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Solid-Phase Synthesis of Chiral Modular Ferrocene-Based Peptides

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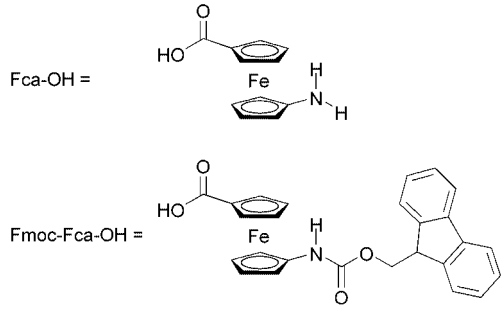
Ferrocene-containing tripeptides with one to two ferrocene building blocks were prepared by solid-phase peptide synthesis (SPPS) using Fmoc-protected 1′-aminoferrocene-1-carboxylic acid (Fca). The conformations of the tripeptides were analysed by spectroscopic and theoretical methods. The immobilised ferrocene compounds can be reversibly oxidised on-bead to positively charged peptides and were analysed for anion-binding affinities by a solid-phase binding assay.

Introduction

In contrast to organic-based receptors[1] with carboxylate binding sites, which are either charged (e.g. guanidinium or pyridinium ions[2]) or uncharged (e.g. pyrroles, amides or diketopiperazines[3]), suitably substituted ferrocene derivatives create the possibility to switch between the neutral ferrocene (low-affinity state) and charged ferricinium (high-affinity state) simply by applying an external voltage or by chemically induced redox reactions (redox control of molecular recognition[4]). Application of the reverse concept for cation binding to a ferrocene derivative has been recently demonstrated.[5]

With this idea in mind we set out to prepare chemically diverse chiral ferrocene-based two-armed receptors preferably in a combinatorial fashion using state-of-the-art solid-phase peptide synthesis methods (SPPS). For this application we have recently prepared a SPPS-compatible ferrocene building block, namely the fluorenylmethoxycarbonyl-protected 1,1′-ferrocene amino acid Fmoc-Fca-OH (Scheme 1).[6]

Peptides incorporating the artificial amino acid Fca have been previously synthesised by Metzler-Nolte, Kraatz, Rapić and by us using solution-phase methods.[7,8] Fca derivatives have been employed as transition-state analogues for formation of catalytic antibodies.[9] Simple neutral ferrocene-containing amides were shown to bind anions with moderate affinity while the charged ferricinium counterparts bind anions much more strongly because of additional electrostatic interactions between host and guest.[10,11] Fca derivatives bind chloride anions in an induced fit binding process.[11]



Scheme 1.

Results and Discussion

Solid-Phase Synthesis of Ferrocene-Containing Peptides

Here we report a general and modular synthetic approach to peptides containing ferrocene in the backbone compatible with standard SPPS based on the Fmoc-protection strategy.[12] As solid support/linker TentaGel-Wang resins turned out to be superior to polystyrene/divinyl benzene-Wang resins both for SPPS and on-bead reactions. After Fmoc removal from the TentaGel support using piperidine, Fmoc-Fca-OH is preactivated with DIC/HOBt and coupled to the free amine (Scheme 2, DIC = 1,3-diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole). Because of the orange colour of the immobilised ferrocene, a Kaiser test for free amines proved impossible. Capping of unreacted amino groups was done using Ac_2O/pyridine (see Experimental Section).

Fmoc deprotection of the immobilised ferrocene occurs under standard conditions, as does DIC/HOBt-promoted coupling with an *N*-acetylated L-α-amino acid. Cleavage of the resulting tripeptide from the support with trifluoroacetic acid (TFA) gives tripeptides **1**–**3**. Attaching Ac-Fca-OH[6] instead of an α-amino acid yields the diferrocene peptide **4**. After chromatographic workup the orange peptides

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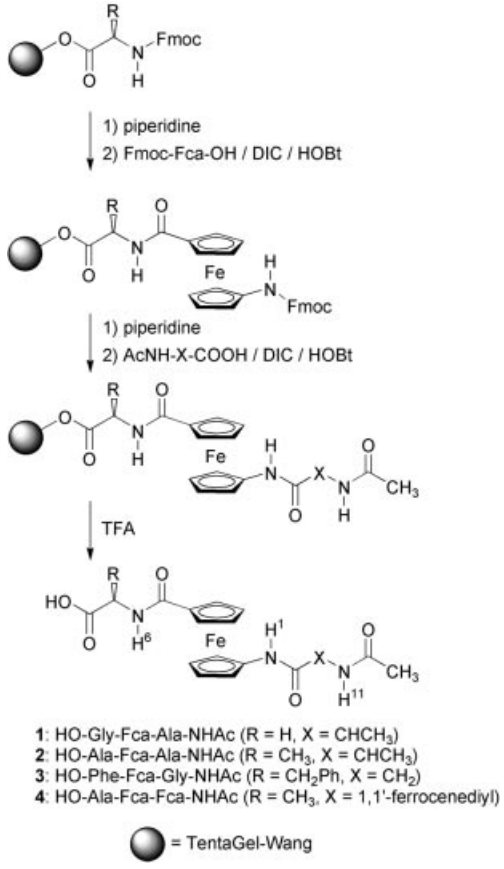
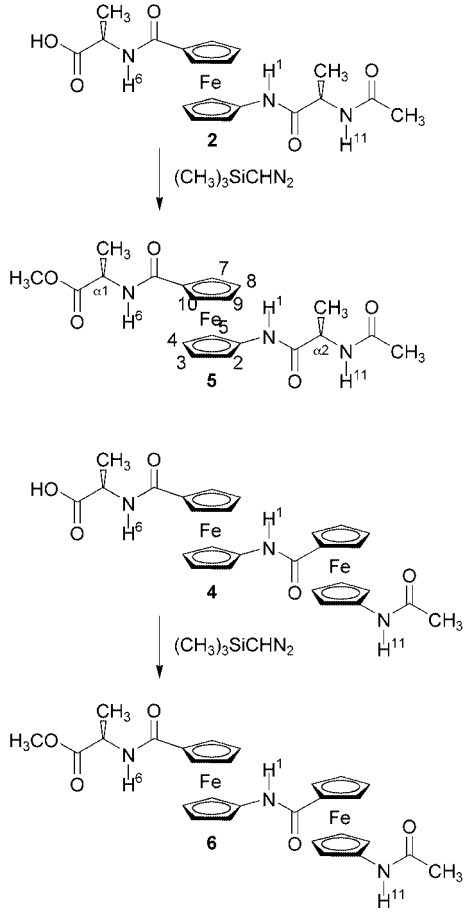


Scheme 2.



