Bioactivities and Secondary Structures of Constrained Analogues of Human Parathyroid Hormone: Cyclic Lactams of the Receptor Binding Region†

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Received October 24, 1996⊗

In a search for analogues of human parathyroid hormone (hPTH) with improved activities and bioavailabilities, we have prepared the following three lactam analogues of hPTH-(1−31)-NH_2 (**1**) or [Leu^{27}]hPTH-(1−31)-NH_2 (**2**): [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2 (**3**), [Leu^{27}]-*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH_2 (**4**), and *cyclo*(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH_2 (**5**). Analogues **1**, **2**, and **5** had seven or eight residues of α-helix, as estimated from their circular dichroism (CD) spectra, in contrast to 12 residues in cyclic analogues **3** and **4**. Thus, lactams **3** and **4** stabilized a helix previously shown to exist within residues 17−29. The adenylyl cyclase activity (EC_{50}), measured in rat osteosarcoma 17/2 cells, of **5** (40.3 ± 2.3 nM) was half that of its linear form **1** (19.9 ± 3.9 nM). The linear Leu^{27} mutant **2** was twice as active (11.5 ± 5.2) as analogue **1**, and lactam analogue **3** was 6-fold more active (3.3 ± 0.3 nM). Lactam analogue **4** had less activity (16.9 ± 3.3 nM) than **2**, its linear form. Peptides hPTH-(1−30)-NH_2 (**6**), [Leu^{27}]hPTH-(1−30)-NH_2 (**7**), and [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−30)-NH_2 (**8**) all had AC-stimulating activities similar to that of **1**. When injected intravenously, with a dose of 0.8 nmol/100 g of analogue in acid saline, hypotensive effects paralleled their adenylyl cyclase activities. They behaved quite differently when applied subcutaneously. Analogues **1**, **5**, and **6**, the weakest, showed about half the drop in blood pressure observed with **3** and **4**, the most active. In contrast, the time required to reach a maximum drop in blood pressure of **4**−**8**, after subcutaneous administration, was 2−4 times that of the other analogues. Thus, the bioavailabilities of the lactam analogues, unlike their adenylyl cyclase-stimulating activities, were highly dependent on the presence or conformation of Val^{31}.

Introduction

Parathyroid hormone (PTH) is a major regulator of extracellular calcium through its action on osteoblast and renal epithelial cell receptors[1] and can have both catabolic and anabolic effects on bone. There has recently been renewed interest in PTH as an anabolic agent for the treatment of osteoporosis. Thus more potent analogues of PTH and analogues with increased bioavailabilities may be very attractive for therapeutic use.

The biological activities of PTH are almost entirely restricted to a 34-residue N-terminal fragment of the 84-residue holohormone.[2] On binding to its receptor, this peptide stimulates both adenylyl cyclase (AC)[1] and protein kinase C's (PKC)[3−5] activities. The latter effect results from a stimulation of phosphatidylinositol-specific phospholipase-Cβ (PLC) with a subsequent stimulation of PKC by diacylglycerols released by PLC-induced phospholipid breakdown. Although even shorter C-terminal truncated fragments have some residual activity,[6,7] the minimal sequence for full AC activity is PTH-(1−28)-NH_2.[7] In contrast, stimulation of PKC's activity has been shown to require only the residue's 29-32 amide.[5] Consequently, the AC-stimulating and PKCs-stimulating activities of PTH can be separated. Thus, hPTH-(1−31)-NH_2 has no PLC/PKC-stimulating activity, but stimulates AC in osteoblasts[5] and bone growth in ovariectomized rats.[8,9]

Early CD studies indicated the presence of some ordered structure within PTH-(1−34).[10] Further CD studies of fragments of PTH-(1−34) and the holohormone, hPTH-(1−84), in neutral, aqueous buffer, showed this α-helical structure to be within residues 16−31.[11,12] NMR studies of fragments of hPTH-(1−84) have shown a general absence of structure in neutral, aqueous buffer, but residues 17−29 formed an α-helix on the addition of about 10% trifluoroethanol (TFE).[14] Higher concentrations of TFE stabilize additional α-helix near the N-terminus of 1−34.[13,15−17] CD studies of PTH fragments in the presence of lipid vesicles further showed the presence of an amphiphilic helix within residues 20−34.[11] This amphiphilic helix partially overlaps the helix that is stable in the absence of lipid. Very recently, an NMR study of hPTH-(1−37) has shown this analogue to have a secondary and tertiary structure in a near neutral solvent.[18]

Receptor-binding studies of PTH fragments have indicated a principal binding region within residues 14−34.[19] We have suggested that the 17−29 α-helix of the residue binds as such to the PTH receptor and that the amphiphilic portion of this α-helix binds with its hydrophobic face to the receptor.[7] This model is consistent with the results of a study of receptor binding region analogues.[12]

† Abbreviations: PTH, parathyroid hormone; hPTH, human parathyroid hormone; CD, circular dichroism; NMR, nuclear magnetic resonance; Fmoc, 9-fluorenylmethoxycarbonyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; NMM, *N*-methylmorpholine; DCM, dichloromethane; AC, adenylyl cyclase; PKC, protein kinase Cs; PIPLC, phosphatidylinositol-specific phospholipase-Cβ; EC_{50}, effective concentration for half-maximal activity; SEM, standard error of the mean; TFE, trifluoroethanol.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

S0022-2623(96)00743-1 CCC: $14.00 Published 1997 by the American Chemical Society

NMR studies have shown that even a model peptide found to be highly helical by CD also populates many nonhelical conformations.[20] Thus, the structure of a receptor-bound peptide hormone, such as PTH, cannot be inferred reliably from its free structure in solution. Constrained analogues of peptide hormones have been used to limit the number of conformational states available to the peptide.[21] Examination of the sequence of hPTH reveals three possible salt bridges within residues 17−29 which could either stabilize or destabilize the α-helix. These are between Glu^{22} and Lys^{26}, and Lys^{26} and Asp^{30}, both of which are expected to stabilize an α-helix, and between Lys^{27} and Asp^{30}, which is expected to destabilize an α-helix.[22] Lactam formation between these residue pairs would restrict the conformations available to hPTH in this helical region. Furthermore, two of these lactams, Glu^{22}−Lys^{26} and Lys^{26}−Asp^{30}, which are expected to stabilize the α-helical structure, are located on the polar face of the amphiphilic portion of the α-helix. The third one, Lys^{27}−Asp^{30}, is expected to at least partially destabilize the α-helix and involves a residue, Lys^{27}, which is on the hydrophobic face of the amphiphilic helix.

