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Exploration of an imide capture/*N*,*N*-acyl shift sequence for asparagine native peptide bond formation

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ABSTRACT

Imide capture of a C-terminal peptidylazide with a side-chain thioacid derivative of an N-terminally protected aspartyl peptide leads to the formation of an imide bond bringing the two peptide ends into close proximity. Unmasking of the N^{α} protecting group and intramolecular acyl migration results in the formation of a native peptide bond to asparagine.

1. Introduction

The design of novel amide bond forming reactions is one of the greatest challenges in synthetic organic chemistry today.[1] In particular, methods enabling the formation of native peptide bonds between peptide segments are of the utmost importance for assembling complex peptide scaffolds or proteins.

The peptide bond can be formed by the direct reaction of a peptide segment featuring a C-terminal activated carbonyl group with the free α-amino group of the second segment.[2–4] Alternately, the peptide bond forming process can be initiated by the selective formation of a non-peptidic bond between the two segments in a first step, which brings the C- and N-terminal ends into close proximity. The native peptide bond is then formed in a subsequent intramolecular rearrangement step.[5] Several native peptide bond forming processes rely on such a capture/intramolecular rearrangement sequence, among which are the native peptide ligation methods used for protein total synthesis starting from unprotected peptide segments, that is, native chemical ligation,[6,7] *bis*(2-sulfanylethyl)amido (SEA) native peptide ligation,[8–12] the traceless Staudinger ligation,[13,14] or the decarboxylative condensation of *N*-alkylhydroxylamines and α-ketoacids.[15]

Thioacids are of interest in this area due to their unique reactivity compared to other functional groups present within polypeptides.[16] Activation of C-terminal peptide thioacids by silver ion,[3,4] Sanger and Mukaiyama reagents,[17] or arylsulfonamides[18] enables the block synthesis of peptides. C-terminal peptide thioacids have been used also in the design of elegant capture/intramolecular rearrangement sequences leading to native or peptidomimetic amide bond formation.[19] In particular, the capture step in these sequences exploits the nucleophilic properties of the thioacid function toward bromoalkyl,[20] disulfide,[21] isocyanate or isothiocyanate,[22] or aziridine functionalities.[23]

The reaction of thioacids with azides is another route to amides. The reaction is particularly effective with electron-poor azides such as sulfonyl azides,[24–30] and gives access to stable acylsulfonamide-linked peptide[28] or protein[30] conjugates of the type frequently used as safety catch linkers.[31–35] We have also recently reported on the potential of the imide ligation, that is, on the reaction of a C-terminal peptidyl thioacid with an azidocarbonyl derivative for the assembly and enzyme-triggered disassembly of peptide-drug conjugates.[36] We describe here our exploration on the potential of a related Asparagine Native Peptide Ligation (AsnNPL), which relies on an imide capture/intramolecular *N*,*N*-acyl shift reaction sequence.

2. Results and discussion

2.1. General strategy

AsnNPL requires a C-terminal peptide azide **1** and an N-terminal thioaspartyl peptide **2** as starting peptide segments (Scheme 1).

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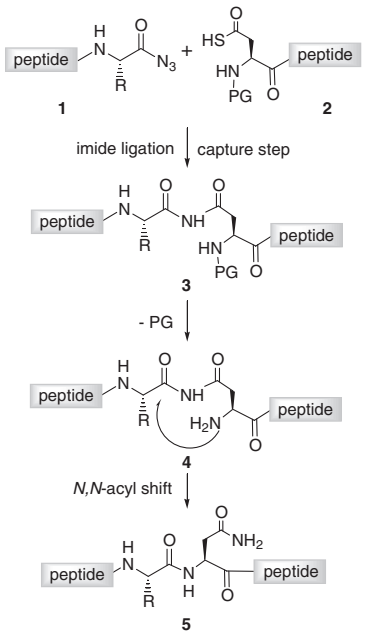


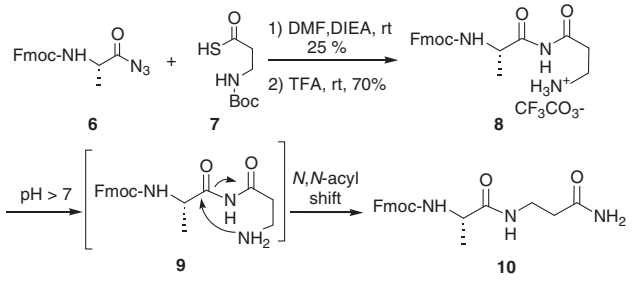
Scheme 1. Asparagine native peptide ligation (AsnNPL) relies on an imide capture/*N*,*N*-acyl shift sequence.

The capture step is an imide ligation between the acyl azide and the thioacid moieties, which results in the formation of imide-linked intermediate **3**. Removal of the temporary protecting group from the N-terminal position of the thioaspartyl segment after the capture step enables the migration of the acyl segment derived from the acyl azide **1** from the carboxamide nitrogen to the α-amino group. This rearrangement is an *N*,*N*-acyl shift which occurs presumably through a 6-membered cyclic intermediate,[37] and is favored by the mild electrophilicity of the imide carbonyl. The rearrangement results in the formation of a native peptide bond to Asn. The carboxamide nitrogen of the final asparagine side-chain derives from the C-terminal azido group of acyl azide segment **1**.

