

***Natural Product Screening and
Hit Characterization using
Affinity Mass Spectrometry-Based
Automated Ligand Identification System (ALIS)***

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Outline

- Introduction to Automated Ligand Identification System (ALIS)
 - Screening tool
 - Affinity triage tool
- Application of ALIS to Natural Products (NP) Research
 - Sample suitability studies for NP extracts
 - Counter screening to isolate specific binders
 - Software tools to prevent replication
 - Triage methods to affinity rank newly discovered compounds
 - ALIS-MS/MS for structural elucidation of unknown NP hits

Automated Ligand Identification System (ALIS)

The ALIS system integrates six fundamental technologies:

Affinity Selection

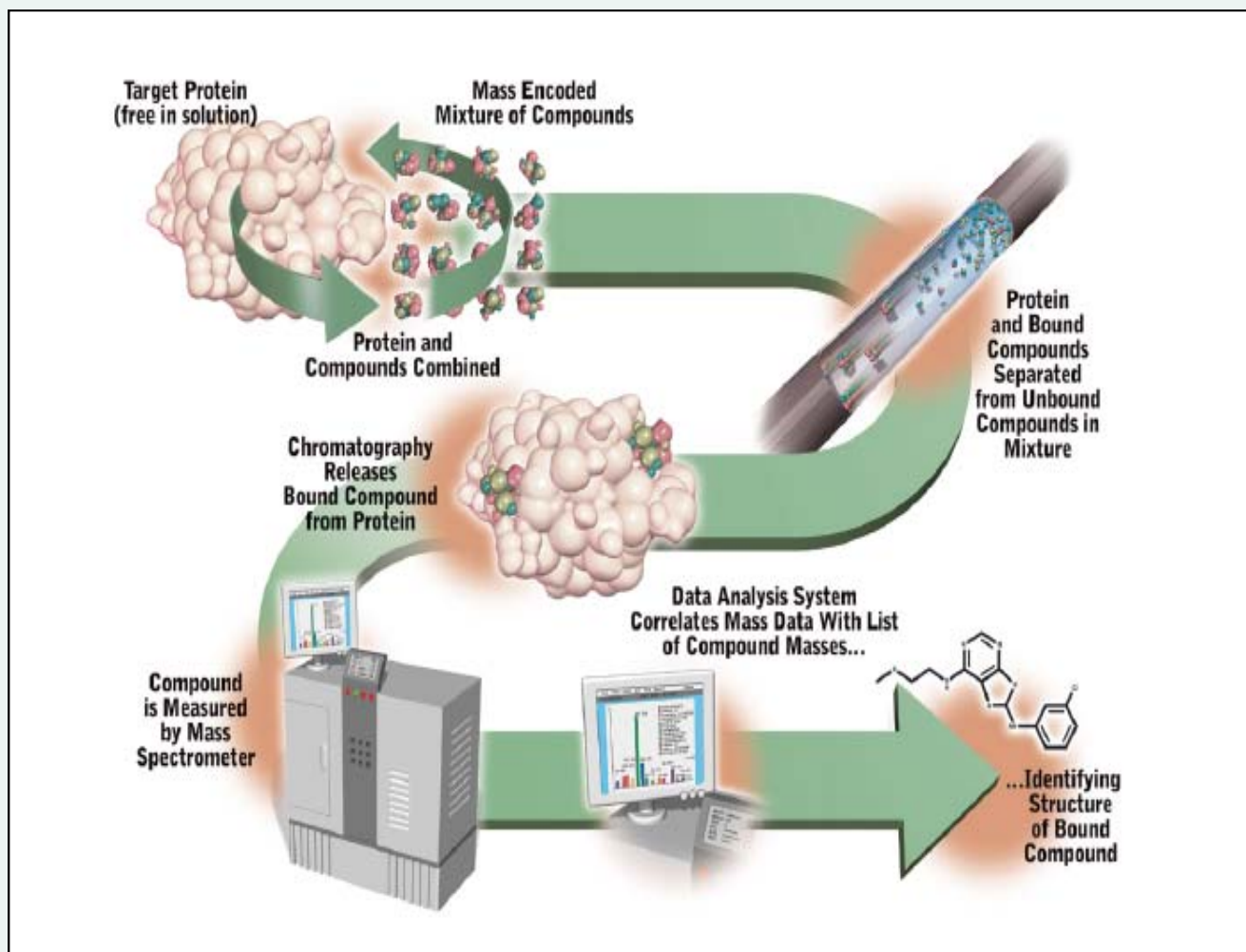
Sample
Automation

Size Exclusion
Chromatography

Reverse Phase
Chromatography

Mass
Spectrometry

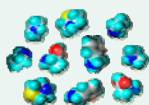
Data Analysis
System



ALIS Workflow

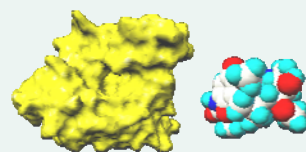
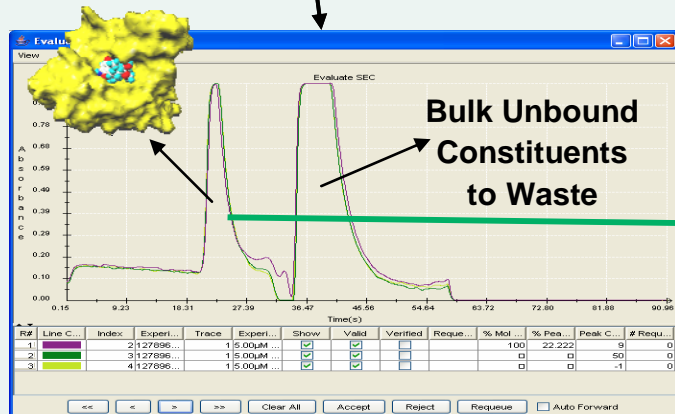


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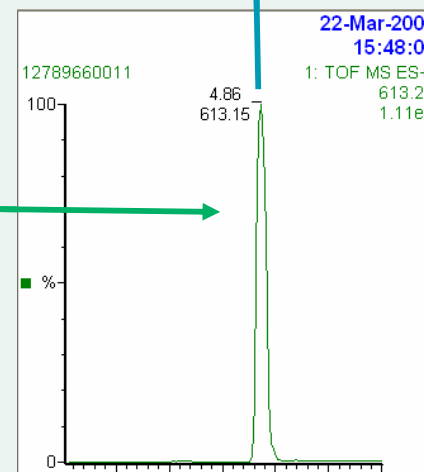


Incubate Target protein
(free in solution) with
Mass-encoded libraries

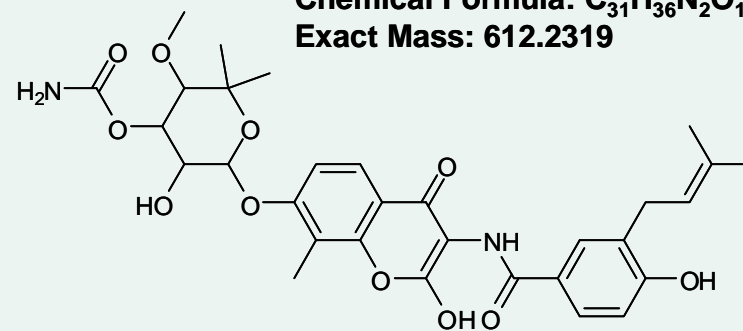
Rapidly separate ligands bound
to target from unbound library
members by automated micro-
scale SEC