In this paper, we present data on the effects of these lactams on structure and bioactivities. We have used CD to monitor the α-helical secondary structure and, for bioactivities, measured AC stimulation and hypotensive activities in rats. These latter measurements give an indication of the relative stabilities and bioavailabilities *in vivo* of these analogues. The data are consistent with hPTH binding to its receptor as an α-helix with its hydrophobic face toward the receptor. Furthermore, the presence of the lactams can profoundly affect their *in vivo* activities and availabilities.

Results

Circular Dichroism Spectroscopy. Three of the lactams used in this study are derivatives of either hPTH-(1−31)-NH_2 (**1**) or [Leu^{27}]hPTH-(1−31)-NH_2 (**2**) (Figure 1). The CD spectra of [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2 (**3**) and [Leu^{27}]*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH_2 (**4**) are shown in Figure 2, together with the spectrum of the parent linear analogue, **2**. The CD spectra of both of the lactams in Figure 2 are substantially different than that of their linear analogue. In contrast, the spectra of the Lys^{27}−Asp^{30} lactam **5** is quite similar to the spectrum of its linear species, **1** (Figure 3).

Interpretation of the CD of small peptides is not straightforward. In part, this is because small peptides usually exist in a multitude of similar conformers.[23] The CD spectrum of a polypeptide is particularly sensitive to the presence of an α-helix, and the inference of the α-helix from the spectrum is most reliable. However, the length dependency of CD signals of peptides and the possible contributions of aromatic residues to the CD signal results in an uncertainty in quantitative interpretations.[24] Since estimating the secondary structure of short peptides from their CD spectra with algorithms which use basis sets derived from protein structures is not valid, we have previously used the ellipticity at 222 nm, $[\theta]_{222}$, to measure the α-helix content in PTH analogues.[7,11,25] This is because the rotational strength of the n−π^* transition at 222 nm is particularly strong in the α-helix[26] as compared to other peptide secondary structures.

Figure 1. Sequences of hPTH analogues: **1**, hPTH-(1−31)-NH_2; **2**, [Leu^{27}]hPTH-(1−31)-NH_2; **3**, [Leu^{27}]*cyclo*(Glu^{22}−Asp^{30})-hPTH-(1−31)-NH_2; **4**, *cyclo*(Lys^{26}−Asp^{30})[Leu^{27}]hPTH-(1−31)-NH_2; **5**, *cyclo*(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH_2; **6**, hPTH-(1−30)-NH_2; **7**, [Leu^{27}]hPTH-(1−30)-NH_2; **8**, *cyclo*(Glu^{22}−Lys^{26})[Leu^{27}]hPTH-(1−30)-NH_2.

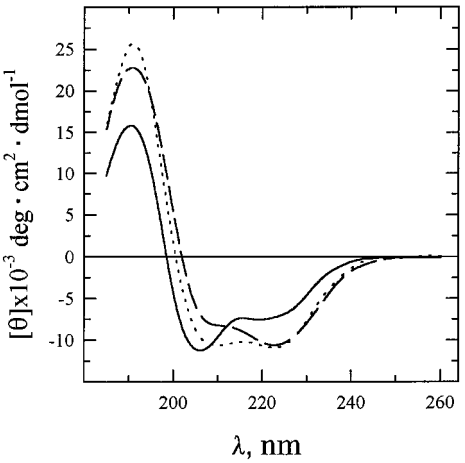

Figure 2. CD spectra of *i*, *i* + *4* lactams. Spectra were taken in 25 mM sodium phosphate, pH 7.2, at 20 °C. Shown are peptides **2** (—), **4** (− − −), and **3** (- - -). Concentrations were 0.35 mg/mL (**2**), 0.22 mg/mL (**3**), and 0.24 mg/mL (**4**).

The two lactams with *i*, *i* + 4 spacings, peptides **3** and **4**, are expected to be helix stabilizing.[21] They have similar values of $[\theta]_{222}$, approximately −10 600 and −12 600 (deg/cm^2)/dmol^{-1}, respectively, and the corresponding estimates of α-helical residues are 11 and 13, compared with eight residues of the α-helix in the linear form, peptide **2**. Although they have similar intensities at 222 nm, the spectra of these two lactams are in other respects quite different. In contrast, the similarity of the CD spectra of **5** to its linear form, **1**, suggests that their average conformations are quite similar. The cyclic form is estimated to have seven residues as the α-helix, slightly less than the estimate of eight for the linear form.

