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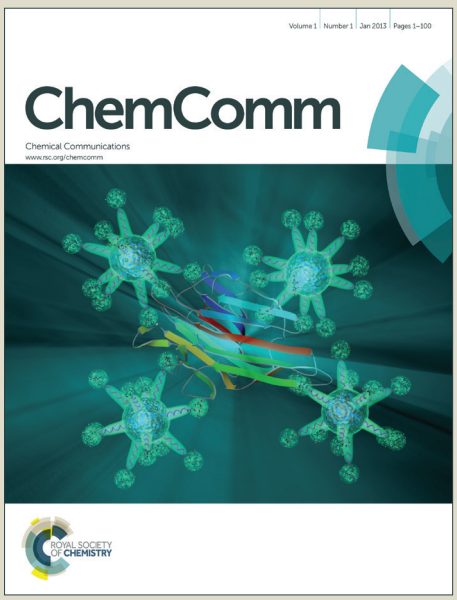

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An Octadentate Bifunctional Chelating Agent for the Development of Stable Zirconium-89 Based Molecular Imaging Probes

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^{89}Zr-based imaging agents hold great promise as novel radiotracers in nuclear medicine. However, insufficient stability of currently used radiometal complexes *in vivo* is a safety concern for clinical applications. We herein report the first octadentate bifunctional chelating agent for the development of ^{89}Zr-labelled (bio)conjugates with improved stability.

The radionuclide zirconium-89 (^{89}Zr) is an emerging radiometal with promising characteristics for diagnostic applications in the field of nuclear medicine using high resolution positron emission tomography (PET).[1] Compared to other common PET radionuclides, ^{89}Zr has a physical half-life ($t_{½}$ = 78.4 h) long enough to match the biological half-life of most antibodies (Ab; $t_{½}$ = several days). Thus, their combination holds great promise for the development of immuno-PET imaging agents. Several preclinical studies and clinical trials have demonstrated the potential of ^{89}Zr-labelled Ab in nuclear oncology.[2] A potential limitation of the use of ^{89}Zr is the lack of appropriate methods for the stable chelation of the metallic radionuclide. To date, radiolabelling of antibodies with ^{89}Zr is solely achieved through bifunctional chelating agents (BFCAs) based on desferrioxamine (DFO; Scheme 1). The use of DFO as a chelator for ^{89}Zr is attractive since it has been safely used in the clinic for the treatment of acute iron poisoning. However, there is preclinical evidence that ^{89}Zr is released to some extent from the chelator *in vivo* and taken up in radiation sensitive bones,[1, 3, 4] which not only reduces the signal-to-background ratio important to imaging but also represents a safety concern in the view of clinical applications.

DFO comprises three hydroxamic acid moieties and thus, two of the eight coordination sites of the oxophilic Zr^{4+} are likely occupied by water molecules, which are relatively labile ligands.[5] It has been postulated that the incomplete coordination of $^{89}Zr^{4+}$ by DFO is responsible for the observed instability of the complex *in vivo*, which eventually leads to mineralization of the osteophilic radiometal in bones (presumably *via* transchelation to transferrin). Taking this into account, we envisioned that a DFO analogue with an additional hydroxamic acid entity (termed DFO*, Scheme 1) would provide an octadentate chelator for the stable complexation of Zr^{4+}. The expected molecular structure involving coordination through the eight oxygen atoms of all four hydroxamic acid moieties was optimized using DFT calculations (Figure 1). The calculated Zr–O bond distances in the range 2.153-2.344 Å (average 2.239 Å) are in agreement with the X-ray and DFT data reported by Guérard et al[6] for Zr(Me-AHA)$_4$ (Me-AHA = *N*-methyl acetohydroxamic acid), which indicates that such a chelator would be able to complex Zr^{4+} through the proposed octadentate coordination, both electronically and sterically.

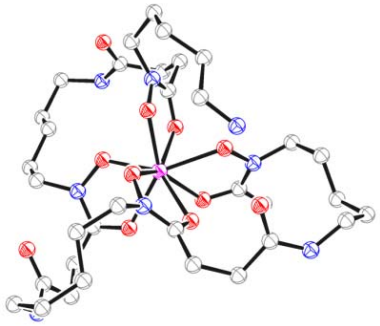
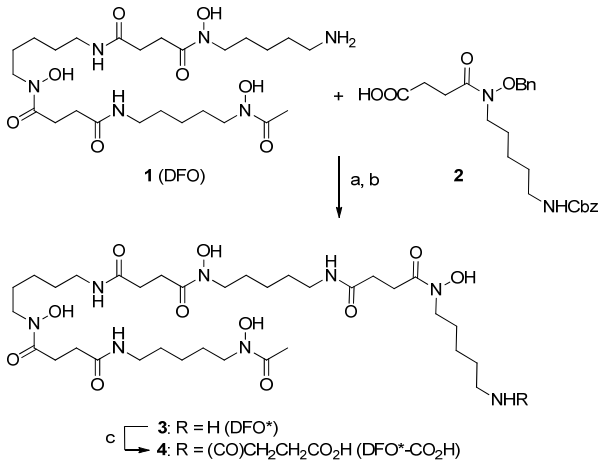


Figure 1. DFT optimized structure of Zr-DFO* with the terminal primary amine protonated (atom colour coding: white = carbon; blue = nitrogen; red = oxygen; magenta = zirconium); hydrogen atoms are omitted for clarity.

