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The Effects of C-Terminal Modifications on the Opioid Activity of [*N*-BenzylTyr^1]Dynorphin A-(1−11) Analogues

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Structural modifications affecting the efficacy of analogues of the endogenous opioid peptide dynorphin (Dyn) A have focused on the N-terminal "message" sequence based on the "message-address" concept. To test the hypothesis that changes in the C-terminal "address" domain could affect efficacy, modified amino acids and cyclic constraints were incorporated into this region of the partial agonist [*N*-benzylTyr^1]Dyn A-(1−11). Modifications in the C-terminal domain of [*N*-benzylTyr^1]Dyn A-(1−11)NH_2 resulted in increased κ opioid receptor (KOR) affinity for all of the linear analogues but did not affect the efficacy of these peptides at KOR. Cyclization between positions 5 and 8 yielded [*N*-benzylTyr^1,*cyclo*(D-Asp^5, Dap^8)]Dyn A-(1−11)NH_2 (zyklophin, **13**) (*J. Med. Chem.* **2005**, *48*, 4500−4503) with high selectivity for KOR. In contrast to the linear peptides, this peptide exhibits negligible efficacy in the adenylyl cyclase (AC) assay and is a KOR antagonist. These data are consistent with our hypothesis that appropriate modifications in the "address" domain of Dyn A analogues may affect efficacy.

Introduction

Narcotic analgesics that target μ opioid receptors (MOR[a]) have been used extensively in the clinical management of pain,[1] but their use is complicated by serious side effects.[2] Thus there has been considerable interest in the development of ligands for κ opioid receptors (KOR) as potential therapeutic agents for pain and other disorders.[3] KOR agonists can produce analgesia with less addiction liability and respiratory depression than MOR agonists,[4,5] and compounds with KOR agonist activity are used clinically (e.g., pentazocine). However, KOR agonists can also cause dysphoria in humans.[6] In addition to the centrally mediated analgesia, KOR agonists can produce antinociception through peripheral KOR[7] and peripherally selective KOR agonists are in clinical trials as analgesics.[8] Moreover, KOR agonists also exhibit anti-inflammatory activity.[9] Other potentially useful therapeutic effects of KOR agonists include anticonvulsant and neuroprotective effects and their ability to down-regulate HIV-1 expression in human microglial and CD_4 cells (see ref 10 for a review). KOR antagonists, initially used only as pharmacological tools, also have potential therapeutic uses in the treatment of depression,[11−13] anxiety,[14,15] and opiate and cocaine addiction.[12,16,17] The KOR selective peptide antagonist arodyn[18] designed in our laboratory suppresses stress-induced reinstatement of cocaine seeking behavior in mice,[17] suggesting its potential use in the development of peptide-based therapeutics for the treatment of cocaine addiction.

The heptadecapeptide dynorphin (Dyn) A (Figure 1) is an endogenous agonist for KOR.[19] Studies of Dyn A and its fragments by Chavkin and Goldstein led them to propose the "message-address" concept in which the four N-terminal amino acids in Dyn A are the "message" sequence responsible for opioid agonist activity and the C-terminal domain is the "address" that enhances the affinity of this peptide for KOR.[20] The entire C-terminal sequence is not required for KOR interaction, however, and the first 11 to 13 residues of Dyn A account for essentially all of the opioid activity of the longer peptide.[20]

We are interested in exploring the structural features of Dyn A that affect efficacy and result in peptide antagonists for KOR. Structural modifications in the N-terminus of Dyn A can alter efficacy as well as potency and selectivity. Alkylation of the N-terminus can influence both KOR affinity and efficacy. While the *N*,*N*-diallylTyr^1 analogues of [D-Pro^{10}]Dyn A-(1−11) and Dyn A-(1−13) exhibit antagonist activity at KOR and MOR[21−23] (but with decreased binding affinity and selectivity for KOR compared to the parent peptides without the N-terminal modification), the monoalkylated derivatives have high KOR affinity and selectivity.[23,24] The N-allyl- and N-cyclopropylmethyl analogues are potent agonists in the guinea pig ileum (GPI) assay, while the N-benzyl analogue exhibits weak agonist activity in the GPI assay[23,24] and is a partial agonist in the adenylyl cyclase (AC) assay using cloned KOR expressed on Chinese hamster ovary (CHO) cells.[25] Other modifications in the N-terminal sequence of Dyn

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[a] Abbreviations: AC, adenylyl cyclase; Boc, *tert*-butyloxycarbonyl; CHO, Chinese hamster ovary; Dap, 2,3-diaminopropionic acid; DOR, δ opioid receptor; DMF, *N*,*N*-dimethylformamide; Dyn, dynorphin; EL, extracellular loop; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; KOR, κ opioid receptor; MOR, μ opioid receptor; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Mtt, 4-methyltrityl; NMM, *N*-methylmorpholine; NMM, *N*-methylmorholine; PAC, peptide acid linker; PAL, peptide amide linker; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sufonyl; PEG-PS, poly(ethylene glycol)-polystyrene; Pip, 2-phenylisopropyl; PyAOP, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Tis, triisospropylsilane; Trt, trityl.

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HTyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys[11]-Leu-Lys[13]-Trp-Asp-Asn-GlnOH

Figure 1. Dynorphin A.

A-(1−11) analogues that result in antagonist activity at KOR are Pro in position 3,[26] and the removal of the basic α-amine of Tyr[1] in analogues such as dynantin.[27] Other analogues with antagonist activity, namely JVA-901,[28] arodyn,[18] and *cyclo*^{N,5}[Trp[3],Trp[4],Glu[5]]Dyn A-(1−11)NH_2,[29] prepared in our laboratory, also lack a basic N-terminus.

