# The Bioactive Conformation of Human Parathyroid Hormone. Structural Evidence for the Extended Helix Postulate

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**Abstract:** Conformational restrictions in the form of [*i*, *i* + 4] lactam bridges were sequentially incorporated into the shortest fragment of hPTH with recognized efficacy in the OVX rat model of osteoporosis, hPTH(1−31)$NH_2$ (**1**). Cyclo(Lys$^{18}$−Asp$^{22}$)[Ala$^1$,Nle$^8$,Lys$^{18}$,Asp$^{22}$,Leu$^{27}$]hPTH(1−31)$NH_2$ (**2**) is a potent agonist of the PTH/PTHrP receptor located on the surface of ROS 17/2.8 cells as measured by its ability to stimulate adenylyl cyclase activity ($EC_{50}$ = 0.29 nM). A second analogue, which constrains the entire C-terminal receptor binding domain, bicyclo(Lys$^{18}$−Asp$^{22}$,Lys$^{26}$−Asp$^{30}$)[Ala$^1$,Nle$^8$,Lys$^{18}$,Asp$^{22}$,Leu$^{27}$] hPTH(1−31)$NH_2$ (**6**), is also shown to be a potent agonist ($EC_{50}$ = 0.13 nM), thus providing further evidence for an extended helix as the relevant bioactive conformation in this region of the hormone. Adjacent lactam bridges were incorporated into the analogue bicyclo(Lys$^{13}$−Asp$^{17}$,Lys$^{18}$−Asp$^{22}$)[Ala$^1$,Nle$^8$,Lys$^{18}$,Asp$^{17,22}$,Leu$^{27}$]hPTH(1−31)$NH_2$ (**7**) to evaluate the receptor's tolerance to conformational restriction in the midregion of the peptide. In fact, peptide **7** is also a highly potent agonist ($EC_{50}$ = 0.43 nM) in the cAMP-based assay, which suggests that at least one bioactive form of the hormone requires a helical conformation extending from residue 13 to residue 22. Incorporation of all three lactam bridges afforded the most conformationally constrained PTH peptide agonist yet reported, tricyclo(Lys$^{13}$−Asp$^{17}$,Lys$^{18}$−Asp$^{22}$,Lys$^{26}$−Asp$^{30}$)[Ala$^1$,Nle$^8$,Lys$^{18}$,Asp$^{17,22}$,Leu$^{27}$]hPTH(1−31)$NH_2$ (**9**). Peptide **9** ($EC_{50}$ = 0.14 nM) forces residues 13−30 into an extended helical conformation and is a more potent PTH receptor agonist than the parent linear hPTH(1−31)$NH_2$ (**1**, $EC_{50}$ = 4.7 nM). Comparative circular dichroism studies indicate that peptide **9** is highly helical even in the absence of TFE, indicating that residues 1−12 are also likely to be helical in the bioactive conformation. Taken together, these results provide strong structural evidence that hPTH binds to its receptor in an extended helical conformation.

## Introduction

Human parathyroid hormone (hPTH) is the major regulator of calcium homeostasis in the body and exerts its biological action by binding to and activating specific receptors located throughout the gastrointestinal tract, the kidney, and bone tissue. The principle PTH receptor, PTHR1, is a member of the seven-helical transmembrane receptor superfamily (7-TMs) and shares modest homology with other receptors within this family such as calcitonin (CT), calcitonin gene-related peptide (CGRP), secretin, and glucagon, among others. Recently, a second PTH receptor subtype (PTHR2) has been isolated from brain tissue although its precise physiological role remains unclear.[1,2]

Human PTH is secreted from the parathyroid gland following posttranslational processing as an 84-amino acid protein; however, the predominant form of the peptide isolated from human plasma is composed of only the 37 N-terminal residues [hPTH(1−37)].[3] It has been established that most of the known biological activities of hPTH including receptor binding and activation is provided by the first 34 N-terminal residues. Primary sequence homology of the N-terminus of PTH is well-preserved across various mammalian and avian species including human, rat, cow, pig, and chicken. Parathyroid hormone-related protein (hPTHrP) is a 139−171-amino acid protein which shares significant N-terminal homology to hPTH; in fact, 9 of the first 14 N-terminal residues of hPTH and hPTHrP are identical. Beginning around position 15, the C-terminal regions of hPTH(1−34) and hPTHrP(1−34) maintain little primary sequence homology; however, Chou−Fasman and helical wheel analyses suggest that these regions are similar in their secondary structure. The observation that both PTH and PTHrP interact with a common PTH/PTHrP receptor with comparable affinity has led to speculation that the bioactive conformations of these two hormones are likely to be similar despite the difference in parts of their primary sequences. Recently, several reviews summarizing the structural aspects of PTH, PTHrP, and their ligand−receptor interactions have appeared in the literature.[4,5]

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(1) Usdin, T. B.; Gruber, C.; Bonner, T. I. *J. Biol. Chem.* **1995**, *270*, 15455−15458.
(2) Usdin, T. B. *Endocrinology* **1997**, *138*, 831−834.
(3) Hock, D.; Magerlein, M.; Heine, G.; Ochlich, P. P.; Forssmann, W.-G. *FEBS Lett.* **1997**, *400*, 221−225.
(4) Chorev, M.; Rosenblatt, M. In *The Parathyroids*; Bilezikian, J. P., Levine, M. A., Marcus, R., Eds.; Raven Press, Ltd.: New York, 1994; pp 139−156.
(5) Chorev, M.; Rosenblatt, M. In *Principles of Bone Biology*; Bilezikian, J. P., Raisz, L. G., Rodan, G. A., Eds.; Academic Press, Inc.: New York, 1996; pp 305−323.

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It is now well-recognized that hPTH and its N-terminal fragment hPTH(1−34) as well as hPTHrP(1−36)[6] possess significant in vivo anabolic activity in rats as well as in humans when administered appropriately, typically as daily subcutaneous injections.[7,8] As such, much effort has been directed toward the development of PTH, PTHrP, and related analogues[9] for the treatment of postmenopausal (type I) and senile (type II) osteoporosis as well as other physiological conditions associated with bone cell regulation.[10,11] Whitfield and co-workers have demonstrated that the shorter fragment, hPTH(1−31)$NH_2$, is the minimum sequence required to elicit the in vivo anabolic effect in the aged ovariectomized (OVX) rat model of osteoporosis even though a fragment as small as hPTH(1−28)-$NH_2$ retains most of the in vitro agonist activity of the full length hormone.[12]

The biological activity of hPTH is reflected in the activation of two secondary messenger systems: the G-protein coupled adenylyl cyclase (AC) and G-protein coupled and uncoupled protein kinase C (PKC) pathways. The observed increase in bone growth has been suggested to be coupled with stimulation of the AC pathway.[13] It has also been established that deletion of up to six amino acid residues from the N-terminus of hPTH-(1−34) markedly decreases the resulting analogue's ability to stimulate AC while having little effect on receptor binding; thus the term "activation domain" is commonly used to refer to the first 6−10 N-terminal residues.[14] Truncation at the C-terminus, or "receptor-binding domain," of PTH(1−34) results in analogues with decreasing ability to bind to the PTH/PTHrP receptor. The smallest such fragment that still maintains significant receptor affinity is the aforementioned hPTH(1−28).

