A novel heterotrifunctional peptide-based cross-linking reagent for facile access to bioconjugates. Applications to peptide fluorescent labelling and immobilisation†‡

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Received 29th April 2008, Accepted 2nd June 2008
First published as an Advance Article on the web 30th June 2008
DOI: 10.1039/b807263a

A convenient, versatile and straightforward synthesis of a novel heterotrifunctional peptide-based linker molecule is described. This generic bio-labelling reagent contains an amine-reactive *N*-hydroxysuccinimidyl carbamate moiety, an aldehyde/ketone-reactive aminooxy group and a thiol group with a propensity to form urea, oxime and thioether linkages respectively. The full chemical orthogonality between the free aminooxy and thiol functionalities was demonstrated through the preparation of a fluorescent reagent suitable for the selective staining of a carboxaldehyde-modified surface by means of oxime ligation. The absence of reactivity of these two functions toward the nucleophile-sensitive active carbamate was obtained by using temporary aminooxy- and thiol-protecting groups removable under mild conditions. The utility of the linker molecule to cross-link three different molecular partners has been illustrated by the preparation of fluorescent tripod-functionalised surfaces which may be useful in developing new peptide microarrays and related immunosensors.

Introduction

Recent advances in the field of bioconjugate chemistry have spurred the construction of novel engineered molecules based on chemoselective coupling reactions either between two (or more) different biopolymers or between a biopolymer and various chemical reporter groups. As the resulting bioconjugates possess the combined and unaltered properties of their individual components, bioconjugation appears to be the best approach to introduce a high level of chemical diversity within complex biopolymers and provide sophisticated chemically engineered biomolecular tools essential for various applications in diagnostics, therapeutics and related biomedical fields.[1]

The conjugation of two or more (bio)molecules is most frequently achieved through two sequential steps: (1) the incorporation of mutually reactive groups into the individual molecular components, and (2) their bio-orthogonal coupling in solution leading to the formation of a stable chemical linkage such as carboxamide, disulfide, hydrazone, oxime, thioether, carbo- or heterocycle (*i.e.*, Diels–Alder cycloadducts, thiazolidine or triazole moieties). The use of a heterobifunctional cross-linking reagent is often required for the implementation of such biocompatible synthetic strategies. Indeed, numerous commercially available heterobifunctional coupling agents enable the conversion of free amino (or sulfhydryl) groups of a biopolymer into carboxamides (or thioethers) bearing various terminal reactive moieties, including activated mixed disulfide, azido, carboxaldehyde, iodoacetyl, maleimide and succinimidyl esters. Thereafter, the resulting adduct is conjugated to other (bio)molecular components through a second chemoselective reaction to give the targeted bioconjugate. However, such a strategy is not suitable for the preparation of bioconjugates resulting from the covalent association of three different molecular partners. In this case, the use of a cross-linking reagent bearing three different orthogonal reactive groups is thus required. These trifunctional building blocks are key components in the preparation of molecular 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;[2] the energy transfer terminators (*i.e.*, dideoxynucleotides labelled with FRET cassettes) for DNA sequencing;[3] and the biopolymer microarrays currently used for the rapid analysis of various biological events.[4] However, only few cross-linkers equipped with a triply orthogonal set of functional groups have been reported to date. Some heterotrifunctional reagents suitable for bio-labelling applications

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† Such heterotrifunctional cross-linking reagents were covered by a patent entitled "Réactif pseudo peptidique trifonctionnel, ses utilisations et applications" filed on 11th July 2007 at the European Patent Office: G. Clavé, P.-Y. Renard, A. Romieu and H. Volland, no. 07/0518.

‡ Electronic supplementary information (ESI) available: Detailed synthetic procedures for compounds **4**, **7**–**13** and **A**; characterisation data for compounds **A**, **B**, **C**, **5**, **19** and **23**. See DOI: 10.1039/b807263a

are commercially available from Pierce (*e.g.*, Sulfo-SBED biotin label reagent **1**, see Fig. 1 for the corresponding structure) but one of the three available functionalities found in these trifunctional reagents is always the biotin moiety, which is used as an affinity probe (through its strong interaction with avidin, neutravidin or streptavidin protein) and not as a reactive group for subsequent reactions with a biopolymer or a reporter group.[5] Recently, Watzke *et al.* reported the design and synthesis of an aromatic building block **2** for C- and N-terminal protein labelling and protein immobilisation.[6] The presence of an azido group, an S-protected cysteine residue and a carboxylic acid onto the benzene ring, enable 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, and 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.

With the goal in mind to develop a universal, versatile and ready-to-use bio-labelling reagent, compatible with fragile biopolymers, we have explored the chemistry of a new family of heterotrifunctional linker molecules based on a dipeptidyl architecture (*i.e.*, lysine–cysteine) that contains an amine-reactive *N*-hydroxysuccinimidyl carbamate, an aldehyde/ketone-reactive aminooxy group,[7] a thiol group and an hydrophilic pseudo-PEG spacer (Fig. 1). In this paper, we describe the general synthesis of a representative heterotrifunctional cross-linking reagent **3**, used in a protected form **5**, and to illustrate its efficacy in coupling a representative biomolecule (*i.e.*, a neuropeptide) to two different molecular partners (namely, a chemical reporter group and a solid phase). The first targeted application is thus the detection of neuropetide substance P (SP) through the original immunoassay SPIT-FRI (for Solid-Phase Immobilised Tripod for Fluorescent Renewable Immunoassay) recently developed by us.[8]

Results and discussion

Considerations for the design and synthesis of heterotrifunctional cross-linking reagents suitable for bio-labelling applications

The two obvious synthetic strategies to obtain heterotrifuntional linkers consist in derivatising either a triply substituted benzene (the strategy used by Watzke *et al.*) or a functionalized amino acid (*e.g.*, aspartic acid, lysine),[9] readily available from commercial sources. As we suspect that an aromatic core could

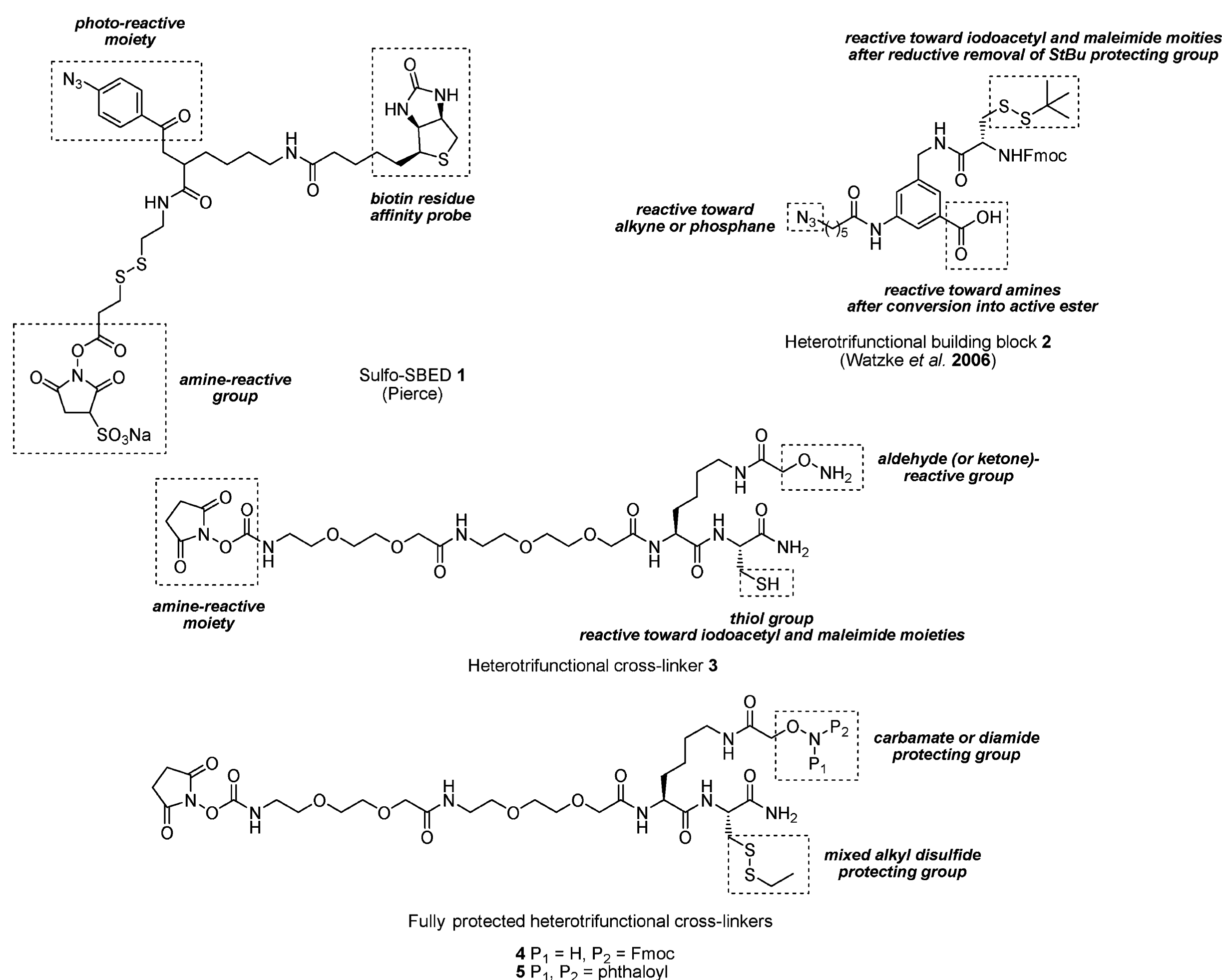

Fig. 1 Structures of Sulfo-SBED biotin label reagent **1**, heterotrifunctional cross-linker **2** developed by Watzke *et al.*[6] and peptide-based cross-linking reagents **3–5** studied in this work.

