Synthesis and Characterization of Octapeptide Somatostatin Analogues with Backbone Cyclization: Comparison of Different Strategies, Biological Activities and Enzymatic Stabilities [1])

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Abstract. Somatostatin octapeptide analogues of the general sequence DPhe^5-Phe^6-Tyr^7-DTrp^8-Lys^9-Val^{10}-Phe^{11}-Thr^{12}-NH$_2$ containing two types of backbone cyclization have been synthesized by the solid phase methodology. Backbone cyclization in these peptides was achieved *via* N-modified phenylalanines in position 6 and 11. The N-modified amino acids were incorporated as dipeptide building units which have been prepared in solution prior to the solid phase synthesis. Two dipeptide units of structure a) Fmoc-aa$_1$ Ψ[CO–N((CH$_2$)$_n$-X)]Phe–OH or b) Fmoc-aa$_1$ Ψ[CH$_2$–N(CO(CH$_2$)$_n$-X)]Phe–OH have been introduced into the peptide sequence. Different resins and linkers were examined for an optimized peptide assembly and monitoring. The synthesized somatostatin analogues are highly resistant against enzymatic degradation as determined *in vitro* by incubation with rat liver homogenate. The biological activity was determined in binding experiments to the somatostatin receptors expressed in CHO- or BON-1 cells. Most analogues show moderate activity without differentiation between the receptor subtypes.

In recent years there was considerable interest in the synthesis of conformationally restricted bioactive peptides [1–3]. Due to the reduced flexibility of the peptide backbone these constrained structures are expected to possess improved potency, receptor subtype selectivity and enhanced resistance against proteolytic degradation. Besides other cyclization approaches backbone cyclization has been proven a promising concept for introducing conformational constraints on peptides without affecting the amino acid side chains [4]. To achieve N-backbone cyclization two amide nitrogens of the peptidic backbone have to be modified by alkylation. A variety of N-functionalized amino acids have been prepared and incorporated in peptide sequences [5–9]. To circumvent difficult coupling steps during peptide synthesis using the solid phase methodology we have developed a method to introduce different types of N-functionalized dipeptide building units which are prepared in solution before their use in SPPS [10, 11]. We were further interested in the application of the method of backbone cyclization to somatostatin analogues to probe our synthetic approach of incorporating preformed di-

peptide units as well as to produce analogues with bioactivity, receptor subtype selectivity and enzymatic stability.

The cyclic tetradecapeptide somatostatin (SST-14, SRIF) which was first isolated from ovine hypothalamus [12] is a potent inhibitor of endocrine and exocrine secretion of several hormones including growth hormone, glucagon, insulin and gastrin [13–15]. It also regulates many other physiological activities and is considered an inhibitor of tumor cell growth [16] through binding to its specific cell surface receptors [17–19]. Since SRIF and somatostatin-28, a 14 residues longer native form [13], bind to the five known receptors with low selectivity extensive structure-activity-studies have been carried out with a variety of synthetic analogues derived from small peptide structures [20–22]. In both native peptides the Phe^7-DTrp^8-Lys^9-Thr^{10}-moiety has been disclosed as the pharmacophore sequence essential for biological activity of the molecule [20]. This core sequence allows further modifications and therefore a substitution of Phe^7 and Thr^{10} by Tyr and Val, respectively, was found out to lead to biologically active com-

pounds, too [23]. Except of that, the side chains of Phe[6] and Phe[11] are considered to play an important role for receptor affinity through hydrophobic interactions of their aromatic groups [24]. A combination of the Tyr[7]-DTrp[8]-Lys[9]-Val[10]-core sequence and *N*-alkylated phenylalanines in position 6 and 11 led to our lead structure for backbone cyclic somatostatin octapeptide analogues DPhe-Phe-Tyr-DTrp-Lys-Val-Phe-Thr-NH$_2$. Cyclization in these peptides was achieved *via* a lactam bridge between the *N*-functionalized phenylalanine residues in position 6 and 11. A similar approach was used by Gilon *et al.* and was recently shown to lead to a receptor selective heptapeptide analogue of somatostatin [25].

We herein report the study of different synthetic strategies, including different protecting group combinations and the use of different resins and linkers, to gain backbone cyclic peptides on the solid phase. These peptides differ in the type of backbone modification containing either an *N*-alkylated peptide bond or an *N*-acylated reduced peptide bond [10, 11] and due to the different chain length of the *N*-functionalities of the modified phenylalanines in the ring size and in the direction of the lactam bridge.

