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**Citation style:** Fulczyk Agnieszka, Łata Eliza, Talik Ewa, Dolnik Milos, Kowalska Teresa, Sajewicz Mieczysław. (2020). Impact of D2O on the peptidization of L-alanine. "Reaction Kinetics, Mechanisms and Catalysis" (Vol. 130 (2020), s. 5–15), doi 10.1007/s11144-020-01783-y



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## Impact of D<sub>2</sub>O on the peptidization of L-alanine

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Received: 24 March 2020 / Accepted: 25 April 2020 / Published online: 5 May 2020

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### Abstract

This is our fifth consecutive study carried out in an order to collect experimental evidence on the impact of heavy water (D<sub>2</sub>O) on the spontaneous peptidization of proteinogenic  $\alpha$ -amino acids and this time its subject matter is L-alanine (L-Ala). Our four earlier studies have been focused on the two sulfur-containing  $\alpha$ -amino acids (i.e., L-cysteine (L-Cys) and L-methionine (L-Met)), and on two structurally related  $\alpha$ -amino acids (i.e., L-proline (L-Pro) and L-hydroxyproline (L-Hyp)). It seemed interesting to assess the effect exerted by D<sub>2</sub>O on L-Ala, the simplest chiral (endogenous and proteinogenic)  $\alpha$ -amino acid with as low molar weight, as 89.09 g mol<sup>-1</sup> only. As analytical techniques, we used high-performance liquid chromatography with the diode array detection (HPLC–DAD), mass spectrometry (MS), and scanning electron microscopy (SEM). The obtained results make it clear that the impact of heavy water on the dynamics of the spontaneous peptidization of L-Ala is even stronger than with the four other  $\alpha$ -amino acids discussed earlier (although in all five cases, heavy water significantly hampers spontaneous oscillatory peptidization). Unlike in the four previous cases, though, the solubility of L-Ala in pure D<sub>2</sub>O is quite low and it takes twice as much time to dissolve it in D<sub>2</sub>O than in MeOH + X, 70:30 (v/v). Consequently, the peptidization of L-Ala in heavy water is even more obstructed than it was the case with the other investigated  $\alpha$ -amino acids and it results in considerable yields of the L-Ala crystals (most probably at least partially deuterated) at the expense of the L-Ala-derived peptides. Perhaps it might be interesting to add that out of five  $\alpha$ -amino acids investigated so far, which can be divided into two groups of endogenous and exogenous species, two endogenous species (L-Cys and L-Pro) undergo spontaneous oscillatory peptidization in an aqueous-organic solvent (i.e., in the absence of D<sub>2</sub>O) following the circadian rhythm, whereas two exogenous ones (i.e., L-Met and L-Hyp) do not. The third endogenous species (L-Ala) first undergoes two initial oscillations which are damped (not periodic) and the oscillatory changes are on a scale of ca. 10 h (as estimated with use of the Fourier transform approach) and after that, the system reaches a steady state.

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s11144-020-01783-y>) contains supplementary material, which is available to authorized users.

**Keywords** L-alanine · D<sub>2</sub>O · Spontaneous oscillatory peptidization · High-performance liquid chromatography · Mass spectrometry · Scanning electron microscopy

## Introduction

As soon as the preparation of heavy water on a larger scale was made possible, its biological effects on living organisms have immediately attracted wide attention of scientists and it happened as early, as before WWII [1–4]. The first experiments were cautiously planned with small organisms (such, as algae, plants, fungi and bacteria), to stepwise switch toward the more complex and warm-blooded ones. For example, Lewis was the first one to publish his results on full inhibition of the tobacco seeds germination in pure heavy water and on considerable retardation of this process by 50% D<sub>2</sub>O only [1]. Pratt was another pioneer of the experiments with heavy water who investigated its strong inhibitory effect on growth of fungus *Erysiphe graminis tritici* [2]. Further systematic investigations have been carried out with selected mammals (e.g., [5–8]) and more recent studies with heavy water often target various different tissue preparations, with an aim to reveal apoptotic potential of D<sub>2</sub>O against the lung, pancreatic and other tumor cells [9–13]. Better understanding of such apoptotic effects might help develop regular curative carcinoma treatments making use of D<sub>2</sub>O. Although the lethal effect induced by heavy water has been confirmed with practically all kinds of living beings, it is obvious that physiological mechanisms of structurally diverse organisms from unicellular to the mammalian ones considerably differ, with probably one common structural denominator shared by all living matter which is the amino acids as elementary building blocks of each single cell.

