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Authors: Uwe Seibold, Björn Wängler, and Carmen Wängler

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Rational Design, Chemical Development and Stability Assessment of a New Macrocyclic, Four-Hydroxamates-Bearing Bifunctional Chelating Agent for ^{89}Zr

Uwe Seibold[a], Björn Wängler[b], Carmen Wängler*[a]

Abstract: ^{89}Zr is a positron-emitting radionuclide being of high interest for medical imaging applications with Positron Emission Tomography (PET). For the introduction of this radiometal into biologically active targeting vectors, the chelating agent desferrioxamine B (DFO) is commonly applied which is however known to form ^{89}Zr-complexes of limited *in vivo* stability. We herein describe the rational design and chemical development a new, macrocyclic, four hydroxamates-bearing chelating agent (CTH36) for the stable complexation of Zr^{4+}. For this purpose, we first performed computational studies to determine the optimal chelator geometry before we developed different synthesis pathways towards the target structures. The best results were obtained using an efficient solution phase-based synthesis strategy towards the target chelating agent. To enable an efficient and chemoselective conjugation to biomolecules, a tetrazine-modified variant of CTH36 was also developed. The excellent conjugation characteristics of the so-functionalized chelator were demonstrated on the example of the model peptide TCO-c(RGDfK). In the following, we determined the optimal ^{89}Zr-radiolabeling parameters for CTH36 as well as its bioconjugate and found that the ^{89}Zr-radiolabeling proceeds efficiently under very mild reaction conditions. Finally, we performed comparative complex stability tests for ^{89}Zr-CTH36-c(RGDfK) and ^{89}Zr-DFO-c(RGDfK), showing an improved complex stability for the newly developed chelator CTH36.

Introduction

^{89}Zr is a radiometal with high relevance for PET imaging applications due to its long half-life of 3.27 days, matching the biological half-life of slowly-accumulating biomolecules such as antibodies. Thus, ^{89}Zr allows for imaging of biological processes at late time-points after tracer application. Furthermore, the relatively low mean energy of the emitted positrons of 0.389 MeV enables well-resolved PET images using this nuclide.[1]

[a] Dr. U. Seibold, Priv.-Doz. Dr. Carmen Wängler
Biomedical Chemistry, Department of Clinical Radiology and Nuclear Medicine
Medical Faculty Mannheim of Heidelberg University
Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany
E-mail: Carmen.Waengler@medma.uni-heidelberg.de
[b] Prof. Dr. Björn Wängler
Molecular Imaging and Radiochemistry, Department of Clinical Radiology and Nuclear Medicine
Medical Faculty Mannheim of Heidelberg University
Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

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Due to these favorable characteristics, ^{89}Zr has a high potential for clinical applications, being reflected in several human studies.[2]

For the radiolabeling of biomolecules with $^{89}Zr^{4+}$, the chelating agent desferrioxamine B (DFO) is commonly applied. However, it has been shown in many studies that the ^{89}Zr-DFO-complex exhibits an only limited stability under *in vivo* conditions.[3] This is a problem as the liberated $^{89}Zr^{4+}$-ion accumulates in mineral bone over time due to its affinity to hydroxylapatite[4] and deposits significant doses there.[4-5]
A reason for the instability of the ^{89}Zr-DFO-complex lies in the incomplete saturation of the coordination sphere of the radiometal: The oxophilic Zr^{4+} ion prefers an octadentate ligand sphere but is stabilized in its DFO-complex by the three hydroxamates (and thus 6 oxygen atoms only) and two additional anions or water molecules, leaving a considerable cleft in the ligand sphere of the complex.[3c, 6] Such clefts in ligand spheres were however shown before to have a strongly adverse effect on the resulting complex stabilities.[7] Consequently, it was proposed that a chelator comprising four hydroxamate units should able to form ^{89}Zr-complexes of significantly higher stability than DFO.[8]
Besides the incomplete saturation of the coordination sphere of the radiometal by DFO, this chelator exhibits a linear structure which cannot take advantage of the stabilizing macrocycle effect upon metal complexation.

An ideal chelator for $^{89}Zr^{4+}$, being able to form highly stable complexes with the radiometal, would be able to completely saturate the octadentate coordination sphere of $^{89}Zr^{4+}$ and exhibit a cavity size matching the size of the central ion. Thus, no cleft would be present in the ligand sphere, enabling a high *in vivo* stability of the complex due to the complete envelopment of the radiometal ion. Furthermore, as macrocyclic ligands in general give more stable complexes than linear ones,[9] a macrocyclic structure of the chelator could also contribute to a high complex stability due to the macrocycle effect upon complexation.

Although several promising new chelators for $^{89}Zr^{4+}$ have been developed in parallel to our attempts presented here[10] – with some of them showing a very high potential for successful *in vivo* application due to a high stability of the complexes even at late time-points after injection as shown by their antibody conjugates which circulate over days before getting metabolized and excreted[10b, 11] – we followed in this work another approach towards a new chelating agent for $^{89}Zr^{4+}$: We intended to design and synthesize a macrocyclic tetra-hydroxamate with optimal

ring size to completely saturate the oxophilic octadentate coordination sphere of $^{89}Zr^{4+}$ and additionally take advantage of the macrocycle effect upon radiometal complexation. Furthermore, the chelator should be applicable for a chemoselective and highly efficient conjugation to biomolecules. The determination of the ideal (matching) ring size aims at the complete envelopment of the central radiometal ion without resulting in ring strain or entropic effects limiting the resulting complex stability. In the following, we developed an efficient chemical synthesis pathway towards the target chelating agent, conjugated it to a model biomolecule, and performed ^{89}Zr-radiolabeling as well as complex stability tests.

Results and Discussion

As already mentioned, a four hydroxamate-bearing macrocycle should offer ideal properties for the stable complexation of Zr^{4+} under *in vivo* conditions as it should on the one hand be able to completely saturate the coordination sphere of the Zr^{4+} ion and on the other hand enable the formation of stable complexes due to the macrocycle effect.
However, these positive effects on stability can only be taken advantage of if a chelator cavity size is found which fits the ion radius of the central metal ion. Otherwise, the complex geometry gets distorted by ring strain (if the cavity is too small) or entropic effects can impede high complex stabilities (if the cavity is too large and the chelator thus folds too flexibly around the radiometal, leaving room for the interaction of the central ion), resulting in a susceptibility of the complex towards complex challenge and ion release. This can be attributed to an increased probability of interaction of the central ion with other ions or challenging molecules present *in vivo* at the site of incomplete ion enclosure.
Thus, we intended to first determine the ideal cavity size of a potential four hydroxamate-bearing macrocycle for Zr^{4+} before commencing the development of a chemical synthesis pathway towards the target chelator.

Rational design of the four hydroxamate-bearing macrocycle by computation studies
This was achieved by computational studies which are able to predict e.g. the lowest energy and thus highest stability of different comparable complexes. The rational design was based on the following assumptions: *i*) the chelator should comprise four hydroxamates as these structure elements are believed to result in exceptionally high complex stabilities as Zr^{4+} is highly oxophilic and four hydroxamates should be able to completely saturate the postulated eight coordination sites, *ii*) it should be macrocyclic to take advantage of the macrocyclic effect, *iii*) it should be rotationally symmetric to enable the formation of a symmetrical complex, limiting the probability of complex challenge as the coordination sphere is uniformly closed around the central ion, *iv*) it should be as hydrophilic as possible to render it highly biocompatible and soluble in aqueous media.
The computational studies were then performed by semi-empiric geometry optimization calculations using the software MOPAC and the parameter set PM6[12] and based on the structures depicted in figure 1, giving the result that the ring has to consist of at least 36 atoms to minimize ring strain (for details, see SI), a result which was also recently confirmed by another group.[13]
Thus, 36 ring atoms should form the optimal cavity size for the Zr^{4+} ion as this size results on the one hand in a minimal ring strain and on the other hand in less entropy effects than larger ring sizes. This result was likewise found for both Zr-complex templates, Zr-**II** and Zr-**III** (figure 1). Due to the assumption that structure **III** should be more favorable than **II** regarding biocompatibility and solubility of the chelator as well as the complex in aqueous media, **III** should represent the most promising molecular design for the target chelator.

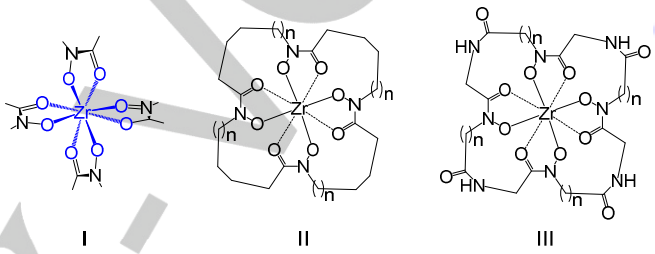


Figure 1. Schematic depiction of the complexation of Zr^{4+} by four isolated hydroxamate units (**I**) and potential macrocyclic, four hydroxamates-comprising chelating agents (**II** and **III**) derived from these isolated hydroxamates. These three structures were used as basis for the semi-empiric MOPAC calculations intended to determine that ring size of **II** and **III** resulting in minimal Zr^{4+}-complex energies and thus maximum complex stabilities. The ideal complex geometry was found for n=4.

In addition, we also performed density functional theory calculations using the 3-21G basis set for the Zr^{4+}-complex of **III** in direct comparison to Zr^{4+}-DFO (for details, see SI) in order to determine if – in contrast to Zr^{4+}-DFO – a complete envelopment of the central metal ion can be achieved using the 36-membered ring. The geometry-optimized structure results of these calculations are depicted in figure 2 and show a completely enveloped Zr^{4+}-ion for the Zr^{4+}-**III**-complex without cleft in the coordination sphere as it is obvious for the Zr^{4+}-DFO-complex.

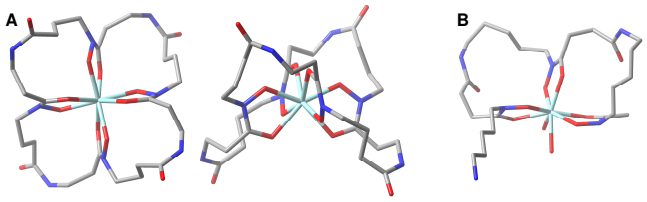


Figure 2. Geometry-optimized structure results of the DFT calculations of the Zr^{4+}-**III**-complex (A) and Zr^{4+}-DFO$(H_2O)_2$ (B). Hydrogen atoms were omitted for clarity.

As consequence of our calculation results and due to the assumption that structure **III** should be more favorable than **II**

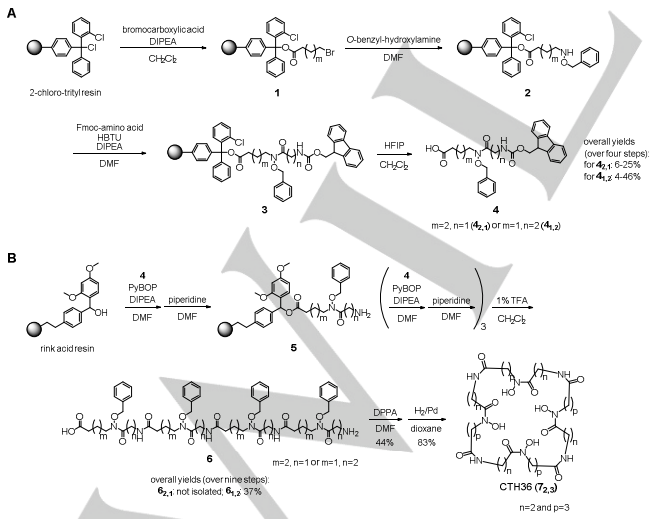
(figure 1) regarding biocompatibility and solubility in aqueous media, we developed in the following a synthesis pathway for the macrocyclic chelator based on structure **III** with a ring size of 36 ring atoms, termed in the following CTH36 (Cyclic Tetra-Hydroxamate with a ring size of 36 atoms).

Synthesis of CTH36 via solid phase-assisted pathways

Initially, we developed a solid phase-assisted synthesis towards CTH36, as by this approach, large excesses of reagent can be applied resulting in principle in high reaction yields and pure products. Furthermore, repeated purifications can be omitted, simplifying and expediting the overall chemical synthesis.

For this solid phase synthesis approach, we intended to develop a procedure for the preparation of a benzyl protected tetra-hydroxamate as a key intermediate for the macrocycle synthesis. This intermediate should in the following be cyclized in solution to the target chelator CTH36. In a first attempt, we tested if it is possible to build the entire hydroxamate tetramer chain by successive coupling of the components 5-bromopentanoic acid, *O*-benzyl-hydroxylamine and Fmoc-glycine on resin. However, this procedure showed to be unsuccessful – despite optimization of several reaction steps – as during every reaction step, side products were formed which resulted in an exponentiating number of side products with increasing chain length.

