DIRECT DETECTION OF N-H···O=C H-BONDS IN A 13C- AND 15N-LABELLED CYCLIC LIPODEPSIPEPTIDE AND THE INVESTIGATION OF ITS SELF-ASSEMBLY

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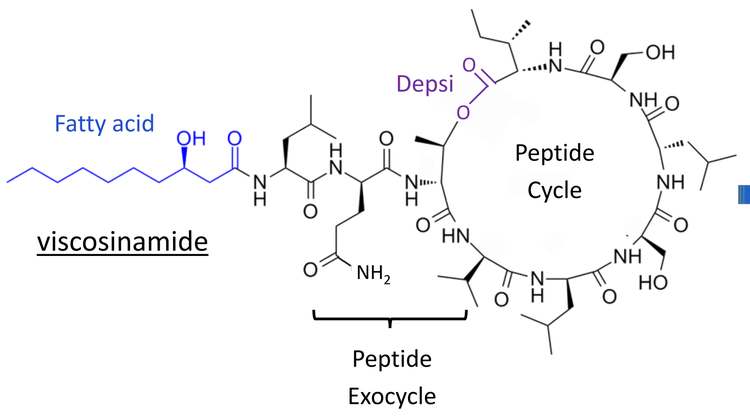
What CLPs are? How do they look like?

- Cyclic lipodepsipeptides (CLPs) are secondary metabolites of *Pseudomonas* and *Bacillus* bacterial species produced via non ribosomal pathways [1]
- They are consisted of a fatty acid moiety linked to the N-terminus of a peptide chain which is cyclized by an ester (or depsi) bond formation between its C-terminus and an OH capped side chain of a Ser or Thr
- Peculiar primary structural features: D-amino acids + alternation of polar and apolar amino acid side chains
- Tertiary structure: backbone conformation assessed:

  alfa-helix + loop → amphipathic character [2]



(*R*)-3-HDA-*L*-Leu1-*D*-Gln2-*D*-allo-Thr3-*D*-Val4-*L*-Leu5-*D*-Ser6-*L*-Leu7-*D*-Ser8-*L*-Ile9

O

Polar side chain: **red** Apolar side chain: **green**

CLP bioactivity

- Bacterial swarming (motility), biofilm formation [1]
- Stimulation of the plant immune system → crop protection [3]
- *In vitro* testing → activity against bacteria, viruses, fungi (non-exhaustive) [1]
- Novel antibiotics: daptomycin (marketed as CUBICIN®) [4]
- Anticancer effects below cytotoxic level (xantholysin, MD0066, viscosin) [5]

Structure – function/mode of action relationships not well understood!

Goals
- More detailed structural information is needed (than ^{1}H-^{1}H distance restraints) →
- Direct evaluation of amide plane orientations and H-bond pattern

I) Intramolecular: peptide conformation in monomeric state *vs* in membrane-mimicking environment [6]

II) Pore formation in low polarity medium [7] → structural characterization of a self-assembly

How?
- Growing *Pseudomonas* DR54 in minimal salt medium → ^{13}C-, ^{15}N-labelled viscosinamide (VA): *first ever* isotope labelled CLP
- J-correlation spectroscopic methods: $^3J_{HNHA}$ [φ] and $^{3h}J_{NC'}$ [r,Θ] → H-bonds
- Complementary *in silico* studies: AMBER molecular dynamic simulations

Results I) Conformational rigidity of VA

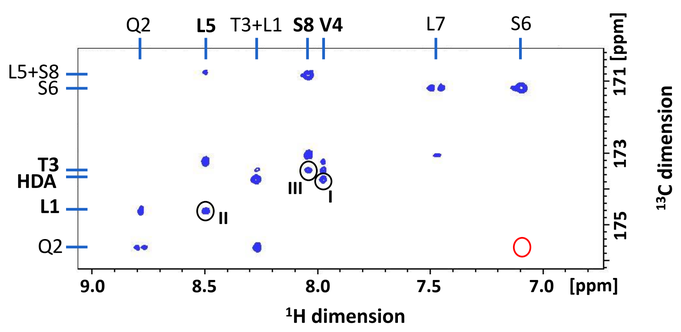
- In polar solvent i.e. AcN-d3: VA is in *monomeric state*
- In aqeuous DPC-d38 solution: VA is *coaggregated* with the DPC molecules (DOSY)
- **VA adopts the 'same' conformation in both states!**
- Experimental assessment:

a) ^{1}H – ^{1}H nOe cross peaks → backbone structure b) Measured $^3J_{HNHA}$ values → In AcN from 1D ^{1}H NMR spectrum (H^N peak fine structure); In aqueous DPC → line broadenings → J-correlated HNHA [8]

