Synthesis, Characterization, and Conformational Analysis of the D/L-Tic^7 Stereoisomers of the Bradykinin Receptor Antagonist D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin

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ABSTRACT: D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin (HOE-140) is a potent (K_i = 0.11 nM) inhibitor of [^{3}H]bradykinin binding to bradykinin B_2 receptors found on human IMR-90 fetal lung fibroblasts. During the synthesis of this compound, we isolated and unambiguously identified the L-Tic^7 stereoisomer (WIN 65365), which exhibits a 2000-fold lower binding affinity (K_i = 130 nM) than HOE-140 to the bradykinin receptor. A similar decrease in potency is observed for WIN 65365 inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux from IMR-90 cells. Both HOE-140 and WIN 65365 appear to be competitive antagonists at the IMR-90 bradykinin receptor. This is the first documentation of bradykinin binding and functional antagonist activity by a bradykinin peptide analogue with an L amino acid replacing Pro^7. In an attempt to rationalize the differences in binding affinities of HOE-140 and WIN 65365, a conformational analysis of the peptides was undertaken using annealed molecular dynamics (AMD). Conformational analysis of HOE-140 reveals a strong preference for the formation of a type II′ β-turn in the carboxy-terminal region. Analogous modeling of WIN 65365 reveals that its conformation is strikingly different from HOE-140 in that the four carboxy-terminal residues of WIN 65365 do not form a β-turn. These differences in low-energy conformations between the two peptides may lead to a better understanding of the molecular interaction of antagonists with the bradykinin receptor.

In general, peptide hormones generate their physiological effects in target cells via unique cell surface receptors. The formation of the hormone–receptor complex is followed by the transduction of information into the target cell by one of many secondary signals/messengers leading to a physiological response (Colquhoun, 1979; Gilman, 1987). Understanding the molecular interactions between a peptide hormone and its receptor in terms of the molecular conformations needed to promote high-affinity binding is key to the design of potent receptor antagonists. An approach to this problem is the synthesis and biological characterization of synthetic peptide analogues which have constrained conformations (Pelton et al., 1986). Analysis of the conformational and dynamic features of such peptide ligands should provide valuable insights into the mechanisms of interaction between hormone and receptor at the molecular level (Kazmierski et al., 1988, 1991).

Bradykinin is a nonapeptide (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) released from kininogens by the actions of plasma kallikreins (Proud & Kaplan, 1989). The physiological effects of bradykinin are mediated through specific cell surface receptors. The first antagonist for the bradykinin B_2 receptor (Vavrek & Stewart, 1985) was a bradykinin analogue wherein D-phenylalanine was substituted for L-proline in the 7 position of bradykinin. This substitution resulted in a series of compounds having antagonist activity in a variety of tissues (Stewart & Vavrek, 1990; Bathon & Proud, 1991). The structure and pharmacological activity of a novel bradykinin antagonist, D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin (HOE-140), have recently been described (Hock et al., 1991). In a variety of bradykinin assays, this compound appears to be a selective B_2 receptor antagonist and is at least 2 orders of magnitude more potent than any of the D-Phe^7-substituted compounds (Lembeck et al., 1991; Wirth et al., 1991). Although HOE-140 is a competitive antagonist in some biological assays, it also demonstrates noncompetitive interactions with the receptor (Rhaleb et al., 1992; Cuthbert et al., 1992).

Chemical synthesis and pharmacological characterization of HOE-140 confirmed that it is a potent inhibitor of [^{3}H]-bradykinin binding to the bradykinin B_2 receptor found on human IMR-90 fetal lung fibroblasts (Sawutz et al., 1992). During the purification of synthetic HOE-140 in our laboratory, a peptide (WIN 65365) identical to HOE-140 except in the stereochemistry at the Tic^7 (1,2,3,4-tetrahydroisoquinolin-2-ylcarbonyl) position was identified. Analytical characterization of WIN 65365 by chiral amino acid analysis and mass spectrometry confirmed the identity of this peptide. In this report, we describe the synthesis and pharmacological characterization of WIN 65365, the L-Tic^7 isomer of HOE-140. In an attempt to understand the differences in biological activity observed for HOE-140 and WIN 65365, we describe conformational analysis of both decapeptides using annealed molecular dynamics (AMD).

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MATERIALS AND METHODS

Materials. [^{3}H]Bradykinin (80–90 Ci/mmol) and $^{45}Ca^{2+}$ (10–75 Ci/g; chloride salt) were obtained from New England Nuclear Corp. (Boston, MA). IMR-90 cells (PDL 24) were obtained from the American Type Culture Collection. Protease-free bovine serum albumin (BSA), bradykinin, captopril, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), and sodium azide were obtained from Sigma (St. Louis, MO). HOE-140 and WIN 65365 were synthesized at Sterling Winthrop Pharmaceuticals, Inc. (see below). Other bradykinin peptide analogues were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Tissue culture reagents were obtained from Gibco. All solvents for peptide synthesis were from Aldrich; the amino acid derivatives and FMOC-Arg-(Mtr)-Wang resin were obtained from Applied Biosystems (Bachem Biosciences, Philadelphia, PA) and Advanced Chemtech, respectively. Marfey's reagent and trifluoroacetic acid were obtained from Pierce Chemical Co.