Results and Discussion

Peptide Synthesis

Two types of backbone modification (Scheme 1) have been introduced in somatostatin octapeptide analogues.

a)

R^2

X = -COOAll, -COO^{t}Bu
Y = -NHAloc, -NHBoc

b)
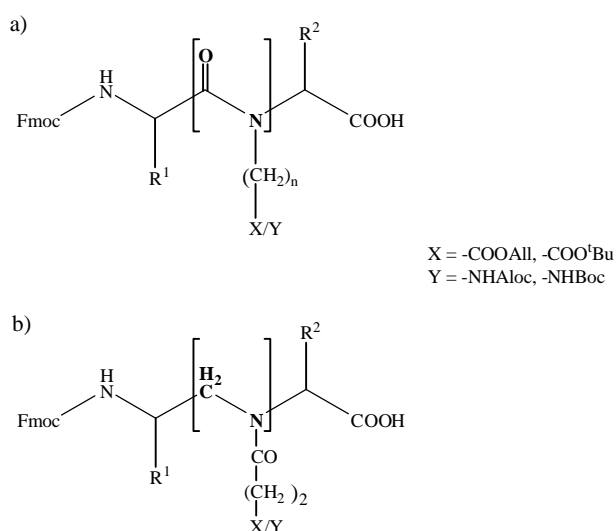
Scheme 1 Structures of *N*-functionalized dipeptide units a) and dipeptide units with an acylated reduced peptide bond b)

For the assembly of the peptides on the solid support the Fmoc-strategy seemed to be the most suited due to milder conditions for repeated cleavage compared to

the Boc-strategy and therefore more gentle for modified peptide bonds. The dipeptide building units were thus prepared carrying the Fmoc-group as the *N*-terminal protection [10, 11].

Since orthogonality of the protecting groups is a prerequisite in solid phase synthesis we had to examine different protecting group combinations for the side chains of the trifunctional amino acids in the sequence and the *N*-functionalities of the building units in position 6 and 11. For this purpose the Bzl/Z, Boc/*t*-Bu or Aloc/All-protection can be applied (Scheme 2). According to our lead structure the peptides should be obtained as *C*-terminal amides. The use of different resins and linkers was therefore also one of our concern.
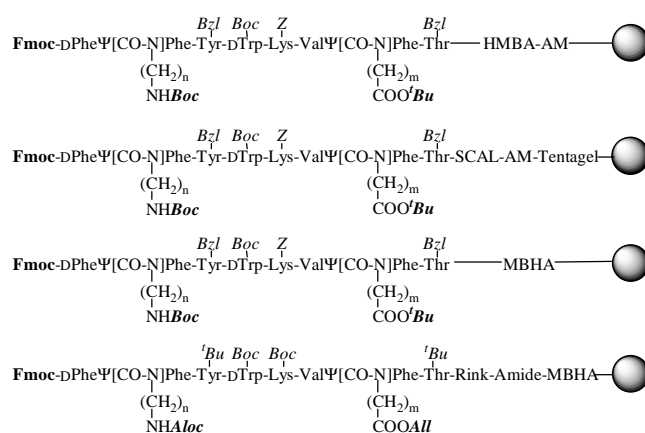
Scheme 2 Different protecting group strategies for the synthesis of somatostatin analogues with *N*-functionalized dipeptide building units

Generally, in peptide synthesis the selection of the linker and the resin as well as the loading capacity of the solid support are very important factors with regard to the assembly of difficult sequences and possible effects of aggregation. In our analogues there is a high potential of amino acids like Val, Phe and bulky side chain protecting groups expected to lead to aggregation during the solid phase synthesis and thus reducing the coupling yield. From the variety of linkers and resins available for the synthesis of peptide amides by the Fmoc-strategy we have chosen the following linker/resin-combinations for our investigation.

1. *HMBA-AM*-resin which allows the preparation of peptide carboxyamides through cleavage with methanolic ammonia [26].

2. *SCAL-AM*-Tentagel, a safety-catch type of acid labile linkage for the synthesis of *C*-terminal amides developed by Patek and Lebl [27], is based on the benzhydrylamine-linker. This linker enables the peptide assembly according to the Fmoc-, Aloc- or Boc-strategy and is here attached to the Tentagel-support, a polystyrene-polyoxyethylene graft copolymer which is sug-

gested for the assembly of difficult sequences because of its physical properties, *e.g.* low loading, good accessibility of growing peptide chains and avoidance of intermolecular migrations and reactions [28]. Additionally, the SCAL-linkage which is stable to basic conditions (piperidine for Fmoc-deprotection) as well as conditions used for Aloc/All-deprotection (Pd⁰-treatment) is suited for our purpose since different groups reported on the lability of *N*-alkylated peptides towards strong acidic cleavage, such as TFMSA or HF [29, 30].

3. *Rink-amide-MBHA*-resin is a resin also recommended for the synthesis of peptide amides by the Fmoc-strategy. Cleavage and deprotection of this kind of resin can be performed using different TFA-mixtures. The MBHA-resin in combination with the Rink-amide linker led to a solid support with excellent physical and swelling properties and was reported to be a good choice for the synthesis of peptide amides [31, 32].