In the course of the last three years, we started systematically examining an inhibitory effect of heavy water (D<sub>2</sub>O) on spontaneous oscillatory peptidization with a number of endogenous and exogenous  $\alpha$ -amino acids, and the obtained results have been published in a series of papers [14–18]. The main analytical tools employed in these investigations were high-performance liquid chromatography (HPLC), mass spectrometry (MS) and scanning electron microscopy (SEM), with an additional contribution from turbidimetry. All these results have unequivocally proved an inhibitory effect of D<sub>2</sub>O on oscillatory peptidization, although the dynamics of this inhibition perceptibly differed from one compound to another. Moreover, it was revealed that oscillatory peptidization of the three endogenous  $\alpha$ -amino acids investigated so far undergoes the circadian rhythm (L-Cys [15], L-Pro [17], and L-Ser [19]), whereas oscillatory peptidization of the three exogenous  $\alpha$ -amino acids does not follow it (L-Met [16], L-Hyp [18], and L-Phe [20]). The phenomenon of spontaneous oscillatory peptidization running in the parallel with spontaneous oscillatory chiral inversion of different proteinogenic  $\alpha$ -amino acids has been extensively discussed in papers [21–26].

It seems noteworthy that our discovery of spontaneous chiral oscillations was confirmed by some other researchers as a reasonable justification of their own, often striking and quite unexpected results (e.g., [27–29]). For example, the authors of paper [27]

have stated that until recently, chiral oscillations have not been reported in any experiment, and hence the possibility of studying their onset in theoretical models (like in the research that they have been conducting themselves) has received sparse attention. Consequently, the experimental evidence of chiral oscillations reported in our publications gave a strong boost to their own studies. The authors of paper [28] from the Caltech and Harvard University units involved in the earth and planetary studies admitted that certain results of their optical investigations on spontaneous transformations of the organic aerosol matter could only be interpreted based on the concept of chiral oscillations provided in our studies. In a recently published PhD thesis focusing on prebiotic evolution of Earth [29], the author benefited from an information originating from our scientific reports as to spontaneous oscillatory peptidization of the sulfur-containing  $\alpha$ -amino acids, presented in papers [30, 31].

This is our fifth consecutive study carried out in an order to collect experimental evidence on the impact of heavy water ( $D_2O$ ) on spontaneous peptidization of L-alanine (L-Ala), an important endogenous  $\alpha$ -amino acid playing many vital roles in human organism, where it is involved in the glucose-alanine cycle [32, 33]. An important role played by L-Ala in the animal metabolic cycles was confirmed with selected mammals also [34]. Although the chiral D-Ala enantiomer has been found to alleviate the symptoms of schizophrenia [35] and currently non-chiral  $\beta$ -Ala is in the main focus of the sports scientists' attention due to its positive physiological effects (like an improvement of muscle performance and reduction of physical fatigue [36–38]), we are going to investigate the impact of  $D_2O$  exclusively on spontaneous peptidization of L-Ala with use of the HPLC, MS and SEM techniques.

## Experimental

### Reagents and samples

In our experiments, we used L-Ala of analytical purity, purchased from Reanal (Budapest, Hungary), methanol (MeOH) of HPLC purity (Merck KGaA, Darmstadt, Germany) and heavy water ( $D_2O$ ) (Cambridge Isotopic Laboratories, Andover, MA, USA; 99% purity). Water ( $H_2O$ ) was de-ionized and double distilled in our laboratory by means of the Elix Advantage model Millipore System (Molsheim, France).

The L-Ala sample prepared for the HPLC–DAD experiment was dissolved at a concentration of  $1 \text{ mg mL}^{-1}$  in  $\text{MeOH} + \text{H}_2\text{O}$ , 70:30 ( $v/v$ ). All L-Ala solutions used for mass spectrometry (MS) and scanning electron microscopy (SEM) were prepared at a concentration of  $1 \text{ mg mL}^{-1}$  either in pure  $D_2O$ , or in the binary liquid mixtures  $\text{MeOH} + X$ , 70:30 ( $v/v$ ), where X: the binary mixture of  $H_2O + D_2O$  in the changing volume proportions of 30:0, 25:5, 20:10, 10:20 and 0:30.

