

Synthesis and structural studies of new analogues of PTH(1–11) containing C α -tetra-substituted amino acids in position 8

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Abstract The N-terminal 1–34 fragment of parathyroid hormone (PTH) is fully active in vitro and in vivo and it can reproduce all biological responses characteristic of the native intact PTH. Recently, analogues of PTH(1–11) fragments with helicity-enhancing substitutions have been demonstrated to yield potent analogues of PTH(1–34). The work describes the synthesis, biological activity and structure of analogues of the best modified PTH sequence H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH₂ (I). In particular, the effect of the Ala/Aib substitution at positions 1 and 3 as well as of the replacement of Nle in position 8 with D-Nle, L-(α Me)-Nle and D-(α Me)-Nle was studied. The resulting peptides were characterized structurally by CD spectroscopy, solution NMR and MD, and in vitro for activity with respect to the cognate receptor, parathyroid hormone receptor.

Keywords SPSS · CD · NMR · MD ·
C α -tetra-substituted Amino Acids · PTH

Introduction

Research over the last 50 years has led to a better understanding of the mechanisms of action, physiology,

pathophysiology and therapeutic significance of the parathyroid hormone (PTH) and its analogues (Potts and Gardella 2008). PTH is an 84-amino acid hormone and is a gland-secreted endocrine hormone (Kronenberg et al. 1997). The parathyroid hormone receptor (PTHr) (Jüppner et al. 1991) is a family-B G-protein-coupled receptor (Chorev and Rosenblatt 1994), is expressed on the surface of bone and kidney target cells, and mediates the biological actions of two ligands, PTH and PTH-related protein (PTHrP). Therefore, it plays critical roles in calcium and phosphate homeostasis, via PTH, and in bone growth and development via PTHrP [Chorev and Rosenblatt 1994; Kronenberg 2006].

Biosynthetic PTH(1–34) increases bone mineral density and bone strength in humans and indeed is now considered one of the most effective treatments for osteoporosis (Tashjian and Gagel 2006). The hypothesis of the mechanism of the interaction between PTH(1–34) and its receptor involves two principal components: an interaction between the C-terminal domain of PTH(1–34) and the N-terminal extracellular domain of the receptor and an interaction between the signalling domain of PTH, which comprises the first 11 amino acids, and the juxtamembrane region of the receptor, which contains the extracellular loops and seven transmembrane helices (Hoare et al. 2001; Castro et al. 2005; Shimizu et al. 2005; Gensure et al. 2005; Wittelsberger et al. 2006; Dean et al. 2008, and references therein).

NMR analyses of PTH(1–34) analogues in a variety of polar and non-polar solvents suggest that the N-terminal portion of PTH, known to be responsible for receptor activation, contains a short α -helical segment from residue 3 to 13. In addition, there is a more stable, C-terminal α -helical segment (from Arg²⁰ to Val³¹), where the principal receptor-binding domain is located.

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Recent studies have demonstrated that enhancement of α -helicity in the PTH(1–11) sequence results in potent PTH(1–11)NH₂ analogues (Tsomaia et al. 2004; Barazza et al. 2005). Based on mutagenesis studies and on the position and shape of the binding sites for residues in position 2, 5 and 8, high helicity has been suggested to be essential for receptor activation (Shimizu et al. 2000; Monticelli et al. 2002). Specifically, the arrangement of residue 8 on the same face of the helix as Ile⁵, as well as the position of Val² projecting toward the third extracellular loop have been hypothesized to be fundamental requirements for receptor activation (Gardella and Jüppner 2001).

Based on the hypothesis of an α -helical N-terminal portion of PTH when bound to the receptor, a series of PTH(1–11) analogues containing sterically hindered and helix-promoting C ^{α} -tetra-substituted amino acids was synthesized to enhance α -helicity of short PTH fragments with PTH/PTH1R (Shimizu et al. 2004).

Introduction of residues conferring conformational constraints, such as α -amino isobutyric acid (Aib), into peptides can improve their activity and receptor binding selectivity (Hirschmann 1991; Gante 1994; Kessler et al. 1995). The Aib-modified PTH(1–14) analogues were found, by circular dichroism spectroscopy, to exhibit more helicity than their Ala-containing counterparts (Shimizu et al. 2001). Moreover, theoretical and experimental studies (Torrás et al. 2008, and references herein; Moretto et al. 2008) have highlighted the strong tendency of Aib to induce folded structures falling in the 3_{10} -/ α -helical region (ϕ , $\psi \approx \pm 60^\circ$, $\pm 30^\circ$) of the conformational space, while semi-extended or fully extended conformations are extremely rare. In comparison, Ala is easily accommodated in both folded and extended structures. Thus, a local increment of steric hindrance around the α -carbon appears to be responsible for an effective promotion of helical arrangements in Aib-containing peptides (Kaul and Balaram 1999; Torrás et al. 2008; Maity and König 2008).

According to this hypothesis, we describe here the synthesis, the biological activity and the structure of a series of analogues of the most active modified PTH(1–11) sequence, H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH₂ (I) (Table 1). We were interested in studying the effect of the introduction of a C ^{α} -tetra-substituted amino acid in position 8. Specifically, we analyzed peptides containing D-Nle, L-(α Me)Nle and D-(α Me)Nle in that position as well as various combinations of Ala/Aib in positions 1 and 3. The final aim of the study is to generate a candidate peptide-based osteoporosis therapeutic drug that, by virtue of enhanced α -helicity, will bind to the target receptor more effectively.

