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Slow *tert*-Butyl Ester Acidolysis and Peptide 3_{10}-Helix to α-Helix Transition in HFIP Solution

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ABSTRACT:

We have already shown by CD and NMR techniques that the terminally protected homo-octapeptides Z (and Ac)-[L-(αMe)Val]$_8$-OtBu undergo a slow and irreversible 3_{10}-helix to α-helix transition when dissolved in 1,1,1,3,3,3-hexafluoroisopropanol. In the present work, we find by HPLC and CD that under the aforementioned experimental conditions, a slow acidolysis of the tert*-butyl ester functionality does take place affording the corresponding octapeptide free acids. The results of our combined chromatographic and spectroscopic experiments are confirmed by a comparison with the properties of independently synthesized and chemically characterized authentic compounds.* © *2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88: 233–238, 2007.*

Keywords: circular dichroism; helix-to-helix transition; HPLC; peptide helices; C^{α}-tetrasubstituted α-amino acid

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INTRODUCTION

The 3_{10}-helix constitutes a small but significant percentage of secondary structural elements in globular proteins.[1,2] According to a general survey of protein structures, about 10% of all helical conformations are 3_{10}-helices, in which an intramolecular H-bond is formed between the C=O group of the *i*th amino acid residue and the N—H group of the $(i + 3)$ residue ($i + 4$ for the closely related α-helix).[3] The 3_{10}-helices are mainly observed at the termini of α-helices, in loops, and as connectors between β-strands, with an average length of 3.3 residues. They play important functional roles in several proteins.[4–8] Some transmembrane channel-forming antibiotics, such as peptaibols, have a significant content of 3_{10}-helices.[9–12] Moreover, the 3_{10}-helix structure has been proposed as an intermediate in the folding of α-helices and observed as picosecond intermediates in simulation studies of α-helix melting.[13–18] To fully understand peptide helix formation processes, including nucleation of nascent helices, theoretical findings should be combined with experimental techniques that have a high sensitivity to the subtle structural differences between 3_{10}- and α-helices.

In this connection, we have reported CD and NMR studies of terminally protected Z (or Ac)-[L-(αMe)Val]$_8$-O*t*Bu [Z, benzyloxycarbonyl; Ac, acetyl; (αMe)Val, C^{α}-methyl valine; O*t*Bu, *tert*-butoxy], which have shown that these two homo-octapeptides undergo an intriguing phenomenon, namely an unexpectedly slow and irreversible conversion from 3_{10}-helix to α-helix in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) solution[19,20] [this conformational transition does not seem to take place, at least within 3 weeks time, in 2,2,2-trifluoroethanol (TFE) or in $CHCl_3$]. Therefore, the terminally protected-[L-(αMe)Val]$_8$-was the only peptide sequence based exclusively on C^{α}-tetrasubstituted α-amino acids known before this

study to be able to fold into an α-helical structure (at least under well specific conditions). Interestingly enough, a fast and reversible 3_{10}-helix/α-helix transition has been published before for two Aib (α-aminoisobutyric acid)—or L-(αMe)Val/Aib-rich co-oligopeptide amides, and found to be temperature—or solvent-driven.[21–23]

More recently, during the initial part of our femtosecond 2D IR investigation on the different spectral signatures of the 3_{10}- and α-helices adopted by Z-[L-(αMe) Val]$_8$-O*t*Bu in HFIP solution,[24] we noticed a previously undetected phenomenon. In this fluoroalcohol, significant changes in the HPLC runs were observed as a function of time. Therefore, we decided to analyze these chemical and conformational changes in more detail using combined chromatographic and spectroscopic techniques.

MATERIALS AND METHODS

Synthesis of Peptides

Melting points were determined using a Leitz (Wetzlar, Germany) model Laborlux 12 apparatus and are not corrected. Optical rotations were measured using a Perkin-Elmer (Norwalk, CT) model 241 polarimeter equipped with a Haake (Karlsruhe, Germany) model D thermostat. Thin-layer chromatography was performed on Merck (Darmstadt, Germany) Kieselgel 60/F_{254} precoated plates with the following solvent systems: (1) (chloroform/ethanol, 9:1) and (2) (1-butanol/acetic acid/water, 3:1:1). The chromatograms were examined by UV fluorescence or developed by the chlorine/starch/potassium iodide chromatic reaction as appropriate. All compounds were obtained in a chromatographically homogeneous state. Mass spectra were obtained using a Perseptive Biosystem (Foster City, MA) Mariner ESI-TOF spectrometer.