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impede some of the physicochemical properties of the resulting bioconjugates (*e.g.*, fluorescence properties), we decided to use a pseudo-peptidyl architecture as the scaffold of our tripodal systems. The choice of its constituting amino acids was guided by the following requirements: (1) their reactive side-chains should be easily and selectively converted into bioconjugatable groups, preferably through standard peptide coupling reactions, (2) the corresponding heterotrifunctional reagents must display good water solubility properties achieved by the presence of hydrophilic moieties within their core structures and/or side-chains, and (3) the synthetic accessibility of the suitably protected building blocks must be as easy as possible, especially for the non-natural amino acids. Thus, we have selected L-cysteine, L-lysine and an original amino-PEG-acid spacer. A key parameter in the design of our heterotrifunctional linker lies in its shape and length versatility: the chosen hydrophilic pseudo-PEG spacer can be introduced between any of the functional groups (and two spacers can be introduced at different positions), and its length can be easily adapted to any targeted use for the linker. Moreover, in order to have a flexible and easy synthetic access to the heterotrifunctional linker, we chose a highly convergent and versatile synthetic strategy (Fig. 2), based on fully protected building blocks and well-established solution-phase peptide coupling reactions. Concerning the choice of the three bioconjugatable groups, we have focused on the well-known bioconjugate chemistry of the aminooxy and thiol groups.[10] The aminooxy group was chosen since we have already experimented in its use in biomolecule immobilisation on the solid phase,[11,12] and since we suspected (and proved in this study, *vide infra*) that its particular "super-nucleophile" character would allow an orthogonal reactivity compared with the sulfhydryl group. We also took advantage of the original and unprecedented "wet chemistry" of the *N*-hydroxysuccinimidyl carbamate moiety, whose potential in the construction of biconjugates (through the formation of urea linkages) has never been reported,[13] although the activated carbamates are more stable than their parent activated esters. The sequential derivatisation of the tri-orthogonal set of functional groups of on reagent **3** should enable the covalent association of three different (bio and/or macro)molecules in a highly efficient manner respectively through chemoselective *N*-acylation reaction (under Schotten–Bauman conditions), Michael addition (or S_N2 reaction) and oxime ligation. Despite the full orthogonality between the aminooxy and thiol groups (*vide infra*), these bioconjugatable moieties were kept protected subsequent to their introduction onto the peptide scaffold, to avoid self-degradation of the heterotrifunctional reagent through side reactions with the reactive electrophilic carbon center of the third functional group. It thus appeared essential to use aminooxy- and thiol-protecting groups removable under mild conditions (aqueous buffers, pH 6–9 and room temperature are preferred) prior to their derivatization in order to keep the integrity of the (bio)molecules previously anchored onto the trifunctional peptide-based linker. The aminooxy phthalimide protection and the sulfhydryl protection as mixed ethyl disulfide (SEt)[14] were used for the temporary masking of these nucleophilic moieties, and so

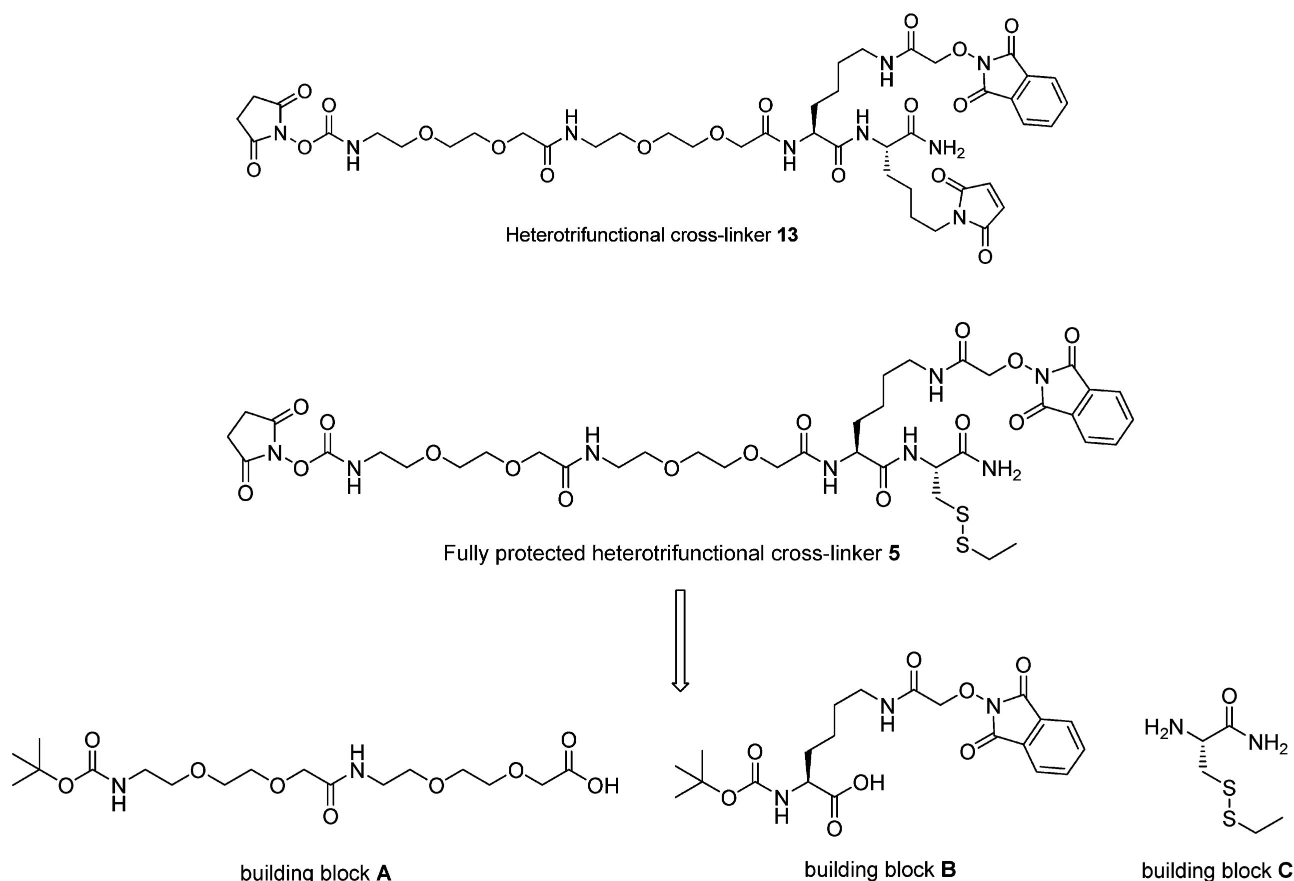


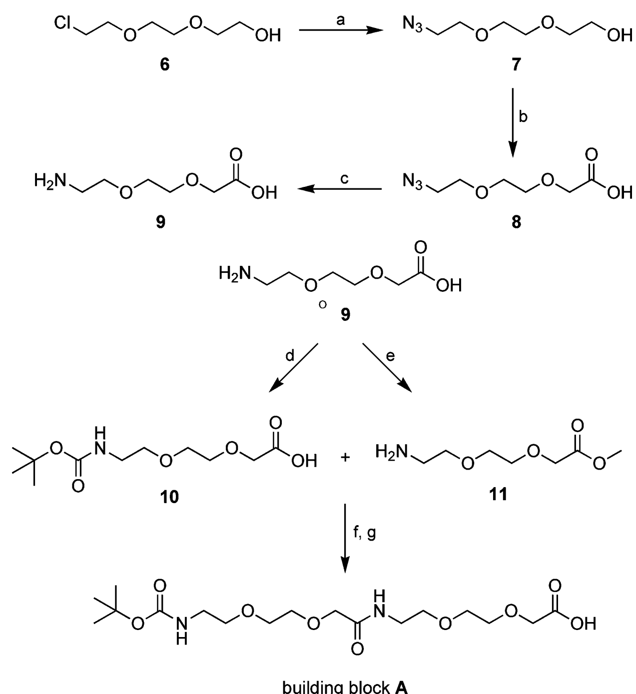
Fig. 2 Building block approach to the solution-phase synthesis of heterotrifunctional cross-linking reagent **5**.

the synthesis of fully protected derivative **5** was achieved§. As illustrated in Fig. 2, the use of three functionalised amino acid building blocks **A–C** enabled us to develop a highly convergent synthetic strategy based on solution-phase peptide couplings, suitable for the generation of a large set of heterotrifunctional peptide based-linkers derived from **5**. Indeed, the re-use of some building blocks avoided having to start the synthesis from the beginning with the commercially available lysine and cysteine derivatives, and additional joining of a pseudo-PEG linker can be achieved on any of the building blocks.

Synthesis of building blocks A–C used for the preparation of heterotrifunctional cross-linking reagents

Synthesis of Boc-protected amino-PEG-acid spacer A. The PEG-based building block was introduced within the peptide scaffold both as a flexible spacer between the anchored biomolecule and the two other molecular components of the resulting bioconjugate, and in order to increase reagent solubility in water and related aqueous buffers. Linker **A** was synthesised in 29% overall yield through a 7-step synthetic procedure depicted in Scheme 1. Commercially available 2-(2-(2-chloroethoxy)ethoxy)ethanol **6** was allowed to react with sodium azide in the presence of sodium iodide as a catalyst, under refluxing ethanol, producing azido compound **7** in quantitative yield. This alcohol was subjected to Jones' oxidation to produce carboxylic acid **8**. Conversion of the azido group into primary amine was achieved by Pd/C catalytic hydrogenation. Two units of the resulting amino acid **9** were assembled by using standard peptide chemistry. The Boc urethane and the methyl ester were chosen respectively as N- and C-terminal protecting functions, and BOP phosphonium salt in the presence of DIEA was used as coupling reagent.[15] As the final step, the methyl ester was removed by short treatment of the fully protected pseudo-PEG linker with 1 M LiOH in H_2O–MeOH, to give the target building block **A**. All spectroscopic data (see ESI‡), in particular NMR and mass spectrometry, were in agreement with the structure assigned.

