Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 4836

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COMMUNICATION

Traceless chemical ligations from *O*-acyl serine sites†

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Received 7th December 2011, Accepted 11th April 2012
DOI: 10.1039/c2ob07050b

Chemical ligation *via* *O*- to *N*-acyl transfer of *O*-acylated serine containing peptides affords serine containing native peptides *via* 8- and 11-membered cyclic transition states opening the door to a wide variety of potential applications to peptide elaboration. The feasibility of these traceless chemical ligations is feasible as supported by computation.

The total chemical synthesis of proteins has already greatly contributed to knowledge of the relationship of protein structure to their function in important biological processes.[1–5] The development of chemical ligation has facilitated the synthesis of large peptides by linking the C-terminus of one unprotected peptide with the N-terminus of another.[1–3] Native chemical ligation (NCL), first reported by Wieland *et al.*[6] and later developed by Kent,[1,2] is a chemoselective and regioselective reaction of a peptide-thioester and a terminal Cys-peptide that results in a native amide bond at the ligation site through a rapid NCL *S*- to *N*-acyl transfer *via* a cyclic transition state.[1–3] The bifunctional nature of the N-terminal cysteine 1,2-mercaptoamine moiety is responsible for the observed chemoselectivity in NCL.[7]

While of great importance, NCL has limitations that include the requirement of a N-terminal cysteine residue at the ligation site to afford a peptide containing an internal cysteine. The low abundance of cysteine in human proteins (1.7% of the residues) also presents another limitation.[8–10] In attempts to overcome the limitation of low abundance of cysteine, considerable effort has been devoted to developing thiol auxiliary groups, but such ligations were found: (i) difficult to complete due to steric hindrance[10–16] and (ii) problematic since extraneous groups were present in the ligated product which can be troublesome to remove.[10–16] Another approach to overcome this limitation involves the conversion of a cysteine residue into a serine residue after NCL,[10b,c] however, this requires post-NCL modifications after NCL peptide synthesis.

To address these limitations, our group developed[17–19] ligations of *S*-acylated cysteine peptides to form native peptides through expanded transition states with 11- and 14-membered rings. The developed methodology required no auxiliary groups and enabled the selective *S*-acylation of cysteine peptides by *N*-acylbenzotriazoles in good yields and under mild conditions followed by microwave-assisted chemical ligations of *S*-acyl isopeptides. However, the challenge of ligation through 8-membered transition state and the low abundance of cysteine remained an obstacle. Our current approach to this limitation is therefore focused on serine which possesses the 1,2-hydroxylamine bifunctionality[7] (mimicking the SH/NH_2 bifunctionality of cysteine) and thus offers the possibility of chemoselective ligations by *O*- to *N*-acyl transfer without the need of cysteine residues.

Initially, two problems existed for the acylation of the hydroxyl group of serine: (i) difficulty in achieving *O*-acylation without epimerization (especially in solid-phase synthesis)[4,5] and (ii) the facile hydrolysis of *O*-acyl serine ester linkages[20] under the aqueous conditions of classical NCL. These problems were successfully overcome by our recently reported methodology for the preparation of chirally pure *O*-acyl isopeptides in a single step (74–91%) and under anhydrous conditions.[20]

Kiso *et al.*[5] had demonstrated that *O*-acyl residues within a backbone significantly altered the secondary native peptide structure. "*O*-Acyl isopeptides" are more hydrophilic and easier to purify by HPLC[5] than their corresponding native peptides. N-Terminal serine isopeptides rapidly generate, by O→N intramolecular acyl migration, the corresponding native peptide *via* a 5-membered transition state (Fig. 1).[21]

We now demonstrate that this classic *O*- to *N*-acyl shift *via* a 5-membered transition state can be extended to eight-membered

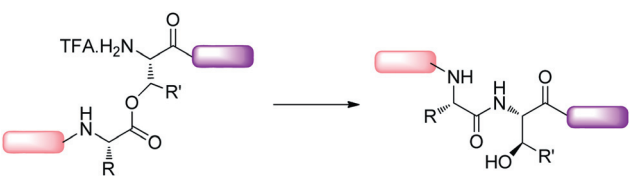

Fig. 1 *O*-Acyl isopeptide methodology.[21]

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†Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR for all compounds and HPLC/Mass-Spectrometry spectra for compounds **4**, **5**, **7** and **8**. See DOI: 10.1039/c2ob07050b

and eleven-membered transition states. Thus, "traceless" chemical ligation involving *O*- to *N*-acyl shift (at a Ser site) involving neither cysteine nor an auxiliary group at the ligation site has been achieved.

Traceless chemical ligation by *O*- to *N*-acyl shift *via* an eight-membered-TS at Ser site was demonstrated in **4a,c**. Protected *N*-(Pg-α-aminoacyl)benzotriazoles **1a–c** were coupled with L-Ser-OH using benzotriazole methodology[22,23] giving intermediates **2a–c**, which on *O*-acylation provided **3a–c**. Deprotection of the Cbz/Boc group of **3** by hydrogenation with Pd/C or by HCl–dioxane afforded *O*-acyl isodipeptides **4a–c** (Scheme 1).

