Synthesis and Opioid Activity of Dynorphin A-(1-13)NH_2 Analogues Containing *cis*- and *trans*-4-Aminocyclohexanecarboxylic Acid†

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It has been proposed that the "message" sequence of dynorphin A (Dyn A) exists in an extended conformation in aqueous solution (Schiller, P. W. *Int. J. Pept. Protein Res.* **1983**, *21*, 307-312). Molecular modeling suggested that *trans*-4-aminocyclohexanecarboxylic acid (*trans*-ACCA) might function as a conformationally constrained replacement for Gly^2-Gly^3 of Dyn A in such an extended conformation. ACCA was synthesized by catalytic hydrogenation of *p*-aminobenzoic acid, and the cis and trans isomers were separated by fractional recrystallization. Analogues of Dyn A-(1-13)-NH_2 containing *cis*- and *trans*-ACCA were prepared by solid-phase peptide synthesis using the Fmoc chemical protocol. Results from radioligand binding assays indicated that the peptides have modest affinity for κ opioid receptors (K_i's = 9.1 and 13.4 nM for [*cis*-$ACCA^{2-3}$]- and [*trans*-$ACCA^{2-3}$]Dyn A-(1-13)NH_2, respectively) and modest κ-receptor selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/13/210 and 1/21/103, respectively). [*cis*-$ACCA^{2-3}$]- and [*trans*-$ACCA^{2-3}$]Dyn A-(1-13)-NH_2 are the first reported Dyn A analogues constrained in the "message" sequence that are selective for κ receptors. The *cis*-ACCA analogue showed very weak opioid activity (IC_{50} = 4.0 μM) in the guinea pig ileum.

Introduction and Rationale

Dynorphin A (Dyn A), a 17-amino acid peptide, has been postulated to be an endogenous κ opioid receptor ligand.[1] Dyn A shares with other mammalian opioid peptides a common N-terminal tetrapeptide "message" sequence which is important for opioid activity, while containing a unique C-terminal "address" sequence which imparts selectivity for κ opioid receptors.[2] The shortened Dyn A-(1-13), with the sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, accounts for essentially all of the biological activity of Dyn A in the guinea pig ileum (GPI) assay.[3]

Since Dyn A is a linear peptide, it can adopt a number of possible conformations in solution, with the conformation dependent on the environment. In aqueous solution results from several spectral techniques, circular dichroism,[4,5] IR spectroscopy,[6,7] Raman spectroscopy,[8] and NMR,[7,9,10] are consistent with a random coil and/or extended β-strand conformation for Dyn A-(1-13). Fluorescent energy transfer experiments with [Trp^4]Dyn A-(1-13) in dilute aqueous solution suggested that the N-terminal portion of Dyn A is in an extended conformation.[11] On the planar surface of a neutral lipid membrane,[6] or in the presence of sodium dodecyl sulfate,[4] however, Dyn A-(1-13) appears to adopt an α-helical structure. Binding to an anionic phospholipid[9] or the addition of cerebroside sulfate[5] does not induce the helical structure in the peptide.

The question remains of whether a conformation observed in solution reflects the conformation Dyn A adopts at opioid receptors. Preparing conformationally constrained derivatives is another way to examine the biologically active conformations of a peptide. The two reported analogues of Dyn A constrained in the "message" sequence, cyclo[D-Cys^2,Cys^5]Dyn A-(1-13)[12] and cyclo-[D-Orn^2,Asp^5]Dyn A-(1-8),[13] show high potency in the GPI, but also exhibit high μ-receptor affinity. The other reported cyclic analogues of Dyn A, cyclized via either a lactam[13] or a disulfide,[14] are constrained in the C-terminal "address" portion of the peptide.

Since the N-terminal "message" sequence appears to be important for the opioid activity of Dyn A, we incorporated conformational constraints into this region of the peptide. Tyr^1 and Phe^4 residues are important for opioid activity and potency,[2] so conformational constraints involving Gly^2 and/or Gly^3, which would affect the relative orientation of the aromatic rings at positions 1 and 4, were examined. Since several of the conformational studies described above, particularly Schiller's fluorescent energy transfer experiments,[11] suggested an extended conformation for the N-terminus of Dyn A, a conformational constraint consistent with this proposed conformation was chosen for preparation.