Z-[L-(αMe)Val]$_8$-OH

Z-[L-(αMe)Val]$_8$-O*t*Bu[25] was dissolved in a 1:1 trifluoroacetic acid (TFA)/CH_2Cl_2 mixture and stirred for 2 h at room temperature. After removal of most of the solvent, the peptide free acid precipitated upon addition of diethyl ether. It was collected by filtration and kept for 12 h in a desiccator over KOH. Yield: 90%. Melting point: 267–269°C (from TFE/water); TLC R_f 1 0.65, R_f 2 0.90; IR (KBr) 3312, 1724, 1654, 1635, 1533 cm^{-1}. ^{1}H NMR [250 MHz, DMSO (dimethylsulfoxide), 10 m*M*] δ 7.88, 7.65, 7.49, 7.43, 7.41, and 7.39 (6s, 6H, 6 NH), 7.37 (5H, Z—CH), 7.35 and 7.33 (2s, 2H, 2 NH), 5.14 (m, 2H, Z—CH_2), 2.28 (m, 1H, 1 β—CH), 2.20–1.85 (7H, 7 β—CH), 1.40–1.20 (24H, 8 β—CH_3), 1.05–0.75 (48H, 16 γ—CH_3). Mass spectrometry: $[M + H]^+_{calcd}$ 1057.4299; $[M + H]^+_{exp}$ 1057.6527.

Ac-[L-(αMe)Val]$_8$-OH

This compound was prepared from Ac-[L-(αMe)Val]$_8$-O*t*Bu[25] as described above for Z-[L-(αMe)Val]$_8$-OH. Yield: 92%. Melting point: 334–335°C (from TFE/water); TLC R_f 1 0.55, R_f 2 0.90; IR (KBr) 3309, 1739, 1652, 1525 cm^{-1}. ^{1}H NMR [250 MHz, DMSO, 10 m*M*] δ 7.98, 7.61, 7.53, 7.47, 7.46, 7.45, 7.41, and 7.36 (8s, 8H, 8 NH), 2.25 (m, 1H, 1 β—CH), 2.15–1.85 (10H, 7 β—CH, 1 Ac—CH_3), 1.40–1.20 (24H, 8 β—CH_3), 1.05–0.75 (48H, 16 γ—CH_3). Mass spectrometry: $[M + H]^+_{calcd}$ 965.3325; $[M + H]^+_{exp}$ 965.6914.

IR Absorption

The IR absorption spectra were obtained using a Perkin-Elmer model 1720X FTIR spectrometer, nitrogen-flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. The spectra were acquired using the KBr disk technique.

Circular Dichroism

The CD spectra were recorded on a Jasco (Tokyo, Japan) model J-715 dichrograph. Cylindrical, fused quartz cells (Hellma, Müllheim, Germany) of 1 and 0.1 mm path lengths were employed. The data are expressed in terms of $[\Theta]_T$, the total molar ellipticity (deg × cm^2 × d mol^{-1}). HFIP, puriss. p. a., ≥ 99.8 (GC), freshly opened bottle, was a Sigma-Aldrich (St. Louis, MO) product. Hexafluoroacetone (HFA) trihydrate, purum, >96.0% (GC), freshly opened bottle, was obtained from Fluka (Buchs, Switzerland). The two fluoroalcohols were used without further purifications.

^{1}H NMR

The ^{1}H NMR spectra were recorded with a Bruker (Karlsruhe, Germany) model AM 250 spectrometer. Measurements were carried out in deuterated DMSO (99.96%, d_6; Acros Organics, Geel, Belgium) with tetramethylsilane as the internal standard.

HPLC

The HPLC measurements were performed using a Gilson (Middleton, WI) apparatus equipped with a UV detector at 226 nm and a Vydac C_{18} (Columbia, MD) column.

