Amino Acid Side Chain Attachment Approach and Its Application to the Synthesis of Tyrosine-Containing Cyclic Peptides

Chiara Cabrele, Michael Langer, and Annette G. Beck-Sickinger*

Department of Pharmacy, Pharmaceutical Biochemistry, ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

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The technique of resin loading by the attachment of the amino acid side chain represents a powerful tool for the synthesis of cyclopeptides by solid phase. We investigated the anchoring of the side chain of *N*-(9-fluorenylmethoxycarbonyl, Fmoc)-tyrosine methyl ester to benzyl-type resins by the Mitsunobu reaction. Satisfactory loading was obtained for HMPB−MBHA and Wang resins. The suitability of the preloaded resins for solid-phase peptide synthesis by using the Fmoc strategy, combined with the head-to-tail cyclization on the solid support, was illustrated by the preparation of three cyclic analogues of neuropeptide Y (NPY), a 36-residue peptide hormone and one of the most abundant neuropeptides in the brain. Each peptide contained the N- and C-terminal tetrapeptide segments of NPY, joined by different spacers: 6-aminohexanoic acid, β-alanine, or Ala-Aib. First the synthesis of the peptide methyl esters was performed, followed by saponification and cyclization on the resin. HOBt/DIC or HOBt/TBTU was used for the ring closure. The CD spectra of the three cyclopeptides in 30% trifluoroethanol showed a type I and III β-turns structure, which was already adopted by the (Ala-Aib)-containing cyclopeptide in water. The CD spectra, together with the biological assays, confirmed the suitability of these cyclopeptides as conformationally restricted peptides that may serve as lead structures in drug development.

Introduction

In the past few years, interest in solid-phase synthesis techniques has increased significantly, especially in conjunction with their application to combinatorial chemistry. Libraries of peptides and organic molecules have proven to be powerful tools for facilitating the discovery process of new lead and therapeutic compounds.[1] Linear peptides built by the natural L-amino acids are generally not suitable for use as drugs because of their poor oral availability and enzymatic degradation. For this reason, much attention is being paid at present to the construction and screening of combinatorial libraries of modified peptides, peptidomimetics, and nonpeptide compounds.[2] In particular, C-terminally modified and cyclic peptides have been shown to be diagnostically and therapeutically useful tools in all areas of biomedical research. In many cases, C-terminal peptide modifications have led to the preparation of potent agonists or antagonists, which are also resistant to proteolytic degradation.[3] Furthermore, the peptide cyclization approach has provided conformationally constrained analogues with suitable properties for investigating ligand−receptor interactions and structure−activity relationships,[4] as well as for developing drugs with increased metabolic stability and receptor selectivity.[5] The importance of the cyclic peptides has necessitated the improvement of the method of their preparation.[6] Classic solid-phase peptide synthesis does not allow the introduction of modifications at the C-terminus or the head-to-tail cyclization of peptides that are still bound to the resin. The protected peptide must first be cleaved from the solid support and then modified or cyclized in solution. This synthetic procedure presents some disadvantages, such as the necessity to isolate the desired peptide from the excess reagents, which leads in some cases to a considerable loss of the product. Furthermore, cyclization in solution requires very diluted concentrations of the linear peptide to minimize the formation of the cyclodimer and oligomers. In the early 1990s, a new strategy has been introduced for peptide synthesis on the solid phase, based on the attachment of the amino acid side chain to the solid support. So far, this method has been applied to aspartic[7,8] and glutamic[8] acids, lysine,[8] serine, and tyrosine[8,9] in the Boc strategy and to aspartic and glutamic acids[10] and lysine[11] in the Fmoc strategy with different C-terminal protecting groups.

* Phone: 41-1-63 56063; secretary: 41-1-63 56061. Fax: ++41-1-63 56884. E-mail: beck-sickinger@pharma.ethz.ch.

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Table 1. Amino Acid Sequences of the Cyclopeptides Synthesized by Combination of the Tyrosine Side Chain Attachment Approach with the Resin-Bound Peptide Cyclization

peptide	amino acid sequence[a]	no. of backbone atoms
10	cyclo-(β-Ala-Tyr-Pro-Ser-Lys-β-Ala-Arg-Gln-Arg-**Tyr**)	32
11	cyclo-(Ahx-Tyr-Pro-Ser-Lys-Ahx-Arg-Gln-Arg-**Tyr**)	38
12	cyclo-(Ala-Aib-Tyr-Pro-Ser-Lys-Ala-Aib-Arg-Gln-Arg-**Tyr**)	36

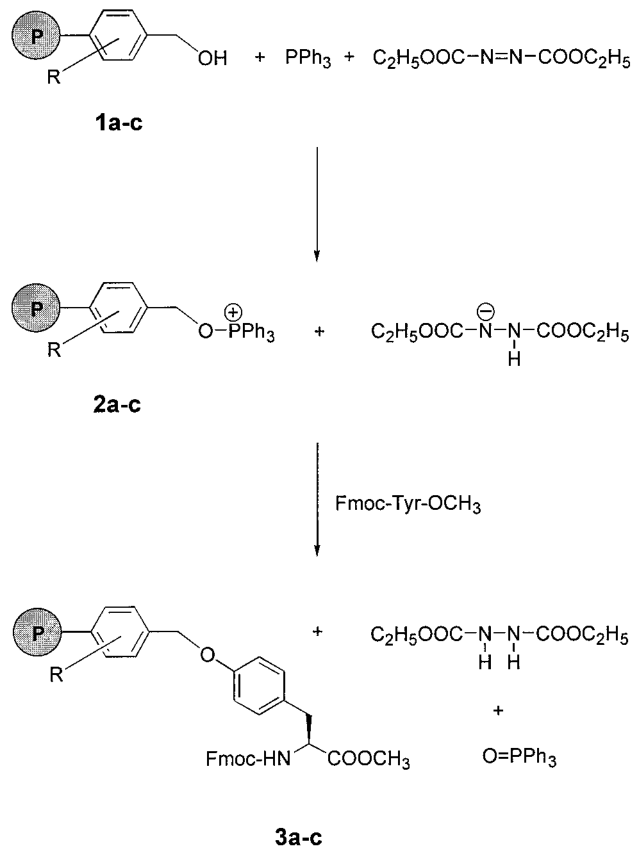
[a] The tyrosine side chain that was attached to the resin is given in bold.