2.2. Proof-of-concept of the imide capture step using model amino acid derivatives

To illustrate the method we first used simple amino acid derivatives, that is, Fmoc-Ala-N_3 **6** and Boc-βAla-SH **7** (Scheme 2), with the latter mimicking the N-terminal thioaspartyl residue of segment **2** (Scheme 1). The alanine derivative was protected by a 9-fluorenylmethoxycarbonyl (Fmoc) group to facilitate the UV detection of the compounds during HPLC. The β-alanine derivative was protected by a *tert*-butyloxycarbonyl (Boc) group since the intermediate imide group was expected to be stable in trifluoroacetic acid (TFA).

Imide ligation of Fmoc-Ala-N_3 **6** with Boc-βAla-SH **7** followed by removal of the Boc group in TFA successfully furnished the ammonium imide intermediate **8**. The modest yield for this step was due to the formation of other imide products which were not isolated. This aspect is discussed in more detail later. This compound proved to be stable in mildly acidic aqueous solution due to the protection of the βAla amino group by protonation, and therefore could be purified by HPLC and fully characterized.[38] In particular, its ^{1}H NMR spectrum showed a peak at δ 11 in accordance with the proposed imide structure.



Scheme 2. Proof-of-concept study using β-alanine as an aspartic acid mimetic.

2.3. Intramolecular *N*,*N*-acyl shift reaction

Imides are mildly activated derivatives of carboxylic acids and therefore have been used as amide prodrugs due to their propensity to be hydrolyzed in biological fluids.[39] In another application, Tsunoda et al., developed a method for the hydrolysis of N-monosubstituted amides based on the formation of acyclic imides followed by an intramolecular *N*,*O*-acyl migration reaction.[40] Recently, we reported on the design of a novel alcohol prodrug system in which the unmasking step involved the intramolecular attack of a primary amine on an imide carbonyl moiety (Scheme 3).[36] In this system, the alcohol moiety acted as a leaving group during the intramolecular *O*,*N*-imide carbonyl migration reaction. To the best of our knowledge, the involvement of imides in intramolecular *N*,*N*-acyl migration reactions resulting in the departure of a primary amide has not been reported in the literature.

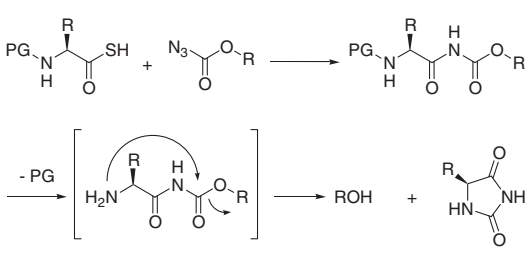
In a first approach, we used imide **8** to probe the feasibility of the intramolecular *N*,*N*-acyl shift (Scheme 2). The pK_a of the imide group in compound **8** was estimated to be 7.8 using the SPARC[41] algorithm. Consequently, this group was expected to be in its protonated form at pH 7 and thus to act as a mildly activated carboxylic acid set up to enable the *N*,*N*-acyl shift to occur through a 6-membered cyclic intermediate.

In agreement with this hypothesis, solubilization of imide **8** in water at pH 7 resulted within 1 min in the clean formation of dipeptide **10** (Fig. 1), which was found to be identical by ^{1}H and ^{13}C NMR spectroscopy with a reference compound prepared by standard Fmoc-solid phase peptide synthesis (SPPS) (see Supplementary data).

2.4. Proof-of-concept of AsnNPL using model peptide segments

2.4.1. Imide capture step

We next examined the imide capture step using model N-terminal thioaspartyl peptide **12** and C-terminal peptide azide **13** (Scheme 4). In a first approach, peptide azide **13a** was prepared using diphenylphosphoroazidate (DPPA) in the presence of triethylamine. DPPA allows the prompt and straightforward preparation of C-terminal peptide azide, but results in the partial racemization of the C-terminal residue (~30% D-Ala for **13a**). Once formed, peptidylazide **13a** was reacted with N-terminal thioaspartyl peptide **12** in DMF[42] at room temperature resulting in the formation of the target imide **14a**, but also unexpectedly of two other products



Scheme 3. An intramolecular *O*,*N*-imide carbonyl shift reaction used in the design of an alcohol prodrug system.[36]

