Amide Activation

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Fmoc Solid-Phase Synthesis of C-Terminal Peptide Thioesters by Formation of a Backbone Pyroglutamyl Imide Moiety**

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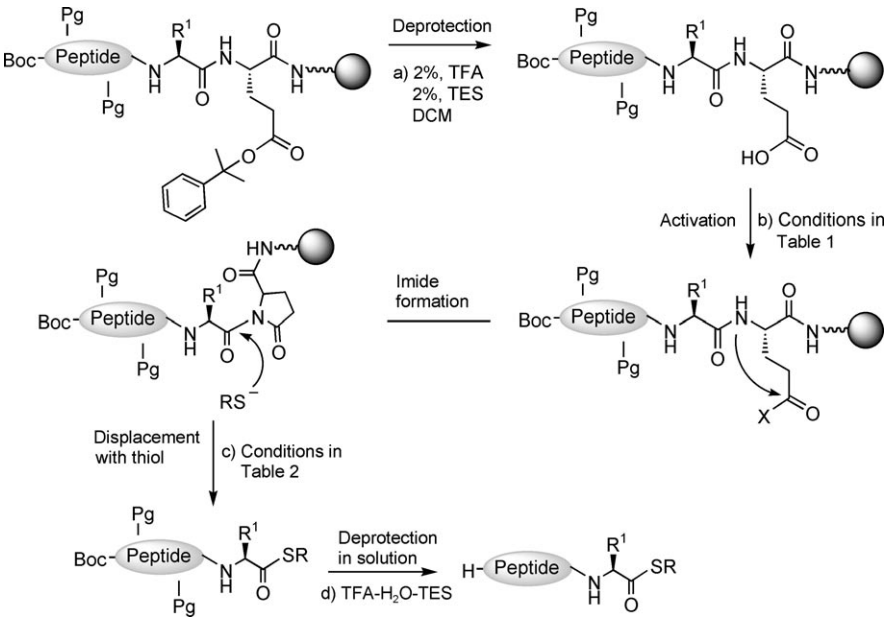
The use of C-terminal peptide thioesters in synthetic protein chemistry has inspired the search for optimal solid-phase peptide synthesis (SPPS) strategies for protein assembly.[1,2] However, peptide thioesters are not directly accessible by Fmoc-SPPS (Fmoc = 9-fluorenylmethoxycarbonyl) owing to the nucleophilicity of the secondary amine required for Fmoc removal. Peptide thioesters can be accessed by Boc-SPPS (Boc = *tert*-butoxycarbonyl), however, the conditions are incompatible with many posttranslational modifications.[3] The development of methods for Fmoc-SPPS of peptide thioesters has therefore been a major challenge over the past decade, and several approaches have been reported,[2] for example, Kenner's safety-catch linker system,[4] as well as other methods.[5–7] Very recently, thiolysis of peptides with a C-terminal peptide N-acylurea unit was described.[8] Peptide thioesters with a C-terminal achiral glycine unit can be conveniently synthesized.[9] However, new, more general methods for such peptides are also of interest.

We reasoned that the activation of a peptide backbone amide bond by increasing its nucleofugality could render the C–N bond susceptible to thiolysis and provide peptide thioesters. Cleavage of the C–N bonds in a backbone amide to provide a thioester can occur biologically in intein-mediated protein splicing.[10]

Herein, we describe an unprecedented method for the activation of a backbone amide in a peptide by formation of a backbone pyroglutamyl imide, which, after displacement by a thiol, provides the peptide thioester (Scheme 1). General Fmoc-SPPS protocols were applied for the synthesis of peptides prior to activation.



Scheme 1. The strategy for the synthesis of peptide thioesters through a backbone amide activation. Pg = protecting group, TES = triethylsilane, TFA = trifluoroacetic acid, R^1 = amino acid side-chain.

