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Synthesis and conformational studies of a Leu-enkephalin amide analogue containing a ferrocene substructure

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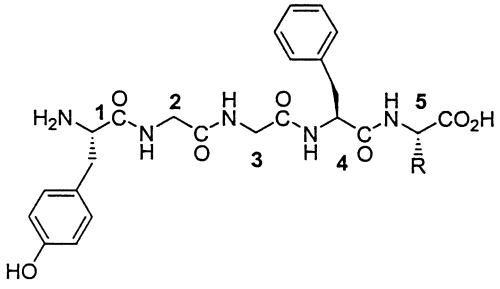
Abstract—Solution phase synthesis of a constrained Leu(5)-enkephalin amide analogue **10** is reported, in which the cyclic ferrocenyl containing subunit **7** was introduced as a mimetic of the tetrapeptide Tyr-Gly-Gly-Phe unit. Temperature dependence of the chemical shift of the amide protons of **10** indicated a hydrogen bond between the same amino acid residues as observed for the natural Leu-enkepalin in the single-bend conformation. The rotational barrier ($\Delta G_c^{\ddagger}$=16.8 kcal/mol) of the C-terminal amide group, which was determined by DNMR spectroscopy, and NOESY experiments indicated that the two termini were more distant as compared to the single-bend conformation of natural Leu-enkephalin amide.

1. Introduction

In 1975, Hughes and Kosterlitz discovered the opioid pentapeptides Met(5)-enkephalin and Leu(5)-enkephalin, which started extensive research on their biology and chemistry.[1] The ratio of the two enkephalins depended on the species in which they were found.[1,2] These neurotransmitters regulate sensory functions including pain and control of respiration in the central nervous system by binding to the G-protein coupled μ- and δ-opioid receptors, respectively.[3,4] The non-specific receptor affinity of the enkephalins has been associated with their large conformational freedom. Therefore, it has been a great challenge to determine the bioactive conformation of the enkephalins. Since the structural determination of the membrane bound opioid receptors is extremely difficult and short linear peptide agonists are very flexible, conformationally restricted mimetics of the agonists are synthesised and tested in order to gain some insight into the binding mode in the receptor. The conformational behaviour of flexible effector molecules has also been studied under various biomimetic conditions for the same purpose.[5] Leu(5)-enkephalin has been found to exist predominantly in three different conformations; extended,[6] single-bend[7,8] and double-bend[9] depending on which biomimetic environment was used (Fig. 2). According to an earlier report,[10] the receptor selectivity of the enkephalins was dependent on the orientation of the two aromatic residues; the μ-receptor required the two aromatic residues to be on the opposite side of the peptide backbone, while the δ-receptor required them to be on the same side. The distances between the centroids of the two aromatic residues were determined to be 11 and 9 Å, respectively.



R=$CH_2CH(CH_3)_2$ [Leu(5)], $CH_2CH_2SCH_3$ [Met(5)]

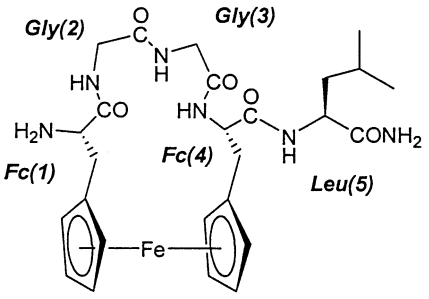


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Figure 1. (a) The two enkephalins, Leu(5)- and Met(5)-enkephalin; (b) The constrained Leu(5)-enkephalin mimetic **10**, in which the Tyr-Gly-Gly-Phe subunit has been replaced by a Gly-Gly-looped 1,1′-ferrocenyl-bis-alanine residue.

Keywords: ferrocene; cyclic peptide; conformational analysis; β-turn mimetic.

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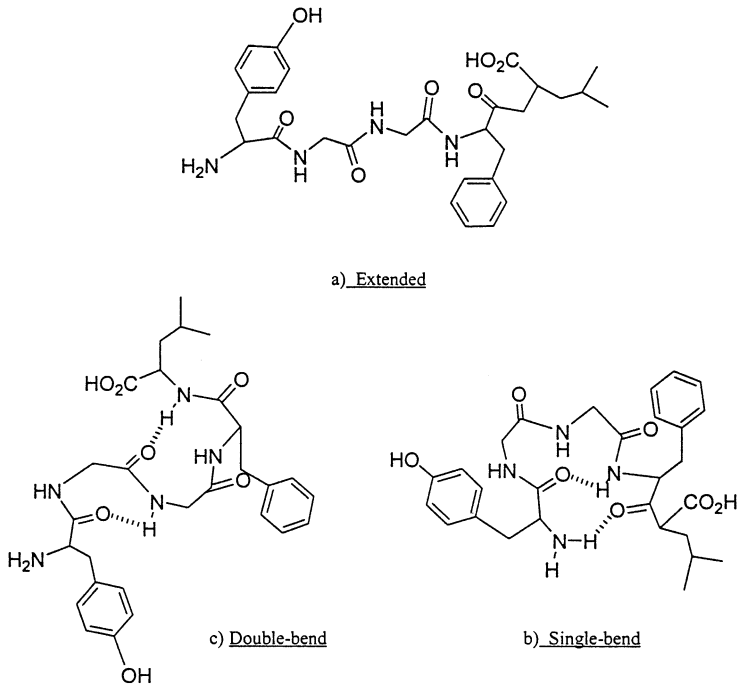


Figure 2. Schematic drawing of the three enkephalin conformations. (a) The extended conformation is usually found in the crystalline state as a two-dimensional infinite antiparallel β-pleated sheet; (b) the single-bend conformation is stabilised by a β-turn, found in the peptide region Fc(4)→Fc(1) or Leu(5)→Gly(2), with two antiparallel hydrogen bonds; c) the double-bend conformation is adopted in biomimetic media where a γ-turn is centred on Gly(2) and a β-turn is centred on Gly(3)-Phe(4).

In this paper, we report the synthesis and conformational studies of a highly constrained cyclic Leu(5)-enkephalin analogue **10**, in which the constraint was introduced by replacing the two aromatic residues with a ferrocenyl moiety (Fig. 1).

2. Results and discussion

The synthetic strategy was chosen as shown in Scheme 1, by taking into account the previous cyclisation studies of peptide analogues made in our group.[19] From these studies, it was obvious that the best cyclisation results were obtained by having the nucleophilic amino moiety positioned close to the aromatic core and the carboxylic acid group on the coupled amino acid part. The reverse positioning of the amino and carboxyl groups was expected to give lower yields also in the present case.

