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Design and Synthesis of Novel Phenothiazinium Photosensitiser Derivatives

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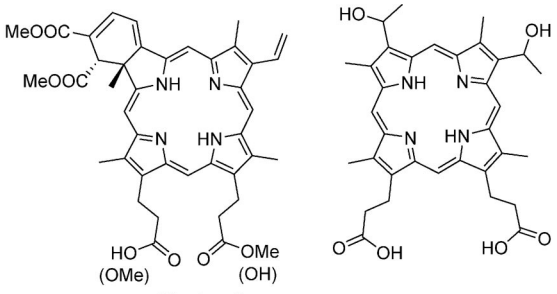
A high-yielding approach towards *N*-(2-aminoethyl)-Azure B has been established and extended towards the preparation of a functionalized peptide-dye synthetic precursor; Boc-protected 3-(alkylamino)phenothiazin-5-ium TFA. This has been utilized in a series of reactions with various amines towards nine novel 3,7-disubstituted phenothiazin-5-ium derivatives. Cleavage of the Boc group in the 3,7-disubstituted salts allowed a further seven novel dyes to be prepared and these have been utilized in a practical methodology designed for the synthesis of 13 novel phenothiazine-peptide conjugates. These peptide-photosensitizer vectors consist of a covalent attachment of a tethered dye to various protected amino acid moieties including the cell-penetrating peptide (CPP) octa-arginine and exhibit intense absorption maxima in the 623–650 nm regions of the visible spectrum. The main goal of the studies is the design of cell-compatible photosensitisers that may be employed in photodynamic therapy.

Introduction

Progress in photodynamic therapy (PDT) has been motivated by a quest to launch new and improved sensitisers. There are four main properties to be considered in the design and synthesis of an ideal candidate for use in photochemical cytotoxicity: i) selective retention or uptake by the tumour or other target tissue, ii) high quantum yields of singlet oxygen production, iii) photostability, and iv) sufficient tissue penetration.[1] For most purposes, red light is used because it penetrates tissue better than blue light and as such a potentially beneficial sensitizer must generally have appreciable absorption of light in the red region of the spectrum.

Verteporfin (**1**), a benzoporphyrin derivative with the trade name Visudyne®, first prepared in our laboratories, has absorption maxima in the range 670–700 nm. Verteporfin in particular has been shown to preferentially accumulate in neoplastic blood vessels and, when stimulated by non-thermal red light with a wavelength of 690 nm in the presence of oxygen, produces highly reactive short-lived singlet oxygen and other reactive oxygen radicals, resulting in local damage to the endothelium and blockage of the vessels.[2]



Visudyne®

1 **2**

It is not surprising that second-generation photosensitizers are mostly based on porphyrins since modern PDT has evolved from the naturally derived porphyrins, for instance hematoporphyrin (Hp) **2** was used in the studies carried out in 1925 by Policard et al., whereby the ability of such compounds, to produce phototoxic effects, was ascertained.[3] Porfimer sodium (Photofrin®), an oligomeric mixture of porphyrins isolated following gel exclusion chromatography to remove monomeric porphyrins, is currently one of only a few photosensitizers being used for the photodynamic therapy of cancers.[4]

Other compounds such as phenothiazinium derivatives have been shown to have higher levels of activity than porphyrin-derived materials in cell culture and in animal testing. The phenothiazinium dyes exhibit intense absorption maxima in the 600–660 nm region of the spectrum (typically $>50000\ L\,mol^{-1}\,cm^{-1}$; $\log\varepsilon_{max} > 4.7$), which is required for efficient tissue penetration by light and thus absorb in the PDT "therapeutic window".[5]

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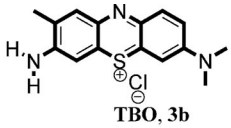
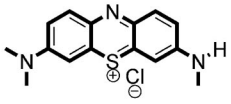
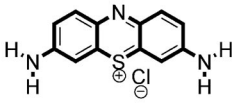
So far, the field of vital staining utilises many commercially available phenothiazines e.g. Methylene Blue (MB) (**3a**), demethylated analogs [for instance toluidine blue (**3b**), azure B, (**3c**) and thionin (**3d**)]. However, in PDT, when activated by visible light, such compounds are known to generate reactive oxygen species, mainly singlet oxygen, through a Type II photodynamic reaction. Studies on the use of phenothiazines in the photodynamic therapy of cancer have concentrated predominantly on Methylene Blue (MB) and its analogs.[6]


MB, 3a


TBO, 3b


Azure B, 3c


Thionin, 3d

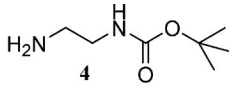
We report here on the design and synthesis of novel phenothiazines incorporating varying amino substituents. In our studies, a modified version of the method described by Strekowski et al. is utilized for their synthesis.[7,8] The potential therapeutic efficacy of several synthetic drug analogs can be limited due to their unfavorable properties. These include: poor solubility, absorption, or distribution and poor cell penetration. An increasingly attractive solution to improving passive membrane diffusion is the administration of the drugs in a vectorized form; for instance the use of peptide vectors in the form of covalent conjugates.[9]

Recently, several classes of "cell-penetrating peptides" CPPs i.e. those that are water-soluble and that have the ability to cross biological membranes, have been identified. These are short polypeptide sequences which can be subdivided into two classes: (i) unstructured highly cationic peptides such as TAT and the penetratins, and (ii) amphipathic α-helical peptides based on protein-signal sequences. Transportan for instance is a CPP that incorporates both elements, but its cationic residues seem most important.[10]Research on the HIV-tat transporter sequence has led to the finding that short oligomers of arginine- (7–9 amino acid units) and, more specifically, guanidinium-based oligomers often exhibit superior membrane translocation activity. These molecules, either alone or covalently linked to "cargo" molecules of therapeutic interest, have been shown to readily cross a variety of biological barriers including penetration of human skin. In contrast to simple solubilising functionalities, a particularly significant property of these transporters is that they both enhance water solubility and facilitate uptake through the nonpolar bilayer of a cell. In fact, porphyrin conjugates bearing HIV-tat 40–60 and octa-arginine peptides were found to be the most effective in delivering the conjugates in the cells when compared to the hydrophobic porphyrins.[11]

We envisaged that it was important to merge this methodology into our studies in order to enable the delivery of the novel phenothiazines. An efficient methodology is reported here towards the synthesis of octa-arginine, pursuing an established efficient segment-doubling strategy demonstrated by Wender et al.[12] It is notable that a pilot scale synthesis of octa-D-arginine amide as the nonahydrochloride utilising an otherwise similar approach to this protocol, with no chromatographic purification employed during any step of the process has been reported and thus contributes in making this methodology extremely cost effective.[13] Thus, in our approach, the efficient conjugation of 9 novel phenothiazines to an arginine octamer is detailed with the goal to improve the biological efficacy of the phenothiazines. More specifically, these peptide-photosensitizer vectors consist of a covalent attachment of a tethered novel phenothiazine dye; resulting in dye-octa-arginine conjugates.

Results and Discussion

In order to prepare the desired phenothiazines we synthesized a common tether suitably functionalized with a protected amine moiety to allow attachment to the carboxy terminal of the peptides. Ethylenediamine was successfully converted into the mono-Boc-protected amine **4** in quantitative yield.[14] In addition to the latter, we selected a commercially available tether **5** designed for the synthesis of *N*-(2-aminoethyl)-Azure B (**8**), a compound reported to be a suitable precursor in dye-peptide conjugates.[15]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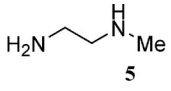
tert-butyl 2-aminoethylcarbamate

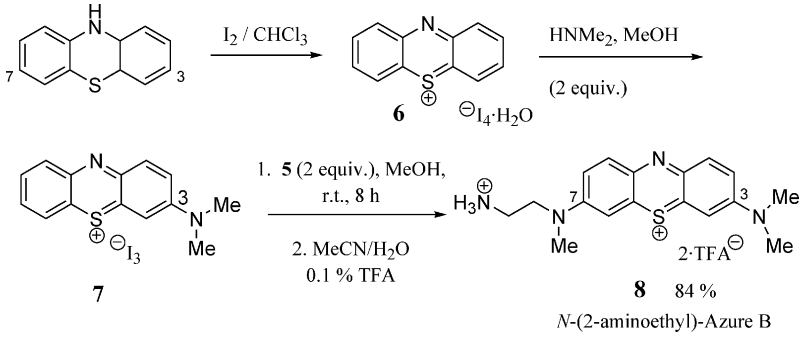

5

N^1-methylethane-1,2-diamine

We expected that attack of the aromatic π-electrons at the methylated terminal of the tether **5** would be most favorable owing to the N-atom's nucleophilicity and thus set out to synthesise compound **8** by the methodology developed by Strekowski et al. towards 3,7-disubstituted phenothiazin-5-ium salts containing two different amino groups (Scheme 1).[7]

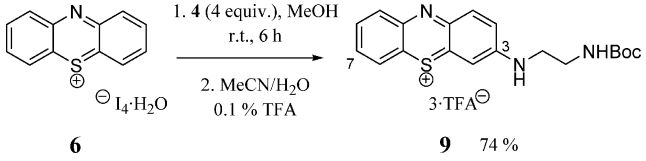
We were delighted that coupling **7** to the amino tether **5**, at room temperature in anhydrous MeOH followed by preparative HPLC purification of the crude mixture, gave the Azure B derivative **8** in 84% yield, the highest reported to date for the synthesis of this compound.

