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Solution phase peptide synthesis with ferrocenyl amino acid derivatives

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Abstract

We report the successful stepwise synthesis of di- and tripeptides starting from ferrocenyl amino acids in solution. This methodology is extended to the synthesis of homo- and hetero-bimetallic peptides. Using the fluorenyl-methoxy-carbonyl (Fmoc) protecting group, the ferrocenyl methyl leucine derivative **2** is obtained quantitatively as a starting material. Coupling to PG-Ala-OH (PG = protecting group, *tert*-butoxy-carbonyl (Boc) or Fmoc) and Boc-Met-Phe-OH yields dipeptides **3a**,**b** and the tripeptide **5** in very good yield. The Fmoc derivative **3b** can be used for further deprotection/coupling cycles and serves as a precursor for the bis(ferrocenyl) tripeptide **7** after deprotection and coupling to Fc-CH_2-Phe-OH. Coupling of Fmoc deprotected **3b** to benzoic acid chromium tricarbonyl yields the hetero-bimetallic dipeptide **8** in good yield. In addition to reversible electrochemistry of the ferrocenyl group, compound **8** shows characteristic IR bands around 2000 cm^{-1} which enable its sensitive detection. All new compounds are completely characterized. Their constitution is established by MS and characteristic NMR spectra. Two isomers are observed at room temperature because rotation about the tertiary amide bond is slow on the NMR time scale ($\Delta G^{\ddagger} = 70 \pm 1$ kJ mol^{-1} by VT ^{1}H-NMR). Cyclic and square wave voltammetry experiments on the bis(ferrocenyl) tripeptide **7** do not indicate an electronic interaction between the two ferrocenyl moieties. Molecular modelling and NMR investigations do not give any indication of a rigid structure in solution but suggest a through-space distance of about 10 Å between the metal centres.

Keywords: Amino acid derivatives; Bio-organometallic chemistry; Biosensors; Electrochemistry; Ferrocene; Peptide synthesis

1. Introduction

Ferrocene is a compound with excellent stability. Unlike many other organometallics, it is completely stable in water and air. In addition, ferrocene has very favourable electrochemical properties. It is reversibly oxidized at a potential of +400 mV versus NHE. The combination of good stability and reversible electrochemistry lead to applications of ferrocene in a number of sensor devices with electrochemical detection. Examples include ferrocene mediated electron transfer in glucose oxidase [1–4], anion and cation sensors [5–7], and DNA biosensors [8,9]. If ferrocene is linked covalently to a biomolecule in these devices, this reaction is usually carried out in the last synthetic step [10–13].

Our group is exploring new ways of linking organometallics to biomolecules like peptides [14–18] and DNA analogues (peptide nucleic acid, PNA) [9,19]. Again, the metal complex is often added at the final synthetic steps. For the synthesis of more sophisticated bioconjugates, it would certainly be desirable to introduce a metal complex at an early synthetic stage. In such a scheme, the metal complex must then withstand all subsequent synthetic steps without decomposition. Given its good chemical stability, ferrocene seems like an ideal first choice for such a project. However, not much is known about the chemical stability of ferrocene or its derivatives under the conditions of peptide synthesis. Sergheraert and coworkers reported profound difficulties in the solid phase synthesis of [Fer[4], Leu[5]]-enkephalin, a neuronally active pentapeptide, where phenylalanine (Phe) in position 4 was replaced by ferrocenylalanine (Fer) [20]. Obviously, the ferrocenyl amino acid did not survive the harsh conditions of Merrifield synthesis.

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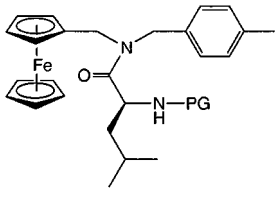
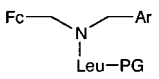
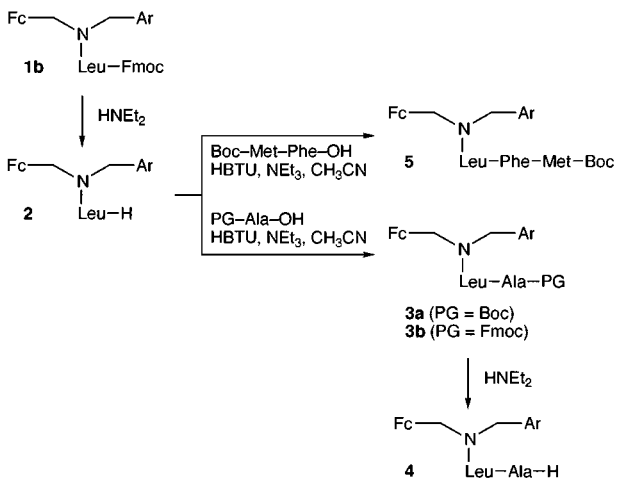
In this communication, we report the successful stepwise synthesis of ferrocenyl peptide derivatives by fluorenyl-methoxy-carbonyl (Fmoc) solution phase chemistry and the comprehensive characterization of the conjugates, including electrochemical and structural data from molecular modelling.



