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A New Triferrocenyl-tris(hydroxymethyl)aminomethane Derivative as a Highly Sensitive Electrochemical Marker of Biomolecules: Application to the Labelling of PNA Monomers and Their Electrochemical Characterization

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Abstract: We have designed and synthesised a new organometallic molecule containing three ferrocene groups for use as a highly sensitive electrochemical marker in biological assays. This trisferrocene derivative was conjugated to different PNA monomers, and the electrochemical activities of the conjugates were extensively investigated in organic solvents, in view of their potential diagnostic applications. The results showed that the introduction of a trisferrocene unit on the PNA monomer triples the current signal in comparison with the monoferrocene-labelled one. Despite their greater molecular complexity, trisferrocene-conjugated PNA monomers are even more electrochemically active than the reference ferrocene. By using differential pulse voltammetry (DPV), the detection limit can reach 10^{-8} M in acetonitrile solution. These results are a good premise for the use of the trisferrocene unit as an effective electrochemical probe for biomolecules.

Keywords: bioorganometallic chemistry · electrochemistry · ferrocene · metallocenes · peptide nucleic acids

Introduction

There is a growing demand for high affinity and specific biomolecular probes capable of detecting selected base sequences in human, viral, bacterial and vegetal nucleic acids.

For human DNA sequences, the objective is to detect the disease-related punctiform mutations responsible for hereditary and other disorders, and the different individual responses to drugs. One promising method for detecting DNA involves the use of peptide nucleic acids (PNAs), nucleic acid mimics introduced by Nielsen in 1991,[1] in which the sugar-phosphate backbone is replaced by a neutral achiral pseudopeptide chain of *N*-(2-aminoethyl)glycine units, with the nucleobases linked to the glycine nitrogen through carbomethylene bridges. PNA oligomers can be prepared in relatively large quantities by using a peptide synthesizer, and are highly stable in biological environments.[2] The nucleobases form highly specific duplex hybrids with complementary DNA sequences based on the Watson–Crick base-pairing rules; these products are much more thermally stable than the analogous DNA–DNA or DNA–RNA duplexes because the PNA chain is neutral and therefore does not give rise to any repulsive electrostatic interstrand interaction with the negatively charged DNA sequence.[3] The stability of a PNA–DNA duplex, measured on the basis of melting temperature (T_m),[4,5] is related to the ability of PNA oligomers (15–20 units) to discriminate even a single mismatched base in a given DNA sequence,[6–8] an ability that is further increased in PNAs containing the so-called "chiral box".[9,10] These characteristics give PNAs great potential as antigene or antisense drugs,[11,12] and as biomolecular probes in biotechnologies,[2,13] thus making it worth trying to equip them with sensitive markers capable of detecting the hybridisation event and converting it into an easily measurable analytical signal.

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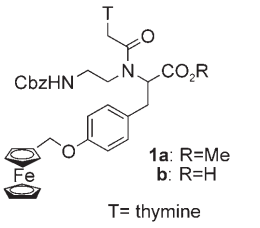
As a part of our research project concerning bioorganometallic chemistry,[14] we have recently been interested in PNA properties and we have synthesised some metal-conjugated PNA monomers[15] with the aim of evaluating their analytical characteristics as a preliminary step to the synthesis of new metal-conjugated PNA oligomers to be used as DNA probes. In the search for new DNA detection methods, the use of an electro-active DNA as analytical probe has been widely investigated,[16] because electrochemistry seems to be a very promising alternative to fluorescence in terms of sensitivity, response times and costs. However, electrochemistry has been much less used for PNA probes.[17] Of the various organometallic complexes, useful electrochemically detectable fragments, ferrocene is known to be one of the most convenient, because it is very stable and undergoes reversible electron oxidation.[18] Both ourselves[15] and other authors[19] have synthesised and electrochemically studied a number of ferrocene-labelled PNA monomers and oligomers; in particular the electrochemical behaviour and efficiency of a monoferrocene end-labelled PNA 10-mer, in a surface immobilized duplex, was recently investigated by Metzler-Nolte.[20]

In a previous work we have synthesised the tyrosine-derived ferrocene-labelled monomer **1a**, which is chiral and has amino and carboxylic functions available for the oligomerization; this could allow the insertion of more than one ferrocene unit into a PNA chain and, in principle, the enhancement of the electrochemical response. Cyclic voltammetry (CV) studies of **1a** led to good results, producing a reversible CV curve, and the product was detected in concentrations as low as 10^{-7} M by using differential pulse voltammetry (DPV).[15,21a]

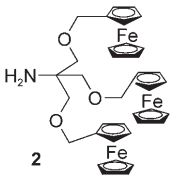


As improving sensitivity is valuable for analytical purposes, the above results convinced us to seek more efficient systems, and thus to design and synthesise a molecule containing multiple ferrocene groups that could act as a flexible, general marker and would have improved electrochemical properties and sensitivity.

In principle, any such molecule should:

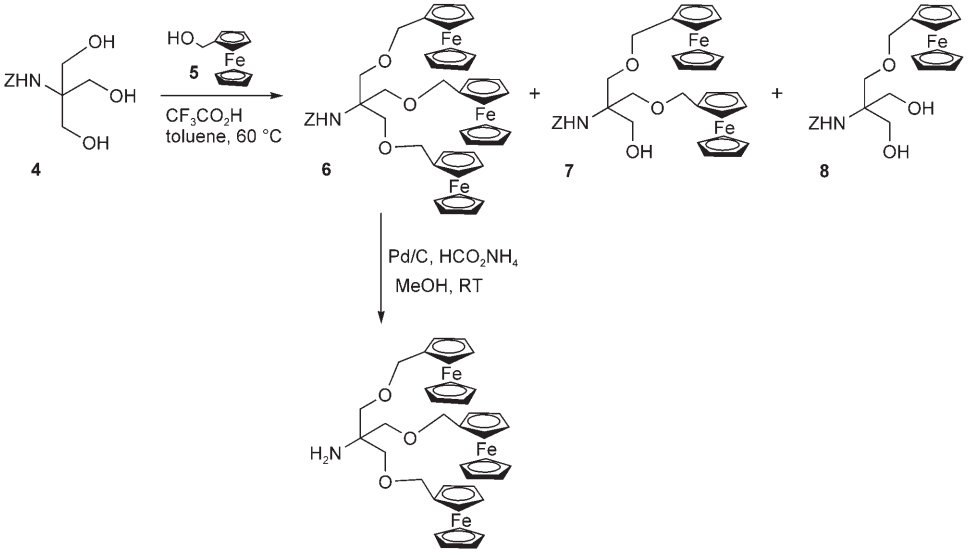
1) Be easy and inexpensive to make.
2) Bear a versatile and reactive functional group for linking the marker to the substrate.
3) Have electrochemically equivalent ferrocene units in order to obtain a single, multiple-independent-electron oxidation wave; this means proportionality between current density and the number of ferrocene groups.

We selected the structure tris(ferrocenemethylenoxymethyl)aminomethane (**2**, Tris-Fc),[21b] because it has a versatile and reactive functional amino group, and the ferrocene units are electronically isolated in the sense that they have no evident interactions other than unpredictable "through space" interactions. These characteristics should produce the electrochemical equivalence of the ferrocene groups. In this paper we report, the synthesis of compound **2**, its use for labelling PNA monomers and the electrochemical characterisation of the conjugates obtained.



Results and Discussion

Synthesis: Tris-Fc **2** was synthesised following the sequence shown in Scheme 1. The amino group of tris(hydroxymethyl)-aminomethane was Cbz-protected to give **4**, a compound that had been previously reported (30% yield),[22] but we improved the yield to 88% by changing the organic solvent from CH_2Cl_2 to AcOEt. Compound **4** was then reacted with an excess of ferrocene methanol **5**[15] by heating to 60°C in toluene in the presence of a catalytic amount of trifluoroacetic acid. After chromatographic purification, the *N*-Cbz protected Tris-Fc **6** was isolated in 73% yield as a yellow solid, together with a small amount (about 10% each) of the di- and monosubstituted derivatives **7** and **8**. The cleavage of the Cbz group in **6** by ammonium formate and Pd/C, in MeOH at room temperature, afforded **2** as a dark yellow solid in 73% yield.



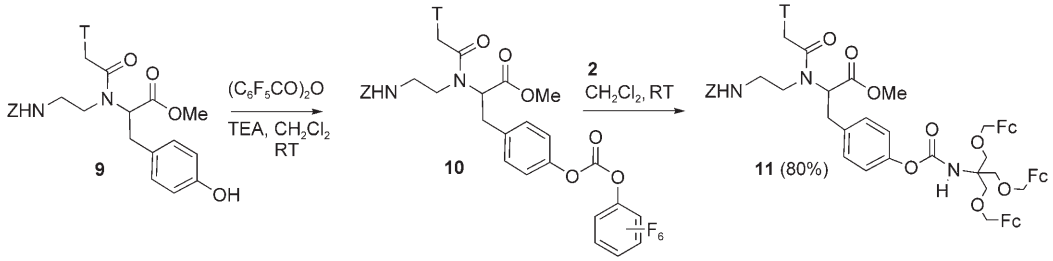
Scheme 1. Synthesis of Tris-Fc **2**.