The straightforward synthesis of novel octadentate DFO* is shown in Scheme 1.[7] Commercial DFO (**1**, mesylate salt) was reacted with protected hydroxamic acid building block **2**, which in turn is readily accessible in five steps following published procedures.[8] Removal of the protecting groups gave DFO* (**3**) in satisfying overall yield. Reaction of DFO* (**3**) with succinic anhydride provided BFCA **4** with a pending carboxylic acid functionality suitable for conjugations to (bio)molecules with a free amine *via* amide bond formation. To the best of our knowledge, compound **4** represents the first example of an octadentate BFCA for the development of ^{89}Zr-based radiopharmaceuticals.

The preparation of complexes of DFO* (**3**) and its BFCA analogue **4** with non-radioactive ^{nat}Zr salts on a macroscopic scale was initially somewhat difficult due to the poor solubility of the chelators and complexes thereof. This observation is in agreement with recently

reported physical properties of similar compounds.[9] However, after some experimentation, suitable conditions for the complexation were identified (e.g., organic solvents, elevated temperature) and ^{nat}Zr-**3** and ^{nat}Zr-**4** could be obtained.[7] Analysis of the complexes by LC-MS confirmed a metal-to-ligand ratio of 1:1 in each case. Further analysis of ^{nat}Zr-**4** by NMR spectroscopy indicated the presence of different isomers. This result is not surprising in light of the fact that acyclic chelating systems can "wrap around" the polyhedron metal centre in different orientations.



Scheme 1. Synthesis of DFO* and its BFCA-analogue. a) HATU, DIPEA, DMF, rt; b) 10% Pd/C, H_2 (1 bar), MeOH, rt; c) succinic anhydride, NEt_3, DMF, rt.

With BFCA **4** in hand, we next set out to evaluate its potential for (bio)conjugations in an effort to provide proof of concept for its usefulness in the development of ^{89}Zr-based radiopharmaceuticals. For demonstration purposes, we chose the peptide bombesin (BBS) as a model compound of medicinal importance. Specifically, we used the modified minimum binding sequence of bombesin, [Nle^{14}]BBS(7-14) (QWAVGHLNle-NH_2), a peptide currently under investigation for the development of tumour-targeting imaging probes and therapeutics with high affinity and specificity towards the gastrin-releasing peptide receptor (GRP-r), which is overexpressed by, e.g., prostate, breast, and small-cell lung cancer.[10] Even though the biological half-life of tumour-targeting small peptides ($t_{½}$ = minutes to hours) is not an ideal match for the physical half-life of ^{89}Zr, examples of ^{89}Zr-labelled peptides and other low molecular weight vectors have been reported.[11, 12] Attachment of DFO* analogue **4** to [Nle^{14}]BBS(7-14) not only served for proof of concept studies but also facilitated the assessment of the formation of ^{89}Zr-complexes since the separation of the free metal from its complexes with chelators alone (e.g., in challenging experiments) can be a difficult analytical endeavour, as we and others have experienced.[6]
Peptide synthesis and the conjugation of DFO*-CO_2H (**4**) were accomplished by solid-phase synthesis using standard Fmoc-peptide chemistry.[7] [Nle^{14}]BBS(7-14) was extended N-terminally with a $(\beta Ala)_3$ spacer to prevent potential interference of the complex with the receptor-targeting properties of the peptide moiety.[10] For a direct side-by-side comparison of DFO*-functionalized BBS **5** with an analogue DFO-conjugate, commercial DFO-*p*-SCN was also coupled to the peptide precursor to provide reference compound **6**. After cleavage from the resin, overall deprotection, and HPLC purificationthe peptide conjugates were obtained in >95% purity and their identity were confirmed by mass spectrometry (Table 1).

Nr.	Conjugate	*m/z*
5	DFO*$(\beta Ala)_3$[Nle^{14}]BBS(7-14)	1978.1 $[M+H]^+$ (calcd. 1977.10)
6	DFO$(\beta Ala)_3$[Nle^{14}]BBS(7-14)	1887.9 $[M+H]^+$ (calcd. 1886.96)

Table 1. Analytical data of chelator-peptide conjugates.

