Modifications to the N-Terminus but Not the C-Terminus of Calcitonin Gene-Related Peptide(8-37) Produce Antagonists with Increased Affinity

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Seventeen novel analogues of human calcitonin gene-related peptide(8-37) (hCGRP(8-37)) were synthesized by solid-phase methods and purified to apparent homogeneity by semipreparative cation exchange and/or reversed-phase high-performance liquid chromatography. The C-terminal Phe was replaced by Gly, cyclohexylalanine (Cha), Tyr, all four isomers of β-methylphenylalanine (β-MePhe), and L- and D-tetrahydroisoquinoline carboxylic acid (Tic), resulting in analogues **3**−**11**. For the synthesis of the β-MePhe-containing analogues **6**−**9**, crystallization was used to separate a mixture of all four isomers of β-MePhe into the erythro pair of enantiomers (2*S*,3*S*, 2*R*,3*R*) and the threo pair of enantiomers (2*S*,3*R*, 2*R*,3*S*), which were then converted to Fmoc derivatives and used in two separate syntheses. Two diastereomeric peptides were obtained from each synthesis and were separated by RP-HPLC to yield enantiomerically pure **6**−**9**. Substitution of Tyr for Phe caused no change in binding affinity at CGRP receptors. All other substitutions for Phe resulted in substantial reductions in binding affinity. Indeed, no binding was observed for analogues **7**, **9**, and **11**, all of which contained a D-amino acid residue in the C-terminal position, and the binding affinities of the remaining analogues were >10-fold lower than that of *h*-α-CGRP(8-37). These data suggest that a conformationally flexible phenyl ring in the C-terminal position of *h*-α-CGRP(8-37) is preferred for high-affinity binding to CGRP receptors. Acetylation, benzoylation, and benzylation of the N-termini of *h*-α-CGRP(8-37) and *h*-β-CGRP(8-37) produced analogues **12**−**14** and **16**−**18**, respectively. A byproduct was isolated by RP-HPLC from the resin-cleaved crude product of each benzylated analogue, which was characterized as the dibenzylated derivative of *h*-α-CGRP-(8-37) and *h*-β-CGRP(8-37) (analogues **15** and **19**, respectively). Amino acid analysis and ^{1}H NMR showed that the second benzyl group was located on the C4 carbon of the imidazole ring of His^{10}. Radioligand binding experiments showed that derivatizing the N-termini substantially increased binding affinities at CGRP receptors. The benzoylated and dibenzylated derivatives had the highest affinities, which were approximately 50-fold greater than those of *h*-α-CGRP-(8-37). Functional experiments confirmed that the N-terminally derivatized analogues of *h*-α-CGRP(8-37) are antagonists that are more potent than *h*-α-CGRP(8-37). In conclusion, these studies underscore the importance of Phe^{37} of *h*-α-CGRP(8-37) for binding to CGRP receptors and have identified the N-terminus and His^{10} as two positions that can be used for the design of antagonists with increased affinity for CGRP receptors.

CGRP is a 37-residue peptide with a disulfide bridge between positions 2 and 7 and with a C-terminal phenylalanylamide residue (Figure 1). In human and rat, CGRP exists in two forms designated α and β that are located throughout the peripheral and central nervous systems[1] and have numerous biological actions.[1−3] While a major physiological role of CGRP is thought to be regulation of blood pressure, its abundance and widespread distribution in the central nervous system (CNS) suggest that it may play a role in olfactory, auditory, and vision systems and contribute to learning, feeding, and other behaviors.[1] Dennis et al. proposed that CGRP elicits its effects through two subtypes of

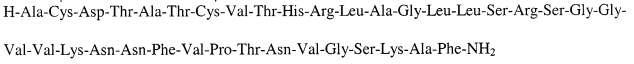

Figure 1. Primary structure of human α-calcitonin gene-related peptide.

CGRP receptors.[4] Pharmacological characterization of the receptor subtypes showed that CGRP1 receptors have a high affinity for the competitive antagonist CGRP(8-37) while CGRP2 receptors are selectively activated by the linear agonists [Cys(ACM)^{2,7}]-*h*-α-CGRP[5] and [Cys(Et)^{2,7}]-*h*-α-CGRP[6] and have a low affinity for CGRP(8-37). $CGRP_1$ receptors have been cloned and expressed.[7,8] To date, CGRP(8-37) is the most common antagonist used as a pharmacological tool to identify CGRP receptor subtypes.

CD[9−11] and NMR[12] experiments showed that the solution structure of *h*-α-CGRP(8-37) is largely disordered at ambient temperatures. Only when the tem-

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Table 1. Observed Masses of *h*-α-CGRP(8-37) and Its Analogues and Their Binding Affinities for CGRP Receptors on Porcine Coronary Arteries

	analogue	ESI-MS (*m*/*z*) observed	ESI-MS (*m*/*z*) calculated	binding affinity IC_{50} (nM)
	h-α-CGRP[a]			0.18 ± 0.12, 5.19 ± 1.6
1	*h*-α-CGRP(8-37)	3125.7	3125.6	14.4 ± 0.38
2	*h*-*β*-CGRP(8-37)	3130.4	3130.6	20.7 ± 3.96
3	[Gly^{37}]-*h*-α-CGRP(8-37)	3035.3	3035.5	25900 ± 840
4	[Cha^{37}]-*h*-α-CGRP(8-37)	3131.9	3131.7	155 ± 88
5	[Tyr^{37}]-*h*-α-CGRP(8-37)	3141.1	3141.6	17.6 ± 8.26
6	[(2*S*,3*S*)-*β*-MePhe^{37}]-*h*-α-CGRP(8-37)	3139.6	3139.7	631 ± 89
7	[(2*R*,3*R*)-*β*-MePhe^{37}]-*h*-α-CGRP(8-37)	3139.6	3139.7	*b*
8	[(2*S*,3*R*)-*β*-MePhe^{37}]-*h*-α-CGRP(8-37)	3139.6	3139.7	434 ± 113
9	[(2*R*,3*S*)-*β*-MePhe^{37}]-*h*-α-CGRP(8-37)	3139.6	3139.7	*b*
10	[Tic^{37}]-*h*-α-CGRP(8-37)	3137.8	3137.6	363 ± 81
11	[D-Tic^{37}]-*h*-α-CGRP(8-37)	3137.9	3137.6	*b*
12	*N*-α-Ac-*h*-α-CGRP(8-37)	3167.6	3167.0	3.05 ± 0.79
13	*N*-α-Bzl-*h*-α-CGRP(8-37)	3229.4	3229.6	0.27 ± 0.00
14	*N*-α-Bn-*h*-α-CGRP(8-37)	3215.6	3215.6	1.58 ± 0.38
15	diBn-*h*-α-CGRP(8-37)	3305.6	3305.5	0.22 ± 0.06
16	*N*-α-Ac-*h*-*β*-CGRP(8-37)	3172.2	3172.5	3.6 ± 1.15
17	*N*-α-Bzl-*h*-*β*-CGRP(8-37)	3233.5	3233.7	0.63 ± 0.09
18	*N*-α-Bn-*h*-*β*-CGRP(8-37)	3221.3	3221.0	3.62 ± 0.8
19	diBn-*h*-*β*-CGRP(8-37)	3310.7	3311.1	0.73 ± 0.14

