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Singlet oxygen-mediated one-pot chemoselective peptide–peptide ligation

Eirini Antonatou, Yentl Verleysen and Annemieke Madder*

We here describe a furan-oxidation based site-specific chemical ligation approach using unprotected peptide segments.

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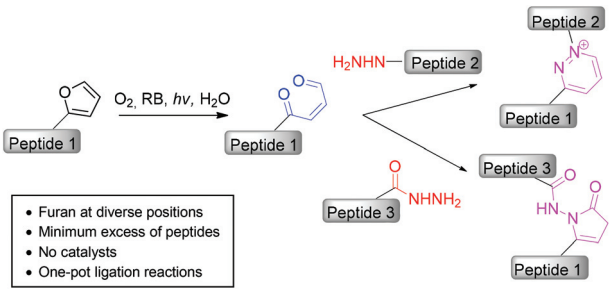

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PAPER

Singlet oxygen-mediated one-pot chemoselective peptide–peptide ligation†

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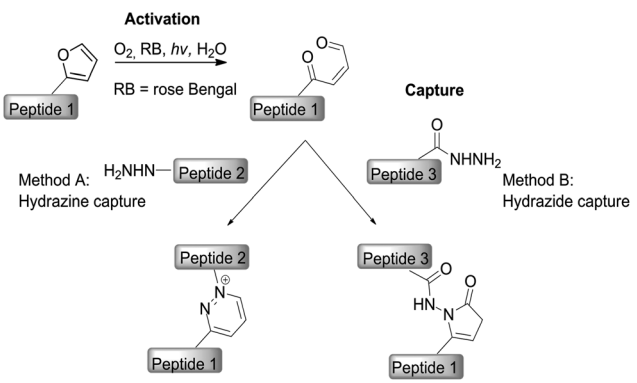
We here describe a furan-oxidation based site-specific chemical ligation approach using unprotected peptide segments. This approach involves two steps: after photooxidation of a furan-containing peptide, ligation is achieved by reaction of the unmasked aldehyde with C- or N-terminal α-nucleophilic moieties of the second peptide such as hydrazine or hydrazide to form a pyridazinium or pyrrolidinone linkage respectively.

Peptide conjugates are useful tools in research for the construction of multifunctional scaffolds, for the chemical synthesis of proteins[1,2] and in various cell applications.[3] Chemoselective coupling reactions have been widely used for the site-selective ligation of biomolecules under physiological conditions.[4–7] Recent advances include native chemical ligation,[2,8–15] Staudinger ligation,[16–18] azide–alkyne cycloaddition,[19–22] thiol–ene coupling reactions,[23] Diels–Alder cycloaddition reactions,[24–28] a photoclick tetrazole–alkene cycloaddition,[29] and olefin metathesis.[30] Additionally, hydrazone and oxime condensations or thiazolidine reactions are extensively utilized in the area of bioconjugation in view of the high chemoselectivity of the reactions.[31–35] Nonetheless, carbonyl ligations suffer from slow kinetics at neutral pH.[36,37] To address these issues, nucleophilic catalysts such as aniline and derivatives have been employed to enhance hydrazone ligation at neutral pH.[38–40] Alternatively, it was shown that by tuning the structure and reactivity of carbonyls and nucleophiles, fast and catalyst-free hydrazone ligations could be achieved at biological pH.[41,42]

Despite the great advancements, there is still need for simple and chemoselective methods for ligating peptides into larger constructs for diverse applications. In line with our previously established methodology,[43] where furan peptides could be efficiently labeled with various fluorescent moieties using α-effect nucleophiles, we envisaged to expand our methodology towards the ligation of unprotected peptide fragments carrying bioorthogonal handles. In this way, a mild and efficient coupling reaction has been developed where unprotected furan peptides are ligated with unprotected hydrazine peptides through a pyridazinium or pyrrolidinone linkage (Scheme 1). Important advantages of this strategy include the easy preparation of peptides containing furan at different positions as well as nucleophilic peptides using commercially available building blocks and the respectable conversions that can be attained with minimum excess of reagents. Photoinitiated furan-hydrazine reaction can be triggered in aqueous solution under physiological conditions by white light irradiation. A furan peptide, when irradiated with white light in presence of rose bengal, forms a reactive 1,4-enedione *in situ* which is further intercepted by α-effect nucleophilic peptides to yield pyridazinium or pyrrolidinone conjugates (Scheme 1). In previous work, the exact type of linkage formed between the oxidized furan moiety and the α-effect nucleophiles, was characterized by extensive NMR analysis.[43]

Our foray began with the reaction between model peptides Ac-Ile-Glu-Lys-Phe-Lys-Fua-NH_2 (**1a**) and α-hydrazinoacetyl-Gly-Arg-Gly-Asp-Ser-NH_2 (**2**).[44] Fua is the three-letter code used here for Fmoc-L-furylalanine residue. The ligation procedure consists of two steps that are carried out in a one-pot fashion.