Scheme 3.

1–**4** were characterised by spectroscopic and mass spectrometric techniques. The data fully prove the composition and sequence of the peptides.

Conformational Analysis of Ferrocene-Containing Peptides

Negative ion ESI mass spectra of **1**–**4** display, in addition to the $[M - H]^-$ peaks, signals corresponding to $[2M - H]^-$ consistent with the formation of carboxylic acid dimers in solution.[13] The dimer motif is also the most stable arrangement in the solid state as the signal for the $C{=}O_{acid}$ stretching vibration is found at 1725 cm^{-1}.[6]

To allow a detailed conformational analysis, the acid groups of **2** and **4** were methylated with (trimethylsilyl)diazomethane, giving esters **5** and **6** (Scheme 3). This increases the solubility of the tripeptides and eliminates the interference of the OH group. The IR spectra of **5** and **6** in CH_2Cl_2 indicate free and hydrogen-bonded NH groups (**5**: $\tilde{\nu}_{NH}$ = 3439, 3353, 3323 cm^{-1}; **6**: $\tilde{\nu}_{NH}$ = 3432, 3373, 3291 cm^{-1}) as well as hydrogen-bonded CO_{ester} groups for **5** but not for **6** (**5**: $\tilde{\nu}_{CO}$ = 1727 cm^{-1}; **6**: $\tilde{\nu}_{CO}$ = 1741 cm^{-1}).

For ester **5** the chemical shifts of the amide protons H^1 and H^6 in CD_2Cl_2 (δ = 9.19, 7.95; for atom numbering see Scheme 3 and Figure 3) show that these two protons engage in (intramolecular) hydrogen bonds while H^{11} (δ = 7.08 ppm) does not. This is also supported by the finding that H^6 exchanges more slowly against deuterium than H^{11}, with half-lives of $\tau_{1/2}(H^6)$ = 6.8 min and $\tau_{1/2}(H^{11})$ = 5.6 min in CD_2Cl_2/D_2O. In addition the chemical shift of proton H^{11} is significantly different in the hydrogen-bond-forming solvent [D$_6$]DMSO as compared to the shift in CD_2Cl_2 [$\Delta\delta(H^{11}$, [D$_6$]DMSO, CD_2Cl_2) = 1.05] while H^1 and H^6 form hydrogen bonds in both solvents [$\Delta\delta(H^1$, [D$_6$]DMSO, CD_2Cl_2) = 0.08; $\Delta\delta(H^6$, [D$_6$]DMSO, CD_2Cl_2) = 0.08].[7]

However, H^{11} forms an intermolecular hydrogen bond in concentrated CD_2Cl_2 solution [$\delta(H^{11})$ = 7.08 in concentrated solution; $\delta(H^{11})$ = 6.39 in diluted solution], at lower temperature, as the proton resonance is strongly temperature-dependent [$\Delta\delta(H^{11})$ = –24 ppb K^{-1} in concentrated CD_2Cl_2 solution and $\Delta\delta(H^{11})$ = –27 ppb K^{-1} in diluted CD_2Cl_2 solution; Figure 1], as well as in the solid state ($\tilde{\nu}_{NH}$ = 3316 cm^{-1}).

NOE contacts *between* the two ferrocene substituents of **5** are observed in CD_2Cl_2 but not in [D$_6$]DMSO, namely cross-peaks between H^6–H^1 and H^6–$H^{\alpha 2}$ (Figure 2). Thus the NMR and IR data of **5** suggest a conformation with two intramolecular hydrogen bonds $H^1\cdots O^4$ and $H^6\cdots O^1$ between the two substituents – a pattern similar to that found in the crystalline state of the tetrapeptide Boc-Ala-Fca-Ala-Ala-OMe.[7] The hydrogen bond between H^1 and O^4 is also found in short dipeptides of the type Ac-Fca-AA-OMe (AA = L-Val, L-Ile).[8] DFT calculations (B3LYP, LANL2DZ) additionally indicate this (*P*)-helical conformation with two

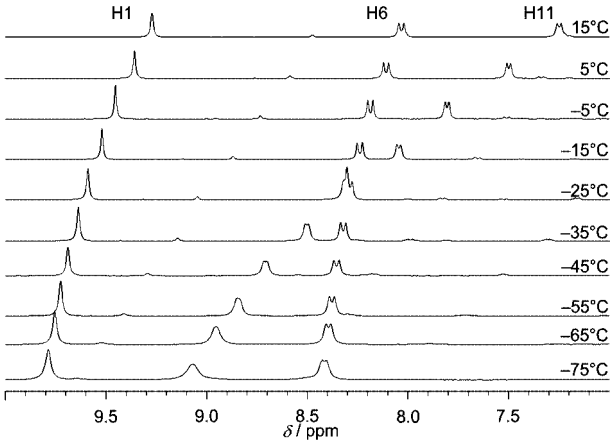


Figure 1. VT ^{1}H NMR spectra of **5** in concentrated CD_2Cl_2 solution.

intramolecular hydrogen bonds to be the global minimum of seven hydrogen-bonded conformations of **2** (Figure 3). Other folded conformations for **1**–**3** with one to three intramolecular hydrogen bonds are calculated higher in energy by 6–39 kJ mol^{-1} (see Supporting Information).

