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Original Article

Photochemical synthesis of peptides, *O*-glycopeptides, and their derivatives

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ABSTRACT

N-acyl-7-nitroindolines are light-responsive compounds capable of acylating nucleophiles in inert, aprotic solvents under neutral conditions when triggered by near UV light. These compounds have previously served as linkers in solid-phase synthesis to generate amides and thioesters. In exploring their potential in glycopeptide synthesis, we constructed two model glycopeptides derived from Mucin 1, featuring tumor-specific antigens GalNAcα (Tn) or NeuNAcα(2,6)GalNAcα (STn). This was achieved via solid phase peptide synthesis (SPPS) utilizing the Fmoc/*t*Bu strategy, employing a 7-nitroindoline linker attached to the resin. This linker facilitated the direct photorelease of protected glycopeptide acids and amides from the solid support into the solution. Alternatively, glycopeptides with a C-terminal 7-nitroindoline moiety were subjected to photochemical reactions in solution, enabling the synthesis of the same peptide derivatives, and additionally, glycopeptide phenyl thioesters. We showcase that *N*-glycopeptidyl-7-nitroindolines can be effectively transformed into protected glycopeptide acids, amides, and thioesters, thereby broadening the applicability of *N*-acyl-7-nitroindoline linkers in SPPS.

KEYWORDS: photoreactive linker, *N*-acyl-7-nitroindoline, solid phase peptide synthesis, glycopeptides, photochemical acylation, MUC1, tumor-specific antigen.

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1. INTRODUCTION

Photochemistry has first been employed in solid phase peptide synthesis (SPPS) in 1973, when Rich and Gurwara introduced a 4-chloromethyl-3-nitropolystyrene resin [1], followed by the development of some other *o*-nitrobenzyl-based resins [2, 3]. They allowed for the photorelease of protected peptides from the solid support by illumination with near UV light at 350 nm. These mild reaction conditions are compatible with the Boc/Bn strategy of SPPS, and somewhat less compatible with the Fmoc/*t*Bu strategy due to the instability of ester bond of the C-terminal amino acid toward piperidine. The formation of diketopiperazine byproducts was also reported.

Before the advent of Native Chemical Ligation (NCL) [4], protected peptide acids were routinely used as building blocks in the synthesis of larger peptides by peptide segment condensation, including convergent SPPS [5]. Today, the synthesis of protected peptide acids and glycopeptide acids still has its place for post SPPS modifications of the C-terminus, *e.g.*, biotinylation, fluorescent labeling, and linker attachment for the construction of chimeras such as proteolysis-targeting chimera (PROTAC) compounds [6].

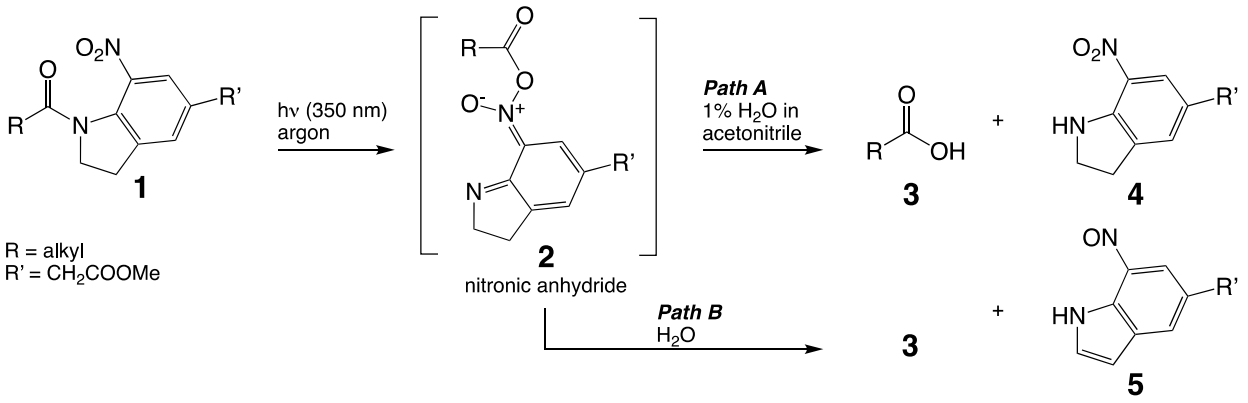
Another class of photoreactive compounds are 7-nitroindolines with *N*-carbonyl moieties, *i.e.*, amides [7-9], ureas and carbamates [10, 11], and thiocarbamates [12]. Both, *N*-acyl-7-nitroindolines and 7-nitroindoline-*S*-thiocarbamates can undergo photolysis by one- and two-photon absorption mechanisms [12-14]. Patchornik and coworkers

demonstrated that *N*-acyl-7-nitroindoline derivatives (**1**) can be used as acylating agents when activated by near UV or indigo light [7, 8]. Corrie and coworkers suggested the photochemical formation of a nitronic anhydride intermediate **2** *in situ* via an *N* to *O* acyl shift, whose fate is solvent-dependent (Scheme 1) [15, 16]. In an inert, aprotic solvent with a small amount of water present, a nucleophilic acyl substitution takes place, resulting in the formation of carboxylic acid **3** and 7-nitroindoline **4** (Path A). In contrast, under aqueous conditions a different photolysis pathway takes place, which results in the formation of a carboxylic acid by an ε-elimination followed by a tautomerization resulting in a carboxylic acid (**3**) and a 7-nitrosoindole **5** (Path B). Depending on the level of water content in the reaction mixture, mixed photolysis mechanisms can take place [15, 16].

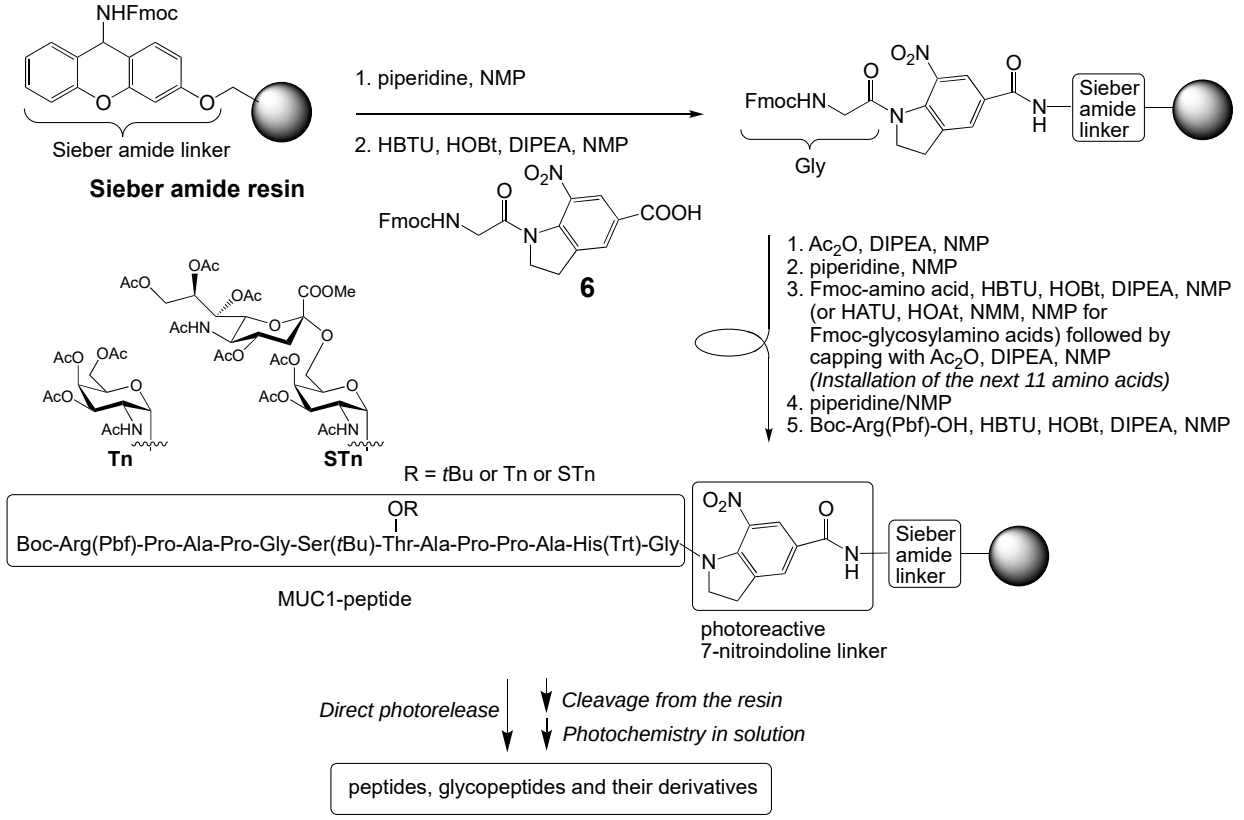
Several different nucleophiles have been photoacylated, *i.e.*, water, alcohols, amines, aminals, hydrazine, and aliphatic and aromatic thiols [8, 9, 17-22]. Various mechanisms have been suggested for the formation of the crucial nitronic anhydride intermediate **2** including an addition-elimination process involving a cyclic intermediate [16], as well as a Norish type I and type II acyl migration from N-1 to the adjacent nitro group [16, 23-26].

