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The first "ready-to-use" benzene-based heterotrifunctional cross-linker for multiple bioconjugation†

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Guillaume Viault,[a] Sébastien Dautrey,[a] Nicolas Maindron,[a] Julie Hardouin,[b] Pierre-Yves Renard*[a] and Anthony Romieu*[a]

The synthesis and applications of the first water-soluble benzene derivative bearing a set of three different and orthogonal bioconjugatable groups (aminooxy, azido and thiol) are described. The combined use of a 5-amino isophthalic acid scaffold and unusual acid-labile protecting groups for temporarily masking aminooxy and thiol moieties has enabled the development of a highly convergent approach towards the synthesis of such a trivalent bioconjugation platform in good yields. The potential utility of this "ready-to-use" cross-linking reagent for creating complex and fragile tri-component (bio) molecular systems was illustrated through (1) the rapid preparation of a three-colour FRET cascade with valuable spectral properties and (2) the luminescent/fluorescent labelling of peptides and peptide–oligonucleotide conjugates. Thus, such (bio)molecular assemblies were readily obtained *via* a three-step process or in a "one-pot" manner, both involving oxime ligation, thiol-alkylation (S_N2 or Michael addition) and copper-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reactions.

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Introduction

Heteromultifunctional molecular scaffolds equipped with three (or more) (bio)orthogonal reactive handles are now regarded as versatile and unique tools to make easier access to a myriad of chemically engineered bioconjugates,[1] through synthetic methods involving a sequential combination of chemoselective ligations.[2,3] Indeed, there is a growing need for such multicomponent biomolecular systems with uniquely combined properties of the individual components, especially for the development of innovative and valuable diagnostic and/or therapeutic tools (*e.g.*, multimodality molecular imaging probes, "smart" drug delivery systems, *etc.*). The vast majority of heterotrifunctional cross-linking reagents (also named by us "tripods") commercially available and/or reported in the literature are based on amino acid or peptidyl scaffolds that contain either a set of two different functional groups and a biotin affinity tag (*e.g.*, Sulfo-SBED, TRICEPS),[4,5] or three different functional groups allowing sequential bioconjugations through biocompatible and/or bioorthogonal "click" reactions.[3,6,7]

It is also worth mentioning the recent work of the Santoyo-Gonzalez group related to the facile synthesis of alkyne-vinyl sulfone derivatised tags (AVST) from a PEGylated bis-vinyl sulfone.[8] Indeed, these non-peptidyl trivalent cross-linkers have enabled the convenient and efficient dual labelling of proteins (*e.g.*, glycoprotein horseradish peroxidase (HRP)) with a biotin and a fluorescent dye, through effective reactions belonging to the "click chemistry" repertoire (*i.e.*, CuAAC and Michael-type addition using a vinyl sulfone as acceptor).

The preparation of such multifunctional architectures relies almost exclusively on classical stepwise peptide synthesis in solution, and so, it is not always easy to rapidly expand their structural diversity (especially for fine-tuning of both physiochemical properties and bioconjugation ability) as well as to greatly simplify their synthetic access. Thus, there is an urgent need for "tripods" derived from structurally simpler molecular scaffolds, and easily accessible using flexible and highly convergent synthetic routes. In that context, our recent research efforts have been focused on design and evaluation of the bioconjugation ability of a non-peptidic cross-linker based on a benzenic core decorated with three different conjugation handles: an aminooxy functional group for oxime ligation, a thiol for reactions with maleimide or α-haloacetyl derivatives, and an azido for copper-mediated azide-alkyne 1,3-dipolar

[a]*Normandie Univ, COBRA, UMR 6014 & FR 3038; UNIV Rouen; INSA Rouen; CNRS, 1 Rue Tesnières, 76821 Mont St Aignan Cedex, France.*
E-mail: pierre-yves.renard@univ-rouen.fr, anthony.romieu@univ-rouen.fr; http://ircof.crihan.fr; Fax: +33 (0)2 35 52 29 71; Tel: +33 (0)2 35 52 24 76 (or 24 27)
[b]*Laboratory PBS UMR 6270, Bât. Chimie, 76821 Mont St Aignan Cedex, France*

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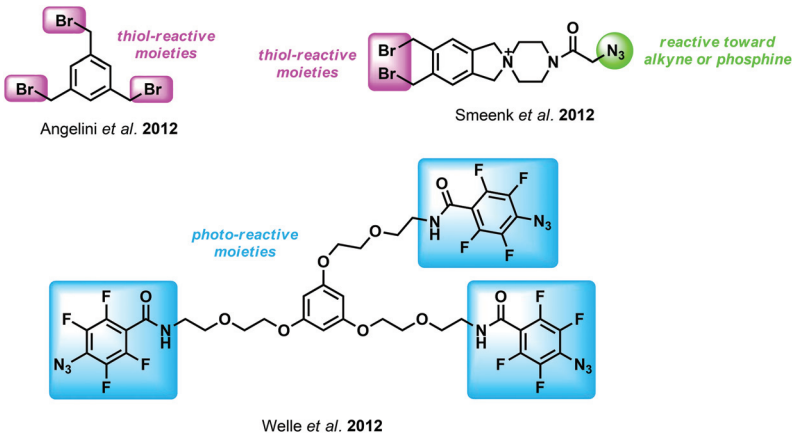


Fig. 1 Examples of trivalent benzene derivatives bearing two or three identical (photo)reactive groups, published in 2012.

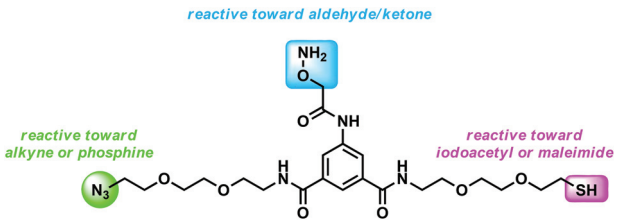


Fig. 2 Structure of heterotrifunctional benzene-based cross-linker **1**.

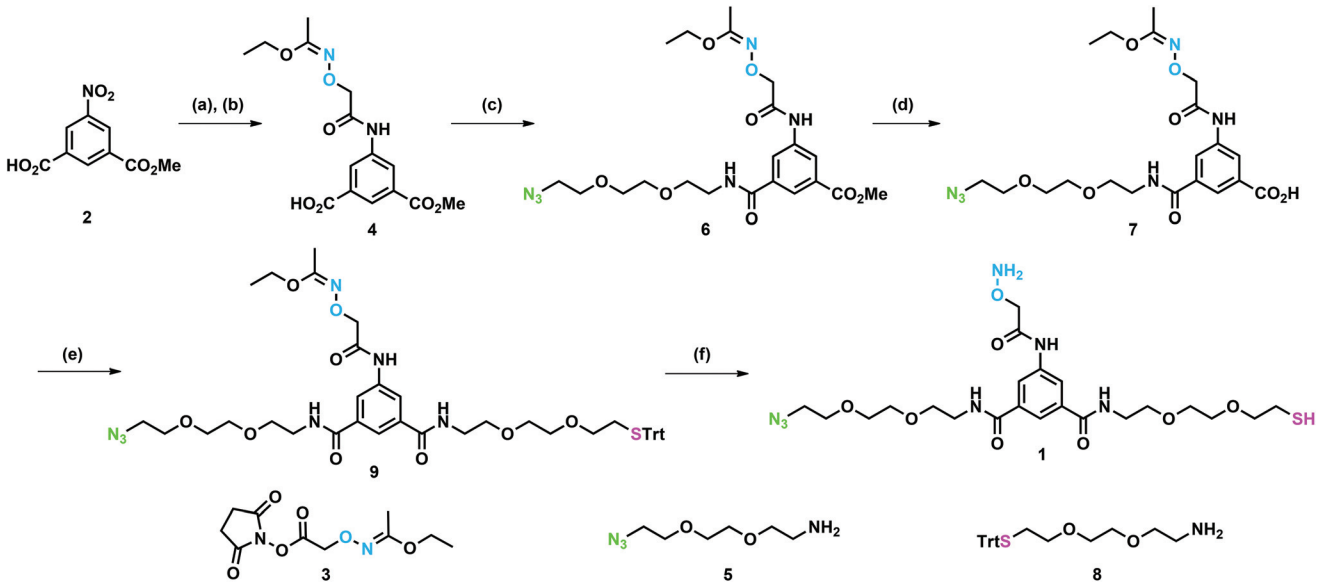
cycloaddition (CuAAC) or Staudinger ligation, and pseudo-PEG moieties as neutral water-solubilising moieties.[9] To the best of our knowledge, only trivalent benzene derivatives bearing two or three identical (photo)reactive groups have already been reported in the literature, especially for applications in peptide/protein bioconjugation (see Fig. 1 for selected examples).[10]

In this article, we report the rapid and straightforward synthesis of the first benzene-based "tripod" **1** (Fig. 2) and its application for the preparation of sophisticated three-component conjugates including a three-colour FRET cascade based on cyanine dyes and luminescent peptide–oligonucleotide conjugates (POCs), through sequential mild and biocompatible reactions performed under mild aq. conditions fully compatible with the moderate stability of selected fluorescent labels and biopolymers. Dual fluorescent labelling of peptides through a sequential one-pot derivatisation of **1** that avoids intermediate chromatographic purifications is also presented.

