Improved acylation of pseudoproline: masked threonine in flow peptide chemistry

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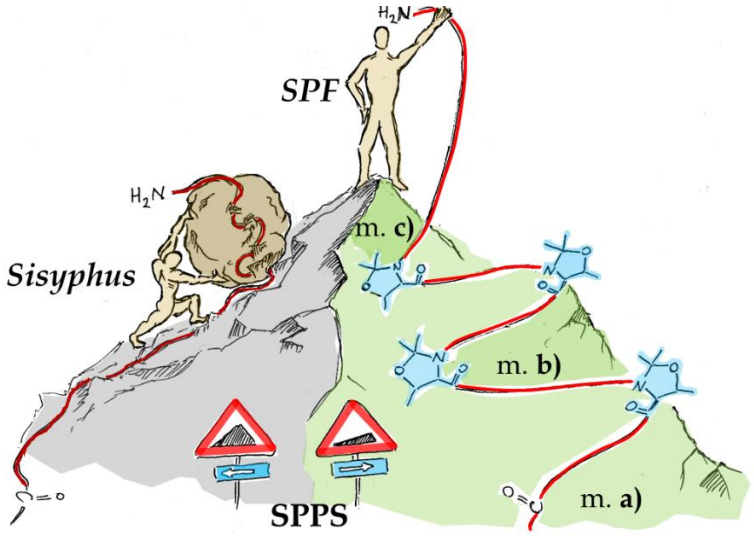
Graphical abstract:



Here we introduce a productive flow chemistry method to acylate H-Thr(ΨPro) derivatives, without double coupling or recirculating.

Abstract:

Pseudoproline derivatives are built into polypeptides as aggregation disrupters during the synthetic process from which during the final step (cleavage from the resin) the unmodified sidechains can be recovered. They are often introduced as expensive dipeptides, H-Xaa-Yaa(ΨPro)-OH (Xaa = any of the 20 proteinogenic amino acid, Yaa = Ser or Thr) as the direct acylation efficacy of hindered pseudo derivatives is too low. Here we show how the acylation of incorporated H-Thr(ΨPro)- (less reactive and thus more challenging than that of H-Ser(ΨPro)-) can be successful in flow peptide chemistry with nearly all proteinogenic amino acids (Xaa or X). The Xaa-Thr(ΨPro) amide bond was created using 3-5 equ. (equivalent) reagent excess, with better than 75% efficiency for most Xaa, and with 70% in case of Met. Only in case of Asp was efficiency too low (8%) (due to succinimide formation) to support use of pseudoproline derivatives.

Introduction

The chemical synthesis of longer polypeptides remains a challenge, as unspecific entanglement and aggregation might occur between polypeptides of elevated hydrophobicity due to their bulky and fatty

sidechain-protecting groups during synthesis. Furthermore, interacting with the surface of the resin, the *N*-terminal amine of the nascent hydrophobic chain becomes gradually more hidden during Fluorenylmethoxycarbonyl (Fmoc) based **S**olid **P**hase **P**eptide **S**ynthesis (SPPS) and thus, more difficult to acylate. These effects strongly hinder the synthesis of longer polypeptides. The introduction of pseudoproline (ΨPro) derivatives, protected residues that mask Ser, Thr or Cys sidechains as proline-like five-membered oxazolidines or thiazolidines -Ser($\Psi^{Me,Me}$pro)- , -Thr($\Psi^{Me,Me}$pro)- , -Cys($\Psi^{Me,Me}$pro)- (referred to as **S(ΨPro)**, **T(ΨPro)** and **C(ΨPro)** respectively)[1,2], offers a solution as they force kinks to the main chain structure, disrupting the unwanted dispersive forces. In this way, making longer polypeptides and achieving higher synthetic yields becomes possible without changing the primary structure, as the isopropilidene protecting group of the pseudoproline rings is hydrolyzed during the cleavage of the polypeptide chain from the resin affording the unmodified sidechains. (**Figure 1**)

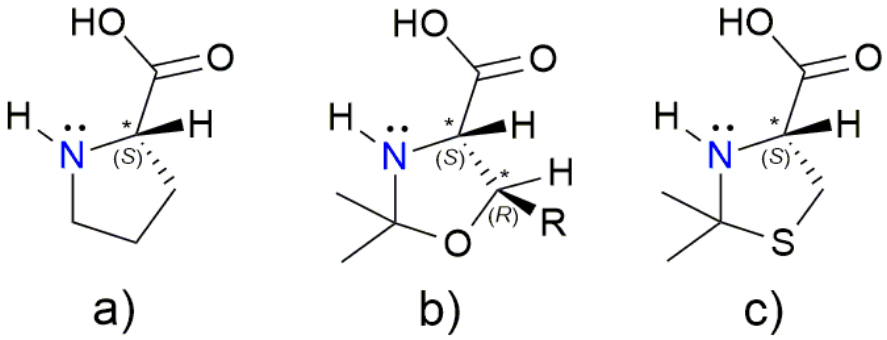


Figure 1. The chemical structure of **a)** H-L-Pro-OH, **b)** H-Ser($\Psi^{Me,Me}$pro)-OH (R_1= H), H-Thr($\Psi^{Me,Me}$pro)-OH (R_1= CH_3) and **c)** H-Cys($\Psi^{Me,Me}$pro)-OH

However, coupling to such sterically hindered residues as 2-aminoisobutyric acid (Aib) or pseudoproline remains a challenge. To overcome the problem of devastatingly weak acylation efficacy of H-L-Yaa($\Psi^{Me,Me}$pro)-, dipeptides of pseudoprolines, Fmoc-Xaa-Yaa($\Psi^{Me,Me}$pro)-OH, are traditionally introduced, which requires however full libraries of Fmoc-Xaa-Ser($\Psi^{Me,Me}$pro)-OH, Fmoc-Xaa-Thr($\Psi^{Me,Me}$pro)-OH and Fmoc-Xaa-Cys($\Psi^{Me,Me}$pro)-OH on stock, making this approach expensive.[3,4]

