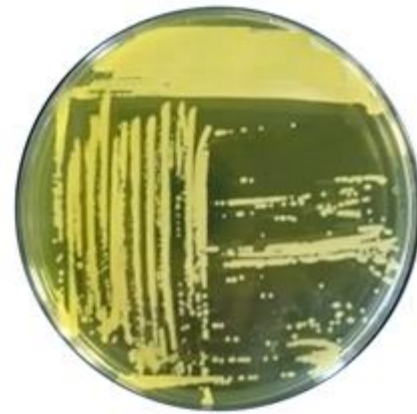FACULTY OF SCIENCES

BREAKING THE LIMITS OF NMR SPECTROSCOPY IN PEPTIDE – MEMBRANE INTERACTION STUDIES USING ISOTOPE LABELLING
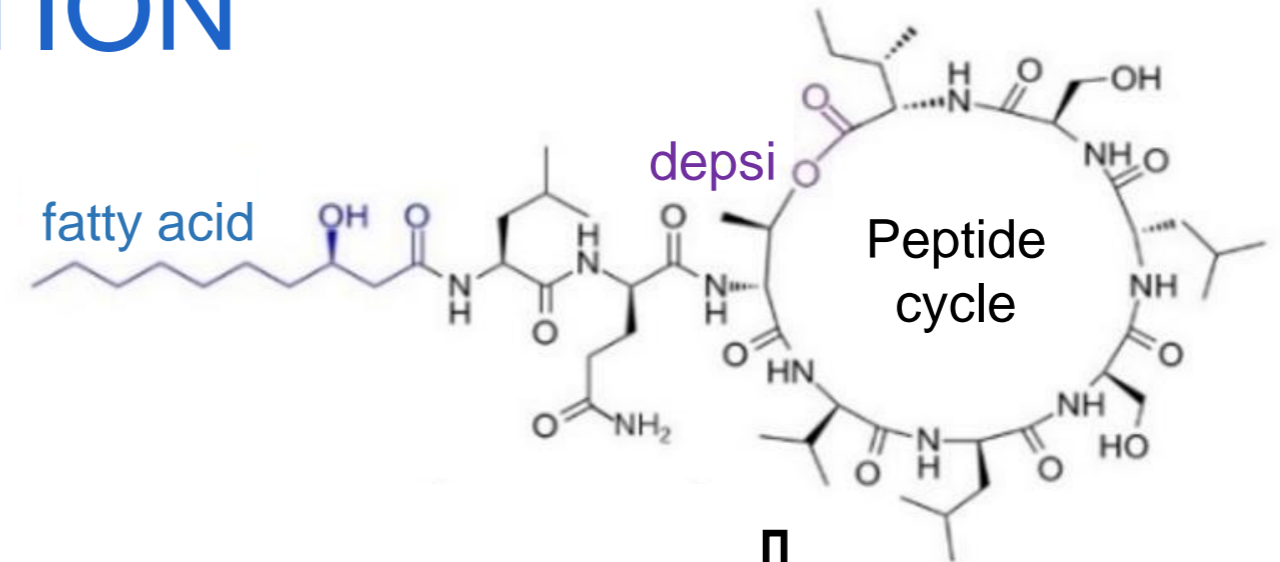
Benjamin Kovacs, NMR & Structure Analysis Unit (NMRSTR), Ghent University

YBMRS, December 6 2018 – Spa

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CLiPs – ORIGIN AND COMPOSITION
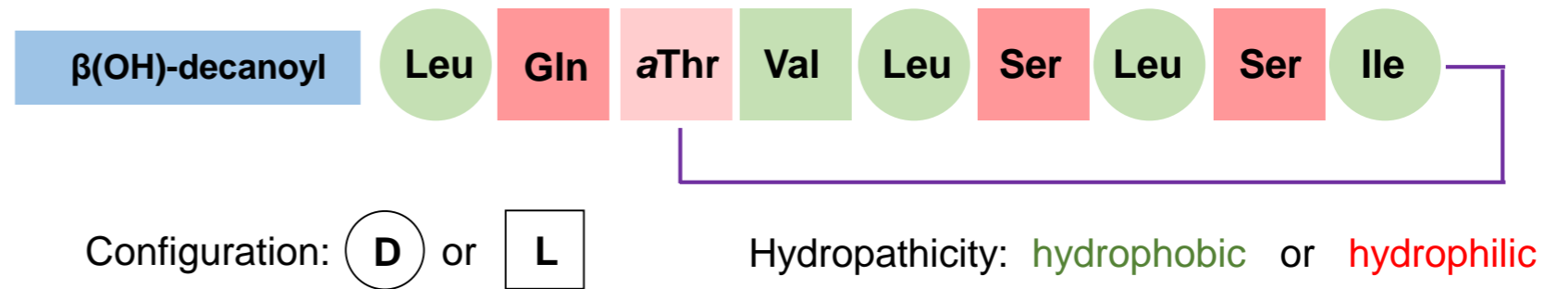
- Cyclic lipodepsipeptides (CLiPs)
- Bacterial origin

  (*Pseudomonas* or *Bacillus* spp.)

  → Produced via non ribosomal pathways

- **Primary structure**:
- Lipid tail + Oligopeptide chain
- → Latter cyclized via ester bond
- → Exotic AA pattern
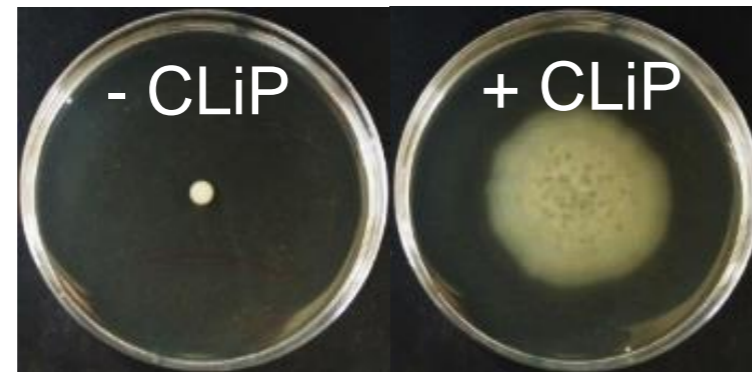- More than 100 natural CLiPs

- **Classification:**

$$\frac{\text{total AA \# } (l)}{\text{AA \# in the cycle } (c)}$$

Viscosinamide (9:7)

simplified representation

| β(OH)-decanoyl | Leu | Gln | *a*Thr | Val | Leu | Ser | Leu | Ser | Ile |

Configuration: (D) or [L]   Hydropathicity: hydrophobic or hydrophilic

CLiP group	*l:c*	CLiP group	*l:c*
Bananamide	8:6	Entolysin	14:5
Syringomycin	9:9	Xantholysin	14:8
Viscosin	9:7	Tolaasin	18:5
Orfamide	10:8	Fuscopeptin	19:5
Amphisin	11:9	Corpeptin	22:5
Putisolvin	12:4	Syringopeptin	22:8 or 25:8

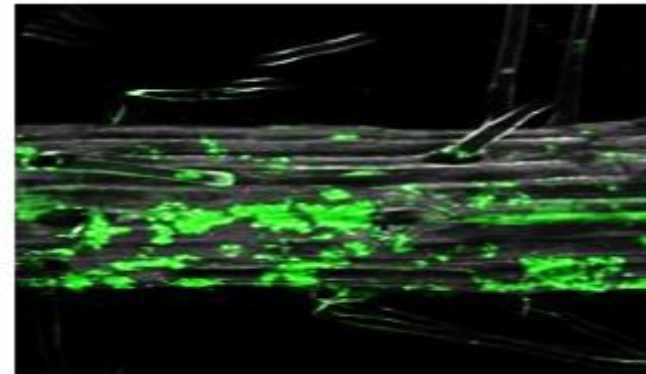# CLiPs – BIOLOGICAL ROLE

- CLiP producers are plant associated ....
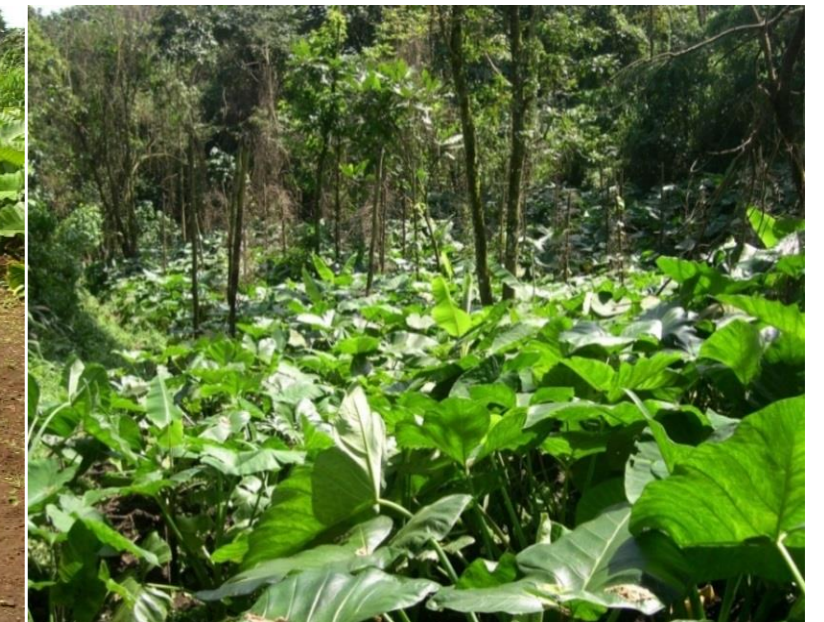
biosurfactants: cell motility
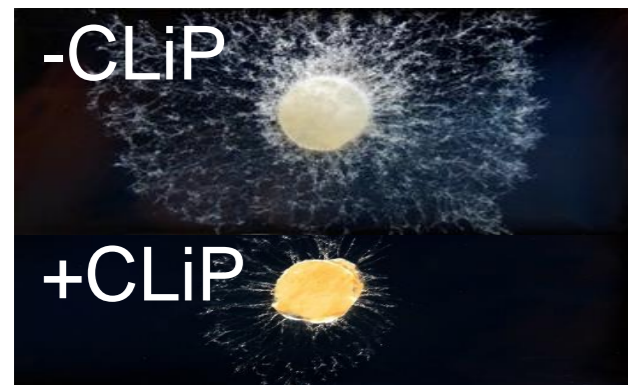
Plant health in the field

antifungal activity

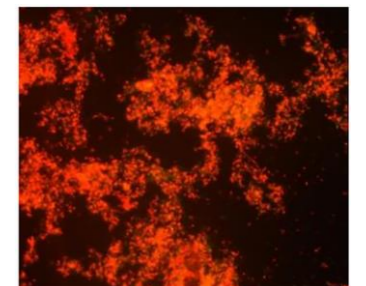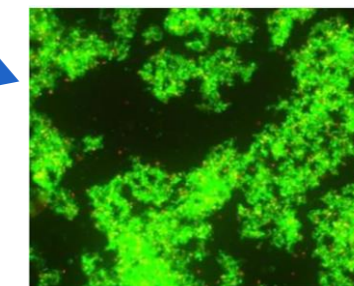**root colonization**

Few CLiP Producers   Many CLiP Producers

Figure courtesy of Feyisara Eyiwumi Olorunleke

.... yet are promising candidates for clinical applications as well!

- **antibacterial** (e.g. *Staphylococcus*)
- **anticancer** (breast, kidney)
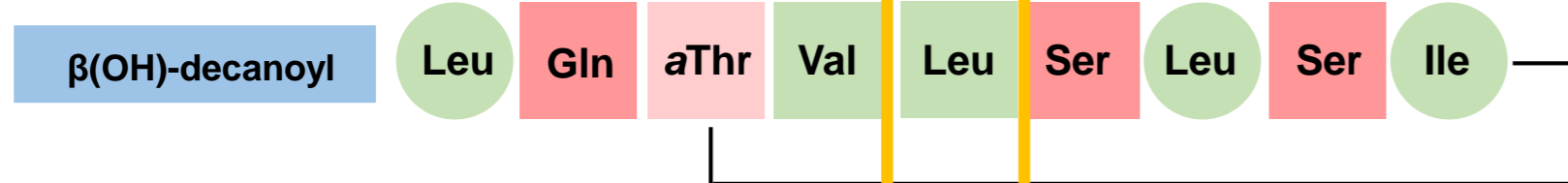- **antiviral** (influenza, hepatitis)

-CLiP   +CLiP

daptomycin *aka* **Cubicin**@

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3

Geudens, N.; Martins, J., *Front. Microbiol.*, 9, 1–18 (2018)

CLiPs – 3D STRUCTURE

- Solution state NMR spectroscopy and X-ray scattering
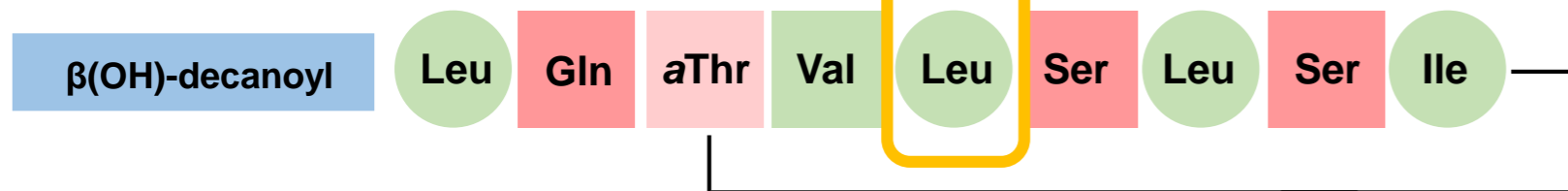
→ Characteristic backbone conformations / CLiP group
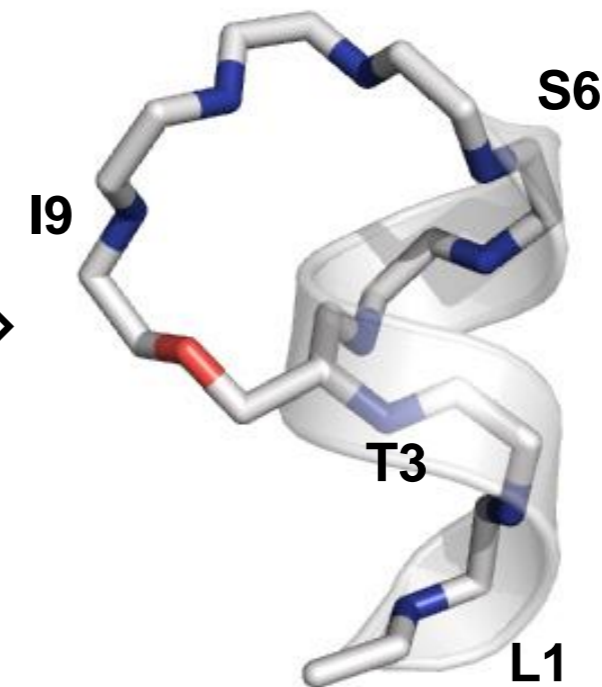

Pseudodesmin A (9:7)

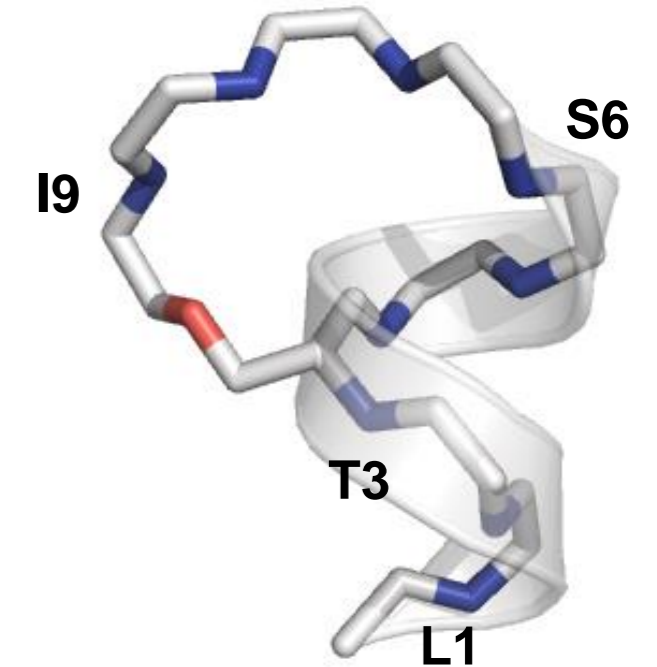| β(OH)-decanoyl | Leu | Gln | aThr | Val | Leu | Ser | Leu | Ser | Ile |

Viscosinamide (9:7)

| β(OH)-decanoyl | Leu | Gln | aThr | Val | Leu | Ser | Leu | Ser | Ile |

Fold →

'stapled helix' conformation:
α_L (L1 → S6) + loop (L7 → I9)

Backbone conformation of Viscosin-CLiPs
(grey: C, blue: N, red: O)

S6
I9
T3
L1

Pseudodesmin A (9:7)

S6
I9
T3
L1

Viscosinamide (9:7)

