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# Synthesis of cyclooctapeptides: constraints analogues of the peptidic neurotoxin, ω-agatoxine IVB – an experimental point of view

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**Abstract:** ω-AGA IVB is an important lead structure when considering the design of effectors of glutamate release inducting P/Q-type calcium channels. The best route to achieve the analogues possessing the three-dimensional arrangement corresponding to the native binding loop was the introduction of constraint by ring formation via side chain to side chain lactamization for suitably protected Lys and Glu residues. Since tryptophane residue located at position 14 of this neuropeptide has been suggested as essential for binding, analogues in which this amino acid was replaced by aza-tryptophane and alanine were synthesized. The synthesis was carried out on various acid-labile resins (BARLOS chlorotrityl, Rink amide, PEG-based or Wang resins), by Fmoc strategy. In this paper, we describe optimization of the peptide cyclization with various protecting groups, and on resin or in solution cyclization experimental parameters. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** P/Q-type calcium channels; peptidic neurotoxins; ω-agatoxin IV B; SPPS; on resin side chain to side chain lactam cyclization

## INTRODUCTION

For several years, our laboratory has been engaged in enantio- and diastereoselective syntheses of neuroexcitatory amino acids and their derivatives, blockers of the transport of excitatory amino acids [1–5]. These analogues of aspartate and glutamate are involved in development of many brain diseases including: ischemies, epilepsy, Huntington's and Alzheimer's diseases [6,7]. Trying to discover new pharmacological tools for cognition studies, we decided to study peptidic neurotoxins which affect glutamate release in the central nervous system (CNS).

As a result of studies aimed at mammalian $Ca^{2+}$ channels antagonist, it was found that the fractions isolated from *Agelenopsis aperta* venom affect responses of chicken and rat synaptosomes. This finding led to the discovery of Type IV of ω-agatoxins, namely ω-AGA IVA, ω-AGA IVB (also called ω-AGA TK) and ω-AGA IVC [8–10]. These toxins are the smallest peptides found in spider venom, and their amino acid sequences are unrelated to previously described Type I–III of ω-agatoxin. However, type IV seemed to be the most important of the four types of ω-agatoxins with regard to the pharmacological studies on glutamate release inducing P/Q-type calcium channels [11] and their availability for neurophysiological studies.

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The main challenge of this work was the choice of minimal sequence, which should assure similar activity to the native agatoxin IVB. Only one type of ω-agatoxin, called ω-agaTK has been successfully synthesized in a biologically active form. The subsequent studies of biological activity showed that inhibition of P-type calcium channels by one of the synthetized products is imcomplete. The analysis of a non-active analogue revealed the modification of the indole moiety of Trp 14 [12,13]. This loop, located between the 11 and 18, included amino acids (Figure 1) stabilized by the two disulfide bridges (12–25 and 19–36). Its conformation is almost identical in both ω-agatoxins IVA and IVB [8–10].

Additionnaly, the ω-aga IVA contains only two aromatic residues (Tyr 9 and Trp 14), which could influence the binding of the toxin to the channel, including Π-interactions (from ring planarity), hydrogen bond (from the indole nitrogen or hydroxyl group). Moreover, the side chain of Trp 14 is positioned on the upper side of the second β-strand situated on the toxin core and well exposed on toxin–channel interactions. The sequence homology of ω-agatoxins IVA, B and C is around 70% and all possess Trp14 (Figure 2).

In order to mimic the spatial arrangement of the natural peptide, the preliminary studies of structural similarities of the octapeptide analogue to the native toxin were performed. The results obtained from molecular mechanics calculations showed that the analogues of the native loop require the necessity of introduction of conformational constraints, which will retain the structural feature of natural toxin.

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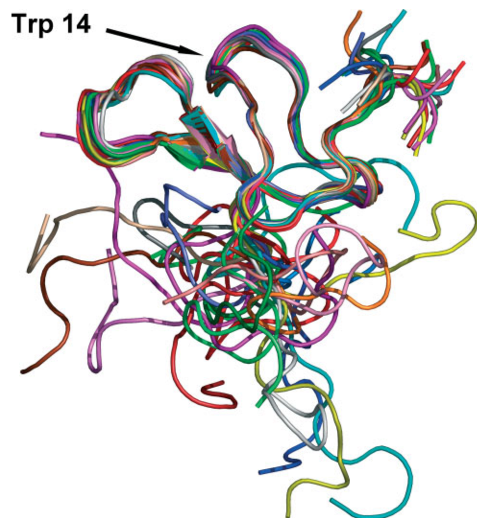



**Figure 1** NMR structure of $\omega$-agatoxin IVB (from Protein Data Bank) [8–10].

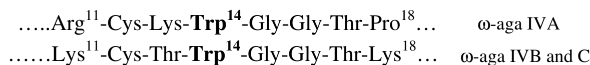



**Figure 2** Comparison of amino acid sequence (11–18) in $\omega$-aga IV A, B and C.

The presence of Lysines 11 and 18 in the native sequence of the binding loop of $\omega$-agatoxins IVB allowed cyclization of both lateral chains via succinic acid spacer. Molecular modelling calculations suggested a three-dimensional structure of the loop was suitably mimicked (Figure 3).

Many efforts have been currently devoted to the solid phase synthesis of cyclic peptides. After specific deprotection of side chains, the amide bond can be formed. However, succinylation and cyclization steps were unsuccessful. However, linear peptides (**EM 1**(Trp[14]) and **EM 2** (Ala[14])) were prepared and purified, and exhibited quite interesting activity.

As described by Davies in 2003 [14–16], the easiest way to achieve side chain to side chain lactamization was between suitably protected Lys and Glu residues. Therefore, this approach was chosen and Lys[11] was replaced by glutamic acid residue [17].

Molecular modelling studies suggested that this change still assures the correct orientation of the lateral chain of Tryp[14].

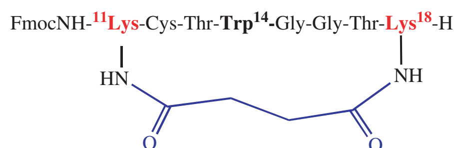
Cyclic peptides, analogues of this important loop, have been designed and obtained by replacement of *L*-Tryptophane by Alanine, *D*-Tryptophane and *L*-7-azatryptophane (Figure 4). The replacement by alanine (peptides **EM 3** and **EM 9)** was chosen in order to prove the importance of the Trp[14] for the toxin binding [12,13]; replacement with (*D*) Trp (peptide **EM 11)** should confirm key structural features of Trp configuration for toxin activity, whereas *L*-7-azatryptophan (peptide **EM 10**) prepared in N-protected form in our laboratory [18] was considered as a fluorescent probe suitable for binding studies.

