A novel heterobifunctional linker for facile access to bioconjugates†

Yashveer Singh, Nicolas Spinelli, Eric Defrancq* and Pascal Dumy

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A convenient synthesis of a novel heterobifunctional linker molecule is described. The linker contains a thiol-reactive nitropyridyl disulfide group (Npys) and an aldehyde-reactive aminooxy group with a propensity to form disulfide and oxime linkages. The utility of the linker molecule to cross-link different biomolecules has been demonstrated by employing it in the efficient preparation of a peptide–oligonucleotide conjugate. The linker reported herein could be a useful tool for cross-coupling of different but appropriately functionalised biomolecules.

Introduction

Bioconjugation has generated significant research interest as it provides an easy way to couple two or more molecules with distinct properties thereby producing a novel complex structure (bioconjugate) that possesses the combined properties of its individual components.[1,2] This usually involves the joining (by chemical or biological methods) of molecules such as antibodies (their fragments), nucleic acids and their analogues, liposomal components, polysaccharides, hormones, proteins and peptides to drugs, radionuclides, toxins, fluorophores or photoprobes, inhibitors, enzymes and haptens.[3] Bioconjugation may also involve the simple joining of two or more different biomolecules to combine their useful properties. Examples in this category include peptide–oligonucleotide,[4] peptide–carbohydrate[5] or oligonucleotide–carbohydrate conjugates[6] and so on. Over the years, several bioconjugates have been prepared for use in research, therapeutics and diagnostics. The results from these studies show that bioconjugation indeed adds significant benefits to the biomolecules being conjugated. For instance, it may result in an increase in stability to chemical or proteolytic degradation, reduced immunogenicity, or improved pharmacokinetics, biodistribution and targeting.[1,2,7]

The conjugation of two or more biomolecules is mostly achieved by incorporating mutually reactive groups into the individual components, followed by their coupling in solution, leading to the formation of stable chemical linkages like amides, thioethers, disulfides or oximes.[1,2] Such strategies have found wide acceptance because of the high coupling efficiency and ease of purification. In this context, the design and synthesis of appropriately derivatised homo/heterofunctional linkers that can be utilized to cross-couple two or more individual components (multiple conjugation) is important. The requirements for bioconjugation are stringent, for instance the use of a largely aqueous medium, physiological pH, ambient temperature, short reaction time, equimolar proportions of the components and formation of stable linkages. This has spurred an interest in the synthesis of bioconjugates based on chemoselective ligation techniques[1–3] and therefore the synthesis of heterobifunctional linkers that can be utilized to cross-link biomolecules through chemoselective linkages has assumed greater significance. Several such cross-coupling agents have been developed and studied earlier for the preparation of bioconjugates.[8] For instance, a linker with reactive groups like succinimidyl ester and maleimide has been used to cross-couple proteins and enzymes.[9] More recently, the conjugation of carbohydrates to proteins was achieved by using an aminooxy-thiol containing linker.[10] Herein, the thiol moiety was reacted to a bromoacetylated protein to form a thioether linkage.

The linker containing a thiol-reactive nitropyridyl disulfide group (Npys) and an aldehyde-reactive aminooxy group is of particular importance because such a linker molecule would provide the possibility of generating two very versatile chemical linkages, disulfide and oxime, in a single molecule. It would be relevant to mention that the oxime bond formation has been extensively used earlier to prepare a wide variety of bioconjugates such as peptide–oligonucleotide,[11] carbohydrate–peptide,[12] and carbohydrate–oligonucleotide conjugates.[13] It has also been used to anchor oligonucleotides and carbohydrates onto glass surfaces with potential applications in the area of microarray technology.[14] The oxime ligation is chemoselective, gives high coupling efficiency without additives and the oxime bond is stable over a pH range and yet can be hydrolysed under harsh conditions. The routine method employed for oxime bond formation involves an addition–elimination reaction between an aminooxy group and an aldehyde/ketone.[15] Similarly, the disulfide bond represents another linkage of choice for the conjugation of biomolecules because it is stable and yet reversible in a reducing environment where it is converted to a free sulfhydryl.[16] This last property allows the selective release of biomolecules in the intracellular medium. A thiol-reactive group is useful as a potential precursor for the formation of the disulfide bond because under physiological conditions a thiol group is more nucleophilic than an amine. Several thiol-reactive reagents have been used earlier to modify peptides and proteins at specific sites[17] but the advantage associated with the use of a pyridyl disulfide group is that it is thiol-specific under acidic as well as basic conditions. Moreover, the pyridyl disulfide undergoes exchange by oxidative coupling to a free sulfhydryl group to provide a disulfide linkage and pyridine thione is released as a side product, which is non-reactive and hence prevents contamination of the disulfide.

