Virtually epimerization-free synthesis of peptide-α-thioesters†

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Under slightly basic or neutral reaction conditions peptide-α-thioesters are photochemically synthesized from peptide-α-nitroindoline precursors, either in solution, or by direct photorelease from a solid support.

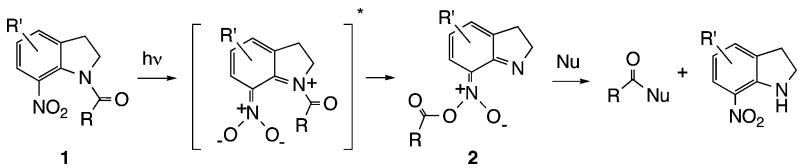
In the past decades solid phase peptide synthesis (SPPS) has significantly advanced due to the development and commercial availability of numerous different linkers on a variety of solid supports, modern coupling reagents, and orthogonally protected amino acids.[1] Yet, one major limitation of SPPS is that it fails in the synthesis of large peptides and proteins. Recently, a novel technique called native chemical ligation (NCL) has emerged that allows for the total chemical synthesis of moderately sized proteins.[2] A peptide with a C-terminal thioester is condensed with a second peptide that has an N-terminal cysteine. The two building blocks undergo *trans*-thioesterification in an aqueous buffer, under denaturing conditions, and the resulting thioester rapidly rearranges to produce a native peptide bond. Peptide-α-thioesters can be easily prepared by SPPS using the Boc protecting group strategy,[3] but recently, alternative methods that do not require acidic deprotection and cleavage with hydrofluoric acid have been reported. By developing methods that made the synthesis of peptide-α-thioesters compatible with Fmoc chemistry,[4] and by developing cysteine-free techniques,[5] NCL could be extended to the generation of large glycopeptides.

While the NCL steps are usually high yielding, overall protein yields often suffer from low yielding peptide-α-thioester syntheses. Although these Fmoc-chemistry compatible preparations have avoided harsh reaction conditions, and allowed for the generation of large peptides and glycopeptides by NCL, their use is often limited due to a) the occurrence of unacceptable levels of C-terminal epimerization; b) hydrolysis of the activated carboxylic acid or thioester under basic conditions; c) or by the necessity of post-chain assembly manipulations prior to thioesterification. In light of the potential impact of NCL on the study of proteomics and glycomics, there is still a great demand for a universal and efficient synthesis of peptide-α-thioesters.

Here we report on a convenient synthesis of peptide-α-thioesters by an unconventional photochemical thioesterification under slightly basic or neutral conditions. Approximately 30 years ago, it was discovered that *N*-acyl-7-nitroindolines undergo photosolvolysis upon illumination, which results in the acylation of a nucleophilic solvent.[6] Mechanistic studies suggest that *N*-acyl-7-nitroindolines (**1**) are latent until activation occurs upon illumination with near UV-light.[7] The resulting nitronic anhydrides (**2**) can serve as powerful acylating agents in inert organic solvents (Scheme 1).[8] For example, we have demonstrated that nitroindoline derivatives of aspartic acid and glutamic acid side chains can acylate glycosylamines forming *N*-glycosylamino acids by phototransamidation.[9] The scope of this chemistry was subsequently extended to the convergent photochemical synthesis of *N*-glycopeptides.[10]

Recently, an *N*-acylated nitroindoline linker attached to aminomethyl polystyrene beads has proven effective in the photochemical release of amides and lactams.[11] We envisioned that attaching a nitroindoline to Rink amide resin would generate a solid support with two orthogonal linkers suitable for SPPS providing chemical flexibility for cleaving the peptides from the solid support. Since the C-terminal amino acid would be attached to the nitroindoline linker *via* an amide bond, peptides could be synthesized by standard Fmoc strategy. Cleavage of the mature peptide with 95% TFA at the Rink linker would afford photoreactive peptide amides, which can subsequently be converted into thioesters in solution. Alternatively, illumination of the beads in the presence of a mercaptan would photorelease peptide-α-thioesters directly. We hypothesized that the proposed photochemical method would generate diastereomerically pure peptide-α-thioesters due to neutral reaction conditions. Thus the problem of base-catalyzed epimerization of the C-terminus would be eliminated.

Polystyrene beads equipped with a Rink linker are commercially available, but 7-nitroindoline building blocks suitable for attachment to a resin are not. Our initial idea was to couple the first



Scheme 1 Photoacylation of nucleophiles in inert solvents.

