Synthesis of dicarba-cyclooctapeptide Somatostatin analogs by conventional and MW-assisted RCM: a study about the impact of the configuration at C^{α} of selected amino acids

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Abstract

This work describes the synthesis of thirteen cyclooctapeptides dicarba-analogues of Somatostatin, containing L- or D-allylglycine (Agl) residues at the termini of the peptide chain, through on resin Ring Closing Metathesis (RCM) of the linear octapeptides. We investigated the influence of the stereochemistry of some strategic amino acids on the propensity to give the cyclic compounds in mild conditions (refluxing DCM). Systematic individual replacement of $Phe^{6,7,11}$ residues with the corresponding enantiomers, strongly favoured the ring closure by conventional heating. The yield of the cyclic products was strictly correlated to the position of this amino acid on the peptide chain. In particular substitution of Phe^{6} by Tyr in peptides which did not give the cyclic compounds, allowed the ring formation. The effect of the phenolic -OH function of Tyr side chain on the proximity of the terminal Agl residue was studied by NMR techniques. All the linear precursors gave cyclic somatostatin dicarba-analogues, in good to high yields and in short reaction times, by microwave-assisted RCM, performed with the 2^{nd} generation Grubbs catalyst. The unsaturated dicarba-tether resulted in a mixture of *E* and *Z* stereoisomers in a variable ratio, depending on the sequence and the cyclization method. The *E* isomer was largely the most abundant in all but one the described product.

Keywords: Cyclooctapeptides, dicarba-analogues of Somatostatin, Ring Closing Metathesis (RCM), Micro-Wave assisted peptide synthesis, Microwaves-driven RCM.

1. Introduction

Cyclic peptide chemistry and biology is an emerging multidisciplinary approach to medicinal chemistry and molecular biology. Native cyclic peptides are present in animals, plants and bacteria and were found in several classes of natural compounds like hormones, antibiotics, and toxins. In

medicinal chemistry, synthetic cyclopeptides showed many advantages over their linear counterparts: increased metabolic stability, better bioavailability and receptor selectivity, higher resistance to enzymatic degradation. Furthermore, due to their restricted conformational freedom, the cyclopeptides are valuable tools in the study of receptor-ligand interactions. Cyclisation methods cover head-to-tail linkage, side-chain and backbone cyclisation with all possible connections.[1] A frequent structural motif found in side-chain-to-side-chain bridged natural peptides is the cystine (S-S) moiety which forms inter- and intra- molecular tethers of fundamental importance for the structure of biomolecules and hence for their activity.[1,2,3,4] Among the natural hormones, somatostatin-14 (SRIF-14), a S-S tethered cyclotetradecapeptide, was discovered to exert anti-secretory and anti-proliferative activity through the binding to five receptor subtypes, $sstr_{1\text{-}5}$.[5] In many cases, especially in oligopeptides, intramolecular disulphide bridges act as a constraint maintaining active secondary structures like helices or turns. However, the S-S bridge is highly sensitive to exo- and endogenous attack of oxidant or reducing agents (i. e. disulfide reductase enzymes). Therefore, the native cystine tether has been substituted, in time, by other linkages like thioether and ester groups, peptide bond, *N*-backbone linkages, or triazolic rings. In the last decade, the substitution of the disulfide group by isosteric, non-reducible, dicarba-bonds has grown of importance after the discovery of the Ru-catalysts mediated Ring-Closing Metathesis (RCM) of alkylidene substrates, proposed by Grubbs and other researchers.[6,7,8,9] Nowadays, the researchers can handle efficient Ru-catalysts representing useful and flexible tools for building a variety of dicarba-bridges in bioactive peptides, intended to stabilize the molecules and/or to rigidify their active conformation.[10,11,12,13,14] Recently, we applied the RCM on hexapeptides containing L-allylglycine residues and, by using 1st or 2nd generation Grubbs catalysts with conventional heating, we successfully installed an intramolecular C=C bridge in the cyclic peptides. Subsequent coupling of the terminal NH_2 with D-Phe afforded a series of robust octreotide (Sandostatin) mimics, containing $C^{\alpha}_{(i)} \rightarrow C^{\alpha}_{(i+5)}$ [m = 4, (*E*)/(*Z*) tethers] especially suitable for ^{99m}Tc or ^{188}Re labeling methods which require reducing media.[15] Some of them, mainly *Z* stereoisomers, showed high affinity and selectivity for sst2/sst5 receptor subtypes.[16,17,18] Studies

performed by Reubi *et al.*[19] on the structure-activity relationship of Somatostatin-14 (SRIF-14) mimic ligands, paid attention to the role of the ring size, and discovered that some disulfide bridged cyclo-octapeptides, rich in aromatic and hetero-aromatics side-chains, showed antagonist activity towards SRIF receptors, making these molecules very interesting for the clinical use as radio-labeled drugs.[20] Two well performing examples are depicted in Figure 1.

