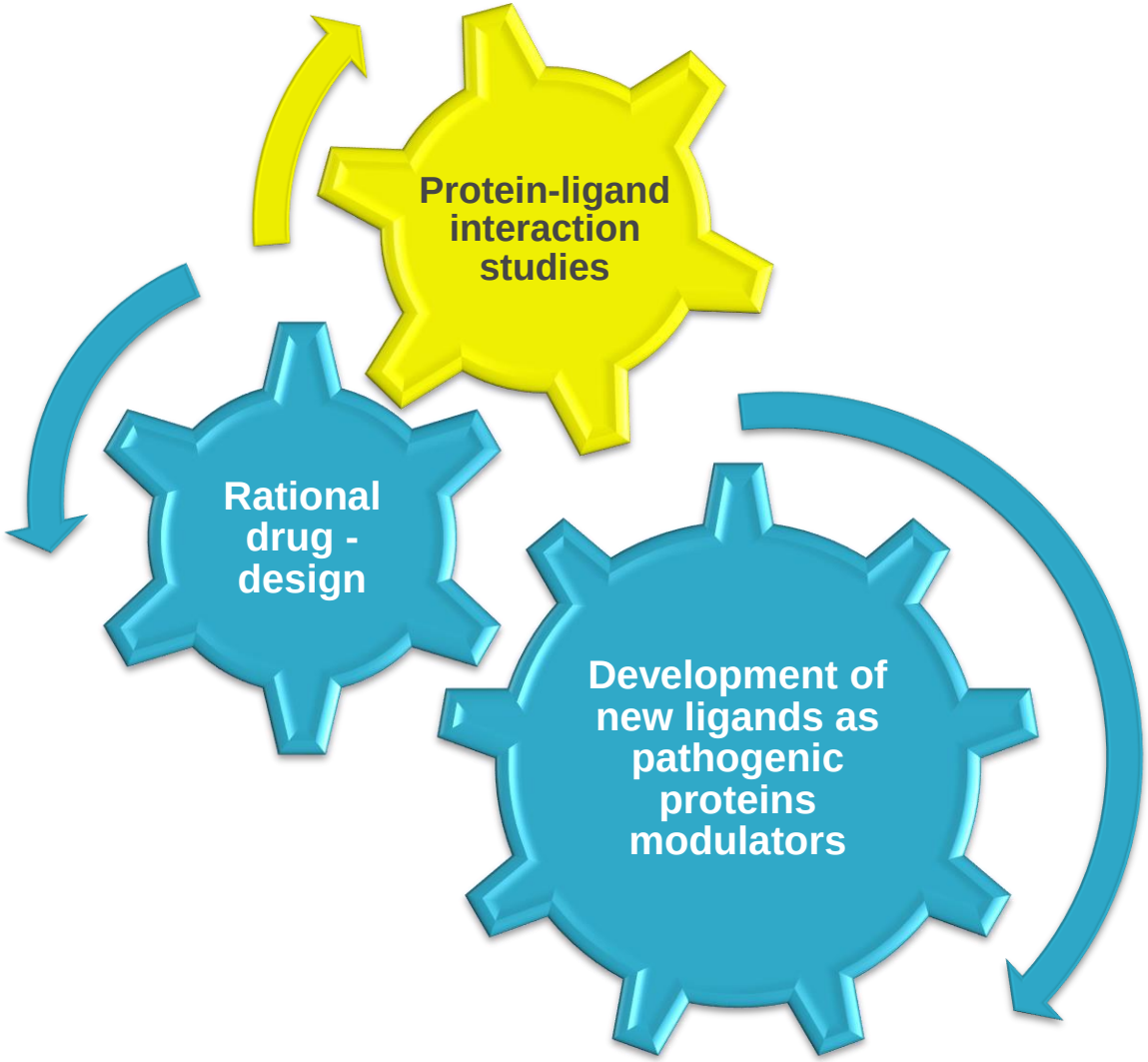
# BioNMR

# Structural Analysis in Biochemistry and Drug Discovery



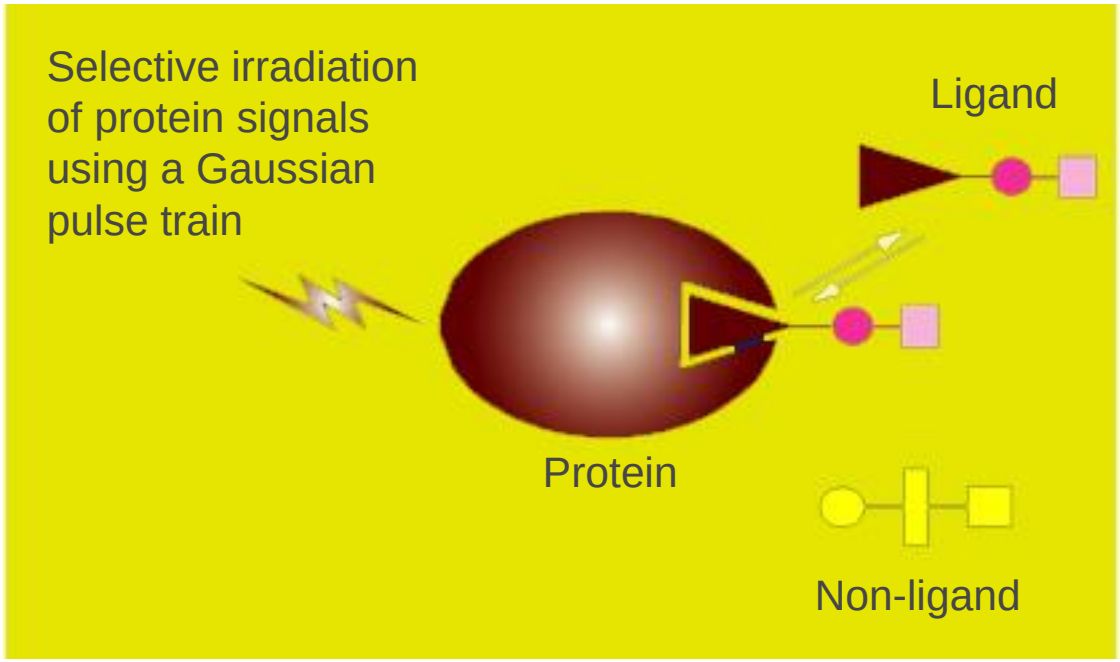
# NMR Techniques for Protein-Ligand Interaction Studies

Three different approaches:

1. experiments based on complex observation
   - solution of the complex structure
   - big amount of $^{13}C$ and $^{15}N$-labelled protein required
   - time-demanding (months)
2. experiments based on protein observation
   - ligand binding-site identification
   - complete protein resonance assignment
   - moderate amount of $^{15}N$-labelled protein
   - several weeks of work
3. experiments based on ligand observation
   - screening of libraries of potential ligands
   - information about ligand binding mode
   - very small amount of non-labelled protein required
   - very fast analysis

# Experiments Based on Ligand Observation

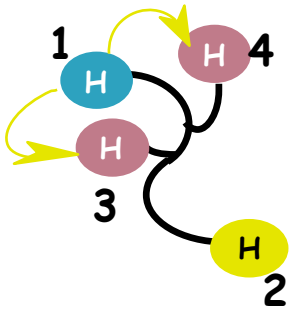
# Saturation Transfer Difference experiments (STD)



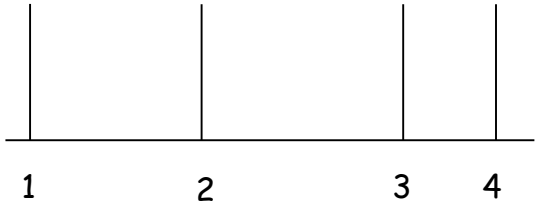

Identification of ligands

Epitope mapping

# Saturation Transfer Difference experiments (STD)

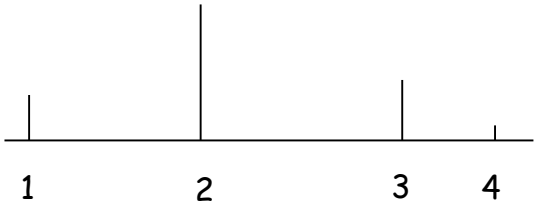



off resonance spectrum




-

on resonance spectrum

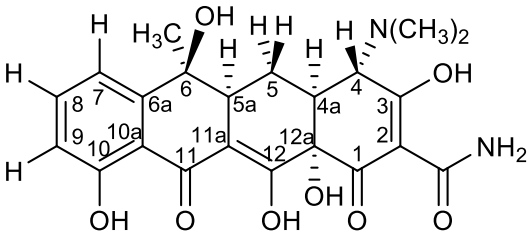



=

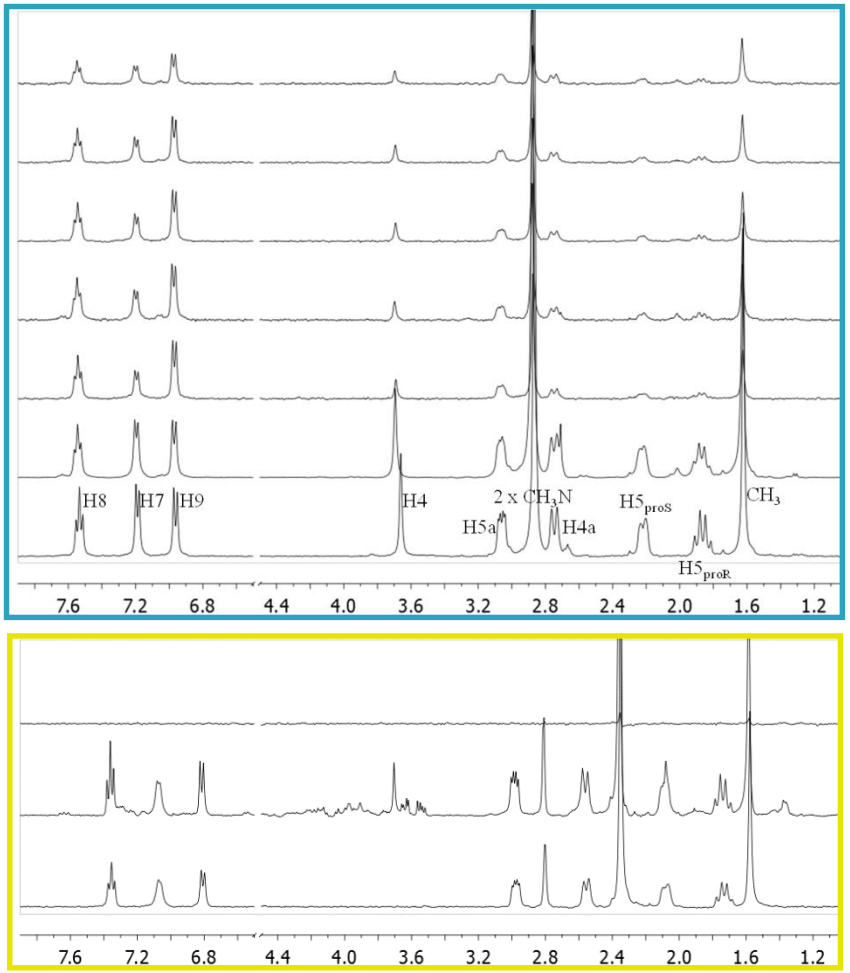
1D-STD spectrum

# Saturation Transfer Difference (STD) NMR experiments

Aβ oligomers
+




+
Aβ monomers

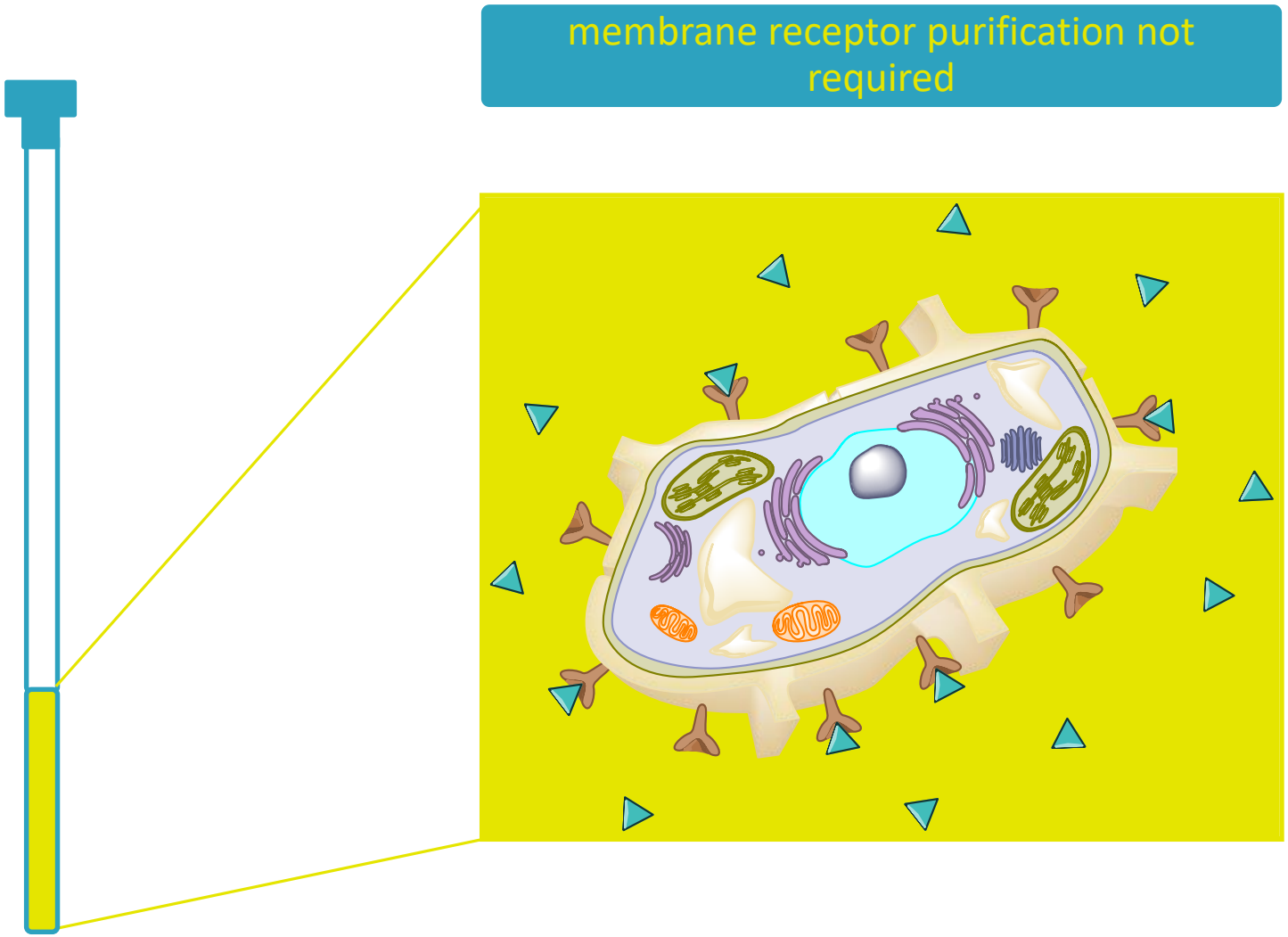



1D STD

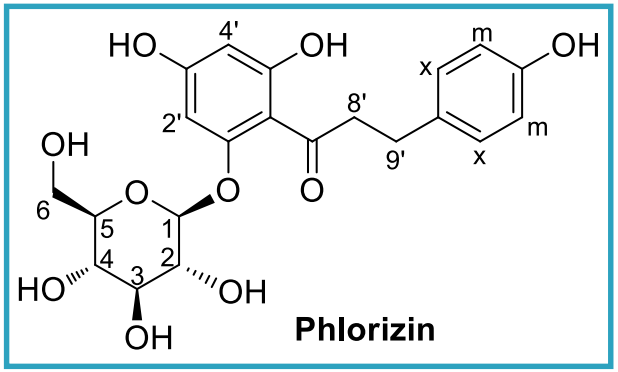
1D STD

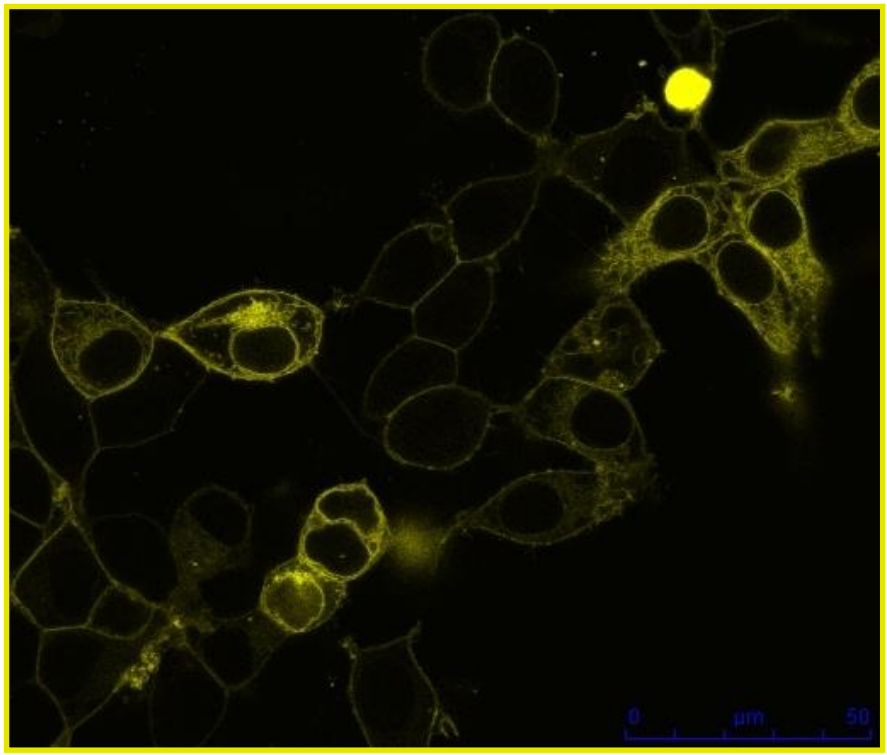
no interaction with Aβ monomers
interaction with Aβ oligomers
but no tetracycline epitope-mapping

