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Synthesis of Fully Active Biotinylated Analogues of Parathyroid Hormone and Parathyroid Hormone-Related Protein as Tools for the Characterization of Parathyroid Hormone Receptors

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ABSTRACT: The synthesis, purification, and characterization of biotinylated analogues of parathyroid hormone (PTH) and PTH-related protein (PTHrP) are described. A novel methodology was developed which allowed the selective biotinylation during solid-phase synthesis of either the Lys^{13} or Lys^{26} residue in PTH/PTHrP sequences. Incorporation of orthogonally protected N^{α}-Boc-Lys(N^{ϵ}-Fmoc) at a selected position in the sequence, followed by selective side-chain deprotection and biotinylation of the ϵ-amino group, permitted modification of the specific lysine only. Biotinylated analogues of [$Nle^{8,18}$,Tyr^{34}]bPTH(1-34)NH_2 (analogue **1a**) were prepared by modification of Lys^{13} with a biotinyl group (analogue **1**) or a biotinyl-ϵ-aminohexanoyl group (analogue **2**) or at Lys^{26} with a biotinyl-ϵ-aminohexanoyl group (analogue **3**). A biotinylated PTHrP antagonist [Leu^{11},D-Trp^{12},Lys^{13}(N^{ϵ}-(biotinyl-β-Ala))]PTHrP(7-34)NH_2 (analogue **5**), was also prepared. In a different synthetic approach, selective modification of the thiol group of [Cys^{35}]PTHrP(1-35)NH_2, in solution, with *N*-biotinyl-*N'*-(6-maleimidohexanoyl)hydrazide, resulted in analogue **4**. The high affinities of the biotinylated analogues for PTH receptors present in human osteosarcoma B-10 cells or in porcine renal cortical membranes (PRCM), were comparable to those of the underivatized parent peptides. The analogues were also highly potent in stimulation of cAMP formation (analogues **1-4**) or inhibition of PTH-stimulated adenylyl cyclase (analogue **5**) in B-10 cells. The most potent analogue (analogue **1**) had potencies in B-10 cells (K_b = 1.5 nM, K_m = 0.35 nM) and in porcine renal membranes (K_b = 0.70 nM) identical or similar to those of its parent peptide, respectively. Furthermore, these high binding affinities were retained in the presence of either avidin, streptavidin, or anti-biotin (1 μM). Radioiodination of PTH analogues (analogues **1-2**) generated highly potent radioligands which bind to a single class (B_{max} = 390-450 fmol/mg of protein) of high affinity (K_d = 0.24-0.61 nM) PTH receptors on renal membranes and which also bind to immobilized streptavidin or anti-biotin. Cross-linking of biotinylated radioligands (analogues **1-3**) and analysis by SDS-PAGE and autoradiography showed specific labeling of renal PTH receptors (M_r = 75 000). The biotinylated analogues prepared in this study, and their radiolabeled derivatives, serve as useful tools for the identification and isolation of PTH receptors.

Biotinylated derivatives of peptide hormones have been useful as specific probes for the localization and visualization of membrane receptors in cells by means of affinity cytochemistry techniques (Childs et al., 1986; Yamasaki et al., 1988; Wilchek & Bayer, 1990) and in the purification of hormone receptors in combination with avidin affinity chromatography (Finn et al., 1984; Kohanski & Lane, 1985; Hazum et al., 1986; Lee et al., 1989). Parathyroid hormone, a linear peptide of 84 amino acids, acts primarily at bone and kidney tissue to regulate calcium levels in blood. Parathyroid hormone-related protein (PTHrP), a 141 amino acid peptide associated with the clinical syndrome of humoral hypercalcemia of malignancy, has been isolated and cloned (Suva et al., 1987; Strewler et al., 1987; Mangin et al., 1988). PTHrP shares considerable sequence homology with PTH within the N-terminal 1-13 region of the peptide: 8 positions contain identical amino acids in the two hormones. There is little homology beyond position 13, including the C-terminal 25-34

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region thought to play an important role in receptor binding. PTHrP and its N-terminal fragments interact with PTH receptors and mimic many of the PTH actions on skeletal and renal tissue (Horiuchi et al., 1987; Kemp et al. 1987; Nissenson et al. 1988; Shigeno et al., 1988). PTH/PTHrP binding to PTH receptors and full agonist activity (in terms of stimulation of cAMP production) are contained in the N-terminal 1–34 sequences of both hormones. [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$, an oxidation-resistant, synthetic analogue of PTH (Rosenblatt et al., 1976), and PTHrP(1–34)NH$_2$ (Horiuchi et al., 1987; Shigeno et al., 1988) are fully active agonists of PTH and PTHrP, respectively. Biotinylated native parathyroid hormone (1–84) has been prepared by random biotinylation in solution and used for histochemical studies (Niendorf et al., 1986, 1988); however, the ligand was found to have weak agonist activity in vitro. More recently, bioactive biotinylated derivatives of PTH(1–34) and its analogues have been prepared by means of postsynthetic random biotinylation of the analogues in solution (Brennan & Levine, 1987; Newman et al., 1989; Abou-Samra et al., 1990). However, the latter method is limited as a general approach for derivatization of PTH/PTHrP. The existence of multiple sites available for acylation in PTH, namely, the N-terminal α-amino group and the ϵ-amino groups of Lys^{13}, Lys^{26}, and Lys^{27}, has led, in these studies, to the generation of a complex mixture of mono- and multibiotinylated peptides. The separation of the desired product from the complex reaction mixture is difficult, and the overall yields are too low to allow a full characterization of the biotinylated analogues.

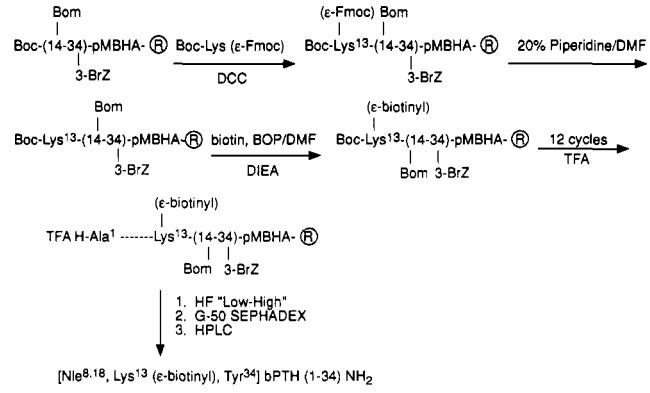
Our interest in the purification of the PTH receptor prompted us to develop strategies for the synthesis of bioactive biotinylated analogues of PTH/PTHrP in large quantities by (1) direct biotinylation at specific sites during solid-phase synthesis or (2) selective postsynthetic modification. Structure–activity studies for PTH/PTHrP in the agonist (1–34) and antagonist (7–34) sequences have shown tolerance of Lys^{13}, Lys^{26} (or Lys^{27}), and Cys^{35} toward structural modification by biotin addition or a variety of acylating or alkylating groups (Shigeno et al., 1989; Abou-Samra et al., 1990; Chorev et al., 1991).