CLiPs – 3D STRUCTURE

- Merit of 'stapled helix' fold: **amphipathicity**

Viscosinamide (9:7)

| β(OH)-decanoyl | Leu | Gln | *a*Thr | Val | Leu | Ser | Leu | Ser | Ile |

Fold
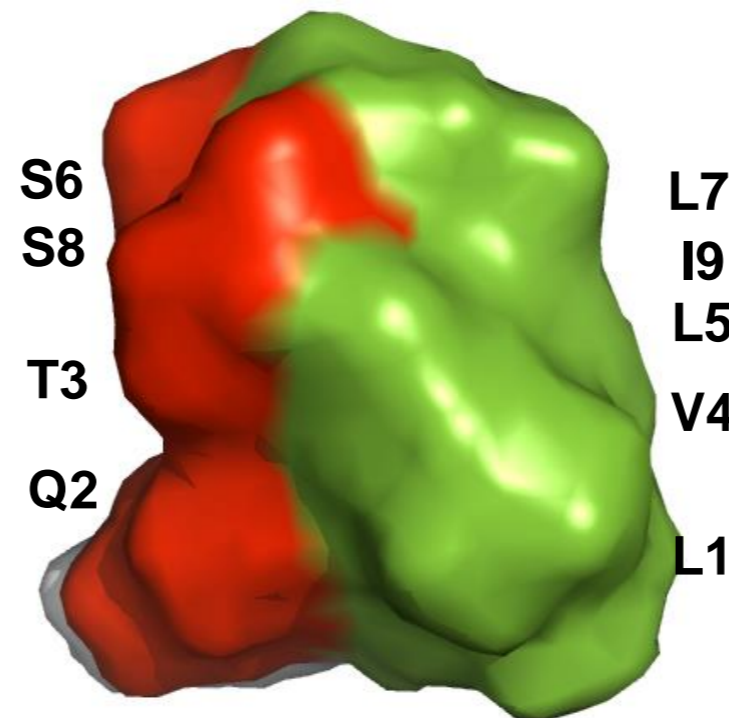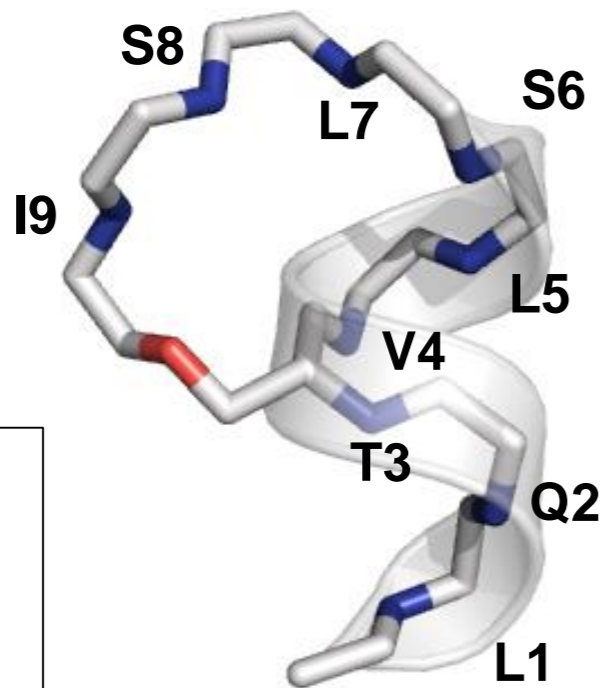
'stapled helix':
α_L (L1 → S6) + loop (L7 → I9)

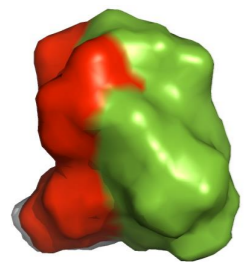**Backbone conformation** (grey: C, blue: N, red: O)

**Surface of side chains** (green: hydrophobic red: hydrophilic)

- Biological membranes are likewise amphipathic...

- (How) do Viscosins interact with them?

VISCOSINS + MEMBRANES

- To mimic real conditions: detergents as model membrane systems

→ Viscosinamide (VA) + zwitterionic DPC (dodecylphosphocholine) comicelles

→ PRE + MD simulation studies

in polar solvents: **free**

Diffusion coefficient of VA (in AcN)

792.2 ± 1.4 µm$^2 \cdot$s^{-1}

+DPC

H$_2$O
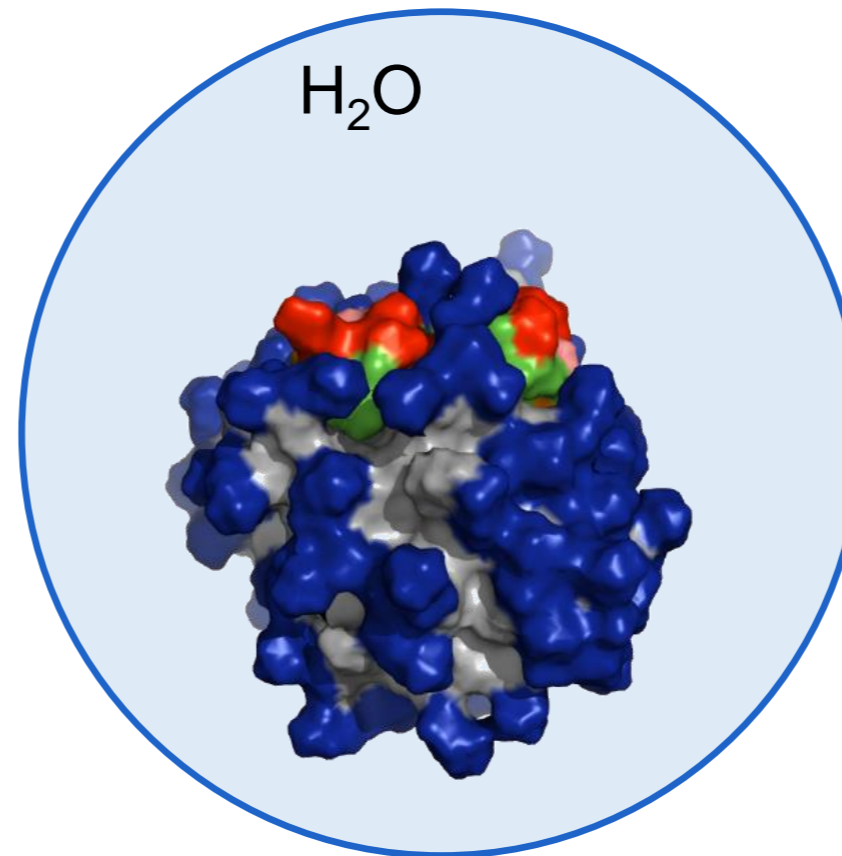
comicellised

Diffusion coefficients

D(VA) = 80.9 ± 0.5 µm$^2 \cdot$s^{-1}

D(DPC) = 79.6 ± 4.0 µm$^2 \cdot$s^{-1}

coherent motion!

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RIGID VISCOSINS! (?)

→ CD: Viscosins retain their 'free state' structure?

Viscosins in TFE

**Viscosins +
PE:PG:cardiolipin vesicules**
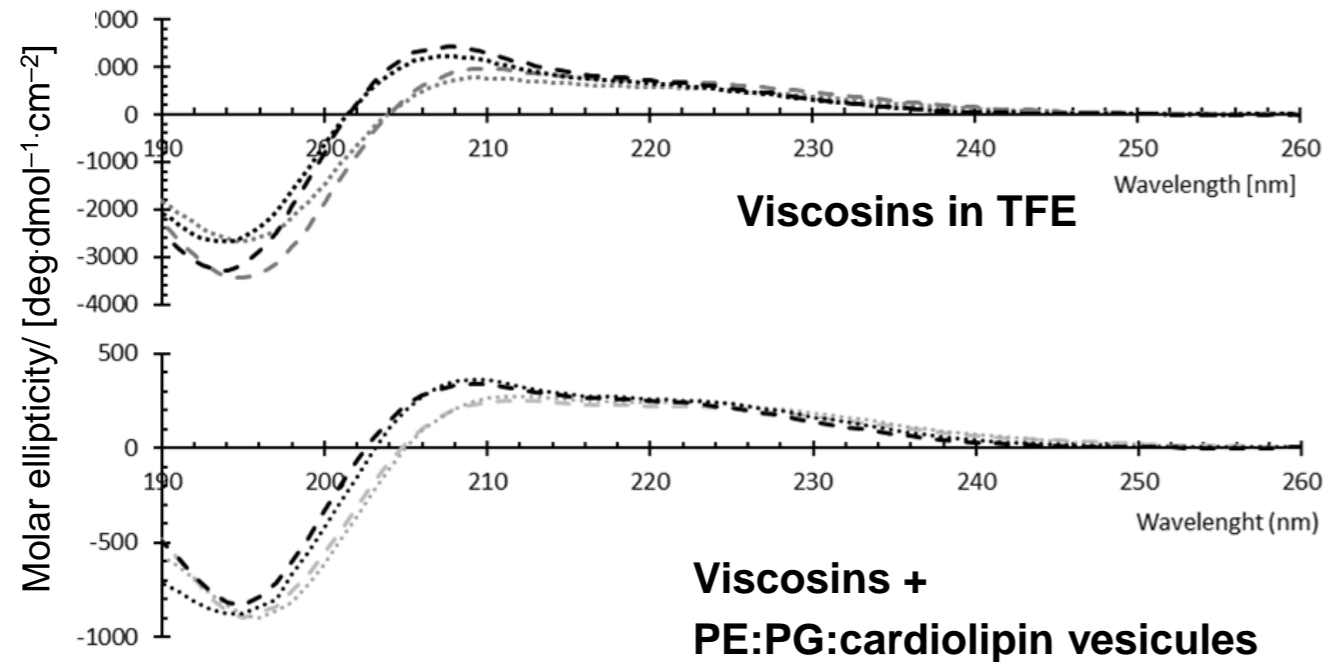
Adapted from Geudens, N.; Nasir, M. *et al.. Biochim Biophys
Acta,*1859,331–339 (2017)

→ Let's go more into details!
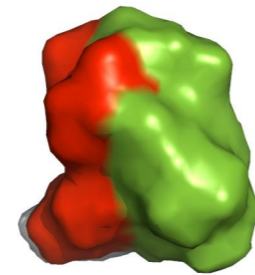
→ NMR: atomic resolution; longer timescale

+DPC

Viscosinamide

free

(in polar organic
solvents)
structurized

Any conformational
change?

comicellised

structurized

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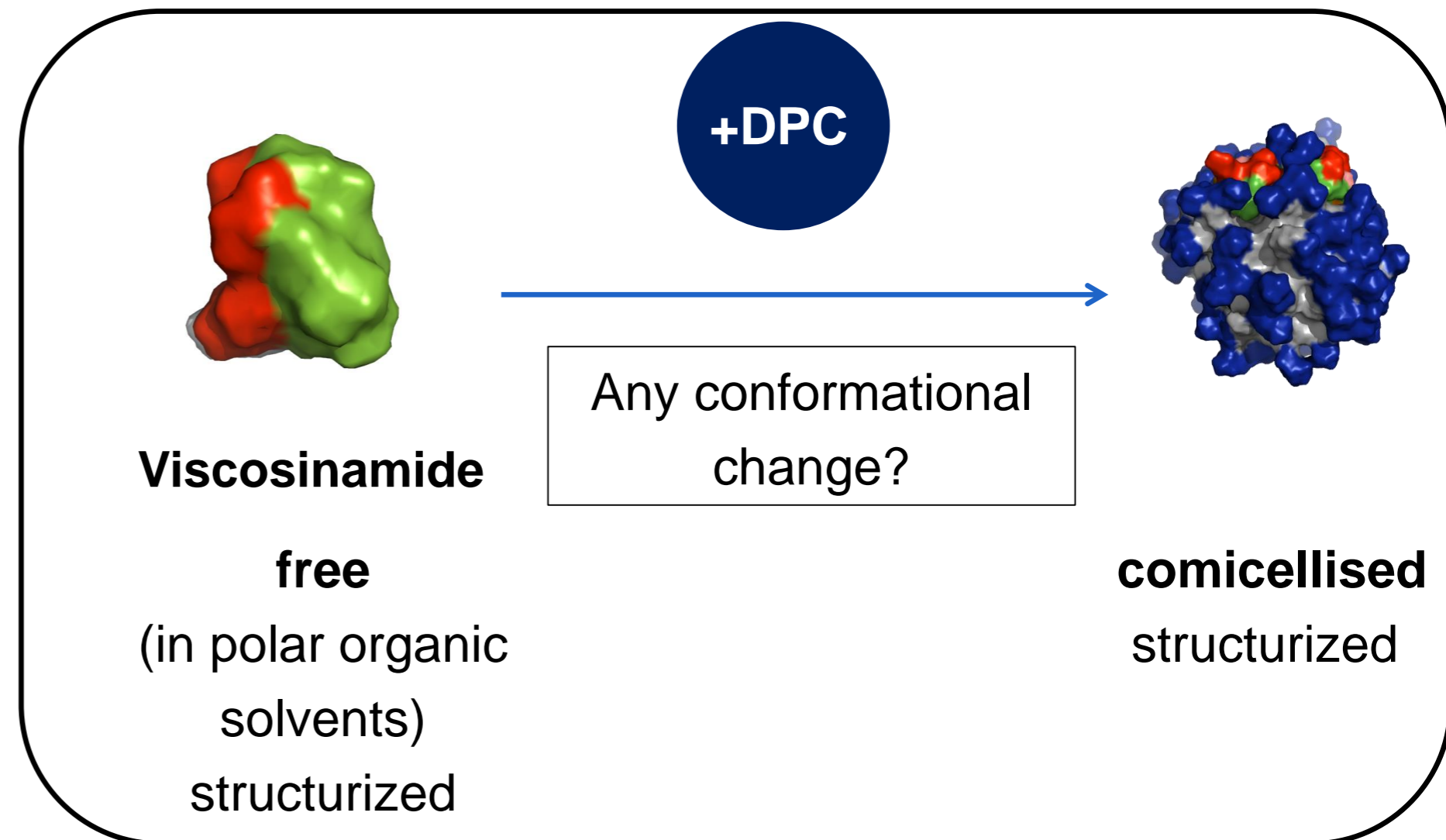
COMMON METHODS IN CLiP RESEARCH: ^{1}H-NMR

1D 1H:

$^3J_{HN-H\alpha}$ AcN-d3

8.6   8.4   8.2   [ppm]
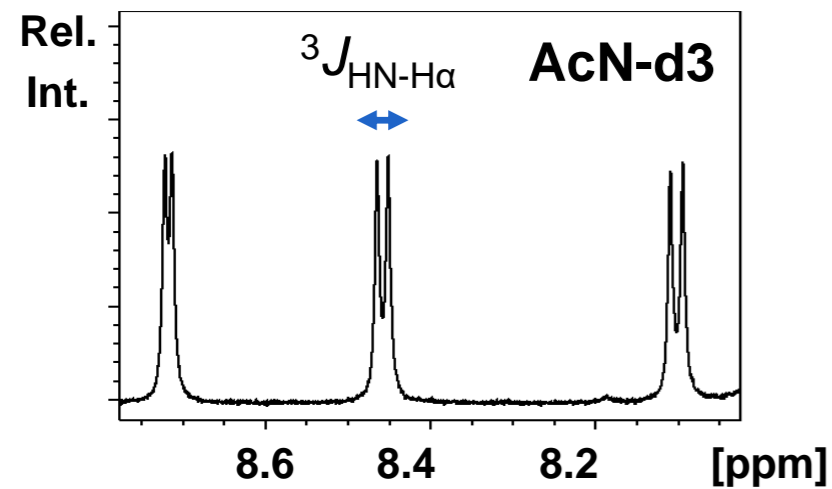
H^N region (part) of viscosinamide

Viscosinamide

free

+DPC

τ_c **increases, enhanced relaxation...**
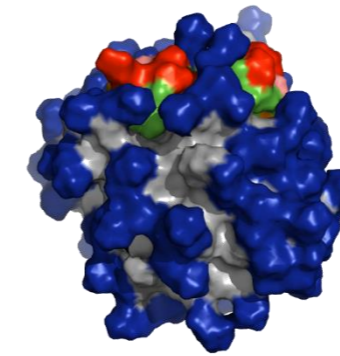
comicellised

1D 1H:

DPC solution

8.6   8.4   8.2   [ppm]
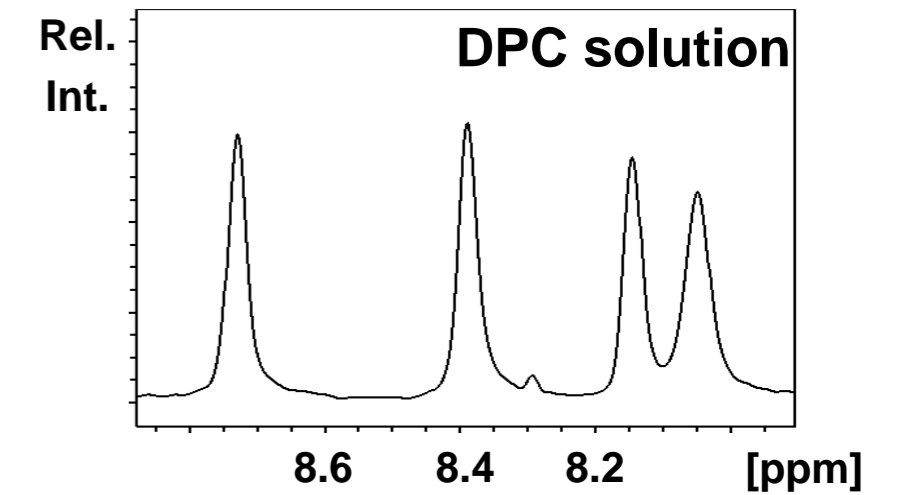
H^N region (part) of viscosinamide
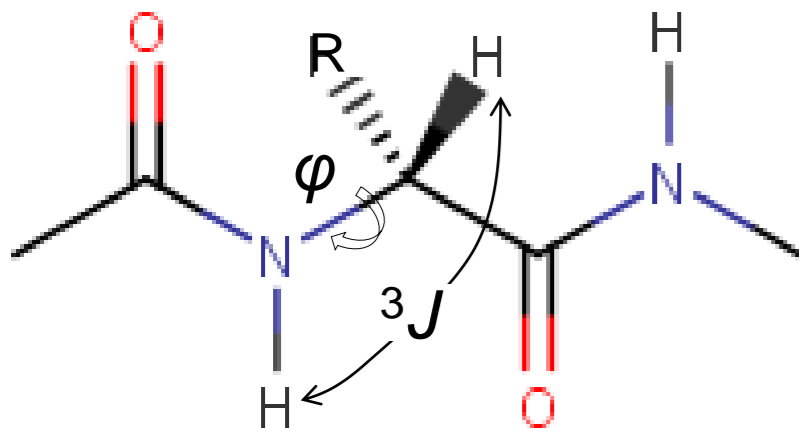
1 φ torsion angle
• Size matters... → **Protein NMR!**
from $^3J_{HN-H\alpha}$ Line broadenings ❌

• Heteronuclear experiments →

2 ^{1}H-^{1}H distance restraints
→ φ,ψ,χ ✅   spin diffusion, bad SNR...
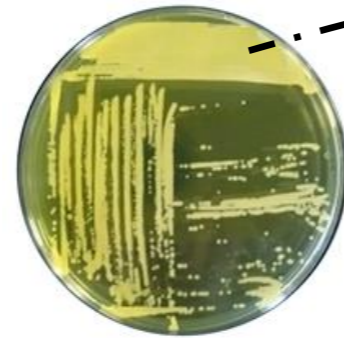from NOESY/ROESY
→ H-bonds!