Results and discussion

Synthesis of heterotrifunctional benzene-based cross-linker 1

Compared to the convergent synthetic strategies previously developed to prepare heterotrifunctional peptide-based templates,[3,6,7] our basic idea is to dramatically simplify the orthogonal protection/deprotection strategy required to ensure the correct assembly of functionalised building blocks. To this end, (1) unusual protecting groups suitable for masking aminooxy and thiol moieties, both labile under the same acidic conditions were used, and (2) a cheap benzenic building block **2** whose two functional groups (*i.e.*, amino and carboxylic acid) can be easily unveiled through standard and easily implemented reactions (*i.e.*, hydrogenation and saponification) was chosen as starting material. Thus, a short six-step synthetic route was designed as depicted in Scheme 1. First, the nitro group of **2** was converted into primary amine by Pd/C cat. hydrogenation, and the resulting aniline was used as an anchoring point to graft the aminooxy-containing building block. Aminooxyacetic acid (Aoaa) *N*-protected with the acid-labile 1-ethoxyethylidene group (Eei) was preferred to the standard di-Boc-derivative because it has been shown recently that this oxime is more adequate for stepwise SPPS of Aoaa-containing peptides, especially in preventing the *N*-overacylation side-reactions frequently encountered during such syntheses.[11] Thus, acylation of the aniline derivative with the known NHS active ester **3**,[11] provided oxime derivative **4** in 55% overall yield for the two steps. Amidification of its carboxylic acid moiety with azido-PEG-amine spacer **5** (for its synthesis, see ESI†) has been readily achieved with BOP/DIEA, in dry CH_3CN to provide **6** in 94% yield. Thereafter, methyl ester **6** was saponificated by treatment with 1.0 M aq. LiOH in MeOH to give the targeted benzoic acid **7**. This acid was coupled to *S*-protected mercaptoamine linker **8** (for its synthesis, see ESI†) under the same conditions used for **5**, to afford fully protected heterotrifunctional cross-linker **9** in 91% yield. Finally, a single treatment of **9** with a mixture of TFA/TES/H_2O has led to heterotrifunctional benzenic derivative **1**. The best way to purify this polar compound is to make semi-preparative RP-HPLC which enables to recover **1** as a TFA salt and in a moderate not yet optimised yield (23%). Indeed, the use of RP-HPLC for purifications (followed by lyophilisation) and the small-scale chosen for the deprotection step induced significant loss of material whereas this reaction was found to be almost complete. But compared to the previously reported peptidyl analogue isolated in a very low yield (<5%),[6] the single deprotection step and the presence of a UV chromophore have made easier the chromatographic purification and helped to

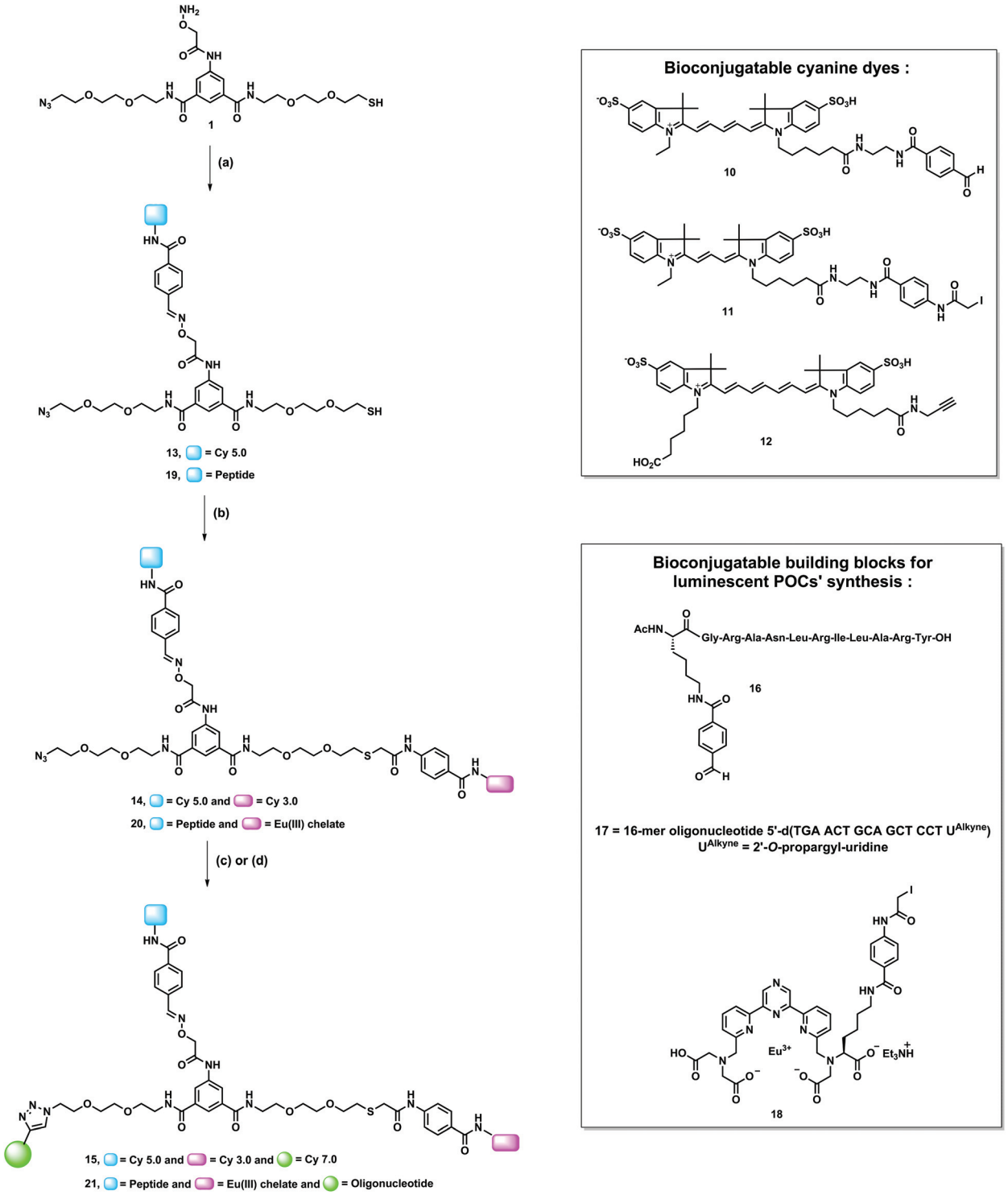


Scheme 1 Synthesis of benzene-based heterotrifunctional bioconjugate linker **1**. *Reagents and conditions*: (a) H_2(g), Pd/C, EtOH, rt, 3 h; (b) **3**, DIEA, CH_3CN, rt, 12 h, 55% for 2 steps; (c) **5**, BOP, DIEA, CH_3CN, rt, 12 h, 94%; (d) 1.0 M aq. LiOH, MeOH, rt, 5 h, 93%; (e) **8**, DIEA, CH_3CN, rt, 3 h, 91%; (f) TFA–TES–H_2O (95 : 2.5 : 2.5, v/v/v), rt, 3 h, 23% after RP-HPLC purification.

dramatically increase the isolated yield. The structure of **1** was unambiguously confirmed by detailed measurements, including ESI-MS and NMR analyses (see ESI†). We have observed that this free aminooxy derivative readily reacts with acetone commonly found in chemistry labs' atmosphere, leading to blocking of its third bioconjugatable group as an unreactive oxime. Consequently, this reagent was stored under an argon atmosphere, and at −20 °C to limit also both partial reduction of its azido group through a thiol-mediated redox process,[12] and oxidative dimerisation to disulfide. Indeed, under these storage conditions, such self-reactivity was highlighted by periodical HPLC analyses of **1** and about 30% degradation occurred within two months (see ESI†). In this context, it is generally better to perform deprotection of **9** only a few days prior to the use of **1** in bioconjugation.