Therefore, we developed an alternative approach for the solid phase-assisted hydroxamate-tetramer synthesis which consisted of two steps: *i*) the preparation of monomeric protected hydroxamate building blocks on solid support (**4**, scheme 1A), and *ii*) their repeated conjugation on solid support to form the linear tetra-hydroxamates which were intended – after cleavage from the resin in hydroxamate-protected form (**6**) – to be cyclized in solution and deprotected to the target macrocycle CTH36 (scheme 1B).



Scheme 1. Schematic depiction of the solid phase-assisted synthesis towards CTH36 (**$7_{2,3}$**). **A** shows the on-resin-synthesis and cleavage of the protected hydroxamate-building block **4** (for m=2 and n=1 (**$4_{2,1}$**): overall yield of 6–25% over four steps; for m=1 and n=2 (**$4_{1,2}$**): overall yield of 4–46% over four steps). **B** shows the tetramerization of **4**, the synthesis of the benzyl-protected hydroxamate-tetramer **6** (for m=2 and n=1 (**$6_{2,1}$**): percentage of product in raw mixture: 20%, product not isolated; for m=1 and n=2 (**$6_{1,2}$**): overall yield of 37% over 9 steps), its cyclization and deprotection to **$7_{2,3}$** (overall yield of 37% over two steps). DIPEA: *N,N*-diisopropylethylamine, DMF: *N,N*-dimethylformamide, DPPA: diphenyl phosphoryl azide, HBTU: *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol.

In the initial attempt, we used the standard α-amino acid glycine for the synthesis of **$4_{2,1}$** (with the intention to later just replace one glycine unit by a branched amino acid to obtain a backbone-functionalized derivative of CTH36), resulting in a monomeric hydroxamate building block exhibiting a short distance (one methylene unit) between the hydroxamate and the amino functionality (scheme 1A, m=2, n=1). This building block could be synthesized using a 2-chloro-trity resin in four steps in varying yields of 6–25%. From **$4_{2,1}$**, the protected hydroxamate tetramer **$6_{2,1}$** could be assembled on solid support in moderate yields of about 20% as determined by analytical HPLC of the crude product mixture. However, **$6_{2,1}$** showed to be highly susceptible to hydrolysis which prevented a successful purification of the product. This observation is in accordance with previously described findings for the synthesis of DFO-analogs, reporting that the use of α-amino acids can result in sequence deletions or the cleavage of the hydroxamate.[14]

As a result, we developed an analogous synthesis approach using β-alanine and 4-bromo butanoic acid in contrast to the previously applied α-amino acid glycine and 5-bromo pentanoic acid to result in an unaltered ring size of 36 atoms while "shifting" the position of the acid amides within the ring (scheme 1B). Following this strategy, again, the respective hydroxamate monomer building block **$4_{1,2}$** was synthesized first and obtained in varying yields of up to 46%. For the subsequent assembly of the protected hydroxamate-tetramer **$6_{1,2}$** on solid support using the new hydroxamate monomer **$4_{1,2}$**, the synthesis parameters were optimized to maximize product yields and purities. One parameter which showed to influence product specifications was the solid support used and the best results were obtained using a rink acid instead of a 2-chloro-trityl resin. Further improvements were made using PyBOP ((benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) instead of HBTU as activation agent during coupling. By these improvements, the overall synthesis yields for **$6_{1,2}$** (over four steps) were increased to 37%. This new tetra-hydroxamate **$6_{1,2}$** also showed to be much more resistant against hydrolysis than its tautomer **$6_{2,1}$** and could be purified by HPLC without any observed fragmentation.

Before the cyclization of **$6_{1,2}$** to the protected macrocycle intermediate could be performed in solution, the formate counterion – introduced during the HPLC purification of **$6_{1,2}$** – was removed by C18 cartridge purification to prevent the formylation of the linear tetra-hydroxamate during the cyclization reaction. The following cyclization was then performed in solution under similar dilution conditions as described for the

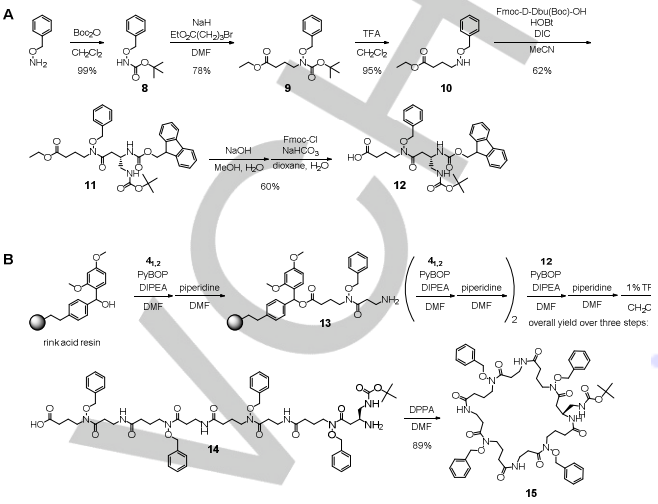
synthesis of the macrocyclic three hydroxamate moieties-containing desferrioxamine-E.[15] We however observed that much longer reaction times of up to 20 days were obligatory to achieve satisfying reaction rates. The following de-benzylation to the target macrocycle CTH36 (**7$_{2,3}$**) (scheme 1B, n=2, p=3) was performed by catalytic hydrogenation under standard conditions. It is noteworthy that the reaction progress had to be monitored carefully as otherwise, – if conducted for long reaction times – a reduction of the hydroxamates took place. This observation is in accordance with former reports.[16]

By this approach, a small amount of the desired macrocyclic target molecule CTH36 (**7$_{2,3}$**) could be obtained. However, the whole multistep preparation of **7$_{2,3}$** unfortunately yielded unsatisfactory low amounts of the target substance due to the very low overall synthesis yield of only 0.3 % (referred to the loading of the starting rink acid solid support). This can first be attributed to the low yields for the solid phase-assisted synthesis of **4$_{1,2}$** and more important to the required excesses of at least 11 equivalents of **4$_{1,2}$** which had to be applied during each step of the tetramerization to **6$_{1,2}$** to achieve complete conversions. Incomplete conversions resulted in product mixtures containing besides the target substance also shorter hydroxamate chains which were nearly inseparable from the target compound.

In parallel, we intended to establish a corresponding synthetic approach towards a backbone-functionalized derivative of CTH36 (fCTH36) (scheme 2B), being applicable for further modification and biomolecule derivatization reactions. For this purpose, an appropriate, branched, hydroxamate-bearing β-amino acid derivative with a suitable side chain functionality for further derivatization and potential conjugation (**12**) had to be synthesized first. This was initially performed analogously to the synthesis of **4$_{1,2}$** on solid support which however showed to be rather inefficient, rendering it difficult to obtain the product in gram scale for the following syntheses. Thus, an alternative solution phase synthesis approach towards the required building block was developed (scheme 2A).

The synthesis of this monomeric hydroxamate building block started from *O*-benzyl-hydroxylamine which was protected to its *N*-(*tert*-butoxycarbonyl) derivative **8** and *N*-alkylated with 4-bromo-butyric acid to **9**, as described before.[17] After Boc-deprotection using dilute TFA, the resulting amine **10** was conjugated to Fmoc-D-Dbu(Boc)-OH similar to a published procedure[18] using HOBt and DIC activation in order to receive the fully protected building block **11** in good yields of 62%. **12** could be obtained from the protected intermediate **11** by treatment with 1M aqueous NaOH solution which removed not only the ethyl ester but also the Fmoc-protecting group which thus had to be re-introduced by reacting the intermediate with Fmoc-Cl in aqueous solution to give **12** in good yields over two steps of 60%.

This building block **12** was in the following applied during the synthesis of the linear, backbone-functionalized tetra-hydroxamate **14** on solid support (scheme 2B) and also during the later on developed solution phase approach (*vide infra*). **14** could be cyclized in solution as established during the synthesis of the non-functionalized macrocycle **7$_{2,3}$** to give the fully protected, backbone-modified macrocycle **15**.



Scheme 2. Schematic depiction of the solution phase synthesis pathway towards the branched, hydroxamate-bearing β-amino acid building block **12** (**A**) and the solid phase-based synthesis towards the protected and backbone-amino-functionalized tetra-hydroxamate macrocycle **15**. DIC: diisopropylcarbodiimide, HOBt: 1-hydroxy-benzotriazole, TFA: trifluoro acetic acid.

Although not only the non-functionalized chelator CTH36 (**7$_{2,3}$**), but also the protected and backbone-functionalized macrocycle **15** could be synthesized using the described solid phase-based synthesis pathway, an alternative, solution phase-based synthesis approach towards CTH36 and fCTH36 had to be developed in order to be able to obtain larger amounts of the target compounds. Of **15**, e.g., only a few milligrams could be obtained although starting with gram amounts of hydroxamate monomers; the obtained product quantities were thus too small for further deprotection, modification and biomolecule derivatization being however the prerequisite for the application of this new chelating agent in molecular imaging.

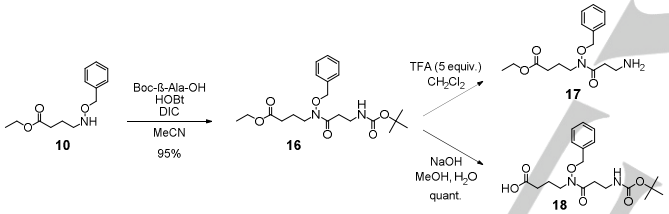
The solid phase-based approach – although being in principle successful – did thus not measure up with our expectations as we initially intended to develop a solid phase-assisted synthesis strategy to achieve high conversion rates and thus high yields for our target compounds, an aim which was not achieved due to a large number of side reactions or required building block excesses.

Solution phase synthesis of CTH36 and its protected, backbone-functionalized analog fCTH36

Thus, a new synthesis route towards CTH36 and its backbone-functionalized analog had to be developed. For this reason, we in the following focussed on the development of a conventional organic liquid phase synthesis – enabling a comparatively convenient upscaling – to significantly improve the synthesis

efficiency and to be able to obtain sufficient amounts of substance for further processing.
A challenge during the development of this new synthesis strategy was the susceptibility of the hydroxamates to various reaction conditions, easily resulting in product fragmentation. Furthermore, a suitable and complementary protecting group chemistry had to be found to enable a chain elongation to the target tetra-hydroxamates without side reactions in order to obtain the desired products with high synthesis efficiency.

The new synthesis strategy comprised – just as the solid phase-based approach – first the synthesis of suitable, protected, hydroxamate monomer building blocks (**17** and **18**, scheme 3) which subsequently can be assembled to the respective hydroxamate di-, tri- and tetramers. Using four non-branched hydroxamate monomer units, the protected, non-backbone-functionalized hydroxamate tetramer **21** (scheme 4) could be obtained. Applying three non-branched hydroxamate monomers and **12**, the protected, backbone-functionalized hydroxamate tetramer **25** (scheme 5) could be synthesized. These protected, linear tetra-hydroxamates were then terminally deprotected and cyclized to the respective protected macrocycles **23** and **26**.
23 was in the following further reacted to the non-functionalized target chelator **24** (CTH36).



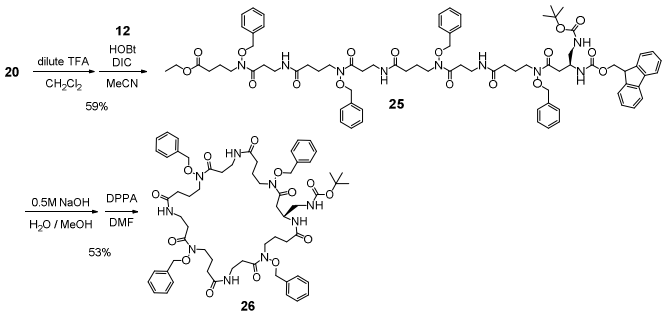
Scheme 3. Schematic depiction of the synthesis of the monomeric hydroxamate building blocks **17** and **18**.

