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NOTE

Revised ^{1}H and ^{13}C NMR Assignments of the Polyene Antibiotic Filipin III

Patrick Rochet and Jean-Marc Lancelin*
Laboratoire de RMN Biomoléculaire associé au CNRS, Université Claude Bernard – Lyon I and Ecole Supérieure de Chimie Physique et Electronique de Lyon, Bâtiment 308, F-69622 Villeurbanne, France

The major polyene antibiotic macrolide isolated from *Streptomyces filipinensis*, filipin III, was reinvestigated in DMSO solution using homonuclear and heteronuclear correlation spectroscopy. In addition to several corrections to previous ^{1}H NMR assignments, the nine exchangeable hydroxylic protons were structure-specifically assigned together with ^{13}C NMR lines using proton-detected HSQC spectroscopy. The magnitudes of the ^{3}J proton–proton couplings indicated a probable constrained geometry of the macrocyclic lactone. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Filipin III is the major polyene macrolide antibiotic isolated from *Streptomyces filipinensis*.[1] In contrast to nystatins, amphotericins and many other polyene macrolides, the important toxicity of filipins against animals cells never made them of practical use in the treatment of pathogenic fungal infections. A great affinity of filipin III towards cholesterol was proven early.[2] This remarkable property is at the origin of the use of filipin III as a histological and cholesterol-specific staining agent.[3] This property is the basis of the clinical diagnosis of Niemann–Pick C disease, an inherited metabolic disease manifested by an excess of lipoprotein-derived cholesterol.[4] This high affinity for cholesterol also makes filipin III an interesting model for studying polyene macrolide antibiotic–sterol interactions.

In 1993, Balakrishnan and Easwaran[5] published a conformational study of filipin III based on circular dichroism spectra and ^{1}H NMR in DMSO-d_6–methanol-d_4 (2:3, v/v). The absolute stereochemistry of filipin III was later and independently described based on chemical derivatizations of filipin III.[6] We have now reinvestigated the ^{1}H NMR spectra of filipin III in neat DMSO-d_6 in order to assign the nine well resolved and exchangeable resonances in the 4.4–5.4 ppm region. These signals were not recognized in the previous work[6] as exchangeable hydroxylic protons in slow exchange on the chemical shift NMR time-scale in DMSO-d_6. During the assignment work, we found several discrepancies in the ^{1}H assignments. We completed the assignment of ^{13}C nuclei using proton-detected ^{1}H–^{13}C correlation spectroscopy.

* *Correspondence to*: J.-M. Lancelin. E-mail: lancelin@ginko.cpe.fr.

EXPERIMENTAL

Filipin III was purchased from Sigma Chemical and used without further purification. Samples were dissolved under argon at 3–5 mM concentration (0.5–1 mg ml^{-1}) in DMSO-d_6 (99.8% D) (CEA-Eurisotope) and sealed in a 5 mm diameter NMR tube. This was found necessary since DMSO solutions of filipin III slowly degraded to products with broad resonances within a few hours upon exposure to air.

NMR spectra were recorded at 25 °C on a Bruker AMX spectrometer operating at 400 MHz for the proton frequency. Chemical shifts are quoted relative to the DMSO resonance fixed at 2.50 ppm for the proton and 39.5 ppm for the carbon. 2D spectra, DQF-COSY (double quantum filtered correlation spectroscopy),[7] TOCSY/HOHAHA (total correlation spectroscopy/homonuclear Hartmann–Hahn spectroscopy),[8,9] NOESY (nuclear Overhauser spectroscopy),[10,11] ROESY (rotating frame Overhauser spectroscopy)[12,13] and HSQC (heteronuclear single quantum coherence spectroscopy)[14] spectra were recorded in the phase-sensitive mode using the hypercomplex method.[15] The Waltz-17 mixing scheme used in TOCSY/HOHAHA experiments was optimized according to the technique known as clean TOCSY.[16] Two-dimensional homonuclear spectra were collected as a 512 (t_1) × 1024 (t_2) complex point time-domain matrix with a spectral width of 2800 Hz in both dimensions and 32 scans per t_1 increment. They were transformed after zero-filling in

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the F_1 dimension, into 1024 (F_2) × 1024 (F_1) real point frequency-domain spectra. HOHAHA spectra were recorded with a mixing time of 45 ms that includes the delays of the clean TOCSY pulse scheme. NOESY and ROESY spectra were recorded with a 300 ms mixing time. Two-dimensional heteronuclear spectra were collected as a 256 (t_1) × 1024 (t_2) complex point time-domain matrix with a spectral width of 2800 Hz in F_2 (^{1}H), 14000 Hz in F_1 (^{13}C) and 256 scans per t_1 increment. The ethylenic carbons were better resolved with an HSQC spectrum centred in F_1 (^{13}C) on the ethylenic region and a 1000 Hz spectral width. They were transformed, after zero-filling in the F_1 dimension, into 1024 (F_2) × 512 (F_1) real point frequency-domain spectra.

