Chapitre 3

Utilisation de la réaction de Ugi dans le design et le développement d'une nouvelle méthode de couplage de fragments peptidiques

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Avant-propos

Ce 3e chapitre de mon mémoire est constitué d'un article scientifique qui a été soumis au journal *Organic and Biomolecular Chemistry* le 27 octobre 2016, accepté le 9 novembre 2016 et publié en ligne le 14 novembre 2016. Par la suite, il a été incorporé au volume 14 de 2016 aux pages 11230 à 11237. Le numéro d'identification du papier est 10.1039/C6OB02342H.

De nombreux auteurs ont contribué au projet. D'abord, Sindy-Marcela Galindo, Xinxia Liang et Simon Vézina-Dawod ont effectué les premiers tests démontrant que la méthode était prometteuse. Ces travaux très préliminaires ont été effectués avant que je travaille sur le projet, me pavant ainsi le chemin vers des découvertes plus importantes.

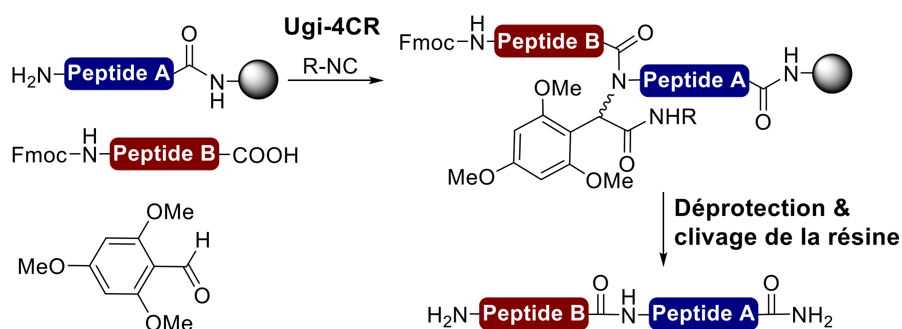
Ensuite, il y a Alexia Méjean qui a été stagiaire sous ma supervision lors de l'été 2016. Sa contribution au projet a été de tester les limites de la méthode et de tenter de comprendre divers phénomènes que l'on observait, mais dont l'explication logique nous échappait. Pour ce travail lors de ses 10 semaines de stage, elle méritait une place de choix dans la liste d'auteurs.

Mon directeur de recherche, Éric Biron, a supervisé et fait le design des travaux en donnant des idées novatrices à expérimenter en laboratoire. Il a également participé très activement à la rédaction du manuscrit.

Pour ma part, j'ai mené la grande majorité des expériences se trouvant dans ce papier, effectué la mise en forme des données expérimentale dans le document du matériel supplémentaire joignant cet article, en plus de rédiger le plan et le brouillon de l'article.

Résumé

Une nouvelle méthodologie pour coupler des fragments peptidiques sur support solide en utilisant la réaction multicomposante de Ugi suivi d'une déprotection est décrite dans ce manuscrit. L'approche utilise les irradiations micro-ondes pour attacher à l'aide d'une réaction multicomposante de Ugi, un fragment peptidique dont l'extrémité C-terminale est libre à un fragment supporté qui porte l'amine libre afin de former un lien amide tertiaire. Par la suite, cette protection de lien amide peut être efficacement retiré par acidolyse avec de l'acide trifluoroacétique sous irradiations de micro-ondes afin de fournir le peptide lié totalement déprotégé. Cette approche rapide et efficace de Ugi/déprotection sur support a été appliquée pour condenser des fragments peptidiques de différentes longueurs et produire une variété de peptides.



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Toward solid-phase peptide fragment ligation by a traceless-Ugi multicomponent approach

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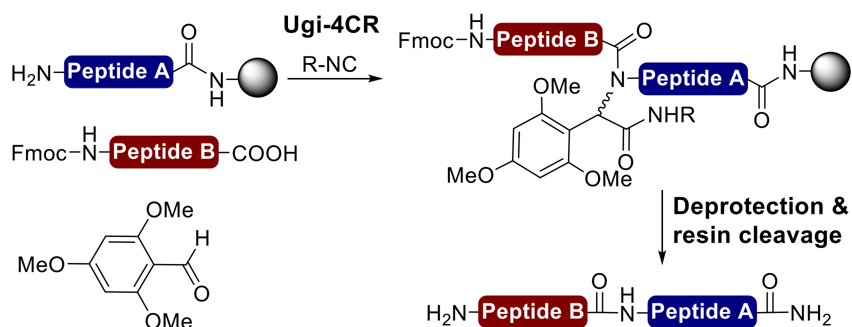
Abstract

A new methodology to couple peptide fragments on solid support using a traceless isocyanide-based multicomponent reaction is described. The approach uses a microwave-assisted on-resin Ugi four-component reaction to attach a carboxyl free peptide to a supported peptide bearing a free N-terminal amine *via* the formation of an *N*-protected amide bond at the ligation site. Afterward, the generated backbone amide protecting group can be efficiently removed by microwave-assisted acidolysis with trifluoroacetic acid to afford a fully deprotected peptide. This straightforward Ugi reaction/deprotection approach was applied to condense various fragment lengths and provided a variety of oligopeptides.



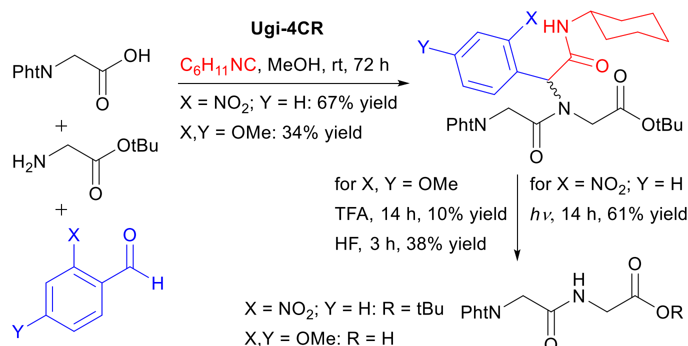
Introduction

Peptides and small proteins are very useful tools in chemical biology and their attractive pharmacological profile has increased their use in drug development and the interest in peptide-based therapeutics.[1,2] The chemical synthesis of peptides has remarkably progressed since the first work of Bruce Merrifield on solid-phase peptide synthesis (SPPS) in 1963.[3] A great number of synthetic improvements including more efficient coupling reagents,[4,5] solid supports,[6–9] linkers,[10,11] and orthogonal protecting groups[12] have emerged to overcome difficulties associated with SPPS and access a wide variety of peptides.[13–15]

Currently, peptides prepared by stepwise amino acid additions *via* SPPS are generally limited to approximately 50 residues.[15,16] The convergent synthesis approach, involving the condensation of peptide fragments, has led to the development of several ligation methods that allowed the preparation of long peptide chains and small proteins.[16–19] Beside fragment condensation with standard coupling reagents, most of these methods such as the Native Chemical Ligation (NCL),[20] *α*-ketoacid-hydroxylamine ligation (KAHA),[21,22] salicylaldehyde (SAL) ester-mediated ligation[23] and traceless-Staudinger ligation[24,25] are compatible with unprotected peptide fragments. But on the other hand, they generally require special C-terminal modifications (*e.g.* thioesterification for NCL and Staudinger ligation), thiol-modified amino acids or a specific N-terminal residue (*e.g.* Cys for NCL or Ser/Thr for SAL) at the ligation site. Based on these ligation strategies, we were looking for a straightforward approach that does not require C- or N-terminal modified fragments, has no or few residue restriction at the ligation site, and could be performed on solid support.

