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Synthesis of an EDTA-like Chelating Peptidomimetic Building Block Suitable for Solid-Phase Peptide Synthesis

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A *novo* trifunctional EDTA-like peptidomimetic amino acid is described. This unique building block, which prepared in a straighforward manner from commercialized starting materials, contains three moieties: a hexadentate chelating unit similar to that present in EDTA, and also, the amino and carboxylic groups, which faciliate its introduction into the backbone of peptides using conventional SPPS. As a proof of concept, this building block is introduced into a cyclic peptide inspired in the family of Gratisin analogues. The designed peptide ~~model~~ contains the amino acid analogue in one of the turns, and chelates Ca^{2+} with nanomolar affinity at physiological pH.

Bifunctional chelating agents (BFCAs) derived from polyamino polycarboxylic ligands are efficient and widely used for the coordination of metal ions.[1] In addition to a chelating moiety, BCFAs have only one functional group, which allows for covalent attachment to biomolecules—mostly peptides. The resulting conjugates attract interest in biomedicine as metal-based pharmaceuticals, with applications such as metal-labeled drugs for diagnostic purposes or radioactively labeled drugs for therapy.[2] In these cases, the metal ion coordination with BCFAs is mostly restricted to the peptide N or C termini. To expand the potential use of these ligands, we sought to design a chelating agent (CA) that can be introduced into the backbone of peptides using conventional SPPS.[3]

As the building block, we envisioned an EDTA-like molecule, which provides all six coordination sites of EDTA and is modified to contain an additional amino and an additional carboxylic groups crafted to the pendant arms of the EDTA-like moiety.

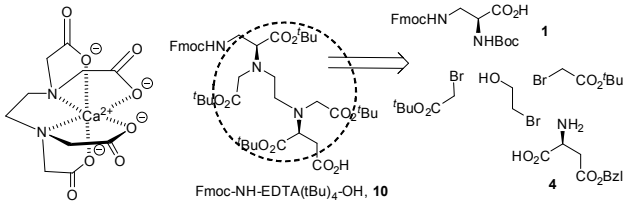

Figure 1. EDTA and Fmoc-NH-EDTA(tBu)$_4$-COOH **10**

Here we report the synthesis of Fmoc-NH-EDTA(tBu)$_4$-COOH **10**, its introduction into a cyclic model peptide, and the characterization of the peptide´s Ca^{2+} chelation at physiological pH by CD, NMR and ITC. Compound **10** has the EDTA substructure running through its backbone, which is, in turn, stable to proteolytic degradation (Figure 1). The functional groups are adequately protected for standard coupling conditions used in solid-phase peptide synthesis (SPPS).

The building block **10** is derived from diamino propionic acid (Dap) **1** and aspartic acid (Asp) **4** derivatives, connected by an ethylene bridge, and decorated with additional carboxymethyl substituents (Figure 1). We attempted several synthetic strategies to prepare **10** from commercially available compounds. However, only one rendered **10** efficiently (Scheme 1). The intermediate bromo-product **8** is described in the literature and can be obtained in 25-30% yield starting from H-Asp(Bzl)-OH after three steps.[4] It represents the Asp-part of the target molecule carrying the terminal bromo-substituted ethylene bridge to be connected to the Dap-part **3**. For the synthesis of enantiomerically pure **2**, we used the $HClO_4$-catalyzed transesterification of *tert*-butylacetate and **1** to satisfy the requirement of epimerization-free conditions for *tert*-butyl ester formation.[5]

The reaction of **2** with *tert*-butylbromoacetate to render **3** by stirring in ACN-phosphate buffer mixture (a heterogenic

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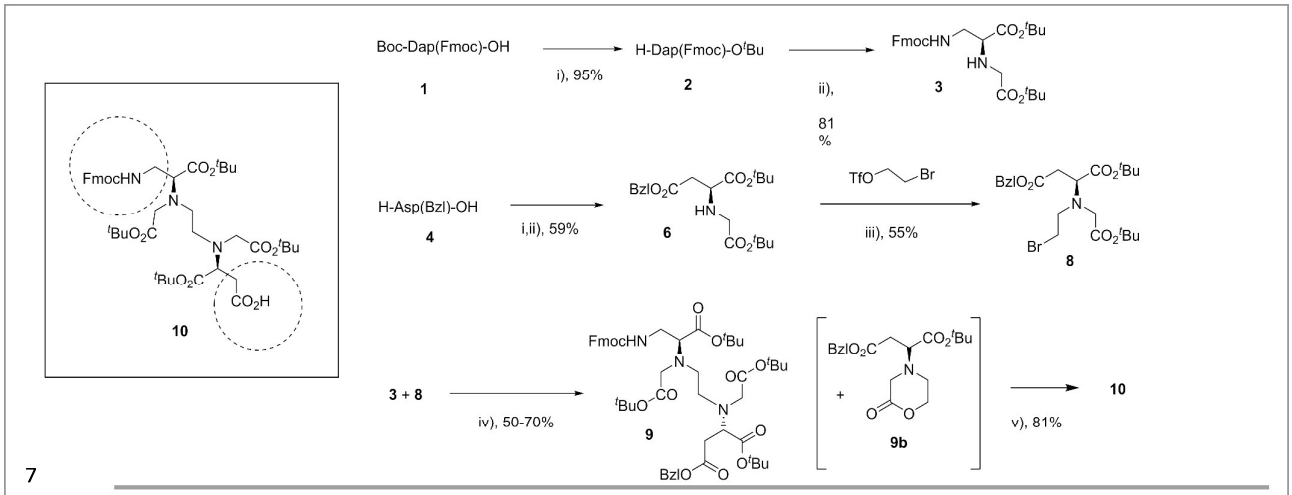