LEDSS, UMR CNRS 5616, ICMG FR 2607, Université Joseph Fourier, 301 rue de la Chimie, BP 53, F 38041, Grenoble Cedex 9, France. E-mail: Eric.Defrancq@ujf-grenoble.fr

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We herein report on the synthesis and characterisation of a new heterobifunctional linker molecule **1** that contains a thiol-reactive nitropyridyl disulfide group and an aldehyde-reactive aminooxy group (Fig. 1). The efficacy of this linker to cross-couple two different biomolecules has been illustrated by employing it for the preparation of a peptide–oligonucleotide conjugate.

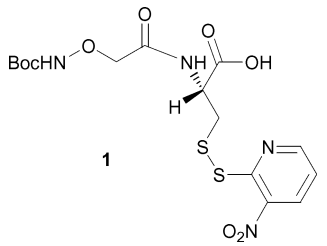
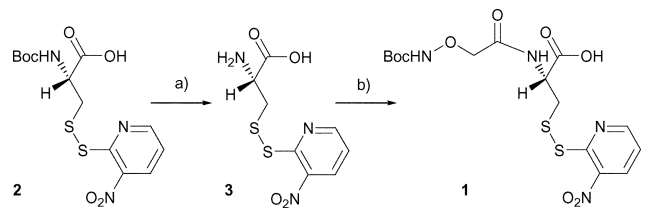


Fig. 1 Heterobifunctional linker **1**.

Results and discussion

Syntheses

The synthesis of linker **1** was carried out in two straight steps starting from the commercially available *N*-α-Boc-*S*-(3-nitro-2-pyridylthio)-L-cysteine **2** (Scheme 1). The Boc protection on the α-amino group in **2** was removed by treatment with 50% aqueous trifluoroacetic acid at room temperature for 4–5 h to obtain **3** after ether precipitation. This derivative was found to be sufficiently pure (on TLC) and hence used in the next step without further purification. The protected aminooxy moiety was incorporated into **3** by an overnight reaction with the *N*-hydroxysuccinimide ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine in the presence of *N*-methyl morpholine in anhydrous DMF at room temperature. It would be relevant to note that the quantity of the base added must be carefully controlled because the Npys group is known to be unstable in basic conditions. The linker **1** was obtained in good yield after purification on a silica gel column and characterized by satisfactory ^{1}H, ^{13}C NMR, mass and elemental data.

