Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 1289–1291

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Facile Synthesis of N-Fmoc-Serine-S-GlcNAc: A Potential Molecular Probe for the Functional Study of O-GlcNAc

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Received 9 March 2000; accepted 4 April 2000

Abstract—A methabolically stable β-*N*-acetylglucosaminyl-1-thio-N-Fmoc-serine (S-GlcNAc-Ser) derivative was synthesized in two procedures: one involving a coupling of a readily obtainable 1-pseudo-thiourea of GlcNAc (S-GlcNAc) and iodo-N-Boc-L-alanine benzyl ester, and the other utilizing a modified Mitsunobu reaction of GlcNAc-SH and a serine derivative.

O-Linked *N*-acetylglucosamine (O-GlcNAc) is an abundant and ubiquitous post-translational modification of Ser/Thr residues in both nuclear and cytoplasmic proteins, and its addition to proteins is thought to be dynamically regulated as is protein phosphorylation.[1–5] Accumulating evidence has suggested a putative functional role for O-GlcNAc[1,3] in a variety of diseases, including cancer, diabetes, and Alzheimer's disease; however, due to the lack of a suitable molecular probe (inhibitor), the role of O-GlcNAc had not yet been fully understood at the molecular level. Unfortunately, site-directed mutagenesis of the Ser/Thr residues which are substituted with GlcNAc does not offer a promising approach to studying this glycosylation, because these residues are also potential phosphorylation sites, as is the case for c-myc.[6,7]

The proteins exist in three different isoforms: the naked (the Ser/Thr-free form), O-glycosylated (O-GlcNAc form), and O-phosphate forms (Fig. 1), and the mechanism of their interconversions is dynamically regulated by corresponding enzymes. The key enzyme in this process is an O-GlcNAc-specific *N*-acetylglucosaminidase (O-GlcNAc'ase), which cleaves O-GlcNAc residues to liberate the OH group of Ser/Thr,[8] which can then be phosphorylated by a kinase(s). We thought that it would be very valuable to prepare a peptide containing an O-GlcNAc equivalent that is resistant to O-GlcNAc'ase digestion, so that we could modulate such dynamic transformations of nuclear protein structures. We chose a 1-thio-β-*N*-acetyl-glucosamine (S-GlcNAc) as an O-GlcNAc'ase-resistant analogue of O-GlcNAc because thio-glycosides are known to be glycosidase resistant.[9] Although several synthetic procedures have been reported for the preparation of neutral sugars thio-linked to amino acids,[10–14] there is still, to our knowledge, none which describes the synthesis of thio-linked GlcNAc to serine and S-GlcNAc-Ser-incorporated peptides. We have examined several synthetic approaches and report herein a facile synthesis of S-GlcNAc-N-Fmoc-Ser, which serves as a building block for the preparation of a peptide bearing S-GlcNAc.

N-Acetylglucosamine was converted to the known 1-isothiourea derivative 1[15,16] (Scheme 1), which was precipitated during the reaction and readily isolated (about 60% overall yield from GlcNAc) by simple filtration after the reaction mixture was cooled. We confirmed the β-stereochemistry of the 1-thio linkage of **1** by ^{1}H NMR spectral analysis with a large $J_{1,2}$ coupling (10.8 Hz) and did not detect any α-isomer.[17] The following coupling reaction between the 1-thio-GlcNAc derivative **2** and the iodo alanine derivative **3**[18] was troublesome because of the competing side reactions under various conditions (Table 1). In a reaction in acetone–water at room temperature,[12] some of the iodo derivative **3** seemed to undergo a β-elimination, giving an α,β-unsaturated ester **5** (evidenced by TLC), which then presumably served as a Michael acceptor to produce a mixture of **4** (L and D, Fig. 2). In a separate experiment, we indeed found that **2** reacted with the unsaturated ester **5** to give a mixture of **4** under the same reaction conditions. Coupling of **2** and **3** in DMSO–H_2O at 0 °C (Monsigny's conditions)[11] gave an almost pure product of **4**. In addition, a biphase reaction using CH_2Cl_2–H_2O in the presence of

