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Early-Stage Incorporation Strategy for Regioselective Labeling of Peptides using the 2-Cyanobenzothiazole/1,2-Aminothiol Bioorthogonal Click Reaction

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Herein, we describe a synthetic strategy for the regioselective labeling of peptides by using a bioorthogonal click reaction between 2-cyanobenzothiazole (CBT) and a 1,2-aminothiol moiety. This methodology allows for the facile and site-specific modification of peptides with various imaging agents, including fluorophores and radioisotope-containing prosthetic groups. We investigated the feasibility of an early-stage incorporation of dipeptide **1** into targeting vectors, such as **c[RGDyK(C)]** and **HER2 pep**, during solid-phase peptide synthesis. Then, the utility of the click reaction to label bioactive peptides with a CBT-modified imaging agent (FITC–CBT, **9**) was assessed. The ligation reaction was found to be highly selective and efficient under various conditions. The fluorescently labeled peptides **2** and **3** were obtained in respective yields of 88 and 82% under optimized conditions.

Low-molecular-weight peptides have received increasing interest as molecular imaging probes and therapeutics for cancer.[1,2] Their short blood half-life, low toxicity of degradative products, and high specificity for biomolecular targets make them attractive candidates for use as diagnostic agents.[3,4] Several short peptides, such as octreotide and BBN(7–14) analogs, have been previously identified as efficient ligands to image overexpressed receptors on tumor cell surfaces.[5–7] Incorporation of a radionuclide or a fluorogenic dye into peptides provides the functionality required for cancer diagnosis using real-time, non-invasive imaging technologies, such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), and fluorescence imaging.[8–10]

For a labeled peptide to be effective, it must maintain affinity and specificity for its target. The most common strategy to incorporate an imaging agent into a peptide-based targeting vector is a post-synthetic modification (Figure 1 A). It consists of a direct-labeling approach mainly based on the side-chain reactivity of lysine (Lys) or cysteine (Cys) residues, where the imaging agent is coupled to the targeting vector through the formation of either a peptide bond, thioether, or thiourea linkage. Direct labeling is often chosen because it allows us to make use of commercially available peptides and imaging agents. However, these conventional conjugations often lack regioselectivity. The functionalized imaging agent (e.g. succinimidyl ester, maleimide, isothiocyanate) can react with multiple residues and generate a mixture of labeled products with varying yields. Labeling on the bioactive portion of the targeting vector may compromise its bioactivity and, therefore, tedious purification may be required to isolate the desired product.[11] Moreover, a distorted stoichiometry, that is, nanomolar quantities of the imaging agent versus micromolar amounts of the targeting peptide, is applied to favor the kinetics of the bioconjugation.[12] Furthermore, the labeling efficiency can be affected by the distribution of the functional groups of the lysine and cysteine residues in the peptide sequence.[13] In some cases, these intrinsic residues can become buried within the secondary and tertiary structural conformation, resulting in

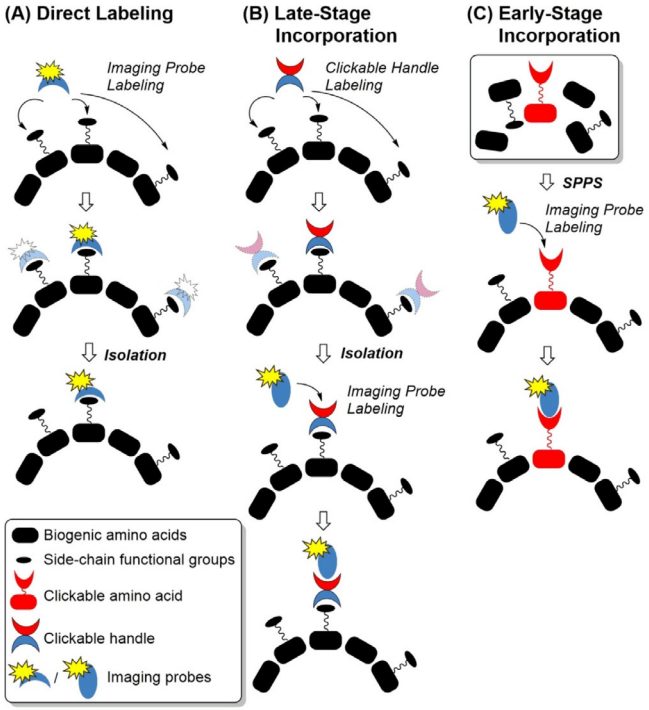

Figure 1. Synthetic strategies for peptide-based imaging probes labeling. A) Direct labeling; B) late-stage incorporation; C) early-stage incorporation.

