ChemPubSoc Europe

DOI: 10.1002/ejoc.201901206

EurJOC European Journal of Organic Chemistry
Communication

Peptide Conjugates | *Very Important Paper* |

Design and Synthesis of New Protease-Triggered CO-Releasing Peptide–Metal-Complex Conjugates

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Abstract: Aiming at the development of novel protease-triggered CO-releasing molecules (PT-CORMs) we designed a series of oxycyclohexadiene-$Fe(CO)_3$ complexes, which are connected through a self-immolative linker with a plasmin-specifying peptide unit. The challenging synthesis of these compounds was elaborated employing a combination of solid-phase peptide synthesis and solution chemical synthesis. Late-stage attachment of the organometallic unit and Pd-catalyzed cleavage of an alloc-protected lysine side chain afforded the target compounds in good yields and high purity.

Introduction

Carbon monoxide (CO), one of the three known gasotransmitters, is endogenously produced in the human body through heme oxygenase, which exists in three isoforms: the constitutive HO-2 and HO-3 and inducible HO-1.[1] As CO has a high affinity to hemoglobin (ca. 230-fold higher than that of oxygen) it causes strong inhibition of O_2 transport throughout the body by formation of carboxyhemoglobin (COHb). This leads to hypoxia and an overall intoxication of the organism.[2] However, more recently it was revealed that CO actually represents an essential signaling molecule[3] and its endogenous actions affect the neuronal, cardiovascular, immune, respiratory, reproductive, and gastrointestinal systems.[4] Thus, CO plays a vital role in many physiological functions in the mammalian body and exerts anti-inflammatory, antiapoptotic, anti-coagulative, anti-hypertensive, antioxidant, and cell-protective effects.[5] In principle, CO has great therapeutic potential against a wide range of human diseases and pathological states including cancer, bacterial infections, inflammatory diseases, cardiovascular disorders, rheumatoid arthritis, cerebral malaria, lung injury, neurodegeneration, kidney and liver dysfunctions, chronic rejection after organ transplantation, and others.[6,7] However, a person would need to inhale a comparably high concentration of the gas to attain a meaningful concentration in the body.[5c] This hampers the clinical use of carbon monoxide gas because of the risk of intoxication.[5c] Therefore, the use of CO as a therapeutic agent requires the development of alternative methods for its delivery. Carbon monoxide releasing molecules (CORMs) are compounds capable to of delivering controlled amounts of CO to the body without affecting the level of COHb produced. They represent potential pharmaceutical agents of high clinical value.

The first generation of CORMs, suggested by Motterlini, mainly included metal carbonyl complexes (based on Cr, Mo, Mn, Re, Fe, Ru) but also other types of compounds such as "boranocarbonate" (CORM A1).[1b,3c,5d,6a] While these first-generation CORMs are stable and storable compounds, a disadvantage is that they spontaneously release CO through ligand exchange upon dissolution in aqueous media.[1b] In contrast, enzyme-triggered CO-releasing molecules (ET-CORMs) allow a controlled intracellular CO delivery as they are activated only by specific enzymes.[8] The first representatives of the ET-CORM family were esterase-activated acyloxydiene tricarbonyl iron complexes (Figure 1 and Scheme 1).[8a–8c] The concept was later expanded to phosphatase- and penicillin G amidase-activated analogs (Figure 1).[8d–8e] Other options to achieve a controlled CO release[9] are based on pH-sensitive CORMs[10] or the use of light[11] or heat[10,12] as external stimuli.

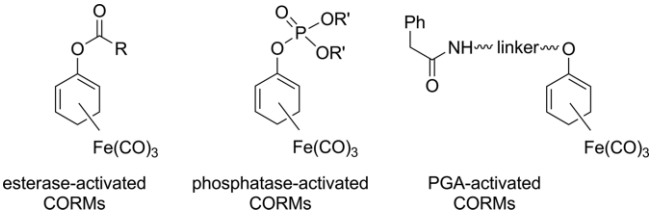

Figure 1. Structures of selected enzyme-triggered CORMs (ET-CORMs).

ET-CORMs are activated by enzyme-induced hydrolysis to form a highly labile dienol-$Fe(CO)_3$ intermediate, which in turn rapidly undergoes oxidation under physiological conditions to liberate up to three molecules of CO as the main bioactive product, besides a Fe^{3+} ion and cyclohexenone (Scheme 1). The

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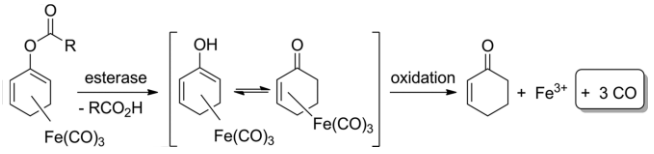


Scheme 1. Mechanism of enzyme-triggered CO release from ET-CORMs.

enzyme-triggered CO release from such complexes was proven by means of the UV-based myoglobin assay and by headspace GC analysis. Also, ET-CORMs showed very promising biological effects in different cell-based assays.[13]

Today's knowledge about the construction of prodrugs with controlled in vivo distribution and activation forms a solid basis for the design of new organometallic CORMs for possible therapeutic applications.[14] In this context, protease-activated prodrugs are particularly promising since they allow drug delivery to tissues with an enhanced expression of certain proteases.[15] Indeed, modifications in proteolytic activity are associated with diseases like cancer, as well as neurodegenerative and cardiovascular disorders.[16] Plasmin represents a serine protease that plays a key role in tumor invasion and metastasis and is known to be overexpressed in tumor tissues.[15a,17] It is essentially an intracellular protease which is rapidly deactivated in the blood circulation.[17] Increased plasmin activity is also strongly associated with rheumatoid arthritis (RA).[15a,18] Therefore, this protease represents a particularly promising enzyme for the development of prodrugs for the treatment of cancer and RA. At the same time, both of these diseases are among the most promising targets for therapeutic application of CO.[6a]

Herein, we report on the design and synthesis of novel protease-activated CORMs (PT-CORMs) which were expected to exhibit improved pharmacological properties (water solubility, biocompatibility, tissue selectivity and cell permeability[15a]) and tunable CO release kinetics.