[a] Reference 24. [b] No inhibition of [^{125}I]-*h*-α-CGRP binding was observed at peptide concentrations up to 10 000 nM.

perature was lowered to 5 °C did *h*-α-CGRP(8-37) exhibit some helical character, which was less than 10% by CD measurements.[11] This was much lower than the 15−20% helical content observed in *h*-α-CGRP at this temperature and suggests that the disulfide bridge of *h*-α-CGRP stabilizes its helical content. No empirical evidence exists for secondary structure in the C-terminal portion of *h*-α-CGRP(8-37). Data from theoretical studies,[13] however, support recent structure−activity studies[14−18] that show that turn structures in the regions of Arg^{18} to Gly^{21} and Val^{32} to Ala^{36} are compatible with high-affinity binding to CGRP receptors.

Other structure−activity studies of CGRP(8-37) have been limited. N-truncated fragments *h*-α-CGRP(19−37) and *h*-α-CGRP(23−37) are antagonists that bind with low affinity to CGRP receptors on guinea pig atria[19] and [Tyr^{0}]rat-CGRP(28-37) is a weak antagonist of CGRP receptors in guinea pig pancreatic acini.[20,21] These results suggest that the C-terminal half of CGRP(8-37) contains structural elements required for it to bind to CGRP receptors and the N-terminal half contains structural elements that are responsible for the high-affinity of CGRP(8-37) for CGRP receptors. One important C-terminal structural element appears to be in position 37. Substitution of Ala for Phe^{37} of *h*-α-CGRP-(8-37) causes loss of antagonistic effects on rat prostatic vas deferens,[17] and modification of the C-terminal amide to a carboxyl or *N*-ethylamide group abolishes antagonistic properties in guinea pig atrium.[6] We now describe the synthesis and pharmacological evaluation of nine analogues (**3**−**11**) of *h*-α-CGRP(8-37) with amino acid substitutions in position 37 in an attempt to define the structural and subsequently conformational components of Phe^{37} that are important for *h*-α-CGRP(8-37) binding to CGRP receptors.

A more detailed structure−activity study of the N-terminus of *h*-α-CGRP(8-37), covering positions 8−12, revealed that Thr^{9} to Arg^{12} were essential for maintaining the peptide's propensity to form an amphipathic α-helix between positions 8 and 18.[22] This secondary structure is considered the N-terminal structural element that confers high affinity for CGRP receptors. To protect the helix from aminopeptidase degradation, we synthesized N-acetylated analogues of *h*-α-CGRP(8-37) and *h*-*β*-CGRP(8-37). These analogues lack the positive charge that is present at the N-terminus of *h*-CGRP(8-37) but is not present at the analogous position between Cys^{7} and Val^{8} of CGRP. We also performed a structure−activity study, synthesizing and pharmacologically evaluating a total of eight N-terminally derivatized analogues of *h*-CGRP(8-37) (**12**−**19**).

Results and Discussion

Peptide Synthesis. Seventeen novel analogues containing modifications to the N-terminus or C-terminus of *h*-α-CGRP(8-37) were synthesized and are listed in Table 1. Analogues **3**−**11** contain amino acid substitutions in position 37, the C-terminal position of *h*-α-CGRP(8-37). Analogues **12**−**19** contain N-α-alkylated or N-α-acylated N-termini. All peptides including *h*-α-CGRP(8-37) were synthesized by solid-phase methods on automated synthesizers. Specifically, for analogues **1**−**9**, Fmoc-amino acids were coupled as hydroxybenzotriazole active esters to polystyrene resin derivatized with the Knorr linker.[23] Peptides were freed of protecting groups and cleaved from the resin with trifluoroacetic acid (TFA) in the presence of thiol scavengers. Analogues **10**−**19** were synthesized by our previously published methods[24] employing Boc-amino acids and the MBHA resin. N-terminally modified peptides were derivatized by either the appropriate acid anhydride or benzyl bromide. All peptides were purified by preparative cation exchange and/or reversed-phase (RP) high-performance liquid chromatography (HPLC),[25] and their purity was judged to be >98% by analytical RP-HPLC under isocratic elution conditions using three different RP-HPLC columns. Electrospray ionization mass spectrometry (Table 1) and amino acid analysis confirmed peptide structure.

Structural Characterization of *β*-MePhe-Containing Analogues 6−9. Further structural characterization of analogues **6**−**9** was required to unambiguously identify the isomers of *β*-MePhe in position 37. An ambitious preliminary synthesis was an attempt to

assemble all four peptides on the same resin using a mixture of the four isomers of Fmoc-*β*-MePhe and to separate **6**−**9** from one another by RP-HPLC. While conductivity monitoring of the coupling reactions indicated that the assembly proceeded smoothly, the crude cleaved product contained only two chromatographically distinguishable prominent peaks. Subsequent experiments showed the earlier eluting peak consisted of a mixture of peptides containing the diastereoisomers (2*S*,3*S*) and (2*S*,3*R*) and the later eluting peak consisted of a mixture of peptides containing the diastereoisomers (2*R*,3*R*) and (2*R*,3*S*). Consequently, RP-HPLC could not be used to separate *β*-MePhe-containing peptide diastereoisomers of *h*-α-CGRP(8-37) that differ in configuration only at the C-3 (*β*-) position of the side chain but can be used when the diastereoisomers differ in configuration at the C-2 (α-) position with the peptide backbone.[26]

A second approach to the synthesis of **6**−**9** involved separating the mixture of all four isomers of *β*-MePhe into two mixtures using a combination of two previously published methods.[26,27] One mixture contained the erythro enantiomers (2*S*,3*S*) and (2*R*,3*R*), and the other contained the threo enantiomers (2*S*,3*R*) and (2*R*,3*S*). Recrystallization from water yielded white crystals 90% enriched with the erythro enantiomers, as determined by RP-HPLC.[26] Conversion of this mixture to the corresponding Fmoc derivatives[28] and repeated recrystallization from ethyl acetate/hexane mixtures yielded diastereomerically pure Fmoc-*erythro*-*β*-MePhe [(2*S*,3*S*) and (2*R*,3*R*)]. Preparative RP-HPLC of the aqueous mother liquour from the first recrystallization of *β*-MePhe yielded diastereomerically pure *threo*-*β*-MePhe [(2*S*,3*R*) and (2*R*,3*S*)]. This was converted to its Fmoc derivative[28] for peptide synthesis.

