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Synthesis and conformation of an analog of the helix-loop-helix domain of the Id1 protein containing the *O*-acyl iso-prolyl-seryl switch motif

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Synthetic peptides reproducing the helix-loop-helix (HLH) domains of the Id proteins fold into highly stable helix bundles upon self-association. Recently, we have shown that the replacement of the dipeptide Val-Ser at the loop–helix-2 junction with the corresponding *O*-acyl iso-dipeptide leads to a completely unfolded state that only refolds after intramolecular *O* → *N* acyl migration. Herein, we report on an Id HLH analog based on the substitution of the Pro-Ser motif at the helix-1–loop junction with the corresponding *O*-acyl iso-dipeptide. This analog has been successfully synthesized by solid-phase Fmoc chemistry upon suppression of DKP formation. No secondary structure could be detected for the *O*-acyl iso-peptide before its conversion into the native form by *O* → *N* acyl shift. These results show that the loop–helix junctions are determinant for the folded/unfolded state of the Id HLH domain. Further, despite the high risk of DKP formation, peptides containing *O*-acyl iso-Pro-Ser/Thr units are synthetically accessible by Fmoc chemistry. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: helix-loop-helix motif; Id proteins; *O*-acyl isopeptide; *O*–*N* acyl migration; conformation switch; diketopiperazine

Introduction

The solid-phase synthesis of large peptides or peptides based on 'difficult sequences' represents often a challenge. One of the aspects negatively affecting the success of a synthesis is the aggregation of the growing peptide chains during assembly on the solid support. As intermolecular hydrogen bond networks favor aggregation, successful strategies aiming at the reduction of intermolecular backbone interactions rely on the conversion of the peptide bond into a non-hydrogen-bond donor. For example, the dipeptide-based building blocks Xaa-ψPro (ψPro: pseudoproline) and Ser/Thr(Xaa) can be used for peptide chain assembly in place of Xaa-Ser/Thr (Figure 1).

ψPro consists of a Ser/Thr-derived oxazolidine with proline-like ring structure [1]. Owing to the preference for a *cis*-amide bond with the preceding residue and to the absence of the amide proton, ψPro can induce a kink of the peptide backbone and suppress hydrogen bonding, thereby preventing β-sheet structure formation and peptide aggregation [2]. The use of ψPro allows for a temporary modification of the peptide during chain assembly, as ψPro is converted into the corresponding Ser/Thr residue upon acidic cleavage of the peptide from the solid support.

The second building block Ser/Thr(Xaa) consists of an *O*-acyl iso-dipeptidyl unit, in which the peptide bond is replaced by an ester bond involving the β-*OH* group of Ser/Thr. Thus, incorporation of one or more *O*-acyl iso-dipeptidyl units significantly reduces the ability of the peptide backbone to form hydrogen bonds. Contrarily to the ψPro residue, the *O*-acyl iso-dipeptidyl unit is stable under acidic cleavage conditions, which allows obtaining the generally more water-soluble and less aggregation-prone *O*-acyl iso-peptide analog as final product. The latter can be then purified and successively converted into the native peptide by triggering an intramolecular *O* → *N* acyl migration under neutral or mild basic conditions [3–9]. Recently, the incorporation of *S*-acyl iso-dipeptides in place of Xaa-Cys has been proposed [10].

In addition to the advantages for the peptide synthesis and purification steps, the *O*-acyl iso-peptide method offers the possibility to observe the conformational transformations accompanying the conversion of the ester bond into the native peptide bond. For example, a precursor of the Alzheimer's disease-related amyloid β-peptide containing an *O*-acyl iso-dipeptide unit has been used to monitor its conformational transitions, oligomerization, and amyloid fibril formation upon changing from acidic to neutral pH [11–13].

