Research Article **CODEN: AJPAD7** **ISSN: 2321 - 0923**

Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry

Journal home page: www.ajpamc.com

https://doi.org/10.36673/AJPAMC.2024.v12.i01.A02

PREPARATION OF COST-EFFECTIVE, ROBUST PROCESS OF B-D-N^4-HYDROXYCYTIDINE-5′-ISOPROPYL ESTER AND ISOLATION OF THEIR INTERMEDIATES AND USAGE OF FLOW REACTOR IN THE SYNTHESIS OF B-D-N^4-HYDROXYCYTIDINE-5′-ISOPROPYL ESTER

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ABSTRACT

β-D-N4-Hydroxycytidine-5′-isopropyl ester is an investigational direct-acting antiviral agent that is under development for the treatment of COVID-19. Given the potential high demand for this compound, it was critical to develop a sustainable and efficient synthesis from commodity raw materials[1]. A cost effective, commercially viable two step synthesis of highly pure β-D-N^4-Hydroxycytidine-5′-isopropyl ester from cytidine is described in this paper. The main contribution for enzymatic synthesis is the cost of the enzyme. After understanding the reaction profile enzyme loading was reduced to one fourth i.e., 5% with respect to N-hydroxy cytidine from 20-200% i.e., 4-40 times higher enzyme loading as reported in[2] and 50-200% i.e., 10-40 times higher enzyme loading as reported in[3] for the formation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester. By this selective process Enzyme loading can be reduced to one fourth i.e., 5% with respect to N-hydroxy cytidine from 20-200% i.e., 4-40 times higher enzyme loading to afford good yield and enhanced good purity of β-D-N^4-Hydroxycytidine-5′-isopropyl ester by controlling all other process related impurities less than 0.10% followed by purification techniques using ionic liquids, water and mixture of both.

KEYWORDS

β-D-N^4-Hydroxycytidine-5′-isopropyl ester intermediates, Ionic liquids, Flow reactor, N-Hydroxy Cytidine, N-Hydroxy Cytidine Oxime ester, Dimer compound and β-D-N^4-Hydroxycytidine-5′-isopropyl ester.

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INTRODUCTION

β-D-N^4-Hydroxycytidine-5′-isopropyl ester is an orally bioavailable prodrug of the ribonucleoside analog β-d-N^4-hydroxy cytidine with broad-spectrum antiviral activity against SARS-CoV-2, MERS-CoV, SARS-CoV and the causative agent of COVID-19.

As per disclosed routes reported[2] in Medicines for All Institute at Virginia Commonwealth University

route initial synthesis overall yield was 17%, 37% and 60% yield with 20-200% i.e., 4-40 times higher enzyme loading. In our synthesis only used one fourth i.e., 5% with respect to N-hydroxy cytidine enzyme loading and achieved with low cost of the β-D-N^4-Hydroxycytidine-5′-isopropyl ester. β-D-N4-Hydroxycytidine-5′-isopropyl ester preparation from N-hydroxy cytidine involves following steps in various schemes. Earlier enzymatic synthesis paper[2] (Scheme No.1) for the formation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer added N-Hydroxy cytidine, 20-200% i.e., 4-40 times higher enzyme loading and acylating agent isobyutyryl oxime ester and in patent [3] (Scheme No.2) for preparation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer added N-Hydroxy cytidine, 50-200% i.e., 10-40 times higher enzyme loading and acylating agent Isobutyric anhydride together.

In our route of synthesis of (Scheme No.3) N-hydroxy cytidine is reacted with Isobutyric anhydride at 25-70^{o}C to form N-Hydroxy Cytidine Oxime ester, then N-Hydroxy Cytidine Oxime ester is reacted with Isobutyric anhydride at 25-70^{o}C in the presence of one fourth i.e., 5% addzyme-015 enzyme with respect to N-hydroxy cytidine to form β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer, then dimer will react with ~ 50% aq. hydroxyl amine solution to give β-D-N^4-Hydroxycytidine-5′-isopropyl ester. Before developing this process, reactions were carried out using different types of enzymes in the Table No.1 below.

