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COMMUNICATION

Aromatic capping surprisingly stabilizes furan moieties in peptides against acidic degradation†

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We herein describe the synthesis of furan containing peptides for further post-synthetic derivatisation in solution through our recently developed furan-oxidation-labeling technology. Previously, it was reported by others that during acidic cleavage of furan-modified peptides, furan moieties can suffer from degradation. We demonstrate here that this degradation is position dependent and can be fully suppressed through introduction of proximate aromatic residues. Versatile introduction of 2-furylalanine at internal, C-terminal as well as the sensitive N-terminal positions has now been proven possible.

Recently our group developed a highly efficient nucleic acid crosslinking methodology based on the selective oxidation of a furan moiety incorporated into oligonucleotides.[1] It was subsequently shown that translation of the methodology to the area of peptide chemistry was possible. Indeed, we further reported on the development of a method for the labeling of peptides on solid support by incorporation of a furan residue into the solid phase bound peptide. Following oxidative ring opening of the furan ring into a highly reactive enal and subsequent reductive amination, labeling could be carried out with the peptide still bound to the solid support.[2] As cleavage of peptides from resins often requires acidic or basic conditions that are not always compatible with the introduced labels, it is of interest to further develop the methodology for post-synthetic furan based labeling in solution. As for the labeling methodology of peptides in solution, the introduction of a fluorescent label at the N-terminus has been widely described. The amine reactive probe FITC can be selectively introduced at the N-terminus under buffered conditions at pH 8.5.[3] Alternatively, peptides can be prepared by using the Boc/Bzl strategy, using a Fmoc-protecting group for lysine residues.[4] Following acidic cleavage, the N-terminus can be selectively labeled with a fluorophore after which the final N-terminally labeled peptide can then be obtained upon lysine deprotection. For C-terminal labeling the carboxylic group can be left to react with a fluorophore amine group such as aminocoumarin using a variety of coupling reagents.[5–8] However, low yields have been observed due to the reduced nucleophilicity of aromatic amines, and epimerization seems to be a severe problem. As for the internal labeling of peptides, mostly the natural available nucleophilic functionalities are used. Amine groups of lysine residues can be modified by reaction with isothiocyanates,[9,10] active esters,[11,12] sulfonyl chlorides[13] or aldehydes,[14,15] while thiols can be modified by reaction with maleimides.[16,17] However, these nucleophilic functionalities are widely occurring in peptides and proteins, rendering it difficult to obtain monolabeled or site-specifically labeled species.

Therefore, it would be of use to introduce a unique electrophilic functionality into the peptide. As has been shown and reported by others earlier, site specific incorporation of an aldehyde functionality still remains a hurdle as the available building blocks require lengthy syntheses, mostly combined with additional protecting group chemistries.[18,19] Introduction of a furan moiety as a masked reactive enal functionality appears to be an attractive alternative to existing methods, since commercially available building blocks such as 3-(2-furyl)-propenoic acid for N-terminal introduction or N-α-Fmoc-β-(2-furyl)-L-alanine for internal incorporation are ready to use in solid phase peptide synthesis.

In continuation of our previous work where a solid-phase based peptide labelling method was developed,[2] exploiting a furan moiety internally incorporated in the peptide, here we set out to explore the translation of our furan-oxidation technology towards solution conditions. However, in the first experiments major difficulties were experienced upon cleavage of the furan-peptides from the solid support. In the context of our research on peptide–DNA binding studies,[20] we envisaged the synthesis of a furan containing analogue of the PhoB peptide, a minimized epitope of the DNA-binding domain of the transcription factor PhoB from *Escherichia coli*, described by Eckel and coworkers.[21] As illustrated in Scheme 1, in a first attempt the DNA-binding PhoB peptide was synthesized introducing a furan moiety through N-terminal capping with 3-(2-furyl)propanoic acid for further DNA binding studies with labeled versions of this peptide. Upon using a standard cleavage cocktail containing trifluoro acetic acid (TFA), dithiothreitol (DTT) and H_2O, cleavage of **1** from the chlorotrityl resin yielded a very complex