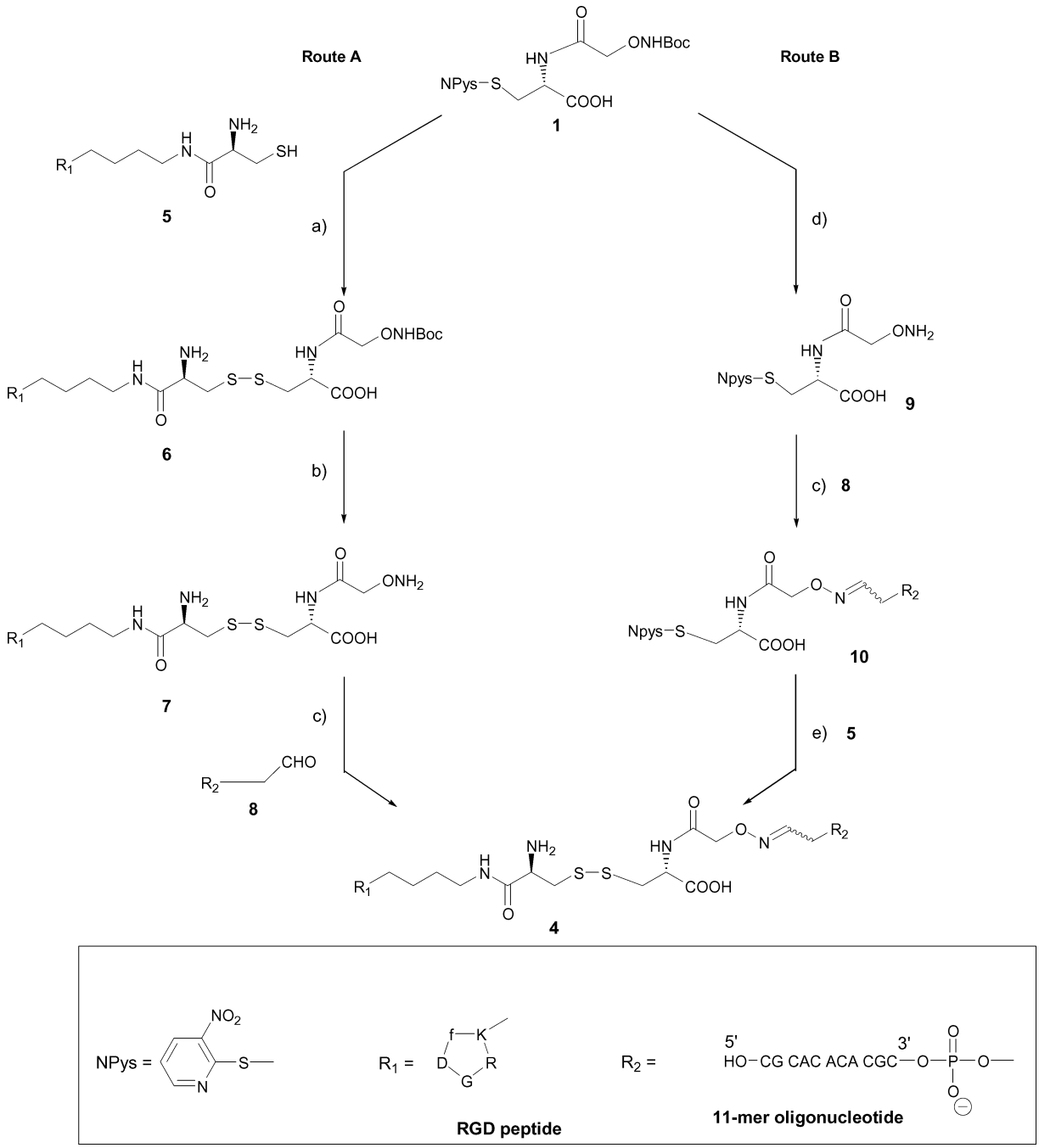


Scheme 1 Preparation of the linker **1**. (a) Aqueous TFA–CH_2Cl_2, r.t., 73%; (b) *N*-hydroxysuccinimide ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine, *N*-methyl morpholine (1 equiv.) in DMF, overnight, r.t., 89%.

The efficiency of the linker **1** to cross-couple different appropriately functionalised biomolecules was investigated next. A peptide–oligonucleotide conjugate **4** was prepared by using the linker **1** (Scheme 2). Peptide–oligonucleotide conjugate was chosen because such conjugates have been reported to enhance cellular uptake efficiency, cell-specific targeting, stability to degradation and binding affinity towards the target sequence of synthetic oligonucleotides.[18] Synthesis of such conjugates is considered important insofar as improvement in the therapeutic properties of synthetic oligonucleotides is concerned. The cyclopentapeptide used in the present work contains an arginine–glycine–aspartic acid (RGD) tripeptide motif and is known for its selective and powerful binding to $\alpha_v\beta_3$ integrin receptors.[19] The oligonucleotide conjugate of this peptide has been investigated for tumour targeting[20] and DNA delivery.[21] The key question was to ascertain whether the disulfide linkage is stable in the reaction conditions employed for oxime bond formation and *vice versa*. It was therefore decided to employ two synthetic routes for the preparation of the conjugate **4**. In the first approach, the linker **1** was coupled to a peptide thiol followed by reaction to an oligonucleotide aldehyde (Route A) whereas in the second approach, linker **1** was first reacted to an oligonucleotide aldehyde followed by reaction to a peptide thiol (Route B). The reaction conditions and their influence on the stability of other linkages were thoroughly investigated.

