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COMMUNICATION

Two bifunctional desferrioxamine chelators for bioorthogonal labeling of biovectors with zirconium-89

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We report two bifunctional chelators, DFO-Cys and DFO-CBT, to label biovectors with zirconium-89 according to the 2-cyanobenzothiazole/1,2-aminothiol cycloaddition. Their features are high labeling yields, rapid and efficient bioconjugation, metabolically stable luciferin-based end products, and applicability to orthogonal two-step labeling of sensitive biomolecules.

Positron emission tomography (PET) is a sensitive and non-invasive molecular imaging technique, which allows for the visualization and quantification of biological processes in living subjects.[1, 2] The success of PET imaging relies on the development of radiotracers that accumulate in the targeted tissue with high specificity and selectivity. Therefore, extensive effort has been made to develop radiolabeled biological molecules, which target oncoproteins overexpressed on the extracellular membrane of cancer cells.[3-5] Of the potential radionuclides, zirconium-89 is an attractive candidate for PET imaging with its half-life of 78.4 h and its positron mean energy of 0.389 MeV.[6, 7] ^{89}Zr is often associated to desferrioxamine B (DFO), a siderophore known to form stable hexadentate complex with Zr(IV).[8] Several investigations were recently conducted to improve the *in vivo* stability of ^{89}Zr-DFO complex and to minimize the accumulation of osteophilic ^{89}Zr(IV) in bones.[9-11] Specifically, Patra et al. have transformed DFO into an octadentate chelator, dubbed DFO*, through the addition of a hydroxamic acid moiety and a desferrioxamine B squaramide ester was prepared by Rudd and coworkers as a putative octadentate ligand for ^{89}Zr(IV).[12, 13] It has been shown that the new ligands reduce to some extent the release of ^{89}Zr in animal models. The instability of the complex is mainly observed in preclinical models, presumably because of a higher rate of transchelation and/or a more pronounced metabolic degradation in rodents than in human subjects.[14] Thus, DFO remains the most prominent chelator for ^{89}Zr-radiopharmaceuticals.

DFO is commercially available under two forms, a variant containing a maleimide functional group for Michael addition with thiols and a *p*-phenyl-isothiocyanate intermediate that reacts with primary amines of lysine residues. None of these precursors allows control over chemical reactivity and selectivity and consequently a major limitation is the lack of regioselectivity during the coupling of the chelator to the biovector. Recently, the ligation between 2-cyanobenzothiazole (CBT) and 1,2-aminothiol (e.g. a *N*-terminal cysteine residue), forming stable luciferin linkage, was reported as an attractive labeling strategy for sensitive biomolecules.[15-17] The reaction was shown to be biocompatible, rapid and orthogonal; and it has been applied to the labeling of peptides and proteins with small ^{18}F-fluorinated prosthetic groups.[18-21] Surprisingly, it has never been applied to the labeling of biomolecules with radiometals; although this click reaction can be performed in aqueous media and without the need of metal catalyst.

Herein, we report the synthesis of two bifunctional chelators (BFCs) and their application to label biovectors with ^{89}Zr via the luciferin-adduct formation. Cyclic RGD peptides were chosen as proof-of-principle biovectors in this study because characterization of ^{89}Zr-bioconjugates is facilitated by using low molecular weight molecules.[12, 22] The arginine–glycine–aspartate (RGD) motif is the natural ligand sequence recognized by several integrins - including $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ - highly up-regulated in cancer, thrombosis, cardiovascular, and inflammatory diseases. Labeled RGD-based soluble peptides have been extensively used to image angiogenesis and to monitor response to antiangiogenic therapies.[23, 24]