Intermediates **4a,c** underwent ligation under microwave irradiation in piperidine–DMF 20 v/v%, 50 °C, 50 W, 1 h (Scheme 2). Anhydrous conditions were chosen to avoid ester hydrolysis.

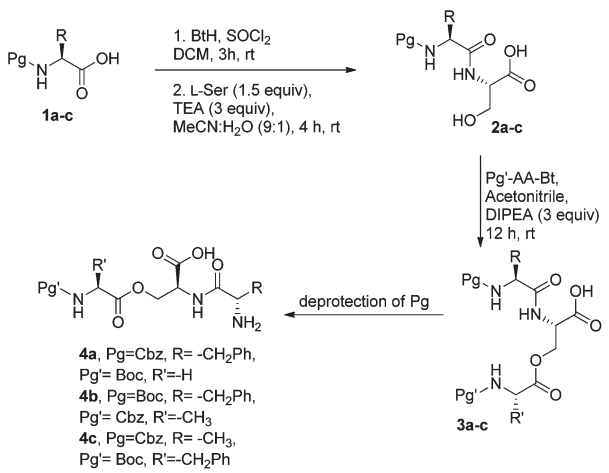
Indeed, HPLC-MS indicated the formation of desired intramolecular ligated products **5a** (57%, retention time 23.07 min) and **5c** (22%, retention time 39.50 min) and the presence of starting materials **4a** (43%, retention time, 19.61 min) and **4c** (78%, retention time, 38.65 min). The retention times and fragmentation patterns of **4a** and **4c** were also studied by control experiments (HPLC-MS of pure **4a** or **4c**). HPLC-MS, *via* (−) ESI-MS/MS, confirmed that compounds **4a** and **5a** with MW 409 have different fragmentation patterns. These data indeed proved the formation of intramolecular ligated products **5a** and **5c**. Moreover, product **5a** was isolated and the structure further confirmed by HRMS.

Traceless chemical ligation by *O*- to *N*-acyl shift from a Ser site *via* an 11-membered TS was achieved in isotripeptides **7a–b**. Amino-unprotected *O*-acyl isodipeptides **4a–b** (Scheme 1) were coupled with Pg″-Gly-Bt to give **6a–b** which after deprotection of the protecting group Pg″ provided *O*-acyl isotripeptides **7a–b** (Scheme 3).

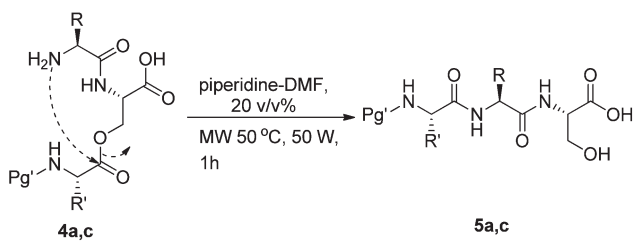
Intermediates **7a–b** underwent ligation (Scheme 3) under anhydrous conditions (piperidine 20 v/v% in DMF, MW 50 °C, 50 W, 1 h (for **7a**) and 3 h (for **7b**)). HPLC-MS showed the formation of expected intramolecular ligated product **8a** (99%, retention time 21.67 min), hydrolysed form **9a** (1%) and none of the intermolecular by-product **10a**. As for ligation on **7b**, HPLC-MS indicated the formation of the desired **8b** (18%, retention time 17.86 min), hydrolysed form **9b** (8%) and intermolecular by-product **10b** (31%). The retention times and fragmentation patterns of **7a** and **7b** were also studied by control experiments (HPLC-MS of pure **7a** and **7b**). HPLC-MS, *via* (−)ESI-MS/MS, demonstrated that products **7a** and **8a**, each of MW 466, produced different fragmentation patterns. In addition, product **10a** was isolated and its structure further confirmed by HRMS.

The ligation of **7a** was also examined under aqueous conditions (pH 7.6, 1 M buffer strength, MW 50 °C, 50 W, 1 h) HPLC-MS of the aqueous product, which disclosed a small amount corresponding to the ligated product **8a**, together with a major peak corresponding to a fragment having MW 366 that corresponds to the removal of the Boc-group either from **7a** or from the ligated product **8a**.