Owing to the vast therapeutic potential of such osteogenic agents, considerable work on the underlying structural issues associated with PTH binding and activation of the PTH/PTHrP receptor has been performed. Barden and Kemp's first NMR-derived structure of PTHrP(1−34) suggested that the hormone existed in solution in a paired-helix or "U-shaped" conformation (Figure 1).[15] This early result inevitably led to the hypothesis that PTH might also interact with its endogenous receptor in this conformation, a supposition that was fully consistent with the hypothetical binding mode for a number of polypeptide hormones and their receptors.[16,17] Several groups have since reported on the IR-,[18] NMR-,[19−21] and circular dichroism (CD)-derived[22] structures of PTH,[23] PTHrP, and their analogues[24−26] in aqueous environment as well as in the presence of salts,[27] lipid micelles,[28−30] and secondary structure-inducing solvents.[31] These investigations have essentially established that the nonhomologous C-terminal receptor-binding regions of hPTH-(1−34) and hPTHrP(1−34) adopt highly helical structures under a variety of solvent conditions. The midregion and N-terminal conformations have proven to be more intractable since these sections of the hormone have shown much less ordered structure by CD and NMR analysis even in the presence of solvent additives and lipid micelles. PTH/PTHrP hybrid peptides[32] and site-directed mutagenesis at both the C- and N-termini of the hormone[33,34] as well as on the PTH/PTHrP receptor[35−42] have

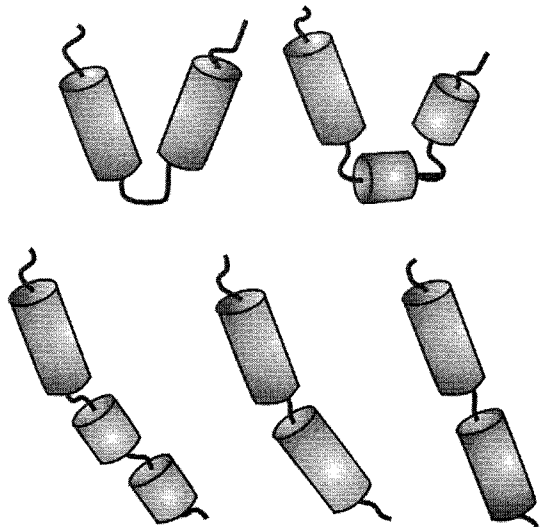

**Figure 1.** Graphical representation of putative bioactive conformations of PTH(1−31): (top) "U-shaped" or paired helix; (bottom) "linear" or extended helix .

(6) Stewart, A. F. *Bone* **1996**, *19*, 303−306.
(7) Reeve, J. *J. Bone Miner. Res.* **1996**, *11*, 440−445.
(8) Dietrich, J. W. *Exp. Opin. Invest. Drugs* **1997**, *6*, 655−663.
(9) Vickery, B. H.; Avnur, Z.; Cheng, Y.; Chiou, S.-S.; Leaffer, D.; Caulfield, J. P.; Kimmel, D. B.; Ho, T.; Krstenansky, J. L. *J. Bone Miner. Res.* **1996**, *11*, 1943−1951.
(10) Morley, P.; Whitfield, J. F.; Willick, G. *Exp. Opin. Ther. Patents* **1998**, *8*, 31−37.
(11) Patel, S. *Exp. Opin. Invest. Drugs* **1996**, *5*, 429−445.
(12) Whitfield, J. F.; Morley, P.; Willick, G. E.; Ross, V.; Barbier, J.-R.; Isaacs, R. J.; Ohannessian-Barry, L. *Calcif. Tissue Int.* **1996**, *58*, 81−87.
(13) Rixon, R. H.; Whitfield, J. F.; Gagnon, L.; Isaacs, R. J.; Maclean, S.; Chakravarthy, B.; Durkin, J. P.; Neugebauer, W.; Ross, V.; Sung, W.; Willick, G. E. *J. Bone Miner. Res.* **1994**, *9*, 1179−1189.
(14) Mahaffey, J. E.; Rosenblatt, M.; Shepard, G. L.; Potts, J. T., Jr. *J. Biol. Chem.* **1979**, *254*, 6496−6498.
(15) Barden, J. A.; Kemp, B. E. *Eur. J. Biochem.* **1989**, *184*, 379−394.
(16) Kirby, D. A.; Koerber, S. C.; Craig, A. G.; Feinstein, R. D.; Delmas, L.; Brown, M. R.; Rivier, J. E. *J. Med. Chem.* **1993**, *36*, 385−393.
(17) Glover, I.; Haneef, I.; Pitts, J.; Wood, S.; Moss, D.; Tickle, I.; Blundell, T. *Biopolymers* **1983**, *22*, 293−304.
(18) McFarlane, D. R.; McFarlane, E. F.; Barden, J. A.; Kemp, B. E. *Biochim. Biophys. Acta* **1993**, *1162*, 187−194.
(19) Barden, J. A.; Kemp, B. E. *Biochemistry* **1993**, *32*, 7126−7132.
(20) Klaus, W.; Dieckmann, T.; Wray, V.; Schomburg, D.; Wingender, E.; Mayer, H. *Biochemistry* **1991**, *30*, 6936−6942.
(21) Wray, V.; Federau, T.; Gronwald, W.; Mayer, H.; Schomburg, D.; Tegge, W.; Wingender, E. *Biochemistry* **1994**, *33*, 1684−1693.
(22) Chorev, M.; Epand, R. F.; Rosenblatt, M.; Caufield, M. P.; Epand, R. M. *Int. J. Pept. Protein Res.* **1993**, *42*, 342−345.
(23) Pellegrini, M.; Royo, M.; Rosenblatt, M.; Chorev, M.; Mierke, D. F. *J. Biol. Chem.* **1998**, *273*, 10420−10427.
(24) Sung, W. L.; Willis, K. J.; Willick, G. E.; Gagnon, L.; Luk, C. L.; Whitfield, J. F. *Protein Pept. Lett.* **1994**, *1*, 163−168.
(25) Barden, J. A.; Cuthbertson, R. M.; Jia-Zhen, W.; Moseley, J. M.; Kemp, B. E. *J. Biol. Chem.* **1997**, *272*, 29572−29578.
(26) Chorev, M.; Behar, V.; Yang, Q.; Rosenblatt, M.; Mammi, S.; Maretto, S.; Pellegrini, M.; Peggion, E. *Biopolymers* **1995**, *36*, 485−495.
(27) Marx, U. C.; Austermann, S.; Bayer, P.; Adermann, K.; Ejchart, A.; Sticht, H.; Walter, S.; Schmid, F.-X.; Jaenicke, R.; Forssmann, W.-G.; Rosch, P. *J. Biol. Chem.* **1995**, *270*, 15194−15202.
(28) Neugebauer, W.; Barbier, J.-R.; Sung, W. L.; Whitfield, J. F.; Willick, G. E. *Biochemistry* **1995**, *34*, 8835−8842.
(29) Oldenburg, K. R.; Epand, R. F.; D'Orfani, A.; Vo, K.; Selick, H.; Epand, R. M. *J. Biol. Chem.* **1996**, *271*, 17582−17591.
(30) Strickland, L. A.; Bozzato, R. P.; Kronis, K. A. *Biochemistry* **1993**, *32*, 6050−6057.
(31) Barden, J. A.; Cuthbertson, R. M. *Eur. J. Biochem.* **1993**, *215*, 315−321.
(32) Gardella, T. J.; Luck, M. D.; Wilson, A. K.; Keutmann, H. T.; Nussbaum, S. R.; Potts, J. T., Jr.; Kronenberg, H. M. *J. Biol. Chem.* **1995**, *270*, 6584−6588.
(33) Gardella, T. J.; Wilson, A. K.; Keutmann, H. T.; Oberstein, R.; Potts, J. T., Jr.; Kronenberg, H. M.; Nussbaum, S. R. *Endocrinology* **1993**, *132*, 2024−2030.
(34) Gardella, T. J.; Axelrod, D.; Rubin, D.; Keutmann, H. T.; Potts, J. T., Jr.; Kronenberg, H. M.; Nussbaum, S. R. *J. Biol. Chem.* **1991**, *266*, 13141−13146.
(35) Mannstadt, M.; Luck, M. D.; Gardella, T. J.; Jüppner, H. *J. Biol. Chem.* **1998**, *273*, 16890−16896.
(36) Zhou, A. T.; Besalle, R.; Bisello, A.; Nakamoto, C.; Rosenblatt, M.; Suva, L. J.; Chorev, M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3644−3649.

been utilized to gain further insight into the specific ligand−receptor interactions required to initiate the two biologically relevant signaling pathways.[43−45] Recently, Pellegrini et al. reported an elegant evaluation of PTHrP(1−34)−receptor interactions garnered from solution-phase NMR analysis, affinity labeling, molecular modeling, and nitrosyl-labeled membrane studies[46−49] concluding that, in contrast to the previously proposed paired-helix motif, PTHrP most likely interacts with its receptor in an extended or "linear" fashion (Figure 1). Structural investigations on a number of medium-length peptide hormones including secretin,[50] human growth hormone-releasing factor (hGHRF),[51−53] and vasoactive intestinal peptide (VIP)[54] have provided strong evidence for extended or partially extended conformations in solution which, as before, have been translated into putative binding conformations between the ligands and their cognate receptors.

