CHEMISTRY
A EUROPEAN JOURNAL

ChemPubSoc
Europe

DOI: 10.1002/chem.201201370

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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Supporting information for this article, including experimental details of peptide elongation by using solid-phase peptide synthesis, is available on the WWW under http://dx.doi.org/10.1002/chem.201201370.

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]

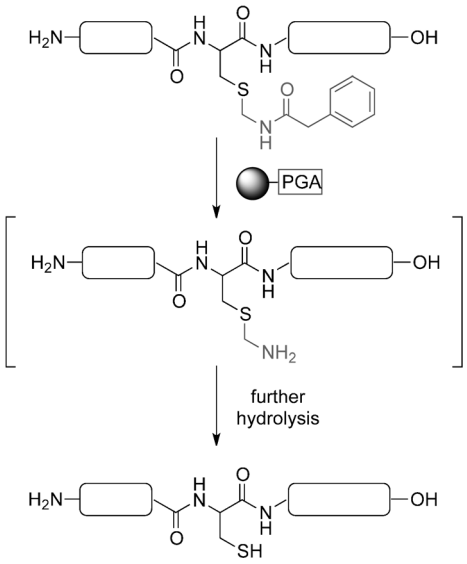
The covalent immobilization of penicillin G acylase (PGA) from *E. coli* (E.C. 3.5.1.1) on an amino-acrylic resin, which recognizes the phenylacetyl moiety, exhibits promising applications in peptide synthesis and opens up new perspectives for further applications.[9] Herein, we explore the power of 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.

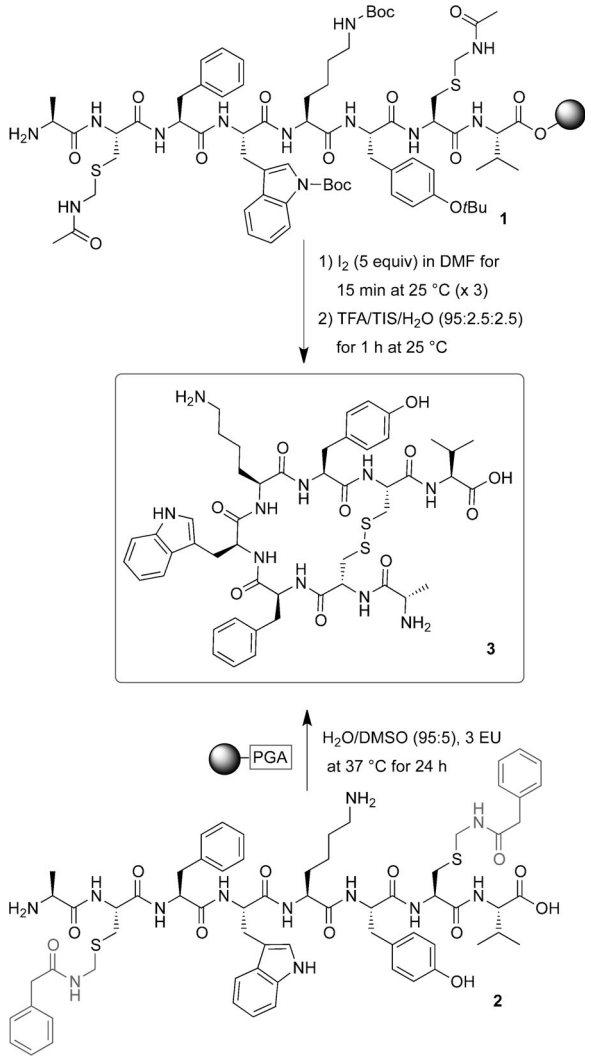
_S_-Phacm as a green alternative to the conventional _S_-Acm group: The *S*-Acm protecting group can be removed to either generate the free thiol (mediated by Hg^{II} or Ag^{I} salts, followed by extensive thiol treatment) or to directly render a disulfide bond through oxidation (mediated by I_2 or Tl^{III} 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



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).

