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Synthesis of a ferrocenyl uracil PNA monomer for insertion into PNA sequences

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ABSTRACT

The deprotection of the *tert*-butyl group of a ferrocenyl uracil Peptide Nucleic Acid (PNA) monomer, Fmoc-aeg(R)-O^{t}Bu (**1**) was achieved using a two step synthesis involving hydrolysis in basic conditions to give first the zwitterion of $^+NH_3$-aeg(R)-O$^-$ (**7**). Compound **7** was reacted *in situ* with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide to obtain the expected compound Fmoc-aeg(R)-OH (**2**) (Abbreviations: Aeg = (2-aminoethyl)-glycine; Fmoc = 9-fluorenylmethoxycarbonyl; O^{t}Bu = *tert*-butyl; R = 5-(*N*-ferrocenylmethylbenzamido)uracyl).

1. Introduction

Insertion of organometallics into peptide nucleic acids (PNA) [1] is currently a field of active research [2–14]. The idea of coupling the remarkable properties of PNA, such as high binding affinity for DNA/RNA strands, high chemical stability and resistance to nucleases [15], with those of organometallics is attractive from the point of view of the development of DNA biosensors [16]. Due to its well established chemistry, availability, reversible electrochemistry and chemical stability [17], ferrocene (Fc) was the first organometallic moiety to be attached to a PNA monomer [10], and then to a PNA sequence [9]. The method used by Metzler-Nolte and co-workers involving the coupling of ferrocenecarboxylic acid to the amino end of a PNA sequence allows insertion of the organometallic moiety at the amino terminus only. Significant flexibility in bioconjugate design could be achieved if the organometallic moiety could be inserted at any selected position within the PNA sequence. From the perspective of electrochemical DNA/RNA biosensor applications, this would allow, for example, the incorporation of a redox-active unit in closer proximity to a base mismatch. In an effort to achieve this insertion, Hudson et al. [8], Maiorana et al. [3–6,13] and our group [18] have reported the synthesis of PNA monomers containing an organometallic moiety that could be potentially inserted anywhere within the PNA sequence. When Fc was used, it was attached either directly to the base uracil [8,18] or to the PNA backbone [4,6,13]. To the best of our knowledge, however, none of these ferrocenyl PNA monomers have been successfully inserted within a PNA oligomer sequence (instead of amino terminus, as reported by Metzler-Nolte et al. [9]). During our attempts to achieve this, we encountered synthetic problems derived from the instability of ferrocene bearing PNA monomers in acidic media. We report the successful synthesis of a ferrocenyl PNA monomer (**2** in Fig. 1) that could be inserted into PNA sequences using standard solid phase protocols and the characterisation of the side-products (**3**–**5** in Fig. 1) isolated during our unsuccessful endeavours to obtain **2**. The choice of an appropriate solid support for ferrocene to be inserted into a PNA sequence is also discussed.

2. Experimental

2.1. Materials

All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or dried over 4 Å molecular sieves or dried following literature procedures [19]. High purity nitrogen gas was used directly from the reticulated system.

2.2. Instrumentation

^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on either a Bruker AC200, a Bruker DPX300 or an Avance DRX400 Bruker spectrometer at 30 °C. The chemical shifts, δ, are reported in ppm (parts per million). Tetramethylsilane (TMS) or the residual solvent peaks have been used as an internal reference.

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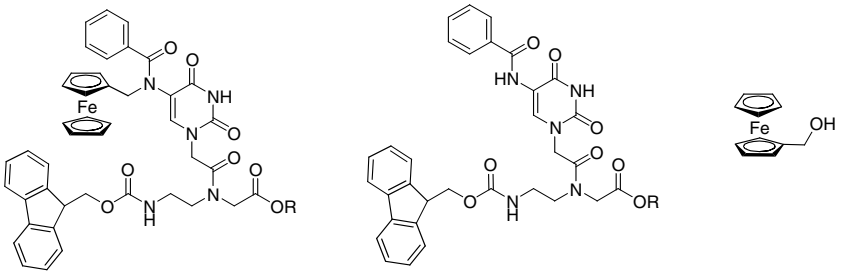


1: R = $C(CH_3)_3$
2: R = H
3: R = $C(CH_3)_3$
4: R = H
5

Fig. 1. Structures of **1**–**5**.