Cross-coupling through disulfide linkage formation followed by oxime linkage formation (Route A). The preparation of RGD peptide **5** containing the thiol functionality has been described earlier.[11a] Similarly, the preparation of oligonucleotide **8** containing the 3′-aldehyde moiety (5′-CGCACACACGCX-3′ where X = aldehydic linker) has also been reported.[11c] The RGD peptide **5** was reacted with a slight excess of linker **1** in DMF–phosphate buffer (pH = 4.8) at room temperature for one hour to obtain linker–peptide **6**. As expected, the reaction was found to be fast and efficient. The HPLC profile of the crude conjugation reaction mixture showed almost complete consumption of the peptide thiol (Fig. 2A). The peptide derivative **6** was used in the next step without further purification. It was treated with 50% trifluoroacetic acid in dichloromethane in the presence of scavengers (triisopropyl silane–water) to remove the Boc protection. The reaction was carried out at room temperature for about an hour and complete removal of the Boc group was achieved. The linker–peptide derivative **7** containing the free aminooxy group was obtained in a satisfactory yield after HPLC purification. The second conjugation to the oligonucleotide was carried out next. The linker derivative **7** was reacted with oligonucleotide-3′-aldehyde **8** in a solution of ammonium acetate buffer (pH = 4.8) at room temperature for 6–8 h. The slightly acidic conditions were used for the reaction because these are conducive to the formation of oxime bonds.[1] The HPLC profile of the conjugation reaction mixture is shown in the accompanying figure and two facts are clearly evident from it (Fig. 2B). First, the conjugation reaction is highly efficient as is known for oxime ligations and second, no unaccounted side products are present (HPLC/mass analysis) thereby suggesting that the reaction conditions used for the oxime bond formation do not influence the disulfide linkage holding the peptide. The conjugate **4** was obtained in a satisfactory yield after HPLC purification. The derivatives **4** and **5–8** described above were characterised by ESIMS analysis and an excellent agreement between the calculated and the observed molecular weight was observed.

Cross-coupling reaction through oxime linkage formation followed by disulfide linkage formation (Route B). Once the synthesis of the peptide–oligonucleotide conjugate was successfully achieved, the opposite route for the synthesis of the conjugate **4** was also attempted. Consequently, the linker **1** was treated with



Scheme 2 Preparation of peptide–oligonucleotide conjugate **4** by using the linker **1**. (a) DMF–phosphate buffer, 1 h, r.t.; (b) TFA–CH_2Cl_2–triisopropyl silane–H_2O, 1 h, r.t.; (c) ODN-3′-aldehyde **8**, ammonium acetate buffer, 6–8 h, r.t.; (d) aqueous TFA, 3 h, r.t.; (e) peptide **5**, DMF–phosphate buffer, 1 h, r.t.

an aqueous solution of trifluoroacetic acid (50%, v/v) at room temperature for 3 h to remove the Boc protecting group masking the aminooxy functionality. The linker **9** with a free aminooxy group was used in the next step without purification. It was reacted with a slight excess of 11-mer-oligonucleotide-3′-aldehyde **8** (5′-CGCACACACGCX-3′) in a solution of ammonium acetate buffer (pH = 4.6) at room temperature for 5–6 h. An efficient conjugation led to the formation of conjugate **10**, which was purified by HPLC (Fig. 3A and 3B). Dual wavelengths of detection were used to monitor this reaction: 260 nm for the oligonucleotide and 400 nm for Npys. The ESIMS analysis however suggested the loss of the Npys group. To ascertain whether the Npys group is lost during the course of the conjugation reaction/purification or during the mass analysis, conjugate **10** was treated with DTT. It was found that peaks corresponding to Npys-on or Npys-off products are well resolved (see ESI†) and hence possibly could not be collected together during the purification of the conjugate **10**. In our case, this loss of the Npys group could probably be due to the basic conditions employed during the ESIMS analysis of the conjugate (CH_3CN–H_2O–Et_3N, 49 : 49 : 2). The

