

# Application of HPLC-NMR, HPLC-MS and MS/MS to Investigate the Environmental Fate of Fluoroquinolone Antibiotics

Cynthia K. Larive and Laurie Harned  
University of California - Riverside

# Environmental Contaminants

## Persistent Organic Pollutants

- Non-polar compounds
  - Pesticides (e.g. atrazine and alachlor)
  - Dioxins, Polychlorinated biphenyls (PCB's)
- Extensive research has been conducted to understand their attenuation and fate

## Emerging Organic Contaminants

- Polar compounds
  - Steroids and hormones
  - Personal care products
  - *Pharmaceuticals*

# Pharmaceuticals: Antibiotics

Application: prevent infection, treat disease, and promote growth

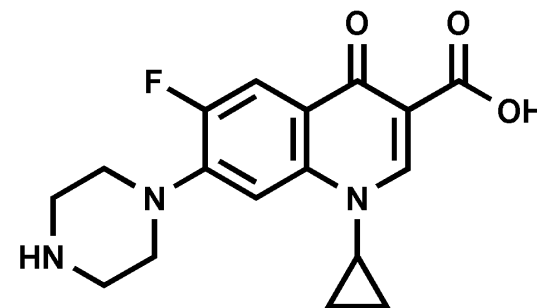
- Clinical medicine
- Veterinary practices
- Agriculture and aquaculture

Implications of persistence or continuous release into the environment

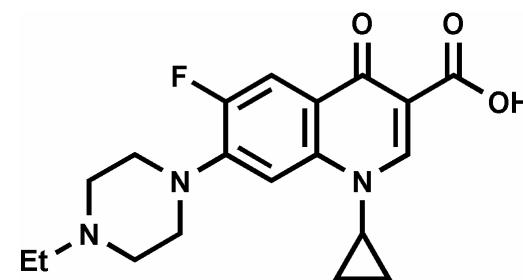
- Increase in antibiotic resistant strains of pathogenic and non-pathogenic bacteria
- Affect balance of microorganisms in the environment

# Fluoroquinolones

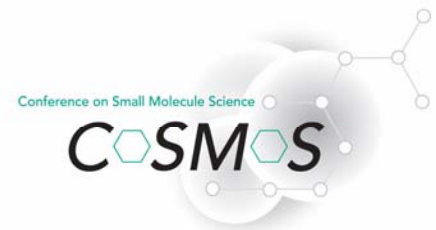
- Synthetic antibiotics
- Prescribed in high dose  
Transformation products
  - Substitution of the fluorine atom
  - Degradation/ functionalization of the piperazine ring
  - Hydroxylation of quinolone ring
- Transformation product standards not commercially available



Ciprofloxacin



Enrofloxacin



# Team Research Goals

- Determine breakdown rates and mechanisms in aquatic ecosystems
- Determine mechanisms from experimental design and the structures of the breakdown products formed
- *Development of antibiotic resistance in non-pathogenic organisms*
- *Effect of these antibiotics on the natural microbial community*

# Lab & Field Scale Studies

## Environmental parameters

- Dissolved organic carbon
- Particulate organic carbon
- Sterile and non-sterile water
- Percent light transmitted



**Laboratory Scale Studies**



**Field Scale Studies: Nelson Environmental Study Area**

August 8-11, 2005 Bristol, Rhode Island

## Experimental design

- Initial [FQ] 250, 25 mg/L
- Sample size: 50, 500 mL
- Prepared using SPE method
- Analyzed by HPLC/PDA/MS

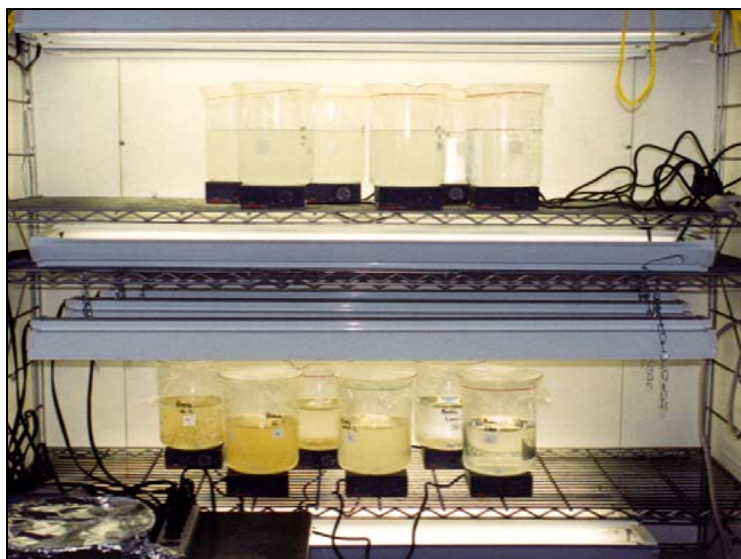
# Attenuation of Ciprofloxacin

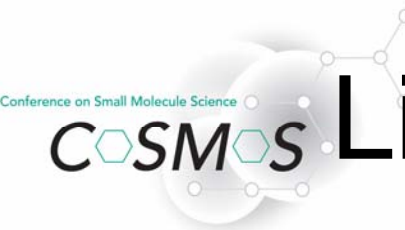
## Laboratory Experiments

Light and Dark

Sterile and Non-sterile

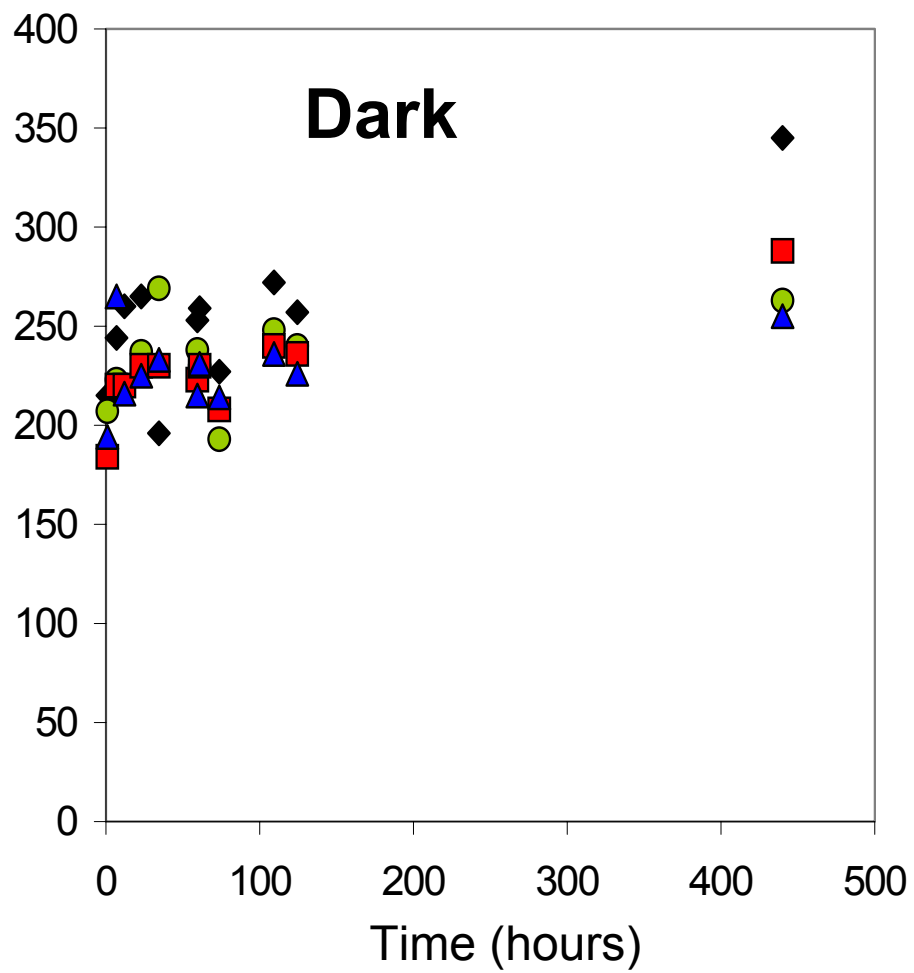
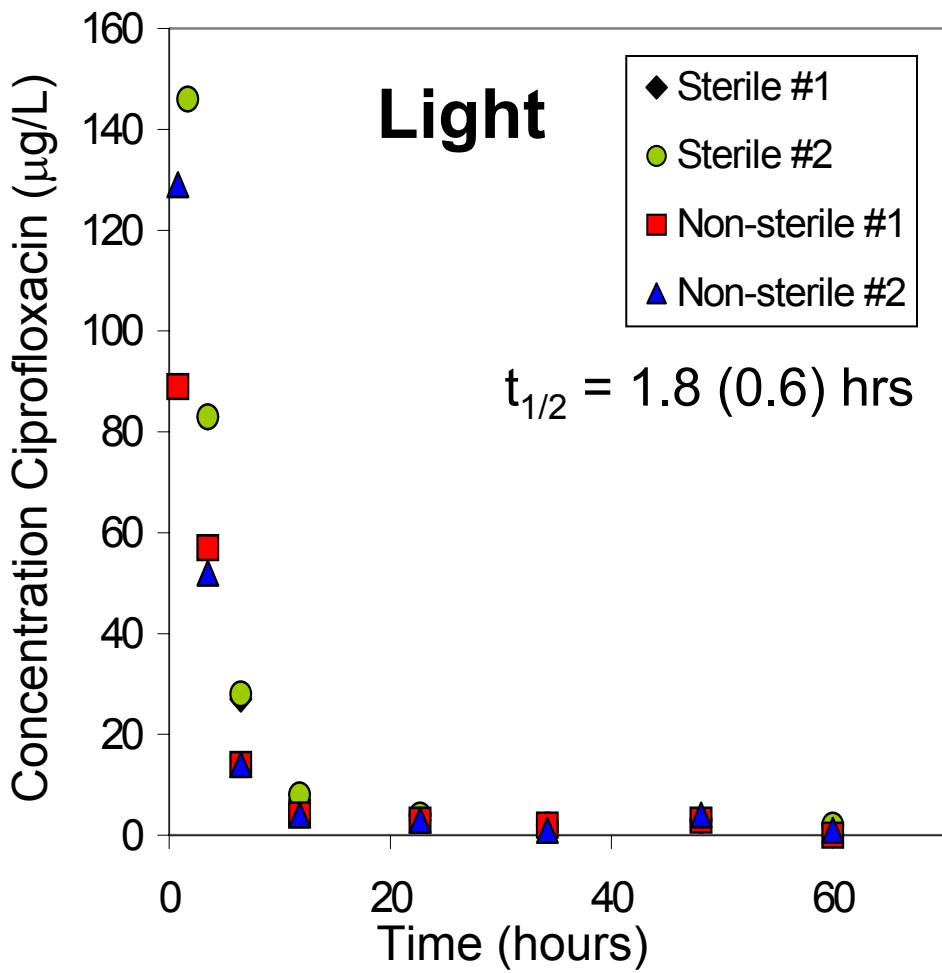
Varied Particulate and Dissolved Organic Carbon