Solid-phase peptide synthesis using Fmoc-*erythro*-*β*-MePhe-OH yielded a mixture of peptide diastereoisomers **6** and **7** that were readily separated by semipreparative RP-HPLC. A sample of each synthetic product was digested with trypsin,[29] and the C-terminal dipeptide fragment H-Ala-*β*-MePhe-NH_2 was isolated by RP-HPLC. After acid hydrolysis of this fragment, the resulting mixture of Ala and *β*-MePhe was subjected to chiral TLC and the *β*-MePhe isomer was identified by comparing its R_f value with previously published values for all four isomers of *β*-MePhe.[30] The earlier eluting peak from semipreparative RP-HPLC was identified as analogue **6** containing the (2*S*,3*S*) enantiomer, and the later eluting peak was analogue **7** containing the (2*R*,3*R*) enantiomer. Analogues **8** and **9** were synthesized and identified in a similar manner using Fmoc-*threo*-*β*-MePhe-OH. Analogue **8** contained the (2*S*,3*R*)-*β*-MePhe isomer and eluted earlier than analogue **9**, which contained the (2*R*,3*S*)-*β*-MePhe isomer. Analogues **6** and **8** had the same retention time on RP-HPLC and coeluted with the earlier eluting peak of the previously described preliminary synthesis. Similarly, analogues **7** and **9** had the same retention time on RP-HPLC and coeluted with the later eluting peak of the preliminary synthesis.

Discovery and Structural Characterization of Dibenzyl CGRP(8-37). *N*-α-Bn-*h*-α-CGRP(8-37) (**14**) was synthesized by benzylating the N-terminal free amino group of the resin-bound protected peptide of *h*-α-CGRP(8-37) with excess benzyl bromide under basic conditions. RP-HPLC analysis of the crude product after cleavage of the peptide from the resin revealed the presence of a more hydrophobic major byproduct that was readily purified by preparative RP-HPLC. The mass of the byproduct was 3229.4 by ESI-MS, which is consistent with the byproduct being a dibenzylated derivative of *h*-α-CGRP(8-37) (theoretical mass of 3229.6). The byproduct is listed in Table 1 as analogue **15**.

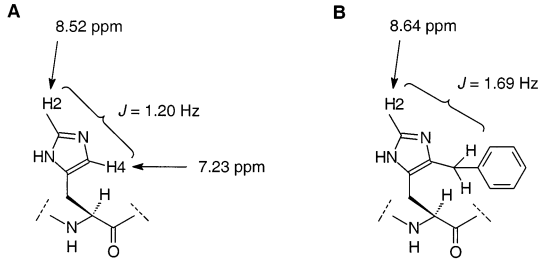

Figure 2. ^{1}H NMR assignments of the (A) His^{10} residue and (B) benzylated His^{10} residue of the tryptic tetrapeptides.

The monobenzylated analogue **14** had a desired mass of 3215.6, but analysis of its amino acid composition revealed a low recovery of Thr and one less Val than expected. Since this phenomenon was not observed for all other non-benzylated analogues of CGRP(8-37), these data suggest that the *N*-α-benzylvaline residue not only is stable to acid hydrolysis conditions but partially protects the Val^{10}−Thr^{11} peptide bond from hydrolysis.

Amino acid analysis of the dibenzylated analogue **15** showed similar low recoveries of Thr and Val. However, His was also not found. Similar results were obtained for benzylated derivatives of *h*-*β*-CGRP(8-37). Benzylation of the resin-bound protected peptide of *h*-*β*-CGRP-(8-37) produced an expected monobenzylated product (analogue **18**) and a dibenzylated product (analogue **19**) (Table 1**)**. The amino acid composition of analogue **18** lacks a Val and has low Thr recovery but contains one His. The amino acid composition of analogue **19** is similar but does not contain a His. These data showed that benzylation of the N-terminal α-amino group of *h*-α/*β*-CGRP(8-37) is accompanied by an irreversible, slower benzylation of the histidyl residue in position 10.

N-terminal tetrapeptides spanning positions 8−11 of *h*-α-CGRP(8-37) and analogue **15** were subjected to NMR analysis to determine the site of benzylation within the His^{10} residue. The tetrapeptides were generated by digestion of *h*-α-CGRP(8-37) (**1**) and analogue **15** with trypsin and purified by RP-HPLC using our previously published methods.[29] Presaturation ^{1}H NMR spectroscopy of *h*-α-CGRP(8−11) in D_2O unambiguously located the His-imidazole ring hydrogens H2 and H4 at 8.52 and 7.23 ppm, respectively. The upfield H4 appeared as a singlet and H2 appeared as a doublet with $J = 1.20$ Hz, both typical in appearance for 5-substituted imidazoles[31] and in coupling constant magnitude for four-bond coupling (Figure 2A**)**. When the complementary experiment was performed using dibenzyl-*h*-α-CGRP(8-11), the presence of two benzyl groups in the tetrapeptide was confirmed by observation of 10 new aromatic hydrogens in the region 7.48−7.24 ppm, a new doubly benzylic methylene group at 7.25 ppm, and a new *N*-benzylmethylene group at 5.31 ppm. The His-imidazole ring hydrogen H2 was retained at 8.64 ppm but

now appeared as a triplet with $J = 1.69$ Hz. The observed change in multiplicity and coupling constant magitude for H2 is appropriate for 5-bond coupling to a methylene group replacing H4, which indeed no longer was apparent (Figure 2B). Consequently, benzylation of His[10] occurs through alkylation of the benzyloxymethyl-protected imidazole ring at carbon C4, presumably by an electrophilic mechanism. Correlation spectroscopy revealed the observed long-range coupling of H2 to be unique to the doubly benzylic methylene group at 7.25 ppm, confirming the site of benzylation to be at C4 of the His-imidazole ring. To the best of our knowledge, this is the first report of the benzylation of the imidazole ring of His as a side reaction during solid-phase peptide synthesis. All benzylated derivatives of *h*-α/*β*-CGRP(8-37) were included in the subsequent pharmacological studies.

Binding Affinities of Position 37 Analogues. A previously described, radioligand binding method was used to determine the affinity of peptides for CGRP1 receptors.[24,32] Membranes were prepared from fresh porcine coronary arteries, and CGRP1 receptors were labeled with [^{125}I]-*h*-α-CGRP. The ability of *h*-α-CGRP-(8-37) and its analogues to compete with [^{125}I]-*h*-α-CGRP for binding to CGRP1 receptors in these membranes was then measured. Table 1 lists the binding affinities (IC_{50} values) of *h*-α-CGRP(8-37) (**1**) and its analogues for CGRP1 receptors. In this study, *h*-α-CGRP(8-37) bound with high affinity ($IC_{50} = 14.4$ nM), in agreement with our previously published data ($IC_{50} = 19$ nM).[24,31] The binding affinity of *h*-α-CGRP is presented for comparison.[24]

Work by our group and others showed that Phe[37] of *h*-α-CGRP is essential for binding of this endogenous agonist to CGRP1 receptors.[29,33−35] Rist et al. showed that the C-terminal Phe of the antagonist [Tyr^0]rat-α-CGRP(28-37) could not be removed or replaced with Ala or D-Phe without losing binding to CGRP1 receptors on SK-N-MC cells.[15] Analogues **3**−**11** were designed to identify structural features of the phenylalanyl residue in position 37 of *h*-α-CGRP(8-37) that are important for high-affinity binding of this antagonist to CGRP receptors.

