New Orthogonal Strategies for Peptide Synthesis

Miriam Góngora Benítez

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Programa de Química Orgánica

Tesis Doctoral

New Orthogonal Strategies for Peptide Synthesis

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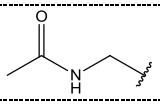
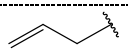
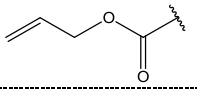
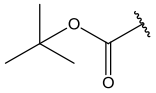
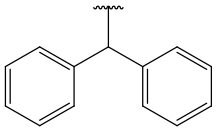
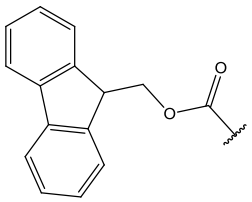
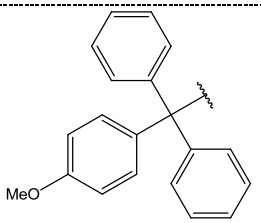
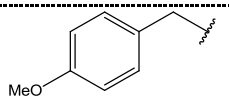
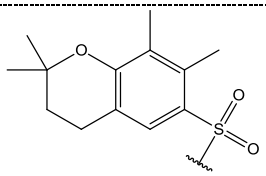
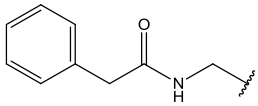
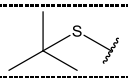
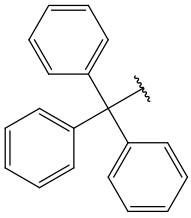
Annex 1. Protecting groups

Symbol	Name	Chemical structure
Acm	Acetamidomethyl	
All	allyl	
Alloc	allyloxycarbonyl	
Boc	*tert*-butyloxycarbonyl	
Dpm	diphenylmethyl	
Fmoc	9-fluorenylmethoxycarbonyl	
Mmt	4-methoxytrityl	
Mob	4-methoxybenzyl or *para*-methoxybenzyl	
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl	
Phacm	phenylacetamidomethyl	
S*t*Bu	*tert*-buthio	
Trt	trityl	

Annex 2. Resins and Handles

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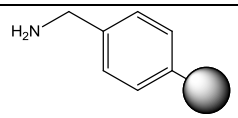
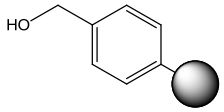
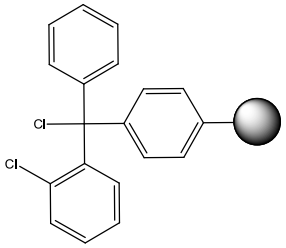
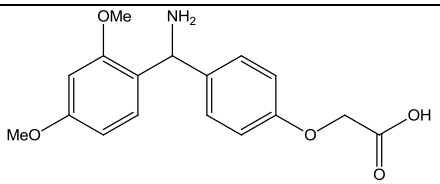
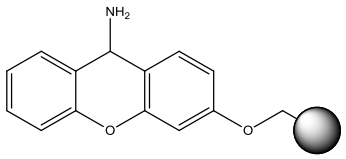
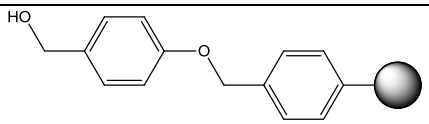
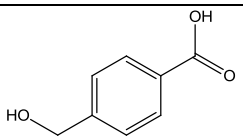
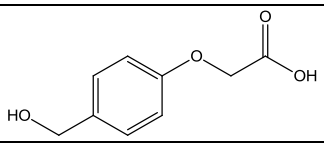
aminomethyl	
hydroxymethyl	
2-chlorotrityl chloride resin	
Rink amide linker	
Sieber amide resin	
Wang resin	
HMPA linker	
HMBA linker	

Annex 3. Coupling reagents and additives

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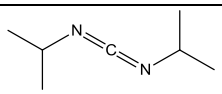
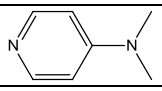
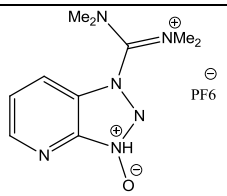
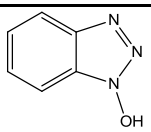
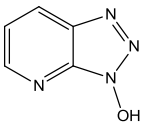
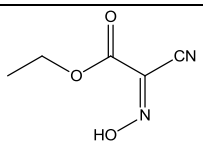
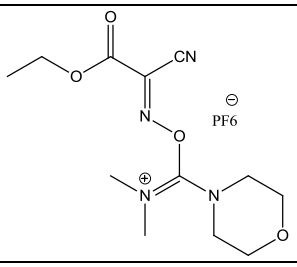
DIPCDI	
DMAP	
HATU	
HOBt	
HOAt	
Oxyma	
COMU	

Annex 4. Abbreviations and Acronyms

Ac	acetyl
DIEA	*N,N*-ethyldiisopropilamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DKP	diketopiperazine
ESMS	electrospray mass spectrometry
equiv	equivalent
FT-IR	Fourier transform infrared spectrometry
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
HPLC	high-performance liquid chromatography
PGA	penicillin G acylase (EC 3.5.1.11)
TFA	trifluoroacetic acid
TIS	triisopropylsilane
t_R	retention time
δ	chemical shift
J	coupling constant
ν	frequency

Annex 5. List of Publications

Publication I. M. Góngora-Benítez, Judit Tulla-Puche, M. Paradis-Bas, O. Werbitzky, M. Giraud, and F. Albericio. Optimized Fmoc solid-phase synthesis of the cysteine-rich peptide linaclotide. *Biopolymers (Pept. Sci.)*, 96, 69–80 (**2011**).

Publication II. M. Góngora-Benítez, A. Basso, T. Bruckdorfer, M. Royo, J. Tulla-Puche, and F. Albericio. Eco-friendly combination of the immobilized PGA enzyme and the *S*-Phacm protecting group for the synthesis of Cys-containing peptides. *Chem. Eur. J.*, 18, 16166–16176 (**2012**).

Publication III. M. Góngora-Benítez, L. Mendive-Tapia, I. Ramos-Tomillero, A.C. Breman, J. Tulla-Puche, F. Albericio. Acid-labile Cys-protecting groups for the Fmoc/*t*Bu strategy: filling the gap. *Org. Lett*., 14, 5472–5475 (**2012**).

Publication IV. M. Góngora-Benítez, M. Cristau, M. Giraud, J. Tulla-Puche, and F. Albericio. A universal strategy for preparing protected *C*-terminal peptides on the solid phase through an intramolecular *click* chemistry-based handle. *Chem. Comm*., 48, 2313–2315 (**2012**).

Publication V. F. Albericio, M. Cristau, M. Giraud, M. Góngora-Benítez, J. Tulla-Puche. Diketopiperazine forming dipeptidyl cleavable linkers and their use in the hydrid solid and homogeneous solution phase peptide synthesis. WO2012055509 A1 20120503 (**2012**).

Publications submitted or in preparation

M. Góngora-Benítez, J. Tulla-Puche, F. Albericio. Constella(EU)–Linzess(US): the last milestone in the long journey of the peptide linaclotide. (*submitted*)

M. Góngora-Benítez, J. Tulla-Puche, F. Albericio. Peptide as Therapeutics. The multifaceted roles of disulfide bonds. (*submitted*)

M. Góngora-Benítez, J. Tulla-Puche, F. Albericio. Handles for Fmoc-solid-phase synthesis of protected peptides. (*in preparation*)

Outline of the Thesis

This thesis is structured as a compendium of publications, being organized around four publications which have been published in international scientific journals, and which compose the core of this research work.

This manuscript is divided into three chapters which include the publications as *Results and Discussion* sections and short reviews related to the specific topics as *Introduction* of each chapter.

Chapter 1 is focused on the peptide Linaclotide, a small, disulfide-rich peptide recently approved by the FDA and EMA agencies. The *Introduction* of this chapter deals with the presentation of Linaclotide as a potent guanylate cyclase type-C agonist for the treatment of gastrointestinal disorders. In collaboration with Dr. M. Giraud and Dr. O. Werbitzky from Lonza AG, diverse synthetic strategies for the preparation of this peptide were evaluated in our lab. This research work is covered in the **Publication I** added in this thesis as *Results and Discussion* of this chapter.

In the light of the results achieved in **Chapter 1**, we were encouraged to explore new alternatives for the synthesis of disulfide-rich peptides, such as Linaclotide. In this regards, **Chapter 2** is focused on disulfide-containing peptides and Cys-protecting groups for the preparation of these tricky macromolecules. In the *Introduction* of this chapter a literature review highlights the importance of disulfide bonds in peptide therapeutics, and lists the synthetic tools available for their synthesis along with the common methods and techniques used for their further characterization. In partnership with Dr. A. Basso from Purolite and Dr. T. Bruckdorfer from Iris Biotech GmbH, the *S*-Phacm protecting group, previously developed in our group, was evaluated as an eco-friendly alternative. Simultaneously, in our group the acid-labile *S*-Dpm protecting group proved to be a useful alternative for the preparation of disulfide-containing peptides. These research works are covered in **Publication II** and **Publication III**, respectively, and are found in the *Results and Discussion* section of this chapter.

In collaboration with Dr. M. Giraud and Dra. M. Cristau from Lonza AG, a novel strategy for the preparation of *C*-terminal protected peptides on solid phase was developed in our group, which is the research core in the **Chapter 3**. The *Introduction* of this chapter is a literature review of handles described for the preparation of protected peptides. The results from this research work is covered in the **Publication IV** placed in this thesis as a *Results and Discussion* of this chapter. This latter research work has led to a **patent** –**Publication V**–.

GENERAL INTRODUCTION

According to the last Peptide Therapeutics Foundation report[1], forty years ago the average frequency of peptides entering clinical studies was just over one per year. In the 80's, about five new peptide molecules per year entered into clinical trials, and this number was increased to about 10 during the 1990s. Presently, close to 20 peptide-based drug candidates per year are estimated to enter into clinical trials.

Given the increasing importance of peptides as therapeutic entities, the development of new synthetic strategies for the convenient access to complex peptides in a simple, rapid, efficient, cost-effective, and eco-friendly way is required. The **solid-phase peptide synthesis** methodology, first described by Merrifield in 1963[2], has represented a revolution in the field of peptide chemistry and has been key in the further introduction of **peptides** as **drugs**. This strategy is based on the anchoring of the *C*-terminal group to a polymeric support, and peptide chain growing in the C-to-N direction. Principally, peptide synthesis relies on an efficient method for the activation of the carboxyl group prior to reaction with the amino terminal along with the appropriate combination of **protecting groups**. Thus, a general solid-phase peptide synthesis scheme includes a N^{α}–amino protecting group (*temporary* protecting group), side-chain protecting groups (*permanent* protecting groups) and a **linker**[3] –a specialized protecting group which attaches the peptide to the support– (**Figure 1**).

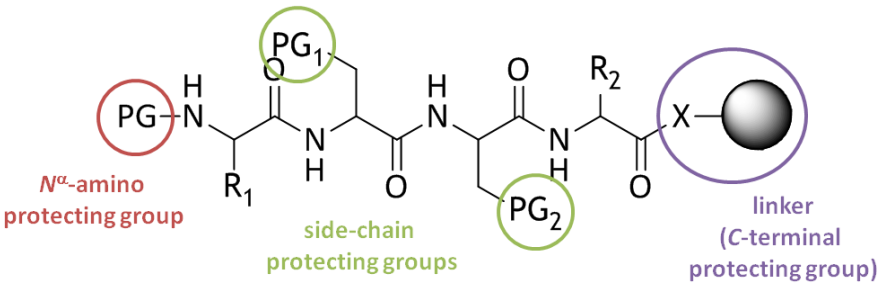


Figure 1. Protecting groups for solid-phase peptide synthesis

Protecting group **orthogonality** is crucial for the successful synthesis of polyfunctional molecules, such as peptides which comprise several distinct functional groups as well as imidazole and indole rings, which should also be protected. In peptide synthesis, orthogonality is described as the property of a set of completely independent classes of protecting groups to be removed by distinct chemical mechanisms, in any order and

in the presence of the rest[4]. On the contrary, **compatible** protecting groups are those used in schemes based on graduated lability to the same type of reagent.

Synthesis of **Cys**-containing peptides has long been posed as an attractive challenge for peptide chemist. Protecting groups for the nucleophilic β-thiol group of Cys residues must be stable during peptide elongation, and can be removed under mild conditions when necessary. Synthesis of peptides with multiple-**disulfide bonds** frequently demands the implementation of additional orthogonal dimensions into the protection schemes. Therefore, combinations of orthogonal and/or compatible protecting groups for each pair of Cys residues enable the regioselective formation of disulfide bridges. The development and study of novel protecting groups for the side-chain of the Cys residue may increase the number of synthetic tools for the efficient synthesis of Cys-containing peptides.

The solid-phase strategy has been applied for the preparation of small, medium-sized peptides. Nevertheless, the elongation of large peptide sequences through a stepwise solid-phase peptide synthesis is limited and convergent strategies, such as fragment condensation approach should be adopted. The preparation of **fully protected peptide fragments** on solid phase for convergent synthesis calls for handles with maintain intact the side-chain protecting groups upon cleavage.

The present thesis has focused on the development and application of novel orthogonal strategies for the synthesis of complex peptides, including Cys-containing peptides and long and difficult peptide sequences by fragment condensation approach. Specifically, different **Cys-protecting groups** have been evaluated and a **handle** has been designed and developed.

The following chapters of this thesis include a specific introduction to the particular topics covered on them as short reviews. As mentioned, the thesis comprises several published scientific articles, and the research work presented in **Chapter 3** has led to a **patent**. All of them are listed as bibliographic references in **Annex 5** (List of Publications).

References

[1] Peptide Therapeutics Foundation, *Development Trends for Peptide Therapeutics Report* (San Diego, 2010).

[2] a) Merrifield, R.B. Solid phase peptide synthesis. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154; b) Merrifield, R.B. Solid-phase syntheses (Nobel lecture). *Angew. Chem. Int. Ed.* **1985**, *24*, 799–810.

[3] Linker and handle terms are used indistinctly to describe the same concept.

[4] a) Barany, G.; Merrifield, R.B. A new amino protecting group removable by reduction. Chemistry of the dithiasuccinoyl (Dts) function. *J. Am. Chem. Soc.* **1977**, *99*, 7363–7265; b) Barany G.; Albericio F. A three-dimensional orthogonal protection scheme for solid-phase peptide synthesis under mild conditions. *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942.

OBJECTIVES

The overall goal of this thesis is to contribute to the development and evaluation of new orthogonal strategies for the efficient preparation of complex peptides mainly based on the Fmoc/*t*Bu strategy. The specific objectives of this thesis are:

- Study and optimization of the synthesis of linaclotide, a Cys-rich peptide recently accepted by the FDA, following Fmoc/*t*Bu chemistry (**Chapter 1**).

- Study and evaluation of the eco-friendly combination of the Cys-protecting group Phacm and the immobilized penicillin G acylase (PGA) enzyme for the synthesis of Cys-containing peptides (**Chapter 2**).

- Synthesis and evaluation of diverse acid-labile protecting groups for the side-chain of the Cys amino acid for their further application in the regioselective construction of disulfide bonds (**Chapter 2**).

- Design and development of a universal strategy for the preparation of protected C-terminal peptides on solid phase through a diketopiperazine (DKP)-based handle (**Chapter 3**).

CHAPTER 1

Linaclotide –a small, disulfide-rich peptide–

Chapter 1. Introduction

Constella(EU)-Linzess(US): The last milestone in the long journey of the peptide linaclotide

Constella(EU)–Linzess(US): the last milestone in the long journey of the peptide linaclotide

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For years, peptides were largely overlooked by large pharmaceutical companies, who argued that these molecular entities lacked drug-like properties. However, specificity, potency, and low toxicity are the main features of peptides as drugs, although their poor oral bioavailability and low biological stability, and as a consequence their short half-life in the body, cannot be disregarded.[1] Peptide therapeutics, many of them containing 10 to 30 amino acids and with one, two, sometimes even three disulfides bonds, are now synthetically manageable at large scales under regulatory compliance. This achievement along with the dramatic reduction in the cost of synthesis over the past decade has helped advance peptides as therapeutic agents. To date, hundreds of synthetic therapeutic peptides are in clinical trial processes, and even more are in advance stages of preclinical studies.[2]

Recently, after a long journey of preclinical studies and clinical trials, linaclotide, a first-in-class GC-C receptor peptide agonist, received Food and Drugs Administration (FDA) approval in August 2012 under the trade name Linzess,[101] and in September 2012 the European Medicines Agency (EMA) recommended the granting of its marketing authorization for the treatment of irritable bowel syndrome with constipation (IBS-C) and chronic idiopathic constipation (CIC) under the trade name Constella,[102] thereby becoming the first medicine approved in Europe for the treatment of IBS-C.

Functional gastrointestinal disorders: a major health and social problem

Functional gastrointestinal disorders (FGIDs) are a heterogeneous group of symptomatically-defined clinical entities characterized by chronic or recurrent gastrointestinal disorders in the absence of any known structural or biochemical abnormalities. FGIDs are influenced by multiple factors, including genetic predisposition, environmental influence, as well as by psychological and social factors. According to Rome III criteria,[3] CIC and IBS-C are two distinct functional bowel disorders[4] associated with constipation. IBS-C is distinguished from CIC based on the presence of any abdominal pain or discomfort associated with disturbed defecation. Thus, these two clinical entities share a number of symptoms, such as straining, hard

or lumpy stools, decreased frequency of bowel movement, and the sensation of incomplete evacuation; however, only those patients experiencing abdominal pain or discomfort are most likely to suffer from IBS-C. Nevertheless, there has been some evidence to suggest a degree of overlap between these two conditions, and an absence of stability in either diagnosis during follow-up, thereby indicating that IBS-C and CIC may be not entirely separate conditions.[5]

The worldwide impact of these two functional bowel disorders is frequently underestimated because of the restricted associated mortality. However, CIC and IBS-C have a significant negative impact on the patient's quality of life**,** and a elevated prevalence in the population which has a significant direct health care cost because of the number of physician visits, unnecessary diagnostic tests, procedures and surgeries, along with indirect expenses as a result of absenteeism and presenteeism, thereby making these conditions a major health and social problem.[6, 7, 8] A recent meta-analysis revealed a global prevalence of 11.2% for IBS,[9] whereas a previous study disclosed a 14% for CIC, along with a significantly elevated presence of CIC individuals with IBS, thus suggesting an overlap between these two conditions.[10]

Emerging therapeutic targets for chronic constipation disorders: current and prospective therapeutic agents for the treatment of CIC and IBS-C

Multiple factors have been considered to play a role in the complex pathophysiology of IBS and CIC disorders.[11, 12] Although recent studies have proposed that CIC and IBS-C are enteric neuropathies,[13] precise pathophysiological mechanisms underlying these disorders remain incompletely understood, and patients not responding to diet and lifestyle changes require effective and safe long-term therapies. As our knowledge of the pathophysiology of these disorders increases, one of the most important things the pharmaceutical industry can offer patients is the development of compounds with proved global symptom relief and minimal side-effects. Traditional therapies are often of limited efficacy in addressing the overall symptom complex, and are usually insufficient in terms of patient satisfaction.[14] Among non-specific laxatives available,

osmotic laxatives, such as PEG and lactulose, have shown efficacy for chronic constipation, although they may not alleviate pain in IBS-C, whereas stimulant laxatives, such as diphenylmethanes (*e.g.* bisacodyl, and sodium picosulfate), have demonstrated effectiveness for chronic constipation. However, no randomized controlled clinical trials have assessed the effectiveness or long-term safety of these drugs.[15]

The potential market for IBS-C and CIC drugs is large, but under-penetrated by the global pharmaceutical industry, and only a few effective therapeutic agents are available to mitigate the predominant bowel symptoms. In the last decade, the discovery of novel therapeutic targets has helped to identify the underlying pathology (*e.g.* abnormal gastrointestinal (GI) motility and secretion in CIC and IBS-C individuals, and visceral hypersensitivity in IBS-C patients) and promote the development of prospective therapeutic agents. Among others, novel therapeutic targets identified for the treatment of chronic constipation disorders include the 5-hydroxytryptamine receptor (5-HT4) receptor, the chloride channel-2 (CLC2), the cholecystokinin receptor-1 (CCK1 or CCKA), the guanylate cyclase C (GC-C) receptor, the ileal bile acid transporter (IBAT), and the Na+/H+ exchanger 3 (NHE3) channel.[16, 103]

The pharmaceutical industry is highly incentivized to invest in the development of more efficacious therapies for CIC and IBS-C. In this regard, several clinical trials are currently evaluating the effectiveness of molecular entities directed at new targets for the treatment of these two conditions (**Table 1**). Furthermore, many pharmaceutical companies are now in the process of developing drugs with indications for both men and women, which is a major improvement for male IBS-C patients because until now most new medications have been tested and approved only for use in women.

The first serotonergic agent for the treatment of chronic constipation disorders was Tegaserod, a 5-HT4 receptor agonist, which received approval from the Food and Drugs Administration (FDA) in 2002 for the treatment of IBS-C in women and CIC. However, this drug was withdrawn from the US market in 2007 after post-marketing analysis demonstrated increased serious cardiovascular effects in some patients. Prucalopride, another 5-HT4 agonist, was approved in Europe (2009), Australia (2011)

and Canada (2011). However, the FDA has recently refused its marketing in the US. At present, diverse pharmaceutical companies are carrying out clinical trials to evaluate potential 5-HT4 receptor agonists. What it is clear is that future 5-HT4 receptor agonists have to provide greater selectivity and safety.

Mechanism of action	**Drug name (brand names)**	**Indication**	**Company**	**Status**
5-HT$_4$ agonist	Tegaserod maleate (Zelmac, Zelmorm)	IBS-C, CIC	Novartis Bristol-Myers Squibb	Withdrawn 2007 (US)
	Prucalopride (Resotran, Resolor)	CIC	Janssen	Launched 2009 (EU)
	Velusetrag	IBS-C, CIC	Theravance	Phase II
	HCP-0613	IBS-C	Hanmi	Phase II
	DA-6650	IBS-C	Dong-A	Phase I
	DSP-6952	IBS-C	Dainippon Sumitomo Pharma	Phase I
CLC2 activator	Lubiprostone (Amitiza)	IBS-C, CIC	Sucampo Takeda	Launched 2006 (US)
CCK1 (CCKA) antagonist	Dexloxiglumide	IBS-C	Rottapharm	Phase III (EU)
GC-C receptor agonist	Linaclotide acetate (Linzess, Constella)	IBS-C, CIC	Forest-Ironwood Almirall	Registered 2012 (US, EU)
	Plecanatide	IBS-C, CIC	Synergy	Phase I
IBAT inhibitor	Elobixibat	IBS-C CIC	Albireo	Phase II Phase III
NHE3 inhibitor	RDX5791	IBS-C	Ardelyx	Phase II

Table 1. Current and prospective therapeutic agents for CIC and IBS-C.[16, 23, 103]

As a unique representative of CLC2 activator, lubiprostone, an analog of prostaglandin E1, was approved by the FDA in 2006 for the treatment of CIC, and later, in 2008, received FDA approval for the treatment of IBS-C in women. Lubiprostone is a selective CLC2 activator that stimulates chloride-fluid secretion into the gastrointestinal tract, softening stools and accelerating transit time. Unfortunately, common side effects reported for this drug include nausea, headaches, and diarrhea.

Given the significant role of CCK1 in gastrointestinal motility, CCK1 antagonists have emerged as potential pharmaceutical agents for the treatment of chronic constipation disorders. Thus, dexloxiglumide, a CCK1 receptor antagonist, underwent clinical trials for the treatment of IBS-C in females. Nevertheless, during phase III studies statistically significant efficiency was not demonstrated, and the development of this drug for the treatment of IBS-C was discontinued in the US in 2003, although phase III clinical trials are still being pursued in Europe.

Linaclotide, an orally administered peptide, is a GC-C receptor agonist that acts locally in the intestine. It shows minimal oral bioavailability to the systemic compartment, eliminating mechanism (GC-C)-independent and off-target adverse effects. In August of the present year, linaclotide received the approval from the FDA, and then, one month later, in September, has been approved by the EMA in Europe. Plecanatide, another GC-C receptor agonist, is currently undergoing phase I clinical trials.

A first-in-class IBAT inhibitor developed for the treatment of CIC and IBS-C, Elobixabat, acts locally in the gut with minimal systemic exposure and modulates the enterohepatic circulation of bile acids by partial inhibition of IBAT, which increases colonic fluid secretion and motility. Elobixabat is currently undergoing phase II trials for the treatment of IBS-C and phase III for CIC.

Another novel therapeutic target for the treatment of chronic constipation is the NHE3 channel, a sodium transporter channel present on the surface of intestinal epithelia. RDX5791 is a potent and selective inhibitor of NHE3 and is currently undergoing phase II clinical trials. It has proved to be well tolerated, and it produces a significant improvement of IBS-C symptoms.

GC-C receptor: a specific target for the treatment of CIC and IBS-C

The GC-C receptor[17] is a member of the guanylyl cyclase family, predominantly expressed in the luminal aspect of intestinal epithelial cells. CG-C is a multi-domain enzyme composed of an extracellular ligand-binding domain, a single transmembrane

region, a domain similar to that of protein tyrosine kinases, and a C-terminal guanylate cyclase catalytic domain, responsible for producing cyclic guanosine monophosphate (cGMP) by ligand-mediated activation (**Figure 1**).

The first ligand identified for GC-C was a member of the heat-stable enterotoxin family (STa), produced by bacteria that colonize the intestine, including *Escherichia coli*, *Enterobacter sp.*, *Klebsiella sp.*, and *Yersinia enterocolitica,* and one of the most common causative agents of secretory diarrhea.[18] Later, two endogenous intestinal paracrine hormones, first guanylin,[19] expressed mainly in duodenum and proximal small intestine, and then uroguanylin,[20] abundant in the colonic epithelium, were identified. The signaling cascade mediated by these two peptide hormones in the intestine is identical to that described for STa. Thus, after binding to the external domain of GC-C at the apical membrane of enterocytes, the ligands stimulate intrinsic guanylyl cyclase catalytic activity, initiating a cascade in which an accumulation of the second messenger cGMP occurs first. This leads to a stimulation of the membrane-associated cGMP-dependent protein kinase II (PKGII), or inhibition of the activity of a cAMP-specific phosphodiesterase (PDE3). The latter hydrolyzes cAMP, thus its inhibition provokes a cAMP accumulation, which, in turn, actives cAMP-dependent protein kinase (PKA). Next, the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) by PKGII or PKA kinases triggers an increase in bicarbonate and chloride-secreting activity, thereby prompting net secretion of salts and water into the intestinal lumen. In addition, cGMP enhances duodenal bicarbonate secretion through an unknown channel in a CFTR-dependent manner and inhibits NHE3, a sodium-hydrogen exchanger, through interaction with PKGII and the regulatory cofactor NHERF2. NHE3 inhibition leads to a marked reduction in Na+ absorption and consequently decreased fluid uptake by the intestinal cells. cGMP can also directly activate cyclic nucleotide gated channels (CNGs), thereby causing Ca2+ influx. Finally, GC-C signaling is terminated by hydrolysis of cGMP into GMP by a cGMP-dependent phosphodiesterase (PDE5) (**Figure 1**).[21]

Overall, the GC-C receptor plays a crucial role in fluid homeostasis, pH control, and electrolyte balance. Thus, it has been proposed that the endogenous ligands guanylin

and uroguanylin regulate intestinal fluid and electrolyte homeostasis involving cGMP as a second messenger, a fundamental phenomenon for the maintenance of gut physiology. Moreover, various studies suggested that administration of STs stimulates intestinal smooth muscle,[22] thereby altering gut motility, and GC-C activation by STs diminishes afferent pain fiber firing,[23] presumably through the release of a specific mediator that stimulates surrounding dendritic nerve endings, thus modifying neural firing rates.

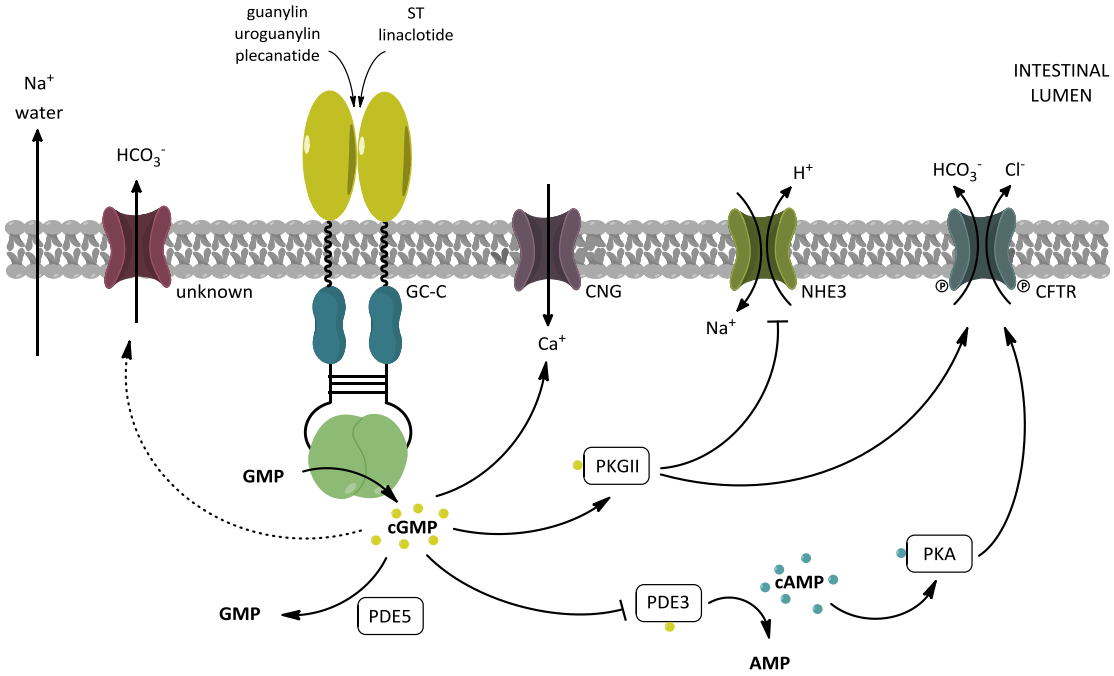

Figure 1. Signaling cascade mediated by GC-C receptor activation.[20, 43]

Therefore, given the participation of the GC-C receptor in the maintenance of fluid and ion homeostasis in the intestine and its alleged implication in intestinal motility and attenuation of afferent pain, and based on the emerging molecular-targeted therapeutic approach, this receptor has become a potential therapeutic target for the treatment of chronic constipation disorders such as CIC and IBS-C. These observations, along with the discovery of the molecular mechanism of action of ST peptides and the endogenous hormones guanylin and uroguanylin, have lead to the development of innovative pro-secretory drugs, such as linaclotide[24] and plecanatide[25].

Linaclotide an analog of the GC-C super-agonist STh enterotoxin

One of the most common STa enterotoxins produced by *E. coli* is STh,[26] a 19-amino acid peptide that contains six Cys residues forming three intramolecular disulfide bonds (Cys-6Cys11, Cys7-Cys15, and Cys11-Cys18) (**Figure 2**). These bonds stabilize the tertiary structure and are required for full biological potency and efficacy.[27] The 13-amino acid sequence from Cys6 to Cys18 of STh enterotoxin was determined as the minimal fragment responsible for full biological activity, referred to as the toxin domain (**Figure 2**).[28] However, a truncated STh variant lacking the *N*-terminal residues Asn-Ser-Ser-Asn was reported to exhibit 10-fold reduction of potency compared to the full-length STh, thereby suggesting that the extra *N*-terminal residues, although required for full potency, are not essential for the biological activity of this peptide.[29] Moreover, the receptor binding region of STh was identified in the highly solvent-exposed β-turn formed by amino acids Asn11-Pro12-Ala13.[30] Point mutation in this region dramatically reduces the receptor binding activity of ST, thus demonstrating the relevance of these residues for interaction with the GC-C receptor.[31]

The toxic domain of STa shows significant homology to the endogenous peptides guanylin and uroguanylin in both amino acid sequence and three-dimensional structure. However, these two hormones contain four Cys residues forming two disulfide bonds with a 1-3/2-4 connectivity (**Figure 2**). Thus they lack one of the bonds present in ST enterotoxins. The two disulfide bonds present in guanylin and uroguanylin are structurally equivalent to the 2-5 and 3-6 disulfide bridges of ST and provide two distinct interconvertible topologies, of which only one isomer, the so-called A-from, is biologically active and meets the structural requirements to bind and activate GC-C receptor.[32, 33] Interestingly, the three-dimensional structure of this bioactive isomer closely resembles the crystal structure of STa. Thus the third disulfide bond found in STa enterotoxins appears to freeze the conformation of the biologically active topological A-isomers.[34]

The relevance of the conserved disulfide bonds, along with the STh-specific disulfide bridge, was studied by Gariépy and coworkers by preparing STh analogs that lack one

or two of the disulfide bonds by replacing the pair of Cys residues with two Ala ones. The Cys7-Cys15 bond was found to be crucial for biological activity, although this bridge alone was not sufficient for binding, while replacement of the Cys6-Cys11 and Cys10-Cys18 bonds resulted in peptides that bind 42000- and 130-fold less strongly to their receptor, respectively.[35] That study reflected the key structural role of the conserved disulfide bonds and demonstrated that the disruption of STh-specific Cys10-Cys18 diminishes toxicity but is not essential for binding.

Moreover, STh and human uroguanylin share features that distinguish these peptides from the human guanylin hormone. For instance, the presence of an extra residue located after the *C*-terminal Cys is a common element found in STh and uroguanylin peptides (a Tyr residue and a Leu residue, respectively), but absent in guanylin (**Figure 2**). Furthermore, human guanylin has a conserved aromatic amino acid Tyr9, instead of the corresponding Asn residue found in STh and human uroguanylin. In the latter two peptides, this residue renders them resistant to proteolytic degradation and inactivation by the endopeptidase chymotrypsine, a digestive enzyme abundant in the intestinal tract (**Figure 2**). Carpick and coworkers[36] demonstrated that guanylin is rapidly hydrolyzed *in vitro* by the action of the enzyme chymotrypsin and proposed that its bioactive form makes it prone to be cleaved by this endopeptidase.

Singularly, human uroguanylin has two Asp residues in the *N*-terminal region that appear to contribute to biological activity and modulate binding affinity to the GC-C receptor in a pH-dependent manner (**Figure 2**).[37] Therefore, a truncated analog of uroguanylin without the last three *N*-terminal residues resulted in an active peptide, although the potency was markedly reduced compared to the native 15-amino acid form. Regarding the pH-dependency, at acidic pH (5) the potency of uroguanylin increased considerably, while guanylin became ineffective at activating the GC-C receptor. In contrast, at basic pH (8) the potency of guanylin increases substantially while diminishing the potency of uroguanylin, thereby suggesting the possible segmental regulation of the intestine by these two endogenous peptides since the pH of the intestinal lumen varies considerably from the stomach to the rectum. Accordingly, the *N*-terminal acidic residues of uroguanylin are required for increased

binding affinities, and thus the enhanced potency of the hormone for activating the receptor under acidic conditions. Regarding the pH-dependence of ST, although the difference was smaller in comparison to the endogenous homologs, ST exhibited slightly more potency in acidic conditions than in basic pH.[37]

Overall, differences in the structural features of STh, guanylin and uroguanylin are reflected in the pharmacodynamic and pharmacokinetic properties of these GC-C receptor ligands. Hence, the reduced conformational space of the constrained STh, compared to that of the endogenous homologues, leads to an enhancement of the receptor-binding affinity and resistance to hydrolytic and enzymatic degradation, and as a consequence, ST is reported to be more potent than guanylin and uroguanylin, regardless of the pH, and it is therefore considered a super-agonist of GC-C.[36, 37]

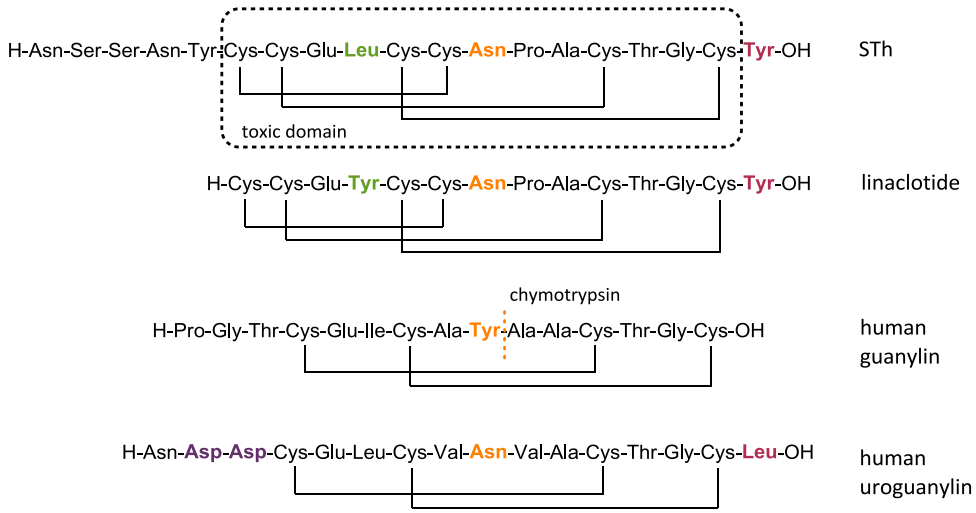

Figure 2. Primary structures of STh enterotoxin, linaclotide, human guanylin, and human uroguanylin peptides.

Linaclotide is a 14-amino acid peptide homolog of STh that contains the three distinctive disulfide bonds present in enterotoxins, thus conferring this peptide higher stability and binding affinity compared with its endogenous two disulfide bond-containing counterparts, guanylin and uroguanylin (**Figure 2**). It is worth noting the single amino acid substitution (Leu8 in STh is replaced with Tyr4 in linaclotide) and the lack of the five *N*-terminal residues present in STh, which modulate the pharmacodynamics and pharmacokinetic stability of linaclotide, as well as the absence

of the two Asp residues of the *N*-terminal region, which are present in uroguanylin, allow linaclotide to act in a pH-independent manner throughout the gastrointestinal tract.

A pharmacokinetic study of linaclotide,[38] revealed that the biologically active parent drug, linaclotide, has a half-life of approximately 3 min in the intestine before the proteolytic action of carboxypeptidase A removes the C-terminal Tyr residue, producing a fully biological active pharmacophore which includes the 13 amino-acid core and the three disulfide bonds (MM-419447). In turn, in the intestine, this active metabolite has a half-life of approximately 10 min before undergoing degradation, which is believed to occur in two phases. First, in the unfolding phase, the pharmacophore would undergo enzymatic reduction of the three disulfide bonds under the action of free luminal glutaredoxin, glutathione as a source of reducing equivalent, and free luminal glutathione reductase for glutathione regeneration. In the second phase, degradation, the reduced and unfolded peptide undergoes complete degradation by the proteolytic action of pancreatin.

Although the degradation pathway reported, orally administered linaclotide led to significant increases of intestinal fluid secretion and rate of intestinal transit accompanied by decreases in visceral hypersensitivity, thus indicating sufficient intestinal residence time for parent drug and/or the active metabolite thereof to bind to GC-C receptor and mediate the therapeutic effects. Therefore, following the same mechanism of action as its homologs ST, guanilyn and uroguanylin, linaclotide activates GC-C, thus leading to the signaling cascade (**Figure 1**) that finally promotes the secretion of chloride ions, bicarbonate, and water into the intestinal tract, acting as a so-called pro-secretory agent. In animals, linaclotide enhances gut secretion and transit,[39] and has anti-nociceptive properties in models of visceral hypersensitivity.[40] In patients with CIC and/or IBS-C, linaclotide provides therapeutic relief of symptoms, including mitigation of abdominal pain associated with IBS-C.[41] According to non-clinical models, orally administrated cGMP reduces response to pain, which may be attributed to the alleviation of abdominal pain associated with IBS-C.[42]

Finally, the minimal oral bioavailability to the systemic compartment of linaclotide eliminate mechanism (GC-C)-independent and off-target adverse effects.

Linaclotide has expanded the range of emerging molecular-based therapeutic options available for the treatment of chronic constipation disorders and is an example of the successful development of a peptide as a therapeutic agent.

Future perspective

Although a considerable number of peptide therapeutics is currently in the market for addressing new therapeutic challenges, there is a remaining issue to be resolved: the administration route of peptide drugs. The non-invasive oral route is often the preferred for drugs administration due to the high patient compliance, the convenience for self-administration, as well as the wide range of dosage adjustment. However, owing to the general low bioavailability of orally administered peptides, most of the approved peptide therapeutics available in the current pharmaceutical market are administered parenterally. The oral administration of peptide drugs is well known to be precluded partly by the harsh hydrolytic environment along with the extensive presystemic proteolytic activity in the gastrointestinal tract, and partly due to their poor absorption across the intestinal epithelium.

Small disulfide-rich peptides, such as linaclotide, possess an extremely compact and constrained architecture which endows these peptides a remarkable stability to thermal and chemical denaturation and extremely resistance against proteolytic degradation. In addition, the reduced conformational space, distinctive characteristic of the multiple disulfide-containing peptides, enhances the binding affinity of these peptides to the therapeutic target. As a result, it can be said that disulfide-rich peptides combine the potency of biologics and the pharmacokinetics of small molecules.

It is not surprising that, at the present, no less than 12 companies are developing macrocycle and constrained-peptide synthesis technologies to improve medicinal

chemistry properties, including cell-penetrating features and drug-like profile. Since 2007, at least 27 drug development collaboration have been announced for using macrocycle technologies and constrained peptide technologies, with several pharmaceutical corporations assaying more than one technology at the same time.[43] Considering all these facts, there is a promising future for innovative disulfide-rich peptide therapeutics in coming years.

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Publication I

Optimized Fmoc solid-phase synthesis of the cysteine-rich peptide linaclotide.

Optimized Fmoc Solid-Phase Synthesis of the Cysteine-Rich Peptide Linaclotide

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ABSTRACT:

Linaclotide, a small 14-mer peptide highly rich in cysteines, is currently in phase III clinical trials for the treatment of gastrointestinal disorders. The challenge in the assembly of linaclotide consists of achieving the correct and clean folding of its three disulfide bridges. For this purpose, a number of regioselective, semiregioselective, and random strategies have been studied. In addition to selecting distinct protecting groups for the thiol function, their position in the sequence, the influence of the neighboring protecting groups, as well as the order in which the disulfides fold were studied. Here we describe an optimized solid-phase synthesis of linaclotide that should allow the production of this peptide in multigram amounts. © *2010 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 96: 69–80, 2011.*

Keywords: protecting group; cysteine; folding; solid phase

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INTRODUCTION

Linaclotide is a 14-residue peptide that is currently undergoing phase III clinical trials for the treatment of gastrointestinal diseases such as chronic constipation (CC) and irritable bowel syndrome (IBS).[1] Linaclotide, which can be administered orally, is an agonist of the guanylate cyclase type-C receptor, found in the intestine.[2,3] From a structural point of view, this small peptide presents a constrained conformation with three disulfide bridges. These lies between Cys1–Cys6, Cys2–Cys10, and Cys5–Cys13 (Figure 1).

To achieve the large amounts required for a marketed peptide, an efficient synthesis must be developed. To optimize the synthesis, its fundamental limitations need to be determined and addressed. In the case of linaclotide, the key points are related to the high density of Cys residues (43% of the sequence, some of them consecutive) present in the peptide for two reasons: the potential risk of racemization upon assembling the linear chain and the misfolding of the three disulfide bridges. To address these points, we studied the concourse of distinct protecting groups and folding conditions. The disulfide bridges in the final folded peptide were also carefully analyzed.

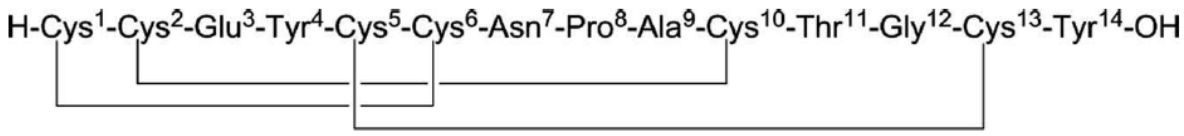

FIGURE 1 Structure of linaclotide.

RESULTS AND DISCUSSION

Disulfide bonds are crucial for maintaining the biological activity and structure of peptides and proteins.[4] The misfolding of proteins has been associated with numerous diseases,[5] one type of misfolding being the mispairing of disulfide bridges. In this regard, partially folded proteins containing only one or two disulfide bridges have been used as probes to study the folding of these molecules.[6] Therefore, the synthesis of disulfide-rich peptides and proteins with proper folding has been the subject of continuous studies,[7,8] since the folding is also sequence dependent.

Several strategies have been examined for the synthesis of linaclotide using distinct Cys-protecting groups, such as [*S-tert*-butyl (*St*Bu),[9] acetamidomethyl (Acm),[10–12] trityl (Trt), methoxytrityl (Mmt),[13] and *p*-methoxybenzyl (*p*MeOBzl)] on Wang and 2-chlorotrityl[14] (CTC) resins. In addition, disulfide formation has been performed in solid phase and solution. In all cases, linear peptides were manually synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry using 1-[bis(dimethylamino)methylene]-6-chloro-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HCTU) and diisopropylethylamine (DIEA) in N,N-dimethylformamide (DMF) for 1 h at 25°C to incorporate all amino acids other than Cys. To prevent racemization,[15,16] Cys was coupled using *N,N′*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) in DMF, with a 5-min preactivation, for 1 h at 25°C. [Analysis of the peptide by enantiomer labeling (ELAB) performed at C.A.T. GmbH & Co. (Tubingen, Germany) determined a racemization of only 1.5% for Cys.]

Random Strategy: [6-Trt]

Disulfide-rich peptides have commonly been synthesized by regioselective methods to assure the correct pairing of Cys.[17–19] Nevertheless, some examples can be found in nature, such as cyclotides[20] (~30 residues), which behave like miniproteins and fall into the right conformation without assistance. To determine whether linaclotide could be included in the latter category and to have a first standard in hand, we studied a random synthetic strategy. The six Cys residues were incorporated with Trt side-chain protection. The protected peptide was cleaved from the CTC resin by a low acid-content cleavage cocktail, followed by a total side-chain deprotection

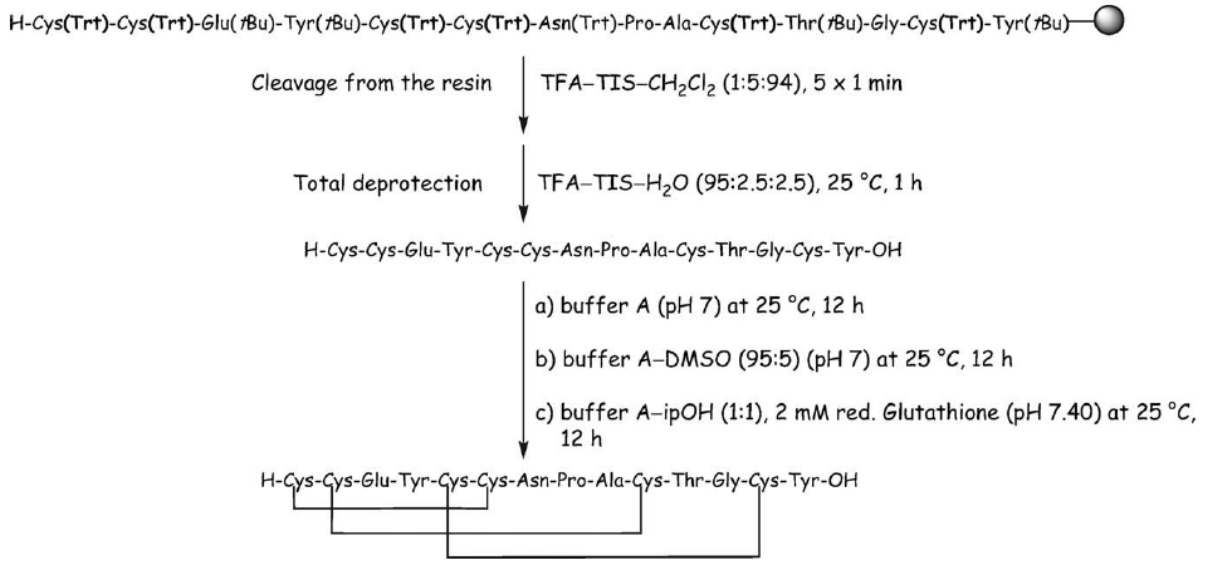

FIGURE 2 Synthetic scheme for linaclotide using all-Trt side-chain protection for Cys.

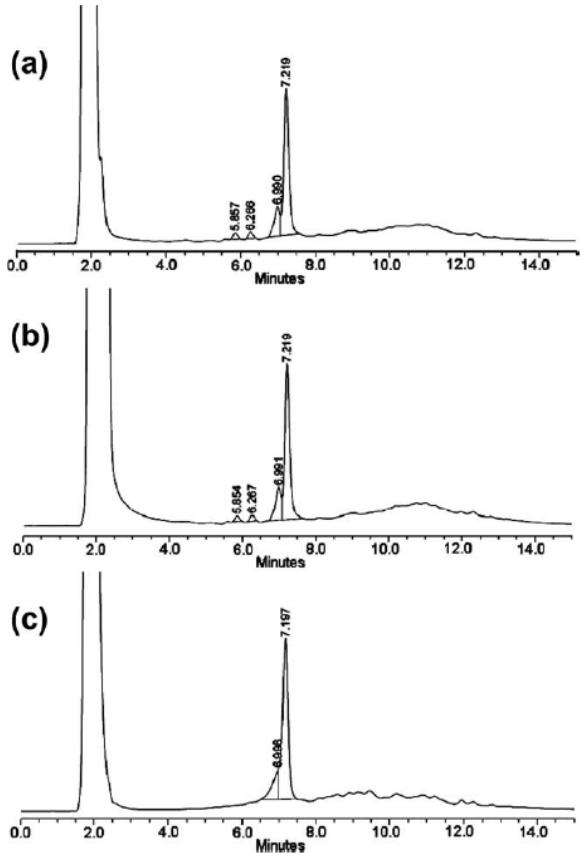

FIGURE 3 Chromatographic profiles of the completely oxidized peptides from the [6-Trt] strategy: (a) buffer A (100 m*M* sodium phosphate, 2*M* guanidine hydrochloride, pH 7.0), 25°C, 12 h; (b) buffer A, 5% DMSO, 25°C for 12 h; and (c) buffer A-2-propanol (1:1), 2 m*M* reduced glutathione (pH 7.4), 25°C, 12 h. Gradient: from 15% to 45% ACN over 15 min.

cleavage step (see Figure 2). In this random strategy, free thiols were cyclized in solution.

The following three conditions were studied for the cyclization: (a) the peptide was dissolved in buffer A (100 m*M* sodium phosphate, 2*M* guanidine hydrochloride, pH 7.0) and the solution was stirred under open atmosphere at 25°C for 12 h; (b) the peptide was dissolved in buffer A, 5% dimethyl sulfoxide (DMSO) was then added,[21] and the solution was stirred under open atmosphere at 25°C for 12 h; and (c) the peptide was dissolved in a mixture of buffer A-2-propanol (1:1), 2 m*M* reduced glutathione was added (pH 7.4), and the solution was stirred under open atmosphere at 25°C for 12 h. In all cases, a negative Ellman's test[22] indicated the absence of free thiols.

All the experiments showed a similar chromatographic profile, with a main peak that corresponded to the completely oxidized peptide, as shown by high-performance liquid chromatography electrospray mass spectrometry (HPLC-ESMS) (+) (see Figure 3). To confirm that the peptide had the correct pairing of Cys residues, an extensive analysis of the disulfide bonds was undertaken.

Disulfide Bond Analysis

The presence of six Cys residues in the 14-residue peptide linaclotide implies that 15 theoretical disulfide pairings can be achieved. To confirm that a proper isomer (Cys1–Cys6, Cys2–Cys10, Cys5–Cys13) had been obtained following the [6-Trt] strategy, we performed a disulfide bond analysis using a modified method of Wu and Watson.[23–25]

The masses of the resulting peptide fragments were related to the location of the paired Cys residues that had undergone reduction, cyanylation, and cleavage (see Figure 4). Fragments expected from the **Cys1–Cys6** disulfide bond had a MW of 643.16 and 955.30; fragments expected from the **Cys2–Cys10** disulfide bond had a MW of 120.04, 570.16, and 925.29; and finally those expected from the **Cys5–Cys13** disulfide bond had a MW of 309.08, 515.15, and 791.25 (see Figure 4).

HPLC-ESMS analysis (see Figure 5) showed the presence of 645 and 957 $[M + H]^+$, which account for the presence of the disulfide bond Cys1–Cys6; 310, 517, and 793 $[M + H]^+$ confirm the pairing of Cys5 and Cys13; and 572 $[M + H]^+$ belongs to disulfide bond Cys2–Cys10. Clearly, this analysis shows how linaclotide folds by itself in the correct and active conformation.

Regioselective and Semiregioselective Strategies

Use of the Acm Group in Linaclotide. The Acm group has been widely used in the synthesis of disulfide-containing peptides. Its main advantage is its resistance toward trifluoroacetic acid (TFA) cleavage and the possibility to perform I_2-mediated disulfide bonds in solution and solid phase. Therefore, the Acm group was a first choice in designing the syntheses of linaclotide. In parallel with the [6-Trt] strategy, a random synthesis using six Acm groups on CTC resin was performed. Although the linear peptide was of good quality, the cyclization in solid phase and solution resulted in scrambling. Nevertheless, less-aggressive regioselective and semiregioselective approaches with this group were examined. Semiregioselective approaches included three [4 Trt +2 Acm] strategies and two [2 Trt +4 Acm] strategies, all performed on CTC resin (see Figure 6).

In the [2 Trt +4 Acm] strategies, when oxidizing the first disulfide bridge with DMSO (either 2–10 or 5–13), a main peak with a correct mass was obtained. However, after I_2 oxidation (5 equiv) in acetic acid (AcOH)/H_2O (4:1) in solution

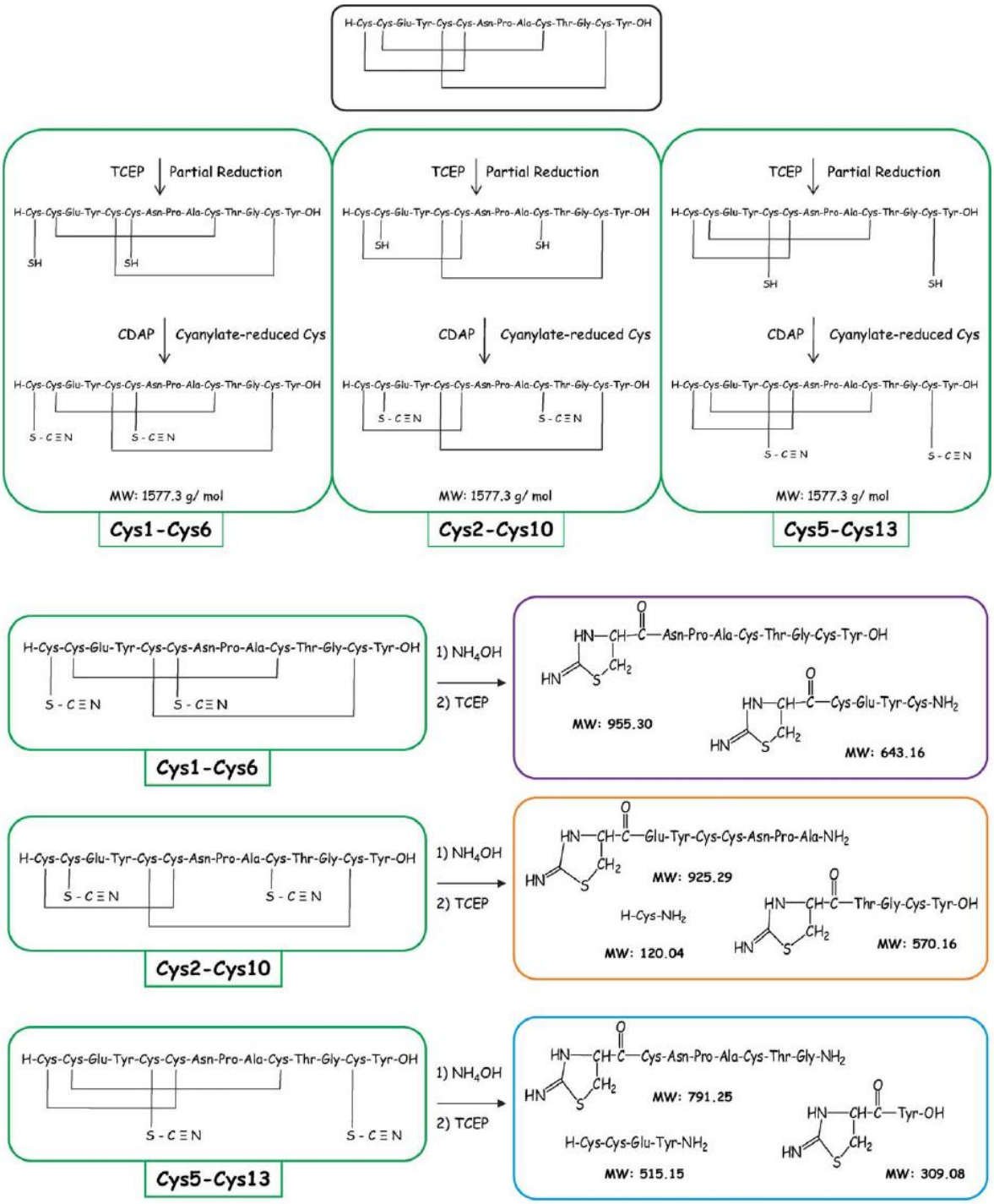

FIGURE 4 Expected masses of the disulfide bond analysis.

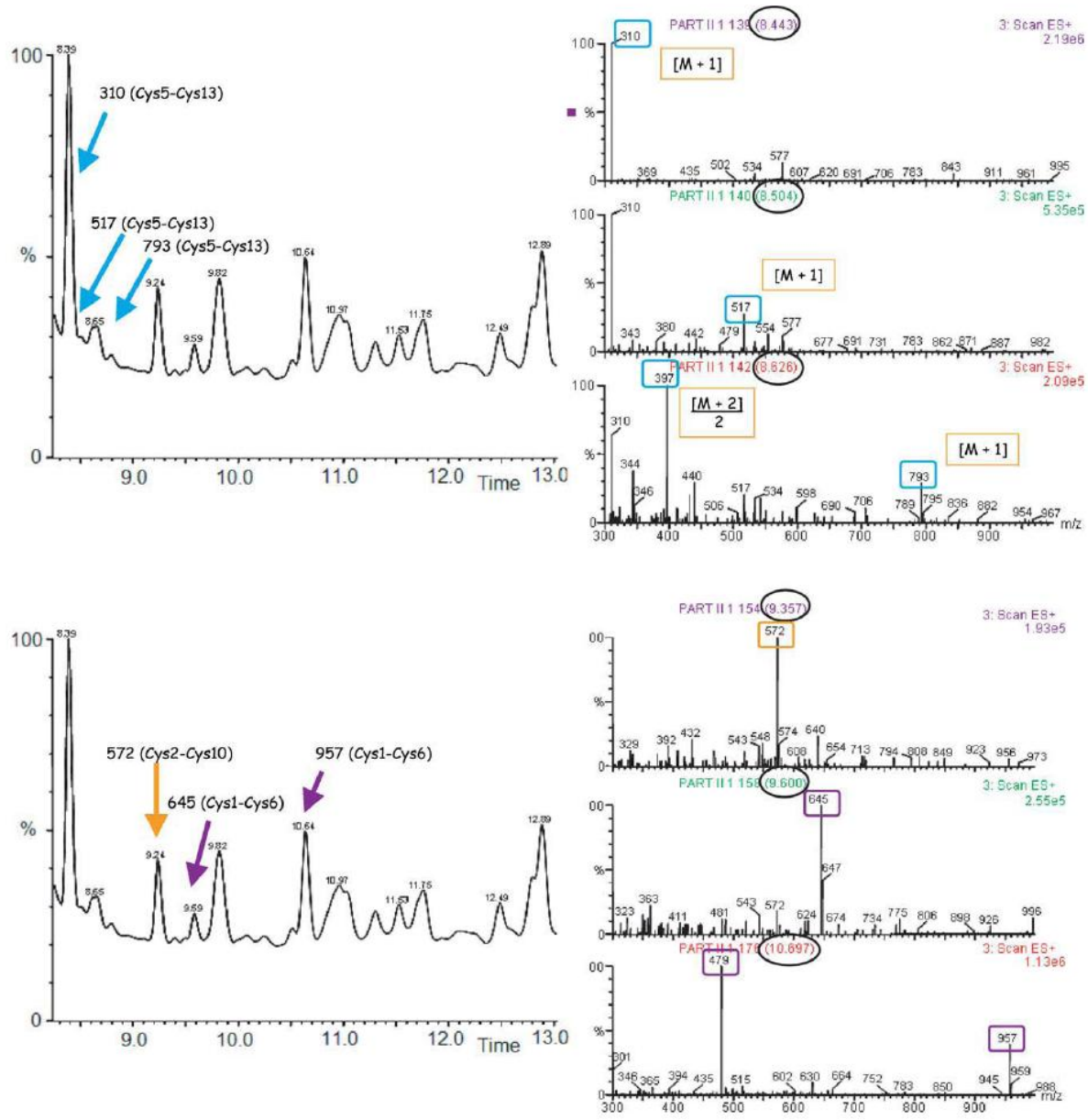

FIGURE 5 HPLC-ESMS(+) of the disulfide bond analysis.

and with subsequent removal of I_2 excess with $CHCl_3$, the purity of the crude peptide decreased considerably. This observation may point to the occurrence of scrambling. Coelution of these final crude products with the correctly folded peptide obtained from the [6-Trt] strategy did not show any coincidence with the [2 Trt (5-13) +4 Acm (1-6, 2-10)] approach; however, one of the peaks of the [2 Trt (2-10) + 4 Acm (1-6, 5-13)] strategies coeluted with the correct peptide. However, since these final crude peptides were of low purity, strategies in which Acm was used to achieve only one disulfide bridge were then studied. In strategies (iii) and (iv), the quality of the first intermediate (two disulfide bonds formed) was good, especially in the [4 Trt (2-10, 5-13)] approach. However, upon addition of I_2 to remove Acm and to perform the last disulfide bridge, considerable scrambling was observed. Coinjection of this crude product with the peptide obtained from the [6-Trt] strategy did not show any coincidence between the main peaks. In strategy (v), after DMSO

2Trt + 4Acm

i) H-Cys(**Acm**)-Cys(**Acm**)-Glu-Tyr-Cys(**Trt**)-Cys(**Acm**)-Asn-Pro-Ala-Cys(**Acm**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

ii) H-Cys(**Acm**)-Cys(**Trt**)-Glu-Tyr-Cys(**Acm**)-Cys(**Acm**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(**Acm**)-Tyr-OH

4Trt + 2Acm

iii) H-Cys(**Acm**)-Cys(**Trt**)-Glu-Tyr-Cys(**Trt**)-Cys(**Acm**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

iv) H-Cys(**Trt**)-Cys(**Acm**)-Glu-Tyr-Cys(**Trt**)-Cys(**Trt**)-Asn-Pro-Ala-Cys(**Acm**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

v) H-Cys(**Trt**)-Cys(**Trt**)-Glu-Tyr-Cys(**Acm**)-Cys(**Trt**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(**Acm**)-Tyr-OH

FIGURE 6 Semiregioselective strategies using Trt- and Acm-protecting groups for Cys.

oxidation of the first two disulfide bridges, the quality of this intermediate was very low, and the strategy was abandoned.

I_2 oxidation can be improved by performing cyclization at low temperature and slowly folding the peptide. For this purpose, in the first four strategies, 0.5 m*M* of the peptide was dissolved in AcOH/H_2O (4:1) and I_2 was added dropwise for 3 h at 0°C. Peptides were further reacted overnight at room temperature. In all four cases, no improvement in purity was observed.

[2 Mmt +2 Acm +2 Trt] and [2 Acm +2 Trt +2 pMeOBzl]: On-Resin Versus Solution Disulfide Formation. The Acm group was also used in two total regioselective strategies. One of these used the Mmt and the Trt groups for the other Cys pairs while the other used the Trt and the *p*MeOBzl groups. When used in conjunction with Wang resin, the Mmt group is compatible with the Trt group and allows the formation of on-resin disulfide bridges. Removal of the Mmt from a peptide-CTC resin was accompanied by partial cleavage of the peptide. With the peptide [Mmt (1-6), Acm (2-10), Trt (5-13)] on Wang resin, and after selectively and carefully removing the Mmt groups with 1% TFA to prevent premature cleavage of the Trt groups, several conditions were tested to oxidize the free thiols. These included the following: (a) 5% DMSO in DMF-phosphate buffer (4:1); (b) Et_3N (6 equiv) in N-methyl-2-pyrrolidone (NMP) with air bubbling; (c) red. glutathione/ox. glutathione (10:1) in DMF-0.2*M* Tris-HCl, 1 m*M* EDTA buffer pH 8.0 (4:1); and (d) CCl_4 (2 equiv), Et_3N (3 equiv) in NMP. The reactions were monitored by analysis of resin-bound thiol groups[26] and analytical HPLC following minicleavage. No on-resin disulfide formation was observed in any case after 10 days and, consequently, the Mmt group was replaced by the *p*MeOBzl group, and all cyclizations were performed in solution.

Therefore, for the strategy with Acm, Trt, and *p*MeOBzl groups, two combinations were assayed. In both approaches, Cys1 and Cys6 were protected with the Acm group, while the difference was in the position of the other two protecting groups (see Figure 7).

After completing the linear assembly of the peptides on 2-CTC resin, the cleavage conditions were studied. When a two-step cleavage was performed [TFA-TIS-CH_2Cl_2 (1:5:94), 5 × 30 s to cleave the peptide from the resin, then TFA-TIS-CH_2Cl_2 (10:5:85) (1 × 15 min) to remove the Trt group], partial cleavage of the *t*Bu groups was observed. Better results were obtained when the cleavage was performed directly from the resin with TFA-TIS-CH_2Cl_2 (10:5:85) (1 × 15 min). The peptide was then evaporated, precipitated with Et_2O, dissolved with acetonitrile (ACN)-H_2O (1:1) and lyophilized. In this case, a clean chromatogram with a major peak with the correct mass was obtained.

When the peptide obtained by strategy (vi) was subjected to oxidation (100 m*M* sodium phosphate, 2*M* Gdn · HCl, pH 7.0, 5% DMSO), 9 days were necessary to completely oxidize the Cys residues. When the oxidation was carried out in the presence of ACN [H_2O/ACN (3:2), pH 8.0 (dil. NH_3), 5% DMSO], oxidation was completed in 36 h (Figures 8a–8c, reduced peptide at 8.14 min, oxidized peptide at 7.87 min);

vi) H-Cys(**Acm**)-Cys(**Trt**)-Glu-Tyr-Cys(***p*MeOBzl**)-Cys(**Acm**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(***p*MeOBzl**)-Tyr-OH

vii) H-Cys(**Acm**)-Cys(***p*MeOBzl**)-Glu-Tyr-Cys(**Trt**)-Cys(**Acm**)-Asn-Pro-Ala-Cys(***p*MeOBzl**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

FIGURE 7 Strategies using *p*MeOBzl-, Trt-, and Acm-protecting groups for Cys.

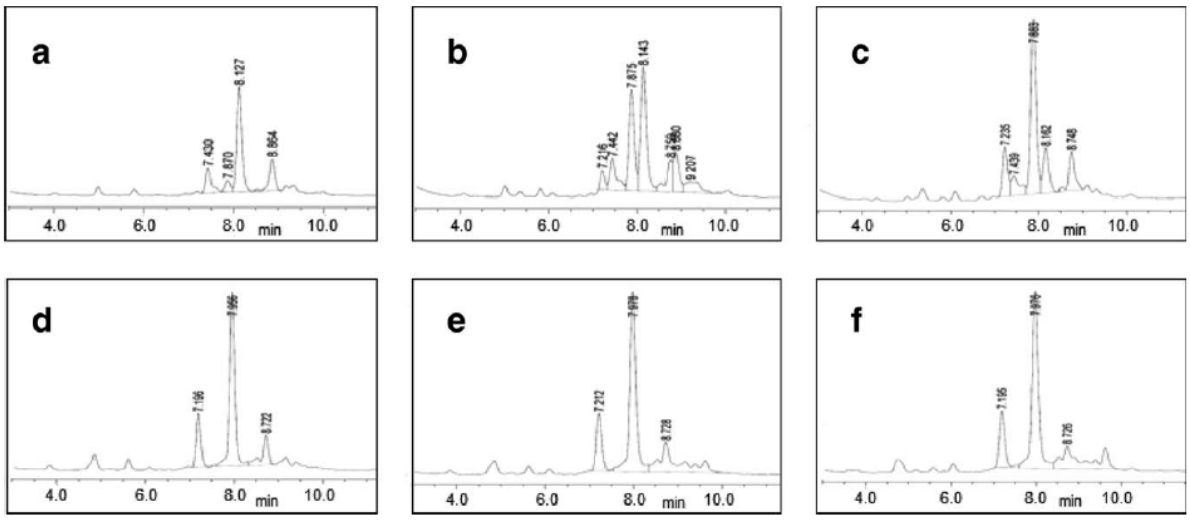

FIGURE 8 Oxidation using (H_2O/ACN (3:2), pH 8.0 (dil. NH_3), 5% DMSO): (a) peptide vi, 4 h; (b) peptide vi 24 h; (c) peptide vi 36 h; (d) peptide vii, 4 h; (e) peptide vii, 24 h; and (f) peptide vii, 36 h; gradient: from 30% to 100% ACN over 15 min.

however, a clean chromatogram was not obtained. Even in these more apolar conditions and after 9 days of reaction, the disulfide bridge **Cys5–Cys13** in strategy (vii) was not obtained (Figures 8d–8f, reduced peptide at 7.98 min). As a result of the unsatisfactory results, these two strategies were abandoned.

From these sets of experiments, we conclude the following: (1) the Acm group is not compatible with linaclotide folding; (2) disulfide formation in solution is better suited to on-resin cyclization; and (3) the disulfide bridge **Cys5–Cys13** formation is not favored and should not be the first to be formed.

[2 StBu +2 Trt +2 pMeOBzl] Strategy: StBu Removal on Solid Phase. After discarding the Mmt and Acm groups, the *St*Bu group was considered in the design of a regioselective strategy for linaclotide that also includes the Trt and *p*MeOBzl groups. In this synthetic scheme, after completing the linear assembly of the peptide, the *St*Bu group was removed on resin but the following sequence of steps were performed in solution. To study whether a cyclization sequence was more favored than the others, the three possibilities listed below were synthesized (see Figure 9).

