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# General strategy for the preparation of membrane permeable fluorogenic peptide ester conjugates for in vivo studies of ester prodrug stability

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**Abstract**—To study ester prodrug stability properties in living cells we have conjugated fluorogenic esters to the cell membrane permeable peptide $Arg_9$. The desired conjugates are prepared by coupling N-maleoyl amino acid esters of monoalkylated fluoresceins or fluorescein to $TyrArg_9Cys$. The photophysical properties of the monoalkylated fluorescein derivatives are described.


## 1. Introduction

Prodrugs are a practical way to increase drug bioavailability and selectivity. In a recent series of papers we have described a new approach for the design of patient specific chemotherapeutic drugs and diagnostic agents based on nucleic acid triggered drug or probe release.[1–3] The basic idea is to use a unique or overexpressed mRNA molecule specific to the disease state to template the association of two complementary nucleic acid analogues, one bearing a prodrug and the other a catalytic group that can trigger the release of the drug or probe. Currently we have been using imidazole as the catalytic group to release phenol-based probes and drugs by hydrolysis of the corresponding phenyl ester. A key requirement of this approach is that the prodrug is stable under physiological conditions, and that it is readily hydrolyzed by the catalytic group when held in proximity by the disease specific nucleic acid sequence.

Usually the stability of a candidate prodrug is evaluated by monitoring the prodrug and its degradation products in physiological buffer solutions and with purified enzymes, or with intestinal juices, serum, and cell or tissue homogenates or lysates.[4–6] We have found that we can dramatically enhance the stability of phenyl ester prodrugs in human serum by substituting the α-position with bulky groups[2] but know nothing about the stability of these phenyl esters in living cells. Rather than use cell lysates, we have decided to develop a general method for conjugating fluorogenic ester prodrugs to cell membrane permeable peptides so that their hydrolysis inside living cells can be monitored by fluorescence microscopy and flow cytometry.

## 2. Results and discussion

### 2.1. Design of the fluorogenic ester peptide conjugates

We designed the fluorogenic ester peptide conjugates so that they could be prepared by a versatile synthetic strategy that would allow for variation of the membrane permeability of the fluorophore to be released, the lability of the ester linkage, and the membrane permeability of the peptide. These criteria could be met as described below through the use of the *N*-maleimido amino acid esters of mono-*O*-alkylated fluoresceins. The alkyl group on the fluorescein could be used to modulate its membrane permeability properties, natural and unnatural amino acids side chains could be used to modulate the rate of enzymatic hydrolysis of the ester, and the maleimido group could be used to couple different permeation peptides through an appended cysteine under neutral conditions.

We chose to make fluorogenic esters based on mono-*O*-alkylated fluoresceins, because their long wavelength absorption and fluorescence properties are similar to

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those of fluorescein itself, which has found wide application as a fluorescent probe in biological systems. While mono-*O*-substituted and unsubstituted fluorescein is in equilibrium with the highly fluorescent carboxylate form under neutral and basic conditions, di-*O*-substituted fluorescein compounds exist in a non-fluorescent lactone form. As a result, di-*O*-substituted fluoresceins have been widely used as fluorogenic substrates for enzyme activity studies. For example, mono-*O*-alkylated fluorescein phosphate has been used as a fluorogenic substrate for the continuous assay of phosphoinositide-specific phospholipase C.[7,8] In another study, *N*-acetyl-β-D-glucosaminides of 6′-*O*-alkylated fluorescein derivatives were used as substrates for a kinetic assay of *N*-acetyl-β-D-glucosaminidase.[9] In these studies, enzyme activities were quantified by analysis of the fluorescence resulting from enzymatic formation of the fluorescent mono-*O*-alkylated fluorescein. The additional advantage of using mono-*O*-substituted fluoresceins as fluorescent probes is that their solubility and membrane permeability following release can be tailored by the nature of the appended side chain. In this study, we have sought to replace the previously used hydrophobic butyl substituent with more water soluble methoxyethoxymethyl (MEM) and ethylmethylether (EME) groups, but in principle, any suitable side chain could be added.

Although some fluorescein esters have shown the ability to disperse into cells passively[10–14] a common carrier would be necessary to transport different fluorogenic esters into cells efficiently and quantitatively so that the hydrolysis rates can be directly compared. We have initially chosen $Arg_9$ to transport the esters into cells because polyarginine peptides show high membrane permeability in many cell lines,[15–19] and are able to transport a variety of molecular cargoes such as drugs, peptides, and enzymes into cells.[20–22] Among oligoarginines in the range 4 to 16 arginines, $Arg_9$, which has similar charge and amino acid composition as HIV Tat peptide 46–57, shows both efficient permeability and synthetic economy.[15,16,19,22]

Because the fluorogenic esters are readily hydrolyzed under basic conditions, we required a method for coupling them to the membrane permeabilizing peptides that takes place efficiently under neutral or acidic conditions. We therefore decided to make use of the same Michael addition of thiol to maleimide that we have previously used to conjugate the base labile *p*-nitrophenyl, hydroxycoumarin, and fluorescein esters to oligodeoxynucleotides at neutral pH[1,2] and PNA,.[3] The required *N*-maleimido amino acid esters are conveniently made from maleic anhydride and amino acids in two steps, and thiolated $Arg_9$ or other peptides can be readily made by appending cysteine to the sequence.

### 2.2. Synthesis of the mono-*O*-alkylated fluoresceins

The mono-*O*-alkylated fluoresceins were synthesized as illustrated in Scheme 1 according to methods previously reported for the butyl derivative.[7,8] In the first step, the disodium salt of fluorescein **1** is prepared by treatment of fluorescein with NaOH. The disodium salt is then alkylated with the appropriate alkyl bromide to give the intermediate ester **2**, which can then be hydrolyzed with base and acidified to provide the desired alkylated fluoresceins **3**. Whereas alkylation to give the butyl and EME derivatives **3a** and **3b** required higher temperatures and longer reaction times, alkylation to give the MEM[23] derivative **3c** only required room temperature and a 30 min reaction time. MEM fluorescein can also be obtained directly from fluorescein upon treatment with MEMCl.