We also applied the *O*-acyl iso-peptide method to examine the impact of the simultaneous peptide backbone and side-chain modification on the conformational stability of the helix-loop-helix (HLH) domains of the Id1 and Id2 proteins [14]. The latter are, together with Id3 and Id4, the members of the so-called Id family that is classified under the large family of the HLH transcription factors [15]. The Id proteins act as inhibitors of DNA binding and cell differentiation and play a crucial role in embryogenesis and tumorigenesis [16]. The Id HLH domain determines the mode of

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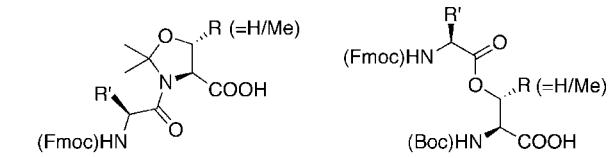


Fmoc-Xaa-Ser/Thr(ψ(Me,Me)pro)-*OH* Boc-Ser/Thr(Fmoc-Xaa)-*OH*

Figure 1. Chemical structures of the dipeptide-based building blocks Fmoc-Xaa-ψ Pro-*OH* and Boc-Ser/Thr(Fmoc-Xaa)-*OH*.

	Helix-1	Loop	Helix-2
Id1 (66–106)	LYD**MN**G**C**Y**S**R**L**K**E****LVP** T**L**P**Q**N**R**K**VS** K**V****EILQ**H**VIDYI**R**DLQ**
Id2 (36–76)	LYN**MN**D**C**Y**S**K**L**K**E****LVP** S**I**P**Q**N**K**K**VS** K**M****EILQ**H**VIDYI**L**DLQ**
Id3 (41–81)	LDD**MN**H**C**Y**S**R**L**R**E****LVP** G**V**P**R**G**T**Q**LS** Q**V****EILQ**R**VIDYI**L**DLQ**
Id4 (65–105)	QCD**MN**D**C**Y**S**R**L**R**R****LVP** T**I**P**P**N**K**K**VS** K**V****EILQ**H**VIDYI**L**DLQ**
identical residues	---**MN**-**C**Y**S**-**L**--**LVP** --**P**-----**S** --**EILQ**-**VIDYI**-**DLQ**

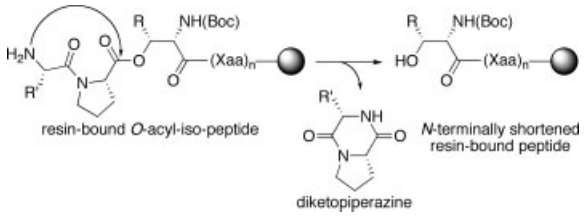
Figure 2. Amino acid sequences of the HLH domains of human Id1-4. The conserved residues are in bold (the Pro-Ser/Thr and Val/Leu-Ser motifs at the helix-1–loop and loop–helix-2 junctions, respectively, are underlined by the gray boxes).

action of these proteins, which is based on the formation of non-DNA-binding heterodimers with basic HLH transcription factors and consequent negative regulation of DNA transcription. The Id HLH domain consists of a 41-residue-long sequence that is conserved within the four Id proteins. In particular, the *N*-terminal and *C*-terminal helical segments (helix-1 and helix-2, 16 residues each) are highly conserved (Figure 2).

Although the loop connecting the two helices shows reduced sequence identity, both loop–helix junctions are characterized by conserved residues: indeed, Pro is followed by Ser/Thr (Id1, 2, and 4) or Gly (Id3) at the helix-1–loop junction, and Ser follows Val/Leu at the loop–helix-2 junction. The structural role of the junctions in the building of the HLH fold is believed to be very important [14,17]. With one exception for the Id3 HLH domain, all other junctions are suitable for the application of the *O*-acyl isodipeptide method. Recently, we have shown that the introduction of the *O*-acyl isodipeptidyl unit Ser(Val) in place of Id1 Val^{89}-Ser^{90} and Id2 Val^{59}-Ser^{60}, both located at the junction between the loop and helix-2, arose two fully unstructured *O*-acyl isopeptide analogs. The native fold reappeared only after induction of the intramolecular $O \rightarrow N$ acyl migration [14]. This confirms that the loop–helix-2 junction is crucial for the formation of secondary and tertiary structures of the Id HLH domain.