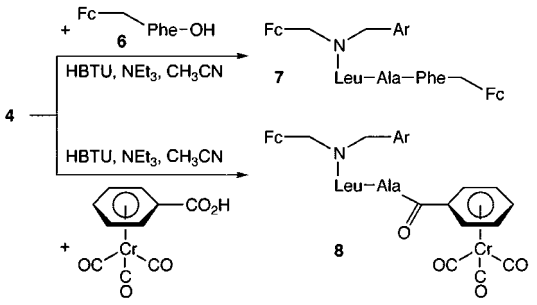
PG	Amino Acid	
Boc	Leu	**1a**
Fmoc	Leu	**1b**



Scheme 1. Ferrocenyl starting material **1** and naming convention used.



Scheme 2. Synthesis of **3**, **4** and **5**.



Scheme 3. Synthesis of bimetallic peptides **7** and **8**.

2. Synthesis and characterization

We recently reported the synthesis of secondary ferrocene amines from ferrocene aldehyde and primary amines like *p*-methyl–benzylamine, followed by reduction with $NaBH_4$ [15]. Also, Kraatz has reported a convenient one-pot synthesis of ferrocenemethylamine from ferrocene aldehyde using a similar reductive amination procedure [21]. To these secondary amines, N-protected amino acids were coupled by DCC/DhbtOH to yield N-protected conjugates like **1** (Scheme 1) [15]. These ferrocenyl amino acid derivatives should be well-suited to further investigate the feasability of typical peptide chemistry like deprotection and coupling reactions on ferrocene derivatives.

In exploratory experiments, the *tert*-butoxy-carbonyl (Boc) protecting group proved unsuitable because the ferrocene moiety did not withstand the cleavage conditions (50% TFA in CH_2Cl_2). Addition of ascorbic acid [20] or working under Ar in degassed solvents did not substantially improve this situation. The Fmoc protecting group, on the other hand, can be cleaved under mildly basic conditions, e.g. by triethylamine. Using this method, compound **2** with a free amino group was obtained in quantitative yield (Scheme 2). From **2**, two dipeptide derivatives **3** were obtained by HBTU-mediated coupling to *N*-Boc- and *N*-Fmoc-alanine (Boc-Ala-OH and Fmoc-Ala-OH) in good yield. Compound **3b** can be Fmoc-deprotected by triethylamine to give a ferrocenyl dipeptide derivative **4** that is again amendable to further peptide coupling reactions (see below). The ferrocene amine **2** can also be coupled directly to the dipeptide *N*-Boc-methionyl-phenylalanine (Boc-Met-Phe-OH) to yield the ferrocenyl tripeptide **5** (Scheme 2). Again, this reaction proceeds in good yield and **5** is isolated and purified by column chromatography.

Ferrocene aldehyde can also be condensed with amino acid esters to yield Schiff bases. After reduction and ester hydrolysis, ferrocenyl amino acids like **6** are obtained (Scheme 3) [17]. These ferrocene compounds with a free acid group are complementary to ferrocene amino acids with free amino groups like **2** and **4**. Condensation of **4** and **6** yields the tripeptide **7**, which carries a ferrocenyl–methyl group on the N- as well as on the C-terminus. A hetero-bimetallic dipeptide **8** is obtained by reaction of **4** with benzoic acid chromium tricarbonyl in the presence of HBTU (Scheme 3). This compound is isolated in 64% yield after column chromatography, demonstrating the good stability of the benzene chromium tricarbonyl moiety.

All new compounds are obtained as yellow–orange solids or oils. The colour is caused by an absorption at 420 nm which is characteristic for ferrocene compounds. In all cases, mass spectra were obtained by electron impact ionization (EI). Under these conditions,

a peak for the molecular ion with appreciable intensity was detected even for compounds with high molecular mass like the tripeptides **5** and **7**. In addition, a peak at $m/z = 199$ is significant for all ferrocene compounds in this study. This peak has a characteristic isotope pattern, which matches the fragment $CpFeC_5H_4–CH_2$ (Cp = η-cyclopentadienyl), and is the base peak for compound **7**. All compounds show characteristic NMR spectra. Hindered rotation about the central amide bond causes all signals to split into two signals with equal intensity. This indicates that there is no energetic preference for either the *s-cis* or the *s-trans* isomer. Variable temperature NMR experiments yield activation barriers of 70 ± 1 kJ mol^{-1} for rotation about the amide bond. The existence of two isomers can even be detected by two singlets for the very remote $S–CH_3$ group of the ferrocenyl tripeptide **5**, which have a ^{1}H-NMR shift difference of about 5 Hz. Typical ^{1}H-NMR signals originating from the ferrocene group are also observed. A singlet at ca. 4.1 ppm (intensity 5H) for the unsubstituted Cp ring is most easily recognized. This signal is inevitably split into two as a consequence of the presence of two rotational isomers. For the bis(ferrocenyl) tripeptide **7**, a second singlet with intensity 5H is observed for the second ferrocene Cp ring around 3.9 ppm, also split into two. The hetero-bimetallic dipeptide **8** shows a number of signals around 5.5 ppm with overall intensity 5H. These signals are characteristic for a benzene ring which is π-complexed to a $Cr(CO)_3$ group. The $Cr(CO)_3$ group is also characterized by two very strong absorptions in the IR spectrum at 1927 and 1899 cm^{-1} with an intensity ratio of ca. 1:2. In an approximate C_{3v} symmetry, these bands correspond to the ν_a and ν_e of the $Cr(CO)_3$ group and are the strongest bands in the spectrum of **8**.

