Facile and Stabile Linkages through Tyrosine: Bioconjugation Strategies with the Tyrosine-Click Reaction

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Supporting Information

ABSTRACT: The scope, chemoselectivity, and utility of the click-like tyrosine labeling reaction with 4-phenyl-3H-1,2,4-triazoline-3,5(4H)-diones (PTADs) is reported. To study the utility and chemoselectivity of PTAD derivatives in peptide and protein chemistry, we synthesized PTAD derivatives possessing azide, alkyne, and ketone groups and studied their reactions with amino acid derivatives and peptides of increasing complexity. With proteins we studied the compatibility of the tyrosine click reaction with cysteine and lysine-targeted labeling approaches and demonstrate that chemoselective trifunctionalization of proteins is readily achieved. In particular cases, we noted that PTAD decomposition resulted in formation of a putative isocyanate byproduct that was promiscuous in labeling. This side reaction product, however, was readily scavenged by the addition of a small amount of 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) to the reaction medium. To study the potential of the tyrosine click reaction to introduce poly(ethylene glycol) chains onto proteins (PEGylation), we demonstrate that this novel reagent provides for the selective PEGylation of chymotrypsinogen, whereas traditional succinimide-based PEGylation targeting lysine residues provided a more diverse range of PEGylated products. Finally, we applied the tyrosine click reaction to create a novel antibody–drug conjugate. For this purpose, we synthesized a PTAD derivative linked to the HIV entry inhibitor aplaviroc. Labeling of the antibody trastuzumab with this reagent provided a labeled antibody conjugate that demonstrated potent HIV-1 neutralization activity demonstrating the potential of this reaction in creating protein conjugates with small molecules. The tyrosine click reaction demonstrated stability to extremes of pH, temperature, and exposure to human blood plasma indicating that this linkage is significantly more robust than maleimide-type linkages that are commonly employed in bioconjugations. These studies support the broad utility of this reaction in the chemoselective modification of small molecules, peptides, and proteins under mild aqueous conditions over a broad pH range using a wide variety of biologically acceptable buffers such as phosphate buffered saline (PBS) and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffers as well as others and mixed buffered compositions.

INTRODUCTION

Bioconjugation reactions exploit either the intrinsic chemical reactivity of the biomolecule or introduce extrinsic functionalities that can then be subsequently reacted in a bio-orthogonal fashion. In recent years a wide range of extrinsic bioorthogonal reactions have been developed (Figure 1a). This two-step approach relies on the introduction of functionalities such as ketones, aldehydes, azides, alkynes, or alkenes into the target protein. In a second step, the introduced extrinsic functionalities are selectively reacted to form oximes/hydrazones, Staudinger ligation products, triazole-Click products, or Diels–Alder, among others. In addition to chemical routes, extrinsic functional groups can be introduced into biomolecules via enzymatic modification or genetic encoding. Thus, extrinsic approaches involve a minimum of two modification steps of the biomolecule. In contrast, intrinsic approaches have the potential to be one-step (Figure 1b). Intrinsic approaches to bioconjugation are, however, limited to the development of chemistry compatible with the 20 naturally occurring amino acids and are challenged by the fact that any given amino acid is likely to occur more than once in a protein. Regardless of the approach, bioconjugation reactions ideally proceed rapidly, selectively, and in high yield under physiological conditions while preserving the target protein’s biological activity.

Much of the chemistry developed to tap intrinsic amino acid reactivity is many decades old and is centered on the modification of lysine and cysteine side chains. The abundance of lysine residues on the protein surface, however, makes site-specific modification difficult. In contrast, cysteine is rare and
most often found in disulfide-linked pairs in native proteins. For cysteine modification, pretreatment by reduction to cleave disulfide bonds is typically required; this is followed by reaction with a reagent like maleimide. Recently, novel mono- and dibromomaleimides have expanded the potential of cysteine-targeted labeling. Less developed are methods for the selective modification of the aromatic amino acid side chains of tryptophan and tyrosine, but a number of promising approaches have been recently reported. Among nucleophilic amino acids, tyrosine has unique reactivity due to the acidic proton of the phenol ring. The alkylation or acylation reaction of tyrosine under basic conditions proceeds at the oxygen. Under acidic conditions, an ene-like reaction occurs at a carbon atom on the aromatic ring. Bioconjugation at carbon in mild, biocompatible, metal-free conditions was reported using Mannich-type additions to imines or cerium(IV) ammonium nitrate (CAN) as an oxidation reagent. However, highly reactive diazodicarboxylate reagents decompose rapidly in aqueous media and stable diazodicarboxyamide reagents do not react efficiently with phenols in aqueous media; thus most diazodicarboxyamides are not suitable as bioconjugation reagents. Although cyclic diazodicarboxylate reagents can be activated by interaction with cationic species such as protons or metals, cyclic diazodicarboxamides like 4-phenyl-3H,1,2,4-triazoline-3,5(4H)-dione (PTAD) are not activated by proton or Lewis acid additives. Our initial study involved a survey of the reactivities and stabilities of diazodicarboxylate and diazodicarboxyamide; these studies led us to develop the tyrosine-click reaction of PTAD with the phenolic side chain of tyrosine (Scheme 1).

The tyrosine click reaction is illustrated in Scheme 1 involving the reaction in this case of N-acyl tyrosine methyl amide 1 with PTAD 2a in mixed organic solvent/aqueous media. In sodium phosphate buffer (pH 7)/acetonitrile (1:1), 1 reacted rapidly (reaction was complete in less than 5 min) with 1.1 equiv of PTAD 2a to provide 3a in quantitative yield. PTAD labeling was more efficient than 4-methyl-3H,1,2,4-triazoline-3,5(4H)-dione (MTAD) labeling; reaction of MTAD 2b with amide 1 gave 3b in 57% isolated yield as electronic modulation of the triazolinedione system is key to controlling this reaction. Here we present a comprehensive study concerning the synthesis and characteristics of versatile triazolinedione-based labeling reagents, studying of the scope, stability, and chemoselectivity of these reagents, and examine their applications in bioconjugation reactions with small molecules, peptides, and proteins.
**EXPERIMENTAL PROCEDURES**

**General.** 1H NMR and C NMR spectra were recorded on Bruker DRX-600 (600 MHz) or Varian MER-300 (300 MHz) spectrometers in the stated solvents using trimethylsilane as an internal standard. Chemical shifts were reported in parts per million (ppm) on the δ scale from an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Coupling constants, J, are reported in Hertz. Mass spectroscopy was performed by The Scripps Research Institute Mass Spectrometer Center. Analytical thin-layer chromatography and flash column chromatography were performed on Merck Kieselgel 60 F254 silica gel plates and Silica Gel ZEOprep 60 ECO 40–63 Micron, respectively. Visualization was accomplished with anisaldehyde or KMnO4.

High performance liquid chromatography (HPLC) was performed on Shimadzu GC-8A using Vydac 218TP C18 GRACE Column (22 mm × 250 mm) for purification and Hewlett-Packard series 1100 using MCI GEL C18 Mitsubishi Chemical Column (4.6 mm × 250 mm) for analysis. Unless otherwise noted, all the materials were obtained from commercial suppliers, and were used without further purification. All solvents were the commercially available grade. All reactions were carried out under argon atmosphere unless otherwise mentioned. Amide starting materials, tyrosine, histidine, tryptophan, serine, cysteine, lysine, and (Ile 3)‐aminoacetophenone, and was used as a model protein for PEGylation.