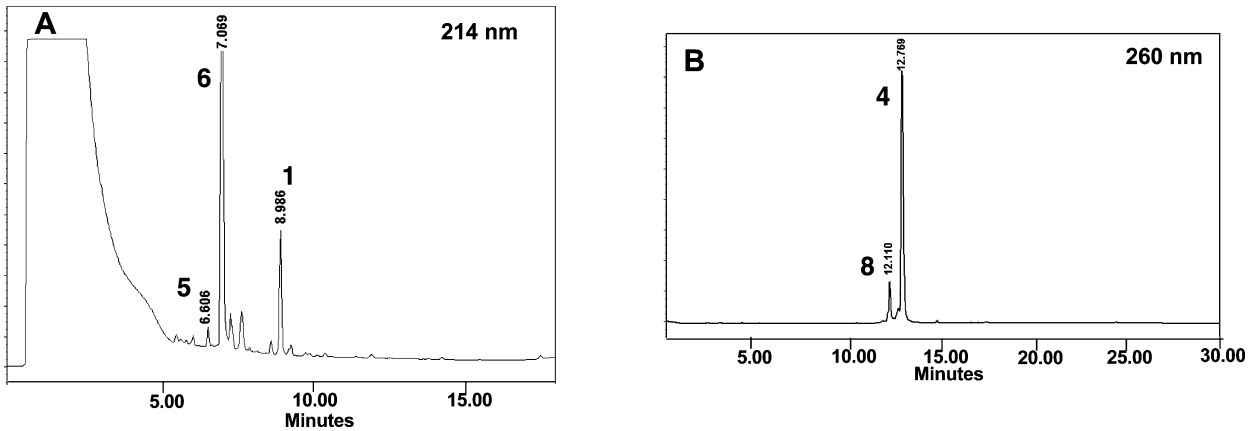


Fig. 2 RP-HPLC profiles of crude conjugation reaction mixtures. (A) Linker **1** with RGD peptide **5** (disulfide bond formation); (B) linker–peptide **7** with oligonucleotide-3′-aldehyde **8** (oxime bond formation).

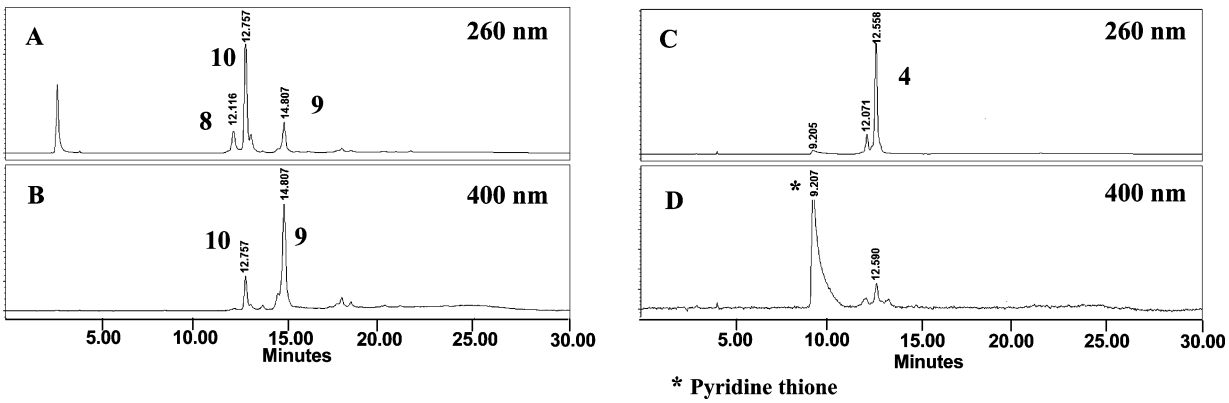


Fig. 3 RP-HPLC profiles of crude reaction mixtures. (A–B) Linker **9** with oligonucleotide-3′-aldehyde **8** (oxime bond); (C–D) peptide **5** with linker–oligonucleotide **10** (disulfide).

linker–oligonucleotide conjugate **10** was next treated with a slight excess of peptide **5** at room temperature for about an hour. An efficient conjugation led to the formation of peptide–linker–oligonucleotide conjugate **4** (Fig. 3C and 3D). Two different wavelengths of detection as described above were also employed to monitor this conjugation reaction. It was not possible to detect the formation of the product at 260 nm but well-resolved peaks for the disappearance of Npys on the oligonucleotide (RT = 12.6 min) and the appearance of a pyridine thione peak (RT = 9.2 min) were obtained when the reaction was monitored at 400 nm. The conjugate **4** was purified by HPLC. The derivatives **4**, **9** and **10** were characterised by satisfactory ESIMS data. This preparation of conjugate **4** conclusively established that an oxime bond can be formed in the presence of a disulfide bond and *vice versa*.

