

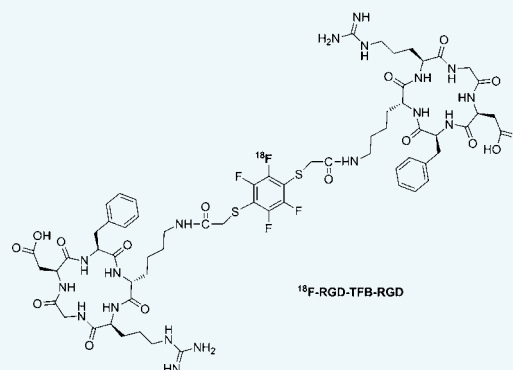
# Novel Method for Radiolabeling and Dimerizing Thiolated Peptides Using $^{18}\text{F}$ -Hexafluorobenzene

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**S** *Supporting Information*

**ABSTRACT:** Hexafluorobenzene (HFB) reacts with free thiols to produce a unique and selective perfluoroaromatic linkage between two sulfurs. We modified this chemical reaction to produce dimeric  $^{18}\text{F}$ -RGD-tetrafluorobenzene (TFB)-RGD, an integrin  $\alpha_v\beta_3$  receptor ligand.  $^{18}\text{F}$ -HFB was prepared by a fluorine exchange reaction using  $\text{K}^{18}\text{F}/\text{K}_{2.2,2}$  at room temperature. The automated radiofluorination was optimized to minimize the amount of HFB precursor and, thus, maximize the specific activity.  $^{18}\text{F}$ -HFB was isolated by distillation and subsequently reacted with thiolated c(RGDfk) peptide under basic and reducing conditions. The resulting  $^{18}\text{F}$ -RGD-TFB-RGD demonstrated integrin receptor specific binding, cellular uptake, and in vivo tumor accumulation.  $^{18}\text{F}$ -HFB can be efficiently incorporated into thiol-containing peptides at room temperature to provide novel imaging agents.



Developing simple synthetic approaches for  $^{18}\text{F}$ -labeling has been the thrust of research in many laboratories.<sup>1</sup> Although there are many different concepts of “simple”, one criterion is the incorporation of  $^{18}\text{F}$ -fluoride in the final radiosynthetic step. Nucleophilic substitution reactions with  $^{18}\text{F}$ -fluoride ion are typically conducted under basic conditions at elevated temperatures. Consequently, many biomolecules may undergo hydrolytic reactions that result in disruption of their biological activity. Because of this,  $^{18}\text{F}$ -labeling of biomolecules is often accomplished through the use of  $^{18}\text{F}$ -labeled prosthetic groups. Prosthetic radiolabeling requires several steps including preparation and purification of the  $^{18}\text{F}$ -prosthetic group by high performance liquid chromatography (HPLC) before conjugation, under mild conditions, to the biomolecules.<sup>1,2</sup> Numerous  $^{18}\text{F}$ -prosthetic groups have been developed for conjugation by chemical reaction at amine- or thiol-functional groups inherent in the biomolecules or small drug-like molecules via acylation, alkylation, amidation, imidation, oxime, or hydrazone formation.<sup>3–6</sup> An additional class of prosthetic groups containing functional groups operative in click-reactions has also been developed for reaction with properly functionalized substrates.<sup>7,8</sup>

Recently, Pentelute and co-workers reported the usage of hexafluorobenzene (HFB) in peptide/small protein stapling.<sup>9</sup> HFB was reacted with free thiols, exclusively by 1,4-disubstitution, to produce a unique and selective perfluoroaromatic linkage between the sulfurs.<sup>9,10</sup> We examined two radiolabeling approaches that utilize this method: (1) nucleophilic radiofluorination on the tetrafluoroaromatic linker, and (2) preparation of <sup>18</sup>F-HFB and subsequent reaction of

thiol containing molecules to simultaneously radiolabel and dimerize.

HFB is chemically inert and highly stable under conditions such as radiation and heat. The strongly electronegative fluorine atoms deactivate the aromatic ring, making it highly susceptible to nucleophilic substitution. Direct displacement of a fluorine anion in HFB is feasible by the usage of strong nucleophilic reagents and the formation of transition metal complexes.<sup>11,12</sup> Substitution of one or more fluorine atoms in HFB took place via reactions with hydroxides, alkoxides, aqueous amines, and organolithium reagents.<sup>11,12</sup> Sequential displacement of two fluorine atoms predominantly provides *para* substitution.

