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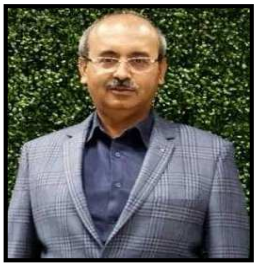


CHEMOSELECTIVE ENZYMATIC HYDROLYSIS AND GREEN CHEMISTRY APPROACH OF DIPENTYLDIACETYL CAPECITABINE

Madhuresh Sethi*[1], Sureshbabu Jayachandra[1], Lakshmana Rao Vadali[1], Sanjay Mahajan[1], Mahesh Gadakar[1], Bhairaiah Mara[1], Ghanshyam Wagh[1], H. Vijayashree[1], Rajakrishna Yerramalla[1], Lakshminarayana Vemula[1], Jayaprakash Thirunavukarasu[1], Jaganmohanarao Bontalakoti[1], Upendranath Veera[1]

[1*]R and D, Mylan Laboratories Ltd., Plot No. 31, 32, 33 and 34 A ANRICH Industrial Estate, Bollaram (Village), Jinnaram (Mandal), Sangareddy (District) 502325, Telangana, India.

ABSTRACT

This manuscript aims to illustrate chemoselective hydrolysis of Di-pentyldiacetyl capecitabine by using Immozyme CAL B -T3-150 therefore preparation of novel Di-pentyloxycarbonyl capecitabine analogue and it's impact on active pharmaceutical ingredient capecitabine.

KEYWORDS

Chemoselective hydrolysis, Enzymatic biocatalyst, Di pentyl diacetyl capecitabine, Immozyme CAL B -T3-150 and Di-Pentyloxycarbonyl capecitabine.

Author for Correspondence:

Madhuresh Kumar Sethi,
API R and D, Mylan Laboratories Ltd,
Plot No. 31, 32, 33 and 34A ANRICH Industrial Estate, Bollaram (Village), Jinnaram (Mandal), Sangareddy, Telangana, India.

Email: madhuresh.sethi@mylan.in

INTRODUCTION

Applications of enzymatic biocatalysts have played a great role in production of pharmaceutical key intermediates in the recent years[1]. Enzymes offer many advantages over conventional chemical catalysts such as cost reduction, improved productivity and higher yields, higher stereo-,regio- and chemo-selectivity leading to improved quality of API/intermediate and environmental friendliness due to waste reduction[2].

Lipases (EC Number 3.1.1.3) are one of the most commonly used classes of enzymes in biocatalysis.

Lipases catalyze the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerol, glycerol and free fatty acids[3]. These biocatalysts offer several advantages over traditional chemical routes such as high efficiency, selectivity and easy separation from unreacted substrates. Lipase catalysts are used in various industrial applications and some of their chemical transformations include enantioselective resolution of esters and amides, acylation of alcohols and amines and kinetic resolution by hydrolysis of racemic esters.

Microbial Lipases are highly appreciated as biocatalysts due to their unique characteristics such as their ability to exhibit a wide range of substrate specificity, high stability in organic solvents, ability to operate in mild conditions and showing high regio- and/or stereo-selectivity in catalysis.

The lipases show its characteristics of the regio-, chemo- and enantioselectivity in the resolution process of racemates, without the use of cofactors. Moreover, this class of enzymes has generally excellent stability in organic solvents also. Some reviews on the preparation of APIs via biocatalysis were also published but involved various types of enzymes and not only lipases.

Lipozyme CALB is a non-specific lipase from *Candida antarctica B* and is stable over a broad pH range and exhibits a high degree of substrate specificity, resulting in highly regio- and enantioselective conversions. CALB has been used extensively in resolution of racemic alcohols, amines and acids[4]. The resulting optically pure compounds are highly difficult to obtain by alternative routes.

Lipozyme TL 100L is a 1, 3 specific lipase which is an effective catalyst for transesterification, inter esterification, ester hydrolysis and desymmetrization of esters. It is used in the commercial manufacture of Pregabalin.