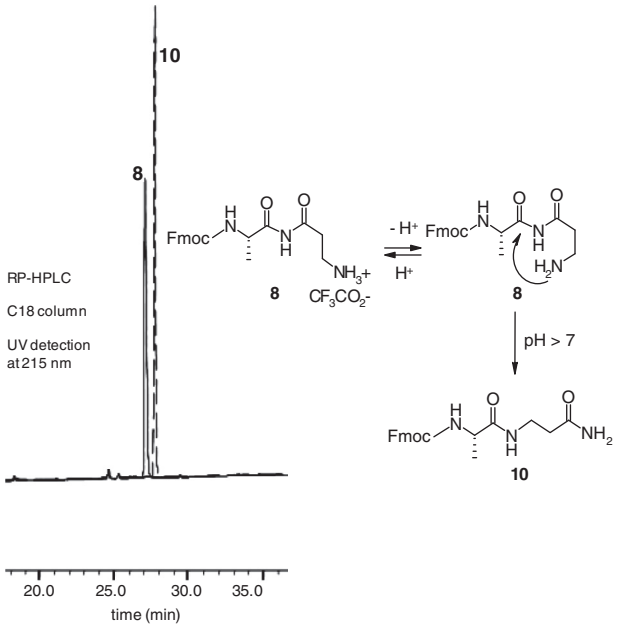
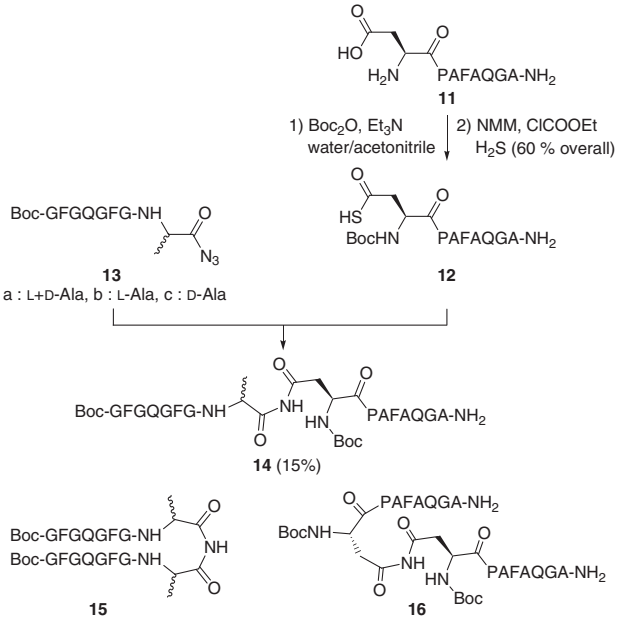


Figure 1. HPLC analysis of the rearrangement of imide **8** into dipeptide **10**. Continuous trace: imide **8**, dashed trace: dipeptide **10** obtained after dissolving imide **8** in water at pH 7.



Scheme 4. Study of imide capture step using model peptides. GFGQGFG is the one-letter code for GlyPheGlyGlnGlyPheGly. PAFAQGA is the one-letter code for ProAlaPheAlaGlnGlyAla.

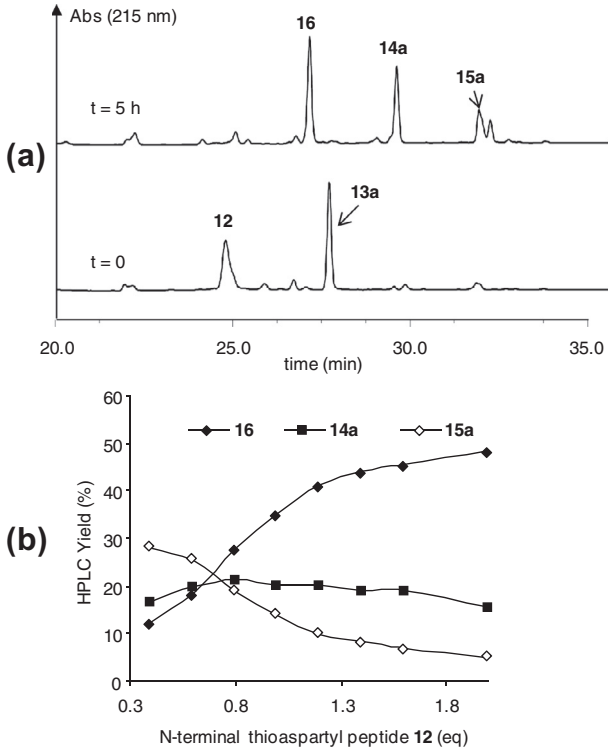


Figure 2. Imide ligation of peptides **12** and **13a**. (a) HPLC analysis of the reaction mixture immediately after mixing ($t = 0$) or after 5 h at rt (**12**/**13a**: 1/1 by mol). (b) Influence of the stoichiometry of the reaction on the proportion of imides **14a**, **15a** and **16**.

