USE OF ELECTRON-SPIN-RESONANCE SPECTROSCOPY FOR STUDYING THE CONFORMATIONAL STATES OF PEPTIDES

GRAMICIDIN S

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UDC 543.422.27+547.964.4

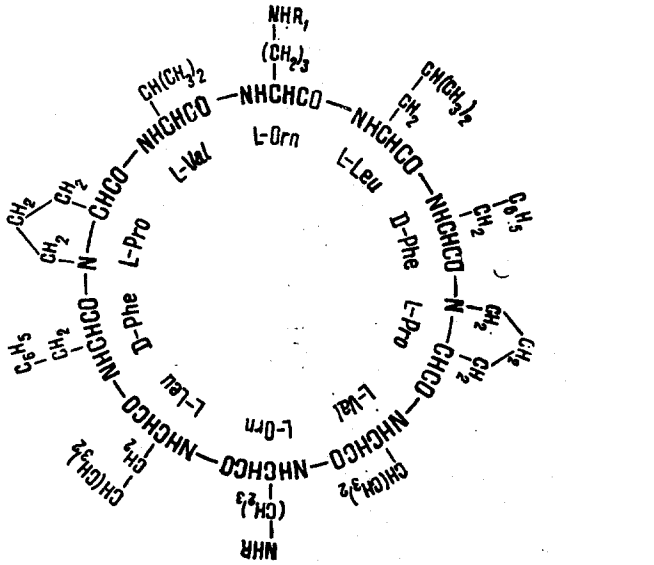
Up to the present time, to study the spatial structure of peptides in solutions use has been made mainly of IR and NMR spectroscopy, optical rotary dispersion, circular dichroism, dipole moments, and theoretical conformational analysis [1-6]. The methods mentioned give fairly complete information on the preferred conformations of the main peptide chain, but, as a rule, the question of the mutual positions of the side chains of the individual amino acid residues which are, in a number of cases, responsible for the appearance of the biological function, remains open. In the present paper, we propose a new approach to the study of the conformational states of peptides which is based on the use of electron spin resonance (spin-label method).

The ESR spectra of spin-labeled proteins and nucleic acids [7-10], under definite conditions, reflect the mobility of the groupings containing the free radical, their steric screening, and their accessibility for interaction with ions of paramagnetic metals. This enables the method to be used for the investigation of microrelief and conformational mobility in biopolymers.

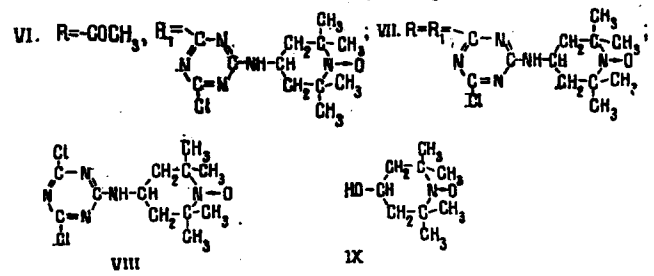
The ESR spectra change their shape substantially when the spins of the radical fragments interact. Consequently, when several spin labels are introduced into the molecules of the substances under investigation, the spectral parameters prove to be extremely sensitive to the distances between the groups bearing the free radicals. Here, interaction may take place by two mechanisms: exchange, and dipole–dipole. The first mechanism is realized in solutions in the direct collision of radical iminoxyl fragments and is shown in the appearance of additional lines in the ESR spectrum [11]. Dipole–dipole interaction appears in its purest form in the ESR spectra of polyradicals in solid matrices. The preparation of calibration ESR spectra of solid solutions of biradicals with a known distance between the iminoxyl groups has been described in the literature [12, 13]. Thus, the investigation of the ESR spectra of peptides bearing two or more spin labels in the side chains in solutions and in solid matrices would, in principle, enable the distances between these groups to be evaluated. The value obtained depends on the conformation of the peptide chain between the corresponding amino acid residues, which permits definite conclusions to be drawn concerning the spatial structure of the peptide molecule as a whole.

