Identification of Potent, Selective, and Metabolically Stable Peptide Antagonists to the Calcitonin Gene-Related Peptide (CGRP) Receptor

Les P. Miranda,*[,†] Jerry Ryan Holder,[†] Licheng Shi,[§] Brian Bennett,[†] Jennifer Aral,[†] Colin V. Gegg,[‡] Marie Wright,[‡] Kenneth Walker,[‡] George Doellgast,[#] Rick Rogers,[#] Hongyan Li,[#] Violeta Valladares,[‡] Kevin Salyers,[#] Eileen Johnson,[§] and Kenneth Wild[§]

Chemistry Research and Discovery, Protein Sciences, Pharmacokinetics and Drug Metabolism, and Neuroscience, Amgen, Inc., One Amgen Center Drive, Thousand Oaks, California 91320

Received July 24, 2008

Calcitonin gene-related peptide (CGRP) is a 37-residue neuropeptide that can be converted to a $CGRP_1$ receptor antagonist by the truncation of its first seven residues. CGRP(8−37), **1**, has a $CGRP_1$ receptor K_i = 3.2 nM but is rapidly degraded in human plasma ($t_{1/2}$ = 20 min). As part of an effort to identify a prolonged in vivo circulating CGRP peptide antagonist, we found that the substitution of multiple residues in the CGRP peptide increased $CGRP_1$ receptor affinity >50-fold. Ac-Trp-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]-CGRP(8−37)-NH_2, **5** (K_i = 0.06 nM) had the highest $CGRP_1$ receptor affinity. Using complimentary in vitro and in vivo metabolic studies, we iteratively identified degradation sites and prepared high affinity analogues with significantly improved plasma stability. Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},2-$Nal^{27,37}$,Asp^{31}, $Oic^{29,34}$,Phe^{35}]CGRP(8−37)-NH_2, **32** (K_i = 3.3 nM), had significantly increased (>100-fold) stability over **1** or **5**, with a cynomolgus monkey and human in vitro plasma half-life of 38 and 68 h, respectively.

Introduction

Calcitonin gene-related peptide (CGRP[a]) is a 37 amino acid neuropeptide produced by tissue-specific alternative mRNA splicing of the calcitonin gene that has vasodilatory, ionotropic, and chronotropic activities.[1] The CGRP peptide signals through a seven-transmembrane G-protein-coupled receptor (GPCR) belonging to the secretin receptor family.[2] Coexpression of the calcitonin-like receptor (CLR) with receptor activity modifying protein-1 (RAMP-1) forms a mature CGRP receptor on the cell membrane surface.[3] CGRP is widely distributed in the peripheral and central nervous systems.[1] In the latter, CGRP is expressed in trigeminal ganglia nerves and when it is released has potent dilator effects on cerebral and dural vessels.[4] Through this mechanism, CGRP is involved in the regulation of blood flow to the brain and pain-sensitive meninges. The pathology of migraine has been associated with the vasodilation effects of CGRP on cerebral circulation.[5,6] Clinical proof-of-concept has been obtained that the inhibition of CGRP binding to its receptor may be a useful approach for therapeutic intervention.[7−10]

The structure−activity relationship of CGRP has been studied by several laboratories. The CGRP peptide contains a disulfide bridge between residues 2 and 7 and an amidated C-terminus. In humans CGRP exists as α and β isoforms differing at residues 3, 22, and 25 (Figure 1).[11] In the α isoform, residues 3, 22, and 25 are aspartic acid, valine, and asparagine, respectively, whereas in the β isoform, residues 3, 22, and 25 are asparagine, methionine, and serine, respectively. Adrenomedullin (AM) shares 24% amino acid sequence homology with CGRP and with some other members of the calcitonin family of regulatory peptides, such as calcitonin and amylin.[12] Adrenomedullin (AM) interacts with two specific receptors formed by the calcitonin-like (CL) receptor and RAMP 2 or 3, known as AM1 and AM2.[1,13] CGRP and AM have limited selectivity at the CGRP, AM1, and AM2 receptors, and AM has appreciable affinity for the $CGRP_1$ receptor,[14] whereas CGRP has been reported to activate AM2.[15,16]

The tertiary structure of CGRP has yet to be conclusively determined; however, solution NMR analysis has identified secondary structure elements.[17] In addition to the N-terminal cyclic structure between cysteine residues 2 and 7, NMR studies indicate the presence of a helical region between residues valine[8] and arginine[18] and the presence of three poorly defined turns at serine[19]−glycine[21], proline[29], and proline[34].[17] Distinct regions in the CGRP peptide responsible for receptor binding and activation have also been identified. The N-terminal region, residues alanine[1]−cysteine[7], has been found to be required for receptor activation,[18] while the valine[8]−arginine[18] and valine[28]−phenylalanine[37] regions are important for receptor affinity.[19,20] It has been shown that removal of the CGRP activation region via N-terminal truncation, for example, human αCGRP(8−37), results in an antagonist to the $CGRP_1$ receptor.[21−23] hαCGRP(8−37) also appears to be a weak antagonist to both AM receptors. Further N-terminal truncation is possible, although the resulting antagonists, such as CGRP(27−37), have low affinity to the CGRP receptor.[24,25] However, the affinity of CGRP(27−37) has been reported to be improved with substitutions at residue positions 29, 31, 34, and 35, for example, [Asp^{31},Pro^{34},Phe^{35}]-human αCGRP(27−37) and [Aib^{29},Asp^{31}, Pro^{34},Phe^{35}]-human αCGRP(27−37).[26] Hydrophobic N-terminally modified analogues of human αCGRP(8−37) have also been reported with high affinity, including [N^{α}-benzoyl] human αCGRP(8−37) and [N^{α}-benzyl] human αCGRP(8−37).[27] Despite improvements in receptor potency, CGRP peptide antago-

* To whom correspondence should be addressed. Phone: 1-805-447-9397.Fax: 1-805-480-3015. E-mail: lesm@amgen.com.
† Chemistry Research and Discovery.
§ Neuroscience.
‡ Protein Sciences.
# Pharmacokinetics and Drug Metabolism.

[a] Abbreviations: DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxocarbonyl; CGRP, calcitonin gene-related peptide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate *N*-oxide; GPCR, G-protein-coupled receptor; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid. Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977).

10.1021/jm8009298 CCC: $40.75
Published on Web 11/20/2008

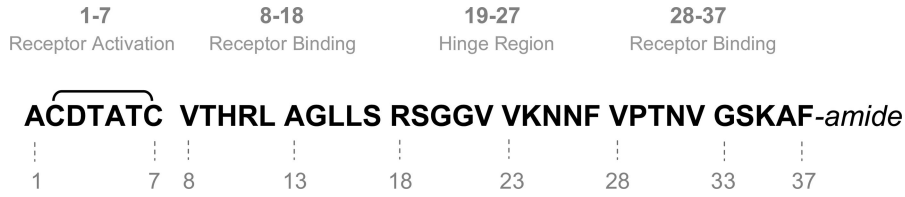


Figure 1. Primary structure of human αCGRP using single letter amino acid codes. Residue numbering and the proposed functional region are indicated.

nists suffer from poor metabolic stability. Human CGRP and CGRP(8−37) both have a short plasma half-lives of approximately 10 min.[21,28,29]

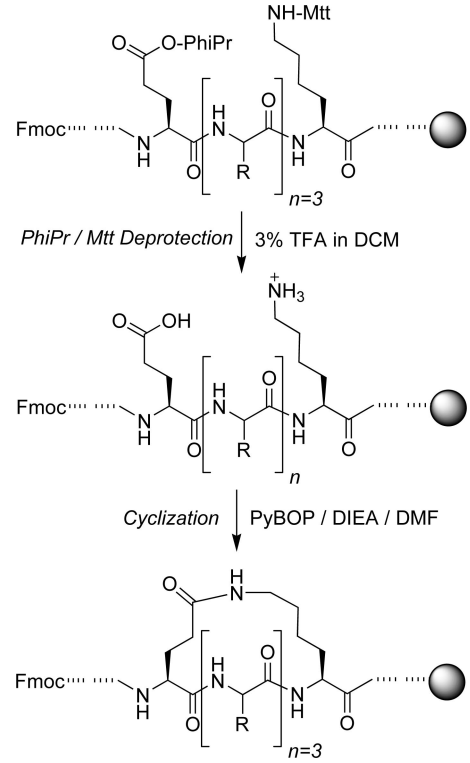
As part of an effort to identify a prolonged in vivo circulating CGRP peptide antagonist, we first sought to improve the metabolic stability, selectivity, and receptor affinity of human αCGRP(8−37) based antagonists. In this article, we report the design and synthesis of high-affinity CGRP antagonist analogues with significantly increased plasma stability, higher selectivity over AM1 and AM2, and their ability to bind and antagonize the human CGRP receptor in vitro.

