Organic LETTERS

Letter

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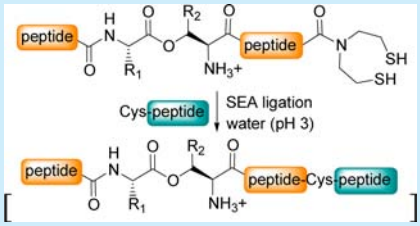
Synthesis of Unprotected Linear or Cyclic *O*-Acyl Isopeptides in Water Using Bis(2-sulfanylethyl)amido Peptide Ligation

Rémi Desmet, Mindaugas Pauzuolis, Emmanuelle Boll, Hervé Drobecq, Laurent Raibaut, and Oleg Melnyk*

UMR CNRS 8161, Université Lille, Pasteur Institute of Lille 59021 Lille, France

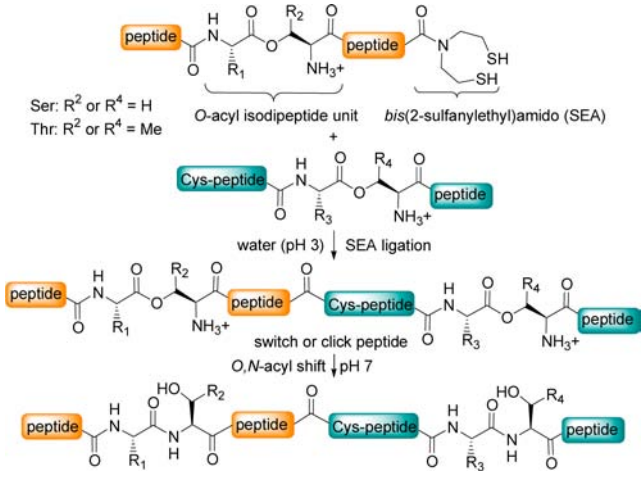
Supporting Information

ABSTRACT: SEA ligation proceeds chemoselectively at pH 3, i.e., at a pH where the *O*-acyl isopeptides are protected by protonation. This property was used for synthesizing unprotected *O*-acyl isopeptides in water, starting from peptide segments which are easily accessible by the Fmoc SPPS.



In serine (Ser) or threonine (Thr) *O*-acyl isopeptides (R = H or Me, respectively, Scheme 1), the peptidyl chain preceding the Ser/Thr residue is connected to this residue through an ester bond involving the Ser/Thr β-hydroxyl group. *O*-Acyl isopeptides spontaneously undergo an oxygen to nitrogen acyl shift at neutral pH, which restores a native peptide backbone structure.

Scheme 1. Synthesis of *O*-Acyl Isopeptides Using SEA Native Peptide Ligation



O-Acyl isopeptides have found a large variety of applications. The kink induced by the *O*-acyl isopeptide bond disfavors the aggregation of the peptide chain during the solid-phase peptide synthesis (SPPS), making this modification useful for improving the synthesis of difficult peptides.[1−3] The additional amino group displayed by the *O*-acyl isopeptides also helps solubilizing hydrophobic peptides such as amyloid β peptide (Aβ) 1−42 in water.[4−6] The coupling of protected peptides featuring a C-terminal *O*-acyl isodipeptide unit also allowed minimization of the epimerization of the C-terminal Ser/Thr during peptide segment condensation or cyclization.[7,8] Importantly, *O*-acyl isopeptides were used for designing a caged chemokine[9] or activatable peptidic scaffolds called switch peptides[10] or click peptides,[11] which are useful for studying the folding or the aggregation of peptides. In this case, the primary α-amino group of the *O*-acyl isodipeptide unit is protected until the *O*,*N*-acyl shift reaction is desirable. The unmasking of the amino group and the rearrangement can be triggered by the pH (PG = H^+),[10,12] by enzymes such as trypsin (PG = Arg)[10] or by UV light (PG = 6-nitroveratryloxycarbonyl, Nvoc).[9,11]

