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A "Ready-To-Use" fluorescent-labelled-cysteine-TBTP (4-thiobutyltriphenylphosphonium) synthon to investigate the delivery of non-permeable PNA (peptide nucleic acids)-based compounds to cells

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Abstract

This paper reports: (i) the facile synthesis of a cysteine synthon incorporating both a fluorescent group and a triphenylphosphonium derivative (TBTP) via the formation of a disulphide bond, which can subsequently undergo facile intracellular scission, (ii) the direct conjugation of this synthon to a non-permeable drug, (a cyclic PNA (peptide nucleic acid)-based compound has been chosen as a model), and (iii) that this conjugation enables the efficient homogenous delivery of the otherwise non-permeable cyclic PNA into the cytoplasm of cells, as demonstrated by fluorescence microscopy. Our results indicate that this fluorescent-labelled cysteine-TBTP synthon can provide a very useful tool for exploring the cellular uptake of a large range of molecules of biological interest, containing only a single reactive function. The preparation of an activated TBTP derivative is also described and this procedure could be widely used to introduce a TBTP cation to any thio-containing molecule.

Keywords: PNA cellular delivery; Lipophilic triphenylphosphonium cation; Fluorescence microscopy

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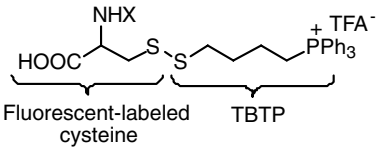
1. Introduction

The development of therapeutics such as anticancer or antiviral drugs (especially antisens oligomers) is often hampered by a poor cellular delivery. A variety of techniques are currently available to enhance the cellular uptake of potentially promising candidates which include the use of liposomes [1–5] or conjugation to cell-penetrating peptides (CPP) [6–9]. A good alternative to the strategies above involves the incoporation of a the cationic and lipophilic triphenylphosphonium (TPP) moiety, which has been shown to facilitate the lipid bilayer transport of neutral molecules as large as 500 Da [10,11]. Recently, Murphy et al. applied this strategy to investigate the cellular delivery of peptide nucleic acids (PNA) antisens [12]. For this purpose, a fluorescent-labelled PNA-cysteine was linked to the 4-thiobutyltriphenylphosphonium cation (TBTP) via a biolabile disulphide bond, which can be rapidly reduced by cytoplasmic glutathione, allowing the fluorescent PNA-cysteine to remain within the cells [13]. This method requires the synthesis of a PNA which incorporates both a fluorescent group at its N-terminal extremity and a cysteine residue at its C-terminal extremity. In order to investigate the cellular uptake of a drug containing only one reactive functional group, we have designed the conveniently prepared cysteine synthons **1** (Fig. 1) incorporating both a fluorescent group (dansyl or fluorescein) and a TBTP moiety connected via a disulphide bond, which can subsequently undergo facile intracellular scission. These synthons can be attached directly to non-permeable drugs containing amino groups and subsequent cytoplasmic reduction results in the release of a fluorescent-labelled drug, allowing its detection and intracellular localization.

Previously, we have elaborated on cyclic PNA-based compounds (such as compound **2a** ; Fig. 2) to target various viral RNA hairpins [14–17]. These compounds do not penetrate cells spontaneously even at high concentrations (up to 1 mM), as evidenced by previous fluorescence microscopy studies using the fluorescein-labelled analogue **2b** (Fig. 2) [18].

To illustrate the usefulness of synthons **1** (Fig. 1), we have conjugated them to the cyclic PNA-based compound **2a**, through the free ε-amino group of its lysine residue which constitutes the only reactive function of the molecule (Fig. 2).

In this paper, we report: (i) the synthesis of fluorescent-labelled-cysteine-TBTP synthons **1** (Fig. 1) and their cyclic PNA conjugates **3** (Fig. 3), and (ii) the successful homogenous cellular uptake of the above, which has been investigated by fluorescence microscopy.



1a X = Fluorescein
1b X = Dansyl

Fig. 1. Structure of synthons **1**.

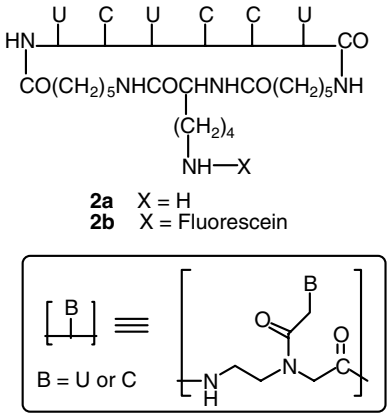


Fig. 2. Structure of the cyclic PNA-based compounds **2**.

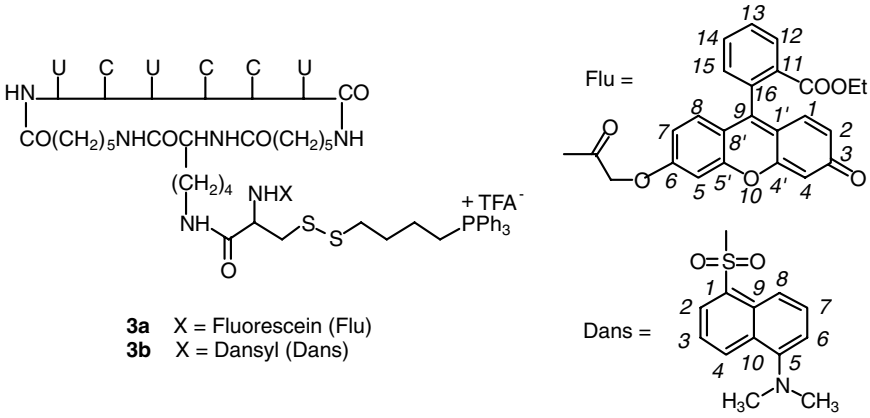
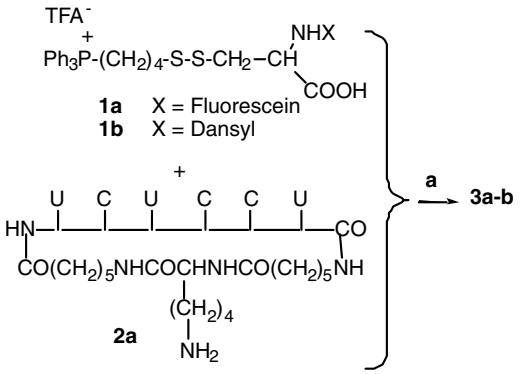


Fig. 3. Structure of the cyclic PNA fluorescent-labelled-cysteine-TBTP conjugates **3**. The atom numbering (*in italics*) is used for the description of the NMR spectra.