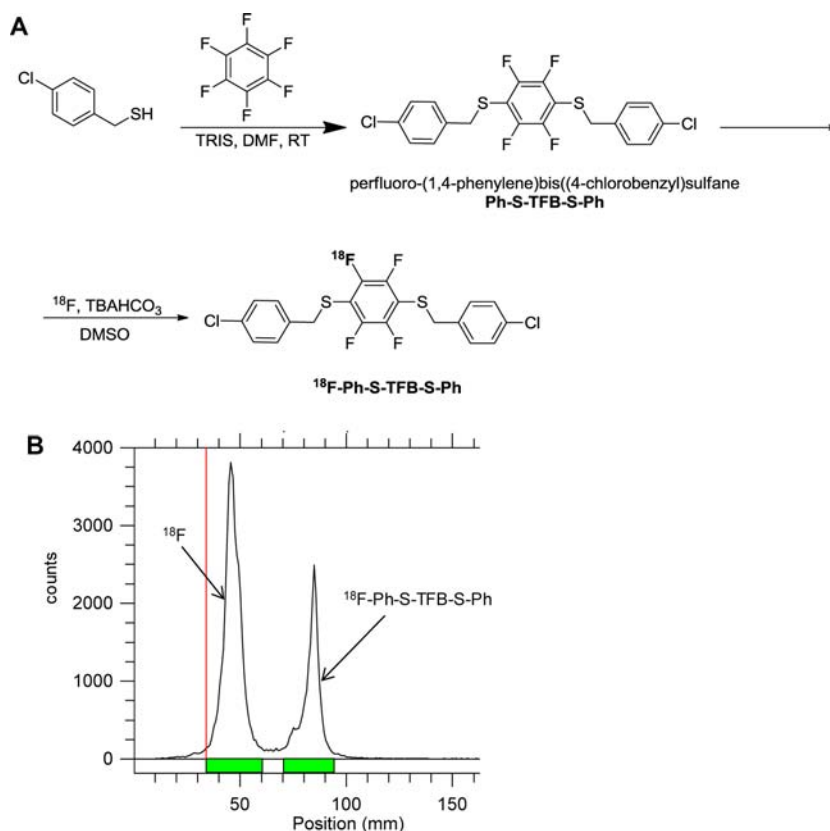
Herein we conducted the direct radiofluorination of a *para*-substituted tetrafluorobenzene. Because the direct nucleophilic exchange conditions were viewed as too harsh for biomolecules, we exploited the second approach. As a proof-of-concept, we demonstrated the dimerization of two thiolated RGD peptides by reaction with  $^{18}\text{F}$ -HFB.

We prepared a tetrafluorophenyl dimer and subsequently conducted a fluoride exchange reaction. The dimer was synthesized by reaction of HFB with 2 equiv of 4-chlorobenzenemethanethiol to give tetrafluoro-(1,4-phenylene)bis((4-chlorobenzyl)sulfane (Ph-S-TFB-S-Ph) in 45% yield (Figure 1A). Successful exchange labeling of one of the four aromatic fluorines on Ph-S-TFB-S-Ph (7–31  $\mu$ mol) was achieved using 1.5 equiv of tetrabutylammonium