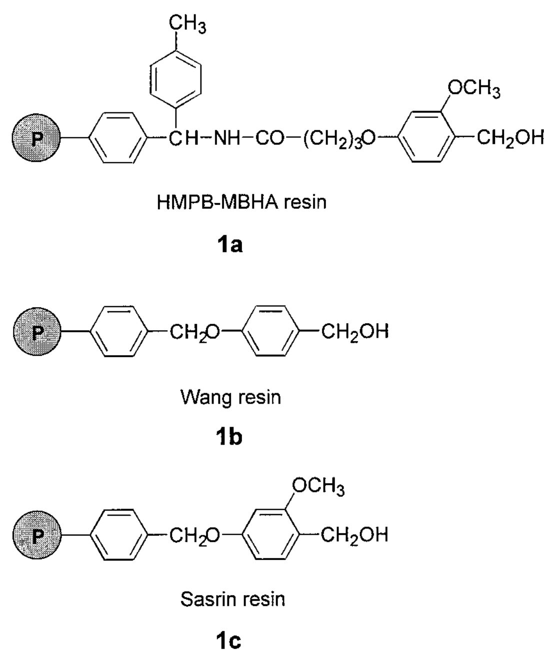
To extend this method to a broader context, we have investigated further N^{α}-Fmoc amino acids that could be suitable to be linked to the resin through their side chains. In the present work, we address the possibility of attaching the phenolic group of the tyrosine side chain to the solid support and of carrying out the synthesis and the head-to-tail cyclization of tyrosine-containing peptides on the solid phase by Fmoc chemistry. We selected three cyclic peptides, corresponding to centrally truncated analogues of the neuropeptide Y (NPY). NPY, a 36-residue peptide amide of the pancreatic polypeptide family (PP family), is widely distributed in the peripheral and central nervous systems and is one of the most abundant neuropeptides in the brain.[12] Physiological effects attributed to NPY include vasoconstriction, stimulation of food intake, inhibition of anxiety, increase of memory performance, presynaptic inhibition of neurotransmitter release, and regulation of the sexual hormones. So far, five receptor subtypes of NPY have been identified and characterized: Y_1, Y_2, Y_4, Y_5, and y_6.[13] They belong to the large superfamily of the G-protein-coupled receptors, and each of them mediates specific biological actions of NPY. It has been found that the N- and C-terminal regions of NPY are involved in the recognition and binding of the ligand to the Y-receptor subtypes.[12] To have insights into the conformational requirements for the receptor affinity and selectivity, we designed three cyclic peptides, **10**−**12**, containing the two NPY segments Tyr^1-Pro^2-Ser^3-Lys^4 and Arg^{33}-Gln^{34}-Arg^{35}-Tyr^{36}, joined by different types of spacers (Table 1). The synthesis of the cyclopeptides was accomplished totally on the solid phase, starting from the chain assembly of the corresponding linear peptide methyl esters on the resins preloaded by the tyrosine side chain attachment, followed by ester hydrolysis and head-to-tail cyclization.

Results and Discussion

Anchoring of *N*-(9-Fluorenylmethoxycarbonyl)-Tyrosine Methyl Ester to the Resin. *N*-(9-Fluorenylmethoxycarbonyl, Fmoc)-tyrosine methyl ester was attached to 4-hydroxymethyl-3-methoxyphenoxybutyric acid 4-methyl benzhydrylamine resin (HMPB−MBHA resin, **1a**), 4-benzyloxybenzyl alcohol resin (Wang resin, **1b**), and 4-benzyloxy-2-methoxybenzyl alcohol resin (Sasrin resin, **1c**) by its phenolic group under the conditions of the Mitsunobu reaction,[14] by using triphenylphosphine and diethyl azodicarboxylate (DEAD) (Scheme 1). The loading of the resin was determined by UV quantification of the Fmoc-piperidine adduct,[15] as well as by a quantitative ninhydrin test.[16]

Scheme 1. Loading of a Benzyl-Type Resin by Side Chain Attachment of Fmoc-Tyrosine Methyl Ester Using the Mitsunobu Reaction





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To improve the efficiency of the reaction, extensive variations of the reaction conditions were made. The influence of the solvent was investigated by comparing the results obtained with dichloromethane, *N*-methylmorpholine, and tetrahydrofuran. Although *N*-methylmorpholine has been reported to give the best results in coupling of the phenolic group of tyrosine to hydroxymethyl polystyrene resin,[17] the substitution levels of the Sasrin and Wang resins obtained in this solvent upon dropwise addition of diluted DEAD at room temperature were not acceptable, as they were below 0.02 mmol/g. When the etherification was repeated in dichloromethane, the yields for the Sasrin and Wang resins were increased to 0.1 mmol/g. The loading of the HMPB−MBHA resin in dichloromethane gave a substitution level of 0.17 mmol/g. It has been reported that the attachment of Boc-tyrosine *p*-nitrobenzyl ester to hydroxymethyl resin in tetrahydrofuran was successful.[8] However, the loading of the Wang resin in this solvent gave no satisfactory results in our case.

It is known that one side reaction of the Mitsunobu etherification is the thermal decomposition of DEAD with the resulting production of ethyl alcohol; the ethyl alcohol is activated by the remaining DEAD and reacts with the phenolic group of the tyrosine, yielding the corresponding ethyl ether.[18] To avoid the formation of this side product, the reaction mixture was cooled at 0 °C during the dropwise addition of DEAD. The loading obtained for the Wang resin in dichloromethane resulted in 0.23 mmol/g. Some attempts were carried out by using the more stable diisopropyl dicarboxylate (DIAD) instead of DEAD. However, the loading of the resin could not be increased.

By varying the *n*-fold excess of the reagents amino acid/triphenylphosphine/DEAD (2- to 5-fold), as well as the order of the reagent addition, no increase of the substitution level of the resins was obtained.

Taken together, these results led to the following conclusions: The efficiency of the Mitsunobu etherification of benzyl-type resins with *N*-(9-fluorenylmethoxycarbonyl)-tyrosine methyl ester depends on the ability of the linker to form the oxyphosphonium salt **2** (Scheme 1). This seemed to be favored for the HMPB−MBHA linker (**1a**), where the methoxy ortho position substituent in the phenoxy ring could stabilize the positive charge on the phosphor atom. An *o*-methoxy group is present also in the Sasrin linker (**1c**); however, the loading of this resin proved to be more difficult, maybe because of steric hindrance. In fact, the flexibility of a linker influences its solvation and therefore its propensity to undergo the reaction. The Sasrin linker is less flexible than the HMPB−MBHA linker, the latter one containing the butyric acid moiety that acts as a flexible spacer between the polymer and the reactive part of the linker. Furthermore, the initial substitution level of the Sasrin resin was higher than that of the HMPB−MBHA resin (1.06 mmol/g versus 0.44 mmol/g), and this could also contribute to the reduction of the mobility of the linker within the polymer.

The attempt to enhance the nucleophilicity of the phenolic group of the tyrosine side chain by choosing *N*-methylmorpholine as the reaction solvent failed, as shown by the unsatisfactory yields of the loading of the Sasrin and Wang resins in this solvent. In contrast, better results were obtained in dichloromethane. In the case of the Wang resin, loading was increased by carrying out the reaction at low temperature, which might prevent the partial thermal decomposition of DEAD. However, the efficiency of the reaction was not dependent on other parameters, such as the alkyl group of the oxidant (DIAD gave no better results than DEAD), the use of the reagents in a large *n*-fold excess, or the sequence of the reagent addition.

