Solution NMR structures of the polyene macrolide antibiotic filipin III[1]

Laurent Volpon, Jean-Marc Lancelin*

Laboratoire de RMN Biomoléculaire associé au CNRS,
Université Claude Bernard–Lyon 1 et Ecole Supérieure de Chimie Physique et Electronique de Lyon, Bâtiment 308G, F-69622 Villeurbanne, France

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Abstract The solution structure of filipin III, an antifungal polyene macrolide biosynthesized by *Streptomyces filipinensis* and widely used for the detection and the quantitation of cholesterol in biomembranes, has been calculated with a set of geometrical restraints derived from ^{1}H NMR in DMSO-d_6 at 25°C. Filipin III appears as a rod-shaped molecule of 18 Å length. Its amphiphilic structure is made of an all-*syn* 1,3-polyol motif, stabilized by intramolecular hydrogen bonds on one side, and a conjugated pentaene moiety on the other side of the molecule. The overall shape is comparable to cholesterol, and the molecular structure of filipin III affords a first molecular basis to the comprehensive understanding of the interactions possible in the filipin III–cholesterol complex which is still unknown at the atomic resolution.

Key words: Antifungal antibiotic; Filipin–cholesterol complex; NMR spectroscopy; Polyene macrolide; Solution structure

1. Introduction

The polyene macrolide antibiotic filipin III has been isolated in 1955 from *Streptomyces filipinensis* cultured from samples of Philippine soil [1]. Its covalent structure (Fig. 1) was first established by a series of chemical degradation studies as a 28-membered lactone ring equipped with a 1,3-polyol motif (eight hydroxyl groups) and a conjugated pentaene [2]. The too strong affinity of the filipin III for cholesterol has disqualified this polyene for antifungal therapies in mammals while other polyene macrolides as nystatin A1 and amphotericin B are classic molecules used in such therapies [3]. This strong affinity for cholesterol rendered however filipin III a useful tool for probing and quantifying histologically the presence of cholesterol in cell membranes [4]. The fluorescent filipin III–cholesterol complex is consequently used in the clinical diagnosis of the type C of the Niemann–Pick inherited disease, for which a resulting deficit of cholesterol in the cytoplasmic membrane is detected [5–7]. To date, no definite structural basis characterizing the polyene macrolide affinities with sterols is available, mainly because such complexes are soluble at a too low concentration (ca. 1 μM) for the NMR analysis in aqueous solution.

Due to the lack of radio-crystallography data, filipin III absolute configuration has been established only recently by means of chemical derivations of the native structure as 1′*R*, 2*R*, 3*S*, 5*S*, 7*S*, 9*R*, 11*R*, 13*R*, 15*S*, 26*S* and 27*R* as shown in Fig. 1 [8]. Based on this assignment, the first total stereoselective synthesis has been published past year by Richardson and Rychnovsky [9,10]. A conformational study of filipin III was also reported recently based on NMR data collected in a mixture of DMSO-d_6 and MeOH-d_4 (2:3, v/v) [11], with number of erroneous structural and spectral assignments [12]. We report herein the first solution structure of a polyene macrolide, the filipin III, based on the revised and complete ^{1}H-^{13}C NMR assignments in DMSO-d_6 [12]. We used the magnitude of ^3J spin–spin coupling constants and ROE cross peaks to derive a set of approximate geometric structural restraints. This restraint set was used to explore the conformational space accessible to filipin III by protocols homologous to those used for biopolymers as proteins and nucleic acids [13,14].

2. Materials and methods

NMR spectra have been collected at 25°C for a 3 mM solution of filipin III (Sigma) in 99.9% DMSO-d_6 (CEA Eurisotope) as previously described [12]. All the interproton distance restraints, derived from a two-dimensional homonuclear ROESY experiment recorded with a 250 ms spin-lock mixing time [12], were classified into three categories depending on their volume integration values using GIFA 4.2 software [15]. Upper limit bounds were fixed at 2.7, 3.3 and 3.9 Å for strong, medium and weak correlations, respectively. The possibility of spin diffusion precludes higher precision in the derivation of distance limits. The intensities of the H17-H19 ROE was considered as a reference intensity for strong correlations. Pseudo-atom corrections of the upper bounds were applied for distance restraints involving the unresolved methylene and methyl protons (+1 Å) [16]. For non-stereospecifically assigned but spectroscopically resolved diastereotopic methylene protons, the interproton distances were treated as single $(\langle r^{-6}\rangle)^{-1/6}$ average distances. Dihedral angle restraints were deduced from the magnitude of $^3J_{H,H}$ coupling constants previously reported [12].