In addition, we embarked on the preparation of a common template 3-(alkylamino)phenothiazin-5-ium dye to which various alkylamino groups could be attached at the 7-phenothiazine position. Initial attempts to optimise the reaction involved reflux conditions and led to a more complex mixture that could not easily be separated. Increasing the quantity of amine in our original conditions, by gradually adding 0.5 equiv. increments to the reaction allowed us to determine the optimum quantity of required amine at 4 equiv. ^{1}H NMR monitoring of the reaction showed a



Scheme 1. Synthesis of Azure B derivative.

maximum of 80% product formation even upon addition of up to 5 equiv. of amine. The final isolation of our novel target compound **9** was achieved with a 74% yield, as a dark green solid, λ_{max} (CH_3CN) = 628 nm (Scheme 2).



Scheme 2. Synthesis of the mono-substituted common precursor.

With precursors **8** and **9** in hand, stored as a light-sensitive material, covered in foil and under argon, we embarked on the synthesis of the desired series of novel 3,7-diaminophenothiazines by reacting with various amines as shown in Table 1 and Scheme 3.

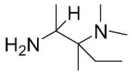
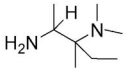
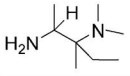
Following the protocol employed for the synthesis of Azure B derivative **8**, analytically pure samples of compounds **10**–**18** were isolated in good to high yields. Most successful were the reactions with secondary amines (compounds **10**–**15**) which gave yields as high as 88% in the case of the butyl derivative **14**. Purification was relatively straightforward, giving samples as bright blue-dark blue solutions. The latter were lyophilized and concentrated to give dark solids of 99+% purity with UV λ_{max} (CH_3CN) ranging from 626–648 nm.

The results in Table 1 suggested that tether **5** could be of greater interest than tether **4** in the synthesis of novel phenothiazines by a common precursor similar to **9**. However, our attempts were met with little success, a complex mixture of inseparable products was formed and none of the "common" precursor was isolated. Our next strategy with these compounds was to cleave the Boc-group by a simple, efficient and mild method.

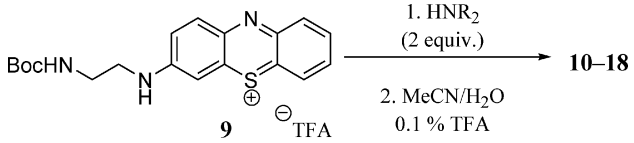
Thus, utilising zinc dibromide in a solution of the Boc-derivative in dichloromethane under an inert atmosphere, quantitative yields of all the amines of compounds **10**–**15** with UV λ_{max} (CH_3CN) = 623–645 nm were obtained, ready to be reacted with the peptides (Scheme 4).[16]

On reacting **9** with primary amines (compounds **16**–**18**) in the presence of molecular sieves, we observed several products varying in color from blue to purple. We concentrated our efforts on the blue samples, because only they

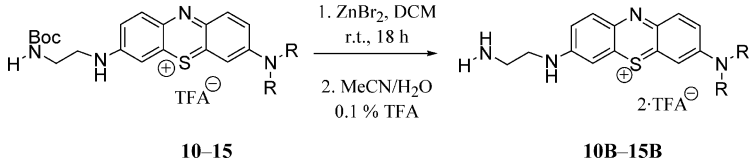
Table 1. 3,7-(Dialkylamino)phenothiazin-5-ium derivatives.

Amine R	Product	% Yield[a]
	**10**	72
	**11**	75
	**12**	74
	**13**	82
	**14**	88
	**15**	71
	**16**	30
	**17**	36
	**18**	21

[a]% Yields are expressed for Boc-protected derivatives only **10B**–**15B**; all deprotection reactions gave quantitative yields.

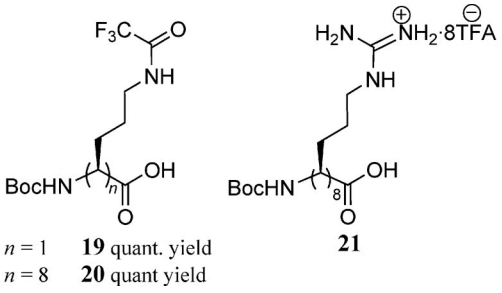


Scheme 3. Synthesis of the desired series of novel 3, 7-diaminophenothiazines.



Scheme 4. Cleavage of Boc-group to functionalise dyes.

had fulfilled the criteria for the PDT "therapeutic window", with UV λ_{max} (CH_3CN) = 626–628 nm as observed for **16**–**18**. To our disappointment, lyophilization of the blue fractions revealed a mixture of products from which **16**–**18** were isolated, purified and lyophilized and obtained in conservative yields as shown in Table 1. The methodology towards the novel series of phenothiazines **10**–**18** is encouraging, especially with the secondary amines.

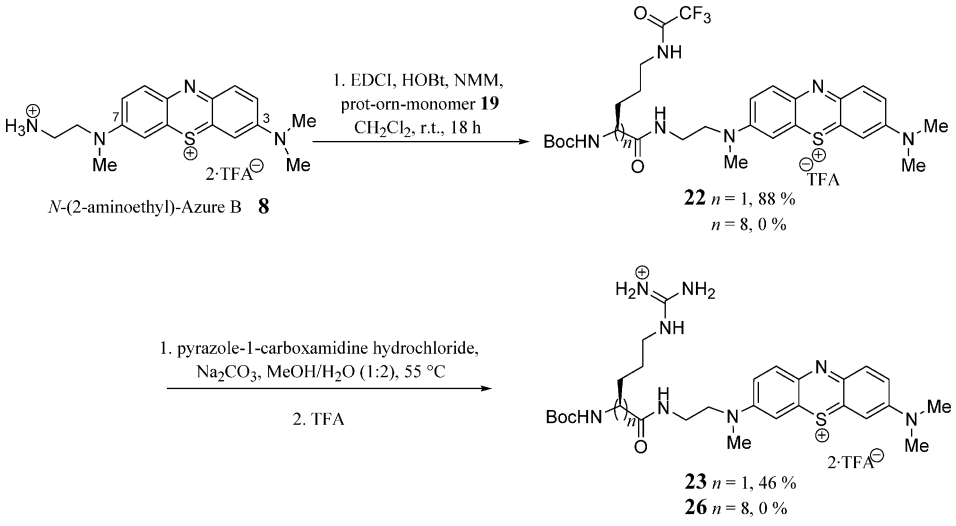


Peptide reactions were performed and a suitable synthesis of the protected ornithine octamer **20** was achieved from protected ornithine monomers e.g. **19**; exactly as described in the literature.[12] We considered performing the mild conversion of the trifluoroacetamides to guanidines once the protected ornithine compounds had been attached to the dye but at this stage our interest was to establish the most efficient methodology towards, dye-octa-arginine conjugates. In addition, we favored this route because we had found the coupling reactions of the protected derivatives to be high yielding. Thus, we explored initial dye-amine and peptide-acid coupling conditions using the protected ornithine monomer **19**, and hence opted to establish the perguanidylation conditions on a simpler system as a model for the octamer synthesis. Later **20** would be converted to **21** following literature protocols.

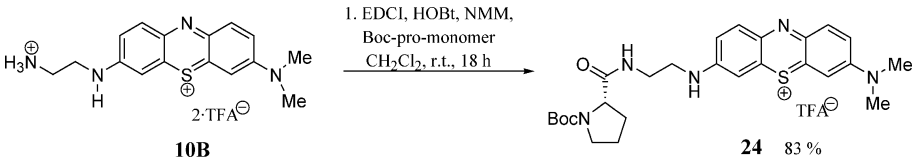
Dye-Peptide Conjugate Synthesis

Our rationale for performing the perguanidylation after attachment of the peptide acid to the dye amine **8** proved to have merit. We had a surplus of the precursor **8** and opted to optimize the reaction using this "model" system (Scheme 5).

Thus the coupling between *N*-(2-aminoethyl)-Azure B (**8**) and protected ornithine acid **19** furnished the novel mono-ornithine derivative **22** in good yields. Subsequent treatment of the latter with sodium carbonate and pyrazole-1-carboxamidine hydrochloride, in aqueous methanol, gave the arginine derivative **23** in 46% isolated yield after purification by RP-HPLC [99+% purity, λ_{max} (CH_3CN) = 623 nm]. Interestingly, λ_{max} dye amine = 628 nm > λ_{max} arg-monomer-dye = 623 nm > λ_{max} protected ornithine-dye-conjugate = 616 nm; indicating that the arginine moiety does not significantly alter the absorption of the dye. When the penetrating ability of the octamer is also considered we see this as an encouraging result in the field of PDT. Hence, the methodology was used for the synthesis of the octamer model system **26**; however, this time without success, all attempts to optimise the reaction were met with failure, as we continually ended up with a complex inseparable mixture. Perplexed, we applied the original conditions on the



Scheme 5. Synthesis of arginine-dye conjugates.