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inefficient couplings.[14] Thus, for the successful development of molecular imaging probes, strategies avoiding non-specific labeling must be emphasized.

An alternative strategy to the direct labeling is to functionalize inherent residues with a clickable handle for late-stage labeling (Figure 1 B). The functionality is used to incorporate an imaging agent through a rapid and chemoselective click reaction. This strategy has been successfully used in the labeling of peptides with sensitive fluorophores or short-lived radionuclides.[15–18] However, similar to the direct-labeling approach, incorporation of the clickable handle is not selective and requires an isolation step prior to the conjugation of the imaging agent. To overcome this critical limitation, an early-stage incorporation strategy (Figure 1 C) has been advanced to enable regioselective labeling of peptide-based molecular imaging probes. With this method, a molecule functionalized with a clickable handle is introduced into the peptide sequence during the synthesis, followed by the chemoselective ligation of an imaging agent containing the complementary click functionality. This strategy not only avoids the formation of multi-labeled products, but also allows for a flexible design where the click handle is incorporated on a non-bioactive region of the peptide, thus preserving its native function.

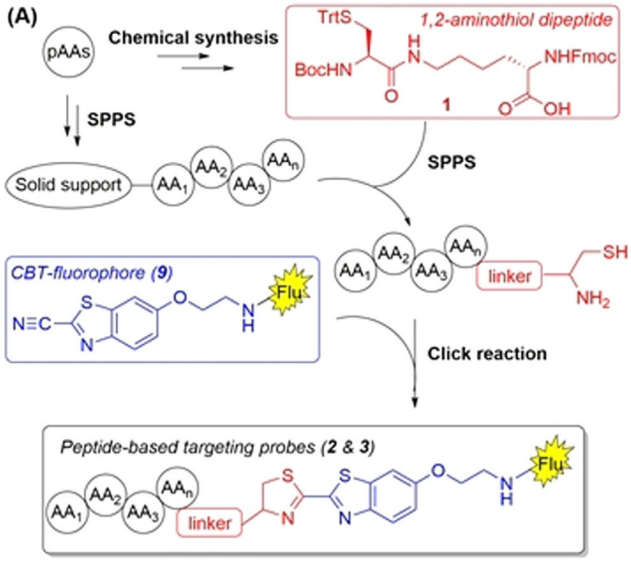
Synthetic approaches to regioselectively label peptides often implement a variety of click reactions, including the Huisgen cycloaddition (azide/alkyne), Staudinger ligation between an azide and phosphite to yield an iminophosphorane, and the inverse electron demand Diels–Alder reaction (tetrazine/*trans*-cyclooctene).[19–21] While these synthetic routes are viable labeling methods, they require the insertion of a non-biogenic functionality in the peptide motif for the click reaction. Such functional groups are not always compatible with reagents used in peptide synthesis (i.e. piperidine and trifluoroacetic acid). Moreover, the click reaction may involve a toxic metal catalyst, considered too slow for short-lived radionuclides or the click reagents might be quite unstable. Consequently, these reactions may not be the most suitable for the early-stage strategy.

Over the last few years, we studied the naturally occurring 2-cyanobenzothiazole (CBT)/1,2-aminothiol click reaction for the labeling of PET imaging probes.[22,23] The bioorthogonality as well as the high selectivity, metabolic stability, and rapid formation of the resulting luciferin conjugate (approximately three orders of magnitude faster than the Staudinger ligation) makes this synthetic approach an attractive option for radiochemistry and for general bioconjugations.[24–27] Indeed, considering the fragile nature of biomolecules and the relatively short half-life of some positron-emitting radionuclides (^{18}F, $T_{1/2}$: 109.8 min; ^{68}Ga, $T_{1/2}$: 67.6 min), the radiolabeling procedures should be rapid and simple. As such, we envisioned to take advantage of the facile ligation between CBT and an *N*-terminal cysteine (Cys) residue, which offers a nearly ideal labeling strategy based on early-stage incorporation for peptides. Insertion of the biogenic cysteine amino acid in a non-bioactive portion of the targeting vector warrants the compatibility with standard solid-phase peptide synthesis (SPPS) reaction conditions and the efficient coupling of the imaging agent.