Reports in the literature examining modifications in the C-terminal or "address" sequence of Dyn A suggest that in selected cases such modifications may affect the efficacy of these analogues at KOR. While analogues of [Phe[1]]Dyn A-(1−11)NH_2 with D-Ala[8] and D-Pro[10] substitutions retain similar KOR affinity (< 50% difference), they exhibit large differences in potency in the GPI[30](almost a 50-fold decrease for the D-Ala[8] analogue compared to the parent peptide). Such discrepancies in affinity and potency suggest a possible role of the C-terminus in the efficiency of receptor activation. A variety of cyclic analogues of Dyn A, cyclized either with a disulfide or lactam linkage, have been prepared to restrict conformational freedom and study possible bioactive conformations (see refs 3 and 31 for reviews). Interestingly, some of these analogues also display discrepancies between KOR affinity and potency in the GPI. Cyclic analogues with disulfide linkages between residues 5 and 11 ([5,11]), which may stabilize a reverse turn in the C-terminal region of Dyn A-(1−13)NH_2, were reported to possess high KOR affinity in brain homogenate but low potency in the GPI assay, which the authors suggested could be due to different interactions with central vs peripheral KOR.[32] Similarly, a series of lactam bridged [5,8] cyclic analogues of Dyn A-(1−13)NH_2 with varying ring sizes prepared in our laboratory exhibit much lower potency in the GPI than anticipated based on their KOR binding affinity.[33] Also, while the [5,8] cyclic analogues exhibit similar binding affinities for KOR, they varied substantially in potency in the GPI (IC_{50} = 530 − >5000 nM). Interestingly, a computational model suggests a possible role for the hydrophobic residues Leu[5] and Ile[8] in the "address" sequence of Dyn A-(1−10) in KOR activation.[34] Molecular simulation of Dyn A-(1−10) bound to KOR suggested that these two residues interact with hydrophobic residues in extracellular loop (EL) 2 of KOR.[34] On the basis of this model, it was proposed that these hydrophobic interactions may play a role in the efficacy and/or affinity of Dyn A for this receptor. In contrast, [6,9] cyclic analogues exhibit similar KOR affinities and are potent agonists in the GPI (IC_{50} = 7−46 nM).[33]

On the basis of these results, we hypothesized that C-terminal residues and/or the conformation of the C-terminal domain of Dyn A, while not a primary determinant, could affect the efficacy of Dyn A analogues. To explore the possible roles of the C-terminus in efficacy, we introduced a series of modifications into [*N*-benzylTyr[1]]Dyn A-(1−11) (Figure 2). This peptide was chosen because [*N*-benzylTyr[1],D-Pro[10]]Dyn A-(1−11) exhibits partial agonist activity in the AC assay (71% relative to Dyn A-(1−13)NH_2),[25] and therefore we could readily detect changes in efficacy. We anticipated that selected changes in the C-terminal domain of the peptide [*N*-benzylTyr[1]]Dyn A-(1−11) could alter the efficacy of these analogues at KOR. Because the basic residues in the C-terminal sequence of Dyn A were thought to be important in KOR interaction,[20] the modifications were mainly carried out in the nonbasic residues in the "address". On the basis of the results with [Phe[1]]Dyn A-(1−11)NH_2 analogues discussed above, we chose D-Ala[8] and D-Pro[10] as modifications to incorporate into the C-terminal domain. To retain the important Arg side chain but introduce a modification that could alter peptide backbone conformation, N^{α}-MeArg was also incorporated in position 7. On the basis of the lactam bridged cyclic Dyn A analogues *cyclo*[D-Asp[5],Dap[8]]- and *cyclo*[D-Asp[6],Dap[9]]Dyn A-(1−13)NH_2 synthesized previously in our laboratory,[33] we also examined cyclic constraints between the fifth and eighth and between the sixth and ninth positions in the C-terminal domain to evaluate their effect on efficacy at KOR. These analogues were evaluated for their opioid receptor affinity in radioligand binding assays and for opioid activity in the AC assay using cloned KOR stably expressed on CHO cells.[25] Among these, the [5,8] cyclic analogue [*N*-benzylTyr[1],*cyclo*(D-Asp[5],Dap[8])]Dyn A-(1−11)NH_2 (which we have named zyklophin) exhibits negligible efficacy at KOR and acts an antagonist in the AC assay at KOR, as we reported in an earlier communication.[35] Here we describe the synthesis and report the pharmacological profiles of the full series of C-terminally modified [*N*-benzyl-Tyr[1]]Dyn A-(1−11) analogues.

1 HTry-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-LysOH

2 HTyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-LysNH_2

3 $PhCH_2$Tyr-Gly-Gly-Phe-*Leu-Arg-Arg-Ile-Arg-Pro-LysOH*

4 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro[10]-LysOH

5 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-LysNH_2

6 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-**N^{α}–MeArg[7]**-Ile-Arg-Pro-LysNH_2

7 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-Arg-**D-Ala[8]**-Arg-Pro-LysNH_2

8 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-**D-Pro[10]**-LysNH_2

9 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-**N^{α}–MeArg[7]-D-Ala[8]**-Arg-Pro-LysNH_2

10 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-**N^{α}–MeArg[7]**-Ile-Arg-**D-Pro[10]**-LysNH_2

11 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-Arg-**D-Ala[8]**-Arg-**D-Pro[10]**-LysNH_2