The 1000-fold reduction in binding affinity of analogue **3** confirms that the benzyl side chain of Phe[37] is essential for binding of *h*-α-CGRP(8-37) to CGRP1 receptors. The planar aromatic phenyl ring of the side chain appears to be optimal for high-affinity binding but not essential. Replacement of the phenyl ring for a puckered, nonaromatic six-membered cyclohexyl ring that is present in analogue **4** causes a 7-fold reduction in binding affinity, whereas the *p*-hydroxy-substituted phenyl ring of analogue **5** resulted in no reduction in binding affinity. Attempts to constrain the flexibility of the side chain produced large reductions in binding affinity. Substitution of (2*S*,3*S*)-*β*-MePhe, (2*S*,3*R*)-*β*-MePhe, and Tic for Phe[37] gave analogues with IC_{50} values of 631, 434, and 363 nM, respectively. No binding was observed with analogues containing the corresponding D-(2*R*) isomers of the above constrained phenylalanine derivatives. As stated previously, substitution of Ala for Phe or changing the C-terminal amide of *h*-α-CGRP(8-37) to a carboxyl group or an ethyl-substituted amide also results in loss of antagonistic properties.[6,17,18] A structure−activity profile for position 37 of *h*-α-CGRP(8-37) is emerging in which a L-amino acid residue with a flexible side chain containing a phenyl ring and a C-terminal amide is required for high-affinity binding to CGRP1 receptors. The structure−activity profile is also consistent with structure−activity relationships of the C-terminal position reported for the smaller antagonist [Tyr^0]rat-α-CGRP(28-37)[15] and the endogenous agonist *h*-α-CGRP.[29] This suggests that CGRP1 receptors have similar requirements of the C-terminal Phe residue for high-affinity binding of agonists and antagonists. It would appear that position 37 of CGRP is not useful for the selective design of agonists or antagonists but is essential for high-affinity binding of both types of ligands to CGRP1 receptors.

Binding Affinities of N-Terminally Derivatized Analogues. A series of analogues of *h*-α-CGRP(8-37) were synthesized with derivatized amino termini to render these peptides stable to degradation by aminopeptidases. The C-terminal amide group of *h*-α-CGRP(8-37), which is essential for its high-affinity binding to CGRP receptors, also renders this peptide stable to degradation by carboxypeptidases. On the basis of previous reports that showed that *h*-*β*-CGRP(8-37) is more proteolytically stable than *h*-α-CGRP(8-37),[36] N-terminally derivatized analogues of *h*-*β*-CGRP(8-37) were also included. Binding affinities of *h*-α-CGRP(8-37) (**1**), *h*-*β*-CGRP(8-37) (**2**), N-terminally derivatized analogues of *h*-α-CGRP(8-37) (**12**−**15**), and N-terminally derivatized analogues of *h*-*β*-CGRP(8-37) (**16**−**19**) for CGRP1 receptors on porcine coronary arteries are listed in Table 1.

h-*β*-CGRP(8-37) had a similar affinity for CGRP1 receptors compared to *h*-α-CGRP(8-37) (Table 1), in agreement with previous reports for these peptides tested on human and rat CGRP receptors.[36] Derivatizing the amino terminus of *h*-α-CGRP(8-37) increased binding affinities at CGRP1 receptors (Table 1, analogues **12**−**14**). The largest increase was observed for the benzoylated analogue **13**, which has a 53-fold greater affinity than *h*-α-CGRP(8-37), whereas acetylating or benzylating the amino terminus (analogues **12** and **14**) produced 5- and 10-fold increases in affinity, respectively. The high binding affinity of **13** shows that the uncharged hydrophobic bulk of a benzoyl group is favored for binding to CGRP1 receptors over the uncharged, more polar acetyl group and the positively charged benzylated secondary amino terminus of **14**. Interestingly, benzylating the His[10] residue of **14** caused a further increase in binding affinity. The dibenzylated analogue **15** has the highest binding affinity of all analogues synthesized, 65-fold higher than that of *h*-α-CGRP(8-37). While previous studies suggested the side chain of His[10] may be important for binding,[22] the substantially increased binding affinity of analogue **15** over analogue **14** proves that position 10 is intimately involved in the recognition and/or binding of *h*-α-CGRP-(8-37) to CGRP receptors. Similar rank orders of increasing affinity were observed for the N-derivatized analogues of *h*-*β*CGRP(8-37) (Table 1), further validating the importance of the N-terminus and position 10 in binding to CGRP receptors.

Functional Effects of N-Derivatized Analogues. Previously, we reported that *h*-α-CGRP potently relaxes

Table 2. Antagonistic Potencies of N-Terminally Modified *h*-α-CGRP(8-37) Analogues on Porcine Coronary Arteries

	analogue	antagonistic potency K_B (nM)
1	*h*-α-CGRP(8-37)	970 ± 300
12	*N*-α-Ac-*h*-α-CGRP(8-37)	29.2 ± 14.4
13	*N*-α-Bzl-*h*-α-CGRP(8-37)	40.4 ± 19.0
14	*N*-α-Bn-*h*-α-CGRP(8-37)	119 ± 56.4
15	diBn-*h*-α-CGRP(8-37)	29.0 ± 6.37

precontracted porcine coronary artery rings in a concentration-dependent manner and that this effect is competitively blocked by *h*-α-CGRP(8-37).[37] N-derivatized analogues of *h*-α-CGRP(8-37) (**12**−**15**) showed no agonist effects as determined by their inability to relax porcine coronary arteries at concentrations up to 10 μM. Not surprisingly though, analogues **12**−**15** did cause parallel rightward shifts of the CGRP concentration−response curve, indicating that they were all competitive antagonists at CGRP receptors. The affinities of analogues **12**−**15** are listed as K_B values in Table 2. All analogues were significantly more potent antagonists than *h*-α-CGRP(8-37).

Derivatization of the N-terminus of peptides is often used to afford protection from aminopeptidase degradation.[38] The increased antagonistic potencies of analogues **12**−**15** may well be due to their inherent stability toward tissue-derived aminopeptidases, which may be present in isolated tissues. Consistent with this hypothesis is that even though analogues **12**−**15** contain structurally diverse derivatizing functional groups at the amino terminus and/or position 10, their K_B values are very similar (Table 2).