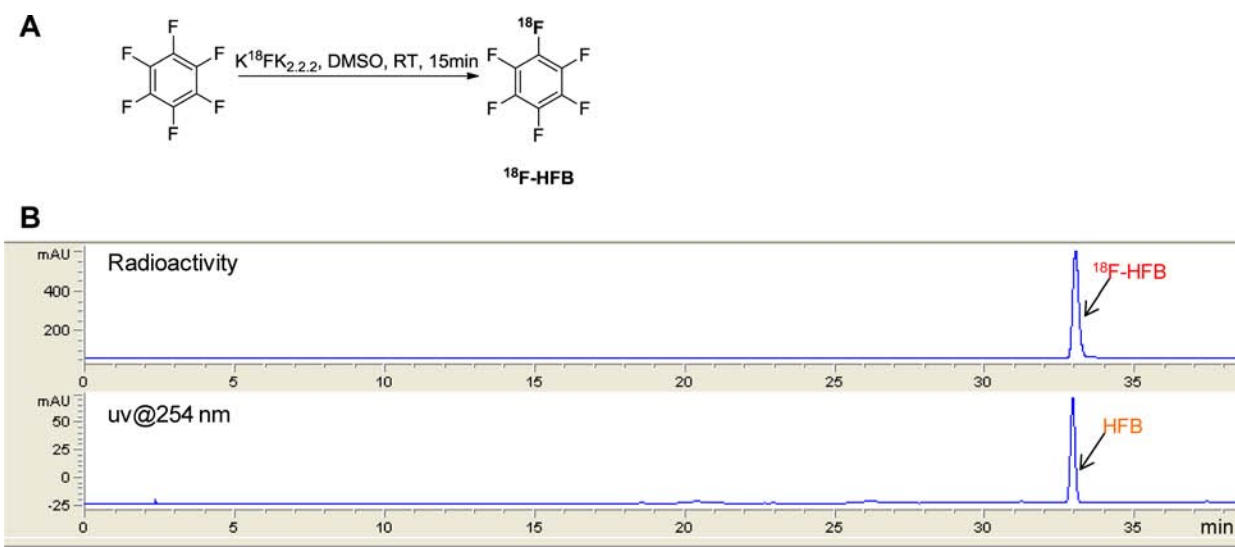
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**Figure 1.** (A) Radiosynthesis of  $^{18}\text{F}$ -Ph-S-TFB-S-Ph from  $^{18}\text{F}$ -HFB. (B) Representative radioTLC of  $^{18}\text{F}$ -Ph-S-TFB-S-Ph crude reaction using normal phase TLC plates and 20% methanol in dichloromethane as a developing solvent.



**Figure 2.** (A) Radiosynthesis route of  $^{18}\text{F}$ -HFB. (B) Representative HPLC radioactive (upper) and UV (lower) chromatograms of isolated  $^{18}\text{F}$ -HFB.

bicarbonate and 8–10 mCi of F-18, providing a 33% radiochemical yield (Figure 1B). This direct labeling method required a reaction temperature of 90 °C for 15 min. Although this method may be applicable for heat-stable small molecules, we deemed these conditions unsuitable for general peptide labeling. We did not exhaustively investigate but directed our attention to the second approach that allows thiol containing molecules to react with  $^{18}\text{F}$ -HFB for simultaneous radiolabeling and dimerization.

The published radiosynthesis of  $^{18}\text{F}$ -HFB (Figure 2)<sup>13</sup> was adapted for automation on a modular system (Eckert and Ziegler). The synthesis was conducted at room temperature (RT) in dimethyl sulfoxide (DMSO) and quantities of  $\text{K}_2\text{CO}_3$ ,  $\text{K}_{2.2.2}$ , and HFB were adjusted in order to achieve good yields using the lowest amount of HFB (Table 1).  $^{18}\text{F}$ -HFB was isolated by distillation with argon flow at 25 °C into a vial containing dimethylformamide (DMF) cooled in dry ice/acetonitrile (−45 °C). Radiochemical yields (RCY) were calculated based on collected  $^{18}\text{F}$ -HFB divided by initial  $^{18}\text{F}$

**Table 1.** Different Reaction Conditions of Automatic Labeling  $^{18}\text{F}$ -HFB in an Eckert and Ziegler Module<sup>a</sup>

$\text{K}_2\text{CO}_3$	$\text{K}_{2.2.2}$	HFB	isolated yield (%)
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	7 mg (37 $\mu\text{mol}$ )	28
0.17 mg (1.2 $\mu\text{mol}$ )	0.9 mg (2.4 $\mu\text{mol}$ )	1.4 mg (7.5 $\mu\text{mol}$ )	15
0.03 mg (0.24 $\mu\text{mol}$ )	0.18 mg (0.48 $\mu\text{mol}$ )	0.24 mg (1.3 $\mu\text{mol}$ )	0
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	1.4 mg (7.5 $\mu\text{mol}$ )	25
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	0.7 mg (3.8 $\mu\text{mol}$ )	25
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	0.5 mg (2.7 $\mu\text{mol}$ )	25
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	0.35 mg (1.9 $\mu\text{mol}$ )	15
0.83 mg (6 $\mu\text{mol}$ )	4.5 mg (12 $\mu\text{mol}$ )	0.2 mg (1 $\mu\text{mol}$ )	0

<sup>a</sup>Isolated yield was calculated from F-18 activity at the start of synthesis to the isolated product after distillation.