The next step was the study of a suitable protocol to link the new marker **2** to the tyrosine PNA monomer **9**,[15] to do this the hydroxyl group of **9** was activated through the formation of the bis-pentafluorophenyl carbonate **10**, which easily reacted with Tris-Fc **2** in CH_2Cl_2 at room temperature, affording triferrocenyl monomer **11** in 80% yield (Scheme 2).

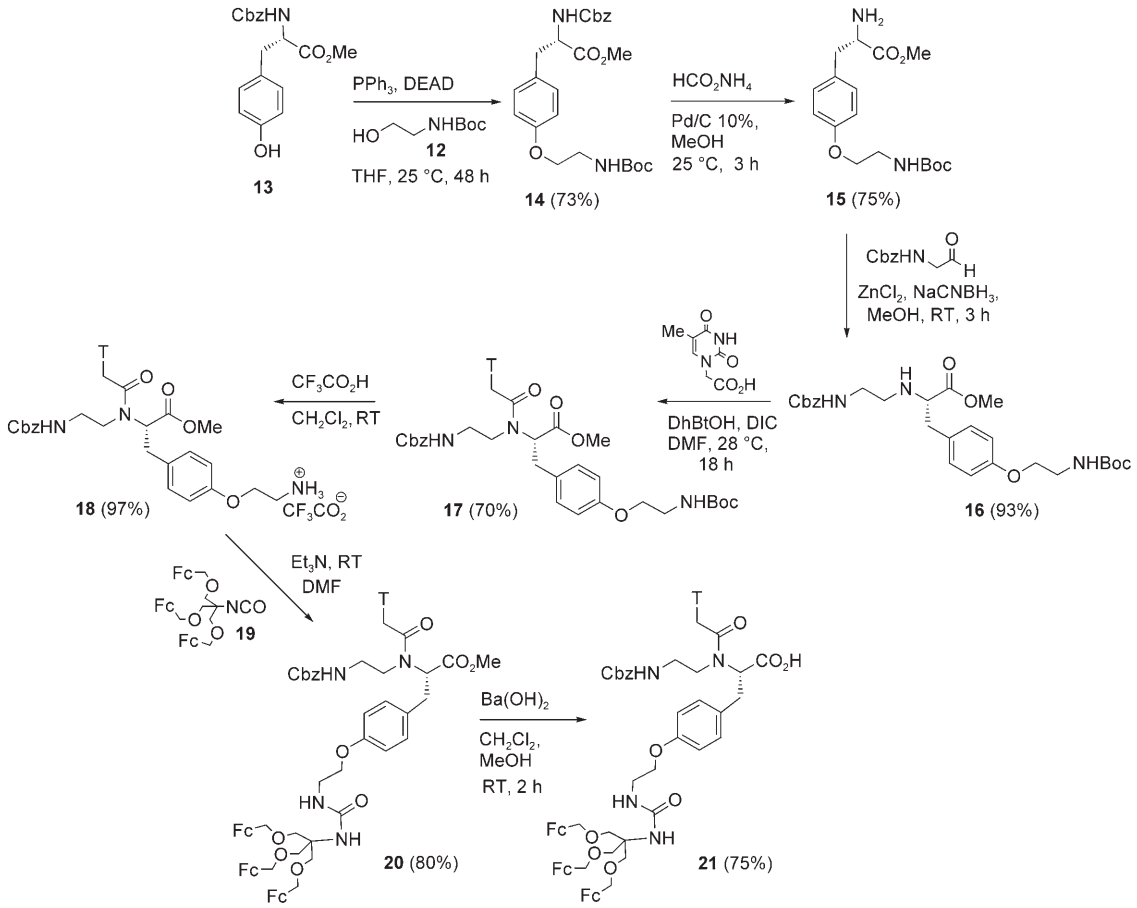
The synthesis of monomer **11** allowed us to quickly reach two objectives, which were firstly, the verification of the good reactivity of the amino group in Tris-Fc **2** and secondly, to check the amplification of the electrochemical signal in monomer **11** (see discussion below) with respect to the monoferrocene-labelled monomer **1a**. To be able to construct PNA oligomers, it was necessary to check the stability of the carbamate group in **11** under the alkaline hydrolysis conditions of the methyl ester[23] but, unfortunately, even the mild treatment of monomer **11** with two equivalents of LiOH at room temperature caused the cleavage of the carbamate group.

Thus, it was necessary to select a linker group stable in basic conditions and we focused on the ureido function. Therefore, we synthesised the monomer **18** bearing an aminoethyl linker at the tyrosine phenol group, to which **2** was connected to give the new trisferrocene-labelled monomers **20** and **21** (Scheme 3).

The synthesis of the tyrosine PNA monomer **18** is depicted in Scheme 3. L-*N*-Cbz tyrosine methyl ester **13** reacted with *N*-Boc-2-amino ethanol **12** in THF, in the presence of



Scheme 2. Synthesis of Tris-Fc-labelled monomer **11**.



Scheme 3. Synthesis of Tris-Fc-labelled tyrosine PNA monomer **21**.

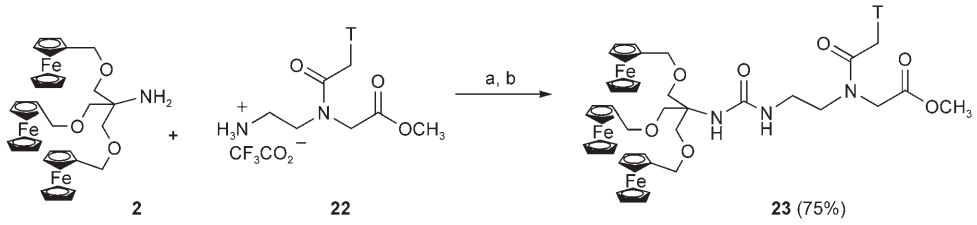
PPh_3 and DEAD, under Mitsunobu conditions at room temperature to give the tyrosine derivative **14** in 73% yield.

The cleavage of the Cbz group with ammonium formate and Pd/C in MeOH at room temperature gave **15**, which was then reacted with *N*-Cbz aminoacetaldehyde under reductive amination conditions ($NaCNBH_3/ZnCl_2$) in methanol at room temperature to afford the corresponding *N*-aminoethyl derivative **16** in very good yield (93%). The subsequent condensation of **16** with 1-carboxymethylthymine was conducted in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhBtOH) and diisopropylcarbodiimide (DIC) to give compound **17** in 70% yield. After cleavage of the Boc-protecting group with TFA, the new monomer **18** was isolated as the trifluoroacetate salt in 97% yield.

Compound **18** was linked to the Tris-Fc isocyanate derivative **19**, which was obtained by treating **2** with Boc_2O in the presence of dimethylamino pyridine,[24] and afforded trisferrocene-labelled monomer **20** in good yield (80%). The subsequent methyl ester hydrolysis was successfully conducted by using $Ba(OH)_2$ and gave acid **21** in 75% yield.

In addition, as a further possibility for labelling a PNA strand, we investigated the reaction of Tris-Fc **2** with the terminal amino group of an aminoethylglycine (aeg) PNA monomer **22**. Tris-Fc **2** was transformed into the intermediate pentafluorophenyl carbamate, which was directly reacted with monomer **22** in the presence of diisopropylamine and gave **23** in 75% yield (Scheme 4). Monomer **23** was also obtained from the reaction of Tris-Fc isocianate **19** and **22** in DMF at room temperature, but in lower yield (58%).

At this stage of the research, after the check of the synthetic efficiency of **2**,[25] it was important to study the electrochemical behaviour of the monomers labelled with Tris-Fc, **11**, **20**, **21** and **23**, in comparison with that of mono-Fc monomers **1a**,**b**.[15] Thus, all compounds were investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV), the results of which are discussed below.



Scheme 4. Synthesis of Tris-Fc-labelled aeg-monomer **23**: a) $(C_6F_5CO)_2O$, DIPEA, DMF; b) **22**, DMF, DIPEA.