In this regard, the Ugi four-component reaction (Ugi-4CR) is very attractive as it offers a great input diversity to access complex molecules with high efficiency and atom economy.[26–31] The Ugi-4CR involves the reaction of isocyanide, carboxylic acid, amine and carbonyl compounds to afford an α-acylamino amide.[30,31] As the generated tertiary amide bond is formed between the amine and the acid components, the Ugi-4CR has been successfully used in the preparation of linear and macrocyclic peptides.[32–40] While most reported methods yield an *N*-substituted peptide bond at the ligation site, utilize C-terminal convertible isocyanide, involve N-terminal isocyanide or afford mixture of diastereoisomers

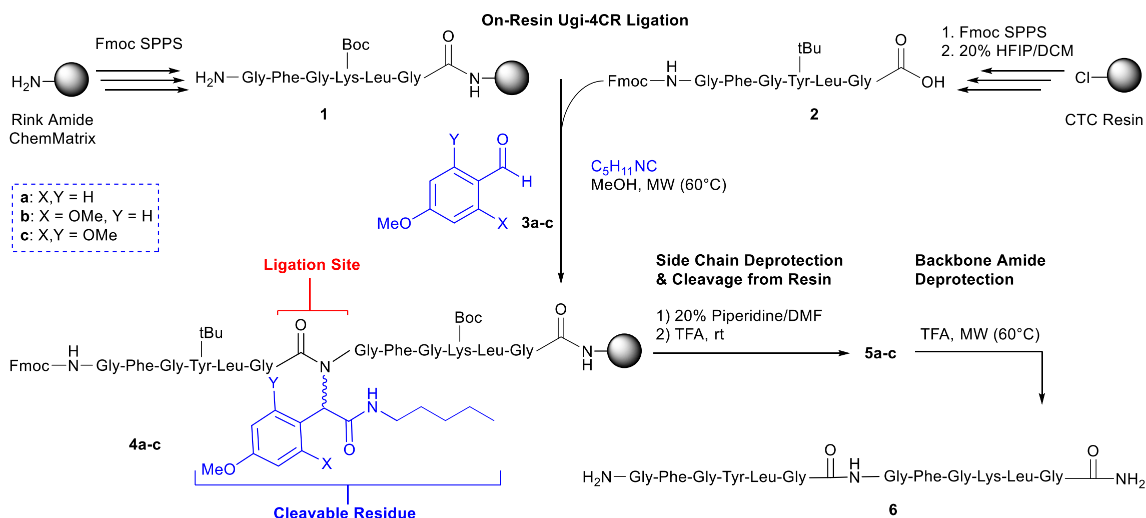
when carbonyl compounds other than formaldehyde are used, a study described by Waki and Meienhofer in 1977 has particularly drawn our attention.[40] In this pioneering work, different benzaldehyde derivatives were used in a Ugi-4CR with a glycine ester, phthalylglycine and cyclohexyl isocyanide to generate a peptide bond bearing an *N*-substituent that could be subsequently cleaved to afford a dipeptide (Scheme **1**).



Scheme 1. Peptide bond formation by a sequential Ugi-4CR and *N*-substituent cleavage described by Waki and Meinhofer.[40]

Acting as a backbone amide protecting group, the *N*-substituent was successfully removed by different cleavage strategies such as photolysis and acidolysis. Another advantage to consider with this approach is that backbone protecting groups have been shown to decrease aggregation during synthesis and increase the solubility of the growing protected peptide on solid support.[13,15] This Ugi-4CR/deprotection approach was very promising to develop a solid-phase fragment coupling method but its application was limited by moderate yields and long reaction times for the Ugi-4CR and *N*-substituent cleavage (Scheme **1**).[40]

In order to improve and accelerate the Ugi-4CR and backbone amide deprotection by acidolysis at the ligation site, our strategy was to use microwave (MW) irradiations in both steps (Scheme **2**). Herein we report our results concerning the development of a solid-phase traceless-Ugi-4CR ligation approach to couple standard unmodified peptide fragments.



Scheme 2. Synthesis of model peptide fragments and solid-phase traceless Ugi-4CR ligation.

Results and discussion

To evaluate the efficiency of the on-resin Ugi-4CR, a first series of N- and C-terminal fragments were synthesized by standard Fmoc/*t*Bu chemistry (Scheme **2**). First, the anchored C-terminal model peptide **1** was prepared on Rink Amide ChemMatrix® (CM) and the Fmoc group removed to yield the free amine. CM resin was selected to perform the ligation because of its compatibility with polar solvents such as MeOH and other alcohols usually used in Ugi-4CR and its ability to form aggregation-disrupting interactions with growing peptides.[7] At the same time, the N-terminal peptide fragment **2** was assembled on 2-chlorotrityl chloride (CTC) resin and cleaved from the resin with a solution of hexafluoroisopropanol (HFIP). After solvent removal, the protected peptide acid fragment **2** was analyzed to ensure sufficient purity and used without further purification. For the benzaldehyde derivatives, the selection was based on their commercial availability, price and acid sensitivity of the benzyl counterpart as protecting group. As several acid labile backbone amide protectors are composed of methoxy-substituted benzyl structures,[12] methoxy-substituted benzaldehyde derivatives **3a–c** were selected to generate the cleavable *N*-substituent and allow acid sensitivity tuning for the identification of the optimal cleavage conditions (Scheme **2**).