To study and compare the different linkers and resins the backbone cyclic octapeptide J1712 (Table 1) was synthesized as an example on these different resins (Scheme 2). In case of the HMBA-linker the peptide was assembled with Boc/*t*-Bu-protection for the *N*-aminoalkyl or *N*-carboxyalkyl chains of the pseudopeptide moieties and Z/Bzl-protection for the side chains of the

trifunctional amino acids. The peptide was removed from the resin with methanolic ammonia and hydrogenolytically deprotected from the Z/Bzl-groups. However, this strategy of assembly and deprotection had some disadvantages. The high concentration of ammonia led to a partial peptide fragmentation with more than one *N*-terminal amino acid as was estimated by Edman degradation.

Compared to the HMBA-linker the SCAL-linker allows a peptide cleavage under very mild conditions $(P(C_6H_5)_3/(CH_3)_3SiCl$ and TFA). Because of a possible O→N migration of the Aloc-group from the Thr-side chain to the *N*-terminus the combination of Fmoc-, Z/Bzl- and Boc/*t*-Bu-protection was used for the peptide assembly on SCAL-AM-Tentagel (Scheme 2). After backbone cyclization and Fmoc-deprotection the peptide was removed from the resin. Unfortunately, a complete cleavage from the resin required long and repeated treatment with $P(C_6H_5)_3$ and $(CH_3)_3SiCl$ followed by a mixture of 50% TFA in methylene chloride. The following hydrogenolytic removal of the benzyl protecting groups using $H_2/Pd(CH_3COO)_2$ in acetic acid could not be performed with satisfying yields. Except of the synthetic problems it was found that the use of Tentagel led to a loss of resin under the shaking condi-
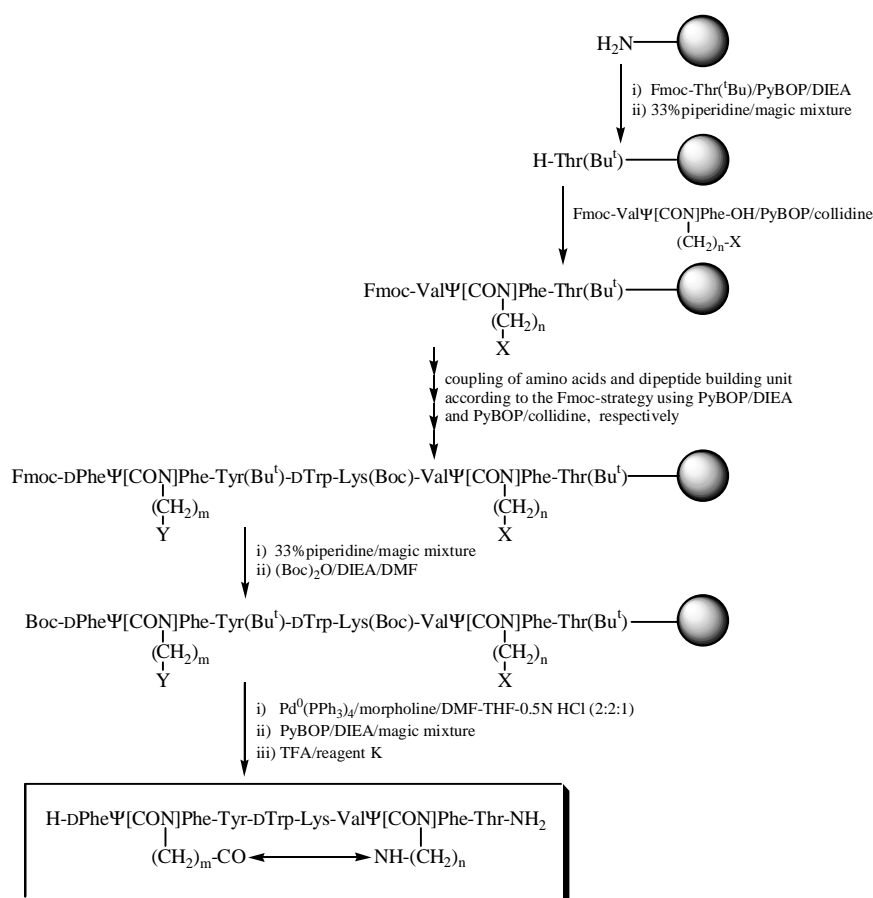
Scheme 3 Solid phase synthesis of a somatostatin analogue containing dipeptide building units of structure (a)

tions of a MPS-396 automated peptide synthesizer probably due to mechanical crush.

The assembly of J1712 on the MBHA-resin was performed with Fmoc-, Z/Bzl- and Boc/*t*-Bu-protection in the same manner as described above (Scheme 1). Because of a degradation of the amino acid Trp by TFM-SA the peptide cleavage had to be performed with HF. The peptide was obtained with good yield, but in some cases by-products which still contained protecting groups were formed.

