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Isolated Gramicidin Peptides Probed by IR Spectroscopy

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We report double-resonant IR/UV ion-dip spectroscopy of neutral gramicidin peptides in the gas phase. The IR spectra of gramicidin A and C, recorded in both the 1000 cm^{-1} to 1800 cm^{-1} and the 2700 to 3750 cm^{-1} region, allow structural analysis. By studying this broad IR range, various local intramolecular interactions are probed, and complementary IR modes can be accessed. Ab initio quantum chemical calculations are used to support the interpretation of the experimental IR spectra. The comparison of the calculated frequencies with the experimental IR spectrum probed via the strong infrared absorptions of all the amide groups (NH stretch, C=O stretch and NH bend), shows evidence for a helical structure in the gas phase, which is similar to that in the condensed phase. Additionally, we show that to improve the spectral resolution when studying large neutral molecular structures of the size of gramicidin, the use of heavier carrier gas could be advantageous.

1. Introduction

Peptides and proteins are responsible for many different and important functions in the living cell, where each protein or protein family has its own specific function. The function of these biomolecules is inherently connected to their three-dimensional structure. For proteins and peptides, this three-dimensional structure depends on the sequence of amino acid residues, their noncovalent intramolecular interactions, and the interplay with the biological and protein environments. Unfortunately, this complex environment of proteins, such as the solvent and intermolecular interactions with neighboring residues, masks many details of the intrinsic structural properties. Investigating the conformational properties demands the study of isolated molecules, which can be achieved by gas phase spectroscopy. This technique has been limited to small molecules for a long time. The Levy group reported the first molecular beam experiments on tryptophan followed by studies on di- and tripeptides.[1–5] Afterwards, the application of laser desorption allowed expansion to study larger, neutral species such as DNA bases,[6,7] rotaxanes,[8,9] and multi-residue peptides.[10–13] A previous paper by de Vries et al. already showed the investigation of isolated, neutral 15-residue peptide gramicidin.[11]

IR/UV ion-dip spectroscopy is an important tool for determining gas-phase structures and for obtaining detailed conformational information of neutral biological molecules.[12–17] The conformations of these biological systems can be sensitively probed via the strong infrared absorptions of the amide groups (NH stretch, C=O stretch and NH bend) which respond to intramolecular interactions. The IR frequencies of these vibrations strongly depend on the molecular conformation and can therefore reveal the secondary structure. Moreover, in combination with quantum chemical calculations, the IR spectra allow for the analysis of the structural properties.[13–21]

Conformation-selective structural identification becomes more challenging for larger peptides for which the resolution of the IR spectra decreases, for example, due to thermal broadening or insufficient cooling. For ions, this problem is addressed with the emergence of the cooled 22-pole ion trap.[21–23] Recent experiments by Rizzo et al. present the high-resolution electronic spectra and conformationally selective vibrational spectra of the doubly protonated cyclo-decapeptide gramicidin S.[22] However, to enhance the spectral resolution when studying large, neutral molecular structures such as the 15-residue peptide gramicidin presented herein, other solutions are required. One possible approach, which we explore here, is to improve the cooling conditions during the expansion by employing heavy carrier gas atoms such as krypton and xenon.

Herein, we report on the structural characterization by IR-UV spectroscopy and high-level DFT calculations of isolated gramicidin, a family of 15-residue peptides with molecular weights of 1845—1898 Da. Gramicidin, which can be obtained from

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the soil-dwelling bacterium *Bacillus brevis*, is a heterogeneous mixture of antibiotic compounds that each differ in one residue.[24,25] Herein we concentrate on gramicidin A and C (see Figure 1). These peptides vary only in the 11th residue: gramicidin A contains tryptophan while for gramicidin C the tyrosine residue occupies this position. In the condensed phase it is found that this peptide forms a helix, which is expected to persist in the gas phase. To determine the structure of isolated gramicidin A and C, we have employed IR-UV ion dip spectroscopy in both the light-atom stretch region (around 3 micron) and the amide I, II and fingerprint region (1000–1800 cm^{-1}). These results show not only 1) that it is possible to bring these peptides into the gas phase intact and 2) that high-resolution spectroscopy can be performed on these large peptides, in spite of the large number of internal degrees of freedom, but also 3) that they adopt unique structures that reveal details of the intramolecular stabilizing forces, and very importantly 4) that they can be characterized with *ab initio* quantum chemical DFT calculations.