The electrochemistry of all compounds was investigated by cyclic voltammetry (CV) and square wave voltammetry (SWV) in CH_2Cl_2 (0.1 M Bu_4NPF_6 as electrolyte). Compounds **1**–**5** all show a reversible one-electron oxidation at a potential of ca. + 50 mV versus Fc/Fc^+. The variation of potentials is too small to enable reliable differentiation between the various species [12]. The square wave voltammogram of **7** can be simulated by overlapping the individual spectra of **3** and **6**. This argues clearly against an electronic interaction between the Fe centres in **7**. In this case, a narrowing of the signal of **7** would have been expected as has been observed in other bis(ferrocenyl) compounds.[22]

3. Molecular modelling and discussion

This work explores the sequential peptide synthesis in solution in the presence of a ferrocenyl substituent. While the strongly acidic conditions of Boc deprotection were unsuitable, Fmoc chemistry was found to be excellently suitable. Using standard peptide synthesis protocols and reagents, ferrocenyl di- and tripeptides were synthesized and characterized comprehensively. Using this chemistry as a starting point, a bis(ferrocenyl) tripeptide **7** with ferrocenyl-methyl groups on the N- and C-terminus was obtained by fragment condensation of two ferrocene containing halves.

The electronic interaction of metal centres in biological systems has raised considerable interest among chemists. Examples include the study of electron transfer in peptides [12,23–27] and DNA [28–35]. In synthetic systems, the interaction of the metal centres linked by a carbon chain has been studied [22,36,37]. Electrochemical data on the bis(ferrocenyl) tripeptide **7** do not support an interaction between the two metal centres. We have tried to use molecular modelling to gain further insight into the structure of **7**. A starting structure was constructed assuming an α-helical peptide backbone, and various orientations of the ferrocenyl substituents were tried. During the energy minimization, the helical structure of the tripeptide backbone was lost in all cases, probably as a consequence of the interaction of the relatively bulky ferrocenyl substituents with the amino acid side chains. After energy minimization, we found a number of minima with very similar energy but different geometry. While the assumption of a helical structure seems not unreasonable for this amino acid sequence, NMR experiments (in particular $^3J(NH–C_\alpha H)$ and NOESY data) did not yield characteristic values for an ordered structure of **7** in solution. It is well established that tripeptides like **7** are fluxional on the timescale of NMR spectroscopy and do not generally adopt a single rigid conformation in solution. This notion is supported by the observation of several energetically similar minima from molecular modelling. However, the metal centres at both ends of the molecule were in a distance of at least 8–10 Å in all minimized structures. A relatively large distance between the metal centres is in accordance with our electrochemical findings, arguing against an electronic interaction.

A second, hetero-bimetallic peptide **8** is obtained from **4** and benzoic acid chromium tricarbonyl. This compound is quite stable without major precautions and can be handled under air at least for short times. In addition to the electrochemistry of the ferrocene moiety, this compound offers a second unique spectroscopic handle, namely infrared spectroscopy (IR). The carbonyl stretching vibrations of the $Cr(CO)_3$ group appear around 1900 cm^{-1}, which is a blank region for almost all organic compounds. This fact has been recognized by Jaouen et al. [38], who developed an immunoassay based on infrared spectroscopy of organometallic carbonyl complexes (carbonyl metallo immuno assay, CMIA) [39–41]. Under proper experimental conditions [41], this technique can match the

sensitivity of radio immuno assays (RIA) without the need to handle 'hot' material. Recently, an extension of CMIA for the simultaneous detection of two or three compounds was published (double [42] and triple [43] CMIA) [41].

4. Conclusions

This work demonstrates for the first time that typical peptide chemistry is possible *in the presence* of organometallic compounds, namely ferrocene. Using Fmoc chemistry and standard peptide coupling reagents, di- and tripeptides were synthesized in a sequential manner in good yield with a ferrocenyl group present at the N-terminus. Using the ferrocenyl dipeptide **4** as a building block, bimetallic di- and tripeptides were synthesized, again in acceptable yield. Naturally, the yield in solution synthesis will hardly match yields of $>99\%$ which are possible in optimized solid phase peptide synthesis. On the other hand, there is generally greater flexibility in solution chemistry and cost-effective synthesis is possible on a larger scale. The compounds presented in this study point the way to the planned, stepwise construction of complicated bioorganometallics. Apart from applications in biosensors, such synthetic systems may facilitate the study of phenomena like electron transfer in biology in more clarity than will ever be possible in modified natural systems because only synthetic systems can be constructed and modified at will exactly in accordance with the experimental needs. Applications in biosensors include new bioconjugates for metallo immuno assays and sensors with electrochemical detection. Modified electroactive proteins in particular may have a very promising application in arrayed protein expression assays ('protein chips'). This area of research is pursued actively in our group.

