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Synthesis and conformational studies of a 1,1′-ferrocenophane lactam mimetic of substance P

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Abstract—The synthesis of a bis-phenylalanine mimetic **6** and its incorporation into Substance P (SP), giving a conformationally constrained organometallic SP analogue **8**, is described. The lactam **6** was synthesized in five steps, via a HWE olefination reaction, enantioselective hydrogenation with [Rh(I)(COD)((*S*,*S*)Et-DuPHOS)]$^+$OTf$^-$ and intramolecular cyclization with PyAOP as a coupling reagent. Comparative CD studies of **8** with native SP indicated that the flexibility around the amide bond of Phe(7)–Phe(8) sequence is crucial for the C-terminal (from residue Gln(4)) to adopt an α-helical conformation in the micellar environment created by SDS or in methanol.

1. Introduction

Substance P (SP), an undecapeptide of the tachykinin family, was discovered by von Euler and Gaddum in 1931.[3] It took another 40 years until the exact structure was determined by Chang and Leeman.[4] SP is involved in several important physiological processes, such as neurogenic inflammation, pain transmission, contraction of smooth muscles, increased salivation and activation of the immune system.[5] The physiological processes of SP are mediated through activation of NK_1-receptors.[6]

SP is well known to exist in aqueous solution as an equilibrium mixture of conformers.[1] Based on two-dimensional NMR spectroscopy experiments, circular dichroism (CD) spectroscopy studies and simulated annealing calculations it was determined that the midregion of SP (Pro(4)-Gln(5)-Gln(6)-Phe(7)-Phe(8)) in the presence of SDS (sodium dodecyl sulfate) micelles in water or dissolved in MeOH has an α-helical structure.[1,2] The main aim of this work was to synthesize a bis-phenylalanine mimetic **6** containing ferrocene, to incorporate it into SP as a replacement of Phe-Phe (Fig. 1) and to compare it spectroscopically with SP.

2. Results and discussion

The unprotected lactam **6** was synthesized from formyl ferrocene **1**, which was available from ferrocene according to our previous report.[7a] In the first step, **1** was condensed with the **12** according to Schmidt et al.[8,9] to give the bis-armed unsaturated derivative **2**. Based on previous experiences of similar couplings we believe that also in this case the olefin had (*Z*)-configuration.[7a,10] The phosphonate **12** was obtained by a three step synthesis from glyoxylic acid.[8] Boc and Bn were chosen as protecting groups on **12** to allow simultaneous deprotection of the desired N- and C-terminals of **3** (step c, Scheme 1). Other protecting groups of **12** may be introduced by deprotection–reprotection.[7b]

Hydrogenation of **2** at 55 psi (3.7 atm) using [Rh(I)-(COD)((*S*,*S*)-Et-DuPHOS)]$^+$OTf$^-$ as catalyst in methanol gave **3** (ee>99%). For the stereochemical analysis of **3** both the racemate and the (*R*,*R*)-isomer were needed. Therefore samples of **2** were hydrogenated using achiral Rh(I)-dppe and [Rh(I)(COD)((*R*,*R*)-Et-DuPHOS)]$^+$OTf$^-$ as catalysts, respectively. HPLC analysis of the racemate using (*R*,*R*)-Whelk-O1 chiral column showed four stereoisomers as separate peaks, although not baseline separated. Single peaks were observed for both (*S*,*S*)-**3** and (*R*,*R*)-**3** when injected separately. When either of those was coinjected with the racemate, an increase of the first and last of the four peaks was observed, respectively. From this we established that compound **3** had ee>99% assuming a <1% detection limit of the HPLC system. Based on the established selectivity of the (*S*,*S*)-Et-DuPHOS ligand we assigned the absolute configuration of **3** as (*S*,*S*).[11] It should be pointed out that the diastereomeric ratio of **3** could not be determined by ^{1}H NMR spectroscopy since **3** and the racemic mixture had identical NMR spectra.

Similar hydrogenation results were obtained for a compound

Keywords: ferrocene 1,1′-bis-alanine lactam; substance P mimetic; solid phase peptide synthesis; conformational analysis; circular dichroism.