RESULTS AND DISCUSSION

Filipin III has the molecular structure shown in Fig. 1.[6] The general appearance of the ^{1}H spectrum, displayed in Fig. 2, is similar to those previously described by Balakrishnan and Easwaran.[5] However, the region 4.1–3.8 ppm integrates to eight protons instead of the seven described, and the resonance at 3.04 ppm integrates to one proton instead of two. The 5.2–4.4 ppm region includes ten resonances, among which nine are fully exchanged upon addition of heavy water.

The DQF-COSY spectrum allowed the unambiguous assignment of H-17. This ethylenic proton is the only one coupled with a methyl group through an allylic ^4J coupling, firmly detected as a cross peak between H-17

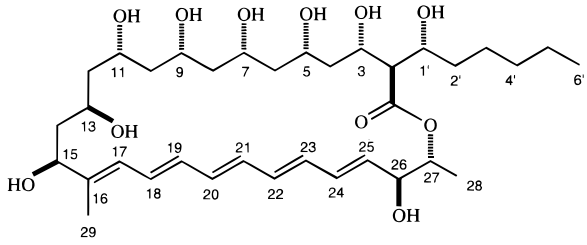

Figure 1. Molecular structure of filipin III.[6]

and 29-CH_3. The assignment of H-25 was also straightforward owing to the unique ethylenic proton (H-25) J-coupled to a CH(O) proton resonance (H-26) in the molecular structure. H-26 is itself coupled to an exchangeable hydroxylic resonance at δ 5.19 ppm. This allowed direct assignments of both H-27 and 28-CH_3. The assignments were extended within the ethylenic region only to H-18 and H-24 due to an extensive spectral overlap in this region at δ 6.30 ppm. The last methyl resonance was assigned to (6′)-CH_3, a triplet at δ 0.83 ppm, which is the most shielded resonance of the spectrum and also identifies the 5′-CH_2 resonance.

Figure 3 shows part of the DQF-COSY spectrum including cross peaks between hydroxylic resonances and CH(O) proton resonances in addition to the correlation between H-27 and H-26.

Another starting point in the assignment was given by the TOCSY correlation between 6′-CH_3 and H-1′ that extended weakly to H-2. This identified readily the coupled hydroxylic proton at the 1′-position and the diastereotopic (H-2′a) and (H-2′b) by COSY correlations from H-1′. In the same way, H-3 and 3-OH are

