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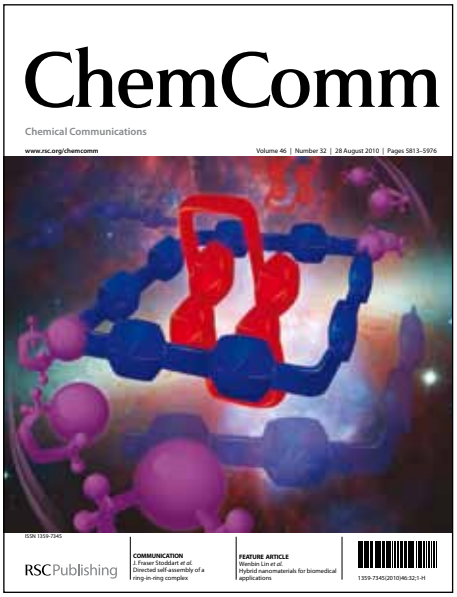

Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Exploiting Furan's Versatile Reactivity in Reversible and Irreversible Orthogonal Peptide Labeling

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

A general method for the facile and versatile decoration of peptides is proposed exploiting furan based cycloaddition and electrophilic aromatic substitution reactions. Given the commercial availability of furylalanine derivatives for peptide synthesis, the current work significantly enlarges the toolbox of available methodologies for site specific labeling and conjugation of peptide probes.

Site-specific and selective biomacromolecule modification has become an area of research with ever-growing importance in biological, biochemical, diagnostic and medicinal developments.[1] Since peptides, proteins and nucleic acids feature a large variety of functional groups with often overlapping reactivity profiles, site-selectivity is a major concern. It drives the quest for the development of compatible and orthogonal chemistries for postsynthetic decoration with fluorescent labels, affinity tags, spin probes and other types of biomolecules (e.g. peptide-oligonucleotide, protein-carbohydrate conjugates).[2]

In this respect, chemoselective derivatisation via the Diels-Alder cycloaddition reaction has naturally attracted attention as a highly promising method. Recent reviews describe the versatility of Diels-Alder ligation for the decoration of peptides[3], proteins[4] and nucleic acids.[5] Frequently employed Diels-Alder partners for peptide and protein modification are the 2,4-hexadienyl or cyclopentadienyl/maleimide[3,4] and the tetrazine/norbornene or cyclooctene couples.[6] Besides these classical dienyl systems,[7] a furan moiety has also been used to partner up with the maleimide dienophile in the case of covalent conjugation of nucleic acids.[8,9] However, to the best of our knowledge, furan-based Diels-Alder conjugations have not been explored yet for the decoration of peptides and proteins, in spite of the fact that β-(2-furyl)-alanine is a commercially available building block.

An explanation for this apparent oversight might be found in the fact that, until recently, incorporation of furan moieties into peptides was thought to present severe hurdles linked to furan instability during resin cleavage and side chain deprotection, as reported by Schulz and coworkers in 2004.[10] In a more recent contribution dealing with the site specific incorporation of furan at various positions in a peptide, we showed that these hurdles can be overcome by slightly adapting the reaction conditions.[11] Additionally, the recently reported procedure of Davis and coworkers on the Suzuki-Miyaura based introduction of furan onto genetically encoded aryl halides[12] opens the way to site-specific furan incorporation into proteins. These recent synthetic