### 2.3. UV–Visible absorption properties of the mono-*O*-alkylated fluoresceins

3′-*O*-Butyl,-EME and-MEM fluoresceins have the same absorption maxima of 450 and 475 nm in the visible spectrum (Fig. 1), which are blue shifted relative to that of 488 nm for fluorescein. The molar extinction coefficients, ε, of the mono-alkylated fluoresceins at 475 nm (Table 1) were 22,300, 18,200 and 16,200 $cm^{-1}$ $M^{-1}$ for the 3′-*O*-butyl,-MEM and-EME derivatives of fluorescein, respectively, at pH 7 compared to 68,000 $cm^{-1}$ $M^{-1}$ for fluorescein at 488 nm. The lower absorptivity of the mono-*O*-alkylated fluoresceins than fluorescein is likely due to lower electron delocalization that results from replacement of a phenolate anion with a phenylether.

### 2.4. Fluorescence excitation and emission spectra

The fluorescence excitation spectra of 3′-*O*-butyl,-EME and-MEM fluoresceins under neutral conditions have similar excitation spectra at pH 7.0 (Fig. 2), with a excitation maximum wavelength of 467 nm, which is blue-shifted compared to that of 492 nm for fluorescein. The emission spectra (Fig. 2B) show a similar emission maximum of 512 nm, though 3′-*O*-butyl fluorescein shows greater fluorescence intensity. The acetyl derivative of the 3′-*O*-butyl,-EME and-MEM fluoresceins **4a**–**c** were not fluorescent in the 400–500 nm range (shown for 6′-*O*-acetyl-3′-*O*-EME-fluorescein, **4b**, Fig.

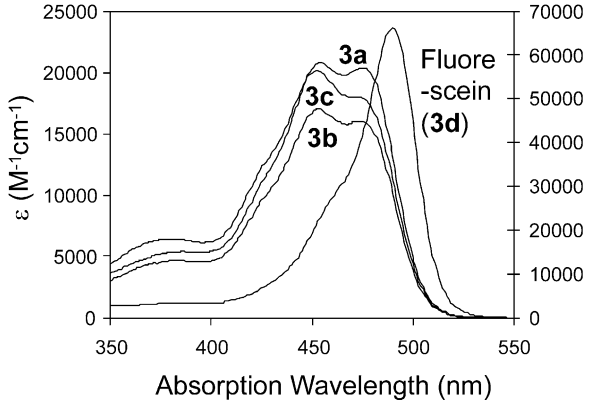
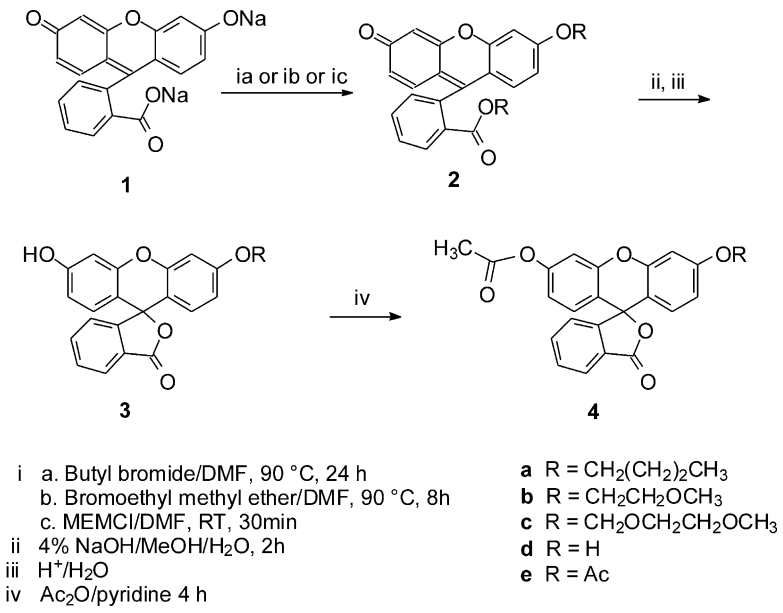



**Figure 1.** The molar extinction curves of the mono-*O*-alkylated fluorescein derivatives in comparison to fluorescein. Absorption spectra for 3′-*O*-butyl fluorescein **3a**, 3′-*O*-EME fluorescein **3b**, 3′-*O*-MEM fluorescein **3c** and fluorescein were obtained at pH 7.0 (30 μM in 10 mM PBS). The scale on the left is for **3a**–**c** and on the right for fluorescein (**3d**).




**Scheme 1.**

**Table 1.** Photophysical properties of mono-*O*-alkylated fluoresceins

| | pH 7.0 | | | pH 9.0 | | |
|---|---|---|---|---|---|---|
| | λ max (nm) excitation | λ max (nm) emission | ε $_{475}$ ($cm^{-1}M^{-1}$) | λ max (nm) excitation | λ max (nm) emission | ε $_{475}$ ($cm^{-1}$ $M^{-1}$) |
| **3a** | 467 | 512 | $2.2\times10^4$ | 467 | 512 | $2.6\times10^4$ |
| **3b** | 467 | 512 | $1.6\times10^4$ | 467 | 512 | $1.8\times10^4$ |
| **3c** | 467 | 512 | $1.8\times10^4$ | 467 | 512 | $1.9\times10^4$ |

1A). Emission from the mono-alkylated fluoresceins upon excitation at 467 nm is only about 15% that of fluorescein excited at 492 nm, which can be explained in part by the lower absorptivity of the mono-alkylated fluoresceins compared to fluorescein (Fig. 1).

### 2.5. pH Dependence of the fluorescence

The fluorescence emission intensity of the alkylated fluoresceins was obtained as a function of pH (Fig. 3) so that their p$K_a$'s could be estimated as well as their fluorescence efficiency under biologically relevant pHs. The mono-*O*-alkylated fluoresceins all have similar p$K_a$ values in the range of 6.0 to 6.3 (Fig. 3), and at pH 7.0 fluoresce with greater than 90% of their maximum value. The excitation and emission spectra acquired at pH 9.0 retain the same features as those observed at pH 7.0 except for a slight increase in intensity (data not shown), due to the exclusive presence of the fluorescent carboxylate form (Table 1).