RESULTS

Figure 1 shows the time-dependent evolution of the CD spectra of Ac-[L-(αMe)Val]$_8$-O*t*Bu in HFIP solution at the peptide concentration 4 × 10^{-3}*M*. The spectrum gradually (within days) changes from having a weak amide n → π^* CD (at about 222 nm), typical of the 3_{10}-helix,[26] to having a strong n → π^* CD (typical of a remarkable population of α-helical species[27]). Figure 2 graphically illustrates the trend of the ratio (*R*) of the ellipticity value at 222 nm over that at 207 nm (parallel component of the amide $\pi \rightarrow \pi^*$ transition) from about 0.4 (3_{10}-helix) to 1.0–1.1 (α-helix)[26] as a function of time after octapeptide dissolution. Similar results were already published by us for the same octapeptide and its Z-protected analogue in the same fluoroalcohol.[19,24] In addition, in the present study, we found that this phenomenon is accelerated by increasing the temperature (in the range 10–25°C) (data not shown). Also, any attempt to reverse the

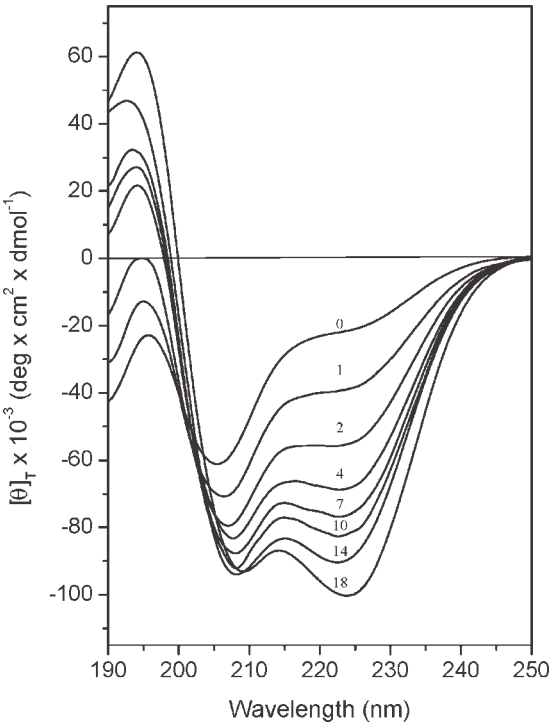

FIGURE 1 Time-dependent evolution of the CD spectra of Ac-[L-(αMe)Val]$_8$-O*t*Bu in HFIP solution (peptide concentration 4 $\times$ 10^{-3}*M*). From top to bottom, the spectra were recorded immediately (0) and 1, 2, 4, 7, 10, 14, 18 days after sample dissolution.

conformational transition from α-helix to 3_{10}-helix (e.g., by evaporating the HFIP solution and let the residue stand in TFE) failed.

In Figure 3, we report the time-dependent % peak (29 min) area evolution from the HPLC runs for the aforementioned octapeptide studied under the same experimental conditions as those of Figures 1 and 2. It is quite clear that two peaks are observed (inset) and the area of the second eluted peak (at about 29 min retention time), shown to be associated with the starting N^{α}-acetylated octapeptide *tert*-butyl ester, decreases slowly with time, with a concomitant increase in the area of a new peak (first eluted, at about 18 min retention time). It is worth pointing out that the CD pattern modification with time discussed above parallels, albeit not strictly, the HPLC area change of the newly observed peak.

Acidolysis represents a classical method to deprotect peptide carboxylic esters in soluble compounds and to remove peptide esters from insoluble matrices in the last step of the solid-phase synthetic methodology.[28] In general, TFA at different concentrations in CH_2Cl_2 solution, is the reagent of choice. However, a careful search in the literature revealed that HFIP-CH_2Cl_2 solvent mixtures are able to completely remove a peptide from the 2-chlorotrityl ester resin in a few minutes at room temperature (unsubstituted trityl esters are only partially deprotected).[29,30] However, the less acid-labile -COO*t*Bu ester moiety seems to be stable under these conditions. The ease of acidolysis appears to be strictly related to the stability of the intermediate carbocation that is formed. Nevertheless, we decided to check whether a prolonged standing of our octapeptide *tert*-butyl ester in HFIP solution would induce ester acidolysis.