Results and Discussion

The aim of this work was to increase potency, selectivity, and metabolic stability of the human αCGRP(8−37)-amide antagonist peptide (**1**). Linear and cyclic CGRP analogues used in this study were prepared by solid-phase peptide synthesis (SPPS) using a N^{α}-Fmoc/*tert*-butyl strategy on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink) resin.[30] Peptide-chain assembly on-resin was carried out using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/diisopropylethylamine (DIEA)/*N,N*-dimethylformamide (DMF) chemistry as previously described.[31,32] For cyclic CGRP analogues, side chain to side chain lactam formation (i to $i + 4$) was carried out on the assembled N-terminally Fmoc-protected resin-bound peptide (Scheme 1). At cyclization sites, side chain amino (Lys) and carboxyl (Glu) functionalities were protected with 4-methyltrityl (Mtt) groups and 2-phenylisopropyl ester (2-Ph^{i}Pr) groups, respectively.[33,34] After deprotection of the Mtt and 2-Ph^{i}Pr groups with dilute TFA, benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) mediated cyclization was used to form the chemically stable side chain to side chain lactam constraint.[35] RP-HPLC retention time and electrospray mass spectrometry were used to confirm the condensation (−18 Da) of the involved side chain groups. CGRP analogues were then purified by preparative RP-HPLC and assessed by analytical HPLC and electrospray mass spectrometry (see Supporting Information).

A human neuroblastoma SK-N-MC cell line endogenously expressing the $CGRP_1$ receptor was used to compare the potency of CGRP peptide antagonists.[36,37] Briefly, upon binding of the αCGRP(1−37) agonist peptide to the $CGRP_1$ G-protein-coupled receptor, cAMP levels increase, mediated by G-protein activation of adenylyl cyclase and cAMP-dependent protein kinase (PKA). An ELISA was used to measure the level of cAMP accumulated in treated SK-N-MC cells as a screening system for the evaluation of CGRP antagonists. Under these assay conditions, the endogenous agonist human αCGRP(1−37) (Figure 1) had an EC_{50} of 0.05 nM whereas the truncated antagonist peptide, human αCGRP(8−37)-amide (**1**), had an CGRP1-receptor IC_{50} of 4.87 nM and K_i of 3.2 nM (Tables 1 and 2). In similar cAMP assays, the functional selectivity of the CGRP antagonist peptide **1** for the $CGRP_1$ receptor over

Scheme 1. Synthesis of i to i+4 Constrained CGRP Analogues via Orthogonally Protected Peptides[a]



[a] For i to $i + 4$ constraints, $n = 3$. R indicates the protected side chain group of L-amino acid residues spanning the cyclization site. Dash lines represent the remaining protected peptide segments of CGRP.

both AM1 and AM2 was approximately 50-fold (Table 3). No $CGRP_1$ receptor agonist activity was observed for **1** or other CGRP analogues reported here. Starting from peptide **1** several modifications were initially made with the objective to improve antagonist potency. In agreement with previous reports, increased hydrophobicity at the N-terminus of the peptide is favorable for receptor interactions, and Trp-CGRP(8−37)-amide, **2**, had an IC_{50} of 0.64 nM. It had been previously reported that aspartic acid[31], proline[34], and phenylanine[35] substitutions in short CGRP peptides spanning residues 27−37 resulted in high-affinity antagonists.[25] When aspartic acid[31], proline[34], and phenylalanine[35] substitutions and N-terminal acetylation were incorporated in the longer CGRP(8−37) peptide, **3**, an IC_{50} of 1.74 nM was obtained. In comparison, [Aib[29],Asp[31],Pro[34],Phe[35]]CGRP(27−37), one of the most high-affinity short peptides reported to date,[26] was found to have an IC_{50} of 740 nM under these assay conditions which use the hαCGRP agonist.[36,37]

The CGRP(8−37)-amide peptide, **1**, has two lysine residues located at positions 24 and 35. The potency enhancing phenylalanine[35] substitution in **3** removed one of these lysine residues, while the lysine residue at position 24 in the putative hinge region remained. Next, we found that substitutions around this

Table 1. List of All Peptides and in Vitro Activities Using SK-N-MC Cells Expressing the Human CGRP Receptor[a]

compd	peptide	antagonist activity, $IC_{50} \pm SD$ (nM)
1	CGRP(8−37)-NH_2 VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-*NH_2*	4.87 ± 0.43
2	Trp-CGRP(8−37)-NH_2 **W**VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-*NH_2*	0.64 ± 0.24
3	Ac-[Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***VTHRLAGLLSRSGGVVKNNFVPT**D**VG**PF**AF-*NH_2*	1.74 ± 0.55
4	Ac-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***VTHRLAGLLSRSGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	0.71 ± 0.39
5	Ac-Trp-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**THRLAGLLSRSGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	0.20 ± 0.05
6	Ac-Trp-[Cit^{11},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLSRSGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	0.57 ± 0.17
7	Ac-Trp-[Cit^{18},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**THRLAGLLS**[Cit]**SGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	0.58 ± 0.04
8	Ac-Trp-[$Cit^{11,18}$,Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	1.73 ± 0.46
9	Ac-Trp-[$Gln^{11,18}$,Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**Q**LAGLLS**Q**SGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	0.53 ± 0.07
10	Ac-Trp-[$hArg^{11,18}$,Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[hArg]**LAGLLS**[hArg]**SGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	2.1 ± 0.16
11	Ac-Trp-[cycloGlu^9-Lys^{13},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**E**H**R LKGLLSRSGGVV**RK**NFVPT**D**VG**PF**AF-*NH_2*	1.82 ± 0.81
12	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPT**D**VG**PF**AF-*NH_2*	1.27 ± 0.12
13	Ac-Trp-[cycloGlu^9-Lys^{13},cycloGlu^{15}-$Lys^{19}$$Arg^{24}$,$Lys^{25}$,$Asp^{31}$,$Pro^{34}$,$Phe^{35}$] CGRP(8−37)-$NH_2$ ***Ac-***W**V**E**HRL**K**G***E***LSR***K***GGVV**[hArg]K**NFVPT**D**VG**PF**AF-*NH_2*	1.02 ± 0.3
14	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Hyp^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Hyp]F**AF-NH_2	4.98 ± 1.19
15	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Aib^{34},Phe^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Aib]F**AF-*NH_2*	3.54 ± 0.1
16	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Thz^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Thz]F**AF-NH_2	1.97 ± 0.9
17	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Oic^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Oic]F**AF-*NH_2*	2.23 ± 0.77
18	Ac-Trp-[$Cit^{11,18}$,Arg^{24},Lys^{25},Asp^{31},Oic^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**RK**NFVPTDVG**[Oic]F**AF-*NH_2*	0.85 ± 0.04
19	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Sar^{34},Phe^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Sar]F**AF-*NH_2*	3.81 ± 1.89
20	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pip^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Pip]F**AF-*NH_2*	11.21 ± 2.12
21	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},$Tp3i^{34}$,Phe^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[Tpi]F**AF-*NH_2*	29.73 ± 5.73
22	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},βhPro^{34},Phe^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**[βhPro]F**AF-*NH_2*	78.9 + 1.2
23	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},D-Pro^{34},Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**pF**AF-*NH_2*	>1000
24	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},1-Nal^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[1-Nal]**AF-*NH_2*	2.18 ± 1.46
25	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},2-Nal^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[2-Nal]**AF-*NH_2*	0.79 ± 0.11
26	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31}, Pro^{34},Bip^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[Bip]**AF-*NH_2*	1.56 ± 0.56
27	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},Igl^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[Igl]**AF-*NH_2*	4.15 ± 0.28
28	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},Tpi^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[Tpi]**AF-*NH_2*	79.34 ± 25.37
29	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},Pro^{34},Tic^{35}]CGRP(8−37)-NH_2 **Ac-W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFVPTDVG**P[Tic]**AF-*NH_2*	89.91 ± 18.93
30	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},Asp^{31},$Oic^{29,34}$,Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**NFV**[Oic]**TDVG**[Oic]F**AF-*NH_2*	1.31 ± 0.33
31	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},2-$Nal^{27,37}$,Asp^{31},$Oic^{29,34}$,Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**N**[2-Nal]**VPTDVG**[Oic]F**A**[2-Nal]**-*NH_2*	3.45 ± 1.43
32	Ac-Trp-[$Cit^{11,18}$,$hArg^{24}$,Lys^{25},2-$Nal^{27,37}$,Asp^{31},$Oic^{29,34}$,Phe^{35}]CGRP(8−37)-NH_2 ***Ac-***W**V**TH**[Cit]**LAGLLS**[Cit]**SGGVV**[hArg]K**N**[2-Nal]**V**[Oic]**TDVG**[Oic]F**A**[2-Nal]**-*NH_2*	3.57 ± 0.04