Structures were calculated using the X-PLOR software version 3.851 [17]. Initial atomic coordinates and parameter structure file for filipin III were generated step by step (given as supporting information) from the X-PLOR libraries and topology files of different parts of other molecules, such as daunomycin or rapamycin, collected from the Protein Data Bank (http://www.rcsb.org/pdb/). The result was visualized using the program MOLMOL version 2.4 [18]. For the widest possible sampling of the conformational space, we used a protocol that starts from fully randomized atomic coordinates as starting points for a simulated annealing algorithm (random SA) under experimental NMR restraints as described previously [13,19,20]. A simplified molecular force field (the *allhdg.pro* force field of X-PLOR) was used. In particular, the non-bonded van der Waals

*Corresponding author. Fax: (33)-4 72 43 13 95.
E-mail: lancelin@hikari.cpe.fr

[1] Web site available at http://sakura.cpe.fr

Abbreviations: DMSO, dimethyl sulfoxide; CD, circular dichroism; MeOH, methanol; ROESY, rotating frame NOESY; DQF-COSY, double quantum filtered correlation spectroscopy; rmsd, root mean square deviation

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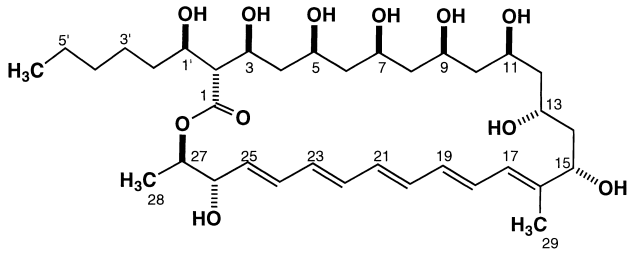

Fig. 1. The molecular structure and absolute configuration of filipin III [6].

interactions were represented by a simple repulsive quadratic potential. The experimental distance restraints were represented as a soft asymptotic potential beyond the upper limit defined, and electrostatic interactions were ignored. The force constant associated with the distance restraints was kept at 209 kJ mol^{-1} Å^{-2} throughout the protocol. One cycle of random SA consisted of 1500 steps of 3 fs at 1000 K followed by 3000 cooling steps of 1 fs from 1000 K to 100 K. At the end each structure was subjected to 1500 steps of conjugate gradient energy minimization.

DQF-COSY were calculated back using the Bruker NMRSim software version 2.7.1 with the same digital resolution as the experimental spectra [12]. The ^3J coupling constants were determined from the average of the coupling constants calculated for each torsion angle of the final structures with the optimized Karplus-type empirical functions [21].

3. Results and discussion

Thirty eight unambiguous distance and 14 dihedral angle restraints (Table 1), derived respectively from ROESY experiments and the magnitude of ^3J spin–spin coupling constants, were used for the structure calculations. From the 50 structures calculated, 29 structures with a total potential energy below a threshold of 13 kJ mol^{-1} were retained due to their double satisfaction of NMR geometric restraints and the back-calculated patterns of the cross peaks in the DQF-COSY spectrum (given as supporting information on the web edition of this article). The filipin III models have no ROE violations exceeding 0.1 Å and no dihedral angle violations exceeding 5°. The structures are almost represented as a single conformation of the macrocycle and superpose to the geometric average using the heavy atoms (C and O) with root mean square atomic deviation (rmsd) of 0.37 Å excluding the C1′-C6′ part (Fig. 2A). Structural statistics of filipin III structures are given in Table 2. The H–C–O–H torsions in the C1′-

Table 1
Angle and interproton distance restraints deduced from spin–spin $^3J_{H,H}$ coupling constants and ROE cross peak volume integrations for filipin III measured at $^1H = 400$ MHz in DMSO-d_6 and 25°C [9]

