RCU - Metabolomics
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High-Resolution Mass Spectrometric Identification of Apyc1-Derived Products from 2′,3′-Cyclic Nucleoside Monophosphates

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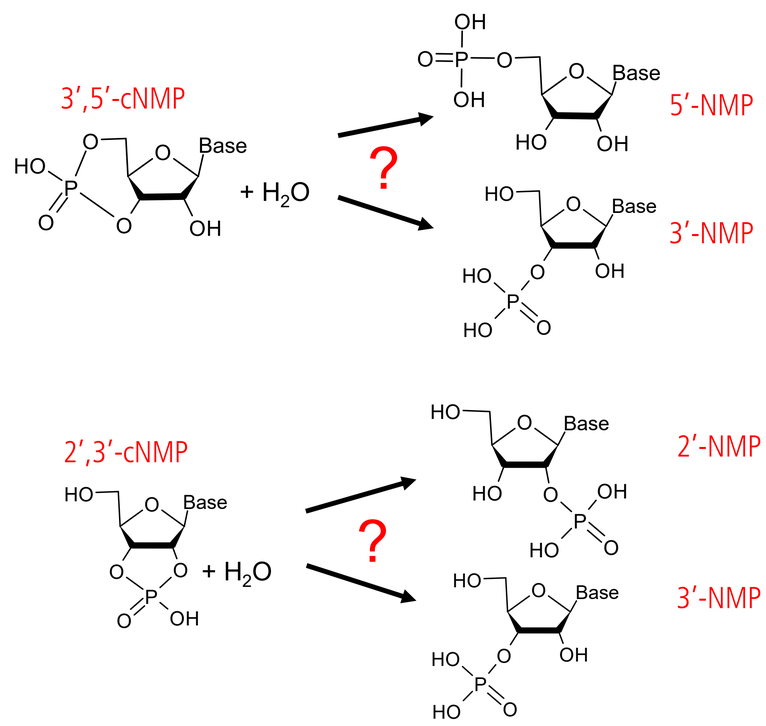
Introduction

The enzyme anti-Pycsar-1 (Apyc1) is a viral phosphodiesterase that plays an important role in evading bacterial immune defense during phage–bacterium interactions. Apyc1 hydrolyzes cyclic nucleotides and thereby contributes to the inactivation of the bacterial defense system Pycsar [1, 2]. In this study, we investigated the activity of Apyc1 toward 2′,3′-cyclic nucleoside monophosphates (2′,3′-cNMPs), with a particular focus on the mass spectrometric identification of the reaction products formed during enzymatic turnover.

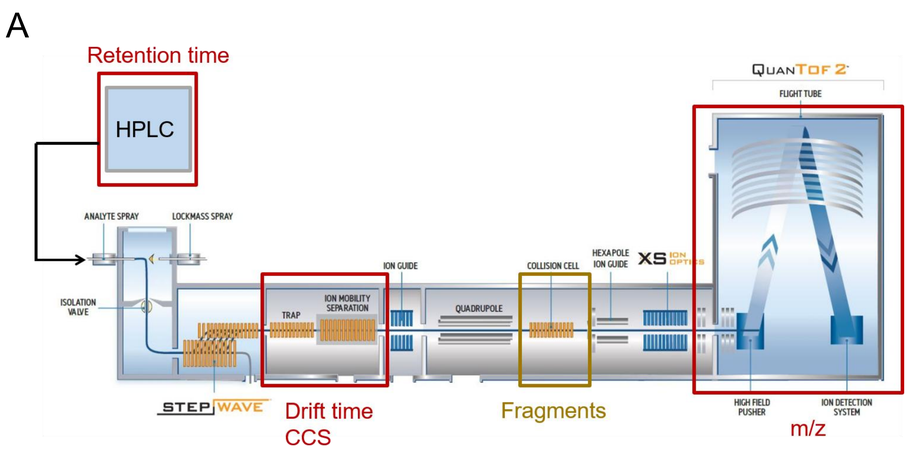
Methods

To investigate whether recombinant Apyc1 can hydrolyze 2′,3′-cNMPs in addition to its known substrates 3′,5′-cCMP and 3′,5′-cUMP, in vitro reactions using 2′,3′-cNMPs were performed and analyzed. Because the expected reaction products were isobaric species, particularly 2′-, 3′-, and 5′-nucleoside monophosphates, a high-resolution mass spectrometric approach was required for their differentiation and identification. Analyses were performed using a reversed-phase liquid chromatography setup coupled to a Vion™ ion mobility separation (IMS)-QTof instrument, equipped with an electrospray ionization (ESI) source. The use of IMS provided an additional analytical dimension, significantly improving the discrimination of structurally related compounds and further increasing confidence in the assignment of isobaric hydrolysis products. The combination of chromatographic retention time, drift time/collision cross section (CCS) information, and high-resolution accurate-mass data enabled unambiguous assignment of the reaction products.



