Chemical ligation from O-acyl isopeptides *via* 8- and 11-membered cyclic transition states

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Abstract
Unprotected O-acylated serine and O-acylated threonine isopeptides have been synthesized, and their conversion to native tripeptides and tetrapeptides by O- to N-terminus transfer investigated. Ligations involving 8- and 11-membered cyclic transition states are shown experimentally and computationally to be more favorable than intermolecular cross-ligations.

Keywords: Ligation, serine, threonine, peptide, benzotriazole

Introduction

The crucial importance of peptides in biological systems and their potent therapeutic activity[1–4] has increased the demand for efficient preparative methods. Synthetic access to proteins allows changes to be made in their covalent structure and enables specific labelling of a protein.[5–7]

Merrifield's linear solid-phase peptide synthesis (SPPS) is commonly used in polypeptide synthesis[9] but, linear SPPS of a large polypeptide can be tedious and costly. Thus, techniques to achieve convergent synthesis from smaller polypeptide fragments are critical in terms of reducing the production costs of peptide therapeutics and the synthesis of proteins.[5–7]

Chemoselective ligations are increasingly the 'key to the success' of protein synthesis.[10–13] Native chemical ligation (NCL), uniquely joins two unprotected peptide segments chemoselectively and regioselectively to afford a native peptide bond.[5,7] As reported in 1953 by Wieland *et al.*[8] NCL can join two peptide fragments through a thioester transesterification step followed by an intramolecular rearrangement involving an S- to N-acyl transfer *via* a cyclic

transition state resulting in the native peptide bond.[7,8] The bifunctional nature of the N-terminal cysteine 1,2-mercaptoamine moiety is responsible for the observed chemoselectivity in NCL.[14]

NCL development as a synthetic tool for building peptides depends on additives to increase ligation rates and yields. Ligations depend on factors such as steric demand, the exogenous thiol reactivity and the nature of the solvent.[14–17] In addition, the low abundance of cysteine (1.7% of the residues in protein sequences) is a major drawback of this methodology since NCL is restricted to Cys residues.

Our focus has been on serine and threonine, each possessing the 1,2-hydroxylamine bifunctionality[14] (corresponding to the SH/NH_2 in cysteine) thus affording chemoselective ligation by O- to N- acyl transfer without the need of cysteine residues. Acylation of the hydroxyl groups of serine and threonine, difficult without epimerization (especially in solid-phase synthesis)[18,19] was recently simplified by a solution-phase entry to enantiopure O-acyl isopeptides using acyl-benzotriazoles.[20]

Our group developed ligations of S-acylated Cys-peptides,[21−23] and N-acylated Trp-peptides.[24] Recently we demonstrated such classic O- to N-acyl migration *via* various transition states in O-acylated serine and tyrosine isopeptides.[25,26]

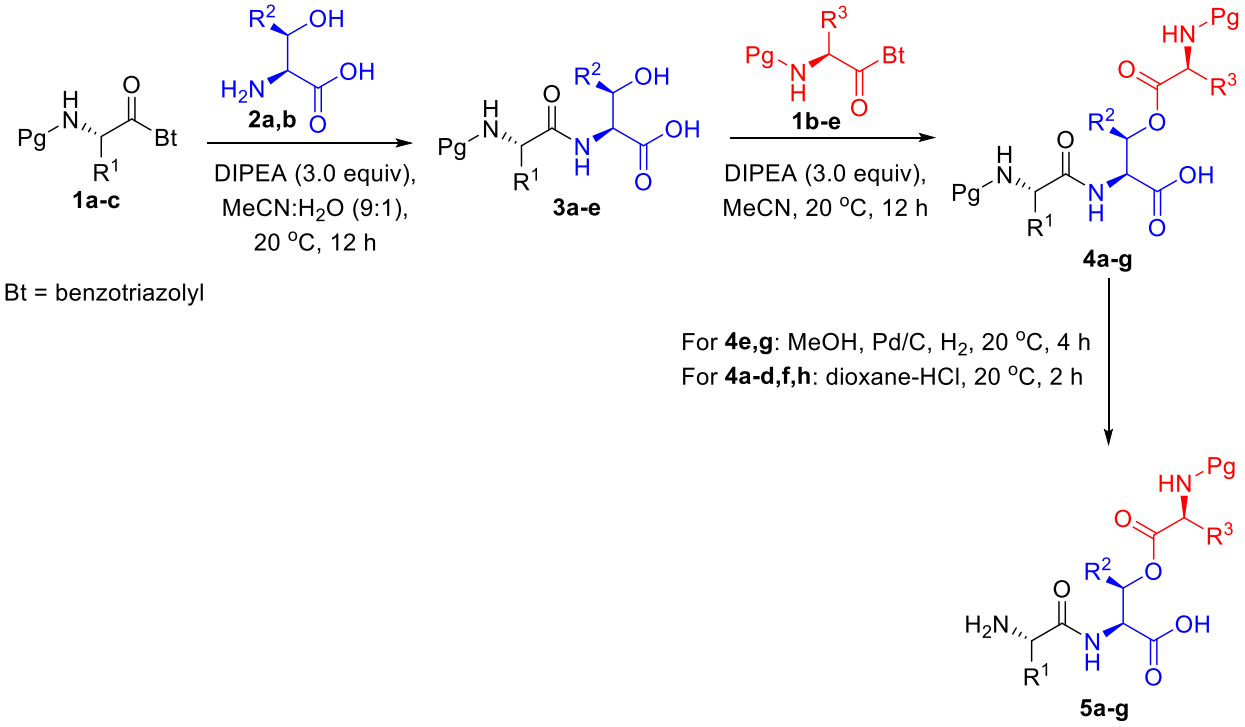
We now report an experimental and computational study of "traceless" chemical ligation involving O- to N- acyl shift (at Ser and Thr sites) with 8- and 11-membered transition states, involving neither cysteine nor an auxiliary group at the ligation site.

Results and Discussion

Monoisotripeptides **5a–g** were synthesized as starting materials to study the possibility of O- to N-acyl migrations *via* 8-membered cyclic transition state. Compounds **5a,f** were used as starting material for the synthesis of monoisotetrapeptide **9a,b** for ligation studies *via* an 11-membered cyclic transition state.

Preparation of the monoisotripeptides 5a–g

N-(Pg-α-Aminoacyl)benzotriazoles **1a–c** were coupled with L-Ser-OH **2a**/L-Thr-OH **2b** using a previously reported method[20] to give the corresponding protected dipeptides **3a–e**. Dipeptides **3a–e** were O-acylated by Cbz-L-Ala-Bt **1b**, Boc-L-Phe-Bt **1c**, Boc-Gly-Bt **1d** or Boc-L-Val-Bt **1e** in the presence of diisopropylethylamine (DIPEA) to provide N-protected monoisotripeptides **4a–g** (71−86%), which after deprotection by hydrogenation with Pd/C in methanol or HCl solution in 1,4-dioxane, yielded the free monoisotripeptides **5a–g** (Scheme 1, Table 1). Peptides **5a,f** were used both directly for ligation studies and also as intermediates to prepare the monoisotetrapeptides **9a,b**.