Results and Discussion

Design Rationale and Synthesis. Molecular modeling with the AMBER[15,16] program was used to examine possible conformational constraints for incorporation into positions 2 and 3 in Dyn A-(1-13)NH_2. These studies suggested that *trans*-4-aminocyclohexanecarboxylic acid (*trans*-ACCA) might replace Gly^2-Gly^3 in an extended conformation. The calculated nitrogen-carbonyl carbon distance was 5.70 Å for *trans*-ACCA in the diequitorial conformation vs 6.12 Å between the nitrogen of Gly^2 and the carbonyl carbon of Gly^3 when the peptide was in an extended conformation. The ACCA dipeptide replacement is equivalent to constraining ψ_2 and ϕ_3 while still allowing free rotation around ϕ_2 and ψ_3.

ACCA was synthesized by hydrogenation of *p*-aminobenzoic acid with PtO_2 as a catalyst[17] (Scheme I), which

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† Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature outlined in *J. Biol. Chem.* (1972, *247*, 977-983). Amino acids are in the L-configuration. Additional abbreviations used are as follows: ACCA, 4-aminocyclohexanecarboxylic acid; Aib, α-aminoisobutyric acid; DAMGO, [D-Ala^2,$MePhe^4$,glyol]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-ON-Su, *N*-(9-fluorenylmethoxycarbonyl)-succinimide; GPI, guinea pig ileum; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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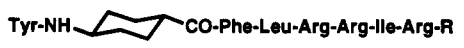
distance was 5.70 Å for *trans*-ACCA in the diequitorial conformation vs 6.12 Å between the nitrogen of Gly^2 and the carbonyl carbon of Gly^3 when the peptide was in an extended conformation. The ACCA dipeptide replacement is equivalent to constraining ψ_2 and ϕ_3 while still allowing free rotation around ϕ_2 and ψ_3.

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-R

Dyn A-(1-13)NH_2



[*trans*-$ACCA^{2-3}$]Dyn A-(1-13)NH_2

R = -Pro-Lys-Leu-$LysNH_2$

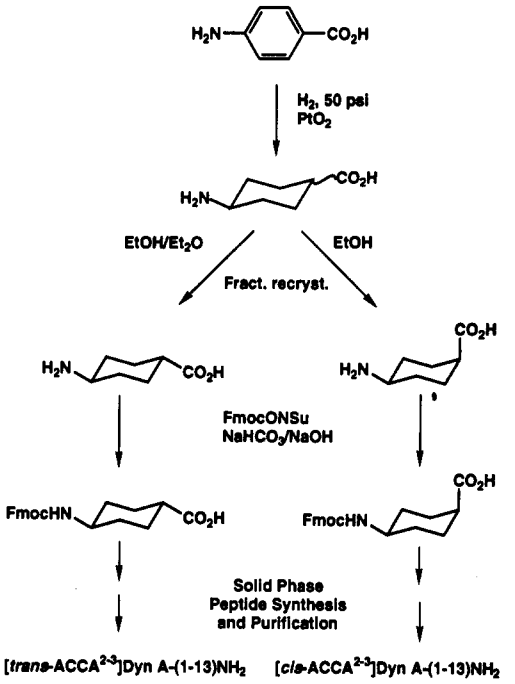
ACCA was synthesized by hydrogenation of *p*-aminobenzoic acid with PtO_2 as a catalyst[17] (Scheme I), which yielded a mixture of cis and trans isomers (approx ratio of 2.5/1 cis/trans). Separation by fractional recrystallization[18] from EtOH yielded the cis isomer, which was pure by ^1H NMR. Subsequent recrystallization from EtOH/ether yielded the trans isomer, but ^1H NMR indicated that the trans isomer contained from 10–15% up to 35% of the cis isomer, depending on the batch. Following fractional recrystallization, each isomer was converted separately to its Fmoc (9-fluorenylmethoxycarbonyl) derivative. Fmoc protection of these branched amino acids proved to be difficult, and literature procedures[19] had to be modified[20] to obtain the desired product (see Experimental Section). HPLC verified that Fmoc-*cis*-ACCA contained only the cis isomer. In the case of Fmoc-*trans*-ACCA, the contaminating cis isomer (t_R = 29.3 min) was not well resolved from the desired Fmoc-*trans*-ACCA (t_R = 29.7 min) by HPLC, so this mixture was used in the synthesis of the *trans*-ACCA analogue of Dyn A-(1–13)-NH_2.