We describe, herein, the preparation of peptide-based imaging probes with an early-stage incorporation strategy through a rapid CBT/1,2-aminothiol click reaction. The scope of this approach was demonstrated through the labeling of two targeting vectors, a cyclic and a linear peptide, with well-established biological activity. Joshi et al. recently identified a peptide sequence that specifically binds to an extracellular domain of the human epidermal growth factor 2 receptor (HER2).[6] HER2 is a member of the ErbB tyrosine kinase family, which is significantly overexpressed in many tumors. A linker containing a terminal lysine residue was introduced onto the HER2 binding sequence for on-resin derivatization of the peptide. A fluorescent probe was installed on the lysine after selective removal of the N^{ε} protecting group. Although this approach enabled selective labeling of the lysine residue, it is not adapted to radioactive labeling. Indeed, deprotection and cleavage of the peptide from the solid support is done post-labeling, the labeling efficiency is quite low, and the reaction time is too long. We instead envisioned that Fmoc—Lys–OH could be functionalized with Boc–Cys(Trt)–OSuc to generate Fmoc–Lys(Boc–Cys(Trt))–OH (**1**) for use directly in SPPS without the need for foreign reagents or expensive protecting groups in our synthesis of **HER2 pep** (**S4**). Analogously, we applied this strategy to the synthesis of **c[RGDyK(C)]** (**S3**), where the RGD motif is a known antagonist of the $\alpha_v\beta_3$ integrin, a transmembrane protein that is also overexpressed in malignant tissue. As an initial proof of concept, we evaluated the kinetics of the CBT/Cys ligation on these two bioactive peptides with a fluorophore functionalized with CBT. We propose that this bioconjugation strategy not only provides maximal versatility and modularity in developing peptide-based imaging probes, but also introduces a new strategy for pre-targeting imaging and therapy.

Our strategy for the development of the peptide-based targeting probes **2** and **3** is illustrated in Figure 2. Preparation of the linear peptide chains was performed by standard Fmoc SPPS.[28] Dipeptide **1** was designed as a Fmoc-compatible substrate. The acid-labile trityl and *tert*-butyloxycarbonyl protecting groups on **1** are easily removed in the final cleavage/deprotection step and release the 1,2-aminothiol click functionality for subsequent coupling to a CBT-bearing imaging agent. Synthesis of dipeptide **1** is outlined in Scheme 1. Activation of the carboxylic acid group of commercially available Boc–Cys(Trt)–OH (**4**) was accomplished through coupling with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC) in ethyl acetate (EtOAc) to yield **5**. The resulting ester **5** was conjugated with Fmoc–Lys–OH in mild basic conditions to produce the CBT-conjugating building block **1** in a satisfying yield of 75 % over the two steps.

Synthesis of the bioactive peptides **c[RGDyK(C)]** (**S3**) and **HER2 pep** (**S4**) containing dipeptide **1** were performed on an automated synthesizer by following a standard Fmoc-based SPPS strategy (structures and experimental details provided in the Supporting Information).[29,30] In the synthesis of the linear portion of **S3**, Fmoc–Gly–OH was selected as the initial residue for loading onto the 2-chlorotrityl chloride solid support (**S1**) to prevent unwanted *C*-terminal epimerization during the final cyclization step (Scheme S1).[31] The following conjugations