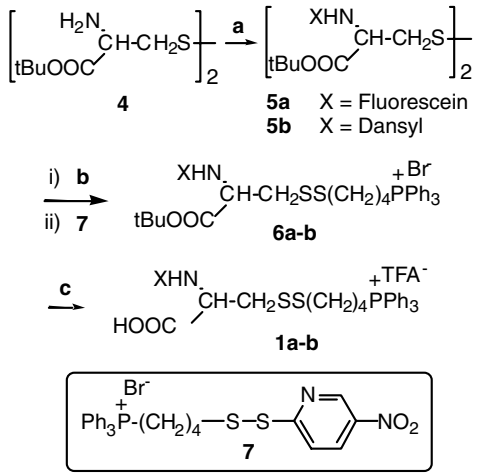
2. Results and discussion

2.1. Synthesis

The cyclic PNA fluorescent-labelled-cysteine-TBTP conjugates **3a** and **3b** were obtained by condensing directly the cyclic PNA-based compound **2a** [18] with the fluorescent-labelled-cysteine-TBTP synthons **1a** and **1b**, respectively (Scheme 1). For this purpose, several uronium (HATU, HBTU, ToPPiPu) and phosphonium (Brop, Bop) reagents were investigated. The coupling of **1a** with **2a** to give the conjugate **3a** was best performed using ToPPiPu whereas Brop was found to be the most efficient for the condensation between **1b** and **2a** to give **3b**. **3a** and **3b** were isolated after semi-preparative HPLC in 31% and 17% yield, respectively. Their purity was checked by analytical HPLC and their identity was confirmed by MALDI-TOF MS.



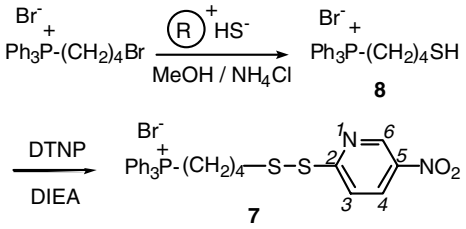
Scheme 1. Synthesis of the cyclic PNA fluorescent-labelled-cysteine-TBTP conjugates **3**. Reagents: (a) for **3a**: ToPPiPu, NMM, DMF; for **3b**: Brop, DIEA, DMF.



Scheme 2. Synthesis of the fluorescent-labelled-cysteine-TBTP synthons **1**. Reagents: (a) for **5a**: Flu-OH, Bop, DIEA, DMF; for **5b**: DansCl, DIEA, CH_2Cl_2; (b) for **6a**: DTT, THF/H_2O, pH 7.4 ($NaHCO_3$), for **6b**: DTT, THF/H_2O, pH 8.5 (NH_4OH); (c) TFA/CH_2Cl_2/anisole (4.5/5/0.5).

The synthetic pathway to the fluorescent-labelled-cysteine-TBTP synthons **1a** and **1b** is illustrated in Scheme 2. 6-*O*-(carboxymethyl)fluorescein ethyl ester (Flu-OH) [19] was condensed with cystine tertiobutyl ester **4** [20] to yield **5a** (78%) by means of the Bop reagent. Similarly, **4** was acylated using dansyl chloride (DansCl) to give **5b** in 96% yield. The cystine reduction in **5a-b** was performed using dithiothreitol (DTT) [21] and the resulting sulphide was then allowed to react with the activated compound **7**, as shown in Scheme 3. The fluorescent-cysteine-TBTP derivatives **6a** and **6b** were obtained in 53% and 44% yield, respectively. Hydrolysis of **6a** and **6b** with trifluoroacetic acid (TFA) in CH_2Cl_2, in the presence of anisole as scavenger, gave synthons **1a** and **1b** in 68 and 63% yield, respectively.

Compound **7** was prepared from 4-thiobutyltriphenylphosphonium bromide **8** and 2,2′-dithio-bis-(5-nitropyridine) (DTNP) in 59% yield (Scheme 3) and compound **8** was obtained in one step and in high yield (96%) from commercial 4-bromobutyltriphenyl-



Scheme 3. Synthesis of compound **7**. The atom numbering (in italics) is used for the description of the NMR spectra.

phosphonium bromide, using a hydrosulphide exchange resin [22] in the presence of an equimolar amount of ammonium chloride. It is noteworthy that this method constitutes a substantial improvement over the method described in the literature, which required three steps and gave only a 30% overall yield [23].

2.2. Cellular uptake

The uptake of the cyclic PNA fluorescent-labelled-cysteine-TBTP conjugates **3a** (Flu) and **3b** (Dans) and of the corresponding synthons **1a** and **1b** in HeLa cells was investigated by confocal microscopy. HeLa cells were incubated at 37 °C for 1 h with a 5 μM 10% FCS/DMEM solution of conjugate **3a-b** or synthon **1a-b**. These adherent cells were then washed with PBS to remove any loosely adsorbed compound, fixed and visualized by confocal microscopy (Fig. 4a–d). For all tested compounds (**1a-b** and **3a-b**), the acquired images show an intense and homogeneous fluorescence mainly located in the cytoplasm and the nucleus. This fluorescence is not associated in a punctate pattern in the cytoplasm, typical of endosomal or mitochondrial accumulation. To ensure that the observed diffuse fluorescence does not result from the cell fixing conditions (3.7% formaldehyde in PBS), the apparent uptake of **3b** was investigated in live HeLa cells (incubation at 37 °C for 1 h with a 5 μM 10% FCS/DMEM solution of **3b**), by fluorescence microscopy (Fig. 4e and f). As obtained with confocal microscopy images, an intense and homogenous distribution of **3b** is observed throughout the cytoplasm and the nucleus. Since conjugates **3a** and **3b** were found to be stable in the extracellular medium (5 μM 10% FCS/DMEM) for more than 24 h, (by HPLC analyses, data not shown), and since no cellular uptake of the cyclic PNA-(fluorescein or dansyl)-labelled-cysteine conjugates (i.e. **3a** and **3b** without the TBTP moiety) was observed under similar conditions (data not shown), these images indicate clearly that synthons **1**, upon direct connection to the cyclic PNA representative **2a**, allow (i) transport of the cyclic PNA into the cells, and (ii) the subcellular localization of the released fluorescent cyclic PNA (resulting from the disulphide bond reduction). Our data confirm the efficiency of the TBTP moiety as a carrier system for the delivery of PNAs into cells [12,13], and suggest that the nature of the fluorescent group (dansyl and fluorescein) has no major impact on internalization, as similar results were obtained with both types of derivatives.