[a] Residues in bold are substitutions from the native residues, and underlined and italic-underlined residues denotes *i, i+4* cyclization.

lysine 24 could alter activity. Specifically, it was found that the substitution of lysine^{24}−asparagine^{25} with arginine^{24}−lysine^{25} to give **4** resulted in slightly improved potency. Combination of the modifications in analogues **2**, **3**, and **4** gave Ac-Trp-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2, **5** (IC_{50} = 0.2 nM, K_i = 0.06 nM), a derivative with approximately 25-fold higher affinity than the starting peptide, **1**. The selectivity of **5** for the AM1 and AM2 receptor was largely unchanged with respect to **1**, although the affinity of **5** to the AM receptors was in fact approximately 25-hold higher overall (Table 3).

CGRP Metabolic Map. To fulfill our ultimate objective of identifying a prolonged in vivo circulating CGRP peptide antagonist, a peptide with high metabolic stability was needed.

Table 2. Binding Assay Data of Human CGRP(8−37) Analogues at the $CGRP_1$ on hαCGRP-Induced cAMP Accumulation in Human Neuroblastoma SK-N-MC Cells

compd	$hCGRP_1$ K_i (nM)
1	3.19 ± 2.22
5	0.06 ± 0.03
8	0.46 ± 0.12
12	0.53 ± 0.18
17	0.73 ± 0.11
24	0.64 ± 0.22
25	0.6 ± 0.16
32	3.3 ± 0.31

Table 3. Antagonist Activities of hCGRP(8−37) Analogues at the Adrenomedullin 1 and Adrenomedullin 2 Receptors[a]

compd	hAM1 IC_{50} (nM) (hAM1/hCGRP selectivity)	hAM2 IC_{50} (nM) (hAM1/hCGRP selectivity)
1	233 (48)	266 (55)
5	8.72 (44)	11.8 (59)
8	133 (77)	108 (62)
12	466 (367)	318 (251)
17	613 (275)	496 (223)
24	554 (450)	375 (305)
25	382 (483)	418 (529)
32	2242 (628)	2003 (561)

[a] Fold selectivity over the $CGRP_1$ receptor IC_{50} is shown in parentheses.

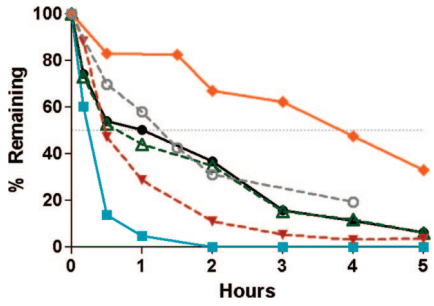


Figure 2. In vitro stability $CGRP_1$ receptor antagonists **5** (closed squares), **8** (closed circles), **9** (open triangles), **10** (closed triangles), **12** (closed diamonds), and **13** (open circles) in 10% human plasma at room temperature. The initial peptide concentrations were 10 μg/mL, and the percentage remaining was determined by quantitative mass analysis of the intact starting peptide.

It had been previously reported that CGRP is a substrate for neutral endopeptidase (EC 3.4.24.11)[38,39] and carboxypeptidases.[40,41] To better understand the metabolic liabilities of our CGRP peptide antagonist, we decided to examine their plasma stability and metabolites. Accordingly, analogues **1** and **5** were incubated in 10% human plasma, and the percentage remaining at $t = 0$, 1, 2, 3, 4, 5, and 6 h was determined by quantitative mass analysis of the intact molecular mass of the starting peptide (data not shown). We found peptides **1** and **5** were rapidly degraded and had an approximate $t_{1/2} = 20$ min under these relatively mild plasma exposure conditions. To better understand the possible metabolic sites within the high-affinity CGRP antagonist **5**, the peptide was first incubated with tryptase (EC 3.4.21.59), an enzyme that according to predicted specificity should cleave CGRP peptides at multiple sites. The enzyme/peptide stoichiometry and time-course were reduced to allow for the observation of first stage peptide digest fragments. Eight major peptide fragments were observed by reversed-phase HPLC analysis (Figure 3). Mass spectrometry peptide mapping was used to identify the major cleavage sites at arginine^{11}−leucine^{12}, arginine^{18}−serine^{19}, lysine^{25}−asparagine^{26}, and arginine^{24}−lysine^{25} (Table 4). Cleavage at the arginine^{18}−serine^{19} site has been reported in rat tissue.[42] Similar degradation products were also observed for the less active CGRP antagonist **1**.

Positions 11 and 18 CGRP Analogues. In view of the poor stability of the CGRP peptide, an amino acid substitution strategy was initiated with the goal of reducing the proteolyic degradation of the CGRP peptide antagonist while maintaining its high-affinity to the $CGRP_1$ receptor and also increasing selectivity over AM1 and AM2. We focused initially on the major N-terminal cleavage sites at arginine residues at positions 11 and 18. It has been previously shown that Arg^{11} plays a critical role for the affinity of CGRP(8−37)-NH_2, whereas Arg^{18} is of less direct significance and is possibly more important for maintenance of the amphipathic nature of CGRP.[16,19,27] The introduction of citrulline, glutamine, and homoarginine residues at these sites was examined for effects on activity and plasma stability. First, we found that the introduction of citrulline residues at either position 11 (**6**, $IC_{50} = 0.57$ nM) or 18 (**7**, $IC_{50} = 0.58$ nM) maintained good antagonist potency. Double citrulline substitution at both residues 11 and 18 (**8**) was also well tolerated giving an IC_{50} of 1.73 nM. Similarly, glutamine and homoarginine residues could also be incorporated at both positions 11 (**9**, $IC_{50} = 0.53$ nM) and 18 (**10**, $IC_{50} = 2.1$ nM) with good antagonist potency. Comparison of the stability of analogues **8**, **9**, and **10** with respect to **5** in 10% human plasma showed that although **8**, **9**, and **10** have improved plasma stability, at the 3 h time point, the majority (∼80%) of each peptide had already been degraded (Figure 2). Although the double homoarginine substituted analogue (**10**) was more stable than **5**, we found the double citrulline (**8**) and glutamine (**9**) substituted CGRP peptides resulted in higher stability. Further derivatives were prepared from compound **8** rather than **10** because of the higher potency of **8** in the rat CGRP antagonist assay, which is relevant to in vivo experimentation. The double citrulline (**8**) and glutamine (**10**) substituted CGRP peptides had IC_{50} values of 8.9 and 20.4 nM at the rat $CGRP_1$ receptor, respectively. In comparison to the potent CGRP analogue **5**, compound **8** had marginally improved selectivity at AM receptors (Table 3).

