A universal and ready-to-use heterotrifunctional cross-linking reagent for facile synthetic access to sophisticated bioconjugates† ‡

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We describe for the first time, the synthesis and some bioconjugation applications of an original heterotrifunctional cross-linking reagent (also named tripod) bearing three different bioorthogonal functional groups which are fully compatible amongst themselves. Contrary to the first generation tripod recently reported by us (*Org. Biomol. Chem.*, 2008, **6**, 3065), the use of an azido group instead of the nucleophile-sensitive active carbamate moiety enables us to reach the targeted chemical orthogonality without the use of temporary aminooxy- and thiol protecting groups. Thus, the preparation of sophisticated bioconjugates through the sequential derivatisation of the tripod by means of copper-mediated 1,3-dipolar cycloaddition, oxime ligation and aqueous compatible mild thiol-alkylation reactions, is significantly simpler and more convenient. The chemoselective bioconjugation protocols were optimised through the preparation of FRET cassettes based on cyanine and/or xanthene fluorescent dye pairs and subsequent anchoring to fragile biomolecules. The applicability of this universal cross-linking reagent was also illustrated by the preparation of biochips suitable for aflatoxin B1 detection through the SPIT-FRI method.

Introduction

During the past decade, considerable efforts have been devoted to the development of efficient, cutting edge and highly practical bioconjugation methods for the chemical modification of biological molecules with various reporter groups (*e.g.*, fluorescent labels, nanoparticles, nanomaterials or other biopolymers).[1,2]

Indeed, there is a growing need of multicomponent biomolecular systems with uniquely combined properties of the individual components, especially in the following disciplines: biotechnology, nano(bio)technology, bio-organic and medicinal chemistry.[3] Among the myriad of chemically engineered biomolecular tools that are currently designed, biosensors, molecular bio-probes and drug vectors are of particular interest for various biomedical applications including diagnostic assays, multimodality optical imaging and drug delivery.[4] The most popular methods to construct such bioconjugates encompass simple second-order reactions that selectively target the functionalities present (or previously introduced) within the native biomolecule/biopolymer.[5] Primary amines and sulfhydryls are the most commonly modified functional groups. The use of a commercially available homo- or heterobifunctional cross-linking reagent often enables to selectively introduce and/or derivatise these bioconjugable groups.

In recent years, new elegant bioconjugation approaches based on original reactions belonging to the "click" chemistry repertoire have been developed.[6] Thus, reactions that meet the "click" chemistry requirements are effective for selective labelling of biomolecules as they are high-yielding under biological conditions and are bioorthogonal since the involved precursors are stable to water and do not react with common functional groups present in biological molecules. These reactions include the very popular azide-alkyne Huisgen cycloaddition, the Diels–Alder-type cycloadditions, the Staudinger ligation of triarylphosphines and azides, and condensation of aldehyde or ketone with α-nucelophiles such as aminooxy and hydrazide derivatives.

The availability of such efficient bioorthogonal reactions and their presumed compatibility with some classic bioconjugation methods should enable us to consider the construction of sophisticated bioconjugates, especially those resulting from the covalent association of three different (bio)molecular partners. Indeed, there is a growing need for three-component biomolecular systems which are useful tools for applications in proteomics and genomics. As illustrative examples, one can mention: the activity-based probes for the identification of enzyme activities from complex proteomes,[7] the energy transfer terminators (*i.e.*, dideoxynucleotides labelled with FRET cassettes) for DNA sequencing,[8] and the biopolymer microarrays currently used for

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† Such heterotrifunctional cross-linking reagents were covered by a patent entitled "Preparation of trifunctional pseudopeptide reagents, especially luminescent reagents, and their bioconjugates, and their use for the functionalization of solid supports for the detection of biomolecules", G. Clavé, P.-Y. Renard, A. Romieu and H. Volland, PCT Int. Appl., 2009, WO 2009043986.

‡ Electronic supplementary information (ESI) available: Detailed synthetic procedures for compound **26**. Characterisation data for compounds **3**, **10** and **22**–**25**. See DOI: 10.1039/c0ob00133c

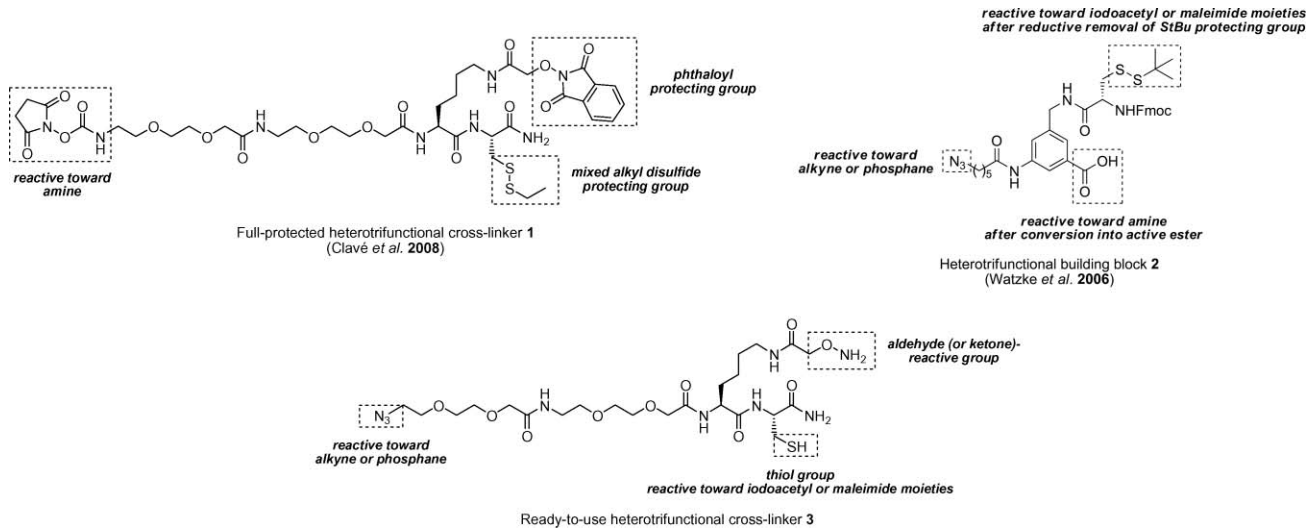


Fig. 1 Structures of heterotrifunctional cross-linkers **1** and **2** developed by us and Watzke *et al.* respectively, and the ready-to-use azido tripod **3** studied in this work.

the rapid analysis of various biological events.[9] However, due to the lack of universal heterotrifunctional cross-linking reagents (*i.e.*, cross-linkers equipped with a triply (bio)orthogonal set of functional groups), synthetic access to these (bio)molecular architectures is still tricky or impossible in most cases. To fill this gap, we have recently reported a heterotrifunctional linker molecule **1** (also named tripod, see Fig. 1 for the corresponding structure) based on a dipeptidyl scaffold (*i.e.*, lysine-cysteine) that contains an amine-reactive *N*-hydroxysuccinimidyl carbamate, an aldehyde/ketone-reactive aminooxy group, a thiol group and a water-solubilising pseudo-PEG spacer.[10] The potential utility of this bioconjugation reagent was demonstrated by the preparation of biochips suitable for substance P and microcystin-LR detection through the SPIT-FRI (for Solid-Phase Immobilised Tripod for Fluorescent Renewable Immunoassay) method.[10,11] However, in this first generation tripod the chemical orthogonality between the active carbamate and the nucleophilic aminooxy and thiol functionalities, and so the chemical stability of the cross-linking reagent was obtained only by using temporary protecting groups for these two latter reactive groups (*i.e.*, phthaloyl (Pht) for aminooxy and SEt for thiol). Thus, bioconjugation schemes involving the use of this reagent always require two additional deprotection steps which must be performed immediately after the acylation reaction of the amine-containing biomolecule with its *N*-hydroxysuccinimidyl carbamate. Since these deprotections require the use of hydrazine monohydrate in MeOH and dithiothreitol (DTT) in aq. buffer, some side-reactions may occur with fragile biomolecules and chemical reporter groups (*e.g.*, fluorophores) exhibiting nucleophile- and/or reductant-sensitive moieties, causing significant losses of bioconjugate material. Furthermore, the multi-step bioconjugation procedures are also complicated by tedious and time-consuming reversed-phase HPLC (RP-HPLC) purifications. Thus, the overall yield of the bioconjugation strategies based on the use and sequential derviatisation of **1** is often poor to modest.

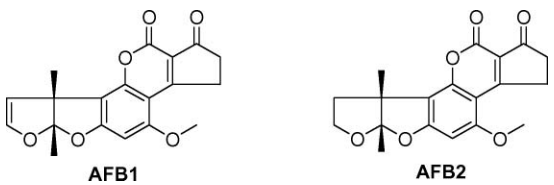
To improve the universality of bioconjugation reagent **1**, we have explored the chemistry of a new tripod bearing three different bioorthogonal functional groups which are expected to be fully compatible amongst themselves without using temporary protecting groups. The availability of such an unprecedented cross-linking reagent should allow straightforward access to sophisticated and fragile bioconjugates because the three bioorthogonal reactions leading to the covalent association of (bio)molecular partners will be performed directly without any previous deprotection step. In this context, we have chosen to replace the nucleophile-sensitive active carbamate moiety of **1** by an azido group aimed at using Staudinger ligation or copper-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) as one of the three derivatisation reactions. Surprisingly, despite numerous recent work about the use and optimisation of "click" chemistry reactions in the context of biomolecule labelling,[6] only one study has been published on the synthesis and applications of heterotrifunctional cross-linking reagents one of whose three free reactive moieties is an azido group (or its complementary functional group: alkyne or triarylphosphine). This work describes the design and synthesis of an aromatic building block **2** for *C*- and *N*-terminal protein labelling and protein immobilisation.[12] The availability of an azido group, an *S*-protected cysteine residue and a carboxylic acid onto the benzene ring, enables both the fluorescent labelling of proteins and their subsequent immobilisation on a phosphane-functionalised surface by means of the Staudinger ligation. However, the full-orthogonality between the three functional groups was not clearly demonstrated; no example of sequential triple derivatisation of reagent **2** with a fluorescent reporter group, a protein and finally a phosphane-modified surface has been reported to date. In the present paper, we want to describe the synthesis of the original azido tripod **3** bearing three free reactive groups that can be directly and sequentially derivatised. To illustrate the versatility of this novel bioconjugation reagent, the construction of three different FRET cassettes based on cyanine and/or xanthene dye pairs and their subsequent grafting to fragile biomolecules (*i.e.*, aflatoxin B2, **AFB2**) and biopolymers (*i.e.*, DNA fragments) were explored. Furthermore, azido tripod **3** was also used to improve the implementation of the original immunoassay format called

SPIT-FRI (for Solid-Phase Immobilised Tripod for Fluorescent Renewable Immunoassay),[13] especially in the context of aflatoxin B1 (**ABF1**) detection.

Results and discussion

General considerations for the design of the second generation tripod suitable for sequential bioconjugation of three different (bio)molecular partners

In the context of peptide bioconjugation, we have recently proved that the aminooxy and thiol groups of tripod **1**, once deprotected, were orthogonal to each other and could be used for a sequential derivatisation of substance P and microcystin-LR analogues. However, the exemplification of the published five-step bioconjugation protocol to other biomolecules (or biopolymers) more fragile than peptides is not trivial and in some cases led to the desired final bioconjugate with a very poor yield. For instance, our attempts to synthesise biochips suitable for detection of aflatoxins, by means of fluorescent labelling and immobilisation of **AFB2** through the sequential tripod derivatisation gave unsatisfactory results. Indeed, we observed that the lactone moiety of this mycotoxin is highly reactive toward hydrazine used for the removal of the Pht aminooxy protecting group, causing significant alteration of the AFB2-tripod conjugate during this first deprotection step and so decreasing the desired product recovery to a *ca.* 40% yield.[14]



In addition to its α-nucleophile character, the contamination of commercial hydrazine solutions with trace amounts of diimine can also negatively affect the structural integrity (and so the corresponding spectral properties) of some fluorescent organic dyes currently used for bio-labelling. As an example, during our synthesis of a cyanine-based amino acid, an unwanted side-reaction of reduction of the polymethene chain of this fluorophore took place during the deprotection of the phthaloyl group according to the hydrazine commercial source.[15] These selected experimental observations have led us to think that the use of hydrazine was not compatible with the development of versatile bioconjugation schemes involving our tripod technology. Consequently, to avoid this non-selective deprotection step, we decided to explore a second generation of heterotrifunctional cross-linking reagents bearing a free aminooxy functional group. Since this α-nucleophile is free to attack the active carbamate found in the structure of the first generation tripods, this latter electrophilic moiety must be replaced by a bioconjugable, bioorthogonal and non-nucleophile sensitive functional group.