BFCs **4** and **10** were synthesized by installing either the 1,2-

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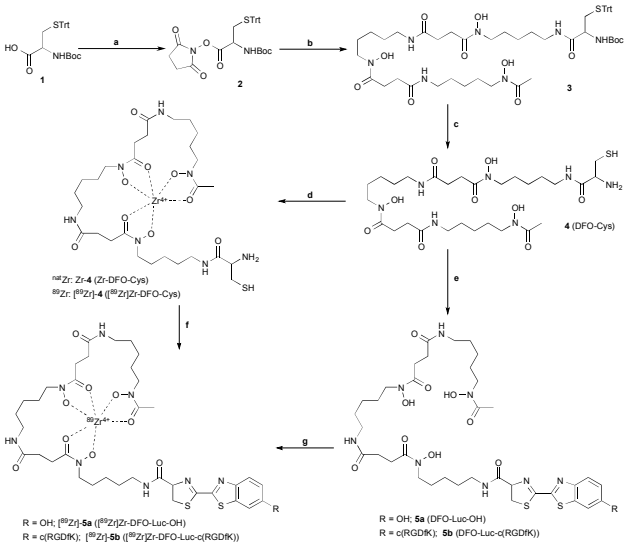
aminothiol or CBT click functionality on the primary amine of DFO. DFO-Cys (**4**) was readily accessible in three steps with a satisfactory overall yield of 30% (Scheme 1). *N*-(*tert*-Butoxycarbonyl)-*S*-trityl-L-cysteine was converted into an activated succinimidyl ester and then reacted with DFO mesylate under basic conditions. Gentle heating was required to aid in the dissolution of DFO and to improve the yield of amide **3**. Removal of the protecting groups provided DFO-Cys (**4**) after HPLC purification. DFO-CBT (**10**) was obtained in a four step straightforward procedure consisting of a Williamson ether synthesis by treatment of 2-cyano-6-hydroxy-benzothiazole with *tert*-butyl bromoacetate, followed by the *tert*-butyl deprotection, activation of the carboxylic acid as succinimidyl ester, and conjugation to the primary amine of desferroxiamine (Scheme 2). DFO-CBT (**10**) was obtained with an overall yield of 8% after HPLC purification. Further details on the synthesis of BFCs **4** and **10** are provided in the ESI.†

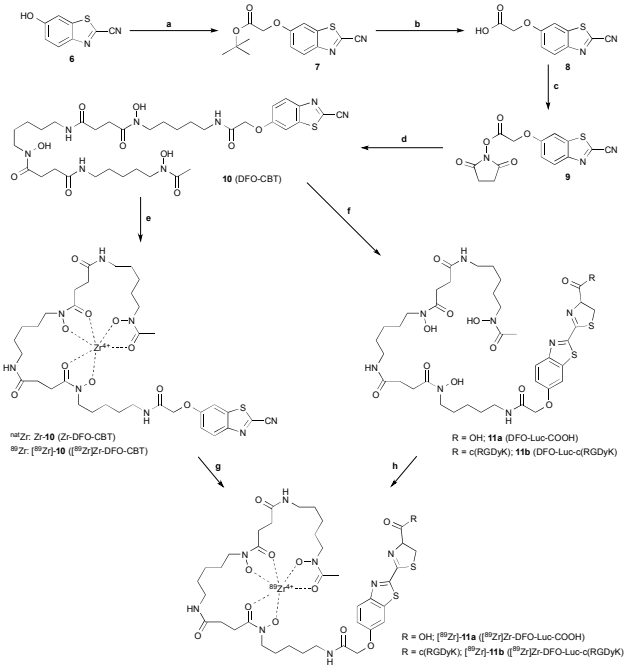
Scheme 1. Synthesis of DFO-Cys (**4**) and the corresponding luciferin adducts [^{89}Zr]-**5a** and [^{89}Zr]-**5b**.



Reaction conditions. (a) NHS, EDC, DMF, rt, 24 h; (b) DFO mesylate, Et_3N, DMF, 45 °C, 18 h; (c) TFA, TIPS, H_2O, rt, 2 h; (d) ^{nat}Zr: $ZrCl_4$, Na_2CO_3, H_2O, rt, 2 h; ^{89}Zr: [^{89}Zr]Zr-oxalate, $NaHCO_3$, HEPES buffer, rt, 1.5 h; (e) TCEP.HCl, 2-cyano-6-hydroxybenzothiazole or c(RGDfK)-CBT, PBS/DMF, rt, 4-6 h; (f) TCEP.HCl, **6** or c(RGDfK)-CBT, PBS/MeOH or DMF, 37 °C, 60-90 min; (g) **5a**, [^{89}Zr]Zr-oxalate, $NaHCO_3$, HEPES buffer, rt, 1.5 h.