## High-performance liquid chromatography with diode array detection (HPLC–DAD)

High-performance liquid chromatography with diode-array detection (HPLC–DAD) was employed to separate the monomeric L-Ala from the peptides. The analyses were carried out using a Varian model 920 liquid chromatograph equipped with a 900-LC autosampler, gradient pump, 330 DAD detector and ThermoQuest Hypersil C18 column (150×4.6 mm i.d.; 5 μm particle size) for L-Ala and Galaxie software for data acquisition and processing. The chromatographic column was thermostated at 35 °C using a Varian Pro Star 510 column oven. The chromatographic analyses were carried out using the 15-μL sample aliquots and an ACN–water (40:60, v/v) mobile phase at a flow rate of 0.40 mL min<sup>-1</sup>. Relatively short sampling time intervals (12 min) were chosen in order to derive quasi-kinetic information about the oscillatory peptidization. For the assessment of quantitative changes of the chromatographic signal valid for the monomeric L-Ala we used the results recorded at 199 nm.

## Mass spectrometry (MS)

All mass spectra for the investigated L-Ala samples were recorded in the positive ionization mode with use of a Varian MS-100 mass spectrometer (extended ESI–MS scan, positive ionization, spray chamber temperature 50 °C, drying gas temperature 250 °C, drying gas pressure 25 psi, capillary voltage 50 V, needle voltage 5 kV). Mass spectrometric detection was carried out straight away after a 7-day sample storage period. Samples were kept in the darkness at 25.0±0.5 °C. Mass spectra were recorded for the solutions which contained the soluble peptide fraction (the insoluble suspensions of microparticles self-separated by sedimentation).

## Scanning electron microscopy (SEM)

The analyses were carried out with use of a JEOL JSM-7600F model scanning electron microscope (SEM) for the six investigated L-Ala samples after one month storage period (at 22.0±0.5 °C). Visualization of the nano- and microparticles was obtained for the respective solutions evaporated to dryness.

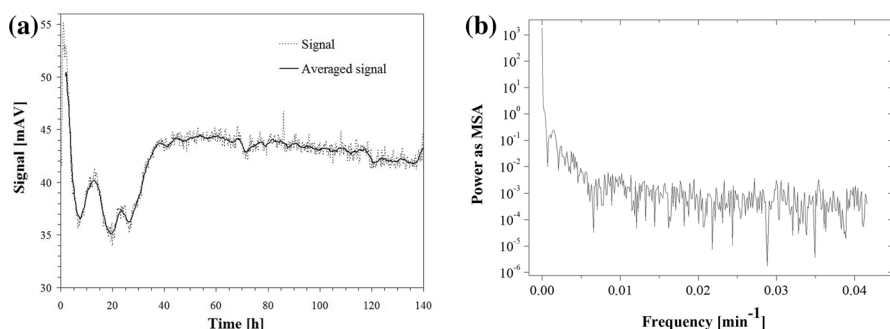
## Results and discussion

The supplementary material contains schematic representation of the processes of spontaneous chiral inversion (Fig. S1a), spontaneous peptidization (Fig. S1b) and of these two processes running in the parallel (Fig. S1c). Schemes given in Fig. S1 illustrate an up-to-date understanding of the molecular-level mechanisms of the aforementioned processes upon an example of L-Ala. In the following

sub-sections, we will discuss the impact of  $D_2O$  on the dynamics of the L-Ala peptidization, as it emerges from the results obtained with use of three different instrumental techniques.

### High-performance liquid chromatography with diode array detection (HPLC–DAD)

Our discussion begins from analyzing the behavior of a freshly prepared L-Ala sample stored for 142 h in MeOH +  $H_2O$  (70:30,  $v/v$ ), as traced in the chromatographic experiment carried out with aid of the achiral HPLC–DAD system. The goal was to separate the monomeric L-Ala from the spontaneously formed peptides and to trace its varying amounts, as represented by the changing peak heights of the L-Ala monomer (with the changes resulting from alternate processes of spontaneous peptidization and hydrolytic degradation of peptides). Fig. 1a shows the changing L-Ala peak heights plotted against sample storage time (the retention time of the monomeric L-Ala was equal to  $t_R \approx 4.43$  min). As a result, the non-linear signal intensity changes in the function of time were observed, equivalent to the respective concentration changes and the plot is labeled as the chromatographic time series of the monomeric L-Ala. To reduce noise and to reveal overall trends in our measurements, we also display a 15-point moving average for the signal given in Fig. 1a. Then we Fourier transformed the time series in order to check the dynamic nature of the spontaneous peptidization process (Fig. 1b). It came out that L-Ala first undergoes two initial oscillations which are damped (not periodic) and the oscillatory changes are on a scale of ca. 10 h and after that time, the system reaches a steady state.