Position	Angle restraint[a]	ROEs[a]
1′	H2 (180 ± 40°)	–
H°1′	–	H2′^{M}
2	H3 (180 ± 40°)	H2′^{W}
3	H°3 (+90 ± 30°)	H3′^{W}, H°5^M
H°3	–	H4^W, H5^W
4	–	H°5^M
5	H°5 (+90 ± 20°)	H°7^M
H°5	–	H6^W, H7^W
6	–	H°7^M
7	H°7 (+90 ± 20°)	–
	H6a (+180 ± 60°)	
H°7	–	H8^W
8	–	H°9^M
9	H°9 (+90 ± 20°)	H°11^M
	H8a (+180 ± 60°)	
H°9	–	H10^W, H11^W
10	–	H°11^M, H13^W, H°13^W
11	H°11 (+90 ± 30°)	H13^W, H°13^W
	H12a (+180 ± 30°)	
	H10a (+180 ± 60°)	
H°11	–	h12^W, H13^W
12	–	H°13^W, H15^W
13	H12b (+180 ± 30°)	H14^W
	H14$^b_{eq}$ (+180 ± 30°)	
H°13	–	H14^W
14	–	H°15^M
15	H14$^b_{ax}$ (+180 ± 30°)	H17^S, H19^W
H°15	–	H17^M
24	–	H26^M, H°26^W
25	–	H°26^M, H27^M
26	–	27CH$^S_3$
H°26	–	H27^S, 27CH$^S_3$

[a]The J coupling and ROE partners are listed only once according to the proton having the lower number. Intensities of the ROEs are strong (^{S}), medium (^{M}) or weak (^{W}).
[b]Refers to the pseudo-axial (ax) or pseudo-equatorial (eq) orientation of these protons relative to the average plane of the macrocycle.

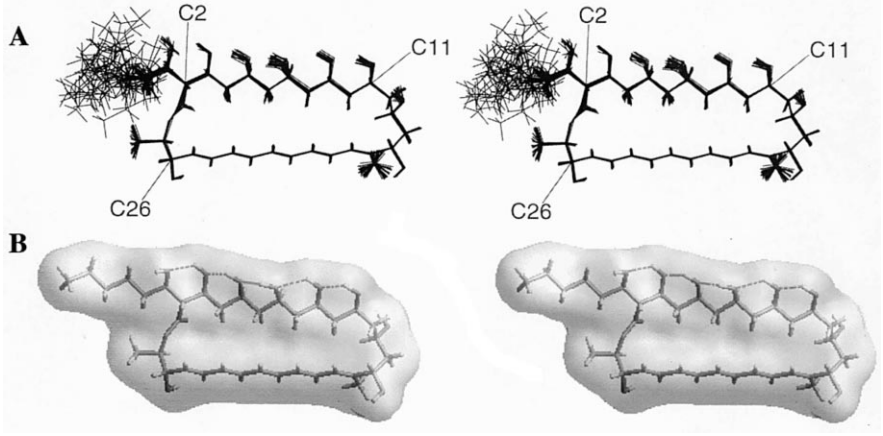

Fig. 2. Stereoviews of (A) 29 NMR structures of filipin III superposed for a minimum root mean square deviation of C and O atoms of C2-C27 segment. The C atoms are labeled according to their structural numbering indicated in Fig. 1. B: Stick representation and accessible surface of the closest structure to the geometric average of the NMR ensemble shown in A of filipin III. Dashed lines represent intramolecular hydrogen bonds between hydroxyl groups.