After synthesizing the linear peptides, solid-phase conditions to remove the *St*Bu group were studied. First, a solution of PBu_3 (100 equiv) in DMF-H_2O (4:1) was applied but after several days of reaction, no deprotection was observed. Neither was any change observed when using a solution of 20% mercaptoethanol in DMF.[27] These conditions were also studied in solution with no success. Finally, with the addition of *N*-methylmorpholine to the solution (20% mercaptoethanol, 0.1*M* *N*-methylmorpholine in DMF), the *St*Bu groups were cleanly removed on solid phase in 16 h. The sequence of steps is shown in Figure 10 for strategy (ix). After removing the *St*Bu groups, treatment with a low acid-content cleavage cocktail detached the peptide from the resin, and the first cyclization was carried out in solution in DMSO conditions. Successive removal of the Trt groups followed by cyclization, one-pot cleavage of the *p*MeOBzl group and cyclization with TFA-DMSO-anisole (94:5:1) at 60°C for 1 h, gave the final peptide.

In strategy (viii) *St*Bu removal was achieved easily, but it was difficult to obtain the disulfide bond (**Cys5–Cys13**), as seen before in the [2 Acm +2 Trt +2 *p*MeOBzl] strategy. The reaction was not complete, and no change was observed in the chromatographic profile some days later. Therefore, this strategy was abandoned.

As for strategy (ix), *St*Bu removal was straightforward and, in this case, the disulfide bridge (**Cys1–Cys6**) was easy to obtain. The reaction was nearly complete (90% conversion) after 1 day. However, after carrying out the two remaining cyclization steps, a mixture of several peptides of the completely oxidized peptide was obtained.

Finally, in strategy (x), high temperature and more time were needed to remove the *St*Bu groups. In contrast, the **Cys2–Cys10** disulfide bond was possible to obtain, although rather slowly. The reaction was complete after 60 h. Nevertheless, as in the previous strategy, a complex mixture of completely oxidized peptides was obtained.

On the basis of the results of these studies, we conclude that linaclotide does not lend itself to a total regioselective

viii) H-Cys(***p*MeOBzl**)-Cys(**Trt**)-Glu-Tyr-Cys(***St*Bu**)-Cys(***p*MeOBzl**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(***St*Bu**)-Tyr-OH

ix) H-Cys(***St*Bu**)-Cys(**Trt**)-Glu-Tyr-Cys(***p*MeOBzl**)-Cys(***St*Bu**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(***p*MeOBzl**)-Tyr-OH

x) H-Cys(**Trt**)-Cys(***St*Bu**)-Glu-Tyr-Cys(***p*MeOBzl**)-Cys(**Trt**)-Asn-Pro-Ala-Cys(***St*Bu**)-Thr-Gly-Cys(***p*MeOBzl**)-Tyr-OH

FIGURE 9 Regioselective strategies using *p*MeOBzl-, Trt-, and S*t*Bu-protecting groups for Cys.

strategy. Although the **Cys1–Cys6** pair is independent from the other two disulfides and is formed easily and may be an intermediate on the folding pathway of linaclotide, the remaining two bridges fold simultaneously. Other information that can be extracted from these experiments is that the removal of the *St*Bu groups can be jeopardized as a result of the proximity of hindered protecting groups. In strategy (x), where the removal of the *St*Bu group was difficult, a Cys(Trt) residue is located next to the sequence. Since the best results were achieved with strategy (ix), we attempted a semiregioselective method with the *St*Bu and Trt groups to see whether the kinetics of the folding would be more favored.

Semiregioselective Strategy: [2 StBu +4 Trt]. Two strategies were studied in which the *St*Bu groups were positioned in Cys1–Cys6 or Cys2–Cys10 (see Figure 11).

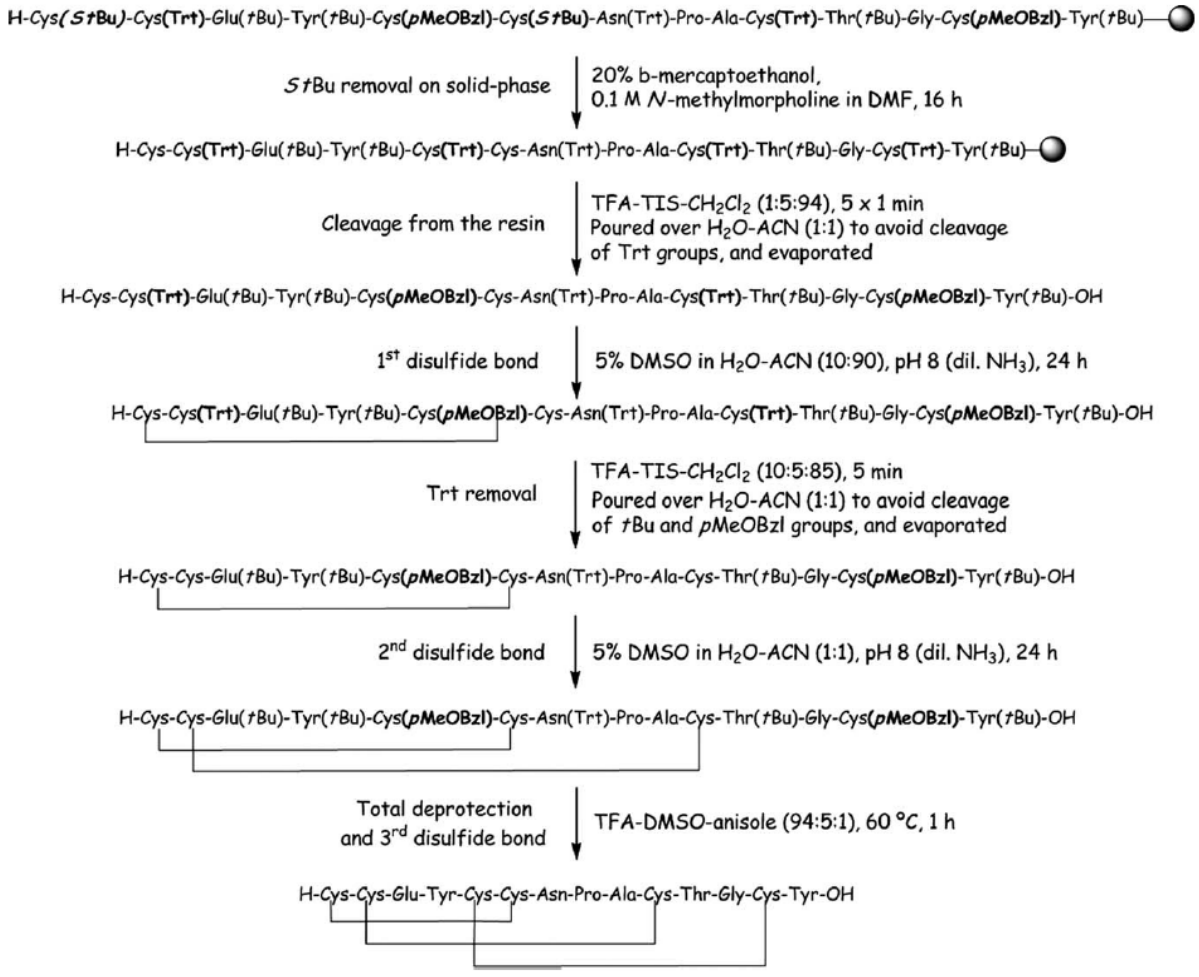

FIGURE 10 Synthetic scheme for linaclotide using S*t*Bu-, Trt-, and *p*MeOBzl-protecting groups for Cys.

xi) H-Cys(***St*Bu**)-Cys(**Trt**)-Glu-Tyr-Cys(**Trt**)-Cys(***St*Bu**)-Asn-Pro-Ala-Cys(**Trt**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

xii) H-Cys(**Trt**)-Cys(***St*Bu**)-Glu-Tyr-Cys(**Trt**)-Cys(**Trt**)-Asn-Pro-Ala-Cys(***St*Bu**)-Thr-Gly-Cys(**Trt**)-Tyr-OH

FIGURE 11 Strategies using *St*Bu- and Trt-protecting groups for Cys.

After completing the linear elongation, the *St*Bu groups were removed on solid phase by treatment with 20% mercaptoethanol and 0.1*M N*-methylmorpholine in DMF (see Figure 12). The peptide was then cleaved from the resin with 1% TFA to prevent premature cleavage of the Trt groups. In this regard, the cleavage mixture was poured over H_2O and the organic phase was evaporated under N_2 to prevent an increase in TFA concentration. Finally, the peptide was dissolved in ACN-H_2O (1:1) and lyophilized. With the peptide in hand, DMSO oxidation was attempted.

When *St*Bu groups were in the **Cys2–Cys10** positions, they could not be removed. This finding confirms that *St*Bu removal depends not only on its position in the sequence,[28] but also on the protecting groups of the nearest residues. (This phenomenon has been previously observed.) When removal of the *St*Bu groups was carried out at high temperature, poor recoveries were obtained as a result of premature cleavage of the peptide from the resin.

In this approach, the best results were achieved with strategy (xi). Despite being located in the **Cys1–Cys6** positions, the *St*Bu groups were more difficult to remove in this case (4 days were required) than in strategy (ix) of [2 *St*Bu +2 Trt +2 *p*MeOBzl] (see Figure 13).

After performing the first cyclization in solution, removal of the Trt groups and total deprotection, and final oxidation of the two remaining disulfide bonds, a main peak with the correct mass was obtained (Figure 14a). This peak coeluted with the oxidized peptide obtained from the [6-Trt] strategy (Figure 14b).

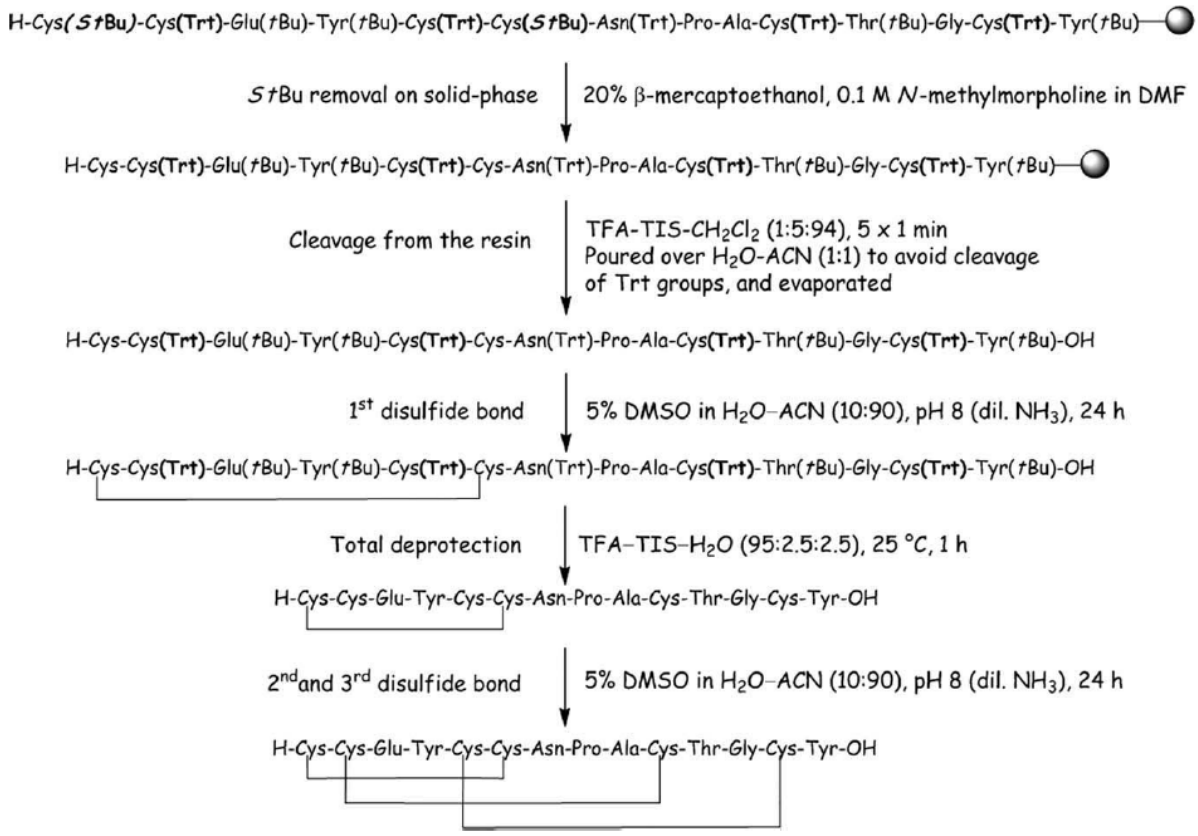

FIGURE 12 Synthetic scheme for linaclotide using *St*Bu- and Trt-protecting groups for Cys.

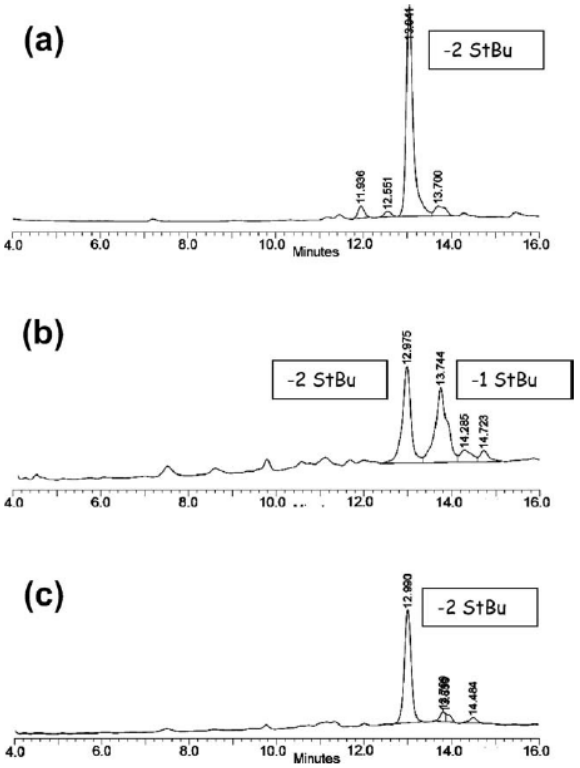

FIGURE 13 S*t*Bu removal in the (a) [2 S*t*Bu +2 Trt +2 *p*MeOBzl] strategy (ix), 12 h, gradient: from 50% to 100% ACN over 15 min.; (b) [2 S*t*Bu +4 Trt] strategy (xi), 12 h, and (c) [2 S*t*Bu +4 Trt] strategy (xi), 4 days, gradient: from 70% to 100% ACN over 15 min.

CONCLUSIONS

Here we synthesized linaclotide following the [6 Trt] and the [2 S*t*Bu^{1,6} + 4 Trt] strategies. Other semi- and regioselective strategies failed or were found to be less suitable. The disulfide bond analysis of the product obtained with the [6 Trt] strategy confirmed that the isomer (Cys1–Cys6, Cys2–Cys10, and Cys5–Cys13) is the major product in both syntheses. Analysis of the 14 strategies tested leads to the following conclusions, which can be extrapolated to other Cys-rich peptides.

(i). The synthesis of peptides, such as linaclotide, with a high density of Cys residues (43% in linaclotide) and side-chain protecting groups (86% in linaclotide) is a challenge.

(ii). The presence of the side-chain protecting groups can jeopardize semi- and regioselective syntheses, because some disulfide formation in these strategies is carried out on the fully protected peptide. Thus, the *St*Bu, whose removal is not always straightforward, can be easily removed from position 1. Removal of the *St*Bu group from Cys6 is easier in strategy (ix) when the protecting group of Cys5 is the *p*-MeOBzl than in strategy (xi), which contains a more hindered Trt for Cys5.

(iii). The Acm group, which is removed by strong conditions, is not suitable for regioselective disulfide bond formation of peptides, such as linaclotide, that have a well-defined structure.

(iv). To perform regioselective synthesis and avoid problems associated with strategies (ii) and (iii), Cys-protecting groups that are easy to remove in mild or controlled conditions should be developed.

(v). In regioselective approaches, it is fundamental to choose the order of Cys residue pairing. Thus, in linaclotide, the Cys1–Cys6 disulfide is the most favored bridge and should be tackled first.

(vi). Oxidation in solution for this kind of peptide is recommended over solid phase.

MATERIALS AND METHODS

CTC resin was from CBL (Patras, Greece), HCTU from Luxembourg Industries Ltd. (Tel Aviv, Israel), and protected Fmoc-amino acid derivatives from IRIS Biotech (Marktredwitz, Germany). Wang resin was from Novabiochem-Merck Biosciences (Darmstadt, Germany). 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), guanidinium hydrochloride (Gdn·HCl), and tris-(2-carboxyethyl) phosphine (TCEP) hydrochloride were from Sigma-Aldrich (St Louis).

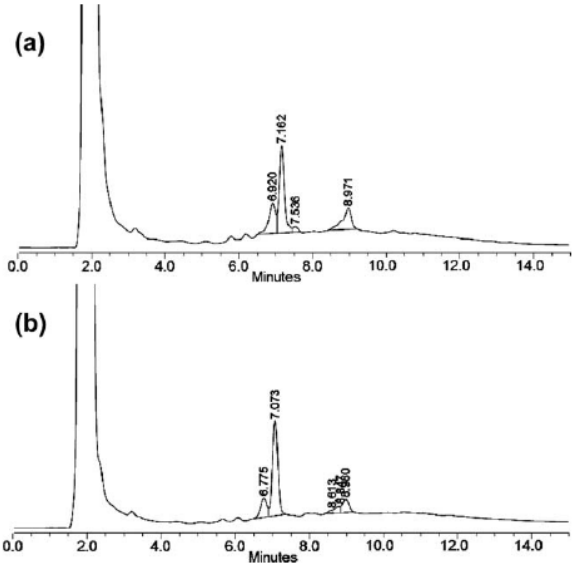

FIGURE 14 (a) Fully oxidized linaclotide from the [2 S*t*Bu +4 Trt] strategy (xi), and (b) coelution with the fully oxidized peptide obtained from the [6-Trt] strategy. Gradient: from 15% to 45% ACN over 15 min.

Solid-phase syntheses were carried out either in glass funnels fitted with a filter or in polypropylene syringes (50 mL) fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling, and, once again deprotection were carried out with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) using 10 mL solvent/g resin each time. Peptide synthesis transformations and washes were performed at 25°C. Syntheses carried out on solid phase were controlled by HPLC of the intermediates obtained after cleaving an aliquot (~2 mg) of the peptidyl resin with TFA-TIS-H_2O (95:2.5:2.5) for 1 h. HPLC reversed-phase columns XTerra® MS C_{18} 4.6 × 150 mm, 5 μm and Symmetry® C_{18} 4.6 × 150 mm, 5 μm were from Waters (Ireland). Analytical HPLC was carried out on a Waters instrument comprising two solvent-delivery pumps (Waters 1525), an automatic injector (Waters 717 autosampler), and a dual wavelength detector (Waters 2487), at 220 nm, and linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were used and are specified in each case. HPLC-ESMS was performed on a Waters Micromass ZQ spectrometer. Linear gradients of ACN (+0.07% formic acid) into H_2O (+0.1% formic acid) were used.

Incorporation of the First Amino Acid on CTC Resin

Fmoc-Tyr(*t*Bu)-OH (1 equiv) was dissolved in CH_2Cl_2 (15 mL/g resin), and DIEA (10 equiv) was added to the resin. The reaction was shaken at 25°C for 1 h. Next, the resin was capped by the addition of MeOH (0.8 μL/mg resin) for 10 min at 25°C, and the resin was washed with CH_2Cl_2 (5 × 1 min) and with DMF (5 × 1 min). Fmoc quantitation gave a loading of 0.72 mmol/g.

Removal of the Fmoc Group

The Fmoc group was removed with piperidine-DMF (1:4) (25 mL/g resin) (1 × 1 min, 2 × 10 min).

Incorporation of Fmoc-AA-OH

(Fmoc-Gly-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Glu(*t*Bu)-OH). Fmoc-AA-OH (3 equiv), HCTU (3 equiv), DIEA (6 equiv) in DMF (15 mL/g resin) were incorporated with preactivation for 30 s at 25°C and left to stand at 25°C for 1 h. No recoupling was required.

Incorporation of Fmoc-Cys(R)-OH

Fmoc-Cys(R)-OH (3 equiv), DIPCDI (3 equiv), and HOBt (3 equiv) in DMF (15 mL/g resin) were preactivated for 5 min at 25°C and then added to the resin and left to stand at 25°C for 1 h. No recoupling was required.

Cleavage and Total Deprotection

After CH_2Cl_2 washing, the resin was first cleaved using 1% TFA in CH_2Cl_2 (5 mL ×5 × 30 s) at 25°C. The mixture was evaporated completely and immediately treated with TFA-TIS-H_2O (95:2.5:2.5; 25 mL) at 25°C for 1 h.

After global deprotection, the resulting solution was evaporated to 5 mL and the crude peptide was precipitated with precooled Et_2O (20 mL). The solid was washed with precooled Et_2O (5 × 20 mL), dissolved in ACN-H_2O (1:1) (25 mL), and lyophilized.

Oxidation

Different oxidation conditions were tested: (a) air oxidation: the peptide (3.0 mg) was dissolved in 100 m*M* NaH_2PO_4, 2 m*M* Gdn · HCl buffer at pH 7.0 (6 mL) and subjected to air oxidation for 12 h; (b) DMSO oxidation: the peptide (3.0 mg) was dissolved in 100 m*M* NaH_2PO_4 and 2 m*M* Gdn · HCl buffer at pH 7.0 (6 mL) containing 5% DMSO and shaken for 12 h; (c) red glutathione oxidation, condition 1: the peptide (3.0 mg) was dissolved in a solution of 2 m*M* red gluthathione in 100 m*M* $(NH_4)_2SO_4$-isopropanol buffer (1:1) at pH 8.5 (6 mL) and shaken for 12 h; and (d) red glutathione oxidation, condition 2: the peptide (3.0 mg) was dissolved in a solution of 2 m*M* red gluthathione in 100 m*M* NaH_2PO_4, 2 m*M* Gdn · HCl buffer-isopropanol (1:1) at pH 8.5 (6 mL) and shaken for 12 h. In any case, to remove the salts from the crude peptide, the mixture was eluted by a column that contained Sephadex G-10 medium. The column was equilibrated with H_2O, and the crude peptide was added to the column and eluted with H_2O. Finally, the fractions were lyophilized. The salts were also removed with diaion resin. In this case, 700 mg of diaion resin was equilibrated with 10 mL MeOH for 10 min at 25°C. The MeOH was filtered and the resin was washed with H_2O (3 × 10 mL) and shaken overnight in H_2O (10 mL) at 25°C. Next, 310 mg of the lyophilized crude (salts + peptide) was dissolved in H_2O (15 mL) and the active resin was added and shaken for 1 h at 25°C. The reaction was monitored by HPLC. The mixture was filtered and the resin was washed with H_2O (4 × 10 mL). Finally, the crude peptide was eluted from the resin with H_2O–ACN (1:1) and lyophilized.

Ellman's Test

Ellman's reagent (50 μL) was added to the reaction mixture (50 μL). The solution was then shaken at room temperature for 3 min. The presence of free thiols was indicated by a yellow-orange color.

Example: Solid-Phase Synthesis of Linaclotide Using the [6-Trt] Strategy

CTC resin (0.2066 mg, 0.32 mmol, 1.55 mmol/g) was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Fmoc-Tyr(*t*Bu)-OH (93.3 mg, 1 mmol) and DIEA (345.1 μL) in CH_2Cl_2 were added to the resin, and the mixture was shaken for 55 min. The resin was capped by adding MeOH (165 μL) for 10 min. After washing the resin with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Cleavage of the Fmoc group was effected by treatment with piperidine-DMF (1:4) (25 mL/g resin) (1 × 1 min, 2 × 10 min). The filtrates were collected and quantified by UV (290 nm) obtaining a loading of 0.72 mmol/g. Based on this loading, the following protected amino acids (3 equiv) were incorporated with HCTU (3 equiv) and DIEA (6 equiv) in DMF, as a coupling system with 30-s preactivation, except for Fmoc-Cys(Trt)-OH (3 equiv), which was incorporated using DIPCDI (3 equiv) and HOBt (3 equiv) in DMF with 5-min preactivation to prevent epimerization. Washes between couplings and deprotections were performed using DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). After finishing the elongation, the peptide was cleaved using a two-step cleavage protocol: in a first treatment with 1% TFA in CH_2Cl_2 (5 mL ×5 × 30 s) at 25°C, the protected peptide

was cleaved from the resin. The mixture was then evaporated completely and immediately treated with TFA-TIS-H_2O (95:2.5:2.5), (25 mL) at 25°C for 1 h. The mixture was partially evaporated, precipitated with cold ether, centrifuged, redissolved in H_2O–ACN (1:1), and lyophilized to obtain the crude linear linaclotide (177.6 mg, 81% yield; 65% purity). The crude peptide was subjected to air oxidation in 100 m*M* NaH_2PO_4 and 2 m*M* Gdn · HCl buffer for 12 h. To remove the salts, the crude peptide was dissolved in H_2O (15 mL) and activated diaion resin (prepared as described earlier) was added, and shaken for 1 h at 25°C. The reaction was monitored by HPLC. The mixture was filtered and the resin was washed with H_2O (4 × 10 mL). Finally, the crude peptide was eluted from the resin with H_2O–ACN (1:1) and lyophilized. The peptide was analyzed by analytical HPLC (t_R = 7.20 min; gradient: from 15% to 45% ACN over 15 min). HR-ESMS calcd for $C_{59}H_{79}N_{15}O_{21}S_6$, 1525.3899; found, 1525.3940 [M^+].

Disulfide Bond Analysis

To denature the peptide, 800 μg (0.5 μmol, 1 m*M*) of peptide was dissolved in citric buffer (0.1*M* citrate, 6*M* Gdn · HCl, pH 3, 500 μL), and the mixture was incubated for 20 min at 65°C. Partial reduction of the disulfide bonds was carried out with 0.1*M* TCEP in citrate buffer, (1 equiv/cystine, 5 μL) and the mixture was incubated for 5 min at 65°C. Next, the free cysteine residues were cyanylated with CDAP (2.5 mg, 10.5 μmol, 20 equiv) and the mixture was incubated at 25°C for 30 min. Partially reduced and cyanylated peptides were separated by HPLC (linear gradient of 0% to 5% ACN over 5 min, then 5% to 50% ACN in 15 min), collected, and lyophilized. CN-induced cleavage of the isolated peptides was performed by addition of 1*M* NH_4OH, 6*M* Gdn · HCl, pH 12 for 1 h at 25°C. Excess of NH_4OH was removed by lyophilization. The resulting fragments were completely reduced by incubation with TCEP (0.1*M*) for 30 min at 37°C.

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CHAPTER 2

Cys-protecting groups

Chapter 2. Introduction

Peptide as therapeutics

The multifaceted roles of disulfide bonds

Peptide as Therapeutics

The multifaceted roles of disulfide bonds

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Abstract

After a long period of lack of interest by a large number of pharmaceutical companies, peptides as therapeutics have re-emerged with force during the past years. Particularly, constrained peptides have generated great expectation as prospective drug candidates owing to the intrinsic high stability and potency associated with these entities. In this review, we have focused on the multifaceted roles of disulfide bonds as constrained structural elements involved in both oxidative folding and conformational stabilization of peptides, along with the participation of these motifs in the improvement of peptide drug-like properties. Moreover, we have laid special emphasis on small, disulfide-rich peptides, privileged architectures with promising pharmacological properties which have gained a foothold as templates in drug design. Finally, we have provided a brief perspective of the major considerations and available tools –strategies and techniques– for the chemical synthesis and successive characterization of disulfide-rich peptides.

The ability of Cys residues to form reversible, covalent intra- and intermolecular disulfide bonds through the side-chain β-thiol group is a distinctive feature of this amino acid. The disulfide bond, after peptide bond itself, is by far the second most widespread covalent bond present in peptides and proteins[1], and one of the most common post-translational covalent modifications that occurs throughout the course of the oxidative folding. These structural elements contribute to the stabilization of the whole three-dimensional arrangement of polypeptides, and therefore, are important for the biological function of these molecular entities. Given the multiple roles played by cystines, we proposed to browse through the most relevant and novel literature reported in recent years regarding disulfide bonds in peptide therapeutics, from their biological role in oxidative folding and structure stability, to their capacity for enhancing drug-like peptide properties. Furthermore, small, disulfide-rich peptides have attracted our attention due to the fact that these naturally occurring molecules have been revealed as privileged structures with promising pharmacological properties, which display high chemical and biological stability. Moreover, some disulfide-rich peptides may address two main unresolved issues for peptides as therapeutics: oral delivery and cell uptake. Additionally, recent studies have also covered the use of some of these structures as scaffolds for peptide drug design and development. Lastly, we pointed out the difficulties that need to be tackled during the chemical synthesis of these complex macromolecules and described the catalogue of the most commonly used synthetic tools, along with the analytical methods and techniques employed for the characterization of multiple-disulfide peptides.

The roles of disulfide bonds in oxidative folding and stability of peptides –and proteins–

Oxidative folding is described as the complex process through which a reduced, unfolded protein or peptide acquires both the formation of the native disulfide bonds along with the proper conformation, thus resulting in the native three-dimensional folded state.[2,3] Disulfide bond formation occurs simultaneously with peptide and

protein folding in both *in vitro* and *in vivo* scenario, thus indicating the importance of these structural elements for the correct course of this process.

The native set of cystines is the outcome of a complex process involving a series of oxidation, reduction and disulfide reshuffling reactions. In general, *in vitro* oxidative folding can be mediated by disulfide-containing small molecules (*e.g.* oxidized glutathione, cystine, cystamine) or electron-accepting reagents (*e.g.* oxygen, H_2O_2, dimethyl sulfoxide) leading to different intermediate species that can render the disulfide bond formation (**Fig. 1**).

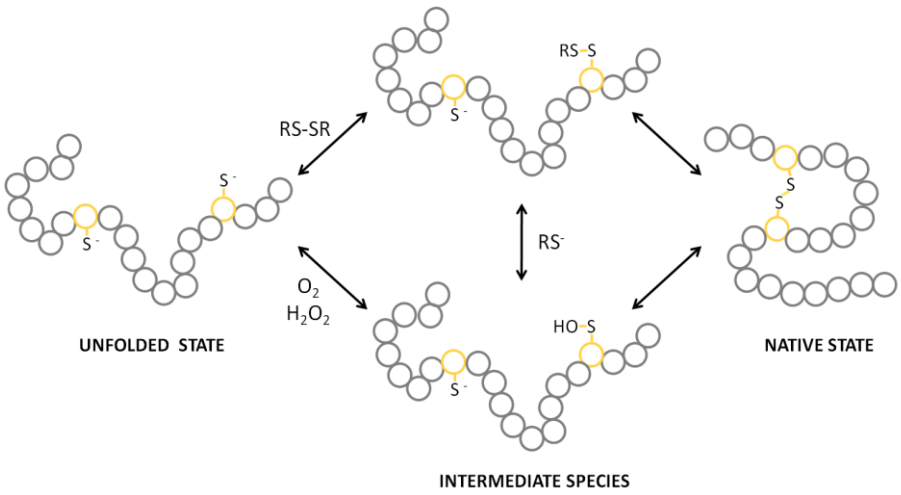

Figure 1. Oxidative folding pathways mediated by disulfide-bond small molecules, or electron-accepting reagents.

Disulfide-mediated oxidative folding is a two-step, reversible process in which two sequential thiol/disulfide exchange reactions are involved. Firstly, the reactive Cys thiolate group attacks the disulfide-containing molecule leading to the formation of a mixed disulfide species, and the rate of this first step is majorly determined by the reactivity of the thiolate group. Next, the asymmetric disulfide experiences another nucleophilic attack from a second Cys thiolate, and consequently, the initial mixed disulfide bond is broken and a new disulfide bond is built, thus generating the native structure. This second step is thermodynamically associated with the conformational features of the peptide or protein. In contrast, oxidative folding mediated by electron-accepting reagents, starts with the oxidation of Cys thiolate to Cys sulfenic acid[4,5]. The sulphur atom of this oxidized Cys intermediate is sufficiently electrophilic to be

susceptible to nucleophilic attack by another Cys thiolate prompting the formation of a disulfide bond. The final nucleophilic attack in both pathways may come from an intramolecular thiolate group or from a thiolate group of another molecule, thus generating intra- and interchain disulfide bonds, respectively. Furthermore, during oxidative folding of multiple disulfide-containing peptides, an already formed disulfide can experience a nucleophilic attack from another thiolate group of the same molecule leading to a rearrangement of disulfide bridges, the so-called scrambling or reshuffling phenomenon.

To date, many oxidative folding studies have revealed an unexpected diversity of disulfide-folding pathways that are influenced by multiple factors[6,7]. Overall, four major factors lead the course of the oxidative folding process, namely, the proximity, reactivity and accessibility of the thiol groups and the intermediate species –either the mixed disulfide or the Cys sulfenic acid–, along with the concentration of the thiolate groups[4]. Nonetheless, the accessibility of the thiol groups to the intermediate species appears to be the most crucial factor, which is determined by the propensity of the peptide backbone to adopt the native conformation.

This process can be kinetically controlled *in vitro* by the thiolate reactivity and by the conformational properties of the peptides, depending on the particular conditions applied. Thus, the concentration of the peptide, the temperature, the ionic strength, the pH, the presence of redox species or electron-accepting reagents and addition of folding additives (*e.g.* surfactants and chaotropic agents such as organic co-solvents, urea or guanidium chloride), are carefully chosen for the proper peptide folding *in vitro*[8] (**Fig. 2**). Disulfide bond formation is mainly dependent on thiolate concentration, and hence, strongly pH-dependent. Thus, oxidation occurs rapidly at pH 9, whereas the process becomes gradually slower at pH values below the pKa (8.7). Moreover, the formation of intra- or intermolecular disulfide bonds is led by the effective concentration of pairs of Cys residues and the total protein concentration in the folding mixture. Therefore, intramolecular disulfide construction requires using a very dilute concentration of peptide (0.001 to 0.1 M) during the oxidative folding course to prevent the accumulation of oligomeric by-products.

In contrast, the folding environment *in vivo* is rather less flexible than in the *in vitro* scenario, and consequently, organisms have developed diverse manners to overcome these limitations. Thus, oxidation of Cys thiolates *in vivo* is assisted by specialized enzymes[9] (*e.g.* endoplasmatic reticulum oxireductin-1 (Ero1), protein disulfide isomerases family (PDI), peroxiredoxin 4 (Prx4), vitamin K epoxide reductase (VKOR), thiol oxidase (Erv2) in eukaryotes, or disulfide bonds enzymes DsbA, DsbB, DsbC and DsbD and analogues thereof in prokaryotes); and mediated by low-molecular-weight oxidants[10] (*e.g.* molecular oxygen, hydrogen peroxide and other reactive oxygen species, ascorbate derivatives, and glutathione) along with changing the electrostatic microenvironment of the reactive groups[11,6] (**Fig. 2**).

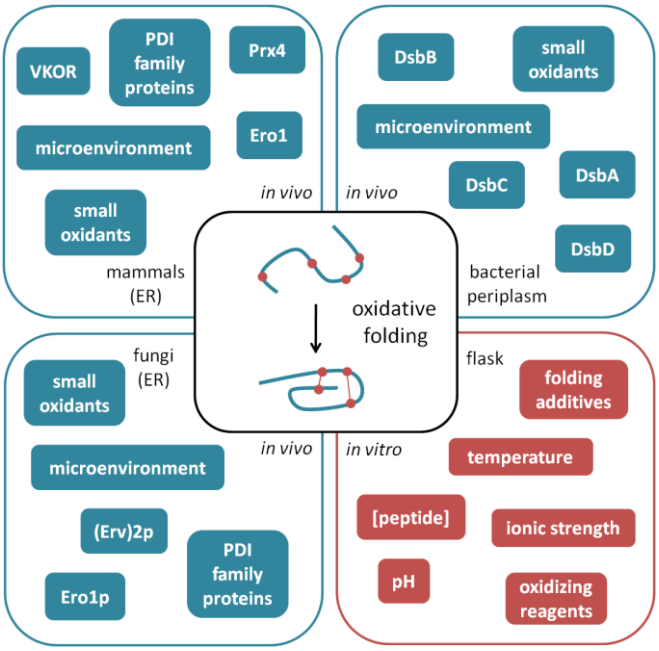

Figure 2. Cell machinery for leading *in vivo* oxidative folding course in mammals, fungi, and bacteria versus oxidative folding factors that govern *in vitro* oxidative folding. (ER = endoplasmatic reticulum)

Disulfide influence on the thermodynamics of the oxidative folding process is exerted by contributing to the stabilization of the native conformation by strongly reducing the entropy of the unfolded form, thus making it less favourable compared to the folded state.[12] According to theoretical studies, by cross-linking distant regions of a polypeptide, disulfide bridges were found to have the ability to decrease the entropy

of the unfolded form, increasing the stability of the native structure in a directly proportional manner to the number of residues between the linked Cys residues. Thus, the larger the number of residues between the Cys, the larger the stability provided to the native structure. Moreover, some studies suggest that the relative position of disulfide bonds directly influenced the kinetics of the protein folding[13]. Therefore, disulfides present in or near the folding nucleus may accelerate the process; whereas those located far from the folding nucleus may slow down the progression of the process up to three-times. In addition, some researches propose that enthalpic effects may also influence the capacity of disulfides to stabilize peptide and protein structures. In this regard, the presence of disulfide bonds may destabilize a denatured peptide state by sterically inhibiting certain potential hydrogen bonding groups from forming satisfied donor-acceptor pairs. Nevertheless, although frequently overlooked, solvation phenomena may also add enthalpic and further entropic effects to proteins under a physiological environment[12].

In addition to keeping protected the nucelophilic β-thiol groups of Cys residues, disulfide bridges are required not only for folding but also for stability and function. According to their function, cystines have been classified as structural, catalytic, or allosteric bonds. Thus, these structural elements are crucial in oxidative folding and contribute to the stabilization of the native conformation of peptides and proteins, besides maintaining peptide integrity by conferring resistance to oxidant agents and proteolytic enzymes. Owing to the reductive environment in the intracellular compartments, disulfide bridges are frequently present in many natural secreted peptides and in these peptides and proteins located on the cell surfaces. On the contrary, catalytic bonds, located in the active site of enzymes (oxidoreductases and isomerases), are involved in thiol/disulfide-exchange systems[14,15,16], while allosteric bonds regulate conformational changes, and therefore protein function, acting as potential redox switches[17]. Furthermore, some natural disulfide linkages are suggested to be strategically located to prevent amyloidogenic regions of peptides and proteins from exploring particularly aggregation-prone conformations[18,19], whereas some other disulfide linkages may be required for protein polymerization[20,21].

Cyclisation through disulfide bond formation for enhancing drug-like peptide properties

Amid many other modifications, cyclisation of peptides is commonly accomplished to improve pharmacological properties and to enhance the drug-like features of their linear counterparts by reducing the available conformational space[22]. Peptide backbone cyclisation can be engineered through head-to-tail amide bond, side chain amide bond, or disulfide bond formation, among others. In nature, the most common way to reduce the conformational flexibility of peptides and proteins is attained by cyclisation through disulfide bond between two Cys residues. Given that disulfide bonds, as mentioned previously, preserve the whole macromolecular conformation, and are crucial for the stability and biological activity of peptides, these structural elements are often found in peptide therapeutics. For instance, insulin, vasopressin, oxytocin, octeotride, calcitocin, terlipressin, and eptifibatide have been in the market for years, whereas several new disulfide-containing peptide therapeutics have been launched after the year 2000 (**Table 1**).

Owing to the main role of disulfide bonds for controlling protein structure, cystines have become an excellent tool to improve drug-like peptide properties. Disulfide bonds can lock the peptide structure into the bioactive conformation, thus enhancing the specificity and potency. Besides, these motifs can lead to a constrained structure which may elevate stability against an extreme harsh chemical and proteolytic environment. Thus, frequently constrained disulfide-containing peptide analogues are significantly more stable by reducing enzymatic degradation, and exhibit enhanced specificity toward a precise target, even discriminating against subtype targets. However, the specific positions of the additional Cys residues should be carefully selected and designed considering spatial requirements in order to prevent both any dramatic perturbation in the native conformation and any considerable decrease in the oxidative folding efficiency[23]. Introduction of disulfide bonds to improve peptide properties is not always a successful strategy; however, many cyclic disulfide peptide analogues have been reported in the literature with varying degrees of success, demonstrating how challenging this task can be. Besides, cyclic disulfide-rich peptides

have been applied as promising scaffolds for installing active sequences in drug design[24].

Drug name	Brand name	Disulfide bonds	Company	Indications	Approval year
atosiban	Tractocile	1	Ferring	premature labor	2000
nesiritide	Natrecor	1	Scios	heart failure, congestive	2001
ziconotide	Prialt	3	Elan	sever and chronic pain	2004
pramlintide	Symlin	1	Amylin	diabetes types 1 and types 2	2005
romidepsin	Istodax	1	Celgene	cutaneous T-cell lymphoma	2009
peginesatide	Omontys	2x1*	Affymax	anemia, renal failure	2012
linaclotide	Linzess	3	Ironwood	chronic constipation, IBS-C	2012

Table 1. Small, disulfide-containing peptide therapeutics approved since 2000. (*A molecule of peginesatide contains 2 copies of a 20-amino acid peptide containing a disulfide bridge each one).

In the last decade, many research groups have explored the insertion of an additional disulfide linkage within peptide sequences for enhancing peptide activity, stabilizing secondary structures, improving specificity, increasing resistance against proteases and elucidating active conformations. Even cyclic disulfide peptides have been applied for grafting active sequences in presentation scaffold constructions (**Fig. 3**). Here, we summarize some representative examples described in the literature during the last years.

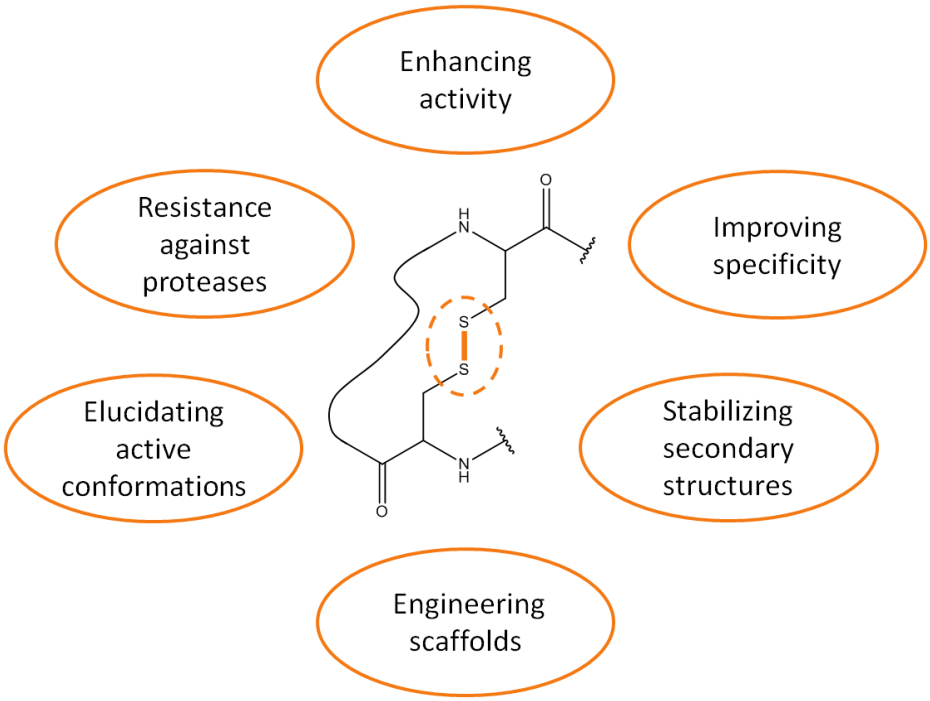

Figure 3. Peptide backbone cyclization through a disulfide bond for multiple purposes.

The selected examples covered below have been divided into several different categories; nonetheless, some of them may be contained in more than one of these categories. It is noteworthy that there are many studies on engineered proteins based on the incorporation of additional disulfide bonds; nevertheless, these are out of the scope of the present manuscript and have not been covered. Moreover, due to the crucial role of disulfides oxidative folding pathway and aggregation processes, these structural elements have been also used to study these important biological processes[25,26], although are not discussed in this review.

Stabilizing secondary structures

Secondary structures within ligand molecules are supposed to stabilize the pharmacophore sort-motif involved in protein-protein recognition interactions and in ligand-receptor or substrate-enzyme complexes[27]. There are numerous examples in the literature in which an additional disulfide bridge within a peptide sequence has contributed to the stabilization of β-turn[28], β-sheet[29,30] and β-hairpin[31], as well as α-helix structures[32,33]. As a first example, a research study demonstrated that disulfide bonds enabled the stabilization of β-hairpin structure depending on the specific

location of the cystine motif within the β-hairpin structure. Therefore, when the disulfide bond was connecting two hydrogen-bonded facing residues, the effect was small or even destabilizing, whereas when the disulfide bonds were linking non-hydrogen-bonded facing residues, then these contributed to the stabilization of this secondary structure[34]. Disulfide linkages were also applied for the stabilization of a β-sheet dimer with a well-defined structure. In this case, two disulfide bonds acted as staples for the generation of a four-stranded β-sheet structure[35]. A recent study demonstrated that a strategic disulfide bond insertion allows generating a stable parallel β-sheet secondary structure, although the resulting parallel sheet could not propagate beyond the disulfide position[36].

Enhancing activity

As a first example of the improved biological activity resulting from the incorporation of an additional cystine motif is illustrated by peptide homologues derived from the E-F loop region of laminin α chain LG4 module. After identifying the active core sequence EF-1 as responsible for promoting the α2β1 integrin-mediated fibroblast attachment, as well as for inhibiting fibroblast attachment to a recombinant laminin α1 chain LG4-5, this active core was cyclized, utilizing two additional Cys residues at both the N- and C-termini through a disulfide bond. The resulting cyclic peptide significantly enhanced integrin-mediated activity, thereby showing the conformation-dependence of the function[37]. In the same year, a research work revealed that while a linear peptide derived from human acidic fibroblast growth factor (aFGF) was inactive and unable to inhibit the binding of human aFGF to cellular receptors, the cyclic disulfide peptide counterpart exhibited a significant inhibitory activity, demonstrating the potential of reducing the conformational space for developing more active compounds[38]. Newly, the cyclisation through a disulfide bond of the N-terminal sequence of the pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon family, allowed confirming the supposed activity of this sequence as a potent agonist for activating the PACAP receptor PAC1. Owing to the fact that the N-terminus sequence is flexible and disordered, the disulfide bridge not only offered stability but also formed a more fixed conformation, which translated into

higher effects of the cyclic analogue upon stimulating cAMP accumulation *in vitro* compared to the effects of the linear counterpart. In addition, the cyclic analogue was able to effectively activate PAC1 receptor *in vivo* and provided further information to address new therapies for diseases related to PACAP peptides[39]. Recently, the same research group has continued evaluating the potential of this novel small cyclic peptide for the treatment of glaucoma or other neurodegenerative diseases[40].

Enhancing selectivity

Although therapeutic peptides possess high selectivity and limited side-effects, occasionally, the insertion of disulfide bonds for improving the specificity may be desirable. For instance, the constraint of a LXXLL nuclear receptor box peptide sequence was exploited to generate a tight and selective peptide ligand to estrogen receptor α when a disulfide bridge combining D-Cys and L-Cys residues was incorporated between i, i+3 positions. This result was significantly better than that observed when a i, i+4 amide bond was inserted within the same peptide sequence. Furthermore, authors provided evidences of α helical conformation for the cyclic disulfide peptide in both X-ray structure and dichroism experiments, suggesting that the D/L-Cys combination at i, i+3 positions may mimic an idealized α-helix[16]. Later on, another example of enhanced selectivity was described. In this case, arenicin-1 (AR-1), a novel 21-residue antimicrobial peptide containing a single intramolecular disulfide bond, displayed undesirable haemolytic activity against human red blood cells. Thus, authors designed a constrained peptide analogue AR-C containing an additional disulfide bridge, which exhibited a two-fold higher antibacterial activity and less haemolytic activity compared to AR-1. It was suggested that the substitution of Val residues with Cys residues reduced the hydrophobicity and the length of β-sheet strand resulting in reduced haemolytic activity[41]. More recently, the construction of diverse disulfide constrained tripeptide analogues of Ang IV, an angiotensin II metabolite was discussed. Two disulfide-containing analogues displayed high efficiency as inhibitors of insulin-regulated aminopeptidase (IRAP), as well as high selectivity for IRAP over aminopeptidase N (AP-N)[42].

Enhancing stability against proteases

The high susceptibility against proteases is one of the main disadvantages of peptides as therapeutics, and a main issue that is commonly addressed by cyclisation techniques. For example, cyclisation of an indolicidin CP-11, an antimicrobial cationic peptide against Gram-negative bacteria, through a disulfide linkage, exhibited higher protease stability in contracts with the linear parent peptide, thus extending the range of clinical applications for this antimicrobial peptide[43]. Afterward, in a research the resistance against enzymatic degradation of three distinct cyclic analogues of a linear sequence of the glycoprotein D of the herpes simplex virus was evaluated. It was determined that cyclisation by amide or disulfide bond improved the resistance against human serum, although a complete stabilization was only achieved by introducing a thioether link[44]. More recently, several Trp- and Arg-rich antimicrobial sequences were decorated with simple modifications, such as end capping and cyclisation. As expected, linear peptides were more susceptible to degradation than their cyclic analogues. However, satisfyingly, by incorporating a disulfide bridge not only was the serum stability improved, but also the microbicidal activity of these peptides. Moreover, remarkably, this study revealed that the shorter the peptide, the more efficient the constraint of the peptide backbone was to enhance resistance to serum proteases[45]. Another interesting example was described recently for the GLP-1 peptide. In this case, a single Cys residue incorporated into the GLP-1 sequence generated a disulfide bond to form a homodimeric analogue of this peptide that retained the therapeutic effect while, at the same time, prolonging its half-life time[46]. Newly, disulfide-bridged cyclic tetrapeptide endomorphin-1 derivatives have been reported to exhibit enhanced metabolic stability towards enzymatic degradation in human plasma in comparison with the native peptide[47].

Elucidating active conformations

The incorporation of disulfide bond may enable the elucidation of the active conformation of a particular peptide or protein since the inherent flexibility of linear peptides hampers this task. For instance, this approach was successfully applied for studying secretin peptide and G protein-coupled receptor interaction. Thus, owing to

the unfeasibility to have a high resolution NMR or a crystal structure of a peptide binding to an intact family B G protein-coupled receptor, authors attempted to constrain the conformation of the N terminus of secretin analogues by means of an intramolecular disulfide bond. After preparing a series of conformationally-constrained secretin peptides, including a disulfide bridge, one of them exhibited a biological activity similar to that of natural secretin, supporting the relevance of this constraint to its active conformation[48]. On the other hand, more recently, the insertion of an intramolecular disulfide linkage into the sequence of pancreatic polypeptides (PP), peptide YY (PYY) and neuropeptide Y (NPY), was performed to lock their tertiary structure into the PP-fold conformation. The cyclic disulfide analogues prepared exhibited both similar *in vivo* activity to their linear counterpart, and affinity for the Y2 and Y4 receptors, thus suggesting that the PP-fold corresponds to the biologically active conformation of these peptides[49]. Interestingly, these studies may reveal crucial information for elucidating the pharmacophore model for further rational design of analogues, or peptidomimetics. For instance, substitution of disulfide bridges by thioethers constitutes a further step in the process of introduction of disulfide bridges with the idea of conferring a higher stability. Although of increasing importance, this flourishing approach is out of the scope of this manuscript as is the review of selenocysteine and selenoether containing peptides[50,51,52,53,54].

Peptide engineering scaffolds

Based on the fact that grafting or integration of a specific binding amino acid sequence with a known function into a structural framework consists of a well-defined folded peptide or protein, scaffold engineering has emerged as a potent strategy to generate novel affinity macromolecular entities for a specific biomolecular recognition. There are many examples in the literature based on small, disulfide-rich peptides that are discussed in the next section.

As an overview, the research studies cited above confirm the latent potential of disulfide bonds for enhancing drug-like profiles of peptide therapeutic candidates, and demonstrate that these linkages may be an excellent tool for studying the underlying pharmacodynamics and improving the pharmacokinetics of these entities. Therefore, it

is not surprising that, currently, many pharmaceutical companies are exploring constrained peptide –cyclisation– synthetic strategies[55,56] and technologies[57,58] for discovering and developing novel peptide therapeutic candidates.

Small, disulfide-rich miniproteins: privileged, highly constrained architectures

Cys is considered a rather unusual amino acid owing to the occurrence of this residue within mammalian proteins is around 2.26%, and even rarer within proteins from simpler organisms[59]. However, the so-called small, disulfide-rich peptides contain in their sequences more than 10% of Cys residues. These singular peptides are constrained structures composed by few residues –less than 100 amino acids– with a frequent deficiency of a widespread hydrophobic core and limited and unusual secondary structure elements, principally stabilized by two or more disulfide bonds. In these constrained architectures, disulfide bonds act as staples that essentially replace the hydrophobic core, thus enabling the peptide to adopt a particular and well-defined folded structure[60].

According to disulfide three-dimensional structures, which are highly preserved in nature, these molecular entities have been clustered in more than 40 different fold groups[60]. These peptides are predominantly extracellular[61], and many diverse biological functions have been described for these peculiar architectures, including cell communication, and structural and enzymatic roles, although communication is the most prevalent by far. Disulfide-rich peptides involved in signalling routes include hormones, growth factors, pheromones, enzyme inhibitors, and ligands of extracellular receptors, among others. Furthermore, a related set of functions includes tasks of an offensive or defensive nature. Small disulfide-rich peptides, including highly knotted antimicrobial defensin peptides[62], plant-derived cyclotides[63], knottins[64], venom-derived peptides[65] such as conotoxins[66,67], and ST enterotoxins [68], among others, are privileged highly constrained peptides with many therapeutic applications[60] (**Fig. 4**).

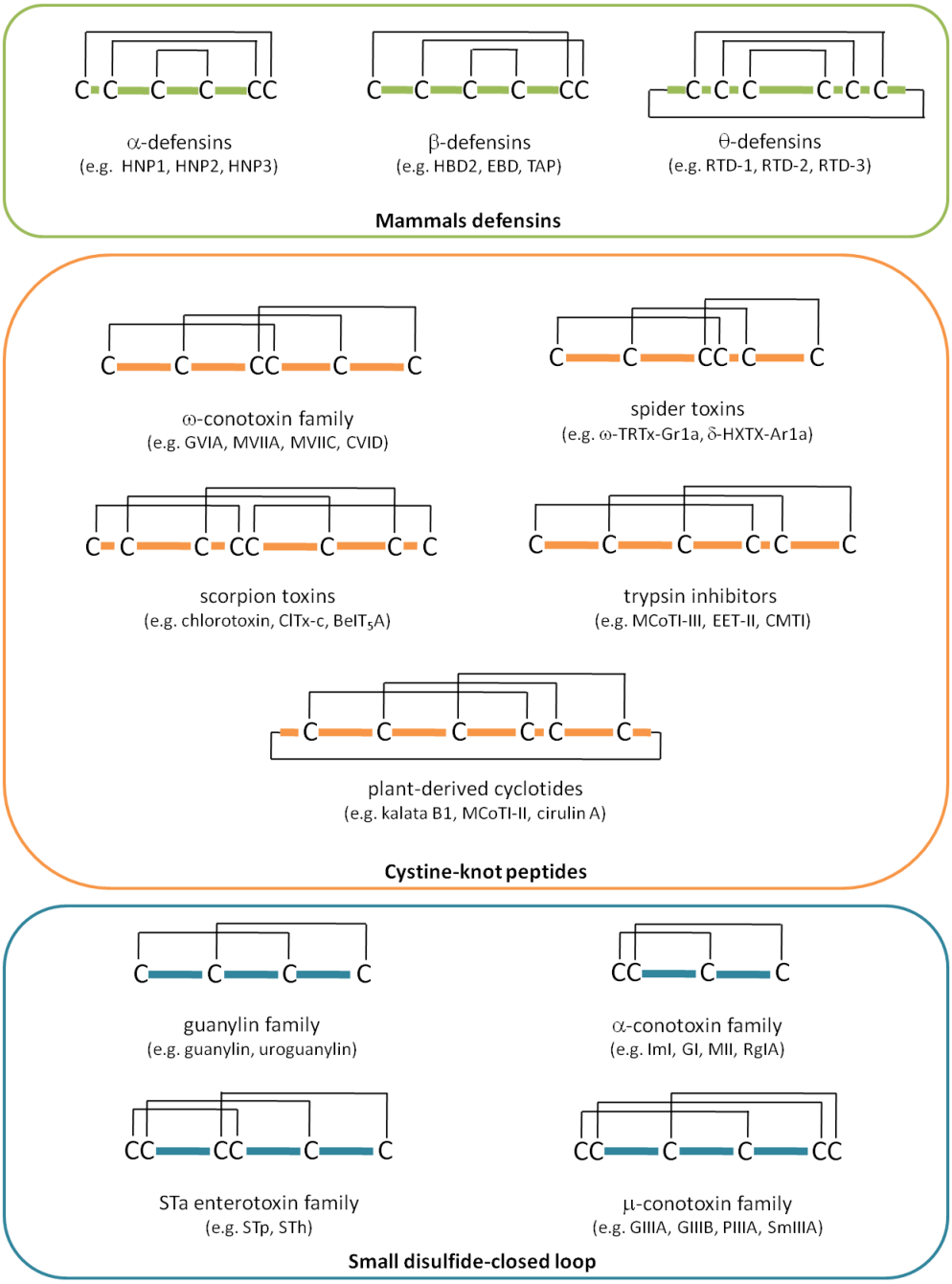

Figure 4. Disulfide bond patterns of some small, disulfide-rich peptides.

The cystine-knot motif[69] is found in a wide range of peptides and proteins from diverse organisms, and is estimated to occur in up to 40% of disulfide-rich peptides and proteins found in nature[60]. Cystine-knot peptides, also called knottins, have six conserved Cys residues that form a characteristic disulfide topology (I-IV, II-V, III-VI), which confer to these structures a remarkable chemical, thermal and proteolytic stability. This is particularly the case of ω-conotoxins, spider and scorpion toxins,

trypsin inhibitors, and cyclotides, among others. Specifically, cyclotides, a family of plant-derived disulfide-rich peptides, is the unique known class of peptides that combines both this topologically complex cystine-knot motif along with a head-to-tail cyclic backbone, which is known as the cyclic cystine knot (CCK) motif. Although the cyclic backbone contributes to the stability and bioavailability of these singular peptides, a recent study determined that, largely, stability is associated with the disulfide arrangement, but not with the cyclic backbone[70]. These peculiar peptides are ultra-stable structures comprising typically around 30 amino acid residues that, apparently, have defence natural functions[71], although a number of cyclotides have shown pharmaceutically relevant activities in various screening studies, including anti-HIV, anticancer, antimicrobial or anthelmintic activities[72]. Nonetheless, it is commonly assumed that cyclotides are most likely to have a prospective therapeutic value as templates for the grafting of bioactive peptide sequences, rather than being used for their inherent biological activities. Therefore, the features of these privileged architectures, together with the possibility to easily introduce combinatorial variation within these sequences, make cyclotides promising scaffolds for designing and developing peptide-based therapeutics. Although, no cyclotide-based drugs have reached clinical trials yet, it is expected that future grafted peptides using these CCK motifs with potential therapeutic applications will emerge in coming years[73].

Novel therapeutic agents targeting protein-protein interactions frequently require larger interaction sites that are difficult to be addressed with small molecular entities. That fact, along with the high specificity, potency and low toxicity associated with peptides, has prompted peptide-based therapeutic agent development during the last decades, although poor chemical and biological stability are the two main drawbacks of peptides as therapeutics. Small, disulfide-rich peptides have emerged as prospective drug candidates addressing the major limitation of peptides as therapeutics, namely their chemical and biological stability. Thus, the constrained structures of these privileged peptides enhance their chemical and biological stability by maintaining the native structure even under extreme pH and temperature conditions, and diminish the susceptible points exposed to enzymatic hydrolysis. In addition, the elevated number of disulfide bonds may reduce the conformational flexibility and fix peptide structure

closer to the bioactive conformation, thereby increasing the binding efficiency and biological activity of these molecules. In turn, this low conformational flexibility increases the specificity of these peptides, which is translated in a reduced promiscuous binding, and therefore, either minimal or no undesired side effects. Thus, it is possible to say that these peptides comprise the pharmacokinetic properties of small molecules, while at the same time exhibiting the potency and specificity of biologics. Ziconoide (Prialt) and linaclotide (Linzess), are two representatives of small, disulfide-rich miniproteins which have reached the market in recent years, while many others are currently undergoing pre-clinical studies or are presently in clinical trials (**Table 2**).

The peptide backbone mostly serves as a scaffold for maintaining the spatial arrangement of amino acid side chains which, in turn, are involved directly in the protein–protein interaction molecular recognition process. Based on that fact, and owing to their constrained structure, it is not surprising that, as mentioned before, some of these particular molecular entities have been used as scaffolds for the optimal presentation of amino acid side chains in a defined three-dimensional arrangement. For instance, the scorpion toxin scyllatoxin was employed as scaffold for the construction and further examination of scyllatoxin-based CD4-mimetic peptides used for viral envelop glycoprotein gp120 recognition studies (HIV-1). This research enabled the authors to determine that although the Phe43 residue in CD4 is important for the high binding affinity in gp120 ligands, it is not essential for prompting conformational isomerization in gp120 involved in the exposure of the binding sites for 17b antibody and CCR5 receptor[74]. Later, the grafting of bioactive peptide fragments comprising poly Arg sequences onto the kalata B1 template as potential anti-angiogenic agents which stabilized VEGF-A antagonists was reported[75]. Moreover, based on trypsin inhibitor-II (MCoTI-II) scaffold, peptides with activity against tryptase and human leucocyte elastase, targets that are involved in many inflammatory diseases were designed[76]. Recently, two different plant-derived disulfide-rich cyclic peptides, MCoTI-II and sunflower trypsin inhibitor-1 (SFTI-1), were used as scaffold for grafting small fragments from laminin and osteopontin, two extracellular matrix proteins, and a sequence from VEGF, which exhibited potent proangiogenic activity. In contrast to the

low stability in human serum of these small sequences, the resultant engineered peptides were significantly more stable in human serum and induced angiogenesis at nanomolar concentration *in vivo*. Thus, this approach made it possible not only to improve the stability, but also to enhance the activity of these fragments[77]. More recently, Craik and coworkers have reported the use of cyclotide kalata B1 for the insertion of the His-Phe-Arg-Trp sequence for the construction of melanocortin receptor 4 (MC4R) agonists. As a result, a grafted peptide was identified that preserved the structural scaffold and exhibited higher affinity for the MC4R than the endogenous agonist, although it resulted to be less potent for activating the receptor. Moreover, the engineered peptide showed high specificity for the MC4C receptor and superior proteolytic stability[78].

Regarding peptides as drug candidates, even when fulfilling the requirement, most peptide drugs must be delivered by injection due to their poor oral bioavailability. Though diminishing, this is considered a drawback of peptides as therapeutics owing to the generally low patient compliance with injection therapies. Orally administered peptides should resist extreme pH conditions and enzymatic hydrolysis throughout the gastrointestinal tract –presystemic conditions–, and should be further absorbed when required. Although not a general property, some of these miniproteins can be orally delivered, this is the case of the already referred peptide linaclotide[79], a modified Vc1.1 conotoxin analogue[80], and two novel engineered BK-peptide analgesics, generated by using kalata B1 framework as scaffold[81]. Even more exciting is the penetrating properties displayed by some of these architectures, including MCoTI-II and kalata B1 cyclotides and sunflower trypsin inhibitor 1 (SFTI-I) peptide[82].

	Drug name	Disulfide bonds	Company	Indications	Phase
in clinical trials	serelaxin (RLX030)	3	Novartis	acute heart failure pre-eclampsia	III I
	plecanatide (SP-304)	2	Synergy	constipation IBS-C*	II/III I
	RPI 78M α-cobratoxin	5	ReceptoPharm	demyelinating diseases herpes virus infection multiple sclerosis	III II II
	χ-CTX MrIA conotoxin (Xen2174)	2	Xenome	pain	II
	RPI MN α-cobrotoxin	4	ReceptoPharm	amyotrophic lateral sclerosis herpes virus infection HIV infection	II II I
	elafin	4	Proteo Biotech	inflammation in esophagus carcinoma	II
	chlorotoxin	4	TransMolecular	glioma	I
	leconotide ω-conotoxin CVID (CNSB004)	3	Relevare Pharmaceuticals	pain	I
	SP-333	2	Synergy	ulcerative colitis	I
in preclinical studies	PcTx1 psalmotoxin	3	Theralpha	chronic pain	preclinical
	APETx2	3	Theralpha	inflammatory pain	preclinical
	NZ2114 plectasin analog	3	Novozyme	antimicrobial	preclinical
	textilinin-1	3	QRxPharma	coagulation disorders	preclinical
	neuropilin 1 antagonist (EG-3287)	2	Ark Therapeutics	cancer stroke	preclinical
	ShK-186	3	Airmid Kineta	multiple sclerosis diabetes type 1	preclinical
	hepcidin (hepc20)	4	INSERM	antibacterial antifungal	preclinical

Table 2. Example of disulfide-rich peptide drugs currently undergoing pre-clinical studies or clinical trials. (*irritable bowel syndrome with constipation)

Synthesis and characterization, a tour de force

To reach the market, peptide therapeutics not only must exhibit excellent pharmacodynamic and pharmacokinetic profiles, but they must also be produced at large scale. Although recombinant technologies and biocatalysis are gaining more popularity, at present, chemical synthesis is the most common way to produce peptides at large scale[83]. As expected, the synthesis of these complex disulfide-rich molecules, along with characterization of their disulfide connectivity, are challenging and demanding tasks. In this regard, the number of possible isomers with different disulfide patterns grows rapidly with the number of Cys residues. Thus, theoretically, for four-Cys-containing peptides, there are three different oxidized isomers; for six-Cys-containing peptides, this number increases up to 15, and for eight-Cys and 10-Cys-containing peptides there are up to 105 and 945 possible disulfide connectivities, respectively.

The success in the synthesis of these particular peptides lies on two main factors namely, suppression or minimization of Cys racemisation during peptide elongation and achievement of the native disulfide bond construction. To address the first point, diverse protecting groups for the β-thiol group of Cys[84], together with precise procedures for Cys incorporation[85,86], have been investigated and applied. To overcome the latter issue, the oxidative folding approach[87] is the most commonly preferred strategy for the synthesis of disulfide-rich peptides[88,89,90,91], although it may result in the formation of native and non-native disulfide bonds depending on the reactivity of the thiolate groups of the Cys and the thermodynamic stability of the native structure. When oxidative folding fails, the presence of different protecting groups for the pairing Cys residues, as well as the implementation of a regioselective protection scheme is required. In this regard, peptide chemists have developed a myriad of suitable protecting groups[92], while sophisticated approaches[93] and semi- and regioselective strategies[94,95] have been designed and successfully applied for the synthesis of disulfide-containing peptides. Owing to the elevated number of Cys residues present in these sequences, the Cys-based native chemical ligation (NCL)[96,97,98] approach is a synthetic strategy to be considered, either to construct long

linear polypeptides –intermolecular NCL– or to generate cyclic backbone –intramolecular NCL–. Specifically, it has been valuable for the synthesis of naturally occurring head-to-tail cyclic cyclotides[99,100], and θ-defensins and for the preparation of disulfide-rich cyclic peptide analogues. What is generally accepted is that there is no universal strategy for the proper synthesis of these disulfide-rich peptides, but there many synthetic tools and strategies that should be carefully selected (**Fig. 5**).

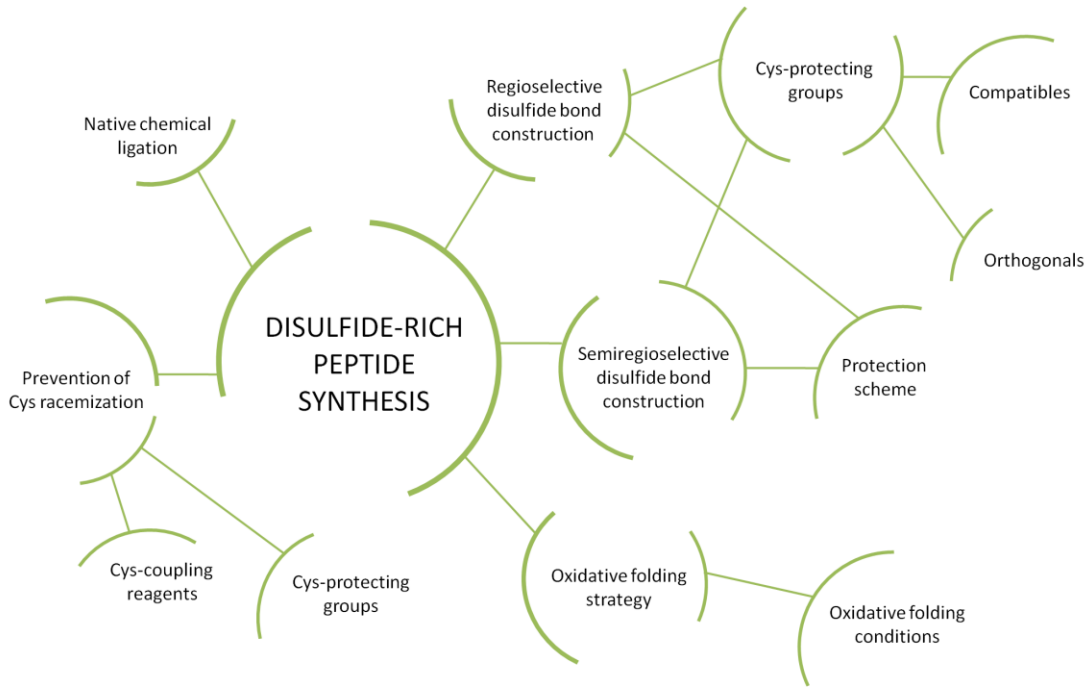

Figure 5. Set of synthetic tools and strategies for overcoming the challenging and demanding chemical synthesis of disulfide-rich peptides.