Synthesis of fluorescent/luminescent (bio)molecular assemblies by sequential triple derivatisation of 1

To demonstrate the facile applicability of the novel bioconjugation reagent **1** toward the construction of three-component (bio)molecular systems under mild conditions, the preparation of a three-colour or triple FRET cascade was first considered (Scheme 2). Such constructs, in which an intermediate energy acceptor relays the energy gained from the donor to a lower-energy acceptor, offer the advantage that they yield information about the position of the three chromophores relative to each other, they extend the working range compared to conventional FRET systems, and the spectral shift between the excitation of the donor and the emission of the lower-energy acceptor is much more pronounced.[13] In addition, fewer labelled sample molecules are required to measure relative distances. Despite these obvious advantages, no general synthetic method for their preparation is now available. Recently, the first triple-colour labelling strategy for proteins, using a combination of site-specific labelling *via* an unnatural amino acid (*para*-acetylphenylalanine for oxime ligation) and statistical labelling of two cysteine residues (by Michael addition), has been reported,[14] but this approach is not entirely satisfactory due to the lack of three independent chemoselective fluorescent tagging reactions easily implemented to a wide range of bioconjugatable fluorophores. Consequently, most three-colour single-molecule FRET studies were performed on fluorescently-labelled nucleic acids (*i.e.*, DNA duplexes or DNA origami).[15] In this context, the sequential derivatisation of **1** with three different complementary fluorophores appears to be a very promising way and still an unexplored approach to achieve this ambitious goal. Sulfoindocyanine dyes Cy 3.0, Cy 5.0 and Cy 7.0 from GE Healthcare (CyDye™) were chosen for the practical implementation of this synthetic strategy.[16] The carboxylic acid function of these dyes was used to introduce the required conjugatable handle α-iodoacetyl (IAc), aldehyde and terminal alkyne respectively (for their synthesis, see ESI†). Despite the drawbacks related to the limited fluorescence quantum yield in aqueous solution and poor (photo)chemical stability of cyanine dye Cy 7.0,[17] we have chosen to use this fragile fluorescent label rather than more stable NIR fluorophores, especially to demonstrate that the use of cross-linker **1** enables the design of effective bioconjugation schemes under very mild aqueous conditions that maintain the structural integrity of each molecular partner involved in the final targeted assembly. Furthermore, the symmetrical dicarboxylic acid cyanine dye Cy 7.0 was used because the unsymmetrical analogue (bearing *N*-ethyl and *N*-carboxypentyl substituents) is prone to aggregation in aqueous environments.[18] As depicted in Scheme 2, sequential derivatisation of **1** was readily and easily achieved under mild aqueous conditions and in the



Scheme 2 Bioconjugation schemes involving **1**. *Reagents and conditions*: (a) 0.1 M aq. NaOAc buffer pH 4.2, Cy 5.0–aldehyde **10**, or peptide–aldehyde **16**, rt, 4 h, 32% and 45%; (b) CH_3CN and 0.1 M aq. $NaHCO_3$ buffer pH 8.5 (1 : 3, v/v), Cy 3.0–IAB **11** or Eu(III) chelate–IAB **18**, rt, 3 h, 36% and 30%; (c) H_2O, 10 mM aq. $CuSO_4$, 10 mM aq. sodium ascorbate, Cy 7.0–alkyne **12**, rt, 2 h, 47%; (d) H_2O, DMSO 10 mM aq. $CuSO_4$, 10 mM aq. sodium ascorbate, 10 mM TBTA, 50 mM aq. DIEA, ODN–alkyne **17**, rt, 24 h, 66%.

following order: (1) oxime ligation with Cy 5.0–aldehyde **10**, (2) S_N2 thio-alkylation with the α-IAc derivative of Cy 3.0 **11** and (3) CuAAC reaction with Cy 7.0–alkyne **12**. To avoid the premature "capping" of an aminooxy moiety of **1** by prolonged exposure to "atmospheric" acetone (*vide supra*), it makes sense to perform oxime ligation prior to the other two reactions. Furthermore, as demonstrated by Galibert *et al.*, it is essential to achieve CuAAC after thiol alkylation to avoid copper-mediated

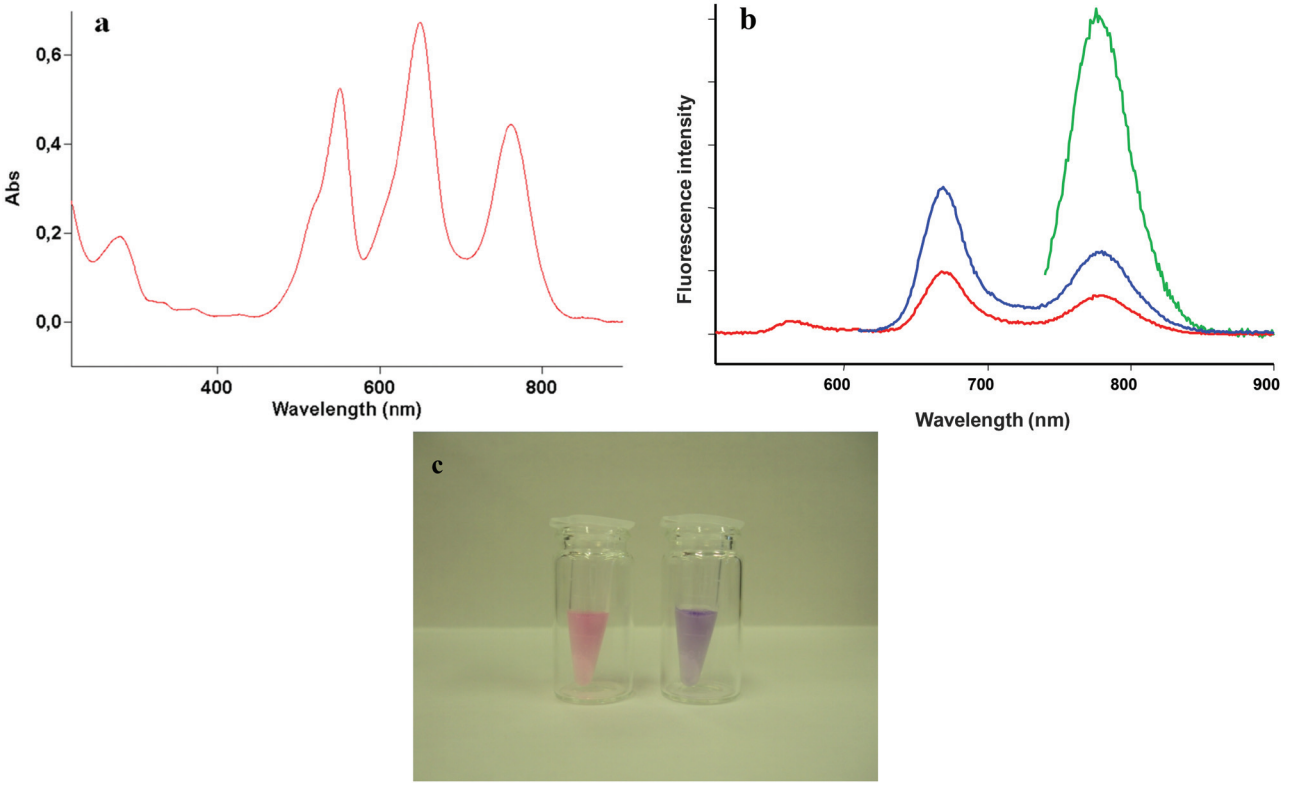


Fig. 3 (a) UV-vis absorption spectrum of **15** in PBS (concentration: 3.5 μM) at 25 °C; (b) normalised fluorescence emission spectra of **15** in PBS at 25 °C upon excitation at 500 nm (—), 600 nm (—) and 730 nm (—); (c) picture of PBS solutions (concentration: 5.0 μM) of Cy 3.0 dye (left) and FRET cascade **15** (right).

oxidation of thiopeptide scaffolds.[3] In addition to the final FRET cascade **15**, we have chosen to isolate the fluorescent derivatives **13** and **14** formed from their ligation, in a pure form to determine their spectral features under physiological conditions. Thus, each fluorescent "tripod" (*i.e.*, monolabelled, FRET pair and FRET cascade) was isolated by semi-preparative RP-HPLC and their structures confirmed by ESI-MS (see ESI†). In order to confirm the presence and integrity of the three cyanine units grafted to the same benzene template, the photophysical properties of **15** were determined in phosphate buffered saline (PBS, pH 7.5). As illustrated in Fig. 3a, **15** exhibits three characteristic absorption maxima at 550, 648 and 761 nm assigned to the three different cyanine labels. Thus, the resulting solution colour of **15** is dramatically different than that of the donor Cy 3.0 unit (Fig. 3c). Furthermore, the absorption ratios are in good agreement with the expected dye : tripod molar ratios equal to 1, and are further evidence for the high purity of this fluorescent three-component conjugate. Upon excitation at 500 nm (Cy 3.0 core), **15** exhibits strong red and NIR emission bands centered at 668 and 779 nm respectively, and a weak green emission band at 561 nm, indicating efficient energy transfer between Cy 3.0 and Cy 5.0 (Fig. 3b), this latter cyanine unit serving as an energy relay toward the final acceptor Cy 7.0. The energy transfer efficiency (E.T.E) was calculated based on the equation: E.T.E = {100 × [1 − (Φ_F (donor Cy 3.0 in the FRET cascade))/(Φ_F (free donor Cy 3.0))]%} and found to be equal to 95% (see ESI† for the determination of the corresponding quantum yield (Φ_F) values of Cy 3.0). Thus, a large pseudo-Stokes' shift (up to 229 nm) was observed, which is, to the best of our knowledge, one of the few examples of FRET-based systems emitting in the NIR region upon green excitation, and particularly valuable for various applications in the field of DNA analysis and molecular imaging.[19,20] Interestingly, the second carboxylic acid function of final acceptor Cy 7.0 remains free for further conjugation of this cassette to a biomolecule or a biological target.