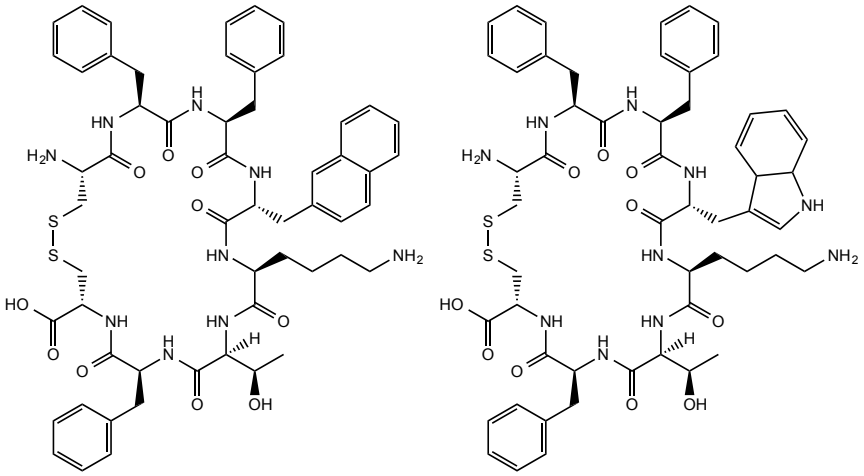


Figure 1. ODN-8 and ODT-8 structures.

Material and methods

General Procedures. Fmoc protected amino acids were purchased from Novabiochem (Laufelfingen, Switzerland) and from Iris Biotech (Marktredwitz, Germany), Rink Amide AM resin was purchased from Iris Biotech. Second generation Grubbs catalyst was obtained from Aldrich (St. Louis, MO, USA). Fmoc-Agl-OH was purchased from Polypeptide Laboratories (Strasbourg, France). Peptide grade DMF was from Scharlau (Barcelona, Spain). Oxyma Pure was purchased from CEM (Matthews, NC, USA) All the other solvents and reagents used for SPPS were of analytical quality and used without further purification. The syntheses of the linear peptides were performed on a CEM Liberty Blue™ automated peptide synthesizer, equipped with a CEM single-mode MW reactor (Matthews, NC, USA). The desired cyclic peptides were obtained by using a CEM

Discovery® microwave reactor (Matthews, NC, USA). Analytical RP-HPLCs were performed on a Thermo Finnigan (Waltham, MA, USA) Surveyor instrument equipped with an UV detector and a Thermo Finnigan LTQ Advantage mass spectrometer.

Micro-Wave assisted synthesis of the linear peptides I-XIII.

The linear peptides **I-XIII** were synthesized on a CEM Liberty Blue™ automated peptide synthesizer, by MW-assisted strategies (Scheme 1) and using the conventional Fmoc/*t*Bu protection scheme.

The reactions were performed in a Teflon vessel and mixed by nitrogen bubbling. Reaction temperature was measured by an internal fiber-optic sensor. The general MW-SPPS protocol was structured as follow: before the coupling of the first amino acid the resin was swelled in DMF for 2 min. Afterwards, two deprotection steps were performed at 75 °C using 155 W for 15 sec for the first and 30 W for 30 sec for the second one. The coupling steps were performed at 90 °C, using 170 W for 15 sec and 30W for 110 sec for all amino acids. After each coupling cycle, the resin was washed tree times with DMF.

The syntheses were performed on Rink-amide AM resin (0.5 mmol/g, 200 mg). During the general coupling cycle, the *N*-terminal Fmoc-protecting group was removed with a solution of 20% piperidine in DMF. Fresh stock solutions of the Fmoc-protected amino acids (0.2 M in DMF) and activators (DIC 0.5 M in DMF, and Oxyma Pure 1.0 M in DMF) were prepared in separated bottles and used as reagents during the SPPS. In particular, for the 0.1 millimolar scale, the coupling cycles were performed using 2.5 mL of Fmoc-protected amino acids solutions, 1.0 mL of DIC solution and 0.5 mL of Oxyma Pure solution.

The synthesis of the linear peptides was monitored by microscale cleavage with TFA/TIS/H_2O/phenol (94:2:2:2) for 3 hours. The resin was filtered off, the solutions were concentrated, the peptide precipitated from Et_2O, centrifuged, dissolved in water and lyophilized. RP-HPLC analysis of the crude products revealed the presence of the linear peptides in approximately 90% purity, without traces of isomers due to amino acids racemization.

RCM by conventional heating (method A).

50 mg of the on resin Fmoc-protected linear peptides **I-XIII** were swollen for 1 h in anhydrous DCM. The vessels were heated, under nitrogen atmosphere, with a conventional oil bath at 50 ºC and the 2nd generation Grubbs catalyst (0.5 mol equiv. calculated on the basis of 0.5 mmol/g of peptide) in anhydrous DCM was added. The suspension was then stirred for 48 hours at reflux temperature, under nitrogen atmosphere. The resin aliquots containing the cyclic-peptides were washed with DMF (×3), MeOH (×3) and DCM (×3), treated with 20% piperidine in DMF (2×10 min) and washed with DMF (×3) and DCM (×3). The cleavage of the crude cyclopeptides was performed using the cleavage mixture TFA/TIS/H_2O/phenol (94:2:2:2, 3h) as previously described. The solutions were concentrated and treated with Et_2O giving the respective crude cyclic dicarba-analogues reported in Scheme 2. The suspensions were centrifuged, dissolved in water and lyophilized. Analytical RP-HPLC and ESI-MS analysis of the crude compounds showed two chromatographic peaks with the same molecular weight, identified as *E* and *Z* isomers on the basis of the ^3J coupling constants of the olefinic protons.