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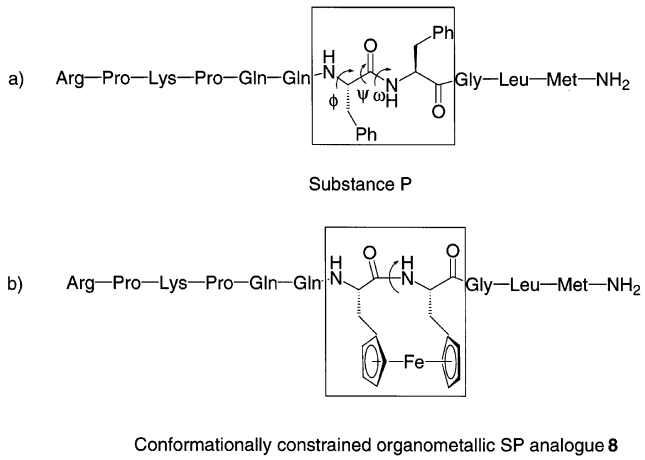


Figure 1. (a) SP and the torsion angles ψ, ω and ϕ. (b) SP analogue **8**; the dipeptidomimetic ferrocenyl bis-alanine lactam (Falc) is marked with a square.

having a TMSE instead of a Bn group of compound **3** using [Rh(I)(COD)((*S*,*S*)-Et-DuPHOS)]$^+$OTf$^-$.[7a]

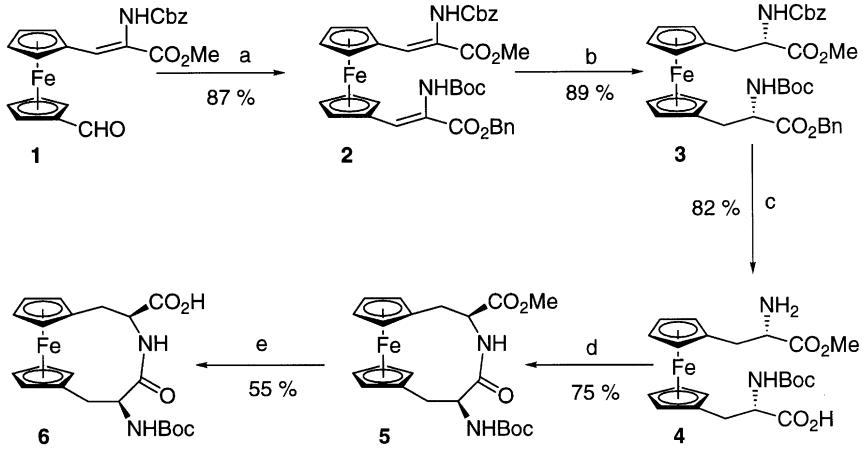
The protecting groups Bn and Cbz of **3** were simultaneously removed by hydrogenation at 1 atm using 5% Pd/C in methanol. The resulting selectively deprotected (*S*,*S*)-ferrocenylbisalanine **4** was then subjected to lactamization conditions intended to give **5**.

Our first choice as a cyclization reagent was HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), since it has proven to be versatile, fast and usually suppressing epimerization of the C-terminal residue in cyclization reactions.[12] The deprotected amino acid **4** in DMF was added over a period of 2 h by a syringe pump to a 2 mM solution of HATU and DIEA in DMF. However, we could not detect any lactam formation. One explanation why the cyclized product was not obtained could be that HATU reacted with the N-terminal amino component leading to a guanidino derivative. This side reaction is known to occur when aminium salt based coupling reagents are used and when activation of the carboxylic acid is slow.[13] To avoid this problem, phosphonium salt based coupling reagents such as PyAOP were recommended.

Therefore our next attempt was to use PyAOP as a cyclization reagent, which gave a 75% isolated yield of lactam **5**. By mass spectroscopic analysis it was confirmed that no more than a few percent of cyclodimerized material was formed. We next tried to minimize the volume of DMF by increasing the concentration of the coupling reagent, so that the reaction would be easier to perform on a lager scale. It turned out that at concentrations higher than 2 mM a poorer yield of the monomer **5** was obtained, due to cyclodimerization, which could be confirmed by mass spectroscopy.

Finally, lactam **5** was hydrolysed using LiOH in THF/water (1:1) to give the corresponding acid **6** in 55%, which was directly used in the synthesis of **8**.

Peptide **8** was synthesized by two different solid-phase methods, using Boc/Cbz protected amino acids. These two already known methods differ from each other by the use of



Scheme 1. *Reagents and conditions*: (a) $(MeO)_2POCH(NHBoc)(CO_2Bn)$ **12**, TMG, THF, 5 h (−70°C), 24 h (rt); (b) [Rh(COD)((S,S)-Et-DuPHOS)]$^+$OTf$^-$, MeOH (degassed 30 min. with Ar(g)), H_2 (55 psi), 24 h, rt; (c) 5% Pd on carbon, MeOH, H_2 (1 atm), 24 h, rt; (d) PyAOP (2 mM in DMF), DIEA, DMF, 2 h, rt; (e) LiOH, THF/H_2O (1:1), 1 h, 0°C. List of abbreviations: TMG=1,1,3,3-tetramethylguanidine, THF=tetrahydrofuran, PyAOP=7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium-hexafluorophophonate, COD=1,5-cyclooctadien, (*S*,*S*)-Et-DuPHOS=(+)-1,2-bis-((2*S*,5*S*)-2,5-diethyl)phospholane, DMF=*N*,*N*-dimethylformamide, DIEA=*N*-diisopropylethylamines.