Complexes of ^{nat}Zr-DFO-Cys and ^{nat}Zr-DFO-CBT were prepared by treatment of **4** and **10** with non-radioactive $ZrCl_4$ under slightly basic conditions (Schemes 1 and 2). After characterization of the complexes by MS and confirmation of a metal-to-chelator ratio of 1:1, they were used as non-radioactive HPLC standards to determine the identity of [^{89}Zr]Zr-DFO-Cys ([^{89}Zr]-**4**) and [^{89}Zr]Zr-DFO-CBT ([^{89}Zr]-**10**) (Fig. S1 and S2 in ESI). Labeling of DFO-Cys and DFO-CBT with [^{89}Zr]Zr-oxalate was carried out in mild conditions. As determined by iTLC chromatography, quantitative labeling yields were obtained after incubation at room temperature for 90 minutes (Table S1 in ESI), and we did not observe any influence of the click functionality on the coordination of zirconium with DFO.

Scheme 2. Synthesis of DFO-CBT (**10**) and the corresponding luciferin adducts [^{89}Zr]-**11a** and [^{89}Zr]-**11b**.



Reaction conditions. (a) *tert*-butyl bromoacetate, K_2CO_3, NaI, acetone, reflux, overnight; (b) TFA, TIPS, $CHCl_3$, rt, overnight; (c) EDC, NHS, DMF, rt, 48 h; (d) DFO mesylate, Et_3N, DMF, 45 °C, 4 h; (e) ^{nat}Zr: $ZrCl_4$, Na_2CO_3, H_2O, rt, 2 h; ^{89}Zr: [^{89}Zr]Zr-oxalate, $NaHCO_3$, HEPES buffer, rt, 1.5 h; (f) L-Cysteine or c[RGDfK(C)], PBS/DMF, rt, 4-6 h; (g) L-Cysteine or c[RGDfK(C)], PBS, 37 °C, 10-90 min; (h) **11a**, [^{89}Zr]Zr-oxalate, $NaHCO_3$, HEPES buffer, rt, 1.5 h.

Subsequently, the bioorthogonal ligation was tested under biologically friendly conditions. Coupling of [^{89}Zr]Zr-DFO-Cys and [^{89}Zr]Zr-DFO-CBT with 2-cyano-6-hydroxy-benzothiazole (6-OH-CBT, **6**) and L-cysteine, respectively, led to the quantitative conversion of [^{89}Zr]-**4** and [^{89}Zr]-**10** into [^{89}Zr]Zr-DFO-Luc-OH ([^{89}Zr]-**5a**) and [^{89}Zr]Zr-DFO-Luc-COOH ([^{89}Zr]-**11a**) after 1 h at 37 °C (Table 1). To confirm the identity of the clicked products [^{89}Zr]-**5a** and [^{89}Zr]-**11a**, we directly labeled DFO-Luc-OH (**5a**) and DFO-Luc-COOH (**11a**) with [^{89}Zr]Zr-oxalate. Identical HPLC retention times were obtained for the radiocomplexes resulting from direct labeling and the corresponding products formed by the cycloaddition reaction (Fig. S3 and S4 in ESI). Thus, generation of radioconjugates can be successfully achieved from DFO-Cys (**4**) and DFO-CBT (**10**) either by the click-labeling (labeling followed by the click reaction) or by the labeling-click synthetic pathway

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(radiolabeling preceded by the bioconjugation). The labeling-click approach may appear less convenient than the click-labeling since a two step radiochemical process is involved. However, owing to the long half-life of ^{89}Zr and the fast kinetics of the CBT/Cys ligation, this two step labeling approach might be more suitable than the click-labeling process when working with sensitive biomolecules or when small quantities of biomolecule are available.

Table 1. Conditions of the click reaction between [^{89}Zr]-**4** or [^{89}Zr]-**10** and their complementary click functionality.

Ligand	[^{89}Zr]-**4**	[^{89}Zr]-**10**
Quantity (nmol)	4.5	4.5
Temperature (°C)	37	37
Pair compound (nmol)[a]	10	10
TCEP.HCl (nmol)	5	--
Reaction time (min)	60	10
pH	7.4	7.4
Yield (%)	99.9	98.4

[a] 6-OH-CBT was used as pair compound for [^{89}Zr]-**4**, while L-cysteine reacted with [^{89}Zr]-**10**.