Scheme 1. Preparation of monoisotripeptide **5a–g**.

Table 1. Preparation of monoisotripeptides

Product **4**	Yield (%)	Mp (°C)	Product **5**	Yield (%)	Mp (°C)
Cbz-L-Phe-Ser(Boc-Gly)-OH, **4a**	86	88–90	**5a**	80	170–172
Cbz-L-Ala-Ser(Boc-L-Phe)-OH, **4b**	73	66–68	**5b**	79	150–152
Cbz-L-Ala-Ser(Boc-Gly)-OH, **4c**	75	58–60	**5c**	85	108–110
Cbz-L-Ala-Ser(Boc-L-Val)-OH, **4d**	71	73–75	**5d**	75	gum
Boc-L-Phe-Ser(Cbz-L-Ala)-OH, **4e**	79	72–73	**5e**	95	103–104
Cbz-L-Phe-Thr(Boc-Gly)-OH, **4f**	80	oil	**5f**	79	148-154
Boc-L-Phe-Thr(Cbz-L-Ala)-OH, **4g**	83	72–74	**5g**	92	93–95

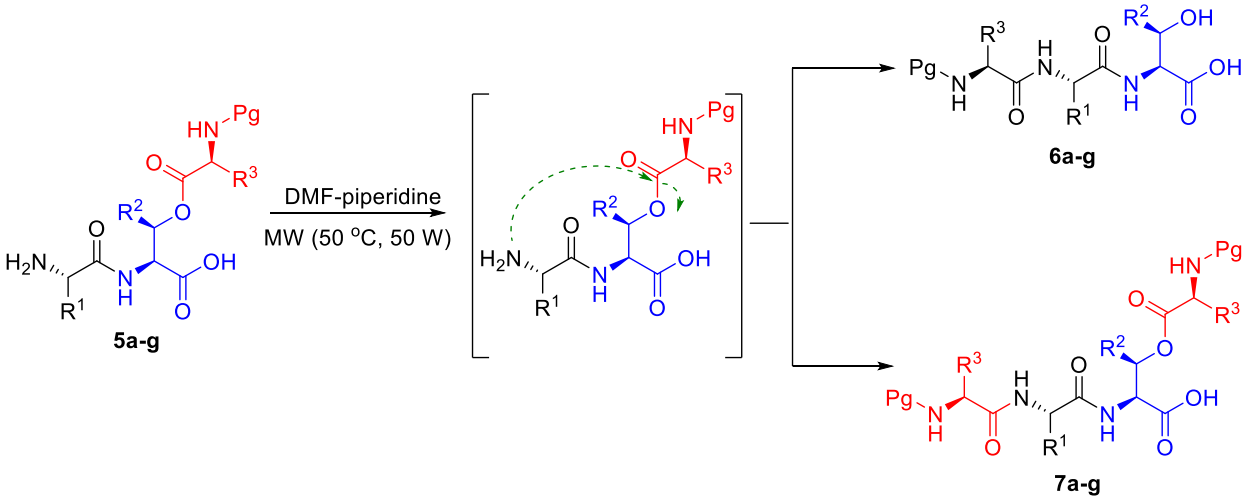
Study of the feasibility of O→N acyl migrations *via* 8-membered cyclic transition state

The O- to N-acyl migration for monoisotripeptides **5a–g** were studied under microwave irradiation in piperidine-DMF (20 v/v%), 50 °C, 50 W, 1 h to generate native peptides. Anhydrous conditions were chosen to minimize ester hydrolysis. HPLC−MS indicated the formation the ligated products **6a–g** along with the bis-acylated product **7a–g**. The results show that the nature of amino acids used has a profound effect on the yield of ligated product (Scheme 2, Table 2).

The desired O- to N-acyl transfer migration was only successful for of **5a**–**c, e**. The retention times and fragmentation patterns of starting material and ligated products were also studied in

control experiments (HPLC−MS of pure monoisotripeptide). Thus HPLC−MS, *via* (−)ESI-MS/MS, confirmed that compounds **5a**–**c, e** have different fragmentation patterns than **6a–c,e** proving the formation of the intramolecular ligated product.

The effect of O- to N-acyl transfer rearrangement using serine versus threonine for the same amino acid sequence was noted, with a 57% yield of native peptide **6a** (L-Ser) but none from **6f** (L-Thr). Microwave irradiation for 18 h at 50 °C, 50 W in piperidine-DMF (20 v/v%) did not alter **5g** significantly and gave no **6g**. The addition of salt, such as NaCl, and microwave irradiation **5g** in piperidine-DMF 20 v/v%, 70 °C, 50 W for 10 h ineffective in the formation of **6g**.



Scheme 2. Acyl migration of O-acyl isotripeptides **5a–g**.

Table 2. Chemical ligation of O-acyl isotripeptides **5a–g**

Entry	Reactant **5**	Ligated product **6** (%)[a]	Bis-acylated **7** (%)[a]
1	L-Phe-Ser(Boc-Gly)-OH, **5a**	57	0
2	L-Ala-Ser(Boc-L-Phe)-OH, **5b**	78	0
3	L-Ala-Ser(Boc-Gly)-OH, **5c**	23	7
4	L-Ala-Ser(Boc-L-Val)-OH, **5d**	0	4
5	L-Phe-Ser(Cbz-L-Ala)-OH, **5e**	8	1
6	L-Phe-Thr(Boc-Gly)-OH, **5f**	0	0.5
7	L-Phe-Thr(Cbz-L-Ala)-OH, **5g**	0	0

[a]Semiquantitative determination HPLC−MS. The area of ion-peak resulting from the sum of the intensities of the $[M+H]^+$ and $[M+Na]^+$ ions for each compound was integrated (corrected for starting material).

Computational rationalization

Conformational preorganization has been shown[27] to be important for internal ligation. In previous publications[20,23] we have demonstrated this principle and shown that sterically hindered and thus poorly preorganized isopeptides gave lower yields.

The reactivity of O-acyl isopeptides **5a–g** also varies with the protecting group and the nature of amino acid adjacent to the protecting group. To rationalize the reactivity patterns of **5a–g**, a previously developed computational protocol[27] were applied, which included a full conformational search and virtual screening based on a purposefully defined scoring function. This function **b**(N-C) is the geometrical distance between the amine nucleophile and the target ester carbon atom. Conformational searches were performed using the MMX force field (as implemented in PCModel v.9.3 software). The **b**(N-C) values are given in Table 3 for all the isopeptides. Generally the scoring function values are in qualitative but not perfect agreement with the yields. This can be explained by the presence of additional degrees of freedom. By analyzing the preorganized structures we found that the nucleophilic attack can be attenuated by hydrogen bond contact between one of the amino group protons and the oxygen atom belonging to the target carbonyl group. These contacts are characterized by bond separations **b**(H-O), which are also listed in Table 3. There is a clear relationship between the yield and the hydrogen bond strength characterized by **b**(H-O): the shorter the **b**(H-O) the lower the yield. The preorganized structures of **5b** and **5f** are shown in Figure 1: structure **5f** forms a distinct hydrogen bond contact (designated with a dashed line) both in terms of **b**(H-O) and the N-H-O angle, whereas **5b** is definitely not in a hydrogen bond configuration, since the NH bonds face away from the acceptor atom (Figure 1).