To provide additional examples of sequential triple derivatisation of cross-linker **1**, we have decided to apply this bioconjugate assembly strategy to the preparation of luminescent peptide–oligonucleotide conjugates (POCs) (Scheme 2). Indeed, it is now well established that linking peptides to ODNs improves some of the desired properties of these DNA fragments to be used as therapeutic agents (cellular delivery, stability to exonucleases, improved binding to complementary sequences and greater rate of hybridization).[21] To gain relevant *in cellulo* or *in vivo* biodistribution data of such potential (antisense) drugs, one of the most practical solutions relies on the use of bis-conjugates of ODNs which combine a luminescent/fluorescent moiety with the grafted peptide sequences, the fluorophore serving as a probe to detect the transport of the POC.[22] The vast majority of that type of optical bioprobes are readily obtained by sequential derivatisation of a 3′,5′-bifunctionalised oligonucleotide (produced by solid-phase synthesis) with a peptide and a fluorescent reporter group, for instance

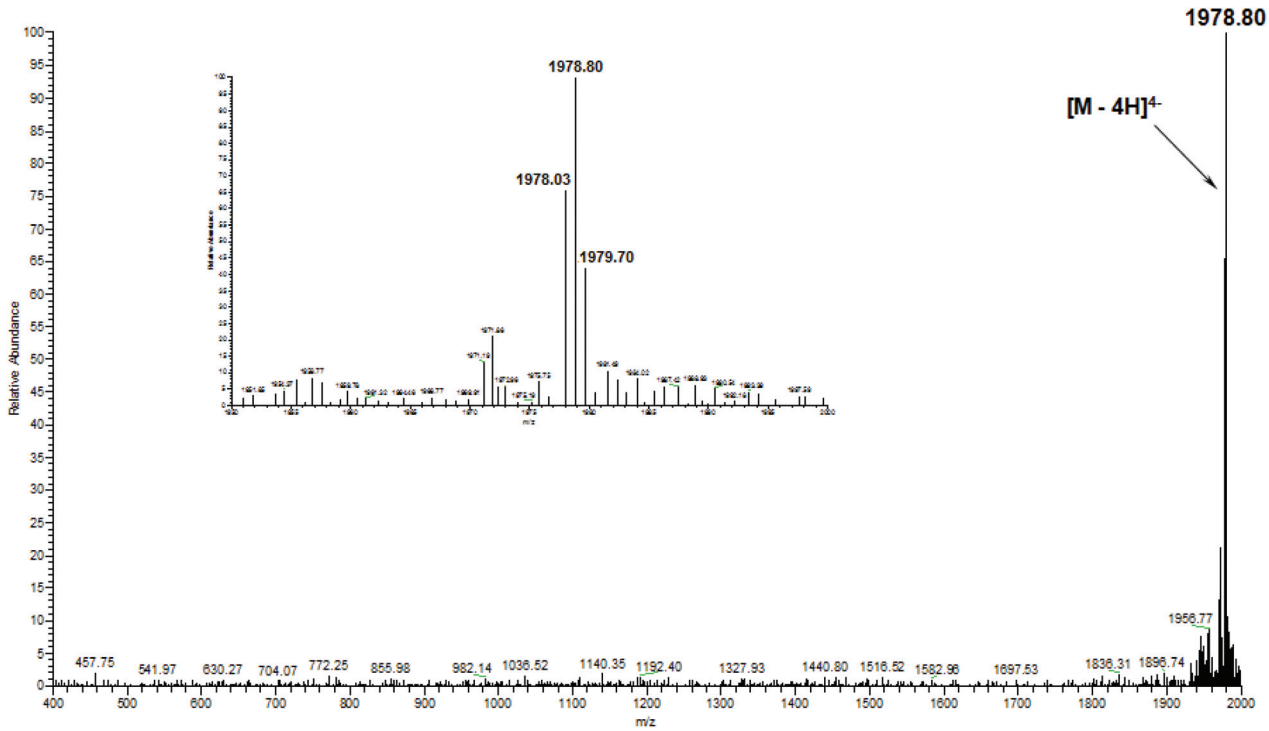
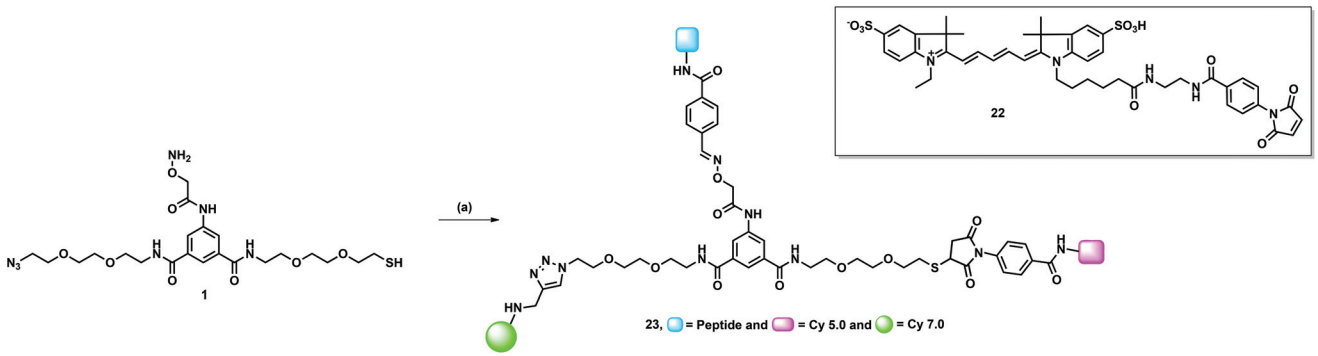


Fig. 4 ESI mass spectrum of luminescent peptide–ODN conjugate **21** recorded in the negative mode (calcd mass 7918.47, found: 7919.20). Under our ionisation conditions, only the −4 charge state was observed (inset: zoom).

through the sequential formation of two oxime bonds.[23] However, the success of this approach has required the use of an acid-labile protecting group to temporarily mask the second reactive functionality of ODN (trityl for the aminooxy moiety or benzaldehyde acetal for the diol used as an aldehyde-precursor). In order to shorten the synthetic routes of these useful bioconjugates, "tripod" **1** appears to be a successful solution to "cross-link" rapidly and effectively the three substrates involved. The dodecapeptide Ac-Lys-Gly-Arg-Ala-Asn-Leu-Arg-Ile-Leu-Ala-Arg-Tyr-OH whose *ε*-amino group was derivatised with the NHS ester of *para*-formylbenzoic acid aldehyde (peptide–aldehyde **16**) and a synthetic 16-mer oligonucleotide (ODN) 5′-d(TGA ACT GCA GCT CCT U) bearing the alkyne group as a nucleotide modification at the 2′-position of uridine (ODN–alkyne **17**) were chosen as representative biopolymers (for their synthesis, see ESI†). As a luminescent tag, an original thiol-reactive Eu(III) bispyridinylpyrazine-based chelate **18** was selected, especially for further time-resolved imaging of such POCs within living cells. This bioconjugatable lanthanide (III) complex was easily synthesised by acylation of the parent amino compound recently reported by us,[24] with the commercially available heterobifunctional cross-linker SulfoSuccinimidyl(4-Iodoacetyl)AminoBenzoate (*i.e.*, Sulfo-SIAB) in aqueous $NaHCO_3$ buffer (pH 8.5), and subsequently purified by semi-preparative RP-HPLC (see ESI†). Since of the three (bio)molecular partners involved in the targeted POC, **16** exhibits the highest chemical stability, oxime ligation was performed first.[25] Thereafter, S_N2 thio-alkylation was readily achieved with luminescent IAc derivative **18**. Finally, CuAAC reaction between the luminescent peptide conjugate and ODN–alkyne **17** was performed in H_2O–DMSO (1 : 1) with the catalytic system ($CuSO_4$–sodium ascorbate) and DIEA and tris-(benzyltriazolylmethyl)amine (TBTA), a well known stabilising ligand for copper(I) species.[26] The corresponding luminescent POC **21** was isolated by RP-HPLC under non-acidic conditions (to avoid premature decomplexation of lanthanide cations) in a good yield (66%) and its structure was unambiguously confirmed by ESI-MS (Fig. 4). The presence and integrity of a grafted Eu(III) chelate were also confirmed by detailed photophysical measurements (see ESI†). Interestingly, a relative quantum yield of 9.5% was found in aqueous buffer. This good value is similar to those previously reported for protein conjugates labeling with the same Eu(III) chelate.[24] This indicates that the benzene core of **1** does not negatively affect the luminescence properties of such grafted labels.