Position 24 CGRP Analogues. To address the cleavage liability in the central region of the peptide around arginine^{24}−lysine^{25}, we investigated substitution of arginine^{24} in compound **8** with a homoarginine residue to give **12**. The homoarginine substitution at position 24 extended the side chain guanido group by a methylene unit from the peptide backbone. Compound **12** retained good antagonist potency with an IC_{50} of 1.27 nM and, importantly, resulted in very good selectivity over the AM1 (>350-fold) and AM2 (>250-fold) receptors. This improved selectivity over AM1 and AM2 was generally observed with other related analogues also possessing a homoarginine residue at position 24 (Table 3). In comparison to the homoarginine^{24} substitution, the introduction of a citrulline, 4-guanidinophenylalanine, or β-guanidinoalanine residue resulted in lower affinity to the $CGRP_1$ receptor (data not shown). To determine the effect of the homoarginine^{24} substitution in combination with the citrulline^{11,18} substitutions on the plasma stability of $CGRP_1$, antagonist **12** was examined (Figure 2). Under these experimental conditions, the half-life of **12** had improved to around 4 h, approximately 4 times longer than **8**, **9**, or **10** and >10 times longer than **5**. Subsequent tandem mass spectrometry analysis of a sample from the plasma stability study of **12** showed the expected presence of the starting peptide but also interestingly revealed an Ac-Trp-[Cit^{11,18},hArg^{24},Lys^{25}, Asp^{31},Pro^{34}]CGRP(8−34) metabolite. This indicated that hy-

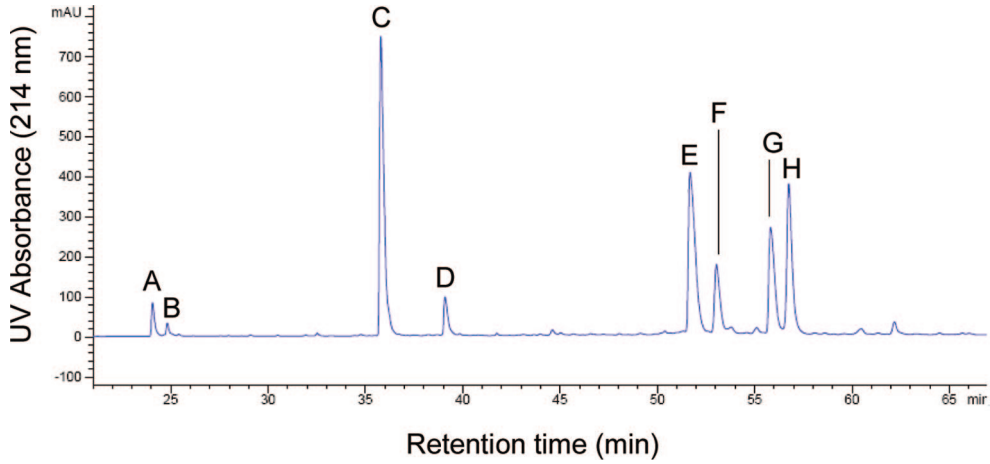


Figure 3. Reversed-phase HPLC trace of the tryptic map of **5**. Peak labels correspond to the peptide fragments shown in Table 4.

Table 4. Observed Masses and Corresponding Peptide Fragments for the Tryptic Digest of **5** Shown in Figure 3

peak	obsd mass (Da)	peptide	residues
A	702.5	SGGVVRK	19−25
B	574.4	SGGVVR	19−24
C	740.4	*Ac*-WVTHR	7−11
D	729.5	LAGLLSR	12−19
E	1994.0	SGGVVRKNFVPTDVGPFAF-NH_2	19−37
F	1437.7	KNFVPTDVGPFAF-NH_2	25−37
G	1451.0	*Ac*-WVTHRLAGLLSR	7−19
H	1309.5	NFVPTDVGPFAF-NH_2	26−37

drolysis was occurring near the C-terminus between proline[34] and phenylalanine[35] during incubation in human plasma.

***i* to *i* + 4 Cyclized CGRP Analogues.** The introduction of an *i* to *i* + 4 side chain to side chain lactam bridge between positions 9−13 and 15−19 was evaluated in conjunction with the homoarginine[24] substitution. To facilitate lactam bond formation in peptides **11** and **13**, residues 9 and 15 were substituted with glutamic acid residues, and residues 13 and 19 were substituted with lysine residues as described above. The side chain to side chain lactam bridge at positions 9−13 spanned the arginine[11] degradation site, whereas the lactam at positions 15−19 spanned the arginine[18] degradation site. We found that the incorporation of a single lactam bridge between residues 9 and 13 (**11**) or two lactam bridges at residues 9−13 and 15−19 (**13**) was very well tolerated and gave potencies of 1.82 and 1.02 nM, respectively. However, the in vitro stability of **13**, which contains two lactam bridges, was actually 2- to 3-fold less than **12** based upon the half-life estimate (Figure 2).

Positions 34 and 35 CGRP Analogues. Additional C-terminal substitutions were made in an effort to further improve stability around the proline[34]−phenylalanine[35] cleavage site. A set of derivatives based upon Ac-Trp-[Cit[11,18],hArg[24],Lys[25],Asp[31],Pro[34],Phe[35]]CGRP(8−37)-amide, **12**, were prepared with additional modifications at residue position 34 or 35. The conformation of the C-terminal region in CGRP peptide antagonists is important for antagonist activity.[26,43] It was recently shown through aza-amino acid and indolizidin-2-one amino acid scanning of the [Asp[31],Pro[34],Phe[35]]-CGRP(27−37)-amide peptide that a type II′ β-turn with Gly[33]-Pro[34] at the *i* + 1 and *i* + 2 positions is a preferred structural element for the antagonist activity at the CGRP receptor.[43] As mentioned above, well tolerated substitutions at position 34 in CGRP antagonist peptides have previously been reported, specifically 4-hydroxyproline (Hyp), [3*S*]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), homoproline, and aminoisobutyric acid (Aib).[26] The introduction of primary amino acids at position 34, such as alanine, has been shown to result in significantly lower affinity to the $CGRP_1$ receptor.[26] Given that proline[34] is situated at the center of a β-turn structural element, we explored the use of secondary, α,α-disubstituted, and cyclical constrained amino acids. In this study, we found that substitution of proline[34] with Hyp (**14**, IC_{50} = 4.98 nM), Aib (**15**, IC_{50} = 3.54 nM), or Sar (**19**, IC_{50} = 3.81 nM) residues resulted in reduced potency. Compared to thiaproline (Thz) and octahydroindole-2-carboxylic acid (Oic), substitutions at position 34 resulted in slightly better retention of antagonist activity, with IC_{50} values of 1.97 nM (**16**) and 2.23 nM (**17**), respectively. The CGRP antagonist **17** also possessed good selectivity (>200-fold) over the AM1 and AM2 receptors with approximately 0.5 μM affinity at these receptors. Other substitutions at position 34, 1,2,3,4-tetrahydronorharman-3-carboxylic acid (Tpi, **21**) and β-homoproline (βhPro, **22**), resulted in an approximate 30- to 80-fold loss of potency. Similarly, incorporation of 4-aminopiperidine-4-carboxylic acid (Pip, **20**) at position 34 resulted in lowered potency, and D-proline (**23** $IC_{50} \geq$ 1000 nM) resulted in no detectable antagonist activity to the $CGRP_1$ receptor.

In an analogous manner, the phenylalanine residue at position 35 in **12** was substituted with several arylamino acids, β-(1-naphthyl)alanine (1-Nal, **24**, IC_{50} = 2.18 nM), β-(2-naphthyl)alanine (2-Nal, **25**, IC_{50} = 0.79 nM), and β-(4-biphenylyl)-alanine (Bip, **26**, IC_{50} = 1.56 nM), that were all well tolerated. In comparison to the above CGRP analogues, **24** and **25** had the most selectivity over the AM1 and AM2 receptors (Table 3). 2-Indanylglycine (Igl, **27**) could also be introduced at position 35 with moderate effect; however, incorporation of Tpi (**28**) or Tic (**29**) at this position led to a >50-fold reduction in potency.

