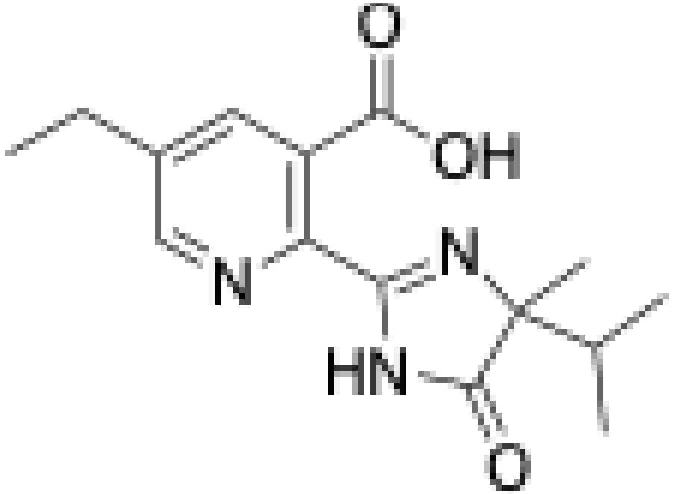
Structural and Kinetic Evidence of Maleamate Amidohydrolase (NicF) in the *Bacillus niacini* Nicotinic Acid Catabolic Pathway

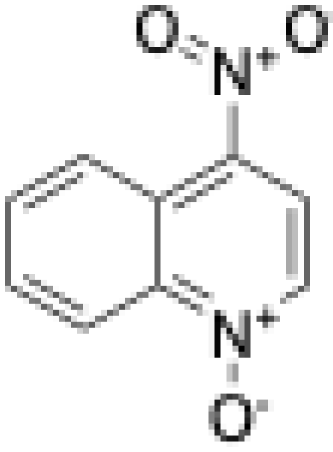
Jake Enzman

N-heterocyclic aromatic compounds (NHACs) are pervasive environmental pollutants

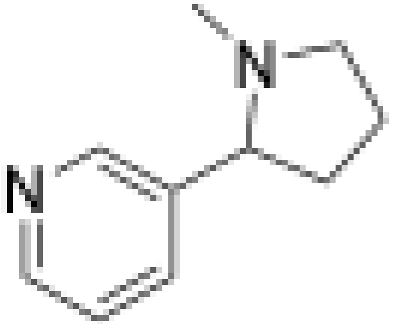
- Pesticides, herbicides, coal gasification sites
- Toxic, carcinogenic, mutagenic



imazethapyr



4-nitroquinoline-N-oxide

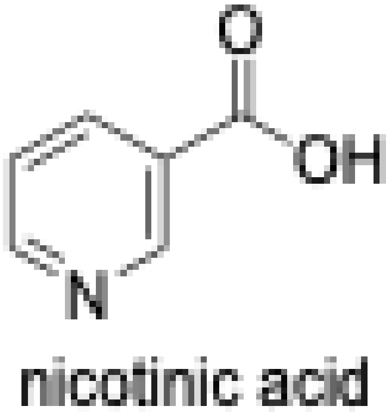


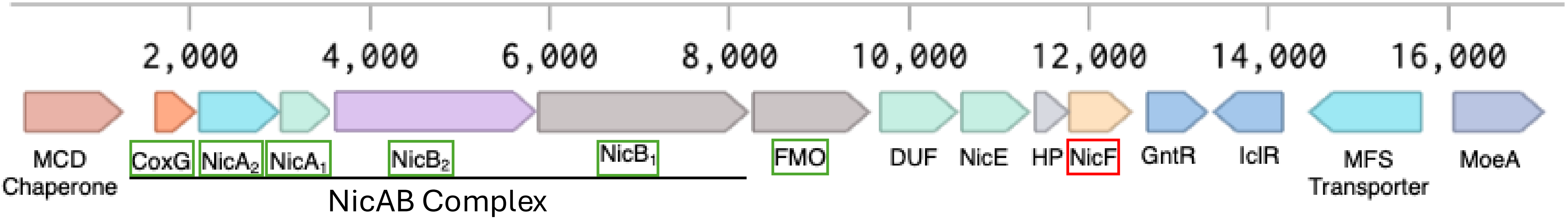
nicotine

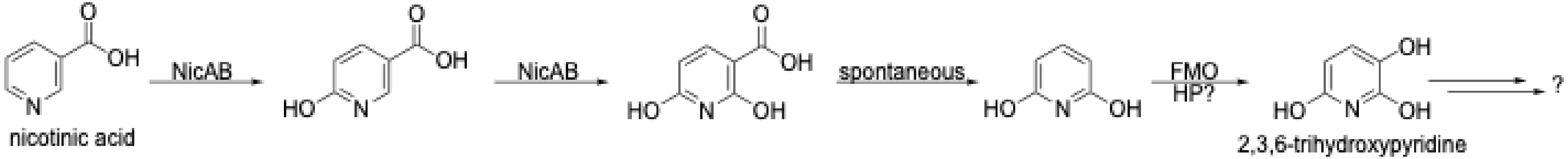
Koutros, S. et al. 2008. *Int. J. Cancer.* **124,** 1206–1212.
Heravi, M. M et al. 2020. V. *RSC Adv.* **10**, 44247–44311.

Microorganisms can be utilized to breakdown NHAC pollutants

- Organism: *Bacillus niacini*
- Nicotinic acid metabolic operon





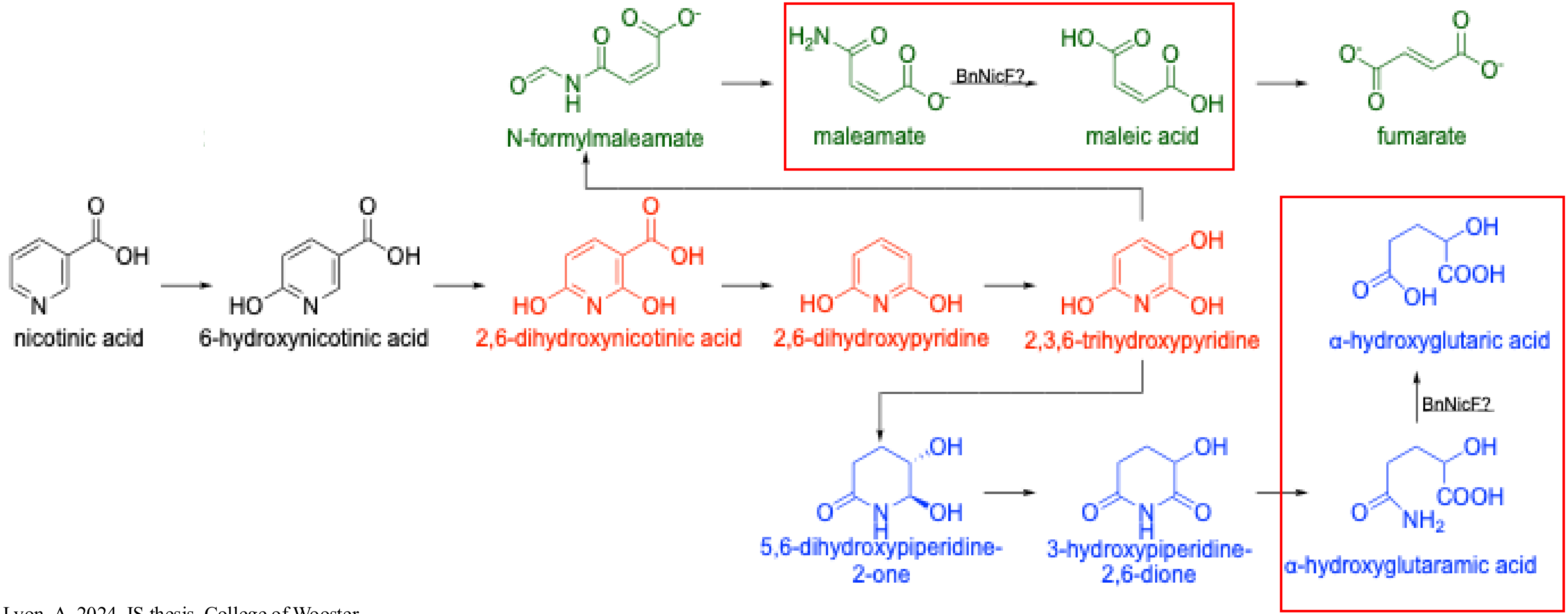


Helton, N. 2020. IS thesis, College of Wooster.
Olson, K. 2024. IS thesis, College of Wooster.