corresponding to the symmetrical imides **15a** and **16** (Fig. 2A). Interestingly, monitoring of the ligation reaction by HPLC revealed the scrambling of the thioacid and azido functionalities, that is, the in situ formation of Boc-GlyPheGlyGlnGlyPheGlyAla-SH and Boc-Asp(N_3)ProAlaPheAlaGlnGlyAla-NH_2 (see Supplementary data) within a few seconds after mixing peptides **12** and **13a**. This scrambling of the thioacid and azido moieties, which explains the formation of symmetrical imides **15a** and **16**, might proceed through the formation of a thioanhydride by nucleophilic attack of the thiocarboxylate group of peptide **12** on the C-terminal carbonyl group of azido peptide **13a**. Subsequent reaction with the released azide anion then gives the scrambled thioacid and azido peptides. A mixture of scrambled imides was also obtained on mixing thiobenzoic acid and 3-phenylpropanoyl azide (see Supplementary data), and it was demonstrated that the reaction of authentic benzoic thioanhydride or 3-phenylpropanoic thioanhydride with sodium azide furnished the corresponding symmetrical imides in 68–75% yield, in support of the proposed reaction pathway. The occurrence of a scrambling process during the reaction of a thiocarboxylic acid with an acyl azide has not been previously discussed to our knowledge. In their pioneering studies, Williams and co-workers described the reaction of thioacids with azides such as benzylazidocarbonate or benzenesulfonylazide.[24,25] Unlike the acyl azides examined in this study, no scrambling reaction was observed with these electron deficient azides. More recently, we examined the reaction of C-terminal peptide thioacids with azidoformates as described in Scheme 3.[36] Here again, no scrambling reaction was observed and imides were isolated in good yield.

In an attempt to improve the proportion of dissymmetrical imide **14a** in the ligation mixture, we varied the stoichiometry of the reaction (Fig. 2B). Unfortunately, the proportion of dissymmetrical imide **14a** did not vary significantly over the stoichiometry range examined. In summary, the imide ligation step proceeds as anticipated but is complicated by a background reaction involving the scrambling of thioacid and azido peptide components.

The imide ligation step also was performed with epimerization free C-terminal azido peptides **13b** (L-Ala) and **13c** (D-Ala) to probe the issue of partial racemization of the C-terminal residue of the peptide azide segment during AsnNPL. Compounds **13b** and **13c** were prepared by reacting the corresponding hydrazides Boc-GlyPheGlyGlnGlyPheGlyAla-$NHNH_2$ or Boc-GlyPheGlyGlnGlyPheGly-D-Ala-$NHNH_2$ with *tert*-butyl nitrite (see Supplementary data). This method is known to be non-racemizing.[43–45] The structure of imides **14a–c** was confirmed by ^1H and ^{13}C NMR spectroscopy (see

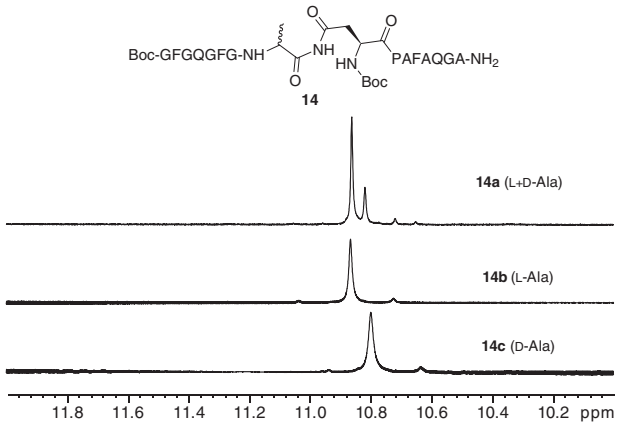
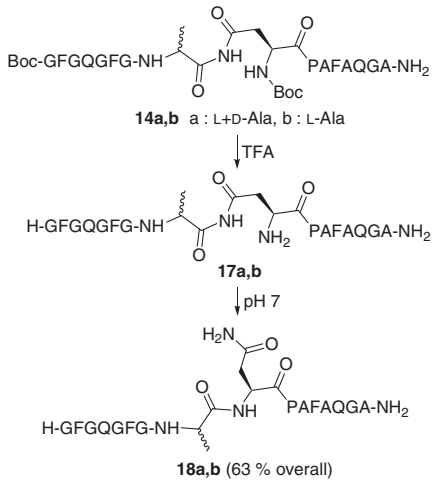


Figure 3. Imide region of the ^{1}H NMR spectra for imides **14a–c** (300 MHz, DMF-d_7, 20 °C, 11 mM).



Scheme 5. *N*,*N*-Acyl shift with model imide peptides **14a**,**b**.

Fig. 3 and Supplementary data), and the imide region of the ^{1}H NMR spectrum showed two main peaks for **14a** at δ 10.81 (0.3 H) and 10.86 (0.7 H), whereas **14b** (L-Ala) and **14c** (D-Ala) showed only one main peak at δ 10.86 and 10.81, respectively. Note that peaks of low intensity relative to the main imide proton are present in the ^{1}H NMR spectra of imides **14b** and **14c**. These minor peaks, which are also present in the ^{1}H NMR spectrum of **14a**, might be due to minor imide conformers.[46,47] Acyclic imides such as imide **14** may adopt three planar conformations, *cis–cis*, *cis–trans*, and *trans–trans*, which differ by the relative orientations of the two carbonyl groups to the central NH bond. Of the three possible conformers, the *cis–trans* is the most favored in simple aliphatic imides. The *trans–trans* form can be present as a minor conformer, whereas the *cis–cis* conformer is usually not observed. Note that unsymmetrical imides such as imide **14** can adopt potentially two *cis–trans* conformations. Consequently, the presence of several isobaric species for imide **14** is not surprising given the known conformational properties of unsymmetrical imides.