# NMR Characterization of Membrane Receptor–Ligand Interaction on Living Cells

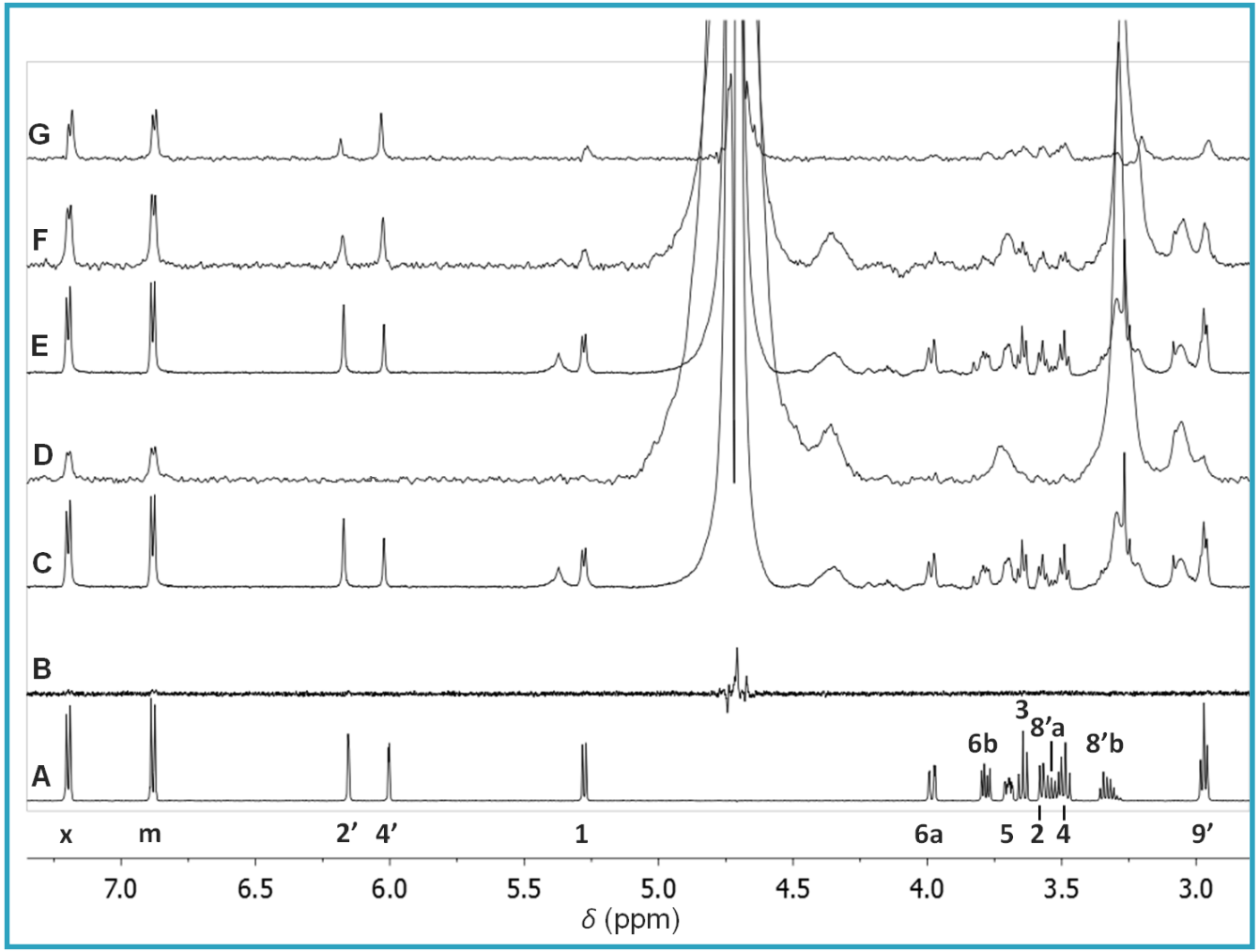


# INTERACTION OF COTRASPORTER SGLT1 WITH PHLORIZIN



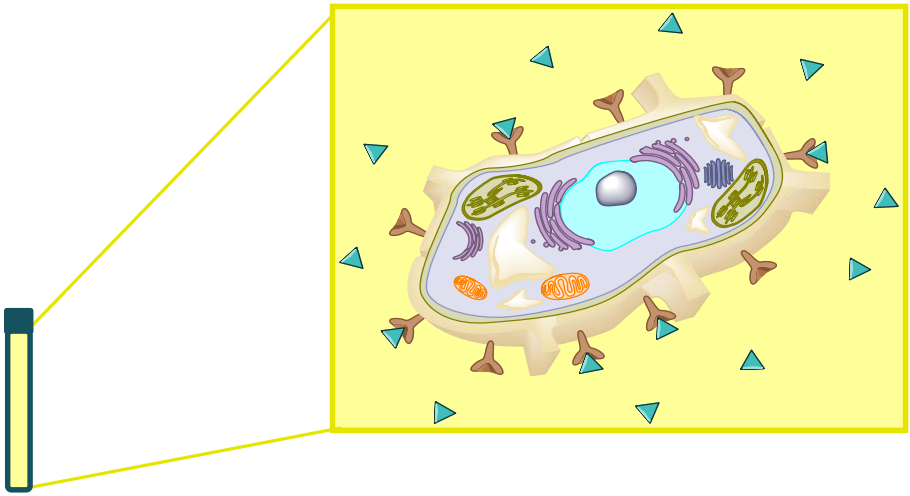
Fluorescence confocal image of living tsA201 cells 48 h after the transfection with EYFP-hSGLT1. Cell culture media is replaced with extracellular control solution before image acquisition.

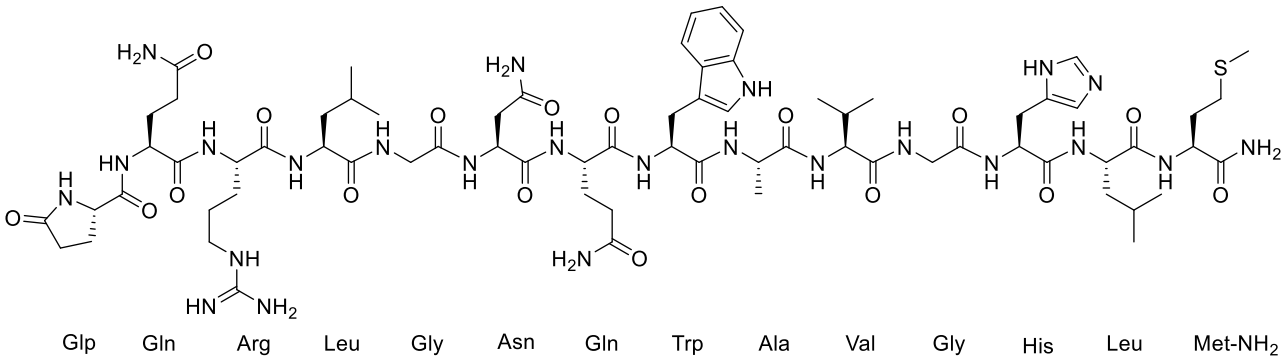



A) $^{1}$H spectrum of a sample containing 6mM phlorizin, deuterated PBS, pH 7.4, 64 scans; B) 1D-STD spectrum of a sample containing 6mM phlorizin, deuterated PBS, pH 7.4, selective irradiation frequency 0.0 ppm, 512 scans, signal enhanced 4x; C) $^{1}$H spectrum of a sample containing 3mM phlorizin and ca. $10^{6}$ tsA201 cells transfected with an empty pcDNA3 plasmid, deuterated PBS, pH 7.4, 64 scans, signal enhanced 2x; D) 1D-STD spectrum of a sample containing 3mM phlorizin and ca. $10^{6}$ tsA201 cells transfected with an empty pcDNA3 plasmid, deuterated PBS, pH 7.4, selective irradiation frequency 0.0 ppm, 512 scans, signal increased 4x; E) $^{1}$H spectrum of a sample containing 3mM phlorizin and ca. $10^{6}$ tsA201 cells transfected with the EYFP-hSGLT1 construct, deuterated PBS, pH 7.4, 64 scans, signal enhanced 2x; F) 1D-STD spectrum of a sample containing 3mM phlorizin and ca. $10^{6}$ tsA201 cells transfected with the EYFP-hSGLT1 construct, deuterated PBS, pH 7.4, selective irradiation frequency 0.0 ppm, 512 scans, signal increased 4x; G) STDD spectrum obtained by subtracting spectrum (D) from (F).

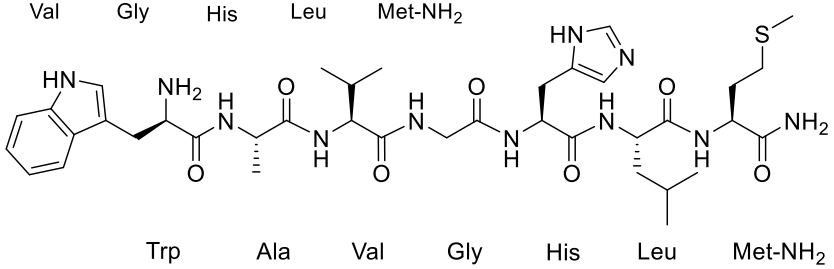
# NMR Binding Studies on Entire Tumor Cells: Bombesin Interaction with Gastrin-Releasing Peptide Receptor

GRPR are **transmembrane G-proteins** coupled receptors that trigger different signaling transduction pathways, resulting, among which, in the stimulation of **cell proliferation**. GRPRs are significantly involved in the pathogenesis of **different human cancers**





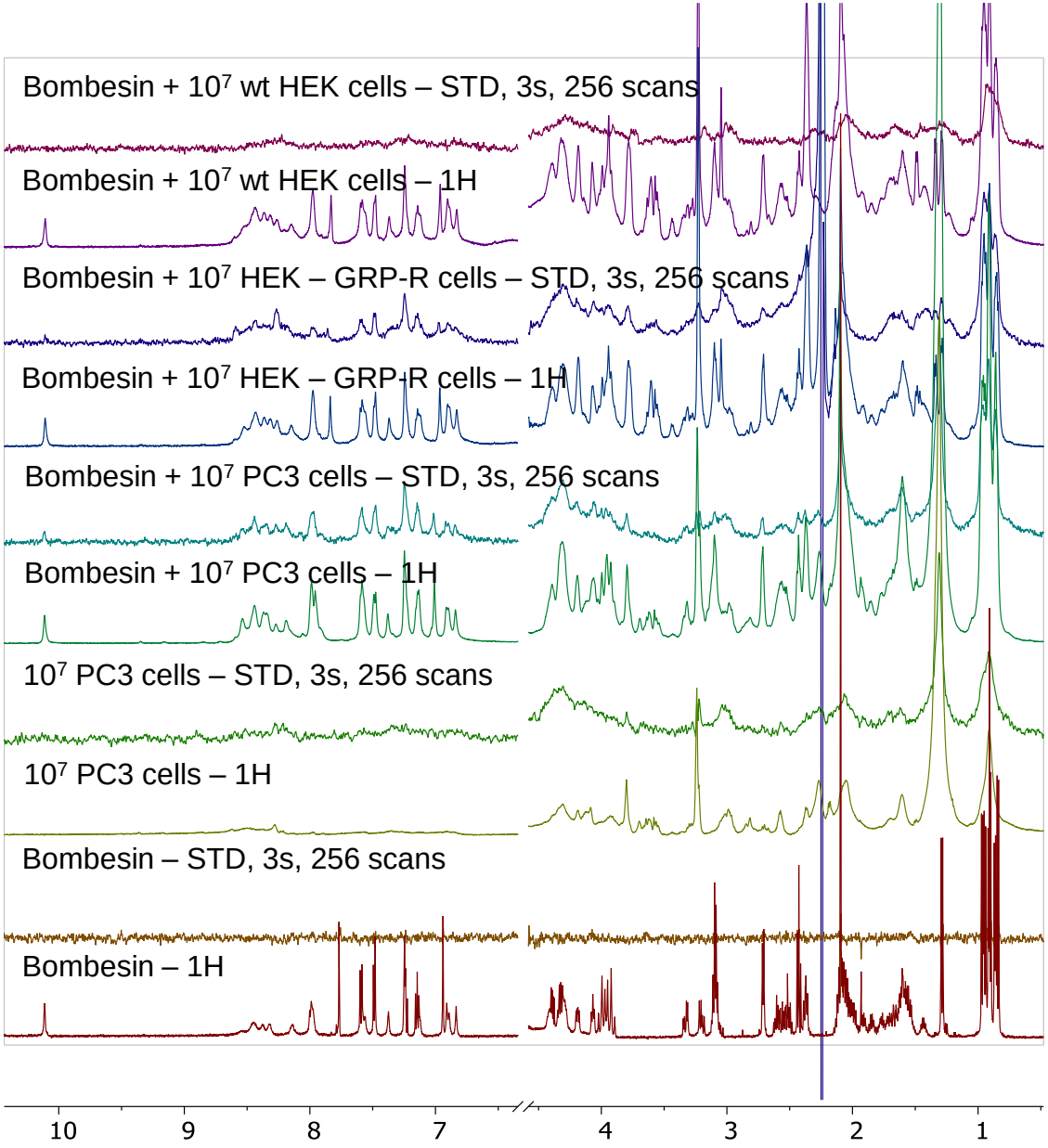

Bombesin



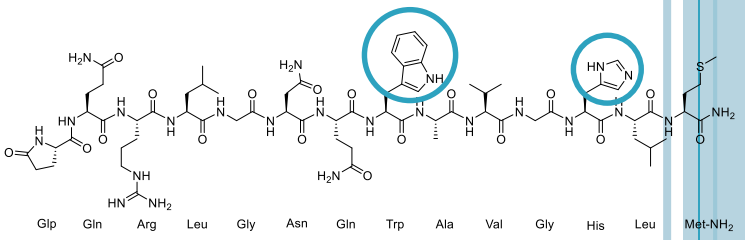

Bombesin (8-14)

AIRC
ASSOCIAZIONE ITALIANA PER LA RICERCA SUL CANCRO

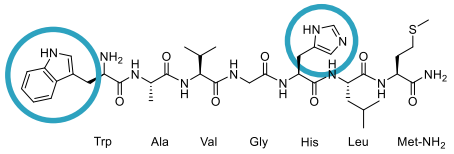
# Bombesin Interaction with Gastrin-Releasing Peptide Receptor: STD on PC3 and HEK Cells




