Synthesis of organometallic PNA oligomers by click chemistry†

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The facile side-specific insertion, on the solid phase, of one or two ferrocene moieties into peptide nucleic acid (PNA) oligomers by click chemistry is presented.

PNAs are neutral, non-natural analogues of DNA/RNA.[1] Their properties include high binding affinity for DNA/RNA strands, high chemical stability, resistance to nucleases, great discrimination of single-base mismatches and fast hybridisation. These favourable characteristics are particularly attractive in the electrochemical biosensing area.[2,3] For this reason, several examples of the labelling of PNA monomers and oligomers with the reversible redox-active moiety ferrocene[2,4] have been reported.[5,6] While structurally diverse ferrocene-containing monomers were reported especially by Baldoli, Maiorana and co-workers, to the best of our knowledge, all the ferrocene-containing PNA oligomers reported to date have their organometallic moiety attached to the N-terminus of the PNA sequence. This is an important limitation as it only allows the insertion of one ferrocene moiety per PNA oligomer, and offers no structural flexibility. To maximise the scope of such organometallic PNAs, it would be particularly attractive to be able to position the redox-active moiety at *any* place within the PNA oligomer. The obvious synthetic strategy to undertake this would be to synthesise the oligomers using ferrocenyl PNA monomers as building blocks. However, this method presents two major disadvantages: (i) the multi-step chemical synthesis of a dedicated ferrocenyl PNA monomer and (ii) the ferrocenyl moiety has to be chemically compatible with all subsequent steps during solid phase peptide synthesis (SPPS) of PNA oligomers. While this may be readily achieved with ferrocenyl monomers as shown herein, it remains a general problem for more sensitive metal compounds, and therefore milder and more general methods for the introduction of metal complexes are always sought after.

With the endeavour of synthesising PNA oligomers containing multiple ferrocene moieties, we searched for a versatile synthetic strategy. Our attention rapidly turned to the click chemistry methodology.[7,8] This term describes a copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition reaction of azides and terminal alkynes yielding 1,4-disubstituted 1,2,3-triazole derivatives.[9] Interestingly for the purpose of our research, click chemistry can proceed in water, under mild conditions and is chemoselective, efficient, reliable and simple. Due to these numerous advantages, the method has already been successfully used for the radiolabelling of biomolecules. An example of this was reported by Schibli *et al.*, who described the insertion of metal chelates into biomolecules such as peptides, phospholipids, carbohydrates and nucleobases using the so called "click-to-chelate" approach.[10] Another significant advantage of this methodology is its possible use on the solid phase. This is particularly attractive in the bioconjugation area as it allows the use of an excess of the cheaper/easier-to-make reagent to give the expected bioconjugates quantitatively and with minimal purification problems.

Herein, we describe, first, the facile synthesis of a new, versatile alkyne-substituted PNA monomer building block (**Fmoc-1-OH**, Fig. 1) that can be inserted at any given place within any desired PNA sequence on an automated DNA/PNA synthesiser.§ We then present, by application of PNA monomer **1**, the first example of the insertion of an organometallic complex, namely ferrocene, into PNA oligomers using the click chemistry methodology. With this work, we thereby contribute to the preparation of new PNA bioorganometallic conjugates that could be employed, for example, as electrochemical probes.

Synthon **Fmoc-1-OH** was readily prepared by reacting the original PNA backbone, *tert*-butyl *N*-[2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl)] glycinate hydrochloride[11] with 4-pentynoic acid to give **Fmoc-1-O^{t}Bu** using the reaction conditions