12 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-Arg-**N^{α}–MeArg[7]-D-Ala[8]**-Arg-**D-Pro[10]**-LysNH_2

13 (zyklophin) $PhCH_2$Tyr-Gly-Gly-Phe-**D-Asp[5]**-Arg-Arg-**Dap[8]**-Arg-Pro-LysNH_2

14 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-**D-Asp[6]**-Arg-Ile-**Dap[9]**-Pro-LysNH_2

15 $PhCH_2$Tyr-Gly-Gly-Phe-**D-Asn[5]**-Arg-Arg-**Dap(Ac)[8]**-Arg-Pro-LysNH_2

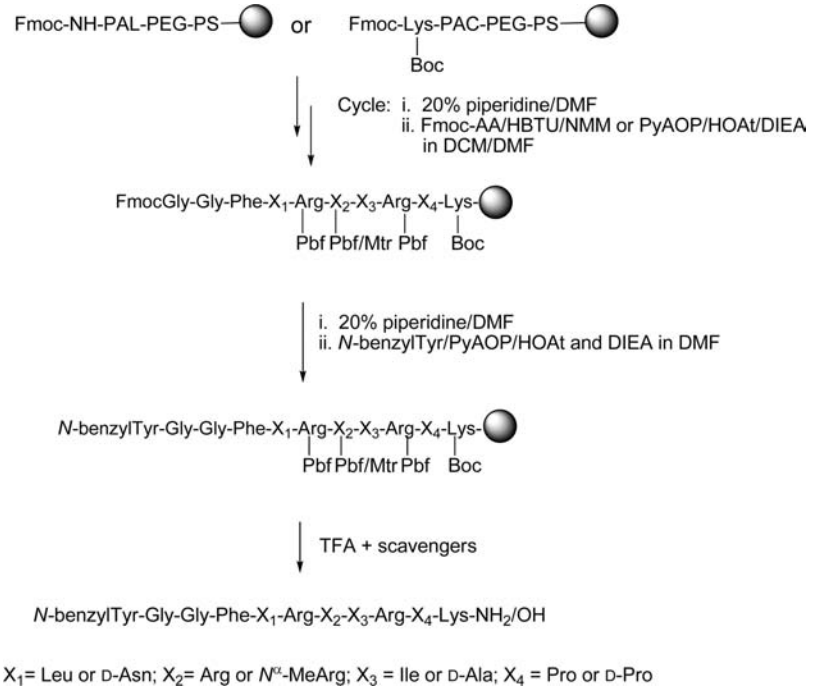
16 $PhCH_2$Tyr-Gly-Gly-Phe-Leu-**D-Asn[6]**-Arg-Ile-**Dap(Ac)[9]**-Pro-LysNH_2

Figure 2. Dyn A-(1−11) and its analogues. Modifications in peptides **4**−**16** were made in the "address" sequence (italicized in **3**). Structural differences from peptide **5** are highlighted in bold in subsequent analogues.

Chemistry

The peptides were synthesized using the Fmoc (9-fluorenylmethoxycarbonyl) solid phase synthetic strategy (Scheme 1). *N*-Benzyltyrosine was synthesized separately in solution and then coupled to the peptide sequences. Selective monobenzylation of tyrosine *tert*-butyl ester was carried out by reductive amination with benzaldehyde using sodium cyanoborohydride in an acidic solution, followed by deprotection of the *tert*-butyl ester using trifluoroacetic acid (TFA) to give *N*-benzyltyrosine. The Dyn A-(2−11) sequences were assembled on a poly(ethylene glycol)-polystyrene (PEG-PS) resin containing the peptide amide linker [PAL,

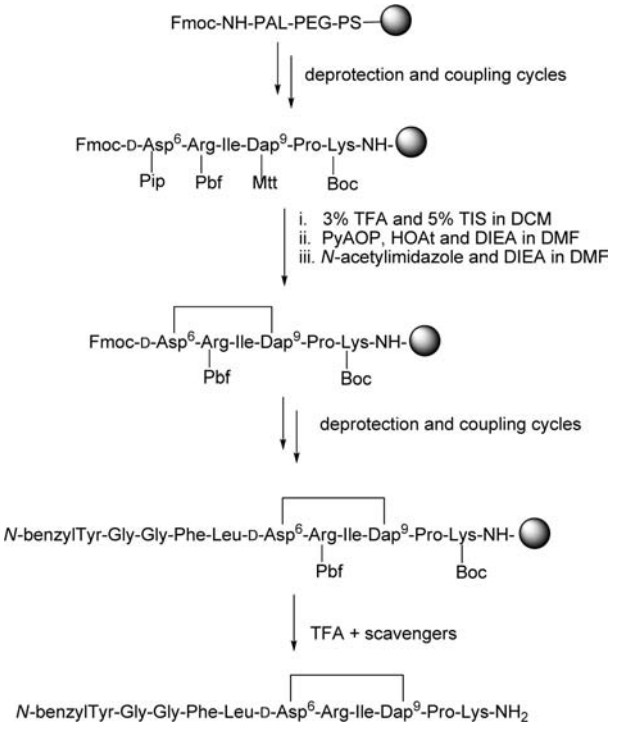
Scheme 1. Solid Phase Synthesis of Linear [*N*-benzylTyr[1]]Dyn A-(1-11) Analogues (**1**−**12**)



5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid linker] for the amides or the peptide acid linker [PAC, (hydroxymethyl)phenoxyacetic acid linker] for the acid derivatives **1**, **3**, and **4**, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-methylmorpholine (NMM) in *N*,*N*-dimethylformamide (DMF) to couple Fmoc-amino acids to the growing peptide chain.[28] The side chain protecting groups used were Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sufonyl) and Mtr (4-methoxy-2,3,6-trimethylbenzenesulfonyl) for the side chains of Arg and N^{α}-Me-Arg, respectively, and Boc (*tert*-butyloxycarbonyl) for Lys. The coupling of Fmoc-Arg(Pbf) at position 6 to N^{α}-Me-Arg(Mtr) at position 7 and the coupling of *N*-benzyltyrosine to the resin-bound Dyn A-(2−11) sequences were carried out using 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP),[36] 1-hydroxy-7-azabenzotriazole (HOAt), and *N*,*N*-diisopropylethylamine (DIEA) in DMF.

