IMPROVEMENT OF HEAD-TO-TAIL RGD PEPTIDE CYCLIZATION EFFICIENCY COMBINING LOW TEMPERATURE AND LITHIUM CHLORIDE

ADRIANA GAUNA [a,b&], FREDYS SANCHEZ-HOYOS [a,b,c&], MAYCOL HUERTA [d], MARCELO J. KOGAN [a,b] AND EYLEEN ARAYA [b,d*]*

[a]*Departamento de Química Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, 8380494, Santiago, Chile.*
[b]*Advanced Center for Chronic Diseases (ACCDiS), Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, 8380494, Santiago, Chile.*
[c]*Grupo de Investigación en Química Orgánica Medicinal. Facultad en Ciencias Farmacéuticas. Universidad de Cartagena, Colombia.*
[d]*Departamento de Ciencias Quimicas, Facultad de Ciencias Exactas, Universidad Andres Bello, Av. Republica 275, Santiago, Chile.*
& These authors contributed equally.

ABSTRACT

RGD-containing peptides (linear and cyclic) have been developed to target some types of cancer cells through interaction with the αvβ3 Integrin. Due to their reduced conformational freedom, cyclic peptides exhibit improved metabolic stability and binding affinity/specificity to their molecular targets. However, macrocyclization is considered a significant synthetic challenge that is affected by the ring size, peptide sequence, and reaction conditions, making tetra- and pentapeptides more difficult to cyclize. In this work, we report some optimized cyclization conditions for the obtention of a cyclic RGDfK peptide that combine the use of low reaction temperature and the addition of LiCl, a combination not yet reported that resulted in a reduction of the formation of oligomers and an improvement of the cyclization efficiency.

Keywords: Cyclic(RGDfK), Head-to-tail cyclization, Integrins, peptide cyclization conditions, Targeting.

1. INTRODUCTION

Peptides are a growing therapeutic class due to their unique spatial characteristics and ability to target protein–protein interactions and surfaces. Despite their advantages, peptides must overcome several key shortcomings, including their high conformational flexibility and susceptibility to proteolytic cleavage. In contrast to linear peptides, cyclic variants are more resistant to both exo- and endoproteases. Furthermore, cyclization may increase not only hydrolytic stability but also binding to the target protein, which explains their significant therapeutic potential [1].

Peptides based on the RGD motif are potent and selective integrin antagonists and have been used for diagnostic imaging and targeted therapy for some types of cancer and tissue engineering [2-5]. Currently, emphasis is being placed on the use of cyclic RGD peptides as they are more stable, potent, and specific than linear RGD peptides, which are highly susceptible to chemical degradation [6].

The cyclic RGDfK [c(RGDfK)] peptide is especially specific towards αvβ3 integrin receptors, which are involved in cell-cell and cell-matrix interactions and tumor angiogenesis and are overexpressed in several types of cancer, including breast, prostate, pancreatic, and brain [7-9]. Therefore, the cRGDfK peptide is a ligand of interest for drug delivery applications [10-13].

However, macrocyclization of peptides has often been perceived as synthetically challenging, and the cyclization yields are affected by several factors, including the ring size, peptide sequence, and reaction conditions, where cyclization of small rings (cyclotetrapeptides and cyclopentapeptides) is considerably more difficult and accompanied by significant cyclic dimer formation [14]. Furthermore, peptides that are difficult to cyclize are generally rich in Lys(Boc) and Arg(Pbf) residues [15]. Based on this, it is not a surprise that cyclization of the RGDfK sequence has a very low yield and is accompanied by significant dimer and oligomers formation. That raises the costs and complexity of production, making the c(RGDfK) peptide difficult to use in the treatment of chronic diseases [16].

Appropriate modification of reaction conditions could improve the cyclization yields of the otherwise poorly cyclizing peptides. Phosphonium salt coupling reagents like PyBOP have acquired broad applications in peptide synthesis. They are very reactive even at low temperatures, and a great advantage of phosphonium salts over aminium/uronium salts is that the phosphonium does not react with the amino function of the incoming moiety, and therefore the phosphonium does not terminate the peptide chain. This is relevant in cyclization when both reactants are in an equimolar relationship and an excess of the coupling reagent could react with the amino component [17].

