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Solid-Phase Synthesis of an "Inaccessible" hGH-Derived Peptide Using a Pseudoproline Monomer and SIT-Protection for Cysteine

Srinivasa Rao Manne, Amit Chakraborty, Karin Rustler, Thomas Bruckdorfer, Beatriz G. de la Torre,* and Fernando Albericio*

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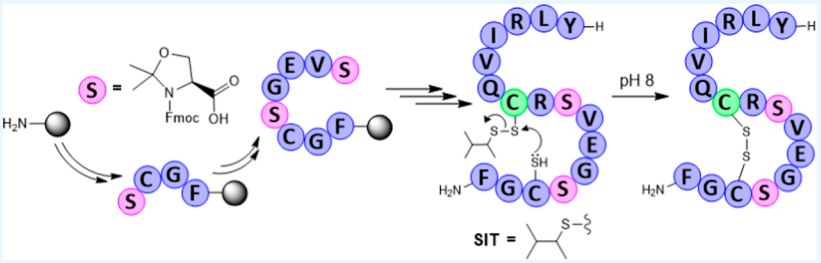

ABSTRACT: The solid-phase peptide synthesis (SPPS) of the C-terminal sequence of hGH with one extra Tyr attached to its N-terminus (total of 16 residues with a disulfide bridge) has been accomplished for the first time by optimizing several synthetic parameters. First of all, the two Ser residues (positions 9 and 13 of the molecule) have been introduced as a single amino acid, Fmoc-Ser($\psi^{Me,Me}$pro)-OH, demonstrating that the acylation of these hindered moieties is possible. This allows us to avoid the use of the corresponding dipeptides, Fmoc-AA-Ser($\psi^{Me,Me}$pro)-OH, which are very often not commercially available or very costly. The second part of the sequence has been elongated via a double coupling approach using two of the most effective coupling methods (DIC-OxymaPure and HATU-DIEA). Finally, the disulfide bridging has been carried out very smoothly by a chemoselective thiol-disulfide interchange reaction between a SIT (*sec*-isoamyl mercaptan)-protected Cys residue and the free thiol of the second Cys. The synthesis of this short peptide has evidenced that SPPS is a multifactorial process which should be optimized in each case.

INTRODUCTION

Solid-phase peptide synthesis (SPPS), using the fluorenylmethoxycarbonyl (Fmoc)-*tert*-butyl (*t*Bu) protection scheme, is the strategy of choice for the preparation of all peptides used in research and of a large majority of those required as active pharmaceutical ingredients for the pharmaceutical industry.[1−3] This approach works very well for small- and medium-sized peptides up to approximately 20 residues, while larger peptides could require a fine-tuning process. The last few years have witnessed the development of solid supports,[4] coupling reagents,[5,6] and protecting groups[7] to facilitate the preparation of the so-called "difficult peptides".[8] Although the steric hindrance of some sequences (e.g., β-branched, α,α-disubstituted, or *N*-methyl amino acids) could explain the failure of some synthesis, the main reasons are intra- and inter-chain interactions promoted by the formation of hydrogen bonds between NH and CO within the peptide chain. In this regard, the development of the backbone protection concept by Sheppard and co-workers was crucial for understanding the interaction phenomenon and improving synthesis.[9] Briefly, this strategy involved the use of a polyalkoxybenzyl protecting group for masking the NH of amino acids, which causes the hydrogen bond formation. These groups and others developed in their shadow are removed during the final global deprotection with trifluoroacetic acid (TFA) (Figure 1).[9−15] At the same time, but independently, Haack and Mutter proposed the use of a dipeptide previously prepared by modifying the residues of Ser, Thr, or Cys for introducing dimethyl-oxazolidines or thiazolidines in the peptide chain for disrupting the aggregation phenomenon as the Pro does by itself (Figure 1). These structures also mimic a *tert*-butyl protection, which—in the case of Ser or Thr—is easily removed during the global deprotection with TFA.[16−19] In the case of the dimethylthiazolidine of Cys, its removal is sequence dependent and very often requires strong acidic conditions.[20] As proposed by Mutter, Ser/Thr($\psi^{Me,Me}$pro) were introduced in the peptide chain as dipeptides due to the intrinsic difficulty of acylation for the incoming protected amino acid during solid-phase synthesis. With the concourse of Fmoc-AA-Ser/Thr[$\psi^{Me,Me}$pro]-OH being commercially available, a large number of difficult peptides have been synthesized in moderate