Then, we proceeded to further investigations on the efficiency of the click reaction between [^{89}Zr]Zr-DFO-Cys and 6-OH-CBT under different reaction conditions. The choice of solvent and reducing agent was based on conditions reported previously.[16, 17] Various pH and reaction temperatures were tested to optimize the conjugation of [^{89}Zr]-**4** with 6-OH-CBT (Table 2). 54% reaction yield was observed when the reaction was carried out at pH 7.4, compared to 16% and 7% at pH 6 and 4, respectively (Fig. S5 in ESI). The increase in bioconjugation yield from pH 4 to 7.4 might be rationalized in terms of conversion of the cationic form of the cysteine present at acidic pH to the neutral form of the cysteine residue at pH above its isoelectric point; making the primary amine more accessible to the coupling reaction. Formation of the luciferin conjugate proceeded optimally at a pH ranging between 7 and 8. Increasing basicity of the solvent had a detrimental effect on the coupling reaction due to the hydrolysis of the cyano group of **6** into the respective carboxamide. Temperature had only a minor effect on the conjugation reaction between [^{89}Zr]-**4** and **6**. An increase of the temperature from 23 to 37 °C resulted in a slight increase of the yield from 46% to 54% after a 10 min incubation time.

To investigate the kinetics of the coupling reactions between [^{89}Zr]-**4** and 6-OH-CBT or [^{89}Zr]-**10** and L-cysteine, the reactions were performed in the optimal pH and temperature conditions (Table S3 in ESI). TCEP·HCl (1.1 eq.) was added to [^{89}Zr]-**4** to prevent oxidation of the thiol group. At specific time points, the reactions were quenched by addition of acetic acid and the conversion yields were determined by analytical radio-HPLC (Fig. S6 and S7 in ESI). [^{89}Zr]-**4** was quantitatively converted into [^{89}Zr]-**5a** in 40 min. However, the reaction between [^{89}Zr]Zr-DFO-CBT and L-cysteine was even faster since the cycloaddition was completed in only 10 min. [^{89}Zr]-**10** reacted more efficiently with its pair compound, presumably because no conditioning of the cysteine is needed after the labeling reaction of **10**.

Table 2. Influence of pH and temperature on the click reaction.

Ligand	[^{89}Zr]-**4**		[^{89}Zr]-**10**	
Temperature (°C)	rt	37	rt	37
pH	Click efficiency (%) [a]			
4	3.6	7.0	-	-
6	14.2	15.8	-	-
7.4	46.2	53.7	96.7	98.4

[a] Click reaction was performed for 10 min. 6-OH-CBT was used for the click reaction with [^{89}Zr]-**4** and L-cysteine with [^{89}Zr]-**10**.

We next turned our attention to address the stability of [^{89}Zr]-**5a** and [^{89}Zr]-**11a** using transchelation experiments (Table 3). In our challenge study, [^{89}Zr]-**5a** and [^{89}Zr]-**11a** were treated with a large excess of cysteine, known to possess metal binding ability. Samples were taken at 1 h and 24 h and analyzed by radio-HPLC. More than 99% of [^{89}Zr]-**5a** and [^{89}Zr]-**11a** remained intact in the presence of an excess of cysteine, which implies the high stability of the radiocomplexes. Stability of [^{89}Zr]-**5a** and [^{89}Zr]-**11a** adducts were then evaluated in PBS buffer and human serum. Both conjugates were stable for up to 12 hours in PBS at 37 °C. High stability was also observed in human serum, as more than 90% of intact labeled complexes were present after a 12 h incubation time. A slow dissociation of zirconium-89 from the ligands was noticed in serum, but the luciferin linkage was found stable. Finally, we looked at the interaction of our radioconjugates with serum proteins. It was determined that binding of [^{89}Zr]-**5a** and [^{89}Zr]-**11a** to serum proteins was relatively weak. Indeed, only a minor fraction of [^{89}Zr]-**5a** and [^{89}Zr]-**11a**, 24% and 19%, were respectively bound to serum proteins after a 4 h incubation in human serum.

Table 3. Stability studies of [^{89}Zr]-**5a** and [^{89}Zr]-**11a**.[a]

Labeled complex	Transchelation		Stability			
	Cysteine		Buffer		Serum	
	1 h	24 h	1 h	12 h	1 h	12 h
[^{89}Zr]-**5a**	> 99	> 99	> 99	> 99	96.3	90.7
[^{89}Zr]-**11a**	> 99	98.4	> 99	> 99	95.8	92.1

[a] Results are expressed as % of intact ligand after incubation.