To circumvent the use of HF or hydrogenation steps and consequently the use of Z/Bzl-protection the change to another protecting group combination for the side chains as well as the dipeptide building units was required. In fact, the unsatisfying results of the first J1712-syntheses and the reported difficulties for a racemization-free synthesis of the *N*-functionalized dipeptide building units [10] led us to the preferation of the Aloc/All-protection for the *N*-functionalized chain of the dipeptide units. In this case the side chains of the trifunctional amino acids can be protected by Boc/*t*-Bu during SPPS (Scheme 2). The first results for the synthesis of J1712 made the Rink-amide-MBHA-resin our preferred resin for the other peptides.

For the new synthetic strategy (Scheme 3) it was necessary to change the *N*-terminal protection prior to the cyclization step which was carried out on the solid phase due to a partial cleavage of the Fmoc-group during Aloc/All-deprotection under basic conditions as was reported by Gothe *et al*. [33]. The remaining cyclic polymer-bound peptide now only contained Boc/*t*-Bu-protecting groups which were removed by treatment with a TFA/reagent K-mixture.

Regarding the tested coupling reagents and solvents (NMP, DMF, magic mixture) best results were obtained with PyBOP and magic mixture, respectively. Magic mixture which is recommended for the synthesis of difficult sequences with low yields caused by aggregation and H-bond formation between peptide chains [34] was used as well as for the Fmoc-deprotection and for the coupling steps on the solid support.

The octapeptides containing lactam bridges between different *N*-functionalized dipeptide units can smoothly be formed using the herein described synthetic strategy not depending on the type of modification, ring size or direction of the lactam bridge.

Analytical Characterization

The synthesized SST-octapeptide analogues are summarized in Table 1.

These peptides have been characterized by HPLC, TLC, MALDI-MS and amino acid analysis (Table 2).

During the semipreparative HPLC-separation of some of the peptides we found fractions of the desired prod-

Table 1 Synthesized backbone cyclic octapeptide analogues of somatostatin

peptide number	structure
J 1709	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ (CH$_2$)$_2$–NH ———————— CO–CH$_2$
J 1712	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ CH$_2$–CO ———————— NH-(CH$_2$)$_3$
J 1715	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ CH$_2$–CO ———————— NH-(CH$_2$)$_2$
J 1719	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ (CH$_2$)$_3$-NH ———————— CO–CH$_2$
J 1729	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ (CH$_2$)$_3$-NH ———————— CO-(CH$_2$)$_2$
J 1729-D[7]	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]DPhe-Thr-NH$_2$ (CH$_2$)$_3$-NH ———————— CO-(CH$_2$)$_2$
J 1738	DPhe Ψ[CO–N]Phe-Tyr-DTrp-Lys-Val Ψ[CO–N]Phe-Thr-NH$_2$ (CH$_2$)$_2$-CO ———————— NH-(CH$_2$)$_3$
J 1742	DPhe Ψ[CH$_2$–N]Phe-Tyr-DTrp-Lys-Val Ψ[CH$_2$–N]Phe-Thr-NH$_2$ CO-(CH$_2$)$_2$-NH ——— CO-(CH$_2$)$_2$-CO
J 1746	DPhe Ψ[CH$_2$–N]Phe-Tyr-DTrp-Lys-Val Ψ[CH$_2$–N]Phe-Thr-NH$_2$ CO-(CH$_2$)$_2$-CO ——— NH-(CH$_2$)$_2$-CO

uct with different retention times but the same molecular weight.

Racemization and isomerization can be indicated by double or multiple peaks in HPLC-analysis. Contrary to Gilon *et al*. [25] who found isomerization of the bond between Val[5] and the *N*-alkylated Phe[6] in their somatostatin-analogue PTR3046 we would exclude a possible *cis/trans*-isomerization based on HPLC-studies at elevated temperatures. In these studies no changes in the peak pattern of the analyzed peptides were observed. We therefore assume that in some of our analogues racemization occurred during the solid phase synthesis. Racemization may occur during the coupling of the dipeptide building units. According to the proposed mechanism for the racemization of *N*-alkyl amino acids [35] the *C*-terminal amino acid in the dipeptide units may partially or completely racemize. The racemization in the described SST-octapeptide analogues is dif-