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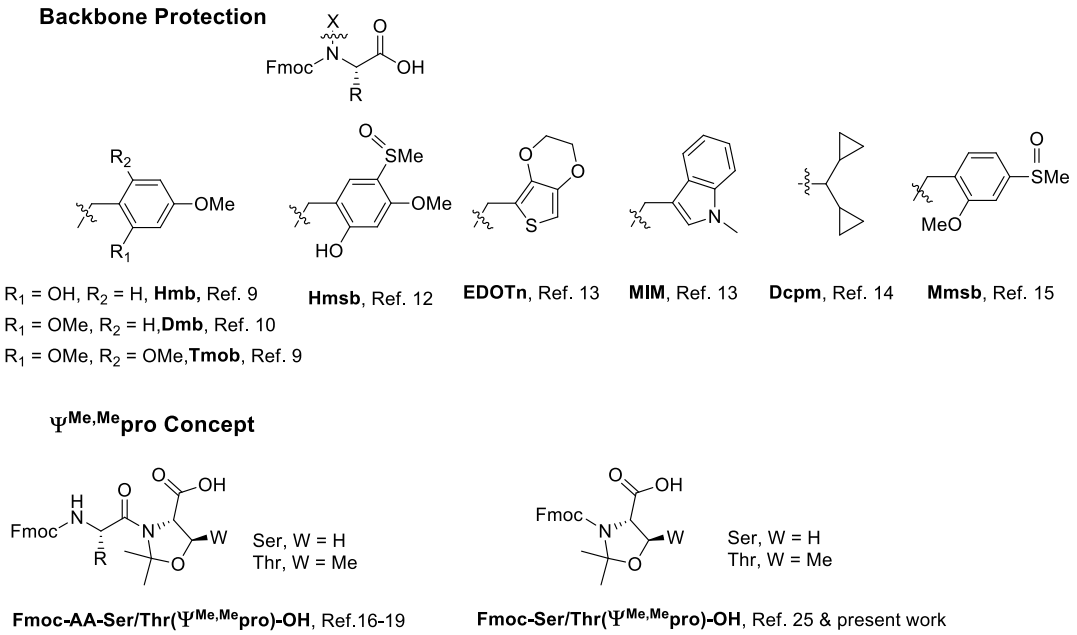
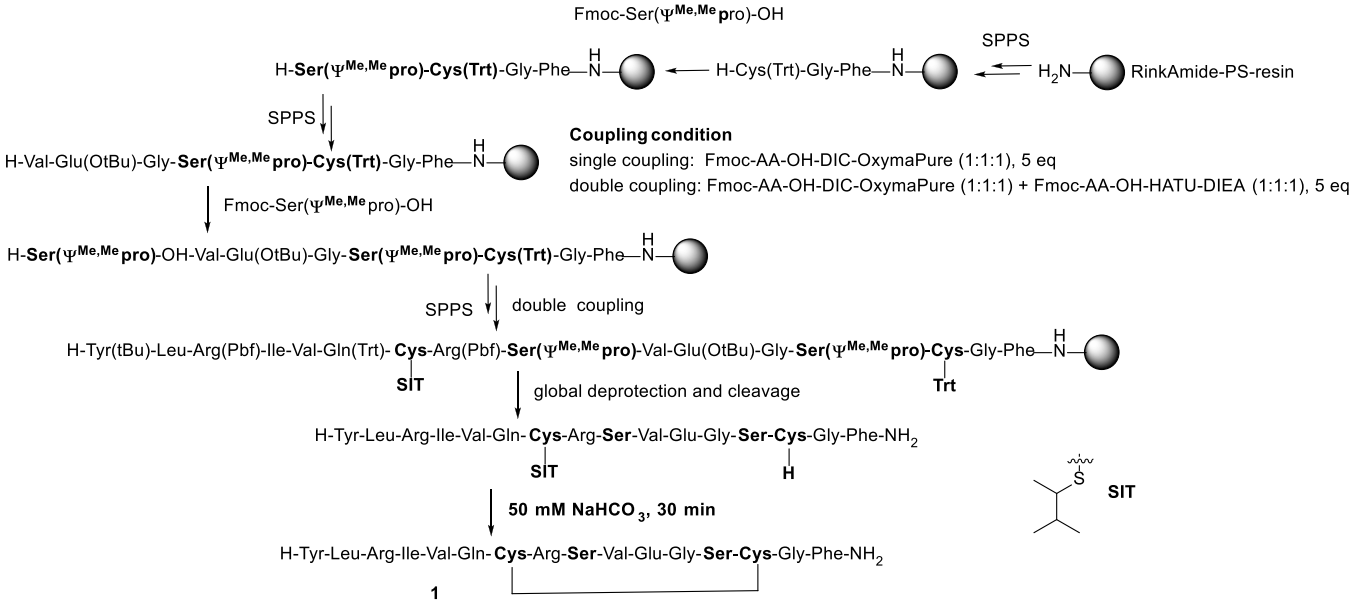