3. Conclusion

In this paper, we describe the ready preparation of cysteine synthons **1** incorporating both a fluorescent group and a TBTP cation via a disulphide bond, which can undergo

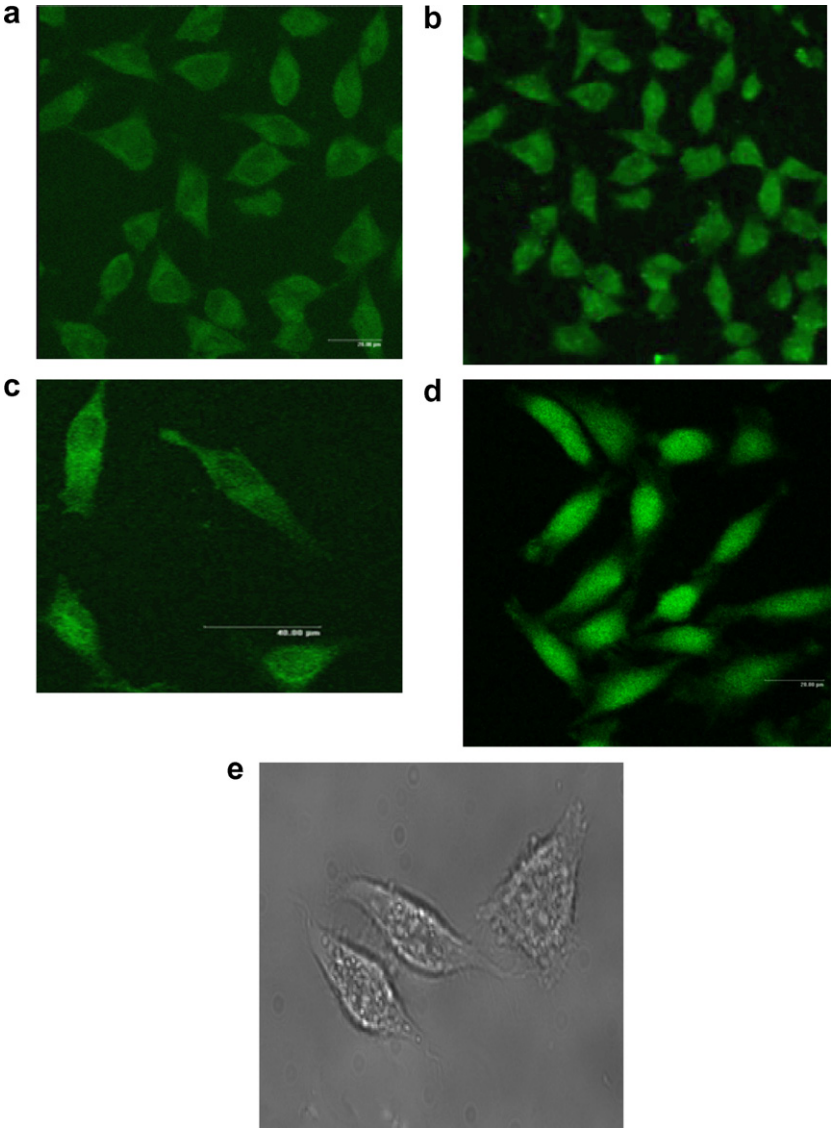


Fig. 4. (a–d) Confocal microscopy images of the uptake by HeLa cells of (a) fluorescein-cysteine-TBTP derivative **1a**, (b) cyclic PNA-fluorescein-cysteine-TBTP conjugates **3a**, (c) dansyl-cysteine-TBTP derivative **1b**, and (d) cyclic PNA-dansyl-cysteine-TBTP conjugates **3b**. HeLa cells were incubated for 1 h at 37 °C with a 5 μM 10% FCS/DMEM solution of **1a-b** or **3a-b** and subjected to fluorescence microscopy analysis after washing and fixation (see Section 4). (e–f) Fluorescence microscopy images of the uptake of **3b** by living (non fixed) HeLa cells, (e) bright-light image and (f) three-dimensional deconvolved image. HeLa cells were incubated for 1 h at 37 °C with a 5 μM 10% FCS/DMEM solution of **3b** and subjected to fluorescence microscopy analysis (see Section 4 for details).

a facile cytoplasmic scission. Conjugation of synthons **1** to a cyclic PNA-based compound **2a**, which has been used as a model since **2a** does not enter cells spontaneously [18], enables the rapid and efficient cellular uptake of **2a** and its homogenous localization inside

the cytosol as visualized by confocal or fluorescence microscopy. Thus, fluorescent-labelled-cysteine-TBTP synthons **1** constitute a very useful tool for exploring the cellular uptake and the intracellular localization of a molecule containing only a single functionalizable amino group. This paper also describes a new one-step procedure to prepare the TBTP cation **8**, which constitutes a substantial improvement over the previously reported method [23] as well as the preparation of the activated-TBTP compound **7** which can be widely used to introduce a TBTP cation into any thio-containing molecule.