Table 2 Analytical characterization of somatostatin analogues

No.	M_W (g/mol) calcd.	found	t_R (min) [a]	R_{fA}	R_{fB}	AAA [b]
J 1709	1219.46	1220.4 (M+H)+	30.47	0.52	0.72	n.d.
J 1712	1233.64	1234.3 (M+H)+	32.61 33.95	0.54	0.78	Thr 1.00 (1), Val 0.94 (1), Tyr 0.49 (1), Phe 0.97 (1), Lys 0.90 (1)
J 1715	1219.46	1219.9 (M+H)+	33.85	0.53	0.71	Thr 1.00 (1), Val 0.97 (1), Tyr 0.52 (1), Phe 0.93 (1), Lys 0.95 (1)
J 1719	1233.64	1234.9 (M+H)+	33.27	0.59	0.73	Thr 1.00 (1), Val 0.96 (1), Tyr 0.47 (1), Phe 0.98 (1), Lys 0.98 (1)
J 1729	1246.65	1270.8 (M+Na)+	31.48 32.45 35.28 35.91	0.52	0.73	Thr 1.00 (1), Val 1.04 (1), Tyr 0.49 (1), Phe 0.89 (1), Lys 1.76 (1)
J 1729-D[7]	1246.65	1270.3 (M+Na)+	31.48 35.13	0.51	0.73	Thr 1.00 (1), Val 1.02 (1), Tyr 0.76 (1), Phe 0.96 (1), Lys 1.65 (1)
J 1738	1246.65	1248.2 (M+H)+	32.78	0.55	0.72	Thr 1.00 (1), Val 1.08 (1), Tyr 0.56 (1), Phe 1.00 (1), Lys 1.82 (1)
J 1742	1261.42	1262.1 (M+H)+	34.26 34.79 35.27 36.09	0.54	0.69	Thr 1.00 (1), Tyr 0.80 (1), Lys 1.71 (1), β-Ala 0.79 (1)
J 1746	1261.42	1262.7 (M+H)+	34.76 35.25 36.35 37.27	0.50	0.65	Thr 1.00 (1), Tyr 0.54 (1), Lys 1.81 (1), β-Ala 0.68 (1)

[a] HPLC-conditions: 10% – 70% acetonitrile/0,1% TFA; flow rate 1.0 ml/min; detection 220 nm. [b] AAA: amino acid analysis; n.d.: not determined

ficult to analyze. Our attempts with chiral columns or formed diastereomers for the separation of *D*- and *L*-isomers of *N*-aminoalkyl or *N*-carboxyalkyl phenylalanine failed. Only the incorporation of the dipeptide building units containing either *D*- or *L*-configuration of the *C*-terminal *N*-alkylated phenylalanine in the octapeptide analogue J1729 gave a HPLC-peak pattern similar in retention times but different in the altitude of the peaks. Collected peaks from the semipreparative HPLC give only single peaks in the analytical HPLC and not the same peak pattern as the starting material. All findings indicate that racemization occured rather than *cis/trans*-isomerization.

We also found that the racemization rate can be reduced by the use of collidine instead of DIEA for the coupling of the dipeptide units. Thus, each dipeptide building unit was coupled with this base.

For the characterization of our backbone cyclic peptides another problem occurred which was the amino acid analysis of *N*-alkylated amino acids and pseudodipeptides with a reduced peptide bond, respectively. After the hydrolysis of octapeptides J1728 and J 1738 for example *N*-aminopropyl and *N*-carboxyethyl Phe were obtained besides the other amino acids. In case of

these peptides an increased value for the amino acid Lys (Table 2) was found. A hydrolysis and amino acid analysis of the corresponding dipeptide building units showed that *N*-aminopropyl Phe was eluted with the same retention time as Lys (Figure 1), whereas no peak could be detected for *N*-carboxyethyl Phe due to its poor solubility and *N*-aminoethyl Phe. *N*-carboxymethyl Phe was found in the amino acid analysis, but could not be quantified.

For the peptides J1742 and J1746 which contain the backbone modification of type b) β-Ala and the pseudodipeptides are additionally obtained as hydrolysis products. Here, also an increased value for Lys was determined in the amino acid analysis (Table 2). Hydrolysis and analysis of the two H-ValΨ[CH$_2$NH]Phe–OH derived dipeptide building units confirmed this finding (Figure 1). A corresponding peak for H-PheΨ[CH$_2$NH]Phe–OH was not detected.