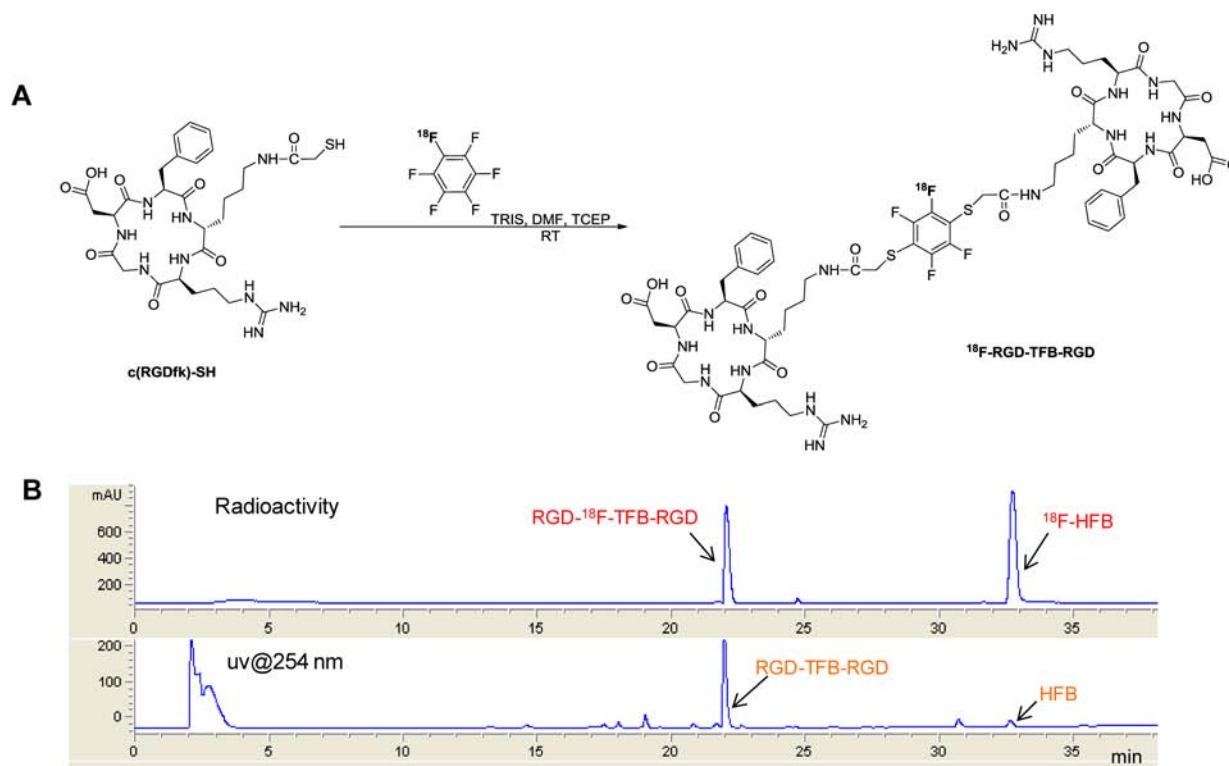
activity (Table 1). The optimized conditions, using only 500  $\mu\text{g}$  (2.7  $\mu\text{mol}$ ) of HFB, provided a  $25 \pm 3\%$  isolated RCY ( $n = 6$ , non-decay-corrected) with specific activity (SA) of 50 mCi/ $\mu\text{mol}$ .