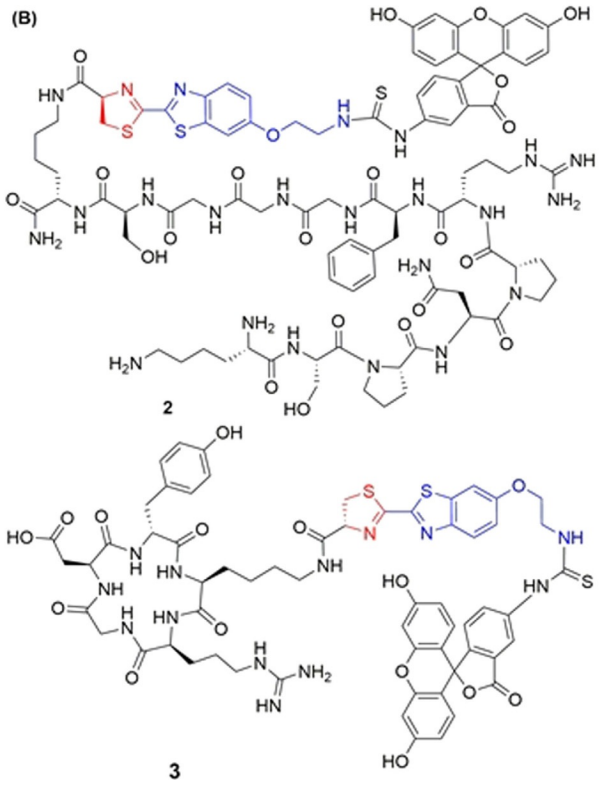
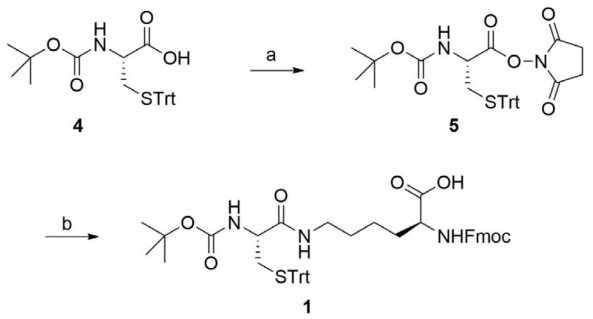


Figure 2. A) General strategy for the development of peptide-based imaging probes through SPPS and CBT/Cys click reaction. AA_n: amino acid, pAAs: protected amino acids. B) Chemical structures of **2** and **3**.

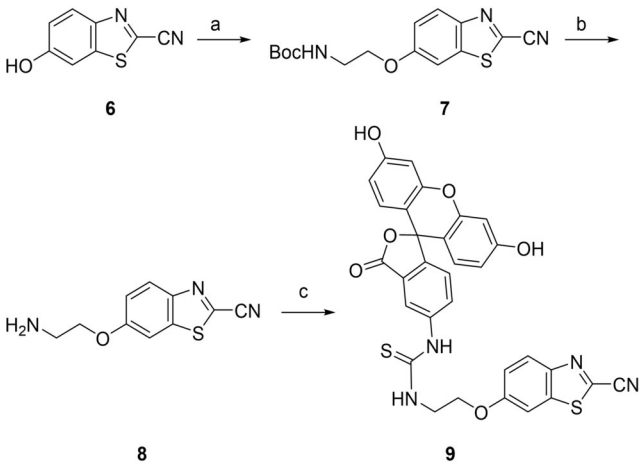
with Fmoc–Arg(Pbf)–OH, **1**, Fmoc–D-Tyr(*t*Bu)–OH and Fmoc–Asp(O*t*Bu)–OH were performed by using HBTU/*N*,*N*-diisopropylethylamine (DIPEA) as coupling reagents. Cleavage of the Fmoc protecting group was accomplished by treatment with 20% piperidine in DMF. The protected linear pentapeptide was selectively cleaved from the resin by using an optimized solution of acetic acid/trifluoroethanol/dichloromethane (v/v/v = 1:1:10). **S2** was obtained in a yield of 89% with all side-chain



Scheme 1. Synthesis of Fmoc–Lys(Boc–Cys(Trt))–OH (**1**). Reagents and conditions: a) *N*-hydroxysuccinimide, DCC, EtOAc, rt, 24 h; b) Fmoc–Lys–OH, triethylamine, ACN/DMF, 40 °C, 16 h, 75% over two steps.

protecting groups intact. Cyclization was carried out by treatment of **S2** with diphenylphosphoryl azide (DPPA) under basic and diluted conditions. Subsequent global deprotection by treatment of the cyclic peptide with a solution of TFA/H_2O/TIPS (v/v/v = 95:2.5:2.5) yielded the $\alpha_v\beta_3$ antagonist **S3** in 7% yield over the last two steps. **S4** was prepared by a similar Fmoc-based SPPS strategy by using a Rink amide MBHA resin. Preparation of **S4** was initiated by loading **1** onto the solid support, followed by a successive coupling/deprotection sequence with the corresponding Fmoc-protected amino acids. After cleavage, **HER2 pep** (**S4**) was isolated in a yield of 68% based on initial resin loading.