Scheme 6. Synthesis of proline-dye conjugate **24**.

novel precursor amine **10B** and used the simple acid Boc-proline to establish coupling conditions for this particular amine precursor.

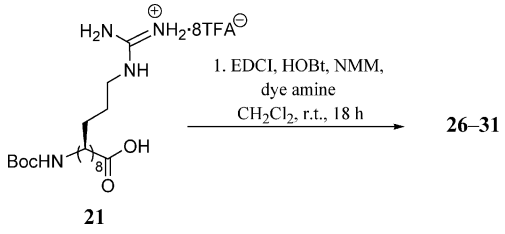
Fortunately, our original conditions readily gave the Boc-pro-conjugate **24** (Scheme 6) in an acceptable 83% yield. However, coupling this to any ornithine residues or perguanidylation failed once again, spectral analysis indicating complex multiple product formation.

Finally, we decided to attempt our "post" perguanidylation methodology on our highest yielding 3,7-(dialkylamino)phenothiazin-5-ium derivative **14** as a means of examining the reactivity of our dye amines in this reaction. Thus, following the literature synthesis of protected Ornithine octamer **20**,[7] coupling to the butylamine dye **14** occurred readily following optimized conditions, furnishing protected octa-orn-dye **25** in a satisfactory 70% yield. Nevertheless, as we had already encountered, "post" perguanidylation towards **30** did not give any products in the "therapeutic" PDT window i.e. those that absorb light in the red region of the spectrum (Scheme 7).

Although we had chosen a cautious route earlier, avoiding imposition of the guanidinium headgroups of arginine in the reaction and taking into account any other complications in the direct coupling of the octa-arginine dye, at this stage we decided to follow the alternative route which involves the direct coupling of the octa-arginine acid **21** to the dye amines (**10**–**17**) that we had in hand.

Thus, once again pursuing the route established by Wender et al. we found the conversion of octa-ornithine trifluoroacetamide carboxylic acid **20** furnished the desired product **21** as a white powder in a reproducible yield, 53% and purity, 99+%.[12] Subsequent treatment of **21** with 4-methylmorpholine (NMM) in anhydrous dichloromethane at 0 °C, followed by 1-hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride allowed formation of the activated ester over a 40 min period. This was followed by cannula addition of a precooled solution of the dye amine deprotonated with NMM in anhydrous dichloromethane at 0 °C while stirring for 4 h and eventually warming to room temperature over a 15 h period.

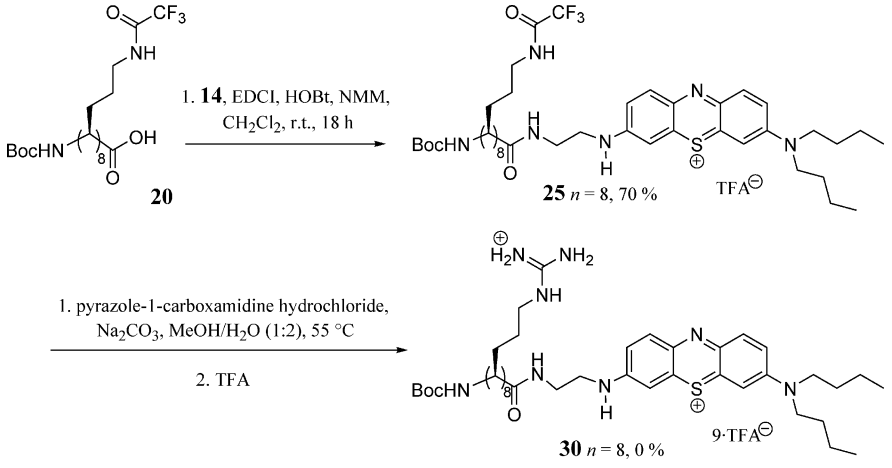
The coupling reagents proved to be appropriate for our synthesis and allowed for an efficient amide bond formation to the dye amines (Scheme 8).



Scheme 8. Synthesis of arginine octamer conjugates.

Furthermore, we found the strongly basic character and tight interaction of the guanidinium ions with TFA counterions caused no problems during the conjugation synthesis. NMM is a good choice of base for the deprotonation of the guanidinium headgroup of arginine and this reduces the possibility of multiple product formation.

The purification of the resulting phenothiazine–peptide dye conjugates **26**–**33** was achieved by RP HPLC and the



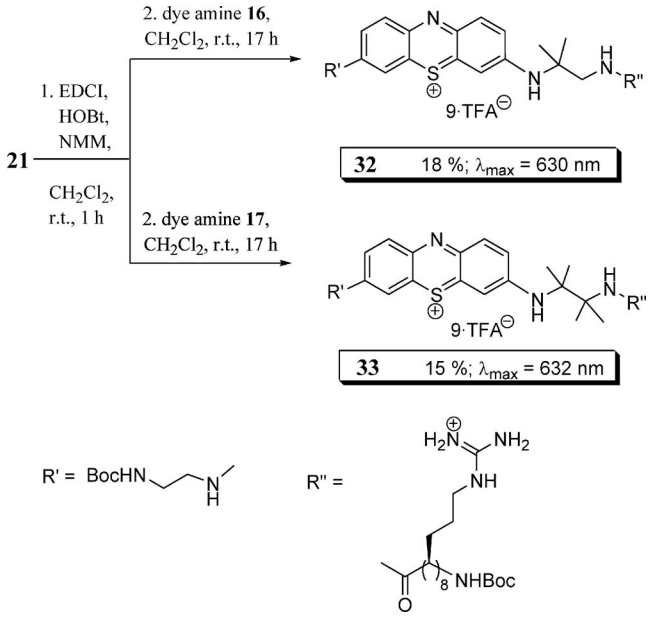
Scheme 7. Syntheses of octamer-dye conjugates.

product-containing fractions were combined and lyophilized yielding the conjugates as dark, hygroscopic powders (Table 2, Scheme 9).

Table 2. Dye-peptide conjugates.

Amine R	Conjugate	% Yield[a] λ_{max}/nm
	**26**	22 623
	**27**	20 646
	**28**	20 623
	**29**	25 644
	**30**	27 650
	**31**	11 642

[a] CH_3CN UV solutions.



Scheme 9. Synthesis of conjugates **32** and **33**.

The arginine conjugates **26**–**33** reveal intense absorption maxima in the 623–650 nm region of the spectrum. The octa-ornithinine conjugate **25** on the other hand may not be suitable as a PDT drug as it absorbs below 600 nm. Although the yields were rather modest ranging from 11–24%, we note that most of the conjugates may potentially be useful in the field of PDT. We attributed the lower yields to the overall steric effects resulting from the "bulky" ligand and some of the amine alkyl groups.

Conclusions

In summary, we have designed cell-compatible photosensitisers that may be employed in photodynamic therapy. Firstly, following a novel higher yielding approach towards Azure B derivative **8**, a handy precursor in the synthesis of dye-peptide conjugates adopted for our "model" studies has been synthesised. We have revealed an optimized practical method for the preparation of 3-(Boc-Ethylamino)phenothiazin-5-ium salt **9** in good yields. The latter was also harnessed as a useful precursor towards analytically pure 3, 7-disubstituted analogs **10**–**18** in modest to good yields, cleavage of the Boc-group (entries **10B**–**15B**) utilising the mild conditions employed with $ZnBr_2$ has unmasked some more novel phenothiazines in quantitative yields.[16] In addition, novel phenothiazine salts **16**–**18** have been synthesized using primary amines and although the yields are low and their purification capricious, the methodology is overall acceptable.

Our goal to establish a practical methodology by reacting the protected ornithine monomer **19** to the novel 3,7-phenothiazine dye, which would enable us to optimise the perguanidylation step and finally translate these results towards the synthesis of the desired arginine octamer was met with partial success. The mono-peptide conjugate was formed in high yield but the synthesis of the desired octapeptide derivatives failed. Following an alternative route, the synthesis of 13 novel conjugates has been reported; 3 novel mono-peptide dyes **22**–**24** that were prepared in order to establish a methodology of coupling octapeptides to the phenothiazines in an attempt to present a "post-perguanidylation" methodology. Our studies progressed to the synthesis of octa-ornithine dye **25** where failed perguanidylation of the latter brought us to the altenative route linking the dye amines **10**–**18** directly to the octa-arginine acid **21** via an EDC/HOBT coupling protocol. Thus, these compounds consist of the covalent attachment of a tethered dye to four different amino acid moieties from proline to octa-arginine, resulting in dye-peptide conjugates, some with intense absorption maxima in the 623–650 nm regions.