Synthesis of aminooxy-containing lysine building block B. The introduction of the super-nucleophile aminooxy moiety within synthetic biopolymers (especially synthetic peptides) is often achieved through coupling reaction between a free amino group on the target (bio)macromolecule and a N-protected derivative of aminooxy acetic acid (Aoaa).[16] Carbamate protecting groups such as Aloc or Boc are often used for amine protection of Aoaa.[17] However, their removal conditions, under Pd(0) catalysis and harsh acidic conditions respectively, are not compatible with the stability of the bioconjugates targeted through the sequential



Scheme 1 *Reagents and conditions*: a) NaN_3 (1.2 equiv), NaI (0.1 equiv), EtOH, reflux, 5 days, quant. yield; b) Jones' reagent (3 equiv), acetone, 4 °C to rt, 1 h, 91%; c) 10% Pd/C, H_2, EtOH, 12 h, quant. yield; d) Boc_2O (1.1 equiv), 2 M aq. NaOH, THF, 4 °C to rt, 63%. e) 2,2-dimethoxypropane, 37% HCl, 1 h, 79%. f) BOP (1 equiv), DIEA (3 equiv), CH_3CN, overnight; g) 1 M aq. LiOH, MeOH, rt, 51% (overall yield for steps f–g). DIEA = *N*,*N*-diisopropylethylamine, Boc_2O = *tert*-butyl dicarbonate, BOP = ((benzotriazole-1-yl)oxy)tris(dimethylamino)phosphonium hexafluorophosphate.

derivatisation of reagent **5**. In this context, we first explored the use of carbamate-type protecting groups removable under mild reducing conditions (*i.e.*, azidomethyloxycarbonyl (Azoc),[18] 2-pyridyldithioethyloxycarbonyl (Pydec)[19]), but the corresponding *N*-protected Aoaa derivatives were found to be too unstable to be easily handled. Thus, we turned our attention to the use of the hydrazine-labile phthalimide protecting group (Pht), which represents a good compromise between stability and lability. The phthaloyl protection of this α-nucleophilic moiety has already been successfully used in the field of bioconjugate chemistry especially for the solid-supported synthesis of oligomeric bioconjugates and for the surface immobilisation of biopolymers through oxime ligation.[20] Furthermore, in contrast to the single carbamate-based protecting groups (*i.e.*, Aloc or Boc), the phthalimide moiety ensures complete protection of the nitrogen of aminooxyacetic acid, therefore preventing the N-overacylation side-reaction frequently encountered during aminooxy peptide synthesis.[21,22] To incorporate the Aoaa moiety within the dipeptidyl architecture of our heterotrifunctional reagent, we designed building block **B**, which corresponds to N^{α}-Boc-L-lysine acylated by Pht-Aoaa-OH **12** (Scheme 2). This *N*-phthaloyl building block was activated as the hydroxybenzotriazole (OBt) ester and subsequently coupled to the free ε-amino group of lysine. This pre-activation procedure avoided an additionnal protection step of the lysine carboxylic acid function. Pht-Aoaa-OH was converted into the corresponding OBt esters by treatment with DCC–HOBt in CH_3CN–DMF

§ Synthesis of *N*-Fmoc aminooxy heterotrifunctional cross-linking reagent **4** was also achieved (see ESI‡). Preliminary bio-derivatisation attempts were performed with this Fmoc derivative, especially through acylation reactions of its active carbamate moiety with various amine-containing peptides, but the resulting conjugates were found to be highly hydrophobic. Thus, the purification and handling of such mono derivatised reagents, especially in aqueous buffers, are not trivial tasks. Furthermore, the premature cleavage of the Fmoc group of reagent **4** was observed during some bioconjugation reactions performed in alkaline buffers. Consequently, in the present article, we have focused on the derivatisation of the phthalimide derivative **5**, but bioconjugate applications of **4** could be envisaged, especially those involving more hydrophilic biopolymers such as nucleic acids.

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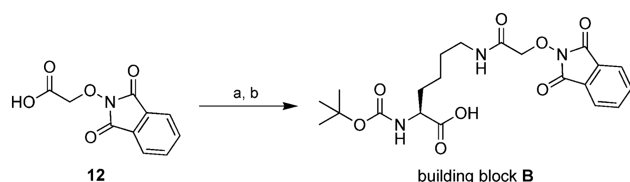
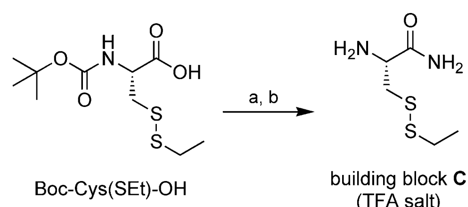


Scheme 2 *Reagents and conditions*: a) DCC (1.2 equiv), HOBt·H_2O (1.2 equiv), CH_3CN–DMF (1 : 1, v/v), rt, 2 h; b) Boc-Lys-OH (1 equiv), DMF, rt, 2 h, quant. yield. DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt·H_2O = hydroxybenzotriazole monohydrate.



Scheme 3 *Reagents and conditions*: a) isobutyl chloroformate (1 equiv), NMM (1 equiv), ethyl acetate, −15 °C, 10 min then 20% aq. NH_3 (3 equiv), 4 °C, 30 min, quant. yield; b) TFA–H_2O (95 : 5, v/v), 4 °C to rt, 1 h, quant. yield. NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid.

(1 : 1).[23] This crude mixture of active esters was directly reacted with Boc-Lys-OH to provide the corresponding building block **B** in quantitative yield. It is important to note that during the course of our work, Dumy *et al.* reported the use of 1-ethoxyethylidene (Eei)-protected Aoaa for the synthesis of aminooxy peptides suitable for oxime ligation.[22,24] The Eei protecting group prevents N-overacylation and can be removed under mild acidic conditions (*i.e.*, aqueous solution of 1% or 5% TFA in CH_3CN). Thus, the use of this Aoaa building block in the synthesis of heterotrifunctional reagent such as **5** could also have been considered, but would have required the choice of an alternative protection scheme for the three building blocks involved in the synthesis of **5** (*e.g.*, Fmoc strategy).

Synthesis of cysteine building block C. In our first attempts to prepare trifunctional-bioconjugable cross-linking reagents, we investigated the synthesis of a pseudo-dipeptide (*i.e.*, lysine–lysine) containing the hydrophilic pseudo-PEG spacer derived from building block **A**, the amine-reactive *N*-hydroxysuccinimidyl carbamate, the Aoaa moiety and a thiol-reactive maleimide group. The corresponding heterotrifunctional reagent **13** was obtained but the yield was impaired by the poor stability exhibited by the maleimide group during the chemical assembly process of building blocks. Indeed, the propensity of the imido group of maleimides to undergo spontaneous hydrolysis or related nucleophilic ring-opening reactions has already been reported in the literature[25] and was observed by us during the coupling reaction between Boc-protected amino-PEG-acid spacer **A** and the two lysine building blocks.

In this context, we decided to use a thiol group as the third bioconjugatable reactive group of our reagents. The commercial availability of N,S-diprotected L-cysteine derivatives has enabled the obtaining of the required building block **C** in only two synthetic steps. The mixed ethyl disulfide (SEt) was used as thiol protection moiety because of its high stability under acidic and basic conditions and its lability under mild reducing conditions fully compatible with sophisticated bioconjugation schemes involving fragile biopolymers. Furthermore, in contrast to the corresponding aryl derivatives (*e.g.*, 3-nitro-2-pyridylthio, Npys), this mixed disulfide is not prone to hydrolytic cleavage under the alkaline conditions (*i.e.*, pH 8–9) currently used in some bioconjugation reactions. As depicted in Scheme 3, the synthesis of building block **C** started with the conversion of the free carboxylic acid function of Boc-Cys(SEt)-OH into the non-reactive carboxamide moiety by treatment of the pre-formed isobutyloxy mixed anhydride with aqueous ammonia.[26] Finally, the Boc group on carboxamide **14** was removed by treatment with trifluoroacetic acid to yield building block **C** as a TFA salt in quantitative yield.

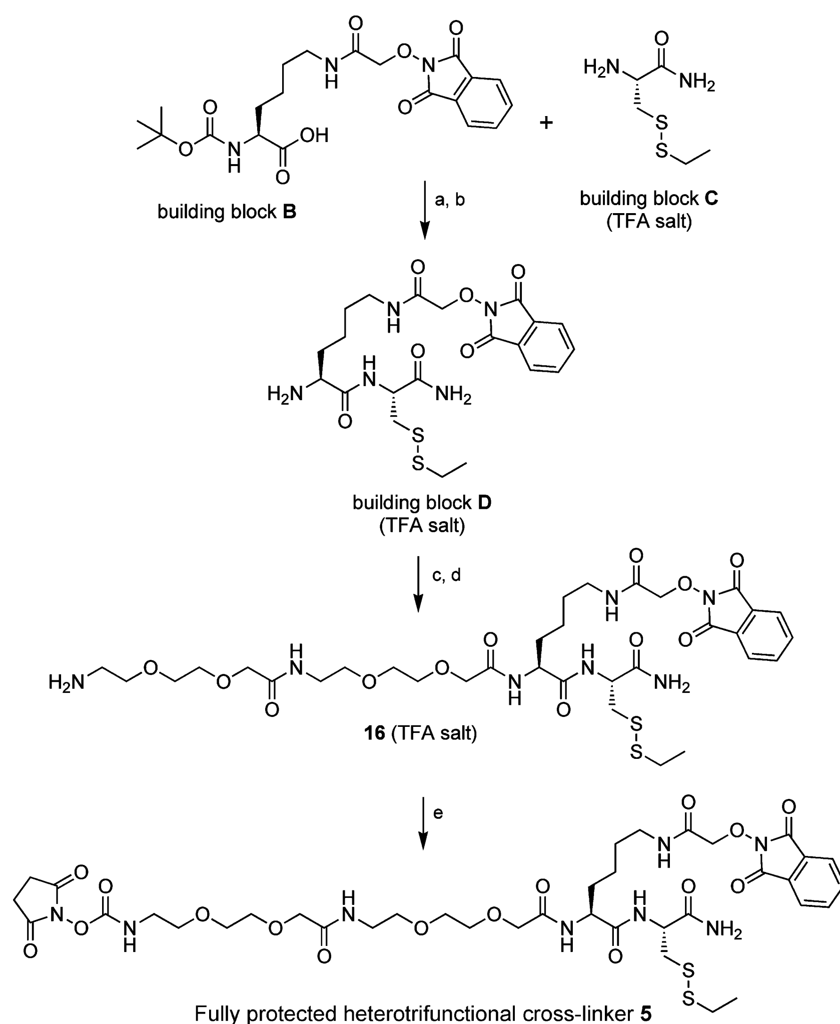
Synthesis of *N*-phthaloyl aminooxy heterotrifunctional cross-linking reagent 5 using amino acid building blocks

Heterotrifunctional reagent **5** was synthesised as shown in Scheme 4. Due to the availability of the three amino acid building blocks **A**, **B** and **C**, a highly convergent solution-phase synthesis from S-protected amino acid H-Cys(SEt)-NH_2 was developed. 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 12% TFA solution in CH_2Cl_2 to give building block **D** in quantitative yield. This latter pseudo-dipeptide was then subjected to the same two-step reaction sequence: BOP-mediated coupling with building block **A** to obtain the fully protected trifunctional pseudo-peptide **15**, and subsequent Boc removal to give the free N-terminal PEG-peptide **16** in 35% overall yield for the two steps. Conversion of the primary amino group into *N*-hydroxysuccinimidyl carbamate was performed with *N*,*N*′-disuccinimidyl carbonate (DSC) and TEA in dry DMF.[27] Purification was achieved by reverse-phase HPLC to give the fully protected heterotrifunctional reagent **5** in quantitative yield. For this chromatographic purification, the use of an acidic mobile phase (*i.e.*, aq. TFA, 0.1%) was found to be essential to prevent premature hydrolysis of the active carbamate moiety. All spectroscopic data, especially NMR and mass spectrometry, were in agreement with the structure assigned (see ESI‡). Interestingly, this reagent could be stored at −20 °C for several months without detectable degradation.