For the NMR configurational analysis, all compounds were pre-purified by SPE, purified by semipreparative RP-HPLC and characterized by ESI-MS. The HPLC purity of each compound studied was >98%, and the isolated compounds showed unique *E* or *Z* configuration, confirmed by NMR analysis. No oligomeric by-products were observed.

Microwaves-driven RCM (method B).

An aliquot of the on-resin Fmoc-protected linear peptides **I-XIII** (50 mg) was placed in a glass vessel (CEM), sealed with a cap and 2 mL of anhydrous DCM was added. The resin was swelled under gentle stirred for 1 hour. The appropriate amount of catalyst (10% mol respect to the loading of the resin) was added and the vessel was introduced in a CEM Discovery® microwave reactor. The vessel was heated at 100 ºC for 3 min with maximum MW power of 300 Watt. Once the set temperature was reached, an air-cooling was applied until the temperature was 95 °C. Each cycle was repeated

for 300 times (for an approx. total time of about 1 h). At the end of the 300 cycles, a fresh solution of Grubbs catalyst in anhydrous DCM was added and another batch of 300 cycles was applied. During the reaction the pressure was monitored and did not exceed 95 psi, while the temperature was monitored by fiber-optic probes at the base of the reaction vessel. At the end of the reaction the solution was cooled rapidly by compressed air, and then the resin was washed with DMF (×3), MeOH (×3) and DCM (×3). After each reaction the instrument was cooled for 10 minutes with compressed air, in order to start the next experiment at the same temperature of the first one. In this way the ramp to reach the experimental temperature and the microwaves applied were approximately the same for all experiments. The on-resin peptides were deprotected from the Fmoc group, cleaved, analysed and purified as described in method A. The corresponding cyclic dicarba-peptides obtained with the two methods showed the same R_t and the same molecular weights.

Chromatography. Semipreparative HPLC was carried out by a Phenomenex Jupiter column C_{18} 180 Å (250 mm × 10 mm, 5 μm,) (Torrance, CA, USA) using a Beckman Gold Nouveau (Brea, CA, USA) instrument equipped with a diode array detector. Analytical HPLC was run on a Phenomenex Jupiter column C_{18} 300 Å (250 mm × 4.6 mm, 5 μm). The solvent systems used for gradients were A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). The flow rates were 1 mL/min for analytical HPLC and 4 mL/min for semipreparative HPLC, with the linear gradients indicated when necessary. HPLC-ESI-MS analyses were performed by a Phenomenex Aqua C_{18} column (250 mm × 4.6 mm, 5 μm) on a Thermo Finnigan Surveyor HPLC system (San Josè, CA, USA) coupled to the ESI-MS, using the same solvent system.

NMR Spectroscopy. The samples for NMR spectroscopy were prepared by dissolving the appropriate amount of peptide in 0.6 mL of DMSO-d_6 to obtain a concentration 1-2 mM of peptides. NMR experiments were recorded on Varian Inova-Unity 500 and 700 MHz. Agl residue spin system

resonances were assigned by ^{1}H-^{1}H COSY spectra. $^{3}J_{CH=CH}$ were measured by 1D ^{1}H NMR spectra after selective homonuclear decoupling of the H_{β}'s proton signals when necessary.

For conformational studies on peptides **VIII** and **XII**, DQF-COSY,[21,22] TOCSY,[23] and NOESY[24] spectra were recorded in the phase-sensitive mode using the method from States.[25] Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run with mixing times in the range of 200-300 ms. The qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra, were obtained using the interactive program package XEASY.[26] $^{3}J_{HN-H\alpha}$ coupling constants were obtained from 1D ^{1}H NMR and 2D DQF-COSY spectra. ^{1}H NMR chemical shift assignments were effectively achieved according to the Wüthrich procedure[27] (Tables S1-S2, Supporting Information).

Results

We firstly attempted to synthesize two dicarba-analogues reported in Figure 1, with Agl^{3} and Agl^{14} replacing the corresponding cysteines and containing unsaturated $C^{\alpha}_{(i)} \rightarrow C^{\alpha}_{(i+7)}$ cycles, [m = 4, (*E*)/(*Z*) tethers], in order to study the influence of the CH=CH bridge on the pharmacological behavior and the affinity pattern towards somatostatin receptors $sst_{1\text{-}5}$. In the past, large rings spanning $i \rightarrow i+7$ amino acid residues and tethered by all-hydrocarbon chains were built by Verdine *et al*. to stabilize the helicity in α-helix conformations.[28,29] The authors noted that the RCM performed by 2nd generation Grubbs catalysts, was successful with relatively high number (m) of the tether C atoms (11-12). Moreover, the formation of large rings in polypeptides chains without helical propensity was known to be disfavored.[30] Indeed, it was not surprising that RCM with 2nd generation Grubbs catalyst, attempted on the linear octapeptides **I** and **VIII** (Table 1 and Table 2) by oil-bath heating (method A), afforded only the starting material, differently from the octreotide dicarba-analogues generally

accessible with this conventional heating method. Conversely, MW-assisted RCM of **I** and **VIII** (method B) was completely successful.

Table 1. *Z/E* ratio of dicarbacyclopeptides (**I′-VII′**) after RCM by conventional heating (A) and MW-assisted cyclization (B).

