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DIPEPTIDE MIMETICS CAN SUBSTITUTE FOR THE RECEPTOR ACTIVATION DOMAIN RESULTING IN HIGHLY POTENT ANALOGUES OF hPTH(1-36) FRAGMENT

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Abstract: A series of hPTH(1-36) analogues were prepared to study the role of the first peptide bond between residues Ser[1]-Val[2]. Some of these analogues were found to show high affinity binding in intact opossum kidney (OK-1) cells and were very active in their ability to stimulate adenylate cyclase production in intact OK-1 cells, rat UMR106-06 osteosarcoma cells, and SaOS-2 human osteosarcoma cells. Copyright © 1996 Elsevier Science Ltd

Parathyroid hormone (PTH) maintains calcium homeostasis through its action on kidney and bone. Constantly elevated plasma levels of (PTH) are known to increase bone resorption, e.g. in primary hyperparathyroidism. Mild forms of this disease are, however, associated with increases in trabecular bone. The anabolic effect of PTH on bone was first described by H. Selye in 1932[1] and reviewed recently[2]. Since then a large number of animal and human studies have shown that intermittent PTH administration produces a net increase in bone mass. Although the full length hormone contains 84 amino acid residues, full biological activity is retained in the N-terminal(1-34) fragment. Within this fragment (1-34), two separate domains have been described for receptor binding and receptor activation. The region defined by the amino acid residues 15-34 is important for high affinity binding to the receptor[3]. Amino acid residues which are essential for PTH receptor activation have also been identified. Removal of the first two amino acid residues reduces the ability to stimulate adenylate cyclase despite a relatively intact binding profile, and truncation beyond position 3 (e.g. PTH-(7-34)-NH_2) leads to potent PTH/PTHrP receptor antagonists[4].

Here we report our studies on the role of the first peptide bond between residues Ser[1]-Val[2] of hPTH-(1-36)-NH_2. This peptide bond was either modified chemically or replaced by an olefin dipeptide isostere within the basic structure of hPTH-(1-36)-NH_2. Analogues were characterized by binding affinity in intact opossum kidney (OK-1) cells and their ability to activate adenylate cyclase (cAMP) in intact opossum kidney (OK-1) cells, UMR106-06 (rat osteosarcoma) and SaOS-2 (human osteosarcoma) cells.

Chemical Synthesis

All N-terminal modified hPTH-analogues were synthesized using a batch of 4.5 mMol (45 g) of side-chain protected hPTH(3-36)-amide-"Rink"-resin. This large-scale synthesis was performed on a semiautomatic Labortec synthesizer equipped with a 1.5 l stirred reactor. The Boc-A^1-A^2-COOH representing isosteres for the

first two amino acids of hPTH were prepared according to Scheme 2 and 3. These derivatives were coupled to the side-chain protected hPTH(3-36)-amide-"rink"-resin to give the fully protected peptide intermediates. Cleavage and simultaneous deprotection by a TFA-scavenger mixture (Scheme 1) gave the desired peptides. All peptides were purified by preparative HPLC and characterized by amino acid analysis and by ion spray mass spectroscopy.

Scheme 1

Boc-A^1-A^2-COOH + H-Ser^3(*t*-Bu)-Glu(*t*-Bu)-Ile-Gln(Trt)-Leu-Met-His(Trt)-Asn^{10}(Trt)-Leu-Gly-Lys(Boc)-His(Trt)-Leu-Asn(Trt)-Ser(*t*-Bu)-Met-Glu(O*t*-Bu)-Arg^{20}(Pmc)-Val-Glu(O*t*-Bu)-Trp(Boc)-Leu-Arg(Pmc)-Lys(Boc)-Lys(Boc)-Leu-Gln(Trt)-Asp^{30}(O*t*-Bu)-Val-His(Trt)-Asn(Trt) Phe-Val-Ala-amide-"Rink"-resin

↓ 1) Coupling with PyBOP
2) TFA/H_2O/Thioanisol 90/5/5
3) Purification

A^1-A^2-Ser^3-Glu-Ile-Gln-Leu-Met-His-Asn^{10}-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg^{20}-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp^{30}-Val-His-Asn-Phe-Val-Ala-NH_2