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PII: S0960-894X(00)00223-7

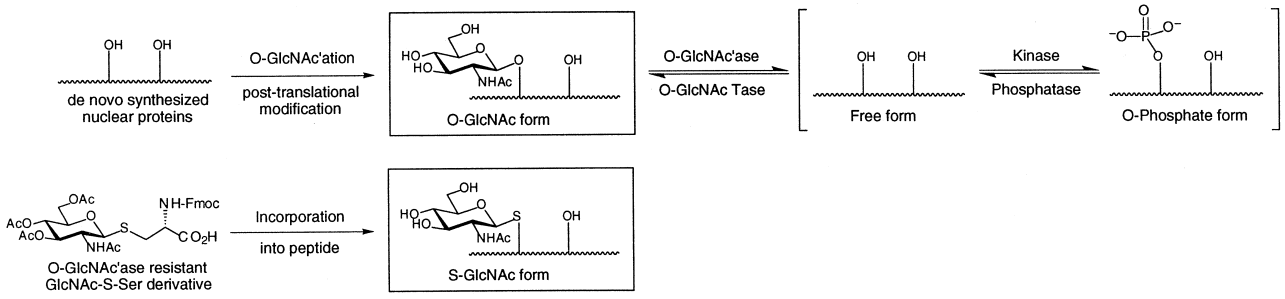
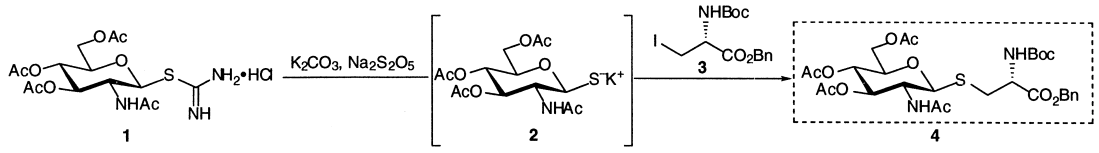


Figure 1. Schematic presentation of the putative role of O-GlcNAc versus O-phosphorylation and the designed O-Glc-NAc'ase resistant S-GlcNAc-Ser.



Scheme 1. Synthesis of N-Boc-S-GlcNAc **4** by alkylation.

Table 1. Coupling reaction of **1** and **3**

Entry	Condition	Ratio (L:D)[a]	Yield (%)
1	$K_2CO_3/Na_2S_2O_5$/acetone/H_2O	5:1	81
2	$K_2CO_3/Na_2S_2O_5$/DMSO/H_2O	>70:1	58
3	$K_2CO_3/Na_2S_2O_5/Bu_4NI/CH_2Cl_2/H_2O$	100:0	34

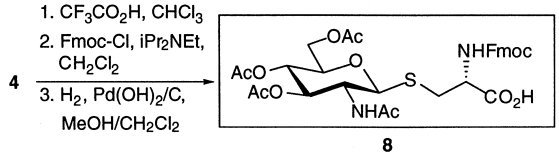
[a]The ratio was determined by ^1H NMR analysis.

Bu_4NI as a phase transfer catalyst also gave **4** without epimerization. Such a coupling reaction of the 1-thio-GlcNAc **2** and the iodo alanine derivative **3** may be suitable for large-scale preparation but is rather laborious; therefore, we searched for a more convenient synthetic route for **4**.[19]

The Toth group has reported a modifed Mitsunobu reaction (Me_3P and 1,1′-(azodicarbonyl)-dipiperidine, ADDP) for the coupling of 1-thio-sugars and alcohols;[20] however, it did not include the coupling reaction of 1-thio-GlcNAc and an amino acid derivative. We therefore examined a direct coupling reaction of the 1-thio-GlcNAc **6** and a serine derivative **7** (Scheme 2). We chose another modified Mitsunobu condition[21] utilizing ADDP and Bu_3P (due to the cost: Me_3P, \$62 for 0.1 mol; Bu_3P: \$3 for 0.1 mol from Aldrich), and found that the coupling of **6** and **7** proceeded smoothly to afford the product **4** in 53% yield after silica gel chromatography (toluene:EtOAc, 1:1) without epimerization.[22]