Results and Discussion

Aiming at the development of novel PT-CORMs based on plasmin as a specifier, we chose a general design in which the plasmin specifier is linked to the CORM unit through a self-immolative linker (Figure 2).

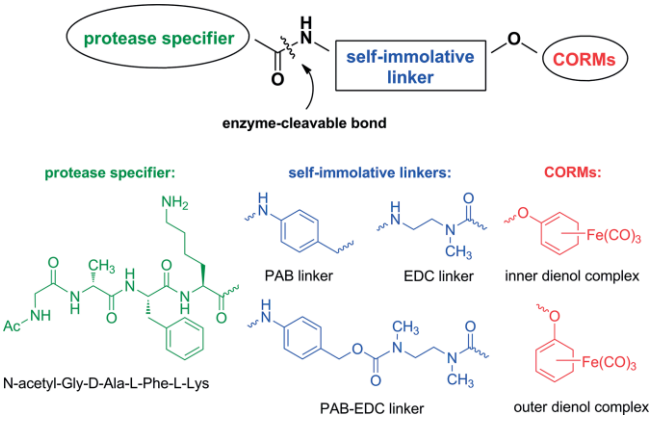


Figure 2. General design of the protease-triggered CORMs.

The remote positioning of the enzyme-cleavable bond relatively to the planar chiral organometallic moiety would suppress kinetic resolution during the enzymatic process, thus eliminating the necessity to control the absolute configuration of the diene-$Fe(CO)_3$ moiety, and conjugation of the enzyme-cleavable moiety through a linker also should facilitate enzymatic hydrolysis.[19] Fine-tuning of CO release kinetics is of high importance

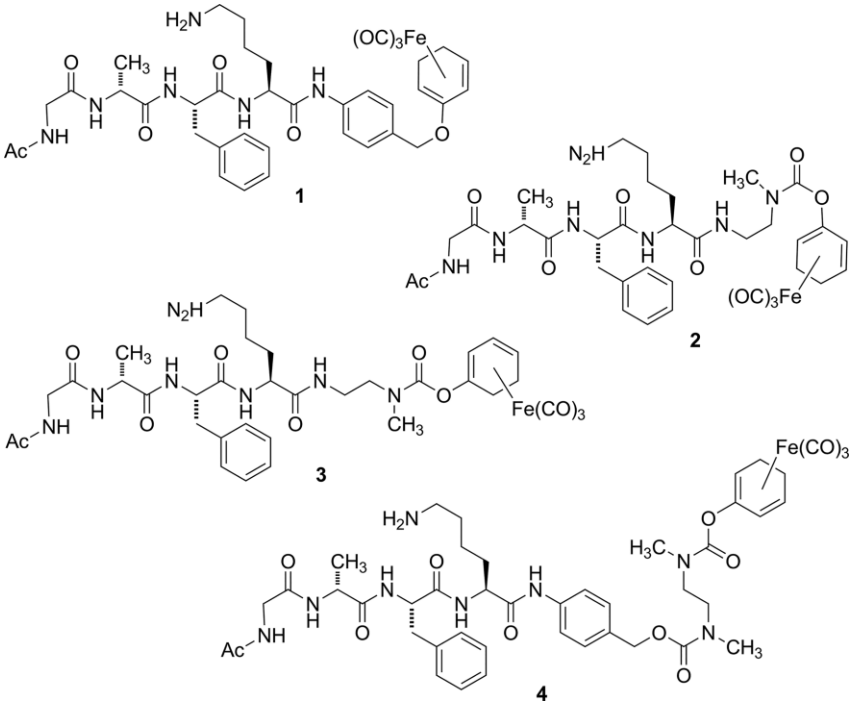
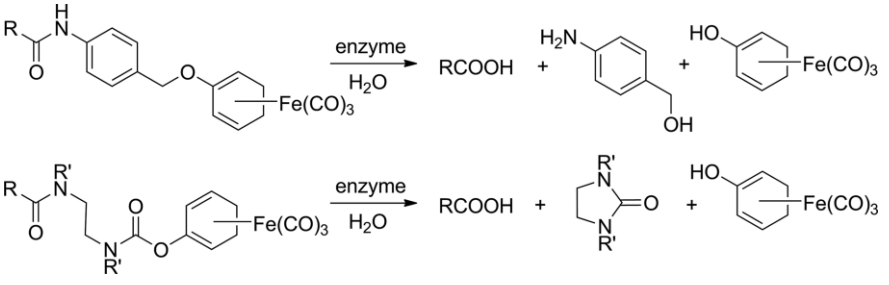


Figure 3. Target plasmin-activated CORMs.

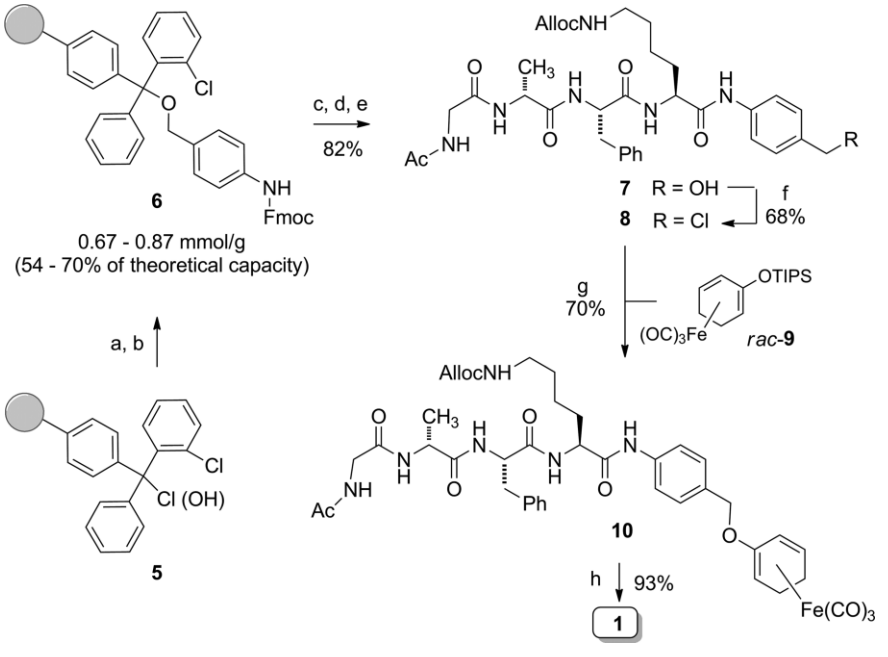
for the development of therapeutically useful CORMs because a fast release rate may lead to cytotoxic levels of CO, while a slow but constant CO release is required to induce a desired biological effect. In the case of PT-CORMs of the general type depicted in Figure 3, the rate of prodrug cleavage and CO release would be determined by superimposition of the rates of the following individual reactions: (1) enzymatic amide bond hydrolysis; (2) linker self-immolation; (3) "decomposition" of the dienol-$Fe(CO)_3$ intermediate. The rate of enzymatic cleavage will depend on the enzyme, the structure of the specifier and probably also on the linker (as it contributes to the stereochemical environment around the cleavable amide bond).