Overall, these data show that the imide capture step is non-racemizing and that the presence of two species in **14a** is due to the partial racemization of C-terminal Ala residue during the preparation of C-terminal azido peptide **13a**.

2.4.2. *N*,*N*-Acyl rearrangement step

Next, the isolated imide-linked peptides **14a** and **14b** were deprotected in TFA and rearranged in water at pH 7 (Scheme 5). As for model imide **8** (Scheme 2), rearrangement of deprotected

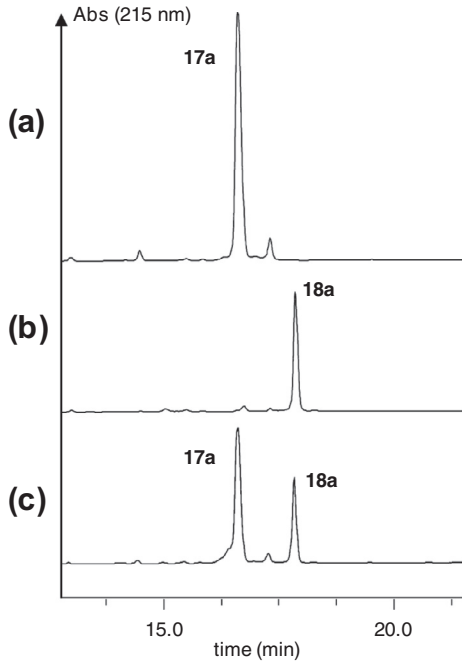


Figure 4. HPLC analysis of: (a) imide **17a**. The peak eluting after **17a** is isobaric to **17a** and might be due to the presence of a minor imide conformer; see main text. (b) Rearranged peptide **18a** formed after dissolving **17a** in water at pH 7 (1 min, rt). Both compounds were co-injected in (c).

imide **17a** proceeded cleanly in less than 1 min to give peptide **18a** (Fig. 4), which was found to be identical by HPLC and capillary electrophoresis to a reference peptide synthesized by standard Fmoc-SPPS. Moreover, automated Edman microsequencing of peptide **18a** showed the signature of an Asn residue at position 9, in between Ala 8 and Pro 10. Importantly, chiral GC–MS analysis of peptide **18b** after acid hydrolysis revealed a D-Ala content of only 0.28%,[48] showing that the imide capture/*N*,*N*-acyl shift sequence proceeded with no significant racemization of C-terminal Ala residue.

3. Conclusions

In conclusion, we have used imide ligation, that is, the reaction of a thioacid with an acyl azide, as a capture step in the design of a novel peptide bond forming reaction. The peptide bond is formed after an intramolecular *N*,*N*-acyl shift reaction involving the nucleophilic attack of an α-amino group on the imide group. The *N*,*N*-acyl shift occurs through a 6-membered cyclic intermediate. An asparagine residue is formed at the ligation junction. The whole process proceeds with no detectable racemization of the preceding residue (Ala in this study). While the scope of the substrates tested in this study in DMF has been limited to those bearing unfunctionalized side chains, the established compatibility of peptidyl thioacids and imides with more complex side chains and aqueous media suggests that the method may have broader scope if and when the issue of thioacid scrambling is solved. Further work in this direction is in progress.

4. Experimental section

4.1. Proof-of-concept of AsnNPL using model amino acids

4.1.1. Synthesis of thioacid 7

To a solution of Boc-βAla-OH (1.89 g, 10 mmol) dissolved in 10 mL of CH_2Cl_2 was added *N*-methylmorpholine (NMM, 11 mmol,

1.22 mL). The reaction mixture was immersed in an ice bath and ethyl chloroformate (12 mmol, 1.15 mL) was added to the mixture which was stirred for 5 min. Hydrogen sulfide was then bubbled for 5 min and 2 equivalents of NMM (22 mmol, 2.44 mL) were added. The reaction mixture was stirred at rt for 30 min. The solvent was then evaporated in vaccuo and the yellow residue obtained was redissolved in 30 mL of deionized water, acidified with 0.1 N HCl to pH 2–3. The aqueous solution was extracted with 3 × 30 mL of ethyl acetate and the organic phase was dried with anhydrous $MgSO_4$, filtered and evaporated in vacuo. Thioacid **7** was isolated as a yellow oil with a yield of 92% (1.97 g) and used directly in the next experiment without further purification.

ESI-MS $[M+H]^+$ m/z calcd (monoisotopic) 206.0, found 206.2.

^1H NMR (300 MHz, $CDCl_3$, sodium trimethylsilylpropionate used as internal reference for chemical shifts) δ 4.87 (s, 1H), 3.32 (q, J = 12.1, 6.1 Hz, 2H), 2.82 (t, J = 5.9 Hz, 2H), 1.37 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 197.10 (s), 155.87 (s), 46.03 (s), 36.27 (s), 28.49 (s).

4.1.2. Synthesis of imide 8

Preparation of Fmoc-Ala-N_3 **6**. Fmoc-Ala-OH (600 mg, 1.9 mmol) was dissolved in 1.8 mL of anhydrous DMF. The solution was cooled in an ice bath. Triethylamine (TEA, 352 μL, 2.50 mmol) and diphenylphosphoroazidate (DPPA, 541 μL, 2.50 mmol) were added to the solution. The reaction mixture was kept under magnetic stirring for 4 h. This solution was used directly in the next step.