The ratio of **b**(N-C):**b**(H-O) gives an important measure of conformational preorganization for binding, as the best performing compounds **5a**–**c** have lower **b**(N-C)/**b**(H-O) values. This NH…O=C is not the only contact that can alter the reactivity: the optimized isopeptide structures disclose additional hydrogen bond contacts capable of locking the structure in an unfavorable conformation. Such conformationally locked structures were found for **5c–g**. The locking hydrogen bonds were characterized not only by bond separations, but also by the H-A…D angles (where A and D stand for acceptor and donor, respectively) are listed in Table 3. Table 3 suggests that hydrogen bond contacts are particularly strong in structures **5c,d,f**. Apparently, such hydrogen bond may be supportive, as, for example, in **5c**, which locks the structure in a favorable conformation, but this is not always the case, as evident from the zero yields found with **5d** and **5f**.

Replacement of Ser by Thr clearly reduces reactivity. This can be deduced by comparing the yields of the target product **6a–g** in **5a** against **5f** (57% vs. 0) and **5e** against **5g** (8% vs. 0). In conformational terms, it follows that the Thr methyl group (absent in Ser) drives the N-terminus slightly farther away from the target carbonyl, which is confirmed by larger **b**(N-C) values in **5f,g** compared with **5a** and **5e**, respectively.

Evidently, the preorganization of the starting O-acyl isotripeptides is an important factor in

the success of intramolecular long range 8-membered transition acyl transfers.

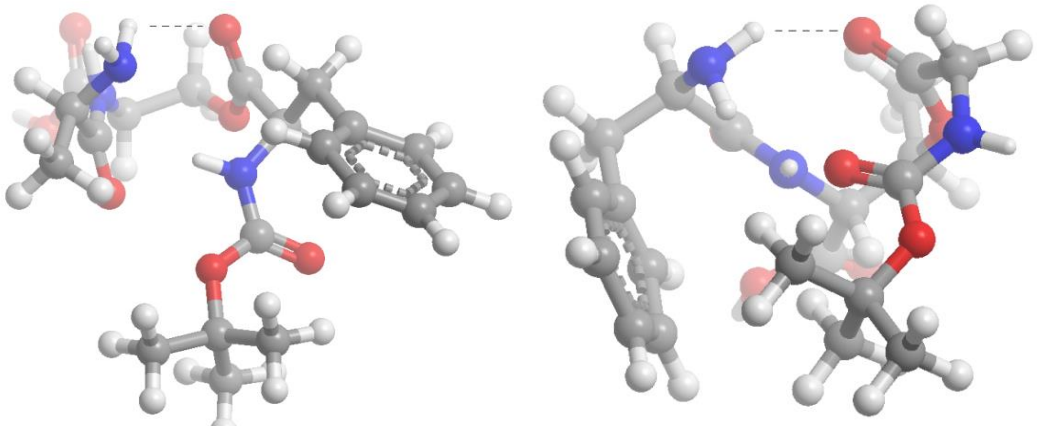


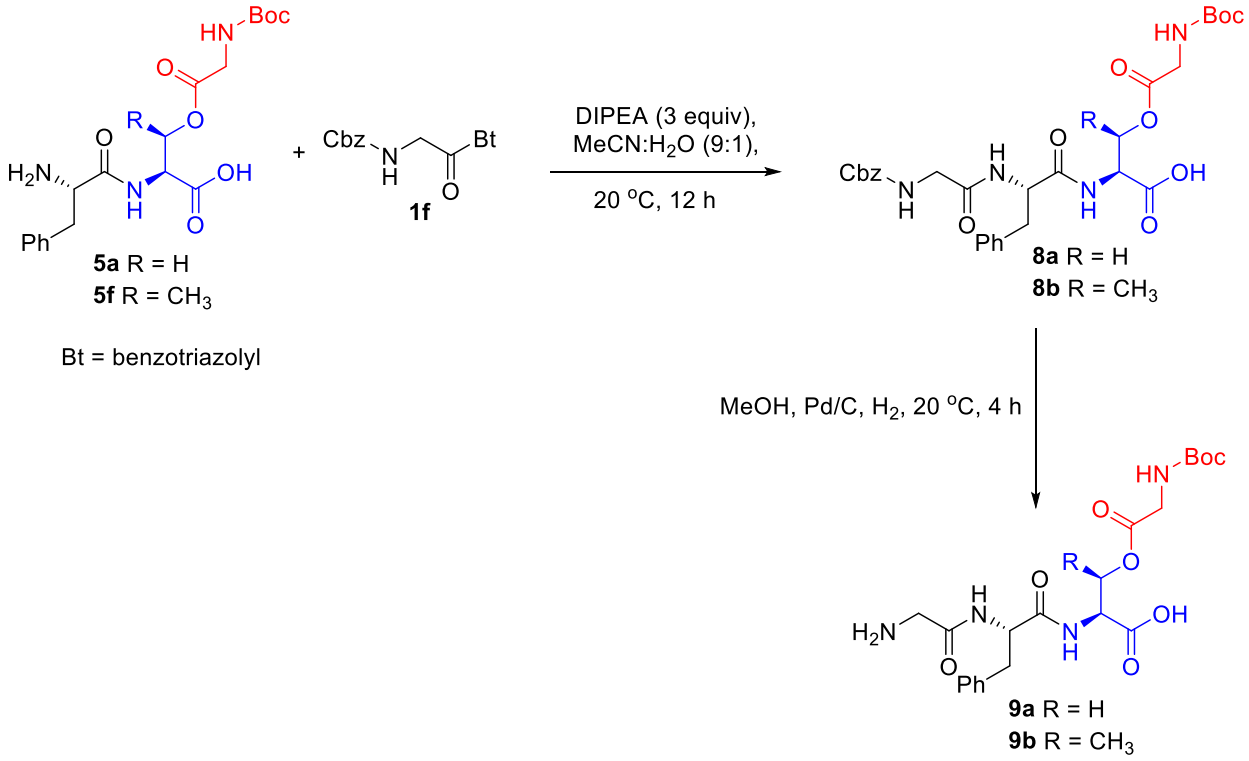
Figure 1. Putative NH…O=C hydrogen bond contact in preorganized structure **5f** (right); no hydrogen bond in **5b** (left).