Bombesin




**His and Trp side chains are essential for binding to GRPR**

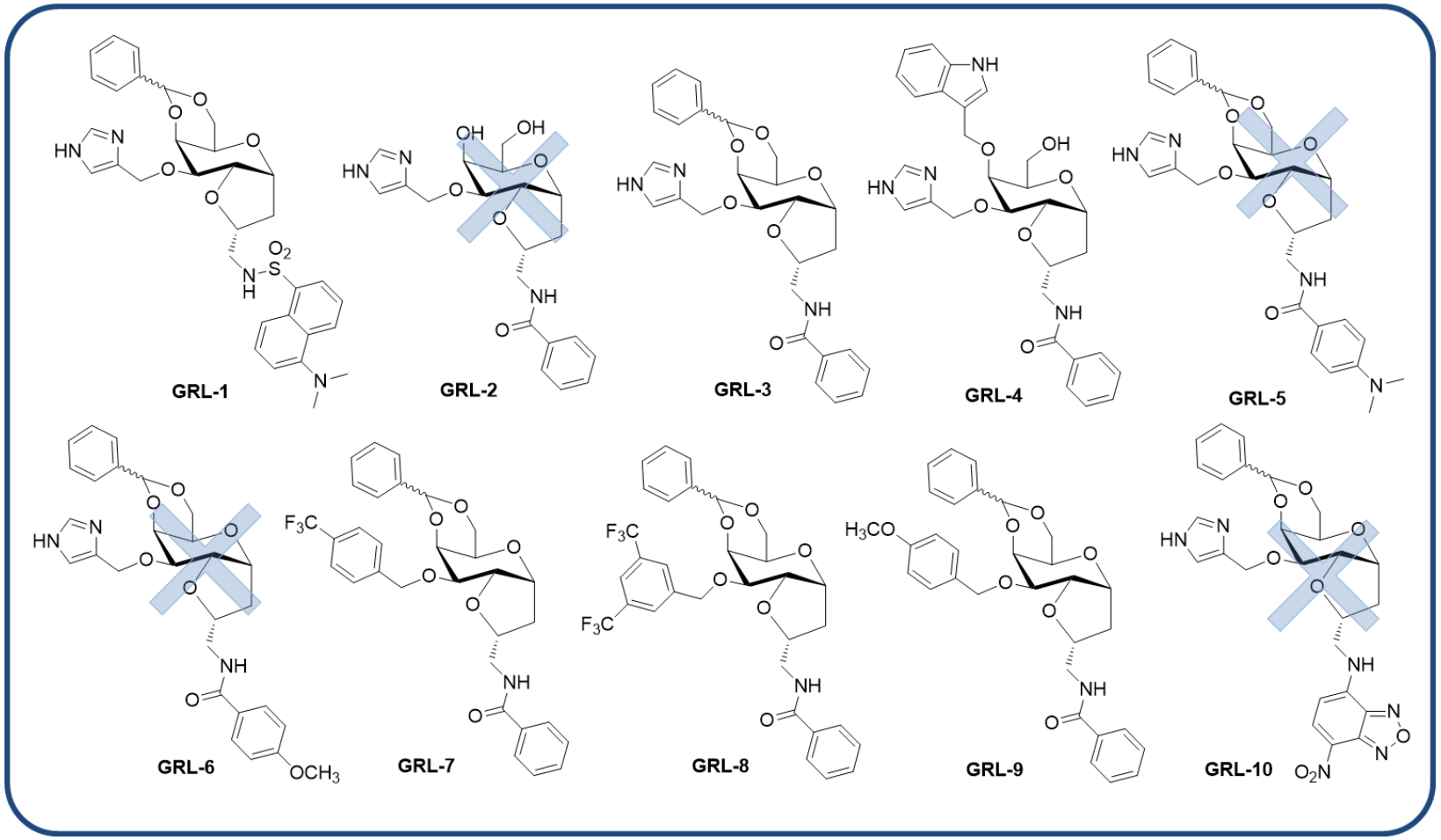



Bombesin (8-14)



*Manuscript in preparation*

# DESIGN AND SYNTHESIS OF NEW VERY POTENT BOMBESIN ANTAGONIST AS POTENTIAL NEW ANTI-TUMOR DRUGS



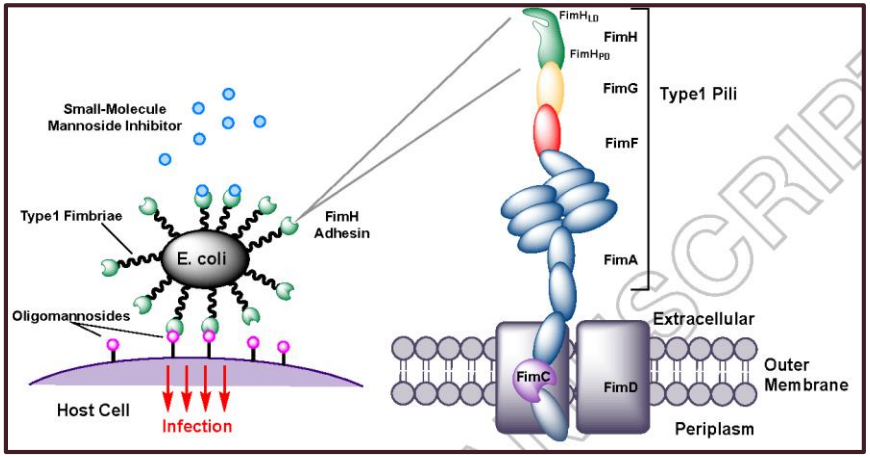

**Compounds inhibit Bombesin-induced increase of cytosolic $Ca^{2+}$ in a human prostate carcinoma cell line (PC-3) over-expressing the GRP receptor.**

**Active in the nM range of concentration**

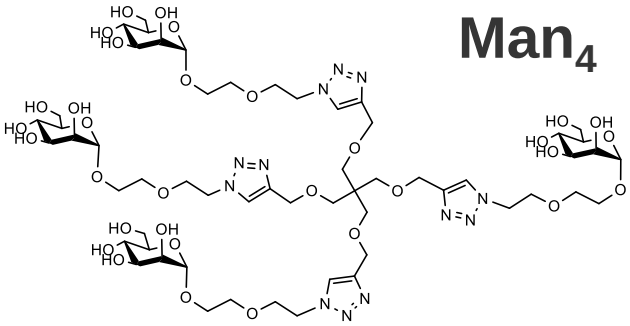
# FimH Multivalent Ligands: New Potential anti-Adhesive Compound

The bacterial adhesin **FimH** is a **virulence factor** and an attractive therapeutic target for **urinary tract infection** and **Crohn's Disease**.

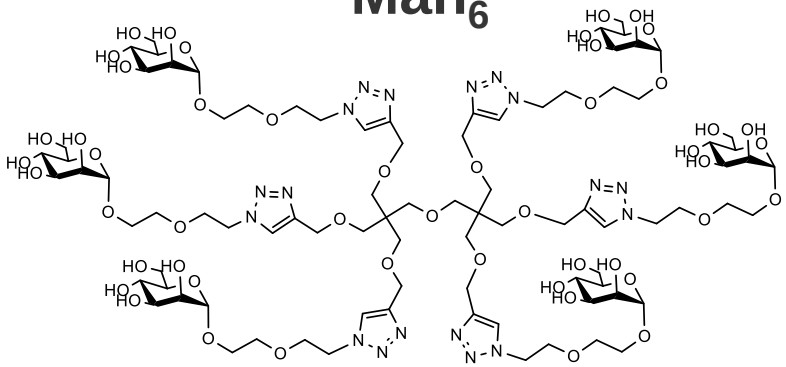
FimHis responsible for **D-Mannose** sensitive adhesion



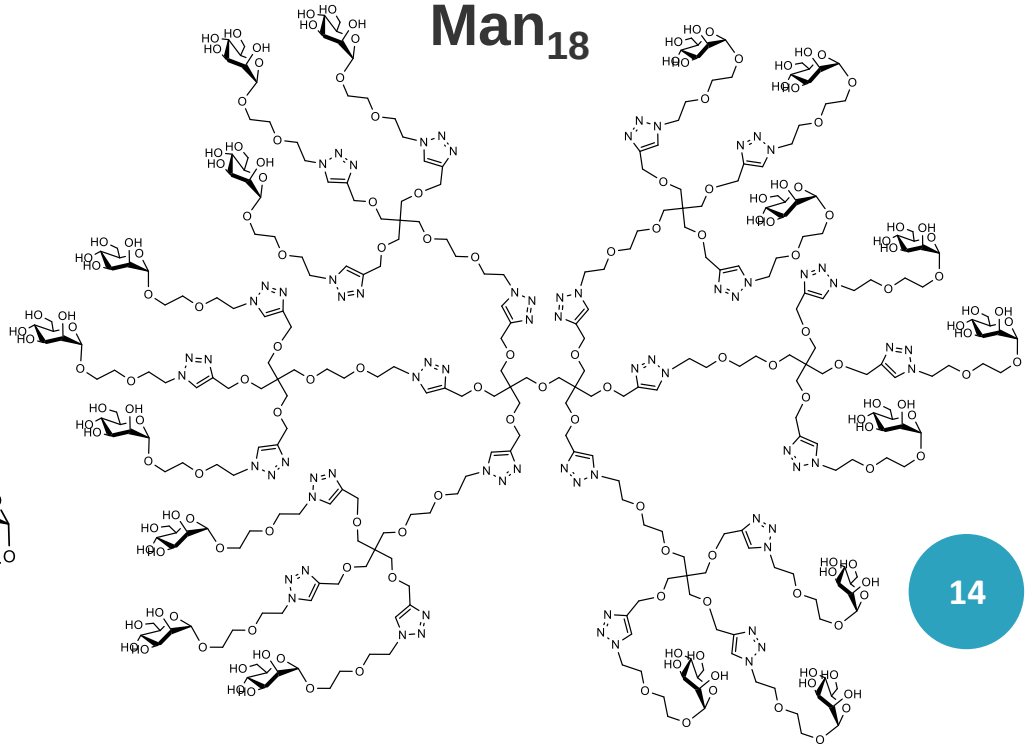

**Man$_4$**



**Man$_6$**

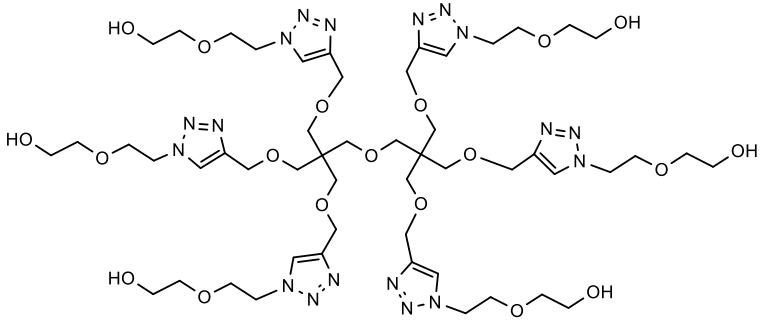


**Man$_{18}$**

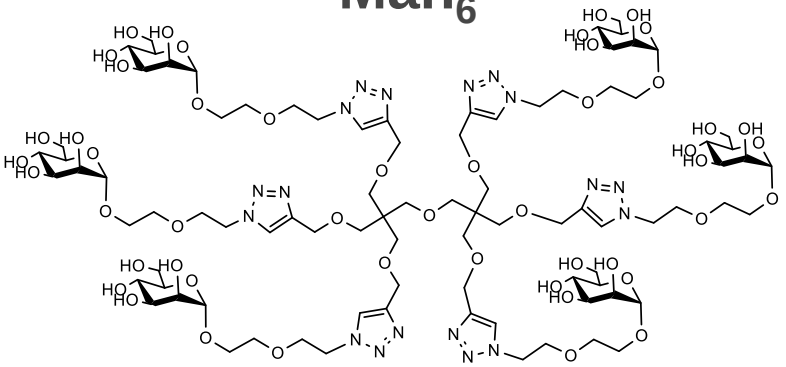


# FimH Multivalent Ligands: STD on *E. coli* Cells

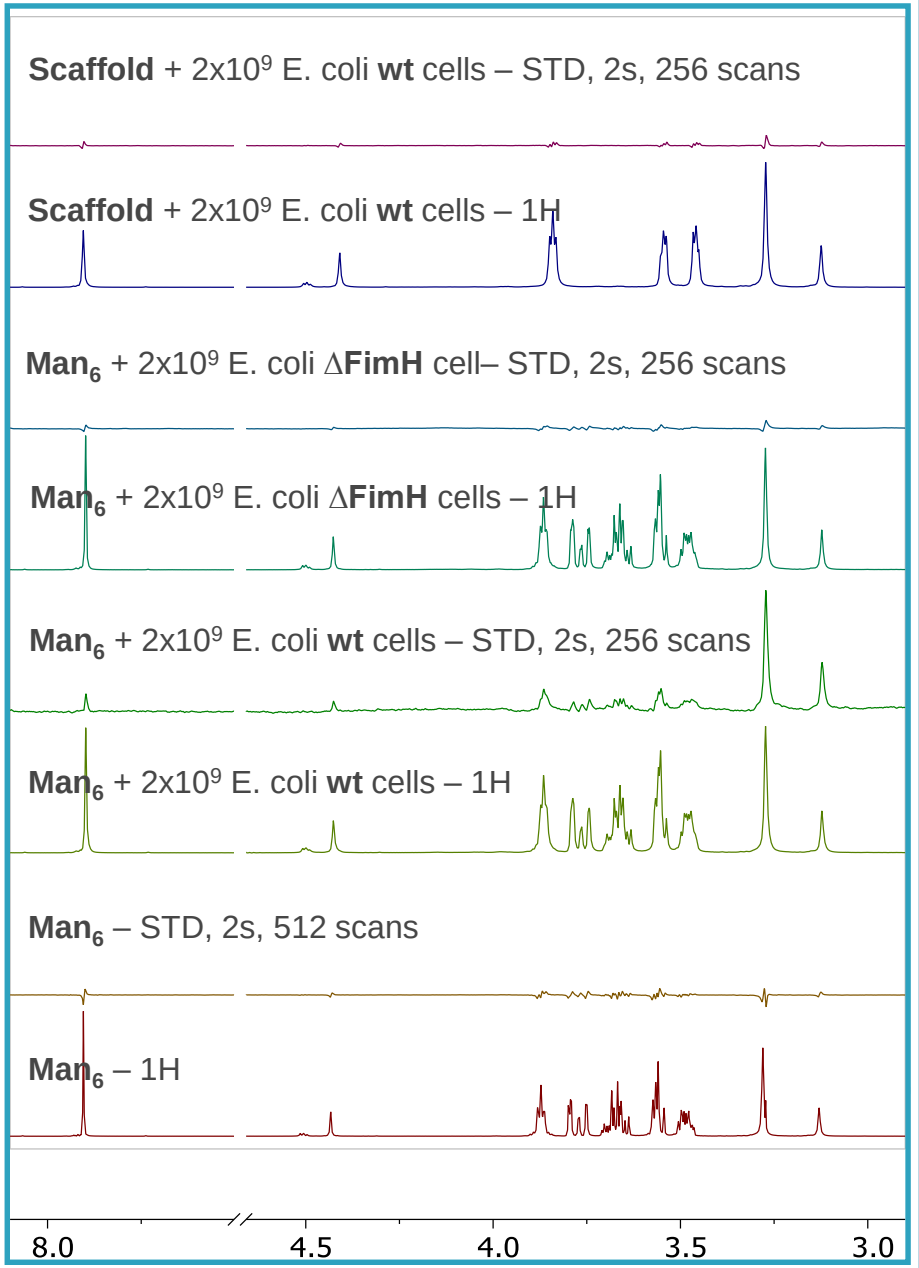
## Scaffold



## $Man_6$

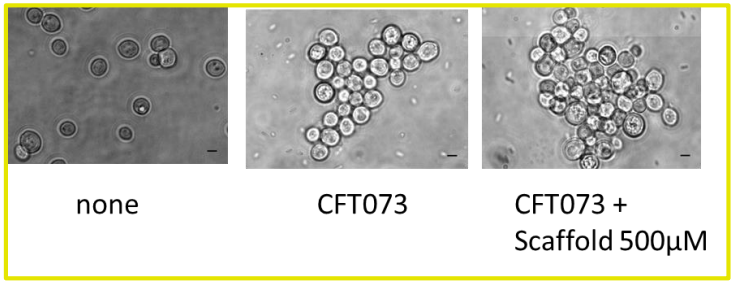


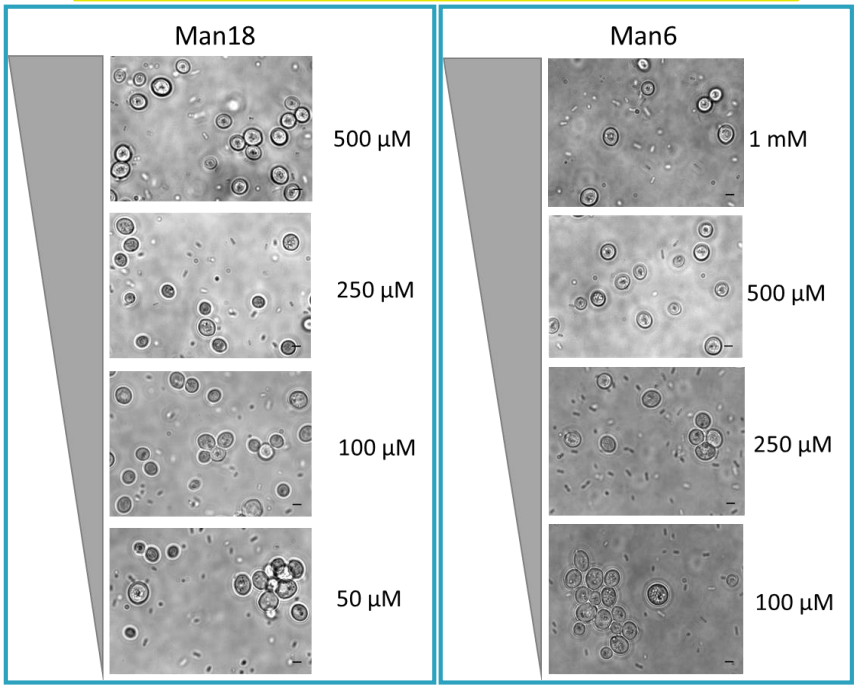
*E. coli* strain: CFT073 (uropathogenic)