Table 2
Structural statistics of filipin III

Cartesian coordinate rmsd (Å) vs. the average geometric structure[a]		
All atoms		0.83 (±0.38)
Excluded the C1′-C6′ segment	all atoms	0.37 (±0.17)
	Heavy atoms	0.16 (±0.08)
Potential energies[b] in kJ mol^{-1} calculated from X-PLOR, *allhdg.pro*		
F_{total}	11.64 (±1.42)	
F_{bond}	0.43 (±0.12)	(0.98×10^{-3})
F_{angle}	7.26 (±0.71)	(0.25)
F_{impr}	2.26 (±0.12)	(0.29)
F_{repel}	0.06 (±0.10)	
F_{noe}	1.61 (±0.36)	(13.96×10^{-3})
F_{cdih}	0.01 (±0.03)	(39.56×10^{-3})
NOE and dihedral violations (average number per structure).		
NOE violations > 0.1 Å	0	
dihedral violations > 5°	0	

[a]Root mean square deviations (rmsd) are calculated for heavy atoms (C, O) of the specified part of the molecule.
[b]F_{bond} is the bond length deviation energy; F_{angle} is the valence angle deviation energy; F_{impr} is the deviation energy for the improper angles used to maintain the planarity of certain groups of atoms; F_{repel} is the repulsive term of the van der Waals energy function; F_{noe} is the experimental NOE function, and F_{cdih} is the experimental function corresponding to the violation of the dihedral angle restraints. In bracket are given the rmsd for certain energetic terms (in Å for F_{bond}, F_{noe} and in degree for F_{angle}, F_{impr}, F_{cdih}).

C11 segment were restrained to +90 ± 20° (Table 1) due to the weak $^3J_{HO,CH}$ observed (< 3 Hz) [12]. H–C–O–H torsions restrained to −90 ± 20° led to structures conflicting with other restraints derived from ROE and the back-calculated DQF-COSY patterns.

The hydroxyl hydrogens are hydrogen bonded to the neighboring 1,3-hydroxyl oxygen from C11 to C1′ (Fig. 2B), though electrostatic and hydrogen bond energetic terms are not taken into account in our calculation protocols (see Section 2). The same arrangement of hydrogen bonds stabilizing *syn* 1,3-polyol motifs has been already observed for a solution in benzene-d_6 of an acyclic C1-C10 polyol fragment of nystatin A1 [22]. *Syn* 1,3-polyols are known to be stabilized in the *trans* carbon chain conformations by intramolecular hydrogen bonds between adjacent 1,3-diol motifs in apolar media [23]. However, these stabilizing intramolecular hydrogen bonds can be strongly competed by interactions between the hydroxyls and polar solvent molecules in polar media, leading to a mixture of *trans* and *gauche* carbon chains [24]. For filipin III, it could be anticipated that the rigidity and the all-*E* C16-C25 conjugated pentaene is an important feature for the macrocycle restricted conformation. This restricted conformation could participate in preserving the C1′-C11 1,3-polyol moiety in an almost all-*trans* conformation with 1,3-hydrogen bonds despite the presence of a polar solvent (DMSO-d_6). Such all-*trans* 1,3-polyol conformation was also characterized for vacidin A and rimocidin polyene macrolides using NMR [25,26].

Filipin III appears in its solution NMR structures, as a rod-shaped molecule of 18 Å length (from H14 to H28, Figs. 1 and 2) flanked by a linear ridge of hydroxyls interacting in a hydrogen bond network from C1′ to C11 (Fig. 2). Cholesterol has a length of about 11 Å for the tetracyclic rigid moiety extended at ca. 18 Å to the end of its lateral aliphatic chain. It is known that exposure of sterol-containing biomembranes to filipin III, causes a filipin-induced extraction of sterols at the origin of a global disorganization of the membrane [27]. The structural features of filipin III and cholesterol lead to imagine possible specific interactions of filipin III and cholesterol that could involve probably hydrophobic interactions along their long axis, completed by a possible hydrogen bond involving the cholesterol hydroxyl and a polar group located at one end of filipin III. Such a hydrogen bond with cholesterol, or in general with sterols, has been demonstrated recently [13] of a crucial importance for bacillomycin *L*, a member of lipidic cyclo-octapeptides biosynthesized by *Bacillus subtilis*. In this family [13], a conserved D-Tyr2 is thought to interact specifically with sterols both by hydrophobic interactions and a specific hydrogen bond involving the D-Tyr2 hydroxyl as the hydrogen donor to the cholesterol hydroxyl as the acceptor of the specific hydrogen bond. The molecular structure and solution conformation is the first structural basis available for a future characterization of the exact nature of the filipin–cholesterol complex at atomic resolution.

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