While the tripeptide *d*-Ala-*l*-Phe-*l*-Lys is a well-established specifier for plasmin-activated anti-cancer prodrugs,[15a,18,19b,20] an even more advantageous *N*-acetyl-Gly-*d*-Ala-*l*-Phe-*l*-Lys sequence was recently reported.[21] As linkers, we selected the well-known *p*-aminobenzyl (PAB) and 1,2-ethylenediaminecarbonyl (EDC) linkers and a combination of *p*-aminobenzyl and ethylenediaminecarbonyl fragments (PAB-EDC), which differ in their mechanism of elimination (1,6-elimination vs. cyclization) (Scheme 2).[19,20,22] The structure of the dienol complex (Figure 2) which is released upon linker self-immolation also has a profound effect on the CO release rate (with the "outer diene" undergoing oxidative decomposition much faster than the "inner diene").[8b]

Taking all the above considerations together, we selected four different target structures for this study (Figure 3). The synthesis of PT-CORM **1** (Scheme 3) started with the preparation of the suitably protected *N*-acetyl-Gly-*d*-Ala-*l*-Phe-*l*-Lys specifier containing the self-immolative PAB linker unit using manual solid phase peptide synthesis.[21] According to a reported procedure commercial 2-chlorotrityl chloride polystyrene resin (2-CTC resin) **5** was first treated with thionyl chloride in the presence of pyridine followed by addition of the *N*-Fmoc-protected *p*-aminobenzyl alcohol to yield the CTC-resin **6**.[21] After Fmoc-deprotection of **6** under basic conditions the subsequent amino acid coupling was carried out using commercially available Fmoc-*l*-Lys-alloc, Fmoc-*l*-Phe, Fmoc-*d*-Ala and Ac-Gly in



Scheme 2. Activation of PT-CORMs with different types of self-immolative linkers.

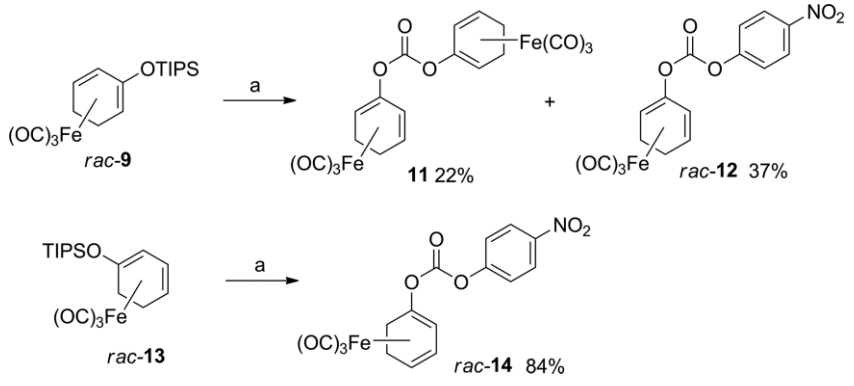


Scheme 3. Synthesis of PT-CORM **1**. Reagents and conditions: (a) $SOCl_2$, pyridine, DCM, 0 °C to r.t., 30 min, then reflux, 4 h; (b) *N*-Fmoc-*p*-aminobenzylic alcohol, pyridine, THF, reflux, 16 h; (c) piperidine, DMF, r.t., 20 min; (d) amino acid, HATU, HOAt, DIPEA, DMF, r.t. (amino acids coupling steps were carried out with Fmoc-*l*-Lys-alloc; Fmoc-*l*-Phe; Fmoc-*d*-Ala; Ac-Gly sequentially); (e) TFA, DCM, r.t.; (f) $SOCl_2$, THF, r.t., 14 h; (g) *rac*-**9**, TBAF, NaH, DMF, 0 °C, 10 min, then r.t., 14 h; (h) $Pd_2(dba)_3{\cdot}CHCl_3$, PPh_3, HCOOH, THF, r.t., 14 h. {HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium-3-oxide hexafluorophosphate; HOAt = 1-hydroxy-7-azabenzotriazole; DIPEA = diisopropylethylamine; Fmoc = fluorenylmethyloxycarbonyl; alloc = allyloxycarbonyl; TFA = trifluoroacetic acid}.