Figure 1. Most used backbone protection groups labile in TFA (Hmsb and Mmsb require a previous reduction) for Ser, Thr, and Cys residues in difficult sequences and the $\psi^{Me,Me}$pro concept, dipeptide, and monomer.

Scheme 1. Synthetic Strategy Used for the Synthesis of the Cyclic hGH Peptide (1)



to excellent yields.[21−24] In a practical manner, the use of this excellent synthetic tool was limited to the presence of Ser or Thr in the sequence and to the commercial availability of the required ψPro dipeptide. Recently, Senko et al.[25] have published the synthesis of the Fmoc monomers (Fmoc-Ser/Thr($\psi^{Me,Me}$pro)-OH) (Figure 1) and the scope and limitations of their use in peptide elongation, allowing a much broader use of this technique.

Herein, we complemented the study of Senko et al.[25] regarding the use of monomeric Fmoc-Ser($\psi^{Me,Me}$pro)-OH and applied it to the synthesis of a so far "inaccessible" peptide, according to the term used by Mutter and co-workers for difficult-to-synthesize peptides.[18]

Peptide 1 (Scheme 1) (H-YLRIVQCRSVEGSCGF-NH_2) is the 15-mer C-terminal sequence of the human growth hormone (hGH) with one extra Tyr attached to its N-terminus (total 16 residues), which is a peptide with therapeutic and potential industrial interest as many of the hGH derivatives.[26,27] The linear precursor was impossible to be obtained by stepwise SPPS [(Fmoc-aa-OH-*N*,*N*′-diisopropylcarbodiimide (DIC)-OxymaPure) (1:1:1), 3 equiv], with sufficient purity that would allow its purification in decent yield.[28] The coupling of Arg^8 to Ser^9 was found to be practically ineffective. The use of the ψPro dipeptide Fmoc-Gly-Ser($\psi^{Me,Me}$pro)-OH at positions 12 and 13 of the sequence renders a complex product mixture, wherein the full sequence could be detected by MALDI-TOF. The use of the second ψPro dipeptide Fmoc-Arg(Pbf)-Ser($\psi^{Me,Me}$pro)-OH was not attempted because it was not commercially available. Using the ChemMatrix resin does not translate into significantly better

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yields. Segment condensation was attempted between the protected Tyr[1]-Arg[8] and Ser[9]-Phe[16] fragments, but it suffers from poor solubility of the fragments as well as high levels of epimerization at Arg[8]. The condensation between the protected fragments Tyr[1]-Gly[12] and Ser[13]-Phe[16] is hampered by the low solubility of the *N*-terminal fragment.[28] Finally, some mg of the peptide with good purity was obtained by a native chemical ligation strategy, which required the preparation of two unprotected fragments, one in the form of a thioester, and even the concourse of Fmoc-Gly-Ser($\psi^{Me,Me}$pro)-OH for the preparation of the C-terminal fragment.[28]