This is in contrast to the substantially different binding affinities observed for these analogues (Table 1). One major difference between the binding experiments and the relaxation experiments is the presence of bacitracin in the binding experiments. Bacitracin is a nonspecific endoprotease inhibitor[39,40] often used to protect peptides from proteolytic degradation.[41,42] Since addition of 0.05% (w/v) of bacitracin to the incubation buffer in the binding experiment caused a significant increase in the IC_{50} value of *h*-α-CGRP(8-37), bacitracin was used in all binding experiments. Consequently, proteolysis of the analogues during binding experiments is thought to be minimized. This is consistent with the observed binding affinities (IC_{50} values) being significantly higher than affinities (K_B values) determined in relaxation experiments. In the absence of proteolytic activity, IC_{50} values are a measure of an analogue's ability to bind to receptors. The substantial variation in IC_{50} values merely reflects the different contributions of the structurally diverse derivatizing functional groups to the analogue's ability to bind to CGRP1 receptors. Unfortunately, in relaxation experiments, bacitracin blocked contraction and relaxation of the porcine coronary artery rings, preventing the determination of K_B values under experimental conditions that are similar to those used in the binding assay.

It is of note that other workers found that acetylation of the amino terminus of *h*-α-CGRP(19-37), a shorter C-terminal fragment of *h*-α-CGRP that is also a weak antagonist, caused a 4-fold increase in antagonistic potency.[19] While no explanation was offered, this observation may also be due to increased stability of the N-terminal-acetylated peptide to aminopeptidase degradation.

Conclusion

Analogues of *h*-α-CGRP(8-37) and *h*-β-CGRP(8-37) containing modifications to the amino terminus and analogues of *h*-α-CGRP(8-37) containing modifications to the C-terminal position 37 were readily synthesized by solid-phase methods and purified by HPLC. All four isomers of the β-MePhe-containing analogues were obtained from two syntheses, one using the diastereomerically pure erythro mixture of (2*S*,3*S*)- and (2*R*,3*R*)-β-MePhe and the other using the diastereomerically pure threo mixture of (2*S*,3*R*)- and (2*R*,3*S*)-β-MePhe. Benzylation of the amino terminus was also accompanied by a novel side reaction involving the benzylation of the BOM-protected imidazole group of His[10] on the C4 carbon. Major structural modifications to position 37 of *h*-α-CGRP(8-37) were not tolerated without substantial reductions in binding affinity. In contrast, derivatizing the α-amino terminus of *h*-α-CGRP(8-37) and *h*-β-CGRP(8-37) increased binding affinity with the benzoylated analogues (**13** and **17**) possessing subnanomolar affinities. Equally high binding affinities were also observed for the dibenzylated analogues **15** and **19**, highlighting the importance of position 10 for the binding of *h*-α-CGRP(8-37) and *h*-β-CGRP(8-37) to CGRP receptors. Functional studies confirmed that the amino terminally modified analogues are potent antagonists at CGRP receptors and are at least 10-fold more potent than *h*-α-CGRP(8-37). These studies show that structure−activity studies of the N-terminal region of CGRP(8-37) may prove to be more fruitful for the development of high-affinity antagonists of CGRP receptors rather than the C-terminal region, which has been the focus of previous studies to date.[14−18,39] The amino terminally derivatized analogues appear to be the first peptide-based antagonists that (i) have affinities for CGRP receptors that are similar if not equal to that of the endogenous ligand *h*-α-CGRP and (ii) are more potent than the prototypical antagonist *h*-α-CGRP(8-37). We make note of exciting reports of non-peptide antagonists with similarly high affinities and potencies at human CGRP receptors.[40−42]

Experimental Section

Commercially available amino acid derivatives were purchased from Applied Biosystems (Foster City, CA), Bachem Inc. (Torrance, CA), and Chem-Impex International (Wood Dale, IL). Diastereomerically pure mixtures of *N*-α-Fmoc-(2*S*,3*S*/2*R*,3*R*)-β-MePhe-OH and *N*-α-Fmoc-(2*S*,3*R*/2*R*,3*S*)-β-MePhe-OH were synthesized using previously published methods.[26−28] NMR spectra were acquired in D_2O at 300 MHz using a Varian Inova spectrometer. Peptides were purified by our previously published methods utilizing preparative cation exchange chromatography on a PolySULFOETHYL Aspartimide column (1 cm × 20 cm) supplied by The Nest Group (Southboro, MA) and/or preparative RP-HPLC on a Vydac 218TP510 C_{18} column (1 cm × 25 cm) from the Separations Group (Hesperia, CA).[25] Buffers for cation exchange chromatography were sodium phosphate (5 mM, pH 3.0) containing sodium chloride (50 mM) and acetonitrile (20%) and sodium phosphate (5 mM, pH 3.0) containing sodium chloride (500 mM) and acetonitrile (20%). Buffers used for RP-HPLC were either 100 mM TEAP, pH 2.5 (buffer A), and a mixture of acetonitrile and buffer A (60/40, v/v) (buffer B) or 0.1% TFA

in water (solvent C) and 0.09% TFA in a mixture of acetonitrile and water (60/40, v/v) (solvent D). Analytical RP-HPLC was performed on a Vydac 218TP54 C_{18} column (0.46 cm × 25 cm), a PLRP-S 300 Å column (0.46 cm × 25 cm) from Polymer Labs (Amherst, MA), and a 300SB-C_8 column (0.46 cm × 25 cm) from MAC-MOD Analytical Inc. (Chadds Ford, PA). The flow rate was 1 mL/min, and the eluent was continuously monitored at 220 nm. Amino acid analyses were performed on a Waters AccQTag system after samples were hydrolyzed in the vapor phase with 6 M constant boiling hydrochloric acid from Pierce (Rockford, IL) at 110 °C for 48 h. ESI-MS was performed on an API150EX from PE-SCIEX (Foster City, CA). Chiral TLC was performed on Macherey-Nagel Chiralplates from Alltech (Deerfield, IL). Spots were visualized by ninhydrin.