Experimental Section

General: Nuclear magnetic resonance spectra (NMR) were recorded with Bruker Avance (400 MHz for ^1H, and 67.5, 90.5 and 125 MHz for ^{13}C) spectrometers as dilute solutions in deuterated solvents as specified. Chemical shifts (δ) are quoted in parts per million (ppm) downfield of tetramethylsilane (TMS) or referenced to residual protonated solvent, as internal standard. Signal multiplicities are designated by the following abbreviations: ^1H spectra: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; app = apparent; br = broad. In ^{13}C spectra the multiplicities were determined using a DEPT sequence with secondary pulses at 90°

and 135° where appropriate. Signal multiplicities are designated by: s = quaternary; d = tertiary methine; t = secondary methylene; q = primary methyl.^{19}F NMR were recorded with a Bruker Avance 400 (376 MHz) spectrometer and are referenced to residual protonated solvent, i.e. CF_3CO_2H. All coupling constants (J) are quoted to the nearest 0.1 Hz.

Ultraviolet-visible spectra were recorded with a Hewlett–Packard 8452 A Diode Array spectrophotometer at room temperature as solutions in spectroscopic grade acetonitrile and are given in nm, with ε in $dm^3 mol^{-1} cm^{-1}$ in parentheses.

Mass spectra were recorded with Varian/MAT CH4B or Kratos/AEI MS-902 using electrospray (ES) or fast atom bombardment (FAB) techniques. Due to the soft nature of these techniques, little fragmentation of the compounds occurred and hence nominal mass and fragmentation data have not been included. High-resolution mass spectra are calculated to 4 decimal places from the molecular formula corresponding to the observed signal using the most abundant isotopes of each element.

Flash chromatography was performed using Merck Kieselgel 60 (230–400 mesh) and the solvents employed were either of analytical grade or were distilled before use according to the technique of Still et al.[17] and elution was performed at ca. 8 psi above atm.

All HPLC analyses were performed using a Beckman Coulter 32 Karat System with a reverse-phase Alltec® C_{18} column (250 × 22 mm internal diameter) or analytical Alltec® C_{18} column (150 × 4.6 mm internal diameter) eluting with a solvent system of water/acetonitrile both containing 0.1% TFA. Simultaneous detections of the products were carried out at several wavelengths using a Beckman Coulter 168 Diode Array UV detector.

Despite many attempts we have been unable obtain consistent elemental-analysis data on the compounds even for those proven, by HPLC, to have a purity ≥ 99%. The compounds we report are salts and clearly do not combust well. Wender et al., who are leaders in this field and whose protocols we have employed, did not provide elemental analyses either, presumably for the same reasons.[12]

Reactions were also monitored by thin layer chromatography (TLC) using Merck DC-Alufolien silica gel 60 F_{254}, 0.2 mm precoated aluminium plates. TLC plates were visualized by quenching of UV fluorescence (λ_{max} = 254 nm) light and were subsequently developed using either acidic ninhydrin solution, acidic alcoholic vanillin solution or a basic potassium permanganate solution as appropriate. All reactions with the dye were performed in Al-foil-protected round-bottomed flasks and dark hoods.

Routinely, organic extracts were dried with anhydrous magnesium sulfate or sodium sulfate. Solvents were removed in vacuo using a Büchi rotary evaporator. Anhydrous organic solvents were stored under an atmosphere of nitrogen and/or over sodium wire. Other organic solvents were dried by distillation as follows: THF and dimethyl ethylene glycol (sodium benzophenone ketyl), and methanol (calcium hydride), acetonitrile (K_2CO_3), under an inert atmosphere. Other organic solvents and reagents were purified according to accepted literature procedures. Where necessary, reactions requiring anhydrous conditions were performed in flame or oven dried apparatus under a nitrogen atmosphere.

Dye Syntheses

BocNH-CH_2CH_2-NH_2 (4): The compound was synthesized in accordance with known literature protocol.[14] A solution of Boc-anhydride (6.1 g, 28 mmol) in dichloromethane (400 mL) was added dropwise to a stirred solution of ethylenediamine (11.2 mL, 166.7 mmol) in dichloromethane (50 mL) over a period of 6 h while the mixture was stirred vigorously at room temperature. The reaction was then stirred for a further 24 h, before the mixture was evaporated in vacuo to leave a crude oily residue, to which aqueous Na_2CO_3 (2 M, 300 mL) was added and the aqueous layer was separated and extracted with dichloromethane (2 × 300 mL). The combined organic extracts were evaporated in vacuo to give the carbamate (4.5 g, quantitative yield) as a colorless viscous liquid. ES-MS (+ ionization) calcd. ($C_7H_{16}N_2O_2$ + H) 161.2; found 161.0. All structural assignments were in agreement with ^{1}H and ^{13}C NMR spectroscopic data available from the literature.[14]

Phenothiazin-5-ium Tetraiodide Hydrate (6): The compound was synthesized in accordance with known literature protocol.[7] A solution of iodine (8.4 g, 33 mmol) in chloroform (175 mL) was added dropwise to a stirred solution of phenothiazine (2.1 g, 11 mmol) in chloroform (75 mL) within 1 h at room temperature. The reaction mixture was then stirred for a further 30 min at 5 °C and the resultant precipitate was filtered, washed with chloroform (2 × 500 mL) and then dried at room temperature for 3 h give the phenothiazine tetraiodide (6.8 g, 74%) as a black solid.^{1}H NMR (300 MHz, [D_6]acetone): δ = 8.07 (m, 2 H), 7.98 (m, 2 H), 7.71 (m, 4 H) ppm. All structural assignments were in agreement with ^{1}H NMR spectroscopic data available from the literature.[7]

3-(Dimethylamino)phenothiazin-5-ium Triiodide (7): The compound was synthesized from **6** in accordance with known literature protocol.[7] The desired product **8** was obtained as a dark solid (340 mg, 28%).^{1}H NMR (300 MHz, [D_6]acetone): δ = 8.22 (dd, J = 8, J = 1.6 Hz, 1 H), 8.17 (dd, J = 8, J = 1.6 Hz, 1 H), 8.10 (dd, J = 8, J = 1.6 Hz, 1 H), 8.10 (d, J = 12 Hz, 1 H), 8.04 (dd, J = 12, J = 1.8 Hz, 1 H), 8.0 (d, J = 2.8 Hz, 1 H), 7.86 (m, 1 H), 3.65 (s, 3 H), 3.62 (s, 3 H) ppm. All structural assignments were in agreement with ^{1}H NMR spectroscopic data available from the literature.[7]

General Procedure A for the Synthesis of Boc-Diamine Dyes: The amine (1.5 equiv., 0.43 mmol) was added dropwise to stirred solution of mono-amine phenothiazine dye **9** (1 equiv., 0.28 mmol) in anhydrous MeOH (1 M) at room temperature under nitrogen. The reaction mixture was stirred for 8 h before concentration in vacuo to leave a dark residue, which was immediately purified by preparative RP-HPLC eluting with a solvent system of H_2O/MeCN 0.1% TFA, with a stepwise gradient 5⟶45% of the water eluent.

3-(2-Aminoethyl)-Azure B (2TFA Salt) (8):[15] Using Procedure A, the major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine TFA salt **8** (130 mg, 84%) as a bluish black solid, t_R = 11.6 min, analytical RP-HPLC (gradient 5⟶95% water, 15 min), t_R = 5.1 min, 99+%. UV (CH_3CN): λ_{max} (ε/$dm^3 mol^{-1} cm^{-1}$) = 628 (15300). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.25 (m, 2 H), 6.75 (m, 2 H), 6.50 (m, 2 H), 3.45 (m, 2 H), 3.35 (m, 2 H), 3.0 (s, 3 H), 2.75 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 38 Hz, TFA C=O), 156.2, 145.2, 144.7, 127.9, 127.5,126.9, 126.3, 119.2, 115.4, 111.2 (q, J = 290 Hz, TFA CF_3), 43.5, 42.4, 38.9, 35.9 ppm. HRMS calcd. (M + H, $C_{17}H_{21}N_4S$) 313.2; found 313.3.

3-(Boc-Ethylamino)phenothiazin-5-ium (3TFA Salt) (9): A solution of Boc-amine **4** (8 mmol) in methanol (10 mL) was added dropwise to a stirred solution of the tetraiodide hydrate **2** (1.44 g, 2 mmol) in methanol (40 mL), over a period of 6 h at room temperature using a syringe pump. The reaction mixture was concentrated in vacuo to leave a dark crude oily residue, which was dissolved in (50 mL) and washed with dilute hydrochloric acid (3 × 30 mL) and dilute sodium hydroxide (30 mL). The organic extracts were concentrated and further purification of the dye was achieved by preparative RP-HPLC as described in the general details eluting with a solvent system of H_2O/MeCN both containing 0.1% TFA, with

a stepwise gradient 5→52% of the water. The major fraction containing the product was collected and lyophilized to yield phenothiazine TFA salt **9** (6.8 g, 74%) as a dark green solid, t_R = 5.3 min, analytical RP-HPLC (gradient 3→97% water, 15 min), t_R = 3.1 min, 97.6% purity. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 628 (14700) nm.. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.72 (m, 5 H), 6.94 (m, 2 H), 3.18 (br.s, 2 H), 3.18 (m, 2 H), 2.85 (m, 2 H), 1.39 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 35 Hz, TFA C=O), 156.4, 155.6, 143.8, 127.9, 127.5, 126.3, 123.4, 115.3, 111.1 (q, J = 290 Hz, TFA CF_3), 79.8, 44.2, 42.5, 28.8 ppm. HRMS calcd. (M + H, $C_{19}H_{22}N_3O_2S$) 357.2; found 358.1.