RESULTS AND DISCUSSION

Evaluation of Using Fmoc-Ser($\Psi^{Me,Me}$pro)-OH as the Monomeric Unit. First of all, the acylation of the two Ser($\psi^{Me,Me}$pro) residues at positions 13 and 9 was studied with different Fmoc-AA-OH, such as Gly (which is in position 12 in the hGH sequence after one Ser), Phe as a non-hindered residue, the two β-branched and hindered amino acids Ile and Thr(tBu), and Arg(Pbf), which can render the δ-lactam in a slow coupling mode with the unproductive consumption of Fmoc-Arg(Pbf)-OH and is at position 8 in the sequence after the second Ser.

First, the tetrapeptide H-Ser($\psi^{Me,Me}$pro)-Cys(Trt)-Gly-Phe-NH-Rink amide-polystyrene (PS)-resin was assembled using the corresponding Fmoc-AA-OH and DIC-OxymaPure (5 equiv each, single coupling for 1 h, with 2 min of preactivation) as the coupling method. Furthermore, acylation on tetrapeptide was studied with different Fmoc-AA-OH [(Gly, Phe, Ile, Thr(*t*Bu), and Arg(Pbf)] using DIC-OxymaPure (5 equiv each, single coupling for 2 h, with 2 min of preactivation) as the coupling method. For acylation with Fmoc-Arg(Pbf)-OH, a coupling without preactivation was also attempted to minimize the δ-lactam formation.[25]

The results of Table 1 show that acylation took place in all cases with excellent yields (>96%). Quantitative yields were obtained with Gly, as expected, and with Arg(Pbf), whose result was surprisingly excellent, even considering its tendency toward the δ-lactam formation.

Table 1. Acylation Efficiency on Ser($\Psi^{Me,Me}$pro)-Cys(Trt)-Gly-Phe-NH-Rink Amide-PS-Resins with Different Amino Acids

#	Fmoc-AA-OH	H-AA-SCGF-NH$_2$ (%)[a]	H-SCGF-NH$_2$ (%)[a]
1	Gly	>99	
2	Phe	96.9	3.1
3	Ile	96.9	3.1
4	Thr	96.2	3.8
5	Arg	>99	
6	Arg*	>99	

[a]% area determined by HPLC, * in situ activation method.

In a similar mode, the acylation efficiency was assessed for the peptide-resin H-Ser($\Psi^{Me,Me}$pro)[9]-Val-Glu(OtBu)-Gly-Ser-($\Psi^{Me,Me}$pro)[13]-Cys(Trt)-Gly-Phe-NH-Rink amide-PS-resin by using the same reaction conditions [Fmoc-AA-OH and DIC-OxymaPure (5 equiv each, single coupling for 2 h, with 2 min of preactivation, including the incorporation of Arg(Pbf))]. The results shown in Table 2 were very similar to the acylation on H-Ser($\Psi^{Me,Me}$pro)[13]-peptide-resin. Once again, the incorporation of Fmoc-Arg(Pbf)-OH was quantitative. Interestingly, in previous work, it was realized that the incorporation of Fmoc-Arg(Pbf)-OH on the Ser(*t*Bu)[9]-peptide-resin was practically inefficient. These better results found herein were the first evidence for the positive effect of Ser($\Psi^{Me,Me}$pro)[13] on the coupling of Fmoc-Arg(Pbf)-OH onto the H-Ser($\Psi^{Me,Me}$pro)[13]-peptide resin. It is important to keep in mind that the beneficial effect of the ψPro moiety is observed four or five residues after its insertion.[8]

Table 2. Acylation Efficiency on H-Ser($\Psi^{Me,Me}$pro)-Val-Glu(OtBu)-Gly-Ser($\Psi^{Me,Me}$pro)-Cys(Trt)-Gly-Phe-NH-Rink Amide-PS-resin with Different Amino Acids

#	Fmoc-AA-OH	H-AA-SVEGSCGF-NH$_2$ (%)[a]	H-SVEGSCGF-NH$_2$ (%)[a]
1	Gly	>99	
2	Phe	95.9	4.1
3	Ile	96.8	3.2
4	Thr	97.8	2.2
5	Arg	>99	

[a]% area determined by HPLC.

