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NMR structure of antibiotics plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A_2

Laurent Volpon[a], Françoise Besson[b], Jean-Marc Lancelin[a,*]

[a] *Laboratoire de RMN Biomoléculaire associé au CNRS, Université Claude Bernard, Lyon 1 and Ecole Supérieure de Chimie Physique et Electronique de Lyon, Bâtiment 308G, F-69622 Villeurbanne, France*
[b] *Laboratoire de Physico-Chimie Biologique associé au CNRS, Université Claude Bernard, Lyon 1, Bâtiment 303, F-69622 Villeurbanne, France*

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Abstract Plipastatins A and B are antifungal antibiotics belonging to a family of lipopeptides capable of inhibiting phospholipase A_2 (PLA_2) and are biosynthesised under certain circumstances by *Bacillus subtilis*. U-^{15}N plipastatins A and B were obtained from cultures of the strain NCIB 8872 on a Landy medium modified for stable-isotope labelling by the substitution of the L-glutamic acid used as the sole nitrogen source, by $^{15}NH_4Cl$. These two lipo-decapeptides, lactonised by esterification of the Ile10 C-terminus with the phenolic hydroxyl of Tyr3, differ only by a D-Ala (plipastatin A)/D-Val (plipastatin B) substitution at the position 6. The ^{1}H- and ^{15}N-nuclear magnetic resonance (NMR) signals of a 4:6 mixture of plipastatins A and B were unambiguously assigned and their structures in dimethylsulfoxide solution were calculated on the basis of a set of NMR-derived restraints. Plipastatins A and B are well-defined structures in solution stabilised by a type 1 β-turn comprising residues 6–9 and several other specific hydrogen bonds. The structures afford a first molecular basis for the future studies of their biological activities both in lipidic layers or on PLA_2.

Key words: Antifungal antibiotic; Plipastatin; Nuclear magnetic resonance spectroscopy; Phospholipase A_2 inhibitor; Solution structure; *Bacillus subtilis*

1. Introduction

Bacillus subtilis strains produce different secondary metabolites in the form of lipopeptides that have specific activities against fungi [1], bacteria [2], erythrocytes [3] and different yeasts [4]. As a consequence, these metabolites are of a high value for biotechnological and pharmaceutical applications. In addition to surfactins, a powerful amphiphilic and surfactant lipopeptide [5], each of these strains specifically produce only one member of the iturinic family comprising bacillomycins, iturins and mycosubtilin (for a review, see [6]) (Fig. 1). However, it was observed, by modifying the culture conditions (nitrogen source, pH), that this bacteria may also produce other compounds as fengycins [7] or plipastatins [8]. These features suggest a high degree of adaptability, by modulation of the genetic expression in the region of the *B. subtilis* genome involved in the synthesis of these two families of compounds [9].

All these antibiotics are either cyclopeptides (iturinics) or macrolactones (surfactins, fengycins and plipastatins) characterised by the presence of L and D amino acids and variable hydrophobic tails [10] (Fig. 1). Iturinics share a common mechanism of action by inserting into the cytoplasmic membrane by their hydrophobic tail and auto-aggregate to form a pore which causes the cellular leakage [11]. The plipastatins, which were isolated from *B. subtilis* [12,13] and *Bacillus cereus* [8], are very similar to fengycins. Their antimicrobial spectrum remains mainly unknown but it was shown to inhibit the phospholipase A_2 (PLA_2) [8], an enzyme involved in a number of physiologically important cellular processes such as inflammation, acute hypersensitivity and blood platelet aggregation [14,15]. As for the other lipopeptides produced by *B. subtilis*, plipastatins are biosynthesised as a mixture of isoforms characterised by variations in both the nature of the hydrophobic tail and the amino acid composition [16]. The hydrophobic tail is a 3(*R*)-hydroxy hexadecanoic acid (plipastatins A1 and B1) or a 14(*S*)-methyl-3(*R*)-hydroxy hexadecanoic acid (plipastatins A2 and B2) while the amino acid sequence differ in position 6 with a D-Ala (plipastatin A1 and A2) substituted by a D-Val (plipastatin B1 at B2). Surprisingly, while attempting to adapt a specific medium of culture for *B. subtilis* NCIB 8872 by substitution of the nitrogen source (L-Glu → $^{15}NH_4Cl$) for isotope labelling of bacillomycin *L* [17], we have obtained two isoforms of plipastatins, plipastatin A and plipastatin B instead of bacillomycin *L* which has not been detected anymore [18]. We report in this paper the detailed nuclear magnetic resonance (NMR) structures of both U-^{15}N plipastatins A and B in dimethylsulfoxide (DMSO) solution obtained after two major revisions of the previously reported ^{1}H-NMR assignment of plipastatin A [19].