- Scheme 1. i) *tert*-butylacetate/70% aqueous $HClO_4$, 15 h, r.t.; ii) *tert*-butyl bromoacetate/ACN/2M phosphate buffer, r.t., 15 h – 3 days; iii) toluene/2,6-lutidine, -15ºC to r.t., 20 h; iv) ACN/2M phosphate buffer, r.t., 3-5 days; v) H_2/Pd-C/EtOH, 1 atm, r.t., 30 min – 3 h.

system, "Rapoport-conditions") proceeded slowly and had to be stopped when *N*-disubstitution started at the expense of *N*-monosubstituted product (monitored by HPLC). The mildly basic conditions do not affect the Fmoc-group.[6]

The reaction between **3** and **8** to prepare **9** using Rapoport-conditions is a slow but continuous process that requires several days. Added excesses of **8** can be partially recovered by column chromatography when isolating **9**. Under these conditions, parts of **8** undergo intramolecular cyclization to render morpholine **9b**, which cannot be separated from **9**.[7]

Among the conditions tested for the removal of the Bzl-group from **9** by catalytic hydrogenation**,** the best results were obtained with Pd-C at atmospheric pressure in EtOH and monitoring the conversion by HPLC-UV**.** Otherwise, hydrogenolysis of the Fmoc-group occurs. After flash chromatography, **10** was obtained with 31-43% yield in 4 steps and starting from **1** and **8**.

Once the building block was obtained, we characterized its incorporation into a cyclic peptide inspired in the antibiotic Gratisin family, well-studied in the literature, and with a high tendency to populate beta-hairpin structures.[8] The sequence of this antibiotic is $(D\text{-Phe-Pro-Val-Orn-Leu})_2$ and is known to populate an amphiphilic beta-sheet conformation in solution, with two hairpins whose formation is favored by the presence of a D-Phe followed by Pro residue in the sequence. We decided to introduce the new amino acid analogue in this scaffold, in the second hairpin, substituting one of the D-Phe-Pro motifs. The logic behind this choice is to introduce an unambiguously measurable effect resulting from the metal binding such as an increase in the flexibility of the peptide directly related to Ca^{2+} coordination. We have also substituted both Orn by Ala residues in positions 2 and 7, in order to exclude unspecific interactions between the NH_2 group of the Orn residues with the carboxylic groups present in the EDTA amino acid analogue. Therefore, the model cyclic peptide (**cP)** sequence is composed by 8 aliphatic amino acids connected to the pendant arm functionalities of **10**, with the substructure of the EDTA analogue running through the peptide backbone. The sequence of the model peptide ensures that at physiological pH, any interaction with metal ions will take place exclusively at the analogue.

The linear precursor of **cP** was synthesized by stepwise standard Fmoc-SPPS on 2-chlorotrityl (2-CTT) resin (Scheme 2). As **cP** is a cyclic peptide, its synthesis can start at any of the amino acids of the sequence. Starting by loading **10** on the 2-CTT resin has several advantages. First, only 1 equiv. of **10** is required for the loading step (introduction in a later position would require excess), and second, the EDTA amino acid does not undergo epimerization when activated for cyclization. The elongation of the peptide chain took place smoothly using DIC-OxymaPure as coupling cocktail,[9] and after removal of the Fmoc of the last residue (Val), the protected peptide was cleaved from the resin with 2% TFA in DCM. Cyclization was carried out using PyBOP and HOBt following the usual conditions developed in our laboratory.[10] Fully protected intermediate **cP** was purified by semi-preparative HPLC. Removal of *tert*-butyl protecting groups from the EDTA moiety was slow (15 hr). After lyophilization, HPLC pure **cP** was obtained in 19% overall yield from the crude linear precursor.

