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Structure-Activity Relationships of the Peptide Kappa Opioid Receptor Antagonist Zyklophin

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Abstract

The dynorphin (Dyn) A analog zyklophin ([*N*-benzyl-Tyr^1-*cyclo*(D-Asp^5,Dap^8)]dynorphin A(1-11)NH_2) is a kappa opioid receptor (KOR) selective antagonist *in vitro*, is active *in vivo* and antagonizes KOR in the CNS after systemic administration. Hence, we synthesized zyklophin analogs to explore the structure-activity relationships of this peptide. The synthesis of selected analogs required modification to introduce the N-terminal amino acid due to poor solubility and/or to avoid epimerization of this residue. Among the N-terminal modifications the *N*-phenethyl and the *N*-cyclopropylmethyl substitutions resulted in the analogs with the highest KOR affinities. Pharmacological results for the alanine-substituted analogs indicated that Phe^4 and Arg^6, but interestingly not the Tyr^1, phenol are important for zyklophin's KOR affinity, and Arg^7 was important for KOR antagonist activity. In the GTPγS assay while all of the cyclic analogs exhibited negligible KOR efficacy, the *N*-phenethyl-Tyr^1, *N*-CPM-Tyr^1 and the *N*-benzyl-Phe^1 analogs were 8- to 24-fold more potent KOR antagonists than zyklophin.

Introduction

While clinically used drugs acting at opioid receptors primarily interact with mu opioid receptors (MOR), extensive research has explored potential therapeutic applications of ligands for other opioid receptors. Kappa opioid receptor (KOR) antagonists were initially used only as pharmacological tools, but recently they have been explored for potential therapeutic use in the treatment of depression,[1–4] anxiety[4–6] and opiate and cocaine addiction.[2, 4, 7, 8] The non-peptide KOR antagonists nor-binaltorphimine (norBNI) and JDTic [(3*R*)-7-hydroxy-*N*-[(2*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide] have been used extensively to study KOR involvement in physiological processes and disease states,[9] and JDTic was examined in a Phase I clinical trial.[10] However, these prototypical KOR selective antagonists exhibit exceptionally long KOR antagonist activity, lasting weeks after a single

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dose,[9] which complicate their use as pharmacological tools and could impact their therapeutic application.

Our research group has designed and synthesized analogs of the endogenous kappa opioid peptide dynorphin (Dyn) A as KOR selective peptide antagonists.[11–13] In early studies on N-terminal alkylated analogs of [D-Pro^{10}]Dyn A-(1-11) we found that despite its high affinity for KOR the *N*-benzyl analog displayed lower agonist potency than other *N*-alkyl derivatives in the guinea pig ileum (GPI) assay,[14, 15] and produced partial agonism in the adenylyl cyclase assay.[16] A series of (5,8) cyclic Dyn A(1-13)NH_2 analogs with varying ring sizes synthesized in our laboratory also displayed very low agonist potencies in the GPI assay.[17] When these structural modifications were combined we obtained the Dyn A-(1-11)NH_2 analog zyklophin (Figure 1) which exhibits high selectivity for KOR and is a KOR antagonist in the adenylyl cyclase assay. [18]

Zyklophin was subsequently shown to also be a selective KOR antagonist *in vivo* with a finite duration of KOR antagonist activity (12-18 h) following systemic administration.[19] Following peripheral subcutaneous (s.c.) administration, zyklophin antagonized the antinociceptive activity of the centrally administered KOR agonist U50,488, suggesting that this peptide crossed the blood-brain barrier to act on KOR in the CNS.[19] This peptide also suppressed stress-induced reinstatement of cocaine seeking behavior in mice following s.c. administration,[19] suggesting its potential as a lead compound for the development of therapeutics for the treatment of cocaine addiction.

We are exploring the structure-activity relationships (SAR) of zyklophin in order to examine its potential interactions with KOR and to enhance its antagonist potency. We initially prepared linear [*N*-benzyl-Tyr^1]Dyn A-(1-11)NH_2 analogs **2** and **3** (Table 1) in which positions 5 and 8 were modified to assess their individual contributions to the activity of zyklophin. We expected that the D-amino acid in position 5 adjacent to the “message” sequence[20] might affect the orientation of pharmacophoric groups in the receptor binding site and therefore could play a significant role in the antagonist activity of zyklophin, while the residue in position 8 was not expected to affect peptide efficacy. In this initial SAR study of cyclic analogs we made three types of modifications: to the N-terminal group (analogs **4–8**, Table 1), amino acid substitutions in the sequence (analogs **9–14**), and to the cyclic constraint (analogs **15–17**). We explored the effect of different N-terminal alkyl substituents on the receptor affinities and KOR activity of zyklophin, based on the hypothesis that the N-terminal alkyl group could affect the interactions of the rest of the peptide with KOR and therefore the ability of zyklophin to bind to this receptor without activating it. We investigated the contribution of different residue side chains to the KOR affinity and antagonist activity of zyklophin by performing an Ala scan of the non-glycine residues of zyklophin (excluding residues 5 and 8) up through residue 9 (Table 1). (Previous studies of linear [*N*-benzyl]Dyn A(1-11)NH_2 analogs suggested that modifications in the C-terminus did not affect peptide efficacy.[21]) In addition, to explore the importance of the phenolic group of Tyr^1 on the activity of zyklophin we synthesized the Phe^1 analog of zyklophin (**14**, Table 1). Finally, we explored the role of ring size and residues involved in the cyclic constraint on the KOR interaction of zyklophin.

Results and discussion

Synthesis

The synthesis of the zyklophin analogs involved synthesizing the N-terminal *N*-alkyl amino acid derivatives in solution and assembly of the peptides on the solid support. Following the solid phase synthesis of the (2-11) peptide fragments, the appropriate N-alkyl amino acid derivatives were coupled to the N-terminus to obtain the protected (1-11) peptides on resin. The final peptides were then obtained by cleavage from the resin.

Amino acid synthesis

Except for *N*-allyl-Tyr the *N*-alkyl amino acid derivatives were synthesized by reductive amination of the amino acid *t*-butyl ester with the appropriate aldehyde and sodium triacetoxyborohydride to yield the *N*-alkyl amino acid *t*-butyl ester, which was subsequently cleaved with 90% TFA and 10% water to yield the *N*-alkyl amino acid (Scheme 1a). *N*-allyl-Tyr (Scheme 1c) was prepared by alkylating H-Tyr-O*t*Bu with 1 equivalent of allyl bromide in the presence of *N*,*N*-diisopropylethylamine (DIEA) in DMF. Using only one equivalent of allyl bromide minimized formation of the diallyl compound; the desired monoallyl amino acid ester was separated from unreacted starting material by silica gel flash column chromatography using EtOAc/hexane. The t-butyl ester was subsequently cleaved with 90% TFA and 10% water.

The resulting *N*-alkyl amino acids, obtained as TFA salts, exhibited low solubility in DMF at room temperature and required heating to >85°C for solubilization (see below). While microwave couplings are routinely performed at ≥ 75°C, racemization at elevated temperatures could be a potential issue during coupling of the *N*-alkyl amino acids to the (2-11) fragments. Initially Fmoc protection of *N*-benzyl-Tyr-O*t*Bu by treatment with Fmoc-Cl was examined, but after removal of the *t*-butyl ester the resulting Fmoc-protected amino acid was not soluble in DCM or DMF at room temperature. Therefore selected *N*-alkyl-amino acid derivatives with low solubility, namely *N*-CPM-Tyr-O*t*Bu and *N*-benzyl-Phe-O*t*Bu, were protected with the Alloc (allyloxycarbonyl) group by treating with allyl chloroformate and DIEA in DCM to afford the Alloc-*N*-alkyl amino acid esters (Scheme 1b); in the case of *N*-CPM-Tyr-O*t*Bu the *bis*-Alloc derivative was obtained. The *t*-butyl esters were subsequently cleaved with 90% TFA/10% DCM to afford the Alloc-protected amino acids (Scheme 1b) which unlike the unprotected *N*-alkyl amino acids were readily soluble in DMF at room temperature. This avoided heating and potential racemization of the amino acids during coupling (see below).

Peptide synthesis

The peptides were synthesized using the Fmoc solid phase synthetic strategy. The (2-11) peptide fragments were assembled on a low load poly(ethylene glycol)-polystyrene (PEG-PS) resin containing the peptide amide linker [PAL, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid linker] using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt) (4 equiv each) and DIEA (10 equiv) in DCM:DMF (1:1) to couple the Fmoc-amino acids (4 equiv) to the growing peptide chain using a custom manual multiple peptide

synthesizer.[22] The side chain protecting groups used were 2,2,4,6,7-pentamethyl dihydrobenzofurane-5-sufonyl (Pbf) for Arg and Boc for Lys.

For the linear analogs **2** and **3** the (2-11) peptide precursors were synthesized similar to the above procedure with the side chains of D-Asn in peptide **2** and Dap (Dap = 2,3-diaminopropionic acid) in peptide **3** protected by trityl (Trt) and 4-methyltrityl (Mtt) groups, respectively. For peptide **3** following the synthesis of the (2-11) linear fragment, the Mtt protecting group on Dap was selectively deprotected using 3% TFA and 5% triisopropylsilane (TIS) in DCM, and the resulting amine was acetylated using acetyl imidazole in DMF.

The (2-11) fragments of the cyclic peptides **4–9** and **14–17** were synthesized by a similar approach (see Scheme 2). The D-Asp and Dap residues in positions 5 and 8 were protected with the hyperacid labile 2-phenylisopropyl (Pip) and Mtt groups, respectively. Following the synthesis of the (5-11) fragments, the Pip and Mtt groups were deprotected by 3% TFA and 5% TIS in DCM. The selective deprotection of the Mtt group on residue 8 was monitored by acetylation of the resulting free amine by acetic anhydride or acetyl imidazole, followed by cleavage of an aliquot of resin and HPLC analysis of the resulting product. Subsequently, the carboxyl group of D-Asp^5 and the amine of Dap^8 were cyclized using 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyClocK) and 1-hydroxy-7-azabenzotriazole (HOAt, 4 equiv each) with DIEA (10 equiv) in a mixture of DCM:DMF (1:1), generally for 20–24 h. The cyclizations were monitored using the qualitative ninhydrin test.[23] As expected for syntheses performed on a low load resin, there was no evidence for cyclodimerization by mass spectrometry. Any remaining unreacted free amine of Dap was acetylated by treatment with acetyl imidazole and DIEA in DMF. Further extension of the peptide up to Gly^2 afforded the (2-11) fragments of the cyclic peptides. For the synthesis of peptides **15** and **16** Dab(Mtt) (Dab = 2,3-diaminobutyric acid) and Orn(Mtt) (Orn = ornithine) were incorporated in position 8, respectively and for peptide **17** L-Asp(Pip) was incorporated in position 5. In the case of these three peptides the cyclizations required longer reaction times (∼ 36 h) to go to completion.

Coupling of the *N*-alkyl amino acids

The coupling of the N-terminal amino acid derivatives to the resin-bound (2-11) peptide sequences was carried out using PyClocK, HOAt and DIEA in DMF. The solutions of the *N*-alkyl amino acids required elevated temperatures (80–85°C for *N*-benzyl-Tyr and *N*-benzyl-Ala and >100°C for the *N*-allyl and *N*-phenethyl Tyr derivatives) to obtain complete dissolution in DMF. For *N*-benzyl-Tyr and *N*-benzyl-Ala the solutions were cooled to room temperature prior to addition of the coupling reagents and base, but the solutions of *N*-allyl- and *N*-phenethyl-Tyr became turbid upon cooling; hence the coupling agents were added to these solutions while they were still hot, followed by cooling the solution, addition of the base, and coupling to the (2-11) fragment. In these cases where the unprotected N-terminal N-alkyl amino acid was coupled to the (2-11) peptides there was no evidence of incorporation of multiple N-alkyl amino acids (i.e. formation of 12-mer peptides); this is likely due to the steric hindrance of the bulky secondary amine and the low (2-fold) excess

of amino acid used in these reactions. *N*-CPM-Tyr-OH did not couple to the (2-11) fragment, probably due to its minimal solubility in DMF.