The identification of peptide disulfide bond arrangement is critical and contributes to the understanding of the structure-function relationship, besides being essential for a rigorous interpretation of bioactivity data. Determination of the disulfide bond arrangement of disulfide-rich peptides, whether synthetic or isolated from natural sources, is often a complex task. Accordingly, many methodologies have been developed for the precise elucidation of the disulfide pattern. Ideally, X-ray crystallography is the method for accurate Cys pairing elucidation. However, peptide crystals are difficult, and, even in some cases, nearly impossible to obtain. On the other hand, it is often complicated by NMR spectroscopy to unequivocally establish the disulfide-bond framework, although the TOCSY experiment for the assignment of

the amino acid spin systems, along with the NOESY experiment for generating distance restraints, have been applied for addressing this issue[101,102]. The most common analytical methods for identifying disulfide bond connectivities are based on chemical or enzymatic fragmentation of the polypeptide while maintaining the cystine bonds intact. The unambiguous assignment following these methods depends on where the disulfide bonds are located in relation to the cleavage points within the polypeptide. In some cases, repetitive or combined cleavage methods are needed, while in some other cases, it is unfeasible to elucidate the disulfide pattern through these procedures. Partial reduction-based protocols[103,104,105] are often useful alternatives. In such cases, tris(2-carboxyethyl)phosphine (TCEP) is employed as a reducing agent in acidic conditions to achieve partial reduction of the disulfide bonds, and then the resulting free-thiol Cys may be alkylated or cyanylated for further fragmentation and analysis. Another analytical method is sequence analysis –Edman degradation–, based on the detection of phenylthiohydantoins (PTH) of either cystine or Cys derivatives. This procedure may be carried out on the whole peptide, although most generally disulfide-containing fragments are subjected to this sort of analysis. Finally, MS spectroscopy analysis is applied in all analytical methods described above for the identification of the diverse resulting fragments. Three main MS techniques are currently being used, fast atom bombardment (FAB-MS), matrix-assisted laser desorption/ionization (MALDI-MS) and electrospray ionization (ESI-MS). In some cases tandem mass spectrometry (MS/MS) is employed as well. Although MS spectroscopy is generally coupled with analytical procedures, diverse methods based on this potent technique have been developed for direct disulfide arrangement elucidation[106,107] (**Fig. 6**).

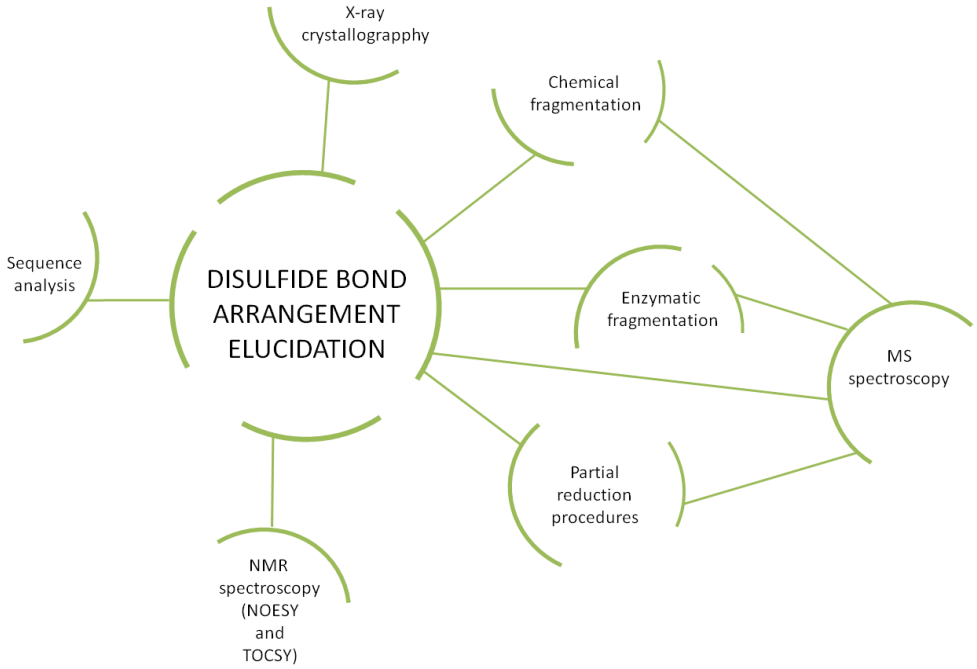

Figure 6. Multiple choices for elucidating the disulfide bond connectivity of small disulfide-rich peptides.

Conclusions and prospects

Here, the versatile roles displayed by the Cys amino acid in therapeutic peptides have been covered, including thiol/disulfide exchange in the oxidative folding process, disulfide contribution into conformational stability of polypeptides and the ability of these linkages for modulating drug-like profiles. Overall, the incorporation of disulfide bridges not only may allow the improvement of diverse pharmacodynamic and pharmacokinetic properties, but may also provide valuable information to understand biological processes and design peptidomimetics. Of special interest are small disulfide-rich peptides, which have also been discussed as valuable therapeutic agents. Together with the increasing significance of these constrained peptides as drugs themselves, these may be used as scaffolds for peptide engineering. Given the important investments of the pharmaceutical industry in constrained peptides, we should expected a great revolution in both scenarios, drug discovery and development of new therapeutics based on disulfide-rich peptides, together with novel and ingenious synthetic strategies for the synthesis of these complex structures in the coming years.

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Competing Financial Interests

The authors declare no competing financial interests.

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Publication II

Eco-friendly combination of the immobilized PGA enzyme and the *S*-Phacm protecting group for the synthesis of Cys-containing peptides

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Eco-Friendly Combination of the Immobilized PGA Enzyme and the *S*-Phacm Protecting Group for the Synthesis of Cys-Containing Peptides

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Abstract: Enzyme-labile protecting groups have emerged as a green alternative to conventional protecting groups. These groups introduce a further orthogonal dimension and eco-friendliness into protection schemes for the synthesis of complex polyfunctional organic molecules. *S*-Phacm, a Cys-protecting group, can be easily removed by the action of a covalently immobilized PGA enzyme under very mild conditions. Herein, the versatility and reliability of an eco-friendly combination of the immobilized PGA enzyme and the *S*-Phacm protecting group has been evaluated for the synthesis of diverse Cys-containing peptides.

Keywords: cysteine · enzymes · green chemistry · peptides · protecting groups

Introduction

The synthesis of complex polyfunctional organic molecules, such as peptides, oligonucleotides, oligosaccharides, and the conjugates thereof, as well as other challenging small compounds, demands the implementation of an efficient protection scheme. The literature describes a myriad of protecting groups, which were usually first developed for peptide chemistry[1] and then rapidly adapted by organic chemists[2] for the pursuit of attractive target molecules. The preparation of polyfunctional molecules, such as peptides, often requires the concourse of a set of orthogonal protecting groups, which are defined as those that are removed in any order by means of a different chemical mechanism.[3] A "plus" for a protecting group is that it should be removable under mild conditions. Nowadays, this term "mild conditions" has a double meaning. Thus, deblocking conditions not only should leave other parts of the molecule unaltered but should also minimize—or avoid—harm to the environment. A paradigm of this ideal protecting group are enzyme-labile protecting groups.[4] These groups offer feasible alternatives to classical chemical methods because they can be removed selectively under mild conditions, thereby installing an additional orthogonal dimension and eco-friendliness in protection schemes.

The amino-acid sequence of a peptide or protein determines its 3D structure and, therefore, its biological activity. In particular, Cys residues are involved in the disulfide-bond scaffold, which is a key structural feature that has been implicated in the folding and structural stability of many natural peptides and proteins. Thus, it is not surprising that Cys is a potential target for incorporating modifications into the peptide structure. For instance, the artificial introduction of extra disulfide bridges into peptides or proteins, thereby allowing the generation of conformational constraints, may improve biological activity, bioavailability, and/or stability. The inclusion of an extra Cys residue is commonly accomplished for further chemoselective derivatization, such as labeling or bioconjugation, by using a variety of electrophilic molecules (haloacetyl groups, maleimides, disulfides, sulfamidates, α,β-unsaturated esters, among others) for many research purposes.[5] Furthermore, the Cys residue is required to perform the native chemical ligation strategy,[6] which is a potent approach for the synthesis of small-to-medium-sized proteins.

Therefore, suitable protection of the nucleophilic thiol group of Cys calls for the preparation of complex Cys-containing peptides, which may require the presence of various Cys-protecting groups for regioselective disulfide-bond formation.

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Over the past few decades, the rapid development of enzyme-immobilization technology has promoted the emergence of enzymatic catalysis in organic synthesis as an attractive and sustainable alternative to the conventional chemical approaches.[7] The activity and stability of enzymes mainly depend on the particular operating and storage conditions and they are strongly influenced by factors such as temperature, pH, ionic strength, and solvent properties. In general, immobilized enzymes have enhanced stability and activity over a broad range of temperatures and pH values, as well as higher tolerance toward organic solvents, compared to soluble enzymes. Furthermore, immobilized enzymes can be easily separated from the reaction media by a simple and rapid filtration, thereby allowing for their reuse and application in continuous processes and in commercial bulk-production processes. Thus, these features lead to significant savings in terms of operating time, enzyme consumption, and production costs by efficient recycling and control of the process.[8]

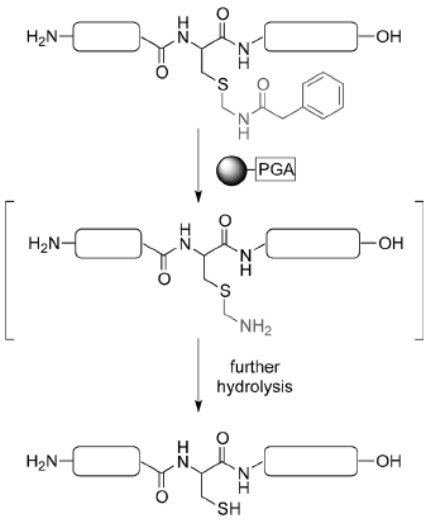
The covalent immobilization of penicillin G acylase (PGA) from *E. coli* (E.C. 3.5.1.1) on an amino-acrylic resin, which recognizes the phenylacetyl moiety, exhibits promising applications in peptide synthesis and opens up new perspectives for further applications.[9] Herein, we explore the power of immobilized-PGA-enzyme catalysis and evaluate several features of this process, including its high efficiency, recyclability, its capacity to operate under mild conditions, and its environmental friendliness, for the synthesis of Cys-containing peptides.

Results and Discussion

Phenylacetamidomethyl (Phacm) is a Cys-protecting group that is compatible with the two major synthetic strategies in peptide chemistry (Boc and Fmoc). Moreover, it can be removed under similar conditions to the acetamidomethyl (Acm) group and, in addition, by the action of the PGA enzyme. Because PGA is selective towards the phenylacetyl moiety, *S*-Phacm can be smoothly deblocked, whilst the resulting thioaminal is hydrolyzed into a free Cys residue (Scheme 1).

Following on from our pioneering work,[10] herein, we examined the combination of immobilized PGA and the *S*-Phacm protecting group for the synthesis of Cys-containing peptides of diverse lengths and structures. The potential scope and limitations of this approach for oxidative cyclization reactions were addressed for peptides with one or two disulfide bridges with various ring sizes, which involved Tyr and Trp residues that were sensitive to oxidation. The reuse of immobilized PGA, as well as the influence of organic solvents, pH, and ionic strength on the biocatalytic reaction, was also evaluated.

S-Phacm as a green alternative to the conventional S-Acm group: The *S*-Acm protecting group can be removed to either generate the free thiol (mediated by Hg^{II} or Ag^{I} salts, followed by extensive thiol treatment) or to directly render a disulfide bond through oxidation (mediated by I_2 or Tl^{III} salts). The requirement of toxic heavy metals and their difficult removal from the crude peptides have made some of these procedures unviable, whilst the iodine-mediated deprotection of *S*-Acm and oxidation has been the cornerstone of numerous successful syntheses in peptide chemistry.[11] Nonetheless, several side-reactions have been reported to occur under *S*-Acm-deblocking conditions, for example, S→N and S→O transfer of the Acm group onto side-chains, the modification of sensitive residues, such as Trp, Tyr, and Met, and the formation of intramolecular tryptophan-2-thioether as a side-product.[12]



Scheme 1. Schematic representation of the removal of *S*-Phacm by the immobilized PGA enzyme.

The removal of *S*-Phacm by immobilized PGA and subsequent disulfide-bridge formation have been thoroughly studied and compared with the commonly used *S*-Acm-protection strategy for the synthesis of the urotensin-related peptide (URP).[13] URP is an eight-residue peptide that contains a disulfide bridge (Cys2–Cys7) and Trp and Tyr residues in its sequence, which are susceptible to side-reactions. Two parallel syntheses of URP, by following the Fmoc/*t*Bu strategy with *S*-Acm- and *S*-Phacm-protection strategies, were carried out. Linear peptides were manually synthesized on a 2-chlorotrityl chloride (2-CTC) resin by using DIPCDI and Oxyma in DMF,[14] with a 5 min pre-activation, for 1 h at 25 °C to incorporate the Cys residues. These conditions assured the absence of racemization.[15] The other amino acids were coupled by using COMU and DIEA in DMF for 1 h at 25 °C.[16]

After peptide elongation on a solid phase, a portion of the peptidyl resin (**1**) from the *S*-Acm strategy was treated three times with I_2 (5 equiv) in DMF at 25 °C for 15 min and the crude peptide was then cleaved from the resin. In contrast, the peptidyl resin from the *S*-Phacm strategy was treated with a mixture of TFA/TIS/water (95:2.5:2.5) for 1 h

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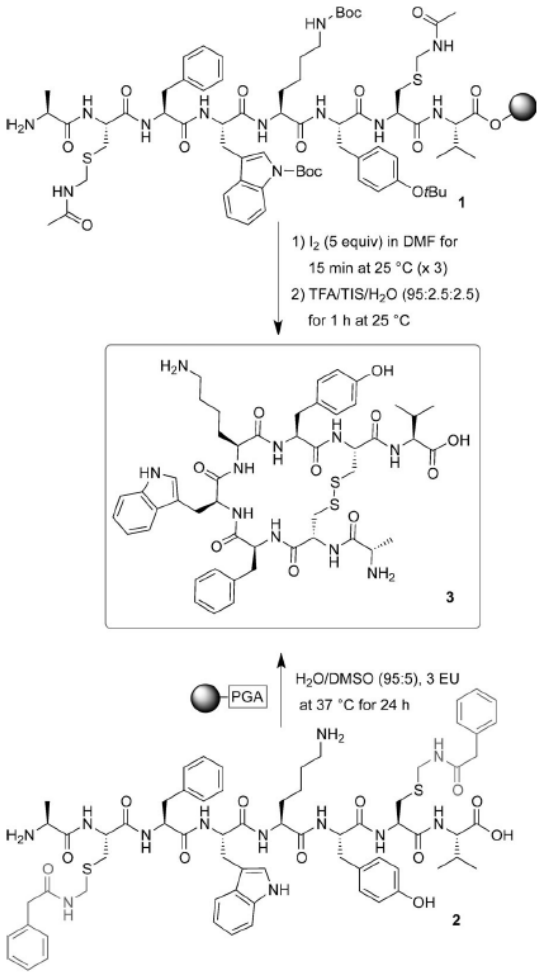

Scheme 2. *S*-Phacm- and *S*-Acm-protection strategies for the synthesis of URP.

at 25 °C and the resulting *S*-Phacm-protected peptide (**2**) was deprotected by immobilized PGA (3 EU) and subsequently oxidized in a mixture of water/DMSO (95:5) at 37 °C for 24 h (Scheme 2).

The quality of the crude oxidized URP product (**3**) by using the *S*-Phacm-protection strategy was clearly superior to that prepared by following the *S*-Acm approach (40% purity by using the *S*-Acm strategy versus 90% purity by using the *S*-Phacm strategy, Figure 1). As expected, a diverse range of side-products were identified by RP-HPLC-MS (ES) analysis of the crude peptide (**3**) that was obtained by using the *S*-Acm strategy. Presumably, some of the extra peaks corresponded to the incorporation of two *S*-Acm groups onto the Trp and Tyr rings (+142 Da), over-oxidation sub-products (+16 and +32 Da), the incorporation of two iodine atoms (+252 Da) combined with over-oxidation (+32 Da), and the formation of intramolecular tryptophan-2-thioether side-products (see the Supporting Information).

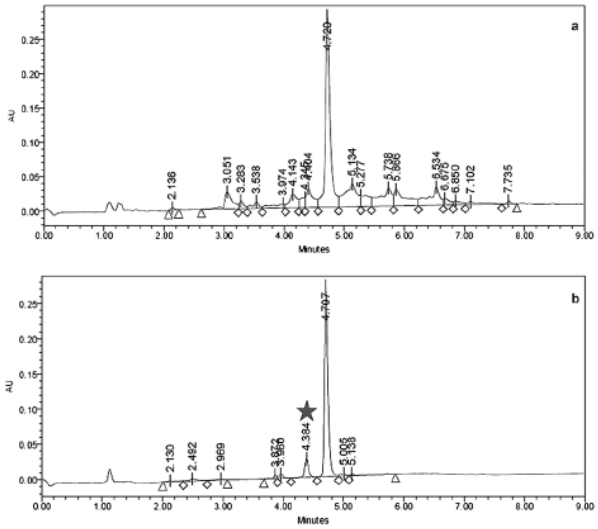

Figure 1. Chromatographic profiles of the oxidized peptide URP (**3**) from the: a) *S*-Acm, and b) *S*-Phacm strategies (phenylacetic acid: ★).

Influence of the co-solvent and pH on the biocatalytic reaction: The use of organic media in enzymatic organic syntheses offers several advantages over enzymatic reactions in aqueous solution. For instance, the solubility of sparingly water-soluble substrates can be greatly enhanced compared to that in water. Thus, the use of co-solvents in aqueous media[17] and the effect of pH were broadly examined for the removal of *S*-Phacm and the further oxidation of URP (Table 1). All of the experiments were carried out by adding immobilized PGA to a solution of the crude peptide (**2**) in the reaction medium and leaving this mixture to stand at 37 °C. The biocatalytic reactions were monitored by reverse-phase HPLC (RP-HPLC).

The enzymatic activity and stability of immobilized PGA remained almost intact in the presence of 5% of an extended variety of organic co-solvents (DMSO, MeCN, DMF, various alcohols, and Et_2O). In some cases, an increase of the ratio of the co-solvent to up to 20% led to a drop in enzymatic activity (MeCN, DMF, EtOH, 2-propanol, and Et_2O), whereas some other co-solvents were well-tolerated by the biocatalyst (DMSO, MeOH, ethylene glycol, and glycerol). Notably, fully oxidized URP (**3**) was obtained when DMSO was present in the reaction media (Table 1, entries 2–3, 23–24, and 28), whilst partially oxidized URP was afforded when either other co-solvents or no co-solvents were used. Therefore, not only is the use of DMSO as a co-solvent better tolerated by immobilized PGA than other organic solvents, but it also promotes the oxidation of thiols to disulfide.

A further advantage of the immobilized enzyme is that it tolerates a relatively wide range of pH values. Although, the optimal conditions were at pH 7.9 in 0.1 mM phosphate buffer/DMSO (95:5; Table 1, entry 23), the use of pH 5.3 also allowed the clean removal of *S*-Phacm (Table 1, entry 28). All of these results demonstrate a high *S*-Phacm-

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Table 1. Influence of the co-solvent and pH on the biocatalytic removal of *S*-Phacm and the further oxidation of URP (**3**).[a]

Entry	Reaction medium	pH	*t* [h]	*S*-Phacm removal [%]	Entry	Reaction medium	pH	*t* [h]	*S*-Phacm removal [%]
1	water	7.0	24	100	15	water/2-propanol (80:20)	7.0	72	10
2	water/DMSO (95:5)	7.0	24	100	16	water/ethylene-glycol (95:5)	7.0	48	100
3	water/DMSO (80:20)	7.0	48	100	17	water/ethylene-glycol (80:20)	7.0	72	100
4	water/MeCN (95:5)	7.0	48	100	18	water/glycerol (95:5)	7.0	24	100
5	water/MeCN (80:20)	7.0	72	10	19	water/glycerol (80:20)	7.0	40	100
6	water/DMF (95:5)	7.0	48	100	20	water/Et_2O (95:5)	7.0	48	100
7	water/DMF (90:10)	7.0	72	100	21	water/Et_2O (80:20)	7.0	72	50
8	water/DMF (80:20)	7.0	72	10	22	0.1 mM phosphate buffer	7.9	16	100
9	water/MeOH (95:5)	7.0	48	100	23	0.1 mM phosphate buffer/DMSO (95:5)	7.9	8	100
10	water/MeOH (80:20)	7.0	72	90	24	0.1 mM phosphate buffer/DMSO (80:20)	7.9	24	100
11	water/EtOH (95:5)	7.0	48	100	25	0.1 mM phosphate buffer/MeCN (95:5)	7.9	16	100
12	water/EtOH (80:20)	7.0	72	45	26	0.1 mM phosphate buffer/MeCN (80:20)	7.9	72	5
13	water/2-propanol (95:5)	7.0	72	100	27	0.1 mM phosphate buffer	5.3	24	100
14	water/2-propanol (90:10)	7.0	72	100	28	0.1 mM phosphate buffer/DMSO (95:5)	5.3	24	100

[a] All experiments were performed at a concentration of 8×10^{-5} M of linear *S*-Phacm-protected URP **2** with 24 mg (3 EU) of SPRIN imibond PGA (hydrolytic activity: 130 $U g_{wet}^{-1}$) at 37 °C. The percentage removal of *S*-Phacm was determined by RP-HPLC analysis.

deblocking efficiency under a wide range of reaction conditions, along with a broad pH-compatible range for the biocatalytic removal of *S*-Phacm by immobilized PGA.

Reuse of the immobilized PGA enzyme: The recycling potential of immobilized PGA was evaluated by reusing the immobilized biocatalyst for the synthesis of peptide URP (**3**) in up to five cycles (for the reaction conditions, see Table 1, entry 2). At the end of each reaction cycle, phenylacetic acid was removed because it is a strong inhibitor of PGA. Therefore, the immobilized enzyme was filtered from the reaction media and then washed three times with a phosphate buffer (20 mM, pH 8.0, three-times the volume with respect to the immobilized PGA), followed by a final washing step with the same reaction medium that was used for the biocatalytic reaction. After five recycling cycles, 100% enzyme activity was fully recovered. Therefore, each biocatalytic cycle exhibited the same kinetics and rendered a similar quality of the crude peptide (Figure 2). These results showed the possibility of applying immobilized PGA in a continuous process with the potential for use in commercial production.

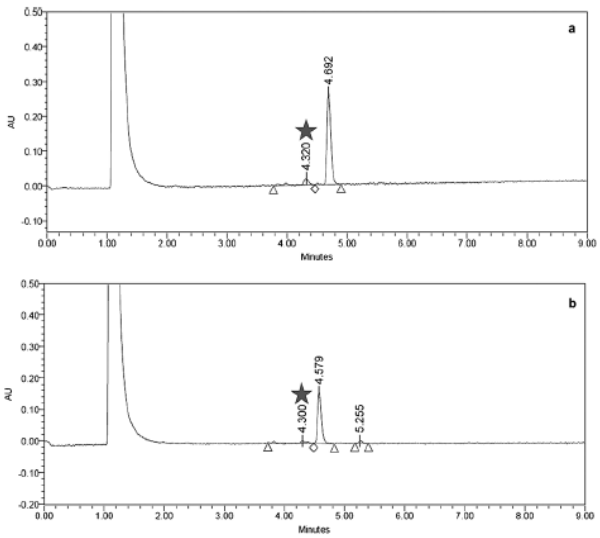

Figure 2. Chromatographic profiles of the oxidized peptide URP (**3**) after: a) cycle 1, and b) cycle 5 (phenylacetic acid: ★).

Regioselective formation of disulfide bonds: The regioselective construction of disulfide bonds is a challenging and desirable goal for the synthesis of complex Cys-containing peptides. Several suitable combinations of orthogonal and/or compatible protecting groups have been described for this purpose.[18] One of the main drawbacks of the synthesis of multiple-Cys-containing peptides is the disulfide-scrambling phenomenon, which can be prevented or minimized by using very mild conditions. The incorporation of *S*-Phacm, which can be removed selectively under mild conditions, installs an additional orthogonal dimension into classical protection schemes and it has been shown to be appropriate for the synthesis of complex Cys-containing peptides.

The compatibility of *S*-Phacm and *S*-Acm has been demonstrated in the preparation of the parallel cyclic dimer (Ac-Cys-Pro-D-Val-Cys-NH_2)$_2$. The tetrapeptide Ac-Cys-(Phacm)-Pro-D-Val-Cys(Acm)-NH_2 was synthesized manually and the protected peptide was then incubated for 30 h with the biocatalyst to obtain the *S*-Acm-protected open dimer. The second disulfide bond was formed upon treatment of the *S*-Acm-protected peptide with I_2 (10 equiv) in AcOH/water (4:1) for 2 h at 25 °C to afford the target peptide in excellent yield.[10]

Herein, we explored the combination of *S*-Trt and *S*-Phacm protecting groups for the regioselective synthesis of diverse peptide sequences.

The use of *S*-Phacm for the synthesis of an oxytocin analogue: First, the orthogonality of *S*-Phacm to the oxidative disulfide formation and the removal of *S*-Phacm in the presence of a disulfide bond was studied in the synthesis of an oxytocin analogue (**6**). Oxytocin is a nine-residue peptide

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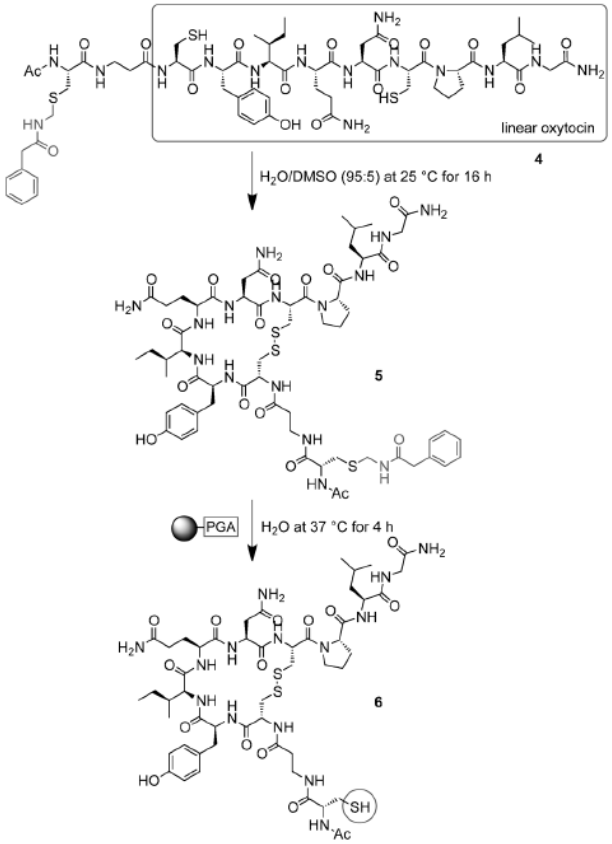
that contains a disulfide bridge (Cys1–Cys6) and a Tyr residue in its sequence. An oxytocin analogue with an extra Cys residue that was linked to the N terminus through a β-alanine residue was prepared by combining *S*-Trt for the native Cys residues and *S*-Phacm for the extra Cys residue. After deblocking, the thiol group of the additional Cys residue could be subsequently derivatized (conjugation, labeling, etc.) for biochemical purposes (Scheme 3).



Scheme 3. Synthesis of an oxytocin analogue that combines *S*-Phacm and 2 *S*-Trt groups.

Linear peptide **4** was manually synthesized on a Rink-Amide AM resin by following the same procedure as that described for the synthesis of linear URP. After the assembly of the peptide chain on the solid phase, the peptide was cleaved from the resin by treatment with TFA, with the concomitant removal of the *S*-Trt protecting groups, and the formation of the disulfide bridge was attempted. Linear oxytocin analogue **4** was not totally soluble in water or in any aqueous buffer; consequently, a co-solvent was required to carry out the oxidation. The use of DMF or DMSO (5-20%) was appropriate for both the solubilization and oxidation of analogue **4**, whereas other co-solvents, such as MeCN, 2-propanol, and MeOH, did not promote its solubility and, hence, its further cyclization. Once the formation of

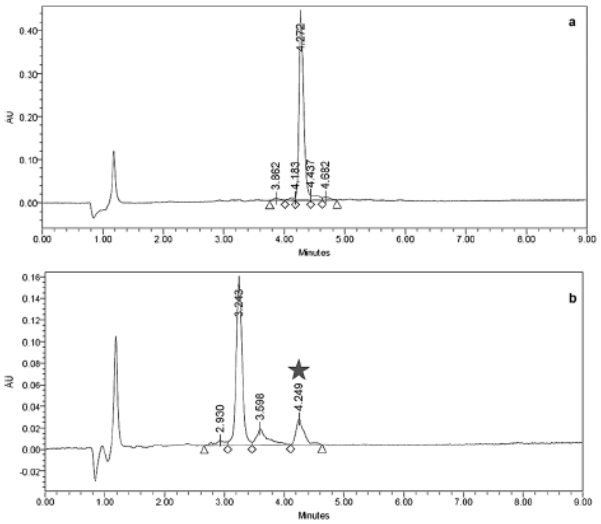


Figure 3. Chromatographic profiles of: a) oxidized *S*-Phacm-protected oxytocin analogue **5**, and b) after the removal of *S*-Phacm (Table 2, entry 9; phenylacetic acid: ★).

Table 2. Disulfide-bond formation and removal of *S*-Phacm in oxytocin analogue (**6**).[a]

Entry	Disulfide-bond formation			Removal of *S*-Phacm			
	Reaction medium	pH	*t* [h]	Reaction medium	pH	*t* [h]	Observations
1	water/DMSO (90:10)	7.0	16	water/DMSO (90:10)	7.0	5	scrambling
2	water/DMF (90:10)	7.0	48	water/DMF (90:10)	7.0	4	some scrambling
3	water/DMSO (80:20)	7.0	16	water/DMSO (80:20)	7.0	5	scrambling
4	water/DMSO (95:5)	7.0	16	water/DMSO (95:5)	7.0	5	scrambling
5	0.1 mM phosphate buffer/DMSO (90:10)	7.0	24	0.1 mM phosphate buffer/DMSO (90:10)	7.0	24	scrambling
6	0.1 mM phosphate buffer/DMF (90:10)	7.0	72	0.1 mM phosphate buffer/DMF (90:10)	7.0	24	some scrambling
7	0.1 mM phosphate buffer/DMSO (90:10)	5.2	72	0.1 mM phosphate buffer/DMSO (90:10)	7.0	24	scrambling
8	0.1 mM phosphate buffer/DMF (90:10)	5.2	120	0.1 mM phosphate buffer/DMF (90:10)	7.0	24	some scrambling
9	water/DMSO (90:10)	7.0	16	water	7.0	4	–
10	water/DMSO (90:10)	7.0	16	0.1 mM phosphate buffer	5.4	4	–
11	water/DMSO (90:10)	7.0	16	0.1 mM phosphate buffer	7.8	4	–
12	water/DMSO (99:1)	7.0	24	water/DMSO (99:1)	7.0	4	some scrambling
13	water/DMSO (97:3)	7.0	24	water/DMSO (97:3)	7.0	4	scrambling

[a] All experiments were performed at a concentration of 8×10^{-5} M of linear *S*-Phacm-protected oxytocin analogue **4** with 24 mg (3 EU) of SPRIN imibond PGA (hydrolytic activity: 130 Ug_{wet}^{-1}) at 37 °C. The progress of the reactions, along with disulfide scrambling, were monitored by RP-HPLC analysis.

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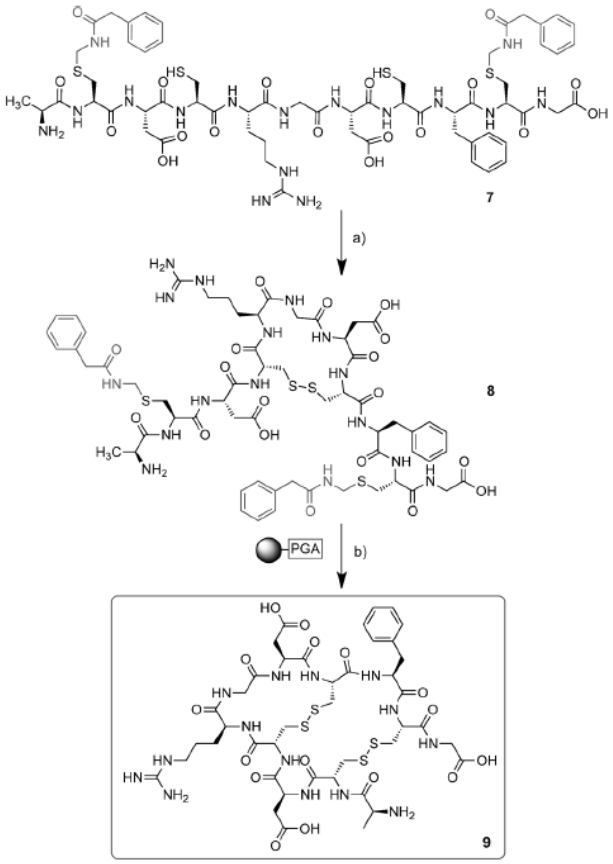
the disulfide had been achieved and without isolation of the *S*-Phacm-protected cyclic peptide (**5**), immobilized PGA was added to remove the *S*-Phacm group. However, when DMF or DMSO was used for *S*-Phacm deblocking, disulfide scrambling occurred. As expected, this phenomenon was more significant in the presence of DMSO than in DMF (Table 2, entries 1, 3–5, and 7 vs. 2, 6, and 8). At this point, a new strategy, which consisted of carrying out the oxidative disulfide formation in the presence of DMSO (10%) in aqueous media, followed by removal of the DMSO by lyophilization, was assayed. The enzymatic cleavage of *S*-Phacm was then accomplished in water or in 0.1 mM phosphate buffer, thereby minimizing disulfide scrambling (Table 2, entries 9–11; Figure 3).

The use of *S*-Phacm for the regioselective synthesis of peptide RGD-4C: The double-cyclic peptide RGD-4C is an 11-residue peptide that contains two disulfide bridges (Cys2–Cys10 and Cys4–Cys8).[19] The linear peptide was manually synthesized on 2-CTC resin by using DIPCDI and Oxyma in DMF, with a 5 min pre-activation, for 1 h at 25°C to incorporate all of the Fmoc-amino acids. After peptide elongation on the solid phase, the peptidyl resin was treated with TFA/TIS/water (95:2.5:2.5) for 1.5 h at 25°C to afford the linear *S*-Phacm-protected peptide (**7**, Scheme 4). The first disulfide-bond formation of this peptide (Scheme 4a) was achieved in either 1 mM or 20 mM phosphate buffer with or

Table 3. The use of *S*-Phacm for the regioselective construction of peptide RGD-4C (**9**).[a]

	Reaction medium	pH	t_A [h]	t_B [h]
1	1 mM phosphate buffer	8.0	240	16
2	1 mM phosphate buffer/DMSO (95:5)	8.0	48	16
3	1 mM phosphate buffer/DMSO (80:20)	8.0	24	16
4	1 mM phosphate buffer	6.9	360	16
5	1 mM phosphate buffer/DMSO (95:5)	6.9	72	16
6	1 mM phosphate buffer/DMSO (80:20)	6.9	24	16
7	20 mM phosphate buffer	8.0	48	16
8	20 mM phosphate buffer/DMSO (95:5)	8.0	24	16
9	20 mM phosphate buffer/DMSO (80:20)	8.0	24	16
10	20 mM phosphate buffer	6.9	240	16
11	20 mM phosphate buffer/DMSO (95:5)	6.9	48	16
12	20 mM phosphate buffer/DMSO (80:20)	6.9	24	16

[a] All experiments were performed at a concentration of 8×10^{-5} M of cyclic *S*-Phacm-protected RGD-4C peptide (**8**) with 24 mg (3 EU) of SPRIN imibond PGA (hydrolytic activity: 130 $U\,g_{wet}^{-1}$) at 37°C. The progress of the reactions was monitored by RP-HPLC analysis (t_A = reaction time for the formation of the first disulfide bond; t_B = reaction time for the deblocking of *S*-Phacm and successive oxidation).



Scheme 4. Synthetic approach for the preparation of peptide RGD-4C (**9**) through a combination of *S*-Phacm and *S*-Trt protecting groups.

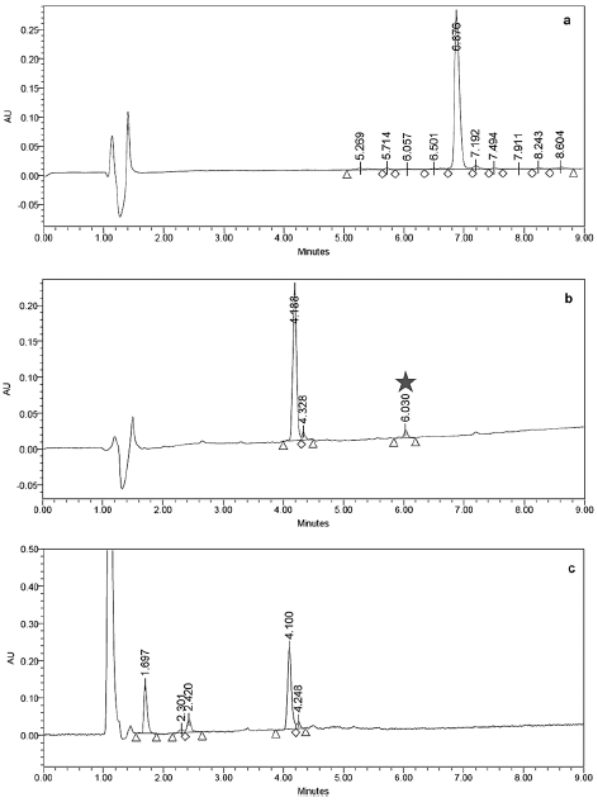


Figure 4. Chromatographic profiles of: a) cyclic *S*-Phacm-protected RGD-4C peptide (**8**), b) final bicyclic peptide **9** from the biocatalytic removal of *S*-Phacm and oxidation (Table 3, entry 1), and c) final bicyclic peptide (**9**) after the removal of *S*-Phacm and oxidation by treatment with I_2 (phenylacetic acid: ★).

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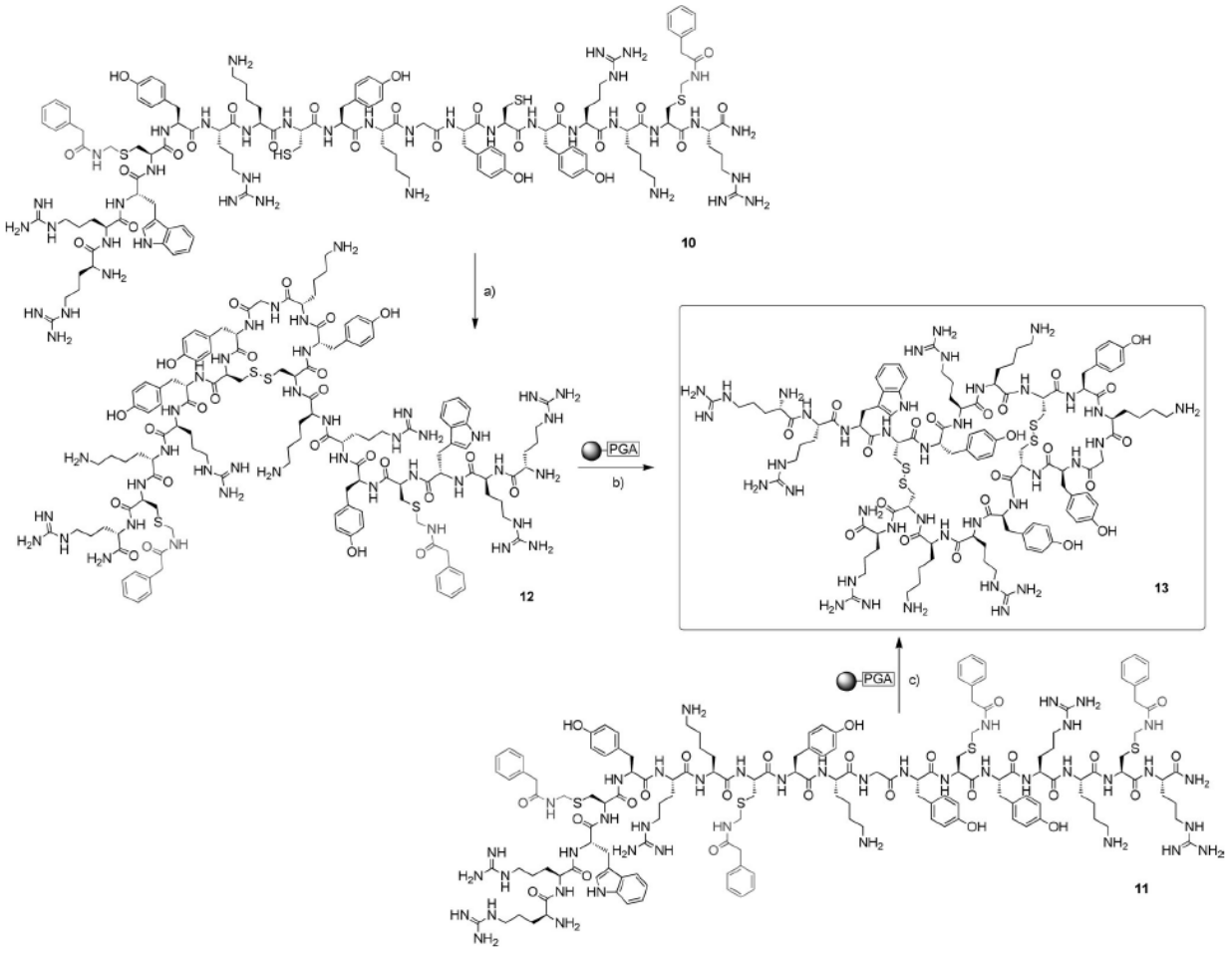
without DMSO as a co-solvent (Table 3, t_A); complete oxidation was faster when 20% DMSO was present in the reaction medium (Table 3, entries 3, 6, 9, and 12). The first disulfide-bridge construction was favored at pH 8 in 20 mM phosphate buffer (Table 3, entry 7), whereas the neat oxidation took much longer when performed at pH 6.9 in 1 mM phosphate buffer (Table 3, entry 4). The disulfide formation was monitored by RP-HPLC analysis until the reaction was completed (Figure 4a). Then, and without isolation of monocyclic peptide **8**, immobilized PGA was added to remove *S*-Phacm, with the concomitant formation of the second disulfide bond (Scheme 4b). *S*-Phacm deblocking and successive oxidation were achieved regardless of the reaction media (Table 3). In all cases, the biocatalytic reaction was complete after 16 h (Table 3, t_B) and the final bicyclic peptide RGD-4C (**9**) was obtained in high purity (Figure 4b).

In addition, the removal of *S*-Phacm and successive oxidation to the disulfide was attempted by treatment with I_2 to compare chemical removal and oxidation with biocatalytic deprotection and successive disulfide-bond formation. Thus, after the formation of the first disulfide bond, the oxidized cyclic *S*-Phacm-protected RGD-4C peptide (**8**) was dissolved in water/MeCN (1:1) and I_2 (5 equiv) was added to the reaction medium. The progress of the I_2-mediated oxidation reaction was monitored by RP-HPLC and the final oxidized peptide (**9**) was obtained in low quality (Figure 4c).

The use of *S*-Phacm for the synthesis of peptide T22: T22 ([Tyr5,12,Lys7]-polyphemusin II) is an 18-residue peptide that contains two disulfide bridges (Cys4–Cys17 and Cys8–Cys13), four Tyr residues, and a Trp residue in its sequence.[20] The *S*-Phacm protecting group was applied for the preparation of the peptide T22 by means of two synthetic approaches that were carried out in parallel: 1) a regioselective strategy that combined *S*-Trt and *S*-Phacm groups (Scheme 5a, b) and 2) a random strategy that used *S*-Phacm as a unique protecting group for Cys residues (Scheme 5c).

Linear peptides **10** and **11** were manually synthesized on an AM ChemMatrix resin by following the same procedure as that for the synthesis of linear peptide RGD-4C. After the assembly of the peptides on the solid phase, the peptidyl



Scheme 5. Synthetic approaches for the synthesis of peptide T22: regioselective strategy that combines the *S*-Phacm and *S*-Trt protecting groups (peptide **10**, steps a and b) and a random strategy that uses *S*-Phacm for the Cys residues (peptide **11**, step c).

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resins were treated with TFA/TIS/water (95:2.5:2.5) for 3 h at 25 °C to assure the complete deprotection of the five Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) protecting groups for the Arg residues.

Whilst the first disulfide bond from the protected *S*-Phacm-protected T22 peptide (**10**, Scheme 5a) was easily and rapidly obtained in water or in low-salt-concentration buffers (0.1 mM and 1 mM), neither total nor partial *S*-Phacm deblocking was observed under these reaction conditions when immobilized PGA was added directly (Table 4, entries 1–4). It is worth noting that increasing the salt concentration enabled the removal of the *S*-Phacm group, thereby demonstrating the main dependence of the biocatalytic reaction on the ionic strength of the medium for this peptide sequence (Table 4). An increase in ionic strength has been reported to promote enzyme–substrate interactions[21] and, in the case of PGA-catalyzed hydrolysis, an increase in ionic strength favors the displacement of PhAcOH from the active site, thus achieving higher yields. In addition, increasing the ionic strength guarantees buffered pH control, thereby preventing the shift to a lower pH, which would cause a decrease in activity and yield.

Table 4. The use of *S*-Phacm for the regioselective construction of peptide T22 (**13**).[a]

	Reaction medium	pH	t_A [h]	t_B [h]	Removal of *S*-Phacm
1	water	7.0	48	72	no
2	water/DMSO (95:5)	7.0	24	72	no
3	0.1 mM phosphate buffer	7.4	24	72	no
4	1 mM phosphate buffer	7.7	24	72	no
5	20 mM phosphate buffer	9.0	72	72	partial
6	20 mM phosphate buffer	8.0	24	72	almost complete
7	20 mM phosphate buffer	7.0	24	72	almost complete
8	20 mM phosphate buffer	6.0	48	72	partial
9	50 mM phosphate buffer	8.1	24	48	complete
10	100 mM phosphate buffer	8.1	24	48	complete
11	20 mM NH_4HCO_3	8.2	24	72	partial
12	50 mM NH_4HCO_3	8.1	24	48	complete
13	100 mM NH_4HCO_3	8.1	24	48	complete

[a] All experiments were performed at a peptide concentration of 8×10^{-5} M cyclic *S*-Phacm-protected T22 peptide (**12**) with 24 mg (3 EU) of SPRIN imibond PGA (hydrolytic activity: 130 $U g_{wet}^{-1}$) at 37 °C. The progress of the reactions was monitoring by RP-HPLC analysis (t_A = reaction time for the formation of the first disulfide bond; t_B = reaction time for the deblocking of *S*-Phacm and successive oxidation).

Therefore, by increasing the salt concentration up to 20 mM, the removal of the *S*-Phacm group by the immobilized PGA was observed. The pH of the reaction media was determinant for the progress of the biocatalytic reaction with this peptide sequence (Table 4, entries 5–8). Thus, although the *S*-Phacm groups were not completely deblocked at pH 7 or 8 after 72 h, the mere partial removal of the protecting group was observed after the same reaction time at pH 6 or 9.

At salt concentrations of 50 mM and 100 mM, the removal of the *S*-Phacm group by the biocatalyst was faster and clean (Table 4, entries 9, 10 and 12, 13) and the oxidized crude peptide T22 (**13**) was superior in quality to those that were rendered at lower salt concentrations (Figure 5a).

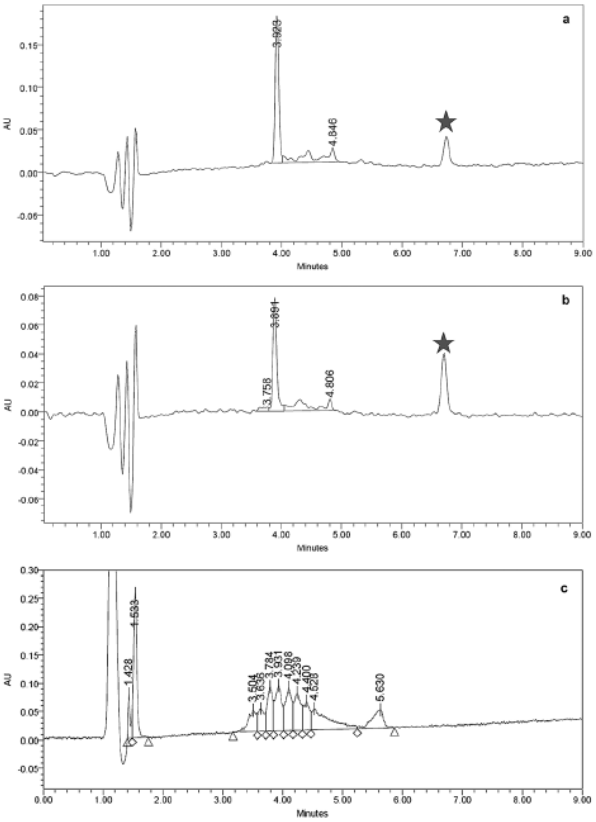

Figure 5. Chromatographic profiles of: a) final bicyclic T22 peptide (**13**) from the regioselective strategy (Table 4, entry 10), b) final bicyclic T22 peptide (**13**) from the random strategy (Table 4, entry 10), and c) final bicyclic T22 peptide (**13**) after the removal of *S*-Phacm and concomitant oxidation of the cyclic *S*-Phacm-protected T22 peptide (**12**) by treatment with I_2 (phenylacetic acid: ★).

Moreover, as for peptide RGD-4C, the removal of *S*-Phacm and the concomitant oxidation of the cyclic T22 intermediate (**12**) by treatment with I_2 were assayed for the regioselective strategy. Therefore, after oxidization, the cyclic *S*-Phacm-protected T22 peptide (**12**) was dissolved in water/MeCN (1:1) and I_2 (5 equiv) was added to the reaction medium. The progress of the reaction was monitored by RP-HPLC, which showed a complex chromatographic profile after 30 min (Figure 5c).

For the random obtaining of peptide T22 (**13**, Scheme 5c), the *S*-Phacm-protected linear T22 peptide (**11**) was treated with immobilized PGA (6 EU) and incubated at 37 °C in the same reaction media (Table 4). Similar behavior was observed in response to salt-buffer concentration and pH. Hence, at higher salt concentrations, *S*-Phacm deblocking and successive random oxidation was accomplished and the final crude product (**13**) was obtained in high quality (Figure 5b).

The use of *S*-Phacm group for the synthesis of peptides through native chemical ligation (NCL): Based on the capacity of immobilized PGA to catalyze the removal of *S*-

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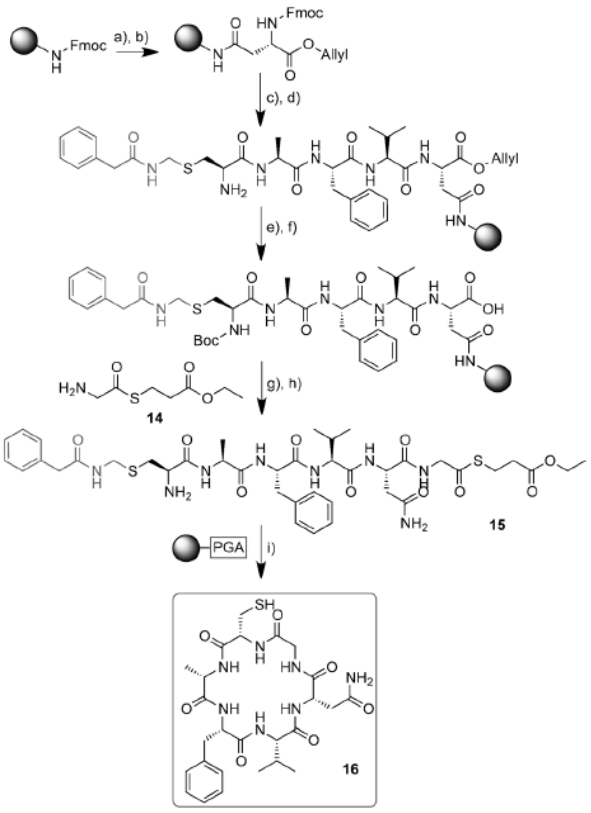
Phacm under a broad range of conditions, we were encouraged to exploit the use of the *S*-Phacm protecting group in the presence of sensitive functional groups, such as thioesters. Therefore, the enzymatic deblocking of *S*-Phacm in the presence of a thioester moiety was applied to the synthesis of a small cyclic peptide by using intramolecular NCL. Thus, linear C-terminal thioester peptide **15** was prepared by using *S*-Phacm as a protecting group for the β-thiol function of the N-terminal Cys residue in a side-chain-anchoring approach[22] where an Fmoc-Asp-OAllyl residue was incorporated onto an Fmoc-Rink-amide AM polystyrene resin and further chain elongation was carried out by using a standard Fmoc strategy with Oxyma and DIPCDI in DMF (Scheme 6a–d). After peptide elongation on the solid phase, the N terminus of the linear peptide was protected with a Boc group before the removal of the allyl protecting group by Pd^0 in the presence of $PhSiH_3$. Subsequently, attachment of previously prepared H-Gly-S$(CH_2)_2CO_2$Et (**14**) was accomplished by using HATU and DIEA as a coupling system. Then, the required linear peptide thioester (**15**) was cleaved from the resin by treatment with TFA/TIS/water (95:2.5:2.5) with the concomitant removal of the N-terminal Boc group (Scheme 6e–h).



Scheme 6. Synthesis of linear thioester-peptide intermediate **15** that features side-chain-anchoring to a Rink-amide AM polystyrene resin and ulterior enzymatic removal of *S*-Phacm with concomitant cyclization by using NCL to obtain the final cyclic peptide (**16**): a) piperidine/DMF (1:4); b) Fmoc-Asp-OAllyl (3 equiv), DIPCDI (3 equiv), Oxyma (3 equiv) in DMF for 1 h at 25 °C; c) standard peptide-chain elongation by using Fmoc chemistry; d) piperidine/DMF (1:4); e) $(Boc)_2O$ (10 equiv), DIEA (10 equiv) in DMF for 1 h at 25 °C; f) $[Pd(PPh_3)_4]$ (0.1 equiv), $PhSiH_3$ (10 equiv) in CH_2Cl_2 (3×15 min); g) H-Gly-S-$(CH_2)_2CO_2$Et (**14**, 10 equiv), HATU (10 equiv), DIEA (20 equiv) in DMF for 30 min at 25 °C; h) TFA/TIS/water (95:2.5:2.5) for 1 h at 25 °C; i) deblocking of *S*-Phacm was performed at a concentration of 8×10^{-5} M of *S*-Phacm-protected thioester-peptide **15** and 24 mg (3 EU) of SPRIN imibond PGA (hydrolytic activity: 130 $U g_{wet}^{-1}$) for 5 h at 37 °C. Fmoc = 9-fluorenylmethoxycarbonyl, Boc = *tert*-butoxycarbonyl, DIPCDI = *N,N'*-diisopropylcarbodiimide, Oxyma = ethyl 2-cyano-2-(hydroxyimino)acetate, DIEA = *N,N*-diisopropylethylamine, HATU = 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

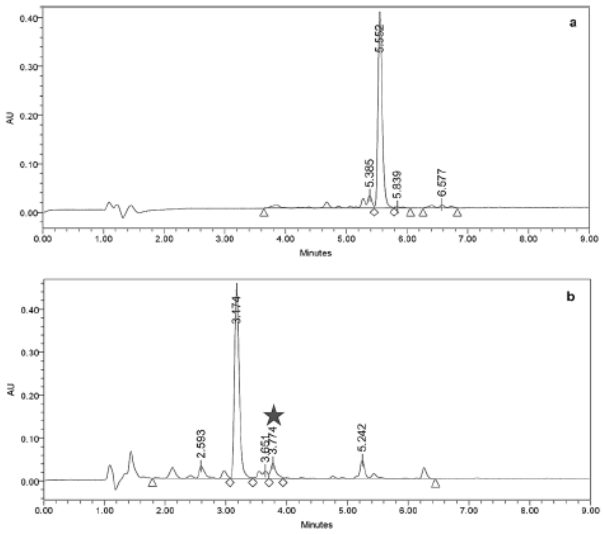


Figure 6. Chromatographic profiles of: a) linear C-terminal thioester peptide **15**, b) final cyclic peptide **16** from the enzymatic removal of *S*-Phacm and concomitant cyclization through an NCL reaction in water at pH 7 (phenylacetic acid: ★).

The formation of linear thioester hexapeptide **15** was determined by analytical RP-HPLC analysis (Figure 6a). Then, the removal of *S*-Phacm and successive head-to-tail cyclization by NCL was attempted under different deblocking conditions to obtain cyclic peptide **16** (Scheme 6i). In all cases, the crude peptide was incubated with the immobilized PGA at 37 °C and enzymatic deprotection and spontaneous cyclization were monitored by RP-HPLC until the reaction was completed. As expected, a clean chromatographic profile was obtained in water or in potassium phosphate buffer (1 mM and 20 mM) at pH 5, 6, and 7, whilst a complex chromatographic profile was observed when the biocatalytic deprotection was performed at pH 8. RP-HPLC analysis of the final crude product at pH 7 in water showed a major peak that corresponded to the desired cyclic peptide (**16**, Figure 6b).

Conclusion

S-Phacm has been proven to be a useful alternative to chemically removable protecting groups such as *S*-Acm. Its

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mild biocatalytic removal by an immobilized PGA enzyme avoids the concourse of harsh reagents, such as toxic heavy metals and I_2, which favor back-alkylation and other modifications of the peptide chain and whose residual products are difficult to remove and are potentially harmful to the environment.

The biocatalytic removal of *S*-Phacm by immobilized PGA, which can be easily separated from the reaction media by a simple and rapid filtration, exhibits the same kinetics and renders the crude peptide with a similar quality after repeated reuse of the immobilized enzyme. The immobilized biocatalyst demonstrates complete recovery of enzymatic activity after each cycle, which opens up the possibility of its implementation in a continuous process.

The removal of *S*-Phacm is sufficiently mild to prevent disulfide scrambling. Therefore, it enables the deblocking of additional Cys residues in peptide analogues for their subsequent derivatization and can be applied in the native chemical ligation strategy. The deblocking of *S*-Phacm in the presence of sensitive functional groups, such as thioesters, was applied successfully to the synthesis of a small cyclic peptide by using NCL.

The combination of *S*-Trt and *S*-Phacm has been successfully applied to the regioselective synthesis of peptides RGD-4C and T22, whilst the use of *S*-Phacm as a unique protecting group has shown notable results for the random synthesis of the bicyclic peptide T22.

One of the main characteristics of the removal of *S*-Phacm by immobilized PGA is that it depends on the peptide sequence. Thus, some sequences tolerate a broad range of conditions (pH, ionic strength, and the presence of organic co-solvents), whereas others require the optimal fine-tuning of the conditions. Thus, we propose the artisanal preparation of multi-Cys-containing peptides, as opposed to conventional methods that apply a general approach to all peptides and, consequently, render unsatisfactory results. In our opinion, the capacity of the immobilized PGA to work under a broad range of conditions is a major advantage and makes the *S*-Phacm protecting group a suitable tool for use in peptide chemistry.

Experimental Section

Fmoc-amino-acid derivatives, Fmoc-Rink-OH linker, 2-CTC resin, and Fmoc-Rink-amide polystyrene resin were obtained from IRIS Biotech (Marktredwitz, Germany). DIEA and DIPCDI were obtained from Aldrich (Milwaukee, WI), TFA was obtained from Scharlau (Barcelona, Spain), Oxyma was obtained from Luxembourg Industries Ltd. (Tel Aviv, Israel), and COMU, KH_2PO_4, and $K_2HPO_4{\cdot}3H_2O$ were obtained from Sigma–Aldrich (St Louis). PGA from *E. coli* (E.C.3.5.1.1) that had been covalently immobilized on an amino-acrylic resin (0.15–0.30 mm (96%), 130 $U\,g_{wet}^{-1}$) was obtained from SPRIN technologies. DMF, CH_2Cl_2, Et_2O, DMSO, piperidine, and MeCN (HPLC grade) were purchased from SDS (Peypin, France). All commercial reagents and solvents were used as received.

Solid-phase syntheses were carried out manually in polypropylene syringes that were fitted with a porous polyethylene disc. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine/DMF (1:4, v/v; 1×1 min, 2×5 min). Washing between the deprotection, coupling, and final deprotection steps were carried out with DMF (5×1 min) and CH_2Cl_2 (5×1 min). Peptide-synthesis transformations and washes were performed at 25 °C.

An XBridge BEH130 C18 RP-HPLC analytical column (4.6 mm × 100 mm, 3.5 μm) was obtained from Waters (Ireland). Analytical RP-HPLC was performed on a Waters instrument that comprised a separation module (Waters 2695), an automatic injector, a photodiode-array detector (Waters 2998), and a system controller (Empower login). UV detection was performed at 220 and 254 nm and linear gradients of MeCN (+0.036% TFA) into water (+0.045% TFA) were performed at a flow rate of 1.0 mL min^{-1} over 8 min. RP-HPLC-MS (ES) was performed on a Waters Micromass ZQ spectrometer with a SunFire™ C18 RP-HPLC analytical column (2.1 mm × 100 mm, 5 μm). Linear gradients of MeCN (+0.07% formic acid) into water (0.1% formic acid) were performed at a flow rate of 0.3 mL min^{-1} over 8 min.

Immobilized PGA: The immobilized PGA was stored in a mixture of 20 mM phosphate buffer/glycerol (20:80) at 4 °C. Before use, the immobilized PGA (1 g) was washed with 20 mM potassium phosphate buffer (pH 8, 5×4 mL), and the glycerol-free immobilized PGA was stored at 4 °C for two months.

Removal of *S*-Acm and the oxidation of URP on a solid phase (3): A portion of peptidyl resin **1** (10 mg) was treated with iodine (5 equiv, 6 mg) in DMF (500 μL) at 25 °C for 15 min and the treatment was repeated two more times. Then, the resin was washed with DMF (5×1 mL× 1 min), piperidine/DMF (1:4) (5×1 mL×1 min), and CH_2Cl_2 to remove any excess iodine from the resin. Next, the resin was treated with a mixture of TFA/TIS/water (95:2.5:2.5, 2 mL) for 1 h at 25 °C to render cyclic URP **3**. The oxidized peptide was obtained in 40% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% MeCN over 8 min; t_R = 4.6 min). RP-HPLC-MS (ES) showed the formation of the target peptide (linear gradient from 0% to 50% MeCN over 8 min; t_R = 7.8 min): *m/z* calcd for $C_{49}H_{64}N_{10}O_{10}S_2$: 1017.2; found: 1018.3 $[M+H]^+$, 509.7 $[(M+2H)/2]^{2+}$ (*M* is the M_W of the oxidized peptide URP **3**).

Removal of *S*-Phacm and the oxidation of URP in solution (3): Linear *S*-Phacm-protected URP **2** (5 mg) was dissolved in a mixture of water/DMSO (95:5, 45 mL, 8×10^{-5} M), immobilized PGA (120 mg, 15 EU) was added, and the reaction was left to stand for 24 h at 37 °C and 50× 10 rpm. Next, the immobilized biocatalyst was removed by filtration from the media and the aqueous mixture was lyophilized. Then, the crude peptide was precipitated with cold Et_2O (10 mL) and centrifuged 3 times to render the cyclic peptide URP (**3**) in 95% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% MeCN over 8 min; t_R = 4.6 min). RP-HPLC-MS (ES) showed the formation of the target peptide (linear gradient from 0% to 50% MeCN over 8 min; t_R = 7.8 min): *m/z* calcd for $C_{49}H_{64}N_{10}O_{10}S_2$: 1017.2; found: 1018.2 $[M+H]^+$, 509.8 $[(M+2H)/2]^{2+}$ (*M* is the M_W of the oxidized peptide URP **3**).

Oxidation of the oxytocin analogue and the removal of *S*-Phacm (6): (Table 2, entry 9) Crude peptide **4** (1 mg) was dissolved in a mixture of water/DMSO (90:10, 9 mL, 8×10^{-5} M) at 25 °C and the disulfide-bond formation was monitoring by RP-HPLC analysis (linear gradient from 20% to 60% MeCN over 8 min; t_R = 4.3). After the oxidation was completed, RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 10% to 60% MeCN over 8 min; t_R = 7.7 min): *m/z* calcd for $C_{60}H_{87}N_{15}O_{16}S_3$: 1370.6; found: 1370.9 $[M+H]^+$, 686.2 $[(M+2H)/2]^{2+}$ (*M* is the M_W of the oxidized *S*-Phacm-protected oxytocin analogue **5**). Next, the mixture was lyophilized to completely remove the DMSO from the reaction media and the crude peptide (**5**) was redissolved in water (9 mL, 8×10^{-5} M). Immobilized PGA (24 mg, 3 EU, 130 $U\,g_{wet}^{-1}$) was added to the reaction mixture, which was left to stand at 37 °C for 4 h to afford the thiol-free oxytocin analogue (**6**). The completion of the reaction was determined by RP-HPLC analysis (linear gradient from 20% to 60% MeCN over 8 min; t_R = 3.2 min). RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 10% to 60% MeCN over 8 min; t_R = 6.9 min): *m/z* calcd for $C_{51}H_{78}N_{14}O_{15}S_3$: 1223.4; found: 1223.6 $[M+H]^+$, 612.6 $[(M+2H)/2]^{2+}$ (*M* is the M_W of oxytocin analogue **6**).

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Regioselective disulfide-bond formation of peptide RGD-4C (9): Crude peptide **7** (1 mg) was dissolved in the reaction medium (9 mL, 8×10^{-5} M; Table 3) at 25 °C and disulfide-bond formation was monitoring by RP-HPLC analysis (linear gradient from 15% to 40% MeCN over 8 min; $t_R = 6.9$). After the oxidation was completed, RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 0% to 70% MeCN over 8 min; $t_R = 6.7$ min): *m/z* calcd for $C_{60}H_{80}N_{16}O_{18}S_4$: 1441.6 $[M+H]^+$; found: 1442.9, 722.2 $[(M+2H)/2]^{2+}$ (M is the M_W of the oxidized *S*-Phacm-protected RGD-4C peptide **8**). Next, the immobilized PGA enzyme (24 mg, 3 EU, 130 U g_{wet}^{-1}) was added to the reaction mixture, which was left to stand at 37 °C for 16 h to afford the bicyclic RGD-4C peptide (**9**). Completion of the reaction was determined by RP-HPLC analysis (linear gradient from 5% to 50% MeCN over 8 min; $t_R =$ 4.1 min). RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 5% to 100% MeCN over 8 min; $t_R =$ 4.6 min): *m/z* calcd for $C_{62}H_{90}N_{14}O_{16}S_4$: 1145.3; found: 1146.3$[M+H]^+$, 573.9 $[(M+2H)/2]^{2+}$ (M is the M_W of the oxidized bicyclic RGD-4C peptide **9**).

Regioselective disulfide-bond formation of peptide T22 (13): Crude peptide **10** (1 mg) was dissolved in the reaction medium (10 mL, 8×10^{-5} M, Table 4) at 25 °C and disulfide-bond formation was monitored by RP-HPLC (linear gradient from 10% to 60% MeCN over 8 min; $t_R =$ 4.2 min). After the oxidation was completed, RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 5% to 100% MeCN over 8 min; $t_R = 4.7$ min): *m/z* calcd for $C_{127}H_{184}N_{40}O_{24}S_4$: 2783.3; found: 929.5 $[(M+3H)/3]^{3+}$, 697.2 $[(M+4H)/4]^{4+}$ (M is the M_W of the oxidized *S*-Phacm-protected T22 peptide **12**). Next, the immobilized PGA enzyme (24 mg, 3 EU, 130 U g_{wet}^{-1}) was added to the reaction mixture, which was left to stand at 37 °C to afford the fully oxidized T22 peptide (**13**). Completion of the reaction was determined by RP-HPLC analysis (linear gradient from 10% to 40% MeCN over 8 min; $t_R = 3.9$ min). RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 0% to 30% MeCN over 8 min; $t_R = 5.7$ min): *m/z* calcd for $C_{109}H_{164}N_{38}O_{22}S_4$: 2487.0; found: 1245.4 $[(M+2H)/2]^{2+}$, 830.6 $[(M+3H)/3]^{3+}$, 623.2 $[(M+4H)/4]^{4+}$ (M is the M_W of the oxidized bicyclic T22 peptide **13**).

Random disulfide-bond formation of peptide T22 (13): Crude peptide **11** (1 mg) was dissolved in the reaction medium (12 mL, 8×10^{-5} M, Table 4) and the immobilized PGA enzyme (48 mg, 6 EU, 130 U g_{wet}^{-1}) was added to the reaction mixture, which was left to stand at 37 °C to afford the fully oxidized T22 peptide (**13**). Completion of the reaction was determined by RP-HPLC analysis (linear gradient from 10% to 40% MeCN over 8 min; $t_R = 3.9$ min). RP-HPLC-MS (ES) analysis showed the formation of the target peptide (linear gradient from 0% to 30% MeCN over 8 min; $t_R = 5.9$ min): *m/z* calcd for $C_{109}H_{164}N_{38}O_{22}S_4$: 2487.0; found: 1245.2 $[(M+2H)/2]^{2+}$, 830.6 $[(M+3H)/3]^{3+}$, 623.2 $[(M+4H)/4]^{4+}$ (M is the M_W of the oxidized bicyclic T22 peptide **13**).

Removal of *S*-Phacm and cyclization through NCL (16): Crude peptide **15** (1 mg) was dissolved in the reaction medium (14 mL, 8×10^{-5} M), the immobilized PGA enzyme (24 mg, 3 EU, 130 U g_{wet}^{-1}) was added, and the reaction mixture was left to stand at 37 °C for 5 h to afford the head-to-tail cyclic peptide (**16**). Completion of the reaction was determined by analytical RP-HPLC (linear gradient from 25% to 50% MeCN over 8 min; $t_R = 3.3$ min). RP-HPLC-MS (ES) showed the formation of the target peptide (linear gradient from 20% to 70% MeCN over 8 min; $t_R =$ 6.4 min): *m/z* calcd for $C_{36}H_{37}N_7O_7S$: 591.68; found: 592.9 $[M+H]^+$ (M is the M_W of the cyclic peptide **16**).