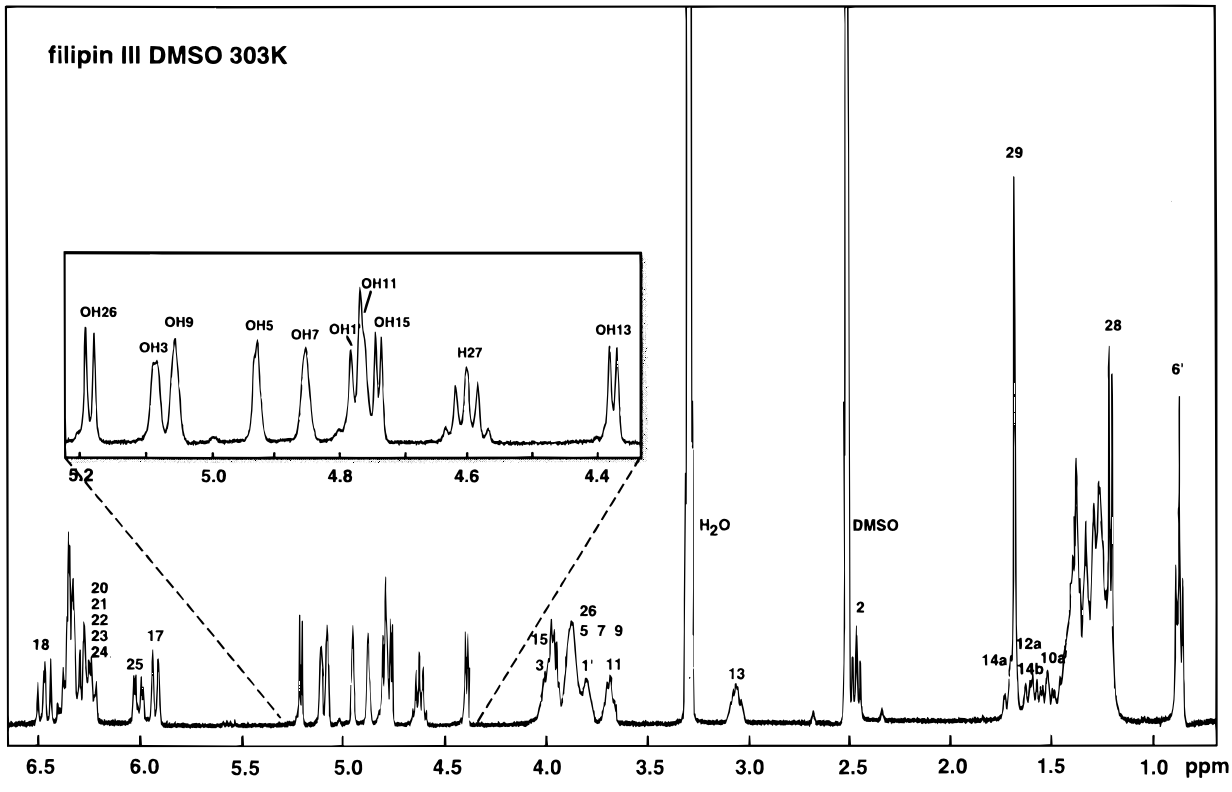

Figure 2. ^{1}H NMR spectrum of filipin III, 3 mM in DMSO-d_6, recorded at 400 MHz and 25 °C. The expanded region contains nine hydroxylic proton resonances that fully exchange with deuterium oxide and correspond to the nine hydroxyl groups of filipin III. No apodization functions were applied prior to the Fourier transformation.

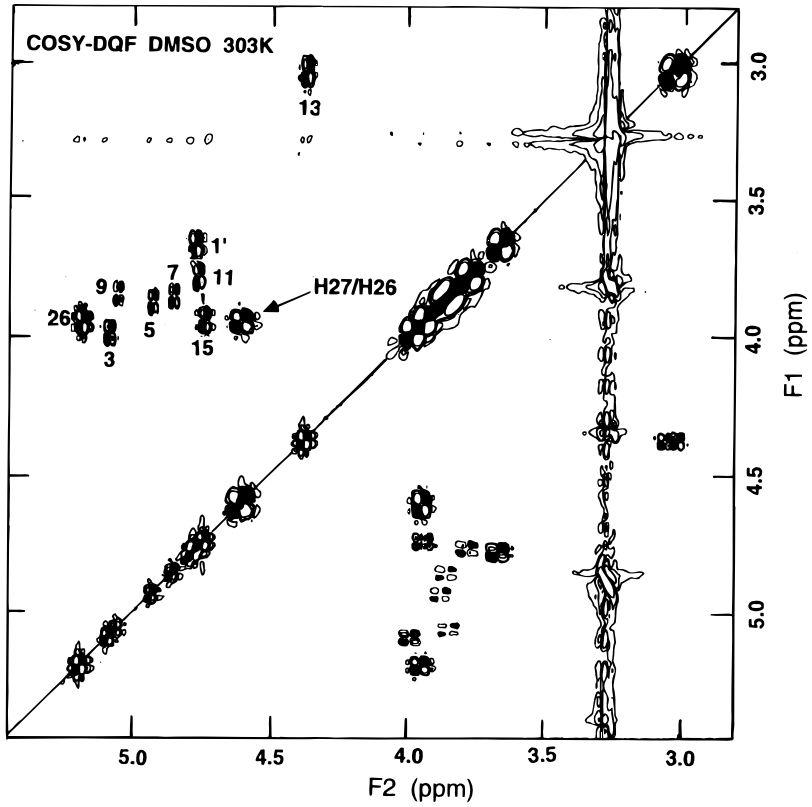

Figure 3. OH/CH(O) (F_2)–OH/CH(O) (F_1) region of the DQF-COSY spectrum of filipin III, 3 mM in DMSO-d_6, recorded at 400 MHz and 25 °C. Open and filled contours represent positive and negative contours, respectively.