The synthesis of the monomeric hydroxamate building blocks **17** and **18** started from the amine **10** (*vide supra*) which was reacted with Boc-β-alanine using HOBt and DIC activation to receive **16** in high yields of 95%. The complementarily protected monomeric hydroxamate building blocks **17** and **18** were obtained from **16** by treatment with dilute TFA (to obtain the carboxyl-reactive monomer **17**) or NaOH (to obtain the amino-reactive monomer **18**) in almost quantitative or quantitative yields. These monomers could in the following be reacted with each other or **12** to achieve the required chain elongation to the desired linear hydroxamate di-, tri- and tetramer intermediates **19**, **20**, **21** and **25** (schemes 4 and 5).
In detail, the non-backbone-functionalized, protected tetra-hydroxamate **21** was obtained by threefold subsequent reaction of **17** with **18** under standard HOBt / DIC conjugation conditions, giving the linear protected di-, tri- and tetra-hydroxamates **19**, **20** and **21** in high yields of 86%, 97% and 90%, respectively.
It should be noted here – although being self-evident – that it is essential for the amino-deprotected intermediates, which were obtained after treatment of the Boc-protected educts with dilute TFA, to remove remains of TFA completely before the next conjugation step can be performed. For this purpose, the crude deprotected amines were thoroughly washed with aqueous carbonate solution. Otherwise, the TFA remains can significantly decrease the yields of the target compounds due to the formation of trifluoro-amides whose removal from the product mixture is furthermore highly intricate.
The linear, terminally protected tetra-hydroxamate **21** was in the following treated first with acid and then base to remove the Boc protecting group as well as the ethyl ester to give the terminally deprotected intermediate **22**. This intermediate interestingly showed to be only moderately stable in aqueous acid and base and attempts to purify larger amounts than a few milligrams were not successful. However, **22** can also be used without further purification for the following cyclization reaction being performed in diluted solution with DPPA over 18 days, giving the protected macrocycle **23** in 20% overall yield over three steps, starting from **21**.
In the final reaction step, the hydroxamate units were de-benzylated by hydrogenation, giving the deprotected, non-backbone-functionalized macrocyclic tetra-hydroxamate CTH36 (**24**) – which demonstrated to exhibit a poor solubility in most organic solvents and in water – in good yields of 79%.

Scheme 4. Schematic depiction of the solution phase synthesis of the deprotected, non-backbone-functionalized macrocyclic tetra-hydroxamate target chelator CTH36 (**24**).

The corresponding protected, backbone-functionalized macrocyclic tetra-hydroxamate pfCTH36 (**26**), whose backbone amino functionality was intended for further derivatization and finally biomolecule-derivatization, was synthesized in a similar way than **23** (scheme 5) and was the highly stable key intermediate for the development of a suitable conjugation

chemistry of the backbone-functionalized tetra-hydroxamate macrocycle. To obtain **26**, first, the protected, branched tetra-hydroxamate **25** was synthesized from **20** by according synthesis protocols as applied for the synthesis of **21** and obtained in good yields of 59% referring to **20**. **25** was then further reacted under basic conditions to simultaneously remove the ethyl ester as well as the Fmoc protecting group and the obtained intermediate was cyclized without previous purification (due to its instability in solution as observed before for **22**) to give the target intermediate pfCTH36 (**26**) in good yields of 53% over two steps, starting from **25**.



Scheme 5. Schematic depiction of the solution-phase synthesis of the protected, backbone-functionalized macrocyclic tetra-hydroxamate pfCTH36 (**26**).

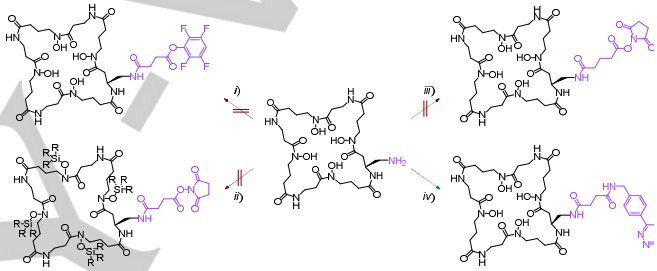
Thus, and although comprising multi-step syntheses and being challenging due to the susceptibility of the hydroxamates, the solution phase-approach towards the target chelators CTH36 and its backbone-functionalized analog pfCTH36 showed to be successful. The synthesis was based on a complementary protecting group chemistry and optimized so that the target chelators could be obtained in satisfactory overall yields.

Development of a suitable conjugation chemistry for the introduction of the backbone-functionalized macrocycle into biomolecules

Next step in the development of a macrocyclic tetra-hydroxamate chelator applicable for the stable ^{89}Zr-labeling of biomolecules was the development of a suitable conjugation chemistry allowing the introduction of a deprotected and activated version of the backbone-modified chelator **26** into biomolecules. This biomolecule conjugation should ideally be able to proceed under mild reaction conditions to principally enable the conjugation not only to relatively robust peptides but also to susceptible biomolecules such as antibodies. Furthermore, the formed conjugates should be stable under *in vivo* imaging conditions. These requirements are fulfilled by different conjugation chemistries and we tested some of them regarding their applicability towards fCTH36 conjugation.

The most established route for DFO introduction into biomolecules is its conjugation via active esters. For this purpose, the terminal amino functionality of DFO is first modified with succinic anhydride to generate a carboxylic acid which can be converted (after temporary blocking of the hydroxamates by Fe^{3+}-complexation) into the respective active ester. After the conjugation of the chelator to the biomolecule, the iron ion can be removed from the complex via an excess of DTPA. This approach – although working quite well for DFO – can however not be adapted to fCTH36 since Fe^{3+} is able to prevent only three of the four hydroxamate units from participating in the activation of the carboxylic acid, resulting in the formation of a significant number of side-products during the formation of an active ester of CTH36.

Our attempts thus focussed on developing another approach to obtain a reactive species of fCTH36 which can be used for the biomolecule conjugation of the chelator. In detail, we tested the following approaches depicted in scheme 6.

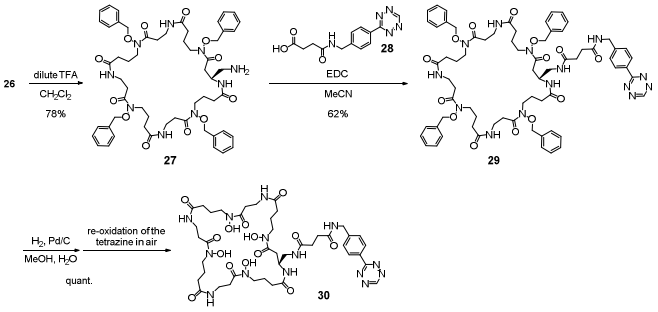


Scheme 6. Schematic depiction of the different attempts made to generate a reactive species of fCTH36 applicable for conjugation of the macrocycle to biomolecules.

Of the studied synthesis pathways, approach *i*) (to activate and conjugate unprotected fCTH36 via its free amino functionality being converted into an active ester *in situ*) was – as expected – not successful due to the formation of a large number of side products; approach *ii*), aiming at the protection of the hydroxamates with highly acid-labile trimethyl-silyl or *tert*-butyl-dimethyl-silyl protecting groups during the conversion of the amino function of the chelator into first an acid and afterwards into an NHS active ester was also not successful as the formation of the target silyl-protected intermediates could neither be proven nor could the products been isolated; approach *iii*), to react the free amino functionality of the chelator with the *bis*-NHS ester di(*N*-succinimidyl) glutarate (with the aim to obtain an NHS active ester derivative of the chelator) demonstrated to be feasible for DFO but not for fCTH36 due to the low solubility of the latter which resulted in such long reaction times of fCTH36 with the *bis*-NHS ester that the formation of a significant number of hydroxamate hydrolysis and other side products was observed.

In contrast to the before mentioned attempts to generate a non-chemoselectively reacting activated species of fCTH36, approach *iv*) finally demonstrated to be successful: the derivatization of fCTH36 with a tetrazine moiety (scheme 7). This activated CTH36 analog is able to react via the inverse

electron demand Diels-Alder (iEDDA) reaction with TCO-modified bioactive molecules (TCO: *trans*-cyclooctene). This iEDDA reaction type has gained increasing popularity over the last years as it proceeds chemoselectively under mild conditions via very fast reactions kinetics and has thus also found widespread application in radiochemistry.[19] Furthermore, another advantage of this tetrazine-derivatized fCTH36 analog is that it does not require blocking or de-blocking steps for the hydroxamate units during and after macrocycle conjugation.



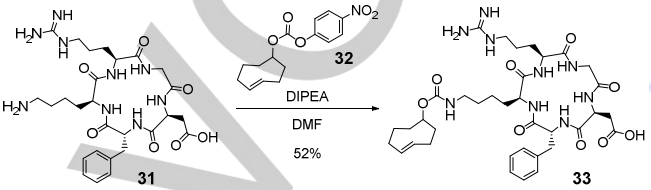
Scheme 7. Schematic depiction of the reaction pathway towards the tetrazine-modified tetra-hydroxamate macrocycle tCTH36 (**30**). EDC: (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide.

In detail, the synthesis of **30** was performed starting from the fully protected backbone-functionalized tetra-hydroxamate intermediate **26** of which the Boc-protecting group was removed by treatment with dilute TFA, yielding **27**. The liberated amino functionality of **27** was in the following reacted with the tetrazine-building block **28**, obtained by the reaction of (4-(1,2,4,5-tetrazin-3-yl)benzylamine with succinic anhydride according to a published procedure.[20] During the conjugation reaction of **28** to **27**, the formation of side products could be observed which likely were formed by rearrangements of **27** under the basic conditions applied. The formed side products could however be separated by semipreparative HPLC from the desired product **29**. In the final reaction step, the benzyl protecting groups were removed from the hydroxamates by hydrogenation, giving **30** in quantitative yields. Interestingly, the tetrazine moiety became also reduced under these conditions (observable by a color shift from pink to colorless during hydrogenation) but readily re-oxidized in air (observable by the reverse color shift from colorless to pink within minutes).

Thus, we were successful in developing an appropriate approach to obtain a highly reactive backbone-functionalized analog of CTH36. With this tetrazine-modified macrocycle tCTH36 at hand, the introduction of the chelator into bioactive molecules became feasible and was performed in the following on the example of a TCO-modified peptide.

Conjugation of tCTH36 to bioactive molecules on the example of TCO-modified c(RGDfK)

To evaluate the chosen tetrazine/*trans*-cyclooctene conjugation method regarding its applicability for the introduction of the newly developed chelator into bioactive molecules, a TCO-modified model peptide was synthesized (TCO-c(RGDfK), **33**). To obtain **33**, first, c(RGDfK) (**31**) was synthesized using standard peptide synthesis methods and the amino functionality of the peptide was in the following reacted with the commercially available TCO-*p*-nitrophenyl active ester **32** (scheme 8). TCO-c(RGDfK) was subsequently used as model reaction partner for tCTH36 (**30**) to show its applicability in biomolecule-derivatization via the chemoselective iEDDA reaction.



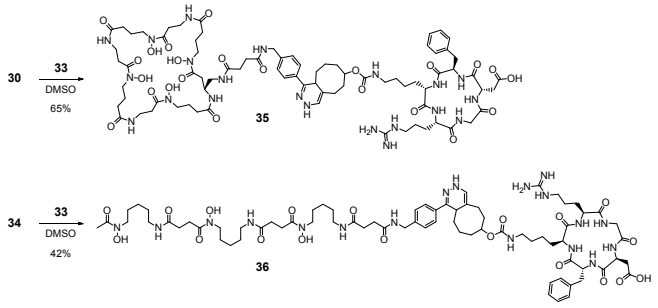
Scheme 8. Schematic depiction of the synthesis of the TCO-modified c(RGDfK) analog **33** used as model biomolecule for the following chelator-conjugation reaction.

Besides evaluation of the chosen approach regarding its applicability to biomolecule modification with tCTH36, we also intended to synthesize a biomolecule conjugate of the chelator to be able to evaluate the ^{89}Zr-radiolabeling properties of the newly developed chelator under radiotracer conditions, where the chelator is usually conjugated to a more complex carrier molecule. Furthermore, a biomolecule conjugate of the chelator further allows to study the ^{89}Zr-complex stability under more realistic radiotracer conditions as if the non-conjugated, "naked" complex were used.

As we further intended to directly compare the ^{89}Zr-complex stability of our new chelator CTH36 to that of DFO, we also synthesized a tetrazine-modified DFO analog tDFO (**34**) which served as the according reaction partner to tCTH36 in the reaction with TCO-c(RGDfK). In detail, the tetrazine-modified DFO analog tDFO (**34**) could be prepared according to published procedures: After reaction of DFO-mesylate with succinic anhydride,[21] the resulting *N*-succinyl-desferrioxamine B was conjugated to the commercially available amino-tetrazine containing building block 4-(1,2,4,5-tetrazine-3-yl)-benzenemethanamine.[22]

Both chelator-tetrazine derivatives tCTH36 (**30**) as well as tDFO (**34**) showed very fast reaction kinetics with TCO-c(RGDfK) (**33**): Both reactions were finished within seconds (comprehensible by means of the immediate decolorization of the solution) and gave the peptide-chelator-conjugates CTH36-c(RGDfK) (**35**) and DFO-c(RGDfK) (**36**) in good yields of 65% and 42%, respectively (scheme 9). As determined by analytical HPLC, the formed products were obtained as a mixture of different isomers and tautomers, an effect having been described for this reaction

type before,[23] and being not adverse especially in case of the in future intended modification of antibodies using this approach.