5. Experimental

All reactions were carried out in ordinary glassware and solvents without further precautions. Chemicals were purchased from Aldrich–Sigma GmbH and used as received, only enantiomerically pure L amino acids were used. Elemental analyses were carried out by H. Kolbe, Analytisches Laboratorium, Mülheim. IR spectra were recorded on a Perkin Elmer System 2000 instrument as KBr disks. Wave numbers ν are given in cm^{-1}. UV–vis spectra were recorded on a Perkin Elmer Lambda 19 spectrometer. Mass spectra were recorded by the mass spectrometry service group, Mülheim, on a MAT 8200 (Finnigan GmbH, Bremen) instrument (EI, 70 eV) or on a MAT95 (Finnigan GmbH, Bremen) instrument (ESI, CH_3OH solution, positive ion detection mode). Only characteristic fragments are given with intensities (%) and possible composition in brackets. Cyclic voltammograms (CV) were obtained with a three-electrode cell and an EG&G Princeton Applied Research model 273A potentiostat. A $Ag \mid AgNO_3$ (0.01 mol/l in $AgNO_3$) reference electrode, a glass carbon disk working electrode of 2 mm diameter and a Pt wire counter electrode was used. Square wave voltammograms (SWV) were recorded with a step height of 1 mV, 25 mV pulse amplitude and 40 Hz frequency. CH_2Cl_2 solutions (ca. 10^{-4} mol l^{-1}) contained 0.1 mol l^{-1} Bu_4NPF_6 as the supporting electrolyte. As an internal standard, ferrocene was added in excess after completion of the experiment and all peaks were referenced against the Fc/Fc^+ couple (0 V). The peak position was read from SWV, whereas electrochemical reversibility was verified by measuring the CV at different scan rates. NMR spectra were recorded in $CDCl_3$ at room temperature (r.t.) on Bruker ARX 250 (^1H at 250.13 MHz and ^{13}C), DRX 400 (^1H at 400.13 MHz, ^{13}C and 2D spectra) and DRX 500 (^1H at 500.13 MHz, ^{13}C, ^{15}N, 2D). ^1H and ^{13}C spectra were referenced to TMS, using the ^{13}C signals or the residual protio signals of the deuterated solvents as internal standards ($CDCl_3 \equiv 7.24$ (^1H) and 77.0 (^{13}C)). Positive chemical shift values δ (in ppm) indicate a downfield shift from the standard, only the absolute values of coupling constants are given in Hz. ^{15}N spectra were referenced to the absolute frequency of 50.6969910 MHz, which was the resonance frequency of neat nitromethane under the same experimental conditions. All resonances were assigned by 2D NMR (H–H-COSY and $^1H-^{13}C$-HMQC for ^1J and long-range couplings). Where unambiguous or proven spectroscopically, the following conventions are used: δ/δ' denotes pairs of signals originating from *s-cis*/*s-trans* isomers, integration '*n*H/2' indicates one signal of one rotational isomer only; 'δ and δ'' denotes pairs of diastereotopic signals. ^{15}N chemical shifts and coupling constants were taken from the F1 projection of indirect detection $^1H-^{15}N$ correlated 2D spectra with 1024/256 data points in F1/F2, processed after applying a matched cosine function and zero filling in both dimensions. Molecular Modelling was carried out with the SPARTAN program, version 5, on a Silicon Graphics Indigo^2 workstation (Wavefunction Inc., 18401 Von Karman Avenue, Suite 370, Irvine, CA).

5.1. Compound **2**

Compound **1b** (0.1 g, 0.15 mmol) was dissolved in 30 ml of $CHCl_3$. After addition of 10 ml of Et_3N, stirring is continued for 2 h at r.t. After removal of all volatiles in vacuo, the residue is chromatographed on silica. After washing with $CHCl_3$, 60 mg of pure **2** are eluted with MeOH (95%). Anal. Found: C, 69.54; H, 7.42; N,

