First synthesis of a fully active spin-labeled peptide hormone

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Abstract For the first time in the electron spin resonance (ESR) and peptide synthesis fields, a fully active spin-labeled peptide hormone was reported. The ESR spectra of this α-melanocyte stimulating hormone (α-MSH) analogue (acetyl-Toac[0]-α-MSH) where Toac is the paramagnetic amino acid probe 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid, suggested a pH-independent conformation and a more restricted movement comparatively to the free Toac. Owing to its equivalent biological potency in a skin pigmentation assay as compared to the native α-MSH and its unique characteristic (paramagnetic, naturally fluorescent and fully active), this analogue is of great potential for investigation of relevant physiological roles reported for α-MSH.
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Key words: Spin-labeled peptide; α-Melanocyte stimulating hormone; Electron spin resonance; Skin pigmentation

1. Introduction

The potentials of the ESR method in peptide chemistry and biology have been the subject of our studies for many years. The initial challenge was to find out a strategy to bind through a peptide bond, a stable and paramagnetic compound (spin label) into a peptide sequence. Thus, our introduction into the realm of ESR application in peptide synthesis method [1–3] initiated almost two decades ago [4] with the synthesis of the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic

acid (Toac) spin probe [5], see below, but protected in its amino group with the acid labile tert-butyloxycarbonyl (Boc) temporary
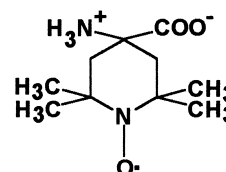
protecting group necessary for peptide chain assembly (Boc-Toac). Unlike most spin-labeling strategies applied so far, where long and flexible nitroxide-containing probes have been routinely used [6,7], the Toac labeling is clearly more advantageous. It binds more rigidly to the target molecule as a consequence of its C^α-tetrasubstituted cyclic structure, where the rotation about side-chain bonds is hampered by incorporation of the nitroxide nitrogen and C^α, C^β and C^γ atoms into the same heterocyclic moiety. Due to these characteristics the probe is highly sensitive to conformational states of the peptide backbone under study.

Hence, the Boc-Toac derivative was initially used for the syntheses of two vasoactive peptide angiotensin II (AII) analogues (Toac[0]- and Toac[1]-AII) but considerable AII activity was lost after the structural alterations [8]. At that time, there was no chemical strategy proposed to introduce the Toac probe at internal positions of the peptide sequence. This shortcoming was due to the irreversible degradation of the nitroxide moiety of the Boc-Toac during the repeated trifluoroacetic acid treatments necessary for removal of Boc group from Toac and from other incoming amino acid residues for further peptide chain elongation with the Boc strategy [1]. Only one decade later [9], we were able to propose an alternative procedure using the base labile 9-fluorenylmethyloxycarbonyl (Fmoc) group [2,3] for the Toac amino group protection and conjugating both Fmoc (for peptide chain elongation) and Boc (for peptide cleavage from the resin) chemistries. Thus, the first synthesis of an internally containing spin probe peptide sequence was achieved with the Toac[7]-AII [9], and more recently other vasoactive peptide (bradykinin, BK) was also internally labeled [10]. However, both Toac[3]-BK and Toac[7]-AII analogues were totally devoid of biological activity, whereas the BK analogue labeled at the N-terminus (Toac[0]-BK) maintained only a partial activity [10]. Additionally, Toac has also been used to label: (i) a seven transmembrane helix AII receptor fragment to investigate its binding to lipid bilayers and micelles [11], (ii) model peptide-resins to evaluate the peptide chain aggregation inside beads during the synthesis [12]. Moreover, the special bend-inducing property of the Toac molecule [13] has permitted in

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recent years the examination of helix-type conformational properties of single and double Toac-labeled model peptide sequences [14–16].