As the substance for testing the proposed approach, we selected the cyclic decapeptide gramicidin S (I) (Fig. 1), which is, at the present time, one of the biologically active peptides that has been studied in most detail from the conformational point of view [14, 16]. The mono- and biradical derivatives (VI) and (VII) were prepared for the investigation. Commercial gramicidin S (I), containing considerable amounts of proline, phenylalanine, ornithine, valine, and leucine as impurities, was converted by treatment with N-(tert-butyloxycarbonyl)succinimide [17] into the N,N-di-tert-butyloxycarbonyl (di-BOC) derivative (II), which was readily separated from by-products of the reaction by passage through ion-exchange resins and by recrystallization. The elimination of the BOC group by the action of trifluoroacetic acid (4 h) and the treatment of the gramicidin S formed (I) with the spin-labeling chloride (VIII) [18] led to the biradical (VII).

M. M. Shemyakin Institute of the Chemistry of Natural Compounds, Academy of Sciences of the USSR. Branch of the Institute of Chemical Physics, Academy of Sciences of the USSR. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 91-98, January-February, 1973. Original article submitted May 17, 1972.



I. R=R$_1$=–H; II. R=R$_1$=–OCOC(CH$_3$)$_3$; III. R=H, R$_1$=–OCOC(CH$_3$)$_3$;
IV. R$_1$=–OCOC(CH$_3$)$_3$, R=–COCH$_3$; V. R=R$_1$=–COCH$_3$;



VI. R=–COCH$_3$, R$_1$=

VII. R=R$_1$=

VIII

IX

Fig. 1. Gramicidin S and some of its derivatives.

The briefer action of CF_3COOH (10 min) formed the mono-BOC derivative (III), which was acetylated with the N-hydroxysuccinimide ester of acetic acid, again treated with CF_3COOH, and caused to react with the chloride (VIII), giving the monoradical derivative (VI).

The ESR spectra of compounds (VI) and (VII) in 96% ethanol, and also that of (VII) in chloroform, at various temperatures are given in Fig. 2. As was to be expected, the spectrum of the derivative (VI) at 59°C (Fig. 2a, curve 2) shows the rapid anisotropic rotation of the radical fragment around the N–O bond (anisotropic rotation is shown by the fact that the left-hand component of the spectrum is stronger than the central component) [19]. At –196°C, compound (VII) shows an ESR spectrum which coincides completely with the spectrum of a frozen ethanolic solution of the iminoxyl radical (IX) (see Fig. 2d).

In spectra of solutions of the biradical derivative (VII) (see Fig. 2b, c), in addition to the three main lines there are two additional ones showing the presence of exchange interaction between the unpaired spins, which appears in the intramolecular collisions of radical fragments. The results of a comparison of the intensities of the additional peaks lead to the conclusion that in ethanolic solution the frequency of the collisions of the radicals (i.e. their approach to a distance of 6 Å) is considerably higher than in chloroform. The evaluation of the frequency of exchange interactions is usually made by means of the formula

$$\Delta T_2^{-1} = \frac{1}{4} a^2 \tau,$$

where ΔT_2^{-1} is the width of the additional line, and a is the hfs constant [11].

Since between the width of the line ΔT_2^{-1} and its amplitude h the relation $\Delta T_2^{-1} \simeq 1/\sqrt{h}$ exists, and h can be measured more accurately than the line width, from the Arrhenius relation $\ln\sqrt{h} = f(1/T_1)$ it is possible to determine the activation energy E of the transition of compound (VII) into the conformation with approximated radical fragments. For an ethanolic solution of spin-labeled gramicidin S (VII) in the 30-62°C region the points lie satisfactorily on an Arrhenius straight line with E = 5-6 kcal/mole. For chloroform, the Arrhenius relationship is satisfied in the 43-55°C range, giving a value of E of 12-13 kcal/mole.