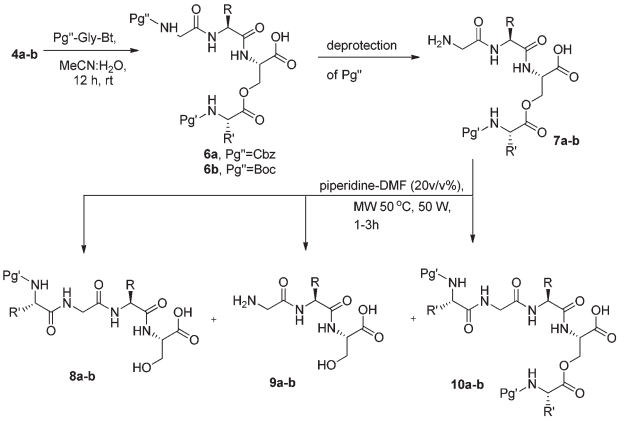
Unlike the sterically hindered and poorly organized for binding eight-membered cyclic transition state in *S*-acyl tripeptides,[18,19] the structurally similar *O*-acyl tripeptide **4a** demonstrated a preferential internal *O*- to *N*-acyl shift. This counter-intuitive reactivity of *O*-acyl tripeptides is however rationalized by the same computational protocol of virtual screening and quantum chemical calculations. The effectiveness of conformational preorganization was defined in terms of the *b*(N–C) scoring function, *i.e.* the geometrical distance between the nucleophilic amine nitrogen and the electrophilic ester carbon atom. A full conformational search was performed using the MMX force field (as implemented in PCModel v.9.3 software), resulting in 572 conformations which were subsequently ranked in descending order of the *b*(N–C) scoring function. The best preorganized conformer shown in Fig. 2 had a value for *b*(N–C) of 3.242 Å, significantly smaller than the value of 3.591 Å found in the similar *S*-acyl structure (the GMMX routine of



Scheme 1 Preparation of *O*-acyl isodipeptides **4a–c**.



Scheme 2 Chemical ligation of *O*-acyl isodipeptides **4a,c**.



Scheme 3 Preparation of *O*-acyl isotripeptides **7a–b** and their ligation to **8a–b**.

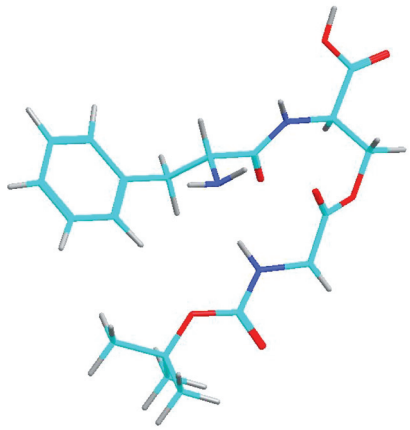


Fig. 2 Preorganised conformer of *O*-acyl peptide **4a**.

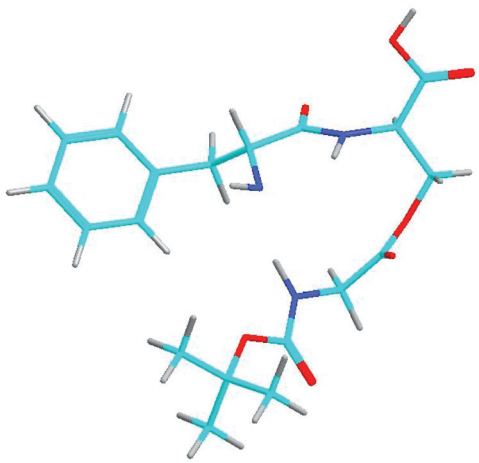


Fig. 3 Geometry optimized conformer of *O*-acyl peptide **4A**.

PCModel and MMX force field were used for scanning all rotatable bonds).[19]

As the *O*- to *N*-shift reaction occurs in the presence of piperidine, we believe general basic catalysis may be involved.

For calculation purposes this means that piperidine scavenges one proton of the free amino group in structure **4a**. We mimic the catalysis conditions by removing the proximal hydrogen atom from the N-terminus. To probe the reactivity, the preorganized deprotonated structure **4A** was subjected to geometry optimization at the HF/6-31+G* level of theory.

The quantum chemical reaction energy E_{react} is defined as $E_{react} = E_2 - E_1$, where E_1 and E_2 are the energies of the starting and final, geometry-optimised structures, respectively. Geometry optimization resulted in $E_{react} = -61.74$ kcal mol^{-1}, which is about 33 kcal mol^{-1} more favorable than the previously studied *S*-acyl structure. The optimized structure is shown in Fig. 3.

Although the optimized structure does not show the ligation completed, the cyclic transition state looks rather more organized than in the corresponding *S*-acyl structure (Fig. 2b in ref. 19). An apparent reason for that is the close hydrogen bond contact made between the Phe donor nitrogen and the Gly donor NH, with the NH···N distance of 1.91 Å. This explains the additional stabilization of structure **4A** as well as the higher yield of the internal ligation product **5a**.

Conclusions

In conclusion, chemical ligation *via* *O*- to *N*-acyl transfer with 8- and 11-membered transition states occurs successfully without the use of either cysteine or an auxiliary group. The reactivity of *O*-acyl peptides in traceless chemical ligation reactions is supported by theoretical and computational studies. Further ligation investigations on other sized transition states as well as ligations *via* threonine are ongoing in our laboratory.

Acknowledgements

We thank the University of Florida, The Kenan Foundation, King Abdulaziz University, Jeddah, Saudi Arabia and the Higher Education Commission, Pakistan for financial support. The authors thank Kind Abdulaziz University for support of this research by grant number 24-3-1432/HiCi. We thank Dr C. D. Hall for helpful discussions, Dr J. Johnson for his help in HLPC-MS analyses, Dr M. C. A. Dancel for her help with HRMS analyses.

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