Probing the Bimolecular Interactions of Parathyroid Hormone with the Human Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor. 1. Design, Synthesis and Characterization of Photoreactive Benzophenone-Containing Analogs of Parathyroid Hormone†

Chizu Nakamoto, Vered Behar, Kingsley R. Chin, Amy E. Adams, Larry J. Suva, Michael Rosenblatt, and Michael Chorev*

Division of Bone and Mineral Metabolism, Harvard-Thorndike and Charles A. Dana Laboratories, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215

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ABSTRACT: Parathyroid hormone (PTH) regulates calcium and phosphate metabolism through a G-protein-coupled receptor which is shared with PTH-related protein (PTHrP). Therefore, structure−activity studies of PTH and PTHrP with their common receptor provide an unusual opportunity to examine the structural elements in the two hormones and their common receptor which are involved in the expression of biological activity. Our approach to studying the nature of the bimolecular interface between hormone and receptor is to use a series of specially designed photoreactive benzophenone- (BP-) containing PTH analogs in "photoaffinity scanning" of the PTH/PTHrP receptor. In this report we describe a series of BP-containing agonists and antagonists which have been synthesized by solid-phase methodology and characterized physicochemically and biologically. Each of the 12 analogs contains a single BP moiety at a different defined position. Examples of BP-containing agonists prepared and studied in human osteogenic sarcoma Saos-2/B-10 cells are [Nle^{8,18},Lys^{13}(ϵ-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (K13) (K_b = 13 nM; K_m = 2.7 nM) and [Nle^{8,18},L-Bpa^{23},Tyr^{34}[bPTH(1−34)NH$_2$ (L-Bpa23) (K_b = 42 nM; K_m = 8.5 nM). Another BP-containing analog, [Nle^{8,18},D-2-Nal^{12},Lys^{13}(ϵ-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(7−34)NH$_2$, was a potent antagonist (K_b = 95 nM; K_i = 72 nM). The amino acids substituted by residues carrying the BP moiety span the biologically active domain of the hormone (Phe^7, Gly^{12}, Lys^{13}, Trp^{23}, and Lys^{26}). Analysis of photo-cross-linked conjugates of PTH/PTHrP receptor with BP-containing PTH analogs should help to identify the "contact points" between ligand and receptor.

Progress in structure−activity studies of parathyroid hormone (PTH)[1] and PTH-related protein (PTHrP) have identified broadly functional domains within the hormones, including receptor binding and activation sequences (Caporale & Rosenblatt, 1986; Rosenblatt, 1986; Kemp et al., 1987; Gardella et al., 1993; Chorev & Rosenblatt, 1994). These studies yielded highly active hormone antagonists, as well as partial agonists, and have delineated the structural requirements for these categories of receptor ligand.

Recent cloning of the PTH/PTHrP receptor (Rc) from several species, including human (Juppner et al., 1991; Abou-Samra et al., 1992; Adams et al., 1993; Schipani et al., 1993), has allowed study of the biological effects of point mutations and deletions in the PTH/PTHrP Rc (Kaufmann et al., 1994; Lee et al., 1994). Chimeric Rcs, composed of portions of PTH/PTHrP Rcs from different species (Kaufmann et al., 1994; Juppner et al., 1993) or combinations of the PTH/PTHrP and evolutionarily related calcitonin Rcs (Juppner et al., 1993) have been assembled. These studies of recombinant Rcs offer important new insights into ligand−receptor interactions for the PTH/PTHrP system.

However, both the hormone structure−activity relations and receptor site-directed mutagenesis approaches have a similar limitation. At best, they can provide only indirect and inferential information about the nature of the bimolecular interaction between hormone and Rc and cannot define precisely the complementary domains in the apposing partner involved in the bimolecular interaction. Ultimately, insights obtained from either approach require independent validation.

One approach to map directly the ligand−receptor bimolecular interface is to identify "contact points" by covalently cross-linking hormone to Rc when the two are in close

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* Address correspondence to this author at the Division of Bone and Mineral Metabolism (RW-563), Beth Israel Hospital, Harvard Medical School, 330 Longwood Ave., Boston, MA 02215.

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[1] Abbreviations used are in accordance with the recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature (1975) *J. Biol. Chem. 250,* 3215−3216 and (1985) *J. Biol. Chem. 260,* 14−42, and IUPAC−CNOC (1979) *Eur. J. Biochem. 86,* 9−25. In addition: Aib, α-aminoisobutyryl; Boc, *N-tert*-butyloxycarbonyl; BP, benzophenone; Bom, benzyloxymethyl; Bpa, *p*-benzoylphenylalanine; *p*Bz$_2$, *p*-benzoylbenzoyl; 2-Br-Z, [(*o*-bromobenzyl)oxy]carbonyl; Bzl, benzyl; 2-Cl-Z, [(*o*-chlorobenzyl)oxy]carbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; FAB-MS, fast-atom bombardment mass spectrometry; FBS, fetal bovine serum; cHex, cyclohexyl; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; *p*MBHA, *p*-methylbenzhydrylamine; Nal, naphthylalanine, NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; PE, petroleum ether; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; Rc, receptor; RP, reversed-phase; RT, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TMS, tetramethylsilane; tosyl, *p*-toluenesulfonyl.

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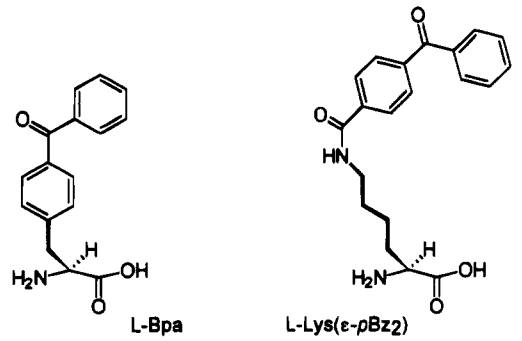

FIGURE 1: Structures of the two BP-containing photoreactive amino acid residues used in this study.