The native chemical ligation (NCL), that is, the chemoselective reaction of a C-terminal peptide thioester with an N-terminal cysteinyl (Cys) peptide, is a powerful tool for accessing large polypeptides or proteins.[13] Not surprisingly, a few reports utilized this reaction for the synthesis of N-protected *O*-acyl isopeptides (Nvoc,[9] azido,[14,15] allyloxycarbonyl[16]). The use of the NCL reaction for accessing *directly* unprotected *O*-acyl isopeptides is far more challenging because they undergo a spontaneous *O*,*N*-acyl shift process in the optimal conditions for the ligation, which occurs in water at neutral pH. Interestingly, Kiso and co-workers[17] showed that the use of short reaction times and *N*,*N*-dimethylformamide as the solvent allowed reduction of the unwanted *O*,*N*-acyl shift reaction to a few percent. Fast kinetic rates were obtained by using an organic base (triethylamine) in combination with an arylthioester peptide segment. Note that in this work the method used for preparing the arylthioester peptide involved a chemical step in water at pH 7. The unprotected *O*-acyl isopeptide unit was thus inserted in the Cys peptide component.

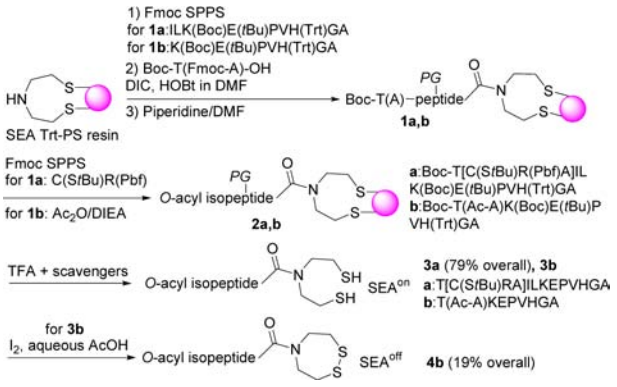
Received: June 2, 2015
Published: June 15, 2015

DOI: 10.1021/acs.orglett.5b01614
Org. Lett. 2015, 17, 3354−3357

Given the importance of unprotected *O*-acyl isopeptides for studying the function of peptides and proteins, we sought to develop a simple method for accessing these compounds by the chemoselective ligation of *unprotected* *O*-acyl isopeptide segments *in water* (Scheme 1). Of importance was the possibility of inserting the *O*-acyl isopeptide unit on both sides of the ligation junction. We show here that SEA ligation[18] can proceed at pH 3, which is a pH level where the *O*-acyl isopeptide is protected by protonation. As a consequence, this reaction proved to be useful for coupling chemoselectively unprotected *O*-acyl isopeptides in water. Keeping the solubilizing effect of the *O*-acyl isopeptide unit(s) throughout the ligation and the purification steps and avoiding a postligation deprotection step as in previous work constitute significant advantages.[16] This strategy allows the *O,N*-acyl shift reaction to be performed at a later stage depending on the final application.

The SEA *O*-acyl isopeptide segments used in this study were prepared using Fmoc SPPS starting from bis(2-sulfanylethyl)-amino polystyrene resin (SEA PS resin) as described in Scheme 2. The Boc-Thr(Fmoc-Ala)-OH *O*-acyl isodipeptide unit was

Scheme 2. Fmoc SPPS of SEA *O*-Acyl Isopeptide Segments



coupled using diisopropylcarbodiimide (DIC)/*N*-hydroxybenzotriazole (HOBt) activation in DMF. For peptidyl resin **2a**, the duration of the piperidine treatment following the coupling of Fmoc-Arg(Pbf)-OH was adapted to minimize diketopiperazine (DKP) formation (20% piperidine in DMF, 2 × 2 min).[3] Then, the coupling of Fmoc-Cys(S*t*Bu)-OH (DIC/HOBt activation in DMF) and the removal of the Fmoc group were performed using standard protocols. Peptidyl resin **2b** was obtained by treating peptidyl resin **1b** with acetic anhydride in the presence of DIEA. Both peptides were deprotected and cleaved from the resin with TFA in the presence of appropriate scavengers. Peptide **3a**, which features a SEA group in the dithiol (SEA^{on}) form, was used directly in the ligation experiments due to the good quality of the obtained product. Peptide **3b** was further oxidized with iodine in aqueous acetic acid and purified by HPLC. The last example shows the compatibility of the free *O*-acyl isopeptide structure with the standard protocols used for the preparation of peptides featuring a C-terminal SEA^{off} group,[19] that is, a SEA group inactivated by formation of a 7-membered ring cyclic disulfide.[20,21] The other SEA^{off} and Cys peptide segments needed for this study are detailed in Table 1 and were produced using standard Fmoc SPPS procedures.