Scheme 9. Schematic depiction of the conjugation of both chelating agents, tCTH36 and tDFO to TCO-modified c(RGDfK), giving the peptide-chelator-conjugates CTH36-c(RGDfK) (**35**) and DFO-c(RGDfK) (**36**).

Thus, it could be demonstrated that tCTH36 is able to react chemoselectively and with extremely high efficiency with appropriately modified biomolecules.

Using **24** and the CTH36- and DFO-chelator-peptide-conjugates **35** and **36**, we in the following performed the ^{89}Zr-radiolabeling and comparative complex stability experiments.

Determination of optimal ^{89}Zr-radiolabeling conditions, pH dependence of the labeling reaction and comparative complex stability

At first, the optimal ^{89}Zr-radiolabeling conditions were determined for the non-functionalized chelator CTH36 (**24**). In these initial experiments, we observed a rapid and almost quantitative complexation of the radiometal within 30 to 60 minutes under mild reaction conditions such as ambient temperature and neutral pH as it was already reported for DFO.[24]

Thus, the cyclization of the tetra-hydroxamate to a macrocycle did not result in slowed complex formation kinetics resulting in prolonged reaction times or the necessity to perform the radiolabeling reaction at elevated temperatures to achieve a complete radiometal incorporation as it is often observed for macrocyclic chelating agents compared to their linear counterparts.

This is an important finding as the long-term objective for the application of tCTH36 is the derivatization and subsequent ^{89}Zr-radiolabeling of antibodies. ^{89}Zr is ideally suited for radiolabeling and molecular imaging purposes using this biomolecule class as the half-life of the radiometal matches the biological half-life of the antibody molecule well. However, antibodies are susceptible to harsh radiolabeling conditions such as elevated reaction temperatures and highly acidic or basic reaction parameters. Thus, the observed mild and efficient ^{89}Zr-labeling of tCTH36-conjugates is well-compatible with antibody-^{89}Zr-labeling.

In the following, we intended to determine the optimal pH range enabling a highly efficient ^{89}Zr-incorporation into CTH36. For this purpose, **24** was incubated with ^{89}Zr-oxalate in HEPES-buffered (HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) solution at different pH values and the progress of the radiolabeling reactions was monitored by analytical radio-HPLC. The results of these experiments are depicted in figure 3 and show the most efficient radiometal incorporation rates in a pH range of 7 to 9, enabling a complexation of ≥90% of the activity within 5 minutes reaction time at ambient temperature.

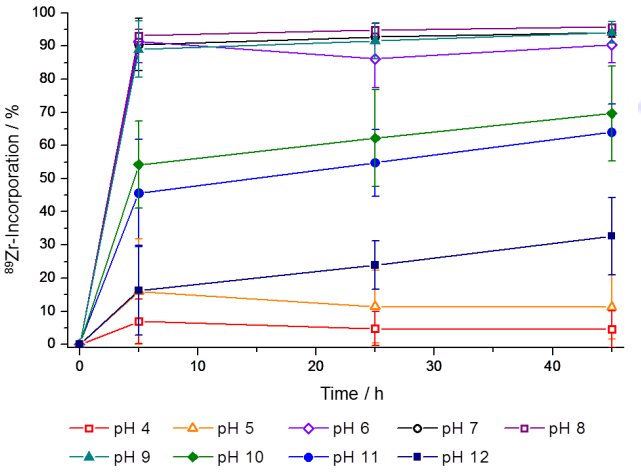


Figure 3. Depiction of the results of the ^{89}Zr-radiolabeling experiments investigating the pH dependence of the CTH36-^{89}Zr-labeling reaction. The values are given ± SD and each experiment was performed thrice.

Next step was the determination of the optimal ^{89}Zr-radiolabeling conditions for the peptide conjugates CTH36-c(RGDfK) (**35**) and DFO-c(RGDfK) (**36**). Both peptide conjugates could be labeled under similar conditions as the non-conjugated chelators at ambient temperature and neutral pH and showed ^{89}Zr-incorporation rates of ≥96% and non-optimized molar activities of 8.4–9.2 GBq/µmol for **[^{89}Zr]35** and 4.0–4.6 GBq/µmol for **[^{89}Zr]36** after a reaction time of one hour, being comparable to the results being recently reported for other ^{89}Zr-bioconjugates.[10, 11b]

With these ^{89}Zr-labeled peptide conjugates, the ^{89}Zr-complex stability of the new chelator CTH36 could be directly compared to that of the commonly used ^{89}Zr-DFO. For this purpose, transchelation challenge experiments were performed: **[^{89}Zr]35** and **[^{89}Zr]36** were incubated with large excesses of EDTA (100-, 1000- and 9000-fold) as competing chelator at neutral pH and ambient temperature. The transchelation rate from **[^{89}Zr]35** and **[^{89}Zr]36** to [^{89}Zr]EDTA was monitored by radio-HPLC and the results of these experiments are depicted in figure 4.

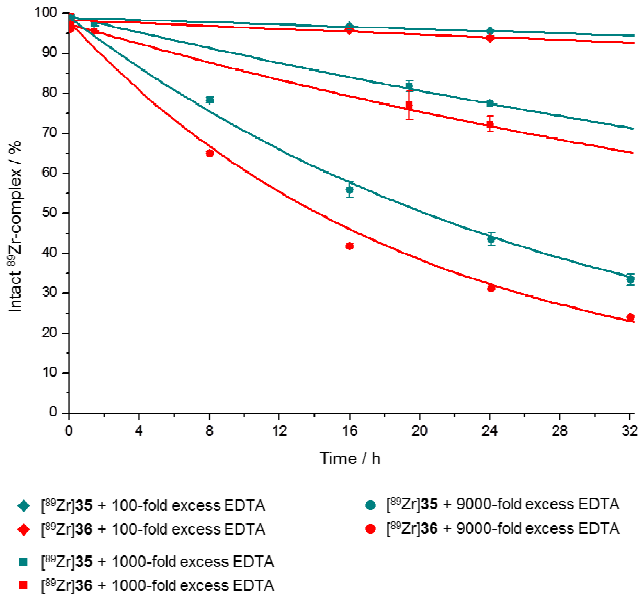


Figure 4. Depiction of the results of the complex challenge experiments of the ^{89}Zr-labeled chelator-peptide conjugates **[^{89}Zr]35** and **[^{89}Zr]36** with 100-, 1000- and 9000-fold excess of EDTA as challenging chelating agent. The graph shows the transchelation rates from **[^{89}Zr]35** and **[^{89}Zr]36** to [^{89}Zr]EDTA as monitored by radio-HPLC. The values are given ± SD and each experiment was performed thrice. Different time points were used for each challenge experiment so that the curves for 100-fold and 1000-fold excess of EDTA were cut off for a clearer overall presentation of the data.

The ^{89}Zr-CTH36-complex in all experiments showed less and slower transchelation than the respective ^{89}Zr-DFO-complex. The difference in transchelation is thereby – as expected – depending on the excess of competing chelator and most pronounced using an excess of 9000 equivalents of EDTA.
These results thus indicate a higher kinetic complex stability for ^{89}Zr-CTH36 compared to ^{89}Zr-DFO. Comparative *in vivo* stability determinations with ^{89}Zr-labeled antibody conjugates of tCTH36 and tDFO and PET imaging studies using these ligands are underway.

Conclusions

We developed a new chelating agent for the complete and symmetrical encapsulation and thus stable complexation of $^{89}Zr^{4+}$. For this purpose, we performed a rational design and determined the optimal target chelator geometry by computational studies. The preparation of the target structures – comprising multi-step syntheses and a sophisticated protecting group chemistry – was successful, showed to be most feasible by solution phase procedures and gave the non- as well as the tetrazine-functionalized chelators CTH36 and tCTH36 in good overall yields. The highly efficient and chemoselective conjugation of tCTH36 to biomolecules could be demonstrated on the example of the bioactive model peptide TCO-c(RGDfK) and the ^{89}Zr-radiolabeling of the new chelator proceeds efficiently under very mild reaction conditions. Complex challenge stability evaluations of ^{89}Zr-CTH36-c(RGDfK) in direct comparison to ^{89}Zr-DFO-c(RGDfK) revealed an improved complex stability for the newly developed chelator ^{89}Zr-CTH36 compared to ^{89}Zr-DFO, indicating its high potential for immuno-PET imaging applications.

Experimental Section

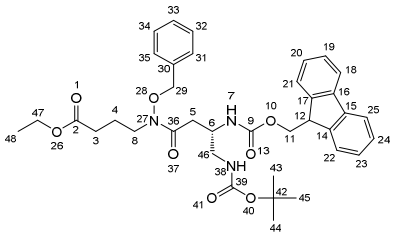
General

All commercially available reagents and solvents were of analytical grade or better and were used without further purification. The resins for peptide synthesis, coupling reagents, and Fmoc-protected amino acids were obtained from NovaBiochem (Darmstadt, Germany). (*S*)-3-(Fmoc-amino)-4-(Boc-amino)butyric acid (Fmoc-D-Dbu(Boc)-OH) was obtained from Iris-Biotech (Marktredwitz, Germany), Desferrioxamine B (DFO) mesylate and Palladium on carbon were obtained from Sigma-Aldrich (Schnelldorf, Germany) and (*E*)-Cyclooct-4-ene *p*-nitrophenyl active ester (**32**) and (4-(1,2,4,5-tetrazine-3-yl)phenyl)methanamine formate were purchased from Sirius Fine Chemicals GmbH (Bremen, Germany). For column chromatography, silica gel 60 for chromatography, 0.040–0.063 mm, 230–400 mesh (Merck, Germany) was used. ^{89}Zr-oxalate solution for radiolabeling experiments was obtained from Cyclotron VU (Amsterdam, Netherlands). The synthons **8**[17a, 17b], **9**[17c], **28**[20] and **34**[3d, 25] were prepared according to published procedures. For analytical and semi-preparative chromatography, a Dionex UltiMate 3000 system was used together with a Chromolith Performance (RP-18e, 100–4.6 mm, Merck, Germany) and a Chromolith (RP-18e, 100–10 mm, Merck, Germany) column, respectively, operated at a flow rate of 4 mLmin^{-1} and H_2O and MeCN +0.1% formic acid as eluents. For radio-HPLC chromatography, a Dionex UltiMate 3000 system was used together with a Phenomenex Gemini (5µm, C18, 250–4.6 mm) column, operated at a flow rate of 2 mLmin^{-1} (5 min at 15% MeCN in H_2O with 0.1% TFA, followed by 15 to 50% MeCN in H_2O with 0.1% TFA within 15 minutes as the gradient). Unless otherwise stated, product purities were ≥95%. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometric data were collected using a Bruker Daltonics Microflex spectrometer (Bremen, Germany). NMR spectra were recorded on a Varian 500 MHz NMR system.

General description of the deprotection and coupling procedure applied during the synthesis of substances 10, 11, 16, 17, 19, 20, 21, 25 and 27. Deprotection: To a solution of Boc-amino ester (**9**, **16**, **19**, **20**, **21** and **26**; 1.0 equiv.) in a small amount of CH_2Cl_2 (just enough to solve the educt), TFA (5–8 equiv.) was added and the mixture was stirred for 5 h at room temperature before being evaporated to dryness. The residue was redissolved in a small amount of CH_2Cl_2 and washed three times with 10% Na_2CO_3-solution to remove the TFA completely. After evaporation of the organic solvent, the resulting deprotected amino ester was used for further coupling without purification. Coupling: Amino ester (**10**, **17**; 1.0 equiv.), protected amino acid (Boc-*β*-Alanine, **12**, **18**; 1.02 equiv.) and 1-hydroxybenzotriazole (0.1 equiv.) were dissolved in ice-cooled, dry acetonitrile. *N,N'*-diisopropylcarbodiimide (1.1 equiv.) was added and the reaction mixture was kept at 0–3°C for 4 h and in the following at ambient temperature overnight. The formed precipitate was removed by filtration and the solution was concentrated *in vacuo* before the product was purified by column chromatography on silica gel. Details for every substance are given in the following.