Electrochemical characterization: The voltammetric features of ferrocene derivatives **2**, **6**, **7** and **8** have already been reported.[21b] Figures 1, 2, 5 and Table 1 summarise the most significant CV features of our six ferrocene-labelled PNA monomers (**1a**, **1b**, **11**, **20**, **21** and **23**) and the reference compound ferrocene (Fc) obtained at potential scan rate $v = 0.2\ V\,s^{-1}$ on GC at 298 K. The effect of the molecular structure of the ester monomers (monoferrocene **1a** and triferrocene derivatives **11**, **20** and **23**) is shown in Figure 1, which contrasts the voltammetric behaviours of the four compounds at $v = 0.2\ V\,s^{-1}$.

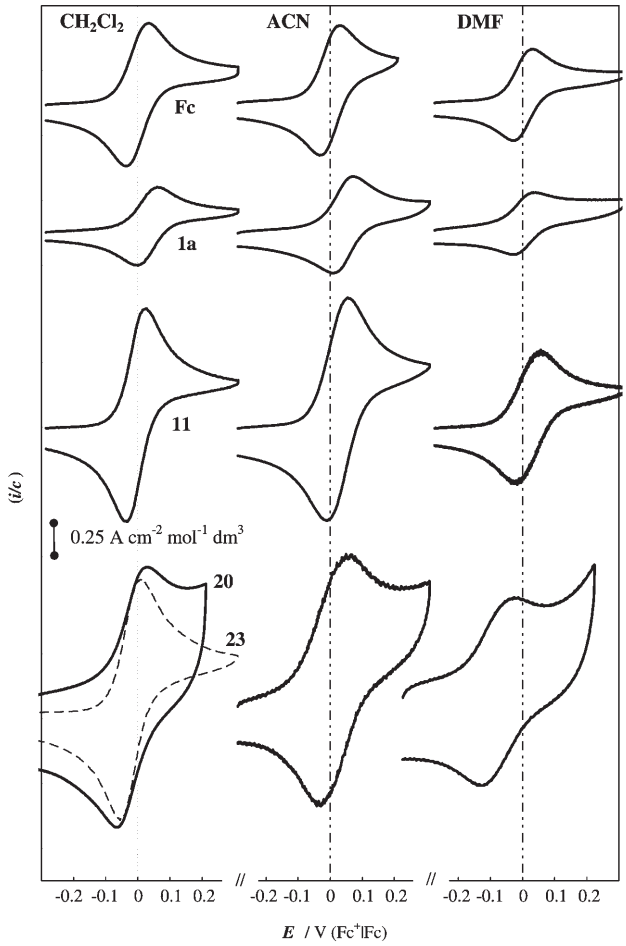


Figure 1. A synopsis of the CV patterns obtained at $v = 0.2\ V\,s^{-1}$ for the four ester substrates **1a**, **11**, **20** and **23** in the three organic solvents CH_2Cl_2, MeCN and DMF, with ferrocene as a reference.

The effect of a free carboxylic function is shown in Figures 2 and 5; these Figures compare the CV features of the two PNA acid monomers **1b** and **21** with their parent esters **1a** and **20**, respectively. The effects of the solvent and molecular structure on peak currents can be seen in Figure 4 in which the squared slopes of the linear characteristics (normalised current densities versus $v^{0.5}$) are plotted as a function of the reciprocal of solvent viscosity.

In the case of a diffusive electrochemically reversible peak and a given number of transferred electrons, such squared slopes are proportional to the substrate diffusion coefficients,[26] and should verify Stokes' law (i.e. the diffusion coefficients should be inversely proportional to solvent

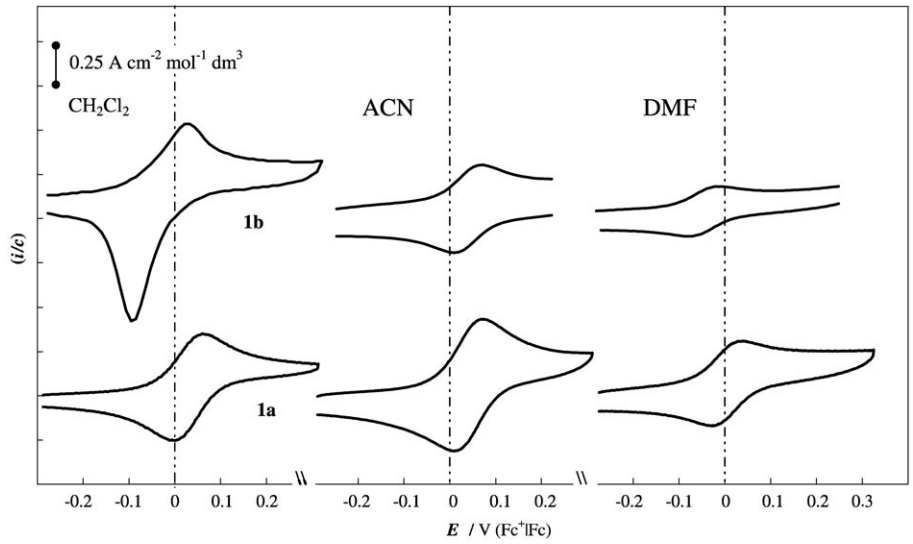


Figure 2. Comparison of the CV patterns obtained at $v = 0.2\ V\,s^{-1}$ in the three organic solvents CH_2Cl_2, MeCN and DMF, for the monoferrocene-labelled ester **1a** and acid **1b**.

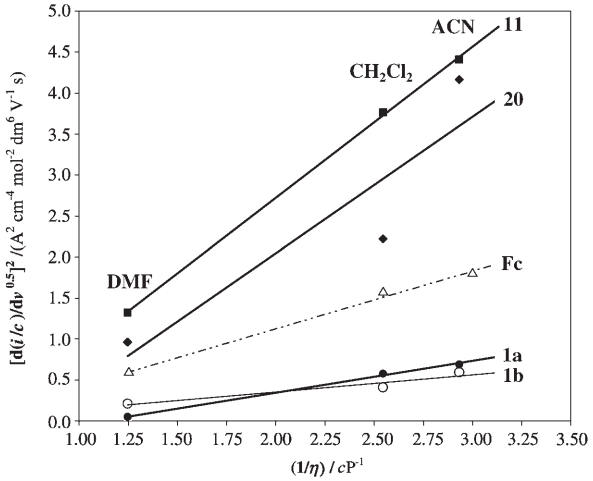


Figure 3. Squared slopes of the normalised current versus $v^{0.5}$ linear characteristics, plotted against the reciprocal of solvent viscosity η (verification of Stokes' law): the reference ferrocene compound (▲); the monoferrocene-labelled ester and acid, **1a** (●) and **1b** (○); and the trisferrocene-labelled esters **11** (■) and **20** (◆).

viscosity). The electrochemical behaviour of the mono- and trisferrocene-labelled series are analysed in detail below.

*Monoferrocene-labelled derivatives **1a** and **1b***: Figure 1 and Table 1 show that, in all solvents, the monoferrocene-labelled ester **1a** has an oxidation peak that is chemically and electrochemically reversible according to all of the criteria (see Experimental Section) and, in the case of acetonitrile (MeCN) and CH_2Cl_2, is located at a slightly more positive potential than the reference ferrocene because of the ferrocene derivatisation with the aryl ether bridge in the β-position. On the whole, both the shape and potential of the peak seem to be almost unaffected by the nature of the working solvent. The currents are lower than those of ferrocene because of the bulkier structure implying lower diffusion coefficients and, as in the case of ferrocene, their modulation by the solvent is consistent with Stokes' law (Figure 3).

The corresponding monoferrocene-labelled acid **1b** (the comparison of CV patterns of **1a** and **1b** is reported in Figure 2) has a slightly wider spread of peak potentials, with a significant anticipation in the case of DMF. Remarkably, in the case of CH_2Cl_2, sharp peak shapes were observed, especially at low scan rates and in relation to the return peak.

Moreover, the return peak had a much larger area (i.e., a larger quantity of charge transferred in the process) than the forward peak, and this asymmetry increased as the potential scan rate decreased, that is, with increasing time between the anode and cathode peak. Furthermore, the return peak markedly increased when the potential scan was paused for different times immediately after the oxidation peak, and when the solution was increasingly stirred during the pause. This phenomenon was studied in detail for the trisferrocene-labelled acid **21** (see Figure 6), which showed the same behaviour as the monoferrocene-labelled acid **1b**. Actually, the CV patterns of both **21** and **1b** in CH_2Cl_2 closely resemble that of stripping voltammetry,[27] the most sensitive voltammetric technique, in which, because its concentration is below the detection limits of CV and DPV (typically, at ppb level), the analyte is preconcentrated on the working electrode. In our case, we could assume that given the presence of the carboxylic group (as the same does not occur with the parent ester **1a**, Figure 2), the oxidation product is so poorly soluble in the hydrophobic CH_2Cl_2 solvent that it films the glassy carbon electrode surface despite its being nearly inert to specific adsorption. This film results in a large-scale increase in the local concentration of the substrate available for the backward reaction, and appears to be conductive (which is consistent with the high density of the ferrocene groups). Accordingly, the peak associated with the stripping-like process is highly intense and very reproducible.