The cyclic peptides were assembled (Scheme 2) similar to the linear analogues except that Fmoc-D-Asp(Pip) (Pip = 2-phenylisopropyl) and Fmoc-Dap(Mtt) (Dap = 2,3-diaminopropionic acid and Mtt = 4-methyltrityl) were used in the positions involved in cyclization. The hyperacid-labile Pip[37] and Mtt[38] protecting groups could be removed by dilute (3%) TFA in the presence of the Boc and other *tert*-butyl type side chain protecting groups that require stronger acidic conditions for deprotection. The cyclizations between the side chain carboxylic acid of D-Asp and the side chain free amine of Dap were carried out using PyAOP, HOAt, and DIEA in DMF. The cyclization between D-Asp in position 6 and Dap in position 9, however, did not go to completion after 48 h, even using 6 equiv of the coupling reagents, so the remaining unreacted amine was acetylated with *N*-acetylimidazole and DIEA in DMF (Scheme 2). After cyclization, the remaining amino acids were then coupled to complete the peptide sequences. For the synthesis of the acetylated linear analogues **15** and **16**, Fmoc-D-Asn(Trt) (Trt = trityl) and Fmoc-Dap(Mtt) were used; following deprotection of the

Scheme 2. Solid Phase Synthesis of Cyclic Peptide **14**



Mtt group with dilute TFA, the free amine of Dap was acetylated using *N*-acetylimidazole in DMF.

The peptides were cleaved from the resin using either reagent B[39] or modified reagent K[40] (when N^{α}-MeArg(Mtr) was present in the peptide sequence). The crude peptides were purified to ≥98% by preparative reversed phase HPLC; they were analyzed for purity by reversed phase HPLC and their molecular weights confirmed by ESI-MS (see Supporting Information).

Table 1. Opioid Receptor Binding Affinities of Dyn A-(1-11)OH Analogues

peptide	K_i (nM) ± SEM KOR	MOR	DOR	K_i ratio KOR/MOR/DOR
1 Dyn A-(1−11)	1.11 ± 0.36	3.50 ± 0.42	8.18 ± 0.09	1/3/7
2 Dyn A-(1−11)NH_2	0.57 ± 0.01	1.85 ± 0.52	6.18 ± 1.01	1/3/11
3 [*N*-benzylTyr^1]Dyn A-(1−11)OH	14.8 ± 4.5	98.1 ± 9.8	345 ± 12	1/7/23
4 [*N*-benzylTyr^1,D-Pro^{10}]Dyn A-(1−11)OH	15.5 ± 1.0	159 ± 30	970 ± 131	1/10/63
5 [*N*-benzylTyr^1]Dyn A-(1−11)NH_2	8.17 ± 2.16	84.9 ± 16.5	474 ± 47	1/10/58
Analogues of [*N*-benzylTyr^1]Dyn A-(1−11)NH_2, **5**				
6 [N^{α}-MeArg^7]	3.99 ± 1.09	49.9 ± 9.7	345 ± 31	1/13/86
7 [D-Ala^8]	4.64 ± 0.91	51.2 ± 3.0	372 ± 25	1/11/80
8 [D-Pro^{10}]	4.56 ± 0.42	45.1 ± 4.9	148 ± 5	1/10/32
9 [N^{α}-MeArg ^{7},D-Ala^8]	3.11 ± 0.64	44.8 ± 7.3	458 ± 130	1/14/147
10 [N^{α}-MeArg ^{7},D-Pro^{10}]	2.17 ± 0.22	36.5 ± 3.6	120 ± 24	1/17/55
11 [D-Ala^8,D-Pro^{10}]	3.10 ± 0.98	46.1 ± 8.6	337 ± 54	1/15/109
12 [N^{α}-MeArg ^{7},D-Ala^8,D-Pro^{10}]	4.40 ± 0.75	69.8 ± 0.7	665 ± 174	1/16/151
13[a] *cyclo*[D-Asp^5,Dap^8] (zyklophin)	30.3 ± 1.9	5880 ± 1420	> 10,000	1/194/ > 330
14 *cyclo*[D-Asp^6,Dap^9]	245 ± 37	1190 ± 270	3660 ± 660	1/5/15
15[a] [D-Asn^5,Dap(Ac)^8]	66.9 ± 5.9	1660 ± 440	> 10,000	1/25/149
16 [D-Asn^6,Dap(Ac)^9]	2840 ± 300	1500 ± 310	4640 ± 340	2/1/3

[a] From ref 35.

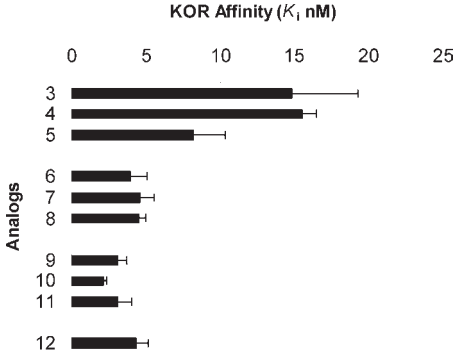


Figure 3. KOR affinities of [*N*-benzylTyr^1]Dyn A-(1−11) Analogues.