General Procedure B for Boc-deprotection of Dyes: $ZnBr_2$ (3 equiv.) was added to the Boc diamine dye (1 equiv.), DCM (3 M) solution, and the mixture was stirred at room temperature for 18 h under nitrogen. The DCM was evaporated in vacuo, to leave the crude amine hydrobromide which was further purified by preparative RP-HPLC eluting with a solvent system of H_2O/MeCN both containing 0.1% TFA, with a stepwise gradient 5→45% of the water eluent.

3-(Boc-Ethylamino)-7-(dimethylamino)phenothiazin-5-ium (1TFA Salt) (10A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **10A** (105 mg, 72%) as a bluish black solid, t_R = 12.6 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.2 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 628 (15300) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.90–7.28 (m, 6 H), 6.50 (br.s, 1 H), 3.18 (br.s, 2 H), 2.85 (m, 2 H), 2.78 (s, 3 H), 2.65 (s, 3 H), 1.39 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 155.9, 155.5, 148.9, 143.7, 127.6, 127.4, 126.6, 116.1, 111.1 (q, J = 290 Hz, TFA CF_3), 78.8, 43.8, 43.1, 39.1, 29.0 ppm. HRMS calcd. (M + H, $C_{21}H_{27}N_4O_2S$) 399.2; found 399.3.

3-(2-Aminoethylamine)-7-(dimethylamino)phenothiazin-5-ium (2TFA Salt) (10B): Using Procedure B, the Boc amine **10A** (100 mg, 0.25 mmol), was first converted into the amine hydrobromide, obtained as an amorphous solid, which was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **10B** (105 mg, quantitative) as a bluish black solid, t_R = 11.8 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 4.88 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 623 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.90–7.50 (m, 6 H), 6.8 (br.s, 1 H), 3.31 (m, 2 H), 2.92 (m, 2 H), 2.80–2.65 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.5 (q, J = 36 Hz, TFA C=O), 156.3, 149.1, 142.4, 127.4,127.3, 127.1, 126.5, 123.8, 115.9, 111.1 (q, J = 290 Hz, TFA CF_3), 44.2, 43.7, 39.4 ppm. HRMS calcd. (M + H, $C_{16}H_{19}N_4S$) 299.1; found 299.4.

3-(Boc-Ethylamino)-7-(diethylamino)phenothiazin-5-ium (1TFA Salt) (11A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **11A** (116 mg, 75%) as a bluish black solid, t_R = 13.1 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.32 min, 99+%. λ_{max} (CH_3CN)/nm 640 (ε/dm^3 mol^{-1} cm^{-1} 12400). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.75–7.0 (m, 6 H), 5.90 (br.s, 1 H), 3.28 (m, 2 H), 3.05–2.97 (m, 6 H), 1.42 (s, 9 H), 1.30–1.27 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 155.8, 155.3, 149.1, 143.9, 128.1, 127.7, 126.5, 114.9, 111.1 (q, J = 290 Hz, TFA CF_3), 78.9, 44.4, 44.2, 42.6, 28.9, 20.5 ppm. HRMS calcd. (M + H, $C_{23}H_{31}N_4O_2S$) 427.2; found 427.5.

3-(2-Aminoethylamine)-7-(diethylamino)phenothiazin-5-ium (2TFA Salt) (11B): Using Procedure B, the Boc amine **11A** (110 mg, 0.23 mmol) was first converted into the amine hydrobromide, obtained as an amorphous solid, that was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **11B** (110 mg, quantitative) as a bluish black solid, t_R = 11.7 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.1 min, 99+%; λ_{max} (CH_3CN)/nm 634 (ε/dm^3 mol^{-1} cm^{-1} 15200). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.12 (s, 1 H) 7.80–7.45 (m, 6 H), 4.10 (m, 4 H), 3.49 (m, 4 H), 1.49 (m, 3 H), 1.30 (m, 3 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.5 (q, J = 36 Hz, TFA C=O), 155.7, 148.5, 144.3, 129.6, 128.1, 127.0, 126.7,124.0, 115.4, 111.1 (q, J = 290 Hz, TFA CF_3), 45.2, 44.0, 43.5, 21.5 ppm. HRMS calcd. (M + H, $C_{18}H_{23}N_4S$) 327.2; found 327.9.

3-(Boc-Ethylamino)-7-(diisopropylamino)phenothiazin-5-ium (1TFA Salt) (12A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **12A** (120 mg, 74%) as a bluish black solid, t_R = 13.9 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.7 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 626 (15300) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.01–6.67 (m, 6 H), 5.49 (br.s, 1 H), 3.10 (m, 2 H), 2.87 (m, 2 H), 2.06 (m, 2 H), 1.43 (s, 9 H), 1.33 (d, J = 6.8 Hz, 6 H), 1.05 (d, J = 6.8 Hz, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 156.5, 155.7, 148.7, 143.6, 127.8, 127.6, 126.5, 115.4, 111.1 (q, J = 290 Hz, TFA CF_3), 79.9, 46.3, 44.4, 43.0, 28.8, 25.4 ppm. HRMS calcd. (M + H, $C_{25}H_{35}N_4O_2S$) 455.3; found 455.9.

3-(2-Aminoethylamino)-7-(diisopropylamino)phenothiazin-5-ium (2TFA Salt) (12B): Using Procedure B, the Boc amine **12A** (120 mg, 0.2 mmol) was first converted into the amine hydrobromide, obtained as an amorphous solid, that was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **12B** (116 mg, quantitative) as a bluish black solid, t_R = 12.4 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.1 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 620 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.01–6.67 (m, 6 H), 5.49 (br.s, 1 H), 3.10 (m, 2 H), 2.87 (m, 2 H), 2.06 (m, 2 H), 1.33 (d, J = 6.8 Hz, 6 H), 1.05 (d, J = 6.8 Hz, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 154.7, 148.5, 143.2, 127.6, 127.3, 126.9, 115.8, 111.1 (q, J = 290 Hz, TFA CF_3), 47.1, 44.2, 43.5, 25.6 ppm. HRMS calcd. (M + H, $C_{20}H_{27}N_4S$) 355.2; found 355.5.

3-(Boc-Ethylamino)-7-(diisobutylamino)phenothiazin-5-ium (1TFA Salt) (13A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **13A** (140 mg, 82%) as a bluish black solid, t_R = 16.8 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 6.2 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 638 (10870) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.30–7.20 (m, 6 H), 3.10 (m, 4 H), 2.87 (m, 4 H), 2.06 (m, 2 H), 1.43 (s, 9 H), 1.04 (d, J = 6.4 Hz, 12 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 157.1, 155.6, 147.6, 143.7, 128.1, 127.7, 126.7, 116.0, 111.1 (q, J = 290 Hz, TFA CF_3), 78.8, 58.7, 44.0, 43.1, 28.9, 28.6, 21.3 ppm. HRMS calcd. (M + H, $C_{27}H_{39}N_4O_2S$) 483.3; found 483.8.

3-(2-Aminoethylamino)-7-(diisobutylamino)phenothiazin-5-ium (2TFA Salt) (13B): Using Procedure B, the Boc amine **13A** (120 mg, 0.2 mmol) was first converted into the amine hydrobromide, obtained as an amorphous solid, that was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **13B** (121 mg, quantitative) as a bluish black solid, t_R = 15.4 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.9 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 631 (11600) nm. ^{1}H NMR

(400 MHz, CD_3OD): δ = 8.25–7.30 (m, 6 H), 6.70 (br.s, 1 H), 3.70 (m, 4 H), 3.2 (m, 4 H), 1.50 (m, 2 H), 1.05 (d, J = 6.8 Hz, 12 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 156.7, 148.4, 143.9, 127.9, 127.5, 126.8, 115.8, 58.9, 44.3, 43.5, 29.0, 22.5 ppm. HRMS calcd. (M + H, $C_{22}H_{31}N_4S$) 383.6; found 383.7.

3-(Boc-Ethylamino)-7-(dibutylamino)phenothiazin-5-ium (1TFA Salt) (14A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **14A** (151 mg, 88%) as a bluish black solid, t_R = 16.5 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 6.1 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 648 (14700) nm. ^1H NMR (400 MHz, CD_3OD): δ = 8.26–7.53 (m, 6 H), 4.23–4.19 (m, 8 H), 1.67–1.60 (m, 4 H), 1.43 (s, 9 H), 1.39–1.24 (m, 4 H), 0.93–0.88 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, $CDCl_3$): δ = 157.6 (q, J = 36 Hz, TFA C=O), 155.9, 155.7, 149.1, 144.1, 128.1, 127.8, 125.9, 113.8, 111.1 (q, J = 290 Hz, TFA CF_3), 79.8, 50.3, 44.4, 43.1, 33.2, 28.9, 21.4, 14.8 ppm. HRMS calcd. (M + H, $C_{27}H_{39}N_4O_2S$) 483.3; found 483.9.