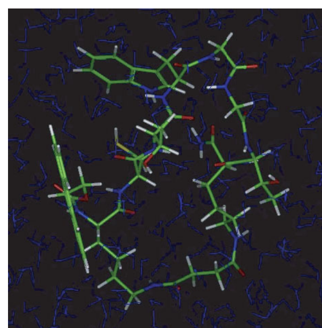
Syntheses were carried out on various acid-labile resins (BARLOS chlorotrityl, Rink amide, PEG-based or Wang resins), by Fmoc strategy and optimization of the cyclization is described in this paper comparing protection, deprotection, and cyclization experimental parameters.

## RESULTS AND DISCUSSION

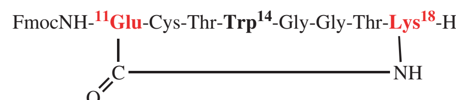
Solid phase synthesis of cyclic peptides was a subject of several reviews [14–16]. The success of this approach depends on loading and nature of the resin, peptidic sequence, nature of reagents used for cyclization. Linear peptides were obtained using standard procedure of automated peptide synthesis

**Hypothesis**



*EM 8*



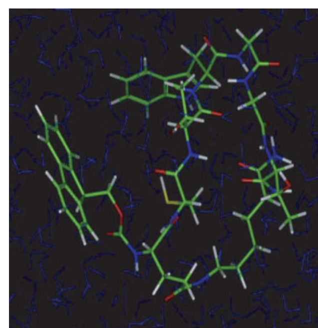



**Figure 3** Molecular modeling: three-dimensional structures of two potential $\omega$-agatoxin IVB analogues.

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according to Fmoc-protocols. Application of three various types of resins, namely, BARLOS chlorotrityl, Rink amide, and PEG-based, yielded the desired on-resin linear peptides with satisfactory yields. The problems appeared upon their protection/deprotection and cyclization processes, and they will be discussed here in some detail.

## Protection/Deprotection Systems

First, the difficulty encountered was the incompatibility between methyl ester deprotection of glutamate residue with alkali metals silanolates and, at the same time, syntheses in batch mode using PS resin [19]. Therefore, the alternative methodology, palladium-labile protecting groups Alloc/Allyl, has been chosen because its removal from Lys and Glu of the peptide attached to the resin was achieved in one step. There is a wide selection among the available scavengers for Pd-based allyl group removal, however, these applying hydride donors seemed to be most appropriate for solid phase technology [20]. As a result of their application, the by-product, propene, is formed. Silicon hydrides and amine–borane complexes revealed to be potent agents preventing formation of allyl amines; they acted rapidly (the deprotection with borane-based scavengers occurs in less than 10 min), and form rapidly removable by-products. It also should be noted that the borane complexes with tertiary amines did not possess the properties of allyl scavengers [21,22].

The deprotection of the linear precursor of **EM 3,** linked to Barlos resin, by phenylsilane ($PhSiH_3$) and $Me_2S \times BH_3$ (borane dimethyl sulfide complex) system in large excess (20 eq) to ensure the total removal of allyl-based groups gave the desired peptide in good yield and without the allylamine side-products. The same deprotection protocol was applied for the peptides attached to the PAL-PEG resins (**EM 4**) and the reaction of simultaneous Alloc/Allyl removal led, like in the case of PS-based resin, to the product with deprotected side chains of Glu and Lys. Phenylsilane used as scavenger in excess (20 eq), ensured the removal of allylic protections from the peptide bound to Rink amide resin, and led to the expected linear product in high yield and excellent purity without the side-reactions related to the Pd-catalysed deprotection. Thus, Alloc/Allyl strategy revealed to be compatible with a variety of resins (Barlos, PAL PEG PS, Rink amide) based on both, PS and PEG matrixes.

We noted that the linear peptide **EM 4** undergoes dimerization through Cys side chain with prolonged storage (6 months) in freezer until 50% and purification was necessary before biological tests.

## On Resin Cyclization

The growing interest in the application of solid phase for peptide cyclization has resulted in the variety of new approaches concerning on-resin synthesis. Beneficial features of ring closing on solid support were undeniable; nevertheless, in some cases the adjustment of certain parameters, depending on characteristics of particular synthesis, was not evident. Numerous studies carried out in order to find the most suitable coupling reagent for cyclization were recently reviewed by Davies [14–16,23].

Application of very effective uronium salts tend to block, irreversibly, the amine function by formation of guanidinium derivative, in case of slow couplings. Additionally, the decomposition of carboxyl moiety inducted by the solvent was observed: for example, carboxyl-activating reagents such as, BOP or HBTU, were useful for cyclization of peptides with high steric hindrance, whereas for peptides where steric hindrance was not a concern, the application of diphenylphosphoryl azide (DPPA) or the DEPBT (3-(diethoxyphosphoryloxy)-(1–3)-benzotriazin-4(3H)-one) led to good results [23]. The first challenge in the synthesis of cyclic analogues of ω-AGA IVB on solid phase concerned the improvement of carboxyl activation. This was done by application of NMP instead of DMF as a reaction solvent, and the

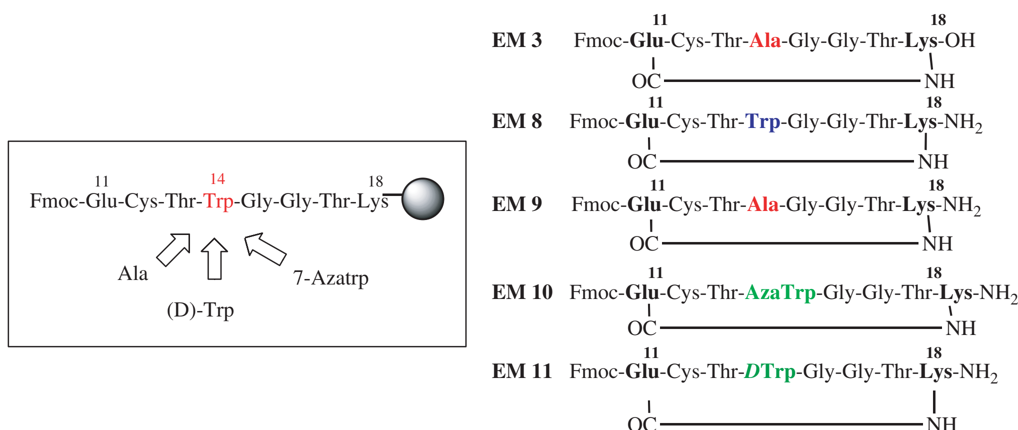


**Figure 4** Linear and cyclic analogues of reduced agatoxin obtained by replacement of $Trp^{14}$ by Ala,, 7-Azatryp, (*D*)Trp.