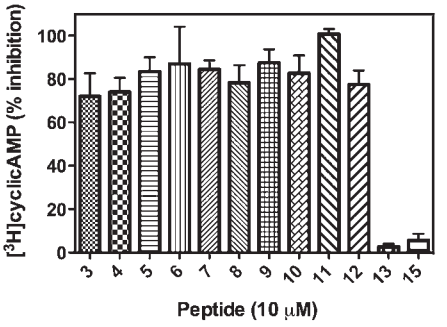


Figure 4. Efficacies of [*N*-benzylTyr^1]Dyn A-(1−11) analogues as inhibitors of adenylyl cyclase (AC) in CHO cells expressing KOR. Percent AC inhibition relative to Dyn A-(1−13)NH_2 (100%).

Results and Discussion

The [*N*-benzylTyr^1] Dyn A-(1−11) analogues were evaluated for KOR, MOR, and δ opioid receptor (DOR) affinity in radioligand binding assays (Table 1 and Figure 3) and for efficacy in the AC assay using cloned rat KOR stably expressed on CHO cells (Figure 4). N-Benzylation of Dyn A-(1−11) and [D-Pro^{10}]Dyn A-(1−11) resulted in peptides **3** and **4**, respectively, with K_i values of 15 nM for KOR using [^{3}H]diprenorphine as the radioligand. These results are comparable to those we reported previously for **4** (K_i = 18 nM).[25] In contrast to other N-alkyl Dyn A analogues, the KOR affinity determined for the [N-benzylTyr^1,D-Pro^{10}]Dyn A-(1−11) (**4**) depends on the radioligand used,[25] and the 14-fold decrease upon N-benzylation observed here was not found when the KOR affinity of **4** was determined using [^{3}H]bremazocine in guinea pig cerebellum.[23,24] N-Benzylation of Dyn A-(1−11) (**1**) and Dyn A-(1−11)NH_2 (**2**) resulted in analogues **3** and **5**, respectively, that exhibit 2- to 3-fold increases in KOR vs MOR selectivity and 3- to 5-fold increases in KOR vs DOR selectivity compared to **1** and **2** (Table 1) due to greater decreases in the affinities for MOR and DOR compared to those for KOR.

We initially prepared both [*N*-benzylTyr^1]Dyn A-(1−11) acid and amide derivatives (**3** and **5**, respectively) for comparison to [*N*-benzylTyr^1,D-Pro^{10}]Dyn A-(1−11) (**4**), which we described previously.[23−25] Compared to peptide **3**, D-Pro in position 10 did not change the KOR affinity for analogue **4** but decreased affinity for DOR, thus improving KOR vs DOR selectivity. Interestingly, converting the C-terminal acid in analogues **3** and **4** to an amide (peptides **5** and **8**, respectively) increased KOR affinity slightly (2- to 3-fold). EL2 of KOR, which has been implicated in Dyn A affinity and selectivity for KOR,[34,41] contains a unique cluster of acidic residues that is not present in human and rodent MOR or DOR.[42,43] An increase in the KOR affinity upon amidation may be due to the neutralization of unfavorable interactions between the C-terminal acid with the acidic residues on the EL2 of KOR. Conversion of the acid **3** to the amide **5** also increased selectivity for KOR over DOR 3-fold. In contrast, the corresponding conversion of the acid in the D-Pro^{10} peptide **4** to give the amide (analogue **8**) increased DOR affinity 6-fold, thus decreasing KOR vs DOR selectivity.

In the linear analogues **6**−**12** modifications that could affect the C-terminal conformation were well tolerated by KOR, with these analogues exhibiting 2- to 4-fold higher KOR affinity than the parent peptide **5** (Table 1 and Figure 3). The introduction of a single modification in the C-terminus of [*N*-benzylTyr^1]Dyn A-(1−11)NH_2 (analogues **6**−**8**) resulted in similar small increases (about 2-fold) in both KOR and MOR affinities regardless of the substitution introduced. The only notable difference in the affinities of these three

analogues was the 3-fold increase in DOR affinity as a result of incorporating D-Pro (analogue **8**). Introduction of an additional modification in the C-terminus yielded analogues **9**−**11** with similar to slightly (up to 2-fold) higher KOR affinity than the monosubstituted analogues **6**−**8**; analogue **12** with all three modifications exhibits KOR affinity similar to the monosubstituted derivatives **6**−**8** (Table 1 and Figure 3).

These modifications affected MOR and DOR affinities differently (Table 1). The MOR affinities of the linear analogues **6**−**12** were similar to one another, regardless of the substitutions, and generally about 2-fold higher than the parent peptide **5**. Because these changes in MOR affinity generally paralleled those for KOR, the selectivity for KOR over MOR varied by only 2-fold among these analogues. The changes in DOR affinity, however, were dependent on the type of substitution. While the N^{α}-MeArg^7 and D-Ala^8 modifications improved selectivity for KOR over DOR slightly, D-Pro^{10} increased DOR affinity in the C-terminal amide series, thereby reducing KOR vs DOR selectivity. In peptides **9**−**12** containing substitutions in addition to D-Pro^{10}, D-Ala^8 but not N^{α}-MeArg^7 alone decreased DOR affinity and restored selectivity for KOR over DOR. Thus, analogue **11** containing D-Ala^8 retained KOR vs DOR selectivity, while the N^{α}-MeArg^7 analogue **10** exhibited higher DOR affinity and therefore lower KOR selectivity. The addition of D-Ala^8 to N^{α}-MeArg^7 and D-Pro^{10}, resulting in analogue **12**, restored the high KOR vs DOR selectivity. Thus, substitution of D-Ala in position 8 appears to be important for maintaining high selectivity for KOR over DOR in these analogues.