2. Materials and methods

2.1. Antibiotic production

The *B. subtilis* strain NCIB 8872 was obtained from Dr. J.B. Barr, Royal Victorial Hospital, Belfast, Northern Ireland. *B. subtilis* was grown at 35°C for 150 h in a Landy medium [20], modified for the nitrogen source, containing 20 g of D-glucose, 1.82 g of $^{15}NH_4Cl$ (Isotec Inc.; instead of glutamic acid), 0.5 g of $MgSO_4$, 0.5 g of

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

Abbreviations: DMSO, dimethylsulfoxide; HSQC, heteronuclear single quantum coherence; PLA_2, phospholipase A_2; SA, simulated annealing; rmsd, root mean square deviation

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KCl, 6.8 g of KH_2PO_4, 1.2 mg of $Fe_2(SO_4)_3$, 0.4 mg of $MnSO_4$, and 1.6 mg of $CuSO_4$ per liter. The pH was adjusted to 7.7 with 10 N NaOH before autoclaving (the solution of D-glucose was sterilised separately) [21,22]. The lipopeptides were then isolated and purified as reported before for bacillomycin *L* [17]. We finally obtained 25 mg of U-^{15}N plipastatins (A and B in a 4:6 ratio determined by NMR, see below) for 1 l of culture from the chromatographic fractions corresponding to the R_F of bacillomycin *L*.

2.2. NMR spectroscopy

This amount was dissolved under dry argon in 550 μl (ca 3.6 mM total concentration) of DMSO-d_6 (CEA-Eurisotop, the solubility in water is too low). NMR spectra were recorded at 22°C on a Bruker *Avance* DRX 500 spectrometer using a 5 mm (^{1}H, ^{13}C, ^{15}N) triple-resonance probe head equipped with a supplementary self shielded *z*-gradient coil. Spectra were processed using Bruker XWINNMR or GIFA V.4 [23] software. Double quantum filtered correlation spectroscopy [24], total correlation spectroscopy (TOCSY) (Hartmann–Hann spectroscopy) [25,26] and nuclear Overhauser enhancement spectroscopy (NOESY) [27,28] experiments were recorded with a 1.5 s recovery delay in the phase-sensitive mode using the States-TPPI method [29] as data matrices of 512 real (t_1)×1024 (t_2) complex data points with accumulation of 32 scans per t_1 increment. Mixing times of 80 ms were used for TOCSY and 350 ms for the NOESY spectra. The spectral width in both dimensions was 5734 Hz. The data were apodised with shifted sine-bell and Gaussian window functions in both F_1 and F_2 dimensions after zero-filling in the t_1 dimension to obtain a final matrix of 1024 (F_1) real×1024 (F_2) complex data points. Proton chemical shifts were referenced to the solvent chemical shift ($\delta(^1H) = 2.49$ ppm). Phase-sensitive ^{15}N-heteronuclear single quantum coherence (HSQC) [30] were recorded with a 1.5 s recovery delay using the echo–antiecho method [31]. The coherence pathway selection was achieved by applying pulsed-field gradients as coherence-filters [32,33]. The FID was collected as a data matrix of 512 (t_1, ^{15}N)×1024 (t_2, ^{1}H) complex data points accumulations and 32 scans per t_1 increment. Spectral widths were 3005 Hz in F_2 and 1773 Hz in F_1 with carrier frequencies at 8.5 and 117.0 ppm, respectively.

2.3. Structure calculations

Interproton-distance restraints were derived from two-dimensional homonuclear NOESY experiments and classified into three categories. Upper bounds were fixed at 2.7, 3.3 and 5.0 Å for strong, medium and weak correlations, respectively. 0.3 Å were added to NOEs involving amide protons. The intensity of the NOE between the two Hε(NH_2) of Gln8 was considered as a reference intensity for strong corrections. Pseudo atom correlations [34] of the upper bounds were applied for distance restraints involving the aromatic protons of Tyr (+2 Å) and unresolved methyl or methylene protons (+1 Å). For non-stereospecifically assigned but spectroscopically-resolved diastereotopic protons, the interproton distances were treated as single $(\langle r^{-6}\rangle)^{-1/6}$ average distances. Dihedral angle restraints of χ_1 were deduced from the stereospecific assignments of diastereotopic β-protons (±40° from the ideal staggered conformation) [35,36].