N-CPM-Tyr-OH and *N*-benzyl-Phe-OH were coupled as their N-protected derivatives which are soluble in DMF at room temperature. Following coupling of the unprotected *N*-benzyl-Phe-OH to the (2-11) fragment, the crude peptide **14** showed a second peak in the HPLC (Figure 3a). ESI analysis of the peptide mixture did not show formation of a higher molecular weight species (i.e. the 12-mer peptide), suggesting that the second peak resulted from *N*-benzyl-Phe-OH undergoing racemization during activation of the amino acid or subsequent coupling to the (2-11) fragment. To avoid potential epimerization and enhance their solubility, the Alloc-protected derivative of *N*-benzyl-Phe-OH, as well as *N*-CPM-Tyr-OH, were prepared as described above. Following coupling of the Alloc derivatives to the (2-11) peptide fragment on the resin, the Alloc group was removed by Pd(0) and phenylsilane.[24] To synthesize peptide **4**, Fmoc-MeTyr(O*t*Bu)-OH was coupled to the (2-11) peptide fragment, followed by Fmoc deprotection. In the case of peptide **8**, Fmoc-Tyr(O*t*Bu)-OH was coupled to the (2-11) peptide fragment by the standard coupling procedure, followed by Fmoc deprotection and subsequent acylation of the N-terminal of Tyr with benzoic anhydride in the presence of DIEA in DMF.

Investigation of potential racemization of the N-terminal residue of zyklophin and analog 14 by HPLC

Because dissolution of the *N*-alkyl amino acids required elevated temperatures, the peptides were examined by HPLC for potential epimerization of the N-terminal residue. To verify that the diastereomeric peptides could be separated by HPLC we synthesized [*N*-benzyl-D-Tyr^1]zyklophin. *N*-Benzyl-D-Tyr-OH was synthesized as described above (Scheme 1a) and coupled to the (2-11) fragment to give the *N*-benzyl-D-Tyr isomer of zyklophin. While a reversed phase HPLC (RP-HPLC) system using a slow gradient of aqueous acetonitrile containing TFA (10–25% over 30 min) did not adequately resolve the diastereomeric mixture of zyklophin and [*N*-benzyl-D-Tyr^1]zyklophin (Figure 2a), a solvent system using aqueous 0.09M triethylammonium phosphate (TEAP, pH = 2.5)[25] in acetonitrile (1–21% over 40 min) did provide baseline resolution of the diastereomers of zyklophin (Figure 2b) with the D-Tyr diastereomer eluting before zyklophin. Zyklophin synthesized by the standard procedure involving solubilization of *N*-benzyl-Tyr at 85°C did not show detectable epimerization when analyzed using the TEAP solvent system (Figure 2c). This HPLC solvent system was used to detect potential epimerization of the N-terminal residue during the synthesis of other zyklophin analogs. None of the peptides analyzed using this system (Table 2) except for peptide **14** showed evidence of epimerization of the N-terminal residue.

The Phe^1 analog of zyklophin **14**, however, showed evidence of epimerization of the N-terminal residue when synthesized by the standard procedure in which *N*-benzyl-Phe in DMF was dissolved at 75–80°C prior to coupling to the (2-11) peptide fragment. Analysis of the crude peptide in the TEAP solvent system (Figure 3a) showed a mixture of two peaks, peak A (t_R = 18.3 min, 17.5%) and peak B (t_R = 20.3 min , 82.5%), for the peptide synthesized by this standard method. However, when synthesized using Alloc-*N*-benzyl-

Phe, peptide **14** did not show detectable epimerization of the N-terminal residue; only peak B (t_R = 20.3 min) was observed in the chromatogram (Figure 3b).

The peptides were purified by preparative RP-HPLC and analyzed by ESI-MS and analytical RP-HPLC. The final purity of all of the peptides by all methods was ≥ 98% (Table 2).

Pharmacological results

The peptides were evaluated for receptor affinities in radioligand binding assays using Chinese hamster ovary (CHO) cells stably expressing opioid receptors (Table 3). Zyklophin showed somewhat lower KOR affinity in the radioligand binding assay than obtained originally (K_i = 30.3 nM).[18] Similarly, the KOR binding affinity of Dyn A(1-11)NH_2 (K_i = 2.6 ± 0.3 nM) was also lower than found previously (K_i = 0.57 ± 0.01 nM),[18] possibly due to subtle differences in assay conditions (e.g. differences in the radioligand, filtration rate, etc.). The MOR affinity found here for zyklophin was similar to that reported previously.[18]

In order to assess the individual contributions of the residues in positions 5 and 8 of zyklophin the linear peptides **2** and **3** were synthesized. Previously we found that the linear zyklophin analog [*N*-benzyl-Tyr^1,D-Asn^5,Dap(Ac)^8]Dyn A(1-11)NH_2 with substitutions in both positions exhibited KOR affinity 2-fold lower (K_i = 66.9 nM) than zyklophin and 8-fold lower than Dyn A(1-11)NH_2.[18, 21] When the peptides with a single modification were evaluated we found that peptide **2** had 4-fold lower KOR affinity (K_i = 412 nM) compared to zyklophin (K_i = 95.2 nM), while peptide **3** exhibited 3-fold higher KOR affinity (K_i = 28 nM). These results suggest that the residue in position 5 had a much larger influence on the KOR affinity of zyklophin than the amino acid in position 8. While zyklophin has a D-Asp involved in the lactam linkage at position 5, peptide **3** has L-Leu in this position as found in the endogenous Dyn A. Hence, the side chain of the residue at position 5 and/or changes in the backbone conformation of the peptide due to the different configuration at residue 5 influenced the KOR affinity of the zyklophin analogs. While peptide **2** had low MOR affinity (K_i = 2440 nM), peptide **3** had 28-fold higher MOR affinity (K_i = 157 nM) than zyklophin (K_i = 4380 nM), suggesting that residue 5 is important for the low MOR affinity of zyklophin. Both linear analogs **2** and **3** displayed similar selectivities for KOR vs. MOR that were lower than that of zyklophin.

Sterically diverse alkyl groups ranging from methyl to phenethyl were incorporated at the N-terminus to examine their effect on the affinity, efficacy and potency of the zyklophin analogs. Peptides **4, 6** and **7** had similar to slightly higher KOR affinities (K_i = 44–160 nM) than zyklophin, suggesting that these *N*-alkyl modifications are tolerated in the KOR binding site. The *N*-allyl analog **5** had the lowest KOR affinity (K_i = 326 nM) among the *N*-alkylated analogs examined. The *N*-benzoyl derivative **8** showed only somewhat lower (3.6-fold) KOR affinity (K_i = 346 nM) compared to zyklophin, indicating that a basic secondary amine at the N-terminus is not essential for maintaining the KOR affinity of zyklophin analogs. All of the analogs displayed low MOR affinity (K_i >1 μM) similar to zyklophin (only the *N*-methyl derivative **4** displayed increased MOR affinity (K_i = 1490 nM) compared to

zyklophin). The increased KOR affinity of analogs **6** and **7** resulted in increases in their KOR vs. MOR selectivities (K_i ratios (MOR/KOR) = 245 and 137), respectively.

An alanine scan of zyklophin of the non-glycine residues up through position 9, excluding the residues in positions 5 and 8 involved in the lactam, was also performed to identify residues important for the KOR affinity of zyklophin. Unexpectedly the Ala[1] analog **9** displayed only a 5-fold decrease in the KOR affinity (K_i = 516 nM) compared to zyklophin, suggesting that Tyr in position 1, while making some contribution to its affinity, is not critical for zyklophin's binding to KOR. Alanine substituted analogs **10** (Ala[4]) and **11** (Ala[6]) showed the largest decreases, 42- and 18-fold (K_i = 4010 and 1750 nM, respectively), respectively, in KOR affinity compared to zyklophin, indicating that the Phe[4] and the Arg in position 6 are critical for maintaining the KOR affinity of zyklophin. Interestingly, peptide **12** (Ala[7]) showed only a 3-fold decrease in KOR affinity (K_i = 293 nM), a much smaller decrease than seen for substitution of Arg[6], suggesting that Arg[7] makes only a minor contribution to the KOR affinity of zyklophin. Analog **13** (Ala[9]) displayed KOR affinity (K_i = 168 nM) within 2-fold of zyklophin, indicating that this positively charged residue is not important for the KOR affinity of zyklophin. Analogs **9–13** all displayed low affinity for MOR (K_i > 2 μM) similar to zyklophin.

The alanine scan of Dyn A(1-13) performed previously[26] revealed that within the Leu-enkephalin core of the peptide Ala[1] and Ala[4] substitution caused dramatic decreases in opioid receptor affinities, indicating that Tyr[1] and Phe[4] residues were critical for opioid receptor binding affinity. Outside the Leu-enkephalin core of Dyn A(1-13), the Ala[6] and Ala[7] analogs showed the largest decreases (3- to 7-fold) in binding affinities, suggesting that Arg[6] and Arg[7] contribute to the opioid receptor affinity of Dyn A(1-13). Ala[9] and Ala[11] analogs showed small decreases (2- to 3-fold) in opioid receptor binding affinity, suggesting that these residues made minor contributions to the opioid receptor affinity of Dyn A(1-13).[26]

Comparison of the alanine scan results for Dyn A(1-13) and zyklophin reveals significant differences in the contributions of some of the residues to the KOR affinities of these two peptides. While Tyr[1] was critical in maintaining the opioid receptor affinity of Dyn A(1-13), it appeared to make a relatively minor contribution to the KOR binding affinity of zyklophin. Phe[4], however, was critical for both the opioid receptor affinity of Dyn A(1-13) and the KOR affinity of zyklophin. Arg[6] contributed to both the opioid receptor affinity of Dyn A(1-13) and the KOR affinity of zyklophin, although the impact of substituting Ala for Arg[6] in zyklophin appeared to be greater (18-fold decrease) than the substitution of the same amino acid in Dyn A(1-13) (7-fold decrease). Arg[7] appeared to make a minor contribution to both the opioid receptor affinity of Dyn A(1-13) and the KOR affinity of zyklophin, while Arg[9] residue did not appear to contribute significantly to the binding affinities of either peptide. The differences in the results for the alanine scan analogs of the two peptides suggest differences in the binding interactions with the kappa opioid receptor. Some differences in receptor interactions would be expected since Dyn A(1-13) is a KOR agonist while zyklophin is a KOR antagonist. Differences in the radioligand binding assays used to evaluate the Ala-substituted analogs of the two peptides, however, limit the comparisons that can be made. The Dyn A(1-13) analogs were evaluated in radioligand binding assays

performed in rat brain homogenates, which contain MOR and delta opioid receptor (DOR) but relatively low levels of KOR,[27] using the non-selective radioligand [^{3}H]etorphine, so that the affinities of the analogs for individual opioid receptors cannot be assessed from those data.

The *N*-benzyl-Phe^1 analog **14** was also synthesized and displayed a slight increase in KOR affinity (K_i = 69 nM) compared to zyklophin, verifying that the phenol of N-terminal Tyr^1 does not contribute to the affinity of zyklophin for KOR. This is in contrast to Dyn A(1-11)NH_2 where substitution of Tyr^1 by Phe resulted in a 23-fold decrease in KOR affinity,[28] demonstrating that the phenol of Tyr is critical for the KOR affinity of this agonist. Peptide **14** also exhibited 3.5-fold higher selectivity for KOR over MOR compared to zyklophin.

Changes in the lactam ring of zyklophin had a minor effect on KOR and MOR affinities. Peptides **15** and **16** with larger ring sizes than zyklophin had KOR affinities 2- to 4-fold lower (K_i = 403 and 222 nM for peptides **15** and **16**, respectively) than zyklophin. Peptide **17** containing L-Asp in position 5 also showed 4-fold lower KOR affinity (K_i = 410 nM); thus a change in the configuration of this amino acid was reasonably well tolerated by KOR without a large loss in affinity. Similar to zyklophin, analogs **15–17** all displayed low (micromolar) affinity for MOR. However, the KOR vs. MOR selectivity varied depending on the analog, with analogs **15** and **17** exhibiting lower (6- to 8-fold) and analog **16** similar (37-fold) selectivities compared to zyklophin (46-fold in these assays).

Only the linear peptide **3** exhibited any appreciable affinity for DOR (K_i = 2290 ± 520 nM). All of the remaining peptides exhibited minimal DOR affinity.