3-(2-Aminoethylamino)-7-(dibutylamino)phenothiazin-5-ium (2TFA Salt) (14B): Using Procedure B, the Boc amine **14A** (130 mg, 0.21 mmol), was first converted into the amine hydrobromide, obtained as an amorphous solid, that was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **14B** (122 mg, quantitative) as a bluish black solid, t_R = 15.2 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.7 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 645 (11200) nm. ^1H NMR (400 MHz, CD_3OD): δ = 7.72–7.66 (m, 6 H), 4.17–4.09 (m, 8 H), 1.65–1.60 (m, 4 H), 1.38–1.24 (m, 4 H), 0.89-.84 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 157.2, 151.1, 147.1, 129.7, 128.5, 127.2, 115.5, 111.1 (q, J = 290 Hz, TFA CF_3), 53.1, 45.5, 43.5, 33.7, 21.6, 19.2 ppm. HRMS calcd. (M + H, $C_{22}H_{31}N_4S$) 383.2; found 383.6.

3-(Boc-Ethylamino)-7-(dihexylamino)phenothiazin-5-ium (1TFA Salt) (15A): The major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine **15A** (133 mg, 71%) as a bluish black solid, t_R = 19.6 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 7.99 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 637 (13300) nm. ^1H NMR (400 MHz, CD_3OD): δ = 7.94 (m, 6 H), 3.35 (s, 1 H), 3.69 (m, 4 H), 1.86–1.79 (m, 4 H), 1.48–1.38 (m, 7 H), 1.39 (s, 9 H), 1.33–1.29 (m, 8 H), 0.89–0.84 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 155.8, 155.5, 149.4, 144.1, 127.7, 127.4, 126.6, 114.8, 111.1 (q, J = 290 Hz, TFA CF_3), 78.8, 51.2, 43.7, 43.5, 34.5, 31.2, 28.9, 27.3, 22.9, 16.3 ppm. HRMS calcd. (M + H, $C_{31}H_{47}N_4O_2S$) 539.3; found 539.6.

3-(2-Aminoethylamino)-7-(dihexylamino)phenothiazin-5-ium (2TFA Salt) (15B): Using Procedure B with the addition of activated type 3A molecular sieves, the Boc amine **15A** (130 mg, 0.21 mmol) was first converted into the amine hydrobromide, obtained as an amorphous solid, that was immediately purified by RP-HPLC. The product was collected and lyophilized to yield the corresponding phenothiazine amine TFA salt **15B** (133 mg, quantitative) as a bluish black solid, t_R = 18.3 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 7.5 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 632 (14300) nm. ^1H NMR (400 MHz, CD_3OD): δ = 8.1–7.6 (m, 6 H), 3.5 (s, 1 H), 3.69 (m, 4 H), 1.86–1.79 (m, 4 H), 1.48–1.38 (m, 7 H), 1.33–1.29 (m, 8 H), 0.89–0.84 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 157.4, 151.5, 148.5, 128.1, 127.5, 126.9, 116.2, 111.1 (q, J = 290 Hz, TFA CF_3), 55.0, 44.5, 44.0, 36.8, 35.3, 29.5, 25.0, 19.3 ppm. HRMS calcd. (M + H, $C_{26}H_{39}N_4S$) 439.3; found 439.6.

3-(1-Amino-2-methylpropylamino)-7-(Boc-Ethylamino)phenothiazin-5-ium (2TFA Salt) (16): Using Procedure A, with the addition of activated type 3A molecular sieves, the major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine TFA salt **16** (58 mg, 30%) as a bluish black solid, t_R = 10.1 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 4.8 min, 98.5%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 619 (13200) nm. ^1H NMR (400 MHz, CD_3OD): δ = 7.0–6.8 (m, 6 H), 3.87 (m, 1 H), 3.52 (m, 2 H), 3.43–3.41 (m, 2 H), 3.20–3.15 (m, 4 H), 2.56 (m, 5 H), 1.43–1.29 (m, 15 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 156.8, 155.5, 151.3, 143.6, 126.4, 126.0, 125.9, 117.1, 111.1 (q, J = 290 Hz, TFA CF_3), 79.7, 65.4, 57.1, 46.7, 44.1, 28.9, 28.4 ppm. HRMS calcd. (M + H, $C_{23}H_{33}N_5O_2S$) 442.2; found 442.5.

3-(3-Amino-2-methylbutan-2-ylamino)-7-(Boc-Ethylamino)phenothiazin-5-ium (2TFA Salt) (17): Using Procedure A, with the addition of activated type 3A molecular sieves, the major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine TFA salt **17** (72 mg, 36%) as a bluish black solid, t_R = 11.9 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 5.7 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 631 (13400) nm. ^1H NMR (400 MHz, CD_3OD): δ = 7.4–7.0 (m, 6 H), 3.89 (m, 1 H), 3.52 (m, 2 H), 3.20–3.15 (m, 4 H), 2.56 (m, 5 H), 1.43–1.29 (m, 21 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 157.2, 155.3, 149.9, 143.7, 128.1, 127.7, 127.5, 116.8, 111.1 (q, J = 290 Hz, TFA CF_3), 78.8, 69.6, 67.5, 46.1, 43.4, 28.9, 23.9, 22.7 ppm. HRMS calcd. ([M – Boc] + H, $C_{25}H_{37}N_5O_2S$) 370.7; found 370.6.

3-(2-Amino-3-methylpentan-2-ylamino)-7-(Boc-ethylamino)phenothiazin-5-ium (2TFA Salt) (18): Using Procedure A, the major fraction containing the product was collected and lyophilized to yield the corresponding phenothiazine TFA salt **18** (42 mg, 21%) as a bluish black solid, t_R = 12.9 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 6.0 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 625 (14230) nm. ^1H NMR (400 MHz, CD_3OD): δ = 7.3–7.7 (m, 6 H), 3.90 (m, 1 H), 3.87 (m, 1 H), 3.52–3.48 (m, 2 H), 3.39–3.36 (m, 2 H), 3.28–3.15 (m, 3 H), 2.58 (m, 6 H), 1.43–1.29 (m, 13 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.6 (q, J = 36 Hz, TFA C=O), 157.8, 155.6, 151.7, 144.1, 128.2, 127.9, 126.6, 118.1, 111.1 (q, J = 290 Hz, TFA CF_3), 79.6, 67.4, 60.3, 46.6, 46.2, 28.9, 28.8, 24.3, 19.4, 13.0 ppm. HRMS calcd. (M + H, $C_{25}H_{37}N_5O_2S$) 470.3; found 469.5.

Ligand Syntheses

BocNH-[Orn($COCF_3$)]$_8$-CO_2H (20): Following the literature conditions described by Wender et al.[11] of octa-ornithine trifluoroacetamide (360 mg, 0.19 mmol), the desired product **20** was obtained as a white powder (340 mg, quantitative yield). ^1H NMR (400 MHz, [D_6]DMSO): δ = 8.71–8.80 (m, 3 H), 7.56–7.69 (m, 4 H), 4.27–4.09 (m, 7 H), 3.22 (br. m, 16 H), 1.75–1.50 (m, 32 H), 1.36 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, [D_6]DMSO): δ = 175.7, 174.9, 174.8, 174.7, 174.2, 173.7, 173.6, 173.5, 158.6 (q, J = 34 Hz), 157.8, 117.1 (q, J = 278 Hz), 81.3, 56.2, 55.6, 55.3, 55.0, 54.5, 54.0, 53.5, 53.1, 40.1 (m), 29.5–25.6 (m) ppm; ppm ES-MS (– ionization) calcd. ($C_{61}H_{83}F_{24}N_{16}O_{19}$ – H) 1798.6; found 1798.2, all data were agreeable with literature.[12].

BocNH-(Arg)$_8$-CO_2H (8TFA Salt) (21): Following the perguanidylation procedure described by Wender et al.[12] for the conversion of octa-ornithine trifluoroacetamide carboxylic acid **20** (340 mg,

0.19 mmol), the desired product **21** was obtained as a white powder (230 mg, 53%); RP-HPLC, t_R = 16.6 min, analytical RP-HPLC (gradient 5→95% water, 13 min), t_R = 4.4 min, 99+% ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.50–8.33 (m, 3 H), 4.25–4.12 (m, 7 H), 3.90 (t, J = 7.0 Hz, 1 H), 3.10–3.02 (m, 16 H), 1.85–1.42 (m, 32 H), 1.27 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, [D$_6$]DMSO): δ = 169.8, 168.1, 168.0, 167.9, 167.8, 157.6 (q, J = 36 Hz, TFA C=O), 152.2, 151.4, 111.1 (q, J = 290 Hz, TFA CF_3), 76.3, 49.3, 48.1, 48.0, 47.2, 35.2, 23.1, 22.4, 22.3, 19.2 ppm. ES-MS (– ionization) calcd. ($C_{53}H_{106}N_{32}O_{11}$ – H) 1365.9; found 1366.3, all data agreeable with literature.[12].