Compound	Sequence	Cyclization method	Linear peptide (%)	Cyclic peptide *Z*/*E*(%)
I	Fmoc-NH-AglAgl-Phe-Phe-D-Trp-Lys-Thr-Phe-AglAgl-$CONH_2$	A	100	-
		B	3	0/97
II	Fmoc-NH-Agl-D-Phe-Phe-D-Trp-Lys-Thr-Phe-Agl-$CONH_2$	A	69	8/23
		B	-	24/76
III	Fmoc-NH-Agl-Phe-D-Phe-D-Trp-Lys-Thr-Phe-Agl-$CONH_2$	A	5	41/54
		B	-	20/80
IV	Fmoc-NH-Agl-Phe-Phe-D-Trp-Lys-Thr-D-Phe-Agl-$CONH_2$	A	29	12/59
		B	-	19/81
V	Fmoc-NH-D-Agl-Phe-Phe-D-Trp-Lys-Thr-Phe-Agl-$CONH_2$	A	100	-
		B	4	15/81
VI	Fmoc-NH-Agl-Phe-Phe-D-Trp-Lys-Thr-Phe-D-Agl-$CONH_2$	A	81	0/19
		B	-	0/100
VII	Fmoc-NH-Agl-D-Phe-Phe-D-Trp-Lys-Thr-D-Phe-Agl-$CONH_2$	A	-	32/68
		B	-	19/81

Method A: 2nd generation Grubbs catalyst (0.5 mol eq), anhydrous DCM, 50 °C, reflux 48h.

Method B: 2nd generation Grubbs catalyst (10% mol), anhydrous DCM, MW cycles (100 °C, 300W, 3 min, cooling). Cleavage: TFA/TIS/H_2O/phenol (94:2:2:2, 3 h).

This finding prompted us to study the behavior of a series of correlated linear octapeptides obtained by changing nature and/or stereochemistry of some amino acids, in order to gain information on the key positions which controlled the viability of the RCM by methods A and B. The variable *E/Z* ratio at the C=C bridge formed by these two methods was also addressed.

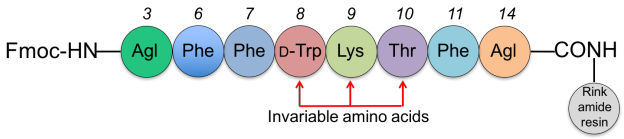
Table 2. *Z/E* ratio of dicarbacyclopeptides (**VIII'-XIII'**) after RCM by conventional heating (A) and MW-assisted cyclization (B).

Compound	Sequence	Cyclization method	Linear peptide (%)	Cyclic peptide Z/E(%)
VIII	Fmoc-NH-Agl-Phe-Phe-D-2-Nal-Lys-Thr-Phe-Agl-$CONH_2$	A	100	-
		B	5	10/85
IX	Fmoc-NH-D-Agl-Phe-Phe-D-2-Nal-Lys-Thr-Phe-Agl-$CONH_2$	A	100	-
		B	-	17/83
X	Fmoc-NH-D-Agl-Phe-Tyr-D-2-Nal-Lys-Thr-Phe-Agl-$CONH_2$	A	100	-
		B	9	34/57
XI	Fmoc-NH-Agl-Phe-Phe-D-2-Nal-Lys-Thr-Phe-D-Agl-$CONH_2$	A	45	15/40
		B	-	20/80
XII	Fmoc-NH-Agl-Phe-Tyr-D-2-Nal-Lys-Thr-Phe-Agl-$CONH_2$	A	9	74/17
		B	0	77/23
XIII	Fmoc-NH-Agl-Phe-Phe-D-2-Nal-Lys-Thr-D-Phe-Agl-$CONH_2$	A	10	24/66
		B	-	33/67

Method A: 2nd generation Grubbs catalyst (0.5 mol eq), anhydrous DCM, 50 °C, reflux 48h.

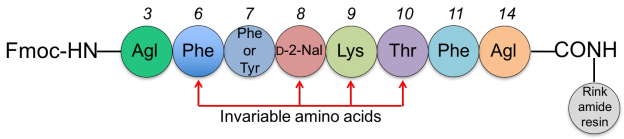
Method B: 2nd generation Grubbs catalyst (10% mol), anhydrous DCM, MW cycles (100 °C, 300W, 3 min, cooling). Cleavage: TFA/TIS/H_2O/phenol (94:2:2:2, 3 h).

Scheme 1. Resin supported protected linear peptides **I-XIII** as starting material for RCM reaction.