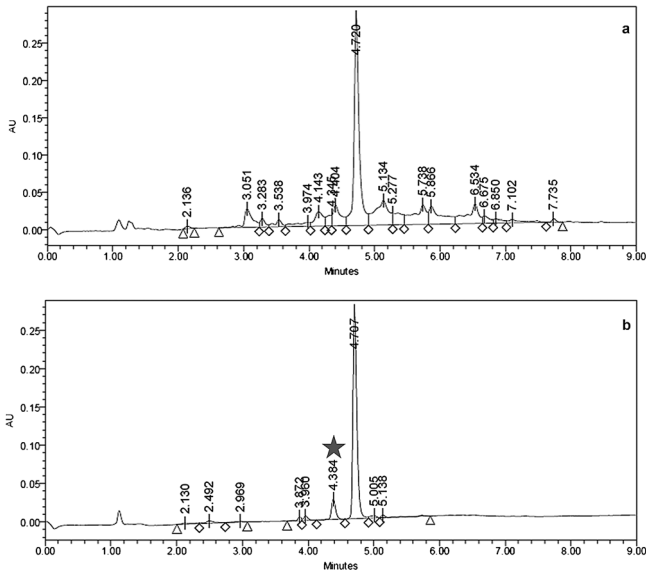


Figure 1. Chromatographic profiles of the oxidized peptide URP (**3**) from the: a) *S*-Acm, and b) *S*-Phacm strategies (phenylacetic acid: ★).

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-

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.

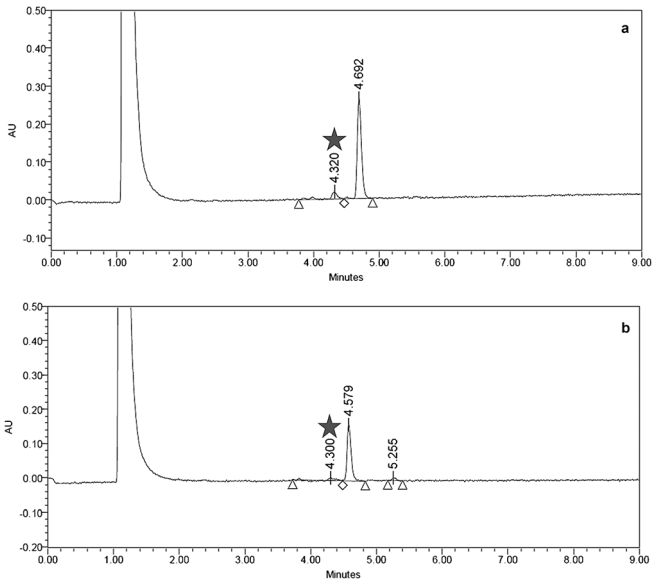


Figure 2. Chromatographic profiles of the oxidized peptide URP (**3**) after: a) cycle 1, and b) cycle 5 (phenylacetic acid: ★).