Metabolically Stable CGRP Antagonists. The three most potent CGRP analogues with these additional C-terminal substitutions at position 34 or 35 were **17**, **24**, and **25**. The stability of these molecules in 10% human plasma was examined. However, we found that the plasma stability of **24** and **25** showed no significant improvement over analogues **9**, **10**, and **13**, whereas peptide **17** was comparable to **12** based upon half-life estimates (data not shown). To complement data on in vitro degradation sites, we proceeded to examine in vivo metabolism in cynomolgus monkeys with the objective of obtaining more insight into the mechanism of CGRP degradation. Accordingly, compound **17** was infused intravenously in cynomolgus monkeys and blood plasma was collected after 30 min and analyzed. Interestingly, in addition to the human in vitro metabolic cleavage sites described above, we also observed

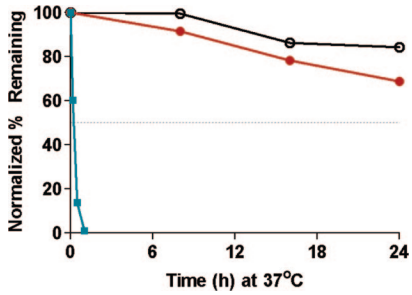


Figure 4. In vitro stability $CGRP_1$ receptor antagonists in 100% plasma at 37 °C: peptide **5** (closed squares) in 100% human plasma; peptide **32** in 100% human plasma (open circles) and also cynomolgus monkey plasma (closed circles). Percentage remaining was determined by sandwich ELISA designed to detect full-length peptides.

cynomolgus monkey in vivo metabolites derived from additional cleavage sites at asparagine^{26}−phenylalanine^{27}, proline^{29}−threonine^{30}, and alanine^{36}−phenylalanine^{37}. The occurrence of these additional C-terminal cleavage fragments was not apparent from in vitro stability experiments with either human or cynomolgus monkey plasma. With the in vivo cynomolgus monkey metabolic information in hand, additional analogues with substitutions at residues 27, 29, and 37 were prepared and examined. Using the structure−activity data at positions 34 and 35, several analogues were prepared containing the substitution of proline^{29} with an Oic, and phenylalanine^{27} and phenylalanine^{37} with 2-Nal. Starting from **17**, it was found that proline^{29} could be substituted with Oic with good retention of potency, **30** (IC_{50} = 1.31 nM). Similarly, both phenylalanine residues 27 and 37 could also be 2-Nal substituted with retention of potency at the $CGRP_1$ receptor, **31** (IC_{50} = 3.45 nM). Combining these modifications at residues 27, 29, and 37 gave **32**, Ac-Trp-[Cit^{11,18},hArg^{24},Lys^{25},2-Nal^{27,37},Asp^{31},Oic^{29,34},Phe^{35}]-CGRP(8−37)-NH_2, a CGRP antagonist peptide **32** with an IC_{50} of 3.57 nM at the human $CGRP_1$ receptor. In the SK-N-MC cell assay, **32** was found to have antagonist activity at the $CGRP_1$ receptor comparable to that of the starting peptide CGRP(8−37)-NH_2, **1**, whereas it was approximately 20-fold less active than the potent, but metabolically unstable, peptide antagonist **5**. Nevertheless, **32** remains a low single digit nanomolar $CGRP_1$ receptor antagonist (K_i = 3.3 nM), and since the primary goal of these multiple C-terminal modifications was to significantly improve stability, it was tested in plasma. Accordingly, we found **32** was very stable and no apparent degradation was observed during the 5 h treatment with 10% human plasma that was used to assess the other peptides reported here. In view of the high stability of **32**, the plasma stability test protocol was changed from the initial relatively mild conditions (10% human plasma at room temperature) to more physiologically relevant conditions, 100% human or cynomolgus monkey plasma at 37 °C for an extended period of time (Figure 4). The percentage of **32** remaining at t = 0, 8, 16, and 24 h was determined by quantitative analysis in a manner analogous to that of the previous plasma stability studies. In this extended human plasma stability experiment, less than 10% degradation of **32** was observed at the 8 h time point and only 30% degradation at the 24 h time point. The estimated $t_{1/2}$ of **32** in 100% human and cynomolgus monkey plasma was 68 and 38 h, respectively. The half-life of **32** is significantly longer (>100-fold) than that of Ac-Trp-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2, **5**, and its selectivity over the AM1 (∼600-fold) and AM2 (∼500-fold) receptors was also significantly improved over the approximately 50-fold selectivity with **1** and **5** (Table 3). Moreover, the CGRP antagonist **32** has >2 μM affinity to AM1 and AM2 compared to approximately 10 nM affinity for **5**. In general, the improved selectivity of **32** and other analogues over the AM1 and AM2 receptor is in part attributed to the introduction of the homoarginine residue at position 24 as well as increased peptide hydrophobicity.

Conclusion

We have found that the substitution of multiple residues within the CGRP(8−37)-NH_2 peptide can be made to increase potency to the $CGRP_1$ receptor by approximately 25-fold. The highest $CGRP_1$ receptor potency was exhibited by Ac-Trp-[Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]CGRP(8−37)-NH_2, **5**, with an IC_{50} value of 0.2 nM compared to 4.87 nM for the starting peptide CGRP(8−37)-amide, **1**. Using a combination of in vitro and in vivo metabolic studies, we were successfully able to identify degradation sites in the CGRP(8−37)-NH_2 peptide antagonists and identify analogues with significantly improved plasma stability (75- to 100-fold) with retained high affinity and potency. Ac-Trp-[Cit^{11,18},hArg^{24},Lys^{25},2-Nal^{27,37},Asp^{31},Oic^{29,34},Phe^{35}]CGRP(8−37)-NH_2, **32**, had the greatest stability in cynomolgus monkey and human plasma of >100-fold over **1** or **5**. These metabolically stable and high affinity $CGRP_1$ receptor antagonists also display >300-fold selectivity over AM1 and AM2 and are useful leads for the identification of a prolonged in vivo circulating peptide antagonist to the CGRP receptor.

Experimental Section

Materials. N^{α}-Fmoc protected amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin were purchased from Novabiochem (San Diego, CA) and Bachem (Torrance, CA). Fmoc-Lys(Mtt)-OH, Fmoc-Dpr(Mtt)-OH, Fmoc-Dab(Mtt)-OH, Fmoc-Glu(2-Ph^{i}Pr)-OH, and Fmoc-Cit-OH were purchased from EMD Biosciences, Inc. (La Jolla, CA). Fmoc-homoArg(Pmc)-OH was purchased from Bachem California, Inc. (Torrance, CA). Fmoc-Oic-OH, Fmoc-homoCit-OH, Fmoc-Pip-OH, Fmoc-Hyp-OH, Fmoc-Aic-OH, Fmoc-Aib-OH, Fmoc-Thz-OH, Fmoc-Sar-OH, Fmoc-Tpi-OH, Fmoc-1-Nal-OH, Fmoc-2-Nal-OH, Fmoc-Bip-OH, Fmoc-Igl-OH, Fmoc-βhomoPro-OH, Fmoc-D-Pro-OH, Fmoc-Tic-OH, and Fmoc-Tpi-OH were purchased from either Advanced Chemtech (Louisville, KY) or Chem-Impex International, Inc. (Wood Dale, IL). *N,N*-Diisopropylethylamine (DIEA), piperidine, acetic anhydride, phenol, triisopropylsilane, HPLC-quality water, and HPLC-quality acetonitrile were purchased from Aldrich (Milwaukee, WI). Dichloromethane (DCM) was from Mallinckrodt Baker, Inc. *N,N*-dimethylformamide (DMF) was purchased from Burdick and Jackson. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was supplied by Matrix Innovation (Montreal, CA), and protease-free bovine serum albumin (BSA), sucrose, sorbitol, sodium azide, trifluoroacetic acid (TFA), and Tris-HCl were from Sigma, St. Louis, MO.

Peptide Synthesis. Peptides **1**, **2**, **13**−**23**, and **26**−**29** were synthesized using Fmoc solid-phase peptide synthesis (SPPS) methodologies on a Symphony automated synthesizer (Protein Technologies, Inc., Washington, DC). Peptides **25** and **30** were synthesized on a Liberty microwave assisted automated synthesizer (CEM Corporation, Matthews, NC). Peptides **3**, **4**, and **24** were synthesized on an ABI 433 automated synthesizer (Applied Biosystems, Foster City, CA). Peptide **11** was synthesized on a CSBio 336 automated synthesizer (CS Bio Company, Inc., Menlo Park, CA). Peptides were assembled on CLEAR-amide-MBHA resin (0.44 mequiv/g substitution), purchased from Peptides International (Louisville, KY). CGRP peptide derivatives were synthesized in a stepwise manner by SPPS using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/*N,N*-diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF) coupling chemistry at 0.2 mmol equiv resin scale. The following side chain protection strategies

were employed for standard amino acid residues: Asp(O^{t}Bu), Arg(Pbf), Cys(Acm), Glu(O^{t}Bu), His(Trt), Lys(N^ϵ-Boc), Ser(O^{t}Bu), Thr(O^{t}Bu), and Tyr(O^{t}Bu). Lys(N^ϵ-Mtt) and Glu(O2-Ph^{i}Pr) were used at cyclization sites. Fmoc deprotections were carried out with two treatments using a 20% piperidine in DMF solution first for 5 min and then for an additional 20 min. For each coupling cycle, 1 mmol N^α-Fmoc-amino acid, 2 mmol DIEA, and 1 mmol equiv of HBTU were used. The concentration of the HBTU-activated Fmoc amino acids was 0.2 M in DMF, and the coupling time was 45 min. The first four residues were coupled with a single 45 min cycle. All other residues were coupled with two succeeding cycles of 45 min each. Residues Val^8, Ser^{17}, Ser^{19}, Val^{22}, Val^{23}, Asn^{26}, Val^{28}, and Val^{32} were double coupled for 2 h per cycle.

