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SYNTHESIS OF α-HELIX SUBSTITUTED ANALOGS OF CALCITONIN GENE-RELATED PEPTIDE

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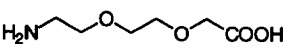
Abstract: *N*-Terminal analogs of calcitonin gene-related peptide (CGRP) were synthesized and assayed for receptor binding affinity. Substitutions in the α-helical region of CGRP with a linear ethylene glycol containing amino acid, or with a β-strand forming sequence resulted in analogs which showed significant affinity for the receptor.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide produced by alternative splicing of the primary transcript of the calcitonin gene.[1] In thyroid carcinoma cells the primary RNA transcripts are processed to messenger RNA (mRNA) for calcitonin, in neural tissue mRNA for CGRP is formed predominantly.[2] CGRP is widely distributed in both the central and peripheral nervous system.[3] Various biological effects have been reported for CGRP, including reduction of gastric acid secretion, peripheral blood vessel dilation, cardiac acceleration, regulation of calcium metabolism and insulin secretion, increase in body temperature, and decrease in food uptake.[4,5] In rabbits, CGRP was found to be involved in ocular neurogenic inflammation, causing vasodilatation in the anterior uvea, breakdown of the blood-aqueous barrier, increase in the intraocular pressure (IOP) and rise in the adenosine 3',5'-cyclic monophosphate (cAMP) content in the aqueous humor.[6,7] An intracameral injection of CGRP, on the other hand, decreases IOP by increasing the outflow facility.[8] In connection with our studies on the determination of how conformation and amino acid sequence of CGRP are related to the binding onto the CGRP receptor and to the biological activity,[9] several CGRP fragments and analogs have been synthesized by solid-phase method using Fmoc/tBu chemistry. In this Letter, we wish to report our findings on selected typical analogs exhibiting significant receptor affinities.

Lynch and Kaiser suggested that the *N*-terminal disulfide bridged loop is followed by an amphiphilic α-helix between residues 8 and 18 terminated by a β-turn in the region 18-23.[10] The presence of the α-helix in aqueous solutions is supported by CD and ^{1}H-NMR spectroscopy.[11-17] Breeze *et* al describe a detailed ^{1}H-NMR study of the solution structure of CGRP, including the α-helical region and

abundant information on the C-terminus.[13] Substitution of this region with an idealized α-helix decreases the binding affinity to one tenth of that observed for CGRP. Studies on analogs of hCGRP(8-37) have shown that the residues 9-12 have more or less critical roles for an effective binding and antagonistic activity to the CGRP receptors.[18] These amino acid residues are involved in stabilizing the amphiphilic α-helix which seems crucial for good receptor affinity. In our modeling studies, we found that a linear ethylene glycol-based amino acid ***1*** could be a possible candidate for replacing the amino acid residues 9-15 still retaining reasonably good ability to bind to the receptor. Due to its amphiphilic nature, the α-helical region of hCGRP could form hydrogen bonds with the *C*-terminal amino acid residues following the β-turn between the residues 17 and 22. Only a structural role has been suggested for the side chains of residues 9, 10 and 12.[18] Interaction of the α-helix and C-terminus in salmon calcitonin, where deletion peptides of the C-terminus retain binding, but lose activity has also been proposed.[19] These facts led us to test the assumption that the entire α-helical region (residues 8-18) of CGRP could be replaced with a sequence having a tendency to form β-strand type secondary structure (***7*** in Table 1), or with a non-proteinogenic amino acid ***1***. According to modeling studies ***1*** would extend the length of the helix, and because of dipolar interactions (C-O bond dipoles would prefer to be antiperiplanar relative to the central C-C bond in the ethylene glycol unit) would tend to exist in an extended conformation.