In this study, we describe the synthesis and full characterization of novel biotinylated, radiolabeled analogues of [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$, [Leu^{11},D-Trp^{12}]PTHrP(7–34)NH$_2$, and [Cys^{35}]PTHrP(1–35)NH$_2$ specifically biotinylated at the ϵ-amino group of either Lys^{13} or Lys^{26} (analogues **1–3** and **5**) or at the thiol group of Cys^{35} (analogue **4**). These bifunctional analogues provide efficient tools for the identification of PTH receptors. Radioiodination of the biotinylated analogues generates ligands with high affinities which can be used in the characterization of PTH receptors and as probes to monitor the various steps included in the receptor purification scheme.

Materials and Methods

Materials. The analogue [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$ and the N^{α}-Boc- and Fmoc-protected amino acids, *N*-Boc-L-Asp(OcHex)-OH, *N*-Boc-L-His(N^{π}-Bom)-OH, *N*-Boc-L-Nle-OH, *N*-Boc-L-Lys(N^{ϵ}-Fmoc)-OH, *N*-Boc-D-Trp(N^{in}-For)-OH, and Fmoc-β-Ala-OH were obtained from Bachem Inc. (Torrance, CA). All other amino acid derivatives, *p*-methylbenzhydrylamine resin hydrochloride (pMBHA-R HCl) (1% cross-linked, 0.72 mmol of nitrogen/g), and SPPS grade reagents and solvents were obtained from Applied Biosystems Inc. (Foster City, CA). (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was purchased from Chemical Dynamics (South Plainfield, NJ).

Scheme I: Synthetic Pathway to Biotinylated Analogue **1**

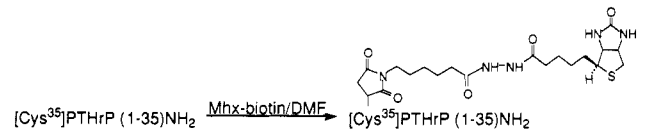


Hydrogen fluoride was purchased from Matheson (Secaucus, NJ). *p*-Cresol, *p*-thiocresol, ethanedithiol (EDT), and methyl sulfide (DMS) were from Aldrich Chemicals Inc. (Milwaukee, WI). D-Biotin and succinimidyl D-biotinyl-ϵ-aminohexanoate (biotinyl-ϵ-Ahx-ONSu) were obtained from Fluka, AG. *N*-biotinyl-*N'*-(6-maleimidohexanoyl)hydrazide (Mhx-biotin), bovine serum albumin, Tris-HCl, Hepes, phosphocreatine, creatine phosphokinase, GTP, Mg-ATP, 3-isobutyl-1-methylxanthine (IBMX), EGTA, aprotinin, and soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO). Avidin-D, streptavidin, and anti-biotin (goat IgG, affinity purified) were purchased from Vector Laboratories (San Mateo, CA). Agarose-immobilized streptavidin, disuccinimidyl suberate (DSS), and Iodogen were purchased from Pierce Chemical Co. (Rockford, IL). Premixed high molecular weight standards for SDS–PAGE and dithiothreitol (DTT) were from Bethesda Research Laboratories (Gaithersburg, MD.). Na^{125}I (2025 Ci/mmol) and [^{3}H]adenine were purchased from Amersham Corp. (Arlington Heights, IL). Canine and porcine kidneys were obtained from Pel-Freeze (Rogers, AR).

Peptide Synthesis and Purification. The peptides were synthesized on an Applied Biosystems 430A automated peptide synthesizer (Applied Biosystems, Foster City, CA), using version 1.2 of the software and a modification of the Merrifield solid-phase synthesis procedure (Merrifield, 1969). The synthesis of bPTH and PTHrP analogues was carried out as previously described (Caulfield et al., 1990), but with the following modifications for PTHrP sequences: recoupling of each of the arginines (residues 19–21) and histidines (residues 25 and 26) was followed by acetylation of the residual free α-amino group using a DCC-mediated acetic acid (114 μL, 2.0 mmol) coupling.

(A) Biotinylation on Solid-Phase Support. Incorporation of N_{α}-Boc-Lys(N$^{\epsilon}$-Fmoc)-OH in position 13 or 26 and the subsequent deprotection and biotinylation of the ϵ-amino group is outlined in Scheme I. The coupling of Boc-Lys(ϵ-Fmoc)-OH (0.94 g, 2.0 mmol) to the free amino terminus of [Nle^{18},Tyr^{34}]bPTH(14–34)pMBHA-R (0.5 mmol), [Tyr^{34}]-bPTH(27–34)pMBHA-R (0.5 mmol), or PTHrP(14–34)-pMBHA-R (0.25 mmol) was carried out by the symmetrical anhydride method, as previously described (Chorev et al., 1991). The recoupling of Boc-Lys(ϵ-Fmoc)-OH was carried out in the presence of 5% DIEA in DMF, followed by two consecutive washes with DCM (1 × 1 min) and DMF (1 × 1 min). The ϵ-Fmoc protecting group was selectively removed by treatment of the resin-bound peptide with 20% piperidine in DMF (1 × 1 min, followed by 1 × 20 min). The resin was consecutively washed with DMF (1 × 1 min), DCM (4 × 1 min), and DMF (2 × 1 min). Coupling of D-biotin (0.49 g,

Scheme II: Synthesis of Biotinylated PTHrP Analogue **4** by Selective Modification at the Thiol Group of Cys^{35} [a]



[a] Mhx-biotin = *N*-(6-maleimidohexanoyl)-*N'*-biotinylhydrazide.