We have naturally chosen the azido group which is known to be inert toward most *N*-, *O*- and *S*-nucleophiles under physiological conditions and easily and selectively derivatisable through Staudinger ligation or CuAAC reaction.[16] Indeed, the expected lack of cross-reactivity between these three bioconjugable handles (*i.e.*, aminooxy, azido and thiol groups) will open up a new avenue for designing an original, easy-to-use and universal bioconjugation strategy leading to the covalent association of three different (bio)molecular partners, through the use of only three reactions belonging to the repertoire of "wet chemistry". Concerning the core structure of our second generation tripod **3**, the previously designed PEG-peptide scaffold based on the (lysine-cysteine) sequence was kept unchanged because it exhibits a good hydrophilic-hydrophobic balance which enables us to get a cross-linking reagent significantly soluble in water and related aq. buffers, a key feature for bioconjugation applications.

Synthesis of the building blocks A–C for the preparation of the second generation tripod

The second generation tripod **3** was synthesised from three different building blocks **A–C** through a highly convergent synthetic strategy similar to this developed for the preparation of **1** (Fig. 2).[10] However, some modifications have been brought to the synthesis of PEG-based building block **A** to incorporate the azido moiety onto the "N-terminal" side of this pseudo-dipeptide. Furthermore, the temporary aminooxy protecting group was revisited because our first synthetic attempts toward the second generation tripod **3** using the first generation building block **B** have shown the non-compatibility of the hydrazine-mediated deprotection of phthaloyl with the reductant-sensitive azido function. Indeed, formation of a significant amount of amino tripod was observed and so we suspected once again that traces of diimine in the hydrazine solution induced this azido reducing reaction. Finally, the structure and so the synthesis of the first generation cysteine-based building block **C** were kept unchanged.

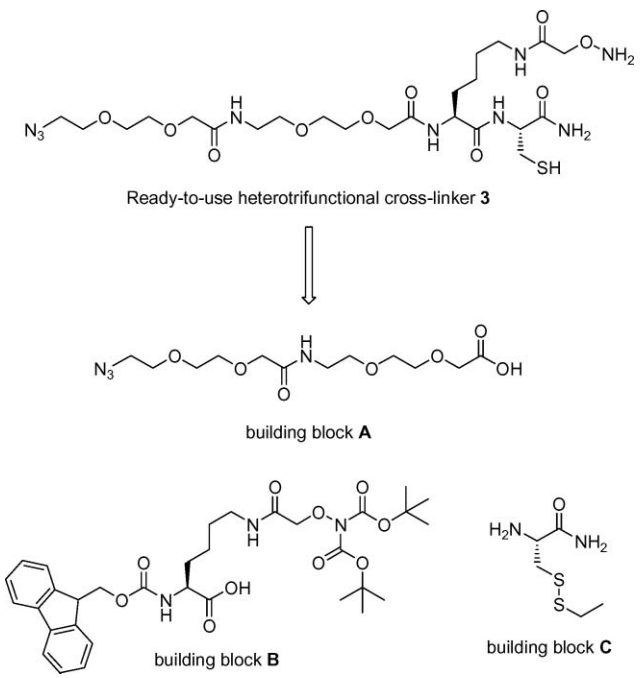
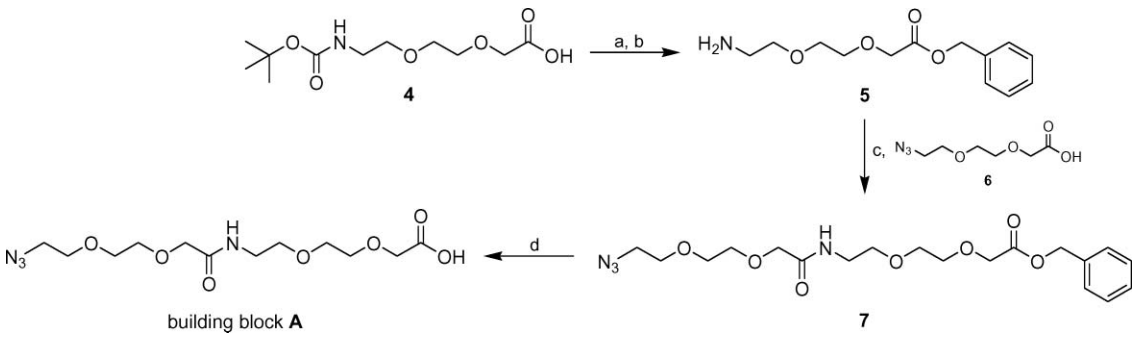


Fig. 2 Building block approach to the solution-phase synthesis of ready-to-use heterotrifunctional cross-linking reagent **3**.

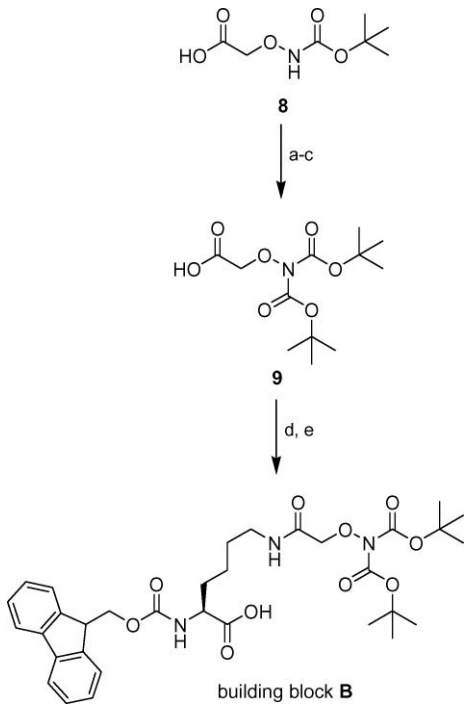
Synthesis of azido-PEG-acid spacer A. Hydrophilic linker **A** was synthesised in 48% overall yield through a four-step procedure depicted in Scheme 1. The main feature of this synthetic route is its shortness made possible by the re-use of two key intermediates involved in the preparation of the first generation



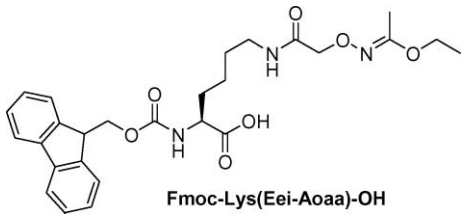
Scheme 1 *Reagents and conditions*: (a) Benzyl chloroformate, TEA, DMAP, CH_2Cl_2, overnight; (b) TFA, CH_2Cl_2, 4 °C to rt, 53% (overall yield for steps a–b); (c) BOP, DIEA, CH_3CN–DMF (1 : 1, v/v), overnight; (d) 1 M aq. LiOH, MeOH, rt, 91% (overall yield for steps c–d). BOP = benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate.

of building block **A** (*i.e.*, *N*-Boc amino- and azido acids **4** and **6**), as starting materials. Firstly, the *N*-Boc amino acid **4** was converted to the corresponding benzyl ester by using unusual conditions reported by Kim *et al.* (*i.e.*, treatment of carboxylic acid with benzyl chloroformate in the presence of TEA and DMAP, in dry CH_2Cl_2).[17] The benzyl moiety was preferred to standard alkyl esters (*i.e.*, ethyl or methyl ester) to get a more hydrophobic compound which was easily purified by conventional liquid–liquid extraction and column chromatography. Further treatment of this resulting benzyl ester with a 20% solution of TFA in CH_2Cl_2 gave the amine **5** in 53% overall yield for the two steps. Amidification of **5** with azido acid **6** has been readily achieved with BOP phosphonium salt in the presence of DIEA, in CH_3CN–DMF (1 : 1).[18] Finally, the benzyl ester was removed by short treatment of the protected pseudo-PEG linker **7** with 1 M LiOH in H_2O–MeOH to give the target building block **A** in 49% overall yield from **4**. All spectroscopic data, in particular NMR and mass spectrometry, were in agreement with the structure assigned.

Synthesis of aminooxy-containing lysine building block B. As mentioned above, the choice of azido moiety as the third bioconjugable function involves the replacement of the phthaloyl aminooxy functionality by another protecting group which enables: (1) the prevention of the *N*-overacylation side-reaction frequently encountered during aminooxy peptide synthesis,[19] and (2) the release of the aminooxy reactive group under conditions compatible with the stability of alkyl azides. Since the azido moiety is stable toward treatment with trifluoroacetic acid, the *N*,*N*-bis-Boc protection of aminooxyacetic acid (Aoaa) was considered. The use of this acid-labile bis-carbamate protecting group led us to modify the original synthetic route leading to the building block **B** (Scheme 2). $(Boc)_2$–Aoaa–OH **9** was readily prepared from commercial mono-Boc building block **8** by using the three-step procedure reported by Brask and Jensen and involving transient protection of the carboxylic acid function of **8** as a benzyl ester.[20] Thereafter, **9** was activated as the *N*-hydroxysuccinimidyl ester (NHS) and subsequently coupled to the free ε-amino group of lysine. This pre-activation procedure avoided an additional protection step of the lysine carboxylic acid function. $(Boc)_2$–Aoaa–OH was converted into the corresponding NHS ester by treatment with DCC-NHS in CH_3CN. This crude mixture of active ester was directly reacted with Fmoc-Lys-OH to provide the corresponding building block **B** in a moderate yield (40%). It is interesting to note that during the course of our work, Foillard *et al.* reported an alternative acide-labile aminooxy protecting group (*i.e.*, 1-ethoxyethylidene, Eei) removable under milder conditions than the bis-Boc moiety (*i.e.*, 1% or 5% TFA solution in CH_3CN-H_2O (1:1)) and more adequate for stepwise SPPS of Aoaa-containing peptides.[21] Thus, the synthesis and use of Fmoc–Lys(Eei-Aoaa)–OH building block could be further considered especially to speed up the production of the targeted heterotrifunctional peptide-based cross-linking reagents through standard automated solid-phase synthesis techniques.

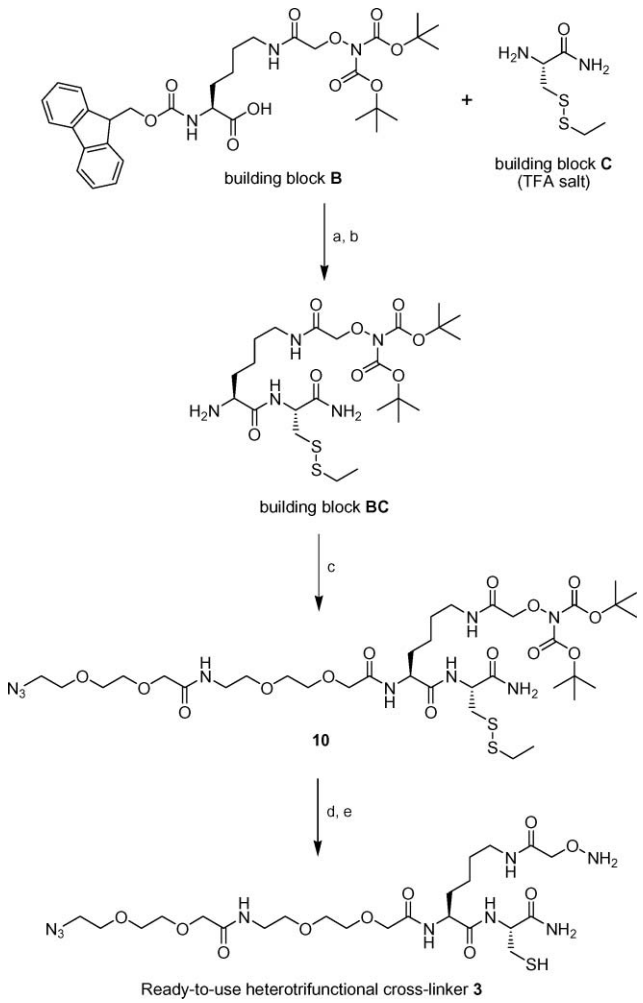


Scheme 2 *Reagents and conditions*: (a) Benzyl bromide, Cs_2CO_3, DMF, rt; (b) Boc_2O, DMAP, CH_3CN, rt; (c) 10% Pd/C, H_2, MeOH, rt, 74% (overall yield for steps a–c); (d) DCC, NHS, CH_3CN, rt, 1h; (e) Fmoc-Lys-OH, CH_3CN, 40% (overall yield for steps d–e).



