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Synthesis of Tryptophan–Folate Conjugates

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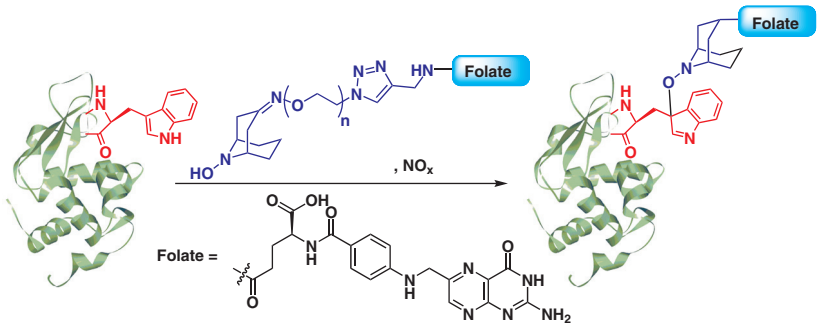

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Abstract A mild protocol for the synthesis of folate–peptide/protein conjugates targeting tryptophan residues is described. This synthetic protocol is advantageous for homogeneous conjugation of chemically sensitive folates to biomolecules, with potential applications in cancer therapy and diagnostics.

Key words bioconjugation, folate, tryptophan, proteins, peptides

Folate (vitamin B_9) is an essential nutrient for cell division and replication. The folate receptor (FR) responsible for folate cellular transport is overexpressed in a variety of human tumor cells but is highly restricted in normal tissues. The FR has long been used as a biomarker for evaluating cancer-cell-targeted delivery, diagnostics and chemotherapy.[1] In particular, folate-conjugated compounds based on small molecules,[2] peptides,[3] proteins,[4] radioactive-metal complexes,[5] oligonucleotides,[6] or antibodies[7] are widely studied and their application in cancer-selective therapy/diagnostics by taking advantage of the strong folate–FR binding affinity (K_d = 0.1–1 nM) is eagerly expected.[8] Because of the sensitivity of folate to various chemical conditions,[9] however, universal synthesis of homogeneous folate conjugates is not a straightforward task.

Protein bioconjugation targeting proteinogenic amino acids is an attractive method for appending artificial functions to biomolecules without reliance on genetic manipulations.[10] We previously reported a metal-free, widely applicable, tryptophan (Trp)-selective protein bioconjugation by using the stable 9-azabicyclo[3.3.1]nonane *N*-oxyl (ABNO) radical.[11] Trp is the least abundant and the least surface-exposed proteinogenic amino acid, and each Trp residue has a variety of solvent accessibilities. Bioconjugation targeting Trp is advantageous in terms of controlling the sites and number of modifications to give homogeneous bioconjugates, unlike conventional bioconjugation methods that target lysine or cysteine. Here, we describe the first feasible synthesis of Trp-targeted folate–peptide/protein conjugates.

In our search for a universal reagent capable of conjugating various functional molecules, we first designed platform compound **7**, in which an ABNO moiety is linked to an azide-terminated oligomeric poly(ethylene glycol) (PEG_n). ABNO-PEG_n–folate (n = 4, 8, 12) reagents could be produced from the appropriate **7** and N^{α}-pteroyl-γ-glutamylpropargylamide (**12**) by Huisgen cycloaddition. Convergent synthesis of **7** might be possible by condensation of an oxime with ω-azido alkoxyamines and keto-ABNO-H (Figure 1).