The synthesis of C-terminal peptide thioesters by this strategy entails anchoring of a C-terminal glutamic acid residue with a selectively removable side-chain protecting group to a solid support (Scheme 1). Upon peptide chain assembly, the glutamic acid side-chain would be selectively deprotected. In the first key step, strong activation of the deprotected carboxylic acid should result in the on-resin formation of the pyroglutamyl (pGlu) imide moiety. The novel formation of the backbone pGlu imide involves the acylation of an amide nitrogen atom, which is a very poor nucleophile, although the reaction is likely to be entropically favored. In the second key step, nucleophilic displacement by treatment with a thiol was expected to release the protected peptide thioester from the solid support, which then would be deprotected in solution.

To implement this strategy for the synthesis of thioesters, a heptamer peptide related to the enkephalins was assembled on-resin to provide the Boc-FY(*t*Bu)GGFAE(Ph*i*Pr)-Rink-

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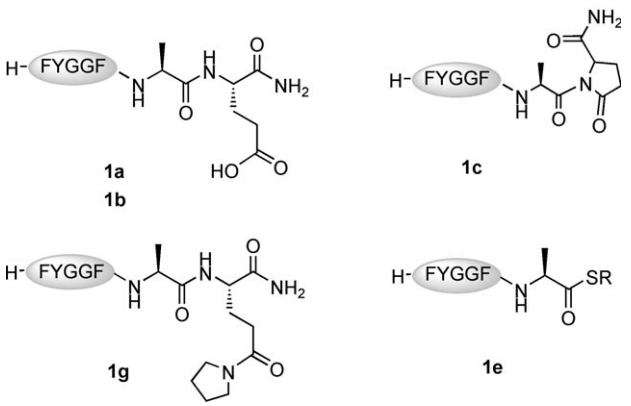


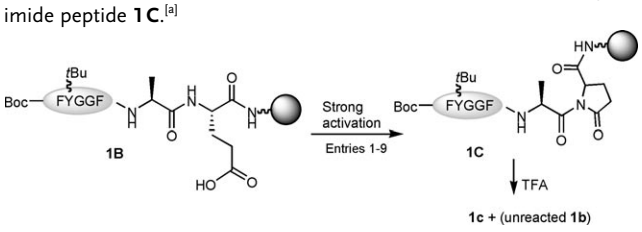
Scheme 2. Structures of peptides released from the solid support. R = $(CH_2)_2$COOEt. Upper case labels refer to resin-bound peptide while the lower case labels refer to peptides after release from the resin. **1a** from peptidyl resin **1A**, **1b** from peptidyl resin **1B**. A = alanine, E = glutamic acid (Glu), F = phenylalanine, G = glycine, Y = tyrosine.

TG resin (**1A**; Scheme 2). This peptide is a double-linker system with a C-terminal Glu(Ph*i*Pr) moiety on a Rink amide linker. The 2-phenylisopropyl (Ph*i*Pr) group was removed with TFA/DCM (1:49) to give the Boc-FY(*t*Bu)GGFAE-Rink-TG resin (**1B**). For the difficult acylation, we chose PyBrOP (bromotris(pyrrolidino)phosphonium hexafluorophosphate) for activation, as it is one of the strongest coupling reagents, which presumably provides the corresponding acyl bromide.[11] In principle, activation of the Glu side-chain carboxylate could lead not only to the desired five-membered ring but also to a six-membered ring by attack on the C-terminal amide nitrogen atom. However, NMR studies showed that the five-membered ring had formed. Importantly, the formation of peptide thioesters (see below) provides chemical proof for the formation of the expected five-membered ring.

To optimize the formation of the pGlu imide, varying reaction conditions were evaluated (Table 1). Initially, DMF was used as solvent but a significant level of competing formylation took place (presumably because of the formic acid present in the DMF); this problem was circumvented by using NMP (*N*-methyl-2-pyrrolidinone) as a solvent. An impurity accounting for 40–60% with a higher HPLC retention time and a mass 53 Da higher than the desired product was observed after the pGlu imide formation. Activation of carboxylates with PyBrOP can give the corresponding pyrrolide as a side-product;[12] in our case **1g** would be formed. PyBrOP was therefore recrystallized to remove pyrrolidine,[13] thus avoiding formation of **1g**. In an additional test, **1g** was expressly prepared. Pyrrolidine was reacted with **1b** using EDC in the presence of DMAP and, after release from the resin, the product was analyzed by LC–MS. The retention time for the impurity (**1g**) was the same as for the expressly synthesized pyrrolidide **1g** (see the Supporting Information).

