

## Tyrosine Bioconjugation through Aqueous Ene-Type Reactions: A Click-Like Reaction for Tyrosine

Hitoshi Ban, Julia Gavrilyuk, and Carlos F. Barbas, III\*

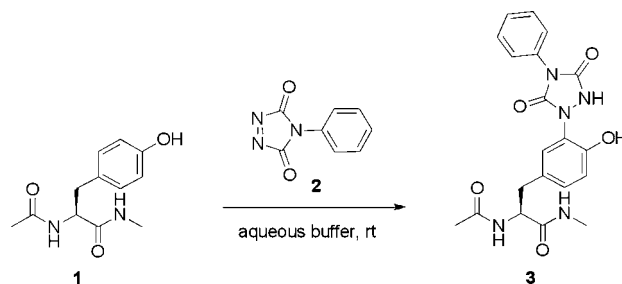
The Skaggs Institute for Chemical Biology and the Departments of Chemistry and Molecular Biology,  
The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received October 23, 2009; E-mail: carlos@scripps.edu

Bioconjugation methods rely heavily on chemoselective modification of native protein functional groups.<sup>1</sup> Lysine and cysteine side chains are the most commonly functionalized amino acids; however, the high abundance of lysine on protein surfaces makes site-specific modification challenging. In contrast, cysteines are rare and most often in disulfide linked pairs in proteins in their natural environment. Labeling at this amino acid typically requires reduction of the target disulfide followed by reaction with a reagent like maleimide. Recently significant attention has been paid to the bioorthogonal modification of the aromatic amino acid side chains of tryptophan<sup>2</sup> and tyrosine.<sup>3</sup> Tyrosine modification in mild, biocompatible, metal-free conditions has been studied using Mannich-type additions to imines.<sup>3a–c</sup> Inspired by these pioneering efforts, we sought to capitalize on the reactivity of diazodicarboxylate-related molecules to create an efficient aqueous ene-type reaction as an orthogonal bioconjugation strategy. Here we present a new and efficient tyrosine ligation reaction (TLR) and demonstrate the utility of the reaction in the preparation of small molecule, peptide, enzyme, and antibody conjugates.

Substituted phenols can react with highly reactive electrophiles such as diazodicarboxylates in organic solvents in the presence of activating protic or Lewis acid additives.<sup>4</sup> Rapid decomposition of diazodicarboxylate reagents in aqueous media and/or low reactivity toward phenols makes them unsuitable for bioconjugation.<sup>5</sup> Acyclic diazodicarboxylate reagents are dramatically activated in ene reactions by interaction with cationic species such as protons or metal ions.<sup>5</sup> Cyclic diazodicarboxamides like 4-phenyl-3*H*-1,2,4-triazole-3,5(4*H*)-dione (PTAD), however, are not similarly activated, and this reactivity difference suggested to us that they might present an opportunity for aqueous chemistry. We conducted a preliminary survey of the reactivity and stability of diazodicarboxylate and diazodicarboxamide reagents for the reaction with *N*-acyl tyrosine methyl amide **1** in aqueous buffer (data not shown). This study revealed that the decomposition of acyclic diazodicarboxylates in aqueous media was faster than the desired reaction with **1**, whereas acyclic diazodicarboxamides were stable but not reactive enough. Ultimately, PTAD **2** provided the desired reactivity and stability (Scheme 1). As a model for peptide labeling, we studied *N*-acyl tyrosine methylamide **1** modification with PTAD **2** in mixed organic/aqueous media necessitated by the solubility characteristics of **1**. In sodium phosphate buffer, pH 7/acetoneitrile (1:1), peptide **1** reacted rapidly (reaction was complete within 5 min) with 1.1 equiv of PTAD to provide **3** in 65% isolated yield. With the addition of 3.3 equiv of PTAD, quantitative modification could be obtained (see Supporting Information). The buffer concentration did not significantly affect the reaction, and notably, the reaction did not proceed in acetonitrile alone. To the best of our knowledge this type of reaction has not been reported to occur in such mild aqueous media.<sup>4</sup>