The above solution of Fmoc-Ala-N_3 in DMF (1.6 mmol) was diluted in 18 mL of DMF. To this solution was added Boc-βAla-SH **7** (328 mg, 1.60 mmol) and TEA (224 μL, 1.60 mmol). The reaction mixture was colored yellow accompanied by gas evolution. The reaction mixture was stirred at rt for 6 h, after which the DMF was evaporated in vacuo. The yellow residue was redissolved in 50 mL of water/acetonitrile: 4/1 by vol containing 0.05% TFA by vol, purified by RP-HPLC and lyophilized. The compound was obtained as a white powder (184 mg, 24%). LC–ESI MS analysis of Boc-protected imide intermediate gave $[M+H]^+$ calcd (monoisotopic) 482.23, found 482.31

Because RP-HPLC purification led to a partial removal of Boc group, compound was further deprotected with TFA. For this, Boc-protected imide intermediate (100 mg, 0.2 mmol) was treated with 20 mL of TFA/CH_2CL_2: 1/1 by vol at rt for 30 min. The solvent was evaporated to dryness in vaccuo. The residue obtained was redissolved in 20 mL of water/acetonitrile: 9/1 by vol containing 0.05% of TFA by vol and purified by RP-HPLC. The fractions containing imide **8** were combined and lyophilized to give the product 72 mg of a white powder (70%).

^1H NMR (300 MHz, DMF) δ 11.07 (s, 1H); 8.50 (s, 1H); 7.96 (d, J = 7.5 Hz, 2H); 7.84 (d, J = 7.3 Hz, 1H); 7.79 (dd, J = 7.2, 4.5 Hz, 2H); 7.47 (t, J = 7.5 Hz, 2H); 7.37 (td, J = 7.5, 1.1 Hz, 2H); 4.56 (p, J = 7.2 Hz, 1H); 4.38–4.23 (m, 3H); 3.65 (s, 1H); 3.39 (t, J = 6.6 Hz, 2H); 3.24 (dd, J = 10.2, 3.9 Hz, 2H); 1.43 (d, J = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 173.94; 172.01; 156.31; 144.25; 141.19; 127.77; 127.18; 125.45; 120.14; 66.41; 51.42; 47.08; 35.09–34.76 (m); 16.86 .

HR-MS analysis of imide **8**. Calcd for $[M+H]^+$ m/z = 382.17613, found 382.17553.

4.1.3. Rearrangement of imide 8 into dipeptide 10

Imide **8** (16 mg) was dissolved in acetonitrile/0.1 M pH 7.3 sodium phosphate buffer: 7/3 by vol (16 mL). After 15 min, the reaction mixture was extracted with ethyl acetate (2 × 20 mL). The organic phase was dried over sodium sulphate, evaporated to dryness, dissolved in 50% by vol aqueous acetonitrile and lyophilized to give 16 mg (∼quantitative) of dipeptide **10**.

Peptide **10** was identical by RP-HPLC and ^1H or ^{13}C NMR to a reference compound produced by conventionnal Fmoc-SPPS (see Supplementary data).

ESI-MS calcd for $[M+H]^+$ m/z = 382.18 (monoisotopic); obsd m/z = 382.2.

HR-MS calcd for $[M+H]^+$ m/z = 382.17613, found 382.17542.

^1H NMR (300 MHz, DMF-d_7, sodium trimethylsilylpropionate used as internal reference) δ 7.94 (d, J = 7.3 Hz, 2H), 7.77 (t, J = 6.3 Hz, 2H), 7.55–7.29 (m, 5H), 4.29 (s, 2H), 4.18 (t, J = 14.5, 7.2 Hz, 1H), 3.41 (d, J = 6.1 Hz, 2H), 2.39 (t, J = 6.7 Hz, 2H), 1.38–1.29 (m, 3H).

^{13}C NMR (75 MHz, DMF-d_7, sodium trimethylsilylpropionate used as internal reference) δ 173.21, 158.69–155.88 (m), 146.70–144.77 (m), 141.75, 128.80, 128.24, 126.54, 121.16, 67.30, 51.80, 48.18, 36.60, 36.16, 19.18.

4.2. Proof-of-concept of AsnNPL using model peptide segments

The procedure is illustrated with the synthesis of peptide imide **14b** and Asn peptide **18b**. Other experimental procedures and characterization are described in the Supplementary data.

4.2.1. Synthesis of peptide 12

Preparation of peptide **11**. The solid phase synthesis of peptide H-DPAFAQGA-NH_2 was performed using the Fmoc/*tert*-butyl strategy on NovaSyn TGR resin (0.2 mmol/g, Novabiochem, 0.5 mmol scale) using an automated peptide synthesizer. The coupling of amino acids was carried out with 2.5 mmol of each amino acid, 2.25 mmol of HBTU activator, and 5 mmol of DIEA. Final deprotection and cleavage of the peptide from the resin were performed with a mixture of TFA/triisopropylsilane (TIS)/H_2O: 95/2.5/2.5 by vol. The resin beads were filtered and the filtrate was precipitated in diethyl ether/heptane: 1/1 by vol. The peptide was then dissolved in water and lyophilized. The peptide was obtained as a white powder with a yield of 87% (386 mg).