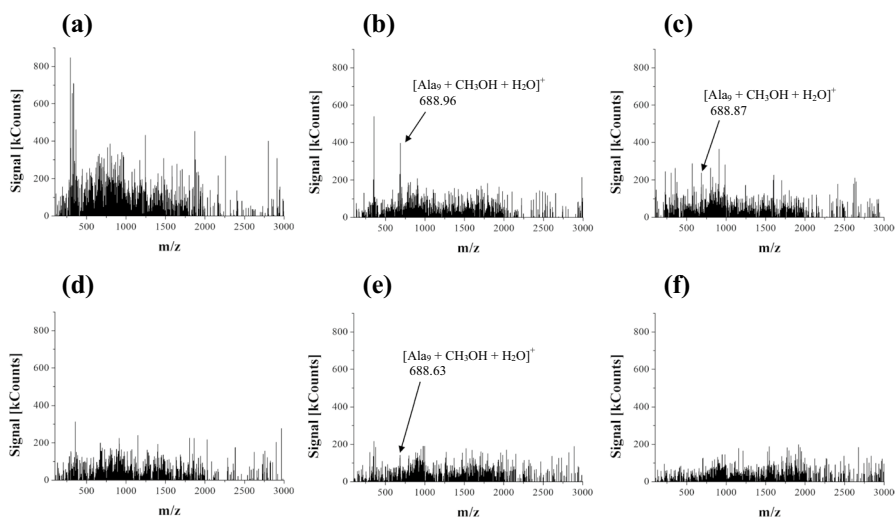


**Fig. 1** **a** Time series of chromatographic peak heights at  $t_R \approx 4.43$  min for the monomeric L-Ala in MeOH +  $H_2O$ , 70:30 ( $v/v$ ) recorded with the DAD detector in the time range from 0 to 142 h sample storage time (the apparatus signal and the averaged signal). **b** Power spectrum calculated from time series of chromatographic peak heights shown in **a**

## Mass spectrometric (MS) tracing of spontaneous peptidization of L-Ala

Mass spectrometry allows recording of the mass spectra for the monomeric L-Ala and the soluble peptides formed during the 7 days of storage period. The insoluble higher peptides which self-separate from the solution by sedimentation were recorded using the scanning electron microscopy (SEM) and these results are going to be discussed in the next sub-section.

Mass spectra recorded for the individual L-Ala solutions are in the first instance regarded as fingerprints (Fig. 2). With the L-Ala sample stored in an absence of heavy water ( $D_2O$ ), the mass spectrum shows significant amounts of high intensity signals, predominantly in the range of the  $m/z$  values below 2000, with the most intense (up to 800 kCounts) signals appearing in the range below  $m/z$  500 (Fig. 2a). The addition of 5%  $D_2O$  causes a visible drop of signal intensities along an entire range of the recorded  $m/z$  values, with the highest intensities of single signals reaching up to 400 or 540 kCounts (Fig. 2b). The contents of 10 and 20%  $D_2O$  in solution (Figs. 2c and 2d, respectively) do not provide any spectacular change of the mass spectroscopic fingerprints, with signal intensities in each consecutive spectrum slightly decreasing, and with the highest signal yields appearing below the  $m/z$  2000 value. The 30% amount of  $D_2O$  in the L-Ala solution results in an even stronger pronounced lowering of signal intensities than it is observed in each preceding case, and now these intensities do not exceed 200 kCounts (Fig. 2e). The mass spectrum recorded for L-Ala dissolved in pure heavy water is very similar to that obtained for 30%  $D_2O$ , yet with a slight drop of signal intensities in the range below  $m/z$  500 (Fig. 2f). Summing up, an increase in the content of  $D_2O$  up to 30% causes a slow and gentle blanking of the mass spectrometric signals, whereas the