Trisferrocene-labelled monomers: The CV characteristics of the trisferrocene-labelled monomers **11**, **20**, **21** and **23** are shown in Figures 1 and 5, together with those of the parent monoferrocene-labelled monomers. On the whole, the triple functionalisation only slightly affects reduction peak potentials and shapes.

One relevant feature is that the three ferrocene groups do not seem to be entirely equivalent in the polar MeCN and

Table 1. Selected cyclovoltammetric features of the investigated PNA monomers and the reference ferrocene compound on a GC electrode at 298 K in different organic media: the anodic peak potentials $E_{p,a}$, anodic half-peak widths, $(E_p - E_{p/2})$, half-wave potentials $E_{1/2} = (E_{p,a} + E_{p,c})/2$, $E'_{1/2}$ and α_{app} parameters from the convolutive analysis (all referring to a scan rate $v = 0.2\,V\,s^{-1}$) and the slopes of the normalised current densities versus $v^{0.5}$ linear characteristics.

Substrate	Solvent	Supporting electrolyte (0.1 M)	c [mol dm^{-3}]	$E_{p,a}$ [V] (Fc^+/Fc)	$(E_p - E_{p/2})$ [V]	$E_{1/2}$ [V] (Fc^+/Fc)	$E'_{1/2}$ [V] (Fc^+/Fc)	α_{app}	$d(i_{p,a}/c)/dv^{0.5}$ [A cm^{-2} mol^{-1} dm^3 V$^{-0.5}$ s$^{0.5}$]
Fc	MeCN	TBAP	0.000770	0.030	0.055	0.001	−0.001	1.04	1.32
	DMF	TBAP	0.000753	0.031	0.057	0.003	−0.003	1.03	0.76
	CH_2Cl_2	TBAP	0.000788	0.032	0.058	−0.001	0.001	0.97	1.25
1a	MeCN	TBAP	0.000730	0.072	0.057	0.043	0.039	0.91	0.77
	DMF	TBAP	0.000761	0.035	0.059	0.005	−0.005	1.03	0.46
	CH_2Cl_2	TBAP	0.000752	0.063	0.062	0.018	0.015	0.91	0.64
1b	MeCN	TBAP	0.000738	0.069	0.056	0.040	0.034	1.14	0.83
	DMF	TBAP	0.000806	−0.015	0.059	−0.044	−0.050	1.08	0.22
	CH_2Cl_2	TBAP	0.000718	0.028	0.059	−0.025	−0.005	0.98	0.76
11	MeCN	TBAP	0.000748	0.056	0.065	0.024	0.022	0.93	2.10
	DMF	TBAP	0.000758	0.057	0.069	0.020	0.015	0.86	1.15
	CH_2Cl_2	TBAP	0.000758	0.026	0.053	−0.002	−0.001	1.09	1.94
20	MeCN	TEATFB	0.000087	0.050	0.088	0.011	0.028	0.68	2.04
	DMF	TEATFB	0.0000927	−0.042	0.079	−0.081	−0.089	0.76	0.98
	CH_2Cl_2	TEATFB	0.0000851	0.027	0.066	−0.012	−0.004	1.01	1.49
21	CH_2Cl_2	TBAP	0.000405	−0.026	0.056	−0.026	−0.030	1.08	6.39
23	CH_2Cl_2	TBAP	0.000387	0.008	0.055	−0.024	−0.021	0.96	2.34

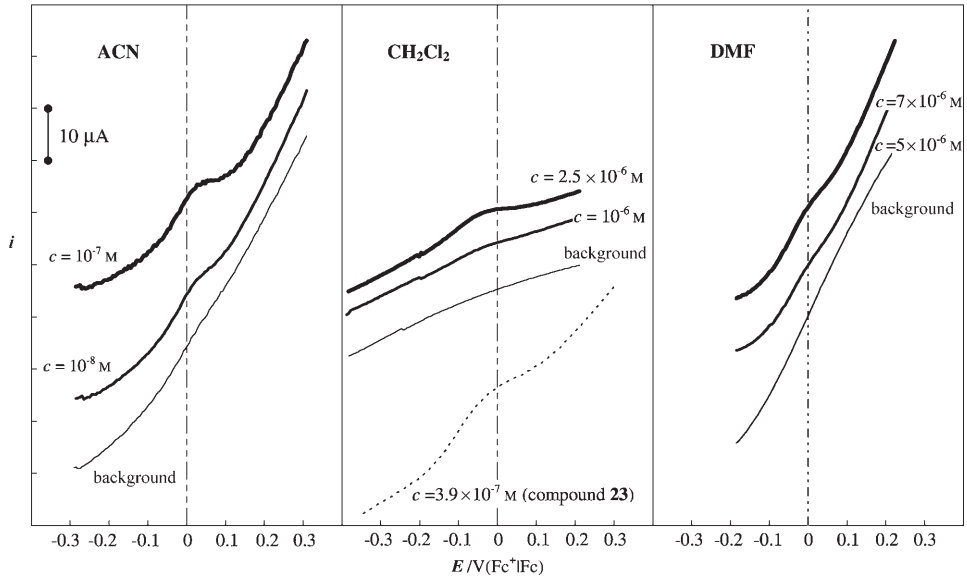


Figure 4. DPV patterns obtained by working with very low concentrations (c) of the Tris-ferrocene-labelled esters **11** in the three organic solvents CH_2Cl_2, MeCN, DMF and **23** in CH_2Cl_2 (dotted line) for the evaluation of the relevant detection limits.

DMF solvents as the oxidation peak is significantly larger than in the case of the monoferrocene-labelled ester **1a** (Figure 1); in other words, although they are not conjugated, the three ferrocene groups apparently maintain some intercommunication in the polar solvents, probably as a result of conformational effects. The same does not apply for CH_2Cl_2, in which the three ferrocene groups seem to be independent, possibly because of strong solvation or the fact that the oxidation product forms a tightly bound ionic couple with the supporting electrolyte anion.[28] However, the effect is slight and, in all three solvents, we obtained currents that were three times higher for the trisferrocene-labelled ester **11** than for the monoferrocene-labelled ester **1a** (a fundamental characteristic in view of our applicative aim), and consistent with Stokes' law (Figure 3).

As a result, despite its bulkiness, **11** has significantly more electrochemical activity than the reference ferrocene compound (Figure 1), affording DPV detection limits in the order of $10^{-8}/10^{-6}$ mol dm^3, depending on the working solvent (Figure 4), even in the absence of amplification steps.

The very similar trisferrocene-labelled ester **20**, which includes a urea-based linker, has oxidation peak potentials that are slightly less positive and have a wider spread than those of **11** (Table 1 and Figure 1). As for the trisferrocene-labelled ester **11**, only in the case of MeCN and DMF (but not CH_2Cl_2), the CV peaks somewhat broaden and reveal some communication among the ferrocene groups. The currents are still very high, and only slightly lower than in the case of **11**, which is consistent with the slightly bulkier structure of **20**.

The NH_2-Tris-Fc labelled ester **23**, (dotted line in Figure 1) produces slightly higher peak currents, with respect to compound **20** (consistently with the lower molecular weight), confirming that triple functionalisation results in a triplication of the electrochemical signal, despite the bulkier structures of the compounds. Accordingly, compound **23** af-

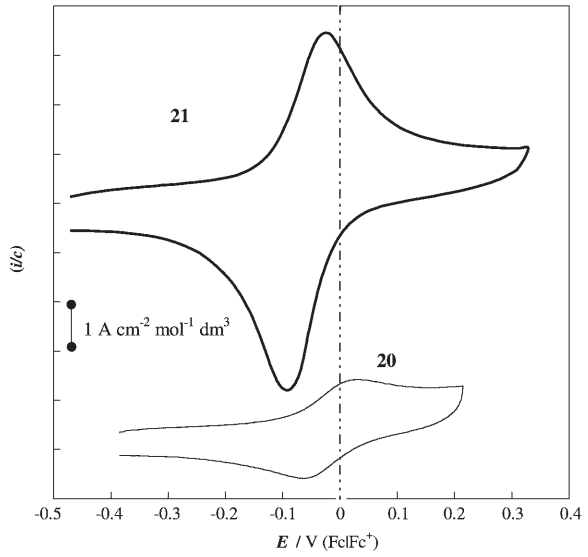


Figure 5. Comparison of the CV patterns obtained at $v=0.2\,V\,s^{-1}$ in CH_2Cl_2 for the Tris-ferrocene-labelled ester **20** and acid **21** derivatives.