Synthesis of Boc-A^1-A^2 -COOH derivatives

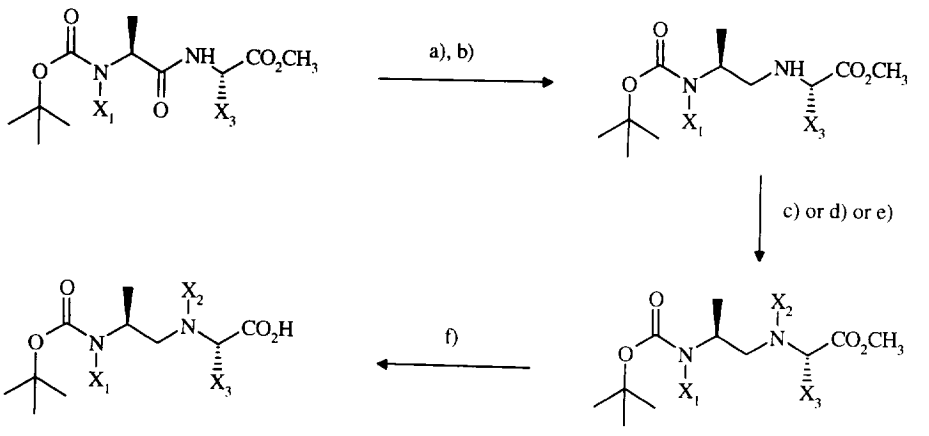
Desoxopeptides:

N-terminal protected dipeptide-methylesters were converted via the corresponding endothiopeptides, using Lawesson-reagent, followed by desulfurization with Nickel boride into the desired desoxopeptides[5]. N-alkylation was performed on the appropriate desoxopeptides by hydrogenation (palladium on carbon) in the presence of acetone or of 37% formaldehyde. For N-acetylation standard acetylation conditions (acetylchloride/triethylamine) were used. All methylesters were saponified with lithiumhydroxide to give the desired free acids. (Scheme 2).

Olefin-Isosteres:

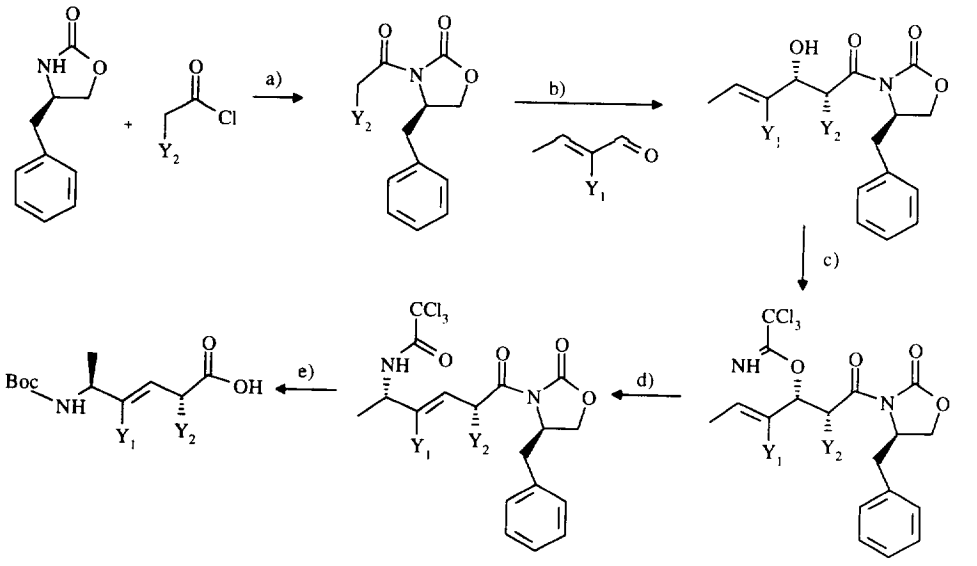
The dipeptideolefin isosteres were synthesized as outlined in Scheme 3[6]. Coupling of the chiral oxazolidinone auxiliary with the appropriate acid chloride followed by condensation of the resulting adduct with an α,β-unsaturated aldehyde resulted in diastereomerically pure aldol-products[7]. Treatment of these aldol-products with trichloracetonitrile in the presence of DBU followed by a thermal aza-Claisen rearrangement[8] of the resulting intermediates, gave the desired dipeptideolefins. After cleavage of the chiral oxazolidinone auxiliary and protecting group exchange on the amino moiety, the acids, were isolated, ready for coupling.