Table 3. Structural characteristics of preorganized conformers **5a–g**

Structure **5**	b(N-C) (Å)	b(H-O) (Å)	Ratio	Yield **6** (%)	Acceptor	Donor	b(D-A) (Å)	$\angle_{HAD}$ (deg.)
5a	3.53	2.38	1.48	57	-	-	-	-
5b	3.48	2.47	1.41	78	-	-	-	-
5c	3.34	2.33	1.43	23	Ala N	Boc NH	1.81	174
5d	3.26	2.17	1.50	-	Ala N	Boc NH	1.86	162
5e	3.85	2.21	1.74	8	Phe O	Cbz NH	2.06	148
5f	3.75	2.06	1.82	-	Boc O	Thr NH	1.98	156
5g	4.25	2.80	1.52	-	Cbz O	Phe NH	2.05	135

Preparation of the O-acyl isotetrapeptides 9a,b

Cbz protected monoisotetrapeptides **9a,b** were synthesized in solution phase, by coupling the benzotriazolides of Cbz protected Glycine **1f** with the unprotected monoisotripeptides **5a,f** at 20 °C in 75–85% yield. The protecting groups of **8a,b** were removed by stirring each with Pd/C in methanol in hydrogen atmosphere for 1 h to afford the unprotected monoisotetrapeptides **9a,b** (Scheme 3). Compounds **8a,b** and **9a,b** were fully characterized by ^{1}H, ^{13}C NMR analysis.

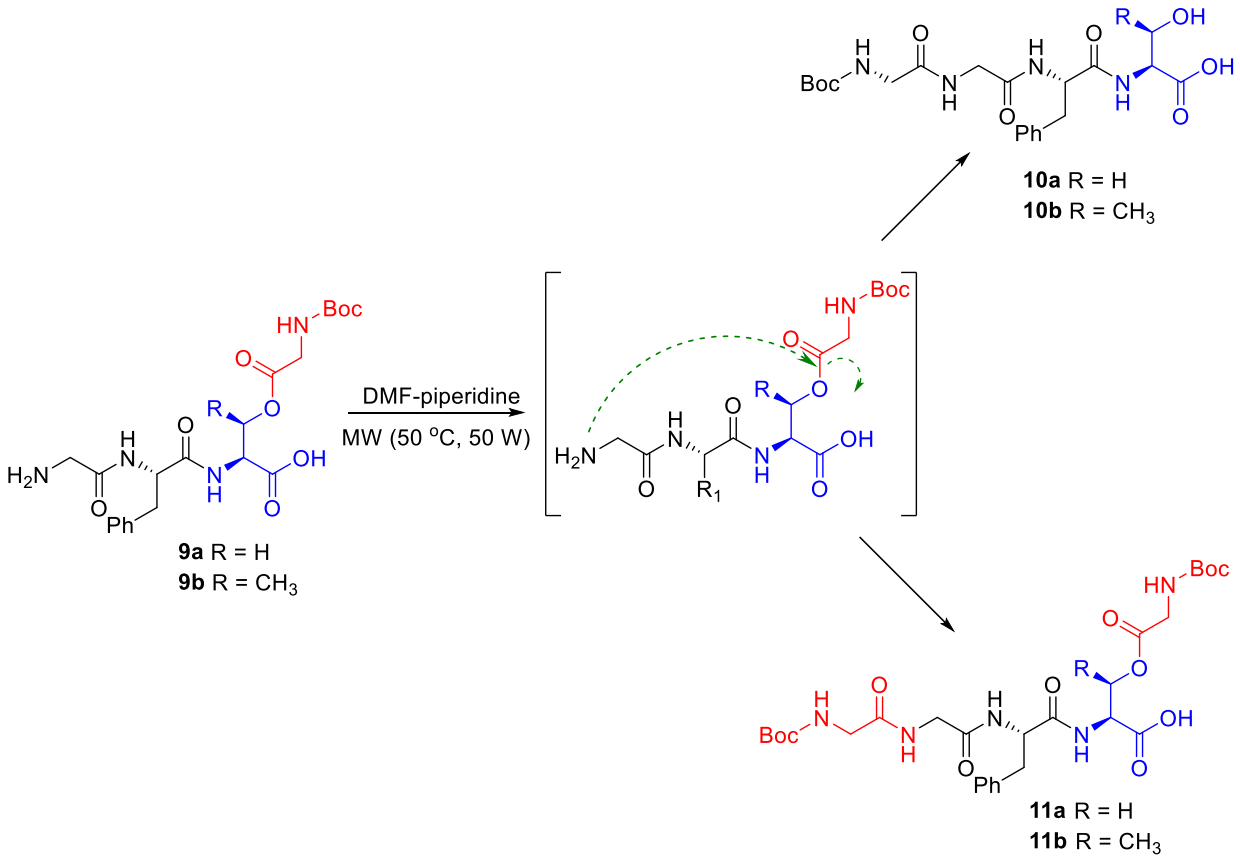


Scheme 3. Synthesis of O-acyl isotetrapeptides **9a,b**.

Study of the feasibility of O→N acyl migrations *via* an 11-membered cyclic transition state

Intermediates **9a,b** underwent ligation (Scheme 4, Table 4) under basic conditions (piperidine 20 v/v% in DMF, MW 50 °C, 50 W, 1 h for **9a** and 1 h, 3 h, and 6 h for **9b**). Unlike the challenges of steric hindrance and poor organization for binding eight-membered cyclic transition state, the expanded eleven transition state afforded O- to N-acyl transfer to give the desired native peptide **10a**. However, threonine remained a challenge in both eight and eleven ring transition states. HPLC-MS showed the formation of the expected intramolecular ligated products **10a**. The retention times and fragmentation patterns of **9a** was studied by control experiments (HPLC-MS of pure **9a**). Different fragmentation patterns in HPLC-MS, *via* (−)ESI-MS/MS, were found for **9a** and **10a**, each of MW 466. In addition, product **10a** was isolated and its structure further confirmed by HRMS.

The ligation of **9a** (eleven transition state) under aqueous conditions (pH 7.6, 1 M buffer strength, MW 50 °C, 50 W, 1 h). HPLC-MS gave a small amount of the corresponding ligated product **10a**, together with a major peak having MW 366 corresponding to the removal of the Boc-group either from **9a** or from the ligated product **10a**.



Scheme 4. Acyl migration of O-acyl isotripeptides **9a,b**.

Table 4. Chemical ligation of O-acyl isotetrapeptides **9a,b**

Entry	Reactant **9**	Ligated product **10** (%)[a]	Bis-acylated **11** (%)[a]
1	Gly-L-Phe-L-Ser(Boc-Gly)-OH, **9a**	99	0
2	Gly-L-Phe-Thr(Boc-Gly)-OH, **9b**	0	0

[a]Semiquantitative determination HPLC−MS. The area of ion-peak resulting from the sum of the intensities of the $[M+H]^+$ and $[M+Na]^+$ ions for each compound was integrated (corrected for starting material).

Conclusions

In summary, the chemical ligation of peptides affording O-acyl isopeptides occurs successfully without the use of cysteine or an auxiliary group. However, conformation preorganization of such peptides is critical for successful O- to N-acyl transfer to afford the corresponding native peptides.

Experimental Section

General. Melting points were determined on a capillary point apparatus equipped with a digital thermometer and are uncorrected. NMR spectra were recorded with TMS for ^{1}H (300 MHz) and ^{13}C (75 MHz) as an internal reference. Starting materials are available commercially and used without further purification. Reaction progress was monitored by thin-layer chromatography (TLC) and visualized by UV light. Elemental analyses were performed on a Carlo Erba EA 1108 instrument. HPLC-MS analyses were performed on reverse phase gradient Phenomenex Synergi Hydro-RP (C18): (2 x 150 mm; 4 um) + C18 guard column (2 x 4 mm), wavelength = 254 nm; flow rate 0.2 mL/min; and mass spectrometry was done with electro spray ionization (ESI).