Peptide Synthesis and Purification. HOE-140 and WIN 65365 were initially synthesized manually using preactivated HOBT esters (Bodanszky et al., 1980). The FMOC-D-Tic used in the initial synthesis was made according to the method of Henke et al. (1989). For confirmatory studies, subsequent quantities of enantiomerically pure peptides were synthesized with an Applied Biosystems 430A automated peptide synthesizer using the HBTU activation protocol (Dourtoglou & Gross, 1984). These secondary lots were made using enantiomerically pure (D and L) FMOC-Tic's, which are commercially available from Bachem Biosciences. FMOC-amino acid side chains were protected using the Mtr and *tert*-butyl protecting groups for guanidino and hydroxyl groups, respectively. The completed peptide was deprotected and cleaved from the resin in 4 h with a solution of TFA–EDT–thioanisole (90:5:5, 25 mL/g of resin, 25 °C). The slurry was filtered, and solvent was removed from the filtrate by rotary evaporation to yield an oil. The peptides were precipitated from this oil with diethyl ether and then purified by reversed-phase HPLC. Resolution of the peptide diasteriomers required the use of an acetonitrile/*tert*-butyl alcohol based system (Bobko & Wolfe, 1993) since they were not resolved using a slow acetonitrile gradient (Figure 1). The two major peaks in the chromatogram were isolated, and their identity was confirmed by FAB-MS and chiral amino acid analysis.

Chiral Amino Acid Analysis. Purified peptides were hydrolyzed in 6 N HCl at 110 °C for 20–24 h, dried, and then redissolved in 0.5 M $NaHCO_3$. An aliquot of each sample was reacted with 1.5 molar equiv of Marfey's reagent in acetone at 40 °C for 90 min and then neutralized with HCl (Marfey, 1984; Szokan et al., 1989). Derivatized D and L standards were used to assign unambiguous retention times for each component by RP-HPLC (Beckman Ultrasphere ODS, 4.6 × 15 cm). RP-HPLC elution of the peptides was done at a flow rate of 1 mL/min while being monitored at 220 nm. The linear elution gradient conditions (pH = 4.0) were 15:5:80 A–B–C to 25:5:70 A–B–C in 50 min, 25:5:70 A–B–C to 47:53 A–C in 1 min, and 47:53 A–C to 53:47 A–C in 39 min, where A = methanol, B = acetonitrile, and C = 20 mM NaOAc, respectively. Minor coelution of D-serine with L-arginine and D-TIC with thienyl-D-alanine standards occurred but did not compromise the results.

IMR-90 Cell Culture. IMR-90 human fetal lung fibroblasts cultured in Dulbecco's modified eagle medium containing fungizone (250 μg/mL), gentamicin sulfate (50 μg/mL), 10% fetal calf serum, and 2 mM L-glutamine were grown to confluence (2–3 days) in an incubator at 37 °C and 7% CO_2. Cells were harvested using 0.25% trypsin/Versine for 5 min and then diluted with fresh medium and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in fresh medium for tissue culture or in ice-cold binding buffer for radioligand binding studies (see below).

[^{3}H]Bradykinin Binding to IMR-90 Cells. Harvested IMR-90 cells were resuspended to the desired cell concentration in ice-cold buffer containing phosphate-buffered saline (PBS), pH = 7.4, sodium azide (0.02%), 0.1% BSA, PMSF (1 mM), DTT (1 mM), and captopril (1 μM). In competitive inhibition binding studies, IMR-90 cells (150 000 cells/tube) were incubated with [^{3}H]bradykinin (1–2 nM) in the absence or presence of increasing concentrations of the competing ligand. The final assay volume was 500 μL. All assay mixtures were incubated for 2 h at 4 °C. The binding assays were terminated by filtration through Whatman GF/B filters on a Brandel cell harvester containing 0.1% BSA. All filters were presoaked in 0.1% poly(ethylenimine) to decrease nonspecific binding.

IMR-90 $^{45}Ca^{2+}$ Efflux Assay. IMR-90 cells were cultured in 12-well tissue culture dishes until confluent. The cells were washed with assay buffer (pH = 7.4) containing DMEM, HEPES (20 mM), captopril (10 μM), BSA (0.1%), and bacitracin (75 μg/mL) and then incubated with $^{45}Ca^{2+}$ (20 μCi/well) for 4 h at 37 °C in 2 mL of assay buffer. After 4 h, each well was washed with 5 × 2 mL aliquots of buffer to remove unincorporated $^{45}Ca^{2+}$ from the plate. The cells were incubated with 0.5-mL aliquots of buffer or antagonist in buffer for 3 min prior to the addition of agonist. A second 0.5-mL aliquot containing bradykinin or buffer (to determine background efflux levels) was added for a second 3-min incubation period to determine agonist-stimulated efflux levels. The assay was terminated by removal of 0.8 mL of the reaction mixture and aspiration of the remaining buffer. Following agonist stimulation (second incubation period), the cells were solubilized for 1 h with 1 mL of 0.2% Triton X-100 to determine the amount of residual $^{45}Ca^{2+}$ left in the cells. The 0.8-mL reaction mixture and the 1-mL Triton X-100 aliquots from each plate were counted in 10 mL of liquid scintillation fluid for 2 min. The counts per minute (cpm) obtained for each were combined to get the total cpm of $^{45}Ca^{2+}$ in each well. This number was then used to calculate the fractional release (cpm released divided by the total cpm) per well. Specific fractional release for each plate is defined as the fractional release induced by agonist minus the fractional release in the absence of agonist.