Bioconjugation applications of fully protected heterotrifuntional cross-linking reagent 5

To demonstrate the potential utility of reagent **3** in the field of bioconjugate chemistry, the development of a reliable protocol enabling the sequential and chemoselective derivatization of the three reactive groups with three different (bio)molecular partners was achieved.

Firstly, we have studied both the chemical compatibility between the free aminooxy and thiol functions and the orthogonality of their respective protecting groups. As mentioned in the introduction, a possible application of our heterotrifunctional reagent concerns biochips and biosensors and thus immobilisation and visualisation of biopolymers on solid surfaces.[28] In order to validate the immobilisation step, we planned the synthesis of the fluorescent aminooxy reagent **19** bearing a rhodamine 6G label and suitable for immobilisation on a aldehyde-functionalised surface by means of oxime ligation (Fig. 3A).[29] To avoid interferences with the active carbamate, *N*-Boc-protected derivative **15** was employed in the corresponding derivatisation reactions. As the



Scheme 4 *Reagents and conditions*: a) BOP (1.1 equiv), DIEA (3.1 equiv), CH_3CN–DMF (1 : 1, v/v), rt, 3 h, quant. yield; b) 12% TFA, CH_2Cl_2, 4 °C to rt, 3 h, quant. yield; c) building block **A** (1 equiv), BOP (1.1 equiv), DIEA (3 equiv), CH_3CN–DMF (1 : 1, v/v), rt, 3 h, 45% after RP-HPLC purification; d) 7.5% TFA, CH_2Cl_2, 4 °C to rt, 1 h, 78%; e) DSC (2.5 equiv), TEA (2.5 equiv), DMF, 2 h, quant. yield after RP-HPLC purification.

iodoacetyl derivatives are known to exhibit excellent selective reactivity toward sulfhydryl groups especially when the corresponding S_N2 alkylation reaction is performed in aqueous media at slightly alkaline pH,[30] we have decided to perform a fluorescent labelling reaction between a iodoacetamide-based fluorescent reagent and the pseudo-peptide **17** bearing free aminooxy and thiol groups (Scheme 5).

Firstly, removal of the phthaloyl and SEt protecting groups were achieved successively under standard conditions: treatment with 1 equiv of hydrazine monohydrate in MeOH and treatment with an excess of dithiothreitol (DTT) in a mixture of NMP and aqueous sodium bicarbonate buffer (pH 8.5) respectively. The resulting *N*-Boc-protected pseudo-peptide **17** was isolated by reverse-phase HPLC in a satisfactory yield (overall yield for the two steps: 45%). The iodoacetamide derivative of rhodamine dye R6G-WS **18** was attached to the free cysteine of the pseudo-peptide **17** through a S_N2 reaction performed in aqueous sodium bicarbonate buffer (pH 8.5). R6G-WS is a water-soluble analogue of rhodamine 6G recently developed by us,[31] whose spectral properties are similar to commercially available Alexa Fluor® 532 and Fluo Probe® 532 labels. The thiol-reactive derivative **18** was prepared by using the three-step synthetic procedure reported by Bouteiller *et al.* for sulfocyanine dyes.[32] The HPLC elution profile of the reaction mixture has clearly shown the absence of the doubly labelled pseudo-peptide resulting from a competitive non-selective anchoring of rhodamine dye onto the aminooxy and thiol functions.[33] The moderate 28% isolated yield is explained by significant losses of fluorescent conjugate during the chromatographic purification. For the immobilisation of the fluorescent reactive label **19** by means of oxime ligation, a silica surface had to be decorated with an appropriately functionalised carboxaldehyde. The introduction of these required aldehyde functions onto solid supports was achieved by using original surface chemistry developed by us.[11,34] For the spotting experiments, the aminooxy reagent **19** (positive control) as well as the non-reactive oxime derivative **20** (obtained by mixing **19** with acetone) as a negative control were dissolved in deionised water at 50 μM. Subsequent to the manual spotting process, the slides were incubated in a humid atmosphere for 15 min. After removal of excess reagent and some washings, the fluorescent

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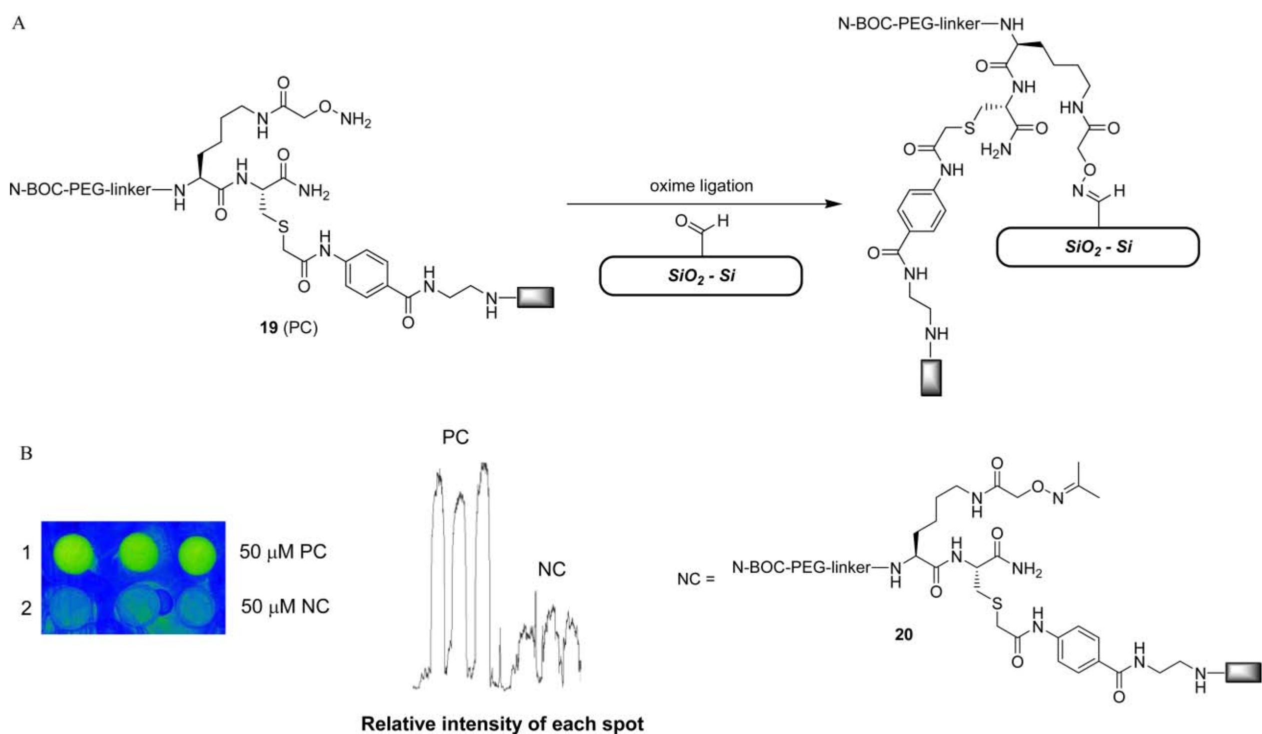
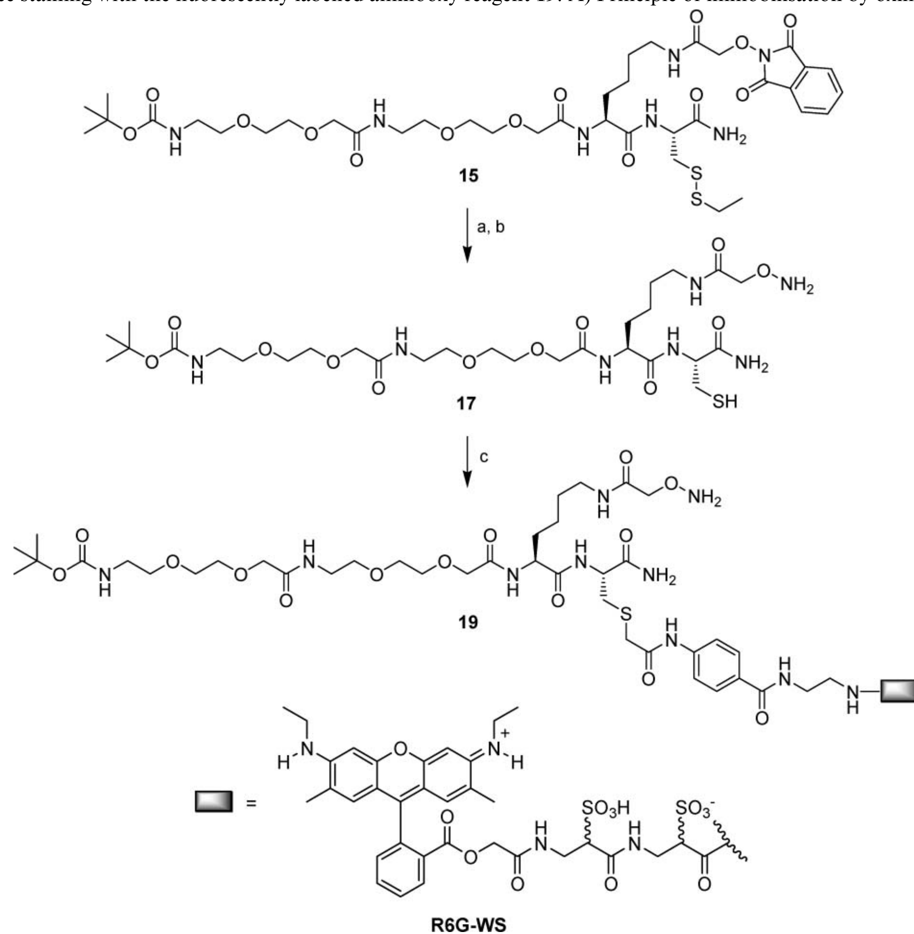


Fig. 3 Silica surface staining with the fluorescently labelled aminooxy reagent **19**. A) Principle of immobilisation by oxime ligation. B) Results.