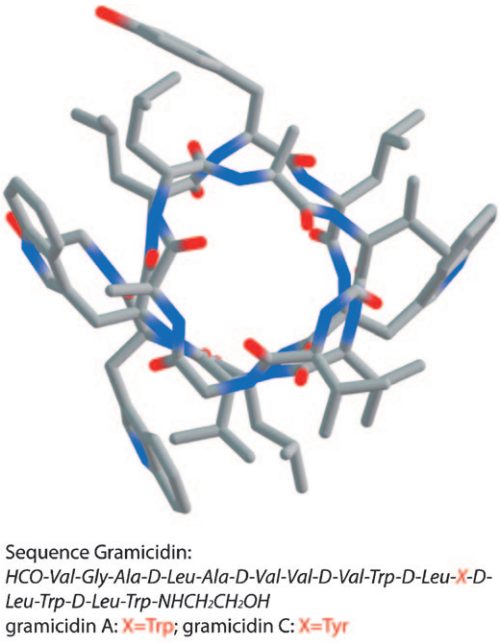

Figure 1. Structure of gramicidin C obtained from the structural optimization step of the quantum chemical calculations. The two types of gramicidin studied herein vary by one residue, namely tryptophan for gramicidin A and tyrosine for gramicidin C.

2. Results and Discussion

2.1. IR Spectroscopy: The Complete Amide A, I and II Regions

The top panels of Figures 2 and 3 show the experimental IR absorption spectra of gramicidin A and gramicidin C (upper trace) in the 1000–1800 cm^{-1} and in the 2700–3750 cm^{-1} region, respectively. The middle and lower part of Figure 2