• Natural abundance: ^{13}C: 1.1%, ^{15}N:0.4%

→ **Isotope enrichment is needed!**

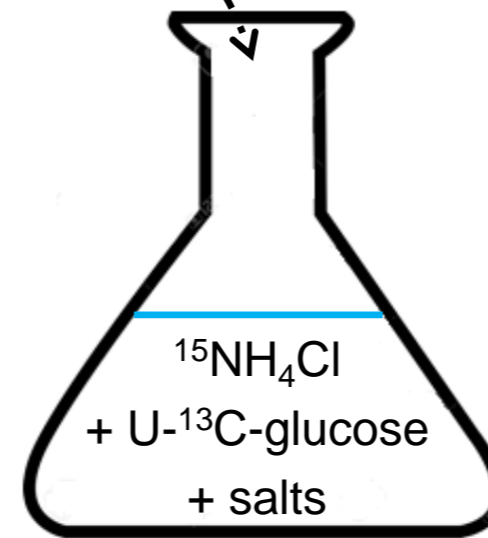# PRODUCTION OF ^{13}C-, ^{15}N-ENRICHED VISCOSINAMIDE
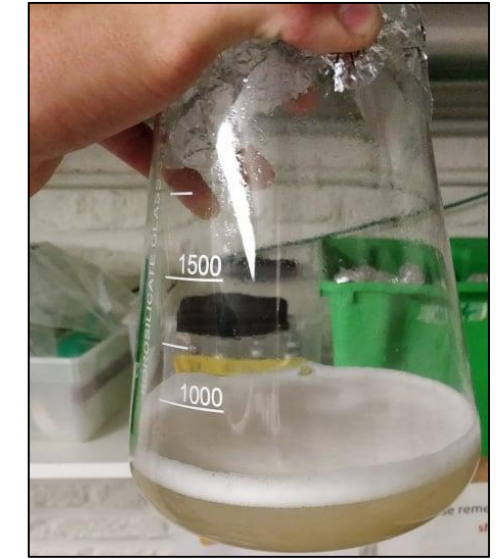
1: Bacterial growth
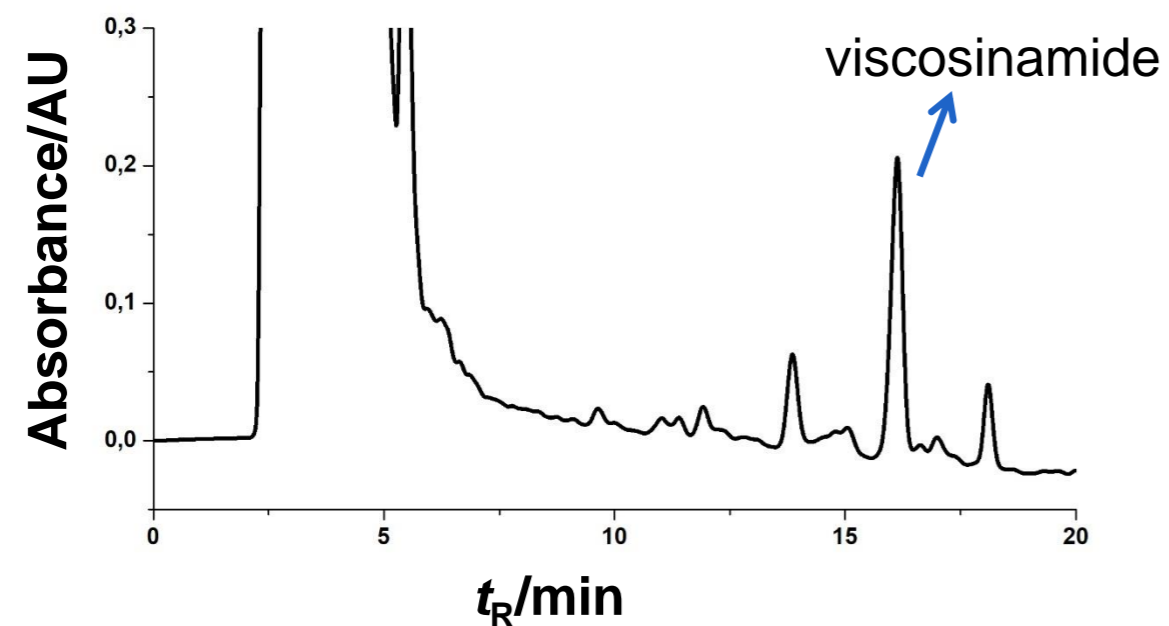
- in minimal salt medium

Pseudomonas
fluorescens DR54

^{15}NH$_4$Cl
+ U-^{13}C-glucose
+ salts

T = 28 ^{o}C for 48 h

3: Purification

- reversed-phased HPLC

viscosinamide

Absorbance/AU

t_R/min

2: Extraction

- both cells and
supernatant with Et-Ac
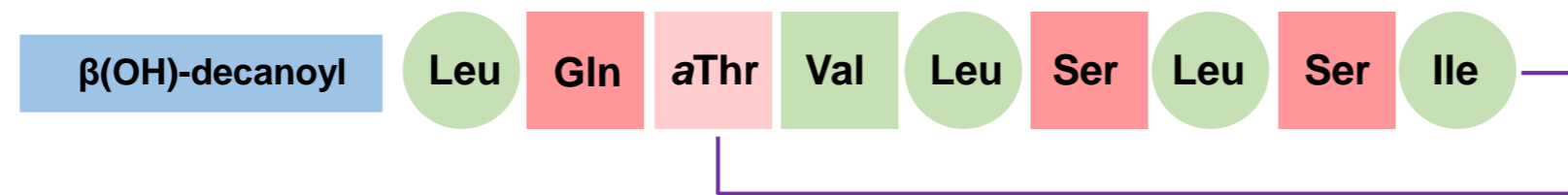
First ^{13}C- and ^{15}N-enriched CLiP

Viscosinamide: $^{12}C_{54}H_{96}O_{15}{}^{14}N_{10} \rightarrow {}^{13}C_{54}H_{96}O_{15}{}^{15}N_{10}$ LC-MS data ✔
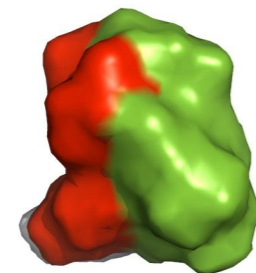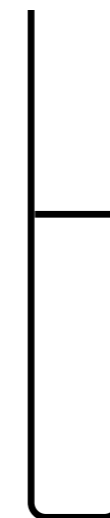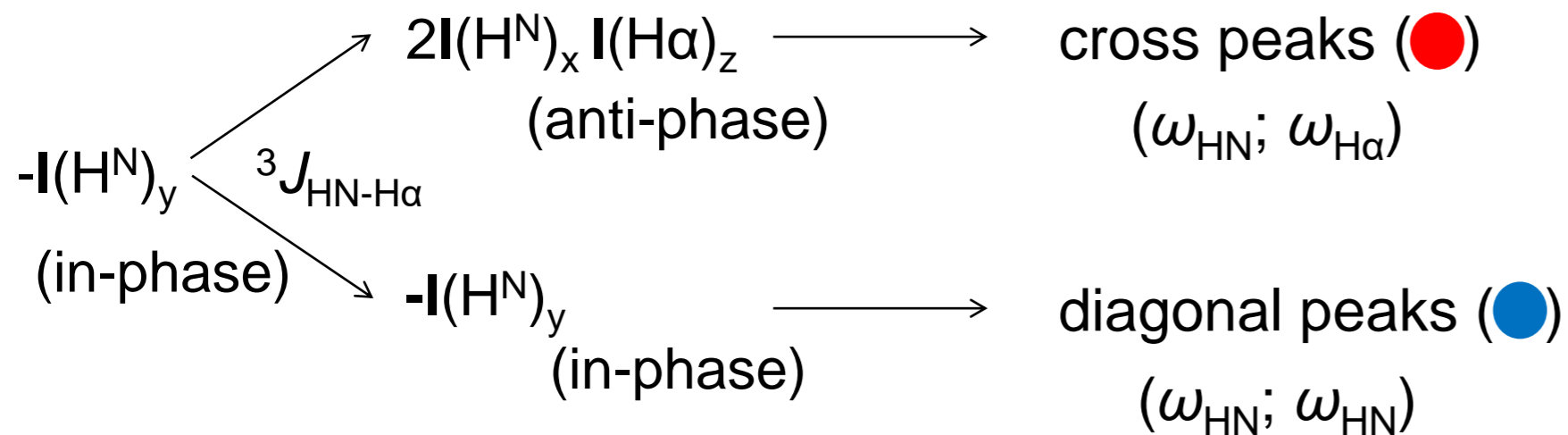
Sample 1

Free state
AcN-d3
[VA] = 2.8 mM
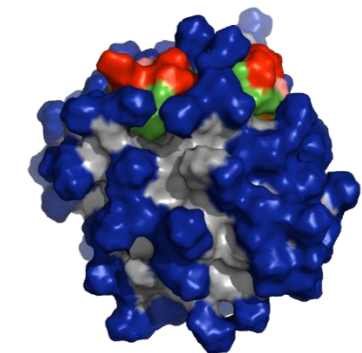
Sample 2

Comicellised state
90% H_2O/10%D_2O + DPC
[VA] = 5.75 mM
[DPC] = 151 mM
$\rightarrow$ ~ 2 peptide/micelle

'states' confirmed by DOSY measurements

PHI TORSION ANGLES

- **HNHA** experiment → $^3J_{HN-H\alpha}$ **[φ]**

$$2I(H^N)_x\, I(H\alpha)_z \longrightarrow \text{cross peaks (●)}$$
$$\text{(anti-phase)} \qquad (\omega_{HN};\, \omega_{H\alpha})$$

$$-I(H^N)_y \xrightarrow{\;^3J_{HN-H\alpha}\;}$$
$$\text{(in-phase)}$$

$$-I(H^N)_y \longrightarrow \text{diagonal peaks (●)}$$
$$\text{(in-phase)} \qquad (\omega_{HN};\, \omega_{HN})$$
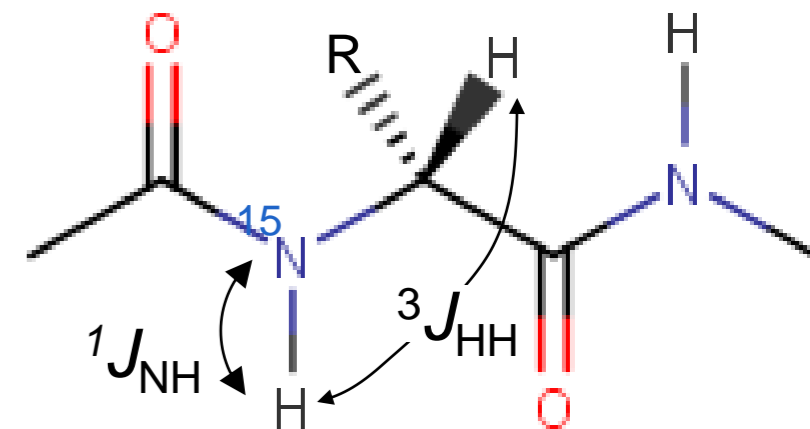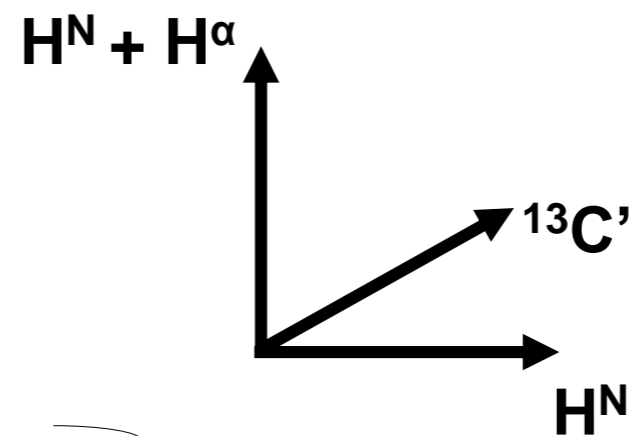
$$S_{cross}/S_{diag.} = -\tan^2(2\pi\,{}^3J_{HN-H\alpha}\,\xi)$$
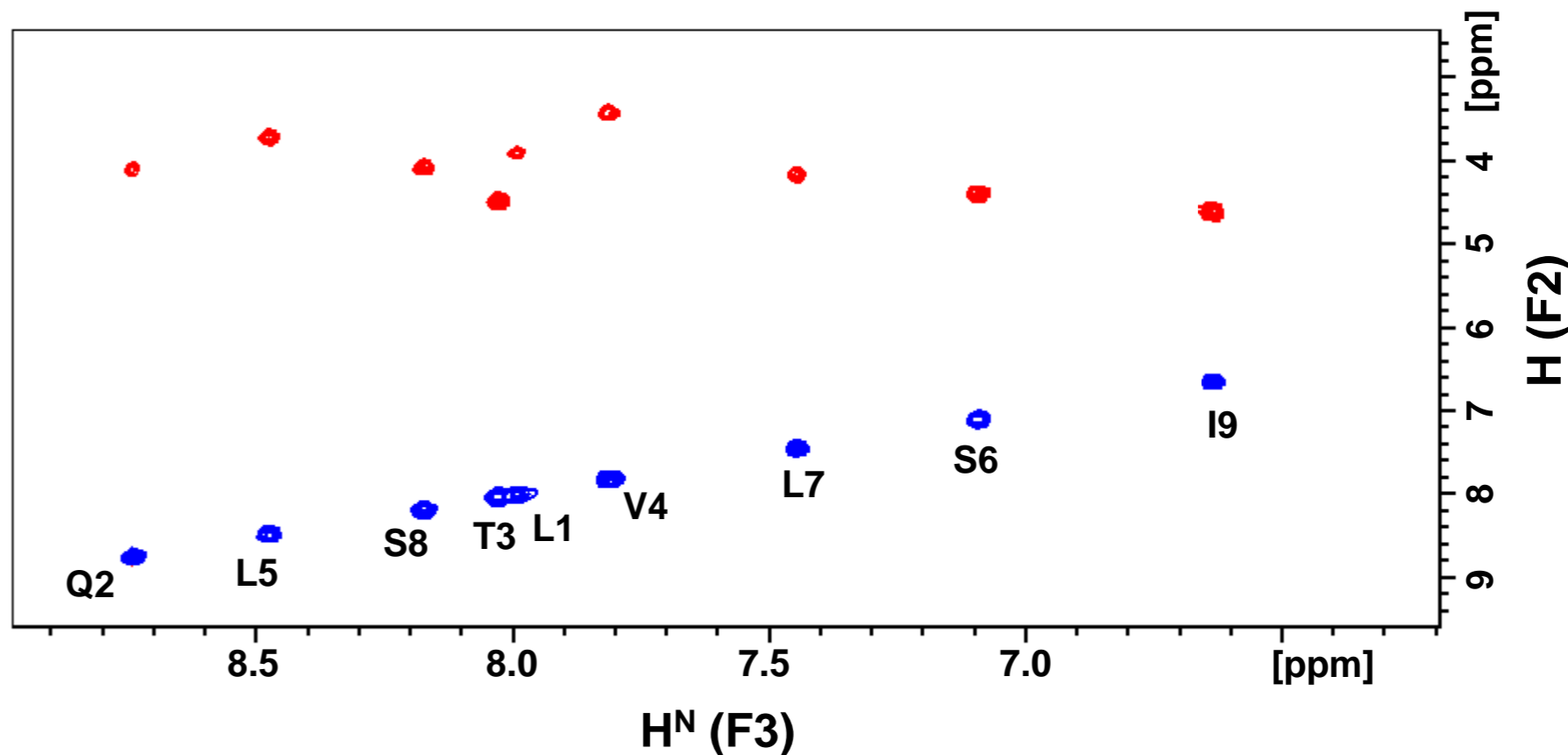
where ξ is the de- and rephasing delay

Note: $\dfrac{1}{T_{2ap}} = \dfrac{1}{T_{2ip}} + \dfrac{1}{T_{1H\alpha}}$ and $T_{1H\alpha} \sim \dfrac{1}{\tau_c}$...

→ uniform correction factor for all $^3J_{HN-H\alpha}$

T_{2ap} : $T2$ relaxation time of $2I(H^N)_x\, I(H\alpha)_z$

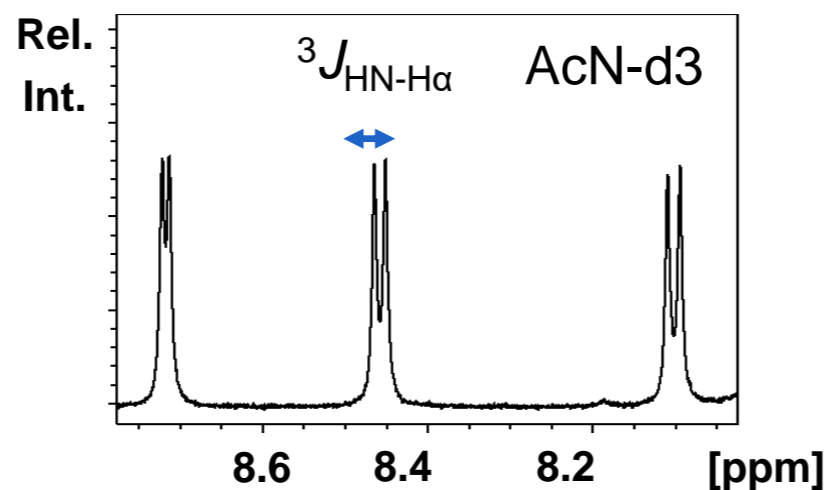T_{2ip} : $T2$ relaxation time of $-I(H^N)$

$T_{1H\alpha}$: selective $T1$ relaxation time of Hα

Vuister, G.; Bax, A., *J. Am. Chem. Soc.*, 115, 7772–7777 (1995)

PHI TORSION ANGLES

H^N region (part) of 1D 1H:

$^3J_{HN-H\alpha}$ AcN-d3

Rel. Int.