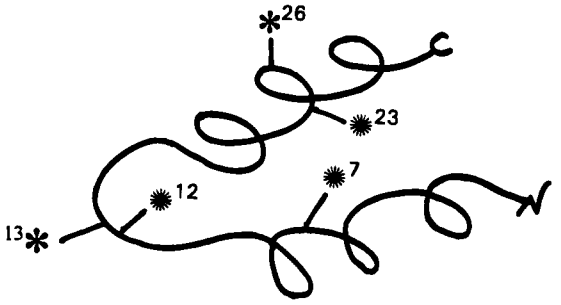

FIGURE 2: Schematic presentation of the positional distribution of BP-containing photoreactive amino acid residues. C and N denote the carboxyl and amino termini, respectively. The locations of the two different photoreactive groups, namely, Bpa and Lys(ϵ-pBz$_2$), are indicated by stick-shaped and oval-shaped asterisks, respectively.

proximity (Shoelson et al., 1993; Keutmann & Rubin, 1993). Photochemical cross-linking provides mild and specific reactions for the formation of discrete bimolecular conjugates (Chowdhry & Westheimer, 1979). Among available photoreactive moieties, aryl ketones possess unique and advantageous properties, including chemical stability of the ketone to a large variety of synthetic conditions including solid-phase peptide synthesis. Aryl ketones also are stable to ambient light conditions. The photoactivated biradical triplet has low reactivity with water and high specificity and efficiency in the photochemical C−H insertion reaction (Dorman & Prestwich, 1994; Breslow, 1980; Helene, 1972; Galardy et al., 1973). Benzophenone- (BP-) substituted ligands have been employed recently in the photoaffinity labeling of several target molecules, including the substance P receptor (Boyd et al., 1991), angiotensin II receptor (Servant et al., 1994), Src homology 2 domain (Williams & Shoelson, 1993), human luteinizing hormone subunits (Keutmann & Rubin, 1993), and insulin receptor (Shoelson et al., 1993).

A new approach for analyzing point-to-point interactions between a peptide and its macromolecular target was developed by Shoelson and his co-workers and termed "photoaffinity scanning" (Williams & Shoelson, 1993). In this approach, almost each one of the amino acid residues in the ligand is substituted (one at a time) by a benzophenone- (BP-) containing moiety. Cross-linking is performed and the conjugate is then isolated, purified, and sequenced in order to unequivocally identify the cross-linking site. In this way, point-to-point contacts are identified and a map of the bimolecular interface can be derived.

To this end, we have designed, synthesized, and biologically characterized a set of BP-substituted PTH analogs, each incorporating a BP moiety at a single defined position (Figures 1 and 2). Two different modes of incorporating the BP moiety were employed: acylation of an ϵ-amino function in a lysyl moiety (Gorka et al., 1989) and substitution of a single amino acid residue by a p-benzoylphenylalanyl (Bpa) (Kauer et al., 1986; O'Neil et al., 1989). One of these photoreactive BP-substituted PTH agonists was cross-linked to native PTH/PTHrP Rcs in human osteosarcoma cells [Saos-2/B-10 (Rodan et al., 1989)] and in both transiently and stably transfected cells expressing cloned human PTH/PTHrP Rcs (Adams et al., 1995). These bioactive photoreactive PTH analogs are essential reagents for undertaking photoaffinity scanning of the human PTH/PTHrP Rc.

MATERIALS AND METHODS

Materials. The analog [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ was prepared as previously reported (Goldman et al., 1988a). N^{α}-Boc-protected Lys-OMe·HCl, Nle-OH, and Asp(OcHex)-OH were purchased from Bachem (Torrance, CA). N^{α}-Boc-2-L-naphthylalanine (Nal) was obtained from SynTech (Albany, OR). N^{α}-Boc-2-D-Nal-OH was obtained from Biomeasure (Milford, MA). N^{α}-Boc-L-p-benzoylphenylalanine (Bpa) and N^{α}-Boc-D-Bpa-OH were purchased from Bachem Bioscience (King of Prussia, PA). The rest of the N^{α}-Boc-protected amino acids, trifluoroacetic acid (TFA), diisopropylethylamine (DIPEA), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxybenzotriazole (HOBt), and the p-methylbenzhydrylamine resin hydrochloride (pMBHA resin) (1% cross-linked, 0.77 mmol of nitrogen/g) were purchased from Applied Biosystems, Inc. (Foster City, CA). Dichloromethane (DCM), N-methylpyrrolidone (NMP) and methanol, all B&J brand, were obtained from Baxter (McGraw Park, IL). Triethylamine (TEA) was purchased from Fisher Scientific (Springfield, NJ). TFA (spectrophotometric grade), acetic anhydride, ethyl acetate (EtOAc), anisole, oxalyl chloride, p-benzoylbenzoic acid, petroleum ether (PE), and anhydrous ether were purchased from Aldrich Chemicals (Milwaukee, WI). Hydrogen fluoride (HF) was purchased from Matheson (Secaucus, NJ). Iodo-Gen was purchase from Pierce Chemical Co. (Rockford, IL). RPMI 1640, Ca^{2+},Mg^{2+}-free Hanks' balanced salt solution, was purchased from Life Technologies (Grand Island, NY). Tissue culture disposables and plasticware were obtained from Corning (Corning, NY). Fetal bovine serum (FBS) and L-glutamine were purchased from Gibco−BRL (Gaithersburg, MD). Adenosine and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Research Biochemical Inc. (Natick, MA). Adenine, cAMP, ADP, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Adenine was purchased from New England Nuclear (Boston, MA). Na^{125}I (2025 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

General Peptide Synthesis and Purification. The peptides were synthesized on a 430A automatic peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using software version 1.40 of the HOBt/NMP cycles. All chiral amino acid derivatives used were of the L-configuration unless otherwise noted. The following side-chain-protected N^{α}-Boc-amino acid derivatives were used in the course of the automated solid-phase peptide syntheses of the different analogs: Arg(N^{G}-tosyl), Asp(O-cHex), Glu(O-Bzl), His(N^{π}-Bom), Lys(N^{ϵ}-2-Cl-Z), Ser(O-Bzl), Thr(O-Bzl), and Tyr(2-

Br-Z). Substitution of Trp^{23} by L-2-Nal or Bpa was performed to simplify synthetic procedures.

Cleavage of the peptide from the *p*MBHA resin with concomitant removal of side-chain protecting groups was achieved by treatment with liquid HF in the presence of 10% anisole (20 mL/g of resin-bound peptide) for 1 h at 0 °C. The mixture of crude peptide and resin obtained following removal of HF under vacuum was washed consecutively with PE and anhydrous ether. The dry mixture of crude peptide and resin was extracted with 50% acetic acid (v/v) and water. The aqueous solution was lyophilized to yield a crude peptide, which was subjected to preparative reversed-phase HPLC purification (PrepPak Vydac C18, 300-Å cartridge, 15 μm, 5.5 × 35 cm) as described elsewhere (Chorev et al., 1990). In all cases the predominant peak in the crude mixture applied for purification corresponded to the anticipated analog.

Synthesis of N^{α}-Boc-Lys(N^{ϵ}-p-Benzoylbenzoyl)-OH [Boc-Lys(ϵ-pBz$_2$)-OH]. (A) A solution of *p*-benzoylbenzoic acid (4.37 g, 19.3 mmol) in DCM (120 mL) was treated with oxalyl chloride (3.37 mL, 38.6 mmol) under N_2 at room temperature for 2 h. The solvent was removed under vacuum. The residue was redissolved in DCM (50 mL) and the solution was evaporated to dryness, yielding the crude acid chloride, which was used in the next step without further purification.