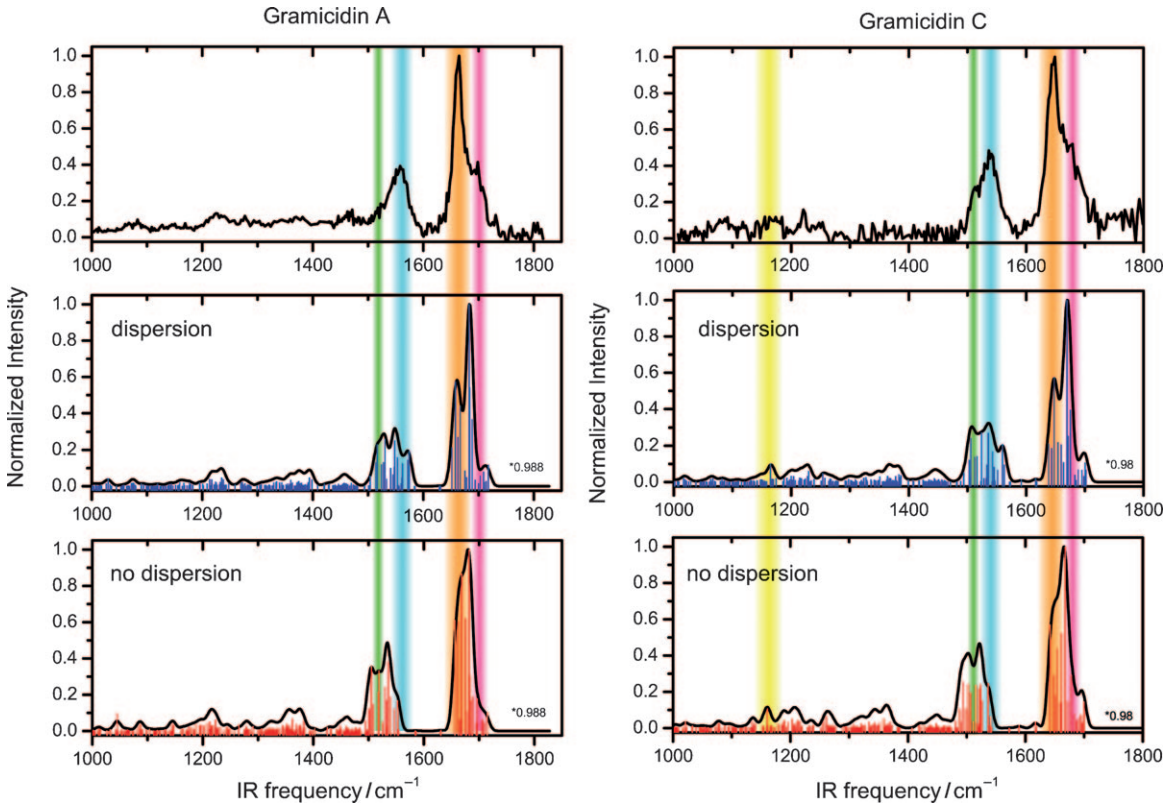

Figure 2. Top panel: IR/UV ion-dip spectra of gramicidin A (left) and C (right) in the mid-IR region, showing the peaks originating from the free and hydrogen-bonded Amide I (pink/orange) and Amide II (green/cyan) vibrations. Middle and lower panels: The calculated IR spectra (scaling factor 0.98) convoluted with a Gaussian with a FWHM of 12 cm^{-1}, with (middle) and without (bottom) dispersion taken into account.

present the calculated stick and convoluted IR spectra with and without dispersion. The experimental spectra of both wavelength regions are compared with the calculated frequencies with and without dispersion. To obtain the IR spectra, the UV laser was tuned to 35400 cm^{-1}. For other UV wavelengths in the region between 35050 to 35450 cm^{-1} we obtained identical IR spectra. This indicates that only one conformer or one family of closely related conformers was present in the molecular beam.

The IR spectra of both gramicidin A and C show a wealth of resolved absorption bands. Here, we first focus on the amide I, the amide II, and the fingerprint region, all between 1000 and 1800 cm^{-1} (see Figure 2). The spectra show two dominant bands around 1650 cm^{-1} and 1525 cm^{-1}. These bands can be assigned to the C=O stretching (orange/pink) and NH bending modes (green/cyan), respectively. The broad C=O band results from the presence of both free and hydrogen-bonded C=O stretching modes. The same holds for the NH bending peak, which is composed of the NH modes of the indole ring of tryptophan, as well as both hydrogen-bonded and free-backbone NH modes.

To further examine the secondary structure of gramicidin, we performed high-level quantum chemical calculations. By comparing the experimental IR-UV spectra in the 1000 to 1800 cm^{-1} region and the calculated frequencies and intensities with and without dispersion, we observe that, especially for gramicidin A but also for gramicidin C, the calculated spectra in which the dispersion is taken into account result in a better match. This can mainly be observed in the spacing between the overall peak of the C=O stretching modes and the NH bending modes, but also in the small details in the fingerprint region. In-depth analysis of the broad C=O band of both gramicidin A and C demonstrates that this band results from two different types of C=O stretching modes. At the blue side of the band, from about 1675 to 1720 cm^{-1}, free C=O stretching modes appear, for which the C=O is pointing out of the gramicidin structure, indicated in Figure 2 in pink. The intense part of the C=O band between 1630 and 1675 cm^{-1} results from hydrogen-bonded C=O stretching modes, for which the C=O is located at the inner part of the peptide (orange band).

The band originating from all the NH bending modes also shows a double-peak structure, which is much more pronounced for gramicidin C than for gramicidin A. This double feature suggests that there are at least two types of NH bending vibrations. The main band is observed between 1530 and 1570 cm^{-1} and a smaller band, which appears as a shoulder at the red side of the main NH bending peak, is located between 1525 and 1500 cm^{-1}. This shoulder results from the presence of a few free NH bending modes (green). The presence of these free NH frequencies is in contrast with earlier findings in the three-micron region. There, it was suggested that all peptide NH groups are hydrogen bonded, leading to an intense red-shifted and broadened band (see ref. [11] and Figure 2 therein). Additionally, the calculations show that the main band between 1530 and 1570 cm^{-1} consists of a number of unresolved bands. The hydrogen bond region (indicated by the cyan band) is split by the NH bending modes of the indole ring. These indole NH bending vibrations result from the tryptophan residues and are located around 1556 cm^{-1}.