To compare these spectra with that of a commonly used α-helix spectrum,[26] we have used the ratios of the absolute values of $[\theta]_{192}$ to $[\theta]_{222}$, and $[\theta]_{222}$ to $[\theta]_{209}$. These ellipticities derive from characteristic minima at 209 and 222 nm, and a maximum at 192 nm for a pure

Table 1. Helical Parameters of hPTH Analogues and a Standard α-Helical Spectrum

analogue[a]	$[\theta]_{222} \times 10^{-3}$	$\lvert[\theta]_{192}/[\theta]_{222}\rvert$	$\lvert[\theta]_{222}/[\theta]_{209}\rvert$
standard α-helix		2.63	1.09
hPTH-(1−31)-NH_2	−7.55	1.26	0.67
[Leu^{27}]hPTH-(1−31)-NH_2	−7.35	2.07	0.72
[Leu^{27}]*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH_2	−10.6	2.14	1.30
cyclo(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH_2	−6.34	1.19	0.69
[Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2	−12.6	2.32	1.03

[a] hPTH analogues were measured in 25 mM sodium phosphate, pH 7.2. [b] Ratios taken from α-helix data of Yang et al.[27]

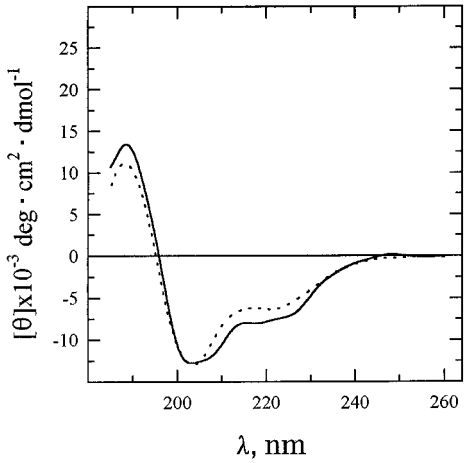

Figure 3. CD spectrum of *i*, *i* + *3* lactam. Spectra were taken in 25 mM sodium phosphate, pH 7.2, at 20 °C. Shown are peptides **1** (—) and **5** (- - -). Concentrations were 0.13 mg/mL (**1**) and 0.35 mg/mL (**5**).

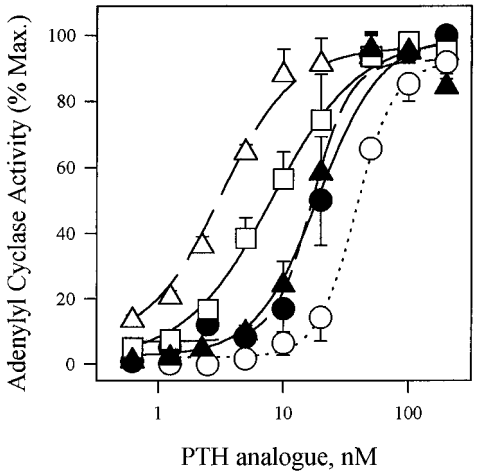

Figure 4. Stimulation of adenylyl cyclase by cyclic hPTH analogues. Shown are peptides **1** (●); **2** (□); **3** (△); **4** (▲); **5** (○).

α-helix. The ratios for the standard spectrum are 2.63 and 1.09 for $[\theta]_{192}/[\theta]_{222}$ and $[\theta]_{222}/[\theta]_{209}$, respectively.[26] In the spectrum of each of **2**, **3**, and **4**, the maximum occurs at about 190 nm, and one minimum is at 222−224 nm. However, the other minimum, which occurs at 203.5 nm in the linear peptide **2**, is shifted to 210.6 nm in the 26−30 lactam **4** and to 210.0 nm in the spectrum of the 22−26 lactam **3**. This minimum is characteristically at 209 nm in an α-helical spectrum. This CD difference between peptide **2** and peptides **3** and **4** could result from less contribution from a more "random" structure, as we have previously speculated,[11] or from the adoption of a more perfect α-helical structure in the region which tends to be α-helical, residues 17−29. Using the characteristic helical parameters mentioned above, these two lactams are definitely more helical or in a more perfect helix than the corresponding linear peptide (Table 1). Furthermore, based on the $[\theta_{222}]$ to $[\theta_{209}]$ ratio, the 22−26 analogue **3** adopts a more perfect helix than the 26−30 analogue **4**. Even though a detailed secondary structure cannot be inferred from these CD spectra, they clearly are in agreement with the expected stabilization of α-helical structure on formation of these lactams. The apparent difference in the conformation stabilized by the lactams of peptides **3** and **4** likely relates to their position in the helix.

Adenylyl Cyclase Activities. We previously reported that [Leu^{27}]hPTH-(1−34)-NH_2 is more active in stimulating AC activity in the ROS cell line than hPTH-(1−34)-NH_2.[27] We have also found that peptide **2** (EC_{50}, 11.5 ± 5.2 nM) is more active than the native sequence **1** (EC_{50}, 19.9 ± 3.9 nM) (Figure 4). Lactam formation between Glu^{22} and Lys^{26} (**3**) induced a still greater AC-stimulating activity, with EC_{50} values of 3.3 ± 0.3 nM (Figure 4). Thus, the net effect of this cyclization and replacement of Lys^{27} with Leu is about a 6-fold increase in activity. In contrast, lactam formation between either Lys^{26} and Asp^{30} (**4**) or Lys^{27} and Asp^{30} (**5**) resulted in a lessening of adenylyl cyclase stimulation, with respect to their parent linear sequences. Thus, the 26−30 lactam (**4**) has slightly less activity than its linear form, with an EC_{50} of 17.0 ± 3.3 nM vs 11.5 ± 5.2 nM for **2**. The 27−30 lactam (**5**) more markedly reduces the activity of the parent linear peptide, having an EC_{50} of 40.3 ± 2.3 nM as compared to 19 ± 3.9 nM for **1**.

We have previously reported that hPTH-(1−30)-NH_2 (**6**) has an AC-stimulating activity (EC_{50}, 20 nM) close to that of analogue **1**. We have now found that [Leu^{27}]-hPTH-(1−30)-NH_2 (**7**) and *cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−30)-NH_2 (**8**) have similar AC-stimulating activities to peptide **6**.