Nonetheless, the use of a potent coupling reagent like PyBOP in combination with some additives like HOBt could yield poor cyclization [15]. But the addition of Li salts in an organic solvent may be useful to enhance the cyclization and the coupling reaction in general [15,18,19], and the use of low temperatures during the reaction could favor amide bond formation in hindered conformations [17,20]. To the best of our knowledge, this is the first study that combines the effect of lithium chloride addition and the low reaction temperatures in the cyclization of two different sequences; once the head-to-tail bonding is accomplished, the cyclic sequence c(RGDfK) is obtained.

The use of two different linear precursors (RGDfK and DfKRG, sequences 1 and 2) permitted us to compare the effect of the amino acids on amide bond formation. By combining different cyclization conditions, this is a comprehensive study of c(RGDfK) synthesis, reaching a considerable improvement in the final product, as shown by HPLC and mass spectrometry analysis.

2. MATERIALS AND METHODS

Chlorotrityl chloride resin, Fmoc-amino acids, N,N'-diisopropylcarbodiimide (DIC), Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), and OxymaPure were obtained from Iris Biotech GmbH (Marktredwitz, Germany).

Fmoc-D-Phe-OH, piperidine, N,N'-dimethylformamide (DMF), dichloromethane (DCM), piperidine, N-ethyldiisopropylamine (DIPEA), acetonitrile (ACN), methanol, ethanol, diethyl ether, trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were obtained from Merck KGaA (Darmstadt, Germany).

Two different linear sequences were synthesized in order so that, once the head-to-tail cyclization is accomplished, the cyclic sequence c(RGDfK) is obtained. These sequences were RGDfK (sequence 1) and DfKRG (sequence 2).

Both sequences were synthesized using standard Fmoc chemistry on chlorotrityl chloride resin (1 meq/g) according to Guzmán et al [21]. Briefly, two coupling mixtures were employed in order to favor reaction completion: (1) a single coupling that consists of HBTU/OxymaPure® activating mix of amino acids, *in situ* neutralization was accomplished with DIPEA, and this mix was used to complete 3 h of total coupling time; and (2) a double coupling that consists of DIC/OxymaPure® activator, with a coupling time of 1 h; both mixes were prepared using DMF as solvent. Coupling monitoring was performed by the ninhydrin test. Fmoc protective group removal was carried out using a solution of 20% piperidine in DMF.

Amide bond formation is not chemoselective, and in-solution cyclization requires side-chain-protected peptides. Thus, peptide cleavage without the removal of side-chain protective groups was accomplished using a solution of

**Corresponding author email: mkogan@ciq.uchile.cl, eyleen.araya@unab.cl*

1% trifluoroacetic acid (TFA) in DCM, followed by water extraction of the acid, where the peptide remains in the organic phase, which was evaporated at 30ºC. After that, a 0.5 mM (high dilution) solution of the linear chain-protected peptide was prepared using a mixture of 30:70 DMF/DCM and three different head-to-tail cyclization conditions were tested to determine the best way to obtain the c(RGDfK) peptide.

Cyclization protocol A: 2 equivalents of PyBOP/HOBt and 1% of DIPEA were used as the C-terminus activating mix, and a chaotropic salt like LiCl (final concentration of 0.168 M) was added. The reaction was performed overnight under agitation at room temperature.

Cyclization protocol B: 2 equivalents of PyBOP/HOBt and 1% of DIPEA were used as the C-terminus activating mix, and the reaction took place overnight under agitation at 4ºC.

Cyclization protocol C: The same activating mix as protocol 1 was used, but the reaction was done overnight under agitation at 4ºC. In this protocol, we could see the effects of both low temperature and the chaotropic salt.