Variation (according to SRIF numbering)

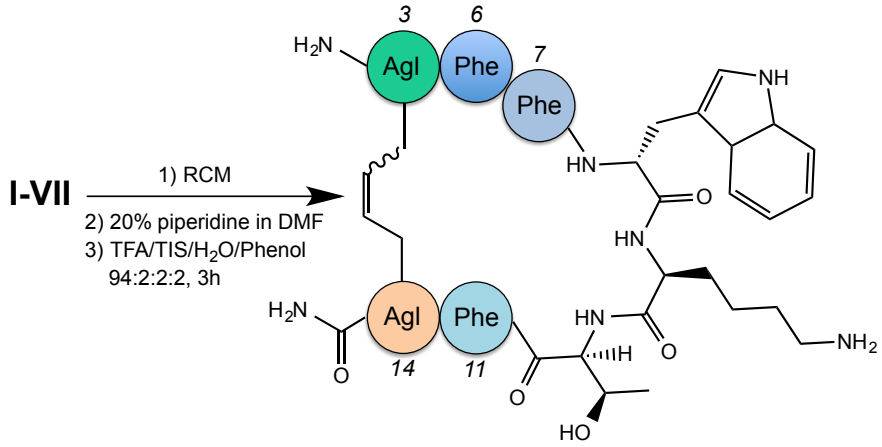
Entry	3	6	7	11	14
I					
II		D-Phe			
III			D-Phe		
IV				D-Phe	
V	D-Agl				
VI					D-Agl
VII		D-Phe		D-Phe	

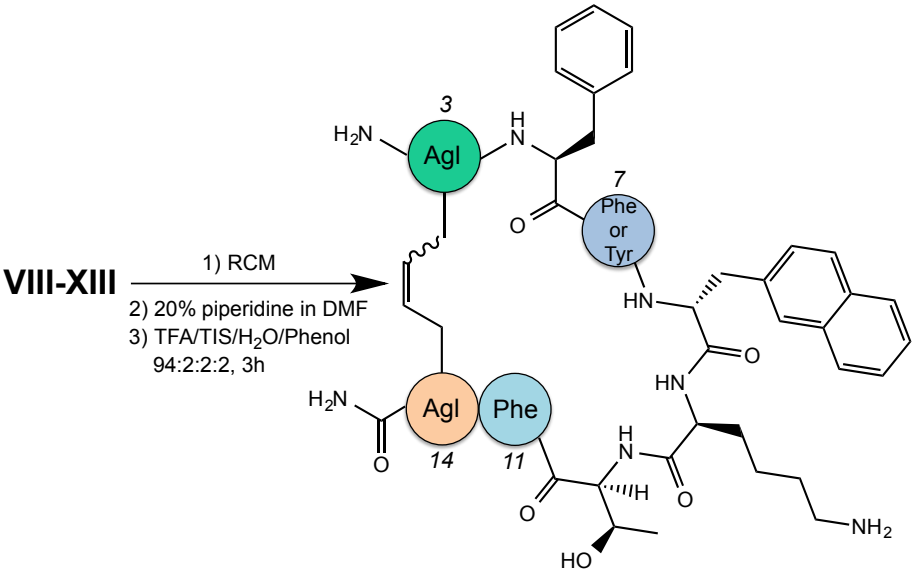


Variation (according to SRIF numbering)

Entry	3	7	11	14
VIII				
IX	D-Agl			
X	D-Agl	Tyr		
XI				D-Agl
XII		Tyr		
XIII			D-Phe	

Scheme 2. RCM reaction for the formation of the dicarba analogues of octreotide **I′-XIII′**.





Mimicking the so called ODT-8 and ODN-8 described in the Reubi's paper, **Errore. Il segnalibro non è definito.** two deprotected linear peptides were initially prepared, des-AA^{1,2,4,5,12,13}[Agl^{3},D-Trp^{8},Agl^{14}]-SS (**I**) and des-AA^{1,2,4,5,12,13}[Agl^{3}, D-2-Nal^{8},Agl^{14}]-SS (**VIII)**, Attempts to obtain the cyclic analogues **I′** and **VIII′** by method A afforded only the starting compounds. On the contrary, MW supported RCM appeared a very efficient method giving the cyclic dicarba-peptides in high chromatographic yields, 97% of **I′** and 95% of **VIII′,** (Table 1 and Table 2). HPLC-MS analysis of the crude compounds did not reveal the presence of oligomers nor of double-bond isomerization. Changing Agl^{3} into D-Agl^{3} for both the sequences (compounds **V** and **IX**) did not prevent the failure of the method A, resulting in 100% of the starting material. On the contrary, method B afforded 96% and 100% of the corresponding cyclopeptides, **V′** and **IX′,** respectively (Table 1 and Table 2). Looking at the D-2-Nal^{8} containing open chain sequences (Table 2), we observed that the analogue **XI**, with D-Agl^{14} instead of Agl^{14}, gave 55% of the cyclic analogue **XI′** by conventional heating while the cognate compound **VI** of Table 1 also afforded the corresponding cyclic peptide **VI′** in the same conditions**,** even if the yield was lower (19%). Unexpectedly, substitution of Phe^{7} with Tyr^{7} in this series (compound **XII**, Table 2), gave high yield of the corresponding cyclic peptide with method A as well as with method B (91% and 96%, respectively). However, if D-Agl^{3} and Tyr^{7} were inserted at the same time (**X**), the cyclic analogue was obtained only by the MW-driven RCM.

Notably, changing in turn the stereochemistry of the aromatic amino acids Phe^{6}, Phe^{7}, and Phe^{11} in the ODT-8 analogues **II**, **III** and **IV** had a significant impact on the yields of the cyclic counterparts obtained with the conventional heating RCM. In fact, **II** gave 31%, **IV** 71% and **III** the 95% of the corresponding cyclic peptides (Table 1). By analogy, 90% of the compound **XIII** (D-Phe^{11}) was converted into the cyclic dicarba-analogue (Table 2). The simultaneous introduction of D-Phe^{6} and D-Phe^{11} in the sequence (compound **VII**, Table 1) even resulted in the complete conversion into the cyclic peptide with the method A as well as with the method B.