Table 1. Standard protocol for the solid phase peptide synthesis using Boc protected amino acids

Function	Time (min)
DCM wash	3×1
TFA/DCM (1:1)+5% 1,2-ethanedithiol deprotection	2+20
DCM wash	3×1
DIEA/DCM (1:9) neutralization	2×2
DCM wash	3×1
DMF wash	3×1
Boc-amino acid (4 equiv.[a]) in DMF/DCM (1:1) DIPCDI (4 equiv.)	5
HOBt (4 equiv.) in DMF/DCM (1:1)	120
DMF wash	3×1

[a] Only 2 equiv. of **6** was used during the coupling.

two different resins, 4-methylbenzhydrylamine (MBHA) resin[14] and hydroxylmethylene resin.[15] The standard coupling procedure was used as described in Table 1. Thus, the crude peptide **8** was obtained in two ways, via trifluoromethane sulfonic acid (TFMSA) cleavage in the first case, and via a mild basic methanolysis followed by ammonolysis and then deprotection of the side chains in the second case. Purification by reversed phase HPLC under acidic conditions gave peptide **8** as the guanidinium salt as the main species as indicated by HRMS analysis.

The conformational behaviour of peptide **8** was studied by CD spectroscopy in water, methanol and in aqueous 30 mM SDS (Fig. 2b). The CD-curves were compared with CD-curves of native SP (Fig. 2a) remeasured in our laboratory

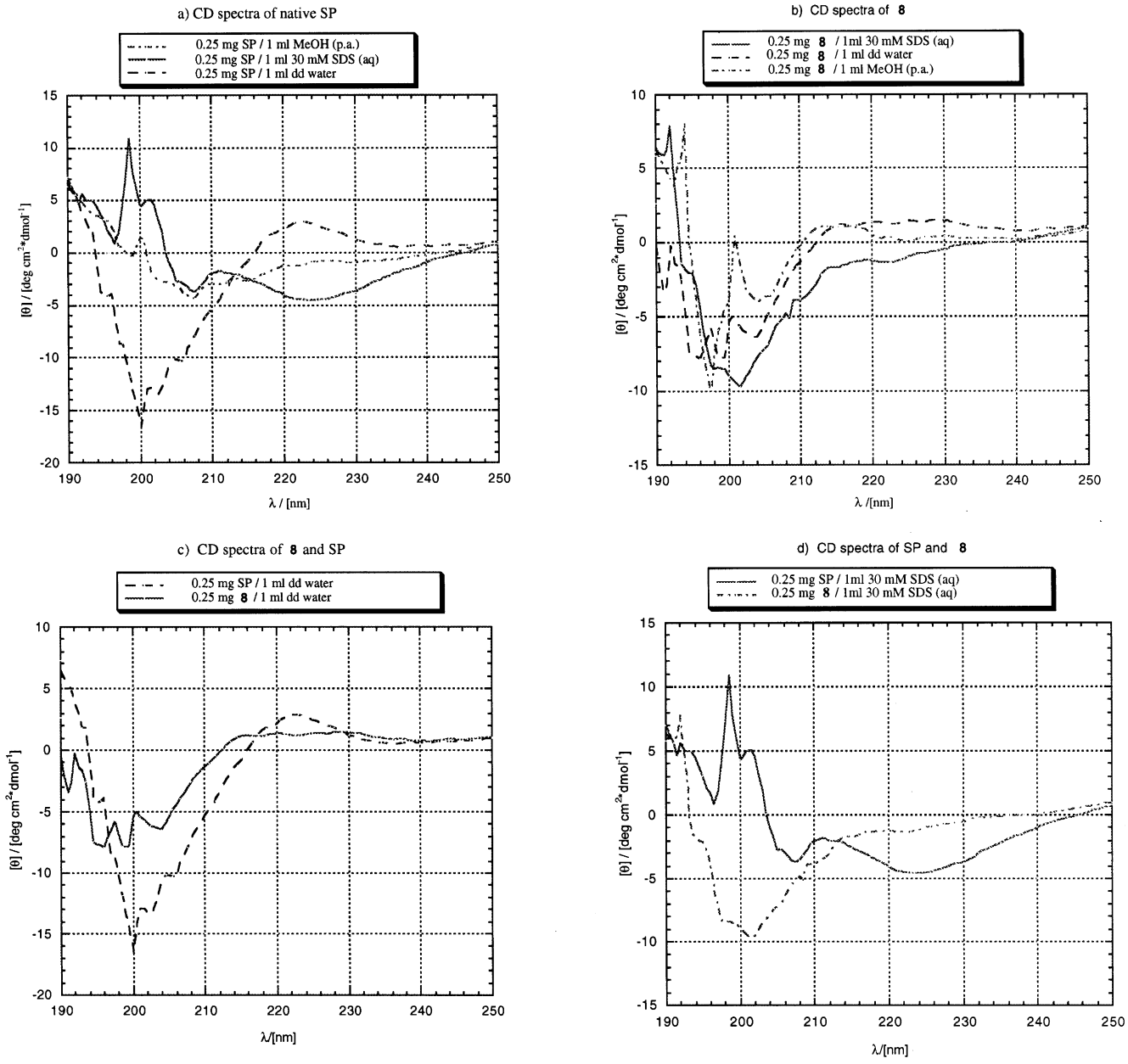
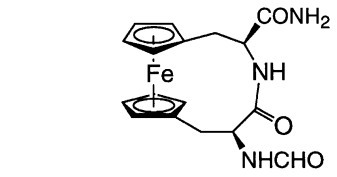