An alternative and perhaps more direct means by which to ascertain the bioactive conformation of a peptide hormone is to impart conformational constraint into the native peptide and then evaluate its ability to bind to and/or activate its endogenous receptor.[55] Maintenance (or improvement) of biological activity by such a conformationally restricted peptide analogue can serve as powerful evidence for the importance of a single, well-defined conformation of its more-flexible, linear parent. One such type of constraining element is the side chain-to-side chain amide bridge formed between a basic lysine residue and an acidic aspartate or glutamate residue. For hPTH[56,57] and hPTHrP,[58−62] several such cyclic and bicyclic analogues have now been described in the literature and their secondary structures investigated by CD and NMR spectroscopy. In this paper, we report our investigations on the activity and structure of a number of novel, biologically active constrained analogues of hPTH(1−31)$NH_2$. With the introduction of one, two, and then three side chain constraints, we have systematically limited the peptide's ability to adopt a paired-helix tertiary conformation in favor of an extended helical form. The resulting bioactivity and circular dichroism measurements provide direct structural evidence that the bioactive 31- and 34-amino acid N-terminal fragments of hPTH(1−84) interact with their receptor in a *linear or extended* helical conformation as opposed to the "U-shaped" or paired-helix hormone−receptor model which has been previously postulated.

## Methods

**Peptide Synthesis and Analysis.** Human parathyroid hormone (1−31) amide and hPTH(1−34)OH were obtained from Bachem Biosciences, Inc. (King of Prussia, PA). All other peptides were prepared by Fmoc-based SPPS using a PS-3 automated peptide synthesizer (Rainin/Varian, Woburn, MA) beginning from Rink Amide MBHA resin (Nova Biochem, La Jolla, CA); Fmoc-amino acids were obtained from Advanced ChemTech, Inc. (Louisville, KY) except for Fmoc-Asp(OAllyl)-OH and Fmoc-Lys(Alloc)-OH, which were purchased from Senn Chemicals AG (Dielsdorf, Germany). The peptides were prepared using Fmoc SPPS incorporating Asp(OAllyl) and Lys(Alloc) when appropriate. Following completion of either the fragments or the full linear sequence, the resin-bound peptide was selectively deprotected using tetrakis(triphenylphosphine)palladium(0)[63] and lactam formation was accomplished in two cycles using HBTU. For polylactam-containing peptides (vide infra), the cyclized fragment was returned to the automated peptide synthesizer for further residue additions and the next lactam bridge was incorporated as described above. After completion of the synthesis, final deprotection and resin cleavage was followed by reversed-phase HPLC purification using a Vydac Protein and Peptide C18 column (10−15 $\mu$m, 300 Å pore size) employing a gradient two-buffer system: buffer A, 0.1% aqueous trifluoroacetic acid; buffer B, 0.1% trifluoroacetic acid in acetonitrile; typical gradient, 0−60% buffer B over 30 min. All peptides were >95% purity by HPLC analysis and were characterized by amino acid (Bachem Biosciences, Inc.) and mass spectral analysis. The position of the lactam bridges was ascertained by sequence analysis, which was performed at the Emory University School of Medicine Microchemical Facility, Winship Cancer Center (Atlanta, GA), and peptide mapping using trypsin (Sigma) following known procedures;[64] the resulting fragments were analyzed by LC−MS using a Micromass Platform II instrument configured to a Hewlett-Packard series 1100 HPLC system. For cyclo(Lys$^{18}$−Asp$^{22}$)[Ala$^{1}$,Nle$^{8}$,Lys$^{18}$,Asp$^{22}$,Leu$^{27}$]hPTH(1−31)$NH_2$ (**2**), the lactam position was also identified by NMR spectroscopy.[65] A complete procedure for the preparation of peptide **2** is contained within the Supporting Information.

(37) Jüppner, H.; Schipani, E.; Bringhurst, F. R.; McClure, I.; Keutmann, H. T.; Potts, J. T., Jr.; Kronenberg, H. M.; Abou-Samra, A. B.; Segre, G. V.; Gardella, T. J. *Endocrinology* **1994**, *134*, 879−884.
(38) Turner, P. R.; Bambino, T.; Nissenson, R. A. *Mol. Endocrinol.* **1996**, *10*, 132−139.
(39) Gardella, T. J.; Luck, M. D.; Fan, M.-H.; Lee, C. W. *J. Biol. Chem.* **1996**, *271*, 12820−12825.
(40) Lee, C.; Luck, M. D.; Juppner, H.; Potts, J. T., Jr.; Kronenberg, H. M.; Gardella, T. J. *Mol. Endocrinol.* **1995**, *9*, 1269−1278.
(41) Lee, C.; Gardella, T. J.; Abou-Samra, A.-B.; Nussbaum, S. R.; Segre, G. V.; Potts, J. T., Jr.; Kronenberg, H. M.; Juppner, H. *Endocrinology* **1994**, *135*, 1488−1495.
(42) Segre, G. V.; Abou-Samra, A.-B.; Gardella, T. J.; Juppner, H.; Kronenberg, H. M.; Nussbaum, S. R.; Potts, J. T., Jr. In *Calcium Regulating Hormones and Bone Metabolism*; Cohn, D. V., Gennari, C., Tashjian, A. H., Jr., Eds.; Elsevier Science Publishers B. V.: Amsterdam, The Netherlands, 1992; pp 92−96.
(43) Huang, Z.; Chen, Y.; Pratt, S.; Chen, T.-H.; Bambino, T.; Shoback, D. M.; Nissenson, R. A. *Mol. Endocrinol.* **1995**, *9*, 1240−1249.
(44) Iida-Klein, A.; Guo, J.; Takemura, M.; Drake, M. T.; Potts, J. T., Jr.; Abou-Samra, A.; Bringhurst, F. R.; Segre, G. V. *J. Biol. Chem.* **1997**, *272*, 6882−6889.
(45) Iida-Klein, A.; Guo, J.; Xie, L. Y.; Juppner, H.; Potts, J. T., Jr.; Kronenberg, H. M.; Bringhurst, F. R.; Abou-Samra, A. B.; Segre, G. V. *J. Biol. Chem.* **1995**, *270*, 8458−8465.
(46) Pellegrini, M.; Bisello, A.; Rosenblatt, M.; Chorev, M.; Mierke, D. F. *Biochemistry* **1998**, *37*, 12737−12743.
(47) Bisello, A.; Adams, A. E.; Mierke, D. F.; Pellegrini, M.; Rosenblatt, M.; Suva, L. J. *J. Biol. Chem.* **1998**, *273*, 22498−22505.
(48) Rötz, C.; Pellegrini, M.; Mierke, D. F. *Biochemistry* **1999**, *38*, 6397−6405.
(49) Mierke, D. F.; Pellegrini, M. *Curr. Pharm. Des.* **1999**, *5*, 21−36.
(50) Gronenborn, A. M.; Bovermann, G.; Clore, G. M. *FEBS Lett.* **1987**, *215*, 88−94.
(51) Clore, G. M.; Martin, S. R.; Gronenborn, A. M. *J. Mol. Biol.* **1986**, *191*, 553−561.
(52) Brunger, A. T.; Clore, G. M.; Gronenborn, A. M.; Karplus, M. *Protein Eng.* **1987**, *1*, 399−406.
(53) Fry, D. C.; Madison, V. S.; Greeley, D. N.; Felix, A. M.; Heimer, E. P.; Frohman, L.; Campbell, R. M.; Mowles, T. F.; Toome, V.; Wegrzynski, B. B. *Biopolymers* **1992**, *32*, 649−666.
(54) Fry, D. C.; Madison, V. S.; Bolin, D. R.; Greeley, D. N.; Toome, V.; Wegrzynski, B. B. *Biochemistry* **1989**, *28*, 2399−2409.
(55) Taylor, J. W.; Ösapay, G. *Acc. Chem. Res.* **1990**, *23*, 338−344.
(56) Barbier, J.-R.; Neugebauer, W.; Morley, P.; Ross, V.; Soska, M.; Whitfield, J. F.; Willick, G. *J. Med. Chem.* **1997**, *40*, 1373−1380.
(57) Neugebauer, W.; Gagnon, L.; Whitfield, J.; Willick, G. E. *Int. J. Pept. Protein Res.* **1994**, *43*, 555−562.
(58) Chorev, M.; Roubini, E.; McKee, R. L.; Gibbons, S. W.; Goldman, M. E.; Caufield, M. P.; Rosenblatt, M. *Biochemistry* **1991**, *30*, 5968−5974.
(59) Maretto, S.; Schievano, E.; Mammi, S.; Bisello, A.; Nakamoto, C.; Rosenblatt, M.; Chorev, M.; Peggion, E. *J. Pept. Res.* **1998**, *52*, 241−248.
(60) Mierke, D. F.; Maretto, S.; Schievano, E.; DeLuca, D.; Bisello, A.; Mammi, S.; Rosenblatt, M.; Peggion, E.; Chorev, M. *Biochemistry* **1997**, *36*, 10372−10383.
(61) Maretto, S.; Mammi, S.; Bissacco, E.; Peggion, E.; Bisello, A.; Rosenblatt, M.; Chorev, M.; Mierke, D. F. *Biochemistry* **1997**, *36*, 3300−3307.
(62) Bisello, A.; Nakamoto, C.; Rosenblatt, M.; Chorev, M. *Biochemistry* **1997**, *36*, 3293−3299.
(63) Kates, S. A.; Daniels, S. B.; Solé, N. A.; Barany, G.; Albericio, F. In *Pept.: Chem., Struct., Biol., Proc. Am. Pept. Symp.*; Hodges, R. S., Smith, J. A., Eds.; ESCOM: Leiden, The Netherlands, 1994; pp 113−115.
(64) Coligan, J. E.; Dunn, B. M.; Ploegh, H. L.; Speicher, D. W.; Wingfiled, P. T. In *Current Protocols in Protein Science*; Chanda, V. B., Ed.; John Wiley & Sons: New York, 1995; Vol. 1, Chapter 11.1.1.
(65) Kumar, N. V.; Krolikowski, P. H. Rhône-Poulenc Rorer, Collegeville, PA, unpublished result.