2.0 mmol) to the free ε-amino group of the resin-bound peptide was carried out with BOP reagent (0.84 g, 2.0 mmol) in DMF (31 mL) in the presence of 1.5% DIEA (0.462 mL, 2.65 mmol), for 2 h. The ninhydrin test (Kaiser et al., 1970) was used to determine completion of the reaction. The resin was washed with DMF (2 × 1 min), DCM (4 × 1 min), and DMF (2 × 1 min). Acetylation of residual ε-amino groups was done with an excess of acetic anhydride (1.0 mmol) preformed with DCC. Incorporation of Fmoc-β-Ala-OH (0.622 g, 2 mmol), on the ε-amino of the resin-bound PTHrP(13–34) fragment, was followed by deprotection and coupling of D-biotin (0.37 g, 1.5 mmol) to the free β-amino group with BOP (0.64 g, 1.5 mmol), in 1.5% DIEA/DMF (21 mL), as described above. Coupling of succinimidyl D-biotinyl-ε-aminohexanoate (biotinyl-ε-Ahx-ONSu), (0.4 g, 0.88 mmol) to the free ε-amino group of the resin-bound peptide was performed in DMF (16 mL) in the presence of DIEA (0.15 mL, 0.88 mmol) at a final pH of 8.5. The reaction was allowed to proceed overnight at room temperature. Consecutive washes and acetylation steps were carried out as described above. Peptides were deprotected and cleaved from the resin using the low–high HF procedure (Tam et al., 1983). The crude synthetic peptides were partially purified on a Sephadex G-50 column (5 × 150 cm) by elution with 50% (v/v) aqueous acetic acid. Further purification was accomplished on a Waters DeltaPrep 3000 HPLC system (Milford, MA) using a PrepPak Vydac Protein C-18 cartridge (15 μm, 5.5 × 35 cm) as previously described in detail (Goldman et al., 1988). The structure and purity of the peptides were established by fast atom bombardment mass spectroscopy (FAB-MS), amino acid analysis, 300-MHz NMR spectroscopy, and Edman degradation sequencing.

(*B*) *Postsynthetic Specific Biotinylation.* PTHrP analogue [Cys^{35}]PTHrP(1–35)NH_2 (analogue **4a**) (20 mg, 4.8 μmol) was reacted with the thiol-selective reagent *N*-biotinyl-*N'*-(6-maleimidohexanoyl)hydrazide (19.4 μmol) in DMF (2 mL) with stirring under N_2 at room temperature (see Scheme II). The progression of the reaction was monitored by C-18 HPLC. After 3 h, another portion of Mhx-biotin (4.7 mg, 9.7 μmol) was added and the reaction was allowed to continue overnight. After evaporation of DMF, the residue was redissolved in 1 M aqueous AcOH/1% DMF (5 mL) and the sample was filtered to remove insoluble material. The crude peptide was purified by HPLC on a semipreparative Vydac protein C-18 column (10 μm, 22 × 25 cm), using a linear gradient of 5%–15% (v/v) of 0.1% TFA–water (A) in 0.1% TFA–acetonitrile (B) for 10 min, followed by a linear gradient of 15%–48% (v/v) of solution A in solution B for 200 min, at a flow rate of 20 mL/min. Fractions containing the pure product were combined, evaporated under reduced pressure, and lyophilized to give 12.8 mg (58% yield) of the peptide. The structure and purity of this peptide were confirmed by the methods described above.

Radioiodination of PTH Analogues. Biotinylated peptides and [$Nle^{8,18}$,Tyr^{34}]bPTH(1–34)NH_2 were radiolabeled by the Iodogen method and purified by HPLC as recently described (Goldman et al., 1988), but with the following modifications: The oxidation reaction was initiated by adding 75 μg of peptide in 40 μL of 0.1 M sodium phosphate buffer, pH 7.4, and 2 mCi (20 μL) of $Na^{125}I$ to an Iodogen-coated borosilicate tube (10 μg). The reaction was continued for 2 min at room temperature; 200 μL of 32% (v/v) acetonitrile–water containing 0.1% TFA was added and the mixture was immediately submitted to HPLC chromatography. Fractions of 0.5 mL were collected in polypropylene tubes containing 0.5 mL of 2% BSA, 2 μM DTT, and 50 mM Hepes, pH 7.4. The radioactive peptides were stored at −70 °C for up to 3 weeks without loss of activity. When used for cross-linking experiments, the radioactive fractions were instead collected in 50 mM Hepes buffer, pH 7.4, brought up to 1% CHAPS, and used on the same day.

Cell Culture. Human osteosarcoma Saos-2/B-10 cells (Rodan et al., 1989) were cultured in RPMI medium supplemented with 10% FBS and 0.1 mg/mL kanamycin at 37 °C in a humidified 95% air/5% CO_2 atmosphere and subcultured every 7 days. Cells were passaged into 24-multiwell plates, prior to each experiment. Confluent cultures were assayed 1–3 days after a change in medium.

PTH Receptor Binding and Adenylyl Cyclase Activity. Canine and porcine renal cortical membranes were prepared according to previously described methods (Goldman et al., 1988). Protein determination of membrane preparations was carried out using the Bio-Rad protein assay kit, with BSA as the standard. Binding studies with renal cortical membranes were performed as previously described using a filter-based method (Caulfield et al., 1990). In binding experiments performed in the presence of either avidin, streptavidin, or anti-biotin (1 μM), the biotin-binding proteins were added to the binding assay at equilibrium, 35 min after initial incubation of the membranes with ^{125}I-labeled peptides, in the presence or absence of increasing concentrations of unlabeled peptides. Incubation of the membranes was then continued for another 35 min at room temperature.

Binding of PTH and PTHrP analogues to Saos-2/B-10 cells was performed as previously described (Chorev et al., 1991) in 24-multiwell plates in Dulbecco's-modified Eagle's (DMEM) medium supplemented with 1% BSA, 20 mM Hepes, pH 7.5, and 0.1% sodium azide. Intracellular cAMP was measured using the procedure as described (Rodan et al. 1983).

Chemical Cross-Linking of ^{125}I-Labeled Biotinylated PTH Analogues to Renal Cortical Membranes and SDS–PAGE Analysis. Purified membranes were resuspended at 1 mg of protein/mL in 50 mM Hepes, pH 7.4, 1 mM $MgCl_2$. Ligand binding was carried out with approximately 100 μg of membrane protein and 500 000 cpm of ^{125}I-labeled peptide/sample, in the absence or presence of an excess of unlabeled peptide (1 μM). After incubation at room temperature for 60 min, 12 μL of 1 mM DSS in 10% DMSO/50 mM Hepes, pH 7.4, was added to the labeled membranes (final concentration of DSS, 100 μM). The reaction was continued for 10 min on ice and stopped by adding 10 μL 1 M Tris-HCl, pH 8.0. Membranes were collected by centrifugation (16000*g*, 5 min, 4 °C) resuspended in 0.5 mL of Tris-HCl, pH 7.4, and centrifuged again, and the pellet was solubilized in SDS–PAGE sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, and 1 mM 2-mercaptoethanol]. The samples (100 μg/lane) were subjected to SDS–PAGE analysis according to the method of Laemmli (1970), using 7.5% or 10% polyacrylamide slab gels as specified in the individual experiments and 4% stacking gel. After electrophoresis at a constant current of 6 mA overnight, the gels were fixed, dried,