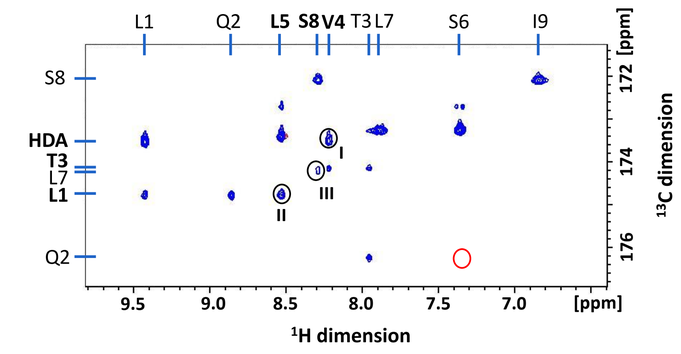
c) Long range (LR) HNCO spectra [9] → intramolecular H-bonds

AA	$^3J_{exp.}$ in AcN/Hz	$^3J_{exp.}$ in aq. DPC/Hz
Q2	4.38	4.31
T3	7.52	7.61
S6	8.28	8.41
L7	5.79	5.93

2D LR HNCO spectrum in AcN / 298 K →



2D LR HNCO spectrum in aqueous DPC / 310 K



Detected H-bonds/$^{3h}J_{NC'}$ values

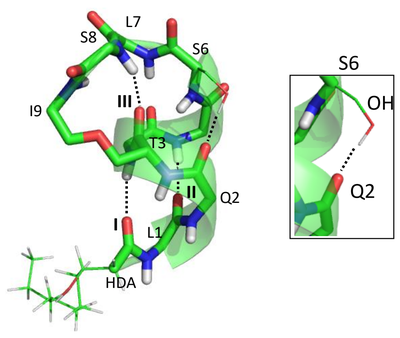
I: V4 (H^N)···(O=C) HDA / -0.30 Hz
II: L5 (H^N)···(O=C) L1 /-0.44 Hz
(S6 (H^N)···(O=C) Q2 is absent: ○) → α_L-helix
III: S8 (H^N)···(O=C) T3 /-0.25 Hz → loop

Same H-bond pattern In both states!

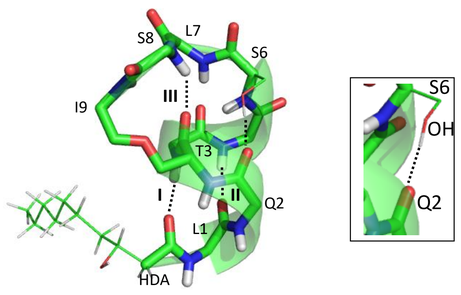
I: V4 (H^N)···(O=C) HDA / no data
II: L5 (H^N)···(O=C) L1 /-0.43 Hz
(S6 (H^N)···(O=C) Q2 is absent: ○) → α_L-helix
III: S8 (H^N)···(O=C) T3 /no data → loop

(V4 H$^{N'}$; HDA C') + (V4 H$^{N'}$; V4 C' ($^2J_{NC'}$))
(S8 H$^{N'}$; T3 C') + (S8 H$^{N'}$; L7 C' ($^1J_{NC'}$))
are overlapping cross peaks

- *In silico* assessment:



VA in explicit AcN solvent box



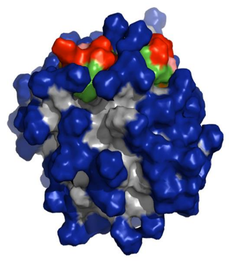
Stripped VA conformation in WAT+DPC

- Input structure: using ^{1}H-^{1}H contacts in CNS [10]
- Refined by AMBER [11] simulations without constraints
- AcN: 100 ns; WAT+DPC: 400 ns