**Circular Dichroism Spectroscopy.** CD spectroscopy was performed on a Jasco J720 Instrument at room temperature in a 0.2-mm path length cell (Wistar Institute, Philadelphia, PA). Doubly distilled water, HPLC grade acetonitrile (ACN), and spectroscopy grade trifluoroethanol (TFE) were used as solvents. Circular dichroism analyses were performed on the linear, monocyclic, bicyclic, and tricyclic peptides at a concentration of ~40 $\mu$M as determined by amino acid analysis and at a pH of 3.5−3.7. Owing to the limited solubility of the polycyclic peptides in water, 20% ACN in water solutions were used to optimize peptide solubilities without altering their secondary structure as observed during TFE titrations. All spectral data were collected at ambient temperature. As was done by Barbier et al.,[56] the relative transition to maximum helicity was monitored by the following: (1) the measured negative ellipticity at 222 nm; and (2) the ratio of the absolute ellipticities measured at 191−193 ($\pi-\pi_\perp$*), 208−210 ($\pi-\pi_\parallel$*), and 222 (n−$\pi$*) nm. These values were then compared with those reported for a standard α-helix of similar length in an aqueous environment (Table 2 and Supporting Information).

**Adenylyl Cyclase Stimulation.** ROS 17/2.8 cells were plated at 1 × $10^5$ cells/well in 24 well plates. After 3−5 days, the culture medium was aspirated and replaced with 0.5 mL of the cAMP assay buffer, which contained Ham's F12 medium, 2 mM IBMX, 1 mg/mL BSA, 35 mM HEPES, and 20 $\mu$g/mL ascorbic acid. The cells were equilibrated in the assay buffer by incubating the cells for 30 min at 37 °C. The cAMP buffer was then aspirated. The PTH analogues were diluted in the assay buffer at several concentrations and added to the wells. The cells were then incubated at 37 °C for 20 min. Following incubation, the cells were solubilized by the addition of 1% Triton. Total cAMP was measured using a cAMP Scintillation Proximity Assay screening system (Amersham, Arlington Heights, IL) and the samples were counted using a Wallac microtiter plate scintillation counter. Data represent mean cAMP values of duplicate samples ± SD. $EC_{50}$ values were defined as the concentration required to elicit half-maximal stimulation and were calculated using a four-parameter fit equation.

**PTH/PTHrP Receptor Binding.** HEK293 cells expressing the recombinant hPTH/hPTHrP receptor (M. Rosenblatt, Harvard Medical School, Cambridge, MA) were plated in poly(D-lysine)-coated 96-well plates (Becton Dickinson, Bedford, MA) at a cell concentration of 4 × $10^4$ cells/well. The cells were maintained in DMEM (Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated FBS, 500 $\mu$g/mL geneticin (G418 continuous selection), and 25 mM HEPES for 18−24 h prior to performing the binding studies. The PTH analogues were diluted in the assay buffer at various concentrations and added to the wells along with 1.8 × $10^5$ dpm of $^{125}$I-labeled [Nle$^{8,18}$,Tyr$^{34}$]hPTH-(1−34) peptide (Amersham Life Sciences). The cells were then incubated for 2 h at 25 °C at which point the reaction mixtures were aspirated and the cells were washed four times with ice-cold buffer solution. Cell-bound radioactivity was counted by the addition of 90 $\mu$L of scintillation cocktail (Packard, Meriden, CT). The plates were sealed and counted for Auger electron radiation in a Microbeta 1450 scintillation counter (EG & G Wallac, Turku, Finland).

**Molecular Modeling.** Interactive modeling utilized InsightII and Discover (MSI Inc., San Diego, CA). The PDB code 1HPH, NMR structure from Marx et al.[27] containing 10 models (Protein Data Bank, Brookhaven), was first used to rationalize the structure−activity relationships that could be deduced from the literature. From these 10 models, which all display a well-defined α-helix in the C-terminal region, it was possible to construct the cyclo(Lys$^{26}$−Asp$^{30}$) lactam bridge as well as the cyclo(Lys$^{27}$−Asp$^{30}$) lactam. This model was also used to evaluate incorporation of the cyclo(Lys$^{18}$−Asp$^{22}$) lactam constraint into the hPTH sequence. The cyclo(Lys$^{13}$−Asp$^{17}$) lactam bridge described by Chorev et al. for the PTHR antagonist, cyclo-(Lys$^{13}$−Asp$^{17}$)hPTHrP(7−34)$NH_2$,[58] however, was clearly incompatible with these NMR-derived models. With the aid of the α-carbon model of hPTHrP(1−34)OH developed by Wallqvist and Ullner,[66] a second model was constructed which included the (Lys$^{13}$−Asp$^{17}$) lactam constrained in a helical conformation. Subsequent models were generated for the polylactam-containing peptides using these localized lactam constraints. Other extended helical conformations of hPTH, hPTHrP, and lactam analogues were built in InsightII and minimized in Discover using the CVFF force field.

## Results and Discussion

Since the first isolation of PTH from parathyroid extracts to the first total synthesis using solid-phase techniques, the relevant bioactive conformation of hPTH(1−34) has remained elusive despite the use of a variety of techniques including NMR spectroscopy, circular dichroism, molecular modeling, and site-directed mutagenesis. Our interest in the bioactive conformation of hPTH evolved from our efforts to develop a novel PTH-derived therapeutic agent capable of eliciting an anabolic response in patients with demonstrated osteoporosis. As such, further resolution of the questions surrounding the bioactive receptor binding conformation of hPTH was expected to critically aid this effort.

