Final Campaign report

Development of a safe and scalable process for

the synthesis of EIDD-2801/MK-4482/Molnupiravir

Submitted to

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Report prepared by Dr. Sarabindu Roy & Dr Ajay K. Yadaw

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1	Dr Chris Senanayake	CEO
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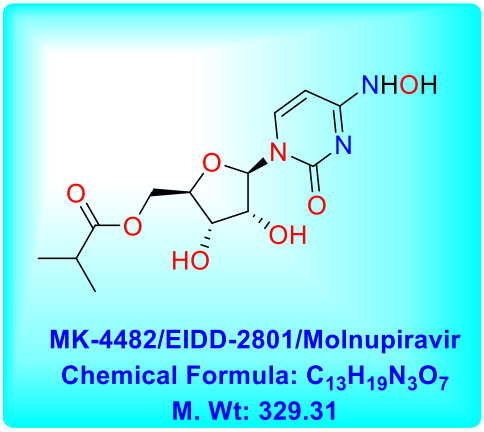
Major Scope of the work:

- Feasibility study of the proposed route
- Analytical method development for in-process control and release of the intermediates as well as final compound
- Optimization of the reaction in terms of yield, quality & reaction time
- Development of suitable isolation and purification protocol to isolate key intermediates as well as final API
- Identification of key process impurities / Impurity profiling
- Synthesis of the final API molnupiravir >1.0 kg scale
- Preparation of Lab development report

Overview:

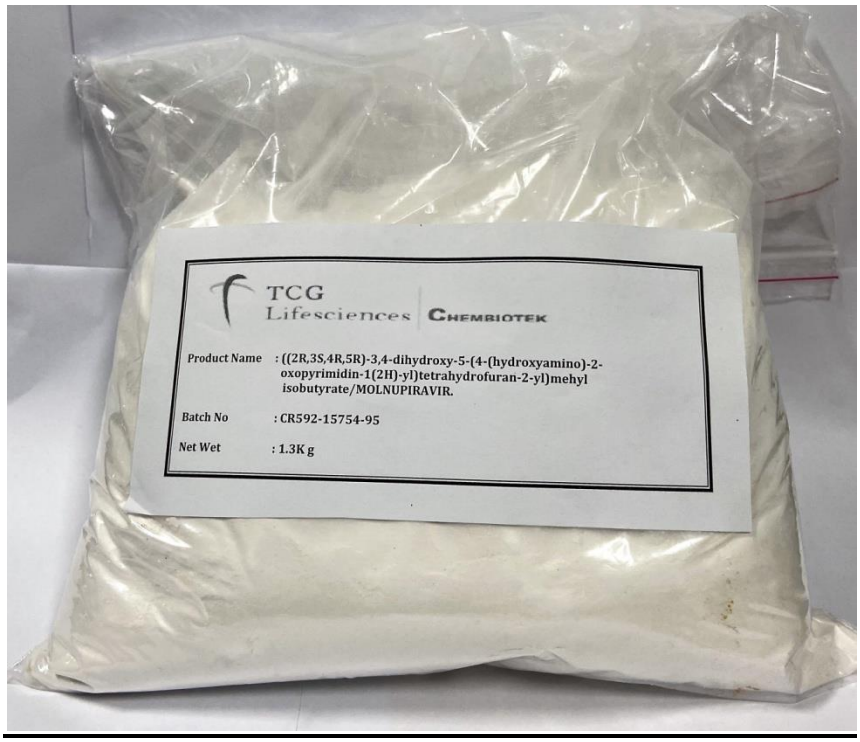
MK-4482/EIDD-2801/Molnupiravir is a final API and an antiviral with emerging potential to treat COVID-19. Structure of the Target compound is shown below.



MK-4482/EIDD-2801/Molnupiravir
Chemical Formula: $C_{13}H_{19}N_3O_7$
M. Wt: 329.31

Summary of work done and major accomplishments

- Various conditions were screened for all the steps and based on the study a safe and scalable protocol has been established for the synthesis of target compound in kg scale using only 10w/w% of enzyme.
    - Lower enzyme loading (5 w/w%) was successfully used on 100.0 g scale
    - Enzyme was re-cycled for 3 times and showed almost similar activity
- Extensive studies have been performed for purification and yield improvement of final API and the results have been summarized in this final report
- Several process impurities have been identified, isolated, synthesized and characterized
- Efficient purification protocol has been established to purge most of the process impurities
- Using optimized process eventually >1.0 kg of final target compound has been synthesized with chemical purity >99.7% (A%) and with assay 98.8%



Certificate of Analysis of Molnupiravir

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CERTIFICATE OF ANALYSIS

Product Name : **((2R,3S,4R,5R)-3,4-dihydroxy-5-(4-(hydroxyamino)-2-oxopyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl isobutyrate/MOLNUPIRAVIR.**

Batch No. : **CR592-15754-95**

Date of Mfg : **MAR 2021**

Quantity : **1.3 Kg**

S.No.	Test	Results	Specification
1.0	Appearance	White color solid	White to light yellow solid
2.0	Identification by		
	i) IR	Graph attached	IR spectrum of sample should match with the IR spectrum of standard.
3.0	Water content by KF (%w/w)	0.32 % w/w	Report the results
4.0	Residue on ignition	0.14 % w/w	Report the results
5.0	Loss on drying (at 105°C)	0.03 % w/w	Report the results
6.0	Chromatographic purity by HPLC (% area)		
	i) Purity	99.70 %	Report the results
	ii) Single maximum unknown impurity	0.25 %	Report the results
7.0	Residual solvent by GC (ppm)		
	i) 2-Methyl THF	Not detected	Not more than 5000
	ii) DCM	Not detected	Not more than 600
	iii) MTBE	28 ppm	Not more than 5000
8.0	Assay by HPLC	99.7 % w/w	Report the results

Prepared By: Amit Ghosh

Date 06 Apr. 2021

Checked By: Soumya Chatterjee

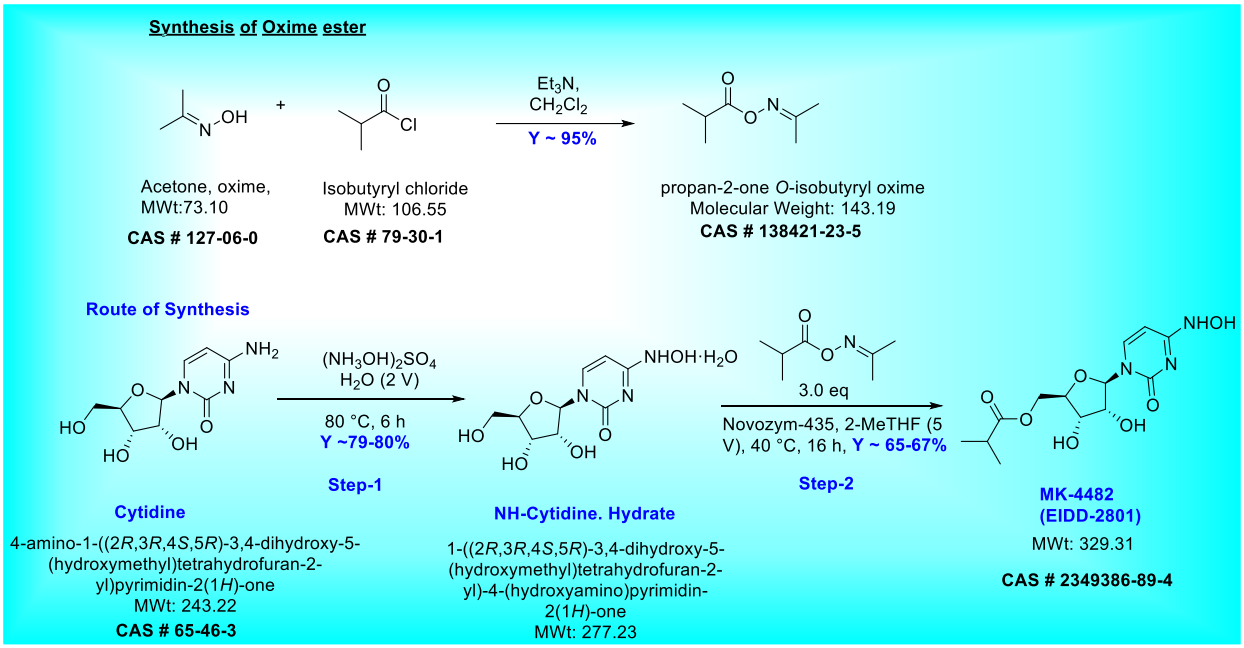
Date 06 Apr 2021

Approved By: Sukanta Kumar Saha

Date 06 Apr 2021

Final optimized synthetic scheme

The final optimized synthetic scheme is shown below.



Step-1 (Intermediate: NHC·H_2O):

List of Raw materials:

i) Cytidine

ii) Hydroxylamine sulfate $(NH_3OH)_2SO_4$

iii) Water

Experimental:

Actual batch size and quantity:

Process Information:

S. No.	Reagent		Unit	Qty	Mol Wt	Mole	Equiv	Source
1	Cytidine		kg	2.0	243.22	8.22	1	Jiangsu Chengyi Pharm. Co. Pvt. Ltd  COA of Cytidine.pdf
2	Hydroxylamine sulfate		g	823.3	164.14	5.01		AVRA Synthesis Pvt. Ltd. Batch No. N2000003
3	Water		L	4.0	18.0		2 V	In-House
4	Water	Lot-1	L	1.0	18.0		0.5 V	
		Lot-2		1.0	18.0		0.5 V	

Process details:

S. No.	Procedure	Qty.	Remarks
1.	Charged cytidine (2.0 kg) into the all glass reactor (AGR-30 L) at 25-30 °C	2.0 kg	Cytidine Batch No:07-0092020-09019
2.	Charged hydroxylamine sulfate (0.61 eq./823.3 g) into the reactor at 25-30 °C	823.3 g	
3.	Added water (4.0 L) into the reactor at 25-30 °C	4.0 L	
4.	Stirred the reaction mass at 25-30 °C		Stirred at ~180 RPM
5.	Slowly increased the reaction mass temperature up to 80-85 °C		Clear solution obtained
6.	Maintained the reaction mass for 6 h at 80-85 °C		
7.	Send the sample for HPLC analysis to check the content of cytidine.		Cytidine content: Results: 6.9%

S. No.	Procedure	Qty.	Remarks
	Sample preparation: Take the sample 0.2 mL directly from reaction mixture and dissolved in 10 mL of water and submit		
8.	After wards slowly cooled the reaction mass at 25-30 °C under stirring		Solid precipitation was observed
9.	Further cooled the RM at -5 to 0 °C and stirred the reaction mass at same temperature for 3 h		
10.	Filtered the RM through nutsche filter under vacuum at 25-30 °C		5-Micron filter cloth was used for filtration
11.	Washed the wet solid with chilled water (10-15 °C) (Lot-1)	1.0 L	
12.	Suck dried the material for 15-30 min		
13.	Again, washed the wet solid with chilled water (10-15 °C) (Lot-2)	1.0 L	
14.	Suck dried for 15-30 min at 25-30 °C		
15.	Unloaded the material and dried under vacuum (NLT 600 mm of Hg) at 55-60 °C for 5-6 h (Till constant weight)		Water content by KF: Results 6.32%
16.	Unloaded the solid: Obtained wt: 1.83 kg (0.91 w/w) Theoretical yield: 2.27 kg Obtained Molar yield: 80.26%		

In process controls data (2.0 kg batch):

IPC data of NHC·H_2O

<table>
<tr><th rowspan="2">S. No.</th><th rowspan="2">Batch Id</th><th colspan="3">IPC-1; Cytidine content by HPLC</th><th rowspan="2">Analytical data</th></tr>
<tr><td>NHC</td><td>Cytidine</td><td>Uridine</td></tr>
<tr><td>1</td><td rowspan="3">CR592-15754-87</td><td>90.08%</td><td>6.91%</td><td>2.06%</td><td>CR592-15754-87</td></tr>
<tr><td rowspan="2">2</td><td colspan="3">IPC-2: water content by KF</td><td rowspan="2">CR592-15754-87-P-kf.pdf</td></tr>
<tr><td colspan="3">Results: 6.32%</td></tr>
</table>

Isolated data of NHC·H_2O (2.0 kg batch):

S. No.	Input (kg)	Output (kg)	Assay based Yield (%)	Analytical data
1	2.0	1.83	80.26%	CR592-15754-87 Purity: 99.31% Cytidine: 0.05%, Uridine: 0.65% Q-NMR assay: 99.88%

IPC-1 data of NHC·H_2O: 3 X 200.0 g batch

S. No.	Batch Id	IPC (HPLC)			Analytical data
		NHC	Cytidine	Uridine	
1	CR592-16022-8	90.74%	5.58%	2.73%	CR592-16022-8
2	CR592-16022-9	90.96%	4.67%	3.43%	CR592-16022-9
3	CR592-15754-82	90.03%	7.00%	2.05%	CR592-15754-82

Isolated data of NHC·H_2O: 3 X 200.0 g batch

S. No	Batch Id	Isolated (HPLC) (%)			Output (g)	QNMR based yield (%)	KF (%)	Q-NMR (%)	Analytical data
		NHC	Cytidine	Uridine					
1	CR592-16022-8	99.44	0.13	0.42	182.0	79.0	6.64	99.07	CR592-16022-8

2	CR592-16022-9	99.19	0.11	0.70	183.0	79.6	6.61	97.58	CR592-16022-9
3	CR592-15754-82	99.55	0.12	0.33	182.0	79.6	6.81	99.64	CR592-15754-82

Stage-2 (Preparation of Intermediate Oxime ester):

List of Raw materials

i) Acetoxime

ii) Dichloromethane (DCM)

iii) Isobutyryl chloride

iv) Triethylamine (Et_3N)

v) Hydrochloric acid (HCl)

vi) Sodium bicarbonate ($NaHCO_3$)

vii) Sodium chloride (NaCl)

viii) Water

ix) Sodium sulfate (Na_2SO_4)

Raw materials:

S. No.	Reagent	Unit	Qty	Mol Wt	Mole	Equiv	Source
1	Acetoxime	kg	2.0	73.10	27.34	1.0 equiv	Deepak Nitrite Ltd. Batch No. 20180753
2	Dichloromethane	L	24.0	84.93		12 V	RANKEM R208A21
3	Triethylamine	L	4.2	101.12	30.0	1.1 equiv	ALFA AESAR LOT: 10227154
4	Isobutyryl chloride	L	3.2	106.55	30.0	1.1 equiv	SAM LABORATORIES

S. No.	Reagent		Unit	Qty	Mol Wt	Mole	Equiv	Source
								Batch No. 854
5	Water	Lot-1	L	5.0	18		2.5 V	IN-HOUSE
	Water	Lot-2		5.0	36.5		2.5 V	
	Water	Lot-3		5.0	18		2.5 V	
6	1N HCl		L	5.0	84		2.5 V	RANKEM K017B21
7	Saturated $NaHCO_3$		L	5.0	18		2.5 V	RANKEM J201K20
8	Brine solution		L	5.0	18		2.5 V	RANKEM J037B21
9	Sodium sulfate (Na_2SO_4)		kg	1.9	142.04		0.5 equiv	RANKEM J240M20

Process details for preparation of Oxime ester:

S.No.	Procedure	Qty.	Remarks
1.	Charged acetoxime into glass lined reactor (GLR-150 L) under nitrogen atmosphere at 25-30 °C	2.0 kg	
2.	Charged DCM into the reactor under nitrogen atmosphere at 25-30 °C	24.0 L	
3.	Stirred the reaction mass at 25-30 °C for 30 min ~180 rpm		Clear solution observed
4.	Slowly added triethylamine into the reactor under nitrogen atmosphere for 30 min at 25-30 °C	4.2 L	
5.	Slowly cooled the reaction mass at -5 to 0 °C for 3-4 h		
6.	Stirred the reaction mass for 1.0 h at -5 to 0 °C		
7.	Slowly added isobutyryl chloride at -5 to 0 °C and U/N_2 for 4-5 h	3.2 L	White fumes appeared and RM became white
8.	After addition completed reaction mass stirred at 25-30 °C for 2-3 h		
9.	Stirred the reaction mass at 25-30 °C for 16 h at 180-200 rpm		Reaction mass became white
10.	Added water (lot-1) into the RM at 25-30 °C	5.0 L	RM became a clear solution
11.	Stirred the RM for 30 min at 25-30 °C		

12.	Stopped the stirring, and settled the layers for 30 min		
13.	Separated the aq. layer		
14.	Organic part again transferred into the reactor		
15.	Added 1N HCl solution into the reactor	5.0 L	HCl (417.0 mL) in 5.0 L
16.	Stirred the RM for 30 min at 25-30 °C		
17.	Stopped the stirring, and settled the layers for 30 min at 25-30 °C		
18.	Separated the layers		
19.	Organic layer again transferred into the reactor		
20.	Added water (Lot-2) into the RM at 25-30 °C	5.0 L	
21.	Stirred the RM for 30 min at 25-30 °C		
22.	Stopped the stirring, and settled the layers for 30 min at 25-30 °C		
23.	Separated the layers		
24.	Organic layer again transferred into the reactor		
25.	Added $NaHCO_3$ solution into the reactor	5.0 L	$NaHCO_3$ (180.0 g) in 5.0 L water
26.	Stirred the RM for 30 min at 25-30 °C		
27.	Stopped the stirring, and settled the layers for 30 min at 25-30 °C		
28.	Removed $NaHCO_3$ solution by separating organic part		
29.	Organic part again transferred into the reactor		
30.	Added water (Lot-3) into the RM at 25-30 °C	5.0 L	
31.	Stirred the RM for 30 min at 25-30 °C		
32.	Stopped the stirring, and settled the layers for 30 min at 25-30 °C		

33.	Removed water by separating the layers		
34.	Organic part again transferred into the reactor		
35.	Added brine solution into the reactor at 25-30 °C	5.0 L	NaCl: 1.8 kg in 5.0 L water
36.	Stirred the RM for 30 min at 25-30 °C		
37.	Stopped the stirring, and settled the layers for 30 min at 25-30 °C		
38.	Removed brine solution by separating the layers		
39.	The organic layer was dried over anhydrous Na_2SO_4	1.9 kg	
40.	Concentrated the organic layer U/V and degassed under vacuum (NLT 600 mm of Hg) at below 45 °C for 3-4 h		Light-yellow oil results of KF: 0.13%
41.	Unloaded the mass and stored at 2-8 °C for next reaction Obtained wt: 3.8 kg Theoretical yield: 3.9 kg Molar yield: 86.5%		

Results of Oxime ester batch: 2.0 kg

S. No	Batch No.	Input (kg)	Output (kg) Assay based		Isolated			Analytical data
				Q-NMR assay	Purity by HPLC	Purity by GC	Water content KF % w/w	
1	CR592-15754-86	2.0	3.8 (Y = 86.5%)	89.21%	96.82%	97.64%	0.13	CR592-15754-86-Analytical data.pdf

Stage-2 (EIDD-2801/MK-4482/Molnupiravir):
List of Raw materials

i) NH-Cytidine hydrate

ii) Novozym-435

iii) Oxime ester

iv) 2-Methyltetrahydrofuran (2-MeTHF)

v) Hydroxylamine solution (50 wt. % in H_2O)

vi) Methyl *tert*-butyl ether (MTBE)

vii) Water

Experimental procedure of EIDD-2801/MK-4482/Molnupiravir:

Raw materials:

S. No.	Reagent		Unit	Qty	Mol Wt	Mole	Equiv/Vol	Source
1	NH-Cytidine hydrate		kg	1.8	277.09	6.49		CR592-15754-87
2	Novozym-435		g	180.0			10 wt%	Novozym-435 Batch No. LC200315
3	Oxime ester		kg	2.8	143.19	19.47	3.0 equiv	CR592-15754-86
4	2-MeTHF	Lot-1	L	6.0	86.13		5 V	SHANDONG YINO BIOLOGIC MATERIALS CO., LTD LOT NO. YINO20200618021
		Lot-2		3.0				
		Lot-3		3.6	86.13		2 V	
		Lot-4		3.6			2 V	
5	Hydroxylamine (50 wt. % in H_2O)		mL	260.0	33.03		3.0 equiv	SYMAX Laboratories, B.No: SHC004
6	MTBE	Lot-1	L	1.8	88.15		1.0 V	Dor Chemicals Ltd. Batch No. DOR0032010
		Lot-2		27.0	88.15		15.0 V	
		Lot-3		3.6	88.15		2.0 V	
		Lot-4		3.6	88.15		2.0 V	
		Lot-5		1.8	88.15		1.0 V	
		Lot-6		1.8	88.15		1.0 V	
7	Water	Lot-1	L	2.7	18		1.5 V	IN-HOUSE
		Lot-2		0.9	18		0.5 V	
		Lot-3		0.9	18		0.5 V	
		Lot-4		2.7	18		1.5 V	
		Lot-5		0.9	18		0.5 V	
		Lot-6		0.9	18		0.5 V	

Process details for the preparation of EIDD:

S.No.	Procedure	Qty.	Remarks
1.	Charged NH-cytidine hydrate into reactor (AGR-30 L) under nitrogen atmosphere at 25-30 °C	1.8 kg	
2.	Charged the washed Novozym-435* into reactor under nitrogen atmosphere at 25-30 °C *Note: washing procedure of the Novozym-435 given below the table	180.0 g	The mixture was stirred with 120 RPM
3.	Charged 2-MeTHF Lot-1 into reactor U/N_2 at 25-30 °C	6.0 L	
4.	Charged above prepared oxime ester into reactor under nitrogen atmosphere at 25-30 °C	2.8 kg	
5.	Charged 2-MeTHF Lot-2 through washing the oxime ester vessel U/N_2 at 25-30 °C	3.0 L	RM became heterogeneous
6.	Increased the RM temperature at 40-45 °C		
7.	Stirred the reaction mass for 16 h at 40-45 °C at 120 RPM and U/N_2		RM became heterogeneous
8.	Sample submitted for HPLC analysis to check the consumption of NHC. **Sample preparation: Take 0.2 mL RM and diluted with 10 mL of water and filtered to separate the solid particles and submitted the filtrate.**		IPC results: NHC 3.39%
9.	Stopped the heating and cooled the reaction mass for 2-3 h to reach temperature 25-30 °C		
10.	Filtered the Novozym-435 through nutsche filter under vacuum at 25-30 °C		5-Micron filter cloth was used for filtration
11.	Washed the Novozym-435 with 2-MeTHF Lot-3	3.6 L	
12.	Washed the Novozym-435 with 2-MeTHF Lot-4	3.6 L	
13.	Charged total organic mass (2-MeTHF) into reactor (AGR-30 L) at 25-30 °C		
14.	Cooled the reaction mass at 15-20 °C		
15.	Charged aqueous hydroxylamine (50 wt. % in H_2O) into the reactor at a time at 15-20 °C	260.0 mL	Depending on % of di-acyl impurity: Calculation: 2.0 eq of di-acyl intermediate

			Results: Di-acyl intermediate 20.27% As the strength of aq. NH_2OH was lower than 50% (~33%)
16.	Stirred the RM for 2-3 h at 15-20 °C		
17.	Sample submitted for HPLC analysis to check the content of di-acyl intermediate. **Sample preparation: Take 0.2 mL RM and diluted with 10 mL of water and submitted.**		Results: Di-acyl intermediate 2.69 %
18.	Distilled the total organic mass (2-MeTHF) under vacuum (NLT 600 mm of Hg) at 40-45 °C		Sticky semi solid mass observed
19.	Added MTBE (Lot-1) and distilled under same temperature still no distillate collected at the receiver.	1.8 L	Sticky semi solid mass observed
20.	Cooled the RM and Charged MTBE (Lot-2) at 25-30 °C	27.0 L	
21.	Stirred the RM for 16 h at 25-30 °C		
22.	Filtered the solid through nutsche filter and washed the wet solid with MTBE (Lot-3)	3.6 L	5-Micron filter cloth was used for filtration
23.	Again, wet solid washed with MTBE (Lot-4)	3.6 L	
24.	Suck dried the solid for 30 min at 25-30°C and then dried under vacuum (NLT 600 mm of Hg) at 45-50 °C for 4h till constant weight		Weight obtained: 1.905 kg
25.	Charged solid crude EIDD into 10 L RBF at 25-30 °C		CR592-15754-89
26.	Charged water (Lot-1) into reactor at 25-30 °C and stirred at 120 RPM	2.7 L	
27.	Increased the temperature of reaction mass at 60-65 °C		RM became homogeneous
28.	When RM inside temperature came under 45-50 °C precipitation was formed		RM slowly became heterogeneous as the product starts to precipitate

29.	Stopped the heating and slowly cooled the RM for 7-8 h to reach temperature 25-30 °C		
30.	RM stirred for 16 h at 25-30 °C		
31.	Further cooled the RM at 10-15 °C and stirred for 3 h		
32.	Filtered the solid at 10-15 °C through Buchner funnel and washed with ice-cold (10-15 °C) water (Lot-2)	0.9 L	5-Micron filter cloth was used for filtration
33.	Washed wet solid with ice-cold (10-15 °C) water (Lot-3)	0.9 L	
34.	Washed the solid with MTBE (Lot-5)	1.8 L	
35.	Suck dried the solid for 30 min at 25-30 °C		
36.	Charged purified EIDD into reactor (10 L RBF) at 25-30 °C		CR592-15754-89
37.	Charged water (Lot-4) into reactor at 25-30 °C and stirred at 120 RPM	2.7 L	
38.	Increased the RM temperature at 60-65 °C		RM became homogeneous
39.	When RM inside temperature came under 45-50 °C precipitation was formed		RM became solid
40.	Stopped the heating and slowly cooled the RM for 7-8 h to reach temperature 25-30 °C		
41.	Stirred the RM for 3 h at 25-30 °C		
42.	Further cooled the RM at 10-15 °C and stirred for 3.0 h		
43.	Filtered the solid at 10-15 °C through Buchner funnel and washed with ice-cold (10-15 °C) water (Lot-5)	0.9 L	5-Micron filter cloth was used for filtration
44.	Washed the wet solid again with ice-cold (10-15 °C) water (Lot-6)	0.9 L	
45.	Followed by washed the wet solid with MTBE (Lot-6)	1.8 L	
46.	Suck dried the solid for 1 h at 25-30 °C		
47.	Dried the wet solid under vacuum (NLT 600 mm of Hg) at 45-50 °C for 5-6 h till constant weight		Off-white solid Water content by KF 0.30%
48.	Unloaded the material Obtained wt: 1.337 kg		

	Theoretical yield: 2.1 kg Molar Yield: 62.53%		

*** Washing procedure of Novozym-435:**

To a clean and dry 2.0 L RBF and charged 2-MeTHF (0.9 L, 5 V) and Novozym-435 (180.0 g). RM stirred at 45-50 °C at 60-70 RPM for 3 h. After 3 h RM was allowed to cool at 25-30 °C and filtered Novozym-435 through Buchner funnel using 5-micron filter cloth, washed with hot 2-MeTHF (45-50 °C) (0.36 L, 2 V) and dried under vacuum for 2-3 h.

IPC-1 data of EIDD-2801 (1.8 kg batch):

S. No.	Batch Id	IPC-1 By HPLC			Analytical data
		NHC	EIDD	Di-acyl	
1	CR592-15754-89	3.39	70.30	20.27	IPCdata-step-2.pdf
2		IPC-2 By HPLC			
		4.22	87.33	2.69	
3		Water content by KF			
		0.30%			

Isolated data of EIDD-2801 (1.8 kg batch):

S. No.	Batch Id	Input (kg)	Isolated & Assay based Yield (%)	HPLC purity	KF	HPLC Assay	Complete Analysis data
1	CR592-15754-89	1.80 QNMR 99.88%	1.337 kg 61.85%	Purity: 99.75%, NHC·H_2O; 0.22%	0.3%	98.8% w/w	CR592-15754-89
The obtained EIDD was having few lumps which were crushed and sieved to afford homogeneous sample and it was sent for complete analysis and generation of Certificate of analysis as batch number CR592-15754-95							

Analysis data of EIDD-2801: 3 X 175.0 g batch

IPC-1 data of EIDD-2801: 3 X 175.0 g batch

S. No.	Batch Id	IPC-1 (HPLC) (%)			Analytical data
		NHC	EIDD	Di-acyl	
1	CR592-15754-83	3.29	71.52	20.02	CR592-15754-83
2	CR592-15754-84	4.43	70.75	19.40	CR592-15754-84
3	CR592-15754-85	3.32	70.86	20.01	CR592-15754-85

IPC-2 data of EIDD-2801: 3 X 175.0 g batch

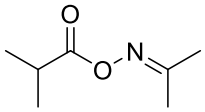
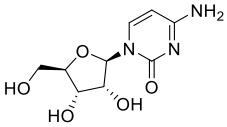
S. No.	Batch Id	IPC-2 (HPLC) (%)			Analytical data
		NHC	EIDD	Di-acyl	
1	CR592-15754-83	4.05	91.36	0.48	CR592-15754-83
2	CR592-15754-84	4.41	89.11	1.51	CR592-15754-84
3	CR592-15754-85	4.48	89.60	1.31	CR592-15754-85-RM2-1.pdf

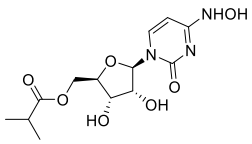
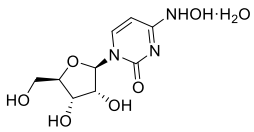
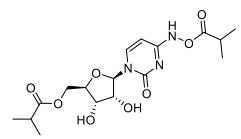
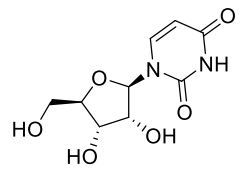
Isolated data of EIDD-2801: 3 X 175.0 g batch

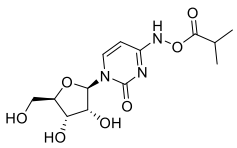
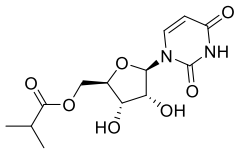
S. No.	Batch Id	Isolated (HPLC) (%)			Output (g)	Yield (%)	Moisture content by KF (%)	HPLC Assay (%)	Analytical data
		NHC·H_2O	EIDD	Di-acyl					
1	CR592-15754-83	0.35	99.61	-	140.0	67.14	0.08	99.0	CR592-15754-83

2	CR592-15754-84	0.36	99.59	0.01	136.5	65.46	0.21	99.2	CR592-15754-84
3	CR592-15754-85	0.31	99.65	-	136.0	65.22	0.07	99.0	CR592-15754-85

Characterization Data of all compounds:

Structure	^{1}H NMR	^{13}C NMR	LCMS	HPLC
 **Propan-2-one O-isobutyryl oxime M.Wt: 143.19**	(400 MHz, DMSO-d_6): δ 2.67-2.60 (m, 1H), 1.94 (d, J = 6.8 Hz, 6H), 1.13 (d, J = 6.8 Hz, 6H). 1H.pdf	(100 MHz, DMSO-d_6): 172.9, 164.2, 32.1, 21.1, 18.6, 16.2. 13C.pdf	LCMS m/z: $[M+H]^+$ Calcd for $C_7H_{14}NO_2$: 144.10; found: 144.24. LCMS.pdf	RT: 16.339 at 205 nm Isolated Purity: 96.53%. HPLC.pdf
 **Cytidine MWt: 243.22**	(400 MHz, DMSO-d_6): δ 7.83 (d, J = 7.6 Hz, 1H), 7.15 (d, J = 12.8 Hz, 2H), 5.76 (s, 1H), 5.70 (d, J = 7.2 Hz, 1H), 5.28 (d, J = 4.4 Hz, 1H), 5.03 (t, J = 5.2 Hz, 1H), 4.98 (d, J = 4.4 Hz, 1H), 3.92 (s, 2H), 3.80 (s, 1H), 3.65-3.62 (m, 1H), 3.54-3.50 (m, 1H). 1H.pdf	(100 MHz, DMSO-d_6): 165.6, 155.6, 141.6, 94.1, 89.2, 84.2, 74.0, 69.5, 60.7. 13C.pdf	LCMS m/z: $[M+H]^+$ Calcd for $C_{19}H_{16}N_3O_5$: 244.09; found: 244.27. LCMS.pdf	RT: 3.656 at 260.0 nm Isolated purity: 99.67%. HPLC.pdf

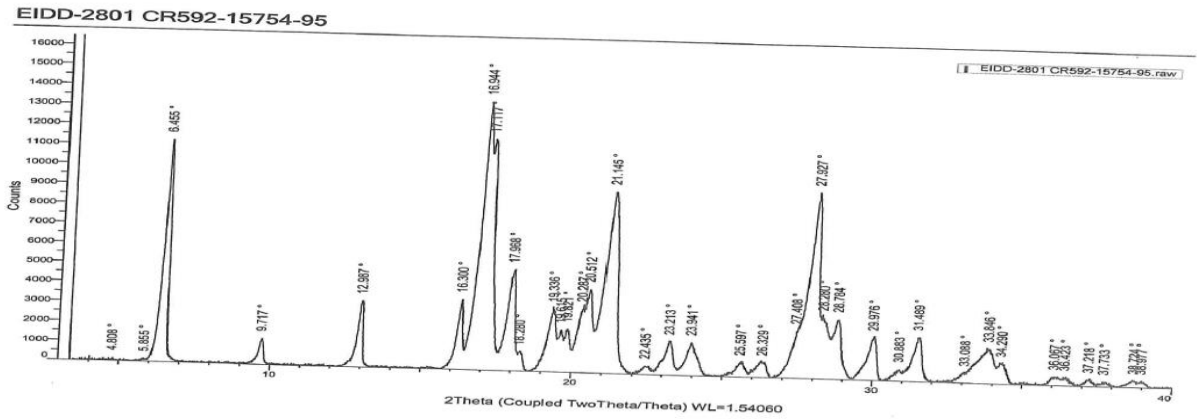
 **MK-4482/EIDD-2801** **Mwt: 329.31**	(400 MHz, D_2O): δ 6.99 (d, J = 8.0 Hz, 1H), 5.88 (d, J = 4.8 Hz, 1H), 5.78 (d, J = 8.0 Hz, 1H), 4.38-4.34 (m, 3H), 4.29 (t, J = 5.2 Hz, 2H), 2.72-2.69 (m, 1H), 1.20-1.18 (m, 6H). 1HNMR-D2O.pdf	(100 MHz, DMSO-d_6): 176.0, 149.5, 143.4, 129.9, 98.8, 87.8, 80.8, 72.2, 72.1, 70.0, 63.9, 33.2, 18.84, 18.81. 13C-DMSO.pdf	LCMS m/z: $[M+H]^+$ Calcd for $C_{13}H_{20}N_3O_7$: 330.13; found: 330.36. LCMS.pdf	RT: 13.565 at 260.0 nm Isolated purity: 99.75%. HPLC
 **1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-(hydroxyamino)pyrimidin-2(1H)-one NH-Cytidine hydrate** **MWt: 277.09**	(400 MHz, D_2O): δ 7.12 (d, J = 8.4 Hz, 1H), 5.92 (d, J = 5.6 Hz, 1H), 5.81 (d, J = 8.4 Hz, 1H), 4.36 (t, J = 5.6 Hz, 1H), 4.25 (t, J = 5.2 Hz, 1H), 4.13-4.10 (m, 1H), 3.91-3.87 (m, 1H), 3.83-3.78 (m, 1H). 1H NMR.pdf	(100 MHz, DMSO-d_6): 149.7, 143.5, 130.1, 98.5, 86.7, 84.5, 72.4, 70.3, 61.4. 13C.pdf	LCMS m/z: $[M+H]^+$ Calcd for $C_{19}H_{14}N_3O_6$: 260.09; found: 260.32. LCMS.pdf	RT: 3.367 at 260.0 nm Isolated purity: 99.05%. HPLC.pdf
 **((2R,3S,4R,5R)-3,4-dihydroxy-5-(4-((isobutyryloxy) amino)-2-oxopyrimidin-1(2*H*)-yl)tetrahydrofuran-2-yl)methyl isobutyrate** **MWt: 399.40**	(400 MHz, D_2O): δ 7.24 (d, J = 8.0 Hz, 1H), 5.87 (t, J = 4.4 Hz, 2H), 4.38-4.34 (m, 3H), 4.28-4.26 (m, 2H), 2.84-2.78 (m, 1H), 2.73-2.66 (m, 1H), 1.24-121 (m, 6H), 1.18-1.13 (m, 6H). 1HNMR	(100 MHz, DMSO-d_6): 175.8, 173.5, 149.2, 148.9, 133.9, 96.4, 87.9, 80.8, 72.1, 69.6, 63.6, 33.0, 31.5, 18.8, 18.6. 13C NMR	LCMS $(M+H^+)$ calcd for $C_{17}H_{26}N_3O_8$: 400.17; found: 400.10. LCMS	RT: 18.478 at 260.0 nm Isolated purity: 99.54%. HPLC
	(400 MHz, D2O): δ 7.91 (d, J = 8.0 Hz, 1H), 5.94 (d, J = 8.0 Hz, 2H), 4.39 (s, 1H), 4.27 (t, J = 5.2 Hz, 1H), 4.18 (s,	(100 MHz, DMSO-d_6): 163.1, 15.0.7, 140.7, 101.7,	LCMS $(M+H^+)$ calcd for $C_9H_{13}N_2O_6$: 245.08; found: 245.20.	RT: 4.186 at 260.0 nm Isolated purity: 99.93%.