Hypotensive Effects of Lactam Analogues. AC activation by PTH is essential for both stimulating bone formation in ovariectomized rats[8,9,28] and reducing blood pressure.[29,30] Indeed, we have recently shown an absolute requirement for AC-stimulating, but not PKC-stimulating, activities for the osteogenic and hypotensive action of PTH.[8,31]

The data of Figure 5 show aspects of the transient hypotensive effect of hPTH analogues when administered either intravenously or subcutaneously. When administered intravenously, all of the analogues dropped the blood pressure by about 30 mmHg in about 1−2 min. However, the blood pressure response to subcutaneously injected analogues was significantly less ($p < 0.05$) than the responses to intravenously injected analogues (Figure 5). In addition, the relative responses of the blood pressure to subcutaneously injected analogues were quite different. Thus, the effectiveness of the 22−26 (**3**) and 26−30 (**4**) lactams were not significantly different ($p > 0.05$) from that of hPTH-(1−34)-NH_2 (Figure 5).

As expected, the time required to reach the minimum blood pressure was not significantly different ($p > 0.05$)

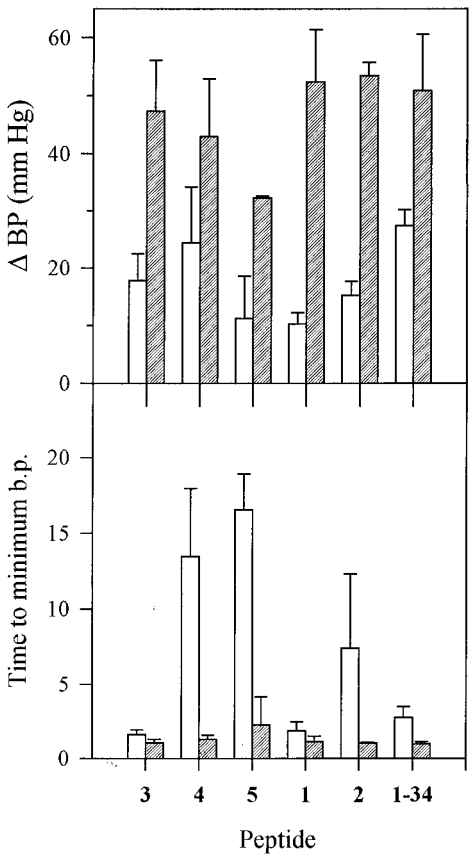

Figure 5. Hypotensive action of linear and cyclic lactam analogues of hPTH-(1−31). Shown are the maximum drops in blood pressure (upper) obtained on injecting the rat with 0.8 nmol/100 g of the analogue and the time taken to attain the maximum drop in blood pressure. The analogues (left to right, peptides **3**, **4**, **5**, **1**, **2**) were injected either intravenously (shaded bar) or subcutaneously (open bar). The graph also includes data for hPTH-(1−34)-NH_2 for comparison.

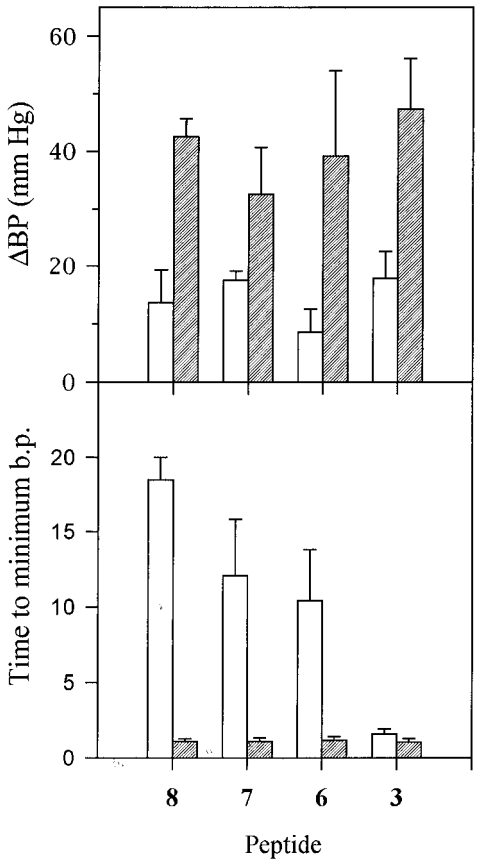

Figure 6. Hypotensive action of linear and cyclic lactam analogues of hPTH-(1−30)-NH_2 (**6**). Shown are the maximum drops in blood pressure (upper) obtained on injecting the rat with 0.8 nmol/100 g of the analogue and the time taken to attain the maximum drop in blood pressure (lower). The analogues (left to right, peptides **8**, **7**, **6**, **3**) were injected either intravenously (shaded bar) or subcutaneously (open bar).

(1−2 min) when the peptides were injected intravenously (Figure 5). In contrast, after subcutaneous injection, the times needed to reach the minimum blood pressure differed significantly ($p < 0.05$) among these analogues. Peptides **1** and **3** and hPTH-(1−34)-NH_2 rapidly decreased the blood pressure, with times of about 1.5−3 min, while peptide **2** took 7 min. In contrast, peptides **4** and **5** took much longer, about 15 min, to reach the target and affect the maximum blood pressure drop. These differences presumably reflect differing abilities of the analogues to escape from their site of injection into the blood and stimulate their vascular smooth muscle cell targets.

The low transit times observed for peptides **1** and **3**, together with the high ones for **4** and **5**, suggested that the presence of Val^{31}, and its correct conformation, might be critical to this transport of the peptide. Removal of Val^{31} resulted in all of analogues **6**, **7**, and **8** having significantly ($p < 0.05$) reduced rates of transport of the subcutaneously injected peptides to the vascular system (Figure 6). Analogues **4** and **5** have lactams involving Asp^{30}, which may restrict the conformations available to this residue. The actual total blood pressure drops of peptides **6** and **8** were, nonetheless, not significantly different ($p > 0.05$) from those of the other analogues, whether subcutaneously or intravenously administered, with the exception of peptide **5**.