In the cyclic peptides there was a marked difference in KOR affinity depending upon the position of the cyclic constraint. As reported previously,[35] the [5,8] cyclic peptide zyklophin (**13**) shows approximately a 4-fold decrease in KOR affinity (K_i = 30 nM) compared to the linear peptide **5**. Cyclization between residues 6 and 9 (cyclic analogue **14**) led to a much larger decrease in KOR affinity (K_i = 245 nM) compared to the linear analogue **5**. Zyklophin and **14** both show drastic decreases in MOR and DOR affinities compared to the linear peptide **5**. The decreases in MOR and DOR affinities were larger as a result of the [5,8] cyclization than due to the [6,9] cyclization. Thus the [5,8] cyclic analogue zyklophin shows much higher affinity for KOR and much lower affinities for MOR and DOR compared to the [6,9] cyclic analogue **14**, resulting in much greater KOR selectivity (Table 1). The affinities for all three opioid receptors for zyklophin (**13**) and analogue **14** with N-benzylation of Tyr^1 are lower than those for the unbenzylated *cyclo*[D-Asp^5,Dap^8]- (**17**) and *cyclo*[D-Asp^6,Dap^9]Dyn A-(1−13)NH_2 (**18**).[33] For the [5,8] cyclic derivative zyklophin the KOR affinity is 4-fold lower than that of **17** (K_i = 8 nM), but for the [6,9] cyclic derivative **14** the KOR affinity is 100-fold lower than that of **18** (K_i = 2.6 nM), suggesting that the steric bulk of the N-benzyl group may have shifted **14** in the KOR binding site relative to **18**, leading to the large decrease in affinity. Interestingly, zyklophin shows much higher KOR selectivity over MOR compared to analogue **17** (K_i ratio (KOR/MOR/DOR) = 1/9/412)[33] that does not possess the benzyl group on its N-terminal amine. Only a modest increase in selectivity for KOR over MOR and DOR upon N-benzylation of Tyr^1 was observed for the cyclic analogue *cyclo*[D-Asp^2,Dap^5]Dyn A-(1−11)NH_2 (K_i = 3.8 nM, K_i ratio (KOR/MOR/DOR) = 1/5/56 for the *N*-benzylTyr^1 derivative vs K_i ratio = 1/1.1/11 for the unbenzylated analogue).[44]

The linear analogues **15** and **16** were designed to evaluate the effect of the substitutions that were used to carry out the cyclizations between positions 5 and 8 or 6 and 9, respectively, on affinity and efficacy. To avoid effects due to the introduction of charged groups in the side chains, Asn and Dap(Ac) were introduced into these positions. Substitution of Asn and Dap(Ac) in positions 5 and 8, respectively, of **5** resulted in an 8-fold decrease in KOR affinity and a much larger decrease in MOR affinity compared to analogue **5**. As reported earlier,[35] the [5,8] cyclization resulted in increases in KOR affinity and KOR vs MOR selectivity compared to the linear analogue **15**. The linear peptide **16** with substitutions in positions 6 and 9 exhibits extremely low affinity for all three opioid receptors (Table 1). However, cyclization between these positions led to an 11-fold gain in KOR affinity for analogue **14** compared to **16**, while the MOR affinity for **14** remained similar to the corresponding linear analogue **16**. Thus the conformation induced by the cyclic constraint does not appear to be responsible for the low KOR affinity of **14**.

These analogues were evaluated for their efficacy in the AC assay using cloned rat KOR stably expressed on CHO cells. At concentrations of 10 μM the linear peptides generally exhibit partial to full agonist activity at KOR (Figure 4), similar to that originally reported for peptide **3** (71% maximum inhibition of AC relative to Dyn A-(1−13)NH_2).[25] The linear analogues with different C-terminal modifications (**3**−**12**) showed very similar maximum inhibition of AC (70−90% relative to Dyn A-(1−13)NH_2) regardless of the type of substitution or number of substitutions. Thus, these changes in the C-terminal domain (N^{α}-MeArg^7, D-Ala^8, D-Pro^{10}, and/or C-terminal amidation) of linear [*N*-benzylTyr^1]Dyn A-(1−11) analogues had little effect on the efficacy of the analogues at cloned KOR in the AC assay. In contrast, the cyclic analogue zyklophin (**13**) and the corresponding linear analogue **15** show minimal efficacy in the AC assay, and as reported previously, zyklophin exhibits antagonist activity (K_B = 84 nM) at KOR in this assay. The linear analogues **14** and **16** were not tested for efficacy due to their low KOR affinities.

Conclusions

Various C-terminal modified analogues of [*N*-benzylTyr^1]Dyn A-(1−11) were synthesized to evaluate the effect of these modifications on opioid receptor affinity and efficacy. The effects on opioid receptor affinity varied depending upon the type of substitution and the receptor type. Interestingly, introducing a C-terminal amide functional group appeared to enhance KOR affinity slightly. While N-benzylation substantially decreased KOR affinity in the linear analogues, incorporation of additional modifications in the C-terminus increased KOR affinity by 2- to 4-fold in peptides **6**−**12** (Table 1 and Figure 3) such that the highest affinity *N*-benzylated analogue had relatively high KOR affinity (K_i = 2.2 nM for peptide **10**). Generally, the effects on MOR affinity were similar so that the selectivity of **6**−**12** for KOR over MOR was within 2-fold that of **5**. The effects of the substituents on DOR affinity were more variable.