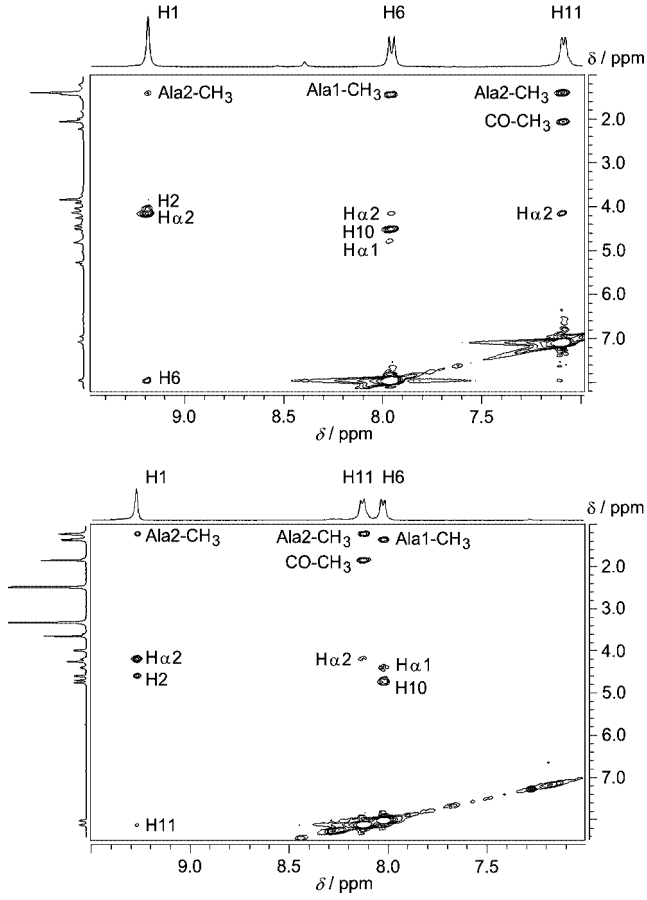


Figure 2. NOESY spectra of **5** in CD_2Cl_2 (top) and in [D_6]DMSO (bottom).

The observed vicinal coupling constants $^3J(H^6\text{-}H^{\alpha1})$ = 7.7 Hz and $^3J(H^{11}\text{-}H^{\alpha2})$ = 6.0 Hz in CD_2Cl_2 and the derived torsion angles[14] C–N–C$^{\alpha1}$–C = –86° and C–N–C$^{\alpha2}$–C = –76° fit well to the DFT calculated angles C–N–C$^{\alpha1}$–C = –90° and C–N–C$^{\alpha2}$–C = –73° of the preferred conformer. In THF similar vicinal coupling constants are observed [$^3J(H^6\text{-}H^{\alpha1})$ = 7.5 Hz and $^3J(H^{11}\text{-}H^{\alpha2})$ = 5.4 Hz] while in [D_6]DMSO the folded structure is completely disrupted [$^3J(H^6\text{-}H^{\alpha1})$ = 6.9 Hz and $^3J(H^{11}\text{-}H^{\alpha2})$ = 6.7 Hz]. These findings support the assumption that even the TentaGel immobilised peptides in THF retain this preferred conformation.

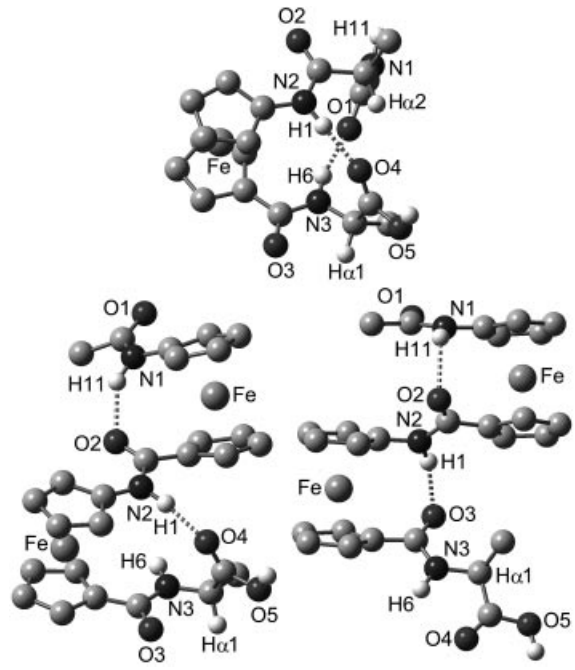


Figure 3. DFT calculated minimum conformations of **2** (top) and **4** (bottom) (Cp and methyl H atoms omitted).

For diferrocene **4** several different conformations were calculated with similar energy (Figure 3, Supporting Information). This is also supported by NMR and IR spectroscopy of ester **6**. The amide proton H^1 engages prevalently in hydrogen bonds (δ = 8.54; $\Delta\delta$ = –10.5 ppb K^{-1}; $\tau_{1/2}$ = 26 min) as compared to H^{11} (δ = 7.61; $\Delta\delta$ = –13.7 ppb K^{-1}; $\tau_{1/2}$ = 5.8 min). However, even H^6 participates in hydrogen bonding (δ = 7.10; $\Delta\delta$ = –14.5 ppb K^{-1}). The finding that all amide protons form hydrogen bonds can only be accomplished by an ensemble of conformations in solution (two of the low-energy conformations of **4** are shown in Figure 3).

Electrochemistry of Ferrocene-Containing Peptides

Monoferrocenes **1**–**3** are reversibly oxidised to the corresponding ferricinium cations around 500 mV versus SCE in CH_3CN almost independently of the α-amino acids employed, while diferrocene **6** displays two oxidation waves at 470 and 590 mV versus SCE (Figure 4). The difference in the redox potentials of **6** ($\Delta E_{1/2}$ = 590 – 470 = 120 mV) is significantly smaller than that observed for Fc-NHCO-Fc-NHAc with differently substituted ferrocene moieties ($\Delta E_{1/2}$ = 305 mV[6]). The similarly substituted ferrocene

units in **6** (in contrast to Fc-NHCO-Fc-NHAc) allow estimation of the comproportionation constant for the monocation $\mathbf{6}^+$ as $K_C = 107$, which characterises $\mathbf{6}^+$ as a class II mixed-valent species with moderate delocalisation of the electron hole.

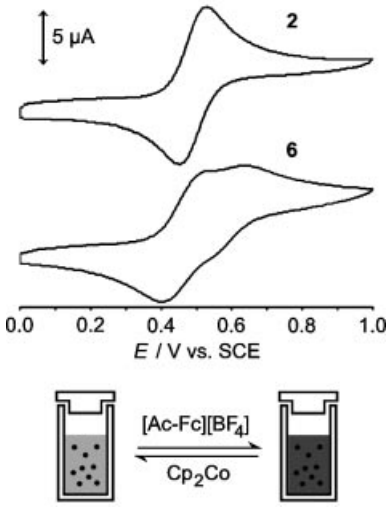


Figure 4. Cyclic voltammograms of tripeptides **2** and **6** (top) and chemical on-bead oxidation/reduction of ferrocene-containing tripeptide **2** (bottom).