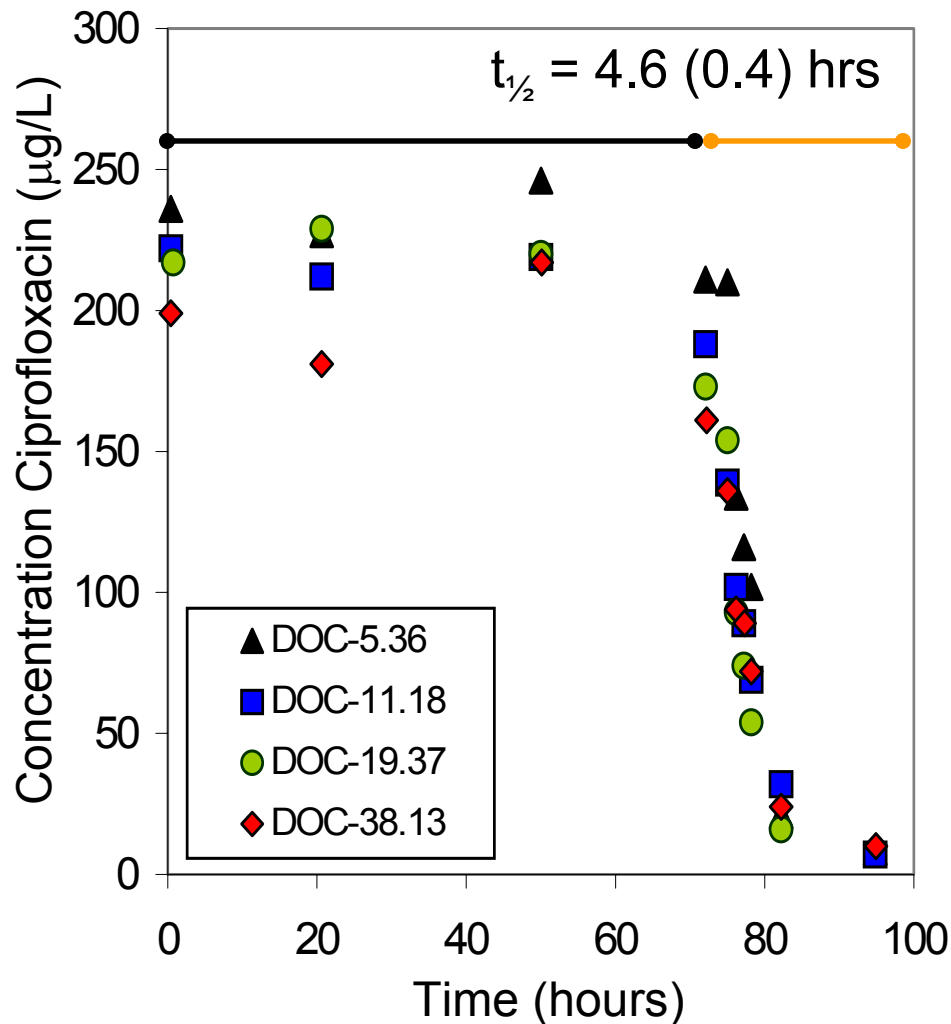
# Light and Dark Attenuation

## Attenuation of ciprofloxacin in sterile and non-sterile water

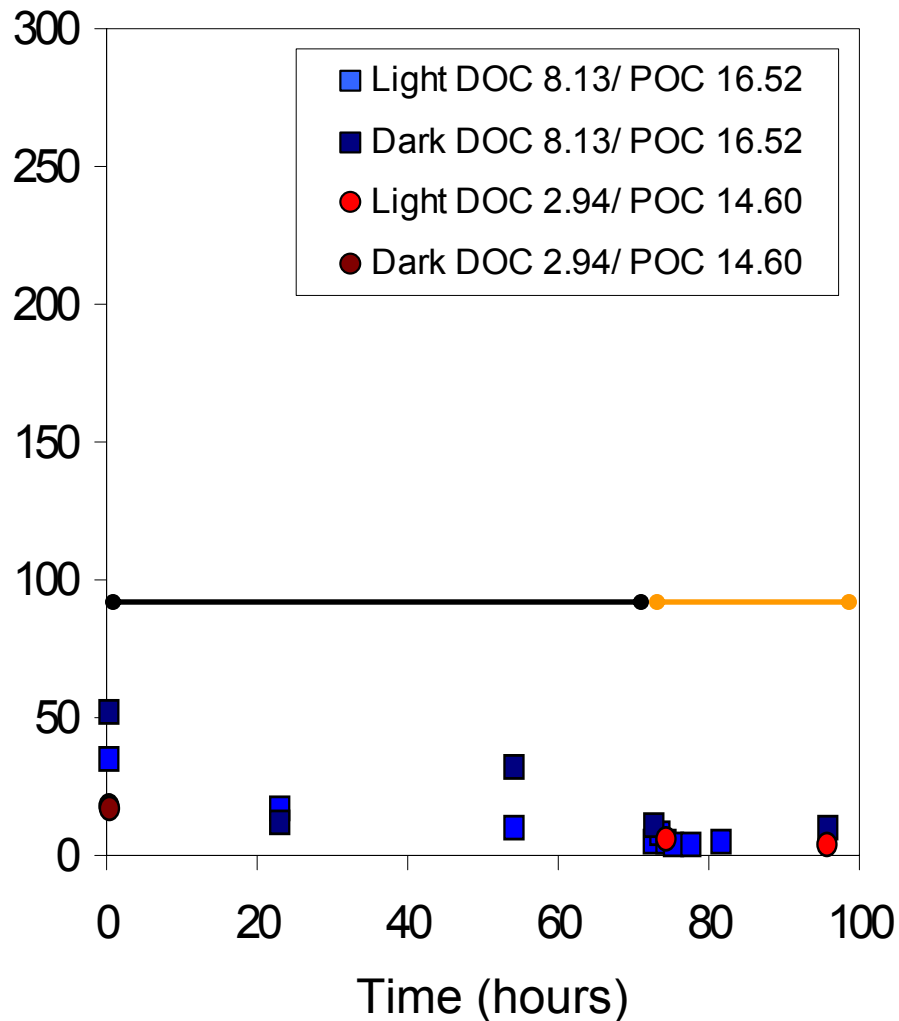


# Effects of DOC and POC

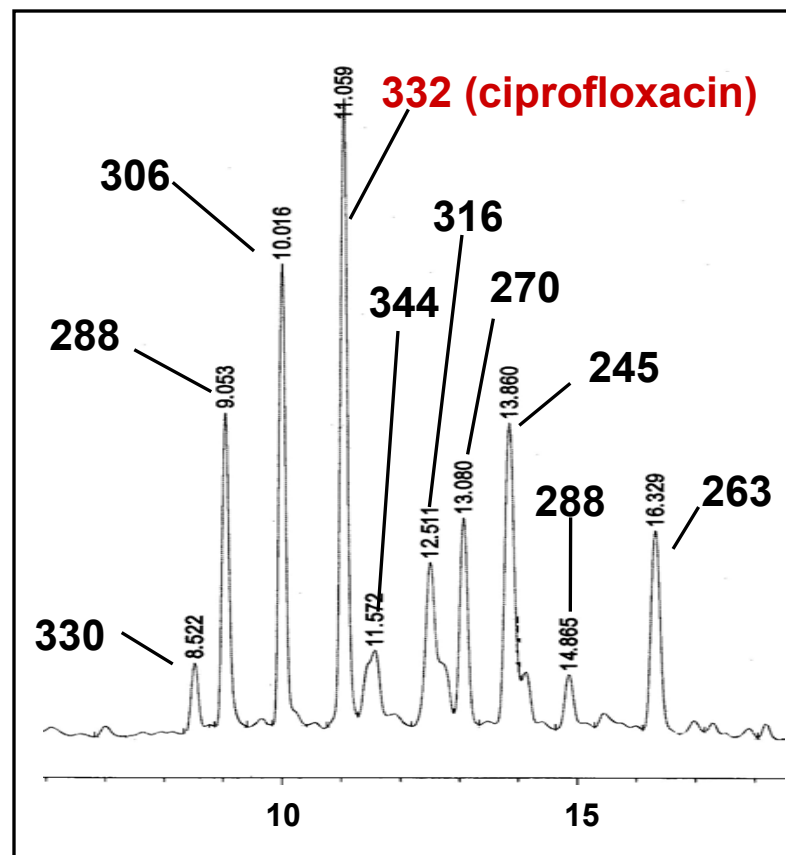
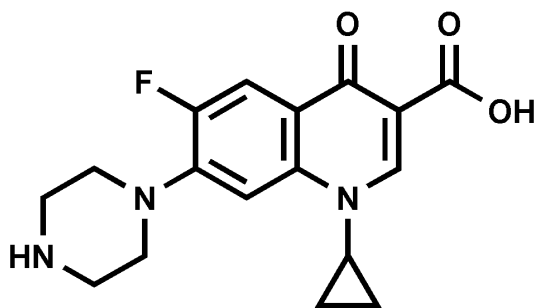
## Varied DOC



## High POC



# Ciprofloxacin Fate



Absorbance vs time

# Product ID Strategies

## NMR

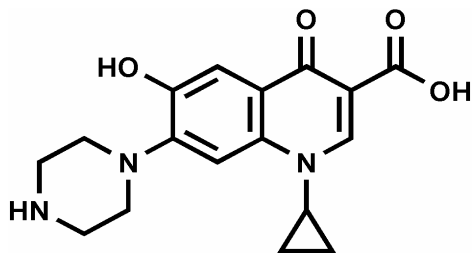
- Connectivity of atoms (coupling)
- Functional group information (chemical shift)
- Structural changes (loss/gain resonances)

- MS

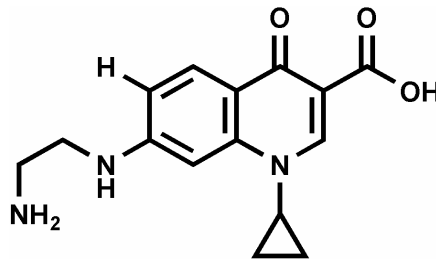
- Molecular weight (exact mass)
- Structural changes (loss/gain mass)
- Fragmentation pattern and neutral loss correlation

# Major Transformation Products

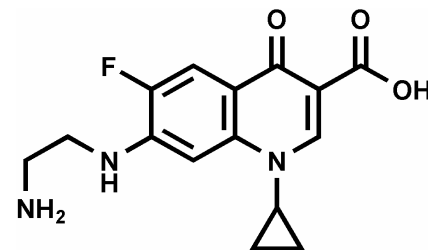
$t_r$ (min)	$[M+H]^+$
8.5	330
9.0	288
10.0	306
11.0	332
11.5	344
12.5	316
13.0	270
13.8	245
14.8	288
16.3	263



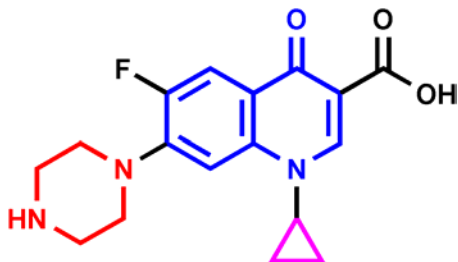
$[M+H]^+ = 330$



$[M+H]^+ = 288$



$[M+H]^+ = 306$



$[M+H]^+ = 332$

?

$[M+H]^+ = 344$

?

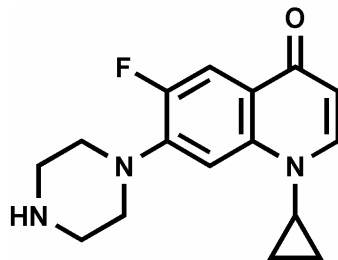
$[M+H]^+ = 316$

?

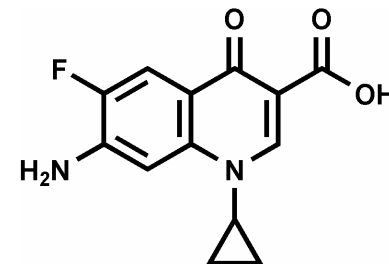
$[M+H]^+ = 270$

?

$[M+H]^+ = 245$

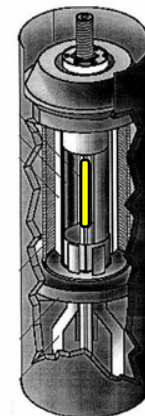
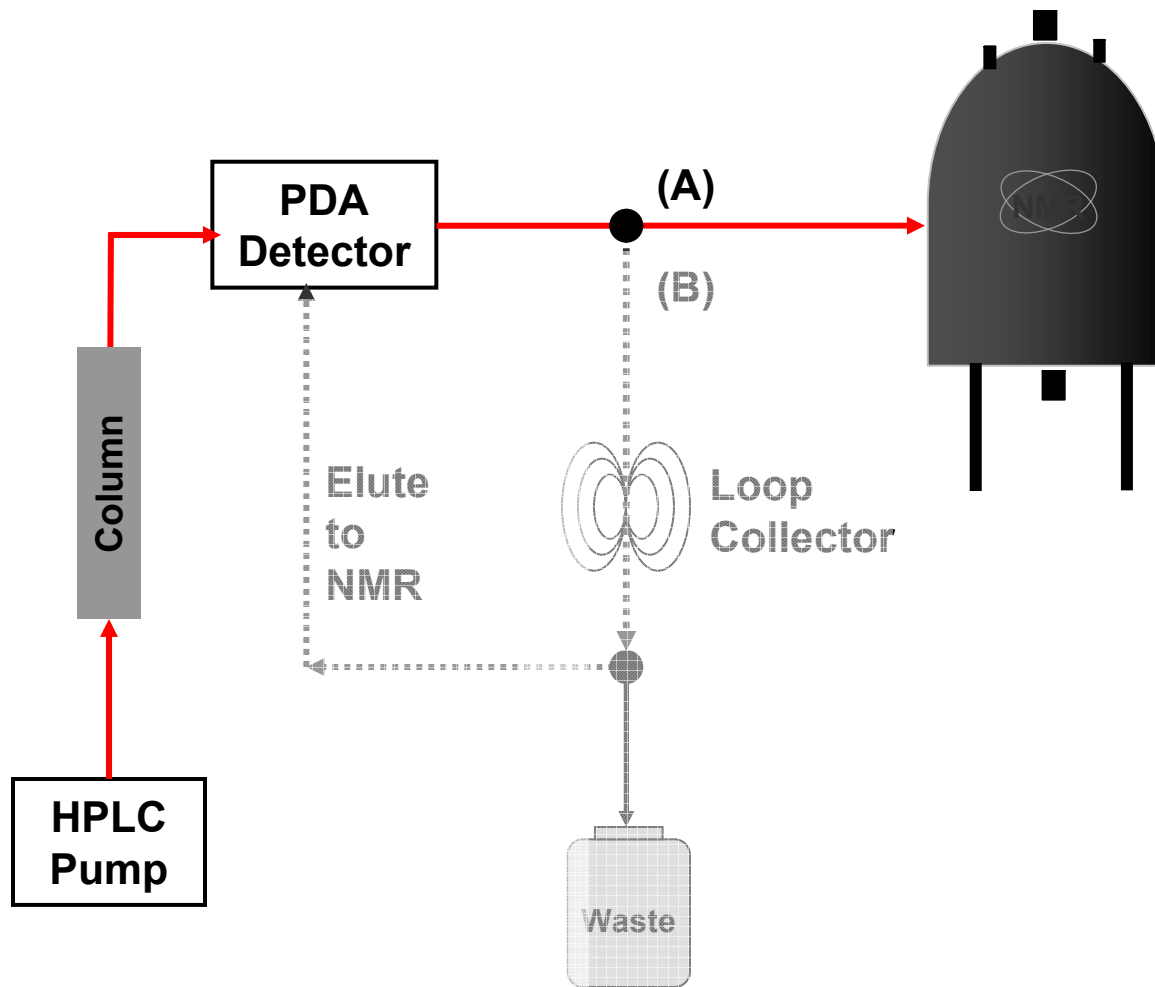
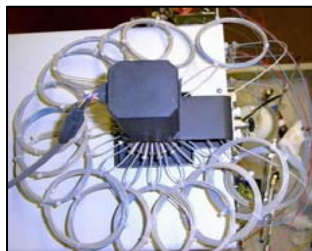
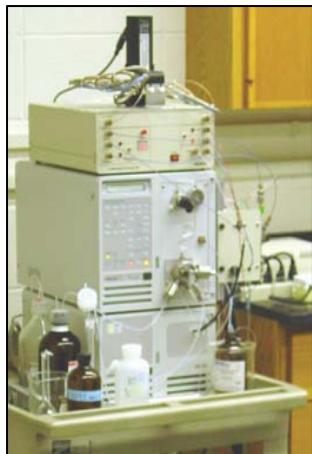