Initial Ugi-4CR ligations were performed with peptides **1** and **2** (1.2 equiv.) in presence of aldehyde **3a** (5 equiv.) and 1-pentyl isocyanide (5 equiv.) in MeOH under MW irradiations in a sealed vial at 60 °C for 1 h (Scheme **2**). After Fmoc removal on supported peptide **4a** and cleavage from the resin with a trifluoroacetic acid (TFA) cocktail, the product **5a** was obtained in 72% yield and analyzed by HPLC-MS. As expected, a mixture of diastereomers was observed since a new racemic stereocenter is formed during the Ugi-4CR (Fig. S3 ESI†).[26–31] However, the impact of this generated chiral center on the final product is minimized by the fact that it will be removed during the backbone amide deprotection step (Scheme **2**). Moreover, the presence of only two diastereomers confirmed that the peptide's stereochemistry is conserved. Afterward, removal of the *N*-substituent at the ligation site was performed by treatment of peptide **5a** with a TFA cocktail with MW heating at 60 °C for 1 h in a sealed vial as reported for various monoalkoxy backbone amide linker.[41] In this case, only partial deprotection of the backbone amide was observed and peptide **6** was obtained in 32% yield (Table **S1** ESI†). Nevertheless, this result was very encouraging and represents a significant improvement compared to the 10% yield previously reported for the cleavage of a dimethoxybenzyl derivative with TFA for 14 h (Scheme **1**).[40]

It is important to notice that compared to standard *N*-benzyl amide cleavage, an additional challenge is faced in this approach during ligation site deprotection due to the substitution of the benzylic position. In this case, the carbenium ion formed during acidolysis is not favorized because of the inductive effect of the carboxamide group (Scheme **2**). As a result, the resonance stability provided by a monomethoxy substituted aromatic ring is insufficient to allow efficient cleavage. Therefore, benzaldehydes bearing two (**3b**) or three (**3c**) methoxy substituents were used to provide more resonance stabilization. After on-resin Ugi-4CR with **1**, **2** and **3b** or **c**, Fmoc removal on **4b** and **4c** and treatment with TFA at room temperature, peptides **5b** and **5c** were obtained as a mixture of diastereomers in HPLC analyses (Fig. **1A** and **S4** ESI†). Surprisingly, a small quantity of fully deprotected peptide **6** was observed in the case of the trimethoxybenzyl analog **4c** (Fig. **1A**). In the presence of TFA with MW heating at 60 °C, compound **5b** was converted into peptide **6** in 69% yield after 1 h (Table **S1** ESI†). On the other hand, the backbone amide deprotection of compound **5c** was completed after 45 min under the same conditions and peptide **6** was obtained in 77% crude purity (Fig. **1B**). The two most important impurities observed in the

HPLC analyses were the precursor peptide **1** and the trimethoxybenzylimine intermediate. As expected, the trimethoxybenzyl derivative **5c** was the most labile and with complete cleavage in 45 min, these results showed that MW irradiations significantly accelerate and improve *N*-substituent acidolysis with TFA. Therefore, 2,4,6-trimethoxybenzaldelhyde **5c** was selected to perform the next experiments.

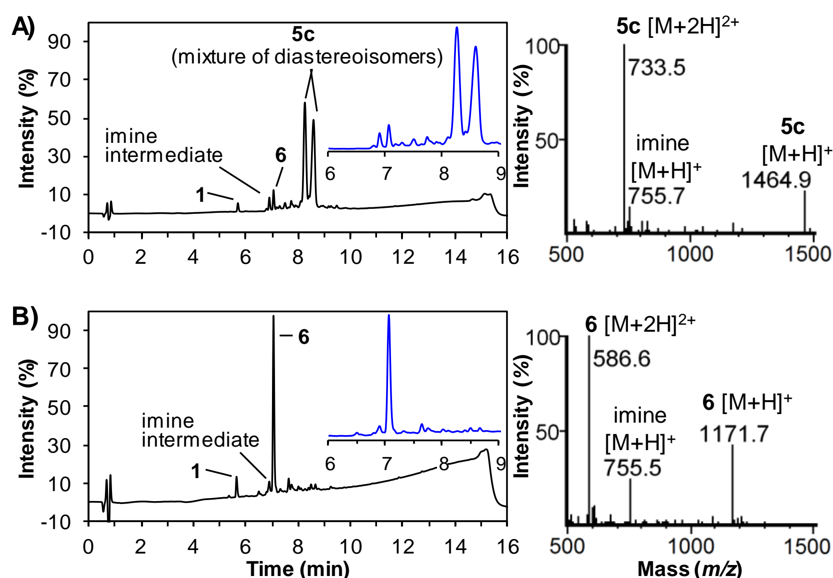


Figure 1. HPLC and MS profiles of crude products after cleavage from resin and deprotection showing Ugi-4CR product **5c** and final peptide **6**. (**A**) After Fmoc removal on **4c** and treatment with TFA for 1 h. (**B**) After treatment of **5c** with TFA under MW heating at 60 °C for 45 min (λ = 220 nm).

Since the solubility of the protected fragments in MeOH can be a limitation, different solvents were evaluated to perform the on-resin Ugi-4CR (Table **1**). The Ugi reaction has been shown to work better in polar protic solvent but a wide variety of solvents have also been successfully used. Unfortunately, besides 2,2,2-trifluoroethanol (TFE) with 78% yield (entry 6), no Ugi products were obtained in solvents with strong dissolving power for poorly soluble protected peptides such as DMF, DMSO and HFIP (entries 3–5). However, solvent mixtures containing MeOH yielded the best results with 89–99% conversion rates and 64–88% crude purities of peptide **6** (Table **1**, entries 7–11). The best result was obtained with MeCN/MeOH (1:1) (entry 11) and the next experiments were made with this mixture.

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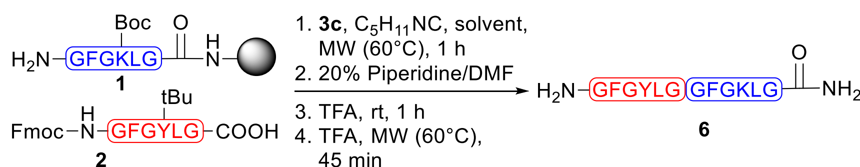
Table 1. Selection of optimal solvent for the on-resin Ugi-4CR



Entry	Solvent	Conversion (%)[a]	Crude purity (%)[b]
1	MeOH	90	77
2	DCM	59	49
3	DMF	0	0
4	DMSO	0	0
5	HFIP	0	0
6	TFE	78	53
7	DCM/MeOH (1:1)	98	73
8	THF/MeOH	89	64
9	$CHCl_3$/MeOH (1:1)	93	73
10	$CHCl_3$/MeOH/TMOF (1:1:1)	94	79
11	MeCN/MeOH (1:1)	99	88

[a]Conversion rate of C-terminal fragment **1** into peptide **6** and [b]crude purities were determined by HPLC.