Figure 2. (a) CD spectra of the native SP in water, methanol and 30 mM aqueous SDS; (b) CD spectra of **8** in water, methanol and 30 mM aqueous SDS; (c) CD spectra of **8** and SP in water; (d) CD spectra of **8** and SP in 30 mM aqueous SDS.



6 a´

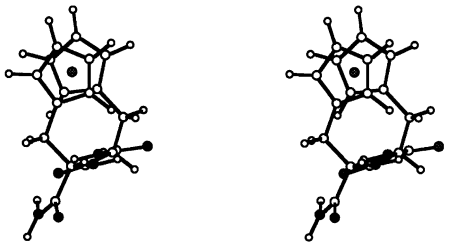


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Figure 3. Model compound **6a**′ (top); The lowest energy conformation **14** (shown as stereoview), which was found by using the MACROMODEL v 6.5 Monte Carlo Amber* minimization method (bottom).

according to Chassaing et al., Mehlis et al. and Schwyzer et al.[1,16,17a] The negative band near 200 nm is characteristic for random coil peptides, the weak maximum around 223 nm reflects the aromatic contributions of the Phe(7)–Phe(8) residue and the two negative bands near 210 and 222 nm are typical for a partial α-helical structure. Earlier CD studies showed that native SP behaves as a random coil in water, but in methanol and in the micellar environment created by SDS it adopts a partial α-helix structure. Contrary to this, SP analogue **8** behaved as a random coil in all three environments. As expected, the CD-curve of **8** did not have the weak maximum near 223 nm, as the CD-curve of SP (Fig. 2c), because the Phe(7)–Phe(8) sequence was replaced by subunit **6**. Otherwise the CD-curves of SP and **8** were similar in water and we concluded that peptide **8** behaved as random coil. On comparing the CD spectrum of **8** with that of SP in the SDS micellar environment we could observe a completely different behaviour (Fig. 2d). Schwyzer et al.[17b] predicted the conformation and orientation of SP in a hypothetic hydrophobic gradient or in a lipid membrane. The C-terminal sequence Pro(4)-…-Met(11) was shown to be placed in the hydrophobic phase as an α-helix, and the N-terminal Arg(1)-Pro(2)-Lys(3) remained in aqueous phase as a random coil. This is in accordance with the α-helix CD-bands at 208 and 222 nm for SP in the SDS micellar environment (Fig. 2d). On the other hand, it seems that the constraints that were introduced in **8** hindered the organization of an α-helix and thereby prevented the peptide to dock into the SDS micelles for stabilization of the secondary structure. Thus, the steric constraint of peptide **8** forced the substance to remain as a random coil also in the presence of SDS micelles.