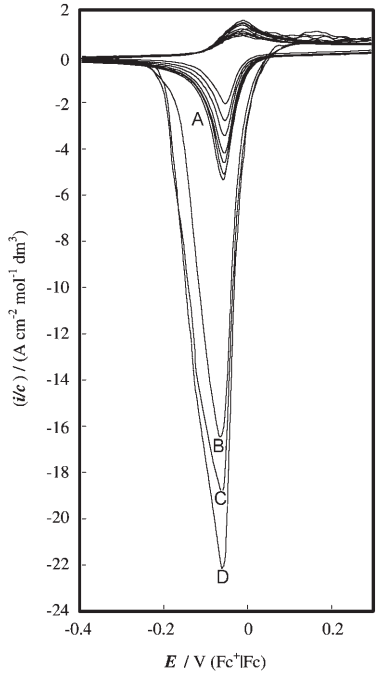


Figure 6. Stripping voltammetry characteristics obtained at $v=0.2\,V\,s^{-1}$ in CH_2Cl_2 with TBAP (0.1 M) for the Tris-Fc **21** (0.0008 M): A) Time of deposition at 0.8 V (SCE) ranging from 0 to 30 s, without stirring, B) 30 s deposition time at 0.8 V (SCE), stirring at 500 rpm, C) 30 s deposition time at 0.8 V (SCE), stirring at 750 rpm and D) 30 s deposition time at 0.8 V (SCE), stirring at 1000 rpm.

fords DPV detection limits in the order of 10^{-7} M in CH_2Cl_2 (Figure 4).

Finally, in comparison with parent ester **20**, the trisferrocene-labelled acid **21** in CH_2Cl_2 (Figures 5 and 6) shows the same slight but significant peak potential anticipation and the same peculiar shape, which indicates the accumulation of the oxidation product on the electrode surface. This, together with the additivity of the three ferrocene groups in terms of current, leads to an exceptionally high peak current for the return peak.

Conclusions

We have designed and synthesised a new organometallic molecule containing three ferrocene groups for use as a highly sensitive electrochemical marker in biological assays.

This trisferrocene-derivative was conjugated to different PNA monomers, and the electrochemical activities of the conjugates were extensively investigated in view of their potential diagnostic applications. All of the synthesised mono- and trisferrocene-labelled PNA monomers have clear electrochemical features and, even in the case of the trisferrocene conjugates, a single, multiple-independent-electron oxidation wave. The increased molecular complexity only implies a limited reduction in current signals in comparison with the reference ferrocene molecule. However, the introduction of three ferrocene units triples the current signal, thus giving the conjugated PNA monomer more electrochemical activity than the reference ferrocene. The detection limits for compound **11** and **23** are therefore 10^{-6}–10^{-8} M, depending on the nature of the solvent, with the best results being observed in acetonitrile (10^{-8} M). The labelling of PNA oligomers with Tris-Fc units is currently in progress in our laboratories with the aim of improving sensitivity in ferrocene-based electrochemical biosensors.

Experimental Section

Materials and methods: All chemicals were purchased from Sigma-Aldrich or Fluka unless otherwise indicated. All reactions were performed under a nitrogen atmosphere by using a standard vacuum line. THF was distilled over sodium benzophenone ketyl, other reagent grade solvents were dried by standard procedures. Column chromatography was performed by using Merck silica gel 60 (70–230 mesh). ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AC 300 and AMX at 300 MHz. IR spectra were recorded on a Perkin–Elmer 1725X FT–IR. MS data were obtained by using a VG 70 EQ Micromass or a Thermo-Finningam mass spectrometer. Melting points were measured with a Büchi B-540 apparatus and are uncorrected. Optical rotations were measured by using a Perkin–Elmer 243 polarimeter.

Electrochemical experiments: The CV investigations were carried out in a cell thermostated at 298 K at scan rates ranging from 0.02–20 $V\,s^{-1}$ in the following: 1) MeCN, DMF and CH_2Cl_2 (Merck, HPLC grade), with tetraethylammonium tetrafluoborate (TEATFB, 0.1 M) or tetrabutylammonium perchlorate (TBAP, Fluka, electrochemical grade) as supporting electrolytes, 2) ultrapure water (Millipore Milli Q system) with $NaClO_4$ (0.1 M) as the supporting electrolyte. The solutions were carefully deaerated by nitrogen bubbling and analyzed using an Autolab PGSTAT 12 or Autolab PGSTAT 30 potentiostat/galvanostat (EcoChemie, The Netherlands) run by a PC with GPES software; the ohmic drop was corrected by means of the positive feedback technique[29] with a glassy carbon GC (Amel, surface 0.071 cm^2) as the working electrode, a platinum counter electrode and an aqueous saturated calomel electrode (SCE) as the operating reference electrode. The formal redox potentials of the ferricinium/ferrocene redox couple recommended by IUPAC for the intersolvental

comparison of potential scales[30] were 0.390, 0.476 and 0.488 V, respectively, when measured against our aqueous SCE in MeCN, DMF and CH_2Cl_2. The optimized polishing procedure for the working GC electrode consisted of surface treatment with diamond powder (Aldrich, diameter 1 μm) on a wet cloth (DP-Nap, Struers).

The chemical and electrochemical reversibility of each well-defined CV peak were checked by means of classical tests,[26] including analyses of 1) half-peak width ($E_p - E_{p/2}$), 2) E_p versus logν characteristics, 3) the distance between the anode and cathode peak potential ($E_{p,a} - E_{p,c}$), 4) I_p versus $\nu^{0.5}$ characteristics and 5) the "stationary" step-like waves obtained by means of convolutive analysis of the original CV characteristics.[31]

***N*-Cbz-Tris(hydroxymethyl)aminomethane (4)**: At room temperature benzylchloroformate (6.0 g, 35 mmol, 5.2 mL) was slowly dropped into a stirred suspension of $NaHCO_3$ (53 mmol, 4.4 g) and tris(hydroxymethyl)aminomethane (5.0 g, 42 mmol) in H_2O (20 mL) and AcOEt (40 mL), keeping the temperature around 20 °C. After stirring for 5 h at room temperature, the solid was filtered off and the layers separated. The aqueous phase was extracted with AcOEt (3×40 mL) and the combined organic layers were washed with H_2O (40 mL). After evaporation of the solvent, the residue was dissolved in diisopropyl ether (20 mL) and cooled in an ice-bath. The white solid was filtered to give **4** (9.5 g, 88 %). M.p. 101–103 °C (lit.[22] 28 %, m.p. 102–104 °C).

***N*-Cbz-Tris(ferrocenylmethylenoxymethyl)aminomethane (6)**: A solution of ferrocene methanol **5**[15] (2.0 g, 9.26 mmol) in toluene (10 mL) and CH_2Cl_2 (3 mL) was heated at 60 °C under N_2. At this temperature a portion of **4** (0.12 g, 0.47 mmol) and CF_3CO_2H (0.2 mL of a 0.65 M toluene solution) was added. The same amount of the two reagents was added every 30 min until a further four additions had been carried out. After two hours, four additions of ferrocenemethanol (0.32 g, 3.8 mmol), each every 4 h, were made, and the mixture was then heated at the same temperature for 8 h. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (20 mL) and washed with saturated $NaHCO_3$ solution (20 mL). The aqueous phase was extracted with CH_2Cl_2 (2×20 mL) and the resulting combined organic phases were washed with water (40 mL), dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica gel (*tert*-butyl methyl ether/petroleum ether 2:8) to afford **6** (1.45 g, 73 %) as an orange powder. Compounds **7** (0.1 g, 10 %), **8** (0.13 g, 13 %) and diferrocenylmethylether (1.5 g) were also recovered.

Compound 6: M.p. 115–117 °C (pentane); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32 (m, 5H; Ph), 5.2 (brs; NH), 5.02 (s, 2H; CH_2Ph), 4.20 (s, 6H; CH_2Fc), 4.16 (t, J = 1.7 Hz, 6H; CH Fc), 4.11 (t, J = 1.7 Hz, 6H; CH Fc), 4.09 (s, 15H; CH Fc), 3.67 ppm (s, 6H; CH_2O); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 156, 137, 128.4, 128.0, 127.9, 83.8, 69.5, 69.0, 68.8, 68.4, 68.2, 66.1, 58.8 ppm; IR (Nujol): $\tilde{\nu}$ = 1723 (NHCO), 1055, 1098 cm^{-1} (Fc); MS (FAB+): m/z: 850 $[M]^+$; elemental analysis calcd (%) for $C_{45}H_{47}Fe_3NO_5$: C 63.63, H 5.58, N 1.65; found: C 63.44, H 5.59, N 1.65.