We next tested different reaction times for the on-resin Ugi-4CR step in the synthesis of peptide **6** at room temperature, under MW irradiations or reflux to identify the optimal reaction conditions (Table **2**). For the MW conditions, the best result was obtained with heating for 1 h (entry 2) as lower conversion was observed with shorter reaction time (entry 1) and lower crude purities were obtained with longer irradiation time (entry 3). No improvement was observed with conventional heating as products with lower purities were obtained (entries 4 and 5). At room temperature, a reaction time of 72 h was necessary to obtain comparable results with a 97% conversion rate and 81% crude purity (entry 8). These results showed that MW irradiations significantly accelerate and improve the Ugi-4CR on solid support and that the reaction time strongly influences the purity of the final compound.

Table 2. Selection of the optimal reaction conditions for the on-resin Ugi-4CR in the synthesis of peptide **6**[a]

Entry	Temperature	Reaction time	Conversion (%)[b]	Crude purity (%)[c]
1	MW (60°C)	0.5 h	90	46
2		1 h	99	88
3		2 h	97	74
4	Reflux	1 h	89	56
5		4 h	91	60
6	rt	24 h	92	55
7		48 h	97	74
8		72 h	97	81

[a]Synthesis was performed as described in Table 1 using MeCN/MeOH 1:1 as solvent and various temperature and reaction time. [b]Conversion rate of C-terminal fragment **1** into peptide **6** and [c]crude purities were determined by HPLC.

The compatibility of other solid supports such as polystyrene and TentaGel resins with the solid-phase Ugi-4CR ligation was addressed using the optimized reaction conditions (Table **S2** ESI†). In this case, conversion rates around 90% were observed for both resins but the final peptide **6** was obtained in 75% and 68% crude purities for Rink Amide polystyrene and TentaGel resins, respectively. These results confirmed that CM resin is the most efficient to perform the Ugi-4CR on solid support under MW irradiations.

To evaluate the applicability of the Ugi-4CR ligation in the synthesis of more complex peptides, the method was applied to longer peptide fragments and repeated to perform multiple fragment condensations. As peptide fragments of 6 to 15 residues have been shown to be optimal in the convergent SPPS,[42] C- and N-terminal fragments of 6, 10 and 15 residues were prepared and used without purification in the ligation reaction. First, to compare the efficiency of our approach to standard fragment coupling reactions, protected peptides **1** and **2** were condensed with coupling reagents including HATU, PyAOP and *N*,*N'*-diisopropylcarbodiimide (DIC) (Table **3**). Surprisingly, compared to the Ugi-4CR ligation, lower crude purities and isolated yields were obtained with the coupling reagents. In this case, peptide **6** was isolated in 45, 33, and 35% yields after fragment coupling with HATU, PyAOP and DIC, respectively. These results showed that the described approach

can be very efficient to couple protected peptide fragments. Next, two on-resin Ugi-4CR were performed back to back with peptide **2** on supported fragment **1** by doing a ligation/deFmoc/ligation sequence. After cleavage from the resin and removal of side chain and backbone protecting groups, peptide **7** was obtained in 55% crude purity and isolated with a 24% yield after HPLC purification. Afterward, longer fragments were used in the Ugi-4CR to prepare 16-mers **8** and **9** (6 + 10), 20-mers **10** and **11** (10 + 10), 21-mer **12** (6 + 15), 25-mer **13** (10 + 15) and 30-mer **14** (15 + 15) peptides.

Table 3. Crude purities and isolated yields for peptides prepared by solid-phase traceless-Ugi-4CR

Oligomer	Sequence[a]	Purity[b] (%)	Yield[c] (%)
6	GFGYLGGFGKLG	88	57
6[d]	GFGYLGGFGKLG	62	45
6[e]	GFGYLGGFGKLG	50	33
6[f]	GFGYLGGFGKLG	65	35
7	GFGYLGGFGYLGGFGKLG	55	24
8	GFGYLGGFGKISGLYG	81	45
9	GFGYLGGFGKLGYIVG	83	46
10	GFGYLGKCFGGFGKISGLYG	89	26
11	GFGYLGKCFGGFGKLGYIVG	85	54
12	GFGYLGGFGVAYKIGLFAPGA	75	69
13	GFGYLGKCFGGFGVAYKIGLFAPGA	43	20
14	GFGAKLYVGICGPAGGFGVAYKIGLFAPGA	33	6

[a]Ligation site is underlined. [b]Crude purities were determined by HPLC. [c]Isolated yield after purification by preparative HPLC. Based on the experimental loading of 0.31 mmol/g for Rink Amide CM. [d-f]Coupling performed with **2** (1.2 equiv) for 3 h using [d]HATU (1.2 equiv) and NMM (2.4 equiv) in DMF; [e]PyAOP (1.2 equiv) and DIPEA (2.4 equiv) in DMF or [f]DIC (1.2 equiv) and 6-Cl-HOBt (1.2 equiv) in NMP.[134]

Beside compound **14**, the peptides were obtained with moderate to good crude purities ranging from 43 to 89% and isolated in 20 to 69% overall yields. While the reaction was very efficient with every tested C-terminal fragments and N-terminal fragments of 6 and 10 residues, the worst result has been obtained with the 15-mer N-terminal fragment. In this case, the HPLC-MS analyses showed a 33% crude purity for peptide **14** with 30% of C-terminal fragment and imine intermediate as the most important impurities. This result

suggests that the solubility of the fully protected peptides in MeCN/MeOH decreases with length and considerably limit the on-resin Ugi-4CR efficiency. Solvent mixtures containing more hydrophobic solvents such as $CHCl_3$ or TFE to increase the solubility of longer fragments were also tested. Unfortunately, no improvements in crude purities and yields have been observed. Other solvents and strategies to improve the solubility of longer fragments are currently under investigation. Nevertheless, it is important to underline that unpurified fragments were used in this study and that fragment purification prior to Ugi-4CR ligation would certainly increase the purity and isolated yield of the final peptides.