Both the cis and trans isomers were incorporated separately into Dyn A-(1–13)NH_2 (Scheme I). The peptides were prepared as amides because of the enhanced metabolic stability of Dyn A-(1–13) amide vs its corresponding acid.[21] The peptides were synthesized on a PAL resin[22] using Fmoc-protected amino acids by procedures described previously.[23] The peptides were deprotected and cleaved from the PAL resin using trifluoroacetic acid (TFA) and purified by preparative reverse-phase HPLC. The purification of [*cis*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 was straightforward, while the purification of the trans analogue proved difficult due to the necessity of separating it from the contaminating cis analogue. This resulted in a very low yield of pure [*trans*-$ACCA^{2-3}$]Dyn A-(1–13)-NH_2.

Opioid Receptor Binding Affinities and Opioid Activity. The peptides were evaluated for opioid receptor affinity at κ receptors by measuring the inhibition of binding of [^3H]bremazocine to guinea pig cerebellar membranes, and for μ and δ receptor affinities in rat forebrain membranes using [^3H]DAMGO ([D-Ala^2,-MePhe^4,glyol]enkephalin) and [^3H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin), respectively (Table I).

Both [*cis*-$ACCA^{2-3}$]- and [*trans*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 bound to κ opioid receptors with modest affinity (K_i = 9–13 nM), with the *cis*-ACCA analogue exhibiting slightly greater affinity than the trans isomer. The affinity of these analogues for κ receptors is $^1/_{60}$ to $^1/_{90}$ that of the parent peptide Dyn A-(1–13)NH_2. Introduction of either isomer of ACCA into Dyn A-(1–13)NH_2 causes an even larger decrease in affinity for μ receptors, resulting in K_i's greater than 100 nM. Therefore, both of these analogues are κ selective, with [*trans*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 showing slightly better κ selectivity (κ/μ ratio = 1/21) than [*cis*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 (κ/μ ratio = 1/13). Both of these peptides had very little affinity for δ receptors (K_i's > 1000 nM).

Scheme I



Both the *cis*- and *trans*-ACCA Dyn A analogues show binding affinity similar to the Dyn A-(1–13)NH_2 analogues containing an L-amino acid at position 2 reported by Story et al.,[24] all of which had K_i's in the 2–20 nM range. The ACCA-substituted peptides, however, have better κ vs μ selectivity than any of these 2-substituted dynorphin analogues. [Aib^2]Dyn A-(1–13),[25] which incorporates an α,α-disubstituted amino acid into position 2 similar to the disubstitution α to the amine of ACCA, surprisingly has lower affinity for κ receptors (K_i = 50 nM). [Aib^2]Dyn A-(1–13) has 10–30-fold higher affinity for μ receptors (K_i = 10 nM) than [*cis*- and [*trans*-$ACCA^{2-3}$]Dyn A-(1–13)-NH_2; thus, the Aib^2-substituted peptide is μ selective while the ACCA-substituted peptides are κ selective.

[*cis*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 was evaluated for opioid activity in the guinea pig ileum (GPI) assay. Its potency (IC_{50} = 4.09 μM, 95% confidence = 2.88–5.80 μM) was much lower than the parent Dyn A-(1–13)NH_2 (IC_{50} = 0.24 nM, 95% confidence = 0.21–0.29 nM), but this analogue exhibited a full dose–response curve and naloxone antagonized its effects (data now shown). The low GPI activity of [*cis*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 parallels the results obtained for the analogues containing L-amino acids in position 2.[24] There was insufficient trans compound to allow for its testing in the GPI.

Conclusions

[*cis*- and *trans*-$ACCA^{2-3}$]Dyn A-(1–13)NH_2 are the first reported Dyn A analogues conformationally constrained in the "message" sequence that are selective for κ opioid receptors. The *cis*- and *trans*-$ACCA^{2-3}$-substituted pep-

Table I. Opioid Receptor Binding Affinities of Dyn A-(1-13)NH_2 and [ACCA]Dyn A-(1-13)NH_2 Analogues

analogue	K_i (nM) [^{3}H]bremazocine (κ)	[^{3}H]DAMGO (μ)	[^{3}H]DPDPE (δ)	ratio $\kappa/\mu/\delta$[a]
Dyn A-(1-13)NH_2	0.146 ± 0.004	0.194 ± 0.002	3.88 ● 0.09	1/1.3/27
[*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2	9.10 ± 0.92	117 ± 2	1910 ● 65	1/13/210
[*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2	13.4 ± 0.6	275 ± 2	1370 ± 53	1/21/103

[a] Ratio of $K_{i_\kappa}/K_{i_\mu}/K_{i_\delta}$.

and selectivity between the κ-selective [*cis*-ACCA$^{2-3}$]- and [*trans*-ACCA$^{2-3}$]Dyn A-(1–13)NH_2 and the μ-selective Dyn A analogue [Aib^2]Dyn A-(1–13)[25] could be due to differences in conformation, the larger size of ACCA vs Aib, or the substitution at the position corresponding to the C_α of Gly^3 in the ACCA-substituted peptides.