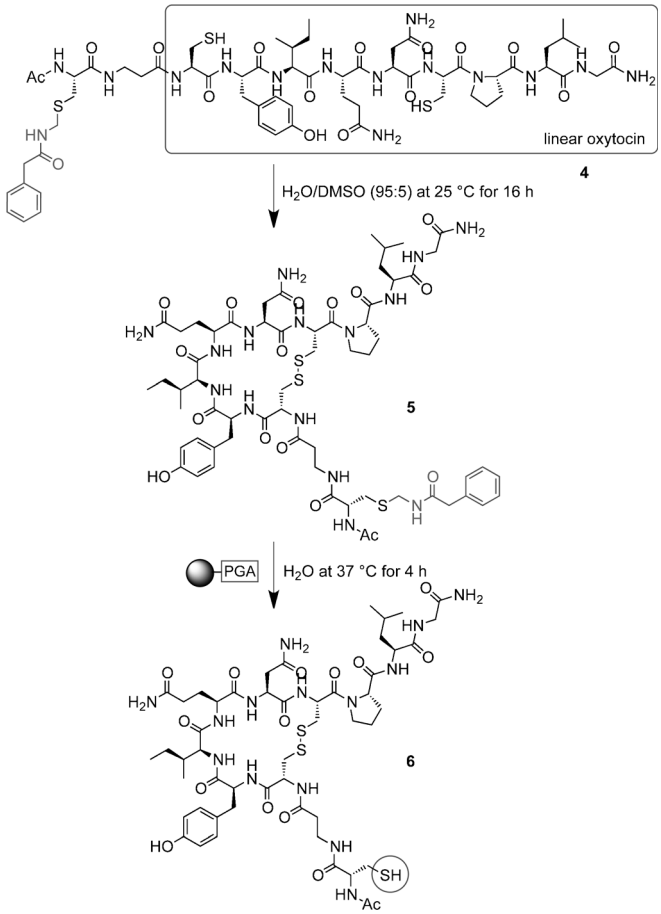
Regioselective formation of disulfide bonds: The regioselective construction of disulfide bonds is a challenging and desirable goal for the synthesis of complex Cys-containing peptides. Several suitable combinations of orthogonal and/or compatible protecting groups have been described for this purpose.[18] 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

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

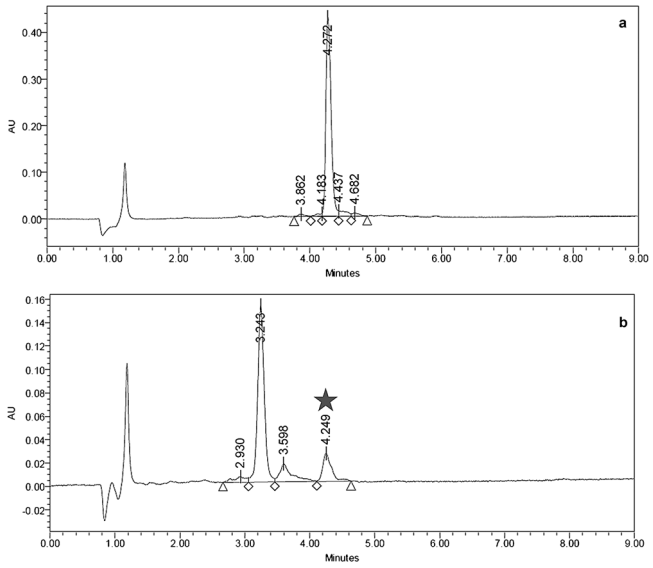


Figure 3. Chromatographic profiles of: a) oxidized *S*-Phacm-protected oxytocin analogue **5**, and b) after the removal of *S*-Phacm (Table 2, entry 9; phenylacetic acid: ★).

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 $U g_{wet}^{-1}$) at 37 °C. The progress of the reactions, along with disulfide scrambling, were monitored by RP-HPLC analysis.