Another drawback commonly observed in solid-phase fragment coupling is C-terminal epimerization at the ligation site.[15,42,44] This problem is mainly due to the N^{α}-substituent of the C-terminal residue. Compared to N^{α}-Fmoc/Boc protected amino acids where the carbamate group prevents the formation of stereochemically labile oxazolone intermediate during coupling, the N^{α}-amino group of the C-terminal residue in peptide fragments is substituted by an acyl group that promotes oxazolone formation.[42,45,46] To evaluate epimerization during the Ugi-4CR on solid support, N-terminal fragments containing a C-terminal L-Phe **15a** or D-Phe **15b** were prepared as described above (Scheme **2**) and used in the on-resin ligation reaction with peptide **1** (Fig. **2A**). A Phe residue was used in the study because it is particularly prone to C-terminal epimerization.[47] Compared to coupling with DIC/6-Cl-HOBt where a 37% epimerized product was obtained, very low

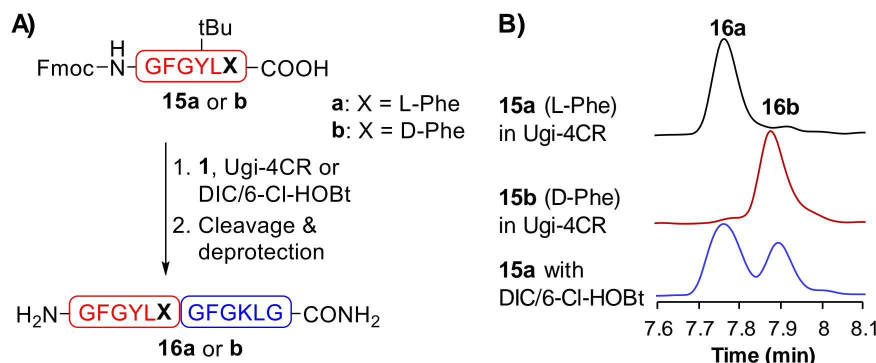


Figure 2.(**A**) Synthesis of peptide **16** by coupling N-terminal fragment **15a** or **b** to peptide **1** and cleavage from resin and deprotection. (**B**) Enlarged HPLC traces (λ = 220 nm) from 7.6 to 8.1 min of products obtained after Ugi-4CR with **15a** (top); Ugi-4CR with **15b** (middle) and coupling of **15a** with DIC/6-Cl-HOBt (bottom).

epimerization (<4%) was observed for peptides **16a** and **16b** with the on-resin microwave-assisted Ugi-4CR (Fig. **2B**). These results suggest that the described approach limits racemization during fragment condensation and could be used with a wide variety of amino acids.

Conclusion

In summary, we report the use of a traceless-Ugi-4CR to efficiently couple peptide fragments on solid support. The study showed that the use of MW irradiations significantly improved and accelerated both on-resin Ugi-4CR coupling and backbone amide deprotection steps. With the help of MW irradiations, the entire ligation, resin cleavage and protecting groups removal cycle could be performed in approximately 3 h. The described approach is straightforward, does not require C- or N-terminal modifications of the fragments prior to ligation, yields unsubstituted peptide bond at the ligation site and seems to avoid C-terminal epimerization. Parameters such as the impact of MW temperature on purity, yield and epimerization; the compatibility and efficiency of C- and N-terminal amino acid residues at the ligation site; and the effect of reagents equivalents are currently under investigation to expend the applicability of the approach. Simple and affordable, the described method is likely to become a useful complementary approach in polypeptide synthesis.

Experimental

Materials and equipment

All the chemical reagents and solvents from commercial sources were used without further purification. Coupling reagents and amino acid derivatives were purchased from Matrix Innovation Inc. (Québec, QC, Canada). Rink Amide ChemMatrix® resin (0.41 mmol g^{-1}) was purchased from PCAS Biomatrix (St-Jean-sur-Richelieu, QC, Canada), Rink Amide AM polystyrene resin (0.56 mmol g^{-1}) from ChemImpex (Wood Dale, IL, USA) and TentaGel S NH2 (130 µm, 0.29 mmol g^{-1}) from Rapp Polymere (Tübingen, Germany). All other reagents and solvents were purchased from Sigma-Aldrich (St Louis, MO, USA). Reactions on solid support were performed in filter columns (2 and 10 mL) from Roland

Vetter Laborbedarf OHG (Ammerbuch, Germany). RP-HPLC analyses were achieved on a Shimadzu Prominence instrument (Columbian, MD, USA) using a Phenomenex Kinetex column (4.6 mm × 100 mm, 2.6 µm XB-C18, 100 Å, 1.5 mL min^{-1}) with a 10.5 min gradient from water (0.1% TFA) and MeCN (0.1% TFA) (MeCN 10–100%) and detection at 220 nm and 254 nm. LC-MS analyses were performed on a Shimadzu Prominence LCMS-2020 equipped with an ESI and APCI ion source. Microwave experiments were conducted on a Biotage Initiator microwave instrument (Charlotte, NC, USA) with 0.2–0.5 and 0.5–2 mL microwave vials. Peptides were synthetized on a Prelude peptide synthetizer from Protein Technologies (Tucson, AZ, USA). High-resolution mass spectrometry was performed on a Waters Synapt G2-Si (Quadrupole/TOF) with a Waters UPLC binary pump and FTN injector. The mass spectrometer was operated in High resolution mode and calibration done with a sodium formate solution and lock-mass correction using a Leucine-enkephaline solution (Waters).

Peptide synthesis

Peptides were synthesized by standard Fmoc solid-phase synthesis.[48,49] Briefly, amino acid couplings were performed with a solution of Fmoc-Xaa-OH (3 equiv.), HCTU (3 equiv.) and *N*-methylmorpholine (NMM) (6 equiv.) in DMF for 20 min. The coupling step was repeated once and the resin washed with DMF (5×). The Fmoc protecting group was removed by treating the resin twice with a solution of 20% piperidine in DMF (v/v) for 8 min followed by washing with DMF (5×).

Preparation of C-terminal peptide fragments.

A Rink Amide resin was swelled in DMF and the first amino acid couple by standard Fmoc solid-phase synthesis. The loading capacity of the resin was determined by the Fmoc dosage assay described by Gude *et al.*[50] and estimated to 0.31 mmol g^{-1} for Fmoc-Gly-Rink Amide ChemMatrix. After peptide synthesis, the N-terminal Fmoc protecting group was removed the resin washed with DMF (5×), DCM (5×), MeOH (5×) and dried *in vacuo*. To characterize the product on resin, a small amount of resin (~10 mg) was treated with a solution of TFA, water and triisopropylsilane (TIPS) (95:2.5:2.5) for 1 h followed by filtration and washing with DCM (2×). The filtrate was evaporated under reduced pressure and the resulting mixture precipitated with cold diethyl ether. The solid was washed twice with diethyl ether and dried under vacuum to be analyzed by HPLC and ESI-MS.

H-Gly-Phe-Gly-Lys-Leu-Gly-NH$_2$ (**1**): 95% crude purity; RP-HPLC t_R = 6.42 min; ESI-MS *m/z*: 577.40 $[M + H]^+$; calcd for $C_{27}H_{45}N_8O_6$: 577.35.

H-Gly-Phe-Gly-Lys-Ile-Ser-Gly-Leu-Tyr-Gly-NH$_2$: 98% crude purity; RP-HPLC t_R = 6.72 min; ESI-MS *m/z*: 997.60 $[M + H]^+$; calcd for $C_{47}H_{73}N_{12}O_{12}$: 997.55.