Reactions were carried out at different temperature to improve yield and to control all other process related compound levels to less than 0.10%. It was observed that whenever reaction mass temperature increased from 26^{o}C to 70^{o}C yield is gradually decreasing along with β-D-N^4-Hydroxycytidine-5′-isopropyl ester crude purity and increase in isobutyryl oxime process related impurity (Figure No.1) level from 0.09-0.48%. After purification of crude β-D-N^4-Hydroxycytidine-5′-isopropyl ester in water isobutyryl oxime process related impurity level controlled by less than 0.10% in a single purification technique whenever isobutyryl oxime process related impurity is less than 0.15% and which leads to increase in yield. But whenever isobutyryl oxime process related impurity level gradually increasing more than 0.15% there is increase in purification steps, and which leads to decrease in yield. Different temperature of the reactions carried out in the Table No.2 below.

In this paper N-hydroxy cytidine (Scheme No.4) and in situ intermediates i.e N-Hydroxy Cytidine Oxime ester (Scheme No.5), β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer (Scheme-6) and β-D-N^4-Hydroxycytidine-5′-isopropyl ester from β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer (Scheme No.7) preparation methods also described.

Ionic liquids are one such alternative that has been found useful to substitute the commonly used bench solvents. Other than their obvious "solvent" property that have been discussed in various publications[4-7], they have also been found to catalyze certain type of reactions in which they participate[8-10]. Moreover, their complete recovery from the reaction is an easy job when juxtaposed with their volatile solvent counterparts. For this reason, an ionic liquid can be re-cycled for multiple batches of reactions.

Another unique property of ionic liquids is that they can be "tailor-made" to suit specific reaction types by playing around with the cation and anion part of them. They are called as "task-specific ionic liquids". These tailored[11] and specially synthesized ionic liquids have more scope of their application in a chemical reaction than just acting as a green solvent.

METHODOLOGY

Experimental Section

Synthesis of N-Hydroxy Cytidine

In a flask, charge cytidine (200g), Hydroxylamine sulfate (121.46g) and water (400ml). Stir and maintain the reaction mass for 4-6 hrs. At 68-72^{o}C and monitor by HPLC showing conversion of N-Hydroxy cytidine-91.98% and Cytidine-4.89%. After reaction complies, cool the reaction mass to 30±5^{o}C and stir for 15 hrs. Overnight. Then cool to 5±3^{o}C and stir for 3-4 hrs. Filter the solid and wash with chilled water (2 x 100ml). Dry the solid under vacuum at 40-50^{o}C to afford desired N-Hydroxy

cytidine (182g) with chromatographic purity-99.62% and was confirmed by ^{1}H NMR and ^{13}C NMR.

Synthesis of N-Hydroxy Cytidine Oxime ester using flow reactor

In a flask, charge N-Hydroxy cytidine (100mg), 1, 4-Dioxane (50ml) and tetrahydrofuran (50ml). Stir for clear solution. Charge Acetone Oxime-O-isobutyryl ester (178mg) and addzyme TL 100L (50mg). Pass the reaction mixture through flow reactor pump at 50^{o}C for 5-6 hrs. The flow rate adjusted to 0.005ml/minute to get N-Hydroxy Cytidine Oxime ester with purity of 94.98% by HPLC.

Synthesis of N-Hydroxy Cytidine Oxime ester

In a flask, charge N-Hydroxy cytidine (10g,), 2-Methyl Tetrahydrofuran (50ml) and isobutyric anhydride (9.15g). Stir and maintain the reaction mass at 45-55^{o}C till reaction complies and monitor the reaction by HPLC after 10-20 hrs. Maintenance showing chromatographic purity - 96.93%. Concentrate the reaction mass under vacuum to remove 2-Methyl Tetrahydrofuran. To the concentrated mass charge Methyl tertiary butyl ether (100ml) and stir for 60-120 min at 5±5^{o}C. Filter and wash with Methyl tertiary butyl ether (50ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford desired NHC-Oxime ester (11.41g) compound with chromatographic purity-95.81% and was confirmed by ^{1}H NMR, ^{13}C NMR and Mass spectra.