To detect and quantify whether **cP** binds Ca^{2+} at pH7, we used Isothermal Titration Calorimetry (ITC) experiments. An aqueous solution of **cP** in Tris-HCl buffer at 25ºC and 37ºC was titrated with increasing amounts of Ca^{2+}, displaying dissociation constants of 40 and 70 nM, respectively (SI).

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The CD spectrum of **cP** showed a minimum at 200 nm and a weak negative band around 220 nm at pH 7.2 (Scheme 2, bottom) in agreement with the presence of β–turn conformations in solution. Interestingly, the addition of Ca^{2+}, (or of other metal salts), the band at 220 nm disappeared and the CD curve observed resembles that of random coil samples. This process was reversible, and the presence of β–turn conformations are detected when the solution was either acidified or in the presence of excess of EDTA.

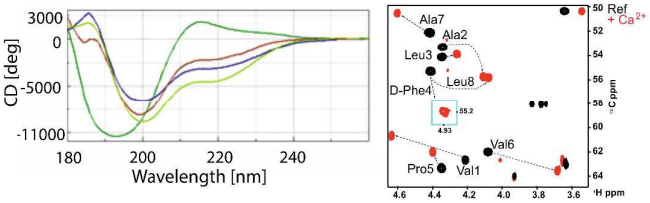
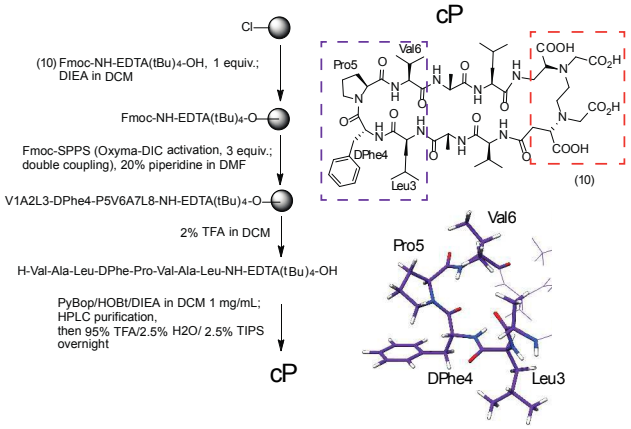


Figure 2. CD and NMR titration of **cP** in the presence or absence of Ca^{2+}. On the left: Light green: **cP** (0.1 mM) in 10 mM phosphate buffer pH 7.2. Dark green: **cP** + 1 equiv. $Ca(tfms)_2$. After addition of $Ca(tfms)_2$, the solution was acidified with $HClO_4$ to pH1 (blue), or EDTA was added (red). On the right 13C-HSQC expansion of **cP**. Changes are indicated with arrows.

The influence of bound Ca^{2+} on the conformational behavior of **cP** was further studied by bidimensional NMR experiments (TOCSY, COSY, ROESY, and ^{1}H ^{13}C HSQC). In the Ca^{2+}-free sample, NMR parameters such as H_α and C_α chemical shifts, $^{3}J_{\alpha NH}$ coupling constants, NH amide temperature coefficients, and NOE patterns, suggest the presence of β–like structures in the Leu3-Ala7 segment as well as backbone flexibility in and around the second hairpin where the aa analogue 10 is introduced, (Supplementary Information).



Scheme 2. SPPS of the peptide **cP,** sequence and hairpins. The DPhe-Pro configuration is trans (highlighted in the 3D representation shown below the **cP** sequence)

Addition of Ca^{2+} induced major changes in the NMR spectra of **cP**. In agreement with the nM affinity obtained by ITC experiments, Ca^{2+}-binding to **cP** showed slow exchange behavior in the NMR time scale. Consistent with the CD results, the NMR analysis also suggests that Ca^{2+}-binding distorts the β-turn-like structure of **cP**, as deduced from C_α chemical shifts and $^{3}J_{\alpha NH}$ coupling constants corroborating that Ca is bound by the BFCA analogue, even under the restraints imposed by its location in the cyclic peptide.

In summary, we describe here a method for preparing the EDTA amino acid building block Fmoc-NH-EDTA(tBu)$_4$-COOH **10** from commercial sources efficiently. This building block can be used in standard SPPS, as we have demonstrated with the cyclopeptide **cP**. We also showed the effectiveness of Ca^{2+} chelation of this analogue, even under severe steric constraints, as those imposed by its location at one of the hairpins of the peptide model used as an example. We believe that the use of **10** as a BFCA will open new possibilities for the preparation of peptide analogs modified to efficiently interact with metal ions along the backbone.

Notes and references

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