### 2.6. Synthesis of *N*-maleimido amino acid esters of the monoalkylated fluoresceins

To test the effects of sterics on the stability of the esters in vivo we prepared a series of substituted amino acid esters in which the α-substituent was varied from hydrogen to methyl to isopropyl to diphenylmethyl. The *N*-maleimido amino acid esters of the mono-*O*-alkylated fluoresceins were synthesized by a two step process. In the first step, the amino acids **5a–d** were treated with maleic anhydride and the resulting *N*-maleoyl amino acids were treated with thionyl chloride to effect formation of the maleimide and simultaneously convert the carboxylic acid to the acid chloride **6a–d** (Scheme 2).[24] The crude acid chlorides were then treated with the appropriate fluorescein derivative to yield the esters **7–10** (Scheme 3). In addition to coupling the *N*-maleimido amino acids to the mono-*O*-alkylated fluoresceins **3a–c**, we also coupled some of them to 3′-*O*-acetyl-fluorescein **4d** and fluorescein **3d**. We expect that the acetyl group of the $Arg_9$ conjugates of the *N*-maleimido amino acid esters of 3′-*O*-acetyl-fluorescein will be rapidly hydrolyzed in vivo to yield the mono amino acid ester of fluorescein. Though the mono ester will be fluorescent, the fluorescence is expected to increase almost 10-fold upon hydrolysis of the ester linkage, the rate of which could be monitored.

### 2.7. Conjugation of the fluorogenic esters to $TyrArg_9Cys$

The $TyrArg_9Cys$ peptide was synthesized by standard Fmoc solid phase methodology and then coupled to a four-fold excess of the maleimido esters **7–10** in methanol for 2 h to give the desired conjugates **11–14** (Scheme 4). Methanol was used as a solvent instead of water because of it was better able to solubilize the reactants, and because the conjugated products could be readily isolated by ether precipitation. All the conjugates were purified by RP-HPLC and characterized by MALDI-TOF MS (Table 2).

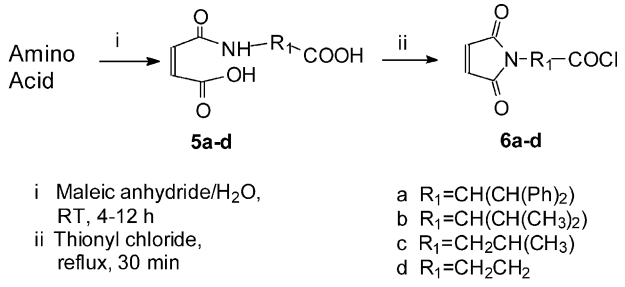
## 3. Conclusion

In summary, we have developed a general method for synthesizing fluorogenic esters and attaching them to a permeation peptide. These fluorogenic ester conjugates should enable the stability of the ester linkage of prodrugs to be evaluated in different cell lines by fluorescence microscopy and flow cytometry, and are currently under study. Fluorogenic ester conjugates with labile ester linkages might also be useful for validating entry of membrane permeabilizing peptides into cells. Other fluorogenic ester conjugates might find use as reporter molecules for disease specific enzymes with esterase activity.

## 4. Experimental

### 4.1. General

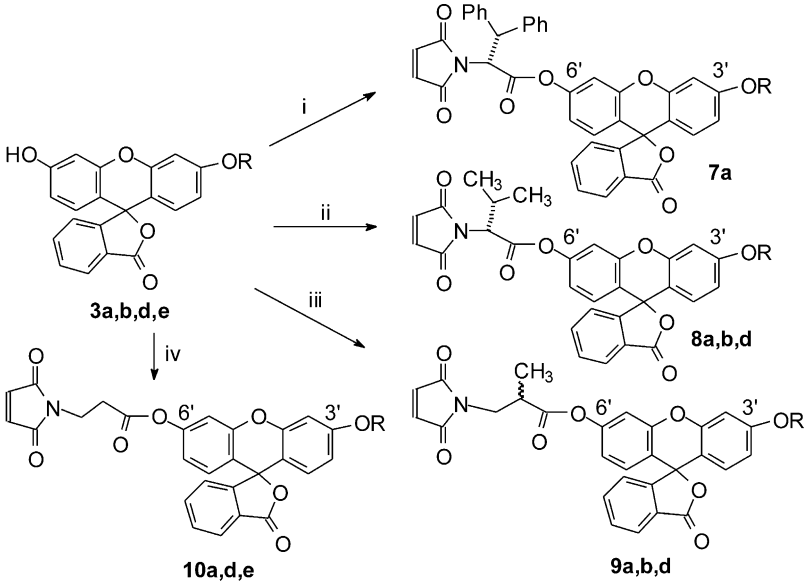
Dichloromethane (DCM) and triethylamine (TEA) were dried by refluxing with $CaH_2$ overnight followed by distillation. Fluorescein, 1-bromobutane, bromoethyl methyl ether, 2-methoxyethoxymethyl chloride, maleic anhydride, thionyl chloride, D-valine, β-alanine, and D,L-3-amino-2-methylpropionic acid were purchased from Aldrich. D-3,3-diphenylalanine was from PepTech Co. Fmoc protected amino acids, resin and other reagents for solid phase peptide synthesis were purchased from NovaBiochem. $^1$H NMR spectra were obtained on a 300 MHz Varian UnityPlus-300 or Varian Mercury-300 spectrometer. Proton chemical shifts are expressed in ppm from tetramethylsilane using residual chloroform (δ = 7.27 ppm), methanol (δ = 4.87 ppm), acetone (δ = 2.05) as an internal standard. Flash chromatography was performed on Selecto Scientific silica gel. TLC and preparative TLC were run on precoated 254-nm fluorescent silica gel sheets manufactured by Alltech Associates. Fluorescence spectra were recorded on a SPEX Fluoromax instrument. UV spectral data were acquired on a Bausch and Lomb Spectronic 1001 spectrophotometer or Varian Cary 100 Bio UV-Visible Spectrophotometer. Matrix-assisted laser desorption ionization (MALDI) mass spectra of peptide conjugates were measured on PerSeptive Voyager RP




**Scheme 2.**

**Table 2.** MALDI-TOF MS of conjugates of fluorogenic esters and $TyrArg_9Cys$ peptide shown in Scheme 4

| | $R_1$ | R | Calcd. MW | Observed (M + 1) |
|---|---|---|---|---|
| **11a** | –CH(CH(Ph)$_2$)-CO— | Butyl | 2381 | 2382.15 |
| **12a** | –CH(CH(CH$_3$)$_2$)-CO— | Butyl | 2257 | 2256.69 |
| **12b** | –CH(CH(CH$_3$)$_2$)-CO— | EME | 2258 | 2259.14 |
| **12d** | –CH(CH(CH$_3$)$_2$)-CO— | H | 2200 | 2201.38 |
| **13a** | –CH$_2$CH(CH$_3$)-CO— | Butyl | 2243 | 2244.26 |
| **13b** | –CH$_2$CH(CH$_3$)-CO— | EME | 2244 | 2243.99 |
| **13d** | –CH$_2$CH(CH$_3$)-CO— | H | 2186 | 2186.97 |
| **14a** | –CH$_2$CH$_2$-CO— | Butyl | 2229 | 2229.80 |
| **14d** | –CH$_2$CH$_2$-CO— | H | 2173 | 2174.14 |
| **14e** | –CH$_2$CH$_2$-CO— | COCH$_3$ | 2215 | 2216.18 |