Uridine **MWt: 244.07**	1H), 3.97-3.91 (m, 1H), 3.86-3.82 (m, 1H). 1H	87.7, 84.8, 73.5, 69.9, 60.8. 13C	LCMS	HPLC.pdf
 **1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-((isobutyryloxy)amino)pyrimidin-2(1*H*)-one** **MWt: 329.12**	(400 MHz, D_2O): δ 7.39 (d, J = 8.0 Hz, 1H), 5.92-5.87 (m, 2H), 4.35 (t, J = 5.2 Hz, 1H), 4.24 (t, J = 5.2 Hz, 1H), 4.12 (d, J = 3.2 Hz, 1H), 3.91-3.88 (m, 1H), 3.82-3.78 (m, 1H), 2.86-2.83 (m, 1H), 1.25 (d, J = 7.2 Hz, 6H). 1H NMR	(100 MHz, D_2O): 177.5, 150.4, 150.1, 134.7, 96.8, 88.3, 84.1, 73.0, 69.6, 60.9, 32.4, 18.2. 13C NMR	LCMS ($M+H^+$) calcd for $C_{13}H_{20}N_3O_7$: 330.13; found: 330.23. LCMS	RT: 13.320 at 260.0 nm Isolated purity: 98.79 %. HPLC
 **((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl isobutyrate** **M.Wt: 314.11**	(400 MHz, D_2O): δ 7.78 (d, J = 8.0 Hz, 1H), 5.95-5.91 (m, 2H), 4.45-4.40 (m, 3H), 4.35-4.30 (m, 2H), 2.76-2.69 (m, 1H), 1.30-1.19 (m, 6H). 1H NMR	(100 MHz, D_2O): 175.9, 162.9, 150.5, 140.6, 101.9, 88.7, 81.0, 72.7, 69.6, 63.5, 33.0, 18.7. 13C NMR	LCMS ($M-H^+$) calcd for $C_{13}H_{17}N_2O_7$: 313.10; found: 313.00. LCMS	RT: 14.369 at 260.0 nm Isolated purity: 94.33%. HPLC

EIDD-2801 synthesized was further characterized by powder XRD which is shown below.

Batch No. CR592-15754-95 (Scale up batch):

EIDD-2801-XRD Test results-TCG Life-09-0



PXRD data of 3-small scale batches of EIDD

CR592-15754-83	CR592-15754-84	CR592-15754-85
PXRD-83 Batch.pdf	PXRD-84 Batch.pdf	PXRD-85 Batch.pdf

Analytical Procedure

Cytidine

Chromatographic purity by HPLC:

Chemicals / Reagent references:

Preparation of Mobile Phase:

Mobile Phase A : 0.1 % OPA in water
Mobile Phase B : Acetonitrile:Water(90:10)v/v
Sample Diluent : WATER

Chromatographic conditions:

Column : XTERRA RP-18 (250X4.6)mm,5µ
Detection : 260 nm
Flow rate : 1.0 mL/min
Injection volume : 10 µL
Run time : 40.0 min
Column Oven Temperature: 35°C
Auto sampler temperature: Ambient

Gradient program:

Time (min)	Flow (ml/min)	A %	B %
0.0	1.0	98	2
5.0	1.0	98	2
25.0	1.0	20	80
30.0	1.0	20	80
31.0	1.0	98	2
40.0	1.0	98	2

Preparation of solutions:

Blank Solution : Diluent

Sample solution:

Accurately weigh about 25 mg of the sample to be examined in a 50 ml. volumetric flask. Dissolve with sufficient diluent, sonicate if necessary; and then make up to volume with diluent.

Procedure:

Inject separately: Blank and sample solution separately.

Record the chromatograms, disregarding peaks due to the blank

Oxime Ester

TEST PROCEDURE:

1) Description:
Take sufficient quantity of test sample in suitable apparatus (test tube/ petri dish) and observe visually against a black/white back ground in diffused light and observe the color and appearance. It should be a clear light yellow to yellow liquid free from extraneous matter.

2) Identification test by GC:
Record the retention times of major peaks of standard and sample obtained from the chromatograms for the related substances by GC. The retention time of major peak in sample should match with that of standard.

3) Water content by KF:
Take about 30-40 mL methanol in titration vessel of Karl Fischer Titrator. Neutralize with Karl Fischer reagent. Accurately transfer 5.0 mL of the sample and transfer immediately into the titration vessel and then titrate with Karl Fischer Reagent. Record the volume of Karl Fischer reagent consumed and calculate the water content of sample as given below.

$$\text{Water content of sample (\% w/v)} = \frac{\text{KFR Factor X Titer Value (mL) X 100}}{\text{Volume of sample (mL) X 1000}}$$

4) Related substances by GC:

Chemicals / Reagent references:
Propan-2-One-O-Isobutyryl Oxime OXIME ESTER standard

Chromatographic conditions:

Column	: DB-624 (30 m x 0.53 mm, 3.0 µm) (Part No: 125-1334)
ISO temperature-1	: 35° C
ISO time-1	: 3.0 minutes
Ramp-1	: 5° C/ minute
ISO temperature-2	: 180° C
ISO time-2	: 2.0 minutes
Ramp-2	: 20° C/ minute
ISO temperature-3	: 240° C
ISO time-3	: 8.0 minutes

Runtime	: 45 minute
Injection temperature	: 240° C
Detector temperature	: 260° C
Volume of injection	: 1.0 µL
Carrier gas	: Helium
Control Mode	: Constant Flow
Column flow	: 3.0 mL/minute
Septum purge flow	: 5.0 mL/min
Split flow	: 30.0 mL/min
Total flow	: 38.0 mL/min.
Split ratio	: 10:1
Hydrogen flow	: 30 mL/minute
Air flow	: 300 mL/minute
Make up flow	: 25 mL/minute

Diluent: Dichloromethane

Blank: Use Diluent

Preparation of standard solution:

Weigh accurately about 200 mg sample into a 10 mL volumetric flask, dissolve and dilute to volume with diluent.
[Concentration: 20000 ppm]

Preparation of sample solution:
Weigh accurately about 200 mg sample into a 10 mL volumetric flask, dissolve and dilute to volume with diluent.
[Concentration: 20000 ppm]

Procedure:
After equilibrating the column, separately inject air as blank and standard solution. If the system suitability criteria pass then inject sample solution as per the sequence given below.

Sequence table:

S. No.	Solution details	No. of injections
1.	Blank (Diluent)	1 (at least)
2.	Standard solution	1
3.	Sample solution	1
4.	Standard solution (Bracketing)	1

System suitability test:

a) No interfering peak should be observed at the retention time of Propan-2-One-O-Isobutyryl Oxime peak, and its known impurity peaks in blank.
b) For standard solution injection, for Propan-2-One-O-Isobutyryl Oxime peak the USP tailing factor should not be more than 2.0 and USP plate count should not be less than 10000.
c) For bracketing standard solution injection, for Propan-2-One-O-Isobutyryl Oxime peak the USP tailing factor should not more than 2.0 and USP plate count should not be less than 10000.

The retention time of Propan-2-One-O-Isobutyryl Oxime peak is about 27.4 min.

Reporting:

Disregard the peaks due to blank and the peaks having area less than 0.05%. Report the chromatographic purity of sample to one decimal point and content of all known impurities, single maximum unknown impurity with RRT and total impurities of sample to two decimal points if less than 1.0% and to one decimal point if equal to or greater than 1.0% by area normalization.

HPLC METHOD FOR OXIME ESTER

Preparation of Mobile Phase:

Mobile Phase A : 0.1 % OPA in water
Mobile Phase B : Acetonitrile:Water(90:10)v/v
Sample Diluent : Acetonitrile

Chromatographic conditions:

Column : XTERRA RP-18 (250X4.6) mm,5µ
Detection : 210 nm
Flow rate : 1.0 mL/min

Injection volume : 10 µL

Run time : 40.0 min

Gradient program:

Time (min)	Flow (ml/min)	A %	B %
0.0	1.0	98	2
5.0	1.0	98	2
25.0	1.0	20	80
30.0	1.0	20	80
31.0	1.0	98	2
40.0	1.0	98	2

Preparation of solutions:

Blank Solution : Diluent

Sample solution:

Accurately weigh about 25 mg of the sample to be examined in a 50 ml. volumetric flask. Dissolve with sufficient diluent, sonicate if necessary; and then make up to volume with diluent.

Procedure:

Inject separately: Blank and sample solution separately.

Record the chromatograms, disregarding peaks due to the blank.

N-Hydroxy Cytidine mono hydrate (NHC·H_2O)

HPLC METHOD FOR NHC·H_2O

Preparation of Mobile Phase:

Mobile Phase A : 0.1 % OPA in water
Mobile Phase B : Acetonitrile:Water(90:10)v/v
Sample Diluent : Water

Chromatographic conditions:

Column : XTERRA RP-18 (250X4.6) mm,5µ
Detection : 260 nm
Flow rate : 1.0 mL/min
Injection volume : 10 µL
Run time : 40.0 min
Column Oven Temperature: 35°C
Auto sampler temperature: Ambient

Gradient program:

Time (min)	Flow (ml/min)	A %	B %
0.0	1.0	98	2
5.0	1.0	98	2
25.0	1.0	20	80
30.0	1.0	20	80
31.0	1.0	98	2
40.0	1.0	98	2

Preparation of solutions:

Blank Solution **:** Diluent

Sample solution:

Accurately weigh about 25 mg of the sample to be examined in a 50 ml. volumetric flask. Dissolve with sufficient diluent, sonicate if necessary; and then make up to volume with diluent.

Procedure:

Inject separately: Blank and sample solution separately.

Record the chromatograms, disregarding peaks due to the blank.

HPLC ASSAY METHOD FOR NHC·H_2O
Preparation of Mobile Phase:

Mobile Phase A	:	0.1 % OPA in water
Mobile Phase B	:	Acetonitrile:Water(90:10)v/v
Sample Diluent	:	Water

Chromatographic conditions:

Column	: XTERRA RP-18 (250X4.6) mm,5µ
Detection	: 260 nm
Flow rate	: 1.0 mL/min
Injection volume	: 10 µL
Run time	: 10.0 min

Column Oven Temperature: 35°C
Auto sampler temperature: Ambient

Mobile Phase: 980 mL of Mobile Phase A and 20 mL of Mobile Phase B transferred into a 1 L bottle, mixed well, degassed by sonication.

Preparation of solutions:

Blank Solution **:** Diluent

Standard solution: Weigh accurately about 10 mg of the NHC standard in a 100 ml. volumetric flask. Dissolve with 50mL of diluent, sonicate if necessary; and then make up to volume with diluents, mixed well.

Sample solution: Weigh accurately about 10 mg of the sample in a 100 ml. volumetric flask. Dissolve with 50mL of diluent, sonicate if necessary; and then make up to volume with diluents, mixed well.

Procedure:

Procedure:

Inject separately: Blank, standard (duplicate) and sample solution separately.

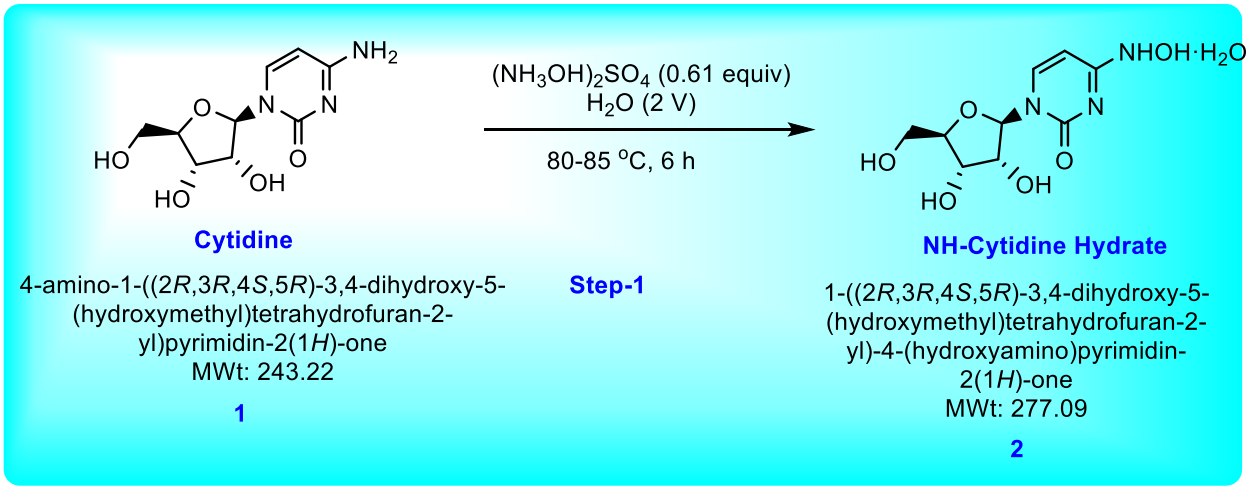
Record the chromatograms, disregarding peaks due to the blank.

METHOD of Analysis of EIDD-2801(MK-4482)

EIDD Method of Analysis.pdf

Results and discussion

Optimization of Step-1:



General protocol: Step-1 (Synthesis of NHC·H_2O)

Charged cytidine (1.0 eq.) and hydroxylamine sulfate followed by the addition of H_2O (2 V). Then, the RM was heated at 80-85 °C and stirred for 6 h. After completion of the reaction, checked by HPLC, RM allowed to slowly cool to ambient temperature (25-30 ºC) over the course of approximately 3 hours and further RM cooled at -5 °C and stirred for 3 h. The solid appeared was isolated by vacuum filtration and washed with ice-cold water (0.5 V X 2). The wet solid was dried u/v at 50-55 °C to obtain the off-white free solid.

Comparative data:

Batch No	Input (g)	Equiv of $(NH_3OH)_2SO_4$	Output (g)/ Yield (%)	Time (h)	IPC Data
CR592-15288-70	10.0	5.0	-	4.0	Cytidine: 0.89%; NHC: 81.37%; Uridine: 13.13%
CR592-15754-47	10.0	3.0	-	4.0	Cytidine: 0.62%; NHC: 80.86%; Uridine: 14.26%
CR592-15754-23	50.0	1.5	38.1 (67)	6.0	Cytidine: 1.81%; NHC: 79.13%; Uridine: 13.97%

CR592-16306-18	25.0	0.90	22.1 (78)	1.5	Cytidine: 3.0%; NHC: 92.82%; Uridine: 3.03%
CR592-15380-71	50.0	0.85	43.0 (75)	6.0	Cytidine: 1.71%; NHC: 91.08%; Uridine: 5.88%
CR592-15380-74	50.0	0.75	46.0 (81)	6.0	Cytidine: 8.68%; NHC: 87.02%; Uridine: 2.36%
CR592-15754-25	50.0	0.61	42.3 (74)		Cytidine (SM) 4.29%, NHC: 93.16%; Uridine: 1.86%
CR592-15288-77	50.0	0.50	-	10.0	Cytidine: 10.16%; NHC: 86.87%; Uridine: 2.1%

As the reaction performed well using 0.61 equiv hydroxylamine sulfate, it was further scaled up and optimized using different volume of H_2O and with respect to time. The reaction mass became heterogeneous after 3.0 h using 2.0 V H_2O at 65-70 °C so temperature increases to 80-85 °C. Details are shown below.

Batch No	**Input (g)**	**Eq. of $(NH_3OH)_2SO_4$**	**Output (g)/ Yield (%)**	**Time (h)**	**Water (V)**	**Data**
CR592-15754-28	100.0	0.61	90.0 (79)	5.5	2.0	IPC: Cytidine: 6.18%; NHC: 88.92%; Uridine: 3.58% Pure: NHC: 99.38%, Uridine: 0.40%
CR592-15754-29	100.0	0.61	90.5 (80)	6.0	2.0	IPC: Cytidine: 4.43%; NHC: 91.74%; Uridine: 2.79% Pure: NHC: 99.40%; Uridine: 0.45%

CR592-15754-30	100.0	0.61	90.0 (79)	5.0	2.0	IPC: Cytidine: 8.04%; NHC: 85.88%; Uridine: 4.21% Pure: NHC: 99.38%, Uridine: 0.43%; Cytidine: 0.13%
CR592-15754-35	250.0	0.61	235.0 (82.4)	6.0	2.0	IPC: Cytidine: 5.49%; NHC: 91.16%; Uridine: 2.28% Pure: NHC: 99.29%, Uridine: 0.65%
CR592-15380-59	250.0	0.61	232.0 (81.4)	6.0	2.0	IPC: Cytidine: 4.09%; NHC: 90.32%; Uridine: 3.71% Pure: NHC: 99.22%, Uridine: 0.49%
CR592-15380-62	250.0	0.61	230.5 (80.8)	6.0	2.0	IPC: Cytidine: 7.94%; NHC: 83.91%; Uridine: 4.87% Pure: NHC: 99.28%, Uridine: 0.55%
CR592-15754-37	250.0	0.61	230.0 (80.7)	6.0	1.5	IPC: Cytidine: 3.2%; NHC: 88.73%; Uridine: 6.1% Pure: NHC: 99.32%, Uridine: 0.68%

The reaction mass became heterogeneous after 3.0 h using 1.5 V H_2O even at 80-85 °C. Therefore, it was assumed that decreasing the hydroxylamine sulfate loading with less volume of water may enhance the reaction yield. Based on this assumption we have carried out more reactions as shown in below.

Batch No	**Input (g)**	**Equiv of $(NH_3OH)_2SO_4$**	**Output (g)/ Yield (%)**	**Time (h)**	**Water (V)**	**Data**
CR592-15288-82	50.0	0.50	-	6.0	1.5	IPC: Cytidine: 9.63%; NHC: 84.91%; Uridine: 3.69%
CR592-15288-83	50.0	0.50	-	6.0	1.0	IPC: Cytidine (SM): 12.86%; NHC: 79.66%; Uridine: 5.11%

The observation showed that the formation of uridine was more instead of consumption of more SM and formation of more NHC. Therefore, the optimized protocol was considered using 2.0 V H_2O and 0.61 equiv hydroxylamine sulfate at 80-85 °C for 6-7 h. More reactions were performed in the optimized conditions to check the further effect on scale-up.

Batch No	Input (g)	Equiv of $(NH_3OH)_2SO_4$	Output (g)/ Yield (%)	Time (h)	Water (V)	Data
CR592-16022-8	200.0	0.61	182.0 (79.9)	6.5	2.0	IPC: Cytidine: 5.58%; NHC: 90.74%; Uridine: 2.73% Isolated: Cytidine: 0.13%; NHC: 99.44%; Uridine: 0.42%
CR592-16022-9	200.0	0.61	183.0 (80.3)	7.0	2.0	IPC: Cytidine: 4.67%; NHC: 90.96%; Uridine: 3.43% Pure: Cytidine: 0.11%; NHC: 99.19%, Uridine: 0.70%
CR592-15754-82	200.0	0.61	182.0 (79.9)	6.5	2.0	IPC: Cytidine: 7.00%; NHC: 90.03%; Uridine: 2.05% Pure: Cytidine: 0.09%; NHC: 99.66%, Uridine: 0.21%
CR592-15754-87	2000.0	0.61	1830.0 (80.26)	6.5	2.0	IPC: Cytidine: 6.9%; NHC: 90.08%; Uridine: 2.06% Pure: Cytidine: 0.05%; NHC: 99.31%, Uridine: 0.65%

To push the complete consumption of Cytidine, top-up of 0.2 equiv hydroxylamine sulfate was used. But, top-up did not improve the consumption of cytidine but increased the formation of Uridine at the cost of NHC. Therefore, we have performed some crossover experiments to check whether cytidine (>99.5% purity) or NHC (>99.0% purity) which one was responsible for the formation of uridine under acidic conditions. The details are shown in below.

Batch No	Input (g)	Protocols	Data
CR592-15288-71	10.0	Cytidine (SM); H_2O (2.0 V); 0.61 equiv	IPC after 7 h: Cytidine: 6.13%; NHC: 80.98%; Uridine: 9.76%

		$(NH_3OH)_2SO_4$; 80-85 °C.	IPC after 2.0 h of first top-up (0.2 equiv): Cytidine: 4.39%; NHC: 72.44%; Uridine: 17.59% IPC after 2.0 h of second top-up (0.2 equiv): Cytidine: 3.34%; NHC: 65.10%; Uridine: 25.03%
CR592-15380-67	2.0	NHC (SM); 0.2 mL conc. H_2SO_4; H_2O (2 V); 80-85 °C	IPC after 16 h: NHC: 63.95%; Uridine: 34.79%
CR592-15380-63	2.0	Cytidine (SM); 0.2 mL conc. H_2SO_4; H_2O (2 V); 80-85 °C	IPC after 5 h: Cytidine: 97.40%; Uridine: 2.37%

From the reaction details it was cleared that NHC and cytidine both decomposes into uridine in acidic conditions. Therefore, it may be the reason that when large excess of hydroxylamine sulfate was used significant amount of uridine was formed in the reaction mixture. Hence we studied the effect of pH change of the reaction after addition of hydroxylamine sulfate.