Solid-Phase Peptide Synthesis Using Fmoc-amino Acid Derivatives. Analogues **1**−**9** were synthesized on an Applied Biosystems 432A "Synergy" peptide synthesizer using the manufacturer's protocols. Reactive side chains of Fmoc-amino acid derivatives were protected as follows: Arg, pentamethylchroman-6-sulfonyl (Pmc); Asn, trityl (Trt); Asp, *tert*-butyl ester (tBu); His, trityl (Trt); Lys, *tert*-butyloxycarbonyl (Boc); Ser, *tert*-butyl ether (tBu); Thr, *tert*-butyl ether (tBu); Trp, *tert*-butyloxycarbonyl (Boc); Tyr, *tert*-butyl ether (tBu). *N*-α-Fmoc-amino acid derivatives (0.075 mmol) were coupled to a polystyrene-based resin derivatized with the Knorr linker[23] for the synthesis of C-terminal amide peptides (0.025 mequiv) using HBTU, HOBt, and DIEA in NMP containing DMSO. The Fmoc group was deprotected in piperidine. Effluent from the deprotection reactions was continuously monitored for conductance resulting from the piperidine carbamate salt. Deprotection times were extended when necessary to ensure complete removal of the Fmoc group. Reaction times for coupling of the subsequent amino acid derivatives were also extended. Single couplings were performed for addition of all amino acid derivatives. TFA in the presence of scavengers was used to cleave side chain protecting groups and the peptide from the resin. Typically, peptide resin (approximately 160 mg) was stirred with the mixture TFA/thioanisole/water/ethanedithiol (1.2 mL, 90/5/2.5/2.5, v/v/v/v) for 3 h at room temperature. Chilled *tert*-butyl methyl ether (12 mL) was added to the reaction mixture to precipitate the peptide that was subsequently removed, with the resin, by filtration. The peptide resin mixture was washed with 10% aqueous acetic acid (3 × 5 mL) and filtered. The pooled filtrates were lyophilized to yield the crude product as a white powder.

***h*-α-CGRP(8-37) 1.** The title peptide was synthesized by methods described above using Fmoc-amino acid derivatives. The crude product was loaded onto the preparative cation exchange HPLC column previously equilibrated with the phosphate buffer (pH 3.0). The product was eluted by increasing the concentration of sodium chloride in the eluent from 50 to 500 mM over 50 min. Fractions containing the product were pooled and loaded directly onto the preparative Vydac C_{18} column previously equilibrated with a mixture of buffers A and B (77/23, v/v). Increasing the concentration of B to 43% over 50 min eluted the product. Fractions containing only the product, as judged by analytical RP-HPLC, were pooled and loaded onto the same Vydac C_{18} column now equilibrated with a mixture of solvents C and D (90/10, v/v). The concentration of solvent D was raised to 100% over 30 min to elute the product. Fractions containing the desired peptide were pooled and lyophilized to yield 21 mg (27%) of *h*-α-CGRP(8-37) as a fluffy white powder.

***h*-β-Calcitonin Gene-Related Peptide (8-37) 2.** Fmoc-amino acid derivatives were used for the synthesis of *h*-β-CGRP(8-37) using methods described above for *h*-α-CGRP(8-37). The crude product was purified by cation exchange chromatography and RP-HPLC. Elution conditions for RP-HPLC were 24−44% buffer B over 50 min and 10−100% solvent D over 30 min. *h*-β-CGRP(8-37) was obtained as a fluffy white powder in a yield of 13 mg (16%).

[Gly^{37}]-*h*-α-CGRP(8-37) 3. The title compound was synthesized using Fmoc-amino acid derivatives. After cation exchange chromatography, the partially purified product was loaded onto the preparative Vydac C_{18} column, which was previously equilibrated with a mixture of solvents C and D (70/30, v/v). The concentration of solvent D was raised to 50% over 50 min to yield 12.7 mg (17%) of [Gly^{37}]-*h*-α-CGRP(8-37) as a fluffy white powder.

[Cha^{37}]-*h*-α-CGRP(8-37) 4. The desired analogue was assembled using Fmoc-amino acid derivatives following methods described above and purified in a manner similar to that for analogue **3**. Elution conditions for preparative RP-HPLC were 32−42% D over 50 min to yield 16.0 mg (20%) of [Cha^{37}]-*h*-α-CGRP(8-37) as a fluffy white powder.

[Tyr^{37}]-*h*-α-CGRP(8-37) 5. [Tyr^{37}]-*h*-α-CGRP(8-37) was synthesized by methods described above utilizing Fmoc-amino acid derivatives and purified in a manner similar to that for analogue **3**. Elution conditions for preparative RP-HPLC were 29−49% D over 50 min to yield 11.0 mg (14%) of the desired peptide as a fluffy white powder.

Separation of [(2*S*,3*S*)-β-MePhe^{37}]-*h*-α-CGRP(8-37) from [(2*R*,3*R*)-β-MePhe^{37}]-*h*-α-CGRP(8-37), Analogues 6 and 7. The title analogues were synthesized by methods described above starting with the racemic mixture Fmoc-(2*S*, 3*S*)/(2*R*, 3*R*)-β-MePhe and purified in a manner similar to that for analogue **1**. No separation of the diastereomeric peptides was observed after preparative cation exchange chromatography. Fractions containing both diastereomeric peptides were pooled and loaded directly onto the preparative RP-HPLC column previously equilibrated with a mixture of buffers A and B (73/27, v/v). The peptide isomers were eluted from the column by changing the concentration of buffer B from 27% to 32% over 20 min, from 32% to 34% over 30 min, and from 34% to 39% over 20 min. Fractions that contained only the earlier eluting diastereoisomer, as judged by analytical RP-HPLC, were pooled and loaded directly onto the same preparative RP-HPLC column previously equilibrated with a mixture of solvents C and D (90/10, v/v). Raising the concentration of solvent D in the eluent to 100% over 30 min rapidly eluted the desalted peptide. Impure fractions from the first RP-HPLC fractionation containing the earlier eluting diastereoisomer were pooled and fractionated on the preparative RP-HPLC column using elution conditions of 35−45% D over 50 min. Fractions from both separations containing only the earlier eluting diastereoisomer were pooled and lyophilized to yield 2.8 mg (4%) of a fluffy white powder designated analogue **6**.

Fractions that contained the later eluting diastereoisomer were pooled and loaded onto the preparative RP-HPLC column previously equilibrated with a mixture of solvents C and D (64/36, v/v). Raising the concentration of D in the eluent to 45% over 50 min eluted the product. Fractions containing the product were pooled and lyophilized to yield 2.4 mg (3%) of a fluffy white powder designated analogue **7**.

Separation of [(2*S*,3*R*)-β-MePhe^{37}]-*h*-α-CGRP(8-37) from [(2*R*,3*S*)-β-MePhe^{37}]-*h*-α-CGRP(8-37), Analogues 8 and 9. The desired analogues were synthesized by methods described above starting with the racemic mixture Fmoc-(2*S*, 3*R*)/(2*R*, 3*S*)-β-MePhe and purified in a manner similar to that for analogue **1**. Once again, no separation of the diastereomeric peptides was observed after preparative cation exchange chromatography. Fractions containing both diastereomeric peptides were pooled and loaded directly onto the preparative RP-HPLC column previously equilibrated with a mixture of buffers A and B (75/25, v/v). Peptide isomers were eluted from the column by changing the concentration of buffer B from 25% to 30% over 20 min, from 30% to 31% over 20 min, holding at 31% B for 20 min, from 31% to 32% over 20 min, and from 32% to 37% over 20 min. Fractions that contained the earlier eluting diastereoisomer were pooled and loaded directly onto the same preparative RP-HPLC column previously equilibrated with a mixture of solvents C and D (75/25, v/v). Raising the concentration of solvent D in the eluent to 55% over 50 min eluted the desalted peptide. Fractions containing only the earlier eluting diastereoisomer were pooled and lyophilized to yield 4.3 mg (6%) of a fluffy white powder designated analogue **8**.