In order to estimate the conformational space of the lactam structure of **6** a conformational search was made on a model

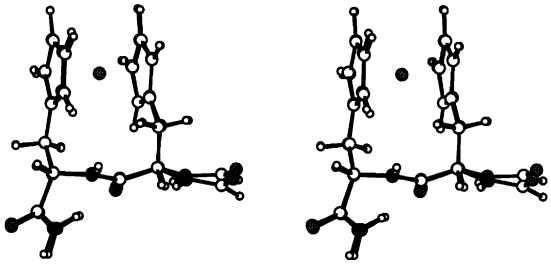

Figure 4. Stereoview of superimposed **14** and **15**.

compound of **6** in which the carboxylic acid was replaced by a carboxamide and the Boc-NH group by a formamide group (**6a**′, Fig. 3). Thus, the global minimum conformation of **6a**′ was explored using MACROMODEL v 6.5 Monte Carlo with random conformation search and Amber* force field (in vacuum) for minimization. Of the 100 Monte Carlo pushes, 32 unique conformations were found. The lowest energy conformation **14** (Fig. 3), found three times, was 5.70 kJ mol^{-1} lower than the next conformation **15**, found once. The next conformation was 11.2 kJ mol^{-1} higher than the global minimum. Conformations **14** and **15** were very similar as shown in Fig. 4 and apparently **6a**′ (as **6**) should be able to adopt a trans amide orientation of the lactam.

3. Conclusion

The synthesis of Phe-Phe mimetic **6** (Scheme 1) and its incorporation into Substance P, thus obtaining a conformationally constrained organometallic SP analogue **8** (Fig. 1) were achieved. By conformational studies with CD spectroscopy it was demonstrated that the constraints imposed by **6** (Fig. 1) did not allow the peptide to adopt the characteristic α-helical secondary structure similar to native SP in a biomimetic SDS micellar environment (Fig. 2). According to minimization using the MACROMODEL v 6.5 Amber* force field the *trans* amide bond of **6** was energetically favoured. The biological activity of the SP analogue **8** and congeners will be published in due time elsewhere.

4. Experimental

4.1. General

(*S*,*S*)-Et-DuPHOS and PyOAP were obtained from Strem Chemicals and Advanced ChemiTech, respectively. The following starting materials were prepared according to literature procedures: **1**,[7] **12**,[8] [Rh(COD)((*S*,*S*)-Et-DuPHOS)]$^{+}$OTf^{-}[11]. All commercial materials were used without further purification. TLC analyses were performed on Merck Silica Gel 60 coated glass plates and for flash chromatography Matrex™ (35–70 μm) silica gel was used. Melting points are uncorrected. ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker DRX 400 spectrometer operating at 400 MHz. CD spectra were recorded on a JASCO spectropolarimeter model J-710/720 at room temperature and constant nitrogen flush. The data are expressed as ellipticity θ in deg cm^2 $dmol^{-1}$ plotted against the wavelength λ in nm.

4.1.1. (*Z*,*Z*)-1-{2-[(Benzyloxycarbonyl)amino]-2-(methoxycarbonyl)ethenyl}-1′-{2-[(*tert*-butoxycarbonyl)amino]-2-(2-benzyloxycarbonyl)ethenyl}ferrocene (2). A solution of compound **1**[7] (853 mg, 1.91 mmol) in THF (14.7 ml) was added to a solution of **12** (959 mg, 2.57 mmol) and TMG (0.309 ml, 2.46 mmol) in THF (14.7 ml) at −70°C and under an inert atmosphere. The mixture was stirred at −70°C for 5 h and then the stirring was continued at room temperature overnight. The reaction mixture was concentrated and the residue was dissolved in EtOAc (150 ml). The organic layer was washed with water (50 ml), brine (50 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent at the reduced pressure followed by chromatography (SiO_2, heptane/EtOAc 1:1) gave 1.157 g (87%; R_f=0.43 heptane/EtOAc 1:1) of **2** as dark red crystals: mp 46–49°C. ^{1}H NMR (50°C, $CDCl_3$): δ 7.29–7.43 (m, 10H), 7.21 (s, 1H), 7.15 (s, 1H), 6.40 (br s, 1H), 5.90 (s, 1H), 5.24 (s, 2H), 5.16 (s, 2H), 4.55–4.57 (m, 4H), 4.36–4.37 (m, 4H), 3.74 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (50°C, $CDCl_3$): δ 165.74, 165.37, 154–156 (br, 2C), 136.68, 136.24, 135.06, 134.59, 128.64, 128.56, 128.31, 128.25, 128.19, 122.72, 122.42, 80.73, 78.20, 78.06, 72.35, 72.26, 72.13, 72.03, 67.38, 67.14, 67.03, 52.35, 28.41. HRMS (FAB^+) *m*/*z* calcd for $C_{37}H_{38}FeN_2O_8$: 694.1978. Found: 694.1984.