→ Rigid backbone/amide plain orientations

→ Experimentally detected H-bond patterns perfectly illustrated

- Representative conformations are displayed

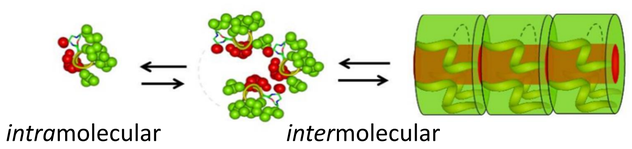


VA + DPC coaggregation = experimental results

VA (red/green) in water + DPC (blue/grey) environment (400 ns)

Results II) Intermolecular interactions in VA self-assembly

- In low polarity solvent i.e. chloroform-d: the amphipathic VA self-assembles



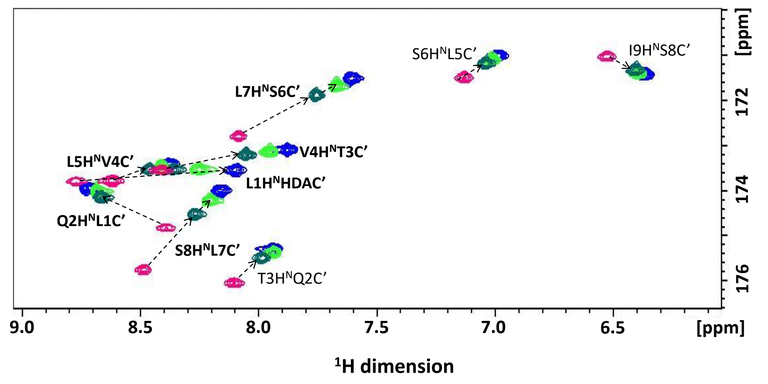
*intra*molecular H-bonds ✓ *inter*molecular H-bonds ?

(→ NMR spectral line broadenings + DOSY)

Plausible model for CLP self-assembly [12]

- LR HNCO did not indicate the intermolecular H-bonds due to the *fast* (>1/$^{3h}J_{NC'}$) exchange between the monomeric and assembled states
- Population averaged amide group chemical shifts → let's influence it!

HNCO cross peaks of VA dissolved in $CDCl_3$ + AcN-d3 mixtures / 278 K



- Solvent polarity to vary

HNCO cross peak color	V/V% of AcN in $CDCl_3$
pink	0.0
dark green	11.7
light green	13.5
blue	17.0

Conclusion and future prospects

- Viscosinamide displays identical conformation in its monomeric state (dissolved in AcN)and in its coaggregated state with real cell membrane-mimicking DPC micelles
- The structural assessment detailed the orientation of the amide planes and of the intramolecular H-bond pattern using J-correlation NMR methods and AMBER molecular dynamic simulations
- The protocol is planned to be applied for larger CLPs e.g. xantholysin, tolaasin
- Interpeptide interactions have been indirectly shown for the self-assembly of viscosinamide. In the future the full structural elucidation of such supramolecular organization will be performed using isotope-filtered NOESY

References

[1] Raaijmakers, J.M. *et. al.*, *Mol. Plant. Microb. Interact.* 2006, **19** (7), 699–710 [2] Jourdan, F.; Lazzaroni, S. *et. al.*, *Proteins: Struct., Funct., Genet.*, 2003, **52**, 534–543 [3] Khong, N.G.; Randoux, B. *et. al.*, *Commun. Agric. Appl. Bio. Sci.,* 2013, 78 (3), 479–487 [4] Fishbach, M.A.; Walsh, C.T.; *Chem. Rev.*, 2006, **106**, 3466–3496 [5] Pascual, J.; Garcia-López, M. *et. al.*, *Syst. Appl. Microbiol.,* 2014, **37**, 412–416 [6] Warschawski, D.E. *et. al.*; *Biochim. Biophys. Acta - Biomembranes* 2011, **1808**, 1957–1974 [7] Lee, T., Hall, K., Aguilar, M., *Curr. Top. Med. Chem.,* 2016, **16**, 25–39 [8] Vuister, G.; Bax, A. J. Am. Chem. Soc. 1993, **115**, 7112–1111 [9] Cordier, F. *et. al.*, *Nat. Protoc.*, 2008, **3**, 235–241 [10] Brunger, A. T.; *Nat. Protoc.*, 2007, **2**, 2728–2733 [11] Salomon-Ferrer, R.; Gotz, A.W. *et. al.*, *J. Chem. Theory Comput.*, 2013, **9**, 3878–3888 [12] Sinnaeve, D.; Hendrickx, P.M.S. *et al.*, *Chem. Eur. J.*, 2009, **15**, 12653–12662

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