Solid-Phase Synthesis of the Peptide Methyl Esters. Three peptides with the amino acid sequence Xxx-Tyr-Pro-Ser-Lys-Xxx-Arg-Gln-Arg-Tyr were chosen for study (Table 1). Tyr-Pro-Ser-Lys and Arg-Gln-Arg-Tyr correspond to the N- and C-terminal tetrapeptide fragments of NPY, respectively. Xxx was incorporated into the sequence as a structural element and differed in each of the peptides. 6-Aminohexanoic acid (Ahx) was chosen to play the role of a flexible long spacer, β-alanine was chosen as a shorter spacer able to induce β-turns,[19] and finally the dipeptide segment alanine-α-aminoisobutyric acid (Ala-Aib) was chosen for its high propensity to build turnlike structures.[20]

The synthesis of the linear peptide methyl esters **4**−**6** was carried out on resins preloaded with the tyrosine side chain attachment, by using the Fmoc strategy and a double coupling protocol with *N*-hydroxybenzotriazole (HOBt) and *N,N*′-diisopropylcarbodiimide (DIC) in DMF (Scheme 2). Peptide ester **4** was synthesized on both the HMPB−MBHA and Wang resins, whereas the peptide esters **5** and **6** were prepared only on the Wang resin.

Hydrolysis of the C-Terminal Methyl Ester on the Resin. When the stepwise solid-phase peptide synthesis was completed, the cleavage of the C-terminal methyl ester was performed on the resin by using methanolic solutions of lithium or sodium hydroxide. Only a partial conversion of the resin bound esters **4a**,**b**, **5b**, and **6b** to the corresponding acids **7a**,**b**, **8b**, and **9b** was obtained with 0.2 M sodium hydroxide in methanol after 2 days or 0.2 M lithium hydroxide in methanol after 4 days. The esters were totally converted to the acids by employing 0.33 M lithium hydroxide in methanol/water 97/3 (v/v) for 6 days or 0.4 M sodium hydroxide in methanol for 3 days. Despite the strong basic conditions and the long reaction times, the hydrolysis of the peptide methyl esters on the resin went smoothly, and no degradation or racemization products were observed (peptide acid **7**, Figure 1A; peptide acids **8** and **9**, data not shown).

Head-to-Tail Cyclization on the Resin. To establish the optimal reaction conditions for cyclization, the resin-bound linear peptide acids **7b**, **8b**, and **9b** were cyclized by using different methods for the activation of the

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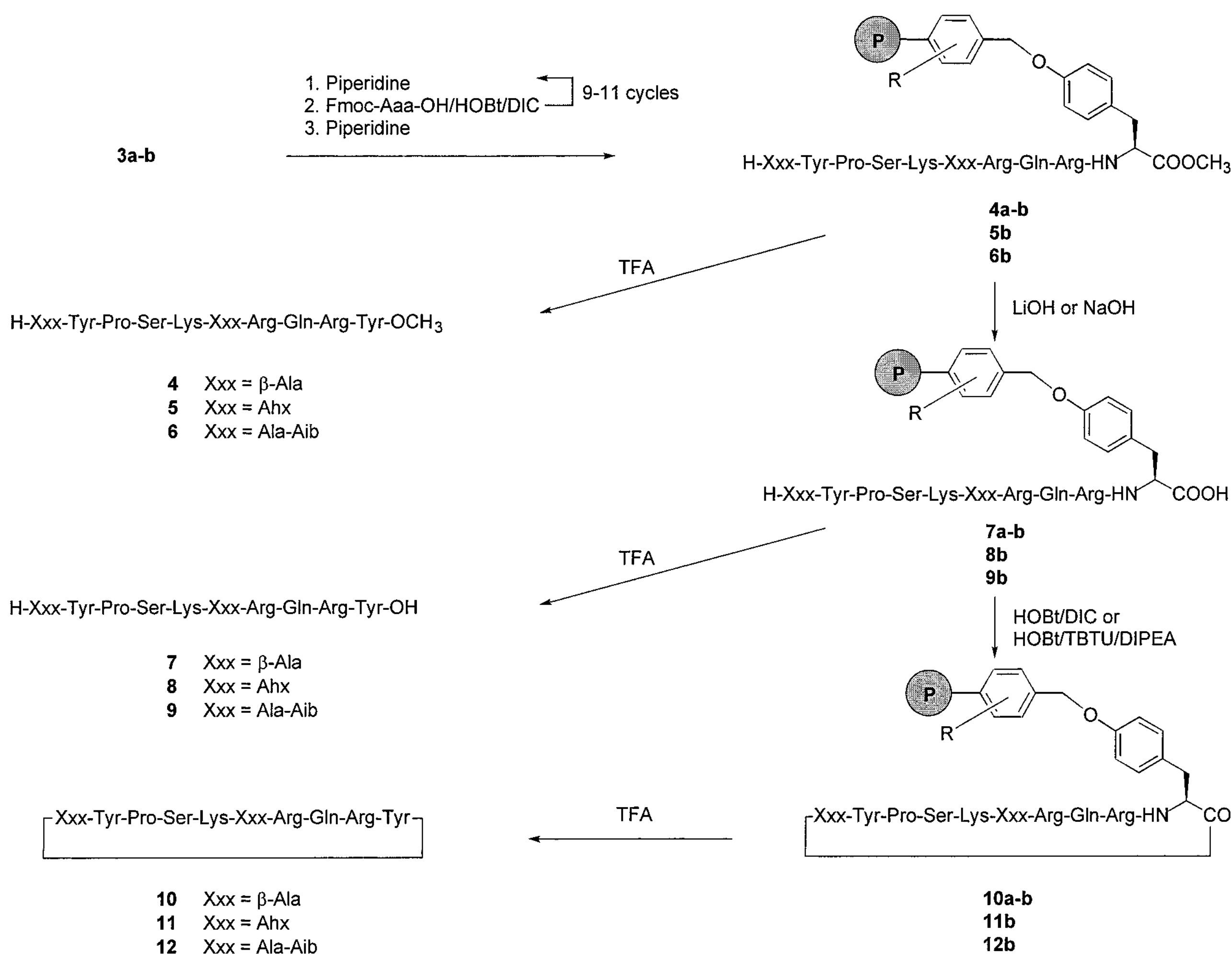
Scheme 2. Solid-Phase Synthesis of Linear and Cyclic Peptides by Amino Acid Side Chain Attachment Approach