Structures were calculated as previously described [17] using the X-PLOR software version 3.851 [37]. The non-standard (hydrophobic tail and Orn2) and modified residues (*Allo*–Thr4 and ester bond between Tyr3 and Ile10) were constructed from the X-PLOR libraries (given as supplementary material). The ester bond between the residues Tyr3 and Ile10 was maintained plane by definition of suitable improper-angle restraints. Starting from fully randomised coordinates, the sampling of the conformational space was performed following a simulated annealing (SA) protocol (random SA) proposed by Nilges et al. [38]. For this calculation, the *allhdg.pro* force field of X-PLOR was used. In a second stage of the calculation, random SA structures with good experimental and geometric energies were further refined using the full CHARMM22 force-field of X-PLOR. An approximate solvent electrostatic screening effect was introduced by using a distance-dependent dielectric constant and by reducing the electric charges of the formally charged amino acid side chains (Glu1, Glu5 and Orn2) to 20% of their nominal charges defined in the CHARMM22 force field. The structures were statistically analysed using X-PLOR routines and the MOLMOL program [39].

3. Results and discussion

3.1. Resonance assignments of the two plipastatin isoforms

The distinction between the two lipopeptides fengycin and plipastatin, which differ only in the stereochemistry of the Tyr residues (L to D diastereoisomers, Fig. 1), is possible only by comparison of their respective ^{1}H chemical shifts in DMSO that were already reported [7,19]. On this basis, plipastatins A and B were unambiguously identified and their spin systems were easily assigned [34] (Table 1). The greatest chemical shift difference between the two isoforms concerns the Hα of the residue 6 (Ala or Val). The assignment of Pro7 was straightforward with strong $d_{\beta\delta}$ and no $d_{\alpha\alpha}$ between Ala6/Val6 and Pro7, indicating that the peptide bond is in a *trans* conformation. Two important discrepancies appeared relative to the previous spectral assignments of plipastatin A1 [19]. In this work, the two broad resonances at δ = 10.47 and 10.67 ppm were assigned to the two COOH protons of Glu1 and Glu5. The HSQC ^{15}N spectrum (Fig. 2) revealed two ^{1}H–^{15}N cross-peaks for the same ^{1}H chemical shifts that precluded any COOH proton assignments for these two resonances. We used the unique Ala6/Val6 and Ile10 spin systems as starting points for the sequential assignments of the two isoforms. The sequence-specific assignment proceeded unambiguously and the two broad resonances at δ = 10.51 and 10.72 ppm corresponds to H^N protons of Orn2 and Glu5, respectively. Due to the broad resonance of Orn2 and Glu5 H^N, only weak

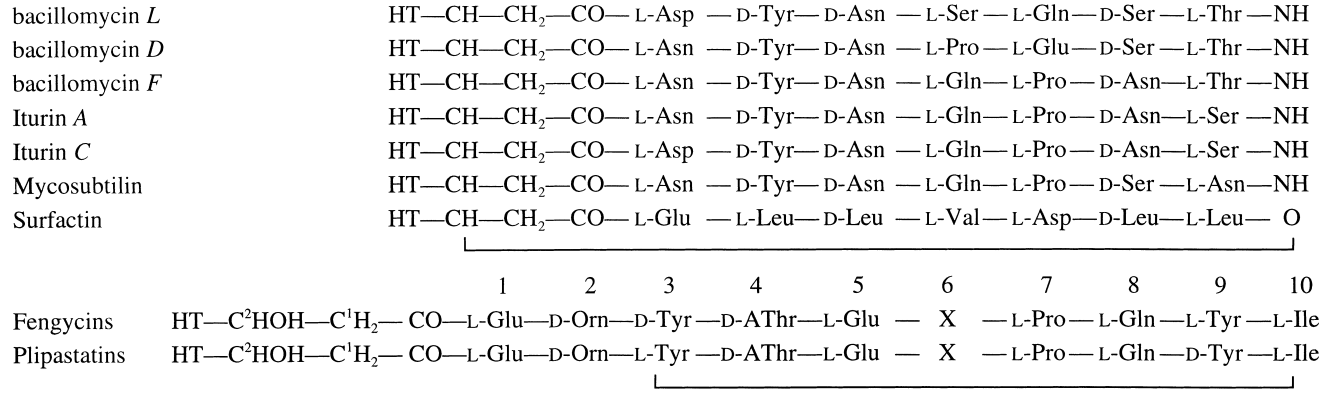

Fig. 1. Peptide sequences of the major antibiotics produced by *B. subtilis*. The hydrophobic tail (HT), is constituted of 11 C to 13 C with *normal*, *iso* or *anteiso* termini. Orn, ornithine; AThr, *Allo*-Threonine. For fengycins and plipastatins only, the carboxyl group of the Ile10 C-terminus is lactonised with the phenolic hydroxyl of Tyr3 to form a macrolactone ring. X = D-Ala and D-Val for isoforms A and B, respectively.