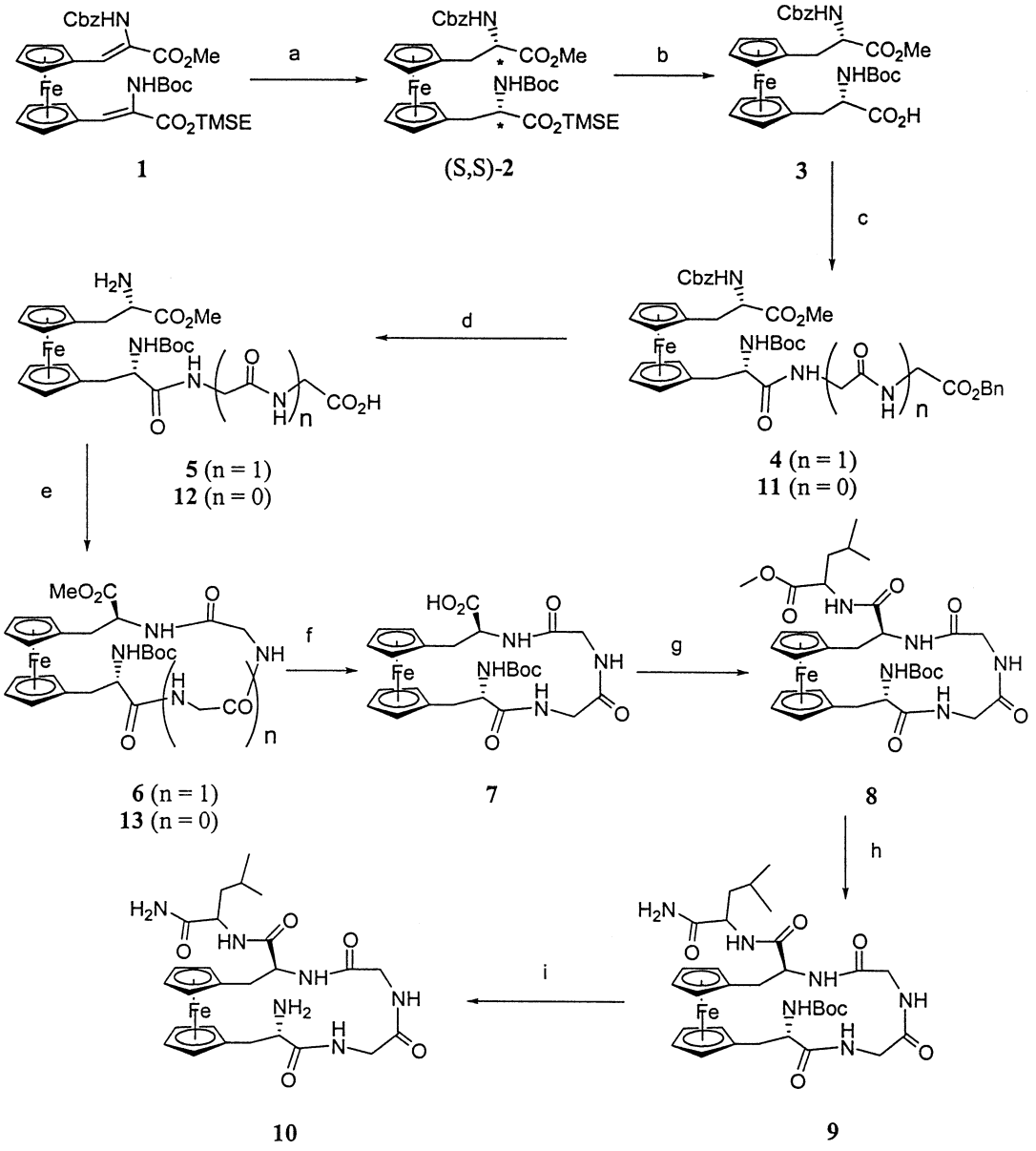
Leu(5)-enkephalin amide **10** was synthesised in nine steps from didehydro ferrocenyl-bis-alanine **1**[11] (Scheme 1). Four different protecting groups (Cbz, Me, Boc, TMSE) were chosen to allow selective removal.[12–17]

Asymmetric hydrogenation of **1** at 60 psi and using [RhCOD((*S*,*S*)-Et-DuPHOS)]$^+$OTf$^-$ as a catalyst gave (*S*,*S*)-**2**. The configuration was assumed to be (*S*,*S*) based on the reported selectivity of the catalyst.[12] Racemic **2**, obtained by using the Rh(I)-dppe catalyst, was resolved into four peaks by HPLC using a (*R*,*R*)-Whelk-O1 chiral column although not with baseline separation. Single HPLC peaks were observed for (*S*,*S*)-**2** and (*R*,*R*)-**2**, obtained using [RhCOD((*R*,*R*)-Et-DuPHOS)]$^+$OTf$^-$ and [RhCOD((*S*,*S*)-Et-DuPHOS)]$^+$OTf$^-$ as catalyst, respectively. The co-injection of (*S*,*S*)-**2** or (*R*,*R*)-**2** with racemic **2** gave an increase in one of the four HPLC peaks. From this we could conclude that compound **2** had ee >99%. By this result, we have improved the enantioselectivity in the asymmetric hydrogenation compared with our earlier results when [RhCOD((*R*,*R*)-DIMPAMP)]$^+$OTf$^-$ was used as a catalyst.[17a]

The C-terminal of **2**, having TMSE as protecting group, was liberated by TBAF in THF to give amino acid **3**. A higher yield was obtained when the solvent was changed from DMF to THF, probably because the decomposition of compounds **2** and **3** observed during the reaction was faster in DMF than in THF. The Gly-Gly sequence was introduced by peptide coupling between carboxylic acid **3** and **18** using EDC/HOBt as coupling reagent in DMF. Pure product **4** was obtained in 85% yield. Next, the Cbz and Bn protecting groups were both removed by hydrogenolysis, which gave deprotected amino acid **5** pure enough for use in the following step. Due to the neutral conditions during the synthetic steps up to this point it seems unlikely that epimerisation had occurred.

The next step was lactam formation of **5**. This synthetic step was performed in 1.37 mM DMF solution of **5** using PyAOP as a cyclisation reagent in presence of DIEA. According to Albericio et al.[18] and our previous experience,[17b] PyAOP was preferred as a cyclisation reagent. Chromatography gave the cyclic Tyr-Gly-Gly-Phe peptide mimetic **6** in 80% yield.

Also the Phe-Gly-Phe mimetic sequence **13** could be synthesised in the same manner as **6** (Table 1 and steps c–e in Scheme 1). *O*-Benzyl-protected glycine (**16**) was