8.6   8.4   8.2   [ppm]

vs 3D HNHA

Hydropathicity: hydrophobic
hydrophilic

Stereochemistry: Ⓓ L̄

$^3J_{HN-HA}$ **in free state**:

R^2=0.9998

$^3J_{HN-H\alpha}$ / Hz
1D 1H

$^3J_{HN-H\alpha}$ / Hz
3D HNHA
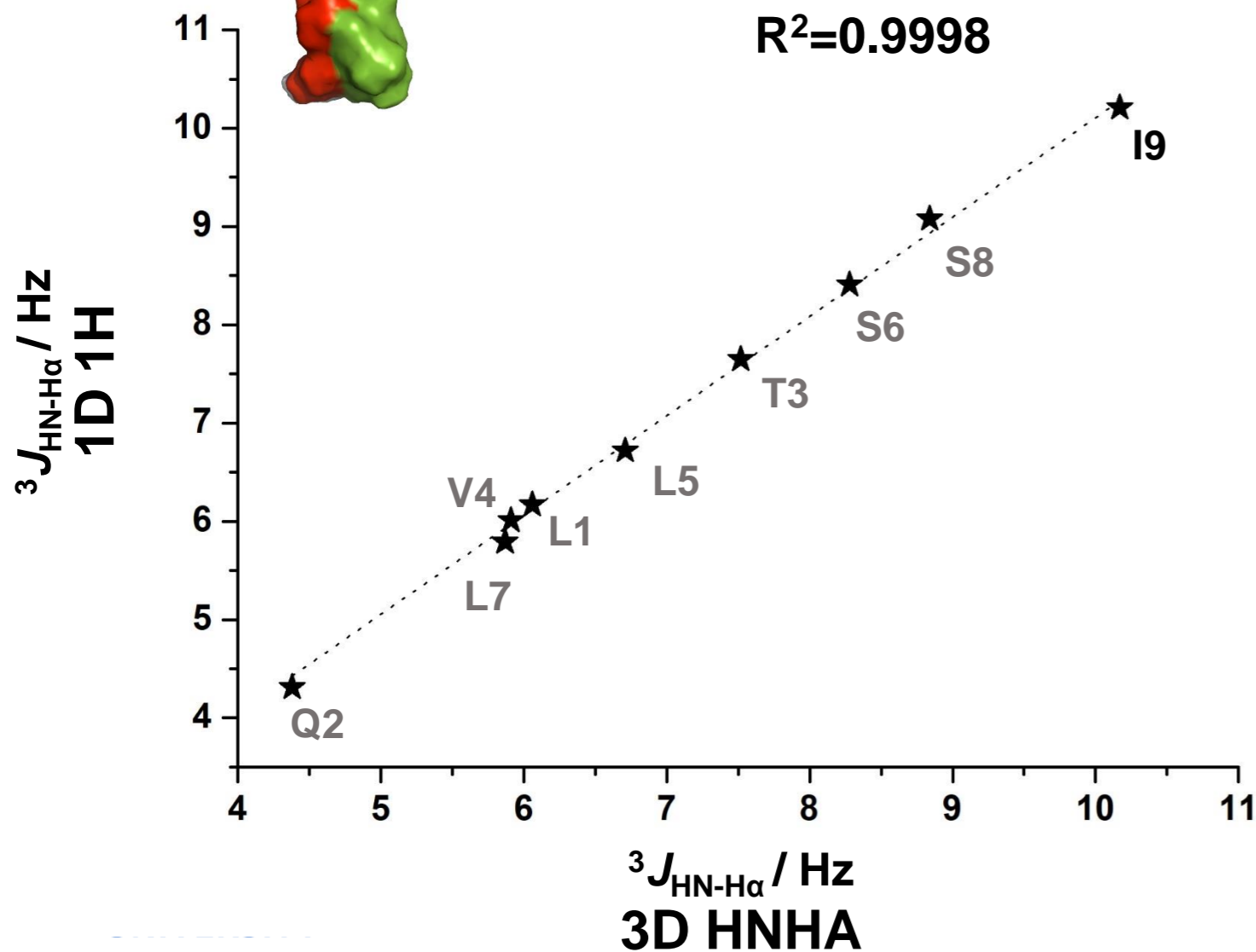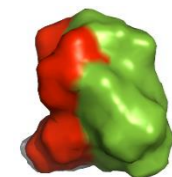
From H^N splitting and 3D HNHA:

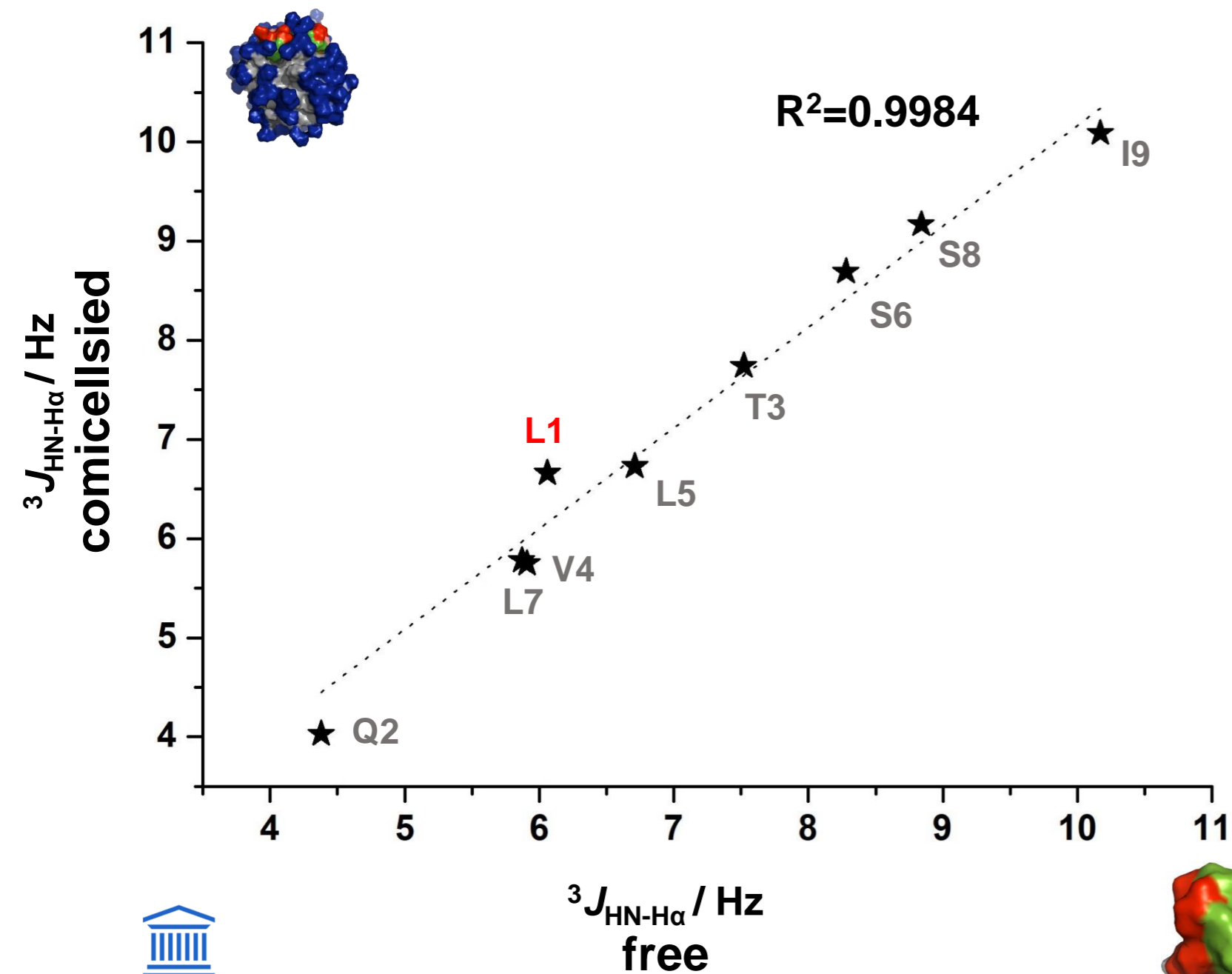| $^3J_{HN-HA}$/Hz	L1	Q2	T3	V4	L5	S6	L7	S8	I9
1D 1H	6.17	4.31	7.65	6.01	6.72	8.41	5.79	9.08	10.21
3D HNHA	6.06	4.38	7.52	5.91	6.71	8.28	5.87	8.84	10.17

- No systematic deviations ($\rightarrow T_{2ap} \sim T_{2ip}$)

- 3D HNHA experiment works

12

PHI TORSION ANGLES

$^3J_{HN-HA}$ in comicellised vs free state:

Hydropathicity: hydrophobic hydrophilic

Stereochemistry: Ⓓ ☐L

R²=0.9984

From 3D HNHA:

$^3J_{HN-HA}$/Hz	L1	Q2	T3	V4	L5	S6	L7	S8	I9
comicellised	6.66	4.03	7.74	5.75	6.73	8.69	5.78	9.17	10.09
free	6.06	4.38	7.52	5.91	6.71	8.28	5.87	8.84	10.17

- Systematic deviations first ($\rightarrow T_{2ap} < T_{2ip}$)

- Correction factor: 1.12

- L1($^3J_{HNHA}$) deviates the most... $\rightarrow$ fatty acid?

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PHI TORSION ANGLES

Hydropathicity: hydrophobic hydrophilic

$^3J_{HN-HA} \rightarrow \varphi$ **in comicellised vs free state**:
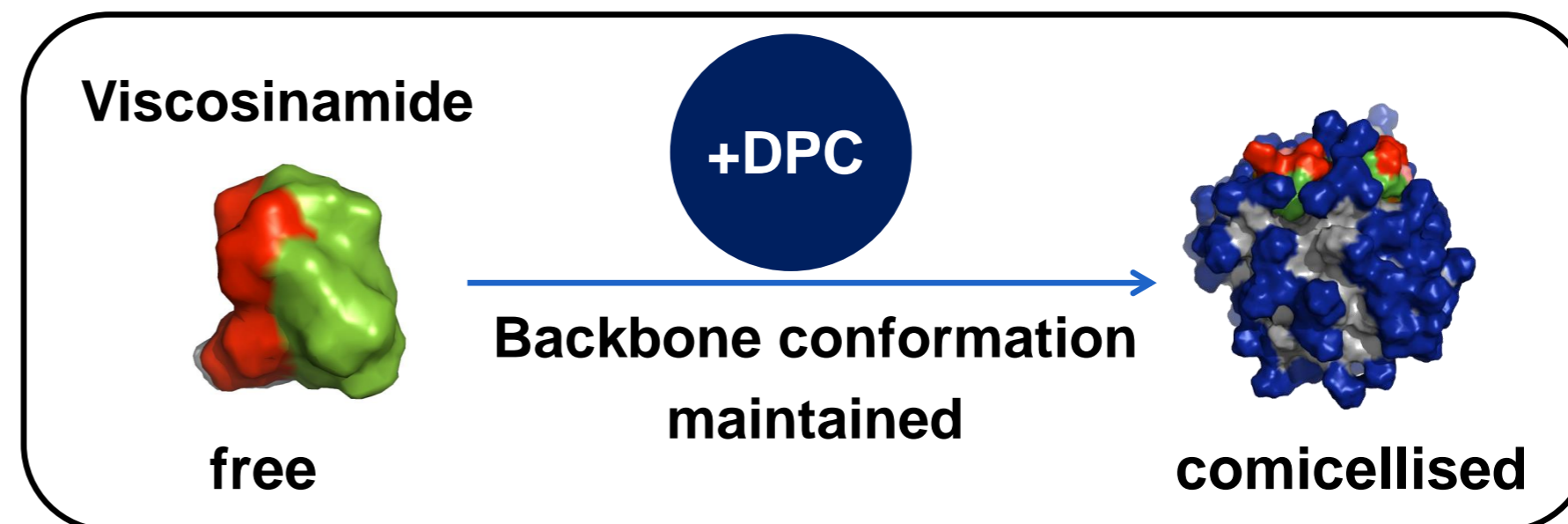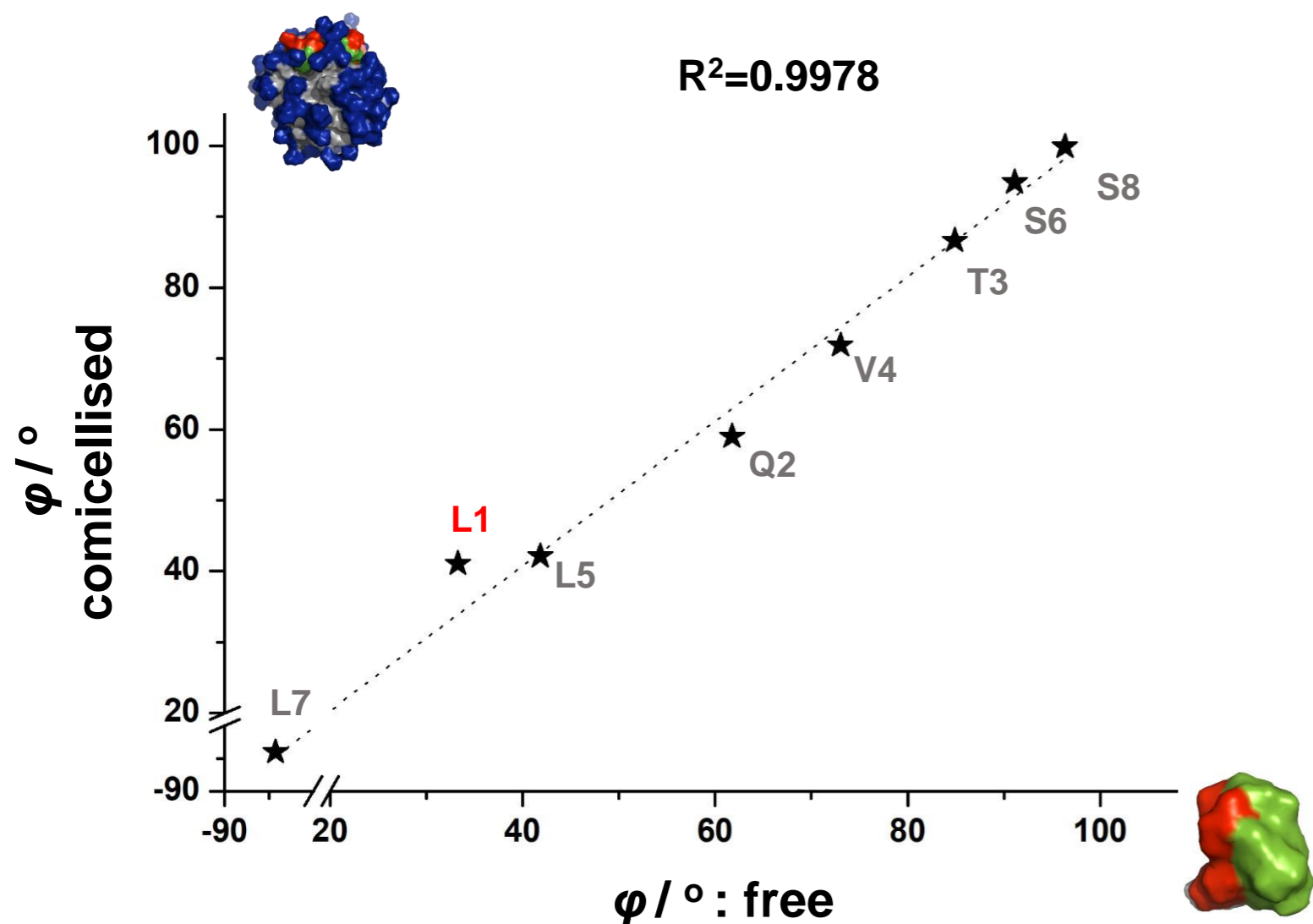
Stereochemistry: Ⓓ ☐L

- **Karplus curves:** Wang, A.,; Bax, A., *J. A. Chem. Soc.,* 118,2483–2494 (1996)

☐**L** : $^3J_{HN-H\alpha} = 6.98 \cos^2(\varphi - 60) - 1.38 \cos(\varphi - 60) + 1.72 \longrightarrow$ max. 10 Hz $\longrightarrow$ $^3J_{HN-H\alpha}$ **(I9)** > 10 Hz

Ⓓ : $^3J_{HN-H\alpha} = 6.98 \cos^2(\varphi + 60) - 1.38 \cos(\varphi + 60) + 1.72$

	L1	Q2	T3	V4	L5	S6	L7	S8
Δφ/°	7.77	-2.75	1.73	-1.15	0.31	3.76	0.65	3.54

- Highest φ change: for Leu1 (7.8°); others φ-s : < |4.0°|

R²=0.9978

- ψ angles? : similarly, from $^3J_{N-H}$ (HNHB experiment)