**Fig. 2** Mass spectra recorded for L-Ala dissolved in MeOH + X, 70:30 (v/v), where X: the binary mixture of  $H_2O + D_2O$  in the changing volume proportions; **a** 0%  $D_2O$ ; **b** 5%  $D_2O$ ; **c** 10%  $D_2O$ ; **d** 20%  $D_2O$ ; **e** 30%  $D_2O$  and **f** L-Ala dissolved in pure  $D_2O$



100% D<sub>2</sub>O content does not result in any further significant change of the MS pattern. Generally, signals from the presented mass spectra are difficult to interpret, but we managed to assign at least one structure to the signal at  $m/z$  689. This signal is well perceptible in the mass spectra recorded for the L-Ala samples containing 5, 10 and 30% D<sub>2</sub>O in solution (Fig. 2b, c, and e), and it most probably originates from the cation built of the nanomeric peptide and the methanol and water adducts, [Ala<sub>9</sub> + CH<sub>3</sub>OH + H<sub>2</sub>O]<sup>+</sup>. As a matter of fact, in the range up to 2000  $m/z$  units and densely populated with signals, it is at least theoretically justified to expect peptides composed even of as many as 28 monomer L-Ala units.

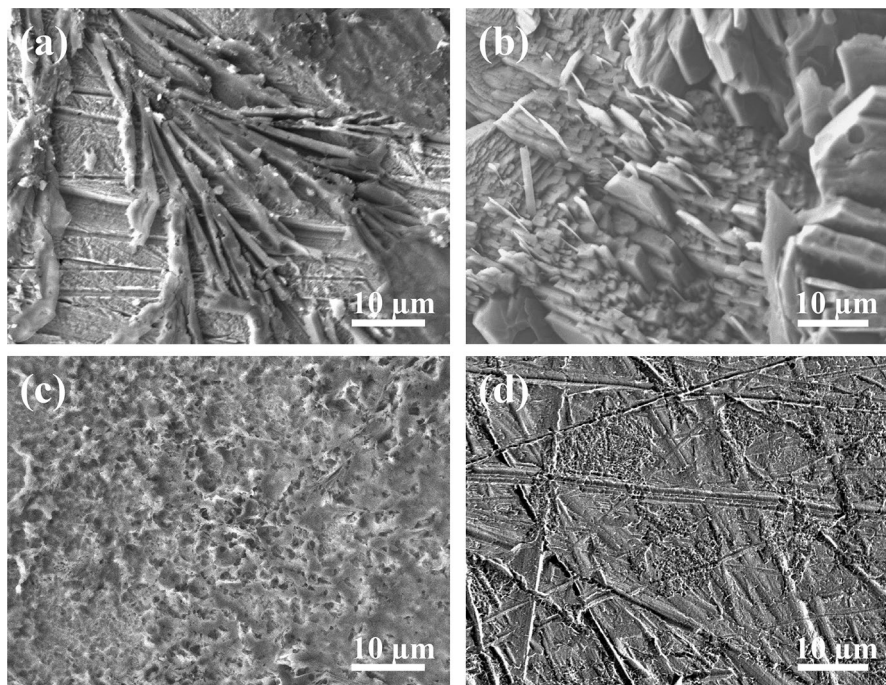
Finally, a comparison can be drawn between the behavior of L-Ala and L-Hyp under the influence of heavy water, with the latter case presented in our earlier study [18]. With L-Hyp, a similar tendency of the decreasing mass spectrometric signal intensities with the increasing contents of D<sub>2</sub>O was observed. Contrary to the case of L-Ala though (where a drop of signal intensities is gradual and smooth), a decrease of signal intensities for L-Hyp was rapid. For example, signal intensities for L-Hyp in the presence of 5% D<sub>2</sub>O reached up to 570 kCounts (Fig. 1b, [18]), and then for the nearest investigated proportion of D<sub>2</sub>O equal to 10% they dropped to the values not exceeding 11 kCounts (Fig. 1c, [18]).