Synthesis of Dimer

In a flask, charge N-Hydroxy Cytidine Oxime ester (5.0g), 2-Methyl Tetrahydrofuran (25ml), addzyme-015 (0.25g, 5%) (Note: Addzyme-015 stir for 10-20 minutes in 5ml of 2-Methyl Tetrahydrofuran at 50^{o}C, filter and wash with 5ml of 2-Methyl Tetrahydrofuran) and isobutyric anhydride (3.60g). Stir and maintain the reaction mass at 45-55^{o}C till reaction complies and monitor the reaction by HPLC, after 10-40 hrs. Maintenance Dimer compound was 94.94%. After completion of the reaction filter the enzyme and wash with 2-Methyl Tetrahydrofuran (5ml). Concentrate the reaction mass under vacuum to remove 2-Methyl Tetrahydrofuran. To the concentrated mass charge Methylene Dichloride (50ml) and dry over silica gel and concentrate under vacuum to get β-D-N^{4}-Hydroxycytidine-5′-isopropyl ester dimer. Purified the β-D-N4-Hydroxycytidine-5′-isopropyl ester dimer compound by using column chromatography using MDC (0.2ml): MeOH (9.8ml) as eluents, followed by ethyl acetate to get pure β-D-N^{4}-Hydroxycytidine-5′-isopropyl ester dimer with purity 98.14% and was confirmed by 1H NMR, 13C NMR and Mass spectra.

Synthesis of crude β-D-N4-Hydroxycytidine-5′-isopropyl ester

In a flask charge Dimer (5.0g) and 2-Methyl Tetrahydrofuran (50ml). Cool the reaction mass temperature to 5±5^{o}C, followed by the addition of 50% aqueous hydroxyl amine solution (3.3g) and stir for 1-2 hrs. Till reaction complies and monitor the reaction by HPLC, after completion of reaction, wash the reaction mass with 20% sodium chloride solution (25ml) and extracted separated aqueous layer with 2-Methyl Tetrahydrofuran (5 x 10ml). Combined organic layer dried over anhydrous sodium sulfate (5.0g) and concentrated under vacuum to remove 2-Methyl Tetrahydrofuran. To the concentrated mass charge Methyl tertiary butyl ether (50ml) and stir for 60-120 minutes at ambient temperature. Filter the solid and wash with Methyl tertiary butyl ether (25ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford desired crude β-D-N^{4}-Hydroxycytidine-5′-isopropyl ester compound (4.0g) with chromatographic purity 98.24%.

Purification of β-D-N4-Hydroxycytidine-5′-isopropyl ester

In another flask charge crude β-D-N^{4}-Hydroxycytidine-5′-isopropyl ester (4.0g) and water (8ml). Stir and maintain the temperature at 65-75^{o}C till clear solution, filter at 65-75^{o}C and wash with hot water (2ml). Cool and maintain the reaction mass temperature at 5±5^{o}C, filter the solid and wash with cold water (2ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford desired pure β-D-N^{4}-Hydroxycytidine-5′-isopropyl ester (3.4g) compound with chromatographic purity 99.88% and was confirmed by 1H NMR and 13C NMR.

Synthesis of β-D-N^4-Hydroxycytidine-5′-isopropyl ester in situ

In a flask, charge N-Hydroxy cytidine (100g), 2-Methyl Tetrahydrofuran (1500ml) and isobutyric anhydride (183g). Stir and maintain the reaction mass temperature at 25-35^{o}C for 6-10 hrs. Charge addzyme-015 (5g, 5%) (Note: Addzyme-015 stir for 10-20 minutes in 50ml of 2-Methyl Tetrahydrofuran at 50^{o}C, filter and wash with 50 ml of 2-Methyl Tetrahydrofuran). Stir and maintain the reaction mass temperature at 30±5oC, after 30 hrs. maintenance, monitor the reaction by HPLC, purity of Dimer compound - 91.51%, β-D-N4-Hydroxycytidine-5′-isopropyl ester - 3.85% and N-Hydroxy Cytidine-0.05% and N-Hydroxy Cytidine Oxime ester-0.82%. After completion of the reaction filter the addzyme-015 and wash with 2-Methyl Tetrahydrofuran (100ml). Cool the reaction mass temperature to 5±5^{o}C followed by the addition of 50% aqueous hydroxyl amine solution (101.84g) and stir for 4 hrs. After that wash with 20% sodium chloride solution (500ml), separate aqueous and organic layers and extract aqueous layer with 2-Methyl Tetrahydrofuran (5 x 200 ml). Combined organic layer dry over anhydrous sodium sulfate (50 g) and concentrated under vacuum to remove 2-Methyl Tetrahydrofuran. To the concentrated mass charge Methyl tertiary butyl ether (1000ml) and stir for 60-120 minutes at ambient temperature. Filter the solid and wash with Methyl tertiary butyl ether (2 x 250ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford desired crude β-D-N^4-Hydroxycytidine-5′-isopropyl ester compound (102.87g). In another flask charge crude β-D-N4-Hydroxycytidine-5′-isopropyl ester (102.87g) and water (200ml). Raise the temperature to 65-75^{o}C till clear solution, filter at 65-75^{o}C and wash with hot water (50ml). Cool and maintain the reaction mass temperature at 5±5^{o}C for 4 hrs, filter the solid and wash with chilled water (50ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford pure β-D-N4-Hydroxycytidine-5′-isopropyl ester (91.7g) compound with chromatographic purity 99.81% with all individual process related compounds are less than 0.10%