To test binding affinities of neutral and charged peptides directly on-resin, it is necessary to chemically oxidise the peptides on the solid support. On-bead oxidation is performed by adding soluble [Ac-Fc][BF_4] ($E_{1/2}$ = 730 mV vs. SCE in CH_2Cl_2[15]) in CH_2Cl_2 solution to the immobilised tripeptide. Progress of the oxidation reaction is monitored UV/Vis-spectrophotometrically as the 633 nm absorption band of $[Ac\text{-}Fc]^+$ disappears while the 449 nm band of Ac-Fc increases (Figure 5, top). The reaction is finished within minutes. EPR spectroscopy[16] confirms the formation of the immobilised ferricinium cation as the typical axial pattern with $g_{\parallel} = 4.30$ and $g_{\perp} = 1.99$ is observed.[17]

Chemical reversibility is checked on-bead by re-reduction to the neutral peptides employing $CoCp_2$ as reductant and UV/Vis-spectrophotometric monitoring (disappearance of the 512 nm band of $CoCp_2$ and development of the 400 nm absorption band of $[CoCp_2]^+$). Reduction requires hours to be completed, probably because of the hydrophobic character of $CoCp_2$ and the hydrophilic nature of the Tentagel resin employed (Figure 5, bottom).

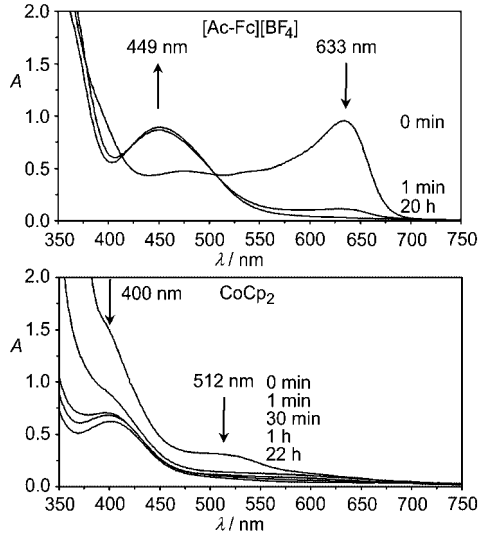


Figure 5. UV/Vis spectra during oxidation of immobilised **2** with [AcFc][BF_4] and during reduction of immobilised $\mathbf{2}^+$ with $CoCp_2$.

Preliminary Solution and On-Bead Binding Studies

The conformation analysis suggests that binding of a guest to Fca peptides should occur by an induced fit process rather than being governed by the static lock and key concept.[11] ESI mass spectra of **5** and an equimolar mixture of five *N*-acetylated α-amino acids (Ac-Gly, Ac-Ala, Ac-Val, Ac-Ile, Ac-Phe; **5**: Ac-NH-CHR-COOH, 10:1) in CH_3CN provide a first hint that Fca peptides bind these guests as carboxylates (*m*/*z* = 559.3, 573.3, 601.2, 615.3 and 649.4 for [**5** + Ac-Gly – H]$^+$, [**5** + Ac-Ala – H]$^+$, [**5** + Ac-Val – H]$^+$, [**5** + Ac-Ile – H]$^+$ and [**5** + Ac-Phe – H]$^+$, respectively; Figure 6; the peak at *m*/*z* = 556.3 corresponds to [**5** + TFA – H]$^+$ with TFA present in the mass spectrometer).

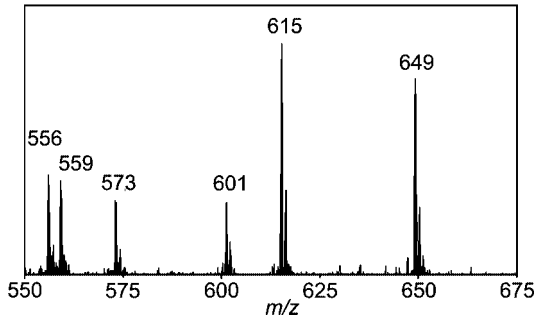


Figure 6. Partial ESI (neg.) mass spectrum of **5** and a mixture of five *N*-acetylated α-amino acids.

On-bead analysis[18] of immobilised peptide **2** using the chromophoric substrates pyrene-$(CH_2)_3$-COOH/P_1tBu [λ_{max} = 344 nm (60000 $M^{-1}cm^{-1}$) in THF] and coumarin-3-COOH/P_1tBu[19] [λ_{max} = 315 nm (12000 $M^{-1}cm^{-1}$) in THF] allowed determination of binding affinities by absorption spectroscopy. Equilibrium is reached after about 10 h in both cases according to spectroscopic measurements (Figure 7). Binding constants K_a were calculated with the assumption of a 1:1 receptor substrate complex by Equation (1).

$$K_a = [RS]/\{([R]_0 - [RS])([S]_0 - [RS])\} \quad (1)$$

where [RS] is the concentration of receptor/substrate complex at equilibrium, $[R]_0$ is the initial receptor concentration and $[S]_0$ is the initial substrate concentration.

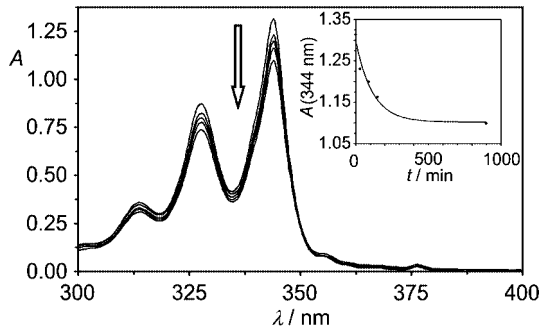


Figure 7. UV/Vis absorption spectra during reaction of immobilised **2** with pyrene-$(CH_2)_3$-COOH/P_1tBu in THF (the inset shows the corresponding t vs. A plot).

Tripeptide **2** binds the coumarin derivative significantly stronger than the pyrene derivative [K_a(coumarin) = 670 M^{-1}, K_a(pyrene) = 200 M^{-1}]. This is probably due to the presence of additional hydrogen acceptors (oxygen atoms) in the coumarin dye as compared to the pyrene dye, which complements the three NH groups of peptide **2** (three-point binding vs. two-point-binding). A detailed analysis of the binding modes of different (chiral) substrates to ferrocene peptides as well as to charged ferricinium peptides is currently in progress. For the latter investigation, redox-stable chromophoric substrates have to be used as the coumarin and pyrene dyes employed in this study undergo chemical degradation when reacted with immobilised $\mathbf{2}^+$.