The stability of the selectively deprotected, resin-bound peptide **1B** was investigated. A significant decrease in the conversion to **1C** was observed when the resin had been stored for 24 h after removal of the Ph*i*Pr group.

Table 1: Reaction conditions for the formation of the resin-bound pGlu imide peptide **1C**.[a]



Entry	Base (equiv)	PyBrOP (equiv)	Reaction conditions	**1b** [%][b]	**1c** [%][c]
1	DIEA (10)	5	O.N., RT	80	15
2	DIEA (20)	10	O.N., RT	62	30
3	DIEA (20), DMAP (2)	10	O.N., RT	13	35
4	collidine (20)	10	O.N., RT	>99	–
5	TEA (20)	10	O.N., RT	>99	–
6	DIEA (20)	10	2×1 h, MW, 60°C	24	70
7	**DIEA (20)**	**10**	**3×1 h, MW, 60°C**	**10**	**80**
8	DBU (20)	10	1×1 h, MW, 60°C	38	46
9	**DIEA (20)**	**10**	**2×2 h, HS, 60°C**	**12**	**78**

[a] Data was obtained by release of a small amount of peptide to give **1c** for LCMS analysis. [b] **1B**: peptidyl–resin [c]**1b**: peptide. The entries in bold represent the optimized reaction conditions. TEA = triethylamine, DIEA = *N*,*N*-diisopropylethylamine, DMAP = 4-(*N*,*N*-dimethylamino)-pyridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, O.N. = overnight (20 hours), MW = microwave heating, HS = heated shaker.

By varying the amounts of PyBrOP, base, and auxiliary nucleophile, and the reaction temperature, the optimal conditions were established (Table 1, entries 7 and 9). The yields for the conversion from **1A** to **1C** of 58% (conditions in Table 1, entry 7) and 67% (conditions in entry 9) were calculated from HPLC data. The yield of peptide **1c** was 30% after purification, and its structure was confirmed by LC–MS (Figure 1) and NMR spectroscopy. Furthermore, explicit

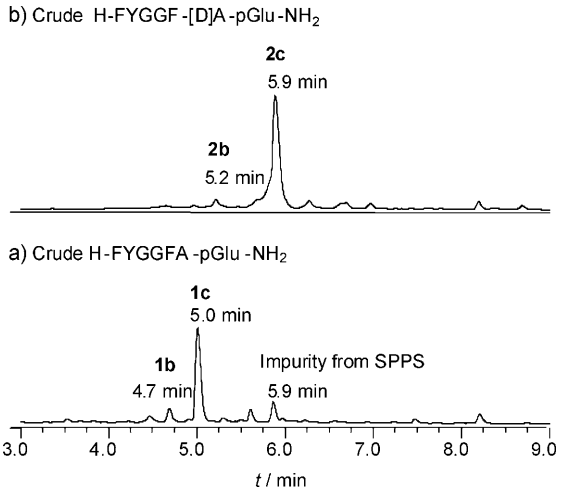


Figure 1. LC–MS chromatogram of pGlu imide formation using reaction conditions in Table 1, entry 9. a) Conversion **1A**→**1C**→**1c**. b) Conversion of the sequence Boc-FY(*t*Bu)GGF-[D]AE(Ph*i*Pr)-Rink-TG resin (**2A**) to the corresponding pGlu imide sequence, **2C**→**2c**. The chromatograms indicated that epimerization was insignificant during this step.

synthesis of the epimeric sequence H-FYGGF-[D]A-pGlu-NH_2 (**2c**) showed no sign of epimerization of the Ala chiral center, according to HPLC analysis.

