Fluorescent labeling of peptides on solid phase†

Alan R. Katritzky,*[a] Megumi Yoshioka,[a] Tamari Narindoshvili,[a] Alfred Chung[b] and Jodie V. Johnson[c]

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N^{α}-Fmoc-N^{ε}-[(7-methoxycoumarin-4-yl)acetyl]-L-lysine (N^{α}-Fmoc-L-Lys(Mca)-OH) **3** is conveniently prepared by benzotriazole methodology (52% over two steps). *N*-Acylbenzotriazoles Mca-Bt **2**, N^{α}-Fmoc-L-Lys(Mca)-Bt **4**, coumarin-3-ylcarbonyl (Cc)-Bt **5**, N^{α}-Fmoc-L-Lys(Cc)-Bt **7** and N^{α}-(Cc)-L-Lys(Fmoc)-Bt **9** enable the efficient microwave enhanced solid-phase fluorescent labeling of peptides.

Introduction

Proteins modified with fluorescent dyes, enzymes, and other reporter groups are valuable tools with widespread uses in immunology and biochemical research. Protein-capture microarrays have become a promising tool for protein analysis in drug discovery, diagnostics and biological research in the last few years.[1a] Fluorescent derivatives of biologically active peptides are useful experimental tools for studying biological structure and function[1b] and for visualization of intracellular processes or molecular interactions.[1c,d]

Matrix metalloprotease (MMP) proteins are implicated in many diseases, including arthritis, periodontal disease, tumor cell invasion, and metastasis.[2–7] The detection of a protein bound by a specific capture agent is key to microarray-based methods, for traditional immunoassays and biosensor applications.[8–12] Synthetic peptide-based assays can differentiate enzyme types and monitor their activities.[13–17] Fluorogenic substrates can be monitored[18] continuously and utilized at low concentrations, thus providing a particularly convenient enzyme assay method.

Fluorescent biosensors can be composed of a binding molecule, such as an antibody or enzyme, derivatized with a single fluorescent probe, which is sensitive to changes in the local environment.[19] Fluorogenic groups can often be attached at the cleavage sites of proteases and esterases. However, this simple approach is not applicable if the enzyme requires binding interactions at both sides of the cleavage site. For such cases, quenched fluorescent peptides are designed as short sequences of amino acids, containing enzyme cleavable specific sites, with fluorescent donor and acceptor probes linked at the N- and C-termini.[13–17]

In other biosensors[20] the binding molecule is labeled with two fluorophores, suitable for fluorescence resonance energy transfer (FRET). The acceptor–quencher pair enables nonradiative energy transfer between an excited donor fluorophore to a proximal acceptor fluorophore.[21] Usually, the donor fluorescence is quenched by the acceptor without subsequent fluorescence emission. Donor and acceptor groups,[13–17,22–24] attached to a synthetic peptide undergo FRET, producing a unique fluorescence spectrum. Enhanced donor fluorescence indicates proteolysis accompanied by the loss of FRET as a result of separation of the donor and acceptor groups.

Many donor and acceptor groups have been incorporated into quenched fluorogenic substrates.[13–24] Coumarins have extensive and diverse applications as fluorescent probes or labels;[25] they exhibit an extended spectral range, are photostable and have high emission quantum yields. 7-Methoxycoumarin-4-ylacetyl (Mca, $\varepsilon_{325} = 14500\ M^{-1}cm^{-1}$ and $\Phi_f = 0.49$) was proposed as a fluorophore for thimet peptidase, pitrilysin and MMP substrates.[14,16a,17] The combination of Mca as a fluorophore and (2,4-dinitrophenyl) (Dnp) as a quencher has several advantages over the more common fluorogenic substrate pair of tryptophan (Trp)/Dnp: the Mca residue is more fluorescent and more chemically stable than Trp, and Mca is efficiently quenched by Dnp.