Synthesis of aminooxy-azido-sulfhydryl-heterotrifunctional cross-linking reagent 3 using amino acid building blocks

Due to the availability of the three amino acid building blocks **A**, **B** and **C**, the azido tripod **3** was synthesised using the same highly convergent solution-phase synthesis that we used to prepare the first generation heterotrifuntional cross-linking reagent **1** (Scheme 3). Firstly, the lysine building block **B** was coupled to *S*-ethylthio cysteine carboxamide **C**, in the presence of BOP-DIEA, and the resulting fully protected dipeptide was treated with a 10% diethylamine solution in CH_3CN to give building block **BC** in 59% overall yield. This latter pseudo-dipeptide was then subjected to a further three-step reaction sequence: (1) BOP-mediated coupling with building block **A** to obtain the fully protected trifunctional pseudo-peptide **10**, (2) subsequent treatment of **10** with a 4.5% solution of TFA in CH_2Cl_2 to remove the two Boc groups, to give the free aminooxy pseudo-peptide, and finally (3) the deprotection of the thiol group by treatment with a large excess of DTT in 0.1 M aq. $NaHCO_3$ buffer. Purification was achieved by RP-HPLC to give the ready-to-use azido tripod **3** as a TFA salt and in a moderate yet not optimised overall yield (5%) from **BC**. Indeed, the use of RP-HPLC for purifications (followed by lyophilisation) and the small-scale chosen for the two latter deprotection steps (<5 mg) induced significant losses of material whereas these reactions were found to be almost complete. Analysis of **3** using ESI mass spectrometry indicated the presence and integrity of the free aminooxy, azido and sulfhydryl reactive groups within the pseudo-peptide architecture (see ESI‡). Interestingly, this reagent could be stored at –20 °C under an argon atmosphere for several months without detectable degradation. The inert atmosphere was found to be essential for long-time storage, to avoid both the oxidative dimerisation of its sulfhydryl moiety and aminooxy carbonatation.



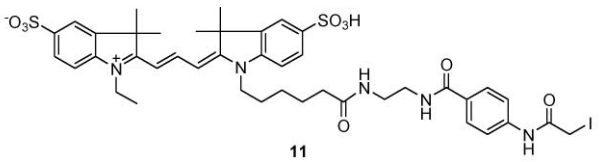
Scheme 3 *Reagents and conditions*: (a) BOP, DIEA, CH_3CN, rt, 2 h, 62%; (b) Et_2NH, CH_3CN, 2 h, 96%.; (c) building block **A**, BOP, DIEA, DMF, rt, 2 h; (d) TFA, CH_2Cl_2, rt; (e) DTT, 0.1 M aq. $NaHCO_3$ (pH 8.5), rt, 5% (overall yield for steps c-e after RP-HPLC purification).

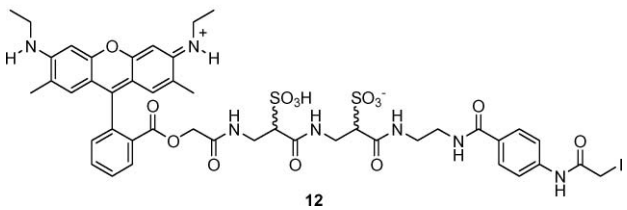
Bioconjugation applications of azido tripod 3

To demonstrate the broad utility of azido tripod **3** in bioconjugation chemistry, it was essential to develop a reliable high-yielding protocol enabling the sequential and chemoselective derivatisation of the three reactive groups with three different (bio)molecular partners. Since the full chemical orthogonality between free aminooxy and thiol functions and the superior chemical inertia of the azido moiety are well established, we planned to make the three reactions, namely, thiol alkylation (through Michael addition or S_N2 reaction), oxime ligation and CuAAC[22] in this order. Furthermore, the choice of a "wet chemistry" sequence involving CuAAC as the ultimate bioconjugation reaction was supported by the fact that: (1) free sulfhydryl reactive groups could be otherwise oxidatively damaged by copper and sodium ascorbate, which are currently used to generate "*in situ*" the catalytically active copper(I) species required in the CuAAC reaction, especially through the generation of reactive oxygen species,[23] and (2) copper salts are known to catalyse the cleavage of the N–O bond and so could lead to the degradation of free aminooxy moieties.[24] To illustrate the efficacy of this three-step bioconjugation procedure, the preparation of FRET cassette bio-probes and immunosensors was considered.

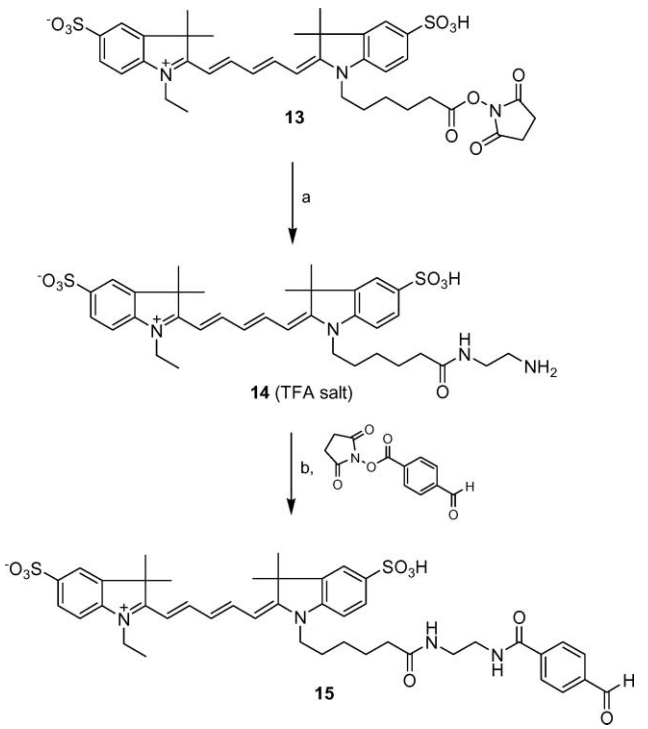
Preparation of FRET cassettes based on cyanine and/or xanthene dyes and their use in labelling of fragile biomolecules and biopolymers. Fluorescent organic dyes are widely used as non-radioactive labels in biological analysis and as a key component of optical bio-probes designed for various bio-imaging applications.[25] However, the undesirable spectral properties of various fluorophores still constrain the full potential of their applications. For instance, many bright organic dyes including cyanine and xanthene derivatives have the serious disadvantage of very small Stokes shifts (typically less than 30 nm), which can lead to significant self-quenching and fluorescence detection errors because of excitation backscattering effects. To overcome such a limitation, the single fluorophore can be replaced by a more sophisticated energy-donor–acceptor architecture based on a fluorescence resonance energy transfer phenomenon.[26] Such FRET dyads (also named

FRET cassettes) result from the covalent association of two complementary dyes through a non-conjugated spacer and exhibit through-space energy transfer. Thus, the pseudo-Stokes shift (*i.e.*, the wavelength discrepancy between the donor absorption and the acceptor emission in an energy transfer system with almost 100% energy transfer efficiency) of FRET-based energy cassettes are larger than the Stokes shifts of either the donor or acceptor dyes. Interestingly, these energy transfer fluorescent labels have found numerous applications in the field of DNA analysis, including sequencing, fragment sizing, and short tandem repeat-based cancer diagnosis.[8] However, the energy transfer fluorescent primers and terminators required for such applications are not synthetically easily accessible. Indeed, their preparation often relies on solution or solid-phase peptide (or oligonucleotide) chemistry involving the use of fluorescent building blocks and harsh conditions (especially for the deprotection steps) not fully-compatible with the moderate stability of some fluorophores.[27] In this context, we wished to use tripod **3** to develop a general and versatile method for FRET cassettes synthesis and their subsequent grafting to biological material. Thus, we planned the synthesis of two different energy transfer dyes through the sequential derivatisation of the two reactive aminooxy and thiol groups of **3**; the azido moiety being free for further grafting to a biomolecule/biopolymer by means of a CuAAC reaction. Either sulfoindocyanine dye Cy 3.0[28] or R6G-WS (a water-soluble analogue of rhodamine 6G recently developed by us)[29] were chosen as the donor and sulfoindocyanine Cy 5.0[28] as the acceptor. The carboxylic acid moieties of these dyes was converted into a function which is able to readily react with thiol or aminooxy reactive groups. Thus, the iodoacetamide derivatives of Cy 3.0 and R6G-WS **11** and **12** were prepared by using the three-step synthetic procedure reported by Bouteiller *et al.*[30]



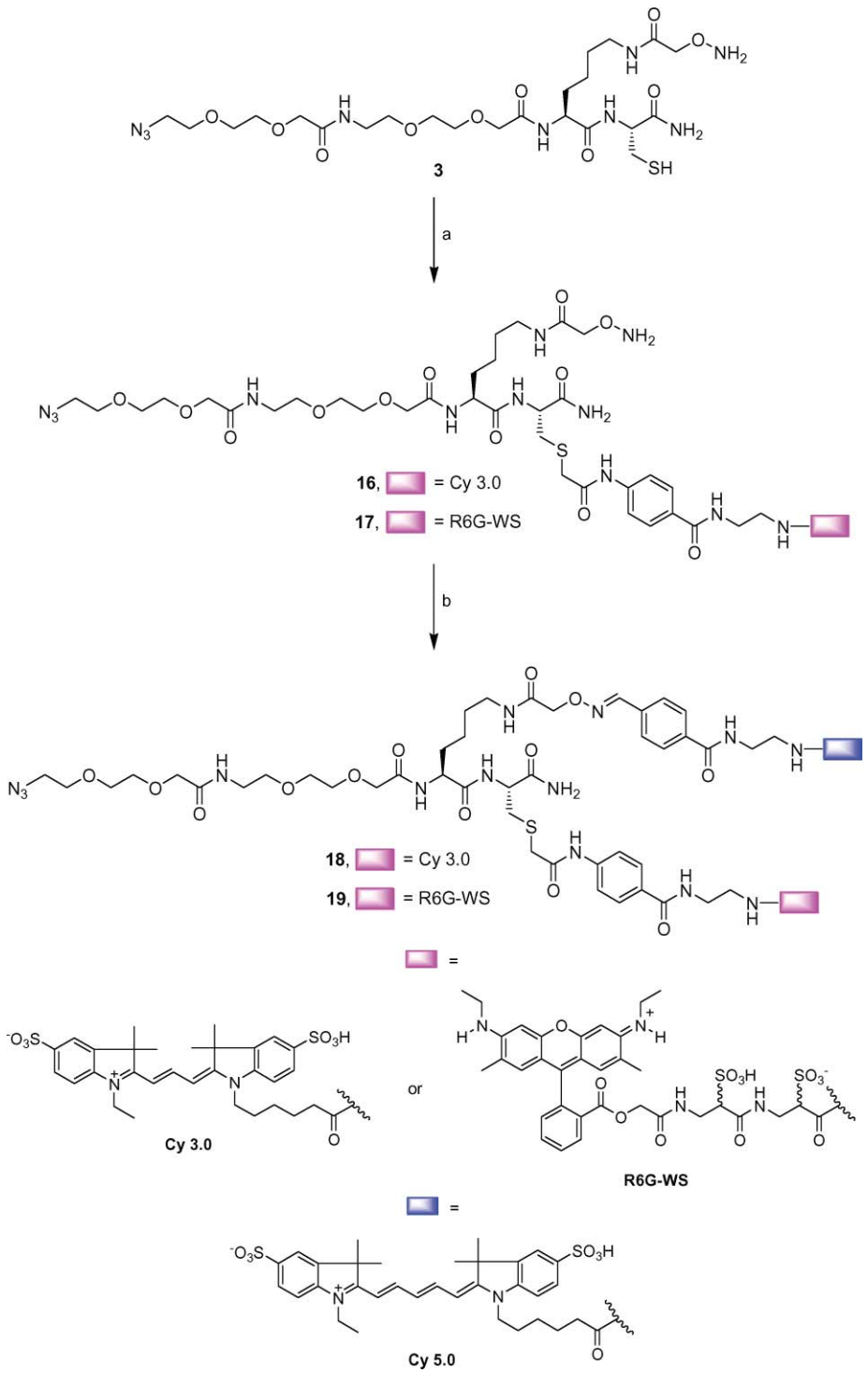


The synthesis of a fluorescent aldehyde **15** derived from Cy 5.0 and reactive toward the aminooxy moiety was also considered. This original compound was readily synthesised by using a two-step method (Scheme 4). Cy 5.0 NHS ester **13** generated by reaction with TSTU uronium salt in the presence of DIEA, in dry NMP, was reacted with ethylenediamine (dihydrochloride salt) under the conditions reported by Gruber *et al.* to give Cy 5.0 amine **14**.[31] Finally, acylation of the primary amino group of **14** with the NHS ester of 4-formylbenzoic acid (generated by treatment with TSTU-DIEA in dry NMP) provided after RP-HPLC purification, the aminooxy-reactive Cy 5.0 analogue **15** (overall yield for the three steps 11%). The easy availability of these monofunctional reactive dyes has enabled us to explore the