Scheme 1 Singlet oxygen-mediated ligation of furan peptides with either hydrazine or hydrazide peptides to form pyridazinium or pyrrolidinone conjugates respectively.

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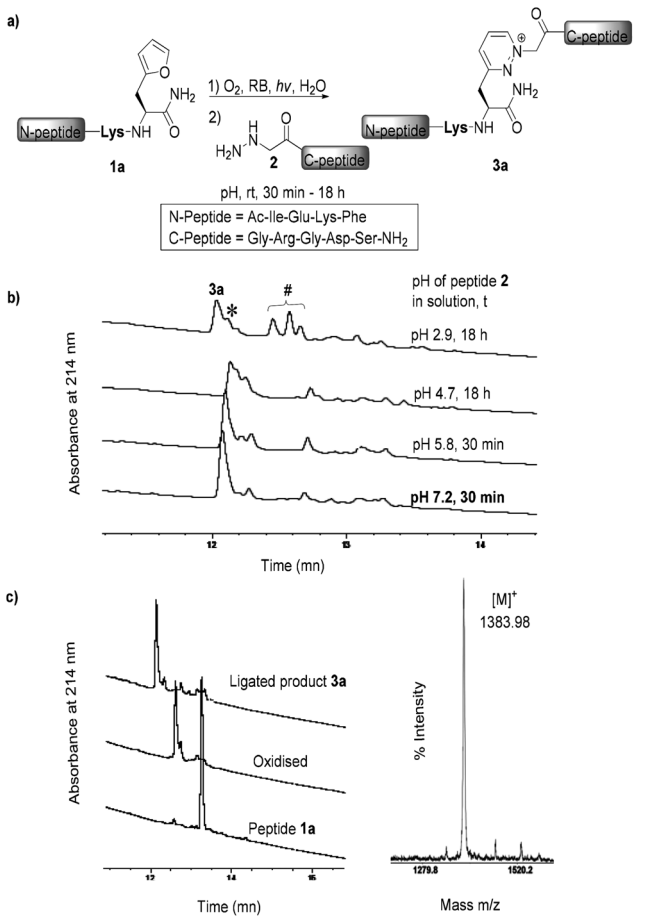
In a first step the furan peptide (0.5 mM) is photooxidised in presence of rose bengal (10 μM) for 40 min in water (pH 4.5). Subsequently the hydrazine peptide is added from its stock solution and the reaction is monitored for 30 min to 18 hours at room temperature. In search for the optimal conditions for coupling a keto–enol-peptide with a hydrazine-containing peptide, it was found that the pH of the stock solution of hydrazine peptide influences the reaction kinetics in that near neutral conditions prompt higher reaction rates. Preliminary experiments where hydrazine peptide **2** (1.0 equiv.) was added from its acidic solution in water (pH 2.9, Scheme 2b), demonstrated low conversion to the conjugate with oxidation and ligation intermediates still remaining after 18 h. Increasing the pH (from 2.9 to 7.2), gradually increased the reaction rate and conversion. Specifically, when hydrazine-peptide was added from a solution at pH 7.2 (1.0 M phosphate buffer), conjugation reached high conversion in only 30 min (Scheme 2b and c). Therefore, the one-pot reactions were all performed at pH 7.2 and rt in subsequent experiments.

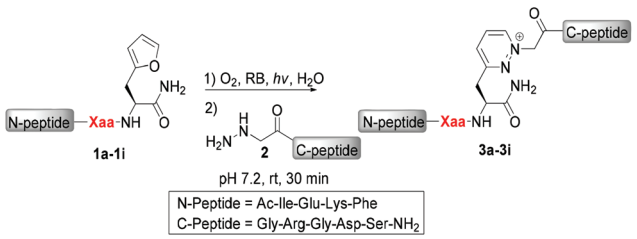


Scheme 2 (a) One-pot pyridazinium-based ligation of furan peptide **1a** with hydrazine peptide **2**. (b) HPLC profiles (λ = 214 nm) for the reaction of oxidised furan **1a** with equimolar peptide **2** added from stock solution of different pH values. Peptide **2** in H_2O: pH 2.9. Buffers used: 0.1 mM acetate buffer pH 4.7 and 1.0 M phosphate buffer pH 5.8 and 7.2. (*) indicates ligation intermediates and (#) oxidized species. (c) HPLC and MALDI-TOF analysis verifying the formation of the ligated peptide **3a** in 30 min at pH 7.2 (obs: 1383.98; calc.: 1383.71).