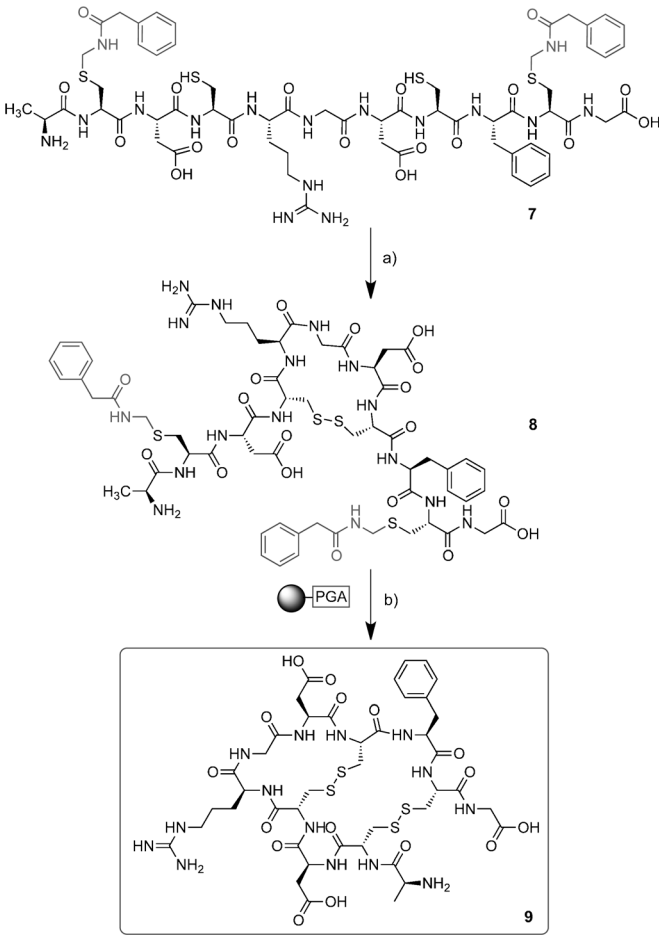
the disulfide had been achieved and without isolation of the *S*-Phacm-protected cyclic peptide (**5**), immobilized PGA was added to remove the *S*-Phacm group. However, when DMF or DMSO was used for *S*-Phacm deblocking, disulfide scrambling occurred. As expected, this phenomenon was more significant 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.