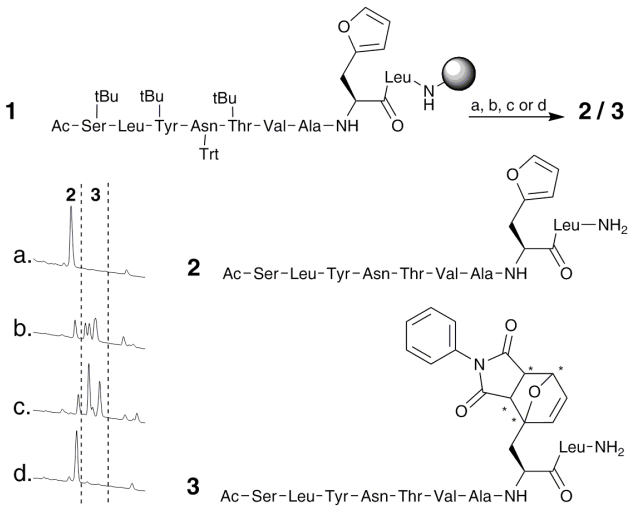

Fig.1 Relevant peptide structures and corresponding HPLC traces of reaction mixtures after cleavage of: a) peptide **1**; b) peptide **1** after treatment with 20 equiv. N-phenylmaleimide in toluene, 48h at RT; c) peptide **1** after treatment with 20 equiv. N-phenylmaleimide in toluene, 24h at 70°C; or d) retro-Diels-Alder after reaction c by heating the solid supported products mixture in toluene for 24h at 70°C. Each reaction was followed by cleavage and deprotection with TFA/TIS/H_2O (95:2,5:2,5), 2h at RT for HPLC analysis.

advances prompted us to explore furan's unique reactivity for biomolecular labeling and conjugation strategies involving peptides. In order to test the feasibility and usefulness of a furan-based Diels-Alder peptide conjugation strategy, model peptide **1** featuring a furylalanine was synthesized on solid support (Figure 1). Inspired by the earlier successes of Graham and coworkers[8] in oligonucleotide labeling, we chose to evaluate maleimide as potential dienophile partner. This choice is even more relevant as numerous maleimide-derivatised fluorophores and other frequently used labels have been developed and commercialized.

The solid supported and fully protected peptide **1** was thus incubated with an excess of N-phenylmaleimide in a minimal amount of toluene at room temperature for 48 hours. The resulting product was analysed after cleavage from the acid labile Rink amide resin and complete deprotection using TFA. As expected, several products were formed, corresponding to the various diastereoisomers formed during the cycloaddition reaction (see Figure 1, HPLC trace b). As can be seen from the HPLC traces in Figure 1, complete conversion to the desired

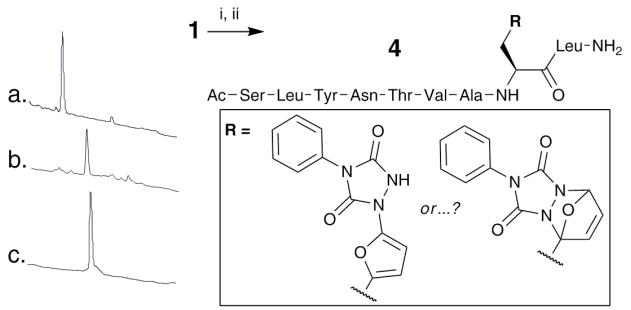

Fig. 2 Reaction conditions: (i) PTAD in DCM, 15 min. at RT; (ii) TFA/TIS/H_2O (95:2,5:2,5), 2h at RT. HPLC traces of reaction mixtures for: a) Peptide **1** after cleavage and deprotection with TFA/TIS/H_2O (95:2,5:2,5); b) Crude HPLC revealed complete conversion; c) Labeled peptide **4** after HPLC purification.

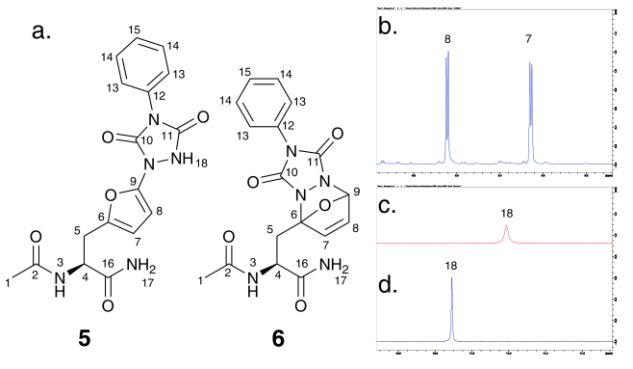

Fig.3 a) Proposed structures for the furylalanine-PTAD adduct; b) 2 doublets between 6,2 and 6,6 ppm; c) broadened signal at 11,5 ppm assigned to exchangeable proton 18 in **5**; d) measurements at -50°C sharpen and move the signal downfield by slowing down exchange.