Batch No	**Cytidine (g)**	**Output (g)/ %**	**$(NH_3OH)_2SO_4$ (eqv.)/Origin**	**pH**			
				Cytidine in 2 V H2O	$(NH_3OH)_2SO_4$ in 2 V H2O	RM at starting	RM after 6 h
CR592-15288-76	10.0	-	0.61 Avra	7.8	2.8	3.8	3.5
CR592-15288-80	10.0	-	0.61 Alfa-aesar	7.8	3.2	5.3	4.6

We have also carried out few reactions through control of pH. We have seen when initial pH was adjusted to 6.5 by adding K_2CO_3 in our optimized reaction conditions, thereafter pH increased to 8.4 at the end of 6 h and 70% cytidine was retained in the RM. Moreover, using saturated K_2CO_3 solution and through adjust the pH=5.5 for the course of 4 h, 97% NHC formation was observed. But yield was getting suppressed. A more detailed observation is shown in below.

Batch No	Cytidine (g)	Qty. of $(NH_3OH)_2SO_4$ (g/eqv.)	pH in Reaction Mass					Remark/Analytical data		
			Initial	2 h	4 h	6 h	8 h	Observation	NHC	Cytidine
CR592-15288-84	10.0	4.1 g **(Alfa-Aesar) 0.61**	6.51*	8.92	8.67	8.52	8.45	After 6 h	28.83	70.45
CR592-16036-9	100.0	67.5 g **(Avra) 1.0**	6.51**	5.5	5.5	-	-	After 4 h	97.05	1.14
								Isolated 70.0 g	99.90	0.04

*After adding all the reagents 1.13 g (0.2 eq) K_2CO_3 was added to take the pH >8

** pH was adjusted ~5.5 by titrating with saturated K_2CO_3 solution

Solubility data of NHC·H_2O:

Temperature (°C)	25	50
Solvent	**Concentration mg/mL**	
Water	14.5	80.0
MeOH	14.2	20.0
THF	4.7	8.2
MeCN	1.25	1.75
DMF	21.2	22.5
EtOH	6.5	11.4
IPA	3.75	5.5
IPAc	2.75	4.25

Purification of NHC·H_2O: Mass Balance on 2.0 kg scale

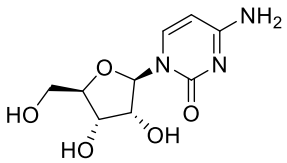
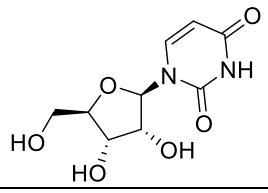
After completion of the reaction, allowed to slowly cool to ambient temperature (25-30 ºC) over the course of approximately 3 hours and further RM cooled at -5 °C and stirred for 3 h. The solid appeared was isolated by vacuum filtration and washed with ice-cold water (0.5 V X 2). The wet solid was dried u/v at 50-55 °C to obtain the off-white free solid.

Result:

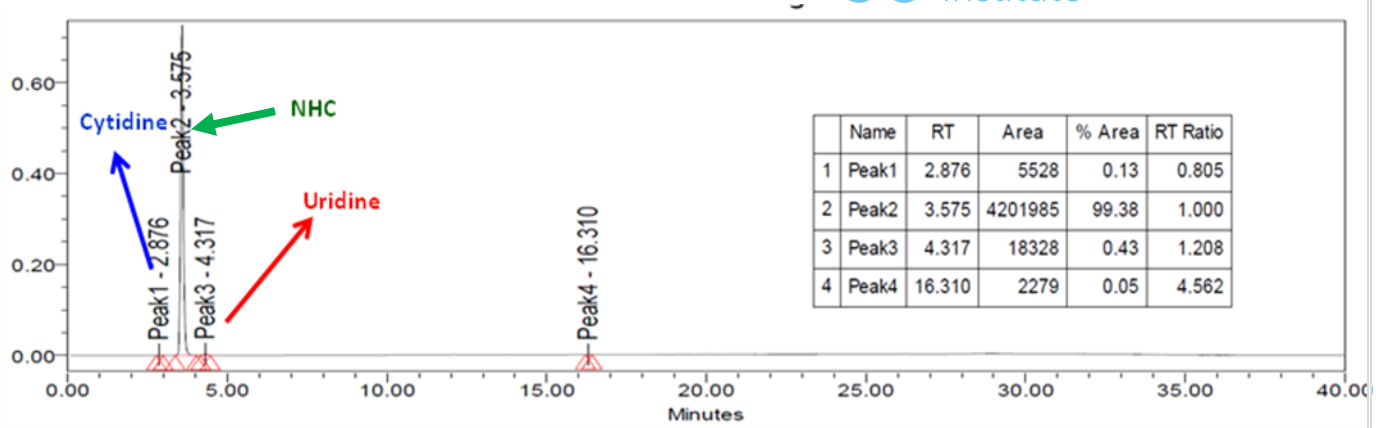
Batch No	Input (kg)	Output (kg)/ Yield	NHC in main aqueous MLR	NHC in 1st water washing (0.5 V/1.0 L)	NHC in 2nd water washing (0.5 V/1.0 L)

<table>
<tr><td rowspan="3">CR592-15754-87</td><td rowspan="3">2.0</td><td rowspan="3">1.83
Assay by HPLC 99.8%
(Y = 80.2%)</td><td colspan="3">Weight of MLR = 3.66 kg
Assay = 1.3%
47.5 g/2.23%</td><td colspan="3">Weight of MLR = 1.63 kg
Assay = 1.0%
16.3 g/0.76%</td><td colspan="3">Weight of MLR = 1.39 kg
Assay = 0.7%
9.7 g/0.45%</td></tr>
<tr><td>Cytidine</td><td>NHC</td><td>Uridine</td><td>Cytidine</td><td>NHC</td><td>Uridine</td><td>Cytidine</td><td>NHC</td><td>Uridine</td></tr>
<tr><td>1.28</td><td>97.30</td><td>1.20</td><td>0.07</td><td>99.53</td><td>0.40</td><td>0.07</td><td>99.50</td><td>0.44</td></tr>
<tr><td colspan="12">Total NHC lost in MLR: 3.44%
Reaction Yield = 80.2 + 3.44 = 83.64 %</td></tr>
</table>

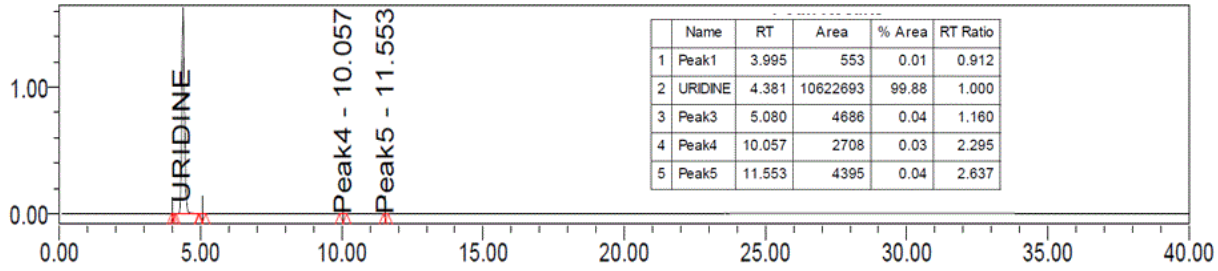
Potential impurities and their origin

Name	Structure	Origin
Cytidine		Process impurity/Un-reacted starting material
Uridine		Process impurity/hydrolyzed product

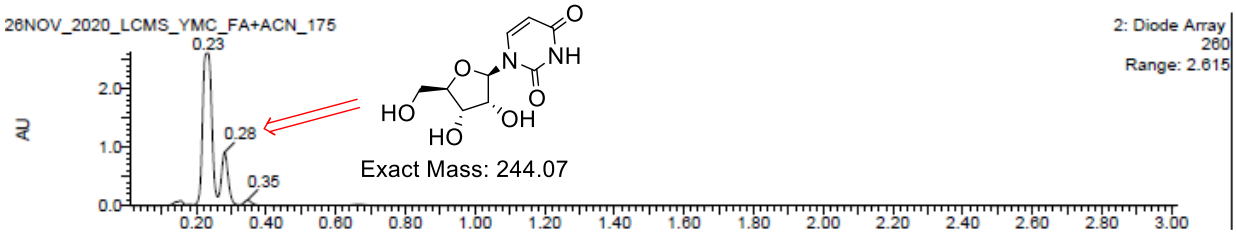
Uridine has been proposed based on the LCMS data.

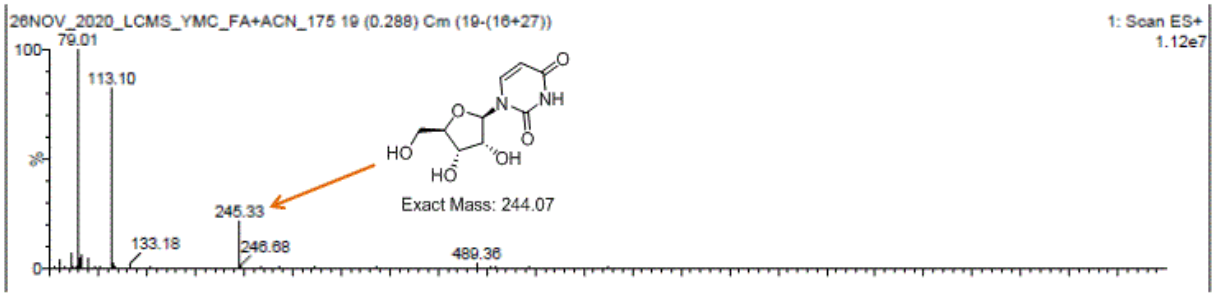


	Name	RT	Area	% Area	RT Ratio
1	Peak1	2.876	5528	0.13	0.805
2	Peak2	3.575	4201985	99.38	1.000
3	Peak3	4.317	18328	0.43	1.208
4	Peak4	16.310	2279	0.05	4.562

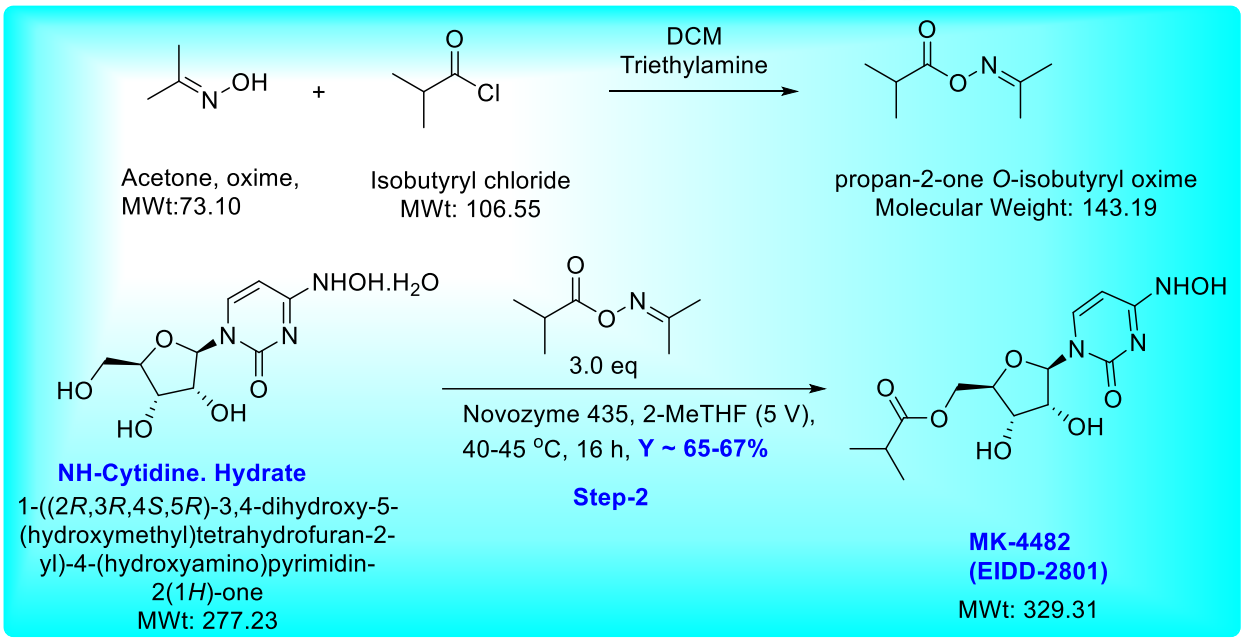


	Name	RT	Area	% Area	RT Ratio
1	Peak1	3.995	553	0.01	0.912
2	URIDINE	4.381	10622693	99.88	1.000
3	Peak3	5.080	4686	0.04	1.160
4	Peak4	10.057	2708	0.03	2.295
5	Peak5	11.553	4395	0.04	2.637





Optimization of Step-2:



General protocol: Synthesis of oxime ester

To a clean RBF, charged acetone oxime (1.0 eq.), DCM (12 V) and triethylamine (1.1 eq.). Then RM was cooled to -5 – 0 °C. Then, isobutyryl chloride (1.1 eq.) was added drop wise by maintaining an internal temperature below 0 °C. The RM was allowed to warm up to 20-25 °C and stirred for 16 h at the same temperature. The RM was washed with H_2O (2.5V), 1N HCl (2.5V), H_2O (2.5 V), saturated solution of $NaHCO_3$ (2.5V), H_2O (2.5V), and brine solution (2.5V). The organic layer was dried over anhydrous Na_2SO_4, evaporated under vacuum in rotavapor to give desired oxime ester as light-yellow oil.

Results:

Batch No	Input Acetoxime (g)	Output (g)/ Yield (%)	Purity (Area%) QNMR (%)	Remark
CR592-15365-40	100.0	192.0 (98)	91.33	KF by moisture content = 0.06% Colour grey **Based on the result another 1.0 kg batch was performed**
CR592-15885-3	1000.0	1840.0 (94.0)	93.20	KF = 0.08% Colour grey

CR592-15380-55	200.0	392.0 (97.61)	88.39 (98.30)	KF = 0.09% Colour grey
CR592-15754-38	500.0	959.8 (98.0)	90.95	KF = 0.09% Colour grey
CR592-15754-86	2000.0	3800.0 (86.5)	96.82 (89.21)	KF = 0.13 Colour grey
CR592-16036-46	500.0	960.0 (97.3)	99.3	KF = 0.04% Colour grey

General protocol: Step-2 (Synthesis of Molnupiravir/EIDD-2801/MK-4482)

To the clean RBF, charged NHC·H_2O (1.0 equiv), oxime ester (3.0 equiv), 2-MeTHF (5 V) and Novozym-435 (10 w/w%). The RM was stirred (~70 RPM) at 40-45 °C for 16 h U/N_2-atmosphere. After 16 h, heating was turned off and the RM was allowed to cool slowly at 25-30 °C for 3 h. The RM was filtered to separate enzyme and the enzyme was washed with 2-MeTHF (2 X 2 V). The combined organic layer was transferred to RBF and charged hydroxylamine 50 wt% in water (2.0 eq of di-acyl intermediate). The RM was stirred at 20-25 °C for 2 h and checked HPLC. Then solvent was distilled, charged with MTBE (15 V) and stirred at 20-25 °C for 5 h. The solid was filtered and washed with MTBE (2 X 2 V). Further wet solid was heated to 60-65 °C in water (1.5 V) to get clear solution, followed by cooling to 20-25 °C and stirred for 16 h at 20-25 °C. Next, cooled to 10-15 °C and hold at same temperature for 2.0 h. Filtered the solid and wet solid washed with chilled water (2 X 0.5 V), followed by MTBE (2 X 1.5 V) and suck dry for 3 h. Next the crude EIDD was dissolved in water (1.5 V) at 60-65 °C and hold for 30 min and cooled to 20-25 °C and stirred (~300 RPM) for 3h at 20-25 °C. Then, cooled to 10-15 °C and hold at same temperature for 2.0 h. Filtered the solid and wet solid washed with chilled water (2 X 0.5 V), followed by MTBE (2 X 1.5 V) and suck dry for 3 h and dried u/v at 50-55 °C to obtain the EIDD-2801 as off-white solid.

Screening of catalyst loading for the acylation reaction:

First, we started our reaction through optimization of Novozym-435 catalyst loading and their detailed study with the impurity profiling as shown in below reaction details it is cleared that the IPC-1 after 16 h data for 2-20% catalyst loading was almost same for smaller batch. Next we have carried out some scale up batch for more clarification about the catalyst loading and impurity profiling which is shown in below.

Batch No (CR592)	Batch Size (g)	Novozym-435 (w/w%)	Operation	NHC	RT 10.6	RT 13.5 *	EIDD	RT 14.7	RT 18.3	Di-acyl	RT 19.2	RT 22.4	RT 22.8
15380-94	50.0	1.0	16 h	21.25	-	9.23	34.44	0.64	0.49	31.51	0.52	0.34	0.47
15380-92	50.0	2.0	16 h	9.43	-	3.67	61.59	0.56	0.67	21.90	0.96	0.24	0.31
15754-57	50.0	5.0	16h	3.85	0.20	1.66	65.03	0.63	1.20	24.16	1.74	0.39	0.54
15754-83	***175.0***	***10.0***	***16 h***	***3.29***	***0.19***	***1.02***	***71.52***	***0.46***	***0.89***	***20.02***	***1.21***	***0.22***	***0.29***
15754-50	180.0	20.0	16 h	4.38	0.21	1.14	72.46	0.49	1.26	16.78	1.99	0.17	0.25

* RT 13.5: NHC-hydroxylamine acylated impurity

From above data it is concluded that 10% catalyst is suitable for further development. In that direction, few more batches ranging from 175.0 g to 1.8 kg were performed and results are shown below.

10 w/w% Novozym-435						
Batch No (CR592)	**Batch Size (g)**	**Operation**	**NHC**	**EIDD (%)**	**Di-acyl (%)**	**Isolated/ Yield (%)**
15754-83	175.0	16 h	3.29	71.52	20.02	140.0 g 67.14
		NH_2OH	4.05	91.36	0.48	
		Pure	0.35	99.61	-	
15754-84	175.0	16 h	4.43	70.75	19.40	136.5 g 65.46
		NH_2OH	4.41	89.11	1.51	
		Pure	0.36	99.59	0.01	
15754-85	175.0	16 h	3.32	70.86	20.01	136.0 g 65.22
		NH_2OH	4.48	89.60	1.31	
		Pure	0.31	99.65	-	
15754-89	1800.0	16 h	3.39	70.30	20.27	1337.0 g 62.53
		NH_2OH	4.22	87.33	2.69	
		Pure	0.22	99.75	-	

The above results show the identical yield and reaction profile for the batches using 10.0 w/w% catalysts loading. The scale up batch 1.8 kg using 10.0 w/w% catalysts proceeded well. Therefore, the catalyst for acylation reaction was optimized to 10.0 w/w% loading. Next, we turned our attention on the optimization of different solvent that is shown in the next table.