My research questions:

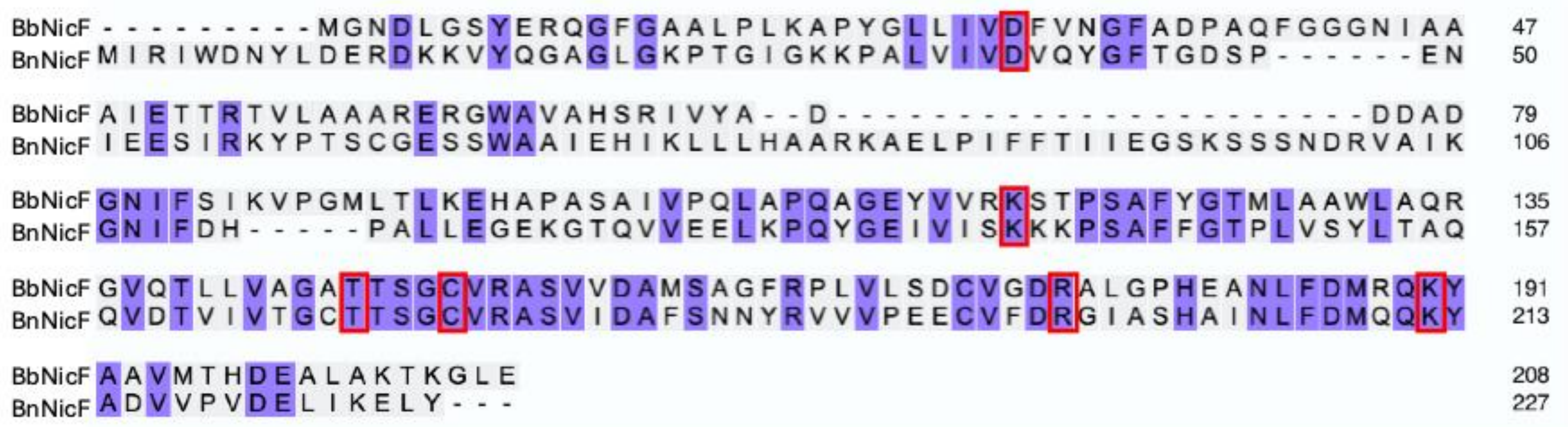
The pathway of nicotinic acid catabolism is unclear in *Bacillus niacini*

1. What is the native substrate of *B. niacini* NicF?
2. What is the overall nicotinic acid catabolic pathway in *B. niacini*?



Lyon, A. 2024. IS thesis, College of Wooster.
Bokor, E. et al. 2022. *Communications Biology.* **5,** 723.
Jimenez, J. et al. 2008. *PNAS.* **105,** 11329-11334.
Ensign, J. and Rittenberg, S. 1964. *The Journal of Biological Chemistry.* **239,** 2285-2291.