With the two modified DFO-based chelators (**4** and **10**) in hand, we next evaluated their potential to label biovectors with ^{89}Zr. We chose two cyclic RGD peptides as model compounds. Peptide syntheses were accomplished by standard Fmoc solid-phase peptide synthesis (SPPS). Introduction of the CBT functional group to the primary amine of the lysine residue was performed post-synthetically after resin cleavage, deprotection and cyclization of the peptide. Functionalization of the lysine with a 1,2-aminothiol group was more amenable to Fmoc-SPPS, as the protected di-amino acid Fmoc-Lys(Boc-Cys(Trt))-OH was introduced *in situ* during SPPS.[17] Peptides were obtained with a chemical purity >95% after

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HPLC purification and their identities were confirmed by mass spectrometry. [^{89}Zr]Zr-DFO-Cys was successfully conjugated to c(RGDfK)-CBT (**12**) in PBS buffer and in presence of TCEP (Fig. S11 in ESI). Solubility of **12** in aqueous media was limited, and therefore DMF was used as co-solvent. The final pH of the reaction mixture was approximately 7.4 and the coupling reaction was carried out at 37 °C for 90 min. We observed that all [^{89}Zr]Zr-DFO-Cys (t_R = 13.5 min) was consumed during the reaction and a new signal corresponding to [^{89}Zr]-**5b** was visualized at 17.5 min on the HPLC chromatogram (Fig. S12 in ESI). Similarly, [^{89}Zr]Zr-DFO-CBT was successfully conjugated to c[RGDyK(C)] (**13**) in PBS buffer (Fig. S13 in ESI). **13** was freshly prepared to prevent the oxidation of the thiol moiety and the cycloaddition reaction was performed under the conditions previously described (Fig. S14 in ESI). More than 98% of [^{89}Zr]Zr-DFO-CBT (t_R = 18.6 min) was consumed during the reaction and [^{89}Zr]-**11b** was observed at 16.8 min. We noticed that the time required for the conjugation of [^{89}Zr]Zr-DFO-Cys or [^{89}Zr]Zr-DFO-CBT to the corresponding RGD peptide is longer than the time needed for the coupling with 6-OH-CBT and L-cysteine. Our results are in accordance with previous data from Jeon et coworkers, who reported 92% yields in 1 and 20 min for the coupling of their ^{18}F-labeled CBT-based prosthetic group to cysteine and a dimeric RGD peptide, respectively.[18]

In summary, two novel DFO-based BFCs were developed for the labeling of biomolecules with zirconium-89 via the bioorthogonal ligation of 2-cyanobenzothiazole and 1,2-aminothiol. Both BFCs were easily prepared and labeled at room temperature with [^{89}Zr]Zr-oxalate. [^{89}Zr]Zr-DFO-Cys and [^{89}Zr]Zr-DFO-CBT have been rapidly and specifically conjugated to RGD peptides at 37 °C and pH 7.4. The luciferin linkage displayed good *in vitro* stability. Successful labeling of the *N*-terminal cysteine residue of **13** with [^{89}Zr]Zr-DFO-CBT indicated that **10** could also be used for chemoselective ^{89}Zr-labeling of other cysteine-bearing biomolecules, such as proteins and antibodies. Moreover, the orthogonality of the reaction, the metabolic stability of the ligation reagents and the end-product suggest that the luciferin-adduct formation might be compatible for pretargeted imaging, provided that reaction kinetics are favorable.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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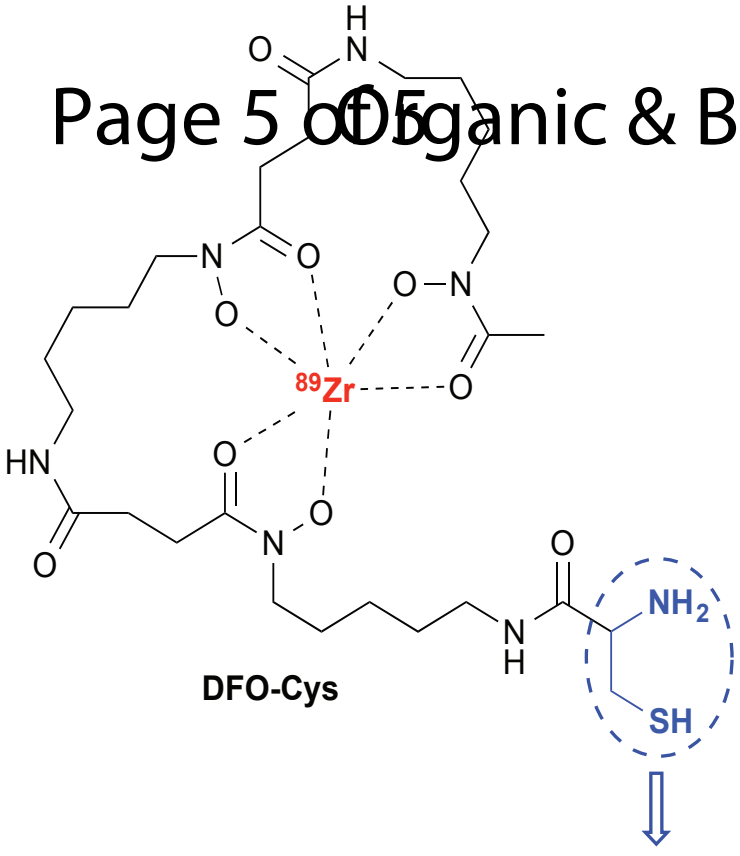