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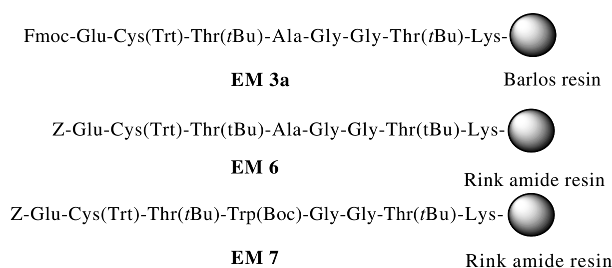


**Figure 5** Improvement of carboxyl activation for on-resin lactamization.

on-resin cyclizations, were carried out overnight with BOP/HOBt/DIEA (3/3/6) system (Figure 5).

In the case of the **EM 3a** on-resin cyclization, the expected product has been obtained, whereas the results of two remaining syntheses led to small amounts of cyclic products (less than 2%). In the case of the syntheses performed on Rink Amide resin with initial substitution of 0.27 mmol/g, cyclic **EM 7** was obtained in 5% yield while cyclization of **EM 6** failed. Regarding the identical conditions of synthesis in these unsuccessful reactions, one of the explanations may be the low probability of the peptide backbone to adopt the correct conformation, in which both reacting functions were in close proximity and thus, promote the reaction [24].The peptide bond reveals the planar geometry in which the carbonyl oxygen and the amide nitrogen were on opposite sides of the bond. Since the energetic barrier required for change of conformation between the *trans* and *cis* forms of peptide bond is in the order of 20 kcal/mol, the peptide backbone possessed the limitations in its geometries, and the extended conformation of the chain was favoured. Rigid, the backbone chain possessed little degree of freedom, and it was not easy to perform the ring closure justifying low yields.

The reaction improvement has been done by changing the nature of the linker; it was reported in the cyclization of tetrapeptide (containing RGD sequence), that the use of short and bulky trityl linker led to better yields (84%) than when longer and more flexible Wang handle (65%) [25] was used. On the other hand, the studies comparing the utility of HMPB-MBHA (4-hydroxymethyl-3-methoxyphenoxybutyric acid 4-methyl benzhydrylamine) *versus* Wang resins for synthesis of cyclic decapeptide analogue of neuropeptide Y gave contradictory results. Good results (65%) were obtained with flexible HMPB-MBHA handle and the application of shorter Wang linker resulted in decrease in the cyclization yield [26]. The choice of suitable linker depends on the length of cyclized peptide; it should be long enough to enable the peptide to adopt the right conformation for intramolecular ring closure, and to assure a good access of reagents. On the other hand, too long a handle provoked the aggregation of peptide leading to the suppression of ring formation or to the inter-chain cyclizations.

Therefore, **EM 8** and **EM 9** linked to the PAL PEG PS resin with equally low charge (0.18 mmol/g) were prepared. Moreover, the choice of PEG-based supports enables application of both protic and aprotic solvents, and assures better solvatation of resin-bound peptides compared to PS-based resins. Final peptides were obtained as *C*-terminal amides after cleavage from resin. Formation of cyclic analogues was detected in all performed reactions (PyBOP/HOBt/DIEA (3/3/5) or BOP/HOBt/DIEA (3/3/5)); but the yields of ring closure were at the limit of detection even when DMF was replaced by NMP/THF mixture, and even if the proper solvatation of peptide-support complex was achieved with both applied solvents. Analysis of the various combinations of tested solvents in the literature [27] led to the conclusion that the best cyclization results might be accomplished in the most polar solvent mixture, and that the peptide solvatation was more important for successful synthesis than the resin swelling. Finally, in order to decrease the viscosity of solvents and insert the additional energy into the system, the reaction temperature has been increased (50 °C) and this parameter did not allow increase in yield.

In brief, none of the on-resin cyclized peptides were obtained after preparative HPLC purification in sufficient quantities and purity required for the biological tests, and therefore, cyclization in solution was envisaged for their preparation.

## Cyclization in Solution

Linear peptides synthesized on the PAL-PEG-PS resin (**EM 8** and **EM 9**), were suitable precursors to studying the cyclization-in-solution procedure because they possessed amidated *C*-terminal carboxylic moiety with *N*-terminal amine being protected with Fmoc moiety. Thus, the only free $NH_2$ and COOH functions provided from Lys 18 and Glu 11 side-chains, respectively, could be involved in ring closure.

The main side reaction occurring during the ring closure in solution is formation of peptide oligomers [cyclodimers, higher aggregates, and also dimers provided from disulfide bridge formation between two $Cys^{12}$ of two peptide molecules (5–10% of the linear peptide)]. Usually, intramolecular cyclizations were favoured by high dilution ($10^{-3}$–$10^{-4}$ M) of the linear counterpart; however, the requirement of the large quantities of solvents is often inconvenient. A good alternative for the use of high amounts of solvent was the prolongation of addition time of linear precursor to the reaction mixture [28]. The prolonged time of addition preserves the conditions of high-dilution, necessary for proper intramolecular ring closure.

In the first attempts of solution-phase cyclization, two different coupling reagents had been compared.

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In the first reaction leading to **EM 9,** BOP/HOBt (5/5 eq.) reagents were applied for the activation of carboxyl function; the second coupling system was the HBTU/HOBt one (5/5 eq.). In both cases, DIEA (10 eq.) was applied as base. Taking into consideration the influence of substrate concentration, the syringe pump was used for the addition of linear precursor to the solution of base and coupling reagents in DMF at the rate of 1.19 ml/h. After one day's stirring, LC-MS analyses revealed the formation of a cyclic product in BOP-based synthesis, and no reaction progress in the HBTU/HOBt system. These findings were in a good correlation with the results obtained in the solid-phase approach, in which cyclizations with uronium-based coupling reagents did not give any product. The long time of reaction of the uronium salts (HBTU) most likely resulted in irreversible blockage of amine function by formation of guanidinium derivative, and addition of HOBt did not diminish this side reaction. Thus, in the following reactions, phosphonium-based coupling reagents were used in order to avoid guanidylation reaction.

Further cyclization steps of **EM 8** and **EM 9** were performed in order to verify if the change in the order of addition of the reagents may influence the cyclization yield. Thus, instead of coupling reagents the linear precursor was placed in the flask and the mixture of base and BOP (or PyBOP) was added to the peptide solution with the syringe. Unfortunately, the results were comparable with those observed in the previous syntheses. The reaction was then performed in DMF with the use of a syringe pump, applying BOP/HOBt/DIEA (5/5/10) or PyBOP/HOBt/DIEA (5/5/10) reagents. In all cases, regardless of the coupling reagent, the obtained results were similar, showing that the primary structure of the linear peptide ($Ala^{14}$ containing **EM 3** and **EM 9** or $Trp^{14}$ **EM 8**) did not influence the cyclization. Since the efficacy of two phosphonium-based coupling reagents, BOP and PyBOP, revealed to be equivalent with regard to the BOP toxicity, PyBOP was chosen for later experiments. Since these reactions were accompanied by the formation of cyclic oligomers, the unambiguous evaluation of monomer/dimer ratio was difficult. The purification of cyclic monomer with use of HPLC preparative was cumbersome; in all cases, the purest product was still the mixture of monomer and dimer, with majority being the monomer molecule (95/5).