Bacillus niacini putative NicF exhibits 38% sequence identity with *Bordetella bronchiseptica* NicF



```
BbNicF ---------MGNDLGSYERQGFGAALPLKAPYGLLIVDFVNGFADPAQFGGGNIAA   47
BnNicF MIRIWDNYLDERDKKVYQGAGLGKPTGIGKKPALVIVDVQYGFTGDSP------EN   50

BbNicF AIETTRTVLAAARERGWAVAHSRIVYA--D----------------------DDAD   79
BnNicF IEESIRKYPTSCGESSWAAIEHIKLLLHAARKAELPIFFTIIEGSKSSSNDRVAIK  106

BbNicF GNIFSIKVPGMLTLKEHAPASAIVPQLAPQAGEYVVRKSTPSAFYGTMLAAWLAQR  135
BnNicF GNIFDH-----PALLEGEKGTQVVEELKPQYGEIVISKKKPSAFFGTPLVSYLTAQ  157

BbNicF GVQTLLVAGATTSGCVRASVVDAMSAGFRPLVLSDCVGDRALGPHEANLFDMRQKY  191
BnNicF QVDTVIVTGCTTSGCVRASVIDAFSNNYRVVVPEECVFDRGIASHAINLFDMQQKY  213

BbNicF AAVMTHDEALAKTKGLE                                         208
BnNicF ADVVPVDELIKELY---                                         227
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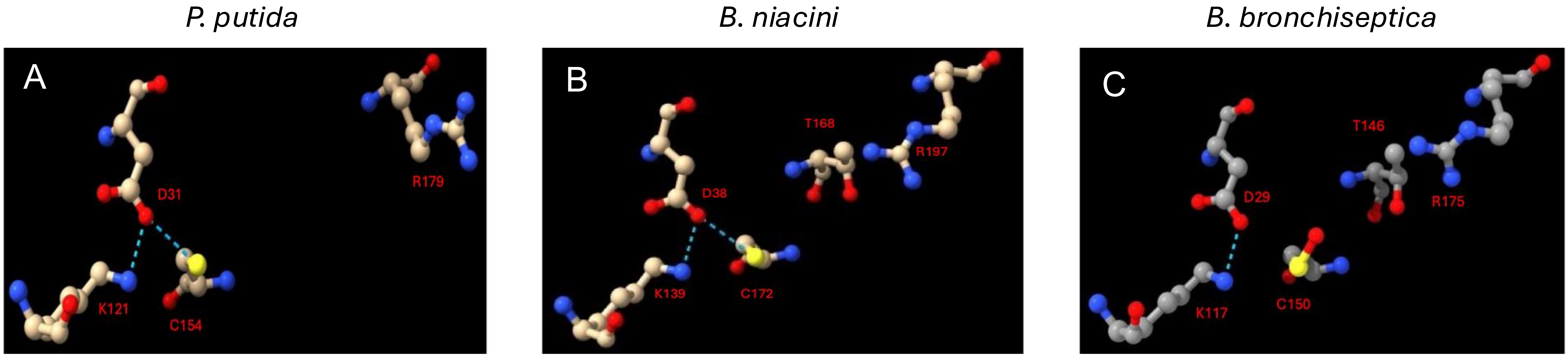
Kincaid, V. et al. 2012. *Biochemistry.* **51,** 545–554.

Structural analysis indicates that BnNicF contains a conserved catalytic triad and nucleophilic cysteine


P. putida

B. niacini

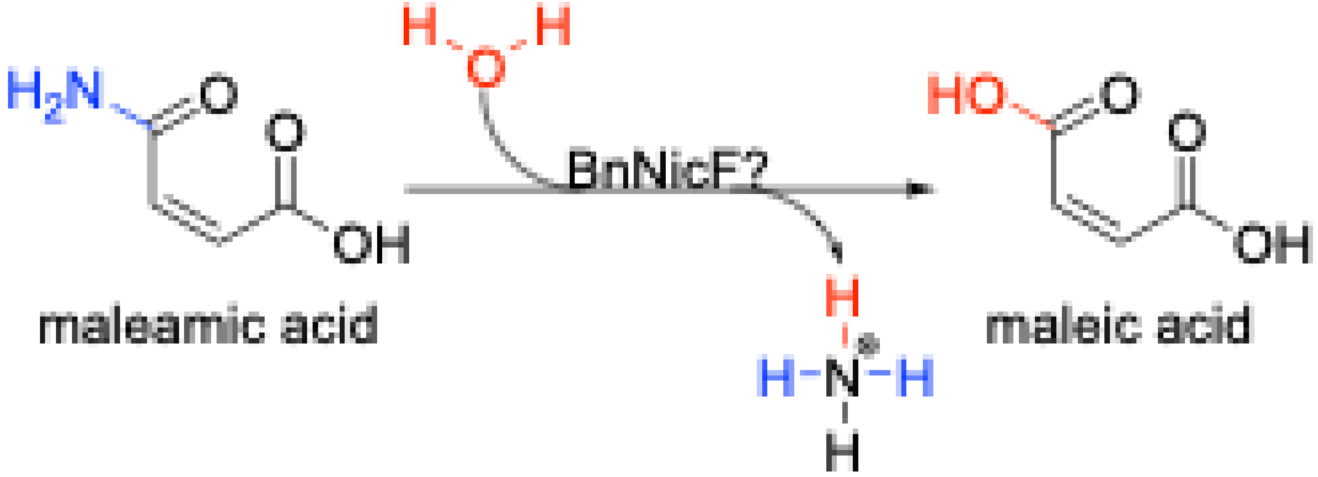
B. bronchiseptica

Hypothesis: BnNicF catalyzes the hydrolytic deamidation of maleamic acid to maleic acid

Aim 1: Clone, recoml

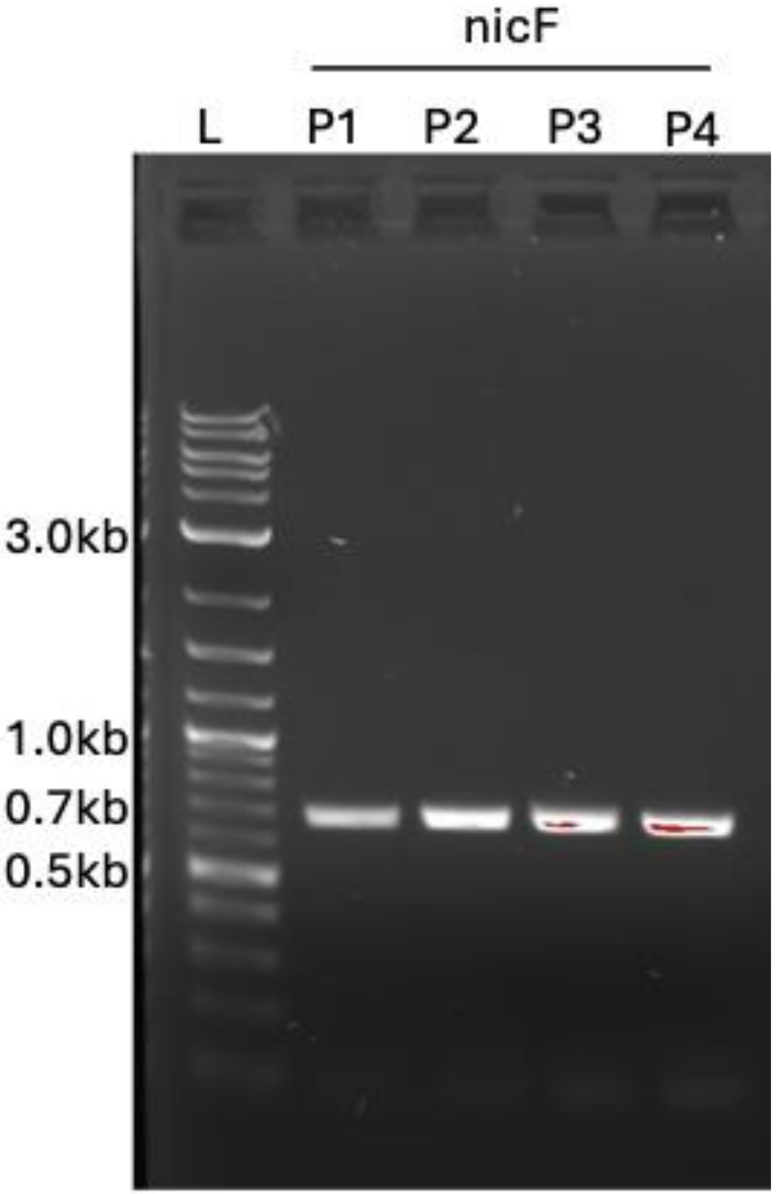
Aim 2: Define the ste … d by BnNicF.

Aim 3: Determine wh … ›us to other NA catabolic pathways.

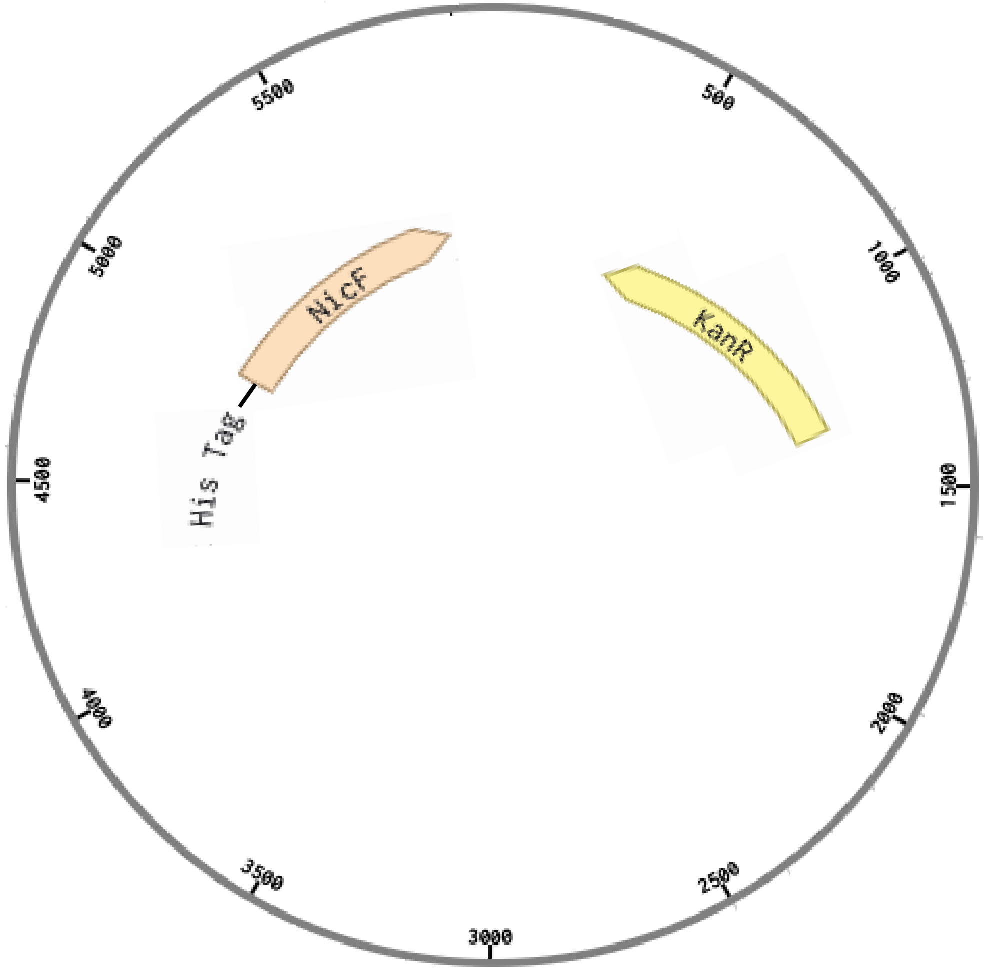


Kincaid, V. et al. 2012. *Biochemistry.* **51,** 545–554.

The nicF gene was amplified via PCR and ligated into the pTHT vector



Bn nicF in pTHT vector



BnNicF was sequenced and subsequently purified by Ni^{2+} affinity chromatography


6X-His tag

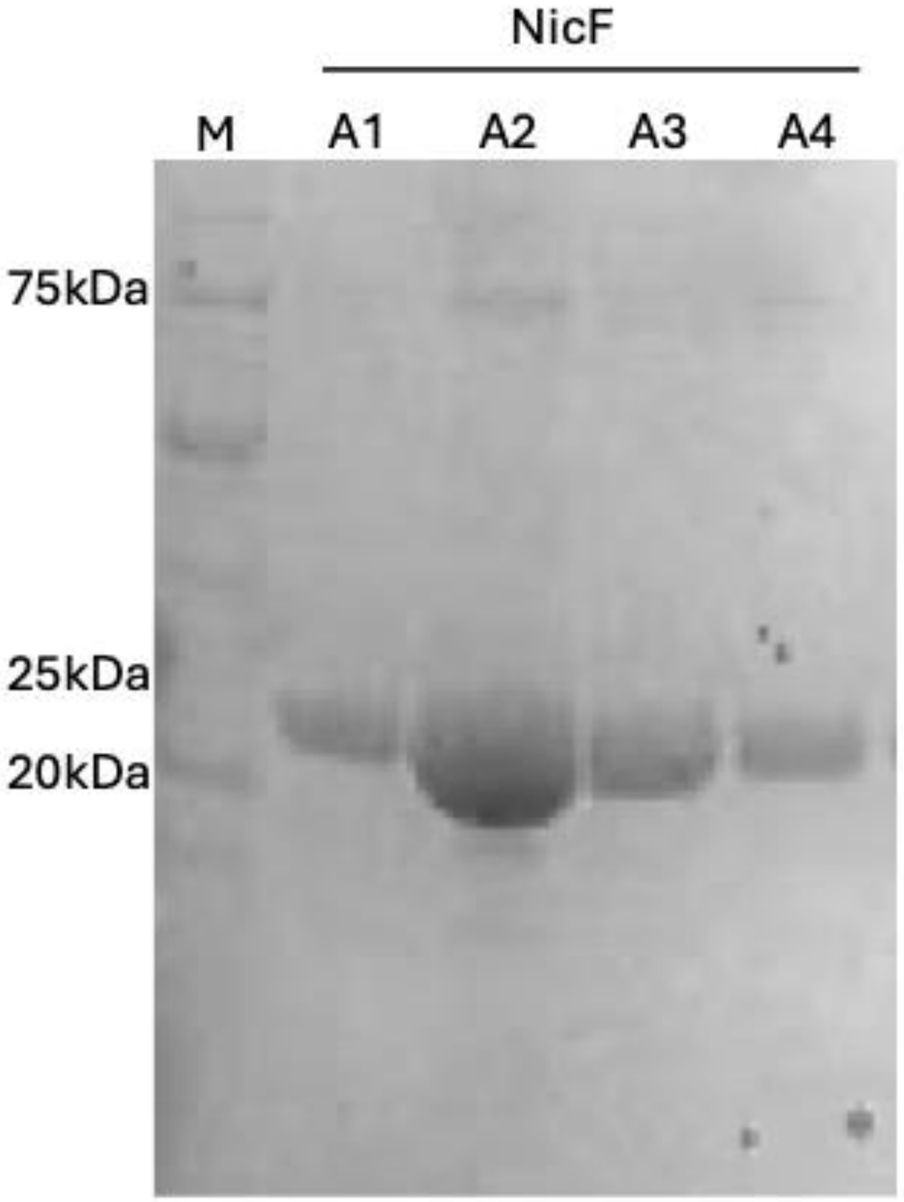
```
B. niacini NicF   1 MGSDKIHHHHHHSSGENLYFQGHMIRIWDNYLDERDKKVYQGAGLGKPTG   50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
     T7 Forward   1 MGSDKIHHHHHHSSGENLYFQGHMIRIWDNYLDERDKKVYQGAGLGKPTG   50