MALDI-Tof (positive mode). Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Calcd for $[M+H]^+$ 775.3 (monoisotopic), found 775.2.

The peptide H-DPAFAQGA-NH_2 (115 mg, 0.13 mmol) was next dissolved in 1.3 mL of a mixture of water/acetonitrile: 1/1 by vol. Boc_2O (41 μL, 0.19 mmol) and TEA (54 μL, 0.39 mmol) was added to this solution. The reaction mixture was stirred for 3 h at rt. The peptide was purified by RP-HPLC and lyophilized. Peptide Boc-DPAFAQGA-NH_2 **11** was obtained as a white powder with a yield of 70% (m = 79 mg).

MALDI-Tof (positive mode). Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Calcd for $[M+H]^+$ 875.4 (monoisotopic), found 875.4; $[M+Na]^+$ 897.4.

Preparation of peptide **12**. Peptide Boc-DPAFAQGA-NH_2 **11** (102 mg, 0.12 mmol) was dissolved in 1.3 mL of DMF/acetonitrile: 1/1 by vol. The solution was cooled to −45 °C under argon, and NMM (14 μL, 0.13 mmol) and ethyl chloroformate (25 μL, 0.24 mmol) were added. After 5 min, the reaction mixture was bubbled with hydrogen sulfide for 5 min. The flow of hydrogen sulfide was maintained during the addition of NMM (29 μL, 0.26 mmol). The reaction mixture was stirred for an additional 15 min still with hydrogen sulfide bubbling. The bubbling of hydrogen sulfide was then stopped and the reaction mixture was allowed to warm at rt and was stirred for 1 h.

The solvents were then evaporated in vacuum and the residue obtained was purified by RP-HPLC using triethylamine acetate buffer (TEAA) buffer. The peptide Boc-D(SH)PAFAQGA-NH_2 **12** was lyophilized twice to remove excess TEAA. It was isolated as a white powder with a yield of 60% (69 mg).

LC–MS analysis of peptide **12**. MS trace (positive mode). Calcd for $[M+H]^+$ 891.4 (monoisotopic), found 891.9.

MALDI-Tof (negative mode). Matrix: 2,5-dihydroxybenzoic acid (DHB). Calcd for $[M-H]^-$ 889.3 (monoisotopic), found 889.4.

4.2.2. Synthesis of peptide 13b

Peptide **13b** was obtained by nitrosation of peptide hydrazide Boc-GFGQGFGA-$NHNH_2$ with *tert*-butylnitrite in acidic media. For this, synthesis of peptide hydrazide H-GFGQGFGA-$NHNH_2$ was performed using the Fmoc/*tert*-butyl strategy on a Fmoc-Ala-Sasrin resin (0.25 mmol scale, Bachem) in an automated peptide synthesizer. The coupling of amino acids was carried out with 1.25 mmol of each amino acid, 1.12 mmol of HBTU activator, and 2.5 mmol of DIEA.

The cleavage of the peptide from the resin was performed using of hydrazine hydrate (20 mL, 20% in *N*,*N*-dimethylacetamide (DMA), 2×24 h). The resin was filtered and washed after each treatment with 10 mL of DMA. The fractions obtained were pooled and evaporated to dryness. The residue obtained was treated for 1 h with 40 mL TFA/TIS: 95/5 by vol. The reaction mixture was evaporated to dryness. The residue was redissolved in 30 mL of water containing 1% TFA by vol and purified by RP-HPLC. The fractions containing the target peptide were pooled and lyophilized. The peptide H-GFGQGFGA-$NHNH_2$ was obtained as a white powder with a yield of 60% (143 mg).

MALDI-Tof analysis of peptide H-GFGQGFGA-$NHNH_2$. Matrix: 2,5-dihydroxybenzoic acid (DHB). Calcd for $[M+H]^+$ 754.3, found 754.2.

Next, the peptide H-GFGQGFGA-$NHNH_2$ (143 mg, 147 μmol) was dissolved in 5 mL of water/acetonitrile: 1/1 by vol. To this solution was added TEA (206 μL, 1.47 mmol) and then $(Boc)_2O$ (33 μL, 147 μmol). The reaction mixture was stirred at rt for 20 h and then evaporated to dryness. The peptide was solubilized in an aqueous solution containing 0.05% TFA by volume, purified by RP-HPLC and lyophilized. The peptide Boc-GFGQGFGA-$NHNH_2$ was obtained as a white powder with a yield of 46% (66 mg).

ESI-MS calcd for $[M+H]^+$ 854.4 (monoisotopic), found 854.7.

MALDI-Tof analysis of peptide Boc-GFGQGFGA-$NHNH_2$. Matrix: DHB. Calculated for $[M+H]^+$ 854.4 (monoisotopic), found 854.0.