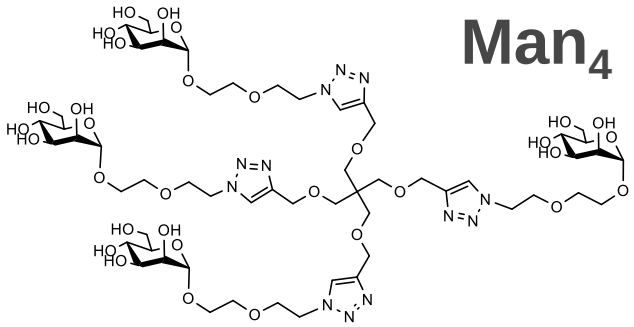
# FimH Multivalent Ligands: Yeast agglutination inhibition assay

| Yeast agglutination assay | |
|---|---|
| **Ligand** | **MIC [µM]** |
| Scaffold | > 500 |
| Man 18 | 63 |
| Man 6 | 250 |
| Man 4 | 500 |

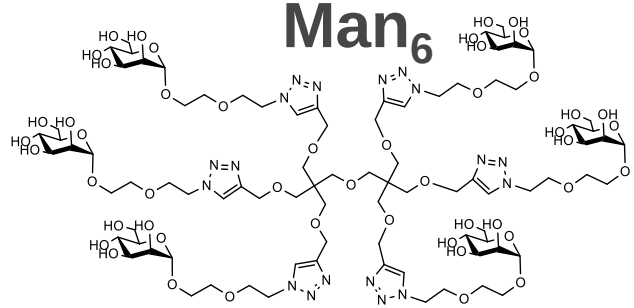


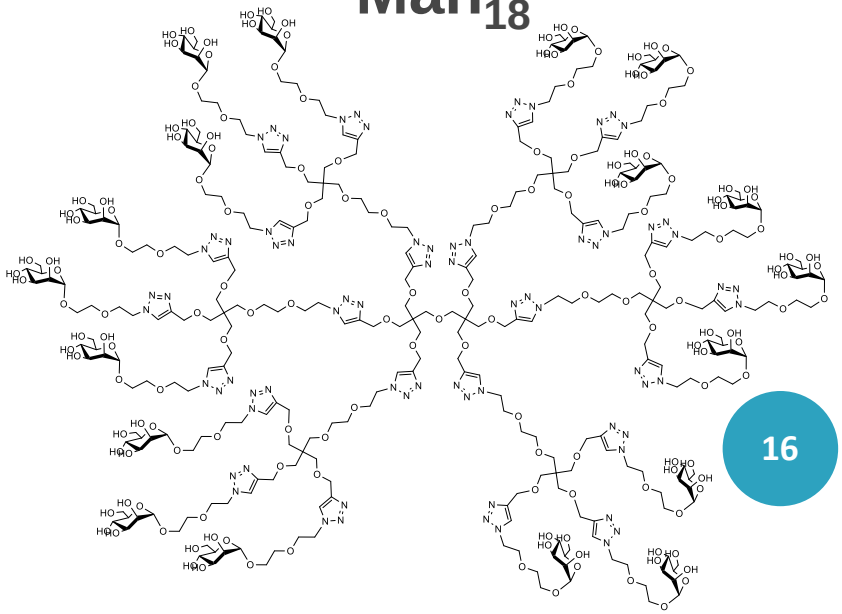

$Man_4$



$Man_6$

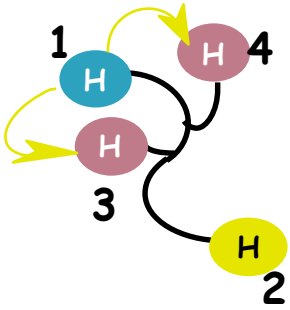


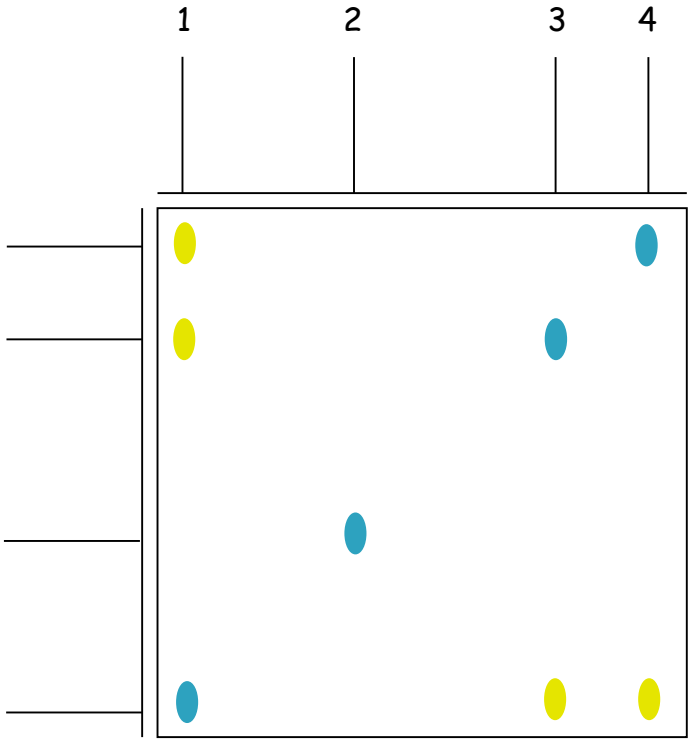
$Man_{18}$



# TRANSFERRED NOESY

$$NOE = \sim f(\tau_c)/ r^6$$

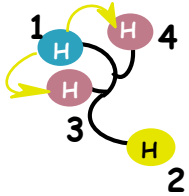


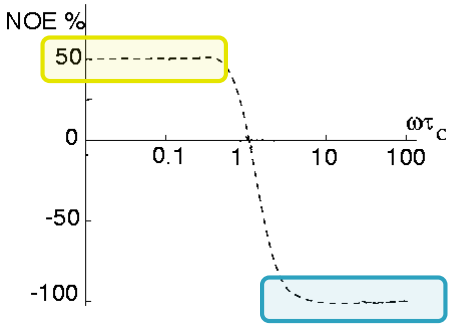

Identification of ligands

Identification of the bioactive conformation

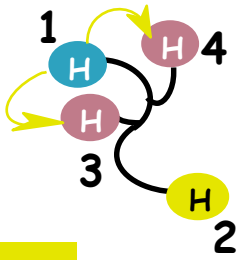
# TRANSFERRED NOESY



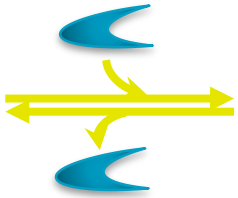

positive NOEs

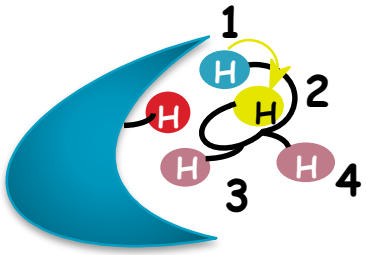



negative NOEs

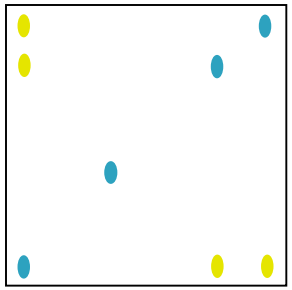



free state

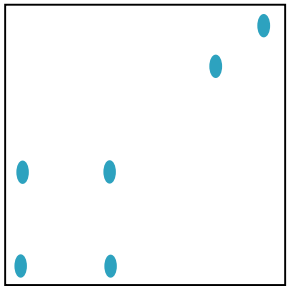





bound state



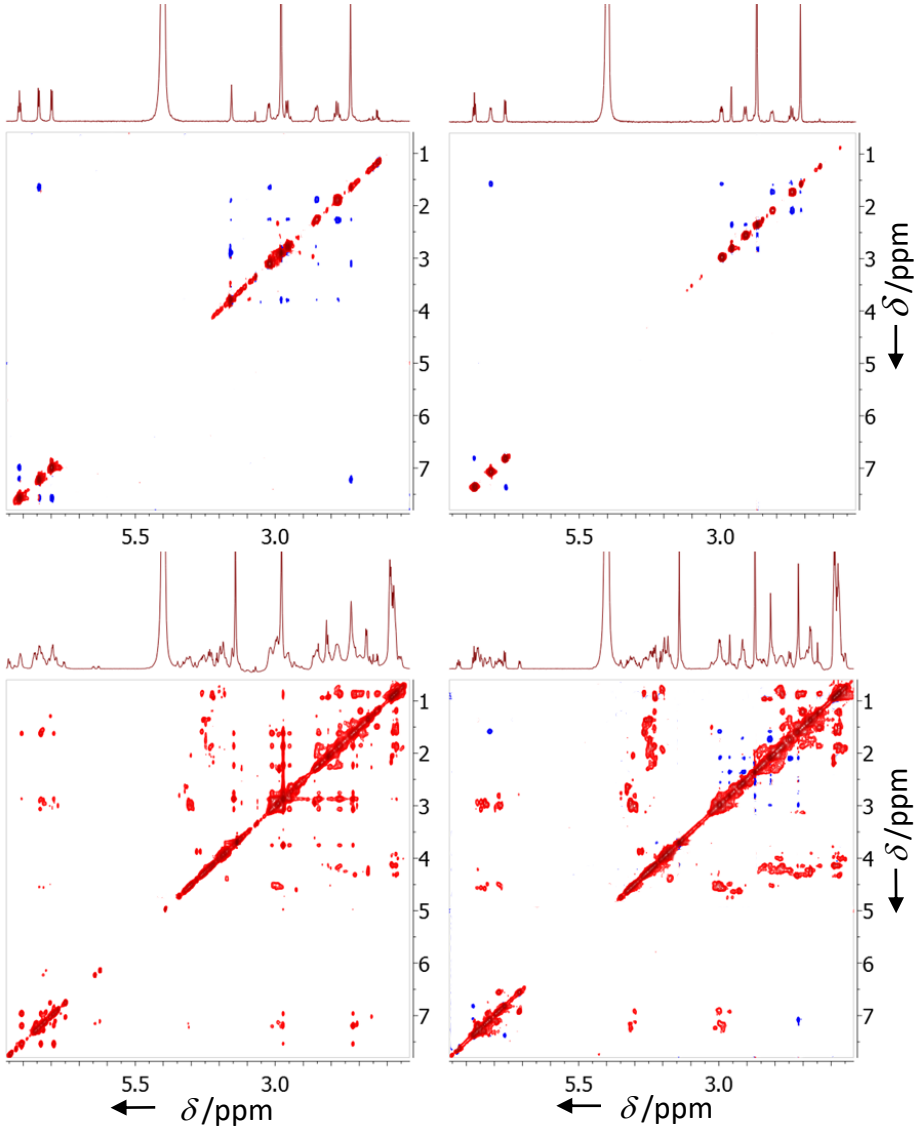




# TRANSFERRED-NOESY NMR EXPERIMENTS

tetracycline alone

tetracycline + Aβ




Aβ oligomers

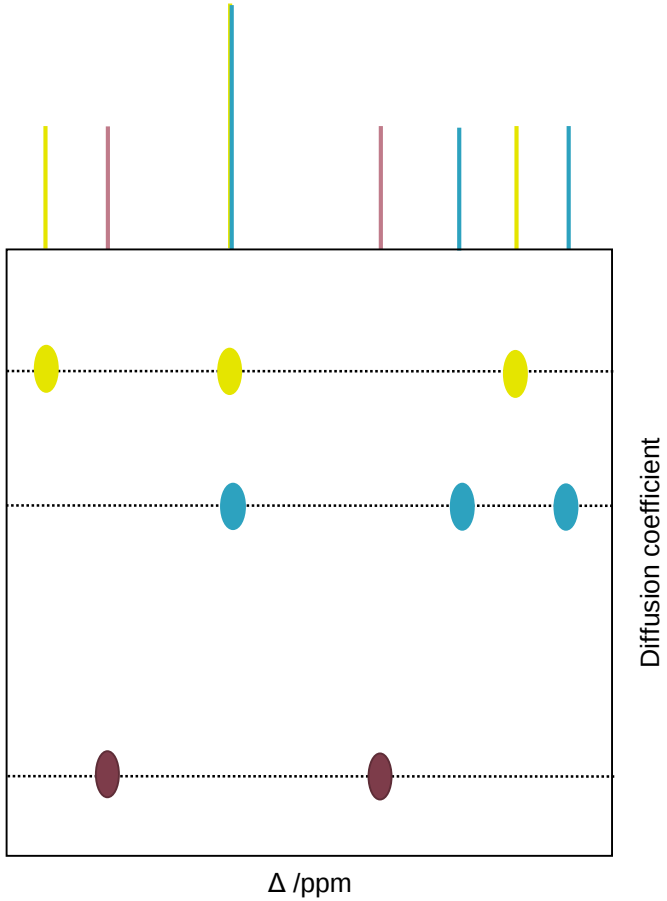
Aβ monomers

no interaction with Aβ monomers

interaction with Aβ oligomers

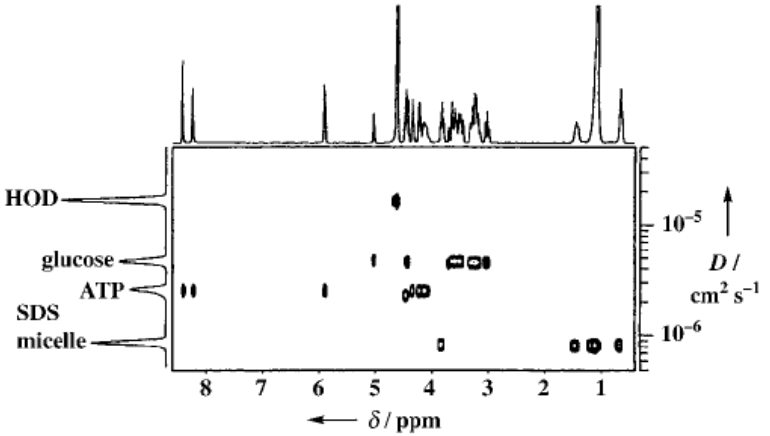
but no binding site on Aβ peptides

# DIFFUSION-ORDERED SPECTROSCOPY (DOSY) NMR




**Separation** of 1D NMR information into the second dimension based on **diffusion coefficient**

"Virtual" separation of compounds mixture by NMR

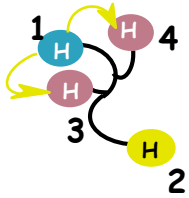


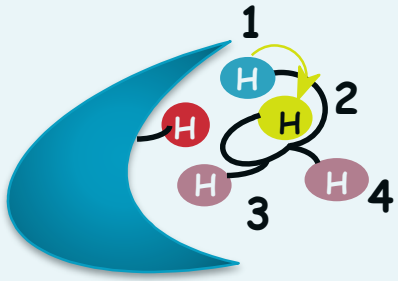

2D DOSY spectrum of a mixture containing HOD, glucose, ATP, and SDS micelles

# DOSY and Ligand Screening

small diffusion coefficient

big diffusion coefficient




small diffusion coefficient

# Tetracycline Diffusion Coefficients in the Absence and the Presence of Aβ Peptides at 5˚C

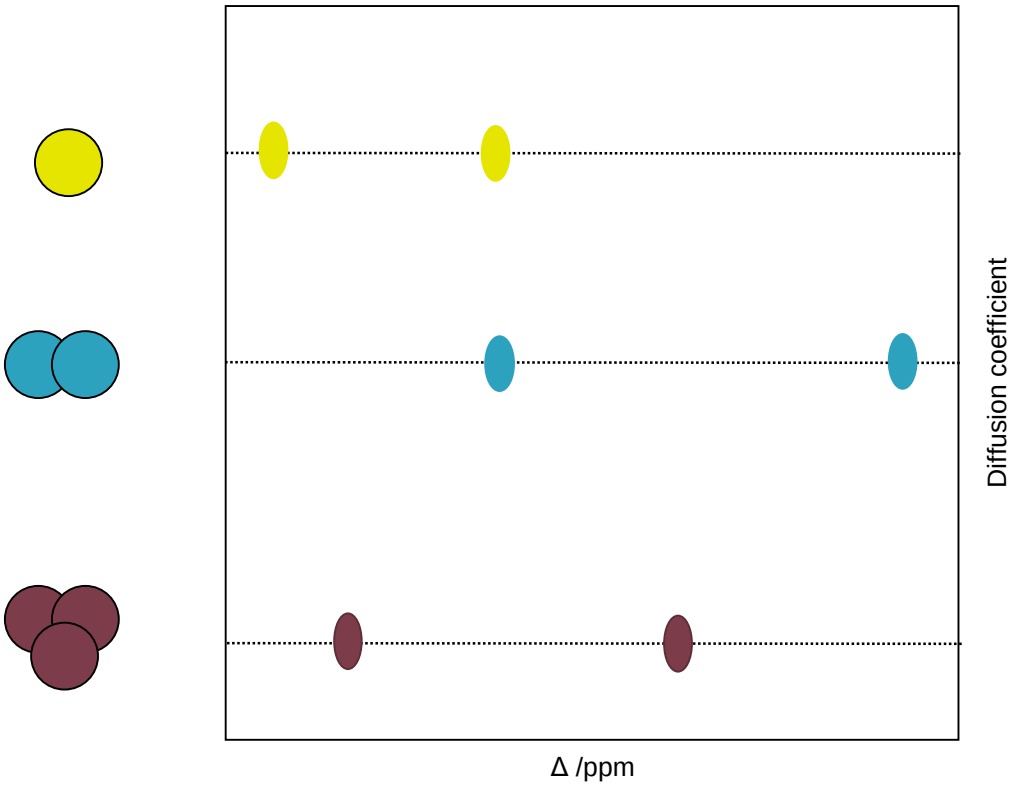
| Aβ sequence | peptide : tetracycline ratio | diffusion coefficient[a] |
|---|---|---|
| | tetracycline alone | $2{,}059*10^{-10}$ m²/s |
| 1-40 oligomers | 1:2 | $1{,}620*10^{-10}$ m²/s |
| 1-42 oligomers | 1:2 | $1{,}589*10^{-10}$ m²/s |
| 1-42 oligomers | 1:4 | $1{,}691*10^{-10}$ m²/s |
| 1-42 oligomers | 1:8 | $1{,}762*10^{-10}$ m²/s |
| 1-40 monomers | 1:2 | $1{,}959*10^{-10}$ m²/s |

[a] uncertainty less than 1%.