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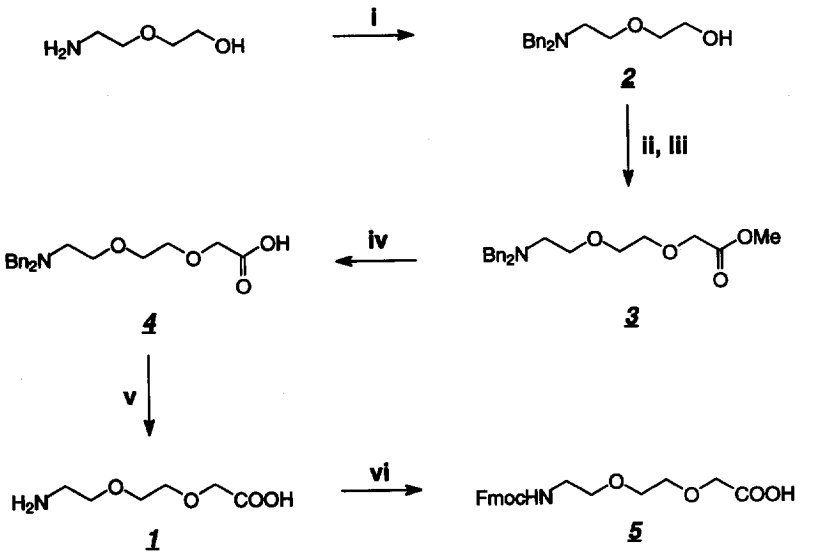
N-Fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic ***5*** acid was synthesized (Scheme 1) starting from 2(2-aminoethoxy)ethanol by first introducing dibenzyl protection on the amino group to give ***2*** as a clear oil. Alkylation of the hydroxyl with sodium hydride and methyl bromoacetate gave ***3***. Ester hydrolysis, and removal of the benzyl groups then gave the protected Fmoc-amino acid ***5*** as a white solid (m.p. 89-90 °C) (Scheme).[20] The free amino acid was not isolated, but turned directly into its Fmoc-derivative, which could then be applied in solid phase peptide synthesis in the usual way. Pentafluorophenyl ester activation was performed for the Fmoc-protected amino acid ***5*** shortly before coupling to the growing peptide chain.[21] Cleavage of the peptides from the resin with 95 % TFA/4.5 % aq. phenol/0.5 % 1,2-ethanedithiol simultaneously removed all the amino acid side chain groups, except Cys(tBu), which was deprotected by first treating with $Hg(OAc)_2$ in TFA, and then with 2-mercaptoethanol at pH 8. The disulfide bond was formed by air oxidation. Deprotection and oxidation can also be accomplished in one step according to Kiso et al. by treating the peptide with methyltrichlorosilane in the presence of diphenylsulphoxide.[22]

Table 1.

Peptide	Sequence	Displacement of [125I]CGRP binding at 4°C IC50 (nM) in rabbit lung	porcine c+i
α-hCGRP	ACDTATCVTHRLAGLNSRSGGVVKNNFVPTNVGSKAF	2-5	2
6	ACDTATCVXLSRSGGVVKNNFVPTNVGSKAF[1]	185	995
7	ACDTATCVHVRVRSGGVVKNNFVPTNVGSKAF-NH2	4	14

1 X = amino acid 1



REAGENTS: I, aq Na_2CO_3, 200 mol-% BnBr, 68 %; ii, NaH, reflux, 75 min; iii, $BrCH_2CO_2Me$, -10 °C, 96 %; iv, NaOH, MeOH/H_2O, 80 %; v, 3 atm H_2, Pd/C, rt, 16 h, 91 %; vi, 10 % Na_2CO_3, dioxane, Fmoc-Cl, 69 %.

Scheme 1.

Lynch and Kaiser suggested residues 8-18 to form an amphiphilic α-helix, and their model peptide having amino acid substitutions generating an idealized α-helix structure, preserved some of the binding affinity and biological activity of rat CGRP.[10] Substitutions in both of the regions 8-14 and 17-25 resulted in loss of affinity, but the model peptide having the intact sequence between the residues 19 and 25 still showed high receptor affinity and activity. They also observed the variations in potency ratios in different tissues, probably resulting from receptor heterogeneity. The importance of the residues 8-12 have been examined by synthesizing *C*-terminal CGRP fragments 9-37, 10-37, 11-

37 and 12-37, and 8-37 with residues Thr9, His10, Arg11 or Leu12 substituted with alanine.[23] Fragments 8-37 and 9-37 are equally potent. Although removal of Arg11 produces the most dramatic decrease in affinity, the analog [Ala11]hCGRP8-37 shows low affinity and antagonistic properties, and thus not even Arg11 is required for binding.