The peaks below 1500 cm^{-1} are mainly due to the CH_2/CH_3 bending modes. However, the band observed in the calculated spectrum at about 1175 cm^{-1} results from the C–O–H mode of the tyrosine residue present only in gramicidin C (yellow). Unfortunately, due to the insufficient signal to noise ratio this band could not be distinguished in the experimental IR spectrum.

Figure 3 shows the IR spectra of gramicidin A and C, obtained from ref. [11], together with the calculated spectra in the 2700–3750 cm^{-1} region. As described by Abo-Riziq et al., various bands are present in the near-IR spectra of both gramicidin A and C: the free indole NH stretching frequencies at 3533 cm^{-1}, a broad band around 3300 cm^{-1} resulting from hydrogen-bonded NH groups of the peptide backbone, and, below 3070 cm^{-1}, the various CH stretching modes. The sequence specificity of this technique is demonstrated by the presence of the free tyrosine OH stretch vibrations at 3666 cm^{-1} for gramicidin C only. The sequences of gramicidin A and C differ in a single residue in that the middle tryptophan residue is exchanged for tyrosine in gramicidin C. The trace of gramicidin A clearly lacks this tyrosine OH stretch frequency. In agreement with the earlier predictions of Abo-Riziq et al., the band between 3074–3120 cm^{-1} indeed corresponds to the first overtone of the hydrogen -bonded NH-bend frequencies located between 1530–1570 cm^{-1} (see Figure 2). As expected, no overtones are observed of the weaker free NH vibrations. Where previously it was suggested that all peptide NH groups of the backbone are hydrogen bonded, the IR spectra in both the 1800–1000 cm^{-1} and the 2700–3750 cm^{-1} region show the presence of free NH groups as well. The small peak at about 3432 cm^{-1} present in the IR spectra of both gramicidin A as C can be assigned to a few free NH groups pointing outwards. This assignment is confirmed by the calculations.

Both the experimental spectra presented in Figures 2 and 3 and the calculated frequencies are consistent with a helical conformation. The tryptophan and tyrosine groups reside outside the helix, since the IR spectra show the free NH and OH frequencies of tryptophan (indole) and tyrosine respectively. Along the helical backbone, most of the peptide NH groups are hydrogen bonded, resulting in an intense and broadened peak for both the NH bending (Figure 2) and the NH stretching (Figure 3) region. The channel-like structure contains three free N–H and C=O groups each, the hydrogen and oxygen atoms pointing away from the channel. The calculated secondary structure corresponds to the experimentally observed structure of the beta sheets coiled into the helical form allowed by the alternating L- and D-residues. Due to hydrogen bonding, the NH bending frequencies are blue-shifted and the NH stretching modes are red-shifted. Additionally, as a result of this strong hydrogen bonding pattern, the overtone of the hydrogen-bonded NH bending mode at about 3100 cm^{-1} appears in the spectra (Figure 3). Moreover, the strong red-shifted band at about 1675 cm^{-1}, resulting from the hydrogen-bonded C=O stretching modes, also indicates the presence of the suggested