Screening of solvent for the acylation reaction:

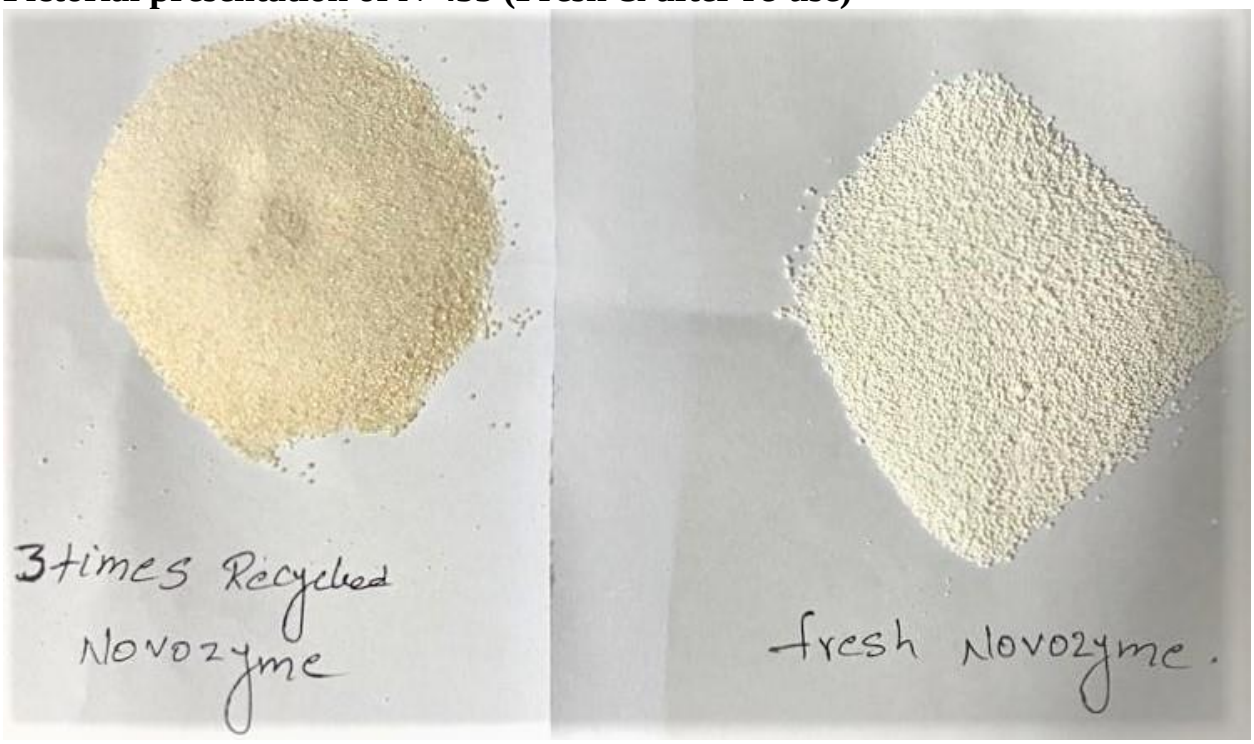
Batch No (CR592)	Batch Size (g)	Solvent (V)	Catalyst (w/w%)	NHC (%)	EIDD (%)	Di-acyl (%)
15380-40	5.0	1,4-Dioxane	200	9.02	78.55	4.16
15380-42	5.0	MeCN	20	15.81	50.01	26.09
15380-69	5.0	Acetone	20	10.82	69.24	5.99
15754-89	1800.0	2-MeTHF	10	3.39	70.30	20.27

2-MeTHF was found to be the best solvent to carry out the reaction on 10 w/w% catalyst loading. Next we focused on recovery of catalyst and their activity for the next subsequent cycles. Hence, we have carried out the reaction using 10 w/w% catalyst loading with top-up of 10% fresh enzyme. A comparison table for recycles batch is shown in the next table.

Recycling of the Novozym-435 catalyst:

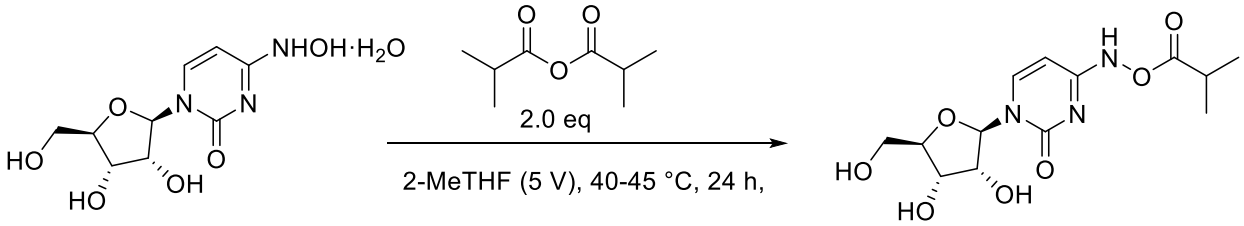
Batch No (CR592)	Batch Size (g)	Novozym-435 (w/w%)	Operation	NHC	RT 10.6	RT 13.7	EIDD	RT 14.7	RT 18.3	Di-acyl	RT 19.2
15380-57	50.0	90% used+ 10% top up 1st recycle	16 h	1.75	-	0.85	66.96	0.52	0.03	26.97	1.11
			NH_2OH	1.49	0.28	-	90.27	0.53	1.44	3.08	2.25
15380-58	50.0	90% used+ 10% top up 2nd recycle	16 h	2.26	0.34	0.92	68.40	0.57	0.83	23.67	1.15
			NH_2OH	2.24	0.05	-	91.63	0.54	2.02	-	2.55
15380-64	50.0	90% used+ 10% top up 3rd recycle	16 h	3.76	2.79	0.79	71.65	0.49	1.13	13.67	1.77
			NH_2OH	5.94	3.55	-	83.98	0.49	1.37	0.11	1.85

Pictorial presentation of N-435 (Fresh & after re-use)



This observation shows, Novozym-435 could be recycled three times but slowly it was transforming to powder like material and hence conversion to EIDD and diacylated EIDD was affected (94% vs 86%).

To monitor the back ground un-catalyzed reaction, this acylation reaction was performed without using Novozym-435 catalyst. But, the reaction was stalled after formation of mono-acylated intermediate. Next, we have synthesized the mono-acyl intermediate and characterized fully by LCMS, HPLC, and NMR. The details and analytical data are shown in below.



NH-Cytidine hydrate

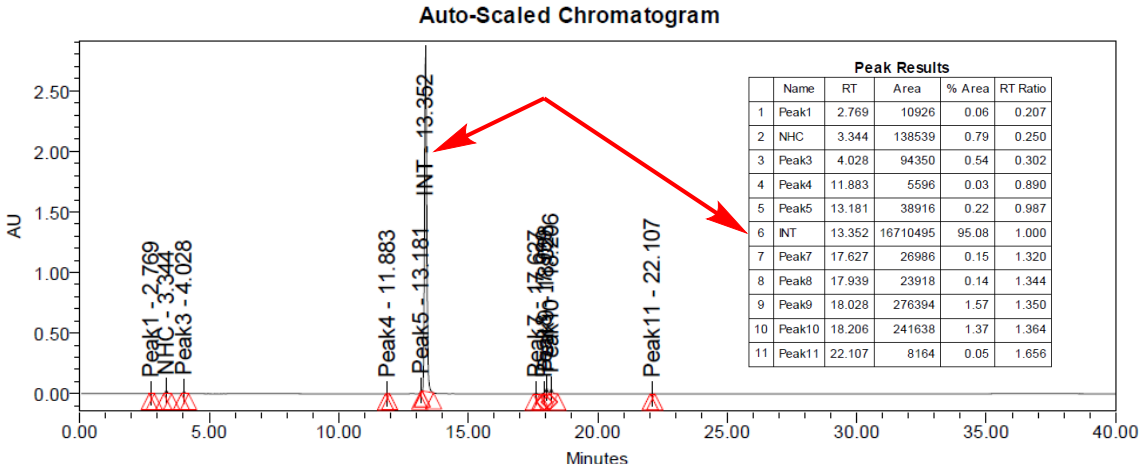
MWt: 277.23

Mono-acylated intermediate

M.Wt: 329.12

Protocol:

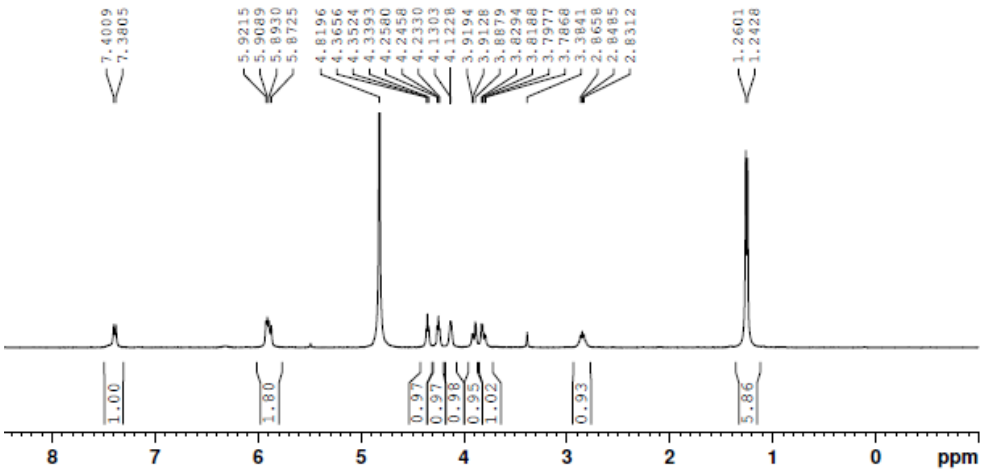
NHC·H_2O (10.0 g, 36.101 mmol) was added to a 100.0 mL clean and dry RBF followed by the addition of 2-MeTHF (5 V, 50.0 mL) and isobutyric anhydride (11.408 g, 72.202 mmol, 2.0 equiv). Then the reaction mixture was stirred continuously at 40 ºC for 24 h using oil bath and magnetic stirrer. Next performed column chromatography.



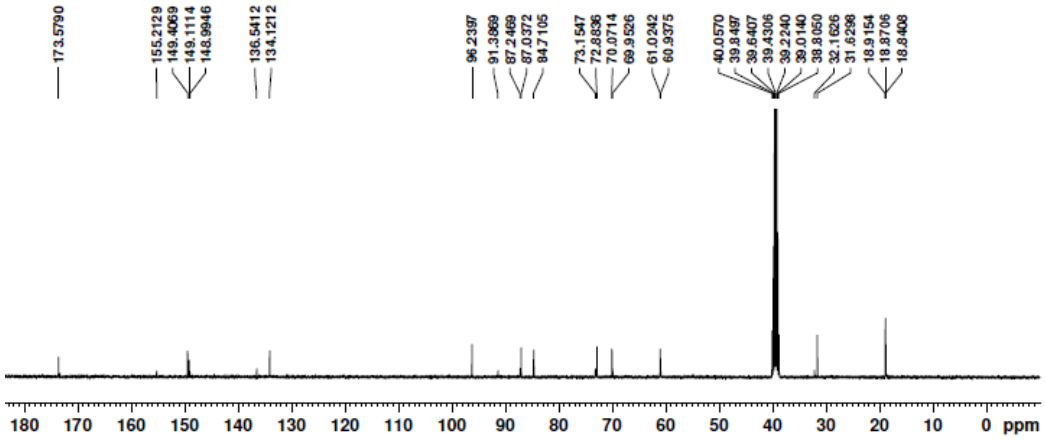
Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	2.769	10926	0.06	0.207
2	NHC	3.344	138539	0.79	0.250
3	Peak3	4.028	94350	0.54	0.302
4	Peak4	11.883	5596	0.03	0.890
5	Peak5	13.181	38916	0.22	0.987
6	INT	13.352	16710495	95.08	1.000
7	Peak7	17.627	26986	0.15	1.320
8	Peak8	17.939	23918	0.14	1.344
9	Peak9	18.028	276394	1.57	1.350
10	Peak10	18.206	241638	1.37	1.364
11	Peak11	22.107	8164	0.05	1.656

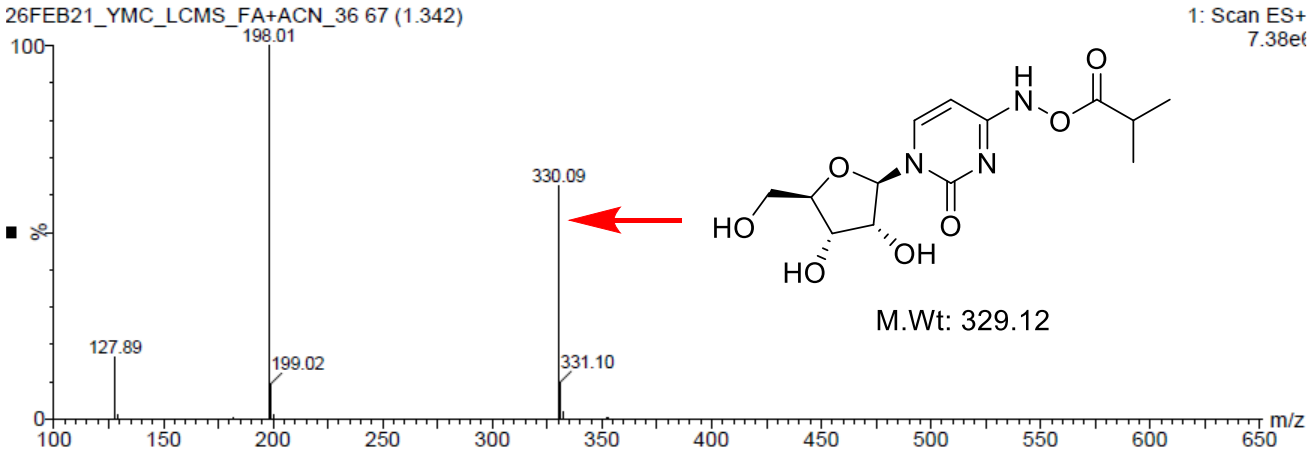
The 1H NMR spectra:



The 13C Spectra:

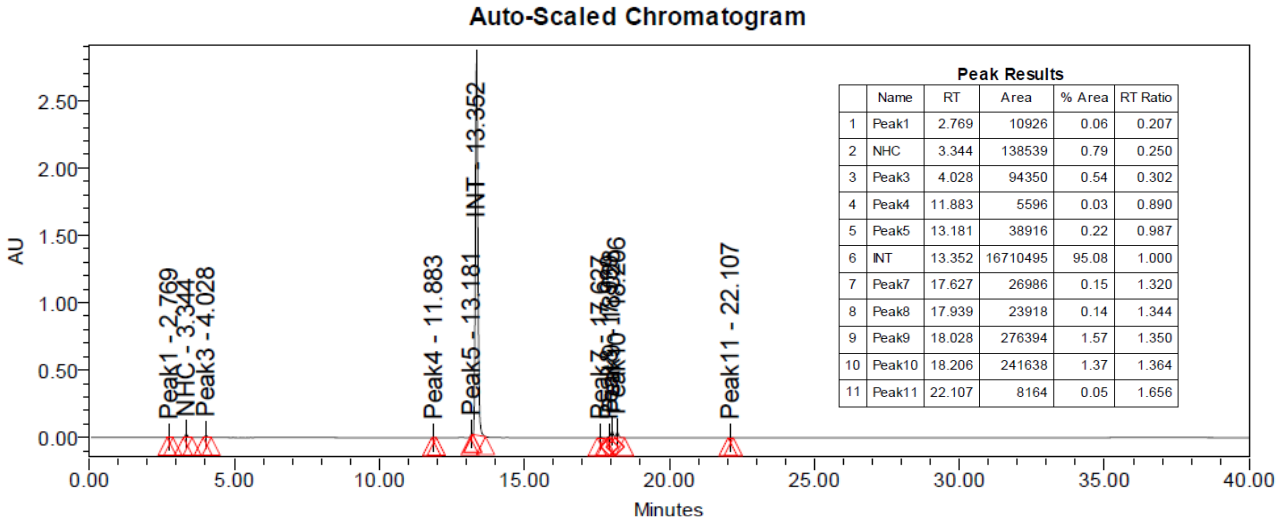


LCMS:



Further reactions of mono-acyl intermediate with 50% aq. NH_2OH solution the mono-acyl derivative completely transformed into NHC·H_2O. The reaction details are shown below.