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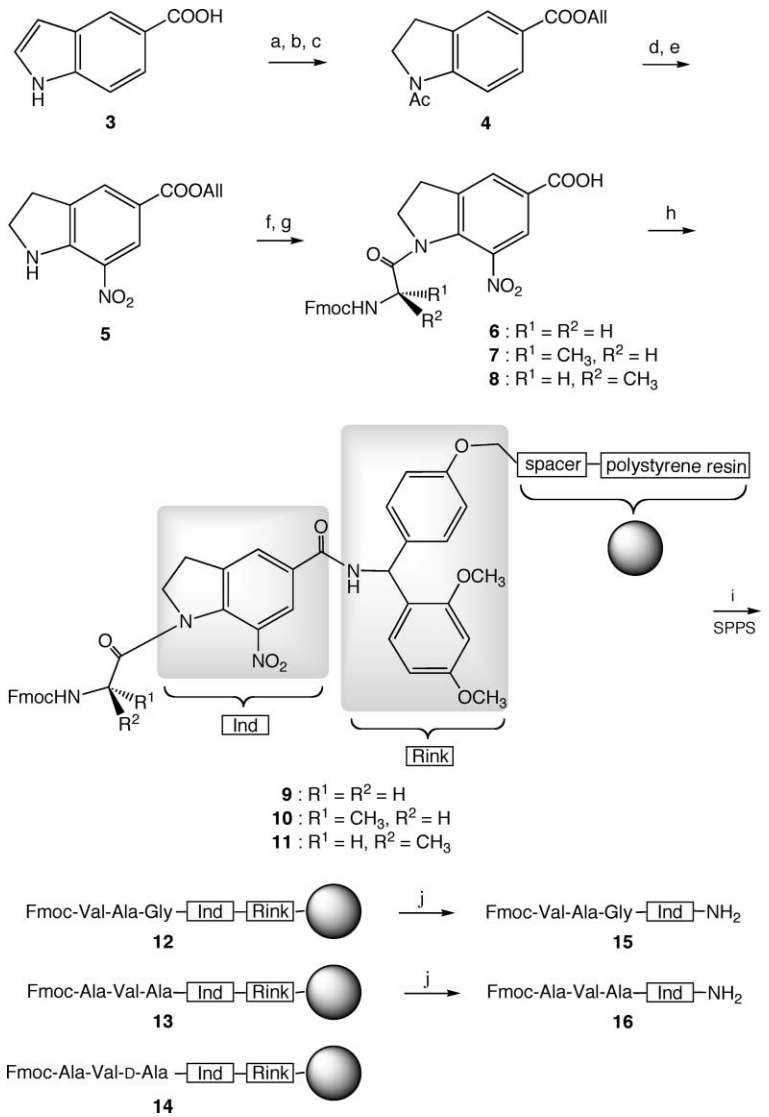
Fmoc amino acid on to a nitroindoline derivatized Rink Amide resin. While 5-carboxylic acid 7-nitroindoline could be condensed with Rink amide resin, coupling of Fmoc-amino acids to this nitroindoline derivatized resin failed.

The strategy was revised to first condensing an Fmoc amino acid with a nitroindoline derivative, followed by deprotection and coupling to Rink amide resin (Scheme 2). Starting from commercially available 5-carboxylic acid indole (**3**), the universal 7-nitroindoline building block **5** was synthesized following a published route.[11] We modified the chemistry and reaction sequence to some extent to make it compatible with Fmoc based peptide chemistry. An important change was the conversion of **3** into an orthogonal allyl ester, followed by double bond reduction with a small excess of $NaCNBH_3$. Acetylation afforded the *N*-acetylindoline derivative **4**, which was then nitrated and deacetylated by acid catalyzed hydrolysis at pH 1. The obtained allyl protected nitroindoline **5** was condensed either with Fmoc-Gly-OH, Fmoc-Ala-OH, or Fmoc-D-Ala-OH in the presence of thionyl chloride followed by Pd(0)-catalyzed deallylation using *N*-methylaniline,[12] to give the photoreactive amino acid building blocks **6–8**. These adducts were then attached to commercially available Rink amide resin with HBTU, HOBt and DIPEA to furnish **9–11**. The photoreactive model peptides **12–14** were constructed by standard SPPS. Cleavage of the photoreactive peptides at the Rink linker allows for their spectroscopic analysis and characterization. Thus, treatment with 95% TFA released peptides **15** and **16** from the resin, each bearing a C-terminal nitroindoline moiety.