The efficacies of the zyklophin analogs were evaluated in the agonist stimulated GTPγS binding assay. All of the cyclic peptides displayed negligible efficacy (<15%) when screened at 10 μM in this assay. Our hypothesis was that the N-terminal alkyl group might shift the peptide in the KOR binding site, thereby changing interactions of other parts of the peptide with the receptor and preventing the peptide from activating the receptor. Among the *N*-alkyl analogs, peptide **4** with an *N*-methyl substitution was expected to cause minimal shift of the peptide in its binding site and hence exhibit agonist activity, but this was not the case. The negligible efficacy of peptides **4–8** and **15–17** in the GTPγS assay suggest that changes in the N-terminal alkyl group, ring size or residue 5 configuration did not increase efficacy compared to zyklophin. As expected, none of the alanine-substituted analogs exhibited appreciable efficacy in this assay, nor did the Phe^1 analog **14**. Only the linear peptide **3** exhibited partial agonist activity at KOR in the GTPγS assay (26% efficacy compared to the full agonist Dyn A(1-13)NH_2, EC_{50} = 39.5 ± 17.4 nM); these results are consistent with those found for [*N*-benzyl-Tyr^1]Dyn A(1-11)NH_2 in the adenylyl cyclase assay.[21]

The KOR antagonist potency of selected analogs was analyzed in the GTPγS assay by Schild analysis against Dyn A(1-13)-NH_2 as the KOR agonist[29] (Figure 4, Table 4). The potency of zyklophin as a KOR antagonist in the GTPγS assay was consistent with its KOR affinity, but somewhat lower than that found previously in the adenylyl cyclase assay (K_B =

84 nM).[21] The *N*-CPM peptide **6** showed potent antagonism in this assay, 28-fold higher than zyklophin, and the *N*-phenethyl peptide **7** exhibited reasonable antagonist potency. The Phe^1 peptide **14** also exhibited potent KOR antagonism, consistent with its binding affinity, indicating that the Tyr phenol is not required for the KOR antagonist potency of zyklophin. The Ala^7 analog **12** did not antagonize KOR, consistent with its lower KOR affinity.

Conclusions

Various linear and cyclic analogs of zyklophin were synthesized in order to examine the SAR of this peptide for KOR interaction and antagonism. During the synthesis there was potential for epimerization of the N-terminal residue because solutions of the N-alkyl amino acid required heating to obtain complete dissolution. An HPLC solvent system that could resolve the diastereomers of zyklophin was identified and used to examine the peptides for potential racemization of the N-terminal residue. Only in the case of *N*-benzyl-Phe was evidence of epimerization detected. These results are not surprising since elevated temperatures (typically 75 °C) are routinely used in microwave-assisted amino acid couplings in peptide synthesis, and phenylalanine is well known to be prone to racemization. To avoid potential epimerization of *N*-benzyl-Phe and to increase the solubility of *N*-CPM-Tyr the Alloc derivatives, which were readily soluble at room temperature, were prepared; this eliminated the epimerization of *N*-benzyl-Phe and increased the yield of the *N*-CPM-Tyr^1 peptide **6**.

The *in vitro* pharmacological evaluation of the linear and the cyclic zyklophin analogs provided initial SAR for the lead peptide zyklophin. The receptor affinities of the linear analogs **2** and **3** suggested that the residue at position 5 has a greater influence on the opioid receptor affinities of zyklophin than residue 8. The L-configuration and/or the hydrophobic side chain of Leu in position 5 of peptide **3** likely contributes to the higher KOR and MOR affinities of this peptide. However, the KOR affinity of the cyclic analog **17** with an L-Asp at position 5 suggested that the configuration of residue 5 has a minor influence on the KOR affinity of cyclic zyklophin analogs. The *N*-phenethyl and the *N*-CPM substitutions resulted in a slightly higher (1.5- to 2-fold) affinity for KOR than zyklophin, while the other N-terminal modifications and ring variations were also well tolerated. The basic amine at the N-terminus of zyklophin is not necessary for maintaining the KOR affinity of zyklophin, as shown by the relatively modest reduction in KOR affinity of the *N*-benzoyl analog **8** compared to zyklophin. These results are consistent with other peptide KOR antagonists based on dynorphin identified in our laboratory.[11–13] The pharmacological results for alanine substituted analogs indicate that Phe^4 and Arg^6 are critical for zyklophin's KOR affinity. Interestingly, the Tyr^1 residue makes a relatively minor contribution to the KOR affinity of zyklophin based on the results for the *N*-benzyl-Ala^1 analog, and the similar KOR affinity of the *N*-benzyl-Phe^1 analog to zyklophin indicates that the phenol of Tyr does not contribute to the KOR affinity of zyklophin. While the loss of KOR affinity with Ala substitution of Phe^4 is similar to that found for Dyn A(1-13),[26] the minimal effect of substitution by Ala or Phe in position 1 are in marked contrast to the effects in Dyn A(1-13) and Dyn A(1-11)NH_2.[26,28] Substitution of Ala at postion 6 markedly decreased KOR affinity, while replacement of residue 7 had a much smaller effect on KOR affinity,

indicating that a basic residue in position 6 is much more important for the KOR affinity of zyklophin than is one in position 7. As expected, the Ala[9] analog displayed affinity comparable to zyklophin, indicating a basic residue at position 9 is not important for the maintaining the KOR affinity of zyklophin.

The results from the agonist stimulated GTPγS assays indicate that all of the zyklophin analogs synthesized in the present study, except for the linear peptide **3**, exhibited negligible efficacy (< 15% compared to full agonist Dyn A(1-11)NH_2) similar to zyklophin. Schild analysis of the analogs with the highest KOR affinities found that the *N*-phenethyl-Tyr[1], *N*-CPM-Tyr[1] and the *N*-benzyl-Phe[1] analogs were more potent KOR antagonists than zyklophin in this assay. These analogs appear to be promising compounds for additional studies towards the potential development of potent peptide KOR antagonists. Further studies of selected zyklophin analogs are underway in our laboratory.

Experimental section

Materials

Fmoc-protected PAL-PEG-PS resin was purchased from Applied Biosystems (Foster City, CA). Standard Fmoc-protected amino acids were obtained from EMD Biosciences (Gibbstown, NJ) and Peptides International (Louisville, KY), Fmoc-D-Asp(Pip)OH was obtained from Bachem (King of Prussia, PA), and Fmoc-Dap(Mtt)OH, Fmoc-Dab(Mtt)-OH and Fmoc-Orn(Mtt)-OH were obtained from EMD Biosciences. Fmoc-*N*Me-Tyr(O*t*Bu)-OH was obtained from APPTec (Louisville, KY), and Tyr-O*t*Bu was obtained from EMD Biosciences. PyBOP and PyClocK were purchased from EMD Biosciences. HOBt was purchased from Peptides International, and DIEA was from Fisher Scientific (Fair Lawn, NJ). Piperidine, THF and anhydrous acetonitrile (SureSeal) were purchased from Sigma-Aldrich (St. Louis, MO). All HPLC-grade solvents (acetonitrile, DMF, DCM, EtOAc, hexane and methanol) used for amino acid and peptide synthesis or HPLC analysis were obtained from Fisher Scientific. TFA for HPLC purification and analysis was purchased from Pierce (Rockford, IL); benzaldehyde and sodium cyanoborohydride from Sigma-Aldrich; and phenylacetaldehyde, allyl bromide, allyl chloroformate and TIS were from Acros Organics (Fairlawn, NJ).

Amino acid synthesis

General procedure for *N*-alkyl-Tyr-OH synthesis—The amino acid *t*-butyl ester (1 equiv) and $NaBH(OAc)_3$ (1 equiv) were dissolved in anhydrous acetonitrile (SureSeal, 5–10 mL/mmol), followed by the addition of the corresponding aldehyde (1.5 equiv) and a catalytic amount of glacial acetic acid (0.2 mL). The reaction was performed under N_2 for 12–18 h at room temperature with continuous stirring. The reaction was monitored by TLC (1:2 EtOAc:hexane plus 1 drop NEt_3). After completion of the reaction the acetonitrile was evaporated *in vacuo*, the pH adjusted to 7.0 with saturated $NaHCO_3$, and the aqueous layer extracted with EtOAc (3 × 10 mL). The combined EtOAc extracts were washed with water, and the organic layer was dried ($MgSO_4$) and evaporated under reduced pressure to obtain crude *N*-alkyl-Tyr-O*t*Bu that was purified by flash silica gel column chromatography (20–30% EtOAc in hexane). The *N*-alkyl-Tyr-O*t*Bu was analyzed for purity by HPLC using a

gradient of 5–50% aqueous acetonitrile with 0.1% TFA over 45 min at a flow rate of 1 mL/min. The *t*-butyl ester on the purified *N*-alkyl-Tyr-O*t*Bu was cleaved by 90% TFA plus 10% water (8–10 mL/mmol) for 12 h. The reaction mixture was concentrated *in vacuo*, and the desired product was obtained by precipitation with diethyl ether.

***N*-Benzyl-Tyr-OH (23a)—**Tyr-O*t*Bu (0.474 g, 2.0 mmol, 1 equiv) was reacted with benzaldehyde and $NaBH(OAc)_3$ in anhydrous acetonitrile (12 mL) for 18 h. The crude product was isolated as described above, and pure *N*-benzyl-Tyr-O*t*Bu (520 mg, 80%) was obtained following flash silica gel column chromatography (25% EtOAc/hexane) as an off-white amorphous solid: ESI-MS *m/z* $[M+H]^+$ 328.1913 (calcd), 328.1784 (observed); HPLC: t_R 24.3 min (purity 100%); ^1H NMR (500 MHz, d_6-acetone) δ 8.14 (s, 1H), 7.35 – 7.15 (m, 5H), 7.04 (d, *J* = 8.2 Hz, 2H), 6.74 (d, J = 8.7 Hz, 2H), 3.82 (d, *J* = 13.3 Hz, 1H), 3.64 (d, *J* = 13.3 Hz, 1H), 3.30 (t, *J* = 6.8 Hz, 1H), 2.88 – 2.71 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.90, 153.14, 136.83, 128.38, 126.42, 126.38, 125.96, 125.20, 113.40, 79.60, 60.52, 49.84, 36.56, 25.98.

N-Benzyl-Tyr-O*t*Bu (200 mg) was cleaved to the corresponding acid using TFA as described above to yield *N*-benzyl-Tyr-OH (160 mg, 68%) as a white amorphous powder: ESI-MS *m/z* $[M+H]^+$ 272.1287 (calcd), 272.1227 (observed).

***N*-Benzyl-D-Tyr-OH (23b)—**D-Tyr-O*t*Bu (0.118 g, 0.5 mmol, 1 equiv) and $NaBH(OAc)_3$ in anhydrous acetonitrile (5 mL) were reacted with benzaldehyde for 18 h. Pure *N*-benzyl-D-Tyr-O*t*Bu (90 mg, 55%) was obtained following flash silica gel column chromatography (20% EtOAc/hexane) as an off-white amorphous solid: ESI-MS *m/z* $[M+H]^+$ 328.1913 (calcd), 328.1890 (observed); ^1H NMR (500 MHz, $CDCl_3$) δ 7.35 – 7.17 (m, 5H), 6.97 (d, *J* = 8.4 Hz, 2H), 6.62 (d, *J* = 8.5 Hz, 2H), 3.80 (d, *J* = 12.8 Hz, 1H), 3.66 (d, *J* = 12.8 Hz, 1H), 3.42 (t, *J* = 6.9 Hz, 1H), 2.95 – 2.77 (m, 2H), 1.39 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.36, 153.37, 137.44, 128.83, 126.84, 126.74, 126.67, 125.59, 113.75, 79.98, 60.98, 50.29, 37.03, 26.43.

N-Benzyl-D-Tyr-O*t*Bu (90 mg) was cleaved with TFA as described above to yield *N*-benzyl-D-Tyr-OH (51 mg, 49%) as a white amorphous powder: ESI-MS *m/z* $[M+H]^+$ 272.1287 (calcd), 272.1251 (observed).

***N*-Phenethyl-Tyr-OH (24)—**Treatment of Tyr-O*t*Bu (0.474 g, 2.0 mmol, 1 equiv) with $NaBH(OAc)_3$ and phenyl acetaldehyde in anhydrous acetonitrile (10 mL) for 16 h yielded pure *N*-phenethyl-Tyr-O*t*Bu (452 mg, 66%) as an off-white oil following flash silica gel column chromatography (25% EtOAc in hexane): ESI-MS *m/z* $[M+H]^+$ 342.2069 (calcd), 342.2004 (observed); HPLC: t_R 27.9 min (purity 100%); ^1H NMR (500 MHz, d_6-acetone) δ 8.13 (s, 1H), 7.32 – 7.11 (m, 5H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.74 (d, *J* = 8.5 Hz, 2H), 3.31 (dd, *J* = 6.4 and 7.6 Hz, 1H), 2.83 – 2.68 (m, 6H), 1.34 (s, 9H); ^{13}C NMR (126 MHz, d_6-acetone) δ 174.49, 156.89, 141.43, 131.36, 129.64, 129.18, 126.82, 115.77, 80.84, 64.77, 50.22, 39.67, 37.42, 28.25.

N-Phenethyl-Tyr-OtBu (165 mg) was cleaved to afford the corresponding acid **24** (125 mg, 65%) as a yellowish-white solid: ESI-MS m/z $[M+H]^+$ 286.1443 (calcd), 286.1432 (observed).