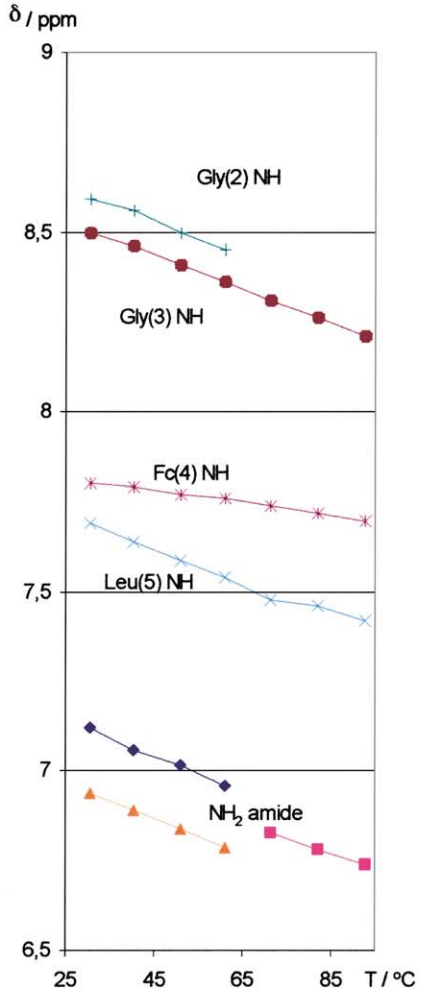
Scheme 1. *Reagents and conditions*: a)[Rh(COD)(DuPHOS)]$^{+}$OTf^{-} **17**, MeOH, H_2 (g, 60 psi), 24 h, rt (94%); (b) TBAF·3H_2O, THF, 0°C→ rt, 3 h (85%); (c) TsOH·NH_2-(Gly)$_n$-CO_2Bn (*n*=1 **16**, *n*=2 **18**), HOBt, DIEA, EDC, DMF, 0°C→rt, (reaction times and yields as shown in entries 1–3, Table 1); (d) 5% Pd/C, MeOH, H_2 (1 atm), 24 h, rt (**4→5**, 90% and **11→12** 87%); (e) reagent (2 mM in DMF), DIEA, DMF, 24 h, rt (reagents and yields as shown in entries 4–6, Table 1); (f) 0.1 M KOH (aq), dioxane, 20 min, rt (63%); (g) HCl·NH_2-Leu-CO_2Me, HOBt, DIEA, EDC, DMF, 0°C→rt, 24 h (65%); (h) NH_3 (g), MeOH, 24 h, rt (71%); (i) TFA (20% in CH_2Cl_2), CH_2Cl_2, 4 h (49%). List of abbreviations: COD, 1,5-cyclooctadiene; (*S*,*S*)-Et-DuPHOS, (+)-1,2-bis-((2*S*,5*S*)-2,5-diethylphospholano)benzene; TBAF, tetra-butylammonium fluoride; HOBt, 1-hydroxybenzotriazole; DIEA, *N*-diisopropylethylamine; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DMF, *N*,*N*-dimethylformamide; PyAOP, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium-hexafluorophosphate; TFA, trifluoroacetic acid.

coupled to **3** using EDC as a coupling reagent. The yield of **11** was improved by increasing the reaction time from 2–3 to 24 h (entries 1 and 2, Table 1). The peptide-coupling step was followed by deprotection, where Cbz and Bn were simultaneously removed by hydrogenolysis. Finally, the cyclisation was preformed using PyAOP. Thus, **13** was obtained in 75% yield, which was better than that obtained when HATU was used (entries 4 and 5, Table 1). In both cases, the monomeric-cyclised product **13** was confirmed by MS. In no case could more than a few percent of cyclodimerised material be detected. As shown by the yields in entries 5 and 6, the length of the amino acid chain was not a restricting factor for peptide cyclisation as was observed in earlier work, where the amino acid analogues were based on a benzene moiety instead of ferrocene.[19]

Table 1. The yields and conditions for steps c–e shown in Scheme 1

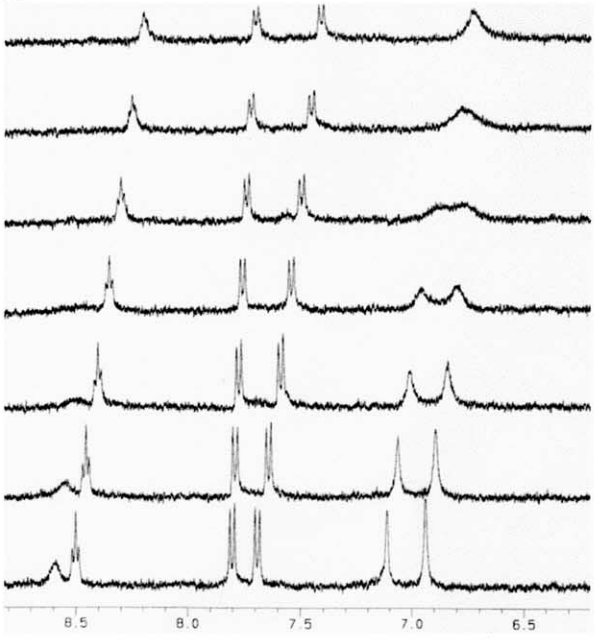
Entry	Precursor	Reagent	Reaction time (h)	Product	Yield (%)
1	**3**	EDC	2–3	**11**	65
2	**3**	EDC	24	**11**	94
3	**3**	EDC	3	**4**	85
4	**12**	HATU	24	**13**	40
5	**12**	PyAOP	24	**13**	75
6	**5**	PyAOP	24	**6**	80

A



NH for	δ[ppm]at 30.6 °C	Δδ/ΔT *10 ^{3}[ppm/(°C)]
Gly(2)	8.6	-4.6
Gly(3)	8.5	-4.5
Fc(4)	7.8	-1.6
Leu(5)	7.7	-4,4
NH_2 amide	7.1, 6.9	-4.8

B



T	k
92.9 °C	420 s^{-1}
82.4 °C	270 s^{-1}
71.7 °C	130 s^{-1}
61.4 °C	55 s^{-1}
50.9 °C	15 s^{-1}
40.2 °C	
30.6 °C	

Figure 3. (a) Shift-temperature coefficients ($\Delta\delta/\Delta T$) for the amide protons of **10** (7 mM in DMSO-d_6); (b) ^{1}H NMR spectra for amide protons of **10** at different temperatures in DMSO-d_6 and the corresponding rate constants.

The C-terminal of **6** was deprotected by 0.1 M KOH in dioxane and after careful acidification with a 5% aqueous solution of $KHSO_4$, carboxylic acid **7** was obtained in 63% yield. A much poorer yield was obtained when the acidification was made with 1 M HCl. It should be emphasised that the ferrocene lactam **6** was very sensitive to both strong acid and base. When the acidification was preformed with stronger acids than those mentioned above, a colour change from yellow to green-blue was observed. According to previous studies made by Rozenkova et al.,[22] a possible cause could be a direct air oxidation of the ferrocene system to ferrocenium, which is blue or green in dilute solutions. Colour change studies of ferrocene-bis-alanine derivatives caused by acidification are under investigation in our laboratory and will be reported in due course.

The last amino acid residue, LeuOMe, was coupled to the C-terminal of **7** to give **8** using EDC as a coupling reagent in the presence of DIEA and HOBt in DMF at room temperature. A better yield was obtained when the reaction time was extended to 24 h instead of 2 h. The peptide-coupling reaction was followed by group transformation of methyl ester **8** to amide **9**, by treating a methanol solution of **8** with NH_3 (g) for 2 h. The ester to amide interconversion was made since, according to a previous report, the amide improved the potency at the μ-opioid receptor by 30% as compared to the ester.[5]

Finally, the Leu(5)-enkephalin amide analogue **10** was obtained after removal of the Boc group using 20% TFA in CH_2Cl_2. During the acidic deprotection, a colour change was again observed from yellow to green. A colour change back to yellow took place during chromatography with a basic eluent. Compound **10** was isolated as a yellow powder. Deprotection attempted with Amberlyst 15 in aqueous acetone caused irreversible binding of the peptide to the ion exchange resin or decomposition, since repeated washings with various acids did not allow isolation of **10**.