Synthesis of the Target Peptide. After the double successful inclusion of Ser($\Psi^{Me,Me}$pro) at positions 9 and 13 of the growing peptide chain and demonstrating that both [Ser($\Psi^{Me,Me}$pro)] can be efficiently acylated, the synthesis of the target peptide (H-YLRIVQCRSVEGSCGF-NH$_2$) following a Fmoc-based SPPS protocol was attempted. At first, the H-Ser($\Psi^{Me,Me}$pro)[9]-Val-Glu(O*t*Bu)-Gly-Ser($\Psi^{Me,Me}$pro)[13]-Cys-(Trt)-Gly-Phe-NH-Rink amide-PS-resin was prepared as described in the acylation study (DIC-OxymaPure, 5 equiv, 2 min preactivation, and 1 h coupling). The remaining residues, using Pbf for Arg, Trt for Gln and Cys, and *t*Bu for Tyr, were incorporated using double coupling (DIC-OxymaPure as previously) to anticipate potential difficulties in the rest of the sequence. After the global deprotection, the crude peptide was evaluated by HPLC and LCMS (Figures 2 and S27), showing a major peak that had the mass of the target linear peptide with a more than decent purity profile (HPLC purity of 42%). This is the first time that a purifiable crude of this peptide has been received in our hands by using a stepwise synthesis.

Considering this and knowing that the cyclization step was also problematic, a new optimized synthesis was carried out. Until Ser($\Psi^{Me,Me}$pro)[9], all amino acids were introduced via single coupling with DIC-OxymaPure as in the previous synthesis, but for the rest of the residues, a double coupling approach was used. Thus, first coupling with in situ activation using DIC-Oxyma Pure (5 equiv for 1 h) and then the second coupling with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (5 eqiuv) in the presence of *N*,*N*-diisopropylethylamine (DIEA) (5 equiv) with 2 min preactivation and 1 h coupling took place.

Furthermore, even though Trt was kept as a side-chain protecting group for Cys[14], Cys[7] was protected with *sec*-isoamyl thiol (SIT), which has recently been developed by our groups.[29] The SIT group is totally stable for the synthesis (elongation and global deprotection) and participates in a chemoselective disulfide formation by the thiol-disulfide interchange,[30] in our case, with the free thiol after global deprotection of the Cys[14]. The SIT-protected peptide showed a much better purity by HPLC (70%) and LCMS (Figures 3 and S29) compared to the first synthetic attempt. The crude

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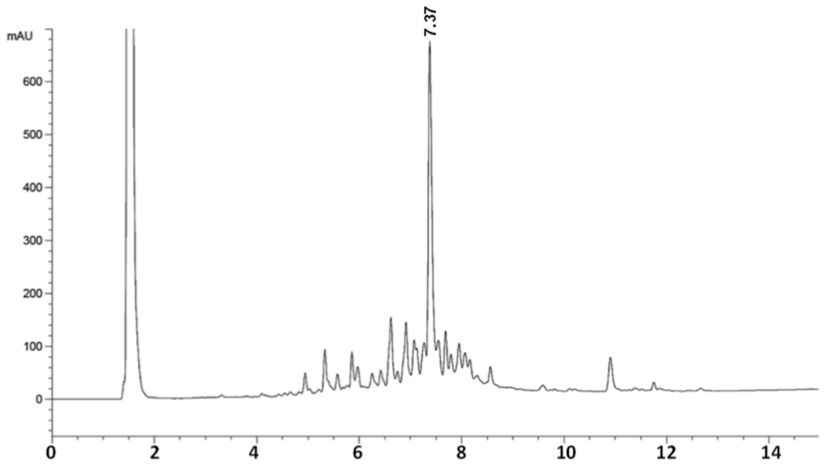

Figure 2. HPLC chromatogram of the linear hGH peptide, double coupling with DIC-OxymaPure [15−60% B (MeCN with 0.1% TFA) into A (H_2O with 0.1% TFA) over 15 min].