**Synthesis of 1,2,4-Triazolidine-3,5-diones 8.**

**Method A:** To a 0.2 M solution of ethyl hydrazinocarboxylate 4 (1.0 equiv) in THF was added 1,1-carbonyldimidazole (CDI, 1.0 equiv) at room temperature. The resulting solution was stirred at room temperature. After 2 h, aniline 5 (1.0 equiv) and Et3N (2.0 equiv) were added at room temperature and stirred overnight. Then, EtOAc and 10% HCl were added. The organic layer was separated and washed once with 10% HCl and water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over MgSO4, and the resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over MgSO4 and concentrated in vacuo. The obtained crude solid was washed with EtOAc, dried, and dissolved in MeOH (0.2 M solution) followed by addition of K2CO3 (3.0 equiv). The calculation was done based on the crudish material. The suspension was stirred under reflux for 3 h. The reaction mixture was mixed with 12 N HCl to pH 2 and then concentrated in vacuo. The obtained white solids were washed with water and Et2O to give 8a. 4-(4-Ethynylphenyl)-1,2,4-triazolidine-3,5-dione (8e). The title compound 8e was prepared from 4-ethylaminocarboxylate and was obtained as white solid (2 steps, 38%). 1H NMR (300 MHz, DMSO-d6): δ 10.6 (br, 2H), 7.60–7.57 (m, 2H), 7.54–7.50 (m, 2H), 4.27 (s, 1H). 13C NMR (75 MHz, DMSO-d6): δ 153.83, 133.36, 133.07, 126.67, 121.59, 83.81, 82.41. HRMS: calcd for C10H9N3O2 (MH+) 202.0611, found 202.0619. 4-(4-Acetylphenyl)-1,2,4-triazolidine-3,5-dione (8f). The title compound 8f was prepared from 4-aminoacetophenone, and was obtained as white solid (2 steps, 15%). 1H NMR (300 MHz, DMSO-d6): δ 10.7 (br, 2H), 8.09–8.06 (m, 2H), 7.71–7.68 (m, 2H), 2.62 (s, 3H). 13C NMR (75 MHz, DMSO-d6): δ 198.18, 153.67, 137.09, 136.26, 129.74, 126.17, 27.76. HRMS: calcd for C10H7N3O3 (MH+) 220.0717, found 220.0713.

**Oxidation of 3H-1,2,4-Triazole-3,5(4H)-diones.** To a 0.05 M solution of compound 8 (1.0 equiv) in CH2Cl2 was added 1,3-dibromo-5,5-dimethylhydantoin 10 (1.0 equiv) at room temperature. The resulting solution was stirred at room temperature. After 2 h, silica sulfuric acid (SiO2, –SO3H, 4 times weight to starting material) was added at room temperature and stirred at room temperature. After 30 min, the silica sulfuric acid was removed by filtration. Then, volatile materials were evaporated in vacuo to give 9. The obtained material was relatively unstable against light and humidity in solution at room temperature. Therefore, it was used for next reaction without additional purification after confirmation of purity by 1H NMR (see SI). 4-(4-(Propargyloxy)phenyl)-3H-1,2,4-triazole-3,5(4H)-dione (9a). The title compound 9a was prepared from 8a (50.0 mg, 0.216 mmol), and was obtained as a deep red solid (42.0 mg, 85%). 1H NMR (300 MHz, CDCl3): δ 7.41–7.37 (m, 2H), 7.15–7.12 (m, 2H), 4.75 (d, J = 3 Hz, 2H), 3.64 (t, J = 3 Hz, 1H). 4-(4-(Azidoethoxy)phenyl)-3H-1,2,4-triazole-3,5(4H)-dione (9b). The title compound 9b was obtained as white solid (2 steps, 12%). This compound was purified by short column chromatography (CHCl3/Methanol). 1H NMR (300 MHz, DMSO-d6): δ 10.4 (br, 2H), 7.31–7.27 (m, 2H), 7.02–6.95 (m, 2H), 4.87 (s, 2H), 2.16 (s, 3H). 13C NMR (75 MHz, DMSO-d6): δ 204.81, 157.96, 154.67, 128.57, 125.75, 115.57, 73.13, 27.16. HRMS: calcd for C10H9N3O3 (MH+) 250.0822, found 250.0826. 4-(4-Azidophenyl)-1,2,4-triazolidine-3,5-dione (8d). The title compound 8d was prepared from 4-azidoaniline and hydrochloride, and was obtained as white solid (2 steps, 35%). 1H NMR (300 MHz, DMSO-d6): δ 10.5 (br, 2H), 7.50 (d, J = 9.0 Hz, 2H), 7.23 (d, J = 9.0 Hz, 2H). 13C NMR (75 MHz, DMSO-d6): δ 154.25, 139.59, 129.76, 128.53, 120.47. HRMS: calcd for C10H7N3O3 (MH+) 219.0625, found 219.0617. General procedure B: To a 0.5 M solution of compound 6 (1.0 equiv) and Et3N (1.8 equiv) in THF (5 mL) was added 4-nitrophenyl chloroformate (1.8 equiv) at 0 °C. The resulting solution was stirred at room temperature overnight. Ethyl hydrazinecarboxylate 4 (2.6 equiv) and Et3N (2.6 equiv) were added at room temperature and stirred at 40 °C for 4 h. Then, EtOAc and water were added. The organic layer was separated and washed once with water. The resulting aqueous layer was combined and extracted twice with EtOAc. The combined organic layer was dried over MgSO4 and concentrated in vacuo. The obtained crude solid was washed with EtOAc, dried, and dissolved in MeOH (0.2 M solution) followed by addition of K2CO3 (3.0 equiv). The calculation was done based on the crude material. The suspension was stirred under reflux for 3 h. The reaction mixture was mixed with 12 N HCl to pH 2 and then concentrated in vacuo. The generated white solids were washed with water and Et2O to give 8. 4-(4-Ethynylphenyl)-1,2,4-triazolidine-3,5-dione (8e). The title compound 8e was prepared from 4-ethynylaniline and hydrochloride, and was obtained as white solid (2 steps, 38%). 1H NMR (300 MHz, DMSO-d6): δ 10.6 (br, 2H), 7.60–7.57 (m, 2H), 7.54–7.50 (m, 2H), 4.27 (s, 1H). 13C NMR (75 MHz, DMSO-d6): δ 153.83, 133.36, 133.07, 126.67, 121.59, 83.81, 82.41. HRMS: calcd for C10H9N3O3 (MH+) 202.0611, found 202.0619. 4-(4-Acetylphenyl)-1,2,4-triazolidine-3,5-dione (8f). The title compound 8f was prepared from 4-aminoacetophenone, and was obtained as white solid (2 steps, 15%). 1H NMR (300 MHz, DMSO-d6): δ 10.7 (br, 2H), 8.09–8.06 (m, 2H), 7.71–7.68 (m, 2H), 2.62 (s, 3H). 13C NMR (75 MHz, DMSO-d6): δ 198.18, 153.67, 137.09, 136.26, 129.74, 126.17, 27.76. HRMS: calcd for C10H7N3O3 (MH+) 220.0717, found 220.0713.
was prepared from 8b (49.0 mg, 0.187 mmol), and was obtained as deep red oil (39.6 mg, 81%).

\[ \text{H NMR (300 MHz, CDCl}_3) \delta 7.40-7.35 \text{ (m, 2H), 7.10-7.06 (m, 2H), 4.20 (t, } J = 3.0 \text{ Hz, 2H), 3.64 (t, } J = 3.0 \text{ Hz, 2H).} \]

\[ 4-(2-Oxopropoxy)-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (9c). \]

The title compound 9c was prepared from 8c (47.0 mg, 0.189 mmol), and was obtained as deep purple solid (34.9 mg, 81%).

\[ \text{H NMR (300 MHz, CDCl}_3) \delta 7.42-7.38 \text{ (m, 2H), 7.05-7.02 (m, 2H), 4.61 (s, 2H), 2.31 (s, 3H). 4-(4-Azidophenyl)-3H-1,2,4-triazole-3,5(4H)-dione (9d).} \]

To a solution of compound 9e (4.43 mg, 0.022 mmol) in MeCN (44 μL) was added and was separated and washed sat. NaHCO₃ aq. and brine. The resulting aqueous layer was dried over Na₂SO₄, and concentrated in vacuo. The crude amine 25 was obtained by silica gel chromatography (CHCl₃/MeOH) to give 29 (158 mg, 75%) as a white solid.