Conclusions

The development of SPPS for ferrocene amino acids, conformational analyses of the peptides, on-bead oxidation procedures and on-bead binding assays paves the way for devising selective redox-controlled receptor and transporter systems, which, by synthetic design, are amenable to parallel solid-phase peptide synthesis and on-bead oxidation and screening assays.

Experimental Section

Unless noted otherwise, all manipulations were carried out under argon by means of standard Schlenk techniques. All solvents were dried by standard methods and distilled under argon prior to use. All other reagents were used as received from commercial sources.

NMR: Bruker Avance DPX 200 at 200.15 MHz and Varian Unity Plus 400 at 399.89 MHz (^{1}H); chemical shifts (δ) in ppm with respect to residual solvent peaks as internal standard: CD_2Cl_2 (^{1}H: δ = 5.32; ^{13}C: δ = 53.5 ppm), [D_8]THF (^{1}H: δ = 1.73, 3.58; ^{13}C: δ = 25.5, 67.7), [D_6]DMSO (^{1}H: δ = 2.49 ppm). IR spectra were recorded with a BioRad Excalibur FTS 3000 spectrometer using CaF_2 cells in CH_2Cl_2 and as CsI disks. Cyclic voltammetry was performed using a glassy carbon electrode, a platinum electrode and a SCE electrode, 10^{-3} M in 0.1 M nBu$_4$NPF$_6$/CH_2Cl_2; potentials are given relative to that of SCE. ESI mass spectra were recorded with a Finnigan TSQ 700 spectrometer. EPR spectra were recorded with a Bruker ELEXSYS E500 spectrometer (X-band). UV/Vis spectra were recorded with a Perkin–Elmer Lambda 19, 0.2-cm cells (Hellma, suprasil). Melting points were determined with a Gallenkamp capillary melting point apparatus MFB 595 010 and are uncorrected.

Computational Method: Density functional calculations were carried out with the Gaussian03/DFT[20] series of programs. The B3LYP formulation of density functional theory was used employing the LanL2DZ basis set.[20] This functional/basis set combination has been successfully applied previously to investigate the conformational preference of substituted ferrocenes.[6,8,11,21,22] All points were characterised as minima (N_{imag} = 0) by frequency analysis.

Solid-Phase Peptide Synthesis of 1–4

TentaGel-Wang-AA^1-Fmoc [1 g; AA^1 = Gly (**1**), Ala (**2**, **4**), Phe (**3**); 0.17–0.24 mmol g^{-1}] was deprotected with 20% piperidine in CH_2Cl_2/DMF (9:1). The loading was determined spectrophotometrically at 290 nm. The resin was washed with DMF (2×), CH_2Cl_2 (1×), DMF (1×) and CH_2Cl_2 (2×). Fmoc-Fca-OH (1.5 equiv.) was activated by using DIC (1.8 equiv.) and HOBt (1.8 equiv.) in CH_2Cl_2/DMF (1:1) for 10 min and added to the resin. The resin was shaken for 12 h, washed with DMF (2×), CH_2Cl_2 (1×), DMF (1×) and CH_2Cl_2 (2×) treated with acetic anhydride/pyridine (10:1) for 30 min and washed with DMF (2×), CH_2Cl_2 (1×), DMF (1×) and CH_2Cl_2 (2×). Fmoc was removed with 20% piperidine in CH_2Cl_2/DMF (9:1). Ac-AA^2-OH [1.5 equiv.; AA^2 = Ala (**1**, **2**), Phe (**3**), Fca (**4**)] activated with HOBt (1.8 equiv.) and DIC (1.8 equiv.) in CH_2Cl_2/DMF (1:1) for 10 min was added and the resin shaken for 12 h. After washing with DMF (2×), CH_2Cl_2 (1×), DMF (1×) and CH_2Cl_2 (2×) the tripeptide was cleaved from the resin with 50% TFA in dichloromethane (1 h) and purified on silica gel (EtOAc/MeOH or EtOAc/THF) to give an orange powder.

Complex 1: 51 mg, 61%; m.p. 128 °C; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.55. ^{1}H NMR ([D_8]THF): δ = 9.17 (s, 1 H, H^1), 7.99 (dvd, 1 H, H^6), 7.78 (m, 1 H, H^{11}), 5.15 (pt, 1 H, H^5), 4.75 (pt, 1 H, H^7), 4.45 (pt, 1 H, H^{10}), 4.35 (pt, 1 H, H^9), 4.33 (dvd, $^2J_{HH}$ = 17.5, $^3J_{HH}$ = 6.8 Hz, 1 H, H$^{\alpha 1}$), 4.20 (pt, 1 H, H^8), 4.14 (pt, 1 H, H^2), 4.09 (q, $^3J_{HH}$ = 6.9 Hz, 1 H, H$^{\alpha 2}$), 4.01 (pt, 1 H, H^3), 3.87 (pt, 1 H, H^4), 3.71 (dvd, $^2J_{HH}$ = 17.5, $^3J_{HH}$ = 5.0 Hz, 1 H, H$^{\alpha 1}$), 1.96 (s, 3 H, $COCH_3$), 1.27 (d, $^3J_{HH}$ = 6.9 Hz, 3 H, Ala2-CH_3) ppm. ^{13}C{^{1}H} NMR ([D_8]THF): δ = 175.7 (s, COOH), 174.6 (s, CO), 171.9 (s, CO), 170.3 (s, CO), 97.7 (s, C_{Cp}-N), 77.6 (s, C_{Cp}-CO), 72.9 (s, C^9), 71.5 (s, C^{10}), 71.0 (s, C^8), 70.5 (s, C^7), 66.1 (s, C^3), 65.7 (s, C^4), 64.6 (s, C^2), 63.4 (s, C^5), 51.2 (s, C$^{\alpha 2}$), 41.5 (s, C$^{\alpha 1}$), 22.7 (s, $COCH_3$), 17.7 (s, Ala2-CH_3) ppm. Complex **1** exists as a mixture of two NMR-discernible conformers; resolved signals for the minor conformer: ^{1}H NMR ([D_8]THF): δ = 8.94 (s, 1 H, H^1), 5.11 (pt, 1 H, H^5), 4.73 (pt, 1 H, H^7), 4.40 (dvd, $^2J_{HH}$ = 17.5, $^3J_{HH}$ = 6.8 Hz, 1 H, H$^{\alpha 1}$), 4.10 (q, $^3J_{HH}$ = 6.9 Hz, 1 H, H$^{\alpha 2}$), 3.78 (dvd, $^2J_{HH}$ = 17.5, $^3J_{HH}$ = 5.0 Hz, 1 H, H$^{\alpha 1}$), 1.95 (s, 3 H, $COCH_3$), 1.29 (d, $^3J_{HH}$ = 6.9 Hz, 3 H, Ala2-CH_3) ppm. IR (CsI): $\tilde{\nu}$ = 3504 (OH), 3373, 3309, 3258 (NH), 1727 (C=O_{acid}), 1666, 1629 (amide I), 1574, 1546 (amide II), 1262 (C–O) cm^{-1}. IR (CH_2Cl_2): $\tilde{\nu}$ = 3687, 3601, 3440, 3365, 3321, 1771, 1732, 1663, 1655, 1605, 1572, 1531, 1522 cm^{-1}. UV (THF): λ_{max} = 445 nm (190 M^{-1} cm^{-1}). CV (CH_3CN, nBu$_4$NPF$_6$): $E_{1/2}$ = 505 mV (ΔE = 80 mV) versus SCE. Neg.-ESI-MS (CH_3CN): m/z (%) = 414.2 (100) [M – H]$^-$, 829.3 (8) [2M – H]$^-$.