Table 1 Library of PTH(1–11) analogues containing D/L-(α Me)Nle

Name	Peptide sequence	CF	MW m.i. ^a	[M + H] ⁺ found	Yield %	R _t ^b (min)	EC ₅₀ (nM) ^c
I	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH ₂	C ₅₉ H ₁₀₃ N ₁₉ O ₁₅	1,317.74	1,318.79	33	16.98	1.0 ± 0.15
II^d	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-D-Nle-His-Gln-Har-NH ₂	C ₅₉ H ₁₀₃ N ₁₉ O ₁₅	1,317.74	1,318.73	39	17.70	2,000 ± 300
III	H-Aib-Val-Ala-Glu-Ile-Gln-Leu-L-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₈ H ₁₀₁ N ₁₉ O ₁₅	1,303.74	1,304.71	17	16.83	Not active
IV	H-Aib-Val-Ala-Glu-Ile-Gln-Leu-D-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₈ H ₁₀₁ N ₁₉ O ₁₅	1,303.74	1,304.75	15	17.10	Not active
V	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-L-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₉ H ₁₀₃ N ₁₉ O ₁₅	1,317.74	1,318.77	21	16.99	20.0 ± 3.0
VI	H-Aib-Val-Aib-Glu-Ile-Gln-Leu-D-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₉ H ₁₀₃ N ₁₉ O ₁₅	1,317.74	1,318.80	19	17.22	270 ± 40
VII	H-Ala-Val-Aib-Glu-Ile-Gln-Leu-L-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₈ H ₁₀₁ N ₁₉ O ₁₅	1,303.74	1,304.72	20	16.44	Not active
VIII	H-Ala-Val-Aib-Glu-Ile-Gln-Leu-D-(α Me)Nle-His-Gln-Arg-NH ₂	C ₅₈ H ₁₀₁ N ₁₉ O ₁₅	1,303.74	1,304.77	19	16.67	Not active

^a Monoisotopic (m/z) molecular weight calculated for chemical formula (CF)

^b R_t was determined with a linear gradient of 10–90% (v/v) B over 30 min (A: water + 0.1% TFA; B: 90% acetonitrile + 0.1% TFA)

^c EC₅₀ is the result of the average on at least three values. EC₅₀ is defined as the half maximal effective concentration and is referred to the concentration of peptide, which induces a response halfway between the baseline and the maximum

^d (Caporale et al. 2010)

Materials and methods

General

Starting materials were obtained from commercial suppliers and used without further purification. Rink Amide MHBA Resin (0.73 mmol/g loading) as a solid support was obtained from Inalco-Novabiochem (Milano, Italy). HBTU, HOBt and Fmoc-protected natural amino acids were obtained from GL Biochem (Shanghai, China). Hexanone and cyanuric fluoride were purchased from Lancaster (Morecambe, England). Fmoc-Aib-OH was purchased from NeoMPs (Strasbourg, France). DMF dried over molecular sieves ($\text{H}_2\text{O} < 0.01\%$) and DIPEA were from Fluka Chemie GmbH (Buchs, Switzerland), and dry dichloromethane was distilled from P_2O_5 and kept over 4 Å molecular sieves. Ammonia solution at 17% was purchased from CarloErba Reagents. Water for reversed-phase high performance liquid chromatography (HPLC) was filtered through a 0.22- μm membrane filter (Millipore, Millipak40). Reversed-phase purification was routinely performed on a Shimadzu LC-8A equipped with a Shimadzu SPD-6A UV detector on a Delta-Pak Waters C18-100 Å silica HPLC column. The operative flow rate was 17 ml/min with a linear gradient of 10–35% (v/v) B over 15 min (A: water + 0.1% TFA; B: 90% acetonitrile + 0.1% TFA). Homogeneity of the products was assessed by analytical reversed-phase HPLC using a Vydac C18 column (218TP510), with a linear gradient of 10–90% (v/v) B in 30 min, a flow rate of 1 ml/min and UV detection at 214 nm. Molecular masses of final peptides were determined by electrospray ionization mass spectrometry (ESI-MS), a Perseptive Biosystems MARINERTM API-TOF spectrometer.

Solid-phase peptide synthesis

Fmoc-protected Rink Amide MHBA Resin (100 mg, 72 mmol) was swelled twice in DMF for 30 min each, treated with 20% piperidine-DMF (5 min and then 25 min), and washed with DMF. The resin was then agitated with Fmoc-Har(Pbf)-OH (4 equiv.), HOBt (4 equiv.), HBTU (4 equiv.) and DIPEA (8 equiv.) in dry DMF (2 ml) for 1 h and finally washed with DMF (3 × 4 ml, 5 min each). The terminal Fmoc group was removed with 20% piperidine-DMF (5 min and then 25 min) and the usual washing procedure was applied again. The following amino acids were coupled in the same way as the first one. The coupling efficiencies were checked with the 2,4,6-trinitrobenzenesulfonic acid (TBNS) test (beads with free amines change from yellow to red–orange when positive). The αMeNle was introduced as Fmoc- αMeNle -F in DMF. A double coupling (2 h) with an excess of 3 equiv. of αMeNle , and 1 equiv. of DIEA was used (Carpino et al. 1991). The

amino acid following αMeNle or other $\text{C}^{\alpha,\alpha}$ -tetrasubstituted amino acids was introduced with the same protocol.