Fractions that contained the later eluting diastereoisomer were pooled and loaded onto the preparative RP-HPLC column previously equilibrated with a mixture of solvents C and D (65/35, v/v). Raising the concentration of D in the eluent to 65% over 50 min eluted the product. Fractions containing the later eluting diastereoisomer were pooled and lyophilized to yield 4.0 mg (5%) of a fluffy white powder designated analogue **9**.

Tryptic Digestion and Identification of *β*-MePhe Isomers of Analogues 6−9. Analogues **6**−**9** (0.5 mg) were each digested with trypsin (2.5 μg) in 50 mM ammonium bicarbonate buffer (0.5 mL, pH 8.5) at 37 °C for 24 h. A 1% aqueous TFA solution (50 μL) was added to terminate the reaction, and the digest mixture was loaded onto an analytical Vydac C_{18} RP-HPLC column previously equilibrated with solvent C. The concentration of solvent D in the eluent was increased from 0% to 10% over 30 min, from 10% to 40% over 30 min, and from 40% to 100% over 30 min. Five fragments for each peptide were collected by hand and identified by comparing their retention times with those of the tryptic fragments of *h*-α-CGRP.[29] The structure of the fragments was confirmed by amino acid analysis. A second aliquot of the acid hydrolysate of fragment H-Ala-*β*-MePhe-NH_2 was subjected to chiral TLC using the solvent system $CH_3CN/CH_3OH/H_2O$ (4/1/1, v/v/v). The isomer of *β*-MePhe was identified by comparison of R_f values with published values.[30] Analogue **6** was [(2*S*,3*S*)-*β*-MePhe[37]]-*h*-α-CGRP(8-37), analogue **7** was [(2*R*,3*R*)-*β*-MePhe[37]]-*h*-α-CGRP(8-37), analogue **8** was [(2*S*,3*R*)-*β*-MePhe[37]]-*h*-α-CGRP(8-37), and analogue **9** was [(2*R*,3*S*)-*β*-MePhe[37]]-*h*-α-CGRP(8-37).

Solid-Phase Peptide Synthesis Using Boc-amino Acid Derivatives. Analogues **10**−**19** were assembled on an Applied Biosystems model 430A peptide synthesizer using solid-phase methods described previously.[24,29] Briefly, *N*-α-Boc-amino acid derivatives (2 mmol) were coupled to MBHA resin (0.5 mequiv) in NMP using HOBt (2 mmol) and DIC (2 mmol) as coupling reagents. The coupling reactions were monitored by the quantitative ninhydrin test.[47] Double couplings were performed at positions 37, 31, and 21−10 to maintain yields in excess of 99% for all couplings. Peptides were freed of side chain protecting groups and cleaved from the resin simultaneously by the low−high TFMSA method of Tam[48] using the scavengers thioanisole, EDT, *m*-cresol, and DMS. Chilled diethyl ether was added to the reaction mixture to precipitate the peptide, which was removed, with the resin, by filtration. The peptide was extracted from the resin with 10% aqueous acetic acid (3 × 5 mL), and the pooled filtrates containing the crude product were subjected to chromatography immediately.

[Tic[37]]-*h*-α-CGRP(8-37) 10. Starting with Boc-L-Tic-OH, analogue **10** was assembled using Boc-amino acid derivatives on the MBHA resin as described above. A portion of the peptide resin (128 mg, 0.025 mequiv) was subjected to low−high TFMSA procedure of Tam to yield the crude lyophilized product, which was dissolved in 5 mL of 0.1% aqueous TFA and desalted on a Sephadex G15 column (1.6 cm × 17 cm) previously equilibrated with 0.1% aqueous TFA. The peptide-containing fraction was loaded directly onto the preparative RP-HPLC column previously equilibrated with a mixture of solvents C and D (70/30, v/v). Raising the concentration of solvent D in the eluent to 50% over 50 min eluted the product. Fractions containing only the product, as judged by analytical RP-HPLC, were pooled and lyophilized to yield 3.1 mg (3.9%) of [Tic[37]]-*h*-α-CGRP(8-37) as a fluffy white powder.

[D-Tic[37]]-*h*-α-CGRP(8-37) 11. The title analogue was synthesized and purified in a manner similar to that for analogue **10** using Boc-D-Tic-OH. After the desalting step, the product was eluted from the preparative RP-HPLC column by increasing the concentration of solvent D from 31% to 51% over 50 min. Fractions containing the product were pooled and lyophilized to yield 12.0 mg (14%) of [D-Tic[37]]-*h*-α-CGRP(8-37) as a fluffy white powder.

***N*-α-Acetyl-*h*-α-CGRP(8-37) 12.** The amino acid sequence of *h*-α-CGRP(8-37) was assembled on MBHA resin using Boc-amino acid derivatives as described above. A portion of the peptide resin (200 mg, 0.04 mmol) was acetylated with acetic anhydride (38 μL, 0.4 mmol) and DIEA (70 μL, 0.4 mmol) in DMF (1.5 mL) for 1 h prior to cleavage from the resin. The crude product from the cleavage reaction was loaded onto the Vydac C_{18} semipreparative column that had been previously equilibrated with a mixture of buffers A and B (71/29, v/v). The product was eluted by raising the concentration of buffer B in the eluent to 49% over 50 min. Fractions containing the product were pooled and loaded onto the same column now equilibrated with a mixture of solvents C and D (90/10, v/v), and the product was eluted by raising the concentration of solvent D in the eluent to 80% over 50 min. Fractions containing only the desired peptide as judged by analytical RP-HPLC were pooled and lyophilized to yield 22 mg (17%) of a fluffy white powder.

***N*-α-Benzoyl-*h*-α-CGRP(8-37) 13.** A portion of the peptide resin (200 mg, 0.04 mmol) described in the synthesis of analogue **12** above was benzoylated with benzoic anhydride (90 mg, 0.4 mmol) and DIEA (70 μL, 0.4 mmol) in DMF (1.5 mL). The crude product from the cleavage reaction was purified by RP-HPLC in a manner similar to that for analogue **12** above. The product was eluted from the Vydac C_{18} column by raising the concentration of buffer B from 31% to 51% over 50 min and the concentration of solvent D from 35% to 55% over 50 min to yield 21 mg (16%) of a fluffy white powder.