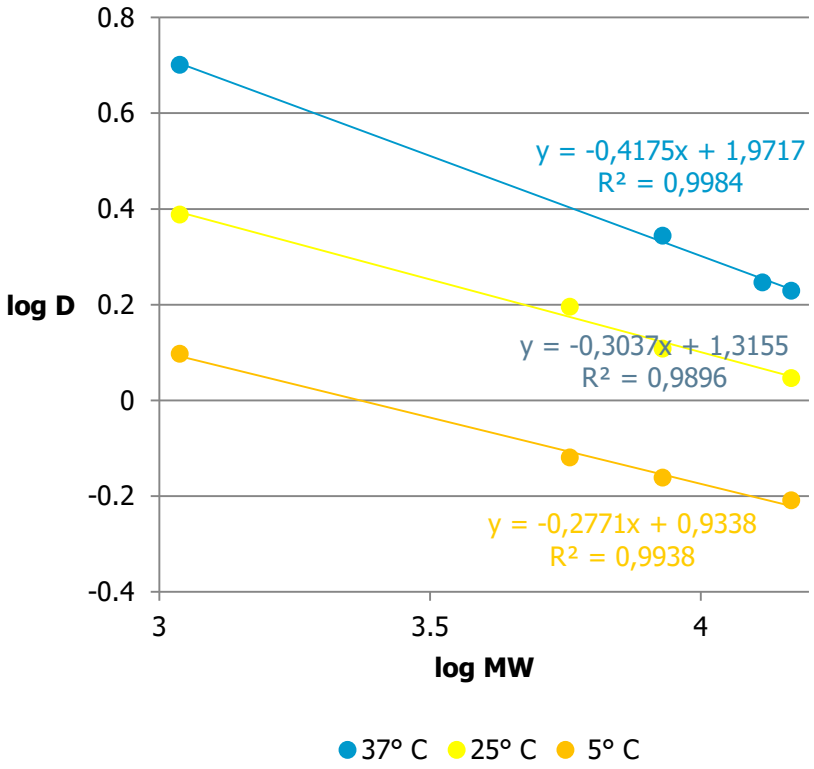
Tetracycline diffusion coefficients decreased significantly at increasing oligomers:drug ratios → interaction with Aβ oligomers

no interaction with monomers

# DOSY and Molecular Aggregation



# Calibration Curves correlating Molecular Weight with Diffusion Coefficient (D)




**Calibration curves** correlating molecular weight with diffusion coefficient (**logD vs logMW**) were used to estimate D values for Aβ peptide monomers and small oligomers at 5, 25, or 37°C.[1]

Protein size standards employed were: bradykinin (1090 Da), bovine insulin (5734 Da, not used for the calibration curve at 37°C),[2] ubiquitin (8500 Da), cytochrome C (13000 Da), lysozyme (14700 Da).



[1] P. Groves, M. O. Rasmussen, M. D. Molero, E. Samain, F. J. Canada, H. Driguez, J. Jimenez-Barbero, *Glycobiology*, **2004**, *14*, 451.
[2] Insulin is a dimer at 37°C, as reported in M. Lin, C. K. Larive, *Anal. Biochem*, **1995**, *229*, 214-220.

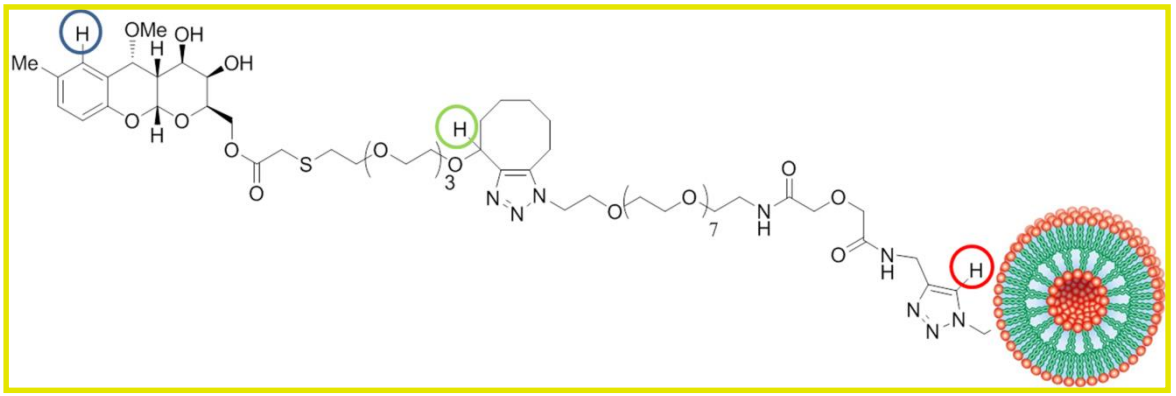
# Aβ1-40 Diffusion Coefficients in solution, pH 7.4

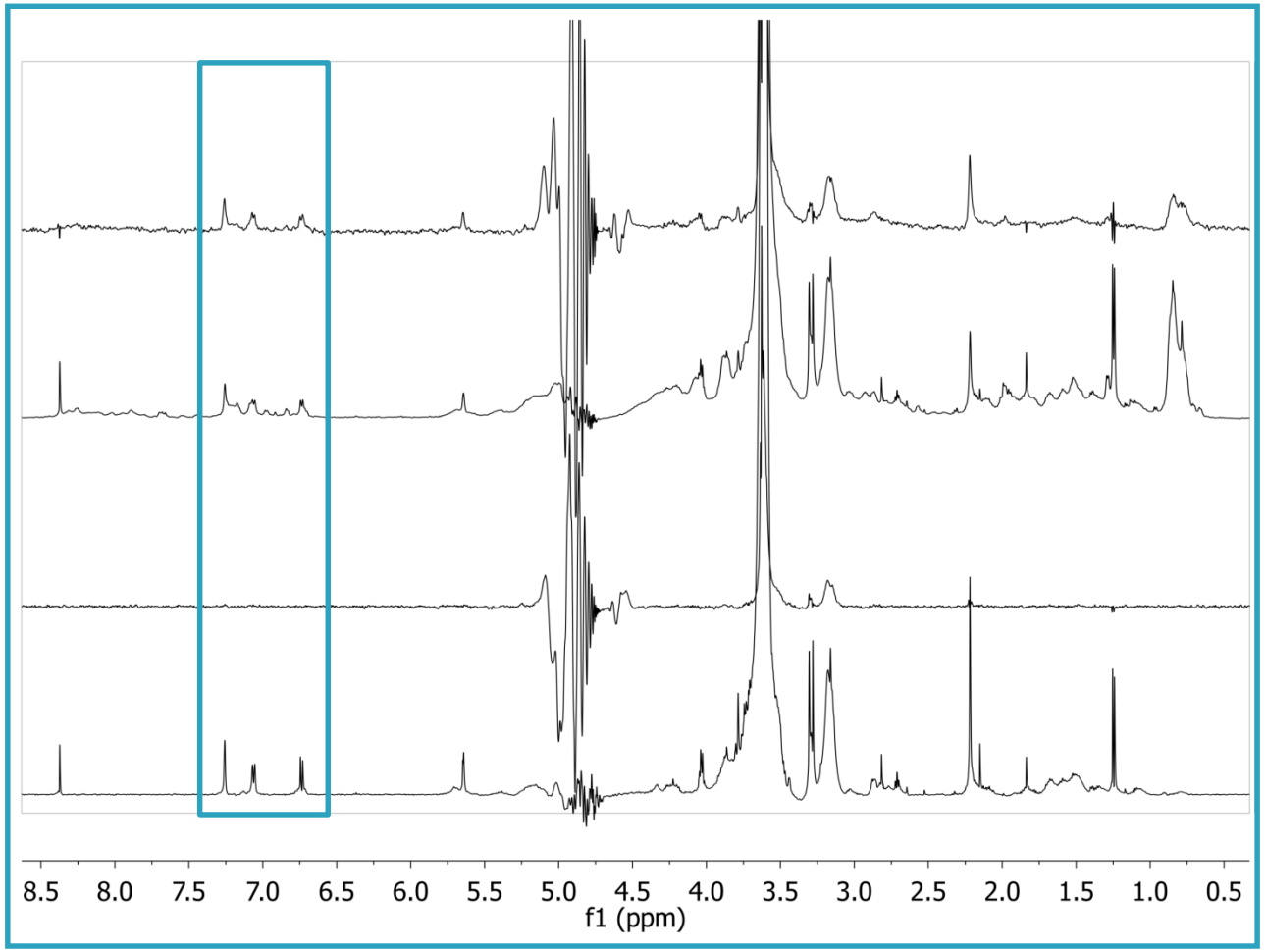
| T (℃) | expected diff. coeff. for the **monomer** | expected diff. coeff. for the **dimer** | expected diff. coeff. for the **trimer** | observed diffusion coefficient [a] |
|---|---|---|---|---|
| 5 | $0.845*10^{-10}$ $m^2/s$ | $0,696*10^{-10}$ $m^2/s$ | $0,622*10^{-10}$ $m^2/s$ | **$0.745*10^{-10}$ $m^2/s$** |
| 25 | $1.626*10^{-10}$ $m^2/s$ | $1.317*10^{-10}$ $m^2/s$ | $1.165*10^{-10}$ $m^2/s$ | **$1.378*10^{-10}$ $m^2/s$** |
| 37 | $2.841*10^{-10}$ $m^2/s$ | $2.127*10^{-10}$ $m^2/s$ | $1.796*10^{-10}$ $m^2/s$ | **$1.862*10^{-10}$ $m^2/s$** |

[a] uncertainty less than 1%.

Measured coefficients at 5°C and 25°C, pH 7.4 consistent with the presence of monomers and small oligomers, with *D* values being lower than expected for the monomer, but higher than expected for the dimer. Diffusion coefficient at 37°C comprised between the values expected from the dimer and the trimer.

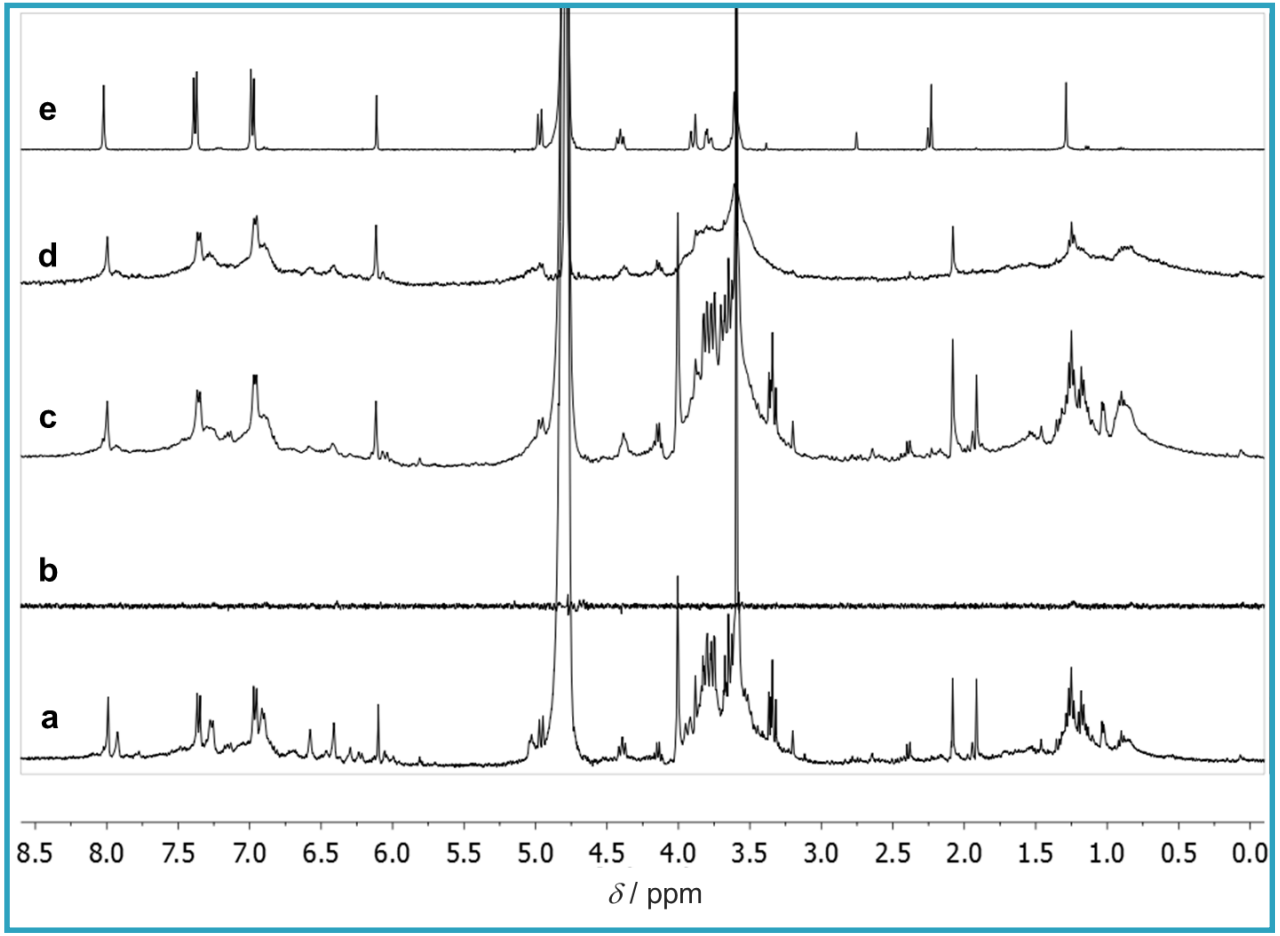
# Binding Studies between Functionalized Liposomes and Aβ oligomers



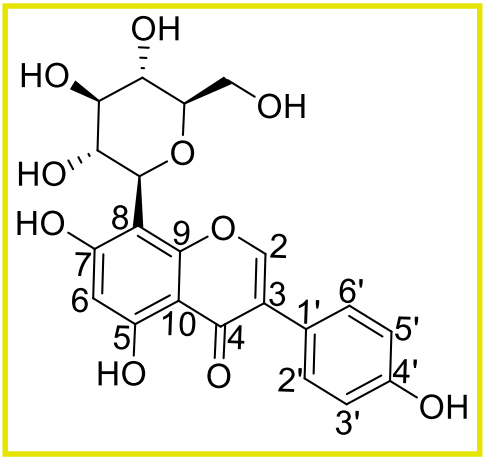




**a, b**) Tricycline-functionalized liposomes, phosphate buffer, pH=7.4, 25^C: 1H NMR spectrum (**a**); waterlogsy NMR spectrum with 1.2 s mixing time (**b**); **c, d**) Abeta1-42 80 μM with tricycline-functionalized liposomes, phosphate buffer, pH=7.4, 25°C: 1H NMR spectrum (**c**); waterlogsy NMR spectrum with 1.2 s mixing time (**d**).