B. niacini NicF  51 IGKKPALVIVDVQYGFTGDSPENIEESIRKYPTSCGESSWAAIEHIKLLL  100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
     T7 Forward  51 IGKKPALVIVDVQYGFTGDSPENIEESIRKYPTSCGESSWAAIEHIKLLL  100

B. niacini NicF 101 HAARKAELPIFFTIIEGSKSSSNDRVAIKGNIFDHPALLEGEKGTQVVEE  150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
     T7 Forward 101 HAARKAELPIFFTIIEGSKSSSNDRVAIKGNIFDHPALLEGEKGTQVVEE  150

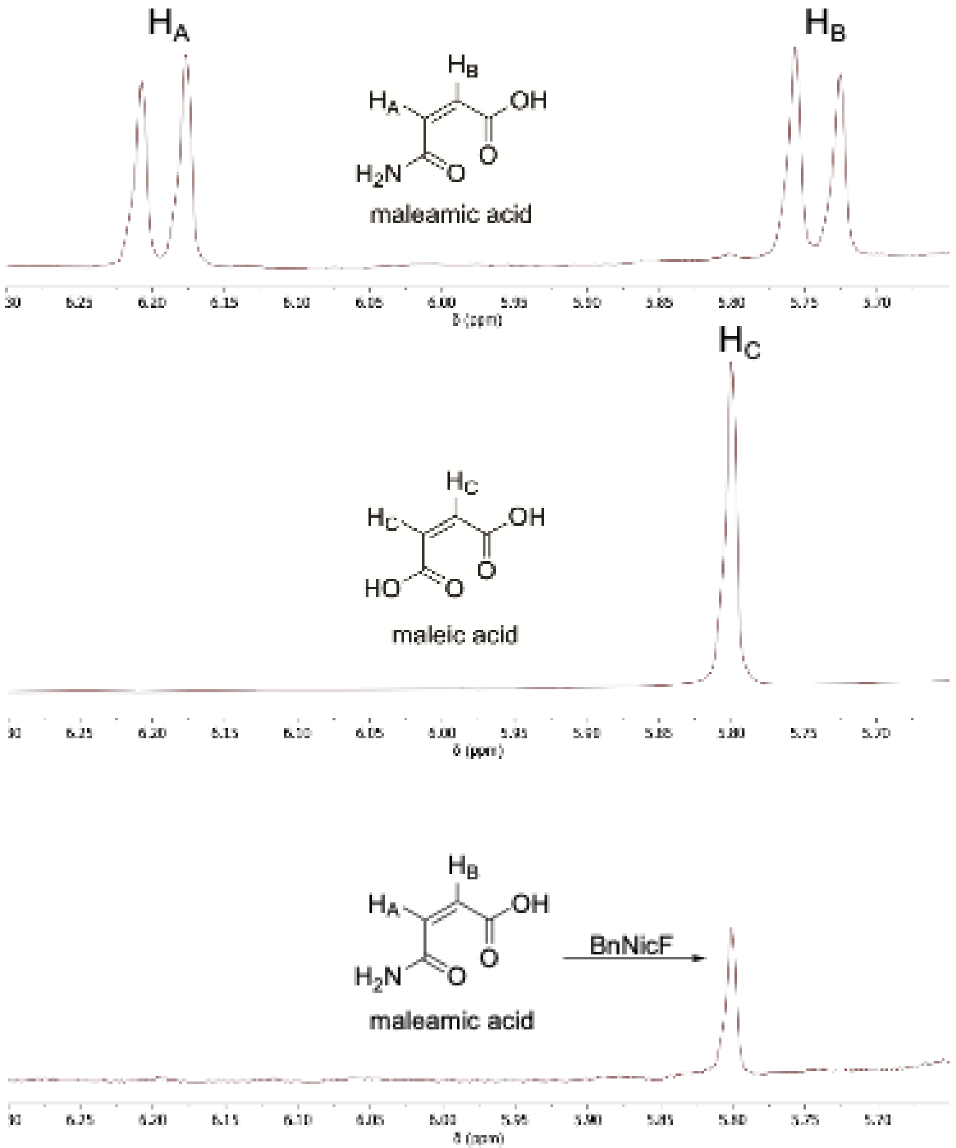
B. niacini NicF 151 LKPQYGEIVISKKKPSAFFGTPLVSYLTAQQVDTVIVTGCTTSGCVRASV  200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
     T7 Forward 151 LKPQYGEIVISKKKPSAFFGTPLVSYLTAQQVDTVIVTGCTTSGCVRASV  200