***N*-Benzyl-Ala-OH (25)—**Ala-O*t*Bu HCl (0.43 g, 2.4 mmol, 1.2 equiv) and benzaldehyde (0.2 mL, 2.0 mmol, 1 equiv) were dissolved in $CHCl_3$ (10 mL), followed by addition of a solution of $NaBH(OAc)_3$ (0.63 g, 3 mmol, 1.5 equiv) in $CHCl_3$ (5 mL). The reaction mixture was stirred at rt for 12 h, water (20 mL) was added, the aqueous layer was separated and extracted with additional $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ layers were washed with water (20 mL), dried over $MgSO_4$ and the solvent evaporated to obtain the product which was purified by flash silica gel column chromatography (15% EtOAc /hexane) to give *N*-benzyl-Ala-O*t*Bu (220 mg, 39%) as a white semisolid: ESI-MS *m/z* $[M+H]^+$ 236.1651 (calcd), 236.1628 (observed); HPLC: t_R 13.08 min (purity 95.8%) ;^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 – 7.23 (m, 5H), 3.82 (d, *J* = 12.7 Hz, 1H), 3.68 (d, *J* = 12.7 Hz, 1H), 3.27 (q, *J* = 7.0 Hz, 1H), 1.86 (s, 1H), 1.51 (s, 9H), 1.30 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.37, 140.14, 128.59, 128.45, 127.19, 81.07, 56.86, 52.15, 28.29, 19.35.

N-Benzyl-Ala-O*t*Bu (120 mg) was cleaved to afford the corresponding acid as a yellowish-white solid (75 mg, 50%): ESI-MS *m/z* $[M+H]^+$ 180.1025 (calcd), 180.1009 (observed).

***N*-CPM-Tyr-O*t*Bu (27)—**Tyr-O*t*Bu (0.237 g, 1.0 mmol, 1 equiv) was reacted with cyclopropylcarboxaldehyde and $NaBH(OAc)_3$ in anhydrous acetonitrile (7 mL) for 12 h, and purified by flash silica gel column chromatography (30% EtOAc in hexane) to give *N*-CPM-Tyr-O*t*Bu (160 mg, 55%) as a white powder: ESI-MS *m/z* $[M+H]^+$ 292.1913 (calcd), 292.1819 (observed); HPLC: t_R 19.10 min (purity 97.4%); ^{1}H NMR (500 MHz, d_6-acetone) 8.05 (s, 1H), 6.97 (dd, *J* = 6.5, 2.0 Hz, 2H), 6.65 (dd, *J* = 6.5, 2.1 Hz, 2H), 3.22 (dd, *J* = 7.6, 6.3 Hz, 1H), 2.79 – 2.67 (m, 1H), 2.66 – 2.59 (m, 1H), 2.38 (dd, *J* = 11.7, 6.1 Hz, 1H), 2.21 (dd, *J* = 11.7, 7.2 Hz, 1H), 1.26 (s, 9H), 0.82 – 0.72 (m, 1H), 0.35 – 0.27 (m, 2H), 0.05-0.01 (dd, *J* = 4.8, 1.8 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.11, 155.11, 130.60, 128.73, 115.55, 81.53, 63.38, 53.28, 38.99, 28.23, 11.10, 3.86, 3.46.

Alloc-*N*-CPM-Tyr(Alloc)-OH (31)—*N*-CPM-Tyr-O*t*Bu (0.08 g, 0.27 mmol, 1 equiv) in anhydrous DCM (2.5 mL) was treated with allyl chloroformate (0.117 mL, 1.08 mmol, 4 equiv) and DIEA (0.189 mL, 1.08 mmol, 4 equiv) for 12 h at room temperature. The DCM was evaporated *in vacuo*, EtOAc (10 mL) was added, and the solution washed with water (10 mL). Following back-extraction of the aqueous layer with additional EtOAc (10 mL), the combined EtOAc fractions were washed with water (10 mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford the product **29** (91 mg, 73%) as a yellowish white semisolid: ESI-MS *m/z* $[M+Na]^+$ 482.2155 (calcd), 482.2087 (observed); HPLC t_R 18.51 min (25–95% of aqueous acetonitrile with 0.1 %TFA over 35 min, purity 95.4%); ^{1}H NMR (500 MHz, $CDCl_3$, 50 °C) δ 7.22 – 7.14 (m, 2H), 7.07 (d, *J* = 8.5 Hz, 2H), 6.03 – 5.87 (m, 2H), 5.46 – 5.18 (m, 4H), 4.74 – 4.48 (m, 4H), 4.08 (m, 1H), 3.23 (m, 3H), 2.56 (m, 1H), 1.44 (s, 9H), 0.69 (m, 2H), 0.35 (m, 2H), 0.05 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.97, 155.99, 153.61, 150.25, 136.79, 131.57, 130.48, 121.02, 119.34, 81.73, 69.17, 66.30, 63.35, 53.64, 29.86, 28.24, 10.34, 4.19, 3.83.

The ester was cleaved with 90% TFA/10% DCM for 12 h at room temperature. The TFA was evaporated, and EtOAc (10 mL) and water (10 mL) were added. The aqueous layer was extracted with EtOAc (10 mL), and the combined organic extracts were washed with water (10 mL), dried with $MgSO_4$ and evaporated *in vacuo* to yield the product (75 mg, 94%) as a yellowish white semisolid: ESI-MS *m/z* $(M+K)^+$ 442.1268. (calcd), 442.1250 (observed); HPLC: t_R 12.05 min (25–95% aqueous acetonitrile with 0.1 % TFA over 35 min, purity 94.5%).

***N*-Benzyl Phe-O *t* Bu (28)—**A solution of $NaBH(OAc)_3$ (1.5 equiv) in $CHCl_3$ (2 mL) was added to a solution of H-Phe-O*t*Bu HCl (0.618 g, 2.4 mmol, 1.2 equiv) and benzaldehyde (1 equiv) in $CHCl_3$ (5 mL), and the reaction mixture stirred at room temperature for 12 h. Water (20 mL) was added, and the aqueous layer extracted with additional $CHCl_3$ (3 × 20 mL). The combined $CHCl_3$ extracts were washed with water (20 mL) and dried over $MgSO_4$, and the crude product was purified by flash silica gel column chromatography (5–10% EtOAc in hexane) to give **28** (335 mg, 44%) as a white semisolid: ESI-MS *m/z* $[M+H]^+$ 312.1964 (calcd), 312.1853 (observed); HPLC: t_R 28.96 min (purity 99.1%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 – 7.18 (m, 10H), 3.86 (d, *J* = 13.0 Hz, 1H), 3.73 (d, *J* = 13.0 Hz, 1H), 3.51 (dd, *J* = 7.6 and 6.6 Hz, 1H), 3.00 (dd, *J* = 13.6 and 6.5 Hz, 1H), 2.91 (dd, *J* = 13.6 and 7.7 Hz, 1H), 1.35 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.82, 138.19, 136.99, 129.47, 128.53, 128.49, 128.31, 127.45, 126.70, 81.65, 62.23, 51.65, 39.21, 27.97.

Alloc-*N*-benzyl-Phe-OH (32)—*N*-Benzyl-Phe-O*t*Bu (0.25 g, 0.8 mmol, 1 equiv) in anhydrous DCM (2 mL) was treated with allyl chloroformate (0.169 mL, 1.6 mmol, 2 equiv) and DIEA (0.278 mL, 1.6 mmol, 2 equiv) for 12 h. Alloc-*N*-benzyl-Phe-O*t*Bu (208 mg, 65%) was isolated as a yellowish semisolid as described above for Alloc-*N*-CPM-Tyr(Alloc)-O*t*Bu: ESI-MS *(m/z)* $[M+Na]^+$ 418.1994 (calcd), 418.2047; HPLC: t_R 18.68 min (25–95% aqueous acetonitrile with 0.1 % TFA over 35 min, purity 96.5%); ^{1}H NMR (500 MHz, $CDCl_3$, 50°C) δ 7.32 – 7.21 (m, *J* = 7.0 Hz, 6H), 7.21 – 7.05 (m, 4H), 6.05 – 5.89 (m, 1H), 5.28 (m, 2H), 4.82 – 4.52 (m, 3H), 4.16 (m, 1H), 3.99 – 3.82 (m, *J* = 13.4 Hz, 1H), 3.32 (dd, *J* = 14.0, 5.8 Hz, 1H), 3.16 (dd, *J* = 14.0, 5.8 Hz, 1H), 1.39 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.47, 156.03, 138.33, 137.51, 132.81, 129.21, 128.33, 128.13, 127.07, 126.36, 117.50, 81.49, 66.26, 62.40, 51.77, 36.24, 27.86.

The ester (120 mg) was cleaved with 90% TFA/10% DCM for 12 h at room temperature and isolated as described above for **31** to yield the acid **32** (100 mg, 98%) as a yellowish white semisolid: ESI-MS *m/z* $(M+Na)^+$ 362.1368 (calcd), 362.1347 (observed); HPLC: t_R 12.14 min (25–95% aqueous acetonitrile with 0.1 % TFA over 35 min, purity 100%).

***N*-Allyl-Tyr-OH (34)—**Tyr-O*t*Bu (0.237 g, 1.0 mmol, 1 equiv) in DMF (2 mL) was cooled to 0–4 °C, and DIEA (0.174 mL, 1.0 mmol, 1 equiv) was added, followed by slow addition of allyl bromide (0.086 mL, 1 mmol, 1 equiv). The progress of the reaction was monitored by TLC (1:3 EtOAc:hexane plus 2 drops NEt_3). After 45 h at room temperature under N_2 a negligible amount of diallyl product was visible on TLC, while a substantial amount of residual starting material was present. The DMF was then removed *in vacuo*, and the crude

product purified by flash silica gel column chromatography (22% EtOAc/hexane) to give *N*-allyl-Tyr-O*t*Bu (80 mg, 29%) as a white solid powder: ESI-MS *(m/z)* $[M\text{-}tBu]^+$ 222.1130 (calcd), 222.1083 (observed); HPLC: t_R 15.91 min (purity 100%); ^{1}H NMR (500 MHz, d_6-acetone) δ 8.19 (s, 1H), 7.05 (d, *J* = 8.1 Hz, 2H), 6.75 (d, *J* = 8.3 Hz, 2H), 5.82 (m, 1H), 5.15 (dd, *J* = 17.2 and 1.3 Hz, 1H), 5.01 (d, *J* = 10.2 Hz, 1H), 3.31 (t, *J* = 6.9 Hz, 1H), 3.19 (ddd, *J* = 77.8, 14.2, 5.9 Hz, 2H), 2.79 (ddd, *J* = 21.1, 13.5, 6.9 Hz, 2H), 1.37 (s, 9H); ^{13}C NMR (126 MHz, d_6-acetone) δ 174.52, 156.89, 138.10, 131.35, 129.53, 115.85, 115.76, 80.94, 63.75, 51.09, 39.67, 28.26.

Pure *N*-allyl-Tyr-O*t*Bu (80 mg) was deprotected as described above to obtain *N*-allyl-Tyr-OH (43 mg, 44%) as a white powder: ESI-MS *(m/z)* $[M+H]^+$ 222.1130 (calcd), 222.1060 (observed).

Peptide synthesis

General procedures for solid phase peptide synthesis

Synthesis of (2-11) peptide fragments: The peptides were assembled on a low load Fmoc-PAL-PEG-PS resin (0.19 mmol/g). Following removal of the Fmoc group from the resin (200 mg, 0.038 mmol) using 20% piperidine in DMF (2 × 20 min) the Fmoc-protected amino acids (4 equiv, 0.152 mmol) were coupled using PyBOP and HOBt (4 equiv each, 0.152 mmol) as the coupling reagents and DIEA (10 equiv, 0.38 mmol) as the base in DCM:DMF (1:1, 3–4 mL) for 2 h (unless otherwise noted) on a manual multiple peptide synthesis apparatus (CHOIR)[22] to afford the linear fragments. The side chains of Lys and Arg were protected by Boc and Pbf, respectively; those of Dap, Dab and Orn were protected by Mtt, and those of D-Asp and L-Asp were protected by Pip.

The (5–11) linear precursors of the cyclic peptides were synthesized by these standard procedures. Selective deprotection of the Pip and Mtt protecting groups on D-Asp and Dap, respectively, was performed using 3% TFA and 5% TIS in DCM (3 × 10–15 min). The cyclizations were performed using PyClocK and HOAt (4 equiv each) with DIEA (10 equiv) as the base in DCM:DMF (1:1) (4 mL for 200 mg peptide-resin) for 24 h, with the coupling reagents refreshed after every 8–12 h unless otherwise noted. The cyclizations were monitored using the qualitative ninhydrin test.[23] Any remaining unreacted free amine of Dap was capped by treatment with acetyl imidazole (16 equiv, 0.61 mmol) with DIEA (8 equiv) as the base in DMF(∼ 3 mL) for 30 min. Further extension of the peptide assembly up to Gly^2 afforded the cyclic (2-11) peptide fragments.