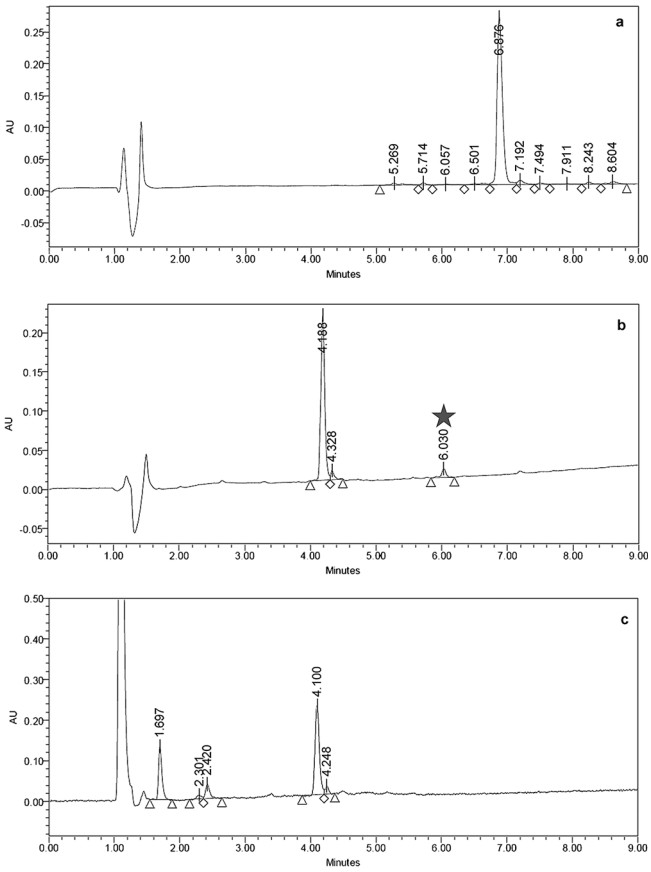


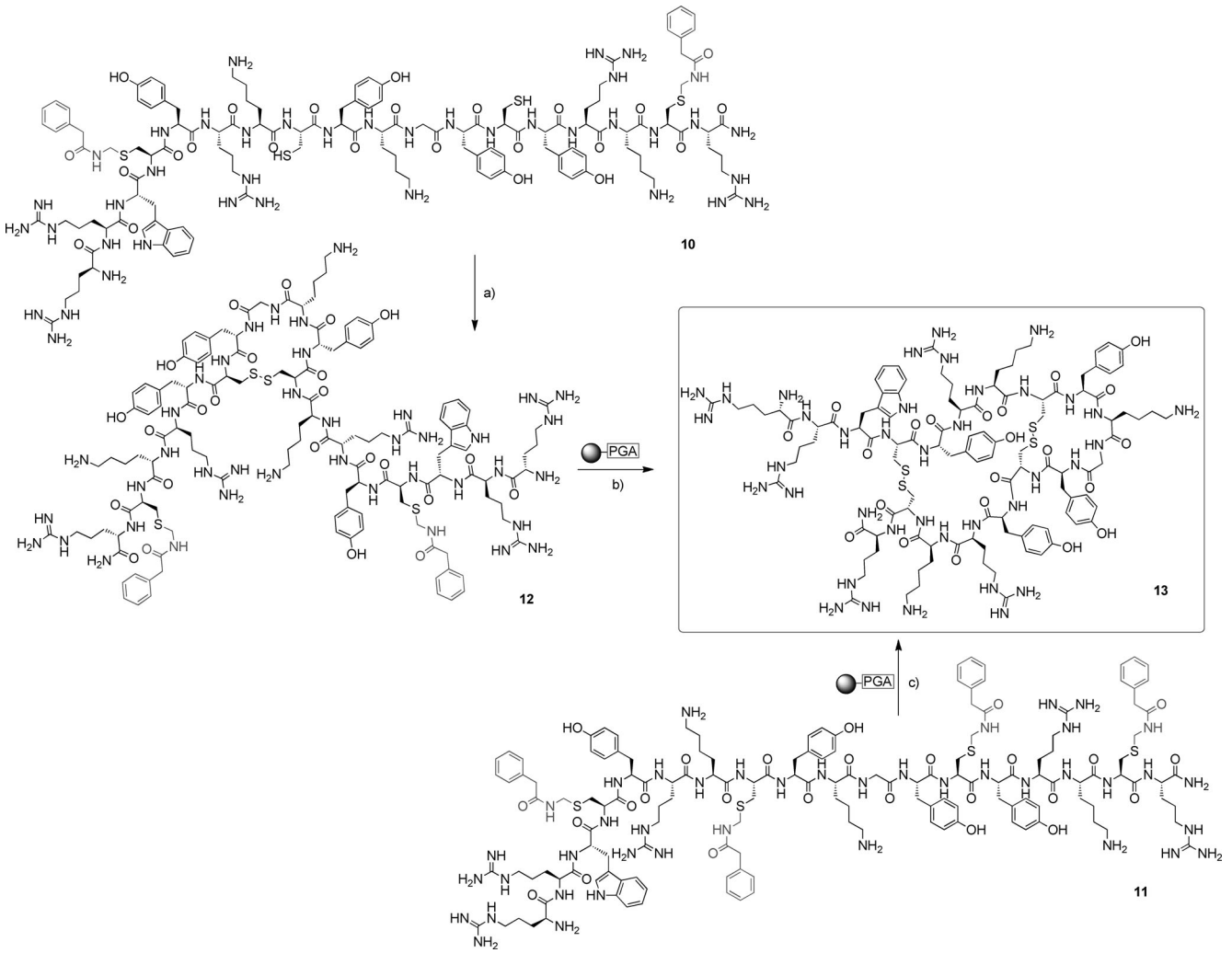
Figure 4. Chromatographic profiles of: a) cyclic *S*-Phacm-protected RGD-4C peptide (**8**), b) final bicyclic peptide **9** from the biocatalytic removal of *S*-Phacm and oxidation (Table 3, entry 1), and c) final bicylic peptide (**9**) after the removal of *S*-Phacm and oxidation by treatment with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

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).

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).