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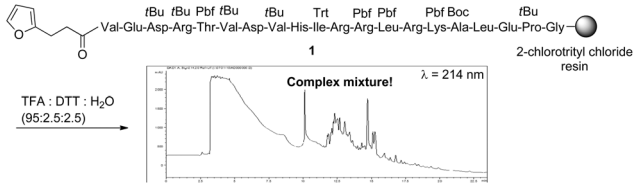
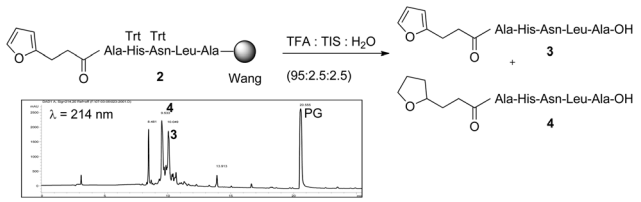
†Electronic supplementary information (ESI) available: Detailed procedures for peptide synthesis and cleavage with relevant LC-MS analysis for peptides **1** and **2**. HPLC and MALDI-TOF analysis for peptides **14–18** and their oxidation products. ESI-MS analyses for peptides **23** and **24**. See DOI: 10.1039/c2ob25548k



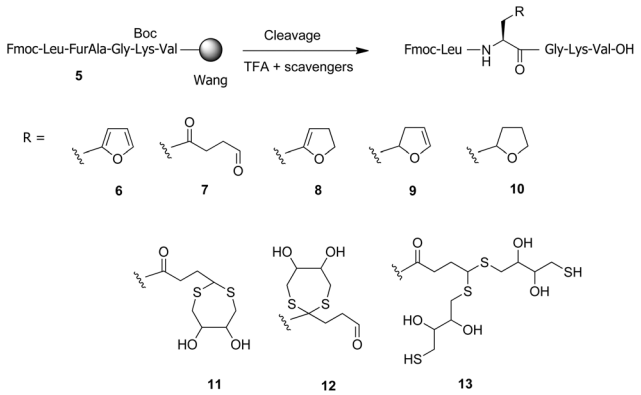
Scheme 1 HPLC analysis after cleavage of N-terminal furan containing PhoB peptide with TFA, DTT and water.



Scheme 2 HPLC-analysis of an N-terminal furan containing peptide pentamer after cleavage with TFA, TIS and water.

reaction mixture as evidenced by RPHPLC (Scheme 1). Furthermore LC-MS analysis showed that none of the mass signals could be correlated to the expected product. As in 2004 Schulz and coworkers already reported the partial degradation of furan as a consequence of using TFA and scavengers,[22] we figured that the main problems occurred during the acidolytic cleavage of the peptide. To analyse the problems in detail and possibly remediate, the simpler pentamer sequence **2**, for which side products are more easily analysed by LC-MS, was prepared as a test peptide on Wang resin. We decided not to use DTT as a scavenger and replaced it by triisopropylsilane (Scheme 2). However, when testing a cleavage cocktail of TFA, TIS and water for the cleavage of **2**, though the situation improved, under these conditions hydrogenation of the aromatic furan ring proved to produce a side product which is difficult to separate from the desired compound. A mixture containing reduced tetrahydrofuranyl peptide **4** as a major compound was obtained.

We reasoned that the situation might improve when the furan moiety is more sterically hindered, *i.e.* located closer to the backbone of the peptide as is the case for a β-(2-furyl)-L-alanine (FurAla) residue. It was therefore analyzed whether incorporating a FurAla residue towards the interior of a peptide would give improvement. A new peptide was therefore conceived on Wang resin containing an internally incorporated FurAla residue. Additionally, to allow evaluation of the influence of nucleophilic side chain functionalities a Lys residue was introduced. The obtained solid phase bound peptide **5** was treated with a range of cleavage cocktails (Scheme 3 and Table 1). Careful LC-MS analysis and characterisation of the cleaved products showed that under the influence of TIS partial or complete hydrogenation of the furan ring can take place (*cf.* compounds **8**, **9** and **10**), while thiol scavengers can lead to thioacetal formation (compounds **11** to **13**). From this comparative study it can be observed that combinations in which either phenol or EDT are used as scavengers lead to further unidentified products and give rather low yields of the desired furan containing peptide. On the other hand, combinations containing either TIS or DTT lead to more favorable ratios of the desired product.



Scheme 3 Products obtained upon cleavage of an internally FurAla modified peptide from the resin.