Coupling of the N-terminal amino acid to the (2-11) peptide: Prior to coupling, complete dissolution of the *N*-alkyl-Tyr-OH (2 equiv) was achieved by heating to 80–120 °C in DMF (1 mL/0.02 mmol of *N*-alkyl amino acid), followed by cooling to rt (unless otherwise indicated) and subsequent addition of PyClock and HOAt (2 equiv each) plus DIEA (6 equiv). The coupling reactions were generally complete after 12 h, as indicated by the qualitative ninhydrin test.

Final deprotection of the peptides: The peptides were cleaved from the resin using Reagent B[30] (87.5% TFA, 5% water, 5% phenol and 2.5% TIS, 4–5 mL/200 mg resin). The

solutions were filtered, diluted with 10% aqueous acetic acid (10–15 mL), and extracted with diethyl ether (3 × 10 mL). The ether extracts were back extracted with 10% acetic acid (10 mL); the combined aqueous solutions were pooled and lyophilized to give the crude peptides.

Synthesis of linear peptides 2 and 3: The linear (2-11) fragments were synthesized as described above under the general procedure except that Fmoc-DAsn(Trt)OH and Fmoc-Dap(Mtt)OH were used in a 2-fold excess for the synthesis of peptides **2** and **3**, respectively. In addition, for peptide **3** the coupling of Fmoc-Dap(Mtt)-OH to the peptide resin was performed using PyClocK and HOAt (2 equiv each) in the presence of DIEA (6 equiv) in DCM:DMF (1:1, 4 mL). For peptide **3**, the Mtt on Dap was selectively deprotected by 3% TFA and 5% TIPS in DCM (3 × 10 min, 3–4 mL each time), followed by acetylating with acetyl imidazole (16 equiv, 0.61 mmol) in the presence of DIEA (8 equiv) in DMF (3 mL) for 1 h. The coupling of *N*-benzyl-Tyr-OH (2 equiv) to the (2-11) fragment was performed according to the general procedure described above.

Synthesis of [*N*-benzyl-D-Tyr^1]zyklophin and peptides 4–9 and 14: The (2-11) cyclic peptide fragment was synthesized according to the general procedure described above. For the D-Tyr derivative of zyklophin *N*-benzyl-D-Tyr (5.16 mg, 0.019 mmol, 2 equiv) was dissolved in DMF (2 mL) by heating to 85 °C, the solution cooled to room temperature followed by addition of PyClocK, HOAt (2 equiv each) and DIEA (6 equiv), and coupled to the (2-11) peptide for 12 h. In the case of peptide **5** *N*-allyl-Tyr-OH (20 mg, 0.076 mmol, 2 equiv) was dissolved in DMF (6 mL) by heating to 105 °C. Since the solution became turbid when it was cooled to ∼70 °C, the coupling reagents were added to the amino acid solution at 105 °C. The solution was then cooled to rt, DIEA (6 equiv) added and the activated amino acid reacted with the (2-11) peptide fragment for 12 h to obtain peptide **5**. For peptide **7** complete dissolution of *N*-phenethyl-Tyr-OH (33 mg, 0.114 mmol, 2 equiv) was achieved by heating to 115 °C in DMF (10 mL). The solution was cooled to ∼70 °C, followed by the addition to PyClocK and HOAt (2 equiv. each). Subsequently, the solution was cooled to rt followed by the addition of DIEA (6 equiv). The amino acid was reacted with the (2-11) peptide fragment for 24 h with reagents replaced after 12 h. In the synthesis of peptide **9**, *N*-benzyl-Ala (2 equiv.) was heated to 85 °C in DMF (3.5 mL), followed by cooling the solution to 60 °C before adding PyClocK and HOAt (2 equiv each) and DIEA (6 equiv). The coupling reaction was performed for 24 h with the amino acid and coupling reagents replaced after 12 h.

For peptide **4** Fmoc-*N*Me-Tyr(O*t*Bu)-OH (54 mg, 0.114 mmol, 2 equiv) was dissolved in DMF (5 mL) at room temperature and coupled to the (2-11) cyclic peptide using PyBOP (60 mg, 0.114 mmol, 2 equiv) and HOBt (16 mg, 0.114 mmol, 2 equiv) in the presence of DIEA (6 equiv), followed by subsequent removal of the Fmoc group to afford peptide **4**. For peptide **8** Fmoc-Tyr(*t*Bu)-OH (52 mg, 0.114 mmol, 4 equiv) was coupled to the (2-11) peptide fragment in the presence of PyBOP (60 mg, 0.114 mmol, 4 equiv), HOBt (15 mg, 0.114 mmol, 4 equiv each) and DIEA (10 equiv) in DCM:DMF (1:1, 3 mL) for 2 h. Following Fmoc deprotection of Tyr, the N-terminus of the Tyr was reacted with benzoic

anhydride (0.025 mg, 0.114 mmol, 4 equiv) and DIEA (8 equiv) in DMF (2–3 mL) overnight.

For peptide **6** Alloc-*N*-CPM-Tyr(Alloc)-OH (23 mg, 0.057 mmol, 2 equiv) was dissolved in DMF (3 mL) at room temperature, and the coupling to the (2-11) peptide performed with PyClocK (31 mg, 0.057 mmol, 2 equiv) and HOAt (8 mg, 0.057 mmol, 2 equiv) in the presence of DIEA (6 equiv) for 1 h, followed by the subsequent removal of the Alloc group (see below) to afford protected peptide **6** on the resin. For peptide **14** Alloc-*N*-benzyl-Phe-OH (20 mg, 0.057 mmol, 2 equiv) was dissolved in DMF (3 mL) at rt with PyClocK and HOAt (2 equiv each) and DIEA (6 equiv) and coupled to the (2-11) peptide-resin. The coupling reaction was performed for 1 h, followed by subsequent removal of the Alloc group (see below) to afford the protected peptide **14** on resin.

Alloc deprotection of peptides 6 and 14: The resin was swollen in DCM (2 × 5 min), phenylsilane (24 equiv/ g of resin) was added, and the mixture was bubbled with N_2 for 5 min. *Tetrakis*-(triphenylphosphine) palladium (0.3 equiv) was added, and a septum with a needle was fixed onto the reaction vessel to allow the evolved carbon dioxide to escape. After shaking for 12 h the solution was drained, and the resin subjected to a series of washes as follows: DCM (4 × 1 min), THF (4 × 1 min), DCM (3 × 1 min), DMF (3 × 1 min), 0.5 % DIEA in DMF (3 × 2 min), 0.2 M sodium diethyldithiocarbamate in DMF (3 × 15 min), DMF (3 × 2 min), DCM (3 × 1 min), and methanol (3 × 1 min), followed by drying of the resin.[24, 31]

Synthesis of cyclic peptides 10–13: The synthesis of peptide **10** was performed according to the general procedure described above with Ala incorporated in position 4. For peptides **11–13** the (5–11) linear peptide fragments with Ala incorporated in the indicated position were synthesized as described under the general procedure. Following selective deprotection by 3% TFA and 5% TIPS (3 × 10 min) in DCM, the cyclization reactions were performed for 24 h using PyClocK and HOAt (4 equiv each) in the presence of DIEA (6 equiv) in DCM:DMF (1:1, 4 mL) with the reagents replaced after 12 h. Following capping with acetyl imidazole the peptides were extended up through Gly^2 as described under the general procedure. The coupling of *N*-benzyl-Tyr-OH to the (2-11) fragments followed the general procedure as described above to obtain peptides **11-13**.

Synthesis of cyclic peptides 15-17: For peptide **15** [D-Asp(OPip)^5,Dab(Mtt)^8]Dyn A-(5-11)-NH_2 was synthesized on resin according to the general procedure described above, except that Fmoc-Dab(Mtt)OH (2 equiv) was coupling three times for 2 h each during the initial two couplings and overnight for the third coupling. Subsequently, the Pip and Mtt protecting groups on D-Asp and Dab, respectively, were selectively deprotected, and the peptide was cyclized for 36 h as described above, with coupling agents replaced every 8 h. Any unreacted free amine of Dab was acetylated as described above for Dap. Further extension of the peptide chain up through Gly^2 was performed as described above under the general procedure. For peptides **16** and **17** the (5-11) linear peptide fragments were synthesized by the general procedure as described above. The Pip and Mtt protecting groups on D-Asp and Orn, respectively (peptide **16**) and L-Asp and Dap, respectively (peptide **17**)

were selectively deprotected using 3% TFA and 5% TIS in DCM (3 × 10 min). The peptides were cyclized for 36 h using PyClocK and HOAt (4 equiv each) in the presence of DIEA (6 equiv.) in DCM:DMF (1:1, 4 mL) with the reagents replaced every 12 h. Following capping with acetyl imidazole the peptide was extended up through Gly^2 as described under the general procedure. Subsequent coupling of *N*-benzyl-Tyr-OH to the corresponding (2-11) fragments was done as described for the general procedure to afford peptides **15–17**.

Purification and analysis of the peptides: The crude peptides were purified by preparative reversed phased HPLC on a Vydac C18 column (10 μ, 300 Å, 22 × 250 mm) equipped with a Vydac C18 guard cartridge using an LC-AD Shimadzu liquid chromatograph system equipped with an SPD-10A VP system controller and SPD-10A VP UV-Vis detector. For purification, a linear gradient of 5–40% aqueous acetonitrile containing 0.1% TFA over 35 min, at a flow rate of 18 mL/min, was used. The purifications were monitored at 214 nm. The purity of the final peptides was verified on a Vydac 218-TP column (5 μ, 300 Å, 4.6 mm x 50 mm) equipped with a Vydac guard cartridge on a Shimadzu LC-10AT VP analytical HPLC liquid chromatograph (equipped with SCL-10A VP system controller and SPD-10A VP UV-Vis detector) or on an Agilent 1200 series liquid chromatograph system equipped with a multiple wavelength UV-Vis detector. Three systems were used for the analyses: 1) a linear gradient of 5–50% solvent B (solvent A = aqueous 0.1% TFA and solvent B = acetonitrile containing 0.1% TFA) over 45 min at a flow rate of 1 mL/min (system 1), 2) a linear gradient of 15–60% solvent B (solvent A = aqueous 0.1% TFA and solvent B = methanol containing 0.1% TFA) over 45 min at a flow rate of 1.0 mL/min (system 2), and 3) a linear gradient of 1–21% solvent B (solvent A = aqueous 0.09 M TEAP, pH = 2.5 and solvent B = acetonitrile) over 40 min. The final purity of all peptides by both analytical systems was ≥ 98% (see Table 2). Molecular weights of the compounds were determined by ESI-MS using a time of flight mass spectrometer analyzer (LCT premier, Waters, Milford, MA).

Pharmacological evaluation

Radioligand binding assays—Radioligand binding assays were performed as previously described[17] using cloned rat KOR and MOR and mouse DOR stably expressed on CHO cells. Incubations with isolated membrane protein were performed in triplicate with concentrations of the cyclic tetrapeptides from 0.1 nM to 10 μM for 90 min in 50 mM Tris, pH 7.4 at 22 °C using [^{3}H]diprenorphine (K_d = 0.45 nM), [^{3}H]DAMGO ([D-Ala^2,NMePhe^4,glyol]enkephalin, K_d = 0.49 nM), and [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin, K_d = 1.76 nM) as radioligands in assays for KOR, MOR, and DOR, respectively (B_{max} = 2.27, 2.20, and 1.45 pmol/mg protein, respectively). Non-specific binding was determined in the presence of 10 μM unlabeled Dyn A(1-13)NH_2, DAMGO and DPDPE for KOR, MOR, and DOR, respectively. Reported K_i values for the analogs are the means ± S.E.M. from at least three independent experiments except where noted.

Agonist stimulated GTPγS assay—The binding of the GTP analog [^{35}S]GTPγS (0.1–0.2 nM) to membranes containing KOR was performed in duplicate for 90 min in 50 mM HEPES, pH 7.4, at 22 °C as described previously.[32] Specific binding was defined as total binding minus nonspecific binding that occurring in the presence of 3 μM unlabeled GTPγS.

At least two independent experiments were performed to evaluate the agonist activity of the analogs at 10 μM compared to the full agonist Dyn A(1-13)NH_2; K_B values from the Schild analysis are the mean ± S.E.M. from at least three independent experiments except where noted.