It is worth mentioning that during HPLC analysis formation of a small amount of side-product could be detected (absorbing only at 254 nm, Fig. 37, ESI†), the mass of which reveals the intramolecular reaction of oxidized furan with the C-terminal amide.[45,46] This side reaction could be circumvented when excess of peptide **2** was added (5.0 equiv.) or when reaction was performed in an all-in-one fashion by photooxidation of furan peptide **1a** in presence of 10 μM of RB and 5.0 equiv. of hydrazine peptide at pH 7.2 (Fig. 44, ESI†). The photooxidation was virtually complete after 15 min of irradiation with the instant formation of ligated product **3a**. Conversion was virtually quantitative after 6 h. Although this all-in-one ligation procedure exhibits practicality, we proceeded with the sequential optimized conditions to allow better evaluation of and control over all aspects of the entire reaction process.

Additionally, MALDI-TOF analysis confirmed the success of obtaining the desired conjugate, yet also showed the formation of pyridazinium and pyridazine fragmentation adducts, formed during the ionization process.[47] This led us to evaluate the stability of the formed conjugate. In this way, potential intermolecular reaction of the conjugate with exogenously added nucleophilic species was evaluated by adding an excess of glutathione (GSH). Following the optimized protocol, *i.e.* photooxidation of **1a** in water and reaction for 30 min with equimolar peptide **2** added from a stock solution of pH 7.2, GSH (10.0 equiv.) was added to the reaction mixture. No obvious degradation of the ligated product was observed after 72 h of incubation. Furthermore, we evaluated the pH sensitivity of the formed conjugate by incubating isolated conjugate **3a** in medium of different pH values of 3, 4, 8 and 9 over time. Interestingly, the resulting conjugate was found to be stable in water at pH 4 and 8, whereas some degradation was observed at pH 3 and 9 respectively (Fig. 46, ESI†). With the optimized conditions in hand, we examined the scope of the ligation of C-terminal furan peptides by varying the identity of the preceding amino acid (Ac-Ile-Glu-Lys-Phe-**Xaa**-Fua-NH_2, Table 1). As prolonged irradiation time can cause damage of peptides, thus affecting successful ligation, we herein also present the optimised oxidation time for each peptide. Gratifyingly, in all but one case, the ligation proved efficient and chemoselective. In particular, Table 1 shows that unprotected nucleophilic residues adjacent to Fua such as Lys, Arg, Ser and Glu are well compatible with the ligation, while Cys led to the formation of a complicated mixture of oxidised species and conjugates (Fig. 61, ESI†). Protecting the cysteine side chain either by disulfide formation or with a stable protecting groups is advisable.[48] A hydrophobic Ala neighbouring residue as well as a sterically hindered Val residue allowed relatively good conversion. Regarding the sensitive amino acids, a Trp containing-peptide could be efficiently ligated to peptide **2** (Fig. 57, ESI†), while reaction with Met-peptide resulted in the formation of the corresponding sulfoxide conjugate. These results suggest that the singlet oxygen-mediated ligation of peptides can toler-

Table 1 Scope of the ligation of C-terminal furan peptides with N-terminal hydrazine peptide **2**



Entry	Xaa	Oxidation time (min)	Conversion[a] (%)
1	Lys	40	62
2	Arg	40	99
3	Ser	40	79
4	Glu	40	72
5	Ala	30	86
6	Val	40	61
7	Trp	30	95
8	Met	15	99
9	Cys	30	n.d.[b]

[a] Conversion was quantified by HPLC as consumption of oxidized species. [b] Conversion not defined due to complicated mixture of products (Fig. 61–63, ESI).

ate a broad range of amino acids thus demonstrating the general applicability of the present methodology.

Subsequently, a positional scan of the furan moiety in diverse peptides was carried out (Table 2). At the outset, internally incorporated furan was tested. When only 1.0 equivalent of peptide **2** was added to the oxidized peptide **4a**, concurrent formation of the intramolecular side-product was observed after 1 h of reaction and no further transformation to the desired conjugate was observed even after 24 h, presumably due to sterical hindrance. However using a slight excess of the hydrazine peptide (5.0 equiv.) drove the reaction to quasi completion in 30 min (Table 2, entries 1–3). Next, we incorporated furylalanine at the N-terminal position to test the conjugation efficiency. Likewise, slight excess of the hydrazine peptide (5.0 equiv.) led to the fast and efficient formation of the desired conjugates in 30 min (Table 2, entries 4 and 5).