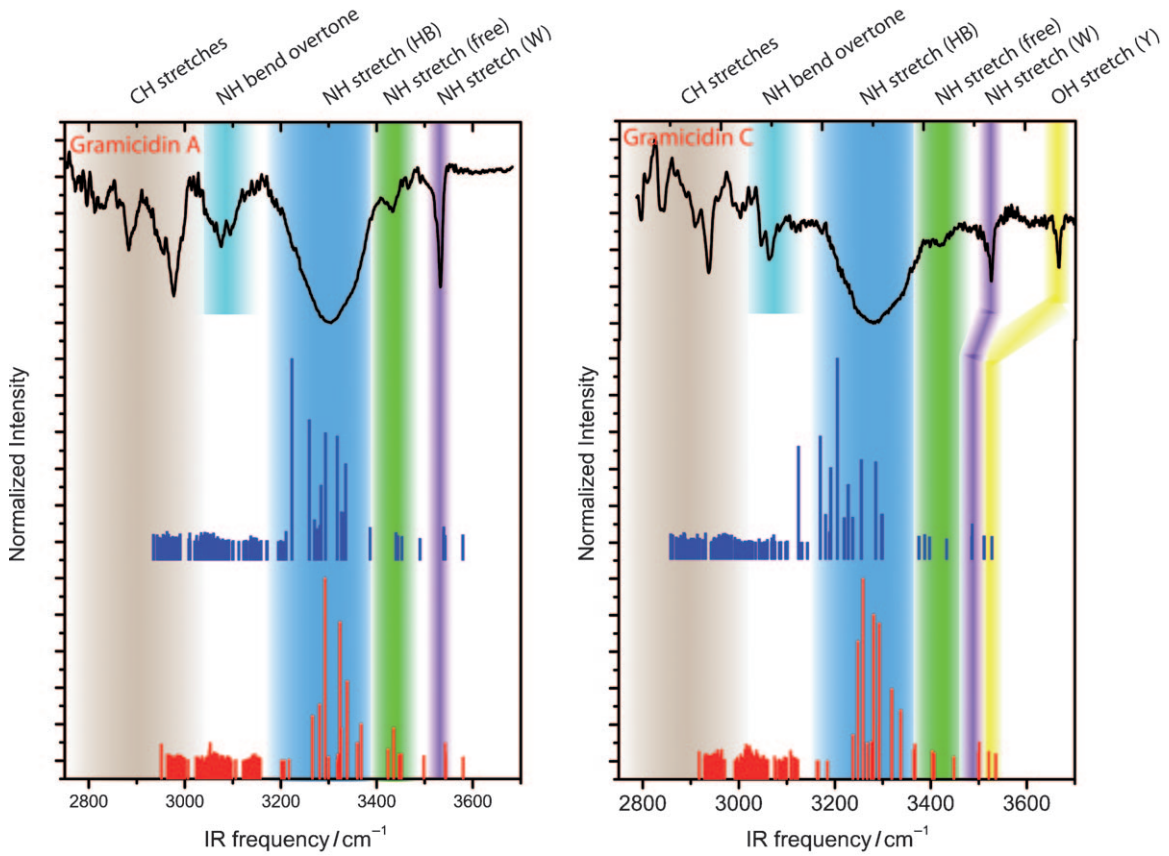

Figure 3. Top panel: IR spectra of gramicidin A (left) and C (right) obtained in the near-IR region. Lower/middle panel: The calculated stick spectra with (middle) and without (bottom) dispersion taken into account.

helical structure. All calculated structures confirm this helical structure.

2.2. IR Spectroscopy: The Influence of the Carrier Gas

To improve the structural resolution when studying large molecular systems such as the 15-residue peptide gramicidin, we recorded the IR-UV ion-dip spectrum of gramicidin C with different seed gases. Under standard conditions, we use argon to cool the desorbed molecules and to produce a molecular beam. However, when studying large peptides, the resolution of the IR spectra is reduced due to thermal broadening, which results from insufficient cooling. One likely reason is conformational broadening. Even if only one dominant low-energy conformation is present in the molecular beam, this may in fact consist of a family of conformations, very similar in both energy and global structure. A lower internal temperature may narrow the population distribution. Therefore, besides argon, we also compare results with krypton and xenon as seed gas.