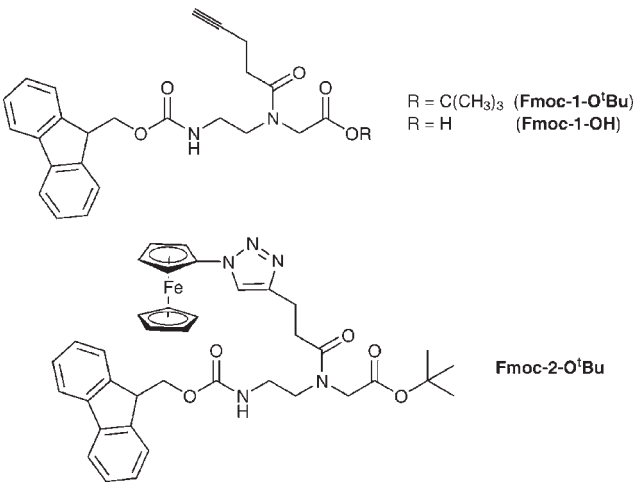

Fig. 1 Structures of **Fmoc-1-O^{t}Bu**, **Fmoc-1-OH** and **Fmoc-2-O^{t}Bu**.

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† Electronic supplementary information (ESI) available: Additional experimental and characterisation details, NMR spectra of **Fmoc-1-O^{t}Bu**, **Fmoc-1-OH** and **Fmoc-2-O^{t}Bu**, MALDI-TOF MS spectra of **Fc-PNA1–3** and **Fc$_2$-PNA4**. See DOI: 10.1039/b805369c

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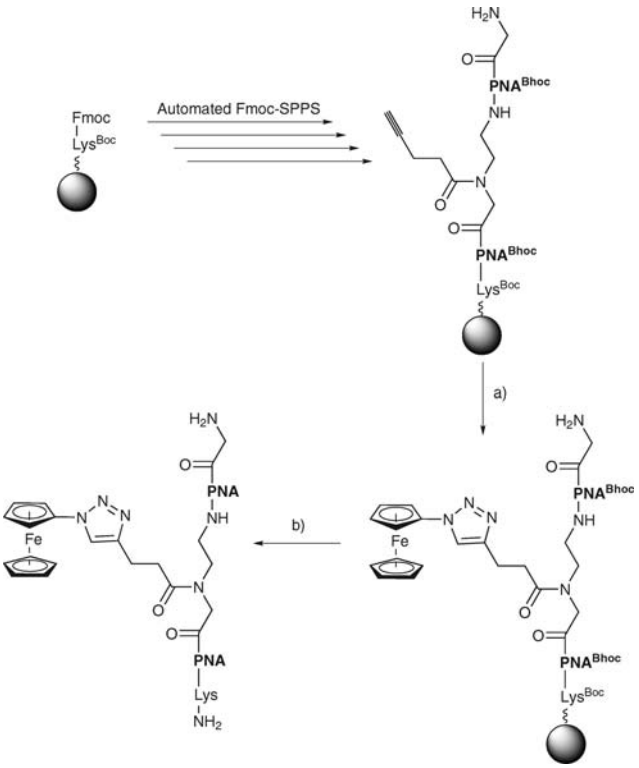
reported by Spiccia *et al.* for a similar coupling.[6] The *tert*-butyl group of **Fmoc-1-O^{t}Bu** was then removed using a mixture of CH_2Cl_2–TFA–triethylsilane 10 : 5 : 2 (v/v/v) to yield **Fmoc-1-OH** (see Fig. 1 and ESI† for further experimental details). An important characteristic of **Fmoc-1-OH** is that it contains a PNA backbone, which should avoid structural distortion when the PNA-hybrid containing monomer **1** is bound to a complementary DNA/RNA strand. Such structural distortion was observed by our group when two amino acids were inserted in the middle of a PNA oligomer.[12] Albeit the number of atoms corresponding to the backbone of these two amino acids was equal to the number in the PNA backbone, a significant destabilisation in the DNA–PNA duplex was observed when there was a DNA base opposite the dipeptide. This feature makes **Fmoc-1-OH** superior to the use of, for instance, commercially available non-natural amino acids containing a triple bond which have been previously employed for coupling glycosides to peptides by click chemistry.[13]