Peptide deprotection, cleavage from the resin and purification

The resin-bound peptides were treated with a deprotection and cleavage solution of TFA/TIS/water (95:2.5:2.5 v/v/v) at room temperature for 2 h. After filtration, the filtrate was concentrated under nitrogen and precipitated with methyl *t*-butyl ether. Peptide purification was performed by reverse-phase HPLC on a Delta Pack Waters C18-100 Å silica column (solvent A: water + 0.1% TFA; solvent B: Acetonitrile 90% + 0.1% TFA) with a linear gradient of 10–35% (v/v) B over 15 min. Peptide homogeneity (>95%) was determined by analytical HPLC on a Vydac C18 (218TP510) column using the same solvents with a linear gradient of 10–90% (v/v) B over 30 min. Molecular masses were determined on a Perseptive Biosystems MARINERTM API-TOF spectrometer.

Synthesis of α -methyl norleucine hydrochloride salt

A solution of ammonia (17% NH_4OH , 150 ml) and one of NaCN (12 g, 245 mmol, dissolved in 28 ml of water) were poured in a reaction vessel kept at 25°C. Acetic acid (14.3 ml) was slowly added with a dropping funnel to avoid a temperature increase above 35°C. To the clear solution, 2-hexanone was finally added under vigorous stirring. The emulsion was stirred overnight at 35°C. The temperature was lowered to 25°C and three dichloromethane extractions were performed. The organic solvent was removed under reduced pressure yielding a yellow oil, which was used in the next step without any other purification. The aminonitrile was dissolved in formic acid (100 ml) at 0°C. Gaseous HCl was bubbled into the solution for 3 h under stirring. The reaction mixture was then stirred overnight at room temperature. Water (4.5 ml) was added to the dark solution. After stirring for additional 10 min, the solution was evaporated to dryness, the residue was taken up three times in toluene and again evaporated. To remove HCl and water completely, the residue was taken up again in diethyl ether. The solid residue was ground with diethyl ether and isolated by filtration and washed with ether. The crude yield was 71.6%. The crude product of the previous reaction (17.36 g, 0.036 mol) was dissolved in 6 N HCl (105 ml) and refluxed for 4 h. The volume was reduced and the precipitate was collected by filtration. The process was repeated. The solid product was ground in toluene to remove HCl in excess. The total yield was 58.3%. ¹H NMR (200 MHz; CDCl_3): 2.20–2.00 (2 m, 2H), 1.60–1.40 (mb, 4H), 1.40 (s, 3H), 1.1–0.9 (m, 3H).

Synthesis of Fmoc-(α Me)Nle-OH

100 mg of HCl*(α Me)Nle (0.14 mmol, 1 equiv.) were suspended in 4 ml of dry DCM under nitrogen atmosphere; 210 μ l of TMS-Cl (0.28 mmol, 2 equiv.) were added and the mixture was refluxed for 2 h. Then, a third equiv. of TMS-Cl was added and reflux continued for another hour. The mixture was then cooled to 0°C and 280 μ l of DIEA (0.42 mmol, 3 equiv.) and 157 mg of Fmoc-Cl (0.15 mmol, 1.1 equiv.) were added. The reaction was followed by TLC (Light Petroleum Ether: Ethyl Acetate 7:3). The solvent was evaporated and the crude material was dissolved in 20 ml of water containing 10% NaHCO₃ and extracted three times with ethyl ether. The organic layers were retro-extracted with a 10% solution of NaHCO₃. The water layers were acidified to pH 2 using conc. HCl, and extracted five times with ethyl acetate. The organic layers were dried over Na₂SO₄. The solvent was evaporated to obtain a yellow oil, which became solid. The yield was 60.0%. The MW was determined by Mass Spectroscopy: calculated 367, found 367.2.

¹H NMR (200 MHz; CDCl₃): 7.8–7.3 (8H, Fmoc); 6.4 (mb, 1H, NH(amide)); 4.4 (d, 2H, CH₂(Fmoc)); 4.25–4.20 (mb, 1H, H-(Fmoc)); 2.20–2.00 (2 m, 2H), 1.60–1.40 (mb, 4H), 1.40 (s, 3H), 1.1–0.9 (m, 3H).

Synthesis of Fmoc-(α Me)Nle-F

To 550 mg of Fmoc-(α Me)Nle-OH (1.5 mmol, 1 equiv.) in 10 ml of DCM, 121 μ l of pyridine (1.5 mmol, 1 equiv.) and 253 μ l of cyanuric fluoride (3.0 mmol, 2 equiv.) were added at 0°C. The mixture was allowed to reach room temperature and after 3 h the mixture was extracted with water and ice (three times). The organic layer was then washed with cold water. The organic layer was dried over Na₂SO₄ and the solvent evaporated under vacuum. The presence of the product was confirmed by IR analysis [1,837 cm⁻¹ (s C–F)].

Circular dichroism

CD measurements were carried out on a PC-controlled JASCO J-715 spectropolarimeter and the CD spectra were acquired and processed using the J-700 program running under Windows. All experiments were carried out at room temperature using HELLMA quartz cells with Suprasil windows and optical path-lengths of 0.01 and 0.1 cm. All spectra were recorded using a bandwidth of 2 nm and a time constant of 8 s at a scan speed of 20 nm/min. The signal-to-noise ratio was improved by accumulating eight scans. Measurements were performed in the 190–250 nm wavelength range and the concentration of the peptides was in the 0.07–1.07 mM range. The peptides were analyzed in

aqueous solution containing 20% (v/v) 2,2,2-trifluoroethanol (TFE). The spectra are reported in terms of mean residue molar ellipticity (deg cm² dmol⁻¹). The helical content for each peptide was estimated according to the literature (Yang et al. 1986).