The next challenge concerning this spin-labeling approach was therefore to demonstrate the feasibility of synthesizing a Toac-containing active peptide that might retain its full biological potency. The tridecapeptide α-melanocyte stimulating hormone, α-MSH [17] containing the sequence acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$ was chosen for the present labeling investigation. This important hormone synthesized in the pituitary gland and skin of vertebrates is involved, among a great variety of physiological processes, in the skin darkening of most vertebrates, including humans [18], erectile function [19] and satiety [20]. Several modifications of the α-MSH primary structure have yielded reasonable elucidation of its structure-activity relationship and the synthesis of a potent and long lasting analogue [21] has been already reported. Owing mainly to its higher stability, most of α-MSH-labeling studies with several markers have been focused on this more potent analogue, with no loss of biological activity [22,23]. Differently, the present report describes the first synthesis of a native α-MSH analogue, labeled with the Toac spin probe (Ac-Toac^0-α-MSH) and that still retained its entire potency.

2. Materials and methods

2.1. Peptide synthesis [1–3]

The native α-MSH was synthesized in 0.1 mmol scale according to the Boc chemistry and using methylbenzohydrylamine-resin (MBHAR) and the following Boc amino acid derivatives: Bzl for Ser, OcHex for Glu, 2-Br-Z for Lys, Tos for His and Arg. Couplings were performed with Boc amino acid/TBTU/HOBt (1:1:1) components in the presence of DIEA in NMP. The synthesized α-MSH was cleaved from the resin with HF:o-cresol:DMS:EDT (8.5:0.5:0.5:0.5, v/v) solution at 0°C for 90 min. After evaporation, the resin was washed with ethyl acetate, dried, and the peptide was extracted into 5% acetic acid in water and lyophilized.

The Ac-Toac^0-α-MSH synthesis followed almost the same protocol applied for the native α-MSH but with some alterations as already reported [9]. The introduction of the Toac probe was performed with its Fmoc derivative, which was removed with the alkaline piperidine/DMF (20%, v/v) treatment for 30 min. The acetylation of the Toac amine group was done with acetic anhydride/DMF (1:4, v/v) solution containing 0.1 ml of pyridine for 30 min. After HF cleavage, the crude spin-labeled peptide was submitted to alkaline treatment (pH 10, for 6 h at 25°C) for complete reversion (monitored by analytical HPLC) of the N-O protonation that occurs during HF treatment.

Both peptides were purified in preparative HPLC (C$_{18}$-column) using aqueous 0.02 M ammonium acetate (pH 5.0) and 60% MeCN solutions as solvents A and B, respectively (linear gradient of 30–70% B in 2 h, flow rate of 10 ml/min). The homogeneity of both peptides was confirmed through analytical HPLC, matrix-assisted laser desorption ionization-mass spectrometry and amino acid analysis. In this latter case, the Toac residue is not determined as it decomposes during the acid hydrolysis.

2.2. Biological assays [24]

The thigh and dorsal skin of the frog was excised, cut in square (2×2 cm^2) pieces, which were placed between two PVC rings and kept for 1 h in Ringer's solution. After this period, the melanin granules were aggregated in the perinuclear region of the melanocytes, which assumed a punctuate state, and the skins become light. Upon addition of α-MSH or the analogue to the medium, the pigment disperses out into the dendritic processes of the cell, resulting in skin darkening. The changes in skin color (decrease in skin reflectance) were monitored with a Photovolt reflectometer and expressed as percent change of the initial value. Dose-response curves and the EC$_{50}$ (the concentration eliciting 50% of the maximal darkening, confidence interval of 95%) were determined for both peptides.

3. Results and discussion

Knowing some of the structural requirements for α-MSH biological activity, the Toac was inserted between the acetyl group and the Ser^1 residue out of the important 4–12 core of its sequence [25]. The chemical strategy to obtain the Ac-Toac^0-α-MSH analogue involved a conjugation of Boc and Fmoc chemistries as already mentioned [9]. The selection of the solvent for the critical coupling reaction step amino acid residues was based upon our recent peptidyl-resin solvation strategy [26] where a novel solvent polarity parameter was also proposed. The polar aprotic 1-methyl-2-pyrrolidinone (NMP) swelled almost 80% of peptide-resin bead volume throughout all chain assembly and was therefore used with success for the synthesis of both peptides. After alkaline reversion of the nitroxide moiety protonation that occurs during HF cleavage of peptide from the resin and HPLC purification, 47 mg and 33 mg of pure α-MSH and Ac-Toac^0-α-MSH were obtained, respectively.