To compare the final CD spectra and HPLC retention times with those of the authentic peptide free acids, we independently synthesized (by TFA treatment of the corresponding peptide *tert*-butyl esters) and chemically characterized Z (and Ac), [L-(αMe)Val]$_8$-OH. Their CD spectra in HFIP solution (Figure 4) and HPLC runs (Figure 5), recorded immediately after sample dissolution, match perfectly the corresponding ones of the final compounds originated from the HFIP acidolyses (for the N^{α}-acetylated octapeptide free acid, see Figures 1 and 3, respectively). Therefore, we conclude that an HFIP treatment of the L-(αMe)Val octapeptide *tert*-butyl ester for sufficiently long times will afford its free acid derivative [using an appropriate Z-protected L-(αMe)Val model compound, we also showed that this urethane function is not acidolyzed upon standing in HFIP solution]. Moreover, the CD properties strongly support the view that

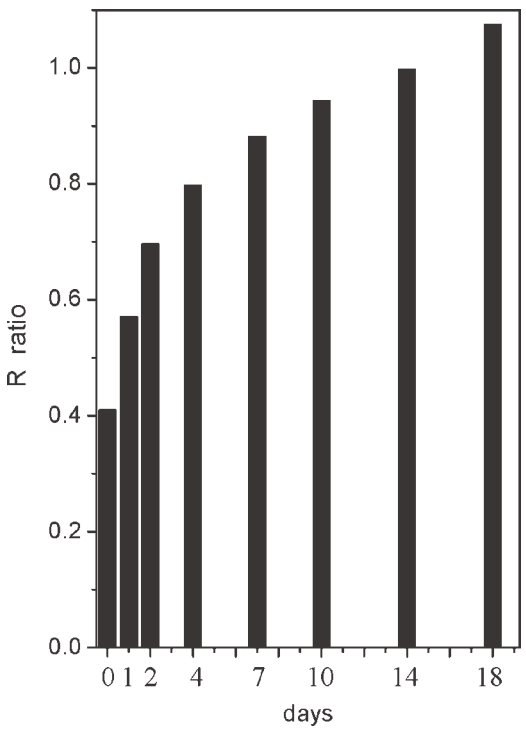

FIGURE 2 Histogram showing the time-dependent evolution of the $[\Theta]_T$ (222 nm)/$[\Theta]_T$ (207 nm) ratio (*R*) in the CD spectra of Ac-[L-(αMe)Val]$_8$-O*t*Bu in HFIP solution (peptide concentration 4 $\times$ 10^{-3}*M*).

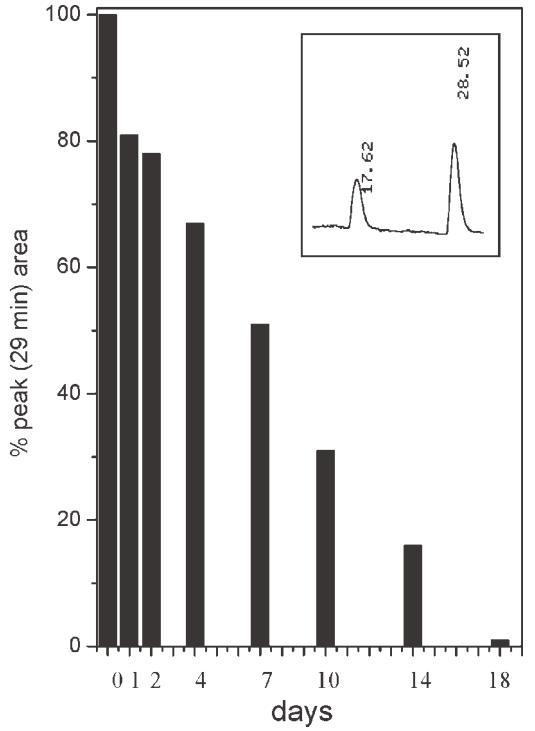

FIGURE 3 Time-dependent evolution of the % peak (29 min) area from the HPLC runs for Ac-[L-(αMe)Val]$_8$-O*t*Bu in HFIP solution (peptide concentration $4 \times 10^{-3}M$). In the inset, HPLC run after 7 days showing the co-elution of the two peaks. HPLC conditions: isocratic gradient, 95/5 CH_3CN/H_2O, 30 min, 1.5 ml/min.