6.51. Calc. for $C_{25}H_{32}N_2OFe$ (432.39 g mol^{-1}): C, 69.45; H, 7.46; N, 6.48%. δ_H 7.13–6.99 (4H, mult.), 4.89/4.11 (1H, d, $J = 14.8$ Hz, diast. CH_2), 4.66/4.03 (1H, d, $J = 14.2$ Hz, diast. CH_2), 4.37 (2H, two overlapping d, diast. CH_2), 4.29/4.00 (1H, d, diast. CH_2), 4.16 (2H, s, H_{Cp}), 4.14 (2H, s, H_{Cp}), 4.09/4.08 (5H, s, H_{Cp}), 3.85/3.53 (1H, dd, $J = 4$ Hz, $C_\alpha H$), 2.33 (3H, s, CH_3), 1.94/1.73 (1H, mult., $C_\gamma H$), 1.40/1.30 (2H, mult., $C_\beta H_2$), 1.01/0.97, 0.83/0.77 (6H, d, $J = 6.5$ Hz, $C_\gamma H_3$); δ_C 137.3, 133.7 (C_{quart}), 129.6, 129.3, 128.0, 126.2 ($C_{Phe}H$), 83.0 (C_{Cp}), 86.7 ($C_{Cp,\,i}$), 68.6/69.7, 69.4/69.3, 68.4, 68.2/67.9 (C_{Cp}), 49.8/49.7 (C_α), 48.7/47.0, 45.1/44.5 (CH_2), 44.7 (C_β), 24.7/24.6 (C_γ), 23.7/23.5, 21.6/21.5 (C_δ), 21.0 (CH_3); ν_{max}/cm^{-1} 2954 (w), 1641 (s); m/z 432 (100, $[M^{+\bullet}]$), 347 (23), 199 (49, $Fc{-}CH_2^+$), 121(36), 86 (81).

5.2. Compounds **3** and **5**

Compound **2** (0.05 g, 0.12 mmol) and Boc–Ala–OH, Fmoc–Ala–OH or Boc–Met–Phe–OH (0.12 mmol each), respectively, were dissolved in CH_3CN. Two equivalents of Et_3N (0.02 g, 0.24 mmol) and HBTU (0.05 g, 0.12 mmol) were added. After 15 min stirring at r.t. 10 ml of brine were added. The organic phase was separated. The aqueous phase was extracted with EtOAc and the combined organic phases were washed with 2 N HCl (3 ×), water, sat. $NaHCO_3$ (3 ×), water and dried over Na_2SO_4. After filtration, the volume was reduced and the residue chromatographed on silica (Et_2O:pentane 3:1).

5.3. Compound ***3a***

Yellow–orange solid, 60 mg (76%); m.p. 67 °C; δ_H 7.11–7.03 (4H, mult.), 6.89/6.76 (1H, d, $J = 8.0$ Hz, NH_{CO}), 5.24/4.87 (1H, mult., $C_\alpha H_{Leu}$), 5.14/5.07 (1H, br, NH_{Boc}), 4.80 /4.52 (1H, d, $J = 14.8$ Hz, diast. CH_2), 4.20 (1H, mult., $C_\alpha H_{Ala}$), 4.43/4.33 (1H, d, $J =$ 14.8 Hz, diast. CH_2), 3.96–4.13 (2H, d, $J = 14.8$ Hz, diast. CH_2), 4.40, 4.14, 4.12 (4H, s, H_{Cp}), 4.09/4.08 (5H, s, H_{Cp}), 2.33 (3H, s, CH_3), 1.75/1.55 (1H, mult., $C_\gamma H_{Leu}$), 1.42/1.41 (9H, s, $CH_{3,\,Boc}$), 1.40/1.30 (2H, mult., $C_\beta H_{2,\,Leu}$), 1.33/1.28 (3H, d, $J = 7$ Hz $C_\beta H_{3,\,Ala}$) 1.06/0.93, 0.76/0.72 (6H, d, $J = 6.4$ Hz, $C_\gamma H_{3,\,Leu}$); δ_C 172.4/172.2, 172.0/171.7 (CO), 155.0 (CO_{Boc}), 137.3/137.0, 133.9/133.1 (C_{quart}), 129.5, 129.3, 127.9, 126.8 ($C_{Ar}H$), 82.6/81.9 (C_{Cp}), 79.8 (C_{Boc}), 68.7/68.6 (C_5H_5), 69.6, 69.4, 69.0, 68.3, 68.0 (C_5H_4), 50.1/50.0 ($C_\alpha H_{Ala}$), 49.1, 46.5, 45.4, 44.1 (all CH_2), 47.6/47.3 ($C_{\alpha,\,Leu}$), 42.9/42.3 ($C_{\beta,\,Leu}$), 28.2 (C_{Boc}), 24.6/24.4 ($C_{\gamma,\,Leu}$), 23.5/23.3, 21.6/21.5 (C_δ), 21.0 (CH_3), 18.6/18.4 ($CH_{3,\,Ala}$); ν_{max}/cm^{-1} 2960 (w), 1716 (m), 1631 (s); m/z 603 (98, $[M^{+\bullet}]$), 438 (39), 360 (48), 199 (100, $Fc{-}CH_2^+$), 121 (25), 105 (57).