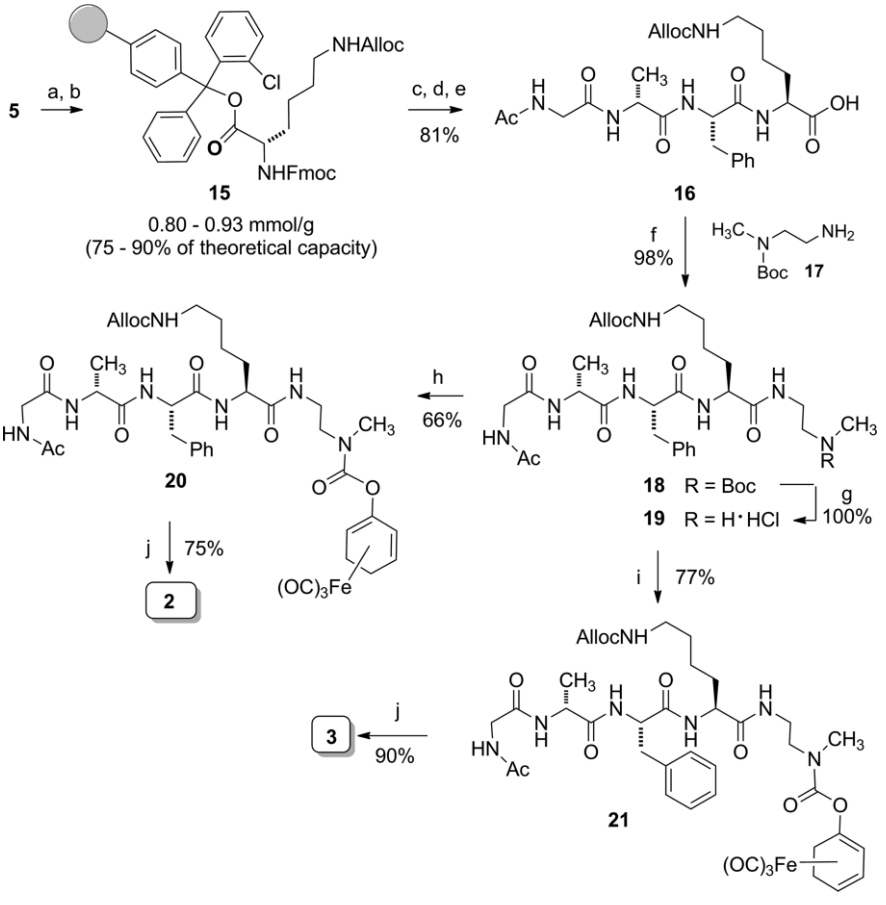
DMF at room temperature.[21] Cleavage of the *p*-aminobenzyl alcohol-peptide conjugate from the resin was carried out by treatment with TFA solution in DCM to provide peptide **7** in 82 % yield (3 steps).

The benzyl alcohol function in **7** was transformed to the chloride **8** by nucleophilic substitution using thionyl chloride in THF. Subsequent conjugation of compound **8** with the cyclohexadiene-$Fe(CO)_3$ complex *rac*-**9** (prepared according to ref.[8b]) in DMF provided organometallic compound **10** in 70 % yield. Alloc-deprotection of complex **10** was finally performed with acetic acid in the presence of $Pd_2(dba)_3$ as a catalyst to give the desired PT-CORM **1** (as the hydroformate salt) in 93 % yield.

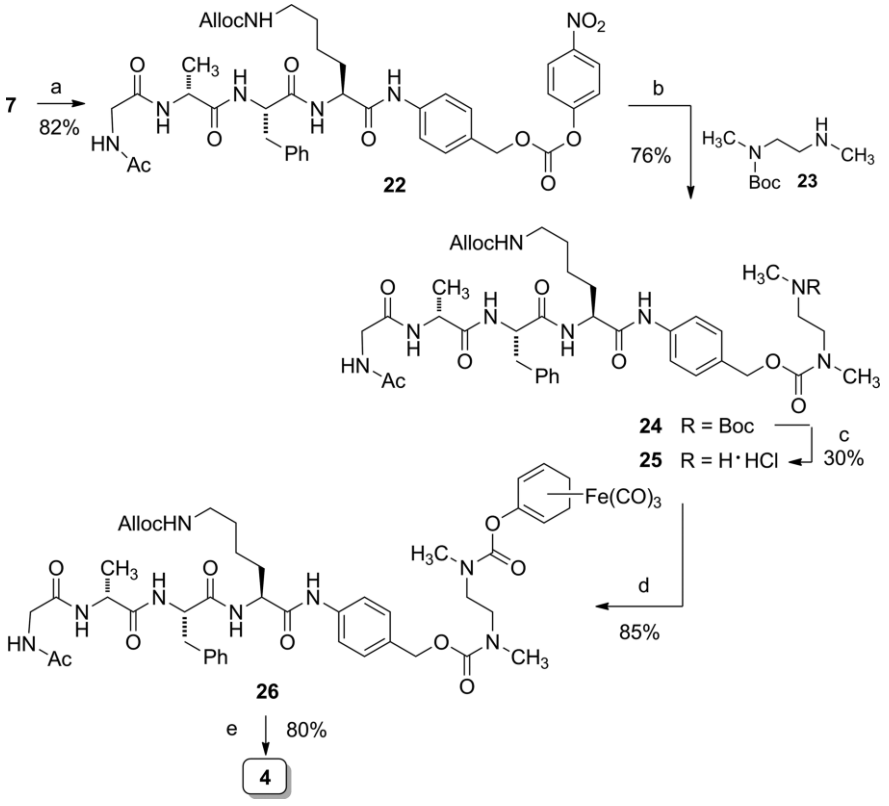
For the synthesis of the target PT-CORMs **2–4** a set of reactive cyclohexadiene-$Fe(CO)_3$ complexes, i.e. **11** and *rac*-**12** with an RO function at the "outer" position of the cyclohexadiene-



Scheme 4. Synthesis of cyclohexadiene-$Fe(CO)_3$ complexes. Reagents and conditions: (a) TBAF, THF, 0 °C, 10 min, then 4-nitrophenyl chloroformate, DIPEA, 1.5 h (TBAF = tetra-*n*-butyl ammonium fluoride).