### Scheme 1. Model Tyrosine Ligation Reaction

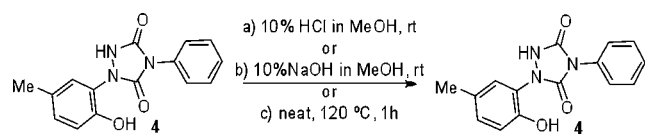


Next we studied the chemoselectivity of this reaction with a defined collection of *N*-acyl methyl amides of histidine, tryptophan, serine, cysteine, and lysine. Significantly, only tryptophan<sup>6</sup> and lysine yielded a product detectable by <sup>1</sup>H NMR. It is important to note that the indole of tryptophan reacted equally sluggish with PTAD when the reaction was performed in neat organic solvent or in mixed aqueous media suggesting that aqueous conditions dramatically activate the phenolic group of tyrosine for reaction. Competition experiments with an equimolar mixture of *N*-acyl methyl amides of tyrosine and tryptophan or tyrosine and lysine resulted in selective modification of tyrosine in 55% and 58% conversion, respectively, with no detectable modification of other amino acid amides (see Supporting Information). Similarly, when an equimolar mixture of all six amino acid amides was treated with PTAD, only tyrosine modification (39% conversion) was observed by <sup>1</sup>H NMR, indicating that this reagent exhibits a high degree of chemoselectivity.

Given the inherent reversibility of the reaction between the related cyclic diazodicarboxamide 4-methyl-1,2,4-triazoline-3,5-dione and indoles,<sup>6</sup> our next concern was the relative stability of the C–N bond formed in our products. To study this, we used *p*-cresol as a model phenol (Scheme 2). Compound **4**, the product of the reaction of *p*-cresol and PTAD, was subjected to both strongly acidic and basic conditions for 24 h at room temperature or high temperature (120 °C) for 1 h. The C–N bond was found to be stable under these conditions, and starting material was recovered in 89% yield following acid treatment and quantitatively recovered following base and heat treatments. These conditions are extremely harsh for a peptide or protein. This study suggests that the 1,2,4-triazolidine-3,5-dione linkage is hydrolytically and thermally stable, more robust than maleimide-type conjugations, which are prone to elimination, or Mannich-type conjugations where retro-Mannich reactions would be expected.

We then evaluated this reaction using peptides to assess the application of this approach in peptide chemistry. The acyclic tripeptide H-Gly-Gly-Tyr-OH reacted rapidly with PTAD **2** in phosphate buffer, pH 7/acetoneitrile (1:1), to provide product **5** in 85% isolated yield (Figure 1). Similarly, reaction of the small cyclic

## Scheme 2. Stability Evaluation



peptide (Ile<sup>3</sup>)-pressinoic acid (tocinoic acid) with PTAD provided product **6** (Figure 1) in 83% isolated yield. No bis-addition products were observed in any of our studies. These experiments demonstrated the chemoselectivity of this reaction and its application to peptide chemistry and suggested that cyclic diazodicarboxamides like PTAD should possess the reactivity and chemoselectivity required for complex protein modification.

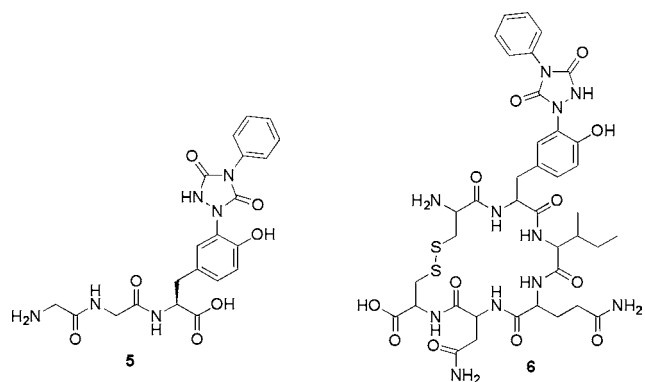


Figure 1. PTAD modification of peptides.