Discussion

The lactam analogues described in this paper are within an amphiphilic helix[11] which is within residues 14−34, the principal binding region of PTH-(1−34).[19] Part of this region, residues 17−29, is part of an α-helix which in hPTH-(1−31)-NH_2 is partially stable in neutral, aqueous buffer.[7] This α-helix has three potential ion pairs. Although there is still debate on the contributions of such ion pairs, especially those exposed to solvent, to the stability of specific structures in peptides and proteins,[32] their presence in many of the NMR-derived models suggested that they may be making contributions to the solution structure of PTH.[18] There are two ion pairs in the C-terminal α-helical region of **1** which may contribute to its stability. These ion pairs, $Glu^{22}\cdots Lys^{26}$ and $Lys^{26}\cdots Asp^{30}$, have i, $i + 4$ spacings, and data exists suggesting pairs of these types are stabilizing.[33] The third, $Lys^{27}\cdots Asp^{30}$, with an i, $i + 3$ spacing, is expected to be destabilizing, but its effect may be minimal since it is at a terminus of the helix.[34] There is some evidence that two of these ion pairs, $Glu^{22}\cdots Lys^{26}$ and $Lys^{26}\cdots Asp^{30}$, are important to stabilizing this α-helix. The NMR structure of hPTH-(1−37) was represented by 10 model structures in the Protein Data Bank.[18] In each structure, the interionic difference of one of these potential ion pairs is 2.5 Å or less. Three models were chosen, each showing a minimal distance between one of the ion pairs, and the lactams were formed, followed by geometry optimization

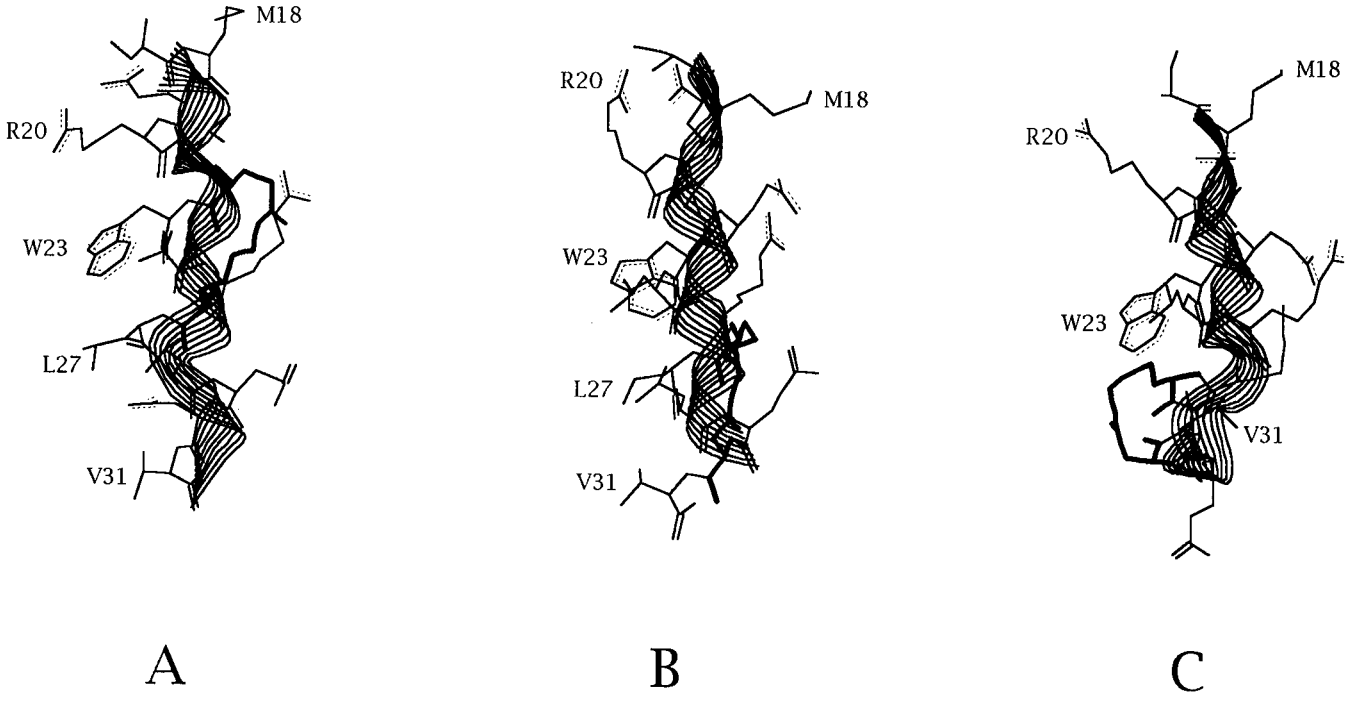

Figure 7. Models showing lactam positions in receptor-binding region of hPTH. The models were adapted from Marx et al.[18] and show the residue 17−31 region of hPTH, after lactam formation and energy minimization: A, peptide **3**; B, peptide **4**; C, peptide **5**.

by molecular mechanics to remove excess strain (Figure 7). In peptide **5** in particular, the twist introduced in the helix by the presence of the *i*, *i* + 3 lactam is obvious (Figure 7). This is consistent with the CD spectrum observed for **5**, and the similarity of the CD spectra of **1** and **5** suggests that the α-helix in the linear form **1** may also be somewhat frayed at the C-terminus. In the absence of the constraints introduced by the lactams, the peptide structure is dynamic, and from time to time various ion pairs exist. Each lactam has the effect of constraining the interatomic distance at the previous ion pair to 1.3 Å, thus reducing the conformational space available in the region of the lactam. The relatively small loss of AC-stimulating activity observed with analogue **5** compared to **1** implies that the receptor-binding conformation is still available after formation of the 27−30 lactam and is thus consistent with the CD observation. In contrast, the CD data of the other two lactams, **3** and **4**, indicated that each had substantially more α-helix than the common linear peptide, [Leu^{27}]-hPTH-(1−31)-NH_2. Peptide **3** had an AC-stimulating activity which was about 2.5-fold greater than the linear form. Presumably, peptide **3**, with the 22−26 lactam, stabilizes an α-helical conformer close to the receptor-binding one. In turn, the Leu^{27} analogue **2** had a greater AC-stimulating activity than the native sequence, **1**, but no increase in α-helix. However, this particular increase in AC-stimulating activity has been ascribed to a replacement of the polar Lys on the hydrophobic face of the helix by a hydrophobic Leu.[27] This result was expected if the hydrophobic face of the helix was directly involved in receptor binding and thus is not necessarily related to a stabilization of helical conformation. In contrast, peptide **4**, the 26−30 lactam of peptide **2**, had less AC-stimulating activity than its parent linear analogue, **2**.