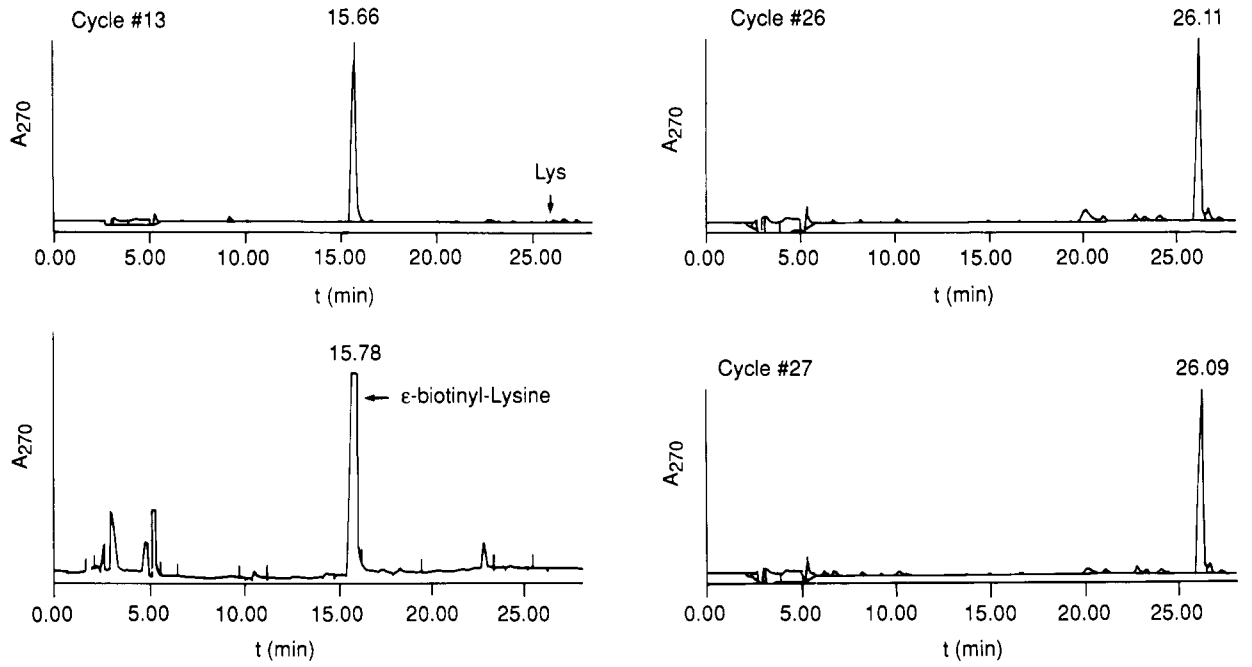


FIGURE 1: Sequence analysis of [Nle^{8,18},Lys^{13}(ε-biotinyl),Tyr^{34}]bPTH(1–34)NH$_2$. Cycles 13, 26, and 27 of the Edman degradation are shown for analogue **1**. The positions of lysine (Lys) and ε-biotinyllysine are indicated by arrows.

and exposed for 7–12 days at −70 °C to Kodak X-Omat AR films using a Cronex Lightning Plus intensifying screen.

Data Analysis. Scatchard analyses of saturation binding experiments were carried out using the Lundon-1 program. Binding constants (K_b), activation constants (K_m), and inhibitory constants (K_i) for PTH-stimulated adenylyl cyclase were calculated according to Cheng and Prusoff (1973). Values represent the mean ± SEM of triplicates from at least three experiments.

Results

Physicochemical Characterization of Analogues. The physicochemical characterization of the biotinylated analogues described in this study is summarized in Table I. The purified peptides displayed final purity greater than 99% on reversed-phase HPLC. Amino acid analyses and determinations of molecular ion masses by fast atom bombardment mass spectroscopy (Table I) corresponded to the theoretical values, confirming the correct compositions of the peptides. The precise location within the peptide sequences of the lysine residue modified by biotin was confirmed by protein sequencing. The biotin residue is destroyed and/or cleaved from the ε-amino group of lysine under the acidic conditions used for peptide hydrolysis. In contrast, conditions of Edman degradation produce no detectable loss of biotin. The sequence analysis obtained for biotinylated analogue **1** shows that the modified lysine at position 13 elutes as a phenylthiohydantoin derivative with a retention time of 15.66 min (Figure 1, cycle 13). This peak coincides with the elution time of the phenylthiohydantoin derivative of ε-biotinyllysine (BK) (r_t = 15.78 min) which was used as a reference standard (Figure 1). The peak of unmodified lysine eluting at 26.1 min was undetectable at cycle 13. Furthermore, analysis of cycles 26 and 27 of the Edman degradation showed that lysines at these two positions (r_t = 26.10 min) were not modified (Figure 1, cycles 26 and 27). Sequence analyses of analogues **2** and **3**, carried out using ε-(biotinyl-ε-aminohexanoyl)lysine as the internal standard, gave results consistent with the expected structures (data not shown).

Radioiodination of Biotinylated PTH Analogues. The radioiodinated peptides were prepared using Na^{125}I in the presence of Iodogen as the solid-phase oxidizing agent (Goldman et al., 1988). The products were purified by C-4 reverse-phase HPLC using a shallow gradient of 0.1% TFA–acetonitrile (32%–35% over 30 min) in 0.1% aqueous TFA. This procedure allowed the separation of the nonreacted, mono-iodinated and di-iodinated species which eluted in that order. Following short incubation times (2 min) with Iodogen and Na^{125}I, [Nle^{8,18},Lys^{13}(N^{ϵ}-biotinyl),Tyr^{34}]bPTH(1–34)NH$_2$ (biotinyl-PTH) generated a major radioiodination product, ^{125}I-biotinyl-PTH, with small amounts (5–10%) of a more polar ^{125}I-labeled product (retention times 14.4 min and 13.2

Table I: Amino Acid Analyses and Fast Atom Bombardment Mass Spectrometry (FAB-MS) of Biotinylated PTH and PTHrP Analogues **1–5**

