RAMAN SPECTRA OF NYSTATIN
INFLUENCE OF IMPURITIES

ALINE COLLINE, JACQUES BOLARD, LAURENT CHINSKY, JIN-RUI FANG and KENNETH L. RINEHART, Jr.

Département de Recherches Physiques, Université Pierre et Marie Curie,
4 Place Jussieu, 75230 Paris Cedex 05, France

Institut Curie, Laboratoire de Physique et Chimie,
11 rue Pierre et Marie Curie, 75005 Paris, France

School of Chemical Sciences, University of Illinois at Urbana-Champaign,
Urbana, Illinois 61801, USA

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The Raman spectra of the polyene antibiotic nystatin have been investigated between 1500 and 1650 cm⁻¹. Only one band is observed in the resonance Raman spectra obtained with a 3045 Å excitation radiation. Two major bands are observed in the preresonance spectra of pure nystatin obtained with a 4545 Å excitation. A supplementary band at 1557 cm⁻¹ observed in the preresonance spectra of samples of commercial origin is ascribed to the presence of heptaene impurities, the Raman spectra of which are preferentially enhanced by resonance, owing to the closer proximity of their absorption to the excitation radiation.

Polyene macrolide antibiotics interact with cell membranes and promote more or less important selective alterations of the membrane permeability leading to cell death. The region of absorption of their polyene part, far from that of the lipid or protein molecules, as well as the large corresponding extinction coefficient make optical spectroscopies particularly appealing for the study of the interaction of these antibiotics with the membranes or their models. Among these methods, resonance or preresonance Raman scattering was used to study the interaction of amphotericin B and nystatin with phospholipid membrane models. The potential usefulness of this method requires, therefore, a good knowledge of the spectral characteristics of the antibiotics.

Nystatin is a tetraene and exhibits around 300 nm the vibronic absorption bands expected for a molecule containing 4 conjugated double bonds. The presence of supplementary weak bands around 400 nm, the intensity of which depends on the sample studied, may be attributed to the presence of the heptaene impurities commonly found in nystatin. In this report we show that the resonance effect in Raman scattering can preferentially enhance the spectra of these impurities and perturb the nystatin spectra, the absorption domain of the heptaene being much nearer to the exciting laser radiation wavelength (4545 Å) than that of nystatin.

Materials and Methods

Commercial nystatin samples were purified by chromatography according to Fang, employing a Waters HPLC system, an Altex Ultrasphere ODS column (5 µm, 10 mm ID × 25 cm), solvent: methanol-water, 70:30; 1.0 ml per minute flow rate and UV detection. Three main peaks observed consisted of nystatin A₃ (18.3 minutes), nystatin A₁ (13.5 minutes) and an unknown tetraene compound (5.1 minutes). When a Zorbox ODS column was employed (MeOH-H₂O, 70:30; flow rate of 5.0 ml/minute) and a 325-nm detector, the respective retention times were 30.0, 20.0 and 12.5 minutes. The components were identified by their FAB mass spectra, with M+H ions at m/z 1,056, 926 and 926, respectively.
tronic absorption spectra were recorded with a Cary 219 spectrophotometer and circular dichroism (CD) spectra with a Jobin-Yvon Mark III dichrograph.

Preresonance Raman spectra were recorded on a Coderg PH$_6$ double monochromator with EMI 95058 photomultiplier. The spectra were excited with the line 4545 Å of an argon ion laser (Spectra Physics 171). The laser power was approximately 190 mW. The Raman frequencies were calibrated to an accuracy of $\pm 1$ cm$^{-1}$ and the split width was 3 cm$^{-1}$. The experimental conditions were exactly the same as in ref 3. Resonance Raman spectra were recorded on a Jobin-Yvon Ramanor HG 2S under the conditions already described$^{(3)}$.

Results

Absorption and CD spectra in dimethyl sulfoxide (DMSO) in nystatin samples from various origins (sample 1, sample 2 and HPLC-purified samples) were recorded. It appeared that samples of identical absorption or CD in the 300-nm region, gave spectra of very different intensities in the 400-nm region although the maxima were located at the same wavelength, namely 417, 392 and 372 nm. The intensity was approximately divided by 10 when going from the commercially available products to the purified samples. Fig. 1 gives an example of such a variation. The absorption and CD spectra in Tris buffer pH 7.4 are concentration-dependent, at least for the 400-nm bands: when going from a $10^{-4}$ M concentration in commercial nystatin to a $10^{-5}$ M concentration, the bands are shifted from 420, 394 and 374 to

![Graph showing absorption and circular dichroism spectra](image-url)
Fig. 2. Raman spectra of nystatin in DMSO between 1650 and 1500 cm⁻¹.

I. Preresonance spectra of 10⁻¹ M nystatin obtained with the 4545 Å radiation of an Ar⁺ laser: upper curve commercial nystatin; lower curve, nystatin A₁ (obtained by HPLC).

II. Resonance spectra of 3 x 10⁻¹ M nystatin recorded with a 3045 Å excitation wavelength obtained by frequency doubling the 606 nm radiation provided by a Chromatix CMX4 tunable pulsed dye laser, using rhodamine 6G as a dye.