***N*-α-Benzyl-*h*-α-CGRP(8-37) 14 and Dibenzyl-*h*-α-CGRP(8-37) 15.** Another portion of the peptide resin (280 mg, 0.056 mmol) from the synthesis of analogue **11** was benzylated with benzyl bromide (67 μL, 0.56 mmol) and DIEA (98 μL, 0.56 mmol) in DMF (1.5 mL). The crude product mixture from the cleavage reaction was purified by RP-HPLC in a manner similar to that for analogue **12** above. Two components eluted from the Vydac C_{18} column when the concentration of buffer B was raised from 24% to 44% over 50 min. The more hydrophilic component was loaded onto the same Vydac C_{18} column now equilibrated with a mixture of solvents C and D (67/33, v/v) and eluted from the column by raising the concentration of solvent D to 53% over 50 min to yield 5 mg (3%) of the monobenzylated product as a white fluffy powder.

The later eluting component was loaded onto the Vydac C_{18} column that was previously equilibrated with a mixture of solvents C and D (64/36, v/v) and eluted from the column by raising the concentration of solvent D to 56% over 50 min to yield 9 mg (5%) of the dibenzylated product as a white fluffy powder.

***N*-α-Acetyl-*h*-*β*-CGRP(8-37) 16.** Boc-amino acid derivatives were used to assemble the amino acid sequence of *h*-*β*-CGRP(8-37) on the MBHA resin. A portion of the peptide resin (200 mg, 0.04 mmol) was acetylated with acetic anhydride (38 μL, 0.4 mmol) and DIEA (70 μL, 0.4 mmol) in DMF (1.5 mL) for 1 h prior to the cleavage reaction. The cleaved crude product was partially purified by cation exchange chromatography and RP-HPLC. The elution conditions for RP-HPLC were 36−56% solvent D in the presence of solvent C over 50 min. Analytical RP-HPLC identified fractions containing only the desired product, which were pooled and lyophilized to yield 14 mg (11%) of **16** as a fluffy white powder.

***N*-α-Benzoyl-*h*-*β*-CGRP(8-37) 17.** A portion of the peptide resin (200 mg, 0.04 mmol) generated in the synthesis of analogue **16** was benzoylated with benzoic anhydride (90 mg, 0.4 mmol) and DIEA (70 μL, 0.4 mmol) in DMF (1.5 mL). The crude product was purified by RP-HPLC in a manner similar to that for analogue **11**. The product was eluted from the Vydac C_{18} column by raising the concentration of buffer B from 27% to 57% over 75 min and the concentration of solvent D from 36% to 56% over 50 min. Analogue **17** was obtained in a yield of 8 mg (6%) as a white fluffy powder.

***N*-α-Benzyl-*h*-*β*-CGRP(8-37) 18 and Dibenzyl-*h*-*β*-CGRP(8-37) 19.** A portion of the peptide resin (400 mg, 0.04 mmol) obtained during the synthesis of analogue **16** was benzylated with benzyl bromide (96 μL, 0.4 mmol) and DIEA (140 μL, 0.4 mmol) in DMF (3 mL). The crude product from the cleavage reaction was purified by RP-HPLC. Two components eluted from the Vydac C_{18} column when the concentration of buffer

B was raised from 27% to 47% over 50 min. The earlier eluting component was loaded onto the same Vydac C_{18} column now equilibrated with a mixture of solvents C and D (69/31, v/v).The component was eluted from the column when the concentration of solvent D was raised to 51% over 50 min and lyophilized to yield 3 mg (2%) of **18** as a fluffy white powder.

The hydrophobic component was loaded onto the Vydac C_{18} column that was previously equilibrated with a mixture of solvents C and D (66/34, v/v). The component was eluted from the column by raising the concentration of solvent D to 54% over 50 min to yield 4 mg (2%) of the dibenzylated product **19** as a fluffy white powder.

Radioligand Binding Assay. *h*-α-CGRP(8-37) and analogues **2**−**17** were tested for their ability to compete with [^{125}I]-*h*-α-CGRP for binding to CGRP receptors in membranes prepared from porcine coronary arteries as previously described.[24,49] Briefly, crude membranes were prepared and incubated with 50 pM [^{125}I]-*h*-α-CGRP in the presence or in the absence of various concentrations of *h*-α-CGRP analogues. Analogue concentrations were calculated from the weight of peptide used in each experiment. Incubations were performed in 50 mM Tris buffer containing 0.1% (w/v) bovine serum albumin and 0.05% (w/v) bacitracin to limit peptidase mediated breakdown of CGRP analogues in the binding assay. Although a variety of other peptidase inhibitors were tested, only bacitracin increased *h*-α-CGRP(8-37) binding affinity without interfering with the binding assay. Nonspecific binding was determined using an excess (1 μM) of *h*-α-CGRP. Bound [^{125}I]-*h*-α-CGRP was separated from free [^{125}I]-*h*-α-CGRP by vacuum filtration through glass fiber filters, and bound radioactivity was counted using a γ-counter.

Coronary Artery Relaxation Assay. All analogues were tested for their ability to relax isolated coronary arteries using methods described by us previously.[37] Analogue concentrations were calculated from the weight of peptide used in each experiment. Briefly, arteries were contracted with KCl and then relaxed with CGRP or one of the analogues. Since peptidase inhibitors blocked KCl-induced contraction and CGRP-induced relaxation, they were not included in the relaxation assay. No analogues caused relaxation and subsequently were tested for antagonist activity. For these studies, arteries were relaxed with *h*-α-CGRP in the absence and in the presence of an antagonist analogue.

Data Analysis. Both functional data and radioligand binding data were analyzed using least sum of squares nonlinear regression curve fitting (Graphpad Inplot 4.0). Radioligand binding data were analyzed using equations that modeled the data to fit to either a one- or two-site competition curve. The best fit of the data was determined using an *F* test that compared the sum of squares and degrees of freedom for each fit. For these comparisons, $p < 0.05$ was accepted as a significant difference between fits. For functional data, complete cumulative concentration−response curves were generated and EC_{50} values were calculated using all points on the relaxation concentration−response curve. K_B values for the antagonist analogues were determined as described by Arunlakshana and Schild.[50]

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Appendix

Abbreviations. Symbols and abbreviations used in this paper conform to the recommendations of the IUPAC−IUB Joint Commission on Nomenclature (*J. Biol. Chem.* **1989**, *264*, 668−673) and/or were recently reported to be in use in peptide science (*J. Pept. Sci.* **1999**, *5*, 465−471). Other abbreviations are the following: Ac, acetyl; Bzl, benzoyl; Bn, benzyl; diBn, dibenzyl; CGRP, calcitonin gene-related peptide; MBHA, *p*-methylbenzhydrylamine; RT-PCR, reverse transcription polymerase chain reaction.

Supporting Information Available: Tables of *k*′ values and amino acid compositions of *h*-α-CGRP(8-37), *h*-β-CGRP-(8-37), and analogues **3**−**19**, RP-HPLC trace of crude cleaved product from synthesis of benzylated analogues **14** and **15**, and NMR spectra of tryptic N-terminal tetrapeptides of *h*-α-CGRP-(8-37) and dibenzylated analogue **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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