(B) To a solution of N^{α}-Boc-Lys-OMe·HCl (5.0 g, 16.8 mmol) in DCM (80 mL) and TEA (4.7 mL, 33.6 mmol), a solution of the crude acid chloride in DCM (30 mL) was added dropwise at 0 °C under N_2. The reaction mixture was allowed to reach room temperature and left for 10 h, during which time the pH was maintained at 7−8 by dropwise addition of TEA. The reaction mixture was washed by water (30 mL). The residue obtained after removal of DCM under vacuum was dissolved in EtOAc and the organic solution was washed consecutively by saturated solution of $KHSO_4$ (3 × 30 mL), $NaHCO_3$ (3 × 30 mL), and brine (2 × 30 mL). The organic phase was dried over $MgSO_4$ (anhydrous) filtered, and evaporated to dryness under vacuum. The solid residue obtained was recrystallized from ether to yield a white powder (6.1 g, 78%): TLC (EtOAc−PE, 2:1) R_f = 0.64; ^{1}H NMR ($CDCl_3$, δ (ppm)/TMS) 7.4−7.9 (m, 9 H, aromatic), 6.4 (d, 1 H, NH amide), 5.1 (d, 1 H, NH carbamate), 4.3 (m, 1 H, $C_{\alpha}H$), 3.7 (s, 3 H, CH_3), 3.4 (quartet, 2 H, $C_{\epsilon}H_2$), 1.4−1.8 (m, 6 H, $C_{\beta}H_2$-$C_{\gamma}H_2$-$C_{\delta}H_2$), and 1.4 [s, 9 H, $C(CH_3)_3$].

(C) To a stirred solution of N^{α}-Boc-Lys(ϵ-pBz$_2$)-OMe (6.1 g, 13.08 mmol) in MeOH−H_2O (80 and 10 mL, respectively), 1 N NaOH (26 mL) was added dropwise within 1 h. After 1 h at room temperature, the MeOH was removed under vacuum and the residual basic aqueous solution was further diluted with water (20 mL) and washed with EtOAc. The ice-cold aqueous phase was acidified to pH 4 with a cold saturated solution of $KHSO_4$ and extracted with EtOAc (3 × 30 mL). The combined organic phase was washed consecutively with water (3 × 30 mL) and brine (30 mL), dried over $MgSO_4$ (anhydrous), and filtered and the solvent was removed under vacuum. The pure N^{α}-Boc-Lys(ϵ-pBz$_2$)-OH (3.4 g, 57%) was obtained as a white powder following recrystallization from ether−PE: TLC (DCM−MeOH, 4:1) R_f = 0.8, mp 67 °C; RP-HPLC on Vydac C18, 300 Å (4.6 × 150 mm), 5 μm at a flow rate of 1 mL/min, monitored at 220 nm, linear gradient of 20−50% B in 30 min [A = 0.1% (v/v) TFA in H_2O and B = 0.1% (v/v) TFA in acetonitrile], T_r = 24.77 min, k' = 11.6; ^{1}H NMR ($CDCl_3$, δ (ppm)/TMS) 7.4−7.9 (m, 9 H, aromatic), 6.65 (t, 1 H, NH amide); 5.25 (d, 1 H, NH carbamate), 4.25 (m, 1 H, $C_{\alpha}H$); 3.5 (quartet 2 H, $C_{\epsilon}H_2$), 2.0−2.6 (m, 4 H, $C_{\beta}H_2$ and $C_{\delta}H_2$), 1.4 [s, 9 H, $C(CH_3)_3$], and 1.1−1.3 (m, 2 H, $C_{\gamma}H_2$); FAB-MS (*m/e*) 477 $[M + Na]^+$, 455 $[M + H]^+$, 421 $[(M - tBu) + Na]^+$, 399-$[(M - tBu) + H]^+$, 355 $[(M - Boc) + H]^+$ and 209 $[C_6H_5$-$COC_6H_4CO + H]^+$.

Synthesis of [Nle^{8,18},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH-(1-34)NH$_2$ (K13) (I). An example of a typical synthesis, with details of recoupling and capping cycles, is given for analog K13. The synthesis of this analog was carried out on a 0.125-mmol scale. The protocol consisted of double couplings which were followed by capping with Ac_2O for the following positions: Ser^{16}-Arg^{25} and Asp^{30}-His^{32}. The crude peptide was purified on a RP-HPLC equipped with a PrePack cartridge, Vydac C18, 300-Å, 15−20-μm column. The eluting solvents used were A = 0.1% TFA in H_2O and B = 0.1% TFA in acetonitrile. The linear gradient employed consisted of 0−15% (v/v) B for 10 min followed by 15−65% (v/v) B for the next 200 min at a flow rate of 70 mL/min and monitored at 220 nm. Purification yielded 44 mg of pure peptide (Table 1).

In addition to the synthesis and purification of analog **I** (K13) described above, the following analogs were also synthesized and HPLC-purified: [Nle^{8,18},L-2-Nal^{23},Lys^{26}(ϵ-pBz$_2$),Tyr^{34}]bPTH(1−34)NH$_2$ (**II**); yield 17 mg; [Nle^{8,18},L-Bpa^{7},L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (**III**), yield 17 mg; [Nle^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (**IV**), yield 28.8 mg; [Nle^{8,18},L-Bpa^{23},Tyr^{34}]bPTH(1−34)NH$_2$ (**V**), yield 200 mg; [Nle^{8,18},D-2-Nal^{12},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]-bPTH(7−34)NH$_2$ (**VI**), yield 67.5 mg; [Nle^{8,18},D-2-Nal^{12},L-2-Nal^{23},Lys^{26}(ϵ-pBz$_2$),Tyr^{34}]bPTH(7−34)NH$_2$ (**VII**), yield 28 mg; [Nle^{8,18},L-Bpa^{7},D-2-Nal^{12},L-2-Nal^{23},Tyr^{34}]bPTH(7−34)-NH$_2$ (**VIII**), yield 24 mg; [Nle^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}]-bPTH(7−34)NH$_2$ (**IX**), yield 34 mg; [Nle^{8,18},D-2-Nal^{12},L-Bpa^{23},Tyr^{34}]bPTH(7−34)NH$_2$ (**X**), yield 52.6 mg; [Arg^{2},Lys^{13}(ϵ-pBz$_2$),Tyr^{34}]hPTH(1−34)NH$_2$ (**XI**), yield 20.8 mg; [Arg^{2},Lys^{7}(ϵ-pBz$_2$),Tyr^{34}]hPTH(1−34)NH$_2$(**XII**), yield 5.8 mg. The analytical analysis of all analogs synthesized is described in detail in Tables 1 and 2.