Previously, we reported on a 7-nitroindoline-based, dual functional, photoreactive linker for the synthesis of aliphatic and aromatic peptide thioesters [27, 28], which can serve as starting materials for NCL. 7-Nitroindoline-glycine derivative **6** (Schemes 2 and 3) was one of the amino acids coupled to Rink amide resin, which installed both the photoreactive linker and the first amino acid on the solid support. This linker was highly compatible with SPPS using the Fmoc/*t*Bu strategy and proved suitable for the photochemical cleavage of protected peptide thioesters in pyridine from the resin under mild conditions [27]. During this process, the nitroindoline moiety remained attached to the resin, while the peptide derivative was released into the solvent. Alternatively, the peptide together with the photoreactive nitroindoline moiety was cleaved from the Rink amide resin with 95% trifluoroacetic acid [27], and side-chain protected peptides with the C-terminal nitroindoline moiety can also be cleaved from the Sieber amide resin using a 1% solution of trifluoroacetic acid in dichloromethane [28]. This produced photoreactive peptides, which underwent photoacylation is solution [27, 28]. Both procedures have proven successful for the synthesis of peptide thioesters in high yields and insignificant levels of epimerization at the C-terminal amino acid [27, 28]. However, SPPS using the photoreactive 7-nitroindoline linker has never been applied to the synthesis of the more delicate glycopeptides and their derivatives, whose SPPS are not always compatible with the Boc/Bn strategy, and therefore are usually synthesized by the Fmoc/*t*Bu strategy. A mild photochemical cleavage and derivatization method would be particularly desirable for the synthesis of sensitive glycopeptides.

Here we demonstrate the usefulness of the photoactive 7-nitroindoline linker by expanding its scope to the SPPS of Mucin 1 (MUC1)-derived peptides and glycopeptides with a terminal carboxylic acid, amide, or thioester. We have chosen a 13-amino acid long peptide sequence, *i.e.*, RPAPGSTAPPAHG (Scheme 2), derived from the 20 amino acid long tandem repeat region of MUC1 [29], as a model peptide/glycopeptide. MUC1 is a heavily *O*-glycosylated protein secreted from the epithelium of gastrointestinal and respiratory tracts. Cancerous cells from a number of different tumor types express hypoglycosylated MUC1, which contains tumor-specific antigens [30, 31]. These glycans are typically truncated and/or prematurely sialylated, *e.g.*, the Tn (GalNAcα) and the STn (NeuNAcα(2,6)GalNAcα) antigen, which are considered tumor cell markers in pancreatic, ovarian and breast cancers. In this work we show the SPPS of a MUC1 peptide and two different glycopeptides on Sieber amide resin with our photoreactive 7-nitroindoline linker incorporated (Scheme 2). The Tn and STn antigens were introduced into the sequence via the glycoamino acid building blocks Fmoc-Thr[Tn$(Ac)_3$]OH [32, 33] and Fmoc-Thr[STn$(Ac)_6$]-OH (methyl ester) (Figure 1). Once the MUC1-derived peptide and glycopeptides were assembled on the solid support, they were either directly photoreleased from the resin and derivatized, or they were cleaved off with dilute



Scheme 1. Solvent-dependent photochemical pathways of *N*-acyl-7-nitroindolines.



Scheme 2. SPPS of a MUC1 peptide and two MUC1 glycopeptides bearing the Tn or STn antigen on Sieber amide resin with a photoreactive 7-nitroindoline linker incorporated, and photochemical manipulations directly on the solid supported (glyco)peptides, or on the photoreactive (glyco)peptides in solution.

trifluoroacetic acid followed by their photochemical derivatization in solution (Scheme 2). We show that the *N*-acyl-7-nitroindoline moiety can be light-activated to produce MUC1-derived (glyco) peptide acids, amides, or thioesters, depending on the reaction conditions.

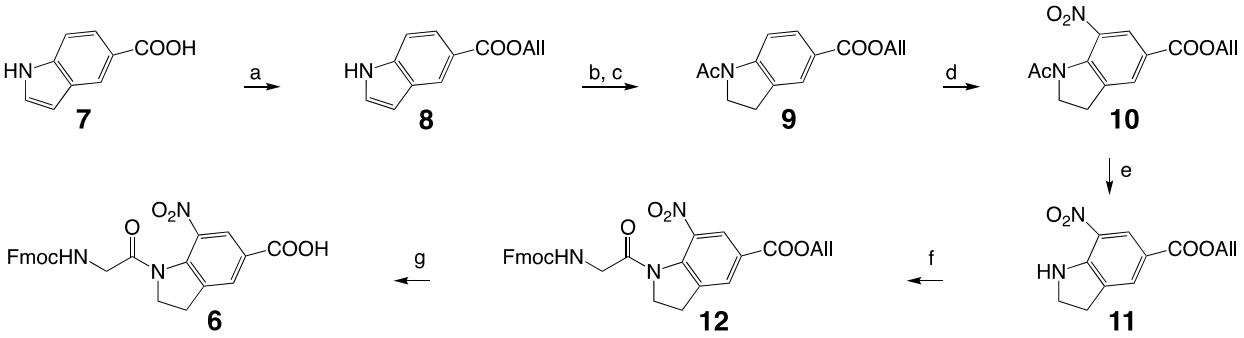
2. MATERIALS AND METHODS

2.1. Chemicals, reagents, and instrumentation

All reagents and solvents used were purchased as reagent-grade from Fisher Scientific or Sigma-Aldrich and used as-is unless otherwise specified. Sieber amide resin, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) and the amino acid derivatives, except for Fmoc-Gly-nitroindoline derivative **6** and the glycosyl amino acids, were purchased from Novabiochem. Peptide and glycopeptide synthesis was performed semi-automatically with a Tribute peptide synthesizer, Protein Technologies, Inc. (USA). Photochemical reactions were carried out in a Rayonet RPR200 Photochemical Reactor equipped with 16 lamps emitting 350 nm. Reversed phase chromatography was performed on the Fast Protein Liquid Chromatography (FPLC) system AKTA Purifier 100 from GE Healthcare Life Sciences. A JEOL AJMS-T100LC system equipped with an Electrospray Ionization source was utilized for high-resolution Time-Of-Flight (TOF) mass spectrometry. External calibration of the instrument was performed using either reserpine (Sigma/Aldrich) or Ultramark 1621® (Alfa Aesar). For protected peptides, a concentration range of 1-20 ppm in methanol/ acetonitrile (1:1) containing 0.1% formic acid was employed for injection. The same solvent composition was utilized for electrospray generation. Unprotected peptides were dissolved in methanol/ water (1:1) containing 0.1% formic acid, with the identical solvent system utilized for electrospray generation. ^{1}H NMR spectra were captured on a JEOL ECA 600 MHz NMR spectrometer at 300K, on a Bruker Avance III HD 400 MHz NMR spectrometer, or on a Bruker 300 MHz NMR spectrometer using either DMSO-d_6 or $CDCl_3$ as solvents. Tetramethylsilane was utilized as an internal standard.