Conformational Analysis Studies. Annealed molecular dynamics (AMD) studies were performed using BIOGRAF following a protocol previously described for a conformational analysis of bradykinin (Salvino et al., 1993). The decapeptides HOE-140 and WIN 65365 were built from a standard library of amino acid residues, except for the two unnatural amino acid residues which were constructed using BIOGRAF's build function. A phenylalanine residue (Phe^5) was used instead of the thienylalanine residue (Thi^5) (Henke et al., 1989). All of the asymmetric centers in the molecule were constrained, and the structures were minimized to an RMS gradient less than 0.1. This resulted in an almost linear starting conformation with good bond length and bond angle geometries. The dynamics were run in the absence of water using a dielectric constant of 4, a value generally regarded as the bulk dielectric of a membrane. An annealing temperature range from 1000 to 0 K was used in order to adequately sample conformational space. The asymmetric centers were constrained to avoid racemization. The starting conformation was, in effect, heated to 1000 K over approximately 5 ps and then cooled to 0 K over approximately 5 ps, resulting in the

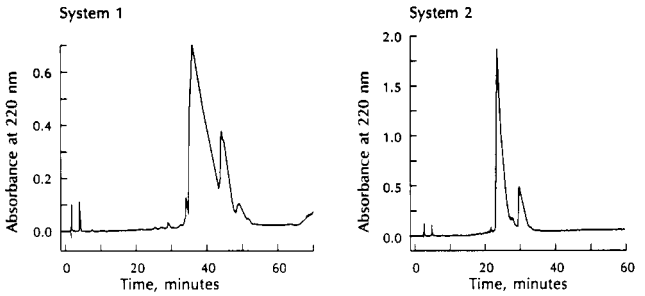

FIGURE 1: HPLC separation of HOE-140 and WIN 65365. The peptides were separated by HPLC on a Vydac C_{18} column (25 × 2.5 cm, 300 Å, 5 μm) under the following gradient conditions: 10–20% solvent B, linear in 40 min, 20–100% solvent B in 10 min, 2 mL/min. System 1: solvent A = H_2O and 0.1% TFA; solvent B = CH_3CN/tBuOH (1:1) and 0.1% TFA. System 2: solvent A = H_2O and 0.1% TFA; solvent B = CH_3CN and 0.1% TFA. In each chromatogram, the major peak is HOE-140 (D isomer) and the second largest peak is WIN 65365 (L isomer).

first conformation. This structure was then used as the starting point for another annealing cycle. The process was repeated altogether 100 times. Each time the structure at the end of the annealing cycle was saved, resulting in a total of 100 conformations. The one-hundredth conformation was the result of about 2000 ps of dynamics. This protocol resulted in a trajectory file containing 100 conformations; the lowest energy conformation was selected and minimized to an RMS gradient less than 0.1 without constraints in BIOGRAF. The lowest energy conformation from the HOE-140 analysis is labeled CONF 18 (see Figure 5); the lowest energy conformation from the WIN 65365 analysis is labeled CONF 56 (see Figure 6). The ϕ, ψ, and χ dihedral angles for the energy-minimized conformations of both peptides are presented in Table 3.

Data Analysis. Analysis of [^{3}H]bradykinin binding data to determine values for K_D, K_i, and B_{max} was performed using LIGAND (Munson & Rodbard, 1980), a nonlinear least squares regression analysis program on an IBM-PC computer. Preliminary IC_{50} values were obtained using the program EBDA (BioSoft, Inc., Princeton, NJ). EC_{50} values for $^{45}Ca^{2+}$ efflux studies were calculated using a four-parameter fitting routine in NLIN as described by DeLean et al. (1978). Schild analysis was done using the Pharmacologic Calculation System, version 4.1, as described by Tallarida and Murray (1987). A two-sample *t*-test was used to determine statistical differences when relevant.

RESULTS

Synthesis and Characterization of HOE-140 and WIN 65365. The use of preformed HOBT esters and HBTU activation of FMOC-amino acids provided a chemically mild and efficient approach to the production of a highly pure peptide. Retrospective analysis of the initial lot of FMOC-D-Tic, relative to commercial batches of the D and L analogues, demonstrated that this lot contained a 3:1 ratio of the D and L enantiomers, respectively ($[\alpha]_D = -16.4°$ compared to $[\alpha]_D = -32.1°$ for FMOC-D-Tic and $[\alpha]_D = +32.6°$ for FMOC-L-Tic; 1% in dioxane). Resolution of the major components in the crude synthetic HOE-140 mixture using standard RP-HPLC acetonitrile gradient systems (Rivier et al., 1982) was not possible. Baseline resolution of the two major components was achieved using a mixture of *tert*-butyl alcohol and acetonitrile as the organic phase (Figure 1). Chromatographic analysis of the hydrolyzed peptides after derivatization with Marfey's reagent confirmed the identity of WIN 65365 as being identical to HOE-140 except for the replacement of an L-Tic residue for a D-Tic residue.

Table 1: Affinity Binding Constants (K_i) for Inhibition of [^{3}H]Bradykinin Binding to Human IMR-90 Cells[a]

peptide	binding K_i (nM)
bradykinin	2.0 ● 0.2
HOE-140	0.11 ± 0.03
WIN 65365	130 ± 60
[D-Phe^7]bradykinin	566 ± 61

[a] Inhibition of [^{3}H]bradykinin binding to human IMR-90 cells is described in Materials and Methods. The binding affinities for the peptides (K_i) were calculated according to the method of Cheng and Prusoff (1973) using IC_{50} values determined by EBDA (BioSoft, Inc.). The K_D for [^{3}H]bradykinin used to calculate the K_i values, determined in separate binding experiments, was 2.1 ± 0.2 nM. The data are from four or more identical binding experiments.