Acknowledgements

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Supporting Information

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Eco-Friendly Combination of the Immobilized PGA Enzyme and the *S*-Phacm Protecting Group for the Synthesis of Cys-Containing Peptides

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S-Acm-protected URP elongation by SPPS (**1**)..... S2

S-Phacm-protected URP elongation by SPPS (**2**)..... S2

Oxytocin analog elongation by SPPS (**4**)..... S2

RGD-4C elongation by SPPS (**7**)..... S3

2 *S*-Phacm-protected T22 elongation by SPPS (**10**)..... S3

4 *S*-Phacm-protected T22 elongation by SPPS (**11**)..... S3

Synthesis of H-Gly-S$(CH_2)_2CO_2Et$ (**14**)..... S3

S-Phacm-protected thioester elongation by SPPS (**15**)..... S4

ESMS analysis of the oxidized URP crude peptide from the *S*-Acm strategy..... S5

***S*-Acm-protected URP elongation by SPPS (1)**: 2-CTC resin (207 mg, 0.11 mmol, 1.57 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min). Fmoc-Val-OH (68 mg, 1 mmol) and DIEA (340 μL, 10 equiv.) in CH_2Cl_2 were added to the resin, and the mixture was shaken for 55 min at 25 ºC. The resin was capped by adding MeOH (160 μL) for 10 min at 25 ºC, and then washed with DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (25 mL/g resin) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). The filtrates were collected and quantified by UV (290 nm), obtaining a loading of 0.55 mmol/g.

Based on this loading, the following protected amino acids (3 equiv.) were incorporated with COMU (3 equiv.) and DIEA (6 equiv.) in DMF, as a coupling system with a 30-s pre-activation, except for Fmoc-Cys(Acm)-OH (3 equiv.), which was incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with a 5-min pre-activation to prevent epimerization. Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). After completing the elongation, a portion of peptidyl-resin **1** (5 mg) was treated with a mixture of TFA-TIS-H_2O (95:2.5:2.5) (1 mL) at 25 ºC for 1 h. The mixture was partially evaporated, precipitated with cold Et_2O (500 μL) and centrifuged twice; then the crude peptide was redissolved in H_2O-ACN (1:1) (500 μL). The linear *S*-Acm-protected URP was obtained in 80% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% ACN over 8 min; t_R : 4.0 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R : 5.4 min; *m/z* calculated for $C_{55}H_{76}N_{12}O_{12}S_2$, 1161.4; found, 1162.2 $[M+H]^+$, 581.6 $[(M+2H)/2]^{2+}$, where M is the MW of the *S*-Acm-protected URP).

***S*-Phacm-protected URP elongation by SPPS (2)**: 2-CTC resin (495 mg, 0.84 mmol, 1.57 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min). Fmoc-Val-OH (163 mg, 1 mmol) and DIEA (870 μL, 10 equiv.) in CH_2Cl_2 were added to the resin, and the mixture was shaken for 55 min at 25 ºC. The resin was capped by adding MeOH (400 μL) for 10 min at 25 ºC, and then washed with DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (25 mL/g resin) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). The filtrates were collected and quantified by UV (290 nm), obtaining a loading of 0.88 mmol/g.

Based on this loading, the following protected amino acids (3 equiv.) were incorporated with COMU (3 equiv.) and DIEA (6 equiv.) in DMF, as a coupling system with a 30-s pre-activation, except for Fmoc-Cys(Phacm)-OH (3 equiv.), which was incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with a 5-min preactivation to prevent epimerization. Washes between couplings and deprotections were performed with DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). After completing the elongation, the peptide was cleaved from the resin by washes with TFA-TIS-H_2O (95:2.5:2.5), (3 x 10 mL x 5 min, and 2 x 5 mL x 5 min) at 25 ºC, and then the filtered washes were left to stand for 1 h at 25 ºC. The mixture was partially evaporated, precipitated with cold Et_2O (25 mL) and centrifuged five times. The crude peptide was then redissolved in H_2O-ACN (1:1) (40 mL) and left to stand for 16 h at 25 ºC to totally deprotect the Trp residue, before being lyophilized.

The linear *S*-Phacm-protected URP **2** (520 mg, 91% yield) was obtained in 91% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% ACN over 8 min; t_R : 6.1 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R : 6.1 min; *m/z* calculated for $C_{67}H_{84}N_{12}O_{12}S_2$, 1313.6; found, 1314.8 $[M+H]^+$, 657.7 $[(M+2H)/2]^{2+}$, where M is the MW of the *S*-Phacm-protected URP **2**).

Oxytocin analog elongation by SPPS (4): Fmoc-Rink-amide AM polystyrene resin (416 mg, 0.29 mmol, 0.7 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Based on the loading, the protected amino acids (3 equiv.) were incorporated with COMU (3 equiv.) and DIEA (6 equiv.) in DMF, as a coupling system with a 30-s pre-activation, except for Fmoc-Cys(R)-OH (3 equiv.), which were incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with a 5-min pre-activation to prevent epimerization. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). After finishing the peptide elongation, the *N*-terminus group was acetylated using DIEA (10 equiv.), acetic anhydride (10 equiv.) in DMF for 30 min at 25 ºC. The acetylation treatment was repeated twice. The peptidyl-resin (203 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5), (15 mL) at 25 ºC for 1 h. The mixture was partially evaporated, precipitated with cold Et_2O (15 mL) and centrifuged three times, and then redissolved in H_2O-ACN (1:1) (20 mL) and lyophilized. The linear Oxytocin analog **4** (56 mg, 59% yield) was obtained in 78% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% ACN over 8 min; t_R : 4.5 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R : 6.6 min; *m/z* calculated for $C_{60}H_{89}N_{15}O_{16}S_3$, 1372.6; found, 1372.8 $[M+H]^+$, 687.2 $[(M+2H)/2]^{2+}$, where M is the MW of the linear Oxytocin analog **4**).

RGD-4C elongation by SPPS (7): 2-CTC resin (411 mg, 0.37 mmol, 1.57 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min). Fmoc-Gly-OH (121 mg, 1 mmol) and DIEA (716 μL, 10 equiv.) in CH_2Cl_2 were added to the resin, and the mixture was shaken for 55 min at 25 ºC. The resin was capped by adding MeOH (330 μL) for 10 min at 25 ºC, and the resin was washed with DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). The filtrates were collected and quantified by UV (290 nm), obtaining a loading of 0.90 mmol/g.
Based on this loading, the protected amino acids (3 equiv.) were incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with 5-min pre-activation for 1 h at 25 ºC. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min).
The peptidyl-resin (509 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5) (30 mL) at 25 ºC for 1 h. The mixture was partially evaporated, precipitated with cold Et_2O (25 mL) and centrifuged five times and then redissolved in H_2O-ACN (1:1) (40 mL) and lyophilized. The linear RGD-4C peptide **7** (190 mg, 67% yield) was obtained in 83% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% ACN over 8 min; t_R : 4.5 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.7 min; *m/z* calculated for $C_{60}H_{82}N_{16}O_{18}S_4$, 1443.7; found, 1445.2 $[M+H]^+$, 722.8 $[(M+2H)/2]^{2+}$, where M is the MW of the linear *S*-Phacm-protected RGD-4C peptide **7**).

2 *S*-Phacm-protected T22 elongation by SPPS (10): AM-ChemMatrix resin (315 mg, 0.21 mmol, 0.66 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min). Before peptide elongation, the resin was washed with TFA-CH_2Cl_2 (1:99) (5 x 5 mL x 1 min), CH_2Cl_2 (3 x 5 mL x 1 min), DIEA-CH_2Cl_2 (5:95) (5 x 5 mL x 1 min), and finally with CH_2Cl_2 (3 x 5 mL x 1 min).
Based on the resin loading, first the Fmoc-Rink-OH linker and then the protected amino acids (3 equiv.) were incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with a 5-min pre-activation for 1 h at 25 ºC. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min).
The peptidyl-resin (502 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5), (15 mL) at 25 ºC for 3 h. The mixture was partially evaporated, precipitated with cold Et_2O (3 x 20 mL), centrifuged, redissolved in H_2O-ACN (1:1) (25 mL) and then lyophilized. The linear 2 *S*-Phacm-protected T22 peptide **10** (79 mg, 25% yield) was obtained in 82% purity, as determined by analytical RP-HPLC (linear gradient from 10% to 60% ACN over 8 min; t_R : 4.3 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.3 min; *m/z* calculated for $C_{127}H_{186}N_{40}O_{24}S_4$, 2785.4; found, 930.1 $[(M+3H)/3]^{3+}$, 697.8 $[(M+4H)/4]^{4+}$, where M is the MW of the linear 2 *S*-Phacm-protected T22 peptide **10**).

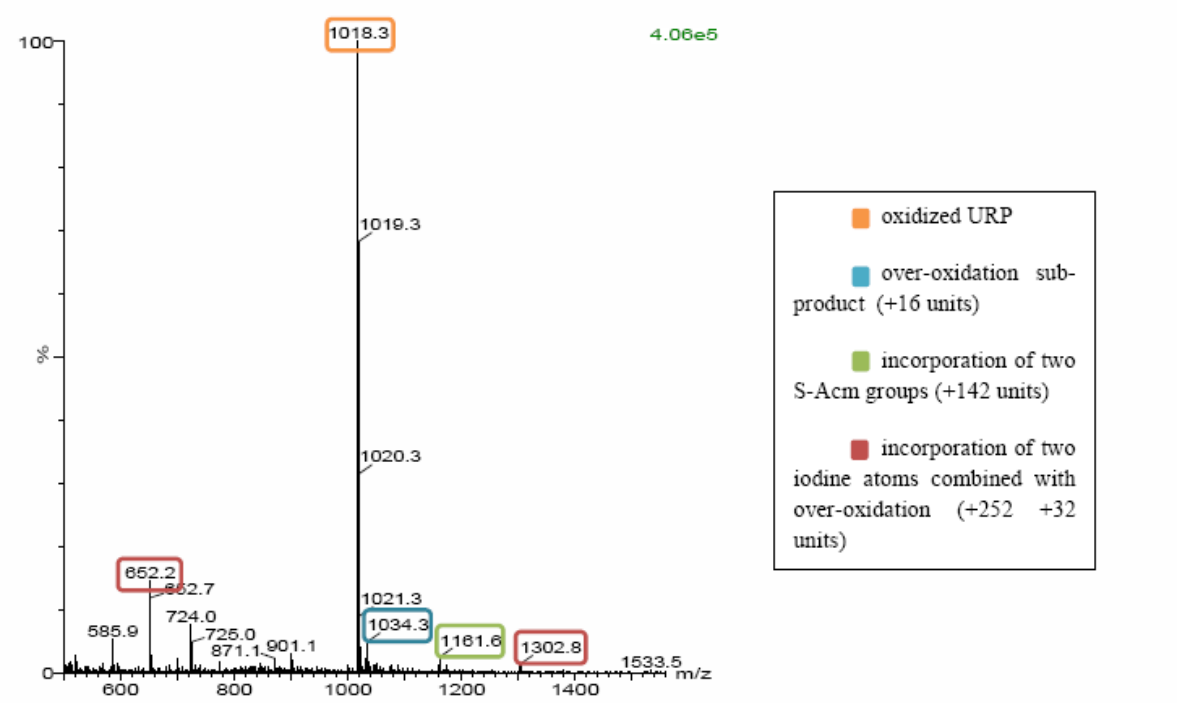
4 *S*-Phacm-protected T22 elongation by SPPS (11): AM-ChemMatrix resin (322 mg, 0.21 mmol, 0.66 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min). Before peptide elongation, the resin was washed with TFA-CH_2Cl_2 (1:99) (5 x 5 mL x 1 min), CH_2Cl_2 (3 x 5 mL x 1 min), DIEA-CH_2Cl_2 (5:95) (5 x 5 mL x 1 min), and finally with CH_2Cl_2 (3 x 5 mL x 1 min).
Based on the resin loading, first the Fmoc-Rink-OH linker and then the protected amino acids (3 equiv.) were incorporated using DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF with a 5-min pre-activation for 1 h at 25 ºC. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min).
The peptidyl-resin (511 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5) (15 mL) at 25 ºC for 3 h. The mixture was partially evaporated, precipitated with cold Et_2O (3 x 20 mL), centrifuged, redissolved in H_2O-ACN (1:1) (25 mL) and then lyophilized. The linear 4-*S*-Phacm-protected T22 peptide **11** (98 mg, 29% yield) was obtained in 69% purity, as determined by analytical RP-HPLC (linear gradient from 10% to 60% ACN over 8 min; t_R : 4.9 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.5 min; *m/z* calculated for $C_{145}H_{204}N_{42}O_{26}S_4$, 3079.7; found, 1028.1 $[(M+3H)/3]^{3+}$, 771.3 $[(M+4H)/4]^{4+}$, where M is the MW of the linear *S*-Phacm-protected T22 peptide **11**).

Synthesis of H-Gly-S$(CH_2)_2CO_2$Et (14): The 3-mercaptopropionic acid (1.7 mL, 19.4 mmol) was dissolved in anhydrous ethanol (35 mL), containing p-toluenesulfonic acid (3.7 g, 19.4 mmol) and the reaction mixture was heated at reflux for 14 h. After being cooled to room temperature, the ethanol solvent was removed under vacuum. The residue was diluted with ethyl acetate (20 mL) and treated with saturated aq. $NaHCO_3$ until pH 8.5. Additional, ethyl acetate was added to extract the desired ethyl ester product (3 x 15 mL). The combined organic layer was washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated to give the expected ethyl 3-

mercaptopropionate (1.6 g, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.1 Hz, 2H), 2.78 (dt, *J* = 8.4, 6.8 Hz, 2H), 2.64 (t, *J* = 6.9 Hz, 2H), 1.64 (t, *J* = 8.3 Hz, 1H), 1.28 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.79, 60.89, 38.72, 19.98, 14.42. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.6 min).
Boc-Gly-OH (2.5 g, 14.2 mmol) was dissolved in CH_2Cl_2 and cooled to 0 ˚C. HOAt (1.9 g, 14.2 mmol), EDC (2.7 g, 14.2 mmol), and DIEA (4.1 mL, 23.7 mmol) were added sequentially to the cooled solution, and the resulting mixture was left to stand for 10 min. At this point, ethyl 3-mercaptopropionate (1.6 g, 11.8 mmol) was added in one portion, and the homogeneous reaction was stirred for 2 h at 0 ˚C and for 14 h at 25 ˚C. The organic reaction mixture was washed with 1 N aq. HCl (2 x 150 mL), 10% aq. Na_2CO_3 (2 x 150 mL), and H_2O (150 mL), dried with $MgSO_4$, and concentrated under vacuum. The resultant yellow oil was purified by column chromatography (hexane/ethyl acetate 5:1) to provide the desired Boc-Gly-S$(CH_2)_2CO_2$Et (1.6 g, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.12 (s, 1H), 4.04 (d, *J* = 6.1 Hz, 2H), 3.15 (t, *J* = 7.0 Hz, 2H), 2.62 (t, *J* = 7.0 Hz, 2H), 1.46 (s, 9H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.29, 171.74, 155.42, 80.64, 61.03, 50.52, 34.44, 28.50, 23.82, 14.38. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.6 min). RP-HPLC-ESMS showed the target compound (linear gradient from 5% to 100% of ACN over 8 min; t_R : 8.7 min; *m/z* calculated for $C_{12}H_{21}NO_5S$, 291.36; found, 291.5 $[M+H]^+$, where M is the MW of the Boc-Gly-S$(CH_2)_2CO_2$Et).
The Boc-Gly-S$(CH_2)_2CO_2$Et intermediate was dissolved in CH_2Cl_2-TFA (3:2) (35 mL), and the deprotection reaction mixture was stirred for 45 min at 25 ˚C. The homogeneous reaction was concentrated under vacuum and the resultant residue was chased with Et_2O (8 x 50 mL, followed by reconcentration) to provide the title product as a white solid (1.5 g, 89% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.14 (q, *J* = 7.1 Hz, 2H), 4.06 (s, 2H), 3.23 (t, *J* = 6.8 Hz, 2H), 2.64 (t, *J* = 6.8 Hz, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.51, 171.73, 61.32, 47.62, 33.98, 24.20, 14.26. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 3.3 min). RP-HPLC-ESMS showed the target compound (linear gradient from 0% to 50% of ACN over 8 min; t_R : 3.9 min; *m/z* calculated for $C_7H_{13}NO_3S$, 191.25; found, 192.2 $[M+H]^+$, where M is the MW of the H-Gly-S$(CH_2)_2CO_2$Et).

***S*-Phacm-protected thioester elongation by SPPS (15):** Fmoc-Rink-amide AM polystyrene resin (0.5 g, 0.23 mmol, 0.45 mmol/g) was washed with DMF (3 x 5 mL x 1 min) and CH_2Cl_2 (3 x 5 mL x 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Based on the loading, the protected amino acids (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with a 5-min pre-activation. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 x 5 mL x 1 min, 2 x 5 mL x 5 min). Washes between couplings and deprotections were performed using DMF (5 x 3 mL x 1 min) and CH_2Cl_2 (5 x 3 mL x 1 min). After finishing the peptides elongation, a Boc group was incorporated in the *N*-terminus using DIEA (10 equiv.), di-*tert*-butyl dicarbonate [$(Boc)_2O$)] (10 equiv.) in DMF for 1 h at 25 ˚C. The treatment was repeated twice. Removal of the allyl protecting group was mediated by $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (10 eq) in CH_2Cl_2 (3 x 15 min), followed by washes with CH_2Cl_2, DMF and sodium *N*,*N*-diethyldithiocarbamate (0.02 M in DMF, 2 x 5 min).
A portion of peptidyl-resin (130 mg) was washed with CH_2Cl_2 (2 x 5 mL x 1 min) and DMF (2 x 5 mL x 1 min). A solution of H-Gly-S$(CH_2)_2CO_2$Et (135 mg, 10 equiv.) in DMF (1 mL) and DIEA (158 mL, 20 equiv.) was added to the peptidyl-resin, and the coupling was initiated by addition of HATU (171 mg, 10 equiv.) in solid form. After 30 min at 25 ˚C, the peptidyl-resin was washed with DMF (2 x 5 mL x 1 min) and CH_2Cl_2 (2 x 5 mL x 1 min). The peptide was cleaved from the resin by treatment with TFA-TIS-H_2O (95:2.5:2.5, 5 mL) at 25 ˚C for 1 h. The mixture was partially evaporated, precipitated with cold Et_2O (15 mL) and centrifuged three times, and then redissolved in H_2O-ACN (1:1) (20 mL) and lyophilized. The linear peptide thioester **15** (32.4 mg, 82% yield) was obtained in 81% purity, as determined by analytical RP-HPLC (linear gradient from 20% to 60% ACN over 8 min; t_R : 5.6 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R : 5.0 min; *m/z* calculated for $C_{40}H_{56}N_8O_{10}S_2$, 873.05; found, 874.3 $[M+H]^+$, where M is the MW of the linear peptide thioester **15**).

ESMS analysis of the oxidized URP crude peptide 3 from the *S*-Acm strategy:



The RP-HPLC-ESMS analysis of the oxidized URP crude peptide obtained from the *S*-Acm strategy showed diverse small peaks whose mass corresponded to the expecting oxidized URP. Presumably, these peaks are related to the formation of intramolecular tryptophan-2-thioether side-products.

Publication III

Acid-labile Cys-protecting groups for the Fmoc/tBu strategy: filling the gap

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Acid-Labile Cys-Protecting Groups for the Fmoc/*t*Bu Strategy: Filling the Gap

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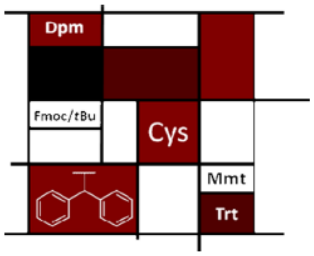
ABSTRACT



To address the existing gap in the current set of acid-labile Cys-protecting groups for the Fmoc/*t*Bu strategy, diverse Fmoc-Cys(PG)-OH derivatives were prepared and incorporated into a model tripeptide to study their stability against TFA. *S*-Dpm proved to be compatible with the commonly used *S*-Trt group and was applied for the regioselecive construction of disulfide bonds.

Since the early days of peptide chemistry, the effective synthesis of natural or non-natural isomers, analogues, or *de novo* designed peptides with complex disulfide bridge patterns has been a demanding task. The oxidative folding[1] of fully deprotected linear peptides is a desirable and commonly applied approach for the synthesis of complex Cys-rich peptides. However, achievement of the desired disulfide bond connectivity through this approach is not always affordable. To overcome these challenging syntheses, a myriad of protecting groups for the β-thiol group of Cys, along with efficient regioselective protection schemes, have been developed.[2]

In recent years, several acid-labile Cys-protecting groups have been developed for the Fmoc/*t*Bu strategy (Figure 1).[3] Most of these are highly sensitive to acid, the *S*-Trt group being one of the most commonly used in the Fmoc/*t*Bu approach. In contrast, the *S*-Mob group requires a high TFA concentration and harsh conditions (high temperature and long reaction times) to be fully removed. In this regard, the current gap between *S*-Trt and *S*-Mob groups captured our attention and prompted us to browse through acid-labile protecting groups to find Cys-protecting groups that, ideally, could be quantitatively removable under mild acidic conditions and, simultaneously, show compatibility with *S*-Trt for their further application in synthetic strategies for the preparation of Cys-rich peptides. Thus, three distinct scaffolds, namely diphenylmethyl, biphenylmethyl, and benzyl groups, were selected and finely tuned for this purpose. Twelve Fmoc-Cys(PG)-OH (**1a**–**l**) were prepared and incorporated into the model tripeptide Fmoc-Ala-Cys(PG)-Leu-NH_2 (**2a**–**l**),

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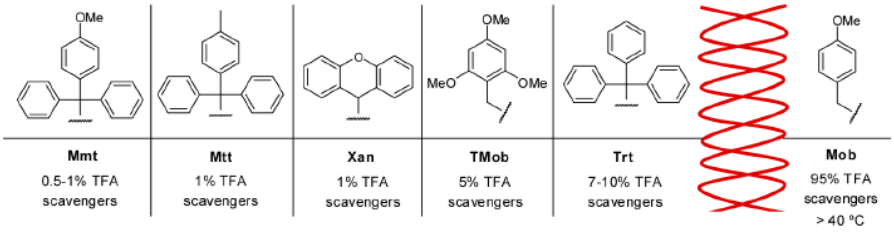
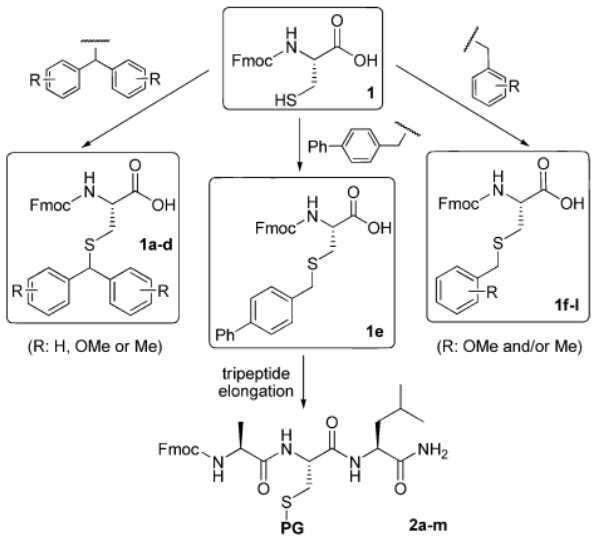


Figure 1. Acid-labile Cys-protecting groups for Fmoc/*t*Bu chemistry.

and their lability against TFA was studied and compared with the *S*-Mob group (**2m**) (Scheme 1).

Scheme 1. Preparation of Fmoc-Cys(PG)-OH Derivatives and Their Successive Incorporation into a Standard Tripeptide

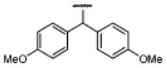
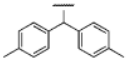
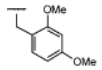
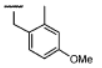
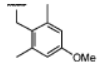
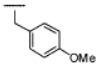


After elongation on a Sieber amide resin, the standard tripeptides were cleaved from the resin and TFA-lability studies were carried out in solution with a range of reaction times and temperatures, in the presence of 2.5% TIS and 2.5% H_2O as scavengers. The tripeptides were then analyzed by RP-HPLC to determine the percentage of deprotected Cys (Table 1).

As expected, the *S*-Mob protecting group was stable against diluted TFA treatments and required a high concentration of TFA, a longer reaction time, and an increase of temperature up to 40 °C to be totally removed (**2m**). Pleasantly, the diphenylmethyl (Dpm) group **1a**, along with two decorated benzyl moieties—**1h** with a *p*-methoxy and an *o*-methyl group and **1i** with two *o*-methoxy groups—exhibited the desired lability against TFA (**2a**, **2h**–**i**), while the others were not labile to TFA treatments (**2d**–**f**, **2j**) or were highly sensitive to TFA (**2b**–**c**, **2g**, **2k**–**l**). Furthermore, the three protecting groups **1a**, **1h**, and **1i** were stable under *S*-Trt cleavage conditions (10% TFA, **2a**, **2h**–**i**). This outcome demonstrates the compatibility of these groups with *S*-Trt for regioselective disulfide construction.

Among the three promising protecting groups for Cys, the easily synthetically accessible *S*-Dpm was chosen as an alternative to the *S*-Mob group for advanced studies.[4]

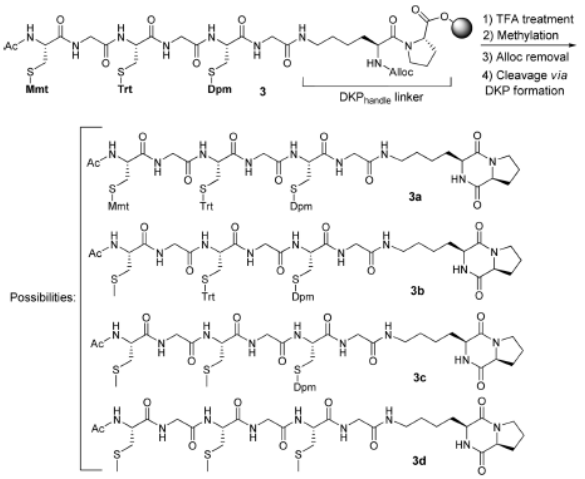
Table 1. TFA-Lability Study of the Tripeptides **2**

	PG	TFA (%)	temp (°C)	reaction time	deprotected Cys (%)
a		10	25	5 min	0
		60	25	1 h	100
b		10	25	5 min	100
c		10	25	5 min	29
		20	25	30 min	100
d		10	25	5 min	0
		95	40	2 h	0
e		10	25	5 min	0
		95	40	2 h	0
f		10	25	5 min	0
		95	40	2 h	0
g		10	25	5 min	17
		20	25	30 min	100
h		10	25	5 min	0
		50	25	1 h	100
i		10	25	5 min	0
		50	25	1 h	100
j		10	25	5 min	0
		95	25	1 h	21
k		10	25	5 min	7
		20	25	30 min	100
l		10	25	5 min	9
		20	25	30 min	100
m		10	25	5 min	0
		95	40	2 h	100

(4) *S*-Mob and *S*-Dpm groups are not compatible. Under the cleavage conditions of *S*-Dpm, 22% *S*-Mob removal was observed.

Although *S*-Dpm was described as a Cys-protecting group by Photaki et al. back in 1970,[5] use of this group in Fmoc/*t*Bu chemistry has not been tackled until now.

Scheme 2. Compatibility Study of Protecting Groups



Before further studies, the absence of racemization during Cys(Dpm) incorporation was proven (see Supporting Information (SI)). Next, the compatibility of *S*-Dpm with *S*-Trt and the highly sensitive acid-labile *S*-Mmt groups were thoroughly examined through a single experiment. Thus, a hexapeptide, which contained three Cys residues, was elongated onto a DKP$_{handle}$ linker, which allowed total free acid cleavage[6] (Scheme 2, hexapeptide **3**). After TFA treatment, the free thiol groups were methylated, and the Alloc group was then removed from the α-*N* of the Lys residue, and the peptidyl-resin was treated with piperidine/THF to render the *C*-terminal DKP$_{handle}$-protected hexapeptides (**3a**–**d**). At 10% TFA in the presence of 2.5% TIS as a scavenger, the *S*-Trt and *S*-Mmt groups were fully removed on solid phase. In contrast, *S*-Dpm was stable under these acidic conditions, requiring up to 90% TFA and 2.5% TIS for its entire removal (Table 2). It is worth mentioning that in any case the selective removal of *S*-Mmt vs *S*-Trt was not achieved, thereby showing the incompatibility of these protecting groups.[7]

After confirming the compatibility of *S*-Dpm with the *S*-Trt and *S*-Mmt groups, we were encouraged to apply the *S*-Dpm/*S*-Trt and *S*-Dpm/*S*-Mmt combinations in the protection scheme for the regioselective construction of intra- and intermolecular disulfide bridges. Thus, the regioselective syntheses of a double-chain bis-cystinyl

Table 2. Lability Study of the Acid-Labile *S*-Mmt, *S*-Trt, and *S*-Dpm Groups on Solid Phase[a]

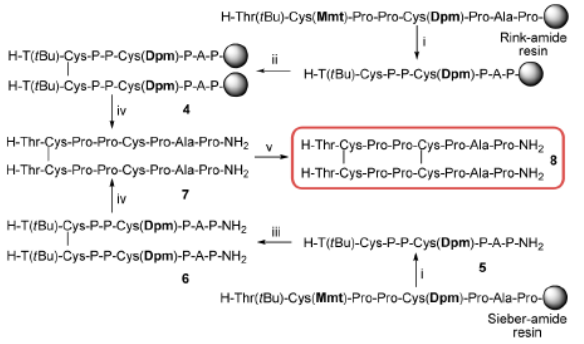
TFA (%)	scavenger	reaction time	**3a** (%)	**3b** (%)	**3c** (%)	**3d** (%)
1	–	3 × 5 min	45	45	10	–
2	–	3 × 5 min	25	50	25	–
10	2.5% TIS	3 × 5 min	–	–	100	–
60	2.5% TIS	1 h	–	–	30	70
90	2.5% TIS	1 h	–	–	–	100

[a] All experiments were carried out with 15 mg of peptidyl-resin.

fragment 225–232/225′–232′ of the human immunoglobulin G1 (IgG1)[1a,8] combining *S*-Mmt and *S*-Dpm, along with the preparation of the α-conotoxin ImI[9] combining the *S*-Trt and *S*-Dpm groups, were carried out.

The hinge fragment of IgG1 was accomplished following two strategies in parallel (Scheme 3). In the first approach (Rink-amide resin), the *S*-Mmt group was selectively removed by diluted TFA treatments and the first disulfide bond was achieved by piperidine/DMF (1:4) on solid phase. The anchored *S*-Dpm-protected dimer **4** was then treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at 25 °C, and the resultant fully deprotected intermediate **7** was redissolved in DMSO/phosphate buffer (1:4) at pH 9 to render the final cyclic parallel dimer **8** (see SI).

Scheme 3. Regioselective Syntheses of Hinge Fragment of IgG1 Combining *S*-Mmt and *S*-Dpm Protecting Groups[a]



[a] (i) TFA/TIS/CH_2Cl_2 (5:2.5:92.5) (5 × 1 min); (ii) piperidine/DMF (1:4); (iii) H_2O/ACN (1:9), 20% DMSO at pH 9; (iv) TFA/TIS/H_2O (95:2.5:2.5) for 1 h at 25 °C; and (v) DMSO/phosphate buffer (1:4) at pH 9 and 25 °C.

In the second approach (Sieber-amide resin), the linear partial *S*-Dpm-protected peptide **5** was obtained by diluted TFA treatments. Subsequently, the first disulfide bond was accomplished in solution to render the protected dimer intermediate **6**. The second disulfide bridge was achieved as described before to render the bis-cystinyl parallel dimer **8** (Figure 2).

(5) Photaki, I.; Taylor-Papadimitrious, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc. (C)* **1970**, 2683.
(6) Góngora-Benítez, M.; Cristau, M.; Giraud, M.; Tulla-Puche, J.; Albericio, F. *Chem. Commun.* **2012**, *48*, 2313.
(7) Although it has been reported in the literature that *S*-Mmt can be selectively removed in the presence of *S*-Trt, actually it has no practical use because the safety window is so narrow that conditions should be carefully optimized and these are, therefore, not of general application.
(8) Wünsch, E.; Moroder, L.; Göhring-Romani, S.; Musiol, H.-J.; Göhring, W.; Bovermann, G. *Int. J. Peptide Protein Res.* **1988**, *32*, 268.
(9) (a) McIntosh, J. M; Yoshikami, D.; Mahe, E.; Nielsen, D. B.; Rivier, J. E.; Gray, W. R.; Olivera, B. M. *J. Biol. Chem.* **1994**, *269*, 16733. (b) Nielsen, J. S.; Buczed, P.; Bulaj, G. *J. Pept. Sci.* **2004**, *10*, 249.

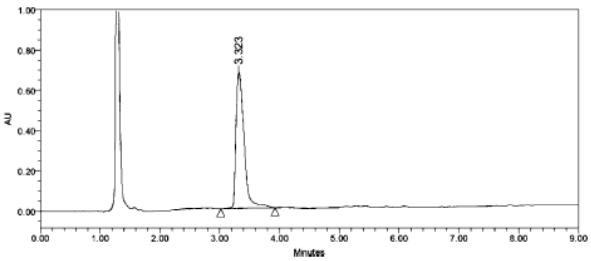
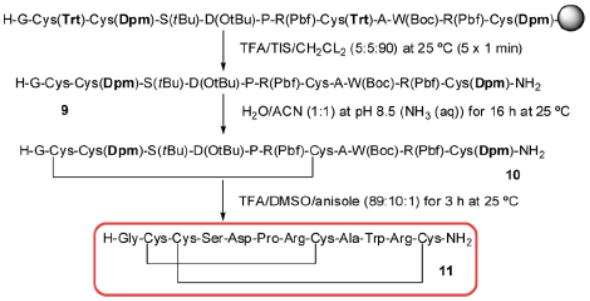


Figure 2. RP-HPLC analysis of the synthesized bis-cystinyl parallel dimer **8** from the Sieber-amide approach.

Although oxidative folding conditions can be carefully refined to provide a major isomer, the regioselective synthesis of the two-disulfide-containing α-conotoxin family[10] members allows the correct construction of disulfide bond pattern present in their biologically active isomers to be ensured.[9,11] Thus, the α-conotoxin ImI, a 12-mer peptide, which contains two disulfide bridges (2Cys-8Cys and 3Cys-12Cys), was prepared by combining two *S*-Dpm and two *S*-Trt for the protection of the Cys residues (Scheme 4). After completion of the peptide elongation

Scheme 4. Regioselective Synthesis of α-Conotoxin ImI Combining *S*-Trt and *S*-Dpm Protecting Groups



on a Sieber-amide resin, the partial *S*-Dpm-protected intermediate **9** was cleaved from the resin by diluted TFA treatments and the construction of the first disulfide bond was achieved in H_2O/ACN (3:7) at pH 8 for 16 h at 25 °C, as determined by RP-HPLC analysis (see SI). At this

(10) (a) Myers, R. A.; Zafaralla, G. C.; Gray, W. R.; Abbott, J.; Cruz, L. J.; Olivera, B. M. *Biochemistry* **1991**, *30*, 9370. (b) Arias, H. R.; Blanton, M. P. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1017. (c) Janes, R. W. *Curr. Opin. Pharmacol.* **2005**, *5*, 280.

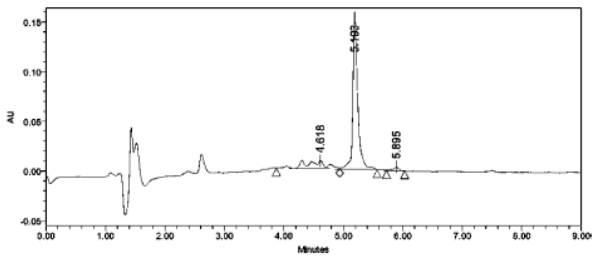


Figure 3. RP-HPLC analysis of the synthesized α-conotoxin ImI **11** combining *S*-Dpm and *S*-Trt protecting groups.

point, various conditions were attempted to obtain the final bicyclic peptide. When the total deprotection and oxidation steps were performed consecutively, a mixture of two isomers was identified by RP-HPLC and RP-HPLC-ESMS analysis, while a single peak corresponding to the expecting isomer **11** was observed when the two steps were performed following a one-pot strategy (Figure 3).

In summary, the *S*-Dpm protecting group is an alternative to the *S*-Mob group. *S*-Dpm can be fully deblocked under the standard conditions used for cleavage and total deprotection steps in Fmoc chemistry, and it is fully compatible with two commonly used acid-labile protecting groups such as *S*-Trt and *S*-Mmt. Here we successfully applied *S*-Dpm for the regioselective synthesis of peptides containing intra- and intermolecular disulfide bonds. These results could be extrapolated to the other two protecting groups **1h** and **1i**.

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Supporting Information Available. Experimental procedures, compound characterization, RP-HPLC analyses, and spectral data of the Fmoc-Cys(PG)-OH derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

(11) (a) Walewska, A.; Bulaj, G. Mechanisms of the Oxidative Folding of Small, Disulfide-Rich Peptides. In *Oxidative Folding of Proteins and Peptides*; Moroder, L., Ed.; Royal Chemical Society: Cambridge, U.K., 2008. (b) Bulaj, G.; Olivera, B. M. *Antioxid. Redox Signal.* **2008**, *10*, 141.

The authors declare no competing financial interest.

Acid-Labile Cys-Protecting Groups for the Fmoc/*t*Bu Strategy: Filling the Gap

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Abbreviations

2MeOBn – 2-methoxybenzyl; 2,4diMeOBn – 2,4-dimethoxybenzyl; 2,6diMeOBn – 2,6-dimethoxybenzyl; 2,6diMeO-4MeBn – 2,6-dimethoxy4-methylbenzyl; 2,6diMe-4MeOBn – 4-methoxy2,6-dimethylbenzyl; 4,4'diMeODpm – 4,4'-dimethoxydiphenylmethyl; 4,4'diMeDpm – 4,4'-dimethyldiphenylmethyl; 9F – 9*H*-fluorenyl; Alloc – allyloxycarbonyl; Boc – *tert*-butoxycarbonyl; Bpm – biphenylmethyl; DIEA – *N,N'*-diisopropylethylamine, DMF – *N,N'*-dimethylformamide, DMSO - dimethyl sulfoxide; DIPCDI – *N,N'*-diisopropylcarbodiimide; Dpm – diphenylmethyl; Fmoc – 9-fluorenylmethyl carbamate; Mob – 4-methoxybenzyl; Oxyma Pure – ethyl 2-cyano-2-(hydroxyimino)acetate; Pbf – 2,2,4,6,7- pentamethyldihydrobenzofurane-5-sulfonyl; PG – protecting group; *t*Bu – *tert*-butyl; TFA – trifluoroacetic acid; TMeb – 2,4,6-trimethylbenzyl.

General information

Benzhydrol, 4-biphenylmethanol, 4,4'-dimethylbenzophenone, 4,4'-dimethoxybenzhydrol, 2,4,6-trimethylbenzaldehyde and 2,6-dimethoxy-4-methylbenzaldehyde were purchased from Alfa Aesar (Karlsruhe, Germany), 9-hydroxyfluorene, 2,4-dimethoxybenzyl alcohol and 2-methoxybenzyl alcohol were procured from Sigma-Aldrich (St Louis, MO), 4-methoxy-2-methylbenzaldehyde from Acros (Geel, Belgium), 2,6-dimetoxybenzaldehy from TCI (Zwijndrecht, Belgium), and 2,6-dimethyl-4-hydroxybenzaldehyde from Fluorochem (Hadfield, UK). Commercial available Fmoc-amino acid derivatives and Fmoc-Rink amide polystyrene resin were acquired from IRIS Biotech (Marktredwitz, Germany), Fmoc-Sieber amide resin was obtained from Merck (Darmstadt, Germany), DIEA, DIPCDI and TFA were procured from Aldrich (Milwaukee, WI), Oxyma Pure from Luxembourg Industries Ltd. (Tel Aviv, Israel), and $NaBH_4$, $(CH_3)_2SO_4$, NH_4HCO_3, KH_2PO_4 and $K_2HPO_4{\cdot}3H_2O$ from Sigma-Aldrich (St Louis, MO). DMF, CH_2Cl_2, Et_2O, DMSO, piperidine and ACN (HPLC grade) were purchased from SDS (Peypin, France), anisole from Fluka Chemika (Buchs, Switzerland), and NH_3 aqueous solution (32%, w/w) from Scharlau (Barcelona, Spain). All commercial reagents and solvents were used as received.

Solid-phase peptide synthesis (SPPS)

Solid-phase syntheses were carried out manually in polypropylene syringes fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine-DMF (1:4, v/v) (1 × 1 min, 2 × 5 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Peptide synthesis transformations and washes were performed at 25 °C.

Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

XBridge™ BEH130 C18 reversed-phase HPLC analytical column (4.6 mm x 100 mm, 3.5 μm) was obtained from Waters (Ireland). Analytical RP-HPLC was performed on a Waters instrument comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller (Empower). UV detection was at 220 nm, and linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at a flow rate of 1.0 mL·min^{-1} over 8 min.

Reverse phase-high performance liquid chromatography-electrospray mass spectrometry (RP-HPLC-ESMS) analysis

SunFire™ C18 reversed-phase HPLC analytical column (2.1 mm x 100 mm, 5 μm) was procured from Waters (Ireland). Analytical RP-HPLC-ESMS was performed on a Waters Micromass ZQ spectrometer comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller MassLynx v. 4.1). UV detection was at 220 nm, mass scans were acquired in positive ion mode, and linear gradients of ACN (+0.07% formic acid) into H_2O (+0.1% formic acid) were run at a flow rate of 0.3 mL·min^{-1} over 8 min.

Semi-preparative RP-HPLC

SunFire™ Prep C18 OBD™ reversed-phase HPLC analytical column (19 x 100 mm, 5 μm) was acquired from Waters (Ireland). Semi-preparative RP-HPLC was performed on a Waters Delta 600 system comprising a sample manager (Waters 2700), a controller (Waters 600), a dual λ absorbance detector (Waters 2487), a fraction collector II, and a software system controller (MassLynx). UV detection was at 220 and 254 nm, and linear gradients of ACN (+0.1% TFA) into H_2O (+0.1% TFA) were run at a flow rate of 16 mL·min^{-1} over 30 min.

CombiFlash Rf 200 system

Normal mode: crude residue and silica media (1.5 mL media/200 mg crude reside) were dissolved in CH_2Cl_2, concentrated, and the resultant solid samples were eluded on a 40 g Redi*Sep* Rf GOLD silica column (Teledyne Isco) on a CombiFlash Rf 200 system (Teledyne Isco, Lincoln). Prior to each run, the column was equilibrated with 5 column volume of hexane. UV detection was at 254 nm, and gradients of ethyl acetate into hexane were run at a flow rate of 40 mL·min^{-1}.

Reverse mode: crude residues and C18 media (1.5 mL media/200 mg crude residue) were dissolved in CH_2Cl_2, concentrated, and the resultant solid samples were eluded on a 13 g Redi*Sep* Rf GOLD C18 column (Teledyne Isco, Lincoln) on a Combi*Flash* Rf 200 system (Teledyne Isco, Lincoln). Prior to each run, the column was equilibrated with 3 column volume of H_2O (+0.045% TFA). UV

detection was at 220 and 254 nm, and gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at a flow rate of 30 $mL \cdot min^{-1}$.

High-resolution mass spectrometry (HRMS) analysis

HRMS analyses were performed on a LTQ-FT Ultra Mass Spectrometer (Thermo Scientific) equipped with an automated nanoelectrospray NanoMate (Advion BioSciences, Ithaca, NY, USA). Spray voltage was 1.75 kV and delivery pressure was 0.50 psi. MS conditions: NanoESI, positive ionization, capillary temperature 200 °C, tube lens 100 V and *m/z* 450-1200 a.m.u. Samples were acquired with Xcalibur software (vs. 2.0SR2), elemental compositions from experimental exact mass monoisotopic values were obtained with Xaclibur software (vs. 2.1.0 SP1), and ion deconvolution to zero charged monoisotopic masses was performed with Xract algorithm in Xcalibur software (vs. 2.1.0 SP1).

Fourier transform Infrared (FTIR) spectrometry

FTIR spectra were acquired on a Thermo Nicolet NEXUS™ 670 Series FTIR spectrometer (Thermo Scientific, Waltham, MA) equipped with OMNIC 6.0 software. Samples were dissolved in CH_2Cl_2 and deposited on a KBr window, the solutions were then evaporated to dryness and the thin films formed on the window were analyzed directly.

Nuclear magnetic resonance (NMR) spectrometry

NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer, operating at 400 and 101 MHz for ^1H and ^{13}C, respectively. Chemical shifts (δ) are reported in ppm relative to residual signals ($CDCl_3$: 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR; DMSO-d_6 : 2.50 ppm for ^1H NMR and 39.52 ppm for ^{13}C NMR). Multiplicities are indicated using the following abbreviations: s = singlet, d = doublet, t = triplet, p = pentet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, qd = quartet of doublets, ddd = doublet of doublet of doublets, tdd = triplet of doublet of doublets, and m = multiplet.

Experimental section

Fmoc-Cys(PG)-OH derivatives (1a-l)

All Fmoc-Cys(PG)-OH compounds were prepared from the Fmoc-Cys-OH **1** and the corresponding alcohol, except for the 4-biphenylmethyl and 2-methoxybenzyl derivatives that were synthesized from the corresponding bromide compound. In any case, the reaction conditions of the protection of the β-thiol function of any Fmoc-Cys(PG)-OH derivative were optimized.

General procedure for the reduction of aldehydes and ketones

To a suspension of aldehyde (6 mmol) in 2-propanol (30 mL) was added $NaBH_4$ (1.6 mmol) and the mixture was stirred at 25 °C for 16 h. After addition of H_2O (2 mL), the mixture was evaporated under reduced pressure and the crude residue was extracted with Et_2O (2 × 20 mL). The organic layer was then washed with H_2O (3 × 10 mL) and brine (3 × 10 mL), dried over $MgSO_4$, filtered and finally the Et_2O was removed under reduced pressure to obtain the desired alcohols.

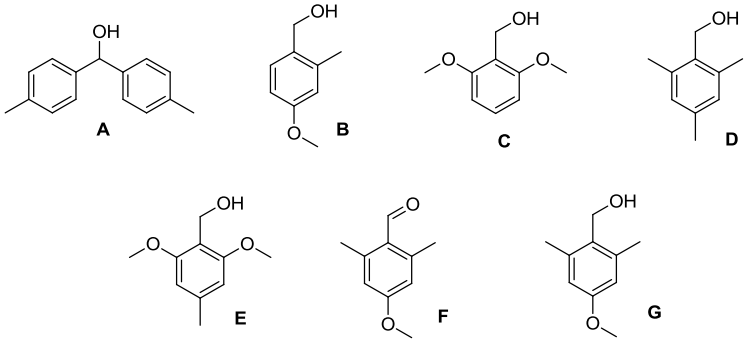
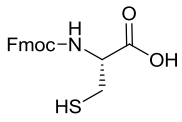


Figure S1. Intermediates involved in the synthesis of the Fmoc-Cys(PG)-OH derivatives.

Preparation of Fmoc-Cys-OH (1)

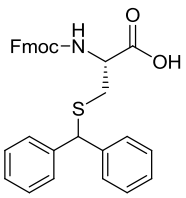


To a suspension of Fmoc-Cys(Trt)-OH (5.0 g, 8.5 mmol) in CH_2Cl_2 (340 mL) was added TIS (10 mL) and TFA (40 mL). The reaction mixture was stirred at 25 °C for 30 min, and the solvent was removed under reduced pressure. The crude was then washed with hexane (120 mL) and centrifuged (× 5) to afford Fmoc-Cys-OH **1** (2.9 g, 98% yield) as a white powder.

^1H NMR (400 MHz, DMSO-d_6): δ 12.79 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.73 (d, *J* = 7.3 Hz, 2H), 7.68 (d, *J* = 8.3 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.32 (td, *J* = 7.4, 1.0 Hz, 2H), 4.31 (d, *J* = 6.5 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 4.12 (td, *J* = 8.4, 4.4 Hz, 1H), 2.89 (ddd, *J* = 13.1,

8.4, 4.4 Hz, 1H), 2.73 (dt, *J* = 13.6, 8.5 Hz, 1H), 2.49 (t, *J* = 8.4 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 171.9, 156.1, 143.8, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 3065, 1717, 1520, 1449, 1335, 1215, 1073, 758, 739. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.1 min). RP-HPLC-ESMS showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.5 min; *m/z* calcd for $C_{18}H_{17}NO_4S$, 343.4; found, 344.5 $[M+H]^+$, where M is the MW of Fmoc-Cys-OH **1**).

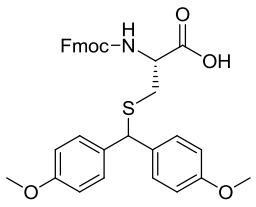
Preparation of Fmoc-Cys(Dpm)-OH (1a)



Fmoc-Cys-OH **1** (2.9 g, 8.4 mmol) and benzhydrol (1.6 g, 8.4 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (25 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 40 g Redi*Sep* Rf GOLD silica column on a Combi*Flash* Rf 200 system following by concentration under reduced pressure to afford Fmoc-Cys(Dpm)-OH **1a** (3.36 g, 78% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.4 Hz, 2H), 7.59 (t, *J* = 6.6 Hz, 2H), 7.37 (d, *J* = 7.0 Hz, 6H), 7.28 (dt, *J* = 7.9, 6.7 Hz, 6H), 7.21 (dd, *J* = 10.5, 3.5 Hz, 2H), 5.54 (d, *J* = 7.9 Hz, 1H), 5.21 (s, 1H), 4.56 (dd, *J* = 12.1, 5.3 Hz, 1H), 4.40 (d, *J* = 6.8 Hz, 2H), 4.22 (t, *J* = 6.7 Hz, 1H), 2.87 (qd, *J* = 13.9, 5.5 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.0, 156.1, 143.8, 141.5, 140.6, 128.8, 128.4, 127.9, 127.6, 127.3, 125.2, 120.2, 67.5, 54.7, 53.4, 47.2, 34.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3062, 3011, 1718, 1515, 1449, 1418, 1336, 1243, 1104, 1077, 1052, 758, 739, 701, 621. **HRMS** calcd for $C_{31}H_{27}NNaO_4S$, 532.15530; found, 532.15646 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.3 min; *m/z* calcd for $C_{31}H_{27}NO_4S$, 509.6; found, 510.3 $[M+H]^+$, where M is the MW of the Fmoc-Cys(Dpm)-OH **1a**).

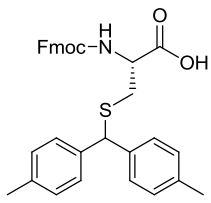
Preparation of Fmoc-Cys(4,4'diMeODpm)-OH (1b)



Fmoc-Cys-OH **1** (1 g, 2.9 mmol) and 4,4'-dimethoxybenzhydrol (700 mg, 2.9 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 80% to 90% ACN) following by lyophilization to afford Fmoc-Cys(4,4'diMeODpm)-OH **1b** (353 mg, 21% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.3 Hz, 2H), 7.60 (t, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.2 Hz, 2H), 7.31 – 7.22 (m, 6H), 6.80 (dd, *J* = 8.3, 3.9 Hz, 4H), 5.56 (d, *J* = 7.9 Hz, 1H), 5.14 (s, 1H), 4.57 (dd, *J* = 12.6, 5.7 Hz, 1H), 4.48 – 4.28 (m, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.74 (s, 3H), 3.73 (s, 3H), 2.85 (qd, *J* = 14.1, 5.6 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.3, 158.9, 156.1, 143.9, 141.4, 132.9, 129.5, 129.5, 127.9, 127.3, 125.2, 120.1, 114.2, 67.6, 55.4, 53.5, 47.2, 34.1. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3065, 2954, 1718, 1607, 1508, 1450, 1301, 1247, 1175, 1033, 759, 740. **HRMS** calcd for $C_{33}H_{31}NNaO_6S$, 592.17643; found, 592.17722 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.6 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.1 min; *m/z* calcd for $C_{33}H_{31}NO_6S$, 569.67; found, 797.2 $[M+C_{15}H_{15}O_2]^+$, where M is the MW of the Fmoc-Cys(4,4'diMeODpm)-OH **1b**).

Preparation of Fmoc-Cys(4,4'diMeDpm)-OH (1c)



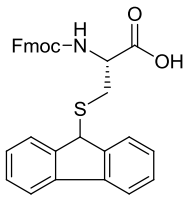
Fmoc-Cys-OH **1** (1.3 g, 3.7 mmol) and the previously prepared 4,4'-dimethybenzhydrol **A** (800 mg, 3.7 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure. The solid crude was then dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 85% to 95% ACN) following by lyophilization to afford Fmoc-Cys(4,4'diMeDpm)-OH **1c** (1.0 g, 52% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (t, *J* = 6.5 Hz, 2H), 7.38 (t, *J* = 7.1 Hz, 2H), 7.33 – 7.20 (m, 6H), 7.07 (d, *J* = 7.6 Hz, 2H), 5.15 (s, 1H), 4.57 (dd, *J* = 12.2, 5.2 Hz, 1H), 4.40 (p, *J* = 10.3 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 1H), 2.95 – 2.80 (m, 2H), 2.27 (s, 3H), 2.27 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.5, 156.2, 143.9, 141.4, 137.8, 137.3, 129.5, 128.2, 127.9, 127.3, 125.2, 120.2, 67.6, 54.2, 53.4, 47.2, 34.1, 21.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3020, 2920, 1719, 1509, 1449, 1418, 1336, 1212, 1106, 1051, 758, 739.

HRMS calcd for $C_{33}H_{31}NNaO_4S$, 560.18660; found, 560.18815 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.8 min; *m/z* calcd for $C_{33}H_{31}NO_4S$, 537.67; found, 733.0 $[M+C_{15}H_{15}]^+$, where M is the MW of the Fmoc-Cys(4,4'diMeDpm)-OH **1c**).

The 4,4'-dimethylbenzhydrol **A** (*Figure S1*) was prepared in 87% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 7.4 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 9.5 min; *m/z* calcd for $C_{15}H_{16}O$, 212.29; found, 195.3 $[M\text{-}OH]^+$, where M is the MW of alcohol **A**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.24 (d, *J* = 8.0 Hz, 4H), 7.13 (d, *J* = 7.9 Hz, 4H), 5.76 (s, 1H), 2.32 (s, 6H), 2.16 (s, *J* = 3.8 Hz, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 141.3, 137.3, 129.3, 126.6, 76.1, 21.2.

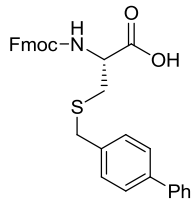
Preparation of Fmoc-Cys(9F)-OH (1d)



Fmoc-Cys-OH **1** (500 mg, 1.4 mmol) and 9-hydroxyfluorene (263 mg, 1.4 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (5 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(9F)-OH **1d** (256 mg, 36% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.69 (t, *J* = 8.5 Hz, 4H), 7.53 (d, *J* = 7.6 Hz, 2H), 7.50 (d, *J* = 8.5 Hz, 1H), 7.45 (t, *J* = 6.7 Hz, 2H), 7.22 (t, *J* = 7.1 Hz, 4H), 7.19 – 7.09 (m, 4H), 5.02 (s, 1H), 4.15 – 4.01 (m, 3H), 3.72 (dd, *J* = 15.5, 7.3 Hz, 1H), 2.36 – 2.32 (m, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 174.2, 156.0, 143.7, 141.5, 140.7, 134.9, 129.2, 128.5, 128.0, 127.9, 127.3, 125.6, 124.5, 120.2, 67.4, 53.0, 48.7, 47.2, 29.7. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3063, 1716, 1507, 1449, 1419, 1335, 1213, 1050, 736. **HRMS** calcd for $C_{31}H_{25}NNaO_4S$, 530.13965; found, 530.14081 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.6 min; *m/z* calcd for $C_{31}H_{25}NO_4S$, 507.60; found, 508.2 $[M+H]^+$, where M is the MW of the Fmoc-Cys(9F)-OH **1d**).

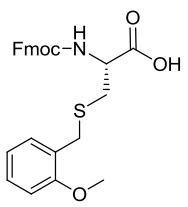
Preparation of Fmoc-Cys(Bpm)-OH (1e)



Fmoc-Cys-OH **1** (747 mg, 2.1 mmol) and 4-(bromomethyl)biphenyl (522 mg, 2.1 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (50 mL). Then, DIEA (2.5 mL, 5%) was added, and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(Bpm)-OH **1e** (418 mg, 39% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.80 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.78 (d, *J* = 8.4 Hz, 1H), 7.74 (d, *J* = 7.4 Hz, 2H), 7.63 (d, *J* = 7.3 Hz, 2H), 7.59 (d, *J* = 8.2 Hz, 2H), 7.47 – 7.29 (m, 9H), 4.36 – 4.16 (m, 4H), 3.81 (s, 2H), 2.86 (dd, *J* = 13.6, 4.6 Hz, 1H), 2.72 (dd, *J* = 13.6, 9.4 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.3, 156.0, 143.8, 140.7, 139.8, 138.7, 137.6, 129.5, 128.9, 127.6, 127.4, 127.1, 126.6, 126.5, 125.3, 120.1, 65.8, 53.7, 46.6, 34.9, 32.3. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3028, 1720, 1516, 1487, 1449.69, 1234.68, 1052.15, 759.66, 738.98, 698.31. **HRMS** calcd for $C_{31}H_{27}NNaO_4S$, 532.15530; found, 532.15729 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.2 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.6 min; *m/z* calcd for $C_{31}H_{27}NO_4S$, 509.62; found, 510.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(Bpm)-OH **1e**).

Preparation of Fmoc-Cys(2MeOBn)-OH (1f)

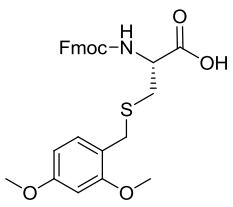


Fmoc-Cys-OH **1** (1.2 g, 3.5 mmol) and 1-(bromomethyl)-2-methoxybenzene (1.1 g, 5.4 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (100 mL). Then, DIEA (5 mL, 5%) was added, and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 65% to 80% ACN) following by lyophilization to afford Fmoc-Cys(2MeOBn)-OH **1f** (345 mg, 21% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.62 – 7.54 (m, 2H), 7.37 (t, *J* = 7.3 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 7.21 (t, *J* = 7.1 Hz, 2H), 6.89 (t, *J* = 7.3 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 5.73 (d, *J* = 7.6 Hz, 1H), 4.62 (d, *J* = 5.5 Hz, 1H), 4.44 (d, *J* = 6.9 Hz, 2H),

4.22 (t, *J* = 6.8 Hz, 1H), 3.77 (s, *J* = 7.1 Hz, 3H), 3.75 (s, 2H), 2.94 (qd, *J* = 14.3, 5.3 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 157.3, 156.3, 143.8, 141.5, 130.6, 128.9, 127.9, 127.2, 126.1, 125.2, 120.9, 120.1, 110.9, 67.3, 55.6, 53.9, 47.3, 33.5, 31.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 3064, 2938, 2938, 1719, 1513, 1493, 1450, 1290, 1245, 1103, 1049, 740. **HRMS** calcd for $C_{26}H_{25}KNO_5S$, 502.10850; found, 502.10898 $[M+K]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.9 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.9 min; *m/z* calcd for $C_{26}H_{25}NO_5S$, 463.55; found, 464.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2MeOBn)-OH **1f**).

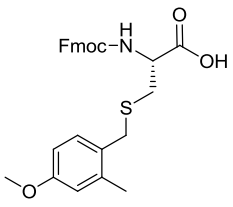
Preparation of Fmoc-Cys(2,4diMeOBn)-OH (1g)



Fmoc-Cys-OH **1** (1.1 g, 3.2 mmol) and 2,4-dimethoxybenzyl alcohol (1.2 g, 7.1 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (25 mL). TFA (50 μL, 0.2%) was then added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(2,4diMeOBn)-OH **1g** (300 mg, 19% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.63 – 7.57 (m, 2H), 7.40 – 7.35 (m, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 7.11 (d, *J* = 8.8 Hz, 1H), 6.42 (d, *J* = 2.1 Hz, 2H), 6.41 (d, *J* = 2.3 Hz, 1H), 5.74 (d, *J* = 7.9 Hz, 1H), 4.63 (dd, *J* = 12.6, 5.8 Hz, 1H), 4.43 (d, *J* = 7.0 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.76 (s, 3H), 3.76 (s, 3H), 3.71 (d, *J* = 1.7 Hz, 2H), 2.93 (qd, *J* = 14.4, 5.7 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 160.5, 158.3, 156.3, 143.8, 141.4, 131.1, 127.9, 127.2, 125.2, 120.1, 118.4, 104.6, 98.9, 67.4, 55.6, 55.5, 53.8, 47.3, 33.4, 30.9. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3065, 2937, 1718, 1611, 1506, 1450, 1329, 1208, 1156, 1040, 740. **HRMS** calcd for $C_{27}H_{27}NNaO_6S$, 516.14513; found, 516.14643 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.8 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.3 min; *m/z* calcd for $C_{27}H_{27}NO_6S$, 493.57; found, 494.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,4diMeOBn)-OH **1g**).

Preparation of Fmoc-Cys(4MeO-2MeBn)-OH (1h)

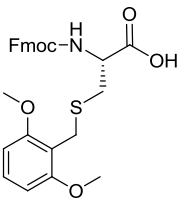


Fmoc-Cys-OH **1** (160 mg, 0.6 mmol) and the previously prepared 4-methoxy2-methylbenzyl alcohol **B** (70 mg, 0.46 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (5 mL). Then TFA (50 μL, 1%) was added, and the mixture was then stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under vacuum and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(4MeO-2MeBn)-OH **1h** (135 mg, 61% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (dd, *J* = 7.4, 3.0 Hz, 2H), 7.63 – 7.54 (m, 2H), 7.41 – 7.34 (m, 2H), 7.32 – 7.26 (m, 2H), 7.07 (d, *J* = 8.3 Hz, 1H), 6.70 (d, *J* = 2.1 Hz, 1H), 6.64 (dd, *J* = 8.2, 1.8 Hz, 1H), 5.58 (d, *J* = 7.8 Hz, 1H), 4.61 (dd, *J* = 12.2, 5.6 Hz, 1H), 4.42 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.74 (s, *J* = 21.7 Hz, 3H), 3.68 (s, 2H), 2.96 (qd, *J* = 14.1, 5.3 Hz, 2H), 2.32 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.2, 159.1, 156.2, 143.7, 141.4, 138.4, 131.1, 127.9, 127.3, 127.3, 125.2, 120.2, 116.7, 111.0, 67.5, 55.3, 53.6, 47.2, 34.7, 33.7, 19.5. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3320, 3065, 2950, 1718, 1608, 1501, 1449, 1256, 1202, 1049, 759, 739. **HRMS** calcd for $C_{27}H_{27}NNaO_5S$, 500.15021; found, 500.15121 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.7 min; *m/z* calcd for $C_{27}H_{27}NO_5S$, 477.57; found, 478.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(4MeO-2MeBn)-OH **1h**).

The 4-methoxy2-methylbenzyl alcohol **B** (*Figure S1*) was prepared in 82% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 4.9 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.6 min; *m/z* calcd for $C_9H_{12}O_2$, 152.19; found, 135.2 $[M-OH]^+$, where M is the MW of alcohol **B**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.22 (d, *J* = 8.2 Hz, 1H), 6.74 (d, *J* = 2.6 Hz, 1H), 6.71 (dd, *J* = 8.2, 2.6 Hz, 1H), 4.62 (s, 2H), 3.79 (s, *J* = 3.0 Hz, 3H), 2.36 (s, *J* = 18.9 Hz, 3H), 1.55 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 159.4, 138.2, 131.3, 129.7, 116.4, 110.9, 63.4, 55.4, 19.1.

Preparation of Fmoc-Cys(2,6diMeOBn)-OH (1i)

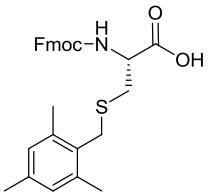


Fmoc-Cys-OH **1** (300 mg, 0.86 mmol) and the previously prepared 2,6-dimethoxybenzyl alcohol **C** (178 mg, 1.0 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (200 μL, 2%) was then added, and the mixture was stirred for 6 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under vacuum and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 13 g Redi*Sep* Rf Gold C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(2,6diMeOBn)-OH **1i** (141 mg, 33% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 8.5 Hz, 1H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.30 (tdd, *J* = 7.4, 2.7, 1.0 Hz, 2H), 7.18 (t, *J* = 8.3 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 2H), 4.35 – 4.25 (m, 2H), 4.26 – 4.20 (m, 2H), 3.73 (d, *J* = 12.7 Hz, 1H), 3.73 (s, 6H), 3.60 (d, *J* = 12.7 Hz, 1H), 2.85 (dd, *J* = 13.8, 4.8 Hz, 1H), 2.64 (dd, *J* = 13.8, 9.6 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.6, 157.6, 156.0, 143.8, 140.7, 128.3, 127.6, 127.1, 125.3, 120.1, 114.5, 103.9, 65.7, 55.7, 53.8, 46.6, 39.5, 32.8, 23.0. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 2938, 2836, 1715, 1595, 1521, 1475, 1257, 1100, 739. **HRMS** calcd for $C_{27}H_{27}NNaO_6S$, 516.14513; found, 516.14612 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{27}H_{27}NO_6S$, 493.57; found, 494.8 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,6diMeOBn)-OH **1i**).

The 2,6-dimethoxybenzyl alcohol **C** (*Figure S1*) was prepared in 68% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 4.9 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.6 min; *m/z* calcd for $C_9H_{12}O_3$, 168.19; found, 151.2 $[M-OH]^+$, where M is the MW of the alcohol **C**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.22 (t, *J* = 8.4 Hz, 1H), 6.56 (d, *J* = 8.4 Hz, 2H), 4.79 (s, 2H), 3.84 (s, 6H), 2.47 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 158.5, 129.3, 117.2, 103.9, 55.9, 54.8.

Preparation of Fmoc-Cys(TMeb)-OH (1j)

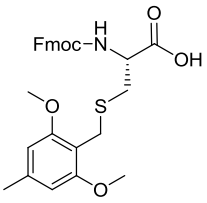


Fmoc-Cys-OH **1** (270 mg, 0.78 mmol) and the previously prepared 2,4,6-trimethylbenzyl alcohol **D** (118 mg, 0.78 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL x 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(TMeb)-OH **1j** (191 mg, 51% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (t, *J* = 6.9 Hz, 2H), 7.62 – 7.54 (m, 2H), 7.37 (dd, *J* = 15.6, 7.9 Hz, 2H), 7.28 (tdd, *J* = 7.5, 2.6, 1.2 Hz, 2H), 6.79 (s, 2H), 5.63 (d, *J* = 7.4 Hz, 1H), 4.67 (d, *J* = 5.8 Hz, 1H), 4.43 (d, *J* = 6.5 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 1H), 3.76 (s, 2H), 3.08 (qd, *J* = 14.2, 4.7 Hz, 2H), 2.31 (s, 6H), 2.22 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 156.1, 143.7, 141.5, 137.1, 137.0, 130.2, 129.2, 127.9, 127.3, 125.2, 120.1, 67.4, 53.8, 47.2, 35.0, 32.1, 21.1, 19.7. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3319, 2948, 1718, 1513, 1449, 1335, 1202, 1051, 853, 739. **HRMS** calcd for $C_{28}H_{29}NNaO_4S$, 498.17095; found, 498.17139 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.4 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.8 min; *m/z* calcd for $C_{28}H_{29}NO_4S$, 475.60; found, 476.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(TMeb)-OH **1j**).

The 2,4,6-trimethylbenzyl alcohol **D** (*Figure S1*) was prepared in 65% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.1 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 8.0 min; *m/z* calcd for $C_{10}H_{14}O$, 150.22; found, 133.2 $[M-OH]^+$, where M is the MW of the alcohol **D**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.86 (s, 2H), 4.68 (s, 2H), 2.38 (s, 6H), 2.26 (s, *J* = 10.6 Hz, 3H), 1.34 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 137.8, 137.4, 133.9, 129.3, 59.3, 21.1, 19.5.

Preparation of Fmoc-Cys(2,6diMeO-4MeBn)-OH (1k)

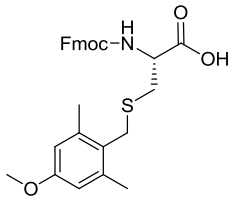


Fmoc-Cys-OH **1** (300 mg, 0.86 mmol) and the previously prepared 2,6-dimethoxy4-dimethylbenzyl alcohol **E** (188 mg, 0.86 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (20 μL, 0.2%) was then added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL × 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k** (175 mg, 40% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.6 Hz, 2H), 7.65 – 7.55 (m, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.32 – 7.25 (m, 2H), 6.35 (s, 2H), 5.88 (d, *J* = 7.8 Hz, 1H), 4.64 (dd, *J* = 11.5, 7.0 Hz, 1H), 4.44 (d, *J* = 6.9 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.85 (d, *J* = 12.6 Hz, 1H), 3.77 (s, *J* = 25.7 Hz, 6H), 3.74 (s, *J* = 8.2 Hz, 1H), 2.99 (dd, *J* = 14.4, 4.1 Hz, 1H), 2.86 (dd, *J* = 14.1, 7.2 Hz, 1H), 2.31 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.6, 158.0, 156.5, 143.8, 141.4, 139.0, 127.8, 127.2, 125.2, 120.1, 111.6, 104.9, 67.3, 55.9, 54.3, 47.3, 33.2, 24.5, 22.3. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3345, 2938, 1721, 1609, 1588, 1450, 1413, 1243, 1186, 1107, 1049, 815, 739. **HRMS** calcd for $C_{28}H_{29}NNaO_6S$, 530.16078; found, 530.16193 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.8 min; *m/z* calcd for $C_{28}H_{29}NO_6S$, 507.60; found, 508.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k**).

The 2,6-dimethoxy4-methylbenzyl alcohol **E** (*Figure S1*) was prepared in 81.2% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.5 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{10}H_{14}O_3$, 182.22; found, 165.2 $[M-OH]^+$, where M is the MW of the alcohol **E**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.38 (s, 2H), 4.74 (s, 2H), 3.82 (s, 6H), 2.34 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 158.3, 139.6, 114.3, 104.8, 55.8, 54.7, 22.3.

Preparation of Fmoc-Cys(4MeO-2,6diMeBn)-OH (1l)



Fmoc-Cys-OH **1** (400 mg, 1.2 mmol) and the previously prepared 4-methoxy2,6-dimethylbenzyl alcohol **G** (194 mg, 1.2 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (30 μL, 0.3%) was then added, and the mixture was stirred for 6 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL x 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l** (400.4 mg, 67% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.94 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.77 (d, *J* = 8.5 Hz, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.30 (qd, *J* = 7.6, 1.0 Hz, 2H), 6.57 (s, 2H), 4.35 – 4.28 (m, 2H), 4.28 – 4.21 (m, 1H), 3.77 (s, 2H), 3.68 (s, 3H), 3.00 (dd, *J* = 13.8, 4.6 Hz, 1H), 2.81 (dd, *J* = 13.8, 9.7 Hz, 1H), 2.30 (s, 6H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.4, 157.7, 156.0, 143.8, 140.7, 138.2, 127.6, 127.1, 125.8, 125.2, 120.1, 113.3, 65.7, 54.8, 54.1, 46.6, 39.5, 33.8, 30.5, 19.4. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3321, 2948, 1718, 1603, 1486, 1321, 1197, 1143, 1062, 740. **HRMS** calcd for $C_{28}H_{29}NNaO_5S$, 514.16586; found, 514.16705 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.4 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.7 min; *m/z* calcd for $C_{28}H_{29}NO_5S$, 491.60; found, 492.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l**).