To investigate the role of the junction between helix-1 and the loop in a similar manner, it is necessary to replace the motif Pro-Ser/Thr with the *O*-acyl isodipeptidyl unit Ser/Thr(Pro). However, one problem related with this building block is the high risk of DKP formation during peptide chain elongation by using Fmoc chemistry, as shown in Scheme 1.

In this work, we present the synthesis and the conformational properties of an analog of the Id1 HLH domain containing the *O*-acyl isodipeptidyl unit Ser(Pro) at the helix-1–loop junction. Our results show that the *O*-acyl isopeptide method can be used to modify the Pro-Ser/Thr dipeptide motif both for synthetic and structural purposes.



Scheme 1. Formation of diketopiperazine with consequent *N*-terminal shortening of a resin-bound peptide containing the prolyl-seryl (R=H)/threonyl (R=Me) *O*-acyl iso-dipeptidyl unit.

Materials and Methods

Synthesis of Boc-Ser(Fmoc-Pro)-*OH*

The synthesis of Boc-Ser(Fmoc-Pro)-*OH* has been previously reported [8]. Briefly, Boc-Ser(Fmoc-Pro)-*OBzl* was synthesized from Boc-Ser-*OBzl* and Fmoc-Pro-*OH* using EDC·HCl and DMAP in dry chloroform. The title compound was obtained after hydrogenolysis with Pd/C in EtOAc under H_2 atmosphere.

Solid-Phase Synthesis of Ac-[*O*-acyl iso-Pro^{81}-Ser^{82}]-(Id1 66–106)-NH_2

The synthesis of the native Id1 HLH peptide 66–106 (*N*-terminally acetylated and *C*-terminally amidated) was described previously [17]. The corresponding *O*-acyl iso-peptide analog was obtained by manual elongation of the Id1 fragment 83–106 that was automatically assembled by following a synthetic protocol reported previously [17]. Briefly, the Id1 fragment 83–106 was synthesized by Fmoc chemistry using the Syro-I peptide synthesizer from MultiSynTech (Witten, Germany) and starting from the Rink amide-MBHA resin (0.34 mmol/g, 100–200 mesh; Novabiochem Merck Biosciences GmbH, Schwalbach/Ts., Germany). Side-chain protecting groups of the Fmoc amino acids were as follows: tBu for Asp, Glu, Ser, Thr, and Tyr; Boc for Lys; trityl for Cys, His, Asn, and Gln; and 2,2,4,6,7-pentamethyldihydro-benzofurane-5-sulfonyl for Arg. Peptide chain assembly was accomplished by double coupling procedure (2 × 40 min) with the mixture Fmoc amino acid/HBTU/HOBt/DIPEA (5 : 5 : 5 : 10 equiv.) in DMF/NMP (70 : 30, v/v), followed by Fmoc removal with 40 and 20% piperidine in DMF/NMP (80 : 20, v/v) for 3 and 10 min, respectively. The *O*-acyl iso-dipeptide Boc-Ser(Fmoc-Pro)-*OH* was attached manually by double coupling (2 × 2.5 h) with the mixture Boc-Ser(Fmoc-Pro)-*OH*/DIC/HOBt (2.5 : 2.5 : 2.5 equiv.) in dry dichloromethane, followed by Fmoc cleavage with 20% piperidine in DMF (2 × 4 min). Fmoc-Val-*OH* was coupled using DIC/HOBt (5 equiv. each) in DMF for 1 h. Afterwards the Fmoc group was removed with a solution of 10% DBU in DMF for 10 s, the resin was immediately washed with 0.2 M HOBt in DMF for 20 s, followed by addition of 5 equiv. Fmoc-Leu-*OH* that had been previously activated for 2 min using 5 equiv. HOBt, 5 equiv. HBTU, and 7.5 equiv. DIPEA in DMF. The coupling reaction was left to run for 30 min. This so-called flash method for Fmoc cleavage and coupling [18] was also used for the two following amino acids Fmoc-Glu(*O*tBu)-*OH* and Fmoc-Lys(Boc)-*OH*. The remaining 11 amino acids were attached by a first coupling using 5 equiv. amino acid, 5 equiv. HOBt, 5 equiv. HBTU, and 10 equiv. DIPEA in DMF for 3 h, followed by a second coupling using 5 equiv. amino acid, 5 equiv. HOBt, and 5 equiv. DIC overnight. The Fmoc cleavage was performed with 40 and 20% piperidine in DMF/NMP (80 : 20, v/v) for 3 and 10 min, respectively. The free *N*-terminus was acetylated using 10 equiv. acetic anhydride and