2.2. Synthesis of the glycine building block with photoreactive linker

Scheme 3 illustrates the synthesis of Fmoc-Gly-nitroindoline derivative **6** which was modified from a procedure by Nicolaou [9]. Experimental details for the synthesis of compound **6** [27] has not been reported. First, commercially available 5-carboxylic acid indole **7** was allylated. The resulting allyl ester **8** was reduced to allyl indoline-5-carboxylate (not shown), which cannot be easily separated from any unreacted starting material due to similar polarities. Therefore, the crude was used for the next reaction step, *i.e.*, the *N*-acetylation. Since indoline is a better nucleophile than indole, selective *N*-acetylation was achieved, and compound **9** could be easily purified by column chromatography on silica gel, followed by nitration to compound **10**. Photochemical deacetylation and attachment of Fmoc-Gly-OH gave the 7-nitroindoline linker **12**, with the first amino acid (glycine) attached. Palladium (0)-catalyzed deallylation without affecting the Fmoc group [34] gave amino acid building block **6** [27].



Scheme 3. Synthesis of the photoreactive amino acid building block **6**. a) Cs_2CO_3, allyl bromide, dry DMF, r.t. 2 h; b) AcOH, $NaBH_3CN$, 10 °C – rt, 2 h; c) Ac_2O, Et_3N, rt, 12 h; d) $NaNO_3$, TFA, 0 °C – rt, 6 h; e) 0.02 M, UV light (350 nm), CH_3CN, 17 h; f) Fmoc-Gly-OH, anhydrous toluene, $SOCl_2$, 70 °C, 19 h; g) $Pd(PPh_3)_4$, *N*-methylaniline, dry THF, rt, 30 min.

2.2.1. Synthesis of *N*-(Fmoc-glycyl)-5-carboxylic acid-7-nitroindoline (6)

Compound **6** was synthesized from allyl ester **12** by Pd(0)-catalyzed deallylation. In a round bottom flask, compound **12** (1.15 mmol, 0.61 g) and tetrakis(triphenylphosphine)palladium (0.12 mmol, 0.13 g) were dissolved in anhydrous tetrahydrofuran (THF, 10 mL) under argon. *N*-methylaniline (11.50 mmol, 1.25 mL) was added to the solution, which immediately turned dark red. The reaction was monitored by TLC until the starting material was consumed (1h). THF was removed under reduced pressure and the remainder was dissolved in ethyl acetate and washed extensively with a 1M HCl solution (10 × 50 mL), followed by water (5 × 50 mL), brine (2 × 50 mL) and dried over anhydrous magnesium sulfate. After filtration, ethyl acetate was removed under reduced pressure to obtain an orange solid (0.56 g, quantitative), and no further purification was required. R_f = 0.18 (MeOH/DCM 5:95). ^{1}H NMR (400 MHz, 295 K, DMSO-d_6) δ 13.44 (s, 1 H, COOH); 8.10 (s, 1 H, H6); 8.08 (s, 1 H, H4); 7.90 (d, 2 H, ^{3}J = 7.4 Hz, Fmoc-arom.); 7.78 (t, 1 H, NH); 7.75 (d, 2 H, ^{3}J = 7.4 Hz, Fmoc-arom.); 7.42 (t, 2 H, Fmoc-arom.); 7.34 (t, 2 H, Fmoc-arom.); 4.33-4.30 (m, 4 H, Fmoc-CH_2, H-2, H-2'); 4.25 [t, 1 H, $^3J_{Fmoc(CH/CH2)}$ = 7.0 Hz, Fmoc (benzylic)]; 4.10 (d, 2 H, $^3J_{H\text{-}\alpha/NH}$ = 6.0 Hz, H-α, H-α'); 3.29 (t, 2 H, $^3J_{H\text{-}2/H\text{-}3}$ = 8.1 Hz, H-3, H-3') ppm; ^{13}C NMR (100 MHz, 295 K, DMSO-d_6) δ 168.2, 156.4, 143.8, 140.7, 139.3, 137.6, 136.5, 129.3, 127.6, 127.0, 125.2, 123.4, 120.0, 65.7, 48.8, 46.6, 43.6, 28.3 ppm. ESI-TOF-MS m/z $[M-H]^-$ calcd. 486.1301; obs. 486.1327. λ_{max} (DCM, 41.1 μM) = 268, 301 and 324 nm, ε268 = 18701 M^{-1} cm^{-1}, ε301 = 5092 M^{-1} cm^{-1}, ε324 = 1935 M^{-1} cm^{-1}. λ_{em} (DCM, 82.1 μM, excited at 324 nm) = 392 nm.

2.2.2. Synthesis of allyl indole-5 carboxylate (8)

Commercially available indole-5-carboxylic acid (**7**) (5.4 g, 33.5 mmol) and cesium carbonate (6.55 g, 20.1 mmol) were dissolved in anhydrous DMF (18 mL) under argon. To this solution, allyl bromide (29 mL, 335 mmol) was added dropwise over 30 min. The solution was allowed to stir at rt for 2 h. The DMF was co-evaporated with toluene, and the resulting gel was redissolved in ethyl acetate. An aqueous workup was performed with water, sodium bicarbonate, then brine. The organic layer was dried over anhydrous magnesium sulfate, filtered through celite, and dried. Product **8** was not further purified. R_f 0.6 (EtOAc/hexanes 1:1). ^{1}H NMR (300 MHz, 23.4 °C, $CDCl_3$) δ 8.66 (br s, 1H, NH), 8.46 (s, 1H, arom.), 7.93 (d, 1H, J = 8.4 Hz, arom.), 7.40 (d, 1H, arom.), 7.26 (d, 1H, H-2), 6.64 (d, 1H, ^{3}J = 4.7 Hz, H-3), 6.08 (m, 1H, CH_2=C<u>H</u>, allyl), 5.43 (dd, 1H, ^{2}J = 1.4 Hz, $^3J_{trans}$ = 17.2 Hz, C<u>H_2</u>=CH), 5.28 (dd, 1H, $^3J_{cis}$ = 10.3 Hz, 1H, C<u>H_2</u>=CH), 4.86 (d, J = 5.5 Hz, 2H, C<u>H_2</u>CH=CH_2) ppm; ^{13}C NMR (75 MHz, 23.4 ºC, $CDCl_3$) δ 167.7, 138.6, 132.8, 127.6, 125.8, 124.0, 123.5, 121.9,118.0, 110.9, 104.1, 65.4 ppm; HR ESI-TOF MS: m/z $[M+H]^+$ cald. = 202.0868, $[M+H]^+$ obs. = 202.0729.