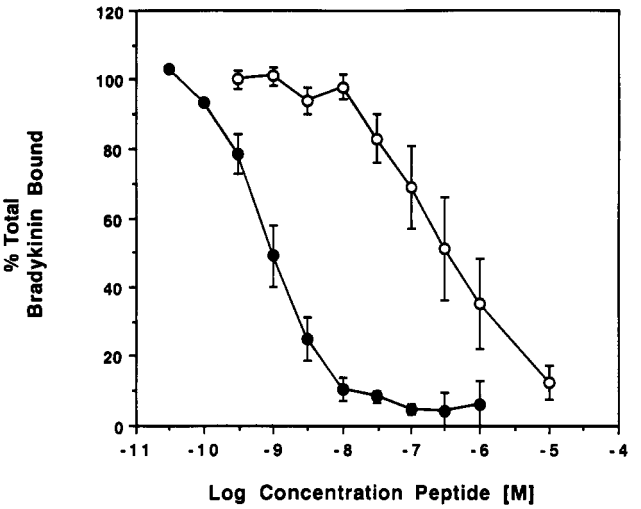

FIGURE 2: Inhibition of [^{3}H]bradykinin binding by HOE-140 and WIN 65365. IMR-90 cells were incubated with [^{3}H]bradykinin (1 nM) and increasing concentrations of HOE-140 (●) or WIN 65365 (○) as described in Materials and Methods. The data are presented as percent total bradykinin bound vs log concentration of peptide (M) and are means (±SE) of two to four identical experiments.

Effect of HOE-140 and WIN 65365 on [^{3}H]Bradykinin Binding. The characterization of [^{3}H]bradykinin binding to intact human IMR-90 fetal lung fibroblasts is described elsewhere (Sawutz et al., 1992). In summary, binding equilibrium is attained by 120 min at 4 °C. [^{3}H]Bradykinin binding is saturable; Scatchard analysis of saturation binding data demonstrates a single binding site having a K_D = 1.8 ± 0.2 nM and a receptor concentration of 17.4 ± 4.0 fmol/10^5 cells. Bradykinin inhibits [^{3}H]bradykinin binding to IMR-90 cells with a binding potency (K_i) of 2.0 nM (Table 1). Both HOE-140 and WIN 65365 inhibit [^{3}H]bradykinin binding dose dependently; HOE-140 demonstrates greater binding affinity for the receptor than either bradykinin or WIN 65365 (Figure 2). The binding potency for WIN 65365 is approximately 2000-fold lower than that of HOE-140. [Des-Arg^9]-bradykinin, a bradykinin B_1 receptor agonist, is essentially inactive in this assay (data not shown), consistent with the fact that both HOE-140 and WIN 65365 are binding to a bradykinin B_2 receptor.

Effect of WIN 65365 on Bradykinin-Stimulated $^{45}Ca^{2+}$ Efflux. We previously reported that HOE-140 attenuates bradykinin-stimulated $^{45}Ca^{2+}$ efflux from IMR-90 cells with an IC_{50} of 2.8 ± 1.9 nM (Sawutz et al., 1992). HOE-140 does not stimulate $^{45}Ca^{2+}$ efflux in the absence of bradykinin, indicating that it is a full antagonist in this system unlike the D-Phe^7-substituted bradykinin analogues which demonstrate agonist activity. WIN 65365 does not stimulate $^{45}Ca^{2+}$ efflux at a concentration of 35 μM, demonstrating that it is also a full antagonist in this system (data not shown).

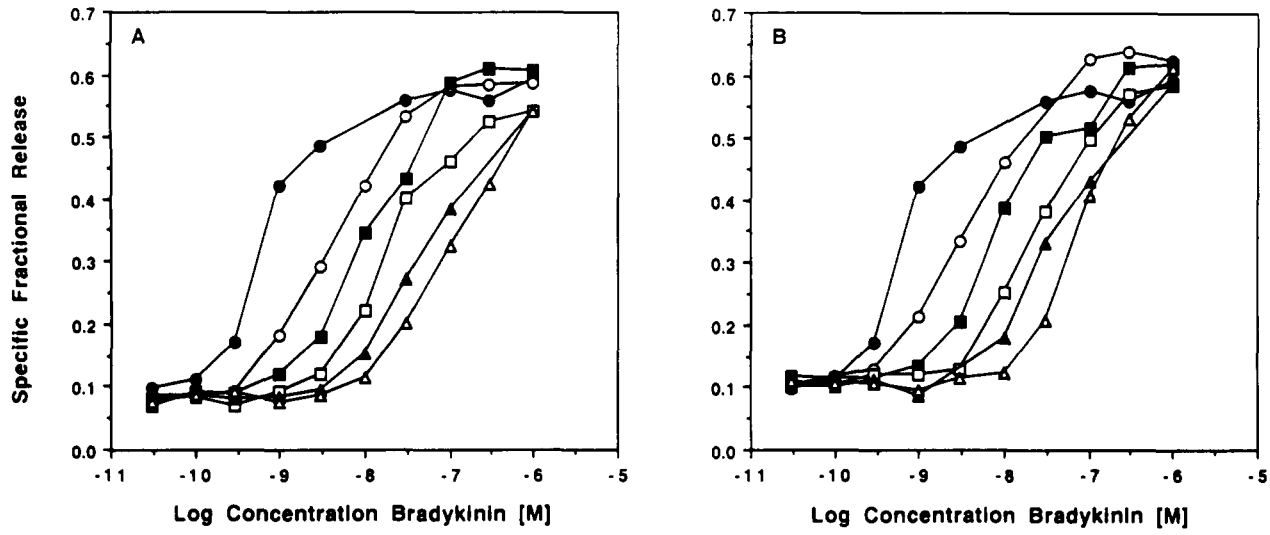

FIGURE 3: Inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux by HOE-140 and WIN 65365. Each concentration of peptide was tested over an agonist concentration range of 30 pM to 1 μM. The results are graphically presented as the fraction of $^{45}Ca^{2+}$ released vs log concentration of bradykinin (M). The data are from one of two identical experiments for each peptide. (A) Bradykinin dose–response curves were constructed in the absence (●) or presence of increasing concentrations of HOE-140 including 0.75 nM (○), 1.5 nM (■), 5.0 nM (□), 15.0 nM (▲), and 30.0 nM (△) peptide. (B) Bradykinin dose–response curves were constructed in the absence (●) or presence of increasing concentrations of WIN 65365 including 0.5 μM (○), 1.5 μM (■), 5.0 μM (□), 15.0 μM (▲), and 30.0 μM (△) peptide.