carboxy group. The main parameters that affect the conversion rate of the resin-bound peptide acid to its reactive form are the efficiency of the activation reagent, the use of the activation reagent in a proper *n*-fold excess, and the steric hindrance of the solid support. To investigate how the efficiency and the amount of the activation reagent could influence the reaction, different *n*-fold excesses of the more efficient HOBt/TBTU (1*H*-benzotriazolium, 1-[bis(dimethylamino)-methylene]-, tetrafluoroborate (1-), 3-oxide, formerly named *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate) activation reagent and of the less effcient HOBt/DIC activation reagent were used. The total conversion of the linear peptides to the corresponding cyclic products was controlled by HPLC and ESI−MS analyses. HOBt/TBTU cyclization was faster than the HOBt/DIC cyclization; however, both reagents could drive the reaction to completion. No epimerization was observed to take place during the activation step of the cyclization reaction. The formation of tetramethylguanidinium side products, which has been reported to occur during HOBt/HBTU side chain to side chain cyclization,[21] could not be detected by ESI−MS analysis. The crude cyclopeptides obtained by the two cyclization methods showed similar HPLC profiles. In each case, the cyclic monomer (**10**−**12**) was present as the major product, whereas the minor products eluting later were attributed to the oligomeric fraction (cyclopeptide **10**, Figure 1B; cyclopeptides **11** and **12**, data not shown). The area percentage corresponding to each cyclopeptide cleaved by TFA in the presence of triisopropylsilane and water (which are shown below to be the most suitable scavengers for minimizing the TFA-promoted side reactions) is reported in the Table 2. It was observed that the faster HOBt/TBTU cyclization did not prove to be more efficient compared to the slower HOBt/DIC cyclization. Cyclization by a 15-fold excess of HOBt/TBTU (5 h) or by a 6-fold excess of HOBt/DIC (22 h) gave over 70% of the cyclic monomer **12** but only about 40% of **10** and **11**. In contrast, cyclization by using a 15-fold excess of HOBt/DIC (19 h) gave over 70% of the cyclopeptides **10** and **11** but only 54% of the cyclopeptide **12**. These results might be explained by the different effects of the activation conditions on the intra- and intermolecular reaction pathways, in conjunction with the different structural features of the spacer Xxx (Scheme 3). In **8** (the corresponding linear form of **11**) the two Ahx units, each containing five methylene groups, confer a relatively high flexibility to the peptide chain, which favors the interchain contacts and therefore the strong competition between monocyclization and oligomerization. Similarly, the two units of *β*-alanine, present in **7** (the corresponding linear form of **10**),

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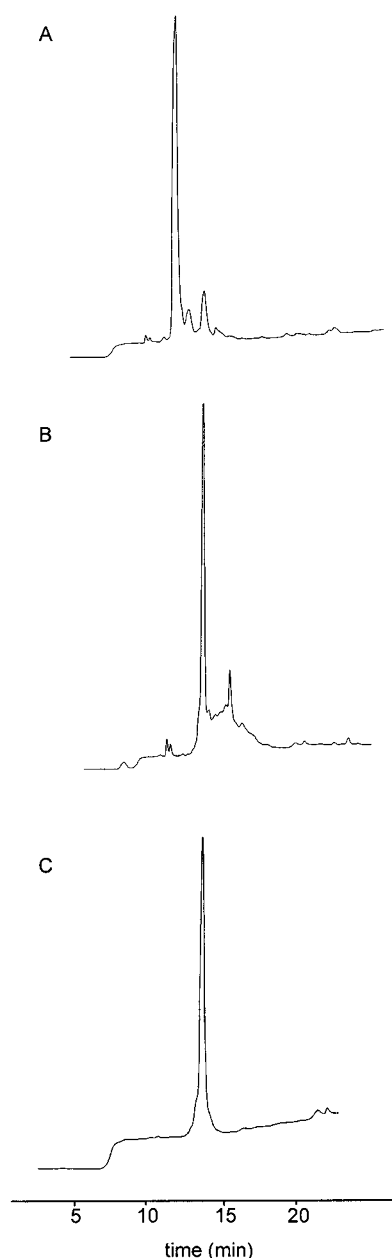

Figure 1. HPLC profiles of the β-alanine-containing peptides synthesized on the Wang resin preloaded by the tyrosine side chain attachment (**3b**) and cleaved by treatment with TFA in the presence of triisopropylsilane and water. Gradient: 0−60% acetonitrile over 30 min; UV detection at 220 nm. A: crude of the peptide acid **7**, obtained by hydrolysis of the ester **4b** with 0.4 M sodium hydroxide in methanol, 3 days. B: crude of the cyclopeptide **10**, obtained by using a 15-fold excess of HOBt/DIC, 19 h. C: Cyclic monomer **10** after purification by semipreparative HPLC.

introduce more mobility into the backbone, especially into the N-terminal region, and the β-alanine in the middle of the amino acid sequence might weakly force the backbone into a loop structure. Because of the low structural constraint of the peptide chains, the cyclization of **7** and **8** could take place by both the intra- and intermolecular mechanisms with the same probability, and the attempt to favor the former mechanism over the latter one might be very challenging. By carrying out the cyclization of **7** and **8** under different reaction conditions to prepare the cyclic peptides **10** and **11**, respectively, we observed that the intra- and intermolecular reaction pathways were highly competitive when either a large excess of HOBt/TBTU (15-fold) or a moderate excess of HOBt/DIC (6-fold) were applied, whereas the use of a large excess of HOBt/DIC (15-fold) favored the monocyclization over the oligomerization. This seems to suggest that if the peptide chain has a low propensity to undergo an intrachain condensation, such as for **7** and **8**, and the interchain contacts are strongly favored (Scheme 3, $k_{\mathrm{intra}} \sim k_{\mathrm{inter}}$), the undesired intermolecular cyclization might be minimized by using reaction conditions that determine an intermediate conversion rate of the peptide to its reactive form. Otherwise, when the peptide is converted to the reactive species too quickly or slowly, the intermolecular mechanism will be more likely to occur. In contrast, if the peptide has a high propensity to undergo an intrachain condensation ($k_{\mathrm{intra}} > k_{\mathrm{inter}}$) like **9** (the corresponding linear form of **12**), which contains two units of the constrained turn-inducing motif Ala-Aib, the ring closure will be favored. Fast or slow conversion rates of the peptide to the reactive form will both be suitable to maintain the selectivity between the intra- and the intermolecular cyclization, and an intermediate conversion rate might equally favor both of the reaction pathways.

On the basis of these results of the cyclization on the resin, a correlation might be supposed to exist between the activation rate (k_a) and the intra- and intermolecular cyclization rates (k_{intra} and k_{inter}), and the success of the cyclization might depend on a combination of reaction parameters that will drive the cyclization through the intramolecular mechanism rather than through the intermolecular.