readily assigned from H-2 using DQF-COSY. The assignment was further extended using the through-space coupling of H-3 to 5-OH by the ROESY experiment. The NOESY spectrum gave only very weak cross peaks, or no cross peaks at all, owing to an unfavourable molecular tumbling correlation time in DMSO solution. In the ROESY spectrum, 29-CH_3 showed a close proximity to two hydroxylic resonances (15- and 13-OH), giving an unambiguous assignment to H-15 or H-13 owing to their different coupling pattern with diastereotopic H-14_a, H-14_b, H-12_a and H-12_b. This gave also the assignment for H-11 and 11-OH. A ROESY connectivity between 11-OH and H-9 completed the assignment as shown in Table 1. Owing to the chemical shift degeneracy between the H-5 and H-7 protons, H-7/7-OH resonances were assigned by default. ^{13}C resonances were easily assigned from the proton-detected ^{13}C HSQC. The HSQC spectrum was particularly useful to assign the CH_2 protons in conjunction with the ROE effects found between OH protons and neighboring diastereotopic CH_2 protons. Figures 4 and 5 exemplify the ^{13}C HSQC spectra obtained.

This assignment affords a number of corrections to the ^1H assignments previously reported for filipin III.[5] Major differences are H-2 at δ 2.44 ppm assigned previously to H-14_a, H-13 at δ 3.04 ppm previously assigned to H-3 and H-9 and H-11 at δ 3.77 ppm previously assigned to H-15. The former assignment was obtained in DMSO-d_6–methanol-d_4 (2:3, v/v), a condition in which the hydroxylic protons were fully exchanged with deuterons. The resonances revised in this work are not affected with respect to chemical shift by the solvent mixture.[5] It is likely that the assignment of the ^1H NMR spectra without the help of hydroxylic resonances information led to numerous ambiguities.

It is noteworthy that both H-13 and 13-OH are the most shielded resonances of the CH(O) and hydroxylic protons. This correlates with the number of ROEs found for these two nuclei and the polyene part, suggesting that H-13 and 13-OH are shielded by the magnetic anisotropy induced by the conjugated polyene. Another interesting feature comes from the hydroxylic proton spin–spin coupling constants. These couplings are <3 Hz for every hydroxyl proton comprising a polyol-1,3 unit from positions 3 to 11, while those for hydroxylic protons at positions 13, 15, 26 and 1′ range from 6 to 4 Hz (see also Fig. 1). Such low $^3J_{HO,CH}$ coupling constants have already been observed for a *syn*-polyol-1,3 unit found in a molecular fragment of nystatin A1[17] in benzene-d_6. This can indicate a restricted torsional H—O—C—H angle of around 90°, favourable for a stabilizing hydrogen bond interaction within the polyol-1,3 groups, possible with the all-*syn* stereochemistry of filipin III.[18] Different conformations or conformational mixtures can be expected for the other hydroxyl groups in filipin III. Since these stabilizing interactions are not favoured in DMSO solution,[19] it is likely that the polyol-1,3 unit in the segment C-3 to C-11 in filipin III is maintained in an extended zig-zag conformation. This interaction is forced as in a model proposed previously for filipin III.[18] The rigidity of the linear conjugated-*trans* pentaene in the C-16 to C-25 structural segment of filipin III segment could be the origin of such a phenomenon.

Derivatization of approximate geometric constraints from J coupling and NOE/ROE are currently being