As determined in preliminary experiments, the approximate IC_{50} for WIN 65365 inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux is 2 μM (data not shown). More detailed studies demonstrate that increasing concentrations of HOE-140 and WIN 65365 produce parallel, rightward shifts in the concentration–response curves for bradykinin-stimulated $^{45}Ca^{2+}$ efflux from IMR-90 cells (Figure 3). There is no indication of a decrease in bradykinin-mediated functional activity in the presence of either peptide, demonstrating that increasing concentrations of bradykinin can surmount the antagonist effect. These data are consistent with competitive antagonism. Schild analysis of the concentration–response curves, to determine the affinity (pA_2, –log of the concentration of antagonist that reduces the effect of a double dose of agonist to that of a single dose) for the receptor and to confirm the mechanism of action of each peptide (Figure 4), results in a calculated pA_2 value for HOE-140 of 9.55 (Table 2). The pA_2 value for WIN 65365 is 6.82; this represents a 500-fold decrease in potency compared to HOE-140. The slope of the Schild plot for HOE-140 (–0.92) is not significantly different from –1, confirming that HOE-140 is a competitive antagonist in this system (Kenakin, 1982). The slope of WIN 65365 is slightly less than unity (–0.86) but not significantly different than unity. These data suggest that WIN 65365 is a competitive antagonist at the IMR-90 bradykinin B_2 receptor.

Conformational Analysis. Analysis of the AMD trajectory file for HOE-140 revealed that greater than 85% of the generated conformations utilize the four carboxy-terminal residues (Ser^{6}D-Tic^{7}Oic^{8}Arg^9) to form a type II′ β-turn (Figure 5). The amino-terminal region (D-Arg^{0}Arg^{1}Pro^{2}Hyp^{3}Gly^4) of the peptide displays significant flexibility, for example, movements of 10–15 Å over the dynamics trajectory. WIN 65365 produces conformations strikingly different from those generated for HOE-140 (Figure 6). An uncharacterizable turn at residues 5–8 does appear in about 50% of the conformations. However, statistically significant, defined structural features are not apparent in WIN 65365 under the annealing model conditions used for the analysis. The conformational analysis of WIN 65365 suggests that this less restricted molecule does not form a β-turn from the last four residues of the carboxy terminus.

It has been suggested that the β-turn structure in the carboxy-terminal region of the peptide (Ser^{6}Pro^{7}Phe^{8}Arg^9)

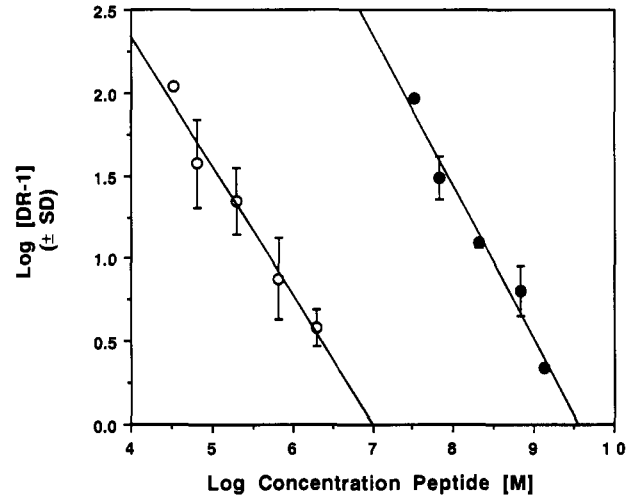

FIGURE 4: Schild analysis of HOE-140 and WIN 65365 inhibition. The dose–response data from Figure 3 were analyzed according to the method of Schild as described in Materials and Methods. Dose ratios (DR) were determined by dividing the ED_{50} of bradykinin obtained with each concentration of antagonist by the ED_{50} of a control concentration–effect curve without antagonist. The log (DR – 1) is plotted vs the log concentration of HOE-140 (●) or WIN 65365 (○). The plots are the best linear regression fits of the data and are means (±SD) of two identical experiments for each peptide. A summary of the data is presented in Table 2.

Table 2: Results from Schild Analysis of HOE-140 and WIN 65365 Inhibition of Bradykinin-Stimulated $^{45}Ca^{2+}$ Efflux from Human IMR-90 Cells[a]

peptide	pA_2	slope
HOE-140	9.55 (0.14)	–0.92 (0.09)
WIN 65365	6.92 (0.10)	–0.86 (0.06)

[a] The data from Figure 4 were analyzed according to the method of Schild (1947) as described in Materials and Methods. The data presented are means (±SE) of the best linear fit for each line. The data are results from two identical experiments for each peptide.

may be the significant recognition element of bradykinin toward its receptor (Kyle et al., 1991). Comparison of the lowest energy conformation of HOE-140 (Figure 7) with an AMD bradykinin model which satisfies available NMR data (Salvino et al., 1993) demonstrates the similarities between the two conformations. Both molecules possess a β-turn in