Fluorescently labeled peptides might be achieved by the reaction of the peptide in solution with an activated form of fluorophore, however a potentially more effective approach is to assemble the peptide chain on a solid phase and incorporate the fluorophore into the peptide attached to the solid support.[26a,26b]

For the solid phase peptide labeling by Mca, the N-termini of peptide–resins have been acylated with 7-methoxycoumarin-4-ylacetic acid using standard synthetic cycles.[14,16a,17] However, inefficient acylation of the peptide-resin led Malkar and Fields to incorporate Mca into N^{α}-Fmoc-lysine molecules by a 4-step method providing N^{α}-Fmoc-L-Lys(Mca)-OH (17% overall).[27]

Coumarin-labeled lysines are of considerable general interest for the design and synthesis of fluorogenic triple-helical substrates for the analysis of matrix metalloproteinase family members.[27–29] Thus, N^{ε}-coumarin-labeled-N^{α}-Fmoc lysines allow the successful labeling of peptide substrates by solid phase peptide synthesis for an extracellular matrix metalloprotease and present a powerful tool for proteolysis monitoring.[27,28]

We have previously reported the extensive use of *N*-acylbenzotriazoles for *N*-acylation,[30] *C*-acylation[31] and *O*-acylation[32] reactions. (N^{α}-Fmoc-aminoacyl)benzotriazoles and their Boc- and Cbz- analogs enabled the preparation of chiral di-, tri- and tetrapeptides in average yields of 88% from natural amino acids

[a]*University of Florida, Gainesville, FL 32611-7200, USA. E-mail: katritzky@chem.ufl.edu; Fax: +1 352-392-9199; Tel: +1 352-392-0554*
[b]*PROTEOMICS, Interdisciplinary Center for Biotechnology, Research University of Florida, Gainesville, FL 32611-1376, USA. E-mail: achung@biotech.ufl.edu*
[c]*Department of Chemistry, University of Florida, Gainesville, FL 32611-7200, USA. E-mail: jvj@chem.ufl.edu*

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in solution phase.[33] Recently we have also prepared tri-, tetra-, penta-, hexa-, and heptapeptides in 71% average crude yields by microwave-assisted solid phase peptide synthesis utilizing (N^{α}-Fmoc-aminoacyl)benzotriazoles.[34] These easily obtained, chirally stable, and moisture insensitive reagents acylate NH_2-peptide-resins without using coupling agents or additives, thus avoiding side reactions and epimerization.[34]

We now report the efficient fluorescent labeling of peptides on solid phase by acylation with benzotriazole activated derivatives of (i) coumarin-3-ylcarboxylic acid and 7-methoxycoumarin-4-ylacetic acid and (ii) coumarin-3-ylcarbonyl (Cc) and 7-methoxycoumarin-4-ylacetyl (Mca) labeled lysines.

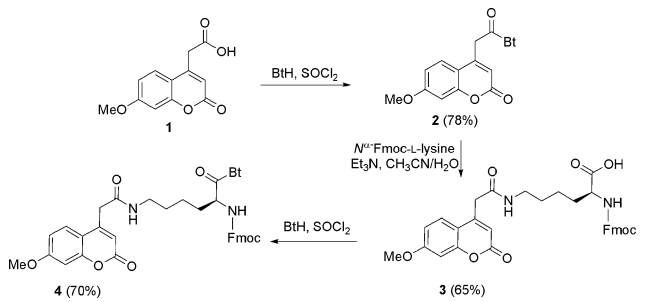
In comparison with the conventional procedures for the preparation of fluorescent peptides on solid phase,[24–26,28] our methodology optimized under mild microwave irradiation, utilizes benzotriazole activated fluorogenic substrates which are stable, moisture insensitive acylation reagents enabling solid phase fluorescent labeling of peptides without using coupling agents or additives and avoiding side reactions and epimerization.

Results and discussion

Benzotriazole activated fluorogenic substrates **2**, **4**, **5**, **7**, **9** were prepared and demonstrated to be useful acylation reagents, for the efficient fluorescent labeling of peptides on solid phase.

N^{α}-Fmoc-N^{ε}-[(7-methoxycoumarin-4-yl)acetyl]-L-lysine (N^{α}-Fmoc-L-Lys(Mca)-OH) 3

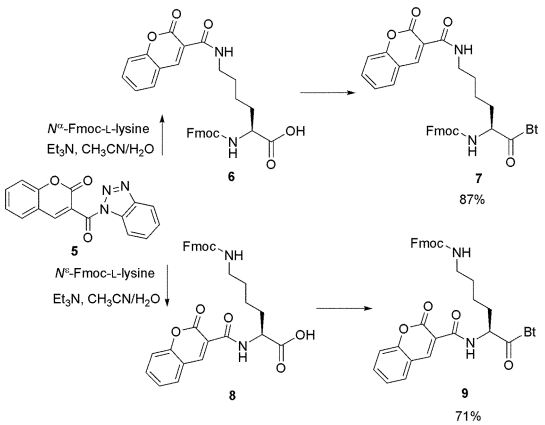
7-Methoxycoumarin-4-ylacetic acid **1** was converted by 1*H*-benzotriazole and thionyl chloride in THF at 20 °C into crystalline, stable 4-(benzotriazole-1-ylacetyl)-7-methoxycoumarin **2** (78%) (Scheme 1); **2** was then coupled with N^{α}-Fmoc-L-lysine in aqueous MeCN in the presence of Et_3N for 20 min, to afford N^{α}-Fmoc-L-Lys(Mca)-OH **3** (overall 51%). In comparison with the recent literature procedure[27] for the preparation of N^{α}-Fmoc-L-Lys(Mca)-OH **3**, our two-step methodology uses N^{α}-Fmoc-L-lysine, offers simple preparative and workup procedures, short times to completion, the use of inexpensive reagents and high yields. Conventional benzotriazole activation of **3** gave N^{α}-Fmoc-L-Lys(Mca)-Bt **4** (70%) (Scheme 1).