Synthesis of fluorescent bioconjugates by sequential one-pot derivatisation of 1

The great utility of the trifunctional scaffold **1** as a "ready-to-use" and user-friendly bioconjugation reagent was ultimately demonstrated through the one-pot sequential chemoselective ligations of peptide **16** and cyanine dyes Cy 7.0–alkyne **12** and Cy 5.0–maleimide **22** (Scheme 3). For this latter fluorescent marker, maleimide was preferred to α-iodoacetyl as the thiol-reactive moiety because the Michael addition (leading to its conjugation to the peptidyl architecture) proceeds rapidly and in good yields in neutral aqueous solutions whereas this is not the case for the S_N2 thio-alkylation involving the



Scheme 3 One-pot bioconjugation scheme involving **1**. *Reagents and conditions*: (a) 0.1 M aq. NaOAc buffer pH 4.2, peptide–aldehyde **16**, rt, 2 h, then Cy 5.0–maleimide **22**, H_2O, rt, 2 h and finally Cy 7.0–alkyne **12**, Cu(0) microsized powder, *t*BuOH, rt, 12 h, 28% overall yield.

corresponding α-IAc fluorescent derivative, which effectively works only in the pH range 8.0–8.5.[27,28] Indeed, for this one-pot approach, we have chosen to conduct the two last derivatisations (Michael addition and CuAAC) at neutral pH because it was not easy to accurately adjust the pH mixture as required by the triptych "oxime ligation (performed at pH 4.2), S_N2 thioalkylation (performed at pH 8.5) and CuAAC (performed at neutral pH)", especially when the reactions were conducted in small volumes and with coloured reagents such as cyanine dyes. Chemoselective ligations were carefully monitored by RP-HPLC analysis (Fig. 5). Oxime ligation between **1** and peptide–aldehyde **16** was found to be complete within 2 h (Fig. 5a). Then, Cy 5.0–maleimide **22** (for its synthesis see ESI†) was added to the reaction mixture. Thiol-mediated Michael addition was observed at rt providing within 2 h the Cy 5.0–labelled peptide (Fig. 5b). For this latter reaction, we have noticed the formation of side-products that result from hydrolysis of maleimide derivatives, which may make the use of this "tripod" system in some practical one-pot biomolecule labelling and cross-linking applications difficult. The implementation of an alternative bioconjugation reaction not involving thiols is probably the best way to solve this problem (*vide infra*, Conclusions section). Finally, CuAAC with Cy 7.0–alkyne **12** was carried out by adding a Cu(0) microsized powder and at rt overnight (Fig. 5c). The expected FRET cassette (Cy 5.0–Cy 7.0) labelled peptide **23** was isolated by RP-HPLC in a satisfying 28% overall yield. The presence and integrity of cyanine labels grafted to the peptide were confirmed by ESI mass spectrometry (Fig. 5e), UV-vis absorption and fluorescence analyses (see ESI†). In the present case, the isolated yield of the three-component bioconjugate is significantly higher than those of compounds **15** and **21** synthesised according to a three-step bioconjugation protocol involving chromatographic isolation of each intermediate (overall yield 5.5% and 9.0% respectively).

Conclusions and future work

In summary, we have described the easy and high-yielding functionalisation of a benzene core to get the first heterotrifunctional aromatic scaffold suitable for various challenging bioconjugation applications. We have demonstrated that the selected set of three reactive groups (aminooxy, azido and thiol) is particularly well-suited for rapid derivatisation/functionalisation of fragile and high-value added biopolymers, through biocompatible reactions easily performed on small scales, either in three steps or in a one-pot sequential manner. We think that this novel cross-linking reagent represents: (1) a significant innovation in the field of chemical biology especially to get highly sophisticated and fragile (bio)conjugates which are not accessible by derivatisation of commercially available bioconjugation reagents (mainly hetero- or homobifunctional cross-linkers), and (2) a valuable tool to make easier the preparation of biological composite nanomaterials through addressing challenges related to the controlled display of biomolecules on nanoparticles.[29] Thus, our current efforts are devoted to expanding the functional diversity of heterotrifunctional benzenic scaffolds for the implementation of new and promising "click" reactions such as tetrazine ligation[30] and related cycloadditions.[31]

Experimental section†

General

Column chromatography purifications were performed on Geduran® Si 60 silica gel (40–63 μm) from Merck. TLC was carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. The spots were visualised by illumination with a UV lamp (λ = 254 nm or 365 nm), by immersion in ninhydrin solution or potassium permanganate solution. Small scale syntheses (*i.e.*, bioconjugation experiments) were performed in standard single-use microtubes (0.5 or 1.7 mL). All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, CH_2Cl_2: distillation over P_2O_5, THF: distillation over Na°/benzophenone). Anhydrous DMF was obtained from Carlo Erba-SdS or Fisher Scientific. TEA was distilled from CaH_2 and stored over BaO. The HPLC-gradient grade acetonitrile (CH_3CN) was obtained from VWR. Buffers and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ cm). Triethylammonium

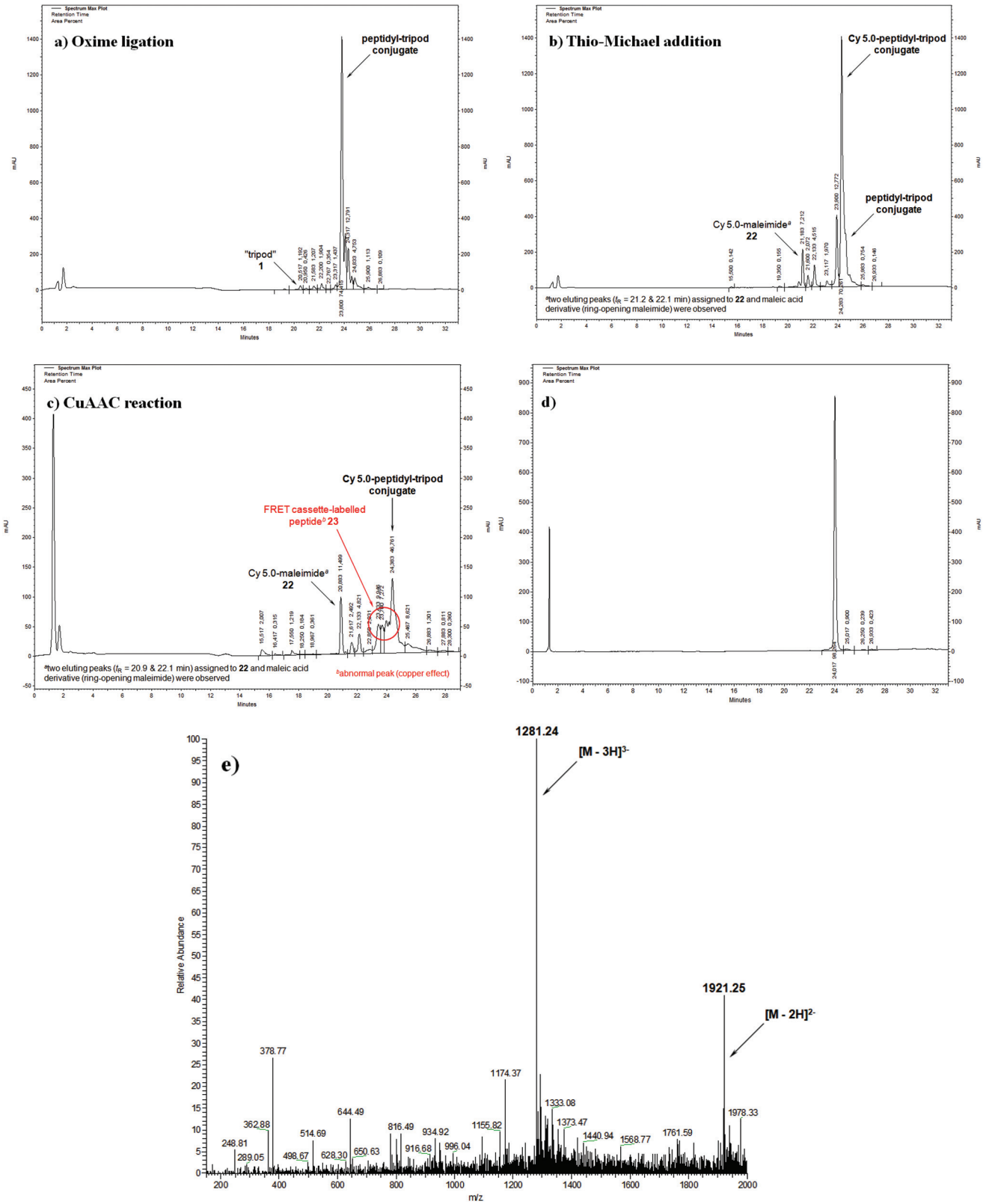


Fig. 5 (a–c) RP-HPLC elution profiles (system K) for the preparation of FRET cassette (Cy 5.0–Cy 7.0) labelled peptide **23** through a sequential one-pot approach; (d) RP-HPLC elution profile (system K) of purified **23**; (e) ESI mass spectrum of purified **23** recorded in the negative mode (calcd mass 3845.73). Under ionisation conditions, only the −2 and −3 charge states were observed.

acetate (TEAA, 2.0 M) and triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid or CO_2 gas.