Table 1 Ratio of products **6–13** formed after cleavage of the internally FurAla modified peptide **5** from the resin

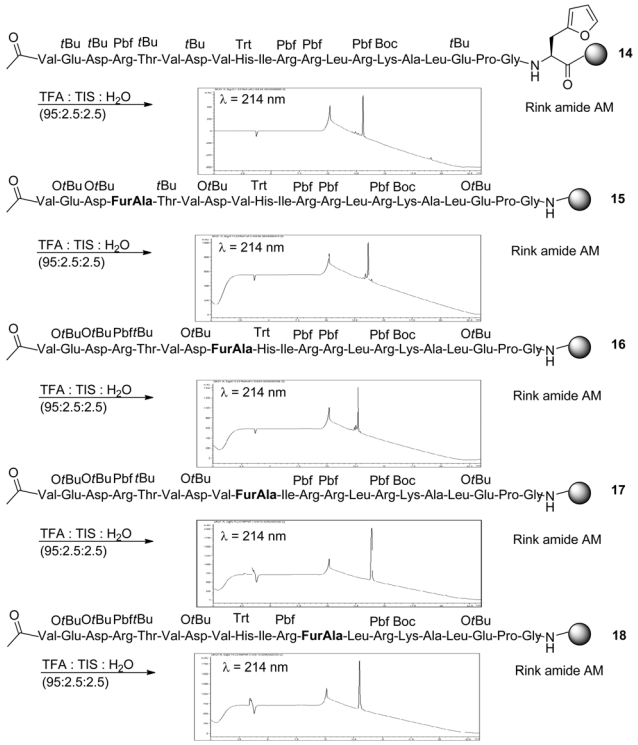
Cleavage cocktail		Products[a]
TFA : H_2O	95 : 5	**6** (50%) + **7**
TFA : TIS	95 : 5	**6** (65%) + **8** + **9** + **10**
TFA : TIS : H_2O	95 : 2.5 : 2.5	**6** (55%) + **7** + **8** + **9** + **10**
TFA : phenol	95 : 5	**6** (60%) + unident. prod.
TFA : phenol : H_2O	95 : 2.5 : 2.5	**6** (30%) + **7** + unident. prod.
TFA : phenol : H_2O : TIS	88 : 5 : 5 : 2	**6** (45%) + **8** + **9** + **10**
TFA : EDT	95 : 5	**6** (25%) + unident. prod.
TFA : EDT : H_2O	95 : 2.5 : 2.5	**6** (25%) + unident. prod.
TFA : DTT	95 : 5	**6** (40%) + **11** + **12** + **13**
TFA : DTT : H_2O	95 : 2.5 : 2.5	**6** (80%) + **11** or **12**

[a] As determined from the crude chromatogram.

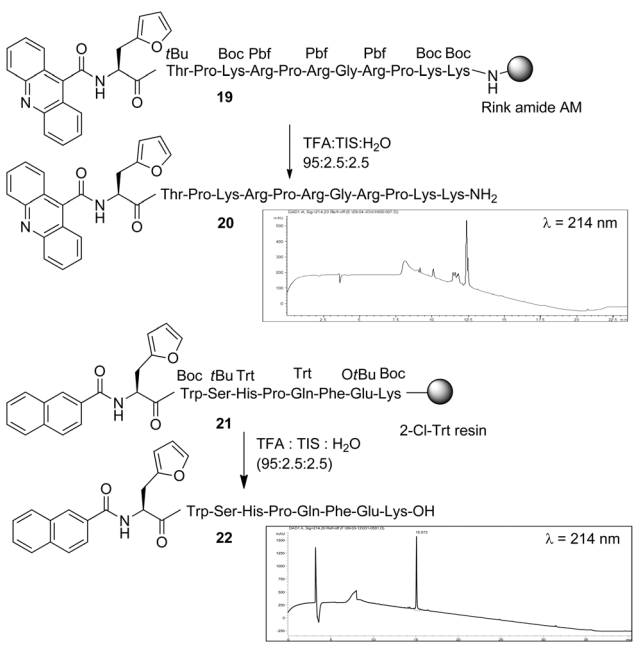
Having performed this comparative analysis, we decided to further investigate the influence of the exact positioning of the furan moiety within the peptide by preparing a series of the originally envisaged PhoB 20-mer peptides (*vide supra*) varying the position of the furylalanine (Scheme 4). A Rink amide linker was chosen because of the more reliable loading of the first residue compared to 2-chlorotrityl linker which was used for peptide **1**. When cleaving 20-mer peptide **14** which contains a C-terminal β-(2-furyl)-L-alanine residue, the furan moiety was found to be completely stable during the acidolytic cleavage with TFA, TIS and water and pure furan-modified peptide was obtained.

Likewise, for all other depicted sequences containing an internal β-(2-furyl)-L-alanine moiety, pure products were obtained corresponding to the desired peptides (Scheme 4, peptides **15–18**). No hydrogenation of the furan ring was observed. However, when N-terminal furan introduction was carried out with Fmoc-Fur-Ala again complete degradation was observed upon cleavage from the solid support similar to the observations previously made with an N-terminal 3-(2-furyl) propanoic acid residue (*cf.* Scheme 2).