In order to gain some insight into the conformational behaviour of **10** in solution, we conducted NMR spectroscopic experiments in DMSO-d_6. A small temperature dependence for amide proton chemical shifts may be taken as an indication of participation in intramolecular hydrogen bonds.[20] This was indeed found for Fc(4)NH, which had a substantially lower temperature coefficient as compared to the

Table 2. Experimentally derived thermodynamic parameters for the chemical exchange observed for the amide protons ($CONH_2$) of **10** and native Leu(5)-enkephalin amide in DMSO-d_6 (7 mM)

Substance (solvent)	T_c (K)	$\Delta G_c^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol K)	$\Delta S^{\ddagger}$ (cal/mol K)
10 (DMSO)	345	16.8	18.0	2.9
Leu(5)-enkephalin amide (DMSO)	346	16.9		
Formamide[a] (MPK)	298	17.8	18.5	2.7

Abbreviations: DMSO, dimethylsulphoxide; MPK, methylpropylketone.
[a] Data taken from Ref. 21a.

other amide protons (Fig. 3). No significant change in chemical shifts of the α-protons was found in this temperature interval. Chemical exchange of the NH_2 amide protons (^{1}H NMR signals at ~7.0 ppm) at the C-terminal of both **10** and Leu(5)-enkephalin was observed on heating (Fig. 3b).

The thermodynamic parameters for the hindered rotation around the C–N bond of the terminal amide group was experimentally determined as shown in Table 2, which agreed well with those usually found for amides (17–20 kcal/mol).[21b] Obviously, the Fc-modification of Leu(5)-enkephalin did not have any influence on the environment around the $CONH_2$ region.

Structural information has also been obtained from COSY and NOESY experiments concerning the assignment of the NMR signals and some relative distances, from which a possible conformation of **10** could be schematically drawn as shown in Fig. 4 (left).

In order to shed some light on the low energy conformation of the β-turn mimetic subunit **7**, a model compound **7′** was used in order to reduce computational time (Fig. 5). The steric energy of compound **7′** was minimised by using Macromodel V6.5, Monte Carlo with random conformation search and the Amber* force field (in vacuum).[23] Of 100 Monte Carlo cycles, 37 unique conformations were found, amongst which two conformations were within 0.95 kcal/mol. The next highest energy conformation was 2.37 kcal/mol above the global minimum. The lowest energy conformation **14**, found once, was 0.95 kcal/mol lower in energy than the next conformation **15**. Due to the small energy difference between **14** and **15**, it is likely that compound **7′** (as **7**) populates both conformations at room temperature. The three amide bonds that are part of the peptide loop, have the same *trans*, *trans*, *trans*, geometries for both **14** and **15**. As seen in Fig. 5, the main difference between these conformers is the orientation of the peptide loop in relation to the ferrocene unit. According to the Monte Carlo search, it was determined that the loop conformation of **14** was stabilised by two hydrogen bonds between Fc(1)CO, Gly(3)NH and Fc(4)NH. However, only one hydrogen bond between Fc(1)CO and Gly(3)NH was stabilising the loop conformation of **15**. In order to satisfy the strongest NOE data between Fc(1)CHα and Fc(4)CHα, a manual construction of **7** followed by minimisation was carried out using the Amber* force field in vacuum and Macromodel V6.5 without random conformation search. A conformation (7.88 kcal/mol above the global minimum) was found that could be amongst the possible conformations in solution (Fig. 4, right). In summary, the Fc-modification induced a β-turn in the same region where the native Leu(5)-enkephalin has a β-turn structure in the single-bend conformation (Fig. 1). However, the NOE-based conformation obtained for subunit Fc(1)-…-Fc(4) did not match well with either of the minimised conformations **14** and **15**. We anticipate that the conformational difference could be due to solvent effects.

3. Conclusions

A constrained ferrocene analogue **10** of Leu(5)-enkephalin was synthesised and its structural features were investigated

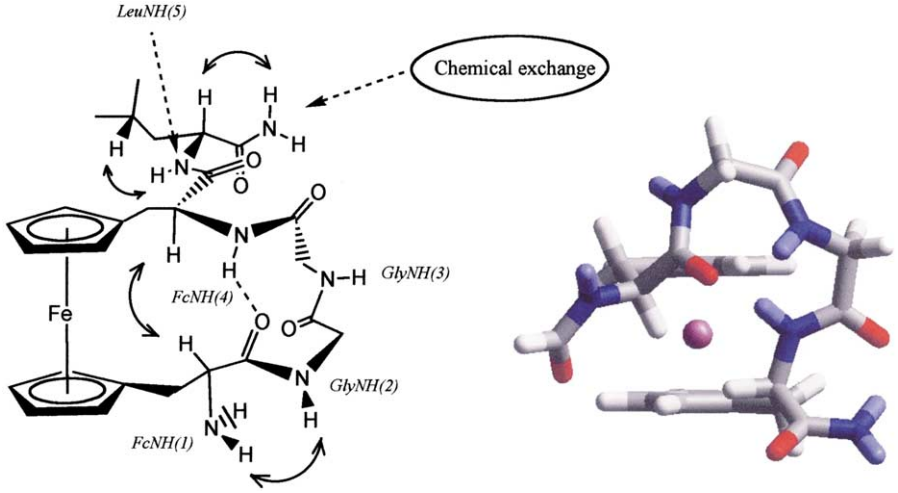


Figure 4. The conformation of 10 that best satisfies the strongest NOE-data found in DMSO solution in which the strongest NOEs are indicated by arrows (left), and a corresponding conformation obtained for model 7′ by using Macromodel V6.5 in vacuum and Amber* as a force field without conformation search (right).

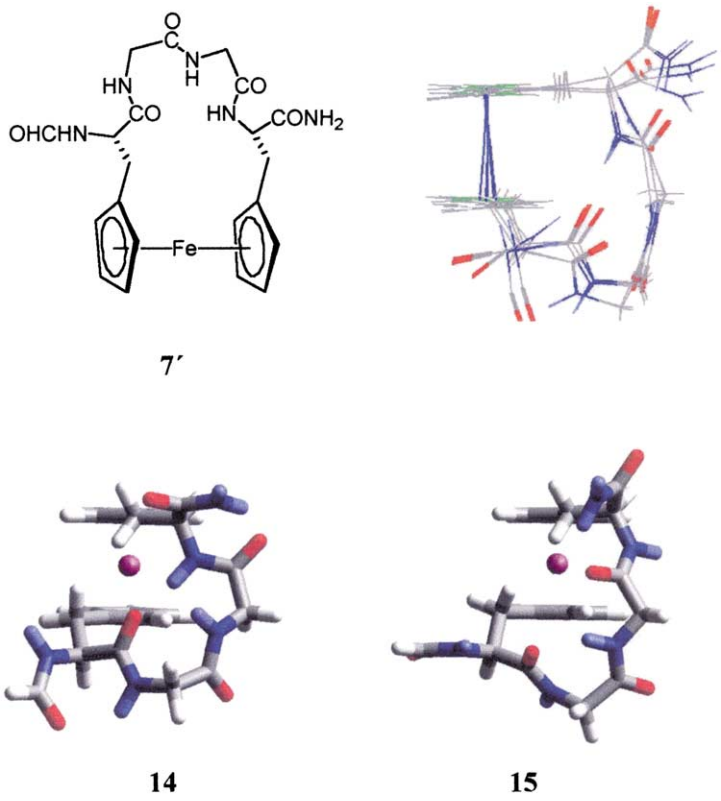


Figure 5. A model compound **7′** (top right). The conformers **14** and **15** were found within 0.95 kcal/mol and a superimposition of the six conformations found within 2.86 kcal/mol of the global minimum are shown top left.

by NMR spectroscopy and computational methods. Temperature-dependent NMR data indicated an intramolecular hydrogen bond between Fc(1)CO and Fc(4)NH thus creating a β-turn. The rotational barrier, $\Delta G_c^{\ddagger}$ of the C-terminal amide bond of **10** was determined to be 16.8 kcal/mol, which is essentially the same as for Leu(5)-enkephalin. A solution structure of **10** is suggested based on the intramolecular hydrogen bond in combination with NOESY and COSY data (Fig. 4). This structure was not reproduced by molecular mechanics computations (Macromodel V6.5, Monte Carlo with random conformation search and Amber* force field in vacuum) on a simplified model, however, presumably because of the solvent effects.