Capture complex & dissociate
ligands from target;
Previously Bound Ligands are
analyzed by Reverse Phase
LC – ESI ToF MS



Novobiocin
Chemical Formula: $C_{31}H_{36}N_2O_{11}$
Exact Mass: 612.2319



ALIS software
uniquely
identifies ligand
structure from
mass information

ALIS: Unique Advantages for Natural Product Screening

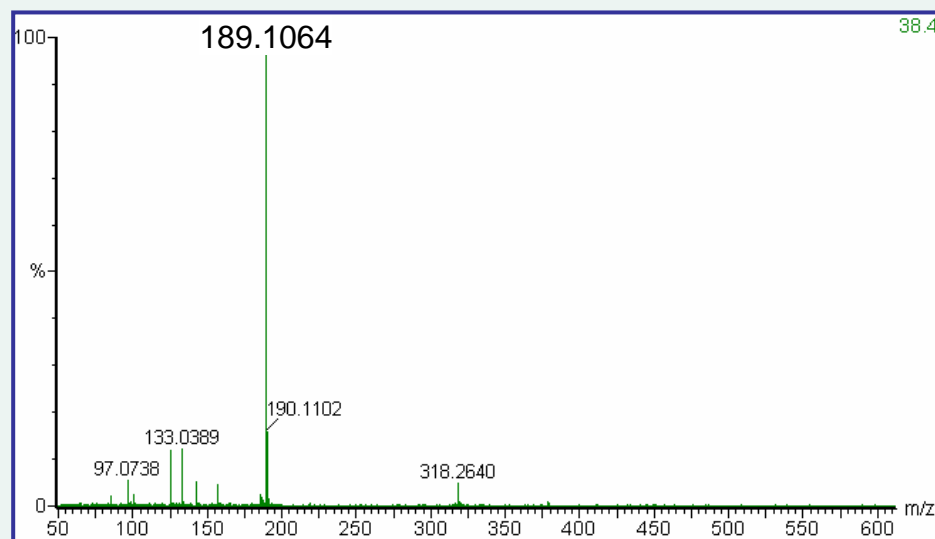
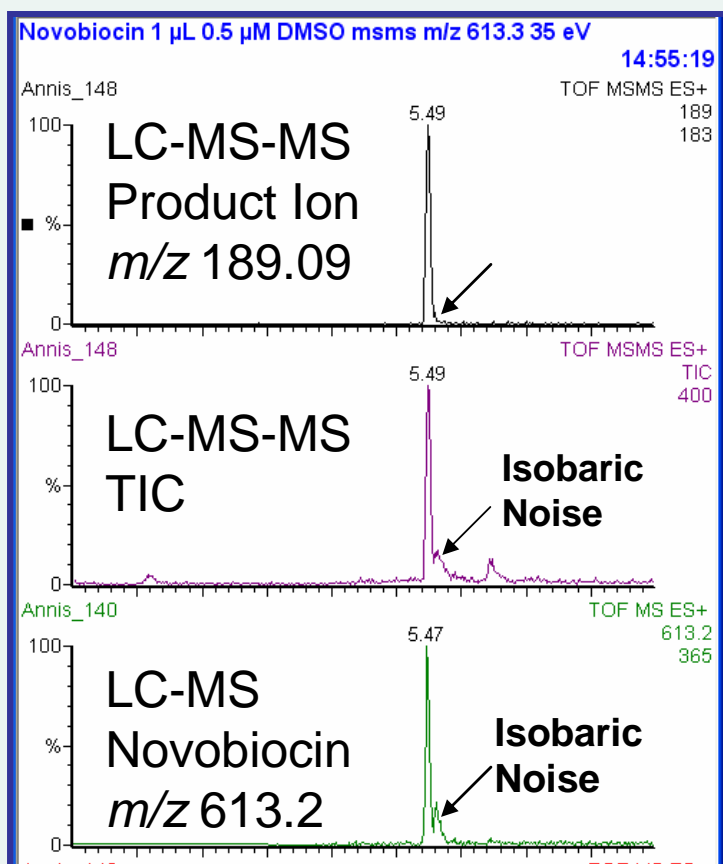
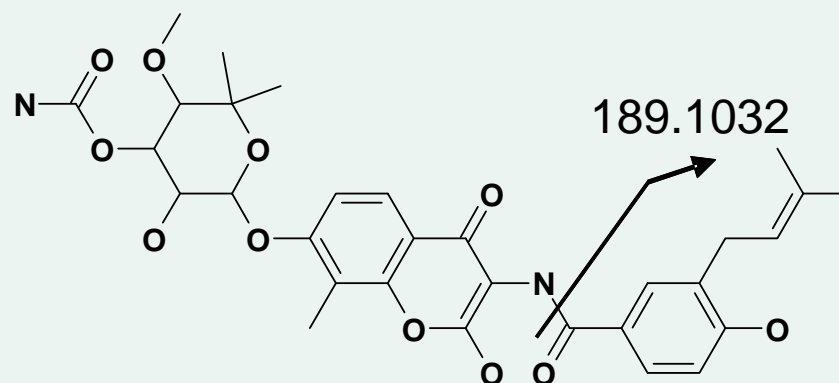
- High sensitivity and broad dynamic range enable drug discovery and characterization from low-level components of complex mixtures
- SEC eliminates false positives due to non-specific binding
- Accurate mass measurements yield empirical formulas for NP database searches
- MS-MS provides structural data and allows MS-triggered purification and fingerprint matching
- Affinity estimates in complex mixtures enable rapid triage
 - **Insensitive to component concentration**
 - **Triage hits before isolation, testing, & structure determination**

Proof of Concept Goal: Determine the Feasibility of Screening Natural Product Extracts with ALIS

- Well-characterized target and known inhibitor
 - Gyrase-B (a Gyrase holoenzyme subunit)
 - Well-known target for antibiotic therapy
 - Soluble protein; Well-behaved in ALIS
 - Novobiocin
 - Aminocoumarin antibiotic isolated from actinomycete in the mid-1950s
 - Binds to the Gyrase-B subunit with nanomolar affinity
 - Inhibits ATPase activity
- Experimental design
 - Linearity and limits of detection of Novobiocin
 - Sample Suitability of Natural Product extracts from multiple sources
 - Analysis of endogenous Novobiocin in active NP extracts
 - Software tools
 - Affinity rank detected components
 - Identify novel components using ALIS-MS/MS