H-Gly-Phe-Gly-Lys-Leu-Gly-Tyr-Ile-Val-Gly-NH$_2$: 99% crude purity; RP-HPLC t_R = 6.94 min; ESI-MS *m/z*: 1009.65 $[M + H]^+$; calcd for $C_{49}H_{77}N_{12}O_{11}$: 1009.58.

H-Gly-Phe-Gly-Val-Ala-Tyr-Lys-Ile-Gly-Leu-Phe-Ala-Pro-Gly-Ala-NH$_2$: 98% crude purity; RP-HPLC t_R = 7.37 min; ESI-MS *m/z*: 1466.80 $[M + H]^+$; calcd for $C_{72}H_{108}N_{17}O_{16}$: 1466.82.

Preparation of N-terminal peptide fragments.

Synthesis of N-terminal peptide fragments bearing a free carboxylic acid was carried out on 2-chlorotrityl chloride (CTC) resin. The first amino acid (3 equiv.) was attached to the resin in presence of diisopropylethylamine (DIEA) (7 equiv.) in DCM (CH_2Cl_2) for 3 h. After filtration the remaining trityl chloride groups were capped by a solution of DCM, MeOH, DIEA (17:2:1; v:v:v) for 15 min. Then, the peptide was assembled by standard Fmoc solid-phase synthesis and the resin washed thoroughly with DMF (5×) and DCM (5×). The fully protected peptide was released from the resin with a solution of 20% HFIP in DCM (v/v) for 30 min. The resin was filtered and the solvent removed under reduced pressure and the resulting mixture precipitated with cold diethyl ether. The solid was washed twice with diethyl ether and dried under vacuum to be analyzed by HPLC and ESI-MS. The peptides were used without further purification in Ugi-4CR ligation.

Fmoc-Gly-Phe-Gly-Tyr(tBu)-Leu-Gly-OH (**2**): 99% purity; RP-HPLC t_R = 11.12 min; ESI-MS *m/z*: 889.55 $[M - H]^-$; calcd for $C_{49}H_{57}N_6O_{10}$: 889.41.

Fmoc-Gly-Phe-Gly-Tyr-Leu-Gly-Lys-Cys-Phe-Gly-OH: 82% crude purity; RP-HPLC t_R = 9.09 min; ESI-MS *m/z*: 1270.55 $[M + H]^+$; calcd for $C_{65}H_{80}N_{11}O_{14}S$: 1270.56.

Fmoc-Gly-Phe-Gly-Ala-Lys-Leu-Tyr-Val-Gly-Ile-Cys-Gly-Pro-Ala-Gly-OH: 72% crude purity; RP-HPLC t_R = 8.93 min; ESI-MS *m/z*: 1631.75 $[M + H]^+$; calcd for $C_{80}H_{111}N_{16}O_{19}S$ 1631.78.

Fmoc-Gly-Phe-Gly-Tyr(tBu)-Leu-Phe-OH (**15a**): 92% crude purity; RP-HPLC t_R = 11.65 min; ESI-MS *m/z*: 979.45 $[M - H]^-$; calcd for $C_{56}H_{63}N_6O_{10}$: 979.46.

Fmoc-Gly-Phe-Gly-Tyr(tBu)-Leu-D-Phe-OH (**15b**): 76% crude purity; RP-HPLC t_R = 11.76 min; ESI-MS *m/z*: 979.45 $[M - H]^-$; calcd for $C_{56}H_{63}N_6O_{10}$ 979.46.

Coupling of peptide fragments by Ugi-4CR

The supported C-terminal fragment bearing a free amino group was first swelled in a minimum of MeCN/MeOH (1:1) (15 μL mg^{-1} of resin) in a microwave vial followed by the addition of 2,4,6-trimethoxybenzaldehyde (5 equiv.). After stirring for 15 min, a solution of the N-terminal fragment bearing the free carboxylic acid (1.2 equiv.) in MeCN/MeOH (1:1) was added and the mixture stirred for 5 min. Finally, pentyl isocyanide (5 equiv.) was added, the microwave vial sealed and the reaction mixture heated for 1 h at 60 °C. The resin was filtered and washed with MeCN/MeOH (1:1) (3×), DMF (3×) and DCM (3×). Following removal of the Fmoc group, the ligated peptide was cleaved from the resin with a solution of TFA/H_2O/TIPS (95:2.5:2.5) for 1 h at room temperature. After filtration and washing with TFA, the filtrate was evaporated under reduced pressure and the resulting mixture precipitated with cold diethyl ether. The solid was washed twice with diethyl ether and dried under vacuum to be analyzed by HPLC and ESI-MS.

H-Gly-Phe-Gly-Tyr-Leu-Gly-(N-(1-(4-methoxyphenyl)-2-oxo-2-(pentylamino)ethyl))Gly-Leu-Gly-Lys-Phe-Gly-NH$_2$ (**5a**): 58% crude purity; RP-HPLC t_R = 8.19 and 8.39 min; ESI-MS *m/z*: 1404.80 $[M + H]^+$; calcd for $C_{71}H_{102}N_{15}O_{15}$ 1404.77.

H-Gly-Phe-Gly-Tyr-Leu-Gly-(N-(1-(2,4-dimethoxyphenyl)-2-oxo-2-(pentylamino)ethyl)) Gly-Leu-Gly-Lys-Phe-Gly-NH$_2$ (**5b**): 63% crude purity; RP-HPLC t_R = 8.95 and 9.20 min; ESI-MS *m/z*: 1434.75 $[M + H]^+$; calcd for $C_{72}H_{104}N_{15}O_{16}$ 1434.78.

H-Gly-Phe-Gly-Tyr-Leu-Gly-(N-(1-(2,4,6-trimethoxyphenyl)-2-oxo-2-(pentylamino)ethyl)) Gly-Leu-Gly-Lys-Phe-Gly-NH$_2$ (**5c**): 62% crude purity; RP-HPLC t_R = 8.24 and 8.58 min; ESI-MS *m/z*: 1464.95 $[M + H]^+$; calcd for $C_{73}H_{106}N_{15}O_{17}$ 1464.79.