NMR measurements

NMR spectra were recorded at 298 K as a 1 mM H₂O solution containing 20% TFE-d₃ (v/v) on a BRUKER AVANCE DMX-600 spectrometer. The water signal was suppressed by pre-saturation during the relaxation delay. The spin systems were determined using standard DQF-COSY (Rance et al. 1983), and CLEAN-TOCSY (Bax and Davis 1985) spectra. In the latter case, the spin-lock pulse sequence was 70 ms long. The sequence-specific assignment was accomplished using the rotating-frame Overhauser enhancement spectroscopy (ROESY) spectrum, using a mixing time of 150 ms.

Spectral processing was performed using the BRUKER XWINNMR software. Spectra were calibrated against the TMS signal. Inter-proton distances were obtained by integration of the ROESY spectra using the AURELIA software package. The calibration of peak-integrals was based on the geminal γ protons of Ile⁵, set to a distance of 1.78 Å. The offset correction was performed setting the B1-field value (2,500 Hz) and the B1-frequency (ca 4.80 ppm), according to the formulas of Bull (1988).

Molecular modelling

The peptide structure determinations were conducted using a simulated annealing (SA) protocol using the X-PLOR-NIH 2.22 program. For distances involving equivalent or non-stereo-assigned protons, r^{-6} averaging was used. The SA protocol consisted of 100 steps of initial minimization followed by 30 ps of high-temperature dynamics at 1,500 K and of 30 ps of cooling from 1,500 to 100 K in 50 K decrements (15,000 cycles, in 2 fs steps). Finally, the calculations were completed with 200 cycles of energy minimization using a force constant of 50 kcal/(mole Å). For each peptide, 150 distance geometry structures were generated, and the 20 minimum energy structures containing no distance constraint violation (<0.5 Å from the integration value) were chosen for conformational studies. The generated structures were visualized and analyzed using the programs VMD (1.8.6.) and MOE2008.10.

Activity assays

Human embryonic kidney (HEK 293) cells stably transfected with recombinant PTH1 receptor (HEK293/C20 cell

line) were used (Pines et al. 1994). The PTH1 receptor couples strongly to the adenylyl cyclase (AC)–protein kinase A (PKA) signalling pathway. In HEK 293 cells, the cAMP response element (CRE) of Luciferase was transfected using CRE-Luc plasmid. This response element (CRE), which is a recognition site of certain transcription factors, interacts with CRE-binding protein (CREB), which is regulated by cAMP. Thus, the activity of the PTH1 receptor is monitored using CRE, positioned upstream of the luciferase gene. Activation of the receptor causes an increase in intracellular cAMP, which is able to activate protein kinase A to phosphorylate CREB. The luciferase concentration within cells is increased when phosphorylated CREB is bound to the CRE consensus sequence, causing an increase in the transcription rate of the luciferase gene (Fan and Wood 2007).

Cell culture and CRE-Luc transfection

HEK293/C20 cell line were cultured at 37°C in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO₂. The cells were subcultured by treatment with Versene every week and the medium was changed every 3–4 days. Twenty-four hours before transfection, the cells were seeded at 10⁵ cells/well in 24-well, collagen-coated plates. On the following day, the cells were treated with FuGENE 6 transfection reagent (1 µl/well), CRE-Luc plasmid (0.2 µg/well) in 0.5 ml/well Opti-Mem I, serum free medium, according with the manufacturer's recommended procedure.

D-MEM, fetal bovine serum, Opti-Mem I, and PBS were from Life Technologies, Inc.; FuGENE 6 Transfection Reagent was purchased from Roche Diagnostic (Indianapolis, IN, USA); Passive Lysis Buffer, 5× from Promega Corporation (Madison, WI, USA); Biocoat Collagen I 24-well plates from Becton-Dickinson (Bedford, MA, USA) while the other tissue culture disposables and plasticware were obtained from Corning (Corning, NY, USA). D-Luciferin, potassium salt was obtained from Molecular Probes (Eugene, OR, USA).

Luciferase assay

About 18 h after CRE-Luc plasmid transfection, the cells were rinsed with PBS buffer and the transfection medium was replaced by 225 µl/well of DMEM. 25 µl/well of peptide solutions at different concentrations (from 10^{−7} to 10^{−3} M to obtain final concentrations between 10^{−8} and 10^{−4} M) in PBS supplemented with 0.1% bovine serum albumin were then added to the wells and incubated at 37°C for 4.5 h, yielding maximal response to luciferase. After this time, the medium was aspirated and the cells lysed by gentle shaking with 200 µl/well of Passive Lysis Buffer. The cells were transferred to labelled low binding Eppendorf tubes, centrifuged for 2 min and 80 µl/tube of supernatant were transferred to individual sample glass tubes. Luciferase activity was measured using a Lumat LB 9507 luminometer (EG&G Berthold). This instrument automatically injects defined volumes of two solutions, A and B, with compositions described below. Initially, a Solution 0 is prepared, containing 25 mM glycylglycine, 15 mM MgSO₄ and 4 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) in deionized water. Solution A is 0.2 mM D-luciferin in Solution 0. Solution B is 0.02 M K₃PO₄, 2.5 mM ATP and 1 mM dithiothreitol in Solution 0. The instrument adds 100 µl of Solution A and 300 µl of Solution B to a sample tube, and performs the measurement for 20 s. All the CRE-Luc experiments were carried out in triplicates.

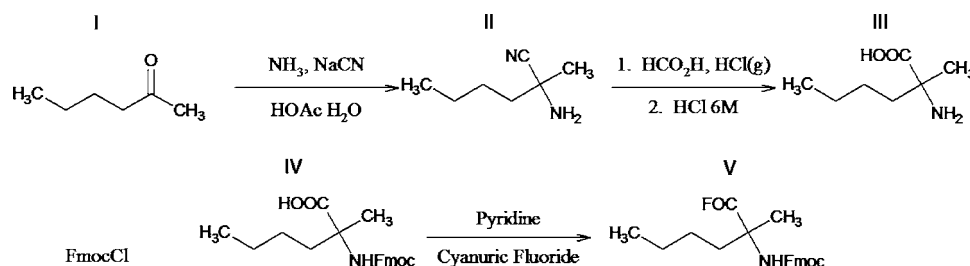
Data calculation

Calculations and data analysis were performed using Microsoft Excel 2000 and GraphPad Prism, Version 3.0.