4.1.2. (+)-(*S*,*S*)-1-{2-[(Benzyloxycarbonyl)amino]-2-(methoxycarbonyl)ethyl}-1′-{[(2-*tert*-butoxycarbonyl)amino]-2-(benzyloxycarbonyl)ethyl}ferrocene (3). A solution of compound **2** (311 mg, 0.45 mmol) in MeOH (11 ml) was degassed for 30 min^{-1}. with Ar(g). [Rh(COD)((*S*,*S*)-Et-DuPHOS)]$^+$OTf$^-$ (15 mg, 0.021 mmol) was added and the reaction mixture was hydrogenated at 3.7 atm (55 psi) for 24 h at rt. The solvent was removed at reduced pressure, the residue was dissolved in EtOAc and passed through a layer of Celite to give **3** (277 mg, 89%) as a yellow viscous mass after evaporation of the solvent: $[\alpha]^{22}_D$=+23 (*c* 1.42, $CHCl_3$). HPLC analysis on a (*R*,*R*)-Whelk-O1 column (flow rate 1.0 ml min^{-1}, eluent: hexane/*i*PrOH 19:1+0.5% of HOAc) at 40°C revealed one peak at R_t=32 min. ^{1}H NMR (110°C, DMSO-d_6): δ 7.26–7.37 (m, 10H), 6.96–6.97 (br d, 1H, *J*=7.3 Hz), 6.44–6.46 (br d, 1H, *J*=7.7 Hz), 5.11 (s, 2H), 5.02 (s, 2H), 4.10–4.18 (m, 2H), 4.05–4.08 (d, 2H, *J*=5.4 Hz), 3.99–4.02 (d, 6H, *J*=5.4 Hz), 3.61 (s, 3H), 2,77–2.84 (ddd, 2H, J_1=14.5 Hz, J_2=5.4 Hz, J_3=2.8 Hz), 2.68–2.75 (ddd, 2H, J_1=14.5 Hz, J_2=8.2 Hz, J_3=3.8 Hz), 1.35 (s, 9H). ^{13}C NMR (110°C, DMSO-d_6): δ 171.25, 170.96, 154.92, 154.29, 136.39, 135.35, 127.57, 127.51, 127.19, 127.05, 126.94, 126.76, 83.43, 83.32, 77.86, 68.97, 68.36, 68.28, 67.48, 67.43, 67.40, 65.28, 65.00, 55.18, 55.04, 50.87, 30.88, 30.77, 27.52. HRMS (FAB^+) *m*/*z* calcd for $C_{37}H_{42}FeN_2O_8$: 698.2291. Found: 698.2299.

4.1.3. (+)-(*S*,*S*)-1-[2-Amino-2-(methoxycarbonyl)ethyl]-1′-[(2-*tert*-butoxycarbonyl)-amino-2-carboxylethyl]ferrocene (4). To a solution of **3** (1.16 g, 1.66 mmol) in MeOH (30 ml) 5% Pd on carbon (582 mg) was added in small portions. The mixture was stirred under 1 atm (14.7 psi) of H_2 for 24 h at rt. The catalyst was removed by filtration through Celite and the filtrate was then concentrated. The residue was purified by triturating a solution of crude product in methanol with ether, which gave compound **4** (644 mg, 82%) as a yellow powder: mp 99–102°C; $[\alpha]^{22}_D$=+37 (*c* 1.43, $CHCl_3$).^{1}H NMR (DMSO-d_6): δ 6.69–6.71 (d, 1H, *J*=8 Hz), 4.75–5.43 (br s, 3H), 3.97–4.10 (m, 8H), 3.85–3.86 (m, 1H), 3.59 (s, 3H), 3.48–3.49 (m, 1H), 2.56–2.78 (m, 4H), 1.35 (s, 9H). ^{13}C NMR: (DMSO-d_6) δ 174.50, 173.70, 155.19, 84.83, 83.18, 77.80, 70.04, 69.59, 69.45, 68.95, 68.14, 68.02, 67.86, 67.80, 55.28, 51.54, 28.23. HRMS (FAB^+) *m*/*z* calcd for $C_{22}H_{30}FeN_2O_6$: 474.1453. Found: 474.1460.