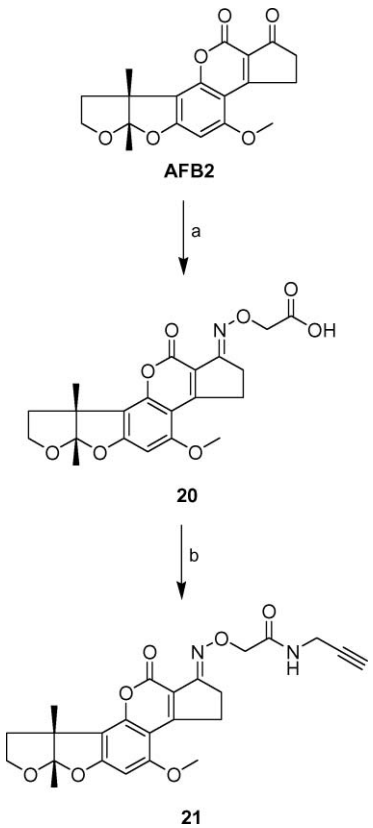
Scheme 4 *Reagents and conditions*: (a) ethylenediamine dihydrochloride, DMF–H_2O (9 : 1, v/v), 4 °C to rt; (b) NMP, 4 °C to rt, 11% (overall yield for steps a–b after RP-HPLC purification).

synthesis and biomolecular labelling of FRET cassettes through the three-step sequential derivatisation described in Schemes 5 and 7. First, cross-linking reagent **3** readily reacted through its free thiol with a slight excess of iodoacetyl derivative **11** (or **12**) in aqueous sodium bicarbonate buffer (pH 8.5) to obtain mono-fluorescently labelled tripod **16** (or **17**). This S_N2 reaction was found to be complete within 1 h and purification by RP-HPLC enabled us to recover **16** (or **17**) in almost quantitative yield. Thereafter, **16** (or **17**) and fluorescent aldehyde **15** were linked together through chemoselective oxime ligation. Such a reaction was performed in aqueous sodium acetate buffer (pH 4.2 in the range 4–5 which is known to be optimal for oxime bond formation)[32] and found to proceed essentially to completion within 3 h to yield exclusively the corresponding FRET cassettes **18** and **19**. These original energy transfer fluorescent azido-containing labels were purified by RP-HPLC and their structures confirmed by ESI mass spectrometry (see ESI‡). Finally, we examined their covalent attachment to biomolecules and biopolymers (Scheme 7). As an example of a fragile biomolecule, we have chosen to work with mycotoxin **AFB2** which exhibits a highly nucleophile-sensitive lactone moiety. Indeed, the preparation of a fluorescent probe of **AFB2** is of particular interest especially to demonstrate the efficacy of our three-step bioconjugation protocol and to get a useful tool for fluorescence immunoassays aimed at detecting aflatoxins. Since **ABF2** possesses only a keto group whose chemical modification through oxime ligation does not alter its biological properties, it was essential to introduce the alkyne moiety onto this position. Thus, we have used a two-step synthetic sequence involving AFB2 carboxylic acid derivative **20** as the key intermediate (Scheme 6). This latter compound was obtained by reaction of commercial **AFB2** with aminooxyacetic acid in



Scheme 5 *Reagents and conditions*: (a) Thiol-reactive fluorophore **11** or **12**, CH_3CN, 0.1 M aq. $NaHCO_3$ (pH 8.5), rt; (b) Cy 5.0 aldehyde **15**, aq. sodium acetate buffer (pH 4.2), rt.

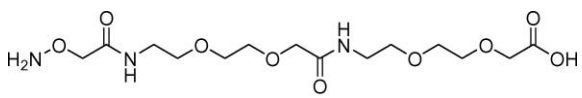
MeOH–H_2O–pyridine (4 : 1 : 1) under reflux, by using a published procedure.[33] Conversion into NHS ester by treatment with TSTU-DIEA in dry NMP and subsequent acylation reaction with propargylamine led to the targeted alkyne derivative of AFB2 **21** in a moderate 32% yield. CuAAC reaction between this latter alkyne and azido FRET cassette **19** was performed under the standard conditions using $CuSO_4$ and sodium ascorbate as the catalytic system. This bioconjugation reaction was found to work well without altering the ABF2 lactone core and the reporter cyanine and xanthene dyes. The corresponding tri-molecular conjugate **22** was isolated in a pure form by RP-HPLC and its structure was confirmed by ESI mass spectrometry (see ESI‡). Interestingly, the success of the present CuAAC reaction enables us to expand the list of clickable fluorophores for biological labelling to cyanine and



Scheme 6 *Reagents and conditions*: (a) Aoaa, MeOH–H_2O-pyridine, reflux; (b) TSTU, DIEA, DMF, rt, 1 h then propargylamine, rt, 1 h, 32% (after RP-HPLC purification). TSTU = *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

xanthene derivatives, recently reported.[34] Furthermore, the mild conditions used for the three derivatisation steps did not lead to significant degradation of intermediary conjugates contrary to the bioconjugation scheme published for the first generation tripod **1**, by avoiding deleterious chemicals such as hydrazine. We then extended the scope of this labelling approach with energy transfer dyes to DNA fragments: a synthetic 16-mer oligonucleotide 5′-d(TGA ACT GCA GCT CCT U) bearing the alkyne group as a nucleotide modification at the 2′-position of uridine (**16-mer ODN**).[35] Indeed, Berndl *et al.* have recently demonstrated that the CuAAC reaction is a valuable tool for the post-synthetic modification of oligonucleotides with brightly emitting fluorophores that are not stable under the typically strong basic conditions involved during DNA cleavage and deprotection.[36] Fluorescent labelling of various synthetic DNA fragments were successfully performed with base-labile phenoxazinium and coumarin dyes but not yet with FRET cassettes. However, such constructions should enable to get more powerful and sophisticated fluorescent labels for nucleic acids in screening assays or cell biology. In this context, we decided to explore the "click" modification of **16-mer ODN** with FRET cassettes **18** and **19**.[37] Thus, CuAAC reactions between 50–100 micromolar concentrations of energy transfer azido dye and alkyne were 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.[38] The corresponding fluorescent oligonucleotides **23** and **24** were isolated by RP-HPLC in almost quantitative yields and their structures confirmed by ESI mass spectrometry (see ESI‡). Furthermore, these two modified oligonucleotides were studied by UV-vis absorption and fluorescence spectroscopy (see ESI‡). The distance between the two cyanine dye molecules within fluorescent conjugate **24** was determined by applying a methodology previously described for a FRET fluorogenic caspase-3 probe[39] and was found to be 44 ± 1 Å (for an energy transfer efficiency $E = 0.75$). These successful examples support the fact that the successive three bioconjugation reactions involving tripod reagent **3** can be performed on each case with high yields, through easily reproducible fool-proof protocols (especially for non-chemists), and at a small scale compatible with highly valuable bio-chemical samples.

Preparation of fluorescent tripod-functionalised surfaces suitable for the detection of aflatoxin B1 through the SPIT-FRI immunoassay. In addition to these bioconjugations performed in homogeneous solution, we planned to use azido tripod **3** to prepare biochips suitable for mycotoxin **AFB1** detection through the SPIT-FRI method. Since, the implementation of such immunoassay requires the fluorescent labelling of **AFB2** (a validated analogue of **AFB1** which is well recognised by *anti*-AFB1 monoclonal antibodies (mAbs)) and its subsequent immobilisation on a glass surface, we have performed the following derivatisations of **3**: (1) S_N2 reaction of its thiol group with iodoacetyl derivative of R6G-WS **12**, (2) oxime ligation between its free aminooxy moiety and an aldehydic glass surface, and finally (3) CuAAC reaction between the grafted azido and the alkyne derivative of AFB2 **21**, directly on solid surface and according to the mild and non-denaturing "click" conditions.[40] However, under these conditions, we have observed the deterioration of the tripod-functionalised surface and severe quenching of R6G-WS fluorescence. Thus, we have modified the chronological sequence of derivatisation events (Scheme 8). After fluorescent labelling, we performed the CuAAC reaction with the tripod bearing the free aminooxy moiety to get **25**. We were pleased to observe the formation of the desired product but the yield was found to be modest (*ca.* 20%) and explained by the formation of side-products probably stemming from the copper-catalysed degradation of the free aminooxy moiety. The resulting fluorescent tripod was efficiently immobilised on an aldehydic glass surface through oxime ligation performed in aqueous sodium acetate buffer (pH 4.2). Before starting SPIT-FRI experiments through fluorescence signal observations, a capping step carried out in the presence of the in-house-developed aminooxy acid pseudo-PEG linker **26** (for its synthesis, see ESI‡) has enabled us to mask the remaining reactive aldehyde functions.



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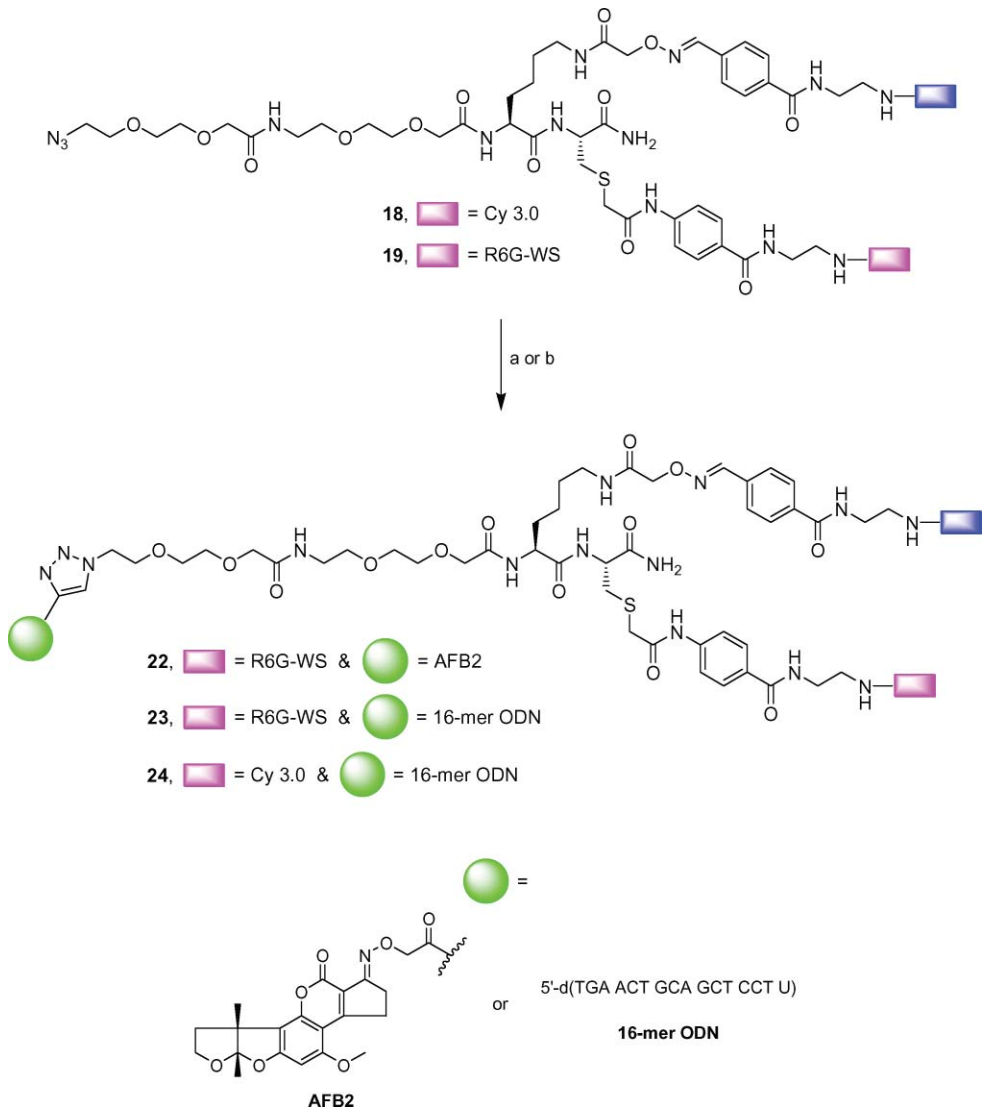
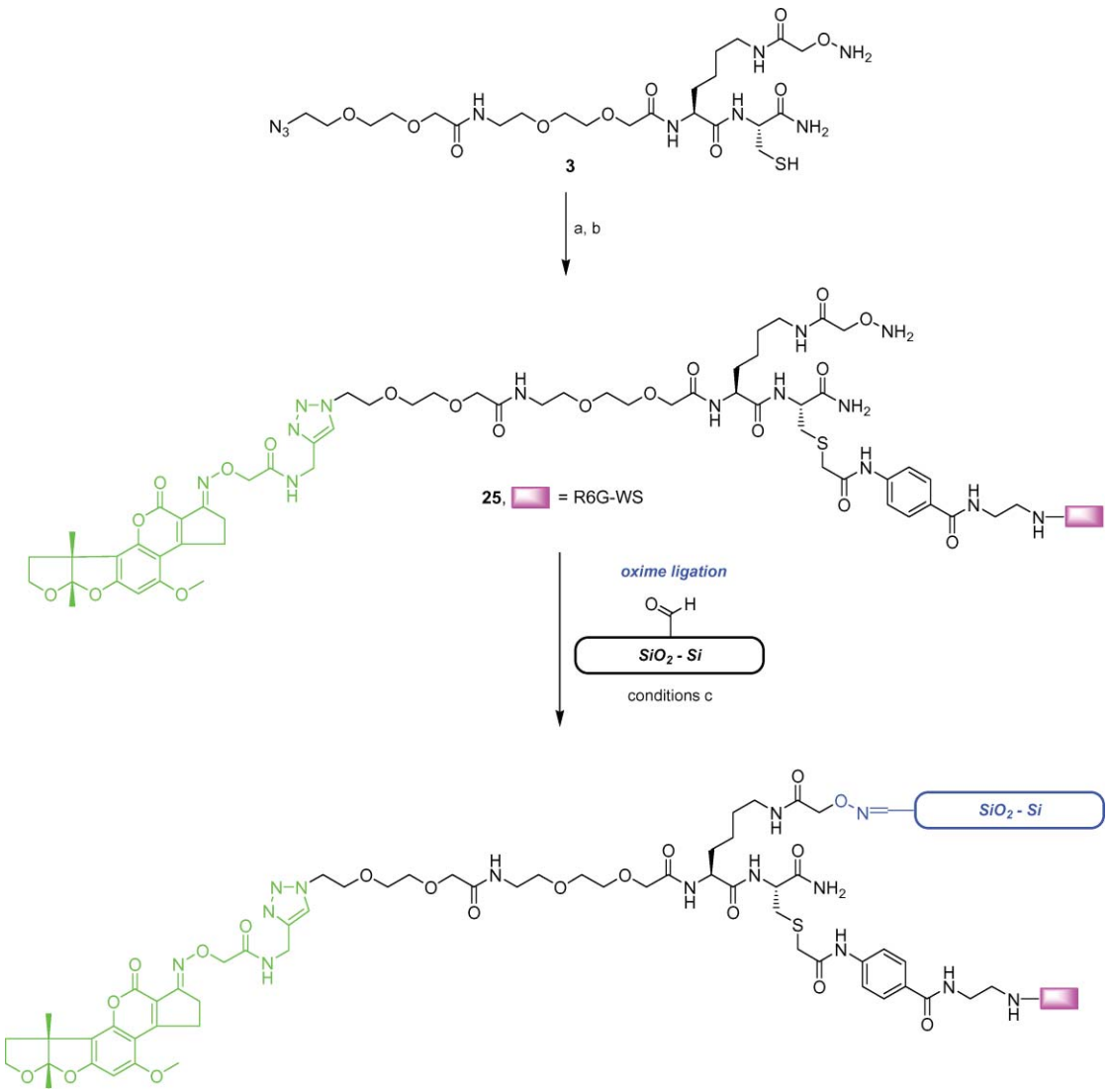
Indeed, we have already encountered some issues when we performed SPIT-FRI experiments after aldehyde-capping with BSA protein, especially non-specific adsorption of mAbs. Therefore, we decided to saturate the surface through the formation of stable oxime covalent linkages. In this context, an aqueous solution of hydroxylamine could be used as an efficient capping agent, but a PEG derivative such as **26** was preferred because some recent work has clearly underlined the positive effect of such a hydrophilic