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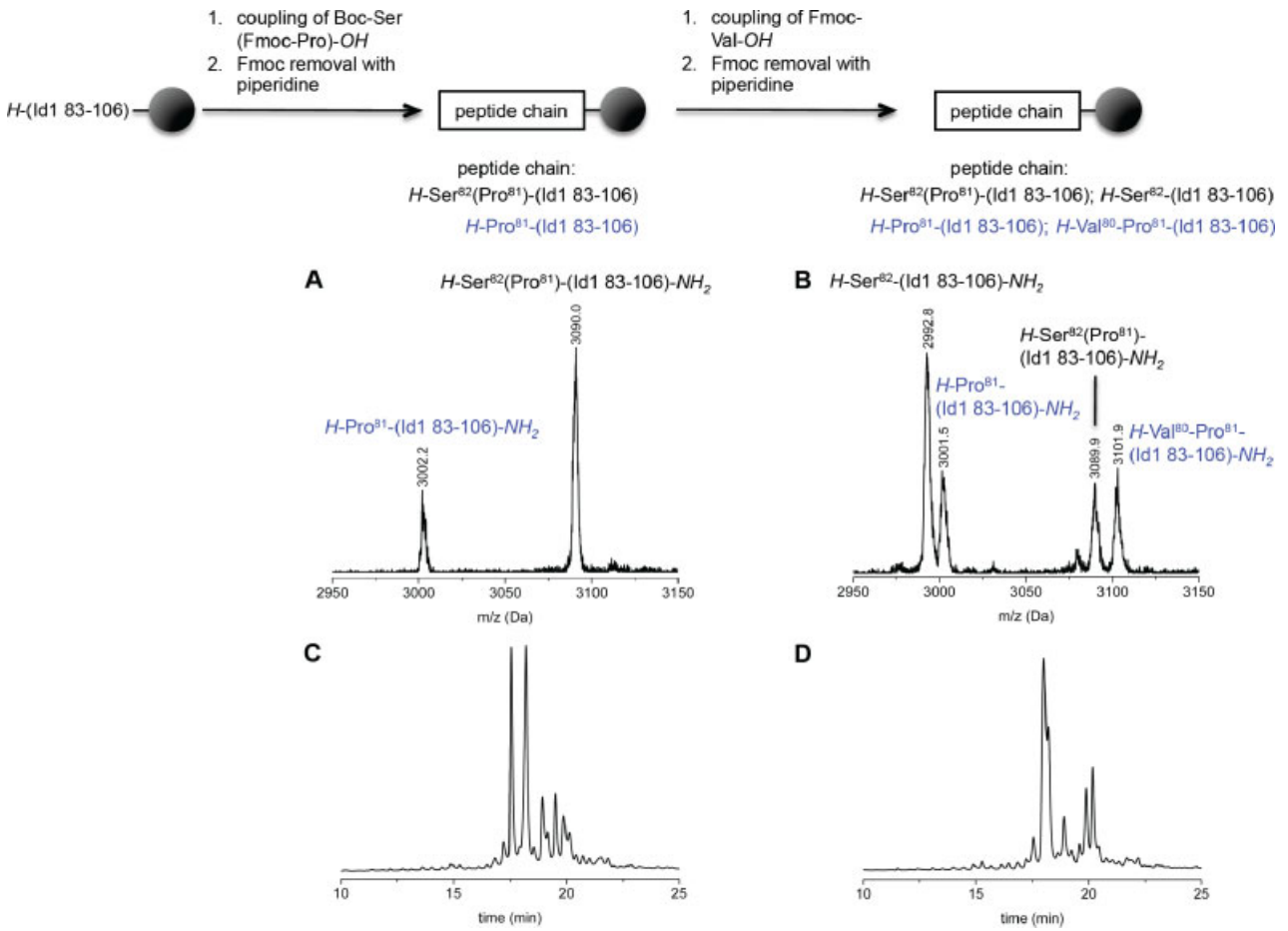