The substitution of residues 9-15 of CGRP with the linear amino acid ***1*** produced an analog ***6*** that shows rather high affinity, but no measurable activity on CGRP receptors in rabbit lung *in vitro* studies. Peptide analogs having residues 10-16 or 11-17 substituted respectively would give more information about the relationship between the amino acid residues Thr9 or His10 and biological activity. A peptide having agonistic properties could possibly be created by connecting the activity inducing *N*-terminus and the *C*-terminal part needed for binding to the receptor with a linear amino acid of a correct length. In our findings the *N*-terminal fragments 1-15, 1-18 or 8-18 have shown a very low affinity to CGRP receptor (data not shown). The fragment 1-15 has been reported to be only about 100 times less active than the natural peptide.[24] It thus seems that also the *N*-terminus has similar although much weaker binding properties as the *C*-terminus. Thus it may be possible to improve the binding of *N*-terminal fragments and/or to design an agonist from the *C*-terminal fragment.

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20. ***2-(N,N-Dibenzyl-2-aminoethoxy)ethanol 2.*** 2-(2-Aminoethoxy)ethanol (3.15 g, 30 mmol), potassium carbonate (8.29 g, 60 mmol) and 8 ml of water were placed in a 100-ml 3-neck flask equipped with a mechanical stirrer and a dropping funnel. Benzylbromide (10.26 g, 60 mmol) was added dropwise over a 50 min period. The mixture was stirred at room temperature overnight. Diethylether (50 ml) was added to the mixture, and the layers were separated. The aqueous phase was extracted with 10 ml of ether, and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave 5.82 g of a clear oil (68 %). R_f= 0.43 (EtOAc/hexane = 1:1). The same yield of the product was obtained if sodium carbonate was used instad of potassium carbonate. ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 1 H), 2.69 (t, 2 H, *J* = 5.9 Hz), 3.47 (t, 2 H, *J* = 4.5 Hz), 3.57 (t, 2 H, *J* = 5.9 Hz), 3.65 (s, 4 H), 7.20-7.38 (m, 10 H). HRMS: $C_{18}H_{23}NO_2$ requires 285.1729, found 285.1720.

N,N-Dibenzyl-8-amino-3,6-dioxaoctanoic acid methyl ester 3. NaH (60 % in mineral oil, 0.96 g, 24 mmol of NaH) was washed with pentane. Compound **2** (5.78 g, 20 mmol) in 15 ml of dry THF was added to a mixture of NaH in dry THF (30 ml). The mixture was refluxed for 1 h 15 min, then cooled to -5 to -10 °C, followed by slow addition of methyl bromoacetate (3.36 g, 22 mmol). After stirring overnight at rt, 10 ml of water was carefully added to the reaction mixture, and THF was evaporated with rotary evaporator. The product was extracted into EtOAc (5 x 20 ml). The combined organic layers were washed with water (2 x 10 ml) and brine (10 ml), backextracting the aq. phases, and dried over Na_2SO_4. Evaporation of the solvent gave a yellow liquid. Yield 6.85 g (96 %). R_f = 0.63 (EtOAc/hexane = 1/1). ^{1}H NMR ($CDCl_3$) δ 2.69 (t, 2 H, *J* = 6.2 Hz), 3.55-3.73 (m, 13 H), 4.14 (s, 2 h), 7.19-7.38 (m, 10 H). HRMS: $C_{21}H_{28}NO_4$ requires 358.2018, found 358.1980.

N,N-Dibenzyl-8-amino-3,6-dioxaoctanoic acid 4. Ester **3** (6.08 g, 17 mmol) was dissolved in 20 ml of MeOH, 19 ml of 1 M NaOH was added, and the mixture stirred for 2 h at rt. After neutralization with 6 M HCl methanol was evaporated in vacuo. The aq. solution was acidified to pH 6.5, and extracted with EtOAc. Drying over Na_2SO_4 and evaporation of the solvent gave the product as an oil, 4.64 g (80 %). Rf = 0.2 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR ($CDCl_3$) δ 2.63 (t, 2 H, *J* = 6.1 Hz), 3.39-3.51 (m, 6 H), 3.60 (s, 4 H), 3.82 (s, 2 H), 5.03 (s, 1 H), 7.16-7.33 (m, 10 H). HRMS: $C_{20}H_{26}NO_4$ requires 344.1862, found 344.1828.