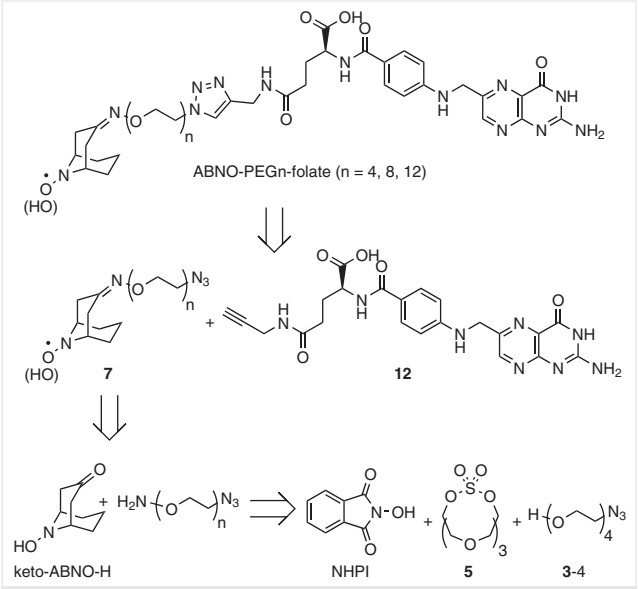

Figure 1 Retrosynthetic analysis of ABNO-PEG_n–folate

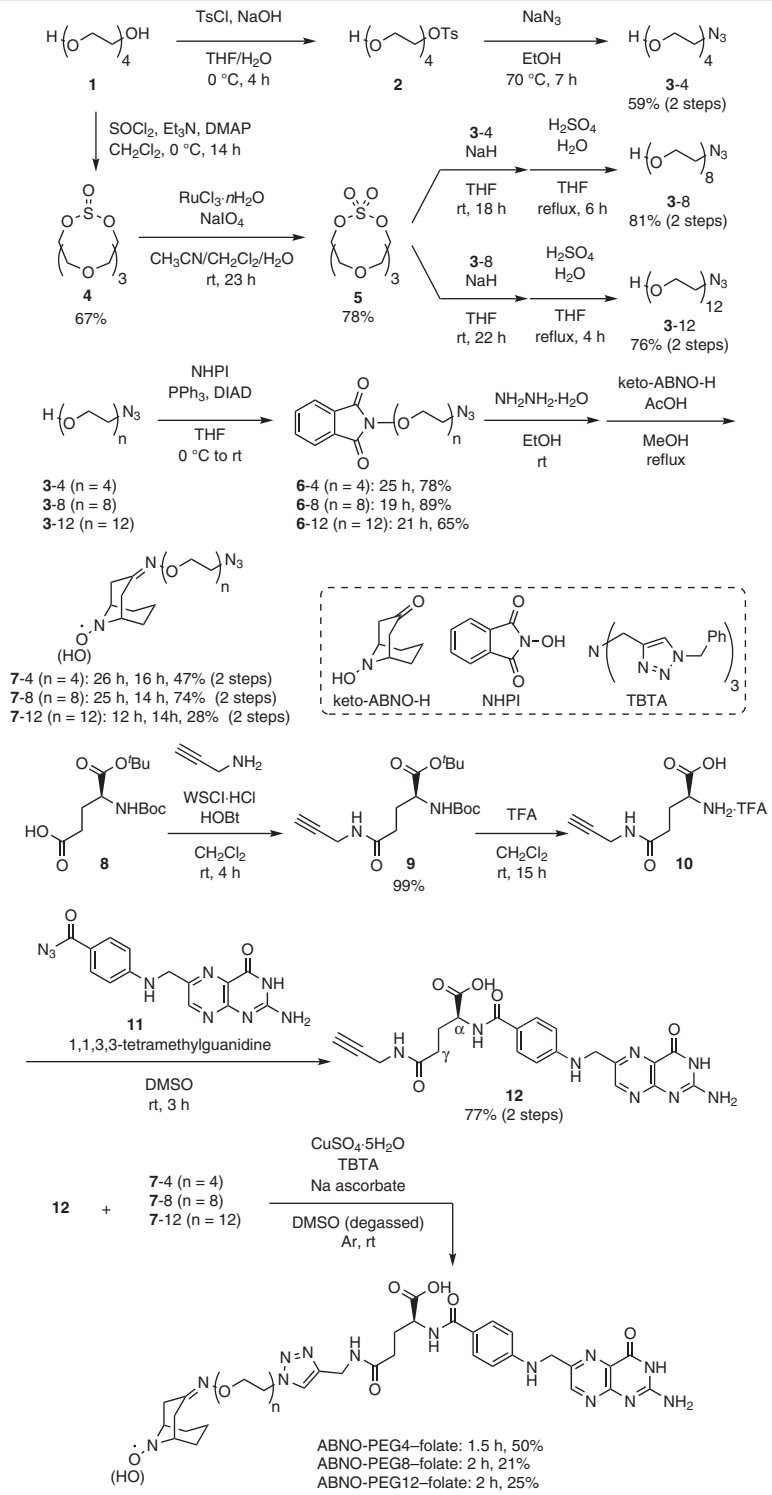

Figure 2 Synthesis of ABNO-PEG$_n$–folates

With some modification of our synthetic route, we synthesized **7**-4,[11a] **7**-8, and **7**-12 (n = 4, 8, and 12, respectively) with various PEG oligomers (Figure 2). Hydroxy azides **3**-4, **3**-8, and **3**-12 were obtained from tetra(ethylene glycol) (**1**) through systematic monodisperse elongation of the PEG chain with the cyclic sulfate **5**.[12] Each azide **3** was subjected