Biological Activity

The affinity of the octapeptide analogues to the somatostatin receptors was determined in a receptor binding assay [25]. In this assay the tested analogues compete
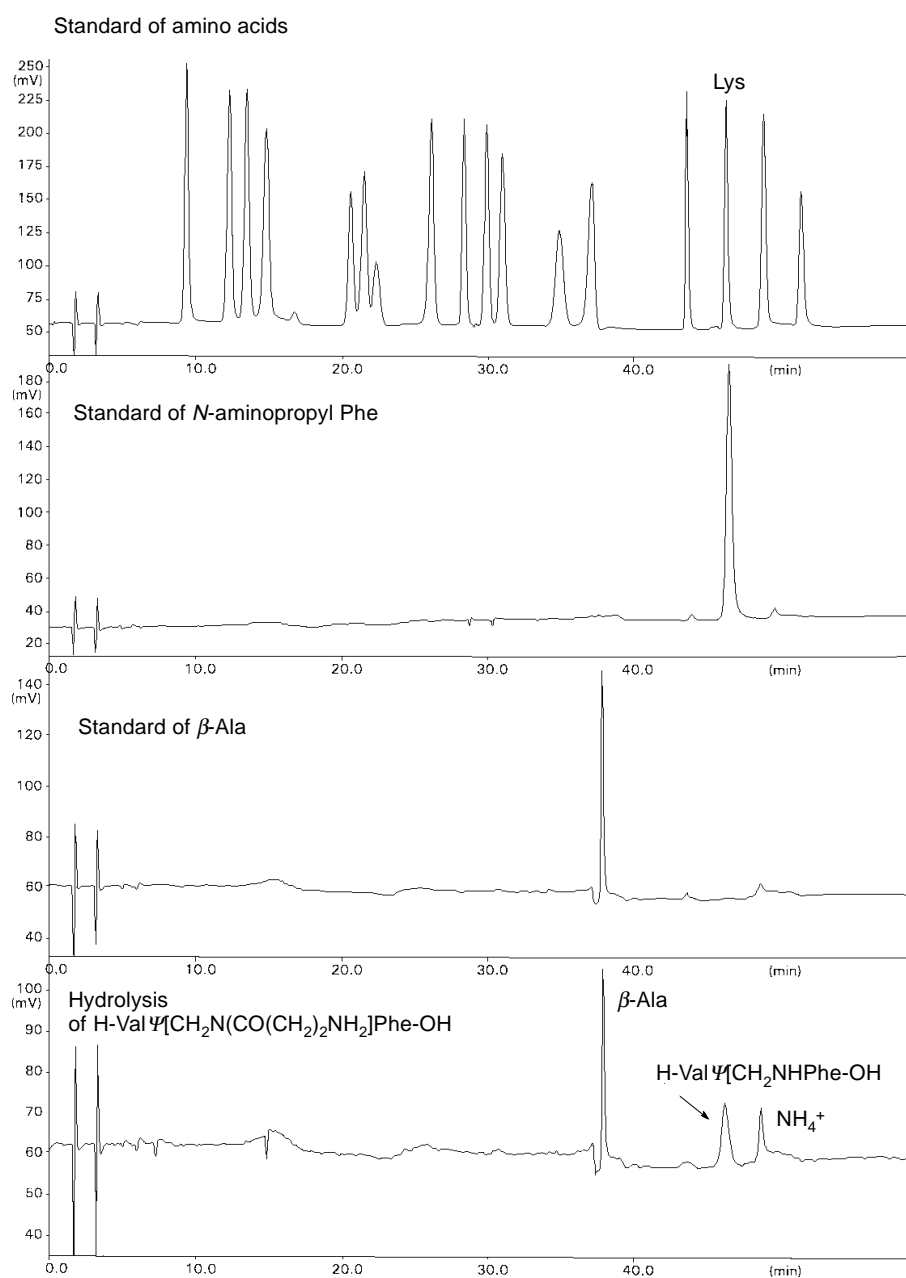
Fig. 1 Amino acid analysis: identification of *N*-aminopropyl Phe and H-ValΨ[CH₂NH]Phe-OH with the retention time of Lys

with the binding of radiolabeled somatostatin-14 ([^{125}I] Tyr[11]-SRIF) to cloned receptors expressed in CHO-cells (containing either sst1, sst2, sst3 or sst5) and in BON-1-cells which naturally contain the receptors sst1, sst2, sst3 and sst5. After incubation of the cell membranes with different concentrations of each peptide and [^{125}I]Tyr[11]-SRIF the binding was determined on the basis of the remaining radioactivity. Nonspecific binding was defined as the amount of radioactivity which remains in the presence of 1 μM unlabeled ligand. The IC$_{50}$-values are those concentrations which are required

to reduce the SRIF binding by 50%. The competitive binding experiments were carried out with three different concentrations (10^{-6}, 10^{-7}, 10^{-8} M) of each peptide fraction.

The estimated IC$_{50}$-values for most of the analogues and receptor subtypes were in between 10^{-5}–10^{-7} M indicating a moderate bioactivity in the range of other reported analogues [24, 25]. However, an excellent receptor subtype selectivity as found for very potent analogues such as *Octreotide* [22] was not observed for our analogues. The highest affinity shows analogue

J1729 on BON-1 cells with an IC_{50}-value of 10^{-7} M. The peptides J1709 and J1712 are more active at the receptor subtypes 1 and 2 than on the others. For J1729 and J1738 IC_{50}-values in the range of 10^{-6} M at the subtypes 3 and 5 and at BON-1 cells were obtained. The analogue containing acylated reduced peptide bonds J1746 has a higher affinity to subtypes 2 and 3 than to the others.

From the obtained values it is difficult to draw conclusions about structure-activity relationships, but it seems that neither the type of backbone modification nor the direction of the lactam bridge have an influence on the affinity and selectivity to the receptor subtypes.

Enzymatic Stability

The proteolytic stability of the somatostatin analogues against proteases of rat liver homogenate was measured with HPLC. The liver homogenate contains a mixture of different proteases and is therefore used for estimation of a more general stability against proteolytic degradation than against distinct proteases. Somatostatin-14 was used to compare the synthesized peptides with the naturally occurring compound which is degraded by proteolytic enzymes within minutes. The proteolytic activity of the liver homogenate was estimated by a modified azocasein test [37] and the protein content of a homogenate probe used for the incubation was determined by the Lowry-method [38].