Scheme 2



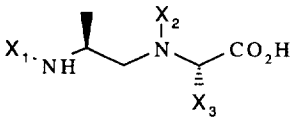
a) Lawesson-reagent; b) $NiCl_2$ $6H_2O/NaBH_4$; c) $H_2/Pd/C/H_2CO$ 37%; d) $H_2/Pd/C$/acetone; e) $AcCl/NEt_3$; f) LiOH

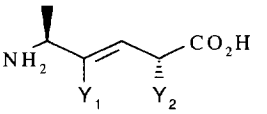
Scheme 3



a) *n*-BuLi/THF/-78°C; b) $TfB(n\text{-}Bu)_2/NEt_3$/-78°C; c) CCl_3CN/DBU; d) Xylene reflux; e) i: $LiOH/H_2O_2$; ii: 6N NaOH; iii: $(Boc)_2O$

A^1-A^2-structures





cpd 11: X_1 = H; X_2 = H; X_3 = isopropyl
cpd 12: X_1 = H; X_2 = acetyl; X_3 = isopropyl
cpd 13: X_1 = H; X_2 = acetyl; X_3 = H
cpd 14: X_1 = H; X_2 = methyl; X_3 = isopropyl
cpd 15: X_1 = methyl; X_2 = H; X_3 = isopropyl
cpd 16: X_1 = methyl; X_2 = methyl; X_3 = isopropyl
cpd 17: X_1 = H; X_2 = isopropyl; X_3 = H

cpd 21: Y_1 = F; Y_2 = methyl
cpd 22: Y_1 = Cl; Y_2 = methyl
cpd 23: Y_1 = F; Y_2 = isopropyl
cpd 24: Y_1 = Cl; Y_2 = isopropyl
cpd 25: Y_1 = methyl; Y_2 = isopropyl
cpd 26: Y_1 = F; Y_2 = benzyl

Table 1: Biological activities of hPTH(1-36)-NH_2 analogues. (i.a. = intrinsic activity)

[A^1-A^2]hPTH-(1-36)-NH_2/ cpd-Nr./Entry	Binding affinity OK-1 pK_D	cyclic AMP OK-1		cyclic AMP UMR		cyclic AMP SaOS	
		EC_{50};(nM)	i.a.	EC_{50};(nM)	i.a.	EC_{50};(nM)	i.a.
Ser-Val / 1	8.6	4.2	1.0	3.3	0.98	2.3	0.96
Ala-Val / 2	8.4	5.9	1.0	3.3	1.0	3.0	0.95
cpd 11 / 3	8.1	7.0	1.0	1.0	0.95	2.4	1.0
cpd 12 / 4	7.9	9.9	0.8	15.0	1.0		
cpd 13 / 5	7.1	116.6	0.2	958	0.8		
cpd 14 / 6	8.4	3.5	1.0	4.4	0.9		
cpd 15 / 7	8.5	7.4	0.9	2.6	1.0	1.6	1.0
cpd 16 / 8	8.5	6.5	1.0	4.9	1.0		
cpd 17 / 9	7.4	97.2	0.5	30.0	0.05		
cpd 21 / 10	8.2	9.1	0.6	13.0	0.95		
cpd 22 / 11	8.9	5.5	1.0	5.0	1.0		
cpd 23 / 12	8.9	4.4	1.0	1.6	1.0	1.5	0.89
cpd 24 / 13	8.9	7.9	1.1	0.65	1.0	0.85	1.0
cpd 25 / 14	9.0	4.4	1.0	1.2	1.0	1.1	1.0
cpd 26 / 15	8.6	>1000	<0.1	26.0	0.08		

Biological Tests

PTH binding studies were performed in intact confluent cultures of opossum (OK-1) kidney cells using [^{125}I]chickPTHrP-(1-36)-NH_2 as radoligand[9]. Cyclic AMP production was measured in confluent cell cultures using a radiometric assay as described by Salomon [10].