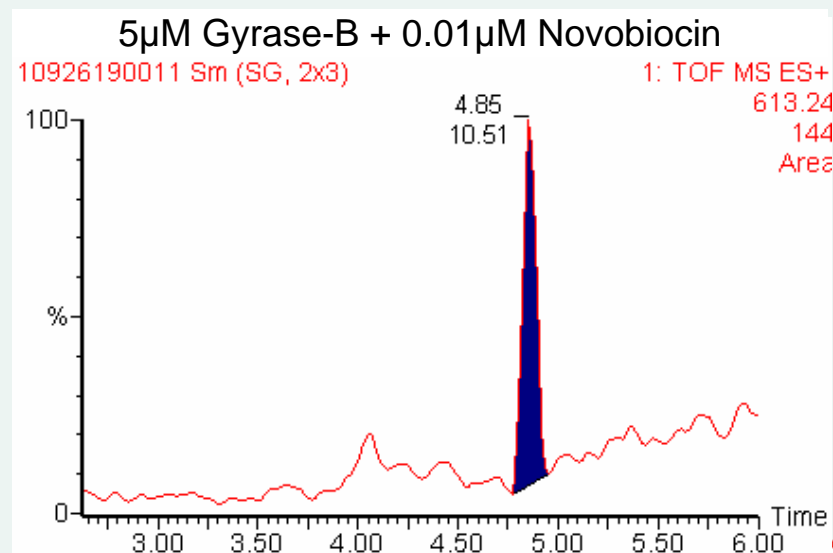
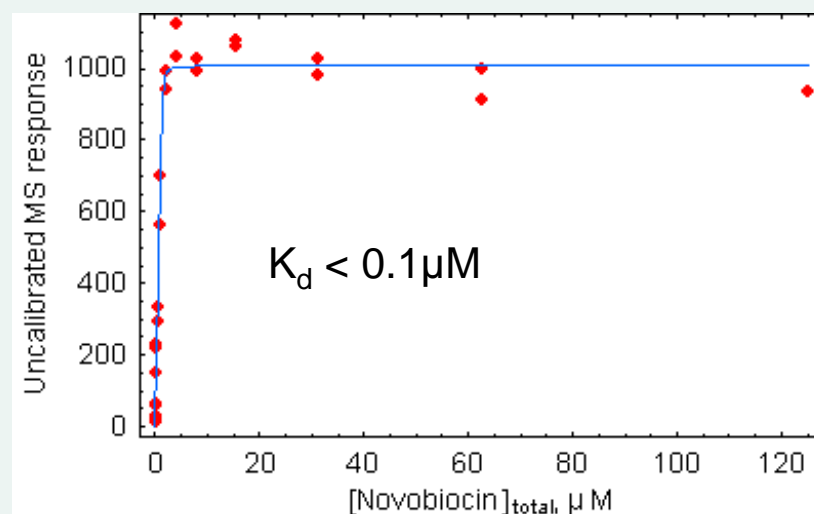
Novobiocin Standard Quantification by LC-MS/MS

- Standard Novobiocin (0.01 – 10 μ M) used to generate MS-MS fingerprint & calibration curve ($R^2 = 0.99957$)



Novobiocin Standard Analysis by ALIS-MS

- K_d determination: 0.01-125 μM
Novobiocin incubated with 2.0 μM Gyrase-B
- ALIS binding conditions: 50 mM TRIS, pH 8.0, 40 mM KCl, 10 mM NaCl, 1 mM EDTA, 2 mM DTT, 4% glycerol.
- SEC (Agilent 1100): F = 450 $\mu\text{L}/\text{min}$, 700 mM Ammonium acetate, pH 8.0; SEC media produced in-house
- RPC (Agilent cap1100): F = 20 $\mu\text{L}/\text{min}$, 0.2% Formic Acid in Water/Acetonitrile; 0.5x50mm Higgins C₁₈ column
- MS: Waters LCT ESI-ToF
- ALIS limit of detection = < 0.008 μM
 - < 5 ng/mL
 - < 10 ppm of total NP extract mass



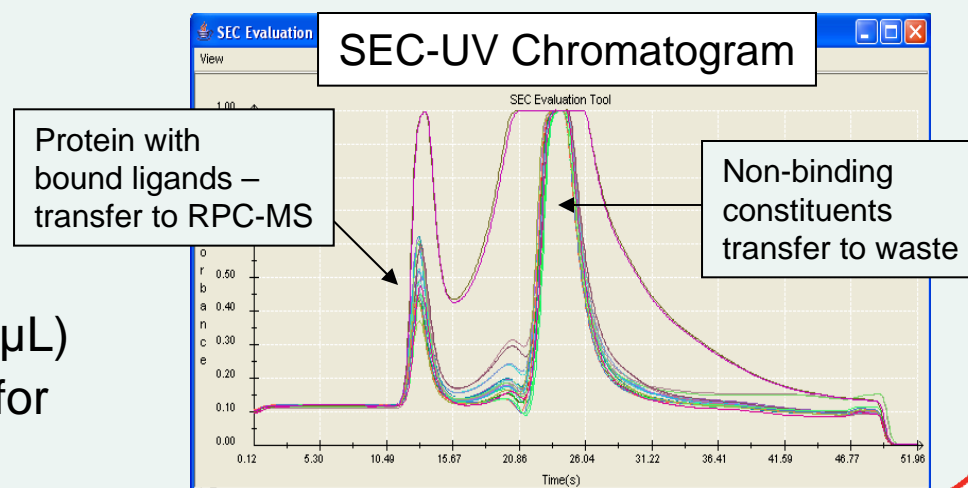
Sample Suitability of NP Extracts for ALIS: Screening Against Gyrase-B