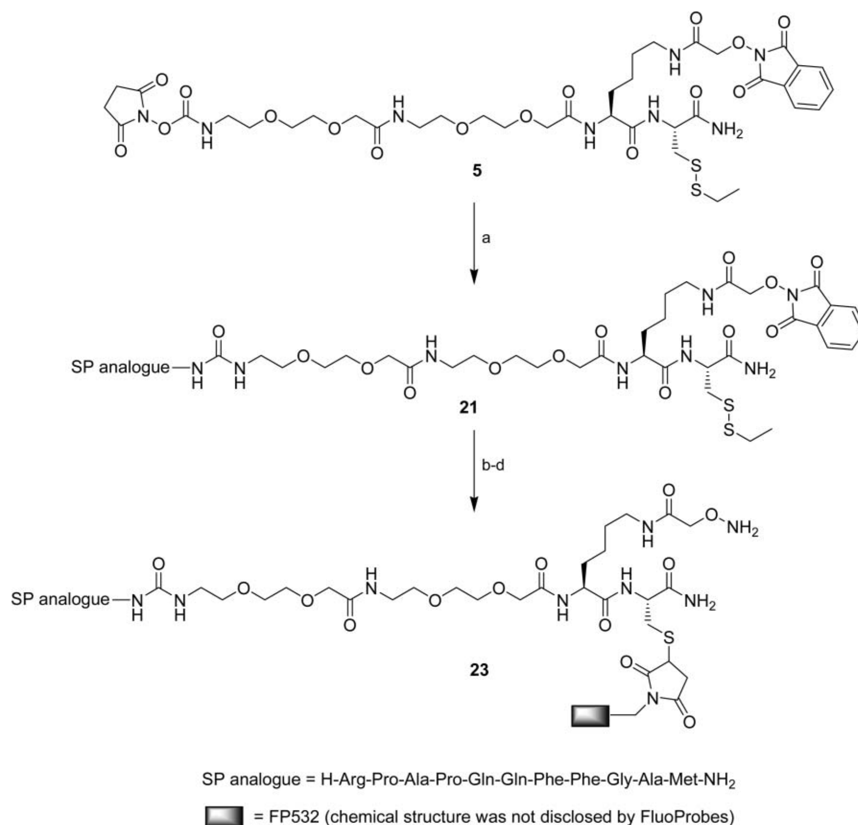


Scheme 5 *Reagents and conditions*: a) Hydrazine monohydrate (1 equiv), MeOH, rt, 1 h, 63% after RP-HPLC purification; b) DTT (30 equiv), 0.1 M aq. $NaHCO_3$ buffer (pH 8.5), NMP, rt, 3 h, 71% after RP-HPLC purification; c) thiol-reactive fluorophore **18** (1 equiv), 0.1 M aq. $NaHCO_3$ buffer (pH 8.5), rt, 1 h, 28%.

signals were recorded and quantified. The obtained results are shown in Fig. 3B. The immobilisation of **19** gave clear fluorescent signals, whereas poor signals were obtained for the negative control. These results demonstrate that the immobilisation of the aminooxy pseudo-peptide **19** onto the aldehyde-modified silica surface was successful.

Furthermore, the full chemical orthogonality between free aminooxy and thiol functions within the same molecule was demonstrated for the first time, and this should be a valuable tool for the design of new bioconjugation strategies.

Preparation of fluorescent tripod-functionalized surfaces suitable for the detection of neuropeptide substance P through the SPIT-FRI immunoassay. In addition to these preliminary experiments and to probe the ability of heterotrifunctional cross-linking reagents such as **3** to conjugate three different molecular partners, we have prepared fluorescent tripod functionalised surfaces suitable for the detection of neuropeptide SP through the original SPIT-FRI immunoassay.[8] SP is a neuropeptide composed of eleven amino acid residues with the following sequence: H-Arg-Pro-Lys-Pro-Glu-Glu-Phe-Phe-Gly-Leu-Met-OH determined by Chang and Leeman.[35] This short peptide is both a neurotransmitter and a neuromodulator. In the central nervous system, SP has been associated with the regulation of mood disorders, anxiety, stress, reinforcement, neurogenesis, respiratory rhythm, neurotoxicity, nausea, emesis and pain. Its detection in complex biological environments has already been validated by several methods including immunoassays.[36] SPIT-FRI detection of this neuropeptide has already been achieved using a peptide construction derived from SP, bearing a fluorescent label and immobilised on solid surface through the neutravidin–biotin interaction.[8] Such a modified peptide was readily prepared by solid-phase synthesis but its non-general structure restricted its use for the preparation of biochips for detection of SP only. To demonstrate the general applicability of our heterotrifunctional reagent, we wished to prepare a similar tripod bearing the SP peptidyl sequence, a fluorescent label and the aminooxy function for subsequent surface immobilisation by means of oxime ligation, through a simple and sequential solution-phase derivatisation approach. As described for the previous SPIT-FRI experiments on SP, we worked with a validated analogue of this neuropeptide in which all reactive amino acid residues were replaced by non-reactive amino acids and the C-terminal carboxylic acid converted into carboxamide: H-Arg-Pro-*Ala*-Pro-*Gln*-*Gln*-Phe-Phe-Gly-*Ala*-Met-NH_2. This latter peptide was reacted with a slight excess of reagent **5** in NMP in the presence of DIEA to obtain linker-peptide **21**. As expected, the acylation reaction was found to be fast and efficient. Purification was achieved by reverse-phase HPLC to give **21** in almost quantitative yield. It is also possible to perform this N-terminal modification reaction in a slightly alkaline buffer (pH 7–8) compatible with the stability of most proteins, but in the present case the resulting urea derivative **21** was obtained in a lower yield. Thereafter, the Pht and SEt protecting groups



Scheme 6 *Reagents and conditions*: a) H-Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Ala-Met-NH_2 (1 equiv), DIEA (6 equiv), NMP, rt, 6 h then acetic acid (4 equiv), 92% after RP-HPLC purification; b) hydrazine monohydrate (1 equiv), MeOH, rt, 1 h, RP-HPLC purification; c) DTT (20 equiv), 0.1 M borate buffer (pH 8.2), NMP, rt, 2 h then acetic acid (2 equiv), RP-HPLC purification; d) FluoProbe® 532A maleimide (1 equiv), DIEA (3 equiv), NMP, rt, 2 h, RP-HPLC purification, 15% (overall yield for b + c + d). NMP = *N*-methylpyrrolidone.

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were cleanly removed by using the two-step synthetic procedure previously used for the preparation of fluorescent aminooxy reagent **19** (see Scheme 6). Finally, the site-selective fluorescent labelling of peptide **22** was achieved by Michael addition of the thiol group to the maleimide of a water-soluble analogue of rhodamine 6G (Fluo Probe® 532, FP532). The rhodamine dye–peptide conjugate **23** was isolated in a pure form by reverse-phase HPLC (overall yield for the three steps: 15%). Its structure was confirmed by ESI mass spectrometry (see ESI‡). Interestingly, as already observed for the iodoacetamide derivative **18**, no further reaction between the thiol-reactive maleimide moiety and the aminooxy group was observed. To obtain biochips suitable for SP detection through SPIT-FRI method, solid-phase immobilisation of fluorescent tripod **23** was performed in the same manner as described for the fluorescent aminooxy reagent **19**. After washings, an intense fluorescence signal was observed and proved the efficacy of the oxime ligation to covalently graft SP related tripods to a solid surface (Fig. 4). In a second step, FP647-labelled anti-SP monoclonal antibodies (mAb) which are able to recognise both SP and its analogue, were added. As Fluo Probe® 647 acts as a quencher of FP532, a decrease of fluorescence emission of FP532 *via* FRET was observed. Competition experiments using original SP aimed at detecting and quantifying this neuropetide are in progress, and the corresponding results will be reported in due course. Indeed, as previously reported with the biotinylated tripod,[8] the added analyte (*i.e.*, SP) will compete with the solid-phase tripod for the binding to the specific antibody, further leading to the displacement of the tripod–antibody complex and thus to an increase of the tripod-related fluorescence proportional to the amount of the analyte.

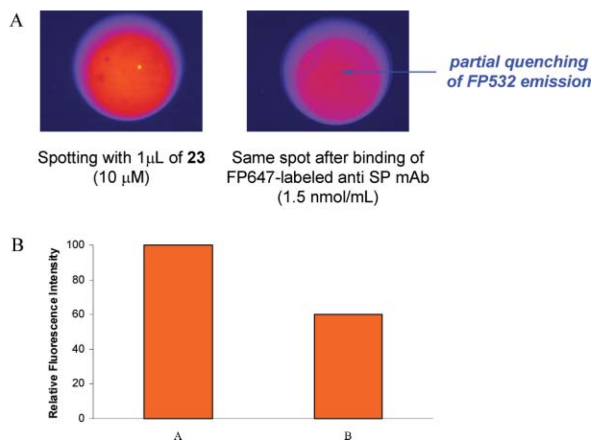

Fig. 4 A) Immobilisation of fluorescent tripod **23** on the aldehyde-functionalised silica surface by oxime ligation as described in Fig. 3A. B) Binding of FP647-labelled anti-SP monoclonal antibodies.

These preliminary results clearly show that fluorescent tripods such as **23**, readily obtained through step-by-step bioconjugation protocols, are promising tools for both immobilisation and visualisation of biopolymers.

Conclusion

A new heterotrifunctional cross-linking reagent based on a hydrophilic peptide scaffold and bearing an original set of three orthogonal reactive groups has been developed. The flexible and efficient methodology for the solution-phase synthesis of this new class of pseudo-peptides relies on the use of three pre-synthesised building blocks. The linker reported herein could be of general utility to cross-couple three different molecular partners and to obtain sophisticated (bio)conjugates that are not accessible by derivatisation of commercially available cross-linking reagents. Thus, it provides a valuable tool for the introduction of different functional groups of interest into biopolymers. This has been demonstrated in the present work by utilising the linker molecule **5** for the covalent attachment of an aminooxy function and a fluorescence marker to the N-terminus of neuropeptide substance P, which could be further immobilised on aldehyde-modified glass slides *via* highly chemoselective oxime ligation. This new strategy for the site-specific immobilisation and visualisation of peptides (and proteins) onto a silica surface is a promising method that may prove useful in the next future for the ready preparation of new generations of peptide (or protein) microarrays suitable for immunoassay applications.[37]