	Amino Acid Analysis[a]				
amino acid	**1**	**2**	**3**	**4**	**5**
Asp, Asn	2.99 (3)	3.12 (3)	3.04 (3)	2.04 (2)	1.96 (2)
Thr	– (–)	– (–)	– (–)	0.92 (1)	1.08 (1)
Ser	3.02 (3)	2.98 (3)	3.05 (3)	1.95 (2)	1.03 (1)
Glu, Gln	5.11 (5)	5.19 (5)	5.13 (5)	4.02 (4)	2.01 (2)
Gly	1.00 (1)	1.09 (1)	1.03 (1)	1.05 (1)	– (–)
Ala	0.98 (1)	1.03 (1)	1.01 (1)	3.03 (3)	2.01 (2)
Val	2.89 (3)	2.98 (3)	3.01 (3)	0.91 (1)	– (–)
Ile	0.96 (1)	0.97 (1)	0.93 (1)	2.95 (3)	2.89 (3)
Leu	4.09 (4)	4.10 (4)	4.12 (4)	5.13 (5)	6.08 (6)
Nle	2.01 (2)	1.96 (2)	1.98 (2)	– (–)	– (–)
Tyr	1.00 (1)	0.96 (1)	0.98 (1)	– (–)	– (–)
Phe	1.03 (1)	1.04 (1)	1.01 (1)	2.08 (2)	2.00 (2)
β-Ala	– (–)	– (–)	– (–)	– (–)	0.94 (1)
ε-Ahx[b]	– (–)	1.05 (1)	1.03 (1)	– (–)	– (–)
His	3.02 (3)	2.89 (3)	3.01 (3)	4.95 (5)	3.98 (4)
Lys	2.98 (3)	2.96 (3)	2.93 (3)	2.08 (2)	1.03 (1)
Arg	1.96 (2)	1.93 (2)	1.91 (2)	3.02 (3)	2.93 (3)

	FAB-MS				
	1	**2**	**3**	**4**	**5**
calcd M_r	4314	4427	4427	4571	3776
$[M]^+$ ion	4315	4427	4426	4570	3775

[a] Peptides were hydrolyzed in 6 M HCl at 110 °C for 70 h, dried under vacuum, and analyzed using a Beckman Model 6300 amino acid analyzer. Results represent the average of analyses performed on hydrolysates of two samples. Trp residues were destroyed upon hydrolysis of the sample. Theoretical values are given in parentheses. [b] ε-Ahx = ε-aminohexanoic acid.

Table II: Biological Activities of Biotinylated PTH and PTHrP Analogues and Their Parent Peptides in Bone Cells[a]

	analogue	binding K_b[b] (nM)	adenylyl cyclase K_m (nM)	adenylyl cyclase K_i[c] (nM)
1a	[Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$	1.88 ± 0.3	0.2 ± 0.01	
1	[Nle^{8,18},Lys^{13}(ε-biotinyl),Tyr^{34}]bPTH(1–34)NH$_2$	1.52 ± 0.4	0.33 ± 0.05	
2	[Nle^{8,18},Lys^{13}(ε-biotinyl-ε-Ahx)),Tyr^{34}]bPTH(1–34)NH$_2$	1.34 ± 0.6	2.75 ± 0.1	
3	[Nle^{8,18},Lys^{26}(ε-(biotinyl-ε-Ahx)),Tyr^{34}]bPTH(1–34)NH$_2$	1.89 ± 0.4	2.91 ± 0.3	
4a	[Cys^{35}]PTHrP(1–35)NH$_2$	37.7 ± 3.1	0.69 ± 0.04	
4	[Cys^{35}(S-Shx-biotin)]PTHrP(1–35)NH$_2$	4.82 ± 0.8	0.36 ± 0.1	
5a	[Leu^{11},D-Trp^{12}]PTHrP(7–34)NH$_2$	27.7 ± 6.1		2.2 ± 0.4
5	[Leu^{11},D-Trp^{12},Lys^{13}(ε-(biotinyl-β-alanyl))]PTHrP(7–34)NH$_2$	37.3 ± 8.7		2.6 ± 0.7

[a] Values are mean ± SEM from at least three experiments. Ahx = 6-aminohexanoyl; Shx = *N*-(6-suffinylhexanoyl)-*N'*-biotinylhydrazide. [b] Inhibition of binding of 95 pM [Nle^{8,18},^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$. [c] Antagonizing 0.25 nM [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$.

Table III: Effect of Avidin, Streptavdin, and Anti-biotin on the Binding Affinities of Biotinylated Analogues of PTH and PTHrP and Their Parent Peptides in Renal Cortical Membranes[a]

	analogue	K_b (nM)	K_b[b] (nM)	K_b[c] (nM)	K_b[d] (nM)
1a	[Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$	0.26 ± 0.03	0.35 ± 0.15	nd	nd
1	[Nle^{8,18},Lys^{13}(ε-biotinyl),Tyr^{34}]bPTH(1–34)NH$_2$	0.71 ± 0.15	0.56 ± 0.18	0.93 ± 0.1	0.91 ± 0.15
2	[Nle^{8,18},Lys^{13}(ε-(biotinyl-ε-Ahx)),Tyr^{34}]bPTH(1–34)NH$_2$	0.71 ± 0.05	1.60 ± 0.1	1.20 ± 0.2	1.43 ± 0.25
3	[Nle^{8,18},Lys^{26}(ε-(biotnyl-ε-Ahx)),Tyr^{34}]bPTH(1–34)NH$_2$	0.67 ± 0.1	3.13 ± 0.3	2.77 ± 0.15	1.90 ± 0.2
4	[Cys^{35}(S-Shx-biotin)PTHrP(1–35)NH$_2$	10.25 ± 1.5	71.8 ± 0.7	150 ± 12	69.5 ± 7.5
5a	[Leu^{11},D-Trp^{12}]PTHrP(7–34)NH$_2$	6.5 ± 1.2	nd	nd	nd
5	[Leu^{11},D-Trp^{12},Lys^{13}(ε-(biotinyl-β-alanyl))]PTHrP(7–34)NH$_2$	3.2 ± 0.4	60 ± 3.5	52 ± 5.0	nd

[a] Ahx = 6-aminohexanoyl; Shx = *N*-(6-succinylhexnaoyl)-*N'*-biotinylhydrazide. Values are mean ± SEM from at least three experiments. The other footnotes represent inhibition of binding of 64 pM [Nle^{8,18},mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$, with binding in the presence of (*b*) avidin (1 μM), (*c*) streptavidin (1 μM), or (*d*) anti-biotin (1 μM); nd = not determined.

min, respectively) (Figure 2A,B). However, when the reaction time was increased to 10 min, the amount of the more polar product generated (peak B′) was found to be considerably higher (>30%) (Figure 2C). The latter product was, therefore, tentatively identified as the oxidized derivative, mono-iodo-biotinyl sulfoxide-PTH (^{125}I-biotinyl(O)-PTH). The minor hydrophobic ^{125}I-labeled products were presumably the di-iodo derivatives of biotinyl(O)-PTH and biotinyl-PTH (retention times 16.9 min and 18.0 min, respectively). Increasing the amount of Na^{125}I used in the reaction resulted in enhanced formation of both the putative oxidized and di-iodo species (data not shown). The controlled conditions described under Material and Methods were used routinely for the radio-iodination of the biotinylated analogues.