Initial attempts to photorelease the ethyl thioesters **17–19** by illumination of the beads with UV-light in the presence of ethylmercaptan–dichloromethane (1 : 6) failed, as the C-terminal peptide acids were obtained almost exclusively. Similar results were observed by others when alcohol nucleophiles were used in the



Scheme 2 Synthesis of Fmoc–amino acid–nitroindolines and their application in the synthesis of photoreactive model peptides. a) i. Cs_2CO_3, DMF; ii. AllBr (91%, crude); b) $NaCNBH_3$, AcOH; c) Ac_2O (76%, two steps); d) $NaNO_3$, TFA (quantitative); e) HCl, H_2O, MeOH, pH 1 (70%); f) Fmoc-Gly-OH, Fmoc-Ala-OH or Fmoc-D-Ala-OH, $SOCl_2$, toluene, 70 °C (61–76%); g) $Pd(PPh_3)_4$, *N*-methylaniline, THF (66–76%); h) Rink amide resin, HBTU, HOBt, DIPEA, NMP (quantitative); i) deprotection of Fmoc: 20% piperidine in NMP; Fmoc–amino acid coupling: HBTU, HOBt, DIPEA, NMP; j) 95% TFA, 2.5% water, 2.5% triisopropylsilane [70% (**15**); 84% (**16**)].

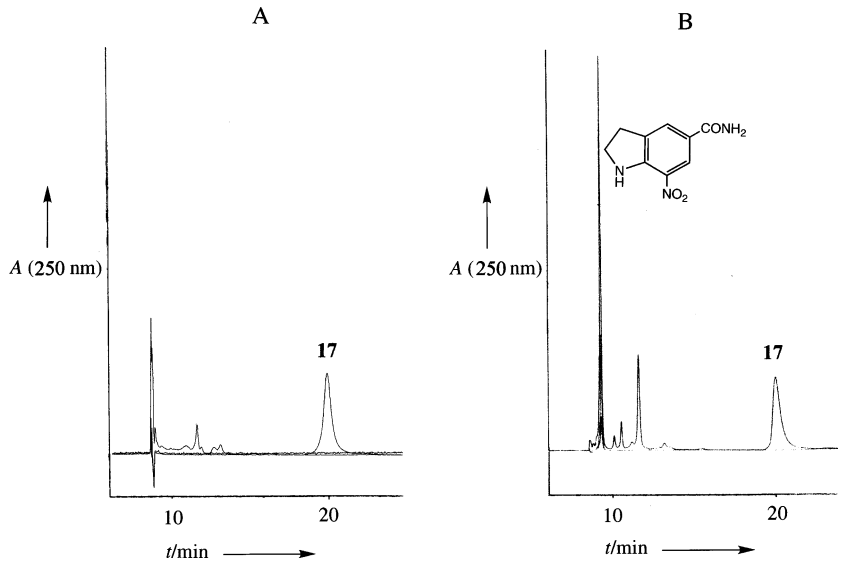
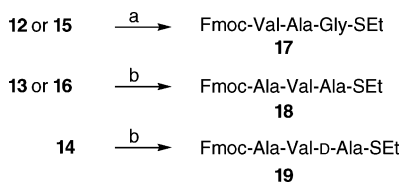


Fig. 1 RP HPLC profile of crude peptide thioester **17**, A) by direct photorelease from **12**, and B) by photoesterification of **15** in solution. A byproduct, Fmoc-Val-Ala-Gly-OH, elutes at 11.5 min.

attempt to generate esters by this photoacylation method.[11,13] We found, however, that by conducting the photoacylation of peptide **12** or **15** in pyridine instead of dichloromethane, the peptide-α-thioester **17** was produced in good yield (Scheme 3, Fig. 1).[14] A small amount of the peptide acid is usually also present. It originates mostly from base catalyzed hydrolysis of thioester **17** upon prolonged exposure to pyridine under anhydrous conditions.



Scheme 3 Photothioesterifications. a) pyridine–ethyl mercaptan 5 : 1, molecular sieves (3 Å), argon, UV light, 30–40 °C, 24 h, yield 70–89%; b) TMU–ethyl mercaptan 5 : 1, HOBt, molecular sieves (3 Å), argon, UV light, 30–40 °C, 19 h, yield 62% based on RP HPLC.

