Herein we report a DOTA-based lanthanide chelating tag (LCT) with rigidified backbone and a reduction-stable linker. The newly developed tag induces strong pseudocontact shifts suitable for paramagnetic protein nuclear magnetic resonance spectroscopy and the obtained anisotropic susceptibility parameters are in the range of the best performing LCTs.

Pseudocontact shifts (PCS) and residual dipolar couplings (RDC) obtained by using lanthanide chelating tags (LCT) yield valuable restraints for investigating protein structures, dynamics and interactions in solution.1–19 The stereo-specifically methyl substituted 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-based chelators provide a sufficiently rigidified scaffold for observation of significant structural restraints.9,20,21 Interestingly, the methyl substituents on the basic macrocyclic scaffold adopt an equatorial-upper position in the final lanthanide complex.22,23 The methyl substituents thereby arrange in the most suitable way to provide a favourable cavity for the lanthanide ion and minimize the steric repulsion between each other. Furthermore, a crucial factor in the design of the LCTs is to obtain a lanthanide complex that shows only one diastereomer and only one conformation of the pendant arms coordinating to the lanthanide ion.17 The conformationally locked and single diastereomeric LCT provides then only one signal set in 1H–15N HSQC experiments and yields strong paramagnetic effects due to its rigidity and immobilization on the proteins surface.17 As shown by Joss et al., introduction of even more bulkier isopropyl substituents on the backbone of the LCT can significantly enhance the tensor parameters when compared to its methyl substituted predecessor.24 In order to circumvent the inherent instability of disulphide linkers towards a reductive environment, various pyridinesulphone- and iodoacetamide linkers have recently been developed. Therefore, we envisioned to synthesize a lanthanide chelating tag offering the combination of a rigidified backbone and a reductively-stable linker (Fig. 1), that enables convenient and fast protein tagging. The resulting thioether linkage is stable under reductive conditions and has been demonstrated to allow for observation of PCS and RDC in intact eukaryotic cells.2,25

In order to synthesize the newly designed tags, we combined the synthetic approaches by Joss et al. (isopropyl-substituted backbone)24 and Müntener et al. (thiazolo linker)18 and developed them further to yield the target molecule.

To explore the performance, i.e. the range of PCS and RDC as well as the associated $\Delta H$ parameters of the Ln-P4T-DOTA tag (P4T-DOTA: $\{2R_2'R_2'R_2'S_0\}-2,2'$-((2S,5S,8S,11S)-2,5,8,11-tetraisopropyl-10-(2-((methylsulphonyl)thiazolo[5,4-$ab$]pyridine-5-yl)methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropionate), the Dy-, Tm- and Lu complexes of P4T-DOTA were synthesized and conjugated to ubiquitin S57C, ubiquitin K48C and to selectively $^{15}$N leucine labelled human carbonic anhydrase S166C. The newly developed LCT was benchmarked by analysing PCS and RDC (Table 1).

P4T-DOTA delivers large PCS on all tested protein constructs exceeding most current high-performance lanthanide chelating tags (Fig. 2, 3 and 5).3,9,11,15,17,18,28,29 The obtained tensor shapes resemble to the ones found for Ln-DOTA-M8-(4R4S)-SSPy and Ln-P4M4-DOTA, i.e. a significantly less rhombic tensor for the thulium complex when compared to

![Fig. 1](image_url)
the tensor of the dysprosium complexes (Fig. 4, 6 and 7). The tensors for Tm-P4T-UBs57C/K48C as well as for Tm-P4T attached to selectively 15N leucine labelled hCA II S166C display a much more favourable motional averaging in comparison to Tm-DOTA-M8-(4R4S)-SSPy, and ensure in this way that the magnetic anisotropy of the lanthanide is efficiently transferred to the protein. This feature can be attributed to the rigid and very short thiazolo-linker, that only enables rotation around the Cthiazolo–SCys bond. Furthermore an orientation of the tag is enforced, so that the large axial lobe of the isosurfaces is colinear with the Cthiazolo–SCys bond and therefore, less diminished by rotational averaging (see Fig. 4 and 6–8).

When compared to the methyl-substituted thiazolo tag described recently by Müntener et al., the isopropyl-substituted thiazolo tag shows an increase in tensor magnitudes due to the sterically more crowded ligand (Table 1). Besides the favourable $\Delta_2$-tensor properties, the reductively stable linker offers new possibilities, as e.g. applications in in-cell NMR, when compared to the Ln-P4M4-DOTA tag, that can only be employed under non-reductive conditions. The strong paramagnetic relaxation enhancement (PRE), e.g. generated for the Dy-P4T-UB/K48C construct leads to relatively few detectable signals. However, the results obtained for the

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**Table 1** Properties of the induced axial and rhombic components of the paramagnetic susceptibility tensors ($\Delta_{\text{ax}}$ and $\Delta_{\text{rh}}$), metal position in PDB coordinate frame ($X_{\text{metal}}$, $Y_{\text{metal}}$, $Z_{\text{metal}}$), Euler angles ($\alpha$, $\beta$, $\gamma$) and quality factor ($Q$, mathematical definition given in ESI) on ubiquitin S57C (pH 6.0), ubiquitin K48C (pH 6.0) and hCA II S166C (pH 6.8) at 298 K.