Side Chain Deprotection and Cleavage from Resin. Following synthesis, crude peptide−resin was washed with DCM and dried under nitrogen prior to cleavage. The peptide−resin (0.2 mmol) was deprotected and released from the resin by treatment with a 20 mL solution of trifluoroacetic acid (TFA)/phenol/triisopropylsilane (TIS)/H_2O (92.5:2.5:2.5:2.5 v/v) for 3 h at room temperature. The resin was removed by filtration and washed with TFA twice. The combined filtrates were concentrated under reduced pressure, followed by peptide precipitation with cold anhydrous diethyl ether (40 mL). The peptide was collected by centrifugation to remove the ether and washed with another 40 mL of diethyl ether. The peptide was centrifuged and dried in vacuo.

General Procedure for the Cyclization via Lactam Formation. Side chain to side chain lactam formation was carried out on the assembled N-terminally Fmoc-protected resin-bound peptide. At cyclization sites, side chain amino and carboxyl functionalities were protected with 4-methyltrityl (Mtt) groups and 2-phenylisopropyl ester (2-Ph^{i}Pr) groups, respectively. In a typical example of cyclic lactam formation on the resin, following assembly of the fully protected peptide on the solid support, the resin was washed with DCM (3 × 2 min). The Mtt and 2-Ph^{i}Pr groups (protecting group at the specified lactam bond forming site) were removed by repeated treatment with 3% TFA in DCM solution containing 5% TIS. Treatment of the peptide−resin with the TFA in DCM solution was repeated 8 times in 30 min increments, and each treatment was followed by extensive DCM washes. The resin was then washed with 2% DIEA solution (2 × 2 min) and DCM (4 × 1 min). The liberated carboxyl and amino groups were then condensed by the addition of 7 equiv of 0.5 M benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and 10 equiv of DIEA in DMF to the peptide resin and left for 24 h. This process was repeated until a negative Kaiser ninhydrin test was attained. The resin was then wash thoroughly with DMF, DCM, and DCM/MeOH and dried.

Reversed-Phase HPLC Analysis, Purification, and Mass Spectrometry. Chromatographic and mass analysis of CGRP products and HPLC fractions were performed on a Waters Acquity UPLC-LCT Premier system Z-spray ionization coupled time-of-flight (TOF) mass spectrometer. Samples (typically 2 μL) were chromatographed on an Waters Acquity BEH column (C18, 2.1 mm × 50 mm, 1.7 μm) using a 10−55% gradient of buffer B versus buffer A in 10 min (buffer A, 0.01% TFA in water; buffer B, 0.01% TFA in acetonitrile). Molecular masses were derived from the observed *m*/*z* values from the LCT Premier TOF mass spectrometer. Preparative reversed-phase high-performance liquid chromatography was performed on Agilent 1100 preparative chromatography system with a photodiode array detector (Agilent Technologies, Inc., Santa Clara, CA) using a 0.25% per min linear gradient over 60 min on a Phenomenex Jupiter C_{18}, 300 Å, 10 μM, 50 mm × 250 mm column at a flow rate of 50 mL/min with UV monitoring at 214 and 230 nm.

CGRP Peptide Stability and Metabolic Identification by LC−MS/MS. CGRP peptide was incubated at room temperature in 10% plasma/water at an initial concentration of 10 μg/mL. Samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, and 5 h time points during the incubation. At each time point, a 100 μL sample (n = 2) was removed from the incubation solution, followed immediately by solid phase extraction (SPE) to quench the incubation and prepare the sample for LC−MS/MS analysis. Trypsin digestions were performed with 0.4 mg/mL peptide in 100 mM Tris, pH 8.5, with a 1:10000 enzyme/substrate ratio at 37 °C. Analytical reversed-phase high-performance liquid chromatography was performed on a Jupiter column (C18, 2.1 mm × 250 mm) using 0.1% TFA as buffer A and 90% acetonitrile/0.1% TFA as buffer B at 50 °C. The HPLC gradient was 0% buffer B to 24% buffer B at 1%/min, followed by 24% buffer B to 45% buffer B at 0.5%/min.

In vitro CGRP peptide stability in plasma was evaluated by a quantitative LC−MS/MS analysis of the intact peptide. An amount of 20 μL of the SPE extract was injected onto a Varian Polaris C18A column (5 μm, 50 mm × 2.1 mm) and separated using a 5−95% B linear gradient elution in 5 min at a flow rate of 400 μL/min. The mobile phases were 0.1% formic acid in 5/95 (v/v) methanol/water (mobile phase A) and 0.1% formic acid in 95/5 (v/v) methanol/water (mobile phase B). MS/MS detection was carried out on an API-4000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, CA) with an electrospray ionization source operated in the positive ion mode. Ion transitions of MS/MS detection for peptide quantitation were optimized for each peptide. The average peak areas of CGRP peptide at each time point were normalized to the average peak area of the peptide at 0 h time point and used to construct the concentration vs time stability profile.

Identification of the degradation pathway of CGRP peptides after incubation in plasma was carried out by a qualitative LC−MS/MS analysis. Chromatographic separation of the SPE extract (20 μL) was performed on a Varian Polaris C18A column (5 μm, 100 mm × 2.1 mm) using a 5−95% B linear gradient elution in 40 min at a flow rate of 300 μL/min. The mobile phases were 0.1% formic acid in 5/95 (v/v) methanol/water (mobile phase A) and 0.1% formic acid in 95/5 (v/v) methanol/water (mobile phase B). MS/MS detection was carried out on a LCQ Deca XP mass spectrometer from Thermo Scientific (San Jose, CA) operating in the data dependent MS/MS scan mode. Peptides relevant to the intact CGRP peptide were identified, and their sequences were constructed from the MS/MS spectra.

CGRP Peptide ELISA. A sandwich ELISA system was used with antibodies specific for each of the respective termini of the peptide and accurately quantifies functional CGRP peptide. Briefly, microplate wells were coated with the capture reagent anti-C-CGRP polyclonal antibody (pAb, 0.050 μg/well incubation overnight at 4 °C) raised against the C-terminal domain of a modified CGRP peptide α-CGGGGGGGPTDVGPFAF-NH_2. The wells were washed and blocked with I-Block buffer (Tropix, Bedford, MA). Samples were pretreated in I-Block buffer 1:10 to achieve 5% or 10% final (rodent or human) serum concentration; sample dilutions were carried out in I-Block, 5% or 10% matching serum. Diluted samples (50 μL/well) were added to anti-C-CGRP coated wells and were incubated at room temperature for 2 h with shaking, after which the wells were washed with I-Block buffer (4 °C) to remove unbound CGRP peptide. Horseradish peroxidase (HRP) labeled α-N-CGRP pAb conjugate, specific for the N-terminal domain of CGRP α-CGGGGGGVTHRLAGLLSRSGGVVKNNFVPTD-VGPFAF-NH_2, was diluted in I-Block buffer and added to the wells and incubated for 1 h (at room temperature with shaking) for detection of captured CGRP peptide. After another washing step, Pico (Bio FX Laboratories) substrate was added to the wells, creating a luminescent signal proportional to the amount of CGRP peptide bound by the capture reagent in the initial step. The intensity of luminescence was measured with a luminometer. Standard (STD) and quality control (QC) samples were made by spiking modified CGRP peptide α-CGGGGGGGPTDVGPFAF-NH_2 into 100% matching serum, were loaded into wells after pretreatment and in I-Block buffer 1:10 and further dilutions in I-Block buffer, and were otherwise assayed in accordance with the ELISA method described above.