Ethyl-(*S*)-4-(*N*-(benzyloxy)-4-(Boc-amino)-3-(Fmoc-amino)-butanamido)-butanoate (11).

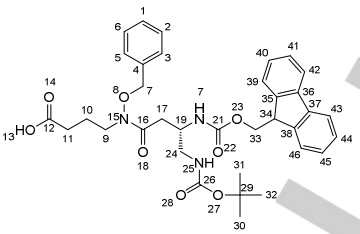
11 was obtained as described before in the general description by deprotection of Boc amino ester **9** (2.64 g, 11.13 mmol) and condensation with (*S*)-3-(Fmoc-amino)-4-(Boc-amino)butyric acid (5.00 g, 11.35 mmol). The product was purified by column chromatography using cyclohexane / ethyl acetate 1:1 as eluent and obtained as colorless oil in 62% yield (4.53 g, 6.90 mmol).



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.76 (d, ^3J(H,H)=7.4 Hz, 2H, H-18 + H-25), 7.60 (d, ^3J(H,H)=7.4 Hz, 2H, H-21 + H-22), 7.39 (td, $^{3,4}J$(H,H)=6.8 + 1.2 Hz, 2H, H-19 + H-24), 7.37 (s$_{broad}$, 5H, H-31 – H-35), 7.30 (td, $^{3,4}J$(H,H)=7.4 + 1.2 Hz, 2H, H-20 + H-23), 5.95 (s$_{broad}$, 1H, H-7), 4.99 (s$_{broad}$, 1H, H-38), 4.80 (s, 2H, H-29), 4.35 (dt, $^{3,4}J$(H,H)=7.6 + 3.2 Hz, 2H, H-11), 4.20 (t, ^3J(H,H)=7.0 Hz, 1H, H-12), 4.10 (q, ^3J(H,H)=7.1 Hz, 2H, H-47), 4.04 (m, 1H, H-6), 3.70 (m, 2H, H-8), 3.33 (m, 2H, H-46), 2.82 (d$_{broad}$, ^2J(H,H)=15.4 Hz, 1H, H-5b), 2.55 (dd, $^{2,3}J$(H,H) = 16.0 + 6.0 Hz, 1H, H-5a), 2.33 (t, ^3J(H,H)=7.2 Hz, 2H, H-3), 1.96 (p, ^3J(H,H)=7.0 Hz, 2H, H-4), 1.43 (s, 9H, H-43 – H-45), 1.21 (t, ^3J(H,H)=7.1 Hz, 3H, H-48). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.00 (C-2 + C-36), 156.24 + 156.74 (C-9 + C-39), 144.12 (C-14 + C-17), 141.40 (C-15 + C-16), 134.17 (C-30), 129.46 (C-32 + C-34), 129.23 (C-33), 128.89 (C-31 + C-35), 127.77 (C-19 + C-24), 127.16 (C-18 + C-25), 125.32 (C-20 + C-23), 120.05 (C-21 + C-22), 79.74 (C-42), 76.47 (C-29), 66.89 (C-11), 60.61 (C-47), 49.29 (C-6), 47.36 (C-12), 44.57 (C-8), 43.82 (C-46), 34.09 (C-5), 31.52 (C-3), 28.49 (C-43 – H-45), 22.25 (C-4), 14.31 (C-48). MALDI-MS (calculated): *m/z*=660.27 [M+H]$^+$ (660.32), 682.32 [M+Na]$^+$ (660.31), 698.40 [M+K]$^+$ (698.28).

(*S*)-4-(*N*-(benzyloxy)-4-(Boc-amino)-3-(Fmoc-amino)-butanamido)-butanoic acid (12).

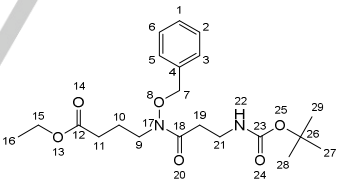
11 (4.53 g, 6.87 mmol) was treated with aqueous sodium hydroxide solution (1M, 2.75 equiv., 18.9 mL, 18.9 mmol) and afterwards, the mixture was diluted with methanol (4 mL) and stirred overnight at ambient temperature to remove the ethyl ester. Under these conditions, the Fmoc-protecting group is removed as well. The resulting suspension was concentrated *in vacuo* and stored at 3 °C for several hours. The precipitate was removed by filtration and rinsed with H_2O (thrice ~10 mL). The resulting, cloudy solution was filtrated over Celite, the Celite was washed with H_2O (twice ~50 mL) and the solution was concentrated to a volume of 40 mL. $NaHCO_3$ (4 g, 47.6 mmol) was added and the mixture was cooled in an ice bath. To this mixture was dropwise added a solution of Fmoc-Cl (2.32 g, 9.00 mmol) in dioxane. After complete addition, the reaction mixture was stirred another two hours at 0–4 °C and overnight at ambient temperature before it was lyophilized to a colorless solid, which was extracted two times with diethylether (100 mL). This solution was further diluted with ethyl acetate (100 mL) and acidified to pH 2 with aqueous sodium hydrogen sulfate solution (20%). The aqueous layer was separated and extracted with ethyl acetate (50 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated to dryness. The product was purified by column chromatography with ethyl acetate / cyclohexane 1:1 containing 1 % acetic acid as eluent and obtained as colorless, non-crystalline solid in 60% yield (2.62 g, 4.12 mmol).



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=8.37 (s$_{broad}$, 1H, H-13), 7.75 (d, ^3J(H,H)=7.4 Hz, 2H, H-42 + H-43), 7.59 (d, ^3J(H,H)=7.4 Hz, 2H, H-39 + H-46), 7.38 (td, $^{3,4}J$(H,H)=7.6 + 1.3 Hz, 2H, H-41 + H-44), 7.35 (s$_{broad}$, 5H, H-1 – H-3 + H-5 + H-6), 7.29 (td, ^3J(H,H)=7.4 + 1.3 Hz, 2H, H-40 + H-45), 6.05 (s$_{broad}$, 1H, H-20), 5.05 (s$_{broad}$, 1H, H-25), 4.79 (s, 2H, H-7), 4.33 (d, ^3J(H,H)=7.2, 2H, H-33), 4.18 (t, ^3J(H,H)=7.0 Hz, 1H, H-34), 4.04 (s$_{broad}$, 1H, H-19), 3.71 (t$_{broad}$, ^3J(H,H)=5.4 Hz, 2H, H-9), 3.31 (s$_{broad}$, 2H, H-24), 2.79 (d$_{broad}$, ^2J(H,H)=15.4 Hz, 1H, H-17b), 2.54 (dd, $^{2,3}J$(H,H)=16.2 + 6.1 Hz, 1H, H-17a), 2.37 (t, ^3J(H,H)=7.0 Hz, 2H, H-11), 1.95 (p, ^3J(H,H)=6.8 Hz, 2H, H-10), 1.42 (s, 9H, H-30 + H-31 + H-32). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=176.99 (C-12), 172.64 (C-16), 156.45 + 157.06 (C-21 + C-26), 144.04 (C-35 + C-38), 141.39 (C-36 + C-37), 134.14 (C-4), 129.45 (C-2 + C-6), 129.25 (C-1), 128.90 (C-3 + C-5), 127.78 (C-41 + C-44), 127.17 (C-42 + C-43), 125.32 (C-40 + C-45), 120.05 (C-39 + C-46), 79.86 (C-29), 76.47 (C-7), 67.00 (C-33), 49.29 (C-19), 47.31 (C-34), 44.41 (C-24), 43.85 (C-9), 34.19 (C-17), 31.30 (C-11), 28.47 (C-30 + C-31 + C-32), 22.06 (C-10). MALDI-MS (calculated): *m/z*=632.34 [M+H]$^+$ (632.30), 654.41 [M+Na]$^+$ (654.28), 670.31 [M+K]$^+$ (670.25).

Ethyl 4-(*N*-(benzyloxy)-3-((*tert*-butoxycarbonyl)-amino)-propanamido)-butanoate (16).

16 was obtained as described before in the general description by deprotection of Boc amino ester **9** (6.93 g, 29.19 mmol) to **10** and condensation with Boc-*β*-alanine (5.53 g, 29.21 mmol). The product was purified by column chromatography using cyclohexane / ethyl acetate 2:1 as eluent and obtained as colorless oil in 95% yield (11.38 g, 27.73 mmol).

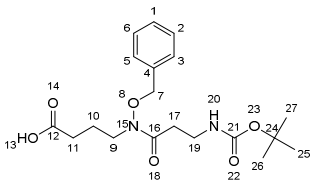


^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.37 (s$_{broad}$, 5H, H-1 – H-3 + H-5 + H-6), 5.16 (s$_{broad}$, 1H, H-22), 4.78 (s, 2H, H-7), 4.10 (q, ^3J(H,H)=7.1 Hz, 2H, H-15), 3.69 (t, ^3J(H,H)=6.7 Hz, 2H, H-9), 3.37 (t, ^3J(H,H)=5.8 Hz, 2H, H-21), 2.60 (t, ^3J(H,H)=5.7 Hz, 2H, H-19), 2.31 (t, ^3J(H,H)=7.3 Hz, 2H H-11), 1.94 (p, ^3J(H,H)=7.2 Hz, 2H, H-10), 1.41 (s, 9H, H-27 – H-29), 1.22 (t, ^3J(H,H)=7.1 Hz, 3H, H-16). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.7 (weak, C-18), 172.91 (C-12), 156.02 (C-23), 134.21 (C-4), 129.37 (C-2 + C-6), 129.17 (C-1), 128.84 (C-3 + C-5), 79.21 (C-26), 77.16 (CDCl$_3$), 76.51 (C-7), 60.54 (C-15), 44.59 (C-9), 36.12 (C-21), 32.81 (C-19), 31.40 (C-11), 28.51 (C-27 – C-29), 22.33 (C-10), 14.31 (C-16). MALDI-MS (calculated): *m/z*=408.98 [M+H]$^+$ (409.23), 430.95 [M+Na]$^+$ (431.22), 447.04 [M+K]$^+$ (447.19).

4-(*N*-(benzyloxy)-3-(Boc-amino)propanamido)butanoic acid (18).

To an ice-cooled solution of **16** (7.50 g, 18.4 mmol) in methanol (100 mL) was slowly added an aqueous sodium hydroxide solution (1M, 20 mL, 20 mmol). After warming to ambient temperature and stirring overnight, the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (50 mL) and washed two times with 20% aqueous sodium

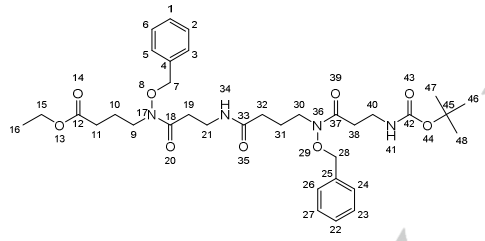
hydrogen sulfate solution. The aqueous phase was extracted with ethyl acetate (20 mL) and the combined organic solutions were dried over $MgSO_4$, evaporated to dryness and afforded the product acid in quantitative yield (7.03 g, 18.4 mmol).



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=8.88 (s, 1H, H-13), 7.37 (s$_{broad}$, 5H, H-1 + H-2 + H-3 + H-5 + H-6), 5.30 (s, 1H, H-20), 4.79 (s, 2H, H-7), 3.71 (t, ^3J(H,H)=6.5 Hz, 2H, H-9), 3.37 (t$_{broad}$, ^3J(H,H)=4.8 Hz, 2H, H-19), 2.61 (t, ^3J(H,H)=5.6 Hz, 2H, H-17), 2.36 (t, ^3J(H,H)=7.2 Hz, 2H-11), 1.95 (p, ^3J(H,H)=7.0 Hz, 2H, H-10), 1.41 (s, 9H, H-25 + H-26 + H-27). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=177.30 (C-12), 173.93 (C-16), 156.18 (C-21), 134.16 (C-4), 129.40 (C2 + C-6), 129.22 (C-1), 128.87 (C-3 + C-5), 79.36 (C-24), 76.59 (C-7), 44.56 (C-9), 36.05 (C-19), 32.77 (C-17), 31.15 (C-11), 28.51 (C-25 + C-26 + C-27), 22.16 (C-10). MALDI-MS (calculated): *m/z*=402.43 [M+Na]$^+$ (403.18), 418.43 [M+K]$^+$ (419.16).

Ethyl 12-(benzyloxy)-3-(3-(Boc-amino)propanoyl)-7,11-dioxo-1-phenyl-2-oxa-3,8,12-triazahexadecan-16-oate, Boc protected amino ester dimer (19).