4. Materials and methods

4.1. Abbreviations

Bop: benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; Brop: bromo-tris-(dimethylamino)phosphonium hexafluorophosphate; C: cytosine; Dans-Cl: dansyl chloride; DIEA: diisopropylethylamine; DMF: dimethylformamide; DTNP: 2,2′-dithio-bis-(5-nitropyridine); DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; FCS: fetal calf serum; FluOH: 6-*O*-(carboxymethyl)fluorescein ethylester; HATU: *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; HBTU: 2-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate; NMM: *N*-methylmorpholine; PBS: phosphate-buffered saline; TBTP: 4-thiobutyltriphenylphosphonium; TFA: trifluoroacetic acid; THF: tetrahydrofuran; ToPPiPu: 2-(2-oxo-1(2*H*)-pyridyl)-1,1,3,3-bis-(pentamethylene)uronium tetrafluoroborate; TPP: triphenylphosphonium; U: uracil.

4.2. General procedures

Reagents and solvents were obtained from commercial sources and used without further purification unless indicated. The cyclic PNA-based compound **2a** was synthesized as described elsewhere [18]. 6-*O*-(carboxymethyl)fluorescein ethyl ester (Flu-OH) was prepared as described in the literature [19]. L-cystine bis-*t*-butyl ester **4** was obtained as reported in the literature [20] and it was used immediately after preparation. Analytical thin-layer chromatography was conduced on Merck precoated silica gel $60F_{254}$ plates and the compounds were visualized with the Ellman reagent and/or by visualization under ultraviolet light (254 nm and/or 365 nm). Chromatography was performed on Merck silica gel 60 (230–400 mesh ASTM) using the solvent systems (v/v ratio) indicated below. Analytical HPLC chromatograms were obtained using a Waters HPLC system (600 E system controller, 996 photodiode array detector or 2487 dual wavelength absorbance detector) and a Beckman (250×4 mm) RP-18 (5 μm) column. The HPLC flow rate was 1 mL min^{-1}, and the elution solvents were water (0.1% TFA) as solvent A, and acetonitrile (0.1% TFA) as solvent B. Optical rotations were measured on a Bellingham & Stanley ADP 220 polarimeter. ^{1}H and ^{13}C NMR measurements were carried out on Brucker AC 200 or AC 500 Fourier transform spectrometers. Chemical shifts (δ) are reported in part per million (ppm); for ^{1}H NMR: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad resonance). For the atom numbering, see Fig. 3 and Scheme 3. ESI mass spectra were recorded with a Bruker Esquire 3000 plus. MALDI-TOF-MS spectra were recorded with an MALDI-TOF 'DE PRO' Applied Biosystem.

4.3. Synthesis

4.3.1. N,N′-Bis(labelled)-L-cystine bis-t-butyl ester (**5a-b**) (Scheme 2)

*4.3.1.1. N,N′-Bis(6-O-(carboxymethyl)fluorescein ethyl ester)-L-cystine bis-t-butyl ester (**5a**).* To a cold solution (0 °C) of compound **4** (510 mg, 1.45 mmol), DIPEA (0.55 mL, 3.19 mmol), and 6-*O*-(carboxymethyl)fluorescein ethyl ester (1.21 g, 2.90 mmol) in DMF (5 mL), was added Bop (1.41 g, 3.19 mmol), followed by stirring for 10 min at this temperature then warming to room temperature (ca. 2 h). The solvent was then evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine and finally dried over Na_2SO_4. The solvent was then removed *in vacuo* and the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 8:2) to afford **5a** (1.30 g, 78%) as an orange amorphous powder. TLC (EtOAc/MeOH 8:2) R_f 0.61. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 22.3$ min ($\lambda_{max} = 228.8$, 251.3, 298.8 and 338.2 nm). $[\alpha]_D^{25}$ −27° (*c* 1, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, 2H, H_{12}, $J = 7.7$ Hz); 7.73 (t, 2H, H_{14}, $J = 7.6$ Hz); 7.67 (td, 2H, H_{13}, $J = 7.7$ Hz, $J = 1.1$ Hz); 7.38 (d, 2H, NH, $J = 7.2$ Hz); 7.29 (m, 2H, H_{15}); 7.00 (m, 2H, H_5); 6.94 (d, 2H, H_8, $J = 8.9$ Hz); 6.86 (dd, 2H, H_1, $J = 9.7$ Hz); 6.81 (dd, 2H, H_7, $J = 8.9$ Hz, $J = 2.3$ Hz); 6.53 (dd, 2H, H_2, $J = 9.7$ Hz, $J = 1.8$ Hz); 6.44–6.40 (m, 2H, H_4); 4.84–4.78 (m, 2H, H_α); 4.69–4.54 (m, 4H, CH_2); 4.08–3.96 (m, 4H, CH_2 OEt); 3.35–3.26 (m, 2H, H_β); 3.22–3.13 (m, 2H, $H_{\beta'}$); 1.46 (s, 18H, OtBu); 1.01–0.94 (m, 6H, CH_3 OEt). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.64 (C_3); 168.80 (C(O)NH); 166.95 (*C*(O)OtBu); 165.34 (*C*(O)OEt); 161.36 (C_6); 158.87 ($C_{4'}$); 154.05 ($C_{5'}$); 150.17 (C_9); 134.13 (C_{16}); 132.76 (C_{14}); 131.39 (C_{12}); 130.84 (C_{11}); 130.53 (C_{15}, C_1); 130.15 (C_2); 129.92 (C_{13}); 129.51 (C_8); 118.44 ($C_{1'}$); 116.26 ($C_{8'}$); 113.26 (C_7); 106.00 (C_4); 102.11, 102.04 (C_5); 83.65 (C tBu); 67.64 (C(O)*C*H_2–O–); 61.49 (CH_2 OEt); 52.52 (C_α); 41.20 (C_β); 28.09 (CH_3 tBu); 13.76 (CH_3OEt). MS (ESI+) Calcd for $C_{62}H_{60}N_2O_{16}S_2$ $[M+H]^+$: 1153.3 and for $[M+Na]^+$: 1175.3. Found: 1153.3 $[M+H]^+$ and 1175.3 $[M+Na]^+$.

