From DNA cross-linking to peptide labeling: on the versatility of the furan-oxidation–conjugation strategy†

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Based on the incorporation of commercially available *N*-Fmoc-furylalanine, a new method for peptide labeling is proposed, relying on the selective oxidative transformation of the furan moiety into a reactive aldehyde and subsequent reductive amination.

Modern chemical biology oriented research frequently relies on the ability to site-selectively modify biomacromolecules such as peptides, carbohydrates and oligonucleotides. Although the so called "click" 1,3-dipolar cycloaddition between alkynes and azides has revolutionized the field of bioconjugation chemistry, it does require the introduction of unnatural functionality in both partners.[1–4] As for methods relying on only one unnatural reaction partner, mostly one makes use of the naturally available nucleophilic biomolecule functionalities such as amines and alcohols. Also, maleimide conjugation with thiols is frequently used,[5,6] but has recently been found to lead to heterogeneous conjugates due to susceptibility of the imido group to spontaneous hydrolysis.[7] Carbonyl condensation reactions represent an often used alternative.[8,9] However, site-selective introduction of selectively modifiable carbonyl moieties into biomolecules is not straightforward, in particular the introduction of aldehydes still represents a major synthetic hurdle.[10]

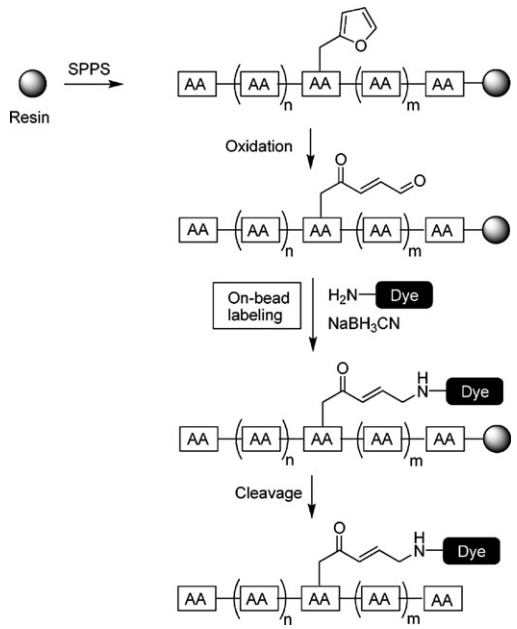
Recently *random* incorporation of aldehyde functionality into proteins has been demonstrated.[11] Existing methods for *site-selective* introduction of aldehyde functionalities are centered around oxidation of N-terminal serine residues.[12] Internal or C-terminal aldehyde functionality can be generated only through the use of building blocks requiring lengthy syntheses and/or making use of orthogonal protection schemes.[13–16]

We earlier developed a methodology for site-selective DNA-cross-linking based on the *in situ* oxidation of an incorporated furan moiety, generating a reactive aldehyde in a site-selective way.[17] We here report on our results indicating that a similar strategy can be applied in a totally different chemical context, namely that of peptide synthesis. We describe the application of this furan-oxidation principle for the site-selective introduction of aldehyde functionality in peptides. As a first proof of principle, we set out to explore the potential of the proposed methodology *via* reductive amination based labeling of solid-phase bound peptide aldehydes. In a peptide labeling context, most existing site-selective techniques rely on amine derivatization, where selectivity issues need to be solved by either specifically modifying N-termini [18] or using carefully balanced orthogonal protection strategies.[19–22]

Recently, a few elegant methodologies have been developed based on orthogonal chemistries such as enzyme-mediated peptide modification[23] or Pd-catalyzed coupling reactions.[24] However, substrate selectivity counteracts generality in the former method while the latter method is limited to arylations. Site-selective labeling of peptides would therefore greatly benefit from an easily applicable inverse chemistry, using nucleophilic labels to react with a specifically located electrophilic moiety in the peptide.

Our proposed strategy for the generation of aldehyde functionality (Scheme 1) involves incorporation of a furan moiety as a masked reactive aldehyde. Oxidative ring opening of the furan moiety generates an enal functionality with various possibilities for conjugation, labeling and cross-linking. The furan moiety can be introduced by capping the sequence with 3-(2-furyl)propionic acid or by using commercially available Fmoc-β-2-furylalanine, allowing introduction at any desired position in the peptide.

First experiments were conducted on TentaGel resin using a photocleavable linker for easy reaction monitoring at every stage. A small test peptide **1** was synthesized and capped with



Scheme 1 General strategy for peptide labeling on solid phase (SPPS = solid phase peptide synthesis).