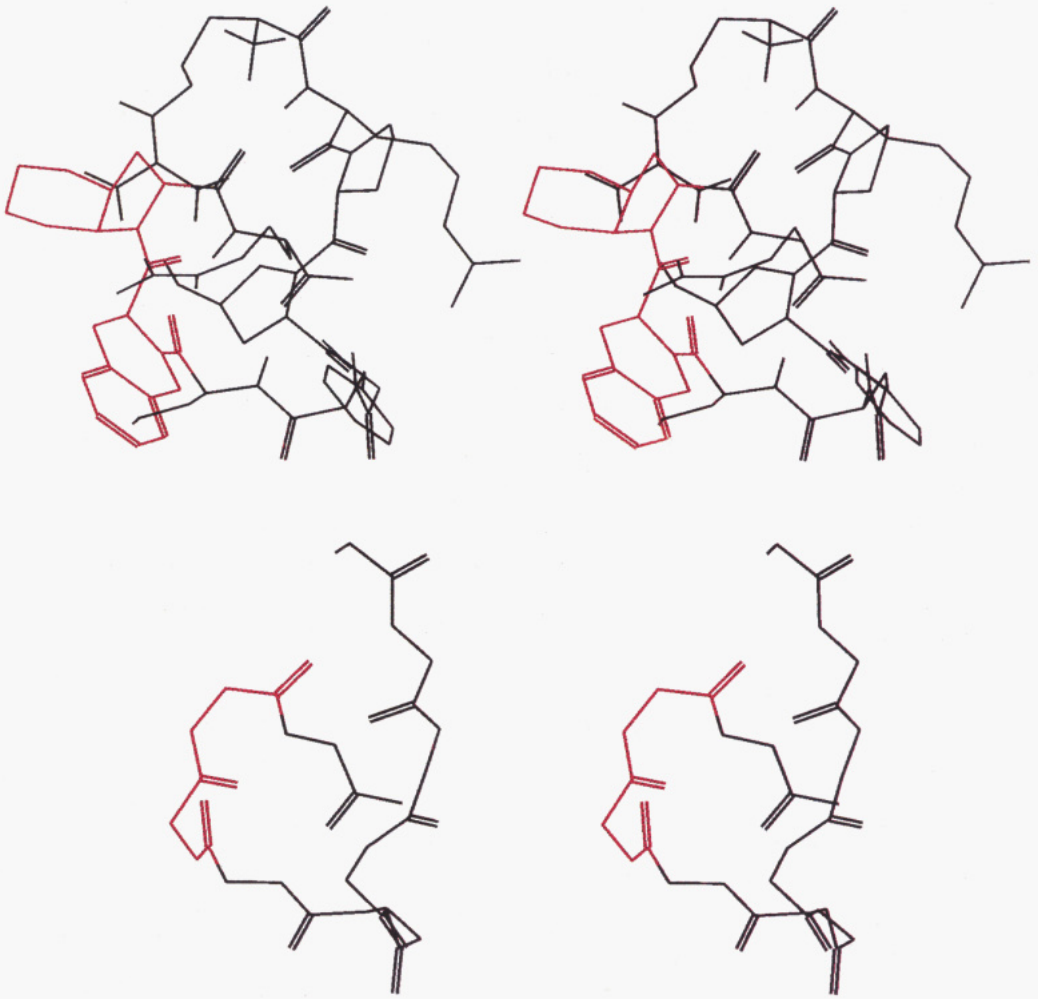

FIGURE 5: Stereo diagrams of the lowest energy conformations of CONF 18 (HOE-140 model) after energy minimization. The upper set of stereo diagrams is the amino acid structures; the side chains for the D-Tic[7] and Oic[8] residues are highlighted in red. The lower set of stereo diagrams is the amino acid backbone conformations only; the D-Tic and Oic residues are highlighted in red.

Table 3: ϕ, ψ, and χ Dihedral Angles for Energy-Minimized Conformations of CONF 18 (HOE-140 Model) and CONF 56 (WIN 65365 Model)

	CONF 18 (HOE-140 model)			CONF 56 (WIN 65365 model)		
residue	ϕ	ψ	χ	ϕ	ψ	χ
D-Arg		44.1	−53.7		−117.6	80.6
Arg	−121.6	143.2	−155.9	−121.4	165.3	−77.8
Pro	−48.4	134.9	−27.3	−43.0	−47.7	−29.2
Hyp	−50.7	118.3	−18.0	−74.9	−2.4	31.0
Gly	−144.1	−56.5		176.8	111.9	
Phe	−90.7	−47.9	−173.6	−104.7	−53.6	−68.9
Ser	−172.0	92.6	−171.8	−72.8	−53.5	−64.7
D/L-Tic	69.1	−109.5	−18.0	−92.5	111.4	36.9
Oic	−61.4	−11.1	25.0	−59.6	116.1	18.9
Arg	−113.6	−20.0	−57.3	−109.0	−58.5	−108.7

the carboxy-terminal region of the peptide, and the amide backbones for the two model structures have a Cα RMS fit of 0.73 Å in this region.

DISCUSSION

D-Arg[0][Hyp[3],Thi[5],D-Tic[7],Oic[8]]bradykinin (HOE-140) is a potent and selective bradykinin receptor antagonist that binds to the bradykinin B_2 receptor with an affinity comparable to or greater than that of the natural hormone bradykinin (Hock et al., 1991). During the purification of synthetic HOE-140 made in our laboratories, we isolated the L-Tic stereoisomer of HOE-140 (WIN 65365). Characterization of WIN 65365 by chiral amino acid analysis and mass spectrometry confirmed the identity of the peptide.