<table>
<thead>
<tr>
<th>Protein mutant</th>
<th>PDB</th>
<th>No.</th>
<th>Ln$^{3+}$</th>
<th>$\Delta_{\text{ax}}$ ($10^{-32}$ m$^3$)</th>
<th>$\Delta_{\text{rh}}$ ($10^{-32}$ m$^3$)</th>
<th>$X_{\text{metal}}$ (Å)</th>
<th>$Y_{\text{metal}}$ (Å)</th>
<th>$Z_{\text{metal}}$ (Å)</th>
<th>$\alpha$ (°)</th>
<th>$\beta$ (°)</th>
<th>$\gamma$ (°)</th>
<th>$Q$ (%)</th>
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<tbody>
<tr>
<td>Ubiquitin S57C</td>
<td>1UBI</td>
<td>50</td>
<td>Dy</td>
<td>54.0</td>
<td>27.0</td>
<td>21.6</td>
<td>14.5</td>
<td>6.0</td>
<td>151.7</td>
<td>85.5</td>
<td>132.4</td>
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<td></td>
<td></td>
<td>72</td>
<td>Tm</td>
<td>39.3</td>
<td>14.6</td>
<td>21.6</td>
<td>14.5</td>
<td>6.0</td>
<td>60.0</td>
<td>34.7</td>
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<td>4.6</td>
</tr>
<tr>
<td>Ubiquitin K48C</td>
<td>32</td>
<td>32</td>
<td>Dy</td>
<td>–53.7</td>
<td>–23.5</td>
<td>20.5</td>
<td>19.9</td>
<td>25.8</td>
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<td>117.4</td>
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<td>Tm</td>
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<td>No.</td>
<td>Ln$^{3+}$</td>
<td>$\Delta_{\text{ax}}$ ($10^{-32}$ m$^3$)</td>
<td>$\Delta_{\text{rh}}$ ($10^{-32}$ m$^3$)</td>
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<td>$Z_{\text{metal}}$ (Å)</td>
<td>$\alpha$ (°)</td>
<td>$\beta$ (°)</td>
<td>$\gamma$ (°)</td>
<td>$Q$ (%)</td>
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**Fig. 2** Overlay of $^1$H–$^{15}$N HSQC spectra of Dy- (blue), Tm- (red), and Lu-P4T-UBs57C (black).

**Fig. 3** Overlay of $^1$H–$^{15}$N HSQC spectra of Dy- (blue), Tm- (red), and Lu-P4T-UBs57C (black).

**Fig. 4** Tensors generated by the dysprosium (left) and thulium (right) complex and their relative orientation to ubiquitin S57C (PCS isosurfaces set to 1.5 ppm (outer layer) and 4.0 ppm (inner layer)).

**Fig. 5** Overlay of $^1$H–$^{15}$N HSQC spectra of Dy- (blue), Tm- (red), and Lu-P4T (black) attached to selectively $^{15}$N leucine labelled human carbonic anhydrase II S166C.
Ln-DOTA-M8-(8S)-SSPy complexes show two conformational isomers depending on the ionic radius of the coordinated lanthanide, whereas the (4R4S) stereoisomer shows exclusively a $A(\delta\delta\delta\delta)$ conformation.\textsuperscript{17,22} Based on the performed calculations with and without implicit solvent model, a clear stabilization of all investigated complexes towards a $A(\delta\delta\delta\delta)$ geometry is observed, a result that matches the outcomes for related tags (Table 2).\textsuperscript{17,18} The obtained stabilization energies for the square antiprism (SAP) conformation of the lanthanide complexes in an implicit water solvent of 17.1 (lutetium), 10.6 (thulium) and 19.3 kJ mol\textsuperscript{−1} (dysprosium) correspond to equilibrium constants of 999, 73 and 2388 towards the favoured SAP conformation. Due to the higher steric demand of the thiazolo ligand close to the ninth coordination site when compared to the amide ligand (dysprosium) correspond to equilibrium constants of 999, 73 and 2388 towards the favoured SAP conformation. Due to the higher steric demand of the thiazolo ligand close to the ninth coordination site, there is no coordination of a water molecule,\textsuperscript{30} no calculations with an explicit water molecule on the ninth coordination site were performed.

Interestingly, from an overlay of the DFT structures of Lu-P4M4 and Lu-P4T, the angle of the LCT to the protein can be estimated (Fig. 8). Two striking differences can be observed: (i) the linker in Ln-P4T, which is rigidified by the non-flexible aromatic system, is significantly shorter and more rigid than the corresponding linker in Ln-P4M4, (ii) while Ln-P4T is attached in a favourable angle to the protein in terms of motional averaging, Ln-P4M4 is more prone to averaging of the magnetic anisotropy.

To conclude, a new, strongly paramagnetic lanthanide chelating tag is presented that yields pseudocontact shifts in a very high range, exhibits large anisotropy tensors for both employed lanthanide ions and forms a reductively stable linkage to the target protein. The newly developed tag was benchmarked on three different protein constructs, ubiquitin S57C, ubiquitin K48C and hCA II S166C. When compared to its predecessors, the presented LCT yields strongly enhanced pseudocontact shifts due to the very rigid and short linker in combination with a highly sterically crowded backbone. In order to enable further studies on large proteins, protein...
complexes and other biomacromolecules by PCS NMR spectroscopy, the development of high-performance LCTs will be continued.

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Conflicts of interest

There are no conflicts of interest to declare.

Notes and references