Scheme 7 *Reagents and conditions*: (a) AFB2–alkyne **21**, H_2O–tBuOH (9 : 1, v/v), 0.1 M aq. $CuSO_4$, 0.1 M aq. sodium ascorbate, rt, 6 h; (b) **16-mer ODN**, H_2O-DMSO (1 : 1, v/v), 10 mM aq. $CuSO_4$, 10 mM TBTA in DMSO, 50 mM aq. DIEA, 10 mM aq. sodium ascorbate, rt, overnight.

spacer in decreasing the non-specific adsorption of proteins on solid supports.[41] After washing, an intense fluorescence signal was observed and proved the efficacy of the oxime ligation to covalently graft ABF1 related tripods to the solid surface (Fig. 3A–B, left). In a second step, FP647-labelled *anti*-AFB1 mAbs, which are able to recognise both **AFB1** and its analogue **AFB2**, were added. As Fluo Probe® 647 acts as a quencher of R6G-WS, a decrease in the fluorescence emission of R6G-WS *via* FRET was observed (Fig. 3A–B, middle). Finally, competition experiments using original **AFB1** aimed at detecting and quantifying this mycotoxin have been done. As expected, the competitive process occurring between the solid-phase tripod and **AFB1** in solution for the binding to the quencher-labelled mAb, led to an increase of the measured fluorescence (Fig. 3A–B, right). We observed partial recovery (around 25%) of the original fluorescence level. In addition to this preliminary experiment, we tried to quantify **AFB1** samples in the context of a continuous-flow sensor compatible with the SPIT-FRI method. Thus, the tripod-functionalised glass surface previously prepared was transposed to a portable biosensor.[42] At the beginning of the experiment the fluorescence signal was very strong and stable despite continuous washing with buffer (Fig. 4), confirming the stability of the oxime linkage on the solid-phase. Then, *anti*-AFB1 mAbs were introduced and we observed a significant decrease of the fluorescence intensity (Fig. 4, activation step). The quenching level is stable during the subsequent washings thanks to the strong affinity of the mAb for **AFB2**. Finally, a solution containing original **AFB1** was used to displace mAbs interacting with the immobilised tripod. The increase of the fluorescence signal is modest but significant enough to conclude the integrity of our biochip (Fig. 4, competition step). Further experiments are in progress to improve this detection-quantification immunoassay method and to expand its scope to a larger number of natural toxins such anatoxins, microcystins and saxitoxins.



Scheme 8 *Reagents and conditions*: (a) Thiol-reactive fluorophore **12**, CH_3CN, 0.1 M aq. $NaHCO_3$ (pH 8.5), rt; (b) AFB2-alkyne **21**, H_2O-DMSO (1 : 1, v/v), 10 mM aq. $CuSO_4$, 10 mM TBTA in DMSO, 50 mM aq. DIEA, 10 mM aq. sodium ascorbate, rt, overnight; (c) aq. sodium acetate buffer (pH 4.2), rt, 2 h.

Conclusion

In this paper, we have described for the first time the synthesis and four applications of an heterotrifunctional cross-linking reagent whose full-orthogonality between its three bioconjugable functions was obtained without using temporary protecting groups. Furthermore, we have demonstrated that the sequential derivatisation of this tripod can be performed with good to high yields under mild conditions fully-compatible with various fragile (bio)molecules and biopolymers. The chosen derivatisation reactions (*i.e.*, S_N2 reaction, oxime ligation and CuAAC reaction) performed in aqueous buffers, are easy to perform and have enabled us to establish an efficient, and reproducible protocol which opens up a new avenue for the synthetic access to highly sophisticated bioconjugates. However, to speed up the three-step bioconjugation methods involving the use of azido tripod **3**, further work is in progress in our lab to perform the three derivatisation reactions (or at least two of them) through a one pot procedure and so avoiding any intermediate RP-HPLC purification. Interestingly, such a one pot synthesis has been recently reported by Mhidia *et al.* for the preparation of peptide-protein conjugates by means of combining two reactions namely amine acylation with *N*-succinimidyl carbamate (NSC chemistry) and α-oxo semicarbazone ligation.[43]

Experimental section‡

General

Column chromatography purifications were performed on Geduran® Si 60 silica gel (40–63 μm) from Merck. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. Compounds were visualised by one or more of the following methods: (1) illumination with a short wavelength UV lamp (*i.e.*, λ = 254 nm), (2) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol, (3) spray with a 3.5% (w/v) phosphomolybdic

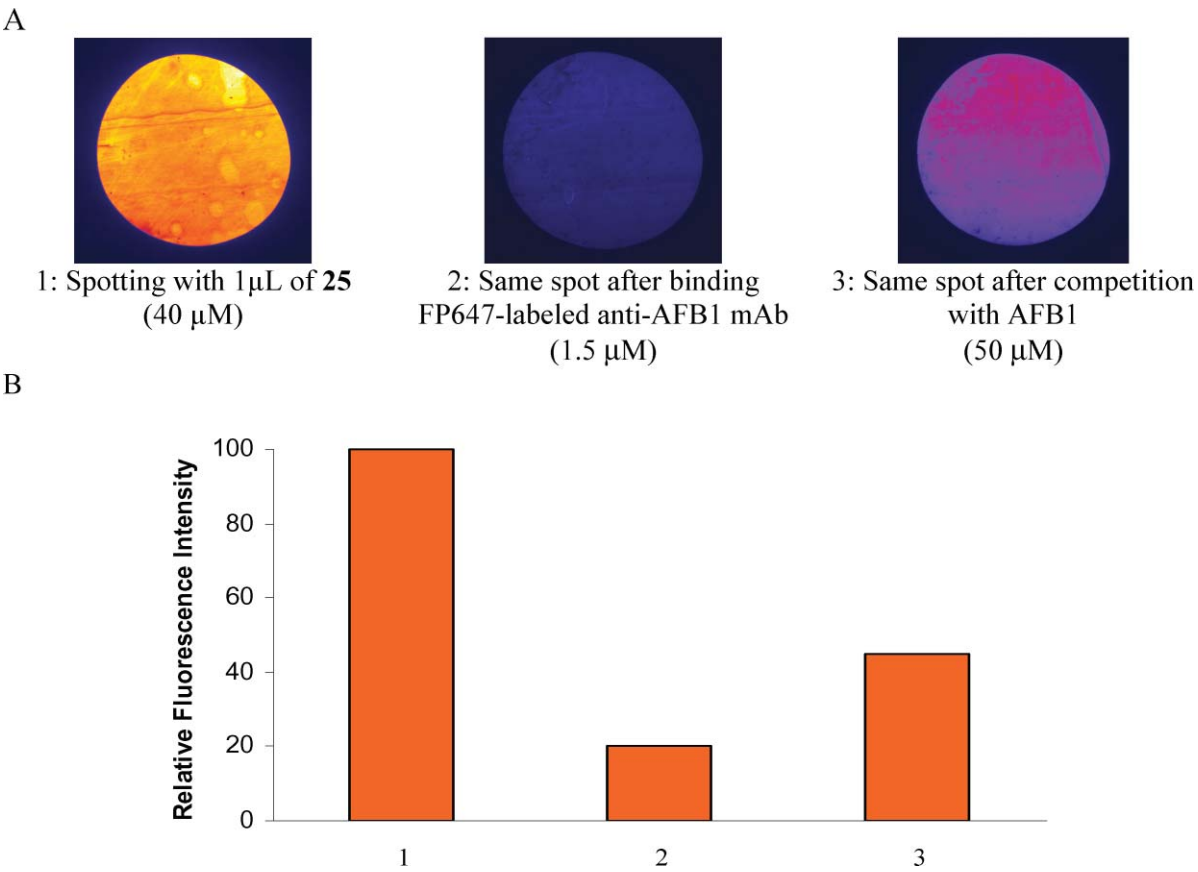


Fig. 3 (A) Immobilisation of fluorescent tripod **25** on the aldehyde-functionalised silica surface by oxime ligation as described in Scheme 8 (left); binding of FP647-labelled *anti*-AFB1 monoclonal antibodies (middle) and competition with AFB1 (right). (B) Relative fluorescence intensity of the spot for each step.