The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). 2D experiments were conducted and analysed on XWinNMR. The ^1H and ^{13}C NMR spectra of compounds **2**–**4**, in particular, were difficult to assign because of the presence of rotamers. This was attempted with the aid of the 2D plots (COSY, HMQC and HMBC), but the assignments should not be treated as definitive. Infrared spectra were recorded using KBr pellets using a Shimadzu FTIR-8400S spectrophotometer at 1 cm^{-1} resolution. Electrospray mass spectra were recorded on Micromass Platform II Quadrupole Mass Spectrometer fitted with an electrospray source. Accurate mass spectra were recorded on a Bruker BioApex II 47e FT-ICR MS fitted with an Analytica Electrospray Source. Samples were introduced by syringe pump at 1 μL/min. A capillary voltage of 200 V was applied.

2.3. Synthesis

2.3.1. tert-Butyl-2-(N-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-(N-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetate (**1**)

Compound **1** was prepared following the procedure published by Spiccia et al. [18]. The spectroscopic data of the product matched that reported previously [18].

2.3.2. 2-(N-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-(N-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetic acid (**2**)

Compound **1** (50.0 mg, 0.06 mmol) was stirred in a mixture of aqueous 2 M NaOH:acetone 1:1 (5 mL of each) for 5 h at r.t. The solution was neutralised with 20% aqueous solution of citric acid and the pH was raised to 8 with an aqueous solution of sat. $NaHCO_3$. Water (5 mL) and acetone (5 mL) were added to the mixture to solubilise the precipitate formed. *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (19.2 mg, 0.06 mmol) was then added and the solution was stirred for 24 h at r.t. The pH was then adjusted to 4 with a 20% aqueous solution of citric acid. Following evaporation of acetone, the resulting aqueous phase was extracted with dichloromethane (150 mL). The organic phase was dried with Na_2SO_4, filtered and evaporated to dryness to give an orange oil. Chromatography on a silica column using a gradient ranging from hexane:ethyl acetate 1:1 to acetone:water 5:1 as the eluent was performed. An orange fraction was collected and evaporated to dryness. The residual solid was dissolved in ethyl acetate (25 mL) and the organic phase washed with water (10 mL), dried with Na_2SO_4, filtered and evaporated to dryness to give **2** as an orange solid. Yield: 18 mg (38%). Selected IR bands (KBr; ν, cm^{-1}): 3396 w br, 3177 w br, 3072 w br, 2924 w br, 1625–1750 s br, 1541 w, 1461 m, 1407 m, 1246 m, 1230 m, 1142 w, 1104 w, 1024 w, 960 w, 793 w, 740 w, 492 w. ^1H NMR Spectrum (acetone-d_6): 3.10–3.45 (m, 4H, NH–CH_2–CH_2 and CH_2–CH_2–N), 3.95–4.00 (m, 4H, 2 × *CH* Cp ring and N–CH_2–CON), 4.05 (s, 5H, 5 × *CH* Cp), 4.10–4.30 (m, 6H, 2 × *CH* Cp, Fmoc–*CH*–CH_2O and 2 × Fmoc–CH–CH_2O and 1 × Cp–CH_2–N), 4.48 (d, ^2J(H–C–H) = 16.3 Hz, 1H, 1 × N–CH_2–COOH), 4.65 (d, ^2J(H–C–H) = 16.3 Hz, 1H, 1 × N–CH_2–COOH), 4.94 (d, ^2J(H–C–H) = 13.5 Hz, 1H, 1 × Cp–CH_2–N), 6.46 (min) and 6.60 (maj) (rotamers, br s, 1H, CH_2–N*H*–COO), 7.08-7.35 (rotamers, m, 10H, 4 × *CH*Fmoc arom and 5 × *CH* arom and C=*CH*—N), 7.57 (m, 2H, *CH*Fmoc arom), 7.75 (m, 2H, *CH*Fmoc arom), 10.14 (min) and 10.17 (maj) (rotamers, s, 1H, CO–N*H*–CO). ^{13}C NMR Spectrum (acetone-d_6): δ 39.96 (NH–CH_2–CH_2), 48.20 (Fmoc–*CH*–CH_2O), 48.43, 48.53 and 49.04 (br, corresponds to Cp–CH_2–N, CH_2–CH_2–N, N–CH_2–CON and N-CH_2–COOH), 67.21 (min) and 67.30 (maj) (rotamers, Fmoc–CH–CH_2O), 69.14 (*C*H Cp), 69.33 (broad, *C*H Cp), 70.87 (*C*H Cp), 83.76 (*C* Cp), 120.90 (*C*H Fmoc), 126.24 (*C*H Fmoc), 128.05 (*C*H arom and *C*H Fmoc), 128.62 (b, *C*H arom and *C*H Fmoc), 130.32 (*C*H arom), 137.73 (rotamers, CO–*C* benzyl), 142.22 (*C*Fmoc), 145.23 (maj) and 145.29 (min) (rotamers, *C*Fmoc), 146.92 (C=*C*H—N), 151.14 (NH–*C*O–N), 157.61 (NH–*C*OO–CH_2), 161.76 (C–*C*O–NH), 167.70 (min) and 167.79 (maj) (N–CH_2–*C*ON), 168.37 (CH_2–*C*OOH), 170.58 (min) and 171.42 (maj) (N–*C*O–benzyl). We were unable to locate the signal for (N—*C*=CH), probably due to a long relaxation time of this carbon. Electrospray mass spectrum (*m*/*z*): Negative mode: 808 $[M-H]^-$ (100%). High Resolution ESI Mass Determination: Found: 832.2046; calcd for $C_{43}H_{39}FeN_5NaO_8$, 832.2038.