Scheme 5. Synthesis of PT-CORMs **2** and **3**. Reagents and conditions: (a) $SOCl_2$, pyridine, DCM, 0 °C to r.t., 30 min, then reflux, 4 h; (b) Fmoc-Lys(Alloc)-OH, DIPEA, DCM, r.t., 14 h; (c) piperidine, DMF, r.t., 20 min; (d) amino acid, HATU, HOAt, DIPEA, DMF, r.t. (amino acid coupling steps were carried out with Fmoc-*l*-Phe; Fmoc-*d*-Ala; Ac-Gly sequentially); (e) TFA, DCM, r.t.; (f) *N*-Boc-*N*-Me-ethylenediamine 17, Py-BOP, HOBt, DIPEA, DMF, r.t., overnight; (g) HCl aq, EtOAc, r.t., 2 h; (h) **11** + *rac*-**12**, DIPEA, DMF, r.t., 1 h; (i) *rac*-**14**, DIPEA, DMF, 0 °C, 30 min; (j) $Pd_2(dba)_3$*$CHCl_3$, PPh_3, HCOOH, THF, r.t., 14 h. {Boc = *tert*-butyloxycarbonyl; Py-BOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; HOBt = 1-hydroxybenzotriazole}.



Scheme 6. Synthesis of PT-CORM **4**. Reagents and conditions: (a) 4-nitrophenyl chloroformate, pyridine, THF; (b) *N*-Boc-*N*,*N*′-dimethyl-1,2-ethylenediamine **23**, DMF, –20 °C, 2 h; (c) HCl aq, EtOAc, r.t., 14 h; (d) *rac*-**12**, Et_3N, DMF, r.t., 10 min; (e) $Pd_2(dba)_3$•$CHCl_3$, PPh_3, HCOOH, THF, r.t., overnight.

$Fe(CO)_3$ moiety and *rac*-**14** with the "inner" position of RO group, were prepared according to Scheme 4. Following our reliable protocols for the selective synthesis of acyloxy-substituted cyclohexadiene-$Fe(CO)_3$ complexes in both regioisomeric series (as racemates) we first prepared the TIPS-protected complexes as storable intermediates.[8b] The isomeric TIPS-protected dienol complexes *rac*-**9**[8b] and *rac*-**13**[8b] were then reacted with 4-nitrophenyl chloroformate in the presence of TBAF and DIPEA in THF to give the compounds **11**, *rac*-**12** and *rac*-**14** (Scheme 4).

PT-CORMs **2** and **3** which contain EDC linker were prepared according to Scheme 5. After pre-activating the 2-CTC-resin **5** with $SOCl_2$ as before (Scheme 3) reaction with Fmoc-Lys(alloc)-OH in the presence of DIPEA in DCM afforded the functionalized resin **15** which was further converted to peptide **16** by successive coupling with the amino acids Fmoc-*l*-Phe, Fmoc-*d*-Ala and Ac-Gly according to Scheme 5 (81 % over three steps).

The linker *N*-Boc-*N*-methyl-ethylenediamine **17** was prepared following a literature protocol[23] and attached to peptide **16** using PyBOP as a coupling reagent to afford **18** in 98 % yield. Subsequent Boc-deprotection gave the peptide **19** which was smoothly converted into the PT-CORM precursors **20** and **21** by reaction with complexes **11**/*rac*-**12** and *rac*-**14**, respectively, in the presence of DIPEA in DMF. The separation of **11** and *rac*-**12** was not necessary as both compounds reacted with **19** to give the same product. Finally, Pd-catalyzed alloc-deprotection afforded the target PT-CORMs **2** and **3** (as hydroformate salts) in an isolated yield of 75 and 90 %, respectively (Scheme 5). The linker *N*-Boc-*N*-Me-ethylenediamine **17** was prepared following a literature protocol[23] and attached to peptide **16** using PyBOP as a coupling reagent to afford **18** in 98 % yield. Subsequent Boc-deprotection gave the peptide **19** which was smoothly converted into the PT-CORM precursors **20** and **21** by reaction with complexes **11**/*rac*-**12** and *rac*-**14**, respectively, in the presence of DIPEA in DMF. The separation of **11** and *rac*-**12** was not necessary as both compounds reacted with **19** to give the same product. Finally, Pd-catalyzed alloc-deprotection afforded the target PT-CORMs **2** and **3** (as hydroformate salts) in an isolated yield of 75 and 90 %, respectively (Scheme 5).

For the preparation of PT-CORM **4** alcohol **7** was activated using 4-nitrophenyl chloroformate in the presence of pyridine to give carbamate **22** in 82 % yield (Scheme 6).[21] *N*-Boc-*N*,*N*′-dimethyl-1,2-ethylenediamine **23**[24] was attached to compound **22** in DMF yielding **24**, which was subjected to Boc deprotection under acidic conditions leading to amine **25** in 23 % yield over two steps. Complex **26** was formed by coupling of the conjugate **25** with the organometallic unit *rac*-**12** in the presence of Et_3N in DMF in 85 % yield. After alloc-deprotection the desired target complex **4** was isolated in 80 % yield, again as the hydroformate salt.

Conclusions

We designed a new chemical architecture for potential tissue-selective protease-activated CO-releasing molecules (PT-CORMs) based on our previous experience with acyloxy-cyclohexadiene-$Fe(CO)_3$ complexes as enzyme-triggered CORMs. For

this purpose we connected a plasmin-specifying peptide sequence to the organometallic CORM unit through a self-immolative linker (*p*-aminobenzyl for PT-CORM **1**), 1,2-ethylenediaminecarbonyl for PT-CORMs **2** and **3**, and a combined linker for PT-CORM **4**. These challenging and non-trivial target structures were then synthesized in a straightforward manner exploiting a combination of solid-phase peptide synthesis and solution chemical synthesis. Late-stage attachment of the (racemic) organometallic unit and Pd-catalyzed cleavage of an alloc-protected lysine side chain afforded the target compounds in good yield, which all were isolated as their hydroformate salts in high purity and proved to be stable under ambient conditions.

While the synthesized compounds are currently under biological evaluation, we are confident that the methodology for their synthesis, which we have disclosed here, provides a valuable basis for the synthesis of the development also of other protease-activated CORM prodrugs with tunable pharmacological properties in the future.