Cell Culture. Human osteoblast-like Saos-2/B-10 cells (Rodan et al., 1989) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every 3−4 days, and the cells were subcultured every week. Adenylyl cyclase and radioreceptor binding assays were performed on confluent cultures, 1−3 days after a change of medium.

Adenylyl Cyclase Assay. Saos-2/B-10 cells were seeded in 24-well plates at 4 × 10^4 cells/well in RPMI1640 medium containing 10% FBS. Cells were washed twice with Ca^{+2}/Mg^{+2}-free Hanks' balanced salt solution and incubated with 0.5 μCi of [^{3}H]adenine in fresh medium at 37 °C for 2 h. The cells were treated with 1 mM IBMX in fresh medium for 15 min and incubated in the presence of PTH agonists for 5 min. Inhibition of PTH-stimulated adenylyl cyclase by PTH antagonists was assayed in cells pretreated in the presence of IBMX and antagonists for 15 min, followed by the addition of agonist. After incubation for 5 min in the presence of agonist, the reaction was terminated by the addition of 1.2 M TCA, followed by neutralization with 4

Table 1: Physicochemical Characterization of Benzophenone-Containing Photoreactive PTH Analogs **I** − **XII**

		I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**
						Amino Acid Analysis[a]							
Asx	calcd	3	3	3	3	3	3	3	3	3	3	4	4
	found	3.07	3.37	3.08	3.12	3.16	2.98	3.13	2.92	3.01	2.96	4.17	4.00
Ser	calcd	3	3	3	3	3	2	2	2	2	2	3	3
	found	2.39	2.27	2.37	2.51	2.62	1.76	1.74	1.6	1.69	1.71	2.44	2.50
Glx	calcd	5	5	5	5	5	3	3	3	3	3	5	5
	found	5.1	5.42	5.37	5.55	5.33	3.21	3.53	3.29	3.37	3.41	5.38	5.33
Gly	calcd	1	1	1		1						1	1
	found	1.03	1.06	0.99		1.03						1.14	1.05
Ala	calcd	1	1	1	1	1							
	found	0.89	0.89	0.86	0.93	0.96							
Val	calcd	3	3	3	3	3	2	2	2	2	2	2	2
	found	2.63	2.74	2.86	2.75	2.79	1.8	1.76	1.99	1.86	1.88	1.75	1.74
Ile	calcd	1	1	1	1	1						1	1
	found	0.8	0.81	0.86	0.92	0.9						0.9	0.91
Leu	calcd	4	4	4	4	4	4	4	4	4	4	5	4
	found	4.11	4.14	4.2	4.09	4.09	4.07	4.13	4.14	4.05	4.08	5.23	4.06
Nle	calcd	2	2	2	2	2	2	2	2	2	2		
	found	2.08	2.21	2.19	2.12	2.18	2.17	2.14	2.08	2.11	2.14		
Tyr	calcd	1	1	1	1	1	1	1	1	1	1	1	1
	found	1.06	0.96	1.12	1.03	0.97	1.08	1.05	1.03	1.01	1	1.03	1.00
Phe	calcd	1	1		1	1	1	1		1	1		
	found	0.92	1.02		0.97	1	1.05	1.03		0.94	0.94		
His	calcd	3	3	3	3	3	3	3	3	3	3	3	3
	found	2.84	2.98	2.92	2.93	2.94	2.87	2.75	2.93	2.89	2.94	3.04	2.82
Lys	calcd	3	3	3	3	3	3	3	3	3	3	3	4
	found	3.07	3.25	3.1	3.07	3.03	3	3.14	3.01	3.05	2.99	2.84	4.01
Arg	calcd	2	2	2	2	2	2	2	2	2	2	3	3
	found	2.01	2.05	2.01	2	1.98	2	1.95	2	1.97	1.97	3.00	2.91
						FAB-MS Analysis							
calcd. MW		4306	4307	4202	4292	4153	3818	3818	3714	3664	3664	4399	4413
$m/z = [M + H]^+$		4308	4309	4204	4294	4156	3821	3821	3717	3667	3667	4398	4413
						RP-HPLC Analysis[b]							
retention time (min)		23.8	20.6	16.5	19.4	16.2	19.2	21	17	21.2	18.4	14.0	13.3

[a] Peptides were hydrolyzed on a Pico-Tag™ instrument and analyzed on an Applied BioSystem Amino Acid Analyzer. [b] Reversed-Phase (RP) HPLC analysis was carried out on a Vydac™ C18, 300Å column (4.6 × 150 mm, 5 μm) at a flow rate of 1 mL/min and monitored at 220 nm. Solvent system employed, in a linear gradient mode, was of 25−50% (v/v) B in A within 30 min, where A is 0.1% (v/v) TFA in H_2O and B is 0.1% (v/v) TFA in acetonitrile.

Table 2: *In Vitro* Activities of Benzophenone-Containing Photoreactive (**I** − **XII**) and Reference PTH Analogs in Human Osteosarcoma Saos-2/B-10 Cells

	PTH analog	K_b[a] (nM)	K_m[b] (nM)	K_i[c] (nM)
	[NIe^{8,18},Tyr^{34}]bPTH(1−34)NH_2	1.3 ± 0.2	1.2 ± 0.3	
I	[NIe^{8,18},Lys^{13}(ε-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH_2	13 ± 3	2.7 ± 0.5	
II	[NIe^{8,18},L-2-Nal^{23},Lys^{26}(ε-*p*Bz$_2$),Tyr^{34}]bPTH(1−34)NH_2	150 ± 70	15.7 ± 2.3	
III	[NIe^{8,18},L-Bpa^{7},L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH_2	40 ± 5	>1000[d]	
IV	[NIe^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH_2	146 ± 30	39 ± 3	
V	[NIe^{8,18},L-Bpa^{23},Tyr^{34}]bPTH(1−34)NH_2	42 ± 2	8.5 ± 0.2	
VI	[NIe^{8,18},D-2-Nal^{12},Lys^{13}(ε-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(7−34)NH_2	95 ± 2.8		72 ± 4
VII	[NIe^{8,18},D-2-Nal^{12},L-2-Nal^{23},Lys^{26}(ε-*p*Bz$_2$),Tyr^{34}]bPTH(7−34)NH_2	no binding		no activity
VIII	[NIe^{8,18},L-Bpa^{7},D-2-Nal^{12},L-2-Nal^{23},Tyr^{34}]bPTH(7−34)NH_2	>1000[e]		150 ± 30
IX	[NIe^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}]bPTH(7−34)NH_2	>1000[f]		>1000[g]
X	[NIe^{8,18},D-2-Nal^{12},L-Bpa^{23},Tyr^{34}]bPTH(7−34)NH_2	>1000[h]		>1000[i]
XI	[Arg^{2},Lys^{13}(ε-*p*Bz$_2$),Tyr^{34}]hPTH(1−34)NH_2	97 ± 20[j]		230 ± 50[j]
XII	[Arg^{2},Lys^{7}(ε-*p*Bz$_2$),Tyr^{34}]hPTH(1−34)NH_2.	165 ± 4[j]		190 ± 70[j]