CGRP$_1$R Antagonist Activity. CGRP peptide analogues were screened in an in vitro CGRP1 receptor mediated cAMP assay to determine intrinsic potency. The in vitro cAMP assay employed a human neuroblastoma-derived cell line (SK-N-MC). SK-N-MC cells

express CRLR and RAMP1, which form functional CGRP1 receptor. Briefly, an amount of 100 μL of SK-N-MC cells was plated in 96-well plates in growth medium (MEM, 10% FBS, 1× glutamine, 1× nonessential amino acid, 1× sodium pyruvate) at 50000/well. The cell plates were incubated at 37 °C in a CO_2 incubator for 2 days. On the day of the assay, cells were rinsed once with PBS followed by the addition of 60 μL of serum free medium for 15 min at room temperature to starve the cells. The test CGRP peptide antagonists were then added at varying concentrations in 20 μL of buffer followed by 5 min of incubation at 37 °C. Subsequently 0.1 nM CGRP agonist, human αCGRP, was added in 20 μL of buffer to each well followed by 5 min of incubation at 37 °C. The medium was then removed, and the cells were lysed with 100 μL of lysis buffer for 30 min at 37 °C. Subsequently 60 μL of the cell lysis from the cell plate was added to the ELISA plate followed by the addition of 30 μL of cAMP-AP conjugate and 60 μL of anti-cAMP antibody provided by a Tropix ELISA kit (Applied Biosystems, Foster City, CA). This mixture was further incubated for 1 h at room temperature with gentle shaking. The ELISA plate was then washed 6× with wash buffer followed by the additions of 100 μL/well of CSPD/sapphire-II RTU substrate/enhancer solution. This mixture was incubated for 30 min at room temperature, and the luminescence signal was read on a MicroBeta Jet or EnVision plate reader.

$CGRP_1$ Ligand Binding Assay. $CGRP_1$ binding assays were set up in 96-well plates at room temperature containing 110 μL of binding buffer (20 mM Tris-HCl, pH7.5, 5.0 mM $MgSO_4$, 0.2% BSA [Sigma], 1 tablet of Complete/50 mL buffer [protease inhibitor]); 20 μL of test compound (10×); 20 μL of ^{125}I-hαCGRP (Amersham Biosciences) (10×); and 50 μL of human neuroblastoma cell (SK-N-MC) membrane suspension (10 μg per well, PerkinElmer). The plates were incubated at room temperature for 2 h with shaking at 60 rpm, then the contents of each well were filtered over 0.5% polyethyleneimine (PEI)-treated (at least one hour) GF/C 96-well filter plates. The GF/C filter plates were washed 6 times with ice-cold 50 mM Tris, pH 7.5, and dried in an oven at 55 °C for 1 h. The bottoms of the GF/C plates were then sealed, and 40 μL Microscint 20 was added to each well. Then the tops of the GF/C plates were sealed with TopSealTM-A, a press-on adhesive sealing film, and the GF/C plates were counted with TopCount NXT (Packard). The K_i values were calculated using the formula $K_i = IC_{50}/(1 + \text{ligand}/K_d)$ with the GraphPad Prism program (GraphPad Software, Inc.).

Adrenomedullin Antagonist Activity. The selectivity of the CGRP peptide analogues on adrenomedullin (AM) receptors was studied using the similar *in vitro* cAMP assay described above. The recombinant AM1 stable cell line was generated in house by cotransfecting CRLR and RAMP2 into HEK-293 cells and AM2 stable cell line was purchased from PerkinElmer by cotransfecting CRLR and RAMP3 into CHO cells. The data were analyzed using the same program.

Cynomolgus Monkey Husbandry. Male Cynomolgus monkeys (*Macaca fasciculari*) weighing 3.2−3.6 kg were used for the pharmacokinetic studies. All animals were housed at Valley Biosystems, Inc. (West Sacramento, CA) according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals in individual cages in unidirectional airflow rooms with controlled temperature (22 ± 2 °C) and relative humidity (30−70%) and 12 h light/dark cycles. Filtered tap water was available ad libitum. Animals were fed a standard animal diet (Harlan Madison, WI). Whenever overnight fasting was employed, food was provided after the 240 min blood sample was obtained on the study day. All animal use was conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee prior to the study. Clinical chemistries and hematology panel were performed before each study; studies were not conducted unless blood parameters were within normal values.

On the study day, each monkey had a catheter temporarily placed in a saphenous vein for drug administration and peripheral samples were taken by direct venous puncture. On each study day, the monkeys were placed in restraining chairs for the first 4 h of the study to facilitate drug administration and blood sampling; monkeys were fasted overnight prior to each study day. On the study day, all monkeys received compound (3 mg/kg) as a 60 min intravenous infusion (6 mL/kg final volume; plasma (100 μL) was isolated by centrifugation and stored at −70 °C until analysis).

Acknowledgment. We gratefully thank Ankita Patel, Jingwen Zhang, Jason Long, Stephanie Diamond, and Linda Huang for their assistance with peptide synthesis and analysis. We also thank Charlotte Toftegaard for editing and proofing the final manuscript.

Supporting Information Available: In vitro functional dose-response assay of human CGRP in human SK-N-MC cell line; in vitro functional dose-response assay of CGRP analogues; list of all compounds with HPLC k' and calculated and experimentally observed molecular weights; and RP-HPLC data for compounds **1**, **5**, **8**, **12**, **17**, **24**, **25**, and **32**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Brain, S. D.; Grant, A. D. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol. Rev.* **2004**, *84*, 903–934.
(2) Brain, S. D.; Cox, H. M. Neuropeptides and their receptors: innovative science providing novel therapeutic targets. *Br. J. Pharmacol.* **2006**, *147*, S202–S211.
(3) Conner, A. C.; Simms, J.; Barwell, J.; Wheatley, M.; Poyner, D. R. Ligand binding and activation of the CGRP receptor. *Biochem. Soc. Trans.* **2007**, *35*, 729–732.
(4) Brain, S. D.; Cambridge, H. Calcitonin gene-related peptide: vasoactive effects and potential therapeutic role. *Gen. Pharmacol.* **1996**, *27*, 607–611.
(5) Doods, H. Development of CGRP antagonists for the treatment of migraine. *Curr. Opin. Invest. Drugs (PharmaPress Ltd.)* **2001**, *2*, 1261–1268.
(6) Goadsby, P. J. Calcitonin gene-related peptide antagonists as treatments of migraine and other primary headaches. *Drugs* **2005**, *65*, 2557–2567.
(7) Fischer, M. J. M.; Koulchitsky, S.; Messlinger, K. The nonpeptide calcitonin gene-related peptide receptor antagonist BIBN4096BS lowers the activity of neurons with meningeal input in the rat spinal trigeminal nucleus. *J. Neurosci.* **2005**, *25*, 5877–5883.
(8) Edvinsson, L.; Petersen, K. A. CGRP-receptor antagonism in migraine treatment. *CNS Neurol. Disord.: Drug Targets* **2007**, *6*, 240–246.
(9) Paone, D. V.; Shaw, A. W.; Nguyen, D. N.; Burgey, C. S.; Deng, J. Z.; Kane, S. A.; Koblan, K. S.; Salvatore, C. A.; Mosser, S. D.; Johnston, V. K.; Wong, B. K.; Miller-Stein, C. M.; Hershey, J. C.; Graham, S. L.; Vacca, J. P.; Williams, T. M. Potent, orally bioavailable calcitonin gene-related peptide receptor antagonists for the treatment of migraine: discovery of *N*-[(3*R*,6*S*)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)piperidine-1-carboxamide (MK-0974). *J. Med. Chem.* **2007**, *50*, 5564–5567.
(10) Troconiz, I. F.; Wolters, J.-M.; Tillmann, C.; Schaefer, H. G.; Roth, W. Modeling the anti-migraine effects of BIBN 4096 BS: a new calcitonin gene-related peptide receptor antagonist. *Clin. Pharmacokinet.* **2006**, *45*, 715–728.
(11) Morris, H. R.; Panico, M.; Etienne, T.; Tippins, J.; Girgis, S. I.; MacIntyre, I. Isolation and characterization of human calcitonin gene-related peptide. *Nature* **1984**, *308*, 746–748.
(12) Wimalawansa, S. J. Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. *Crit. Rev. Neurobiol.* **1997**, *11*, 167–239.
(13) Hay, D. L.; Conner, A. C.; Poyner, D. R. Calcitonin gene-related peptide and adrenomedullin receptors. *Encycl. Biol. Chem.* **2004**, *1*, 212–216.
(14) Entzeroth, M.; Doods, H. N.; Wieland, H. A.; Wienen, W. Adrenomedullin mediates vasodilation via CGRP1 receptors. *Life Sci.* **1995**, *56*, PL19–PL25.
(15) Hay, D. L.; Conner, A. C.; Howitt, S. G.; Smith, D. M.; Poyner, D. R. The pharmacology of adrenomedullin receptors and their relationship to CGRP receptors. *J. Mol. Neurosci.* **2004**, *22*, 105–113.
(16) Howitt, S. G.; Poyner, D. R. The selectivity and structural determinants of peptide antagonists at the CGRP receptor of rat, L6 myocytes. *Br. J. Pharmacol.* **1997**, *121*, 1000–1004.
(17) Breeze, A. L.; Harvey, T. S.; Bazzo, R.; Campbell, I. D. Solution structure of human calcitonin gene-related peptide by ^{1}H NMR and distance geometry with restrained molecular dynamics. *Biochemistry* **1991**, *30*, 575–582.