to a Mitsunobu reaction with *N*-hydroxyphthalimide (NHPI) and subsequent removal of the phthaloyl group to produce the corresponding ω-azido alkoxyamines. These crude products were linked to keto-ABNO-H[13] to give the platform intermediates **7**-4, **7**-8, and **7**-12. Direct amidation of folate with the amine groups using a condensation reagent such as DCC generally produced inseparable mixtures containing α-glutamylamide, γ-glutamylamide, and α,γ-glutamylbisamide.[4a] Because the folate α-glutamylamide form is irrelevant for FR binding,[14] a selective synthesis of γ-glutamylamide is preferred. By following the protocol reported by Fuchs *et al.*,[15] pteroyl azide (**11**), prepared from folic acid, was treated with γ-glutamylpropargylamide (**10**) to give N^{α}-pteroyl-γ-glutamylpropargylamide (**12**) selectively. This product was subjected to Cu/tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)-catalyzed Huisgen cycloaddition[16] with intermediates **7**. The resulting ABNO-PEG$_n$-folates (n = 4, 8, 12) were purified by HPLC.

The ABNO-PEG$_n$–folates were used to synthesize peptide/protein–folate conjugates (Figure 3). First, the reaction was applied to leuprorelin, a peptide drug (Figure 3a). By using two equivalents of the appropriate ABNO-PEG$_n$–folate and 1.2 equivalents of $NaNO_2$, we obtained the corresponding folate–leuprorelin conjugates in moderate yields (41–78%).[17] However, the product contained HPLC-separable nitrosated or benzyl-cleaved conjugates as byproducts (ratio = 0.9:1 to 4.1:1), possibly as a result of the presence of the reactive aniline functionality of the folate moiety. A longer reaction time did not improve the chemical yield and merely resulted in decomposition of the starting peptide and the product.[18] We then applied our protocol to a protein (lysozyme) by using five equivalents of the ABNO-PEG$_n$–folate and three equivalents of $NaNO_2$ (Figure 3b). Analysis of the crude mixture by deconvoluted ESI-MS clearly showed the presence of the folate–lysozyme conjugates, demonstrating that folate conjugation is feasible, even at the protein level. The folate-to-protein ratio (N) ranged from 0.24 to 0.72.[19] This N value was lower than that of previously synthesized fluorescein conjugates.[11] This was rationalized in terms of the NO_x-scavenging effect of folate,[18] which might reduce the concentration of the NO_x activator and retard the reaction.

In conclusion, we have established a protocol for synthesizing tryptophan-targeted folate–peptide/protein conjugates. Although the current protocol is limited by concomitant nitrosation and a low reaction efficiency, the extraordinarily mild conditions that permit homogeneous conjugation of the chemically sensitive folate molecules are noteworthy. Applications of this protocol in the design of cancer therapeutics and diagnostics are expected in the near future.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0039-1691735.