Surprisingly, while studying in parallel a few other DNA-binding HMG-I(Y)-peptides, which were capped with 9-acridine-carboxylic acid in an attempt to enhance the stability of the peptide–DNA complex, we discovered that the stability of the

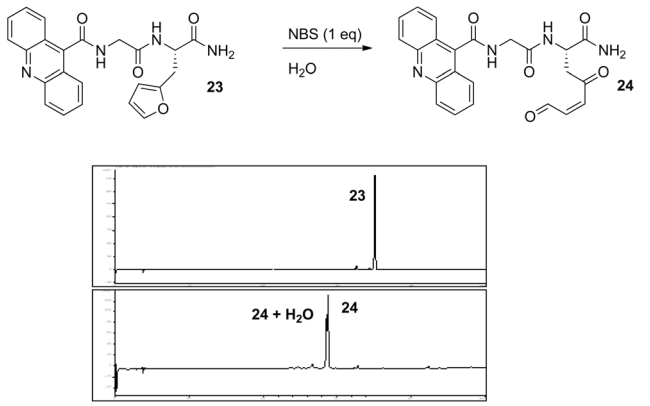


Scheme 4 HPLC-analysis after cleavage of C-terminally and internally furan-modified peptides with TFA, TIS and water (95 : 2.5 : 2.5). The position of the FurAla residue is indicated in bold.



Scheme 5 HPLC-analysis after cleavage of N-terminally furan-modified peptides capped with an aromatic moiety.

N-terminal furan ring can be enhanced by capping the N-terminus with an aromatic moiety (Scheme 5). Two test peptides were prepared with an N-terminal furylalanine residue which was further capped with an aromatic unit. The first peptide **19** was capped with acridine-9-carboxylic acid, while the second peptide **21** was capped with 2-naphthoic acid. Both peptides were cleaved from the solid phase with a mixture of TFA, TIS and water and could be obtained pure. The furan moiety remained intact during the acidic cleavage. A tentative explanation for this unexpected stability of the N-terminal furan ring capped with an aromatic moiety can be found in a π–π stacking of the aromatic moiety with the furan ring, possibly protecting this moiety against side reactions during acidic cleavage.



Scheme 6 HPLC-analysis of oxidation of furan-containing peptide **23** in solution.

Having established suitable synthesis and cleavage conditions for furan-peptides, with variable positioning of the furan moiety throughout the peptide, we further explored various oxidation conditions for generation of the desired reactive unsaturated aldehyde functionality. Fast scanning of oxidation conditions was carried out on the short FurAla containing peptide **23**. One of the above used N-terminal capping moieties was included to ascertain and show that the acridine moiety does not negatively influence the outcome of the oxidation reaction.

During the optimisation studies, it was shown that treatment of furan-peptides with 1 equivalent of NaOCl did not lead to reaction and upon increasing the amount of oxidant, only partial oxidation could be observed next to peptide degradation products and remaining starting material. Next, a combination of Rose Bengal and visible light irradiation, known to generate singlet oxygen, was applied. Though most of the starting material could be converted into the desired enal, next to remaining starting material also products of over oxidation could be detected.

However, the use of a single equivalent of NBS led to clean conversion into the desired furan-oxidized peptide **24** in 1 hour. As evidenced from the RPHPLC analysis in Scheme 6, an extra product was visible. Through MS analysis (see ESI†) it was shown that the originally obtained reactive aldehyde was accompanied by its hydrate as can be expected in aqueous solution. It was further established that also for the furan-containing PhoB 20-mer peptides (*vide supra*, Scheme 4) oxidation could be achieved using NBS (see ESI†). MALDI-TOF analysis of the oxidation reactions revealed a concomitant loss of H_2O in case of peptides obtained from resins **14** and **17**, resulting from an intramolecular imine formation with the proximate lysine residues, illustrating the availability of the unsaturated aldehyde for reaction with nucleophiles.

Conclusions

In summary, the introduction of a furan moiety in peptides *via* solid phase peptide synthesis and subsequent release of the furan containing peptides in solution was optimized. Next to C-terminal and internal modification of the peptide with a furan moiety proceeding smoothly using N-α-Fmoc-β-(2-furyl)-L-alanine, N-terminally furan-modified peptides can be obtained in pure form through capping of the peptide with an aromatic moiety in order to protect the furan ring against degradation during acidolytic cleavage. It was further shown that the furan moiety in the resulting peptides can be selectively converted into a reactive enal upon oxidation with NBS. In that sense current methodology allows for easy and site selective introduction of a caged electrophilic functionality into peptides allowing oxidation triggered activation at any desired moment for further labeling or conjugation purposes.

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