Figure 3. Mass spectra and HPLC profiles of the peptides obtained by small-scale TFA cleavage after coupling of Boc-Ser(Fmoc-Pro)-*OH* to *H*-(Id1 83–106)-Rink amide resin and Fmoc removal with 20% piperidine (A and C), followed by coupling of Fmoc-Val-*OH* and Fmoc removal with 20% piperidine (B and D).

10 equiv. DIPEA in DMF for 30 min. Finally, the crude *O*-acyl isopeptide was simultaneously fully deprotected and cleaved from the solid support using a cleavage mixture consisting of 87% TFA, 5% TIS, 5% H_2O, and 3% thioanisole (2.5 h). The crude peptide was precipitated from ice-cold diethyl ether and recovered by centrifugation at 3 °C for 8 min. Several washing cycles were carried out to efficiently remove the scavengers. Finally, the peptide was purified by semipreparative HPLC (15% yield) and characterized by MALDI-TOF-MS (Bruker) and analytical HPLC (Agilent) using a Nucleosil C18 column from Macherey-Nagel (Düren, Germany), 100 Å, 5 µ, 250 × 4 mm. $MW_{calc.}/MW_{found}$ (Da): 4916.8/4914 (matrix: α-cyano-4-hydroxycinnamic acid).

CD Spectroscopy

Each peptide was dissolved both in water and phosphate buffer (100 mM, pH 7). Peptide concentration was determined spectrophotometrically by measuring the tyrosine absorbance at 280 nm ($\varepsilon = 1480\ M^{-1}$/cm for each tyrosine residue [19]). The CD measurements were recorded at 25 °C on a Jasco J-715 spectropolarimeter using a quartz cell with a path length of 0.1 cm. For each CD spectrum, six scans were accumulated using a step resolution of 0.5 nm, a bandwidth of 1 nm, a response time of 1 s, and a scan speed of 20 nm/min. The CD spectrum of the solvent was subtracted from that of the sample to eliminate interferences from cell, solvent, and optical equipment. Noise reduction was performed with a Fourier transform filter from the Origin program (OriginLab Corporation, Northampton, MA, USA). The ellipticity is expressed as the mean residue molar ellipticity $[\Theta]_R$ in deg·cm^2/dmol.

Results and Discussion

The following synthetic study is based on the building block Boc-Ser(Fmoc-Pro)-*OH* and on its incorporation at positions 81–82 of the Id1 HLH sequence 66–106. After automatic peptide chain assembly of the Id1 fragment 83–106 on Rink amide resin, Boc-Ser(Fmoc-Pro)-*OH* was attached manually by double coupling upon activation with DIC/HOBt in dichloromethane. In a first trial, the Fmoc group was cleaved using two treatments (4 min each) with 20% piperidine in DMF. A small-scale TFA cleavage was performed to record a mass spectrum of the product. Beside the expected mass, 3090.0 Da ($MS_{calc.}$: 3088.6 Da), an MS peak with a difference of −88 Da was detected (Figure 3A). This was attributed to the lack of Ser^{82} ($MS_{calc.}$: 3001.6 Da). The side reaction leading to this side product is known and the proposed mechanism involves a β-elimination occurring on the activated *O*-acyl iso-dipeptide, followed by the formation of a mixed anhydride between the Fmoc amino acid (in this case Fmoc-proline) and Boc-dehydroalanine [4]. Based on this mechanism, the side product lacking Pro^{81}