We next turned our attention to the preparation of the CBT-linked fluorophore **9**, outlined in Scheme 2. Our synthesis was initiated by the *O*-alkylation of 6-hydroxy-2-cyanobenzothiazole (**6**) with *N*-Boc-2-bromoethyl-amine under basic conditions to afford the *N*-Boc-protected CBT **7** in 83% yield. Initial attempts to remove the *N*-Boc protecting group with TFA in dichloromethane (DCM) at room temperature were unsuccessful. We presumed that alkylation of the cyano moiety was occurring because of the presence of the *tert*-butyl cation, followed by a transformation into *N*-*tert*-butylamide side product via hydrolysis. To overcome the degradation of the cyano group



Scheme 2. Preparation of FITC–CBT (**9**). Reagents and conditions: a) *N*-Boc-2-bromoethyl-amine, NaI, K_2CO_3, acetone, reflux, 24 h, 83%; b) TFA, thioanisole, DCM, 0 °C to rt, 4 h, 92%; c) Fluorescein isothiocyanate, DMF, rt, 4 h, 68%.

during deprotection, thioanisole was utilized in the reaction as a cation scavenger. After optimization of the reaction conditions, the amino derivative **8** was obtained in 92% yield with no indication of degradative product. **8** was then conjugated to fluorescein isothiocyanate (FITC) to produce the FITC–CBT analogue **9** in 68% yield.

With the fluorescent probe **9** in hand, we studied the reactivity of CBT derivatives **6** and **9** against 1,2-aminothiol to form luciferin conjugates. We first determined the second-order rate constant of the reaction between **6** and L-cysteine by using a high-performance liquid chromatography (HPLC)-based assay.[24] The assay was performed at pH 6.0, 7.4, and 9.0 in phosphate-buffered saline (PBS) and gave rate constants of 0.004, 4.5, and 18.6 $M^{-1}s^{-1}$, respectively. The value at pH 7.4 is consistent with what was previously reported by Godinat et al. (4.5 vs. 3.2 $M^{-1}s^{-1}$; Figure S1).[26] The results indicate that the chemoselective ligation is pH dependent, as reaction rates at neutral and basic pH conditions are 1125 and 4650 times higher than the rate of conjugation under slightly acidic conditions. From the mechanistic point of view, the thiol group is initially involved in the attack of the CBT cyano group for the formation of the luciferin ring. Our observation implied that the increasing nucleophilicity of the thiol group could accelerate the first step of the cyclization.[32]

We then analyzed the second-order kinetics of the reaction between **9** and L-cysteine, which exhibited a rate constant of 2.1 $M^{-1}s^{-1}$ at pH 7.4 in PBS (Figure S2). The data indicate that **6** and **9** have similar reactivity towards free cysteine. It suggests that modification at the 6 position has no detrimental effect on the reactivity of CBT. Therefore, it is possible to exploit this position to develop a wide range of highly reactive CBT-based reagents for the bioorthogonal ligation with 1,2-aminothiol containing molecules for biochemical and pharmacological studies.

To illustrate the applicability of our methodology, we investigated the bioconjugation reaction between **9** and our synthetic peptides. **9** was found to be stable between pH 7.0 and 9.0 in PBS or DMF-containing DIPEA, but completely decomposed at a higher pH in PBS after 1 h incubation at room temperature (Figure S3). Thus, the coupling reactions were evaluated in different neutral to slightly basic conditions. A mixture of **9** (1.0 equiv) and **HER2 pep** (1.0 equiv) at pH 7.4 in PBS and in the presence of tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl; 2.0 equiv) was monitored (Table 1, entry 1). As shown in Figure 3, after 1 h at room temperature, the starting material **9** was completely consumed. Formation of FITC–**HER2 pep** (**2**) was observed by reverse-phase HPLC (RP-HPLC) and confirmed by electrospray ionization mass spectrometry (ESI-MS). The reaction mixture was then purified by RP-HPLC to give **2** in 71% yield. Purity of the bioconjugate was estimated to be higher than 95% by HPLC (Figure S4). Next, we evaluated the click reaction at pH 9.0 in PBS. As previously observed with L-cysteine, the reaction of **9** with **HER2 pep** was faster at pH 9.0 than pH 7.4. Total consumption of **9** was observed after 30 min of reaction, resulting in a similar yield of **2** (78%) after purification by RP-HPLC (Table 1, entry 2). To mimic in vivo conditions, the reaction was performed in the presence of 10% human serum in PBS. Despite the complex medium, the bioconjugation reaction was extremely efficient. **2** was isolated with a yield of 73% after 1 h incubation (Table 1, entry 3). Similar yields were obtained for the CBT/Cys click reaction both in the presence or absence of human serum, thereby illustrating the high degree of selectivity of CBT-based reagents towards the *N*-terminal cysteine residue. Importantly, this highlights the potential of this bioorthogonal reaction for in vivo applications, such as a pre-targeting methodology. The reaction yields (88%) were increased by using DMF as the solvent and DIPEA as the base (Table 1, entry 4). This improvement is likely the result of a better solubility of **2** in DMF than in aqueous solution. FITC–**c(RGDyK)** (**3**) was synthesized under the optimized conditions (Table 1, entry 5) in a yield of 82% after HPLC purification.