Nevertheless, application of pseudoproline containing dipeptides has proved quite beneficial in a number of greatly varied problematic synthetic cases. Sélambarom *et al*. have prepared temporarily masked pseudoproline dipeptides by condensing L-Ser or L-Thr with paraformaldehyde, which could be efficiently converted to their *N*-Acyl-oxazolidinones.[5] Others use pseudoproline dipeptides at the *C*-terminal to assist condensation of liraglutide and semaglutide, preventing aggregation to the resin.[6] Liu and Tam made the in-situ formation of pseudoproline derivatives during ligation, where at the *C*-terminal an α-acyloxyacetaldehyde and at the *N*-terminal unprotected Ser, Thr, and Cys residues were introduced, followed by the *O*-to-*N* acyl transfer, resulting in the desired peptide bond. Using this method, they successfully synthesized a 50 residue

long epidermal growth factor-like polypeptide.[7] Similarly, the MUC1 80^{mer} glycopeptide was prepared from two polypeptide fragments *via* Ser-ligation, where the *C*-terminal was modified to salicilycaldehyde esters (SAL) and condensed to Ser of the *N*-terminal. (Note that this ligation method also leads to the formation of a pseudoproline derivative).[8] The synthesis of the 37-residue long human Amylin and its amyloidogenic 1−37 fragment[9] was completed successfully using pseudoproline dipeptides, both Xaa-Ser(ΨPro) and Xaa-Thr(ΨPro). During this synthesis, pseudoproline derivatives were inserted at two positions, as Fmoc-A-T(ΨPro)-OH dipeptide was used to form the ^{8}Ala-^{9}Thr, while Fmoc-Ser-Ser(ΨPro)-OH to synthesize the ^{19}Ser-^{20}Ser subunits. However, in order to achieve an acceptably high purity (~70%) the authors had to use, pseudoproline dipeptides and thus, separating the critical acylation step of Ser(ΨPro) from the SPPS. Recently, Manne and co-workers successfully synthetized a nearly inaccessible human Growth Hormone (hGH)-derived polypeptide using Ser(ΨPro), and JR10 using Thr(ΨPro) [10a,b], based on previous work of Senko and co-workers.[11] As they pointed out the usage of pseudoproline is an excellent tool to prevent aggregation, and the single amino-acid coupling makes the synthesis of difficult oligo- and polypeptides more cost effective.

The usefulness of individual, single residue pseudoproline derivatives during polypeptide synthesis was first confirmed by Senko and co-workers. They showed that acylation of S(ΨPro) is superior to that of T(ΨPro), while very low efficiency was reported for C(ΨPro), resulting in an overall product purity of ~1%. Therefore, we chose to enhance the yield of T(ΨPro) acylation, as optimizing that of S(ΨPro) is not strictly necessary, while reaching synthetically relevant efficiency in case of C(ΨPro) seems unrealistic. T(ΨPro) conversion was described to depend on the chemical nature of the acylating residue, varying between 14% and 99%.[11] However, the efficiency of 14 proteinogenic amino acids did not reach 75% and in case of 10 residues the reported efficiency was lower than 50%.

As a coupling agent N,N′-Diisopropylcarbodiimide/ Ethyl cyano(hydroxyimino)acetate (DIC/OxymaPure) surpasses 1–[Bis(dimethylamino)methylene]– 1H–1,2,3–triazolo[4,5–b]pyridinium 3–oxide hexafuorophosphate / N,N-Diisopropylethylamine (HATU/DIPEA), as it is ~10 times more cost effective, it is not categorized as explosive substance, and its use results in lower rates of racemization.[12] We have successfully used DIC/OxymaPure and found that it is an adequate activating compound for **S**mart **P**eptide Chemistry in **F**low (SPF) synthesis[13].

Therefore, here we focused our attention on DIC/OxymaPure activation and optimized the present screening accordingly. Our goal was to test the applicability of T(ΨPro) using our previously published, fully automated continuous flow method operating on commercially available High Performance Liquid Chromatography (HPLC) modules,[13,14] targeting the following key criteria: The developed SPF-based protocol ***i)*** must acylate T(ΨPro) efficiently, ***ii***) must exclude both double coupling and recirculation, ***iii***) should be cost and time efficient, and ***iv***) should operate as environmentally friendly as possible. To achieve these goals, we have optimized the following parameters: flow rate, reagent concentration, and excess reagent quantities.

Results and Discussion

A decapeptide, that of -IYDP**ET**GTWI-, (CLN005 peptide, described by Honda et al.)[17]was chosen as the model system for protocol optimization, as it is a relatively short peptide that nevertheless forms a well resolved β-hairpin[15] secondary structure (in water). We chose to introduce a single mutation (Tyr2Phe) to enhance its hydrophobic character to create an even greater challenge for the SPF protocol. The resulting in the H-I**F**DPE[5]**T**GTWI-NH_2 decapeptide, referred to as **CLN005:Y2F (Pep)** peptide in this paper became our test system, and within this sequence we optimized the acylation of T(ΨPro) at position 6 for all 20 proteinogenic amino acids. Thus, the shorthand notation of the 20 mutants is as follows: **Pep:E5X,** indicating that Glu^5 is systematically replaced (X= G, A, V, I, …., W). Although Pep:E5X contains two Thr residues, position 6 and 8, only the -X^5-T^6- bond formation was challenged. Note that Thr is known to be a favourable residue at the (*i*+2) position of a β-turn[16] therefore a contributor to overall stability. In summary, we systematically mapped and optimized the acylation potential of X with respect to T(ΨPro) in peptide Pep:E5X. (**Figure 2** and **Table 1**)