\[ \text{Synthesis of Aplaviroc-Urazole. Aplaviroc-alkyne (26): To a solution of Azido-alkyne (See SI) (200 mg, 0.670 mmol) in diethyl ether (2 mL) was added triphenylphosphine (264 mg, 1.00 mmol) at 0 °C and stirred at room temperature for 3 h. Then, deionized water 200 mL was added to reaction mixture and stirred for 12 h. 10 % HCl aq. was added, followed by wash with diethyl ether. The aqueous layer was basified to pH 10 with 5 N NaOH and extracted with dichloromethane/PrOH (4:1) 5 times.} \]

The organic layer was dried over Na₂SO₄ and concentrated in vacuo. The crude amine 25 was used to the next reaction without further purification. To a solution of Aplaviroc 24⁻⁸⁻⁻prepared by the previously reported method (300 mg, 0.513 mmol) in DMF (10 mL) was added BOP (174 mg, 0.639 mmol), triethylamine (362 mL, 2.596 mmol), and amine-alkyne 25 (174 mg, 0.639 mmol) at room temperature and stirred for 12 h. Then, dichloromethane were added and was separated and washed sat. NaHCO₃ aq. and brine. The organic solution was dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by silica gel chromatography (Dichloromethane/MeOH = 9:1) to give 26 (181 mg, 42%) as thin yellow oil.

\[ \text{H NMR (400 MHz, CDCl}_3) \delta 7.83-7.79 \text{ (m, 2H), 7.34-7.28 (m, 2H), 7.02-6.96 (m, 5H), 6.47 (br t, 1H), 6.42 (br s, 1H), 4.01 (dd, } J = 1.4, 5.7 \text{ Hz, 1H), 3.68-3.60 (m, 10H), 3.54 (t, } J = 4.8 \text{ Hz, 2H), 3.46-3.38 (m, 4H), 2.97-2.84 (m, 4H), 2.74-2.69 (m, 2H), 2.54-2.47 (m, 2H), 2.41-2.27 (m, 2H), 2.20-2.07 (m, 4H), 2.02 (t, } J = 2.6, 1H), 1.98-1.89 (2H), 1.73-1.53 (m, 8H), 1.40-1.13 (m, 8H), 0.96-0.85 (t, } J = 7.2, 4H). 13C NMR (125 MHz, MeOD-d₄) \]

\[ \delta 173.02, 171.97, 168.41, 165.92, 160.57, 156.64, 132.35, 129.57, 119.81, 117.95, 82.69, 78.94, 70.50, 70.27, 70.23, 69.69, 69.64, 69.55, 61.42, 59.37, 56.98, 53.93, 49.77, 47.27, 42.87, 40.10, 39.98, 39.42, 36.09, 34.98, 32.17, 31.65, 31.54, 30.02, 29.63, 26.53, 26.02, 20.46, 14.75, 13.30. HRMS: calc'd for C₂₉H₂₇N₁₁O₁₂ (MH⁺) 832.4855, found 832.4854. \]

\[ \text{Aplaviroc-urazole (27): To a solution of 8b (20 mg, 0.763 mmol) and 27 (70 mg, 0.0839 mmol) in tert-}BuOH/H₂O (3 mL/1 mL) was added THPTA⁻⁵⁻⁻(458 mL, 0.0229 mmol), 50 mM solution in H₂O, copper sulfate 5 hydrate (114 mL, 0.0229 mmol, 50 mg/mL solution in H₂O), and sodium ascorbate (91 mL, 0.0229 mmol, 50 mg/mL solution in H₂O) at room temperature and stirred for 30 min. Then, chloroform was added and washed with sat. NaHCO₃ aq. and brine. Combined organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by silica gel chromatography (EtOAc/MeOH = 4/1) to give 27 (43 mg, 52%) as thin yellow solid.

\[ \text{H NMR (400 MHz, MeOD-d₄) \delta 7.82-7.80 (m, 3H), 7.45-7.43 (m, 2H), 7.26-7.24 (m, 2H), 7.03-7.01 (m, 4H), 6.97-6.95 (m, 2H), 4.74 (m, 2H), 4.48 (m, 2H), 4.13 (m, 1H), 3.95 (m, 2H), 3.68-3.60 (m, 10H), 3.54 (t, } J = 4.8 \text{ Hz, 2H), 3.46-3.38 (m, 4H), 2.97-2.84 (m, 2H), 2.74-2.69 (m, 2H), 2.54-2.47 (m, 2H), 2.41-2.27 (m, 2H). 2.20-2.07 (m, 4H), 2.02 (t, } J = 2.6, 1H), 1.98-1.89 (2H), 1.73-1.53 (m, 8H), 1.40-0.85 (t, } J = 7.2, 4H). 13C NMR (125 MHz, MeOD-d₄) \]

\[ \delta 193.13, 191.18, 180.40, 165.80, 160.35, 158.03, 157.15, 155.25, 154.88, 52.16, 46.76, 132.64, 129.53, 128.05, 123.27, 119.67, 118.08, 115.07, 79.13, 70.53, 70.27, 70.13, 69.54, 69.47, 66.93, 56.87, 49.92, 49.88, 40.1221, 39.96, 39.13, 35.30, 31.49, 26.49, 25.96, 21.46, 20.41, 13.15. HRMS: calc'd for C₁₉H₁₇N₁₁O₁₂ (MH⁺) 832.4850, found 832.4853. \]

\[ \text{Preparation of product. Labeled peptide (11a). To a 2 mM solution of custom-synthesized peptide 10 (1.82 mL, 3.82 μmol) in 100 mM pH 7.0 NaH₂PO₄/Na₂HPO₄ buffer, 100 mM solution of PTAD 9a (114 μL, 11.5 μmol) in MeCN was added (9.55 μL x 12 times, interval 1 min.) at room temperature. The resulting solution was stirred at room temperature for 30 min. The crude reaction was analyzed directly by ESI-LC/MS at 254 nm UV absorption and corresponding MS. The reaction mixture was then diluted with MeCN (1.00 mL). The obtained crude material was purified by reversed phase HPLC (mobile phase; gradient of MeCN/0.1% TFA wa ter, 30:70 to 50:50 over}
30 min, Rt; 14.5 min, detection; UV 254 nm) to give 11a (4.40 mg, 61%) as white amorphous solid. HRMS: calcd for C_{27}H_{39}N_{13}O_{17} (MH^+) 1536.7131, found 1536.7125. Reversed phase HPLC purity 95.1% (mobile phase; gradient of MeCN/0.1% TFA water, 10:90 to 100:0 over 30 min, Rt 15.8 min, detection; UV 254 nm). Labeled peptide (11b). The compound 11b was prepared from custom-synthesized peptide 10 (5 mg, 3.82 \mu mol) and 9b (2.99 mg, 11.5 \mu mol), and was obtained as white amorphous solid (4.40 mg, 60%). Reverse phase HPLC conditions for isolation: mobile phase; gradient of MeCN/0.1% TFA water, 30:70 to 50:50 over 30 min, Rt 15.2 min, detection at UV 254 nm). HRMS: calcd for C_{72}H_{94}N_{24}O_{17} (MH^+) 1554.7236, found 1554.7220. Reversed phase HPLC purity 93.6% (mobile phase: gradient of MeCN/0.1% TFA water, 0:100 to 100:0 over 30 min, Rt 15.9 min, detection at UV 254 nm). Labeled peptide (11c). The compound 11c was prepared from custom-synthesized peptide 10 (5 mg, 3.82 \mu mol) and 9c (2.84 mg, 11.5 \mu mol), and was obtained as white amorphous solid (4.60 mg, 61%). Reverse phase HPLC conditions for isolation: mobile phase; gradient of MeCN/0.1% TFA water, 30:70 to 50:50 over 30 min, Rt 15.6 min, detection at UV 254 nm). HRMS: calcd for C_{73}H_{95}N_{21}O_{18} (MH^+) 1536.7131, found 1536.7125. Reversed phase HPLC purity 95%. MALDI-TOF MWav = 5921. Pegylation of Chymotrypsinogen A. Synthesis of PEG-urazole (22): In the 1.5 mL Eppendorf tube were mixed 5k PEG-alkyne 21 (See SI) (15 \mu L of 48 mM solution in DMF, 0.72 \mu mol, 1 equiv) and 1,2,4-triazolidine-3,5-dione azide 20 \text{cyclic} (30 \mu L of 24 mM solution in DMF, 0.72 \mu mol, 1 equiv) followed by addition of a small piece of copper wire and copper sulfate (0.72 \mu L, 100 mM solution in DI water). The reaction mixture was vortexed gently and kept at 37 °C for 2 h with intermittent vortexing. Copper wire was removed and copper ions were scavenged from the reaction mixture using “CupriSorb” resin (Seachem) overnight at room temperature. The Cuprisorb resin was filtered and product polymer was precipitated out with cold ether, centrifuged, and ether decanted. The resulting white solid as PEG-urazole (22) was washed with cold ether two times and dried. Isolated yield 4.0 mg, 95%. MALDI-TOF MWav = 5921. Pegylation of Chymotrypsinogen A with PTAD-PEG: To the 0.65 mL Eppendorf tube containing 5k PEG-PTAD precursor 22 (50 \mu L, 10 mM solution in DMF) was added 1,3-dibromo-5,5-dimethylimidazolidine-2,4-dione (0.49 \mu L, 100 mM solution in DMF). The reaction mixture was vortexed gently and formation of the light cranberry red color was observed, characteristic for the presence of desired PTAD reagent. The reagent was kept on ice and used for the protein modification immediately. To the 1.5 mL Eppendorf tube containing chymotrypsinogen A (MW 25 kDa purchased from ImmunO) (50 \mu L of 1 mg/mL solution; 2 N, 1 equiv) was added freshly prepared 3.92 \mu L of 100 mM solution in DMF). The reaction mixture was vortexed briefly and kept at room temperature for 30 min. The product pegylated chymotrypsinogen A 23 was purified by gel filtration using 7k MWCO Zeba Spin Desalting column (Pierce) and characterized by MALDI-TOF and gel electrophoresis.