# Identification of an new Aβ Peptide Ligand on *Genista Tenera* ethyl acetate extract by STD-NMR and tr-NOESY exps



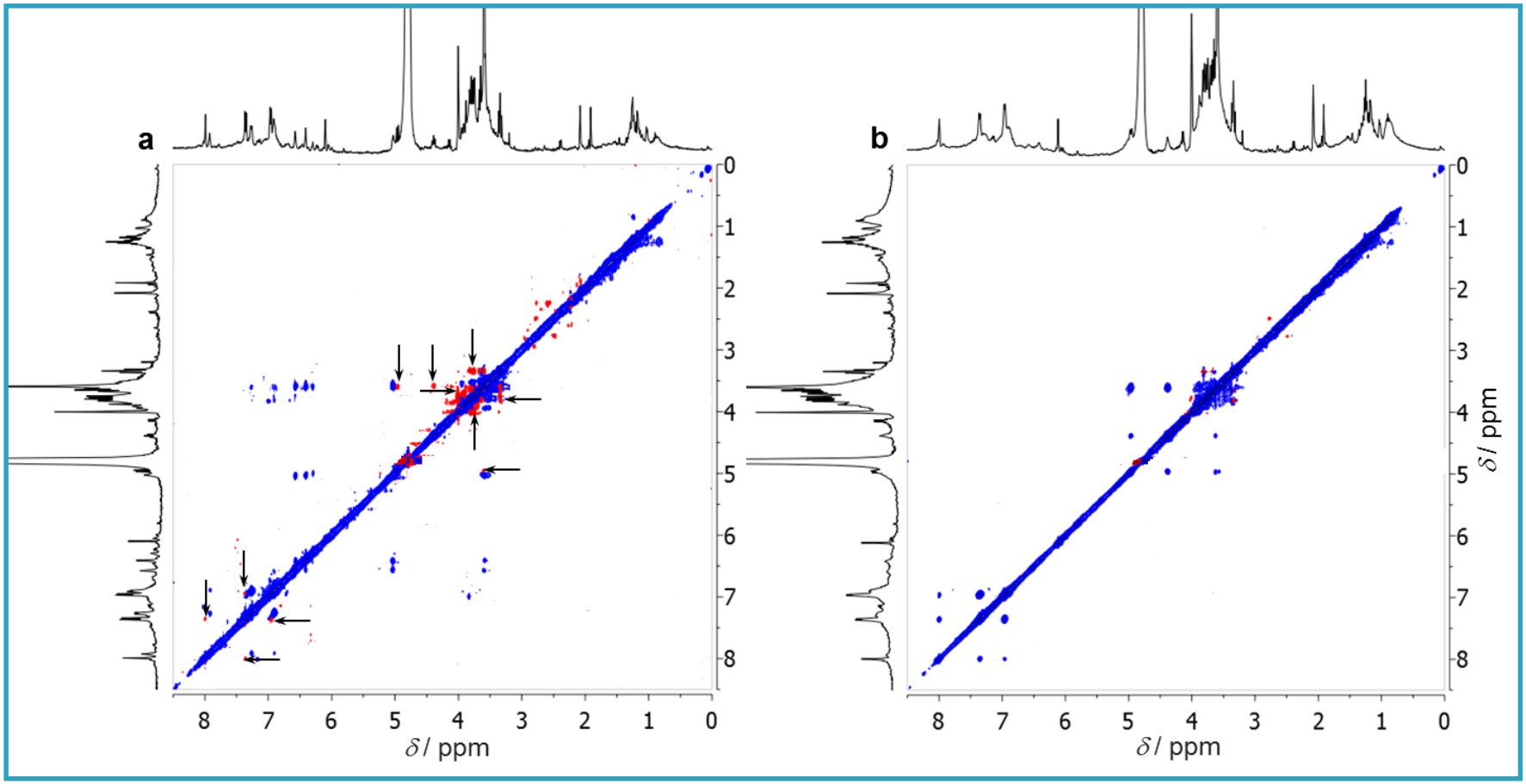

**a)** $^{1}$H NMR spectrum of the 1.5 mg ethyl acetate extract of *G. tenera*; **b)** blank STD-NMR spectrum at 2s saturation time of the same sample; **c)** $^{1}$H NMR spectrum of the mixture containing Aβ1-42 (80 uM) and 1.5 mg of the ethyl acetate extract of *G. tenera*; **d)** STD-NMR spectrum of this mixture at 2s saturation time; **e)** $^{1}$H NMR spectrum of 2 mM Genistein-8-*C*-glucoside. All the samples were dissolved in deuterated PBS, pH 7.5, 25°C. The spectrometer frequency was 400 MHz.




**Genistein-8-*C*-glucoside**

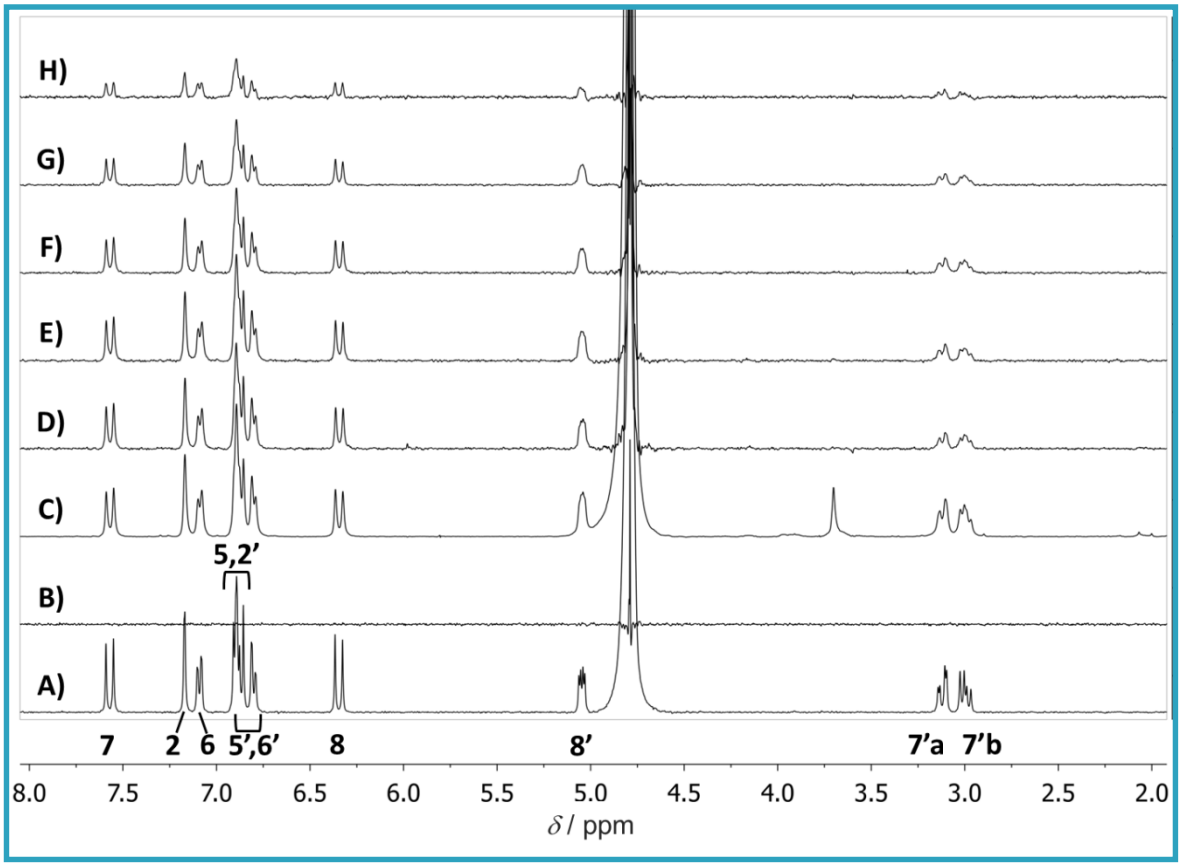
Grases Santos Silva Rauter A. P., et al., New C-glycosylpolyphenol antidiabetic agents, effect on glucose tolerance and interaction with beta-amyloid. Therapeutic applications of the synthesized agent(s) and of Genista tenera ethyl acetate extracts containing some of those agents, **2012**, PT106202.

# Identification of an new Aβ Peptide Ligand on *Genista Tenera* ethyl acetate extract by STD-NMR and tr-NOESY exps

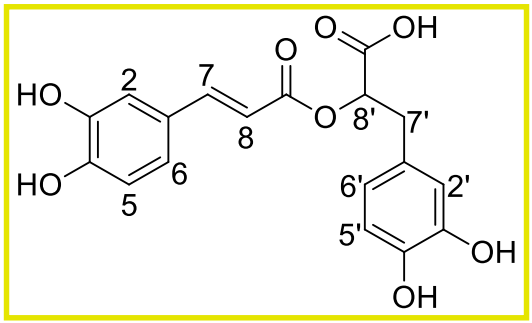



**a)** 400 MHz 2D-NOESY spectra of 1.5 mg of the ethyl acetate extract of *G. tenera*, with a mixing time of 0.8 s. **b)** trNOESY of the mixture containing Aβ1-42 (80 uM) and 1.5 mg of the extract of *G. tenera*, with a mixing time 0.3 s. Both samples were dissolved in deuterated PBS, at pH 7.5 and 25°C. The cross peaks in the free state corresponding to the strongest ones found in the bound state are indicated by arrows. Positive cross-peak are in red; negative, in blue.

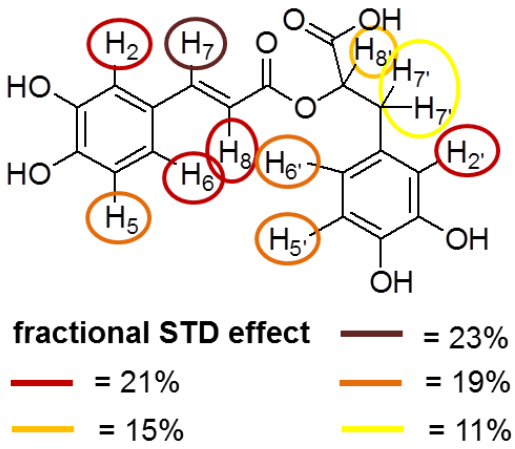
# Molecular recognition of *Salvia sclareoides* extract and its major component, Rosmarinic Acid, with Aβ1-42 peptide




A) 1H NMR spectrum of 2 mM RA; B) blank STD-NMR spectrum of the same sample acquired with a saturation time of 2s; C) 1H NMR spectrum of the mixture containing 80 uM Aβ1-42 and 2 mM RA; D-H) STD-NMR spectra of the same mixture acquired with different saturation times. (D, 3.0 s; E, 2.0 s; F, 1.3 s; G, 0.8 s; H, 0.3 s). Both samples were dissolved in deuterated PBS, pH 7.5, 25°C. The spectra were recorded at 400 MHz.



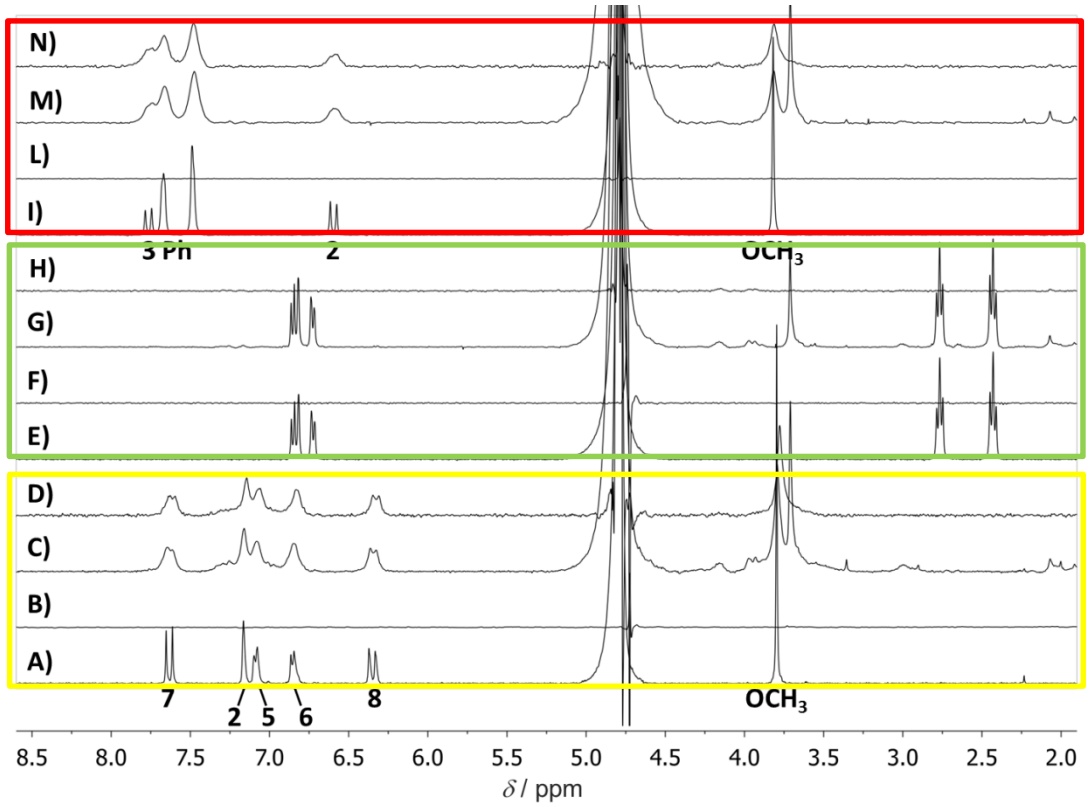

**Rosmarinic acid**

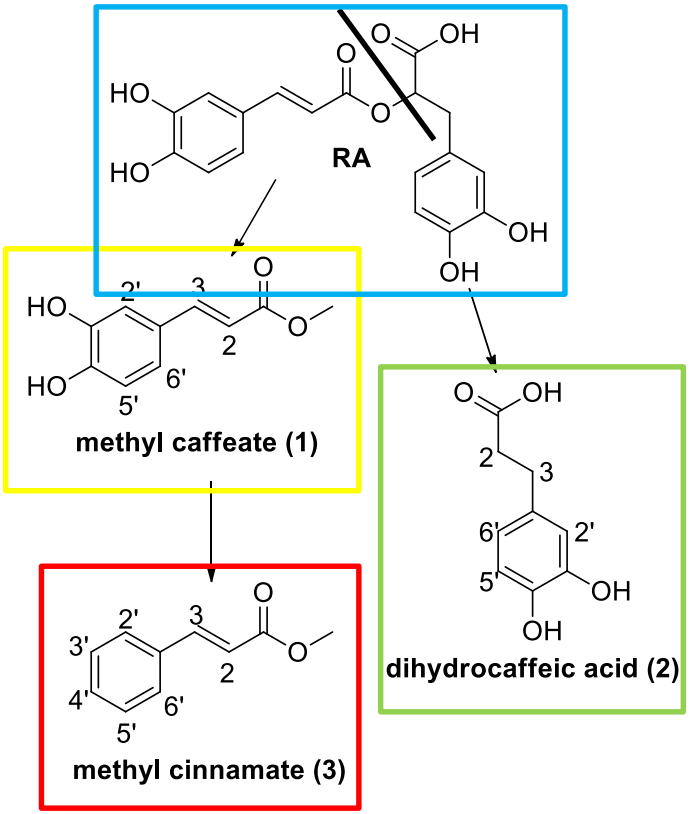


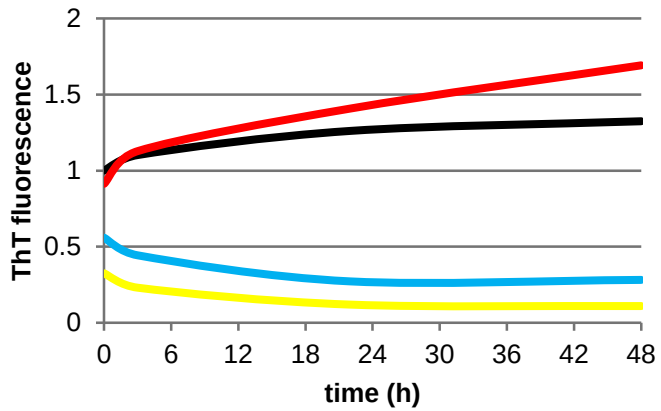



*Airoldi C., Sironi E., Dias C., Marcelo F., Martins A., Rauter A. P., Nicotra F., Jimenez-Barbero J., Chem. Asian J.,* ***2013****, 8, 3, 596-602.*