adduct was not achieved. In an effort to drive the reaction to completion, a fresh reaction mixture was heated to 70°C for 24 hours. Again, similar yields of conversion were observed, albeit with higher selectivity (see Figure 1, HPLC trace c). The obtained conversion of ~85% is consistent with earlier reported experiments.[13] The apparent reluctance of the reaction to achieve completion could be explained by its reversible nature. The retro-Diels-Alder reaction has been illustrated in various cases and even quantitatively exploited for deprotection or cleavage purposes.[14] Indeed, we found that heating of the resin-bound labeled peptide **3** to 70°C for 24 h in toluene, resulted in a complete retro-Diels-Alder conversion. As can be derived from Figure 1 (HPLC trace d) the retro reaction regenerates the starting material with quasi-unaltered purity.

As such, this furan-maleimide Diels-Alder based methodology allows for peptide labeling and conjugation purposes in a way that is completely orthogonal to conventional peptide synthesis strategies. Given the range of commercially available maleimide derivatives, this Diels-Alder strategy thus significantly enlarges the application potential of the maleimide-derivatives based toolbox. The reversibility of this furan-maleimide Diels-Alder reaction has recently been exploited, both in polymer synthesis for the generation of thermoreversible materials[15] as well as in the construction of controlled-release systems for drug delivery[16] or triggered removal of fluorescent labels.[17] This reversible peptide conjugation methodology thus offers interesting perspectives in the area of controlled- or slow-release peptide drugs.

The furan-maleimide reaction, while offering certain advantages in terms of reversibility, still suffers from a non optimal conversion (vide supra, ~85%). Therefore, our attention next turned towards cycloadditions using the more reactive 1,2,4-triazole-3,5-diones (TADs) from which the synthesis of fluorescent derivatives was recently described.[18] TADs have previously shown their use in Diels-Alder type reactions with a range of dienes.[19] As for the reactivity with respect to the furan diene, though Diels-Alder reactions have been described with dialkyl-azo-dicarboxylates and the resulting Diels-Alder adducts unambiguously characterized,[20] to the best of our knowledge only one example of a TAD-furan combination has been reported.[21] Considering the application of TAD derivatives for selective reaction with furylalanine within a peptide containing otherwise natural amino acids, concerns may be raised regarding potential side-reactions with other aromatic side chain functionalities.

Indeed, Barbas and coworkers, in a sole example of the use of 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) in a peptide context,[18] recently showed PTAD to chemoselectively react with unprotected tyrosine giving rise to a formal ortho substituted electrophilic aromatic substitution product. We thus set out to explore the potential usefulness of a furan-based Diels-Alder labeling of furylalanine containing peptides with PTAD. Use of peptide **1**, containing a tyrosine residue, allows direct verification of possible selectivity problems versus natural aromatic side chain residues.

Using only 3 equivalents of 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) in dichloromethane, complete reaction was observed with solid supported peptide **1** in only 15 minutes at room temperature. Judging from HPLC analysis (Figure 2), only a single product is formed. Considering the potential tyrosine versus β-(2-furyl)-alanine selectivity issue the resulting peptide was studied by detailed NMR analysis at 700 MHz. The main concerns are possible side-reactions at the tyrosine residue and the actual structure of the cycloaddition product with furan. Although the tyrosine side chain is protected during the labeling reaction on support, PTAD substitution on that residue cannot be excluded and should thus be verified. Indeed, all aromatic protons of the tyrosine residue are observed via their characteristic doublets, thus eliminating the possibility of reaction at the phenolic side chain (Figure S55). The inability to locate the resonance for the bridgehead proton of the expected oxabicyclic system casts considerable doubts on the supposed Diels-Alder type structure (Figure 2). Therefore, to afford unambiguous elucidation of the structure of the formed product, the spectral complexity was reduced by repeating the reaction on a single, solid phase supported β-2-furyl-alanine residue. By treating solid-supported N-acetyl-β-2-furylalanine with 3 equivalents of PTAD for 15 minutes in DCM, modified amino acid **5**/**6** (Figure 3a) was obtained and further analyzed by NMR spectroscopy. Protons at C-7 and C-8 are observed as mutually coupled doublets, indicating there are no other neighbouring protons (Figure 3b). Moreover, a signal broadened through exchange with residual water in DMF-d7, can be seen at 11,61 ppm (Figure 3c), favoring structure **5** over the Diels-Alder adduct **6**, since only the former features an exchangeable hydrogen in the side chain. This hypothesis was confirmed by measurements at -50°C, where the exchange broadening is sufficiently reduced (Figure 3d) to allow