Both HOE-140 and WIN 65365 recognize the bradykinin receptor on the basis of their ability to inhibit the binding of bradykinin to its receptor. The binding affinity of WIN 65365 for the human bradykinin B_2 receptor is lower than the affinity of HOE-140 by approximately 3 orders of magnitude (Table 1). The binding potency of WIN 65365 is, however, approximately 5-fold better than that of [D-Phe[7]]bradykinin (130 vs 566 nM). Functional studies demonstrate that WIN 65365 is an antagonist at the bradykinin B_2 receptor. HOE-140 and WIN 65365 competitively inhibit a bradykinin-mediated functional response in IMR-90 cells and demonstrate potencies consistent with their respective binding affinities. In addition, neither peptide demonstrates agonist-like activity when tested at concentrations 100-fold greater than their respective binding affinities. The results of WIN 65365 are significant in that they represent the first report of antagonist activity resulting from the replacement of Pro[7] with an L amino acid.

It has been established that a D amino acid in the $i + 1$ position of a β-turn in an amino acid sequence stabilizes the formation of a type II′ β-turn (Rose et al., 1985). The primary origin of this preference is steric, arising from unfavorable nonbonded interactions between the side chains of the residues in the $i + 1$ and $i + 2$ positions and either the carbonyl of residue $i + 1$ or the NH of residue $i + 2$. HOE-140 contains a D-Tic and an adjacent Oic (L-[(3a*S*,7a*S*)-octahydroindol-2-ylcarbonyl) group at positions 7 and 8, respectively, in the bradykinin sequence. The conformation analysis studies indeed suggest that HOE-140 prefers a distinct type II′ β-turn

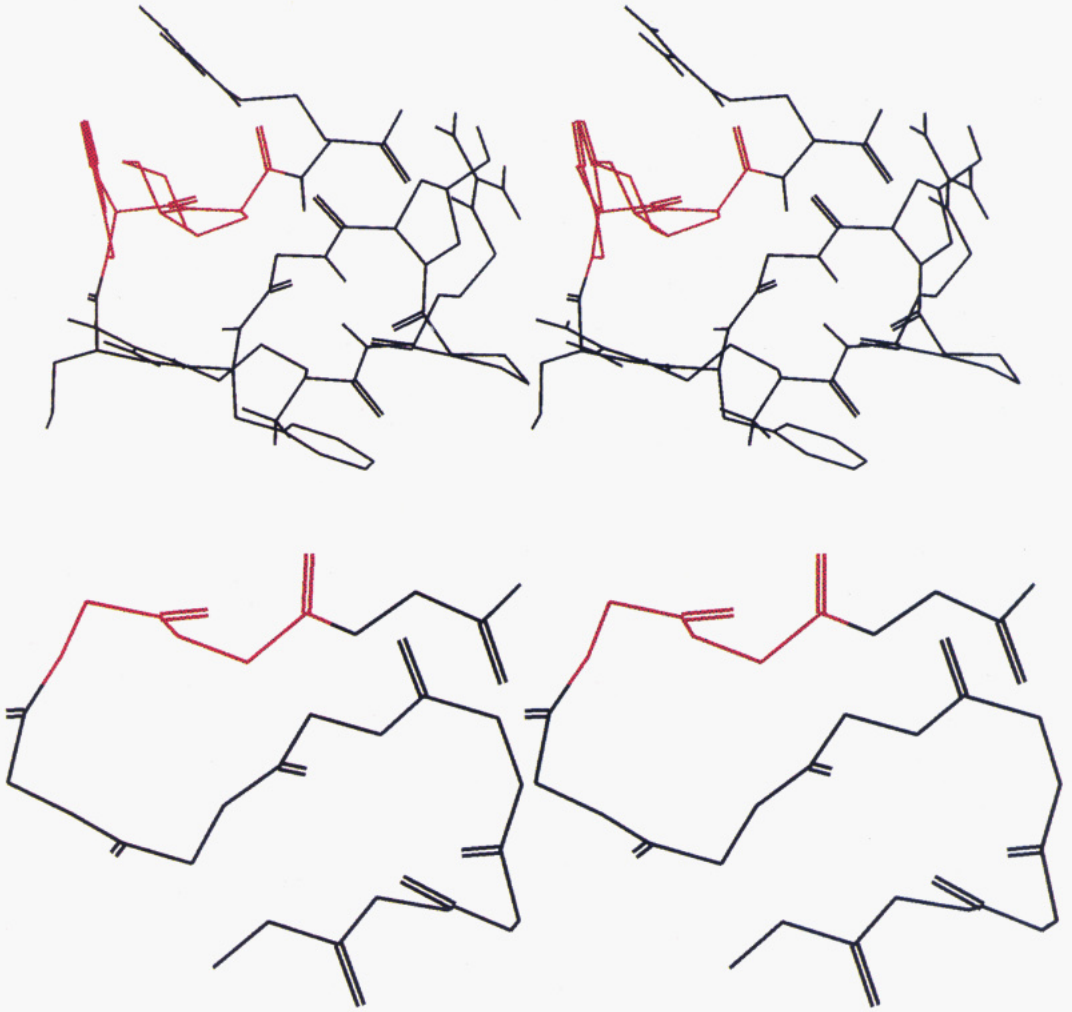

FIGURE 6: Stereo diagrams of the lowest energy conformations of CONF 56 (WIN 65365 model) after energy minimization. The upper set of stereo diagrams is the amino acid structures; the side chains for the L-Tic[7] and Oic[8] residues are highlighted in red. The lower set of stereo diagrams is the amino acid backbone conformations only; the L-Tic and Oic residues are highlighted in red.