2.3.3. 2-(N-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-benzamido-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetic acid (**4**)

Compound **1** (50.0 mg, 0.06 mmol) was dissolved in CH_2Cl_2 (2.5 mL) and a solution of 4 M HCl in dioxane (0.35 mL) was added to the orange solution. The solution was stirred for 48h at r.t. A green solution and a white precipitate were obtained. Cold diethyl ether (5 mL) was added to the mixture to complete the precipitation of product. The white solid, 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-benzamido-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (**4**), was then filtered, washed with ether (3 × 5 mL) and dried. Yield: (24 mg, 67%). Selected IR bands (KBr; ν, cm^{-1}): 3391 w br, 3181 w br, 3072 w br, 2922 w br, 1625–1750 s br, 1542 m, 1462 m, 1416 w, 1384 m, 1244 m, 1230 m, 1196 w, 1153 w, 1104 w, 1001 w, 888 w, 759 w, 742 w, 707 w, 622 w. ^1H NMR Spectrum (acetone-d_6): 3.15–3.55 (m, 4H, NH–CH_2–CH_2 and CH_2–CH_2–N), 4.07 (maj) (rotamers, s, N–CH_2–CON), 4.15–4.23 (m, Fmoc–*CH*–CH_2O and rotamers (min) N–CH_2–CON), 4.30 (min) and 4.32 (maj) (rotamers, m, 2H, Fmoc–CH–CH_2O), 4.65 (min) and 4.80 (max) (s, 2H, N–CH_2–COOH), 6.42 (min) and

6.66 (maj) (rotamers, br s, 1H, CH_2-N*H*–COO), 7.18–7.31 (m, 4H, C*H* Fmoc arom), 7.38–7-47 (m, 3H, C*H* arom), 7.56–7.61 (m, 2H, C*H* Fmoc arom), 7.70-7.83 (m, 4H, 2 × C*H* arom and 2 × C*H* Fmoc arom), 8.39 (min) and 8.41 (maj) (rotamers, s, 1H, C═C*H*—N), 8.46 (min) and 8.48 (maj) (rotamers, s, 1H, Ar–CO–N*H*), 10.54 (s, 1H, CO–N*H*–CO). ^{13}C NMR Spectrum (acetone-d_6): 39.53 (min) and 39.90 (maj) (rotamers, NH–CH_2–CH_2), 48.13 (Fmoc-CH–CH_2O), 48.50 (maj) and 48.61 (min) (rotamers, CH_2–CH_2–N), 49.68 (br, corresponded to two different carbons, N–CH_2–CON and N–CH_2–COOH), 67.08 (maj) and 67.27 (min) (rotamers, Fmoc–CH–CH_2O), 114.94 (maj) and 115.02 (min) (rotamers, N—*C*═CH), 120.82 (*C*H Fmoc arom), 126.20 (*C*H Fmoc arom), 127.99 (corresponded to two different carbons, *C*H Fmoc arom and *C*H arom), 128.52 (*C*H Fmoc arom), 129.62 (*C*H arom), 132.80 (*C*H arom), 133.40 (min) and 133.68 (maj) (rotamers, C═*C*H—N), 134.19 (min) and 135.20 (maj) (rotamers, CO–*C* arom), 142.14 (*C*Fmoc), 145.23 (*C* Fmoc), 150.13 (NH–*C*O–N), 161.22 (NH–*C*OO–CH_2), 165.98 (HC═C—*C*O—NH), 168.18 (N–CH_2–*C*ON), 168.69 (NH–*C*O–benzyl), 171.09 (CH_2–*C*OOH). Electrospray mass spectrum (*m*/*z*): 610 $[M-H]^-$ (100%). High Resolution ESI Mass Determination: Found: 610.1961; calcd for $C_{32}H_{28}N_5O_8$, 610.1938.