$[M+H]^+ = 288$

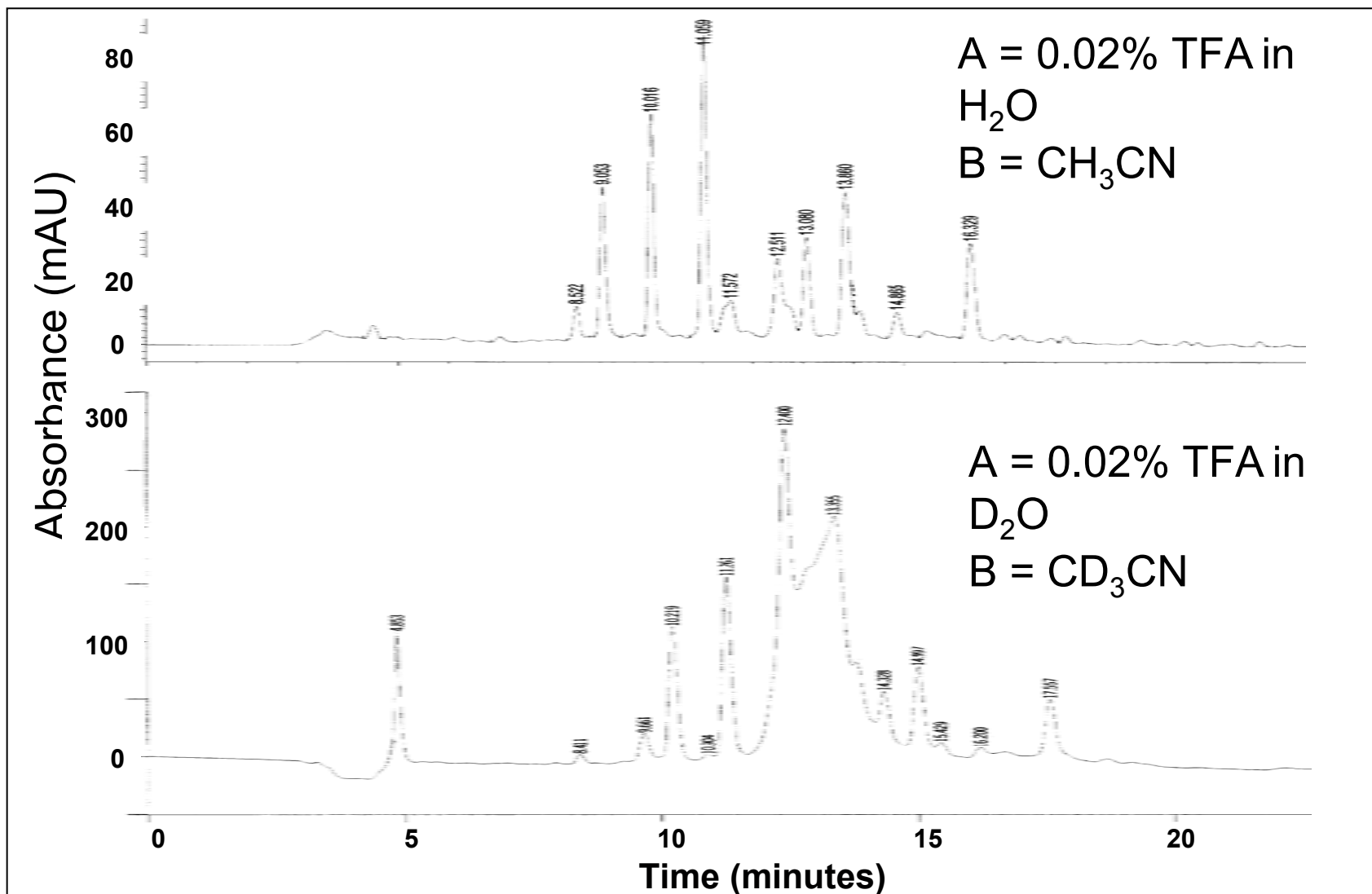


$[M+H]^+ = 263$

# HPLC-NMR

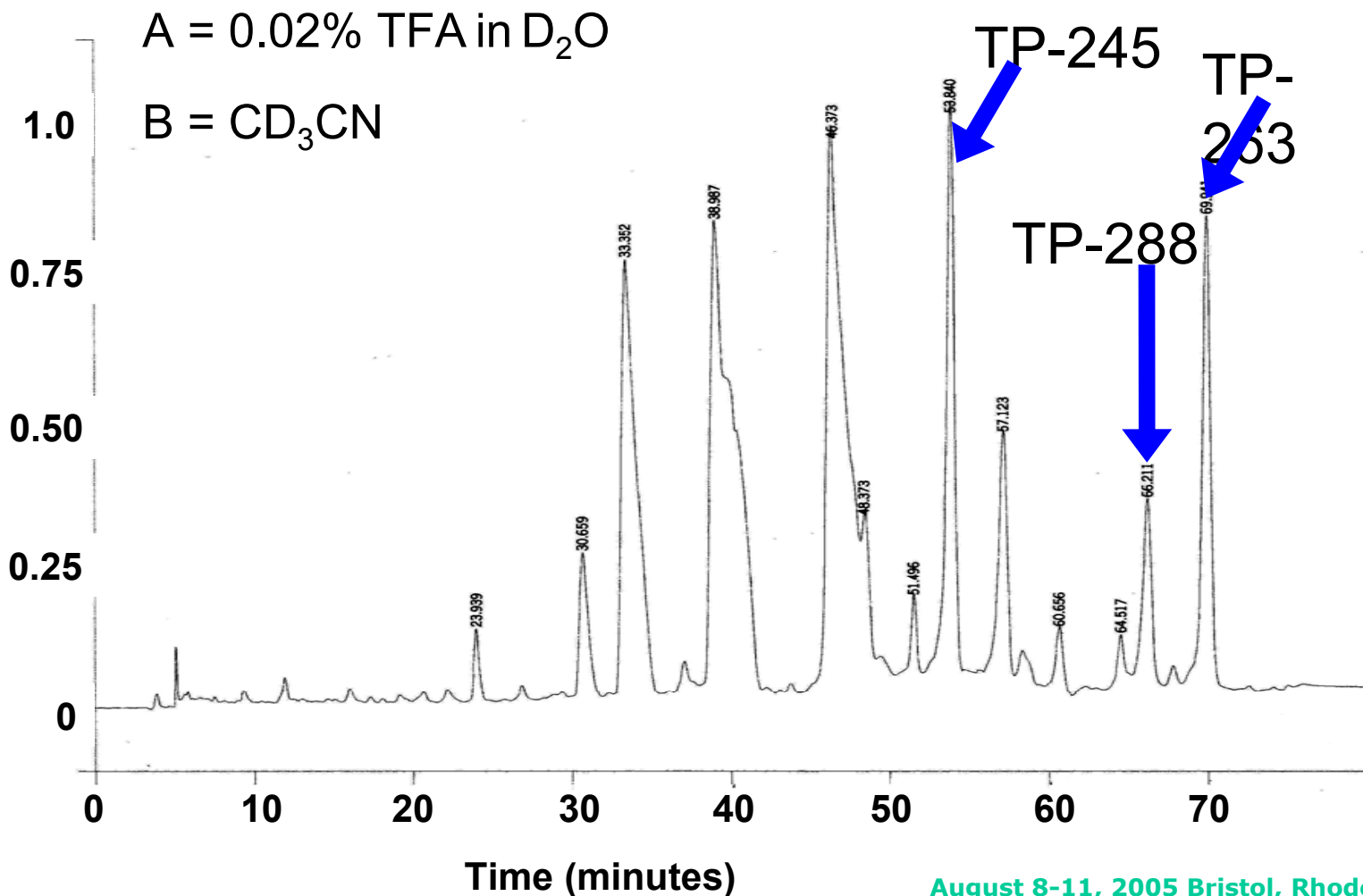


# Separation with Deuterated Solvents

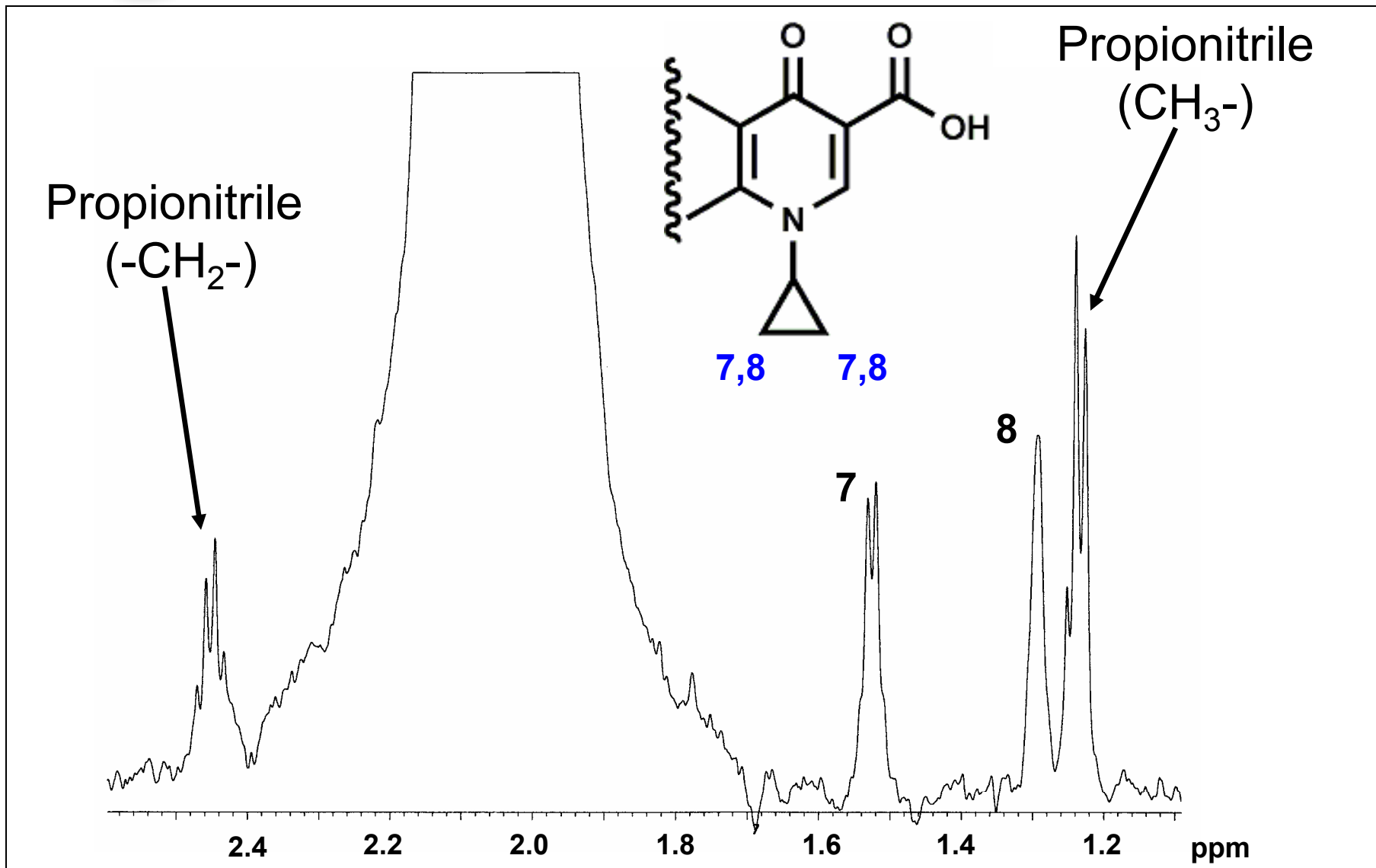


# Modified Method for Deuterated Solvents

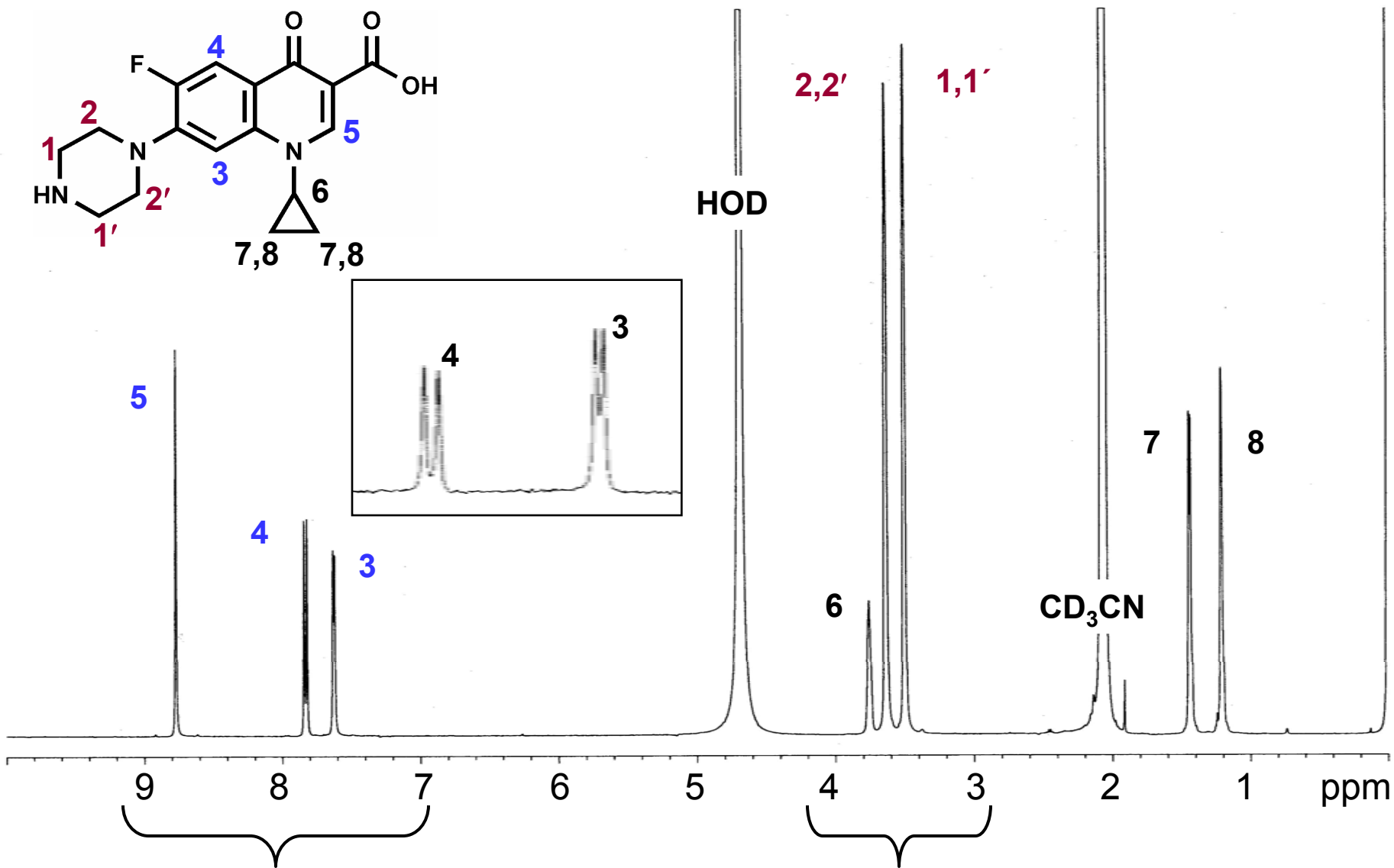
AU



# Solvent Impurity: Propionitrile



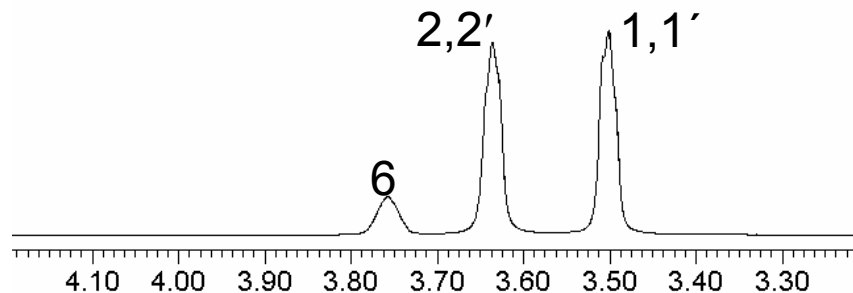
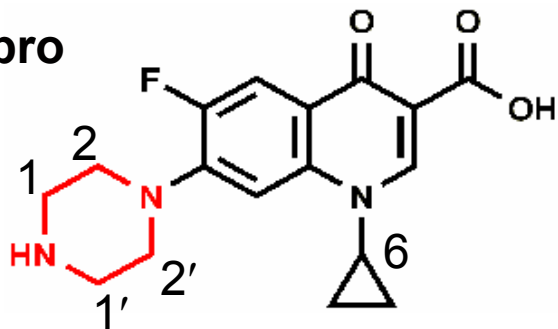
# Ciprofloxacin <sup>1</sup>H NMR



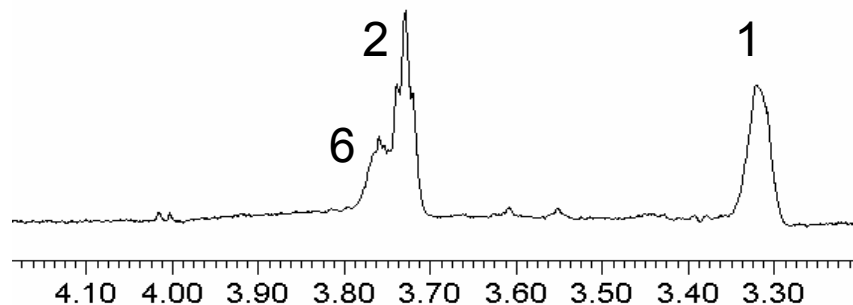
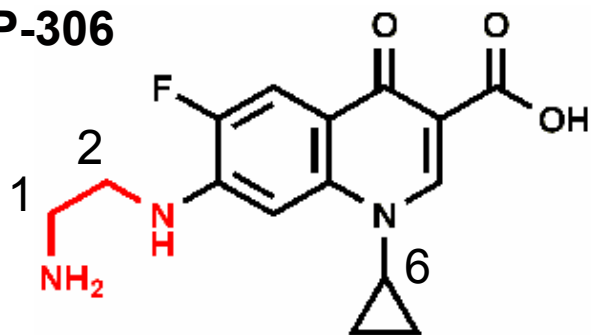