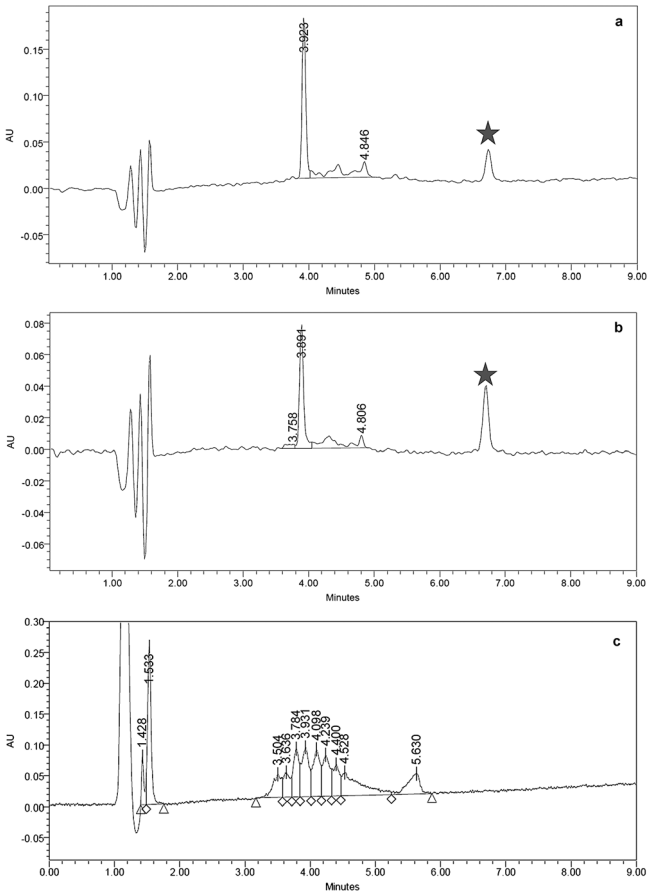


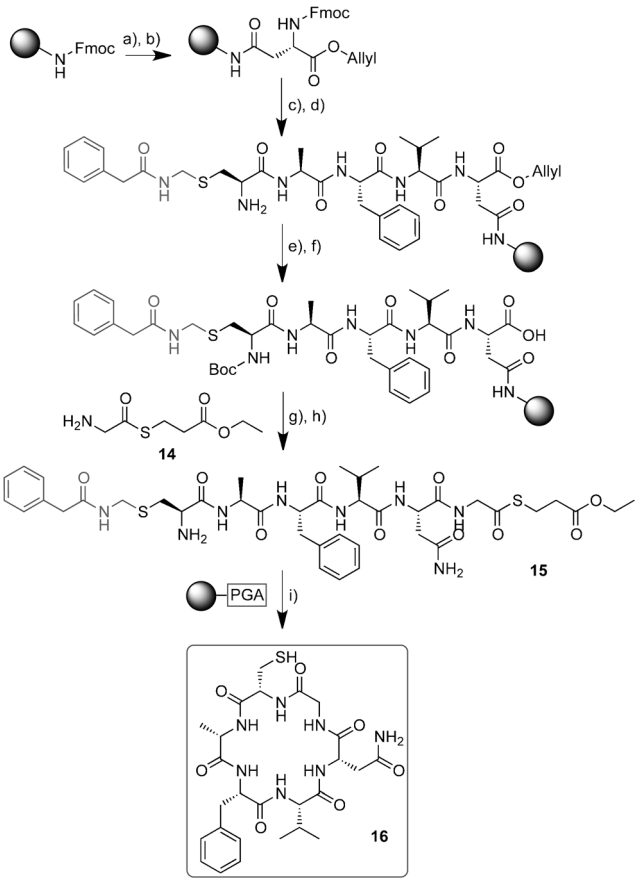
Figure 5. Chromatographic profiles of: a) final bicyclic T22 peptide (**13**) from the regioselective strategy (Table 4, entry 10), b) final bicyclic T22 peptide (**13**) from the random strategy (Table 4, entry 10), and c) final bicyclic T22 peptide (**13**) after the removal of *S*-Phacm and concomitant 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*-

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.