The CD data further indicated a difference in the conformations populated by the two *i*, *i* + 4 spaced lactams even though they apparently had the same α-helical content. We surmised above that the 22−26 lactam analogue **3** had a more perfect helix than the 26−30 one, **4**, and this in turn may be correlated to the relative positions of each in the α-helix. Since the helical region has been shown to include residues 17−29, the Glu^{22}−Lys^{26} lactam is near the center, whereas the Lys^{26}−Asp^{30} analogue is on the periphery. It is also possible that these analogues may have differing tendencies to aggregate and that this could explain the spectral differences between peptides **3** and **4**.

The different AC-stimulating activities of peptides 3 and 4 were reflected in their hypotensive effects. When administered intravenously, each analogue elicited relative hypotensive activities not significantly different from those observed for AC-stimulating activities. This suggests that the receptors in the ROS cell line and the vascular tissue are the same. Additionally, the times to minimum blood pressure did not significantly differ. However, the results were quite different when the analogues were administered subcutaneously. Here, peptides **1**, **2**, and **5** elicited significantly lower drops in blood pressure than did hPTH-(1−34)-NH_2 and peptides **3** and **4**. Perhaps of more interest are the times observed for attaining maximal drop in blood pressure after subcutaneous administration of the various analogues. Here, the 22−26 analogue **3** reached its target much faster than either **4** or **5**, a result consistent with their data on receptor activation. The lower rates of transport of peptides **6**, **7**, and **8** further suggested that Val^{31} and the correct conformation of the peptide about Val^{31} are critical for binding to a possible transporter. The loss of charge associated with formation of the lactams at Asp^{30} is not likely to be important, since an Asp^{30} to Lys mutation was observed to have no effect on receptor-binding.[12]

The hypotensive effect measurements, especially when the analogues are administered subcutaneously, can be

affected by several variables not present in the AC-stimulating experiment. These include relative stabilities of the analogues to proteolytic degradation and transport of the analogues to the target receptor. Several workers have demonstrated increased stability to protease attack as a result of cyclization by lactam formation.[35,36] In some cases this stabilization extends beyond the lactam ring whereas in others stabilization is limited to the immediate region of the lactam ring.[36]

When administered intravenously, the relative behavior of the analogues was similar to that observed when the AC-stimulating activities were measured. Presumably, this was because a direct interaction with the vascular receptor was being measured in this case. Here, such variables as transport into the vascular system and stability were not important. However, when measurements were taken after subcutaneous injection, the relative behavior of the analogues suggested that they may be interacting with a carrier molecule during the transport process. Whatever may be the reason, it appears that formation of lactams near the C-terminus of the α-helix, such as in **4** and **5**, results in a very much increased transit time from the site of subcutaneous injection to the target receptor(s). The data demonstrate clearly that activities *in vivo* include numerous parameters not found in the *in vitro* experiment. Thus, it is valuable to have a rapid means of establishing *in vivo* capabilities, such as the hypotensive effect measurements performed in this work and previously.[31]

The PTH receptor belongs to a family of G protein-linked receptors which include such hormones as vasoactive intestinal peptide (VIP), secretin, glucagon, and calcitonin.[37] Although the peptide hormones bear no sequence similarities, the structure of the peptide as bound to the receptor may be similar. Thus, an analogue of the 28-residue VIP, *cyclo*(Lys^{21}−Asp^{25})-VIP, has more α-helix than the linear peptide and retains full bioactivity.[38] This lactam is also on the polar face of a potential amphiphilic α-helix. Interestingly, it seems that VIP and PTH may even cross-react with each other's receptors.[39] Lactam-bridged analogues of calcitonin have also been shown to have increased activities, in both *in vitro* and *in vivo* experiments. It was also stated that these increased pharmaceutical activities were compatible with an amphiphilic α-helix.[40] For this family of peptide hormones, conformational restraint from lactamization in the C-terminal region may be a general strategy for improving activities and/or bioavailabilities.

Conclusions

This work demonstrates that formation of an *i*, *i* + 4 lactam between residues 22 and 26 on the hydrophilic face of a C-terminal amphiphilic α-helix and substitution of a hydrophobic amino acid, Leu, for a Lys on the hydrophobic face of the helix increase the ability of hPTH-(1−31)-NH_2 to stimulate rat osteoblast adenylyl cyclase activity. This lactam stabilizes the α-helix. However, both an α-helix stabilizing lactam and a helix-destabilizing *i*, *i* + 3 lactam reduced the adenylyl cyclase-stimulating ability relative to the parent linear analogue. These differences in bioactivity were paralleled by the different abilities of the hPTH-(1−31)-NH_2 lactams to reduce rat blood pressure, an action that is mediated by PTH receptor-induced stimulation of vascular smooth muscle adenylyl cyclase. The data support the concept that the hormone reacts with its receptor via the hydrophobic face of an amphiphilic α-helix.