Bioconjugation of Herceptin with Aplaviroc-PTAD. To the 0.65 mL Eppendorf tube containing urazole-Aplaviroc 27 (10 \mu L, 6 mM solution in DMF) was added 1,3-dibromo-5,5-dimethylimidazolidine-2,4-dione (10 \mu L, 6 mM solution in DMF). The reaction mixture was vortexed gently and formation of the light cranberry red color was observed, characteristic for the presence of desired PTAD reagent. The reagent was kept on ice and used for the protein modification immediately. To the 0.65 mL Eppendorf tube containing 70 \mu L of 1.0 mg/mL Trastuzumab (herceptin) in 100 mM pH 7.4 Na phosphate buffer was added freshly prepared 3.92 \mu L of 3 mM PTAD-Aplaviroc in DMF in 5 aliquots at 2 s intervals (use fresh pipet tip for each addition). After 15 min, the resulting solution was purified by gel filtration using 7k MWCO Zeba Spin Desalting column (Pierce). The concentration of recovered protein was 0.75 mg/mL (NanoDrop, IgG mode). The increase of molecular weight of 1376 was observed by MALDI-TOF MS analysis, that corresponds to an average 1.3 aplaviroc molecules per molecule of herceptin. Herceptin–aplatiroc conjugate (28) was used for HIV neutralization assay without additional purification.

HIV Neutralization Assay. Replication-incompetent HIV-1 enveloped pseudovirus was generated by cotransfection of 293T cells with JR-FL HIV-1 Env-expressing plasmid and pSG3ΔEnv as previously described. Serial dilutions of samples (50 \mu L) along with wt b12, 2D7, 2G12, and an
isotype control antibody, DEN3, were added to TZM-bl target cells (50 μL) and preincubated for 1 h at 37 °C. Following incubation 250 TCID50 of pseudovirus (100 μL) was added to each well and incubated at 37 °C. Luciferase reporter gene expression was evaluated 48 h post infection. The percentage of virus neutralization at a given antibody concentration was determined by calculating the reduction in luciferase expression in the presence of antibody relative to virus-only wells. The antibody dilution causing 50% reduction (50% inhibitory concentration [IC50]) was calculated by regression analysis using GraphPad Prism.

Chemical Stability Study. Acidic or Basic Conditions.

The solution of compound 29 or 30 (0.0353 mmol) in 10% HCl (0.5 mL) in MeOH (1.5 mL) and in 10% NaOH (0.5 mL) in MeOH (1.5 mL) was stirred at room temperature for 12 h, respectively. Then, EtOAc and water were added. In the case of basic condition, EtOAc was added after acidification with 10% HCl up to pH 3. The organic layer was separated and washed once with water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over MgSO4 and concentrated in vacuo. The residue was purified by silica gel chromatography (EtOAc) to recover compounds as white solids. The recovery of 29 (8.9 mg, 89%) or 30 (9.0 mg, 86%) under acidic conditions and 29 (10.2 mg, quant.) under basic conditions. 30 decomposed under basic hydrolysis conditions within 15 min, as monitored by TLC. The decomposed compounds were purified and analyzed by 1H NMR and LC-MS. Hydrolysis products constituted the yield of 85% (see the structures above).

Stability Study of a Modified p-Cresol in Thermal Condition.

Compound 29 (4.00 mg, 0.0141 mmol) or 30 (4.00 mg, 0.0135 mmol) was heated at 120 °C for 1 h according to literature. The recovery amounts were both 4.00 mg (quant.). No decomposition has been detected by 1H NMR. Physiological stability in human blood plasma:

RESULTS AND DISCUSSION

In order to develop a versatile family of PTAD based conjugation chemistries, we have focused on the design, preparation, and utility of PTAD analogues possessing readily derivatizable linker arms compatible with widely used bioorthogonal coupling chemistries; click chemistry, Staudinger ligation chemistry, and oxime/hydrazone chemistry. The coupling reaction between ethyl hydrazinecarboxylate 4 and anilines 5 or 6 was performed by Method A or B (Scheme 2) depending on the nucleophilicity of aniline. The anilines used were commercially available or were synthesized from commercially available compounds by the methods detailed in Scheme 2. Method A was used in the reaction of 4 with aniline 5: After activation of 4 by treatment with CDI, aniline 5 was reacted with the activated ester in THF at room temperature to afford coupling intermediate 15. The reaction of the less nucleophilic aniline 6 was carried out via Method B. First, 6 was converted to the corresponding activated ester using 4-nitrophenyl chloroformate, then reacted with 4 to

Scheme 2. Synthesis of PTAD Derivatives
afford coupling intermediate 7 in THF at room temperature. Obtained intermediate 7 was cyclized in the presence of K₂CO₃ in MeOH under reflux without isolation. Finally triazolidine 8 was converted to desired triazole 9 by oxidation of the N–N single bond to an N–N double bond with 1,3-diboromo-5,5-dimethylhydantoin 10 according to a literature procedure.⁵⁷

Side products derived from 10 and unreacted starting material were removed by scavenging with silica sulfuric acid (SiO₂−H₂SO₄), because PTAD products 9 were unstable during silica-gel column chromatography. The oxidation reaction was readily monitored by observing the change in the reaction mixture from colorless to deep red. Reagents, 9a, 9b, 9c, and 9d were obtained as solids or oils but solutions were relatively unstable. The products 9e and 9f were unstable to isolation at room temperature. Purity of products was confirmed by ¹H NMR.

The reactivity of the PTAD analogues was evaluated in reaction with N-acyl tyrosine methyl amide 1, and results are shown in Table 1. All reactions were complete in less than 5 min based on color change; however, the reaction products were characterized after a more extended reaction time of 30 min. Electronically neutral reagents 9d and 9e gave the same results as PTAD with comparable percent conversions and without generation of side products. In contrast, significant amounts of uncharacterized side products were generated in the reaction with electronically poor reagent 9f. The reagents substituted with electronically rich moieties, 9a, 9b, and 9c, efficiently modified 1. These results suggest that an electron donating substitution on the phenyl ring provides stability in water without compromising reactivity toward the phenolic side chain of tyrosine.