In order to investigate the potential occurrence of epimerization, two tripeptides, **13** and **14**, which differ only in their C-terminal configuration, were prepared (Scheme 2). Photothioesterification in pyridine gave 86% yield, but resulted in partial epimerization (~11%). We found that epimerization could be largely suppressed by conducting the photothioesterification in a non-basic solvent in the presence of HOBt. Although substantial amounts of the undesired peptide acids formed when DMSO was used as a solvent, these reaction mixtures proved useful for studying epimerization on two levels. Fig. 2 illustrates that L-configured **13** reacts to the L-configured peptide acid, and the L-configured thioester **18**. Likewise, D-configured **14** reacts to the D-configured peptide acid and the D-configured thioester **19**. The epimerization levels are less than 5%.

A better solvent than DMSO is *N*,*N*,*N*′,*N*′-tetramethylurea (TMU). Photoreactive peptide **16** underwent thioesterification in TMU, ethyl mercaptan, and HOBt, to afford ethyl thioester **18** in 62% yield with 4% epimerization.[15]

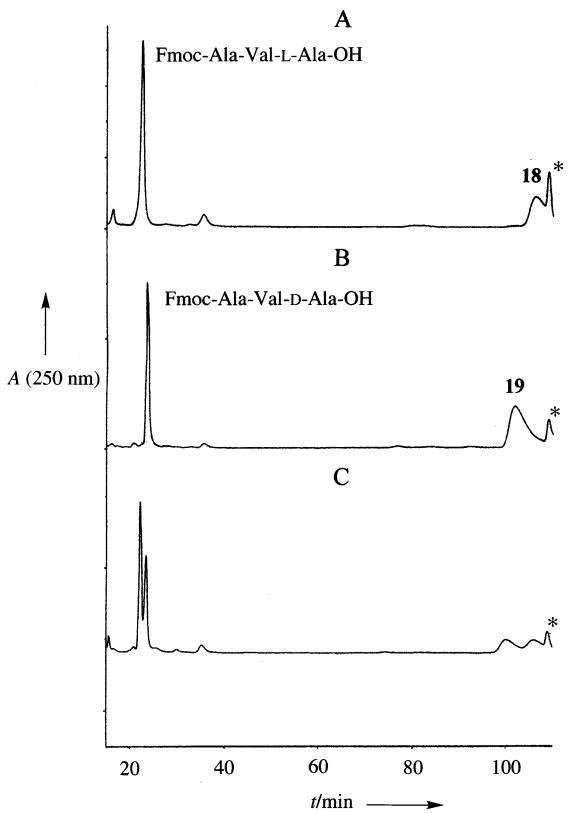


Fig. 2 RP HPLC profiles of crude photothioesterifications in DMSO. A) photorelease of **18** from **13**; B) photorelease of **19** from **14**; C) coinjection of both reaction mixtures. The asterisk depicts material eluting at the beginning of the column wash with 85% acetonitrile.

In summary, Fmoc chemistry based SPPS was applied on Rink amide resin with an additional photoreactive nitroindoline linker. In the presence of ethyl mercaptan, UV light photoreleases peptide-α-thioesters. Alternatively, TFA treatment cleaves off the peptide with an intact photoreactive moiety at the C-terminus,

allowing for analysis and photochemical thioesterification in solution. Neutral reaction conditions keep C-terminal epimerization to a minimum. The direct photorelease from the resin might be particularly useful for the preparation of highly acid-sensitive glycopeptide thioesters.