5.4. Compound ***3b***

Yellow–orange solid, 70 mg (78%); m.p. 86 °C. Anal. Found: C, 71.1; H, 6.5; N, 5.6. Calc. for $C_{43}H_{47}N_3FeO_4$ (725.7 g mol^{-1}): C, 71.2; H, 6.5; N, 5.8%. δ_H 7.74, 7.58, 7.38, 7.30 (8H, mult., H_{Fmoc}), 7.13–7.04 (4H, mult. H_{Ar}), 6.75/6.62 (1H, d, $J = 8.3$ Hz, NH_{CO}), 5.44/5.49 (1H, br, NH_{Fmoc}), 5.24/4.90 (1H, mult., $C_\alpha H_{Leu}$), 4.30/4.25 (1H, mult., $C_\alpha H_{Ala}$), 4.20 (2H, mult., $CH_{2,\,Fmoc}$), 4.84/4.07 (1H, d, $J = 14.8$ Hz, diast. CH_2), 4.57/4.04 (1H, d, $J = 14.8$ Hz, diast. CH_2), 4.47/4.40 (1H, d, $J = 14.8$ Hz, diast. CH_2), 4.36/4.03 (1H, d, diast. CH_2), 4.05–4.5 (4H, s, H_{Cp}), 4.11/4.08 (5H, s, H_{Cp}), 2.32 (3H, s, CH_3), 1.75/1.55 (1H, mult., $C_\gamma H_{Leu}$), 1.65/1.55 (2H, mult., $C_\beta H_{2,\,Leu}$), 1.40/1.33 (3H, $C_\beta H_{2,\,Ala}$) 1.07/0.92 (3H, d, $J = 6.5$ Hz, $C_\gamma H_{3,\,Leu}$), 0.75 (3H, overlapping d, $C_\gamma H_{3,\,Leu}$); δ_C 172.2/172.0, 171.8/171.7 (CO), 141.3 (CO_{Fmoc}), 137.5/137.1, 133.9/133.0 (C_{quart}), 129.6 (C_{Fmoc}), 129.4, 127.9, 126.8 ($C_{Phe}H$), 127.7, 127.1, 125.1, 119.9 ($C_{Fmoc}H$), 82.6/81.9 (C_{Cp}), 68.7/68.6 (C_5H_5), 69.7, 69.6, 69.5, 69.1, 68.9, 68.4, 68.1, 68.0 (C_5H_4), 50.6 ($C_{\alpha,\,Ala}$), 49.1/46.6, 45.5/44.1 (all CH_2), 47.9/47.6 ($C_{\gamma,\,Leu}$), 47.2 ($CH_{2,\,Fmoc}$) 42.9/42.3 ($C_{\beta,\,Leu}$), 24.8/24.6 ($C_{\gamma,\,Leu}$), 23.5/23.3, 21.6/21.5 (C_δ), 21.1 (CH_3), 18.9 ($C_{\beta,\,Ala}$); δ_N −292 (N_{Fmoc}), −262; ν_{max}/cm^{-1} 2956 (w), 1723 (m), 1631 (s); m/z 725 (8, $[M^{+\bullet}]$), 529 (78), 464 (39), 386 (31), 199 (47, $Fc{-}CH_2^+$), 165 (66), 121 (27), 105 (100).

5.5. Compound ***5***

Yellow–orange solid, 70 mg (71%); m.p. 73–74 °C; δ_H 7.22/7.00 (9H, mult.), 6.8/6.70 (1H, d, $J = 8.0$ Hz, NH), 6.55/6.65 (1H, d, $J = 8.0$ Hz, NH) 5.15/4.82 (1H, mult., $C_\alpha H_{Leu}$), 4.65 (1H, mult. $C_\alpha H_{Phe}$), 4.39, 4.18, 4.15, 4.13 (4H, s, H_{Cp}), 4.25 (1H, mult., $C_\alpha H_{Met}$), 4.10 (5H, s, H_{Cp}) 4.81/3.96 (1H, d, diast. CH_2), 4.52/4.04 (1H, d, diast. CH_2), 4.43/4.34 (1H, d, diast. CH_2), 4.30/3.93 (1H, d, diast. CH_2), 3.05 (2H, mult., $C_\beta H_{2,\,Phe}$), 2.49 (2H, mult. $C_\gamma H_{2,\,Met}$), 2.33 (3H, s, CH_3), 2.06/2.05 (3H, s, $CH_{3,\,Met}$), 1.9/2.1 (2H, mult, $C_\beta H_{2,\,Met}$), 1.65/1.25 (2H, mult, $C_\beta H_{2,\,Leu}$), 1.65/1.45 (1H, mult., $C_\gamma H_{Leu}$), 1.41/1.40 (9H, s, $CH_{3,\,Boc}$), 1.40/1.30 (2H, mult., $C_\beta H_{2,\,Ala}$), 1.05/0.91, 0.74/0.72 (6H, d, $J = 6.4$ Hz, $C_\gamma H_{3,\,Leu}$); δ_C 171.9, 171.4, 171.2, 169.7 (CO), 155.4 (CO_{Boc}), 137.5/137.1, 136.0/135.9, 133.9/133.0 (C_{quart}), 129.6, 129.4, 129.3, 128.6, 128.0, 126.9, 126.8 ($C_{Ar}H$), 81.9 (C_{Cp}), 80.2 (CO_{Boc}), 68.7 (C_5H_5), 69.7, 69.5, 69.2, 69.0, 68.6, 68.4, 68.2 (C_5H_4), 54.4/54.1 ($C_{\alpha,\,Phe}$), 53. 8 ($C_{\alpha,\,Met}$), 47.8/47.6 ($C_{\alpha,\,Leu}$), 49.0/46.5, 45.4/44.2 (CH_2), 42.9/42.3 ($C_{\beta,\,Leu}$), 38.1/37.9 ($C_{\beta,\,Phe}$), 31.6 ($C_{\beta,\,Met}$), 30.1 ($C_{\gamma,\,Met}$), 28.3 ($CH_{3,\,Boc}$), 24.6/24.4 ($C_{\gamma,\,Leu}$), 23.5/23.3, 21.6/21.5 ($C_{\delta,\,Leu}$), 21.6/21.5 (CH_3), 15.3 ($SCH_{3,\,Met}$); δ_N −259.6, −264.2; ν_{max}/cm^{-1} 2958 (w), 1637 (s); m/z 810 (11, $[M^{+\bullet}]$), 199 (24, $Fc{-}CH_2^+$), 105 (100).