Purification of β-D-N^4 - Hydroxycytidine - 5′ - isopropyl ester using Ionic liquids and, mixture of Ionic Liquids/water

Purification of β-D-N^4 - Hydroxycytidine -5′ - isopropyl ester using Ionic liquid 1-Butyl-3-Methylimidazolium Tetra Fluoroborate

In a flask charge crude β-D-N^4-Hydroxycytidine-5′-isopropyl ester (5g) contains chromatography purity 96.80% and 1-Butyl-3-Methylimidazolium Tetra Fluoroborate (25ml). Stir and maintain the reaction mass temperature at 45-55^{o}C for 60 minutes. Cool to 25^{o}C and charge water (50ml). Extract with 2-Methyl Tetrahydro furan (5 x 25ml). Combined organic mass dry over anhydrous sodium sulfate and distilled-off under vacuum completely. Cool to 25^{o}C and charge Methyl Tertiary Butyl Ether (100ml). Stir for 60 minutes, filter the solids and wash with Methyl Tertiary Butyl Ether (25 ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford pure β-D-N^4-Hydroxycytidine-5′-isopropyl ester (3.0g) with chromatographic purity 99.81%.

Purification of β-D-N^4-Hydroxycytidine-5′-isopropyl ester using Ionic liquid 1-Butyl-3-Methylimidazolium Chloride and water

In a flask charge water (10ml) and 1-Butyl-3-Methylimidazolium Chloride (2.5g) and crude β-D-N4-Hydroxycytidine - 5′ - isopropyl ester (5g) contains chromatography purity 96.80%. Stir and maintain the reaction mass temperature at 65-75^{o}C for 30 minutes until clear solution. Filter at 65-75^{o}C. Cool to 25^{o}C and then cool to 5±5^{o}C. Stir for 60 minutes at 5±5^{o}C. Filter and wash with chilled (5±5^{o}C) water (10ml). Dry the obtained solid compound under vacuum at 40-50^{o}C to afford pure β-D-N^4-Hydroxycytidine-5′-isopropyl ester (3.83g) with chromatographic purity 99.41%.

Purification of β-D-N^4 – Hydroxycytidine - 5′ - isopropyl ester using Ionic liquid 1-Butyl-3-Methylimidazolium Methane Sulfonate and water

In a flask charge water (10ml) and 1-Butyl-3-Methylimidazolium Methane Sulfonate (2.5g) and crude β-D-N4-Hydroxycytidine-5′-isopropyl ester (5g) contains chromatography purity 96.80 %. Stir and maintain the reaction mass temperature at 65-

75°C for 30 minutes until clear solution. Filter at 65-75°C. Cool to 25°C and then cool to 5±5°C. Stir for 60 minutes at 5±5°C. Filter and wash with chilled (5±5°C) water (10ml). Dry the obtained solid compound under vacuum at 40-50°C to afford pure β-D-N^4-Hydroxycytidine-5′-isopropyl ester (3.68g) with chromatographic purity 99.51%.