*2.3.4. tert-Butyl-2-(N-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino) ethyl)-2-(5-benzamido-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetate (**3**) and hydroxymethylferrocene (**5**)*

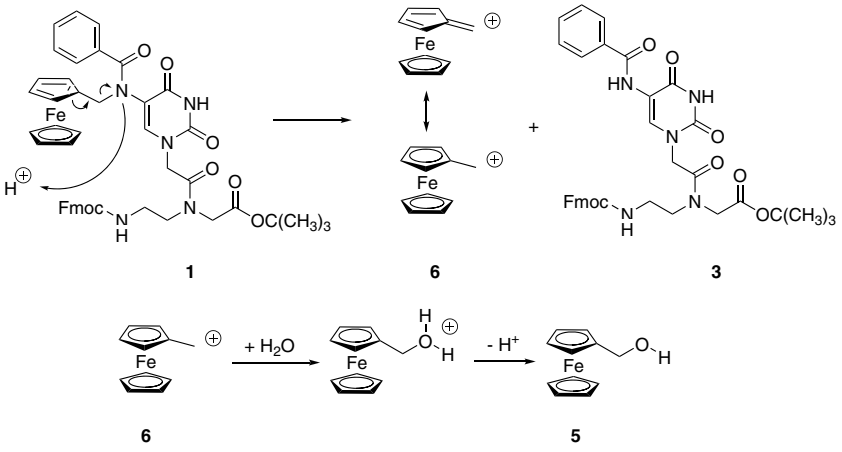
The filtrate obtained in the synthesis of **4** was evaporated to dryness and the orange residual solid purified by column chromatography on silica with a gradient from hexane:ethyl acetate 2:1 to hexane:ethyl acetate 1:3 as the eluent. An orange solid, hydroxymethylferrocene (**5**) (R_f(**5**) = 0.76 in hexane:ethyl acetate 1:1), and a white solid, (**3**) (R_f(**3**) = 0.72 in hexane:ethyl acetate 1:3) were isolated and identified. Estimated yields for **5** (3 mg, 24%) and for **3** (4 mg, 10%). *Characterisation of **3***. Selected IR bands (KBr; *ν*, cm^{-1}): 3200 w br, 3049 w, 2924 m, 2860 w, 1625–1750 s br, 1544 m, 1456 s, 1411 m, 1383 m, 1246 m, 1154 m, 1104 w, 1027 w, 800 w, 760 w, 742 w, 705 w. ^{1}H NMR Spectrum (acetone-d_6): 1.33 (maj) and 1.42 (min) (s, 9H, C(CH_3)$_3$), 3.10–3.55 (m, 4H, NH–CH_2–CH_2 and CH_2–CH_2–N), 3.95 (maj) (rotamers, s, N–CH_2–CON), 4.10-4.20 (m, Fmoc–C*H*–CH_2O and rotamers (min) N–CH_2–CON), 4.21 (min) and 4.31 (maj) (rotamers, m, 2H, Fmoc–CH–CH_2O), 4.63 (maj) and 4.79 (min) (rotamers, s, 2H, N–CH_2–COOC(CH_3)$_3$), 6.41 (min) and 6.64 (maj) (rotamers, br s, 1H, CH_2–N*H*–COO), 7.18–7.32 (m, 4H, C*H* Fmoc arom), 7.39–7.49 (m, 3H, C*H* arom), 7.57–7.62 (m, 2H, C*H* Fmoc arom), 7.71–7.83 (m, 4H, 2 × C*H* arom and 2 × C*H* Fmoc arom), 8.39 (maj) and 8.41 (min) (rotamers, 1H, C═C*H*—N), 8.46 (min) and 8.48 (maj) (rotamers, s, 1H, Ar–CO–N*H*), 10.53 (s, 1H, CO–N*H*–CO). ^{13}C NMR Spectrum (acetone-d_6): δ 28.57 (COO–C(CH_3)$_3$, 39.91 (min) and 40.31 (maj) (rotamers, NH–CH_2–CH_2)), 48.48 (Fmoc-CH–CH_2O), 48.88 (maj) and 48.92 (min) (rotamers, CH_2–CH_2–N, 49.04 (maj) (rotamers, N–CH_2–CON), 50.05 (maj) and 50.15 (min) (N–CH_2–COOC(CH_3)$_3$), 51.58 (min) (rotamers, N–CH_2–CON), 67.38 (maj) and 67.54 (min) (rotamers, Fmoc–CH–CH_2O), 82.30 (COO–*C*(CH_3)$_3$), 115.25 (maj) and 115.33 (min) (N—*C*═CH), 121.10 (min) and 121.13 (maj) (*C*H Fmoc), 126.50 (*C*H Fmoc), 128.33 (*C*H Fmoc), 128.84 (*C*H Fmoc), 129.94 (*C*H arom, corresponds to two different carbons), 133.10 (*C*H arom), 133.78 (min) and 134.10 (maj) (rotamers, C═CH—N), 135.27 (CO–*C* arom), 142.49 (*C*Fmoc), 145.55 *C*Fmoc), 150.44 (NH–*C*O–N), 161.58 (NH–*C*OO–CH_2), 165.88 (C–*C*O–NH), 168.38 (N–CH_2–*C*ON), 169.00 (NH–*C*O–benzyl), 169.62 (CH_2–*C*OO–C(CH_3)$_3$). Electrospray mass spectrum (*m*/*z*): 690 $[M+Na]^-$ (100%). High Resolution ESI Mass Determination: Found: 690.2535; calcd for $C_{36}H_{37}N_5O_8Na$, 690.2540. *Characterisation data of **5***. The analytical data of the products matched that reported previously [20].