**Scheme 3.**

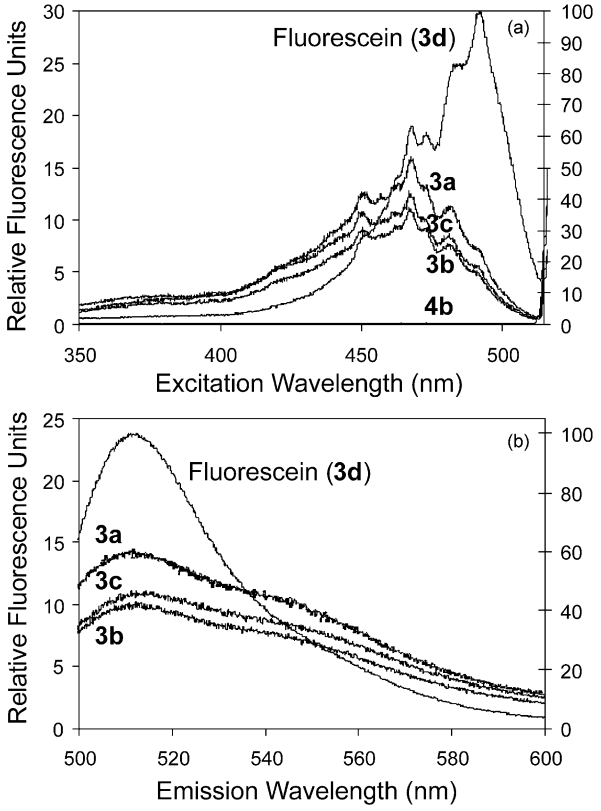



**Figure 2.** Fluorescence spectra of the fluorescein derivatives in comparison to fluorescein. A. Excitation spectra of 3′-*O*-butyl fluorescein **3a**, 3′-*O*-EME fluorescein **3b**, 3′-*O*-MEM fluorescein **3c** and 6′-*O*-acetyl-3′-*O*-EME fluorescein **4b**, (1 μM in 10 mM pH 7.0 PBS, detection at 518 nm) B. Emission spectra of butyl fluorescein **3a**, EME fluorescein **3b**, and MEM fluorescein **3c**. (1 μM in 10 mM pH 7.0 PBS, excitation at 467 nm). The emission intensity was normalized to 100 for the maximum emission intensity of fluorescein (excitation at 492). The scale is on the left for **3a–c**, and **4b**, and on the right for fluorescein (**3d**).

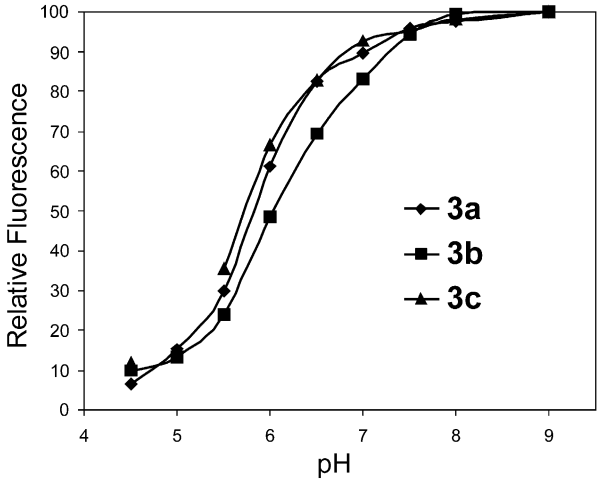
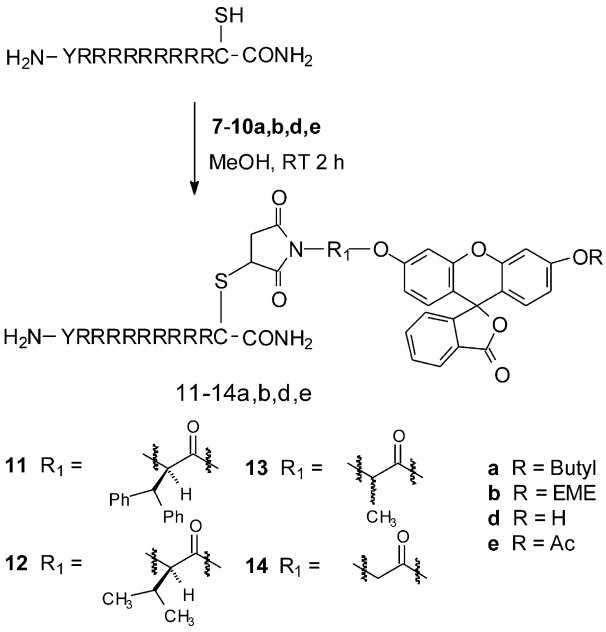



**Figure 3.** Relative fluorescence as a function of pH for the fluorescein derivatives. The derivatives were excited at 467 nm and detected at 518 nm in 10 mM PBS at the indicated pH and normalized to the maximum intensity for each derivative.




**Scheme 4.**

MALDI-time of flight (TOF) mass spectrometer. HR-FAB mass spectra were obtained on a MS-50TA (Ion Spec B126). HPLC were carried out on Beckman Coulter System Gold 126 with RP C18 semi preparative columns.

**4.1.1. 3′-*O*-Butyl-fluorescein (3a).** 1-Bromobutane (2.8 mL, 26.5 mmol) was added dropwise with stirring to a suspension of the disodium salt of fluorescein **1**[8] (2.5 g, 6.6 mmol) in DMF (60 mL) at RT and then heated at 90–100 °C with stirring for 24 h. The DMF was removed from the mixture by evaporation under reduced pressure and the residue treated with 60 mL of 4% NaOH in MeOH–water (3:1, v/v) and stirred for 2 h. The solution was then acidified by dropwise addition of 3 N aqueous HCl until a pH of about 2. The aqueous solution was extracted with ethyl acetate (3×50 mL) and the combined ethyl acetate fractions were dried over $Na_2SO_4$, and evaporated to give the crude butyl substituted fluorescein. Further purification by flash chromatography on silica gel using hexane/ethyl acetate (2:1) gave 1.23 g (48% yield) of **3a** as a yellow powder. $^1$H NMR ($CD_3COCD_3$) δ 0.96 (t, $J$ = 7.5 Hz, 3H), 1.43–1.56 (m, 2H), 1.72–1.81 (m, 2H), 4.08 (t, $J$ = 6.5 Hz, 2H), 6.64–6.66 (m, 2H), 6.68–6.71 (m, 2H), 6.84–6.85 (m, 1H), 6.75 (d, $J$ = 2 Hz, 1H), 7.27(d, $J$ = 8 Hz, 1H), 7.73 (td, $J$ = 7.5, 1 Hz, 1H), 7.81 (td, $J$ = 7.5, 1 Hz, 1H), 7.98 (d, $J$ = 7.5 Hz, 1H), 9.05 (s, 1H); HR FAB MS ($m/z$), calculated for $C_{24}H_{20}O_5Li$ ($M+Li^+$) 395.1471, found 395.1472.