The most drastic changes in opioid receptor affinities occurred with the [5,8] and [6,9] cyclic analogues. Modifications in positions 5 and 8 were tolerated by KOR but not by MOR or DOR, leading to a large increase in KOR selectivity for zyklophin (**13**). The KOR affinity of zyklophin is only 4-fold lower compared to the cyclic peptide **17** without an N-benzyl group. In contrast, cyclization between positions 6

and 9 led to a very large decrease in KOR affinity. Peptide **18** without an N-benzyl group exhibits nanomolar affinity for KOR (K_i = 2.6 nM), suggesting that N-terminal alkylation shifts the peptide in the binding site, resulting in the lower KOR affinities of **14** and **16**.

There was a marked difference in the effects of the modifications in the linear analogues **3**−**12** vs peptides **13** and **15** on efficacy in the AC assay. The linear analogues **3**−**12** all exhibit similar efficacies regardless of the modifications in positions 7, 8, and/or 10 that could affect peptide conformation. In contrast, the modifications in positions 5 and 8 in peptides **13** and **15** eliminated efficacy at KOR. These results are consistent with our hypothesis that appropriate modifications in the C-terminal domain can alter the efficacy of Dyn A analogues at KOR. To further elucidate the role of the conformational constraint and stereochemistry on efficacy, we are preparing a variety of analogues of zyklophin.

Because of their target specificity and high potency, opioid peptides and their analogues could be useful lead compounds for the development of therapeutic agents. The therapeutic use of peptide-based drugs has been limited because of their low oral bioavailability. However, alternative routes of administration such as inhalation[45] are being explored that could increase the acceptance of agents that are not taken orally. Structural modifications to the endogenous peptides are necessary to impart sufficient metabolic stability for systemic administration and for the development of clinically useful peptides. Incorporation of unnatural amino acids along with conformational constraints, as are found in zyklophin, provides the improved enzymatic stability needed for in vivo studies. Metabolically stable, highly potent, and KOR-selective peptide analogues can be used for a variety of central or peripheral effects depending upon their penetration of the blood−brain barrier.[10] Dyn A-(1−11) analogues synthesized in our laboratory have shown blood−brain barrier penetration in the in vitro bovine brain microvessel endothelial cell (BBMEC) model.[46] KOR selective peptide antagonists such as zyklophin can serve as lead compounds for the development of potential treatments for opiate and cocaine addiction. Additional studies with this promising lead peptide antagonist are ongoing in our laboratories.[47]

Experimental Section

Materials. The PAC-PEG-PS resin, PAL-PEG-PS resin, HOBt, and DIEA were purchased from Applied Biosystems (Foster City, CA). HBTU was purchased from EMD Biosciences (San Diego, CA); HOAt and PyAOP were purchased from Applied Biosystems. Standard Fmoc-protected amino acids were purchased from Applied Biosystems, EMD Biosciences, and Bachem Bioscience, Inc. (King of Prussia, PA). Fmoc-N^{α}-MeArG(Mtr), Fmoc-Dap(Mtt), and Tyr*O*t*Bu were purchased from EMD Biosciences. *N*-Benzyltyrosine was synthesized as described in the Supporting Information. Piperidine, triisopropylsilane (TIS), and TFA were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents (AcOH, MeCN HPLC grade, DMF, dichloromethane (DCM), and MeOH (HPLC grade)) used for peptide synthesis or HPLC analysis were obtained from either Burdick and Jackson, Inc. (Muskegon, MI) or EM Sciences (Gibbstown, NJ). TFA for HPLC analysis was purchased from Thermo-Fisher (Rockville, IL). Molecular weights of the compounds were determined by ESI−MS using a Thermoquest LCQ mass spectrometer (University of Maryland Baltimore) and/or by fast atom bombardment mass spectrometry (FAB-MS) on a Kratos MS50RF mass spectrometer (Oregon State University).

Purification and Analysis of the Peptides. The crude peptides were purified by preparative reversed phase HPLC (Rainin HPXL HPLC system equipped with a Shimadzu SPD-10A detector) on a Vydac C18 column (10 μ, 300 Å, 21 mm × 250 mm) equipped with a Vydac guard cartridge. For purification, a linear gradient of 10−60% MeCN containing 0.1% TFA over 50 min, at a flow rate of 20 mL/min, was used. The purification was 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 × 250 mm) equipped with a Vydac guard cartridge using a Beckman System Gold analytical HPLC, consisting of a model 126 programmable solvent module and a model 168 diode array detector module. Two systems, a linear gradient of 10−60% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) over 50 min, at a flow rate of 1 mL/min (system 1), and a linear gradient of 5−80% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeOH containing 0.1% TFA) over 50 min, at a flow rate of 1.5 mL/min (system 2), were used for the analysis. The final purity of all peptides by both analytical systems was generally ≥98% (see Supporting Information).