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14 In the presence of molecular sieves (3 Å) compound **15** (9 mg, 13.7 μmol) and 0.6 mL pyridine–EtSH (5 : 1) were illuminated with $\lambda >$ 310 nm) under argon at 33 °C. After, 20 h RP HPLC indicated the consumption of **15** and the formation of 75% of **17**. The crude was dried in vacuum, dissolved in $CHCl_3$–MeOH 9 : 1, and filtered. The crude was then chromatographed on silica with ethyl acetate–hexanes 1 : 1, R_f 0.22, yield 5 mg, 72%. ^{1}H NMR (DMSO-d_6, 298 K, 500 MHz) δ 8.53 (m, 1H, NH-Gly); 8.04 (d, 2H, NH-Ala, $^3J_{NH,\alpha}$ 7.4); 8.10 (d, 1H, NH-Ala, $^3J_{NH,\alpha}$ 8.3 Hz); 7.88 (d, 2H, Fmoc, ^3J 7.7 Hz); 7.23 (t, 2H, Fmoc); 7.43–7.38 (m, 3H, NH-Val, Fmoc); 7.31 (t, 2H, Fmoc); 4.36 (m, 1H, Hα-Ala); 4.32–4.18 [m, 3H, CH, CH_2 (Fmoc)]; 4.04 (dd, 1H, Hα-Gly, $^2J_{\alpha,\alpha'}$ 17.5 Hz, $^3J_{\alpha,NH}$ 6.4 Hz), 3.93–3.86 (m, 2H, Hα′-Gly, Hα-Val), 2.78 [q, 2H, CH_2 (Et), ^3J 7.4 Hz]; 1.98 (m, 1H, Hβ-Val); 1.26 (d, 3H, 3 × Hβ-Ala); 1.12 [t, 3H, CH_3 (Et)]; 0.85 (d, 3H, 3 × Hγ-Val, $^3J_{\beta,\gamma}$ 6.7 Hz); 0.83 (d, 3H, 3 × H-γ′-Val, $^3J_{\beta,\gamma}$ 7.1 Hz) ppm; ^{13}C NMR (DMSO-d_6, 298 K, 126 MHz) δ 198.4; 172.7; 170.8; 156.1; 143.9; 143.8; 140.7; 127.6; 127.0; 125.3; 120.1; 65.6; 59.9; 48.7; 47.9; 46.7; 30.3; 29.0; 22.1; 19.2; 18.1; 14.6 ppm. FAB MS: m/z [M + H]$^+$ 512.3, [M + Li]$^+$ 518.3, [M + Na]$^+$ 534.3.

15 0.6 mL of TMU–EtSH (5 : 1) were added to an NMR-tube, which contained a mixture of **16** (10 mg, 0.015 mmol) and freshly activated molecular sieves (3 Å) under argon. The sample was illuminated with $\lambda >$ 310 for 19 h at 40 °C. The, crude reaction mixture was filtered and purified by RP-HPLC, which gave 3 mg, 43% of **18**. ^{1}H NMR (DMSO-d_6, 298 K, 500 MHz) δ 8.57 (d, 1H, NH-Ala-2, $^3J_{NH,\alpha}$ 7.1 Hz); 7.88 (d, 2H, Fmoc, ^3J 7.4 Hz); 7.70 (m, 2H, Fmoc); 7.64 (d, 1H, NH-Val, $^3J_{NH,\alpha}$ 9.4 Hz); 7.55 (d, 1H, NH-Ala-1, $^3J_{NH,\alpha}$ 8.1 Hz); 7.41 (t, 2H, Fmoc, ^3J 7.4 Hz); 7.32 (t, 2H, Fmoc); 4.36 (m, H-α-Ala-2); 4.31–4.18 (m, 4H, H-α-Val, CH-Fmoc, CH_2–Fmoc); 4.13 (m, 1H, H-α-Ala-1); 2.76 (q, 2H, CH_2-SEt, ^3J 7.4 Hz); 2.06 (m, 1H, H-β-Val); 1.25 (d, 3H, 3 × H-β-Ala-2, $^3J_{\alpha,\beta}$ 7.1 Hz); 1.20 (d, 3H, 3 × H-β-Ala-1, $^3J_{\alpha,\beta}$ 7.1 Hz); 1.12 (t, 3H, CH_3–SEt); 0.87 (d, 3H, 3 × H-γ-Val, $^3J_{\alpha,\beta}$ 6.7 Hz); 0.84 (d, 3H, 3 × H-γ′-Val, $^3J_{\alpha,\beta}$ 6.7 Hz) ppm; ^{13}C NMR (DMSO-d_6, 298 K, 126 MHz) δ 201.5; 191.8; 177.8; 171.0; 159.8; 155.6; 147.3; 144.9; 143.9; 140.7; 131.2; 127.6; 127.1; 125.2; 120.1; 65.6; 56.8; 54.7; 50.0; 46.6; 38.2; 30.8; 22.3; 19.4; 18.2; 17.5; 17.4; 14.6 ppm. FAB MS: m/z [M + H]$^+$ 526.2, [M + Li]$^+$ 532.2, [M + Na]$^+$ 548.2.