2.2.3. Synthesis of allyl *N*-acetylindoline-5-carboxylate (9)

Allyl indole-5 carboxylate (**8**) (201.2 mg, 1 mmol) and acetic acid (2 mL) were stirred at rt for 15 min and then the solution was placed in an ice bath. Sodium cyanoborohydride (157.14 mg, 2.5 mmol) was added and the solution was allowed to stir at 10 ºC. The reaction was quenched after 5 h by addition of 50% sodium hydroxide solution. The solution was extracted with ethyl acetate (3 × 5 mL). The combined organic phases were washed with brine and dried over anhydrous magnesium sulfate. After filtration and concentration, the solid crude product **9** was obtained, R_f 0.52 (EtOAc/hexanes 1:1). Since its purification by column chromatography is challenging, we decided to perform the next step, *i.e.*, the acetylation, on the crude reduction product. Allyl indoline-5-carboxylate (**8**) (crude, 33.5 mmol) was dissolved in anhydrous DCM. Triethylamine (7 mL, 50.25 mmol) and acetic anhydride (31.7 mL, 335 mmol) were added, and the reaction was allowed to stir at rt for 12 h. The reaction was diluted further with DCM followed by an aqueous extraction (water, saturated sodium bicarbonate solution (3x), and brine). The organic phase was dried over anhydrous magnesium sulfate, filtered through celite, and dried. The solid was then purified by column chromatography on silica gel (EtOAc/ hexanes 1:1). 4.35 g of compound **9** (17.74 mmol, 53% yield over 3 steps) were obtained. R_f 0.25 (EtOAc/hexanes 1:1); ^{1}H NMR (600 MHz, 23.4 °C, $CDCl_3$) δ 8.19 (d, *J* = 8.9 Hz,

1H, arom.), 7.89, (d, *J* = 6.9 Hz, 1H, arom.), 7.78 (s, 1H, arom.), 6.02 (m, 1H, CH_2=C<u>H</u>), 5.39 (dd, 1H, C<u>H_2</u>=CH), 5.27 (dd, 1H, C<u>H_2</u>=CH), 4.78 (d, 2H, CH_2-O), 4.04 (t, 2H, *J* = 8.2 Hz, H-2, H-2'), 3.16 (t, *J* = 8.9 Hz, 2H, H-3, H-3'), 2.18 (s, 3H, CH_3) ppm; ^{13}C NMR (600 MHz, 23.4 °C, CDCl3) δ 169.3, 165.9, 146.9, 132.4, 131.5, 130.1, 125.9, 125.0, 118.1, 116.0, 65.4, 49.11, 27.5, 24.3 ppm.

2.2.4. Synthesis of allyl-7-nitro-N-acetylindoline-5-carboxylate (10)

Allyl *N*-acetylindoline-5-carboxylate (**9**) (0.2 g, 0.8 mmol) was dissolved in 2.5 mL trifluoroacetic acid (TFA) and cooled to 0 ºC. Sodium nitrate (0.68 g, 8 mmol) was added in 8 portions over 10 min. The reaction was then brought to rt and allowed to stir for 2.5 h. It was then diluted with DCM and extracted with water, saturated sodium bicarbonate solution (3 ×), and brine. The organic phase was dried over anhydrous magnesium sulfate, filtered through celite, and concentrated. The resulting solid was then purified by column chromatography on silica gel (acetone/hexanes 1:3), yielding 0.164 g of compound **10** in the form of a pale yellow colored solid (71% yield). R_f 0.25 (acetone/hexanes 1:1). ^{1}H NMR (600 MHz, 23.4 °C, $CDCl_3$) δ 8.31 (s, 1H, arom.), 8.05 (s, 1H, arom.), 6.03 (m, 1H, CH_2=C<u>H</u>), 5.42 (dd, 1H, *J* = 4.1 Hz, 19.0 Hz, C<u>H_2</u>=CH), 5.33 (dd, 1H, *J* = 1.4 Hz, 10.2 Hz, C<u>H_2</u>=CH), 4.83 (d, *J* = 6.9 Hz, 2H, C<u>H_2</u>CH=CH_2), 4.31 (t, *J* = 8.2 Hz, 2H, H-2, H-2'), 3.30 (t, 2H, H-3, H-3'), 2.28 (s, 3H, Ac) ppm.

2.2.5. Synthesis of allyl-7-nitroindoline-5-carboxylate (11)

Allyl 7-nitro-*N*-acetylindoline-5-carboxylate (**10**), (0.159 g, 0.548 mmol) in non-anhydrous acetonitrile (20 mL), placed in a round bottom flask inside the Rayonet UV reactor, and illuminated with 350 nm light for 7.5 h under argon and water-cooling to maintain a temperature of approx. 25 ºC. The acetonitrile was then evaporated under reduced pressure, and the resulting crude was purified by column chromatography on silica gel (EtOAc/ hexanes 1:1). Product **11** was obtained in the form of an orange colored solid, 0.088 g (65% yield). R_f 0.35 (acetone/hexanes 1:3). ^{1}H NMR (600 MHz, 23.4 °C, $CDCl_3$) δ 8.57 (s, 1H, arom.), 7.80 (s, 1H, arom.), 7.12 (br s, 1H, NH), 6.03 (m, 1H, CH_2=C<u>H</u>), 5.41 (dd, *J* = 1.4 Hz, *J* = 17.1 Hz, 1H, C<u>H_2</u>=CH, trans), 5.30 (dd, *J* = 1.4 Hz, *J* = 10.3 Hz, 1H, C<u>H_2</u>=CH, cis), 4.79 (d, *J* = 5.5 Hz, 2H, C<u>H_2</u>CH=CH_2), 3.99 (t, *J* = 8.2 Hz, 2H, H-2, H-2'), 3.24 (t, J = 8.9 Hz, 2H,H-3, H-3') ppm.

2.2.6. Synthesis of allyl *N*-(Fmoc-glycyl)-5-carboxylate-7-nitroindoline (12)

5-Allyl carboxylate-7-nitroindoline (**11**) was reacted with Fmoc-Gly-OH in the presence of $SOCl_2$ similar to a reported procedure [8]. Fmoc-Gly-OH (4.00 mmol, 1.189 g) and derivative **11** (2.00 mmol, 0.496 g, 1 equiv.) were suspended in anhydrous toluene (15 mL) under argon and warmed up to 70 °C. $SOCl_2$ (10.00 mmol, 0.73 mL) was added dropwise, and after 60 min a clear solution was obtained. The reaction was monitored by thin layer chromatography (TLC) until near completion (40 h). The solution was diluted with ethyl acetate (200 mL) and washed with a saturated solution of $NaHCO_3$ (3 × 100 mL), water (3 × 100 mL) and brine (2 × 100 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. Product **12** was purified by silica column chromatography (EtOAc/hexanes 2:1). An orange solid was obtained, 0.981 g, 93% yield. R_f = 0.13 (EtOAc/hexanes 1:1). ^{1}H NMR (600 MHz, 296 K, $CDCl_3$) δ 8.34 (s, 1 H, H-6); 8.08 (s, 1 H, H-4); 7.75 (d, 2 H, ^{3}J = 7.5 Hz, Fmoc, arom.); 7.59 (d, 2 H, ^{3}J = 7.5 Hz, Fmoc, arom.); 7.38 (t, 2 H, Fmoc, arom.); 7.30 (t, 2 H, Fmoc, arom.); 6.06-5.99 (m, 1 H, CH_2=C<u>H</u>-); 5.81 (t, 1 H, NH); 5.42 [dd, 1 H, $^3J_{trans}$ = 17.2 Hz, ^{2}J = 1.3 Hz, olef. terminal CH (trans)]; 5.32 [dd, 1 H, ^{3}Jcis = 10.4 Hz, olef. terminal CH (cis)]; 4.84 (d, 2 H, $^3J_{CH2,\ aliph./CH,\ olef.}$ = 5.2 Hz, allyl, aliph. CH_2); 4.37 (d, 2 H, $^3J_{CH/CH2}$ = 6.9 Hz, Fmoc-CH_2); 4.28 (t, 2 H, $^3J_{H\text{-}2/H\text{-}3}$ = 8.1 Hz, H-2, H-2'); 4.22-4.19 (m, 3 H, Fmoc (benzylic CH), H-α, H-α'); 3.29 (t, 2 H, H-3, H-3') ppm. ^{13}C NMR (150 MHz, 296 K, $CDCl_3$) δ 167.3, 163.9, 156.3, 143.7, 141.2, 140.1, 137.5, 136.7, 131.6, 129.4, 127.7, 127.2, 127.1, 125.1, 125.0, 120.0, 119.1, 67.3, 66.3, 49.0, 47.0, 44.3, 28.7. HR-ESI TOF-MS m/z $[M+Na]^+$ calcd. 550.1590, obs. 550.1597; $[M+K]^+$ calcd. 566.1330, obs. 566.1333. λ_{max} (DCM, 37.9 μM) = 258, 267 and 324 nm, ε258 = 19461 M^{-1} cm^{-1}, ε267 = 17445 M^{-1} cm^{-1}, ε324 = 2192 M^{-1} cm^{-1}. λ_{em} (DCM, 75.9 μM, excited at 324 nm) = 390 nm, tailing weakly through the visible.