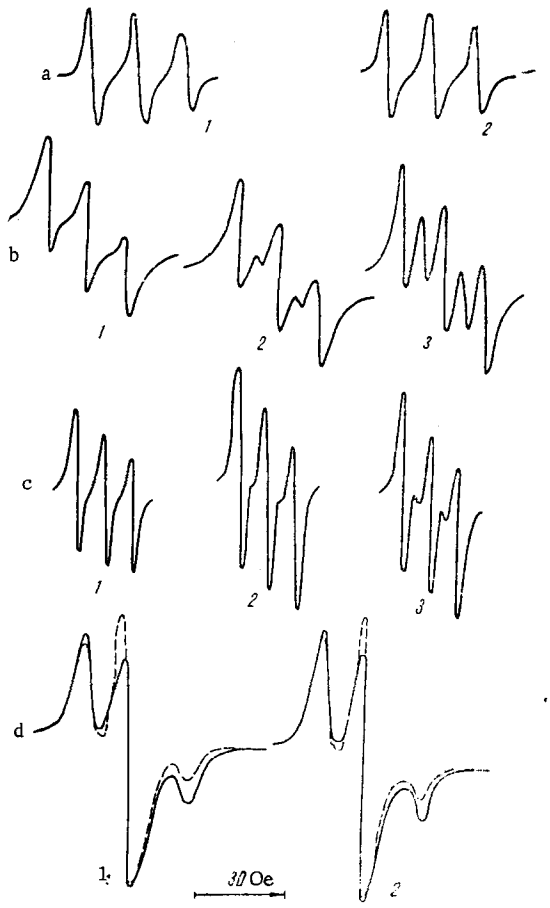


Fig. 2. ESR spectrum of a solution of spin-labeled gramicidin S (VI) in ethanol at 32°C (1) and 59°C (2) (a); of (VII) in ethanol at 20°C (1), 33°C (2), and 62°C (3) (b); of (VII) in chloroform at 20°C (1), 43°C (2), and 55°C (3) (c); and of (VII) at T = 77°K in ethanol (1) and in chloroform (2) [the dashed line shows the spectrum of the radical (IX) in ethanol] (d).

Thus, in solutions of spin-labeled gramicidin S (VII) the possibility exists of collisions between the iminoxyl fragments of the modified ornithine groups, while in ethanolic solutions the frequency of collisions is higher than in chloroform. Judging from the activation energies, the difference in the energies of the two conformers in chloroform is greater than in ethanol by 6-8 kcal/mole.*

The results obtained are in complete harmony with the "pleated-sheet" model of gramicidin S proposed in 1958 by Hodgkin, Oughton, and Schwyzer [20, 21] and definitively proved in 1970 by means of spectral and theoretical methods [14, 15]. In actual fact, a characteristic feature of the structure of the antibiotic in all the solvents investigated is an exceptionally rigid conformation of the main peptide chain stabilized by four intramolecular hydrogen bonds and the location of the side chains of the ornithine residues on the same side of the central plane of the ring, ensuring their easy approach in polar solvents both in the case of gramicidin S (I) itself and in that of its derivative (VII) (conformation A, Fig. 3).† In the case of nonpolar solvents [14], for example in chloroform solution, N,N'-diacetylgramicidin S (V) forms additional hydrogen bonds between the α-NH groups of the ^{2}Orn and ^{7}Orn residues and also between the carbonyl groups of the acetylornithine side chains. The lower frequency of collision of the radical fragments in chloroform than in ethanol that has been found from the ESR spectra shows the realization in compound (VII) in chloroform of analogous H bonds between the leucine carbonyl groups and the NH groups of the ornithine side chains (conformation C, Fig. 2d).

The groupings bearing iminoxyl radicals bound to them prove to be fixed at a considerable distance from one another, which excludes the possibility of collisions. The conclusion drawn is confirmed by the above evaluation of the difference in the activation barriers in chloroform and in ethanol, amounting to ~8 kcal/mole and approximately corresponding to the energy of two hydrogen bonds of the CO···NH type (see, for example, [5]).

Because of the marked restriction of the rotation of the iminoxyl fragments in frozen solutions, the ESR spectra of compound (VII) taken at −196°C (see Fig. 2d) have a different shape from the spectra taken at 38-62°C (Fig. 2b, c). The difference between these spectra and the ESR spectra of the free label (IX) (broken line in Fig. 2d) and the spectrum of the monoradical (VI) in ethanol, which coincides with them, shows the existence of an ion−dipole interaction between the labels. The practically indistinguishable spectra of compound (VII) in chloroform and ethanol are close to those of biradicals in which the distance between the iminoxyl radicals is 12-14 Å [12, 13]. A value of 15 Å is obtained from an estimate of the distances by means of the parameter $d_1/d_2 = 0.72$ (ratio of the sums of the intensities of the extreme peaks to the intensity of the central peaks) [13]. The value of the increment of the second moment $\Delta M_2 = 190$ Oe^2

*We assume that the measured activation energies E are close to the difference in energies between the conformers with remote and close radicals, since the depth of the potential trough of the approached state cannot be large because of steric repulsion.

† This is shown by the results of Schwyzer et al., who synthesized ^{2}Cys,^{7}Cys-gramicidin S (an analog in which the ornithine side chains $-CH_2-CH_2-CH_2-NH_2$ have been replaced by cysteine side chains $-CH_2-SH$) and demonstrated the ease of closure of a S−S bridge without a substantial change in the conformation of the cyclodecapeptide [16].

