Synthesis of the Trifunctional Agent, NBzM

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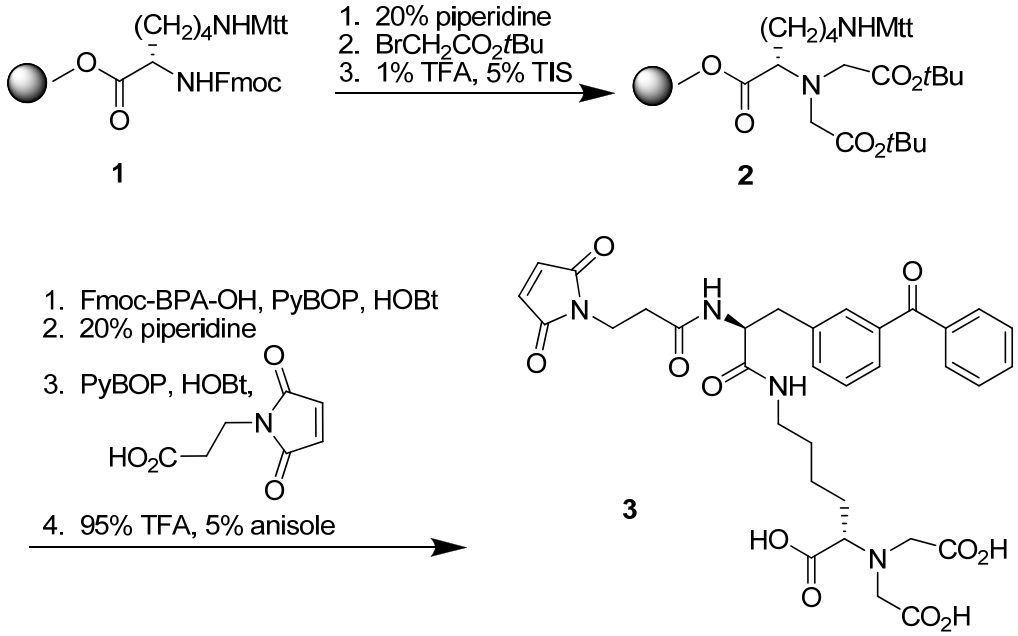
Background

The trifunctional agent NBzM (**3**) is useful for the site selective labeling of 'polyhistidine' or 'His-tag' proteins which in turn are often incorporated near the C-terminal of recombinant proteins to aid in purification processes. The His-tag moiety binds to Ni(II) and nitrilotriacetic acid (NTA), and thereby allows a means of purification by selective affinity chromatography. The presence of the C-terminal His-tag allows for the site-specific labeling of recombinant proteins by using a modified NTA derivative for affinity binding and which further incorporates two chemically reactive groups; in the case of NBzM, one photoreactive and one reactive with sulfhydryl groups. The approach also incorporates regiospecific and stoichiometric control over modification of recombinant proteins.

Chemistry

Synthesis of NBzM (**3**) was performed using the method as essentially described by Meredith et al.[1] Specifically, Fmoc-Lys(Mtt)-Wang resin was first deprotected with 20% piperidine, N-dialkylated with acetate groups at the backbone amino group and then deprotected with 1% TFA followed by elongation with benzoyl-L-phenylalannine (BPA) and maleimido segments at the side chain. Owing to the low coupling efficiency, each coupling step required a double coupling in the presence of a 5- to 40- fold excess of coupling reagents. After cleavage from the resin with simultaneous removal of the *tert*-butyl protecting group, the crude final product **3** was obtained and purified by HPLC.

Scheme 1. Synthesis of NBZM



Experimental

General. Mass spectra were recorded on an API 2000 system. ^{1}H-NMR data was obtained on a Bruker 360MHz model. Chemical shifts are reported in parts per million (δ) and are referenced to tetramethylsilane (TMS). A Waters Atlantis 4.6 x 50mm and a Waters Atlantis 19x50mm preparative column, respectively, were used for HPLC. All chemicals are commercially available from Sigma-Aldrich or Bachem.