In order to explore the potential of the tyrosine click reaction using these reagents for labeling of more complicated targets, we synthesized dodecapeptide 10, H₂N-VWSQKRHFG-Y-CO₂H, which has a tyrosine at the C-terminus and contains the potentially reactive amino acids Trp, Ser, Glu, Lys, Arg, and His (Scheme 3). Because this peptide is designed to present amino acids with potentially reactive and competing side-chains, it serves as a stringent test of the chemoselectivity of this reaction. The reaction was performed using 3.0 equiv of PTAD or PTAD derivative in 6% MeCN/phosphate buffer (pH 7) at room temperature. After purification using reversed-phase HPLC, labeled peptides were obtained in approximately 60% yield (Scheme 6). Significantly, a single Tyr modified compound was observed in each reaction by LC-MS (Supporting Information) and HRMS and MS/MS data indicated tyrosine-selective modification. In MS/MS analyses, products obtained using all tested PTAD analogues showed similar fragmentation patterns and all daughter ions contained the ions of modified tyrosine peptide (Supporting Information).

In our initial report of this reaction,⁴⁵ we had shown that both Trp and Lys can react, albeit inefficiently, with PTAD in 50% MeCN/phosphate buffer (pH 7) at room temperature when studied in isolation. However, when N-acyl tyrosine methyl amide 1 was mixed with the corresponding Trp and Lys amino acid derivatives and reacted with PTAD under these conditions, only Tyr modification was observed. Other competitive labeling studies provided the same outcome: selective Tyr labeling (see SI, ref 45). These studies, together with the study of labeling of peptide 10 (which bears potentially competing functional groups in the same molecule) demonstrate here that the highly selective reaction of Tyr with PTAD derivatives is significantly favored under aqueous buffered conditions. As we initially reported, labeling with PTAD derivatives is effective in PBS, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and a variety of other buffers. In some cases, for example, where tyrosines of the target compound or protein are less accessible or reactive, we have noted that an isocyanate decomposition product of the PTAD may be formed that is promiscuous in its labeling and that the products of this type of side reaction are observed. This is the case for chymotrypsigen labeling with PTADs (see SI). This problem, however, is readily solved by using Tris buffer or by simply adding a small quantity of Tris to form a mixed buffered solution. The primary amine of the Tris buffer we believe then acts to scavenge the isocyanate decomposition product of PTADs to minimize production of the side-reaction product (see Supporting Information).

In our earlier study, we established the efficiency of protein labeling using a PTAD-rhodamine dye derivative and found that bovine serum albumin (BSA) was efficiently labeled in buffered solutions containing minimal organic cosolvent at pH ranging from 2 to 10 with 37–98% labeling efficiency (see ref 45, SI). To explore the potential of the tyrosine click for protein multifunctionalizations, here we studied trifunctionalization at tyrosine, cysteine, and lysine residues of bovine serum albumin (BSA) and human serum albumin (HSA). BSA contains 60

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**Table 1. Reactivity of PTAD Derivatives with N-Acyl Tyrosine Methyl Amide 1**

| Entry | Reagent | Conversion (%)
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>9a</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>9b</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>9c</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>9d</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>9e</td>
<td>67</td>
</tr>
<tr>
<td>6</td>
<td>9f</td>
<td>-</td>
</tr>
</tbody>
</table>

*Reactions were performed in 100 mM NaH₂PO₄−Na₂HPO₄ (pH 7)/MeCN (1:1) at room temperature. *Percent conversion was determined by ¹H NMR. °0.5 M PTAD in MeCN mixture solution. *Reaction conditions were 100 mM NaH₂PO₄−Na₂HPO₄ (pH 7)/MeCN (1:1.5).
lysines, 21 tyrosines, and 35 cysteines, whereas HSA has 55 lysines, 19 tyrosines, and 35 cysteines. Cysteine and lysine were modified with a fluorescein maleimide and 11-(dansylamino) undecanoic acid, respectively (Scheme 4). Labeling of albumins at lysine was performed using 50 equiv of 11-(dansylamino) undecanoic acid and 100 equiv of N-[3-(dimethylamino)-propyl]-N′-ethylcarbodiimide hydrochloride (EDC HCl) in water at 37 °C for 14.5 h. After the gel filtration, MALDI TOF analysis revealed that the modified HSA (12) and modified BSA (13) had 3.8 and 4.1 dansyl residues, respectively (Table 2). Next, tyrosines were reacted in 1% MeCN/100 mM phosphate buffer (pH 7.4) using 30 equiv of PTAD derivatives 9a, 9b, and 9c gave products with 4 to 8 modified residues (Table 2). Final labeling at cysteine in SSC buffer (pH 7.0) was achieved using 1 mM fluorescein-5-maleimide in DMSO at room temperature for 2 h (Table 2). As noted in Table 2, each of the desired labels could be efficiently installed onto the target proteins. Fluorescence properties and molecular masses of the modified BSA and HSA are given in the Supporting Information. Thus, PTAD derived reagents bearing bioorthogonal alkyne, azide, and ketone groups were readily prepared and efficiently modified small molecules, peptides, and proteins that can subsequently be further functionalized using click
One of the most important protein conjugation reactions in the pharmaceutical industry concerns the introduction of poly(ethylene glycol) chains onto proteins (PEGylation) to modify their pharmacokinetic properties.62,63 Protein PEGylation is most commonly employed using maleimide- or N-hydroxysuccinimide-based PEGylation reagents. The high abundance of the lysine moieties on protein surfaces often results in generation of multiple PEG addition products and necessitates complicated separation procedures. Conjugation at cysteine residues typically requires the introduction of surface accessible cysteine residues by mutation of the parental protein sequence. To explore the potential of the tyrosine click reaction for protein PEGylation we prepared a 5-kDa PEG-PTAD precursor reagent 22 (Scheme 5, see details in Supporting Information). We used this reagent to study the modification of Chymotrypsinogen A, which contains four tyrosine and fourteen lysine residues. Reactions were performed in buffer (pH 7.4) with 10 equiv of freshly oxidized PEG-PTAD or freshly dissolved PEG-NHS. Reactions were kept at room temperature for one hour with intermittent mixing. Excess reagents were removed using 7-kDa MWCO Zeba Spin Desalting columns, and the products were characterized by gel electrophoresis and MALDI-TOF MS (Supporting Information). We observed formation of mono-, bis-, tri-, and tetra-PEG addition products upon NHS conjugation and predominant formation of mono-PEG addition products upon PTAD conjugation. We have noted low reactivity of the chymotrypsinogen tyrosine residues accounting for the limited labeling of this protein (Supporting Information). Starting material was not completely consumed in either reaction but could be recovered and recycled.

Because of the significance of antibody–drug conjugates in cancer and other therapeutic applications,64–66 we studied the conjugation of the CCR5 antagonist aplaviroc (24) with a monoclonal antibody. As a model monoclonal antibody, we used the well-characterized antibody trastuzumab.67 Aplaviroc was prepared as we previously reported.58 The carboxylic acid moiety of aplaviroc was condensed with an alkyne linker (25) to give aplaviroc having alkyne moiety (26), and then the click reaction with azide-urazole (9c) gave the PTAD-aplaviroc precursor (27). This precursor was oxidized to produce the PTAD moiety, then immediately used for labeling of trastuzumab in 0.1 M phosphate buffer (pH 7) at room temperature. After the removal of the small molecule by gel...
filtration, a product with a single aplaviroc was observed by MALDI-TOF MS spectrum (Supporting Information).

In order to study the bioactivity of conjugated 28, HIV neutralization activity of the aplaviroc–trastuzumab conjugate (28) was tested in TZM-bl cells expressing CCR5 and clade B pseudovirus JR-FL as shown in Figure 2. The IC_{50} value of aplaviroc–trastuzumab conjugate 28 was 11.3 nM; trastuzumab alone had no neutralization activity. Interestingly, this value was very close to that of aplaviroc (24) alone (5.6 nM) indicating that the tyrosine click conjugation chemistry did not negatively impact the activity of the drug. No significant loss in trastuzumab binding was noted as determined by ELISA. This study and our earlier study concerning the bioconjugation of a peptide to trastuzumab indicates that the tyrosine click reaction is applicable the preparation of antibody–drug conjugates and supports a chemical approach to multispecific antibodies.