Finally, the Boc group of **4** was removed with 50% CF_3CO_2H–$CHCl_3$ and the resulting amino compound was treated with Fmoc-Cl to give the N-Fmoc derivative, which was subsequently hydrogenated over $Pd(OH)_2/C$ and purified on SiO_2 chromatography[23] to afford building block **8**[24] (Scheme 3).

In summary, we have established a facile synthetic route to an O-GlcNAc'ase resistant S-GlcNAc-Ser as a building block for a solid-phase synthesis of S-GlcNAc-containing peptides for use in studying the role of O-GlcNAc in O-GlcNAc-bearing nuclear proteins. We are currently preparing a peptide with Ser-S-GlcNAc from **8** using the solid-phase FMOC chemistry and the biological evaluation of such glycopeptide will be published elsewhere.



Scheme 3.

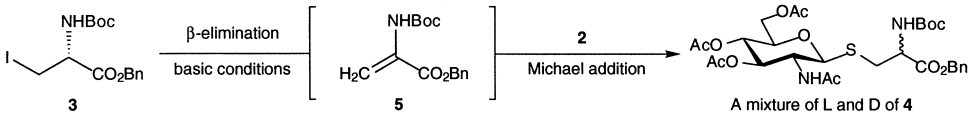
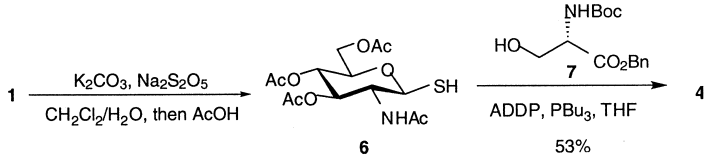


Figure 2. Side reaction observed during the alkylation.



Scheme 2. Synthesis of **4** by a Mistunobu reaction.

Acknowledgements

The NMR studies were performed in the Biochemistry NMR Facility at Johns Hopkins University, which was established by a grant from the National Institutes of Health (GM 27512) and a Biomedical Shared Instrumentation Grant (1S10-RR06262-0). This research was partly supported by the National Institutes of Health (GM 52324).