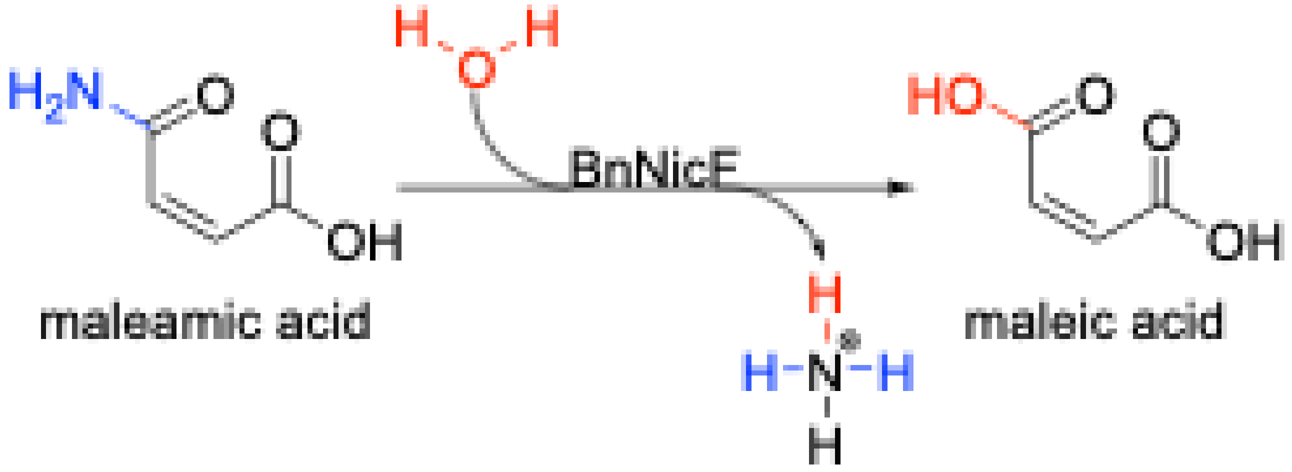
B. niacini NicF 201 IDAFSNNYRVVVPEECVFDRGIASHAINLFDMQQKYADVVPVDELIKELY  250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
     T7 Forward 201 IDAFSNNYRVVVPEECVFDRGIASHAINLFDMQQKYADVVPVDELIKELY  250
```



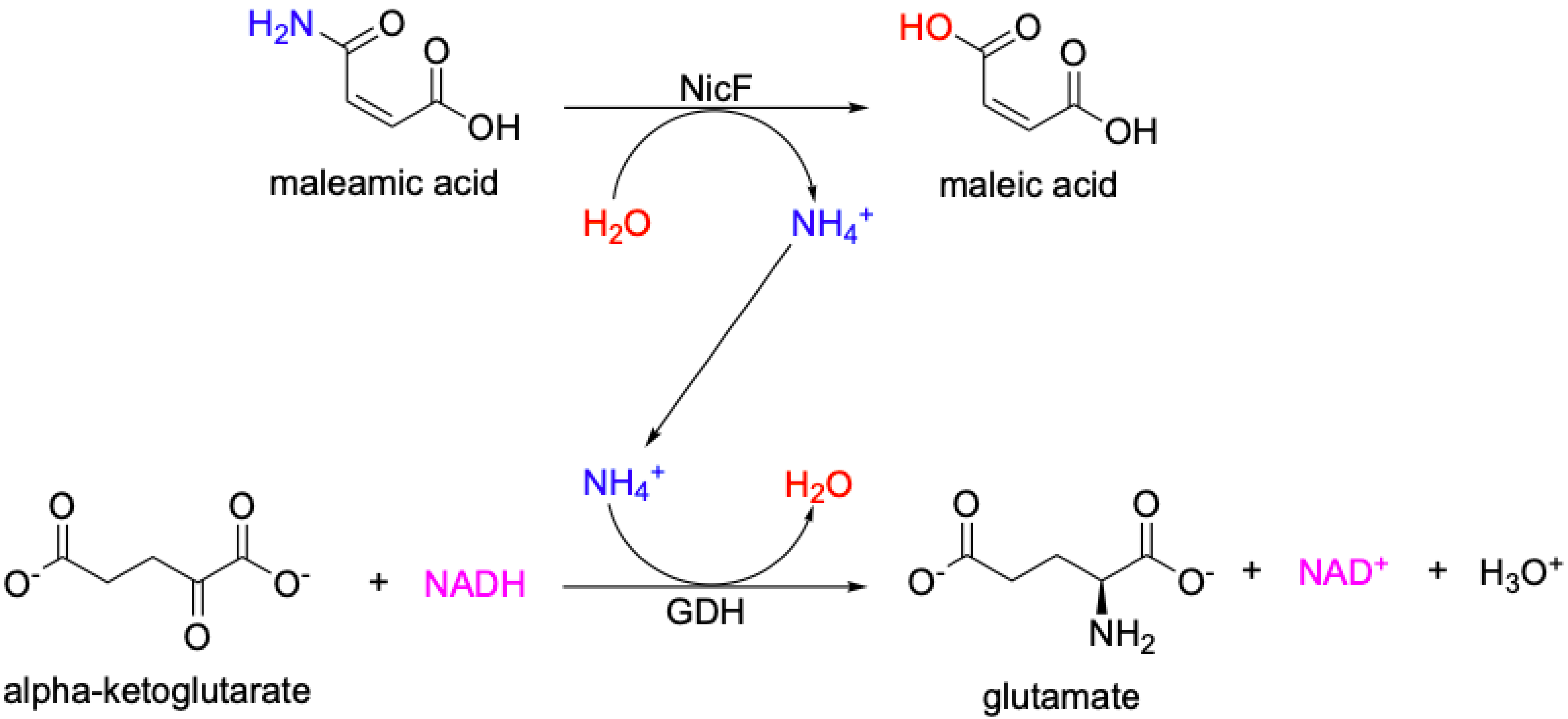
NicF monomer ~28kDa

^{1}H-NMR demonstrates that BnNicF catalyzes the hydrolytic deamidation of maleamic acid to maleic acid

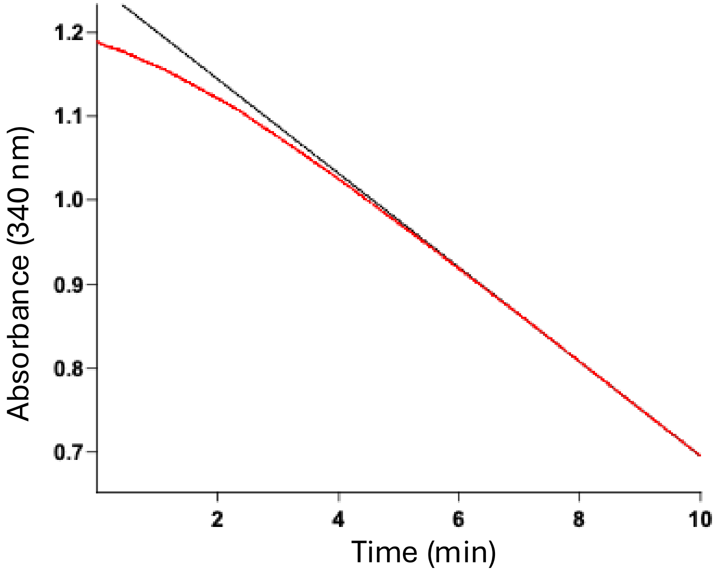


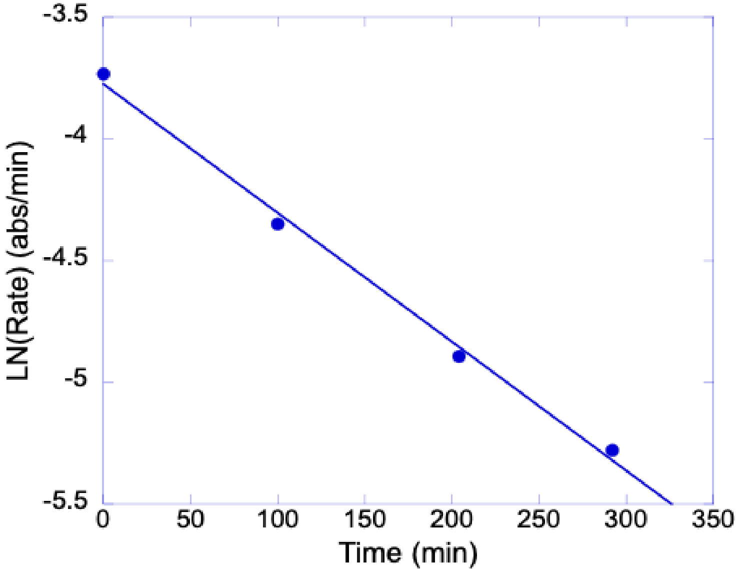


A linked-enzyme assay is utilized to determine the NicF kinetic parameters



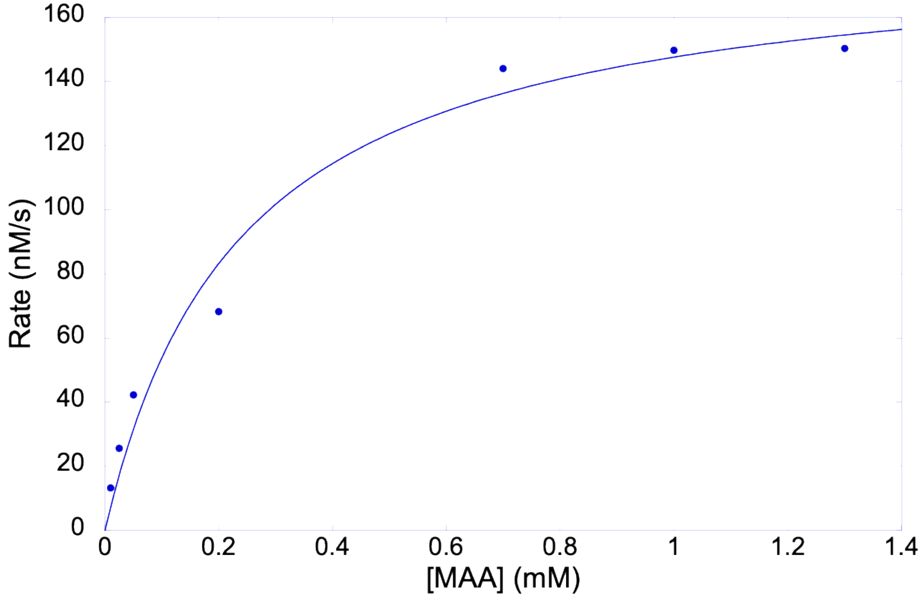
UV-Vis spectrophotometry was used to define physiologically relevant steady-state kinetic parameters





$t_{1/2}$ = 131 minutes

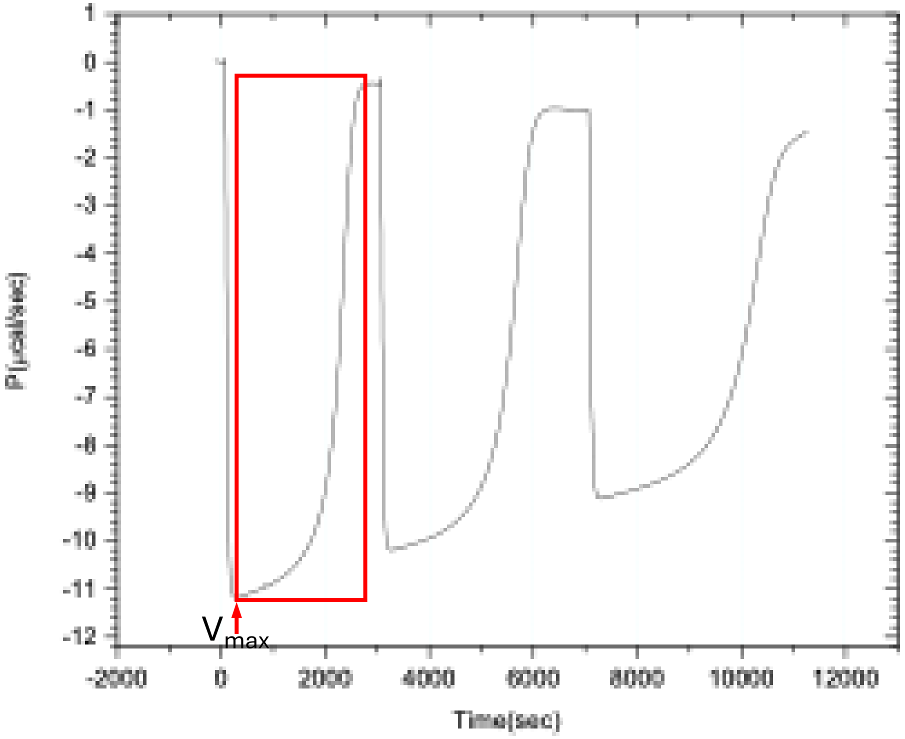
$$\text{Rate} = \frac{V_{\max} \times [S]}{K_M + [S]}$$



NicF Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
B. bronchiseptica	128 ± 6	11.7 ± 0.2	(9.1 ± 0.5) × 10^4
B. niacini (UV-Vis)	150 ± 80	26 ± 11	(1.9 ± 0.8) × 10^5

Kincaid, V. et al. 2012. *Biochemistry.* **51,** 545–554.

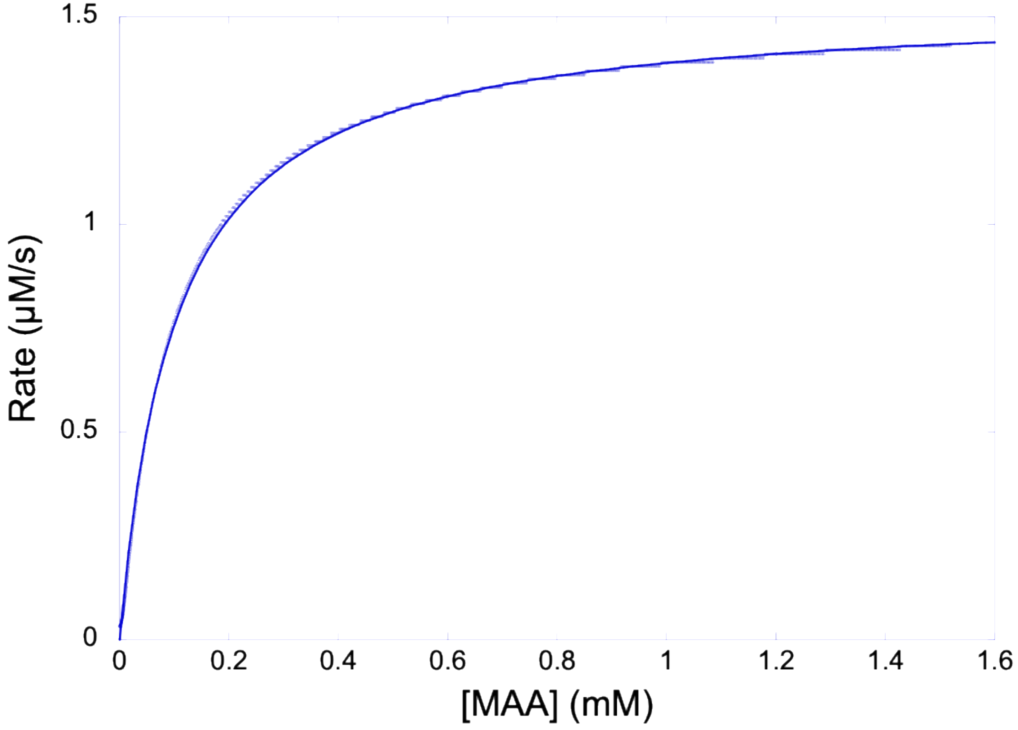
ITC was used to validate steady-state kinetic parameters obtained by UV-Vis and to reduce error between independent experiments



$$\Delta H_{app} = \frac{1}{[S]_{Total} * V} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} dt$$

$$\text{rate} = \frac{d[P]}{dt} = \left(\frac{1}{V \Delta H_{app}}\right)\left(\frac{dQ}{dt}\right)$$

$$\text{Rate} = \frac{V_{max} \times [S]}{K_M + [S]}$$



Technique	K_M (µM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
(UV-Vis)	150 ± 80	26 ± 11	$(1.9 \pm 0.8) \times 10^5$
(ITC)	100 ± 20	13 ± 2	$(1.3 \pm 0.3) \times 10^5$

Kincaid, V. et al. 2012. *Biochemistry.* **51,** 545–554.

The *B. niacini* nic operon encodes an enzyme with 59% sequence identity with *B. bronchiseptica* NicE