It is well proven that the influence of a solvent factor on synthesis of cyclic peptides has significant importance [29]. For example, syntheses of cyclic depsipeptide aureobasidin revealed that when cyclization was carried out in DMF, no cyclic product was obtained, while the use of DCM led to a cyclic product in levels of good yields. The results of NMR experiments on aureobasidin suggested that the linear peptide adopted an extended conformation in DMF (due to solvating effect of the solvent), whereas conformation of linear peptide in DCM favoured the bent alignment. Moreover, the initial step of carboxyl activation with phosphonium salts was an anion–cation reaction and was favoured in the less polar solvents.

The influence of solvent nature on the cyclization rate has been studied by applying a mixture of THF and DMF in the ratio of 1 : 2. In this approach, the peptide was added to the mixture of base and coupling reagent (PyBOP/HOBt/DIEA) in the corresponding ratio 5 : 5 : 10, using a syringe pump. Similarly, as in the previous syntheses, the changes in the order of addition of substrates did not make any improvement in the cyclization yield; HPLC analyses of crude products showed the complete disappearance of linear precursor and the formation of cyclic monomer. Nevertheless, LC/MS characterizations revealed presence of the dimer peptide, but the evaluation of monomer/dimer ratio was impossible with regard to the partial precipitation of the dimer peptide in HPLC elution solvent.

The reaction conditions described above appeared as the most suitable for the formation of our analogues of ω-agatoxin IVB, regardless of the peptide sequence. Thus, when all further cyclizations were performed with the PyBOP/HOBt/DIEA (5/5/10) coupling system, high-dilution conditions were achieved by the application of a syringe pump and the application of THF/DMF (1/2) mixture as a solvent. The main problem, related with the synthesized products, was their purification. Although a variety of HPLC conditions were tested (different gradients, enlargement of separation time, variation of sample amount, isocratic mode), the resulting product was often recovered as the mixture of monomer and dimer peptides as 95% monomer and 5% of dimer; fortunately, pure compounds suitable for biological tests can be obtained in correct amounts.

## Constraints Analogues Preparation

*L*-7-azatryptophan has been prepared in N-protected form in our laboratory [18] and is considered as a fluorescent probe suitable for binding studies. The strategy of synthesis was different: the tetrapeptide Fmoc-Gly-Gly-Thr(tBu)-Lys(Alloc)-Resin was synthesized on PAL-PEG-PS resin in an automated way. Then, after having checked loading of resin by the quantitative Kaiser test, Fmoc-*L*-7-azatryptophan-OH was manually coupled until the negative Kaiser test. Further synthesis was performed in an automated way as previously described.

During lactamization in solution, slow addition of linear precursor to the mixture of PyBOP/HOBt/DIEA in THF/DMF resulted in total disappearance of starting peptide. Unfortunately, the crude product was of lower purity than its parent compound containing tryptophane. Quite interestingly no dimer formation was observed among the minor impurities. Application

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of preparative HPLC gave a pure cyclic monomer **EM 10**.

The main reason for replacement of L-tryptophan by D-tryptophane in studied peptides was related to the structure–activity relationship with these $\omega$-agatoxin analogues. As already mentioned, due to the existence of a *trans* form of the peptide bond, the extended conformation of a backbone chain was favoured, consequently, the parameters that force bending of the chain were indispensable. The studies on these parameters revealed that placement of turn-inducting residues such as D-amino acid, could have a significant effect on the conformational changes of linear precursor [30]. Additionally, once the peptide chain is bent, the $\beta$-turns are stabilized by intramolecular hydrogen bonds between amide hydrogen and oxygen of neighbouring carbonyl group (Figure 6), if one of the residues possesses D-configuration.

The cyclization of linear analogue possessing D-Trp residue was performed using standard conditions (PyBOP/HOBt/DIEA; THF/DMF; slow addition). The reaction resulted in the formation of a monomer molecule, and the LC/MS analyses did not reveal the presence of any dimeric peptide. The introduction of D-tryptophan residue enables the spatial arrangement of linear peptide, which results in only intramolecular cyclization. The pure product, **EM 11** was recovered after the HPLC purification in isocratic mode.

In conclusion, the most suitable conditions for the solution-phase cyclization of linear analogues of $\omega$-agatoxin IVB were the use of PyBOP/HOBt/DIEA in 5 : 5 : 10 ratio coupling system and the mixture of THF/DMF (1/2) as solvent. This preparation mixture enabled correct solvatation of linear precursor and ensured good solubility of all reagents. Application of a syringe pump enabled to achieve high dilution conditions required for the proper intramolecular ring formation. However, it would be desirable to perform further studies concerning the oligomerization of these peptides. On account of the difficulties of analytical separation and dimer precipitation, the evaluation of exact monomer/dimer ratio in the lactamization mixtures of agatoxin analogues was impossible.

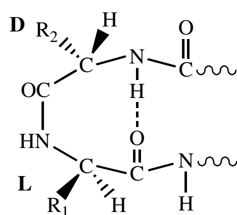


**Figure 6** Hydrogen bond forming so-called $C_8$ conformation.

As shown in this study, the successful synthesis of cyclic peptides depends on many variables, and required suitable correlation of the multiple factors such as amino acids sequence and arrangement of linear precursor, site of ring closure, nature of applied coupling reagents and solvents, concentration of reagents, temperature, nature of the used base, additives, ratio of substrates, and reaction time. Out of consideration, and because of the involvement of all these factors, every synthesis becomes exceptional and needs to be optimized individually.

## Biological Activities

The P-type channels play a critical role in neurotransmitter release from pre-synaptic nerve terminals. Hippocampal cultures were used to study cyclic and linear analogues of $\omega$-AGA IVB synthesized during this work by patchclamp methodology on whole-cell and $Ca^{2+}$ imaging for intracellular $[Ca^{2+}]$ calculation based on the measurement of the changes in fluorescence of an indicator of calcium ions (fura-2).

The results are briefly described in Figure 7.

These results indicate that the presence of $Trp^{14}$ in the sequence is fundamental for activity: **EM 2, EM 4** and **EM 5** containing $Alanine^{14}$ exhibit no activities at all towards calcium channels.