# Degradation: Piperazine Ring

$^1\text{H}$  NMR Aliphatic region (3.2 - 4.2 ppm)

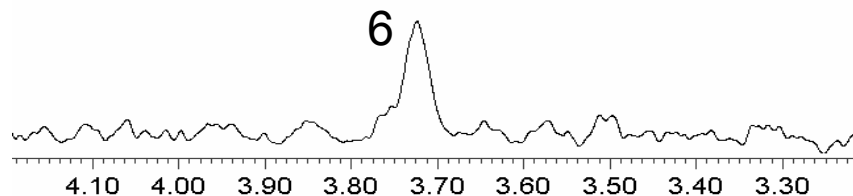
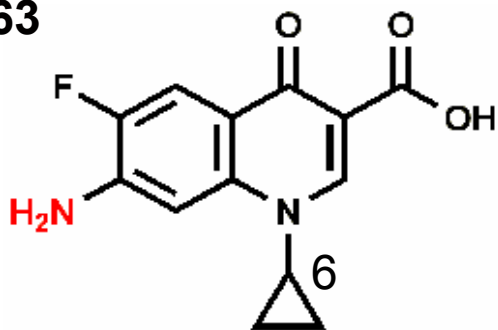
**Cipro**



**TP-306**



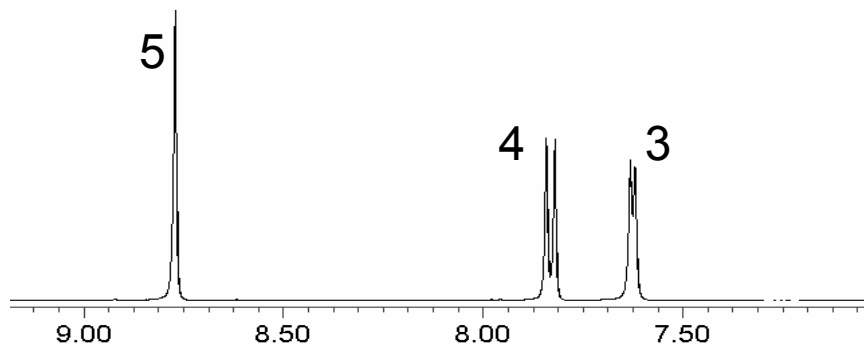
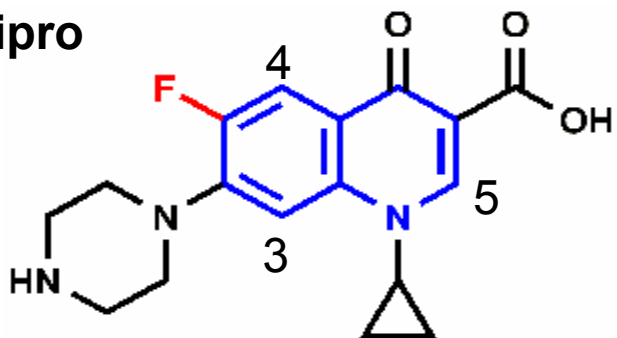
**TP-263**



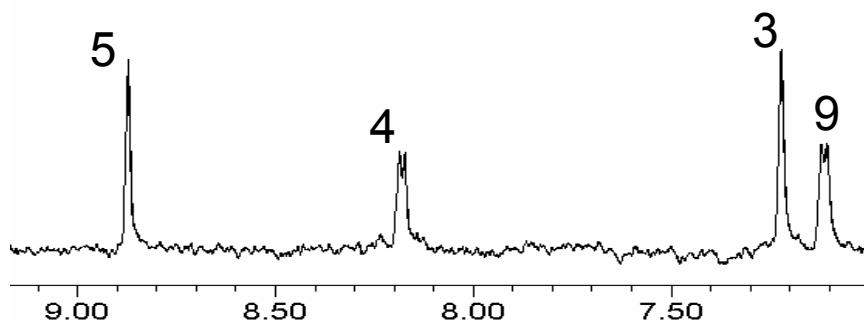
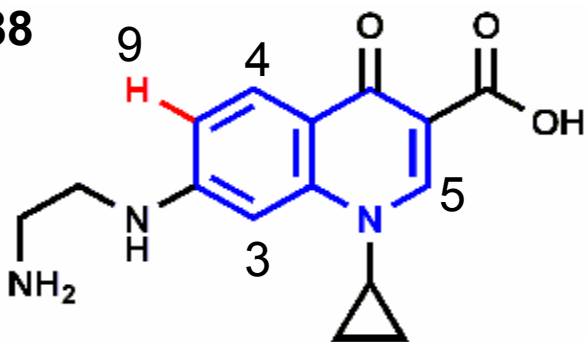
# Substitution of Fluorine

$^1\text{H}$  NMR Aromatic region (7.0 - 9.2 ppm)

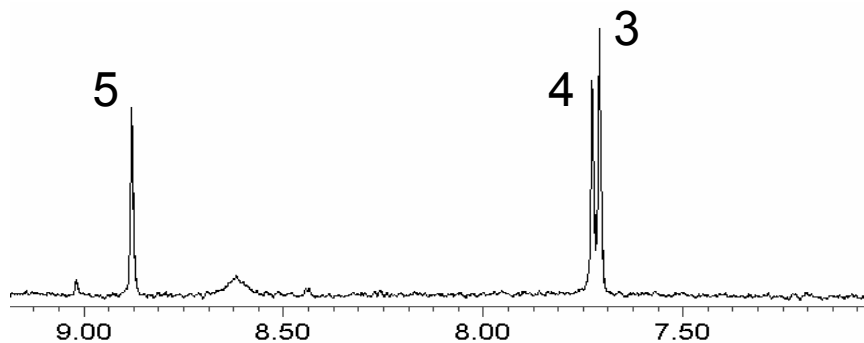
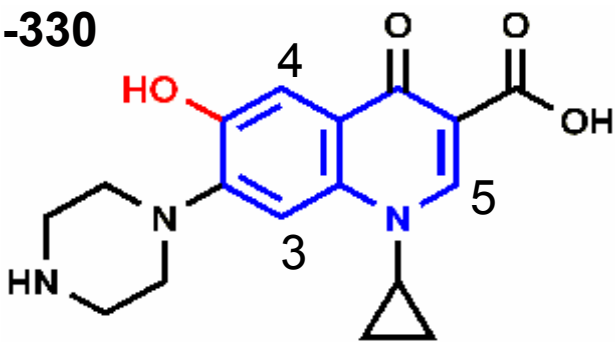
**Cipro**