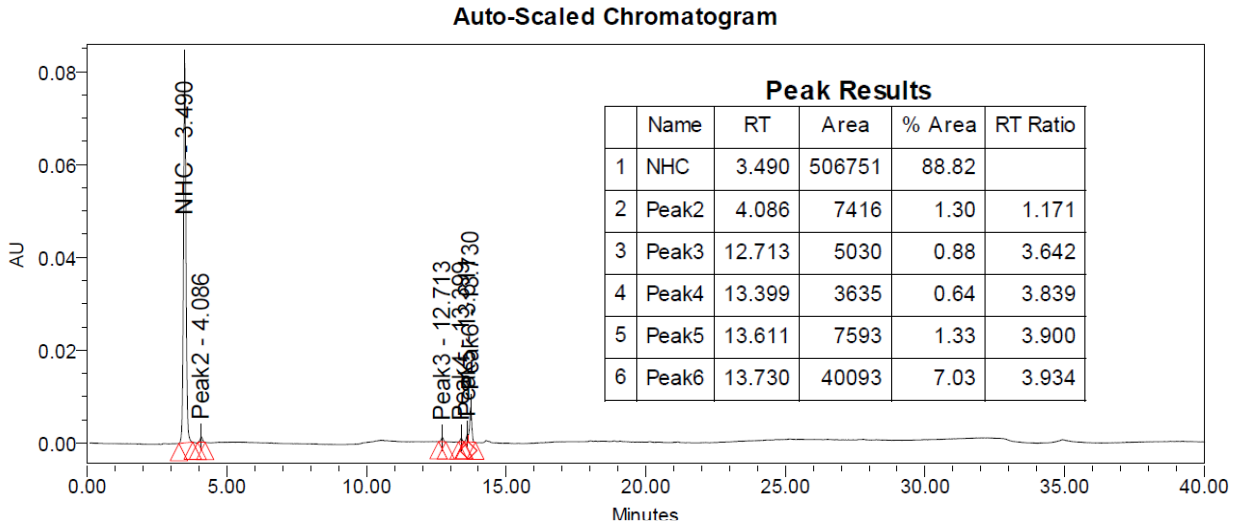
Before reaction:



Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	2.769	10926	0.06	0.207
2	NHC	3.344	138539	0.79	0.250
3	Peak3	4.028	94350	0.54	0.302
4	Peak4	11.883	5596	0.03	0.890
5	Peak5	13.181	38916	0.22	0.987
6	INT	13.352	16710495	95.08	1.000
7	Peak7	17.627	26986	0.15	1.320
8	Peak8	17.939	23918	0.14	1.344
9	Peak9	18.028	276394	1.57	1.350
10	Peak10	18.206	241638	1.37	1.364
11	Peak11	22.107	8164	0.05	1.656

After addition of 50% aq. NH_2OH solution:

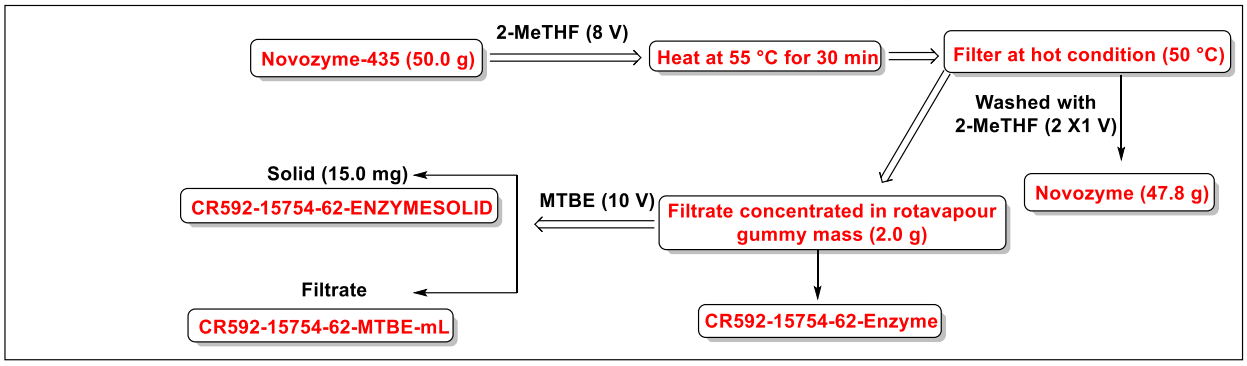


Peak Results

	Name	RT	Area	% Area	RT Ratio
1	NHC	3.490	506751	88.82	
2	Peak2	4.086	7416	1.30	1.171
3	Peak3	12.713	5030	0.88	3.642
4	Peak4	13.399	3635	0.64	3.839
5	Peak5	13.611	7593	1.33	3.900
6	Peak6	13.730	40093	7.03	3.934

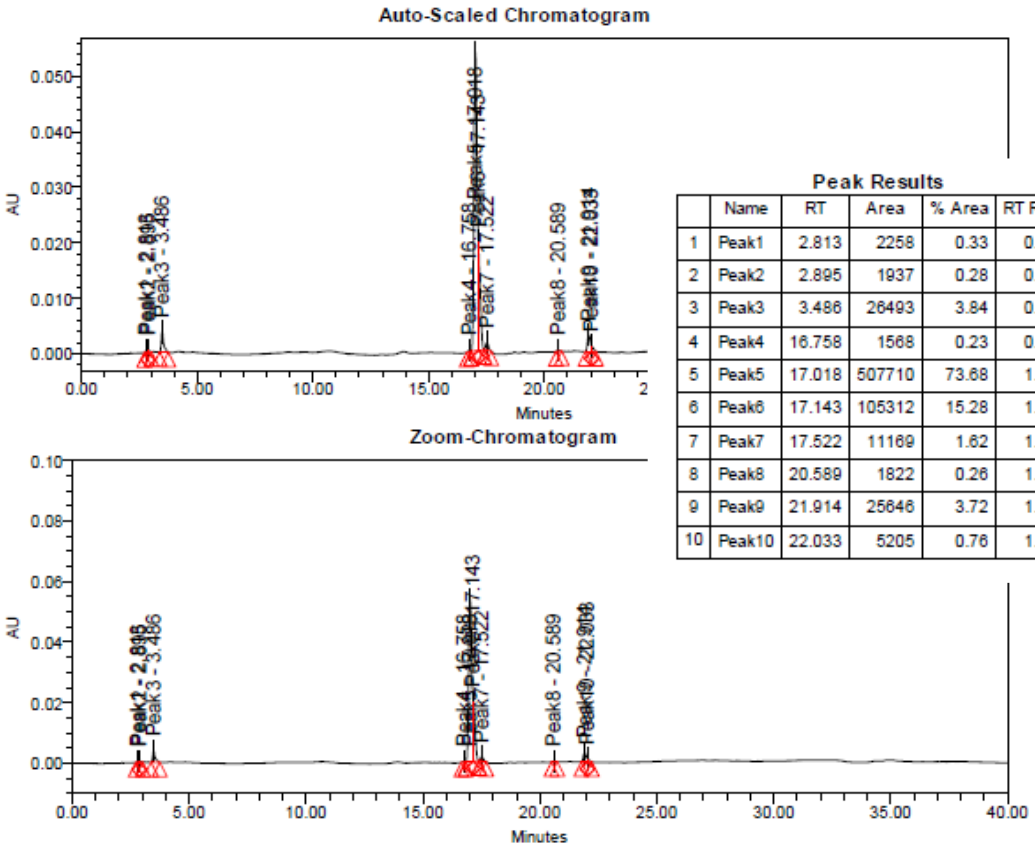
Novozym-435 washing:

As Novozym-435 is an immobilized lipase enzyme on a polymeric support, there is always possibility to leaching of active enzyme as well as support material to the reaction mass. So 50.0 g of N-435 was treated with reaction solvents and filtered to recover the supported catalyst. Organic solvents were evaporated to afford the leached material which was characterized by NMR, LCMS, and HPLC. Details are shown below. But it was observed that most of the leached material are purged off during MTBE washing.



SAMPLE INFORMATION

Sample Name:	CR592-15754-62-ENZYMESOLID	Acquired By:	ah0113531
Sample Type:	Unknown	Sample Set Name:	TCGLS_190221
Vial:	2	Acq. Method Set:	EIDD_2801_OPA
Injection #:	1	Processing Method	EIDD
Injection Volume:	10.00 ul	Channel Name:	260.0nm
Run Time:	40.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 260.0 nm (2998
Date Acquired:	19-02-2021 14:21:29 IST	Column Name:	XTERRA RP 18 (250X4.6)mm,5u
Date Processed:	20-02-2021 11:15:43 IST		



Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	2.813	2258	0.33	0.165
2	Peak2	2.895	1937	0.28	0.170
3	Peak3	3.486	26493	3.84	0.205
4	Peak4	16.758	1568	0.23	0.985
5	Peak5	17.018	507710	73.68	1.000
6	Peak6	17.143	105312	15.28	1.007
7	Peak7	17.522	11169	1.62	1.030
8	Peak8	20.589	1822	0.26	1.210
9	Peak9	21.914	25646	3.72	1.288
10	Peak10	22.033	5205	0.76	1.295

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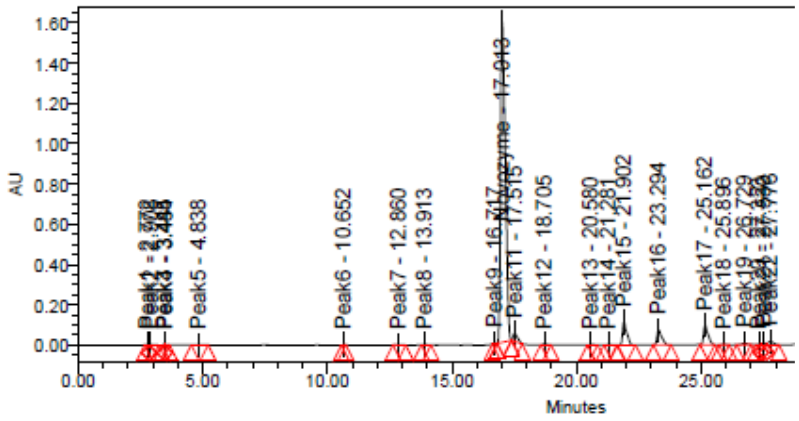
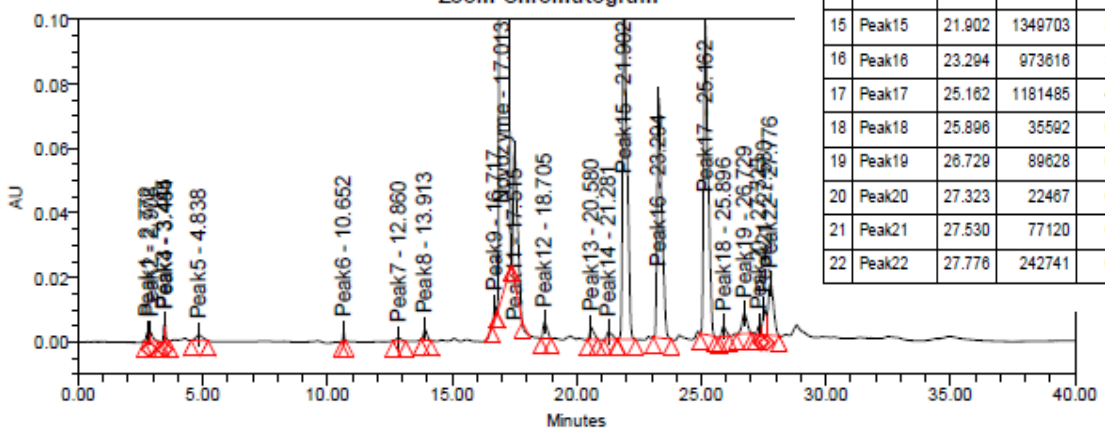
SAMPLE INFORMATION

Sample Name:	GR592-15754-62-Enzyme	Acquired By:	ah0113531
Sample Type:	Unknown	Sample Set Name:	TCGLS_180221
Vial:	15	Acq. Method Set:	EIDD_2801_OPA
Injection #:	1	Processing Method:	EIDD
Injection Volume:	10.00 ul	Channel Name:	260.0nm
Run Time:	40.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 260.0 nm (2998
Date Acquired:	18-02-2021 23:03:58 IST	Column Name:	XTERRA RP 18 (250X4.6)mm,5u
Date Processed:	19-02-2021 06:59:38 IST		



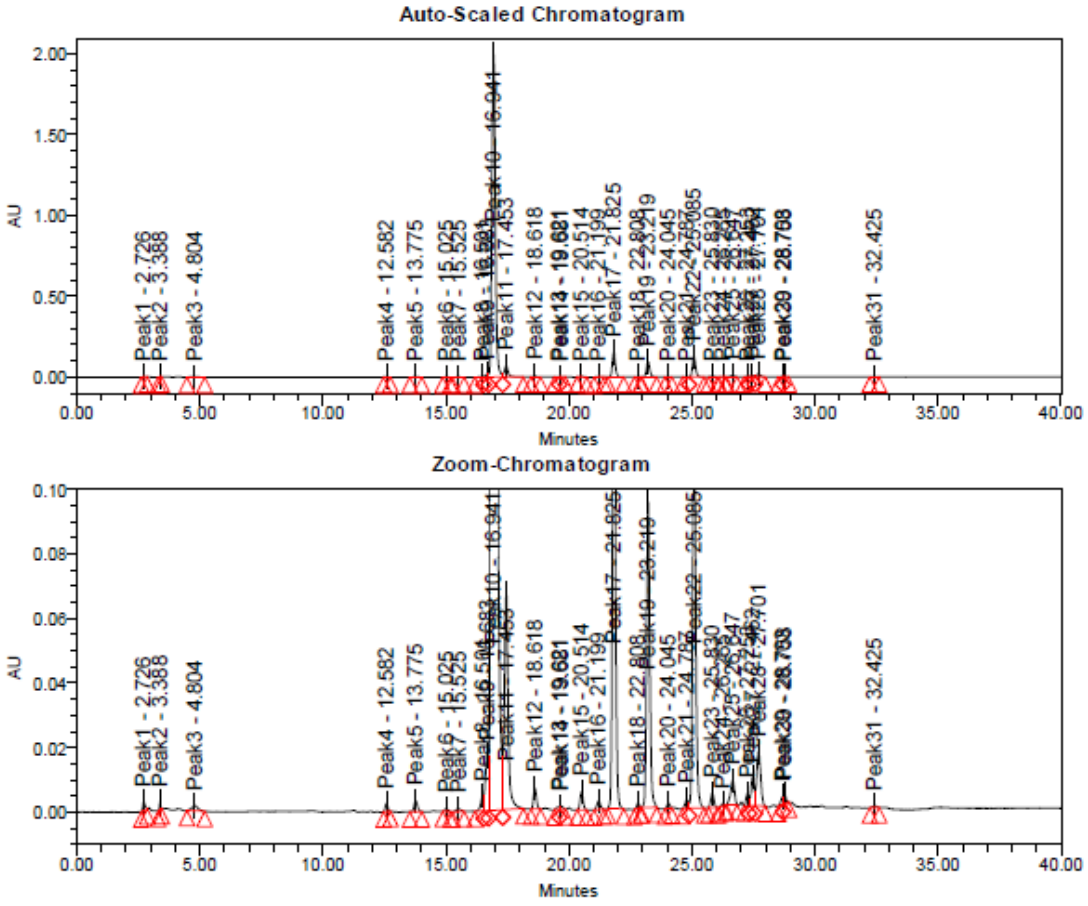


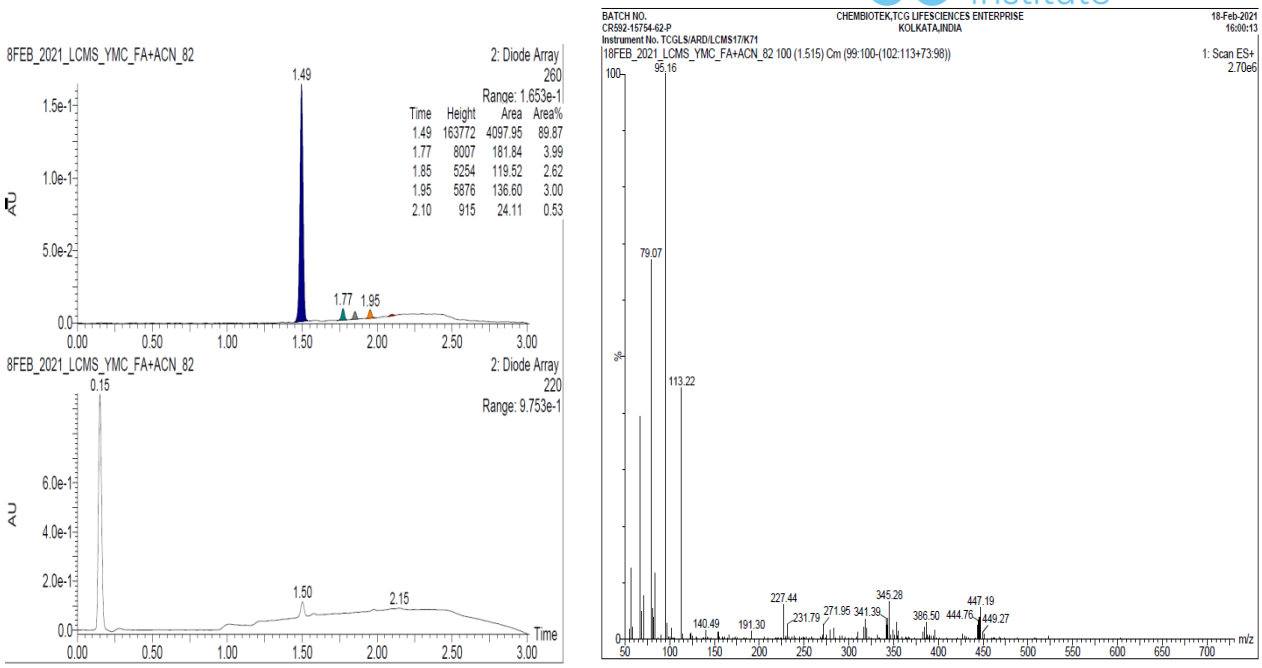
Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	2.772	19049	0.07	0.163
2	Peak2	2.908	25749	0.10	0.171
3	Peak3	3.445	15900	0.06	0.203
4	Peak4	3.484	15849	0.06	0.205
5	Peak5	4.838	32511	0.13	0.284
6	Peak6	10.652	8980	0.03	0.626
7	Peak7	12.860	14250	0.05	0.756
8	Peak8	13.913	30910	0.12	0.818
9	Peak9	16.717	22794	0.09	0.983
10	Novozyme	17.013	21221645	81.62	1.000
11	Peak11	17.515	486981	1.87	1.030
12	Peak12	18.705	57194	0.22	1.099
13	Peak13	20.580	40598	0.16	1.210
14	Peak14	21.281	34566	0.13	1.251
15	Peak15	21.902	1349703	5.19	1.287
16	Peak16	23.294	973616	3.74	1.369
17	Peak17	25.162	1181485	4.54	1.479
18	Peak18	25.896	35592	0.14	1.522
19	Peak19	26.729	89628	0.34	1.571
20	Peak20	27.323	22467	0.09	1.606
21	Peak21	27.530	77120	0.30	1.618
22	Peak22	27.776	242741	0.93	1.633

SAMPLE INFORMATION

Sample Name:	CR552-15754-62-MTBE-ML	Acquired By:	PG0112811
Sample Type:	Unknown	Sample Set Name:	TCGLS_190221
Vial:	16	Acq. Method Set:	EIDD_2801_OPA
Injection #:	1	Processing Method	EIDD
Injection Volume:	10.00 ul	Channel Name:	260.0nm
Run Time:	40.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 260.0 nm (2998
Date Acquired:	19-02-2021 22:21:56 IST	Column Name:	XTERRA RP 18 (250X4.6)mm,5u
Date Processed:	20-02-2021 10:14:19 IST		





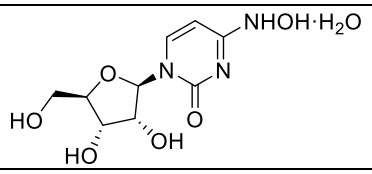
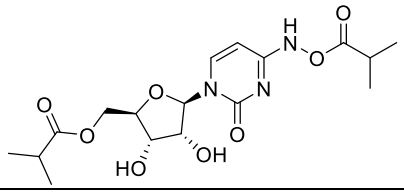
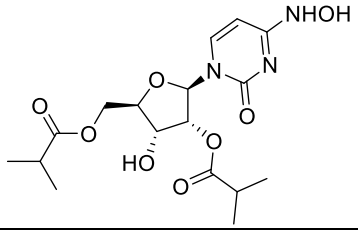
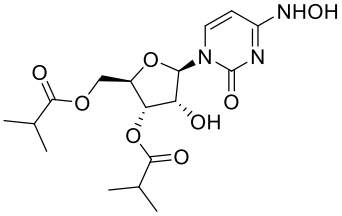
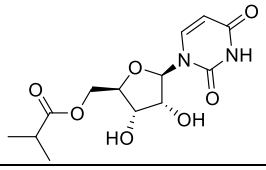
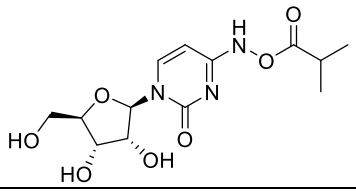
Hence N-435 was washed using 2-MeTHF and suck dried before using in any reaction.