[a] Competitive inhibition of binding of mono-^{125}I-[NIe^{8,18},Tyr^{34}(3-^{125}I)]bPTH(1−34)NH_2(^{125}I-PTH) by either PTH agonists (reference and *I*−*V*) or antagonists (*VI*−*XII*). [b] Agonist-stimulated adenylyl cyclase activity. [c] Inhibition of [NIe^{8,18},Tyr^{34}]bPTH(1−34)NH_2-stimulated adenylyl cyclase (5×10^{-9} M). [d] At 1000 nM, stimulation of adenylyl cyclase was less than 35%. [e] At 1000 nM, it competes for less than 52% of ^{125}I-PTH binding. [f] At 1000 nM, it competes for less than 58% of ^{125}I-PTH binding. [g] At 1000 nM, it inhibits only 30% of the response to 5 nM of [NIe^{8,18},Tyr^{34}]bPTH(1−34)NH_2-stimulated adenylyl cyclase response. [h] At 1000 nM, it competes for less than 45% of ^{125}I-PTH binding. [i] At 1000 nM, it inhibits only 41% of the response to 5 nM of [NIe^{8,18},Tyr^{34}]bPTH(1−34)NH_2-stimulated adenylyl cyclase response. [j] The bioactivity of the parent analog [Arg^{2},Tyr^{34}]hPTH(1−34)NH_2 under identical experimental conditions was K_b = 70 ± 20 nM and K_i = 230 ± 110 nM.

N KOH. Cyclic AMP was isolated by the two-column chromatographic method (Solomon et al., 1974). Radioactivity was counted in a scintillation counter (Packard 2200CA liquid scintillation counter, Downers Grove, IL).

PTH/PTHrP Receptor Binding Assay. Saos-2/B-10 cells were incubated for 2 h at RT (to achieve equilibrium binding conditions) (McKee et al., 1988) in fresh RPMI 1640 containing 5% FBS with 1×10^5 cpm of mono-^{125}I-[Nle^{8,18},-Tyr^{34}]bPTH(1−34)NH_2 (Goldman et al., 1988a) in the presence or absence of unlabeled competing ligand. Following incubation, cells were washed four times with ice-cold PBS and lysed with 0.1 M NaOH. The radioactivity

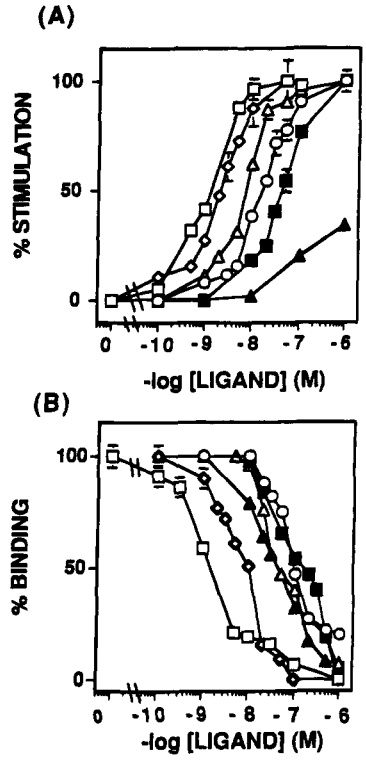

FIGURE 3: Dose–response curves for stimulation of adenylyl cyclase (panel **A**) and binding competition curves (panel **B**) for BP-containing PTH analogs **I-V** in Saos-2/B-10 cells. Panels **A** and **B** include the following 1–34 sequences: reference analog [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$, □; [Nle^{8,18},Lys^{13}(ϵ-*p*Bz$_2$),L-2-Nal^{23},-Tyr^{34}]bPTH(1–34)NH$_2$ (**I**), ◇; [Nle^{8,18},L-2-Nal^{23},Lys^{26}(ϵ-*p*Bz$_2$),-Tyr^{34}]bPTH(1–34)NH$_2$ (**II**), ○; [Nle^{8,18},L-Bpa^{7},L-2-Nal^{23},Tyr^{34}]-bPTH(1–34)NH$_2$ (**III**), ▲; [Nle^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}] bPTH(1–34)NH$_2$ (**IV**), ■; [Nle^{8,18},L-Bpa^{23},Tyr^{34}]bPTH(1–34)NH$_2$ (**V**), △.

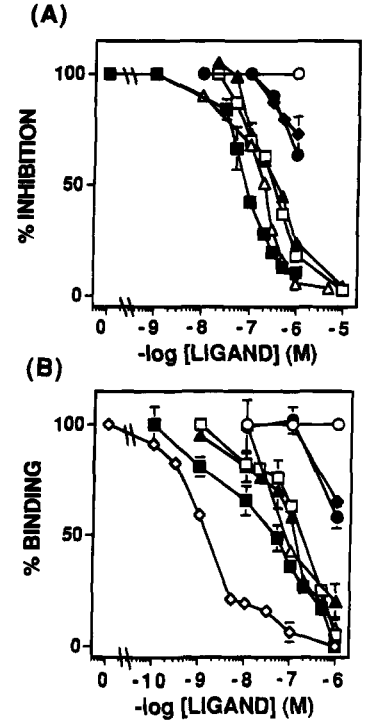

FIGURE 4: Dose–response curves for inhibition of 5 × 10^{-9} M [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$-stimulated adenylyl cyclase (panel **A**) and binding competition curves (panel **B**) for BP-containing PTH analogs **VI-XII** in Saos-2/B-10 cells. Panels **A** and **B** include the following 7–34 and 1–34 sequences (analogs **VI-X** and **XI-XII**, respectively): [Nle^{8,18},D-2-Nal^{12},Lys^{13}(ϵ-*p*Bz$_2$),L-2-Nal^{23},Tyr^{34}]-bPTH(7–34)NH$_2$ (**VI**), ■; [Nle^{8,18},D-2-Nal^{12},L-2-Nal^{23},Lys^{26}(ϵ-*p*Bz$_2$),Tyr^{34}]bPTH(7–34)NH$_2$ (**VII**), ○; [Nle^{8,18},L-Bpa^{7},D-2-Nal^{12},L-2-Nal^{23},Tyr^{34}]bPTH(7–34)NH$_2$ (**VIII**), △; [Nle^{8,18},D-Bpa^{12},L-2-Nal^{23},Tyr^{34}]bPTH(7–34)NH$_2$ (**IX**), ●; [Nle^{8,18},D-2-Nal^{12},L-Bpa^{23},-Tyr^{34}]bPTH(7–34)NH$_2$ (**X**), ◆; [Arg^{2},Lys^{13}(ϵ-*p*Bz$_2$),Tyr^{34}]hPTH-(1–34)NH$_2$ (**XI**), ▲; [Arg^{2},Lys^{7}(ϵ-*p*Bz$_2$),Tyr^{34}]hPTH(1–34)NH$_2$ (**XII**), □. Panel **B** includes a reference analog, [Nle^{8,18},Tyr^{34}[bPTH-(1–34)NH$_2$, ◇.

associated with the cells was counted in a scintillation counter (McKee et al., 1988).