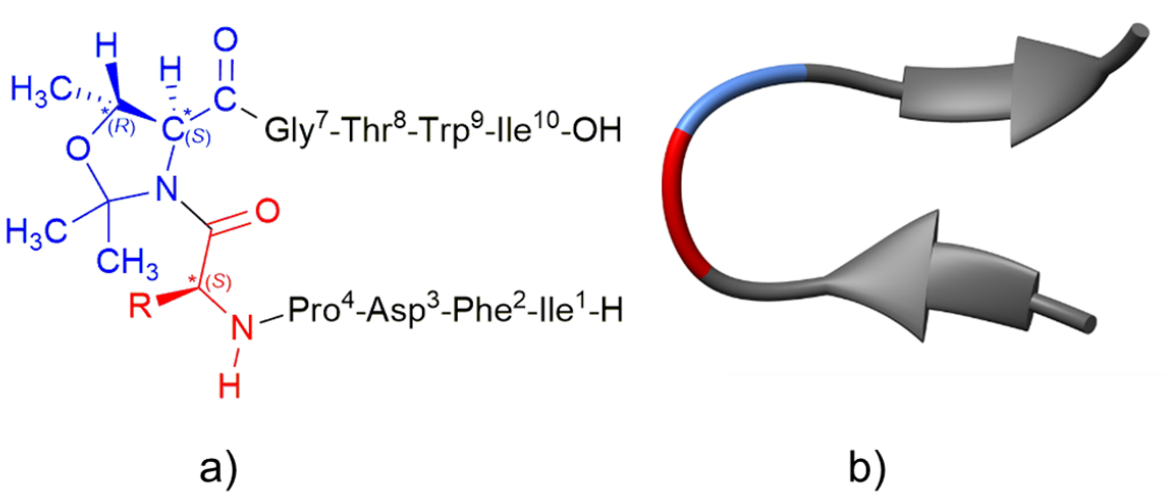


Figure 2. **a)** The primary sequence of the Pep:E5X peptide: H-Ile-Phe-Asp-Pro-**Xaa**-**Thr**-Gly-Thr-Trp-Ile-NH_2 abbreviated as Pep:E5X. At the 5th position Xaa is one of the 20 L-α-amino acids (coloured red), while Thr of 6th position is masked as T(ΨPro) (coloured blue). **b)** The β-hairpin ribbon model based on the NMR structure of the original decapeptide, GYDPETGTWG (Honda et al 2004, CLN peptide, PDB entry 1UAO)[17].

In line with our protocol, initially the first five (-T(ΨPro)GTWI[10]-) and last four (-I[1]FDP[4]-) residues were coupled by using the mixture of Fmoc-Xaa-OH/OxymaPure/DIC using 3/3/6 equivalent. The complete coupling cycle of only 7.5 min includes the acylation, Fmoc deprotection using 30% piperidine/N,N-Dimethylformamide (DMF) and washing steps. (**Table 1)**. The acylation reaction used for residue X (the 5[th] amino acid) as forming the X-T(ΨPro) amide bond was optimized along other selected parameters such as reagent equivalent, flow rate and concentration. In total, the overall synthesis time of a peptide Pep:E5X was less than 2 hours (110 min).

Table 1.: Coupling protocols applied to make H-Ile-Phe-Asp-Pro-**Xaa**-**Thr**-Gly-Thr-Trp-Ile-NH_2 using SPF.

Optimized parameters		**RTC**[a]		
		method **a)**	method **b)**	method **c)**
Fmoc-Xaa-OH[b]	eq.	3	**5**[c]	5
OxymaPure	eq.	3	**5**	5
DIC	eq.	6	6	**10**
Volume of injection	ml	1	0.8	0.8
Flow rate	ml/min.	**0.3**	0.3	**0.15**
Cycle time	min	12	12	18

[a] Residence Time Control (RTC) (see methods for details), [b] all amino acids, including T(ΨPro), were coupled as indicated in the text (standard method), except those of position 5 (Fmoc-Xaa-OH). [c] Numbers highlighted bold indicate changes compared to the one on the left.

Carrying out three parallel set of syntheses using different residues, we obtained a considerable number of observations concerning the acylation ability of X on T(ΨPro). Except for that of X[5], all couplings were completed using 3, 3 and 6 equivalent of Fmoc-Xaa-OH, OxymaPure and DIC (standard method). At first this protocol (named method **a)**) was used to produce the X-T(ΨPro) linkage too giving satisfactorily good results for 11 amino acids. (**Table 2**) The method **a)** is rapid (cycle time of 12 min), efficient, and greener due to using less solvent than by alternatives methods that were published in the lietrature. Though the purity of most Pep:E5X decapeptides, -IFDP**XT**GTWI- was acceptable, far better in fact than those of any previous attempts[11] (**Table 2, Table 3**), two major truncated side products were regularly detected. One of these is the **5**[**mer**] by-product (*Rt*: 4.11 min, **Figure 3.**) with primary sequence of H-T(ΨPro)GTWI-OH, where the T(ΨPro) remained in its oxazolidine form by the Mass Spectroscopy (MS) measurements even after acidic cleavage. The condition used to cleave the peptides from the resin (95% trifluoroacetic acid (TFA)/ 2.5% water/ 2.5% triisopropylsilane (TIS)) is acidic enough to open the oxazolidine ring too. This was clearly demonstrated by T. Wöhr et al.[2] who studied the ring opening reactions of T(ΨPro) and found that the cleavage of isopropylidene group (opening of the ring) with 90% of TFA takes only minutes. Thus, using the standard cleavage conditions (3 hours) the appearance of the intact pseudoproline ring containing 5[mer] was indeed unexpected. The second by-product is a 9[mer], H-IFDP~~X~~***T***GTWI-OH, Pep:E5~~X~~, (*Rt*: 5.37 min, **Figure 3**) systematically identified within the raw material, in which residue X is simply missing from peptide. Pep:E5~~X~~ emerges in cases when the acylation reaction with X on T(ΨPro) fails, but acylation with the forthcoming Pro[4] on T(ΨPro) was successful. This can easily be explained, if we consider the fact that the relative concentration

of Fmoc-Pro-OH is higher to that of the free T(ΨPro). The appearance of both by-products, 5^{mer} and 9^{mer}, was noticed for most X residues probed. (**Table 2, Table 3** and **Figure 3**)