Biophysical studies

A linker employed to cross-couple biomolecules could be considered efficient if it does not diminish the useful and characteristic biological features of the individual components. For instance, in the present case, it is of the utmost importance to ascertain whether the presence of the linker **1** in the conjugate induces an instability and/or conformational perturbations in oligonucleotide duplexes. This is crucial because oligonucleotide conjugates showing duplex instability and/or conformational perturbations may not be desirable for various therapeutic applications where a high degree of sequence specificity and affinity is demanded. Consequently, the oligonucleotide duplexes with the complementary sequence were prepared and investigated by thermal denaturation and circular dichroism studies. The T_m value for the duplex formed by the bioconjugate **4** with the complementary sequence was estimated as 62.1 °C whereas the T_m value for the natural duplex was found to be 61 °C (see ESI†). This suggests that conjugation of an oligonucleotide to a peptide through the linker molecule stabilizes the duplex slightly ($\Delta T_m = 1.1$ °C). A small to moderate increase in the stability of the duplex has been observed earlier with various other peptide–oligonucleotide conjugates.[18] It is therefore difficult to ascertain whether the slight increase in stability is due to the presence of the peptide or the linker. However, it is important to note that the linker does not destabilize the duplex. Similarly, the CD spectrum for the duplex formed by the conjugate **4** was found to be identical to the CD spectrum of the natural duplex. The individual spectrum showed negative and positive excitations respectively at 255 nm and 275 nm thereby suggesting an unperturbed B-type duplex conformation (see ESI†). An unperturbed duplex conformation is consistent with several earlier studies with peptide–oligonucleotide conjugates[13a,15]

and the presence of linker **1** does not appear to have any significant influence.

Conclusion

Thus, an efficient preparation of a novel heterobifunctional linker with a propensity to generate disulfide and oxime linkages is reported. The linker reported herein could be of general utility to cross-couple potentially any two appropriately functionalised biomolecules. This has been demonstrated in the present work by utilizing the linker molecule for the preparation of a peptide–oligonucleotide conjugate. The disulfide linkage could be formed first followed by the formation of the oxime linkage and *vice versa*. The introduction of a linker does not influence the stability and/or the conformation of the duplex. The linker **1** also contains a free carboxylic group, which can be used for on-support modification of peptides and this makes it compatible with solid phase synthetic procedures. The heterobifunctional linker reported herein could be further explored to cross-couple various other biomolecules such as proteins, carbohydrates, enzymes, drugs or fluorescent labels and hence could be of immense use in the field of bioconjugation.

Experimental

General

All solvents and reagents used were of the highest purity available. *N*-α-Boc-*S*-(3-nitro-2-pyridylthio)-L-cysteine was obtained from Bachem. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 300 spectrometer. Mass spectra (DCI mode) for **1** and **3** were recorded on a Thermoquest spectrometer. ESI mass spectra were measured on an Esquire 3000 spectrometer from Bruker. The analysis was performed in positive mode for **5–7** and in negative mode for **4**, **8–10**. Aqueous acetonitrile (50%) was used as the eluent for **5–7** and 2% Et_3N was added for **4**, **8–10**. RP-HPLC analysis and purification were performed on a Waters RP-HPLC system equipped with a dual wavelength detector. For RP-HPLC analysis of **1**, **5–7** and **9** a Nucleosil C_{18} column (Macherey Nagel, 30 × 4 mm, 3 μm) was used with the following solvent system. Solvent A, 0.1% TFA in water; solvent B, acetonitrile containing 10% water and 0.1% TFA; flow rate of 1.3 mL min^{-1}; a linear gradient of 5–100% B was applied in 15 minutes. The RGD peptide **5** and peptide–linker conjugate **7** were purified using a Delta Pak C_{18} column (Waters, 25 × 200 mm, 15 μm) at a flow rate of 22 mL min^{-1}. RP-HPLC analysis of **4**, **8** and **10** was performed on a Nucleosil C_{18} column (Macherey Nagel, 250 × 4.6 mm, 5 μm) using the following solvent system. Solvent A, 20 mM ammonium acetate buffer containing 5% acetonitrile; solvent B, acetonitrile containing 5% water; flow rate of 1 mL min^{-1}; a linear gradient of 0–30% B was applied in 20 minutes. These compounds were purified on a Nucleosil C_{18} column (Macherey Nagel, 10 × 250 mm, 7 μm) at a flow rate of 4 mL min^{-1}.

Syntheses

Amino-*S*-(3-nitro-2-pyridylthio)-L-cysteine (3). **2** (1.0 g, 2.66 mmol) was dissolved in CH_2Cl_2 (20 mL) and 50% aqueous TFA (40.0 mL) was added. The reaction mixture was stirred for 4–5 h (until the reaction shows complete consumption of the starting material on TLC) at ambient temperature. The reaction mixture was concentrated under vacuum and further evaporated with toluene to completely remove the TFA from the reaction mixture. The residue so obtained was re-dissolved in acetonitrile and precipitated with ether. The precipitate was collected and dried in vacuum. This crude product (0.85 g, 73%) was found to be sufficiently pure and was hence used in the next step without further purification. *m*/*z* (DCI) 276 (20%, $(M + H)^+$), 157 (100), 139 (100).