Although these peptides behave as expected for the above model, their rates of escape from subcutaneous sites of injection follow a much different pattern. Only the linear hPTH-(1−31)-NH_2, and the lactam derivative between Glu^{22}, Lys^{26} of [Leu^{27}]hPTH-(1−31)-NH_2 are able to escape rapidly from their subcutaneous sites of injection into the vascular system. Val^{31} and the conformation about Val^{31} are important for this escape. These observations may be particularly important to potential therapeutic uses of these peptides in the treatment of osteoporosis.

Experimental Section

Chemicals. *N*-α-Fmoc-L-amino acids were obtained from Novabiochem (La Jolla, CA). The following protection groups were used: Lys(Boc), Arg(Pmc), Glu(OtBu), Asp(OtBu), Ser(tBu), His(Trt), Asn(Trt), Gln(Trt), and Trp(Boc). *N*-α-Fmoc-Asp(All) and *N*-α-Fmoc-Lys(Allow) were obtained from Perceptive (Framingham, MA). Tentagel-R RAM (0.2 mmol/g) was purchased from Rapp Polymere, Tübingen, Germany. Tetrekis(triphenylphosphine)palladium(0) and sodium diethyl dithiocarbamate were from Aldrich Chemical Co. (Milwaukee, WI). Syntheses were performed on a continuous-flow peptide synthesizer (Perceptive Biosystems Model 9050 Plus). Amino acid analyses were performed with an Applied Biosystems 420H amino acid analyzer.

hPTH-(1−31)-NH_2 (1). This peptide was synthesized by a Fmoc protocol as previously described.[7] Briefly, The C-terminal Val was coupled manually to Tentagel R (0.5 g, 0.2 mmol/g). Fmoc-Val (4× molar excess) was dissolved in DMF (3.2 mL) containing 0.3 M TBTU, 0.3 M HOBt, and 0.45 M NMM, and the mixture was stirred at 45 °C for 30 min. The remaining synthesis was performed with TBTU/HOBt/NMM activation, in which the Fmoc amino acid was dissolved in 1.5 mL of DMF to a final concentration of 0.3 M and the TBTU, HOBt, and NMM concentrations were 0.26, 0.26, and 0.39 M, respectively. Flow rates were 3 mL/min. The Asn, Gln, His, Val, and Ile residues were double coupled, and a 4-fold excess of activated amino acids was used. The coupling times for Arg and Gly additions were increased from 30 to 60 min. After Fmoc removal from the N-terminal Ser, the peptide resin was washed with DCM (13 mL) and then cleaved from the resin by shaking with 7.5 mL of reagent K (6.19 mL of TFA, 0.38 mL each of water, 90% phenol/water, thioanisole, and 0.19 mL of 1,2-ethanedithiol) for 4 h at 20 °C. The cleaved peptide mixture was removed by filtration and precipitated by addition to *tert*-butyl methyl ether.

The crude product was dissolved in 14 mL of 15% acetonitrile/water and 0.1% TFA and chromatographed on a Vydac C_{18}-column (10 μm, 1 × 25 cm), using a 0.5%/min gradient (15−50%) of acetonitrile in 0.1% TFA/water. The purity of the final product was estimated by analytical HPLC on a Vydac C_{18} column (10 μm, 0.4 × 25 cm), using a 0.5%/min gradient of acetonitrile in 0.1% TFA/water. The estimated purity was >99%. The amino acid analysis is included in Table 2. The molecular weight by electrospray mass spectrometry was 3717.77(±0.13) (calculated for M + 1, 3717.14).

[Leu^{27}]hPTH-(1−31)-NH_2 (2). The peptide was synthesized and purified as above, to an estimated purity of >97%. The molecular weight was 3702.03(±1.07) (calculated for M + 1, 3703.12).

[Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2 (3). This peptide was synthesized as described for hPTH-(1−31)-NH_2, with Lys-Alloc and Glu-OAll substituted at positions 26 and 22, respectively. After completion of the addition of Fmoc-Ser^{17}, the peptide-resin was removed from the column to a reaction vial (Minivial, Applied Science), suspended in 1.7 mL of a solution of tetrekis(triphenylphosphine)palladium(0) (0.24 mmol), 5% acetic acid, and 2.5% NMM in DCM under argon,

Table 2. Amino Acid Analyses of Peptides

	observed (expected)							
residue	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Arg	2.3 (2)	2.2 (2)	2.1 (2)	2.2 (2)	2.3 (2)	2.1 (2)	2.1 (2)	2.2 (2)
Asp + Asn	3.4 (3)	3.4 (3)	2.9 (3)	2.9 (3)	2.9 (3)	3.0 (3)	2.6 (3)	3.1 (3)
Gly	1.1 (1)	1.1 (1)	1.2 (1)	1.1 (1)	1.2 (1)	1.0 (1)	1.1 (1)	1.0 (1)
Glu + Gln	5.2 (5)	5.3 (5)	4.7 (5)	4.7 (5)	4.6 (5)	4.6 (5)	4.3 (5)	5.0 (5)
His	2.2 (2)	2.1 (2)	2.3 (2)	2.2 (2)	2.1 (2)	2.0 (2)	2.4 (2)	2.2 (2)
Ile[a]	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
Leu	5.2 (5)	6.0 (6)	5.9 (6)	6.0 (6)	5.0 (5)	5.0 (5)	6.1 (6)	5.7 (6)
Lys	3.3 (3)	2.1 (2)	2.1 (2)	1.9 (2)	2.9 (3)	3.1 (3)	2.0 (2)	2.0 (2)
Met	2.0 (2)	1.9 (2)	1.8 (2)	1.9 (2)	1.9 (2)	2.0 (2)	1.8 (2)	1.9 (2)
Ser	2.6 (3)	2.6 (3)	2.6 (3)	2.6 (3)	2.7 (3)	2.3 (3)	2.6 (3)	2.5 (3)
Trp	nd[b] (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)
Val	3.0 (3)	3.0 (3)	2.9 (3)	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)	2.0 (2)