Compound 7: Orange oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.35–7.38 (m, 5H; Ph), 5.4 (brs; NH), 5.04 (s, 2H; *CH*$_2$Ph), 4.27 (d, J = 11.4 Hz, 2H; O*CH*$_2$Fc), 4.21 (d, J = 11.4 Hz, 2H; O*CH*$_2$*Fc*), 4.17 (t, J = 1.76 Hz, 4H; CH Fc), 4.12 (t, J = 1.76 Hz, 4H; CH Fc), 4.10 (s, 10H; CH Fc), 3.77 (d, J = 6.6 Hz, 2H; *CH*$_2$OH), 3.69 (d, J = 9.16 Hz, 2H; *CH*$_2$OCH$_2$Fc), 3.63 (brs; OH), 3.48 ppm (d, J = 9.16 Hz, 2H; C*H*$_2$OCH$_2$Fc); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 156, 136, 128.5, 128.07, 128.0, 83.3, 69.71, 69.56, 69.01–69.08, 69.49, 68.38, 66.6, 65.1, 59.1 ppm; IR (neat): $\tilde{\nu}$ = 3414 (OH), 1714 cm^{-1} (CONH); MS (ESI): m/z: 651.3 $[M]^+$, 674.3 $[M+Na]^+$; elemental analysis calcd (%) for $C_{34}H_{37}Fe_2NO_5$: C 60.94, H 6.00, N 3.09; found: C 60.54; H 5.60, N 3.06.

Compound 8: Orange solid; m.p. 113–115 °C (decomp) (pentane); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.35 (m, 5H; Ph), 5.74 (s; NH), 5.08 (s, 2H; CH_2Ph), 4.3 (s, 2H; O*CH*$_2$Fc), 4.18 (t, J = 1.8 Hz, 2H; CH Fc), 4.16 (t, J = 1.8 Hz, 2H; CH Fc), 4.09 (s, 5H; CH Fc), 3.72 (d, J = 11.6 Hz, 2H; C*H*$_2$OH), 3.58 (s, 2H; C*H*$_2$OCH$_2$Fc), 3.53 ppm (d, J = 11.6 Hz, 2H; C*H*$_2$OH); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 156.5, 136.0, 128.5, 128.2, 128.0, 83.0, 70.8, 69.9, 68.7, 67, 64.5, 59.1 ppm; IR (Nujol): $\tilde{\nu}$ = 3300 (OH), 1685 cm^{-1} (CONH); MS (ESI): m/z: 453.5 $[M]^+$, 476.4 ppm $[M+Na]^+$; elemental analysis calcd (%) for $C_{23}H_{27}FeNO_5$: C 60.94, H 6.00, N 3.09; found: C 60.90, H 5.90, N 3.06.

Tris(ferrocenemethylenoxymethyl)aminomethane (2): Pd/C (10 %, 0.3 g) and HCO_2NH_4 (0.3 g, 4.7 mmol) were added, at room temperature under nitrogen, to a stirred solution of *N*-Cbz Tris-ferrocene **4** (0.85 g, 1.0 mmol) in MeOH (10 mL) and CH_2Cl_2 (18 mL). After 90 min, the mixture was filtered through a pad of celite and the solvent evaporated, the residue was then dissolved in CH_2Cl_2 (40 mL), washed with brine (2× 40 mL) and the aqueous phase was extracted with CH_2Cl_2 (3×40 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica gel (R_f = 0.25, AcOEt) affording **2** as an orange powder (0.650 g, 90 %). M.p. 105–107 °C (pentane); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.24 (s, 6H; OCH_2Fc), 4.18 (t, J_{ortho} = 1.8 Hz, 6H; Fc), 4.13 (t, J_{meta} = 1.8 Hz, 6H; Fc), 4.10 (m, 15H; Fc), 3.45 (s, 6H; CC*H*$_2$O), 2.28 ppm (brs, 2H; NH_2); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 83.84, 71.48, 69.45, 69.08, 68.4, 68.2, 56.51 ppm; IR (Nujol): $\tilde{\nu}$ = 3093 cm^{-1} (NH_2); MS (EI): m/z: 715 $[M]^+$, 199 (CH_2Fc), 121; elemental analysis calcd (%) for $C_{37}H_{41}Fe_3NO_3$: C 62.13, H 5.78, N 1.96; found: C 62.76, H 5.75, N 1.95.

Tris-Fc monomer 11: D,L-Tyrosine monomer **9**[15b] (0.5 g, 0.93 mmol) was added at 0 °C to a solution of bis(pentafluorophenyl)carbonate (0.38 g, 0.94 mmol) in CH_2Cl_2 (5 mL). Diisopropylamine (DIPEA, 0.16 mL, 0.93 mmol) was then added to the resulting suspension, and the clear solution was stirred at 0 °C for 2 h. After this time, the solution was diluted with CH_2Cl_2 (15 mL), washed with $NaHCO_3$ (5 %, 2×10 mL) and the aqueous phase was extracted with CH_2Cl_2 (2×15 mL). The organic phase, after washing with 20 mL of H_2O, was dried over Na_2SO_4, concentrated to 2 mL and added to a solution of **2** (0.5 g, 0.75 mmol) and DIPEA (0.16 mL, 0.92 mmol) in CH_2Cl_2 (5 mL) cooled at 0 °C. The resulting orange solution was stirred at room temperature for 5 h. After this time, the mixture was washed with $NaHCO_3$ (10 %, 2×10 mL) and the aqueous phase was extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were then washed with H_2O (15 mL), dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography (AcOEt) to give **11** (0.4 g, 80 %) as an orange solid. M.p. 98–103 °C (pentane); ^{1}H NMR (300 MHz, $CHCl_3$): δ = 8.01 (s, 1H; NH T), 7.25–7.35 (m, 5H; Ph), 7.10 (d, J = 8.47 Hz, 2H; PhO), 7.02 (d, J = 8.47 Hz, 2H; PhO), 6.58 (s, 1H, CH=), 5.61 (brs, 1H; NH Cbz), 5.46 (brs, 1H; NH), 5.19 (d, J = 12 Hz, 1H; PhC*H*$_2$O), 4.99 (d, J = 12 Hz, 1H; PhC*H*$_2$O), 4.52 (d, J = 16.2 Hz, 1H; CH_2CO), 4.24 (s, 6H; CH_2Fc), 4.19 (s, 6H; CH_{ortho} Fc), 4.14 (s, 6H; CH_{meta} Fc), 4.11 (s, 15H; Fc), 3.82–3.86 (m, 2H; CH+CH_2CO), 3.77 (s, 3H; CO_2CH_3), 3.67 (brs, 6H; *CH*$_2$OCH$_2$Fc), 3.00–3.10 (m, 4H; CH_2), 2.60 (d, 1H, J = 10.5 Hz; C*H*$_2$NCH), 1.84 ppm (s, 3H; CH_3); ^{13}C NMR (300 MHz, $CHCl_3$): δ = 172, 166.95, 164.1, 156, 155, 151.4, 141.0, 136, 129.9, 128.4, 127, 122.14, 110.8, 83, 69.6, 69.2, 68.6, 68.5, 66.9, 63.6, 60, 50.34, 49.5, 48.7, 39.03, 33.50, 12.3 ppm; IR (Nujol): $\tilde{\nu}$ = 1670 cm^{-1} (CO); UV (CH_2Cl_2): λ_{max} (ε) = 261 (20690 mol^{-1} dm^3 cm^{-1}); HRMS (ESI): m/z: 1279.30186 $[M]^+$.

Tyrosine derivative 14: *N*-Boc ethanolamine **12** (0.78 mL, 5.0 mmol), followed by PPh_3 (1.85 g, 7.0 mmol) was added to a solution of L-*N*-Cbz tyrosine methylester **13** (1.98 g, 6 mmol) in anhydrous THF (50 mL). The mixture was cooled to −30 °C and DEAD (3.2 mL of 20 % toluene solution) was added. The mixture was allowed to warm at room temperature and was then stirred for 48 h. After this time, the solvent was evaporated and the residue dissolved in AcOEt (100 mL) was washed with NaOH (0.1 M, 2×50 mL), followed by brine (2×50 mL). The organic phase was dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography (R_f = 0.47, AcOEt/petroleum ether 1:1) to give **14** (73 %) as a white solid. M.p. 79–81 °C (pentane); $[\alpha]_D^{20}$ = +38.8 (c = 0.23 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.33 (brs, 5H; Ph Cbz), 6.98 (d, J = 8.5 Hz, 2H; PhO), 6.78 (d, J = 8.5 Hz, 2H; PhO), 5.29–4.98 (m, 4H; CH_2-Cbz + NH-Cbz + NH-Boc), 4.62–4.59 (m, 1H; CH), 3.96 (t, J = 5.1 Hz, 2H; CH_2O), 3.71 (s, 3H; OCH_3), 3.45–3.53 (m, 2H; C*H*$_2$NHBoc), 3.09–2.97 (m, 2H; CH_2Ph), 1.44 ppm (s, 9H; *t*Bu); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 171.9, 157.4, 155.8, 155.5, 136.1, 130.1, 128.5–127.9, 114.3, 79.4, 66.8–66.6, 54.8, 52.02, 39.9, 37.0, 28.2 ppm; IR ($CHCl_3$): $\tilde{\nu}$ = 1708–1718 cm^{-1} (NHCO); MS (ESI): m/z: 495.3 $[M+Na]^+$; elemental analysis calcd (%) for $C_{25}H_{32}N_2O_7$: C 63.95, H 6.83, N 5.93; found: C 63.76, H 6.85, N 5.94.