**TP-288**

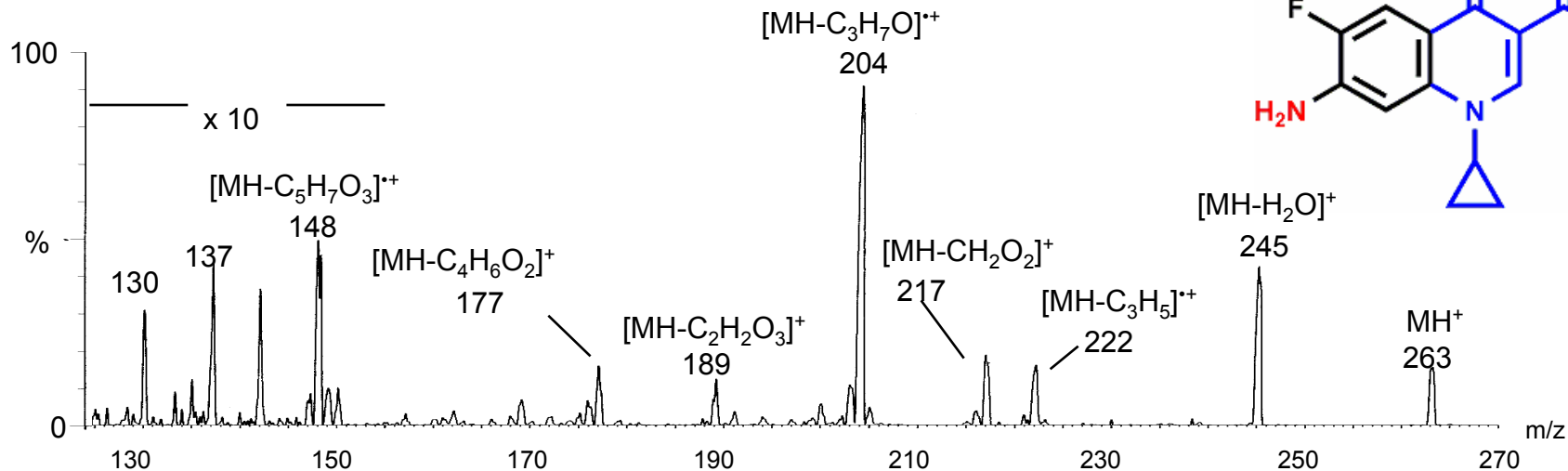


**TP-330**

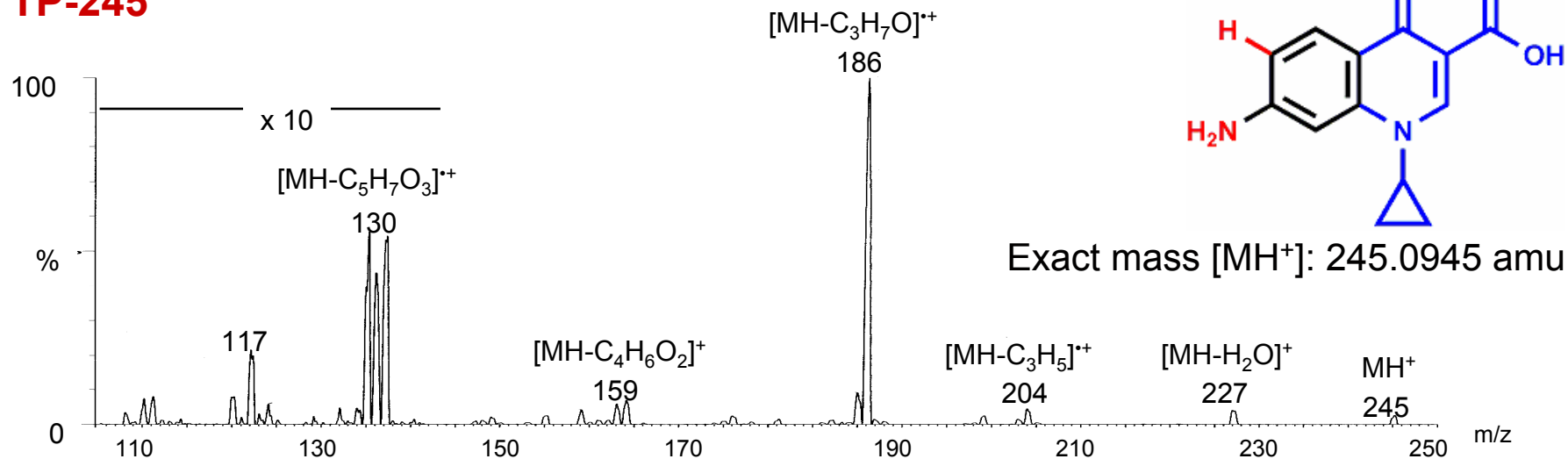


# Product Ion Spectra

## TP-263

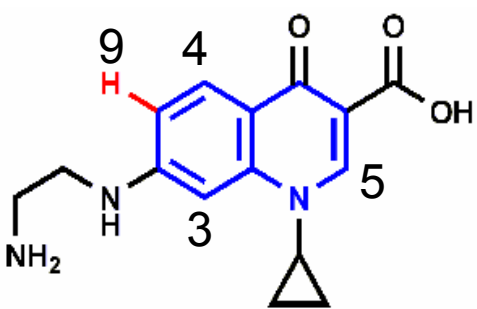
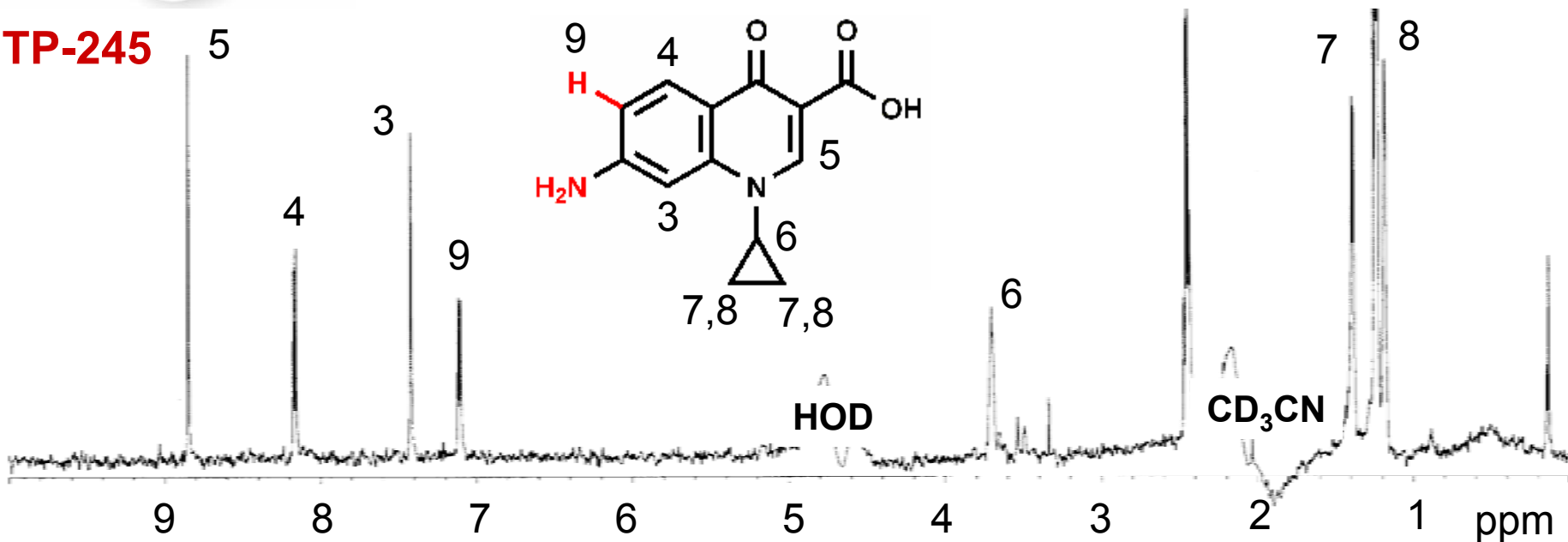