Preparation of 2,2'-((S)-5-((S)-3-(3-benzoylphenyl)-2-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1 yl)propanamido)-1-carboxypentylazanediyl)triacetic acid (NBzM) (3). Fmoc-Lys(Mtt)-Wang Resin (**1**) (1.37g, 1.00 mmol) was swollen and Fmoc deprotection was performed by double treatment of the resin in the presence of 20% piperidine in DMF (7 mL) for 20 minutes each. To the resin was added $BrCH_2CO_2{}^{t}Bu$ (6.4 mL, 40 mmol), DIPEA (7.2 mL, 41 mmol), DMA (2 mL) and the reaction vessel (50 mL) was shaken at 350 rpm at 45°C for 24 hours. A chloranil test indicated that the reaction was incomplete. The resin was then filtered and a double coupling was performed under the above conditions in order to complete the reaction. The Mtt group was cleaved using five, three-minute, treatments with 1%TFA/5%TIS/CH_2Cl_2 solution (5 mL) to obtain resin-bound (**2**). To half of resin (**2**) (0.5 mmol) was added Fmoc-BPA-OH (1.229

g, 2.5 mmol), HOBt (338 mg, 2.5 mmol), PyBOP (1.301 g, 2.5 mmol), DIPEA (0.87 mL, 5.0 mmol) and dimethylacetamide (4 mL)/CH_2Cl_2 (1 mL) solution. The reaction mixture was shaken for 7 hours followed by the double coupling for a period of 4 hours. After deprotection of Fmoc group, to the resin was added β-maleimido-propionic acid (422 mg, 2.5 mmol), HOBt (338 mg, 2.5 mmol), PyBOP (1.301 g, 2.5 mmol), DIPEA (0.87 mL, 5.0 mmol) and DMA (4 mL)/CH_2Cl_2 (1 mL) solution. The first coupling was performed for 20 hours and the second coupling for 5 hours. After washing with DMF (5x8 mL) and CH_2Cl_2 (5x8 mL), the resin was briefly dried under vacuum. The peptide was cleaved from the resin with a 5-minute treatment with a 95%TFA/5% anisole solution (5x8 mL). The solvents were removed and the residue was precipitated with ice-cold diethyl ether and dried under high vacuum to give the product (**3**) as a white powder. The crude white powder was dissolved in 1% TFA/DMSO solution (4 mL) and filtered through a 0.2 μm PTFE Acrodisc Syringe Filter. This crude NBzM solution was injected onto a Waters preparative HPLC system equipped with a Waters Atlantis 19x50mm column, run at a flow rate of 24.0 ml/min, and a Waters micromass ZQ MS detector. Solvents were water and acetonitrile, both containing 0.1% TFA, and the sample was run for 8 minutes under the following gradient: Initial flow rate of 4% CH_3CN to 22% CH_3CN over 0.5 min; to 32% over 5.5 min; to 99% over 0.5 min; to 4% over 1.0 min; 4% for 1.0 min. The pure product was dried by lyophilization to give NBzM (79 mg, overall yield 24%) as a white powder. ^{1}H NMR (d^6-DMSO, 360 MHz) δ 8.27 (d, 1H, J = 8.4 Hz), 7.97 (t, 1H, J = 5.4 Hz), 7.70-7.52 (m, 7H), 7.38-7.36 (m, 3H), 6.95 (s, 1H), 4.4.8 (m, 1H), 3.54-3.43 (m, 7H), 3.06-2.89 (m, 3H), 2.82 (dd, 1H, J = 9.4 Hz, J = 13.5 Hz), 2.41-2.26 (m, 2H), 1.65-1.47 (m, 2H), 1.40-1.20 (m, 4H). MS (m/z): 663.2 $[M-H]^-$.

Reference

1. Meredith GD, Wu HY, Allbritton NL. Targeted protein functionalization using His-tags. Bioconjug Chem. 2004 Sep-Oct;15(5): 969-82. PMID 15366949