To a suspension of 2,6-dimethyl-4-hydroxybenzaldehyde (1 g, 6.7 mmol) and K_2CO_3 (1.1 g, 8.0 mmol) in ACN (15 mL) was added $(CH_3)_2SO_4$ (940 μL, 9.9 mmol) and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the product was extracted with ethyl acetate (100 mL). The organic layer was washed with H_2O (2 × 25 mL) and brine (25 mL), dried over $MgSO_4$, filtered and the ethyl acetate was then removed under reduced pressure to render the 4-methoxy-2,6-dimethylbenzylaldehyde **F** (*Figure S1*) (1.1 g, 100% yield). Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.7 min), and RP-HPLC-ESMS analysis showed the target aldehyde (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.2 min; *m/z* calcd for $C_{10}H_{12}O_2$, 164.20; found, 165.2 $[M+H]^+$, where M is the MW of the 2,6-diMe4MeOBnCHO **F**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 10.48 (s, 1H), 6.59 (s, 2H), 3.84 (s, 3H), 2.61 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 191.7, 162.9, 144.6, 126.1, 115.0, 55.4, 21.2. Next, the 4-methoxy-2,6-dimethylbenzyl alcohol **G** (*Figure S1*) was prepared in 81% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.3 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{10}H_{14}O_2$, 166.22; found, 149.2 $[M-OH]^+$, where M is the MW of the 2,6-diMe4-MeOBnOH **G**).

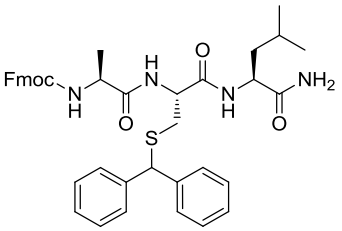
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.58 (s, 2H), 4.66 (s, 2H), 3.77 (s, 3H), 2.39 (s, *J* = 0.5 Hz, 6H), 1.32 (s, 1H). **^{13}C NMR** (101 MHz, $DCDl_3$): δ 159.0, 139.1, 129.4, 113.7, 59.0, 55.2, 19.8.

Fmoc-Ala-Cys(PG)-Leu-NH_2 tripeptides (2a-m): syntheses and TFA-lability studies

All tripeptides were synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (0.69 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treatments with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the homemade protected Cys residues (2 equiv.) were incorporated with DIPCDI (2 equiv.) and Oxyma Pure (2 equiv.) in DMF, with a 5-min pre-activation, for 2 h at 25 °C; while protected Cys(Mob) (3 equiv.), DCys(Trt) (3 equiv.), Ala (3 equiv.) and Leu (3 equiv.) residues were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. These conditions assured the absence of racemization.[1] Cleavage of the Fmoc group was achieved by treatment with piepridine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min). After completing the elongation, the tripeptides were cleaved from the resin by washes with a solution of TFA-CH_2Cl_2 (2:98) (5 × 3 mL × 1 min) at 25 °C, and the filtered washes were poured over H_2O (3 mL). The final mixtures were evaporated and the crude peptides were directly dissolved in H_2O-ACN (1:2) (20 mL) and lyophilized.

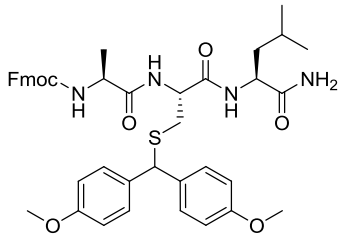
All TFA-lability studies were carried out with the lyophilized tripeptide crudes (1 mg) in glass tubes. To the acidic mixture were added H_2O (2.5%) and TIS (2.5%) as a scavengers, and CH_2Cl_2 was used as a solvent when it was required. After the acidic treatment, the mixtures were completely evaporated by bubbling N_2 (g) and the resultant residue was re-dissolved in H_2O-ACN (3:7, 2mL) and analyzed by RP-HPLC to determine the % of deprotected tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.6 min; *m/z* calcd for $C_{27}H_{34}N_4O_5S$, 526.7; found, 527.2 $[M+H]^+$, where M is the MW of the free-thiol tripeptide Fmoc-Ala-Cys-Leu-NH_2).

Dpm tripeptide (2a)



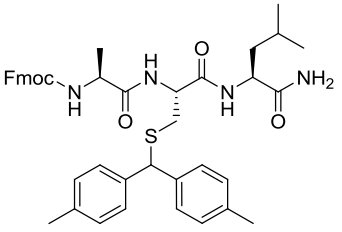
Fmoc-Ala-Cys(Dpm)-Leu-NH_2 tripeptide was obtained in 83% yield and 98% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{40}H_{44}N_4O_5S$, 692.9; found, 694.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{45}N_4O_5S$, 693.31052; found, 693.31138 $[M+H]^+$.

4,4'diMeODpm tripeptide (2b)



Fmoc-Ala-Cys(4,4'diMeODpm)-Leu-NH_2 tripeptide was obtained in 29% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.2 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.9 min; m/z calcd for $C_{42}H_{48}N_4O_7S$, 752.9; found, 754.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{42}H_{49}N_4O_7S$, 753.33165; found, 753.33182 $[M+H]^+$.

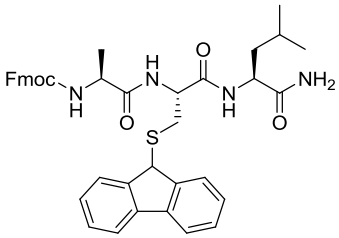
4,4'diMeDpm tripeptide (2c)



Fmoc-Ala-Cys(4,4'diMeDpm)-Leu-NH_2 tripeptide was obtained in 61% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.5 min; *m/z* calcd for $C_{42}H_{48}N_4O_5S$, 720.9; found, 722.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{42}H_{49}N_4O_5S$, 721.34182; found, 721.34279 $[M+H]^+$.

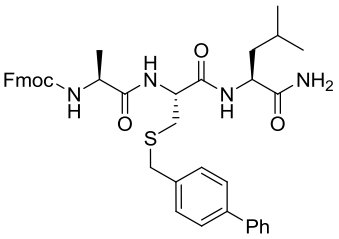
1 (a) Han, Y; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, 4307-4312; (b) Angell, Y.M.; Alsina, J.; Albericio, F.; Barany, G. *J. Peptide Res.* **2002**, *60*, 292-299.

9F tripeptide (2d)



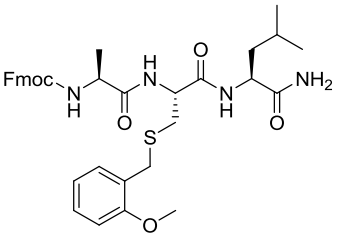
Fmoc-Ala-Cys(9F)-Leu-NH_2 tripeptide was obtained in 57% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.5 min; *m/z* calcd for $C_{40}H_{42}N_4O_5S$, 690.9; found, 691.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{43}N_4O_5S$, 691.29487; found, 691.29548 $[M+H]^+$.

Bpm tripeptide (2e)



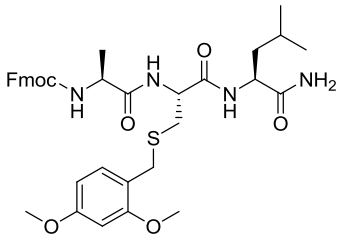
Fmoc-Ala-Cys(Bpm)-Leu-NH_2 tripeptide was obtained in 76% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.6 min; *m/z* calcd for $C_{40}H_{44}N_4O_5S$, 692.9; found, 694.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{45}N_4O_5S$, 693.31052; found, 693.31111 $[M+H]^+$.

2MeOBn tripeptide (2f)



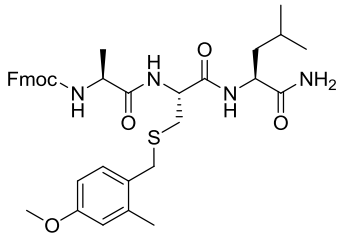
Fmoc-Ala-Cys(2MeOBn)-Leu-NH_2 tripeptide was obtained in 25% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min; *m/z* calcd for $C_{35}H_{42}N_4O_6S$, 646.8; found, 647.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{35}H_{43}N_4O_6S$, 647.28978; found, 647.29035 $[M+H]^+$.

2,4diMeOBn tripeptide (2g)



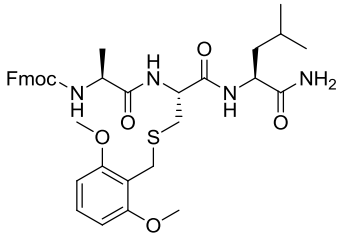
Fmoc-Ala-Cys(2,4diMeOBn)-Leu-NH_2 tripeptide was obtained in 32% yield and 99% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.4 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.3 min; *m/z* calcd for $C_{36}H_{44}N_4O_7S$, 676.8; found, 678.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_7S$, 677.30035; found, 677.30131 $[M+H]^+$.

4MeO-2MeBn tripeptide (2h)



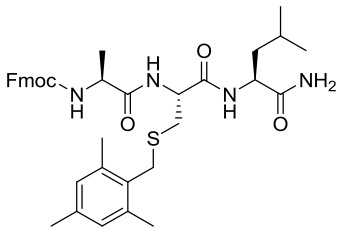
Fmoc-Ala-Cys(4MeO-2MeBn)-Leu-NH_2 tripeptide was obtained in 64% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min; *m/z* calcd for $C_{36}H_{44}N_4O_6S$, 660.8; found, 661.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_6S$, 661.30543; found, 661.30588 $[M+H]^+$.

2,6diMeOBn tripeptide (2i)



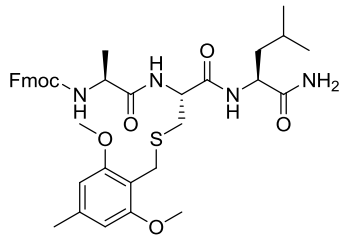
Fmoc-Ala-Cys(2,6diMeOBn)-Leu-NH_2 tripeptide was obtained in 58% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.8 min; *m/z* calcd for $C_{36}H_{44}N_4O_7S$, 676.8; found, 677.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_7S$, 677.30035; found, 677.30104 $[M+H]^+$.

TMeb tripeptide (2j)



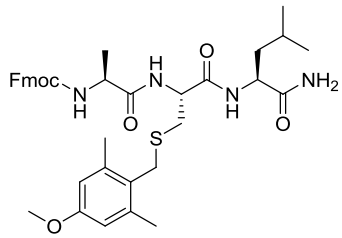
Fmoc-Ala-Cys(TMeb)-Leu-NH_2 tripeptide was obtained in 69% yield and 95% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.7 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.8 min; *m/z* calcd for $C_{37}H_{46}N_4O_5S$, 658.9; found, 659.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_5S$, 659.32617; found, 659.32666 $[M+H]^+$.

2,6diMeO-4MeBn tripeptide (2k)



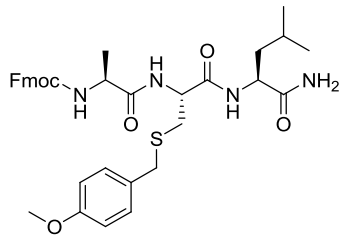
Fmoc-Ala-Cys(2,6diMeO-4MeBn)-Leu-NH_2 tripeptide was obtained in 59% yield and 95% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.0 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.8 min; *m/z* calcd for $C_{37}H_{46}N_4O_7S$, 690.9; found, 692.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_7S$, 691.31600; found, 691.31685 $[M+H]^+$.

4MeO-2,6diMeBn tripeptide (2l)



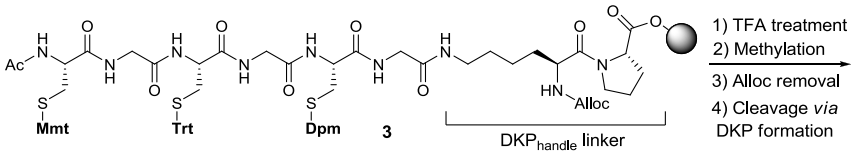
Fmoc-Ala-Cys(4MeO-2,6diMeBn)-Leu-NH_2 tripeptide was obtained in 69% yield and 99% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.8 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min; *m/z* calcd for $C_{37}H_{46}N_4O_6S$, 674.9; found, 676.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_6S$, 675.32108; found, 675.32171 $[M+H]^+$.

Mob tripeptide (2m)



Fmoc-Ala-Cys(Mob)-Leu-NH_2 tripeptide was obtained in 36% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.3 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min; *m/z* calcd for $C_{35}H_{42}N_4O_6S$, 646,8 ; found, 647.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{35}H_{43}N_4O_6S$, 647.28978; found, 647.29037 $[M+H]^+$.

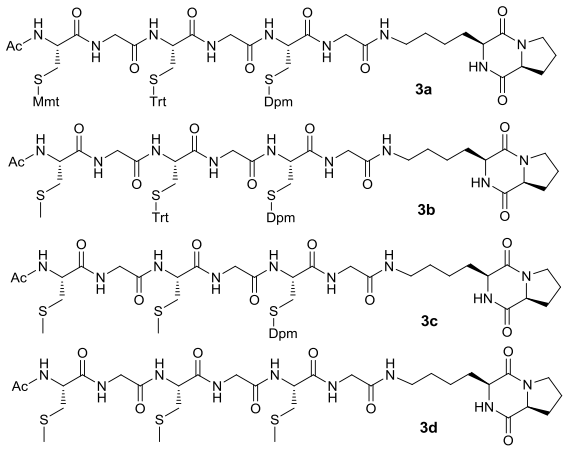
DKP_{handle} experiment (3)



Dipeptydil linker was constructed manually on a HMPS resin (150 mg, 0.98 mmol/g). After washing the resin with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), a mixture of Fmoc-D-Pro-OH (199 mg, 4 equiv.) and DIPCDI (45.7 μL, 2 equiv.) in CH_2Cl_2-DMF (15:1 (v/v), 800 μL) was added to the resin. DMAP (7.3 mg, 0.4 equiv.) in CH_2Cl_2 (200 μL) was then added, and the mixture was left to stand for 2 h at 25 °C. Consecutively, Fmoc-D-Pro-OH was re-coupled following the same procedure for 16 h at 25 °C. The resin was washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min) and successively capped using acetic anhydride (150 μL, 10 equiv.) and DIEA (256 μL, 10 equiv.) in DMF (500 μL) for 30 min at 25 °C. After capping, the resin was washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1min), and the Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid of the DKP_{handle} linker, a mixture of Alloc-Lys(Fmoc)-OH (200 mg, 3 equiv.), Oxyma Pure (63 mg, 3 equiv.) and DIPCDI (68.5 mL, 3 equiv.) in DMF (1 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand for 16 h at 25 °C and the resin was then washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min), and the Fmoc group was removed.

On the previously prepared resin was elongated the hexapeptide. Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min). Finally, the *N*-terminus was acetylated using acetic anhydride (10 equiv.) and DIEA (10 equiv.) in DMF (1 mL) for 30 min at 25 °C (× 2).

After completing the elongation by SPPS, the TFA-lability study on solid phase was carried out. Thus, different peptidyl-resins (20 mg) were treated with diverse solutions of TFA in CH_2Cl_2 with or without the presence of TIS as scavenger, following by methylation using CH_3I (7.5 μL, 5 equiv.) and DIEA (7 μL, 5%) in CH_2Cl_2 (135 μL) for 2 h at 25 °C. Ellman's test for detection of thiol was then performed; if the test was positive an extra treatment with CH_3I in basic conditions was carried out. Subsequently, peptides were cleaved from the resin by Alloc removal from the L-Lys residue of the dipeptidyl linker by treatment with $Pd(Ph_3)_4$ (1 mg, 0.1 equiv.), $PhSiH_3$ (10 μL, 10 equiv.) in CH_2Cl_2 (3 × 15 min), and posterior DKP formation by treatment with piperidine-THF (5:95, 2 × 5 min). THF was removed by evaporation under reduced pressure, and the residual crude peptides comprising the DKP *C*-terminal protecting moiety were analyzed by RP-HPLC.



Ac-Cys(Mmt)-Gly-Cys(Trt)-Gly-Cys(Dpm)-Gly-DKP_{handle} (**3a**)

RP-HPLC analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 6.7 min. RP-HPLC-ESMS analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 11.4 min; *m/z* calcd for $C_{80}H_{85}N_9O_{10}S_3$, 1428.9; found, 1431.0 $[M+H]^+$, where M is the MW of the fully protected peptide.

Ac-Cys(Me)-Gly-Cys(Trt)-Gly-Cys(Dpm)-Gly-DKP_{handle} (**3b**)

RP-HPLC analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 3.7 min. RP-HPLC-ESMS analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 4.9 min; *m/z* calcd for $C_{61}H_{71}N_9O_9S_3$, 1170.4; found, 1171.5 $[M+H]^+$, where M is the MW of the target peptide.

Ac-Cys(Me)-Gly-Cys(Me)-Gly-Cys(Dpm)-Gly-DKP$_{handle}$ (**3c**)

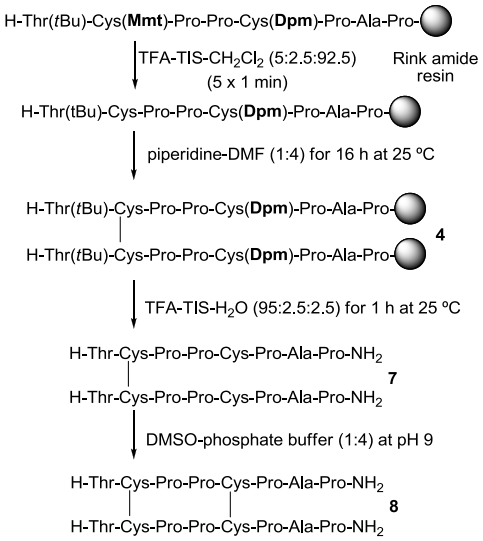
RP-HPLC analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 5.3 min. RP-HPLC-ESMS analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{43}H_{59}N_9O_9S_3$, 942.2; found, 943.1 $[M+H]^+$, where M is the MW of the target peptide.

Ac-Cys(Me)-Gly-Cys(Me)-Gly-Cys(Me)-Gly-DKP$_{handle}$ (**3d**)

RP-HPLC analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 3.6 min. RP-HPLC-ESMS analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 5.7 min; *m/z* calcd for $C_{31}H_{51}N_9O_9S_3$, 790.0; found, 790.9 $[M+H]^+$, where M is the MW of the target peptide.

Regioselective synthesis of the hinge fragment of human IgG1 (8)

Rink-amide approach



Linear peptide was synthesized manually on Fmoc-Rink-amide AM-polystyrene resin (300 mg, 0.45 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piepridine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min).

After completing the elongation by SPPS, a portion of peptidyl-resin (1 mg) was cleaved from the resin by treating with TFA-TIS-H_2O (95:2.5:2.5) (1 mL) for 1 h at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC. H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 was obtained in 91% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 50% ACN over 8 min at 60 °C; t_R : 4.3 min). RP-HPLC-ESMS showed the fully deprotected target peptide (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{33}H_{53}N_9O_9S_2$, 783.9; found, 785.0 $[M+H]^+$, where M is the MW of the target peptide). The RP-HPLC analysis showed two peaks when it was performed at 25 °C due to the existing conformational species of the free linear monomer (linear gradient from 5% to 50% ACN over 8 min; t_R : 3.5 and 3.7 min).

S-Mmt removal and posterior disulfide bond construction on solid phase (**7**)

A portion of peptidyl-resin was treated with TFA-TIS-CH_2Cl_2 (5:2.5:92.5) (5 × 1 min) and then washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Subsequently, the peptidyl-resin was treated with piperidine-DMF (1:4) at 25 °C and the oxidation was monitored by Ellman's test and RP-HPLC analysis until completion, when the crude peptide was cleaved from the resin with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C. The acidic mixture was evaporated, the crude peptide precipitated with pre-cooled Et_2O, centrifuged and finally lyophilized, and analyzed by RP-HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.7 min). RP-HPLC-ESMS showed the fully deprotected mono-disulfide bond dimer (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.6 min; m/z calcd for $C_{66}H_{104}N_{18}O_{18}S_4$, 1565.9; found, 784.1 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate).

A one-pot approach for the total deprotection and second disulfide bond formation was assayed. Thus, after first disulfide bond formation the peptidyl-resin was treated with TFA-DMSO-TIS (89:10:1). However, although, the fully *S*-Dpm removal was achieved after 1 h, the subsequent disulfide oxidation was not complete under acidic conditions.

2nd disulfide bond construction in solution (**8**)

The resultant mono-disulfide bond intermediate **7** was then dissolved in DMSO-phosphate buffer (20 mM) (1:4) at pH 9 and 25 °C to render the totally oxidized parallel dimer in 95% purity, as determined by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.3 min). RP-HPLC-ESMS showed the parallel dimer (linear gradient from 0% to 30% ACN over 8 min; t_R : 6.8 min; m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1563.9; found, 783.2 $[M+2H/2]^{2+}$, where M is the MW of the target peptide).

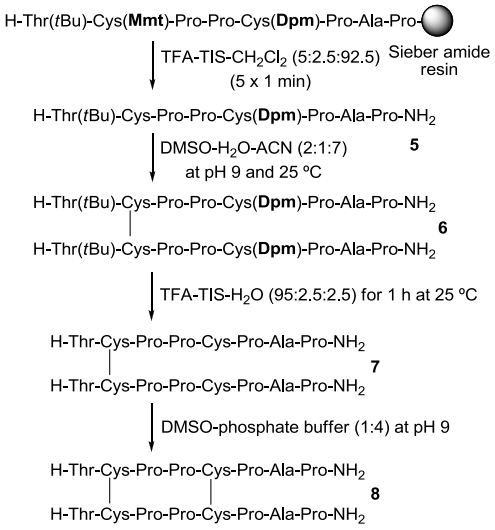
Random approach

A portion of peptidyl-resin from the Rink-amide approach (62.5 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5) (3 mL) for 1 h at 25 °C, and the cleavage mixture was then evaporated and the crude peptide was precipitated with pre-cooled Et_2O (2 × 5 mL), centrifuged and lyophilized to render the fully deprotected linear peptide H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 (10.5 mg, 74%

yield) in 91% purity, as determined by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.5 and 3.7 min). **HRMS** analysis m/z calcd for $C_{33}H_{53}N_9O_9S_2$, 784.34804; found, 784.34965 $[M+H]^+$.

A portion of the linear monomer (1 mg) was dissolved in a 20 mM phosphate buffer (425 μL, 3 mM) at pH 6.8 and 25 °C and the oxidative folding was monitored by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.3 min). The main peak corresponded to the parallel cyclic dimer, as determined by RP-HPLC-ESMS (linear gradient from 0% to 30% ACN over 8 min; t_R : 6.7 min; m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1563.9; found, 783.2 $[M+2H/2]^{+2}$, 1565.0 $[M+H]^+$, where M is the MW of the target peptide). **HRMS** analysis m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1562.64968; found, 1562.62109 $[M+H]^+$.

Sieber-amide approach



Linear peptide was synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (200 mg, 0.69 mmol/g) following the same procedure described in the Fmoc-Rink-amide approach.

After completing the elongation by SPPS, a portion of peptidyl-resin (1 mg) was cleaved from the resin by treating with TFA-TIS-H_2O (95:2.5:2.5) (1 mL) for 1 h at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC.

H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 was obtained in 91% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 50% ACN over 8 min at 60 °C; t_R : 4.3 min). RP-HPLC-ESMS showed the fully deprotected target peptide (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{33}H_{53}N_9O_9S_2$, 783.9; found, 785.0 $[M+H]^+$, where M is the MW of the target peptide).

Cleavage from the resin and concomitant S-Mmt removal (**5**)

A portion of peptidyl-resin (122 mg) was treated with TFA-TIS-H_2O-CH_2Cl_2 (5:2.5:2.5:90) (1 × 30 min, 3 × 1 min; 6 mL each) at 25 °C and the resultant resin was then washed with CH_2Cl_2 (3 × 1 min). The filtered solutions were poured over H_2O (15 mL) and the organic solvents were then concentrated under reduced pressure. To the final aqueous solution was added ACN (15 mL) and the mixture was then lyophilized. The resultant oil was precipitated with pre-cooled Et_2O (1 × 10 mL, 1 × 5 mL) and centrifuged to render the desired *S*-Dpm protected monomer intermediate **5** (50 mg, 89% yield) in 95% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.1 min). RP-HPLC-ESMS showed the target *S*-Dpm protected monomer intermediate **5** (linear gradient from 5% to 80% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{50}H_{71}N_9O_9S_2$, 1006.3; found, 1007.4 $[M+H]^+$, where M is the MW of the target intermediate).

1st disulfide bond construction in solution (**6** and **7**)

The partial protected monomer was dissolved in H_2O-ACN (1:9) (2 mL), and DMSO (500 μL, 20%) was added as an oxidant. The pH was adjusted to 9 using DIEA (45 μL) and the mixture was stirred at 25 °C. The disulfide bond formation was monitored by analytical RP-HPLC until completion to render the *S*-Dpm protected dimer **6** in 81% purity, as determined by RP-HPLC HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.6 min). RP-HPLC-ESMS showed the target *S*-Dpm protected dimer intermediate **6** (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.7 min; *m/z* calcd for $C_{100}H_{140}N_{18}O_{18}S_4$, 2010.6; found, 1006.9 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate). Next, the solvent was removed by lyophilization and the total deprotection of the dimer intermediate **6** was then achieved by treating the crude peptide with TFA-TIS-H_2O (95:2.5:2.5) for 2 h at 25 °C. The mixture was evaporated by bubbling N_2 (g) and the crude peptide was then precipitated with pre-cooled Et_2O, centrifuged (× 3) and lyophilized to render the mono-disulfide bond dimer **7** in 85% purity, as determined by RP-HPLC analysis HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.6 min) and RP-HPLC-ESMS (linear gradient from 5% to 80% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{66}H_{104}N_{18}O_{18}S_4$, 1565.9; found, 1567.8 $[M+H]^+$, 784.9 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate).

2nd disulfide bond construction in solution (**8**)

The formation of the 2nd disulfide bond in solution was accomplished following the same procedure indicated for the Rink-amide approach. **HRMS** analysis m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1562.64968; found, 1562.65136 $[M+H]^+$.

Regioselective synthesis of α-conotoxin ImI (11)

Linear peptide was synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (202 mg, 0.69 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min).
After completing the elongation by SPPS, a portion of peptidyl-resin (5 mg) was cleaved from the resin by treating with TFA-CH_2Cl_2 (1:99) (1 mL) for 1 min at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC. The fully protected peptide H-Gly-Cys(Trt)-Cys(Dpm)-Ser(*t*Bu)-Asp(O*t*Bu)-Pro-Arg(Pbf)-Cys(Trt)-Ala-Trp(Boc)-Arg(Pbf)-Cys(Dpm)-NH_2 was obtained as determined by analytical RP-HPLC (linear gradient from 80% to 100% ACN over 8 min; t_R : 6.7 min), and RP-HPLC-ESMS analysis (linear gradient from 70% to 100% ACN over 8 min; t_R : 8.69 min; *m/z* calcd for $C_{155}H_{186}N_{20}O_{23}S_6$, 2889.6; found, 1447.1 $[M+2H/2]^{2+}$, where M is the MW of the target peptide).

Cleavage from the resin and selective S-Trt removal (**9**)
A portion of peptidyl-resin (95 mg) was treated with TFA-TIS-CH_2Cl_2 (5:5:90) (3 min × 5 mL, 2 × 1 min × 5 mL) and washed with CH_2Cl_2 (3 × 1 min × 3 mL). The filtered mixtures were poured over H_2O (5 mL) and evaporated and the crude peptide was then lyophilized to render de partial *S*-Dpm protected intermediate **9** (40.8 mg, 80% yield) in 80% purity, as determined by analytical RP-HPLC (linear gradient from 70% to 100% ACN over 8 min; t_R : 4.8 min). RP-HPLC-ESMS showed the linear 2 *S*-Dpm deprotected peptide **9** (linear gradient from 70% to 100% ACN over 8 min; t_R : 5.3 min; *m/z* calcd for $C_{117}H_{158}N_{20}O_{23}S_6$, 2405.0; found, 1204.5$[M+2H/2]^{2+}$, where M is the MW of the target peptide).

1st disulfide bond construction in solution (**10**)
A portion of crude peptide (1 mg) was dissolved in H_2O-ACN (1:1) (7 mL) and the pH was adjusted up to 8.5 with an aqueous NH_3 solution (1 M) and the mixture was left to stand for 24 h at 25 °C and lyophilized to afford the oxidized 2 *S*-Dpm intermediate **10** in 80% purity, as determined by analytical RP-HPLC (linear gradient from 70% to 100% ACN over 8 min; t_R : 5.7 min) and RP-HPLC-ESMS analysis (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.2 min; *m/z* calcd for $C_{117}H_{156}N_{20}O_{23}S_6$, 2403.0; found, 1203.4$[M+2H/2]^{2+}$, where M is the MW of the target peptide).

2nd disulfide bond construction in solution (**11**)
The cyclic 2 *S*-Dpm intermediate **10** was then treated with TFA-DMSO-anisole (89:10:1) (2 mL) for 3 h at 25 °C and the mixture was evaporated under reduced pressure and lyophilized to render the α-conotoxin ImI in 80% purity, as determined by analytical RP-HPLC (linear gradient from 10% to 35% ACN over 8 min; t_R : 5.2 min). RP-HPLC-ESMS showed the fully deprotected target peptide **11** (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.6 min; *m/z* calcd for $C_{52}H_{78}N_{20}O_{15}S_4$, 1351.6; found, 1352.8 $[M+H]^+$, 677.2 $[M+2H/2]^{2+}$, where M is the MW of the target peptide). **HRMS** calcd for $C_{52}H_{78}N_{20}O_{15}S_4$, 1350.48329; found, 1350.48492 [M].

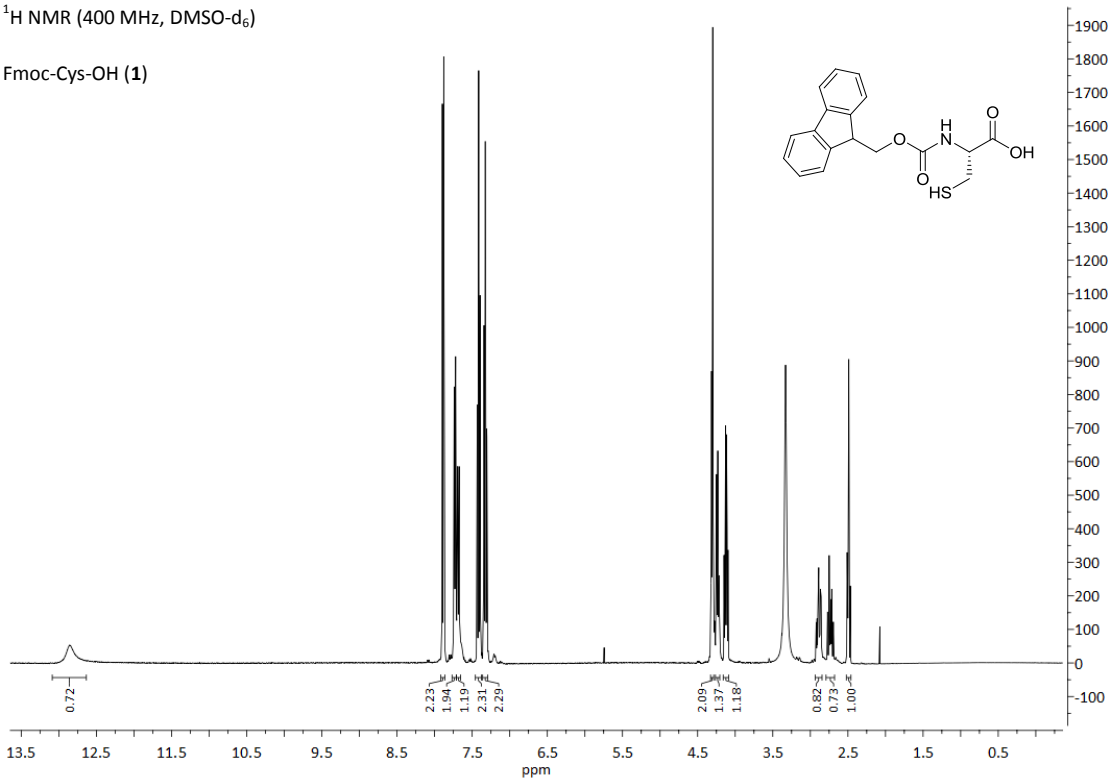
Random approach
A portion of peptidyl-resin (54 mg) was treated with a solution of TFA-TIS-H_2O (95:2.5:2.5) (5 mL) for 1 h at 25 °C and the mixture was then evaporated by bubbling N_2 (g) and the crude peptide was precipitated with pre-cooled Et_2O (10 mL) and centrifuged (×3) to render the fully deprotected linear peptide H-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2 (17 mg, 98% yield).

a) A portion of the resultant crude (1 mg) was re-dissolved in H_2O (2 mL) and the pH was adjusted up to 8 with an aqueous NH_3 solution (1 M) and the oxidative folding was monitored by RP-HPLC analysis. After 18 h, two main peaks corresponded to the structural isomers were observed (linear gradient from 10% to 35% ACN over 8 min; t_R : 4.7 and 5.2 min).
b) A portion of the resultant crude (1 mg) was re-dissolved in H_2O-2-propanol (1:1) (2 mL) and the pH was adjusted up to 10 with DIEA and the oxidative folding was monitored by RP-HPLC analysis. After 18 h, two main peaks corresponded to the structural isomers were observed (linear gradient from 10% to 35% ACN over 8 min; t_R : 4.7 and 5.2 min).

Spectral data of prepared Fmoc-Cys(PG)-OH

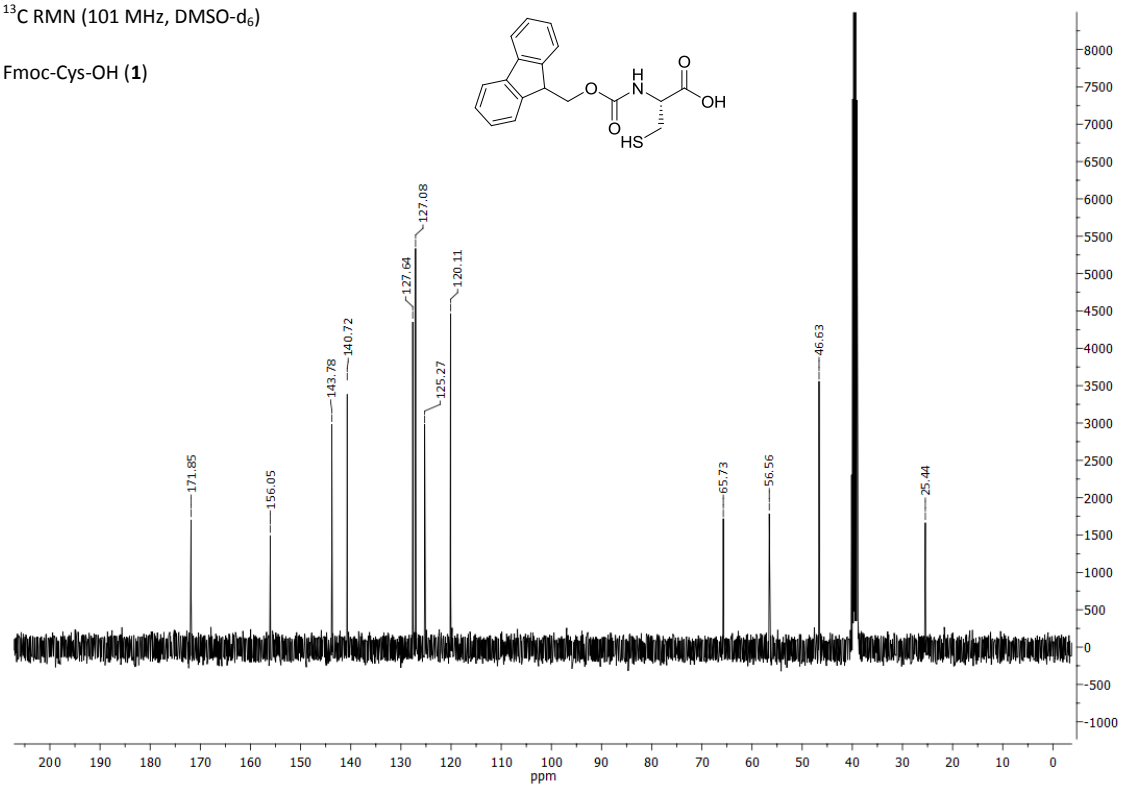
^{1}H NMR (400 MHz, DMSO-d_6)

Fmoc-Cys-OH (**1**)



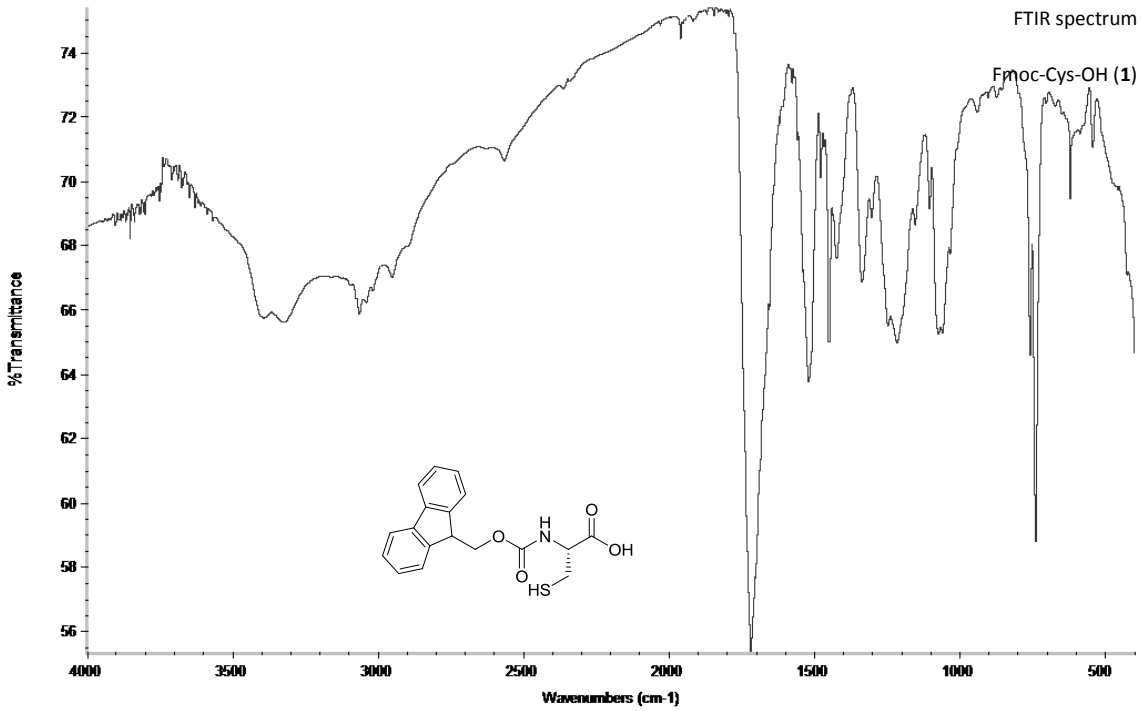
^{13}C RMN (101 MHz, DMSO-d_6)

Fmoc-Cys-OH (**1**)



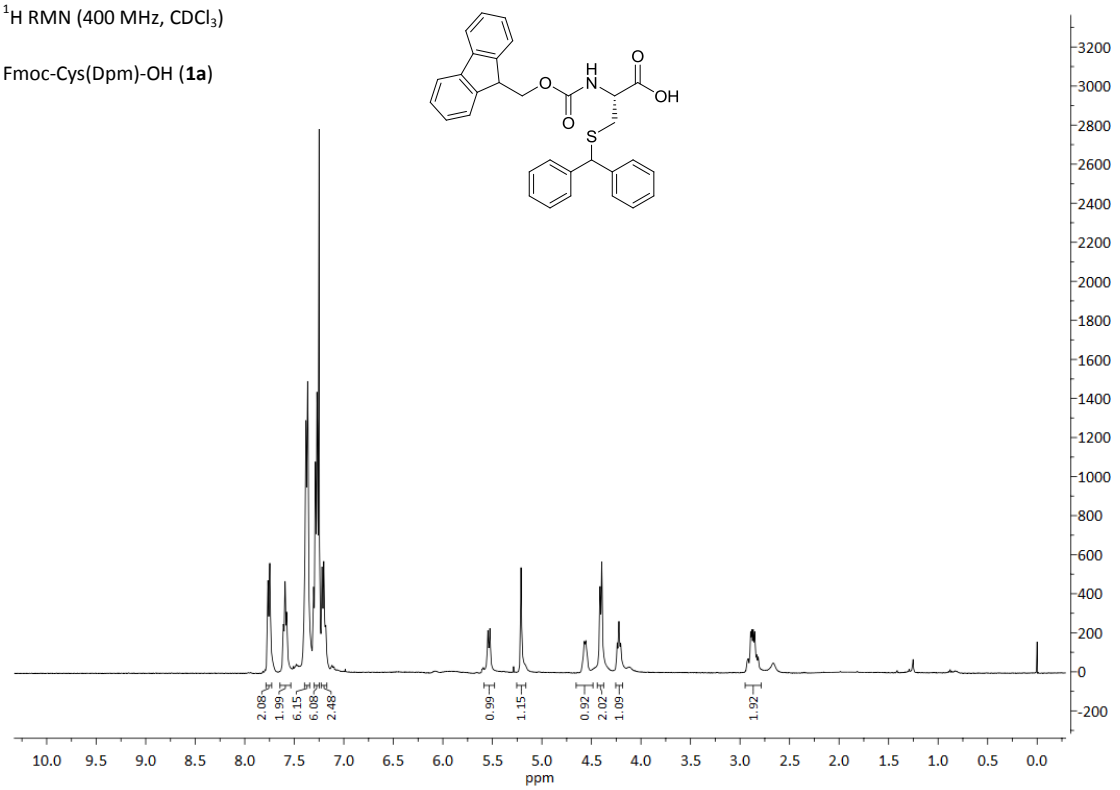
FTIR spectrum

Fmoc-Cys-OH (**1**)



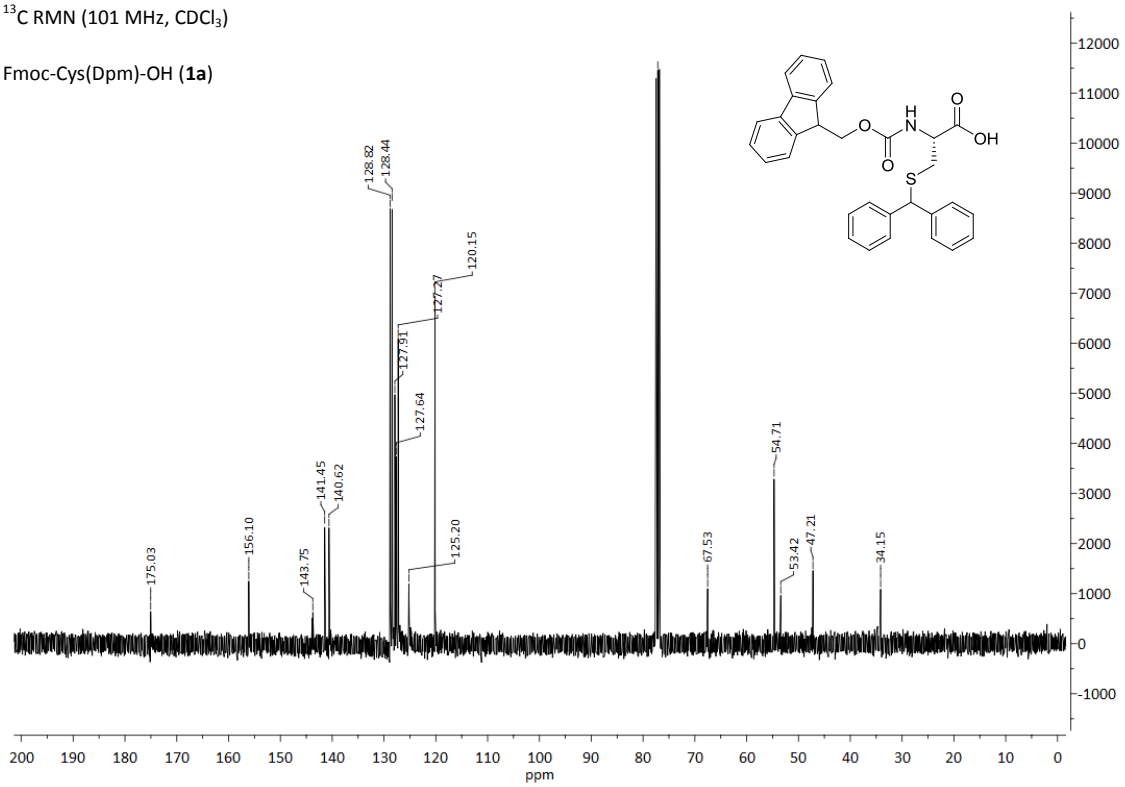
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(Dpm)-OH (**1a**)



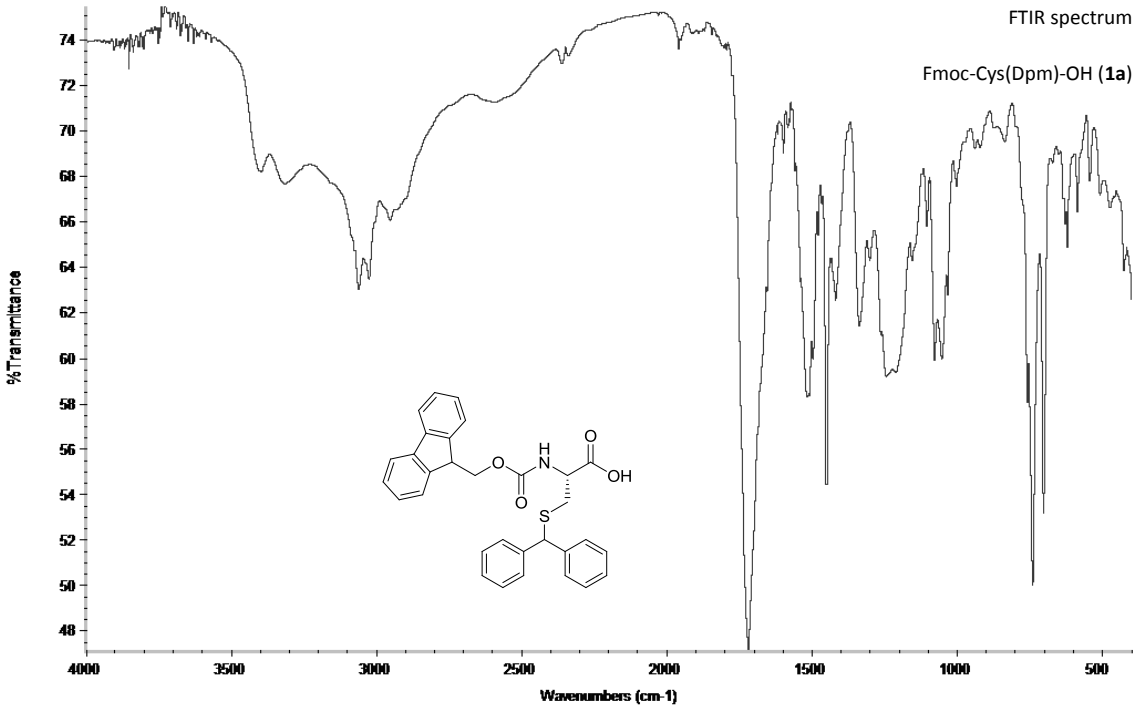
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(Dpm)-OH (**1a**)



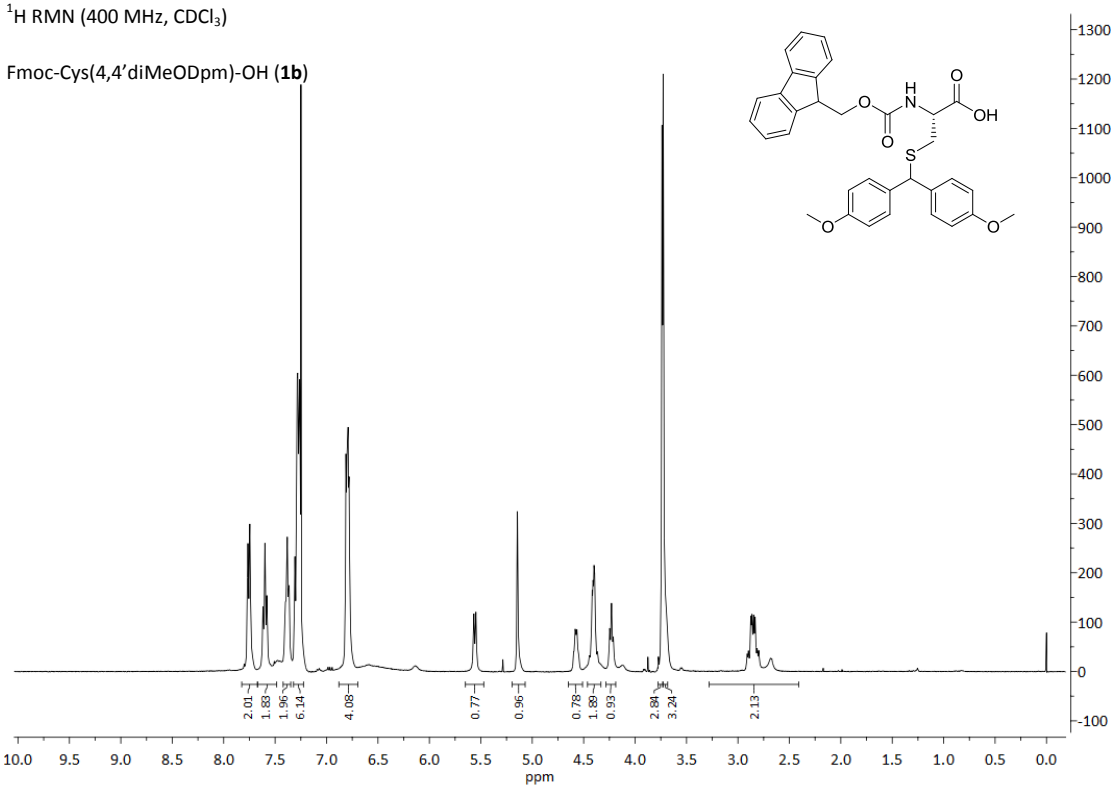
FTIR spectrum

Fmoc-Cys(Dpm)-OH (**1a**)



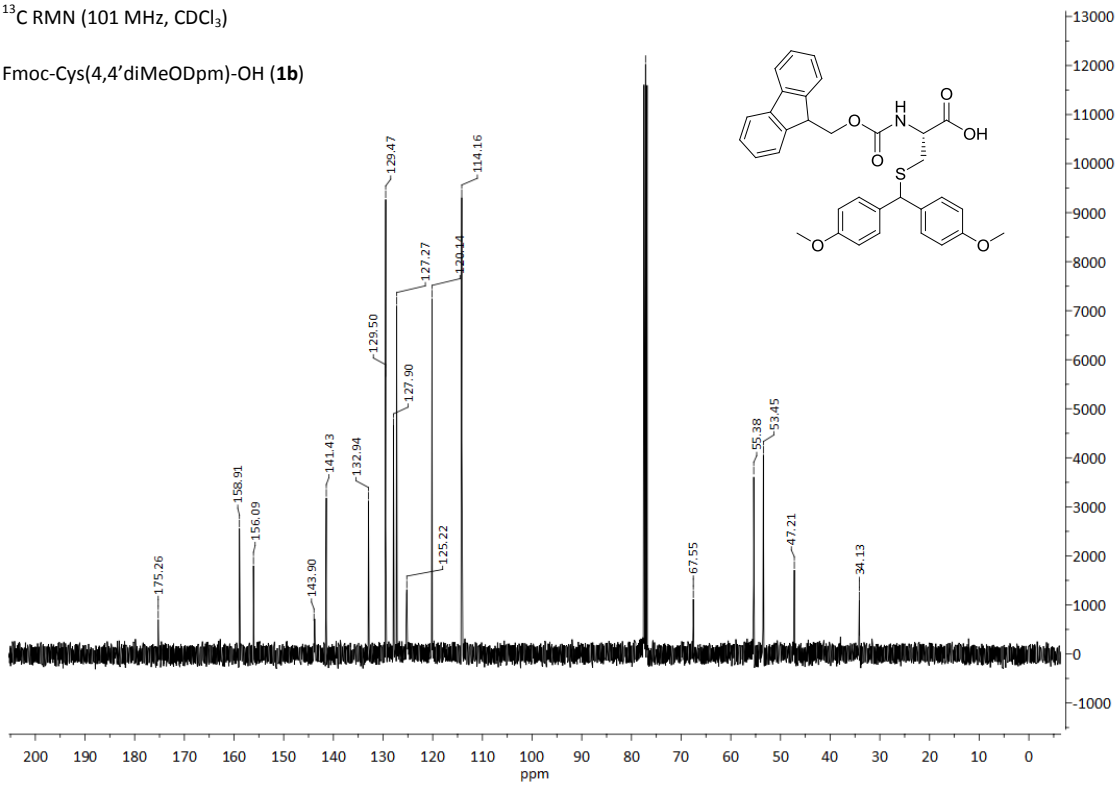
^{1}H RMN (400 MHz, $CDCl_3$)

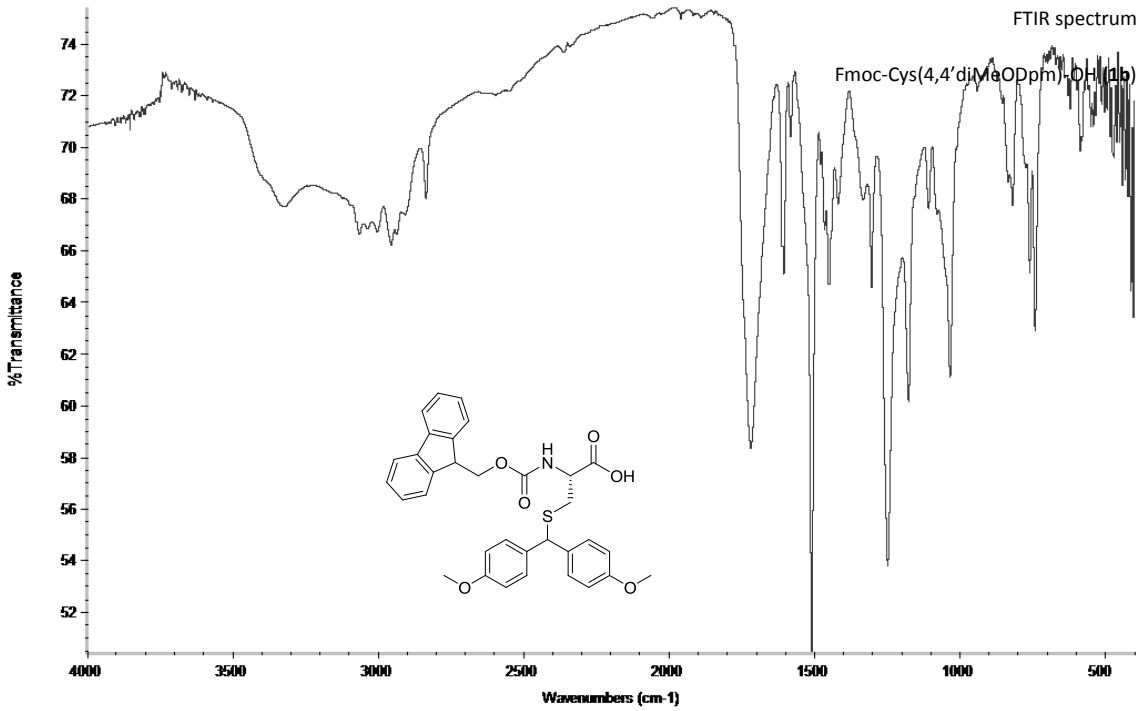
Fmoc-Cys(4,4'diMeODpm)-OH (**1b**)



^{13}C RMN (101 MHz, $CDCl_3$)

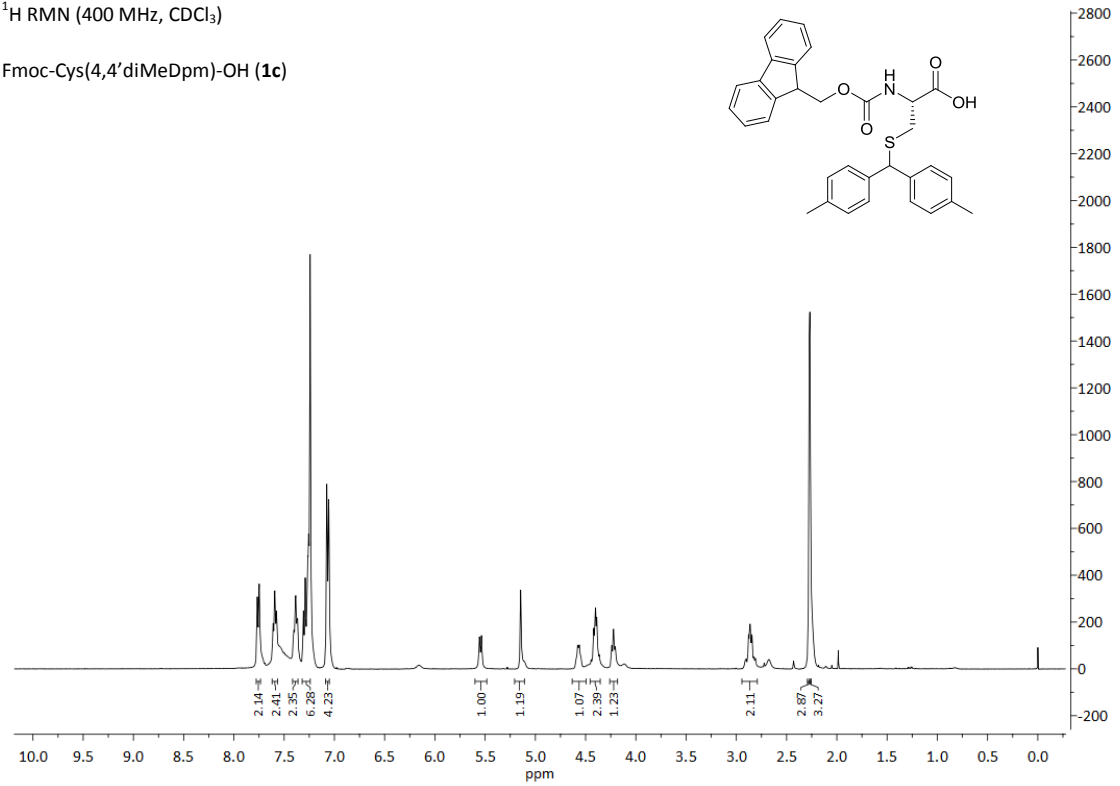
Fmoc-Cys(4,4'diMeODpm)-OH (**1b**)




FTIR spectrum
Fmoc-Cys(4,4'diMeODpm)-OH (1b)
%Transmittance
74
72
70
68
66
64
62
60
58
56
54
52
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
H
N
O
OH
O
O
S
O
O

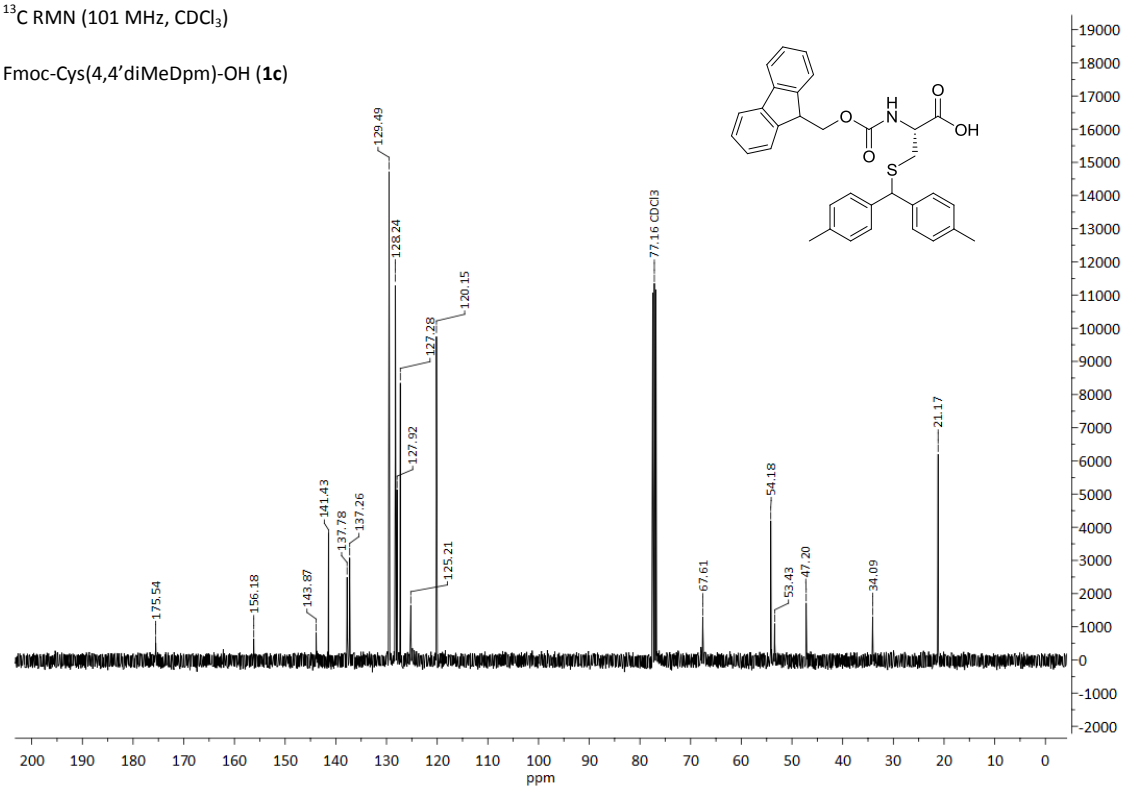
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(4,4'diMeDpm)-OH (**1c**)



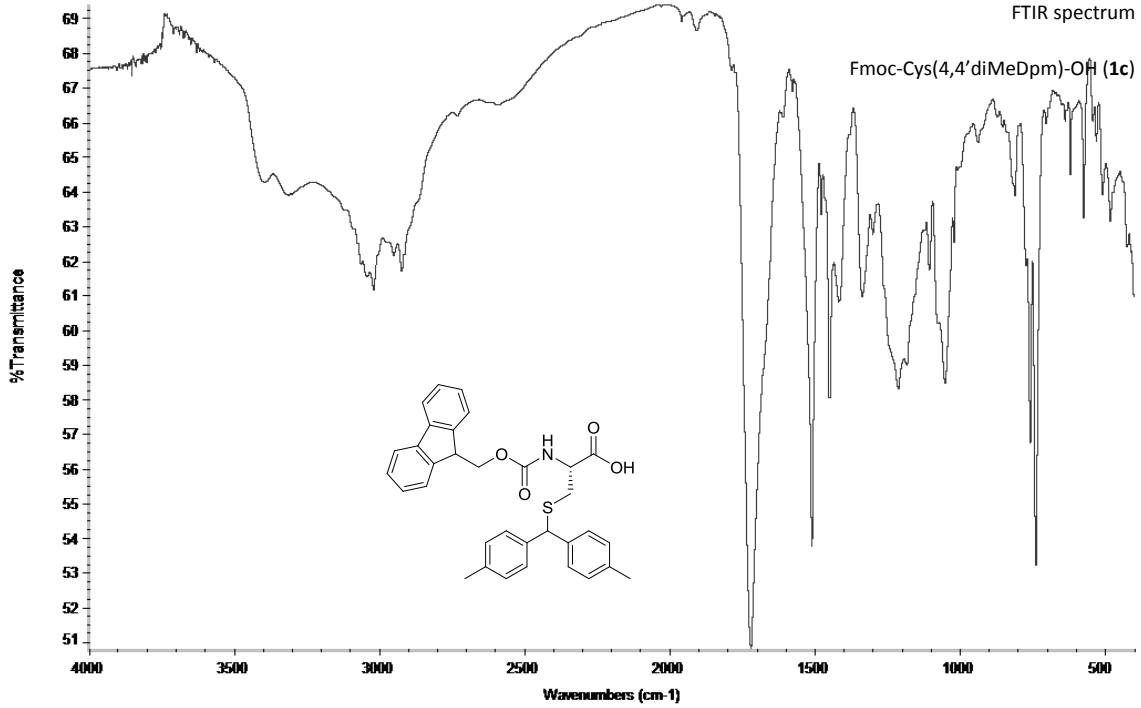
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(4,4'diMeDpm)-OH (**1c**)



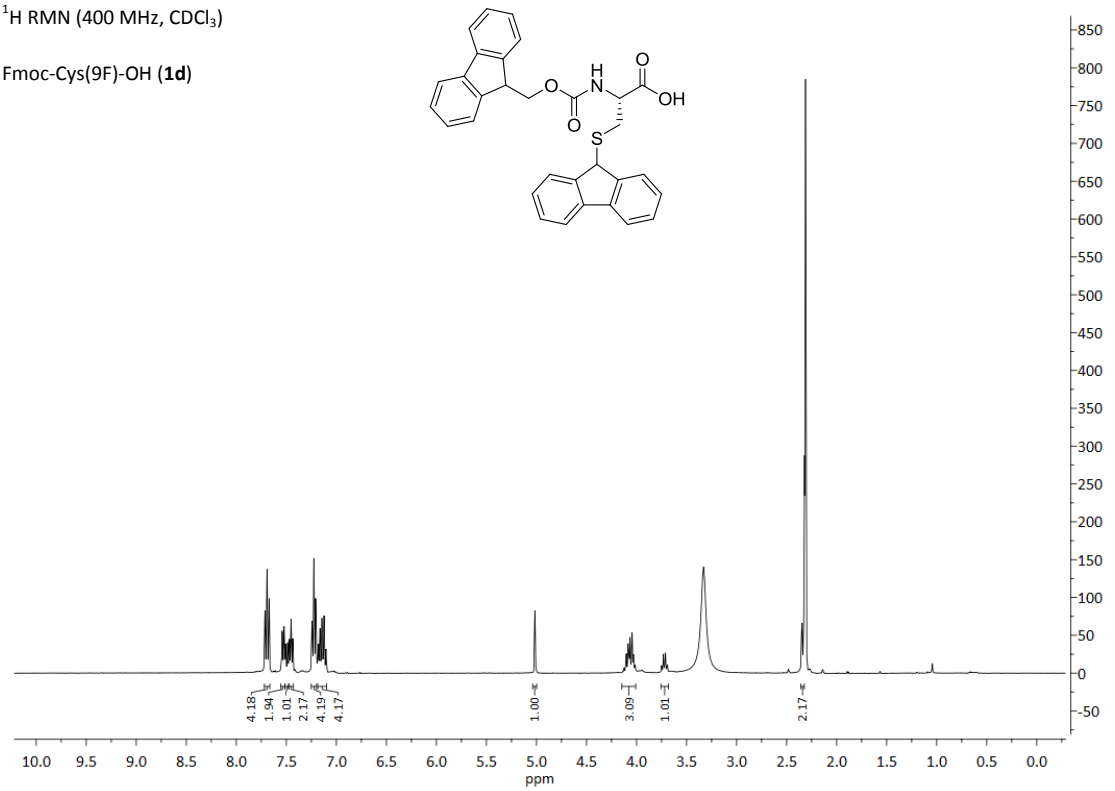
FTIR spectrum

Fmoc-Cys(4,4'diMeDpm)-OH (**1c**)



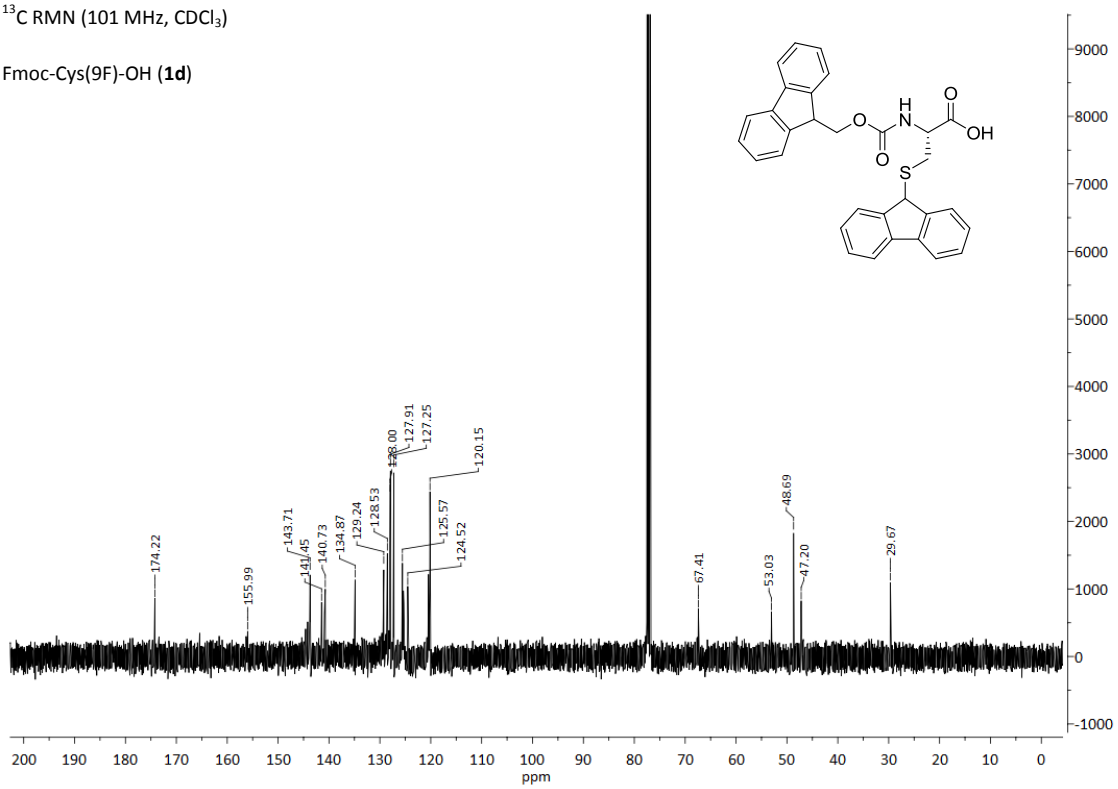
^{1}H RMN (400 MHz, $CDCl_3$)