Peptide Synthesis: General Procedure for Peptides 1−12, 15, and 16. All of the peptides were synthesized by coupling a 4-fold excess of Fmoc-protected amino acids (200 mM) on a Symphony multiple peptide synthesizer (Rainin, Inc.) using DMF as the solvent or a 3-fold excess of Fmoc-protected amino acids using a 1:1 mixture of DCM and DMF on a manual multiple peptide synthesizer ("CHOIR") constructed in house.[48] Peptides **2**, **5**−**12**, **15**, and **16** were assembled on a high load PAL-PEG-PS resin (0.4 mmol/g, 200 mg), and peptides **13** and **14** were assembled on the low load PAL-PEG-PS resin (0.17 mmol/g, 200 mg). Peptides **1**, **3**, and **4** were assembled on an Fmoc-Lys-(Boc)-PAC-PEG-PS (0.19 mmol/g, 200 mg). The side chains of the amino acids Lys, Arg, and N^{α}-MeArg were protected by Boc, Pbf, and Mtr groups, respectively. Following removal of the Fmoc protecting group using 20% piperidine in DMF for 20 min, the amino acids were coupled using HBTU (equimolar to the amino acid) and NMM (4-fold excess relative to the amino acid) as the coupling reagents for 2 h unless otherwise noted. *N*-Benzyltyrosine (1.5−3 equiv) was coupled to the resin-bound peptides using PyAOP, HOAt, and DIEA (1:1:2 relative to the amino acid) in DMF (2−3 mL). Prior to coupling, complete dissolution of *N*-benzyltyrosine was achieved by warming the solution to 90 °C, followed by cooling to about 50−60 °C and then adding PyAOP, HOAt, and DIEA. Completion of the coupling reactions was indicated by the qualitative ninhydrin test and/or chloranil test.[49] During the synthesis of the Dyn A-(2−11) sequences, coupling of Fmoc-Arg(Pbf)OH (6-fold excess) in position 6 to N^{α}-MeArg(Mtr) at position 7 of the Dyn A-(7−11) segment was carried out manually using PyAOP, HOAt, and DIEA (1:1:2 relative to the amino acid) in a 1:1 mixture of DMF and DCM. The coupling was complete in 12−24 h, as indicated by the chloranil test.

For peptides **15** and **16**, after coupling Fmoc-Dap(Mtt) at position 8 or 9, respectively, the Mtt group was selectively removed from the Dap side chain as follows: after swelling of the peptide-resin for 5 min in DCM (5 mL), the resin was treated with a mixture of 3% TFA and 5% TIS in DCM (3 × 10 min, 5 mL each). The resin was subsequently washed first with DCM and then with DMF (5 × 10 mL each). The free amine of the Dap side chain was then acetylated using *N*-acetylimidazole (10-fold excess) and DIEA (2-fold excess) in DMF:DCM (3:1, 5 mL) for 1 h. The completion of the acylation was confirmed by the qualitative ninhydrin test. Fmoc-D-Asn(Trt) was incorporated in positions 5 or 6, respectively, for peptides **15** and **16**. After removal of the N-terminal Fmoc protecting group using 20% piperidine in DMF, the rest of the amino acids were then coupled as described in the general procedure to complete the assembly of the peptides.

The peptides were generally cleaved from the resin using Reagent B[39] (88.5% TFA containing 2.5% water, 2.5% phenol and 5% TIS as scavengers) except for peptides containing N^{α}-MeArg. For these peptides containing the Mtr side chain protecting group on N^{α}-MeArg, 5% thioanisole and 2.5% 1,2-ethanedithiol in TFA[40] were used along with 5% TIS and 2.5% phenol as scavengers. The peptides were filtered from the resin, and the filtrates diluted with 10% acetic acid (about 10−15 mL) and extracted with diethyl ether (3 × 20 mL). The ether extracts were back extracted with 10% acetic acid (20 mL). The combined aqueous extracts were pooled and lyophilized to give the crude peptides.

Synthesis of Compounds 13 and 14. Peptide **14** was synthesized using the same synthetic strategy as peptide **13** described previously.[35] The cyclic peptides were assembled similar to the linear analogues except that Fmoc-D-Asp(Pip) and Fmoc-Dap(Mtt) were used in the positions involved in the cyclizations. For **13**, the amino acids from positions 5−11 and for **14**, the amino acids from positions 6−11 were coupled manually on the "CHOIR" as described above using HBTU and NMM as the coupling reagents. The Pip and Mtt groups were then selectively deprotected with a mixture of 3% TFA and 5% TIS in DCM (3 × 10 min). The cyclization was carried out by mixing the resin with a mixture of PyAOP, HOAt, and DIEA (3:3:6 relative to the resin substitution) dissolved in 1:1 DMF: DCM (4 mL) twice for a total of 24 h for **13** and three times for a total of 48 h for **14**. Any remaining unreacted free amine groups on Dap were acetylated as described above, and the rest of the peptides were assembled as described in the general procedure.

Pharmacological Assays. Radioligand binding assays were performed as previously described[33] using cloned rat KOR and MOR and mouse DOR stably expressed on CHO cells. [^{3}H]Diprenorphine (K_D = 0.45 nM), [^{3}H]DAMGO ([D-Ala^2,*N*-MePhe^4,glyol]enkephalin, K_D) (K_D = 0.49 nM), and [^{3}H]-DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin, K_D) (K_D = 1.76 nM) were used as radioligands in assays for KOR, MOR, and DOR, respectively. Results are mean ± SEM obtained from at least three independent experiments.

Adenylyl cyclase assays were performed as previously described[25] using cloned rat KOR stably expressed on CHO cells. Peptides were evaluated at 10 μM compared to reference agonist Dyn A-(1−13)NH_2 (100 nM) to determine efficacy. Results are mean ± SEM obtained from at least three independent experiments.

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Supporting Information Available: Details of the synthesis of *N*-benzyltyrosine and analytical data for the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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