Complex 2: 71 mg, 83%; m.p. 113 °C; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.32. ^{1}H NMR ([D_8]THF): δ = 9.39 (s, 1 H, H^1), 7.98 (br. s, 1 H, H^6), 7.99 (m, 1 H, H^{11}), 5.16 (pt, 1 H, H^5), 4.77 (pt, 1 H, H^7), 4.71 (q, $^3J_{HH}$ = 7.5 Hz, 1 H, H$^{\alpha 1}$), 4.60 (pt, 1 H, H^{10}), 4.36 (pt, 1 H, H^9), 4.23 (q, $^3J_{HH}$ = 7.0 Hz, 1 H, H$^{\alpha 2}$), 4.20 (pt, 1 H, H^8), 4.14

(pt, 1 H, H^2), 4.00 (pt, 1 H, H^3), 3.86 (pt, 1 H, H^4), 1.97 (s, 3 H, $COCH_3$), 1.42 (d, $^3J_{HH}$ = 7.5 Hz, 3 H, Ala1-CH_3), 1.28 (d, $^3J_{HH}$ = 7.0 Hz, 3 H, Ala2-CH_3) ppm. $^{13}C\{^1H\}$ NMR ([D$_8$]THF): δ = 172.3 (s, COOH), 171.7 (s, CO), 170.7 (s, CO), 170.6 (s, CO), 97.7 (s, C_{Cp}-N), 77.4 (s, C_{Cp}-CO), 72.7 (s, C^9), 71.8 (s, C^{10}), 70.9 (s, C^8), 70.6 (s, C^7), 66.0 (s, C^3), 65.7 (s, C^4), 64.7 (s, C^2), 63.5 (s, C^5), 51.3 (s, $C^{\alpha 2}$), 48.2 (s, $C^{\alpha 1}$), 22.7 (s, $COCH_3$), 17.6 (s, Ala2-CH_3), 17.5 (s, Ala1-CH_3) ppm. IR (CsI): $\tilde{\nu}$ = 3518 (OH), 3310 (NH), 1725 (C=O$_{acid}$), 1656, 1637 (amide I), 1570, 1544 (amide II), 1262 (C–O) cm^{-1}. IR (CH_2Cl_2): $\tilde{\nu}$ = 3687, 3600, 3434, 3408, 3318, 1773, 1724, 1684, 1668, 1653, 1605, 1570, 1539, 1523 cm^{-1}. UV (THF): λ_{max} = 444 nm (290 M^{-1} cm^{-1}). CV (CH_3CN, nBu$_4$NPF$_6$): $E_{1/2}$ = 490 mV (ΔE = 73 mV) versus SCE. Neg.-ESI-MS (MeOH): *m*/*z* (%) = 428.2 (100) [M – H]$^-$, 857.3 (6) [2M – H]$^-$.

Complex 3: 36 mg, 37%; m.p. 125 °C; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.59. ^1H NMR ([D$_8$]THF): δ = 8.99 (s, 1 H, H^1), 7.72 (dvd, 1 H, H^6), 7.96 (m, 1 H, H^{11}), 7.35 (m, 2 H, Phe-CH_{ortho}), 7.23 (m, 2 H, Phe-CH_{meta}), 7.14 (m, 1 H, Phe-CH_{para}), 4.93 (pt, 1 H, H^5), 4.88 (m, 1 H, $H^{\alpha 1}$), 4.66 (pt, 1 H, H^7), 4.59 (pt, 1 H, H^{10}), 4.32 (pt, 1 H, H^9), 4.26 (pt, 1 H, H^2), 4.18 (pt, 1 H, H^8), 4.04 (m, 1 H, $H^{\alpha 2}$), 3.97 (pt, 1 H, H^3), 3.86 (pt, 1 H, H^4), 3.91 (m, 1 H, $H^{\alpha 2}$), 3.23 (m, 1 H, Phe-CH_2), 3.11 (m, 1 H, Phe-CH_2), 2.01 (s, 3 H, $COCH_3$) ppm. $^{13}C\{^1H\}$ NMR ([D$_8$]THF): δ = 176.8 (s, COOH), 171.8 (s, CO), 170.5 (s, CO), 168.6 (s, CO), 139.4 (s, Phe-CH), 130.2 (s, Phe-CH), 129.2 (s, Phe-CH), 127.3 (s, Phe-CH), 97.5 (s, C_{Cp}-N), 77.7 (s, C_{Cp}-CO), 72.5 (s, C^9), 71.4 (s, C^{10}), 71.1 (s, C^8), 70.5 (s, C^7), 66.2 (s, C^3), 65.9 (s, C^4), 64.7 (s, C^2), 63.3 (s, C^5), 55.1 (s, $C^{\alpha 1}$), 45.0 (s, $C^{\alpha 2}$), 37.8 (s, Phe-CH_2), 22.8 (s, $COCH_3$) ppm. Complex **3** exists as a mixture of two NMR-discernible conformers; resolved signals for the minor conformer: ^1H NMR ([D$_8$]THF): δ = 8.83 (s, 1 H, H^1), 3.75 (m, 1 H, $H^{\alpha 2}$), 3.64 (m, 1 H, $H^{\alpha 2}$), 2.88 (m, 1 H, Phe-CH_2), 2.77 (m, 1 H, Phe-CH_2), 1.94 (s, 3 H, $COCH_3$) ppm. IR (CsI): $\tilde{\nu}$ = 3511 (OH), 3323 (NH), 1725 (C=O$_{acid}$), 1657, 1637 (amide I), 1560, 1544 (amide II), 1263 (C–O), 703 (Ph$_{def}$) cm^{-1}. IR (CH_2Cl_2): $\tilde{\nu}$ = 3687, 3601, 3447, 3322, 1723, 1696, 1668, 1656, 1605, 1571, 1522 cm^{-1}. UV (THF): λ_{max} = 444 nm (170 M^{-1} cm^{-1}). CV (CH_3CN, nBu$_4$NPF$_6$): $E_{1/2}$ = 515 mV (ΔE = 79 mV) versus SCE. Neg.-ESI-MS (CH_3CN): *m*/*z* (%) = 490.3 (100) [M – H]$^-$, 981.3 (7) [2M – H]$^-$.