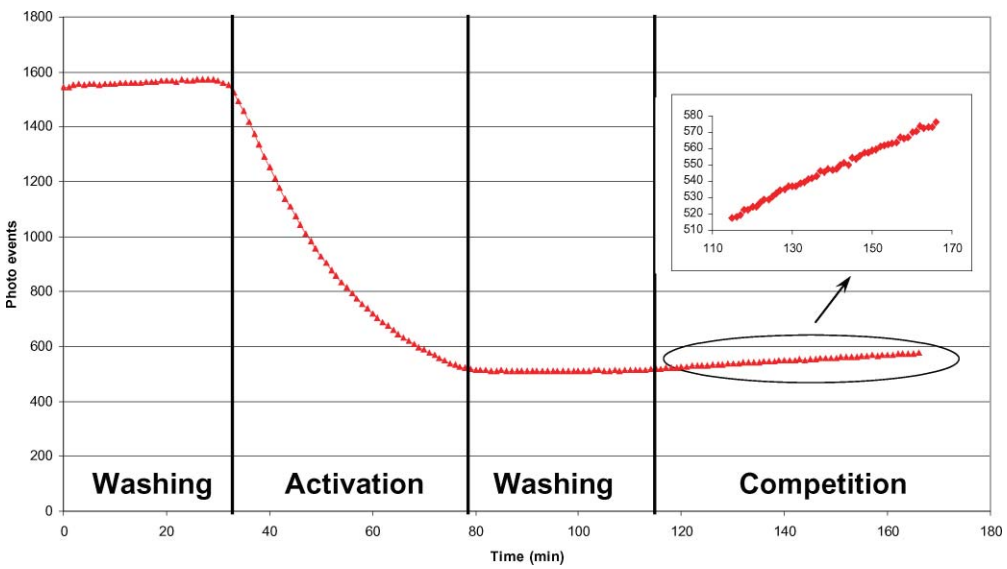


Fig. 4 SPIT-FRI experiment in the context of a continuous-flow sensor: step 1 = washing with EIA buffer (0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.1% tween 20, 0.1% PVP and 0.1 M sodium azide); step 2 = activation with a 1.5 μM solution (in EIA buffer) of *anti*-AFB1 mAbs; step 3 = washing with EIA buffer: step 4 = competition with a 0.5 μM solution (in EIA buffer) of AFB1. Inset: zoom for the competition step.

acid solution in absolute ethanol. All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, CH_2Cl_2: distillation over P_2O_5, DMF and NMP: distillation over BaO). DIEA and TEA were distilled from CaH_2 and stored over BaO. *N*-Sulfosuccinimidyl-(4-iodoacetyl)-aminobenzoate (sulfo-SIAB) and tris-(benzyltriazolylmethyl)amine (TBTA) were purchased from Pierce and Sigma-Aldrich respectively. N^{α}-(9-Fluorenylmethoxycarbonyl)-L-lysine (Fmoc–Lys–OH) was prepared by TFA-mediated removal of the N^{α}-Boc-protecting group of commercially available N^{α}-(9-fluorenylmethoxycarbonyl)-N^{ε}-(*tert*-butyloxycarbonyl)-L-lysine (Fmoc–Lys(Boc)–OH, Iris Biotech). $(Boc)_2$-Aoaa-OH, AFB2 carboxylic acid derivative **20**, and the sulfoindocyanine dyes Cy 3.0 and Cy 5.0 were synthesised following the literature procedures.[20,28,33] Cysteine building block **C**, pseudo-PEG derivatives **4** and **6**, the water-soluble analogue of rhodamine 6G (R6G-WS) and its thiol-reactive derivative **12** were prepared according to protocols recently reported by us.[10,29] The HPLC-gradient grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from Acros or Fisher Scientific. Buffers and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm). ^1H 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 $CDCl_3$ ($\delta_H = 7.26$, $\delta_C = 77.36$) or CD_3OD ($\delta_H = 3.31$, $\delta_C = 49.00$).[44] *J* values are in Hz. Infrared (IR) spectra were recorded as a thin-film on sodium chloride plates or KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer with frequencies given in reciprocal centimetres (cm^{-1}). UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorophore-containing compounds were quantified by UV-visible spectroscopy at the λ_{max} using the corresponding tabulated molar extinction coefficient (*i.e.*, 65 000 $dm^3\ mol^{-1}\ cm^{-1}$ for R6G-WS, 150 000 $dm^3\ mol^{-1}\ cm^{-1}$ for Cy 3.0 and 250 000 $dm^3\ mol^{-1}\ cm^{-1}$ for Cy 5.0). Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. Analytical HPLC was performed on a Thermo Electron Surveyor instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Finnigan SpectraSYSTEM liquid chromatography system equipped with UV-visible 2000 detector. Mass spectra were obtained with either a Thermo Finnigan LCQ Advantage Max (ion-trap), or a LCQ Duo (ion-trap), or a LTQ Orbitrap XL apparatus equipped with an electrospray source. The iodoacetamide derivative of sulfocyanine dye Cy 3.0 **11** and the intermediates NHS ester and Cy 3.0 amine involved in its synthesis were characterised by MALDI-TOF mass spectrometry on a Voyager DE PRO in the reflector mode with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Modified oligonucleotide **16-mer ODN** was characterised by MALDI-TOF mass spectrometry measurements on a Brucker Biflex spectrometer using 3-hydroxypicolinic acid as a matrix. The monoclonal antibodies *anti*-AFB1 were obtained as previously described and the corresponding cross-reactivity experiments were performed by using standard conditions.[45]

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, 10 × 250 mm) with CH_3CN and 0.1% aq. acetic acid (aq. AcOH, 0.1%, v/v, pH 3.3) as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 30% (30 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. Dual UV detection was achieved at 220 and 260 nm. **System B:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (30 min) then from 60 to 90% (10 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Triple UV detection was achieved at 210, 260 and 285 nm. **System C:** RP-HPLC (Waters XTerra MS C_{18} column, 5 μm, 7.8 × 100 mm) with CH_3CN and aq. TFA, 0.1% as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (40 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. Dual UV-visible detection was achieved at 230 and 550 nm. **System D:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and aq. TFA, 0.1% as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 20% (5 min) then from 20 to 60% (40 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. Visible detection was achieved at 675 nm. **System E:** system D with visible detection at 550 nm. **System F:** system D with dual visible detection at 550 and 675 nm. **System G:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 100 mm) with CH_3CN and aq. triethylammonium acetate buffer (aq. TEAA, 25 mM, pH 7.0) as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 20% (5 min) then from 20 to 60% (40 min) of CH_3CN] at a flow rate of 3 mL min^{-1}. Triple UV-visible detection was achieved at 220, 550 and 675 nm. **System H:** system B with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (40 min) of CH_3CN] at a flow rate of 1 mL min^{-1}. Visible detection was achieved at 550 nm. **System I:** system C with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 30% (15 min) then from 30 to 80% (100 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. **System J:** system I with the following gradient [5% CH_3CN (5 min), followed by linear gradient from 5 to 45% (40 min) of CH_3CN]. **System K:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 100 mm) with CH_3CN and aq. TEAA 25 mM as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Triple UV-visible detection was achieved at 260, 550 and 650 nm. **System L:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 × 50 mm) with CH_3CN and aq. AcOH, 0.1% as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 80% (40 min) of CH_3CN] at a flow rate of 0.2 mL min^{-1}. Triple UV-visible detection was achieved at 220, 550 and 650 nm. ESI-MS detection was achieved both in negative and positive modes. **System M:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 100 mm) with CH_3CN and aq. TFA, 0.1% as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 20% (5 min) then from 20 to 60% of CH_3CN] at a flow rate of 2.5 mL min^{-1}. Triple UV-visible detection was achieved at 220, 550 and 650 nm. **System N:** system C with the following column Thermo Hypersil GOLD C_{18} (5 μm, 10 × 100 mm).

Oligonucleotide synthesis

The synthesis of oligonucleotide **16-mer ODN** was carried out on an Applied Biosystems 392 DNA/RNA synthesizer using the phosphoramidite chemistry on a scale of 1 μmol. The

insertion of 2′-*O*-propargyl-uridine residue was performed by using the corresponding phosphoramidite unit prepared following the previously described methodologies.[46] Upon completion, the alkyne function-containing oligonucleotide has been deprotected in concentrated aqueous ammonia for 16 h at 55 °C. After speed-vac evaporation of ammonia, the crude 5′-DMTr oligonucleotide was detritylated and purified on-line by RP-HPLC using a polymeric support.[47] After desalting by size exclusion chromatography, the oligonucleotide was then quantified by UV measurements at 260 nm. The purity and the integrity of the synthetic DNA oligomer were checked by RP-HPLC analyses together with MALDI-TOF mass measurements. Finally the alkyne-modified DNA fragment was lyophilised and freezed at –20 °C until use in the "click" reaction.

(2-(2-Aminoethoxy)ethoxy)acetic acid benzyl ester (5)

(a) Esterification. Boc-protected amino acid **4** (600 mg, 2.28 mmol) was dissolved in dry CH_2Cl_2 (10 mL). The resulting solution was cooled to 4 °C, and DMAP (17 mg, 0.14 mmol), TEA (216 µL, 1.54 mmol) and benzyl chloroformate (239 µL, 1.68 mmol) were sequentially added. The resulting reaction mixture was stirred at room temperature overnight and checked for completion by TLC (CH_2Cl_2–AcOEt, 9 : 1, v/v). Volatiles were removed by evaporation under reduced pressure; the resulting residue was taken up in AcOEt (30 mL) and washed by aq. 10% citric acid (30 mL), aq. sat. $NaHCO_3$ (30 mL) and brine (30 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (10 g) with a mixture of CH_2Cl_2–AcOEt (9 : 1, v/v) as the mobile phase, giving benzyl ester as colorless oil (414 mg, 1.2 mmol, yield 53%). R_f (CH_2Cl_2–AcOEt, 9 : 1, v/v) 0.18; ν_{max}(neat)/cm^{-1} 699, 755, 864, 1121, 1149, 1251, 1365, 1391, 1455, 1517, 1711, 1755, 2926, 2925, 3370 (br); δ_H(300 MHz; $CDCl_3$) 1.41 (s, 9H, tBu), 3.26–3.30 (m, 2H, CH_2 PEG), 3.0 (t, J = 5.1 Hz, 2H, CH_2 PEG), 3.60–3.63 (m, 2H, CH_2 PEG), 3.68–3.73 (m, 2H, CH_2 PEG), 4.16 (s, 2H, CH_2 PEG), 5.04 (bs, 1H, NH), 5.16 (s, 2H, CH_2 Bn), 7.32 (s, 5H, Bn); δ_C(75.5 MHz; CD_3OD) 28.4, 40.3, 66.6, 68.6, 70.3 (2C), 70.9, 79.1, 128.5, 128.6, 135.4, 156.0, 170.3; MS (ESI+): m/z 353.93 [M + H]$^+$, 376.07 [M + Na]$^+$, calcd for $C_{18}H_{27}NO_6$: 353.18.

(b) Boc removal. Benzyl ester obtained previously (287 mg, 0.81 mmol) was dissolved in CH_2Cl_2 (20 mL). The solution was cooled to 4 °C and TFA (4.8 mL, 65 mmol) was added dropwise. The mixture was stirred at room temperature for 1 h and checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). The mixture was evaporated to dryness; the resulting residue was taken up in deionised water (20 mL) and lyophilised to give the compound **5** as a yellow oil (quantitative yield). This compound was used in the next coupling reaction step without further purification or analysis. R_f (CH_2Cl_2–MeOH, 9 : 1, v/v) 0.41.

Azido-PEG-benzyl ester spacer (7). Benzyl ester **5** (297 mg, 0.81 mmol) and (2-(2-azidoethoxy)ethoxy)acetic acid **6** (153 mg, 0.81 mmol) were dissolved in a mixture of DMF–CH_3CN (1 : 1, v/v, 8 mL). BOP reagent (430 mg, 0.97 mmol) and DIEA (402 µL, 2.43 mmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 12 h and checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). The mixture was evaporated to dryness, taken up with AcOEt (10 mL), washed by aq. 10% citric acid (10 mL), aq. sat. $NaHCO_3$ (10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (10 g) with a mixture of CH_2Cl_2–MeOH (98 : 2, v/v) as the mobile phase, to give azido-PEG-benzyl ester **7** as a yellow oil (314 mg, 0.74 mmol, yield 91%). R_f (CH_2Cl_2–MeOH, 9 : 1, v/v) 0.47; ν_{max}(neat)/cm^{-1} 593, 700, 753, 846, 1121, 1201, 1284, 1346, 1456, 1542, 1664, 1751, 2109, 2874, 3411 (broad); δ_H(300 MHz; $CDCl_3$) 3.38–3.76 (m, 16H, CH_2 PEG), 4.00 (s, 2H, CH_2 PEG), 4.19 (s, 2H, CH_2 PEG), 5.19 (s, 2H, CH_2 Bn), 7.36 (s, 5H, Bn); δ_C(75.5 MHz; $CDCl_3$) 38.6, 50.6, 66.6, 68.7, 69.9, 71.2, 70.3, 70.7, 70.9 (2C), 128.5, 128.6, 128.7, 135.4, 169.9, 170.3; MS (ESI+): m/z 425.13 [M + H]$^+$, calcd for $C_{19}H_{28}N_4O_7$: 424.19.