**4.1.2. 3′-*O*-EME-fluorescein (3b).** Bromoethyl methyl ether (1 mL, 1.48 g, 10.6 mmol) was added dropwise to a suspension of the disodium salt of fluorescein **1**[8] (1.0 g, 2.7 mmol) in DMF (25 mL) at RT and stirred for 8 h at 90 °C. The DMF was removed under reduced pressure and 20 mL of 4% NaOH in MeOH and water (3:1, v/v) was added to the residue and stirred for 2 h. The reaction was worked up as described for **3a** to give 0.50 g (48% yield) of **3b** as a yellow powder following flash

chromatography with hexane–ethyl acetate (1:1). $^1$H NMR ($CD_3COCD_3$) δ 3.36 (s, 3H), 3.73 (t, $J$ = 4.5 Hz, 2H), 4.21 (t, $J$ = 4.5 Hz, 2H), 6.62–6.79 (m, 5H), 6.85–6.89 (m, 1H), 7.28 (d, $J$ = 7.5 Hz, 1H), 7.81 (t, $J$ = 7.5 Hz, 2H), 7.73 (t, $J$ = 7.5 Hz, 1H), 8.00 (d, $J$ = 7.5 Hz, 1H), 9.05 (s, 1H); HR FAB MS ($m/z$) calculated for $C_{23}H_{18}O_6Li$ ($M + Li^+$) 397.1263, found 397.1273.

**4.1.3. 3′-*O*-MEM-fluorescein (3c).** MEMCl (121 μL, 1.1 mmol) was added dropwise to a suspension of the disodium salt of fluorescein **1**[8] (200 mg, 0.53 mmol) in DMF (25 mL) and stirred for 30 min at rt. The DMF was removed under reduced pressure and 20 mL of 4% NaOH in MeOH and water (3:1, v/v) was added and stirred for 2 h. The reaction was worked up as described for **3a** to give 159 mg (71% yield) of **3c** as a yellow powder following purification by flash chromatography on silica gel using hexane–ethyl acetate (1.5:1–1:1). $^1$H NMR ($CD_3COCD_3$) δ 3.27 (s, 3H), 3.52 (t, $J$ = 4.5 Hz, 2H), 3.80 (t, $J$ = 4.5 Hz, 2H), 5.34 (s, 2H), 6.60–6.69 (m, 3H), 6.73–6.81 (m, 2H), 7.00 (d, $J$ = 2.5 Hz, 1H), 7.26–7.31 (m, 1H), 7.70–7.84 (m, 2H), 7.96–8.02 (m, 1H), 9.08 (s, 1H); HR FAB MS ($m/z$) calculated for $C_{24}H_{20}O_7Li$ ($M + Li^+$) 427.1369, found 427.1373.

**4.1.4. 6′-Acetyl-3′-*O*-butyl-fluorescein (4a).** A mixture of acetic anhydride–pyridine–DCM (1.5 mL, 1:1:2, v/v) was added to 3′-*O*-butyl-fluorescein **3a** (26 mg, 0.067 mmol) in 4 mL of DCM and stirred for 4 h at rt. The DCM was removed under reduced pressure and the residue was purified by preparative TLC with hexane/ethyl acetate (1:1) to afford 22 mg (76% yield) of **4a** as a white solid. $^1$H NMR ($CD_3COCD_3$) δ 0.97 (t, $J$ = 7.5 Hz, 3H), 1.44–1.57 (m, 2H), 1.72–1.83 (m, 2H), 2.28 (s, 3H), 4.09 (t, $J$ = 6.5 Hz, 2H), 6.71–6.79 (m, 2H), 6.87–6.93 (m, 3H), 7.16–7.19 (m, 1H), 7.34 (d, $J$ = 7 Hz, 1H), 7.76 (t, $J$ = 7 Hz, 1H), 7.83 (t, $J$ = 7 Hz, 1H), 8.02 (d, $J$ = 7 Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{26}H_{22}O_6Li$ ($M + Li^+$) 437.1576, found 437.1577.

**4.1.5. 6′-acetyl-3′-*O*-EME-fluorescein (4b).** Acetic anhydride (65 mg, 0.64 mmol, 50 μL) and pyridine (51 mg, 0.64 mmol, 34 μL) were added to 3′-*O*-EME-fluorescein **3b** (25 mg, 0.064 mmol) in 3 mL DCM and stirred for 4 h at rt. The product was isolated and purified as described for **4a** to give 11 mg (39% yield) of **4b** as a white solid. $^1$H NMR ($CD_3COCD_3$) δ 2.40 (s,3H), 3.36 (s, 3H), 3.72–3.76 (m, 2H), 4.20–4.24 (m, 2H), 6.73–6.78 (m,3H), 6.84–6.95 (m, 3H), 7.08 (s, 2H), 7.34 (d, $J$ = 7.5 Hz, 1H), 7.76 (td, $J$ = 7.5, 1 Hz, 1H), 7.83 (td, $J$ = 7.5, 1 Hz, 1H), 8.02 (d, $J$ = 7.5 Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{25}H_{20}O_7Li$ ($M + Li^+$) 439.1369, found 439.1369.