Heterobifunctional linker (1). **3** (250 mg, 0.64 mmol) was dissolved in anhydrous DMF (12.0 mL) and *N*-methyl morpholine (65 mg, 70 μl, 0.64 mmol) was added followed by the *N*-hydroxysuccinimide ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine (222.5 mg, 0.77 mmol). The reaction mixture was stirred overnight at ambient temperature under anhydrous conditions. Afterwards, the reaction mixture was concentrated under vacuum to remove the DMF and the residue so obtained was re-dissolved in CH_2Cl_2. The organic layer was washed with 0.1 N HCl and dried over anhydrous sodium sulfate. The linker **1** (258 mg, 89%) was obtained after purification by column chromatography on a silica gel column using 10–20% MeOH in CH_2Cl_2 as the eluent (found: C, 38.25; H, 4.25; N, 11.65. $C_{15}H_{19}N_4O_8S_2Na$ requires C, 38.30; H, 4.1; N, 11.1%); δ_H(300 MHz, CD_3CN–D_2O 6 : 1) 8.87 (1H, dd, *J* 1.5, 4.6 Hz, CH_{Npys}=N), 8.71 (1H, dd, *J* 1.5, 8.2 Hz, CH_{Npys}–C(NO_2)=), 7.50 (1H, dd, *J* 4.6, 8.2 Hz, CH=CH_{Npys}–CH), 4.77 (1H, dd, *J* 5.3, 7.9 Hz, C*H*(CO_2H)–NH), 4.28 (2H, 2 x d, *J* 16.1 Hz, CH_2–O–NH), 3.40 (1H, dd, *J* 5.3, 14.1 Hz, S–CH_A(H_B)–CH), 3.22 (1H, dd, *J* 7.9, 14.1 Hz, S–CH_B(H_A)–CH), 1.45 (1H, s, OC(CH_3)$_3$); δ_C (300 MHz, CD_3CN) 170.4 (C_q), 168.8 (C_q), 157.4 (C_q), 155.4 (C_q), 153.8 (CH), 142.8 (C_q), 134.0 (CH), 121.5 (CH), 81.8 (C_q), 74.9 (CH_2), 51.1 (CH), 39.1 (CH_2), 27.1 (CH_3); *m*/*z* (DCI) 448.8 (60%, M^-), 392.8 (100, $(M - t\text{Bu})^-$), 348.8 (86, $(M - \text{Boc})^-$).

Peptide RGD–cysteine (5). The procedure to prepare this peptide has been described earlier.[11a] Linear peptide H-Asp(O*t*Bu)-Phe-Lys(Aloc)-Arg(Pmc)-Gly-OH was prepared by using standard solid-phase peptide synthesis protocols. Head to tail cyclization was effected in the presence of PyBOP in DMF. The Aloc moiety was then cleaved by using the standard Pd^0 procedure and the peptide was purified by HPLC. Finally, the peptide was reacted with Fmoc-Cys(Trt)–OH/PyBOP to introduce the cysteine group. The Fmoc group was removed with piperidine in DMF. The *t*Bu- and Pmc-protecting groups were removed by using 90% TFA with ethanedithiol as a scavenger to obtain **5**.

Boc-aminooxy linker–peptide (6). **5** (10.0 mg, 0.014 mmol) was dissolved in DMF–phosphate buffer (75 : 25 v/v; 1.0 mL; pH = 4.8). A solution of linker **1** in DMF (0.015 mmol, 6.9 mg) was added. The reaction mixture was stirred at room temperature under argon for 3 h (monitored by HPLC). After, the reaction mixture was concentrated and used in the next step without further purification. *m*/*z* (ESI) 999.3 $(M + H)^+$.

Aminooxy linker–peptide (7). The crude **6** obtained from the above step was dissolved in a solution of 50% TFA in CH_2Cl_2 containing 5% triisopropylsilane and 5% water as scavengers. The reaction mixture was stirred at room temperature for 1 h (monitored by HPLC). The reaction mixture was concentrated in a speed vac and the residue was re-dissolved in 50% aqueous

acetonitrile. The crude product was purified by HPLC. The pure product (5.2 mg, 41% with respect to **5**) was obtained as a white powder after freeze-drying the appropriate fractions. *m/z* (ESI) 899.2 $(M + H)^+$.