For each cyclic peptide, the HPLC chromatogram showed two peaks with the same MW, corresponding to the geometric isomers. The HPLC purity of each compound studied was >98%, and the isolated compounds showed unique *E* or *Z* configuration, confirmed by NMR analysis. $^{3}J_{CH=CH}$ were >15 Hz for all the major isomers, in agreement with an (*E*)-geometry of the double bond (*E*-isomer from 67% to 100%) except for compound **XII′** (see below). As an example, selectively decoupled spectrum of the olefinic protons of major isomer **IV′** is reported in Figure 2.

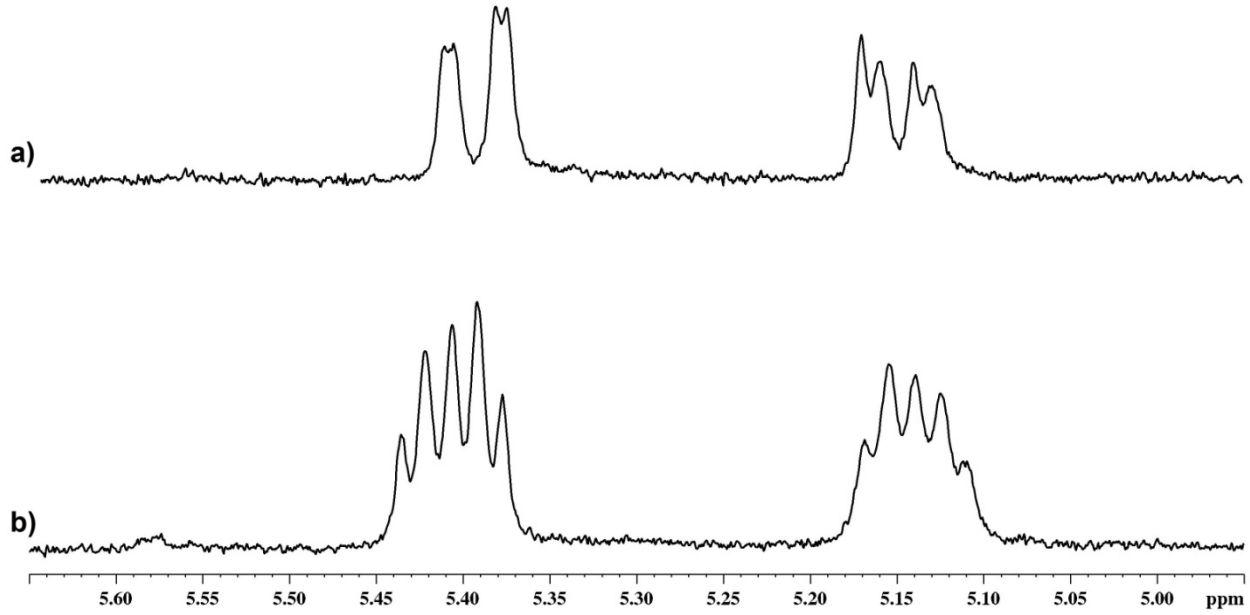


Figure 2. Olefinic protons signals in the ^{1}H NMR spectrum of **IV′** (b) and in the corresponding selectively decoupled spectrum (a).

The peptide **XII′** was purified, both the geometric isomers were isolated and analyzed trough ^{1}H NMR spectra. Unexpectedly, the NMR analysis revealed that in this case the major product was the *Z*-isomer instead of *E*-isomer. The $^{3}J_{CH=CH}$ was 15.5 Hz for the *E*-isomer, while the *Z*-isomer showed at 5.33 ppm a unique and overlapped signal for the olefinic protons (Figure 3).