Cleavage of the backbone amide protecting group

The backbone amide protected peptide obtained by Ugi-4CR was dissolved in a solution of TFA/H_2O/TIPS (95:2.5:2.5) and the mixture heated for 45 min at 60 °C in a sealed microwave vial. Afterward, the solvent was evaporated under reduced pressure and the resulting mixture precipitated with cold diethyl ether. The solid was washed twice with diethyl ether and dried under vacuum to be analyzed by HPLC-MS and HRMS. Compounds **6–14** were purified by RP-HPLC on a Phenomenex Kinetex® EVO C18 column (250 × 21.2 mm, 300 Å, 5 μm) using 0.1% TFA/H_2O (A) and 0.1% TFA/MeCN (B), with a linear gradient of 10% to 100% (B) for 22 min at 14 ml min^{-1} and UV detection at 220 nm and 254 nm. The collected fractions were freeze dried to afford the desired peptide as white powder. Isolated yields were calculated from 50 mg of resin with an experimental loading of 0.31 mmol g^{-1}.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Leu-Gly-Lys-Phe-Gly-NH$_2$ (**6**) (white powder, 9.3 mg, 57% isolated yield): 88% crude purity; RP-HPLC t_R = 7.05 min; HRMS (ESI-TOF) *m/z*: 1171.6355 $[M + H]^+$; calcd for $C_{57}H_{83}N_{14}O_{13}$ 1171.6259.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Leu-Gly-Lys-Phe-Gly-NH$_2$ (**7**) (white powder, 4.0 mg, 24% isolated yield): 55% crude purity; RP-HPLC t_R = 8.50 min; HRMS (ESI-TOF) *m/z*: 1765.9084 $[M + H]^+$; calcd for $C_{87}H_{121}N_{20}O_{20}$ 1765.9061.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Phe-Gly-Lys-Ile-Ser-Gly-Leu-Tyr-Gly-NH$_2$ (**8**) (white powder, 4.9 mg, 45% isolated yield): 81% crude purity; RP-HPLC t_R = 7.50 min; HRMS (ESI-TOF) *m/z*: 1591.8297 $[M + H]^+$; calcd for $C_{77}H_{111}N_{18}O_{19}$ 1591.8267.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Phe-Gly-Lys-Leu-Gly-Tyr-Ile-Val-Gly-NH$_2$ (**9**) (white powder, 6.6 mg, 46% isolated yield): 83% crude purity; RP-HPLC t_R = 7.65 min; HRMS (ESI-TOF) *m/z*: 1603.8645 $[M + H]^+$; calcd for $C_{79}H_{115}N_{18}O_{18}$ 1603.8631.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Lys-Cys-Phe-Gly-Gly-Phe-Gly-Lys-Ile-Ser-Gly-Leu-Tyr-Gly-NH$_2$ (**10**) (white powder, 4.3 mg, 26% isolated yield): 89% crude purity; RP-HPLC t_R = 11.83 min; HRMS (ESI-TOF) *m/z*: 1014.0192 $[M + 2H]^{2+}$; calcd for $C_{97}H_{141}N_{23}O_{23}S$ 1014.0140.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Lys-Cys-Phe-Gly-Gly-Phe-Gly-Lys-Leu-Gly-Tyr-Ile-Val-Gly-NH_2 (**11**) (white powder, 4.5 mg, 54% isolated yield): 85% crude purity; RP-HPLC t_R = 7.77 min; HRMS (ESI-TOF) *m*/*z*: 1020.0392 [M + 2H]$^{2+}$; calcd for $C_{99}H_{145}N_{23}O_{22}S$ 1020.0322.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Gly-Phe-Gly-Val-Ala-Tyr-Lys-Ile-Gly-Leu-Phe-Ala-Pro-Gly-Ala-NH_2 (**12**) (white powder, 7.9 mg, 69% isolated yield): 75% crude purity; RP-HPLC t_R = 7.92 min; HRMS (ESI-TOF) *m*/*z*: 1031.0591 [M + 2H]$^{2+}$; calcd for $C_{102}H_{147}N_{23}O_{23}$ 1031.0515.

H-Gly-Phe-Gly-Tyr-Leu-Gly-Lys-Cys-Phe-Gly-Gly-Phe-Gly-Val-Ala-Tyr-Lys-Ile-Gly-Leu-Phe-Ala-Pro-Gly-Ala-NH_2 (**13**) (white powder, 3.3 mg, 20% isolated yield): 43% crude purity; RP-HPLC t_R= 7.93 min; HRMS (ESI-TOF) *m*/*z*: 1248.6575 [M + 2H]$^{2+}$; calcd for $C_{122}H_{176}N_{28}O_{27}S$ 1248.6485.

H-Gly-Phe-Gly-Ala-Lys-Leu-Tyr-Val-Gly-Ile-Cys-Gly-Pro-Ala-Gly-Gly-Phe-Gly-Val-Ala-Tyr-Lys-Ile-Gly-Leu-Phe-Ala-Pro-Gly-Ala-NH_2 (**14**) (white powder, 1.2 mg, 6% isolated yield): 24% crude purity; RP-HPLC t_R = 8.91 min; HRMS (ESI-TOF) *m*/*z*: 1429.2590 [M + 2H]$^{2+}$; calcd for $C_{137}H_{207}N_{33}O_{32}S$ 1429.2648.

H-Gly-Phe-Gly-Tyr-Leu-Phe-Gly-Leu-Gly-Lys-Phe-Gly-NH_2 (**16a**): 82% crude purity; RP-HPLC t_R = 7.75 min; ESI-MS *m*/*z*: 1261.70 [M + H]$^{+}$; calcd for $C_{64}H_{89}N_{14}O_{13}$ 1261.65.

H-Gly-Phe-Gly-Tyr-Leu-D-Phe-Gly-Leu-Gly-Lys-Phe-Gly-NH_2 (**16b**): 54% crude purity; RP-HPLC t_R = 7.86 min; ESI-MS *m*/*z*: 1261.70 [M + H]$^{+}$; calcd for $C_{64}H_{89}N_{14}O_{13}$ 1261.65.

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Notes and references

1. K. Fosgerau and T. Hoffmann, *Drug Discov. Today*, **2015**, 20, 122-128.

2. A. A. Kaspar and J. M. Reichert, *Drug Discov. Today*, **2013**, 18, 807-817.

3. R. B. Merrifield, *J. Am. Chem. Soc.*, **1963**, 85, 2149-2154.

4. A. El-Faham and F. Albericio, *Chem. Rev.*, **2011**, 111, 6557-6602.

5. E. Valeur and M. Bradley, *Chem. Soc. Rev.*, **2009**, 38, 606-631.

6. W. Rapp, in *Combinatorial Peptide and Nonpeptide Libraries*, ed. G. Jung, Wiley-VCH, Weinheim, **2007**, pp. 425-464.