Purification of β-D-N^4-Hydroxycytidine-5′-isopropyl ester using Ionic liquid 1-Butyl-3-Methylimidazolium Tetra Chloro Aluminate and water

In a flask charge water (10ml) and 1-Butyl-3-Methylimidazolium Tetra Chloro Aluminate (2.5g) and crude β-D-N^4-Hydroxycytidine-5′-isopropyl ester (5g) contains chromatography purity 96.80%. Stir and maintain the reaction mass temperature at 65-75°C for 30 minutes until clear solution. Filter at 65-75°C. Cool to 25°C and then cool to 5±5°C. Stir for 60 minutes at 5±5°C. Filter and wash with chilled (5±5°C) water (10ml). Dry the obtained solid compound under vacuum at 40-50°C to afford pure β-D-N^4-Hydroxycytidine-5′-isopropyl ester (3.98g) with chromatographic purity 99.48%.

These results are indicating that using ionic liquids and mixture of Ionic Liquids/water are the best alternative purification/crystallization techniques to enhance good purity of β-D-N^4-Hydroxycytidine-5′-isopropyl ester.

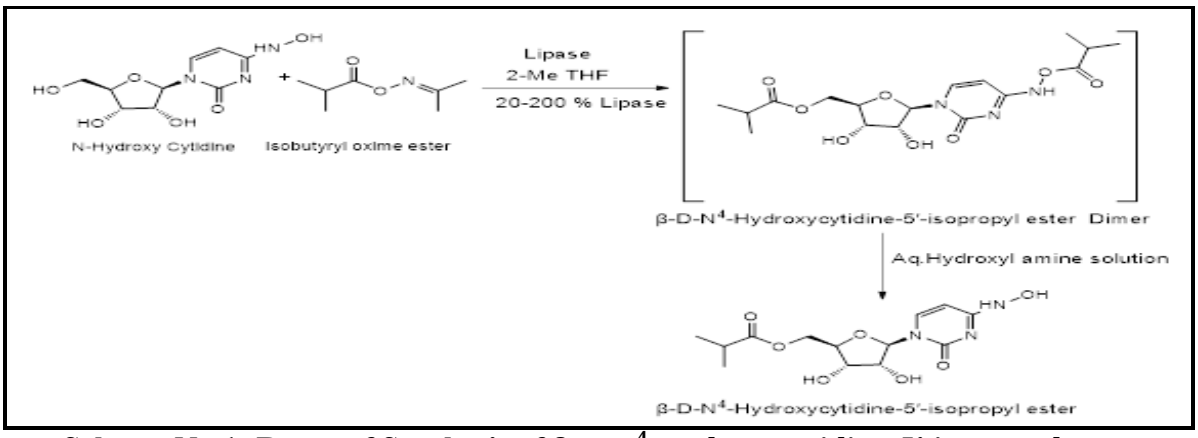
Table No.1: Reactions of β-D-N^4-Hydroxycytidine-5′-isopropyl ester using different types of enzymes

S.No	Name of the enzyme	β-D-N4-Hydroxycytidine-5′-isopropyl ester	N-Hydroxy Cytidine	N-Hydroxy Cytidine Oxime ester	Dimer (It can easily convert to β-D-N4-Hydroxycytidine-5′-isopropyl ester in presence of ~ 50 % aqueous hydroxyl amine)
1	Addzyme-015	6.14 %	0.06 %	0.75 %	90.17 %
2	Addzyme TL-100	8.07 %	57.66 %	24.26 %	5.25 %
3	CALA L Novozyme	5.36 %	83.79 %	3.07 %	1.30 %
4	Palatase 20000	28.48 %	58.32 %	0.57 %	0.15 %
5	Immozyme CALB-T2-150	7.05 %	88.47 %	Not Detected	Not Detected
6	Novozyme 435	5.87 %	91.55 %	1.10 %	1.11 %