Azido-PEG-acid spacer (A). Azido-PEG-benzyl ester spacer **7** (75 mg, 0.18 mmol) was dissolved in MeOH (2 mL) and aq. 1.0 M LiOH (720 µL, 0.72 mmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). The solution was acidified to pH ~ 4 by adding aq. 1.0 M $KHSO_4$. Thereafter, volatiles were removed by evaporation under reduced pressure and the resulting aq. residue was lyophilised to give a white powder. This amorphous powder was suspended in CH_2Cl_2 and filtrated through a Celite® 545 pad. The solvent was removed by rotatory evaporation to give the azido pseudo-PEG-acid spacer **A** as colorless oil (58 mg, 0.17 mmol, quantitative yield). This compound was used in the next coupling reaction step without further purification. R_f (CH_2Cl_2–MeOH, 9 : 1, v/v) 0.20. δ_H(300 MHz; CD_3OD) 3.25–3.27 (m, 2H, CH_2 PEG), 3.35–3.42 (m, 4H, CH_2 PEG), 3.53 (t, J = 5.5 Hz, 2H, CH_2 PEG), 3.59–3.66 (m, 10H, CH_2 PEG), 3.96 (s, 2H, CH_2 PEG), 4.02 (s, 2H, CH_2 PEG); δ_C(75.5 MHz; $CDCl_3$): δ 39.6, 51.7, 69.4, 70.4, 71.1, 71.2, 71.3, 71.3, 71.6, 72.0, 172.8, 174.5; MS (ESI-): m/z 333.47 [M – H]$^-$, 667.00 [2M – H]$^-$, calcd for $C_{12}H_{22}N_4O_7$: 334.14.

Fmoc-Lys((Boc)$_2$-Aoaa)-OH (B). (Boc)$_2$-Aoaa-OH **9** (300 mg, 1.03 mmol) was dissolved in dry CH_3CN (10 mL). *N*-hydroxysuccinimide (130 mg, 1.13 mmol) and DCC (233 mg, 1.13 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 1 h. Thereafter, a solution of Fmoc-Lys–OH (417.4 mg, 1.13 mmol) in dry CH_3CN was added and the resulting reaction mixture was stirred at room temperature. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 96 : 4, v/v) and evaporated to dryness. The resulting residue was taken up with AcOEt (15 mL), washed by aq. 10% citric acid (15 mL) and brine (15 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (40 g) with a step gradient of MeOH (0–8%) in CH_2Cl_2 as the mobile phase, giving the targeted building block **B** as a white foam (266 mg, 0.42 mmol, yield 40%). R_f (CH_2Cl_2–MeOH, 96 : 4, v/v) 0.19; ν_{max}(KBr)/cm^{-1} 641, 732, 846, 912, 1041, 1125, 1350, 1455, 1538, 1670, 2254, 2938, 2983, 3321; δ_H(300 MHz; $CDCl_3$) 1.36–2.06 (m, 24H, CH_2 β, δ, γ Lys, 2 x tBu), 3.29 (t, J = 6.2 Hz, 2H, CH_2 ε Lys), 4.19 (t, J = 6.8 Hz, 1H, CH α Lys), 4.36 (d, 2H, J = 7.2 Hz, CH_2 Fmoc), 4.46 (s, 2H, CH_2 Aoaa), 5.74 (d, J = 7.9 Hz, 1H, CH Fmoc), 7.26–7.91 (m, 8H, Fmoc); δ_C(75.5 MHz; $CDCl_3$) 22.4, 28.0, 28.8, 31.7, 38.8, 47.1, 53.7, 67.1, 76.4, 85.4,

120.0, 125.2, 127.1, 127.7, 141.3, 143.8, 144.0, 150.5, 156.3; MS (ESI-): m/z 640.47 $[M - H]^-$, calcd for $C_{33}H_{43}N_3O_{10}$: 641.29.

Fmoc-Lys((Boc)$_2$-Aoaa)-Cys(SEt)-NH$_2$ (BC). Lysine building block **B** (247 mg, 0.41 mmol) and the TFA salt of H-Cys(SEt)–NH_2 **C** (134 mg, 0.46 mmol) were dissolved in dry CH_3CN (3 mL). BOP reagent (224 mg, 0.51 mmol) and dry DIEA (159 µL, 0.92 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature. After 5 min, dry NMP was added (300 µL) to dissolve a newly formed precipitate and the reaction stirred for a further 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v) and evaporated to dryness. The resulting residue was taken up with AcOEt (10 mL), washed by aq. 10% citric acid (10 mL), aq. sat. $NaHCO_3$ (10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (20 g) with a step gradient of MeOH (0–8%) in CH_2Cl_2 as the mobile phase, to give full-protected dipeptide **BC** as a white foam (208 mg, 259 µmol, yield 62%). R_f (CH_2Cl_2–MeOH, 8 : 2, v/v) 0.48; ν_{max}(KBr)/cm^{-1} 647, 733, 847, 911, 1034, 1122, 1371, 1455, 1530, 1693, 1756, 1790, 2249, 2870, 1932, 2981, 3064, 3322 (br); δ_H(300 MHz; $CDCl_3$) 1.24 (t, J = 7.2 Hz, 3H, CH_3 SEt), 1.41–1.91 (m, 24H, CH_2 β, δ, γ Lys, 2 x tBu), 2.66 (q, J = 7.4 Hz, 2H, CH_2 SEt), 3.00–3.35 (m, 4H, CH_2 β Cys, CH_2 ε Lys), 4.17–4.53 (m, 4H, CH α Lys, CH Fmoc, CH_2 Fmoc), 4.74–4.80 (m, 1H, CH α Cys), 7.27–7.76 (m, 8H, Fmoc); δ_C(75.5 MHz, $CDCl_3$) 14.3, 19.0, 22.4, 27.9, 29.0, 31.3, 32.3, 32.4, 38.2, 39.5, 47.0, 52.0, 52.5, 53.5, 55.3, 67.1, 71.1, 76.5, 77.6, 85.2, 85.4, 119.9, 125.1, 127.1, 127.7, 141.2, 143.68, 143.73, 150.4, 156.7; MS (ESI+): m/z 804.07 $[M + H]^+$, calcd for $C_{38}H_{53}N_5O_{10}S_2$: 803.32.

Fully-protected heterotrifunctional cross-linker (10)

(a) Fmoc removal. Fully-protected dipeptide **BC** (104 mg, 129 µmol) was dissolved in dry CH_3CN (3 mL). Diethylamine (300 µL, 2.9 mmol) was added dropwise and the resulting mixture was stirred at room temperature for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v) and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (10 g) with a step gradient of MeOH (0–50%) in CH_2Cl_2 as the mobile phase, giving amine building block as a white foam (72 mg, 124 µmol, yield 96%). This intermediate compound was immediately used without further analyses. R_f (CH_2Cl_2–MeOH, 8 : 2, v/v) 0.43.

(b) Peptide coupling. Free N-terminal dipeptide (72 mg, 124 µmol) and azido-PEG-acid spacer **A** (50 mg, 149 µmol) were dissolved in dry DMF (2 mL). BOP reagent (66 mg, 149 µmol) and dry DIEA (43 µL, 248 µmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v) and the mixture was evaporated close to dryness. The resulting residue was taken up with AcOEt (10 mL), washed by aq. 10% citric acid (10 mL), aq. sat. $NaHCO_3$ (10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was firstly purified by chromatography on a silica gel column (10 g) with a step gradient of MeOH (0–10%) in CH_2Cl_2 as the mobile phase but the purity of recovered product was not satisfied. Then, this product was dissolved in a mixture of CH_3CN–H_2O (2 : 1, v/v, 3 mL) and purified by semi-preparative RP-HPLC (system A, 2 injections). The product-containing fractions were lyophilised to give fully-protected tripod **10** as a white amorphous powder (22 mg, 24 µmol, yield 20%). δ_H(300 MHz; $CDCl_3$) 1.31 (t, J = 7.4 Hz, 3H, CH_3 SEt), 1.48–2.01 (m, 24H, CH_2 β, δ, γ Lys, 2 x tBu), 2.71 (q, J = 7.4 Hz, 2H, CH_2 SEt), 3.03–3.20 (m, 2H, CH_2 β Cys), 3.30 (q, J = 6.6 Hz, 2H, CH_2 ε Lys), 3.41–3.71 (m, 16H, CH_2 PEG), 4.00 (s, 2H, CH_2 PEG), 4.04 (s, 2H, CH_2 PEG), 4.35–4.42 (m, 3H, CH α Cys, CH_2 Aoaa), 4.68–4.75 (m, 1H, CH α Cys), 5.50 (bs, 1H, NH), 6.72 (bs, 1H, NH), 7.40 (d, J = 7.0 Hz, 1H, NH), 7.67–7.71 (m, 1H, NH); δ_C(75.5 MHz; $CDCl_3$) 14.5, 23.0, 28.2, 29.1, 31.2, 32.6, 38.6, 38.8, 39.3, 50.7, 52.5, 53.7, 70.2, 70.3, 70.5, 70.7, 70.9, 71.1, 85.4, 150.6, 167.9, 170.3, 171.1, 172.0, 172.4; MS (ESI+): m/z 898.33 $[M + H]^+$, 920.20 $[M + Na]^+$, calcd for $C_{35}H_{63}N_9O_{14}S_2$: 897.39.

Azido-tripod (3)

(a) Boc removal. Fully-protected tripod **10** (5.5 mg, 6 µmol) was dissolved in dry CH_2Cl_2 (800 µL) and the solution was cooled to 4 °C. TFA (37 µL, 480 µmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system B) and then evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give the free aminooxy tripod (4.9 mg, 6 µmol, quantitative yield) as a yellow oil. This compound was used in the next deprotection step without further purification or analysis.

(b) Removal of the SEt group. Aminooxy pseudo-peptide (4.9 mg, 6 µmol) was dissolved in aq. 0.1 M $NaHCO_3$ buffer (pH 8.5, 500 µL). DTT (18 mg, 120 µmol) was added and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system B). 0.1% aq. TFA (2 mL) was added and the resulting aq. solution was purified by semi-preparative RP-HPLC (system C, 2 injections). The product-containing fractions were lyophilised to give the targeted azido tripod **3** (0.9 mg, 1.4 µmol, yield 24%). MS (ESI+): m/z 638.33 $[M + H]^+$, 1275.00 $[2M + H]^+$, calcd for $C_{23}H_{43}N_9O_{10}S$: 637.28.

Preparation of thiol-reactive Cy 3.0 derivative (11)

(a) Preparation of Cy 3.0 carboxylic acid, succinimidyl ester. Cy 3.0 carboxylic acid (5.4 mg, 8.5 µmol) was introduced into a Reacti-Vial™ and dissolved in 145 µL of dry NMP. TSTU reagent (2.55 mg, 8.5 µmol) and DIEA (3 mL, 16.9 µmol) in solution in 75 µL of dry NMP were added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system H) and the resulting N-hydroxysuccinimidyl ester was used in the next step without further purification. HPLC (system H): t_R = 26.1 min.

(b) Synthesis of Cy 3.0 amine. Ethylenediamine dihydrochloride (131 mg, 0.98 mmol) was dissolved in a mixture of DMF–H_2O (85 : 15, v/v, 10.2 mL). The crude reaction mixture containing the NHS ester of Cy 3.0 and a solution of DIEA (82.4 µL, 0.47 mmol) in DMF (0.8 mL) were sequentially added. The resulting reaction mixture was protected from light and stirred at room temperature for 30 min. The reaction was checked for completion by

RP-HPLC (system H) and the mixture was evaporated to dryness. The resulting residue was dissolved in 0.1% aq. TFA (3 mL) and purified by RP-HPLC (system I, 2 injections). The product-containing fractions were lyophilised to give Cy 3.0 amine as a pink amorphous powder. HPLC (system H): t_R = 21.1 min, purity > 95%; MS (MADI-TOF+): *m/z* 673.40 $[M + H]^+$, calcd for $C_{33}H_{44}N_4O_7S_2$: 672.26.

(c) Preparation of Cy 3.0 SIAB derivative. Cy 3.0 amine (1.0 mg, 1.35 μmol) was dissolved in 0.1 M aq. borate buffer (100 μL, 50 mM + 5 mM EDTA, pH 8.2). A solution of sulfo-SIAB reagent (0.96 mg, 1.92 μmol) in 0.1 M aq. borate buffer (50 μL) was added. The resulting reaction mixture was protected from light and stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system H). A further amount of sulfo-SIAB reagent (0.35 mg, 0.69 μmol) in 0.1 M aq. borate buffer (30 μL) and borate buffer (100 μL) were added and the mixture was stirred again for 1 h. Finally, the reaction mixture was quenched by dilution with 0.1% aq. TFA (3 mL) and purified by RP-HPLC (system J, 2 injections). The product-containing fractions were lyophilised to give the thiol-reactive Cy 3.0 SIAB derivative **11** as a pink powder (0.5 mg, 0.52 μmol, overall yield for the three steps 3.5%). HPLC (system H): t_R = 24.9 min, purity > 95%; MS (MADI-TOF+): 960.23 $[M + H]^+$, calcd for $C_{42}H_{50}IN_5O_9S_2$: 959.20.