Following pGlu imide formation, the nucleophilic release was tested (Table 2). The resin **1C** was treated with varying thiol/base mixtures and solvents at different temperatures, in order to release the peptide into solution as a peptide thioester (**1d**). The solvent was then removed and the protected peptide thioester was dried thoroughly before treatment with a TFA cocktail to remove the side-chain protecting groups and give deprotected peptide thioester **1e**, which was analyzed by HPLC (Figure 2).

Initially, DMF was chosen as the solvent for thiolysis, however, thorough studies showed that approximately 20% epimerization occurred; removal of the DMF also required high vacuum. We subsequently performed the reaction in neat thiol, as the solvent removal step would not be required. Different bases, temperatures, and reaction conditions were tested with the thiol acting as solvent. Two problems occurred using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and no solvent: 1) an apparent retro-Michael reaction that gave a considerable thioacid byproduct and 2) up to 40% epimerization. As an alternative, different volatile and polar solvents were screened in combination with sodium thiolate. The use of acetonitrile gave reasonable yields and limited the epimerization to less than 2%. The final yields were low as the sodium thiolate was not completely dissolved in acetonitrile. However, addition of crown ether ([15]crown-5) helped dissolve the thiolate and yields were increased. Temperatures higher than 40–45 °C gave rise to increased epimerization at the C-terminal chiral center.

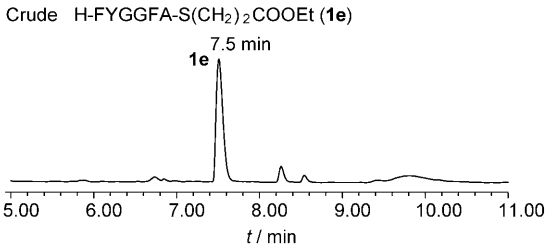
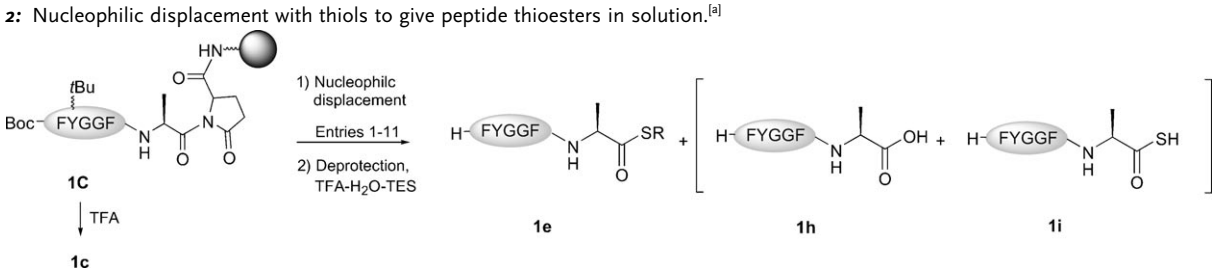


Figure 2. LC–MS chromatogram of crude H-FYGGFLA-S$(CH_2)_2$COOEt prepared using the reaction conditions in Table 2, entry 11.

The diastereomer H-FYGGF-[D]A-S$(CH_2)_2$COOEt (**2e**) of **1e** was synthesized using the optimal conditions (Figure 3). The two diastereomers were not separated by HPLC and a thiol exchange was carried out by adding sodium 2-mercaptoethane sulfonate (MESNa) in aqueous buffer at pH 7.5, in order to evaluate the degree of epimerization. The corresponding peptide thioesters **1f** and **2f** were separated by HPLC. As a result, the use of optimal reaction conditions (Table 2, entry 11) gave the products **1e** and **2e** without any trace of epimerization at the chiral center (Figure 3).