Optimization of Oxime ester quantity:

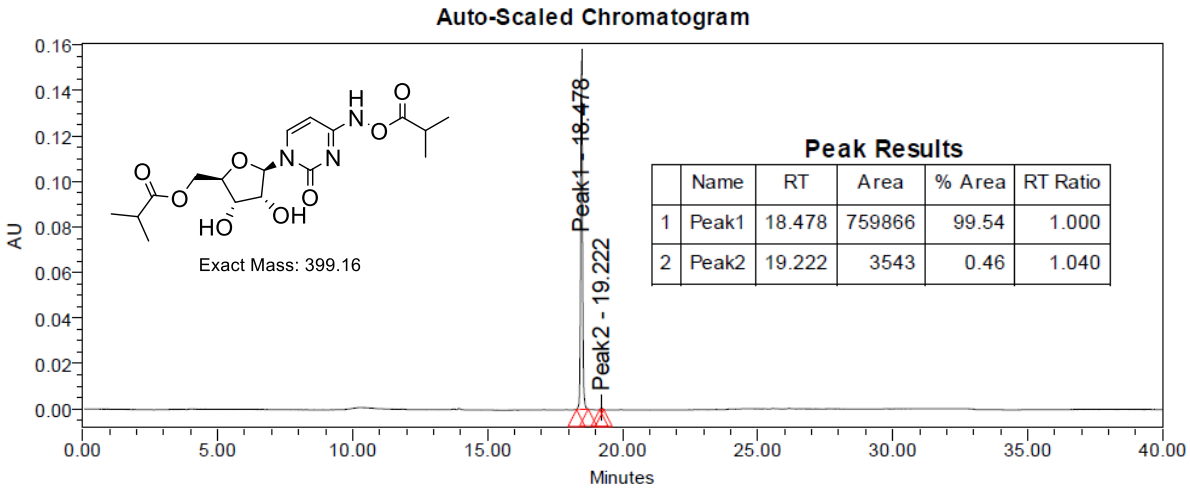
We have also tried the acylation reaction using less equivalent of oxime ester. However, the reaction did not proceed well in comparison to regular 3.0 equiv oxime ester. We have shown the details in below.

Batch No (CR592)	Batch Size (g)	Oxime ester (equiv)	IPC after 16h		
			NHC (%)	EIDD (%)	Di-acyl (%)
15288-75	10.0	1.5	57.23	38.03	1.21
15754-89	1800.0	3.0	3.39	70.30	20.27

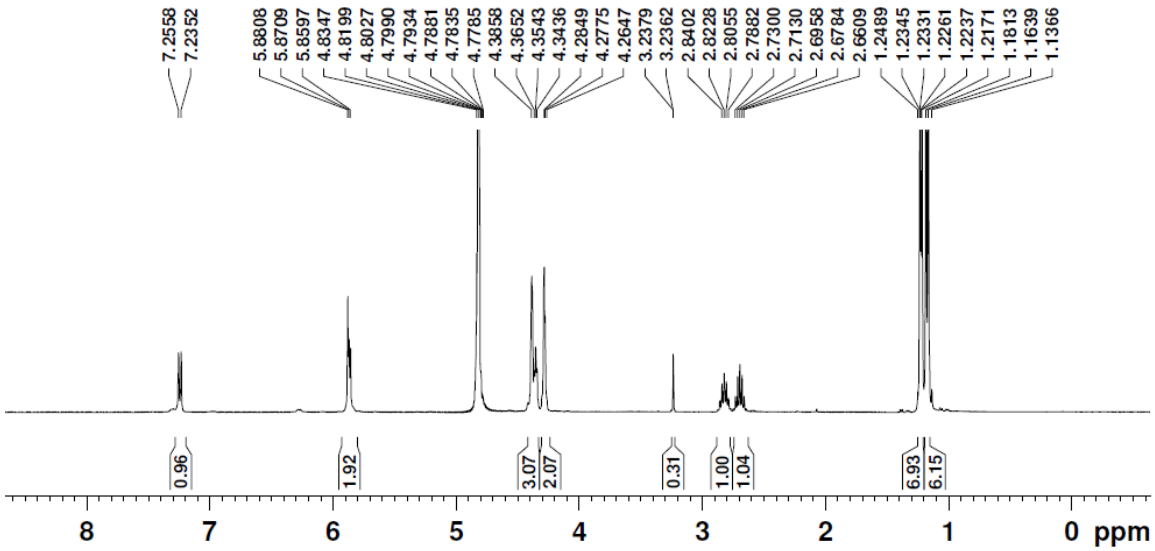
Potential impurities and their origin:

Name	Structure	Origin
NHC·H_2O		Process impurity/Un-reacted starting material
Di-acyl intermediate (1)		Process impurity/O-acylated product
Di-acyl intermediate (2)		Process impurity/O-acylated product
Di-acyl intermediate (3)		Process impurity/O-acylated product
Uridine acylation		Process impurity from carry forward Uridine in NHC
Mono-acyl intermediate		Process impurity/O-acylated product

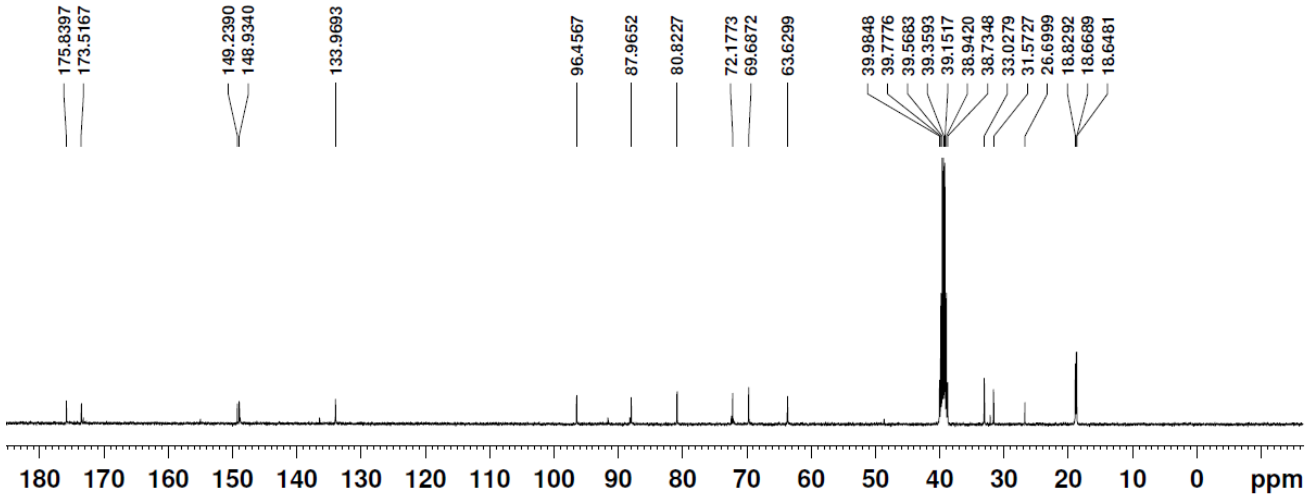
Di-acyl intermediate (1):



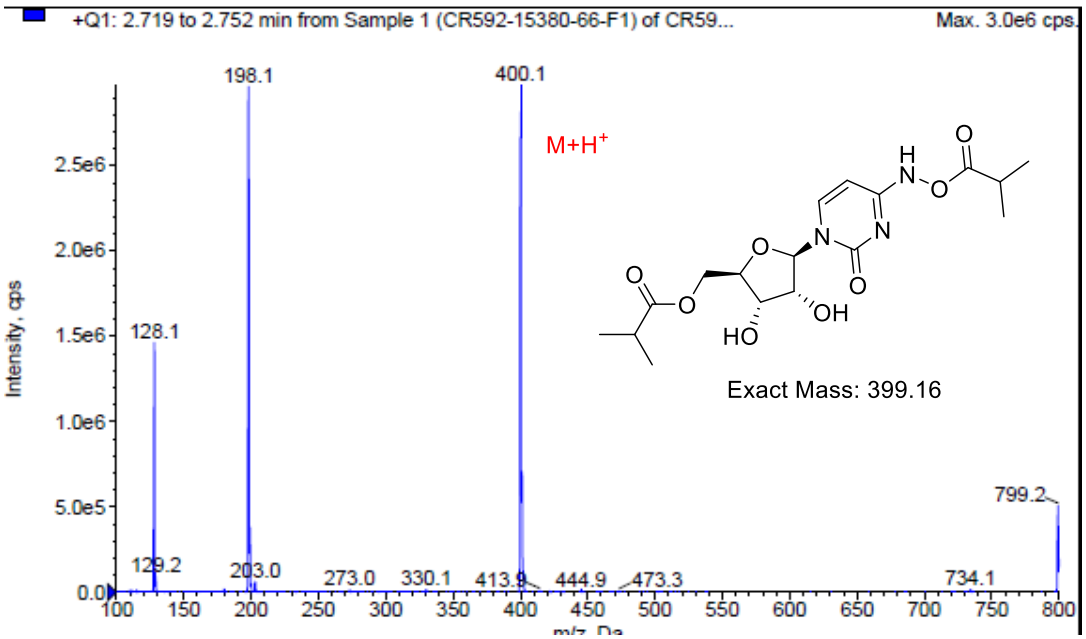
1H NMR:



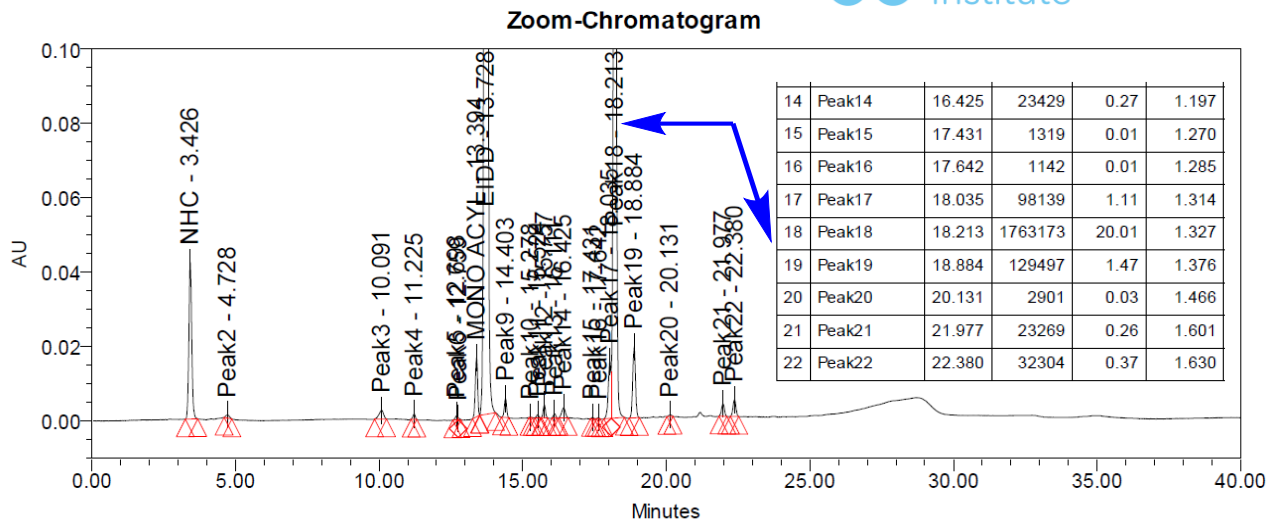
13C NMR:



LCMS:

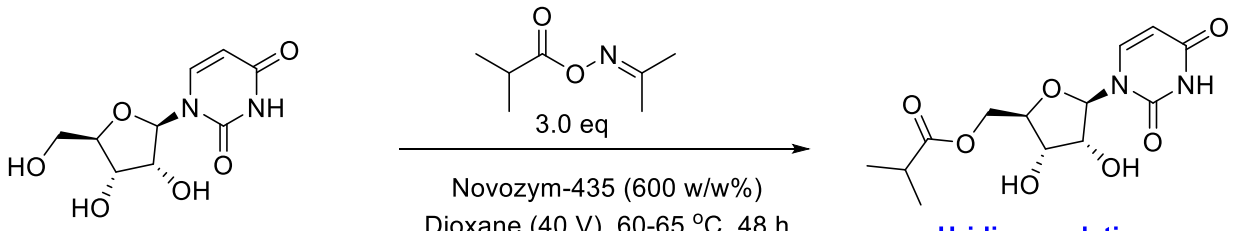


Identification of Di-acyl intermediate in the reaction mass:



14	Peak14	16.425	23429	0.27	1.197
15	Peak15	17.431	1319	0.01	1.270
16	Peak16	17.642	1142	0.01	1.285
17	Peak17	18.035	98139	1.11	1.314
18	Peak18	18.213	1763173	20.01	1.327
19	Peak19	18.884	129497	1.47	1.376
20	Peak20	20.131	2901	0.03	1.466
21	Peak21	21.977	23269	0.26	1.601
22	Peak22	22.380	32304	0.37	1.630

Uridine acylation:



Uridine
Exact Mass: 244.07

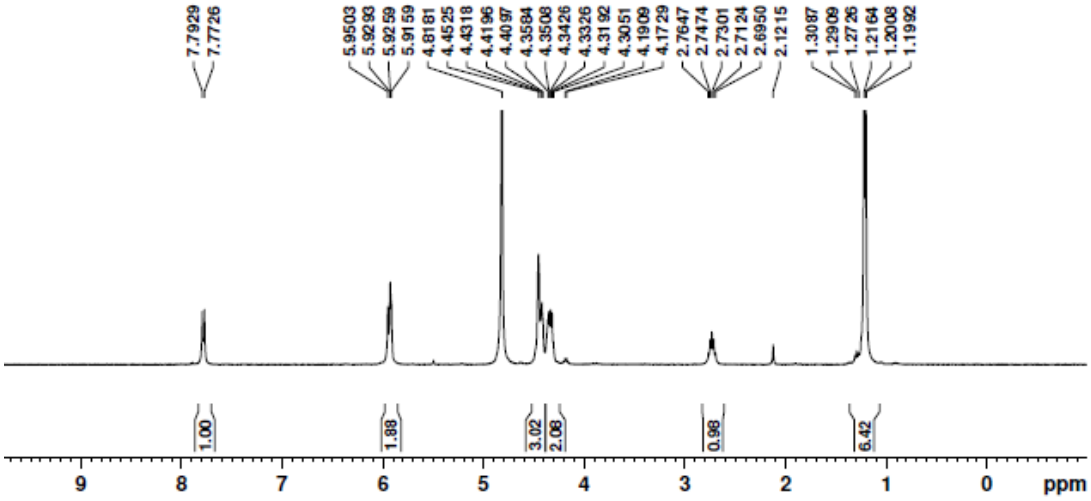
Uridine acylation
Exact Mass: 314.11

Protocol:

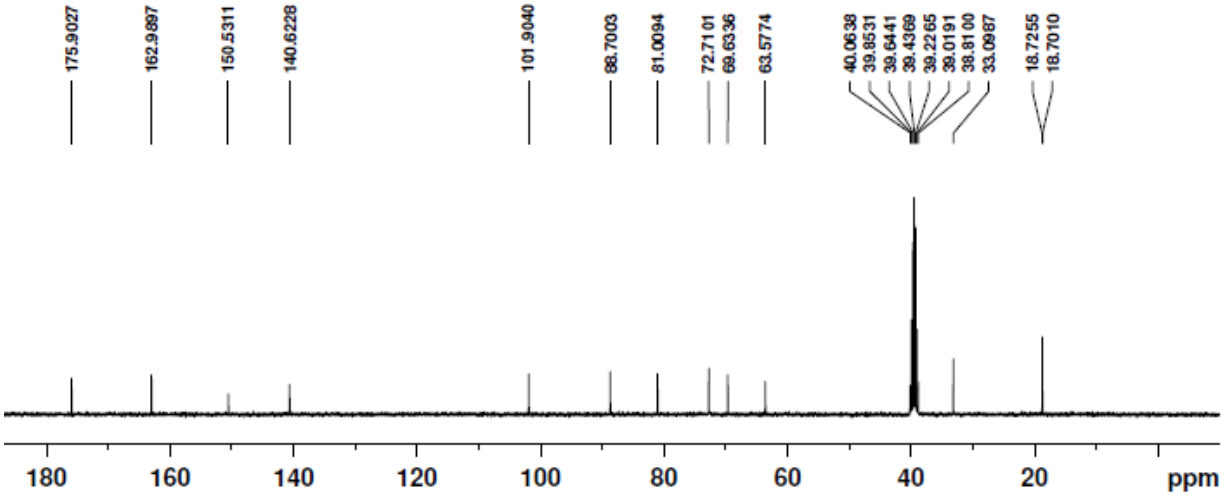
To a suspension of uridine (2.0 g, 8.197 mmol) in 1,4-dioxane (80 V, 40 mL) was added oxime ester (3.5 g, 24.5 mmol), and Novozym-435 (600 w/w%, 12.0 g). The reaction mixture was then stirred at 60 °C for 48 h. The mixture was then filtered to remove enzymes and washed with 1,4-dioxane (2 X 1V). Then distilled 1,4-dioxane and performed column chromatography.

Ref: *Macromolecules* **1999**, *32*, 8725-8731.