Suprisingly, some of our peptides appeared to be agonists (calcium channels activators) as the linear peptide **EM 1** and the cyclic one, **EM 8**. Remarkably, the only activators of calcium channels described concerns the L-Type $Ca^{2+}$ channels. The dihydropyridine derivatives, including BAYK8644, are known to act as agonist of L-type as they provoked the enhancement of the current calcium flux reaction by this type of calcium channel [31].

| Peptides | Effect towards P/Q type calcium channels | Evaluation of opening or closing calcium channel compared to the commercially available ω–agaIVA effect |
|---|---|---|
| ω–agaIVA | Antagonist | Closed at 100% : baseline level |
| EM 1 | Agonist | Opened + 40% |
| EM 2, EM 4, EM 5 | none | - |
| EM 8 | Agonist | Opened + 40% |
| EM 10 | Antagonist | Closed at 40% |
| EM 11 | Antagonist | Closed at 70% |

**Figure 7** Preliminary biological results by patch clamp method and $Ca^{2+}$ imaging: activity of described peptides.

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The cyclic octapeptides containing an azatryptophane (**EM 10**) or a D-Tryptophane (**EM 11**), enhanced antagonist activities (by 40 and 70%, respectively). Further experiments concerning the exact determination of toxin-channel binding site could be useful for better understanding of mechanisms concerning the modulation of calcium channel activity. The synthesized analogues containing, for example, 7-azatryptophane, which reveals significant absorbance between 310 and 320 nm, may serve as a photophysical probe for such studies.

This work opens a variety of perspectives concerning the synthetic and biological approach. The synthesized agonists might be useful for the development of treatment for patients with calcium channelopathies, like migraine, related to decreased calcium influx [32].

## EXPERIMENTAL

### Solvents, Reagents, Analyses

All amino acids were of the L-configuration, except where indicated otherwise. All amino acids, resins, chemicals and solvents were purchased from commercial suppliers (Advanced ChemTech, NovaBiochem, Bachem, PerSeptive Biosystems, Lancaster, Fluka, Aldrich, Avocado and VWR International) and used without further purification. DMF was dried and stored over 4 Å molecular sieves. RP-HPLC analyses were carried out on Waters equipment (solvent delivery module 510) fitted with an autosampler system (Autosampler 717+) and coupled with a UV-*vis* diode array detector operating at 214 nm. Waters Symmetry Shield $RP_{18}$ (3.5 µm, 4.6 × 50 mm) cartridge was used as a stationary phase. The mobile phase was composed of a mixture of acetonitrile/water containing 0.1% TFA introduced at a flow rate of 1 ml/min. The elutions were performed generally using a gradient from 0 to 100% $CH_3CN$ in 15 min. The analytical system was controlled with Millennium 32 software. Peptide purifications were achieved with a preparative HPLC (Waters Delta Prep 4000 system) fitted with Waters 486 UV-*vis* detector operating at 214 nm. Waters Symmetry Shield $RP_{18}$ (3.5 µm, 19 × 100 mm) was used as a reversed-phase column. The binary solvent system (A/B) was as follows : 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) at a flow rate of 20 ml/min with manual injections. ESI mass spectra were recorded on a Platform II Quadrupole Mass Spectrometer (Micromass, Manchester, UK) fitted with an electrospray ion source. The mass spectrometer was calibrated in the positive ion mode using a mixture of NaI and CsI. Voltages were set at +3.5 kV for the capillary and +0.5 kV for the skimmer lens. The source was heated at 120 °C. Nitrogen was used as a nebulizing and drying gas at 15 and 250 l/h, respectively. An Alliance 2690 (Waters, Milford, MA, USA) LC system equipped with an autosampler was used to deliver the mobile phase, continuously degassed, at a flow rate of 0.6 ml/min. The eluent was then split prior to the mass spectrometer entrance to reach 60 µl/min of mobile phase infused in the source. In all experiments, 3 µl of samples were injected at 25 °C. Flow injection analysis (FIA) mass spectrometric data were acquired in the scan mode from 100 to 800 $m/z$ in 2 s. Five scans were summed to produce the final spectrum. LC/ESI-MS experiments were carried out by implementing a XTerra MS C18 cartridge (2.1 × 30 mm) packed with 3.5 µm particles (Waters, Milford, MA, USA) between the LC pumping system set up at 0.6 l/min and the split which reduced the flow rate in the source at 60 µl/min. A linear gradient from 0 to 100% of acetonitrile in water containing 0.1% of formic acid was applied in 5 min. The column was washed during 2 min at the final gradient condition, brought back to the initial condition in 1 min and equilibrated during 3 min. The sampling cone voltage was adjusted according to the samples. Data acquisition and processing were performed using MassLynx 4.0. All molecular mechanics calculations were done with the use of Insight 2005 (Accelrys, Inc., San Diego) molecular modelling program package. Starting structure was obtained by appropriate modification of native peptide (Protein Data Bank ID Code 1AGG) in Builder module. Molecules were soaked in water (a layer of 5 water molecules was generated). Structure optimizations were done using Discover program with CF97 force field and conjugated gradient minimizer. Minimizations were done upto RMS derivative 0.1 kcal $mol^{-1}$. Linear peptides were synthesized on an Applied Biosystems 431A or Pioneer Perseptive Biosystems automated peptide synthesizers according to Fmoc-protocols. For 431A model NMP was used as a solvent, and for the Pioneer model, reactions were performed in DMF. In both apparatuses, a HBTU/HOBt coupling system was used for activation. In a typical experiment, each peptide coupling was carried out with fourfold Fmoc-amino acid excess. After washing with DMF (or NMP), the Fmoc group is cleaved with a solution of 20% piperidine in NMP and the next coupling cycle was repeated. Manual solid-phase peptide syntheses were carried out in a sterile plastic syringe or in a glass reactor containing a porous polypropylene frit. The volumes of containers were 10 or 20 ml depending on the resin quantity. All reactions were performed using mechanical stirring (at slow speeds).

Solvents were evaporated under reduced pressure at ±35 °C with a rotary evaporator connected to a water pump (~16 mmHg) unless otherwise stated.

### Protocol A: Rink Amide MBHA Resin Loading

The Rink Amide resin was swollen in DCM (1 h), and then the excess solvent was removed by filtration. The Fmoc group was removed (Protocole E). Fmoc-protected amino acid, HBTU, HOBt dissolved in DMF and DIEA were added to the swollen resin. After gentle shaking at room temperature (3 h) the resin was filtered off and washed. The remaining free amino groups were acetylated with acetic anhydride in the presence of DIEA in dichloromethane (1 h). The resin was washed with DMF (×3), DCM (×3), isopropanol (×3), $Et_2O$ (×3), and vacuum dried. The substitution level of the loaded resin was determined spectrophotometrically by Fmoc cleavage (dibenzofulven dosage) and/or Kaiser test.