Scheme 1

N^{α}-Fmoc-N^{ε}-(coumarin-3-ylcarbonyl)-L-lysine benzotriazolide (N^{α}-Fmoc-L-Lys(Cc)-Bt) 7 and N^{α}-(coumarin-3-ylcarbonyl)-N^{ε}-Fmoc-L-lysine benzotriazolide (N^{α}-Cc-L-Lys(Fmoc)-Bt) 9

3-(Benzotriazole-1-ylcarbonyl)chromen-2-one[35] **5** was coupled with commercially available N^{α}-Fmoc-L-lysine and N^{ε}-Fmoc-L-lysine in aqueous MeCN at 20 °C in the presence of Et_3N to provide lysine-scaffold based fluorescent building blocks **6** (see ref. 35) and novel **8** (87 and 79%), respectively (Scheme 2), convertible conveniently into the corresponding *N*-acylbenzotriazoles **7** (see ref. 36) and **9** (87 and 71%).



Scheme 2

Solid Phase Peptide Synthesis

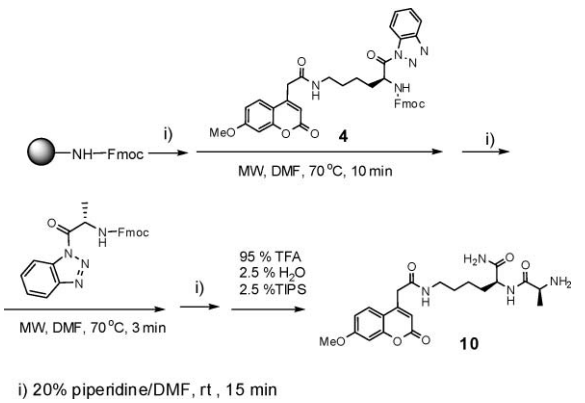
Solid phase peptide synthesis, microwave-assisted as optimized previously in our laboratory,[34] enables efficient acylation of NH_2-groups on solid phase by the benzotriazole activated fluorogenic substrates **2**, **4**, **5**, **7**, **9**. We used **2** or **5** to couple the fluorophore directly to diverse peptides. Alternatively, we used **4**, **7**, or **9** to couple the fluorophore already attached to a lysine moiety to the peptides. Coumarin-labeled peptides were synthesized as *C*-terminal amides using Fmoc solid-phase methodology under microwave irradiation.

Solid phase fluorescent labeling with 4 and preparation of labeled dipeptide H-L-Ala-L-Lys(N^{ε}-Mca)-NH_2 10

The convenience of **4** for fluorescent labeling on solid phase was demonstrated for a model dipeptide H-L-Ala-L-Lys(N^{ε}-Mca)-NH_2 **10**. Compound **10** was synthesized using microwave-assisted SPPS conditions.[34] After initial removal of the Fmoc protecting group, free Rink resin-NH_2 was coupled with **4** in DMF under microwave irradiation for 10 min at 70 °C. The second coupling was performed with the (N^{α}-Fmoc-aminoacyl)benzotriazole reagent derived from Fmoc-L-Ala, finally the desired peptide was cleaved from the resin to produce peptide amide **10** (26%) (Scheme 3, Table 1, Fig. 1). Conditions were optimized to maximize the rate while avoiding epimerization.