Results

The synthesis of (αMe)Nle was carried out following Strecker's amino acid synthesis, starting from the corresponding ketone (Fig. 1). Strecker's synthesis yields the racemic mixture of D/L αMeNle, which was resolved by

Fig. 1 Strecker's synthesis is not enantioselective



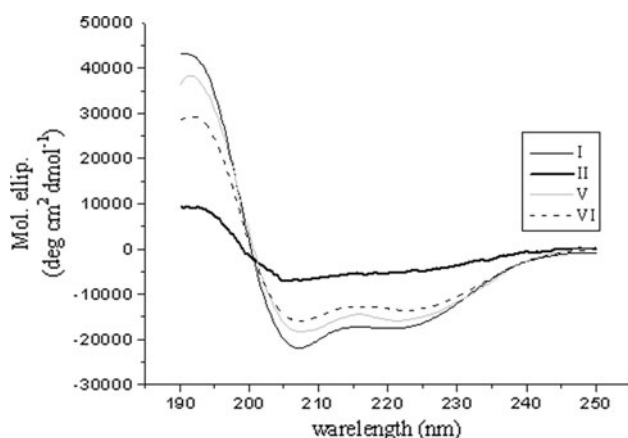


Fig. 2 CD spectra at ~ 1 mM peptide concentration in aqueous solution containing 20% TFE (v/v)

HPLC after the complete synthesis of the PTH(1–11) analogues with good yield and purity.

The peptides containing α -methyl derivatives were synthesised by SPPS employing Fmoc-protected amino acids using a combination of common solid-phase coupling reagents (HBTU/HOBt/DIPEA) and acyl fluoride derivatives to improve the yields.

The conformational properties of the series of peptides were initially investigated by CD in 20% TFE/water (at 1 mM peptide concentration), as in our previous works on potentially bioactive PTH-derived peptides (Caporale et al. 2009a). The CD spectra of all analogues exhibit the typical shape usually associated with the α -helical conformation, showing two negative bands of comparable magnitude near 222 and 208 nm and a stronger positive band near 190 nm (Fig. 2), with a helix content in the range 35–55% [calculated according to reference (Yang et al. 1986)]. In our experimental conditions, no concentration dependence of the CD profiles was observed for any of these analogues (data not shown).

NMR spectra were recorded at 298 K in a 1 mM solution containing 20% TFE- d_3 (v/v). A complete proton resonance assignment was performed using the standard procedure (Wüthrich 1986). The spin systems of all amino acid residues were identified using standard DQF-COSY and CLEAN-TOCSY spectra. The sequence-specific assignment was accomplished using ROESY spectra.

The secondary chemical shifts of the α CH protons of analogues **I**, **II**, **V** and **VI** are shown in Fig. 3. The same data for the inactive analogues **III**, **IV**, **VII** and **VIII** are reported in the Supplementary Information (Figure S3). A general stabilization of the C-terminal helical structure when compared with PTH(1–11) can be seen for all analogues containing an (α Me)Nle residue in position 8. In agreement with CD data, the substitution of L-Nle⁸ with D-Nle⁸ results in a sizeable loss of helical content throughout the sequence. The introduction of Ala in place of either Aib¹ or Aib³ causes an important reduction of the N-terminal helix, although the C-terminus remains ordered. Similar peaks were detected in analogues that differ only for the presence of D- or L-(α Me)Nle⁸, independent of the configuration.

The distance restraints obtained from the ROESY spectra were included in the SA protocol. The 20 lowest energy structures for each analogue were accepted. The analysis of the ensemble of structures, performed with MOE2008.10, confirmed good convergence for each family of conformations, presenting low values of RMSD for each ensemble (Table 2). Superimposition of the ensembles of the low energy structures resulting from molecular dynamics calculations clearly indicated good convergence towards the helical structure from **III** to **VIII** (see Figure S5 in the Supporting Information). The Ramachandran plot underlines the good agreement of φ and ψ angles with the helical conformation for all analogues, especially for the C-terminal segment Glu⁴–Gln¹⁰ (Fig. 4). Moreover, peptides **I**, **V**, **VI**, which contain Aib in position 1 and 3, show similar plots and do not present residues in forbidden areas.

Fig. 3 Secondary chemical shifts of the α CH protons of the active analogues, compared to the reference peptide