### Protocol B: Barlos (2-Chlorotrityl Chloride) Resin Loading

The resin (stored under Ar) was swollen in anhydrous DCM (1 h) and the solvent was removed by filtration. The Fmoc-protected amino acid and DIEA were added to the resin in DCM and mixed (3 h). Addition of methanol (0.5 ml)

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and additional stirring enable end-capping of unreacted chloride functions. After 30 min, the resin was washed with DMF (×3), DCM (×3), isopropanol (×3), $Et_2O$ (×3), and vacuum dried. The substitution level of the loaded resin was determined spectrophotometrically by Fmoc cleavage (dibenzofulven dosage) and/or Kaiser test.

## Protocol C: Resin Loading Evaluation

***Dibenzofulven-piperidine adduct.*** The substitution level was determined spectrophotometrically by Fmoc cleavage: accurately weighed Fmoc-protected resin was introduced into a test tube and a solution of 20% piperidine in DMF was added (solution alone = blank test). The test tube with the resin, and the blank, were stirred for 20 min to make sure all the resin had come in contact with the cleavage solution. The blank was used to zero the UV spectrophotometer at 301 nm. The absorbance of the resin-containing solution is measured. The substitution level was calculated from the following formula:

$$\text{Loading (mmol/g)} = \text{Abs}_{\text{sample}} \times D/7800 \times m_{\text{sample}}(\text{mg});$$

where $D$ is the dilution factor. Generally, an average of two or three measurements was taken.

**Ninhydrin procedure – Kaiser test**

Three solutions were prepared:

- Solution A: ninhydrin in ethanol (5% in ethanol),
- Solution B: phenol in ethanol (40% in ethanol),
- Solution C: aqueous solution of potassium cyanide in pyridine (2 ml of aqueous 0.001 M KCN in 100 ml pyridine)

A sample of dry resin of pre-determined weight was placed in a glass test tube. 75 µl of A and C solutions, and 100 µl of solution B were added to the resin, and the mixture was stirred thoroughly (solutions alone = blank test). Both tubes were heated at 110 °C for 5 min (during longer heating, pyridine causes partial Fmoc removal), after cooling, aqueous solution of ethanol 60% was added to the tubes. Absorbance of the sample with comparison to the blank solution is measured at 570 nm. The level of free $NH_2$ may be estimated from the following equation:

$$NH_2\ (\mu\text{mol/g}) = (\text{Abs}_{\text{sample}} \times D/15\,000 \times m_{\text{sample}}(\text{mg})) \times 10^6$$

This test was also applied for qualitative determination of the free amine groups. A few beads of resin were placed in a glass test tube, 2 drops of each solution were added and the mixture was heated at 110 °C for 5 min. Blue colour indicates the presence of $NH_2$ moiety.

## Protocol D: Alloc/Allyl Removal

The peptide-resin was dried over $P_2O_5$ under vacuum and swollen in dry DCM (15 min) under Ar. The scavenger $Me_2S \times BH_3$ and/or $PhSiH_3$ were added (10 equiv.) and the resin was shaken (5 min). Palladium complex $Pd(PPh_3)_4$ was weighed under Ar (0.1 equiv.), dissolved in dry DCM immediately before use and added to the resin. After overnight stirring in darkness and under argon, the solution was removed and the peptide-resin was washed (×5) with a solution of DIEA in DMF (0,5% (v/v)), with solution of sodium diethyldithiocarbamate in THF (0,5% (w/v)) (×10), followed by washings with DMF (×3), DCM (×3), isopropanol (×3), $Et_2O$ (×3), and under vacuum drying.

## Protocol E: FMOC Removal

Fmoc removal was achieved by resin treatment with a solution of 20% piperidine in DMF (3 × 10 min), followed by resin washing with DMF (×3), DCM (×3), isopropanol (×3) and $Et_2O$ (×3). After vacuum drying, the remaining $NH_2$ groups were determined with one of the tests of protocol C.

## Protocol F: Methyl Ester Removal

The peptide-resin was dried over $P_2O_5$ under vacuum and swollen in dry THF under Ar (15 min). 1 M $NaOSiMe_3$ solution in DCM was added to resin and the mixture was stirred overnight under Ar. The resin was filtered off, washed with DMF (×3), DCM (×3), isopropanol (×3) and $Et_2O$ (×3) and vacuum dried [17].

## Protocol G: Methyl Trityl(Mtt) Removal

The resin was treated with 1% TFA solution and scavenger TIPS in DCM, 6 cycles of 3 min, until the yellow-red color disappeared. The resin was filtered off and washed with DCM (×3), DMF with 2 DIEA drops (×3), DMF (×3), isopropanol (×3) and finally, with $Et_2O$ (×3).

## Protocol H: Resin Cleavage

The peptide-resin was in contact (2.5 h) at RT with the cleavage mixture : TFA/EDT/$H_2O$/TIPS (94/2.5/2.5/1). Then the resin was filtered off and washed with neat TFA, TFA/DCM (50/50). the resulting solution was evaporated under reduced pressure at ±35 °C and the peptide was precipitated from cold diethyl ether.

## Synthesis of EM 1: Fmoc-Lys-Cys-Thr-Trp-Gly-Gly-Thr-Lys-$NH_2$

The peptide was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (1 g, loading 0.36 mmol/g). The side-chain protections were as followed: Fmoc-Lys(Boc)-Cys(Trt)-Thr(tBu)-**Trp**(Boc)-Gly-Gly-Thr(tBu)-Lys(Boc)-*PAL-PEG-PS resin*. The side-chain protections and resin cleavage according to Protocol H were performed twice. $MS_{calc.}$ 1101.28; $t_R = 8.35$ min; LC/MS $[M + H^+]$: 1101.42. The overall yield in 33% (120 mg, 0.11 mmoles) after preparative HPLC. The crude product was lyophilized.

## Synthesis of EM 2: $NH_2$-Lys-Cys-Thr-Ala-Gly-Gly-Thr-Lys-$NH_2$

Like previously for **EM 1**, compound **EM 2** was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (1 g, loading 0.36 mmol/g). The side-chain protections were as follows: Fmoc-Lys(Boc)-Cys(Trt)-Thr(tBu)-Ala-Gly-Gly-Thr(tBu)-Lys(Boc)-*PAL-PEG-PS resin.* Final Fmoc deprotection was included in synthesizer run. The side-chain protections and resin cleavage according to protocol H was performed twice.

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$MS_{calc.}$763.91; $t_R$ = injection peak; LC/MS [M + H$^+$]: 764.29. **EM 2** was purified using preparative HPLC and lyophilized (117 mg, 0.153 mmoles, overall yield : 42.5%).