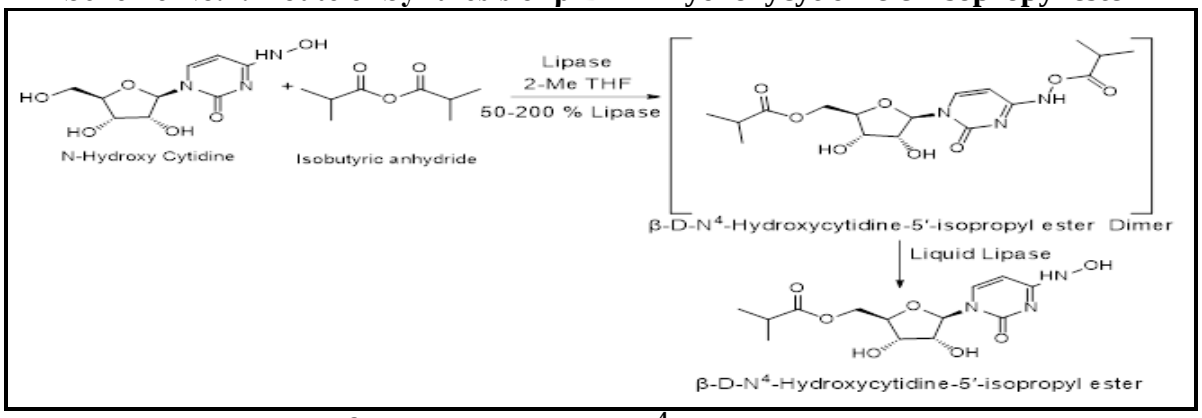
Table No.2: Reactions of β-D-N4-Hydroxycytidine-5′-isopropyl ester at different temperature

S.No	Temperature of the reaction mass	β-D-N^4-Hydroxycytidine-5′-isopropyl ester Crude Yield (w/w)	β-D-N4-Hydroxycytidine-5′-isopropyl ester Crude Purity	Isobutyryl Oxime process related impurity
01	26°C	1.05	99.27%	0.09%
02	30°C	1.02	99.59%	0.14%
03	50°C	1.00	98.70%	0.29%
04	60°C	0.96	97.82%	0.44%
05	70°C	0.94	97.50%	0.48%

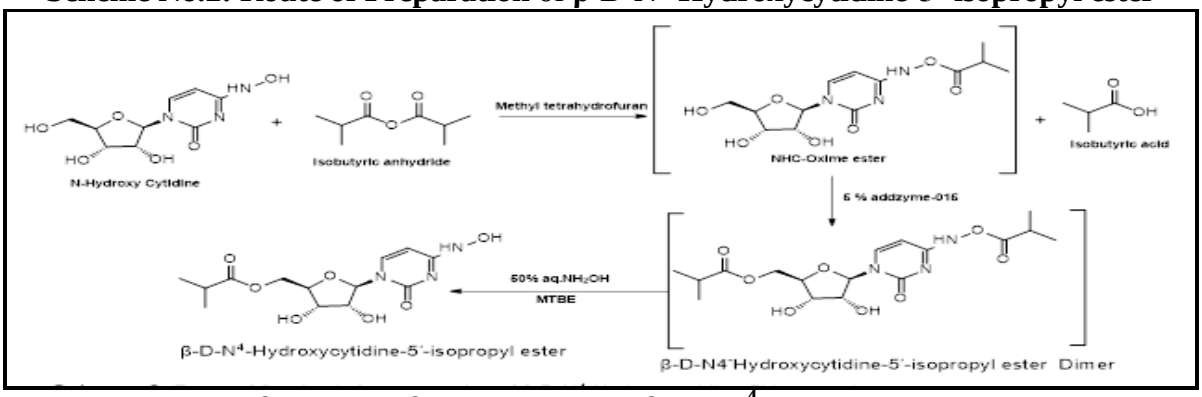
Reaction Scheme



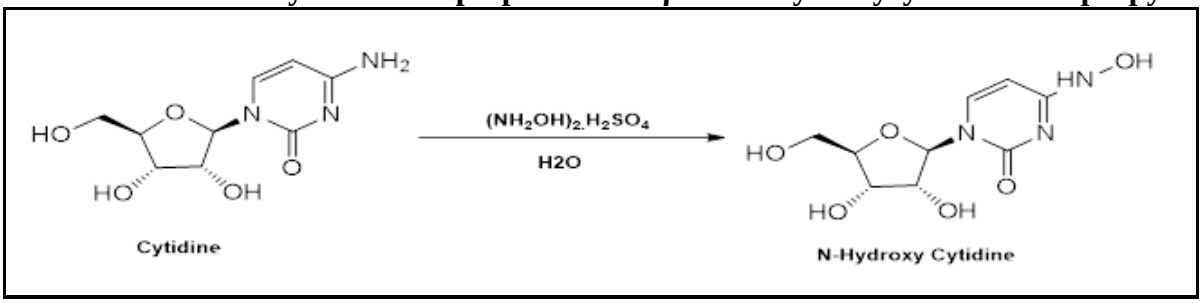
Scheme No.1: Route of Synthesis of β-D-N^4-Hydroxycytidine-5′-isopropyl ester