For the dimerization reaction, an aliquot of the product containing 1 equiv of  $^{18}\text{F}$ -HFB (5–10 mCi, 0.1–0.2  $\mu\text{mol}$ ) was reacted with 2 equiv of thiolated c(RGDfK) peptide (0.44–0.73  $\mu\text{mol}$ ), excess amount of TRIS base (24 equiv), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2.5 equiv), a reducing agent, at RT in DMF. After reaction for 20–25 min, there was about 50% conversion, based on HPLC, into the desired dimerized product, RGD- $^{18}\text{F}$ -TFB-RGD (Figure 3). The product was isolated in  $40 \pm 2\%$  radiochemical yield ( $n =$

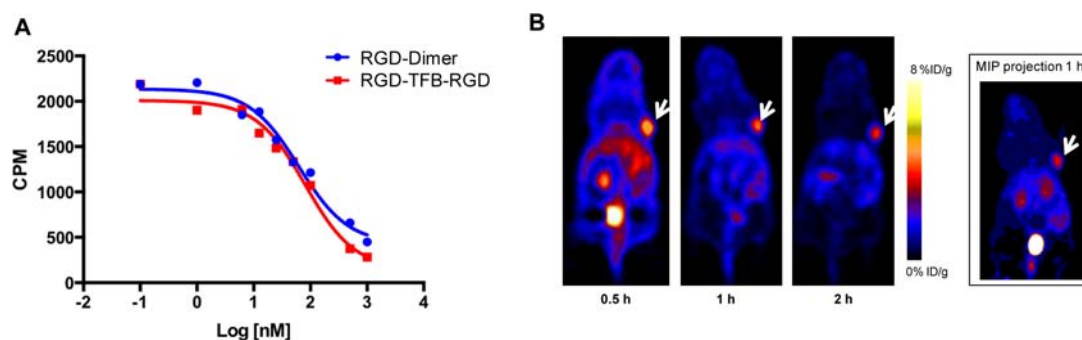
4) with SA of 15 mCi/ $\mu\text{mol}$ . During the dimerization reaction, each thiol attack increases the probability that the radioactive fluorine atom will be displaced from HFB. Attempts to conduct this reaction without TCEP resulted in a higher amount of undesired disulfide dimer, presumably from oxidative coupling (Supporting Information Figure S1). The structure was confirmed by comparison of HPLC profile to that of authentic standard characterized by LC-MS analysis (Supporting Information Figure S2).

Biologically, introduction of HFB group into thiolated c(RGDfK) peptide provided a dimer that displayed a similar  $\text{IC}_{50}$  value in  $\alpha_v\beta_3$  expressing U87MG cells compared to the known dimer E[c(RGDfK)]<sub>2</sub> (81 nM vs 62 nM, Figure 4A). Furthermore, PET imaging, following intravenous injection of RGD- $^{18}\text{F}$ -TFB-RGD (10–20 nmol injected mass dose) to a U87MG xenograft model, showed good tumor uptake ( $\sim 4\%$  ID/g at 1 h postinjection) and high tumor-to-background contrast (tumor/muscle = 9.8, Figure 4B). This tumor uptake was comparable to dimeric RGD peptides radiolabeled by other  $^{18}\text{F}$ -synthons, such as *N*-succinimidyl 4- $^{18}\text{F}$ -fluorobenzoate ( $^{18}\text{F}$ -SFB). RGD-dimer conjugated to  $^{18}\text{F}$ -SFB was reported to have tumor uptake of  $3.8 \pm 0.8\%$  ID/g at 70 min post-injection.<sup>14</sup> No significant defluorination was observed up to 2 h post-injection (Table 2). The specificity of RGD- $^{18}\text{F}$ -TFB-RGD was confirmed by blocking studies (Supporting Information Figure S3). Excess amount of c(RGDfK) peptide significantly blocked RGD- $^{18}\text{F}$ -TFB-RGD uptake in the tumor (Supporting Information Figure S3).

In general, radiochemical syntheses are conducted to obtain the highest possible specific activity. Most biological targets of interest for biomedical imaging have limited numbers per cell and their successful imaging requires high affinity ligands with



**Figure 3.** (A) Radiosynthesis of  $^{18}\text{F}$ -RGD-TFB-RGD from  $^{18}\text{F}$ -HFB. (B) Representative HPLC chromatogram (radioactivity, upper; and UV, lower) of crude aliquot from the labeling of  $^{18}\text{F}$ -RGD-TFB-RGD.



**Figure 4.** (A) Competition cell binding assay of RGD-TFB-RGD and RGDfκ-dimer against  $^{125}\text{I}$ -echistatin in U87MG  $\alpha_v\beta_3$  expressing cells. The results are shown as average of triplicates  $\pm$  standard deviation. (B) Representative coronal PET images of mouse bearing U87MG tumor (marked with white arrows) at 0.5, 1, and 2 h post-injection of  $^{18}\text{F}$ -RGD-TFB-RGD and MIP projection image of the same mouse at 1 h post-injection.