To explore the potential of this reaction for protein functionalization, we prepared and studied several functionalized PTAD analogues (Figure 2). Azide containing linkers **7** and **8** were prepared as stable and synthetically versatile precursors with utility in click chemistry<sup>7</sup> and as intermediates in the synthesis of **9** and **10** (see Supporting Information). Differentially functionalized PTAD reagents were chosen to study whether the reactivity of these reagents could be tuned with electronic effects. Reduction of the azide functionality and reaction with the commercially available NHS-activated 5- and 6-carboxy-X-rhodamine (ROX) provided the corresponding amide products. Oxidation to the corresponding cyclic diazodicarboxamides **9** and **10** (Figure 2) was done with NBS and pyridine in DMF. To evaluate nonspecific and noncovalent attachment of highly hydrophobic ROX reagents to protein, the nonreactive rhodamine alkyne **11** was prepared and used as a negative control reagent.

Chymotrypsinogen A, bovine serum albumin (BSA), and myoglobin from equine heart were chosen as model protein systems, as these proteins have different tyrosine and tryptophan contents and side chain accessibilities. We studied protein labeling at the physiological pH 7.4 in phosphate buffer with a minimal amount of DMF, needed to prepare and deliver the labeling reagent. The final concentration of DMF in the reaction mixture was 1 to 5%. PTADs **9** and **10** were studied at concentrations ranging from 1 to 10 mM (Table 1). Assessment of the reaction conversion was done by UV analysis following dialysis in buffer to remove unbound dye. Reagent **9** provided up to 81% labeling of chymotrypsinogen A and up to 96% labeling of BSA (Table 1). Myoglobin was labeled with reagent **9** at 6–8%. The background nonspecific and noncovalent association of rhodamine dye **11** to proteins accounted for 3–4% labeling in this assay. Reagent **10** was expected to be more reactive and less stable in aqueous media than **9** given the electron-withdrawing linker; it yielded 60% labeling of chymotrypsinogen

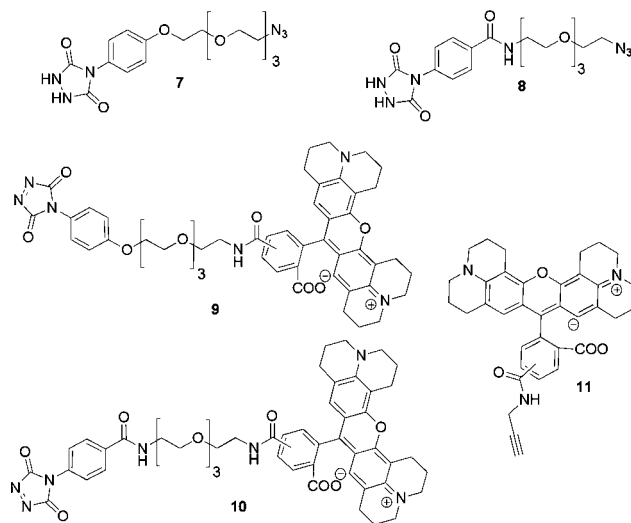


Figure 2. Linkers and rhodamine dye reagents.

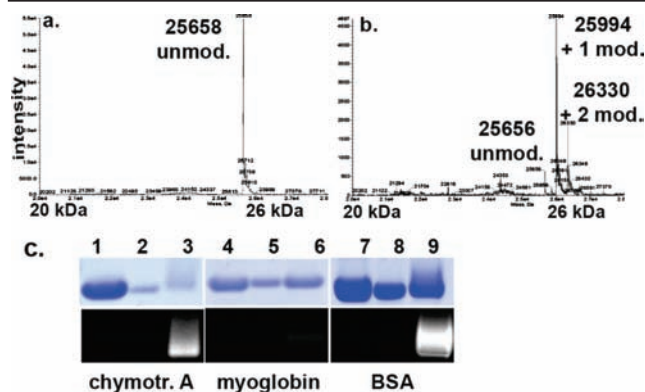
Table 1. Protein Modification Study

No.	Protein <sup>a</sup>	Reagent concn, mM	Labeling with reagent <b>9</b> , % <sup>b</sup>	Labeling with reagent <b>10</b> , % <sup>b</sup>
1	Chymotrypsinogen A	1	56	35
2	Chymotrypsinogen A	5	72	54
3	Chymotrypsinogen A	10	81	60
4	Myoglobin	1	6	13
5	Myoglobin	5	6	13
6	Myoglobin	10	8	16
7	BSA	1	85	53
8	BSA	5	96	65
9	BSA	10	96	68
10	Chymotrypsinogen A	10 <sup>c</sup>	3	3
11	Myoglobin	10 <sup>c</sup>	3	3
12	BSA	10 <sup>c</sup>	4	4