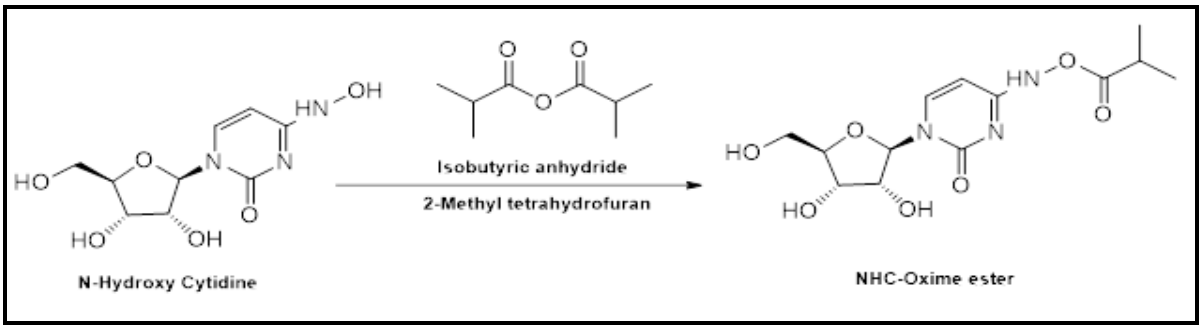
Scheme No.2: Route of Preparation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester



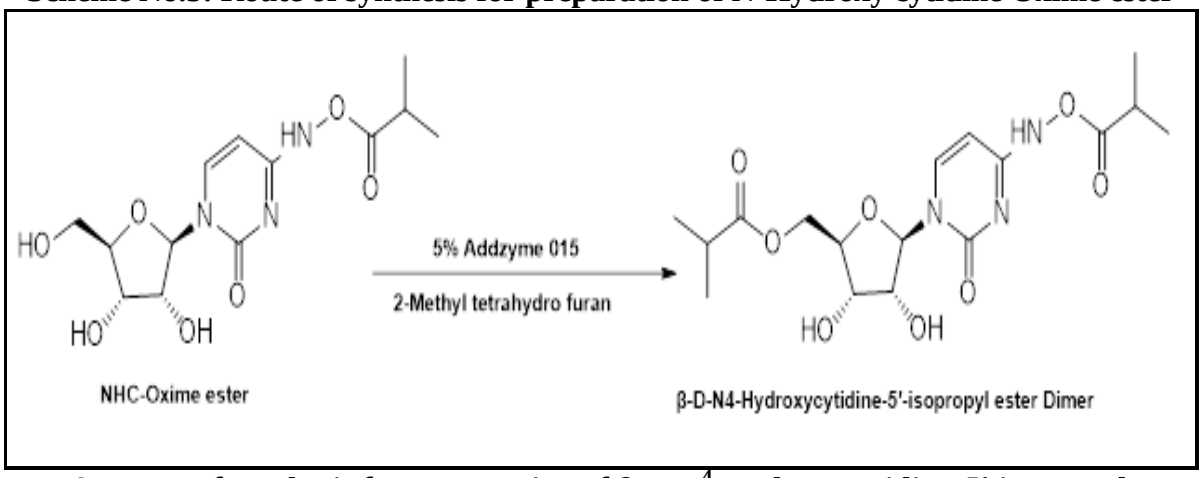
Scheme No.3: Route of synthesis for preparation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester



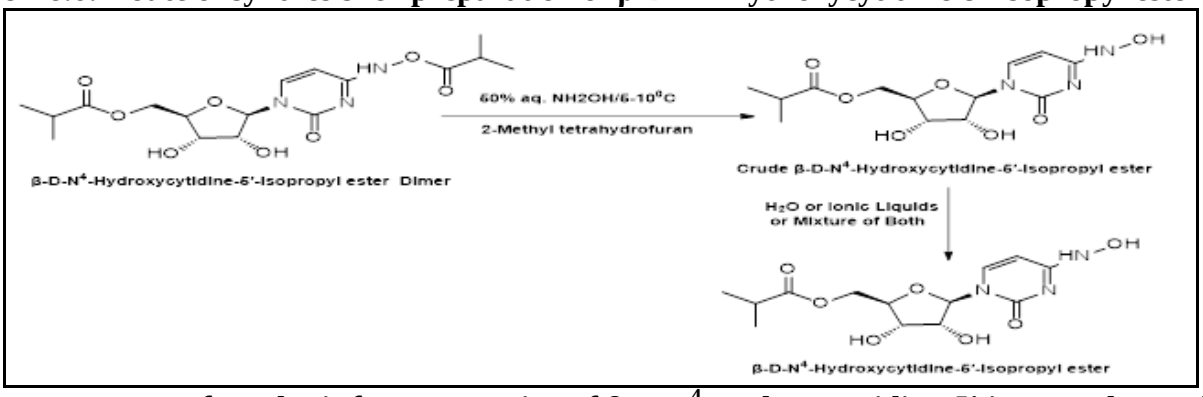
Scheme No.4: Route of synthesis for preparation of N-Hydroxy cytidine



Scheme No.5: Route of synthesis for preparation of N-Hydroxy cytidine Oxime ester



Scheme No.6: Route of synthesis for preparation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester dimer



Scheme No.7: Route of synthesis for preparation of β-D-N^4-Hydroxycytidine-5′-isopropyl ester from β-D-N^4-Hydroxycytidine-5′-isopropyl ester Dimer

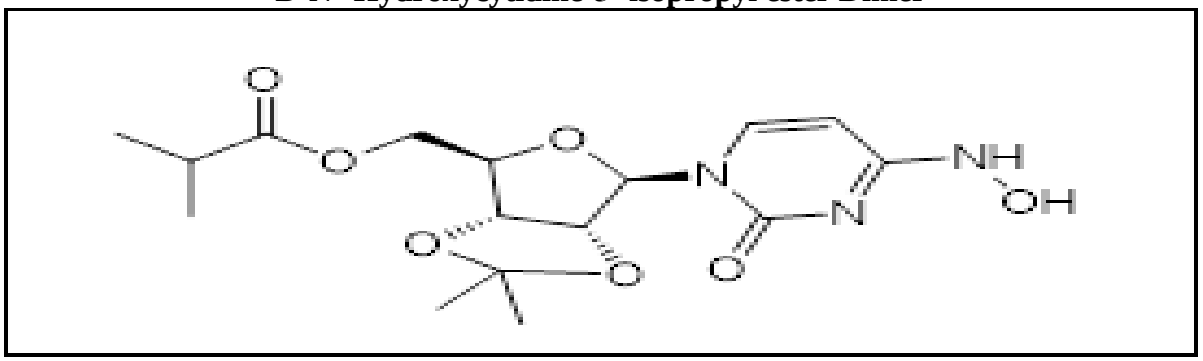


Figure No.1: Isobutyryl oxime process related compound

CONCLUSION

It can be determined from the above study that, formation of N-Hydroxy Cytidine Oxime Ester in neat reaction is the main key role to reduce enzyme loading to one fourth i.e., 5% with respect to N-hydroxy cytidine from 20-200% i.e., 4-40 times higher enzyme loading and 50-200% i.e., 10-40 times higher enzyme loading. Alternatively performed purification techniques using ionic liquids and, mixture of Ionic Liquids/water. By this selective process we achieved cost-effective, robust process with good yield and good purity of β-D-N^4-Hydroxycytidine-5′-isopropyl ester by controlling all other process related impurity levels to less than 0.10%.

ACKNOWLEDGEMENT

Our group is thankful to Department of Scientific and Industrial Research India, Sanjeev Sethi; Jyothi Basu Abbineni {Chief Operating Officer (COO) – Tec Ops}; Dr. Dharmendra Singh Kushwah (Head of Analytical Dept); Dr Narahari and Jayaprakash; as well as analytical development team of Tianish Laboratories Pvt. Ltd for their encouragement and support.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Madhuresh Kumar Sethi *et al*. Preparation of cost effective, robust process of B-D-N^4-Hydroxycytidine-5′-isopropyl ester and isolation of their intermediates and usage of flow reactor in the synthesis of B-D-N^4-Hydroxycytidine-5′-isopropyl ester, *Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry*, 12(1), 2024, 25-32.