## TP-245

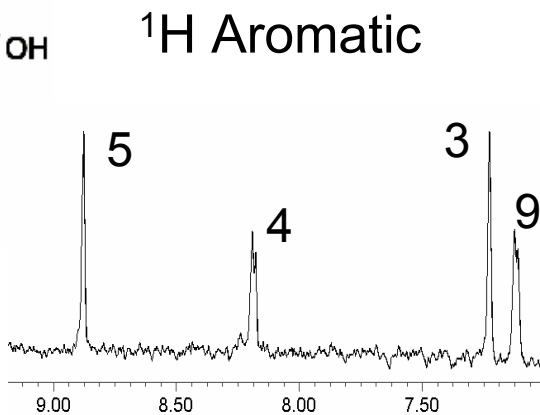


# TP-245: $^1\text{H}$ NMR

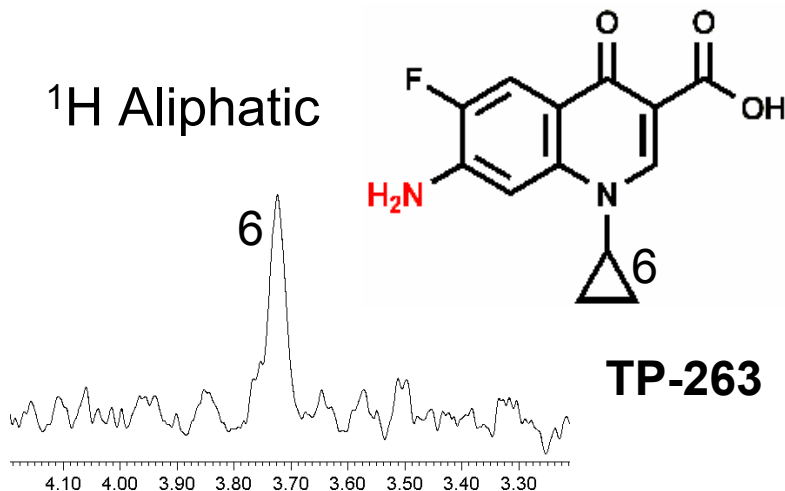
**TP-245**



**TP-288**



$^1\text{H}$  Aliphatic

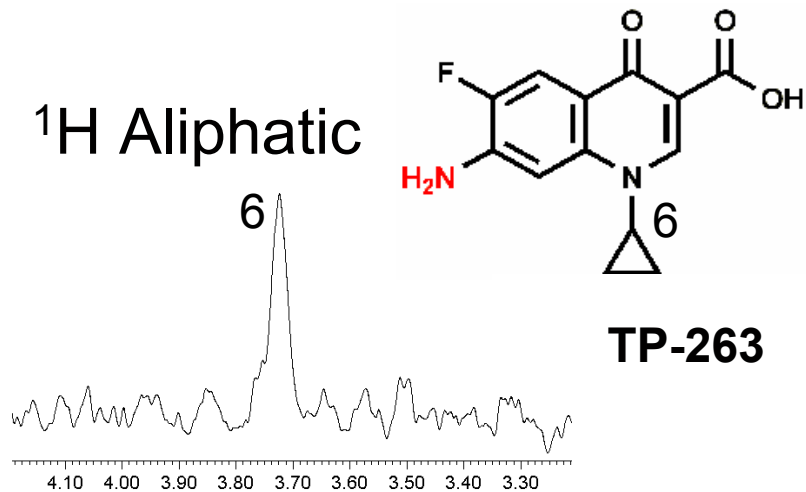
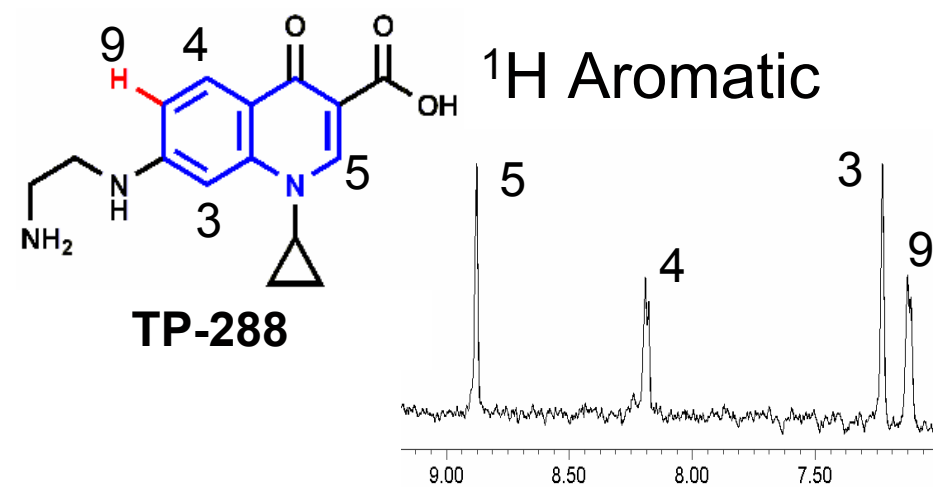
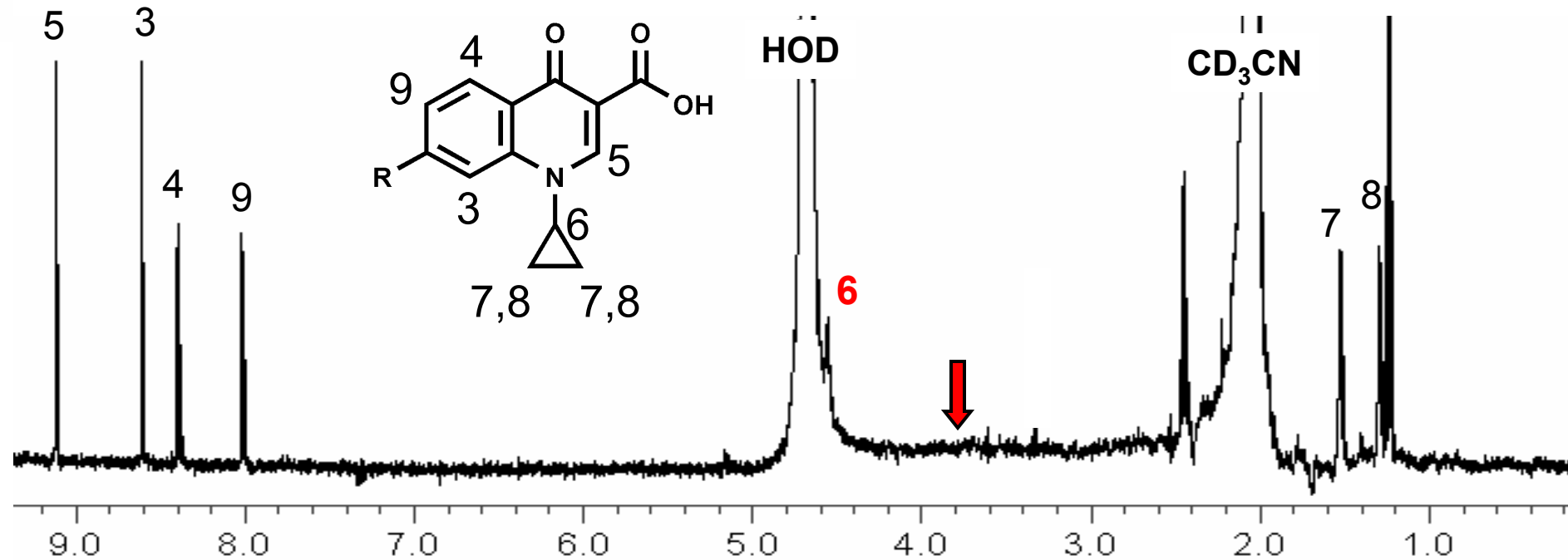


**TP-263**

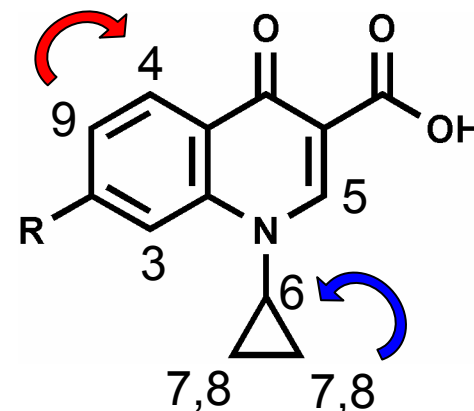
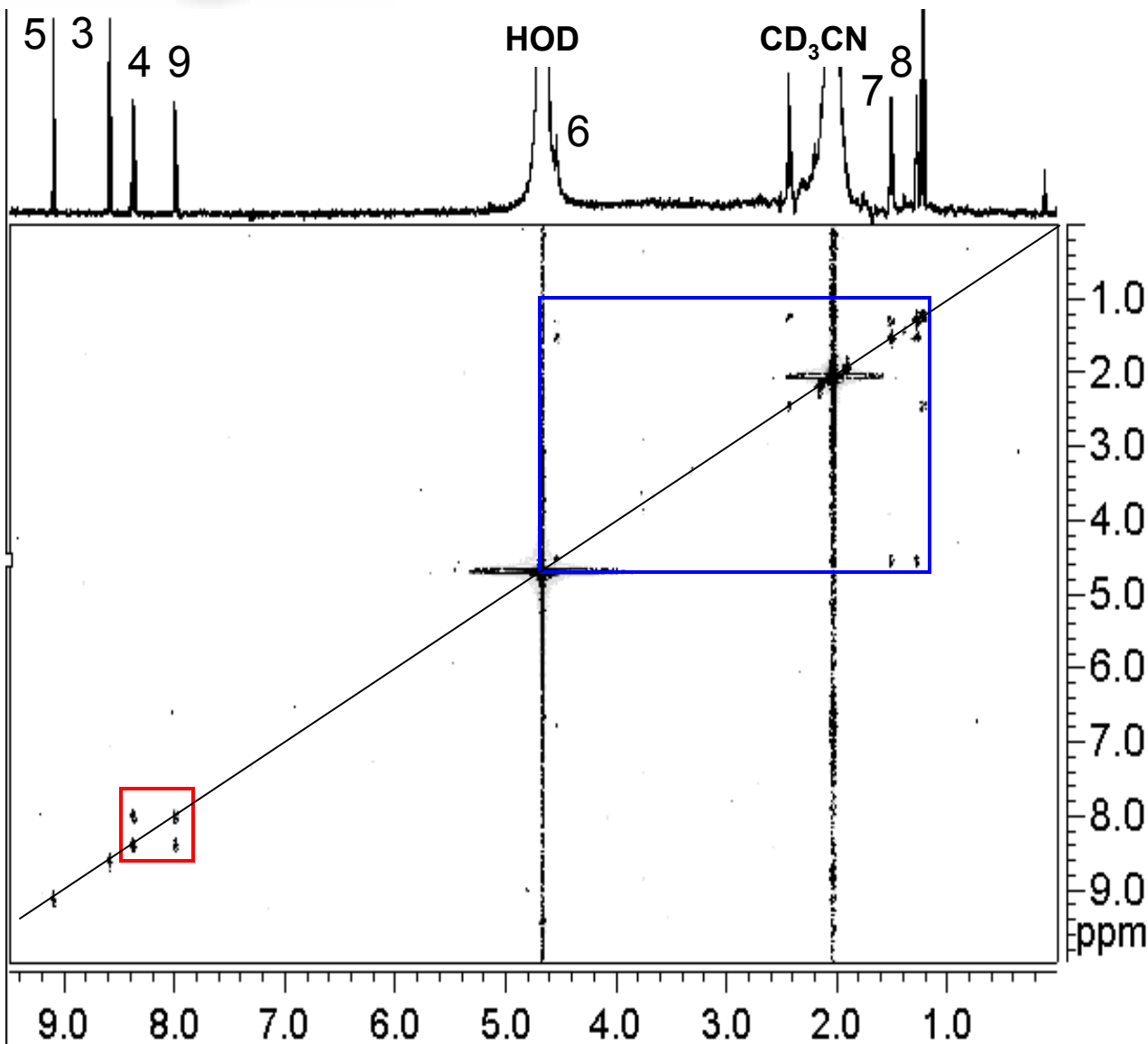
# TP-270: $^1\text{H}$ NMR

Conference on Small Molecule Science

COSMOS



# TP-270 COSY Spectrum

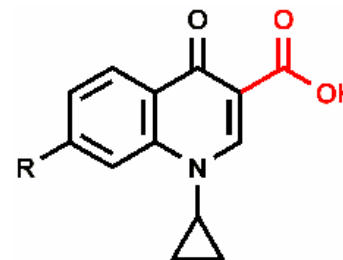
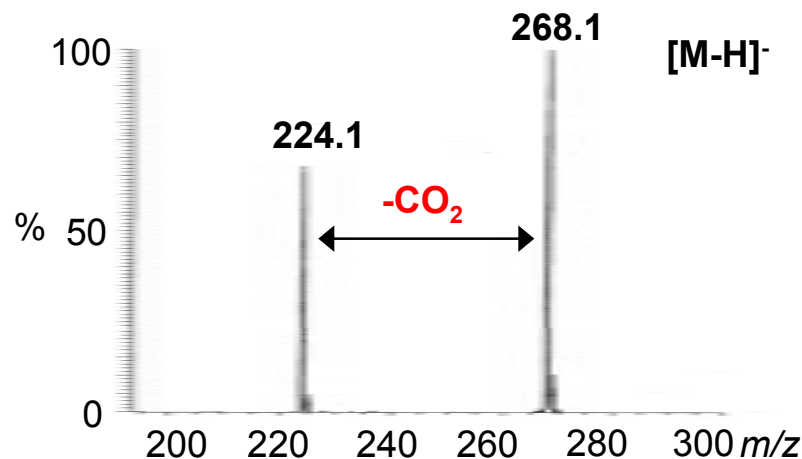


## Confirms

- ✓ Presence of H<sub>6</sub> HOD signal
- ✓ J<sub>H-H</sub> of H<sub>4</sub> and H<sub>9</sub>

# Additional Information

- ESI-MS/MS using QIT: Negative



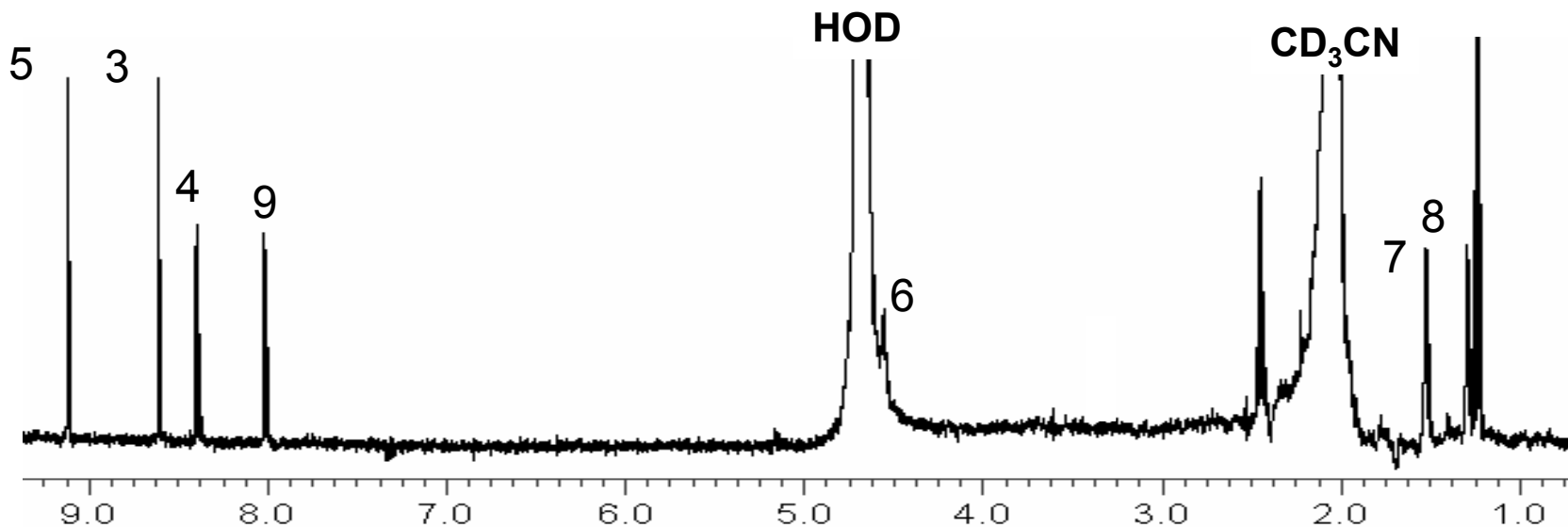
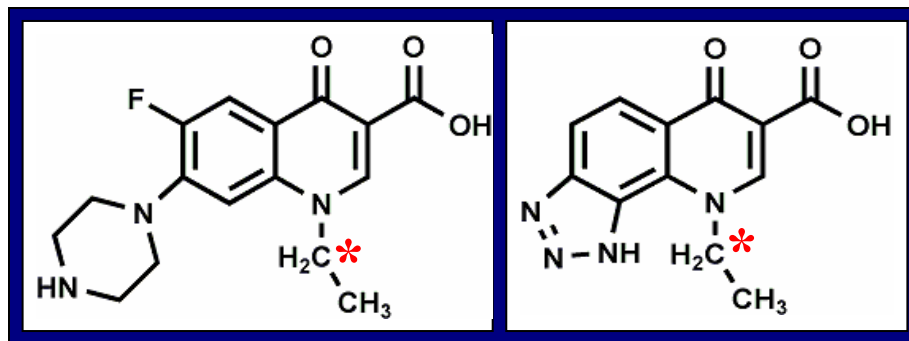
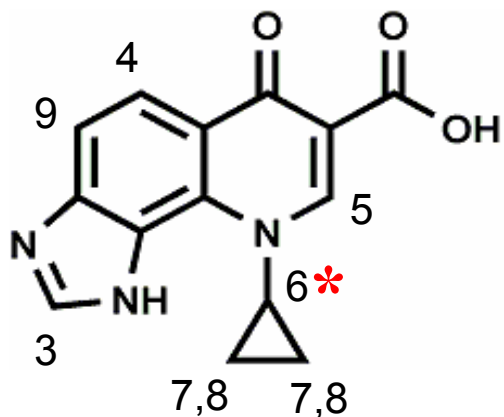
- MS/MS using QqQ and Q-TOF
  - Fragments are similar to TP-263 and TP-245
  - Confirm quinone backbone
- Exact mass measurements using sector MS
 