```
PpNicE MGSSHHHHHHSSGLVPRGSHMVEAMKVVRVGQIVPSSNITMETEIPALLKARELVAP  57
BnNicE -----------------------MKKEYRLGLIVPSSNTTMEKEIPAMLLSREQIEP  34

PpNicE -ERFTFHSSRMRMKHVTKEELARMDGDSDRCALELSDARVDVMGYACLVAIMSMGHG  113
BnNicE QETFTFHSSRMRMMHVTKEELAKMDTASDRCALELSDARCDALAYACLVAIMSQGPG  91

PpNicE YHRVSAERLRNVTENNDAATPIITSAGALIDGIRALGAKRVAVVTPYMKPLTELVVD  170
BnNicE YHCISEDRLSAIAKENGTAVPVVSSAGALIEGINALEAKKVAIITPYMKPLTKLVVD  148

PpNicE YIRHEGIEVGDYRALEISDNLAVAAHDPMNLPGIIASMRTDDVDAIVISACVQMPSL  227
BnNicE YIESTGIEVTDSISLEVSDNLEVGKLDPLNLINIVDNLNISNADAVVLSACVQMQSL  205

PpNicE NAITMVEAQTRKPVISAAVATTWAMLTALDLPTRVPGGGTLLSGAYLEHHHHHH     281
BnNicE PAIQKVQDKINLPVLSAATSTVYKLLKELKLNPIVPNAGYLLSRGY--------     251
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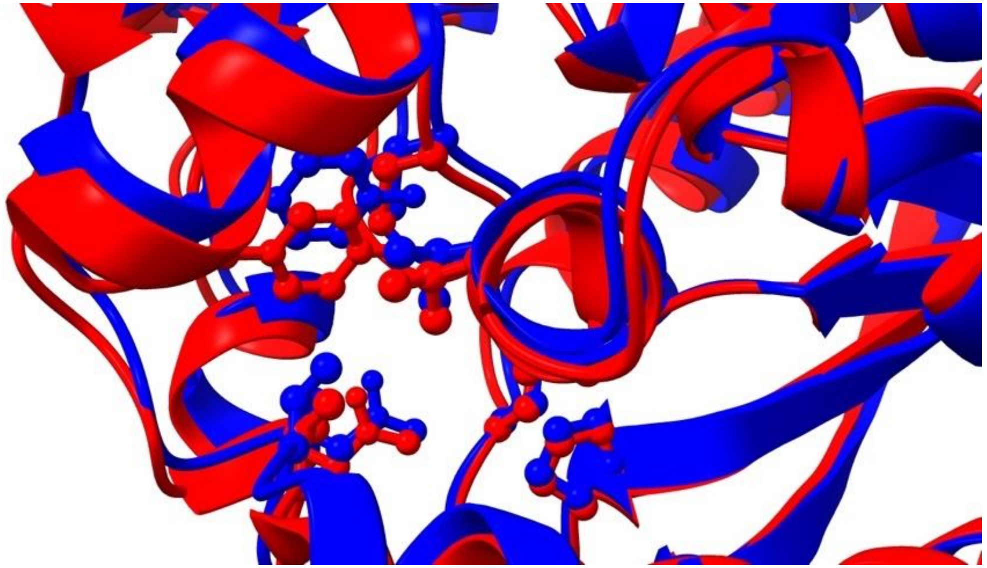
Red = *P. putida* NicE; Blue = *B. niacini* NicE



Chen, D. et al. 2013. *Molecular Microbiology.* **87,** 1237-1244.

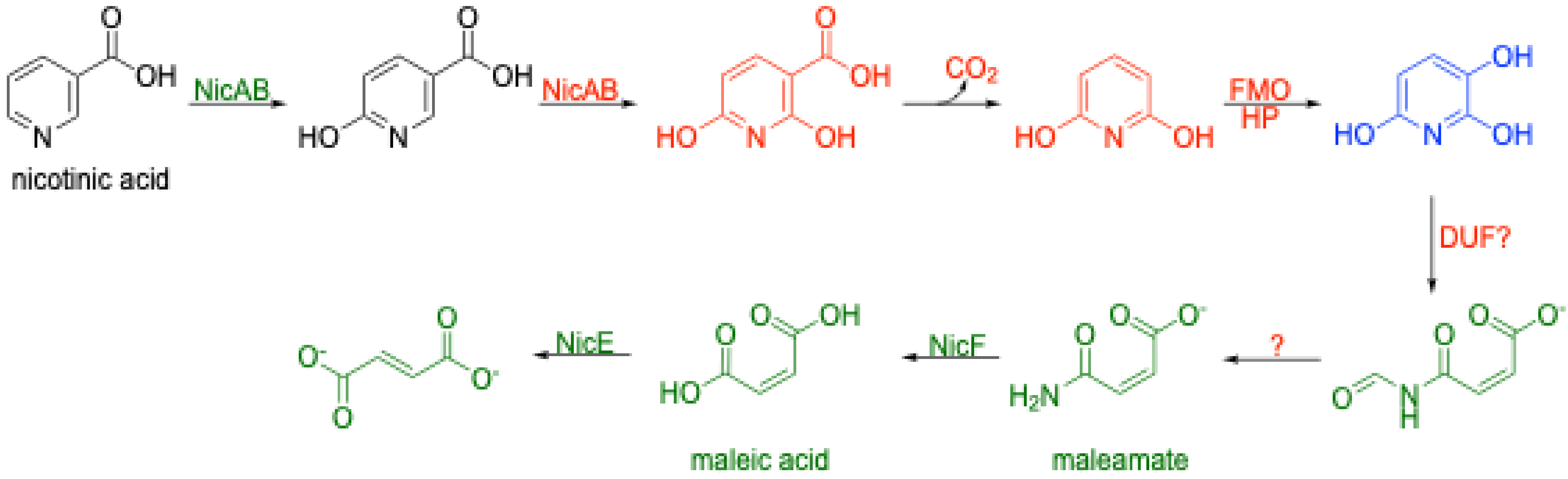
The proposed *B. niacini* NA catabolic pathway contains metabolites found in *A. nidulans* and *B. bronchiseptica*