Data Analysis. Binding constants (K_b), activation constants (K_m), and inhibitory constants (K_i) for PTH-stimulated adenylyl cyclase were calculated (Cheng & Prusoff, 1973). Values represent the means ± SEM of triplicates from at least three individual experiments.

RESULTS

Analysis of Peptides. The purified peptides were analyzed by analytical reversed-phase HPLC (RP-HPLC). Retention times and elution conditions are reported in Table 1. Amino acid analyses and molecular weights determined by fast-atom bombardment mass spectrometry (FAB-MS) are also reported (Table 1). These data are consistent with the theoretical values and validate the structural authenticity of the analogs. Based on analytical RP-HPLC and amino acid analysis, the purity of all analogs exceeds 98%.

Binding Affinity and Adenylyl Cyclase Activity of Benzophenone-Containing PTH Analogs. Table 2 and Figures 3 and 4 summarize the *in vitro* biological characterization of the photoreactive analogs. BP incorporation at position 13 leads to the most potent photoreactive agonist (analog **I**, K_b = 13 nM and K_m = 2.7 nM) and the only potent antagonist (analog **VI**, K_b = 95 nM and K_i = 72 nM). Substitution of position 23 by either L-2-Nal or L-Bpa has no detrimental effect on either binding affinity or activation (inhibition for antagonists) of adenylyl cyclase (e.g., analogs **I** and **V**, K_b = 13 and 42 nM and K_m = 2.7 and 8.5 nM, respectively). Substitution of the Phe^7 by Bpa in either the agonist or antagonist ligands (**III** and **VIII**, respectively) provides unusual properties. Analog **III** has high binding affinity but low efficacy (K_b = 40 nM and K_m > 1000 nM). On the other hand, in the antagonist series, analog **VIII** displays low binding affinity but reasonable efficacy (K_b > 1000 nM and K_i = 150 nM).

In human Saos-2/B-10 cells, both BP-substituted Arg^2 analogs **XI** and **XII** and the parent nonphotoreactive Arg^2-substituted analog (Gardella et al., 1991) display high binding affinity (K_b ~ 100 nM) and antagonist properties (K_i ~ 200 nM) for PTH-stimulated adenylyl cyclase (see Table 2).

The criteria for selecting BP-containing PTH analogs for the forthcoming photoaffinity scanning studies will be affinity and avidity which are similar to the parent peptides lacking the BP modification. In all cases studied, introduction of BP moieties leads to a reduction of *in vitro* potencies. The extent of this reduction varies from 10-fold to 100-fold in affinity for the agonist series (see Table 2, analogs **I–V**). The effect is much more pronounced for the antagonist series (analogs **VI–X**), in which only analog **VI** possess activities in the ~100 nM range. We believe that potential ligands for photoaffinity scanning should have affinities in the 1–50 nM range.

DISCUSSION

In this study, either Bpa (Kauer et al., 1986) or Lys(ϵ-pBz$_2$) (Gorka et al., 1989) was incorporated directly into peptides during solid-phase synthesis as N^{α}-Boc-protected amino acids. In Bpa, the BP moiety is linked to the β-carbon and therefore positioned close to the peptide backbone. In Lys(ϵ-pBz$_2$), the BP group is attached by acylation through the side chain and therefore is remote from the backbone. We predict that positioning the BP moiety at different distances from the peptide backbone should allow detailed analysis of ligand−receptor contact points. However, the possibility that cross-linking will occur at sites remote from the hormone−receptor interface cannot be excluded. In view of this possibility, selective postsynthetic modification of the ϵ-amino of a discrete Lys residue will provide the means to "tag" the BP moiety by either [^{3}H]pBz$_2$ or p-(4-^{125}I-benzoyl)-benzoic acid. The colocalization of both the photoreactive group and the radioactive tag on the same residue in the ligand should prevent dissociation of the radiolabel from the conjugate during fragmentation.

The positions chosen for introduction of the photoreactive moiety were selected to span the sequence of the PTH ligand in order to permit subsequent systematic photoaffinity scanning of the receptor. Each analog was modified at a single site which was chosen for its likelihood to tolerate alteration based on previous extensive structure−activity studies (Chorev & Rosenblatt, 1994) (Figure 2). In addition, BP-containing residues were positioned within two functionally important domains (Chorev & Rosenblatt, 1994). In the C-terminal domain, PTH(25−34) is critical for receptor binding; the N-terminal PTH(1−14) domain is critical for activation of Rc signal transduction. The residues Phe^7, Gly^{12}, Lys^{13}, Trp^{23}, and Lys^{26} were replaced individually by BP-containing residues.

In all peptides analogs except **XI** and **XII**, Trp^{23} was substituted by either L-2-Nal (analogs **I**−**IV** and **VI**−**IX**) or Bpa (analogs **V** and **X**). Such a replacement simplifies synthetic procedures such as liquid HF-mediated deprotection of side-chain protecting groups concomitant with cleavage of the peptide from the resin and subsequent purification. By analogy with previously reported replacements of Trp by Nal in other bioactive peptide systems, as in GnRH (Horvath et al., 1982; Hahn et al., 1985), somatostatin (Murphy et al., 1987), gramicidin (Ranjalahy-Rasoloarijao et al., 1989), bombesin (Mahmoud et al., 1991; Orbuch et al., 1993), and enkephalin (Mierke et al., 1990), we find this substitution to be well-tolerated in PTH. The agonist [Nle^{8,18},Aib^{12},L-2-Nal^{23},Tyr^{34}]bPTH(1−34)NH$_2$ is equipotent to the parent PTH agonist [Nle^{8,18},Tyr^{34}]bPTH(1−34)NH$_2$ (C. Nakamoto and M. Chorev, unpublished results).