8-Amino-3,6-dioxaoctanoic acid 1. Compound **4** (3.43 g, 10 mmol) was dissolved in 100 ml of methanol, and 0.7 g 5 % Pd/C was added. Hydrogenation was done with Parr hydrogenation apparatus under 3 atm pressure at room temperature in 16 h. Pd/C was filtered off, and the filtrate evaporated giving an oil 1.49 g (91 %). ^{1}H NMR (D_2O) δ 2.89 (t, 2 H, *J* = 5.3 Hz), 3.59 (t, 2 H, *J* = 5.3 Hz), 3.64 (s, 4 H), 3.89 (s, 2 H). HRMS: $C_6H_{14}NO_4$ requires 164.0923, found 164.0930.

N-Fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic acid 5. Amino acid *1* was suspended in a mixture of dioxane (10 ml) and 10 % Na_2CO_3 (20 ml). Fluorenylmethyl chloroformate (2.46 g, 9.5 mmol) in 10 ml of dioxane was added dropwise over 15 min to the reaction mixture at 0-5 °C. The ice bath was removed and stirring continued for 2 h at rt Water (90 ml) was added, and the mixture was extracted with diethyl ether (3 x 30 ml). The aq. layer was the acidified with 6 M HCl (cooling in ice) to pH 2, and the product was extracted into ethyl acetate (4 x 30 ml). The combined EtOAc phases were washed with water and brine, then dried over Na2SO4. Evaporation of the solvent gave a clear oil, which crystallized overnight. 2.35 g (68 %). Recrystallization from ethyl acetate with pentane gave white crystals, 2.21 g (64 %); mp 89-90 °C. Elemental analysis: calc. for $C_{21}H_{23}NO_6$: C 65.44, H 6.02, N 3.63; found: C 65.00, H 6.20, N 3.59. FABMS 386 (M + H)+; calc. 385 (M). ^{1}H NMR ($CDCl_3$) δ 3.40-3.77 (8 H, m), 4.16 (2 H, s), 4.10-4.50 (3 H, m), 5.22 (1 H, broad s), 7.25-7.78 (8 H, m).

21. Synthesis of CGRP fragments and analogs were carried out by solid-phase method using Fmoc/tBu chemistry on 2,4-dimethoxy-benzhydrylamine resin (RapidAmide) purchased from Du Pont de Nemours. Peptides were synthesized on a Du Pont RaMPS Multiple Peptide Synthesis System. All amino acids are of the L-configuration. Fmoc-Ala, -Arg(Mtr), -Asn, -Asp(OtBu), -Cys(tBu), -Glu(OtBu), -Gln, -Gly, -His(Trt), -Ile, -Leu, -Lys(Boc), -Phe, -Pro, -Ser(tBu), -Thr(tBu), and -Val were purchased from Du Pont de Nemours (Deutschland) GmbH, Germany and Bachem AG, Switzerland. Pentafluorophenol, diisopropylcarbodiimide, piperidine, trifluoroacetic acid, 2(2-aminoethoxy)ethanol, diphenylsulfoxide, anisole, 1,2-ethanedithiol, 2-mercaptoethanol and methyltrichlorosilane were obtained from Aldrich. 9-Fluorenylmethyl chloroformate and 1-hydroxybenzotriazole hydrate were from Lancaster. Purity of the peptides was checked by thin layer chromatography (TLC, Merck silica gel 60 F254) and reversed phase HPLC (RP-HPLC). In RP-HPLC gradient elution of water and acetonitrile containing 0.1 % TFA was used. The gradient system consisted of Waters Model 660 Solvent Programmer, two Waters 501 HPLC Pumps, Waters 486 Tunable Absorbance detector, and Waters 746 Data Module. Detector wavelength was set at 215 nm. HPLC grade S acetonitrile from Rathburn Chemicals Ltd. was used as eluent. Ultra pure water was obtained from distilled water by a Water-I Model D 2200 (Barnstead, Division of Sybron Corp.). For analytical purposes Nucleosil ODS (particle size 5μ, 4.6 x 250 mm) or μBondapak C-18 (particle size 10μ, 3.9 x 300 mm) columns and a Rheodyne injector having a loop volume of 20 μl, and for semipreparative scale purification of peptides a Nucleosil ODS (particle size 5μ, 10 x 250 mm) column and a injector loop of 300 μl were used.

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