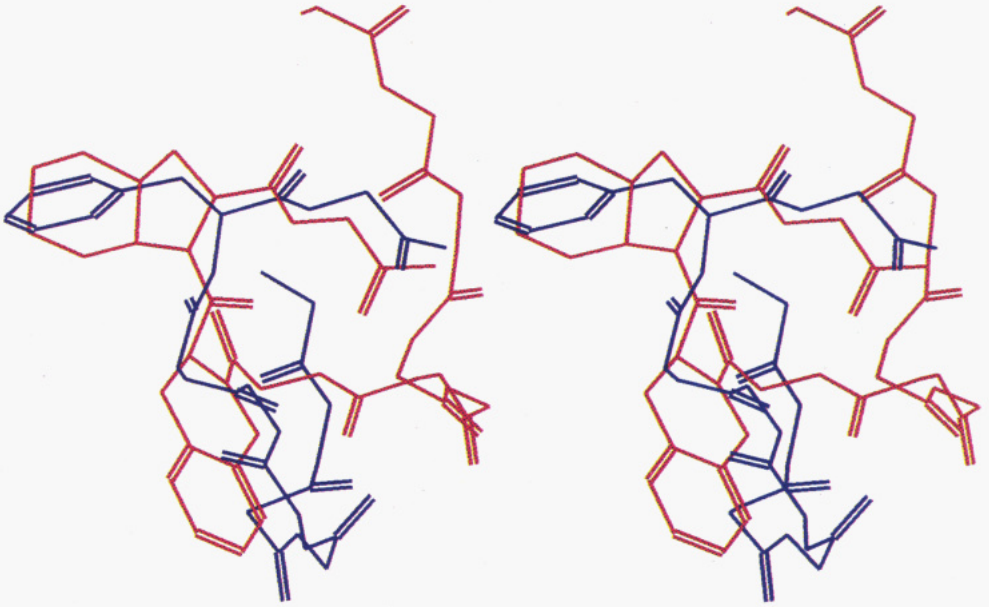

FIGURE 7: Stereo diagram of the overlay of the amide backbone conformation of CONF 18 (HOE-140 model; red) with a conformation model of bradykinin (blue) that satisfies available NMR data (see text). The side chains for D-Tic[7] and Oic[8] in HOE-140 and for Phe[8] in bradykinin are included as points of reference in the stereo diagrams.

where the D-Tic and Oic residues are in the $i + 1$ and $i + 2$ positions, respectively, of the β-turn. Presumably, the use of bulky and highly constrained D-Tic and Oic residues exaggerates this preference and results in a conformationally rigid carboxy-terminal region of the peptide.

Our data have been confirmed independently by recent work of Kyle et al. (1993). Using systematic conformational analyses by molecular mechanics calculations and solution conformations based on 2D NMR data, these authors demonstrate that the carboxy-terminal tetrapeptide regions

of three high-affinity bradykinin receptor antagonists [Ser-D-Phe-Oic-Arg, Ser-D-Tic-Oic-Arg, and Ser-D-Hyp(*trans*-propyl)-Oic-Arg] all adopt β-turns as their primary conformations in aqueous solution. All three tetrapeptides have the conformationally restricted Oic residue in the $i + 2$ position and unique constrained residues in the $i + 1$ position. Bradykinin peptide analogues containing alkyl ethers of D-4-hydroxyproline in position 7 of the native peptide antagonize bradykinin binding with potencies comparable to those of HOE-140 (Kyle et al., 1991). Our data are consistent with these results and imply that the carboxy-terminal four amino acid residues in the full-length HOE-140 peptide appear to be predominantly in a β-turn conformation.

Comparison of HOE-140 to [D-Phe^7]bradykinin is noteworthy in that they both contain D-amino acid residues at position 7, which correspond to the $i + 1$ position in a β-turn. Unlike HOE-140, [D-Phe^7]bradykinin does not have a conformationally restricted amino acid in the $i + 2$ position and should be much less restricted in its conformation. Amelioration of this conformational restriction results in a molecule which has a binding potency for the receptor 3 orders of magnitude less than HOE-140. This observation, coupled with the fact that HOE-140 binds to the bradykinin receptor with an affinity comparable to the native ligand itself, supports the hypothesis that the β-turn in the carboxy-terminal region of the peptide may be part of a recognition element for high-affinity binding to the bradykinin receptor. An overlay of the β-turn region of bradykinin with HOE-140 (Figure 7) suggests that the hydrophobic residue in the $i + 2$ position of the β-turn of HOE-140 (Oic) may engage the same hydrophobic binding pocket at the bradykinin receptor normally used by Phe^8 of the native ligand. It is conceivable that this putative hydrophobic binding pocket could accommodate either aromatic residues (present data) or hydrophobic amino acids that do not contain aromatic moieties (Stewart et al., 1993).

Conformational analysis of WIN 65365 suggests that its conformation is strikingly different from HOE-140 or bradykinin. A well-defined β-turn in the carboxy-terminal region is not observed in conformational studies. This observation supports the hypothesis that the propensity to adopt a carboxy-terminal β-turn is critical for high-affinity bradykinin receptor binding activity. Consistent with this hypothesis, WIN 65365 inhibits bradykinin binding with modest potency (K_i = 130 nM). However, WIN 65365 appears to be a competitive antagonist, suggesting that it associates with the receptor differently than other peptide ligands yet is still able to antagonize functional activity. This is supported by the observation that, although WIN 65365 and [D-Phe^7]bradykinin have similar receptor binding affinities, WIN 65365 is clearly an antagonist and [D-Phe^7]bradykinin is an agonist in this system. We previously reported that D-Phe^7-substituted bradykinin analogues are agonists and that the agonist activity of [D-Phe^7]bradykinin can be attenuated by HOE-140 (Sawutz et al., 1992). This makes WIN 65365 an attractive alternative template from which to design novel antagonists to further characterize the molecular interaction of bradykinin with its receptor.

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