Biological Properties of Biotinylated PTH/PTHrP Analogues. Table II summarizes the biological activities of the biotinylated analogues of bPTH/PTHrP and the corresponding unmodified parent peptides in human osteosarcoma Saos-2/B-10 cells in inhibiting PTH binding and stimulating adenylyl cyclase or antagonizing PTH-stimulated adenylyl cyclase. Analogue **1**, which was modified at Lys^{13} by biotinylation, was highly active and equipotent to the parent peptide [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$ (analogue **1a**) in inhibition of PTH binding and stimulation of adenylyl cyclase. Modifications of the lysine 13 or lysine 26 by addition of the biotinyl-ε-aminohexanoyl moiety generated analogues **2** and **3**, respectively, which displayed equivalent potencies in inhibiting PTH binding and decreased potencies (8–9-fold relative to that of analogue **1**) in stimulating adenylyl cyclase. PTHrP analogue **4** modified at the sulfhydryl group of Cys^{35} by *N'*-(6-maleimidohexanoyl)-*N*-biotinylhydrazide (Mhx-biotin) was 8-fold more potent than the parent peptide **4a** in inhibiting PTH-binding and about 2-fold more potent in stimulating adenylyl cyclase. Modification of the Lys^{13} residue by biotinyl-β-alanyl in the PTHrP antagonist [Leu^{11},D-Trp^{12}]PTHrP(7–34)NH$_2$ **5a** generated analogue **5**, which was found to be as potent as its parent peptide in inhibiting PTH binding and antagonizing PTH-stimulated adenylyl cyclase. Similar to its parent peptide, analogue **5** displayed no residual agonist activity when tested in a sensitive adenylyl cyclase system using ROS 17/2.8 cells (data not shown). The biological activities of the biotinylated bPTH/PTHrP analogues in inhibiting the binding of PTH to porcine renal cortical membranes (PRCM) and the effect of exposure to avidin, streptavidin, or anti-biotin on these activities are summarized in Table III. Analogues **1–3** have potencies comparable to the unmodified parent peptide **1a** in competing with [Nle^{8,18},^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ (^{125}I-PTH) for binding to PRCM. These analogues, however, displayed diverging potencies when tested in the presence of avidin, streptavidin, or anti-biotin. The binding activity of analogue **1** was unaf-

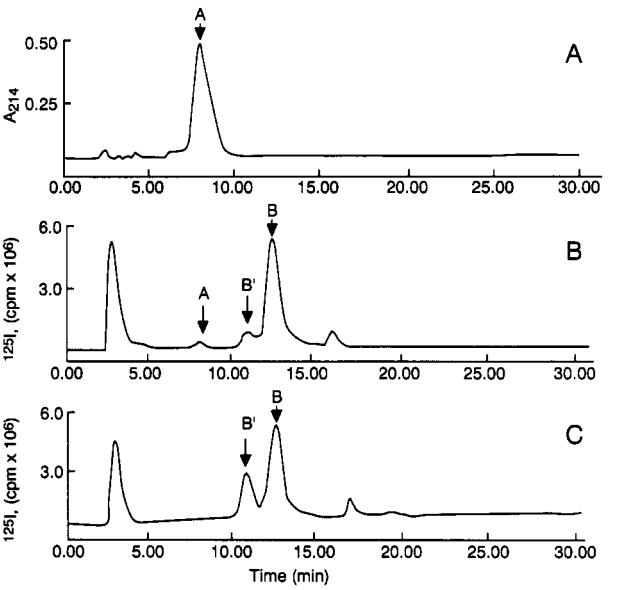


FIGURE 2: Radioiodination of [Nle^{8,18},Lys^{13}(ε-biotinyl),Tyr^{34}]-bPTH(1–34)NH$_2$. Following either a 2-min (panel B) or a 10-min (panel C) incubation of the peptide with Na^{125}I and Iodogen, the reaction mixture was chromatographed by HPLC (as described in Materials and Methods), and the absorption (panel A) and radioactivity (panels B and C) were monitored. Peaks A and B indicate the retention times of [Nle^{8,18},Lys^{13}(ε-biotinyl)Tyr^{34}]bPTH(1–34)NH$_2$ and [Nle^{8,18},Lys^{13}(ε-biotinyl),mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$, respectively. Peak B′ was putatively identified as the sulfoxide of [Nle^{8,18},Lys^{13}(ε-biotinyl),mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$.

Table IV: Binding Affinities of ^{125}I-Labeled Biotinylated PTH (**1**) and Its Parent Peptide in Canine and Porcine Renal Cortical Membranes[a]

	analogue	CRCM K_b[b] (nM)	PRCM K_b[b] (nM)
1	[Nle^{8,18},Lys^{13}(ϵ-biotinyl),Tyr^{34}]bPTH(1–34)NH$_2$	0.35 ± 0.1[c]	0.68 ± 0.2[c]
		0.82 ± 0.1[d]	0.71 ± 0.1[d]
1a	[Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$	0.26 ± 0.03[d]	0.38 ± 0.1[d]

[a] Values are mean ± SEM from at least three experiments. [b] The other footnotes represent the inhibition of binding of 64 pM (*c*) [Nle^{8,18},Lys^{13}-(ϵ-biotinyl),mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ or (*d*) [Nle^{8,18},mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$.

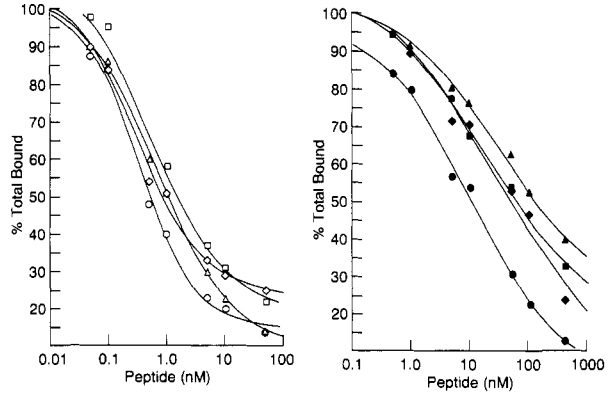


FIGURE 3: Inhibition of [Nle^{8,18},mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ binding by either [Nle^{8,18},Lys^{26}(ϵ-biotinyl-ϵ-Ahx)),Tyr^{34}]bPTH(1–34)NH$_2$ (left panel, ○) or [Cys^{35}(S-Shx-biotin)PTHrP(1–35)NH$_2$ (right panel, ●) in the presence of avidin (□, ■), streptavidin (△, ▲), and anti-biotin (◇, ◆). The value for each concentration was determined in triplicate. The data represent the mean ± SEM of three separate experiments.