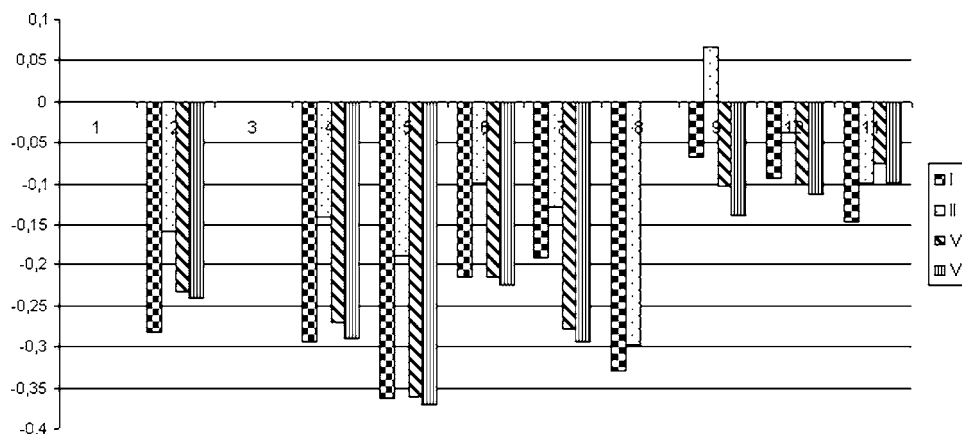


Table 2 Molecular descriptors

	I	II	III	IV	V	VI	VII	VIII
DR	79	83	77	86	82	90	92	89
RMSD [#]	0.546	0.266	0.266	0.762	0.551	0.266	0.358	0.631
E_{tot}	55.3	79.6	75.1	139.8	57.0	93.2	119.1	69.0
ASA	1,475.7	1,509.9	1,509.5	1,458.3	1,475.2	1,509.6	1,424.4	1,388.7
ASA+	963.8	1,003.9	1,004.1	950.5	963.2	1,004.2	952.5	935.1
ASA−	512.0	506.1	505.4	507.8	511.9	505.4	471.8	453.5
ASA H	690.7	717.3	717.6	694.8	690.4	717.7	703.2	704.1
ASA P	785.1	792.7	791.9	763.6	784.8	791.9	721.2	684.6
Vol	1,242.0	1,242.7	1,242.7	1,216.3	1,241.2	1,242.8	1,216.9	1,216.1

DR number of distance restraints introduced in the SA protocol, RMSD[#] root mean square deviation of the ensemble of structures for each analogue, E_{tot} average total energy, ASA accessible surface area using a water molecule as a probe, ASA+/ASA− water accessible surface area of all atoms with positive/negative partial charge, ASA H/P water accessible surface area of all hydrophobic/polar atoms, vol van der Waals volume. Analogues **I** and **V** show strong similarity in the solvent accessible surface descriptors.

To interpret the difference in activity of peptides presenting a notable geometrical similarity, several molecular descriptors were calculated: accessible surface areas (ASA, ASA+, ASA−, ASA H, ASA P), Sterical descriptors (Sterimol L, B1, B2, B3, B4) and the number of backbone hydrogen bonds (Baker and Hubbard 1984). ASAs are commonly used in structure activity relationship studies in medicinal chemistry as accurate descriptors able to characterize molecular surfaces [Connolly 1983; Stanton and Jurs 1990].

Analogue **V** shows a prominent analogy with the reference peptide (**I**) for these conformation-dependent descriptors (ASA, ASA+, ASA−, ASA H, ASA P), indicating a very similar surface behaviour. The partial loss of activity of **VI**, in comparison with **V**, can be explained by an increase of ASA+ due to the different chirality.

In drug discovery, it is important to determine if and to what extent candidate compounds inhibit or induce any activity. Luciferase activity is measured in the presence of ATP (the required luciferase substrate) so that light output varies with ATP concentration (Cali et al. 2008). As a general approach, light intensity is correlated to the chemical concentration of components of luciferase pathway reactions. When the experiment is designed properly, the light intensity can be used to associate an observable parameter with a molecular process. Biological assays were carried out on all peptides after a preliminary structural control by CD and the results are reported in Table 1. Peptide **II**, containing D-Nle, is 2,000 times less active than analogue **I**, containing L-Nle. Analogue **V**, containing Aib in position 1 and 3 and L-(α Me)Nle in position 8, is 20 times less active than analogue **I**. In addition, analogue **VI**, containing Aib in position 1 and 3 and D-(α Me)Nle in position 8, is 270 times less active than analogue **I**. It is interesting to observe that analogues **III** and **IV** and

analogues **VII** and **VIII**, which have the same sequence but differ for the chirality of (α Me)Nle, are not active. They differ from analogues **V** and **VI** for the substitution of Aib in position 1 or 3 with Ala. This small change seems critical in that it alters the correct orientation of the strategic residue Val² (Shimizu et al. 2001).

Discussion

We were interested in studying and comparing the effects of the replacement of Met⁸ with various analogues of the Nle residue on the structural properties and on the biological activity of PTH(1–11). Met⁸ was replaced with L- or D-Nle and L- or D-(α Me)Nle.

It is known that replacement of Met⁸ with the isosteric L-Nle is well tolerated in PTH, with no loss of binding affinity (Rosenblatt et al. 1976). The common use of this substitution stems from the fact that replacement of Met⁸ with Nle⁸ prevents methionine oxidation, which would result in a decrease in the biological response (Frelinger and Zull 1984). The hydrophobic side chain of Nle⁸ appears to be critical for the interaction with the receptor and, in the computer-based models for the PTH/PTH1R complex, residue 8 is found in a deep hydrophobic cleft (Monticelli et al. 2002). α -Methyl norleucine [(α Me)Nle] was inserted at position 8 to enhance the α -helicity in the C-terminal segment of PTH(1–11).