From the outset, we chose the 31-residue peptide for further modification since Whitfield and co-workers had demonstrated that hPTH(1−31)$NH_2$ (**1**) could stimulate bone growth in the OVX rat model of osteoporosis.[12,67] Our design efforts began with the recently published NMR-derived solution structure of hPTH(1−37) by Marx and co-workers which presented the total peptide in the paired-helix context with the two helices separated by a turn region defined by residues 10−17.[27] In addition, five specific residues were selected for replacement: the methionine residues at positions 8 and 18 (Met$^8$ to Nle$^8$ and Met$^{18}$ to Lys$^{18}$) because oxidation of these residues has been associated with reduced in vitro activity;[68] Lys$^{27}$-to-Leu$^{27}$ substitution since this has been shown to be an effective strategy to increase receptor binding and activation by stabilizing the hydrophobic face of the C-terminal amphiphilic helix;[57] replacement of Ser$^1$ with Ala$^1$ to increase AC activity in the in vitro assay and to further simplify substitution at this position; and, Glu$^{22}$ to Asp$^{22}$ to afford better stability of the putative α-helix (vide infra).[69]

To further stabilize or even enhance the desired helical elements at the C-terminus of hPTH(1−31)$NH_2$, we focused our strategy on the introduction of a side chain-to-side chain lactam bridge between residues Lys$^{18}$ (mutated from Met$^{18}$) and Asp$^{22}$ (mutated from Glu$^{22}$).[70] Lactam bridge stabilization is a well-known method by which the bioactive conformation of peptides can be deduced.[16,53,55,71] Chorev and co-workers demonstrated that cyclization between the naturally occurring Lys$^{13}$ and Asp$^{17}$ residues of hPTHrP is an effective strategy to stabilize a potent antagonist of the PTH/PTHrP receptor, cyclo-(Lys$^{13}$−Asp$^{17}$)hPTHrP(7−34)$NH_2$,[58] and recently applied this strategy to a number of agonist ligands.[62] Whitfield and co-workers prepared other lactam-stabilized analogues of hPTH based on the positioning of putative salt bridges within the natural sequence such as cyclo(Glu$^{22}$−Lys$^{26}$)-, cyclo(Lys$^{26}$−Asp$^{30}$)-, and cyclo(Lys$^{27}$−Asp$^{30}$)hPTH derivatives.[56] We selected the cyclo(Lys$^{18}$−Asp$^{22}$) bridge owing to its location at the proximal end of the C-terminal helix defined by the Marx NMR structure (Figure 2). In addition, researchers at Sandoz demonstrated that this midregion of the peptide is quite tolerant toward alanine substitution; thus, we hypothesized that a lactam

(66) Wallquist, A.; Ullner, M. *Proteins: Struct., Funct., Genet.* **1994**, *18*, 267−280.

(67) Whitfield, J. F.; Morley, P.; Willick, G. E.; Ross, V.; Langille, R.; MacLean, S.; Barbier, J.-R.; Isaacs, R. J.; Ohannessian-Barry, L. *Calcif. Tissue Int.* **1997**, *61*, 322−326.

(68) Zull, J. E.; Smith, S. K.; Wiltshire, R. *J. Biol. Chem.* **1990**, *265*, 5671−5676.

(69) Ösapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 6966−6973 and references therein.

(70) Houston, M. E., Jr.; Gannon, C. L.; Kay, C. M.; Hodges, R. S. *J. Pept. Sci.* **1995**, *1*, 274−282.

(71) Kapurniotu, A.; Taylor, J. W. *J. Med. Chem.* **1995**, *38*, 836−847.

**Table 1.** Biological Activities of hPTH(1−34)OH and hPTH Analogues **1**−**9**

| peptide | AC activity $EC_{50}$, nM | receptor binding $IC_{50}$, nM |
|---|---|---|
| hPTH(1−34)OH | 3.3 | |
| hPTH(1−31)$NH_2$ (**1**) | 4.7 | |
| cyclo(Lys[18]−Asp[22])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−31)$NH_2$ (**2**) | 0.29 | 11.3 ± 5.2 |
| [Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−31)$NH_2$ (**3**) | 0.6 | 16.3 ± 10.4 |
| cyclo(Lys[18]−Asp[22])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−34)$NH_2$ (**4**) | 1.6 | 10.4 ± 4.7 |
| cyclo(Lys[18]−Asp[22])[Lys[18],Asp[22]]hPTHrP(1−34)$NH_2$ (**5**) | 0.3 | 6.8 ± 5.1 |
| bicyclo(Lys[18]−Asp[22],Lys[26]−Asp[30])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−31)$NH_2$ (**6**) | 0.13 | 7.9 ± 7.9 |
| bicyclo(Lys[13]−Asp[17],Lys[18]−Asp[22])[Ala[1],Nle[8],Lys[18],Asp[17,22],Leu[27]]hPTH(1−31)$NH_2$ (**7**) | 0.43 | 6.2 ± 3.3 |
| bicyclo(Lys[13]−Asp[17],Lys[26]−Asp[30])[Ala[1],Nle[8,18],Asp[17],Leu[27]]hPTH(1−31)$NH_2$ (**8**) | 0.35 | 9.0 ± 3.1 |
| tricyclo(Lys[13]−Asp[17],Lys[18]−Asp[22],Lys[26]−Asp[30])[Ala[1],Nle[8],Lys[18],Asp[17,22],Leu[27]]hPTH(1−31)$NH_2$ (**9**) | 0.14 | 20.8 ± 13.0 |

**Table 2.** Measured Ellipticities for Human Parathyroid Hormone Analogues at 0 and 40% TFE[a,b]

| peptide | % TFE[c] | $[\Theta]_{192} \times 10^3$ | $-[\Theta]_{209} \times 10^3$ | $-[\Theta]_{222} \times 10^3$ | $\lvert[\Theta]_{192}/[\Theta]_{222}\rvert$ | $\lvert[\Theta]_{222}/[\Theta]_{209}\rvert$ |
|---|---|---|---|---|---|---|
| ideal helix[d] | | | | | 2.63 | 1.09 |
| hPTH(1−31)$NH_2$ (**1**) | 0 | 23.40 | 21.73 | 19.35 | 1.209 | 0.8905 |
| | 40 | 55.70 | 34.09 | 31.20 | 1.785 | 0.9152 |
| cyclo(Lys[18]−Asp[22])-**2** | 0 | 30.55 | 21.61 | 20.51 | 1.490 | 0.9491 |
| | 40 | 56.12 | 33.47 | 29.89 | 1.878 | 0.8930 |
| [Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−31)$NH_2$-**3** | 0 | 12.76 | 9.379 | 6.649 | 1.910 | 0.7089 |
| | 40 | 65.70 | 37.13 | 33.37 | 1.969 | 0.8987 |
| bicyclo(Lys[18]−Asp[22],Lys[26]−Asp[30])-**6** | 0 | 30.52 | 20.41 | 19.07 | 1.600 | 0.9343 |
| | 40 | 52.65 | 29.81 | 27.57 | 1.910 | 0.9249 |
| bicyclo(Lys[13]−Asp[17],Lys[18]−Asp[22])-**7** | 0 | 34.69 | 22.04 | 20.43 | 1.698 | 0.9270 |
| | 40 | 55.65 | 31.41 | 28.44 | 1.957 | 0.9054 |
| tricyclo(Lys[13]−Asp[17],Lys[18]−Asp[22],Lys[26]−Asp[30])-**9** | 0 | 79.66 | 37.78 | 35.66 | 2.234 | 0.9439 |
| | 40 | 103.4 | 47.88 | 44.54 | 2.322 | 0.9302 |

[a] Reported as deg·cm$^2$·dmol$^{-1}$. [b] For a complete listing of measured ellipticity values at 0, 10, 20, 30, and 40% TFE, see Supporting Information. [c] Spectra were measured in 20% ACN/water at ∼40 $\mu$M, ambient temperature. [d] See: Barbier et al. *J. Med. Chem.* **1997**, *40*, 1373−1380 and references therein.