Experimental Section

Synthesis of Complex 1: The TIPS-protected complex *rac*-**9** (2 equiv., 0.9 mmol, 353 mg) was dissolved in anhydrous DMF (4.5 mL) under argon atmosphere and the resulting solution was cooled to 0 °C. TBAF (1 M solution in THF, 2.2 equiv., 0.986 mmol, 986 μL) and sodium hydride (60 wt.-% dispersion in mineral oil, 2.2 equiv., 0.986 mmol, 39 mg) were sequentially added and the reaction mixture was stirred for 10 min at 0 °C. Then, a solution of peptide **8** (1 equiv., 0.448 mmol, 300 mg) in anhydrous DMF (4.5 mL) was added, the ice bath was removed and the mixture was stirred overnight. After removing the solvents in vacuo the product was purified by flash chromatography (SiO_2, DCM/MeOH, 15:1) to give crude **10** (275 mg, 70 %) as a pale brownish solid. A flame-dried Schlenk flask was charged with $Pd_2(dba)_3$*$CHCl_3$ (0.1 equiv., 0.020 mmol, 20 mg) and PPh_3 (0.4 equiv., 0.078 mmol, 20 mg) before anhydrous THF (2 mL) and HCOOH (10 equiv., 1.95 mmol, 73.5 μL) were sequentially added. The mixture was stirred for 10 minutes at room temperature before a suspension of **10** (1 equiv., 0.195 mmol, 170 mg) in anhydrous THF (8 mL) was transferred to the stirred mixture in the Schlenk flask containing the Pd-catalyst. The resulting orange-yellow suspension was stirred overnight to form a yellowish gel. The latter was diluted with THF (10 mL) and the precipitate was filtered off using glass frit and washed with THF and Et_2O. The precipitate was left on the air for ca. 30 minutes before the product was washed off the glass frit with MeCN/MeOH (1:1 v/v, 50 mL). The solvents were removed under reduced pressure to give complex **1** (149 mg, 93 %) as a pale yellow solid which did not show any impurities in NMR. **^{1}H NMR** (600 MHz, CD_3OD): δ = 8.56 (s, 1H), 7.62 (d, ^3J = 8.6 Hz, 2H), 7.35 (d, ^3J = 8.6 Hz, 2H), 7.29–7.14 (m, 5H), 5.39 (dd, ^3J = 6.7 Hz, ^4J = 2.2 Hz, 1H), 4.96 (d, ^2J = 11.1 Hz, 1H), 4.70 (d, ^2J = 11.1 Hz, 1H), 4.54 (dd, ^3J = 9.9 Hz, ^3J = 4.9 Hz, 1H), 4.48 (dd, ^3J = 9.5 Hz, ^3J = 4.8 Hz, 1H), 4.21 (q, ^3J = 7.1 Hz, 1H), 3.80 (d, ^2J = 16.5 Hz, 1H), 3.71 (d, ^2J = 16.5 Hz, 1H), 3.51 (dd, ^3J = 5.3 Hz, ^4J = 2.2 Hz, 1H), 3.26 (dd, ^2J = 14.1 Hz, ^3J = 4.9 Hz, 1H), 2.99 (dd, ^2J = 14.1 Hz, ^3J = 9.9 Hz, 1H), 2.96–2.89 (m, 2H), 2.87–2.82 (m, 1H), 2.01–1.91 (m, 1H), 1.95 (s, 3H), 1.90–1.80 (m, 1H), 1.77–1.64 (m, 4H, 24-H), 1.62–1.40 (m, 4H, 23-H), 1.20 (d, ^3J = 7.1 Hz, 3H). **^{13}C NMR** (126 MHz, CD_3OD): δ = 212.9, 175.7, 173.9, 173.7, 172.1, 171.8, 140.4, 139.4, 138.5, 133.6, 130.2, 129.8, 129.5, 127.8, 121.4, 70.4, 70.2, 56.7, 55.1, 52.7, 51.0, 43.5, 40.6, 37.8, 32.1, 28.2, 25.8, 24.5, 23.8, 22.5, 17.4. **HRMS** (ESI): calcd. for $C_{38}H_{47}FeN_6O_9$ [M – HCOO]$^+$ 787.2748, found 787.2749. **IR** (ATR): $\tilde{\nu}$ = 2037 (s, $Fe(CO)_3$), 1952 (s, $Fe(CO)_3$) cm^{-1}.

Synthesis of the Acyloxydiene-Fe(CO) Building Blocks 11/rac-12: A solution of *rac*-**9** (1 equiv., 5 mmol) in anhydrous THF (50 mL) was cooled down to 0 °C and TBAF (1 M in THF, 1.05 equiv., 5.25 mmol, 5.25 mL) was added dropwise. The mixture was stirred for 10 min before DIPEA (4 equiv., 20 mmol, 3.39 mL) and 4-nitrophenyl chloroformate (4 equiv., 20 mmol, 4.04 g) were sequentially added. After stirring the suspension for 1.5 h at 0 °C the solvent was removed under reduced pressure and the residue was subjected to column chromatography on silica gel (Cy/EtOAc, 5:1) to yield compound **11** (550 mg, 1.11 mmol, 22 %) as a yellowish solid and compound *rac*-**12** (750 mg, 1.87 mmol, 37 %) as a brown solid. **Analytical data for 11**: **^{1}H NMR** (300 MHz, $CDCl_3$): δ = 5.67 (ψ s, 2H), 3.51 (ψ s, 2H), 3.02–2.76 (m, 2H), 1.96–1.67 (m, 4H), 1.70–1.38 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ = 210.3, 129.2, 79.5, 57.9, 52.0, 25.0, 23.5. **HRMS** (ESI): calcd. for $C_{19}H_{15}O_9Fe_2$ [M + H]$^+$ 498.9409, found 498.9413; calcd. for $C_{19}H_{14}O_9Fe_2Na$ [M + Na]$^+$ 520.9229, found 520.9232. **IR** (ATR): $\tilde{\nu}$ = 2042 (s, $Fe(CO)_3$), 1950 (s, $Fe(CO)_3$), 1772 (C=O) cm^{-1}. **Analytical data for *rac*-12: ^{1}H NMR** (300 MHz, $CDCl_3$): δ = 8.31 (d, ^3J = 9.1 Hz, 2H), 7.45 (d, ^3J = 9.1 Hz, 2H), 5.72 (dd, ^3J = 6.5 Hz, ^4J = 2.5 Hz, 1H), 3.55 (ψ q, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 1H), 2.92 (dt, ^3J = 6.5 Hz, ^3J = 2.5 Hz, 1H), 1.96–1.69 (m, 2H), 1.68–1.36 (m, 2H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ = 210.2, 155.2, 151.4, 145.9, 129.2, 125.6, 121.8, 79.6, 57.9, 57.9, 24.9, 23.51. **MS** (EI, 70 eV): *m/z* (%) = 473 ([M – CO]$^+$), 345 ([M – 2CO]$^+$), 317 ([M – 3CO]$^+$, 100 %), 285, 261 ([M – $Fe(CO)_3$]$^+$). **IR** (ATR): $\tilde{\nu}$ = 2060 (s, $Fe(CO)_3$), 1969 (s, $Fe(CO)_3$), 1762 (C=O), 1520 and 1348 (m, NO_2) cm^{-1}.