### Scanning electron microscopic (SEM) tracing of spontaneous peptidization of L-Ala

With the mass spectra recorded for the samples with the increasing proportions of D<sub>2</sub>O in solution, a general trend is observed of the lowering yields of the soluble L-Ala-derived peptides (Fig. 2). Also the micrographs of the higher and mostly insoluble peptides derived from L-Ala show a decrease in peptide yields with the increasing amounts of D<sub>2</sub>O in solution. For each sample, a series of micrographs was taken at different magnifications and from different sample locations on the test pin, and selected micrographs illustrating the observed regularities and trends are given in Figs. 3 and S2 (Supplementary material).

In Fig. 3a and S2a, we present micrographs recorded for the L-Ala sample which underwent peptidization in an absence of D<sub>2</sub>O and the resulting peptide structures resemble elongated and outstretched filaments. The addition of 5% D<sub>2</sub>O changes the pattern of the resulting peptides which now become partially segmented plates of different sizes, tightly overlapping, irregular and noticeably perforated (Figs. 3b and S2b). With 10% D<sub>2</sub>O in solution, the micrographs show the previously observed 'rocky' structures which are now crushing on the edges (this effect is best viewed in Fig. S2c<sub>3</sub>). In that way, the jagged formations arise with a spongy structure and lots of perforations (Figs. 3c, S2c<sub>1</sub> and S2c<sub>2</sub>). With the D<sub>2</sub>O content rising to 20%, single and elongated structures are observed, surrounded by numerous small and granular formations (Fig. S2d). On the micrographs obtained for 30% D<sub>2</sub>O (Figs. 3d and S2e), rare clusters of the fine-grained peptidization products can be seen. The micrographs recorded for L-Ala dissolved in pure D<sub>2</sub>O (Fig. S2f) show quasi-spherical formations evenly scattered across an entire field of view of the microscope. It seems probable that these formations are the non-peptidized (and at least partially





**Fig. 3** Scanning electron micrographs recorded for the L-Ala-derived peptides retrieved from the samples dissolved in MeOH + X, 70:30 (v/v), where X: the binary mixture of H<sub>2</sub>O + D<sub>2</sub>O in the changing volume proportions; **a** 0% D<sub>2</sub>O, ×2000; **b** 5% D<sub>2</sub>O, ×2000; **c** 10% D<sub>2</sub>O, ×2000; **d** 30% D<sub>2</sub>O, ×2000

deuterated) L-Ala crystals. This assumption seems to be supported by a difficulty in dissolving L-Ala in pure D<sub>2</sub>O (its dissolution lasted ca. twice as long as with the remaining L-Ala samples in the MeOH + X solvents). An additional support comes from careful visual inspection of these quasi-spherical formations. In fact, many of them are not spherical, but rather hexagonal, resembling the shape of the L-Ala crystals given in Fig. 1b [39]. The same hexagonal form of the face (011) of the L-Ala crystal is also given in paper [40].

## Conclusion

This is the fifth consecutive report from our series focused on investigating the impact of heavy water on oscillatory peptidization of proteinogenic  $\alpha$ -amino acids, and this time the examined species is L-Ala, the simplest chiral (endogenous and proteinogenic)  $\alpha$ -amino acid with as low molar weight, as 89.09 g mol<sup>-1</sup>. In the previous four cases, we targeted two endogenous (L-Cys and L-Pro) and two exogenous (L-Met and L-Hyp)  $\alpha$ -amino acids. Once again, the experimental evidence collected for L-Ala exposes a hampering effect of D<sub>2</sub>O on the oscillatory peptidization of the proteinogenic  $\alpha$ -amino acid. Unlike in the four previous cases though, dissolution of L-Ala in pure D<sub>2</sub>O is quite difficult and it takes twice as much time as dissolution

of L-Ala in MeOH + X, 70:30 (v/v). Consequently, peptidization of L-Ala in heavy water is even more obstructed than with the other investigated  $\alpha$ -amino acids and it results in some yields of the L-Ala crystals (most probably at least partially deuterated) at an expense of the L-Ala-derived peptides. Perhaps it is noteworthy that with L-Ala, the dynamics of its spontaneous peptidization in an aqueous-organic solvent (i.e., in an absence of D<sub>2</sub>O) differs from that observed in the earlier four cases. The L-Ala solution first undergoes two initial oscillations which are damped (not periodic) and the oscillatory changes are on a scale of ca. 10 h. After that time, the system reaches a steady state, while with L-Cys and L-Pro, the circadian rhythm of oscillations was established, and with L-Met and L-Hyp, neither periodicity of the oscillations, nor a steady state was observed.

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