Acknowledgments

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ABBREVIATIONS

Alloc	allyloxycarbonyl
CHO	Chinese hamster ovary
CPM	cyclopropylmethyl
Dab	2,3-diaminobutyric acid
DAMGO	([D-Ala^2,NMePhe^4,glyol]enkephalin
Dap	2,3-diaminopropionic acid
DIEA	*N*,*N*-diisopropylethylamine
DOR	delta opioid receptor
DPDPE	(*cyclo*[D-Pen^2,D-Pen^5]enkephalin
Dyn	dynorphin
GPI	guinea pig ileum
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
JDTic	(3R)-7-hydroxy-*N*-[(2*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide
KOR	kappa opioid receptor
MOR	mu opioid receptor
Mtt	4-methyltrityl
norBNI	nor-binaltorphimine
Orn	ornithine
PAL	peptide amide linker
Pbf	2,2,4,6,7-pentamethyldihydrobenzofurane-5-sufonyl
PEG-PS	poly(ethylene glycol)-polystyrene
Pip	2-phenylisopropyl

PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyClocK	6-chlorobenzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate
RP-HPLC	reversed phase HPLC
s.c.	subcutaneous
TEAP	triethylammonium phosphate
TIS	triisopropylsilane
Trt	trityl

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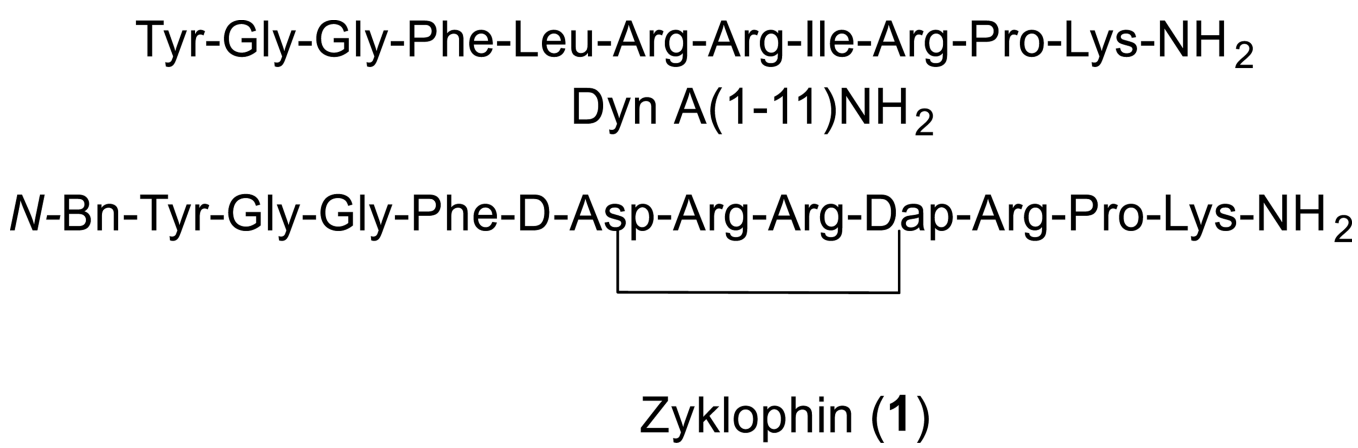
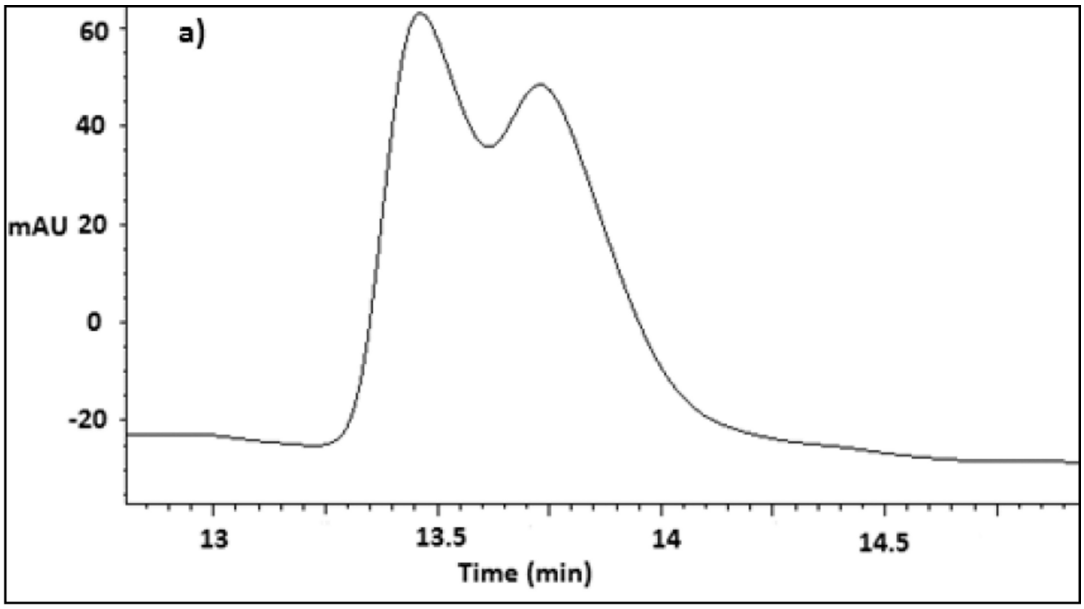
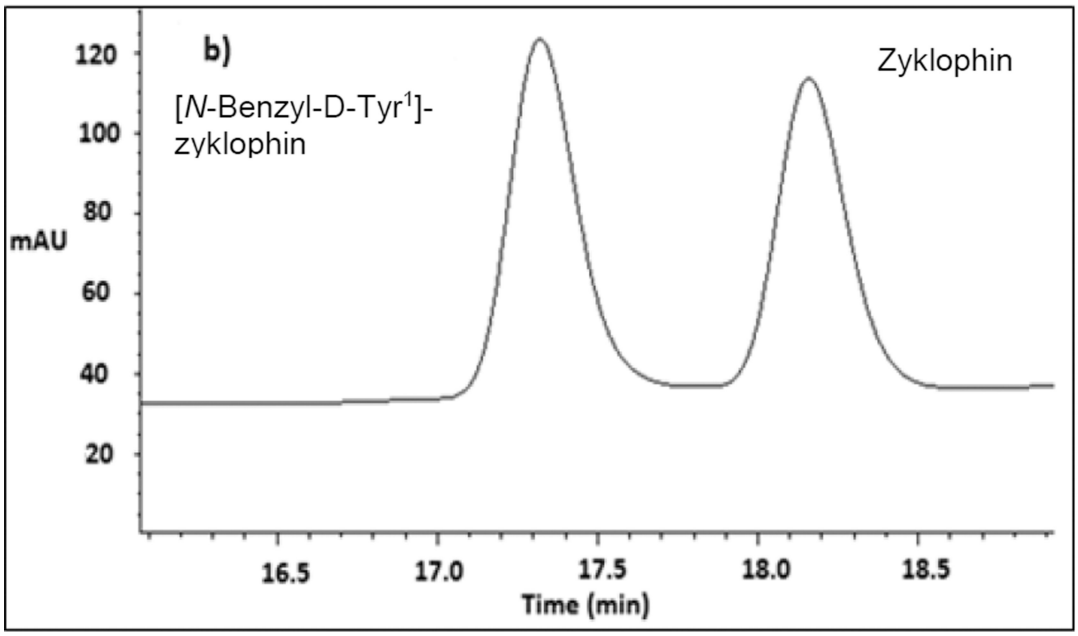


Figure 1.
Structures of Dyn A(1-11)NH_2 and zyklophin (**1**)





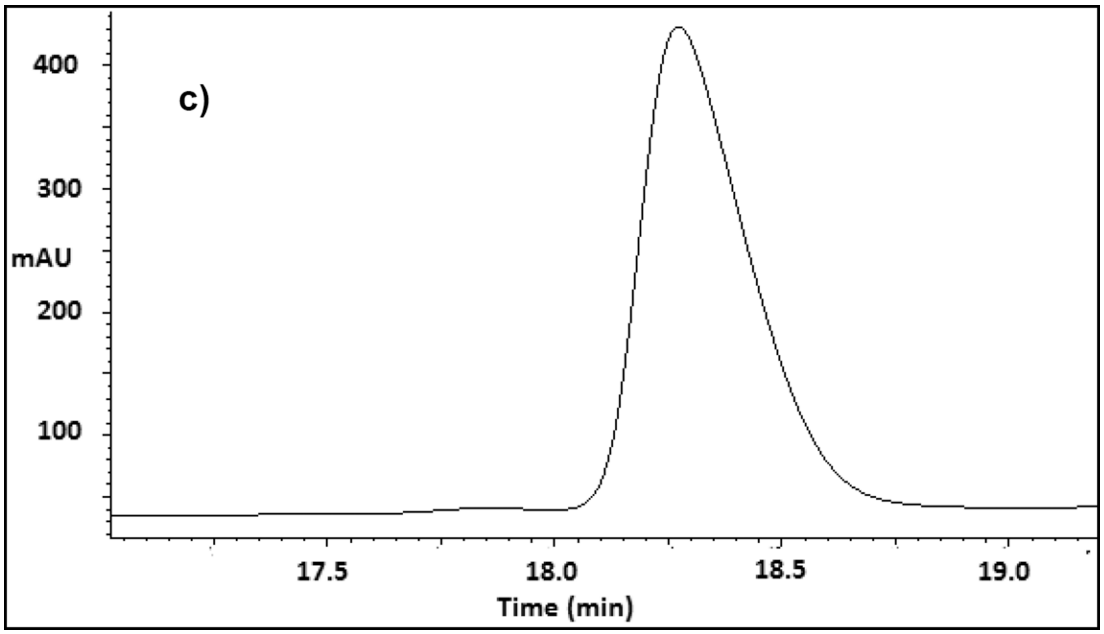


Figure 2.

Separation of a diastereomeric mixture containing [*N*-benzyl-D-Tyr[1]]zyklophin and zyklophin using a) a linear gradient of aqueous acetonitrile containing 0.1% TFA (solvent A = aqueous 0.1% TFA and solvent B = acetonitrile containing 0.1% TFA, 10–25% solvent B over 30 min) and b) a linear gradient of TEAP (solvent A = aqueous 0.09 M TEAP, pH 2.5, and solvent B = acetonitrile, 1–21% solvent B over 40 min). t_R for the D-Tyr diastereomer of zyklophin = 17.3 min and for zyklophin = 18.2 min. c) Zyklophin (t_R = 18.3 min) synthesized by the standard procedure and analyzed using the TEAP solvent system showed no detectable epimerization.

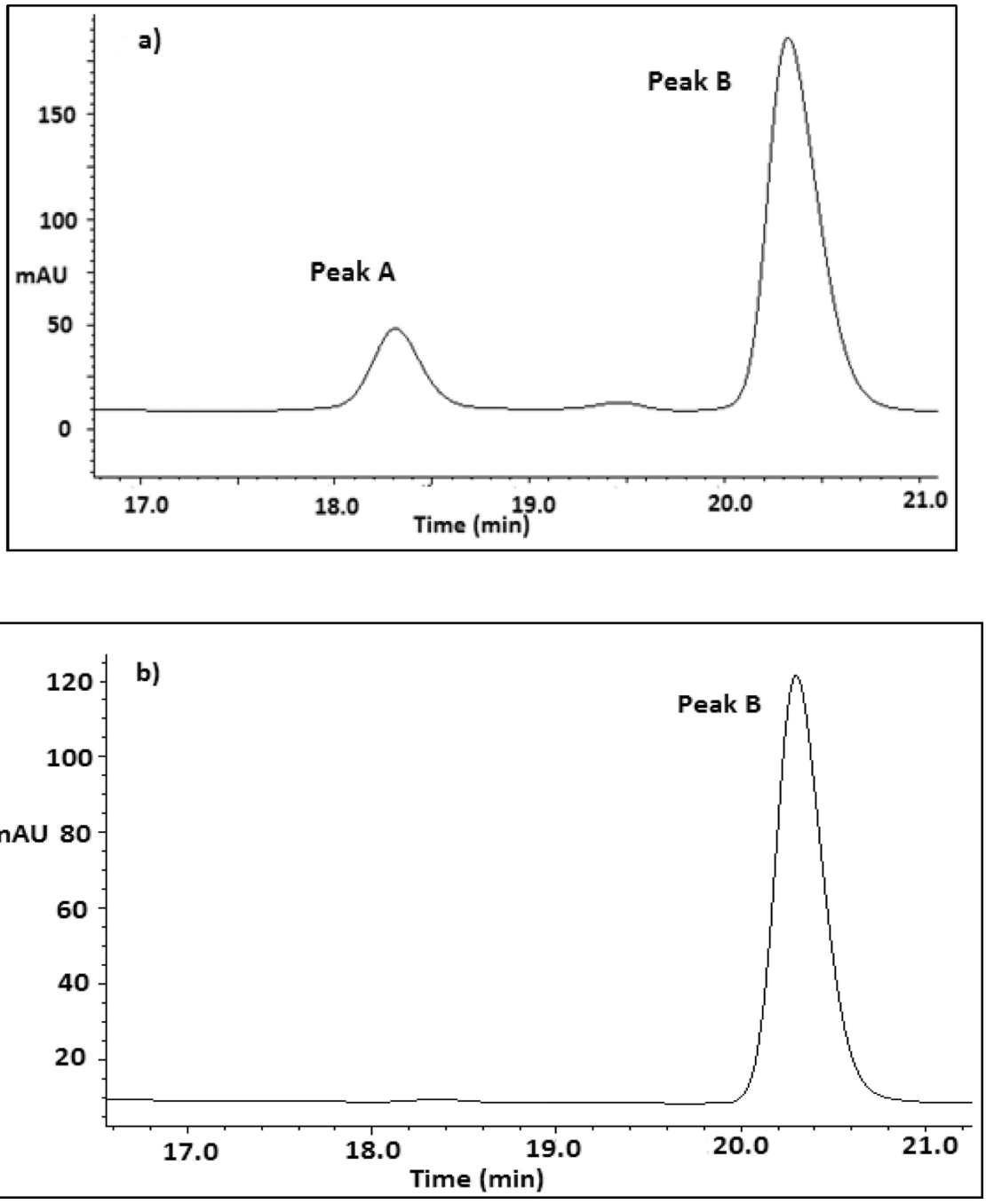


Figure 3.