4. Experimental

4.1. General

TLC analyses were performed on Merck Silica Gel 60 coated glass plates. For column chromatography, Matrex™ (35–70 μm) silica gel was used. Melting points are uncorrected. IR spectra were recorded on a Shimadzu 8300 FTIR Instrument. ^{1}H NMR, ^{13}C NMR, COSY and NOESY spectra were recorded on a Bruker DRX 400 spectrometer. The following starting materials were prepared according to literature procedures: **1**,[11] **17**,[12] **18** (from Gly-Gly),[13] **16** (from Gly).[13] (*S*,*S*)-Et-Duphos, PyAOP and the remaining chemicals were purchased from Strem Chemicals, Advanced ChemiTech and Aldrich, respectively. Deuterated solvents, DMSO-d_6 (99.8 at.% D) and $CDCl_3$ (99.8 at.% D), were used as received. Temperature calibration of the NMR instrument was made by the use of a copper-constantan thermocouple. Adaptation of the NMR spectra were made by visual matching of calculated spectra according to literature procedure.[21c] The derived rate constants were plotted against temperature according to the Eyring equation ($\ln(k/T)$; $1/T$),[21d] to afford the rate constants and the relevant thermodynamic parameters.

4.1.1. (+)-(*S*,*S*)-1-[2[(Benzyloxycarbonyl)amino]-2-(methoxycarbonyl)ethyl]-1′-[2-(*tert*-butoxycarbonyl)amino]-2-[[(2-trimethylsilyl)ethoxycarbonyl]ethyl]ferrocene (2). A solution of **1**[11] (1.33 g, 1.85 mmol) in methanol (140 ml) was purged for 30 min with N_2 (g). [Rh(COD)(*S*,*S*)-Et-Duphos)]$^+$OTf$^-$ (0.053 g, 0.074 mmol) was added and the reaction mixture was hydrogenated at 4 atm (60 psi) for 24 h at room temperature, thereafter MeOH was removed at reduced pressure. The residue was dissolved in EtOAc and passed through a layer of silica (heptane/EtOAc 1:1) to give **2** (1.26 g, 94%) as a yellow syrup. The enantiomeric purity was determined by HPLC analysis on an (*R*,*R*)-Whelk-O1 column (flow rate, 1.0 ml/min; eluent, hexane/*i*PrOH, 19:1+0.5% of HOAc) at 40°C, which revealed only one peak at R_t=49 min. The ee was found to be >99% and the dr was >99.5:0.5. $[\alpha]_D^{21}$=+28 (*c* 0.8, $CHCl_3$) (lit.:[11] $[\alpha]_D^{22}$=+21 (*c* 2.0, $CHCl_3$)); NMR data were identical to those previously described.[11]

4.1.2. (+)-(*S*,*S*)-1-[2-[(Benzyloxycarbonyl)amino]-2-(methoxycarbonylethyl]-1′-[[(2-*tert*-butoxycarbonyl)-amino]-(2-carboxy)ethyl]ferrocene (3). A solution of TBAF·3H_2O (317 mg, 1.01 mmol) in THF (1 ml) was

added to a mixture of **2** (456 mg, 0.629 mmol) in THF (18 ml) at 0°C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 4 h and then the solvent was removed at reduced pressure. The residue was dissolved in EtOAc (11 ml), washed with 0.5 M HCl (1×6 ml), dried (Na_2SO_4) and the solvent was removed at reduced pressure. The crude product **3** (374 mg, 96%) was obtained as a yellow oil, which was used without further purification in the next step: $[\alpha]_D^{21}=+33$ (*c* 0.950, $CHCl_3$); IR (neat) $\tilde{\nu}_{max}$ 3334.7, 1718.5 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.32 (s, 9H), 2.58–2.75 (m, 4H), 3.60 (s, 3H), 3.8–3.9 (m, 1H), 3.97–4.09 (m, 9H), 4.98 (s, 3H), 6.90–6.92 (d, *J*=8.2 Hz, 1H), 7.28–7.33 (m, 5H), 7.66–7.68 (d, *J*=8.04 Hz, 1H), 12.5 (bs, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 39.7, 52.8, 56.3, 56.8, 66.3, 68.8, 68.8, 68.9, 69.5, 69.6, 71.0, 78.9, 84.8, 85.5, 128.5, 128.6, 129.2, 137.8, 156.2, 156.8, 173.3, 174.5; HRMS (FAB^+) *m/z* calcd for $C_{30}H_{36}FeN_2O_8$ 608.1821, found: 608.1830. Anal. calcd for $C_{30}H_{36}FeN_2O_8$: C, 59.22; H, 5.96; Fe, 9.18; N, 4.60; O, 21.04. Found: C, 59.38; H, 6.08; Fe, 9.08; N, 4.58.

4.1.3. (+)-(*S*,*S*)-1-[2-[(Benzyloxycarbonyl)amino]-2-(methoxycarbonyl)ethyl]-1′-[[(2-*tert*-butoxycarbonyl)-amino]-2-(2-benzyloxycarbonylmethyl)amino acetyl-amino carbonylethyl]ferrocene (4). Crude acid **3** (620 mg, 1.02 mmol) was dissolved in DMF (18 ml) together with HOBt (363 mg, 2.68 mmol), triethylamine (498 mg, 3.59 mmol) and glycylglycine benzyl ester hydrotosylate (1.23 g, 3.12 mmol) at room temperature and under a nitrogen atmosphere. The resulting mixture was cooled to 0°C thereafter EDC (315 mg, 1.64 mmol) was added. The temperature of the reaction mixture was allowed to reach room temperature and was then stirred for 3 h. The mixture was worked up as follows: removal of the solvent at reduced pressure, dissolution of the residue in EtOAc (95 ml), washing of the EtOAc solution sequentially with water (6×10 ml), aqueous sat. $NaHCO_3$ (1×20 ml), water (1×10 ml) and brine (1×45 ml), followed by drying (Na_2SO_4). Removal of the solvent at reduced pressure gave 668 mg (85%) of crude product **4** as a yellow oil, which was used without further purification in the next step: $[\alpha]_D^{21}=+31$ (*c* 0.550, $CHCl_3$); IR (neat) $\tilde{\nu}_{max}$ 3332.8, 1681.8 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.32 (s, 9H), 2.65–2.77 (m, 4H), 3.62 (8s, 3H), 3.75–3.78 (m, 2H), 3.92–4.12 (m, 12H), 5.00 (s, 2H), 5.14 (s, 2H), 6.87–6.89 (d, *J*=8.04 Hz, 1H), 7.31–7.38 (m, 10H), 7.69–7.71 (d, *J*=8 Hz, 1H), 8.12 (t, *J*=5.71 Hz, 1H), 8.22 (t, *J*=5.68 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 41.5, 42.7, 52.7, 56.8, 57.0, 63.8, 66.3, 66.7, 68.7, 68.8, 68.9, 68.95, 69.5, 69.8, 70.9, 71.0, 79.0, 84.7, 85.5, 127.3, 127.5, 128.5, 128.6, 128.8, 128.9, 128.9, 129.2, 129.3, 136.7, 137.8, 156.2, 156.8, 170.2, 170.5, 172.8, 173.2; HRMS (FAB^+) *m/z* calcd for $C_{41}H_{48}FeN_4O_{10}$ 812.2720, found: 812.2747. Anal. calcd for $C_{41}H_{48}FeN_4O_{10}$: C, 60.59; H, 5.95; Fe, 6.87; N, 6.89; O, 16.69. Found: C, 60.65; H, 6.10; Fe, 6.80; N, 6.95.