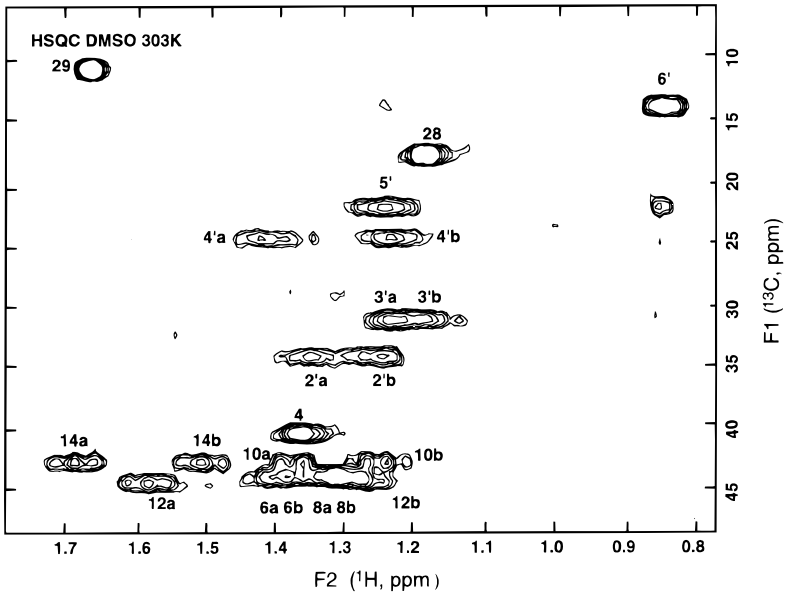

Figure 4. Aliphatic protons (F_2)–aliphatic carbons (F_1) region of the HSQC spectrum of filipin III, 4 mM in DMSO-d_6, recorded at 25 °C. Diastereotopic methylene protons are specified as a and b.

Table 1. Proton and carbon chemical shifts (ppm) of filipin III in DMSO-d_6 solution at 303 K[a]

Position	δ ^{1}H	δ ^{13}C
2	2.44	58.1
3	3.98, 5.08 (OH)	69.9
4	1.36, 1.36	40.0
5	3.87, 4.93 (OH)	69.4
6	1.30, 1.30	43.4
7	3.85, 5.85 (OH)	69.4
8	1.30, 1.38	43.4
9	3.84, 5.06 (OH)	69.9
10	1.26, 1.36	42.3
11	3.77, 4.76 (OH)	68.3
12	1.28, 1.58	44.3
13	3.04, 4.37 (OH)	64.7
14	1.49, 1.68	42.4
15	3.94, 4.74 (OH)	71.0
17	5.91	125.4
18	6.44	127.7
19	6.22	131.0
20	6.30	128.8*
21	6.30	131.0*
22	6.30	132.0*
23	6.30	132.7*
24	6.30	132.8*
25	5.98	134.8
26	3.94, 5.19 (OH)	73.0
27	4.60	72.6
28	1.18	17.0
29	1.67	10.0
1′	3.66, 4.77 (OH)	69.5
2′	1.25, 1.34	33.6
3′	1.18, 1.22	30.6
4′	1.24, 1.42	24.0
5′	1.23	21.3
6′	0.83	12.9

[a] Asterisked resonances can be interchanged. Quaternary C-1 and C-16 are not assigned.

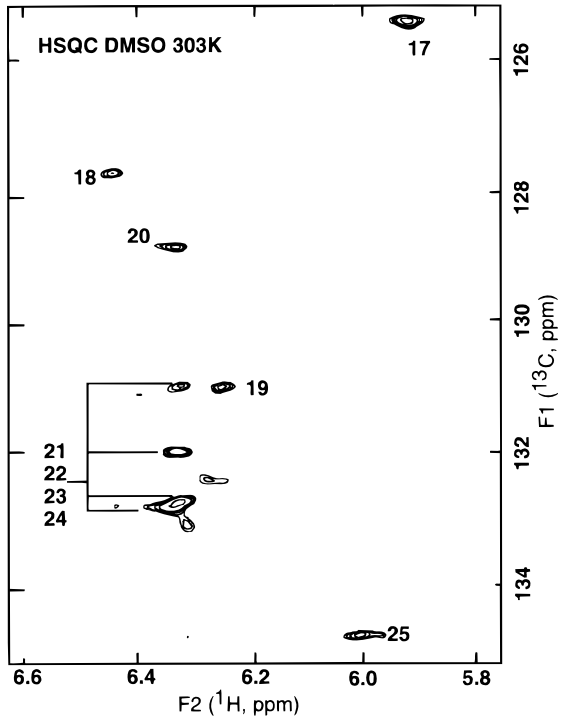

Figure 5. Ethylenic protons (F_2)–ethylenic carbons (F_1) region of the HSQC spectrum of filipin III, 4 mM in DMSO-d_6, recorded at 25 °C.

used to explore the conformational space for filipin III using XPLOR protocols.[20]

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