**Empirical formula:  $[C_{14}H_{12}N_3O_3]^+$**

  - $[MH]^+$  exact mass:  $270.0867 \pm 0.0013$  amu

# TP-270 Proposed Structure

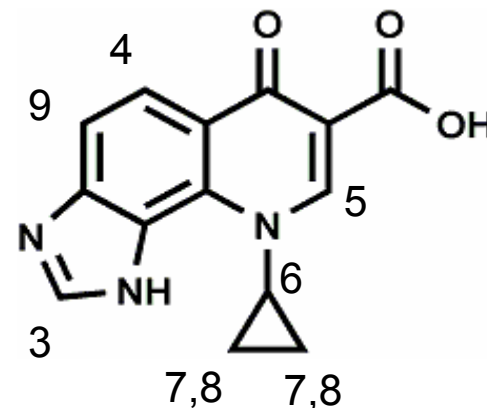
## TP-270



# Structural Information

- Loss of piperazine ring
- Loss of fluorine
- Gain of an aromatic proton
- Retained cyclopropyl group
- Shift in aromatic & aliphatic regions
  - Methine (H<sub>6</sub>) proton
  - Aromatic protons H<sub>3</sub>, H<sub>4</sub>, and H<sub>9</sub>
  - Shifted down-field

- Parent Mass
  - [MH]<sup>+</sup> = 270.3
  - [M-H]<sup>-</sup> = 268.1
- Exact Mass (empirical formula)
- Fragments
  - CO<sub>2</sub> loss (m/z 224)
  - C<sub>3</sub>H<sub>4</sub> loss (m/z 230)
  - No loss for HF+ H<sub>2</sub>O
- Nitrogen rule odd MW



# Conclusion

- NMR

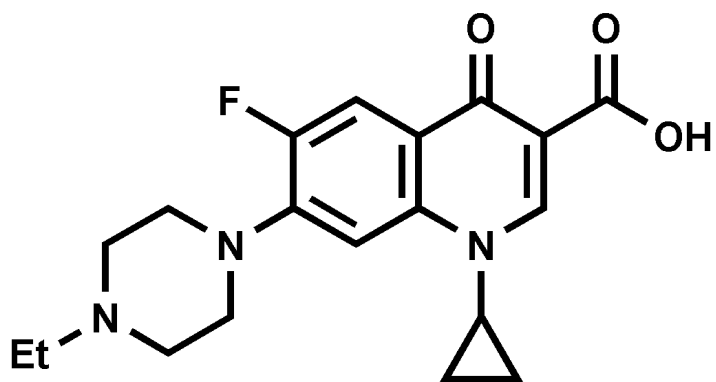
- Established strategies for transformation product identification
- Transformation of piperazine and quinolone rings as well as loss of the fluorine atom observed

- MS

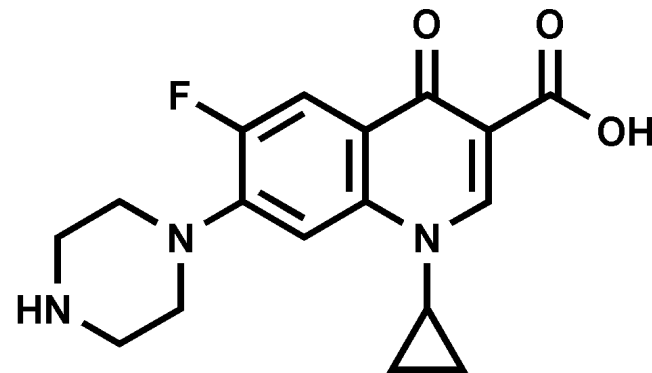
- Parent ions and neutral losses
- Methods optimized with ciprofloxacin do not always show similar neutral losses in products even when moiety is present

# Enrofloxacin Transformation

## Degradation of enrofloxacin to ciprofloxacin

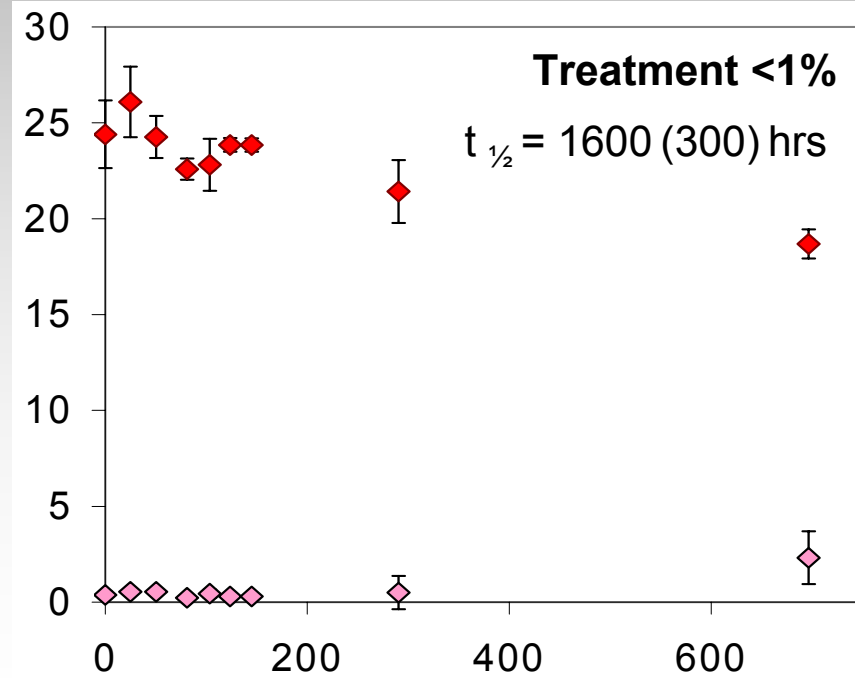
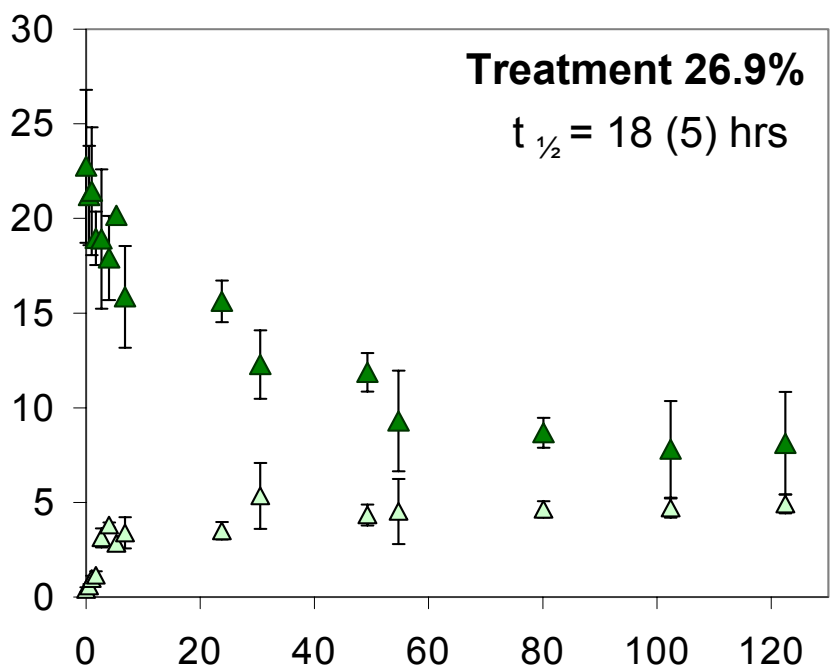
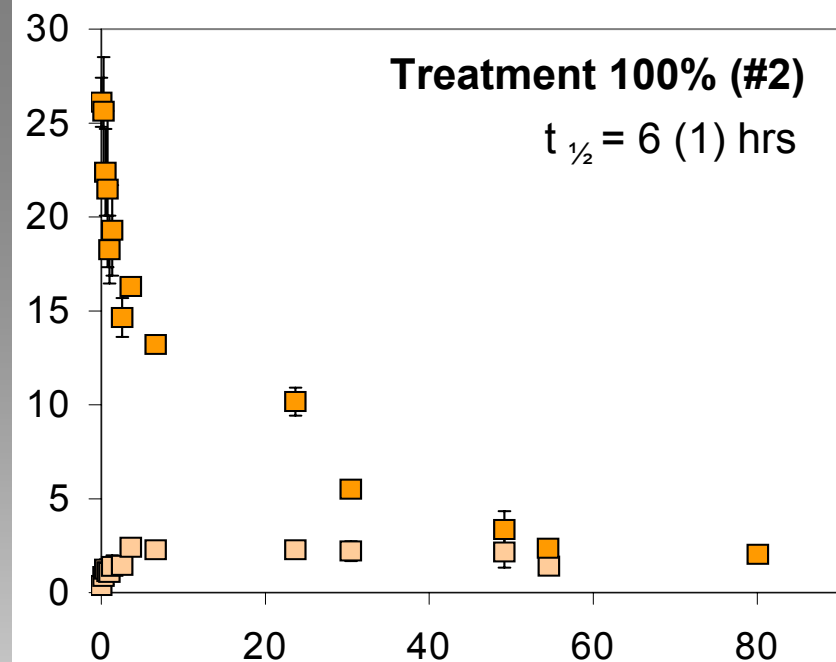
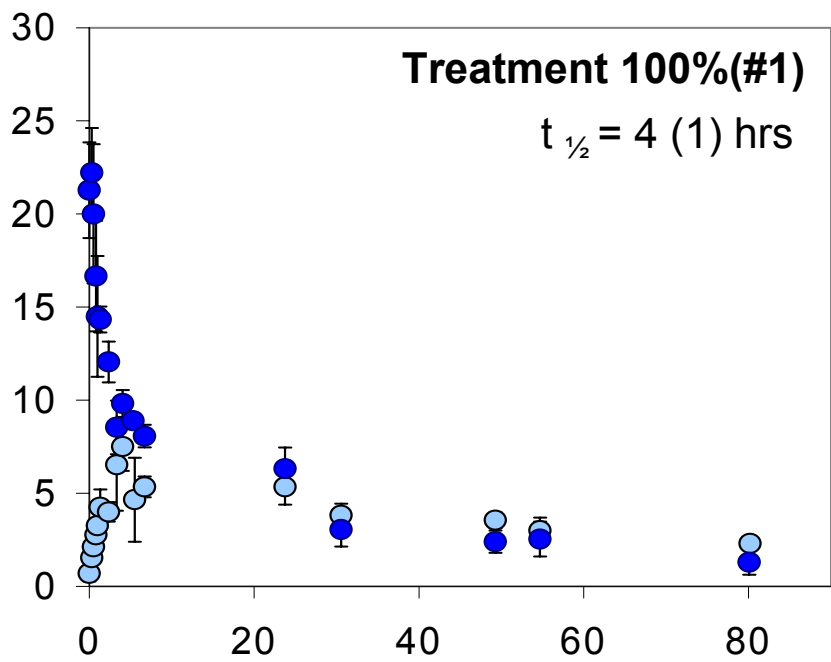


Enrofloxacin



Ciprofloxacin

Fluoroquinolone Concentration ( $\mu\text{g/L}$ )