Figure 4 shows the IR spectra of gramicidin C seeded in argon, krypton and xenon. It is likely that collisions with heavier noble gas atoms with the laser-desorbed gaseous sample molecules will lead to internally colder sample molecules. The IR spectra recorded in the 1400 to 1800 cm^{-1} region show the Amide I (C=O stretch) and Amide II bands (NH bend). The IR spectrum recorded with argon as a carrier gas roughly shows a double-peak structure for both bands, which arises from free and hydrogen-bonded C=O and NH groups respectively. This double-peak structure is more pronounced when using krypton and even more clear for xenon. However, the shape and position of the bands remain identical, implying that the same general family of conformations is present in all cases. The IR spectrum obtained with xenon as carrier gas shows a narrow peak at 1650 cm^{-1} with some structure at higher frequencies. Additionally, the double peak is partly resolved with maxima at 1520 and 1545 cm^{-1}. Gramicidin consists of 15 amino acid residues with at least fifteen NH bending and C=O stretching modes in the peptide backbone. Since each residue has a different peptide environment and undergoes different interactions, they all have a slightly different IR absorption frequencies, resulting in a very congested IR spectrum. Here, the resolution of the FELIX light source becomes the limiting factor. The individual IR absorption frequencies cannot be observed, but rather the envelope of peaks composed of similar types of C=O and NH vibrations, respectively. Still, with sufficient cooling, the hydrogen-bonded modes can be distinguished from the free vibrations for both the C=O stretching and the NH bending modes.

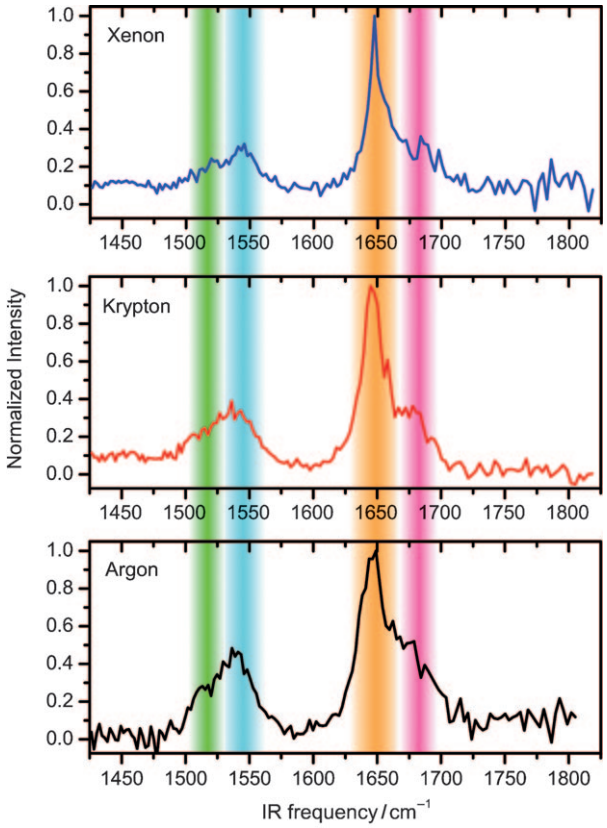

Figure 4. IR spectra of gramicidin C seeded in argon (bottom), krypton (middle) or xenon (top).

3. Conclusions

We have measured the IR absorption spectra of the 15-residue peptide gramicidin A and C by IR-UV ion-dip spectroscopy under isolated conditions. We have recorded these IR spectra in both the 1000–1800 cm^{-1} and the 2700-3750 cm^{-1} regions, which allows the structural analysis based on the NH/OH stretching modes, the C=O stretch vibrations, the NH bending vibrations, and the fingerprint region. This broad range makes it possible to probe different local interactions that together provide complementary information about the conformational structure of gramicidin A and C.

We have performed, for the first time, a computational analysis for a molecule of this size, including both structure optimization and the calculation of the vibrational frequencies. The comparison of the calculated frequencies with the experimental spectrum shows that a sophisticated computational approach, which includes the empirical dispersion correction term, is required to match the structure of gramicidin. The experimental spectra in combination with the calculated geometries and frequencies confirm the presence of a helical structure, whereby most of the peptide backbone amino groups are involved in stabilizing the helical structure, while the tryptophan and tyrosine groups point outwards.

The present study has demonstrated that we can indeed extend the gas-phase IR-UV ion-dip technique to characterize large peptides, allowing us to unravel their inherent structural properties in absence of interfering environmental elements. In future experiments, mass-selected clusters with water can be produced to determine the role of the solvent at the molecular level. To improve the spectral resolution when studying large, neutral molecular structures of the size of gramicidin, the use of heavier carrier gas can be beneficial.