Tyrosine derivative 15: Ammonium formate (0.5 g, 7.9 mmol) and Pd/C 10% (150 mg) were added to a solution of **14** (1.5 g, 3.2 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 3 h, then filtered over a pad of celite. The solvent was evaporated and the residue was dissolved in AcOEt (100 mL) and washed with brine (60 mL). The organic phase was dried over Na_2SO_4 and evaporated. The crude product **15** (colourless oil, 75%) was used for the subsequent reaction without any further purification. $R_f = 0.23$ (AcOEt); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.10$ (d, $J = 8.5$ Hz, 2H; Ph), 6.81 (d, $J = 8.5$ Hz, 2H; Ph), 5.00 (br s, 1H; NH-Boc), 3.97 (t, $J = 5.1$ Hz, 2H; CH_2O), 3.80–3.71 (m, 4H; OCH_3 + CH), 3.53–3.48 (m, 2H; CH_2NH-Boc); 3.12–2.70 (m, 4H; CH_2Ph + NH_2), 1.43 ppm (s, 9H; *t*Bu); MS (ESI): *m*/*z*: 339 $[M+1]^+$.

Tyrosine backbone 16: A mixture of $ZnCl_2$ (0.18 g, 1.32 mmol) and $NaCNBH_3$ (0.165 g, 2.65 mmol) in dry MeOH (1 mL) was slowly added at 0°C, to a solution of **15** (0.82 g, 2.42 mmol) and *N*-Cbz-2-amino acetaldehyde (0.425 g, 2.42 mmol) in dry MeOH (4 mL). The mixture was then stirred at room temperature for 2 h. After evaporation of the solvent, the residue was dissolved in AcOEt (100 mL), washed with H_2O (2 × 50 mL) and the aqueous phase was extracted with AcOEt (3 × 20 mL). The combined organic phases were washed with H_2O (50 mL), dried over Na_2SO_4 and evaporated. The crude product **16** was recovered as a colourless oil (93%). $R_f = 0.52$ (AcOEt); $[\alpha]_D^{20} = -6.60$ ($c = 0.076$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.30$ (d, $J = 8.5$ Hz, 2H; PhO), 6.76 (d, $J = 8.5$ Hz, 2H; PhO), 5.20–4.90 (m, 4H; N*H*Cbz + O*CH*$_2$Ph, NHBoc), 3.90 (br t, $J = 4.85$ Hz, 2H; PhO*CH*$_2$), 3.60 (s, 3H; OCH_3), 3.50–3.40 (m, 3H; *CH*$_2$NHBoc + CH), 3.20–3.10 (m, 2H; *CH*$_2$NHCbz), 2.90–2.70 (m, 3H; CH_2PhO + *CH*$_2$NH), 2.60–2.50 (m, 1H; *CH*$_2$NH), 1.84 (s, 3H, CH_3), 1.80 (br s, 1H; NH*CH*), 1.43 ppm (s, 9H; *t*Bu); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 174.8$, 157.4, 156.4, 155.7, 136.6, 130.1, 129.5, 128.4, 127.9, 114.3, 79.4, 67.0, 66.5, 62.5, 51.6, 47.13, 40.6, 40.0, 38.0, 28.3 ppm; IR (neat): $\tilde{\nu} = 1718$ cm^{-1} (NHCO); MS (ESI): *m*/*z*: 538 $[M+Na]^+$, 516 $[M+1]^+$; elemental analysis calcd (%) for $C_{27}H_{37}N_3O_7$: C 62.90, H 7.23, N 8.15; found: C 63.01, H 7.22, N 8.16.

PNA monomer 17: At room temperature *N*,*N*-diisopropyl carbodiimide (0.48 mL, 3.1 mmol) was added to a solution of 1-carboxy methyl thymine (0.57 g, 3.1 mmol) in dry DMF (4 mL). DhBtOH (0.5 g, 3.1 mmol) was then added and the precipitation of *N*,*N*-diisopropyl urea (DIU) was observed. After the mixture had been stirred at room temperature for 2 h, a solution of **16** (0.9 g, 1.75 mmol) in dry DMF (7 mL) was added. The mixture was stirred for a further 3 h at room temperature, and then the solvent was evaporated. The resulting residue was dissolved in CH_2Cl_2 (10 mL), the DIU was filtered off over a pad of celite and the solvent was evaporated. Finally, the residue was dissolved in AcOEt (50 mL), washed with saturated $NaHCO_3$ (4 × 30 mL) and then with water (50 mL), dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica gel (AcOEt/light petroleum 8:2, $R_f = 0.23$) to afford the PNA monomer **17** (70%) as a white solid. M.p. 128–132°C (pentane); $[\alpha]_D^{20} = -82.4$ ($c = 0.074$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.80$ (s, 1H; NH), 7.40–7.20 (m, 5H; Ph), 7.04 (d, $J = 8.5$ Hz, 2H; PhO), 6.82 (d, $J = 8.5$ Hz, 2H; PhO), 6.60 (s, 1H; CH=), 5.50 (br s, 1H; NHCbz), 5.16 (d, $J_{gem} = 12.2$ Hz, 1H; $PhCH_2O$), 5.00 (d, $J_{gem} = 12.2$ Hz, 1H; $PhCH_2O$), 4.50 (d, $J_{gem} = 16.2$ Hz, 1H; CH_2CO), 3.96 (t, $J = 5.18$ Hz, 2H; CH_2O), 3.90–3.76 (m, 5H; OCH_3 + CH + CH_2CO), 3.50–3.00 (m, 7H; CH_2N + CH_2PhO + CH_2NHCbz + CH_2NHBoc), 2.60 (br d, $J_{gem} = 15.5$ Hz, 1H; C*H*$_2$N), 1.84 (s, 3H; CH_3), 1.44 ppm (s, 9H; *t*Bu); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 170.7$, 167, 164.5, 157.4, 156.4, 155.7, 151.6, 141, 136.6, 130.2, 129.5, 128.4–128.2, 114.7, 110.3, 79.4, 66.9, 66.7, 63.5, 52.6, 48.7, 48.2, 39.9, 39.0, 33.1, 28.3, 12.2 ppm; IR (Nujol): $\tilde{\nu} = 1664$–1719 cm^{-1} (NHCO); MS (ESI): *m*/*z*: 704.2 $[M+Na]^+$; elemental analysis calcd (%) for $C_{34}H_{43}N_5O_{10}$: C 59.90, H 6.36, N 10.27; found: C 61.02, H 6.35, N 10.25.

PNA monomer 18: At room temperature a solution of TFA (1 M, 0.35 mL, 4.4 mmol) in CH_2Cl_2 was added to a solution of **17** (0.5 g, 0.73 mmol) in CH_2Cl_2 (3 mL), and the mixture was stirred at room temperature for 48 h. After this time, the solvent was evaporated and the residue was dissolved in AcOEt (30 mL) and evaporated. The crude product was precipitated from pentane (5 mL) and filtered to give **18** (97%). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 7.4$–7.2 (m, 6H; Ph + CH=), 7.17 (d, $J = 8.5$ Hz, 2H; PhO), 6.93 (d, $J = 8.5$ Hz, 2H; PhO), 5.05 (d, $J_{gem} = 12.2$ Hz, 2H; $PhCH_2O$), 4.65 (d, $J = 16.4$ Hz, 1H; CH_2CO_{Tym}), 4.34 (d, $J = 16.4$ Hz, 1H; CH_2CO_{Tym}), 4.20–4.10 (m, 3H; CH_2O + CH), 3.90–3.76 (br s, 3H; OCH_3), 3.50–3.00 (m, 7H; C*H*$_2$NCH + CH_2Ph + CH_2NHCbz + $CH_2NH_3^+$), 2.60 (m, 1H; CH_2N), 1.84 ppm (s, 3H; CH_3); ^{13}C NMR (300 MHz, CD_3OD); $\delta = 172.2$, 169.4, 167, 158.4, 152.8, 143.2, 138.2, 132.2, 131.8, 128.5–128.1, 115.8, 110.9, 67.7, 65.3, 65.0, 52.9, 40.4, 40.1, 34.4, 12.2 ppm; IR (Nujol): $\tilde{\nu} = 1660$–1719 cm^{-1} (NHCO); MS (ESI): *m*/*z*: 582.3 $[M+Na]^+$.