Key to many bioconjugation chemistries is the stability of the designed linkage. To further stability of the tyrosine click reaction in bioconjugation chemistry, we studied the stability of C–N linkage installed using PTAD reagents in comparison with the more commonly utilized C–S linkage provided by maleimide coupling chemistry (Scheme 7). As noted in our original communication on the PTAD adduct to p-cresol (29), it is stable to acidic or basic conditions at room temperature for 24 h or at 120 °C for 1 h. On the other hand, 30, containing an S–C bond, was stable in acidic conditions, but was hydrolyzed within 5 min in basic condition, although the S–C bond was not cleaved. 30 demonstrated a thermal stability similar to that of 29 after heat treatment for 1 h. This study suggests that the 1,2,4-triazolidine-3,5-dione linkage is hydrolytically and thermally stable, whereas the maleimide linkage is unstable in basic conditions. Next, stability was evaluated in human blood plasma in anticipation of the use of the tyrosine click reaction in protein conjugates, specifically antibody–drug conjugates (ADCs). For this purpose, we studied the stability of 29 and 30 by incubation in fresh human blood plasma at 37 °C for one week (Figure 3). At various time points the reaction was quenched by precipitation with MeCN and analyzed by reversed-phase HPLC (Supporting Information). Compound 29 was found to be completely stable over the course of the 7-day experiment, while 30 decomposed after hours. This data demonstrates that the Tyr click linkage is significantly more...
stabile in human blood plasma than the maleimide linkage and is consistent with reports that protein conjugates prepared with maleimide undergo maleimide exchange with reactive thiols in albumin, free cysteine, or glutathione.\textsuperscript{71,72} Furthermore, thiol-maleimide is prone to oxidation, and this facilitates the retro-Michael reaction and subsequent decomposition.\textsuperscript{73} In view of our studies here and reports concerning maleimide based linkages, the tyrosine click reaction provides a more robust linkage for bioconjugation than maleimide based connections.

\section*{CONCLUSIONS AND IMPLICATIONS}

The studies described herein indicate that the tyrosine click reaction of PTAD derivatives is a highly efficient and chemoselective strategy for small molecule, peptide, and protein conjugation. The reactions of PTAD and designed derivatives developed here were selective for the phenolic side chain of tyrosine residues and proceeded in buffered aqueous media over a broad pH range without the requirement of added heavy metals or other reagents. The C−N linkage that is the product of the tyrosine click reaction is stable to extremes of pH, temperature, and in human serum for extended periods of time. The stability profile of this C−N linkage is significantly better than the stability profile we determined for a model maleimide linkage. While tyrosine residues are commonly found in proteins, surface accessible tyrosines are less common and provide attractive opportunities for minimal labeling using this approach. Because this reaction is highly chemoselective for phenols, it can be applied to couple small molecules, peptides, and poly(ethylene glycol) chains (PEGylation) onto proteins without issues of self-reaction provided that one coupling partner lacks free phenolic groups. This provides an attractive new strategy for the preparation of protein conjugates, including drug conjugates and PEGylated products. We expect that this new methodology and the reagents developed here will find broad utility in production of novel biomolecules, labeled peptides and proteins, and a new chemistry for protein immobilization. Through an agreement with Sigma-Aldrich, alkyne 9a is now commercially available (product number: T-511544).

\section*{ASSOCIATED CONTENT}

\textsuperscript{2} Supporting Information

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.
Genetically encoded tetrazine amino acid directs rapid site-specific cellular protein labelling via a rapid bioorthogonal reaction. 


Facile and Stabile Linkages through Tyrosine: Bioconjugation Strategies with the Tyrosine-Click Reaction

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Supporting Information

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1. Coupling of N-acyl tyrosine methylamide with PTAD or MTAD

**Compound (3a).** To a solution of tyrosine 1 (14.2 mg, 0.060 mmol) in 100 mM pH 7.0 NaH₂PO₄/Na₂HPO₄ buffer (1.5 mL) - MeCN (1.5 mL) was added the 0.5 M solution of PTAD 2a (0.132 mL, 0.066 mmol) in MeCN at room temperature. The resulting solution was stirred at room temperature for 30 min. The reaction mixture was acidified with 12N HCl (0.249 mL) and then concentrated in vacuo. The obtained crude material was purified by flash column chromatography (CHCl₃/MeOH) to give 3a (16.0 mg, 65%) as a white solid.

\[ \begin{align*}
\text{1H NMR (300 MHz, DMSO-d6): } & \delta 11.57 (br, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.90 (q, J = 4.3 Hz, 1H), 7.74 (d, J = 1.7 Hz, 1H), 7.63 – 7.51 (m, 2H), 7.43 (t, J = 7.8 Hz, 2H), 7.34 – 7.21 (m, 1H), 6.83 (dd, J = 8.2, 2.0 Hz, 1H), 6.68 (d, J = 8.2 Hz, 1H), 4.33 (m, 1H), 2.85 (dd, J = 13.5, 5.1 Hz, 1H), 2.63 (dd, J = 13.7, 9.2 Hz, 1H), 2.55 (d, J = 4.5 Hz, 3H), 1.78 (s, 3H). \\
\text{13C NMR (150 MHz, DMSO-d6): } & \delta 172.64, 170.02, 153.90, 150.86, 148.44, 135.37, 129.22, 129.02, 126.96, 126.48, 126.03, 122.72, 117.74, 55.47, 38.23, 26.51, 23.56. \\
\text{HRMS: calcd for C}_{20}H_{22}N_{5}O_{5} (MH^⁺) 412.1615, found 412.1615. 
\end{align*} \]

**Compound (3b).** The compound 3b was prepared from tyrosine 1 (14.2 mg, 0.060 mmol) and 0.5 M solution of MTAD 2b (0.438 mL, 0.132 mmol), and was obtained as white amorphous solid (11.9 mg, 57%).

\[ \begin{align*}
\text{1H NMR (300 MHz, DMSO-d6): } & \delta 10.51 (br, 1H), 8.05 (d, J = 8.4 Hz, 1H), 7.86 (q, J = 4.4 Hz, 1H), 7.21 (s, 1H), 7.02 (dd, J = 1.9, 8.4 Hz, 1H), 6.77 (d, J = 8.3 Hz, 1H), 4.28 (m, 1H), 2.92 (s, 3H), 2.82 (dd, J = 13.8, 4.8 Hz, 1H), 2.60 (dd, J = 13.5, 9.9 Hz, 1H), 2.53 (d, J = 4.5 Hz, 3H), 1.75 (s, 3H). \\
\text{13C NMR (75 MHz, DMSO-d6): } & \delta 172.28, 169.81, 154.68, 153.39, 152.07, 150.78, 130.59, 129.42, 124.27, 117.14, 54.98, 37.38, 26.20, 25.44, 23.22. \\
\text{HRMS: calcd for C}_{15}H_{15}N_{5}O_{5} (MH^⁺) 350.1459, found 350.1460. 
\end{align*} \]
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S4
2. Synthesis of the PTAD analogs


4-Propargyloxy aniline sulfate (4a). A solution of N-(4-(propargyloxy)phenyl)acetamide A (650 mg, 3.44 mmol) in 4 M H$_2$SO$_4$ (10 mL) was stirred under reflux for 3 h. Obtained white crystals were filtered and washed with Et$_2$O to give 4a (577 mg, 68%).