410, 385 and 366 nm. In the 300-nm region no change occurs. Such concentration dependence is not observed with the purified nystatin for which the bands are always observed at 410, 385 and 366 nm.

Preresonance Raman spectra (4545 Å excitation) of commercial nystatin and purified samples exhibit bands at 1557, 1607 and 1632 cm⁻¹ and weaker bands around 1100 cm⁻¹. The intensity of the 1557 cm⁻¹ band as compared to that of the other bands was highly variable according to the sample. In Fig. 2 (I) we give an example of such a variation and in Fig. 3 we have plotted the ratios of the Raman peak heights at 1607 cm⁻¹ and 1557 cm⁻¹ as a function of the ratios of the electronic absorption peak heights at 416 nm and 309 nm for the different samples studied.

Resonance Raman spectra (3045 Å excitation) of all the samples of nystatin studied exhibit only one band between 1500 and 1650 cm⁻¹ (Fig. 2 (II)).

Interpretation

In the previous nystatin Raman scattering study the weak absorption bands near 400 nm were considered as originating from a II→II⁺₁A₁→₁A₁⁺ dipole-forbidden transition to an excited state involving a doubly excited configuration.

However, the energy difference between the lowest excited electronic states (₁A₁⁺ and ₁B₁⁺) in unsubstituted linear octatetraenes is 4495 cm⁻¹ in methanol, or even less if a substituted tetraene is considered while in the observed spectra of nystatin the energy gap, in the same solvent, between the first of the strong bands (304 nm) and the first of the weak bands (405 nm) is 8200 cm⁻¹.

As on the other hand, the intensity of the weak bands depends on the origin of the sample, and their wavelength region is that of heptaenes, we think that the origin of the weak bands is to be found in heptaene impurities, the presence of which was already demonstrated. The expected ε in DMSO is therefore about 10⁴, from which it can be inferred that the impurities account for approximately 2% of the commercial products.

This would explain the concentration dependence of the nystatin absorption spectra in aqueous buffer: when going from 10⁻⁴ M to 10⁻⁵ M in commercial nystatin, namely 5 x 10⁻⁶ to 5 x 10⁻⁷ M in im-
purity, the weak bands are blue shifted while the location of the strong bands is invariant. As a matter of fact above $10^{-6}$ M the polyene antibiotics in aqueous solvents are generally in self-associated form while below they are present as monomers. Therefore, when going from $10^{-4}$ M to $10^{-5}$ M, nystatin remains in a self-associated form or a heptaene-tetraene associated form while this is not the case for the impurity. As for the purified nystatin, the amount of impurity is such that it is always in monomeric form and absorbs at 410, 385 and 366 nm.

From the absorption wavelength in 95% ethanol (408, 384 and 364 nm) some candidates may be proposed as representing the impurity: amphotericin B, candidin, perimycin and candimycin, the other heptaenes absorbing at different wavelengths. Amphotericin B may be eliminated because the corresponding intense excitonic doublet normally found in aqueous buffer is not observed here. Candidin may be the impurity because it is chemically closely related to nystatin and is susceptible to being co-produced with nystatin.

The nystatin Raman spectra are easily interpreted in terms of this impurity. In the resonance Raman spectra of the linear polyenes, two strong bands are observed: one corresponds to the C=C stretching near $1500 \sim 1600$ cm$^{-1}$ and another at $1150 \sim 1200$ cm$^{-1}$ results from the admixture of C=C and C=C stretching with C-H bending. The frequency of $\nu$ (C=C) depends on the number of double bonds and decreases as the chain becomes progressively longer. In contrast, the frequency of the bands around $1150 \sim 1200$ cm$^{-1}$ is invariant. From the relationship obtained from the plot of $\nu$ (C=C) against the number of double bonds, $\nu$ (C=C) is expected to be around $1600$ cm$^{-1}$ in tetraenes and $1550$ cm$^{-1}$ in heptaenes.

Indeed in the resonance Raman spectra (3045 Å excitation) we observe one band between 1500 and 1650 cm$^{-1}$ at 1610 cm$^{-1}$. On the other hand, in the preresonance condition in which the spectra are obtained, the Raman spectrum of the heptaene impurity is expected to be much more enhanced by resonance than that of nystatin, the wavelengths of absorption of the heptaenes being much nearer to that of the exciting radiation (4545 Å) than that of the tetraenes. For this reason, even if the heptaene represents only 2% of the commercial nystatin, it is possible to observe its spectrum superimposed on that of nystatin, and the band at $1557$ cm$^{-1}$ corresponds to $\nu$ (C=C) of the heptaene and the band at $1607$ cm$^{-1}$ to $\nu$ (C=C) of the tetraene nystatin. In purified nystatin the band at $1557$ cm$^{-1}$ is much less intense, as expected. The band observed at $1632$ cm$^{-1}$ has been tentatively assigned to $\nu$ (C=O) as in the case of amphotericin B. In conclusion it appears that the only difference between the Raman spectra of pure nystatin and that of amphotericin B is the expected shift of $\nu$ (C=C). No confirmation of the proposed existence of two nystatin conformers can be obtained from $\nu$ (C=O) consideration.

References

5) MECHLINSKI, W. & C. P. SCHAFFNER: Separation of polyene antifungal antibiotics by high speed liquid