Sample Preparation of Extracts

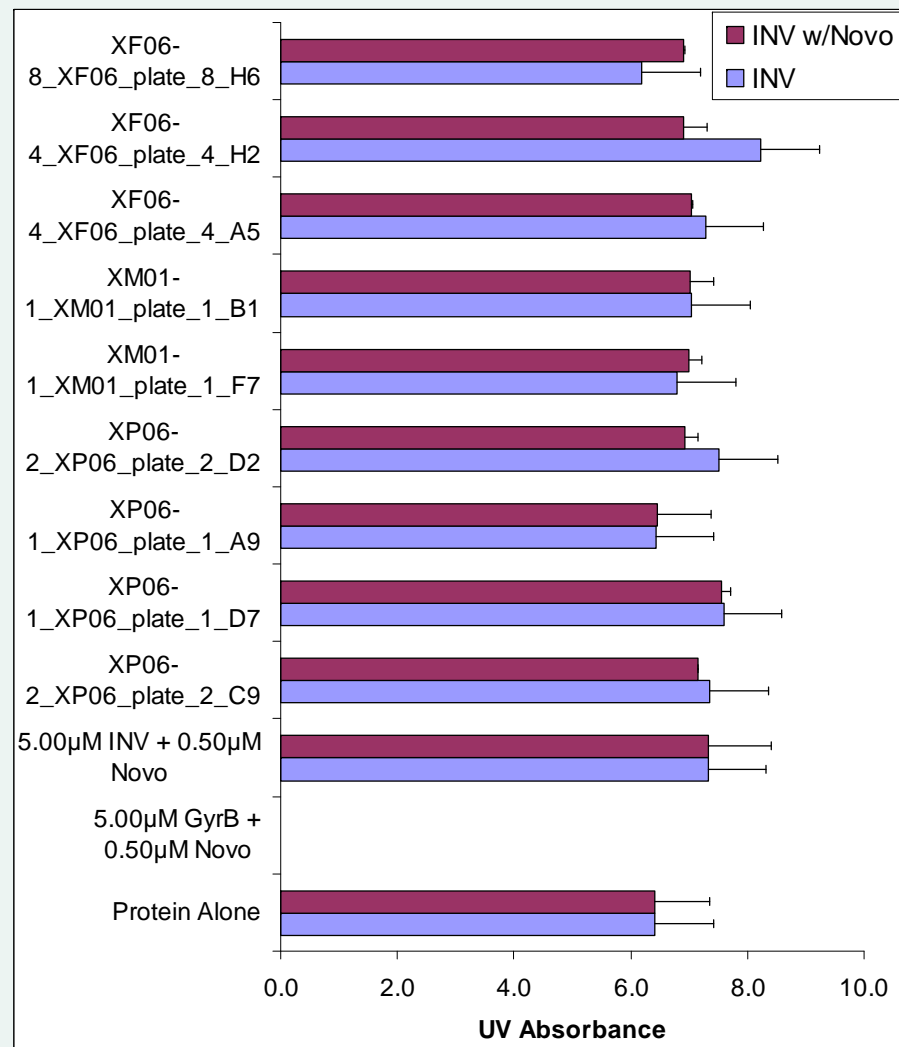
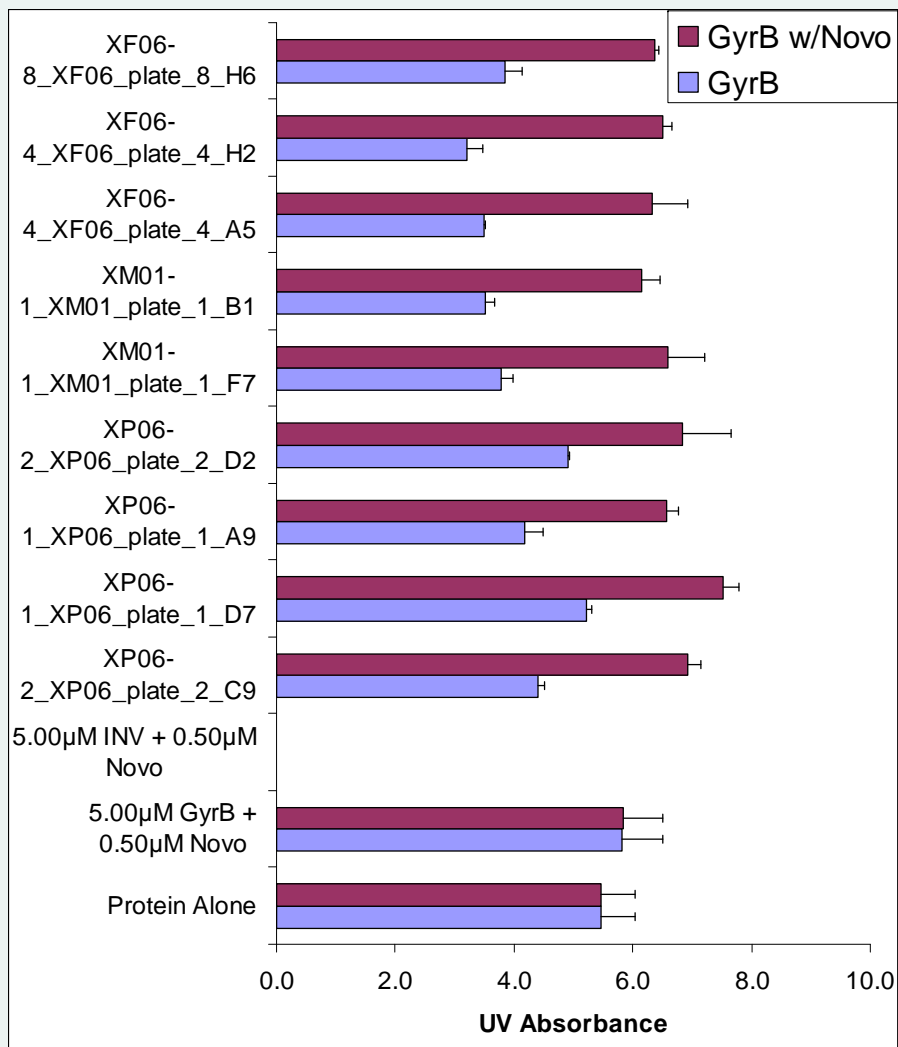
- 1-mg samples of NP extracts dissolved in 40 μL DMSO
 - 25 mg/mL final concentration
- ALIS sample preparation:
 - 1:20 buffer dilution, centrifugation
 - 1:1 dilution with buffer containing 5-10 μM Protein
 - Overall 1:40 dilution
- NP extract total mass = 0.625 mg/mL in 2.5% DMSO
 - 500 MW compd @ 100 ppm total mass = 0.125 μM (250 fmol in 2 μL)
 - ALIS limit-of-detection \approx 50 fmol for most drug-like molecules

Robustness Set

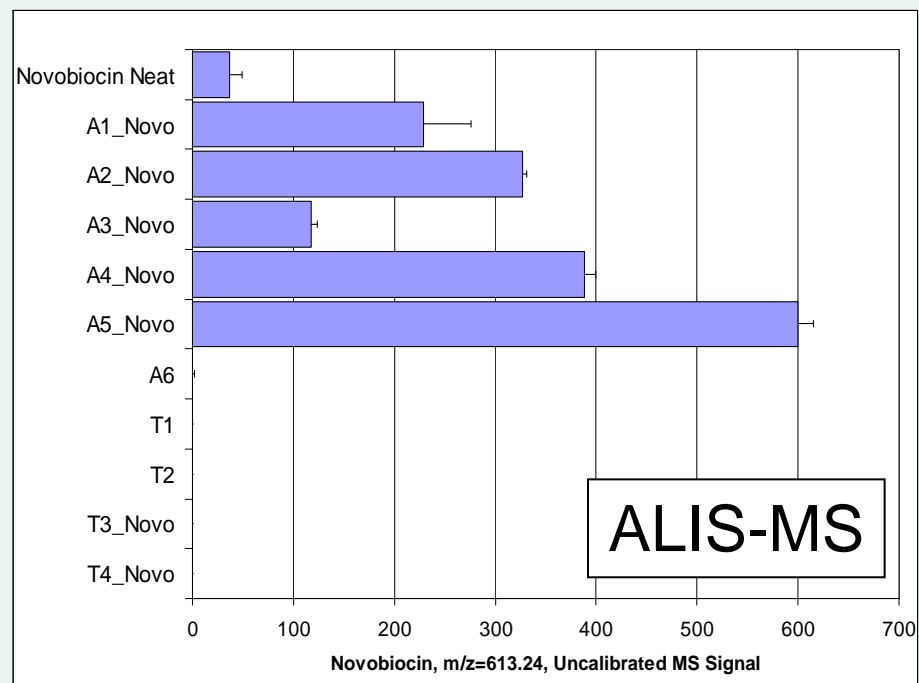
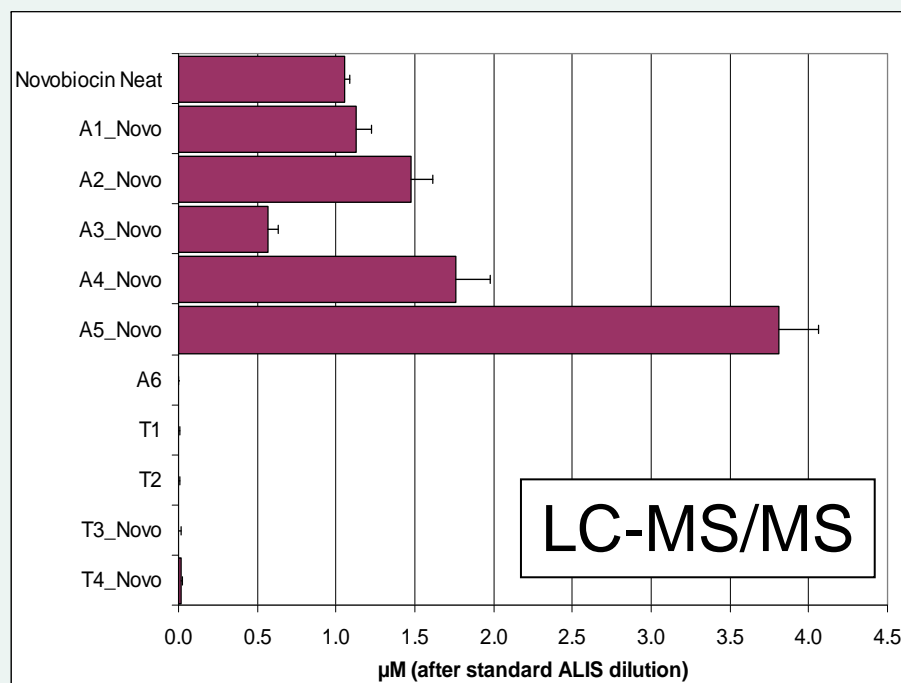
- Sampling of 1500-member robustness set (no endogenous Novobiocin) was chosen at random, equally weighted to different sources
- Screened \pm novobiocin at 0.5 μM
- Invertase screened side-by-side with Gyrase-B samples