$^1$H NMR (300 MHz, DMSO-d6): δ 8.18 (br, 2H), 6.97-6.90 (m, 4H), 4.73 (d, $J = 3.0$ Hz, 2H), 3.55 (t, $J = 3.0$ Hz, 1H). $^{13}$C NMR (75 MHz, DMSO-d6): δ 153.92, 134.71, 120.94, 117.17, 80.61, 79.20, 57.06. HRMS: calcd for C$_9$H$_{10}$NO(MH$^+$) 148.0757, found 148.0754.
4-N-Boc-(2-bromoethoxy)benzene (C). A suspension of 1-(2-bromoethoxy)-4-nitrobenzene B (1.00 g, 4.06 mmol) and 10% Pd/C (100 mg) in THF (20 mL) was stirred at room temperature for 3 h under a hydrogen atmosphere. Hydrogen was replaced with argon, and a solution of (Boc)₂O (708 mg, 4.06 mmol) in THF (5 mL) was added. After overnight, the catalyst was removed by passing through Celite. After evaporation, the obtained solids were washed with Hexane/Et₂O to give C (742 mg, 58%) as white solid.

¹H NMR (300 MHz, CDCl₃): δ 7.28-7.25 (m, 2H), 6.87-6.84 (m, 2H), 6.41 (br, 1H), 4.25 (t, J = 6.0 Hz, 2H), 3.61 (t, J = 6.0 Hz, 2H), 1.51 (s, 9H).

¹³C NMR (75 MHz, CDCl₃): δ 154.36, 153.39, 132.52, 120.80, 115.66, 80.67, 68.65, 29.53, 28.69. HRMS: calcd for C₁₃H₁₈BrNNaO₃ (M⁺Na⁺) 338.0362, found 338.0366.

4-N-Boc-(2-azidoethoxy)benzene (D). A suspension of compound C (1.64 g, 5.19 mmol) and NaN₃ (1.68 g, 25.9 mmol) in DMF (25 mL) was stirred at 50°C for 3 h. Then, EtOAc and water were added. The organic layer was separated and washed once with water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over MgSO₄, and concentrated in vacuo. The residue was purified by short silica gel chromatography (Hexane/EtOAc) and washing with Hexane/Et₂O to give D (1.24 g, 86%) as white crystals.

¹H NMR (300 MHz, CDCl₃): δ 7.29-7.26 (m, 2H), 6.87-6.84 (m, 2H), 6.43 (br, 1H), 4.11 (t, J = 6.0 Hz, 2H), 3.57 (t, J = 6.0 Hz, 2H), 1.51 (s, 9H). ¹³C NMR (75 MHz, CDCl₃): δ 154.51, 153.41, 132.42, 120.75, 115.36, 80.63, 67.64, 50.49, 28.67. HRMS: calcd for C₁₃H₁₈N₄NaO₃ (M⁺Na⁺) 301.1271, found 301.1258.

4-Propargyloxy aniline sulfate (4a). A solution of N-(4-(propargyloxy)phenyl)acetamide A (650 mg, 3.44 mmol) in 4 M H₂SO₄ (10 mL) was stirred under reflux for 3 h. Obtained white crystals were filtered and washed with Et₂O to give 4a (577 mg, 68%).

¹H NMR (300 MHz, DMSO-d₆): δ 8.18 (br, 2H), 6.97-6.90 (m, 4H), 4.73 (d, J = 3.0 Hz, 2H), 3.55 (t, J = 3.0 Hz, 1H). ¹³C NMR (75 MHz, DMSO-d₆): δ 153.92, 134.71, 120.94, 117.17, 80.61, 79.20, 57.06. HRMS: calcd for C₉H₁₀NO (MH⁺) 148.0757, found 148.0754.
4-N-Boc-(2-oxopropoxy)benzene (F). To a suspension of 4-N-Boc-aminophenol E (4.00 g, 18.2 mmol), K$_2$CO$_3$ (3.05 g, 22.1 mmol) and KI (1.22 g, 7.36 mmol) in acetone (40 mL) was added chloroacetone (0.703 mL, 8.83 mmol) under reflux. After 2 h, additional chloroacetone (0.703 mL, 8.83 mmol) was added. The resulting suspension was stirred under reflux for 2 h. Then, EtOAc and water were added. The organic layer was separated and washed once with water. The resulting aqueous layer was extracted once with EtOAc. The combined organic layer was dried over MgSO$_4$, and concentrated in vacuo. The generated white solids were washed with Hexane/Et$_2$O to give F (1.63 g, 83%).

$^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 9.13 (br, 1H), 7.33-7.30 (m, 2H), 6.82-6.78 (m, 2H), 4.71 (s, 2H), 2.13 (s, 3H), 1.45 (s, 9H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$206.25, 153.96, 153.37, 132.78, 120.81, 115.23, 80.65, 73.72, 28.65, 26.92. HRMS: calcd for C$_{14}$H$_{20}$N$_2$O$_4$ (M+Na$^+$) 288.1206, found 288.1199.

4-(2-Oxopropoxy)aniline hydrochloride (4c). A solution of compound F (400 mg, 1.51 mmol) in 4 M HCl/dioxane (10 mL) was stirred at room temperature for 3 h. Solvent was removed in vacuo and resulting pale brown solids were washed with EtOAc to give 4c (303 mg, quant.).

$^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 10.3 (br, 2H), 7.35-7.32 (m, 2H), 7.02-6.99 (m, 2H), 4.87 (s, 2H), 2.16 (s, 3H). $^{13}$C NMR (75 MHz, DMSO-d$_6$): $\delta$204.68, 158.20, 125.42, 116.32, 73.17, 28.65, 26.92. HRMS: calcd for C$_9$H$_{12}$NO$_2$ (MH$^+$) 166.0863, found 166.0867.

2-2. Synthesis of Aplaviroc derivatives

![Chemical structure of Aplaviroc derivatives]

**Compound I:**
To a solution of azido-amine G (Aldrich, 468 mg, 2.391 mmol) in acetonitrile (5 mL) was added pentynoic acid succinimide ester H (474 mg, 2.391 mmol) at room temperature and stirred for 12 hours. Then, dichloromethane were added and was separated and washed 0.5 M HCl, sat.NaHCO₃ aq. and brine. Combined organic layer was dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by silica gel chromatography (EtOAc) to give I (620 mg, 87%) as a white solid.

$^1$H NMR (400 MHz, CDCl₃): δ 6.30 (br s, 1H), 3.75-3.57 (m, 10H), 3.60-3.57 (m, 2H), 3.52-3.47 (m, 2H), 3.49-3.41 (m, 2H), 2.57-2.53 (m, 2H), 2.45-2.41 (m, 2H), 2.04 (t, $J = 5.3$ Hz, 1H), $^{13}$C NMR (125 MHz, MeOD-d4): δ 173.09, 82.78, 72.70, 70.67, 70.65, 70.62, 70.60, 70.56, 70.52, 70.48, 70.42, 70.32, 70.12, 69.60, 61.26, 50.81, 48.19, 39.48, 35.47. HRMS: calcd for C₁₃H₂₂N₄O₄ (MH⁺) 299.1714, found 299.1715.

3. NMR-charts of the PTAD analogs
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$\text{S9}$
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$S_{12}$

![Chemical Structure](image)

![NMR Spectra](image)
Ban, Nagano, Gavrilyuk, Hakamata, Inokuma, and Barbas, III

$S13$
8e
Ban, Nagano, Gavrilyuk, Hakamata, Inokuma, and Barbas, III

$S_{23}$

![Chemical structure and NMR spectrum](image-url)
Ban, Nagano, Gavrilyuk, Hakamata, Inokuma, and Barbas, III

S25
Ban, Nagano, Gavrilyuk, Hakamata, Inokuma, and Barbas, III
S26
4. The reactions of tyrosine derivative with PTAD analogs

$^1$H NMR of the mixture of the reaction

Effect of buffer concentration

TLR in pH 7phosphate buffer/MeCN

![Chemical Structures and NMR Spectra](Image)
Ban, Nagano, Gavrilyuk, Hakamata, Inokuma, and Barbas, III

S28
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$S29$
100 mM Na phosphate buffer (pH 7)

MeCN (1:1), r.t.
5. Peptide modification with PTAD analogs

5-1 HPLC and MS chart

HPLC

Crude reaction LC/MS

10 peak 1307.7
1 mod. compound peak 1536.7
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S32

MS of peptide (10)          MS of modified peptide (11a)
Crude reaction LC/MS
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MS of peptide (10)  

MS of modified peptide (11b)
Crude reaction LC/MS

HPLC

SM peak 1307.