Instruments and methods

The synthesis of dodecapeptide Ac-Lys-Gly-Arg-Ala-Asn-Leu-Arg-Ile-Leu-Ala-Arg-Tyr-OH was carried out on an Applied Biosystems 433A synthesizer using the standard Fmoc/*t*Bu chemistry[32] as previously described by us.[33] The synthesis of ODN–alkyne **17** was carried out on an Applied Biosystems 392 DNA/RNA synthesizer as previously described by us.[6] ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) and relative to tetramethylsilane from $CDCl_3$ (δ_H = 7.26, δ_C = 77.16) or D_2O (δ_H = 4.79).[34] Infrared (IR) spectra were recorded with a universal ATR sampling accessory on a Perkin Elmer FT-IR Spectrum 100 spectrometer. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-visible 2000 detector. Low-resolution mass spectra (LRMS) were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source. High-resolution mass spectra (HRMS) were recorded on an LTQ Orbitrap Elite (Thermo Scientific). UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer by using a rectangular quartz microcell (Hellma, 108.002-QS, light path: 10 mm, 500 μL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed with a Varian Cary Eclipse spectrophotometer with a fluorescence quartz ultra-microcell (Hellma, 105.251-QS, light path: 3 × 3 mm, 45 μL). Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (see Table S1,† excitation and emission filters: auto, excitation and emission slit = 5 nm) in PBS. Relative quantum yields were measured in PBS at 25 °C by a relative method using a suitable standard (see Table S1†). The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

where A is the absorbance (in the range 0.01–0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively.

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps: *System A*: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 × 100 mm) with CH_3CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220–750 nm). *System B*: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 21.2 × 250 mm) with CH_3CN and aq. TFA (0.1%, v/v, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 20% (12.5 min) and 20 to 60% (80 min) of CH_3CN] at a flow rate of 15 mL min^{-1}. Dual UV detection was achieved at 240 and 297 nm. *System C*: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 100 mm) with CH_3CN and aq. TFA (0.1%, v/v, pH 2.2) as eluents [100% TFA (5 min), followed by linear gradient from 0 to 25% (17.5 min) and 25 to 75% (100 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. Dual UV detection was achieved at 240 and 330 nm. *System D*: system A with CH_3CN and aq. triethylammonium acetate buffer (TEAA, 25 mM, pH 7.0) as eluents. Visible detection at 268, 343 or 646 nm. *System E*: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 100 mm) with CH_3CN and aq. triethylammonium bicarbonate buffer (TEAB, 50 mM, pH 7.5) as eluents [100% TEAB (5 min), followed by linear gradient from 0 to 20% (10 min) and 20 to 80% (120 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. Dual visible detection was achieved at 520 and 610 nm. *System F*: system E with the following gradient [100% TEAB (5 min), followed by linear gradient from 0 to 20% (10 min) and 20 to 40% (80 min) of CH_3CN]. Dual visible detection was achieved at 647 and 700 nm. *System G*: system C with the following gradient [100% TFA (5 min), followed by linear gradient from 0 to 20% (12.5 min) and 20 to 40% (60 min) of CH_3CN]. Dual UV detection was achieved at 225 and 280 nm. *System H*: system E with the following gradient [100% TEAB (5 min) followed by linear gradient from 0 to 25% (10 min) and 25 to 70% (100 min) of CH_3CN]. Dual UV detection was achieved at 230 and 340 nm. *System I*: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 100 mm) with CH_3CN and TEAA (100 mM, pH 7.0) as eluents [100% TEAA (10 min), followed by linear gradient from 0 to 100% (25 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV detection was achieved at 260 nm. *System J*: LC-MS under the following conditions: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 × 150 mm) with CH_3CN and TEAB (25 mM, pH 7.5) as eluents [100% TEAB (5 min) followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. Dual UV detection was achieved at 260 and 345 nm. ESI-MS detection in the negative mode (full scan, 150–1500 a.m.u., data type: centroid, sheat gas flow rate: 60 arb unit, aux/sweep gas flow rate: 20, spray voltage: 4.5 kV, capillary temp: 270 °C, capillary voltage: −10 V, tube lens offset: −50 V). *System K*: system A with the following gradient [100% TFA (5 min), followed by linear gradient from 0 to 100% (35 min) of CH_3CN] at a flow rate of 0.25 mL min^{-1}. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220–750 nm). *System L*: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 100 mm) with CH_3CN and aq. TFA (0.1%, v/v, pH 2.2) as eluents [100 TFA (5 min), followed by a linear gradient from 0 to 20% (10 min) and 20 to 60% (80 min) of CH_3CN]. Visible detection was achieved at 630 and 700 nm.

Synthesised compounds

3-Amino-5-(methoxycarbonyl)benzoic acid was synthesised from nitrobenzene derivative **2** by using a recent literature procedure.[35] An *N*-protected derivative of aminooxyacetic acid (Eei-Aoaa-OH) and its NHS active ester were synthesised according to published procedures.[11] See ESI† for the synthesis of bioconjugatable cyanine dyes **10–12** and **22**, peptide–aldehyde **16** and thiol-reactive Eu(III) bispyridinylpyrazine-based chelate **18**.

3-(2-(1-Ethoxyethylideneaminooxy)acetamido)-5-(methoxycarbonyl)benzoic acid (4). To a stirred solution of 3-amino-5-(methoxycarbonyl)benzoic acid (1.23 g, 6.31 mmol) in dry CH_3CN (50 mL) were added NHS ester of Eei-Aoaa-OH **3** (2.0 g, 7.75 mmol) and DIEA (3.3 mL, 18.9 mmol). The resulting reaction mixture was stirred at rt for 12 h before it was concentrated *in vacuo* and diluted with EtOAc (50 mL). The organic layers were washed with 10% aq. citric acid (3 × 20 mL), dried (over anhydrous Na_2SO_4) and concentrated *in vacuo*. Flash-column chromatography (silica gel, CH_2Cl_2–MeOH, 9 : 1, v/v) afforded benzoic acid **4** as a colourless solid (1.2 g, yield 56%). R_f 0.30 (CH_2Cl_2–MeOH, 9 : 1, v/v); ν_{max}(neat)/cm^{-1} 3248, 2928, 1736, 1695, 1679, 1639, 1546, 1416, 1309, 1211, 1105, 885, 747, 702; δ_H(300 MHz; $CDCl_3$) 10.48 (bs, 1 H), 8.50–8.45 (m, 3 H), 4.51 (s, 2 H), 4.00 (q, *J* 7.1, 2 H), 3.94 (s, 3 H), 2.06 (s, 3 H), 1.27 (t, *J* 7.1, 3 H); δ_C(75 MHz; $CDCl_3$) 170.0, 169.4, 165.8, 165.3, 137.9, 131.6, 130.8, 127.0, 125.6, 125.4, 73.0, 63.0, 52.6, 14.2, 14.1; HRMS (ESI+): *m*/*z* 339.1190 $[M + H]^+$, calcd for $C_{15}H_{19}N_2O_7^+$ 339.1192.