CAL A lipase is a non-specific lipase originating from *Candida antarctica A*. It is an extremely thermostable enzyme that can catalyze desired reactions in the esterification of sterically hindered substrates.

Hydrolysis of racemic or prochiral esters catalyzed by lipases for preparing an enantiomerically pure drug intermediate is a well-established method in organic chemistry[5]. Nevertheless, improved techniques and new applications for bettering the efficiency of both Lipase catalyzed hydrolysis and alternative synthon routes for chemical methods using Lipase catalysts, are being continuously proposed.

Crizotinib is currently one of the most popular anti-cancer drugs. An impediment to its synthesis process has been the synthesis of its key chiral intermediate, named (S)-1-(2, 6-dichloro-3-fluorophenyl) ethanol. The most common chemical methods for chiral alcohol synthesis include addition of carbonyl compounds, hydrogenation reduction of ketone and oxidative kinetic resolution[6]. Chemical catalysis is always considered to be one of high pollution, high costs and difficulty in separation of final products. Also, oxidative kinetic resolution of secondary alcohol substrates always brings some side reactions.

An extensive screening among commercial lipases and esterases was carried out by researchers at Agouron Pharmaceuticals for the preparation of (S)-1-(2, 6-dichloro-3-fluorophenyl) ethanol from its corresponding racemic ester. Highly enantioselective hydrolysis (E > 100) was observed with different commercial lipases (CAL-B and *R. delemar* lipase) and provided the (S)-ester and (R)-alcohol with *ee* ranging from 80-97%. Hydrolysis of the (S)-ester provided the desired product (S)-1-(2, 6-dichloro-3-fluorophenyl) ethanol in high yield.

The drug (S)-Piperoxan is an α-adrenergic receptor antagonist and (S)-Doxazosin is a drug used in the treatment of hypertension. All (S)-isomers are more effective than the corresponding (R)-isomers. The above drugs were synthesized by hydrolytic kinetic resolution of the racemic intermediate methyl-1, 4-benzodioxan-2-carboxylate in the presence of lipase from whole cells of wild species of *Arthrobacter*.

Ezetimibe is a drug used in the reduction of cholesterol and blood lipids. As three asymmetric carbons in the Ezetimibe molecule give rise to 8 stereoisomers, the synthesis of the final products with the required stereochemistry is of significant challenge[7]. The synthesis of this drug requires the enantiopure 3-[5-(4-fluorophenyl)-5(S)-

hydroxypentanoyl]-4(S)-4-phenyl-1, 3-oxazolidin-2-one ((S)-FOP alcohol) as a key intermediate. Kinetic resolution of the diastereoisomeric mixture of FOP acetates was assessed using several commercial lipases to finally obtain a (S)-FOP acetate with *de* 98.5% with lipase from *Candida rugosa*.

Nebracetam, a nootropic drug from the racetam family, is used as an antidepressant. It also reduces dopaminergic and serotonergic uptake and inhibits intracellular calcium flux in response to glutaminergic stimulation. Inspite of these unique properties, Resolution of the diastereomeric ammonium salts using L-tartaric acid is the only known route to obtain enantiopure Nebracetam[8] and even then, its absolute configuration could not be fully determined.

Its key intermediate, (5S)-1-benzyl-5-hydroxy-1, 5-dihydropyrrol-2-one could be obtained by the kinetic resolution of the racemic hydroxylactam (1-benzyl-5-hydroxy-1, 5-dihydropyrrol-2-one). The acetylation was performed in the presence of lipase from *Burkholderia cepacia,* vinyl acetate as the acyl donor, 1, 4-dioxane as the organic solvent. The corresponding (R)-acetate and (S)-alcohol were obtained with excellent enantioselectivity and conversion (>99% *ee,* c 49% and E > 200)[9]. After a few chemical steps, the (5S)-alcohol was converted into (S)-Nebracetam.

Capecitabine, the chemical name being 5'-deoxy-5-fluoro-N-[(pentyloxy) carbonyl]-cytidine with a molecular weight of 359.35 is a ribofuranose based nucleoside and an orally based chemotherapeutic agent that can be converted *in vivo* to 5-FU fluoropyrimidine carbamate deoxynucleoside, capable of inhibiting cell division, interfering with RNA and protein synthesis and used mainly for the treatment of advanced primary or metastatic breast cancer, colorectal cancer and colon cancer[10].