After extensive exploration of ligation reactions with N-terminal hydrazines, we wished to explore the possibility of

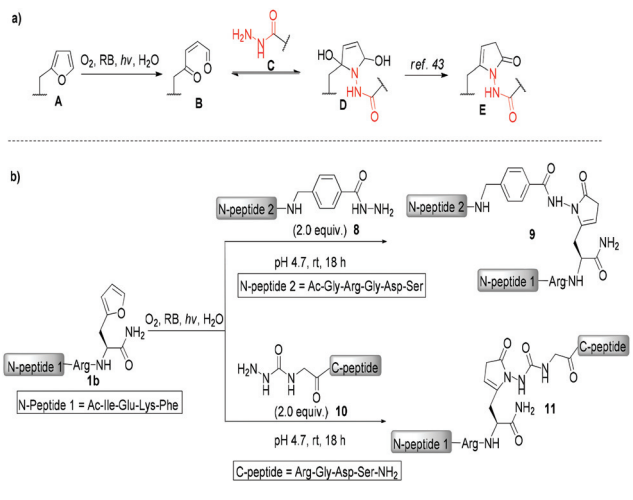
Table 2 Pyridazinium-based ligation of internal and N-terminal furan peptides with N-terminal hydrazine peptide **2**[a]

Entry	Furan peptides	Conversion[b] (%)
1	Ac-Ile-Glu-Lys-Phe-**Fua**-Gly-NH_2 (**4a**)	62 (**6a**)
2	Ac-Ile-Glu-Lys-Gly-**Fua**-Gly-NH_2 (**4b**)	73 (**6b**)
3	Ac-Ile-Glu-Lys-His-**Fua**-Lys-NH_2 (**4c**)	77 (**6c**)
4	Ac-**Fua**-Ile-Glu-Lys-Phe-Gly-NH_2 (**5a**)	87 (**7a**)
5	Ac-**Fua**-Gly-Glu-Lys-Phe-Gly-NH_2 (**5b**)	63 (**7b**)

[a] All peptides were photooxidised for 40 min followed by addition of 5.0 equiv. of hydrazine peptide **2**. Peptide **2** was dissolved in 1.0 M phosphate buffer pH 7.2. [b] Conversion was quantified by HPLC as consumption of oxidized species after 30 min of reaction.

performing coupling of the furan peptides with a C-terminal nucleophilic peptide. To the best of our knowledge, the introduction of a hydrazine moiety at the peptide C-terminus through solid phase synthesis has not yet been described, in contrast with C-terminal hydrazides which are widely employed.[49,50] Based on our previous study,[43] it was anticipated that reacting with a hydrazide peptide would not lead to the formation of pyridazinium products, but to the corresponding pyrrolidinones of type **E** (Scheme 3a). C-Terminal furan-peptide Ac-Ile-Glu-Lys-Phe-Arg-Fua-NH_2 (**1b**) was photooxidised in water followed by addition of C-terminal benzoic hydrazide **8** (2.0 equiv.) added from a stock solution at pH 4.7 where hydrazone reactions are fastest (Scheme 3b).[37,39] The ligation product **9** was almost completely formed in 2 h, however the reaction was left for 18 h to ensure complete transformation of ligation intermediates. Further successful illustration of our methodology was provided when a peptide bearing a semicarbazide moiety at the N-terminus,[51,52] was incorporated into the cascade reaction sequence. Accordingly, peptide **1b** was efficiently ligated with semicarbazide **10** under the same conditions (2.0 equiv., pH 4.7, Scheme 3b).

In summary, we have developed an expedient and site-specific methodology for the singlet oxygen-mediated ligation of furan and nitrogen-nucleophilic peptides. Reactions proceed smoothly under physiological conditions with satisfactory conversions. The procedure was found to be suitable for a range of peptides featuring different amino acids next to the C-terminal furan moiety. The possibility of incorporating furan at any desired position within a peptide and ligating it with hydrazine peptides offers flexibility in the developed methodology. Additionally, pyrrolidinone-based peptides could be efficiently obtained by using peptide hydrazides, providing alternative routes for ligation with furan peptides. It is thus envisioned that singlet oxygen-mediated furan bioconjugation



Scheme 3 (a) General concept for the formation of pyrrolidinone-based conjugates. (b) Pyrrolidinone-based conjugation of a C-terminal furan-peptide to a C-terminal benzoic hydrazide peptide and to an N-terminal semicarbazide peptide.

reactions could be useful for the fast and efficient construction of multi-functional and branched peptide-conjugates for various biochemical studies and applications.

Conflicts of interest

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