3. Results and discussion

When using the Fmoc (9-fluorenylmethoxycarbonyl)/Bhoc (benzhydryloxycarbonyl) strategy to prepare peptide nucleic acids (PNAs), the primary amino group of the PNA monomers is protected with a Fmoc group and the exocyclic amino group of the adenine (A), guanine (G) and cytosine (C) monomers with the Bhoc group [21]. The carboxylic acid group is unprotected, ready to be activated by an activator agent in the presence of a base (generally a mixture of diisopropylamine and lutidine). Optimisation of the synthesis protocols found that *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) gave the highest average coupling yields and therefore HATU is now generally preferred over others activator agents, such as *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) for the synthesis of PNAs [22]. Comprehensive reports on the different PNA synthesis methods, as well as appraisals of their advantages and disadvantages can be found in the literature [21–24]. We recently reported a new ferrocenyl uracil PNA monomer, *tert*-butyl-2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-(*N*-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetate (**1** in Fig. 1), which was developed to incorporate ferrocenyl units into PNA sequences. Cleavage of the *tert*-butyl group of **1** to give 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-(*N*-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (**2**) (Fig. 1) is necessary before it can be used in either automated PNA synthesis [21] or manual solid phase synthesis (for a recent example of manual synthesis of metal-labelled PNAs, see [25]).

The first synthetic method used to cleave the *tert*-butyl group of **1** was a mixture of trifluoroacetic acid (TFA):*m*-cresol 4:1 (v/v), which is typically employed during standard PNA synthesis procedures that cleave both the PNA from the resin and the Bhoc groups from the exocyclic amine of the base [21]. However, when applied to **1**, our mixture turned from orange to green, indicative of oxidation to the ferricenium ion. Milder acidic conditions, such as diluting the TFA in dichloromethane or the use of another scavenger (triethylsilane) did not yield the desired product; either the initial orange mixture turned green or only the starting material was recovered. Oxidation of ferrocenyl compounds in biomolecules has previously been reported by Tartar et al., when they synthesised a ferrocene containing pentapeptide on an automated peptide synthesiser [26]. These workers showed that the addition of ascorbic acid facilitated the formation of the desired ferrocenyl peptide [26]. In our case, the addition of even a large excess of antioxidant agents, such as ascorbic acid and Na_2SO_3, prevented the mixture from turning green but in all cases the expected product **2** was not obtained.