Solid phase fluorescent labeling with 7 to synthesize labeled peptides 11–14

In a similar manner, microwave-assisted SPPS was achieved (3 min coupling time for each step) with **7** to obtain the fluorescently labeled di, tri-, tetra- and hexa-peptides: H-L-Ala-L-Lys(N^{ε}-Cc)-NH_2 **11**, H-L-Pro-L-Phe-L-Lys(N^{ε}-Cc)-NH_2 **12**, H-L-Trp-L-Lys(N^{ε}-Cc)-L-Met-L-Phe-NH_2 **13** and H-L-Lys(N^{ε}-Cc)-L-Pro-Gly-L-Leu-L-Met-L-Trp-NH_2 **14** in yields of 18–45% (after HPLC purification) (Table 1). The successive coupling steps



Scheme 3

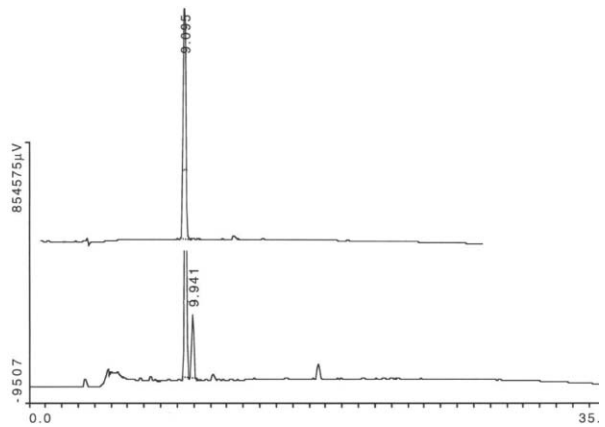


Fig. 1 Bottom: the HPLC profile of crude peptide **10** (H-L-Ala-L-Lys(N^{ε}-Mca)-NH_2). Top: the HPLC profile of peptide **10** after purification.

utilized the appropriate *N*-acylbenzotriazoles derived from Fmoc-protected L-Met, L-Trp, L-Phe, L-Leu, L-Pro and Gly prepared by our previously published procedures.[34] The mild synthetic conditions allowed utilization of the unprotected indole-NH of L-Trp, and no complications were observed with L-Met or any other of the amino-*N*-protected amino acids utilized.

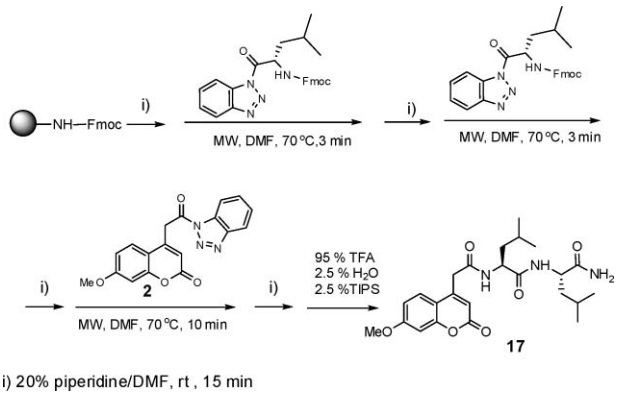
Solid phase fluorescent labeling with 9 to synthesize labeled tripeptide 15

Microwave-assisted SPPS (3 min coupling time for each step) and Fmoc strategy also successfully allowed fluorescent labeling by coupling with N^{α}-coumarin-attached lysine **9** through its free N^{ε}-position to prepare labeled tripeptide H-L-Phe-L-Leu-L-Lys(N^{α}-Cc)-NH_2 **15** (35%) (Table 1).

Solid phase fluorescent labeling with 5 to synthesize labeled dipeptide (Cc)-L-Leu-L-Leu-NH_2 16 and labeling with 2 to synthesize labeled dipeptide (Mca)-L-Leu-L-Leu-NH_2 17

Fluorescent labeling with benzotriazole-activated coumarin-3-ylcarboxyl acid **5** was demonstrated by the preparation of coumarin-3-ylcarbonyl-labeled dipeptide (Cc)-L-Leu-L-Leu-NH_2 **16**. After initial removal of the Fmoc protecting group from Rink resin, Fmoc-L-Leu-Bt was utilized for each of two successive coupling steps (3 min coupling time for each step). After the final coupling with **5** (10 min coupling time), the desired fluorescent-labeled peptide **16** (29%) (Table 2) was cleaved from the resin.

Fluorescent labeling with benzotriazole activated 7-methoxy-coumarin-4-ylacetic acid **2** was demonstrated to allow the preparation of 7-methoxycoumarin-4-ylacetyl-labeled dipeptide (Mca)-L-Leu-L-Leu-NH_2 **17** (26%) (Scheme 4, Table 2), under similar conditions to those utilized for the preparation of **16**.