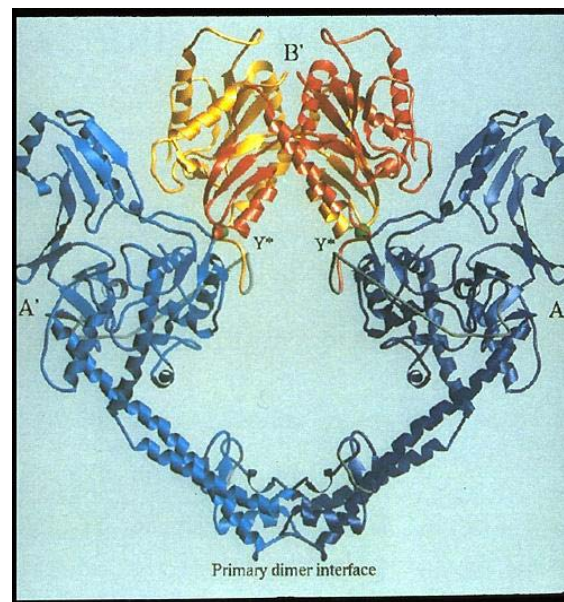
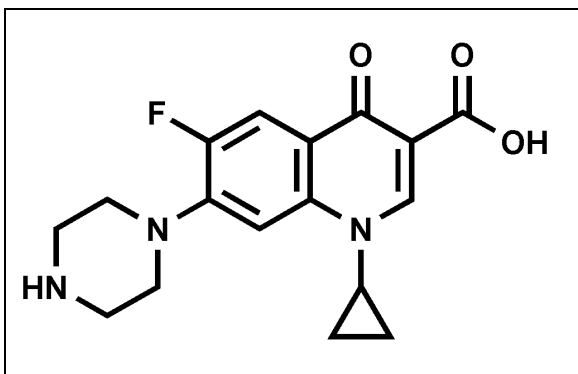


# FQ Attenuation

## Effects of water parameters

- Photodegradation
  - Ciprofloxacin photodegradation is rapid in direct sunlight ( $t_{1/2} \approx 1$  hrs)
  - Enrofloxacin ( $t_{1/2} \approx 5$  hrs) degrades at a slower rate
  - Numerous TPs generated
- No effect of DOC on ciprofloxacin attenuation rate in light or dark waters
- Rapid adsorption with high POC levels; No TPs detected

# Environmental Effects of Enrofloxacin Exposure



# Enrofloxacin Attenuation

Field Scale Experiments  
Varied light transmittance  
Varied microbial community  
size



<1%



26.9%



100% (#1)



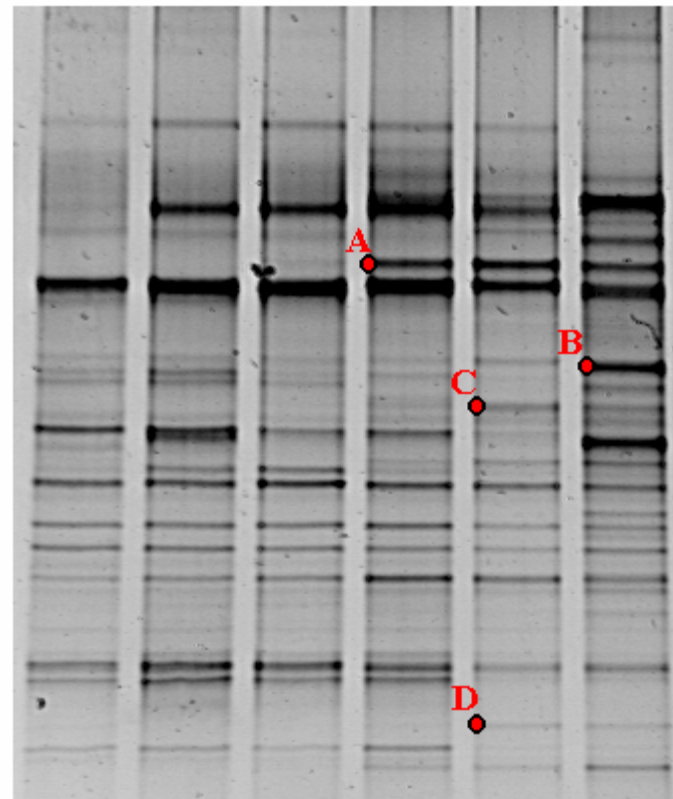
100% (#2)



# Changes in Microbial Community Structure

-16 -8 -2 5 12 26

- 16S rRNA DGGE banding patterns used to compare differences before and after FQ addition.
- No major patterns of change in composition or diversity
- *P. Putida* and *Cyanthothece* sp. Showed some positive selection



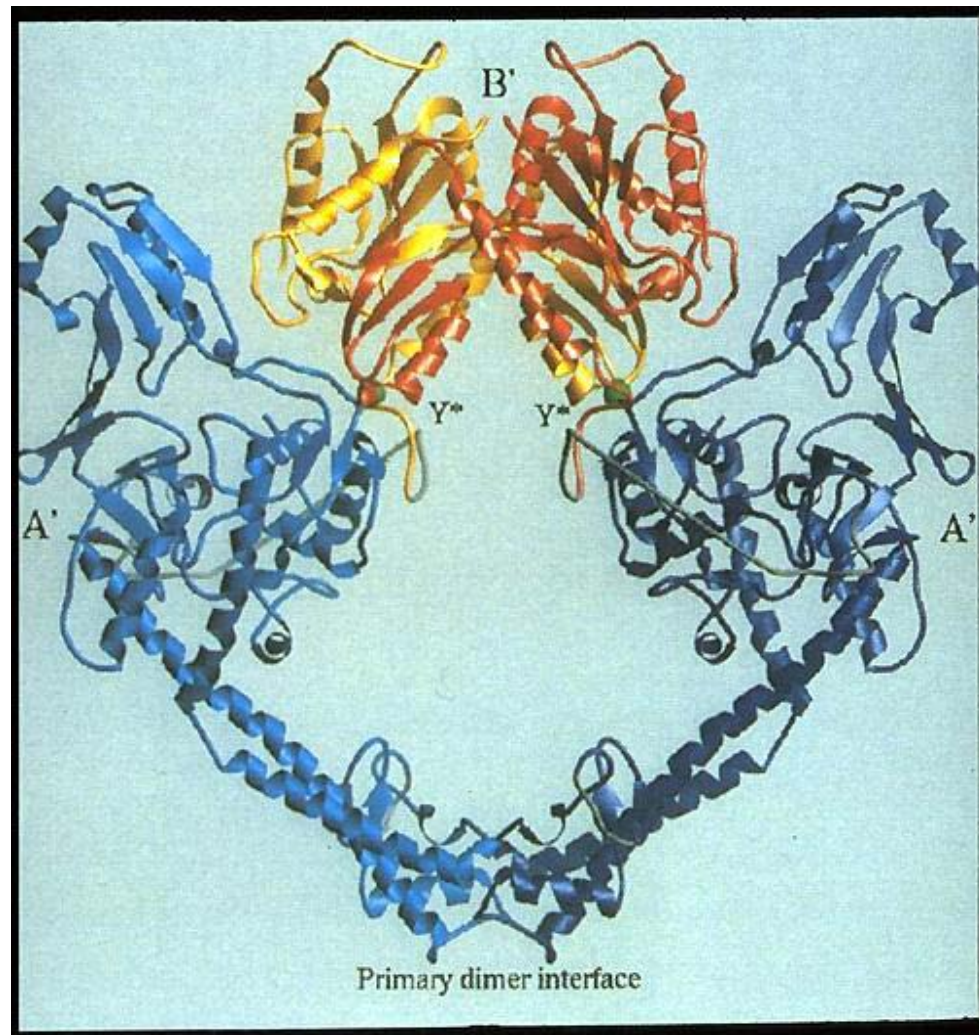
DGGE analysis of 16S rRNA PCR products from mesocosms. Red letters indicate excised bands.

# Changes in Microbial Community Structure

Experiment	Band ID	Identity in blastn	Phylogenetic Group	%
100 % Light	A	<i>Pseudomonas putida</i>	Gamma proteobacteria	97
	B	<i>Alcaligenes latus</i>	Beta proteobacteria	94
	C	Uncultured CFB group bacterium	Bacteroides	98
	D	<i>Acidovorax delafieldii</i>	Beta proteobacteria	97
28 % Light	E	<i>Cyanothece</i> sp.	Cyanobacteria	98
	F	<i>Flexibacter sancti</i>	Sphingobacteria	95
0.5 % Light	G	<i>Synechococcus</i> sp.	Cyanobacteria	96
	H	<i>Flavobacterium aquatile</i>	Flavobacteria	97

# DNA Gyrase

- Fluoroquinolones bind to DNA gyrase and prevent replication
- DNA gyrase is a type II topoisomerases
- Catalyzes DNA supercoiling/relaxation, catenation/decatenation, knotting/unknottting
- Affinity of ciprofloxacin is 100 times greater for bacterial DNA gyrase than for the human protein

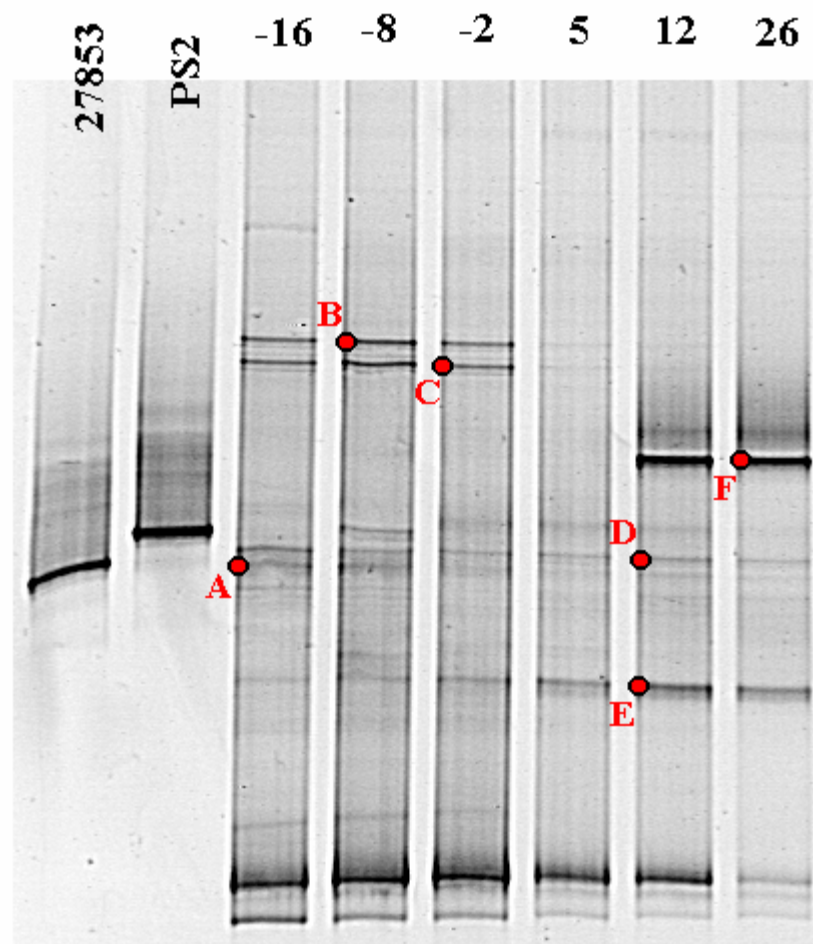


<http://cmgm.stanford.edu/biochem201>

# DNA Gyrase A Mutations

Antibiotic resistance develops through mutations in the C-terminal Quinolone Resistance Determining Region (QRDR) of the gyrase.

QRDR primers were used to amplify a 469 bp fragment DGGE analysis of *P. aeruginosa gyrA* products from 100 % light mesocosms. Red letters indicate excised bands.



# Mutations in DNA Gyrase

<b>Band ID</b>	<b>Closest Identity in blastx</b>	<b>Amino Acid Variations</b>
A	<i>Magnetospirillum magnetotacticum</i>	M89L, <b>V90A</b> , <b>A93V</b> , N95D, F96W, V103I, <b>G114P</b> , <b>P116A</b>
B	<i>Novosphingobium aromaticivorans</i>	A62S, <b>A73V</b> , A83S, <b>T93V</b> , <b>P116A</b> , S119A
C	<i>Cytophaga hutchinsonii</i>	K61R, <b>A62P</b> , M102L, S116N
D	<i>Cytophaga hutchinsonii</i>	<b>A62P</b> , S83A, M92L, E95D, M102L, V112M
E	<i>Bacteroides fragilis</i>	K61R, <b>F87D</b> , <b>A88T</b> , A97S, M98L, S116N

# Conclusions

- Environmental effects on bacteria negligible in these experiments
- Although DGGE bands reflecting possible enrofloxacin resistance are noted, few bands unambiguously appear post-addition.
- No major alterations of the microbial community diversity or composition could be detected as a result of enrofloxacin addition.
- This does not rule out effects that might occur with larger pulsed exposure or with continuous long-term exposures as might be expected in wastewater impacted waters.

# Acknowledgements



- Collaborators
  - David Graham and Chuck Knapp
- NMR
  - David VanderVelde, Sarah Neuenswander, and Martha Morton
- MS
  - Todd Williams and Bob Drake
  - Heather Desaire and Mary Bandu

- Laurie Cardoza Harned
- EPA, NSF for Funding