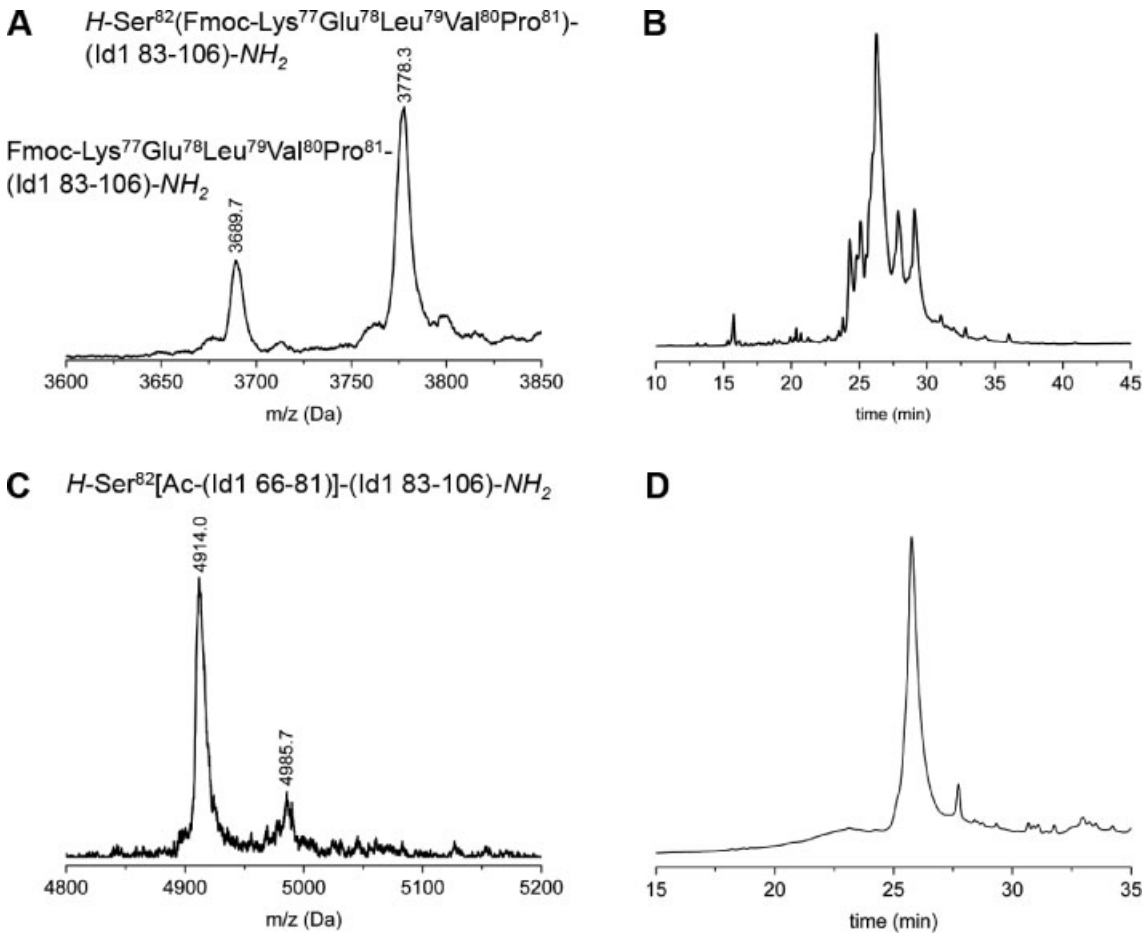

Figure 4. Mass spectrum (A) and HPLC profile (B) of an *N*-terminally Fmoc-protected peptide intermediate obtained by small-scale TFA cleavage upon elongation of *H*-(Id1 83–106)-Rink amide resin with the flash method (an additional MS peak was found at 3874 Da, which was attributed to a trifluoroacetylated species of the desired product). Other minor MS peaks were at 3190, 3206, and 3989 Da. Mass spectrum (C) and HPLC profile (D) of the purified final product Ac-[*O*-acyl iso-Pro^{81}-Ser^{82}]-(Id1 66–106)-NH_2.

and bearing the dehydroalanyl residue (or the corresponding 2-oxopropionyl residue) in place of Ser^{82} is also expected to be formed; however, this side product was not detected by MS analysis. An alternative mechanism might be hypothesized to take place during coupling of the *O*-acyl iso-dipeptide with DIC/HOBt; indeed, HOBt might directly attack the ester bond, thus forming Fmoc-Pro-*O*Bt that would acylate the free amino group of the growing peptide chain or reacylate the serine side chain. Two other species with mass of 3190 and 3206 Da were revealed by MS analysis, whose chemical nature is unclear.

The peptide chain was further elongated with Fmoc-Val-*OH* using DIC/HOBt. Then, the Fmoc group was removed using again two treatments (4 min each) with 20% piperidine in DMF. This procedure resulted in the formation of (Val^{80}-Pro^{81})-DKP, as supported by MS analysis that revealed the presence of the shortened product lacking the *N*-terminal dipeptide Val^{80}-Pro^{81} with a mass of 2992.8 Da (MS_{calc}: 2991.5 Da) (Figure 3B). The single coupling of Fmoc-Val-*OH* resulted to be incomplete, as indicated by the MS peaks both of the Val^{80}-lacking peptide at 3089.9 Da (MS_{calc}: 3088.6 Da) and of the