# Molecular recognition of *Salvia sclareoides* extract and its major component, Rosmarinic Acid, with Aβ1-42 peptide



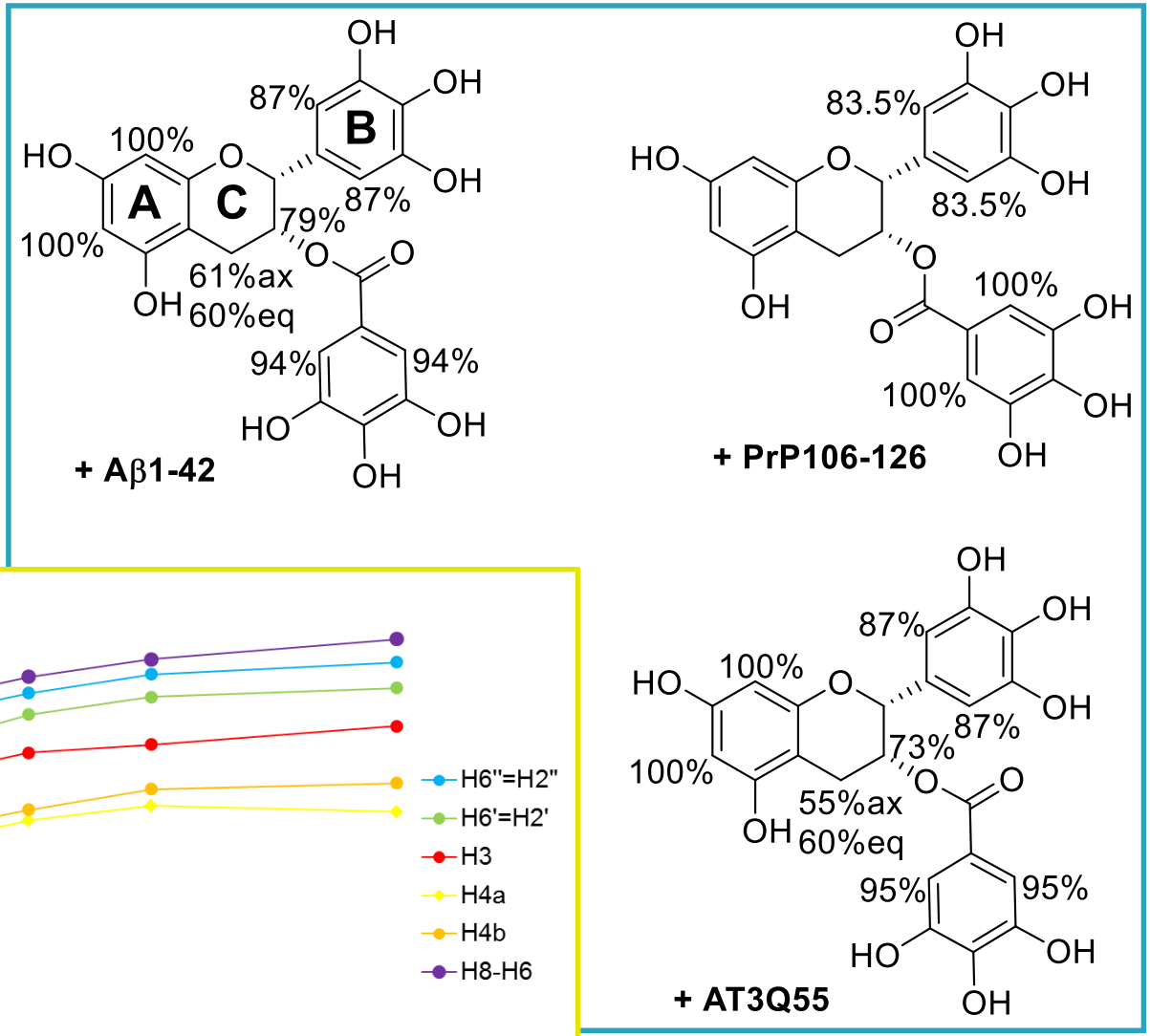



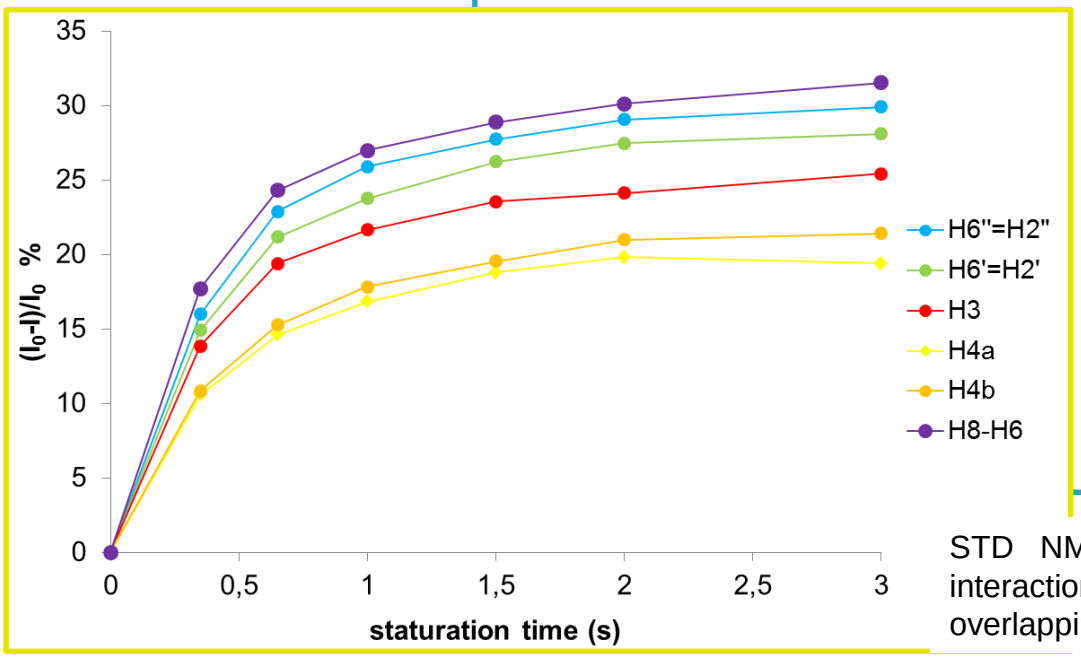




**RA and methyl caffeate inhibit Aβ aggregation**

**Methyl cinnamate induces Aβ aggregation**

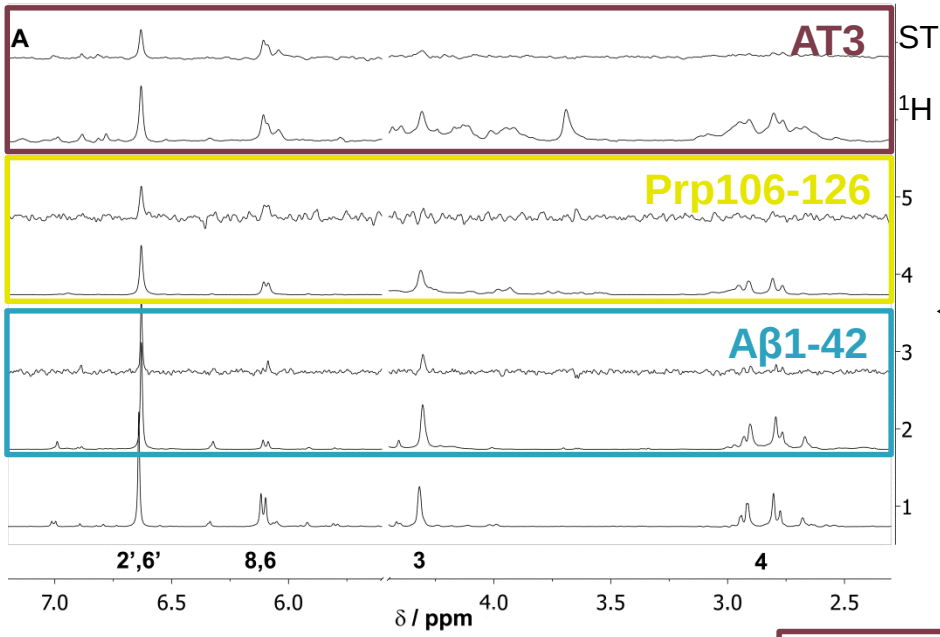
# Binding epitopes of EGCG calculated for the interaction with Aβ1-42, PrP106-126 and AT3Q55 oligomers

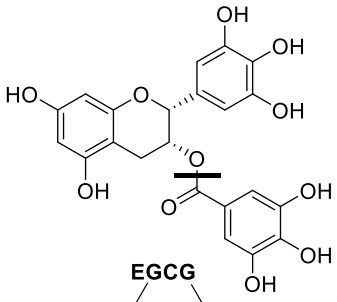


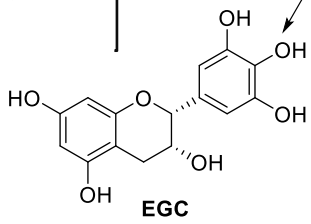



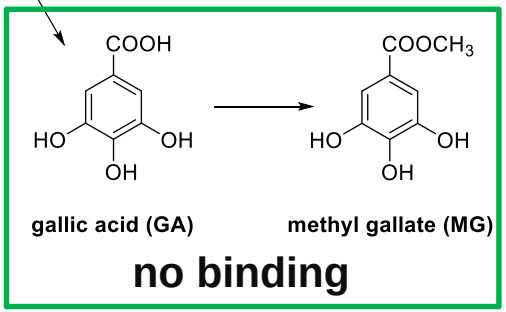

STD NMR characterization of EGCG-Aβ1-42 interaction. **Fractional STD effect** for each non-overlapping signal of EGCG.

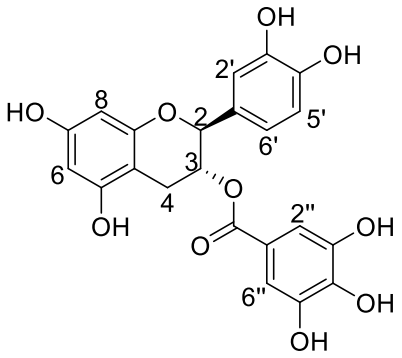
# Screening of Epigallocatechine, Gallic Acid and Methyl Gallate as Ligands of Aβ1-42, PrP106-126 and Ataxin-3 oligomers

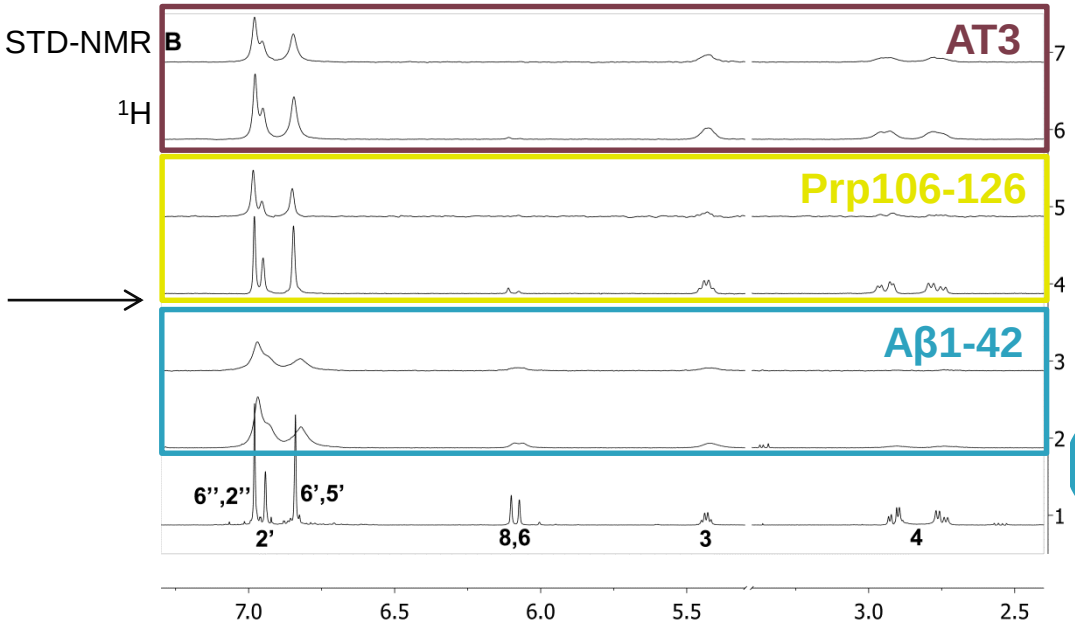


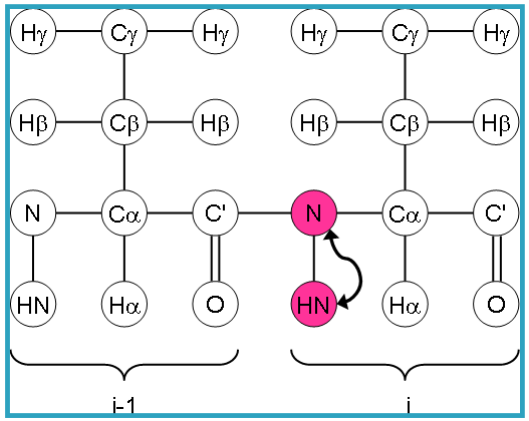



# Experiments Based on Protein Observation

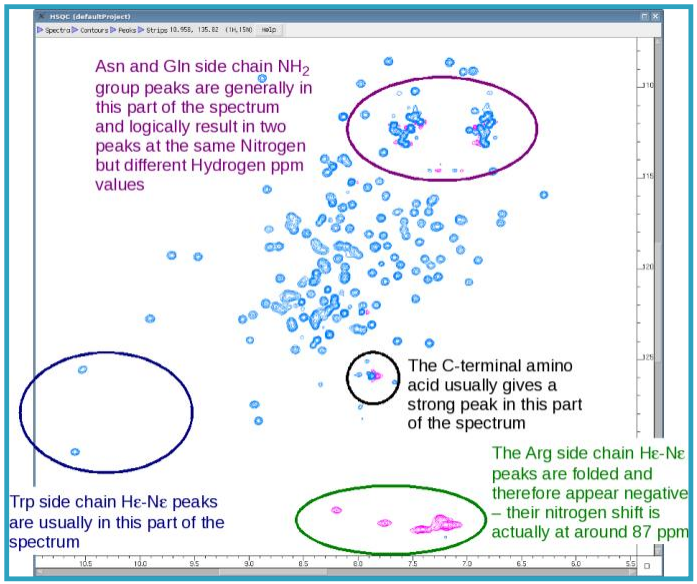
# $^{15}$N, $^{1}$H HSQC Spectra are Fingerprints of Proteins



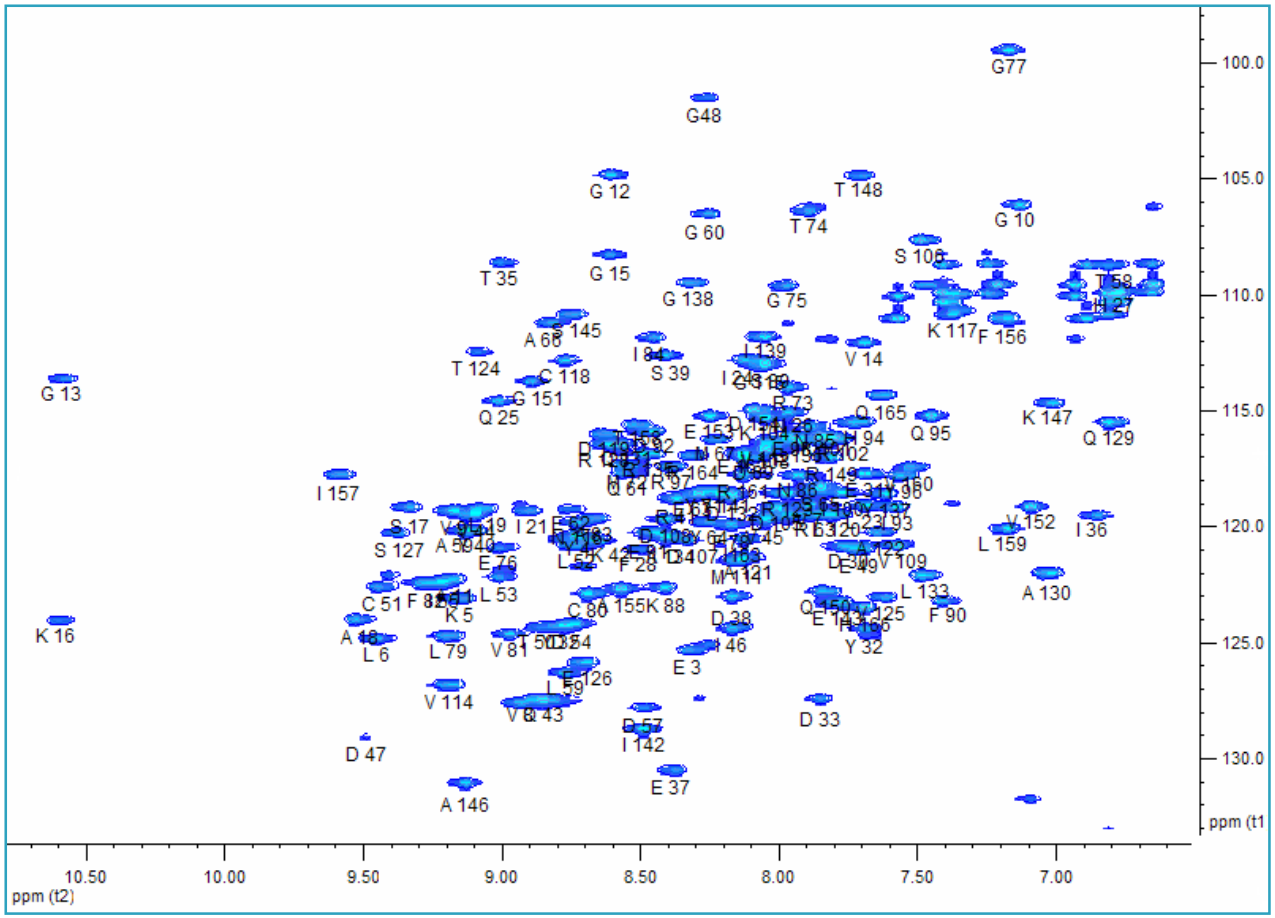

**Protein $^{15}$N-labelling required**

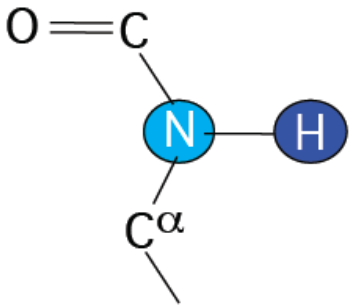
Magnetization is transferred from hydrogen to attached $^{15}$N nuclei via the J-coupling. The chemical shift is evolved on the nitrogen and the magnetisation is then transferred back to the hydrogen for detection.