5.6. Compound **4**

Amine **3b** (0.3 g, 0.4 mmol) was stirred with 10 ml Et_3N in 20 ml of CH_2Cl_2 for 2 h at r.t. The solution was evaporated to dryness and the resulting solid was purified by chromatography on silica. After washing with $CHCl_3$, 160 mg of pure **4** were eluted with MeOH (78%). δ_H 7.76/7.66 (1H, br, NH_{Leu}), 7.13 (2H, mult.), 7.06 (2H, mult.), 5.25/4.89 (1H, mult., $C_\alpha H_{Leu}$), 4.85 (1H, d, CH_2), 4.59 (1H, d, CH_2), 4.46 (1H, d, CH_2), 4.38 (1H, d, diast. CH_2), 4.01 (1H, d, CH_2), 4.08 (1H, d, CH_2), 4.44 (1H, s, H_{Cp}), 4.17 (1H, s, H_{Cp}), 4.15 (1H, s, H_{Cp}), 4.12 (1H, s, H_{Cp}), 4.10/4.08 (5H, s, H_{Cp}), 4.00 (1H, d, CH_2), 3.51/3.42 (1H, mult., $C_\alpha H_{Ala}$), 2.33/2.32 (3H, s, CH_3), 1.75/1.55 (1H, mult., $C_\gamma H_{Leu}$), 1.65 (2H, mult., $C_\beta H_{2,\,Leu}$), 1.35/1.29 (3H, d, $J = 7.0$ Hz, $C_\beta H_{3,\,Ala}$) 1.08/0.96 (3H, d, $J =$ 6.5 Hz, $C_\gamma H_{3,\,Leu}$), 0.79/0.75 (3H, d, $J = 6.0$ Hz, $C_\gamma H_{3,\,Leu}$); m/z 503 (100, [$M^{+\bullet}$]), 438 (66), 360 (61), 199 (77, Fc–CH_2^+).

6. Compound 7

Fc-CH_2-Phe-OH **6** (0.1 g, 0.28 mmol) and 0.14 g (0.28 mmol) of the amine **4** were dissolved in 30 ml of CH_3CN. After addition of Et_3N (0.055 g, 0.6 mmol) and HBTU (0.11 g, 0.28 mmol), stirring was continued for 15 min at r.t. Brine (10 ml) was added, the organic phase was separated and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with 2 N HCl (3 ×), water, sat. $NaHCO_3$ (3 ×), water and dried over Na_2SO_4. After filtration and evaporation on an oil pump, pure **7** was obtained as a yellow–orange solid (0.14 g, 58%). M.p. 72–73 °C. Anal. Found: C, 67.7; H, 6.7; N, 6.7. Calc. for $C_{48}H_{56}Fe_2N_4O_3$ (848.69 g mol^{-1}): C, 67.9; H, 6.7; N, 6.6%. δ_H 7.84/7.76 (1H, d, $J = 7.9$ Hz, NH_{Ala}), 6.82/6.73 (1H, d, $J = 8.6$ Hz, NH_{Leu}), 7.32–7.03 (10H, several mult., H_{Ar}), 5.22/4.89 (1H, mult., $C_{\alpha,\,Leu}H$), 4.83 and 4.06, 4.60 and 4.02, 4.37 and 4.03 (1H each, all pairs of diastereotopic N–CH_2), 4.53 (1H, s, N–CH_2), 4.45 (1H, $C_{\alpha,\,Ala}H$), 4.40 (1H, Cp), 4.15–3.94 (7H, Cp), 4.09/4.08 (5H, Cp), 3.91/3.89 (5H, Cp), 3.46 (1H, mult., $C_{\alpha,\,Phe}H$), 3.44/3.20 (2H, CH_2), 2.33 (s, Ar–CH_3), 1.67/1.52 (1H, mult., $C_{\gamma,\,Leu}H$), 1.63 and 1.45/1.50 and 1.30 (2H, mult., $C_{\beta,\,Leu}H$), 1.36/1.31 (3H, d, $J = 7.0$ Hz, $C_{\beta,\,Ala}H$), 1.03, 0.91, 0.76, 0.71 (3H/2 each, d, $J = 6.4$ Hz, 6.5 Hz, 6.4 Hz, 6.2 Hz, $C_{\delta,\,Leu}H$). δ_C 173.6/173.5 (CO_{Phe}), 172.2/171.8 (CO_{Leu}), 171.7/171.4 (CO_{Ala}), 137.4 ($C_{i,\,Phe}$), 137.3/137.0, 133.9/133.1 ($C_{quart.,\,Ar}$), 129.5, 129.3, 129.0, 128.8, 127.9, 127.0, 126.8 (aromatic C), 86.2/86.1 (Phe-CH_2-Cp_i), 82.6/82.0 (Cp_i), 68.8/68.6, 68.1/68.2 (10H, Cp), 69.6–67.6 (all Cp), 63.6/63.5 ($C_{\alpha,\,Phe}$), 48.4/48.3 ($C_{\alpha,\,Ala}$), 47.8/47.6 ($C_{\alpha,\,Leu}$), 47.7/47.6 (Phe-CH_2-Cp), 49.0, 46.5, 45.4, 44.0 (all remaining CH_2), 43.5/42.2 ($C_{\beta,\,Leu}$), 39.4 ($C_{\beta,\,Phe}$), 24.8/24.6 ($C_{\gamma,\,Leu}$), 23.6, 23.4, 21.6, 21.6 ($C_{\delta,\,Leu}$), 21.1/21.0 (Ar–CH_3), 18.4/18.0 ($C_{\beta,\,Ala}$); δ_N −262, −263; ν_{max}/cm^{-1} 3092 (w), 3026 (vw), 2956 (m), 2927 (m), 2868 (w), 1638 (vs); m/z 848 (19, [$M^{+\bullet}$]), 783 (10), 705 (17), 199(100, Fc–CH_2^+).