Previous reports showed the effectiveness of SEA ligation at neutral or mildly acidic pH (pH 5.5) in the presence of 4-mercaptophenylacetic acid (MPAA[22]).[23,24] However, it was unknown if this reaction could proceed below pH 4 to allow the protection of the *O*-acyl dipeptide units by protonation. Interestingly, we found that the reaction of SEA^{off} peptide ILKEPVHGA-SEA^{off} **4c** with Cys peptide CILKEPVHGA-NH_2 **5a** at pH 3.1 and 37 °C in the presence of MPAA (30 mM), TCEP (125 mM) and guanidinium hydrochloride (Gdn.HCl, 0.6 M) proceeded to completion within 24 h. Gratifyingly, application of these conditions to SEA and/or Cys *O*-acylisopeptide segments successfully yielded the *O*-acylisopeptides shown in Table 1. The ligation of SEA peptide **4d** with Cys *O*-acyl isopeptide segment **5b** to produce *O*-acyl isopeptide **7** (40% isolated by HPLC, entry 1, Table 1) constitutes a typical example. The HPLC of the crude reaction mixture after 48 h (Figure 1B) highlights the efficiency of the ligation process, while the formation of the native peptide **8** by spontaneous *O,N*-acyl shift remained within acceptable levels (**7**/**8** = 17, ratio of peak areas). Unexpectedly, while *O*-acylisopeptides are highly stable in water at pH < 4, some rearrangement occurred during SEA ligation at pH 3.1. However, the rearranged peptide represented only a few percent after 24−48 h.

Table 1. Synthesis of *O*-Acyl Isopeptides by SEA Ligation[a]

entry	SEA peptide	Cys peptide	product	yield (%)
1	ILKEPVHGY-SEA^{off} **4d**	T[C(S*t*Bu)RA]ILKEPVHGA-NH_2 **5b**	T(ILKEPVHG**YC**RA)ILKEPVHGA-NH_2 **7**	40
2	**4d**	S[C(S*t*Bu)RA]ILKEPVHGA-NH_2 **5c**	S(ILKEPVHG**YC**RA)ILKEPVHGA-NH_2 **9**	35
3	ILKEPVHGA-SEA^{off} **4c**	S(CA)ILKEPVHGA-NH_2 **5d**	S(ILKEPVHG**AC**A)ILKEPVHGA-NH_2 **10**[b]	27
4	ILKEPVHGC(S*t*Bu)-SEA^{off} **4e**	**5c**	S(ILKEPVHG**CC**RA)ILKEPVHGA-NH_2 **12**	31
5	T(AcAla)KEPVHGA-SEA^{off} **4b**	**5b**	T[T(AcAla)KEPVHG**AC**RA]ILKEPVHGA-NH_2 **13**[b]	29
6	**4b**	**5c**	S[T(AcAla)KEPVHG**AC**RA]ILKEPVHGA-NH_2 **15**[b]	23

[a]Gdn·HCl 0.63 M citrate−phosphate buffer pH 3.1 or pH 3.5 (entry 3), TCEP 125 mM, MPAA 30 mM, 1.5 equiv of the Cys peptide, 37 °C, 48 h. [b]Peptide **10** was rearranged into peptide **11**, peptide **13** into peptide **14**, and peptide **15** into peptide **16**; see the Supporting Information.