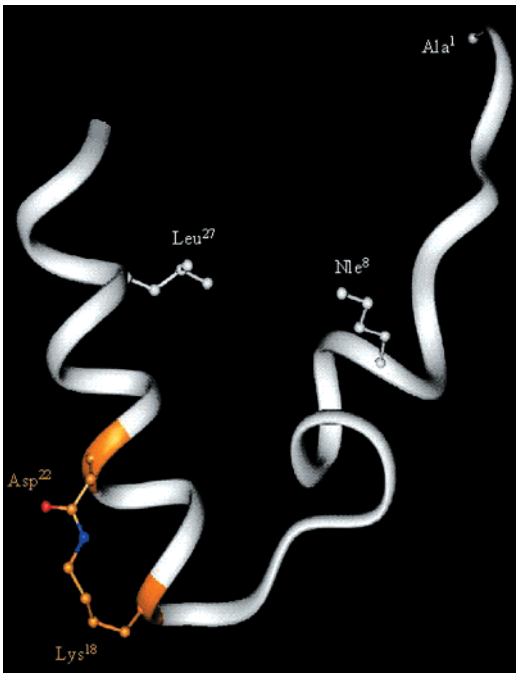


**Figure 2.** Schematic representation of cyclo(Lys[18]−Asp[22])-**2**. The peptide backbone is shown as a white ribbon with the five point mutations indicated. The lactam bridge between residues Lys[18] and Asp[22] (orange) was constructed using model 5 from Marx et al.[27] and locally minimized.

constraint would be similarly well-tolerated.[72] We recognized that the naturally occurring residues, Met[18] and Glu[22], are incapable of stabilizing an intramolecular salt bridge and as such the placement of this conformational constraint was considered to be novel.

Cyclo(Lys[18]−Asp[22])[Ala[1],Nle[8],Lys[18],Asp[22],Leu[27]]hPTH(1−31)$NH_2$ (**2**) is a potent agonist of the PTH/PTHrP receptor displayed on the surface of rat osteosarcoma cells (ROS 17/2.8) with an $EC_{50}$ of 0.29 nM (Table 1). By comparison, hPTH-(1−34)OH and hPTH(1−31)$NH_2$ (**1**) were less potent in this ROS cell-based assay ($EC_{50}$'s = 3.3 and 4.7 nM, respectively). This result clearly attests to the tolerance of the receptor to the five point mutations and (Lys[18]−Asp[22]) lactam bridge present in this analogue.

To establish whether this increase in agonist activity is, at least in part, due to the presence of the conformational constraint, we prepared the linear analogue **3** that incorporates each of the five mutations from the natural sequence. Peptide **3** is also a potent agonist ($EC_{50}$ = 0.6 nM) although moderately less active than its cyclized counterpart **2**. Comparison of the CD spectra of **1** and peptide **3** reveals that the average solution conformation of **3** is significantly less helical in 0 and 10% TFE in 20% ACN/water (Supporting Information). This loss of helical character ($[\Theta]_{222}$ = −6649 deg·cm$^2$·dmol$^{-1}$ for **3** vs $[\Theta]_{222}$ = −19 350 deg·cm$^2$·dmol$^{-1}$ for **1**, Table 2) is accompanied by a blue shift in the negative $\pi-\pi_{||}$* transition band at ∼209 nm, which may represent contributions from alternative solution conformations.

The mutation of Lys[27] to Leu[27] is known to increase the helical character of hPTH analogues in solution owing to its position on the hydrophobic face of the C-terminal amphiphilic helix.[73] The mutations, Glu[22] to Asp[22], Met[18] to Nle[18], and Ser[1] to Ala[1], were not anticipated to have any dramatic effects on

(72) Gombert, F. O.; Gamse, R.; Feyen, J. H. M.; Cardinaux, F. In *Pept.: Chem., Struct., Biol., Proc. Am. Pept. Symp.;* Kaumaya, T. P. P., Hodges, R. S., Eds.; Mayflower Scientific Ltd.: Kingswinford, U.K., 1996; pp 661−662.

(73) Surewicz, W. K.; Neugebauer, W.; Gagnon, L.; MacLean, S.; Whitfield, J. F.; Willick, G. In *Pept.: Chem., Struct., Biol., Proc. Am. Pept. Symp.*; Hodges, R. S., Smith, J. A., Eds.; ESCOM: Leiden, The Netherlands, 1993; pp 556−558.

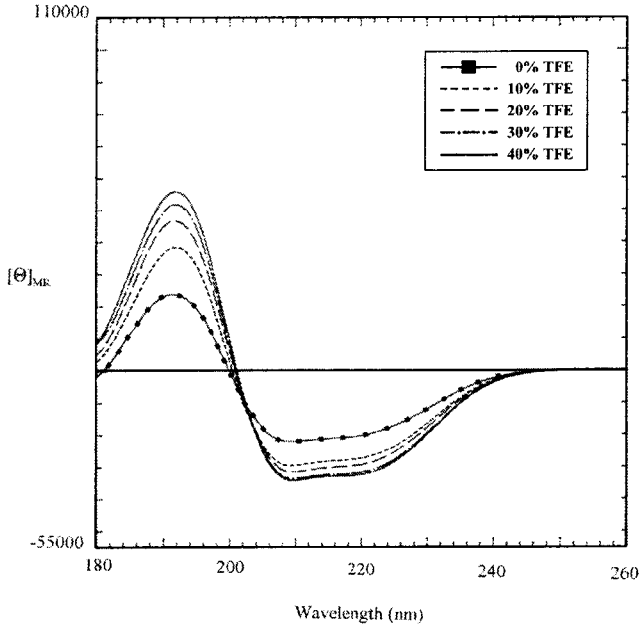


**Figure 3.** CD spectra of hPTH(1−31)$NH_2$ (**1**) in 20% ACN/water at various TFE concentrations (% v/v as indicated). Peptide concentration ~40 $\mu$M. Mean residue ellipticity, [Θ], reported in deg·cm$^2$·dmol$^{-1}$.

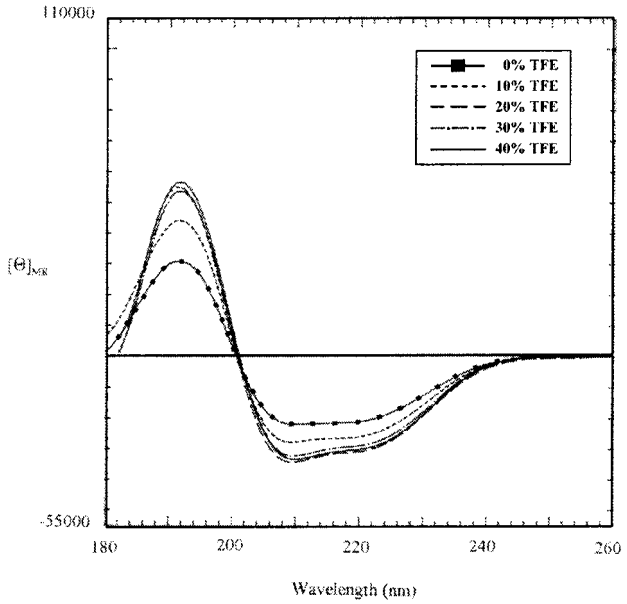


**Figure 4.** CD spectra of cyclo(Lys$^{18}$−Asp$^{22}$)-**2** in 20% ACN/water at various TFE concentrations (% v/v as indicated). Peptide concentration ~40 $\mu$M. Mean residue ellipticity, [Θ], reported in deg·cm$^2$·dmol$^{-1}$.

either solution conformation or agonist activity owing to the isosteric and isoelectronic equivalence of these residue pairs. Incorporation of a Lys residue at position 18 may be less tolerated however; the natural hormone contains the hydrophobic Met residue at this position. It has been suggested that the hydrophilic face of the C-terminal α-helix is partly stabilized by an intramolecular hydrogen-bonding network established by polar residues including Arg$^{20}$, Asp$^{22}$, Arg$^{25}$, Lys$^{26}$, Lys$^{27}$, and Asp$^{30}$. In fact, an analogue of hPTHrP(1−34), which has established anabolic activity in the OVX rat model, RS-66271,[74] replaces the amphiphilic α-helix with the model amphiphilic helix (MAP) Glu$^{22}$-Leu-Leu-Glu-Lys-Leu-Leu-Glu-Lys-Leu$^{31}$ amide, which places alternating charged residues on the solvent-exposed face of the C-terminus to stabilize the helical structure of the receptor binding domain. Lys$^{18}$ may disrupt this charge network and thus contribute to the higher degree of disorder observed in the CD spectrum of **3** at low TFE concentrations.