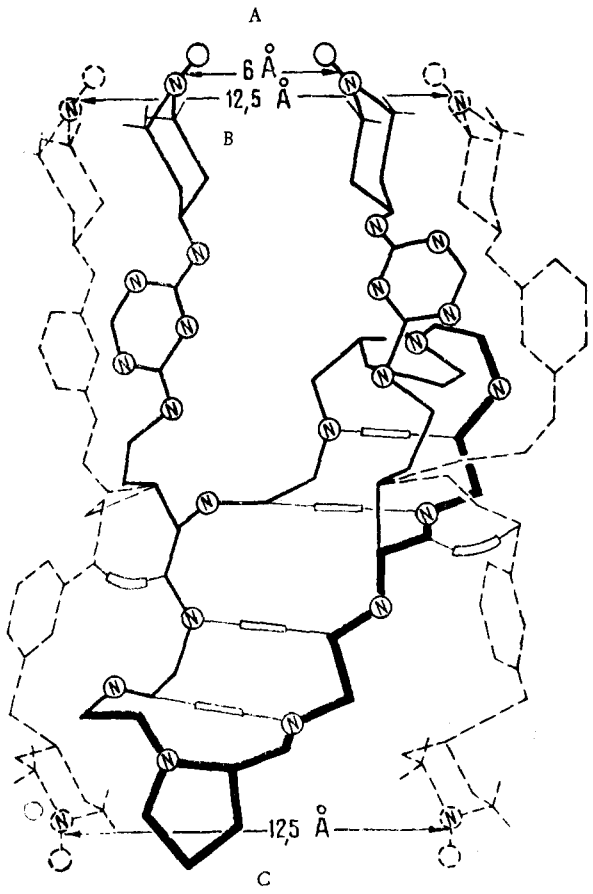


Fig. 3. Conformations of spin-labeled gramicidin S (VII) in different solvents.

gives a distance of 10 Å [12]. Thus, both in ethanol and in chloroform the unpaired spins of the radical fragments are present at a distance of 10-15 Å relative to one another. It follows from this that in conformation C (see Fig. 3), which exists in chloroform, the heterocyclic nuclei are located below the central plane of the ring, since with other orientations of the side chains of the modified ornithine residues the distance between the labels exceeds 15 Å. In ethanolic solutions, where the exocyclic hydrogen bonds are unstable, apparently, conformations of type B, intermediate between A and C, predominate (see Fig. 3).

Thus, the study of spin-labeled derivatives of gramicidin S has enabled new information to be obtained on the conformational possibilities of the molecule of this antibiotic and its analogs. The methods used for the present work can be extended to other biologically active peptides in order to determine (a) the ease of approach of the labels to a distance of ~6 Å in solutions, and (b) the mean distance between the labels in the predominating conformation of the peptide (by means of spectra taken with deep cooling); the range of values capable of determination is 6-20 Å [12, 13]. The high sensitivity of the method and the possibility of performing investigations in a wide range of solvents and temperatures also show its promising nature.

EXPERIMENTAL

All the melting points are uncorrected. The individuality of the compound obtained was checked by TLC on Eastman Kodak, USA, silica gel plates.

Di-tert-butyloxycarbonylgramicidin S (II). To a solution of 1.5 g of commercial gramicidin S in 10 ml of dry pyridine was added 1.1 g of N-(tert-butyloxycarbonyl)succinimide [17], and the mixture was stirred at 20°C for 48 h. Then the pyridine was evaporated off, and the residue was dissolved in 150 ml of ethanol–water (2:1) and passed successively through a column (10 × 1.5 cm) containing Dowex-50 × 1 (H^+ form) and Dowex-1 × 1 (OH form). The eluate was evaporated, and the residue was crystallized from aqueous ethanol. Mp 289-290°C, $[\alpha]_D^{20}$ −196° (c 1; ethanol).