(18) Saha, S.; Waugh, D. J. J.; Zhao, P.; Abel, P. W.; Smith, D. D. Role of conformational constraints of position 7 of the disulfide bridge of h-alpha-CGRP derivatives in their agonist versus antagonist properties. *J. Pept. Res.* **1998**, *52*, 112–120.

(19) Howitt, S. G.; Kilk, K.; Wang, Y.; Smith, D. M.; Langel, U.; Poyner, D. R. The role of the 8−18 helix of CGRP8−37 in mediating high affinity binding to CGRP receptors; coulombic and steric interactions. *Br. J. Pharmacol.* **2003**, *138*, 325–332.

(20) Conner, A. C.; Hay, D. L.; Howitt, S. G.; Kilk, K.; Langel, U.; Wheatley, M.; Smith, D. M.; Poyner, D. R. Interaction of calcitonin-gene-related peptide with its receptors. *Biochem. Soc. Trans.* **2002**, *30*, 451–455.

(21) Chiba, T.; Yamaguchi, A.; Yamatani, T.; Nakamura, A.; Morishita, T.; Inui, T.; Fukase, M.; Noda, T.; Fujita, T. Calcitonin gene-related peptide receptor antagonist human CGRP-(8−37). *Am. J. Physiol.* **1989**, *256*, E331–335.

(22) Dennis, T.; Fournier, A.; St. Pierre, S.; Quirion, R. Structure−activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 718–725.

(23) Mimeault, M.; Fournier, A.; Dumont, Y.; St-Pierre, S.; Quirion, R. Comparative affinities and antagonistic potencies of various human calcitonin gene-related peptide fragments on calcitonin gene-related peptide receptors in brain and periphery. *J. Pharmacol. Exp. Ther.* **1991**, *258*, 1084–1090.

(24) Rist, B.; Lacroix, J. S.; Entzeroth, M.; Doods, H. N.; Beck-Sickinger, A. G. CGRP 27−37 analogs with high affinity to the CGRP1 receptor show antagonistic properties in a rat blood flow assay. *Regul. Pept.* **1999**, *79*, 153–158.

(25) Rist, B.; Entzeroth, M.; Beck-Sickinger, A. G. From micromolar to nanomolar affinity: a systematic approach to identify the binding site of CGRP at the human calcitonin gene-related peptide 1 receptor. *J. Med. Chem.* **1998**, *41*, 117–123.

(26) Lang, M.; De Pol, S.; Baldauf, C.; Hofmann, H.-J.; Reiser, O.; Beck-Sickinger, A. G. Identification of the key residue of calcitonin gene related peptide (CGRP) 27−37 to obtain antagonists with picomolar affinity at the CGRP receptor. *J. Med. Chem.* **2006**, *49*, 616–624.

(27) Taylor, C. K.; Smith, D. D.; Hulce, M.; Abel, P. W. Pharmacological characterization of novel alpha-calcitonin gene-related peptide (CGRP) receptor peptide antagonists that are selective for human CGRP receptors. *J. Pharmacol. Exp. Ther.* **2006**, *319*, 749–757.

(28) Struthers, A. D.; Brown, M. J.; Macdonald, D. W.; Beacham, J. L.; Stevenson, J. C.; Morris, H. R.; MacIntyre, I. Human calcitonin gene related peptide: a potent endogenous vasodilator in man. *Clin. Sci.* **1986**, *70*, 389–393.

(29) Kraenzlin, M. E.; Ch'ng, J. L.; Mulderry, P. K.; Ghatei, M. A.; Bloom, S. R. Infusion of a novel peptide, calcitonin gene-related peptide (CGRP) in man. Pharmacokinetics and effects on gastric acid secretion and on gastrointestinal hormones. *Regul. Pept.* **1985**, *10*, 189–197.

(30) Rink, H. Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methyl ester resin. *Tetrahedron Lett.* **1987**, *28*, 3787–3790.

(31) Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: Oxford, U.K., 1989; 203 pp.

(32) Wellings, D. A.; Atherton, E. Standard Fmoc protocols. *Methods Enzymol.* **1997**, *289*, 44–67.

(33) Aletras, A.; Barlos, K.; Gatos, D.; Koutsogianni, S.; Mamos, P. Preparation of the very acid-sensitive Fmoc-Lys(Mtt)-OH. Application in the synthesis of side-chain to side-chain cyclic peptides and oligolysine cores suitable for the solid-phase assembly of MAPs and TASPs. *Int. J. Pept. Protein Res.* **1995**, *45*, 488–496.

(34) Yue, C.; Thierry, J.; Potier, P. 2-Phenylisopropyl esters as carboxyl terminus protecting groups in the fast synthesis of peptide fragments. *Tetrahedron Lett.* **1993**, *34*, 323–326.

(35) Coste, J.; Le-Nguyen, D.; Castro, B. PyBOP: a new peptide coupling reagent devoid of toxic byproduct. *Tetrahedron Lett.* **1990**, *31*, 205–208.

(36) Vine, W.; Beaumont, K.; Gedulin, B.; Pittner, R.; Moore, C. X.; Rink, T. J.; Young, A. A. Comparison of the in vitro and in vivo pharmacology of adrenomedullin, calcitonin gene-related peptide and amylin in rats. *Eur. J. Pharmacol.* **1996**, *314*, 115–121.

(37) Van Valen, F.; Piechot, G.; Jurgens, H. Calcitonin gene-related peptide (CGRP) receptors are linked to cyclic adenosine monophosphate production in SK-N-MC human neuroblastoma cells. *Neurosci. Lett.* **1990**, *119*, 195–198.

(38) Davies, D.; Medeiros, M. S.; Keen, J.; Turner, A. J.; Haynes, L. W. Endopeptidase-24.11 cleaves a chemotactic factor from alpha-calcitonin gene-related peptide. *Biochem. Pharmacol.* **1992**, *43*, 1753–1756.

(39) Mentlein, R.; Roos, T. Proteases involved in the metabolism of angiotensin II, bradykinin, calcitonin gene-related peptide (CGRP), and neuropeptide Y by vascular smooth muscle cells. *Peptides (Tarrytown, N. Y.)* **1996**, *17*, 709–720.

(40) Lundy, F. T.; Salmon, A. L.; Lamey, P.-J.; Shaw, C.; Linden, G. J. Carboxypeptidase-mediated metabolism of calcitonin generelated peptide in human gingival crevicular fluid—a role in periodontal inflammation. *J. Clin. Periodontol.* **2000**, *27*, 499–505.

(41) Lundy, F. T.; Shaw, C.; McKinnell, J.; Lamey, P. J.; Linden, G. J. Calcitonin gene-related peptide in gingival crevicular fluid in periodontal health and disease. *J. Clin. Periodontol.* **1999**, *26*, 212–216.

(42) Mulderry, P. K.; Ghatei, M. A.; Bloom, S. P. In vitro production and characterization of low molecular weight forms of calcitonin gene-related peptide immunoreactivity from rat thyroid. *Biochem. Biophys. Res. Commun.* **1987**, *144*, 883–890.

(43) Boeglin, D.; Hamdan, F. F.; Melendez, R. E.; Cluzeau, J.; Laperriere, A.; Heroux, M.; Bouvier, M.; Lubell, W. D. Calcitonin gene-related peptide analogues with aza and indolizidinone amino acid residues reveal conformational requirements for antagonist activity at the human calcitonin gene-related peptide 1 receptor. *J. Med. Chem.* **2007**, *50*, 1401–1408.

JM8009298