Results and Discussion

hPTH-(1-36)-NH_2 showed a strong binding affinity (Table 1, entry 1, pK_D=8.6) and a concentration dependent accumulation of cyclic AMP with the apparent EC_{50} value of 4.2 nM on OK-1 cells. Substitution of Ser^1 to Ala^1 (Entry 2) slightly decreased the binding affinity with no significant change in cyclic AMP production.

Reduction of the first peptide-bond between Ala^1 and Val^2 to the methyleneamine isostere (Entry 3) perserved high affinity binding and the capacity to activate adenylate cyclase in all three cell lines tested. Methylation of the newly formed amino-group (Entry 6) showed no significant change in binding and activity. However acetylation of the same amino-group (Entry 4) decreased binding affinity, bioactivity, and introduced partial agonism (i.a.=0.8) in OK-1 cells. Dimethylation of the methyleneamine isostere did not alter the profile of the compound (Entry 8). This result is in line with the observation that N-terminal methylation does not alter the binding and activity profile of hPTH-(1-36)-NH_2. Moving the isopropyl side chain from the carbon to the nitrogen in the second amino acid moiety (Entry 9) resulted in a significant loss of binding affinity potency, and the appearance of partial agonism with apparent intrinsic activities of 0.5 in OK-1 and 0.05 in UMR cells. A similar profile was observed with a second "Gly^2" analogue in which the second nitrogen was acetylated (Entry 5). The most potent analogue in this series was the N-terminus methylated methyleneamine isostere (Entry 7).

In order to retain the geometry of the amide bond we replaced this bond by the Z configurated fluoro-, chloro- or methyl-olefines. The Z configuration of the olefines was proven by Proton Magnetic Resonance spectra[6]. The fluoro-olefin (Entry 12) showed a twofold increase in binding affinity. A similar increase was seen in the potency to stimulate cyclic AMP in UMR and SaOS but not in OK-1 cells. The substituents on the olefin, chlorine (Entry 13) or methyl (Entry 14) did not change this pattern. The Z configurated fluoro-olefin representing an isostere of the Ala^1-Ala^2 dipeptide had reduced binding affinity and potency, and it behaved as a partial agonist (i.a.=0.6) in OK-1 cells (Entry 10). Replacing fluorine by chlorine (Entry 11) restored full agonistic activity. Exchange of the isopropyl-group by a benzyl-group, leading to an Ala^1-Phe^2 isostere (Entry 15) not only decreased potency but almost removed intrinsic activity. Despite the drop in potency, the binding affinity remained unchanged. Therefore this analogue could serve as a lead for a potential PTH antagonist.

Conclusions

Combining fragment synthesis in solution with solid-phase peptide synthesis procedures provided an efficient synthetic route to this series of backbone-modified analogues of hPTH-(1-36)-NH_2.

From our chemical modifications on the first N-terminal peptide bond of hPTH-(1-36)-NH_2 it can be concluded that this bond is not critical for binding affinity or activation of the PTH/PTHrP receptor. Even total replacement of the Ala^1-Val^2 dipeptide by an olefin-isostere does not interefere with the binding and receptor activation of parathyroid hormone. In contrast, the results with Gly^2-analogues indicate that the amino acid side-chain at position 2 is important for both receptor binding and receptor activation. A shift of the isopropyl side-chain from C^α to N^α of position 2, a 'Val-N-peptoid', results in low binding affinity and partial agonism. This may be due to effects on the secondary structure of the hormone which extends into the binding domain and can, therefore interfere with receptor contact. A benzyl-group at position 2 can substitute for the isopropyl-group in binding, but not for agonistic activity.

In conclusion, the chemical modifications around the first peptide bond at the N-terminal side of hPTH-(1-36)-NH_2 have contributed to a better understanding of the structure activity relations of this part of the hormone and new, highly potent hPTH-(1-36)-NH_2 analogues were obtained.

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