*4.3.1.2. N,N′-Bis(dansyl)-L-cystine bis-t-butyl ester(**5b**).* To a cold solution (0 °C) of compound **4** (836 mg, 2.37 mmol) and DIEA (0.91 mL, 5.22 mmol) in CH_2Cl_2 (4 mL) was added dansyl chloride (1.28 g, 4.74 mmol). The mixture was stirred for 15 min at this temperature then allowed to warm to room temperature (ca. 2 h). The residue was dissolved in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine and finally dried over Na_2SO_4. The solvent was then removed *in vacuo* and the residue purified by column chromatography (Cyclohexane/EtOAc 9:1 to 8:2) to afford **5b** (1.86 g, 96%) as a green fluorescent amorphous powder. TLC (cyclohexane/EtOAc 1:1) R_f 0.82. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 22.5$ min ($\lambda_{max} = 226.5$, 254.8 and 288.1 nm). $[\alpha]_D^{25}$ 85° (*c* 1, MeOH). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.53 (d, 2H, H_2, $J = 8.5$ Hz); 8.33 (d, 2H, H_8, $J = 8.6$ Hz); 8.24 (d, 2H, H_4, $J = 7.3$ Hz); 7.57 (m, 2H, H_7); 7.49 (m, 2H, H_3); 7.17 (d, 2H, H_6, $J = 7.2$ Hz); 5.79 (d, 2H, NH, $J = 8.5$ Hz), 4.08 (m, 2H, H_α); 3.07 (dd, 2H, $H_{\beta'}$, $J = 13.9$ Hz, $J = 5.5$ Hz); 2.94 (dd, 2H, H_β, $J = 13.9$ Hz, $J = 5.8$ Hz); 2.85 (s, 12H, $N(CH_3)_2$); 1.08 (s, 18H, OtBu). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 168.47 (*C*(O)OtBu); 152.00 (C_5); 134.87 (C_1); 130.81 (C_2); 129.9, 129.8 (C_9, C_{10}); 129.67 (C_4); 128.67 (C_7); 123.24 (C_3); 119.18 (C_8); 115.49 (C_6); 83.28 (C tBu); 56.01

(C_α); 45.49 ($N(CH_3)_2$); 43.01 (C_β); 27.57 (CH_3 tBu). MS (ESI+) Calcd for $C_{38}H_{50}N_4O_8S_4$ $[M+H]^+$: 819.2 and for $[M+Na]^+$: 841.2. Fund: 819.2 $[M+H]^+$ and 841.2 $[M+Na]^+$.

*4.3.2. [Ph_3P^+–$(CH_2)_4$–S–S–(5-nitro)-pyridine]Br (**7**) (Scheme 3)*

*4.3.2.1. 4-Thiobutyltriphenylphosphonium bromide (**8**).* Preparation of hydrosulphide exchange resin: IRA-400 (chloride form) (5 g, 19 mmol) was added to a solution of $NaSH{\cdot}H_2O$ (850 mg, 15 mmol) in MeOH (50 mL) and the mixture was shaken for 2 h. The resin was filtered off and washed with distilled water until it was free from NaCl and excess of NaSH. The resin was then washed with methanol, diethyl ether and dried under vacuum.

Synthesis of compound (**8**): To a suspension of the resin prepared previously, in 25 mL of MeOH, was added ammonium chloride (111 mg, 2.10 mmol) and 4-bromobutyltriphenylphosphonium bromide (1.0 g, 2.10 mmol). The mixture was stirred for 18 h at room temperature. The resin was then filtered off and washed with MeOH. The extract was evaporated under reduced pressure and the residue taken up in a saturated aqueous NaBr solution followed by extraction of the aqueous layer with CH_2Cl_2. The organic extract was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to afford **8** (860 mg, 95%) as a white resin. TLC (CH_2Cl_2/MeOH 9:1) R_f 0.38. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 19.8$ min ($\lambda_{max} = 228.8$ and 266.7 nm). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.03–7.54 (m, 15H, PPh_3); 3.92–3.63 (m, 2H, P–CH_2); 2.74 (t, 2H, CH_2–SH, $J = 7.2$ Hz); 2.10–1.89 (m, 2H, CH_2–CH_2–SH); 1.87–1.56 (m, 2H, P–CH_2–CH_2). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 135.12 (d, $3CH_{para}$, $J = 2.9$ Hz); 133.77 (d, $6CH_{meta}$, $J = 10.0$ Hz); 130.60 (d, $6CH_{ortho}$, $J = 12.4$ Hz); 118.15 (d, 3C (Ph), $J = 86.0$ Hz); 37.95 (CH_2–SH); 29.71 (d, CH_2–CH_2–SH, $J = 16.8$ Hz); 22.41 (d, P–CH_2, $J = 50.5$ Hz); 21.34 (d, P–CH_2–CH_2, $J = 4.0$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.29. MS (ESI+) Calcd for $C_{22}H_{24}PS$ $[M]^+$: 351.1. Found: 350.4.

*4.3.2.2. [Ph_3P^+–$(CH_2)_4$–S–S–(5-nitro)-pyridine]Br (**7**).* To a cold solution (0 °C) of compound **8** (270 mg, 0.63 mmol) in 3 mL of CH_2Cl_2 was added DIPEA (120 μL, 0.69 mmol) and DTNP (388 mg, 1.25 mmol). The mixture was stirred for 15 min at this temperature then allowed to warm to room temperature (ca. 6 h). The residue was taken up in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, a saturated aqueous NaBr solution and finally dried over Na_2SO_4. The solvent was then removed *in vacuo* and the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 9:1) to afford **7** (215 mg, 59%) as a yellowish resin. TLC (EtOAc/MeOH 8:2) R_f 0.27. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 20.8$ min ($\lambda_{max} = 202.9$, 273.8 and 321.5 nm). ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.11 (d, 1H, H_6, $J = 2.4$ Hz); 8.40 (dd, 1H, H_4, $J = 8.9$ Hz, $J = 2.4$ Hz); 7.97–7.54 (m, 16H, H_3 and PPh_3); 4.09–3.84 (m, 2H, P–CH_2); 3.05 (t, 2H, CH_2–S, $J = 6.9$ Hz); 2.05–1.92 (m, 2H, CH_2–CH_2–S); 1.90–1.60 (m, 2H, P–CH_2–CH_2). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 168.80 (C_2); 144.88 (C_6); 142.04 (C_5); 135.19 (d, $3CH_{para}$, $J = 2.9$ Hz); 133.76 (d, $6CH_{meta}$, $J = 10.2$ Hz); 132.08 (C_4); 130.61 (d, $6CH_{ortho}$, $J = 12.4$ Hz); 119.84 (C_3); 118.15 (d, 3C (Ph), $J = 85.4$ Hz); 37.70 (CH_2–S); 28.95 (d, CH_2–CH_2–S, $J = 16.8$ Hz); 22.37 (d, P–CH_2, $J = 50.5$ Hz); 21.04 (d, P–CH_2–CH_2, $J = 4.0$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.68. MS (ESI+) Calcd for $C_{27}H_{26}N_2O_2PS_2$ $[M]^+$: 505.1. Found: 505.3 $[M]^+$.