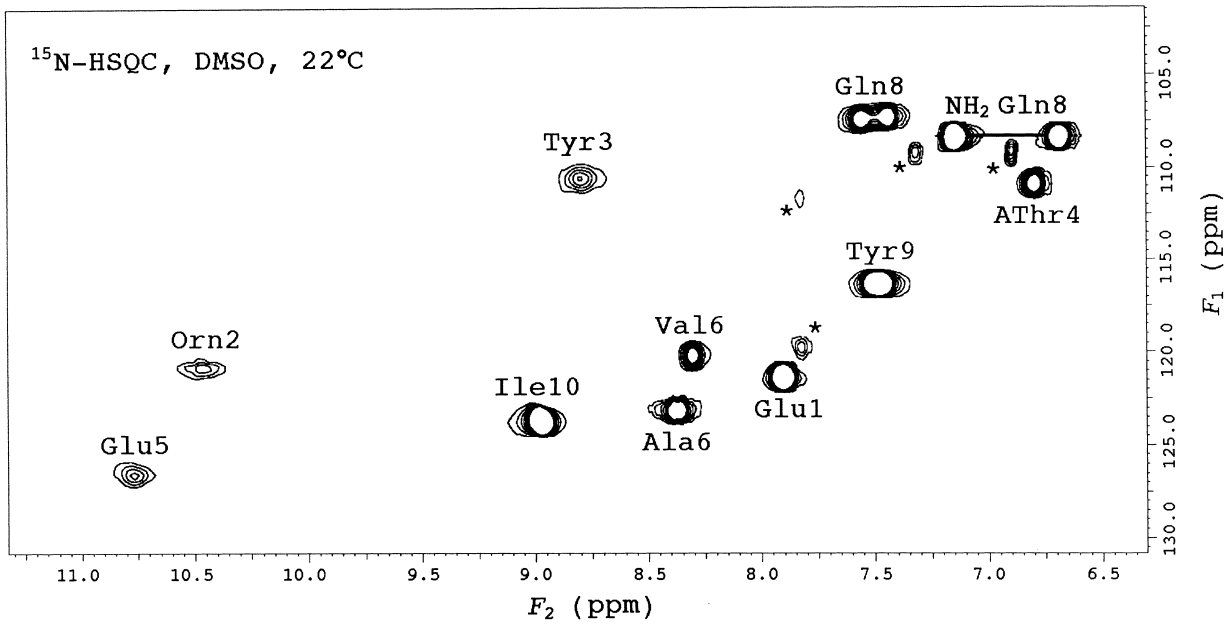

Fig. 2. ^{1}H (F_2)–^{15}N (F_1) HSQC spectrum of plipastatins recorded at 295 K. The cross-peaks labelled with an asterisk correspond to non-identified impurities.

TOCSY transfers were observed from the H^N to the Hα and Hβ.

3.2. NMR restraints and structure calculations of plipastatin A

A total of 125 (28 intra-residue, 64 sequential (*i*, *i*+1) and 33 medium-range (*i*, *i*+*n*, $2 \leq n \leq 4$) structurally relevant distance restraints were used for the structure calculation of plipastatin A and B. In addition, five χ_1 angle restraints were introduced in the restraint set. From the 40 initial structures calculated using consecutively the force fields *allhdg.pro* and