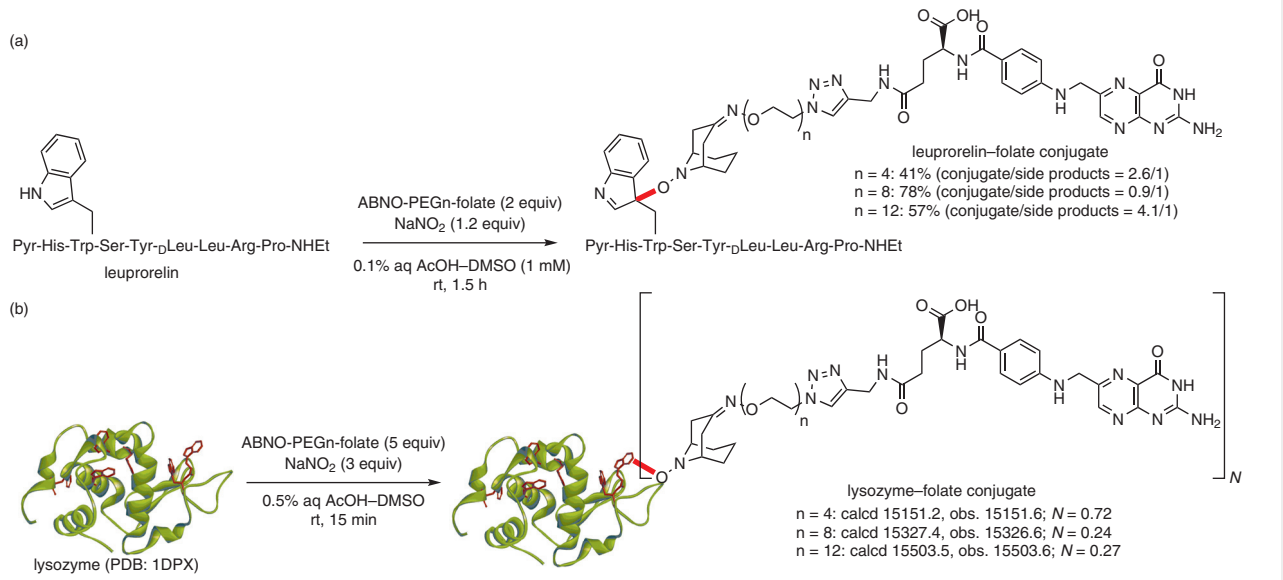

Figure 3 Synthesis of Trp-targeted peptide/protein–folate conjugates[20]

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(19) The folate-to-protein ratio (*N*) was determined by using a calibration curve based on the specific absorbances of folate (A347) and the absorbances of both folate and protein (A278). See the Supporting Information for details.

(20) **Trp-Targeted Conjugation of an ABNO-PEG–Folate to a Peptide; Typical Procedure**

Leuprorelin acetate (25.4 μg, 0.02 μmol, 2.54 μL of 10 mg/mL stock solution), ABNO-PEG_4-folate (34.0 μg, 0.04 μmol, 1.70 μL of 20 mg/mL stock solution), $NaNO_2$ (1.66 μg, 0.024 μmol, 1.66 μL of 1 mg/mL stock solution), and AcOH (0.02 μL, 2.00 μL of 0.01 μL/μL stock solution) were dissolved in H_2O (12.10 μL; final volume 20 μL), and the resulting mixture was stirred at rt for 90 min. The reaction was quenched by the addition of 20 mM phosphate-buffered saline (PBS; pH 7.4) and the mixture was analyzed by HPLC and LC/MS. The yield was calculated to be 41% from the HPLC peak area of the product at 254 nm. HPLC: [YMC-Triart C18 (4.6 × 150 mm); linear gradient B/A 0–100% (A = 0.1% aq TFA, B: MeCN) during 2–42 min at a flow rate of 1 mL/min]; t_R = 21.6 min. ESI-MS: *m/z* calcd $[M + 2H]^{2+}$ for $C_{97}H_{135}N_{29}O_{22}$: 1029.5; found: 1029.6

Trp-Targeted Conjugation of an ABNO-PEG–Folate to a Protein; Typical Example

Lysozyme (358 μg, 0.02 μmol, 7.15 μL of 50 mg/mL stock solution), ABNO-PEG_4-folate (85.0 μg, 0.1 μmol, 4.25 μL of 20 mg/mL stock solution), $NaNO_2$ (4.14 μg, 0.06 μmol, 4.14 μL of 1 mg/mL stock solution), and AcOH (0.1 μL, 1.00 μL of 1 mg/mL stock solution) were dissolved in H_2O (3.46 μL, final volume 20 μL), and the resulting mixture was stirred at rt for 15 min. The mixture was then transferred into 20 mM PBS (pH 7.4, 200 μL) to quench the reaction. Small molecules were removed by ultrafiltration (Amicon Ultra, 10 K) (10×; 200 μL of PBS was added each time). The final volume was adjusted to ~ 250 μL, and the UV/Vis spectrum was recorded. The folate-to-protein ratio (*N*) was calculated to be 0.72 from the absorbances at 347 and 278 nm, based on the Beer–Lambert law. The solution (80 μL) was exchanged with H_2O by ultrafiltration (Amicon Ultra, 10 K), and the final volume was ~ 40 μL. The resulting solution was analyzed by LC-MS. ESI-Q-TOF MS: *m/z* $[M]^+$ calcd for $C_{651}H_{1008}N_{206}O_{195}S_{10}$: 15151.2; found: 15151.6.