The ability of **Fmoc-1-O^{t}Bu** to be used in the click reaction was investigated with the known azidoferrocene[14] (**3**) (see ESI† for experimental details). A slightly different procedure to the one used by Sharpless *et al.*[8] (1 : 1 azide : alkyne stoichiometry in the presence of an aqueous solution of sodium ascorbate and $CuSO_4{\cdot}5H_2O$ in a 2 : 1 v/v mixture of acetone–H_2O)[15] afforded the orange ferrocenyl PNA-like monomer **Fmoc-2-O^{t}Bu** (Fig. 1). Its presence was confirmed from ESI-MS with two peaks at m/z 703.2 and 726.2 corresponding to $[M]^+$ and $[M+Na]^+$, respectively. As expected, the presence of two rotamers made the assignment of both ^1H and ^{13}C spectra complicated, but this was overcome with the use of 2D NMR spectra (COSY, HMQC and HMBC) (see ESI for full details).†

PNA oligomers containing **1** were synthesised on TentaGel R Fmoc-Lys(Boc)-RAM resin, using Fmoc/Bhoc-protected PNA monomers on a standard oligonucleotide synthesiser (see Scheme 1 for a general description of the synthesis of modified PNA oligomers). The C-terminal lysine residue was introduced in order to enhance solubility and cellular uptake.[12,16] A glycine was also inserted at the N-terminus of the PNA sequence in order to avoid *N*-acyl transfer reactions.[17] Two identical sequences were always run in parallel on the automated oligonucleotide synthesiser: one, which was cleaved with a mixture of TFA–TIS–H_2O 95 : 2.5 : 2.5 (v/v), to determine whether the introduction of **Fmoc-1-OH** was successful, and the other one to be used for the subsequent coupling with azidoferrocene. As summarised in Table 1, **Fmoc-1-OH** was successfully introduced at the N-terminus (**PNA1**), at the C-terminus (**PNA3**) and in the middle of the sequence (**PNA2**) of a 10-mer PNA sequence. As expected, the MALDI-TOF mass spectra of each of the sequences, **PNA1**, **PNA2**, **PNA3**, were similar (single peak corresponding to m/z $[M+H]^+$). In order to make a double functionalisation possible, two **Fmoc-1-OH** synthons were also successfully inserted, by automation, at the N-terminus and in the middle of the PNA oligomer (**PNA4**; Table 1). **PNA4** shows a peak in the MALDI-TOF mass spectrum corresponding to m/z 3306 $[M+H]^+$. The automated synthesis of **PNA1**, **PNA2**, **PNA3**, and **PNA4** illustrates clearly that the easily synthesised synthon **1** can be inserted anywhere within a given PNA sequence.

With the alkyne-containing PNA sequences (**PNA1**, **PNA2**, **PNA3**, **PNA4**) in hand, we investigated the feasibility of the



Scheme 1 General synthesis of ferrocene–PNA conjugates in this work. (a) CuI (2.5 eq.), **3** (1.5 eq.), DIPEA (150 eq.), DMF; (b) TFA–phenol–TIS 85 : 10 : 5 (v/v/v).

click reaction with **3** on the solid phase. Due to shrinkage of the Tentagel R Fmoc-Lys(Boc) RAM resin in the acetone–water mixture, which was used for the synthesis of **Fmoc-2-O^{t}Bu**, and the insolubility of sodium ascorbate and copper sulfate in the common organic solvents, different reaction conditions needed to be explored. A similar procedure to the one used by Meldal *et al.*[7] with a polyethylene glycol polyacrylamide resin ($PEGA_{800}$) was applied to our system, namely the use of CuI as catalyst, DMF as the solvent and a large excess of DIPEA (150 eq. in our case) to allow the copper(I) salt to dissolve (see ESI† for further details). Under these conditions, the azidoferrocene was successfully reacted with **PNA1** and **PNA2** to give **Fc-PNA1** and **Fc-PNA2**, respectively. After shaking the mixture for 2–3 days, the resin was washed with DMF and CH_2Cl_2, shrunk with MeOH, dried and the oligomer was cleaved from the resin with a mixture of TFA–phenol–TIS 85 : 10 : 5 (v/v/v). To our