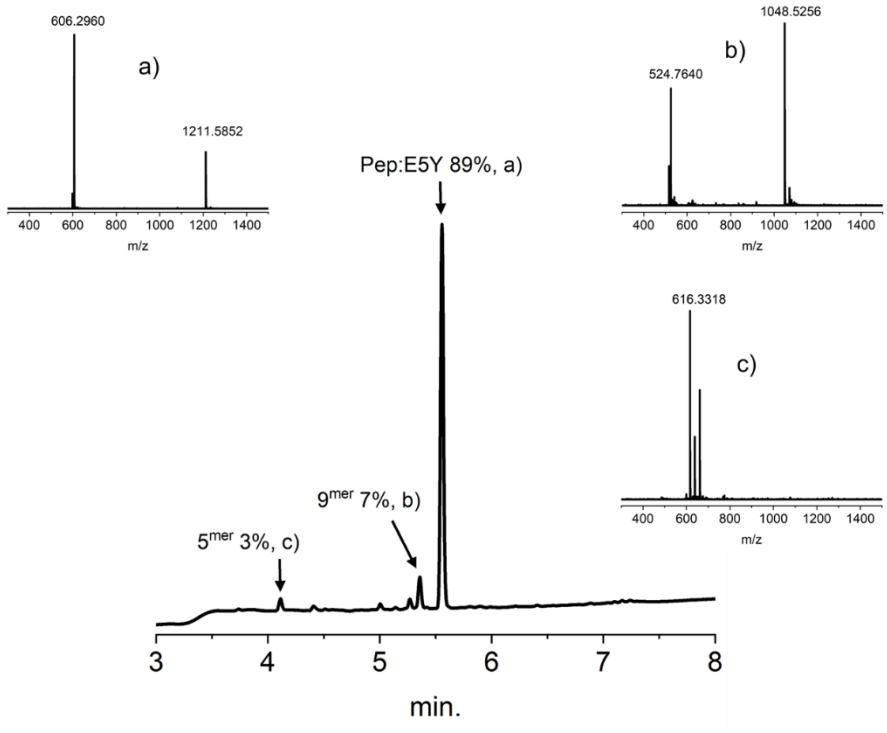


Figure 3. The UPLC (Ultra Performance Liquid Chromatography) chromatogram of the raw Pep:E5Y decapeptide made by SPF, with the identified main (a) and two side products (b and c). The main product (yield) is Pep:E5Y:= H-IFDPYTGTWI-OH (89%), the 9^{mer} or Pep:E5~~Y~~:= H-IFDP~~Y~~TGTWI-OH (7%) and the 5^{mer} := H-T(ΨPro)GTWI-OH (3%), where the latter one has the "intact" oxazolidine ring. Inserts: LCMS results: MS chromatogram of **a)** Pep:E5Y, $[M+2H]^{2+}$: 606.2960, $[M+H]^{+}$: 1211.5852; **b)** 9^{mer} $[M+2H]^{2+}$: 524.7640, $[M+H]^{+}$: 1048.5256 and **c)** 5^{mer} $[M+H]^{+}$: 616.3318.

The obtained purity of the main Pep:E5X using method **a) (Figure 8)** varied in the range of 8% to 94%. For 14 X residuess (out of 20), an average of 32% increase of main product yield was obtained compared to Senko et al.[10] We found that there are 11 Xs, which can easily acylate T(ΨPro) and give a main product purity exceeding 75%, those of G, H, Y, F, I, Q, P, K, L, A and C. (**Table 2**) When comparing the amount of the main product to that of the sum of the two side products, Σ(5^{mer} % + 9^{mer} %), a significant anticorrelation (R^2= 0.92) was established (**Figure 4,** slope is negative: *m*= -0.639). In other words, when the % of both H-IFDP~~X~~TGTWI-OH and H-T(ΨPro)GTWI-OH side-products is low, then that of the main product yield is high. Furthermore, when the side product ratio is elevated, then that of the main product is reduced (**Figure 4**). In this way, we concluded that success relies on the critical step of acylation of T(ΨPro): it should be as complete as possible. For example, when Met acylates T(ΨPro), (Pep:E5M) the main product yield is low (21%), giving rise to the formation of a large amount of 9^{mer} plus 5^{mer} side-products: 58% (51 + 7). On the contrary, when Tyr acylates T(ΨPro), Pep:E5Y, the main product yield is high (89%), and only a small

amount of 9^{mer} and 5^{mer} forms: 10% (7 + 3). Note that if X= Tyr, then the sum of the major and the two side products is 99% (**Table 2**), giving no room for any additional side-products to form. Therefore we can calculate the acylation efficacy as the purity percentage of Pep:E5X peptide.

Table 2. Those 11 Fmoc-Xaa-OH residues with which we were able to acylate T(ΨPro) using method **a)**, making the main product yield (Pep:E5X) between 94 and 75% (reported in a decreasing order). Using UPLC and LC-MS both the 5^{mer} and 9^{mer} side products were quantitatively determined.