**4.1.6. 6′-acetyl-3′-*O*-MEM-fluorescein (4c).** 3′-*O*-MEM-fluorescein **3c** (25 mg, 0.058 mmol) was dissolved in 3 mL of DCM and $Ac_2O$ (59 mg, 0.58 mmol, 43 μL) and pyridine (46 mg, 0.58 mmol, 30 μL) was added and stirred for 4 h at rt. The desired product was isolated and purified by a similar procedure as described for **4a** to give 22 mg (80% yield) of **4c** as a white solid. $^1$H NMR ($CD_3COCD_3$) δ 2.29 (s, 3H), 3.27 (s, 3H), 3.53 (t, $J$ = 4.5 Hz, 2H), 3.80 (t, $J$ = 4.5 Hz, 2H), 5.35 (s, 2H), 6.77–6.95 (m, 4H), 7.02–7.08 (m, 1H), 7.17–7.25 (m, 1H), 7.32–7.41 (m, 1H), 7.72–7.88 (m, 2H), 7.98–8.08 (m, 1H); HR FAB MS ($m/z$), calculated for $C_{26}H_{22}O_8Li$ ($M + Li^+$) 469.1475, found 469.1480.

**4.1.7. 3′-*O*-acetyl-fluorescein (4d).** Acetic anhydride (0.28 mL, 0.31 g, 3 mmol) and TEA (0.30 g, 0.42 mL, 3 mmol) in 10 mL DCM were added dropwise to fluorescein **3d** (1 g, 3 mmol) suspended in 10 mL of DCM and stirred for 4 h at rt. The solvent was removed under reduced pressure and the residue purified by silica gel flash chromatography (hexane-ethyl acetate, 1:1.5) to afford 0.45 g (40% yield) of 4d as an off yellow solid. $^1$H NMR ($CD_3COCD_3$) δ 2.28 (s, 3H), 6.64–6.73 (m, 2H), 6.79 (d, $J$ = 2.5 Hz, 1H), 6.86–6.96 (m, 2H), 7.15–7.22 (m, 1H), 7.34 (d, $J$ = 7.5 Hz, 1H), 7.76 (td, $J$ = 7.5, 1 Hz, 1H), 7.83 (td, $J$ = 7.5, 1 Hz, 1H), 8.01 (d, $J$ = 7.5 Hz, 1H), 9.05 (s, 1H); HR FAB MS ($m/z$), calculated for $C_{22}H_{14}O_6Li$ ($M + Li^+$) 381.0950, found 381.0955.

**4.1.8. *N*-Maleoyl-D-diphenylalanine (5a).** Maleic anhydride (203 mg, 2.1 mmol) was added all at once to D-diphenylalanine (500 mg, 2.1 mmol) in 4 mL of a 3:1 mixture of water and DMF, and stirred overnight at rt. The resulting white solid was filtered, washed with water (3×10 mL), followed by anhydrous ethanol (3×10 mL) and then anhydrous ether (3×10 mL) to give 0.524 g (75% yield) of **5a** as a white powder. $^1$H NMR (300 MHz, $CD_3OD$) δ 4.38 (d, $J$ = 11 Hz, 1H), 5.29 (d, $J$ = 11 Hz, 1H), 6.11 (d, $J$ = 13 Hz, 1H), 6.18 (d, $J$ = 13 Hz, 1H), 7.11 (t, $J$ = 7.5 Hz, 2H), 7.17–7.23 (m, 4H), 7.28–7.31 (m, 4H); HR FAB MS ($m/z$), calculated for $C_{19}H_{18}O_5N$ ($M + H^+$) 340.1185, found 340.1186.

**4.1.9. *N*-Maleoyl-D,L-3-amino-2-methylpropionic acid (5c).** Prepared by the procedure previously described for **5a**[1] giving **5c** as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 1.08 (d, $J$ = 7 Hz, 3H), 2.55–2.64 (m, 1H), 3.16–3.25 (m, 1H), 3.35–3.43 (m, 1H), 6.25 (d, $J$ = 12.5 Hz, 1H), 6.42 (d, $J$ = 12.5 Hz, 1H), 9.08 (t, $J$ = 6.5 Hz, 1H); HR FAB MS ($m/z$): calculated for $C_8H_{11}O_5N$ ($M + H^+$) 202.0637, found 202.0716.

**4.1.10. 6′-*O*-(*N*-Maleimido-D-diphenylalanyl)-3′-*O*-butyl-fluorescein (7a).** *N*-Maleoyl-D-diphenylalanine **5a** (100 mg, 0.29 mmol) was dissolved in 5 mL of thionyl chloride and heated at reflux until gas evolution had ceased at which point the excess thionyl chloride was removed under reduced pressure. The residual thionyl chloride was removed by dissolving the residue in carbon tetrachloride and then removing the solvent under reduced pressure. The crude acid chloride was dissolved in 5 mL of $CH_2Cl_2$ and was slowly added to a stirred mixture of 3′-*O*-butyl-fluorescein **3a** (50 mg, 0.13 mmol) and triethylamine (22 μL, 16 mg, 0.16 mmol) in 5 mL of $CH_2Cl_2$ at 0 °C. The reaction mixture was allowed to warm to rt. After stirring overnight, the reaction mixture was diluted with 20 mL of $CH_2Cl_2$, washed with brine and water, dried over $Na_2SO_4$, and concentrated in vacuo. The residue was purified by silica gel TLC (1:2 ethyl acetate–hexane) to afford 26 mg (29% yield) of **7a** as white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.97 (t, $J$ = 7.5 Hz, 3H), 1.44–1.56 (m, 2H), 1.72–1.82

(m, 2H), 4.07 (t, $J=6.5$ Hz, 2H), 5.17 and 5.23 (d, $J=12$ Hz, 1H), 5.91 and 5.97(d, $J=12$ Hz, 1H), 6.62–6.97 (m, 5H), 7.15–7.40 (m, 12H), 7.60–7.66 (m, 2H), 7.74 (t, $J=7.5$ Hz, 1H), 7.80 (t, $J=7.5$ Hz, 1H), 8.00 (d, $J=7.5$ Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{43}H_{34}O_8N$ ($M+H^+$) 692.2284, found 692.2259.