In-depth study of capecitabine is of great significance. In the conventional chemical method employed to produce capecitabine, the penultimate step possesses a possibility of dicarbamoylation leading to the formation of a Dipentyl Diacetyl capecitabine moiety therefore further converted in to formation of Di-pentyloxycarbonyl capecitabine. Unfortunately, the use of conventional chemical catalysts could not provide the selective hydrolysis required to produce the same. Therefore, our objective was to utilize the selectivity and hydrolytic property of Lipase enzymes to produce our target Di-Pentyloxycarbonyl capecitabine.

MATERIAL AND METHODS

Reagents and chemicals

Experimental Section unless stated, all reagents and solvents used in this study were commercially available. During development, reactions were monitored by TLC using commercial silica gel plates/HPLC. The 1H and 13C NMR spectra were recorded in DMSO d6/CDCl3 at 300 MHz and 75 MHz on Bruker 300 MHz Avance NMR spectrometer with Tetramethyl silane as an internal reference. Mass spectra were recorded on Waters Xevo G2-XS Q-TOF LC/MS/MS system. Or Agilent 1100 Series LC-MSD-TRAP-SL system.

METHODOLOGY

Experimental Section

Step I

Preparation of 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy) carbonyl] cytidine (dipentyl diacetyl Capecitabine).

To a solution of 2', 3'-di-O-acetyl-5'-deoxy-5-fluorocytidine (15g) in dichloromethane (75ml) at room temperature with stirring, Pyridine (6.1ml) is added and the mixture is cooled to -5°C to 10□C. N-pentylchloroformate (10.23ml) is added slowly below 10ºC over 20 minutes and the mixture is stirred for 30 minutes at room temperature. Methanol (0.9ml) and water (30ml) are added and stirred for 15 minutes. The layers are separated, and the obtained organic layer is washed with water (30ml). The organic layer is concentrated completely under vacuum at 40-45°C and then diisopropyl ether (2*30ml) is charged and distilled completely, to obtain 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-di [(pentyloxy) carbonyl]-cytidine (dipentyl diacetyl Capecitabine). and was further purified by using column chromatography in ethyl acetate and n-heptane, The column fractions were concentrated to get pure 2', 3'-di-O-acetyl-5'-deoxy-

5-fluoro-N, N-di [(pentyl oxy) carbonyl]-cytidine (dipentyl diacetyl Capecitabine).

Step II

Preparation of Di-pentyloxycarbonyl Capecitabine

To a flask, charged water (70ml) and sodium dihydrogen ortho phosphate mono hydrate (11.0g). Stir and adjust the pH of the buffer solution to 6.0-6.5 by using 7% sodium bi carbonate solution (Prepare using 3.5g Sodium bicarbonate dissolved in 50 ml of water). To this buffer charge 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-di [(pentyloxy) carbonyl]-cytidine (dipentyl diacetyl Capecitabine) (1.0g) followed by 1g CAL-B T3-150 (Immobilized). Stirred the reaction mass for 36 hrs. Meanwhile monitor the reaction by TLC. After completion of the reaction, charged methanol (60ml) and dichloromethane (150ml) mixture to the reaction mass and stirred for 15 min, adjusted the pH ~ 6.0 using ~ 35%. Aqueous hydrochloric acid (2.5ml). Stir for 15 min and filter the enzyme, washed the enzyme with dichloromethane (15ml) and methanol (5ml) mixture. Stir, settle and separate the layers. Dry the organic layer over anhydrous sodium sulphate. The organic layer was then concentrated under vacuum till almost no solvents. Then purified by using column chromatography in ethyl acetate and heptane, concentrate the product fractions under reduced pressure to give pure (Di-pentyloxycarbonyl Capecitabine) (0.35g).