Sample Suitability of NP Extracts for ALIS: SEC Peak Quantification



Endogenous Novobiocin in Active NP Extracts: LC-MS/MS Quantification and ALIS-MS Screening with Gyrase-B



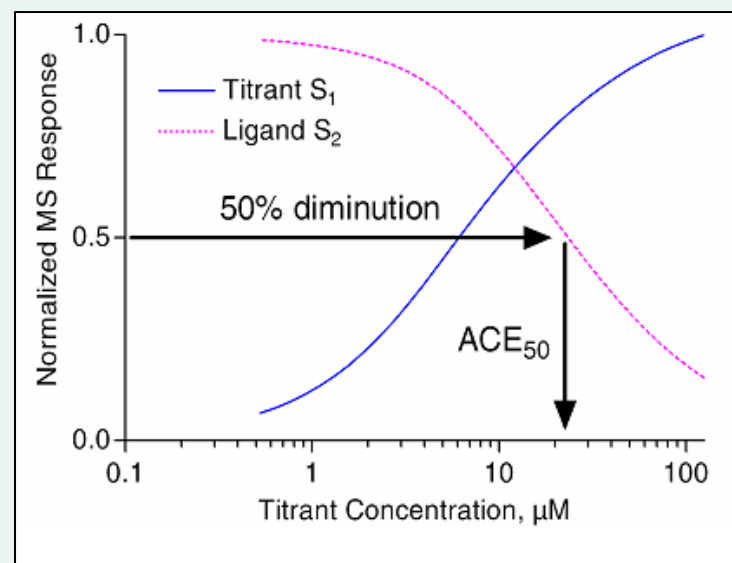
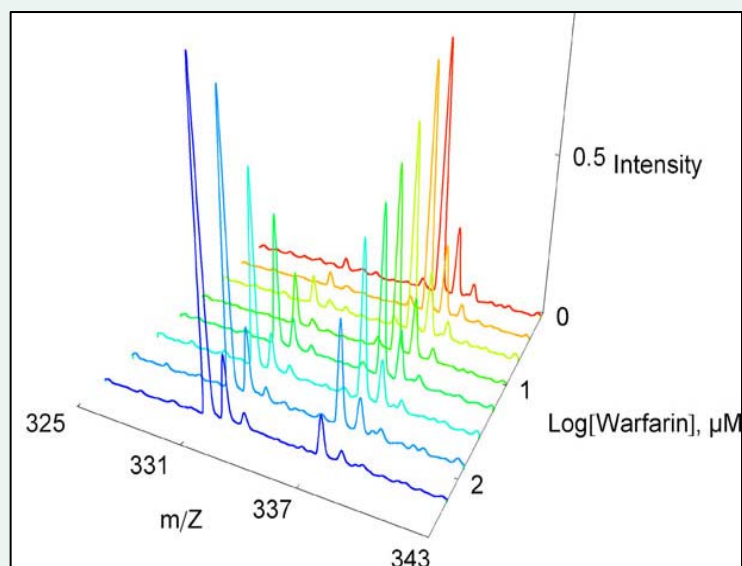
- Endogenous Novobiocin detected in active NP extracts at levels from 0.5 to 6 μM (0.3 to 3.6 $\mu\text{g}/\text{mL}$) after standard ALIS 1:40 dilution
- Novobiocin recovery in ALIS parallels its concentration in extracts
- Two samples purported to contain novobiocin are below LODs of both methods
- Novobiocin was not detected in active samples containing other NP

ALIS Screening of Gyrase-B against Active Extracts

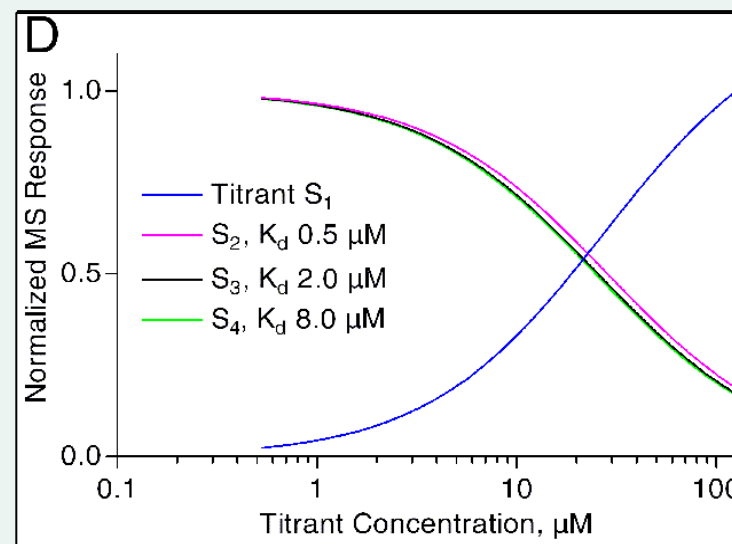
NP_extract	Target	Trunc_mass	measured_m/z	signal_strength
A2_Novo	Invertase			
	Gyrase B	→ 599	599.2291	531.43
			599.2309	544.85
		→ 613	613.2415	4731.3
			613.2416	4955.63
		→ 643	643.2570	1844.96
			643.2571	1811.32

- Side-by-side screening of breakthrough control protein (Invertase) indicates very little SEC “breakthrough” or non-specific binding by NP extract components
- Hits present in both samples are not of interest
- Comparison enables identification of specific hits (e.g., novobiocin, 613) and detection of unknown entities (e.g. m/z 599 and 643)