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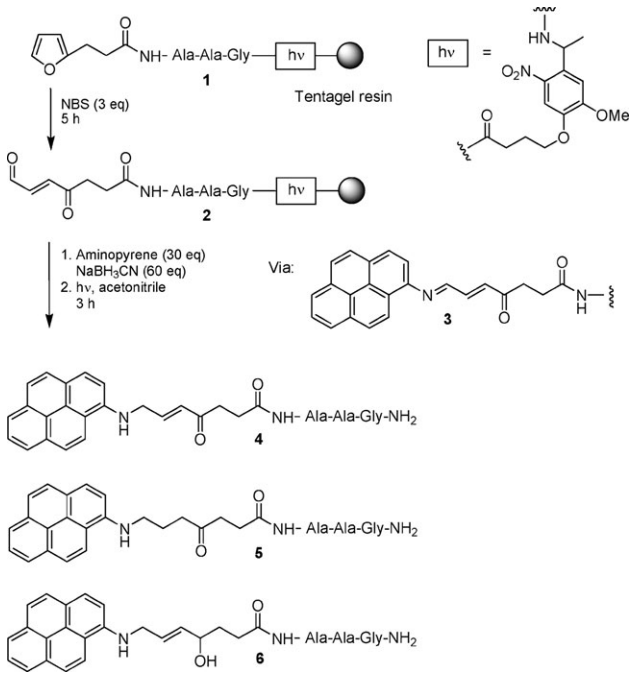
† Electronic supplementary information (ESI) available: Experimental procedures, RP-HPLC and MS data for all labelled peptides. NMR data for labelled peptide **8**. See DOI: 10.1039/b817447d



Scheme 2 Labeling of TentaGel bound peptides and photolytic cleavage.

3-(2-furyl)propionic acid (Scheme 2). In the earlier reported DNA cross-linking strategy *N*-bromosuccinimide (NBS) was used for the oxidative transformation of the furan moiety.[17]

This reagent seems unsuitable in this peptide context as it was described in the early 1960s for fragmentation of polypeptides particularly at the level of tryptophan and tyrosine residues.[25] It was however later shown that in fully protected peptides bound to a hydrazide resin, selective oxidation of the hydrazide linkage is possible under mild NBS conditions.[26] The method was even shown to be compatible with oxidation-sensitive residues, provided suitable protecting groups and oxidative conditions are used.[27]

In test peptide **1**, the furan moiety could be selectively oxidized without side reactions using only 3 equiv. of NBS in THF–acetone–water (5 : 4 : 2). ESI-MS analysis after cleavage confirmed the presence of an oxidation product with

Table 1 Optimization of labeling conditions on TentaGel

Entry	Equiv. AP	Equiv. $NaBH_3CN$	Reaction time	Solvent[a]	Products[b]
1	30	60	20 h	A	**4** (16%) **5** or **6** (84%)
2	30	60	4 h	A	**3** (15%) **4** (60%) **5** or **6** (25%)
3	30	60	20 h	B	**3** (22%) **4** (58%) **5** or **6** (20%)
4[c]	30	60	4 h	A	Complex
5[c]	1	1	1 h	A	**8** (83%)

[a] Solvent A = THF–H_2O–15% aq. CH_3COOH (3 : 1 : 1); solvent B = THF–H_2O (3 : 1). [b] Percentages refer to relative amounts of labeled peptides as determined *via* HPLC. [c] Experiments on Rink amide AM resin involving acidic release of peptides.

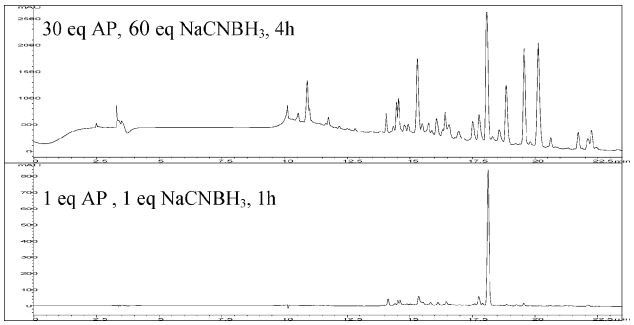


Fig. 1 Labeling on Rink resin: HPLC analysis of crude cleaved peptide. Initial conditions (top) and optimized conditions (bottom).

a MW corresponding to **2**. Reductive amination was then performed with 30 equiv. of 1-aminopyrene (AP) and 60 equiv. of sodium cyanoborohydride in THF–water–15% aq. acetic acid (3 : 1 : 1) for 20 h. Cleavage of the resulting product and LC-MS analysis showed that a labeled product was formed.

Under these initial conditions, however, this turned out to be a product of over-reduction, where next to the imine functionality in **3**, 1,2- and 1,4-reduction had occurred yielding a mixture of **4**, **5** and **6** (see Scheme 2 and Table 1, entry 1). When reaction times were reduced (entry 2) or the reaction was run in the absence of acetic acid (entry 3), higher yields of amine **4** were obtained, but next to products of over-reduction the unreduced imine **3** was also still present. In all cases low recovery was a serious problem and we reasoned this was caused by quenching of the light by the excess of pyrene label used, thus interfering with the photolytic cleavage from the resin.