4.1.4. (2*S*,5*S*)-2-[Methoxycarbonyl]-3-aza-4-oxo-5-[(*tert*-butoxycarbonyl)amino]-[6][1,1′] ferrocenophane (5). A solution of **4** (0.049 g, 0.103 mmol) in DMF (10 ml) was added by a syringe pump to a stirred solution of PyAOP (119 mg, 0.228 mmol) and DIEA (52 μl, 0.304 mmol) in DMF (190 ml) over a period of 2 h at rt under Ar(g). The reaction mixture was concentrated at reduced pressure and the residue was dissolved in $CHCl_3$/H_2O 1:1. The resulting organic phase was dried (Na_2SO_4), filtered and concentrated. Purification by flash chromatography (SiO_2, $CHCl_3$/MeOH/AcOH 120:2:1) gave **5** (0.035 g, 75%, R_f=0.25) as a yellow viscous mass: $[\alpha]^{22}_D$=+82 (*c* 0.055, $CHCl_3$). ^{1}H NMR (60°C, DMSO-d_6): δ 8.44–8.46 (d, 1H, *J*=7.6 Hz), 6.43 (br s, 1H), 4.60 (br m, 1H), 4.27 (br s, 1H), 4.02–4.20 (m, 8H), 3.67 (s, 3H), 2.93–3.03 (m, 2H), 2.64–2.71 (m, 2H), 1.41(s, 9H).^{13}C NMR (27°C, DMSO-d_6): δ 171.31, 169.61, 154.48, 85.19, 83.39, 78.39, 69,10, 68.05, 66.88, 66.32, 66.26, 66.21, 66.11, 65.70, 51.56, 49.79, 27.80. HRMS (FAB^+) *m*/*z* calcd for $C_{22}H_{28}FeN_2O_5$: 456.1348. Found: 456.1342.

4.1.5. (2*S*,5*S*)2-[Carboxyl]-3-aza-4-oxo-5-[(*tert*-butoxycarbonyl)amino]-[6][1,1′] ferrocenophane (6). At 0°C a solution of LiOH (28.8 mg, 0.686 mmol) in water (1 ml) was added dropwise to a stirred solution of lactam **5** (131 mg, 0.287 mmol) in THF (1 ml) and the stirring was continued at 0°C for 1 h. The solvent was evaporated to give a residue, which was dissolved in water (10 ml). The aqueous solution was washed with ether (10 ml), acidified with 0.5 M $KHSO_4$ and extracted twice with 10 ml portions of EtOAc. The collected organic extract was dried (Na_2SO_4), filtered and concentrated. The residue was purified by dissolution in THF followed by precipitation by the addition of heptane. Compound **6** (0.070 g, 55%) was obtained as a light yellow powder: mp 141–144°C; $[\alpha]^{22}_D$=+79 (*c* 0.635, $CHCl_3$). ^{1}H NMR (DMSO-d_6): δ 8.38 (br s, 1H), 6.84 (br s, 1H), 4.43 (br s, 1H), 4.25 (br s, 1H), 3.98–4.18 (dd, 8H), 3.00–3.75 (br s, 1H), 2.81–2.86 (m, 2H), 2.61–2.73 (m, 2H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6): δ 171.91, 170.33, 155.62, 186.73, 84.87, 79.46, 69.81, 69.19, 68.28, 67.77, 67.42, 67.14, 66.80, 54.48, 51.39, 68.99. HMRS (FAB^+) *m*/*z* calcd for $C_{21}H_{26}FeN_2O_5$: 442.1191. Found: 442.1186.

4.1.6. Preparation of Cbz-Arg(HCl)-Pro-Lys(Cbz)-Pro-Gln-Gln-Falc-Gly-Leu-Met-MBHA-resin (7). The protected peptide was synthesized by the standard solid-phase procedure and the MBHA-Merrifield resin was used as a solid support. Boc-Met-MBHA-resin (0.057 g, 57 μmol) was placed in a reaction vessel made of Teflon and the peptide coupling was preformed according to Table 1. When the synthesis was completed the protected peptide

resin **7** (105 mg) was obtained after drying at reduced pressure at 40°C for 24 h.