By performing the cyclization on the solid phase, the properties of the solid support certainly play a crucial role.[6] The steric hindrance of the solid support was evaluated by comparing the cyclization of **7** by HOBt/TBTU on the HMPB−MBHA versus the Wang resins. Under conditions of a 15-fold excess of HOBt/TBTU for 5 h, the two resins yielded 65% and 36% of the cyclic monomer **10**, respectively. The higher percentage of the cyclic monomer obtained on the HMPB−MBHA resin might be attributed to the higher flexibility of the HMPB−MBHA linker compared to that of the Wang linker, which increases the solvation of the peptide chain and favors the contact of the reactive sites (the N- and C-termina). Moreover, the lower substitution level of the peptide on the HMPB−MBHA resin compared to that on the Wang resin (ca. 0.13 mmol/g versus 0.17 mmol/g) could provide a large distance between the peptide chains, which favors the intramolecular cyclization over the intermolecular. In fact, it has been reported that the resin loading can be proportionally correlated to the amount of oligomerization in the case of large loop cyclopeptides.[22] Furthermore, it must be stated that, in the case of the peptide bound to the HMPB−MBHA resin, the hydrolysis of the methyl ester did not go to completion; therefore the ratio between the activated carboxy groups and the free amino groups during the cyclization was lower than 1:1.

Cleavage of the Peptides from the Resin. The peptides **4**, **7**, and **10** were cleaved from the HMPB−MBHA resin using 96% TFA in the presence of thioanisole and thiocresol. The analysis of the crude peptides by HPLC and ESI−MS did not reveal the presence of

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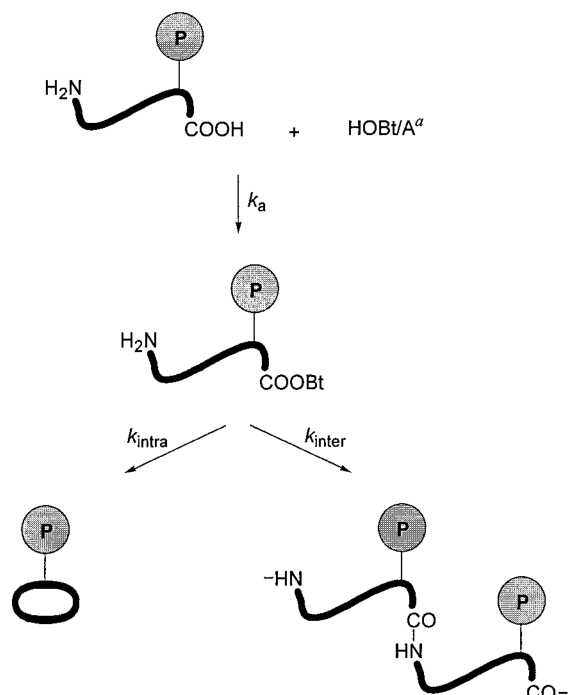
Table 2. Cyclization and TFA-Cleavage Conditions Used for the Synthesis of the Cyclopeptides 10–12 on the Wang Resin

cyclization conditions			TFA-cleavage conditions	cyclic monomer (%)[a]		
reagent	*n*-fold excess	time (h)	scavengers	**10**	**11**	**12**
HOBt/TBTU	15	5	triisopropylsilane/water	36	45	74
HOBt/TBTU	6	4	triisopropylsilane/water/phenol	27	23	38
HOBt/DIC	15	19	triisopropylsilane/water	85	72	54
HOBt/DIC	6	22	triisopropylsilane/water	45	40	72
HOBt/DIC	6	22	thioanisole/phenol	16	18	16

[a] The percentage of the cyclic monomer was estimated from the analytical HPLC profile at 220 nm with a gradient of 0–50% acetonitrile over 30 min.

Scheme 3. Intra- and Intermolecular Reaction Pathways during Cyclization on the Resin



[a] A: DIC or TBTU

byproducts, confirming that the cleavage of the tyrosine side chain from the hyperacid sensitive linker went smoothly. When the peptides **4–12** were cleaved from the Wang resin using 90% TFA and the thioanisole and thiocresol scavenger mixture, a major side product was obtained, corresponding to 35% of the yield and having a mass difference of +106. This additional mass was attributed to the *p*-hydroxybenzyl group, derived from the degradation of the Wang linker, which is likely to occur under strong acid conditions. This observation led us to conclude that the etherification of the Wang linker by the tyrosine side chain strongly destabilized this linker upon TFA treatment, much more than the esterification by the carboxy group of the amino acid. To avoid or at least to minimize the amount of the side products derived from the degradation of the Wang linker, we performed the TFA cleavage in the presence of other scavengers. The following combinations were used: triisopropylsilane/water, triisopropylsilane/water/phenol, and thioanisole/phenol. The first mixture gave the best results, drastically reducing the formation of byproducts. In contrast, the presence of aromatic compounds as scavengers during the TFA cleavage led to a high amount of side products (Table 2).

Biological Activity of the Cyclopeptides 10–12. To characterize the biological properties of the compounds, cyclopeptides **10–12** were tested for their ability to compete with [^{3}H-propionyl]NPY at Y_1-, Y_2-, or Y_5-receptor subtype expressing cells. No displacement was found at Y_2-receptor expressing cells. This is in agreement with previous papers that showed that modifications at the C-terminal amide are not tolerated.[23] Only moderate displacement was found at Y_5-receptor expressing cells. In contrast, 35–41% of radioactivity was displaced at Y_1-receptor expressing cells in the presence of 1 μM of the cyclopeptides, suggesting micromolar affinity of the compounds. Although the compounds **10–12** reveal only moderate affinity, they show that only eight amino acids, which correspond to the N- and the C-terminal segments of NPY, are required to displace the native ligand. Furthermore, these cyclopeptides confirm the existence of a discontinuous binding site at the Y_1-receptor subtype and, as they selectively bind to Y_1-receptor versus Y_2- and Y_5-receptors, could serve as new lead compounds for the development of Y_1-receptor selective ligands.

Circular Dichroism of the Cyclopeptides 10–12. An evaluation of the conformational structure adopted by the three cyclopeptides **10–12** in solution was made by using circular dichroism (CD) spectroscopy. In 20 mM phosphate buffer, pH 7, the spectra exhibit a maximum at 230 nm, a negative shoulder at 216 nm, and a minimum between 190 and 200 nm (Figure 2A). A decrease in intensity of the positive and negative bands and an increase in intensity of the shoulder are observed in the order **11**, **10**, **12**. The absorption at 230 nm can be attributed to the tyrosine ring.[24] It is reasonable to suppose that the two tyrosines in the peptide sequence are involved in electronic interactions that give rise to the splitting of the absorption band into a couplet characterized by two extrema with opposite sign, positive at 230 nm and negative at 216 nm. In the case of the cyclopeptide **12**, the negative counterpart is more intense than the positive one, indicating that there is an additional contribution of the peptide backbone in this region, characteristic of a type I β-turn.[25] The CD spectra of **10** and **11** show a reduced intensity in this spectral region and an increased intensity below 200 nm. This suggests that an unordered structure is dominant in these two peptides. The CD spectra of **10–12** were also recorded in the presence of 30% 2,2,2-trifluoro-