**Table 2. Biodistribution of RGD- $^{18}\text{F}$ -TFB-RGD in Normal Mice at 0.5 and 2 h Post-Injection (%ID/g)<sup>a</sup>**

	0.5 h		2 h	
	Average	STD	Average	STD
Heart	0.50	0.21	0.18	0.02
Lung	1.08	0.42	0.48	0.04
Liver	0.74	0.27	0.43	0.06
Spleen	0.53	0.15	0.32	0.05
Stomach	0.39	0.14	0.34	0.09
Intestine	0.73	0.20	0.57	0.19
Kidneys	1.84	0.57	1.04	0.16
Muscle	0.20	0.08	0.10	0.01
Bone	0.48	0.20	0.27	0.01
Blood	0.62	0.61	0.12	0.03

<sup>a</sup>Results are shown as average of 4 mice  $\pm$  STD.

high specific activity.<sup>15</sup> The tracer principle also requires a low occupancy of receptors to avoid physiological perturbation. However, in some cases the biological target can still be imaged at somewhat lower specific activity. In a human study of somatostatin receptor imaging, the high specific activity tracer was trapped by the off-target binding; optimal images were obtained by pre-injection of a small amount of unlabeled peptide prior to injection of radiotracer.<sup>15</sup> We observed a similar phenomenon when studying high specific activity  $^{18}\text{F}$ -SFB labeled T140 peptide. This peptide showed undesired binding to off-target sites on red blood cells that inhibited the specific binding to CXCR4 positive tumor. The ability to image the tumor was overcome by lowering the SA through addition of a small amount of unlabeled T140 peptide.<sup>16</sup> Another example reported in the literature for antibodies or macromolecules suggested that highest specific activity was not optimal, because a low amount of tracer might be removed rapidly from the circulation and result in low uptake in tumors.<sup>17</sup> Thus, the specific activity of a molecular imaging tracer needs to be appropriate for its application, not necessarily as high as possible.<sup>17</sup> Our approach of  $^{18}\text{F}$ -labeling via  $^{19}\text{F}$ – $^{18}\text{F}$  exchange reaction with moderately high specific activity would be suitable for certain biological applications where the cell receptor density is high and/or the target binding is highly sensitive to the amount of radioligand administered.

To analyze the specificity of HFB displacement reaction toward free thiols rather than free amines that are present on lysine residues, HFB was reacted with T140, a small peptide that has two Cys, one L-Lys, and one D-Lys (Supporting Information Figure S4). Excess of HFB (2.5–25 equiv) was

reacted with reduced T140 peptide for several hours to give the corresponding cyclic product T140-TFB (Supporting Information Figure S4). The major byproduct of this reaction was the formation of cyclic T140 peptide with disulfide bond as determined by LC-MS (Supporting Information Figure S5). Similarly, when the reduced T140 was reacted with  $^{18}\text{F}$ -HFB there was only 8–13% conversion by HPLC (Supporting Information Figure S6). Attempts to add TCEP in order to prevent disulfide bond, as was done for the RGD dimer, did not increase the yield, perhaps because the formation of the disulfide bond by the T140 Cys groups is an intramolecular and not intermolecular manner. It is important to emphasize that we could not detect any byproduct in which the  $^{18}\text{F}$ -HFB reacted with the amine of the Lys.

Other methods of  $^{18}\text{F}$  incorporation employed in the final radiochemical step, such as formation of SiFA,  $\text{BF}_3$ , and  $\text{AlF}$ ,<sup>18–24</sup> have been reported.  $\text{BF}_3$  and  $\text{AlF}$  methods are conducted under acidic conditions, with or without heating. Our approach presented in this paper is a complementary method that allows the unique formation of cyclic and dimeric products using mildly basic conditions at room temperature. As such, it provides uniquely radiolabeled products not easily attainable by any reported method.

In conclusion, we reported a novel prosthetic group for labeling and dimerizing both peptides and small molecules. This reaction, which can be conducted either in one step under heating condition or in two steps at room temperature, provides a unique method for preparing radiolabeled dimeric biomolecules.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental section. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00278.

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### Notes

The authors declare no competing financial interest.

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