*4.3.3. N-(labelled)-Cys(TBTP)-OH·TFA (**1a–b**) (Scheme 2)*

*4.3.3.1. N-(6-O-(Carboxymethyl)fluorescein ethyl ester)-cys(TBTP)-OtBu (**6a**).* To a cold solution (0 °C) of compound **5a** (300 mg, 0.26 mmol) in 4 mL of THF/H_2O (3/1, v/v, pH 7.4 adjusted with $NaHCO_3$) was added, under an inert atmosphere of nitrogen, DTT (42 mg, 0.27 mmol). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 3 h). Then, compound **7** (322 mg, 0.55 mmol), dissolved in 3 mL of degassed THF, was added and the mixture was stirred for 45 min at room temperature. The solvents were evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, a saturated aqueous NaBr solution and finally dried over Na_2SO_4. The solvent was then removed *in vacuo* and the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 8:2) to afford **6a** (280 mg, 53%) as an orange oil. TLC (EtOAc/MeOH 8:2) R_f 0.48. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 21.3$ min ($\lambda_{max} = 202.9$, 227.7 and 298.8 nm). $[\alpha]_D^{25}$ −21° (*c* 1, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (d, 1H, H_{12}, $J = 7.8$ Hz); 8.10, 8.06 (d, 1H, NH, $J = 7.5$ Hz); 7.88–7.60 (m, 17H, PPh_3, H_{14}, H_{13}); 7.29–7.23 (m, 1H, H_{15}); 6.98 (m, 1H, H_5); 6.93–6.80 (m, 3H, H_8, H_7, H_1); 6.48 (d, 1H, H_2, $J = 9.7$ Hz); 6.36–6.32 (m, 1H, H_4); 4.81–4.70 (m, 2H, C(O)CH_2–O); 4.69–4.64 (m, 1H, H_α); 4.05–3.92 (m, 2H, OCH_2–CH_3); 3.89–3.72 (m, 2H, P–CH_2); 3.28–3.18 (m, 2H, H_β); 2.87–2.74 (m, 2H, CH_2–S); 2.11–2.01 (m, 2H, CH_2–CH_2–S); 1.84–1.67 (m, 2H, P–CH_2–CH_2); 1.42 (s, 9H, OtBu); 0.97–0.89 (m, 3H, OCH_2–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.62 (C_3); 168.98 (C(O)NH); 167.28, 167.25 (*C*(O)OtBu); 165.33 (*C*(O)OEt); 162.08, 162.04 (C_6); 158.93 ($C_{4'}$); 154.02 ($C_{5'}$); 150.53 (C_9); 135.15 (d, $3CH_{para}$, $J = 2.9$ Hz); 134.13 (C_{16}); 133.74 (d, $6CH_{meta}$, $J = 10.0$ Hz); 132.73, 132.71 (C_{14}); 131.29 (C_{12}); 130.67 (C_{11}); 130.59 (d, $6CH_{ortho}$, $J = 12.6$ Hz); 130.46, 130.44 (C_{15}, C_1); 129.91 (C_2); 129.80 (C_{13}); 129.34 (C_8); 118.21 (d, 3C (Ph_3), $J = 86.0$ Hz); 117.94 ($C_{1'}$); 115.70 ($C_{8'}$); 113.80, 113.72 (C_7); 105.71 (C_4); 101.90, 101.84 (C_5); 82.92, 82.89 (C tBu); 67.59 (C(O)CH_2O); 61.40 (OCH_2-CH_3); 52.86 (C_α); 40.85 (C_β); 37.69 (CH_2–S); 29.38 (d, CH_2–CH_2–S, $J = 17.0$ Hz); 28.05 (CH_3OtBu); 22.41 (d, P–CH_2, $J = 50.5$ Hz); 21.08 (P–CH_2–CH_2); 13.70 (OCH_2–CH_3). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.33. MS (ESI+) Calcd for $C_{53}H_{53}NO_8PS_2$ $[M]^+$: 926.3. Found: 926.3 $[M]^+$.

*4.3.3.2. N-Dansyl-cys(TBTP)-OtBu (**6b**).* Under an inert atmosphere of nitrogen, DTT (112 mg, 0.72 mmol) was added to a cold solution (0 °C) of compound **5b** (540 mg, 0.66 mmol) in 5 mL of degassed THF/H_2O (3/1, v/v, pH 8.5 adjusted with NH_4OH). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 3 h). Then, compound **7** (706 mg, 1.20 mmol), dissolved in 3 mL of degassed THF, was added and the mixture was stirred for 45 min at room temperature. The solvents were evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, a saturated aqueous NaBr solution and finally dried over Na_2SO_4. The solvent was then removed *in vacuo* and the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 8:2) to afford **6b** (490 g, 44%) as a green fluorescent oil. TLC (EtOAc/MeOH 8:2) R_f 0.30. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 22.1$ min ($\lambda_{max} = 222.9$ and 266.7 nm). $[\alpha]_D^{25}$ 62° (*c* 1, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, 1H, H_2, $J = 8.5$ Hz); 8.35 (d, 1H, H_8, $J = 8.6$ Hz); 8.17 (dd, 1H, H_4, $J = 7.3$ Hz, $J = 0.9$ Hz); 7.89–7.64 (m, 15H, Ph_3P); 7.58 (dd, 1H, H_7, $J = 8.6$ Hz, $J = 7.5$ Hz); 7.49 (dd, 1H, H_3,