7. F. García-Martín, *et al.*, *J. Comb. Chem.*, **2006**, 8, 213-220.

8. J. Rademann, M. Grøtli, M. Meldal and K. Bock, *J. Am. Chem. Soc.*, **1999**, 121, 5459-5466.

9. S. A. Kates, B. F. McGuinness, C. Blackburn, G. W. Griffin, N. A. Solé, G. Barany and F. Albericio, *Peptide Science*, **1998**, 47, 365-380.

10. M. Góngora-Benítez, J. Tulla-Puche and F. Albericio, *ACS Comb. Sci.*, **2013**, 15, 217-228.

11. U. Boas, J. Brask and K. J. Jensen, *Chem. Rev.*, **2009**, 109, 2092-2118.

12. A. Isidro-Llobet, M. Álvarez and F. Albericio, *Chem. Rev.*, **2009**, 109, 2455-2504.

13. M. Paradis-Bas, J. Tulla-Puche and F. Albericio, *Chem. Soc. Rev.*, **2016**, 45, 631-654.

14. R. Behrendt, P. White and J. Offer, *J. Pept. Sci.*, **2016**, 22, 4-27.

15. X. Guan, P. K. Chaffey, C. Zeng and Z. Tan, in *Protein Ligation and Total Synthesis II*, ed. L. Liu, Springer International Publishing, Cham, **2015**, pp. 155-192.

16. M. Chen, P. Heimer and D. Imhof, *Amino Acids*, **2015**, 47, 1283-1299.

17. S. Bondalapati, M. Jbara and A. Brik, *Nat. Chem.*, **2016**, 8, 407-418.

18. T. J. R. Harmand, C. E. Murar and J. W. Bode, *Curr. Opin. Chem. Biol.*, **2014**, 22, 115-121.

19. P. Thapa, R.-Y. Zhang, V. Menon and J.-P. Bingham, *Molecules*, **2014**, 19, 14461-14483.

20. P. Dawson, T. Muir, I. Clark-Lewis and S. Kent, *Science*, **1994**, 266, 776-779.

21. I. Pusterla and J. W. Bode, *Nat. Chem.*, **2015**, 7, 668-672.

22. J. W. Bode, R. M. Fox and K. D. Baucom, *Angew. Chem. Int. Ed.*, **2006**, 45, 1248-1252.

23. Y. Zhang, C. Xu, H. Y. Lam, C. L. Lee and X. Li, *Proc. Natl. Acad. Sci. U. S. A.*, **2013**, 110, 6657-6662.

24. E. Saxon, J. I. Armstrong and C. R. Bertozzi, *Org. Lett.*, **2000**, 2, 2141-2143.

25. B. L. Nilsson, L. L. Kiessling and R. T. Raines, *Org. Lett.*, **2000**, 2, 1939-1941.

26. A. Váradi, T. Palmer, R. Notis Dardashti and S. Majumdar, *Molecules*, **2016**, 21, 19-.

27. G. Koopmanschap, E. Ruijter and R. V. A. Orru, *Beilstein J. Org. Chem.*, **2014**, 10, 544-598.

28. B. H. Rotstein, S. Zaretsky, V. Rai and A. K. Yudin, *Chem. Rev.*, **2014**, 114, 8323-8359.

29. A. Dömling, W. Wang and K. Wang, *Chem. Rev.*, **2012**, 112, 3083-3135.

30. A. Dömling, *Chem. Rev.*, **2006**, 106, 17-89.

31. A. Dömling and I. Ugi, *Angew. Chem. Int. Ed.*, **2000**, 39, 3168-3210.

32. L. A. Wessjohann, M. C. Morejon, G. M. Ojeda, C. R. Rhoden and D. G. Rivera, *J. Org. Chem.*, **2016**, 81, 6535-6545.

33. A. V. Vasco, C. S. Pérez, F. E. Morales, H. E. Garay, D. Vasilev, J. A. Gavín, L. A. Wessjohann and D. G. Rivera, *J. Org. Chem.*, **2015**, 80, 6697-6707.

34. A. P. Treder, J. L. Hickey, M.-C. J. Tremblay, S. Zaretsky, C. C. G. Scully, J. Mancuso, A. Doucet, A. K. Yudin and E. Marsault, *Chem. Eur. J.*, **2015**, 21, 9249-9255.

35. M. G. Ricardo, F. E. Morales, H. Garay, O. Reyes, D. Vasilev, L. A. Wessjohann and D. G. Rivera, *Org. Biomol. Chem.*, **2015**, 13, 438-446.

36. F. E. Morales, H. E. Garay, D. F. Muñoz, Y. E. Augusto, A. J. Otero-González, O. Reyes Acosta and D. G. Rivera, *Org. Lett.*, **2015**, 17, 2728-2731.

37. G. P. Liao, E. M. M. Abdelraheem, C. G. Neochoritis, K. Kurpiewska, J. Kalinowska-Tłuścik, D. C. McGowan and A. Dömling, *Org. Lett.*, **2015**, 17, 4980-4983.

38. R. Hili, V. Rai and A. K. Yudin, *J. Am. Chem. Soc.*, **2010**, 132, 2889-2891.

39. O. E. Vercillo, C. K. Z. Andrade and L. A. Wessjohann, *Org. Lett.*, **2008**, 10, 205-208.

40. M. Waki and J. Meienhofer, *J. Am. Chem. Soc.*, **1977**, 99, 6075-6082.

41. S. Claerhout, T. Duchene, D. Tourwe and E. V. Van der Eycken, *Org. Biomol. Chem.*, **2010**, 8, 60-65.

42. F. Albericio, P. Lloyd-Williams and E. Giralt, in *Methods Enzymol.*, ed. G. B. Fields, Academic Press, New York, **1997**, vol. 289, pp. 313-336.

43. B. Bacsa, K. Horváti, S. Bõsze, F. Andreae and C. O. Kappe, *J. Org. Chem.*, **2008**, 73, 7532-7542.

44. T. Wang and S. J. Danishefsky, *Proc. Natl. Acad. Sci. U. S. A.*, **2013**, 110, 11708-11713.

45. N. L. Benoiton, *Biopolymers*, **1996**, 40, 245-254.

46. N. Sewald and H.-D. Jakubke, in *Peptides: Chemistry and Biology*, Wiley-VCH, Weinheim, **2009**, pp. 175-315.

47. J. Kovacs, in *The Peptides*, eds. E. Gross and J. Meienhofer, Academic Press, New York, **1980**, vol. 2, pp. 485-539.

48. D. A. Wellings and E. *Atherton, in Methods Enzymol.,* ed. G. B. Fields, Academic Press, New York, **1997**, vol. 289, pp. 44-67.

49. G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, **1990**, 35, 161-214.

50. M. Gude, J. Ryf and P. D. White, *Lett. Pept. Sci.,* **2002**, 9, 203-206.

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