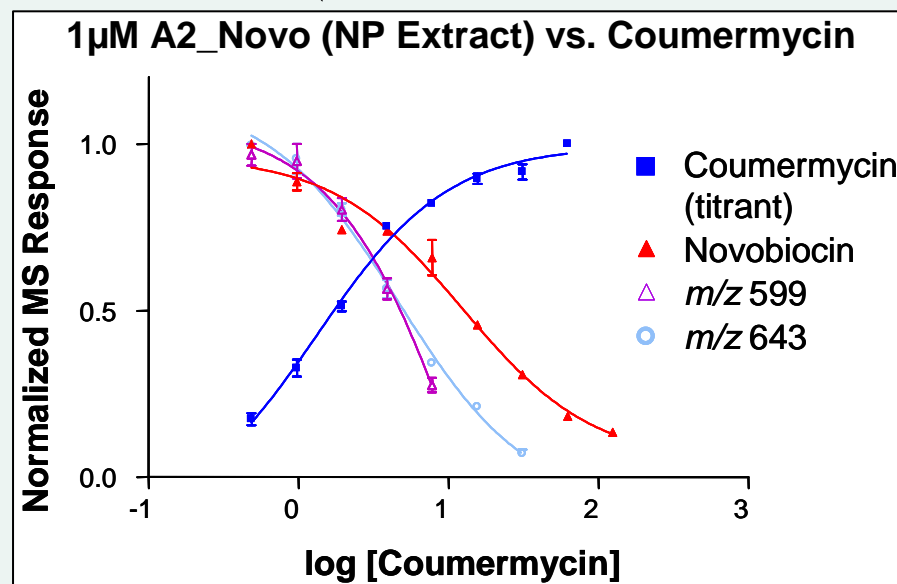
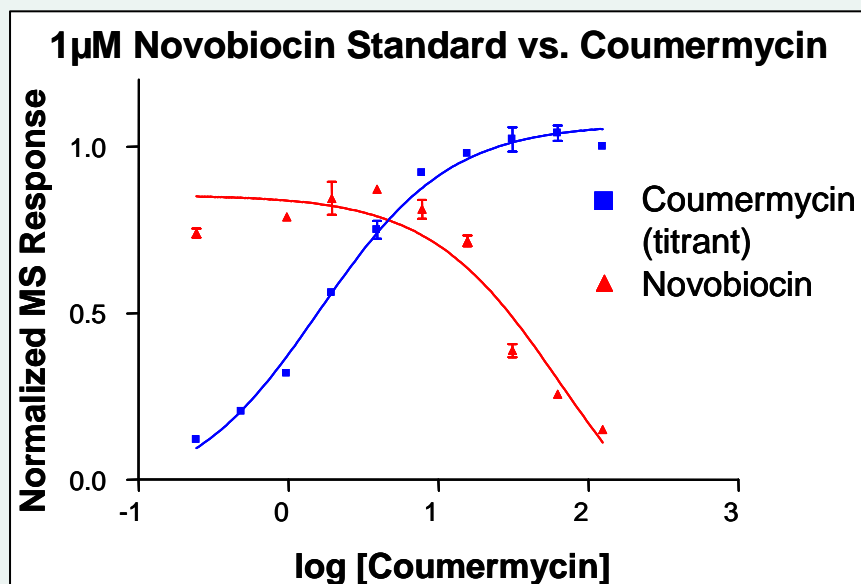
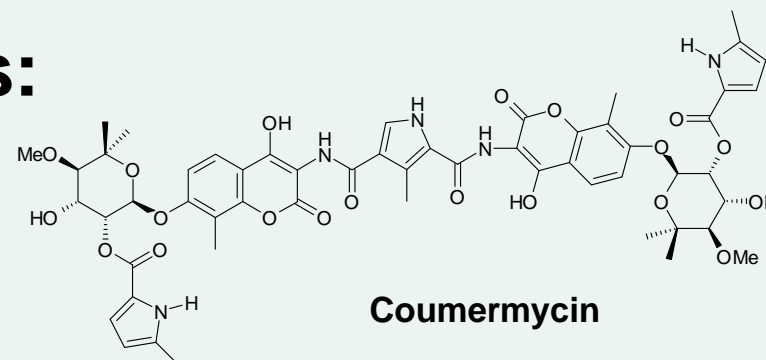
ACE₅₀ Method: ALIS Competition Experiments



- ACE₅₀ value of the ligand is the [titrant] at which the ligand recovery is reduced by 50%
- ACE₅₀ values depend on the K_d of the titrant and the ligand
- If the receptor is present in excess, the ACE₅₀ value is insensitive to the concentration of the unknown



Gyrase-B ACE₅₀ Experiments: Standards and Active NP Extract

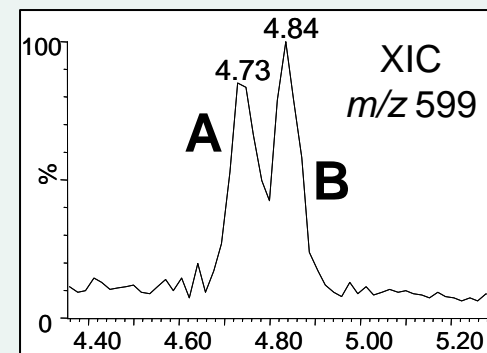
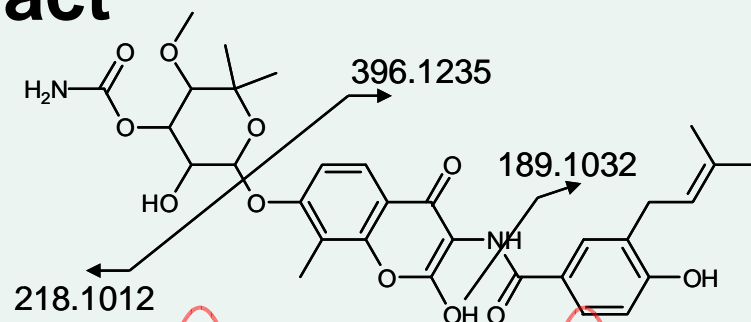


- Coumermycin is directly competitive with Novobiocin
- Two new hits detected in active extract with weaker affinity than Novobiocin