4.1.4. (+)-(*S*,*S*)-1-[[2-Amino-2-(methoxycarbonyl)]-ethyl]-1′-[[(2-*tert*-butoxycarbonyl)amino]-[2-(carboxyl-methyl)amino acetylaminocarbonyl]ethyl]ferrocene (5). Pd/C (5%, 182 mg) was carefully added (Warning! fire hazard) to a solution of **4** (365 mg, 0.621 mmol) in methanol (30 ml). The mixture was stirred under 1 atm (14.7 psi) of H_2 (g) for 24 h at room temperature. The catalyst was then removed by filtration through Celite. The filtrate was then concentrated to give 237 mg (90%) crude **5** as a yellow syrup, which was used without further purification in the next step: $[\alpha]_D^{21}=+37$ (*c* 0.500, $CHCl_3$); IR (neat) $\tilde{\nu}_{max}$ 3294.2, 1651.0 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.31 (s, 9H), 2.50–2.56 (m, 1H), 2.82–2.85 (m, 3H), 3.60–4.16 (m, 17H), 5.8–6.2 (bs, 3H), 6.86–6.88 (d, *J*=8.2 Hz, 1H), 7.53 (bs, 1H), 8.35 (m, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 43.0, 43.2, 49.5, 52.7, 55.3, 57.3, 68.5, 68.57, 68.8, 68.9, 70.4, 71.1, 71.3, 78.9, 83.0, 85.6, 156.2, 169.2, 173.2; HRMS (FAB^+) *m/z* calcd for $C_{26}H_{36}FeN_4O_8$ 588.1883, found: 588.1876. Anal. calcd for $C_{26}H_{36}FeN_4O_8$: C, 53.07; H, 6.17; Fe, 9.49; N, 9.52; O, 21.75. Found: C, 53.18; H, 6.10; Fe, 9.59; N, 9.43.

4.1.5. (2*S*,11*S*)-2-[Methoxycarbonyl]-3,6,9-triaza-4,7,10-trioxo-11-[(*tert*-butoxycarbonyl)amino]-[12][1,1′]ferrocenophane (6). DIEA (142 μl, 0.830 mmol) and PyAOP (187 mg, 0.359 mmol) were added to a solution of amino acid **5** (222 mg, 0.377 mmol) in DMF (275 ml) at room temperature and under a nitrogen atmosphere. The resulting mixture was stirred at room temperature for 24 h and then the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (100 ml) and worked up as follows: washing of the organic phase sequentially with water (3×20 ml) and brine (1×40 ml), followed by drying (Na_2SO_4) and removal of the solvent under reduced pressure. The residue (204 mg) was column chromatographed (SiO_2, $CHCl_3$/MeOH 10:1) to give **6** (171 mg, 80%) as a yellow powder: mp 187.5–191.6°C; $[\alpha]_D^{21}+20$ (*c* 0.550, $CHCl_3$); IR (neat) $\tilde{\nu}_{max}$ 3301.9, 1741.6, 1668.3, 1218.9 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.37 (s, 9H), 2.15–2.20 (dd, J_1=15.4 Hz, J_2=5.16 Hz, 1H), 2.63–2.67 (dd, J_1=14.6 Hz, 1H), 3.01–3.07 (dd, J_1=14.0 Hz, J_2=10.2 Hz, 1H), 3.19–3.23 (dd, J_1=11.9 Hz, J_2=4.04 Hz, 1H), 3.53–3.57 (m, 2H), 3.61 (s, 3H), 3.71–3.95 (m, 4H), 3.98–4.20 (m, 8H), 4.34 (m, 1H), 7.20–7.22 (d, *J*=8.56 Hz, 1H), 7.79–7.81 (d, *J*=7.56 Hz, 1H), 8.57–8.58 (m, 1H), 8.71–8.72 (m, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 43.5, 44.9, 52.9, 55.1, 55.8, 67.5, 67.9, 68.4, 69, 70.1, 70.7, 71.8, 79.1, 85.3, 88.1, 156.1, 170.1, 171.3, 172.7, 174.2; HRMS (FAB^+) *m/z* calcd for $C_{26}H_{34}FeN_4O_7$ 570.1777, found: 570.1771. Anal. calcd for $C_{26}H_{34}FeN_4O_7$: C, 54.75; H, 6.01; Fe, 9.79; N, 9.82; O, 19.63. Found: C, 54.66; H, 6.07; Fe, 9.89; N, 9.68.

4.1.6. (2*S*,11*S*)-2-[Carbonyl]-3,6,9-triaza-4,7,10-trioxo-11-[(*tert*-butoxycarbonyl)amino]-[12][1,1′]ferrocenophane (7). A 0.1 M solution of KOH in water (109 μl) was added dropwise to a mixture of lactam **6** (0.039 g, 0.701 mmol) in dioxane (150 μl) at 0°C under a nitrogen atmosphere. The resulting mixture was stirred at 0°C for 20 min. The reaction mixture was diluted with EtOAc/water 1:1 (10 ml), followed by dropwise addition of a 5% solution of $KHSO_4$ in water until the water layer became acidic (pH 2–3) and the layers were then separated. The aqueous layer was extracted with EtOAc (3×10 ml) and the combined organic extracts were dried (Na_2SO_4). Concentration gave 24 mg (63%) of crude **7** as a yellow powder, which was used without purification in next step: mp 196.4–203.4°C; $[\alpha]_D^{21}=+22$ (*c* 0.450, $CHCl_3$); IR

(neat) $\tilde{\nu}_{max}$ 3300.0, 1710.7, 1651.0 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.39 (s, 9H), 2.47–2.48 (dd, J_1=16.3 Hz, J_2=5.38 Hz, 1H), 2.70–2.73 (d, J=14.8 Hz, 1H), 3.0–3.10 (t, J=10.8 Hz, 1H), 3.20–3.28 (dd, J_1=16.0 Hz, J_2=3.99 Hz, 1H), 3.56–3.61 (m, 2H), 3.70–4.00 (m, 1H), 3.82–3.86 (m, 2H), 3.94–4.19 (m, 8H), 4.34–4.40 (m, 1H), 7.19–7.22 (d, J=9 Hz, 1H), 7.70–7.76 (d, J=8.7 Hz, 1H), 8.56 (t, J=5.17 Hz, 1H), 8.66 (t, J=6.1 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 43.6, 44.9, 55.2, 55.8, 67.4, 67.5, 68.0, 68.4, 68.9, 70.1, 70.8, 71.8, 79.1, 85.7, 88.1, 156.04, 169.6, 171.1, 173.7, 174.1; HRMS (FAB^+) *m/z* calcd for $C_{25}H_{32}FeN_4O_7$ 556.1620, found: 556.1647. Anal. calcd for $C_{25}H_{32}FeN_4O_7$: C, 53.97; H, 5.80; Fe, 10.04; N, 10.07; O, 20.13. Found: C, 53.88; H, 5.69; Fe, 10.15; N, 10.01.