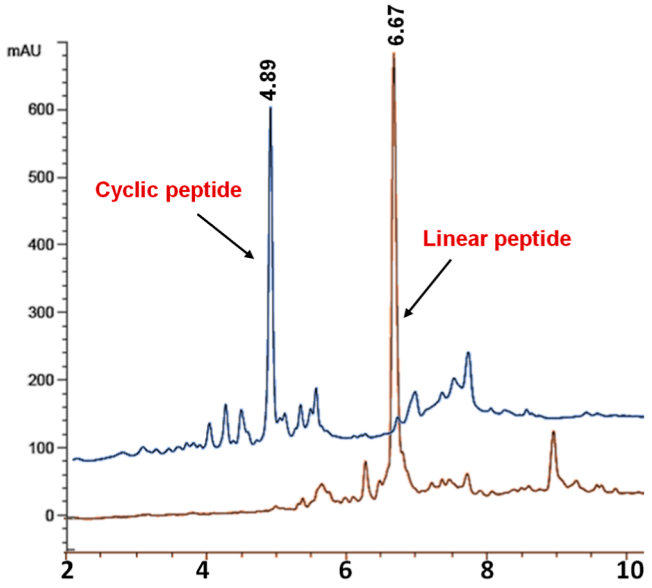

Figure 3. HPLC chromatogram of the linear and cyclic hGH peptide [15−85% B (MeCN with 0.1% TFA) into A (H_2O with 0.1% TFA) over 15 min].

peptide was lyophilized, dissolved in H_2O and 50 mM $NaHCO_3$, and added till the pH was around 8. The cyclization took place very smoothly within 30 min, showing again a good purity by HPLC (54%) and LCMS (Figures 3 and S31). Scheme 1 resumes the synthetic strategy followed.

CONCLUSIONS

Taking as a target, the "inaccessible" 15-mer C-terminal sequence of human growth hormone (hGH) with one extra Tyr attached to its *N*-terminus, it has been demonstrated once again that the use of ΨPro is an excellent tool to disrupt aggregation and facilitate the elongation of the peptide chain. In two different parts of the sequence, the monomer Fmoc-Ser($\psi^{Me,Me}$pro)-OH has been incorporated and its acylation using different Fmoc-AA-OH has been demonstrated to be successful using single coupling conditions with DIC-OxymaPure with 2 min preactivation as a coupling reagent. The use of just the monomer instead of ΨPro [Fmoc-AA-Ser($\psi^{Me,Me}$pro)-OH] opens new avenues for the synthesis of difficult peptides in two directions: (i) with just one Fmoc-derivative, all sequences could be attempted, increasing the synthetic flexibility of this methodology; and (ii) the cost of the synthesis using the monomer should be lower than when the dipeptides are used.

The total sequence of the target peptide has been obtained with good purity by double coupling using two different coupling methods for the last residues of the sequence. In addition to DIC-OxymaPure with in situ activation, the second coupling has been carried out with HATU-DIEA with 2 min preactivation. Although comparative studies have not been carried out, the use of two different coupling methods in the case of double coupling could improve the yield of the final product. Formation of the disulfide bridge has been carried out using one Trt and one SIT-protecting group for each of the two Cys residues. After the global deprotection and cleavage, the disulfide formation takes place chemoselectively by a thiol-disulfide interchange in 30 min by adding 50 mM of $NaHCO_3$ to the aqueous solution of the crude peptide.