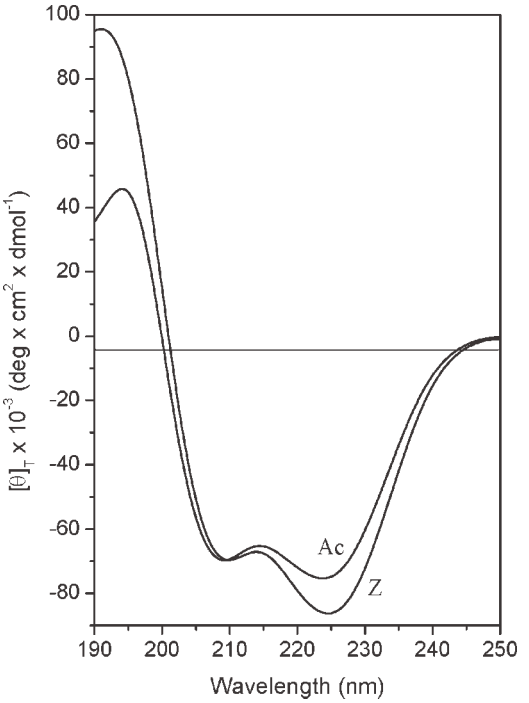

FIGURE 4 CD spectra of the independently synthesized Z-[L-(αMe)Val]$_8$-OH (Z) and Ac-[L-(αMe)Val]$_8$-OH (Ac) in HFIP solution (peptide concentration $4 \times 10^{-3}M$) recorded immediately after sample dissolution.

the octapeptide free acids adopt immediately the α-helical conformation in HFIP solution.

We also investigated by CD the effect of a solvent (HFA trihydrate) more acidic than HFIP[31] in inducing the 3_{10}-helix to α-helix transition of the L-(αMe)Val octapeptide. Figure 6 clearly shows that the conformational change is much faster as the acidity of the fluoroalcohol is remarkably enhanced (a concomitant, extremely fast, acidolysis of the *tert*-butyl ester does occur; not shown).

Conversely, no acidolysis of our octapeptide was seen after 2 weeks in a HFIP solution to which a base (triethylamine) had been added in a 1:1 mol/mol ratio to the fluoroalcohol, unambiguously showing that HFIP is acting as the acid in the solvolysis.

To check whether the *tert*-butyl ester acidolysis is restricted to an L-(αMe)Val homopeptide with a main-chain length of eight residues, we performed similar, time-dependent, HPLC experiments in HFIP solution on the shorter oligomers Z-[L-(αMe)Val]$_n$-O*t*Bu ($n = 5$, 2, and 1)[25] (results not shown). Interestingly, after a week from dissolution in the fluoroalcohol, only the homopentapeptide provided evidence for a sizable formation of the corresponding free acid

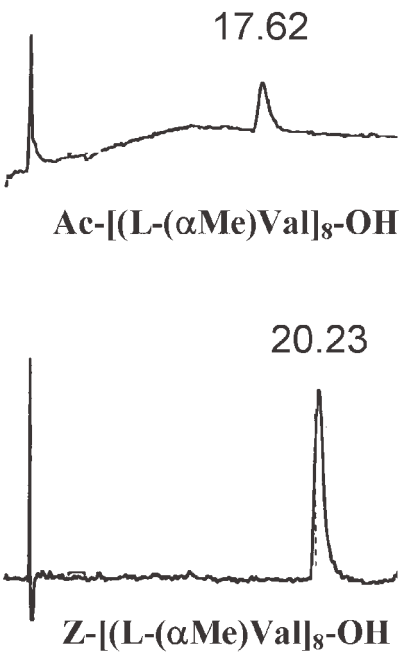

FIGURE 5 HPLC runs of the independently synthesized Z-[L-(αMe)Val]$_8$-OH and Ac-[L-(αMe)Val]$_8$-OH performed immediately after sample dissolution (isocratic gradient, 95/5 CH_3CN/H_2O, 30 min, 1.5 ml/min).