Synthesis of Complex 2: Peptide **19** (1 equiv., 0.61 mmol, 390 mg) was dissolved in anhydrous DMF (6 mL) under argon. Then a solution of a mixture of **11** (0.45 equiv., 0.273 mmol, 136 mg) and *rac*-**12** (0.86 equiv., 210 mg, 0.524 mmol) in anhydrous DMF (6 mL) was added followed by DIPEA (10 equiv., 6.1 mmol, 1.06 mL). The resulting dark orange solution was stirred for 1 h at room temperature. Then DMF was removed under reduced pressure (vacuum line, bath temperature 45–50 °C) and the residue was subjected to column chromatography on silica gel (DCM/MeOH, 10:1) to yield **20** (350 mg, 66 %, as a 1:1 mixture of epimers) as a white solid. A flame-dried Schlenk flask was charged under argon with $Pd_2(dba)_3$*$CHCl_3$ (0.1 equiv., 0.05 mmol, 52 mg) and PPh_3 (0.4 equiv., 0.2 mmol, 52 mg) before anhydrous THF (5 mL) and HCOOH (10 equiv., 5 mmol, 189 μL) were sequentially added, and the mixture was stirred for 10 minutes at room temperature. Then, a suspension of **20** (1 equiv., 0.5 mmol, 433 mg) in anhydrous THF (15 mL) was transferred to the stirred mixture in the Schlenk flask containing the Pd-catalyst. The resulting orange-yellow suspension was stirred overnight to form a brown gel-like mixture. The latter was diluted with MeOH (10 mL), and after addition of silica gel the solvents were evaporated to yield the crude product directly absorbed on silica gel. The product was finally isolated using column chromatography on silica gel (EtOAc/MeCN/MeOH/HOH, 3:1:1:1 + 0.05 vol.-% HCOOH). The solvents were removed under reduced pressure using MeCN for azeotropic removal of water. The resulting solid was dissolved in HPLC-grade MeOH/MeCN (1:1 v/v) and the solution was filtered through a syringe filter (0.2 μm, PTFE). After removal of the solvents and drying under reduced pressure for 8 h product **2** (302 mg, 73 %) was obtained as a pale yellowish solid (mixture of epimers at the planar chiral unit). **^{1}H NMR** (500 MHz, CD_3OD): δ = 8.48 (s, 1H), 7.80–7.67 (m, 4H), 7.35–7.16 (m, 1H), 5.77–5.70 (m, ≈ 0.5H), 5.70–5.60 (m, ≈ 0.5H), 4.56–4.48 (m, 1H), 4.35–4.28 (m, 1H), 4.22–4.13 (m, 1H), 3.82 (s, 2H), 3.57–3.22 (m, 6H, 12-H_a), 3.02–2.84 (m, 7H), 2.00 (s, 3H), 1.94–1.36 (m, 10H), 1.21–1.16

(m, 3H). **^{13}C NMR**: Multiple signals sets are observed due to the presence of diastereomers and amide rotamers; see the SI. **HRMS** (ESI): calcd. for $C_{35}H_{48}N_7O_{10}Fe$ [M – HCOO]$^+$ 782.2807, found 782.2817; calcd. for $C_{35}H_{47}N_7O_{10}FeNa$ [M – HCOOH + Na]$^+$ 804.2626, found 804.2628.). **IR** (ATR): $\tilde{\nu}$ = 3269 (s br, NH), 2044 (s, $Fe(CO)_3$), 1961 (s, $Fe(CO)_3$), 1635 and 1612 (s, C=O), 1548 (s, C=O) cm^{-1}.

Synthesis of the Acyloxydiene-Fe(CO) Building Block rac-14: A solution of *rac*-**13** (1 equiv., 1 mmol) in anhydrous THF (10 mL) was cooled to 0 °C and TBAF (1 M in THF, 1.05 equiv., 1.05 mmol, 1.05 mL) was added dropwise. After 10 min, DIPEA (4 equiv., 4 mmol, 0.68 mL) and 4-nitrophenyl chloroformate (4 equiv., 4 mmol, 0.81 g) were sequentially added and the resulting suspension was stirred for 1.5 h at 0 °C. After removal of the solvent under reduced pressure the residue was subjected to column chromatography on silica gel (Cy/EtOAc, 5:1) to yield *rac*-**14** (335 mg, 84 %) as a brownish solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ = 8.28 (d, ^3J = 9.1 Hz, 2H), 7.37 (d, ^3J = 9.1 Hz, 2H), 5.55 (d, ^3J = 4.4 Hz, 1H), 5.16 (ψ t, ^3J = 5.1 Hz, 1H), 3.18 (dt, ^3J = 5.1 Hz, ^3J = 2.0 Hz, 1H), 2.43–2.19 (m, 1H), 2.04–1.62 (m, 3H, 6-H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ = 210.8, 155.4, 150.8, 145.7, 125.5, 122.0, 105.0, 81.3, 79.7, 61.1, 26.4, 24.0. **IR** (ATR): $\tilde{\nu}$ = 2047 (s, $Fe(CO)_3$), 1970 (s, $Fe(CO)_3$), 1764 (C=O), 1530 and 1347 (m, NO_2), 1207 (s, C-O) cm^{-1}.