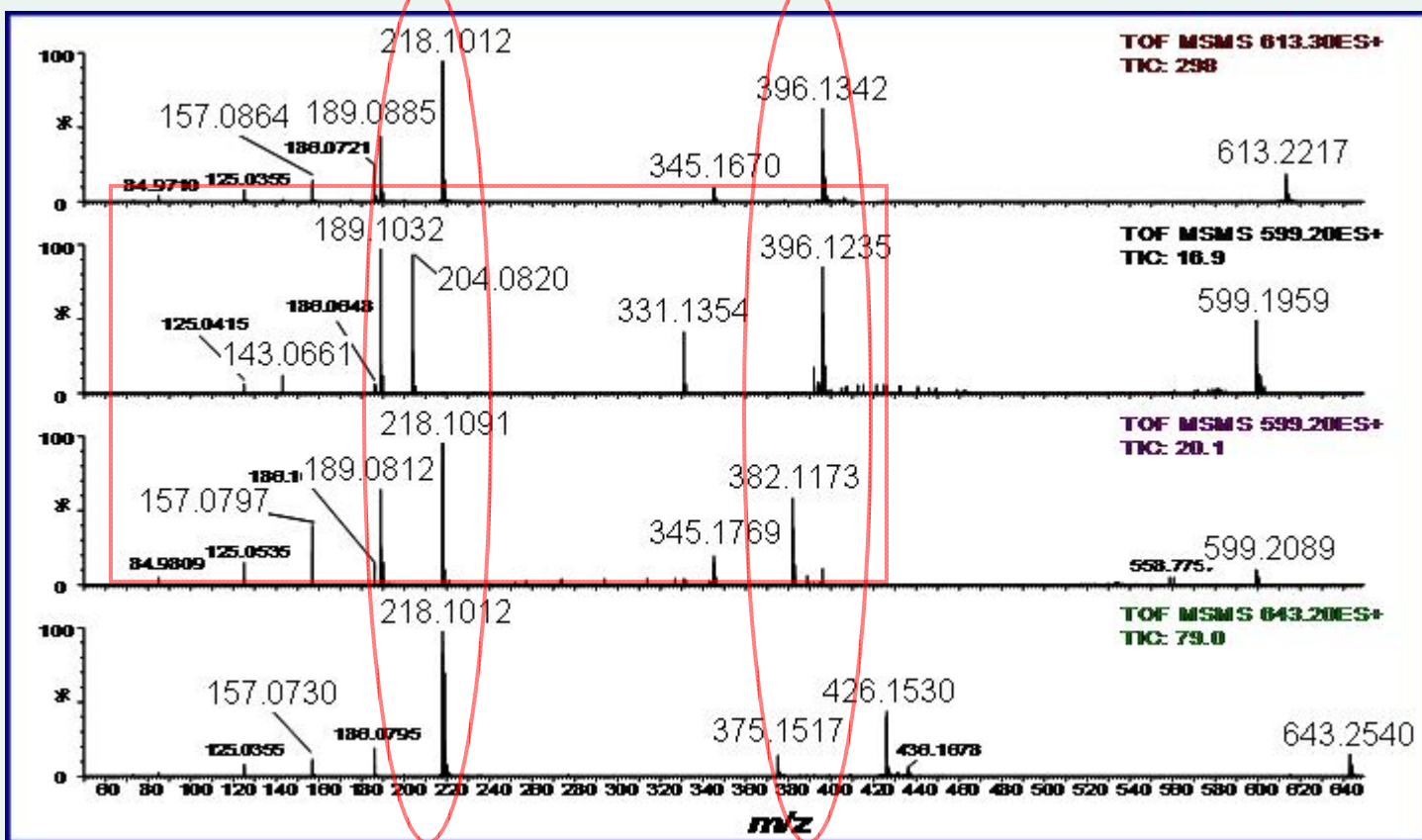
–*m/z* 599.2241,
 –Novobiocin – CH₃ ?
 – Δ ppm = -6.3 ± 10.2 , n = 13

–*m/z* 643.2503
 – Novobiocin + CH₃O ?
 – Δ ppm = 0.0 ± 11.3 , n = 19

ALIS-MS/MS Identification of New Hits from Active NP Extract



Novobiocin

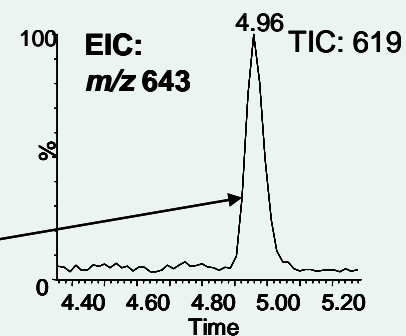
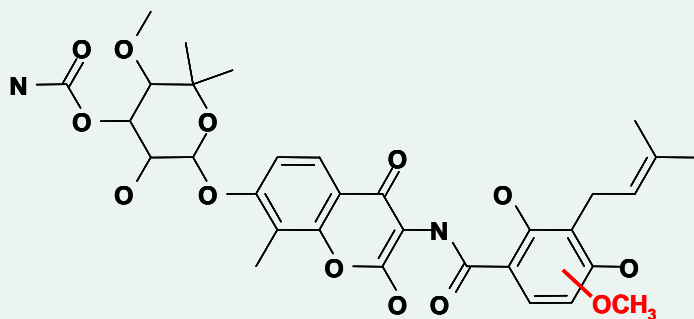
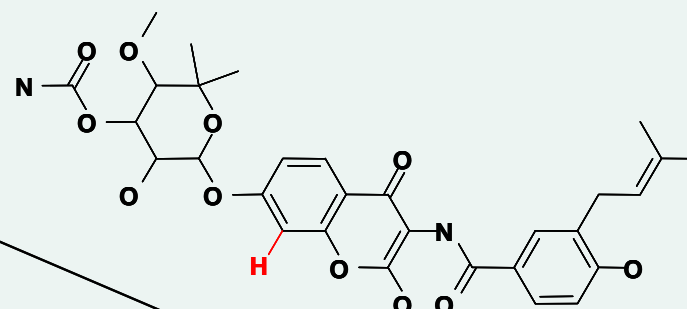
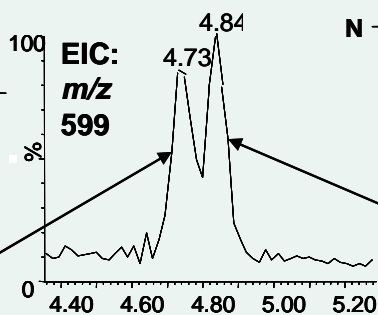
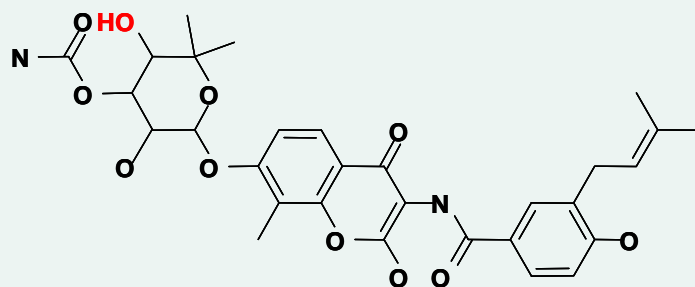
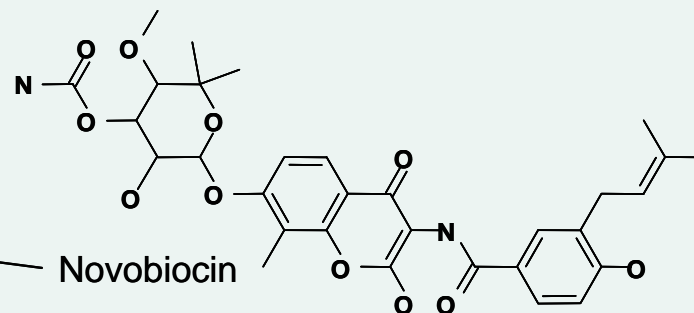
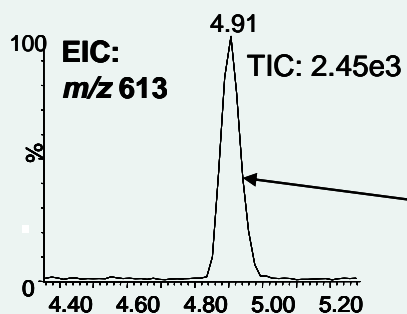