Methods

Experimental Section: Gramicidin A and gramicidin C were obtained from Sigma–Aldrich and Fluka respectively. Both were used without further purification. Gramicidin consists of a mixture of four components that each differ in one amino acid residue, with gramicidin A the most abundant (about 80%) conformer. To obtain a better signal-to-noise ratio for gramicidin C, it was used in a purified form (Fluka).

The experimental setup has been described in detail elsewhere.[26] Briefly, a thin layer of sample is desorbed from a graphite sample bar, which is placed just in front of a pulsed nozzle.[27] The desorption laser, a Nd:YAG operating at 10 Hz, pulse duration of 10 ns, and about 2 mJ per pulse, is mildly focused to a spot of approximately 0.5 mm diameter within 1–2 mm in front of the nozzle orifice. The sample bar is translated in order to provide fresh sample to successive laser shots. The nozzle consists of a pulsed valve with a nozzle diameter of 0.5 mm and a backing pressure of 3 bar of inert seeding gas (argon, krypton or xenon). The neutral gramicidin molecules are skimmed with a diameter of 2 mm and then ionized by a frequency-doubled dye laser. We perform mass selected spectroscopy by two-photon ionization, detecting the ions in a reflectron-type time-of-flight mass spectrometer. The IR spectrum is obtained by using IR-UV ion-dip spectroscopy. The ions are constantly produced from ground-state molecules using a two-photon resonant ionization scheme. Preceding the excitation and ionization laser beam by about 500 ns, an IR laser interacts with the molecular beam. If the IR laser is resonant with a vibrational transition, population is transferred from the ground state in to a vibrationally excited state, thereby depleting the ground-state population. This depletion causes a dip in the number of produced ions. By measuring the ion yield of the mass of interest, while varying the IR wavelength, an IR ion-dip spectrum is obtained.

We recorded the IR absorption in the frequency range between 1000 and 1800 cm^{-1}, produced by the free -electron laser FELIX, showing the Amide I (C=O stretch), Amide II (NH bend) and the fingerprint region (below 1500 cm^{-1}). FELIX produces pulses with a pulse duration of about 5 μs, pulse energies of about 100 mJ, and a spectral line width of typically 0.5% of the IR frequency.[28] Both the molecular beam and the UV laser beam are running at 10 Hz, while FELIX is running at 5 Hz. In order to minimize signal fluctuations due to long-time drifts in the UV laser power or source conditions, a normalized ion-dip spectrum is obtained by recording separately the alternating IR-off and IR-on signals. Additionally, the IR spectra are corrected for the intensity variations of the IR power over the complete wavelength range.

To record the IR-UV ion dip spectra in the 2700–3750 cm^{-1} region, the same method was used as described above for the FELIX experiments. However, a YAG-pumped IR OPO/OPA (Laser Vision) was used operating at 10 Hz producing pulses with typical energies of about 12 mJ and a bandwidth of 4 cm^{-1}.

Computational Methods: The 2D NMR structure (PDB Code 1J03)[29] of gramicidin B was mutated in the position of the 11th amino

acid to form the structures of gramicidin A and C. These three structures were optimized at the DFT/SV(P) level of theory employing a TPSS functional[30] with and without the empirical correction dispersion term[31] using the Turbomole 6.0 program package.[32] The resolution of identity (RI-) approximation was used.[33] Harmonic vibration frequencies were evaluated numerically at the same basis set and level of theory. The frequencies were scaled by a universal factor of 0.988 to fit the experimental values in the mid-IR region.

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Keywords: density functional calculations · gramicidin · IR spectroscopy · protein folding · structural analysis

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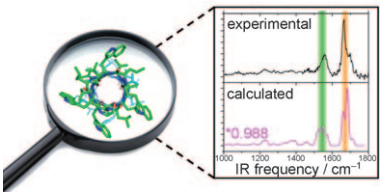
Through the (IR) looking glass: IR spectra of isolated neutral gramicidin are obtained in the mid and near-IR spectral range (see picture) and compared with high-level quantum chemical calculations. The gas-phase spectra reveal a helical structure, similar to that in the condensed phase.



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