Aminooxy-reactive Cy 5.0 derivative (15)

(a) Synthesis of Cy 5.0 amine. Cy 5.0 carboxylic acid (7.5 mg, 11 μmol) was dissolved in dry NMP (400 μL). TSTU reagent (7 mg, 22 μmol) and DIEA (8 μL, 44 μmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 1 h. Meanwhile, ethylenediamine dihydrochloride (65 mg, 0.49 mmol) was dissolved in a mixture of DMF–H_2O (9 : 1, v/v, 10 mL) and DIEA (174 μL, 1 mmol) was then added. Thereafter the mixture was cooled to 4 °C and NHS ester of Cy 5.0 **13** was added dropwise. The reaction was checked for completion by RP-HPLC (system B) and then quenched by addition of glacial acetic acid to reach pH 3-4. The mixture was evaporated to dryness and then dissolved in aq. TFA 0.1%. The resulting solution was purified by RP-HPLC (system D, 2 injections). The product-containing fractions were lyophilised to give Cy 5.0 amine **14** as a blue amorphous powder. The compound was used in the next step without further purification or analysis.

(b) Preparation of Cy 5.0 aldehyde. 4-Formylbenzoic acid (3.7 mg, 25 μmol) was dissolved in dry NMP (300 μL). TSTU reagent (7.5 mg, 25 μmol) and DIEA (17 μL, 100 μmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 1 h. Cy 5.0 amine was dissolved in dry NMP (100 μL) and then cooled to 4 °C. The mixture containing the NHS ester of 4-formylbenzoic acid was added dropwise to the Cy 5.0 amine solution. The reaction was checked for completion by RP-HPLC (system B) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system D, 2 injections). The product-containing fractions were lyophilised to give Cy 5.0 aldehyde **15** as a blue amorphous powder (0.93 mg, 1.2 μmol, overall yield for the two steps 11%). HPLC (system K): t_R = 22.8 min, purity > 95%; MS (ESI+): *m/z* 831.40 $[M + H]^+$, calcd for $C_{43}H_{50}N_4O_9S_2$: 830.30.

AFB2-alkyne (21). AFB2 carboxylic acid derivative **20** (12 mg, 31 μmol) was dissolved in dry DMF (800 μL). TSTU reagent (18.6 mg, 62 μmol) and DIEA (11 μL, 62 μmol) were sequentially added. The mixture was stirred at room temperature for 1 h. Thereafter, propargylamine (4 μL, 62 μmol) was added and the mixture was stirred at room temperature for a further 1 h. Aq. AcOH 0.1% was added to quench the reaction (1.2 mL) and then the mixture was purified by RP-HPLC (system A, 2 injections). The product-containing fractions were lyophilised to give AFB2-alkyne **21** as a white powder (5 mg, 12 μmol, yield 32%). MS (ESI+): *m/z* 425.13 $[M + H]^+$, 447.27 $[M + Na]^+$, calcd for $C_{22}H_{20}N_2O_7$: 424.12.

FRET cassette (R6G-Cy5.0) labelled AFB2 (22)

(a) S_N2 reaction with iodoacetyl derivative of R6G-WS. Azido tripod **3** (1.5 mg, 2.3 μmol) was dissolved in a mixture of CH_3CN and 0.1 M aq. $NaHCO_3$ buffer (1 : 3, v/v, 800 μL). Thiol-reactive R6G-WS derivative **12** (2.8 mg, 2.6 μmol) was added and the reaction mixture was stirred at room temperature for 4 h. The reaction was checked for completion by RP-HPLC (system B) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system E, 2 injections). The product-containing fractions were lyophilised to give the fluorescent aminooxy tripod **17** as a pink amorphous powder. MS (ESI+): *m/z* 807.60 $[M + 2H]^{2+}$, 1613.20 $[M + H]^+$, calcd for $C_{68}H_{92}N_{16}O_{24}S_3$: 1612.56.

(b) Oxime ligation with Cy 5.0 aldehyde. Fluorescent tripod **17** (0.5 mg, 310 nmol) was dissolved in acetate buffer (pH 4.2, 350 μL). Cy 5.0 aldehyde (0.42 mg, 620 nmol) was added and the resulting mixture was stirred at room temperature for 4 h. The reaction was checked for completion by RP-HPLC (system B) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system F, 2 injections). The product-containing fractions were lyophilised to give the energy transfer azido dye **19** as a purple amorphous powder. MS (ESI+): *m/z* 1214.53 $[M + 2H]^{2+}$, calcd for $C_{111}H_{140}N_{20}O_{32}S_5$: 2424.85.

(c) CuAAC reaction with AFB2-alkyne. FRET cassette **19** (22 μg, 9 nmol) and AFB2-alkyne (5 μg, 12 nmol) were dissolved in H_2O–*t*BuOH (9 : 1, v/v, 100 μL). 10 μL of a 0.1 M aq. $CuSO_4$ solution and 10 μL of a 0.1 M aq. sodium ascorbate solution were added. The reaction mixture was stirred at room temperature for 6 h, with further additions of 10 μL of aq. sodium ascorbate solution every 90 min. The reaction was checked for completion by RP-HPLC (system B) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system F, 2 injections). The product-containing fractions were lyophilised to give the fluorescent conjugate of AFB2 **22** as a purple amorphous powder (16.2 μg, 8.1 nmol). MS (ESI-): *m/z* 1424.73 $[M - 2H]^{2-}$, calcd for $C_{133}H_{162}N_{22}O_{39}S_5$: 2850.99.

FRET cassette (R6G-WS-Cy5.0) labelled oligonucleotide (23)

CuAAC reaction with alkyne-oligonucleotide. FRET cassette **19** (11.1 μg, 5 nmol) and alkyne-oligonucleotide **16-mer ODN** (39 μg, 8 nmol) were dissolved in H_2O-DMSO (1 : 1, v/v, 80 μL). 8 μL of a 10 mM aq. $CuSO_4$ solution, 8 μL of 10 mM TBTA solution in DMSO, 4 μL of a 50 mM aq. DIEA solution, and 8 μL of 10 mM aq. sodium ascorbate solution were sequentially added.

The resulting reaction mixture was stirred at room temperature for 90 min. Thereafter, further amounts of $CuSO_4$ (8 μL) and sodium ascorbate (8 μL) were added. The reaction mixture was stirred at room temperature overnight. Further amounts of $CuSO_4$ (8 μL) and sodium ascorbate (8 μL) were added again and the reaction was stirred at room temperature for 90 min. The crude was desalted by size exclusion chromatography on a NAP-5 column with deionised water as eluent. The fractions containing coloured product were dried in a speed-vac for 4 h. The resulting residue was taken up in a mixture of CH_3CN and 0.1 M aq. TEAA buffer pH ~ 7 (1 : 9, v/v, 1 mL) and purified by RP-HPLC (system G, 1 injection). The product-containing fractions were dried in a speed-vac to give the FRET cassette labelled oligonucleotide as a purple amorphous powder (16.2 μg, 8.1 nmol). HPLC (system K): t_R = 21.8 and 21.9 min (mixture of two racemic diastereomers), purity > 95%; MS (ESI–): *m/z* 728.76 $[M - 10H]^{10-}$, 809.84 $[M - 9H]^{9-}$, 911.20 $[M - 8H]^{8-}$, calcd: 7298.97.

FRET cassette (Cy3.0-Cy5.0) labelled oligonucleotide (24)

(a) S_N2 reaction with iodoacetyl derivative of Cy 3.0. Azido tripod **3** (0.38 mg, 0.6 μmol) was dissolved in a mixture of CH_3CN and 0.1 M aq. $NaHCO_3$ buffer (1 : 3, v/v, 500 μL). Thiol-reactive Cy 3.0 derivative **11** (0.5 mg, 0.52 μmol) was added and the reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by LC-MS (system L) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system M, 2 injections). The product-containing fractions were lyophilised to give the fluorescent aminooxy tripod **16** as a pink amorphous powder. MS (ESI+): *m/z* 850.4 $[M + 2H + 2TFA]^{2+}$, calcd for $C_{65}H_{92}N_{14}O_{19}S_3$: 1468.58.

(b) Oxime ligation with Cy 5.0 aldehyde. Fluorescent tripod **16** was dissolved in acetate buffer (pH 4.2, 350 μL). Cy 5.0 aldehyde **15** (62.3 μg, 75 nmol) was added and the resulting mixture was stirred at room temperature for 4 h. The reaction was checked for completion by LC-MS (system L) and then quenched by addition of aq. TFA 0.1% (2 mL). The mixture was purified by RP-HPLC (system M, 2 injections). The product-containing fractions were lyophilised to give the energy transfer azido dye **18** as a purple amorphous powder (2.6 μg, 8.2 nmol). MS (ESI+): *m/z* 1141.61 $[M + 2H]^{2+}$, calcd for $C_{108}H_{140}N_{18}O_{27}S_5$: 2280.87.

(c) CuAAC reaction with alkyne-oligonucleotide. The same conditions as those described for the synthesis and purification of fluorescent oligonucleotide conjugate **23** were used. HPLC (system K): t_R = 19.5 min, purity > 95%; MS (ESI-): *m/z* 714.36 $[M - 10H]^{10-}$, 793.96 $[M - 9H]^{9-}$, 893.45 $[M - 8H]^{8-}$, 1021.09 $[M - 7H]^{7-}$, calcd: 7154.92.

Fluorescent labelling and immobilisation of mycotoxin AFB2 on silica surfaces

(a) Preparation of the aldehyde-functionalised silica surface. This was achieved from a silicon substrate (square, 10 × 10) doped with a thick SiO_2 layer (thickness 500 nm) by using experimental conditions already reported by us.[10]

(b) CuAAC reaction with alkyne derivative of AFB2. R6G-WS fluorescent labelled tripod **17** (100 μg, 60 nmol) and AFB2-alkyne **21** (34 μg, 80 nmol) were dissolved in H_2O-DMSO (1 : 1, v/v, 20 μL). 10 μL of a 10 mM aq. $CuSO_4$ solution, 10 μL of a 10 mM TBTA solution in DMSO, 4 μL of a 50 mM aq. DIEA solution, and 10 μL of 10 mM aq. sodium ascorbate solution were sequentially added. The resulting reaction mixture was stirred at room temperature for 90 min. Further amounts of $CuSO_4$ (10 μL) and sodium ascorbate (10 μL) were added and the reaction was stirred at room temperature overnight. Further amounts of $CuSO_4$ (10 μL) and sodium ascorbate (10 μL) were added again and the reaction was stirred at room temperature for a further 90 min. 0.1% aq. TFA (1 mL) was added and the crude product was purified by RP-HPLC (system M, 1 injection). The product-containing fractions were lyophilised to give the fluorescent aminooxy derivative **25** as a pink amorphous powder. HPLC (system N): t_R = 28.0 min, purity 93%; MS (ESI+): *m/z* 1039.64 $[M + H + K]^{2+}$, calcd for $C_{90}H_{112}N_{18}O_{31}S_3$: 2036.69

(c) Immobilisation of fluorescent AFB2 conjugate. A 40 μM solution of the aldehyde-reactive fluorescent AFB2 derivative **25** was prepared in sodium acetate buffer and 1 μL of this solution was put over the freshly prepared aldehydic surface. After 2 h of incubation in a humid atmosphere at room temperature, the reaction solution was carefully removed and the surface was washed with deionised water, 0.2% aq. SDS, deionised water and dried. Fluorescence scanning was performed with an Olympus inverted microscope model IX71 (4X objective) equipped with a camera PCO 1600, and the resulting images were analysed using ImageJ software (http://rsbweb.nih.gov/ij/).

SPIT-FRI experiment

The detection of **AFB1** through the SPIT-FRI method was performed by using the thick layer previously prepared (*vide supra*) and a continuous-flow device (flow rate of 100 μL min^{-1}, thermostated at 22 °C) previously described.[42] Once the layer fixed into the device, the EIA buffer (0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.1% BSA and 0.01% sodium azide) was pumped through the microchannels for 30 min. Then, the activation step was performed by passing the *anti*-AFB1 mAb solution (1.5 μM in EIA buffer) for 40 min. A further washing using EIA buffer was done for 40 min. Finally, the competition step was performed by passing the AFB1 solution (0.5 μM in EIA buffer) was performed for 50 min.

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