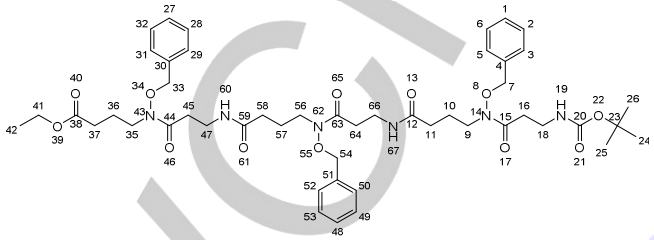
19 was obtained as described before in the general description by deprotection of **16** (0.95 g, 3.09 mmol) to **17** and condensation with 4-(*N*-(benzyloxy)-3-(Boc-amino)propanamido)butanoic acid (**18**) (1.23 g, 3.24 mmol). The product was purified by column chromatography using first cyclohexane / ethyl acetate 1:3 and in the following 100% ethyl acetate as eluents to obtain the product as colorless oil in 86% yield (1.78 g, 2.66 mmol).



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.36 (s$_{broad}$, 10H, H-1 – H-5 + H-6 + H-22 – H-24 + H-26 + H-27), 6.76 (s$_{broad}$, 1H, H-34), 5.31 (s$_{broad}$, 1H, H-41), 4.78 + 4.79 (2s, 4H, H-7 + H-28), 4.10 (q, ^3J(H,H)=7.1 Hz, 3H, H-15), 3.69 (q, ^3J(H,H)=5.7 Hz, 4H, H-9 + H-30), 3.44 – 3.55 (m, 2H, H-21), 3.35 (t, ^3J(H,H)=5.6 Hz, 2H, H-40), 2.62 (q, ^3J(H,H)=6.2 Hz, 4H, H-19 + H-38), 2.30 (t, ^3J(H,H)=7.2 Hz, 2H, H-11), 2.16 (t, ^3J(H,H)=7.1 Hz, 2H, H-32), 1.88 – 2.01 (m, 4H, H-10 + H-31), 1.41 (s, 9H, H-46 – H-48), 1.22 (t, ^3J(H,H)=7.3 Hz, 2H, H-16). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.75 (C-18 + C-37), 172.39 + 172.90 (C-12 + C-33), 156.06 (C-42), 134.18 (C-4 + C-25), 129.42 + 129.43 (C-2 + C-6 + C-23 + C-27), 129.19 (C-1 + C-22), 128.86 (C-3 + C-5 + C-24 + C-26), 79.30 (C-45), 76.46 + 76.52 (C-7 + C-28), 60.60 (C-15), 44.53 (C-9 + C-30), 36.19 (C-21), 34.87 (C-40), 32.30 + 32.69 + 33.08 (C-19 + C-32 + C-38), 31.45 (C-11), 28.53 (C-46 – C-48), 23.03 (C-10), 22.29 (C-31), 14.33 (C-16). MALDI-MS (calculated): *m/z*=671.41 [M+H]$^+$ (671.80), 693.66 [M+Na]$^+$ (693.35), 709.58 [M+K]$^+$ (709.32).

Ethyl-12,21-*bis*(benzyloxy)-3-(3-(Boc-amino)propanoyl)-7,11,16,20-tetraoxo-1-phenyl-2-oxa-3,8,12,17,21-pentaazapentacosan-25-oate, Boc protected amino ester trimer (20).

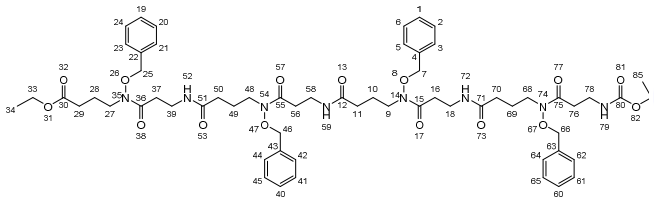
20 was obtained as described before in the general description by deprotection of **19** (1.33 g, 2.18 mmol) and condensation with 4-(*N*-(benzyloxy)-3-(Boc-amino)propanamido)butanoic acid (**18**) (0.87 g, 2.29 mmol). The product was purified by column chromatography using first cyclohexane / ethyl acetate 1:3 and in the following ethyl acetate / ethanol 9:1 as eluents to obtain the product as colorless oil in 97% yield (1.96 g, 2.11 mmol).



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.36 (s$_{broad}$, 15H, H-1 – H-6 + H-27 – H-29 + H-31 + H-32 + H-48 – H-50 + H-52 + H-53), 6.93 (s, 2H, H-60 + H-67), 5.33 (s$_{broad}$, 1H, H-19), 4.78 + 4.79 (2s, 6H, H-7 + H-33 + H-54), 4.09 (q, ^3J(H,H)=7.1 Hz, 2H, H-41), 3.69 (t$_{broad}$, 6H, H-9 + H-35 + H-56), 3.49 (s$_{broad}$, 4H, H-47 + H-66), 3.35 (t, ^3J(H,H)=5.7 Hz, 2H, H-18), 2.58 – 2.65 (3t, ^3J(H,H)=5.6 Hz, 6H, H-16 + H-45 + H-64), 2.30 (t, ^3J(H,H)=7.2 Hz, 2H, H-37), 2.18 (q, ^3J(H,H)=7.3 Hz, 4H, H-11 + H-58), 1.86 – 2.02 (m, 6H, H-10 + H-36 + H-57), 1.41 (s, 9H, H-24 – H-26), 1.22 (t, ^3J(H,H)=7.1 Hz, 3H, H-42).^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.68 + 173.76 (C-15 + C-44 + C-63), 172.87 (C-38), 172.47 (C-12 + C-59), 156.06 (C-20), 134.16 (C-4 + C-30 + C-51), 129.43 (C-2 + C-6 + C-28 + C-32 + C-49 + C-53), 129.18 (C-1 + C-27 + C-48), 128.86 (C-3 + C-5 + C-29 + C-31 + C-50 + C52), 79.29 (C-23), 76.41 + 76.49 (C-7 + C-33 + C-54), 60.60 (C-41), 44.52 (C-9 + C-35 + C-56), 36.22 (C-18), 34.89 (C-47 + C-66), 33.08 + 33.21 (C-11 + C-58), 32.31 + 32.51 + 32.68 (C-37 + C45 + C-64), 31.44 (C-16), 28.54 (C-24 – C-26), 23.10 (C-10 + C-57), 22.28 (C-36), 14.33 (C-42). MALDI-MS (calculated): *m/z*=933.63 [M+H]$^+$ (933.50), 955.74 [M+Na]$^+$ (955.50), 971.86 [M+K]$^+$ (971.45).

Ethyl-12,21,30-*tris*(benzyloxy)-3-(3-(Boc-amino)propanoyl)-7,11,16,20,25,29-hexaoxo-1-phenyl-2-oxa-3,8,12,17,21,26,30-heptaaza-tetratriacontan-34-oate, Boc protected amino tetramer ethyl ester (21).

21 was obtained as described before in the general description by deprotection of **20** (570 mg, 684 µmol) and condensation with 4-(*N*-(benzyloxy)-3-(Boc-amino)propanamido)butanoic acid (**18**) (276 mg, 725 µmol). The product was purified by column chromatography using acetone / CH_2Cl_2 3:2 containing 1% H_2O as eluent and obtained as colorless oil in 90% yield (737 mg, 616 µmol).

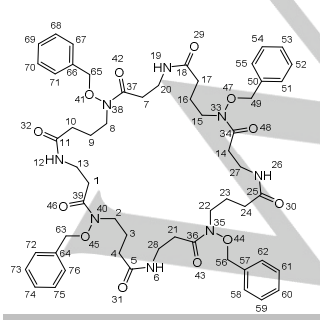


^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.35 + 7.36 (4 s$_{broad}$, 20H, H-1 – H-3 + H-5 + H-6 + H-19 – H-21 + H-23 + H-24 + H-40 – H-42 + H-44 + H-45 + H-60 – H-62 + H-64 + H-65), 6.38 (s$_{broad}$, 3H, H-52 + H-59 + H-72), 5.36 (s$_{broad}$, 1H, H-79), 4.74 – 4.82 (4s, 8H, H-7 + H-25 + H-46 + H-66), 4.09 (q, ^3J(H,H)=7.1 Hz, 2H, H-33), 3.68 (t$_{broad}$, 8H, H-9 + H-27 + H-48 + H-68), 3.50 (t$_{broad}$, 6H, H-18 + H-39 + H-58), 3.34 (t, ^3J(H,H)=5.6 Hz, 2H,

H-78), 2.58 – 2.66 (m, 8H, H-16 + H-37 + H-56 + H-76), 2.16 – 2.38 (m, 8H, H-11 + H-29 + H-50 + H-70), 1.83 – 2.04 (m, 8H, H-10 + H-28 + H-49 + H-69), 1.40 (s, 9H, H-84 – H-86), 1.21 (t, ^{3}J(H,H)=7.2 Hz, 3H, H-34). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.40 + 173.51 + 173.62 + 173.71 + 173.75 (C-15 + C-30 + C-36 + C-55 + C-75), 172.86 + 173.07 + 173.13 (C-12 + C-51 + C-71), 156.08 (C-80), 134.12 (C-4 + C-22 + C-43 + C-63), 129.42 (C-2 + C-6 + C-20 + C-24 + C-41 + C-45 + C-61 + C-65), 129.19 (C-1 + C-19 + C-40 + C-60), 128.85 (C-3 + C-5 + C-21 + C-23 + C-42 + C-44 + C-62 + C-64), 79.33 (C-83), 76.40 + 76.43 + 76.49 + 76.51 (C-7 + C-25 + C-46 + C-66), 60.59 (C-33), 44.46 + 44.50 + 44.53 + 44.58 (C-9 + C-27 + C-48 + C-68), 36.27 (C-78), 35.07 + 35.12 + 35.16 (C-18 + C-39 + C-58), 32.19 + 32.34 + 32.49 + 32.66 + 32.90 + 33.02 (C-11 + C-16 + C-37 + C-50 + C-56 + C-70 + C-76), 31.43 (C-29), 28.46 + 28.58 (C-84 – C-86), 23.16 (C-10 + C-49 + C-69), 22.27 (C-28), 14.36 (C-34). MALDI-MS (calculated): *m/z*=1195.32 [M+H]$^+$ (1195.63), 1217.15 [M+Na]$^+$ (1217.61), 1233.11 [M+K]$^+$ (1233.58).

1,10,19,28-tetrakis(benzyloxy)-1,5,10,14,19,23,28,32-octaaza-cyclohexatriacontan-2,6,11, 15,20,24,29,33-octaone, benzyl protected CTH36 (23).

21 (138 mg, 115 µmol) was deprotected as described before in the general description of deprotection and condensation. The resulting, Boc-deprotected intermediate was dissolved in methanol (2.5 mL) and treated with aqueous sodium hydroxide solution (0.5M, 690 µL, 345 µmol, 3 equiv.) and reacted overnight at ambient temperature. In the following, the solvent was removed by evaporation, the residue was taken up with H_2O and lyophilized to give a colorless powder. The deprotected intermediate was purified by semipreparative HPLC (10 minutes gradient from 20 to 70% MeCN in H_2O with 0.1% formic acid as additive, R_t=5.52 min) and subsequently lyophilized. Afterwards, formic acid was removed by cartridge purification. For this purpose, the intermediate product was dissolved in a mixture of MeCN (500 µL) and phosphate buffer (0.05M, pH 7.2, 50 mL) and divided into three portions which were independently purified using Waters Sep-Pak tC18 Plus Short cartridges by first trapping the product on the cartridge, washing the cartridge with phosphate buffer (0.05M, pH 7.2, 3 mL) and elution of the product with MeCN (15 mL). The combined product solution was concentrated, diluted with H_2O and lyophilized, giving tetramer **22** as colorless solid in 45% yield (55 mg, 51.8 µmol). To an ice-cooled solution of this intermediate **22** in dry DMF (40 mL) were added *N,N*-diisopropylethylamine (DIPEA, 9.5 µL, 55.8 µmol, 1.1 equiv.) and DPPA (diphenyl phosphoryl azide, 13.1 µL, 60.8 µmol, 2.75 equiv.) and the mixture was kept at 3 °C for 18 days. The solvent was removed *in vacuo* and the residue was taken up in MeCN / H_2O 1:1 (1 mL) and lyophilized. The product was precipitated in MeCN (300 µL) at 3 °C for three days. The precipitate was washed four times with cold MeCN (50 µL each) and dried before the product was purified by column chromatography with acetone / CH_2Cl_2 3:1 containing 5% H_2O as eluent, giving the desired macrocycle **23** as colorless solid in 20% overall yield (23.5 mg, 23 µmol) over three steps, starting from **21**.