Azido derivative (6). To a stirred solution of benzoic acid **4** (1.1 g, 3.2 mmol) in dry CH_3CN (30 mL) were added azido amino-PEG linker **5** (0.57 g, 3.2 mmol), BOP reagent (1.44 g, 3.2 mmol) and DIEA (1.7 mL, 9.7 mmol). The resulting reaction mixture was stirred at rt for 3 h before it was concentrated *in vacuo* and diluted with EtOAc (50 mL). The organic layers were washed with 10% aq. citric acid (3 × 20 mL), sat. aq. $NaHCO_3$ (3 × 20 mL), brine (20 mL), dried (over anhydrous Na_2SO_4) and concentrated *in vacuo*. Flash-column chromatography (silica gel, CH_2Cl_2–MeOH, 99 : 1, v/v) afforded azido derivative **6** as a colourless oil (1.37 g, yield 84%). R_f 0.1 (CH_2Cl_2–MeOH, 99 : 1, v/v); ν_{max}(neat)/cm^{-1} 3315, 2864, 2098, 1723, 1642, 1538, 1450, 1306, 1259, 1116, 1092, 1018, 968, 883, 825; δ_H(300 MHz; $CDCl_3$) 8.44 (s, 1 H), 8.31 (s, 1 H), 8.11 (s, 1 H), 8.05 (s, 1 H), 7.07 (bs, 1H), 4.38 (s, 2H), 3.90 (q, *J* 7.1, 2H), 3.81 (s, 3H), 3.60–3.55 (m, 10 H), 3.26 (t, *J* 5.1, 2 H), 1.95 (s, 3 H), 1.17 (t, *J* 7.1, 3 H); δ_C (75 MHz; $CDCl_3$) 169.1, 166.2, 165.9, 164.9, 138.0, 135.7, 131.1, 123.4, 123.3, 122.9, 72.9, 70.4, 70.2, 69.9, 69.6, 62.8, 52.3, 50.5, 39.9, 14.2, 13.9; HRMS (ESI+) 495.2205 $[M + H]^+$, calcd for $C_{21}H_{31}N_6O_8^+$ 495.2203.

Benzoic acid derivative (7). To a stirred solution of azido derivative **6** (110 mg, 0.22 mmol) in MeOH (5 mL) was added 1.0 M aq. LiOH (1 mL). The resulting reaction mixture was stirred at rt for 5 h before it was concentrated *in vacuo* and diluted with CH_2Cl_2 (10 mL) and 1.0 M aq. NaOH (10 mL). The aqueous layer was washed with CH_2Cl_2 (2 × 10 mL), acidified with 1.0 M aq. HCl (15 mL) and diluted with CH_2Cl_2 (10 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (4 × 10 mL). The combined organic layers were dried (over anhydrous Na_2SO_4) and concentrated *in vacuo* to afford benzoic acid **7** as a colourless oil (100 mg, yield 93%) directly used without purification. ν_{max}(neat)/cm^{-1} 3330, 2936, 2108, 1644, 1546, 1451, 1308, 1098; δ_H(300 MHz; $CDCl_3$) 9.85 (bs, 1 H), 8.56 (s, 1 H), 8.42 (s, 1 H), 8.27 (s, 1 H), 8.18 (s, 1 H), 7.72 (s, 1 H), 4.42 (s, 2 H), 3.91 (q, *J* 6.9, 2 H), 3.70–3.55 (m, 10 H), 3.30–3.25 (m, 2 H), 1.97 (s, 3 H), 1.18 (t, *J* 6.9, 3 H); δ_C(75 MHz; $CDCl_3$) 169.4, 168.2, 166.5, 165.0, 137.9, 135.3, 131.1, 124.1, 124.0, 123.7, 72.9, 70.2, 69.9, 62.8, 50.5, 40.0, 14.2, 14.0; HRMS (ESI+): *m*/*z* 481.2045 $[M + H]^+$, calcd for $C_{20}H_{29}N_6O_8^+$ 481.2047.

Full-protected tripod (9). To a stirred solution of benzoic acid **7** (280 mg, 0.58 mmol) in dry CH_3CN (10 mL) were added *S*-trityl amino-PEG linker **8** (240 mg, 0.58 mmol), BOP reagent (260 mg, 0.59 mmol) and DIEA (0.3 mL, 1.7 mmol). The resulting reaction mixture was stirred at rt for 3 h before it was concentrated *in vacuo* and diluted with EtOAc (20 mL). The organic layers were washed with 10% aq. citric acid (3 × 10 mL), sat. aq. $NaHCO_3$ (3 × 10 mL), brine (20 mL), dried (over anhydrous Na_2SO_4) and concentrated *in vacuo*. Flash-column chromatography (silica gel, CH_2Cl_2–MeOH, 98 : 2, v/v) afforded the full-protected tripod **9** as a colourless oil (460 mg, yield 91%). R_f 0.15 (CH_2Cl_2–MeOH, 98 : 2, v/v); ν_{max}(neat)/cm^{-1} 3306, 2867, 2099, 1645, 1596, 1540, 1444, 1306, 1098, 744, 700; δ_H(300 MHz; $CDCl_3$) 8.55 (s, 1 H), 8.22–8.18 (m, 2 H), 7.97 (s, 1 H), 7.45–7.40 (m, 6 H), 7.30–7.20 (m, 11 H), 4.51 (s, 2 H), 4.03 (q, *J* 7.0, 2 H), 3.70–3.60 (m, 16 H), 3.50–3.45 (m, 2 H), 3.40–3.30 (m, 4 H), 2.43 (t, *J* 6.4, 2 H), 2.09 (s, 3 H), 1.30 (t, *J* 6.9, 3 H); δ_C(75 MHz; $CDCl_3$) 169.1, 166.3, 164.9, 144.7, 138.0, 135.6, 129.5, 127.8, 126.6, 121.1, 73.0, 70.5, 70.3, 70.2, 70.1, 70.0, 69.7, 69.6, 66.6, 62.9, 50.6, 40.0, 31.6, 14.3, 14.1; HRMS (ESI+): *m*/*z* 870.3885 $[M + H]^+$, calcd for $C_{45}H_{56}N_7O_9S^+$ 870.3860.

Benzenic "tripod" (1). The full-protected tripod **9** (130 mg, 0.15 mmol) was dissolved in 5 mL of TFA–TES–H_2O (95 : 2.5 : 2.5, v/v/v) and the resulting reaction mixture was stirred at rt for 3 h. Thereafter, the product was isolated after removal of volatiles under reduced pressure, precipitation from Et_2O and semi-preparative RP-HPLC purification (system B) to yield the TFA salt of tripod **1** as a white amorphous powder (30 mg, yield 23%). IR (neat) ν_{max}(neat powder)/cm^{-1} 3316, 2872, 2109, 1645, 1596, 1547, 1447, 1344, 1295, 1201, 1132, 896, 836, 799, 721; δ_H(300 MHz; D_2O) 7.95 (s, 2 H), 7.86 (s, 1 H), 4.74 (s, 2 H), 3.67–3.50 (m, 20 H), 3.33 (t, *J* 5.1, 2 H), 2.55 (t, *J* 6.2, 2 H); δ_C(75 MHz; $CDCl_3$) 168.8, 167.7, 162.8 (q, $^2J_{CF}$ 35.5, TFA), 137.1, 135.0, 134.9, 122.7, 122.4, 116.3 (q, $^1J_{CF}$ 291.8, TFA), 72.1, 72.0, 69.5, 69.4, 69.2, 69.1, 68.7, 68.6, 50.0, 39.6, 23.0; HPLC (system A): t_R = 23.8 min (purity 91%); LRMS (ESI+): *m*/*z* 558.20 $[M + H]^+$, 1115.07 $[2M + H]^+$; HRMS (ESI+): *m*/*z* 558.2350, calcd for $C_{22}H_{36}N_7O_8S^+$ 558.2346.

Preparation of three-colour FRET cascade (15)

(a) Oxime ligation with Cy 5.0 aldehyde: Benzenic "tripod" **1** (2.5 mg, 4.48 μmol) was dissolved in 0.1 M aq. NaOAc buffer

(pH 4.2, 1.0 mL). Cy 5.0 aldehyde **10** (2.8 mg, 3.38 μmol) was added and the resulting mixture was stirred at rt for 4 h. The reaction was checked for completion by RP-HPLC (system A) and then quenched by addition of aq. TFA 0.1% (2 mL). Then, the mixture was purified by semi-preparative RP-HPLC (system C, 2 injections). The product-containing fractions were lyophilised to give the red-fluorescent tripod **13** as a blue amorphous powder (1.5 mg, yield 32%). HPLC (system D): t_R = 28.2 min (purity > 95%); LRMS (ESI−): *m*/*z* 1368.40 $[M - H]^-$, calcd for $C_{65}H_{83}N_{11}O_{16}S_3$ 1369.52; λ_{max} (PBS) nm 658 (ε/dm^3 mol^{-1} cm^{-1} 250 000); λ_{max} em (PBS) nm 668 (Φ_F 0.40 in PBS + 5% BSA).

(b) S_N2 reaction with an iodoacetyl derivative of Cy 3.0: Cy 5.0–labelled tripod **13** (1.5 mg, 1.1 μmol) was dissolved in a mixture of CH_3CN and 0.1 M aq. $NaHCO_3$ buffer (1 : 3, v/v, 1.0 mL, pH 8.5). Thiol-reactive Cy 3.0 derivative **11** (2.1 mg, 2.2 μmol) was added and the resulting reaction mixture was stirred at rt for 3 h. The reaction was checked for completion by RP-HPLC (system D) and the mixture was dissolved in aq. TEAB (5 mL, 50 mM, pH 7.5) and purified by semi-preparative RP-HPLC (system E). The product-containing fractions were lyophilised to give the doubly fluorescently-labelled azido tripod **14** as a dark blue amorphous powder (0.86 mg, yield 36%). HPLC (system D): t_R = 27.3 min (purity >95%); LRMS (ESI−): *m*/*z* 1099.93 $[M - 2H]^{2-}$, calcd for $C_{107}H_{132}N_{16}O_{25}S_5$ 2200.81; λ_{max} (PBS) nm 549 (ε/dm^3 mol^{-1} cm^{-1} 150 000) and 653 (ε/dm^3 mol^{-1} cm^{-1} 250 000), λ_{max} em (PBS) nm 565 and 670. Please note: semi-preparative purification was performed under non-acidic conditions (*i.e.*, TEAB) to avoid severe degradation of the Cy 3.0 unit.