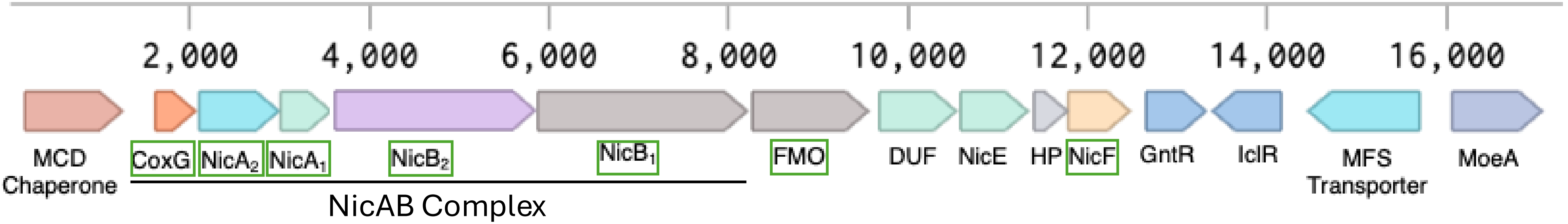
Black = shared

Green = *Pseudomonas*

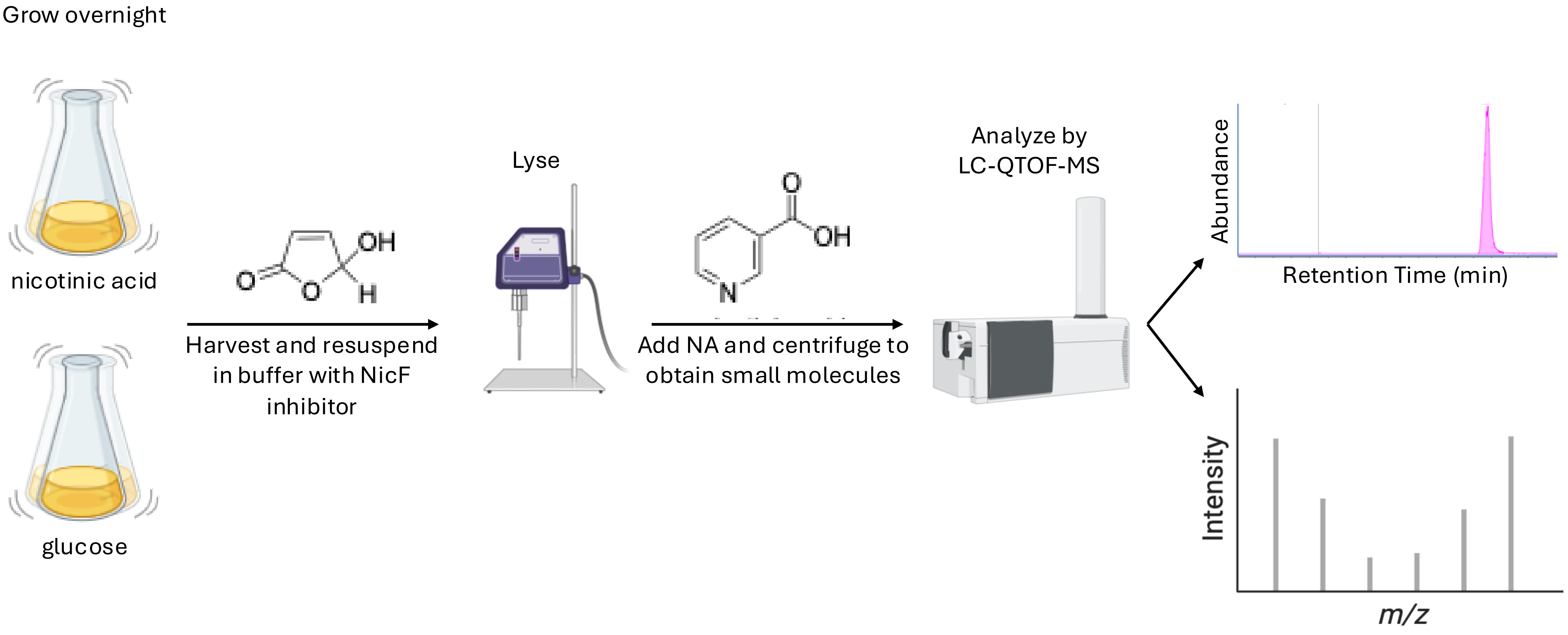
Red = unique

Blue = *Aspergillus*





LC-QTOF-MS can be used to identify other intermediates of NA catabolism in *B. niacini*



Grow overnight

nicotinic acid

glucose

Harvest and resuspend in buffer with NicF inhibitor

Lyse

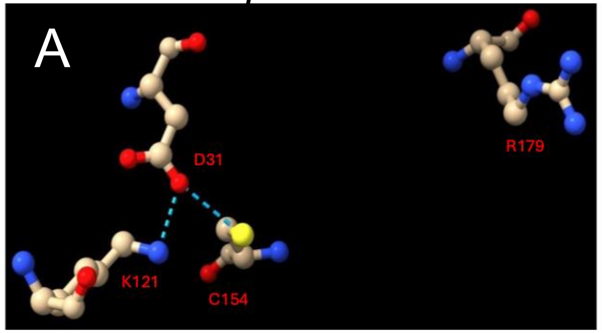
Add NA and centrifuge to obtain small molecules

Analyze by LC-QTOF-MS

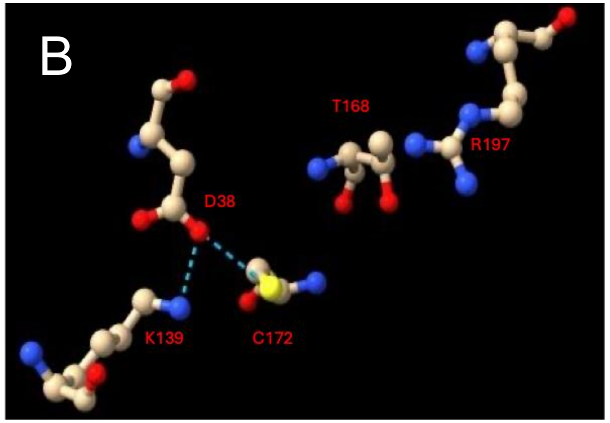
Olson, K. 2024. IS thesis, College of Wooster.

Structural analysis indicates that homotetrameric BnNicF contains a conserved catalytic triad and non-proline *cis*-peptide bond