Capecitabine synthesis involves reaction of 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro -D-cytidine with pentyl chloroformate to get a 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro- N-[(pentyloxy) carbonyl] cytidine (stage -1 intermediate). The 5'-Deoxy-2'3'-di-O-acetoxy-5-fluoro-N^4-n-pentyloxy carbonyl cytidine (stage -I intermediate) is further subjected for O-deacetylation deprotection reaction at Stage -II, preferably by using a basic condition to get Capecitabine (API)[11].

RESULTS AND DISCUSSION

At stage-1, there is a possibility of formation of 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyl diacetyl capecitabine) due to side reaction of NH with one more molecule of n-pentyl choro formate (Figure No.3 stage I). The 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyl diacetyl capecitabine) will further result into formation of 5'-deoxy-5-fluoro-N, N-bis[(pentyloxy)carbonyl] cytidine (Di-pentyloxycarbonyl Capecitabine) after removal of di-O-acetyl group during deacetylation deprotection reaction at stage -II.

However, the 5'-deoxy-5-fluoro-N, N-bis [(pentyloxy) carbonyl] cytidine (Di-pentyloxycarbonyl Capecitabine) and its synthesis is not reported in literature. Attempts were made for synthesis of Dipentyloxycarbonyl Capecitabine as it could be one of the possible process impurities. Therefore, initially 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyl diacetyl capecitabine) was synthesized and characterized.

Based on the reaction mechanism the obtained 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyl diacetyl capecitabine) was further subjected for hydrolysis reaction for deacetylation by using the conventional methods of basic hydrolysis as well as acid catalyzed hydrolysis.

However, during the conventional acetyl deprotection methods for the deacetylation of 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyldiacetyl capecitabine) it was observed that along with the deprotection of O-acetyl groups one pentyloxycarbonyl group as well as both the Dipentyloxycarbonyl groups were also getting deprotected by hydrolysis along with other degradation product because of competing reaction.

Hence synthesis of target Di-pentyloxycarbonyl capecitabine was difficult, and it was challenging to deprotect the acetyl groups selectively by keeping both the Dipentyloxycarbonyl groups unaffected. Therefore, our attempts were directed for deprotection of -O-diacetyl of 2', 3'-di-O-acetyl-5'-deoxy-5-fluoro-N, N-Bis[(pentyloxy)carbonyl] cytidine (dipentyl diacetyl capecitabine) by using Enzymes.

We screened about 13 variants of the Enzyme Lipase in which one Particular variant is found to be more selective towards deprotection of carbonate hydroxyl protecting groups without affecting carbamoyl groups and leads to successful formation of Di-Pentyloxycarbonyl Capecitabine with good yield.

Immozyme CALB-T3-150 Lipase variant was effective in synthesis of our novel Di-Pentyloxycarbonyl Capecitabine with 40% yield.

Screening of Lipase Enzymes for Preparation of Di-Pentyloxycarbonyl Capecitabine

Table No.1: Selection of Lipase Enzyme Variants for Synthesis of Di-Pentyloxycarbonyl Capecitabine

S.No	Name of the Variant	Result	Mass
1	CALB EX10000	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
2	Immozyme CALA T2-150	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
3	Immozyme CALB T1-60	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
4	Immozyme CALA T1-500	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
5	Immozyme CALB T1-1500	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
6	Immozyme CALB T2-350	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
7	Immozyme CALB-y	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
8	Immozyme CALBY-T2-150	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
9	Immozyme CALBY-T1-1500	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
10	Immozyme CALBY-T3-150	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24
11	Immozyme CALB-T3-150	Product Formation Observed	Required Di-Pentyloxycarbonyl Capecitabine Mass Obtained 473.22
12	CALB TA-10000	Product Formation Observed	Required Di-Pentyloxycarbonyl Capecitabine Mass Obtained 473.22
13	Advanced Enzyme TL 165G	Product Formation Not Observed	Starting Raw Material Mass Obtained 557.24