Radiolabelling of DFO*-conjugate **5** and reference compound **6** with ^{89}Zr was investigated by reported protocols.[13] In brief, peptide conjugates **5** and **6** were incubated at room temperature with aqueous solutions of $^{89}Zr^{4+}$ oxalate at neutral pH for various time points and samples were analysed by γ-HPLC.[7] Quantitative complexation of ^{89}Zr was observed for both compounds after approx. 1.5-2 h. Specific activities (A_s) for ^{89}Zr-**5** and ^{89}Zr-**6** were in the range of 5-6 GBq/μmol, which could be improved by extending the incubation time overnight (not optimized). Importantly, radiolabelling of DFO*-BBS conjugate **5** with a clinically useful A_s was achieved at room temperature, a prerequisite for applications to delicate biomolecules (e.g., Ab). While γ-HPLC analysis confirmed unambiguously the absence of free radiometal, an unusual "fronting" of peaks that corresponded to the radiolabelled peptide conjugates was observed. The "fronting" of HPLC peaks was more pronounced for the DFO conjugate **6** than for DFO* compound **5**. We tentatively ascribe this observation to the presence of different isomers of the radiometal complexes, which is in accordance to the NMR data obtained for non-radioactive ^{nat}Zr-**4**.
To assess the stability of ^{89}Zr-**5** and ^{89}Zr-**6**, transchelation challenging experiments were performed. For this purpose, we chose DFO (**1**, mesylate salt) over other potential challenging ligands (e.g., EDTA, DTPA) because it is the most potent chelator currently known for $^{89}Zr^{4+}$. Thus, solutions of ^{89}Zr-**5** and ^{89}Zr-**6** were incubated at room temperature with 300 to 3000-fold molar excess of DFO (**1**). Samples were taken at various time points and analysed by γ-HPLC. As predicted on a theoretical base, DFO* radioconjugate ^{89}Zr-**5** showed a remarkably improved stability in comparison to the DFO analogue ^{89}Zr-**6** (Figure 2). While substantial transchelation of the radiometal to the challenging DFO ligand was observed for reference compound ^{89}Zr-**6** within hours, ^{89}Zr-**5** remained largely intact even after 24 h in the presence of 3000-fold excess of DFO. This data clearly demonstrates the outstanding stability of DFO*-derived compound ^{89}Zr-**5** in comparison to the DFO reference conjugate ^{89}Zr-**6**.

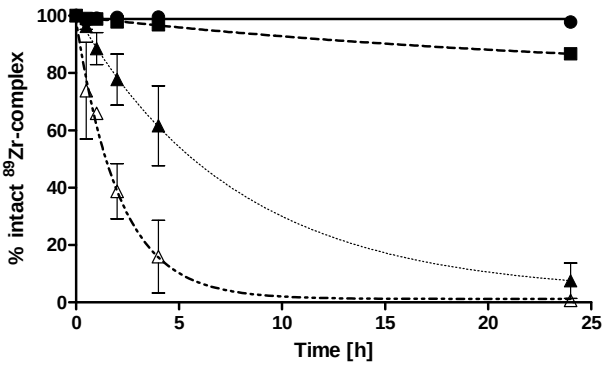


Figure 2. Stability of ^{89}Zr-complexes in DFO challenging experiments. (●): ^{89}Zr-**5**, 300-fold excess DFO; (▲): ^{89}Zr-**6**, 300-fold excess DFO; (■): ^{89}Zr-**5**, 3000-fold excess DFO; (Δ): ^{89}Zr-**6**, 3000-fold excess DFO (n= 2-3, presented as mean ± SD).

The choice of the chelator can influence the biological properties of a radiometal-labelled compound. We therefore investigated further the physico-chemical properties of the ^{89}Zr-labelled peptides.[7] The lipophilicity of ^{89}Zr-**5** and ^{89}Zr-**6**, as determined by the shake flask method, were found to be in the same range (logD = -1.6±0.1 and -1.5±0.2, respectively). Cell internalisation and receptor saturation binding assays were performed with GRP-r over expressing PC-3 cells. Both compounds exhibited similar cell internalisation profiles in terms of the extent and rate of receptor-specific uptake. Approximately 30-40% of the applied radioactivity was internalised within 1 h. Receptor affinities were also comparable with determined dissociation constants (K_d) for both compounds in the single-digit nanomolar range. The *in vitro* data obtained are comparable to those reported in the literature for related radiometal-labelled bombesin derivatives[10, 14] and thus, the different chelators did not influence the properties of the peptidic vector. Because the two chelators behave similarly when conjugated to a small peptide, the replacement of DFO by DFO* in larger immuno conjugates is not expected to influence their properties either. The development of DFO*-based BFCAs with improved solubility and their application in the development of immuno-PET imaging agents is currently ongoing.

The clinical need for new chelating systems, which provide ^{89}Zr-based radiopharmaceuticals with an improved stability *in vivo* has stimulated research worldwide. During the preparation of this manuscript, two other groups have independently reported novel octadentate chelators for the complexation of ^{89}Zr and demonstrated their superior stability in comparison to DFO.[9, 15] However, we herein report for the first time a bifunctional version of such an octadentate chelating agent suitable for conjugations to (bio)molecules. Using the peptide bombesin as a model compound for applications to tumour-targeting molecules, we were able to show *in vitro* that the replacement of the traditionally used chelator DFO by the novel DFO* ligand system in radioconjugates holds great promise to provide new PET imaging agents with superior stability profiles.

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Notes and references

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Electronic Supplementary Information (ESI) available: experimental details, characterisation of intermediates, and final compounds by NMR, MS, and HPLC. See DOI: 10.1039/c000000x/

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