After incubation of each peptide with the liver homogenate and several work-up procedures the samples were analyzed by HPLC. Figure 1 shows the biostabil-

Fig. 2 HPLC-analysis of somatostatin-14 and octapeptide J1738 after treatment with rat liver homogenate

ity of the somatostatin analogues exemplified on J1738 compared to somatostatin-14. In contrast to the native hormone which is completely degraded at least after 1 h no degradation was found for our analogues even after an incubation time of 15 h. This study indicates that backbone cyclic peptides containing different types of backbone modification (Scheme 1) are highly resistant to proteolytic degradation.

Conclusion

An optimized synthetic strategy for the preparation of backbone cyclic somatostatin octapeptide analogues on the solid phase has been developed. By comparison of different strategies using different protecting group combinations, different linkers and resins the best results were obtained by the Fmoc-strategy with Aloc/All-protection for the *N*-functionalized peptide bonds, Boc/*t*-Bu-protection for the side chains of trifunctional amino acids and by the use of Rink-amide-MBHA-resin. To avoid incomplete couplings of amino acids to *N*-alkyl amino acids preformed dipeptide building units with an *N*-functionalized peptide bond or an acylated reduced peptide bond were successfully used to synthesize the octapeptides. This strategy allows monitoring of the assembly at every step by removing an analytical amount of peptide from the resin. Coupling of the *N*-modified dipeptide units may lead to racemization of the *C*-terminal amino acid. The lactam bridges in the octapeptides between *N*-functionalized dipeptide units can be formed smoothly not depending on the type of modified peptide bond, ring size or direction of the lactam bridge.

As an important result of this study it was found that backbone cyclization stabilizes the tested octapeptides against enzymatic degradation. Even in a mixture of different proteases existing in rat liver homogenate they are stable over a period of at least 15 h.

The biological activities which were tested in radioligand binding studies on cell lines with different somatostatin receptor subtypes are in the same range as found for other published analogues, but do not show a sufficiently pronounced subreceptor selectivity.

Based on the very high stability against enzymatic degradation we are interested to extend the structure-activity-studies with new backbone cyclic octapeptides and with selective pharmacological tests to develop more potent and selective analogues.
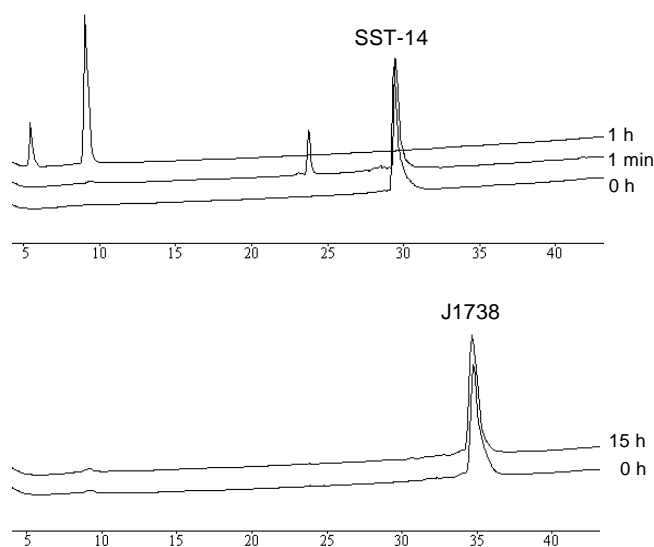
Experimental

Materials

Peptide assembly was performed on Rink-amide-MBHA-resin purchased from Novabiochem by the solid phase method on a semiautomated Bachem Peptide Synthesizer SP 650. Amino acid derivatives and coupling reagents were purchased from Orpegen (Heidelberg, Germany) and Novabiochem (Bad Soden, Germany). All materials and solvents were of reagent grade and were used without further purification with the following exceptions: DMF was first dried over molecular sieves and distilled from phthalic anhydride, DCM was stored over molecular sieves. The *N*-functionalized dipeptide building units were prepared as described elsewhere [10, 11].

Thin layer chromatography of peptides was performed on precoated glass plates (silica gel 60 F_{254} Merck, Darmstadt, Germany) using the solvent systems A) *n*-butanol/acetic acid/water (4:1:1) and B) *n*-propanol/25% ammonia (7:3). The peptides were visualized with UV and ninhydrine.