4.1.7. (2*S*,11*S*)-2-[Methoxy-(α-(2-methylprop-1-yl)acetyl)-aminocarbonyl]-3,6,9-triaza-4,7,10-trioxo-11-[(*tert*-butoxy-carbonyl)amino]-[12][1,1′]ferrocenophane (8). A solution of leucine methyl ester hydrochloride (164 mg, 0.903 mmol) and DIEA (138 μl, 0.807 mmol) in DMF (2.4 ml) were added to a mixture of crude acid **7** (171 mg, 0.307 mmol) and HOBt (99 mg, 0.731 mmol) in DMF (2.4 ml) at 0°C and under a nitrogen atmosphere. Then EDC (171 mg, 0.892 mmol) was added. The reaction mixture was allowed to warm to room temperature and was stirred for 24 h. Work-up was performed as follows: removal of the solvent under reduced pressure, dissolution of the residue in EtOAc (20 ml), washing of the EtOAc solution sequentially with water (5×5 ml), aqueous sat. $NaHCO_3$ (1×5 ml), water (1×5 ml) and brine followed by drying (Na_2SO_4) and removal of the solvent at reduced pressure. The residue (137 mg, 65%) was column chromatographed (SiO_2, $CHCl_3$/MeOH 10:1) to give **8** (99 mg, 47%) as a yellow powder: mp 160.5–163.5°C; $[\alpha]_D^{21}$=+40 (*c* 0.450, $CHCl_3$); IR (neat) $\tilde{\nu}_{max}$ 3267.2, 1651.0, 1629.7, 1215.1 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 0.81–0.89 (dd, J_1=23.5 Hz, J_2=6.4 Hz, 6H), 1.40 (s, 9H), 1.49–1.59 (m, 3H), 2.15–2.28 (dd, J_1=15.7 Hz, J_2=5.33 Hz, 1H), 2.53–2.56 (d, J=13.1 Hz, 1H), 2.91–3.01 (dd, J_1=14.5 Hz, J_2=10.4 Hz, 1H), 3.15–3.21 (dd, J_1=17.0 Hz, J_2=5.14 Hz, 1H), 3.59–3.69 (m, 5H), 3.81–3.85 (m, 2H), 3.97–4.18 (m, 7H), 4.24–4.25 (m, 2H), 4.38 (m, 1H), 7.14–7.16 (d, J=8.6 Hz, 1H), 7.66–7.68 (d, J=8.2 Hz, 1H), 8.22–8.24 (d, J=7.4 Hz, 1H), 8.52–8.55 (t, J=5.5 Hz, 1H), 8.60–8.61 (t, J=5.6 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 22.2, 23.6, 25.0, 29.1, 43.7, 44.8, 51.1, 52.7, 55.2, 55.8, 67.2, 67.5, 67.8, 68.5, 68.9, 70.2, 70.7, 71.9, 79.1, 85.7, 87.8, 156.0, 169.7, 171.1, 172.3, 173.2, 173.9; HRMS (FAB^+) *m/z* calcd for $C_{32}H_{45}FeN_5O_8$ 683.2618, found: 683.2645. Anal. calcd for $C_{32}H_{45}FeN_5O_8$: C, 56.23; H, 6.64; Fe, 8.17; N, 10.25; O, 18.72. Found: C, 56.17; H, 6.71; Fe, 8.25; N, 10.18.

4.1.8. (2*S*,11*S*)-2-[Amino-(α-(2-methylprop-1-yl)acetyl)-aminocarbonyl]-3,6,9-triaza-4,7,10-trioxo-11-[(*tert*-butoxycarbonyl)amino]-[12][1,1′]ferrocenophane (9). A solution of methyl ester **8** (0.068 g, 0.102 mmol) in MeOH (26 ml) was purged with NH_3 (g) at room temperature for 2 h. Then the reaction mixture was stirred at room temperature for 24 h and worked up as follows: removal of the solvent under reduced pressure, dissolution of the residue with EtOAc (30 ml), washing of the EtOAc solution with water (4×10 ml) followed by drying (Na_2SO_4) and removal of the solvent under reduced pressure. The residue (0.056 g) was column chromatographed (SiO_2, $CHCl_3$/MeOH 10:1) to give **9** (0.047 g, 71%) as a yellow oil: $[\alpha]_D^{21}$=+16 (*c* 0.750, MeOH); IR (neat) $\tilde{\nu}_{max}$ 3294.2, 1651.0 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 0.78–0.85 (dd, J_1=20.6 Hz, J_2=6.44 Hz, 6H), 1.37 (s, 9H), 1.39–1.43 (m, 2H), 1.53 (m, 1H), 2.20–2.22 (dd, J_1=15.6 Hz, J_2=5.3 Hz, 1H), 2.54–2.57 (dd, J_1=13.6 Hz, J_2=1.3 Hz, 1H), 2.90–2.98 (dd, J_1=14.2 Hz, J_2=10.2 Hz, 1H), 3.15–3.23 (dd, J_1=15.0 Hz, J_2=3.5 Hz, 1H), 3.57–3.66 (m, 2H), 3.81 (m, 2H), 3.94–4.16 (m, 9H), 4.34 (m, 1H), 6.95 (s, 1H), 7.11–7.13 (m, 2H), 7.67–7.69 (d, J=8.3 Hz, 1H), 7.76–7.78 (d, J=8.2 Hz, 1H), 8.51 (t, J=5.6 Hz, 1H), 8.58 (t, J=6.8 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 22.4, 23.9, 25.0, 29.1, 43.8, 44.8, 51.8, 55.6, 55.8, 67.3, 67.8, 67.9, 68.5, 68.8, 70.3, 70.7, 71.2, 79.1, 85.8, 87.7, 156.0, 169.3, 171.1, 171.7, 173.6, 174.9; HRMS (FAB^+) *m/z* calcd for $C_{31}H_{44}FeN_6O_7$ 668.2621, found: 668.2615. Anal. calcd for $C_{31}H_{44}FeN_6O_7$: C, 55.69; H, 6.63; Fe, 8.35; N, 12.75; O, 16.75. Found: C, 55.74; H, 6.66; Fe, 8.28; N, 12.41.