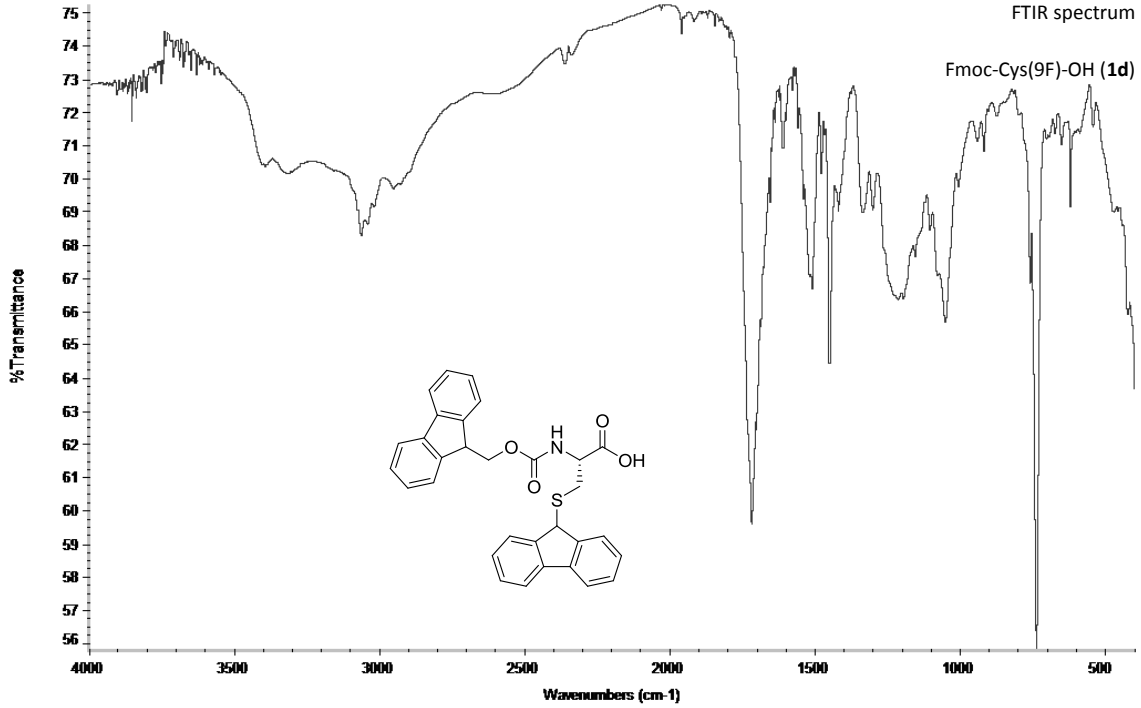
Fmoc-Cys(9F)-OH (**1d**)



^{13}C RMN (101 MHz, $CDCl_3$)

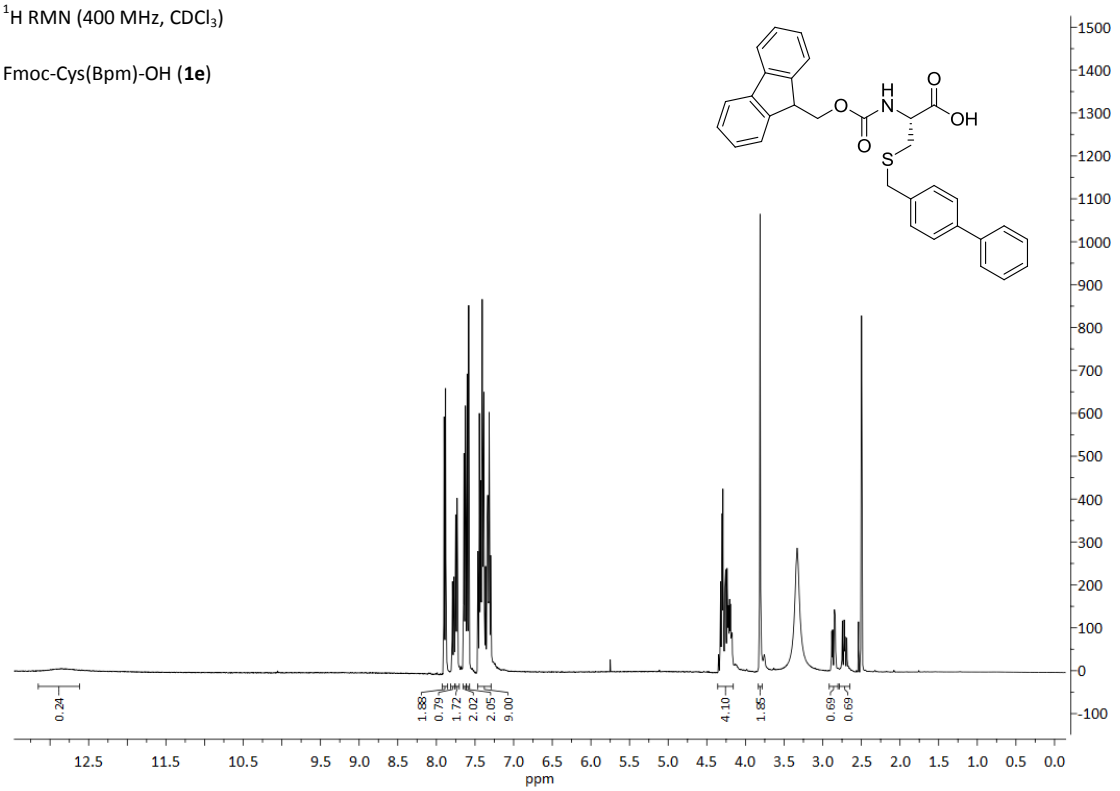
Fmoc-Cys(9F)-OH (**1d**)




FTIR spectrum
Fmoc-Cys(9F)-OH (1d)
%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500
O
H
N
O
OH
O
S

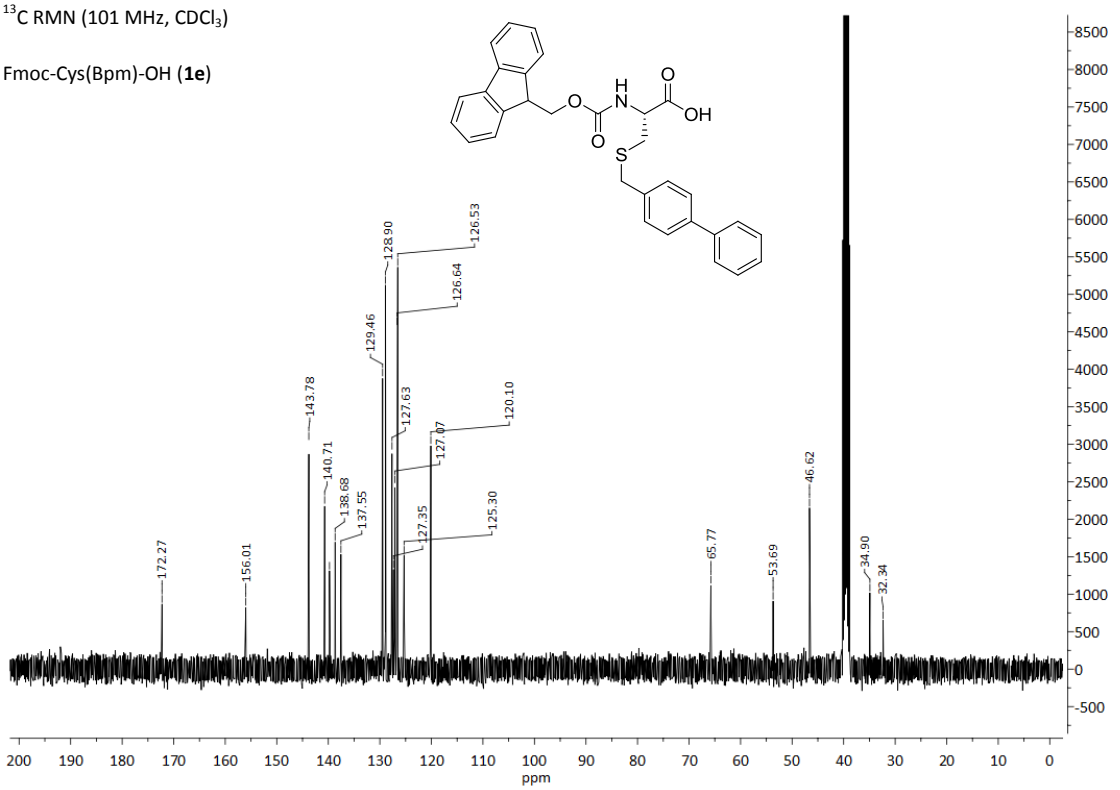
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(Bpm)-OH (**1e**)



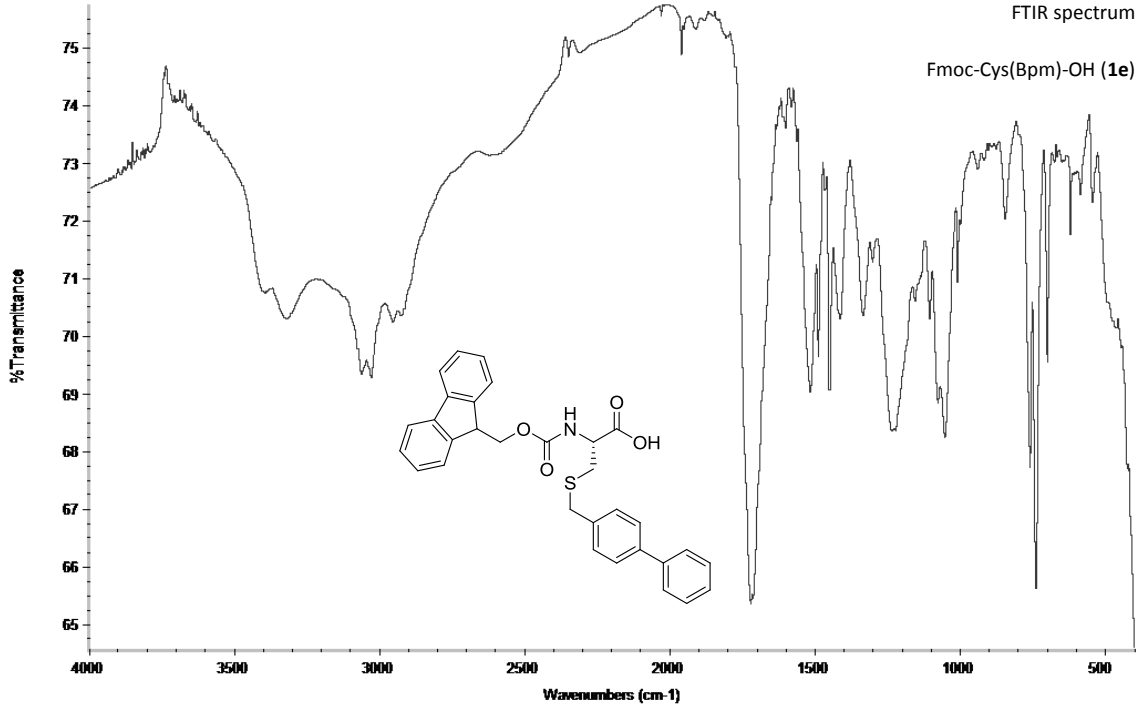
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(Bpm)-OH (**1e**)



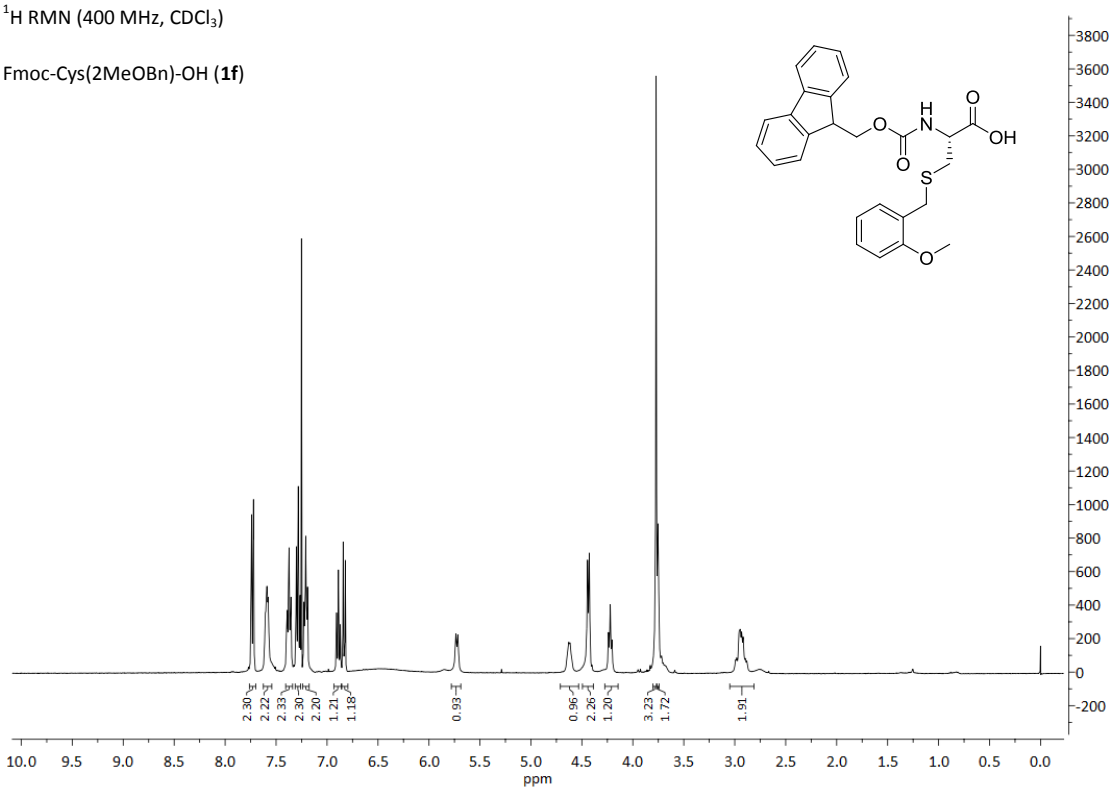
FTIR spectrum

Fmoc-Cys(Bpm)-OH (**1e**)



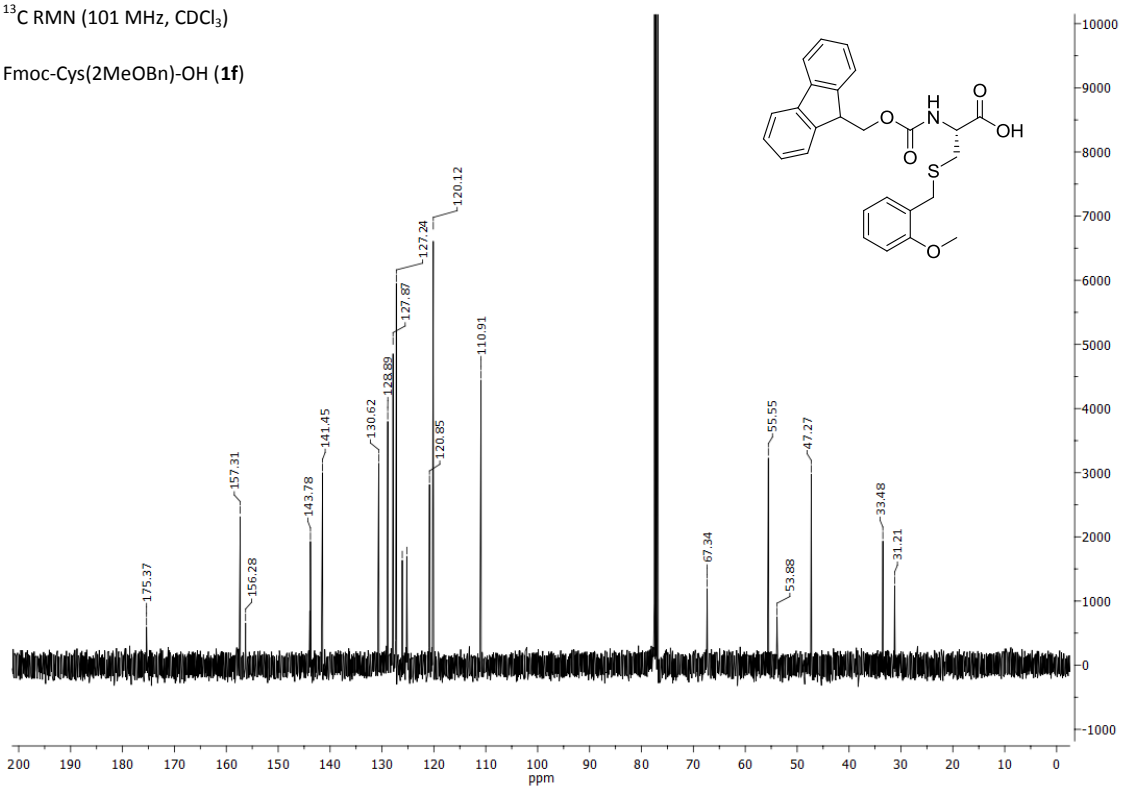
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(2MeOBn)-OH (**1f**)



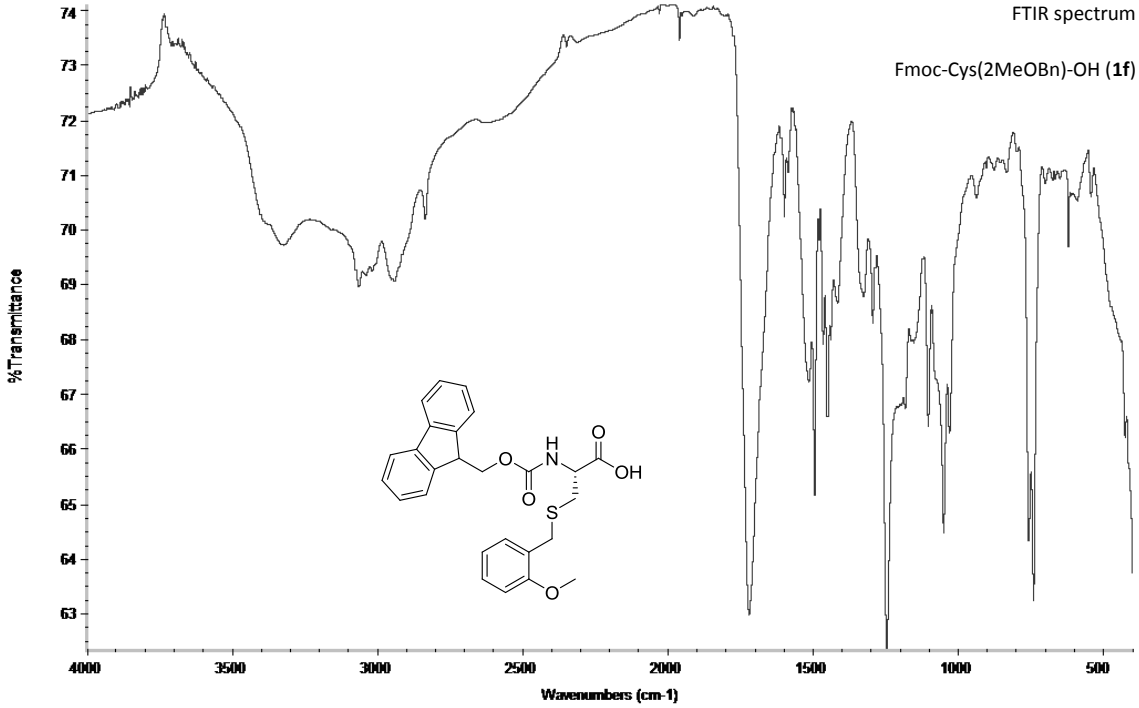
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(2MeOBn)-OH (**1f**)



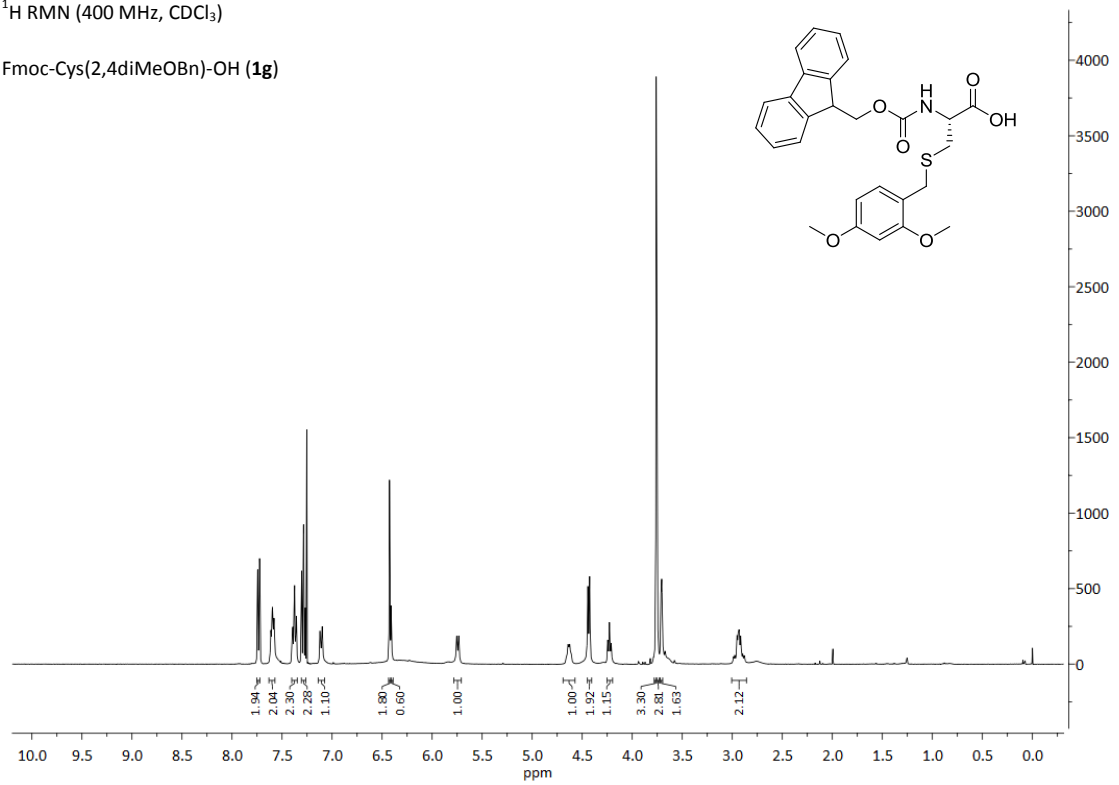
FTIR spectrum

Fmoc-Cys(2MeOBn)-OH (**1f**)



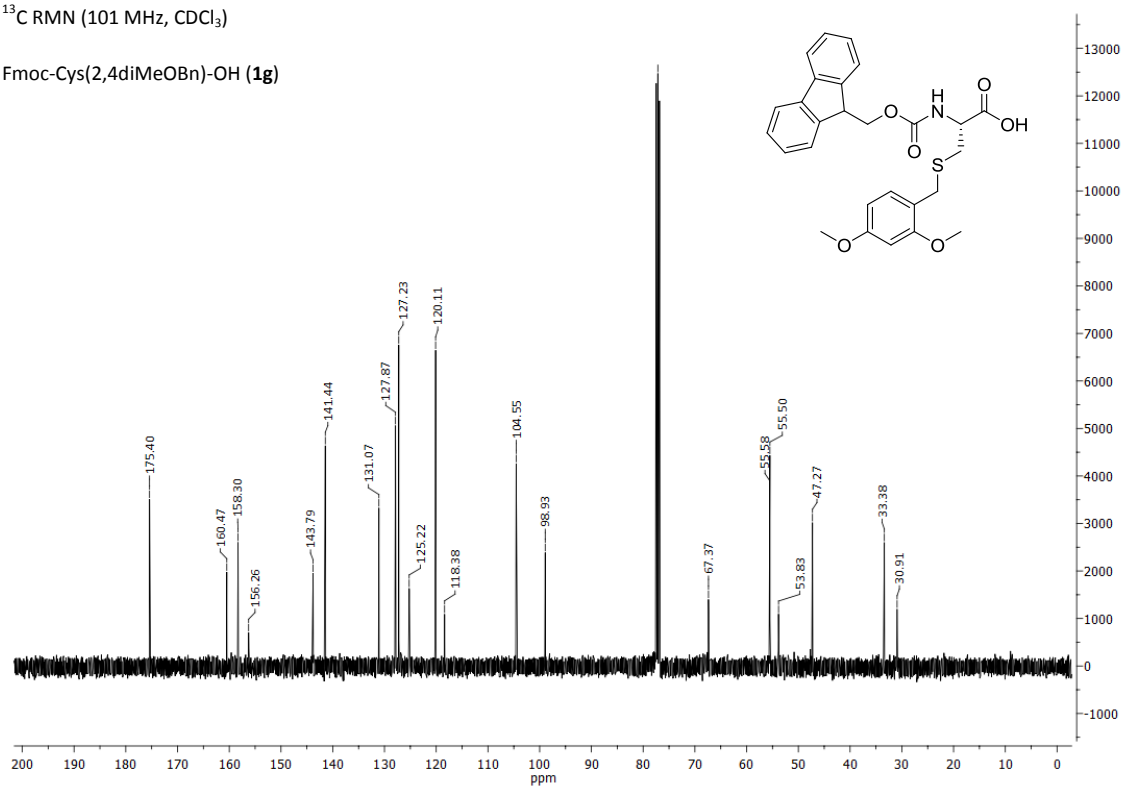
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(2,4diMeOBn)-OH (**1g**)



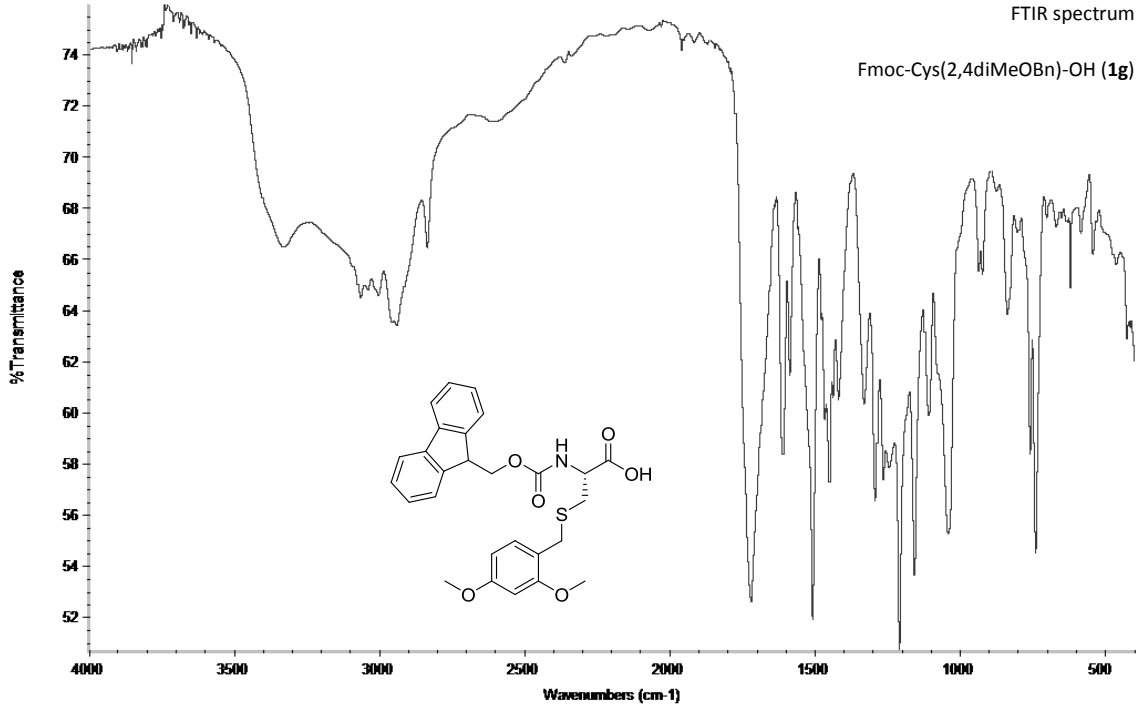
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(2,4diMeOBn)-OH (**1g**)



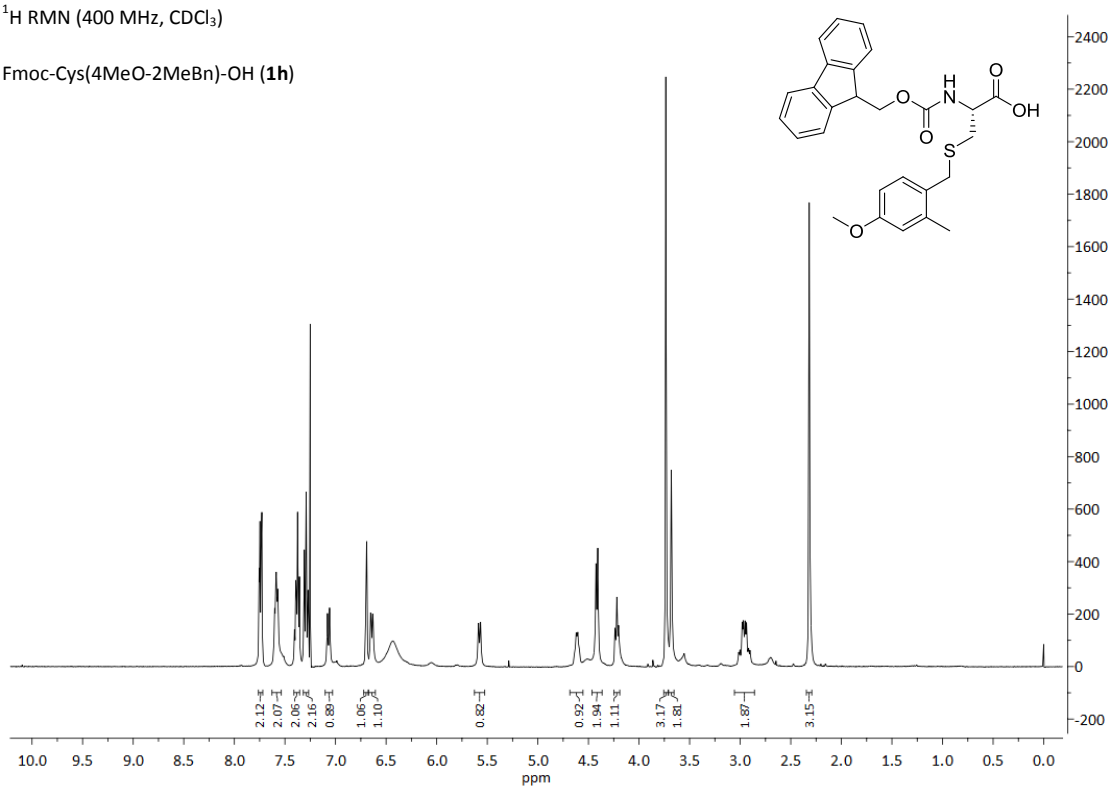
FTIR spectrum

Fmoc-Cys(2,4diMeOBn)-OH (**1g**)



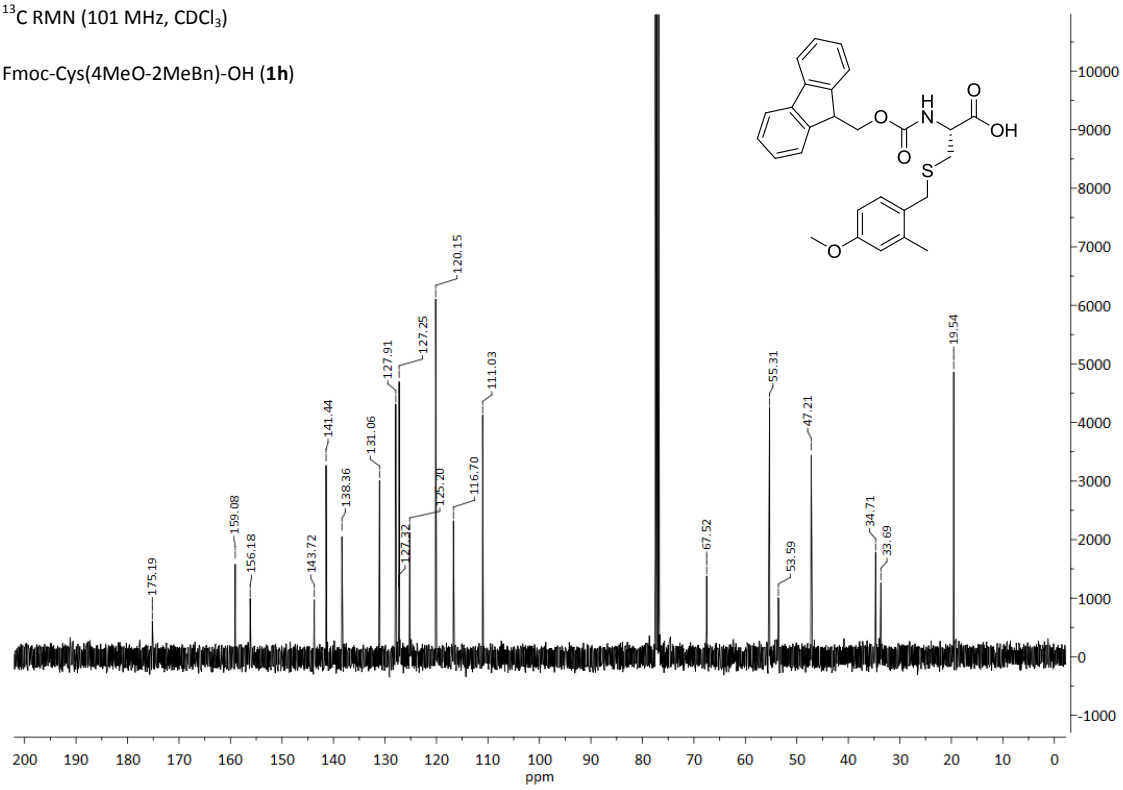
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(4MeO-2MeBn)-OH (**1h**)



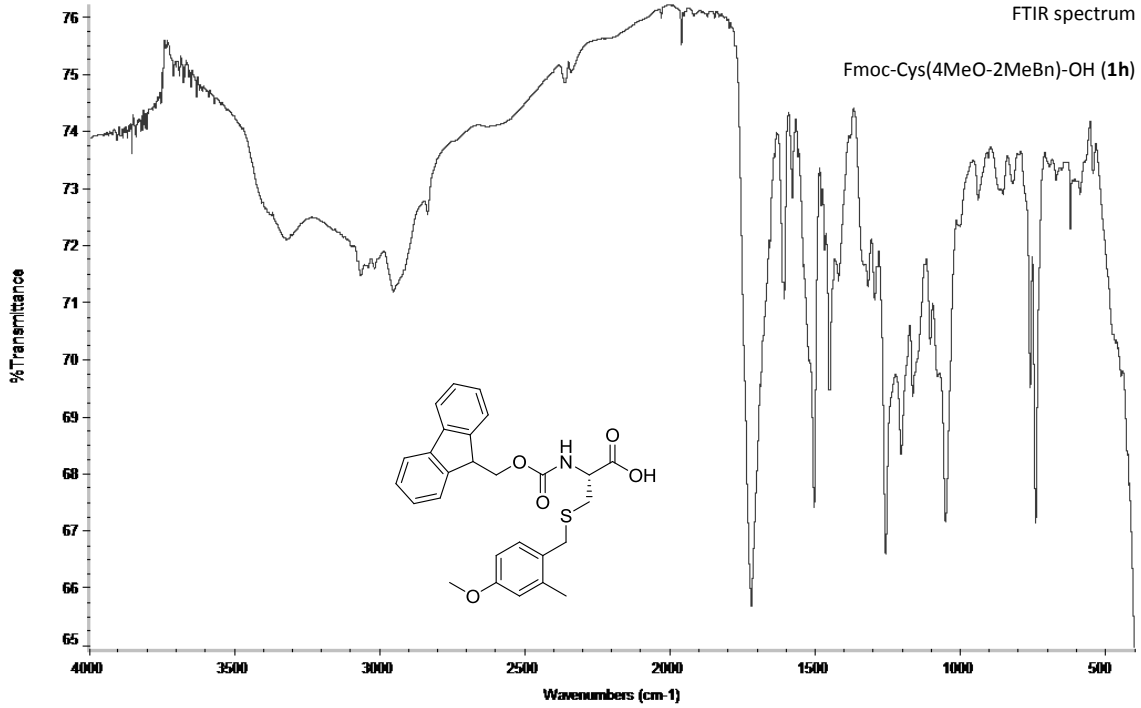
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(4MeO-2MeBn)-OH (**1h**)



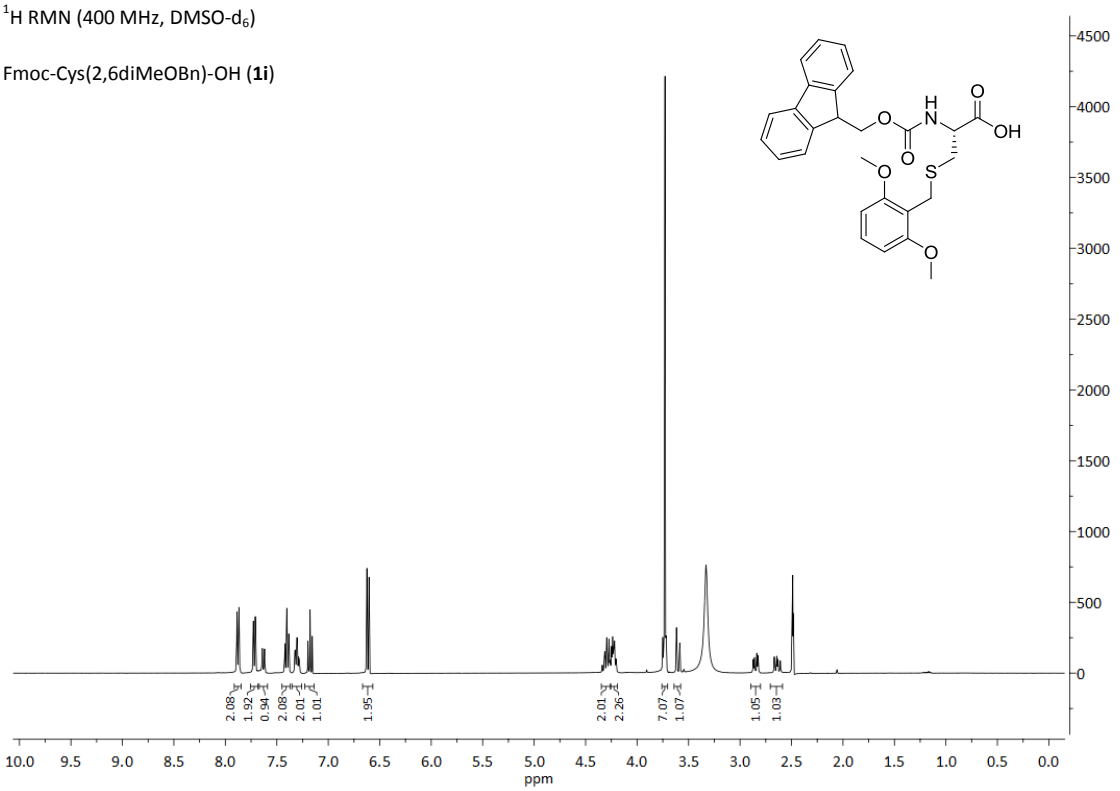
FTIR spectrum

Fmoc-Cys(4MeO-2MeBn)-OH (**1h**)



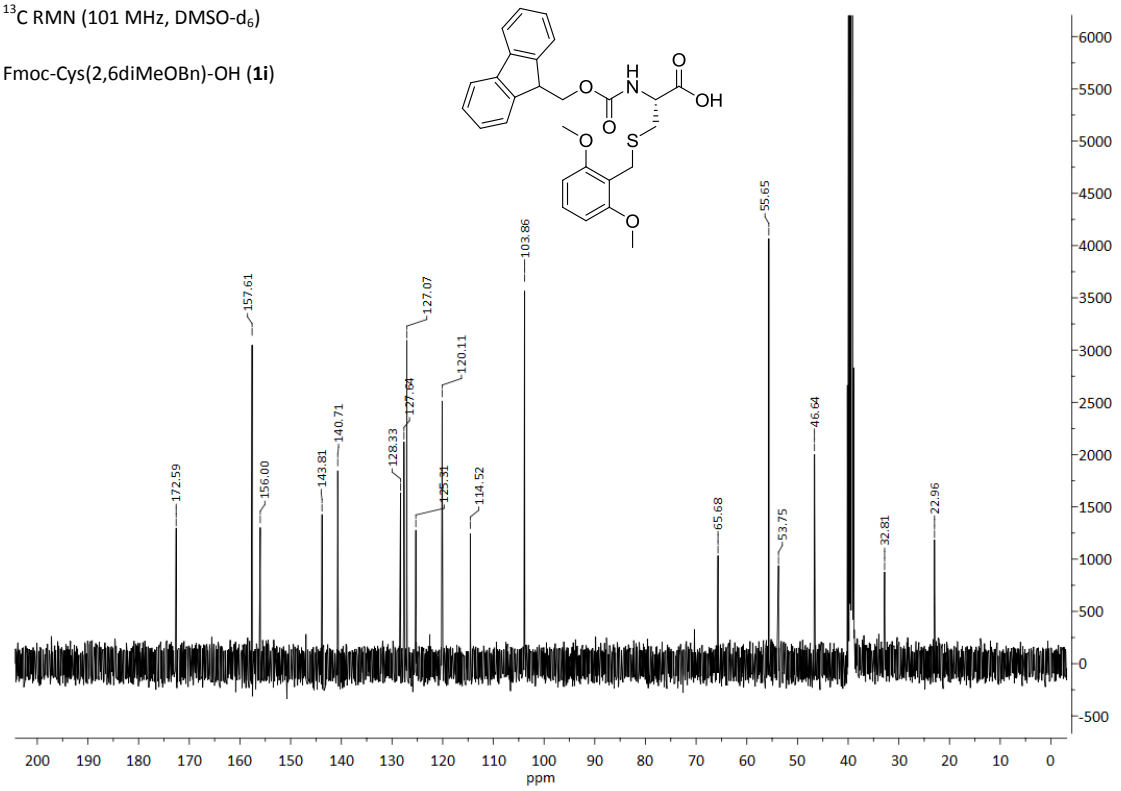
^{1}H RMN (400 MHz, DMSO-d_6)

Fmoc-Cys(2,6diMeOBn)-OH (**1i**)



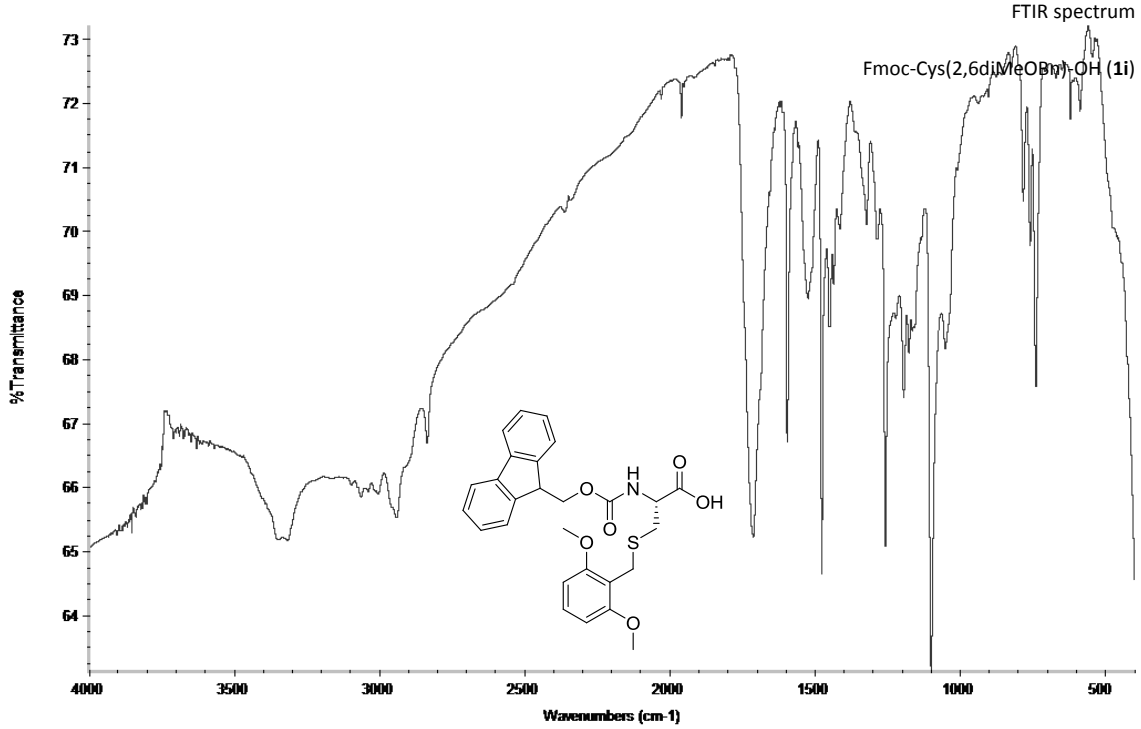
^{13}C RMN (101 MHz, DMSO-d_6)

Fmoc-Cys(2,6diMeOBn)-OH (**1i**)



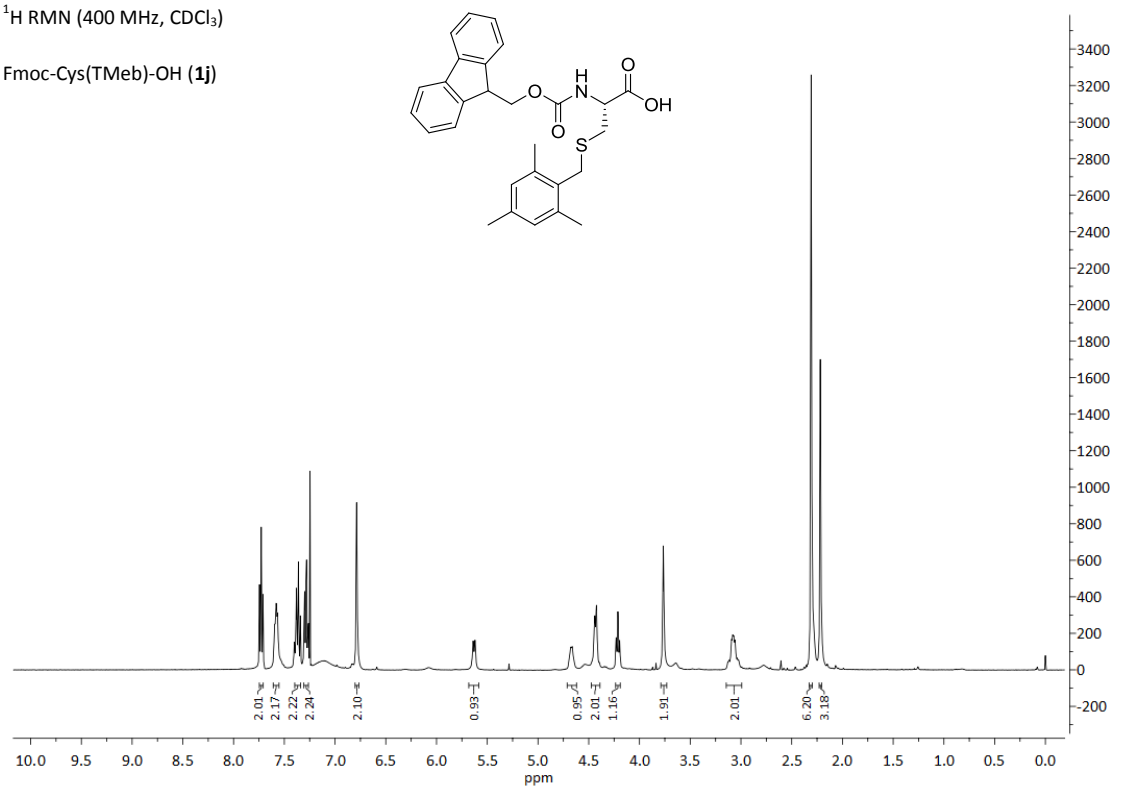
FTIR spectrum

Fmoc-Cys(2,6diMeOBn)-OH (**1i**)



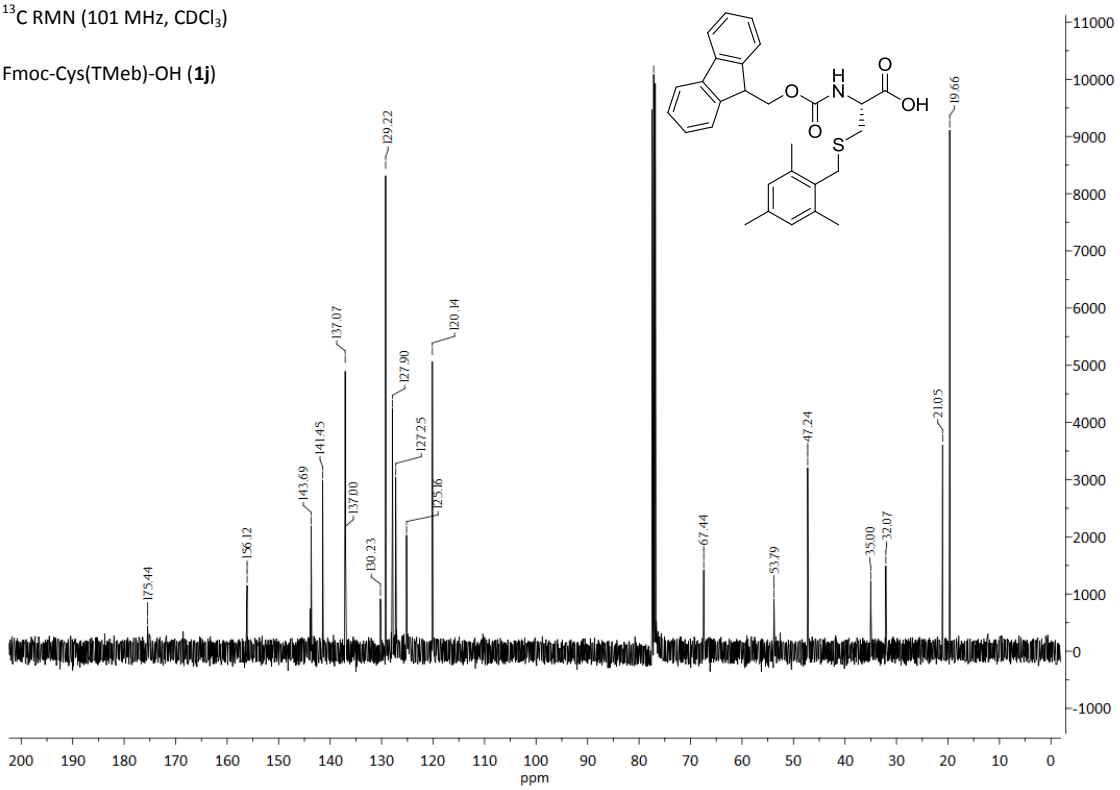
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(TMeb)-OH (**1j**)



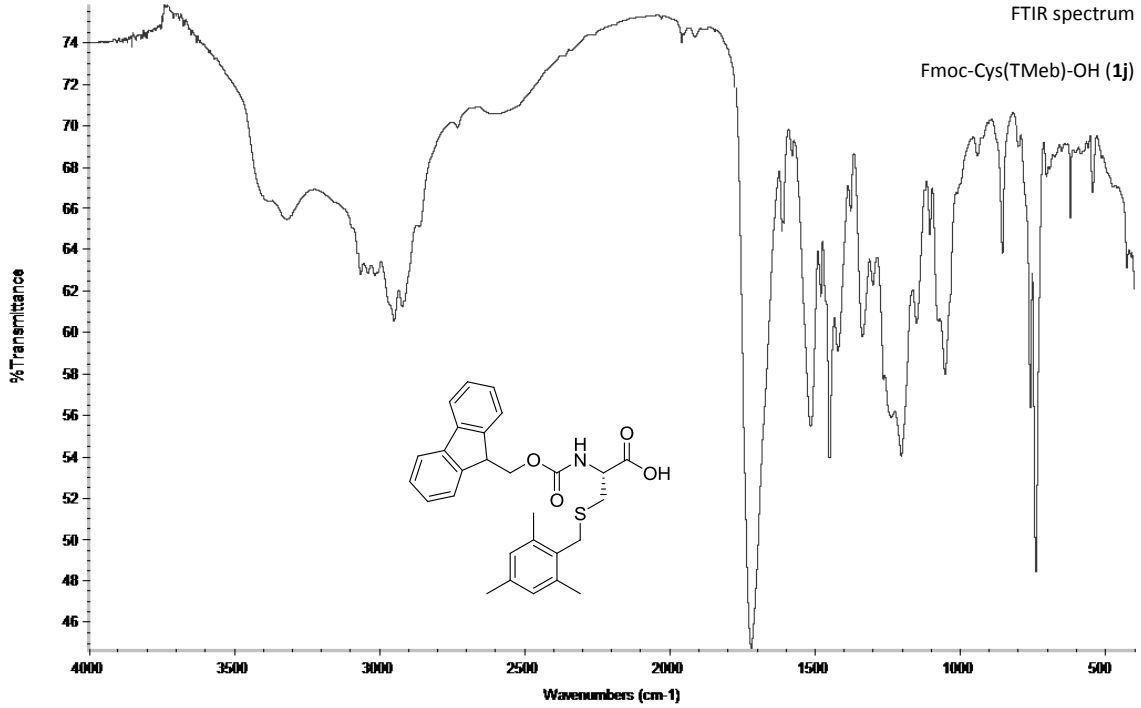
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(TMeb)-OH (**1j**)



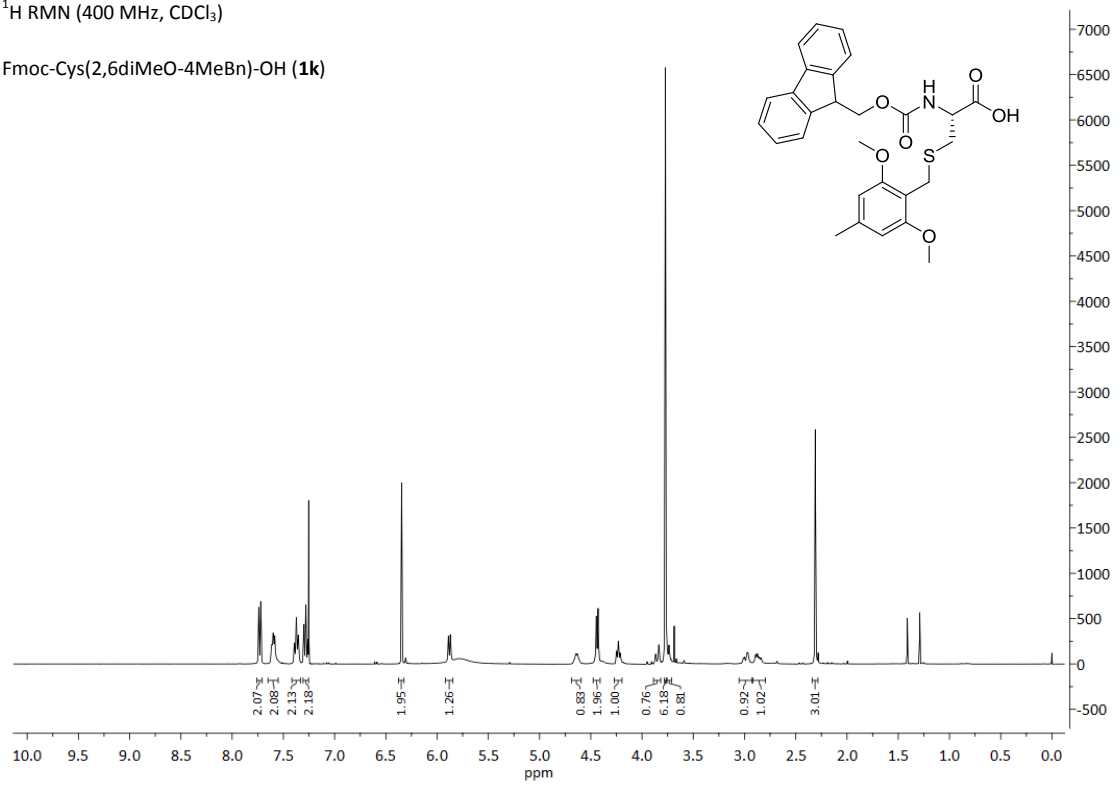
FTIR spectrum

Fmoc-Cys(TMeb)-OH (**1j**)



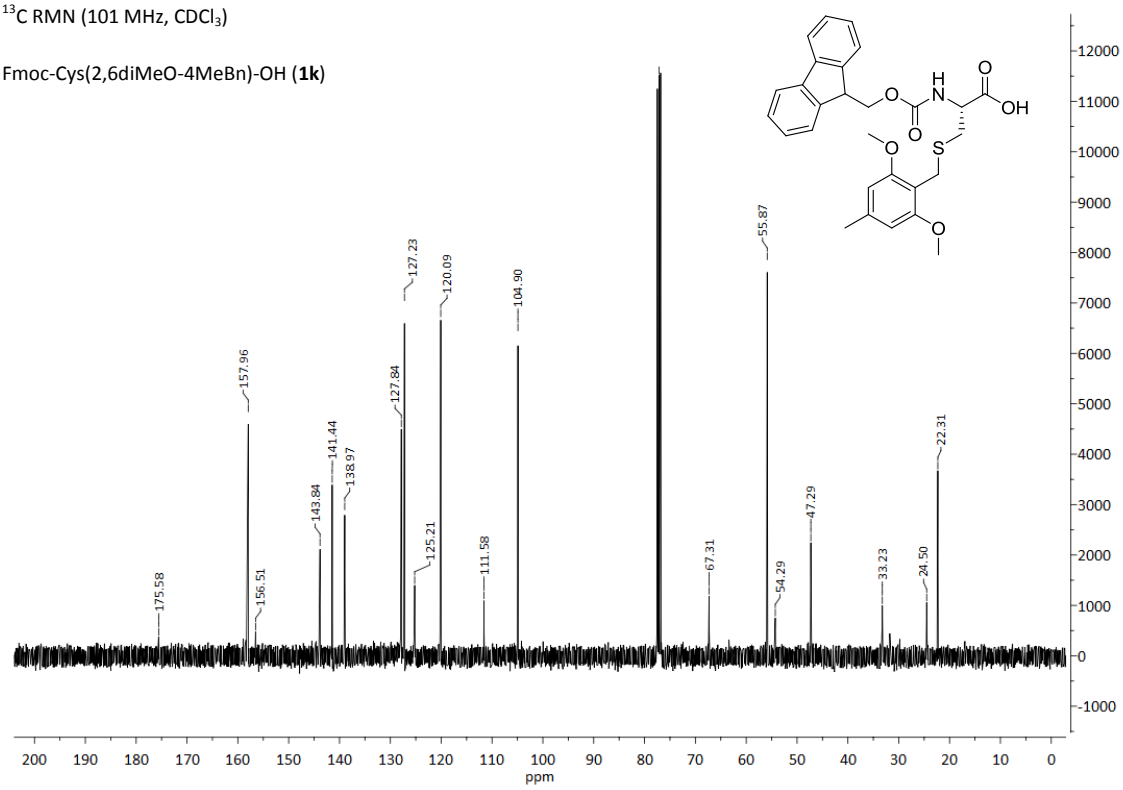
^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)



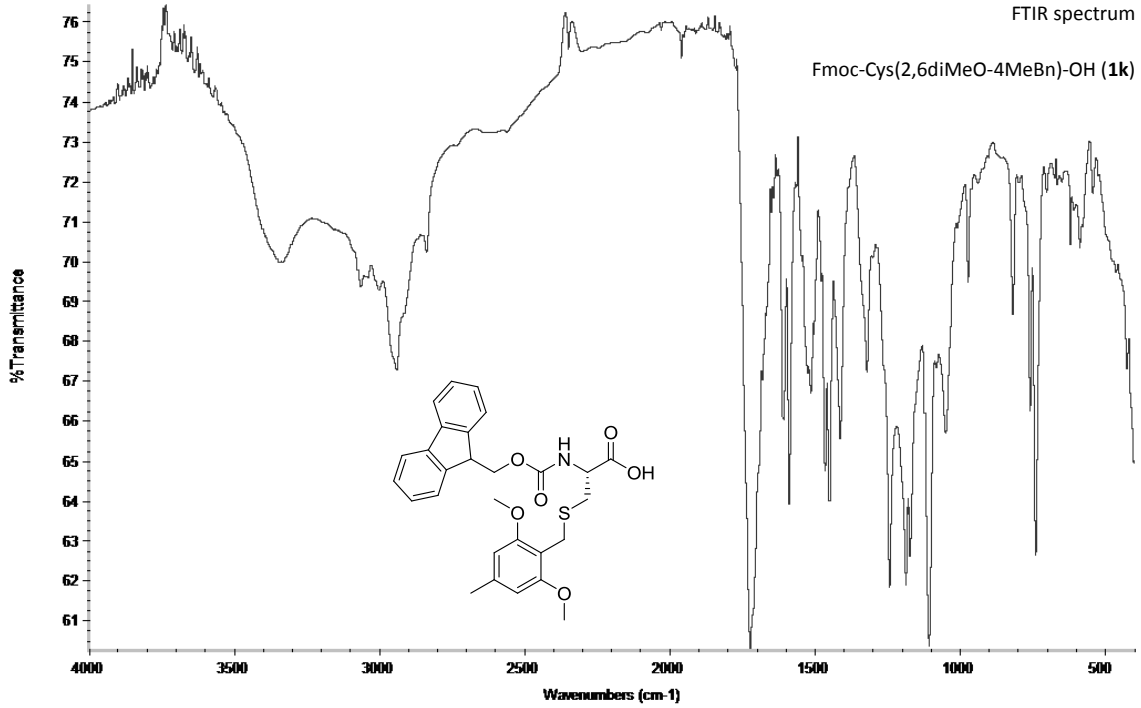
^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)



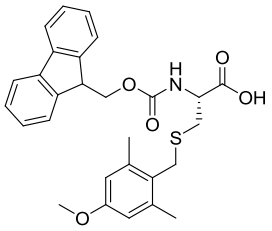
FTIR spectrum

Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)



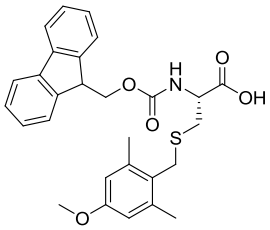
^{1}H RMN (400 MHz, DMSO-d_6)

Fmoc-Cys(4MeO-2,6diMeBn)-OH (**1l**)



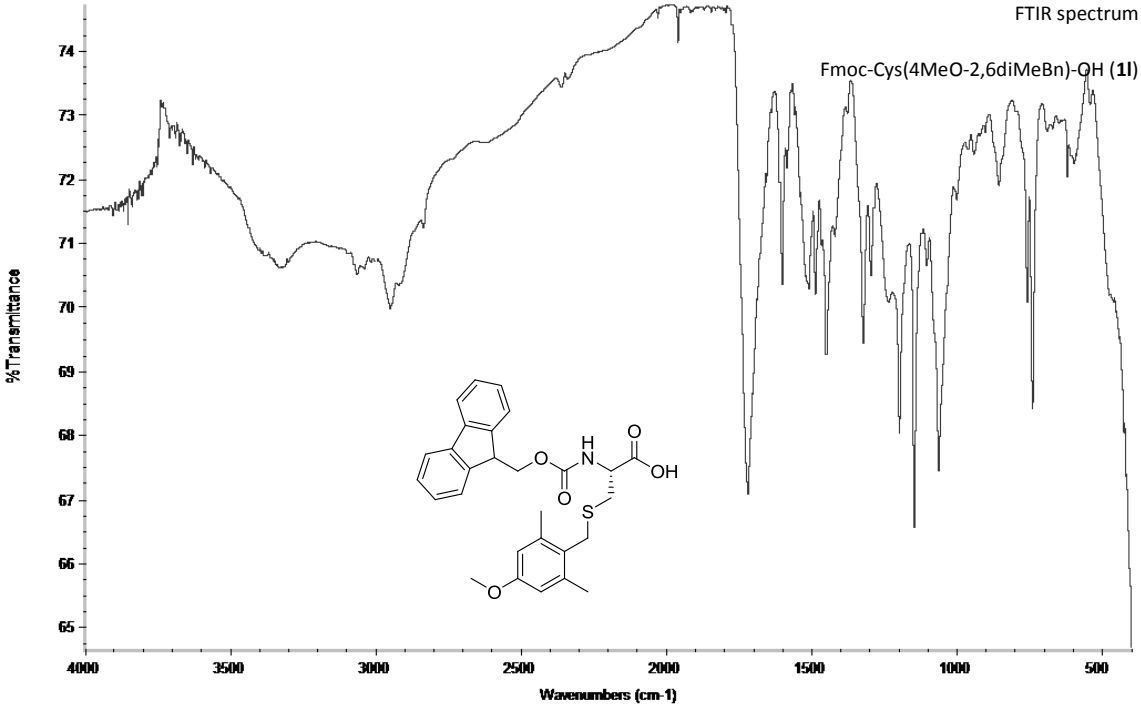
^{13}C RMN (101 MHz, DMSO-d_6)

Fmoc-Cys(4MeO-2,6diMeBn)-OH (**1l**)

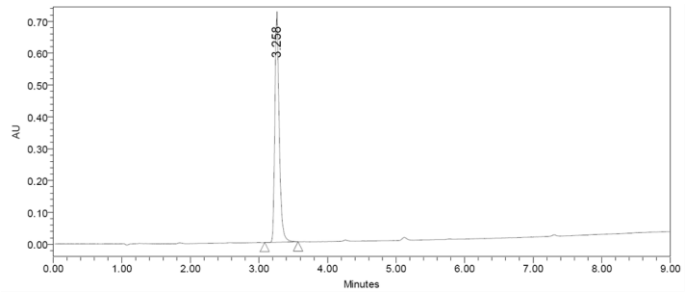


FTIR spectrum

Fmoc-Cys(4MeO-2,6diMeBn)-OH (**1l**)

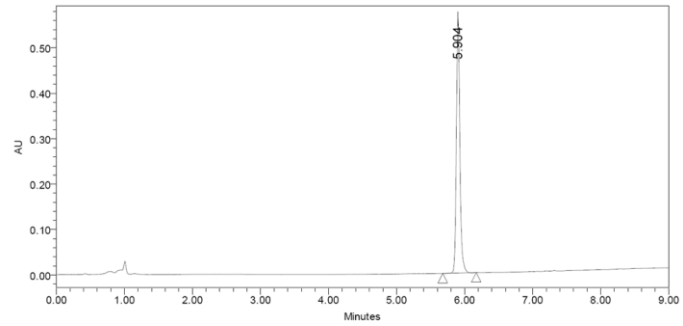


RP-HPLC analysis of the Fmoc-Cys(PG)-OH derivatives (1a-l)



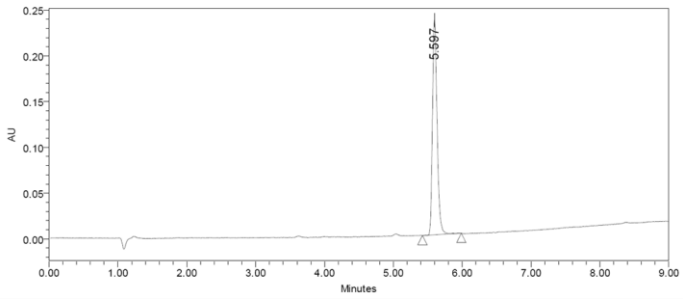
Fmoc-Cys-OH **1**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



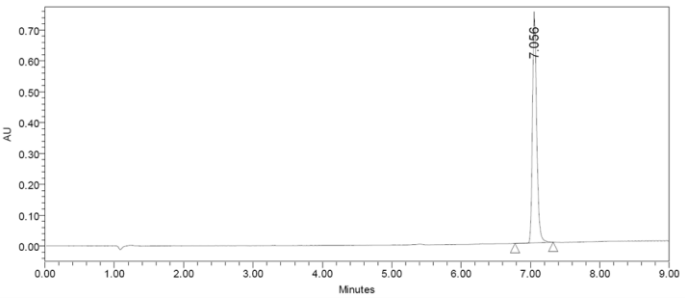
Fmoc-Cys(Dpm)-OH **1a**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



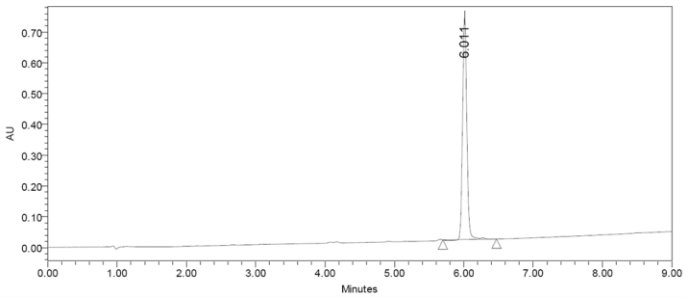
Fmoc-(4,4'diMeODpm)-OH **1b**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



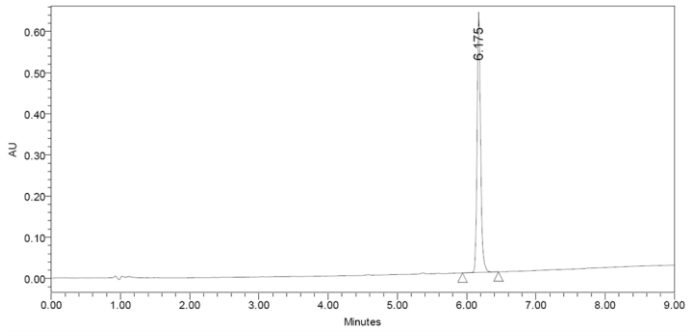
Fmoc-Cys(4,4'diMeDpm)-OH **1c**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



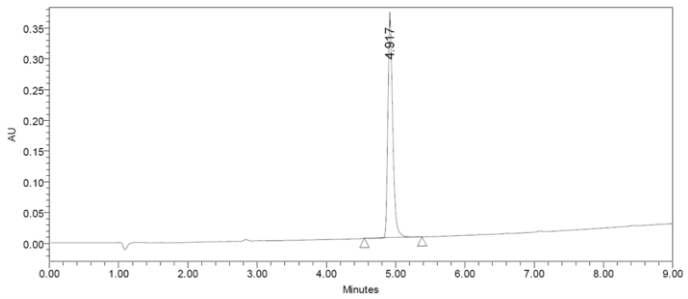
Fmoc-Cys(9F)-OH **1d**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