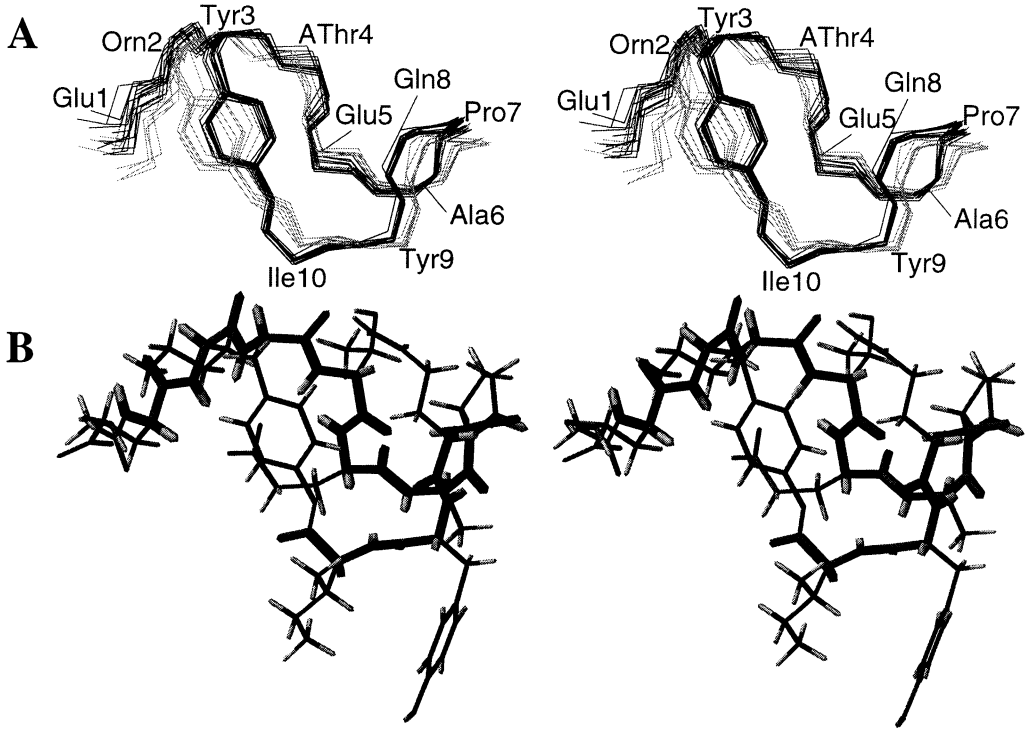

Fig. 3. A: Stereoviews of the 19 NMR structures of plipastatin A (peptidic (N, Cα, C) backbone and Tyr3 side chain heavy atoms) superimposed for a minimum root mean square deviation (rmsd) using the (N, Cα, C and Cβ, Cγ, Cδ, Cε, Cη, Oη of Tyr3) atoms from residue Tyr3 to Ile10, the P1 family of conformers (see the text) is represented with grey lines and the P2 family with black lines. Amino acids are labelled according to the amino acid sequence. B: Stereoviews of the closest structure to the geometric average for the P2 family (all-atom representation). The hydrophobic tail is not represented.

Table 1
^{1}H- and ^{15}N-NMR resonance assignments (ppm) of plipastatins A and B in DMSO solution at 295 K

Residue	H^N	N	Hα	Hβ	Others
Glu1	7.92	121.9	4.52	1.84, 1.96*	Hγ: 2.13
Orn2	10.54	120.9	4.12	1.76, 1.87	Hγ: 1.64; Hδ: 2.68
Tyr3	8.75	110.7	4.74	2.37*, 3.27	Hδ: 6.88; Hε: 6.83
AThr4	6.82	111.1	4.27	3.68	Hγ: 0.96
Glu5	10.73 (10.71)	126.5 (126.5)	3.29 (3.33)	2.02, 2.37	Hγ: 1.54, 1.59
Ala6 (Val6)	8.40 (8.33)	123.4 (120.3)	4.98 (4.50)	1.22 (2.06)	(Hγ,γ′: 0.87)
Pro7			4.19 (4.23)	1.93, 2.20	Hγ: 1.83, 2.05; Hδ: 3.51, 3.97
Gln8	7.58 (7.46)	107.6 (107.8)	4.27	1.38, 2.38* (1.37, 2.39)	Hγ: 2.09, 2.30; NH_2: 6.70, 7.18 N: 108.6
Tyr9	7.49 (7.53)	116.5	4.56 (4.57)	2.91 (2.92)	Hδ: 7.07; Hε: 6.63
Ile10	8.99 (8.98)	123.7 (123.8)	3.98	2.00 (1.75)	$H\gamma^{CH_3}$: 0.91 (0.86); $H\gamma^{CH_2}$: 1.16 $H\delta$: 0.75
Fatty acid	H2	H3	H4	H5–H15	methyl groups
	2.23	3.72	1.12, 1.28	1.05	0.85, 0.89

An asterisk indicates the $H^{\beta 2}$ proton when the β methylene protons were stereospecifically assigned. The numbers in parentheses correspond to the value for plipastatin B. Chemical shifts are given ±0.01 ppm for ^{1}H and ±0.05 ppm for ^{15}N.