The frog (*Rana catesbeiana*) skin bioassay was performed in vitro as previously described [24]. Ac-Toac^0-α-MSH was a full agonist on the frog melanocyte, promoting a dose-dependent skin darkening (Fig. 1) with the same potency as the native α-MSH. The concentration eliciting 50% of the maximal darkening (EC$_{50}$) and the confidence interval of 95% (CI) were for the labeled analogue and the native hormone, respectively 2.70×10^{-10} M (1.02–7.10) and 1.96×10^{-10} M (0.72–5.34). In addition, after removal of the agonist, and several Ringer's rinses, the reversal of the maximal response to Ac-Toac^0-α-MSH was achieved after 180 min, at the same rate exhibited by α-MSH (Fig. 2). These results show, for the first time, that the spin labeling of the native sequence of α-MSH was successfully performed, full agonism being retained, with no loss of potency as compared to the native hormone. It also means that, in some circumstances, the most serious criticism of the ESR method, i.e. the lack of meaning in studying a spin-la-
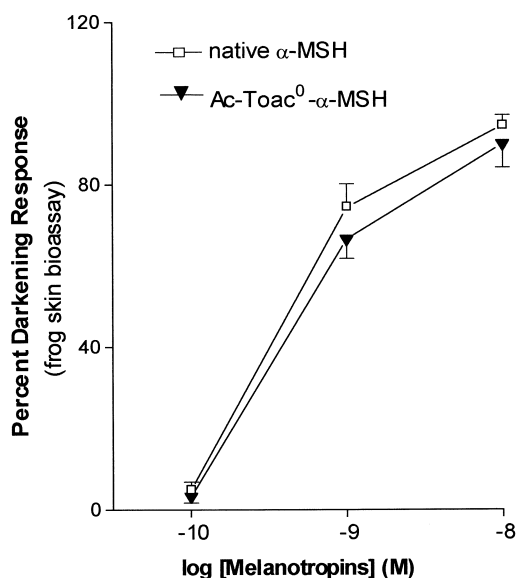

Fig. 1. Dose-response curves to Ac-Toac^0-α-MSH as compared to the native α-MSH, in the frog *Rana catesbeiana* skin bioassay. Each point represents the mean ($n = 10$) ±S.E.M. (standard error of the mean) darkening response at the concentrations noted.
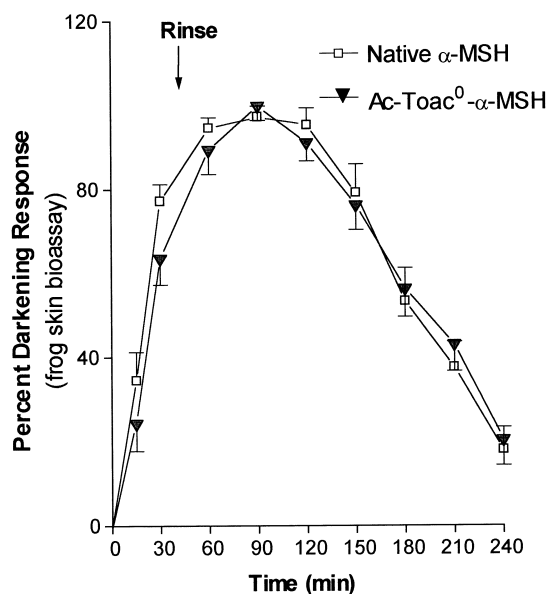
Fig. 2. Reversal of the maximal responses to the native hormone and to Ac-Toac[0]-α-MSH (10^{-8} M) after removal of the peptides and rinsing of the preparation (arrow). Each point is the mean ($n = 10$) ±S.E.M., *Rana catesbeiana* skin darkening at the times noted.

Fig. 3. ESR spectra of 10^{-4} M Toac and Ac-Toac[0]-α-MSH in aqueous solutions (pH 5.0).

beled system due to the introduction of a non-natural component in its structure, may be not acceptable.