Lactamization at 18−22 removes the potentially destabilizing charged residue at position 18 and restores the helical nature of the peptide as observed by CD spectroscopy. In fact, the CD curves of both hPTH(1−31)$NH_2$ (**1**) and cyclo(Lys$^{18}$−Asp$^{22}$)-**2** are nearly identical across the TFE titration (Figures 3 and 4, respectively). We have attributed therefore the improved in vitro performance of **2** primarily to the five point mutations present in peptide **3**. Intramolecular (Lys$^{18}$−Asp$^{22}$) cyclization can reestablish the helical character at the C-terminus of **2** as observed by CD spectroscopy and may also contribute to nucleation of the secondary structure through the midregion of the peptide toward the N-terminus.

The incorporation of the cyclo(Lys$^{18}$−Asp$^{22}$) lactam bridge into the two full-length 34-mers, cyclo(Lys$^{18}$−Asp$^{22}$)[Ala$^1$,Nle$^8$,-Lys$^{18}$,Asp$^{22}$,Leu$^{27}$]hPTH(1−34)$NH_2$ (**4**, $EC_{50}$ = 1.6 nM) and cyclo(Lys$^{18}$−Asp$^{22}$)[Lys$^{18}$,Asp$^{22}$]hPTHrP(1−34)$NH_2$ (**5**, $EC_{50}$ = 0.3 nM), also provided potent receptor agonists. Recently, Mierke and Pellegrini investigated the bioactive conformation of hPTHrP(1−34) using a variety of techniques including synthetic analogues, photoaffinity cross-linking, and molecular simulations, thus providing greater insight into the bioactive conformation of this peptide.[49] They concluded, on the basis of the binding and receptor activation for a number of linear and constrained PTH/PTHrP receptor agonists and antagonists, that hPTHrP interacts with its receptor through a series of helical domains separated by well-defined hinge regions such as the one speculated to occur around Arg$^{19}$ in hPTHrP. Of particular note therefore is the highly potent hPTHrP analogues that places the putative hinge residue, Arg$^{19}$, within the confines of the Lys$^{18}$−Asp$^{22}$ lactam bridge, demonstrating that potent agonists can be generated by reducing the flexibility of the peptide within this area.

Barbier et al. demonstrated that cyclization of the side chain residues of Lys$^{26}$ and Asp$^{30}$ in [Leu$^{27}$]hPTH(1−31)$NH_2$ results in a peptide, cyclo(Lys$^{26}$−Asp$^{30}$)[Leu$^{27}$]hPTH(1−31)$NH_2$, with modestly reduced in vitro activity when compared with its noncyclized counterpart ($EC_{50}$ = 17.0 ± 3.3 vs 11.5 ± 5.2 nM, respectively) despite its apparent increase in helicity as observed by CD spectroscopy in aqueous solution.[56] The combination of the 18−22 lactam with the previously demonstrated Lys$^{26}$−Asp$^{30}$ lactam was expected to lock the entire C-terminal receptor-binding domain into a helical conformation. In the absence of TFE, the absorption at 222 nm observed for bicyclo-(Lys$^{18}$−Asp$^{22}$,Lys$^{26}$−Asp$^{30}$)-**6** is comparable to that observed for **1** (Supporting Information). Titration with TFE affords almost identical CD spectra for these two analogues. The ability of bicyclic analogue **6** to act as an agonist ($EC_{50}$ = 0.13 nM) demonstrates the high level of conformational constraint tolerated in the receptor-binding domain of the hormone.

We next moved the constraining element toward the midregion of the peptide. Like the N-terminus, the conformation of the midregion of hPTH(1−34) that connects the receptor-binding and activation domains has been the subject of much conjecture and recent debate. Nuclear magnetic resonance-derived structures have provided some support for a $\beta$-turn or flexible-linker region existing between residues 10 and 17 while others have argued against these conclusions.[20] Chorev et al. prepared highly potent antagonists of the PTH/PTHrP receptor which incorporate a side chain-to-side chain lactam bridge from

(74) Vickery, B. H.; Avnur, Z.; Cheng, Y.; Chiou, S.-S.; Leaffer, D.; Caulfield, J. P.; Kimmel, D. B.; Ho, T.; Krstenansky, J. L. *J. Bone Miner. Res.* **1996**, *11*, 1943−1951.

Lys[13] to Asp[17] of PTHrP(7−34)$NH_2$.[58] Using CD and NMR spectroscopy, they observed a bend motif centered about residues Asp[17]-Leu[18]-Arg[19]-Arg[20] in addition to the C-terminal helix and midregion helix promoted by the lactam bridge.[61] From NMR measurements and distance geometry calculations, they proposed the hinge regions about Arg[19] and Gly[12] as determining factors for the optimization of ligand binding and receptor activation for both linear and constrained agonists and antagonists.[60]

We prepared the second bicyclic analogue, bicyclo(Lys[13]−Asp[17],Lys[18]−Asp[22])-**7**, to evaluate the effect of adjacent lactam constraints within this critical region of PTH and PTH-related peptides. Peptide **7**, possessing 10 contiguous residues under conformational constraint, is fully capable of eliciting a potent response in the ROS cAMP-based assay ($EC_{50} = 0.43$ nM) and displays excellent binding affinity ($IC_{50} = 6.2 \pm 3.3$ nM).

Surprisingly, although this analogue is nearly 1 order of magnitude more potent than linear **1** in the AC-based assay, its solution conformation as measured by CD spectroscopy is nearly superimposable with the linear peptide **1** (Supporting Information). In fact, comparison of the CD spectra recorded for peptides **1**, **2**, **6**, and **7** reveals remarkably similar behavior in 20% ACN/water as well as in the presence of differing concentrations of TFE despite the apparent increased in vitro activity for the lactam-containing analogues. This observation underscores the limited utility of overall helicity, as determined by CD spectroscopy, as the sole predictor of in vitro AC activity in the ROS cell-based assay and, thus, as the primary determinant of bioactive conformation. The combination of structural constraint, in vitro agonist activity, and CD analysis must be used cooperatively to ascertain the important structural elements required for biological activity (vide infra).

Moving one of the lactam constraints toward the C-terminus of the molecule affords bicyclo(Lys[13]−Asp[17],Lys[26]−Asp[30])-[Ala[1],Nle[8,18],Asp[17],Leu[27]]hPTH(1−31)$NH_2$ (**8**, $EC_{50} = 0.35$ nM), which maintains much of the activity of peptide **7**. Interestingly, after this investigation, Bisello et al. reported the biological activity of a similarly constrained analogue of hPTHrP, bicyclo(Lys[13]−Asp[17],Lys[26]−Asp[30])[Lys[26],Asp[30]]-hPTHrP(1−34)$NH_2$, to be 2−3 times more efficacious than the linear parent peptide for agonist-stimulated adenylyl cyclase activity in Saos-2/B-10 cells and nearly equipotent to the monocyclic (Lys[13]−Asp[17])-containing analogue.[62] They thus classified the C-terminal (Lys[26]−Asp[30]) lactam bridge as a "silent" modification in PTHrP since it imparted no greater agonist activity to the peptide when compared with the midregion lactam-containing analogue.