2.3. Glycoamino acid building blocks

Fmoc-Thr[Tn(Ac)$_3$]-OH, and Fmoc-Thr[STn(Ac)$_6$]-OH (methyl ester) (Figure 1) were synthesized by methods described in the literature [33, 35-38].

2.4. Solid phase peptide synthesis (SPPS) of resin-bound peptide 19 and glycopeptides 20, and 21

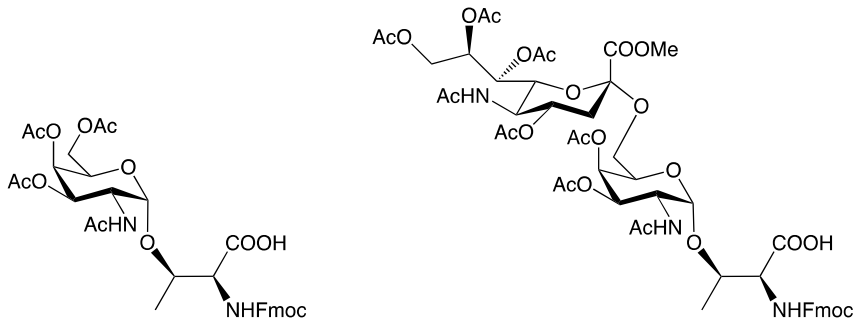
SPPS followed the Fmoc/*t*Bu strategy as described in [39] and was performed semi-automatically using a Tribute peptide synthesizer from Protein Technologies Inc., U.S.A., *i.e.*, each amino acid, coupling reagent, auxiliary nucleophile, and the non-nucleophilic base were dissolved in *N*-methyl-2-pyrrolidone (NMP) and manually added to the resin in a peptide synthesis vessel. Peptides were synthesized on highly acid-labile Sieber amide resin [40] with a loading capacity between 0.65 and 0.69 mmol/g, which allowed for peptide cleavage under mild conditions (1% TFA in DCM) [40]. The fist amino acid (photoreactive compound **6**) was used in only 1.5 molar excess with respect to the number of amines on the resin, and was coupled with 3 equiv. HBTU, 3 equiv. hydroxybenzotriazole (HOBt), and 6 equiv. of *N*,*N*-diisopropylethylamine (DIPEA). The coupling time was 1 h to achieve approximately 50% coupling efficiency to avoid peptide crowding and aggregation on the beads. Unreacted amino groups were capped with 10% acetic anhydride in the presence of 5% DIPEA in NMP. Fmoc group removal was performed with 20% piperidine in NMP. Amino acid couplings were carried out typically with 5 equiv. of the Fmoc or Boc amino acid, and HBTU in the presence of hydroxybenzotriazole (HOBt), and 10 equiv. of DIPEA in NMP as described in [39]. The average coupling time was approximately 45 min, and each coupling was monitored by the bromophenol blue test. Glycoamino acid **13** (2 equiv.) was coupled for 8 h using 2.4 equiv. hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU), 2.4 equiv. 1-hydroxy-7-azabenzotriazole (HOAt), and 5 equiv. *N*-methylmorpholine (NMM) in NMP. Glycoamino acid **14** (1.7 equiv.) was coupled for 30 min at 50 ºC with 2.5 equiv. HOAt, 2.5 equiv. HATU, and 5 equiv. NMM in NMP. After each amino acid coupling, capping was performed with 10% acetic anhydride in the presence of 5% DIPEA in NMP.

2.5. Synthesis of an amine-functionalized biotin

Scheme 4 illustrates the synthesis of the amine-functionalized biotin **18**.

2.5.1. Synthesis of biotin-NH-(CH_2CH_2O)$_2$-CH_2CH_2-NH-Tr (17)

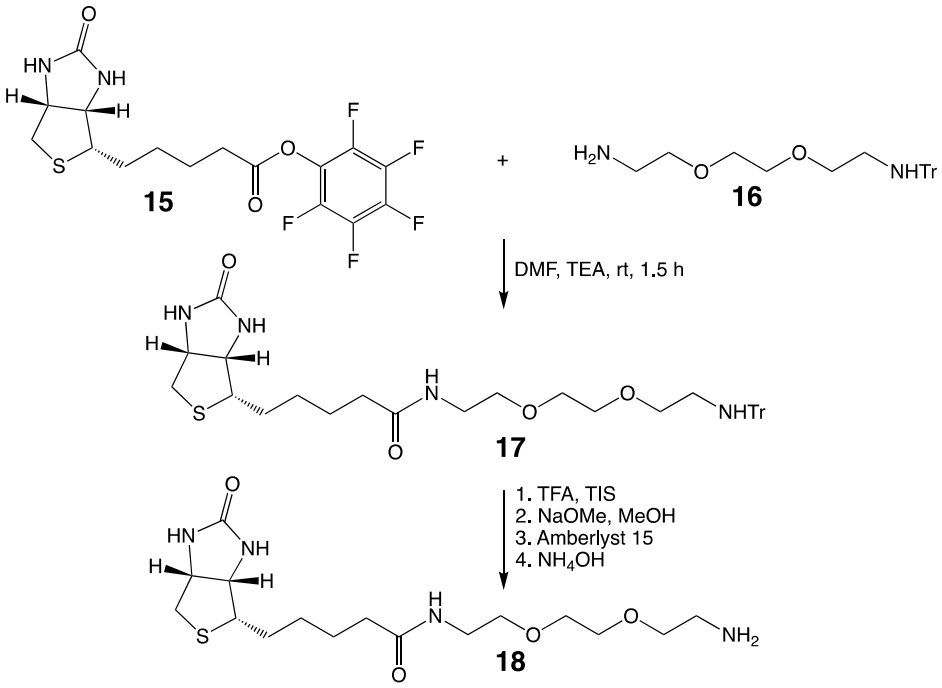
A solution of biotin-pentafluorophenyl ester **15** [41] (384 mgs, 0.936 mmol) in 10 mL anhydrous DMF was added to a solution of *N*-triphenylmethyl-2,2'-(ethylene-dioxy)bis(ethylamine) (**16**) [42], crude, 0.851 mmol, and triethylamine (TEA) (0.13 mL, 0.936 mmol) in 5 mL anhydrous DMF. This mixture was allowed to stir at rt for 90 min. DMF was then co-evaporated with toluene and



13
Fmoc-Thr[**Tn**(Ac)$_3$]-OH

14
Fmoc-Thr[**STn**(Ac)$_6$]-OH (methyl ester)

Figure 1. Glycoamino acid building blocks used for the SPPS of MUC1 glycopeptides.



Scheme 4. Synthesis of an amine-functionalized biotin.

the remaining crude was chromatographed on silica gel (DCM/MeOH 15:1 to 9:1), yielding 424 mgs (0.687 mmol) of product **17**. R_f 0.25 (DCM/MeOH 9:1). HRMS (ESI-TOF): m/z $[M+H]^+$calcd. 617.3162, $[M+H]^+$ obs. 617.1964.