Experimental‡

General

Column chromatography purifications were performed on silica gel (40–63 µm) from SdS. 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.*, $\lambda = 254$ nm), (2) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol, (3) spray with a 3.5% (w/v) phosphomolybdic 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: distillation over BaO). Anhydrous ethanol was obtained by storing absolute ethanol (EtOH, VWR PROLABO, AnalaR NORMAPUR) over anhydrous Na_2SO_4. Ethyl acetate (AcOEt, Riedel de Haën, extra pure) was dried by storage over 4 Å molecular sieves. DIEA and TEA were distilled from CaH_2 and stored over BaO. The water-soluble analogue of rhodamine 6G (R6G-WS) was prepared by using literature procedures.[31] *N*-Sulfosuccinimidyl[4-iodoacetyl]aminobenzoate (Sulfo-SIAB) and FluoProbes® 532A maleimide were purchased from Pierce and Interchim respectively. N^α-(*tert*-Butyloxycarbonyl)-L-lysine (Boc-Lys-OH) was prepared by hydrogenolysis of commercially available N^α-(*tert*-butyloxycarbonyl)-N^ε-(benzyloxycarbonyl)-L-lysine (Boc-Lys(Z)-OH, Bachem). *N*-(*tert*-Butyloxycarbonyl)-*S*-(ethylthio)-L-cysteine dicyclohexylammonium salt (Boc-Cys(SEt)-OH). DCHA was purchased from Bachem. Synthetic analogue of substance P was synthesised in the "Laboratoire d'Etudes et de Recherche en Immunoanalyse" using a ABI 433A apparatus (Applied Biosystems) and standard Fmoc chemistry. The HPLC-gradient grade solvents (acetone, MeOH and CH_3CN) were obtained from Acros or Fisher Scientific. Buffers and aqueous 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) relative to the residual solvent signal: CD_3CN ($\delta_H = 1.96, \delta_C = 1.79$ (CH_3), 118.26 (CN)), $CDCl_3$ ($\delta_H = 7.26, \delta_C = 77.36$), CD_3OD ($\delta_H = 3.31$, $\delta_C = 49.00$ (CH_3), or D_2O ($\delta_H = 4.79$).[38] *J*

values are in Hz. Infrared (IR) spectra were recorded as 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. Chromophore-containing compounds were quantified by UV-visible spectroscopy at the λ_{max} using the corresponding tabulated molar extinction coefficient. 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-vis 2000 detector. Mass spectra were obtained with a Thermo Finnigan LCQ Advantage Max (ion-trap) apparatus equipped with an electrospray source or a Voyager DE PRO MALDI-TOF mass spectrometer (reflector mode, by using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix).

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, 4.6 × 150 mm) with CH_3CN and 0.1% aqueous trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.0) as the eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (30 min) of CH_3CN] at a flow rate of 1 mL min^{-1}. Triple UV detection was achieved at 210, 260 and 285 nm. **System B**: System A with the following gradient [20% CH_3CN (5 min), followed by linear gradient from 20 to 90% (35 min) of CH_3CN]. UV detection was achieved at 254 nm. **System C**: System A with visible detection at 530 nm. **System D**: RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2 × 250 mm) with CH_3CN and deionised water as eluents [10% CH_3CN (5 min), followed by linear gradient from 10 to 40% (15 min) and 40 to 70% (40 min) of CH_3CN] at a flow rate of 20 mL min^{-1}. Dual UV detection was achieved at 254 and 305 nm. **System E**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and 0.1% aq. TFA as eluents [10% CH_3CN (5 min), followed by linear gradient from 10 to 40% (15 min) and 40 to 70% (40 min) of CH_3CN] at a flow rate of 5 mL min^{-1}. Dual UV detection was achieved at 254 and 305 nm. **System F**: System E with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 30% (30 min), then from 30 to 60% (15 min) of CH_3CN] at a flow rate of 4 mL min^{-1}. Visible detection was achieved at 550 nm. **System G**: 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}. UV detection was achieved at 220 and 260 nm. **System H**: RP-HPLC (MS C_{18}, Waters XTerra, 5 μm, 7.8 × 100 mm) with CH_3CN and aq. TFA as eluents [0% CH_3CN (5 min), followed by linear gradient from 0 to 50% (50 min) of CH_3CN] at a flow rate of 2 mL min^{-1}. UV detection was achieved at 305 nm. **System I**: System H with the following gradient [0% CH_3CN (5 min), followed by linear gradient from 0 to 60% (40 min) of CH_3CN]. Visible detection was achieved at 550 nm.

***N*^α-(*tert*-Butyloxycarbonyl)-*N*^ε-(phthalimidooxyacetyl)-L-lysine (B).** Phthalimidooxyacetic acid **12** (259 mg, 1.1 mmol) was dissolved in a mixture of dry CH_3CN–DMF (1 : 1, v/v, 10 mL). Hydroxybenzotriazole monohydrate (162 mg, 1.2 mmol) and DCC (250 mg, 1.1 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 1 h under an argon atmosphere. Thereafter, a solution of N^{α}-Boc-L-lysine (317 mg, 1.2 mmol) in dry DMF (1 mL) was added and the resulting reaction mixture was stirred at room temperature. The reaction was checked for completion by RP-HPLC (system A) and TLC (CH_2Cl_2–MeOH, 8 : 2, v/v). The mixture was evaporated to dryness. The resulting residue was taken up with ethyl acetate, washed by 10% aq. citric acid and brine. 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 with a step gradient of MeOH (0–5%) in CH_2Cl_2 as the mobile phase, to give the lysine building block **B** as a white foam (512 mg, 1.1 mmol, quantitative yield). R_f (CH_2Cl_2–MeOH, 8 : 2, v/v) 0.68; IR (KBr): ν_{max} 518, 588, 703, 784, 878, 912, 964, 1030 (broad), 1081, 1164 (broad), 1249, 1368, 1469, 1520, 1732, 1792, 2936, 3357 (broad) cm^{-1}; ^1H NMR (300 MHz, CD_3OD): δ 1.59–1.87 (m, 15H, CH_2 β, δ, γ Lys, tBu), 3.31–3.37 (q, J = 6.3 Hz, 2H, CH_2 ε Lys), 4.25–4.29 (t, J = 5.5 Hz, 1H, CH α Lys), 4.72 (s, 2H, CH_2 phthalimidooxy-acetyl), 7.74–7.87 (m, 4H, phthalimide), 10.74 (s, 1H, CO_2H); ^{13}C NMR (75.5 MHz, CD_3OD): δ 22.5, 28.3, 28.7, 32.0, 39.1, 53.3, 53.5, 80.0, 124.1, 128.5, 135.2, 163.2, 167.8, 175.6; MS (ESI+): m/z 449.33 $[M + H]^+$, 472.13 $[M + Na]^+$, calcd for $C_{21}H_{27}N_3O_8$ 449.46.

***N*-(*tert*-Butyloxycarbonyl)-*S*-(ethylthio)-L-cysteine carboxamide (14).** Boc-Cys(SEt)-OH. DCHA (0.5 g, 1.08 mmol) was dissolved in dry ethyl acetate (15 mL) and the resulting solution was cooled to −15°C (bath of ethylene glycol–dry ice). NMM (119 μL, 1.08 mmol) and isobutyl chloroformate (140 μL, 1.08 mmol) were sequentially added and the resulting mixture was stirred at −15 °C for 10 min under an argon atmosphere. To this mixed anhydride solution was added 20% (v/v) aq. ammonia solution (0.38 mL, 3.24 mmol) and the mixture was stirred at 4 °C for 30 min. Thereafter, the reaction mixture was evaporated to dryness; the resulting residue was taken up in ethyl acetate and washed with deionised water. The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. 302 mg (1.08 mmol) of Boc-Cys(SEt)-NH_2 **14** was obtained as a white solid (quantitative yield). R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.47; IR (KBr): ν_{max} 623, 653, 714, 759, 778, 822, 860, 947, 969, 1021, 1046, 1115, 1169, 1255, 1270, 1320, 1370, 1393, 1429, 1517, 1662, 1686, 2781, 2871, 2930, 2975, 3186, 3342, 3389 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.24–1.29 (t, J = 6.8 Hz, 3H, CH_3(SEt)), 1.39 (s, 9H, tBu), 2.63–2.70 (q, J = 7.1 Hz, 2H, CH_2(SEt)), 2.99–3.01 (d, J = 6.0 Hz, 2H, CH_2 β), 4.36–4.39 (m, 1H, CH α), 5.25–5.28 (d, J = 9.0 Hz, 1H, NH), 5.63 (bs, 1H, NH), 6.31 (bs, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.8, 27.8, 32.0, 39.9, 53.0, 80.1, 155.1, 172.6; MS (MALDI-TOF, positive mode): m/z 303.13 $[M + Na]^+$, calcd for $C_{10}H_{20}N_2O_3S_2$ 280.41.

***S*-(Ethylthio)-L-cysteine carboxamide (C).** Boc-Cys(SEt)-NH_2 **14** (336 mg, 1.2 mmol) was slowly dissolved in a stirred TFA–H_2O mixture (95 : 5, v/v, 14 mL) while being warmed from 4 °C to rt over 1 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v) and the mixture was evaporated almost to dryness. Deionised water was added and the resulting solution was lyophilised to give the cysteine building block **C** as a white powder (353 mg, 1.2 mmol, quantitative yield). R_f

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(CH_2Cl_2–MeOH, 9 : 1, v/v) 0.09; IR(KBr): ν_{max} 582, 676, 725, 801, 844, 869, 955, 1136, 1185, 128, 1302, 1346, 1441, 1531, 1574, 1615, 1682, 2361, 2979, 3188, 3314, 3391 cm^{-1}; ^{1}H NMR (300 MHz, D_2O): δ 1.26–1.31 (t, J = 7.1 Hz, 3H, CH_3(SEt)), 1.39 (s, 9H), 2.72–2.80 (q, J = 7.1 Hz, 2H, CH_2(SEt)), 3.25–3.26 (d, J = 6.0 Hz, 2H, CH_2 β), 4.30–4.35 (m, 1H, CH α); ^{13}C NMR (75.5 MHz, D_2O): δ 13.8, 30.1, 38.3, 52.2, 170.8; MS (MALDI-TOF, positive mode): m/z 181.00 [M + H]$^+$, calcd for $C_5H_{12}N_2OS_2$ 180.29.