General procedure for the preparation of dipeptides (3a–e). *N*-(Pg-α-Aminoacyl)benzotriazoles (1.0 mmol) in MeCN (5 mL) was added dropwise to a solution of free amino acid (1.5-2.0 equiv) and DIPEA (3.0 equiv) in MeCN (sometimes MeCN/H_2O 9:1, 15 mL) at the room temperature and stirred until all the *N*-(Pg-α-aminoacyl)benzotriazoles were consumed (12 h). MeCN was evaporated and the residue dissolved in EtOAc (50 mL) and washed with 3 N HCl (5 x 50 mL). The organic portion was dried over anhyd. Na_2SO_4, filtered and concentrated to give the desired peptide fragment. No further purification was required in all cases.

((Benzyloxy)carbonyl)-L-phenylalanyl-L-serine (Cbz-L-Phe-L-Ser-OH, 3a). White solid (85%); mp 156–157 ºC; ^{1}H NMR (CD_3OD) δ 8.16 (d, *J* 7.8 Hz, 1H), 7.38–7.20 (m, 10 H), 5.05–4.80 (m, 2H), 4.52–4.42 (m, 2H), 3.95–3.80 (m, 2H), 3.23–3.16 (m, 1H), 2.90–2.81 (m, 1H); ^{13}C NMR (CD_3OD) δ 174.3, 173.2, 158.4, 138.7, 138.2, 130.5, 129.6, 129.0, 128.8, 127.8, 67.7, 63.0, 57.9, 56.2, 39.3. Anal. Calcd for $C_{20}H_{22}N_2O_6$: C, 62.17; H, 5.74; N, 7.25; Found: C, 62.47; H, 5.82; N, 7.21.

((Benzyloxy)carbonyl)-L-alanyl-L-serine (Cbz-L-Ala-L-Ser-OH, 3b). White solid (81%); 195–197 ºC; (lit.[28] mp 192–194 ºC); ^{1}H NMR (DMSO-d_6) δ 7.99 (d, *J* 7.8 Hz, 1H), 7.46 (d, *J* 7.8 Hz, 1H), 7.36–7.29 (m, 5H), 5.02 (s, 2H), 4.29–4.23 (m, 1H), 4.17–4.10 (m, 1H), 3.72 (dd, *J* 11, 5 Hz, 1H) 3.62 (dd, *J* 11, 4 Hz, 1H), 1.21 (d, *J* 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 172.5, 171.9, 155.6, 137.0, 128.3, 127.8, 127.7, 65.4, 61.3, 54.6, 49.8, 18.3.

(*tert*-Butoxycarbonyl)-L-phenylalanyl-L-serine (Boc-L-Phe-L-Ser-OH, 3c). White solid (80%); mp 63–65 ºC; ^{1}H NMR ($CDCl_3$) δ 7.47–7.28 (m, 5H), 5.02 (br s, 2H), 4.29–4.22 (m, 1H), 4.17–4.10 (m, 1H), 3.76–3.71 (m, 1H), 3.66–3.59 (m, 1H), 1.23–1.20 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 172.7, 172.1, 156.1, 136.3, 129.4, 128.5, 126.9, 80.8, 62.6, 55.5, 54.7, 38.7, 28.2, 28.0. Anal. Calcd for $C_{17}H_{24}N_2O_6$: C, 57.94; H, 6.86; N, 7.95; Found: C, 57.83; H, 7.34; N, 7.47.

((Benzyloxy)carbonyl)-L-phenylalanyl-L-threonine (Cbz-L-Phe-L-Thr-OH, 3d). White solid (89%); mp 51–53 ºC; ^{1}H NMR (CD_3OD) δ 7.40–7.20 (m, 10H), 4.55–4.46 (m, 2H), 4.34 (br s, 1H), 4.10–4.06 (m, 1H), 3.30–3.18 (m, 1H), 2.95–2.88 (m, 1H), 1.96 (br s, 1H), 1.20–1.15 (m, 3H); ^{13}C NMR (CD_3OD) δ 174.1, 173.1, 157.8, 138.1, 137.7, 130.1, 129.0, 128.5, 128.3, 127.3,

68.3, 67.2, 58.7, 57.4, 38.7, 20.2. HRMS *m/z* for $C_{21}H_{24}N_2O_6$ $[M+Na]^+$ calcd. 423.1527, found 423.1514.

(*tert*-Butoxycarbonyl)-L-phenylalanyl-L-threonine (Boc-L-Phe-L-Thr-OH, 3e). Yellow gel[29] (78%); ^{1}H NMR ($CDCl_3$) δ 7.57 (d, *J* 7.9 Hz, 1H), 7.25–7.19 (m, 5H), 5.70 (d, *J* 7.5 Hz, 1H), 4.75–4.25 (m, 3H), 3.25–3.08 (m, 1H), 2.99–2.81 (m, 1H), 1.33 (s, 9H), 1.17 (d, *J* 5.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 173.4, 173.1, 156.1, 136.5, 129.5, 128.5, 126.9, 80.5, 68.1, 57.6, 28.3, 19.5.

General procedure for the preparation of O-acyl isopeptides 4a–g. Compound **3** (1.0 mmol) was added to a solution of *N*-(Pg-α-aminoacyl)benzotriazoles (1.0 mmol) (1.0 mmol) and DIPEA (3.0 mmol) in MeCN (20 mL) at room temperature and stirred for 12 h. MeCN was evaporated and the residue dissolved in EtOAc (50 mL) and washed with 2 N HCl (3 x 50 mL). The organic portion was dried over anhyd. Na_2SO_4, filtered and concentrated to give **4a–g**.

***N*-(((Benzyloxy)carbonyl)-L-phenylalanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-serine (Cbz-L-Phe-L-Ser(Boc-Gly)-OH, 4a)**. White solid. (86%); mp 86–88 ºC; ^{1}H NMR (CD_3OD) δ 8.35 (d, *J* 8.1Hz, 1H), 7.33–7.17 (m, 10 H), 5.01–4.98 (m, 2H), 4.77–4.73 (m, 1H), 4.57 (dd, *J* 11.4, 3.6 Hz, 1H), 4.48–4.36 (m, 2H), 3.78 (s, 2H), 3.18 (dd, *J* 13.8, 4.5 Hz, 1H), 2.89–2.80 (m, 1H), 1.42 (s, 9H); ^{13}C NMR (CD_3OD) δ 174.3, 171.8, 158.6, 158.3, 138.6, 138.2, 130.5, 129.5, 129.0, 128.8, 127.8, 80.9, 67.7, 65.0, 57.8, 53.0, 43.0, 39.2, 28.9. Anal. Calcd for $C_{27}H_{33}N_3O_9$: C, 59.66; H, 6.12; N, 7.73; Found C, 59.62; H, 6.13; N, 6.96.