## Synthesis of EM 3: Fmoc-c(Glu-Cys-Thr-Ala-Gly-Gly-Thr-Lys)-OH

Barlos resin (250 mg, loading 1.5 mmol/g) was manually pre-loaded according to Protocol B in DCM (10 ml) (3 h). Peptide was synthesized using a general SPPS procedure on 431A synthesizer on previously pre-loaded resin (200 mg, 1.33 mmoles). The side-chain protections were as follows: Fmoc-Glu(OAllyl)-Cys(Trt)-Thr(tBu)-Ala-Gly-Gly-Thr(tBu)-Lys(Alloc)-*Barlos-Resin*. The side-chain protections and resin cleavage was performed according to Protocol G.

On-resin removal of Alloc/Allyl protections was performed according to Protocol D.

$MS_{calc.}$1112.2; $t_R$ = 11.1 min; LC/MS [M + H$^+$] : 1112.4

***On-resin cyclization.*** Resin was swollen in DMF and washed with NMP. All reactants (HOBT/BOP/DIEA) (3/3/5) were added to resin/NMP and stirred (48 h). The side-chain protections and resin cleavage was performed according to Protocol H. $MS_{calc.}$970.06; $t_R$(HPLC) = peak from 11.00 to 15.5 min; LC/MS [M + H$^+$]: 970.8. Titration of crude product with diethyl ether resulted in detection of peak corresponding to the cyclic peptide **EM 3.** $t_R$ = 10.9 min; LC/MS [M + H$^+$]: 970.8. After washing the resin with DMF (×3), DCM (×3) and isopropanol (×3), the new coupling reagents were added and the mixture was stirred for an additional 48 h. No reaction progress was observed. **EM 3** was detected but not isolated.

## Synthesis of EM 4: Fmoc-Glu-Cys-Thr-Ala-Gly-Gly-Thr-Lys-$NH_2$

**EM 4** was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (1 g, loading 0.36 mmol/g). The side-chain protections were as follows: Fmoc-Glu(tBu)-Cys(Trt)-Thr(tBu)-Ala-Gly-Gly-Thr(tBu)-Lys(Boc)-*PAL-PEG-PS resin*. The side-chain protections and resin cleavage were performed according to Protocol H. $MS_{calc.}$987.09; $t_R$ = 8.9 min; LC/MS [M + H$^+$]: 987.4. **EM 4** was purified using preparative HPLC (75% A ($H_2O$) to 60% A in 20 min) and lyophilized, yielding 35% (124 mg, 0.12 mmoles). **EM 4** undergoes dimerization through Cys side-chain with prolonged storage (6 months) in a freezer at a rate of 50%.

$t_{R\ (EM\ 4\ dimer)}$ = 9.6 min; LC/$MS_{(EM\ 4\ dimer)}$[M + 2H$^{++}$] : 987.0.

## Synthesis of EM 5: Fmoc-c(Glu-Cys-Thr-Ala-Gly-Gly-Thr-Lys)-$NH_2$

Rink amide resin (1 g, loaded 0.7 mmol/g) was manually preloaded according to Protocol A, reaction was carried out in DMF (10 ml) with coupling reagents listed below.

The capping step was performed in $Ac_2O$/DIEA (10/10) mixture. After determination of resin substitution applying C Protocol, new charge of support was ~0.7 mmol/g, linear peptide was synthesized using a general SPPS procedure (Pioneer apparatus) on pre-loaded resin (1 g, 0.7 mmol)). The side-chain protections were as follows: Fmoc-Glu(Allyl)-Cys(Trt)-Thr(tBu)-Ala-Gly-Gly-Thr(tBu)-Lys(Alloc)-*Rink-amide-Resin*. On-resin removal of Alloc/Allyl protections (Protocol D) and side-chain deprotection and resin cleavage (Protocol H) were performed. $MS_{calc.}$987.09; $t_R$ = 9.1 min; LC/MS [M + H$^+$]: 987.3. Crude linear peptide yielded 77% (533 mg, 0.54 mmoles) was used directly for cyclization.

***Cyclization in solution.*** The linear peptide (315 mg, 0.32 mmoles) was dissolved in a mixture of THF/DMF (1/2; 10 ml) and transferred into a syringe. Coupling reagents (HOBt/PyBOP : 5/5) and DIEA (10 equiv.) were dissolved in THF/DMF (1/2 (v/v); 100ml) mixture and purged under Argon. The peptide solution was added to coupling reagents/DIEA solution, using a syringe pump system (1.19 ml/h). After complete addition, the mixture was stirred for 24 h. After solvent evaporation under reduced pressure, **EM 5** was purified using preparative HPLC (gradient : 70 to 50% $H_2O$ in 30 min) and lyophilized. The dimer was present in the mixture and not separable from the monomer even after preparative HPLC optimization. Dimer partially precipitated in HPLC solvent elution. $MS_{calc.}$969.07; $t_R$ = 9.5 min; LC/MS [M + H$^+$]: 969.4. $t_{R\ (EM\ 5\ dimer)}$ = 10.1 min; LC/$MS_{(EM\ 4\ dimer)}$[M + 2H$^{++}$]: 968.9.

## Synthesis of EM 6: Z-c(Glu-Cys-Thr-Ala-Gly-Gly-Thr-Lys)-$NH_2$

Rink amide resin was manually pre-loaded with Fmoc-Lys(Mtt)-OH according to Protocol A; new resin charge was 0.27 mmol/g. On previously pre-loaded resin (500 mg, 0.135 mmoles) the peptide was synthesized using a general SPPS procedure on 431A synthesizer. The side-chain protections were as followed: Z-Glu(OMe)-Cys(Trt)-Thr(tBu)-Ala-Gly-Gly-Thr(tBu)-Lys(Mtt)-*Rink-amide-Resin*. The side-chain protections and resin cleavage were performed according to Protocol H. $MS_{calc.}$913.01; $t_R$ = 10.46 min; LC/MS [M + H$^+$]: 913.4. Deprotection of methyl ester was performed according to Procol F[9]. $MS_{calc.}$898.98; $t_R$ = 9.12 min; LC/MS [M + H$^+$]: 900.2. In order to remove Mtt protection **EM 6**-*resin* entity was treated as described in Protocol G.

***On-resin cyclization.*** **EM 6**-*Resin* was swollen in DMF and washed with NMP. All reactants (HOBT/BOP/DIEA) (3/3/5) were added to resin/NMP and stirred (48 h).

The side-chain protections and resin cleavage were performed according to Protocol H; expected **EM 6** was not recovered: $MS_{calc.}$880.96. The following non-identified products were observed: $t_R$ = 8.8 min; LC/MS [M + H$^+$]: 1005.3; $t_R$ = 9.2 min; LC/MS [M + H$^+$]: 1001.3.