$J = 8.5$ Hz, $J = 7.3$ Hz); 7.20 (d, 1H, H_6, $J = 7.5$ Hz); 6.29 (d, 1H, NH, $J = 8.6$ Hz), 4.03–3.96 (m, 1H, H_α); 3.96–3.76 (m, 2H, P–CH_2); 3.05–2.95 (m, 2H, H_β); 2.90–2.76 (m, 2H, CH_2–S); 2.86 (s, 6H, $N(CH_3)_2$); 2.13–2.04 (m, 2H, CH_2–CH_2–S); 1.84–1.72 (m, 2H, P–CH_2–CH_2); 0.99 (s, 9H, OtBu). ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.50 (*C*(O)tBu); 151.58 (C_5); 135.08 (C_1); 135.06 (d, $3CH_{para}$, $J = 3.0$ Hz); 133.86 (d, $6CH_{meta}$, $J = 10.0$ Hz); 130.62 (C_2); 130.57 (d, $6CH_{ortho}$, $J = 12.9$ Hz); 129.88 (C_9); 129.71 (C_{10}); 129.50 (C_4); 128.65 (C_7); 123.38 (C_3); 119.60 (C_8); 118.39 (d, 3C, $J = 85{,}5$ Hz); 115.65 (C_6); 83.13 (*C* OtBu); 56.39 (C_α); 45.57 ($N(CH_3)_2$); 43.37 (C_β); 37.45 (CH_2–S); 29.22 (d, $J = 16.8$ Hz, *C*H_2–CH_2–S); 27.51 (CH_3 OtBu); 22.21 (d, P–CH_2, $J = 50.5$ Hz); 21.07 (d, P–CH_2–*C*H_2, $J = 3.9$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.73. MS (ESI+) Calcd for $C_{41}H_{48}N_2O_4PS_3$ $[M]^+$: 759.2. Found: 759.2 $[M]^+$.

*4.3.3.3. N-[6-O-(Carboxymethyl)fluorescein ethyl ester]-cys(TBTP)-OH·TFA (**1a**).* Compound **6a** (300 mg, 0.30 mmol) was dissolved in 5 mL of a solution of TFA/CH_2Cl_2/anisole (45:50:5, v/v/v) at 0 °C. The mixture was stirred at room temperature for 3 h. The solvents were removed under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 1:1) to afford **1a** (200 mg, 68%) as an orange resin. TLC (EtOAc/MeOH 1:1) R_f 0.23. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 18.1$ min ($\lambda_{max} = 201.7$, 227.7 and 297.6 nm). $[\alpha]_D^{25}$ −50° (*c* 1, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, 1H, H_{12}, $J = 7.8$ Hz); 7.98 (d, 1H, NH, $J = 6.1$ Hz); 7.89–7.62 (m, 17H, PPh_3, H_{14}, H_{13}); 7.28 (d, 1H, H_{15}, $J = 7.5$ Hz); 6.93 (m, 1H, H_5); 6.89 (d, 1H, H_8, $J = 8.9$ Hz); 6.84 (d, 1H, H_1, $J = 9.6$ Hz); 6.83–6.78 (m, 1H, H_7); 6.50 (dd, 1H, H_2, $J = 9.7$ Hz, $J = 1.8$ Hz); 6.39–6.34 (m, 1H, H_4); 4.61–4.54 (m, 3H, C(O)CH_2–O and H_α); 4.07–3.94 (m, 2H, OCH_2–CH_3); 3.91–3.54 (m, 3H, P-CH_2 and H_β); 3.22–3.18 (m, 1H, H_β); 2.94–2.87 (m, 1H, CH–S); 2.79–2.69 (m, 1H, CH–S); 2.10–1.98 (m, 1H, C*H*–CH_2–S); 1.95–1.86 (m, 1H, C*H*–CH_2–S); 1.82–1.67 (m, 2H, P–CH_2–CH_2); 0.99–0.91 (m, 3H, OCH_2CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.82 (C_3); 172.79 (C=O acid); 166.43 (C(O)NH); 165.46, 165.41 (*C*(O)OEt); 162.03, 162.00 (C_6); 158.97 ($C_{4'}$); 154.03, 154.01 ($C_{5'}$); 150.21 (C_9); 135.17 (d, $3CH_{para}$, $J = 3.1$ Hz); 134.27, 134.23 (C_{16}); 133.81 (d, $6CH_{meta}$, $J = 10.3$ Hz); 132.72 (C_{14}); 131.38 (C_{12}); 130.64 (d, $6CH_{ortho}$, $J = 12.6$ Hz); 130.47 (C_{15}, C_1); 130.13 (C_2); 129.82 (C_{13}); 129.43, 129.41 (C_8); 118.55 (d, 3C (Ph_3), $J = 86{,}0$ Hz); 118.17 ($C_{1'}$); 115.87 ($C_{8'}$); 113.35, 113.22 (C_7); 105.91 (C_4); 102.32, 102.26 (C_5); 67.91 (C(O)*C*H_2O); 61.50 (O*C*H_2–CH_3); 54.30 (C_α); 44.00 (C_β); 37.11, 37.06 (CH_2–S); 29.47, 29.43, 29.34, 29.30 (d, *C*H_2–CH_2-S, $J = 16.1$ Hz); 21.48 (d, P–CH_2, $J = 50.5$ Hz); 20.95 (P–CH_2–*C*H_2); 13.75 (OCH_2–*C*H_3). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.41. MS (ESI+) Calcd for $C_{49}H_{45}NO_8PS_2$ $[M]^+$: 870.2. Found: 870.2 $[M]^+$.