2.5.2. Synthesis of biotin-NH-$(CH_2CH_2O)_2$-CH_2CH_2-NH_2 (18)

TFA (0.8 mL) was added dropwise to a solution of compound **17** (318 mgs, 0.516 mmol) and triisopropylsilane (TIS) (0.127 mL, 0.619 mmol) in 23 mL of anhydrous DCM. The mixture was stirred at rt for 10 min, at which point the acid was neutralized with NaOMe/MeOH solution until the pH was 10. Then 8 g of Amberlyst 15 was added (0.5 g at a time) until compound **18** could no longer be observed on TLC. The reaction mixture was stirred for 2.5 h, and was then filtered, washed with MeOH (3 ×), and the Amberlyst 15 (now containing biotin-ethylene glycol-NH_3^+) was transferred to a larger container. 25 mL of 30 % aq. ammonium hydroxide was added and the reaction mixture was agitated gently for 1 h. Then the reaction mixture was filtered, the Amberlyst 15 resin was washed with water (3 ×), and the combined filtrate was frozen and lyophilized giving 121 mg of a solid (0.324 mmol, 63% yield). R_f 0.25 (DCM/MeOH 1:1). NMR data matched reference [43]. HRMS (ESI-TOF): m/z $[M+H]^+$ calcd. 375.2066, $[M+H]^+$ obs. 375.1178.

2.6. Direct photorelease of peptide acid 22 and glycopeptide acids 23 and 24 from the solid support

Upon completion of the SPPS, the resin containing peptides and glycopeptides was thoroughly washed with NMP and DCM. In a round bottom flask under an argon atmosphere, the beads were suspended in a mixture of 10% water in THF, and placed into a Rayonet photoreactor, where they were irradiated at 350 nm for 15 h under stirring and water cooling (~25 ºC). The peptide or glycopeptide acid (**22**, **23**, or **24**) was then filtered, concentrated, and purified by column chromatography on silica gel.

2.7. Direct photorelease of peptide and glycopeptide amides 25, 26, and 27 from the solid support (biotinylation)

Upon completion of the SPPS, the resin containing peptides and glycopeptides was

thoroughly washed with NMP and DCM, and co-evaporated with anhydrous benzene to remove traces of water. The resin was then placed into a vacuum desiccator for 12 h. The amount of peptide attached was determined based on the weight of the resin. The auxiliary nucleophile *N*-hydroxysuccinimide (HONSu) was kept in a vacuum desiccator under argon. The amine-derivatized biotin **18** was dried via co-evaporation with anhydrous benzene and lyophilized from benzene, and further dried in a vacuum desiccator for 12 h. HONSu (3 equiv.) and **18** (1.2 equiv.) were dissolved in a solvent mixture consisting of 15% DMSO and 85% THF, and transferred to a dried, argon flushed test tube containing the dried resin **19**, **20**, or **21**. Powdered, freshly activated molecular sieves (4Å) were added, the test tube was sealed, and the mixture was stirred for 10 min. Then the test tube was placed into a Rayonet photoreactor where it was irradiated at 350 nm under water cooling (~25 ºC) and stirring for 15 h. Then the reaction mixture was filtered through a cotton plug, the filtrate was concentrated and dried. The crude biotinylated peptide **25**, and biotinylated glycopeptides **26** and **27** were purified by column chromatography on silica gel.

2.8. Cleavage of fully protected, photoreactive *N*-peptidyl- and *N*-glycopeptidyl-7-nitroindolines 28, 29, and 30 from the solid support

Since Sieber amide resin is a hyper-acid labile solid support [40], the cleavage of *N*-peptidyl- and *N*-glycopeptidyl-7-nitroindolines was performed with 1% trifluoroacetic acid (TFA) in DCM exactly as described in [39]. After precipitation of the peptides and glycopeptides, they were subjected to column chromatography on silica gel (DCM/MeOH 9:1 to 6:1). The purity of **28**, **29**, and **30** was confirmed by FPLC, and they were characterized by high resolution ESI-MS.

2.9. Photochemical deprotection of the peptide's and glycopeptides' photoreactive C-termini (28, 29, and 30) in solution to give the peptide and glycopeptide acids 22, 23, and 24

Peptides and glycopeptides **28**, **29**, and **30** were dissolved in THF/Water (9:1) to achieve a concentration of 10 mM. The solutions were irradiated in a Rayonet photoreactor at 350 nm for 4 h. Then the reaction mixtures were concentrated, and the peptide and glycopeptide acids were purified by column chromatography on silica gel.

2.10. Photochemical conversion of 28, 29 and 30 to the peptide and glycopeptide amides 25, 26, and 27 in solution (biotinylation)

Each peptide or glycopeptide (**28**, **29**, or **30**) and the amine-containing biotin derivative **18** were co-evaporated with anhydrous benzene to remove trace amounts of water, followed by lyophilization from benzene. They were further dried in a vacuum desiccator over phosphorus pentoxide for 12 h, along with HONSu. The photoreactive peptide or glycopeptide (1 equiv.), biotin linker (1.2 equiv.) and HONSu (3 equiv.) were transferred to a dried, argon-flushed test tube and dissolved in an anhydrous mixture of THF and DMSO (17:3). Freshly activated, powdered molecular sieves (4Å) were added, the test tube sealed, and the reactants were allowed to stir for 10 min. The test tube was then irradiated with 350 nm light in a Rayonet photoreactor for 4 h under water cooling (~ 25 ºC). The reaction mixture was then filtered, dried, and compounds **25**, **26**, and **27** were purified by column chromatography on silica gel.

2.11. Photochemical conversion of 28, 29 and 30 to the peptide and glycopeptide phenylthioesters 31, 32, and 33 in solution

Each peptide or glycopeptide (**28**, **29**, or **30**) (1 equiv.) and HONSu (2 equiv.) were dried as described above. The dried mixture was transferred to a dried, argon flushed test tube and dissolved in anhydrous DMF to achieve a concentration of 50 mM. Freshly activated, powdered molecular sieves (4Å) was added, the test tube sealed, and the reactants were allowed to stir for 10 minutes. The test tube was then irradiated with 350 nm light in a Rayonet photoreactor for 2 h under water cooling (~ 25 ºC). Then 10 equiv. of thiophenol were added and the mixture was agitated without UV light. After 2 h, the mixture was filtered, and the thioesters **31**, **32**, or **33** were precipitated using cold diethyl ether, then purified by reverse phase FPLC.

2.12. Deprotection of the amino acid side chains and the *N*-terminal Boc group

The deprotection of the *N*-terminal Boc group and the amino acid side chains of compounds **22**-**27**, and **31-33** was accomplished with a solution of 95% TFA/2.5% triisopropylsilane (TIS), and 2.5% water followed by precipitation exactly as described in [39].