## Synthesis of EM 7: Z-c(Glu-Cys-Thr-Trp-Gly-Gly-Thr-Lys)-$NH_2$

For the synthesis of linear peptide, the same conditions were used as in **EM 6**. The side-chain protections were as follows: Z-Glu(OMe)-Cys(Trt)-Thr(tBu)-Trp(Boc)-Gly-Gly-Thr(tBu)-Lys(Mtt)-*Rink-amide-Resin*. The side-chain protections and resin cleavage were performed according to Protocol H. $MS_{calc.}$1028.14; $t_R$ = 8.75 min; LC/MS [M + H$^+$]: 1028.2; by-product without *N*-terminal Z moiety:

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$t_R = 6.53$ min; LC/MS [M + H$^+$]: 894.3. Deprotection of methyl ester was performed according to Protocol F and the side-chain protections and resin cleavage were performed according to Protocol H. MS$_{calc.}$1014.1; $t_R = 8.6$ min; LC/MS [M + H$^+$]: 1014.3. In order to remove Mtt protection **EM 7**-*Resin* was then treated as in Protocol G.

***On-resin cyclization.*** **EM 7**-*Resin* was swollen in DMF and washed with NMP. All reactants (HOBT/BOP/DIEA) (3/3/5) were added to resin/NMP and stirred (48 h). After the side-chain protections and resin cleavage, according to Protocol H, the HPLC analyses revealed multitude of non-identified by-products. MS$_{calc.}$996.1; $t_R$ = from 10.6 to 15.5 min. Ether precipitation was performed and the expected product was detected and yielded in 5%. $t_R = 8.8$ min; LC/MS [M + H$^+$]: 996.2. The attempts of separation of masses containing the cyclic **EM 7** by preparative HPLC failed.

## Synthesis of EM 8: Fmoc-c(Glu-Cys-Thr-Trp-Gly-Gly-Thr-Lys)-NH$_2$

The peptide was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (0.698 g, loading 0.43 mmol/g). The side-chain protections were as follows: Fmoc-Glu(Allyl)-Cys(Trt)-Thr(tBu)-Trp(Boc)-Gly-Gly-Thr(tBu)-Lys(Alloc)-*PAL-PEG-PS resin*. On-resin removal of Alloc/Allyl protections was performed according to the protocole D. The side-chain protections and resin cleavage were performed according to Protocol H. MS$_{calc.}$1102.22; $t_R = 9.6$ min; LC/MS [M + H$^+$]: 1102.3. The crude product was lyophilized and used for further cyclization (297 mg, 0.27 mmoles, yield = 94%). Linear product was purified twice using preparative HPLC (gradient : 70 to 50% $H_2O$ in 30 min) and lyophilized. The cyclization in solution was performed in coupling mixture (HOBT/PyBOP/DIEA) (5/5/10) as described for **EM 5** synthesis. Dimerization was observed during the cyclization step. **EM 8**: MS$_{calc.}$1084.2; $t_R$ = 10.1 min; LC/MS [M + H$^+$]: 1084.6. **EM 8**$_{dimer}$: MS$_{calc.}$1083.2; $t_R = 11.5$ min; LC/MS [M + 2H$^+$]: 1083.8. The dimer partially precipitated in HPLC solvent system. Determination of dimerization ratio was impossible. **EM 8** was purified twice using preparative HPLC in isocratic mode (80% $H_2O$) and lyophilized. Final monomer pure product was obtained in 2% overall yield (21 mg, 0.02 mmole).

## Synthesis of EM 10: Fmoc-c(Glu-Cys-Thr-AzaTrp-Gly-Gly-Thr-Lys)-NH$_2$

Linear **EM 10** was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (0.698 g, loading 0.43 mmol/g). The side-chain protections were as followed: Fmoc-Gly-Gly-Thr(tBu)-Lys(Alloc)-*PAL-PEG-PS-Resin.* MS$_{calc.}$666.72; $t_R = 10.2$ min; LC/MS [M + H$^+$]: 667.1. Resin-bound tetrapeptide was treated like described in Protocol E. New resin substitution was estimated (Protocol C) (loading 0.35 mmol/g). After Fmoc deprotection Fmoc-AzaTrp-OH was manually bound. The reagents were dissolved DMF (5 ml) and added to swollen resin (FmocAzaTrp-OH/PyBop/HOBT/DIEA) (2.5/5/5/10). The mixture was stirred (3.5 h). MS$_{calc.}$853.92; $t_R = 9.2$ min; LC/MS [M + H$^+$]: 854.4. Further synthesis was performed in an automated way (Pioneer apparatus). The side-chain protections were as follows: Fmoc-Glu(Allyl)-Cys(Trt)-Thr(tBu)-AzaTrp-Gly-Gly-Thr(tBu)-Lys(Alloc)-*PAL-PEG-PS-Resin*. On-resin removal of Alloc/Allyl protections was performed according to Protocol D and the side-chain protections and resin cleavage according to Protocol H. MS$_{calc.}$1103.21; $t_R$ = 8.6 min; LC/MS [M + 2H$^{++}$]: 552.1. Linear product was purified using preparative HPLC in isocratic mode (70% $H_2O$) and lyophilized. Solution phase cyclization of AzaTrp-containing peptide was carried out on the remaining part of the linear precursor in HOBT/PyBOP/DIEA (5/5/10) mixture. MS$_{calc.}$1085.19; $t_R = 9.1$ min; LC/MS [M + H$^+$]: 1084.4. Cyclic peptide was purified using preparative HPLC in isocratic mode (70% $H_2O$) and lyophilized (3 mg, 7.9 µmoles, yield: 2.3%). The **EM 10**$_{dimer}$ was not detected.

## Synthesis of EM 11: Fmoc-c(Glu-Cys-Thr-(D)Trp-Gly-Gly-Thr-Lys)-NH$_2$

Linear **EM 11** was synthesized using a general SPPS procedure (Pioneer apparatus) with commercially available Fmoc-PAL-PEG-PS resin (1 g, loading 0.35 mmol/g). The side-chain protections were as follows: Fmoc-Glu(Allyl)-Cys(Trt)-Thr(tBu)-*D*-Trp(Boc)-Gly-Gly-Thr(tBu)-Lys(Alloc)-*PAL-PEG-PS resin*. On-resin removal of Alloc/Allyl protections was performed according to Protocol D, and the side-chain protections and resin cleavage according to Protocol H. MS$_{calc.}$1102.22; $t_R$ = 6.81 min; LC/MS [M + H$^+$] 862.5 (loss of Fmoc and subsequent pGlu formation); $t_R = 9.573$ min; LC/MS [M + H$^+$]: 1102.3. Linear product was purified using preparative HPLC (from 80 to 55% $H_2O$ in 35 min) and lyophilized. The cyclization in solution was performed in HOBT/PyBOP/DIEA (5/5/10) mixture. MS$_{calc.}$1084.2; $t_R = 11$, 17 min; LC/MS [M + H$^+$]: 1084.2. **EM 11** was purified using preparative HPLC in isocratic mode (65% $H_2O$) and lyophilized. The **EM 11**$_{dimer}$ was not detected.


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