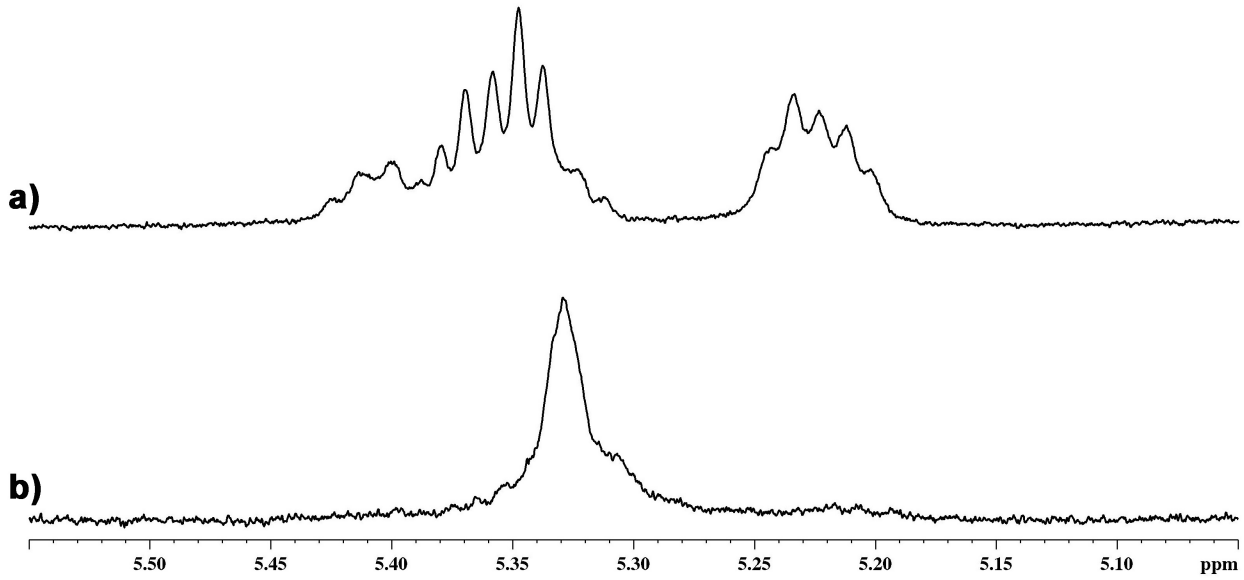


Figure 3. Olefinic protons signals in the ^{1}H NMR spectrum of **XII′** (upper panel, *E* isomer – lower panel, *Z* isomer).

Since the replacement of Phe^{7} with a Tyr residue in linear compounds **VIII** and **XII** changed both the reactivity and the configuration of the cyclization products, those compounds were more deeply analyzed by solution NMR. In particular, 2D DQF-COSY, TOCSY, and NOESY experiments were acquired for both compounds in $DMSO_{d6}$ solution. Complete assignment was achieved by well-established protocols (Tables S1-S2, Supporting Information).[27] NMR parameters of the two peptides were generally similar (Tables S1-S2) but exchangeable -OH signal of Thr^{10} showed a different behavior. In fact, in peptide **VIII**, Thr^{10} -OH resonance displayed vicinal coupling (doublet with ^{3}J=7.0 Hz) with H_{β} proton signal, as expected in a DMSO solution, while the corresponding resonance in peptide **XII** is a broad signal which doesn't show any scalar coupling. Considering dipolar couplings (Figure S1 and S2, Supporting Information), Thr^{10} -OH signal of peptide **VIII** was coupled with Lys^{9} amine protons resonance, while Thr^{10} -OH signal of peptide **XII** displayed dipolar couplings with the phenol OH resonance of Tyr^{7} and with NH_2 signals of terminal Agl^{3}.

Discussion

Microwave-assisted organic synthesis has gained considerable importance in the last two decades, enhancing speed and yield of an huge number of organic reactions.[31,32] Regardless of the large body of published works on microwave chemistry, the exact reasons why microwave heating enhance chemical processes are still not well clarified.[33] However, it is now common opinion that microwave energy acts by thermal effects on the solvent and not by electromagnetic field.[34,35] SPPS method was also greatly improved by this technique.[36,37] In particular, when the carbocyclic peptide formation from on-resin long peptide chains is difficult or impossible with conventional heating, probably owing to intramolecular aggregations and unfavourable secondary structures,[38,39] MW-supported RCM or insertion of turn inducing amino acids, like Pro, or both[39,40] often afforded the expected carba-rings. The open chain peptides **I-XIII** which we studied had significantly long peptide sequences (Table 1 and Table 2) and did not contain turn-inducing residues like Pro. However, the expected dicarba-tethers were always obtained by MW-assisted RCM, even without the addition of chaotropic salts.[30] Moreover, some of them underwent cyclisation also by conventional heating. Without conformational data on the linear sequences **I-XIII** in the reaction solvent we were not able to predict their ability to form turns favouring the proper orientation of the Agl termini for the formation of the ruthenacyclobutane intermediates.[41] Therefore, we undertake a try-and-see study on the relationship between the nature and stereochemistry of some amino acids of the chains and the propensity of the linear sequences to give the dicarba-cyclopeptides. This task was accomplished by selective changing the nature or, mainly, the stereochemistry of backbone selected amino acids. The substitution of D-Trp[8] with D-2-Nal, did not affect the ability of the linear peptides to give the corresponding cycles. The dicarba-analogues **I′** and **VIII′,** in fact, were obtained from the linear counterparts **I** and **VIII** (sequences of Table 1 and Table 2, respectively) only by the method B. Attempt to gain the proper orientation of the allylic side-chains, by the introduction of the enantiomer D-Agl[3] (compounds **V** and **IX**) did not work with the method A though this amino acid was located far from the steric hindrance of the Rink-amide resin and hence more accessible to the catalyst.[39]

Surprisingly, the replacement of Phe^7 with Tyr^7 in the sequence **XII** strongly supported the formation of the dicarba-tether, also with the conventional method. This might be due to hydrogen bonds formed by the -OH group of Tyr, which helped the appropriate orientation of the adjacent Agl side chains. In fact, the absence of the phenolic -OH in compound **VIII** or the entry of D-Agl^3 in the Tyr^7 containing **X** prevented the formation of the cycle unless the MW irradiation support was used (Table 2). To investigate the existence of such hydrogen bonds, linear peptide **XII** was studied by solution NMR in DMSO, together with compound **VIII** used for comparison. Dipolar couplings of the OH signal of Tyr^7 with NH_2 protons of Agl^3 as well with the hydroxyl group of Thr^{10} in the NOESY spectrum of peptide **XII** confirmed the spatial proximity of these groups. Differently, the OH signal of Thr^{10} showed inter-residual NOE only with Lys^9 ε-NH_2 protons signals. Hence, Tyr^7 phenol OH really promotes the cyclization reaction upon conventional heating by approaching N- and C-terminal sides of the peptide.