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.36 (s$_{broad}$, 20H, H-51 – H-55 + H-58 – H-60 + H-61 + H-62 + H-67 – H-76), 7.06 (s$_{broad}$, 4H, H-6 + H-12 + H-19 + H-26), 4.78 (s, 8H, H-49 + H-56 + H-63 + H-65), 3.69 (t, ^{3}J(H,H)=6.1 Hz, 8H, H-2 + H-8 + H-15 + H-22), 3.49 (dt, $^{3,3}J$(H,H) = 5.8 + 5.1 Hz, 8H, H-13 + H-20 + H-27 + H-28), 2.64 (dd, $^{3,3}J$(H,H)=5.8 + 4.9 Hz, 8H, H-1 + H-7 + H-14 + H-21), 2.14 (t, ^{3}J(H,H)=6.6 Hz, 8H, H-4 + H-10 + H-17 + H-24), 1.95 (p, ^{3}J(H,H)=6.0 Hz, 8H, H-3 + H-9 + H-16 + H-23). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=174.05 (C-5 + C-11 + C-18 + C-25), 172.43 (C-34 + C-36 + C-37 + C-39), 134.14 (C-50 + C-57 + C-64 + C-66), 129.42 (C-52 + C-54 + C-59 + C-61 + C-68 + C-70 + C-73 + C-75), 129.23 (C-53 + C-60 + C-69 + C-74), 128.89 (C-51 + C-55 + C-58 + C-62 + C-67 + C-71 + C-72 + C-76), 76.41 (C-49 + C-56 + C-63 + C-65), 44.24 (C-2 + C-8 + C-15 + C-22), 34.80 (C-13 + C-20 + C-27 + C-28), 33.25 (C-4 + C-10 + C-17 + C-24), 32.51 (C-1 + C-7 + C-14 + C-21), 23.16 (C-3 + C-9 + C-16 + C-23). MALDI-MS (calculated): *m/z*=1049.43 [M+H]$^+$ (1049.53), 1071.40 [M+Na]$^+$ (1071.52), 1087.23 [M+K]$^+$ (1087.30).

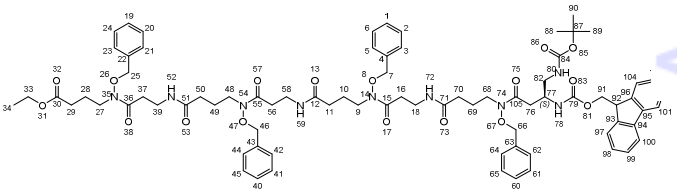
1,10,19,28-tetrahydroxy-1,5,10,14,19,23,28,32-octaaza-cyclohexatriacontan-2,6,11,15,20, 24,29,33-octaone, CTH36 (24).

To a solution of the benzyl protected macrocycle **23** (27 mg, 25.7 µmol) in a mixture of methanol (5 mL), H_2O (1 mL) and formic acid (60 µL), was added 10% Pd/C (2 mg) and the mixture was stirred under a hydrogen atmosphere for 3.5 h. The suspension was centrifuged and the remaining solid was extracted with MeCN / H_2O 2:1 (5 mL). The methanol fraction was dried *in vacuo*, the residue was taken up with the MeCN / H_2O fraction and the resulting mixture was lyophilized to afford the product as white powder in 96% yield (17 mg, 24.7 µmol) and a purity of 78%. This material was further purified by semipreparative HPLC (10 minutes gradient from 0 to 20% MeCN in H_2O with 0.1% formic acid as additive, R_t=7.28 min) and subsequent lyophilization, giving the pure product (determined by analytical HPLC) in 79% yield (14.1 mg, 20.5 µmol).

The product could not be characterized by NMR due to a very limited solubility of the substance in organic solvents as well as aqueous media, preventing the achievement of the required substance concentration in the NMR sample. MALDI-MS (calculated): *m/z*=689.17 [M+H]$^+$ (689.35), 711.18 [M+Na]$^+$ (711.33), 727.3 [M+K]$^+$ (727.15).

Ethyl-(*S*)-12,21,30-*tris*(benzyloxy)-3-(3-(Fmoc-amino)-4-(Boc-amino)butanoyl)-7,11, 16,20,25,29-hexaoxo-1-phenyl-2-oxa-3,8,12,17,21,26,30-heptaazatetratriacontan-34-oate, Boc/Fmoc *bis*protected tetramer ethyl ester (25).

25 was obtained as described before in the general description by deprotection of **20** (238 mg, 285 µmol) and condensation with (*S*)-4-(*N*-(benzyloxy)-4-(Boc-amino)-3-(Fmoc-amino)-butanamido)-butanoic acid **12** (180 mg, 285 µmol). The product was purified by column chromatography using acetone / CH_2Cl_2 1:1 containing 1% H_2O as eluent and obtained as colorless, non-crystalline solid in 59% yield (245 mg, 168 µmol).

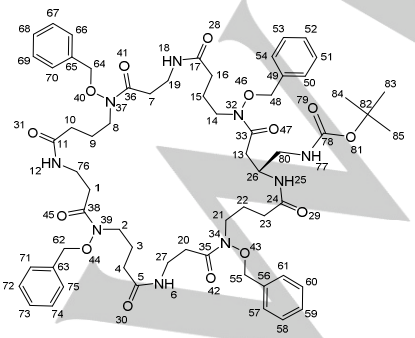


^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.73 (d, ^{3}J(H,H)=7.5 Hz, 2H, H-100 + H-101), 7.57 (d, ^{3}J(H,H)=7.4 Hz, 2H, H-97 + H-104), 7.31 – 7.40 (m, 22H, H-1 – H-3 + H-5 + H-6 + H-19 – H-21 + H-23 + H-24 + H-40 – H-42 + H-44 + H-45 + H-60 – H-62 + H-64 + H-65 + H-99 + H-102), 7.28 (t, ^{3}J(H,H)=7.5 Hz, 2H, H-98 + H-103), 6.08 (s$_{broad}$, 1H, H-78), 5.24 (s$_{broad}$,

1H, H-83), 4.97 (s_{broad}, 3H, H-52 + H-59 + H-72), 4.70 – 4.84 (2s, 8H, H-7 + H-25 + H-46 + H-66), 4.32 (d, ^3J(H,H)=7.5 Hz, 2H, H-91), 4.16 (t, ^3J(H,H)=7.0 Hz, 1H, H-92), 4.08 (q, ^3J(H,H)=7.1 Hz, 2H, H-33), 3.95 – 4.06 (m, 1H, H-77), 3.67 ($4t_{broad}$, 8H, H-9 + H-27 + H-48 + H-68), 3.49 ($3t_{broad}$, 6H, H-18 + H-39 + H-58), 3.28 (t_{broad}, 2H, H-82), 2.72 – 2.84 (m, 1H, H-76b), 2.47 – 2.70 (m, 7H, H-16 + H-37 + H-56 + H-76a), 2.18 – 2.34 (m, 8H, H-11 + H-29 + H-50 + H-70), 1.85 – 2.02 (m, 8H, H-10 + H-28 + H-49 + H-69), 1.40 (s, 9H, H-88 – H-90), 1.21 (t, ^3J(H,H)=7.1 Hz, 3H, H-34). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=173.11 + 173.17 + 173.28 + 173.46 + 173.57 (C-15 + C-30 + C-36 + C-55 + C-75), 171.80 + 172.42 + 172.88 (C-12 + C51 + C-71), 156.88 (C-79), 156.26 (C-84), 144.01 + 144.04 (C-93 + C-96), 141.34 (C-94 + C-95), 134.10 (C-4 + C-22 + C-43 + C-63), 129.40 + 129.47 (C-2 + C-6 + C-20 + C-24 + C-41 + C-45 + C-61 + C-65), 129.18 (C-1 + C-19 + C-40 + C-60), 128.84 (C-3 + C-5 + C-21 + C-23 + C-42 + C-44 + C-62 + C-64), 127.15 + 127.76 (C-99 – C-102), 125.26 (C-98 + C-103), 120.02 (C-97 + C-104), 79.70 (C-87), 76.35 + 76.41 + 76.43 + 76.48 (C-7 + C-25 + C-46 + C-66), 66.83 (C-91), 60.59 (C-33), 49.31 (C-77), 47.30 (C-92), 43.78 + 44.14 + 44.57 (C-9 + C-27 + C-48 + C-68 + C-82), 35.07 + 35.22 (C-18 + C-39 + C-58), 34.15 (C-76), 32.19 + 32.31 + 32.50 + 32.78 + 32.89 + 33.04 (C-11 + C-16 + C37 + C-50 + C-56 + C-70), 31.42 (C-29), 28.44 + 28.53 (C-88 – C-90), 23.08 + 23.19 + 23.27 (C-10 + C-49 + C-69), 14.27 (C-34), 22.27 (C-28). MALDI-MS (calculated): *m/z*=1447.39 $[M+H]^+$ (1446.72), 1469.87 $[M+Na]^+$ (1468.71), 1485.57 $[M+K]^+$ (1484.68).

1,10,19,28-Tetrakis(benzyloxy)-4-((Boc-amino)methyl)-1,5,10,14,19,23,28,32-octaaza-cyclohexatriacontan-2,6,11,15,20,24,29,33-octaone, Boc/benzyl protected functionalized CTH36 (26).

To an ice-cooled solution of *bis*-protected tetramer **25** (227 mg, 157µmol) in methanol (3.4 mL) was slowly added an aqueous sodium hydroxide solution (0.5M, 940 µL, 470 µmol, 3 equiv.). After warming up to ambient temperature and stirring overnight, the solvent was removed *in vacuo*. The obtained residue was taken up in H_2O (1 mL) the precipitate was removed by filtration and washed twice with H_2O (0.5 mL each portion). The aqueous solution was lyophilized and the residue (201 mg) was used without further purification for the next reaction step. To an ice-cooled solution of this intermediate in dry DMF (50 mL) was added DPPA (42 µL, 195 µmol, 1.24 equiv.) and the mixture was stirred at 3°C for two hours. The solvent was removed *in vacuo*, the residue was taken up in MeCN (5 mL) and the precipitate was removed by filtration. Further purification was performed by column chromatography with acetone / CH_2Cl_2 / H_2O 20:10:1 as eluent. After isolation of the product by evaporation, it was redissolved in MeCN / H_2O 1:1 and lyophilized to afford the desired macrocycle **26** as colorless solid in 53% overall yield (97.4 mg, 83 µmol) over two steps, starting from **25**.



^{1}H NMR (300 MHz, [D$_3$]CDCl$_3$, 25°C): δ=7.73 (s_{broad}, 4H, H-6 + H-12 + H-18 + H-25), 7.35 + 7.36 ($2s_{broad}$, 20H, H-50 – H-54, H-57 – H-61, H-66 – H-75), 5.78 (s, 1H, H-77), 4.76 – 4.81 (4s, 8H, H-48 + H-55 + H-62 + H-64), 4.35 (s, 1H, H-26), 3.56 – 3.92 (m, 8H, H-2 + H-8 + H14 + H-21), 3.36 – 3.56 (m, 6H, H-19 + H-27 + H-76), 3.28 (d_{broad}, ^3J(H,H)=5.7 Hz, 2H, H-80), 2.50 – 2.81 (m, 8H, H-1 + H-7 + H-13 + H-20), 2.11 – 2.33 (m, 8H, H-4 + H-10 + H-16 + H-23), 1.94 (p_{broad}, ^3J(H,H)=6.4 Hz, 8H, H-3 + H-9 + H-15 + H-22), 1.40 (s, 9H, H83 + H-84 + H-85). ^{13}C NMR (75 MHz, [D$_3$]CDCl$_3$, 25°C): δ=172.51 + 173.13 – 173.77 (C-5 + C-11 + C-17 + C-24 + C-33 + C-35 + C-36 + C-38), 156.71 (C-78), 134.16 (C-49 + C-56 + C-63 + C-65), 129.44 (C-51 + C-53 + C-58 + C-60 + C67 + C-69 + C-72 + C-74), 129.20 (C-52 + C-59 + C-68 + C-73), 128.87 + 128.91 (C-50 + C-54 + C-57 + C-61 + C-66 + C-70 + C-71 + C-75), 79.50 (C-82), 76.37 + 76.43 (C-48 + C-55 + C-62 + C-64), 47.44 (C-26), 43.78 + 44.04 + 44.09 + 44.41 (C-2 + C-8 + C-14 + C-21 + C-80), 35.03 + 35.17 (C-19 + C-27 + C-76), 34.52 (C-13), 32.30 + 32.43 + 32.81 (C-1 + C-4 + C-7 + C-10 + C-16 + C-20 + C-23), 28.54 (C-83 – C-85), 23.07 + 23.35 (C-3 + C-9 + C-15 + C-22). MALDI-MS (calculated): *m/z*=1178.48 $[M+H]^+$ (1178.61), 1200.21 $[M+Na]^+$ (1200.60).