Oligonucleotide-3′-aldehyde (8). The preparation of the 11-mer oligonucleotide (5′-CGCACACACGC-3′) containing 3′-aldehyde functionality has been described by us.[11d] Briefly, automated synthesis of the oligodeoxynucleotide (ODN) was carried out on an Expedite DNA synthesiser (Perkin-Elmer) by using standard β-cyanoethyl nucleoside phosphoramidite protocols at a 1 μM scale. The automated synthesis was carried out on a modified solid support, (3-[(4,4′-dimethoxytrityl)-glyceryl-1-succinyl]) long chain alkylamino controlled pore glass obtained from Eurogentec. This support introduces a protected diol group (an aldehyde precursor) into ODNs. Afterwards, the ODNs were cleaved from the solid support and released into the solution by treatment with 28% ammonia (1.5 mL) for 2 h and were finally deprotected by keeping in the ammonia solution for 16 h at 55 °C. The oligonucleotides containing free 3′-diol groups were obtained after HPLC purification. The 5′-dimethoxytrityl group was removed by treatment with 80% aqueous acetic acid for 1 h at room temperature (standard procedure). These ODN-3′-diols were subjected to oxidative cleavage with aqueous sodium-*m*-periodate to obtain ODNs **8** with 3′-aldehyde functionality after HPLC purification.

Aminooxy linker (9). Linker **1** (20 mg, 44.6 μmol) was dissolved in a solution of 50% TFA in water (1 mL). The reaction mixture was stirred at room temperature for 3 h (monitored on HPLC). After, the reaction mixture was concentrated and the residue was re-dissolved in water. The aminooxy linker **9** was obtained after freeze-drying and the crude product was used in the next step without further purification. *m/z* (ESI) 346.8 $(M + H)^+$.

Linker–oligonucleotide conjugate (10). Oligonucleotide 3′-aldehyde **8** (0.36 mg, 0.105 μmol) was dissolved in 0.1 M ammonium acetate buffer (95 μl; pH = 4.6) and an aqueous solution of aminooxy linker **9** was added (10 μl, 0.195 μmol). The reaction mixture was stirred at room temperature for 8 h (monitored by HPLC). The linker–oligonucleotide conjugate **10** (0.26 mg, 67% with respect to **8**) was obtained after RP-HPLC purification. *m/z* (ESI) 3721.2 $(M - H)^-$, 3565.0 $(M - Npys)^-$ (see ESI†).

Peptide–linker–oligonucleotide conjugate (4).

A) From linker–peptide conjugate (7). Oligonucleotide aldehyde **8** (0.7 mg, 0.206 μmol) was dissolved in a solution of 0.1 M ammonium acetate buffer (0.7 mL, pH = 4.6) and an aqueous solution of linker–peptide conjugate **7** (0.74 mg, 0.824 μmol) was added. The reaction mixture was stirred at ambient temperature for 7–8 h (monitored by RP-HPLC). The peptide–linker–oligonucleotide conjugate **4** (0.48 mg, 55%) was obtained after RP-HPLC purification. *m/z* (ESI) 4273.5 $(M - H)^-$.

*B) From linker–oligonucleotide conjugate (**10**).* A solution of peptide **5** (100 μl, 0.118 μmol) in phosphate buffer (0.1 M, pH = 4.6) was added to the linker–oligonucleotide conjugate **10** (0.36 mg, 0.097 μmol). The reaction mixture was stirred at room temperature for 1 h (monitored by HPLC) and conjugate **4** (0.17 mg, 41%) was obtained after RP-HPLC purification.

Thermal denaturation (T_m) studies

The melting curves (absorbance *versus* temperature) were recorded at 260 nm on a UV–visible spectrophotometer equipped with a temperature controller. The melting experiments were carried out by mixing the equimolar amount of two oligonucleotide strands in a solution of sodium phosphate buffer (10 mM, pH 7.0) containing EDTA (1 mM) and NaCl (100 mM). The ODN concentration was kept at 12 μM. The absorbance was recorded in the temperature range of 10–80 °C at a sweep rate of 1 °C min^{-1}. All experiments were done in triplicate.

Circular dichroism (CD) studies

The CD experiments were carried out by using similar solutions as described above for the thermal denaturation studies.

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