4.1.9. (2*S*,11*S*)-2-[Amino-(α-(2-methylprop-1-yl)acetyl)-aminocarbonyl]-3,6,9-triaza-4,7,10-trioxo-11-[amino]-[12][1,1′]ferrocenophane (10). A 20% solution of TFA in CH_2Cl_2 (2.81 ml) was added to a suspension of **9** (41 mg, 61.4 mmol) in CH_2Cl_2 (1.41 ml) at 0°C and under a nitrogen atmosphere. The resulting mixture was stirred at 0°C for 4 h and then concentrated. The residue was column chromatographed (SiO_2, $CHCl_3$/MeOH/Et_3N 10:1:0.2) to give **10** (17 mg, 49%) as a yellow powder: mp 191.9–193.3°C; $[\alpha]_D^{21}$=+18 (*c* 0.100, MeOH); ^1H NMR (400 MHz, DMSO-d_6) δ 0.78–0.85 (dd, J_1=19.9 Hz, J_2=6.5 Hz, 6H), 1.40–1.43 (m, 2H). 1.50–1.60 (m, 1H), 1.90–2.10 (bs, 2H), 2.20–2.35 (dd, J_1=14.7 Hz, J_2=3.8 Hz, 1H), 2.53–2.56 (d, J=12.4 Hz, 1H), 2.85–3.10 (m, 2H), 3.63–4.17 (m, 15H), 6.93 (s, 1H), 7.13 (s, 1H), 7.71–7.73 (d, J=8.3 Hz, 1H), 7.81–7.83 (d, J=8.2 Hz, 1H), 8.51–8.54 (t, J=5.6 Hz, 1H), 8.70 (bs, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 22.4, 23.9, 25.0, 41.7, 43.9, 44.7, 51.8, 54.9, 56.3, 67.8, 67.9, 68.30, 68.5, 68.7, 70.0, 70.1, 10.9, 86.0, 87.1, 169.9, 170.5, 171.7, 174.9, 178.7; HRMS (FAB^+) *m/z* calcd for $C_{26}H_{36}FeN_6O_5$ 568.2097, found: 568.2109.

4.1.10. (+)-(*S*,*S*)-1-[2-[(Benzyloxycarbonyl)amino]-2-(methoxycarbonyl)ethyl]-1′-[[(2-*tert*-butoxycarbonyl)-amino]-2(2-benzyloxycarbonylmethyl)amino carbonyl-ethyl]ferrocene (11). Crude acid **3** (351 mg, 0.577 mmol) was dissolved in DMF (11 ml) along with HOBt (207 mg, 1.53 mmol), triethylamine (282 mg, 2.03 mmol) and glycine benzyl ester hydrotosylate (569 mg, 1.77 mmol) at room temperature and under a nitrogen atmosphere. The resulting mixture was cooled to 0°C thereafter EDC (176 mg, 0.928 mmol) was added. The reaction mixture was allowed to warm to room temperature and was then stirred for 24 h. Work-up was performed as follows: removal of the solvent under reduced pressure, dissolution of the residue in EtOAc (54 ml), washing of the EtOAc solution sequentially with water (6×5 ml), aqueous sat. $NaHCO_3$ (1×10 ml), water (1×5 ml) and brine (1×25 ml), followed by drying (Na_2SO_4). Removal of the solvent under reduced pressure gave 384 mg (94%) of crude **11** as a yellow oil, which was used without purification in next

step: $[\alpha]_D^{21}=+35$ (*c* 0.450, MeOH); IR (neat) $\tilde{\nu}_{max}$ 3334.7, 1732.0 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.33 (s, 9H), 2.62–2.80 (m, 4H), 3.62 (s, 3H), 3.88–4.11 (m, 13H), 5.00 (s, 2H), 5.14 (s, 2H), 6.79 (d, *J*=8.54 Hz, 1H), 7.30–7.39 (m, 10H), 7.68 (d, *J*=7.78 Hz, 1H), 8.28 (t, *J*=5.75 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 29.1, 41.7, 52.7, 56.8, 66.3, 66.65, 68.7, 68.8, 68.9, 68.95, 69.5, 69.7, 70.9, 71.0, 78.9, 84.7, 85.5, 128.5, 128.7, 128.8, 128.9, 129.2, 129.3, 136.8, 137.9, 156.0, 156.8, 170.6, 173.5, 173.2; HRMS (FAB^+) *m/z* calcd for $C_{39}H_{45}FeN_3O_9$ 755.2505, found: 755.2509.

4.1.11. (+)-(*S*,*S*)-1-[[2-Amino-2-(methoxycarbonyl)]-ethyl]-1′-[[(2-*tert*-butoxycarbonyl)amino]-[2-(carboxylmethyl)amino carbonyl]ethyl]ferrocene (12). Pd/C (5%, 162 mg) was carefully (Warning! fire hazard) added to a solution of **11** (317 mg, 0.420 mmol) in methanol (26 ml). The mixture was stirred under 1 atm (14.7 psi) of H_2 (g) for 24 h at room temperature. The catalyst was removed by filtration through Celite. The filtrate was then concentrated to give 222 mg (99%) of crude **12**, as a yellow powder, which was used without purification in next step: mp 127.8–130.2°C; $[\alpha]_D^{20}=+37$ (*c* 0.400, MeOH); $\tilde{\nu}_{max}$ 3415.7, 1651.0 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.35 (s, 9H), 2.51–2.76 (m, 2H), 2.89–2.94 (m, 2H), 3.78 (bs, 3H), 3.86–3.89 (m, 2H), 4.0–4.14 (m, 10H), 5.62 (bs, 3H), 6.43 (m, 1H), 7.90 (t, *J*=7.04 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 28.2, 41.4, 51.5, 55.4, 56.0, 67.8, 67.9, 68.1, 68.9, 69.4, 69.6, 70, 78.0, 83.3, 84.8, 155.2, 171.1, 171.1, 174.6; HRMS (FAB^+) *m/z* calcd for $C_{24}H_{33}FeN_3O_7$ 531.1668, found: 531.1673.

4.1.12. (2*S*,8*S*)-2-[Methoxycarbonyl]-3,6-diaza-4,7-dioxo-8-[(*tert*-butoxycarbonyl)amino]-[9][1,1′]ferrocenophane (13). DIEA (50 μl, 0.292 mmol) and PyAOP (66 mg, 0.126 mmol) were added to a solution of amino acid **12** (50 mg, 0.094 mmol) in DMF (98 ml) at room temperature and under a nitrogen atmosphere. The resulting mixture was stirred at room temperature for 24 h and then the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (50 ml) and worked up as follows: washing of the EtOAc solution sequentially with water (3×10 ml) and brine (1×20 ml), followed by drying (Na_2SO_4) and removal of the solvent under reduced pressure. The residue (65 mg) was column chromatographed (SiO_2, $CHCl_3$/MeOH 10:1) to give **13** (33 mg, 75%) as a yellow syrup: $[\alpha]_D^{21}=+11$ (*c* 0.500, $CHCl_3$); ^1H NMR (400 MHz, DMSO-d_6) δ 1.36 (s, 9H), 2.75–2.95 (m, 4H), 3.33 (bs, 5H), 3.93–4.51 (m, 9H), 4.52 (m, 1H), 6.39 (d, *J*=9.0 Hz, 1H), 7.93 (d, *J*=10.2 Hz, 1H), 8.57–8.58 (m, 1H), 8.71–8.72 (m, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 27.7, 42.8, 51.2, 51.8, 53.1, 66.6, 66.9, 67.1, 68.8, 69.5, 69.5, 69.6, 78.1, 82.9, 84.2, 154.2, 168.7, 171.0, 171.8; HRMS (FAB^+) *m/z* calcd for $C_{24}H_{31}FeN_3O_6$ 513.1562, found: 513.1571.

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