Use of 4 M HCl in dioxane further diluted in CH_2Cl_2 led to the isolation of *tert*-butyl 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-benzamido-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetate (**3**) and 2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-benzamido-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (**4**) (Fig. 1), indicative of the cleavage of the ferrocenyl moiety from the molecule. Evidence of the presence of **3** and **4** were given by ESI–MS spectrometry with a peak at *m*/*z* = 690 and 610, corresponding to $[M+Na]^+$ and $[M-H]^-$, respectively, and the concordance of the High Resolution ESI-Mass spectra. The cleavage of the ferrocenyl moiety was further confirmed by the disappearance of characteristics ^{1}H and ^{13}C NMR signals (see SI for ^{1}H NMR spectra of **3** and **4**). Furthermore, the two signals in the ^{1}H spectrum corresponding to the magnetically inequivalent protons of the CH_2 linking the ferrocene to the amino uracil derivative [18] (in particular, a signal at

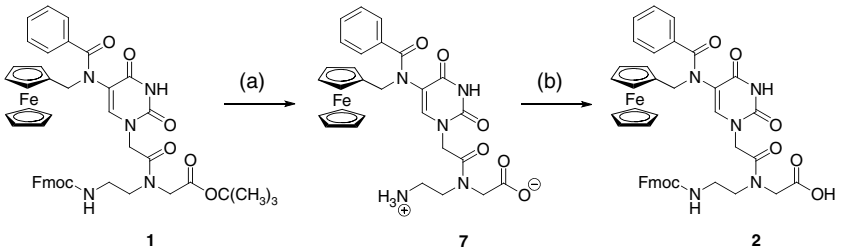


Scheme 1. Proposed mechanism of the formation of **3** and **5** from **1**.

ca. 5 ppm with a geminal coupling constant of 13.7 Hz) were no longer present in the spectra of both **3** and **4**. The main difference between the ^{1}H spectrum of **3** and **4** are the two singlets at 1.33 (maj. rotamer) and 1.42 ppm (min. rotamer) corresponding to the protons of the *tert*-butyl group in **3** that are absent in **4**. An interesting feature in the ^{1}H NMR spectra of **3** and **4** is the presence of two singlets at 8.39 and 8.41 ppm (rotamers) corresponding to the C*H* proton on the uracil base. These resonances are shifted significantly downfield upon the removal of the ferrocenyl moiety compared to the ^{1}H NMR spectrum of **1**, where they appear at 7.11 and 7.19 ppm [18].

The isolation of **3** and **4** implies the formation of other ferrocenyl derivatives. In fact, the known ferrocenyl compound, hydroxymethylferrocene (**5** in Fig. 1) was isolated from the reaction mixture. Another less polar ferrocenyl compound was also present but could not be identified. Interestingly, we recently found that **5** was a side-product of the coupling of (ferrocenylmethyl)trimethylammonium iodide and 1,4,7-(triformyl)-1,4,7,10-tetraazacyclododecane to give 1-(ferrocenemethyl)-4,7,10-(triformyl)-1,4,7,10-tetraazacyclododecane in aqueous solution [27]. A possible pathway for the formation of **3**–**5** is presented in Scheme 1 and involves the simultaneous formation of **3** and the carbocation **6** which has been previously reported to be stabilised in acidic media [28–31]. Interestingly, the fact that both **3** and **4** were formed suggests that the cleavage of ferrocenyl group is faster than the cleavage of the *tert*-butyl group as **2** would have been isolated if the cleavage of the ester was faster. The π-system of the ferrocene in **1** is probably responsible for the (hyper-) sensitivity to hydrolysis. The carbocation **6** is then highly susceptible to reaction with nucleophiles and will react readily with a water molecule to give **5**. The hygroscopic character of the HCl in dioxane solution would provide sufficient water for this reaction to take place.