**4.1.11. 6′-*O*-(*N*-Maleimido-D-valyl)-3′-*O*-butyl-fluorescein (8a).** *N*-Maleoyl-D-valine **5b**[1] (153 mg, 0.71 mmol) was treated with thionyl chloride as described for the preparation of **7a** to give the crude acid chloride which was dissolved in 3 mL of $CH_2Cl_2$ and was slowly added to a stirred mixture of 3′-*O*-butyl-fluorescein **3a** (200 mg, 0.52 mmol) and triethylamine (0.19 mL, 1.4 mmol) in 5 mL of $CH_2Cl_2$ at 0 °C. The reaction was worked up as described for 7a, and the residue was flash chromatographed on silica gel (1:2 ethyl acetate–hexane) to afford 108 mg (37% yield) of **8a** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.97 (t, $J=7.5$ Hz, 3H), 0.93 (d, $J=6.5$ Hz, 3H), 1.12 (d, $J=6.5$ Hz, 3H), 1.43–1.56 (m, 2H), 1.72–1.82 (m, 2H), 4.08 (t, $J=6.5$ Hz, 2H), 4.78 (d, $J=7$ Hz, 1H), 6.71–6.78 (m, 2H), 6.84–6.94 (m, 3H), 7.05 (s, 2H), 7.08–7.13 (m, 1H), 7.31–7.35 (m, 1H), 7.76 (td, $J=7.5$, 1 Hz, 1H), 7.82 (td, $J=7.5$, 1 Hz, 1H), 8.01 (m, 1H); high-resolution FAB mass spectrometry ($m/z$) calculated for $C_{33}H_{29}O_8NLi$ [$M+Li^+$] 574.2053, found 574.2053.

**4.1.12. 6′-*O*-(*N*-Maleimido-D-valyl)-3′-EME-fluorescein (8b).** The title compound was made from *N*-maleoyl-D-valine **5b**[1] (70 mg, 0.33 mmol) and 3′-*O*-EME-fluorescein **3b** (90 mg, 0.23 mmol) as described above for **7a**. The crude product was purified by silica gel TCL (1:20, ethyl acetate–DCM) to afford 35 mg (27%) of **8b** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.93 (d, $J=6.5$ Hz, 3H), 1.12 (d, $J=6.5$ Hz, 3H), 2.59–2.69 (m, 1H), 3.36 (s, 3H), 3.72–3.75 (m, 2H), 4.21–4.24 (m, 2H), 4.78 (d, $J=7$ Hz, 1H), 6.74–6.78 (m, 2H), 6.85–6.98 (m, 3H), 7.03–7.16 (m, 3H), 7.34 (d, $J=7$ Hz, 1H), 7.76 (td, $J=7.5$, 1 Hz, 1H), 7.83 (td, $J=7.5$, 1 Hz, 1H), 8.02 (d, $J=7.5$ Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{32}H_{28}O_9N$ ($M+H^+$) 570.1765, found 570.1757.

**4.1.13. 3′-*O*-(*N*-Maleimido-D-valyl)-fluorescein (8d).** The title compound was prepared from *N*-maleoyl-D-valine **5b** (65 mg, 0.3 mmol) and fluorescein **3d** (100 mg, 0.3 mmol) as described for **7a**. Silica gel column chromatography (1:1–1.5:1, ethyl acetate–hexane) gave 54 mg (35% yield) of **8d** as an off yellow powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.94 (d, $J=7$ Hz, 3H), 1.12 (d, $J=7$ Hz, 3H), 2.58–2.70 (m, 1H), 4.78 (d, $J=7$ Hz, 1H), 6.64–6.73 (m, 2H), 6.79 (d, $J=2$ Hz, 1H), 6.86–6.93 (m, 2H), 7.05 (s, 2H), 7.07–7.13 (m, 1H), 7.34 (d, $J=7.5$ Hz, 1H), 7.76 (td, $J=7.5$, 1 Hz, 1H), 7.83 (td, $J=7.5$, 1 Hz, 1H), 8.01 (d, $J=7.5$ Hz, 1H), 9.10 (s,1H); HR FAB MS($m/z$), calculated for $C_{29}H_{21}O_8NLi$ ($M+Li^+$) 518.1427, found 518.1423.

**4.1.14. 6′-*O*-(*N*-Maleimido-D,L-3-amino-2-methylpropionyl)-3′-*O*-butyl-fluorescein (9a).** The title compound was prepared from *N*-maleoyl-D,L-3-amino-2-methylpropionic acid **5c** (60 mg, 0.30 mmol) and 3′-*O*-butyl-fluorescein **3a** (60 mg, 0.16 mmol) as described for **7a**. Silica gel TLC (1:2 ethyl acetate–hexane) gave 52.2 mg (61% yield) of **9a** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.97 (t, $J=7$ Hz, 3H), 1.26 (d, $J=7$ Hz, 3H), 1.44–1.58 (m, 2H), 1.73–1.83 (m, 2H), 3.06–3.18 (m, 1H), 3.72–3.98 (m, 2H), 4.09 (t, $J=6.5$ Hz, 2H), 6.70–6.79 (m, 2H), 6.88–6.98 (m, 5H), 7.21–7.24 (m, 1H), 7.35 (d, $J=7.5$ Hz, 1H), 7.76 (td, $J=7.5$, 1 Hz, 1H), 7.83 (td, $J=7.5$, 1 Hz, 1H), 8.02 (d, $J=7.5$ Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{32}H_{27}O_8NLi$ ($M+Li^+$) 560.1897, found 560.1906.

**4.1.15. 6′-*O*-(*N*-Maleimido-D,L-3-amino-2-methylpropionyl)-3′-*O*-EME-fluorescein (9b).** The title compound was made from *N*-maleoyl-D,L-3-amino-2-methylpropionic acid **5c** (52 mg, 0.26 mmol) and 3′-*O*-EME-fluorescein **7b** (100 mg, 0.26 mmol) as described for **7a**. The crude product was purified by silica gel TCL (1:5, ethyl acetate–DCM) to afford 41 mg (29% yield) of **9b** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 1.26 (d, $J=7$ Hz, 3H), 3.08–3.18 (m, 1H), 3.37 (s, 3H), 3.72–3.81 (m, 3H), 3.88–3.98 (m, 1H), 4.22–4.27 (m, 2H), 6.73–6.78 (m, 2H), 6.88–6.98 (m, 5H), 7.23–7.29 (m, 1H), 7.35 (d, $J=7$ Hz, 1H), 7.77 (td, $J=7.5$, 1 Hz, 1H), 7.84(td, $J=7.5$, 1 Hz, 1H), 8.03 (d, $J=7.5$ Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{31}H_{26}O_9N$ ($M+H^+$) 556.1608, found 556.1627.