The good discrimination of κ vs μ receptors exhibited by the *cis*- and *trans*-ACCA derivatives of Dyn A-(1–13)-NH_2 is encouraging and suggests that further modification at positions 2 and/or 3 might lead to Dyn A analogues with higher κ receptor affinity and opioid potency.

Experimental Section

Materials. The reagents and instrumentation used were those described previously.[23] Elemental analysis was performed by MWH Laboratories, Phoenix, AZ. NMR spectroscopy was done on a 400-MHz Bruker NMR in the Department of Chemistry at Oregon State University.

Hydrogenation of *p*-Aminobenzoic Acid to ACCA.[17] *p*-Aminobenzoic acid (Aldrich, 1.056 g, 7.71 mmol) in 30% EtOH (100 mL) was hydrogenated under 50 psi of H_2, with PtO_2 (278 mg) as a catalyst, at room temperature overnight. This was repeated six times and the solutions combined. After removing the platinum by filtration, the filtrate was concentrated in vacuo until crystals appeared. The crystals were dissolved in H_2O and boiled, and the solution was treated with charcoal. ACCA was precipitated by the addition of 55 mL of EtOH/ether (10/1), followed by additional ether until no more crystals appeared. This yielded 4.916 g (70.7%) of ACCA as a mixture of cis and trans isomers; TLC: R_f (C_{18}, reverse phase, MeOH/H_2O, 60/40) 0.33.

Separation of Cis and Trans Isomers of ACCA.[18] The crystals were dissolved in water, and EtOH was added to crystallize *cis*-ACCA. The crystals were collected, and the process was repeated three times to obtain additional cis isomer. The remaining filtrate was concentrated and 55 mL of EtOH/ether (10/1) added to crystallize the *trans*-ACCA. The crystals were collected, and the process was repeated three times with the addition of excess ether. The isomers were then recrystallized from H_2O/EtOH (cis) or H_2O/EtOH/ether (trans) to yield 3.125 g (44.9%) of *cis*-ACCA and 1.259 g (18.1%) of *trans*-ACCA. Data for *cis*-ACCA follow. Mp: 258–264 °C. FAB-MS: *m/z* 144 (M + 1). ^{1}H NMR (*d*-TFA): δ 1.76–2.34 (m, 8 H, $-CH_2CH_2-$), 2.85 (m, 1 H, $CHCO_2H$), 3.53 (m, 1 H, $CHNH_2$). Anal. ($C_7H_{13}NO_2 \cdot 0.4H_2O$): C, H, N. HCl salt mp: 207–208 °C (lit. mp:[18] 217 °C). Data for *trans*-ACCA follow. Mp: 262–267 °C. FAB-MS: *m/z* 144 (M + 1). ^{1}H NMR (*d*-TFA): δ 1.60–1.70 (m, 4 H, $-CH_2-$), 2.33 (m, 4 H, $-CH_2-$), 2.52 (m, 1 H, $CHCO_2H$), 3.46 (m, 1 H, $CHNH_2$). Anal. ($C_7H_{13}NO_2 \cdot 0.4H_2O$): C, H, N. HCl salt mp: 272–273 °C (lit. mp:[18] 273 °C).

Fmoc-*cis*- and Fmoc-*trans*-ACCA.[20] *cis*-ACCA (393 mg, 2.73 mmol) and NaOH (109 mg, 2.73 mmol) were dissolved in H_2O (1.97 mL). A suspension of Fmoc-ON-Su (Bachem, 737 mg, 2.18 mmol) in THF (1.97 mL) was added to the ACCA solution, followed by additional H_2O and THF (1.97 mL of each). After 5 min $NaHCO_3$ (229 mg, 2.73 mmol) and THF (1.97 mL) were added; all reactants went into solution after 25 min. The reaction was stirred until TLC ($CHCl_3$/MeOH/AcOH, 89/10/1) indicated that all of the Fmoc-ON-Su had reacted (overnight). The solution was then acidified with excess 1.5 N HCl and the product, which precipitated out, dissolved in EtOAc. The HCl layer was extracted with additional EtOAc. The combined EtOAc extracts were then washed with 1.5 N HCl (2×), H_2O (2×), and saturated NaCl and dried over anhydrous $MgSO_4$ or Na_2SO_4. The solvent was evaporated in vacuo to give a thick oil. Dissolution in EtOAc, precipitation with hexane, and evaporation of solvent yielded 737 mg (92.6%) of Fmoc-*cis*-ACCA as a glassy solid. Mp: 128–130 °C. FAB-MS: *m/z* 366 (M + 1). HPLC[26] (0 to 75% B over 50 min, 1.5 mL/min): t_R = 29.3 min. ^{1}H NMR (d_6-DMSO): δ 1.40–1.58 (m, 6 H, $-CH_2CH_2-$), 1.85 (m, 2 H, $-CH_2-$), 2.38 (br s, 1 H, $CHCO_2H$), 3.41 (m, 1 H, $-CHNH-$), 4.22 (m, 3 H, Fmoc $-CHCH_2O-$), 7.29 (d, 1 H, $-NH-$), 7.31 (t, 2 H, Ar), 7.40 (t, 2 H, Ar), 7.69 (d, 2 H, Ar), 7.87 (d, 2 H, Ar). Anal. ($C_{22}H_{23}NO_4$): C, H, N.