We contend that this bioconjugation strategy not only provides maximal versatility and modularity in developing peptide-based theranostic agents, but also introduces a new strategy for the generation of imaging or therapeutic conjugates for pre-targeting applications in biological systems. The direct application of **1** into routine Fmoc-based SPPS synthesis makes it an ideal candidate for the construction of bioactive peptides that, after resin cleavage and deprotection, will yield a reactive target molecule that does not require further derivatization

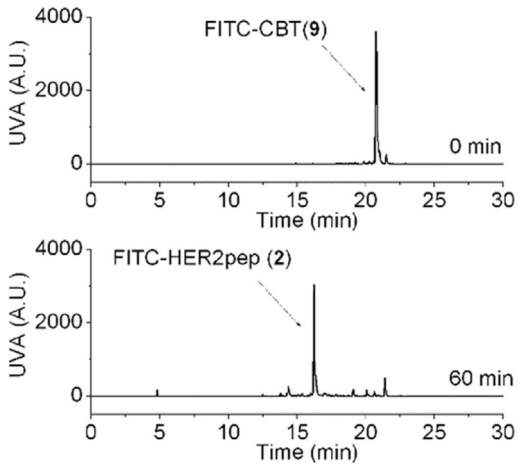


Figure 3. HPLC monitoring of the click reaction between FITC–CBT (**9**) and **HER2 pep**. UVA: UV absorption at 254 nm. A.U.: absorption units.

Table 1. Evaluation of the click reaction between **9** and peptides under varying conditions

Entry	Conditions	Product	Yield [%][a]
1	**9**, **HER2 pep**, TCEP, PBS, pH 7.4, 1 h	**2**	71
2	**9**, **HER2 pep**, TCEP, PBS, pH 9.0, 0.5 h	**2**	78
3	**9**, **HER2 pep**, 10% human serum, PBS, pH 7.4, 1 h	**2**	73
4	**9**, **HER2 pep**, DIPEA, TCEP, DMF, 1 h	**2**	88
5	**9**, **c[RGDyK(C)]**, DIPEA, TCEP, DMF, 1 h	**3**	82

[a] Isolated yield.

prior to conjugation with an imaging agent. Moreover, the incorporation of the fluorophore at the 6 position of CBT did not hamper its reactivity towards 1,2-aminothiol. It confirms the possibility to functionalize CBT with other imaging agents, including radio-metal chelators for PET or SPECT imaging. To our delight, the bioconjugation proceeded extraordinarily well under biological conditions. Even in the presence of human serum, the specificity and efficiency of the CBT/1,2-aminothiol ligation was unaltered. Potential of this bioorthogonal cycloaddition for in vivo applications has been highlighted and efforts are underway to optimize the kinetics of the ligation and to apply this methodology to the preparation of theranostics by conjugation of a chelator, enabling the coordination of an imaging and a therapeutic radio metal.