Xaa type	**Pep:E5X yield (%)[a]**	**9^{mer} yield (%)[b]**	**5^{mer} yield (%)[c]**	**The 3 products yield** (%)	**SFigure**
Gly	94	2	2	98	6
His(Trt)	90	<1	7	98	7
Tyr(*t*Bu)	89	7	3	99	Fig.3
Phe	87	4	2	93	5
Ile	86	11	3	99	8
Gln(Trt)	82	9	5	96	14
Pro	82	2	2	**86**[d]	13
Lys(Boc)	80	9	4	93	9
Leu	78	10	3	91	10
Ala	77	6	3	**86**[d]	1
Cys(Trt)	75	10	5	90	2

[a] Except those of Xaa^5 all couplings were completed by using the standard method (3, 3 and 6 equivalent of Fmoc-Xaa-OH, OxymaPure and DIC, respectively). The X^5-T(ΨPro) amide bond was made *via* method **a)**. [b]H-IFDP~~X~~**T**GTWI-OH and [c] H-T(ΨPro)GTWI-OH, [d] Only coupling with Ala and Pro on T(ΨPro) results in significant amounts of by-products different from the 5^{mer} and 9^{mer}.

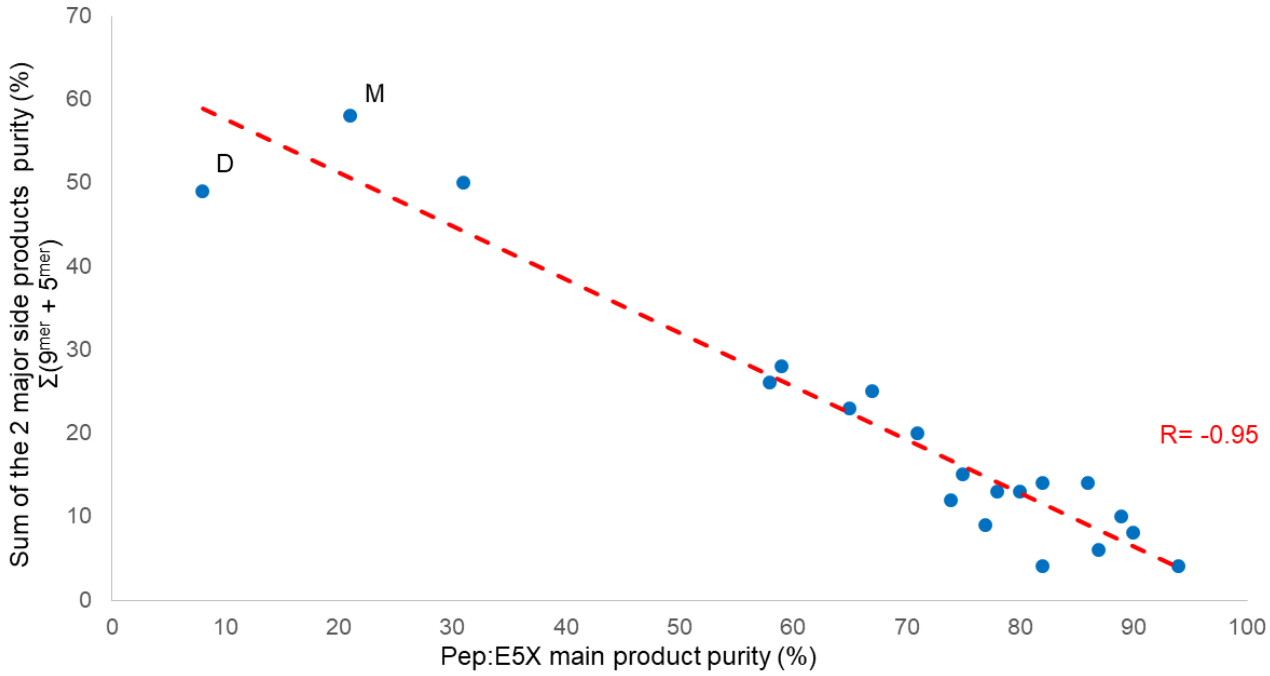


Figure 4. Anticorrelation of the main product yield, Pep:E5X (%) to the sum of the two major side products yield, Σ(5mer % + 9mer %). (For synthesis details see Table 2.) The significant anticorrelation (R^2= 0.92, R= -0.95) indicates that for most Xaa, if the main product yield is high (*e.g.* G, F, Ile), then the sum of the two side product yield is low: the slope (m= -0.639) is negative and Pep:E5X %s anticorrelate with Σ(5^{mer} % + 9^{mer} %). Only Pep:E5D and Pep:E5M have other side products then the 5^{mer} and 9^{mer}.

Starting with those residues for which method **a)** (**Figure 8**) gave lower than 75% main product yield (X^5 = V, S, T, R, N, E, W, M and D), (**Table 3**) we have set out to design a better performing approach. To achieve this, besides altering the flow rate (which in the means of residence time control, or RTC for short), the concentration and excess of the reagents were changed too. First, we have increased the concentration and the amount of reagents of both Xaa and that of the coupling reagent (Oxyma Pure) (method **b)**, **Table 3**). By slightly reducing the injection volume from 1.0 to 0.8 mL and increasing from 3 to 5 eq. both the concentration of Fmoc-Xaa-OH and Oxyma Pure, a net improvement was observed.