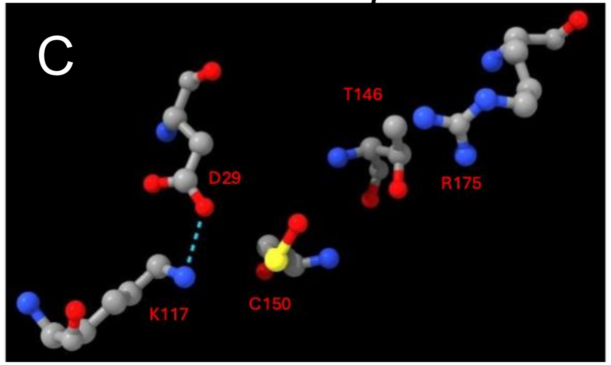
P. putida

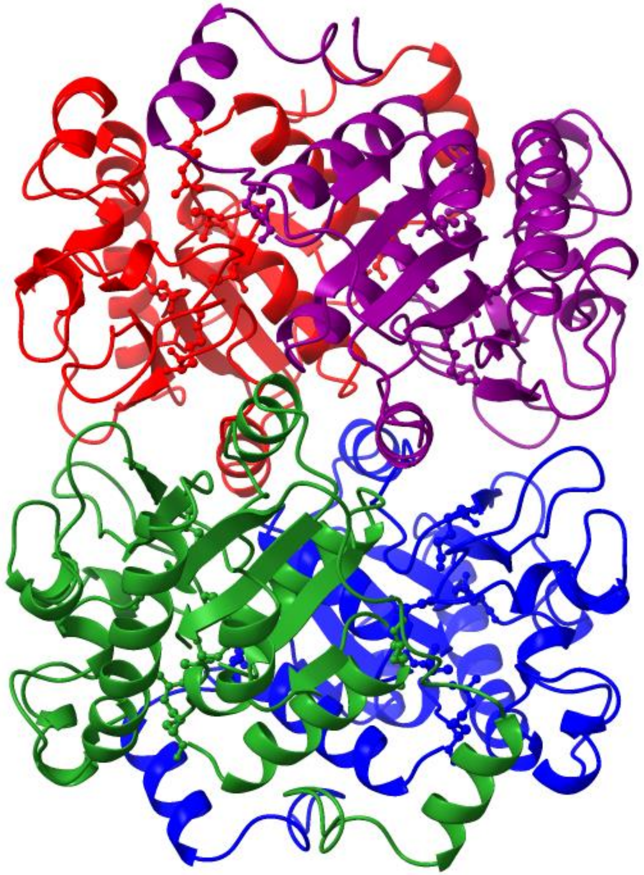


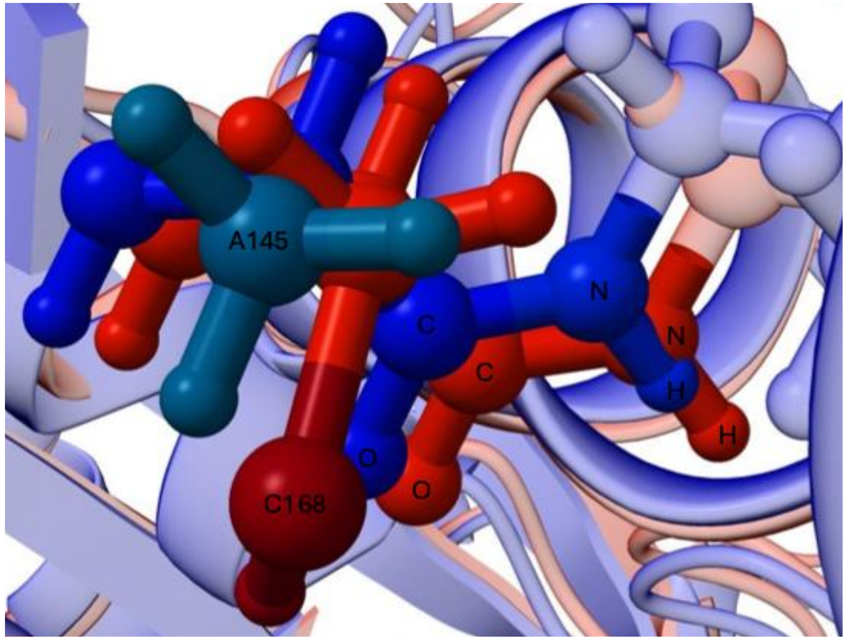
B. niacini



B. bronchiseptica

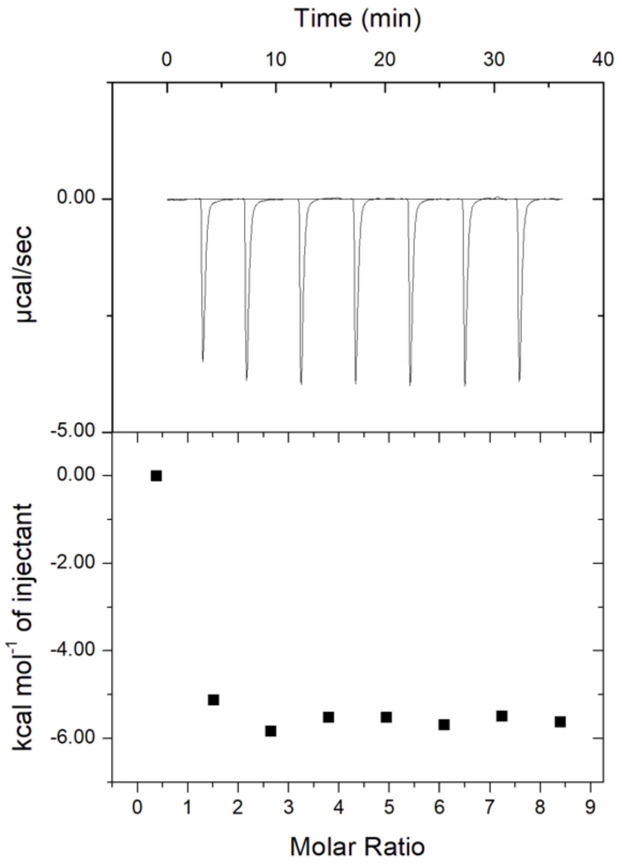






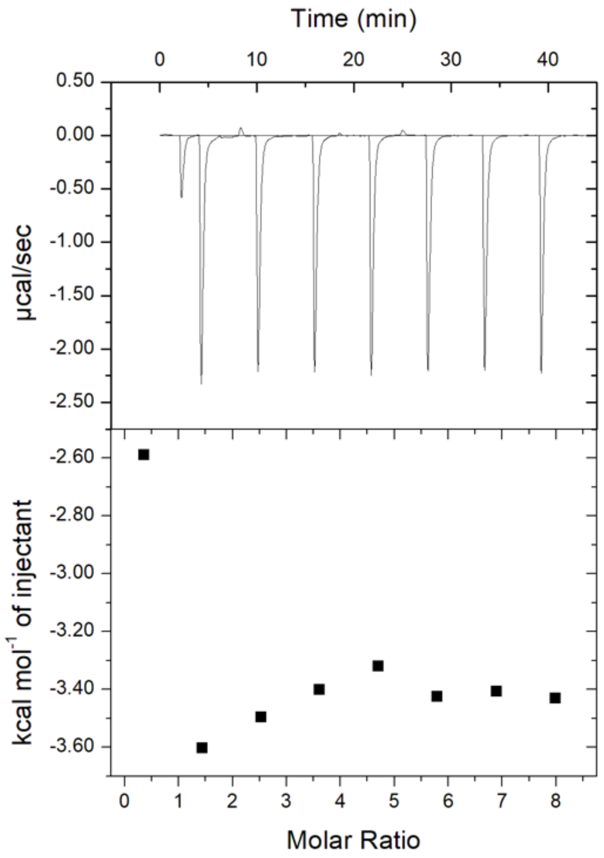
The $\Delta H_{apparent}$ of the BnNicF catalyzed hydrolytic deamidation in different buffers suggests that the mechanism involves proton buffer-exchange

Sodium Phosphate



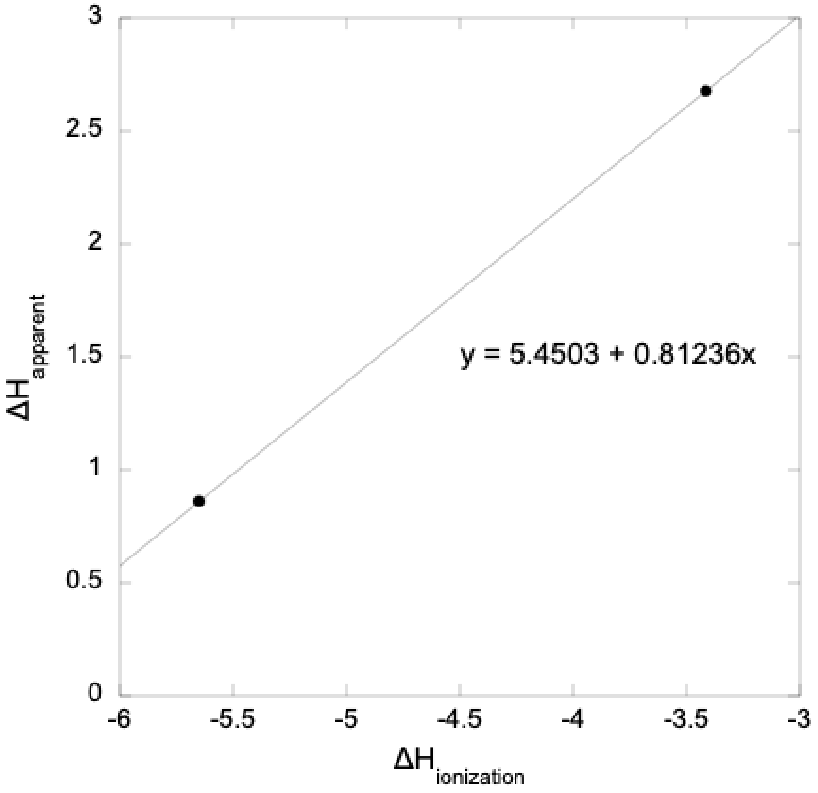
ΔH_{app}= -5.62 ± 0.13 kcal/mol

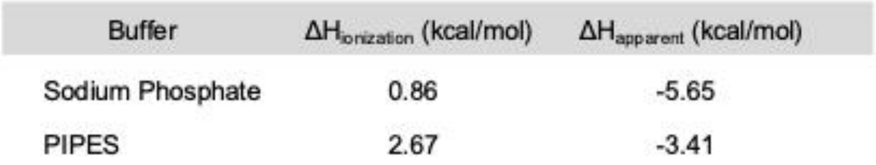
PIPES



ΔH_{app}= -3.41 ± 0.06 kcal/mol

$$\Delta H_{app} = \frac{1}{[S]_{Total*V}} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} dt \qquad (2)$$





Buffer	$\Delta H_{ionization}$ (kcal/mol)	$\Delta H_{apparent}$ (kcal/mol)
Sodium Phosphate	0.86	-5.65
PIPES	2.67	-3.41

BnNicF catalysis likely follows a nucleophilic addition-elimination sequence