Experimental Section

General Information

All chemicals were obtained from commercial suppliers and used without further purification. All solvents were anhydrous grade unless indicated otherwise. Hexanes are mixture of isomers. All non-aqueous reactions were performed in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) on Merck aluminum-backed pre-coated plates (Silica gel 60 F254), and visualized with ultraviolet light or by staining with 10% phosphomolybdic acid in neat ethanol. Flash chromatography was performed on silica gel of 40–63 μm particle size. Concentration refers to rotary evaporation. RP-HPLC was carried out on an Agilent 1200 series system equipped with a diode array detector. Yields are reported for spectroscopically pure compounds. NMR spectra were recorded in D_2O, $CDCl_3$, or CD_3OD in diluted solutions on a Bruker AVANCE 400 at ambient temperature. Chemical shifts are given as δ values in ppm and coupling constants J are given in Hz. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), and br (broad signal). Low-resolution ESI mass spectra were recorded on a Bruker HCT spectrometer. Fmoc-based SPPS was conducted on an Aapptec Focus Xi automated peptide synthesizer.

HPLC Conditions

The analyses of FITC-labeled CBT or peptides were performed by HPLC on an analytical RP-C18 column (Aqua®, Phenomenex, 5 μm, 4.6×250 mm) with a gradient elution of acetonitrile (ACN; 10% to 90% in H_2O, containing 0.1% TFA) at a flow rate of 1 mL min^{-1} over 30 min. The products were monitored at 254 nm by a UV detector. The purification of the FITC-labeled peptides was performed on a semi-preparative RP-C18 column (Luna®, Phenomenex, 5 μm, 10.0×250 mm) at a flow rate of 3 mL min^{-1} over 45 min. The products were monitored at 254 nm by a UV detector.

Chemical Synthesis

Fmoc–Lys(Boc–Cys(Trt))–OH (1)

N-Hydroxysuccinimide (1.35 g, 11.8 mmol) and DCC (2.67 g, 12.9 mmol) were added to a solution of Boc–Cys(Trt)–OH (**4**) (5.0 g, 10.7 mmol) in EtOAc (100 mL). The mixture was stirred at room temperature for 24 h. The reaction mixture was filtered off and the solvent layers were concentrated and purified by flash column chromatography (EtOAc/hexanes = 1:4, silica gel) to give **5** as a white solid (6.1 g, 10.8 mmol, quantitative yield). The crude product **5** was directly used for the next step without further purification. Compound **5** (6.1 g, 10.8 mmol) and triethylamine (2.0 mL, 14.3 mmol) were added to a suspension of Fmoc–Lys–OH (4.80 g, 13.0 mmol) in ACN (50 mL) and DMF (50 mL). The reaction mixture was stirred at 40 °C. After stirring for 16 h, the reaction mixture was diluted with EtOAc (200 mL) and washed with 1.0 N $HCl_{(aq)}$ (2× 300 mL) and H_2O (2×300 mL). The organic layers were dried over $MgSO_4$, concentrated, and purified by column chromatography (EtOAc/hexanes = 2:1, silica gel) to give **1** as a white solid (6.5 g, 7.9 mmol, 75% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, 2H, J = 7.5 Hz, ArH), 7.62 (d, 2H, J = 7.0 Hz, ArH), 7.19–7.43 (m, 19H, ArH), 6.46 (br, 1H, NH), 5.82 (br, 1H, NH), 5.36 (br, 1H, NH), 4.35–4.42 (m, 3H), 4.21 (t, 1H, J = 6.9 Hz), 3.89 (br, 1H), 3.19 (m, 2H), 2.49–2.72 (m, 2H), 1.82–1.94 (m, 1H), 1.72–1.79 (m, 1H), 1.36–1.55 (m, 2H), 1.42 (s, 9H, RC(CH_3)$_3$). LC-MS (ESI) m/z: $[M-H]^-$ 812.5.

tert-*Butyl (2-((2-Cyanobenzo[d]thiazol-6-yl)oxy)ethyl)carbamate (7)*

The mixture of 6-hydroxy-2-cyanobenzothiazole (**6**, 668 mg, 3.76 mmol), NaI (168 mg, 1.13 mmol), and K_2CO_3 (1.29 g, 9.41 mmol) in anhydrous acetone (20 mL) was stirred at room temperature and treated with *N*-Boc-2-bromoethyl-amine (1.26 g, 5.64 mmol). The reaction mixture was heated under reflux for 24 h. After reaction was completed (monitored by TLC), the reaction mixture was concentrated, extracted with EtOAc (100 mL) and washed with H_2O (2×200 mL). The organic layer was dried over $MgSO_4$ and concentrated. Purification by column chromatography (100% DCM to EtOAc/DCM = 1:1, silica gel) afforded compound **7** as a white solid (1.0 g, 3.13 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, 1H, ^{meta}J = 8.8 Hz, ArH), 7.33 (d, 1H, ^{para}J = 2.4 Hz, ArH), 7.39 (dd, 1H, $^{para,meta}J$ = 2.4, 8.8 Hz, ArH), 4.96 (br, 1H, NH), 4.10 (t, 2H, ^3J = 5.2 Hz, OCH_2R), 3.58 (q, 2H, ^3J = 5.2 Hz, RCH_2N), 1.43 (s, 9H, RC(CH_3)$_3$). LC-MS (ESI) m/z: $[M+Na]^+$ 342.1.