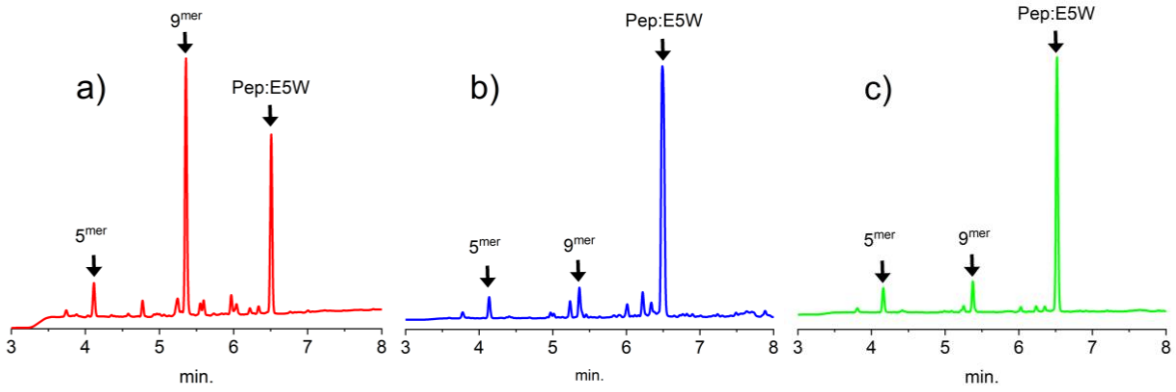


Figure 5. The UPLC chromatograms of the raw Pep:E5W by using method **a)**, method **b)** and method **c)**.

Note that the improved protocol (method **b) / Figure 8**) uses elevated concentration only for the critical coupling step (1 out of the 9) and thus it remains eco-friendly while significantly increases the yield of the main products Pep:E5X - specifically by ~14% on average (**Table 3**). In line with the anticorrelation established between the main and side reactions, by increasing the amino acids concentration of X (and keeping the residence time on the same level), the ratio of faulty acylations should decrease. This way we were able to avoid implementing the commonly applied "double coupling" strategy. By slightly lengthening the overall synthesis time (plus 4.5 min in total / cycle) we were able to preserve both cost effectiveness and the green concept (**Table 3**). For example, acylating with Trp to make Pep:E5W, method **a)** performed poorly (31%), however method **b)** was significantly more effective (69%), while the ratio of the two major side products, 5^{mer} and 9^{mer},was significantly reduced (10%) (**Figure 5/b**). Note that the contribution of the 9^{mer} side-product decreased nearly ten-fold: 44→6% (**Table 3**).

Method **c)** was developed in order to couple the most problematic amino acids: X= V, T, T, M and D. Acylation with these five residues was the most challenging, giving the lowest main product yield when using

either method **a)** and **b)**. Method **b)** was further optimized by reducing the flow rate from 0.3 to 0.15 mL/min during coupling. With this modification, we have further increased the main product yield of V, T, W and M respectively (**Table 3** and **Figure 6)**. For example, in case of Met (Pep:E5M) the main product yield, increases from 21 to 70% when changing from method **a)** to **c)**, parallel with the significant reduction of the 9^{mer} side-product from 51 to 7% (**Table 3**).

Table 3. Coupling efficiencies fine-tuned for the 9 most difficult residues, when acylating T(ΨPro) to make Pep:E5X.

Xaa	method **a)**			method **b)**			method **c)**			SFigure
	Pep:E5X (%)	**9^{mer} (%)**	**5^{mer} (%)**	**PepE5X (%)**	**9^{mer} (%)**	**5^{mer} (%)**	**Pep:E5X (%)**	**9^{mer} (%)**	**5^{mer} (%)**	
Val	72 [a]	8	4	70	17	4	**91**	<1	4	17a/b/c
Ser(*t*Bu)	71	16	4	**85**	2	<1	-	-	-	15a/b
Thr(*t*Bu)	67	20	5	70	14	5	**77**	<1	8	16a/b/c
Arg(Pbf)	65	19	4	**91**	<1	2	-	-	-	14a/b
Asn(Trt)	59	22	6	**78**	<1	3	-	-	-	11a/b
Glu(O*t*Bu)	58	23	3	**81**	3	4	-	-	-	3a/b
Trp(Boc)	31	44	6	69	6	4	**80**	9	8	Fig.5
Met	21	51	7	24+10*	44	8	**62+8***	7	8	10a/b/c
Asp(O*t*Bu)	8	45	4	8	-	3	8	-		Fig.7

[a] yields were established by integrating UPLC peak integrals. * Beside the main Met product, Pep:E5M, its sulfoxide derivative was also determined.

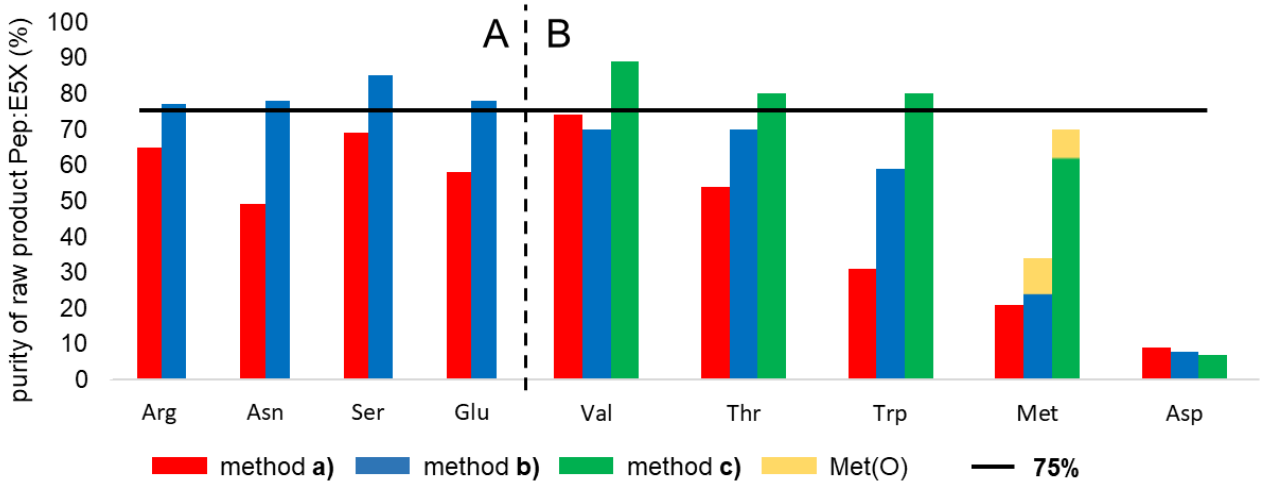


Figure 6. Comparison of the main product yield (%) of Pep:E5X for the most difficult 10 Xaa(s) as function of the fine-tuned and applied coupling RTC methods: **A)** Acylation efficiency of X(s) for which increased concentration of reagents (*method **b)***) successfully improved the main product yield above 75% (blue). **B)** Coupling efficiency improved above 75% (except Met, and Asp) by using increased residence time (*method **c)***, green).