DOI: 10.1021/acs.orglett.5b01614
Org. Lett. 2015, 17, 3354−3357

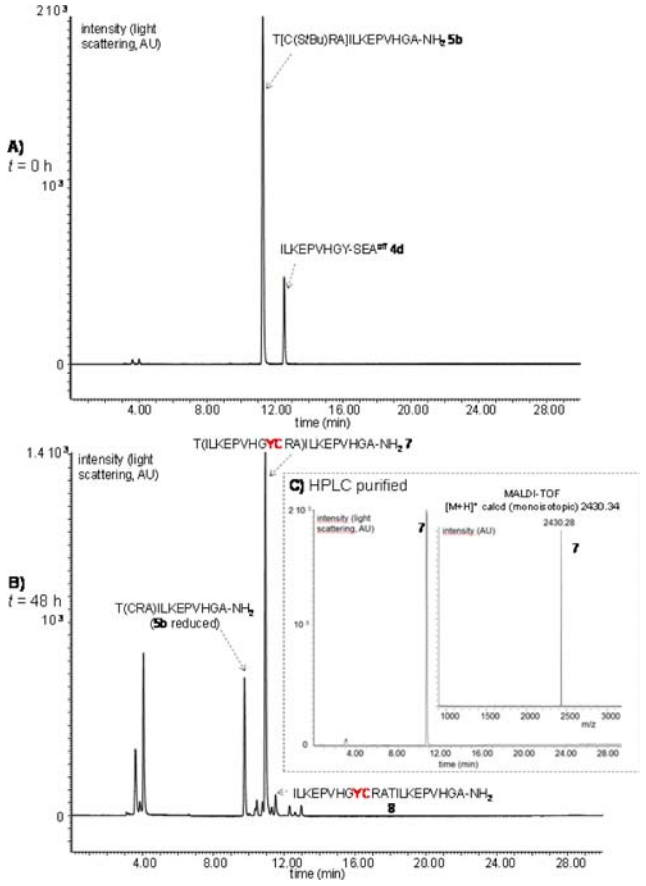


Figure 1. Synthesis of *O*-acyl isopeptide **7**. HPLC of the reaction mixture after mixing peptides **4d** and **5b** (A), after 48 h (B), and after purification (C). MPAA was extracted before analysis.

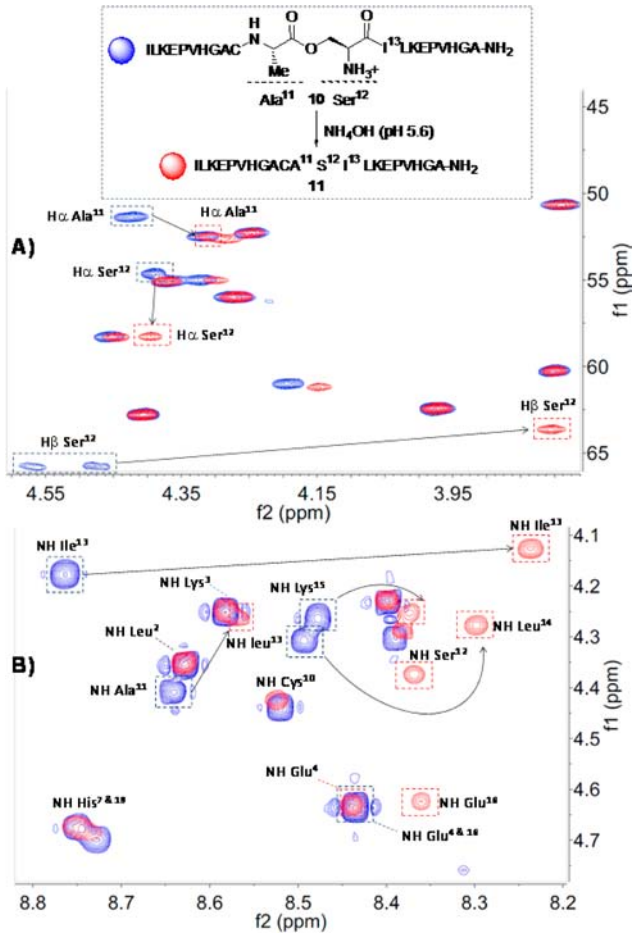


Figure 2. NMR analysis of *O*-acyl isopeptide **10** (in blue). Comparison with the native peptide **11** (in red) produced from **10** upon addition of NH_4OH. $^1H-^{13}C$ HSQC (A) and TOCSY (B) spectra in H_2O/D_2O: 9/1 by volume (^1H 600 MHz spectrometer).