2.13. Deprotection of the carbohydrate moieties

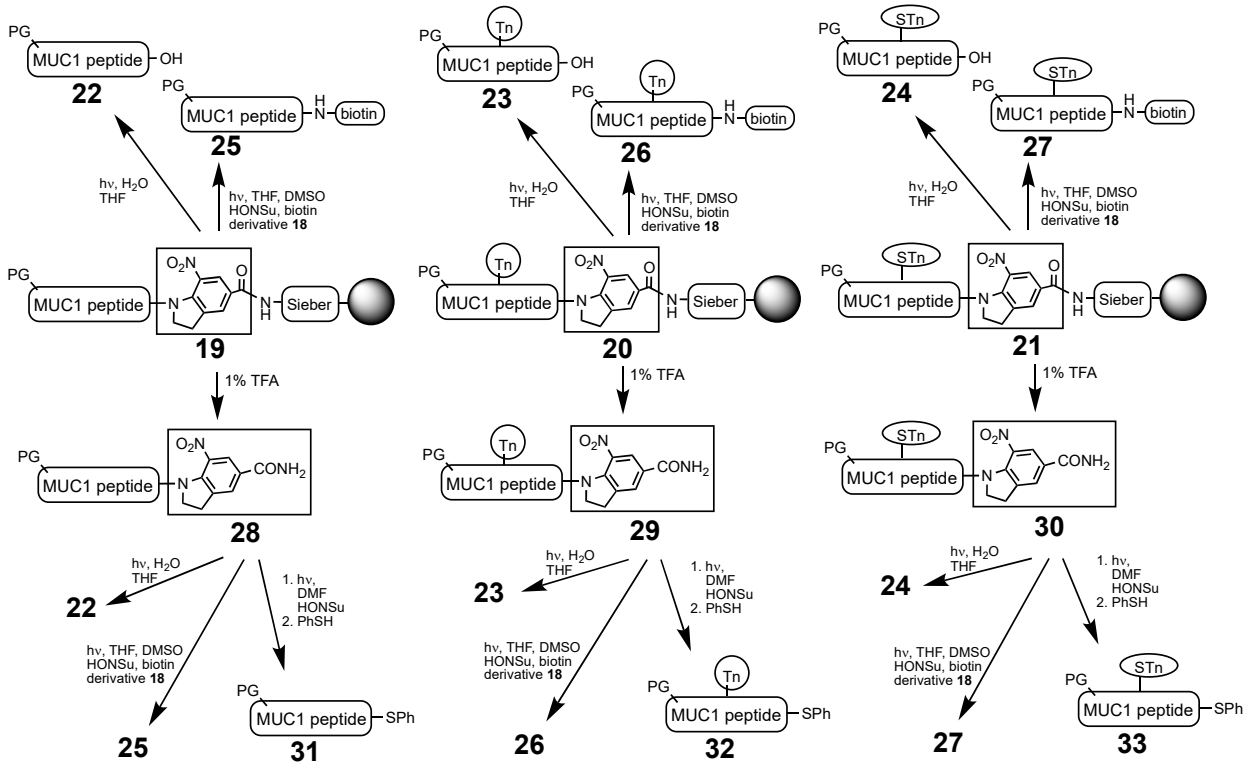
The Tn-containing glycopeptides **35** and **38** were dissolved in methanol (5 mL) and a solution of NaOMe was added until the pH reached 10. This solution was stirred at rt for 40 h, at which point it was neutralized with acetic acid and precipitated with cold diethyl ether. Purification by reverse phase FPLC gave glycopeptides **43** and **44**. The STn-containing glycopeptides **36** and **39** were dissolved in MeOH with 1M NaOH (1% v/v) and stirred at rt for 2.5 h. The mixture was then neutralized with 90 mM aq. AcOH to a pH of 6, and the solvent was evaporated. The glycopeptides were then dissolved in 5 mM aq. NaOH and stirred at rt for 3 h 45 min. The solution was again neutralized to a pH of 6 with 90 mM aq. AcOH. The solution was then frozen and lyophilized. The glycopeptides were purified by reverse phase FPLC, resulting in glycopeptides **45** and **46**.

3. RESULTS AND DISCUSSION

A MUC1-derived peptide, and two *O*-glycopeptides with the glyco antigens Tn and STn were synthesized by SPPS applying the Fmoc/*t*Bu strategy [39] on a dual functional resin that contained both the acid-labile Sieber amide linker, as well as the photoreactive *N*-acyl-7-nitroindoline linker [14, 27], see constructs **19**, **20**, and **21** in Scheme 5. The photoreactive linker was introduced by coupling the Fmoc-protected amino acid **6** to commercial Sieber amide resin [40], as shown in Scheme 2. The carbohydrate moieties Tn and STn were introduced via the Fmoc-glycosyl threonine derivatives **13** and **14** (Figure 1). When constructs **19**, **20**, and **21** were agitated under near UV light (350 nm) irradiation for many hours in THF/water (9:1), the peptide **22** and the glycopeptides **23** and **24** with free C-termini were directly photoreleased from the solid support, most likely by mixed photolysis mechanisms corresponding to Paths A and B shown in Scheme 1. When the photolysis occurred under inert, aprotic conditions (THF/DMSO) in the presence of amine-derivatized biotin **18** and HONSu, a transamidation occurred resulting in the biotinylated peptide amides **25**, **26**, and **27**. The auxiliary nucleophile HOSu was added as a catalyst. In addition, it allowed for the photochemical formation of an intermediate activated ONSu ester, which is relatively stable toward nucleophilic attack by water but reacts readily with primary amines.

Instead of directly photoreleasing peptides from the resin, we have also cleaved the peptides and glycopeptides under dilute acidic conditions, taking advantage of the hyper-acid-labile Sieber amide linker [40]. This produced fully protected peptide **28** and glycopeptides **29** and **30** as photoreactive *N*-peptidyl-7-nitroindolines. After purification, these compounds were suitable for photochemical derivatizations in solution. When **28**, **29**, and **30** were irradiated at 350 nm in water and THF for a few hours, the corresponding peptide and glycopeptide acids **22**, **23**, and **24** were obtained.

We also investigated the ability of peptides and glycopeptides **28**, **29**, and **30** to undergo a phototransamidation in solution, a type of reaction first reported on simple *N*-peptidyl-7-nitroindolines [8]. Irradiating compounds **28**, **29**, and **30** at 350 nm in the presence of biotin derivative **18** and HONSu in THF and DMSO resulted in the peptide and glycopeptide amides **25**, **26**, and **27**. Lastly, we explored if this photochemical acylation method could be used to synthesize glycopeptide thioesters, which are more challenging to synthesize than peptide thioesters by conventional methods due to the acid sensitivity of the glycosidic linkages. Therefore, a method that can install C-terminal thioesters on glycopeptides under neutral conditions is attractive. Our previous work on peptide thioesters showed that the desirable phenyl thioesters cannot be installed directly by photoactivation of *N*-peptidyl-7-nitroindolines in the presence of thiophenol. Instead, we developed a one-pot-two-reaction procedure, in which an auxiliary nucleophile reacted with the photoactivated *N*-peptidyl-7-nitroindoline to form a peptide activated ester.



Scheme 5. Overview of all photochemical reactions performed on (glyco)peptides directly on the solid support, or in solution. For simplification, protecting group (PG) and linker details were omitted.

Thiophenol was then added in the absence of UV light, and the desired phenyl thioester formed efficiently [28]. When **28**, **29**, and **30** were irradiated at 350 nm in DMF in the presence of HONSu, followed by addition of thiophenol, the protected peptide and glycopeptide thioesters **31**, **32**, and **33** were obtained, but thioester formation failed or was inefficient when we attempted to perform a light-induced one-pot-two-reaction sequence directly from the solid support. Since these attempts produced significant quantities of the free acids **22**, **23**, and **24**, one reason could be that a significant amount of water was present. Another potential reason could be that the reaction Path B took place (Scheme 1), possibly due to acidic conditions [25].

Table 1 summarizes the reaction yields of the various photochemically synthesized peptide and glycopeptide derivatives (**22**-**27**, **40**-**42**). In case of the peptide/glycopeptide acids and peptide/ glycopeptide amides, the direct photoreleases from the solid support could be compared with the photochemical derivatizations in solution. The reaction yields of the direct photoreleases refer to the amount of loading of the first amino acid (compound **6**), via spectrophotometric quantification of dibenzofulvene and its piperidine adduct upon removal of this amino acid's Fmoc group [39]. Thus, the yield reported for compounds **22**-**27** by direct photorelease refers to the overall yield that includes all the steps of SPPS. In contrast, the yields reported for compounds **22**-**27**, where the photochemical conversion was performed in solution, refer to only one reaction. In case of phenyl thioesters **40**-**42**, the reported yields refer to the thioester formation by a one pot-two reaction sequence since the activated ONSu ester was not isolated, and the deprotection of the amino acid protecting groups with TFA. In general, all peptide and glycopeptide acid yields are very high and mostly comparable by both methods, indicating that all peptide coupling, Fmoc removal, and photorelease/ photodeprotection steps were very efficient. In case of the peptide and glycopeptide amides **25**-**27**, the phototransamidations in solution were high-yielding (Table 1), and the phototransamidation

Table 1. Reaction yields of chromatographed peptide/glycopeptide derivatives after photochemical modifications performed on the solid support, and in solution.