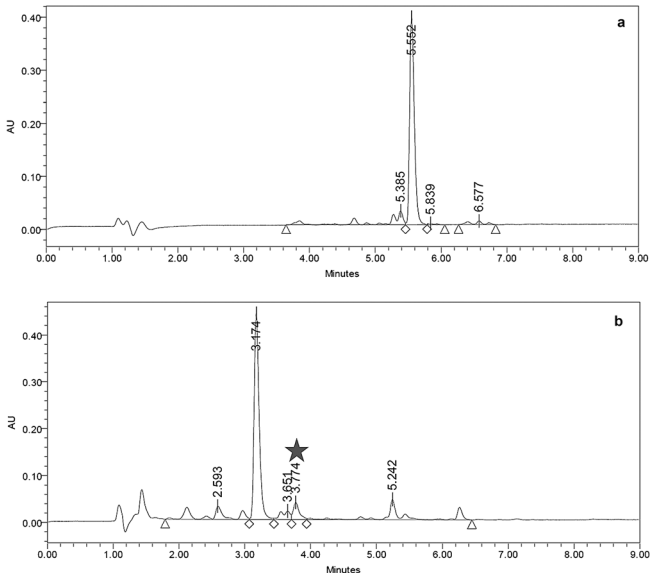


Figure 6. Chromatographic profiles of: a) linear C-terminal thioester peptide **15**, b) final cyclic peptide **16** from the enzymatic removal of *S*-Phacm and concomitant cyclization through an NCL reaction in water at pH 7 (phenylacetic acid: ★).

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

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**).

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 $C_{42}H_{60}N_{14}O_{16}S_4$: 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 $C_{26}H_{37}N_7O_7S$: 591.68; found: 592.9 $[M+H]^+$ (M is the M_W of the cyclic peptide **16**).

Acknowledgements

The work has been financed by the CICYT (CTQ2009-07758), the Generalitat de Catalunya (2009SGR1024), the Institute for Research in Biomedicine Barcelona (IRB Barcelona), and the Barcelona Science Park.