**4.1.16. 3′-*O*-(*N*-Maleimido-D,L-2-amino-1-methylpropionyl)-fluorescein (9d).** The title compound was made from *N*-maleoyl-D,L-2-amino-1-methylpropionic acid **5c** (60 mg, 0.3 mmol) and fluorescein **3d** (100 mg, 0.3 mmol) using the same methods described for **8e**. The crude product was purified by silica gel column chromatography (1:1–1.5:1, ethyl acetate–hexane) to afford 77 mg (52% yield) of **9d** as a yellow powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 1.26 (d, $J=7$ Hz, 3H), 3.06–3.18 (m, 1H), 3.72–3.79 (m, 1H), 3.89–3.96 (m, 1H), 6.65–6.73 (m, 2H), 6.80 (d, $J=2$ Hz, 1H), 6.88–6.96 (m, 4H), 7.21–7.23 (m, 1H), 7.35(d, $J=7.5$ Hz, 1H), 7.76 (t, $J=7.5$ Hz, 1H), 7.83 (t, $J=7.5$ Hz, 1H), 8.02 (d, $J=7.5$ Hz, 1H), 9.12 (s,1H); HR FAB MS ($m/z$), calculated for $C_{28}H_{20}O_8N(M+H^+)$ 498.1190, found 498.1191.

**4.1.17. 6′-*O*-(*N*-Maleimido-β-alanyl)-3′-*O*-butyl-fluorescein (10a).** The title compound was prepared from *N*-maleoyl-β-alanine **5d**[2] (50 mg, 0.27 mmol) and 3′-*O*-butyl-fluorescein **3a** (50 mg, 0.13 mmol) as described for **7a**. Silica gel TLC (1:1 ethyl acetate–hexane) gave 48 mg (71% yield) of **10a** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 0.97 (t, $J=7.5$ Hz, 3H), 1.44–1.57 (m, 2H), 1.73–1.82 (m, 2H), 2.88–2.96 (m, 2H), 3.85–3.96 (m, 2H), 4.09 (t, $J=6.5$ Hz, 2H), 6.71–6.79 (m, 2H), 6.88–6.98 (m, 5H), 7.21–7.24 (m, 1H), 7.34 (d, $J=7.5$ Hz, 1H), 7.76 (td, $J=7.5$, 1 Hz, 1H), 7.83 (td, $J=7.5$, 1 Hz, 1H), 8.02 (d, $J=7$ Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{31}H_{25}O_8NLi$ ($M+Li^+$) 546.1740, found 546.1733.

**4.1.18. 3′-*O*-(*N*-Maleimido-β-alanyl)-fluorescein (10d).** The title compound was prepared from *N*-maleimido-β-alanine **5d**[2] (28 mg, 0.15 mmol) and fluorescein **3d** (50 mg, 0.15 mmol) as described for **8e**. The crude product was purified by silica gel TLC (1:1 ethyl acetate–hexane)

to afford 25 mg (36% yield) of **10d** as a yellow powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 2.92–2.96 (m, 2H), 3.88–3.97 (m, 2H), 6.62–6.72 (m, 2H), 6.78–6.81 (m, 1H), 6.88–6.94 (m, 3H), 6.95–6.98 (m, 1H), 7.19–7.23 (m, 1H), 7.34 (d, $J$ = 7.5 Hz, 1H), 7.76 (tt, $J$ = 7.5, 1.2 Hz, 1H), 7.83 (tt, $J$ = 7.5, 1 Hz, 1H), 8.01 (d, $J$ = 7.5 Hz, 1H), 9.08 (s, 1H); HR FAB MS ($m/z$), calculated for $C_{27}H_{17}O_8NLi$ ($M + Li^+$) 490.1114, found 490.1109.

**4.1.19. 6′-*O*-(*N*-Maleoyl-β-alanyl)-3′-*O*-acetyl fluorescein (10e).** The title compound was prepared from *N*-maleoyl-β-alanine **5d**[2] (37 mg, 0.2 mmol) and 3′-*O*-acetyl-fluorescein **3d** (50 mg, 0.13 mmol) as described for **7a**. The crude product was purified by silica gel TLC (1:1 ethyl acetate–hexane) to give 50 mg (74% yield) of **10e** as a white powder. $^1$H NMR (300 MHz, $CD_3COCD_3$) δ 2.29 (s, 3H), 2.88–2.96 (m, 2H), 3.89–3.97 (m, 2H), 6.91–6.99 (m, 6H), 7.21–7.28 (m, 2H), 7.41 (d, $J$ = 7.5 Hz, 1H), 7.78 (t, $J$ = 7.5, 1H), 7.85 (t, $J$ = 7.5, 1H), 8.04 (d, $J$ = 7.5 Hz, 1H); HR FAB MS ($m/z$), calculated for $C_{29}H_{19}O_9$ NLi ($M + Li^+$) 532.1220, found 532.1233.

**4.1.20. Synthesis of TyrArg$_9$Cys.** The oligopeptide $H_2N$-Tyr-$(Arg)_9$-Cys-$CONH_2$ was synthesized by standard solid phase Fmoc peptide synthesis methodology. Fmoc protected Rink amide resin (100 mg, substitution = 0.61 mmol/g) was used as the solid support. After deprotection of the resin with 20% piperidine in DMF (v/v) and washing with MeOH and DMF alternately, FmocNH-Cys(Trt)-OH (2.5 equiv) was coupled on the resin in presence of PyBOP (2.5 equiv) and DIPEA (4 equiv) in 3 mL of DMF. This was followed by nine cycles with FmocArg(Pbf)-OH and one cycle with FmocTyr(But)-OH. After removal of the terminal Fmoc group and washing, the resin was dried and cleaved with 4 mL of 95% TFA containing 0.5% TIS, 2.5% EDT and 2.5% $H_2O$ (v/v) for 12 h at rt. The resin was removed by filtration and the crude product was precipitated from solution by addition of 10 parts of ethyl ether. The oligopeptide was purified by semi-preparative RP-HPLC with a 30 min 0–40% gradient of acetonitrile containing 0.1%TFA in water containing 0.1% TFA at a flow rate of 1.0 mL/min and detection at 270 nm. The calculated MW of product is 1689 and the MALDI-TOF MS found ($M + 1$): 1689.8.

**4.1.21. General method for conjugating TyrArg$_9$Cys to the fluorogenic esters (11–14).** The maleimido fluorescein derivatives **7–10** (4 equiv) in 50 μL acetonitrile or MeOH were added separately to TyrArg$_9$Cys in 0.5 mL of deoxygenated MeOH and then shaken under $N_2$ for 2–4 h at rt. Ethyl ether (10 fold excess) was then added to precipitate the products. Purification was carried out by semi-preparative RP-HPLC with a 1.0 mL/min 0–40% 30 min gradient of B (acetonitrile containing 0.1% TFA) 0–40% in A (water containing 0.1% TFA) with detection at 270 nm. The products were characterized by MALDI-TOF MS (Table 2).

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