***N*-(((Benzyloxy)carbonyl)-L-alanyl)-*O*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-L-serine (Cbz-L-Ala-L-Ser(Boc-L-Phe)-OH, 4b).** White solid (73%); mp 66–68 ºC; ^{1}H NMR ($CDCl_3$) δ 7.89 (br s, 2H), 7.40–7.16 (m, 10H), 5.66–5.56 (m, 1H), 5.21–5.10 (m, 2H), 5.08–4.98 (m, 2H), 4.81–4.23 (m, 3H), 3.22–2.88 (m, 2H), 1.40–1.28 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 172.4, 172.1, 171.3, 156.4, 155.7, 136.7, 135.8, 129.3, 128. 5, 128.2, 128.1, 126.7, 80.3, 67.3, 63.7, 56.8, 51.8, 48.9, 38.3, 28.2, 17.5. Anal. Calcd for $C_{28}H_{35}N_3O_9$: C, 60.31; H, 6.33; N, 7.54; Found C, 60.34; H, 6.74; N, 7.37.

***N*-(((Benzyloxy)carbonyl)-L-alanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-serine (Cbz-L-Ala-L-Ser(Boc-Gly)-OH, 4c).** White solid (75%); mp 58–60 ºC; ^{1}H NMR (DMSO-d_6) δ 8.24 (d, *J* 7.2 Hz, 1H), 7.45 (d, *J* 7.5 Hz, 1H), 7.36–7.30 (m, 5H), 7.21 (t, *J* 12.0 Hz, 1H), 5.01 (s, 2H), 4.5 (s, 1H), 4.32–4.24 (m, 2H), 4.13–4.08 (m, 1H), 3.68 (t, *J* 10.5 Hz, 2H), 1.45–1.23 (m, 9H), 1.20 (d, *J* 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 172.7, 170.5, 170.2, 155.8, 155.6, 137.0, 128.3, 127.8, 78.3, 65.4, 63.5, 50.9, 49.8, 41.7, 28.2, 18.2. Anal. Calcd for $C_{21}H_{29}N_3O_9$: C, 53.96; H, 6.25; N, 8.99; Found C, 53.28; H, 6.49; N, 7.81.

***N*-(((Benzyloxy)carbonyl)-L-alanyl)-*O*-((*tert*-butoxycarbonyl)-L-valyl)-L-serine (Cbz-L-Ala-L-Ser(Boc-L-Val)-OH, 4d).** White solid (71%); mp 73–75 ºC; ^{1}H NMR ($CDCl_3$) δ 7.33–7.28 (m, 5H), 5.24 (d, 1H), 5.15–5.03 (m, 2H), 4.84 (br s, 1H), 4.54 (br s, 1H), 4.42–4.07 (m, 2H), 2.13–2.04 (m, 1H), 1.43–1.38 (m, 12H), 0.89 (dd, *J* 16.4, 6.6 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 173.3, 172.3, 171.5, 156.3, 136.3, 128.6, 128.3, 128.2, 80.5, 67.2, 63.8, 58.9, 51.9, 50.7, 30.9, 28.4, 18.8, 17.8. Anal. Calcd for $C_{24}H_{35}N_3O_9$: C, 56.57; H, 6.92; N, 8.25; Found C, 56.92; H, 7.35; N, 8.02.

***O*-(((Benzyloxy)carbonyl)-L-alanyl)-*N*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-L-serine (Boc-L-Phe-L-Ser(Cbz-L-Ala)-OH, 4e).** White solid. (79%); mp 72–73 ºC; ^{1}H NMR ($CDCl_3$) δ 7.85 (br s, 2H), 7.36–7.16 (m, 10H), 5.65 (br s, 1H), 5.21–4.98 (m, 2H), 4.80–4.69 (m, 2H), 4.53–4.23 (m, 3H), 3.24–2.88 (m, 2H), 1.39–1.28 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 172.4, 172.1, 171.3, 156.4, 155.7, 136.7, 135.8, 129.3, 128.5, 128.2, 128.1, 126.7, 80.3, 67.3, 63.7, 55.8, 51.8, 49.9, 38.3, 28.2, 17.5. Anal. Calcd for $C_{28}H_{35}N_3O_9$: C, 60.31; H, 6.33; N, 7.54; Found C, 60.05; H, 6.77; N, 7.39.

***N*-(((Benzyloxy)carbonyl)-L-phenylalanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-threonine (Cbz-L-Phe-L-Thr(Boc-Gly)-OH, 4f).** Yellow oil (80%);^{1}H NMR ($CDCl_3$) δ 7.30–7.17 (m, 10H), 6.28 (br s, 3H), 5.80–5.75 (m, 1H), 5.44–5.40 (m, 1H), 5.32–5.30 (m, 1H), 5.10–4.95 (m, 2H), 4.80–4.75 (m, 1H), 4.70–4.54 (m, 2H), 3.80–3.78 (m, 2H), 3.15–3.13 (m, 1H), 3.07–2.98 (m, 1H), 2.04 (br s, 2H), 1.41 (br s, 9H), 1.27–1.23 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 172.4, 172.2, 171.7, 171.3, 156.4, 136.3, 129.4, 128.6, 128.5, 128.2, 128.0, 127.0, 80.5, 67.2, 60.5, 56.2, 55.7, 42.3, 28.3, 17.1, 14.2. Anal. Calcd for $C_{28}H_{35}N_3O_9$: C, 59.04; H, 6.43; N, 7.38; Found C, 59.40; H, 6.76; N, 7.00.

***O*-(((Benzyloxy)carbonyl)-L-alanyl)-*N*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-L-threonine (Boc-L-Phe-L-Thr(Cbz-L-Ala)-OH, 4g).** White solid (83%); mp 72–74 ºC; ^{1}H NMR ($CDCl_3$) δ 10.24 (s, 1H), 7.30–7.16 (m, 10H), 5.44 (dd, *J* 6.4, 3.8 Hz, 1H), 5.14–5.07 (m, 2H), 4.80 (dd, *J* 8.9, 3.5 Hz, 1H), 4.60–4.47(m, 1H), 4.32–4.24 (m, 1H), 3.23–2.94 (m, 2H), 1.43–1.23 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 173.1, 173.0, 171.8, 156.5, 156.3, 136.9, 136.3, 129.6, 128.7, 128.3, 127.0, 80.5, 71.7, 67.4, 55.8, 49.9, 38.1, 28.4, 18.6, 17.9, 17.3. Anal. Calcd for $C_{29}H_{37}N_3O_9$: C, 60.93; H, 6.52; N, 7.35; Found C, 61.24; H, 6.64; N, 7.55.

General procedure for the preparation of unprotected O-acyl isopeptides 5a–g

For deprotection of the Cbz- protecting group. Compound **4a-d**, **4f** and **4g** (1.0 mmol) was dissolved in anhydrous MeOH (30 mL) and stirred under an atmosphere of hydrogen in the presence of a catalytic amount of Pd/C for 4 h. Filtration through a bed of celite and evaporation afforded **5a–d**, **5f** and **5g**. Compound **4f** was deprotected and used as crude to make compound **8b**.