6.1. Compound **8**

Benzoic acid chromium tricarbonyl (0.2 g, 0.77 mmol) and amine **4** (0.39 g, 0.77 mmol) were dissolved in 50 ml of CH_3CN. After addition of Et_3N (0.78 g, 0.77 mmol) and HBTU (0.29 g, 0.77 mmol), stirring was continued for 15 min at r.t. Brine (10 ml) was added, the organic phase was separated and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with 2 N HCl (3 ×), water, sat. $NaHCO_3$ (3 ×), water and dried over Na_2SO_4. After filtration, the organic solution was evaporated on an oil pump. The residue was chromatographed on silica (ether:pentane 3: 1) and pure **8** was obtained as a yellow–orange solid (0.36 g, 64%). δ_H 7.55/7.20 (1H, br, NH), 7.11/7.01 (4H, mult., H_{Ar}), 5.92–5.20 (5H, several mult., H_{Ar}), 5.21/4.90 (1H, mult., $C_{\alpha,\,Leu}H$), 4.82 and 4.09, 4.60 and 4.01, (1H each, all pairs of diastereotopic N–CH_2), 4.35 (2H, s, N–CH_2), 4.49/4.40 (1H, $C_{\alpha,\,Ala}H$), 4.40/4.12 (4H, Cp), 4.08 (5H, Cp), 2.33 (s, Ar–CH_3), 1.55/1.25 (2H, mult., $C_{\beta,\,Leu}H$), 1.51/1.22 (1H, mult., $C_{\beta,\,Leu}H$), 1.40 (3H, d, $J = 7.0$ Hz, $C_{\beta,\,Ala}H$), 1.05, 0.92, 0.75, 0.72 (3H/2 each, d, $J = 6.4$ Hz, 6.5 Hz, 6.4 Hz, 6.2 Hz, $C_{\delta,\,Leu}H$). δ_C 231.2 (Cr–CO), 172.4, 172.1, 164.2 (all CO), 137.4/137.0, 133.6/132.9 ($C_{quart.,\,Ar}$), 129.5/129.3, 127.6, 126.6 (aromatic C), 95.4, 93.8, 92.8, 90.3 ($CH_{Ar,\,Cr}$), 82.4/81.7 (Cp_i), 68.9/68.7 (5C, Cp), 69.6–68.0 (all Cp), 49.6/49.4 ($C_{\alpha,\,Ala}$), 47.2 ($C_{\alpha,\,Leu}$), 49.1, 47.8, 46.8, 45.6 (all CH_2), 44.2/41.8 ($C_{\beta,\,Leu}$), 24.6/24.5 ($C_{\gamma,\,Leu}$), 23.4, 23.2, 21.5, 21.4 ($C_{\delta,\,Leu}$), 21.0 (Ar–CH_3), 18.6 ($C_{\beta,\,Ala}$); δ_N −262, −263; ν_{max}/cm^{-1} 2958 (w), 1972 (s), 1899 (s), 1631 (m); m/z 743 (5, [$M^{+\bullet}$]), 607 (71), 542 (54), 464 (100), 199(85, Fc–CH_2^+), 121 (25), 105 (67).

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