4.1.7. Acidic cleavage and purification (MBHA resin): H-(H-Arg)$^+$-Pro-Lys-Pro-Gln-Gln-Falc-Gly-Leu-Met-NH_2 (8). To cleave off the peptide, the resin (20 mg) was mixed with 1,2-ethanedithiol (20 μl) and thioanisole (40 μl) and was then suspended in TFA (0.40 ml) under N_2. After stirring the mixture for 5 min at 0°C TFMSA (40 μl) was added dropwise. A green colour developed gradually. After stirring for another 1 h at rt, the dark green mixture[18] was filtered to remove the resin, which was washed with a small amount of TFA. Ether (10 ml) was then added to the combined filtrates to precipitate the crude peptide, which was washed with ether (3×10 ml) by centrifugation, and then dissolved in water (1 ml). The dark green solution was passed through a Dowex 1X8 (OH^- form, 1×10 cm) anion exchange column to remove trifluoroacetate and triflate ions. A yellow solution of the crude peptide was obtained after elution with water (10–15 ml). The volume of the eluate was reduced to 0.5 ml at reduced pressure and a few drops of acetic acid were added to ensure complete dissolution of the crude peptide. Purification by HPLC using a 10×250 mm KR100-5-C18 column with gradient elution (linear gradient from 85:15 to 60:40 water/MeCN+0.1% TFA during 40 min) gave the pure peptide (3.7 mg, 13%, R_t=6.98 min) as a dark green powder after lyophilization. HMRS (FAB^+) *m/z* calcd for $C_{61}H_{96}FeN_{18}O_{13}S$ (M+H): 1377.6555. Found: 1377.6572.

4.1.8. Preparation of Cbz-Arg(HCl)-Pro-Lys(Cbz)-Pro-Gln-Gln-Falc-Gly-Leu-Met-hydroxymethylresin (9). The protected peptide was synthesized by the standard solid-phase procedure, using hydroxylmetylresin as a solid support. Boc-Met-Resin (70.6 mg, 0.057 mmol) was placed in a reaction vessel made of Teflon and the peptide coupling was preformed according to Table 1. When the synthesis was completed the resin was washed with *iso*-propanol and methanol and dried overnight in vacuo at +40°C. The resulting orange resin **9** weighed 85.5 mg.

4.2. Basic methanolytic cleavage of the hydroxymethyl resin

4.2.1. Preparation of protected peptide methyl ester: Cbz-Arg(HCl)-Pro-Lys(Cbz)-Pro-Gln-Gln-Falc-Gly-Leu-Met-OMe (10). The peptide resin **9** (35.5 mg) was suspended in a mixture of DMF (0.36 ml) and methanol (0.36 ml). Then freshly distilled triethylamine (36 μl) was added and the suspension was gently stirred at rt overnight. After filtration, the filtrate was concentrated under reduced pressure and the methyl ester **10** was obtained as an orange viscous mass (17 mg, 44%), which was not further purified.

4.2.2. Preparation of protected peptide amide: Cbz-Arg(HCl)-Pro-Lys(Cbz)-Pro-Gln-Gln-Falc-Gly-Leu-Met-NH_2 (11). The protected ester **10** (17 mg, 0.10 μmol) was dissolved in methanol (2.0 ml) and gaseous ammonia was bubbled through the stirred solution for 3 h at rt. The stirring was continued for 2 days. Evaporation of the solvent gave the protected peptide amide **11** (14 mg, 83%) as an orange viscous mass, which was used directly in the next step.

4.2.3. Preparation and purification of H-(H-Arg)$^+$-Pro-Lys-Pro-Gln-Gln-Falc-Gly-Leu-Met-NH_2 (8). The orange di-(Cbz)-protected peptide **11** was dissolved in a mixture of TFA (0.84 ml) and thioanisole (0.30 ml) at rt and the mixture was stirred overnight. By ether addition the light green compound **8** was precipitated and washed with ether (3×10 ml) by centrifugation. Crude peptide **8** was then dissolved in water and the dark green solution[18] was purified by HPLC using a 10×250 mm KR100-5-C18 column with gradient elution (linear gradient from 85:15 to 60:40 water/MeCN+0.1% TFA during 40 min), which gave the pure peptide (5.1 mg, 44%, R_t=6.98 min) as a dark green powder after lyophilization. HMRS (FAB^+) *m/z* calcd for $C_{61}H_{96}FeN_{18}O_{13}S$ (M+H): 1377.6555. Found: 1377.6572.

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18. The dark green colour may be caused by protonation directly on iron of ferrocene ('endo-protonation') to form the ferrocenonium ion; see: (a) Karlsson, A.; Broo, A.; Ahlberg, P. *Can. J. Chem.* **1999**, *77*, 628–633. (b) Mueller-Westerhoff, U. T.; Haas, T. J.; Swiegers, G. F.; Leipert, T. K. *J. Organomet. Chem.* **1994**, 229–246. (c) Floris, B.; Illuminati, G.; Ortaggi, G. *Tetrahedron Lett.* **1981**, *22* (28), 2627–2628. (d) Ceruchelli, G.; Illuminati, G.; Ortaggi, G. J. *J. Organomet. Chem.* **1977**, *127*, 357–-370. (e) Olah, G. A.; Mo, Y. K. *J. Organomet. Chem.* **1973**, *60*, 311–321.