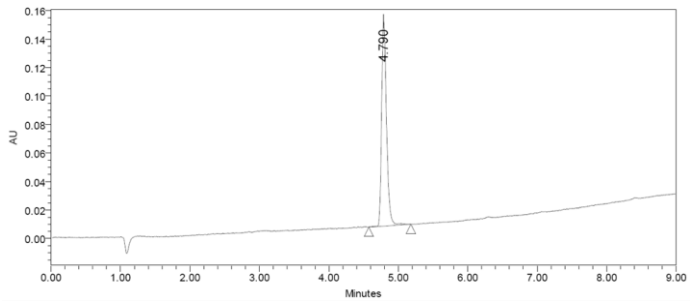
Fmoc-Cys(Bpm)-OH **1e**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



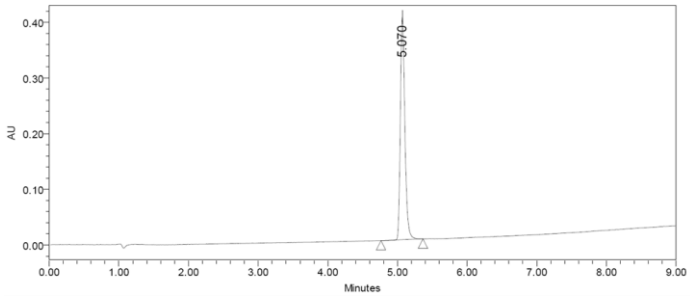
Fmoc-Cys(2MeOBn)-OH **1f**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



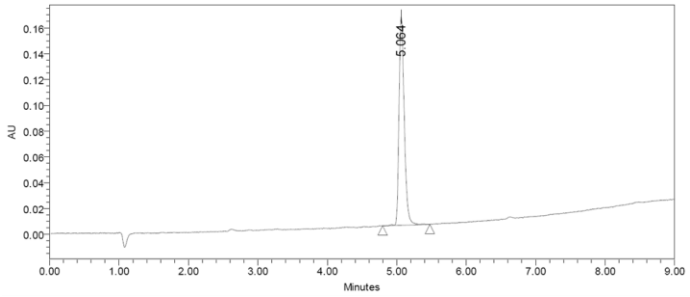
Fmoc-Cys(2,4diMeOBn)-OH **1g**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



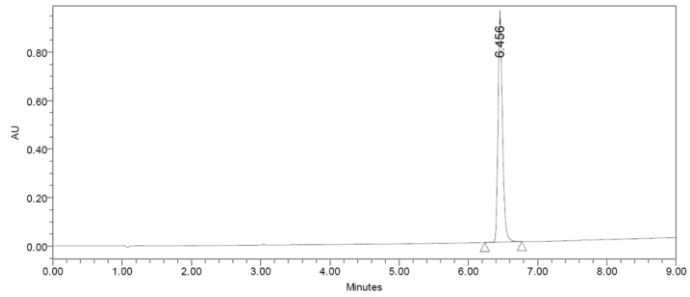
Fmoc-Cys(4MeO-2MeBn)-OH **1h**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



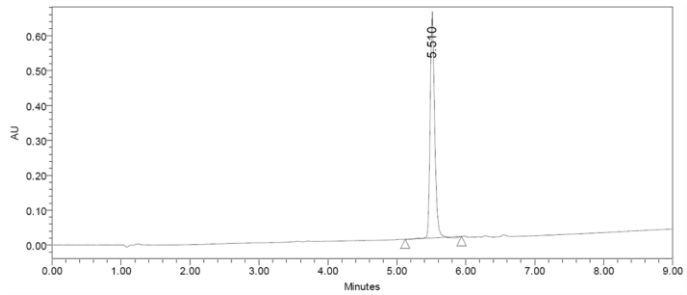
FmocCys(2,6diMeOBn)-OH **1i**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



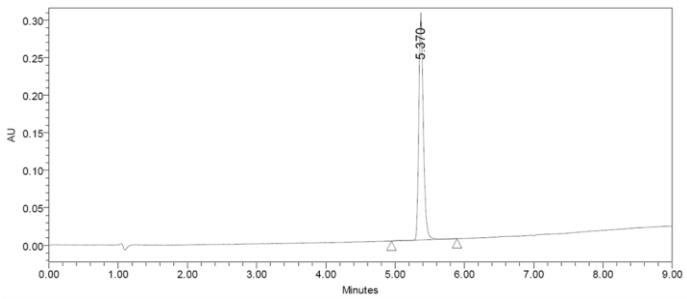
Fmoc-Cys(TMeb)-OH **1j**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k**

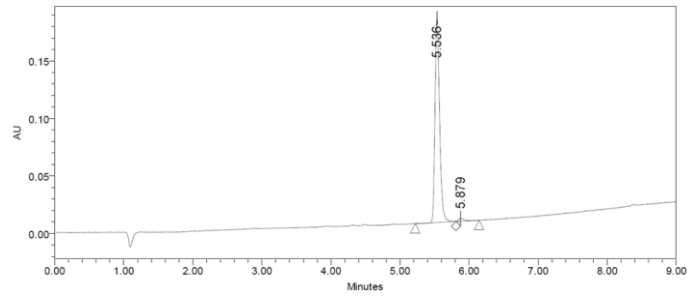
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l**

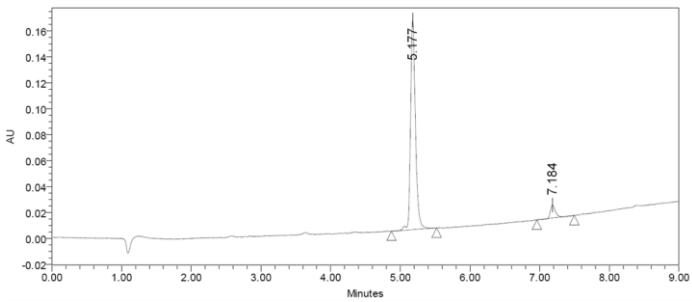
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of the Fmoc-Ala-Cys(PG)-Leu-NH_2 tripeptides (2a-m)



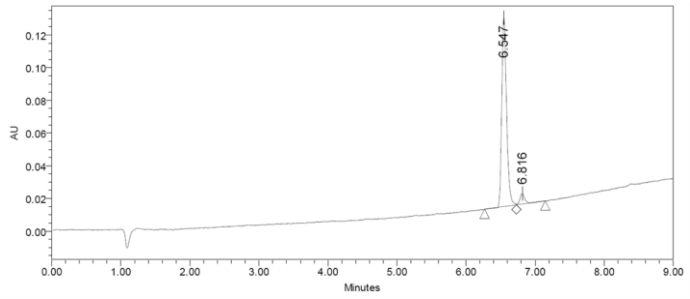
Cys(Dpm) tripeptide **2a**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



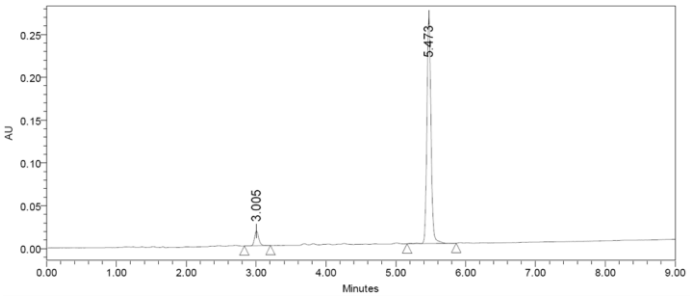
Cys(4,4'diMeODpm) tripeptide **2b**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



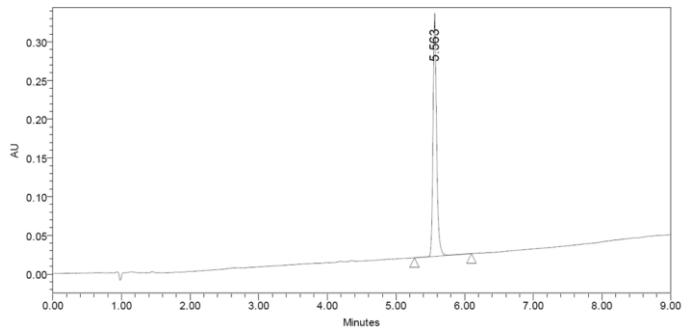
Cys(4,4'diMeDpm) tripeptide **2c**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



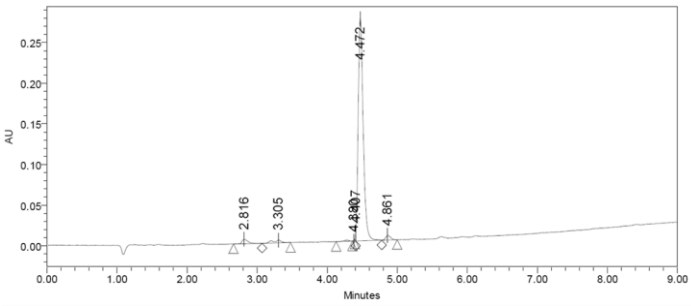
Cys(9F) tripeptide **2d**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



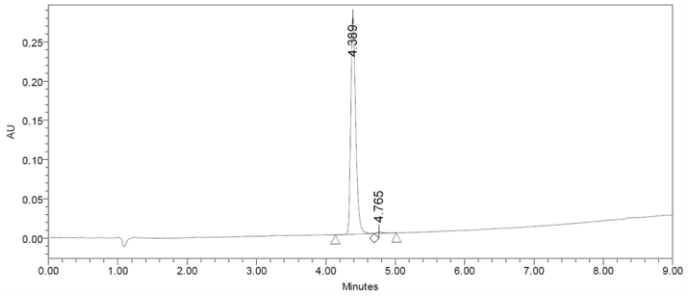
Cys(Bpm) tripeptide **2e**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



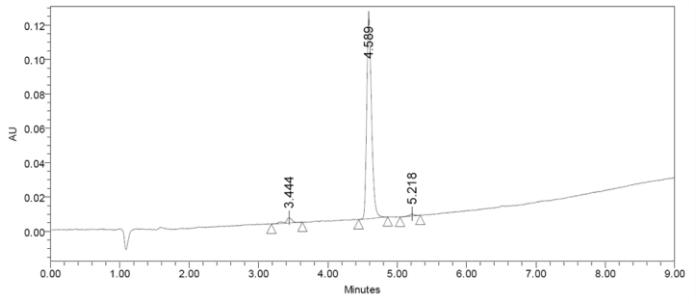
Cys(2MeOBn) tripeptide **2f**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



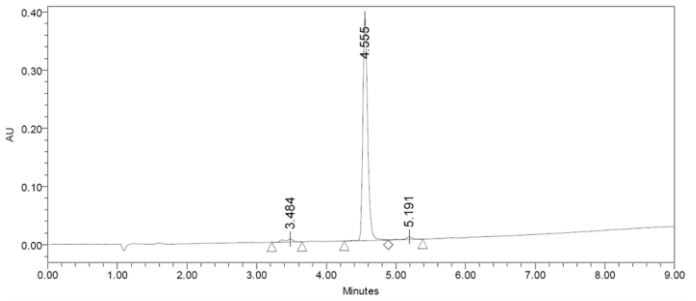
Cys(2,4diMeOBn) tripeptide **2g**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



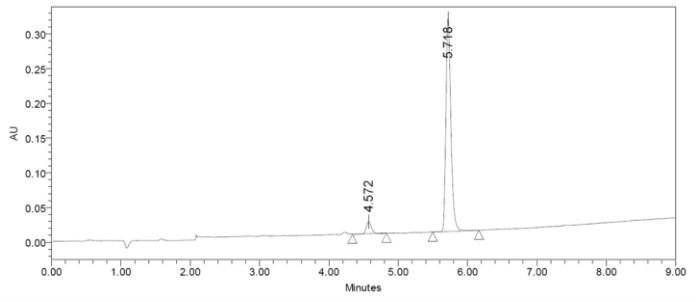
Cys(4MeO-2MeBn) tripeptide **2h**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



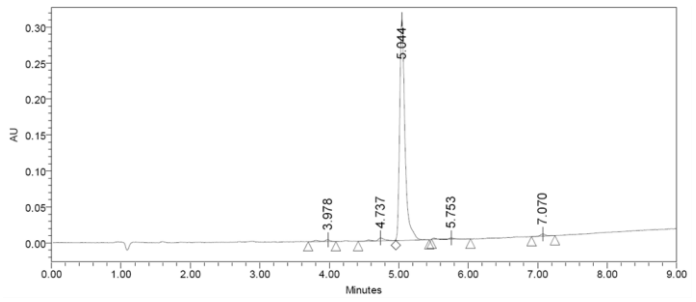
Cys(2,6diMeOBn) tripeptide **2i**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



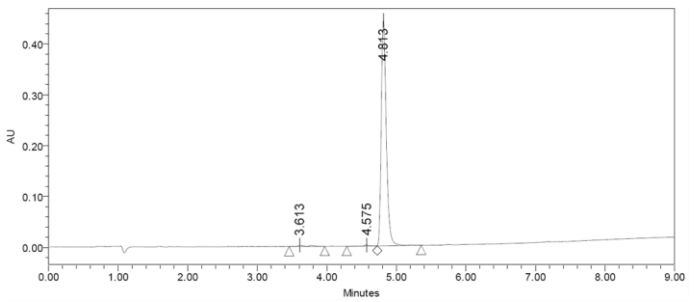
Cys(TMeb) tripeptide **2j**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



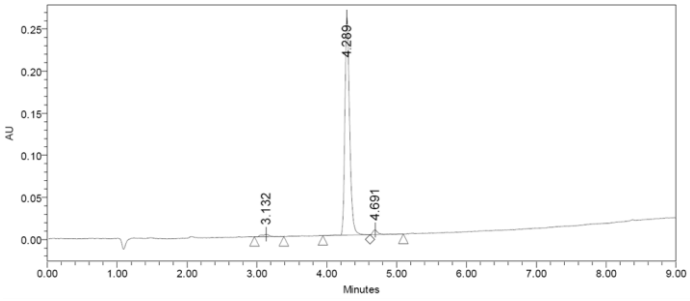
Cys(2,6diMeO-4MeBn) tripeptide **2k**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Cys(2,6diMe-4MeOBn) tripeptide **2l**

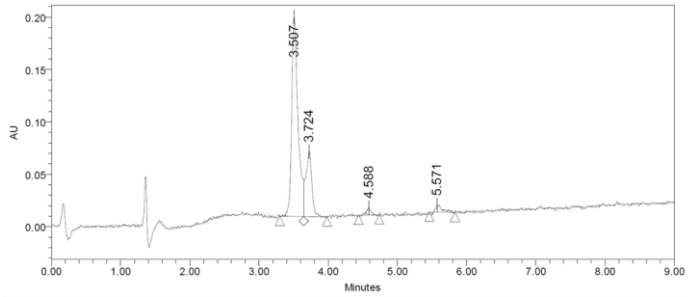
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Cys(Mob) tripeptide **2m**

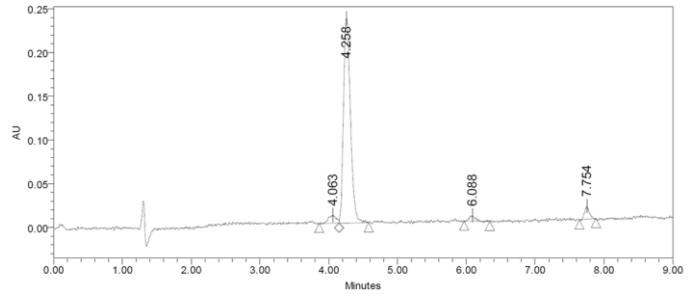
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of the hinge fragment of human IgG1 (8) and intermediates



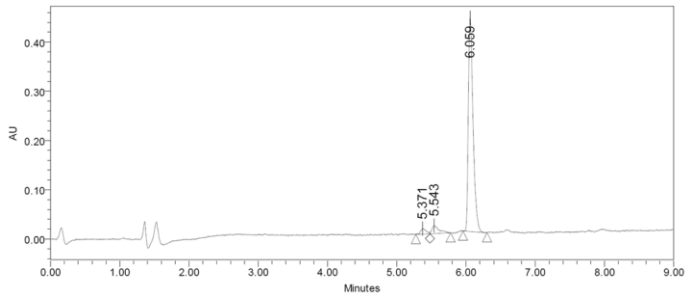
Fully deprotected linear monomer
H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2
(Rink-amide approach)

Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



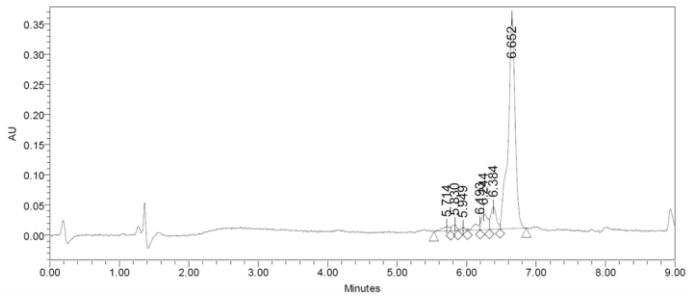
Fully deprotected linear monomer
H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2
(Rink-amide approach)

Gradient: 5% to 80% ACN at 60 °C
Wavelength: 220 nm



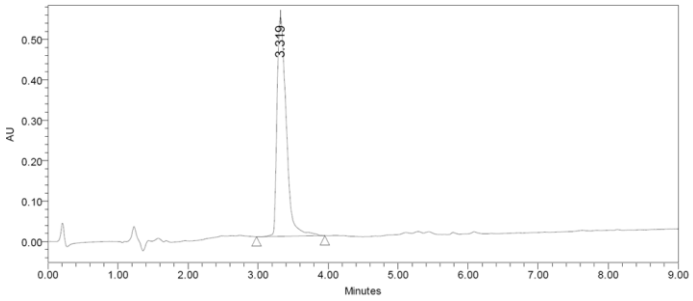
Partial deprotected linear monomer **5**
H-Thr(*t*Bu)-Cys-Pro-Pro-Cys(Dpm)-Pro-Ala-Pro-NH_2
(Sieber-amide approach)

Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



Protected dimer **6**
[H-Thr(*t*Bu)-Cys-Pro-Pro-Cys(Dpm)-Pro-Ala-Pro-NH_2]$_2$
(Sieber-amide approach)

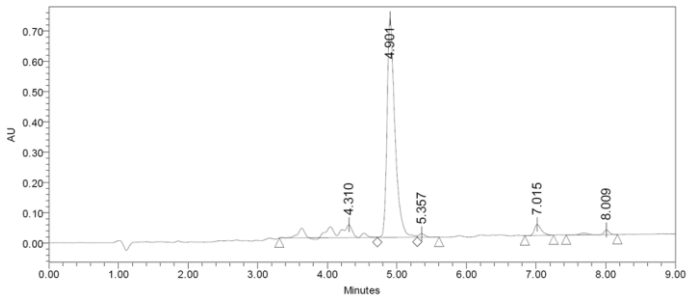
Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



Final parallel dimer **8**
(Random approach)

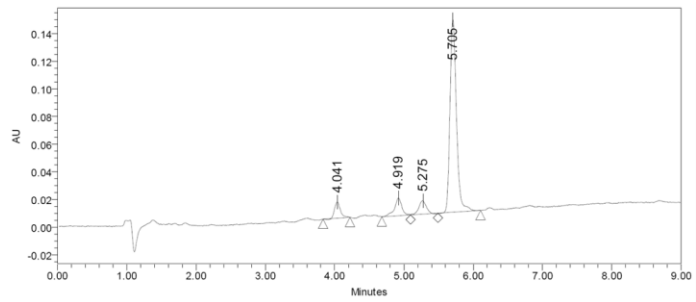
Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of α-conotoxin ImI and intermediates



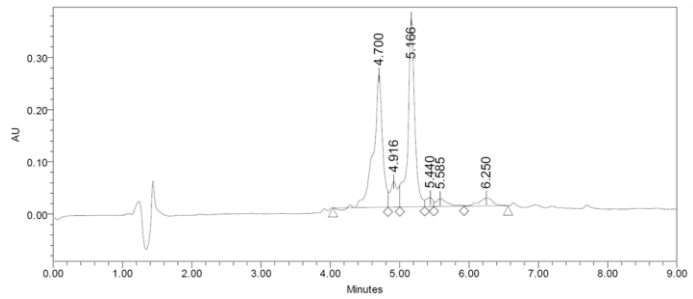
Reduced *S*-Dpm-protected intermediate **9**

Gradient: 70% to 100% ACN at 25 °C
Wavelength: 220 nm



Oxidized *S*-Dpm-protected intermediate **10**

Gradient: 70% to 100% ACN at 25 °C
Wavelength: 220 nm

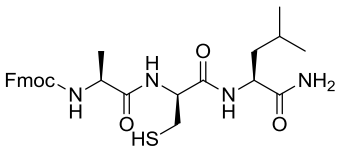


Final bicylic α-conotoxin ImI **11**
(Random strategy a)

Gradient: 10% to 35% ACN at 25 °C
Wavelength: 220 nm

Racemization study of Cys(Dpm)

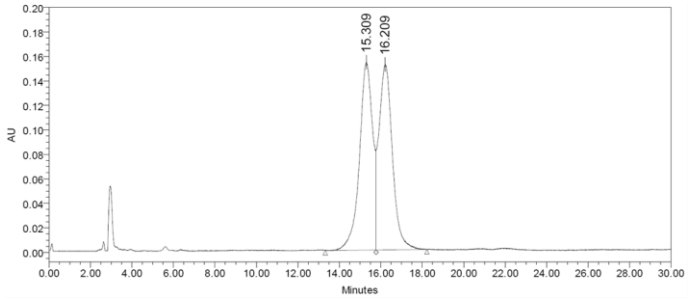
Fmoc-Ala-DCys-Leu-NH_2



Fmoc-Ala-DCys-Leu-NH_2 was synthesized as described for all tripeptides. After elongation, a portion of peptidyl-resin (5 mg) was cleaved and deprotected by using TFA-TIS-H_2O (95:2.5:2.5) for 10 min at 25 °C. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.6 min; *m/z* calcd for $C_{27}H_{34}N_4O_5S$, 526.7; found, 527.2 $[M+H]^+$, where M is the MW of the target tripeptide).

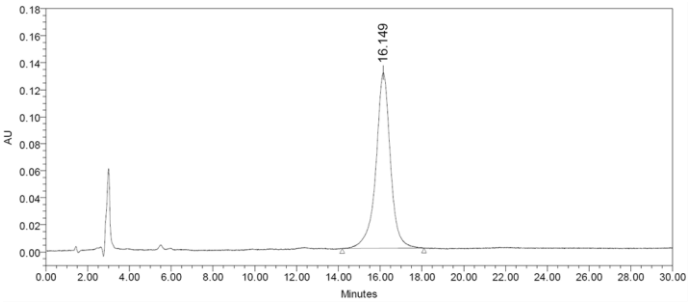
HPLC analysis of Fmoc-protected tripeptides: Fmoc-Ala-DCys-Leu-NH_2 and Fmoc-Ala-Cys-Leu-NH_2

Fmoc-Ala-DCys-Leu-NH_2 was prepared as described above and Fmoc-Ala-Cys-Leu-NH_2 was obtained by treating the Cys(Dpm) tripeptide with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C. Both thiol-free tripeptides were analyzed and compared with each other by RP-HPLC analysis on a PLRP-S reversed-phase analytical column (4.6 × 250 mm, 10 μm) from Varian.



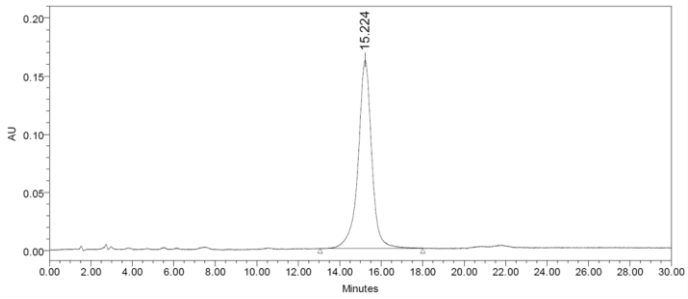
DCys and Cys
Fmoc-protected tripeptides

Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm



Fmoc-Ala-DCys-Leu-NH_2

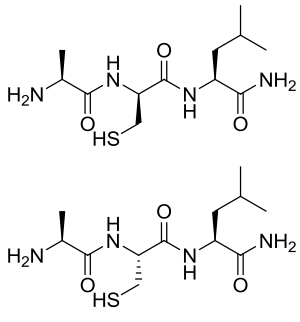
Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm



Fmoc-Ala-Cys-Leu-NH_2
from Cys(Dpm) tripeptide

Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm

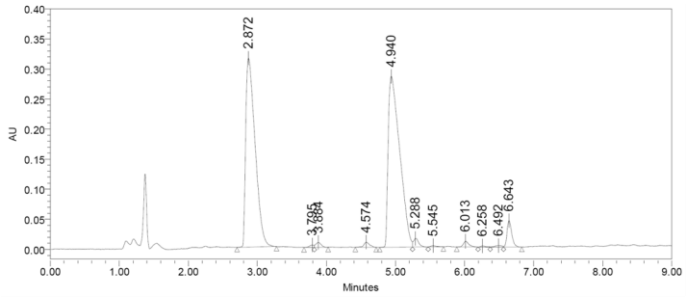
H-Ala-DCys-Leu-NH_2 and H-Ala-Cys-Leu-NH_2



Because a clear separation was not achieved for these Fmoc-protected tripeptides, we decided to remove the Fmoc group first. Thus, a portion of DCys tripeptide anchored on the resin (5 mg) was treated with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min) and consecutively cleaved and deprotected by using TFA-TIS-H_2O (95:2.5:2.5) for 10 min at 25 °C to render the H-Ala-DCys-Leu-NH_2 tripeptide. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 0% to 50% ACN over 8 min; t_R : 1.2 min; *m/z* calcd for $C_{12}H_{24}N_4O_3S$, 304.4; found, 305.1 $[M+H]^+$, where M is the MW of the target tripeptide). On the other hand, the Fmoc group form the Cys(Dpm) tripeptide was removed in solution. Therefore, the solid tripeptide (2 mg) was dissolved in piperidine-ACN (1:40) (2 mL) and left to stand for 30 min at 25 °C. Then, the solvent was removed under vacuum and the crude residue was treated with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C to render the H-Ala-Cys-Leu-NH_2 tripeptide. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 0% to 50% ACN over 8 min; t_R : 1.2 min; *m/z* calcd for $C_{12}H_{24}N_4O_3S$, 304.4; found, 305.1 $[M+H]^+$, where M is the MW of the target tripeptide).

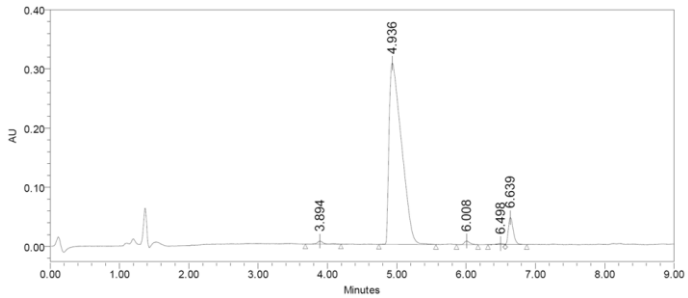
HPLC analysis of Fmoc-deprotected tripeptides: H-Ala-DCys-Leu-NH_2 and H-Ala-Cys-Leu-NH_2

Both thiol-free tripeptides were analyzed and compared with each other by RP-HPLC analysis on a XBridge™ BEH130 C18 reversed-phase HPLC analytical column (4.6 mm x 100 mm, 3.5 μm) from Waters.



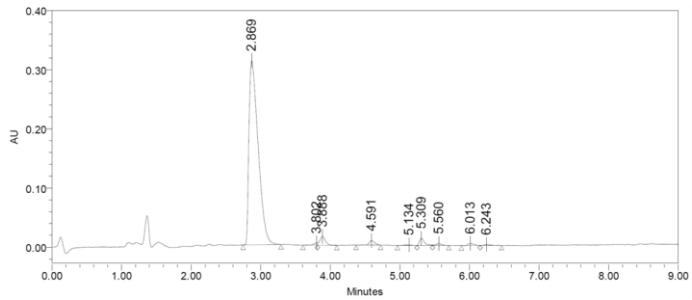
DCys and Cys
Fmoc-deprotected tripeptides

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm



H-Ala-DCys-Leu-NH_2

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm



H-Ala-Cys-Leu-NH_2
from Cys(Dpm) tripeptide

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm

No racemization occurred during Fmoc-Cys(Dpm)-OH coupling under neutral conditions, as determined by HPLC analysis.

CHAPTER 3

DKP-based handle

Chapter 3. Introduction

Handles for Fmoc-solid-phase synthesis of protected peptides

Handles for Fmoc-solid-phase synthesis of protected peptides

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A linker or handle is described as a bifunctional chemical moiety attaching a compound to a solid or soluble support, which can be cleaved to release compounds from the support. The careful choice of linker allows cleavage to be performed under appropriate conditions compatible with the stability of the compound and assay method [1].

In solid-phase peptide synthesis (SPPS), ideally, a linker must be orthogonal or compatible, this means inert under peptide elongation conditions –avoiding the premature cleavage of the peptide during coupling and deprotection cycles–, and should allow the controlled release of the substrate under mild conditions. In general, protecting-group chemistry has been the basis of the development of novel handles and specific cleavage conditions, enabling the release of the target molecule without degradation occurring, and free of by-products. Based on protecting group strategies, linker design has expanded the initial purpose of connect substrate and support, and has moved a step further for encompassing novel concepts and functionalities. For instance, may handles have been designed and developed for the preparation of *C*-terminal modified peptides by mean of diverse concepts and strategies [2]. This is the case of backbone amide attachment (BAL) concept, side-chain anchoring, or inverse solid-phase peptide synthesis, among others, which are commonly used as synthetic tools incorporated in peptide synthetic schemes. Although acid-labile linkers are, by far, the most widely used in peptide chemistry, a myriad of handles that can be cleaved by means of different mechanisms have been described. Depending on the specific cleavage conditions, linkers may be divided into different categories, including acid-labile, base-labile, nucleophile-labile, enzyme-labile, photo-labile, labile to reducing agents, labile to oxidizing agents, thiol-labile, hydrazine-labile, palladium-labile, and the most sophisticated traceless, safety-catch or cyclative cleavage linkers.

Although several handles have been design and applied for the preparation of modified peptides, we have focused on these linkers described for the synthesis of protected peptides on solid phase. Preparation of protected peptide fragments is demanded for their further application in the fragment condensation approach, and the synthesis of protected peptides is often required for the head-to-tail cyclization of

peptides in solution. For those readers interested in handles used for preparing modified peptide by SPPS, several reviews and books have covered this field in the last decade [2–3–4–5].

Stepwise *vs.* convergent approach

Stepwise SPPS, pioneered by Merrifield in 1963 [6], represented a revolution in the art of peptide synthesis. Nowadays, SPPS is a well-established methodology for the synthesis of small- to medium-sized peptides. Originally based on the combination of Boc group as temporary protecting group for the N α-amino function and benzyl-type groups as permanent protecting groups for side-chains, currently, peptides are mostly synthesized by using Fmoc/*t*Bu orthogonal strategy.

SPPS methodology generally results efficient for the elongation of peptide sequences, although the ability to assemble a peptide in a stepwise manner is limited by the synthesis efficiency of each step –including coupling and deprotection–. Thus, for instance, the synthesis of a 100-residue peptide with 99% efficiency at each step provides a 90% overall yield of the target peptide, while 97% efficiency renders a 5% overall yield [7]. Difficult couplings and deprotection during SPPS are commonly associated with poor solvation of the elongating strand anchored onto the solid support, high tendency of growing hydrophobic peptide sequences to aggregate [8], or the presence of consecutive hindered amino acids in the sequence.

The development of novel coupling reagents [9], handles [2-5] and protecting groups [10], the identification and diminishment of detrimental side reactions, the use of DMSO [11] or “magic mixtures” [12], or the addition of chaotropic salts [13] during coupling/deprotection cycles, along with the emergence of innovative PEG-based resins, such as SPOCC [14] and ChemMatrix [15-16], have proved to significantly improve the efficiency of stepwise SPPS approach. Moreover, the use of additional synthetic tools, including the incorporation of pseudoprolines [17–18–19–20–21–22] or *N*-2-hydroxy-4-methoxybenzyl (Hmb) [23–24] and dicyclopropylmethyl (Dcpm) [25] for backbone protection, as well as the implementation of an *O*-acyl isopeptide

method [26–27–28] have been successfully applied to increase the overall synthesis efficiency. Nevertheless, although all these advances, stepwise SPPS approach is limited for the synthesis of polypeptides longer to 50 residues owing to the accumulation of undesirable by-product derived from no quantitative coupling and deprotection reactions during peptide elongation –which lead to truncated or deletion sequences–, and convergent approaches should be addressed.

Convergent approaches enable greater control of the synthesis due to fragment peptide intermediates can be accurately analyzed –by RP-HPLC and mass-spectrometry techniques– and may be purified before fragment combination when necessary. Moreover, synthesis of shorter peptide fragments might allow the rapid identification of difficult sequences, double coupling required, truncated sequences, and side-reactions, as well as enable to adopt the appropriate procedures for preventing or minimizing deletions and/or partial N^{α}-protecting group removal. Convergent approaches, including i) fragment condensation strategy on solid phase –classically called convergent approach- or in solution –hybrid approach–, and ii) chemoselective ligation approaches[29] (such as original Native Chemical Ligation (NCL) [30], and variant thereof [31–32], hydrazine ligation [33], and α-ketoacid-hydroxylamine (KAHA) ligation [34–35], among others), have been developed to address the synthesis of large polypeptides and difficult peptide sequences.

Fragment –or segment– condensation strategy relies on the preparation of polypeptides by assembling protected peptide fragments (**Figure 1**). Thus, acyl-acceptor-peptide fragment (*C*-terminal protected segment) and acyl-donor-peptide fragment (*N*-terminal protected segment) are elongated on solid phase, and then, the resulting protected segments can be coupled on-resin (solid phase fragment coupling) or in solution (hybrid strategy), followed by the fully removal of the protecting groups. While acyl-donors may be directly used in the fragment coupling reaction in solution, acyl-acceptor fragments frequently required additional protection transformation of the *C*-terminal group.

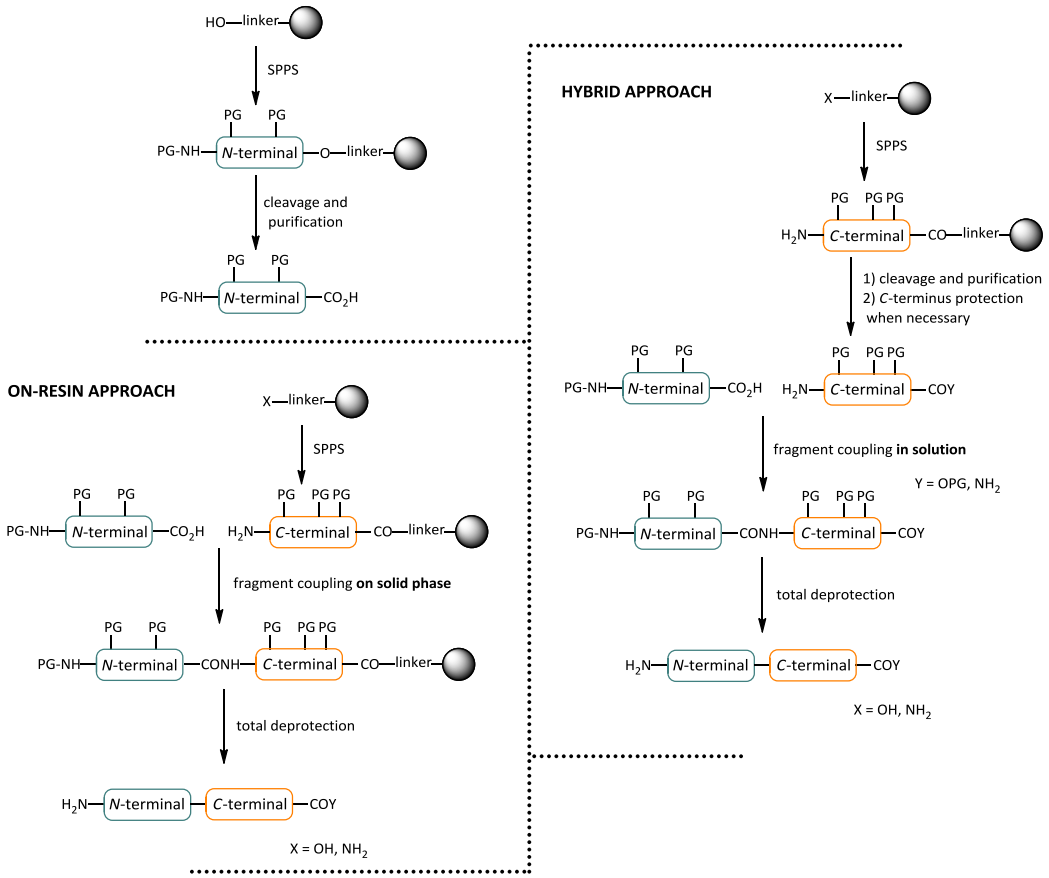


Figure 1. Schematic representation of the fragment condensation approaches for the synthesis of peptides.

Fragment condensation approach has been successfully applied for the syntheses of medium-sized peptides, including exenatide [36], hirudin [37], Mdm2 ring finger domain [38], and T20 [39] peptides. Hybrid convergent strategy has proved to be effectively for the preparation of miniproteins, such as ribonuclease A [40] and green fluorescent protein [41]. Moreover, the solid-phase assembly of protected segments has been reported for β-amyloid protein [42], the *N*-terminal domain of γ-zein protein [43], or the prothymosin α [44], among others.

For the final success of this synthetic strategy many consideration need to be address, including the epimerization susceptibility during the activation at the *C*-terminus, the poor solubility of long protected peptide sequences in aqueous media for RP-HPLC purification, as well as the reactivity of the functional groups involved in the generated amide bond. Thus, to overcome the racemization issue is crucial that in the retrosynthetic design the *C*-terminal residue of the *N*-fragment peptide preferred must

be Gly, Pro, or pseudoprolines [45] due to absence of racemization of these residues, while Cys and His, amino acids prone to epimerize, must be avoided. A low solubility may not only hamper fragment condensation, but also purification and characterization of the protected fragments. Furthermore, the efficiency of the fragment condensation depends on the *N*-terminal amino acid of the *C*-fragment intermediate involved, so if possible, sterically hindered amino acids should be excluded.

As mentioned before, fragment coupling reaction may be carried out on solid-phase or in solution. When fragment combination is performed on-resin, the *C*-fragment peptide is typically used in excess to assure complete condensation reactions. For large-scale productions, the requirement of a *C*-component in excess is economically unfeasible owing to the *C*-fragment peptide used is not recoverable. On the other hand, when fragment condensation is performed in solution, equimolecular quantities of both fragments are applied, so that this approach is preferred at large scale. In the **Table 1**, the advantages and disadvantages of both stepwise SPPS and fragment condensation approach are summarized.

	stepwise SPPS	**fragment condensation approach**
pros	straightforward synthesis well-established protocols	high quality peptide fragments minimized accumulative effects of stepwise synthetic errors
cons	accumulative effects of synthetic inefficiencies insolubility of growing peptides difficult purification aggregation of the peptide by-products limited for long peptide synthesis	solubility of fragments in aqueous media racemization during fragment coupling efficiency of fragment coupling

Table 1. Advantages and disadvantages of both stepwise SPPS strategy and fragment condensation approach.

Fragment condensation approach requires rapid access to pure protected peptide fragments, which are needed as building blocks. Thus, efficient synthesis of protected *N*- and *C*-peptide intermediates demand for handles which maintain intact the side-chain protecting groups after cleavage.

Handles for Fmoc-solid-phase synthesis of protected peptides

Synthesis of protected peptides by SPPS relies on the proper choice of orthogonal/compatible set of protecting groups and linkers. Orthogonal protection schemes comprise two or more classes of protecting groups which may be removed by different chemical mechanisms, in any order an in the presence of other classes, while compatible protecting groups are those belonging to a similar class of protecting groups cleaved by means of similar chemical mechanisms with different reaction rates.

Then, we have covered a wide range of handles described for the preparation of protected peptides on solid phase so far, with the final purpose of having an overview on this topic. Although some of the compiled linkers here have been reported to be orthogonal and/or compatible with both Boc/Bzl and Fmoc/*t*Bu chemistry, we have focused on those linkers described for Fmoc/*t*Bu strategy.

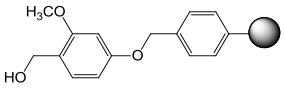
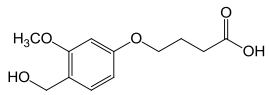
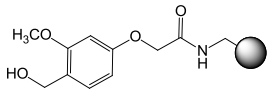
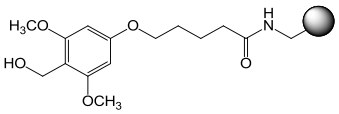
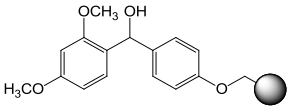
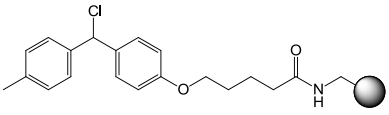
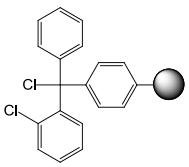
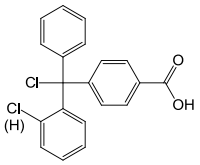
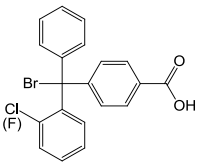
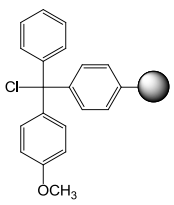
Highly acid-labile handles are, by far, the most commonly used linkers for the preparation of protected peptides by Fmoc/*t*Bu procedure. These handles are preferred, since management of the cleavage step is easier, and generally good yields and purities are obtained. Nevertheless, other linkers including fluoride-labile, palladium-labile, photo-labile, safety-catch handles, BAL-based linkers, oxidation-labile, hydrazine-labile, and cyclization/cleavage handles have been described for this purpose.

Mostly acid-labile linkers are based on benzyl, benzhydryl and trityl systems, frequently cleaved by using trifluoroacetic acid (TFA). Acid lability is related with the stability of the carbocation generated under acidic treatment, and can be fairly modulated by incorporating *p*- and/or *o*-electron-donating or –withdrawing

substituents to the aromatic rings. Thus, the carbocations of trityl structures are more stable than those of benzhydryl moieties, which in turn, are more stable than those derived of benzylic structures. Moreover, addition of further *p*- and/or *o*-methoxy groups results in linkers with enhanced acid sensitivities.

For the synthesis of protected peptide acids several linkers and resin have been described, including SASRIN resin (**1**), HMPB linker (**2**), Sheppard linker (**3**) and HAL linker (**4**), which are based on benzyl moiety; Rink acid resin (**5**), and a Rink chloride analog (**6**) based on benzhydryl scaffold; along with 2-CTC resin (**7**) and various trityl system-based analogues (**8**-**10**). More recently, linker THAL (**11**) was developed in our group based on the electron-rich 3,4-ethylenedioxythenyl (EDOT). Among all of them, 2-CTC is the most widespread used resin owing to its extensive availability and lower cost. In addition, 2-CTC resin offers other synthetic advantages such as minimization of diketopiperazine (DKP) formation due to of the steric hindrance of trityl groups [46], low epimerization during incorporation of the first amino acid [47], as well as the possibility of being reused [48]. 2-CTC resin, besides allow protected fragment to be released by low-acidic mixture of 1-2% TFA in CH_2Cl_2, cleavage can be accomplished in milder conditions by using trifluorethanol (TFE)-CH_2Cl_2 or hexafluoroisopropanol (HFIP)-CH_2Cl_2 mixtures [49]. All these resins and linkers render protected fragment acids with the *C*-terminus unprotected. These protected segments may be directly used in the fragment coupling reaction when they participate as the *N*-terminal fragment. However, normally, when these fragments corresponded to the *C*-terminal intermediates, then the *C*-terminal carboxylic acid should be previously protected in an additional step before fragment coupling in solution is performed.

On the other hand, general highly acid-labile linkers for the synthesis of protected peptide amides are based on xanthenyl moiety, such as Sieber amide resin (**12**), XAN linkers (**13**), and a Sieber-based analog (**14**), although a trityl-based linker (**15**) has been also described. Ramage and coworkers reported the preparation of linker (**16**) based on the dibenzocyclohepta-1,4-diene moiety. Complete release of peptide amides is achieved with diluted TFA treatments (**Table 2**).

	Name	Structure	Cleavage conditions	Refs.
1	Sarsin resin		1% TFA (acids)	[50]
2	HMPB (HMBA) linker		1% TFA (acids)	[51–52]
3	Sheppard linker		1% TFA (peptide acids)	[53]
4	HAL linker		0.1% TFA (acids)	[54]
5	Rink acid resin		1% TFA, 10% AcOH (acids)	[55]
6			1% TFA (acids)	[56]
7	2-CTC resin		1% TFA (acids)	[57–58]
8	2-CTC linker variants		(peptide acids)	[46]
9	2-CTC linker variants		(acids)	[46]
10	4-MeO trityl resin		(acids)	[59]

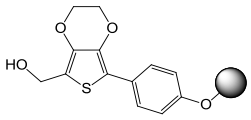
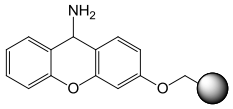
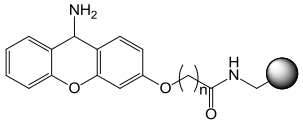
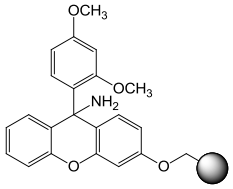
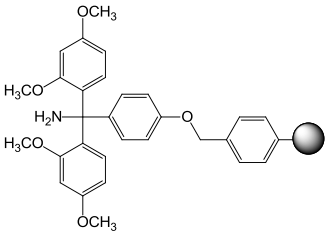
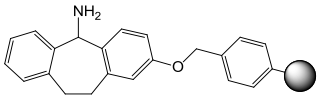
11	THAL		(acids)	[60]
12	Sieber amide		1-5% TFA (amides)	[61]
13	XAL linkers		1% TFA (amides)	[62]
14			(amides)	[63]
15	trityl-based		(amides)	[64]
16			3% TFA	[65]

Table 2. Acid-labile linkers for protected peptide synthesis on solid phase.

Acid- and base-stable allylic linkers such as HYCRAM (**17**), β-Ala containing analog thereof (**18**), and HYCRON (**19**) linker allow peptide liberation under almost neutral conditions through a palladium(0)-catalyzed allyl transfer to scavenger nucleophiles. Boc-groups, *t*Bu-esters and *t*Bu-ethers remained intact after cleavage treatment (**Table 3**).

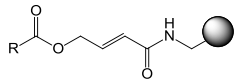
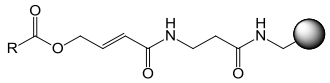
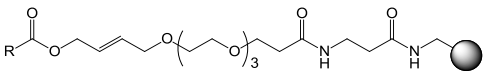
	Name	Structure	Cleavage conditions	Refs.
17	HYCRAM		Pd(0) + scavenger	[66]
18	HYCRAM with β-Ala		Pd(0) + scavenger	[66]
19	HYCRON with β-Ala		Pd(0) + scavenger	[67]

Table 3. Palladium-labile linkers for protected peptide synthesis on solid phase.

Given the stability of typical side-chain protecting group used in Fmoc-SPPS chemistry to fluoridolysis, several silicon-based handles have been developed. These linkers enable the release of protected peptides under either basic or neutral conditions. For instance, the so-called "silico Wang" linker Pbs (**20**), developed by Barany's group, demonstrated to yield protected peptides by brief treatments of TBAF in DMF with the presence of appropriate scavengers. The SAC linker (**21**), stable under typical peptide synthesis conditions permitted to generate protected peptide acids using either fluoride ions or 1% TFA in CH_2Cl_2. Other silicon-based handles were described by Ramage et al. (**22**) and Flitsch, Turner and coworkers (**23**). After peptides elongation, cleavage from **22** was performed with TBAF in DMF, while release from **23** could be carried out using either TBAF or CsF. Moreover, an interleukin-2 fragment was prepared using a novel silyl linker (**24**) which demonstrated its compatibility with Fmoc-SPPS procedures. This latter linker enabled the release of the protected glycopeptides fragment by treatments with CsF–AcOH. More recently, (2-phenyl-2-trimethylsilyl)ethyl linker (PTMSEL) (**27**) demonstrated to be more sensitive towards fluoridolysis, thus permitting protected peptide release by using $TBAF{\cdot}3H_2O$ in CH_2Cl_2 under almost neutral conditions (**Table 4**).

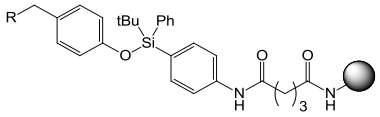
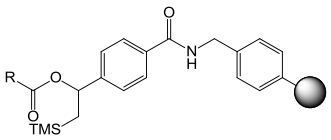
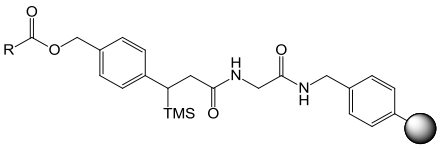
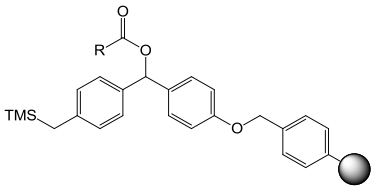
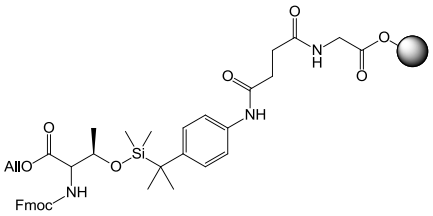
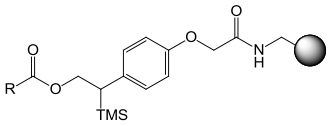
	Names	Structures	Cleavage conditions	Refs.
20	Pbs handle		TBAF	[68]
21	SAC linker		TFAB 1% TFA in CH_2Cl_2	[69]
22	Ramage linker		TBAF	[70]
23	Flitsch and Turner linker		TBAF or CsF	[71]
24	Nakahara linker		TBAF	[72]
25	PTMSEL linker		TBAF	[73–74]

Table 4. Fluoride-labile linkers for protected peptide synthesis on solid phase. (TMS = trimethylsilyl)

Backbone amide linker (BAL) approach, developed by Albericio and Barany, relies on the peptide anchored to the support through a backbone nitrogen, thus allowing the preparation of *C*-terminal-modified peptides. The original BAL linker (**26**) was treated with 95% TFA for the proper release of the target peptide, although a later study demonstrated that both original BAL and *o*-BAL (**27**) linkers are surprisingly highly acid-labile. Thus, peptide may be cleaved from the resin by using 1-5% TFA, and therefore can be used for the preparation of protected peptides.

More recently, a series of BAL linkers based on various alkoxynaphthalene core structures were designed. From this study, the tetraalkoxynaphthalene NAL-4 structure (**28**) was revealed as a very high acid-labily, releasing a *t*Bu protected dipeptide by treating with 0.5% TFA in CH_2Cl_2. Recently, the same research group designed a novel TBAL linker (**29**) based on the EDOT scaffold which exhibited a very high acid-labily (**Table 5**). Due to the ability of BAL-based handles to prepare *C*-terminal-modified peptides, these linkers would allow the preparation of protected peptide fragments on solid phase with the *C*-terminal acid function already protected.

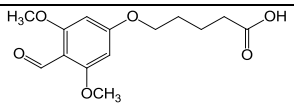
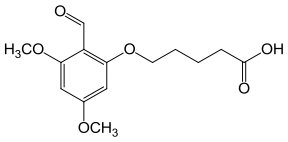
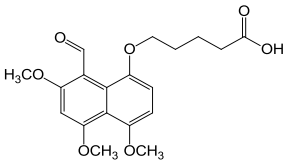
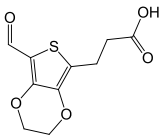
	Names	Structures	Cleavage conditions	Refs.
26	BAL		95% TFA (1-5% TFA)	[75] [76]
27	o-BAL		1-5% TFA	[76]
28	NAL-4		0.5% TFA	[77]
29	T-BAL		1% TFA	[78]

Table 5. Backbone acid-labile linkers for protected peptide synthesis on solid phase.

Of special interest, safety-catch linker approach is ideally, owing to the particular the activation and further cleavage and release. In this case, the linkage between the carboxyl group and the solid support is stable under SPPS conditions, until the safety-catch system is activated by a specific chemical reaction leading to the release of the compounds. Thus, these linkers require the previous activation before product release, thereby allowing employment of conditions that would otherwise cleave the substrate

from the solid support. Some handles based on this concept have been developed for the release of fully protected peptides. For instance, Pascal *et al.* reported the used of Dpr(Phoc) safety-catch linker (**30**) for the synthesis of protected segments by using Fmoc chemistry. Originally reported for Boc chemistry [79], the corresponding cyclic acylurea (Imc = 2-oxo-imidazolidine-4-carboxylic acid) demonstrated to be stable under typical Fmoc chemistry procedures and was applied for the preparation of protected peptides. After peptide elongation, release was performed by using NaOH and $CaCl_2$ in iProOH-water.

4-sulfamylbutyryl linker (**31**), also called alkanesulfonamide safety-catch linker described by Ellman and coworkers, is a variant of the previous Kenner's sulfonamide safety-catch linker [80]. This handle is stable to both basic and strongly nucleophilic reaction conditions, and after treatment with iodoacetonitrile provides *N*-cyanomethyl derivatives that then can be cleaved by nucelophiles under mild reaction conditions to release the target peptide. This linker has been recently employed for the preparation of a small cyclic peptide [81] (**Table 6**).

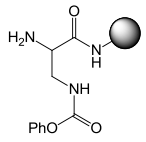
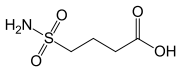
	Names	Structures	Cleavage conditions	Refs.
30	Dpr(Phoc) linker		activation: PhONa–PhOH release: NaOH, $CaCl_2$	[82–83]
31	4-sulfamylbutyryl linker		activation: iodoacetonitrile	[84]

Table 6. Safety-catch linkers for protected peptide synthesis on solid phase.

Giese and coworkers designed a photolabile linker based on the 2-pivaloylglycerol moiety (**32**) for the solid-phase synthesis of peptide acids. A protected peptide was successfully cleaved from this linker in high yields and purities in THF by irradiation at 320-340 nm. Recently, Copley *et al.* have design a photocleavable linker based on the 3',5'-dimethoxybenzoin moiety in which the carbonyl group is protected as a dimethyl

ketal (**33**). This handle is compatible with the Fmoc/*t*Bu strategy and can be quantitatively removed by 3% TFA in CH_2Cl_2, as well as by irradiation at 350 nm in aqueous or partially aqueous media (**Table 7**).

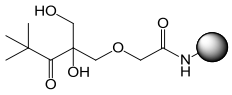
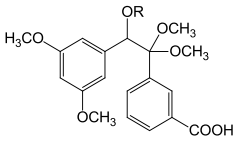
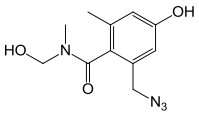
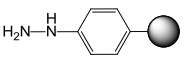
	Names	Structures	Cleavage conditions	Refs.
32	Pivalopylglycol moiety		at 320-340 nm	[85]
33	dimethyl ketal-protected benzoin-based linker		at 350 nm 3% TFA	[86]

Table 7. Photolabile linkers for protected peptide synthesis on solid phase.

(Azidomethyl)benzamide linker **(34)** was developed for use in solid-phase peptide synthesis which allows a protected peptide to be cleaved from the resin under neutral conditions by using Bu_3P in a mixture DMF-imidazole buffer at pH 7, while retaining intact *t*Bu, Boc and Fmoc protecting groups. Hydrazine linker (**35**) is a versatile handle which allow the preparation of a wide variety of *C*-terminal-modified peptides. This linker is stable to acids and bases and is cleaved with high specificity under mild oxidative conditions in the presence of nucleophiles and basic media, yielding a broad range of *C*-terminal functionalities. Hydrazine linker is oxidized to acyldiazene by either O_2 in the presence of Cu(II) salts and a nucleophile, or using by *N*-bromosuccinimide (NBS) followed by a nucleophilic cleavage. This latter procedure is preferred when oxidative-sensitive residues such as Trp, Tyr and Cys are contained in the peptide sequence. A selenyl liker (**36**) was designed for the solid-phase synthesis of protected peptides which contain a dehydroalanine residue. Thus, after peptide elongation by using Fmoc/*t*Bu procedure, an oxidative cleavage by treatment with H_2O_2 in THF led to the fully protected dehydropeptide. Dde-based linker (**37**) is stable to standard basic conditions employed in the Fmoc/*t*Bu strategy, and can be easily cleaved in 2% hydrazine monohydrate. This linker was successfully applied for the synthesis of side-chain protected and unprotected peptides. More recently, a triazene function was

used to anchor a Phe residue to a solid support through its side chain (**38**). This versatile triazene linkage allowed the preparation of Phe-containing cyclic, *C*-modified, and protected peptides by using 2-5% TFA in CH_2Cl_2 and successive reduction of the resulting diazonium salt by using $FeSO_4 \cdot 7H_2O$ in DMF for the release of the target products.

Cyclative cleavage –or cyclorelease strategy– relies on the release of a compound generated by a intramolecular nucleophilic displacement at the linker, which in turn results in a cyclized product. Following this approach, we have recently described a novel handle for the preparation of fully protected peptides based on the formation of a DKP moiety (**39**). Firstly, a dipeptidyl moiety is incorporated to hydroxymethyl resin followed by the assembly of a bifunctional linker. Next, after peptide elongation by Fmoc-SPPS, the α-N-amino protecting group (PG) comprised on the dipeptidtyl moiety is selectively removed and then treatments with piperidine or pyrrolidine promote the DKP formation –intramolecular cyclization– and concomitant release of the fully protected peptide. Singularly, the DKP moiety remains as a *C*-terminal protecting group which can be selectively removed when necessary. This versatile handle is so designed that can be incorporated any bifunctional linker between the dipeptidyl moiety and the target peptide, thereby allowing further selective deprotection of the *C*-terminal DKP-moiety under specific conditions (**Table 8**).

	Name	Structures	Cleavage conditions	Refs.
34	(Azidomethyl)benzamide linker		Bu_3P in DMF-imidazole buffer at pH 7	[87]
35	Aryl hydrazine		O_2 + Cu(II) salts + Nu^- or NBS + Nu^-	[88–89]

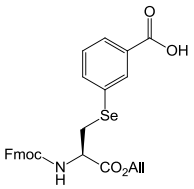
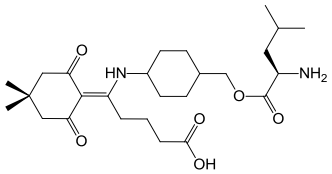
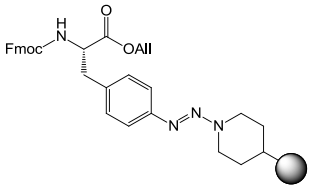
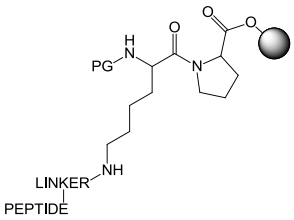
36	Seleno-based linker		H_2O_2 in THF (dehydropeptides)	[90]
37	Dde linker		NH_2-NH_2	[91]
38	Phe-triazene linker		2-5% TFA + $FeSO_4 \cdot 7H_2O$	[92]
39	DKP handle		PG removal + basic treatments (*C*-terminal protected)	[93]

Table 8. Several linkers for protected peptide synthesis on solid phase.

Orthogonal combinations of Fmoc/*t*Bu strategy with diluted-acid-labile, palladium-labile, silicon-based, BAL approach, photolabile, and safety-catch linkers, among others, have been designed and developed for the synthesis of protected peptides for their further application in the fragment condensation approach. A further step have been achieved with the DKP-based handle (**38**), which allows the preparation of *C*-terminal protected fragment by SPPS that can be used directly for fragment coupling in solution. Although highly-acid-labile handles are frequently employed for this purposed, many innovative linkers have been described and successfully applied, thus expanding the available synthetic tools for SPPS.

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Publication IV

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COMMUNICATION

A universal strategy for preparing protected C-terminal peptides on the solid phase through an intramolecular *click* chemistry-based handle†

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A new universal strategy exploits DKP formation in a dipeptide moiety whose C-terminal residue is blocked by a leaving group. It enables both synthesis of C-terminal protected peptides that are useful for convergent synthesis of large peptides and use of a C-terminal permanent protecting group that can be cleaved by catalytic hydrogenation to release the peptide.

The past decade has witnessed the so-called *peptide revolution*, in which peptides have been promoted from mere biochemical tools to real alternatives to small-molecule drugs.[1] Furthermore, peptides are becoming cornerstones of emerging fields such as drug delivery, nanotechnology and materials.[2] This is partly due to the explosion of solid-phase peptide synthesis (SPPS), first developed by Nobel Laureate Bruce Merrifield and then fine tuned by numerous research groups.[3] This method is now used to prepare all peptides used in research as well as commercial peptides comprising more than a few amino acids.

All synthetic strategies are based on the appropriate combination of protecting groups together with an efficient method for activating the carboxyl group prior to peptide coupling. While extensive studies have yielded myriad protecting groups for both the α-amino and the side-chain functions, which allow synthesis of cyclic and/or complex peptides, far less effort has been dedicated to the C-terminal function, which is blocked with the insoluble polymer resin. Removal of the C-terminal protecting group and concomitant liberation of the peptide from the resin afford a free C-terminal functional group, usually an acid or amide. However, a semi-permanent protecting group for the C-terminal function would be desirable, as it would enable cleavage of the peptide from the insoluble polymer resin to render the C-terminal function protected for further manipulation in solution.

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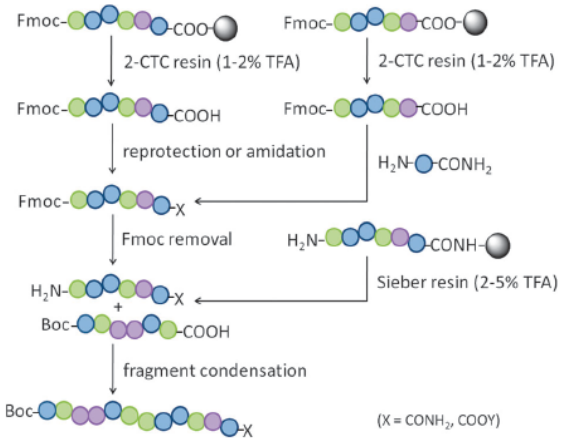

Scheme 1 Classical convergent strategy for peptide synthesis.

Chemical synthesis of proteins and industrial preparation of peptides longer than 20–30 residues are performed by a convergent strategy in which different protected fragments are first constructed on the solid phase and subsequently coupled together in solution (Scheme 1).[4]

Protected peptides are currently prepared on a 2-Cl–Trityl–Cl (2-CTC) resin using an Fmoc–*t*Bu strategy. This resin enables liberation of the protected peptide using 1–2% TFA solution.[5] A drawback of this methodology is the preparation of C-terminal fragments whose C-terminal function requires to be blocked. In the case of C-terminal acid peptides, this requires reprotection of the C-terminal carboxyl group in solution, with a consequent risk of racemization and low yields. For C-terminal amide peptides, in addition to amidation or incorporation of the amide form of the last residue, superlabile amide peptide resins such as the Sieber-resin can be used.[6] However, liberation of protected peptides from these resins requires 2–5% TFA solution, which is not totally compatible with all side-chain protecting groups (*e.g.* the Trt of His).

Herein is described a new universal strategy for preparing C-terminal protected peptides on solid phase. The strategy was exemplified in two applications: (i) synthesis of C-terminal protected peptides useful for assembly of large peptides (convergent approach); and (ii) use of a permanent C-terminal protecting group that can be liberated by catalytic hydrogenation.

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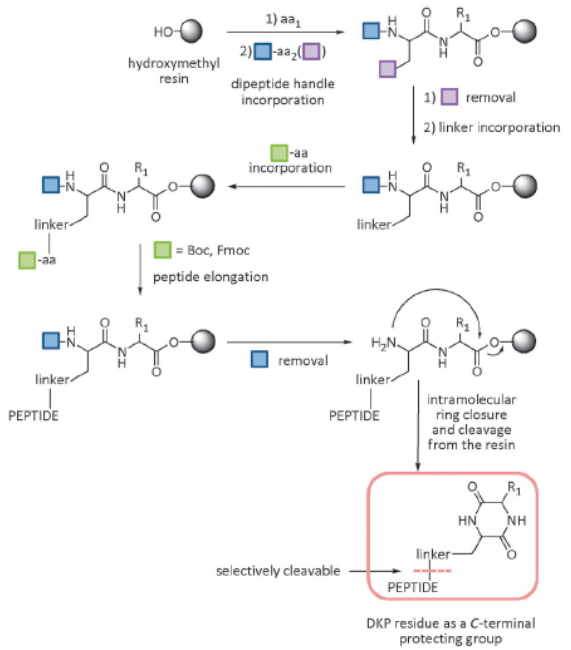


Scheme 2 Schematic representation of the universal DKP *click* handle strategy.

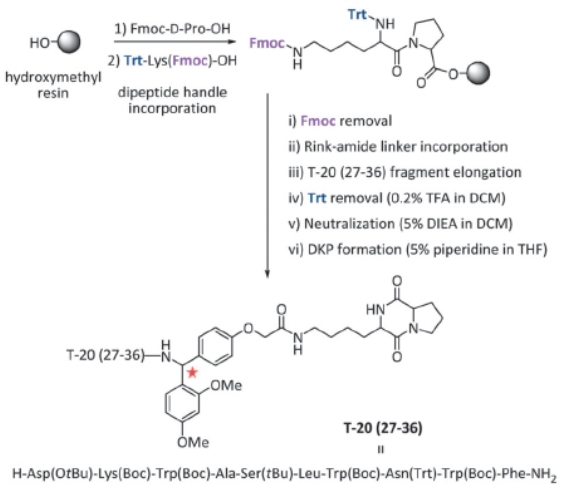
Click reactions were defined by K.B. Sharpless as "*those inspired in nature, that give very high yields, generate inoffensive side-products, are stereospecific, exhibit a large thermodynamic driving force, have simple reaction conditions...*"[7] In peptide chemistry, the reaction that fits this definition is diketopiperazine (DKP) formation, which often jeopardizes preparation of simple C-terminal acid peptides as well as complex ones that contain *N*-alkyl amino acids.[8] After pioneering works of Geysen *et al.*[9] and Atrash and Bradley,[10] we have further elaborated the DKP cleavage concept by incorporating a selectively cleavable linker group in the DKP *click* handle (Scheme 2).

The present strategy exploits DKP formation in dipeptide moieties whose C-terminus is blocked by a leaving group. As the DKP itself becomes part of the permanent protecting group of the C-terminal carboxyl function, the dipeptide should contain a function to facilitate its connection to the C-terminal fragment, such as the amino side-chain of Lys.‡ Considering that DKP formation is favored by the presence of an *N*-alkyl amino acid, which favors the *cis*-conformation, and an L/D combination of amino acids, which stabilizes the 6-member ring DKP,[11] a D-Pro as the other component was chosen.§ This D-Pro is attached directly to a hydroxyl resin (*e.g.* hydroxy Merrifield resin). Finally, the connection between the C-terminal fragment and the dipeptide moiety would be achieved through a bifunctional linker (*e.g.* Rink-amide, Wang-type or benzyl-type handles), which acts as a permanent protecting group, and liberates the peptide with the expected functionality (acid or amide) during the final global deprotection.

The cornerstone of this process is the protecting group of the α-amino of the penultimate amino acid (—in Scheme 2), which dictates the conditions for releasing the protected peptide from the resin. Thus, if Trt is used, cleavage is accomplished by treatment with 0.1–0.3% TFA; if Alloc is used, then liberation of the protected peptide is carried out with Pd(0); and if pNZ is used, then cleavage is performed with Sn^{2+}.[12] At this stage, DKP formation does not take place. Moreover, during successive treatments with 5% DIEA in DCM, which enables washing/neutralization of the resin before liberation of the protected peptide, DKP formation is negligible. This feature is extremely important when Pd(0) is used, because extra washing facilitates removal of any Pd salts, which can contaminate the final product.

The absence of DKP formation during treatment of the resin with DIEA is intriguing because, as it is well-known, in a Boc/Bzl strategy any C-terminal sequence Aa–Pro can easily render quantitative DKP formation after removal of the Boc group of the Aa residue and subsequent neutralization with 5% DIEA in DCM.[13] According to Goolcharran and Borchardt,[14] the differences observed in the rate of DKP formation can be explained by various factors: differences in the pK_a values of the terminal α-amino groups of the analogs, the steric bulk, the ability of the Aa–Pro peptide bond to undergo *cis–trans* isomerization, and/or the conformational stability of the resulting DKPs. In our case, the presence of the target peptide and the linker as the side-chain substituent of one of the DKP components can affect said parameters—namely, by adding bulkiness to the system and decreasing the polarity of the medium, thereby resulting in the need for a stronger base; hampering *cis–trans* isomerization of the Aa–Pro bond; and/or compromising the conformational stability of the resulting DKP.

As a model to test our strategy, the fragment H-(17–36)-NH_2 of the drug Fuzeon® (T-20) was chosen. The fragment is synthesized in solution, by coupling of fragments Fmoc-(17–25)-OH and H-(26–36)-NH_2. The former is prepared using a 2-CTC resin, and the latter, by coupling of H–Phe–NH_2 to pre-formed Fmoc-(26–35)-OH (also obtained using 2-CTC resin) in solution.[15] Scheme 3 shows the one-pot SPPS of C-terminal protected fragment H-(27-36)-DKP_{handle}, using the new DKP *click* handle strategy. The dipeptide Lys-D-Pro, the Fmoc-Rink-amide linker, as well as the 10 Fmoc-amino acids of the



Scheme 3 Synthesis of C-terminal protected T-20 fragment H-(27–36)-DKP_{handle} using the DKP *click* handle strategy.