***N*^{1}-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*^{4}-((5,14,23,32-tetrakis-(benzyloxy)-4,9,13,18,22,27, 31,36-octaoxo-1,5,10,14,19,23,28,32-octaaza-cyclohexatriacontan-2-yl)methyl)succinamide, benzyl protected tetrazine-modified CTH36 (29).**

26 (12.0 mg, 13.9 µmol) was deprotected as described before in the general description of deprotection and condensation, giving the deprotected aminomethyl-CTH36 derivate **27** (11.6 mg, 10.8 µmol) which was dissolved together with 4-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-4-oxobutanoic acid (**28**, 3.1 mg, 10 µmol)[20] in MeCN (800 µL). To this mixture was added EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 2.1 mg, 11 µmol, 1.1 equiv.) and the mixture was stirred at ambient temperature overnight. The solvent was evaporated, the residue was dissolved in CH_2Cl_2 (1 mL) and washed with aqueous sodium hydrogen sulfate solution (10%, 100 µL). After evaporation of the solvent, the purification of the product was performed by semipreparative HPLC (15 minutes gradient from 35 to 40% MeCN in H_2O with 0.1% formic acid as additive, R_t=11.08 min). After lyophilization, the pure product (determined by analytical HPLC) was obtained as pink-colored powder in 48% overall yield (7 mg, 4.8 µmol) over two steps, starting from **26**.

The product could not be characterized by NMR due to a very limited solubility of the substance in organic solvents as well as aqueous media, preventing the achievement of the required substance concentration in the NMR sample. MALDI-MS (calculated): *m/z*=1347.63 $[M+H]^+$ (1347.65), 1369.76 $[M+Na]^+$ (1369.63), 1385.65 $[M+K]^+$ (1385.61).

***N*^{1}-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*^{4}-((5,14,23,32-tetrahydroxy-4,9,13,18,22,27,31,36-octaoxo-1,5,10,14,19,23,28,32-octaazacyclohexatriacontan-2-yl)methyl)succinamide, tetrazine-modified CTH36 (tCTH36, 30).**

To a solution of **29** (6.5 mg, 5.0 µmol) in a mixture of methanol (3 mL), H_2O (30 µL) and formic acid (1 µL) was added Pd/C (10%, 2mg) and the mixture was stirred under a hydrogen atmosphere for 3.5 h. The catalyst was removed by filtration and the solution was evaporated to dryness. The residue was dissolved in MeCN / H_2O 1:1 (1 mL) and lyophilized before the product was purified by semipreparative HPLC (10 minutes gradient from 10 to 20% MeCN in H_2O with 0.1% formic acid as additive, R_t=8.40 min) and subsequent lyophilization. The pure product (determined by analytical HPLC) was obtained in quantitative yield (4.9 mg, 5.0 µmol).

The product could not be characterized by NMR due to a very limited solubility of the substance in organic solvents as well as aqueous media, preventing the achievement of the required substance concentration in the NMR sample. MALDI-MS (calculated): *m/z*=987.28 $[M+H]^+$ (987.46).

c(RGDfK) Peptide (31).

The cyclic pentapeptide c(RGDfK) was synthesized in 0.2 mmol scale by solid phase peptide synthesis on solid support using the standard Fmoc-strategy on commercially available H-Asp(*t*Bu)-2-Chlortrityl-resin (loading: 0.80 mmol/g). For amino acid conjugation, 3.9 equiv. HBTU (*N*,*N*,*N*',*N*'-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluoro-phosphate), 4.0 equiv. of Fmoc protected amino acids and 4.0 equiv. of DIPEA were used in DMF as solvent. Coupling times were 30 minutes for each amino acid. After coupling of the last amino acid and Fmoc-removal, the linear, protected peptide was cleaved from the resin using 1% TFA in CH_2Cl_2. The crude intermediate was isolated by evaporation of the volatile components of the mixture and was in the following dissolved in dry DMF (170 mL). To this solution, DIPEA (119 µL, 700 µmol, 3.5 equiv.) was added and the solution was cooled to 0-4°C before DPPA (54 µL, 250 µmol, 1.25 equiv.) were added. The mixture was allowed to warm to ambient temperature and reacted for three days until the cyclisation was complete. The volatile components of the mixture were removed *in vacuo* and the residue was treated for 2.5h with a mixture of TFA / TIS (triisopropylsilane) 97.5:2.5 (5 mL) to completely deprotect the peptide. After concentration *in vacuo* and precipitation of the product in cooled diethylether (25 mL), the crude product was obtained by centrifugation, washing with diethylether (5 mL) twice and drying of the obtained solid. Purification of the product was performed by semipreparative HPLC (6 minutes gradient from 0 to 15% MeCN in H_2O with 0.1% formic acid as additive, R_t=3.65 min). The product was obtained after lyophilization as white solid in 69% overall yield (90 mg, 138 µmol).

MALDI-MS (calculated): *m/z*=604.11 $[M+H]^+$ (604.32), 626.29 $[M+Na]^+$ (626.30), 642.17 $[M+K]^+$ (642.28).

TCO-modified c(RGDfK) derivative (33).

To a solution of **31** (15.3 mg, 20 µmol) and DIPEA (6.8 µL, 40 µmol) in DMF (400 µL) was added a solution of *trans*-cyclooct-4-en-1-yl p-nitrophenyl carbonate (**32**) (5.8 mg, 20 µmol) in DMF (50 µL). After 6h reaction at ambient temperature, the solvent was removed *in vacuo* and the obtained yellow-colored solid was washed twice with acetone (200 µL) and dried. The product was purified by semipreparative HPLC (10 minutes gradient from 0 to 50% MeCN in H_2O with 0.1% formic acid as additive, R_t=8.46 min) and obtained after lyophilization as white solid in 52% yield (7.8 mg, 10.4 µmol).

MALDI-MS (calculated): *m/z*=755.95 $[M+H]^+$ (756.40), 778.03 $[M+Na]^+$ (778.39).

CTH36-c(RGDfK) conjugate (35).

To a solution of the tCTH36 **30** (6.30 mg, 6.38 µmol) in DMSO (100 µL) was added in portions a solution of the TCO-modified c(RGDfK) peptide **33** (3.25 mg, 4.31 µmol) in DMSO (135 µL). The pink color of **30** disappeared during the reaction. The mixture was evaporated to dryness and the product was purified by semipreparative HPLC (10 minutes gradient from 0 to 40% MeCN in H_2O with 0.1% formic acid as additive, retention times of isomers: R_{t1}=8.14 min, R_{t2}=9.39 min and R_{t3}=9.58 min). The product was obtained after lyophilization as a mixture of isomers and colorless solid in 65% yield (4.77 mg, 2.80 µmol).

MALDI-MS (calculated): *m/z*=1714.96 $[M+H]^+$ (1714.86), 1737.08 $[M+Na]^+$ (1736.84).

DFO-c(RGDfK) conjugate (36).

To a solution of tDFO **34** (5.92 mg, 7.13 µmol) in DMSO (200 µL) was added in portions a solution of the TCO-modified c(RGDfK) peptide **33** (3.78 mg, 5.00 µmol) in DMSO (140 µL). The pink color of **34** disappeared during the reaction. The mixture was evaporated to dryness and the product was purified by semipreparative HPLC (10 minutes gradient from 0 to 50% MeCN in H_2O with 0.1% formic acid as additive, retention times of isomers: R_{t1}=8.56 min, R_{t2}=9.38 min and R_{t3}=9.58 min). The product was obtained after lyophilization as a mixture of isomers and colorless solid in 42% yield (3.30 mg, 2.1 µmol).

MALDI-MS (calculated): *m/z*=1558.24 $[M+H]^+$ (1557.84).

Radiolabeling of CTH36 (24) with $^{89}Zr^{4+}$ and determination of the optimal pH for radiometal incorporation.

First, a stock solution of the radiolabeling precursor **24** (220 µg, 320 nmol) in H_2O (320 µL) was prepared. For the radiolabeling experiments, ^{89}Zr-oxalate solution (0.1M, 8–12 µL, 13.5–18.5 MBq) was added to HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffers of different pH (0.25M, 150 µL, pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and the pH was controlled and if necessary adjusted to the initial pH by addition of NaOH (0.1M). Aliquots of the stock solution of **14** (40 µL, 40 nmol) were added to each ^{89}Zr solution and the radiolabeling reaction mixtures were analyzed by radio-HPLC after 5, 25 and 45 minutes. Each experiment was performed thrice.

Radiolabeling of tCTH36- and tDFO-peptide conjugates 35 and 36 with $^{89}Zr^{4+}$.

First, stock solutions of the radiolabeling precursors **35** and **36** in H_2O (1 nmol/µL) were prepared. For the radiolabeling experiments, ^{89}Zr-oxalate solution (0.1M, 16–25 µL, 20–23 MBq) was added to HEPES buffer (0.25M, 150 µL, pH 9) and the pH of the solution was controlled and if necessary adjusted to 7.0 by addition of NaOH (0.1M, ~20 µL). To this mixture was added **35** (2.5 µL of the before prepared stock solution, 2.5 nmol) or **36** (5 µL of the before prepared stock solution, 5 nmol) and reacted for 60 minutes at ambient temperature. The radiolabeling reaction mixtures were analyzed by radio-HPLC and showed ^{89}Zr-incorporation rates of ≥96%. **[^{89}Zr]35** and **[^{89}Zr]36** were thus obtained in radiochemical yields of ≥96% and non-optimized molar activities of 8.4–9.2 GBq/µmol for **[^{89}Zr]35** and 4.0–4.6 GBq/µmol for **[^{89}Zr]36**.

Challenge experiments of ^{89}Zr-labeled peptide conjugates [^{89}Zr]35 and [^{89}Zr]36 with EDTA.

First, stock solutions of the radiolabeling precursors **35** and **36** in H_2O (1 nmol/µL) were prepared. For the radiolabeling experiments, ^{89}Zr-oxalate solution (0.1M, 40 µL, 49 MBq) was added to HEPES buffer (0.25M, 225 µL, pH 9) and the pH of the solution was controlled and if necessary adjusted to 7.0 by addition of NaOH (0.1M, ~25 µL). To this mixture was added **35** or **36** (15.4 µL of the before prepared stock solution, 15.4 nmol) and reacted for 70–90 minutes at ambient temperature. The radiolabeling reaction mixtures were analyzed by radio-HPLC and showed ^{89}Zr-incorporation rates of ≥98 %.

Three aliquots of 99 µL each (containing 5 nmol **35** or **36**) were taken from the radiolabeling reaction mixtures and added to HEPES buffer (0.25M, 401 µL, pH 7) containing EDTA (ethylenediaminetetraacetic acid) (0.5, 5 or 45 µmol). The process of transchelation was determined by radio-HPLC at different time points. Each experiment was performed thrice.

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Keywords: Chelates • Macrocyclic ligands • Radiochemistry • Macrocyclic tetra-hydroxamate • ^{89}Zr

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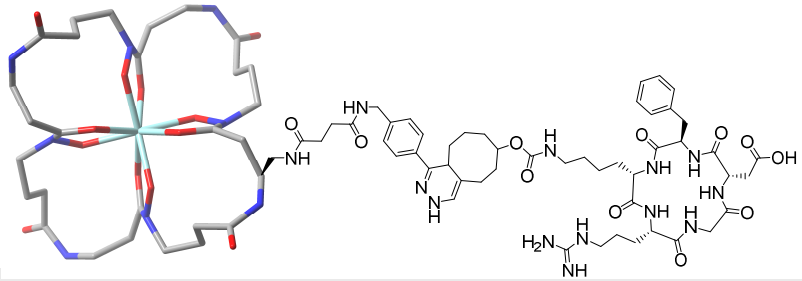

*Uwe Seibold, Björn Wängler, Carmen Wängler**

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Rational Design, Chemical Development and Stability Assessment of a New Macrocyclic, Four-Hydroxamates-Bearing Bifunctional Chelating Agent for ^{89}Zr

Complex four-leaf clover: Symmetry meets high complex stability.
Here we present the development of a macrocyclic tetra-hydroxamate ^{89}Zr-chelator, applicable in bioconjugation and symmetrically encapsulating the radiometal without cleft in the ligand sphere for high ^{89}Zr-complex stability.