m/z 599 A

m/z 599 B

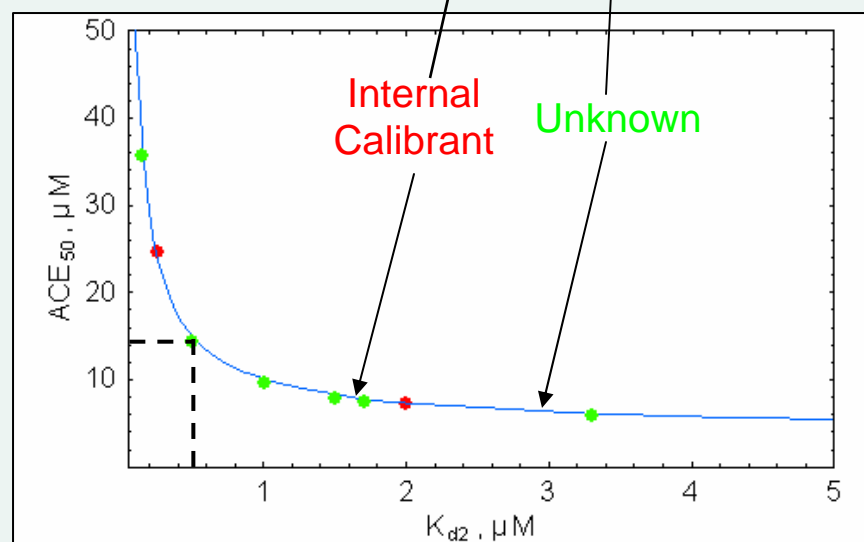
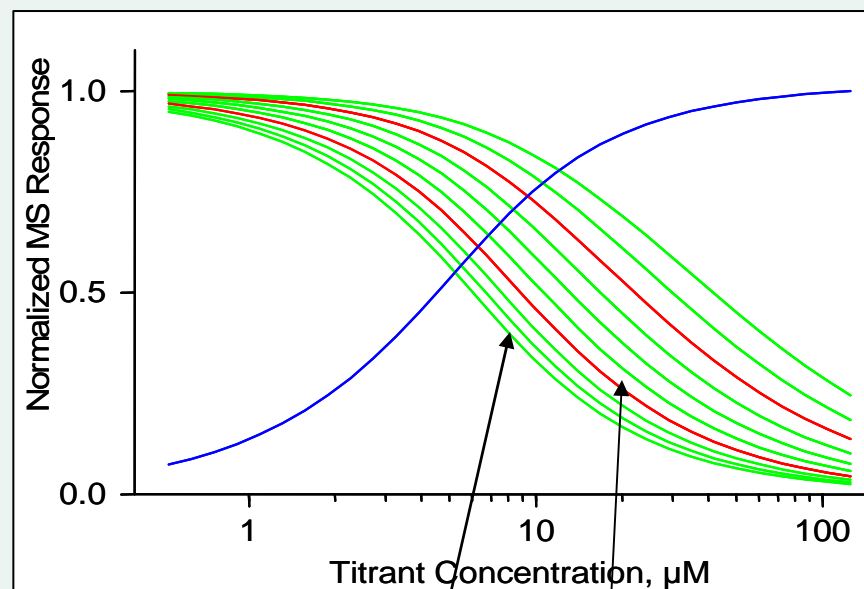
m/z 643

ALIS-MS/MS Identification of New Hits from Active NP Extract



ALIS-based Affinity Ranking: Absolute K_d Determination

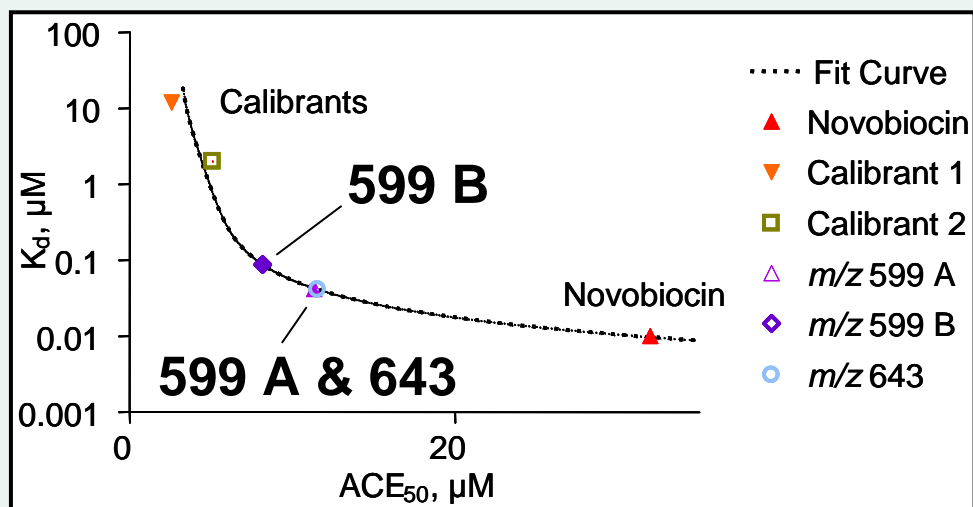
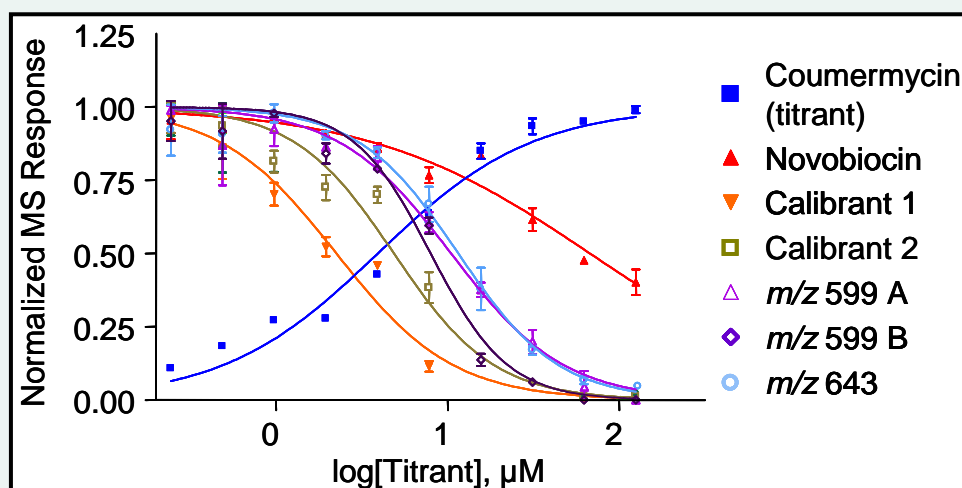
- ACE_{50} curves enables K_d estimates
- Spike internal calibrants of known K_d into mixture (e.g. NP extract)
- Compare the calibrant ACE_{50} values to those of the other mixture components
- Plotting calibrant ACE_{50} values versus their known K_d values yields absolute K_d values of unknown



Calibrants Spiked into NP Extract to Determine Absolute K_d for New Hits

- By plotting known K_d values vs. ACE_{50} values, it is possible to calculate the K_d values of the unknowns
- All 3 unknowns K_d values are $\sim 0.1\mu\text{M}$
- This ALIS ACE_{50} method distinguishes between two unknowns with the same molecular weight and different affinities

Gyrase-B ACE_{50} Experiment
1 μM Calibrant Mix + 1 μM A2_Novo vs. Coumermycin



Conclusions

- ALIS can identify target-specific ligands from Natural Product extracts
 - Sample preparation is simple; Amenable to automation
 - Protein SEC behavior is acceptable
 - Individual component limit-of-detection is 10-100 ppm of crude extract
- Ability to select of target-specific ligands via counter-screening
 - Eliminates non-specific hits
 - Conserves resources
- Hit triage is demonstrated by ALIS ACE₅₀ experiments prior to purification and using minimal amounts of protein and crude extract
 - Determination of relative affinity
 - Accurate mass information and MS/MS characterization
 - Affinity ranking with internal calibrants allows absolute K_d determination
- **Successful Proof of Concept: ALIS is a unique technology with several advantages for Natural Product drug discovery**

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