Methods

General SPPS protocol

The octapeptide analogues of somatostatin were synthesized by the Fmoc-strategy using PyBOP as the coupling reagent. The Fmoc-group was removed with 33% piperidine/magic mixture (1% Triton X 100 in piperidine/DMF/NMP, 1:1:1) twice (10 and 20 min). Couplings were performed using 4 equiv. of Fmoc-amino acid and 4 equiv. of PyBOP in the presence of 8 equiv. DIEA in magic mixture (DCM/DMF/NMP, 1:1:1, containing 1% Triton X 100 and 2M ethylene carbonate) for 4 h and repeated in case of incomplete couplings. The dipeptide building units were coupled in 2 fold excess with 2 equiv. of PyBOP and 4 equiv. of collidine. The assembly was monitored by the qualitative Kaiser-test and HPLC-analysis of cleaved probes. Remaining unreacted amino groups after two couplings were capped with 2 equiv. of Z(2Cl)–OSu and DIEA in DCM/NMP (1:1) for 30 min. All deprotection and coupling steps were followed by intensive washings using DMF and DCM. After the final Fmoc-deprotection the Boc-group was introduced with 5 equiv. of (Boc)$_2$O and 10 equiv. of DIEA in DMF for 2 h. The loaded resin was then washed and dried in vacuo for the following deprotection of the Aloc/OAll-groups which was carried out using 2 ml of DMF, 2 ml of THF, 1 ml of 0.5N HCl, 900 µl of morpholine and Pd^0(PPh$_3$)$_4$ as the catalyst for 500 mg resin. The reaction time was 16 h. The catalyst was removed by washing the resin with DCM, DMF and ethanol. For the cyclization 6 equiv. of PyBOP and 12 equiv. of DIEA in magic mixture were used with a cyclization time of 2–4 h. In some cases cyclization had to be repeated. Cleavage and deprotection of the peptide from the resin were performed according to the reagent K/TFA-cleavage protocol (for 500 mg resin: 375 mg phenol, 250 µl thioanisol, 125 µl 1,2-ethandithiol, 250 µl water and 5 ml TFA) for 2 h. The crude peptides were precipitated in cold diethyl ether, centrifugated, dissolved in 80% *tert*-butanol and lyophilized. The purified peptides were obtained in yields varying between 15–35%.

Purification of Peptides

The crude peptides were purified by semipreparative HPLC on a Shimadzu LC-8A equipped with a Vydac 218TP column (10 µm particle size, 300 Å pore size, 22 × 250 mm). The elution gradient was 15–65% B in 120 min at a flow rate of 10 ml/min, where A was 0.1% TFA in water and B 0.1% TFA in 90% acetonitrile; detection at 220 nm. Collected fractions were concentrated and lyophilized from water.

Analytical RP-HPLC

HPLC-analysis for monitoring and purity control was performed on a Shimadzu LC-10AT chromatograph with a Vydac 218TP column (5 µm particle size, 300 Å pore size, 4.6 x 25 mm). Peptides were eluted with the gradient 10–60% B in 50 min at a flow rate of 1.0 ml/min, A was 0.1% TFA in water and B 0.1% TFA in acetonitrile; detection at 220 nm.

Mass Spectrometry

The molecular weights of the octapeptides were determined by MALDI-TOF-mass spectrometry on a Laser Tec Research mass spectrometer (PerSeptive Biosystems) using α-cyano-4-hydroxycinnamic acid as matrix.

Amino Acid Analysis

Peptides were hydrolyzed with 6N HCl at 110 °C for 24 h. The hydrolysates were analyzed in an amino acid analysator LC 3000 (Eppendorf Biotronik).

Edman Degradation

Octapeptides containing dipeptide building units of the conventional type were additionally analyzed by Edman degradation on a protein sequencer LF 3000 (Beckman) with an online HPLC injection system. The phenylthiohydantoin (PTH) derivatives were identified by RP-HPLC using a supersher RP-8 column (4 µm particle size, 1.6 × 250 mm) (MZ Analysentechnik) at a flow rate of 0.2 ml/min and a temperature of 54 °C. The PTH amino acids were eluted with a four step gradient of solvent A (0.12M sodium acetate buffer, pH 3.85, containing 1 g/l octanesulfonic acid and 32.5 ml/l THF) and B (100% acetonitrile).

Metabolic Stability

Determination of Proteolytic Activity

The proteolytic activity of the liver homogenate was determined according to the literature [36]. 0.2% azocasein was incubated with liver homogenate at 37 °C for 30 min. The reaction was stopped by the addition of 10% TCA. Proteolytic activity was determined by measuring the absorbance of the degraded products at 336 nm.

Proteolytic Assay

The proteolytic stability of the octapeptide analogs against proteases of the liver was measured as follows: liver homogenate was prepared at 0–4 °C according to the procedure of Rapoport and Radebrecht [39]. The liver of Wistar rats were finely minced with surgical scissors and perfused using 0.25M saccharose buffer. The tissue (5 g) was then homogenized in 10 ml of homogenization buffer (0.1 mM sodium phosphate buffer, pH 6.5) with a Potters homogenizer (Braun). 0.5 mg

of the peptide was dissolved in 10 µl of DMSO and 490 µl of the phosphate buffer (pH 6.5) were added. The mixture was then incubated with 500 µl of the liver homogenate (protein content after Lowry [38]: 15.65 mg) at 37 °C. Incubation was stopped after different times by the addition of 20% TFA. After centrifugation the supernatant is used for HPLC-analysis.

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