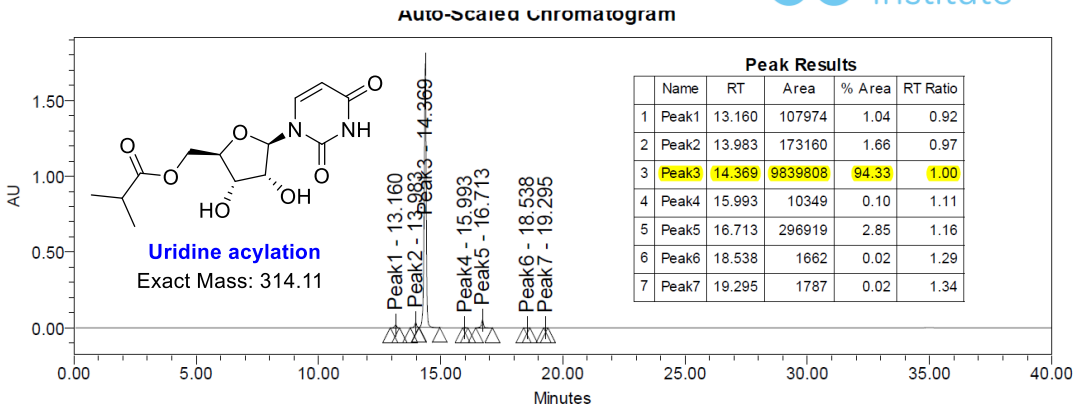
1H NMR:


7.7929
7.7726
5.9503
5.9293
5.9259
5.9159
4.8181
4.4525
4.4318
4.4196
4.4097
4.3584
4.3508
4.3426
4.3326
4.3192
4.3051
4.1909
4.1729
2.7647
2.7474
2.7301
2.7124
2.6950
2.1215
1.3087
1.2909
1.2726
1.2164
1.2008
1.1992
1.00
1.88
3.02
2.06
0.98
6.42
9
8
7
6
5
4
3
2
1
0
ppm

13C NMR:


175.9027
162.9897
150.5311
140.6228
101.9040
88.7003
81.0094
72.7101
69.6336
63.5774
40.0638
39.8531
39.6441
39.4369
39.2265
39.0191
38.8100
33.0987
18.7255
18.7010
180
160
140
120
100
80
60
40
20
ppm

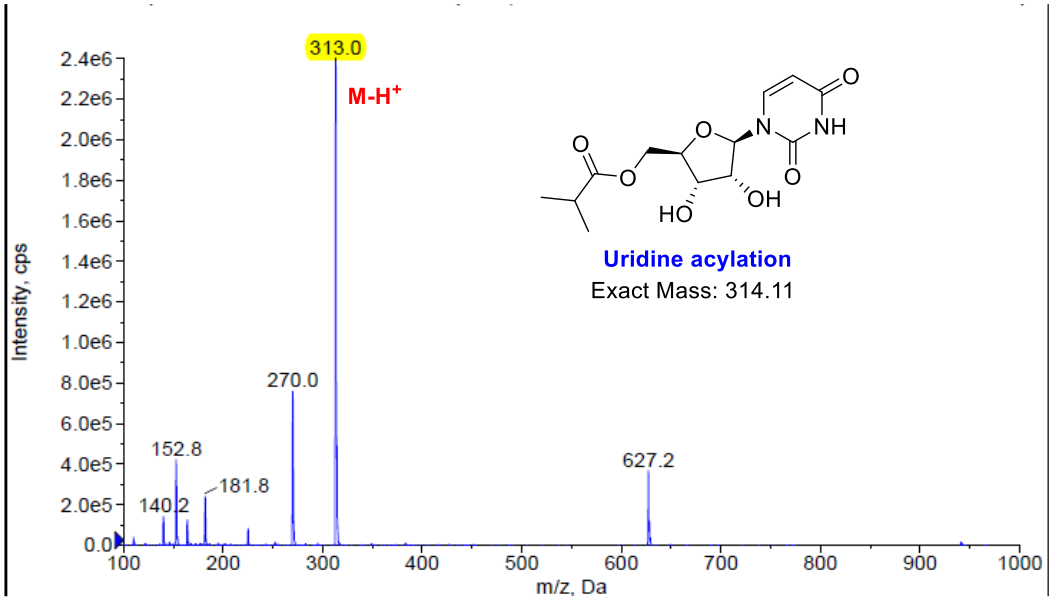
HPLC:



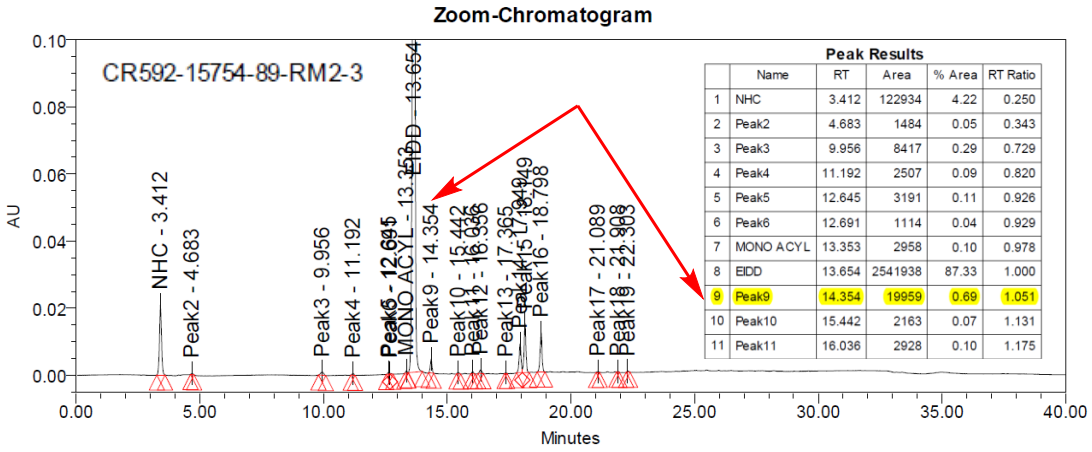
Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	13.160	107974	1.04	0.92
2	Peak2	13.983	173160	1.66	0.97
3	Peak3	14.369	9839808	94.33	1.00
4	Peak4	15.993	10349	0.10	1.11
5	Peak5	16.713	296919	2.85	1.16
6	Peak6	18.538	1662	0.02	1.29
7	Peak7	19.295	1787	0.02	1.34

LCMS:



Identification of uridine acylation in the reaction mass (1.8 Kg batch):



Alternate of 50% aq NH_2OH solution:

We have carried out a few reactions taking the 2-MeTHF containing stage-2 reaction mixture and added different quantity of base to convert the di-acylated intermediate into EIDD. The details are shown in the next table.

Batch No	Using base	Input purity	Remark/Analytical data
CR592-15754-58	$NH_2OH{\cdot}HCl$	NHC: 4.14%; EIDD: 51.27%; Di-acyl: 38.85%	EIDD: 56.56%; NHC: 2.99; Di-acyl: 35.47%
CR592-15380-83	$(NH_3OH)_2SO_4$		EIDD: 53.65%; NHC: 3.03; Di-acyl: 37.24%
CR592-15380-84	$NH_4CH_3CO_2$		EIDD: 84.91%; NHC: 6.1; Di-acyl: 2.49%
CR592-15380-85	NH_4Cl		EIDD: 52.65%; NHC: 3.53; Di-acyl: 36.54%
CR592-15380-86	$NH_3(Aq)$		EIDD: 85.57%; NHC: 7.8; Di-acyl: 2.03%

Remark: Ammonium acetate and aq. ammonia worked in the similar way to NH_2OH solution but after distillation of 2-MeTHF layer free solid EIDD not appeared that makes it difficult in purification of molnupiravir.

Optimization of other organic solvents after distillation of 2-MeTHF instead of MTBE:

Protocol: Taken stage-2 reaction mass after complete distillation of 2-MeTHF and added 15 V of solvent and stirred for 15 h at 25-30 °C. Next it was filtered, washed with the same solvent and dried u/vacuum at 45-50 °C.

Batch No	In put (g)	Solvent	Output (g)	Remark/Analytical data
CR592-15754-74	CR592-15754-72 (NHC:-5.91% EIDD:-85.06% Di-acyl:-02.89% SMI:-2.15)	EtOAc	21	NHC: 4.57%; EIDD: 84.34%; Di-acyl: 3.74%; SMI: 2.65% & 2.05%
CR592-15754-75		IPAc	21.5	NHC: 4.51%; EIDD: 85.72%; Di-acyl: 2.59%; SMI: 2.77% & 1.81%
CR592-15754-76		$^{i}Pr_2O$	22	NHC: 5.58%; EIDD: 92.58%; Di-acyl: 0.33%; SMI: 0.31% & 0.23%

Remarks: This observation shows $^{i}Pr_2O$ is the best choice among EtOAc and IPAc as all the non-polar impurities were purge out through the washing of solvents. But the data of $^{i}Pr_2O$ and MTBE are identical. Therefore we have used MTBE as an optimized solvent for this operation.

Solubility of EIDD-2801:

We have also checked the solubility of EIDD in different solvent as shown in the next table.

Temperature (°C)	5	10	25
Solvent	Concentration mg/mL		
Water	36	40	67
ACN	11	14	28
IPA	10	15	24

Based on the observation we have performed many purifications using different kind of organic solvents by re-crystallization method to improve the yield and purity of final API molnupiravir. The details and protocol for purification is shown below.

EIDD purification using 5% NaCl solution:

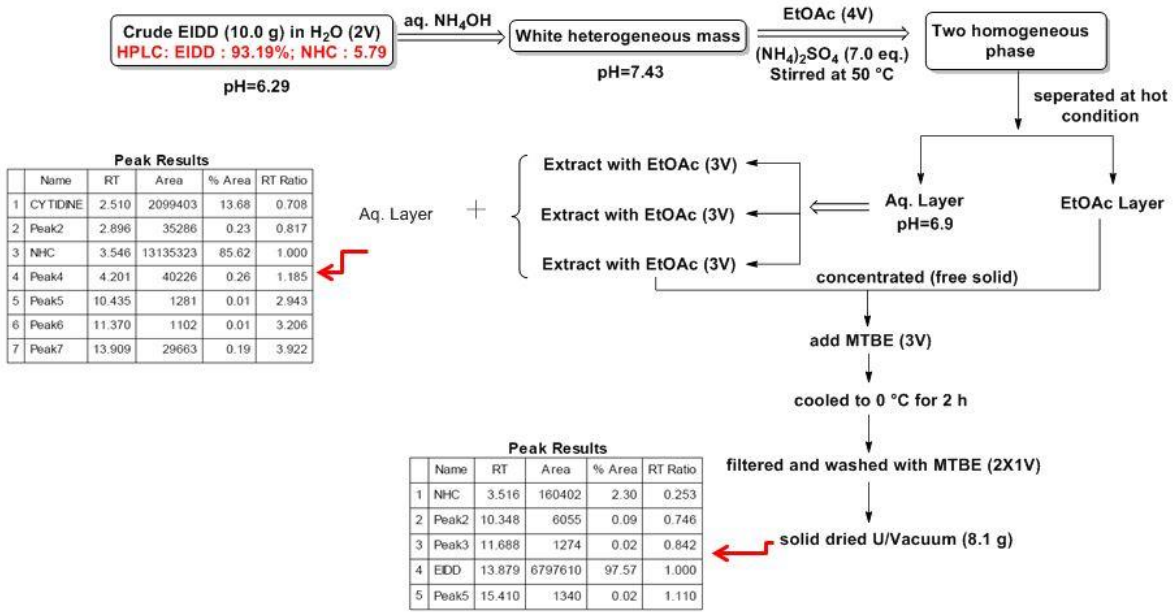
Protocol: To a clean RBF, charged crude EIDD and 5% NaCl solution (3 V) and stirred for 1 h at 65-70 °C to get a clear solution. Then, RM cooled to 25-30 °C for 1 h and stirred for 3 h at that temperature. Afterwards cooled to 15-20 °C and stirred for 2 h and filtered the solid and wet solid washed with 15-20 °C water (2 X 0.5 V) and dried u/v at 50-55 °C for 1 h to afford the off-white solid.

<table>
<tr><th>Batch No.</th><th>Input (g)/Purity (%)</th><th>Output (g/%)</th><th colspan="3">Pure EIDD after crystallization from 5% NaCl Sol (3.0 V)</th></tr>
<tr><td rowspan="4">CR592-15754-43</td><td rowspan="4">10.0

NHC:0.71%
EIDD:98.50
Di-acyl: 0.11%</td><td rowspan="4">8.4/84</td><td colspan="3">99.69/84.4</td></tr>
<tr><td>EIDD</td><td>NHC</td><td>Di-acyl</td></tr>
<tr><td>99.34%</td><td>0.21%</td><td>0.03%</td></tr>
<tr><td colspan="3">Assay w/w only 84%
Chloride content: 7%</td></tr>
</table>

Although recovery looks better but inorganic contamination was higher. Therefore, it was not suitable for purification of API at final stage.

Purification of EIDD using EtOAc (Merck protocol):



	Name	RT	Area	% Area	RT Ratio
1	CYTIDINE	2.510	2099403	13.68	0.708
2	Peak2	2.896	35286	0.23	0.817
3	NHC	3.546	13135323	85.62	1.000
4	Peak4	4.201	40226	0.26	1.185
5	Peak5	10.435	1281	0.01	2.943
6	Peak6	11.370	1102	0.01	3.206
7	Peak7	13.909	29663	0.19	3.922

	Name	RT	Area	% Area	RT Ratio
1	NHC	3.516	160402	2.30	0.253
2	Peak2	10.348	6055	0.09	0.746
3	Peak3	11.688	1274	0.02	0.842
4	EIDD	13.879	6797610	97.57	1.000
5	Peak5	15.410	1340	0.02	1.110

Purification from organic solvents:

Protocol: In a clean and dry RBF, charged crude EIDD and **solvent** at 25-30 °C and heated to 70-75 °C either to get a clear solution or aged for 3 h. Next heating was tuned off and slowly cooled the RM to 20-25 °C and stirred at same temp for 3 h. Filtered the solid and wet solid washed with same solvent (0.5 V X 2) (25-30 °C). Next the wet solid was dried u/vacuum at 40-45 °C for 3 h.

Batch No.	Crude Input (g)	Output EIDD (g)	Solvent (V)	Temp	RM status	Crude EIDD data	Isolated EIDD after purification
CR592-16036-31	25.0	24.0	IPAc (10)	70-75 °C	Aged for 3 h heterogeneous	CR592-16036-29-CR-1	EIDD: 95.01%; NHC: 4.70%; Di-acyl: 0.07
CR592-16036-38	25.0	18.0	EtOH (6.5)	70-75 °C	Clear solution		EIDD: 96.67%; NHC: 3.33%

CR592-16036-42	10.0	9.0	Water saturated IPA (10)	70-75 °C	Clear solution	EIDD: 95.24%; NHC: 4.46%; Di-acyl: 0.16%	EIDD: 96.67%; NHC: 3.33%
CR592-16036-34	25.0	24.0	MTBE (10)	50-55 °C	Aged for 3 h heterogeneous		EIDD: 95.11%; NHC: 4.64%; Di-acyl: 0.06%
CR592-16036-40	25.0	3.0	MeOH (3)	70-75 °C	Clear solution		EIDD: 98.46%; NHC: 1.54%

There was no improvement on the purity of final API by using organic solvents hence we optimized the volume of H_2O for final purification and provided detailed mass balance of EIDD to calculate the loss during re-crystallization as shown in the next table.

Mass balance of EIDD to calculate the loss of EIDD during purification (1.8 kg batch):

Protocol: The solid EIDD from MTBE layer was filtered and washed with MTBE (2 X 2 V). Further wet solid was heated to 60-65 °C in water (1.5 V) to get clear solution, then slowly cooled to 20-25 °C and stirred for 16 h at 20-25 °C. Further, it was cooled to 10-15 °C and hold at same temp for 2 h. Filtered the solid and wet solid washed with chilled water (2 X 0.5 V), followed by MTBE (2 X 1.5 V) and suck dry for 3 h. Next the crude EIDD was dissolved in water (1.5 V) at 60-65 °C and hold for 30 min and cooled to 20-25 °C and stirred (~300 RPM) for 3 h at 20-25 °C. Again it was cooled to 10-15 °C and hold at same temp for 2 h. Filtered the solid and wet solid washed with chilled water (2 X 0.5 V), followed by MTBE (2 X 1.5 V) and suck dry for 3 h and dried u/v at 50-55 °C to obtain the EIDD-2801 as off-white solid.

EIDD after MTBE wash			**EIDD in MTBE MLR**			**Reaction Yield (%)**	**Remark**
Wt. (kg)	Assay (w/w%)	Yield of EIDD (%)	Wt. (kg)	Assay (w/w%)	Loss of EIDD (%)		
1.905	88.6	*78.9*	2.337	5.9	*6.4*	*85.3*	*6.4% Loss in MTBE washing*

	EIDD in water MLR

EIDD 1st Crystallization from water			Main MLR			1st washing			2nd washing		
Wt (kg)	Assay (w/w%)	Yield of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)
1.483	98.2	***67.87***	2.175	5.2	***5.28***	1.105	4.4	***2.27***	1.085	3.8	***1.92***

EIDD in MTBE MLR						**Total EIDD loss in MLR (%) during 1st Crystallization**
1st washing			2nd washing			
Wt (g)	Assay (w/w%)	Loss of EIDD (%)	Wt (g)	Assay (w/w%)	Loss of EIDD (%)	
6.0	97.8	***0.2***	7.0	95.7	***0.3***	***5.28+2.27+1.92+0.2+0.3 = 9.97***

EIDD after 2nd Crystallization from water			**EIDD in water MLR**								
			Main MLR			1st washing			2nd washing		
Wt (kg)	Assay (w/w%)	Yield of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)	Wt (kg)	Assay (w/w%)	Loss of EIDD (%)
1.337	99.1	***61.97***	1.531	3.6	***2.5***	0.911	3.9	***1.6***	0.885	4.3	***1.7***

EIDD in MTBE MLR						**Total EIDD loss in MLR (%) during 2nd Crystallization**
1st washing			2nd washing			
Wt (g)	Assay (w/w%)	Loss of EIDD (%)	Wt (g)	Assay (w/w%)	Loss of EIDD (%)	
4.5	98.9	***0.2***	4.0	99.6	***0.18***	***2.5+1.6+1.7+0.2+0.18 = 6.18***

Purification using different volume of water:

Protocol: In a clean and dry RBF, charged crude EIDD and water at 25-30 °C and then heated to 70-75 °C to get a clear solution. Next heating was tuned off and slowly cool the RM up to 20-25 °C. The RM further cooled to 10-15 °C and hold at same temp for 3 h. Filtered the solid and wet solid washed with chilled water (0.5 V) (10-15 °C). Next the wet solid was suck dry and further dried u/vacuum at 50-55 °C.

Batch No.	Crude Input (g)	Output EIDD (g)	Water volume	Stirring time at RT (h)	Isolated EIDD after purification
CR592-16306-13	50.0 CR592-16036-25-CR1 EIDD: 93.17; NHC: 6.01; Di-acyl: 0.09; **assay: 92.2%**	32.0	3.5 (reaction) 1 X 0.5 V (washing)	3.0	EIDD: 99.18%; NHC: 0.52%; Di-acyl: 0.04%
CR592-16306-12	50.0 CR592-16306-4-Cr-1 EIDD: 94.21; NHC: 5.10; Di-acyl: 0.16; assay: 85.5%	39.2 g after 1st purification	1.5	16.0	EIDD: 97.89%; NHC: 1.56%; Di-acyl: 0.08%
		38.0 g after 2nd purification	1.0	3.0	EIDD: 99.35%; NHC: 0.52%; Di-acyl: 0.02%
CR592-16036-43	100.0 CR592-16306-41-CR EIDD: 96.02; NHC: 3.83; Di-acyl: 0.05;	86.0 g after 1st purification	1.5	16.0	NHC: 0.96%; EIDD: 99.01%
		79.0 g after 2nd purification	1.5	3.0	EIDD: 99.50%; NHC: 0.50%

Decomposition study of Cytidine, NHC·H_2O, Oxime ester and EIDD-2801 by DSC

Compound/Batch No	DSC-Observation	DSC spectra
Cytidine/ CR592-15380-14-Cytidine	Melting starts at 207°C and decomposition starts at 229°C and heat of decomposition ~380 J/g	Cytidine.pdf
NHC·H_2O/CR592-15754-25-P	Melting starts at 131°C and decomposition starts at 193°C and heat of decomposition ~915 J/g	NHC.pdf
Oxime ester/ CR592-15380-38-P	Decomposition starts at 165°C and heat of decomposition ~570 J/g	Oxime ester.pdf
EIDD-2801/CR592-15380-33-P	Melting starts at 160°C and decomposition starts at 218°C and heat of decomposition ~240 J/g	EIDD.pdf

Further areas of developments / improvements

- Although the process has been scaled up to kg scale, there is still scope for further improvements.
- Step-1 reaction needs to be further optimized to increase the product formation as only ~85% product formation was observed.
- Telescoping the Oxime ester synthesis in 2-MeTHF followed by step-2 can reduce the number of solvents as well as improve the recovery of the solvent.
    - An inorganic base as an alternative to organic base Et_3N can minimize the generation of organic waste.
    - To improve the quality of oxime ester as well as minimize the carryforward impurities into the API, after proper safety assessment suitable purification technique such as distillation can be developed.
- Isolation of Molnupiravir is still sub optimized. Reaction yield ~95% *vs*. isolated yield 63-67%. Need to study the nucleation pattern and time of crystal growth to optimize the isolation procedure.
    - As it is an orally given drug candidate, polymorphism as well as particle size distribution also needs to be studied.
    - Development of suitable analytical method for the estimation of residual enzyme on the API and analysis of the API for the same needs to be done.

Acknowledgement: TCG would like to thank VCU to provide an opportunity to work on such a challenging development project as well as providing valuable comments, continuous guidance, encouragement throughout the campaign.

Looking forward to continue our collaboration further and to strengthen our relationship with VCU