Viscosinamide

+DPC

Backbone conformation maintained

free → comicellised

H-BOND PATTERN

- **long range HNCO** experiment → N–H[···]O=C H-bonds

- <u>HNCO</u>: $H^N \xrightarrow{^1J_{\text{H-N}}} {}^{15}N \xrightarrow{^1J_{\text{N-C'}}} {}^{13}C' \xrightarrow{^1J_{\text{N-C'}}} {}^{15}N \xrightarrow{^1J_{\text{H-N}}} H^N$ (detection)
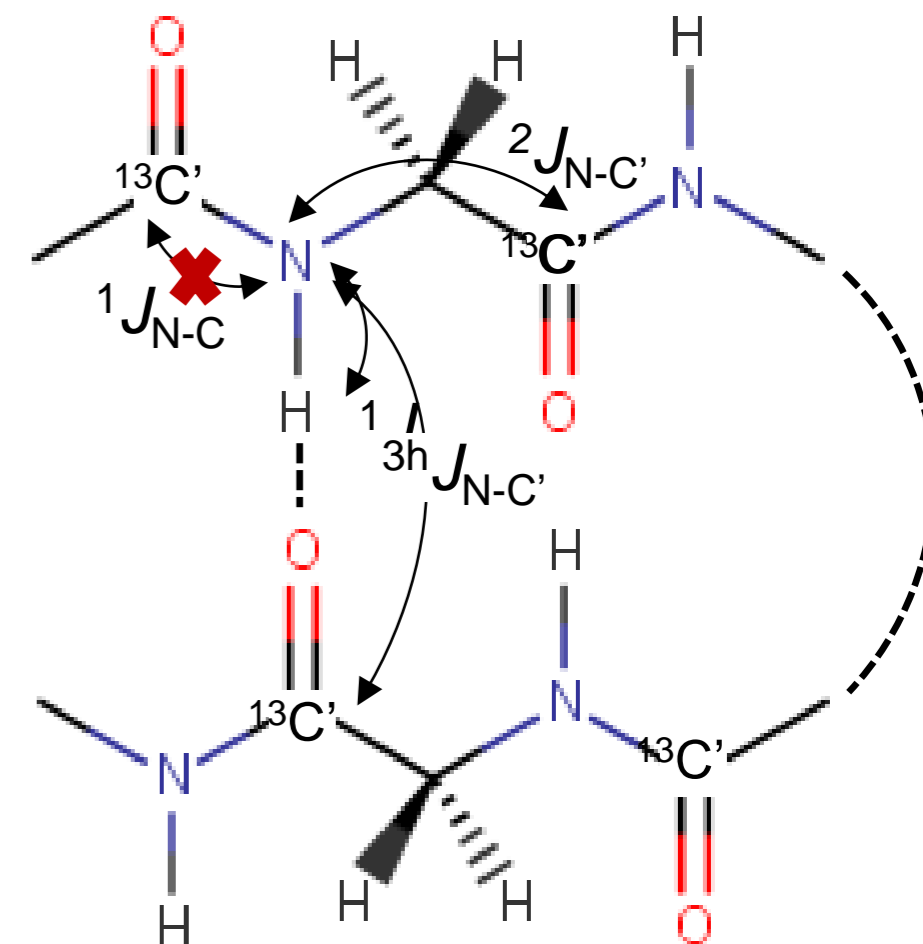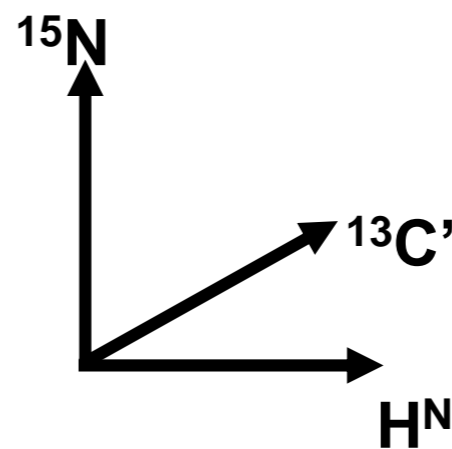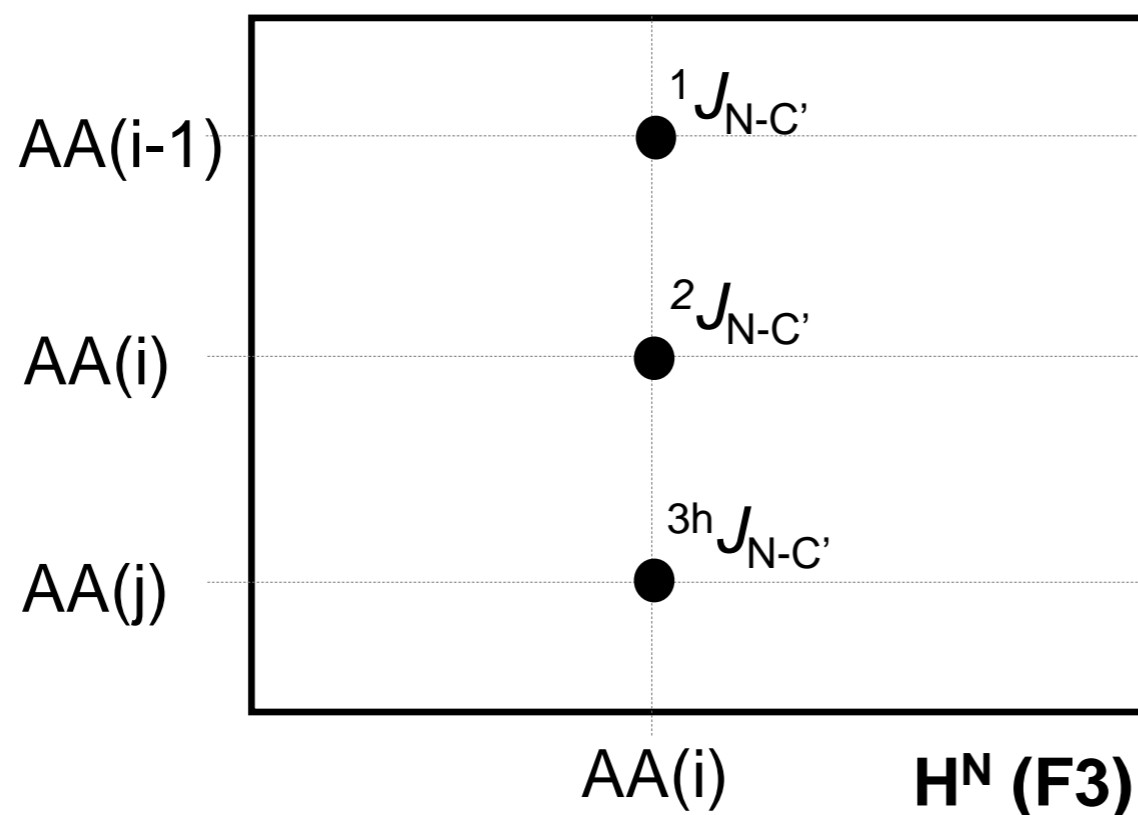
- If 'long' $\quad {}^{15}N \to {}^{13}C'$ INEPT
  $\quad\quad\quad {}^{13}C' \to {}^{15}N$ rev-INEPT

delays → effect of
- weak $^{3h}J_{\text{N-C'}}$ active
- strong $^1J_{\text{N-C'}}$ drops
- (weak $^2J_{\text{N-C'}}$ also visible)

<u>H^N-C' 2D plane of
LR HNCO spectrum:</u>

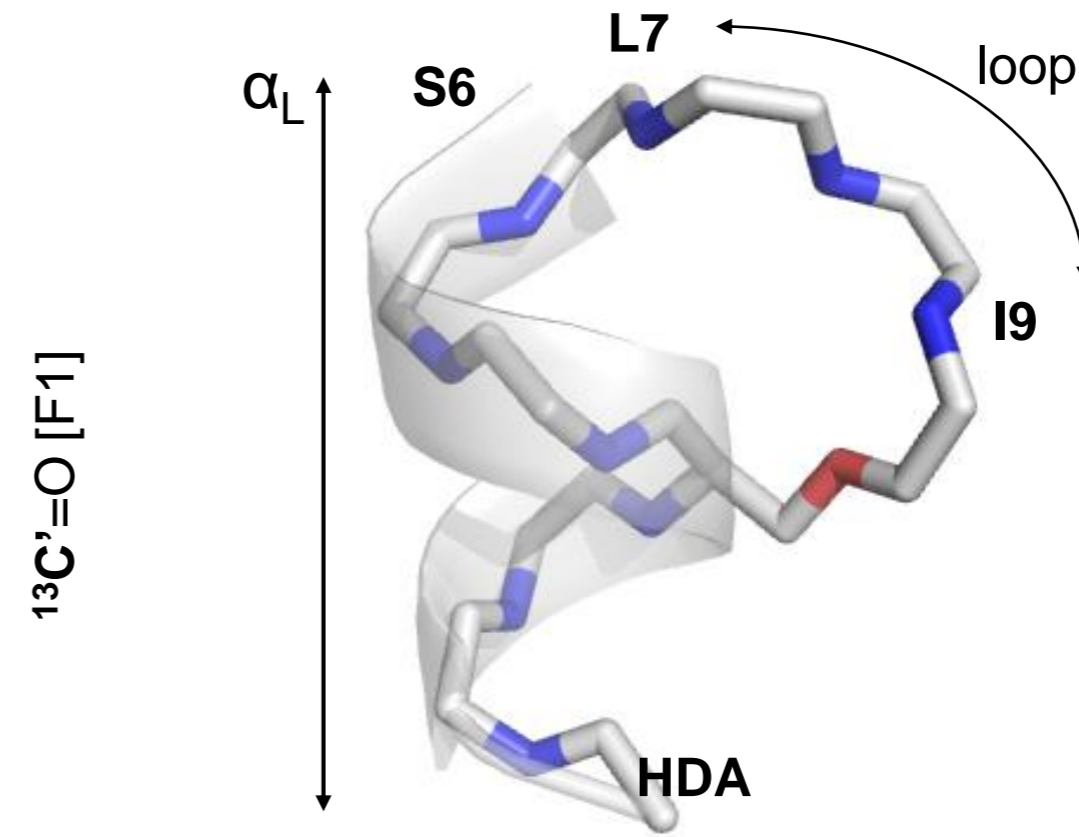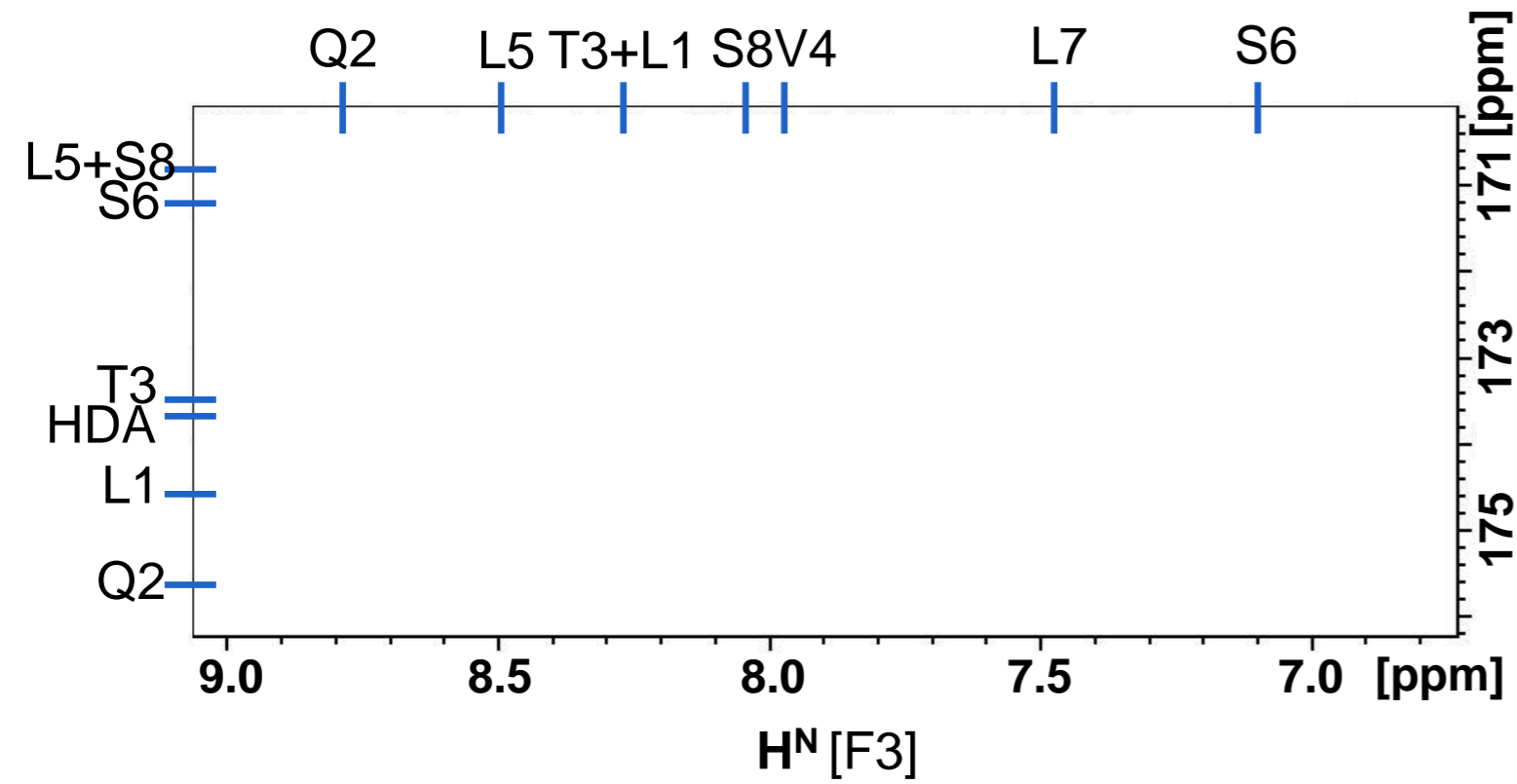- Max. 3 C' cross peaks to each H^N
  (effect of $^1J_{\text{N-C'}}$ breaks through)
- Here: N–H(i)[···]O=C(j) H-bond

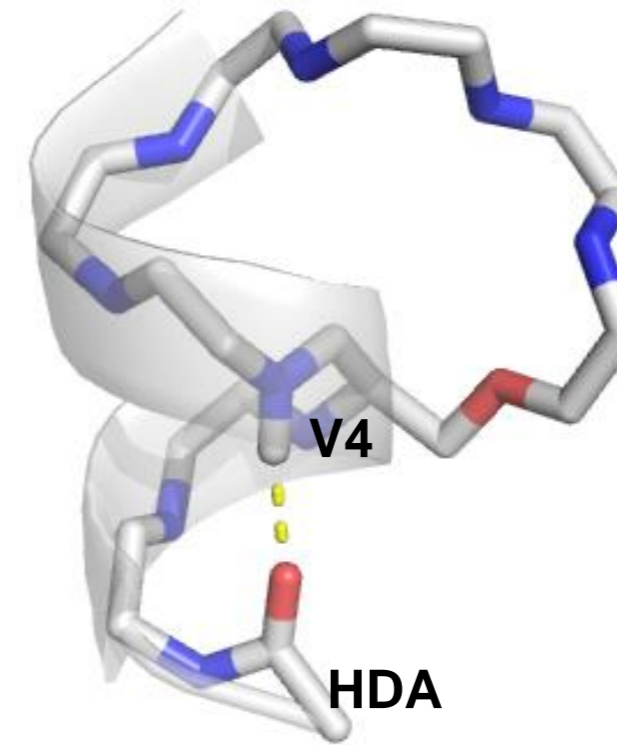- Cordier, F. *et. al.*, *Nat. Protoc.*, 3,235–241 (2008)

H-BOND PATTERN
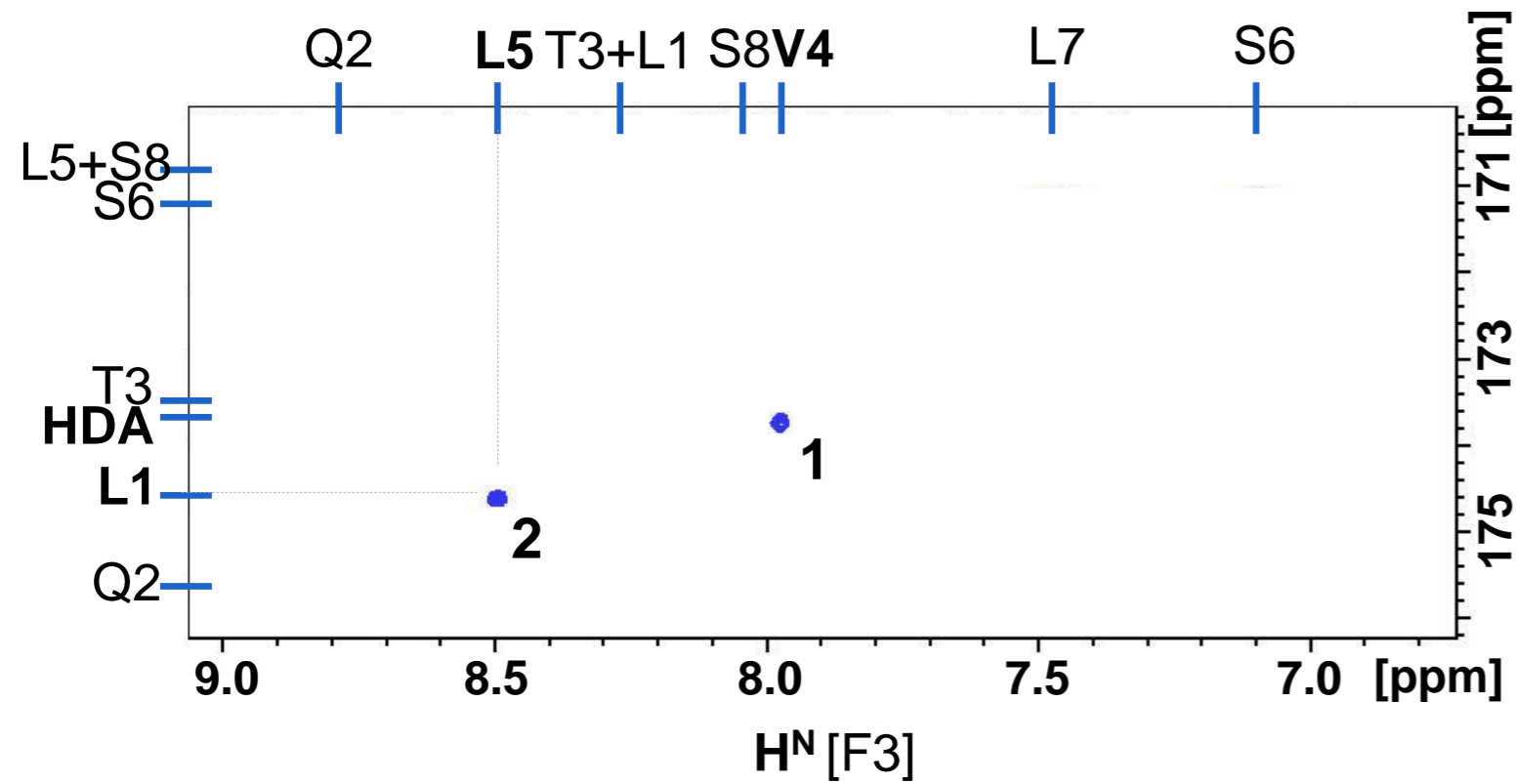
- **VA** in **free** state:

H-BOND PATTERN

- **VA** in **free** state:
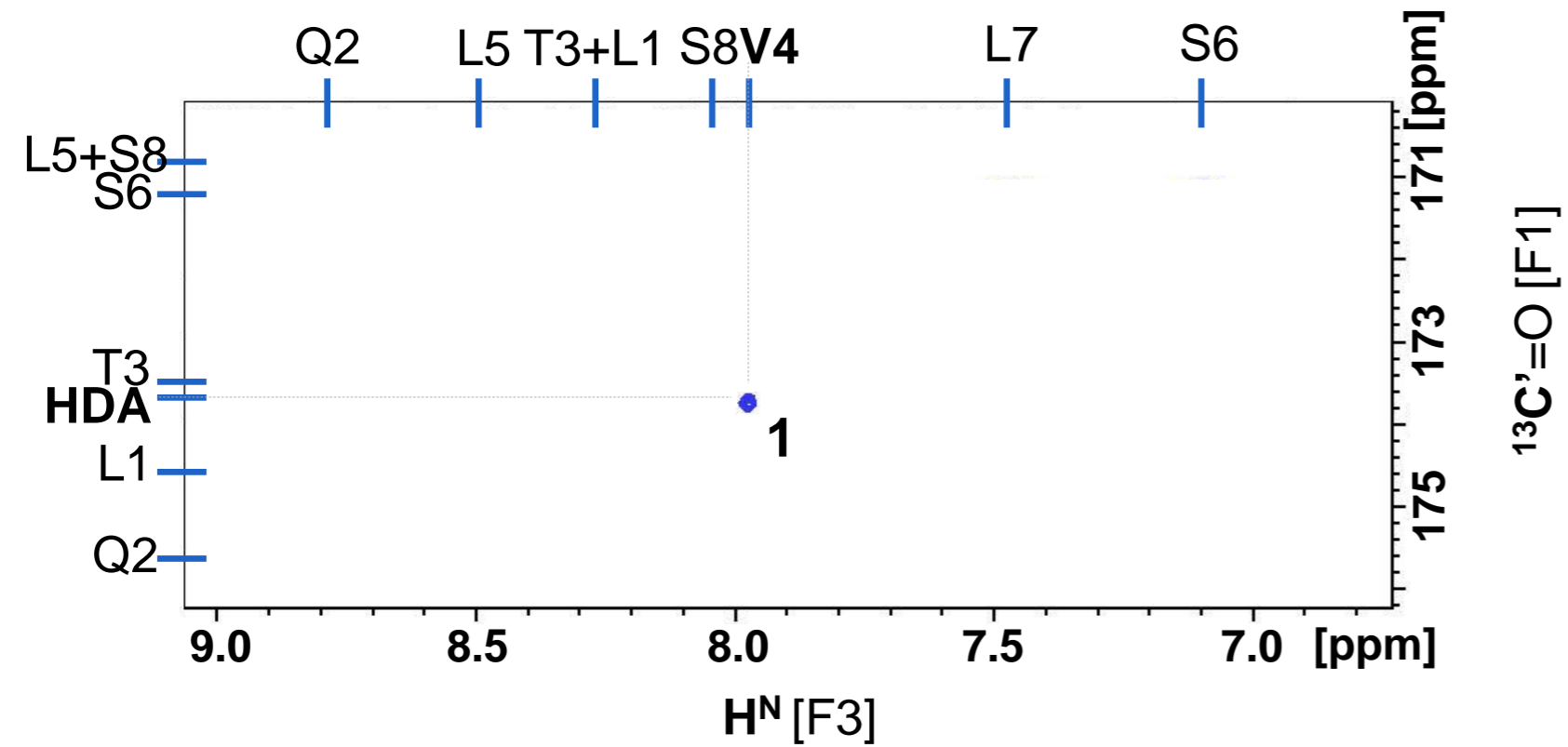
N-H[···]O=C #	AA-s
1	**V4**(H^N) $\rightarrow$ O=C **HDA**

H-BOND PATTERN

- **VA** in **free** state:
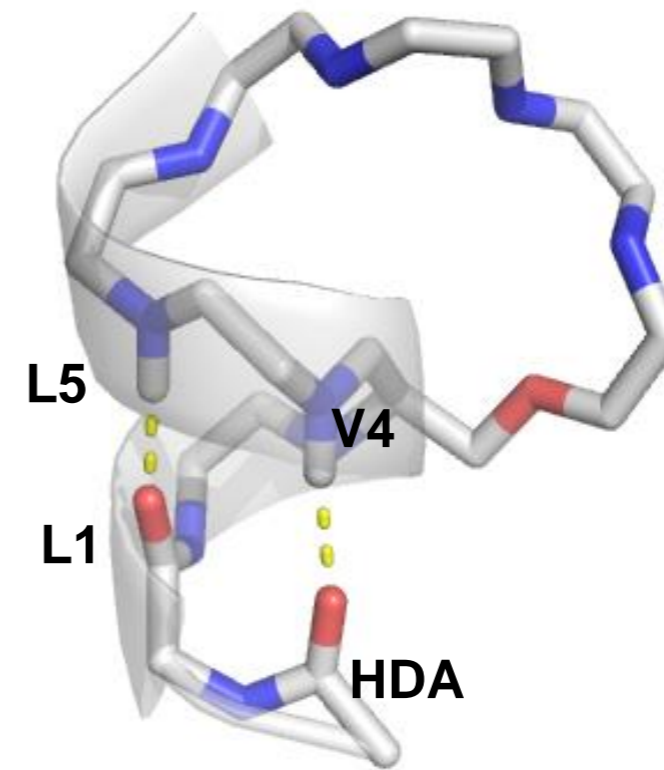
N-H[···]O=C #	AA-s
1	**V4**(H^N) → O=C **HDA**
2	**L5**(H^N) → O=C **L1**

H-BOND PATTERN

- **VA** in **free** state:
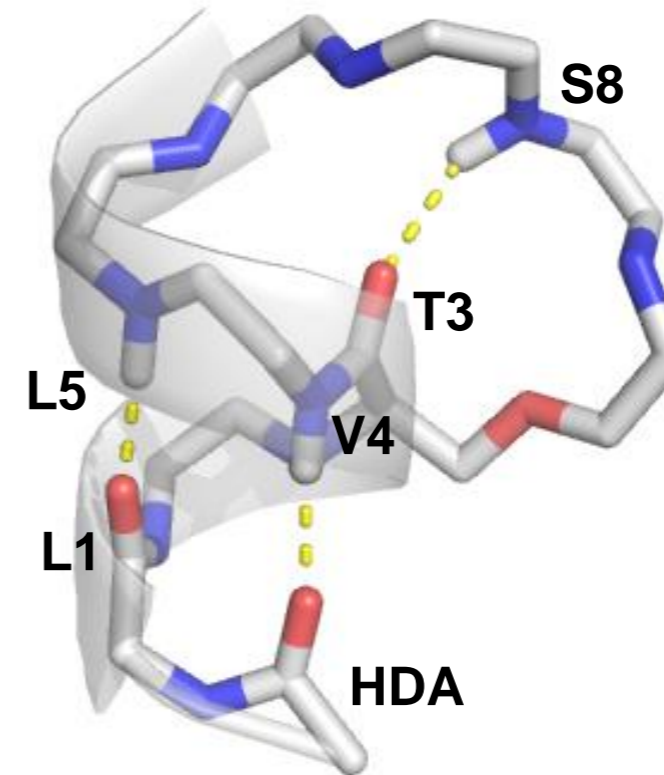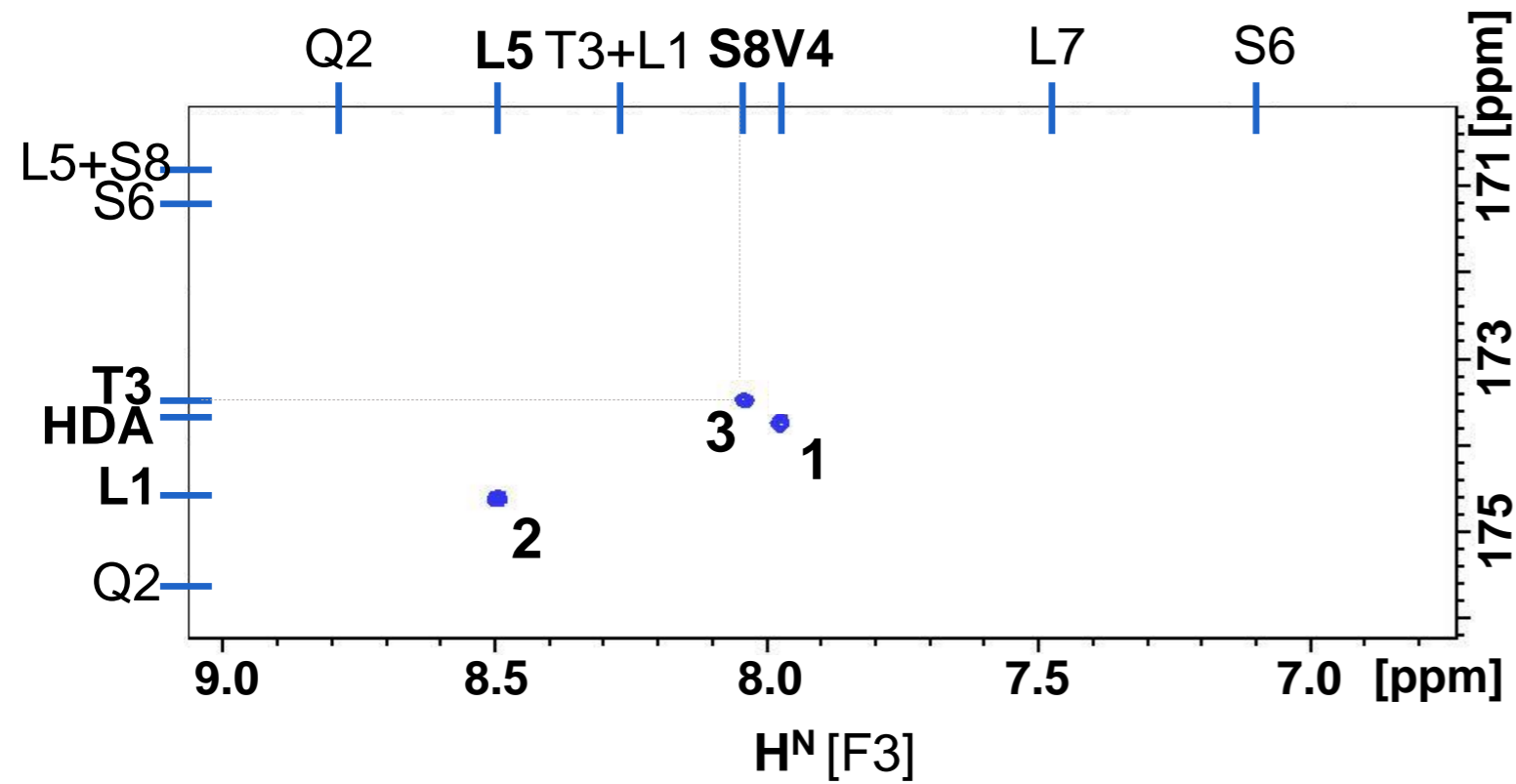
N-H[⋯]O=C #	AA-s
1	**V4**(H^N) $\rightarrow$ O=C **HDA**
2	**L5**(H^N) $\rightarrow$ O=C **L1**
3	**S8**(H^N) $\rightarrow$ O=C **T3**

H-BOND PATTERN
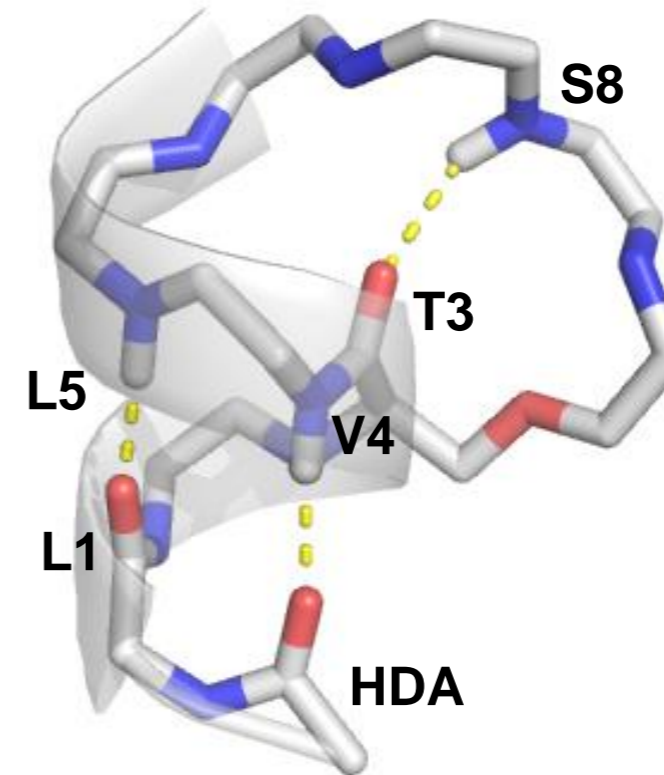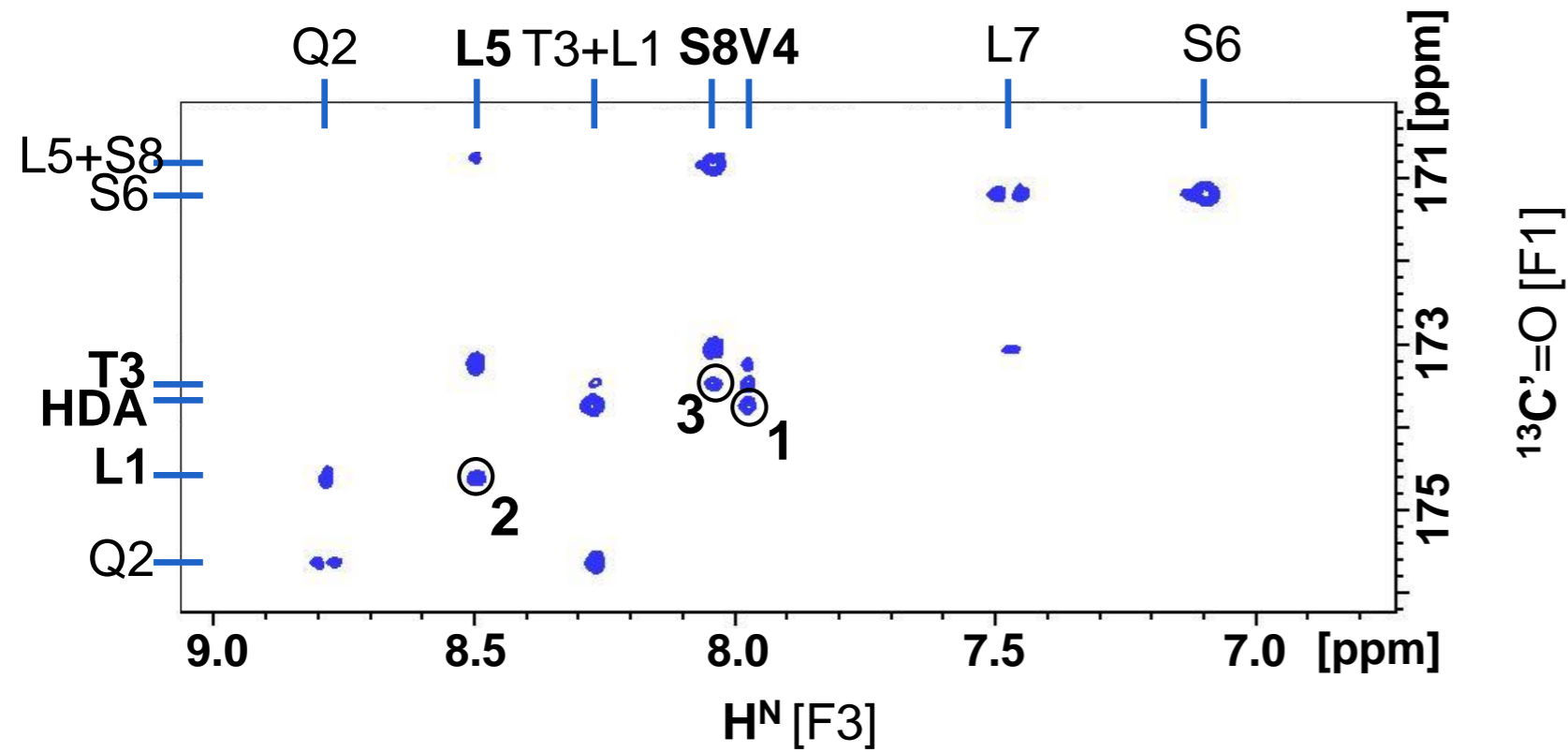
- **VA** in **free** state:

- $^{2}J_{N-C'}$ and $^{1}J_{N-C'}$ cross peaks are also visible

N-H[···]O=C #	AA-s
1	**V4**(H^N) $\rightarrow$ O=C **HDA**
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3	**S8**(H^N) $\rightarrow$ O=C **T3**

H-BOND PATTERN

- **VA** in **free** state:

Q2   **L5** T3+L1 **S8V4**   L7   S6
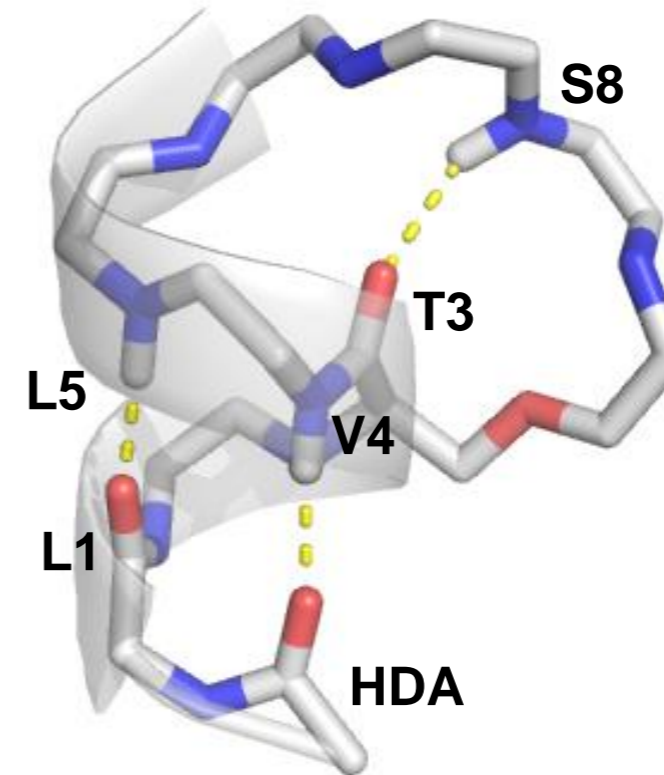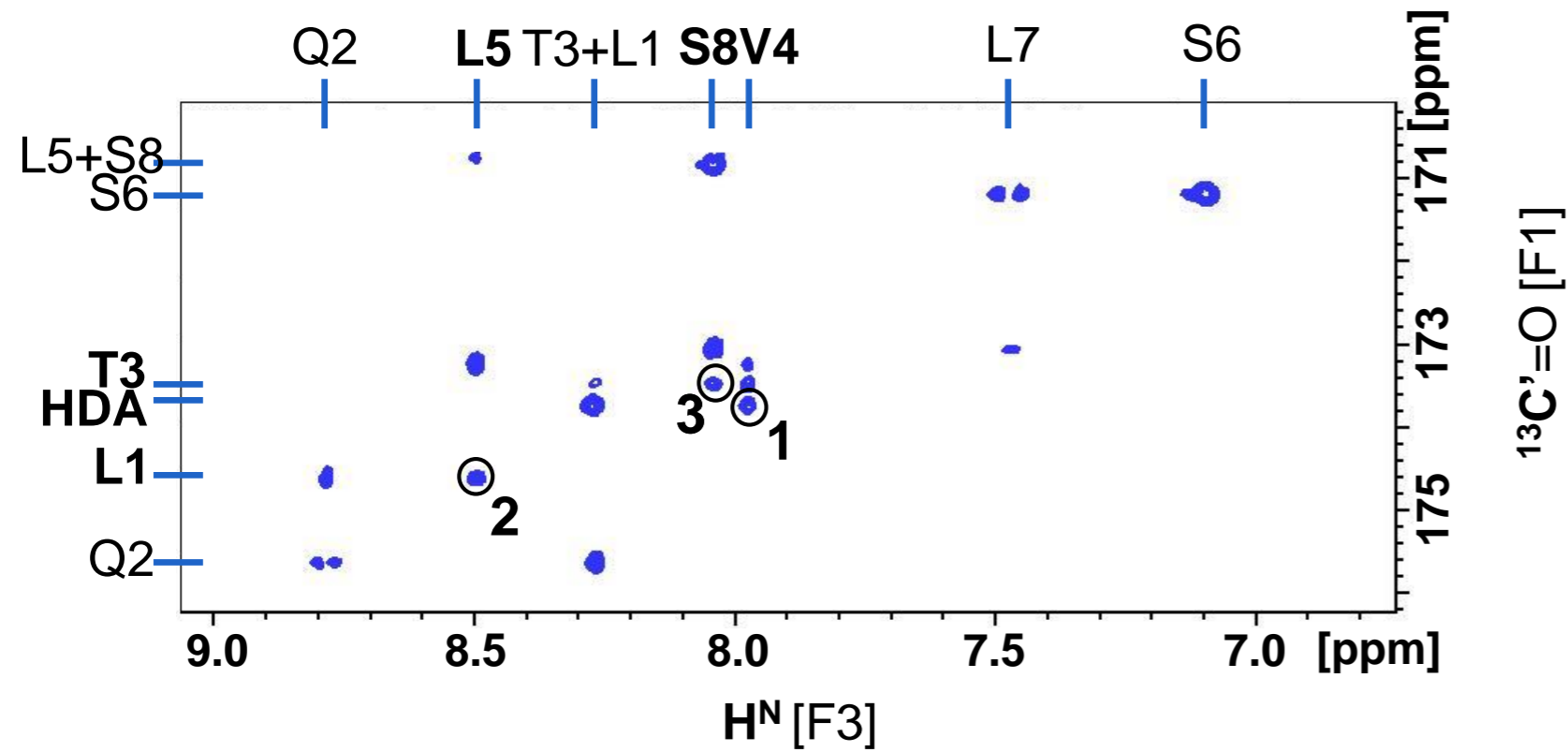
lifetime of H-bond:

$$\tau \geq \frac{1}{\left|{}^{3h}J_{N-C'}\right|} \sim s$$

- ${}^{2}J_{N-C'}$ and ${}^{1}J_{N-C'}$ cross peaks are also visible

N–H[···]O=C #	AA-s	${}^{3h}J_{N-C'}$/ Hz
1	**V4**(H^N) → O=C **HDA**	−0.30
2	**L5**(H^N) → O=C **L1**	−0.44
3	**S8**(H^N) → O=C **T3**	−0.25

From reference spectrum: ${}^{1}J_{NC'}$ = −15 Hz is assumed

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H-BOND PATTERN
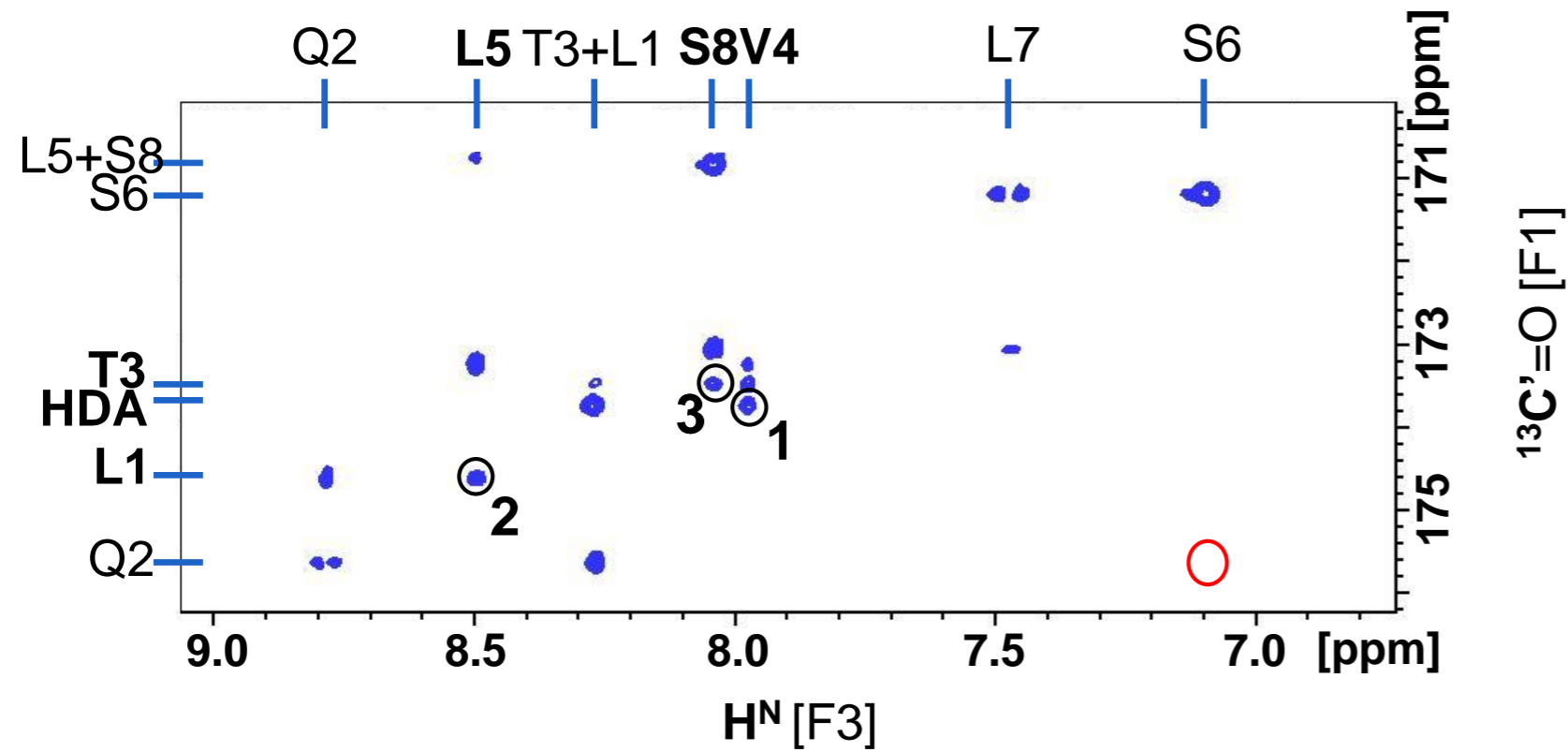
- **VA** in **free** state:

? S6 H^N → O=C Q2

- $^2J_{N-C'}$ and $^1J_{N-C'}$ cross peaks are also visible
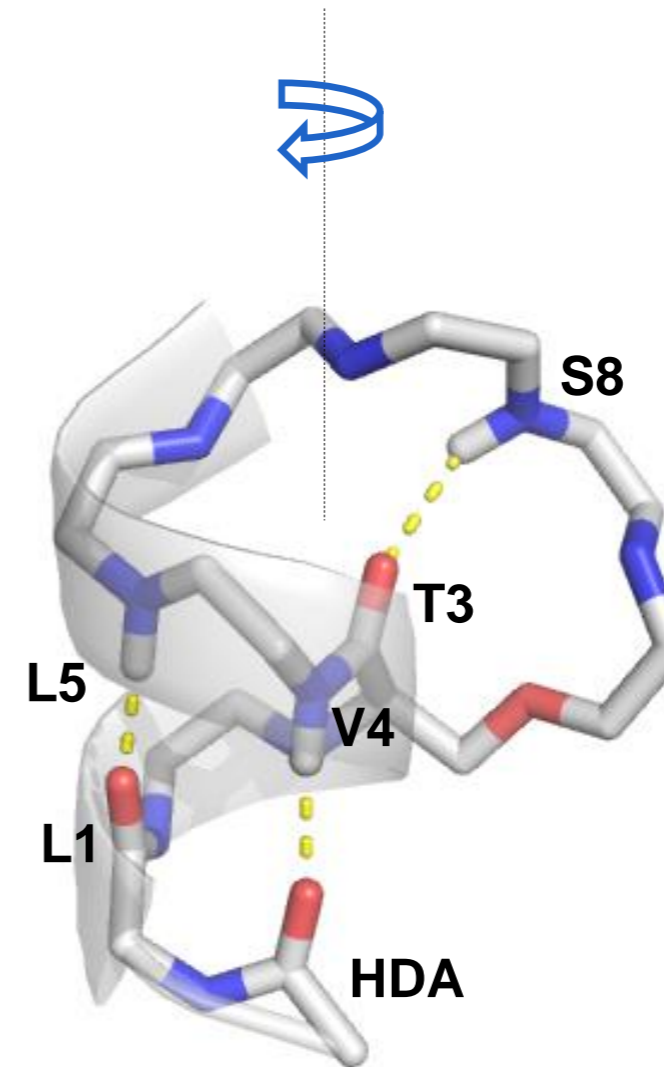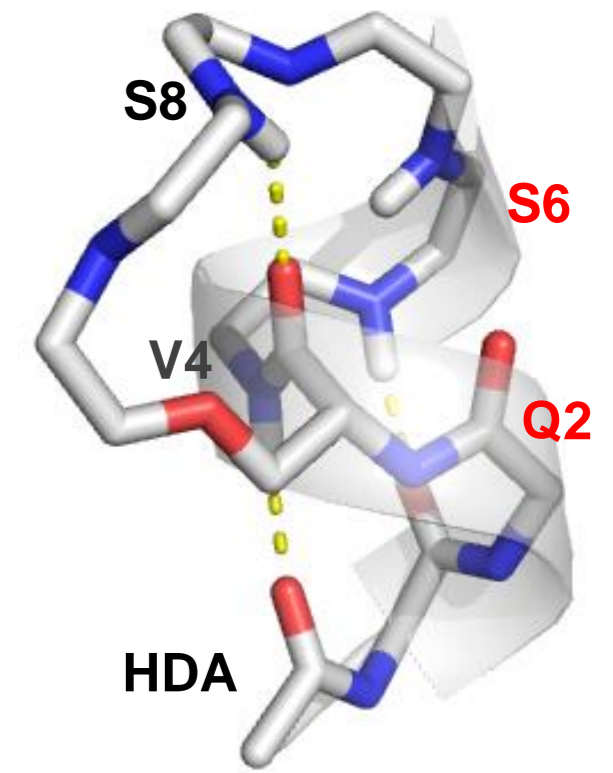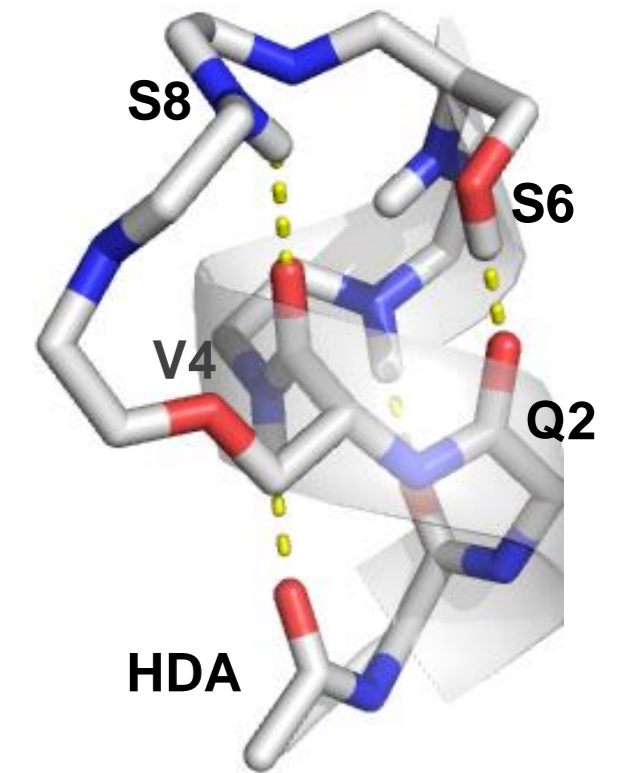
MD simulation in AcN

N-H[···]O=C #	AA-s	$^{3h}J_{N-C'}$/ Hz
1	**V4**(H^N) → O=C **HDA**	−0.30
2	**L5**(H^N) → O=C **L1**	−0.44
3	**S8**(H^N) → O=C **T3**	−0.25

H-BOND PATTERN

- **VA** in **free** state:

S6 OH → O=C Q2

MD simulation in AcN

- $^2J_{N-C'}$ and $^1J_{N-C'}$ cross peaks are also visible

N-H[···]O=C #	AA-s	$^{3h}J_{N-C'}$/ Hz
1	**V4**(H^N) → O=C **HDA**	−0.30
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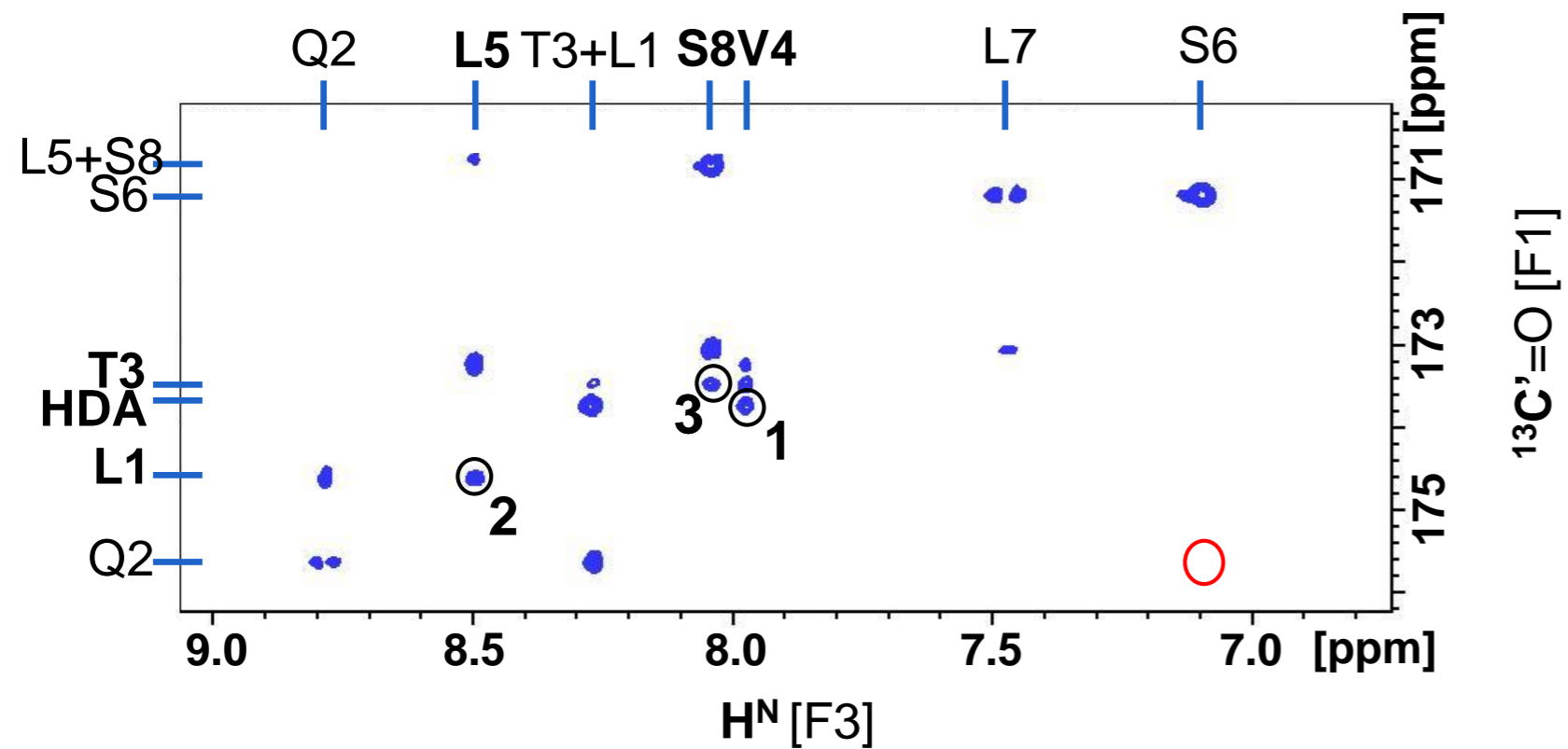
H-BOND PATTERN

- **VA** in **free** state:

S6 OH → O=C Q2
S8 OH → OH S6
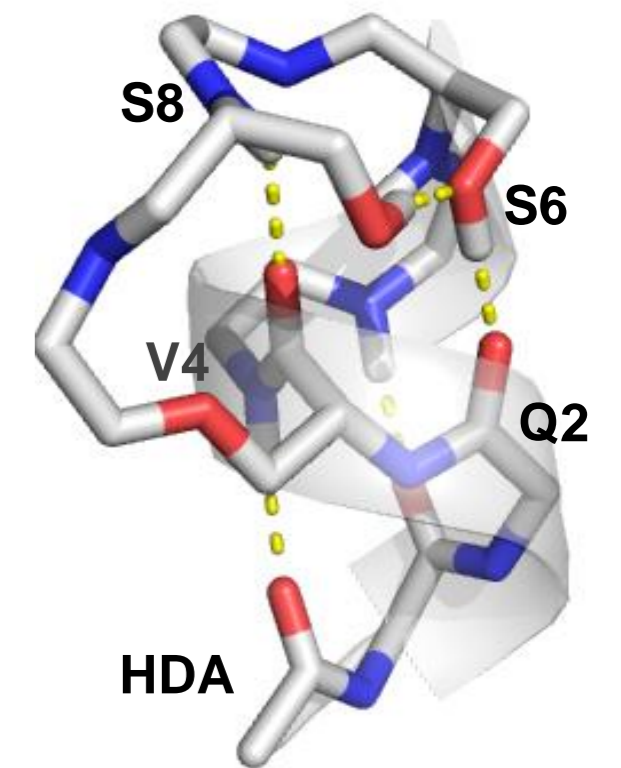
**MD simulation
in AcN**

↓

rigid!

- $^2J_{N-C'}$ and $^1J_{N-C'}$ cross peaks are also visible

N-H[···]O=C #	AA-s	$^{3h}J_{N-C'}$/ Hz
1	**V4**(H^N) → O=C **HDA**	−0.30
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H-BOND PATTERN

- **VA** in **free** state:
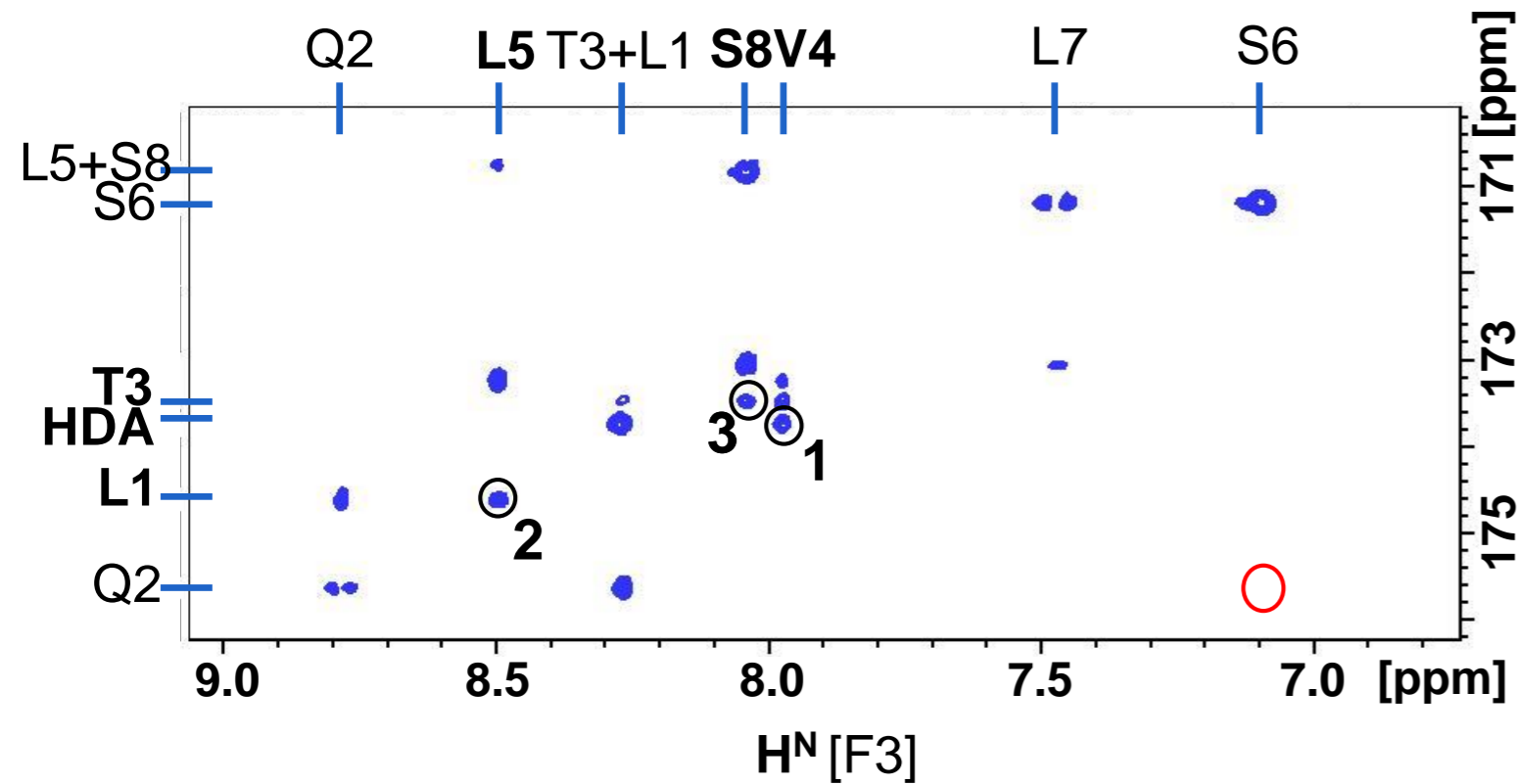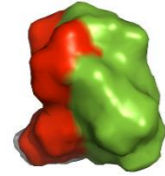
- **VA** in **comicellised** state:

- $^2J_{N-C'}$ and $^1J_{N-C'}$ cross peaks are also visible

N-H[···]O=C #	AA-s	$^{3h}J_{N-C'}$/ Hz : free	$^{3h}J_{N-C'}$/ Hz : comicellised
1	**V4**(H^N) → O=C **HDA**	−0.30	observed
2	**L5**(H^N) → O=C **L1**	−0.44	−0.43
3	**S8**(H^N) → O=C **T3**	−0.25	observed

* Quantification not possible due to peak overlaps

H-BOND PATTERN

- Experimental data: N–H [···] O=C H-bond pattern of VA is rigid upon transition

- MD simulations: Ser side-chain H-bonds pattern of VA is rigid upon transition
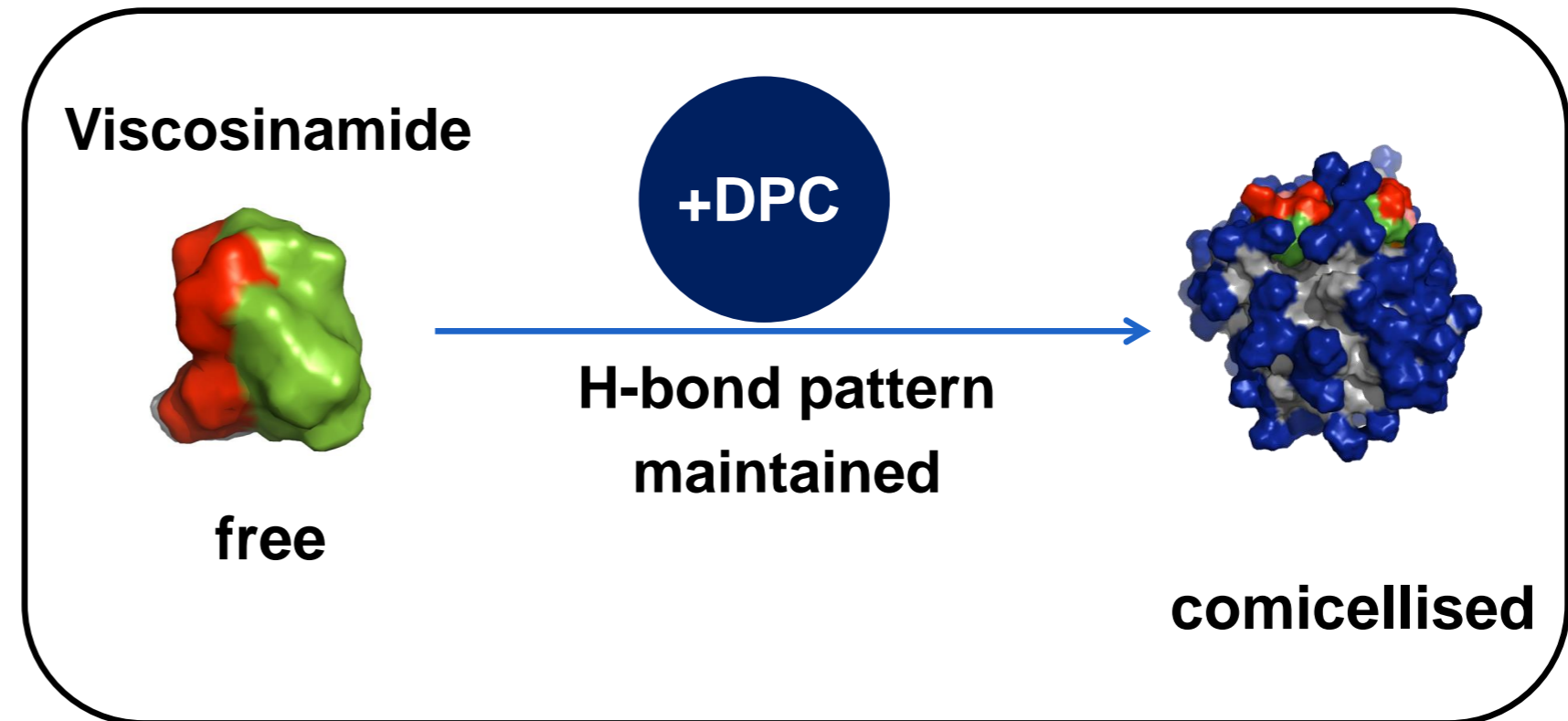

Viscosinamide

+DPC

H-bond pattern maintained

free

comicellised

N-H[···]O=C #	AA-s	$^{3h}J_{N-C'}$/ Hz : free	$^{3h}J_{N-C'}$/ Hz : comicellised
1	**V4**(H^N) → O=C **HDA**	−0.30	observed
2	**L5**(H^N) → O=C **L1**	−0.44	−0.43
3	**S8**(H^N) → O=C **T3**	−0.25	observed

SUMMARY

- CLiPs possess a wide bioactivity range → membrane interactions?

- VA structure upon interaction with model membrane system

→ Heteronuclear NMR spectroscopy

- Isotope enrichment: first ^{13}C-, ^{15}N-labelled CLiP

Viscosinamide

+DPC

Backbone conformation
H-bond pattern
maintained

free   **comicellised**
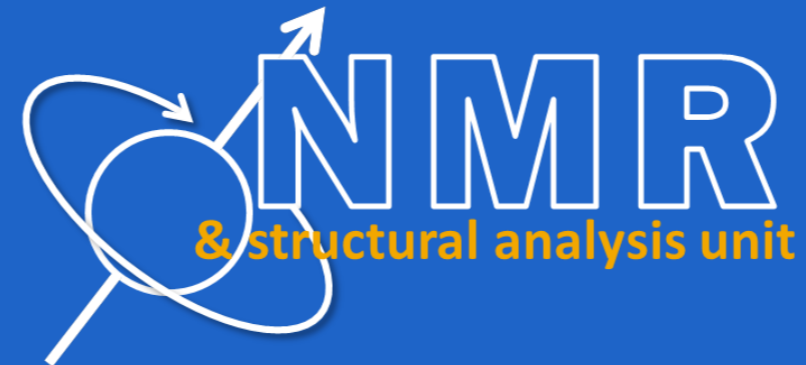
ACKNOWLEDGEMENTS
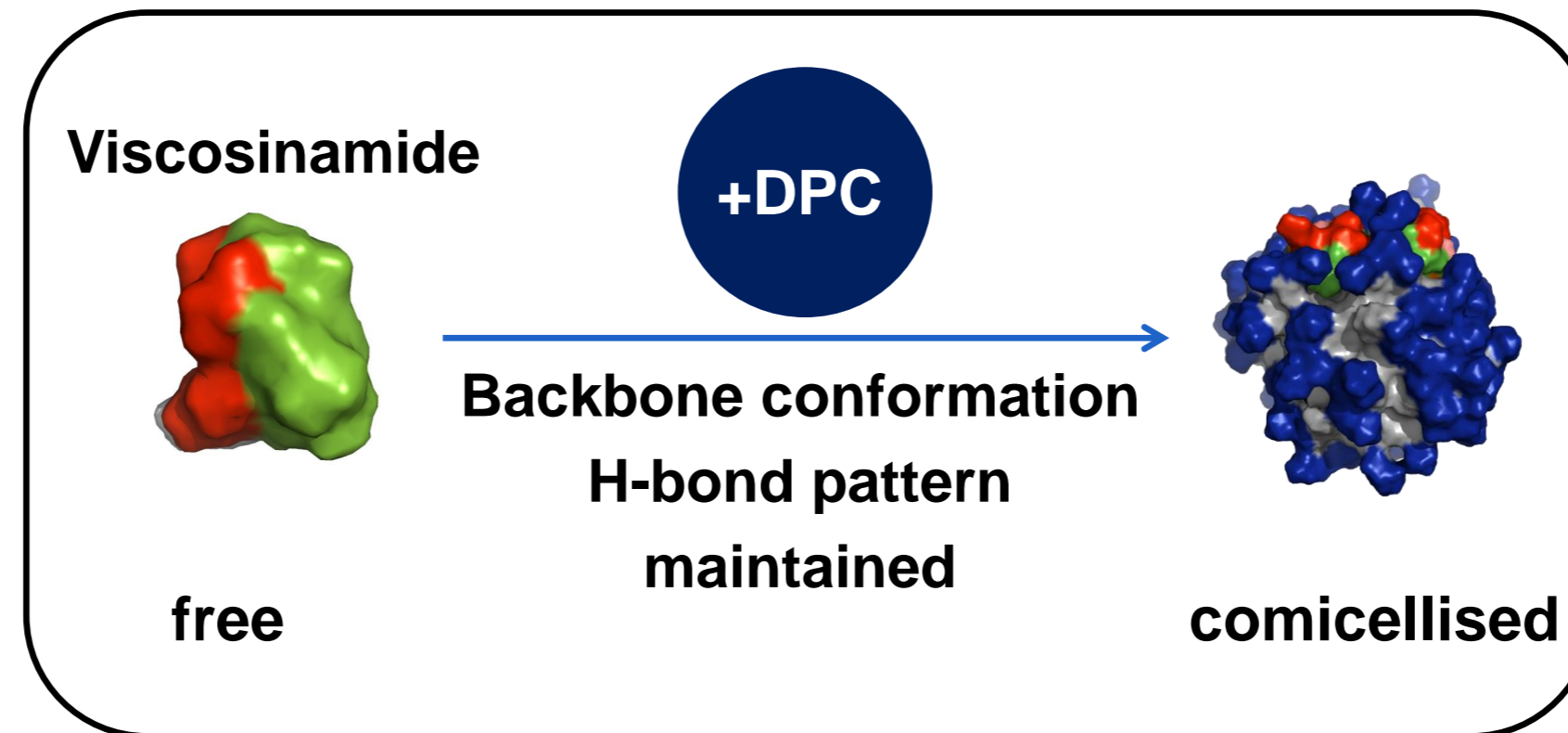
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https://www.ugent.be/we/orgchem/nmr-structure-analysis/en