Val^{80}-Ser^{82}-lacking side product at 3001.5 Da (MS_{calc}: 3001.6 Da). Again, the two unassigned MS peaks mentioned above were detected. In this case, the MS peak at 3190 Da might have been easily attributed to the desired product, assuming only partial DKP formation. However, this was excluded by the two following observations: (i) this MS peak was already found before the coupling of Val^{80} and (ii) the attempt of elongation with Leu^{79} only enriched the species derived from DKP formation with the mass of 2992 Da (data not shown). Therefore, we attribute the MS peak at 3190 Da to the species that was already found after the coupling of the *O*-acyl iso-dipeptide, together with the species with the mass of 3206 Da.

Beyermann and coworkers reported recently on the solid-phase synthesis of cyclodepsipeptides [18] and proposed a so-called flash method to reduce the risk of DKP formation. The advantages of this method are the fast removal of the Fmoc protecting group from the growing peptide chain and the immediate coupling of a preactivated amino acid. In this procedure, the Fmoc group is removed with a solution of 10% DBU in DMF for 10 s. The resin is immediately washed with 0.2 M HOBt in DMF for 20 s, followed by addition of the next amino acid that has been previously activated for 2 min with HOBt/HBTU/DIPEA in DMF. Thus, in a second synthetic trial the cleavage of the Fmoc group of Val^{80} and the coupling of the preactivated Fmoc-Leu^{79}-*OH* were performed by using the flash method. The same procedure was applied to couple Fmoc-Glu^{78}(*O*tBu)-*OH* and Fmoc-Lys^{77}(Boc)-*OH*. As shown by MS and HPLC analyses (Figure 4A,B), the flash method proved to be successful and the peptide chain could be elongated. The remaining *N*-terminal amino acids were attached by double