<sup>a</sup> Protein concentration was kept at 30  $\mu$ M in 0.1 M phosphate buffer, pH 7.4. <sup>b</sup> Conversion was calculated based on UV-vis absorption for extensively desalted and dialyzed sample. Average conversion of two independent experiments is shown. <sup>c</sup> Reagent **11** was used as a negative control.

A, 68% labeling of BSA, and 16% modification of myoglobin. Tryptic digest and subsequent ESI-MS analysis of the fragments of all proteins modified with reagents **9** and **10** confirmed primary sites for covalent modification of chymotrypsinogen A at Y228 and BSA at Y355 and Y357. Additional modification sites for BSA were identified to be Y393 and Y424 in the reaction with 10 mM reagent **9**. In the doubly modified chymotrypsinogen, Y171 was additionally labeled (Figure 3). Reagent **10** modified myoglobin at W15 at a very low level, consistent with results of our small molecule study. Although we detected some modification of myoglobin with **9**, the degree of labeling was too low to identify the site. Covalent modification of proteins was confirmed using a gel-based assay, MALDI-TOF, and ESI analysis (Figure 3 and Supporting Information). Chymotrypsinogen A retained its enzymatic activity following labeling consistent with the mild nature of the reaction (see Supporting Information).

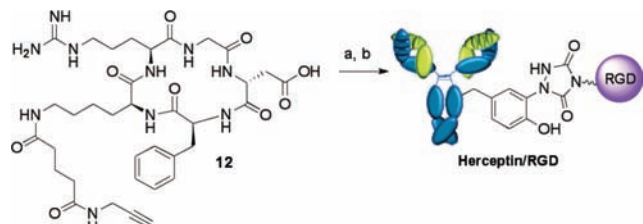
We studied BSA labeling with **9** over a wide pH range (pH 2 to pH 10) and found significant protein labeling at all pH's. Up to 54% labeling was observed at pH 2 with labeling ranging from 85% to 98% between pH 7 and 10 (see Supporting Information). Thus the tyrosine ligation reaction is applicable over a wide pH range.



**Figure 3.** ESI-MS analysis of purified samples containing (a) unmodified chymotrypsinogen A and (b) chymotrypsinogen A modified with oxidized linker 7. (c) Gel stained with coomassie blue (top) and under UV light (bottom): lane 1, unmodified chymotrypsinogen A; lane 2, chymotrypsinogen A/11; lane 3, chymotrypsinogen A/9; lane 4, unmodified myoglobin; lane 5, myoglobin/11; lane 6, myoglobin/9; lane 7, unmodified BSA; lane 8, BSA/11; and lane 9, BSA/9.

We envision that this tyrosine ligation reaction might be used for the bioconjugation of a wide variety of functionalities onto protein surfaces. To test this hypothesis, an integrin binding cyclic RGD peptide containing an alkyne, **12**, was prepared. Subsequent Cu(I)-mediated click reaction with intermediate **7** followed by oxidation with NBS/Py provided the labeling reagent that was then reacted with the therapeutic antibody herceptin<sup>8</sup> (Scheme 3). The

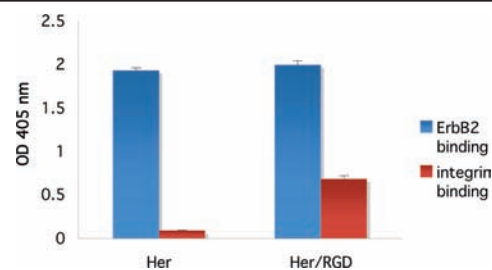
#### Scheme 3. Preparation of Her/RGD Construct<sup>a</sup>