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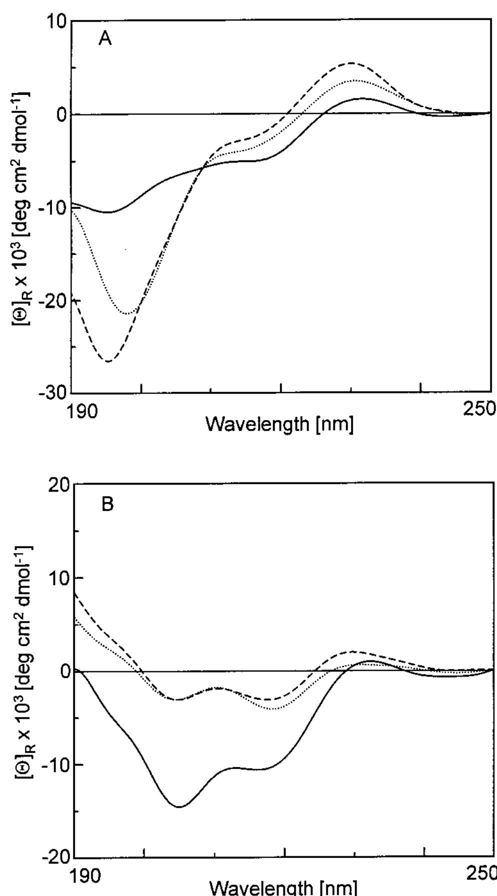

Figure 2. CD spectra of the cyclic peptides **10** (····), **11** (- - -) and **12** (—) at the concentration of 0.1 mM. A: phosphate buffer, 20 mM, pH 7.0. B: 30% trifluoroethanol.

ethanol (Figure 2B). By addition of this solvent, the negative band below 200 nm, characteristic of the CD spectra in water, vanishes, and two new bands are present, a minimum at 205 nm and a maximum near 190 nm. Furthermore, a well-defined negative band is now present at 218 nm, which is red-shifted and more intense compared to the shoulder observed in water. The trifluoroethanol-induced changes in the CD curves indicate that the cyclopeptides adopt a more ordered structure, which may be interpreted in terms of a mixture of type I and type III β-turns. The spectra of **10** and **11** are very similar, whereas the spectrum of **12** is much more intense in the region of 200−225 nm. This observation suggests that the β-turns conformation is more prominent in **12** than in **10** and **11**. Interestingly, **12**, which is the cyclopeptide containing the turn-inducing sequence Ala-Aib, already shows a major propensity to form β-turns in water.

Conclusions

Our work proves the suitability of the amino acid side chain attachment approach to the synthesis of cyclic peptides on the solid phase, using Fmoc chemistry. In particular, we have illustrated the anchoring of Fmoc-tyrosine side chain to benzyl-type resin linkers by the Mitsunobu reaction and the use of the preloaded resins for the preparation of three cyclic analogues of NPY, with ring sizes of 32, 36, and 38 atoms. The head-to-tail cyclization was carried out on the resin by using HOBt/DIC or HOBt/TBTU. No epimerization was observed to occur during the condensation reaction. Tetramethylguanidinium derivatives that would arise by transfer of the bis(dimethylamino)-methylene moiety from TBTU to the terminal α-amino group of the peptide could not be detected. We observed that the success of the cyclization could depend on the activation conditions, as well as on the structural features of the peptide backbone. Specifically, the optimal reaction conditions for the monocyclization of the two peptides containing β-alanine and Ahx, respectively, were found to be suboptimal for the peptide containing the segment Ala-Aib. The removal of the cyclic peptides from the solid support could be achieved by TFA treatment. To minimize the side products following the degradation of the Wang linker, the triisopropylsilane/water scavenger mixture was shown to be superior to the phenol-, thioanisole-, and thiocresol-containing mixtures. The CD spectra in water and in 30% trifluoroethanol confirmed that the more conformationally restricted Aib-containing cyclic peptide has a higher propensity to build β-turns than the other two peptides, which contain more flexible spacers. Furthermore, the cyclopeptides were identified to serve as lead structures for the development of Y_1-receptor selective ligands.

The attachment of the N^α-Fmoc-protected tyrosine methyl ester to acid-labile resins through its side chain provides the opportunity to perform solid-phase reactions that involve selectively the α-amino and the carboxy groups of the aromatic amino acid. For example, after completion of the peptide chain elongation, the C-terminal carboxy group can be selectively deprotected and subjected to the desired chemical modification. Therefore, the Fmoc-tyrosine side chain attachment approach will prove to be valuable for the preparation of C-terminally modified and cyclic peptides that contain this residue in at least one position within their primary sequence, by carrying out each step of the synthesis on the solid support, using the Fmoc strategy. Moreover, this method should contribute to the broadening of the combinatorial chemistry in the field, which is focused largely on the generation of libraries of peptides either chemically modified at the C-terminus or conformationally constrained by backbone cyclization. To date, syntheses of cyclic peptide libraries accomplished totally on the solid support have been reported using the Boc strategy combined with the attachment of the aspartic acid side chain[1,7b,26] or the oxime resin.[27] However, the high interest in modified peptides, cyclopeptides, and peptidomimetics as lead compounds for the discovery of more potent, stable, and orally available drugs requires future efforts. These include the application of the tyrosine side chain attachment to the combinatorial library approach in the Fmoc chemistry, for the preparation of head-to-tail cyclized peptides or peptides modified at the C-terminus upon reaction with amines, alcohols, and other organic compounds, as well as for the synthesis of tyrosine-based nonpeptide molecules. Furthermore, we are investigating the side chain attachment of other N^α-Fmoc-protected trifunctional amino acids, such as serine, threonine, histidine, and arginine, to the solid support, which would rapidly increase the versatility of this synthetic approach for the solid-phase technique.

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Experimental Section

Materials. The *N*-(9-fluorenylmethoxycarbonyl, Fmoc)-protected natural amino acids and 1*H*-benzotriazolium, 1-[bis(dimethylamino)-methylene]-, tetrafluoroborate (1-), 3-oxide (TBTU) were purchased from Alexis (Läufelfingen, Switzerland). Side chain protecting groups were *tert*-butyl for Ser and Tyr, Boc for Lys, trityl for Gln, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. The Fmoc-protected β-Ala, 6-aminohexanoic acid (Ahx), and α-aminoisobutyric acid (Aib), the 4-benzyloxybenzyl alcohol (Wang) resin, and the 4-hydroxymethyl 3-methoxyphenoxybutyric acid 4-methylbenzhydrylamine (HMPB−MBHA) resin were purchased from Novabiochem (Läufelfingen, Switzerland). The 4-benzyloxy-2-methoxybenzyl alcohol (Sasrin) resin was obtained from Bachem (Bubendorf, Switzerland). Tyrosine methyl ester hydrochloride (Novabiochem) was Fmoc-protected in our laboratory by using 9-fluorenylmethyl succinimidyl carbonate[28] (Novabiochem). *N*-Hydroxybenzotriazole (HOBt), *N,N*-diisopropylethylamine (DIEA), TFA, thioanisole, triisopropylsilane, piperidine, pyridine, 4-methylmorpholine, 1-methyl-2-pyrrolidinone, *tert*-butyl alcohol, 2,2,2-trifluoroethanol, triphenylphosphine, DEAD, DMF (puriss.), phenol (puriss.), sodium hydroxide, sodium hydrogenphosphate, and potassium dihydrogenphosphate, ninhydrin, potassium cyanide were obtained from Fluka (Buchs, Switzerland). *N,N*-diisopropylcarbodiimide (DIC) and *p*-thiocresol were purchased from Aldrich (Buchs, Switzerland). DMF (pure), dichloromethane, methanol, and diethyl ether were purchased from Scharlau (La Jota, Barcelona, Spain). Chloroform, ethanol, *n*-hexane and acetonitrile were obtained from Romil (Cambridge, England). Lithium hydroxide and acetic anhydride were purchased from Merck (Darmstadt, Germany). All material used for cell culture was purchased from Gibco. ^{3}H-propionyl-NPY was purchased from Amersham.