*4.3.3.4. N-Dansyl-cys(TBTP)-OH·TFA (**1b**).* Compound **6b** (490 mg, 0.58 mmol) was dissolved in 5 mL of a solution of TFA/CH_2Cl_2/anisole (45:50:5, v/v/v) at 0 °C. The mixture was stirred at room temperature for 3 h. The solvents were removed under reduced pressure and the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 8:2) to afford **1b** (300 mg, 63%) as a green fluorescent resin. TLC (EtOAc/MeOH 1:1) R_f 0.43. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 17.1$ min ($\lambda_{max} = 222.9$ and 266.7 nm). $[\alpha]_D^{25}$ 9° (*c* 1, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (d, 1H, H_2, $J = 8.3$ Hz); 8.35 (d, 1H, H_8, $J = 8.5$ Hz); 8.16 (d, 1H, H_4, $J = 7.3$ Hz); 7.85–7.59 (m, 15H, PPh_3); 7.56–7.35 (m, 2H, H_7 and H_3); 7.28 (d, 1H, H_6, $J = 7.5$ Hz); 6.46 (br s, 1H, NH); 4.04 (br s, 1H, H_α); 3.40–3.24 (m, 2H, P–CH_2); 3.22–3.15 (m, 1H, H_β); 3.07–2.89 (m, 1H, $H_{\beta'}$); 2.96 (s, 6H, $N(CH_3)_2$);

2.78–2.58 (m, 2H, CH_2–S); 1.95–1.81 (m, 2H, CH_2–CH_2–S); 1.78–1.63 (m, 2H, P–CH_2–CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.61 (C=O acid); 149.12 (C_5); 135.34 (d, $3CH_{para}$, $J = 2.3$ Hz); 134.97 (C_1); 133.51 (d, $6CH_{meta}$, $J = 9.2$ Hz); 130.65 (d, $6CH_{ortho}$, $J = 12.6$ Hz); 129.96 (C_2); 129.69 and 129.63 (C_{10},C_9); 129.21 (C_4); 128.36 (C_7); 123.98 (C_3); 120.92 (C_8); 118.03 (d, 3C, $J = 86.0$ Hz); 116.28 (C_6); 55.83 (C_α); 45.79 ($N(CH_3)_2$); 44.02 (C_β); 37.01 (CH_2–S); 29.67 (d, CH_2–CH_2–S, $J = 17.2$ Hz); 21.73 (d, P–CH_2, $J = 51.6$ Hz); 20.89 (d, P–CH_2–CH_2, $J = 3.7$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ 23.31. MS (ESI+) Calcd for $C_{37}H_{40}N_2O_4PS_3$ $[M]^+$: 703.1. Found: 703.1 $[M]^+$.

*4.3.4. N-(labelled)-Cys(TBTP)-[K(UCCUCU)]$_c$ (**3a-b**) (Scheme 1)*

*4.3.4.1. N-[6-O-(Carboxymethyl)-fluorescein ethyl ester]-cys(TBTP)-[K(UCCUCU)]$_c$ (**3a**).* To a cold solution (0 degrC) of compound **2a** (20.0 mg, 8.62 μmol), NMM (4 μL, 36.40 μmol), and compound **1a** (9.4 mg, 9.55 μmol) in DMF (1 mL), was added ToPPiPu (3.6 mg, 9.54 μmol). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 2 h). The solvent was then evaporated under reduced pressure. The crude product was triturated in water and after filtration, the solid was washed with water and CH_2Cl_2. It was further purified on a reverse phase HPLC column (elution with 20% acetonitrile in water (0.1% TFA) to 100% acetonitrile over 30 min at flow rate of 1 mL/min) to give **3a** (8.5 mg, 31%) as a green fluorescent solid after lyophilisation. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 16.4$ min ($\lambda_{max} = 202.9$ and 260.8 nm). MALDI-TOF-MS Calcd for $C_{127}H_{152}N_{32}O_{31}PS_2[M]^+$: 2715 (63%), 2716 (100%), 2717 (88%), 2718 (56%), 2719 (28%), 2720 (12%). Found $[M]^+$ 2714.6 (60%), 2715.6 (100%), 2716.6 (87%), 2717.6 (60%), 2718.6 (38%), 2719.6 (17%).

*4.3.4.2. N-Dansyl-cys(TBTP)-[K(UCCUCU)]$_c$ (**3b**).* To a cold solution (0 °C) of compound **2a** (17.0 mg, 7.33 μmol), DIPEA (13 μL, 7.33 μmol), and compound **1a** (6.6 mg, 8.10 μmol) in DMF (1 mL), was added PyBrop (3.8 mg, 8.10 μmol). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 2 h). The solvent was then evaporated under reduced pressure. The crude product was triturated in water and after filtration, the solid was washed with water and CH_2Cl_2. It was further purified on a reverse phase HPLC column (elution with 20% acetonitrile in water (0.1% TFA) to 100% acetonitrile over 30 min at flow rate of 1 mL/min) to give **3b** (4.0 mg, 17%) as a white solid after lyophilisation. HPLC (A/B 80:20 to 0:100 over 30 min) $R_t = 15.2$ min ($\lambda_{max} = 204.1$ and 266.7 nm). MALDI-TOF-MS Calcd for $C_{115}H_{147}N_{33}O_{27}PS_3$ $[M]^+$ 2548 (69%), 2549 (100%), 2550 (85%), 2551 (53%), 2552 (27%), 2553 (11%). Found $[M]^+$ 2547.9 (74%), 2548.9 (100%), 2549.9 (90%), 2550.9 (53%), 2551.9 (30%), 2552.9 (12%).

4.4. Cell culture

HeLa (human epithelial cervical carcinoma) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

4.5. Microscopy

HeLa cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin until 80% confluence. Cells were suspended using tryp-

sin/EDTA and counted. Cells were seeded in 24-well plates at a density of 10^5 cells per well and incubated overnight. After medium removal, cells were pre-incubated for 30 min at 37 °C with 600 μL of fresh 10% FCS/DMEM. Subsequently, the medium was discarded and the cells were incubated at 37 °C for 1 h with a 5 μM 10% FCS/DMEM solution of fluorescent compound.

For confocal microscopy, cells were rinsed with PBS (phosphate-buffered saline) solution and fixed with 3.7% (v/v) formaldehyde in PBS for 10 min at room temperature. For direct fluorescence detection, cells were washed three times with PBS before being processed in mounting FluoG medium. The distribution of fluorescence was analysed with an inverted Leica SP2 confocal microscope (Leica, Wetzlar, Germany) equipped with argon ion, helium–neon, and green neon lasers and images were processed with Leica and NIH Image/ImageJ software.

For microscopy in live HeLa cells, cell images were recorded by using the 63× oil immersion objective lens. Image acquisition and analysis were performed using the Applied Precision Deltavision system (Applied Precision, Issaquah, WA) built on an Olympus IX-70 base. Excitation and emission filters used for dansyl were FITC and DAPI, respectively. Images were processed with NIH Image/ImageJ software.

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