Finally, the peptide Boc-GFGQGFGA-$NHNH_2$ (30 mg, 32 μmol) was dissolved in 195 μL of anhydrous DMF. The solution was placed under argon at −25 °C. The solution was acidified with 40 μL of 4 N HCl in 1,4-dioxane. *tert*-Butylnitrite (39 μL of a 10% solution in DMF, 35 μmol) was added. The reaction mixture was stirred for 15 min and added dropwise to 20 mL of diethyl ether to precipitate peptide azide **13b**. The white suspension was centrifuged, washed twice with 10 mL of diethyl ether and dried under vacuum. The peptide **13b** was stored at −20 °C and used immediately without further purification.

4.2.3. Synthesis of imide 14b

The C-terminal azido peptide **13b** (35 μmol) was dissolved in DMF (200 μL). The N-terminal thioaspartyl peptide **12** (31 mg, 35 μmol) was dissolved in DMF (400 μL) and added to this solution. The reaction mixture was stirred for 24 h at rt, then diluted with 20 mL of water containing 0.05% TFA, and purified by RP-HPLC to give peptide imide **14b** with a yield of 19% (11.4 mg).

MALDI-Tof (positive mode). Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Calcd for $[M+Na]^+$ 1717.8 (monoisotopic), found 1717.9.

^{1}H NMR (300 MHz, DMF-d_7) δ 10.71 (s, 1H), 8.38–8.29 (m, 1H), 8.23–7.97 (m, 6H), 7.96–7.82 (m, 8H), 7.82–7.74 (m, 1H), 7.72–7.58 (m, 2H), 7.30 (s, 1H), 7.28–6.98 (m, 23H), 6.87 (s, 1H), 6.81–6.60 (m, 3H), 4.70 (d, J = 7.0 Hz, 1H), 4.57–4.31 (m, 4H), 4.29–4.06 (m, 6H), 4.06–3.95 (m, 1H), 3.88–3.52 (m, 17H), 3.23–2.96 (m, 5H), 2.91–2.78 (m, 4H), 2.24–2.07 (m, 5H), 2.07–1.68 (m, 9H), 1.28–1.03 (m, 40H).

^{13}C NMR (75 MHz, DMF-d_7) δ 174.87 (d, J = 6.4 Hz), 174.53 (s), 173.92 (s), 173.47 (s), 173.12 (s), 172.70 (s), 172.50 (s), 172.33 (s), 172.21 (s), 172.07 (s), 171.72 (s), 170.65 (s), 170.05 (s), 169.94 (s), 169.42 (s), 169.08 (s), 156.60 (s, J = 20.5 Hz), 155.99–155.78 (m), 138.46 (s), 138.35 (s), 138.28–138.23 (m), 129.60 (d, J = 7.1 Hz), 128.50 (s), 126.65 (s), 78.93 (s), 78.59 (d, J = 8.5 Hz), 61.73 (s), 55.61 (s, J = 19.1 Hz), 55.35 (s), 54.15 (s), 53.69 (s), 50.88 (s), 50.05 (s), 49.07 (s), 43.77 (s), 43.12 (s), 42.90 (s), 42.72–42.49 (m), 37.62 (s), 37.34 (s), 31.92 (s), 28.06 (s), 27.76 (d, J = 12.7 Hz), 25.11 (s), 17.95 (s), 17.13 (s), 16.73 (s).

4.2.4. Synthesis of peptide 17b

The deprotection of peptide **14b** (11 mg, 6.4 μmol) was performed with a mixture of TFA/CH_2Cl_2/TIS: 50/49/1 by vol. The reaction mixture was stirred for 40 min at rt. The solvent was then evaporated in vaccuo and the residue obtained was redissolved in deionized water (30 mL), desalted with SPE C18 Column and lyophilized. Peptide **17b** was obtained as a white powder with a yield of 98 % (11 mg).

MALDI-Tof (positive mode). Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Calcd for $[M+H]^+$ 1495.71 (monoisotopic), found 1495.7.

4.2.5. Synthesis of peptide 18b

Peptide **17b** (8 mg, 4.7 μmoL) was dissolved in phosphate buffered saline (0.01 M, pH 7.4, 10 mL). The reaction mixture was kept at rt for 1 h and then purified by RP-HPLC. The fractions containing peptide **18b** were combined and lyophilized to give 5.1 mg of a white powder (63%).

MALDI-Tof (positive mode). Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Calcd for $[M+H]^+$ 1495.71 (monoisotopic), found 1495.6.

Chiral GC–MS analysis of peptide **18b** after acid hydrolysis revealed a D-Ala content of 0.28% (CATT, Germany).

The same imide capture/*N*,*N*-acyl shift sequence allowed the synthesis of peptide **18a**. Peptide **18a** was identical by HPLC and capillary electrophoresis to a reference compound produced by conventional Fmoc-SPPS. Edman microsequencing showed the presence of Asn 9 residue in between Ala 8 and Pro 10, in accord with the proposed structure.

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Supplementary data

Supplementary data (experimental procedures and characterization data for all compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.053.

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37. Note that the nucleophilic attack of the N-alpha amino group on the other imide carbonyl would proceed through a 4-membered ring intermediate and thus is not favored.
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