N^{α}-(Phthalimidooxyacetyl)-L-lysinyl-S-(ethylthio)-L-cysteine carboxamide (D). ***(a) Coupling reaction***: Lysine building block **B** (215 mg, 0.48 mmol) and TFA salt of H-Cys(SEt)-OH **C** (141 mg, 0.48 mmol) were dissolved in a mixture of dry CH_3CN–DMF (1 : 1, v/v, 5 mL). BOP reagent (233 mg, 0.53 mmol) and freshly distilled DIEA (275 µL, 1.5 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 3 h under an argon atmosphere. The reaction was checked for completion by RP-HPLC (system A) and TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). Thereafter, the mixture was evaporated to dryness. The resulting residue was taken up with ethyl acetate, washed by 10% aq. citric acid, aq. sat. $NaHCO_3$ and brine. 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 with a step gradient of MeOH (0–5%) in CH_2Cl_2 as the mobile phase, to give the coupling product as a white foam (288 mg, 0.47 mmol, quantitative yield). R_f (CH_2Cl_2–MeOH, 9 : 1, v/v) 0.58; IR (KBr): ν_{max} 518, 588, 702, 786.2, 846, 878, 912, 1030 (broad), 1082, 1132, 1167 (broad), 1251, 1288 (broad), 1367, 1467, 1524 (broad), 1667 (broad), 1737, 1792, 2868, 1929, 3321 (broad) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27–1.32 (t, 3H, J = 7.3 Hz, CH_3(SEt) Cys), 1.32–2.03 (m, 15H, CH_2 β, δ, γ Lys, tBu), 2.66–2.74 (q, 2H, J = 7.3 Hz, CH_2(SEt) Cys), 3.05–3.19 (m, 2H, CH_2 β Cys), 3.29–3.45 (m, 2H, CH_2 ε Lys), 3.99–4.05 (m, 1H, CH α Lys), 4.70 (s, 2H, CH_2 phthalimidooxy-acetyl), 4.70–4.77 (m, 1H, CH α Cys), 5.52–5.54 (d, J = 5.6 Hz, 1H, NH), 5.75 (bs, 1H, NH), 6.95 (bs, 1H, NH), 7.79–7.83 (m, 4H, phthalimide); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.4, 22.7, 28.4, 28.9, 29.8, 32.5, 38.4, 39.5, 52.4, 53.6, 124.3, 128.5, 135.3, 163.9, 167.4, 172.6; MS (ESI+): m/z 611.67 [M + H]$^+$, 629.00 [M + H_2O]$^{+\bullet}$, calcd for $C_{26}H_{37}N_5O_8S_2$ 611.74. ***(b) Removal of the Boc group***: Fully protected dipeptide (223 mg, 0.36 mmol) was dissolved in dry CH_2Cl_2 (12 mL) and the solution was cooled to 4 °C. TFA (1.6 mL, 21.9 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 3 h. The reaction was checked for completion by TLC (CH_2Cl_2–MeOH, 9 : 1, v/v) and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and the resulting aqueous solution was lyophilised to give the dipeptide building block **D** as a white amorphous powder (221 mg, 0.35 mmol, quantitative yield). This compound was used in the next coupling reaction step without further purification.

Fully protected heterotrifunctional cross-linker (5). ***(a) Coupling reaction***: The TFA salt of dipeptide **D** (269 mg, 0.43 mmol) and Boc-protected amino-PEG-acid spacer **A** (175 mg, 0.43 mmol) were dissolved in dry CH_3CN–DMF (1 : 1, v/v, 5 mL). BOP reagent (209 mg, 0.47 mmol) and dry DIEA (222 µL, 1.29 mmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 3 h under an argon atmosphere. The reaction was checked for completion by RP-HPLC (system A) and TLC (CH_2Cl_2–MeOH, 9 : 1, v/v). Thereafter, the reaction mixture was evaporated to dryness. The resulting residue was taken up with ethyl acetate, washed by 10% aq. citric acid, sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtrated and evaporated to dryness. The resulting residue was dissolved in a mixture of CH_3CN–H_2O (2: 1, v/v, 4.5 mL) and purified by RP-HPLC (system D, 3 injections, t_R = 31.3–32.6 min). The product-containing fractions were lyophilised to give the coupling product **15** as a white amorphous powder (133 mg, 0.15 mmol, yield 45%). R_f (CH_2Cl_2–MeOH, 9 : 1, v/v) 0.50; IR (KBr): ν_{max} 703, 755, 878, 1127 (broad), 1253, 1367, 1456, 1339, 1662.3 (broad), 1733, 1791, 2926, 3344 (broad) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27–1.32 (t, 3H, J = 7.3 Hz, CH_3(SEt) Cys), 1.43–1.95 (m, 15H, CH_2 β, δ, γ Lys, tBu), 2.66–2.73 (q, 2H, J = 7.3 Hz, CH_2(SEt) Cys), 3.04–3.15 (m, 2H, CH_2 β Cys), 3.39–4.02 (m, 18H, CH_2 ε Lys + 8 × CH_2 linker), 4.03 (s, 4H, 2 × CH_2 linker), 4.39–4.45 (m, 1H, CH α Lys), 4.68–4.72 (m, 3H, CH_2 phthalimidooxy-acetyl + CH α Cys), 5.23 (bs, 1H, NH), 6.00 (bs, 2H, NH_2), 6.16 (bs, 1H, NH), 6.97 (bs, 1H, NH), 7.46 (bs, 2H, 2 x NH), 7.79–7.83 (m, 4H, phthalimide); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.4, 22.8, 28.5, 28.8, 31.4, 32.5, 38.8, 38.9, 39.2, 40.5, 52.6, 53.5, 70.1, 70.3, 70.4, 71.1, 71.2, 124.3, 128.6, 135.3, 163.9, 167.5, 171.0, 171.2, 172.0, 175.7; HPLC (system A): t_R = 26.9 min; MS (ESI+): m/z 902.00 [M + H]$^+$, 919.00 [M + H_2O]$^{+\bullet}$, 924.33 [M + Na]$^+$, calcd for $C_{38}H_{59}N_7O_{14}S_2$ 902.06. ***(b) Removal of the Boc group***: Fully protected pseudo-peptide **15** (25 mg, 28 µmol) was dissolved in dry CH_2Cl_2 (2 mL). The solution was cooled to 4 °C and TFA (164 µL, 2.2 mmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A) and the mixture was evaporated to dryness. A minimum of deionised water was added (*ca.* 3 mL) and the resulting aq. solution was purified by RP-HPLC (system D, 2 injections, t_R = 31.4–35.0 min). The product-containing fractions were lyophilised to give the PEG-peptide **16** as a white amorphous powder (20 mg, 22 µmol, yield 78%). HPLC (system A): t_R = 22.7 min, purity 95%; MS (ESI+): m/z 802.60 [M + H]$^+$, calcd for $C_{33}H_{51}N_7O_{12}S_2$ 801.94. ***(c) Conversion into N-hydroxysuccinimidyl carbamate***: TFA salt of PEG-peptide **16** (20 mg, 22 µmol) was dissolved in dry DMF (500 µL). TEA (3.7 µL, 26.4 µmol) and 50 µL of a solution of DSC reagent in dry DMF (14 mg, 55 µmol) were sequentially added and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system A). Finally, the reaction mixture was quenched by dilution with aq. TFA 0.1% (pH 2, *ca.* 2 mL) and purified by RP-HPLC (system E, 2 injections, t_R = 25.9–27.8 min). The product-containing fractions were lyophilised to give **5** as a white amorphous powder (20 mg, 21 µmol, quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23–1.27 (t, 3H, J = 7.2 Hz, CH_3(SEt) Cys), 1.44–1.93 (m, 6H, CH_2 β, δ, γ Lys), 2.65–2.73 (q, 2H, J = 7.2 Hz, CH_2(SEt) Cys), 2.82 (s, 4H, 2 × CH_2 succinimide), 2.98–3.18 (m, 2H, CH_2 β Cys), 3.33–3.77 (m, 18H, CH_2 ε Lys + 8 × CH_2 linker), 4.03 (s, 4H, 2 × CH_2 linker), 4.43–4.45 (m, 1H, CH α Lys), 4.68–4.71 (m, 3H, CH_2 phthalimidooxy-acetyl + CH α Cys), 6.06 (bs, 1H, NH), 6.90 (bs, 1H, NH), 7.00–7.04 (m, 3H, NH + NH_2), 7.78–7.83 (m, 4H, phthalimide); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.4, 22.8, 25.6, 28.8, 31.5, 32.5, 38.9, 39.0, 39.2, 41.9, 52.6, 53.3, 69.5, 70.1, 70.2, 70.3, 71.0, 71.1, 77.4, 124.2, 128.6, 135.3, 152.0, 163.9, 167.7, 170.4, 171.3, 171.3, 172.1, 173.5; HPLC (system A):

t_R = 25.2 min, purity 92%; MS (ESI+): *m/z* 943.27 [M + H]$^+$, 965.20 [M + Na]$^+$, calcd for $C_{38}H_{54}N_8O_{16}S_2$ 943.03.

Sulfhydryl aminooxy pseudo-peptide (17). ***(a) Removal of the phthaloyl group***: Fully protected pseudo-peptide **15** (11.0 mg, 12.2 μmol) was dissolved in MeOH (1.5 mL) and a solution of hydrazine monohydrate (12.2 μmol) in MeOH (250 μL) was added. The resulting reaction mixture was stirred at room temperature for 1 h. The deprotection reaction was checked for completion by RP-HPLC (system A). Thereafter, volatiles were removed under reduced pressure without warming. 0.1% aq. TFA (pH 2, 1 mL) and CH_3CN (1 mL) were added and the resulting solution was purified by RP-HPLC (system G, 2 injections, t_R = 32.8–39.2 min). The product-containing fractions were lyophilised to give aminooxy pseudo-peptide as a white amorphous powder (5.9 mg, 7.6 μmol, yield 63%). ^{1}H NMR (300 MHz, D_2O): δ 1.23–1.28 (t, 3H, J = 7.3 Hz, CH_3(SEt) Cys), 1.40–1.86 (m, 15H, CH_2 β, δ, γ Lys, *t*Bu), 2.67–2.74 (q, 2H, J = 7.3 Hz, CH_2(SEt) Cys), 3.19–3.27 (m, 4H, CH_2 β Cys, CH_2 ε Lys), 3.44–3.48 (t, J = 5.5 Hz, 2H, CH_2 linker), 3.56–3.60 (t, J = 5.3 Hz, 2H, CH_2 linker), 3.64–3.74 (m, 8H, 4 × CH_2 linker), 4.00 (s, 4H, 2 × CH_2 linker), 4.06 (s, 2H, CH_2 linker), 4.15 (s, 2H, CH_2 linker) 4.10 (s, 2H, CH_2 linker), 4.35–4.37 (t, J = 2.0 Hz, 1H, CH α Lys), 4.40–4.63 (t, J = 4.6 Hz, 1H, CH α Cys); HPLC (system A): t_R = 23.7 min. MS (ESI+): *m/z* 772.00 [M + H]$^+$, calcd for $C_{30}H_{57}N_7O_{12}S_2$ 771.96. ***(b) Removal of the SEt group***: Aminooxy pseudo-peptide (5.9 mg, 7.6 μmol) was dissolved in a mixture of 0.1 M aq. $NaHCO_3$ buffer (pH 8.5, 500 μL) and NMP (400 μL). DTT (20.0 mg, 130 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 A). Further amount of DTT (15.0 mg, 97 μmol) was added and the mixture was stirred for 2 h. Finally, the reaction mixture was quenched by dilution with 0.1% aq. AcOH (pH 3.3, 10 mL) and the solution was lyophilised. The resulting residue was dissolved in a mixture of CH_3CN–0.1% aq. AcOH (1 : 2, v/v, 3 mL) and purified by RP-HPLC (system G, 2 injections, t_R = 31.5–34.7 min). The product-containing fractions were lyophilised to give the sulfhydryl aminooxy pseudo-peptide **17** as a white powder (3.0 mg, 4.2 μmol, yield 71%). MS (ESI+): *m/z* 719.33 [M + Li]$^+$, calcd for $C_{28}H_{53}N_7O_{12}S$ 711.84.