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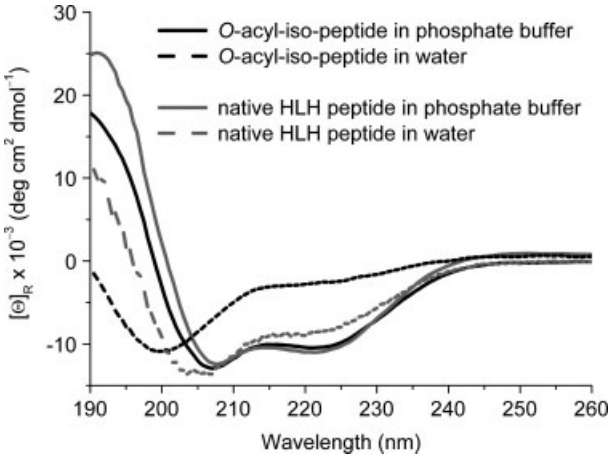

Figure 5. CD spectra of the native Id1 HLH domain (53 and 58 μM) and its *O*-acyl iso-peptide analog (47 and 45 μM) in water (pH 4.5) and in 100 mM phosphate buffer (pH 7). In phosphate buffer, the *O*-acyl iso-peptide analog undergoes intramolecular $O \rightarrow N$ acyl shift and folds into the native structure.

coupling (first with HOBt/HBTU/DIPEA for 3 h, then with DIC/HOBt overnight). The Fmoc group was removed by using piperidine. Finally, the crude peptide was cleaved from the resin, purified by semipreparative HPLC, and characterized by MS and analytical HPLC (Figure 4C,D).

The CD spectrum of the *O*-acyl iso-peptide was recorded in water (pH 4.5) and compared with the CD curve of the native Id1 HLH peptide. Whereas the latter adopts a α-helical conformation, the corresponding *O*-acyl iso-peptide is mainly flexible (Figure 5). In contrast, in 100 mM phosphate buffer (pH 7), in which the intramolecular $O \rightarrow N$ acyl migration occurs, the CD spectra of the native and rebuilt HLH peptide are superimposable. It should be noted that the peptide obtained after $O \rightarrow N$ acyl shift corresponds to the Ser→Thr^{82} mutant of the Id1 HLH peptide. However, the fact that the CD spectra of the native and mutated Id1 HLH sequences are superimposable indicates that the highly conserved single-point mutation is structurally fully tolerated.

The behavior of the *O*-acyl iso-peptide presented in this work resembles that of the previously reported analog containing the reversible modification at the junction between the loop and helix-2 [14]. This suggests that the two junctions play a crucial role in the formation of the Id HLH fold. The $O \rightarrow N$ acyl shift and the following conformational change at pH 7 were found to be fast for both *O*-acyl iso-peptides, since the CD curves of their freshly prepared samples already revealed the presence of the folded native peptides. It will be interesting to study the kinetics of the conformational transition upon $O \rightarrow N$ acyl migration. However, this will require a different strategy based, e.g., on the use of an orthogonal *N*-protection of Ser for slow release of the free amino group.

Conclusions

The HLH domain of the Id proteins contains the conserved Pro-Ser/Thr and Val-Ser motifs at the junctions between helix-1 and the loop and between the loop and helix-2, respectively. We previously showed that the Id1 HLH analog containing the *O*-acyl iso-dipeptide Val-Ser was devoid of the ability to fold into the native structure until the intramolecular $O \rightarrow N$ acyl migration was allowed to occur under neutral pH values [14]. In this work, we have synthesized the Id1 HLH analog containing the *O*-acyl iso-dipeptide Pro-Ser by applying a procedure suppressing DKP formation as reported by Beyermann and coworkers [18]. Again, the Id1 HLH iso-peptide analog lost any propensity to build secondary and tertiary structures. This suggests that the loop–helix junctions are determinant for the fully folded and unfolded states of the Id HLH domain.

Further, we have shown that *O*-acyl iso-Pro-Ser building blocks can be useful synthetic tools in Fmoc-based peptide chemistry, provided a procedure to prevent DKP formation is applied during the peptide chain assembly.

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