Replacements of Phe^7 with L-Bpa (analogs **III** and **VIII**) or Lys(ϵ-pBz$_2$) (analog **XII**) in PTH were carried out with the intention to conserve the aromatic and hydrophobic nature of the side chain at this site.

In most of the antagonists (analogs **VI**−**X**), as well as the agonist (**IV**), Gly^{12} was replaced by an aromatic D-amino acid residue, such as Bpa (**IV** and **IX**) or 2-Nal (**VI**−**VIII** and **X**). Previous studies identified position 12 as one tolerant of some structural latitude (Chorev et al., 1990).

On the basis of previous studies (Roubini et al., 1992; Chorev et al., 1991, 1992, 1993; Abou-Samra et al. 1989; Coltrera et al., 1981; Shigeno et al., 1989), we anticipated that the introduction of Lys^{13}(ϵ-pBz$_2$), as in analogs **I**, **VI**, and **XI**, would also be accommodated. Similarly, previous structure−activity studies (Brennan & Levine, 1987; Abou-Samra et al., 1989; Newman et al., 1989; Roubini et al., 1992) directed at the Lys residues in positions 26 and 27 suggest that the addition of pBz$_2$-OH to the ϵ-amino of Lys^{26}, as in analogs **II** and **VII**, would generate potent BP-containing PTH analogs.

In the agonist series of photoreactive BP-containing analogs **I**−**V**, our structural modifications were well-tolerated and provided a potentially useful set of bioactive and photoreactive analogs (Figure 3A) for future conduct of photoaffinity scanning. In contrast, loss of binding affinity and inhibitory potency characterize many of the BP-substituted PTH(7−34) antagonist analogs (**VII**−**X**) (Figure 4A,B). Therefore, leads for rational design of PTH(7−34)-based antagonists generated in previous structure−activity studies (Chorev et al., 1990, 1991; Goldman et al., 1988) cannot be readily applied to BP substitutions. In the antagonist series, combinations of single silent or potentiating modifications are, in most cases, mutually exclusive in terms of their antagonist activity (Figure 4B). Nevertheless, we did obtain one highly potent BP-containing PTH(7−34) antagonist, namely, [Nle^{8,18},D-2-Nal^{12},Lys^{13}(ϵ-pBz$_2$),L-2-Nal^{23},Tyr^{34}]bPTH(7−34)NH$_2$ (**VI**) (Table 2).

Analogs **XI** and **XII** are derived from the parent compound, [Arg^2,Tyr^{34}]hPTH(1−34)NH$_2$ (Gardella et al., 1991), by substituting Lys^{13} or Phe^7 with Lys(ϵ-pBz$_2$). Arg^2 is a substitution that has attracted considerable interest for use in studying PTH−receptor interactions. [Arg^2,Tyr^{34}]hPTH-(1−34)NH$_2$ was reported to be a weak partial agonist with high binding affinity for rat osteosarcoma (ROS17/2.8) cells but a full agonist in opossum kidney (OK) cells (EC_{50} in OK cells is 3 orders of magnitude lower than in ROS17/2.8 cells) (Gardella et al., 1991). The parent Arg^2-containing analog and its two BP-substituted analogs **XI** and **XII** prepared by us are potent antagonists in the human Saos-2/B-10 cells (Figure 4). Introduction of the BP moiety does not change the bioactivity profile in human Saos-2/B-10 cells.

In summary, we report a series of bioactive BP-containing PTH analogs in which the position of the photoreactive moiety spans the two most interesting functional domains in the ligand, namely, the "binding" (e.g., analogs **II** and **V**) and "activation" regions (e.g., analogs **I**, **III**, **IV**, and **XII**). These analogs represent the first set of molecular reagents designed for photoaffinity scanning of the hPTH/PTHrP Rc. As demonstrated in the accompanying paper (Adams et al., 1995), we are able to photo-cross-link one of the BP-substituted PTH analogs (K13) to endogenously expressed and stably transfected human PTH/PTHrP Rc. In the future, we plan to isolate and analyze the cross-linked photoconjugates in an effort to map the hormone−receptor contact points. Such information promises to generate a molecular model which may provide insights for understanding the mechanism of PTH/PTHrP signal transduction and facilitate rational receptor-based design of novel PTH/PTHrP-related pharmaceuticals.

REFERENCES

Abou-Samra, A.-B., Freeman, M., Juppner, H., Uneno, S., & Serge, G. V. (1989) *J. Biol. Chem. 264*, 16359.

Abou-Samra, A.-B., Juppner, H., Force, T., Freeman, M. W., Kong, X.-F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M., & Segre, G. V. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2732.

Adams, A. E., Chorev, M., Rosenblatt, M., & Suva, L. J. (1993) *J. Bone Miner. Res. 8* (Suppl. 1), S195 (abstr).

Adams, A. E., Pines, M., Nakamoto, C., Behar, V., Yang, Q. M., Bessalle, R., Chorev, M., Rosenblatt, M., Levine, M. A., & Suva, L. J. (1995) Probing the Bimolecular Interactions of Parathyroid Hormone and the Human Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor. 2. Cloning, Characterization, and Photoaffinity Labeling of the Recombinant Human Receptor, *Biochemistry 34*, 10553−10559.

Boyd, N. D., White, C. F., Cerpa, R., Kaiser, E. T., & Leeman, S. E. (1991) *Biochemistry 30*, 336.

Brennan, D. P., & Levine, M. A. (1987) *J. Biol. Chem. 262*, 14795.

Breslow, R. (1980) *Acc. Chem. Res. 13*, 170.

Caporale, L. H., & Rosenblatt, M. (1986) in *Phosphate and mineral homeostasis* (Massry, S. G., Olmer, M., & Ritz, E., Eds.) pp 315−327, Plenum Publishing Corp., New York.

Cheng, Y., & Prusoff, W. H. (1973) *Biochem.* Pharmacol. *22*, 3099.

Chorev, M., & Rosenblatt, M. (1994) in *The Parathyroids* (Bilezikian, J. P., Levine, M. A., & Marcus, R., Eds.) pp 139−156, Raven Press, Ltd., New York.

Chorev, M., Goldman, M. E., McKee, R. L., Roubini, E., Levy, J. J., Gay, C. T., Reagan, J. E., Fisher, J. E., Caporale, L. H., Golub, E. E., Caulfield, M. P., Nutt, R. F., & Rosenblatt, M. (1990) *Biochemistry 29*, 1580.

Chorev, M., Roubini, E., McKee, R. L., Gibbons, S. W., Reagan, J. E., Goldman, M. E., Caulfield, M. P., & Rosenblatt, M. (1991) *Peptides 12*, 57.