6-(2-Aminoethoxy)benzo[d]thiazole-2-carbonitrile (8)

7 (791 mg, 2.48 mmol) and thioanisole (2.5 mL) in DCM (5 mL) was stirred at 0 °C for 3 min. Trifluoroacetic acid (2.5 mL) in DCM (5 mL) was slowly added into the solution. The reaction mixture was warmed to room temperature and stirred for 4 h. The reaction mixture was concentrated and purified by column chromatography (EtOAc/hexanes = 1:4 to 100% EtOAc to EtOAc/MeOH = 4:1, silica gel) to afford **8** as a yellow solid (500 mg, 2.28 mmol, 92%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.11 (d, 1H, ^{meta}J = 9.2 Hz, ArH), 7.74 (d, 1H, ^{para}J = 2.4 Hz, ArH), 7.40 (dd, 1H, $^{para,meta}J$ = 2.4, 9.2 Hz, ArH), 4.36 (t, 2H, ^3J = 7.8 Hz, OCH_2R), 3.44 (t, 2H, ^3J = 7.8 Hz, RCH_2N). LC-MS (ESI) m/z: $[M+H]^+$ 220.2.

1-(2-((2-Cyanobenzo[d]thiazol-6-yl)oxy)ethyl)-3-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thiourea (9)

To a solution of **8** (60 mg, 0.27 mmol) in DMF (4 mL) was added fluorescein isothiocyanate (100 mg, 0.25 mmol) at room temperature. The reaction mixture was stirred in the dark. After stirring for 4 h, the crude product was concentrated, re-dissolved in MeOH and then co-evaporated with silica gel for coating. The crude com-

pound on silica gel was purified by column chromatography (EtOAc/DCM = 1:4 to 100% EtOAc to EtOAc/MeOH = 1:9 to 1:4) to give **9** as an orange solid (112 mg, 0.18 mmol, 68%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.13 (d, 1H, J = 2.0 Hz, ArH), 8.07 (d, 1H, J = 9.2 Hz, ArH), 7.71–7.75 (m, 2H, ArH), 7.39 (dd, 1H, J = 2.4, 9.2 Hz, ArH), 7.14 (d, 1H, J = 8.4 Hz, ArH), 6.63–6.67 (m, 4H, ArH), 6.53 (dd, 2H, J = 2.4, 8.4 Hz, ArH), 4.38 (t, 2H, ^3J = 5.6 Hz, OCH_2R), 4.10 (t, 2H, ^3J = 5.6 Hz, RCH_2N). LC-MS (ESI) m/z: $[M-H]^-$ 606.9.

FITC–HER2 pep (2)

The HER2 peptide (**S4**) (3.3 mg, 2.5 μmol), DIPEA (9.5 μL, 25 μmol) and TCEP·HCl (1.4 mg, 5.0 μmol) were dissolved in DMF (2.0 mL) and incubated for 30 min at room temperature. **9** (1.6 mg, 2.5 μmol) was then added into the above solution, and stirred at room temperature for 1 h. The reaction was monitored and purified by semi-preparative RP-C18 HPLC (retention time, t_R = 16.1 min) to give compound **2** as a yellow solid (4.2 mg, 2.2 μmol, 88%). LC-MS (ESI) m/z: $[M+2H]^{2+}$ 963.5 and $[M+3H]^{3+}$ 642.6.

FITC–c(RGDyK) (3)

The synthesis of **3** was carried out from **S3** (1 mg, 1.4 μmol), as described for the preparation of **2** (yield of **3**: 1.5 mg, 1.1 μmol, 82%). LC-MS (ESI) m/z: $[M+H]^+$ 1314.6.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biorthogonal chemistry · click reactions · fluorescent probes · peptide modifications · solid-phase peptide synthesis

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