Table 1 Sequences of **PNA1**, **PNA2**, **PNA3**, and **PNA4** prepared in this study. Small letters a, c, g, t denote PNA monomers, standard three-letter codes are used for amino acids

PNA	Sequence
PNA1	H-Gly-**1**-ggg-tc-agc-tt-Lys-NH_2
PNA2	H-Gly-ggg-tc-**1**-agc-tt-Lys-NH_2
PNA3	H-Gly-ggg-tc-agc-tt-**1**-Lys-NH_2
PNA4	H-Gly-**1**-ggg-tc-**1**-agc-tt-Lys-NH_2
Fc-PNA1	H-Gly-**2**-ggg-tc-agc-tt-Lys-NH_2
Fc-PNA2	H-Gly-ggg-tc-**2**-agc-tt-Lys-NH_2
Fc-PNA3	H-Gly-ggg-tc-agc-tt-**2**-Lys-NH_2
Fc_2-PNA4	H-Gly-**2**-ggg-tc-**2**-agc-tt-Lys-NH_2

satisfaction, the reaction proceeded cleanly so that no further purification of the metallated PNA oligomers was required. Importantly, full conversion was observed for both sequences (no peaks corresponding to **PNA1** and **PNA2** were observed by MALDI-TOF MS).† For both cases, a peak in the MALDI-TOF mass spectrum corresponding to $[M+H]^+$ as well as a peak corresponding to $[M+Cu]^+$ was observed.

Even more challenging was the click chemistry reaction between **3** and **PNA3**. Due to the close proximity of the alkyne group in **1** to the resin, it was anticipated that the azidoferrocene might not readily be reacted with **PNA3**. Nevertheless, as for **Fc-PNA1** and **Fc-PNA2**, **PNA3** was fully converted to **Fc-PNA3** using the same reaction conditions. Finally, a double functionalisation of **PNA4** with **3** was attempted by simply doubling the stoichiometry used for the single functionalisation. Again, full conversion of **PNA4** to **Fc_2-PNA4** was observed. The PNA oligomer **Fc_2-PNA4** is thus the first PNA oligomer with two organometallic groups at two different positions within the PNA sequence.

The potential of the versatile, easy-to-make alkyne PNA monomer **Fmoc-1-OH** for the facile insertion of one or two organometallic ferrocenyl moieties into PNA oligomers has been demonstrated in this work. Click chemistry has recently been used to link cell uptake-promoting peptides to PNA oligomers in solution.[18] To the best of our knowledge, our work presents the first functionalisation of PNA oligomers by click chemistry on solid support. Unlike in all other steps during SPPS, where a three-to-five fold excess is used, the high yield in the click reaction allows us to use only a slight excess of the metal complex. The scope of the reaction is demonstrated by the insertion of electrochemically active ferrocene groups. Further research into the coupling of organometallics other than ferrocene, as well as the electrochemical biosensing capability of our PNA bioconjugates, is currently under way in our laboratory and will be published in due course.

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Notes and references

§ Abbreviations: peptides and PNA oligomers are written from the N- to C-terminus in standard peptide-like nomenclature (*e.g.* Fmoc-Lys-OH indicating Fmoc-protected lysine). According to common convention, the same four letter code is used for PNA as for DNA, small letters however indicate PNA oligomers. Bhoc: benzhydryloxycarbonyl; Boc: *tert*-butyloxycarbonyl; Fmoc: 9-fluorenylmethoxycarbonyl; DIPEA: diisopropylethylamine; ESI-MS: electrospray ionisation mass spectrometry; HMBC: heteronuclear multiple bond correlation; HMQC: heteronuclear multiple quantum correlation; MALDI-TOF: matrix-assisted laser desorption/ionisation time-of-flight; PNA: peptide nucleic acid; SPPS: solid phase peptide synthesis; TFA: trifluoroacetic acid; TIS: triisopropylsilane.

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