(c) CuAAC reaction with Cy 7.0–alkyne. Doubly fluorescently-labelled azido tripod **14** (0.86 mg, 0.39 μmol) and Cy 7.0–alkyne **12** (0.26 mg, 0.33 μmol) were dissolved in ultrapure water (0.4 mL). 10 μL of a 10 mM aq. $CuSO_4$ solution and 10 μL of a 10 mM aq. sodium ascorbate solution were added. The resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system D) and then quenched by addition of aq. TEAB (2 mL, 50 mM, pH 7.5). The mixture was purified by semi-preparative RP-HPLC (system F, 2 injections). The product-containing fractions were lyophilised to give the three-colour FRET cascade **15** as a dark blue amorphous powder (0.45 mg, yield 47%). HPLC (system D): t_R = 26.1 min (purity > 95%); LRMS (ESI−): *m*/*z* 1001.73 $[M - 3H]^{3-}$, 1502.80 $[M - 2H]^{2-}$, calcd for $C_{149}H_{183}N_{19}O_{34}S_7$ 3006.12; λ_{max} (PBS) nm 550 (ε/dm^3 mol^{-1} cm^{-1} 150 000), 650 (ε/dm^3 mol^{-1} cm^{-1} 250 000) and 761 (ε/dm^3 mol^{-1} cm^{-1} 200 000); λ_{max} em (PBS) nm 560, 670 and 776. See Table S1† for Φ_F and E.T.E. Please note: semi-preparative purification was performed under non-acidic conditions (*i.e.*, TEAB) to avoid severe degradation of the Cy 7.0 unit.

Preparation of luminescent POC (21)

(a) Oxime ligation with peptide–aldehyde: Benzenic "tripod" **1** (2.5 mg, 4.48 μmol) was dissolved in 0.1 M aq. NaOAc buffer (pH 4.2, 1.0 mL). Peptide–aldehyde **16** (2.8 mg, 1.74 μmol) was added and the resulting mixture was stirred at rt for 4 h. The reaction was checked for completion by RP-HPLC (system A) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by semi-preparative RP-HPLC (system G, 2 injections). The product-containing fractions were lyophilised to give the peptide–tripod conjugate **19** as a white amorphous powder (1.7 mg, yield 45%). HPLC (system D): t_R = 27.2 min (purity > 95%); LRMS (ESI+): *m*/*z* 715.53 $[M + 3H]^{3+}$, 1072.60 $[M + 2H]^{2+}$, calcd for $C_{95}H_{150}N_{30}O_{25}S$ 2143.11.

(b) S_N2 reaction with an iodoacetyl derivative of Eu(III) chelate: Peptide–tripod conjugate **19** (4.0 mg, 1.87 μmol) was dissolved in a mixture of CH_3CN and 0.1 M aq. $NaHCO_3$ buffer (1 : 3, v/v, 1.0 mL, pH 8.5). Thiol-reactive Eu(III) chelate **18** was added and the reaction mixture was stirred at rt for 3 h. The reaction was checked for completion by RP-HPLC (system D) and the mixture was dissolved in 50 mM aq. TEAB (2 mL) and purified by semi-preparative RP-HPLC (system H). The product containing fractions were lyophilised to give the luminescent peptide–tripod conjugate **20** as a white amorphous powder (0.23 mg, yield 30%). HPLC (system D): t_R = 26.6 min (purity > 95%); MS (ESI−): *m*/*z* 1522.48 $[M - 2H]^{2-}$, calcd for $C_{132}H_{1842}EuN_{38}O_{35}S$ 3046.27; λ_{max} (H_2O) nm 262 and 347; λ_{max} em (H_2O) nm 615 (only the most intense emission band corresponding to the $5D_0 \rightarrow 7F_2$ transition is reported, Φ_F 0.080 in H_2O). Please note: semi-preparative purification was performed under non-acidic conditions (*i.e.*, TEAB) to avoid premature decomplexation of lanthanide cations.

(c) CuAAC reaction with ODN–alkyne: Luminescent peptide–tripod conjugate **20** (30 μg, 10 nmol) and ODN–alkyne **17** (82 μg, 17 nmol) were dissolved in H_2O–DMSO (1 : 1, v/v, 160 μL). 16 μL of a 10 mM aq. $CuSO_4$ solution, 16 μL of a 10 mM TBTA solution in DMSO, 8 μL of a 50 mM aq. DIEA solution, and 16 μL of 10 mM aq. sodium ascorbate solution were sequentially added. The resulting reaction mixture was stirred at rt for 5 h. The reaction was checked for completion by RP-HPLC (system I). Thereafter, further amounts of ODN–alkyne **17** (82 μg, 17 nmol), $CuSO_4$ solution (16 μL) and sodium ascorbate solution (16 μL) were added. The reaction mixture was stirred at rt overnight. Further amounts of ODN–alkyne **17** (82 μg, 17 nmol), $CuSO_4$ solution (16 μL) and sodium ascorbate solution (16 μL) were added again and the reaction mixture was stirred at rt for a further 2 h. The resulting residue was taken up in 0.1 M aq. TEAA buffer pH ~ 7 (3 mL) and purified by RP-HPLC (system I, 1 injection). The product-containing fractions were dried using a concentrator (GeneVac miVac) to give luminescent peptide–ODN conjugate **21** as a white amorphous powder (52 μg, yield 66%). LC-MS (system J): t_R = 22.7 min; MS (ESI−): *m*/*z* 1978.03, 1978.80 and 1979.70 $[M - 4H]^{4-}$, found 7919.20, calcd 7918.47; λ_{max} (TEAA) nm 262 and 343; λ_{max} em (TEAA) nm 615 (only the most intense emission band corresponding to the $5D_0 \rightarrow 7F_2$ transition is reported, Φ_F 0.095 in TEAA). Please note: semi-preparative purification was performed under non-acidic conditions (*i.e.*, TEAB) to avoid premature decomplexation of lanthanide cations.

Sequential one-pot approach – Preparation of FRET cassette (Cy 5.0–Cy 7.0) labelled peptide (23). Oxime ligation with

peptide aldehyde: Benzenic "tripod" **1** (0.7 mg, 1.2 μmol) was dissolved in 0.1 M aq. NaOAc buffer (pH 4.2, 0.4 mL). Peptide aldehyde **16** (2.0 mg, 1.2 μmol) was added and the resulting reaction mixture was stirred at rt for 2 h. The reaction was checked for completion by RP-HPLC (system K). Then, Michael addition with Cy 5.0–maleimide: To the previous mixture, a solution of Cy 5.0–maleimide **22** (1.2 mg, 1.3 μmol) in ultrapure water (0.3 mL) was added and the resulting reaction mixture was stirred at rt for 2 h. The reaction was checked for completion by RP-HPLC (system K) and ESI-MS; LRMS (ESI+): *m*/*z* 1014.84 $[M + 3H]^{3+}$, 1521.28 $[M + 2H]^{2+}$, calcd for $C_{141}H_{201}N_{35}O_{35}S_3$ 3040.42. Finally, CuAAC reaction with Cy 7.0–alkyne. To 100 μL of the previous mixture, a solution of Cy 7.0–alkyne **12** (0.5 mg, 0.6 μmol) in ultrapure water (40 μL), *t*BuOH (100 μL) and Cu(0) microsized powder (5 equiv.) were added. The resulting reaction mixture was stirred at rt for 12 h. The reaction was checked for completion by RP-HPLC (system K) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system L, 2 injections). The product-containing fractions were lyophilised to give the FRET cascade (Cy 5.0–Cy 7.0) labelled peptide **23** as a dark green amorphous powder (0.19 mg, yield 28%). HPLC (system K): t_R = 24.0 min (purity >95%); LRMS (ESI−): *m*/*z* 1281.24 $[M - 3H]^{3-}$, 1921.25 $[M - 2H]^{2-}$, calcd for $C_{183}H_{252}N_{38}O_{44}S_5$: 3845.73; λ_{max} (H_2O) nm 649 (ε/dm^3 mol^{-1} cm^{-1} 250 000) and 758 (ε/dm^3 mol^{-1} cm^{-1} 200 000); λ_{max} em (PBS) nm 669 and 773.

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