Synthesis of Complex 3: Peptide **19** (1 equiv., 0.61 mmol, 320 mg) and *rac*-**14** (1.2 equiv., 0.574 mmol, 230 mg) were dissolved in anhydrous DMF (10 mL) under argon and the solution was cooled to 0 °C. Then, trimethylamine (5 equiv., 2.39 mmol, 333 μL) was added and the mixture allowed to stir for 30 min before the solvent was removed under reduced pressure. Purification by chromatography on silica gel (DCM/MeOH, 10:1) yielded **21** (320 mg, 77 %) as a white solid. Following the same alloc deprotection protocol as described above in the synthesis of complex **2** 0.404 mmol of **21** were reacted to give **3** (250 mg, 90 %) as a colorless solid. **^{1}H NMR** (400 MHz, [D$_6$]DMSO): δ = 8.61–7.90 (m, 6H, NH), 7.33–7.09 (m, 5H), 5.65 (d, ^3J = 4.3 Hz, 1H), 5.36 (ψ s, 1H), 4.47 (m, 1H), 4.30–4.06 (m, 1H), 3.66 (m, 2H), 3.35–3.14 (m, 4H), 3.09 (dd, ^2J = 13.9 Hz, ^3J = 3.9 Hz, 1H), 2.97–2.62 (m, 5H), 2.21–2.00 (m, 1H), 1.93–1.71 (m, 4H), 1.71–1.16 (m, 8H), 0.98 (d, ^3J = 6.9 Hz, 3H). **^{13}C NMR**: Multiple signal sets are observed due to the presence of diastereomers and amide rotamers; see the SI. **HRMS** (ESI): calcd. for $C_{35}H_{48}N_7O_{10}Fe$ [M – HCOO]$^+$ 782.2807, found 782.2809; calcd. for $C_{35}H_{47}N_7O_{10}FeNa$ [M – HCOOH + Na]$^+$ 804.2626, found 804.2620. **IR** (ATR): $\tilde{\nu}$ = 3268 (s br, NH), 2044 (s, $Fe(CO)_3$), 1962 (s, $Fe(CO)_3$), 1662 and 1635 and 1602 (s, C=O), 1548 (s, C=O) cm^{-1}.

Synthesis of Complex 4: To a solution of peptide **25** (1 equiv., 0.074 mmol, 60 mg) in anhydrous DMF (1.6 mL) stirred under argon at room temperature were sequentially added *rac*-**12** (1.5 equiv., 0.111 mmol, 45 mg) and trimethylamine (2 equiv., 0.148 mmol, 21 μL). After 10 minutes the solvent was removed under reduced pressure and the resulting solid was purified by column chromatography on silica gel (DCM/MeOH, 10:1) to give **26** (71 mg of still containing traces of DMF and *p*-nitrophenol; i.e. 64.5 mg, 85 %) as a white solid which was further used following the same alloc deprotection protocol as described above in the synthesis of complex **2**. 0.058 mmol of **26** were reacted to give **4** (46 mg, 80 %) as a pale brownish solid. **^{1}H NMR** (500 MHz, CD_3OD): δ = 8.52 (s, 1H), 7.63 (d, ^3J = 7.7 Hz, 2H), 7.36 (d, ^3J = 7.7 Hz, 2H), 7.29–7.14 (m, 5H), 5.61–5.50 and 5.39–5.32 (both m, overall 1H), 5.24–5.19 and 5.14–5.01 (both m, overall 2H), 4.54 (dd, ^3J = 10.0 Hz, ^3J = 4.8 Hz, 1H), 4.47 (dd, ^3J = 9.8 Hz, ^3J = 4.7 Hz, 1H), 4.21 (q, ^3J = 7.0 Hz, 1H), 3.81 (d, ^2J = 16.4 Hz, 1H), 3.77–3.58 (m, 2H), 3.58–3.20 (m, 5H), 3.04–2.77 (m, 10H), 2.01–1.62 (m, 7H), 1.58–1.37 (m, 6H), 1.20 (d, ^3J = 7.0 Hz, 1H). **^{13}C NMR**: Multiple signal sets are observed due to the presence of diastereomers and amide rotamers; see the SI. **HRMS** (ESI): calcd. for $C_{44}H_{57}N_8O_{12}Fe$ [M – HCOO]$^+$ 945.3440, found 945.3446; calcd. for $C_{44}H_{56}N_8O_{12}FeNa$ [M – HCOOH + Na]$^+$ 967.3259, found 967.3252. **IR** (ATR): $\tilde{\nu}$ = 3268 (s br, NH), 2043 (s, $Fe(CO)_3$), 1961 (s, $Fe(CO)_3$), 1724 and 1698 (m, C=O), 1635 and 1603 (s, C=O), 1544 (s, C=O) cm^{-1}.

Supporting Information (see footnote on the first page of this article): For further experimental details including NMR signal assignments, see the supporting information.

Acknowledgments

This work was supported by the Alexander von Humboldt foundation (postdoctoral fellowship to N. S.), the Deutsche Forschungsgemeinschaft (Project SCHM857/20-1) and the Russian Foundation for Basic Research (Project №18-503-12087).

Keywords: CO releasing molecules · Peptides · Iron complexes · Enzymes · Protecting groups

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Received: August 16, 2019

Peptide Conjugates

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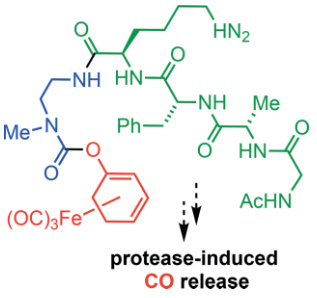
VIP

Design and Synthesis of New Protease-Triggered CO-Releasing Peptide–Metal-Complex Conjugates



Potentially tissue-selective protease-activated CO-releasing molecules (PT-CORMs) were designed and synthesized by conjugating a protease-specifying peptide to an oxy-cyclohexadiene-$Fe(CO)_3$ unit through a self-immolative linker.

DOI: 10.1002/ejoc.201901206