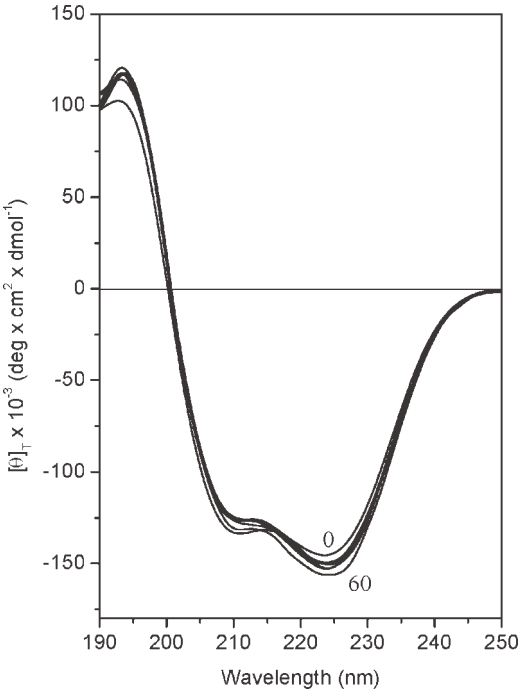

FIGURE 6 Time-dependent evolution of the CD spectra of Ac-[L-(αMe)Val]$_8$-O*t*Bu in HFA hydrate solution (peptide concentration 4×10^{-3}*M*). From top to bottom, the spectra were recorded immediately (0) and 5, 10, 15, 20, 30, and 60 min after sample dissolution.

(approximately to the same extent as that shown by the homo-octapeptide). Also, no acidolysis was observed during the same time in the case of the simple, nonpeptide, *tert*-butyl ester *tert*-butylphenylacetate.

DISCUSSION

In this work, using the HPLC technique, we have been able to show for the first time that a peptide *tert*-butyl ester is slowly converted into its corresponding free acid upon standing in HFIP solution for long times. Under these conditions, HFIP is acting as an acid strong enough to facilitate the solvolytic cleavage.

Even more intriguing is the observation that N$^{\alpha}$-protected (or blocked) octapeptide *tert*-butyl esters based exclusively on the helicogenic C$^{\alpha}$-methylated α-amino acid L-(αMe)Val slowly give a CD pattern change typical of a 3_{10}-helix to an α-helix conformational transition in HFIP solution, whereas this latter chirospectroscopic pattern is produced by the corresponding peptide free acids immediately after dissolution in this fluoroalcohol. Therefore, our published CD and NMR findings[19,20] on Z (or Ac) -[L-(αMe)Val]$_8$-O*t*Bu in HFIP solution are the result of a slow ester acidolysis and a concomitant rapid α-helix formation by the octapeptide free acids. In this connection, not unexpectedly, the more acidic solvent HFA trihydrate seems to produce analogous chemical and conformational consequences as HFIP, but with faster kinetics.

In our view, there are several factors (even in combination), which may play a role in this complicated process, the most evident of which are: (i) A peptide free acid has one additional H-bonding donor, the -OH group of the carboxylic acid function, if compared to the corresponding ester. It is well established that in this peptide main-chain range (6–9 residues), the addition of one intramolecular H-bonding donor may significantly increase the relative stability of the α- versus the 3_{10}-helix.[32,33] (ii) Peptide *tert*-butyl esters are characterized by a significant bulkiness at their C-terminus. A similar type of steric hindrance is known to affect the relative stabilities of 3_{10}- and α-helices favoring the former[34] (in any case, our present results do not allow us to exclude the possibility that an L-(αMe)Val octapeptide with a very bulky, but not acidolyzable, C-protection might undergo this same evolution, albeit more slowly). Support for this latter conclusion arises from our observation of the lack of a strict going together between the rate of *tert*-butyl ester acidolysis (as determined by HPLC) and the (faster) increase of the absolute negative ellipticity at 222 nm (as determined by CD).

The rationale for *tert*-butyl ester acidolysis in HFIP solution was carefully explored. Competition experiments between L-(αMe)Val peptide esters clearly indicate that only the longest homo-oligopeptides (penta- and octa-peptides) undergo acidolysis to a measurable extent after a week. In contrast, the shortest members of the series (homodipeptide and amino acid derivative) and a nonpeptide model ester are not acidolyzed. The significant preferential acidolysis undergone by the longest L-(αMe)Val oligopeptide *tert*-butyl esters, the only two among those investigated, which may adopt a helical structure in solution, points to the helix dipole as the main factor responsible for the increased propensity for protonation of the negatively charged, C-terminal, peptide ester moiety (the first step of the acidolytic process) relative to that of other, non helical, esters.

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