<sup>a</sup> (a) **7**, Cu, CuSO<sub>4</sub>. (b) (i) NBS, Py, DMF; (ii) herceptin in phosphate buffer, pH 7.4.

resulting herceptin/RGD conjugate was purified and characterized by MALDI-TOF MS. ErbB-2 and integrin  $\alpha v\beta 3$  binding ELISA (Figure 4) demonstrated that modification of the antibody herceptin through tyrosine conjugation did not impair its ability to bind to ErbB-2, while introduction of the cyclic RGD peptide allowed the antibody conjugate to bind integrin  $\alpha v\beta 3$ , thereby providing a new chemical route to antibodies with multiple specificities.<sup>9</sup>

In summary, we have developed a new and versatile class of cyclic diazodicarboxamides that react selectively with phenols and the phenol side chain of tyrosine through an ene-like reaction. This



**Figure 4.** Normalized ELISA for Her/RGD bispecific Ab.

mild aqueous reaction works over a broad pH range and expands the repertoire of aqueous chemistries available for small molecule, peptide, and protein modification. We believe this reaction will find broad utility in protein chemistry and in the chemistry of phenol-containing compounds.

**Acknowledgment.** We thank Prof. Phil Baran for useful discussions. This study was supported by the Skaggs Institute for Chemical Biology.

**Supporting Information Available:** Full experimental procedures and characterization data are available for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

#### References

- (1) (a) Aslam, M.; Dent, A. *Bioconjugation. Protein Coupling Techniques for Biomedical Sciences*; Grove's Dictionaries Inc.: New York, NY, 1998. (b) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974–6998.
- (2) (a) Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256–7. (b) Antos, J. M.; McFarland, J. M.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2009**, *131*, 6301–6308.
- (3) (a) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 15942–3. (b) McFarland, J. M.; Joshi, N. S.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 7639–44. (c) Minakawa, M.; Guo, H. M.; Tanaka, F. *J. Org. Chem.* **2008**, *73*, 8669–72. (d) Kodadek, T.; Duroux-Richard, I.; Bonnafous, J. C. *Trends Pharmacol. Sci.* **2005**, *26*, 210.
- (4) (a) Schroete, S. *J. Org. Chem.* **1969**, *34*, 4012–14. (b) Mitchell, H.; Leblanc, Y. *J. Org. Chem.* **1994**, *59*, 682–687. (c) Leblanc, Y.; Boudreault, N. *J. Org. Chem.* **1995**, *60*, 4268–4271. (d) Yadav, J. S.; Reddy, B. V. S.; Kumar, G. M.; Madan, C. *Synlett* **2001**, 1781–1783. (e) Bombek, S.; Lenarsic, R.; Kocevar, M.; Saint-Jalmes, L.; Desmurs, J. R.; Polanc, S. *Chem. Commun.* **2002**, 1494–1495. (f) Yadav, J. S.; Reddy, B. V. S.; Veerendhar, G.; Rao, R. S.; Nagaiyah, K. *Chem. Lett.* **2002**, 318–319. (g) Kinart, W. J.; Kinart, C. M. *J. Organomet. Chem.* **2003**, *665*, 233–236. (h) Chee, G. L. *Synth. Commun.* **2006**, *36*, 2151–2156. (i) Brandes, S.; Bella, M.; Kjoersgaard, A.; Jorgensen, K. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 1147–1151.
- (5) Desimoni, G.; Faita, G.; Righetti, P. P.; Sfulcini, A.; Tsyganov, D. *Tetrahedron* **1994**, *50*, 1821–1832.
- (6) Baran, P. S.; Guerrero, C. A.; Corey, E. J. *Org. Lett.* **2003**, *5*, 1999–2001.
- (7) (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599. (b) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057–3064. (c) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047. (d) Ning, X. H.; Guo, J.; Wolfert, M. A.; Boons, G. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253–2255.
- (8) Goldenberg, M. M. *Clin. Ther.* **1999**, *21*, 309–18.
- (9) Gavriluyuk, J. I.; Salahuddin, S.; Goswami, R. K.; Sinha, S. C.; Barbas, C. F., III. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3716–3720.

JA909062Q