The ligation of SEA peptide **4c** with Cys *O*-acylisopeptide segment **5d** to produce *O*-acylisopeptide **10** also proceeded well (27% isolated, entry 3, Table 1). This example shows that the method works satisfactorily with an *O*-acylisopeptide segment which can potentially decompose through DKP formation. This side reaction was indeed observed but did not preclude the ligation to go to completion thanks to the slight excess of Cys peptide segment **5b** used in this experiment and to the fact that the Cys-Ala DKP cannot react with the SEA peptide to produce additional side products. Importantly, multidimensional NMR analysis of the isolated *O*-acyl isopeptide **10** demonstrated the presence of the *O*-acyl isopeptide motif (for example Hβ of Ser12 are found at ~4.5 ppm, Figure 2A) and of a native peptide bond to cysteine in its structure (NH Cys[10] at 8.52 ppm, Figure 2B). Moreover, the rearrangement of *O*-acyl isopeptide **10** into native peptide **11** upon addition of ammonia resulted in a upfield shift of Hβ protons of Ser[12] (Figure 2A) and in the appearance of a novel peptide bond to Ser[12] as well as in a significant upfield shift of several amide protons upward this residue, notably, Ile,[13] Leu,[14] and Lys[15] (Figure 2B). Finally, the rearranged peptide **11** was found to be identical by HPLC and NMR to a reference peptide produced by conventional SPPS (see the Supporting Information). The example described in entry 4 of Table 1 shows that the method is compatible with the presence of an internal Cys residue, while those presented in entries 5 and 6 of Table 1 show that the method can be used for producing peptides featuring multiple *O*-acyl isodipeptide units on both sides of the ligation junction. Both *O*-acyl isopeptides **13** and **15** were rearranged successfully into the corresponding native peptides **14** and **16**, respectively, in water at pH > 5 (see the Supporting Information).

O-Acyl isopeptides display a typical fragmentation pattern when analyzed by MALDI LIFT TOF/TOF mass spectrometry in comparison with the corresponding native analogues. Indeed, the ester moiety undergoes a facile McLafferty rearrangement in the gas phase, which yields a peptide acid and another fragment featuring an N-terminal α,β-dehydroamino acid in place of Ser or Thr. The breakdown of the two *O*-acyl isopeptide bonds present in peptides **13** or **15** is clearly seen in their MALDI LIFT TOF/TOF spectra (see the Supporting Information). This feature constitutes an additional proof of identity, since these rearrangements are not observed in the corresponding native peptides.

In the last experiment, the method was used to cyclize *O*-acyl isopeptide **3a**, which features an N-terminal Cys residue and a C-terminal SEA functionality (Scheme 3). Here again, MALDI LIFT TOF/TOF spectra of peptide **17** confirmed the presence of the *O*-acyl isopeptide motif and its cyclic structure (see the Supporting Information). The cyclic *O*-acylisopeptide **17** was subsequently rearranged in the presence of ammonia into the cyclic analogue **18** with a native peptide bond to Thr.

In conclusion, SEA ligation at pH 3 enables the facile synthesis of unprotected *O*-acylisopeptides and the introduc-

DOI: 10.1021/acs.orglett.5b01614
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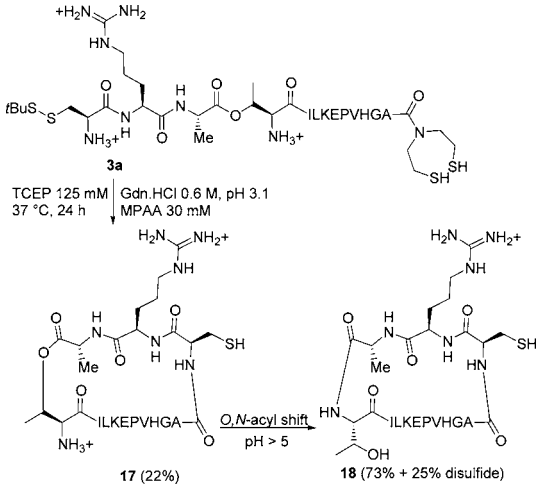
Scheme 3. Synthesis of a Cyclic *O*-Acyl Isopeptide Using SEA Ligation at pH 3



tion of the *O*-acylisopeptide units on both sides of the ligation junction. This method also enables the synthesis of cyclic *O*-acyl isopeptides and more generally should facilitate the access to sophisticated *O*-acyl peptide scaffolds of interest for studying the function of peptides. The fact that SEA ligation proceeds in a large range of pH (from 3 to 7) brings a lot of flexibility to the synthesis of complex polypeptides and raises significant questions regarding the mechanism of this reaction.

■ ASSOCIATED CONTENT

Supporting Information

Procedures and characterization for all new compounds. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01614.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: oleg.melnyk@ibl.cnrs.fr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank SIRIC OncoLille and Région Nord Pas de Calais for financial support. We thank O. El-Mahdi for useful discussions and the CSB facility for technical help.

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DOI: 10.1021/acs.orglett.5b01614
Org. Lett. 2015, 17, 3354−3357