Scheme 4

Absorption ($\lambda_{Abs.}$) and fluorescence ($\lambda_{Em.}$) wavelength maxima were measured of all synthesized fluorescent peptides **10–17**. (Table 3) (Figures showing fluorescence are included in the ESI.†)

Conclusions

In conclusion we have described the convenient and efficient preparation of a variety of coumarin fluorescent probes or

Table 1 Preparation of fluorescent peptides **10–15**

Labeled peptide	Structure (N–C terminus)	Purified peptide			
		Yield (%)[a]	Purity (%)[b]	t_R[c]/min	HRMS[d] $[M + H]^+$
10	H-L-Ala-L-Lys(N^{ε}-Mca)-NH_2	26	99	9.10	433.2103
11	H-L-Ala-L-Lys(N^{ε}-Cc)-NH_2	45	99	9.82	389.1825
12	H-L-Pro-L-Phe-L-Lys(N^{ε}-Cc)-NH_2	23	99	13.38	562.2680
13	H-L-Trp-L-Lys(N^{ε}-Cc)-L-Met-L-Phe-NH_2	18	94	18.02	782.3328
14	H-L-Lys(N^{ε}-Cc)-L-Pro-Gly-L-Leu-L-Met-L-Trp-NH_2	20	99	17.00	902.4212
15	H-L-Phe-L-Leu-L-Lys(N^{α}-Cc)-NH_2	35	99	14.28	578.2987

[a] Isolated yields after HPLC purification; [b] Purity after HPLC purification; [c] t_R = retention time. For conditions see experimental. [d] For the calculated values, see the ESI.†

Table 2 Preparation of fluorescent peptides **16** and **17**

Labeled peptide	Structure (N–C terminus)	Purified peptide			
		Yield (%)[a]	Purity (%)[b]	t_R[c]/min	HRMS[d] $[M + H]^+$
16	(Cc)-L-Leu-L-Leu-NH_2	29	> 99	20.67	416.2223
17	(Mca)-L-Leu-L-Leu-NH_2	26	> 99	17.47	460.2455

[a] Isolated yields after HPLC purification; [b] Purity after HPLC purification; [c] t_R = retention time. For conditions see experimental. [d] For the calculated values, see the ESI.†

Table 3 Absorption and fluorescence data of fluorescent labeled peptides **10–17**

Entry	Compound	$\lambda_{Abs.}$[nm][a]	$\lambda_{Em.}$ [nm][a]
1	**10**	323	383
2	**11**	294	409
3	**12**	295	407
4	**13**	289	409
5	**14**	290	405
6	**15**	299	407
7	**16**	295	413
8	**17**	322	383

[a] Determined in 95% methanol.

labels, including coumarin-3-carbonyl and 7-methoxycoumarin-4-yl-acetyl labeled lysines as fluorogenic substrates in solution phase and demonstrated that their benzotriazole derivatives are appropriate materials for peptide labeling thus enabling efficient peptide α-amino group acylation under microwave irradiation on solid phase without using coupling agents or additives and without side reactions or epimerization.

Experimental

General Methods

Reagents were purchased from Peptides International or Aldrich and used without further purification. Rink-amide-MBHA resin (200–400 mesh, 0.35 meq/g) was purchased from Peptide International (Louisville, KY, USA). Melting points were determined on MEL-TEMP II apparatus. NMR spectra were recorded on OXFORD 300 in $CDCl_3$ with TMS as the internal standard for ^1H (300 MHz) or a solvent as the internal standard for ^{13}C NMR (75 MHz). UV and fluorescence measurements were recorded on Cary 100 UV-Vis and FluoroMax spectrophotometers respectively. Elemental analyses were performed on a Carlo Erba-1106 instrument. MALDI analyses were performed on Bruker Reflex II TOF mass spectrometer retrofilled with delayed extraction.

Analytical reversed-phase HPLC was performed on a Rainin HPXL system with a Vydac C-18 (5 μm, 2.1 × 250 mm) silica column at a 1ml/min flow rate. Peptides were eluted using a 10–80% gradient of solvent B (0.1% TFA in acetonitrile) *vs* solvent A (0.1% TFA in water) and peaks were detected at a wavelength of 214 nm. The identification of the products was achieved by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, ABI 4700 Proteomics Analyzer) with α-cyano-4-hydroxy cinnamic acid as the matrix. Synthesis of the peptides was performed in a Discover BenchMate peptide synthesizer from CEM (Matthews, NC, USA). The conditions for a variety of coupling steps were optimized to increase rate and eliminate epimerization. Single mode irradiation with monitoring of temperature, pressure, and irradiation power *versus* time was used throughout, making the procedure highly reproducible. To customize, we varied T_{max} from 50 to 70 °C, power from 80–100 W, and time from 3–10 min. The conditions (T_{max} = 70 °C, P_{max} = 100 W, and time = 3–10 min) were found to be optimal.