General Methods. The assembly of the linear peptides was performed on an automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum). Analytical and semipreparative reverse-phase HPLC was performed on a LiChrospher RP-18 column (5 μm, 3 mm × 125 mm, Merck, Darmstadt, Germany) and on a Delta-Pak C_{18} column (15 μm, 300 Å, 8 mm × 100 mm, Waters), respectively, using a Merck-Hitachi L-7100 liquid chromatograph. The following gradients were used: from 0% to 60% A over 30 min or from 0% to 50% A over 30 min at the flow rate of 0.6 mL/min on the analytical column and from 5% to 36% A over 23 min at the flow rate of 3 mL/min on the semipreparative column. The binary solvent system (A/B) was as follows: 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B). The absorbance was detected at 220 nm. Electrospray ionization mass spectrometry (ESI−MS) was performed on a Finnigan mass spectrometer. Radioactivity was determined by using a Beckman LS-6500 β^- counter. Circular dichroism was recorded using a JASCO model J720 spectropolarimeter. The CD spectra were measured over 250−190 nm at 20 °C in a N_2 atmosphere. The cyclopeptides were dissolved in 20 mM phosphate buffer, pH 7.0, at the concentration of 0.1 mM. Each measurement was performed four times using a thermostatable sample cell with a path of 0.02 cm. Response time was set at 2 s at a scan speed of 20 nm/min, a sensitivity range of 10 mdeg, and a step resolution of 0.2 nm. High-frequency noise was reduced by means of a low-path Fourier transform filter. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent, and optical equipment. The values for the mean-residue molar ellipticity $[\Theta]_R$ were expressed in deg cm^2 dmol^{-1}.

Preparation of Cells. SK-N-MC cells were grown in minimum essential medium (MEM) with Earl's salts containing 10% fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. SMS-KAN cells were grown under the same conditions using Dulbecco's MEM/nutrient mix F12 1:1 with 15% fetal calf serum.[29] BHK cells were cultured in Dulbecco's MEM with 10% fetal calf serum, 5% Penstrep, and 0.1% Geneticin. Cells were grown to confluency at 37 °C and 5% CO_2. The growth medium was removed, and the cells were washed with Dulbecco's phosphate-buffered saline (PBS). After incubation at room temperature for 3 min with PBS containing 0.02% EDTA, the cells were detached by mechanical agitation and suspended in new medium or incubation buffer.

Binding Assays. Assays were performed using as incubation buffer MEM with Earl's salts containing 0.1% Bacitracin, 50 μM Pefabloc SC, and 1% bovine serum albumin (BSA). The growth medium was removed, and cells were washed if necessary and resuspended in incubation buffer. A 200 μL portion of the suspension containing ca. 300 000 cells was incubated with 25 μL of 10 nM [^{3}H-propionyl]NPY and 25 μL of 10 μM NPY or cyclopeptide (**10**−**12**). Nonspecific binding was defined in the presence of 1 μM cold NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 2000 × *g* and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS, and mixed with scintillation cocktail. Radioactivity displacements by the cyclopeptides **10**−**12** were measured at the human Y_1-, Y_2-, and Y_5-receptors. Specific binding (i.e., total minus unspecific binding in the presence of 1 μM NPY) was equated to 100%. Each experiment was performed in triplicate, and data are given ±SEM. At the Y_1-receptor, the following displacements were shown: 37% ± 3 by **10**, 35% ± 2 by **11**, and 41% ± 4 by **12**. A displacement $<$20% was observed at the Y_2- and Y_5-receptors by all three peptides.

Anchoring of *N*-(9-fluorenylmethoxycarbonyl)-tyrosine methyl ester to the resins 1a−c was accomplished by using the general procedure described below for the preparation of the resin **3a**.

Resin 3a. The HMPB−MBHA resin **1a** (100 mg; loading, 0.44 mmol/g) was swollen in 2 mL of dichloromethane for 15 min, and then the excess solvent was removed by filtration. *N*-(9-Fluorenylmethoxycarbonyl)-tyrosine methyl ester (96 mg, 0.23 mmol) and triphenylphosphine (72 mg, 0.27 mmol) were dissolved in 2 mL of dichloromethane and added to the swollen resin. A 42 μL (0.27 mmol) portion of DEAD was diluted to 200 μL with dichloromethane and added dropwise to the mixture at room temperature. The reaction was run overnight. The mixture was then removed, and the resin was washed sequentially with dichloromethane (3 ×), DMF (3 ×), methanol (2 ×), DMF (2 ×), and finally diethyl ether and then dried in vacuo. The substitution level of the loaded resin **3a**, determined spectrophotometrically by Fmoc cleavage[15] and ninhydrin assay,[16] was 0.17 mmol/g. The remaining free hydroxy groups on the resin were acetylated with acetic anhydride (26 μL, 0.28 mmol) in the presence of DIEA (48 μL, 0.28 mmol) in dichloromethane (0.5 mL) for 20 min.

Resin 3b. The Wang resin **1b** (237 mg; loading, 0.96 mmol/g) was treated following the protocol described above for the resin **1a**, with the only variation being that the DEAD solution in dichloromethane was added dropwise at 0 °C. The mixture was kept at 0 °C over 1 h after DEAD addition, and then it was left to warm to room temperature. The substitution level of the loaded resin **3b** was 0.23 mmol/g.

Resin 3c. The Sasrin resin **1c** (100 mg; loading, 1.06 mmol/g) was loaded with *N*-(9-fluorenylmethoxycarbonyl)-tyrosine methyl ester by repeating twice the procedure outlined above for the loading of **1a**. The first time, *N*-methylmorpholine was used as the reaction solvent, whereas dichloromethane was used the second time. The final amino acid substitution on the loaded resin **3c** was determined to be 0.10 mmol/g.

Synthesis of the Resin-Bound Peptide Methyl Esters 4a,b, 5b, and 6b. The peptide ester **4** was synthesized on both resins **3a** and **3b**. The peptide esters **5** and **6** were synthesized once starting from the resin **3b**. The chain assembly was performed by the Fmoc strategy using HOBt/DIC as the activation reagents. The Fmoc protecting group was removed by treatment with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min, and finally 40% piperidine for 5 min. Each

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amino acid was introduced by a double coupling (2 × 36 min) using a 10-fold excess of the Fmoc-amino acid and of the activation reagents.