For deprotection of the Boc-protecting group. Compounds **4e** and **4h** (1.0 mmol) was dissolved in either HCl-dioxane (4.0 M HCl in dioxane, 15 mL) or freshly prepared HCl-MeOH (prepared by bubbling HCl in MeOH) (15 mL) and stirred for 2 h. Solvent is evaporated, and ether was added to the residue and stirred for 2 h. Filtration gave a white solid **5e** and **5h** (when sticky solid resulted, decantation of ether several times was performed instead).

***N*-(L-Phenylalanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-serine (L-Phe-L-Ser(Boc-Gly)-OH 5a).** White solid (80%);mp 170–172 ºC; ^{1}H NMR (CD_3OD) δ 7.35–7.24 (m, 5H), 4.60–4.50 (m, 1H), 4.39 (s, 2H), 4.25–4.18 (m, 1H), 3.77 (s, 2H), 3.30–3.00 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (CD_3OD) δ 174.2, 172.8, 169.5, 158.8, 135.5, 130.7, 130.3, 129.0, 81.5, 66.5, 55.9, 55.6, 43.1, 38.2, 28.8. HRMS *m/z* for $C_{19}H_{28}N_3O_7$ $[M+H]^+$ calcd. 410.1922, found 410.1909.

***N*-(L-Alanyl)-*O*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-L-serine (L-Ala-L-Ser(Boc-L-Phe)-OH, 5b).** White solid (79%); mp 150–152 ºC; ^{1}H NMR (CD_3OD) δ 7.28–7.17 (m, 5H), 4.53–4.33 (m, 4H), 3.18 (dd, *J* 13.9, 4.7 Hz, 1H), 2.87 (dd, *J* 13.9, 9.5 Hz, 1H), 1.53 (d, *J* 6.7 Hz, 3H), 1.36 (s, 9H); ^{13}C NMR (CD_3OD) δ 174.2, 173.4, 170.8, 157.8, 138.5, 130.5, 130.3, 129.4, 127.7, 80.6, 66.5, 56.5, 55.6, 50.4, 38.4, 28.7, 17.5. Anal. Calcd for $C_{20}H_{29}N_3O_7$: C, 56.73; H, 6.90; N, 9.92; Found C, 56.61; H, 7.33; N, 9.18.

***N*-(L-Alanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-serine (L-Ala-L-Ser(Boc-Gly)-OH, 5c).** White solid (85%); mp; 108–110; ^{1}H NMR (CD_3OD) δ 4.42–4.30 (m, 2H), 3.85–3.83 (m, 1H), 3.63 (br s, 2H), 3.20–3.14 (m, 1H), 1.38 (d, *J* 7.2 Hz, 3H), 1.29 (s, 9H); ^{13}C NMR (CD_3OD) δ 174.5, 172.3, 171.0, 158.7, 80.8, 66.5, 55.7, 43.1, 28.9, 17.7. Anal. Calcd for $C_{13}H_{23}N_3O_7$: C, 46.84; H, 6.95; N, 12.61; Found C, 46.36; H, 7.34; N, 12.08.

***N*-(L-Alanyl)-*O*-((*tert*-butoxycarbonyl)-L-valyl)-L-serine (L-Ala-L-Ser(Boc-L-Val)-OH, 5d).** Gum (75%); ^{1}H NMR (CD_3OD) δ 4.67–4.38 (m, 3H), 4.10–3.28 (m, 2H), 2.21–2.03 (m, 1H), 1.56 (d, *J* 6.1 Hz, 3H), 1.44 (s, 9H), 0.92 (dd, *J* 11.3, 6.8 Hz, 6H); ^{13}C NMR (CD_3OD) δ 173.6, 170.8, 158.4, 80.7, 66.2, 60.7, 50.5, 42.2, 31.8, 28.9, 19.8, 18.5. HRMS *m/z* for $C_{16}H_{29}N_3O_7$ $[M+H]^+$ calcd. 376.2010, found 376.2039.

***N*-(L-Phenylalanyl)-*O*-(((benzyloxy)carbonyl)-L-alanyl)-L-serine (L-Phe-L-Ser(Cbz-L-Ala)-OH, 5e).** White microcrystals (95%); mp 103–104 ºC; ^{1}H NMR (DMSO-d_6) δ 9.15 (d, *J* 8.1 Hz, 1H), 8.37 (br s, 3H), 7.82 (d, *J* 7.2 Hz, 1H), 7.39–7.23 (m, 10H), 4.99 (dd, *J* 15.7, 12.6 Hz, 2H), 4.65–4.59 (m, 1H), 4.36 (dd, *J* 11.3, 4.7 Hz, 1H), 4.27 (dd, *J* 11.3, 5.9 Hz, 1H), 4.16–4.05 (m, 2H), 3.20 (dd, *J* 14.3, 5.7 Hz, 1H), 3.03 (dd, *J* 14.3, 7.5 Hz), 1.29 (d, *J* 7.4 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 172.6, 170.0, 168.2, 155.9, 136.9, 134.8, 129.7, 128.5, 128.4, 127.8, 127.1, 66.4, 65.6, 53.2, 51.2, 49.3, 36.7, 16.9. Anal. Calcd for $C_{46}H_{58}N_6O_{15}$: C, 54.93; H, 5.81; N, 8.35; Found C, 54.63; H, 6.27; N, 8.02.

***N*-(L-Phenylalanyl)-*O*-((*tert*-butoxycarbonyl)glycyl)-L-threonine (L-Phe-L-Thr(Boc-Gly)-OH, 5f).** Used as crude to make compound **8b**. See **8b**.

***N*-(L-Phenylalanyl)-*O*-(((benzyloxy)carbonyl)-L-alanyl)-L-threonine (L-Phe-L-Thr(Cbz-L-Ala)-OH, 5g).** White solid (92%); mp 93–95 ºC; ^{1}H NMR (CD_3OD) δ 7.35–7.28 (m, 10H), 5.46–5.38 (m, 1H), 5.07 (s, 2H), 4.70 (d, *J* 5.0 Hz, 1H), 4.30 (dd, *J* 8.3, 5.7 Hz, 1H), 4.23–4.15(m, 1H), 3.34 (dd, *J* 9.8, 4.4 Hz, 1H), 3.08 (dd, *J* 14.3, 8.4 Hz, 1H), 1.37 (d, *J* 7.3 Hz, 3H), 1.31 (d, *J* 6.4 Hz, 3H); ^{13}C NMR (CD_3OD) δ 173.8, 171.6, 170.3, 158.6, 138.2, 135.5, 130.8, 130.2, 129.6, 129.2, 129.0, 128.8, 72.0, 67.8, 57.5, 55.7, 51.2, 38.6, 17.6, 17.5. Anal. Calcd for $C_{48}H_{62}Cl_2N_6O_{15}$: C, 55.76; H, 6.04; N, 8.13; Found C, 55.59; H, 6.35; N, 7.98.

General procedure for the preparation of unprotected O-acyl isopeptides 8a–c

Compounds **8a–c** were prepared by following the same procedure followed for **4a–g**.