CHARMM22 of X-PLOR, 19 were retained due to their low experimental and non-experimental potential energies. The statistics of the final structures are given in Table 2.

3.3. Structural analysis of plipastatin A and B

Fig. 3 shows the ensemble of the 19 final NMR structures of plipastatin A. This ensemble contains clearly two groups of conformers which include 7 (P1 family) and 12 (P2 family) structures, mainly characterised by the two opposite spatial positions of the ester group between Tyr3 and Ile10. This alternative induced only a local perturbation of the ϕ dihedral angle of Glu5 (from −60° for P2 to −120° for P1). The rest of the main chain is very close in the two families of conformers.

Based on the analysis of the ϕ and ψ angles [40], a type I β-turn was systematically identified for residues Ala6 to Tyr9 in both P1 and P2 families. The β I-turn involves a hydrogen bond between H^N of Tyr9 and CO of Ala6. Other hydrogen bonds stabilise the models: $6H^N$–9CO, $8H^N$–4CO (only for the P2 family) and $4H^O$–3CO. Hydrogen bonds $6H^N$–9CO and $9H^N$–6CO were previously proposed for plipastatin A1 and match the observed temperature dependence of the H^N chemical shifts in DMSO [19]. Moreover, the Oε atom of Glu5 is hydrogen bound to the H^N protons of both Orn2 and Glu5 in 19 and 14 models, respectively. Such interactions, together with the vicinity of the large ring-current effects of Tyr3, could explain the downfield chemical shifts observed for the H^N protons of these two residues (Fig. 2). The same phenomenon was observed by NMR for instance in PLA_2 [41], chymotrypsin [42], triosephosphate isomerase [43] for amide protons involved in strong hydrogen bonds. The H^N resonances of Orn2, Tyr3, and Glu5 are additionally quite broad. These broad lines could possibly originate from a dynamic averaging at intermediate frequencies between conformers with lifetimes in the 20–200 μs range.

The solution structure of plipastatin B with a D-Val replacing the D-Ala6 does not differ from plipastatin A due to the solvent exposure of the side chain of D-Ala6 (Fig. 3) that can in consequence accommodate easily a larger side chain of a valine without conformational changes. The well-defined structures of plipastatins A and B in DMSO solution will help, in the future, the understanding of both their behaviour

Table 2
Structural statistics of plipastatin A

	Backbone atoms[a]	Heavy atoms
Cartesian coordinate rmsd (Å) versus the average geometric structure		
All (without the fatty chain)	0.59 (±0.25)	0.79 (±0.36)
3–10	0.59 (±0.24)	0.64 (±0.33)
	X-PLOR – *allhdg.pro*	X-PLOR – CHARMM22
Potential energies[b] (kcal mol^{-1}) calculated from X-PLOR		
F_{total}	14.51 (±1.33)	−72.48 (±14.29)
F_{bond}	0.31 (±0.13)	6.60 (±0.30)
F_{angle}	11.50 (±0.31)	24.21 (±1.37)
F_{impr}	2.40 (±0.45)	1.33 (±0.53)
$F_{Coulombic}$	–	−152.60 (±10.55)
F_{L-J}	0.08 (±0.09)	−8.06 (±4.77)
F_{noe}	0.22 (±0.35)	0.36 (±0.17)
NOE violations (as average number per structure)		
violations > 0.1 Å	0.1	0.1
violations > 0.2 Å	0.0	0.0

[a]rmsd are calculated for backbone heavy atoms (C, N, C^α of amino acids and Cβ, Cγ, Cδ, Cε, Cη, Oη of Tyr3).
[b]F_{bond} is the bond-length deviation energy; F_{angle} is the valence angles deviation energy; F_{impr} deviation energy for the improper angles used to maintain the planarity of certain groups of atoms; $F_{Coulombic}$ is the Coulombic energy contribution to F_{total}; F_{L-J} is the Lennard–Jones van der Waals energy function (in the case of the *allhdg.pro* force field, only the repulsion term is given); F_{noe} is the experimental NOE function calculated using a force constant of 25 kcal mol^{-1} $Å^{-2}$ in the case of the CHARMM22 force field.

once inserted in lipidic layers, or how plipastatins can inhibit the PLA_2 activity.

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