Complex 4: 65 mg, 56%; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.48. ^1H NMR ([D$_6$]DMSO): δ = 10.24 (s, 1 H, H^1), 9.74 (s, 1 H, H^{11}), 7.41 (d, $^3J_{HH}$ = 6.5 Hz, 1 H, H^6), 4.96 (br. s, 1 H), 4.89 (br. s, 2 H), 4.68 (br. s, 2 H), 4.60 (br. s, 2 H), 4.52 (br. s, 1 H), 4.29, 4.22, 4.20 (m, 5 H), 3.99 (br. s, 2 H), 3.93 (br. s, 2 H), 1.86 (s, 3 H, $COCH_3$), 1.32 (d, $^3J_{HH}$ = 6.8 Hz, 3 H, Ala-CH_3) ppm. Poor solubility prevented recording of ^{13}C NMR spectra. IR (CsI): $\tilde{\nu}$ = 3509 (OH), 3341 (NH), 1733 (C=O$_{acid}$), 1642 (amide I), 1546 (amide II), 1262 (C–O) cm^{-1}. UV (THF): λ_{max} = 447 nm (360 M^{-1} cm^{-1}). Neg.-ESI-MS (CH_3CN): *m*/*z* (%) = 584.2 (100) [M – H]$^-$, 1169.1 (8) [2M – H]$^-$.

Synthesis of 5 by Methylation of 2

(Trimethylsilyl)diazomethane (0.1 mL; 2 M in Et_2O, 0.2 mmol) was added to **2** (50 mg, 0.117 mmol) in MeOH (10 mL) and the mixture was stirred at 25 °C for 2 h. The solvent was removed in vacuo and the residue was purified on silica gel (THF/PE, 7:3) to give **5** as an orange powder.

Complex 5: 47 mg 90%; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.66. ^1H NMR (CD_2Cl_2): δ = 9.19 (s, 1 H, H^1), 7.95 (d, $^3J_{HH}$ = 7.7 Hz, 1 H, H^6), 7.08 (d, $^3J_{HH}$ = 6.0 Hz, 1 H, H^{11}), 5.27 (pt, 1 H, H^5), 4.82 (br. s, 1 H, H^7), 4.77 (q, $^3J_{HH}$ = 6.3 Hz, 1 H, $H^{\alpha 1}$), 4.51 (br. s, 1 H, H^{10}), 4.43 (br. s, 1 H, H^9), 4.26 (br. s, 1 H, H^8), 4.16 (q, $^3J_{HH}$ = 6.9 Hz, 1 H, $H^{\alpha 2}$), 4.14 (br. s, 1 H, H^3), 4.04 (br. s, 1 H, H^2), 3.92 (br. s, 1 H, H^4), 3.84 (s, 3 H, OCH_3), 2.07 (s, 3 H, $COCH_3$), 1.44 (br. s, 3 H, Ala1-CH_3), 1.42 (br. s, 3 H, Ala2-CH_3) ppm. $^{13}C\{^1H\}$ NMR (CD_2Cl_2): δ = 177.4 (s, COOMe), 171.6 (s, CO), 170.9 (s, CO), 170.1 (s, CO), 95.6 (s, C_{Cp}-N), 75.7 (s, C_{Cp}-CO), 72.5 (s, C^9), 71.0 (s, C^{10}), 70.5 (s, C^8), 69.7 (s, C^7), 65.8 (s, C^3), 65.3 (s, C^4), 64.2 (s, C^2), 62.8 (s, C^5), 52.8 (s, OCH_3), 50.8 (s, $C^{\alpha 2}$), 48.2 (s, $C^{\alpha 1}$), 22.6 (s, $COCH_3$), 17.2 (s, Ala2-CH_3), 16.7 (s, Ala1-CH_3) ppm. ^1H NMR ([D$_8$]THF): δ = 8.94 (s, 1 H, H^1), 7.97 (d, $^3J_{HH}$ = 7.5 Hz, 1 H, H^6), 7.77 (d, $^3J_{HH}$ = 5.4 Hz, 1 H, H^{11}), 5.15 (pt, 1 H, H^5), 4.74 (pt, 1 H, H^7), 4.72 (q, $^3J_{HH}$ = 7.6 Hz, 1 H, $H^{\alpha 1}$), 4.56 (pt, 1 H, H^{10}), 4.36 (pt, 1 H, H^9), 4.18 (pt, 1 H, H^8), 4.12 (q, $^3J_{HH}$ = 6.8 Hz, 1 H, $H^{\alpha 2}$), 4.07 (pt, 1 H, H^2), 4.02 (pt, 1 H, H^3), 3.86 (pt, 1 H, H^4), 3.79 (s, 3 H, OCH_3), 1.96 (s, 3 H, $COCH_3$), 1.41 (d, $^3J_{HH}$ = 7.6 Hz, 3 H, Ala1-CH_3), 1.30 (d, $^3J_{HH}$ = 6.8 Hz, 3 H, Ala2-CH_3) ppm. ^1H NMR ([D$_6$]DMSO): δ = 9.27 (s, 1 H, H^1), 8.13 (d, $^3J_{HH}$ = 6.7 Hz, 1 H, H^{11}), 8.03 (d, $^3J_{HH}$ = 6.9 Hz, 1 H, H^6), 4.76 (br. s, 1 H, H^{10}), 4.70 (br. s, 1 H, H^7), 4.61 (br. s, 1 H, H^5), 4.59 (br. s, 1 H, H^2), 4.40 (q, $^3J_{HH}$ = 7.0 Hz, 1 H, $H^{\alpha 1}$), 4.26 (br. s, 2 H, $H^{9,10}$), 4.19 (q, $^3J_{HH}$ = 6.8 Hz, 1 H, $H^{\alpha 2}$), 4.00, 3.98 (br. s, 1 H, $H^{3,4}$), 3.65 (s, 3 H, OCH_3), 1.85 (s, 3 H, $COCH_3$), 1.37 (d, $^3J_{HH}$ = 7.0 Hz, 3 H, Ala1-CH_3), 1.23 (d, $^3J_{HH}$ = 6.8 Hz, 3 H, Ala2-CH_3) ppm. $^{13}C\{^1H\}$ NMR (CD_2Cl_2): δ = 173.8 (s, COOMe), 171.4 (s, CO), 169.4 (s, CO), 169.2 (s, CO), 96.3 (s, C_{Cp}-N), 76.0 (s, C_{Cp}-CO), 71.9, 71.8 (s, $C^{8,9}$), 69.4 (s, C^7), 69.1 (s, C^{10}), 66.0, 65.9 (s, $C^{3,4}$), 62.3, 62.1 (s, $C^{2,5}$), 52.0 (s, OCH_3), 49.1 (s, $C^{\alpha 2}$), 47.9 (s, $C^{\alpha 1}$), 22.6 (s, $COCH_3$), 18.1 (s, Ala2-CH_3), 17.0 (s, Ala1-CH_3) ppm. IR (CsI): 3316 (NH), 1743 (C=O$_{ester}$), 1657, 1638 (amide I), 1570, 1543 (amide II), 1263 (C–O) cm^{-1}; N-deuterated analogue: $\tilde{\nu}$ = 2455 (ND) cm^{-1}. IR (CH_2Cl_2): $\tilde{\nu}$ = 3439, 3353, 3323 (NH), 1727 (C=O$_{ester}$), 1690, 1668, 1651 (amide I), 1571, 1527 (amide II) cm^{-1}. UV (THF): λ_{max} = 443 nm (310 M^{-1} cm^{-1}). Pos.-ESI-MS (CH_3CN): *m*/*z* (%) = 444.3 (39) [M + H]$^+$, 466.3 (100) [M + Na]$^+$, 482.2 (10) [M + K]$^+$, 887.1 (3) [2M + H]$^+$, 909.8 (39) [2M + Na]$^+$, 925.0 (4) [2M + K]$^+$.