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17. Spectral data for **1**: ^{1}H NMR (300 MHz, D_2O): δ 5.42 (1H, d, J = 10.8 Hz, H-1), 5.53 (1H, dd, J = 9.8, 9.6 Hz, H-3), 5.15 (1H, dd, J = 9.8, 9.6 Hz, H-4), 4.40–4.15 (4H, m, H-2,5,6a, and 6b), 2.10, 2.07, 2.04 (3H each, s, OAc), 1.97 (3H, s, NAc); ^{13}C NMR (75 MHz, D_2O) δ 177.2, 176.2, 175.5, 175.2, 170.5, 84.7, 78.4, 75.6, 70.6, 64.6, 54.4, 24.5, 22.7.
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19. Procedure for the coupling reaction of **2** and **3** in DMSO–H_2O: To a cooled (0 °C) and a stirred solution of K_2CO_3 (93.7 mg, 0.68 mmol) and $Na_2S_2O_5$ (104.0 mg, 0.55 mmol) in H_2O (3 mL) was added **1** (299.5 mg, 0.68 mmol) in DMSO (3 mL) and **3** (274.8 mg, 0.68 mmol) in DMSO (5 mL). The reaction mixture was stirred for 1.5 h at 0 °C, and then poured into ice-water. The products were extracted with $CHCl_3$, and the organic layer was washed with 0.5 M $KHSO_4$, H_2O, and brine. The combined organic layer was dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by SiO_2 column chromatography (toluene:EtOAc, 5:1 to 1:2) to afford **4** (252.5 mg, 58%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.37–7.35 (5H, m, aromatic), 6.13 (1H, d, J = 9.0 Hz,-NH), 5.89 (1H, d, J = 7.5 Hz,-NH), 5.24–5.03 (4H, m), 4.64–4.55 (2H, m), 4.22–4.09 (5H, m), 3.73 (1H, m, H-5), 3.31 (1H, dd, J = 14.5, 3.3 Hz,-CHa), 2.95 (1H, dd, J = 14.5, 7.5 Hz,-CHb), 2.05 (3H, s,-OAc), 2.02 (6H, s,-OAc×2), 1.94 (3H, s, NAc), 1.45 (9H, s, *tert*-Bu); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.8, 170.6, 170.5, 170.2, 169.1, 135.1, 128.6, 128.5, 128.4, 128.1, 83.6, 80.1, 77.2, 75.8, 73.6, 68.3, 67.2, 62.1, 53.4, 52.5, 28.2, 23.0, 20.5.
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22. Procedure for coupling of **2** and **6** by a Mitsunobu reaction: To a cooled (0 °C) and stirred bi-phase solution of K_2CO_3 (1.52 g, 11.0 mmol) and $Na_2S_2O_5$ (1.74 g, 9.2 mmol) in CH_2Cl_2:H_2O (1:1, 80 mL) was added **1** (4.06 g, 9.2 mmol). The reaction mixture was stirred for 2 h at room temperature. The solution was cooled to 0 °C, then acetic acid was added (0.63 mL, 11.0 mmol), and the resulting mixture was stirred for 10 min at 0 °C. The mixture was diluted with CH_2Cl_2, washed successively with H_2O, brine, and dried over $MgSO_4$, and concentrated to give **6** (ca. 2.95 g) which was used in the next step without further purification. To a cooled (0 °C) and stirred solution of ADDP (2.9 g, 11.3 mmol) in dry THF (50 mL) was added Bu_3P (2.9 mL, 11.3 mmol) under argon. The mixture was then warmed to room temperature and stirred for 1 h. The solution was re-cooled to 0 °C, and a solution of **3** (3.3 g, 11.3 mmol) in dry THF (25 mL) and a solution of above compound **6** (ca. 2.95 g, 8.1 mmol) in dry THF (30 mL) were added to the solution. The reaction mixture was stirred overnight at room temperature. Any precipitate was filtered, and the filtrate was concentrated in vacuo. The residue was further precipitated from EtOAc:hexane, filtered, and concentrated. The residue was diluted with CH_2Cl_2, washed with H_2O, brine, and dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (toluene:EtOAc, 1:1) to give **4** (3.1 g, 53%).
23. Even a small amount of contaminating diastereomer could be eliminated during this purification step: SiO_2 column chlomatography, eluted with $CHCl_3$:EtOAc:MeOH (5:1:1).
24. Analytical data for compound **8**: ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.96–7.30 (8H, m, aromatic), 5.10 (1H, dd, J = 9.8, 9.6 Hz, H-3′), 4.68 (1H, dd, J = 9.8, 9.6 Hz, H-4′), 4.77 (1H, d, J = 10.5 Hz, H-1′), 4.29–3.85 (8H, m), 3.14 (1H, dd, J = 13.8, 4.2 Hz, H-3a), 2.81 (1H, dd, J = 13.8, 9.6 Hz, H-3b), 1.99, 1.97, 1.92 (3H each, s, OAc), 1.75 (3H, s, NAc); ^{13}C NMR (DMSO-d_6, 300 MHz): δ 172.0, 169.9, 169.5, 169.20, 169.17, 143.7, 140.6, 127.6, 127.0, 125.1, 120.0, 83.1, 74.6, 73.5, 68.5, 66.3, 65.7, 62.0, 54.2, 52.0, 46.5, 30.7, 28.9, 22.5, 20.3; HRMS (FAB) m/z calcd for $C_{32}H_{37}O_{12}N_2S$ $(M+H)^+$ 673.2067, found 673.2065.