The peptides containing α -methyl derivatives were synthesised by SPPS employing Fmoc-protected amino acids. In our experience, the correct peptide was not detectable in the crude product (by mass spectroscopy analysis) when HATU and HOAt (Carpino et al. 1994; 1995) were used as coupling reagents to introduce (α Me)Nle (data not shown). The complexity of the synthesis is caused by the sterical

Fig. 4 Structural analysis of the PTH(1–11) analogues. For each peptide, the backbone structure of the lowest energy conformation and the Ramachandran Plot of the entire ensemble of low energy conformations are reported. The Ramachandran plot underlines the good agreement of φ and ψ angles with the helical conformation for all analogues, especially for the C-terminal segment Glu⁴–Gln¹⁰

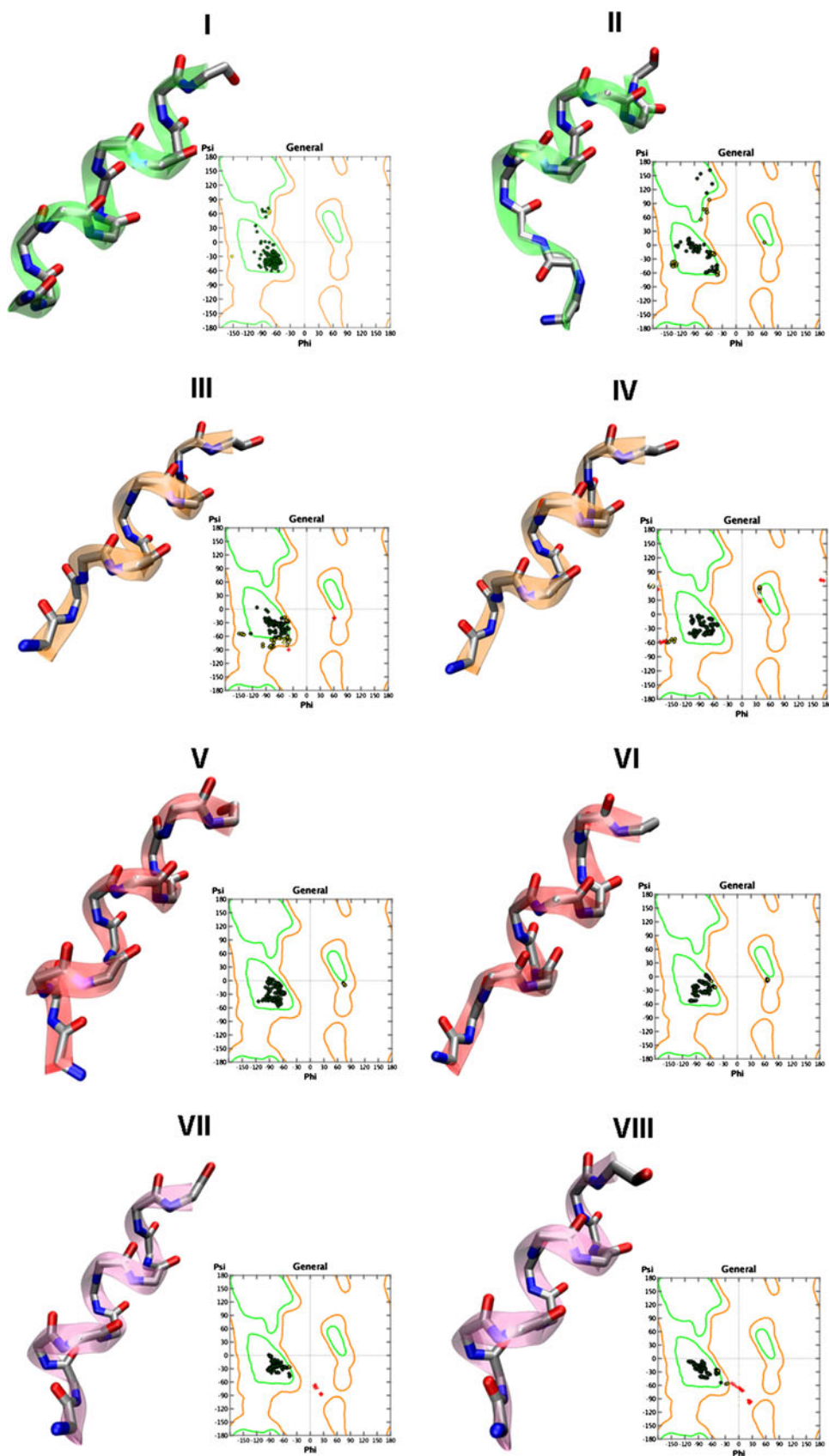


Table 3 Frequency of hydrogen bonds in each analogue ensemble

	I	II	III	IV	V	VI	VII	VIII
1–5							15%	15%
2–6			10%				100%	25%
3–7				60%		10%		
4–8	45%	80%	10%	5%	50%	100%		45%
5–9			75%					
6–10		15%	90%			15%		
7–11	60%		95%		60%		10%	
1–4	10%				10%	10%	95%	95%
2–5					10%		80%	80%
3–6	80%		15%	50%	80%			10%
4–7								
5–8								
6–9	100%	50%	50%		100%	50%		
7–10	100%			100%	100%		5%	
8–11				100%			15%	

The 20 lower energy structures were analyzed using 3.4 Å as donor–acceptor distance threshold and 40° as tolerance windows around the donor–hydrogen–acceptor ideal angle. Only backbone interactions were considered

hindrance typical of (α),(α)-dialkylamino acids, which decreases the reactivity of the terminal amino group and requires a stronger activation protocol. To solve this problem, we decided to use the acyl halide technique (Wenschuh et al. 1994), frequently recommended in peptide coupling reactions of extremely hindered amino acids. The acyl fluoride method has the great advantage of facile preparation and utilization in laboratory practice. Fmoc-amino acid fluorides have been shown to be excellently suited for the rapid solid-phase peptide synthesis of medium-sized peptides. The most impressive property of the Fmoc-amino acid fluorides is their ability to couple sterically hindered C $^{\alpha}$ -tetrasubstituted amino acids, such as Aib, to similarly hindered amino acids. Cyanuric fluoride easily converts