Synthesis of 6 by Methylation of 4

(Trimethylsilyl)diazomethane (0.1 mL; 2 M in Et_2O, 0.2 mmol) was added to crude **4** (50 mg, 0.085 mmol) in MeOH (10 mL) and the mixture was stirred at 25 °C for 2 h. The solvent was removed in vacuo and the residue was purified on silica gel (EtOAc/MeOH, 7:3) or alternatively by TLC (EtOAc/MeOH, 7:3) to give **6** as an orange powder.

Complex 6: 40 mg, 79%; R_f (SiO_2, EtOAc/MeOH, 7:3) = 0.72. ^1H NMR (CD_2Cl_2): δ = 8.54 (s, 1 H, H^1), 7.61 (s, 1 H, H^{11}), 7.10 (d, $^3J_{HH}$ = 7.1 Hz, 1 H, H^6), 4.79 (br. s, 1 H), 4.67 (br. s, 1 H), 4.49 (br. s, 1 H), 4.47 (br. s, 1 H) [$H^{2,2',5,5'}$], 4.75 (br. s, 1 H), 4.69 (br. s, 2 H), 4.61 (br. s, 1 H) [$H^{7,7',10,10'}$], 4.62 (m, 1 H, $H^{\alpha 1}$), 4.42 (br. s, 2 H), 4.41 (br. s, 2 H) [$H^{8,8',9,9'}$], 4.15 (br. s, 2 H), 4.13 (br. s, 2 H) [$H^{3,3',4,4'}$], 3.76 (s, 3 H, OCH_3), 2.00 (s, 3 H, $COCH_3$), 1.51 (d, $^3J_{HH}$ = 7.2 Hz, 3 H, Ala-CH_3) ppm. $^{13}C\{^1H\}$ NMR (CD_2Cl_2): δ = 71.6, 71.5, 71.3, 71.2 (s, $C^{8,9,8',9'}$), 70.0, 69.9, 69.8, 69.6 (s, $C^{7,10,7',10'}$), 66.4, 66.3, 66.2, 66.1 (s, $C^{3,4,3',4'}$), 65.2, 64.5, 64.4, 64.3 (s, $C^{2,5,2',5'}$), 52.5 (s, OCH_3), 48.6 (s, C^{α}), 23.8 (s, $COCH_3$), 17.7 (s, Ala-CH_3) ppm. Poor solubility prevented observation of all quaternary C signals. IR (CsI): $\tilde{\nu}$ = 3299 (NH), 1742 (C=O$_{ester}$), 1654, 1638 (amide I), 1560, 1542 (amide II), 1262 (C–O) cm^{-1}. IR (CH_2Cl_2): $\tilde{\nu}$ = 3432, 3373, 3291 (NH), 1741 (C=O$_{ester}$), 1676, 1655, 1606 (amide I), 1557, 1538, 1517 (amide II) cm^{-1}. CV (CH_3CN, nBu$_4$NPF$_6$): $E_{1/2}$ = 470 mV, 590 mV versus SCE. Pos.-ESI-MS (CH_3CN): *m*/*z* (%) = 600.3 (100) [M + H]$^+$, 622.2 (30) [M + Na]$^+$, 638.3 (6) [M + K]$^+$, 1199.3 (2) [2M + H]$^+$, 1221.4 (2) [2M + Na]$^+$.

Supporting Information (see also the footnote on the first page of this article): Selected NMR spectra of **3**, temperature-dependent NMR shifts of NH protons of **1**–**3**, **5** and **6**, ^1H NMR spectra during reaction of **5** with D_2O in CD_2Cl_2, time dependence of

H/D exchange of **5** in CD_2Cl_2/D_2O, X-band EPR spectrum of immobilised $\mathbf{2}^+$, DFT-calculated minimum structures and relative energies of **1**–**3** and **4**.

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