Dye-Conjugate Syntheses: Model System

General Procedure C for the Synthesis of Dye-Conjugates: 1-Hydroxybenzotriazole (82 mg, 0.6 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.1 equiv.) were added consecutively in one portion to a stirred solution of the Boc-protected amino acid (1 equiv.) and 4-methylmorpholine (1 equiv.) in anhydrous dichloromethane (0.5 M) at 0 °C under nitrogen. The mixture was stirred at 0 °C for a further 40 min before a precooled solution of the dye amine (2 equiv.) and 4-methylmorpholine (2 equiv.) in anhydrous dichloromethane (1 M) at 0 °C was added to it dropwise. The mixture was stirred at 0 °C for 4 h and then warmed to room temperature over 15 h. The reaction mixture was quenched with H_2O: 0.1% TFA and MeCN: 0.1% TFA and concentrated in vacuo. The purification of the phenothiazine–amino acid dye conjugates was achieved by RP HPLC (eluent: H_2O/MeCN 0.1% TFA, gradient: 90% – 5%). The product-containing fractions were combined and lyophilized yielding the conjugates as dark, hygroscopic powders.

Boc-[Orn($COCF_3$)(ethyl)(methyl)amino]-7-(dimethylamino)phenothiazin-5-ium (TFA Salt) (22): The residue obtained from following Procedure C with Azure B amine derivative **8** (40 mg, 0.07 mmol), was purified and gave the conjugate **22** (46 mg, 88%) as a bluish black solid, RP-HPLC t_R = 6.9 min,(gradient 0→40% water, 20 min), analytical, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 616 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 9.39 (t, J = 3.4 Hz, 1 H), 7.90–7.50 (m, 6 H), 6.8 (br.s, 1 H), 3.88–3.85 (m, 1 H), 3.31 (m, 4 H), 3.19–3.15 (m, 2 H), 2.92 (s, 3 H), 2.80–2.65 (s, 6 H), 1.69–1.51 (m, 4 H), 1.39 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.5 (q, J = 36 Hz, TFA C=O), 156.8, 156.4, 156.2 (q, J = 35 Hz), 155.7, 145.2, 144.7, 127.9, 127.5, 126.3, 119.2, 116.0 (q, J = 286 Hz), 111.1 (q, J = 290 Hz, TFA CF_3), 78.1, 53.2, 43.5, 42.4, 38.9, 37.3, 35.9, 28.2, 25.2 ppm. ES-MS (+ ionization) calcd. ($C_{29}H_{38}N_6O_4S$ + H) 623.3; found 623.8.

Boc-arg(ethylmethylamino)-7-(dimethylamino)phenothiazin-5-ium (2TFA salt) (23): Following the perguanidylation conditions described in the procedure by Wender et al.[12] for the conversion of mono-ornithine trifluoroacetamide phenothiazine conjugate **22** was readily converted into the conjugate **23** (20 mg, 46%) and was isolated as a bluish black solid, t_R = 11.4 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 3.9 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 623 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 8.89 (t, J = 3.8 Hz, 1 H), 7.55 (m, 2 H), 6.53 (m, 2 H), 6.48 (m, 2 H), 3.73 (m, 1 H), 3.41 (m, 2 H), 3.29 (m, 2 H), 3.13 (m, 2 H), 2.91 (s, 3 H), 2.73 (s, 6 H), 1.65 (m, 4 H), 1.40 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 157.8, 157.6 (q, J = 36 Hz, TFA C=O), 156.5, 156.2, 155.9, 145.4, 144.5, 128.1, 127.7, 126.5, 119.5, 111.2 (q, J = 290 Hz, TFA CF_3), 78.3, 53.4, 44.1, 43.4, 38.9, 36.7, 36.2, 28.7, 25.6 ppm. ES-MS (+ ionization) calcd. ($C_{28}H_{42}N_8O_3S$ + H) 569.3; found 569.0.

Boc-Pro(ethylamino)-7-(dimethylamino)phenothiazin-5-ium (TFA Salt) (24): The residue obtained from following Procedure C with phenothiazine amine **10B** (30 mg, 0.06 mmol) was purified and gave the conjugate **24** (30 mg, 83%) as a bluish black solid, t_R = 8.0 min, analytical RP-HPLC (gradient 0→40% water, 20 min), 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 636 (10600) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.90–7.50 (m, 6 H), 6.8 (br.s, 1 H), 4.22 (m, 1 H), 3.40–3.30 (m, 2 H), 3.31 (m, 2 H), 2.92 (m, 2 H), 2.80–2.65 (m, 6 H), 1.95–1.70 (m, 2 H), 1.64–1.54 (m, 2 H), 1.40 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, $CDCl_3$): δ = 157.9, 157.6 (q, J = 36 Hz, TFA C=O), 156.4, 156.3, 149.5, 143.1, 127.5, 127.2, 126.8, 123.9, 115.4, 111.1 (q, J = 290 Hz, TFA CF_3), 79.8, 63.1, 48.2, 44.5, 44.3, 37.7, 29.3, 28.6, 22.7 ppm. ES-MS (+ ionization) calcd. ($C_{26}H_{34}N_5O_3S$ + H) 496.2; found 497.2.

Dye-Octamer Conjugate Syntheses

Boc-Octa-[Orn($COCF_3$)]$_8$-amino-7-(dibutylylamino)phenothiazin-5-ium Iodide (25): The residue obtained from following Procedure C with phenothiazine amine **14B** (40 mg, 0.07 mmol), was purified by routine column chromatography on silica gel, eluting with pentane/ethyl acetate (3: 7) and the conjugate **25** (110 mg, 70%) was isolated as a bluish black solid, analytical RP-HPLC (gradient 0→40% water, 20 min), t_R = 13.6 min, 98.99%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 583 (10900) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.72 (m, 3 H), 7.62 (m, 3 H), 4.24 (m, 6 H), 3.88 (m, 2 H), 3.64 (m, 1 H), 1.86–1.64 (m, 24 H), 1.51–1.32 (m, 30 H), 1.02–0.88 (m, 25 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 175.9, 174.7, 174.5, 174.3, 173.9, 173.7, 173.5, 158.4 (q, J = 34 Hz), 158.1, 157.4 (q, J = 35 Hz, TFA C=O), 157.2, 156.5, 151.7, 148.5, 129.5, 128.2, 127.9, 117.3 (q, J = 278 Hz), 115.5, 111.1 (q, J = 290 Hz, TFA CF_3), 82.4, 56.7, 55.5, 55.3, 54.9, 54.3, 54.0, 53.8, 53.5, 53.0, 46.2, 44.6, 41.9 (m), 36.7, 29.0–25.5 (m), 22.5, 19.8 ppm. ES-MS (+ ionization) calcd. ($C_{83}H_{111}F_{24}N_{20}O_{18}S$ + H) 2163.8; found 2164.5.

Boc-Octa-arg-7-(dimethylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (26): Using Procedure C with (30 mg, 0.06 mmol) phenothiazine amine **10B**, the conjugate **26** (35 mg, 22%) was isolated as a bluish black solid, t_R = 16.2 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 6.0 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 623 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 8.20 (m, 2 H), 7.6–7.4 (m, 3 H), 3.70 (m, 6 H), 3.10–2.98 (m, 32 H), 1.89–1.38 (m, 30 H), 1.40 (s, 9 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 167.9, 167.6, 167.4, 167.3, 157.5 (q, J = 36 Hz, TFA C=O), 156.9, 156.5, 152.7, 152.0, 150.0, 141.9, 127.0, 126.8, 126.7, 124.0, 116.4, 111.0 (q, J = 290 Hz, TFA CF_3), 76.5, 49.5, 48.4, 48.1, 47.5, 44.0, 43.9, 40.0, 35.8, 24.5, 23.6, 22.5, 19.9 ppm. ES-MS (+ ionization) calcd. ($C_{69}H_{131}N_{36}O_{10}S$ – H) 1646.0; found 1646.6.

Boc-Octa-arg-7-(diethylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (27): Using Procedure C with (50 mg, 0.09 mmol) phenothiazine amine **11B**, the conjugate **27** (48 mg, 20%) was isolated as a bluish black solid, t_R = 17.7 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 7.1 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 646 (12700) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.99 (s, 5 H), 7.90 (d, J = 8.4 Hz, 3 H), 7.75 (d, J = 8.4 Hz, 3 H), 7.56 (m, 5 H), 7.07 (s, 1 H), 3.75 (m, 6 H), 3.23 (m, 8 H), 3.0 (m, 18 H), 2.23–1.70 (m, 13 H), 1.40 (m, 43 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD):δ = 169.5, 169.0, 168.5, 168.2, 157.5 (q, J = 36 Hz, TFA C=O), 156.7, 156.0, 153.0, 151.5, 148.5, 144.5, 129.8, 128.0, 127.0, 115.6, 111.1 (q, J = 290 Hz, TFA CF_3), 77.0, 49.5, 48.2, 47.6, 47.4, 45.6, 44.1, 43.8, 35.6, 23.9, 23.0, 22.5, 21.8, 19.7 ppm. ES-MS (– ionization) calcd. ($C_{71}H_{135}N_{36}O_{10}S$ – H) 1675.0; found 1675.3.