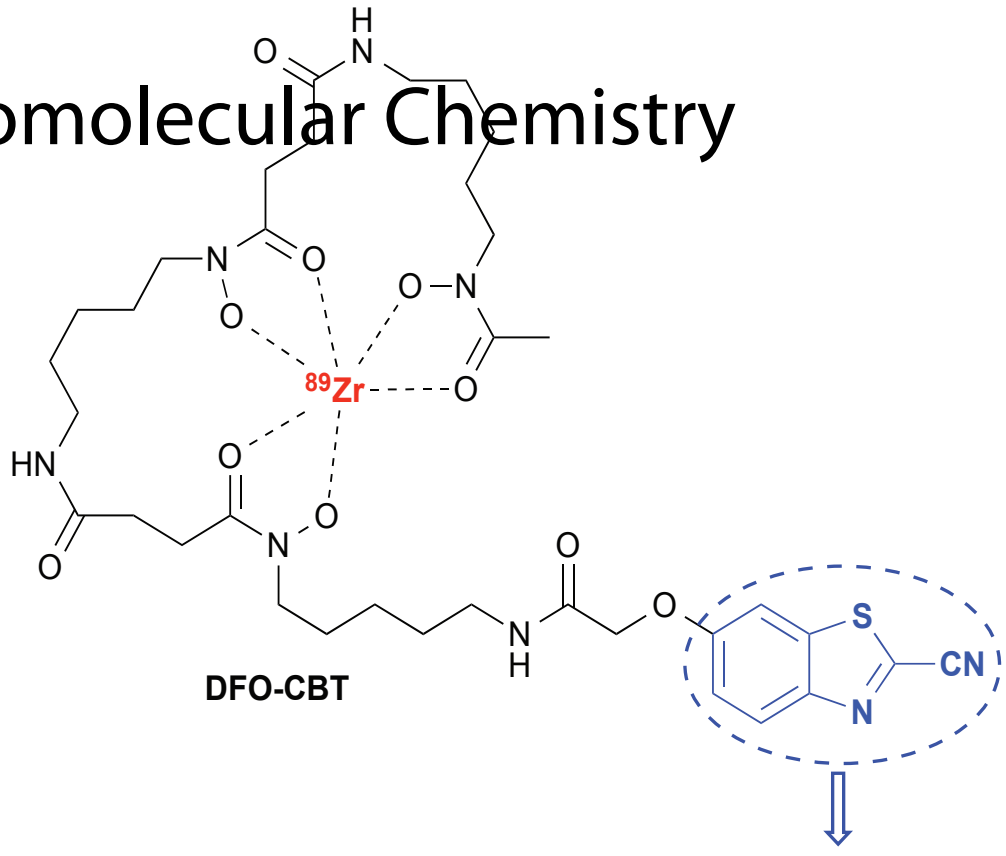

Bioorthogonal ligation to biomolecules containing the complementary click functionality