Decomposition of ferrocenyl amino acid derivatives in a mixture of 50% TFA in CH_2Cl_2, despite the use of ascorbic acid and working under argon in degassed solvent, was previously reported by Metzler-Nolte et al., but the decomposition compounds were not reported [32]. Maiorana et al. recently reported similar difficulties to ours when synthesising a triferrocenyl thymine PNA monomer. The instability of ferrocene in acidic media led this group to use basic conditions [13]. In light of these findings, we decided to change our synthetic strategy. The new approach was similar to that reported by Metzler-Nolte et al. during their first description of the automated synthesis of a ferrocenyl PNA sequence [9], in which acidic conditions were not used because they employed highly cross-linked polystyrene beads as the solid support that was functionalised with a glycine moiety via a *p*-hydroxymethylbenzoic acid linker. Importantly, the removal of the PNA sequence from this solid support does not require TFA, but methanolic ammonia which was tolerated by their ferrocenyl PNA sequence. This same strategy was used to incorporate an amino acid bearing ferrocenyl unit into oligopeptides [33]. With this in mind, aqueous 2 M NaOH diluted in acetone was employed to cleave the *tert*-butyl group of **1**. This also cleaved the base sensitive Fmoc protecting group, forming the zwitterion of 2-(*N*-(2-aminoethyl)-2-(5-(*N*-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (**7**) when neutralised to pH 7 with aqueous 20% citric acid solution (Scheme 2). Compound **7** was not isolated but was directly reacted with one equivalent of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) at pH 8 (adjusted with saturated $NaHCO_3$ (aq) as reported by Coull et al. for an adenine PNA monomer [34]). The expected compound **2** was obtained after purification by column chromatography on silica (see SI). Evidence of the successful synthesis of **2** is given by the ESI–MS with a peak at *m*/*z* 808 corresponding to the $[M-H]^-$ ion, the concordance of the High Resolution ESI–MS data (found: 832.2046; calcd for $C_{43}H_{39}FeN_5NaO_8$, 832.2038) and the disappearance in the ^{1}H and ^{13}C NMR spectra of the proton and carbon signals, respectively, corresponding to



Scheme 2. (a) i. 2M NaOH(aq): acetone 1:1 (v/v), 5 h, r.t.; ii. 20% citric acid aqueous solution until pH 7; (b) sat. $NaHCO_3$(aq), Fmoc-OSu, 24 h, r.t., Total yield. 38% over 2 steps.

the *tert*-butyl group. As for **3** and **4**, a very broad band between 1625 and 1750 cm^{-1} is observed in the IR spectrum of **2**, which is attributed to the ν_{CO} stretch of several different types of carbonyl functional groups present in the compound. The presence of rotamers, which were detected by NMR spectroscopy, gives rise to additional carbonyl vibrations and adds complexity to this region of the spectrum. The ^{1}H NMR spectrum exhibits some other interesting features. Contrary to that observed for **3** and **4** (see above), no singlets at 8.39 and 8.41 ppm (rotamers) corresponding to the alkene C*H* proton on the uracil base are observed for **2**. They appear in the multiplet at 7.05–7.35 ppm. This is consistent with that found for **1** for which the peaks were observed at 7.11 and 7.19 ppm [18]. As for **1**, the two protons of the CH_2 linking the ferrocene to the uracil base are not magnetically equivalent and therefore appeared as two doublets with a high value geminal constant (~14 Hz). Contrary to what was found for **1**, the N–CH_2–COOH protons in **2** appeared as two doublets (at 4.48 and 4.65 ppm with a geminal coupling constant of about 16 Hz), and not as a single singlet [18]. The assignment of these two protons was aided by 2D NMR spectroscopy which indicated that these two protons correlated to each other (COSY) and to the same carbon (HMQC).

4. Conclusion

A new ferrocenyl PNA monomer (**2**) that could potentially be inserted at any chosen position within a PNA sequence has been prepared via a one-pot two step synthesis involving the simultaneous saponification of the *tert*-butyl group and the cleavage of the Fmoc protecting group of *tert*-butyl-2-(*N*-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-2-(5-(*N*-ferrocenylmethylbenzamido)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetate (**1**), followed by the reprotection of the amino end of the PNA backbone. Initial attempts using acidic conditions were found to be unsuccessful, leading to either no reaction or to ferrocenylmethyl decomposition and indicated that the introduction of a ferrocenyl PNA monomer into a PNA sequence by automation and the subsequent cleavage from the resin is a real chemical challenge. While the ferrocenyl PNA monomer **2** is unsuitable for use in PNA synthesis that applies the XAL/PAL resin [21], these limitations may be overcome by using Fmoc/*N*-acyl protected PNA monomers [35,36] and highly cross-linked polystyrene beads functionalised with a glycine moiety as the solid support, as reported by Metzler-Nolte et al. [9] This approach allows basic conditions (ethanolic ammonia) to be used to cleave the PNA from the resin.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2008.05.009.

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