***C*-terminal modification**	**Compound # (with Thr side chain specification)**	**Yield after the direct photorelease from the solid support**	**Yield after the peptide derivatization step in solution**
None (free acid)	**22** (*t*Bu)	87%	73%
	23 (Tn)	92%	90%
	24 (STn)	84%	92%
Amide (with biotin attached)	**25** (*t*Bu)	64%	80%
	26 (Tn)	67%	78%
	27 (STn)	76%	79%
Phenyl Thioester	**40** (OH)*	-	77%*
	41 (Tn)*	-	84%*
	42 (STn)*	-	79%*

*All amino acid side chain protecting groups and the *N*-terminal Boc group had been removed with TFA.

yields directly from the solid support are somewhat lower, possibly due to some hydrolysis byproducts.

After the photochemical reactions, and purifications, the resulting peptide and glycopeptide derivatives were subjected to TFA treatment to remove all acid-labile protecting groups. Figure 2 lists the peptides and glycopeptides whose *N*-termini and amino acid side chains had been deprotected (**34**-**46**). The compounds were purified by FPLC and characterized by mass spectrometry. Four of the side chain deprotected glycopeptide derivatives (**35**, **36**, **38**, and **39**) were subjected to deprotection of the carbohydrate moieties. Deacetylation of glycopeptides **35** and **38** gave the completely deprotected glycopeptides **43** and **44**, respectively. Hydrolysis of the methyl ester and simultaneous deacetylation of the protected STn moieties in **36** and **39** produced the completely deprotected glycopeptides **45** and **46**, respectively. The deprotection of the NeuNAc methyl ester in *O*-glycopeptides is challenging due to its stability, and the possible β-elimination of the entire *O*-glycan under basic conditions. Table 2 shows the mass spectrometric data for derivatives **22**-**46.**

O-Glycopeptide synthesis poses significant challenges due to their susceptibility to both acid and base sensitivity, stemming from the presence of glycosidic linkages and potential glycan loss through β-elimination. Hence, methodologies facilitating chemical manipulations under neutral reaction conditions are invaluable in glycopeptide synthesis. Notably, the *N*-acyl-7-nitroindoline group emerged as a promising photoreactive entity. It served dual functions as an orthogonal protecting group and as an activator of carboxylic acids for nucleophilic attack under near UV light irradiation [8]. This versatility rendered *N*-acyl-7-nitroindolines useful linkers in SPPS of glycopeptides.

However, glycopeptide thioesters present a challenge as they are incompatible with both the Boc/Bn and conventional Fmoc/tBu SPPS strategies. Although here we have demonstrated elegant photorelease chemistry for glycopeptide acids and amides, the direct photorelease of glycopeptide thioesters failed. Nevertheless, by incorporating an additional step - cleaving the *N*-glycopeptidyl-7-nitroindoline from the solid support - it became feasible to achieve high-yielding photothioesterification in solution. This

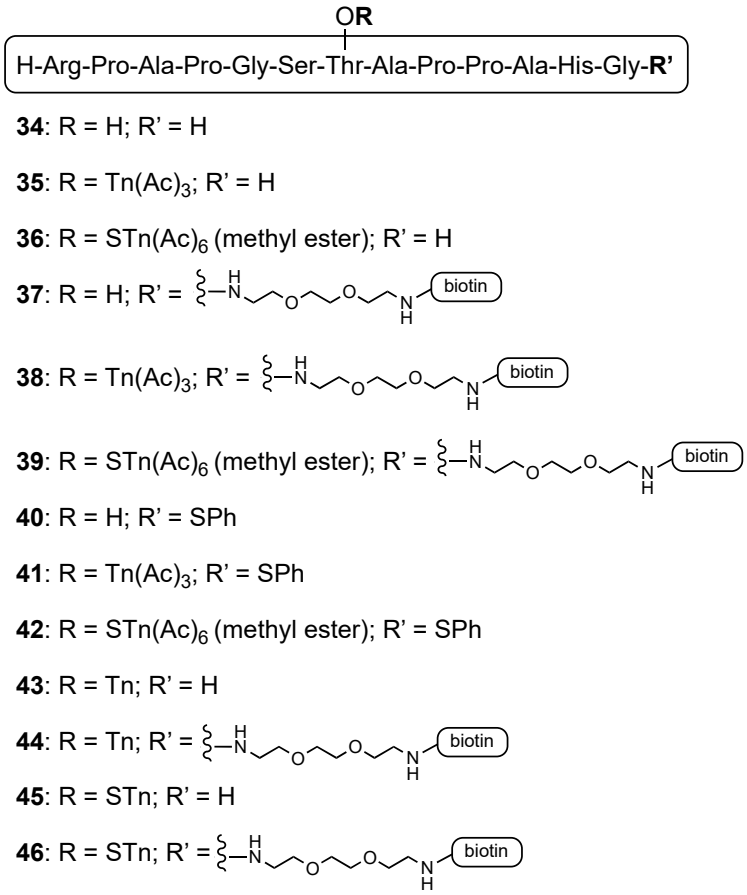


Figure 2. Partially and fully deprotected peptide and glycopeptide derivatives.

Table 2. Mass spectrometric characterization of peptide and glycopeptide derivatives.

Compound	*m/z* $[M+H]^+_{calcd.}$	*m/z* $[M+H]^+_{obs.}$
22	1922.9960	1922.9785
23	2196.0445	2196.0463
24	2627.1872	2627.2588
25	2279.1842	2279.2160
26	2552.2327	2552.3072
27	1492.1916*	1492.1912*
28	2112.0498	2112.0489
29	2385.0983	2385.0644
30	2817.2444	2817.1709
31	1307.6319	1307.6332
32	1636.7430	1636.7344

Table 2 continued..

Compound	*m/z* $[M+H]^+_{calcd.}$	*m/z* $[M+H]^+_{obs.}$
33	2068.8891	2068.8662
34	1215.6234	1215.6190
35	1544.7345	1544.7241
36	1976.8806	1976.8564
37	1571.8117	1571.8037
38	1901.9261	1901.9340
39	2333.0688	2333.0372
40	1307.6332	1307.6319
41	1636.7344	1636.7430
42	2068.8891	2068.8662
43	709.8553*	709.8558*
44	887.9494*	887.9442*
45	855.4030*	855.3970*
46	1033.4973*	1033.5018*

* m/z value of $[M+2H]^{2+}$ species.

approach facilitated the production of glycopeptide thioesters suitable for NCL applications.

4. CONCLUSIONS

This study has significantly broadened the application of the *N*-acyl-7-nitroindoline linker within the Fmoc/*t*Bu strategy of SPPS to encompass *O*-glycopeptides. These glycopeptides could be directly and highly efficiently photoreleased from the solid support, yielding protected glycopeptide acids primed for subsequent C-terminal derivatization in solution. Moreover, our investigation revealed an additional capability: the C-terminus of the glycopeptide could undergo direct derivatization during the photorelease process when light irradiation of the beads occurred in the presence of a nucleophilic primary amine and HONSu. This led to phototransamidation, releasing a glycopeptide amide into the solution. Furthermore, we have successfully achieved glycopeptide thioesterification under light activation using a one-pot-two-reaction strategy in solution. Overall, this research demonstrated the remarkable versatility of the photoactive nitroindoline linker as a potent tool for peptide and glycopeptide C-terminal modifications, whether on solid support or in solution. Its ability to react with various nucleophiles in different solvents enabled the generation of diverse functional groups under neutral reaction conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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