This is the most standard experiment and shows all H-N correlations. Mainly these are the backbone amide groups, but Trp side-chain Nε-Hε groups and Asn/Gln side-chain Nδ-Hδ2/Nε-Hε2 groups are also visible. The Arg Nε-Hε peaks are in principle also visible, but because the Nε chemical shift is outside the region usually recorded, the peaks are folded/aliased (this essentially means that they appear as negative peaks and the Nε chemical shift has to be specially calculated). If working at low pH the Arg Nη-Hη and Lys Nζ-Hζ groups can also be visible, but are also folded/aliased. The spectrum is rather like a fingerprint and is usually the first heteronuclear experiment performed on proteins. From it you can assess whether other experiments are likely to work and for instance, whether it is worth carbon labelling the protein before spending the time and money on it. Or if your protein is reasonably large you might be able to judge whether deuteration might be necessary.



# $^{15}$N, $^{1}$H HSQC Spectra are Fingerprints of Proteins



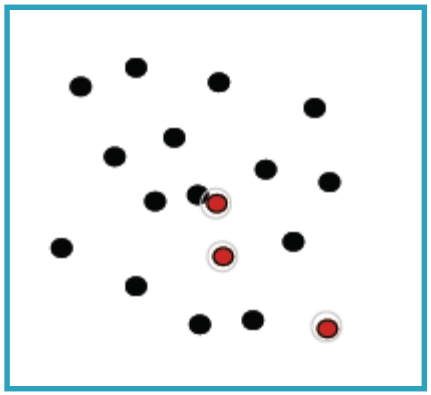




$^{15}$N, $^{1}$H HSQC spectrum of Ras 1-166

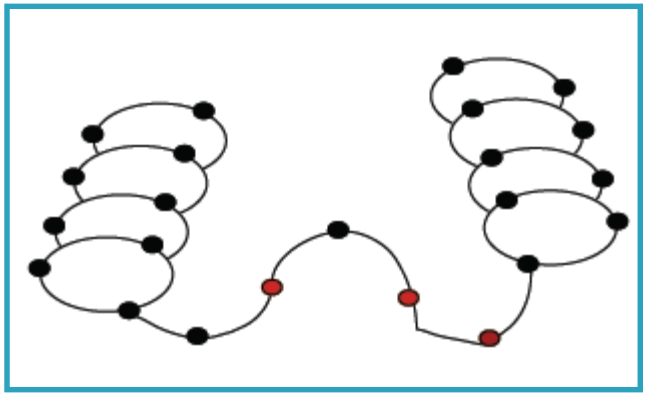
# Detecting Ligand Binding using $^{1}H,^{15}N$ NMR

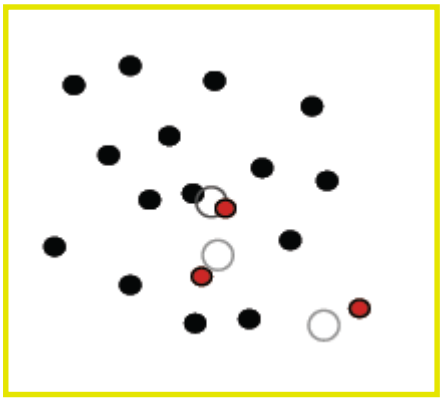
$^{15}N$, $^{1}H$ correlations

3D Structures of the polypeptide chain

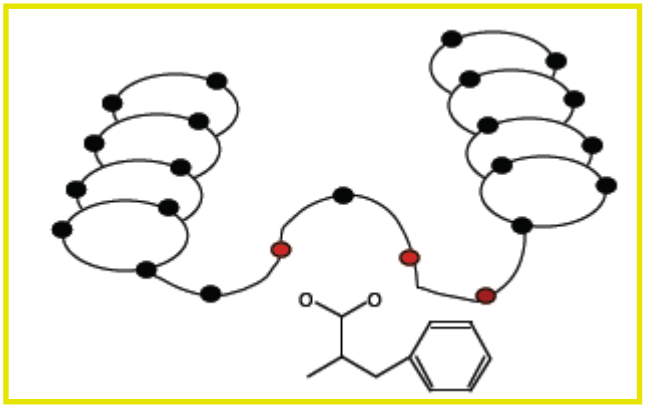


without ligand

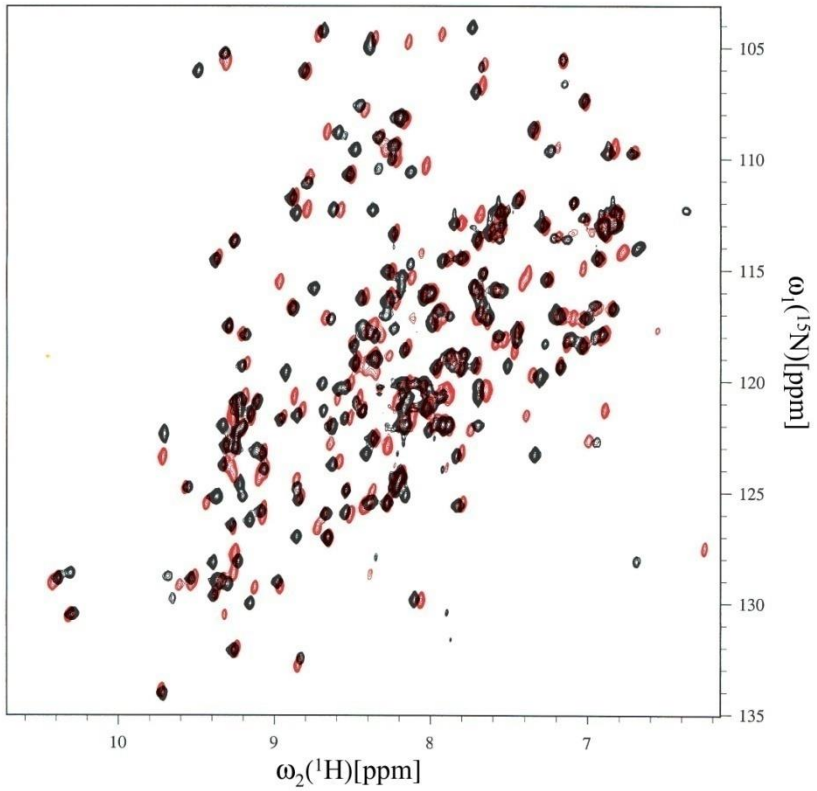


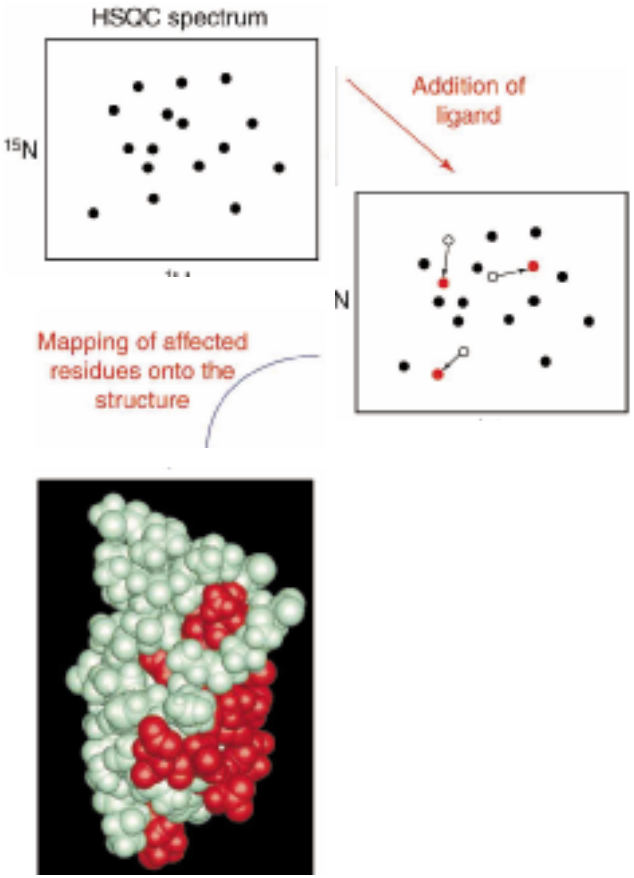


with ligand



# Detecting Ligand Binding using $^{1}H,^{15}N$ NMR

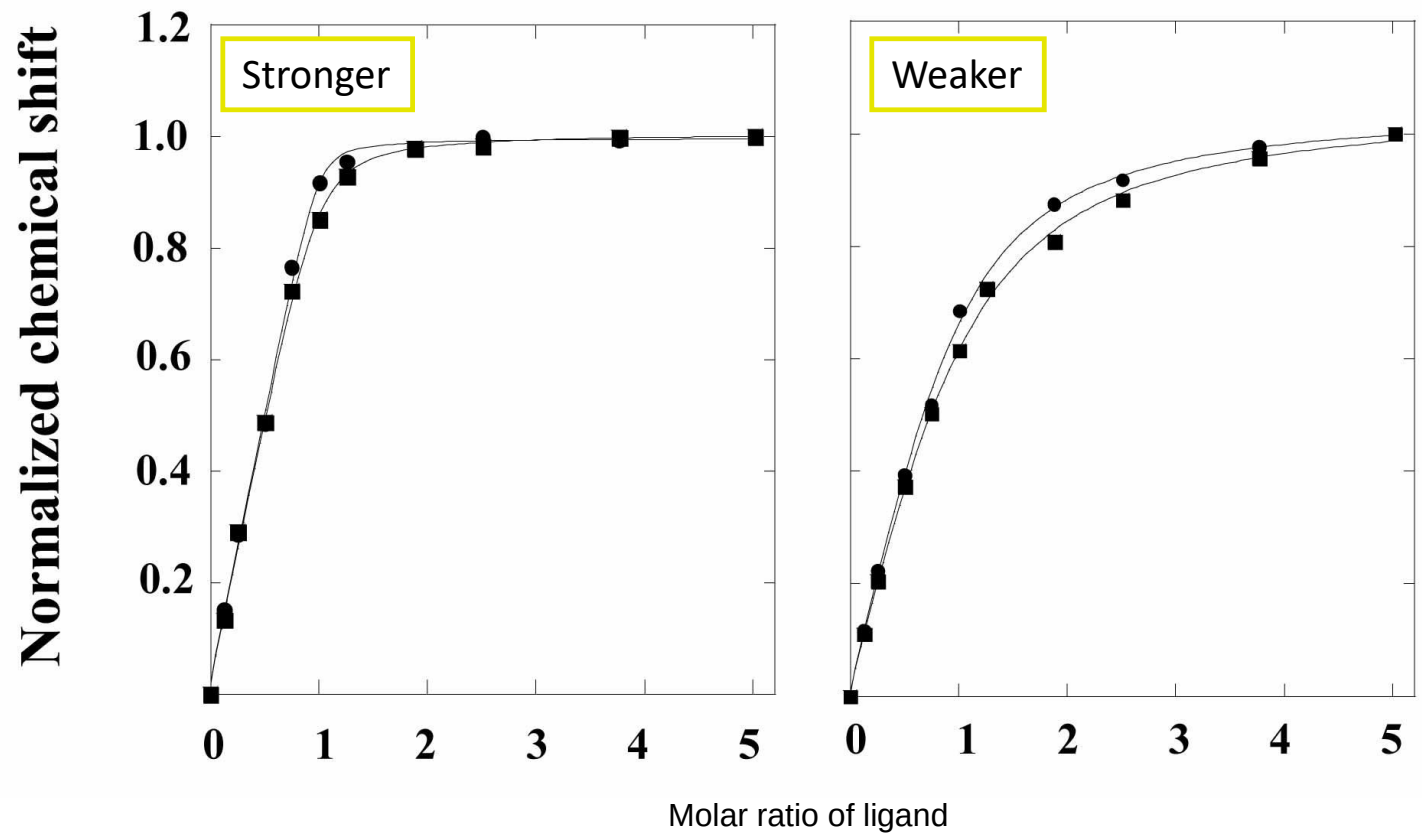



Identification of ligands

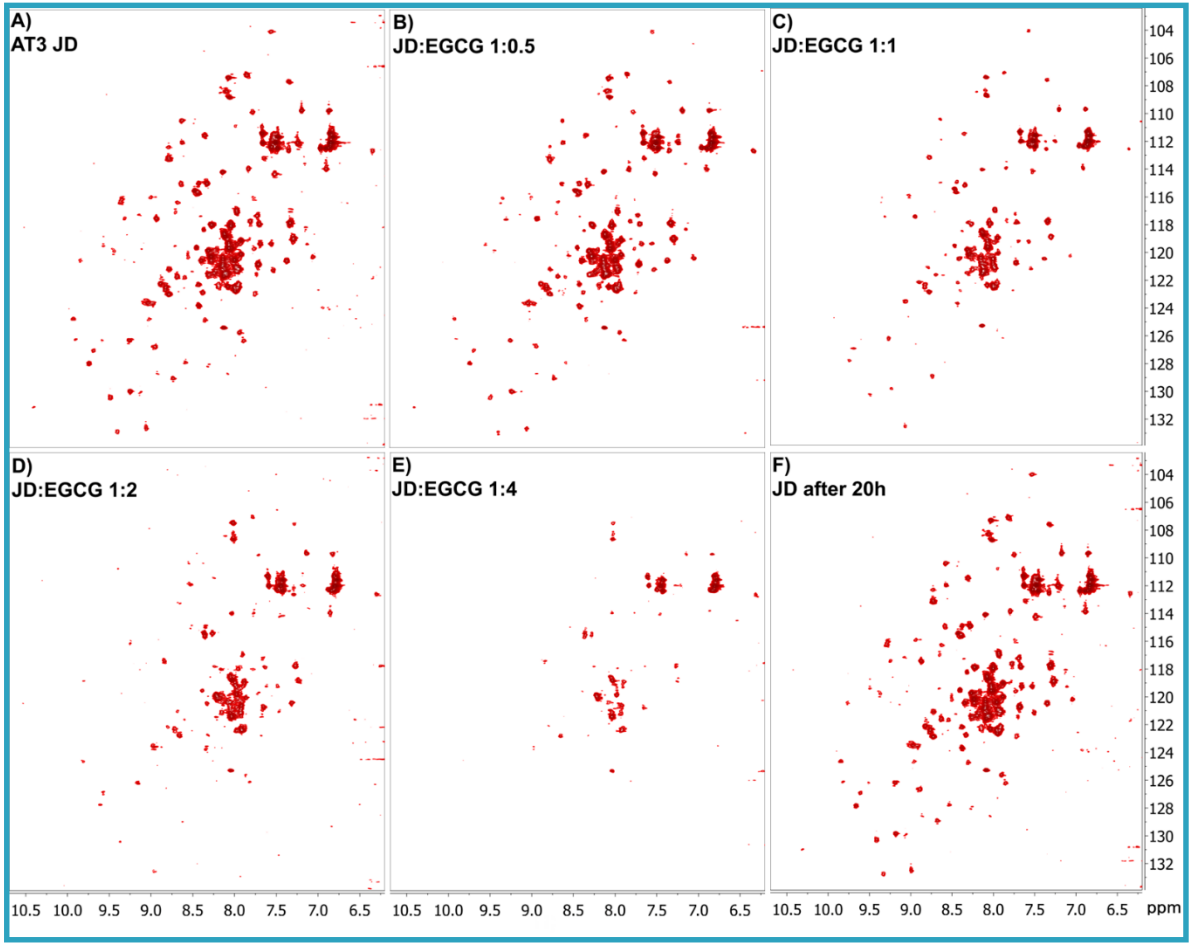
Identification biding site

# Binding Constants from Chemical Shift Changes




Fit change in chemical shift to binding equation

# Binding of Epigallocatechin gallate (EGCG) to Ataxin 3 – The Receptor point of view




**$^{15}$N-SOFAST-HMQC titration experiments for the characterization of EGCG interaction with JD monomer.**


**EGCG induces AT3 unfolding and precipitation**

Elena Regonesi

Antonino Natalello

Bonanomi M., Visentin C., Natalello A., Spinelli M., Vanoni M., Airoldi C., Regonesi M. E., Tortora P., *Chem. Eur. J.*, **2015**, 21, 50, 18383–18393.