Product yield remained below 75% (even applying method c)) for only two residues, those of Met (70%) and Asp (8%). In the case of Met to we suspected that the presence of the sulfur might be causing the difficulties.

To decipher the role of S-atom, we replaced it by norleucine (Nle), which has the same sidechain length as Met, but the S-atom is replaced by a -CH_2 group. Using method **a)** the main product yield of Pep:E5Nle[method **a)**] increased to 77%, from the 21% achieved in case of Pep:E5M[method **a)**]. This improvement makes it clear, that the S-atom of Met itself is the origin and cause of the problem.

Finally, Pep:E5D remains the worst case, as the main product yield remains as low as 8%, regardless of the method applied (**Table 3** and **Figure 6)**. The UPLC chromatograms have revealed that there are additional side products, which couldn't be eliminated by using method **b)** or **c)**. This signals that the major cause of "failure" is not the incomplete acylation with Asp, but a residue specific side reaction. In case of Asp this can safely proposed to be its well-known succinimide formation temperature[18-22], which is especially rapid at higher. MS analysis has revealed a side product of 30% with n molecular weight 18 Da lower than that of the expected Pep:E5D. **Figure 7** Considering the fact that succinimide formation is only possible if the acylation of T(ΨPro) with Fmoc-Asp(O*t*Bu)-OH is successful, we can conclude that the overall acylation efficiency in this case is around 38%. This led to the conclusion, that the parameters that favour the acylation, also support aspartimide and succinimide ring formation in case of X=Asp.

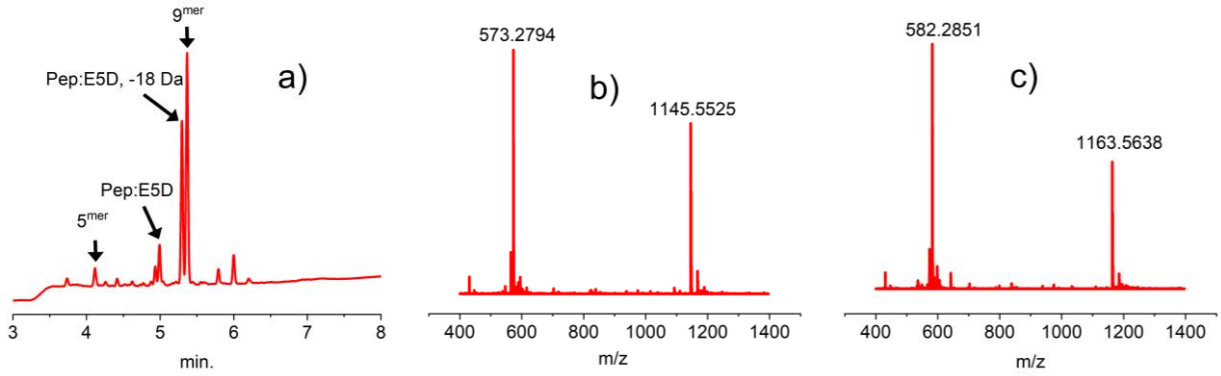


Figure 7. **a)** UPLC Chromatogram of the raw products of Pep:E5D, among which the expected product remains low (9%) due to the side reaction of succinimide formation, which is especially high at elevated temperature, such as 80°C, **b)** MS spectrum of the side product Pep:E5D -18 Da ([M]: 1144.56 Da) at 5.30 min., **c)** MS spectrum of the Pep:E5D (at 4.99 min.).

Conclusion

1. We have shown that SPF and its RTC modified protocols, produces satisfactory results when an Fmoc-protected proteinogenic amino acid is coupled to pseudoproline, with the exception of Fmoc-Asp(O*t*Bu)-OH. Here we show fine-tuned data obtained for the acylation of T(ΨPro). The synthetic protocols were worked out for the oxazolidine derivative of Thr.
2. Acylation of T(ΨPro) is an achievable task with method **a)** (with 12 min long cycle time) is already suitable for as many as 16 Xaa(s), resulting in better main product yields than those published to date.

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3. If X is either Ser, Arg, Asn or Glu we recommend using method **b)** to achieve success. Although this approach uses a somewhat elevated reagent excess (5 eq. instead of 3 eq.) for X, it remains eco-friendly, fast and robust.
4. If X is either Val, Thr, or Trp, we propose the use of method **c)**. For these challenging residues, besides the elevated X concentration, the use of a slower coupling flow rate (0.15 mL/min) is recommended. In this way the desired 75-85% coupling efficacy is achievable.
5. In the case of Met we have shown an acceptable method (method **c)**) to reach a satisfactory yield of crude product. Comparing results obtained using Met with those of Nle (norleucine), which only differs from Met by the replacement of –S– by a $–CH_2–$ group, we concluded that the sulphur atom is the source of the challenge: in the presence of S-atom the coupling efficiency drastically decreased. Interestingly, the yield of methionine sulphoxide side-product is limited and it appears only if method **b)** or **c)** is in use.
6. Acylation of T(ΨPro) with Asp remains the most challenging reaction due to its high potential to form succinimide. This spontaneous reaction is especially rapid at elevated temperatures, such as the 80 °C of methods. Thus, Asp is the only N-terminal neighbour of T, which would prevent the use of T(ΨPro) in support of the synthesis of a long or otherwise challenging peptide.
7. As in most cases, the conversion of the acylation of the pseudoproline residue is not better than 99%, thus after the coupling step an acetylation capping step could be necessary to reduce side product formation.