MS/MS peptide fragmentation was obtained on the crude peptides by way of low resolution MS and tandem mass spectrometry (MSn) data obtained *via* HPLC/UV/(+)ESI-MS and –MSn on a ThermoFinnigan (San Jose, CA) LCQ Classic quadruple ion trap mass spectrometer in electrospray ionization (ESI) mode. High resolution mass spectrometry (HRMS) *via* flow-injection positive [(+)ESI]-time of flight (TOF) was obtained on an Agilent 1200 series spectrometer.

(*S*)-2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-6-(2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)acetamido)pentanoic acid (N^{α}-Fmoc-L-Lys(Mca)-OH) (3). Compound **2** (0.36 g, 1.1 mmol) was added in one portion to a solution of N^{α}-Fmoc-L-lysine (0.57 g, 1.8 mmol) in MeCN–H_2O (24 mL:5 mL), in the presence of Et_3N (0.75 mL, 5.4 mmol). The reaction mixture was stirred at 20 °C for 20 min. A solution of 6M HCl (2 mL) was then added and the MeCN was removed under reduced pressure. The residue was extracted with EtOAc (100 mL) and the organic extract was washed with 6M HCl (2 × 50 mL), brine (50 mL) and dried over $MgSO_4$. Evaporation of the solvent gave white microcrystals of **3** (0.41 g, 65%), which were recrystallized from EtOAc–hexanes. mp 184.9–185.7 °C. ^1H NMR (300 MHz, DMSO-d_6) δ 1.20–1.46 (m, 4H), 1.46–1.80 (m, 2H), 3.02–3.15 (s, 2H), 3.70 (s, 2H), 3.88 (s, 3H), 3.90–3.98 (m, 1H), 4.20–4.36 (m, 3H), 6.28 (s, 1H), 6.97–7.06 (m, 2H), 7.36 (t, J = 7.2 Hz, 2H), 7.45 (t, J = 7.2 Hz, 2H), 7.64–7.74 (m, 2H), 7.76 (d, J = 7.4 Hz, 2H), 7.93 (d, J = 7.4 Hz, 2H), 8.24–8.31 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 23.1, 28.5, 30.4, 46.6, 53.8, 55.9, 65.6, 100.9, 112.2, 112.6, 112.7, 120.1, 125.3, 126.5, 127.1, 127.7, 140.7, 143.8, 143.9, 151.2, 154.9, 156.2, 160.1, 162.4, 167.4, 174.0. Found: C, 67.55; H, 5.60; N, 4.40. Anal. calcd. for $C_{33}H_{32}N_2O_8$: C, 67.80; H, 5.52; N, 4.79%.

General procedure for the synthesis of fluorescent peptides on solid phase

Labeled peptides were synthesized using Fmoc solid-phase methodology as C-terminal amides utilizing Rink-amide-HMBA resin. The commercial Rink-amide-HMBA resin was deprotected by 20% piperidine/DMF (15 min, 20 °C) (0.05 mmol) and then coupled with 5 eq. of benzotriazole activated fluorogenic substrate (**2**, **4**, **5**, **7**, **9**) or with N^{α}-Fmoc-protected (aminoacyl)benzotriazole reagent derived from Fmoc-protected amino acids, prepared

following previously published procedures,[34] (for the sequences see Tables 1 and 2) in DMF, under microwave irradiation for 10 min for fluorescent labeling and 3 min for other couplings. When complete coupling was verified by a negative Kaiser (ninhydrin) test (10 min), the solid resin was washed with DMF (3 × 5 mL) and DCM (3 × 5 mL) followed by another coupling. After the coupling step, the desired peptide was cleaved from the resin using established cleavage cocktails: (i) TFA–anisole–thioanisole-2,3-dimercaptopropanol (from Alfa Aesar, Ward Hill, MA USA) (90:2:3:5) (for peptide sequences including Trp or Met) or (ii) TFA:water:TIPS (95:2.5:2.5) (for the other peptide sequences) at 20 °C for 2 h. The resin was filtered, then the cocktail was concentrated under nitrogen, and cold diethyl ether was added to achieve precipitated peptide (**10–17**), under conditions optimized to increase rate and eliminate epimerization.

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