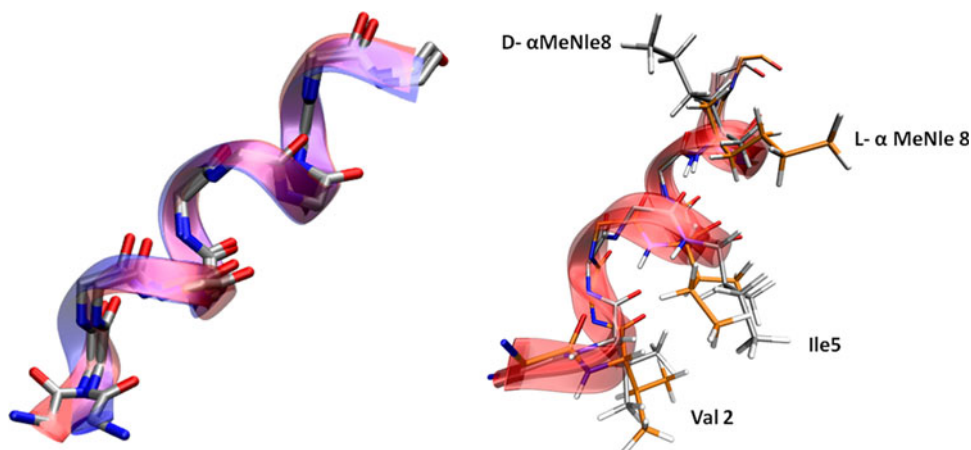
amino acids into the corresponding acid fluorides, which show better stability towards moisture and acid-labile functional groups than amino acid chlorides (Carpino et al. 1998). We obtained good crude products, as checked by mass spectroscopy analysis and analytical HPLC before purification. The mixture of analogues containing D/L- α MeNle was resolved by HPLC after the complete synthesis of PTH(1–11) analogues with good yield and purity.

The sequence-specific assignment was accomplished using ROESY spectra, which also yielded the absolute configuration of (α Me)Nle. ROESY cross-peaks between Ile⁵ α CH and both β CH₂ protons of (α Me)Nle were detected for analogues containing L-(α Me)Nle, and ROESY interactions between Ile⁵ α CH and the α -methyl protons of (α Me)Nle were detected for analogues containing D-(α Me)Nle (Belvisi et al. 2002).

A preliminary structure analysis was carried out through circular dichroism (Barazza et al. 2005; Caporale et al. 2009a, b). The intensities of the band at 220 nm can be rationalized in terms of the sequence modifications introduced. With reference to Fig. 2, which reports only the peptides that exhibit some biological activity, it can be seen that the introduction of an α -methyl group on Nle⁸, preserving the L chirality (analogue V), causes a minor disruption of the α -helix and a corresponding reduction in activity. Switching the two side chains (methyl and *n*-butyl) to yield analogue VI, which contains a D-(α Me)Nle⁸, results in a more sizeable reduction in both CD intensity and biological activity. The removal of the α -methyl group from D-(α Me)Nle⁸, leaving D-Nle⁸ (analogue II), causes the maximal reduction in both CD intensity and biological activity.

The lack of activity of analogues III, IV, VII, and VIII can be ascribed to the modifications introduced at the N-terminus. Specifically, the presence of Aib in positions 1 and 3 has been previously described to improve potency and helicity in PTH(1–11) analogues (Shimizu et al. 2001).

Fig. 5 Left the superimposition of the reference peptide I (blue) and V (red) shows a notable similarity between the backbones. Right the superimposition of V and VI underlines the different orientation of the (α Me)Nle side chains



The presence of a C $^{\alpha}$ -tetrasubstituted amino acid in position 8 seems to exacerbate the difference in biological activity while the effect on the CD intensity is less pronounced (see Figure S2 in the Supporting Information). After CD studies, we carried out the structural analysis using analogue **I** as a reference.

The structures derived from the SA protocol for analogues **III–VIII** are in good agreement with the CD and the chemical shift difference analyses. The analogues containing (α Me)Nle⁸ show a clear tendency toward the helical conformation. The analysis of the backbone geometries confirms good convergence towards a C-terminal α -helical structure for analogues **III** to **VIII** while only analogues **V** and **VI** show a clear N-terminal α -helix (Fig. 4). Clearly, the presence of both a methyl and a butyl side chain in position 8 supports the α -helix, irrespective of the absolute configuration of the amino acid. The presence of C $^{\alpha}$ -tetrasubstituted amino acids in positions 3 is necessary to preserve the N-terminal helix. These observations are supported by an analysis of the intra-molecular backbone hydrogen bonds (Table 3). In this analysis, analogue **V** displays a similar network of backbone interactions to that of the reference peptide, **I**. Analogue **II** lacks a continuous H-bond network, in line with its scarcely ordered structure.

Conclusions

The results presented in this work indicate that the presence of an (α Me)Nle in position 8 can enhance the α -helical structure, as can be seen from the superimposition of the lowest energy structures of analogues **I** and **V** (Fig. 5, left). This result can probably be ascribed to the reduced spatial freedom of C $^{\alpha}$ -tetrasubstituted amino acids. Notably, this result is independent of the absolute configuration of (α Me)Nle, as can be seen from the superimposition of the lowest energy structures of analogues **V** and **VI** (Fig. 5, right). The reduced bioactivity of analogue **VI** relative to analogue **V** can be ascribed to the incorrect orientation of the butyl side chain brought about by the configuration of (α Me)Nle. The introduction of D-Nle⁸ (analogue **II**) caused not only a strong reduction in bioactivity, but also a decrease in the α -helical content.

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