Tris(ferrocenemethylenoxymethyl)methylisocyanate (19): 4-Dimethylaminopyridine (45 mg, 0.35 mmol) was added to a solution of **2** (0.250 g, 0.35 mmol) in anhydrous THF (15 mL). The mixture was cooled to −15°C and a solution of Boc_2O (0.095 g, 0.43 mmol) in THF (3 mL) was slowly added. The mixture was allowed to warm at room temperature and was stirred for 20 h, then the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and purified by column chromatography (AcOEt/light petroleum 3:7) to afford isocyanate **19** as an orange oil (76%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.25$–4.10 (m, 33H; Fc + OCH_2Fc), 3.46 ppm (s, 6H, CH_2O); IR (Nujol): $\tilde{\nu} = 2294$ cm^{-1} (NCO); MS (ESI): *m*/*z*: 741 $[M]^+$; elemental analysis calcd (%) for $C_{38}H_{39}Fe_3NO_4$: C 61.57, H 5.30, N 1.89; found: C 61.60, H 5.28, N 1.88.

Tris-Fc tyrosine ester monomer 20: At room temperature DIPEA (0.25 mL, 1.45 mmol) was added to a solution of Tris-isocyanate **19** (0.535 g, 0.72 mmol) and PNA monomer **18** (0.335 g, 0.48 mmol) in anhydrous DMF (6 mL), and the mixture was stirred at room temperature for 24 h. After this time, CH_2Cl_2 (20 mL) was added and the solution was washed with H_2O (2 × 15 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 15 mL) and the combined organic layers were treated with $KHSO_4$ (0.3 M, 2 × 15 mL). After the organic phase had been washed with H_2O (20 mL), it was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (AcOEt/light petroleum 4:6 then AcOEt/MeOH 9:1) to afford monomer **20** (84%) as a yellow solid and unreacted isocyanate **19** (30%). $R_f = 0.45$ (AcOEt); m.p. 178–180°C (pentane); $[\alpha]_D^{20} = -67.2$ ($c = 0.064$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.80$ (s, 1H; NH_{Tym}), 7.40–7.20 (m, 5H; Ph), 7.03 (d, $J = 8.5$ Hz, 2H; PhO), 6.81 (d, $J = 8.5$ Hz, 2H; PhO), 6.60 (s, 1H; CH=), 5.5 (br s, 1H; CbzNH), 5.16 (d, $J_{gem} = 12.2$ Hz, 1H; $PhCH_2O$), 5.00 (d, $J_{gem} = 12.2$ Hz, 1H; $PhCH_2O$), 4.50 (br d, 1H; CH_2), 4.30–4.00 (m, 33H; OCH_2Fc + Fc), 3.96–3.76 (m, 7H; OCH_3 + CH + CH_2CO + CH_2O), 3.70 (s, 6H; $C(CH_2)_3O$), 3.50–3.00 (m, 7H; CH_2N + CH_2Ph + CH_2NHCbz + $CH_2NHCONH$), 2.60 (br s, 1H; CH_2N), 1.84 ppm (s, 3H, CH_3); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 170.7$, 167, 164.1, 157.7–156.4, 151.7, 141, 136.6, 130.2, 129.4, 128.4, 128.3, 128.2, 114.9, 110.3, 83.6, 69.6, 69.3–68.2, 67.5, 66.9, 63.5, 58.8, 52.6, 48.9, 48.2, 39.6, 39.0, 33.1, 12.2 ppm; IR (CH_2Cl_2): $\tilde{\nu} = 1688$, 1716 cm^{-1} (NHCO, COOMe); MS (ESI): *m*/*z*: 1345 $[M+Na]^+$; elemental analysis calcd (%) for $C_{67}H_{74}Fe_3N_6O_{12}$: C 60.83, H, 5.64, N 6.35; found: C 61.02, H 5.65, N 6.37.

Tris-Fc tyrosine acid monomer 21: At room temperature $Ba(OH)_2 \cdot 8H_2O$ (0.090 mg, 0.28 mmol) was added to a solution of **20** (0.15 g, 0.11 mmol) in CH_2Cl_2 (1 mL), MeOH (0.5 mL) and H_2O (0.10 mL). The mixture was vigorously stirred for 2 h at the same temperature, and then CH_2Cl_2 (20 mL) was added and the organic phase was washed with $KHSO_4$ (0.3 M, 2 × 15 mL) (pH 2). The organic phase was then washed again with H_2O, dried over Na_2SO_4 and evaporated. The yellow residue produced was dissolved in pentane (5 mL), cooled and filtered (96%). M.p. 225–230°C; $[\alpha]_D^{20} = -32.26$ ($c = 0.062$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 10.2$ (s, 1H; NH), 7.40–7.20 (m, 5H; Ph), 7.20–6.95 (m, 2H; PhOH), 6.90–6.70 (m, 3H; CH= + PhOH), 6.10 (br s, 1H; NH), 5.60 (br s, 1H; NH), 5.10–4.90 (m, 3H; $PhCH_2O$ + NH), 4.50–3.76 (m, 38H; CH + CH_2CO + CH_2O, CH_2Fc + Fc), 3.70–2.30 (m, 14H; CH_2N + CH_2Ph + CH_2NH-Cbz + $CH_2NHCONH$; $C(CH_2)_3O$), 1.84 ppm (s, 3H; CH_3); ^{13}C NMR (300 MHz, $CDCl_3$); $\delta = 170.7$, 167.0, 164.1, 158.2, 157.5, 156.6, 151.9, 141, 136.4, 130.2, 129.4, 128.4–128.1, 114.7, 110.3, 83.6, 69.5, 69.2–68.4, 67.3, 66.8, 63.3, 58.8, 48.7, 39.6, 38.6, 33.1, 12.2 ppm; IR (CH_2Cl_2) $\tilde{\nu} = 3050$ cm^{-1} (COOH), 1688, 1716 cm^{-1} (NHCO); MS (ESI): *m*/*z*: 1308 $[M]^+$, 1331 $[M+Na]^+$; elemental analysis calcd (%) for $C_{66}H_{72}Fe_3N_6O_{12}$: C 60.57, H 5.54, N 6.42; found: C 60.53, H 5.55, N 6.44.

Tris-Fc aminoethylglycine monomer 23: A solution of **2** (0.21 g, 0.30 mmol) and DIPEA (0.13 mL, 0.74 mmol) in DMF (0.5 mL) was added at 0 °C to a solution of pentafluorophenyl carbonate (0.115 g, 0.30 mmol) in *N*-methyl pyrrolidone (1.5 mL) and the mixture was stirred for 1 h at the same temperature. This solution was then slowly added to a solution of monomer **22**[32] (0.070 g, 0.17 mmol) and DIPEA (0.05 mL, 0.3 mmol) in DMF (1 mL). The solution was heated at 45 °C for 2 h, followed by dilution with CH_2Cl_2 (25 mL). This mixture was washed with a saturated solution of $NaHCO_3$ (3×15 mL) and the aqueous phase was extracted with CH_2Cl_2 (20 mL). The combined organic layers, after washing with H_2O (20 mL), were dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (AcOEt/light petroleum 4:6, increasing progressively the polarity of the solvent) to afford **23** (75%) as a yellow solid. M.p. 176–178 °C (pentane); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.11 (br s, 1H; NH), 6.76 (s, 1H; CH=), 5.70 (br s, 2H; NH), 4.28 (s, 2H; CH_2CO), 4.20 (s, 6H; CH_2Fc), 4.17 (t, 6H; Fc), 4.12 (t, 6H; Fc), 4.10 (s, 15H; Fc), 3.96 (s, 2H; CH_2COO), 3.72 (s, 3H; OCH_3), 3.62 (s, 6H; $C(CH_2)_3$), 3.40–3.30 (m, 4H; CH_2CH_2), 1.80 ppm (s, 3H; CH_3CH=); ^{13}C NMR (300 MHz, $CDCl_3$); δ = 170.1, 167.6, 163.9, 157.2, 150.9, 141.9, 110.0, 83.5, 69.7, 69.4, 69.3, 68.5–68.4, 58.9, 52.5, 49.5, 49.0, 48.8, 37.7, 12.3 ppm; MS (ESI): *m*/*z*: 1039 $[M]^+$, 1062 $[M+Na]^+$; IR (Nujol): $\tilde{\nu}$ = 1651 (CO), 1455 cm^{-1} (NHCONH); elemental analysis calcd (%) for $C_{50}H_{57}Fe_3N_5O_9$: C 57.77, H 5.53, N 6.74; found: C 60.10, H 5.52, N 6.71.

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