Finally, a liquid-liquid extraction was carried out using a 5% $NaHCO_3$ solution and then saturated solutions of NH_4Cl and NaCl were subsequently used [22]. The cyclic side-chain-protected peptide remained in the organic phase, which was evaporated at 30ºC. The removal of the side-chain protective groups was done with a cocktail of TFA/TIS/Water of 95:2.5:2.5, and the resulting peptide was precipitated, washed with cold diethyl ether, and lyophilized. The molecular mass and purity of peptides were confirmed by electrospray ionization mass spectrometry (UPLC Aquity/Xevo G2XS QToF) and RP-HPLC (JASCO Corp., Tokyo, Japan), respectively.

3. RESULTS

We propose three different cyclization conditions for two different linear precursor sequences that, after the head-to-tail amide bond formation, yield the same cyclic peptide, c(RGDfK). In this way, we had six different products to be compared in terms of identity by mass spectrometry, which indicated if the cyclization was achieved and the side products obtained, and purity by HPLC, which revealed how abundant the different species were. Based on the results, three species were obtained: the desired cyclic monomer and two very common side products in the head-to-tail cyclization reaction: the linear peptide (and its oligomers) and the cyclic dimer. Table 1 shows the obtained products for both sequences (1 and 2) cyclized using protocols A (PyBOP, HOBt, 1% DIPEA, and 0.168M LiCl at room temperature), B (PyBOP, HOBt, and 1% DIPEA at 4ºC), and C (PyBOP, HOBt, 1% DIPEA, and 0.168M LiCl at 4ºC). Chromatograms and mass spectra for each product are in Supporting information.

Table 1. Products obtained for sequences 1 and 2 cyclization using protocols A, B, and C. Their retention time observed by reverse phase chromatography and main peaks by ESI-MS are indicated.

Protocol	Conditions	Sequence	Products	HPLC t_R (min)	MS ions (m/z)
A	PyBOP HOBt 1%DIPEA 0.168 M LiCl Room T	1	Linear peptide	4.6	M+1= 622 M+2= 311
		2	Linear peptide	4.6	M+1= 622 M+2= 311
B	PyBOP HOBt 1%DIPEA 4ºC	1	Linear peptide	4.6	M+1= 622 M+2= 311
			Cyclic peptide	4.9	M+1= 604 M+2= 302
		2	Linear peptide	4.6	M+1= 622 M+2= 311
			Cyclic peptide	4.9	M+1= 604 M+2= 302
			Cyclic Dimer	5.1	M+2= 604 M+4= 302
C	PyBOP HOBt 1%DIPEA 0.168 M LiCl 4ºC	1	Cyclic peptide	4.9	M+1= 604 M+2= 302
			Cyclic Dimer	5.1	M+2= 604 M+3= 403 M+4= 302
		2	Cyclic Dimer	5.1	M+2= 604 M+3= 403 M+4= 302

Products obtained following Protocol A showed that the single addition of LiCl in the presence of HOBt did not favor the cyclization of the peptide, which was hindered due to the small size of the sequence. Thus, mainly the linear peptide and other impurities like oligomers were obtained, as observed by mass spectrometry and reverse phase chromatography (Figure 1). The chromatogram showed a predominant peak at 4.6 min, corresponding to the linear sequence and other impurities that could be oligomers. By mass spectrometry, two main peaks were observed at 622 and 311 m/z, corresponding to the ions of the linear peptide and oligomers.