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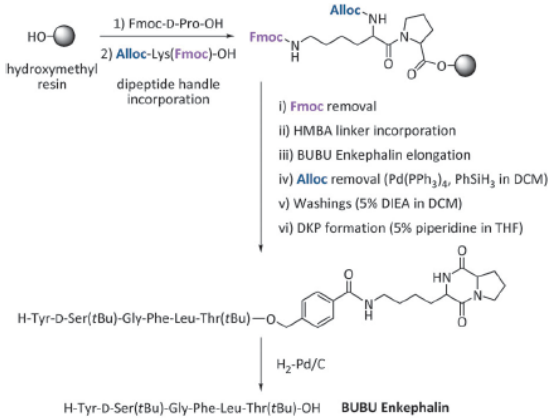

Scheme 4 Synthesis of BUBU Enkephalin using the DKP *click* handle strategy.

fragment, were all incorporated smoothly using conventional SPPS protocols (see ESI†). After elimination of the Fmoc group from the last amino acid, the Trt group was removed. The peptidyl-resin was neutralized with 5% DIEA in DCM, and then the protected peptide was liberated by treatment with 5% piperidine in THF¶, which was removed under reduced pressure. The protected peptide was then washed with pre-cooled Et_2O. The protected T-20 fragment H-(27–36)-DKP_{handle} was ready to be coupled with the fragment Boc-(17–26)-OH, which was prepared using the 2-CTC resin, saving the two steps of the classical convergent approach (incorporation of H–Phe–NH_2 and removal of Fmoc in solution). Coupling of the fragments using DIPCDI/HOBt, followed by global deprotection with TFA–DMB(1,3-dimethoxybenzene)–TIS (92.5 : 5 : 2.5), afforded the desired product, unprotected H-(17–36)-NH_2, in a good purity (see ESI†).

Incorporation of a benzyl-type protecting group as a selectively cleavable linker enables peptide synthesis through *a totally acid-free* strategy, which has been exemplified by the synthesis of BUBU Enkephalin, a highly potent and selective δ-opioid agonist whose D-Ser^2 and Thr^6 hydroxyl groups are *tert*-butylated.‖ In this *totally acid-free* strategy, the α-amino function (■—in Scheme 2) of Lys was protected with the Alloc group (Scheme 4). After removal of Fmoc, 4-hydroxymethylbenzoic acid (HMBA) was incorporated and the peptide sequence was elongated (see ESI†). After cleavage of the protected peptide from the resin, the benzyl-type protecting group was removed by H_2–Pd/C.

In conclusion, a new concept for protection of the C-terminus of peptides has been developed. It overcomes some of the drawbacks associated with SPPS, such as preparation of the C-terminal fragment in a convergent strategy. Furthermore, it introduces more flexibility into SPPS, by enabling the use of benzyl-type protecting groups, which can be removed by catalytic hydrogenation. Finally, it may enable better control in the solid-phase strategy, by allowing further manipulation of the molecule in solution after release. We are presently extending this strategy to side-chain anchoring, which can involve other functional groups (*e.g.* hydroxyl, thiol and carboxylic acid).

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Notes and references

‡ Lysine was chosen due to its lower cost, but other amino acids with amino side-chains were also used (see ESI†).
§ Although D-Pro is an optimal component of the dipeptide part of the handle, it can be replaced with L-Pro and other *N*-alkyl amino acids (results not shown), which give similar results. Furthermore, γ-amino-Pro, which contains the two main features needed for this strategy (an *N*-alkyl amino acid and an amino side-chain), can be used.
¶ Pyrrolidine can be used instead of piperidine.
‖ BUBU Enkephalin was used to demonstrate the feasibility of this strategy; however, this peptide can be synthesized using the 2-CTC resin.

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Universal strategy for preparing protected *C*-terminal peptides on solid phase through an intramolecular *click* chemistry-based handle

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GENERAL

General methods: Protected amino acid derivatives, Fmoc-Rink-OH, HOBt and 2-CTC resin were obtained from Iris Biotech (Marktredwitz, Germany). HMPS resin, HMBA and HMPA linkers were obtained from Novabiochem (Läufelfingen, Switzerland). DIEA and DIPCDI were obtained from Aldrich (Milwaukee, WI), TFA was from Scharlau (Barcelona, Spain), and HCTU and Oxyma were from Luxembourg Industries (Tel Aviv, Israel). DMF, DCM, Et_2O, piperidine and acetonitrile (HPLC grade) were obtained from SDS (Peypin, France). All commercial reagents and solvents were used as received. THF was obtained from Scharlau and purified using a Pure-Solv MD-2 solvent system (Innovative Technology, Inc.)

Solid-phase syntheses were performed in polypropylene syringes fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Removal of the Fmoc group was carried out with piperidine–DMF (1:4, v/v) (1 × 1 min, 2 × 10 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min). Peptide synthesis transformations and washes were performed at 25 °C.

SunFire® C18 reversed-phase HPLC analytical column (4.6 mm × 100 mm, 3.5 μm), and XBridge BEH130 C18 column, (4.6 mm x 100 mm, 3.5 μm) were obtained from Waters (Ireland). Analytical RP-HPLC was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998), and system controller (Empower login). UV detection was at 220 and 254 nm, and linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 1.0 mL·min^{-1} flow rate over 8 min. RP-HPLC-ESMS was performed on a Waters Micromass ZQ spectrometer. Linear gradients of ACN (+0.07% formic acid) into H_2O (0.1% formic acid) were run at 0.3 mL·min^{-1} flow rate over 8 min.

SPPS of T-20 fragment H-(27-36)-DKP$_{handle}$ using as attachment to the resin a DKP *click* handle (0.1 mmol scale): Trt-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (106.2 mg, 0.98 mmol/g) was swelled with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (135 mg, 4 eq) and DIPCDI (31 µL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were then added to the resin. DMAP (4.9 mg, 0.4 eq) in DCM (0.5 mL) was then added, and the mixture was left to stand at 25 °C for 2 h. A recoupling of Fmoc-D-Pro-OH was carried out for 16 h. The resin was washed with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each). The resin was then capped using acetic anhydride (47 µL, 5 eq) and DIEA (85 µL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.95 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Lys) of the dipeptidyl linker, a mixture of Trt-L-Lys(Fmoc)-OH (198 mg, 3 eq), HOBt (50 mg, 3 eq) and DIPCDI (50 µL, 3 eq) in DMF (2 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 16 h. No recoupling was required according to the chloranil test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

Incorporation of a Rink-amide handle group

Fmoc-Rink-OH (175 mg, 3 eq), HOBt (50 mg, 3 eq) and DIPCDI (50 µL, 3 eq) in DMF (2 mL) were shaken for 5 min at 25 °C, and then added to the previously described resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

T-20 fragment H-(27-36)-DKP$_{handle}$ elongation by SPPS

The first amino acid, Fmoc-^{36}Phe-OH, was coupled to the resin comprising the dipeptidyl linker and a Rink-amide handle group described above, and then the following amino acids were coupled. A mixture of Fmoc-Xaa-OH (3 eq), HCTU (140 mg, 3 eq), DIEA (110 µL, 6 eq) in DMF (2 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h, and then a ninhydrin test was performed. When the ninhydrin test was positive, a recoupling was carried out (using the same coupling conditions). The resin was then capped using acetic anhydride (47 µL, 5 eq) and DIEA (85 µL, 5 eq) in DMF (2.5 mL) for 15 min at 25 °C. The Fmoc group was then removed. Washes between coupling,

deprotection and capping steps were performed using DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each).

Analysis - Cleavage of T-20 fragment H-(27-36)-NH_2 from the Rink-amide handle group

A small portion of peptidyl-resin (5 mg) was treated with 1 mL of a mixture TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C in order to cleave the peptide from the Rink-amide handle group and to fully remove the amino acid side-chains. The unprotected peptide was obtained in 83% purity, as determined by analytical RP-HPLC (linear gradient from 25% to 50% ACN over 8 min; t_R = 5.25 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.47 min; *m/z* calculated for $C_{68}H_{86}N_{16}O_{14}$, 1351.5; found, 1352.2 $[M+H]^+$, 676.9 $[(M+2H)/2]^{2+}$, where M is the MW of the fully unprotected T-20 fragment H-(27-36)-NH_2).

Trt protecting group removal of the L-Lys residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

In a first step, the Trt protecting group of the L-Lys residue of the dipeptidyl linker was removed by treating the peptidyl-resin (15 mg) with 0.2% (v/v) TFA in DCM (2 × 5 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then neutralized by washing with 5% (v/v) DIEA in DCM (2 × 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show peptide cleavage from the resin or from the Rink-amide handle group, and after the second step, almost negligible peptide comprising the DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). Finally, the fully protected peptide comprising the DKP *C*-terminal protecting moiety was obtained by treating the peptidyl-resin from the second step with 5% (v/v) piperidine in THF at 25 °C (5 × 5 min, 2 mL each). This step can also be performed with 5% (v/v) piperidine in DMF, 5% (v/v) pyrrolidine in THF or 5% (v/v) pyrrolidine in DMF. THF was then removed by evaporation under reduced pressure and the resulting fully protected peptide comprising the DKP *C*-terminal protecting group was obtained as a diastereomeric mixture (1:1) due to the Rink-amide handle group. The mixture was analyzed by RP-HPLC (linear gradient from 80% to 100%; t_R = 4.20 min and 4.63 min) and RP-HPLC-ESMS (linear gradient from 80% to 100% ACN over 8 min; t_R = 2.64 min; *m/z* calculated for $C_{143}H_{181}N_{19}O_{28}$, 2614.1; found, 2616.2 $[M+H]^+$, 1308.0 $[(M+2H)/2]^{2+}$, where M is the MW of the fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP moiety).

In order to quantify the amount of the remained target peptide attached to the resin via the DKP *click* handle, the resin was treated with 2 mL of TFA–TIS–H_2O (92.5:2.5:2.5) for 1 h at 25 °C. RP-HLPC analysis showed that there was less than 1% left of target fragment on the resin (linear gradient from 5% to 100% ACN over 8 min).

SPPS of T-20 fragment H-(27-36)-DKP$_{handle}$ using as attachment to the resin a DKP *click* handle (5 mmol scale): Trt-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The SPPS was performed manually using HMPS resin (5.0977 g, 0.98 mmol/g). Introduction of the dipeptidyl linker, Rink-amide handle group, first amino acid, and coupling cycles were performed as described in the previous synthesis except for Fmoc-^{28}Lys(Boc)-OH, which was coupled as follows: a mixture of Fmoc-^{28}Lys(Boc)-OH (3 eq), DIPCDI (2.3 mL, 3 eq), HOBt (2.3 g, 3 eq) in DMF (100 mL) was shaken for 5 min at 25 °C, and then added to the resin prepared according to the preceding step in the elongation sequence. The mixture was left to stand at 25 °C for 16 h. A recoupling was carried out using HCTU (6.2 g, 3 eq), DIEA (5.2 mL, 6 eq) in DMF (100 mL). This mixture was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 2 h. The resin was washed with DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each), and then was capped using acetic anhydride (2.4 mL, 5 eq), DIEA (4.4 mL, 5 eq) in DMF (100 mL) for 1 h at 25 °C. The resin was washed with DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each), and then the Fmoc group was removed.

At this point, an aliquot of peptidyl-resin (5 mg) was cleaved from the Rink-amide handle group by treating the resin with 1 mL of a mixture of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C. The unprotected peptide was obtained in 72% purity, as determined by analytical RP-HPLC (linear gradient from 25% to 50% of ACN over 8 min, t_R = 5.25 min).

In this case, the Trt protecting group of the L-Lys of the dipeptidyl linker was removed by treating the peptidyl-resin (1.57 g) with 0.5% (v/v) TFA in DCM (2 × 5 min; 20 mL each) at 25 °C. Neutralization was performed as described earlier by washing the peptidyl-resin with 5% (v/v) DIEA in DCM (2 x 5 min; 20 mL each) at 25 °C. Diketopiperazine formation was carried out by treating the peptidyl-resin with 5% (v/v) piperidine in THF (5 × 5 min; 20 mL each). THF was removed under reduced pressure and the resulting crude was washed with

pre-cooled (4 °C) Et_2O (3 × 50 mL). 642.5 mg of the fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP *C*-terminal protecting moiety were obtained.

SPPS of T-20 fragment Boc-(17-26)-OH

SPPS of T-20 fragment Boc-(17-26)-OH was performed manually by linear Fmoc SPPS. Only the last amino acid was Boc-protected (Boc-Glu(*t*Bu)-OH).

2-CTC resin (5.0054 g) was swelled with DCM (5 × 1 min; 50 mL each) and DMF (5 × 1 min; 50 mL each) at 25 °C. The first amino acid, Fmoc-^{26}Leu-OH (1.8 g, 1 eq) and DIEA (8.7 mL, 10 eq) in DCM (50 mL) were added to the resin, and then the mixture was left to stand at 25 °C for 1h. The resin was then capped by adding MeOH (0.8 µL/mg resin; 4 mL) to the resin for 15 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 50 mL each) and DMF (5 × 1 min; 50 mL each). The Fmoc group was then removed and a 0.89 mmol/g resin loading was determined by UV quantification.

To elongate the peptide, a mixture of Fmoc-Xaa-OH (3 eq), Oxyma (1.9 g, 3 eq), DIPCDI (2.3 mL, 3 eq) in DMF was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 16 h. No recouplings were required according to the ninhydrin test. Washes between couplings and deprotection steps were performed using DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each).

Analysis - Cleavage of T-20 fragment H-(17-26)-OH from the 2-CTC resin

An aliquot of peptidyl-resin (5 mg) was treated with 1 mL of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C in order to cleave the peptide from the 2-CTC resin and to fully remove the amino acid side-chains. The T-20 fragment H-(17-26)-OH was obtained in 85.7% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R = 3.90 min). The peptide was analyzed by RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.71 min; *m/z* calculated for $C_{53}H_{89}N_{13}O_{21}$, 1244.5; found, 1244.7 $[M+H]^+$, where M corresponds to the fully unprotected T-20 fragment H-(17-26)-OH).

Cleavage of the fully protected T-20 fragment Boc-(17-26)-OH

The peptidyl resin (1.094 g) was treated with 1% (v/v) TFA in DCM (5 × 1 min; 50 mL each) at 25 °C, all 5 mixtures were poured into H_2O (20 mL). This aqueous mixture was then evaporated under reduced pressure and the crude was lyophilized. The fully protected T-20 fragment Boc-(17-26)-OH was obtained (510 mg) and analyzed by RP-HPLC-ESMS (linear

gradient from 95% to 100% of ACN over 8 min; t_R = 7.84 min; *m/z* calculated for $C_{117}H_{165}N_{13}O_{25}$, 2153.6; found, 2153.8 $[M+H]^+$, where M corresponds to the fully protected T-20 fragment Boc-(17-26)-OH). Analytical RP-HPLC showed one peak with 85.7% purity (linear gradient for 95% to 100% of ACN over 8 min; t_R = 6.06 min).

No partial deprotection of the fully protected T-20 fragment Boc-(17-26)-OH was observed by RP-HPLC-ESMS (linear gradient from 50% to 100% ACN over 8 min).

Assembly of T-20 fragment H-(17-36)-NH_2 by fragment coupling in solution

Fully protected T-20 fragment Boc-(17-26)-OH (10 mg, 4.6 µmol), HOBt (2.2 mg, 3 eq) were dissolved in DCM (350 µL) and DIPCDI (2.2 µL, 3 eq) was then added to the mixture. The mixture was shaken for 5 min at 25 °C, and then added to a solution of fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP *C*-terminal protecting moiety (12 mg, 4.6 µmol) in DCM (350 µL). The resulting mixture was stirred at 25 °C for 16 h. The fragment coupling was monitored by analytical RP-HPLC (linear gradient from 95% to 100% ACN over 8 min); no starting material was observed after 16 h.

The solvent was evaporated under reduced pressure yielding the fully protected T-20 fragment Boc-(17-36)-DKP_{handle} whose *C*-terminal function was protected with the DKP moiety. An aliquot of the crude peptide (1 mg) was treated with 1 mL of TFA–DMB–TIS (92.5:5:2.5) for 1 h at 25 °C. The mixture was evaporated and a 0.5% (v/v) aqueous NH_3 (1 mL) solution was added and left to stand for 16 h at 25 °C to remove the remained *N*-carboxy groups from the side chains of the Trp residues. The fully unprotected T-20 fragment H-(17-36)-NH_2 was obtained with 60.2% purity, as determined by analytical RP-HPLC (linear gradient from 30% to 40% ACN over 8 min; t_R = 5.94 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 30% to 40% of ACN over 8 min; t_R = 6.84 min; *m/z* calculated for $C_{121}H_{173}N_{29}O_{34}$, 2577.8; found, 1290.0 $[(M+2H)/2]^{2+}$, where M is the MW of the T-20 fragment H-(17-36)-NH_2).

SPPS of the tripeptide H-Phe-Ala-Leu-OH using as attachment to the resin a DKP *click* handle: Trt-Dap(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (103.6 mg) was swelled with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (132 mg, 4 eq) and DIPCDI (30 μL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were then added to a resin. DMAP (4.8 mg, 0.4 eq) in DCM (0.5 mL) was then added to the resin, and the mixture was left to stand at 25 °C for 2 h. A recoupling of Fmoc-D-Pro-OH was carried out for 16 h at 25 °C. The resin was washed with DCM (5 x 1 min) and DMF (5 x 1 min). The resin was then capped using acetic anhydride (46 μL, 5 eq) and DIEA (86 μL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Dap) of the dipeptidyl linker, a mixture of Trt-L-Dap(Fmoc)-OH (173 mg, 3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 mL, 3 eq) in DMF (2 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required according to the chloranil test. The resin was washed with DMF (5 x 1 min) and DCM (5 x 1 min), and then the Fmoc group was removed.

Incorporation of HMPA handle group

A mixture of HMPA (55 mg, 3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 μL, 3 eq) in DMF (2 mL) was added to the previously described resin, and then left to stand at 25 °C for 1 h. The resin was then washed with DMF (5 x 1 min; 3 mL each) and DCM (5 x 1 min; 3 mL each). No recoupling was required, according to the ninhydrin test.

Tripeptide elongation by SPPS

Fmoc-Leu-OH (144 mg, 4 eq) and DIPCDI (30 μL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were added to the resin comprising the dipeptidyl linker and the HMPA handle group described above. DMAP (4.8 mg, 0.4 eq) in DCM (0.5 mL) was then added and the mixture was left to stand at 25 °C for 2 h. The amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DMF (5 x 1 min; 3 mL each) and DCM (5 x 1 min; 3 mL each). The resin was then capped using acetic anhydride (46 μL, 5 eq) and DIEA (86 μL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.94 mmol/g resin loading was determined by UV quantification. Next, a mixture of the respective Fmoc-

Xaa-OH (3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 μL, 3 eq) in DMF (2 mL) was added to the resin, and then left to stand at 25 °C for 1 h. No recoupling was required, according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

Analysis - Cleavage of the tripeptide from HMPA handle group

A small portion of resin (5 mg) was cleaved from the HMPA handle group by treating the resin with 1 mL of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C. RP-HLPC-ESMS analysis confirmed the identity of Phe-Ala-Leu (linear gradient from 5% to 100% of ACN over 8 min; t_R = 5.53 min; *m/z* calculated for $C_{18}H_{27}N_3O_4$, 349.4; found, 350.3 $[M+H]^+$, where M is the MW of the tripeptide).

Trt protecting group removal of the L-Dap residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

In a first step, the Trt protecting group of the L-Dap of the dipeptidyl linker was removed by treating the peptidyl-resin (15 mg) with 0.2% (v/v) TFA in DCM (2 × 5 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then neutralized by washing with 5% (v/v) DIEA in DCM (2 × 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show any peptide cleavege from the resin or from the HMPA handle group, and after the second step, no peptide comprising a DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). Finally, the peptide comprising the diketopiperazine *C*-terminal protecting moiety was obtained by treating the peptidyl-resin from the second step with 5% (v/v) piperidine in THF at 25 °C (5 × 5 min; 2 mL each). THF was then removed by evaporation under reduced pressure and the resulting peptide comprising the DKP *C*-terminal protecting group was analyzed by RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; $t_{R[M]}$ = 4.62 min, $t_{R[M+HMPA]}$ = 5.31 min). Over-incorporation of the HMPA handle group was determined by analytical RP-HPLC-ESMS (linear gradient from 5% to 100% ACN over 8 min; $t_{R[M+H]}$ = 5.90 min, $t_{R[M+HMPA+H]}$ = 6.41 min; *m/z* calculated for $C_{35}H_{46}N_6O_8$, 678.8; found, 679.4 $[M+H]^+$, 843.4 $[M+HMPA+H]^+$; where M is the MW of tripeptide comprising the DKP moiety).

SPPS of BUBU Enkephalin using as attachment to the resin a DKP *click* handle: Alloc-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (312.1 mg) was swelled with DCM (5 × 1 min; 5 mL each) and DMF (5 × 1 min; 5 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (396 mg, 4 eq) and DIPCDI (93 µL, 2 eq) in DCM-DMF (15:1 (v/v); 3 mL) were added to the resin. DMAP (15 mg, 0.4 eq) in DCM (0.5 mL) was then added to the resin, and the mixture was left to stand at 25 °C for 2 h. The first amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DCM (5 x 1 min; 5 mL each) and DMF (5 x 1 min; 5 mL each), and then capped using acetic anhydride (150 µL, 5 eq) and DIEA (270 µL, 5 eq) in DMF (3 mL) for 30 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 5 mL each) and DMF (5 × 1 min; 5 mL each). The Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Lys) of the dipeptidyl linker, a mixture of Alloc-L-Lys(Fmoc)-OH (390 mg, 3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 6 eq) in DMF (3 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required, according to the chloranil test. The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each), and then the Fmoc group was removed.

Introduction of HMBA handle group

A mixture of HMBA (137 mg, 3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 3 eq) in DMF (3 mL) was added to the resin, and the mixture was left to stand at 25 °C for 1 h. No recoupling was required, according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each).

BUBU Enkephalin elongation by SPPS

Fmoc-Thr(*t*Bu)-OH (487 mg, 4 eq) and DIPCDI (93 µL, 2 eq) in DCM-DMF (15:1 (v/v); 3 mL) was added to the resin comprising the dipeptidyl linker and a HMBA handle group describe above. DMAP (15 mg, 0.4 eq) in DCM (0.5 mL) was then added, and the mixture was left to stand at 25 °C for 2 h. The amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DCM (5 x 1 min; 5 mL each) and DMF (5 x 1 min; 5 mL each). The resin was then capped using acetic anhydride (150 µL, 5 eq) and DIEA (270 µL, 5 eq) in DMF (3 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each). The Fmoc group was then removed and a 0.94 mmol/g resin loading was determined by UV quantification. Next, a mixture of the respective Fmoc-

Xaa-OH (3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 3 eq) in DMF (4 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. When the ninhydrin test was positive, a recoupling was carried out (using the same conditions). The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each), and then the Fmoc group was removed.

Alloc protecting group removal of the L-Lys residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

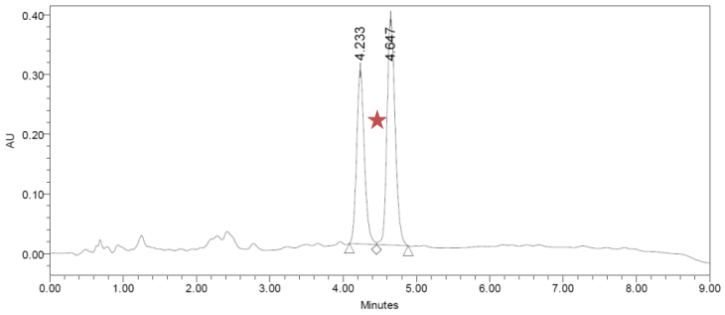
To remove the Alloc group, the peptidyl-resin (15 mg) was treated with $Pd(PPh_3)_4$ (0.1 eq) and $PhSiH_3$ (10 eq) in DCM (3 × 15 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then washed with 5% (v/v) DIEA in DCM (2 x 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show cleavage from the resin or from the HMBA handle group, and after the second step, almost negligible peptide comprising a DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). The BUBU Enkephalin peptide comprising the DKP *C*-terminal protecting moiety was obtained by treatment with 5% (v/v) piperidine in THF (2 × 5 min; 2 mL each). THF was removed by evaporation under reduced pressure and the resulting crude was analyzed by RP-HPLC (linear gradient from 30% to 60% of ACN over 8 min; $t_{R[M]}$ = 5.43 min, $t_{R[M+HMBA]}$ = 5.78 min). Over-incorporation of the HMBA handle group was determined by analytical RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; $t_{R[M+H]+}$ = 6.61 min, $t_{R[M+HMBA+H]+}$ = 6.70 min; *m/z* calculated for $C_{60}H_{85}N_9O_{13}$, 1140.3; found, 1140.7 $[M+H]^+$, 1274.6 $[M+HMBA+H]^+$; where M is the MW of the BUBU Enkephalin peptide comprising the DKP moiety).

Diketopiperazine *C*-terminal protecting group removal by catalytic hydrogenation

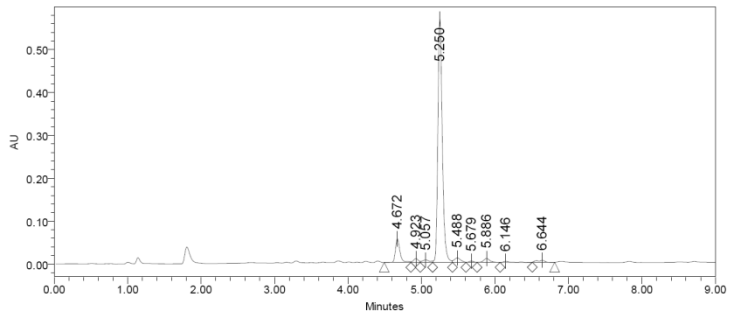
A stirred solution of *C*-terminal protected BUBU Enkephalin and Pd/C (10% w/w) in MeOH was hydrogenated under H_2 atmosphere for 2 h. The mixture was filtered over a Celite pad, washed with MeOH, evaporated under reduced pressure and analyzed by RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.45 min; *m/z* calculated for $C_{41}H_{62}N_6O_{10}$, 798.9; found, 799.56 $[M+H]^+$, where M is the MW of BUBU Enkephalin peptide).

RP-HPLC of T-20 fragments

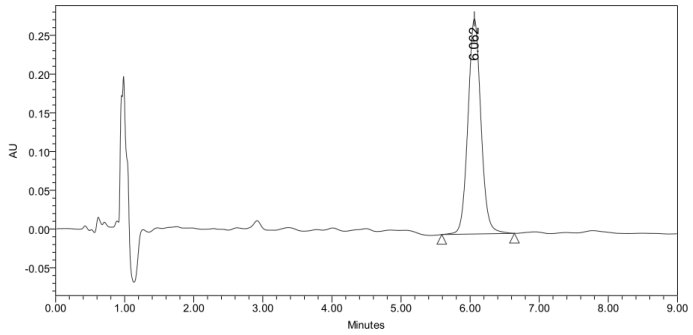
a) fully protected T-20 fragment H-(27-36)-DKP_{handle}



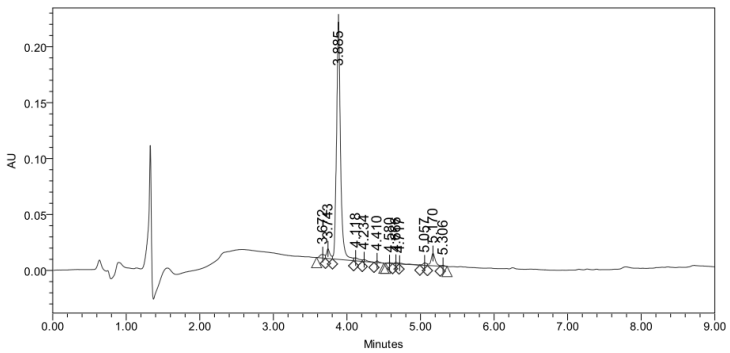
b) unprotected T-20 fragment H-(27-36)-NH_2



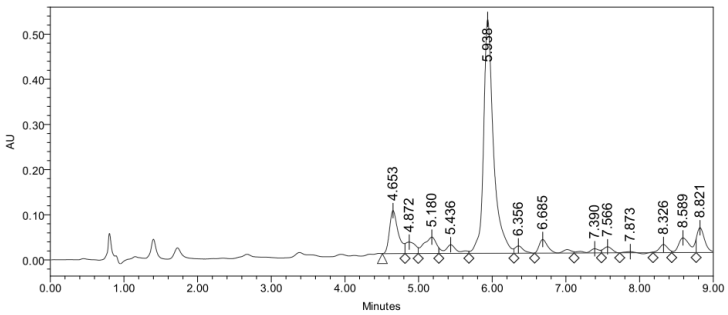
c) fully protected T-20 fragment Boc-(17-26)-OH



d) unprotected T-20 fragment H-(17-26)-OH

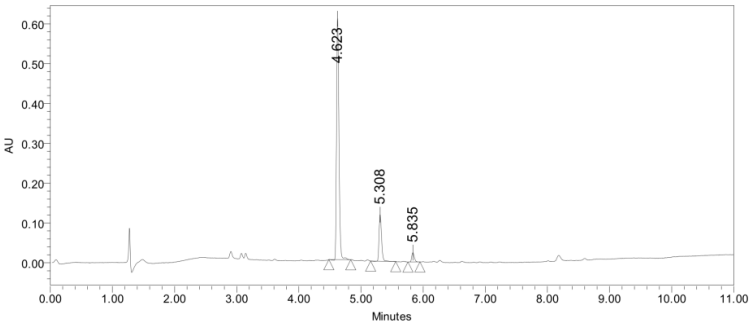


e) unprotected T-20 fragment H-(17-36)-NH_2



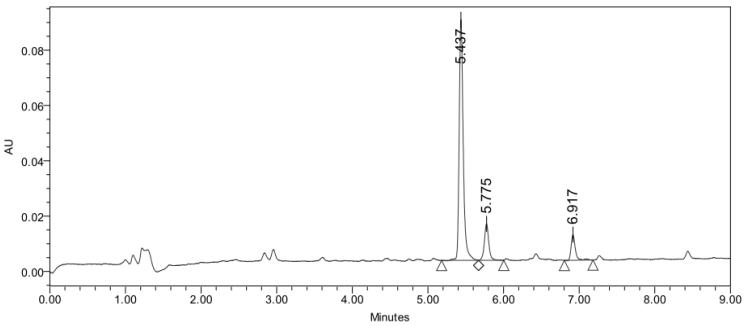
RP-HPLC of Phe-Ala-Leu

Phe-Ala-Leu comprising the diketopiperazine *C*-terminal protecting moiety



RP-HPLC of BUBU Enkephalin

BUBU Enkephalin comprising the diketopiperazine *C*-terminal protecting moiety



GENERAL CONCLUSIONS

As discussed in this thesis, we are witnessing the re-emergence of peptides as prospective therapeutics. As a result, the pharmaceutical industry calls for new methodologies to address the efficient synthesis of these chemical entities. The research covered in this thesis has contributed to the expansion of synthetic tools available for the effective preparation of complex peptides.

According to the proposed objectives, the main conclusions of this thesis are:

Chapter 1

Several regioselective and semi-regioselective strategies, along with a random approach were evaluated for the synthesis of the small, disulfide-rich peptide linaclotide. This constrained peptide was successfully synthesized by following a random approach (6 *S*-Trt group) and a semi-regioselective strategy (2 S*t*Bu (Cys1-Cys6) + 4 *S*-Trt), while other semi- and regioselective strategies failed or were found to be less suitable. The disulfide bond analysis by a partial reduction method enabled to determine that the major product obtained in the random approach corresponded to the native isomer (Cys1-Cys6, Cys2-Cys10, and Cys5-Csy13).

From detailed analysis of all the synthetic approaches used for the synthesis of linaclotide, it can be extracted valuable information for the further synthesis of other disulfide-rich peptides.

Chapter 2

S-Phacm protecting group, which can be removed by the action of an immobilized PGA enzyme under a broad range of mild conditions, proved to be a useful alternative to chemically removable protecting groups such as *S*-Acm for the synthesis of Cys-containing peptides. The combination of *S*-Trt and *S*-Phacm was successfully applied to the regioselective synthesis of disulfide bridges, whilst the use of *S*-Phacm as a unique protecting group through a random approach for preparing a

two-disulfide peptide was also explored. Interestingly, *S*-Phacm can be cleaved in the presence of sensitive functional groups, such as thioesters, and may be applied in the powerful Native Chemical Ligation strategy.

The immobilized PGA enzyme, which can be easily separated from the reaction medium by a simple and rapid filtration, demonstrated complete recovery of enzymatic activity after repeated reuse, which opens up the possibility of its implementation in a continuous process.

After synthesizing and evaluating diverse Fmoc-Cys(PG)-OH, the acid-labile *S*-Dpm protecting group was identified as an alternative to the *S*-Mob group. *S*-Dpm, which was coupled without racemization, can be fully deblocked under the standard conditions used for the cleavage and total deprotection steps in Fmoc chemistry, and it is fully compatible with both *S*-Trt and *S*-Mmt groups. *S*-Dpm was successfully applied for the regioselectivie synthesis of peptide containing intra- and intermolecular disulfide bonds.

Chapter 3

A universal handle for preparing protected *C*-terminal peptides by SPPS was designed and developed. Based on the fast and efficient diketopiperazine (DKP) formation, the DKP_{handle} overcomes some of the drawbacks associated with SPPS, such as preparation of the *C*-terminal fragment in a hybrid convergent strategy. The possibility to incorporate any bifunctional spacer between the DKP moiety and the growing peptide introduce more flexibility into SPPS schemes, by enabling the use of bezyl-type protecting groups, which can be removed by catalytic hydrogenation.

This strategy may be extended to other side-chain anchoring, which can involve other functional groups (*e.g.* hydroxyl, thiol and carboxylic acid).

RESUMEN

CAPÍTULO 1. Linaclotide

1. Introducción

Recientemente, el péptido linaclotide ha sido aprobado por la *Food and Drug Administration* (FDA) y por la *European Medicines Agency* (EMA) para el tratamiento de enfermedades gastrointestinales tales como el estreñimiento crónico (EC) y el síndrome del intestino irritable (SII)[1]. Linaclotide es administrado oralmente, y es un potente agonista selectivo del receptor de la guanilato ciclasa tipo C (GC-C) localizado en el intestino.

Desde un punto de vista estructural, este péptido de cadena corta presenta un elevado número de residuos de cisteínas que le confieren una conformación bien definida debido a la presencia de tres enlaces disulfuro intramoleculares entre los residuos Cys1-Cys6, Cys2-Cys10 y Cys5-Cys13 (**Figura 1**).

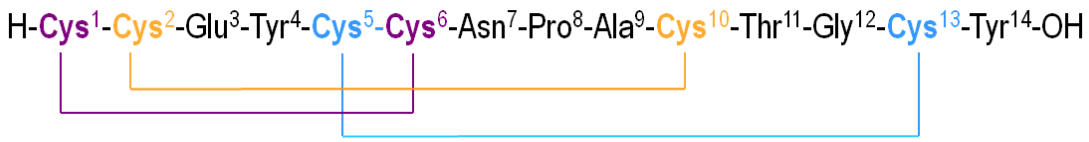


Figura 1. Estructura del péptido linaclotide.

El diseño y la optimización de una estrategia sintética eficiente para la obtención de cantidades que permitan satisfacer el mercado en un futuro, se presenta como un reto debido a la presencia de sus tres puentes disulfuro. Para la optimización de dicha síntesis es fundamental establecer y discutir sus dos principales limitaciones: el riesgo de racemización de los residuos de cisteína durante la síntesis lineal de la cadena peptídica; y el posterior plegamiento y formación de los enlaces disulfuros correctamente.

Se diseñaron y estudiaron diversas estrategias regioselectiva, semi-regioselectiva y se exploró la posibilidad de preparar el péptido linaclotide siguiendo una estrategia aleatoria, utilizando un esquema de protección de los residuos de cisteína no selectivo. La elección de los grupos protectores del grupo tiol de las cisteínas, su posición en la

secuencia, la influencia de los grupos protectores vecinos, además del orden de plegamiento y formación de los enlaces disulfuro fueron objeto de estudio.

Finalmente, el péptido completamente plegado y oxidado se analizó detenidamente con el fin de confirmar que la conectividad establecida entre los residuos de cisteína coincidían con los enlaces disulfuro nativos presentes en Linaclotide.

2. Objetivo

Estudio y optimización de la síntesis del péptido linaclotide en fase sólida siguiendo la estrategia Fmoc/*t*Bu.

3. Resultados y discusión

La correcta formación de los enlaces disulfuro es, a menudo, la etapa crucial en una síntesis peptídica, es por eso que existen un gran número de procedimientos que permiten establecer los enlaces disulfuro, ya sean intra- o intermoleculares, de manera selectiva.

Con el fin de explorar la síntesis de linaclotide se llevaron a cabo diversas estrategias sintéticas basadas en la síntesis de péptidos en fase sólida y el posterior plegamiento oxidativo en solución, utilizando diferentes grupos protectores para la cadena lateral de los residuos de cisteína tales como S-*tert*-butilo (S*t*Bu)[2], tritilo (Trt) y *p*-metoxibenzilo (*p*MeOBzl). La combinación de éstos dio lugar a estrategias regioselectivas (2+2+2; utilización de tres grupos protectores), y estrategias semi-regioselectivas (2+4; utilización de dos grupos protectores). Paralelamente, se exploró una estrategia no selectiva, donde la utilización de un único grupo protector permitió estudiar el control termodinámico del plegamiento y formación de los enlaces disulfuro.

En todos los casos, el péptido lineal se sintetizó manualmente utilizando la resina de cloruro de 2-clorotritilo (2-CTC)[3] siguiendo la estrategia Fmoc, utilizando 1-

[bis(dimetilamino)metilene]-6-cloro-1H-benzotriazolium hexafluorofosfato 2-oxido (HCTU) y diisopropiletilamina (DIEA) en dimetilformamida (DMF) durante 1 h a t.a. para incorporar todos los aminoácidos excepto los residuos de cisteína. Para prevenir su racemización,[4,5] los residuos de cisteína fueron incorporados utilizando N,N'-diisopropilcarbodiimida (DIPCDI) y 1-hidroxibenzotriazol (HOBt) en DMF, con una preactivación de 5 min, durante 1 h a t.a.

3.1. Estrategia 6 Trt

Los 6 residuos de cisteína fueron incorporados con el grupo Trt como grupo protector de la cadena lateral. El péptido totalmente protegido fue escindido de la resina 2-CTC con una solución diluida de ácido trifluoroacético (TFA), seguido de un paso de desprotección total de las cadenas laterales. La formación de los enlaces disulfuro se llevó a cabo en solución; para esta etapa de oxidación se estudiaron diversas condiciones:

a) El péptido se disolvió en el tampón A (fosfato de sodio (100 mM), hidrocloruro de guanidinio (Gdn.HCl) (2 M), pH 7.0) y la solución se agitó a t.a. durante 12 h.

b) El péptido se disolvió en el tampón A y se añadió DMSO (5%)[6]; la solución se agitó a t.a. durante 12 h.

c) El péptido se disolvió en una mezcla de tampón A y 2-propanol (1:1), se añadió glutatión reducido (2 mM) (pH 7.4) y la solución se agitó a t.a. durante 12 h.

En todos los casos, el test de Ellman[7] resultó negativo transcurridas 12 h, indicando la ausencia de grupos tiol libres. Todos los experimentos mostraron perfiles cromatográficos similares, y el análisis por RP-HPLC-ESMS mostró que el producto mayoritario presentaba la masa correspondiente al péptido completamente oxidado.

Con el fin de confirmar que el péptido obtenido mayoritariamente correspondía con el isómero estructural deseado se llevó a cabo un exhaustivo análisis de los enlaces disulfuro.

3.2. Análisis de los enlaces disulfuro

La presencia de 6 residuos de cisteína en el péptido linaclotide implica que pueden existir, teóricamente, un total de 15 isómeros estructural con patrones de pares Cys-Cys diferentes. Para establecer la conectividad entre los residuos de cisteína del péptido obtenido mayoritariamente siguiendo la estrategia 6 Trt se llevó a cabo un análisis de los puentes disulfuro utilizando un método modificado del descrito por Wu y Watson.[8,9] Este análisis confirmó que linaclotide es capaz de plegarse por sí mismo adoptando una conformación adecuada para favorecer la formación de sus tres puentes disulfuro nativos correctamente.

3.3. Estrategias regioselectivas

Se llevaron a cabo diversas estrategias semi- y regioselectivas para la preparación de linaclotide, aunque en ningún caso el resultado fue e igual o superior al obtenido mediante la aproximación termodinámica. Concretamente, la combinación de los grupos protectores Trt, S*t*Bu y *p*MeOBzl no es adecuada para la preparación del de este péptido, y además se observó que la desprotección de los grupos S*t*Bu, etapa crucial de estas estrategias, se ve afectada por la proximidad de grupos protectores voluminosos en los residuos adyacentes. Por otro lado, se determinó que el enlace disulfuro Cys1-Cys6 se forma más fácilmente en primer lugar, por lo que se presenta como un buen intermedio del camino de plegamiento del péptido linaclotide y favorecería el plegamiento y simultánea formación de los dos puentes disulfuro restantes. A partir de la información extraída siguiendo esta metodología, se decidió explorar estrategias semi-regioselectivas utilizando combinaciones de los grupos protectores ortogonales S*t*Bu y Trt para los residuos de cisteína.

3.4. Estrategias regioselectivas: 2 S*t*Bu + 4 Trt

Considerando los resultados obtenido en las estrategias regioselectivas 2 S*t*Bu + 2 Trt + 2 *p*MeOBzl, se plantearon dos combinaciones semi-regioselectivas 2 S*t*Bu + 4 Trt, desestimando la posibilidad de realizar el enlace disulfuro Cys5-Cys13 en primer lugar. Así, se exploraron dos estrategias diferentes donde los grupos protectores S*t*Bu, se encontraban localizados en el par Cys1-Cys6 o en las posiciones Cys2-Cys10.

Una vez obtenido el péptido lineal, los grupos S*t*Bu fueron eliminados en fase sólida mediante un tratamiento con una mezcla de BME (20%), NMM (0.1 M) en DMF. A continuación el péptido fue escindido de la resina mediante tratamientos ácidos de TFA en CH_2Cl_2. Finalmente, el péptido se disolvió en una mezcla de H_2O/MeCN (1:1) y se liofilizó. La formación del primer puente disulfuro se llevó a cabo en solución en presencia de DMSO a pH 8.

Cuando los grupos protectores se encontraban en las posiciones Cys2-Cys10, no fue posible su desprotección. Este hecho confirma que la eliminación del grupo S*t*Bu no depende únicamente de su posición dentro de secuencia, sino de los grupos protectores presentes en los residuos adyacentes. Cuando la desprotección de los grupos S*t*Bu se llevó a cabo a elevada temperatura, la recuperación de crudo fue pobre como consecuencia de una escisión prematura del péptido de la resina.

En esta aproximación, los mejores resultados se obtuvieron cuando los grupos S*t*Bu estaban localizados en las posiciones Cys1-Cys6, a pesar de ser necesarios 4 días para la completa desprotección de los grupos S*t*Bu. Después de la primera oxidación en solución, la desprotección total de los grupos protectores y la oxidación final de los dos puentes disulfuros restantes, se obtuvo un producto mayoritario cuya masa correspondía con el péptido completamente oxidado. Para confirmar que se trataba del isómero deseado se realizó una coelución de éste con el péptido completamente oxidado obtenido a partir de la estrategia 6 Trt.

3. Conclusiones

Varias estrategias semi- y regioselectivas, además una aproximación no selectiva fueron evaluadas para la síntesis de péptido rico en residues de Cys linaclotide. Este complejo péptido fue obtenido siguiendo las estrategia no selectiva (6 Trt) y una estrategia semi-regioselectiva (2 S*t*Bu (Cys1-Cys6) + 4 *S*-Trt). Otras estrategias semi- y regioselectivas fallaron o no fueron suficientemente adecuadas para la síntesis del linaclotide. El análisis de los puentes disulfuro mediante un método, basado en la reducción parcial de éstos, permitió determinar que el producto mayoritario obtenido

en la estrategia no selectiva correspondía con el isómero nativo (Cys1-Cys6, Cys2-Cys10, and Cys5-Csy13).

A partir del análisis detallado de todas las aproximaciones sintéticas llevadas a cabo para la síntesis del péptido linaclotide puede extraerse información valiosa a tener en cuenta en futuras síntesis de péptidos ricos en puentes disulfuro.

CAPÍTULO 2. Péptidos que contienen puentes disulfuro y grupos protectores para el aminoácido Cys

1. Introducción

Una de las modificaciones postranduccionales más frecuentes presentes en péptidos y proteínas son los enlaces disulfuro entre dos residuos de Cys. Estos elementos estructurales son responsables de la preservación de la conformación global de péptidos y proteínas, y por lo tanto son cruciales para la estabilidad y actividad biológica de estas macromoléculas. Así, no es de extrañar su elevada prevalencia en péptidos terapéuticos, como por ejemplo en la insulina, vasopresina, oxitocina, octeotrida, terlipressin, y eptifibatida que llevan años en el mercado, o nuevos pépidos con actividad terapéutica que al alcanzado el mercado en la última década, como por ejemplo atosiban, nesiritide, ziconotide, pramlintide, romidepsin, peginesatide y el ya mencionado linaclotide.

Para alcanzar el mercado, los péptidos con actividad terapéutica, además de demostrar excelentes cualidades farmacodinámicas y farmacocinéticas, deben poder ser producidos en gran escala. A pesar de la aparición de nuevas tecnologías, actualmente la síntesis en fase sólida es la forma más común de producir péptidos a gran escala. El éxito en la síntesis de estos péptidos recae básicamente en dos factores principalmente, primero en la supresión o minimización de la racemización del residuo

de Cys durante la síntesis lineal en fase sólida, y la correcta construcción de los enlaces disulfuro nativos. Para abordar el primer punto, se han investigado y desarrollado diversos grupos protectores para el β-tiol de la Cys, junto con el desarrollo de protocolos convenientes para su incorporación. Para la formación de los puentes disulfuro, la aproximación no selectiva, bajo control termodinámico, acostumbra a ser la estrategia preferida, sin embargo ésta no siempre dar lugar a la formación de los puentes disulfuro nativos. Cuando la aproximación termodinámica no resulta adecuada, es necesario implementar una estratégica de protección regioselectiva utilizando combinaciones de grupos protectores para los pares de residuos de Cys. En este sentido, los químicos de péptidos han desarrollado un gran número de grupos protectores, además de diseñar estrategias semi- y regioselectivas más o menos sofisticadas para la síntesis de péptidos con múltiples enlaces disulfuro.

2. Objetivos

Desarrollar y evaluar nuevos grupos protectores para el grupo β-tiol de la cadena lateral del aminoácido Cys para su posterior utilización en síntesis regioselectivas de péptidos con múltiples puentes disulfuro.

3. Resultados y discusión

3.1. *S*-Phacm, grupo protector lábil a la enzima penicilina P acilasa (PGA)

El grupo protector *S*-Phacm para Cys, desarrollado en nuestro grupo de investigación[10], es compatible con las estrategias Boc/Bzl y Fmoc/*t*Bu y puede ser eliminado en condiciones similares al acetamidometil (Acm) y, también por la acción del enzima penicilina G acilasa (PGA). Con el fin de evaluar la robustez y versatilidad de la enzima inmovilizada PGA en combinación con el grupo protector *S*-Phacm para síntesis de péptidos que contienen puentes disulfuro se llevaron a cabo diversos estudios.

Son muchos los estudios que han tratado la modificación de residuos susceptibles de reacciones secundarias tales como Tyr, Trp y Met durante la eliminación del grupo *S*-Acm. En consecuencia, se decidió llevar a cabo un estudio comparativo de las estrategias de protección S-Phacm y S-Acm para la síntesis del péptido relacionado con la urotensina II (URP). URP es un péptido de 8 residuos que contiene un puente disulfuro (Cys2-Cys7) y presenta en su secuencias residuos de Tyr y Trp. Se llevaron a cabo dos síntesis en paralelo del péptido URP siguiendo la estrategia Fmoc/tBu, una con *S*-Phacm y otra con *S*-Acm como grupos protectores de los residuos de Cys, respectivamente. Los péptidos lineales se sintetizaron manualmente sobre la resina 2-CTC utilizando DIPCDI y Oxyma en DMF, con preactivación de 5 min, durante 1 h para incorporar los residuos de Cys. Estas condiciones aseguran la ausencia total de racemización. El resto de aminoácidos fueron acoplados utilizando COMU y DIEA en DMF durante 1 h a 25 °C. Para la formación del enlace disulfuro siguiendo la estrategia *S*-Acm, el péptido anclado en la resina se trató con iodo en DMF, mientras que *S*-Phacm se desprotegió de manera selectiva utilizando la enzima inmovilizada PGA. La calidad del crudo resultante utilizando *S*-Phacm fue superior a la obtenida con *S*-Acm.

Por otro lado, se examinó extensamente la utilización de co-solventes en medio acuoso[11] y el efecto del pH sobre la actividad enzimática para la síntesis del péptido URP (**Tabla 1**).

	medio de reacción	pH	*S*-Phacm eliminación	formación puente disulfuro
1	H_2O	7.0	OK	-
2	H_2O-DMSO (95:5)	7.0	OK	OK
3	H_2O-ACN (95:5)	7.0	OK	-
4	H_2O-MeOH (95:5)	7.0	OK	-
5	H_2O-isopropanol (95:5)	7.0	OK	-
6	H_2O-DMF (95:5)	7.0	OK	-
7	tampón 0.1 mM fosfato	7.8	OK	-
8	tampón 0.1 mM fosfato-DMSO (95:5)	7.8	OK	OK
9	tampón 0.1 mM fosfato	5.3	OK	-
10	tampón 0.1 mM fosfato-DMSO (95:5)	5.3	OK	OK

Tabla 1. Todos los experimentos se realizaron a una concentración de péptido de 8 x 10^{-5} M, 25 mg (3 UE) de SPRIN imibond PGA (actividad hidrolítica 130 U/g) a 37° C durante 24 h.

La estabilidad y la actividad catalítica de la enzima PGA inmovilizada se mantiene intacto en la presencia de un amplia variedad de co-solventes orgánicos. Además, la reacción enzimática muestra una elevada tolerancia a la variación del pH.

Posteriormente, se evaluó la reutilización de la enzima inmovilizada para la preparación del péptido URP en H_2O-DMSO (95:5) a 37 °C y 24 h. Después de cada ciclo biocatalítico, la enzima inmovilizada se filtró, se lavó con tampón fosfato (20 mM, pH 8) y se utilizó directamente en la siguiente transformación enzimática. Después de cinco ciclos, tanto la cinética de la reacción, como la calidad del producto final se mantuvieron intactas, demostrando así su posible utilización en procesos continuos y en producciones a escala industrial.

La combinación de los grupos protectores *S*-Phacm y *S*-Trt se empleó en la preparación de un análogo del péptido oxitocina con un residuo de Cys adicional en su extremo *N*-terminal y en la síntesis regioselectiva de los péptidos bicíclicos RGD-4C y T22.

Después de su elongación en fase sólida, la oxitocina lineal, parcialmente protegida con el grupo *S*-Phacm en la Cys *N*-terminal, fue oxidada y posteriormente el *S*-Phacm fue eliminado biocatalítciamente en condiciones suficientemente suaves como para minimizar reacciones no deseadas de intercambio de puentes disulfuro. La presencia

de DMF o DMSO durante la eliminación del grupo S-Phacm provocó el intercambio entre los puentes disulfuro, por lo que se evitó su utilización durante esta etapa.

Respecto a la síntesis de los péptidos bicíclico RGD-4C y T22, posteriormente a su elongación en fase sólida, los péptidos lineales fueron escindidos de la resina y el primer puente disulfuro se construyó en solución seguido de la desprotección de los grupos *S*-Phacm por acción de la enzima inmovilizada y posterior oxidación del segundo puente disulfuro. En el caso del péptido RGD-4C, tanto la formación del primer enlace disulfuro, como la eliminación de los grupos *S*-Phacm y oxidación posterior para la obtención del péptido bicíclico final resultaron ser independientes de las condiciones del medio de reacción. La calidad del producto final obtenido siguiendo este esquema sintético fue elevada y superior a la oxidación mediante la utilización de iodo. Por el contrario, la formación del primer puente disulfuro del péptido T22 se logró en diferentes condiciones de reacción, mientras que la eliminación de los grupos *S*-Phacm resultó ser dependiente de la concentración de sales y del pH del medio de reacción. Así, en ausencia de sales o a concentraciones bajas de las mismas (0.1 y 1 mM) la reacción enzimática no tuvo lugar, mientras que la eliminación de los grupos *S*-Phacm fue parcial a concentraciones más elevadas del tampón (20 mM). La eliminación total y posterior oxidación del segundo puente disulfuro para el péptido T22 se consiguió de manera satisfactoria a concentraciones de sales de 50 y 100 mM, y la calidad del producto final obtenido siguiendo esta esquema sintético fue elevada y superior a la oxidación mediante la utilización de iodo. En paralelo, el *S*-Phacm se aplicó como único grupo protector para la síntesis del péptido T22. Como en el caso anterior, las condiciones de eliminación del grupo protector *S*-Phacm resultó ser dependiente de la concentración de sales del medio y del pH de la reacción. La calidad del producto final fue similar a la obtenida mediante el esquema sintético regioselectiva.

Finalmente, mediante la síntesis de un péptido modelo cíclico se pudo demostrar la compatibilidad de nuestro grupo *S*-Phacm con el grupo funcional tioester, clave en la estratégica sintética *Native Chemical Ligation*[12] (NCL). Este último resultado abre la

posibilidad a futuras aplicaciones de este grupo protector de Cys en la potente estrategia sintética NCL para la síntesis de pequeñas proteínas.

3.2. Grupos protectores lábiles a ácidos

Durante los últimos años se han diseñado diversos grupos protectores lábiles a ácido para la cadena lateral del aminoácido Cys. La mayor parte de éstos son muy sensibles a ácido (Mmt, Mtt, Xan, Tmob), siendo el grupo *S*-Trt el más comúnmente utilizado. En el otro extremo se encentra el grupo *S*-Mob (también conocido como PMB) que requiere una elevada concentración de ácido trifluoroacético (TFA), tiempos largos de reacción y elevada temperatura para su completa eliminación (**Figura 2**). A partir de este análisis, nos propusimos desarrollar nuevos grupos protectores lábiles a ácido que, por una parte, puedan ser desbloqueados en condiciones más suaves que las requeridas por el grupo *S*-Mob, y que a su vez, sean compatibles con el grupo *S*-Trt para su posterior aplicación en la síntesis regioselectiva de péptidos que contengan más de un enlace disulfuro.

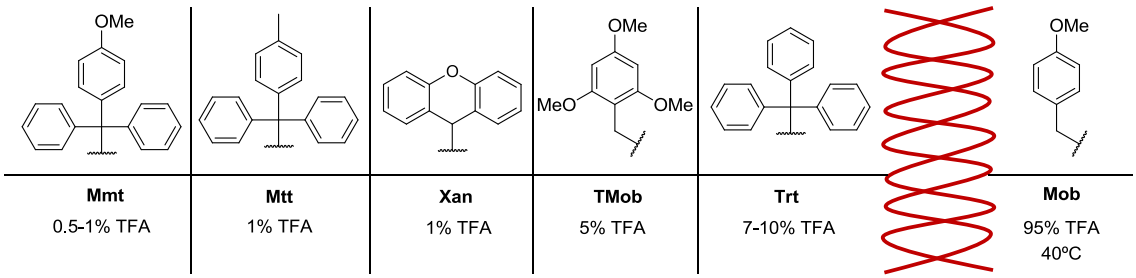


Figura 2. Grupos protectores desarrollados para la cadena lateral del aminoácido cisteína lábiles a ácidos utilizados en la estrategia Fmoc/*t*Bu.

Se prepararon y posteriormente fueron incorporados al tripéptido modelo Fmoc-Ala-Cys(R)-Leu-NH_2 hasta 12 Fmoc-Cys(R)-OH para su posterior estudio de labilidad del grupo protector R frente a diferentes concentraciones de TFA y se compararon con el grupo protector *S*-Mob. Como puntos de partida para la preparación de los grupos protectores se eligieron derivados sustituidos de los grupos bencilo, difenilo y el grupo bifenilo. La síntesis de los tripéptidos se llevó a cabo manualmente siguiendo la estrategia Fmoc/*t*Bu sobre la resina Sieber amida utilizando DIPCDI y Oxyma en DMF,

con preactivació de 5 min, durante 1 h a 25 °C. Los estudios de estabilidad se realizaron a concentraciones de 1 y 10 mM, en presencia de triisopropilsilano (TIS) y H_2O como capturadores de carbocationes y con diversas soluciones de TFA en CH_2Cl_2 (**Tabla 2**).

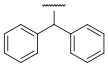
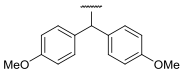
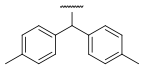
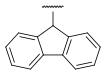
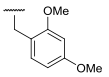
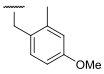
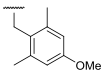
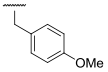
	Grupo Protector (R)	TFA (%)	T (°C)	tiempo de reacción	% Cys desprotegida
a		10	25	5 min	0
		60	25	1 h	100
b		10	25	5 min	100
c		10	25	5 min	29
		20	25	30 min	100
d		10	25	5 min	0
		95	40	2 h	0
e		10	25	5 min	0
		95	40	2 h	0
f		10	25	5 min	0
		95	40	2 h	0
g		10	25	5 min	17
		20	25	30 min	100
h		10	25	5 min	0
		50	25	1 h	100
i		10	25	5 min	0
		50	25	1 h	100
j		10	25	5 min	0
		95	25	1 h	21
k		10	25	5 min	7
		20	25	30 min	100
l		10	25	5 min	9
		20	25	30 min	100
m		10	25	5 min	0
		95	40	2 h	100

Tabla 2. Estudio de labilidad de los grupos protectores para cisteína.

El análisis por RP-HPLC determinó que el grupo protector *S*-Mob (# **m**; **Taula 2**) requiere 2 h y aumentar la temperatura hasta 40 °C para su total eliminación, mientras que tres de los grupos protectores evaluados mostraron unas condiciones de eliminación más suaves y compatibles con las condiciones estándares de eliminación del grupo *S*-Trt (# **a**, **h** y **i**; **Tabla 2**). Otros de los grupos evaluados resultaron ser muy lábiles a ácido (# **b-c, g,** y **k-l**; **Tabla 2**), mientras que el resto de grupos no mostraron ser lábiles a tratamientos ácidos (# **d-f** y **j**; **Tabla 2**). En este punto se eligió el grupo *S*-

Dpm, sintéticamente más accesible, para continuar con los experimentos adicionales de compatibilidad y su posterior aplicación en esquemas sintéticos regioselectivos. A pesar de ser descrito por primera vez por Zervas el año 1970[13], este grupo protector de Cys no ha sido utilizado en química Fmoc/*t*Bu para la síntesis regioselectiva de péptidos con puentes disulfuro hasta el momento.

Después de confirmar la ausencia de racemización durante la incorporación del Cys(Dpm) durante la síntesis del tripéptidos modelo, y la compatibilidad de éste con los grupos protectores *S*-Trt y *S*-Mmt, se decidió aplicar combinaciones de estos en esquemas de protección para la construction regioselectiva de puntos disulfuro intra- e intermoleculares. Así, combinaciones de los grupos *S*-Mmt y *S*-Dpm se emplearon en la síntesis regioselectiva de el fragmento bisagra (225–232/225'–232') de la inmunoglobulina humana (IgG1), mientras que la α-conotoxina ImI se preparó combinando los grupos *S*-Trt y *S*-Dpm.

El fragmento bisagra de la IgG1 humana se obtuvo siguiendo dos estrategias diferentes en paralelo. En la primera aproximación (resina Rink amida), el grupo *S*-Mmt fue selectivamente eliminado con tratamientos diluidos de ácido y posteriormente se llevó a cabo la construcción del primer puente disulfuro en fase sólida mediante un tratamiento de piperidina-DMF (1:4). A continuación, el dímero protegido y anclado a la resina se trato con una mezcla de TFA-TIS-H_2O (95:2.5:2.5) durante 1 h a 25 °C seguido de un tratamiento con DMSO-tampón fosfato (1:4) a pH 9 para la formación del segundo enlace disulfuro en solución. En la segunda aproximación sintética (resina Sieber amida), después de la elongación del péptido en fase sólida se formó el primer puente en solución a partir del péptido lineal parcialmente protegido, y el dímero totalmente oxidado se obtuvo con una elevada pureza siguiendo las mismas condiciones descritas para la primera aproximación.

La α-conotoxina ImI, un péptido de 12 residuos que contiene en su secuencia dos enlaces disulfuro (2Cys-8Cys y 2Cys-12Cys), se obtuvo combinando dos grupos *S*-Dpm y dos *S*-Trt para la protección de los residuos de Cys. Después de la elongación del péptido lineal sobre la resina Sieber amida, el péptido parcialmente protegido fue escindido de la resina y se realizó la construcción del primer puente disulfuro en H_2O-

ACN (3:7) a pH 8 durante 16 h a 25 °C y posteriormente la mezcla fue liofilizada. El crudo resultante fue tratado directamente con una mezcla de TFA-DMSO-anisol (89:10:1) durante 2 h a 25 °C para obtener el péptido final con una elevada pureza.

4. Conclusiones

El grupo protector *S*-Phacm, que puede ser eliminado por la acción de la enzima inmovilizada PGA bajo un amplio rango de condiciones, ha demostrado ser una alternativa muy útil al grupo protector *S*-Acm para la síntesis de péptidos con puentes disulfuro. La cominación de los grupos *S*-Trt y *S*-Phacm ha sido aplicada con éxito en la síntesis regioselectiva de puentes disulfuro. La utilización del grupo *S*-Phacm como único grupo protector también ha sido explorado con buen resultado. Éste grupo puede ser eliminado bajo condiciones suaves en la presencia de grupos funcionales sensibles, tales como el enlace tioester, lo que permite su utilización en la estrategia síntetica de NCL. Finalmente, la enzima inmovilizada ha demostrado gran robustez en un amplio rango de condiciones experimentales, y puede ser reciclada con facilidad.

Después de sintetizar y evaluar diferentes Fmoc-Cys(PG)-OH, el grupo *S*-Dpm lábil a ácido es una alternativa prometedora al grupo S-Mob. S-Dpm, que es incorporado sin racemización, puede ser completamente eliminado en las condiciones estándares utilizadas durante la escisión y desprotección de la química Fmoc. Asimismo, este grupo es completamente compatible con los grupos S-Trt y S-Mmt, y ha sido utilizado con éxito en la síntesis regioselectiva de péptidos con puentes disulfuro intra- e intermoleculares.

CAPÍTULO 3. Nuevo espaciador basado en la formación de dicetopiperazinas (DKP)

1. Introducción

La síntesis de péptidos en fase sólida (SPPS), descrita por Merrifield en 1963[14], representó una auténtica revolución en el arte de la síntesis de péptidos. Esta metodología resulta eficiente y está bien establecida para la síntesis de péptidos de cadena corta y media. Sin embargo, la posibilidad de hacer crecer un péptido incorporando aminoácido, tras aminoácido está limitado por la eficiencia de cada etapa –tanto de acoplamiento como de desprotección–. El desarrollo de nuevos agentes de acoplamiento[15], espaciadores y grupos protectores[16], además de la aparición de resinas innovadoras basadas en polietilenglicol (PEG), como son ChemMatrix[17,18] o SPOCC[19] ha permitido mejorar de forma significativa la eficiencia de esta metodología. Asimismo, la incorporación de pseudoprolinas[20] y/o grupos protectores para el esqueleto peptídico, tales como Hmb[21] y Dcpm[22], han sido utilizadas para reducir la propensión de algunas cadenas hidrofóbicas a agregar, y de esta manera incrementar la eficiencia de la síntesis. A pesar de todos estos avances, para la síntesis de péptidos que contienen más de 50 residuos, es necesario adoptar estrategias de síntesis convergentes.

Las aproximaciones convergentes permiten tener mayor control de la síntesis, ya que los fragmentos peptídicos intermedios pueden ser purificados antes de su utilización. Entre las estrategias convergentes más utilizadas se encuentra la estrategia de condensación de fragmentos en fase sólida, la condensación de fragmentos en solución –también conocida como híbrida–, y aproximaciones basadas en ligaciones quimioselectivas, siendo la más importante la *Native Chemical Ligation* (NCL)[23].

Entre las estrategias convergentes basadas en la condensación de fragmentos, la aproximación híbrida resulta muy adecuada, ya que esta combina las ventajas de la SPPS y la síntesis en solución. Esta aproximación incluye la preparación de los fragmentos peptídicos protegidos sobre un soporte polimérico y el posterior acoplamiento de estos en solución.[24]

Las estrategias basadas en la condensación de fragmentos peptídicos, requieren la preparación de segmentos peptídicos protegidos. En este sentido, la síntesis de estos fragmentos con una elevada pureza y buenos rendimientos siguiendo la estrategia sintética Fmoc/*t*Bu, frecuentemente, se lleva sobre resinas de elevada sensibilidad a ácido. Así, para la preparación de fragmentos peptídicos amida protegidos se utiliza la resina Sieber amida, sin embargo durante la etapa de escisión puede generar problemas de desprotección parcial de las cadenas laterales. Por otro lado, la resina 2-CTC se aplica en la síntesis de fragmentos peptídicos ácido protegidos, y en este caso la etapa de escisión da lugar al fragmento peptídico con el grupo ácido terminal libre. Si éste último así obtenido es el fragmento *C*-terminal y la etapa de acoplamiento se realiza en solución, generalmente, el grupo ácido terminal libre debe ser protegido en una etapa anterior a la condensación de fragmentos.

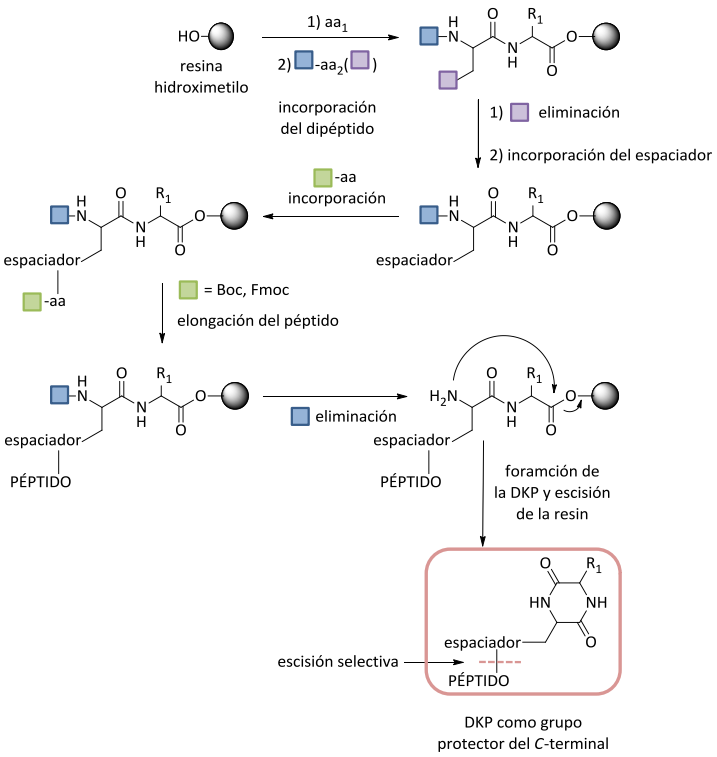
2. Objetivo

Diseño y evaluación de una nueva metodología para la preparación en fase sólida de péptidos y fragmentos peptídicos con el extremo *C*-terminal protegido temporalmente.

3. Resultados y discusión

Se ha diseñado y desarrollado una nueva metodología para la síntesis de péptidos con el extremo C-terminal protegido. Se fundamente en la idea de un nuevo espaciador basado en la eficiente reacción de formación de dicetopiperainas (DKP) del dipéptido PG_1-Lys(PG_2)-DPro y la incorporación de un espaciador bifuncional temporal entre la cadena lateral de la Lys contenida en el grupo dipeptídilo y la secuencia peptídica a sintetizar (**Esquema 1**). Esta nueva metodología permite la preparación en fase sólida de péptidos y fragmentos peptídicos con el extremo *C*-terminal protegido temporalmente para su posterior utilización en la síntesis convergente de péptidos

complejos, además, permite la síntesis de péptidos en fase sólida mediante un procedimiento totalmente libre de tratamientos ácidos.



Equema 1. Representación esquemática de la estrategia basada en la formación de DKP para la síntesis en fase sólida de péptidos y fragmentos peptídicos con el extremo *C*-terminal protegido.

Siguiendo esta nueva metodología se sintetizó el fragmento H-(17-36)-NH_2 del péptido T-20 (Fuzeon®). Este fragmento se preparó en solución a través del acoplamiento del fragmento Boc-(17-26)-OH, sintetizado en fase sólida sobre la resina 2-CTC y el fragmento *C*-terminal protegido H-(27-36)-DKP_{handle} sintetizado en fase sólida mediante el espaciador basado en DKP. Como etapa final, el fragmento totalmente protegido fue tratado con una mezcla de TFA-DMB(1,3-dimetoxibenceno)-TIS (9.2.5:5:2.5) para la obtención del fragmento H-(17-36)-NH_2 totalmente desprotegido con una elevada pureza. Para la síntesis del fragmento *C*-terminal protegido se empleó espaciador el dipéptido Trt-Lys-DPro-OH. Así, después de la elongación del péptido, el N^{α}-amino de la Lys se desprotegió selectivamente con una solución de TFA (0.2%) en CH_2Cl_2, mientras que la ciclación –formación de la DKP– y escisión del péptido

totalmente protegido fue favorecida mediante tratamientos de piperidina–THF (20:80).

Por otro lado, la incorporación del derivado Alloc-Lys(Fmoc)-OH como componente del dipéptido, y del espaciador bifuncional ácido 4-hidroximetilbenzoico (HMBA) permitió preparar el péptido BUBU Enkephalin H-Tyr-DSer(*t*Bu)-Gly-Phe-Leu-Thr(*t*Bu)-OH sobre el espaciador basado en la formación de DKP mediante un proceso totalmente libre de tratamientos ácidos. En este caso, después de la eliminación del grupo Alloc, el péptido se escindió mediante un tratamiento básico para dar lugar a la formación de la DKP. Finalmente, el extremo *C*-terminal fue desprotegido en solución mediante hidrogenación catalítica con Pd/C para obtener el producto final con una elevada pureza.

4. Conclusiones

Se ha desarrollado una nueva metodología para la síntesis en fase sólida de péptidos y fragmentos peptídicos con el extremo *C*-terminal protegido de forma temporal. Esta metodología ha permitido superar uno de los inconvenientes asociados con la SPPS como es la preparación de los fragmentos *C*-terminales para su posterior uso en estrategias convergentes. Además, este nuevo espaciador introduce más flexibilidad en la SPPS, incorporando la utilización de grupos protectores del tipo bencilo, que pueden ser eliminados mediante hidrogenación catalítica.

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