After the peptide chain was completed, a small-scale cleavage of the peptide esters **4**−**6** was performed to characterize them as follows. Some peptide resin beads were treated with 0.1 mL of the cleavage mixture TFA/triisopropylsilane/water (93/3.5/3.5 v/v) for 3 h, and then 1 mL of ice-cold ether was added to precipitate the peptide. The suspension was centrifuged, the ether was decanted, and the product was suspended again in ice-cold ether. The washing was repeated three times. The peptide was dissolved in 0.1 mL of *tert*-butyl alcohol/water 4/1 (w/w), and the resin was removed by centrifugation. The peptide esters were characterized by analytical HPLC and ESI−MS. Peptide **4**: t_R 15.26 min; MS calcd for $C_{56}H_{88}N_{18}O_{15}$ $[M + H]^+$ 1254.4, found 1254.3. Peptide **5**: t_R 15.84 min; MS calcd for $C_{62}H_{100}N_{18}O_{15}$ $[M + H]^+$ 1338.6, found 1338.2. Peptide **6**: t_R 16.45 min; MS calcd for $C_{64}H_{102}N_{20}O_{17}$ $[M + H]^+$ 1424.6, found 1424.1.

Hydrolysis of the Resin-Bound Peptide Methyl Esters. General Procedure for the Synthesis of 7a,b, 8b, and 9b. The peptide resins **4a**,**b**, **5b**, and **6b** (each 20 mg) were suspended in 0.5 mL of methanol for 15 min, and then the solvent was removed by filtration. The hydrolysis was performed using 0.6 mL of one of the two following solutions: 0.33 M lithium hydroxide in methanol/water (97/3 v/v) for 6 days or 0.4 M sodium hydroxide in methanol for 3 days. After removal of the hydrolysis mixture, the resin was washed with methanol (3 ×), DMF (3 ×), dichloromethane (2 ×), DMF (2 ×), methanol (2 ×), DMF (2 ×), and finally diethyl ether and then dried in vacuo. To characterize the peptide acids **7**−**9**, a small-scale cleavage was carried out as described above for the characterization of the peptide esters **4**−**6**. The peptide acids were characterized by analytical HPLC and ESI−MS. Peptide **7**: t_R 12.10 min; MS calcd for $C_{55}H_{86}N_{18}O_{15}$ $[M + H]^+$ 1240.4, found 1240.4. Peptide **8**: t_R 12.62 min; MS calcd for $C_{61}H_{98}N_{18}O_{15}$ $[M + H]^+$ 1324.6, found 1324.4. Peptide **9**: t_R 12.90 min; MS calcd for $C_{63}H_{100}N_{20}O_{17}$ $[M + H]^+$ 1410.6, found 1410.5.

Cyclization of the Resin-Bound Peptide Acids. General Procedure for the synthesis of 10a,b, 11b, and 12b. The peptide resins **7a** (26 mg), **7b**, **8b**, and **9b** (each 20 mg) were swollen in 0.6 mL of DMF, and then the excess solvent was removed by filtration. The cyclization was carried out by using two different coupling reagents in different *n*-fold excess and over different reaction times. **A:** HOBt/TBTU/DIEA/DMF (0.05 mmol/0.05 mmol/0.09 mmol/0.7 mL), corresponding to 15-fold excess of condensing reagents and 26-fold excess of the base, 5 h. **B:** HOBt/TBTU/DIEA/DMF (0.02 mmol/0.02 mmol/0.04 mmol/0.5 mL), corresponding to a 6-fold excess of condensing reagents and a 12-fold excess of the base, 4 h. **C:** HOBt/DIC/DIEA/DMF (0.05 mmol/0.05 mmol/0.05 mmol/0.6 mL), corresponding to a 15-fold excess of condensing reagents and of the base, 19 h. **D:** HOBt/DIC/DIEA/DMF (0.02 mmol/0.02 mmol/0.02 mmol/0.7 mL), corresponding to a 6-fold excess of condensing reagents and of the base, 22 h. The free amine content on the resin was controlled by a qualitative ninhydrin assay. The coupling mixture was removed, and the resin was washed with DMF (3 ×), dichloromethane (2 ×), DMF (2 ×), methanol (2 ×), DMF (2 ×), and finally diethyl ether and then dried in vacuo.

Cleavage of the Cyclopeptides 10−**12 from the Wang Resin 1b. General Procedure.** The dried peptide resins **10b**, **11b**, and **12b** (each 20 mg) were treated for 3 h with 0.6 mL of one of the following cleavage mixtures: TFA/triisopropylsilane/water (90/7/3 v/v); TFA/triisopropylsilane/water/phenol (93.5/2.6/2.6/1.3 w/w); TFA/thioanisole/phenol (90.5/4.2/5.3 w/w). Then the resin was filtered off and washed with neat TFA (2 × 0.2 mL). The filtrates were pooled and the peptide was recovered by addition of 4 mL of ice-cold ether. The suspension was centrifuged, the ether was decanted, and the peptide was washed with ice-cold ether until removal of the scavengers was complete. After drying under a stream of N_2, the peptide was dissolved in *tert*-butyl alcohol/water 4/1 (w/w) and lyophilized. The crude product was purified by semipreparative HPLC and characterized by analytical HPLC and ESI−MS. Peptide **10**: 4.0 mg (55% overall yield); t_R 13.48 min (95.1% purity); MS calcd for $C_{55}H_{84}N_{18}O_{14}$ $[M + H]^+$ 1222.4, found 1222.2. Peptide **11**: 3.0 mg (35% overall yield); t_R 14.46 min (95.6% purity); MS calcd for $C_{61}H_{96}N_{18}O_{14}$ $[M + H]^+$ 1306.6, found 1306.2. Peptide **12**: 3.1 mg (36% overall yield); t_R 14.10 min (95.5% purity); MS calcd for $C_{63}H_{98}N_{20}O_{16}$ $[M + H]^+$ 1392.6, found 1392.3.

Cleavage of the linear peptides 4−**9 and cyclopeptides 10**−**12** in the presence of the scavengers thioanisole and thiocresol was accomplished by following the procedure described below for the cleavage of **10** from the HMPB−MBHA resin **1a**.

Cleavage of the Cyclopeptide 10 from the Resin 1a. The dried peptide resin **10a** (10 mg) was treated with 0.3 mL of the cleavage mixture TFA/thioanisole/thiocresol (96/2/2 v/v) for 3 h. The resin was removed by filtration, and the peptide **10** was recovered by addition of ice-cold ether and centrifugation. The analytical HPLC and ESI−MS of the purified cyclopeptide were identical to those reported above for the same peptide cleaved from the Wang resin **1b**.

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