1 mod. compound 1555.
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MS of decapeptide (10)  MS of 1 modified peptide (11c)
5-2. MS/MS analysis of labeling peptides
6. Albumin modification through three different orthogonal reactions

1) Reaction of albumins with dansyl derivative

Overlay of MALDI-TOF MS charts of HSA (red) and 12 (blue); molecular weight increase 1592.
Overlay of MALDI-TOF MS charts of BSA (red) and 13 (blue); molecular weight increase 1706.

Fluorescence intensity of dansyl moiety of HSA, BSA, 12, and 13.
2) Reaction of dansyl-albumins with PTAD derivative.

Overlay of MALDI-TOF MS charts of 12 (blue) and 14a (red). Molecular weight increase 1540.

Overlay of MALDI-TOF MS charts of 12 (blue) and 14b (red). Molecular weight increase 1681.
Overlay of MALDI-TOF MS charts of 12 (blue) and 14c (red). Molecular weight increase 1130.

Overlaid MALDI-TOF MS chart of 13 (blue) and 15a (red). Molecular weight increase 1708.
Overlay of MALDI-TOF MS charts of 13a (blue) and 15b (red). Molecular weight increase 1681.

Overlay of MALDI-TOF MS charts of 13a (blue) and 15c (red). Molecular weight increase 969.
3) Reaction of dual labeled albumins with fluorescein-5-maleimide.

Overlay of MALDI-TOF MS charts of 14a (blue) and 16a (red). Molecular weight increase 966.

Overlay of MALDI-TOF MS charts of 14b (blue) and 16b (red). Molecular weight increase 568.
Overlay of MALDI-TOF MS charts of 14c (blue) and 16c (red). Molecular weight increase 1142.

Overlay of MALDI-TOF MS charts of 15a (blue) and 17a (red). Molecular weight increase 492.
Overlay of MALDI-TOF MS charts of 15b (blue) and 17b (red). Molecular weight increase 755.

Overlay of MALDI-TOF MS charts of 15c (blue) and 17c (red). Molecular weight increase 682.
Fluorescence intensity of fluorescein moiety of 14a-c, 15a-c, 16a-c and 17a-c.
7. Chymotrypsinogen PTAD labeling buffer study

We noted an experimental artifact in our original PTAD labeling study of chymotrypsinogen (ref. 1, figure 3B), namely the addition of a mass of approximately 336 daltons to the protein. It is known that PTADs can decompose to form isocyanates and a mass addition of 336 is consistent with isocyanate formation of the PTAD azide derivative used in the original study and its subsequent reaction with various nucleophilic functionalities present on proteins like amines and hydroxyls. The simplest PTAD shown above is expected to decompose to an isocyanate of mass 119 (see above). Reaction of isocyanate derivatives with a protein is expected to be promiscuous whereas direct reaction with PTADs is highly selective for the phenolic side chain of tyrosine as we have proven in many cases. We suspect that with slow reacting proteins with no or poorly exposed/reactive tyrosine residues like chymotrypsinogen, decomposition of PTADs produces isocyanates that then promiscuously label the protein.

In our initial disclosure of PTAD labeling, we had documented that labeling is effective in a wide variety of buffered solutions, including 2-amino-2-hydroxymethyl-propane-1,3-diol or Tris buffered solution. Since Tris buffer contains a primary amine, we studied the potential of using Tris buffer as an isocyanate scavenger in PTAD labeling reactions of chymotrypsinogen. As shown in the ESI-MS charts that follow, chymotrypsinogen labeling in phosphate buffer revealed significant isocyanate labeling. Addition of as little as 5% volume Tris to the PBS solution, however, acted to significantly scavenge the isocyanate decomposition product, while addition of 25% volume Tris nearly completely removed detectable isocyanate-derived protein labeling as effectively as performing the reaction in Tris buffer itself. Thus for the PTAD labeling of particular proteins, an exploration of buffers, including mixed PBS/Tris buffered media is recommended to ensure tyrosine selective labeling when Tyr specificity is critical. PBS buffer is often fine.
Study of Chymotrypsinogen labeling with PTAD in PBS/Tris buffered conditions:

To the 1.7 ml microcentrifuge tube was added chymotrypsinogen solution (60.6 µM protein in 98 µL mixed buffer prepared by volumetric mixing of PBS (10 mM, pH 7.4) and Tris (1.0 M, pH 7.4) buffers) followed by addition of PTAD (100 mM in DMF, 2 µL). The reaction mixture was allowed to stand at room temperature for 15 min before the unreacted small molecules were removed using Zeba spin desalting column (7k MWCO). ESI-MS was then obtained and are given below.

3. We thank Drs Edmund Graziani and Qi-Yang Hu for also bringing the artifact at ref. 1, Fig. 3B to our attention and Qi-Yang Hu for additional discussion.

ESI-MS charts for Chymotrypsinogen labeling with PTAD

Unmodified protein
Labeling in PBS

+ PhNCO

+ PhNCO (x2)

+ PhNCO + PTAD

Labeling in PBS/Tris (95/5)
Labeling in PBS/Tris (85/15)
Labeling in PBS/Tris (75/25)

Labeling in Tris
8. PTAD mediated PEGylation reaction

Preparation of PEG-PTAD

In the 1.5 ml Eppendorf tube were mixed 5k PEG-NHS (NOF Corporation, 98% end group reactivity) (38 ul of 50 mM solution in DMF, 1.89 umoles, 1 eq) and propargyl amine (1.89 ul of 1M solution in DMF, 1.89 umoles, 1 eq). The reaction mixture was vortexed gently and kept at room temperature for 3 hours with intermittent vortexing. Methyl amine (5 ul, neat) was added to the reaction to make sure all the activated ester groups were consumed; reaction was vortexed and kept at room temperature for 30 min. The product polymer was precipitated out with cold ether, centrifuged and ether decanted. The resulting white solid was washed with cold ether two times and dried. Isolated yield 9.1 mg, 93%. MALDI-TOF $M_{av} = 5656$. 

PEG-NHS (starting material):

PEG-alkyne:
Overlay of PEG-NHS starting material (red) with PEG-alkyne (blue):

*Synthesis of PEG-urazole (23):* In the 1.5 ml Eppendorf tube were mixed 5k PEG-alkyne (NOF Corporation, 98% end group reactivity) (15 µl of 48 mM solution in DMF, 0.72 µmoles, 1 eq) and 1,2,4-triazolidine-3,5-dione azide 14 (30 µl of 24 mM solution in DMF, 0.72 µmoles, 1 eq) followed by addition of a small piece of copper wire and copper sulfate (0.72 µl, 100 mM solution in DI water). The reaction mixture was vortexed gently and kept at 37 °C for 2 hrs with intermittent vortexing. Copper wire was removed and copper ions were scavenged from the reaction mixture using “CupriSorb” resin (Seachem) over night at room temperature. The Cuprisorb resin was filtered and product polymer was precipitated out with cold ether, centrifuged and ether decanted. The resulting white solid (23) was washed with cold ether two times and dried. Isolated yield 4.0 mg, 95%. MALDI-TOF $M_{\text{wav}} = 5921$.  

PEG-alkyne:

Overlay of PEG-NHS (red) with PEG-PTAD(green).
MALDI-TOF:
Chymotrypsinogen A:

Chymotrypsinogen-PEG (PTAD)
Chymotrypsinogen-PEG(NHS):
Comparison of products of reaction of Chymotrypsinogen A with 5-kDa PEG-PTAD and 5-kDa PEG-NHS. Products were separated on a NuPage 4-12% Bis-Tris gel (Invitrogen) and the gel was Coomassie stained: L, molecular weight ladder; lane 1, Chymotrypsinogen A; lane 2, reaction with 10 eq. PEG-PTAD; lane 3, reaction with 10 eq. PEG-NHS.

9. Trastuzumab (Herceptin) conjugation with Aplaviroc-PTAD

![Diagram of the conjugation process]
10. Stability study in human plasma

HPLC charts of the analyzed compounds.