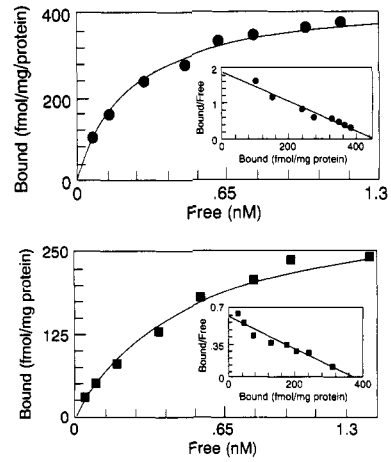


FIGURE 4: Scatchard analysis of saturation binding of [Nle^{8,18},Lys^{13}(ϵ-biotinyl),mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ (●) and [Nle^{8,18},mono^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ (■) to PTH receptors on porcine renal cortical membranes. Each value is the mean of triplicate determinations of a representative experiment.

fected by either avidin, streptavidin, or anti-biotin, whereas analogues **2** and **3**, containing a longer ϵ-aminohexanoyl spacer arm, showed 2–5-fold decreases in potencies, with the larger decline observed for analogue **3** modified by Lys^{26} (see Figure 3, left panel). PTHrP analogue **4**, modified at position Cys^{35} and containing an aminohexanoyl spacer group, displayed a 7–15-fold decrease in potency in inhibiting the binding of PTH to PRCM when tested in the presence of either avidin, streptavidin, or anti-biotin (Figure 3, right panel). The biotinylated PTHrP antagonist analogue **5** although 2-fold more potent than its parent peptide **5a**, showed a 16–19-fold decrease in potency in the presence of avidin or streptavidin.

Radioiodination of analogue **1** generated a ligand, ^{125}I-biotinyl-PTH, with high binding affinity for PTH receptors in both canine and porcine renal cortical membranes (Table IV). Analogue **1** displays potencies comparable to those of the unmodified parent peptide in inhibiting the binding of ^{125}I-biotinyl-PTH or ^{125}I-PTH in both renal membrane preparations. Saturation binding experiments carried out in PRCM with ^{125}I-biotinyl-PTH and ^{125}I-labeled [Nle^{8,18},Lys^{13}(N$^{\epsilon}$-(biotinyl-ϵ-aminohexanoyl),Tyr^{34}]bPTH(1–34)NH$_2$ (^{125}I-biotinyl^{13}-Ahx-PTH) are depicted in Figure 4. ^{125}I-Biotinyl-PTH and ^{125}I-biotinyl^{13}-Ahx-PTH interact with a single class of high-affinity binding sites, as shown by Scatchard analysis. The observed K_d values of 0.24 nM and 0.61 nM for ^{125}I-biotinyl-PTH and ^{125}I-biotinyl^{13}-Ahx-PTH, respectively, are close to those determined for the corresponding unlabeled ligands **1** and **2**. The maximal binding capacity (B_{max}) of 450 fmol/mg of protein for ^{125}I-biotinyl-PTH was very close to that determined for ^{125}I-biotinyl^{13}-Ahx-PTH, namely B_{max} = 390 fmol/mg of protein. Similar results were recently reported by us for ^{125}I-PTH (B_{max} = 395 fmol/mg of protein) in canine renal membranes (Goldman et al., 1988).

The binding of ^{125}I-biotinyl-PTH to streptavidin–agarose or anti-biotin–agarose compared to that of the nonbiotinylated ligand, ^{125}I-PTH, showed that only ^{125}I-biotinyl-PTH bound

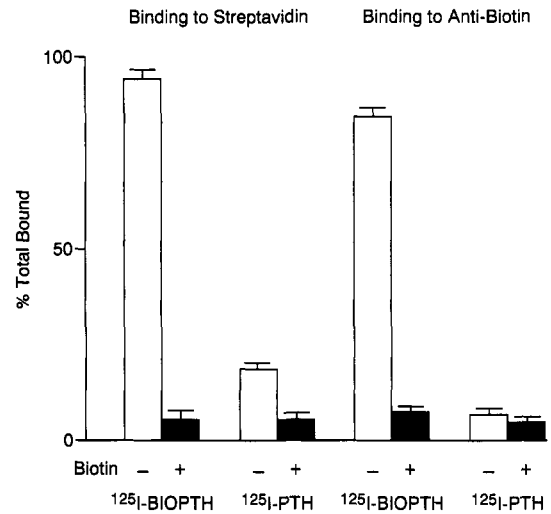


FIGURE 5: Binding of [Nle^{8,18},Lys^{13}(ϵ-biotinyl),^{125}I-Tyr^{34})NH$_2$ (^{125}I-BIOPTH) and [Nle^{8,18},^{125}I-Tyr^{34}]bPTH(1–34)NH$_2$ (^{125}I-PTH) to streptavidin–agarose and anti-biotin–agarose. Binding of the radioligands (2 × 10^6 cpm) to the immobilized streptavidin or anti-biotin was tested in the absence or presence of 2 mM biotin. The results represent the means ± SEM of at least three experiments.

specifically to streptavidin–agarose or anti-biotin–agarose (Figure 5). However, due to the extremely high affinity of avidin for biotin ($K_b = 10^{-15}$ M), ^{125}I-biotinyl-PTH was found to bind to streptavidin–agarose in a nonreversible manner. In contrast, ^{125}I-biotinyl-PTH when bound to anti-biotin–agarose could be specifically dissociated, albeit slowly ($t_{1/2}$ = 45 min), upon incubation with 2 mM biotin (data not shown). The latter property is essential for affinity chromatography.

Affinity Labeling of Renal Membrane PTH Receptors. Chemical cross-linking experiments of the ^{125}I-biotinyl-PTH analogues (analogues **1–3**) to porcine renal membranes with DSS identified one specific band of 75 kDa, as determined by autoradiography (Figure 6). The intensity of this band was diminished in experiments in which cross-linking was

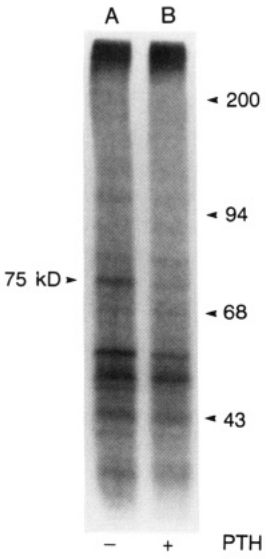


FIGURE 6: Electrophoretic analysis of affinity-labeled PTH receptors. Autoradiography of 7.5% SDS–polyacrylamide gel electrophoresis of affinity-labeled PTH receptors on purified porcine renal membranes (PRCM) was performed as described in Materials and Methods. Lanes A and B represent PRCM labeled with ^{125}I-biotinyl-PTH in the absence (A) or presence (B) of 1 μM unlabeled [Nle^{8,18},Tyr^{34}]-bPTH(1–34)NH$_2$. Molecular weight standards include myosin (200 000), phosphorylase *b* (97 400), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), and β-lactoglobulin (18 400).

conducted in the presence of an excess of unlabeled PTH analogue **1a**.