In a second series of experiments we therefore turned to the use of the acid labile Rink amide resin. Initially, labeling was performed using 30 equiv. of aminopyrene and 60 equiv. of $NaBH_3CN$. After cleavage a complex reaction mixture was observed (entry 4 and Fig. 1, top). Since the reaction product contained doubly labeled products as well as products of over-reduction, reaction times and the number of equiv. aminopyrene and $NaBH_3CN$ added were gradually lowered. Surprisingly, reductive amination with only 1 equiv. of aminopyrene and 1 equiv. of $NaBH_3CN$ in THF–water–15% aq. acetic acid (3 : 1 : 1) for a period of 1 h led to a perfectly clean labeled product as evidenced by the crude chromatogram in Fig. 1 (bottom).

Upon ESI-MS analysis (Fig. 2), the molecular weight of the labeled peptide turned out to be 18 mass units lower than

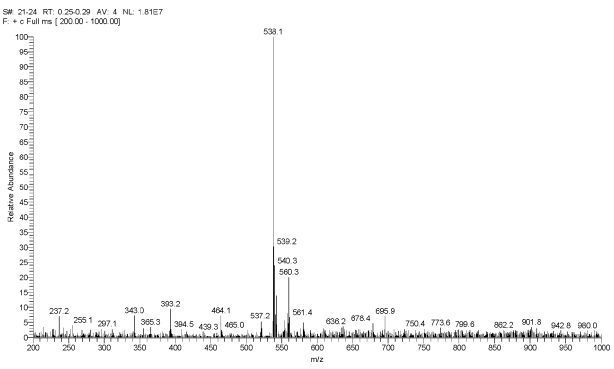
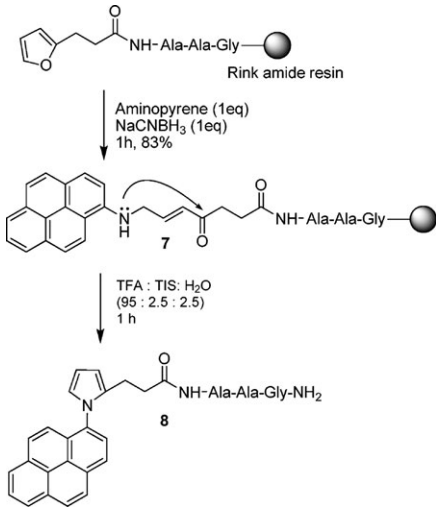


Fig. 2 ESI-MS analysis of crude labeled peptide **8**.



Scheme 3 Labeling and pyrenylpyrrole formation on Rink amide AM resin.

Table 2 Labeling tests on functionalized resins

Entry	Peptide	Resin[a]	Purity[b]
1	Ac–FurAla–Ala–Ala–Gly	R	80%
2	Fur–Ala–Cys(S*t*Bu)–Gly	R	70%
3	Fur–Ala–Met–Gly	R	85%
4	Fur–Ala–Arg(Pbf)–Gly	R	68%
5	Fur–Ala–Ser(*t*Bu)–Gly	R	80%
6	Fur–Ala–Trp(Boc)–Gly	R	64%
7	Fur–Ala–Tyr(*t*Bu)–Gly	R	78%
8	Fur–Phe–Gln(Trt)–Gly–Ile–Ile	W	93%
9	Fur–Ile–Leu–Pro–Glu(*t*Bu)–Ile	W	79%
10	Fur–Ala–His(Trt)–Asn(Trt)–Leu–Ala	W	80%
11	Ac–Leu–FurAla–Gly–Lys(Boc)–Val	W	71%

[a] R: Rink amide AM resin; W: Wang resin. [b] As determined from the crude chromatogram.

expected. Mechanistically, attack of the amine on the enone functionality is possible, yielding a cyclized product which can aromatize upon loss of H_2O (see Scheme 3). The structure of the obtained material was confirmed by ^{1}H-NMR analysis of the cleaved and isolated peptide **8** (see ESI†). Upon $NaBH_4$ treatment of the resin before cleavage, pyrenylpyrrole formation was suppressed and the labeled peptide can be cleaved as the aminopyrene derivative.

To demonstrate the scope of this approach in a more general context, a series of peptides was made, incorporating different functionalized amino acids (see Table 2). Incorporation of an internal furan moiety (using *N*-Fmoc-furylalanine) evenly provided labeled peptides in good yield. As for labeling using amines other than aminopyrene, peptides labeled with 7-amino-4-methylcoumarine have been generated with similar purity (see ESI†).

In summary we have developed a novel strategy for site-selective fluorescent labeling on solid phase *via* transformation of an incorporated furan moiety into an aldehyde functionality. Selective oxidation of the furan moiety can be conducted using 3 equiv. of NBS and releases the caged electrophilic keto-enal at any required position in very short time. The methodology is very straightforward in terms of the building blocks used (commercially available *N*-Fmoc-2-furylalanine). More importantly, incorporation of this residue can be easily achieved at any position, including the C-terminus. Furthermore, the current labeling technique is orthogonal to earlier developed methods based on N-terminal labeling or incorporation of protected lysine derivatives. For this and the previous reason, the current method offers attractive opportunities for the straightforward synthesis of doubly labeled peptides which are often used in FRET based screening strategies.

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