsed to take the images.

Fluorescence microscopy. Data were obtained with a Leica microscope (*DM6000B*) equipped with a *Hamamatsu Orca-ER C10600* and an A4 Leica filter.

Degradation studies with human plasma: Resistance to proteases was evaluated for both peptides **9f** and **9f'**. Enzimatic degradation in human plasma was analysed using commercially available serum from human male AB plasma. Peptides **9f** and **9f'** were suspended in a solution of 20% human serum in water at 37 ºC and the degradation of the peptides was determined at 0.5, 1,5 and 4 h (time in which these peptides are incubated with HeLa cells), by using UPLC-micrOTOF-Q (column: ACQUITY UPLC BEH C18 1.7; diameter: 2.1 mm and length: 100 mm), eluting a mixture of 5 mL of the peptide-serum solution in 795 mL of water and employing an isocratic method (70% H_2O, 30% acetonitrile, temperature: 40 ºC).

Cytotoxicity assay. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) hydrolysis method (MTS-based CellTiter® 96. AQueous Assay; Promega Corp., Madison, WI) was used to determine the cell viability as an indicator of HeLa cells sensitivity to the peptides. 50 µL of exponentially growing cells were seeded at a density of $1.5x10^3$ cells per well, in a 96-well flatbottomed microplate in growing media. 24 h later cells were incubated with the corresponding peptides at final concentration in sextuplicates ranged from 7,8 µM to 200 µM. After 2, 24 and 72 h at 37 °C, 20 µL of MTS was added and plates were incubated for 1 h at 37 °C. Optical density was measured at 490 nm using a 96-well multiscanner autoreader (POLARstar Omega, BMG Labtech; Germany).

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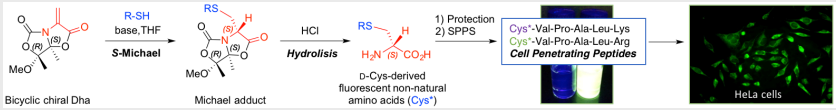

This work describes the efficient synthesis of enantiopure and fluorescent D-cysteines, using highly chemo- and stereoselective *S*-Michael additions of thiol-containing dyes on a chiral bicyclic dehydroalanine. Their use as fluorescent labels was validated by incorporation into cell penetrating pentapeptides, which were treated with different HeLa cell lines, monitoring their internalization by fluorescence microscopy.

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Cell Penetrating Peptides containing Fluorescent D-Cysteines