Two additional thioesters were synthesized by this strategy, H-FYGGFL-SR (**3e**) and H-ALGFYGGFA-SR (**4e**). Peptide **3e** provided information on a different C-terminal residue and **4e** on a longer sequence (see the Supporting Information). In order to verify the usability of the peptide

Table 2: Nucleophilic displacement with thiols to give peptide thioesters in solution.[a]



Entry	Thiol (equiv)	Base (equiv)	Solvent	Reaction Conditions	**1e** yield [%][b]	**1h** yield [%]	**1i** yield [%]	**1i** epimerization [%]
1	RSH (50)	PhSNa (10)	DMF	4 h, MW, 60 °C	25 (≈35)	<2	–	20
2	RSH (50)	PhSNa (10), DIEA (5)	DMF	4 h, MW, 60 °C	– (≈39)	<7	–	20
3	RSH (50)	PhSNa (10)	DMF	2×4 h, MW, 60 °C[c]	41 (≈55)	<4	–	20
4	RSH (200)	PhSNa (20)	–	3 h, MW, 60 °C	– (≈5)	–	–	–
5	RSH (200)	DIEA (20)	–	3 h, MW, 60 °C	–	–	–	–
6	RSH (200)	DBU (5)	–	O.N. HS, 40 °C	40 (≈50)	–	<10	40
7	RSH (50)	PhSNa (10)	CH_3CN	O.N. HS, 40 °C	20 (≈30)	<5	-	<1
8	RSH (50)	PhSNa (10)	THF	O.N. HS, 40 °C	– (≈20)	<5	–	<2
9	RSH (50)	PhSNa (10)	Dioxane	O.N. HS, 40 °C	– (<5)	–	–	–
10	RSH (50)	PhSNa (10), [15]crown-5	CH_3CN	O.N. HS, 40 °C	45 (≈50)	–	–	<2
11	**RSH (50)**	**PhSNa (2), [15]crown-5**	**CH_3CN**	**O.N. HS, 40 °C**	**45 (≈60)**	**–**	**–**	**<1**

[a] R = $(CH_2)_2$COOEt. All HPLC analyses were carried out with detection at 280 nm using **1a** as standard. [b] Yields were calculated relative to the HPLC-derived yield of **1C**. The main value is the yield of purified product. The HPLC-quantified yield of crude product is given in parentheses. [c] The resin was treated twice with the thiol/DMF mixture; the combined yield is that for the two treatments. The entry in bold represents the optimized reaction conditions.

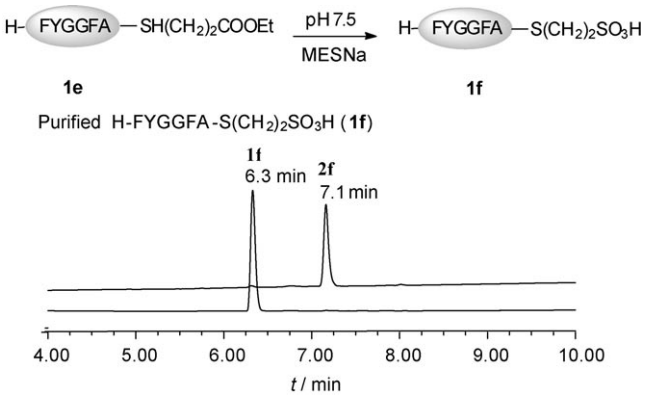


Figure 3. LC–MS chromatogram of purified **1f** after addition of MESNa to **1e** in aqueous buffer at pH 7.5. The overlay shows the spectrum of the corresponding diastereomer **2f**.

thioesters synthesized by this new strategy, **1e** was ligated with H-CGERGFFY-NH_2 (**5**) in 0.1M phosphate buffer (pH 7.5) containing 6M guanidine hydrochloride (GuHCl) and 50 mM MESNa to give H-FYGGFACGERGFFY-NH_2 (**6**; see the Supporting Information).

We have introduced a new concept in peptide chemistry, that is, backbone amide activation, and have demonstrated its use for efficient synthesis of peptide thioesters. The method relies on a simple glutamic acid linker system which, following activation, renders the C–N bond susceptible to thiolysis and provides the formation of peptide thioesters. Application to other C-terminal modifications can readily be envisioned.

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