Methodology and Results

Identification of 3′,5′-cNMP and 2′,3′-cNMP hydrolysis products



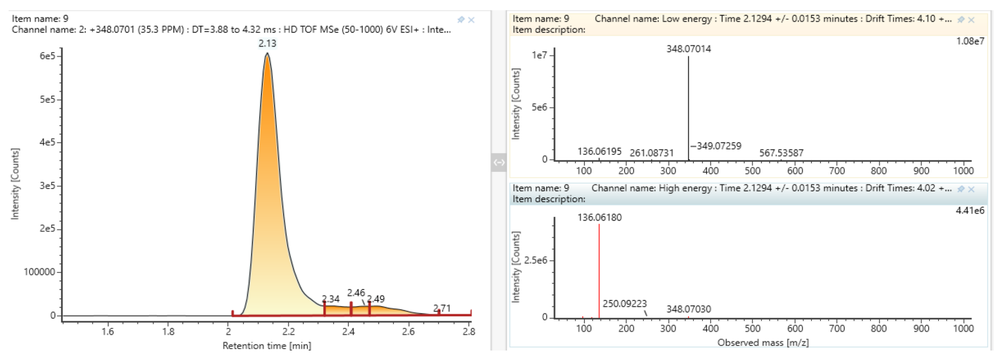


Fig 1: (A) Schematic setup of a LC-IMS-QTOF-MS (Vion™, Waters) [3]: It combines liquid chromatographic separation with ion mobility separation and high-resolution accurate-mass detection: The reaction products are first separated by LC, generating retention time information. After ionization, ions are separated in the ion mobility cell. The resulting drift time can be converted into a collision cross section (CCS). Finally, QTOF-MS provides accurate *m/z* values and high-resolution mass spectra. The combination of LC retention time, ion mobility drift time, CCS, and accurate mass enables multidimensional characterization and improved differentiation of isobaric or structurally related compounds. MS/MS fragment spectra were acquired for selected features to provide additional structural information and support compound annotation.
(B) Representative LC-IMS-QTOF-MS/MS analysis of 5′-AMP. The extracted chromatographic signal (left) illustrates the retention behavior of the compound, while the fragment spectrum (right) provides additional structural information for compound annotation.

compound	m/z	RT (min)	Drift time (ms)	CCS (Å²)
2′-AMP	348.070	4.93	4.17	169.63
3′-AMP	348.070	3.03	4.24	171.38
5′-AMP	348.070	2.14	4.07	167.23
2′-CMP	324.059	2.69	3.96	164.96
3′-CMP	324.059	2.00	4.02	166.21
5′-CMP	324.059	1.56	3.93	164.10
2′-GMP	364.065	5.45	4.38	174.64
3′-GMP	364.065	3.55	4.61	180.06
5′-GMP	364.065	2.37	4.45	176.24
2′-UMP	325.042	2.70	3.90	163.47
3′-UMP	325.042	2.45	3.96	164.85
5′-UMP	325.042	1.75	3.88	162.95

Tab 1.: Analytical results of 2′-, 3′-, and 5′-NMP-standards. Summarized are all LC-IMS-QTOF-MS parameters (m/z, retention time, drift time, and CCS values) used for compound identification. Analysis was performed in positive ESI mode.

Tab. 2: LC-IMS-QTOF-MS feature identification results of the hydrolysis of 3′,5′-cNMPs showing best matching reference standards as deviations from experimental values.

hydrolysis product of	m/z	RT (min)	Drift time (ms)	CCS (Å²)	Identified compound	Δ m/z (ppm)	Δ RT (min)	Δ Drift time (ms)	Δ CCS (Å²)
3′,5′-cAMP	348.070	2.13	4.10	168.81	**5′-AMP**	0.07	0.01	0.3	0.42
3′,5′-cCMP	324.059	1.56	3.97	165.83	**5′-CMP**	0.03	0	0.04	1.73
3′,5′-cGMP	364.065	2.36	4.50	178.13	**5′-GMP**	0.84	0.01	0.05	1.89
3′,5′-cUMP	325.043	1.71	3.90	164.31	**5′-UMP**	3.07	0.04	0.02	1.36

Tab. 3: LC-IMS-QTOF-MS feature identification results of the hydrolysis of 2′,3′-cNMPs showing best matching reference standards as deviations from experimental values.

hydrolysis product of	m/z	RT (min)	Drift time (ms)	CCS (Å²)	Identified compound	Δ m/z (ppm)	Δ RT (min)	Δ Drift time (ms)	Δ CCS (Å²)
2′,3′-cAMP	348.070	3.06	4.24	171.92	**3′-AMP**	0	0.03	0	0.54
2′,3′-cCMP	324.059	2.02	4.05	167.63	**3′-CMP**	0.31	0.02	0.03	1.42
2′,3′-cGMP	364.065	3.60	4.59	180.33	**3′-GMP**	0	0.05	0.02	0.27
2′,3′-cUMP	325.043	2.44	4.00	166.62	**3′-UMP**	2.15	0.01	0.04	1.77

Conclusion and Outlook:

- The hydrolysis products formed in the reactions of Apyc1 with **3′,5′-cNMPs** and **2′,3′-cNMPs** were unambiguously identified as **5′-NMPs** and **3′-NMPs**, respectively (based on accurate mass, retention time, ion mobility, CCS, and MS/MS data).
- Future work will focus on quantitative analysis of hydrolysis products, the evaluation of substrate specificity under different reaction conditions, and kinetic investigations.

References
[1] Tal *et al.* (2021). *Cell*, *184*(23), 5728-5739.e16.
[2] Hobbs *et al.* (2022). *Nature*, *605*(7910), 522–526
[3] www.waters.com

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