[a] Amino acid analyses ratioed to Ile. [b] Not determined.

and then shaken at 20 °C for 6 h to remove the allyl and Alloc protecting groups.[41,42] The peptide resin was then washed with 0.5% DEDT and 0.5% NMM in DMF (50 mL), followed by DMF (50 mL) and DCM (50 mL). The peptide (0.06 mmol) was cyclized by shaking with 0.06 mmol HOAt/0.12 mmol NMM in 2 mL of DMF for 14 h at 20 °C.[42,43] The peptide-resin was filtered, washed with DMF, and repacked into the column. The Fmoc-peptide was cleaved from the resin with reagent K. After the first HPLC, the Fmoc group was removed, and purification was completed by further HPLC. The purity was >99%, and the product had a mass of 3685.46(±0.46) (expected M + 1 = 3685.12). The position of the lactam was confirmed by sequencing.

[Leu^{27}]*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH$_2$ (4). The synthesis was performed in a manner analogous to that of [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH$_2$. The product had an estimated purity of >95%, with a molecular weight of 3685.61(±0.36) (expected M + 1 = 3685.12). The peptide was also sequenced to confirm the lactam position.

***cyclo*(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH$_2$ (5).** The synthesis was performed in a manner analogous to that of [Leu^{27}]-*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH$_2$. The product had an estimated purity of >95%, with a molecular of 3700.64(±0.38) (expected M + 1 = 3700.14). The peptide was sequenced to confirm the lactam position.

hPTH-(1−30)-NH$_2$ (6). This peptide was synthesized in the same way as analogue **1**, without manual addition of Val to the support. The product had an estimated purity of >99%, with a molecular mass of 3619.05(±0.48) (expected for M + 1 = 3619.01).

[Leu^{27}]hPTH-(1−30)-NH$_2$ (7). The synthesis was analogous to peptide **6**. The product had an estimated purity of >99% and a mass of 3603.98(±0.19) (expected M + 1 = 3603.99).

[Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−30)-NH$_2$ (8). This peptide was synthesized as for peptide **3**, without manual addition of Val to the support. The product had an estimated purity of >97%, with a molecular mass of 3586.14(±0.19) (expected M + 1 = 3685.99).

Molecular Modeling. Preliminary structures were selected from NMR data for hPTH-(1−37) stored in the Brookhaven Protein Data Bank.[18] From the models provided, selections were made of those having side-chain COOH···NH$_2$ distances close to that of the desired lactams. Specifically, models 1, 3, and 4 were used for analogues **3**, **4**, and **5**, respectively. Manipulations on these data were performed with Hyperchem release 4 for Windows (Hypercube, Inc., Waterloo, ON, Canada). Residues 32−37 were removed and the molecule converted to a C-terminus amide. The models were subjected to a molecular mechanics energy minimization using an AMBER force field and a distance dependent electrostatic potential. These models are at a nearby local energy minimum to the initial structure and are useful in understanding the restraints imposed by the lactams. They cannot be inferred to describe the receptor-bound conformation of the analogue.

Circular Dichroism Spectroscopy. Spectra were obtained on a JASCO J-600 spectropolarimeter at 20 °C. At least four spectra were averaged and the data smoothed by the JASCO software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Peptide concentrations were calculated from the absorption at 280 nm, using an extinction coefficient of 5700 M^{-1} for the single tryptophan. Data are expressed per peptide bond.

Adenylyl Cyclase Activities. Adenylyl cyclase activities of 4−5-day cultures of ROS 17/2 cells in 24-well plates were estimated from the rate of formation of [^{3}H]cAMP from the cellular ATP pool, which had been labeled with [^{3}H]adenine before exposure to hPTH or its analogues.[5] Cells were incubated for 10 min after addition of analogues, before stopping the reaction with 10% trichloroacetic acid, followed by separation and measurement of [^{3}H]cyclic AMP. Each experiment was done at least in duplicate, and the standard errors were determined. The values for the concentrations of half-maximal activities (EC_{50}) were determined by fitting the data to a sigmoidal function (Table Curve, Jandel, San Rafael, CA). Errors for each EC_{50} were estimated by fitting to the limits of the standard errors.

Blood Pressure Assay. Female Sprague−Dawley rats (weighing over 290 g) were anesthetized with intraperitoneally injected sodium pentobarbital (65 mg/kg of body weight).[31] Rectal temperature was monitored with a YSI402 thermistor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and maintained between 36.0 and 38.5 °C throughout the experiment. Ear pinna temperature was also monitored using a YSI banjo thermistor. The tail artery was exposed and cannulated with a Jelco 25-g IV catheter (Johnson and Johnson Medical Inc., Arlington, TX) and connected to a Statham pressure transducer, the signals from which were recorded digitally with a Biopac Systems MP100 monitor (Harvard Instruments, Saint Laurent, QC, Canada). For intravenous injection of PTH or one of its fragments, a femoral vein was also exposed. After surgery, the blood pressure was allowed to stabilize for 8 min, after which PTH or one of its fragments (dissolved in acidified saline containing 0.001 N HCl) was injected into the femoral vein or under the skin of the abdomen. Data were collected for 12 min after intravenous injection or for 22 min after subcutaneous injection.

Blood pressure data were accumulated and averaged, using AcqKnowledge software (Biopac Systems, Santa Barbara, CA), to determine the mean changes in five animals. The standard errors were calculated from these data sets. Blood pressure data are expressed as means ± SEM's.[9] Statistical comparisons were made by one-way analysis of variance (ANOVA). When significant differences were observed, Scheffe's test was used for multiple comparisons; $p < 0.05$ was considered to be significant. No detectable change was observed when animals were injected with vehicle alone.

Acknowledgment. We thank D. Krajcarski for mass spectra and D. Watson for peptide sequencing.

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JM960743O