Boc-Octa-arg-7-(diisopropylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (28): Using Procedure C with (50 mg, 0.09 mmol) phenothiazine amine **12B**, the conjugate **28** (48 mg, 20%) was iso-

lated as a bluish black solid, t_R = 18.3 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 7.89 min, 99+%; UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 623 (15688) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.99 (s, 4 H), 7.89 (d, J = 8.0 Hz, 2 H), 7.76 (d, J = 8.0 Hz, 3 H), 7.57 (m, 5 H), 7.15–6.85 (m, 3 H), 3.76 (m, 6 H), 3.10 (m, 7 H), 2.99 (s, 9 H), 2.86 (s, 9 H), 1.92–1.50 (m, 7 H), 1.43 (s, 38 H), 0.9 (m, 4 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 168.1, 168.0, 167.9, 167.8, 157.6 (q, J = 36 Hz, TFA C=O), 156.5, 154.7, 152.2, 151.4, 148.5, 143.2, 127.6, 127.3, 126.9, 115.8, 111.1 (q, J = 290 Hz, TFA CF_3), 76.3, 49.3, 48.1, 48.0, 47.2, 47.1, 44.2, 43.5, 35.2, 25.6, 23.1, 22.4, 22.3, 19.2 ppm. ES-MS (– ionization) calcd. ($C_{73}H_{139}N_{36}O_{10}S$ – H) 1703.1; found 1703.9.

Boc-Octa-arg-7-(diisobutylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (29): Using Procedure C with (60 mg, 0.1 mmol) phenothiazine amine **13B**, the conjugate **29** (68 mg, 25%) was isolated as a bluish black solid, t_R = 20.4 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 9.02 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 644 (14000) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 8.2 (m, 3 H), 7.98 (d, J = 8.0 Hz, 2 H), 7.90 (d, J = 8.0 Hz, 1 H), 7.54 (m, 3 H), 4.5–3.9 (m, 6 H), 3.74–3.67 (m, 6 H), 3.34–3.20 (m, 15 H), 3.0 (m, 10 H), 2.86 (m, 4 H), 1.95–1.71 (m, 24 H), 1.37 (s, 17 H), 0.98 (m, 8 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 168.3, 168.1, 168.0, 167.8, 157.6 (q, J = 36 Hz, TFA C=O), 156.6, 156.3, 152.8, 150.9, 148.7, 144.0, 128.2, 127.9, 127.5, 116.0, 111.0 (q, J = 290 Hz, TFA CF_3), 77.9, 58.7, 49.5, 48.5, 48.2, 47.6, 44.5, 43.0, 34.9, 29.7, 23.5, 23.0, 22.8, 22.0, 19.8 ppm. ES-MS (– ionization) calcd. ($C_{75}H_{143}N_{36}O_{10}S$ – Boc) 1631.1; found 1631.5.

Boc-Octa-arg-7-(dibutylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (30): Using Procedure C with (40 mg, 0.07 mmol) phenothiazine amine **14B**, the conjugate **30** (51 mg, 27%) was isolated as a bluish black solid, t_R = 19.8 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 8.9 min, 99+%. λ_{max} (CH_3CN)/nm 650 (ε/dm^3 mol^{-1} cm^{-1} 11200). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.94 (d, J = 8.5 Hz, 1 H), 7.87 (s, 1 H), 7.72 (d, J = 8.5 Hz, 3 H), 7.51 (t, J = 7.0 Hz, 2 H), 7.39 (d, J = 7 Hz, 3 H), 7.17 (s, 1 H), 5.41 (br. m, 4 H), 3.10 (m, 25 H), 2.88 (s, 9 H), 1.62–1.16 (m, 44 H), 0.88 (m, 3 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 168.0, 167.8, 167.5, 167.3, 157.4 (q, J = 35 Hz, TFA C=O), 157.0, 156.5, 152.0, 151.5, 151.0, 146.8, 129.5, 128.8, 127.9, 115.4, 111.0 (q, J = 290 Hz, TFA CF_3), 76.5, 53.0, 49.0, 48.0, 47.5, 47.3, 45.8, 43.7, 35.5, 34.1, 23.4, 23.0, 22.5, 22.0, 19.7, 19.5 ppm. ES-MS (– ionization) calcd. ($C_{75}H_{143}N_{36}O_{10}S$ – H) 1730.1; found 1730.7.

Boc-Octa-arg-7-(dihexylamino)-(3-ethylamino)phenothiazin-5-ium (9TFA Salt) (31): Using Procedure C with (60 mg, 0.09 mmol) phenothiazine amine **15B**, the conjugate **31** (28 mg, 11%) was isolated as a bluish black solid, t_R = 20.9 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 10.03 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 642 (12900) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.98 (s, 4 H), 7.93 (d, J = 8.4 Hz, 2 H), 7.76 (d, J = 8.4 Hz, 3 H), 7.57 (m, 5 H), 3.72 (m, 4 H), 3.25 (m, 5 H), 3.0 (m, 16 H), 2.85 (s, 13 H), 1.72–1.66 (m, 9 H), 1.4 (m, 40 H), 0.93–0.88 (m, 6 H) ppm. ^{13}C NMR (90.5 MHz,CD_3OD): δ = 168.2, 167.8, 167.8, 167.5, 157.6 (q, J = 36 Hz, TFA C=O), 157.5, 156.5, 152.0, 151.8, 151.5, 149.0, 128.0, 127.9, 127.5, 115.7, 111.0 (q, J = 290 Hz, TFA CF_3), 76.4, 54.9, 49.5, 48.0, 47.5, 47.3, 45.0, 44.7, 37.2, 36.0, 35.4, 29.6, 25.5, 23.0, 22.5, 22.3, 19.6, 19.3 ppm. ES-MS (+ ionization) calcd. ($C_{79}H_{151}N_{36}O_{10}S$ + H) 1788.1; found 1788.6.

3-(2-Amino-2-methylpropylamino)-7-(ethylamino)(Boc-Octa-arg)-phenothiazin-5-ium (9TFA Salt) (32): Using Procedure C with (100 mg, 0.15 mmol) phenothiazine amine **16**, the conjugate **32** (75 mg, 18%) was isolated as a bluish black solid, t_R = 18.6 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 7.6 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 630 (14300) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.98 (m, 2 H), 7.89 (d, J = 8.4 Hz, 5 H), 7.75 (d, J = 8.4 Hz, 4 H), 7.57–7.47 (br. m, 9 H), 6.97 (br. m, 5 H), 3.75 (m, 6 H), 3.25 (m, 9 H), 3.02 (m, 3 H), 2.97 (m, 1 H), 1.69–1.43 (m, 10 H), 1.38 (m, 44 H), 0.94 (m, 2 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 168.0, 167.9, 167.7, 167.5, 157.6 (q, J = 36 Hz, TFA C=O), 156.8, 156.2, 152.5, 151.5, 151.2, 144.1, 127.5, 127.0, 126.8, 117.3, 111.0 (q, J = 290 Hz, TFA CF_3), 80.0, 77.0, 65.5, 57.5, 49.7, 48.5, 48.3, 47.5, 47.0, 44.0, 35.8, 29.5, 29.0, 23.5, 23.0, 22.8, 19.7 ppm. ES-MS (+ ionization) calcd. ($C_{76}H_{144}N_{37}O_{12}S$ + H) 1791.1; found 1790.2.

3-(2-Amino-2-methylbutan-2-ylamino)-7-(ethylamino)(Boc-Octa-arg)phenothiazin-5-ium (9TFA Salt) (33): Using Procedure C with (100 mg, 0.14 mmol) phenothiazine amine **17**, the conjugate **33** (58 mg, 15%) was isolated as a bluish black solid, t_R = 20.1 min, analytical RP-HPLC (gradient 5→95% water, 15 min), t_R = 9.51 min, 99+%. UV (CH_3CN): λ_{max} (ε/dm^3 mol^{-1} cm^{-1}) = 632 (14900) nm. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.98 (m, 3 H), 7.88 (d, J = 8.4 Hz, 5 H), 7.75 (d, J = 8.4 Hz, 4 H), 7.57–7.47 (br. m, 10 H), 6.97 (br. m, 3 H), 3.75 (m, 9 H), 3.25 (m, 10 H), 3.02 (m, 8 H), 2.01–1.43 (m, 9 H), 1.38 (m, 55 H) ppm. ^{13}C NMR (90.5 MHz, CD_3OD): δ = 168.9, 168.5, 168.0, 167.8, 157.6 (q, J = 36 Hz, TFA C=O), 157.5, 156.5, 156.0, 153.5, 151.0, 150.4, 143.5, 128.0, 127.9, 127.5, 116.4, 111.1 (q, J = 290 Hz, TFA CF_3), 79.2, 77.0, 69.5, 67.8, 49.0, 48.7, 48.5, 47.9, 46.5, 43.5, 36.2, 29.0, 23.4, 23.2, 22.9, 22.5, 22.3, 19.7 ppm. ES-MS (+ ionization) calcd. ($C_{78}H_{148}N_{37}O_{12}S$ + H) 1819.1; found 1819.4.

Supporting Information (see also the footnote on the first page of this article): Experimental details; ^{1}H and ^{13}C NMR, HRMS, ES-MS and UV spectra for compounds **24** and **26**–**33**. UV, ^{1}H and ^{13}C data tables for dyes 8 and 10–18 and conjugates **24** and **26**–**33**.

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