Preparation of thiol-reactive R6G-WS derivative (18).

(a) N-Hydroxysuccinimidyl ester of R6G-WS. The sulfonated analogue of rhodamine 6G (5.3 mg, 8.7 μmol) was dissolved in dry NMP (200 μL). A solution of TSTU reagent (3.0 mg, 10 μmol) in dry NMP (38 μL) and a solution of DIEA (2.7 μL) in dry NMP (17 μL) were sequentially added. The resulting reaction mixture was protected from light and stirred at room temperature for 75 min. The reaction was checked for completion by RP-HPLC (system C). The resulting *N*-hydroxysuccinimidyl ester was used in the next step without further purification. HPLC (system C): t_R = 26.9 min (compared to t_R = 25.9 min for R6G-WS carboxylic acid); MS (ESI−): *m/z* 870.40 [M - H]$^-$, calcd for $C_{38}H_{42}N_5O_{15}S_2$ 870.8.

(b) R6G-WS amine. Ethylenediamine dihydrochloride (96.7 mg, 738 μmol) was dissolved in a mixture of DMF-H_2O (9 : 1, v/v, 10 mL). The crude reaction mixture containing the *N*-hydroxysuccinimidyl ester and a solution of DIEA (64 μL) in DMF (150 μL) were sequentially added and 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 C) and the mixture was evaporated to dryness. The resulting residue was dissolved in 0.1% aq. TFA (pH 2, *ca.* 3 mL) and purified by RP-HPLC (system F, 2 injections, t_R = 25.7–29.2 min). The product-containing fractions were lyophilised to give R6G-WS amine as a pink powder. HPLC (system C): t_R = 25.7 min, purity > 95%; MS (ESI+): *m/z* 817.27 [M + H]$^+$, calcd for $C_{36}H_{43}N_6O_{12}S_2$ 815.24.

(c) R6G-WS SIAB derivative. R6G-WS amine (3.4 μmol) was dissolved in 0.1 M aq. borate buffer (500 μL). A solution of Sulfo-SIAB reagent (4.2 mg, 8.4 μmol) in 0.1 M aq.borate buffer (50 μL, 50 mM + 5 mM EDTA, pH 8.2) was added. 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 C). Further amount of Sulfo-SIAB reagent (4.2 mg, 8.4 μmol) was added and the mixture was stirred for one more hour. Finally, the reaction mixture was quenched by dilution with 0.1% aq. TFA (pH 2, *ca.* 3 mL) and purified by RP-HPLC (system F, 2 injections, t_R = 22.8–26.4 min). The product-containing fractions were lyophilised to give the thiol-reactive R6G-WS derivative **18** as a pink powder (2.5 mg, 2.2 μmol, over yield for the three steps 27%). HPLC (system C): t_R = 27.7 min, purity 93%; MS (ESI+): *m/z* 1104.07 [M + H]$^+$, calcd for $C_{45}H_{50}IN_7O_{14}S_2$: 1103.97.

R6G-WS labelled aminooxy reagent (19). Sulfhydryl aminooxy pseudo-peptide **17** (3.0 mg, 4.7 μmol) was dissolved in 0.1 M aq. $NaHCO_3$ buffer (400 μL) and a solution of thiol-reactive R6G-WS derivative **18** (5.3 mg, 4.7 μmol) in 0.1 M aq. $NaHCO_3$ buffer (pH 8.5, 400 μL) was added. The resulting reaction mixture was protected from light and stirred at room temperature for 1 h. Finally, the reaction mixture was quenched by dilution with 0.1% aq. AcOH (pH 3.3, 2 mL) and purified by RP-HPLC (system F, 2 injections, t_R = 50.1–55.6 min). The product-containing fractions were lyophilised to give the fluorescent aminooxy reagent **19** as a pink powder. Quantification was achieved by UV-vis measurements at λ_{max} = 531 nm of R6G-WS by using the ε value 65000 L mol^{-1} cm^{-1} (yield estimated after RP-HPLC purification 28%). HPLC (system B): t_R = 27.6 min, purity 97%; MS (ESI−): *m/z* 1686.47 [M − H]$^-$, 1670.73 [M − NH_2]$^-$, 834.87 [M − NH_2 − H]$^{2-}$, calcd for $C_{73}H_{102}N_{14}O_{26}S_3$ 1687.90.

Substance P-tripod conjugate (21). *N*-Hydroxysuccinimidyl carbamate cross-linking reagent **5** (5.0 mg, 5.4 μmol) and undecapeptide H-Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Ala-Met-NH_2 (8 mg, 5.4 μmol) were dissolved in dry NMP (400 μL). DIEA (3.8 μL, 21.6 μmol) was added and the resulting reaction mixture was stirred at room temperature for 4 h. Further DIEA (1.9 μL, 10.8 μmol) was added and the mixture was stirred for 2 h. The reaction was checked for completion by RP-HPLC (system A). Acetic acid (1.2 μL, 21.6 μmol) and 0.1% aq. TFA (pH 2, 1 mL) were sequentially added and the aqueous mixture was purified by RP-HPLC (system H, 2 injections, t_R = 32.5–33.2 min). The product-containing fractions were lyophilised to give peptide conjugate **21** as a white powder (10.3 mg, 5.0 μmol, yield 92%). HPLC (system B): t_R = 26.2 min; MS (ESI+): *m/z* 1056.4 [M + 2H_2O + 2H]$^{2+}$, calcd for $C_{91}H_{136}N_{24}O_{26}S_3$ 2078.44.

FluoProbes® 532A labelled substance P-tripod conjugate (23). ***(a) Removal of the phthaloyl group***: Substance P-tripod conjugate

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21 (10.3 mg, 5 μmol) was dissolved in MeOH (500 μL) and a solution of hydrazine monohydrate (5 μmol) in MeOH (100 μL) was added. The resulting reaction mixture was stirred at room temperature for 1 h. The deprotection reaction was checked for completion by RP-HPLC (system A). Thereafter, 0.1% aq. TFA (pH 2, 2 mL) was added and the resulting solution was purified by RP-HPLC (system H, 2 injections). The product-containing fractions were lyophilised to give the aminooxy tripod as a white powder. ***(b) Removal of the SEt group***: Aminooxy-peptide (3.8 mg, 1.8 μmol) was dissolved in dry NMP (100 μL). A solution of DTT (5.6 mg, 36 μmol) in 0.1 M aq. borate buffer (pH 8.2, 300 μL) was added and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system A). Acetic acid (0.2 μL, 3.6 μmol) and 0.1% aq. TFA (pH 2, 500 μL) were sequentially added and the aqueous mixture was purified by RP-HPLC (system I, 2 injections). The product-containing fractions were lyophilised to give the sulfhydryl tripod **22** as a white powder. ***(c) Fluorescent labelling***: Sulfhydryl tripod **22** (1.5 mg, 0.7 μmol) was dissolved in dry NMP (300 μL). A solution of FluoProbes® 532A maleimide (0.5 mg, 0.7 μmol) in dry NMP (200 μL) and a solution of DIEA (0.7 μmol) in dry NMP (50 μL) were sequentially added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The labelling reaction was checked for completion by RP-HPLC (system C). After 2 h, further DIEA (1.4 μmol) in dry NMP (41 μL) was added. Finally, 0.1% aq. TFA (pH 2, *ca.* 2 mL) was added and the aqueous mixture was purified by RP-HPLC (system I, 2 injections, t_R = 26.5–27.3 min). The product-containing fractions were lyophilised to give fluorescent tripod **23** as a pink amorphous powder. Quantification was achieved by UV-vis measurements at λ_{max} = 532 nm of FluoProbes® 532A by using the ε value 115000 L mol^{-1} cm^{-1} (overall yield for the three steps estimated after RP-HPLC purification 15%). HPLC (system B): t_R = 23.8, 24.3, 24.6 and 25.3 min (several peptide rotamers), purity 98%; MS (ESI+): m/z 1347.5 $[M + 2H_2O + 2H]^{2+}$, calcd mass for fluorescent labelled substance P-tripod conjugate 2656.21.

Functionalisation and peptide immobilisation 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.[34]

(b) Immobilisation of fluorescent aminooxy reagent 19. This aldehyde-reactive label was prepared as a 50 μM solution in deionised water and some drops of the resulting solution were put over the freshly prepared aldehydic surface. After 15 min 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 a four-color microarray GeneTAC scanner (Genomic Solutions) and filter for Cy 3.0 dye (Ex./Em. 550/570 nm). The same experimental procedure was followed for negative control **20**.

(b′) Immobilisation of fluorescent labelled SP tripod 23. This fluorescent aminooxy pseudo-peptide was prepared as a 10 μM solution in immobilisation buffer (potassium phosphate 0.1 M, pH 7.4) and 1 μL of the resulting solution was put over the freshly prepared aldehydic surface. After 4 h of incubation in the dark and in a humid atmosphere at room temperature, the reaction solution was carefully removed and the surface was washed for 10 min with phosphate buffer (10 mM + 0.05% Tween, pH 7.4). Finally, slide was stored with EIA buffer (0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.1% BSA and 0.01% sodium azide) overnight before observation. Fluorescence scanning was performed with an Olympus inverted microscope model IX71 (4X objective) equipped with a camera PCO 1600. Thereafter, FP647-labelled anti-SP mAb (1.5 nmol mL^{-1}) was added and after 30 min of incubation, fluorescence scanning of plots was again performed.

Acknowledgements

This work was supported by the Agence Nationale de la Recherche (Programme Emergence et Maturation 2005), Commissariat à l'Energie Atomique and Institut Universitaire de France (IUF). We thank Elisabeth Roger (INSA de Rouen) for IR measurements, Dr Jérôme Leprince (U 413 INSERM-IFRMP 23) for MALDI-TOF mass spectrometry measurements, Dr Hervé Bernard (INRA) for peptide synthesis, Dr Christophe Portal (QUIDD) for English corrections of the manuscript and QUIDD company for the open access to their HPLC instruments.

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