***N*-((Benzyloxy)carbonyl)glycyl-L-phenylalanyl-*O*-((*tert*-butoxycarbonyl)glycyl)-L-serine (Cbz-Gly-L-Phe-L-Ser(Boc-Gly)-OH, 8a).** White solid (89%), converted to compound **9a** after checking NMR. **8a**: mp 180–181 ºC (decomposed). ^{1}H NMR (CD_3OD) δ 7.39–7.22 (m, 10H), 5.08 (s, 2H), 4.75–4.63 (m, 2H), 4.57–4.49 (m, 1H), 4.43–4.37 (m, 1H), 3.79–3.66 (m, 4H),

3.30–3.06 (m, 1H), 2.97–2.77 (m, 4H), 1.42 (s, 9H); ^{13}C NMR (CD_3OD) δ 177.0, 173.6, 173.4, 172.1, 171.9, 171.8, 159.1, 138.3, 130.5, 129.6, 129.1, 129.0, 127.9, 127.2, 80.9,74.3, 68.0, 65.0, 55.7, 53.0, 45.0, 44.0, 43.0, 38.7, 28.6. HRMS *m/z* for $C_{29}H_{36}N_4O_{10}$ $[M+H]^+$ calcd. 601.2441, found 601.2402.

***N*-((Benzyloxy)carbonyl)glycyl-L-alanyl-*O*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-L-serine (Cbz-Gly-L-Ala-L-Ser(Boc-L-Phe)-OH, 8b).** Colorless oil (89%); converted to compound **9b** after checking NMR. **8b**:^{1}H NMR ($CDCl_3$) δ 9.70 (br s, 2H), 7.34 (d, *J* 7.4 Hz, 1H), 7.25–7.07 (m, 10H), 6.03 (d, *J* 7.4 Hz, 1H), 5.07–4.57 (m, 4H), 4.29–4.14 (m, 2H), 3.84–3.55 (m, 3H), 3.12–2.89 (m, 2H), 1.36 (d, *J* 2.8 Hz, 3H), 1.32 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.5, 170.3, 170.0, 169.4, 155.5, 155.1, 135.1, 134.9, 128.2, 127.5, 127.4, 127.1, 127.0, 125.9, 124.4, 66.1, 62.6, 53.0, 50.9, 48.8, 42.8, 36.5, 29.3, 27.2, 16.4. HRMS *m/z* for $C_{30}H_{38}N_4O_{10}$ $[M+H]^+$ calcd. 615.2584, found 6159.2601.

***N*-((Benzyloxy)carbonyl)glycyl-L-phenylalanyl-*O*-((*tert*-butoxycarbonyl)glycyl)-L-threonine (Cbz-Gly-L-Phe-L-Thr(Boc-Gly)-OH, 8c).** Colorless oil (82%); ^{1}H NMR (CD_3OD) δ 7.37–7.20 (m, 10H), 5.10–5.08 (m, 2H), 4.13–4.08 (m, 1H), 3.83–3.29 (m, 3H), 2.01–1.97 (m, 8H), 1.44 (br s, 9H), 1.23 (t, *J* 7.2 Hz, 3H); ^{13}C NMR (CD_3OD) δ 175.4, 174.5, 174.0, 173.8, 173.2, 138.3, 130.5, 129.6, 129.2, 129.1, 129.0, 129.0, 128.0, 80.7, 68.0, 67.8, 61.7, 55.1, 45.0, 43.3, 42.9, 38.9, 38.5, 28.8, 21.0, 20.9, 14.6. HRMS *m/z* for $C_{21}H_{30}N_4O_8Na$ $[M+Na]^+$ calcd. 489.1956, found 489.1965. HRMS *m/z* for $C_{30}H_{38}N_4O_{10}$ $[M+H]^+$ calcd. 615.2584, found 6159.2596.

General procedure for the preparation of unprotected O-acyl isopeptides 9a,b

Compounds **9a,b** were prepared by following the same procedure followed for **5a–g**.

***O*-((*tert*-Butoxycarbonyl)glycyl)-*N*-glycyl-L-phenylalanyl-L-serine (Gly-L-Phe-L-Ser(Boc-Gly)-OH, 9a).** White solid (85%) yield; mp 168–173 ºC; ^{1}H NMR (CD_3OD) δ 7.41–7.11 (m, 5H), 4.78–4.63 (m, 1H), 4.62–4.49 (m, 2H), 4.43–4.33 (m, 1H), 3.93–3.63 (m, 3H), 3.27–3.18 (m, 1H), 3.01–2.63 (m, 4H), 1.43 (s, 9H); ^{13}C NMR (CD_3OD) δ 174.9, 173.2, 172.2, 167.8, 158.7, 138.5, 130.5, 129.7, 128.0, 80.9, 66.0, 56.6, 54.7, 44.8, 43.1, 38.8, 28.9. HRMS *m/z* for $C_{21}H_{30}N_4O_8Na$ $[M+Na]^+$ calcd. 489.1956, found 489.1965.

***O*-((*tert*-Butoxycarbonyl)glycyl)-*N*-glycyl-L-phenylalanyl-L-threonine (Gly-L-Phe-L-Thr(Boc-Gly)-OH, 9b).** Sticky yellow solid (75%); ^{1}H NMR ($CDCl_3$) δ 7.30–7.13 (m, 5H), 5.14–4.49 (m, 1H), 3.75–3.70 (m, 2H), 3.10–3.06 (m, 1H), 1.80–1.50 (m, 3H), 1.45–1.10 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 171.7, 170.1, 168.5, 152.0, 151.4, 132.0, 125.5, 125.3, 124.6, 123.1, 76.3, 52.0, 50.5, 50.3, 48.3, 38.3, 34.4, 33.9, 29.6, 24.3, 22.2. HRMS *m/z* for $C_{22}H_{32}N_4O_8$ $[M+H]^+$ calcd. 481.2320, found 481.2334.

(*tert*-Butoxycarbonyl)glycyl-L-phenylalanyl-L-serine (Boc-Gly-L-Phe-L-Ser-OH, 6a). Compound **5a** (20 mg, 0.05 mmol) was dissolved in piperidine 20 v/v% in DMF (1 mL) and stirred at 50 ºC and 50 W for 1h. The mixture was then evaporated and purified by HPLC to give ligated product **6a** (57%); The sample was analyzed *via* reverse phase gradient C18 HPLC/UV/(-)ESI-MSn to give a retention time of 23.07 min. To confirm structure, HRMS for **6a** *m/z* for $C_{19}H_{26}N_3O_7$ $[M-H]^+$ calcd. 408.1776, found 408.1794.

(*tert*-Butoxycarbonyl)glycylglycyl-L-phenylalanyl-L-serine (Boc-Gly-Gly-L-Phe-L-Ser-OH, 10a). Compound **9a** (20 mg, 0.04 mmol) was dissolved in piperidine 20 v/v% in DMF (1 mL) and stirred at 50 ºC and 50 W for 1h (3h for **9b**). The mixture was then evaporated and purified by HPLC to give ligated product **10a** (99.39%). The sample was analyzed *via* reverse phase gradient C18 HPLC/UV (254 nm/ESI-MSn gave a retention time of 21.67 min (for **10a**). To confirm structure, HRMS for **11a** *m/z* for $C_{21}H_{29}N_4O_8$ $[M-H]^+$ calcd. 465.2064, found 465.1992.

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