Chorev, M., Caulfield, M. P., Roubini, E., McKee, R. L., Gibbons, S. W., Leu, C.-T., Levy, J. L., & Rosenblatt, M. (1992) *Int. J. Pept. Protein Res. 40*, *445*.

Chorev, M., Epand, R. F., Rosenblatt, M., Caulfield, M. P., & Epand, R. M. (1993) *Int. J. Pept. Protein Res. 42*, 342.

Chowdhry, V., & Westheimer, F. H. (1979) *Annu. Rev. Biochem. 48*, 293.

Coltrera, M. D., Potts, J. T., Jr., & Rosenblatt, M. (1981) *J. Biol. Chem. 256*, 10555.

Dorman, G., & Prestwich, G. D. (1994) *Biochemistry 33*, 5661.

Galardy, R. E., Craig, L. C., & Printz, M. P. (1973) *Nature 242*, 127.

Gardella, T. J., Axelrod, D., Rubin, D., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., & Nussbaum, S. R. (1991) *J. Biol. Chem. 266*, 13141.

Gardella, T. J., Wilson, A. K., Keutmann, H. T., Oberstein, R., Potts, J. T., Jr., Kronenberg, H. M., & Nussbaum, S. R. (1993) *Endocrinology 132*, 2024.

Goldman, M. E., Chorev, M., Reagan, J. E., Nutt, R. F., Levy, J. J., & Rosenblatt, M. (1988a) *Endocrinology 123*, 1468.

Goldman, M. E., McKee, R. L., Caulfield, M. P., Reagan, J. E., Levy, J. J., Gay, C. T., DeHaven, P. A., Rosenblatt, M., & Chorev, M. (1988b) *Endocrinology 123*, 2597.

Gorka, J., McCourt, D. W., & Schwartz, B. D. (1989) *Pept. Res. 2*, 376.

Hahn, D. W., McGuire, J. L., Vale, W. W., & Rivier, J. (1985) *Life Sci. 37*, 505.

Helene, C. (1972) *Photochem. Photobiol. 16*, 519.

Horvath, A., Coy, D. H., Nekola, M. V., Coy, E. J., Schally, A. V., & Teplan, I. (1982) *Peptides 3*, 969.

Juppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Jr., Hock, J., Potts, J. T., Jr., Kronenberg, H. M., & Segre, G. V. (1991) *Science 254*, 1024.

Juppner, H., Flannery, M. R., McClure, I., Abou-Samra, A. B., Gardella, T. J., & Goldring, S. R. (1993) *J. Bone Miner. Res. 8*, S183 (abstr).

Juppner, H., Schipani, E., Bringhurst, F. R., McClure, I., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., Abou-Samra, A. B., Serge, G. V., & Gardella, T. J. (1994) *Endocrinology 134*, 879.

Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., & DeGrado, W. F. (1986) *J. Biol. Chem. 261*, 10695.

Kaufmann, M., Muff, R., Born, W., & Fischer, J. A. (1994) *Mol. Cell. Endocrinol. 104*, 21.

Kemp, B. E., Moseley, J. M., Ebeling, P. R., Wettenhall, R. E. H., Stapleton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G., & Martin, T. J. (1987) *Science 238*, 1568.

Keutmann, H. T., & Rubin, D. A. (1993) *Endocrinology 132*, 1305.

Lee, C., Gardella, T. J., Abou-Samra, A.-B., Nussbaum, S. R., Segre, G. V., Potts, J. T., Jr., Kronenberg, H. M., & Jueppner, H. (1994) *Endocrinology 135*, 1488.

Mahmoud, S., Staley, J., Taylor, J., Bogden, A., Moreau, J., Coy, D. H., Avis, I., Cuttitta, F., Mulshine, J. L., & Moody, T. W. (1991) *Cancer Res. 51*, 1798.

McKee, R. L., Goldman, M. E., Caulfield, M. P., DeHaven, P. A., Levy, J. J., Nutt, R. F., & Rosenblatt, M. (1988) *Endocrinology 122*, 3008.

Mierke, D. F., Said-Nejad, O. E., Schiller, P. W., & Goodman, M. (1990) *Biopolymers 29*, 179.

Murphy, W. A., Lance, V. A., Moreau, S., Moreau, J., & Coy, D. H. (1987) *Life Sci. 40*, 2515.

Newman, W., Beall, L. D., Levine, M. A., Cone, J. L., Randhawa, Z. I., & Bertolini, D. R. (1989) *J. Biol. Chem. 264*, 16359.

O'Neil, K. T., Erickson-Viitanen, S., & DeGrado, W. F. (1989) *J. Biol. Chem. 264*, 14571.

Orbuch, M., Taylor, J. E., Coy, D. H., Mrozinski, J. E., Mantey, S. A., Battey, J. F., Moreau, J., & Jensen, R. T. (1993) *Mol. Pharmacol. 44*, 841.

Pines, M., Adams, A. E., Stueckle, S., Bessalle, R., Rashti-Behar, V., Chorev, M., Rosenblatt, M., & Suva, L. J. (1994) *Endocrinology 135*, 1713.

Ranjalahy-Rasoloarijao, J., Lazaro, R., Daumas, P., & Heitz, F. (1989) *Int. J. Pept. Protein Res. 33*, 273.

Rodan, S. B., Wesolowski, G., Ianacone, J., Thiede, M. A., & Rodan, G. A. (1989) *J. Endocrinol. 122*, 219.

Rosenblatt, M. (1986) *N. Engl. J. Med. 315*, 1004.

Roubini, E., Duong, L. T., Gibbons, S. W., Leu, C. T., Caulfield, M. P., Chorev, M., & Rosenblatt, M. (1992) *Biochemistry 31*, 4026.

Schipani, E., Karga, H., Karaplis, A. C., Potts, J. T., Jr., Kronenberg, H. M., Segre, G. V., Abou-Samra, A.-B., & Juppner, H. (1993) *Endocrinology 132*, 2157.

Servant, G., Bosse, R., Guillementte, G., & Escher, E. (1994) in *Peptides* (Hodges, R. S., & Smith, J. A., Eds.) pp 698−703, ESCOM, Leiden, The Netherlands.

Shigeno, C., Hiraki, Y., Keutmann, H. T., Stern, A. M., Potts, J. T., Jr., & Segre, G. V. (1989) *Ann. Biochem. 179*, 268.

Shoelson, S. E., Lee, J., Lynch, C. S., Backer, J. M., & Pilch, P. F. (1993) *J. Biol. Chem. 268*, 4085.

Solomon, Y., Londos, C., & Rodbell, M. A. (1974) *Anal. Biochem. 58*, 541.

Williams, K. P., & Shoelson, S. E. (1993) *J. Biol. Chem. 268*, 5361.

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