Finally, we incorporated all three [*i*, *i* + 4] lactam constraints into a single peptide, tricyclo(Lys[13]−Asp[17],Lys[18]−Asp[22],Lys[26]−Asp[30])-**9**. Peptide **9** represents the most structurally constrained analogue of PTH yet reported. Its activity in the ROS 17/2.8 cell-based AC assay parallels that of the bicyclic (Lys[18]−Asp[22],−Lys[26]−Asp[30])-containing derivative (**6**) and as such is one of the most potent analogues discovered in this investigation ($EC_{50} = 0.14$ nM). Peptide **9** is 2−3 times more potent than either the monocyclic (Lys[18]−Asp[22])-containing peptide (**2**), or the two bicyclic analogues, bicyclo(Lys[13]−Asp[17],Lys[18]−Asp[22])-**7** and bicyclo(Lys[13]−Asp[17],Lys[26]−Asp[30])-**8**, which suggests an underlying cooperativity between the 18−22 and 26−30 lactam bridges. The observation that the 13−17 bridge within the hPTH series, as opposed to the hPTHrP series (vide supra), takes on the role as the "silent" modification may suggest a subtle conformational distinction between the agonist binding modes of these two similar hormones.

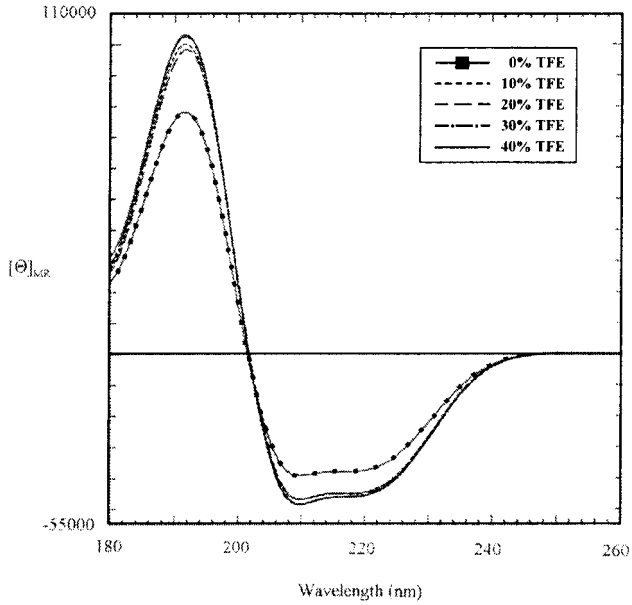


**Figure 5.** CD spectra of tricyclo(Lys[13]−Asp[17],Lys[18]−Asp[22],Lys[26]−Asp[30])-**9** in 20% ACN/water at various TFE concentrations (% v/v as indicated). Peptide concentration ∼40 $\mu$M. Mean residue ellipticity, [Θ], reported in deg·cm$^2$·dmol$^{-1}$.

To further evaluate the solution structure of this analogue, we compared the CD spectra of linear **1** with that obtained for the tris-lactam-containing peptide **9** in 20% ACN/water and at various concentrations of TFE (Figures 3 and 5, respectively). The CD spectra for linear peptide **1** presents the classical pattern of a linear peptide undergoing a two-state random coil-to-helix transition with increasing amounts of TFE as evidenced by the established isodichroic point throughout the titration. Perhaps not surprisingly, this amphiphilic peptide displays a good deal of helical character even in 20% ACN/water in the absence of TFE; however, upon the portionwise addition of TFE, the helical nature of the peptide is reinforced in an almost linear fashion, as measured by the increase in the negative n−$\pi$* absorption at 222 nm, until saturation is finally achieved around 30% TFE. At 0% TFE, peptide **9** appears to be much more helical when comparing not only the negative ellipticity at 222 nm but also the absolute ratio of magnitudes for the $\pi-\pi$* and n−$\pi$* absorptions at 190 and 222 nm, respectively. Upon the addition of only 10% TFE, the peptide achieves maximum helical character which is unaffected by the further addition of TFE.

Because the reduced flexibility of constrained analogues makes them superior reporters of the biologically relevant conformations in ligand−receptor interactions, the improved agonist activity achieved by peptide **9** and the high degree of helicity measured by CD spectroscopy provides direct synthetic evidence that hPTH must bind to its receptor in an extended helical conformation (Figure 6) as opposed to the "U-shaped" or paired-helix conformation as previously described (Figure 2). These observations together with those reported for bicyclic peptides **6**, **7**, and **8** provide strong support for a single extended helical bioactive conformation of hPTH, extending from the N-terminus to the C-terminus of the peptide, and further strengthens the proposal that hPTHrP(1−34) binds in a similar fashion.

## Conclusion

Owing to the therapeutic potential of the osteogenic peptides, PTH and PTHrP, as a treatment for type I osteoporosis, a detailed understanding of their active conformations is a

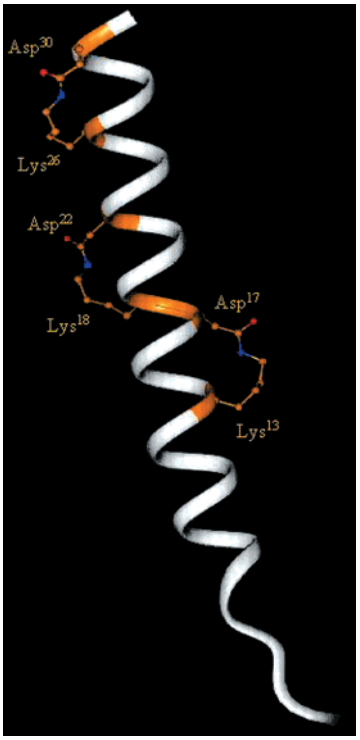


**Figure 6.** Schematic representation of tricyclo(Lys$^{13}$−Asp$^{17}$,Lys$^{18}$−Asp$^{22}$,Lys$^{26}$−Asp$^{30}$)-**9**. The peptide backbone is shown as a white ribbon. The three lactam bridges are indicated in orange.

prerequisite for defining their mechanism(s) of action as well as for the development of subsequent agents with improved pharmacological profiles. The isolation of a second, PTH-selective receptor (PTHR2) suggests that other isoforms of the PTH/PTHrP receptor may yet to be discovered. How these peptides interact across a variety of receptor subtypes, therefore, will be important for understanding their recognized biological activities.[75] Although solution-based structural studies in the absence and presence of membrane mimics are a valuable tool to gain insight into the receptor-bound conformation of medium-sized, linear peptides, the use of structurally restricted analogues provides a more in-depth appreciation of the receptor's acceptance or rejection of specific conformational motifs encoded by the peptide's primary sequence. This investigation has provided a number of unique, conformationally constrained analogues of hPTH(1−31)NH$_2$ (**1**), the shortest fragment of PTH with recognized osteogenic activity. From in vitro analysis in the ROS cell line, we have determined that the receptor is fully activated by a peptide (**9**, $EC_{50}$ = 0.14 nM) that effectively restricts residues 13−30 by three [*i*, *i* + 4] side chain-to-side chain lactam bridges. Spectroscopic (CD) analysis has determined that the peptide is highly helical without TFE and apparently maximally helical in 10% TFE solution or at the quartz−water interface,[76] which affords further support for the extended helix as the relevant bioactive conformation for hPTH. Further in vitro and in vivo analysis of the more highly constrained analogues should provide a greater appreciation for receptor selectivity (PTHR1, PTHR2), signal transduction mechanisms (AC, PLC/PKC), and osteogenesis.

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**Supporting Information Available:** Full analytical data for peptides **2**, **3**, **6**, **7**, and **9** including amino acid analysis and peptide mapping via tryptic digest (Tables 3 and 4); a complete listing of the mean residue ellipticities measured at 191−193, 209, and 222 nm as well as the absolute ratios, $[\Theta]_{192}/[\Theta]_{222}$ and $[\Theta]_{222}/[\Theta]_{209}$, for peptides **1**, **2**, **3**, **6**, **7**, and **9** at various concentrations of TFE (Table 5); CD spectra at increasing concentrations of TFE for peptides **3**, **6**, and **7;** and the complete experimental procedure for the preparation of peptide **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(75) Benzinger, T. L. S.; Braddock, D. T.; Dominguez, S. R.; Burkoth, T. S.; Miller-Auer, H.; Subramanian, R. M.; Fless, G. M.; Jones, D. N. M.; Lynn, D. G.; Meredith, S. C. *Biochemistry* **1998**, *37*, 13222−13229.

(76) Taylor, J. W. In *The Amphipathic Helix*; Epand, R. M., Ed.; CRC Press, Inc.: Boca Raton, FL, 1993; pp 285−311.