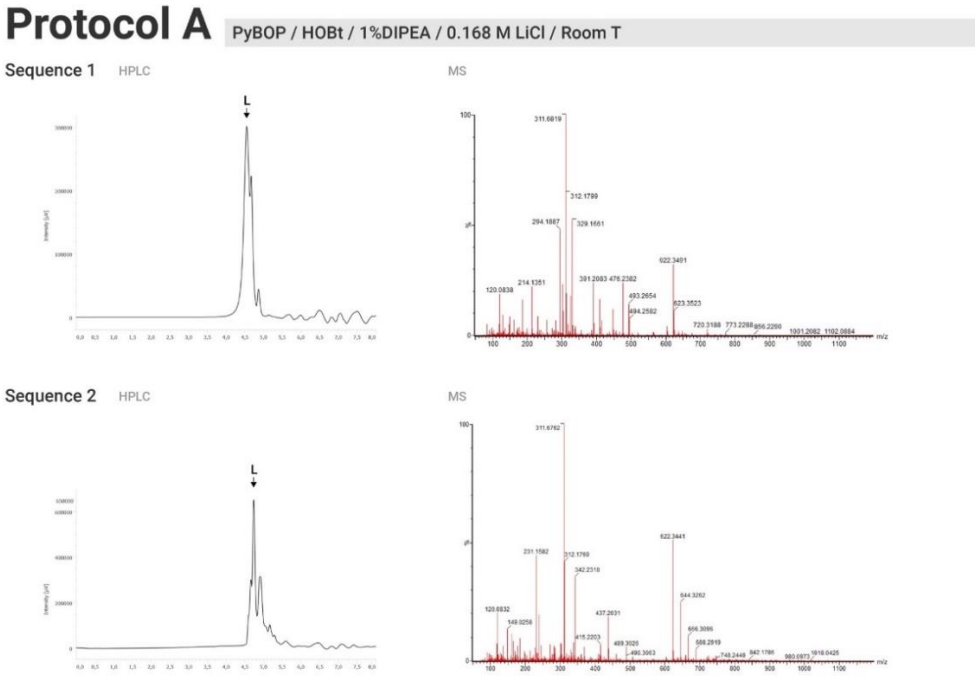

Figure 1: Protocol A product characterization by HPLC and ESI-MS

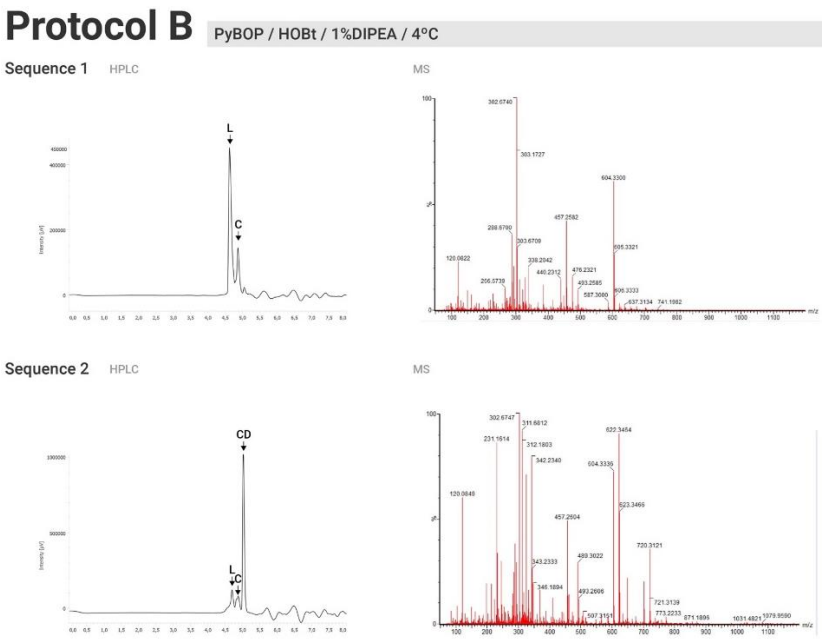

Figure 2: Protocol B product characterization by HPLC and ESI-MS

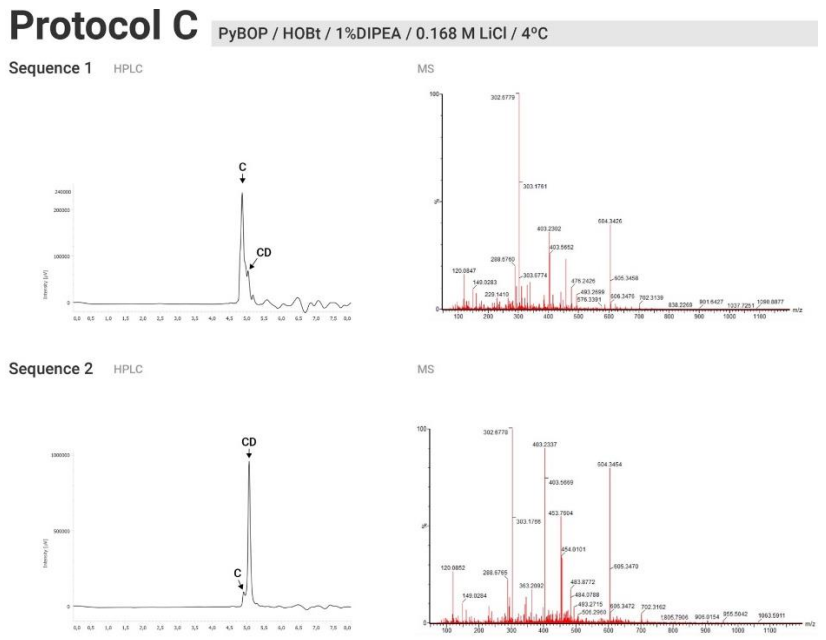

Figure 3: Protocol C product characterization by HPLC and ESI-MS

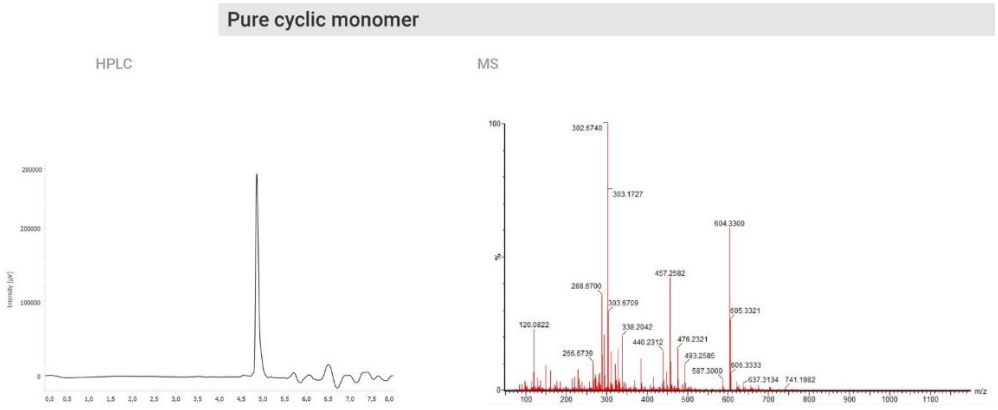

Figure 4: Pure cyclic monomer characterization by HPLC and ESI-MS

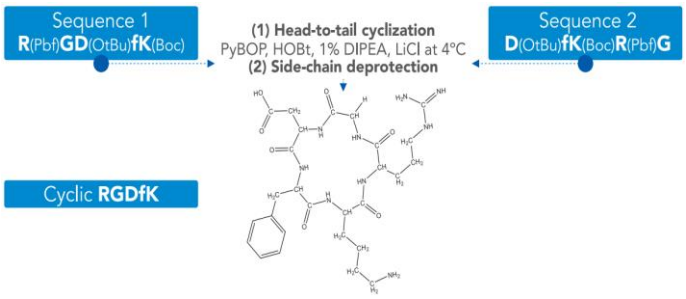

Figure 5. Linear sequences and cyclic product.

In order to try restricting the degrees of freedom of the molecules and promoting torsional conformations that would allow cyclization, the reaction temperature was lowered to 4°C. In doing so, a partial cyclization of the peptide was finally achieved, but the linear peptide was still the main product in the case of sequence 1, and the formation of the cyclic dimer was observed for sequence 2. This was corroborated by the retention times in reverse phase chromatography, which were 4.6 min, 4.9 min, and 5.1 min for the linear, cyclic, and cyclic dimer peptides, respectively. According to the HPLC results, mass spectrometry showed two major peaks for both sequences, corresponding to the M+1 and M+2 ions of the linear peptides (311 and 622 m/z), and two additional peaks that were common for both cyclic species, monomer and dimer (604 and 302 m/z) (Figure 2).