Discussion

The strategy employed in the present study for designing bioactive biotinylated PTH/PTHrP analogues was to specifically biotinylate certain residues which were previously identified as biologically "silent". Recently, we have shown that analogues of bPTH(7–34)NH$_2$ modified at Lys^{13} with 3-phenylpropanoyl or N^{ϵ},N^{ϵ}-diisobutyl addition were highly potent PTH antagonists (Chorev et al., 1991). Photoreactive analogues of PTH have been prepared (Coltrera et al., 1981; Goldring et al., 1984). The most potent derivative was found to be modified with 4-azido-3-nitrophenyl at the ϵ-amino of Lys^{13} (Shigeno et al., 1988, 1989). In recent studies (Newman et al., 1989; Abou-Samra et al., 1990), biotinylated derivatives of [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$ were prepared by post-synthetic modification of the peptide in solution, which results in a statistical distribution of biotinylation across available amino groups. It was suggested that the biotinylated analogues maintain bioactivity when modified at the lysines at either position 13, 26, or 27 but are totally inactive when modified at the α-amino group of the N-terminal alanine. However, due to its lack of selectivity, this method of biotinylation suffers from the following drawbacks: (1) formation of a highly complex mixture of closely similar products from which the desired product is isolated in low yield and (2) ambiguity in the exact position of modification of Lys^{26} versus Lys^{27} in PTH.

In the present work, we prepared monobiotinylated analogues of PTH/PTHrP (analogues **1–3** and **5**) modified selectively at either Lys^{13} and Lys^{26} residues during solid-phase synthesis. The N-terminal biotinylation of a peptide bound to resin is straightforward and generally compatible with the subsequent steps of deprotection and cleavage. In contrast, selective biotinylation at an internal position, such as the ϵ-amino group of lysine, requires the development of alternative strategies. The orthogonal combination of N^{α}-Boc/N^{ϵ}-Fmoc protecting groups was used during synthesis for the specific lysine residue to be modified. Introduction of N^{α}-Boc-Lys-(N^{ϵ}-Fmoc)-OH into a desired position permitted subsequent selective removal of the Fmoc group followed by biotinylation of the unprotected ϵ-amino group of lysine while on the solid-phase support. An alternative approach was used for the synthesis of the biotinylated PTHrP analogue **4**. In this case, [Cys^{35}]PTHrP(1–35)NH$_2$ was modified postsynthetically while in solution by reaction with a maleimidobiotin derivative. This method takes advantage of the particular specificity and high reactivity of the sulfhydryl function of cysteine residues toward maleimido derivatives (Koshland et al., 1963; Chorev et al., 1992).

The above synthetic methods allowed the preparation of specific monobiotinylated analogues in large quantities and high purity. This in turn, enabled the unequivocal assessment of their structure and biological activity.

The biotinylated analogues retained full biological activity in binding to PTH receptors using both human osteosarcoma (Saos-2/B-10) cells and PRCM; they also displayed high potency in stimulating cAMP increases in human osteosarcoma cells. Specifically, insertion of a 6-aminohexanoyl spacer arm between biotin and the ϵ-amino of lysine 13 or 26 was found to be compatible with retention of high biological activity (analogues **2** and **3**). These results strongly suggest that the ϵ-amino group of both Lys^{13} and Lys^{26} residues is not directly involved in interaction with the PTH receptor and/or stabilization of the bioactive conformation of the peptide. Interestingly, modification of Lys^{13} or Lys^{26} with the biotinyl-ϵ-aminohexanoyl group did not affect the affinity of the peptides for the PTH receptor but lowered their potency in stimulating adenylyl cyclase activity in Saos-2/B-10 cells. However, modification of Lys^{13} with the biotinyl group itself resulted in no apparent loss in affinity or biological activity. The reason for this apparent discrepancy in agonist potencies is not known. It is possible that introduction of the aminohexanoyl spacer at position 13 introduces steric hindrance and/or local conformational changes which selectively affect agonist efficacy but not affinity for the receptor.

Modification of Cys^{35} in PTHrP by the maleimidobiotin derivative containing the 6-aminohexanoyl spacer arm is well-tolerated, resulting in increased affinity for the PTH receptor and no apparent loss of biological activity.

The biotinylated PTH analogues **1–3** were shown to possess high binding affinities for PTH receptors in PRCM in the presence of a large excess (1 μM) of either avidin, streptavidin, or anti-biotin. Under these conditions, the rank order of potencies of PTH analogues was **1a** = **1** > **2**, **3** ≫ **4** > **5**, with the largest change occurring with PTHrP analogues **4** and **5**, which displayed a 7–20-fold decrease in potency, whereas, no apparent loss in affinity was observed for analogue **1**. These results suggest that analogues **1–3** can interact specifically with the biotin-binding proteins while they maintain reasonable affinities for the receptor. In contrast, the large decline in affinity observed for analogues **4** and **5** suggest that these ligands, once bound to avidin or anti-biotin, are not available for an effective interaction with the PTH receptor.

Radioiodination of biotinylated analogues of PTH (analogues **1–3**) generated ligands which efficiently interact with PTH receptors on PRCM and bind specifically to immobilized streptavidin or anti-biotin. Scatchard analysis revealed specific binding to a single class of high-affinity sites on renal membranes, with binding constants similar to those observed for ^{125}I-labeled [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$ (Goldman et al., 1988). Furthermore, the biotinylated radioligands specifically labeled renal PTH receptors (estimated M_r = 75 000 as de-

termined by chemical cross-linking with DSS). These results closely correlate with previous studies which reported similar M_r (70 000–80 000) for PTH receptors in renal membranes (Karpf et al., 1988) using bPTH(1–34) as the radiolabeled ligand and succinimidyl 4-azidobenzoate (HASB) as the cross-linking reagent and observations reported with rat osteosarcoma ROS 17/2.8 cells and either chemical cross-linking of radiolabeled [$Nle^{8,18}$,Tyr^{34}]bPTH(1–34)NH_2 (Wright et al., 1987) or photoaffinity labeling with 4-nitrophenyl azide derivatized radiolabeled [$Nle^{8,18}$,Tyr^{34}]bPTH(1–34)NH_2 (Shigeno et al., 1988).

In conclusion, a novel synthetic methodology has been developed for the preparation of selectively modified monobiotinylated analogues of PTH/PTHrP during solid-phase synthesis or in solution. Furthermore, the general approach described here should be applicable to other peptide hormones.

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