Chromatograms of peptide **14** synthesized by a) the standard procedure and b) using Alloc-*N*-benzyl-Phe using the TEAP solvent system.

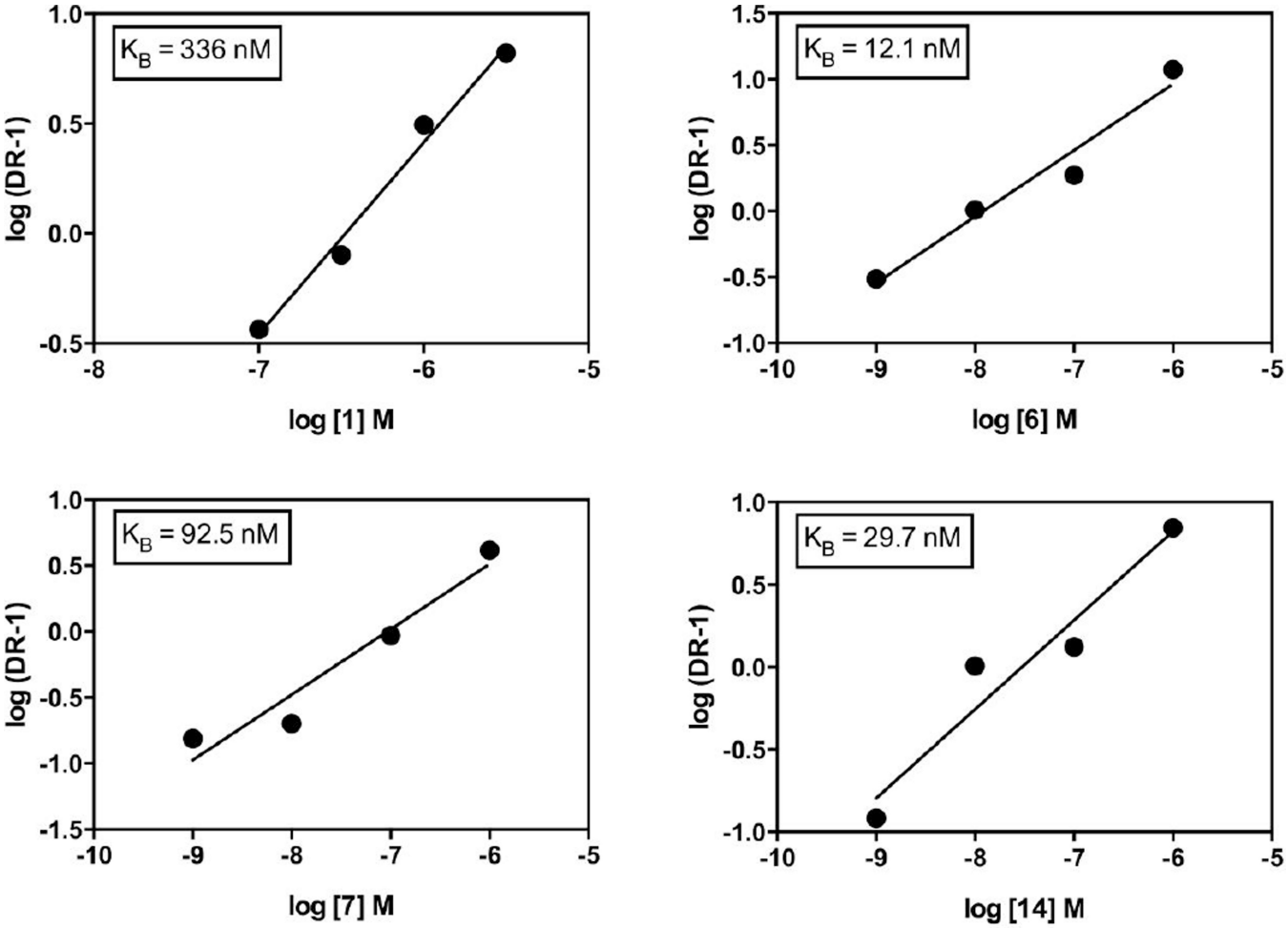
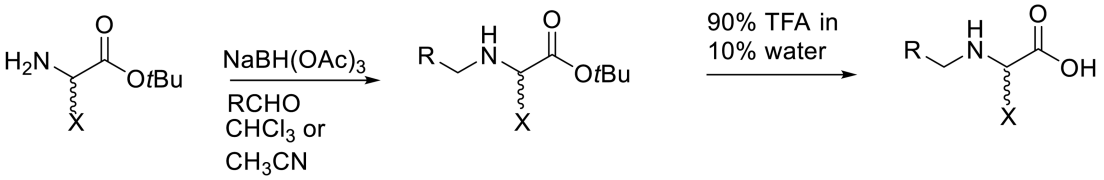


Figure 4.

Schild regression analysis of the ability of zyklophin (**1**) and peptides **6, 7** and **14** to produce rightward shifts in the Dyn A(1-13)-NH_2 concentration-response relationships for stimulation of [^{35}S]GTPγS binding. Data depicted were pooled from 2–5 experiments for each peptide. The 95% confidence intervals for the K_B values are listed in Table 4.

a) Synthesis of *N*-alkyl-amino acid derivatives by reductive amination



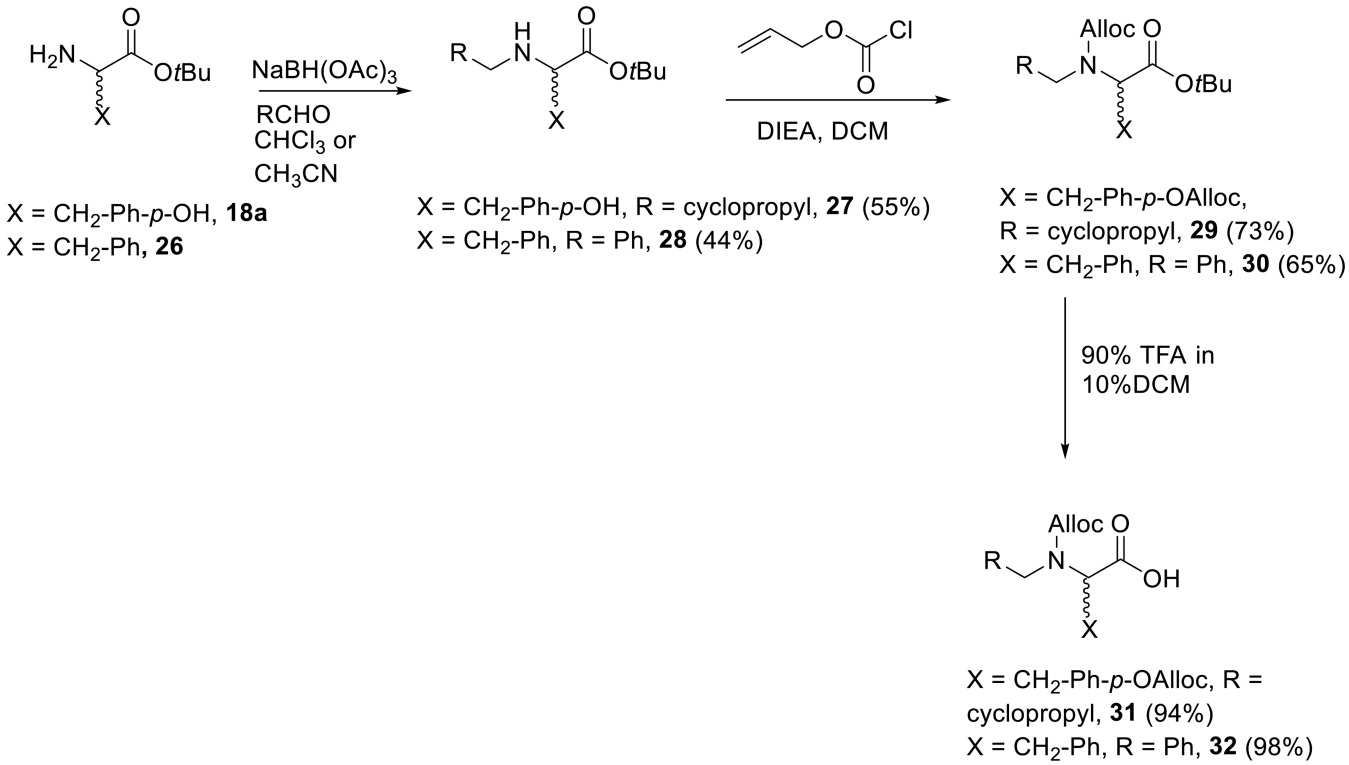
X = CH_2-Ph-*p*-OH, **18a**
X = CH_2-Ph-*p*-OH, **18b***
X = CH_3, **19**

X= CH_2-Ph-*p*-OH, R = Ph, **20a** (80%)
X= CH_2-Ph-*p*-OH, R = Ph, **20b** (55%)*
X= CH_2-Ph-*p*-OH, R = CH_2-Ph, **21** (66%)
X = CH_3, R = Ph, **22** (39%)

X = CH_2-Ph-*p*-OH, R = Ph, **23a** (68%)
X = CH_2-Ph-*p*-OH, R = Ph, **23b** (49%)*
X = CH_2-Ph-*p*-OH, R = CH_2-Ph, **24** (65%)
X = CH_3, R = Ph, **25** (50%)

* D-isomer

b) Synthesis of Alloc-*N*-alkyl amino acid derivatives



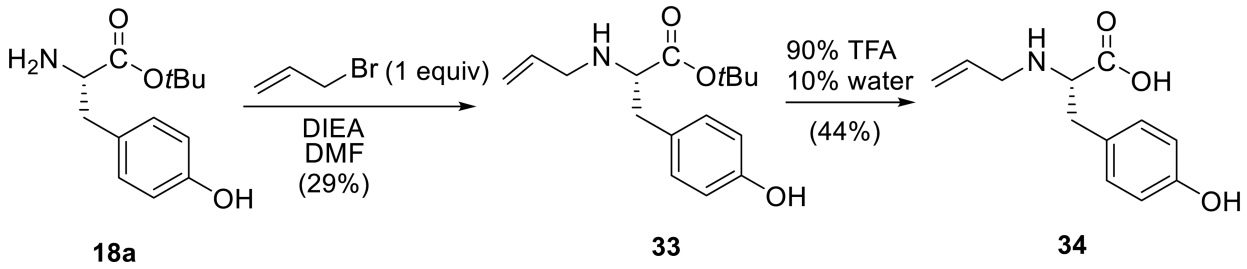
X = CH_2-Ph-*p*-OH, **18a**
X = CH_2-Ph, **26**

X = CH_2-Ph-*p*-OH, R = cyclopropyl, **27** (55%)
X = CH_2-Ph, R = Ph, **28** (44%)

X = CH_2-Ph-*p*-OAlloc,
R = cyclopropyl, **29** (73%)
X = CH_2-Ph, R = Ph, **30** (65%)