Protection of the trans isomer (429 mg, 3 mmol) as described for *cis*-ACCA yielded 760 mg (86.7%) of Fmoc-*trans*-ACCA. FAB-MS: *m/z* 366 (M + 1). HPLC[26] (0 to 75% B over 50 min, 1.5 mL/min): t_R = 29.7 min (Fmoc-*trans*-ACCA, 66%), t_R = 29.3 min (Fmoc-*cis*-ACCA, 33%). Data for an analytical sample recrystallized from THF/H_2O follow. Mp: 220–227 °C. ^{1}H NMR (d_6-DMSO): δ 1.19–1.35 (m, 4 H, $-CH_2CH_2-$), 1.85–1.94 (m, 4 H, $-CH_2CH_2-$), 2.08 (br t, 1 H, $CHCO_2H$), 3.18 (m, 1 H, $-CHNH-$), 4.20 (m, 1 H, Fmoc $-CHCH_2O-$), 4.26 (m, 2 H, Fmoc $-CHCH_2O-$), 7.22 (d, 1 H, $-NH-$), 7.32 (t, 2 H, Ar), 7.40 (t, 2 H, Ar), 7.67 (d, 2 H, Ar), 7.87 (d, 2 H, Ar). Anal. ($C_{22}H_{23}NO_4$): C, H, N.

Peptide Synthesis Using Fmoc-Protected Amino Acids. Both peptides were synthesized, cleaved from the resin, and purified by procedures described previously.[23] Before removing the Fmoc group from ACCA, the entire coupling sequence for that amino acid was repeated to ensure complete coupling. Following cleavage from the resin the peptides were purified by preparative reverse-phase HPLC[27] (0 to 50% B over 75 min, 20 mL/min for the cis analogue, and 15 to 45% B over 60 min, 20 mL/min for the trans analogue). Data for [*cis*-ACCA$^{2-3}$]Dyn A-(1–13)NH_2 follow. Yield: 30 mg (24%). FAB-MS: *m/z* 1614 (M + 1). HPLC[26] (0 to 75% B over 50 min, 1.5 mL/min): t_R = 19.5 min. Amino acid analysis: Tyr (1) 0.93, Phe (1) 0.99, Leu (2) 2.02, Arg (3) 3.15, Ile (1) 0.97, Pro (1) 0.97, Lys (2) 2.01. Data for [*trans*-ACCA$^{2-3}$]Dyn A-(1–13)NH_2 follow. Yield: 4.4 mg (3.3%). FAB-MS: *m/z* 1614 (M + 1). HPLC[26] (15 to 35% B over 40 min, 1.5 mL/min): t_R = 14.4 min. Amino acid analysis: Tyr (1) 0.79, Phe (1) 1.05, Leu (2) 2.02, Arg (3) 3.12, Ile (1) 0.99, Pro (1) 1.02, Lys (2) 2.02.

Binding and Guinea Pig Ileum Assays. Guinea pig cerebellar membranes and rat forebrain membranes were prepared and binding assays performed as previously described,[24] except that 100 nM DAMGO was included in the incubation mixtures of [^{3}H]bremazocine with guinea pig cerebellar membranes (κ binding assays). Guinea pig ileum assays were performed as previously described.[24] In experiments involving naloxone, the antagonist was added to the tissue bath 10 min prior to determination of the agonist dose–response curve.

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(26) Vydac 214TP54 column, solvent A = 0.1% TFA in H_2O, solvent B = 0.1% TFA in AcCN.
(27) Protein Plus preparative column, solvents A and B are as above.