Monoradical Derivative of Gramicidin S (VI). Compound (II) (100 mg) was dissolved in 5 ml of anhydrous trifluoroacetic acid; after 10 min, the solution was evaporated at 20°C. The residue was treated with 10 ml of toluene and the mixture was again evaporated in vacuum. The residual product was dissolved in 50 ml of methanol–water (2:1) and passed through a column (10 × 1 cm) of Dowex 50 × 1 (H^+ form). The column was eluted with 250 ml of methanol–water (1:1), and the aqueous ethanolic eluate was evaporated. Compound (II) was isolated from the residue. The gramicidin S (I) and the monosubstituted derivative (III) were eluted with a 2 N solution of ammonia in ethanol. The ammoniacal eluate was evaporated, the residue was dissolved in 10 ml of pyridine, and the solution was treated with 0.5 g of the N-hydroxysuccinimide ester of acetic acid. After 48 h (20°C), the solution was evaporated, the residue was washed on the filter with citric acid solution and with water, and it was dried in vacuum over P_2O_5. The mixture of mono- and diacetylated derivatives of gramicidin S (IV) and (V) was dissolved in 20 ml of trifluoroacetic acid and the solution was evaporated in vacuum over 4 h (20°C). The residue was dried over KOH, dissolved in 50 ml of ethanol–water (2:1), and chromatographed on Dowex 50 × 1 (H^+ form) as described above. The aqueous ethanolic eluate yielded the diacetyl derivative of gramicidin S (V) (mp 305-306°C, from aqueous ethanol); $[\alpha]_D^{20}$ −302° (c 1.5, 70% ethanol), and the ammoniacal eluate yielded 12 mg of the electrophoretically homogeneous [formic acid–acetic acid–methanol–water (1:3:6:10) system, 4 h,

500 V, spots revealed with ninhydrin] monoacetyl derivative of gramicidin S. To a solution of 10 mg of the monoacetyl derivative so obtained in 10 ml of 96% ethanol were added 0.01 ml of triethylamine and 10 mg of the chloride (VIII). The solution was stirred at 20°C for 60 h and evaporated in vacuum, and the residue was dissolved in methanol and chromatographed on a column of Sephadex LH-20 (150 × 1 cm). The fraction containing the monoradical derivative (VI) was evaporated, and the residue was reprecipitated from ethanol with ether. This gave 9 mg of compound (VI) with mp 241-243°C, showing no reaction with ninhydrin, R_f 0.72 in the benzene–dioxane (2:1) system.

The Biradical Derivative of Gramicidin S (VII). Compound (II) (25 mg) was dissolved in 10 ml of trifluoroacetic acid, and after 4 h the solution was evaporated in vacuum. The residue was dried over KOH and was then dissolved in 10 ml of 96% ethanol and treated with 0.02 ml of triethylamine and 24 mg of the chloride (VIII). The solution was stirred at 20°C for 60 h and evaporated, and after working up and chromatography as described for the monoradical derivative (VI), 10 mg of a chromatographically homogeneous compound with mp 279-280°C was obtained which gave no reaction with ninhydrin, R_f 0.6 in the benzene–dioxane (2:1) system.

The ESR spectra were taken on a ÉPR-2 spectrometer. To measure the spectra at 30-62°C, the solution was placed in a cell thermostated with a mixture of silicone oil and decane (1:1); the accuracy of the temperature measurement was 0.5°C. Measurements at the boiling point of liquid nitrogen were performed in a quartz Dewar vessel. The concentration of the radical was determined by comparing the area under the integral curve of the sample being investigated with the area of a sample having a known concentration of the radical. For the monoradical derivative of gramicidin S (VI) we obtained $c_r = 5.8 \cdot 10^{-4}$ M (concentration of free radical), $c_1 = 7 \times 10^{-4}$ M [concentration of the monoradical derivative (VI)], i.e. $c_r/c_1 \simeq 1$; and for the biradical derivative of (VII), $c_r = 1.35 \cdot 10^{-4}$, $c_2 = 7.2 \cdot 10^{-4}$ M* [concentration of the biradical derivative (VIII)], i.e. $c_r/c_2 \simeq 2$.

The absence of intermolecular interactions in all cases was confirmed by measuring the spectra at several concentrations. The spectra at room temperature were taken for solutions with concentrations of the gramicidin derivatives $c \sim 10^{-3}$ M, and the spectra at −196°C in ethanol were taken at $c \sim 10^{-4}$ M.

SUMMARY

1. A new method has been proposed for studying the conformational states of peptides in solutions which is based on a consideration of the ESR spectra of their spin-labeled derivatives.

2. The synthesis of derivatives of gramicidin S containing one and two iminoxyl radicals in the ^{2}Orn and ^{7}Orn side chains has been effected.

3. On the basis of an analysis of the ESR spectra of the compounds obtained at −196°C and 30-62°C, conformations of the side chains of modified ornithine residues in chloroform and ethanol have been proposed.

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