[1] A. Isidro-Llobet, M. Álvarez, F. Albericio, *Chem. Rev.* **2009**, *109*, 2455–2504.
[2] T. W. Greene, P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 3rd ed., Wiley, New York, **1999**.
[3] G. Barany, F. Albericio, *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942.
[4] a) T. Pathak, H. Waldmann, *Curr. Opin. Chem. Biol.* **1998**, *2*, 112–120; b) T. Pathak, H. Waldmann, in *Stereoselective Biocatalysis*, (Ed.: R. N. Patel, M. Dekker), CRC, New York, **2000**, pp. 775–797; c) D. Kadereit, H. Waldmann, *Chem. Rev.* **2001**, *101*, 3367–3396.
[5] G. T. Hermanson, *Bioconjugate Techniques*, 2nd ed., Academic Press, **2008**.
[6] a) M. Schnolzer, S. B. H. Kent, *Science* **1992**, *256*, 221–25; b) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776–79.
[7] J. Brask, in *The Power of Functional Resins in Organic Synthesis* (Eds.: J. Tulla-Puche, F. Albercicio), Wiley-VCH, Weinheim, **2008**, pp. 365–380.
[8] a) L. Cao, *Carrier-bound Immobilized Enzymes: Principles, Application and Design*, Wiley-VCH, Weinheim, **2005**; b) R. Wohlgemuth, *Curr. Opin. Biotechnol.* **2010**, *21*, 713–724.
[9] a) A. Basso, P. Braiuca, S. Cantone, C. Ebert, P. Linda, P. Spizzo, P. Caimi, U. Hanefeld, G. Degrassi, L. Gardossi, *Adv. Synth. Catal.* **2007**, *349*, 877–886; b) A. Basso, L. De Martin, C. Ebert, L. Gardossi, P. Linda, *J. Mol. Catal. B Enzym.* **2001**, *16*, 73–80.
[10] M. Royo, J. Alsina, E. Giralt, U. Slomcyznska, F. Albericio, *J. Chem. Soc. Perkin Trans. 1* **1995**, 1095–1102.
[11] a) K. Adermann, K. Barlos, in *Oxidative Folding of Peptides and Proteins*, (Eds.: J. Buchner, L. Moroder), RSC, **2009**, pp. 297–317; b) B. Kamber, A. Hartmann, K. Eisler, B. Riniker, H. Rink, P. Sieber, W. Rittel, *Helv. Chim. Acta* **1980**, *63*, 899–915; c) C. S. Samuel, F. Lin, M. A. Hossain, C. Zhao, T. Ferraro, R. A. D. Bathgate, G. W. Tregear, J. D. Wade, *Biochemistry* **2007**, *46*, 5374–5381.
[12] a) H. Lamthanh, C. Roumestand, C. Deprun, A. Menez, *Int. J. Pept. Prot. Res.* **1993**, *41*, 85–95; b) H. Lamthanh, H. Virelizier, D. Frayssinhes, *Pept. Res.* **1995**, *8*, 316; c) M. Engebretsen, E. Agner, J. Sandosham, P. M. Fischer, *J. Pept. Res.* **1997**, *49*, 341–346; d) N. M. Alexander, *J. Biol. Chem.* **1974**, *249*, 1946–1952; e) P. Sieber, B. Kamber, B. Riniker, W. Rittel, *Helv. Chim. Acta* **1980**, *63*, 2358–2363.
[13] D. Chatenet, C. Dubessy, J. Leprince, C. Boularan, L. Carlie, I. Ségalas-Milazzo, L. Guilhaudis, H. Oulyadi, D. Davoust, E. Scalbert, B. Pfeiffer, P. Renard, M. C. Tonon, I. Lihrmann, P. Pacaud, H. Vaudry, *Peptides* **2004**, *25*, 1819–1830.
[14] R. Subirós-Funosas, R. Prohens, R. Barbas, A. El-Faham, F. Albericio, *Chem. Eur. J.* **2009**, *15*, 9394–9403.
[15] a) Y. Han, F. Albericio, G. Barany, *J. Org. Chem.* **1997**, *62*, 4307–4312; b) Y. M. Angell, J. Alsina, F. Albericio, G. Barany, *J. Peptide Res.* **2002**, *60*, 292–299.
[16] A. El-Faham, R. Subirós-Funosas, R. Prohens, F. Albericio, *Chem. Eur. J.* **2009**, *15*, 9404–9416.
[17] a) M. G. Kim, S. B. Lee, *J. Mol. Catal. B Enzym.* **1996**, 181–190; b) M. G. Kim, S. B. Lee, *J. Mol. Catal. B Enzym.* **1996**, 201–211.
[18] a) D. Andreu, F. Albericio, N. A. Sole, M. C. Munson, M. Ferrer, G. Barany, in *Peptide Synthesis Protocols* (Eds.: M. W. Pennington, B. M. Dunn), Humana, New York, **1994**, pp. 91–169.
[19] N. Assa-Munt, X. Jia, P. Laakkonen, E. Ruoslahti, *Biochemistry* **2001**, *40*, 2373–2378.
[20] H. Nakashima, M. Masuda, T. Murakami, Y. Koyanagi, A. Matsumoto, N. Fujii, N. Yamamoto, *Antimicrob. Agents Chemother.* **1992**, *36*, 1249–1255.
[21] K. Kuttiyawong, S. Nakapong, R. Pichyangkura, *Carbohydr. Res.* **2008**, *343*, 2754–2762.
[22] J. Tulla-Puche, I. V. Getun, J. Alsina, F. Albericio, G. Barany, *Eur. J. Org. Chem.* **2004**, 4541–4544.

Received: April 21, 2012
Published online: October 18, 2012