Differently from what it could be expected on the basis of the location of Agl^{14} near to the bulky resin linker, in both the series (Table 1 and Table 2), the stereochemical inversion of this amino acid residue (compounds **VI** and **XI**), afforded the corresponding cycles also without the support of the microwaves. Worth of note is the positive effect that the stepwise inversion of stereochemistry of the $Phe^{6,7,11}$ residues exerted on the propensity to give the cyclic peptides. This behaviour of the linear chains strongly supported the idea that the orientation of the phenylalanine side-chains should have a crucial impact on the pre-organization of the peptide backbone, as previously suggested for the synthesis of analogues of mahafacyclin.[42] In the cyclic peptide SRIF-14, aromatic rings interactions between Phe^6 and Phe^{11} were firstly proposed by Veber *et al.* and recently highlighted by NMR conformational data when Phe residues in native somatostatin were substituted with Msa side-chains.[43] Finally, the influence of the π-π stacking in our SRIF mimicking chains was substantiated by the fact that the **VII**, encompassing both D-Phe^6 and D-Phe^{11} which are located four residues away each other, favored so much the proper allylic side-chains orientation to give 100% of the cyclic dicarba-analogue **VII′** with the method A as well as with the method B. Nevertheless, the *Z/E* isomers

ratio at the level of the tether double bond in our cyclo-octapeptides, requires a brief comment. We isolated the *E* isomer as the most abundant one, or even the only species, doesn't matter what method has been used, except for compound **XII′** containing Tyr^7, where the *Z*-isomer was predominant (Table 2). At the variance, replacing Agl^3 with D-Agl in this sequence (compound **X**) hindered the cycle formation with the method A, but the MW cyclization afforded again *Z/E* isomers in a 34/57 ratio. In the absence of catalyst-induced stereoselective RCM,[44] the overall results seem in agreement with the thermodynamic control of the metathesis products. Moreover, it is also in accordance with the behavior of the 2nd generation Grubbs catalysts and with the large ring size of our compounds.[44,45,46,47,48] However, recently Riera *et al.* published the MW supported synthesis of the first dicarba-analogue of SRIF-14.[49] The authors claimed that in the RCM conditions very similar to those we used, the *Z*-isomer was by far the major species obtained despite the fact that the dicarba-SRIF-cycle is larger than our cyclo-octapeptides and contains the same aromatic side-chains pattern in positions 6-11 of the compounds **I-XIII** here described.

Conclusion.

The results of the RCM reaction on the thirteen linear octapeptides studied revealed that some sequences had more propensity to give the dicarba-cycles in mild conditions respect to others, and that the stereochemistry of some amino acids located in specific positions played a fundamental role in favoring the correct orientation of the allylic side chains respect to the Grubbs catalyst. In particular, it appeared that inversion of the stereochemistry at the $Phe^{6,7,11}$ residues strongly favored the correct pre-organization of the linear chains, giving high yields of the dicarba-cycles even without the intervention of the MW heating. Furthermore, replacement of Phe^7 with a Tyr residue also allowed cyclization by conventional heating. A network of hydrogen bonds, revealed by NMR spectroscopy, can account for this observation. As expected, the MW supported cyclization afforded the dicarba-cyclo analogues of all the sequences from high to 100% yields. RCM reaction proceeded with high *E*-double bond prevalence (*E*-isomer from 67% to 100%) for all but one the linear precursors. This is

in contrast with our preceding results obtained by studying the smaller cycle of the octreotide dicarba-analogues, where a net preference of the *Z* isomer was observed.[17] From this study it appears that the increased di-carbacycles dimension in passing from the octreotide ring closure ($C^{\alpha}_{(i)} \rightarrow C^{\alpha}_{(i+5),}$ 20-membered rings) to the cyclic octapeptides ($C^{\alpha}_{(i)} \rightarrow C^{\alpha}_{(i+7),}$ 26-membered rings) clearly favored the thermodynamically more stable *E* configuration.

Abbreviations

Agl	Allylglycine
COSY	Correlation Spectroscopy
D-2-Nal	(2-naphthyl)-D-alanine
DIC	*N,N'*-Diisopropylcarbodiimide
DMF	N,N-Dimethylformamide
HPLC-ESI-MS	High Pressure Liquid Chromatography - Electrospray Ionization - Mass Spectrometry
Msa	3-mesityl alanine
MW	microwave
MW-SPPS	Microwave Assisted – Solid Phase Peptide Synthesis
ODN-8	des-AA^{1,2,4,5,12,13}[D-2Nal^{8}]-somatostatin-14
ODT-8	des-AA^{1,2,4,5,12,13}[D-Trp^{8}]-somatostatin-14
Oxyma	Ethyl(2-cyano-2-(hydroxyimino)acetate)
RCM	Ring Closing Methatesis
RP-HPLC	Reverse Phase - High Pressure Liquid Chromatography
SPPS	Solid Phase Peptide Synthesis
SRIF	Somatotropin Release-Inhibiting Factor
TFA	trifluoroacetic acid
TIS	Triisopropyl silane

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