To conclude, along these lines one can successfully acylate T(ΨPro) and thus build in T(ΨPro) at the (*i*+1) position of any polypeptide for any residue at *i*, but Asp. Using the standard SPF method **a)**, or its variants good conversion can be ensured. Acylation of T(ΨPro) can be carried out in a straightforward way, almost as successful as coupling to any other amino acids. Thus, introducing main chain kinks *via* T(ΨPro) to nascent polypeptides to improve the synthesis of longer chains and even shorter protein domains is feasible, while preserving the eco-friendly approach of the peptide-synthesis-in-flow chemistry.

Experimental

The commercially available flow peptide apparatus (HPPS-4000, METALON Ltd., Hungary) consists of a conventional Jasco LC-4000 series HPLC system, except the PU-4180 HPLC pump, modified with an additional valve, allowing for recirculation and regulation of solvent flow (*e.g.,* cleavage mixture). ChromNAV2 software ensures the fully automated process. The autosampler injects the reagent solutions from a 1 mL sample vial, placed in the sample rack. The polyetheretherketone (PEEK) chromatography column was used as a fixed bed reactor tube for the resin and DMF was used as eluent. 150 mg of Fmoc-Rink amide TentaGel resin (0.24 mmol/g) was used. The Tentagel S Resin was purchased from Rapp Polymere GmbH. The Fmoc-Thr($\Psi^{Me,Me}$pro)-OH was purchased from Iris Biotech GmbH.

The reagent solutions were injected on the resin-filled column. For Fmoc-deprotection, the cleavage solution consisted of 30 v/v % piperidine in DMF. In the vials, protected amino acids with coupling reagents were dissolved in N-Methylpyrrolidone (NMP). The activating agent (DIC) was added promptly before the coupling and was injected with the auto-sampler. During the synthesis, the pressure varied between 3 and 5 bar, with the use of a backpressure regulator. The flow parameters of the coupling protocols for **standard** method and **method a)**, **b)**, **c)** are depicted in flowchart **Figure 8**.

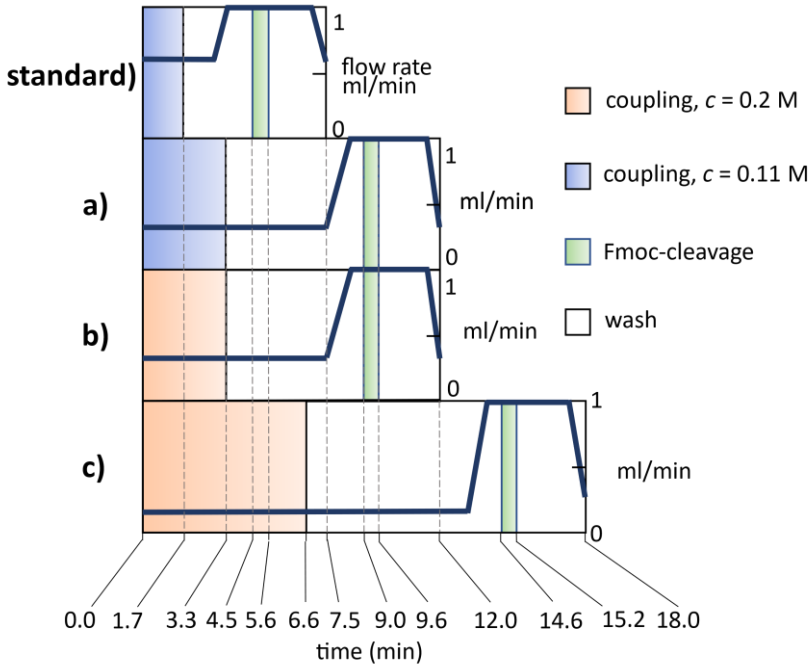

Figure 8. Flowcharts of coupling method **a), b)** and **c)**, respectively.

After peptide synthesis, the resin was washed with DCM and then dried in vacuum. Cleavage from the resin was accomplished with TFA/H_2O/TIS (95:2.5:2.5) stirring for 3 h. and in case of Pep:E5H, Pep:E5M, Pep:E5R with more scavengers TFA/H_2O/thioanisole/1,2-ethanedithiol (EDT)/TIS/phenol (5 mL/250 µL/250 µL/125 µL/60 µL/250 mg), stirring for 4.5 h. Then, the solution was filtered and freed from TFA via a rotary vacuum evaporator. The peptide was washed with diethyl ether and dried in vacuum, solubilised in water and liophilised.

Cleavage products were analysed by Ultra Performance Liquid Chromatography – Mass Spetrometer (UPLC-MS) on ACQUITY UPLC® HSS T3 1.8µm column (Waters, 100 × 2.1 mm, 100 Å) using gradient elution, consisting of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) with a gradient of 25% to 65% B over 8 min and the column temperature was set to 45 °C. The flow rate was 0.18 mL/min, and the absorbance was detected at λ = 220 nm. LC−MS analysis of the compounds was performed on a Waters Select Series Cyclic IMS Mass Spectrometer connected directly to a Waters Acquity I-Class Plus UPLC. Data were analysed by Waters MassLynx V4.2 (Waters Corporation, Milford, UK).

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information: UPLC chromatograms and MS spectrum for all synthesized peptides. This material available free of charge via internet at http://pubs.acs.org

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