In order to promote the yield for the cyclization reaction, the chaotropic agent LiCl was incorporated in addition to lowering the reaction temperature. Under these conditions, the formation of the cyclic peptide and cyclic dimer was observed, as denoted by the presence of a signal at 403 m/z in the mass spectrum for both sequences, corresponding to M+3 in the case of the cyclic dimer, in addition to the peaks at 302 and 604 m/z that were common to the cyclic monomer and dimer. However, in sequence 1, a predominance of the cyclic monomeric species was observed, while in sequence 2, the formation of the cyclic dimer prevailed (Figure 3). Finally, purification of the desired monomeric cyclic peptide was achieved from the cyclized sequence 1 by reverse phase chromatography, with a retention time of 4.9 min, while the cyclic dimer showed a retention time of 5.1 min. (Figure 4).

4. DISCUSION

Regarding the effect of the sequence used for cyclization, the obtained products were almost the same for each protocol, with slight differences in the relative abundance of the species depending on the sequence. Sequence 1 implied amide bond formation between the sterically hindered residues Arg(Pbf) and Lys(Boc), with Pbf being the larger protective group typically used in Fmoc synthesis. On the other hand, sequence 2 implied amide bond formation between Asp(OtBu) and Gly, whose side chain consisted of just one hydrogen atom, having plenty of space and rotational freedom. However, it must be noted that these less sterically hindered residues could facilitate the reaction for both inter- and intramolecular bond formation, not necessarily favoring cyclization. Indeed, our best result was obtained using sequence 1 as a linear precursor, which involved amide bond formation between Arg(Pbf) and Lys(Boc), even when these protective groups were larger than those involved in the cyclization of sequence 2. Instead, sequence 2 favored cyclic dimer formation.

Based on our results, cyclization reaction conditions are more determinant than the linear precursor. This result was expected since the synthesis of cyclic peptides can be difficult to achieve because a defined pre-cyclization conformation must be formed -an entropically unfavorable process– before the desired intramolecular reaction can occur [23]. Therefore, we had to set reaction conditions that favored the desired conformation. Lithium salts are believed to influence the conformation of the peptide and break hydrogen bonds involving the carbonyl oxygens. Therefore, in the presence of Li, unnatural conformations could be generated, and the N- and C-termini of the peptide could end up near each other, allowing cyclization [18]. Furthermore, a global analysis of peptide cyclization efficiency made by Thakkar et al. indicated that the poorly cyclizing peptides are generally rich in Lys(Boc) and Arg(Pbf), which apparently form bidentate hydrogen bonds with the C-terminal carboxyl group and reduce their reactivity. Thus, they found that the addition of LiCl can break the hydrogen bonding network and improve cyclization efficiency [15].

In our case, the best result was observed when the reaction was accomplished at a lower temperature in the presence of lithium chloride, where both conditions are essential for obtaining the majority of the cyclic monomer peptide (Figure 5). LiCl could have influenced the conformation of the peptide and, at the same time, acted as a chaotropic agent that can break the hydrogen bonding network. Finally, low temperatures seem to restrict the degree of freedom of the backbone, giving the amide bond the necessary time for its proper formation when the peptide has the appropriate conformation. To the best of our knowledge, this is the first report of the use of LiCl in combination with a low reaction temperature to successfully improve the head-to-tail cyclization efficiency of a short peptide.

In our case, sequence 1, which implied amide bond formation between the sterically hindered residues Arg(Pbf) and Lys (Boc), favored obtaining the desired cyclic monomer, allowing us to obtain the pure desired product after reverse phase chromatography.

CONCLUSION

Using mild conditions as an efficient coupling reagent like PyBOP, in the presence of HOBt, DIPEA, and LiCl at 4ºC, we could improve the head-to-tail cyclization of linear precursor sequences, obtaining a product that could be easily purified to acquire the cyclic peptide c(RGDfK), widely used in biomedicine for diagnostic imaging and targeted therapy for some types of cancer and in tissue engineering. Furthermore, these conditions could be applied for the cyclization process of other small peptides which can be difficult due to their size and sequence.

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