As a final conclusion, once again it has been demonstrated that SPPS is a multifactorial process, where a proper choice of reagents and conditions can have a great impact on the purity of the final product. In this case, the combination of Fmoc-Ser($\psi^{Me,Me}$pro)-OH and Fmoc-Cys(SIT)-OH together with two of the most potent coupling methods (DIC-OxymaPure and HATU-DIEA) allowed for the synthesis of a short peptide that has so far only been synthesized by native chemical ligation.[28]

The use of the sterically more hindered monomer Fmoc-Thr($\psi^{Me,Me}$pro)-OH requires a deeper fine-tuning and will be reported elsewhere.

MATERIAL AND METHODS

General Information. All solvents and reagents used in the experiments were bought from commercial suppliers and were used further without any purification unless otherwise indicated. Fmoc amino acids and Fmoc Rink amide PS-resin (0.74 mmol/g) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Fmoc-Ser($\psi^{Me,Me}$pro)-OH was from Iris Biotech GmbH (Marktredwitz, Germany). DIC and OxymaPure were gifts from Luxembourg Bio-Technologies and Ness Zion, respectively, and piperidine was supplied by Sigma-Aldrich (St. Louis, Missouri, USA). DMF and HPLC-

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quality CH_3CN were purchased from SRL (CRD-SRL, India). Milli-Q water was used for RP-HPLC analyses. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex AerisTMC18 (3.6 μm, 4.6 × 150 mm) column, with a flow rate of 1.0 mL/min and UV detection at 220 nm. Chemstation software was used for data processing. Buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN. LCMS was performed on an Ultimate 3000 using an AerisTM 3.6 μm wide pore column from Phenomenex C_{18} (4.6 mm ×150) (system 2). Buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in CH_3CN, flow 1.0 mL/min, UV detection 220 nm.

Solid-Phase Peptide Synthesis. All peptides were synthesized following the standard Fmoc/*t*Bu-based solid-phase synthesis protocol (SPPS). The Fmoc Rink amide PS-resin (0.74 mmol/g) was used as a solid support for the peptides. Initially, the resin was washed using DMF (3 × 1 min), DCM (3 × 1 min), and DMF (3 × 1 min). The Fmoc group was removed by treatment of the resin with 20% piperidine/DMF (1 × 1 and 1 × 7 min), followed by washing with DMF. The protected Fmoc amino acids (5 equiv) were incorporated using DIC-OxymaPure (5:5) or HATU-DIEA in DMF as coupling reagents at rt. Fmoc from the last coupled amino acid was removed as explained above. After drying the peptidyl resin, cleavage was performed by treating with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was then precipitated with Et_2O and centrifuged, and the pellet was re-dissolved in H_2O/MeCN (1:1) for analysis by HPLC and LCMS.

For cyclization, the peptide crude obtained after cleavage was dissolved in H_2O−MeCN, followed by the addition of 50 mM aqueous solution of $NaHCO_3$ to achieve pH 8. The mixture was stirred vigorously at rt and monitored timewise using HPLC and LCMS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03261.

HPLC and LCMS characterization data of peptides (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Beatriz G. de la Torre − *KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa;* orcid.org/0000-0001-8521-9172; Email: garciadelatorreb@ukzn.ac.za

Fernando Albericio − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, 4000 Durban, South Africa; Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain;* orcid.org/0000-0002-8946-0462; Email: albericio@ukzn.ac.za

Authors

Srinivasa Rao Manne − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, 4000 Durban, South Africa*

Amit Chakraborty − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, 4000 Durban, South Africa;* orcid.org/0000-0002-1244-304X

Karin Rustler − *Iris Biotech GmbH, 95615 Marktredwitz, Germany*

Thomas Bruckdorfer − *Iris Biotech GmbH, 95615 Marktredwitz, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c03261

Author Contributions

The strategy was designed by all of the authors. Experimental work was performed by S.R.M and A.C. All of the authors discussed the results and prepared the article. All authors approved the final version of the article.

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Notes

The authors declare the following competing financial interest(s): K.R. and T.B. work for Iris Biotech, which is planning to commercialize the Fmoc-pseudoproline, The rest of authors declare no competing financial interest.

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