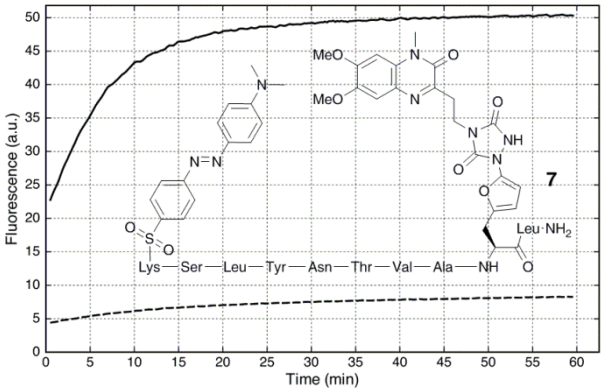

Fig. 4 FRET-probe **7** with alpha-chymotrypsin (solid) and control without enzyme (dashed) for 1h at 37°C in 0,02 M NH_4HCO_3 buffer.

the surrounding structure to be mapped through both nOe and heteronuclear $^nJ_{CH}$ correlation techniques (cfr. Electronic Supplementary Information). Having established the furan-PTAD aromatic substitution as a useful tool for quantitative site-selective and irreversible peptide labeling, we further explored the application potential of the newly developed methodology in dual orthogonal peptide labeling. Indeed, given the recent development of fluorescent TAD derivatives,[18] current furan-PTAD conjugation allows for convenient construction of FRET-probes. A simple FRET-probe **7** was synthesized using the commercially available TAD based compound DMEQ-TAD (4-[2-(3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)-ethyl]-3H-1,2,4-triazole-3,5(4H)-dione). Following completion of the peptide synthesis and labeling through reaction between DMEQ-TAD and the β-2-furylalanine containing peptide, subsequent Fmoc deprotection and labeling with the DABSYL quencher was carried out. The resulting peptide **7** was cleaved from the resin and purified by HPLC (Figure 4). Enzymatic digestion of the peptide induced by alfa-chymotrypsin was monitored by fluorescence spectrometry at 440 nm during 1 hour.[22] In the short dead time between enzyme addition and recording of the first measurements, a significant increase in fluorescence was already observed compared to the control sample (without enzyme treatment). This increase in fluorescence continued for 30 minutes during which the control sample exhibited by comparison only negligible amounts of fluorescence (Figure 4). An additional advantage of this type of DMEQ-TAD conjugation, apart from ease of synthesis, is the fluorogenic nature of the labeling procedure. The fluorescence arises only after conjugation, thus avoiding any problems associated with background fluorescence caused by remaining excess reagent.

Conclusions

In conclusion, as the incorporation of furan moieties in peptides and proteins has recently been firmly established in various contexts (vide supra),[11,12] the current methodology ideally complements the toolbox of bio-orthogonal labeling reactions. In conjunction with our previously developed furan-oxidation based peptide labeling[23] and nucleic acid crosslinking methodologies[24] the current work again testifies of the usefulness and versatile application of a simple and small aromatic furan moiety for the decoration and conjugation of different biomacromolecules.

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, HPLC and NMR data. See DOI: 10.1039/b000000x/

‡ We thank Prof. Dr. Alain Krief for valuable suggestions and BOF-UGent (01J06111) for financial support. The 700 MHz equipment is part of the Interuniversitary NMR Facility funded in part by the FFEU-ZWAP initiative of the Flemish Government.

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