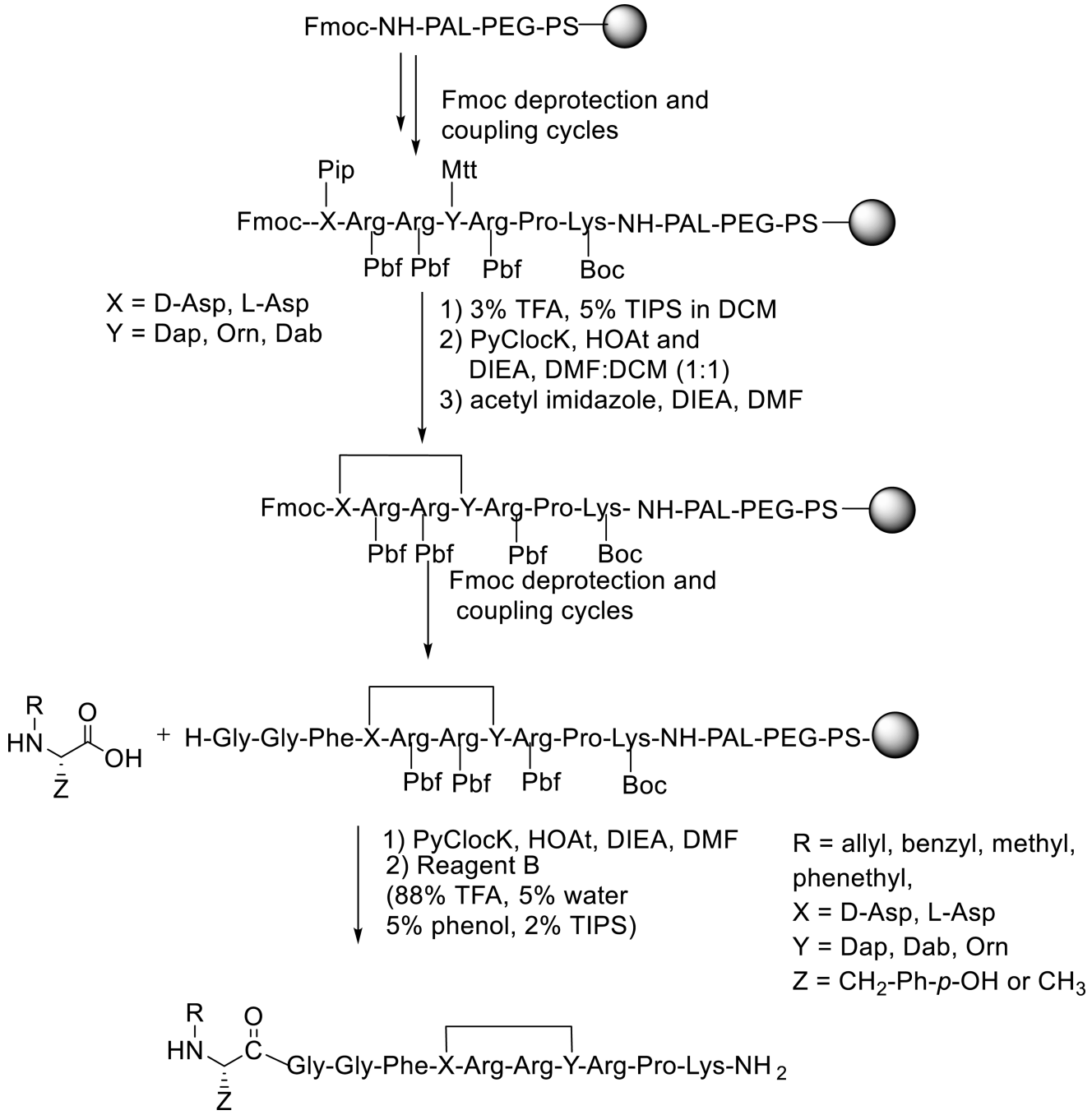
X = CH_2-Ph-*p*-OAlloc, R = cyclopropyl, **31** (94%)
X = CH_2-Ph, R = Ph, **32** (98%)

c) Synthesis of *N*-allyl-Tyr



18a **33** **34**

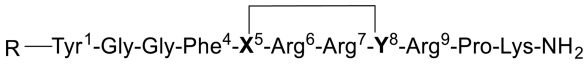
Scheme 1.
Synthesis of amino acid derivatives



Scheme 2.
Solid phase synthesis of cyclic peptides **4, 5, 7–9** and **15–17**

Table 1

Analogs of zyklophin; changes from zyklophin are shown in bold.

A) Linear analogs				
2	*N*Bn-Tyr^1-Gly-Gly-Phe-**DAsn^5**-Arg-Arg-**Ile**-Arg-Pro-Lys-NH_2			
3	*N*Bn-Tyr^1-Gly-Gly-Phe-**Leu**-Arg-Arg-**Dap(Ac)^8**-Arg-Pro-Lys-NH_2			
B) Cyclic analogs				
				
	R	**X^5**	**Y^8**	Other substitutions
1 (Zyklophin)	Bn	D-Asp	Dap	
N-terminal modifications				
4	**Me**	D-Asp	Dap	
5	**CH_2=CH-CH_2**	D-Asp	Dap	
6	**CPM**	D-Asp	Dap	
7	**Phenethyl**	D-Asp	Dap	
8	**Bz**	D-Asp	Dap	
Amino acid substitutions				
9	Bn	D-Asp	Dap	**Ala^1**
10	Bn	D-Asp	Dap	**Ala^4**
11	Bn	D-Asp	Dap	**Ala^6**
12	Bn	D-Asp	Dap	**Ala^7**
13	Bn	D-Asp	Dap	**Ala^9**
14	Bn	D-Asp	Dap	**Phe^1**
Ring variants				
15	Bn	D-Asp	**Dab**	
16	Bn	D-Asp	**Orn**	
17	Bn	**L-Asp**	Dap	

Bn = benzyl

Bz = benzoyl

Table 2

Analytical data for the peptides

Peptides	HPLC t_R (min)			ESI-MS(*m/z*)	
	System 1[a] (% Purity)	System 2[b] (% Purity)	System 3[c] (% Purity)	Calcd	Obsd
2	15.4 (>99.5)	18.0 (>99.5)		$[M + 3H]^{3+}$ = 484.9	$[M + 3H]^{3+}$ = 484.9
				$[M + 2H]^{2+}$ = 726.9	$[M + 2H]^{2+}$ = 726.9
3	17.7 (99.4)	23.1 (>99.5)		$[M + 3H]^{3+}$ = 489.6	$[M + 3H]^{3+}$ = 489.6
				$[M + 2H]^{2+}$ = 733.9	$[M + 2H]^{2+}$ = 733.9
4	11.3 (>99.5)	13.1 (99.4)		$[M + 3H]^{3+}$ = 444.9	$[M + 3H]^{3+}$ = 444.9
				$[M + 2H]^{2+}$ = 666.8	$[M + 2H]^{2+}$ = 666.9
5	12.1 (>99.5)	14.2 (>99.5)	11.9 (>99.5)	$[M + 3H]^{3+}$ = 453.5	$[M + 3H]^{3+}$ = 453.6
				$[M + 2H]^{2+}$ = 679.8	$[M + 2H]^{2+}$ = 679.9
6	13.1 (>99.5)	14.4 (99)	13.5 (>99.5)	$[M + 3H]^{3+}$ = 458.2	$[M + 3H]^{3+}$ = 458.2
				$[M + 2H]^{2+}$ = 686.8	$[M + 2H]^{2+}$ = 686.8
7	17.0 (>99.5)	15.3 (>99.5)	21.52 (98.9)	$[M + 3H]^{3+}$ = 474.9	$[M + 3H]^{3+}$ = 474.9
8	18.1 (>99.5)	23.3 (>99.5)		$[M + 3H]^{3+}$ = 474.9	$[M + 3H]^{3+}$ = 474.9
				$[M + 2H]^{2+}$ = 711.8	$[M + 2H]^{2+}$ = 711.9
9	13.3 (>99.5)	13.3 (>99.5)	11.58 (>99.5)	$[M + 3H]^{3+}$ = 439.5	$[M + 3H]^{3+}$ = 439.6
10	10.1 (>99.5)	8.0 (>99.5)		$[M + 3H]^{3+}$ = 444.9	$[M + 3H]^{3+}$ = 444.9
				$[M + 2H]^{2+}$ = 666.8	$[M + 2H]^{2+}$ = 666.9
11	15.1 (>99.5)	18.5 (>99.5)		$[M + 3H]^{3+}$ = 441.9	$[M + 3H]^{3+}$ = 441.9
				$[M + 2H]^{2+}$ = 662.3	$[M + 2H]^{2+}$ = 662.4
12	14.8 (99.0)	15.2 (98.9)		$[M + 3H]^{3+}$ = 441.9	$[M + 3H]^{3+}$ = 441.9
				$[M + 2H]^{2+}$ = 662.3	$[M + 2H]^{2+}$ = 662.4
13	14.8 (>99.5)	14.6 (>99.5)		$[M + 3H]^{3+}$ = 441.9	$[M + 3H]^{3+}$ = 441.9
				$[M + 2H]^{2+}$ = 662.3	$[M + 2H]^{2+}$ = 662.3
14	17.0 (98.1)	15.6 (>99.5)	20.1 (98.7)	$[M + 3H]^{3+}$ = 464.9	$[M + 3H]^{3+}$ = 464.9
15	15.3 (>99.5)	18.5 (>99.5)		$[M + 3H]^{3+}$ = 474.9	$[M + 3H]^{3+}$ = 474.9
				$[M + 2H]^{2+}$ = 711.9	$[M + 2H]^{2+}$ = 711.9
16	15.1 (>99.5)	18.1 (>99.5)		$[M + 3H]^{3+}$ = 479.6	$[M + 3H]^{3+}$ = 479.6
				$[M + 2H]^{2+}$ = 718.9	$[M + 2H]^{2+}$ = 718.9
17	13.2 (99.1)	15.2 (99.0)		$[M + 3H]^{3+}$ = 470.2	$[M + 3H]^{3+}$ = 470.2
				$[M + 2H]^{2+}$ = 704.8	$[M + 2H]^{2+}$ = 704.8

[a] Aqueous acetonitrile containing 0.1% TFA, λ = 214 nm;

[b] aqueous methanol containing 0.1% TFA λ = 220 nm;

[c] aqueous acetonitrile containing 0.09 M TEAP, λ = 214 nm. See the experimental section for details.

Table 3

KOR and MOR affinities and selectivities of zyklophin analogs.

Peptide	KOR K_i (nM ± SEM)[a]	Relative KOR affinity[b]	MOR K_i (nM ± SEM)[a]	K_i ratio[c,d]
2, D-Asn^5	412 ± 60	0.23	2440 ± 570	6
3, Dap(Ac)^8	28.6 ± 0.6	3.3	157 ± 25	5
4,*N*-Me	160 ± 18	0.60	1490 ± 410	9
5,*N*-Allyl	326 ± 72	0.29	11000 ± 4,000	34
6,*N*-CPM	44.4 ± 16.3	2.1	10900 ± 2700	245
7,*N*-Phenethyl	62.0 ± 19.9	1.5	8470 ± 560	137
8,*N*-Benzoyl	346 ± 21	0.28	10600 ± 1700	31
9, Ala^1	516 ± 82	0.18	>10000	>19
10, Ala^4	4010 ± 360	0.02	> 10,000 (2)	>2.5
11, Ala^6	1750 ± 540	0.05	11500 ± 2600	7
12, Ala^7	293 ±118	0.32	>10000	>34
13, Ala^9	168 ± 50	0.57	2550 ± 640	15
14, Phe^1	68.7 ±18.1	1.4	11300 ± 1980	164
15, Dab^8	403 ± 91	0.24	3200 ± 850	8
16, Orn^8	222 ± 5	0.42	8260 ± 1870	37
17, L-Asp^5	410 ± 17	0.23	2610 ± 780	6
1, (zyklophin)	95.2 ± 28.8[e]	1.0	4380 ± 560	46

[a] K_i values for KOR and MOR were obtained from n ≥ 3 independent experiments except where noted.

[b] Compared to zyklophin (1.0).

[c] K_i (MOR)/K_i (KOR)

[d] The analogs exhibited minimal DOR affinity (<20% inhibition of binding when screened against DOR at 10 μM, K_i > 5 μM) except for **3** (K_i = 2290 ± 520 nM).

[e] 95% confidence interval 30–160 nM (n = 10).

Table 4

KOR antagonist potencies of selected zyklophin analogs in the Dyn A(1-13)NH_2 stimulated GTPγS assay

Peptide	K_B (95% Confidence interval), nM[a,b]
6 [*N*-CPM]	12.1 (0.8–61)
7 [*N*-Phenethyl]	92.5 (9.0–6110)
14 [Phe^1]	29.7 (0.5–1320)
1 (zyklophin)	336 (180–535)

[a] K_B values derived from Schild analysis in the GTPγS assay. Values for the peptides are the average from 2–5 independent experiments. For reference the norBNI K_B = 0.074 nM

[b] Peptide **12** did not cause a significant shift in the Dyn A(1-13)NH_2 concentration-response curve at concentrations up to 10 μM.