The ESR spectra demonstrate the different mobility of the free and peptide-bound Toac (Fig. 3). Both compounds display narrow lines, as expected for small molecules tumbling in a non-viscous solvent. However, the correlation times (τ) for α-MSH-bound Toac are one order of magnitude higher than those obtained for free Toac (Table 1). The different rotational correlation time τ_B and τ_C values obtained for the labeled peptide and not for Toac indicate an anisotropic movement [27] for the former. Preliminary results with Ac-Toac[0]-α-MSH also suggest that its conformation does not depend on the pH of the media, as its ESR parameters have not changed in acid or alkaline solutions (Table 1). Moreover, equivalent isotropic

Table 1
ESR data for Toac and Ac-Toac[0]-α-MSH

System	a_0 (G)	τ_B (ns)	τ_C (ns)
Toac			
pH 5.0	16.32	0.036	0.036
Ac-Toac[0]-α-MSH			
pH 3.0	16.29	0.324	0.396
pH 5.0	16.29	0.313	0.382
pH 9.0	16.26	0.305	0.372

The estimated errors in the values of a_0 and τ are around ±0.03 G and 0.005 ns, respectively. ESR measurements were performed at 25°C with a Bruker EMX spectrometer. A field-modulation amplitude of 0.5 G and microwave power of 5 mW was used. The temperature was controlled to about 0.2°C with a Bruker BVT-2000 variable temperature device. The spectral parameters were found by fitting each line to a Gaussian-Lorentzian sum function taking advantage of the fact that the sum function is an accurate representation of a Gaussian-Lorentzian convolution, the Voigt function [31]. The hyperfine splitting, a_0, was taken to be one-half the difference in the resonance fields of the high- and low-field lines. The intrinsic (Lorentzian) line widths and the line heights were determined from the fits and rotational correlation times, τ_B and τ_C were calculated [27].
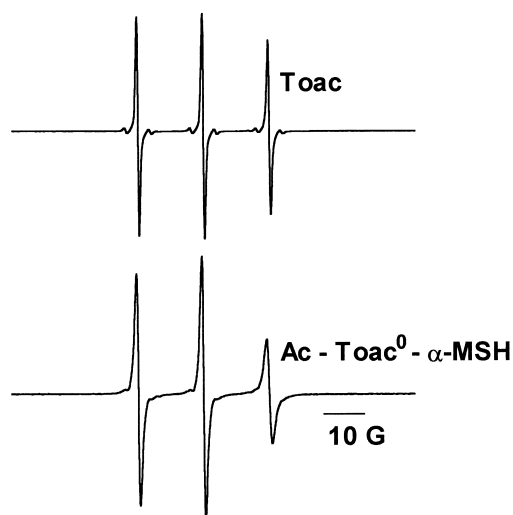
hyperfine parameter (a_0) values indicate similar microenvironment for Toac either free or bound to the peptide, at all pH values.

In conclusion we herein described the first synthesis of a fully active spin-labeled peptide hormone. The availability of such unique analogue (Ac-Toac[0]-α-MSH) that is paramagnetic, naturally fluorescent due to the tryptophan residue of its structure and fully active, is clearly of great potential. Besides the expected more complete ESR studies in solution, the use of this paramagnetic peptide may allow for instance, the inversion of the more common strategy used up to now for peptide binding studies in membrane-mimetic systems. In the place of spin labeling the lipid bilayer and evaluating the binding of the agonist, as already described for the α-MSH itself [28], the labeling site is now located within the native hormone structure as already reported with external fragments of some seven transmembrane helix proteins [11,29]. The same approach would be also further extended for the approach [30] which evaluates interaction features of transmembrane helix segments with model membrane. Moreover, one can also simultaneously spin label both the agonist and the system under study, for structure and binding investigation through the assessment of spin-spin interactions phenomenon [31] that may occur depending upon the average distance between probes. The α-MSH binding assay to lipid bilayers has already been examined by fluorescence, monitoring the native Trp[9] residue of the hormone [32]. Complementarily, the use of the paramagnetic hormone may provide an alternative route to that investigation due to the well-known fluorescence quenching effect [33] of the nitroxide function. The secondary structure of nicotinic acetylcholine receptor inside the membrane was for instance investigated through the quenching phenomenon [34]. Therefore, the use of this special quenching agonist may be also valuable for further imaging and quantification studies of melanotropin binding to α-MSH receptors present in normal melanocytes or melanoma cell lines [35,36].

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