Table No.2

S.No	Position	^{1}H	δ (ppm)	J (Hz)	^{13}C δ (ppm)	DEPT
1	1	-	-	-	-	-
2	2	-	-	-	152.08	-
3	3	-	-	-	-	-
4	4	-	-	-	154.03, d(14.1)	-
5	5	-	-	-	139.91 d(240.3)	-
6	6	1 H	8.48	d(5.4)[1]	133.30, d(32.9)	-CH
7	7	1 H	5.70	d(1.8)[1]	92.13	-CH
8	8	1 H	4.09-4.11	m	74.03	-CH
9	9	1 H	3.65-3.72	m	74.08	-CH
10	10	1 H	3.93-4.00	m	79.59	-CH
11	11	1 H (-OH)	4.50-6.10	b	-	-
12	12	1 H (-OH)		b	-	-
13	13, 19	-	-	-	150.03	-
14	14, 20	4 H	4.23	t(6.3)	68.06	-CH2
15	15, 21	4 H	1.54-1.63	m	27.50	-CH2
16	16, 22	4 H	1.18-1.29	m	27.31	-CH2
17	17, 23	4H	1.18-1.29	m	21.65	-CH2
18	18, 24	6 H	0.84	t(6.8)	13.79	-CH3
19	25	3 H	1.35	d(6.3)	17.98	-CH3
20	26	-	-	-	-	-

s- Singlet, d-Doublet, m- Multiplet, b-Broad singlet,[1] Coupling Constant

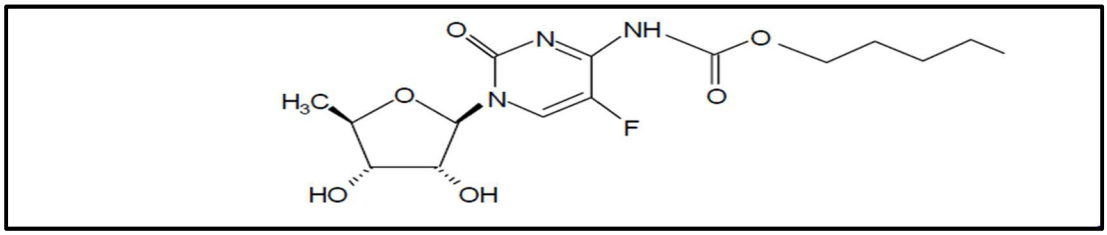


Figure No.1: Structural Formula of Capecitabine

Reaction Scheme for synthesis of Capecitabine

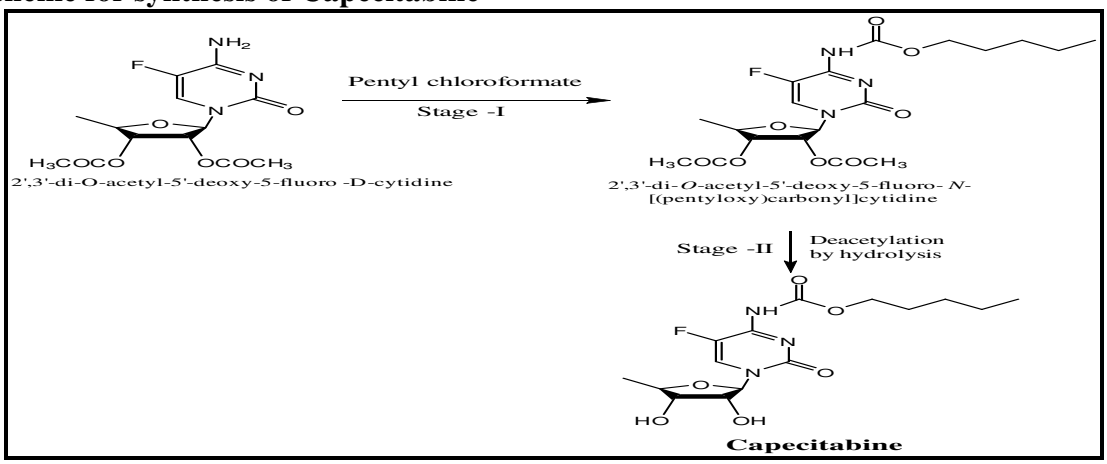


Figure No.2: Reaction Scheme for Capecitabine Synthesis

By-Product formed using conventional Acetyl Deprotection Methods

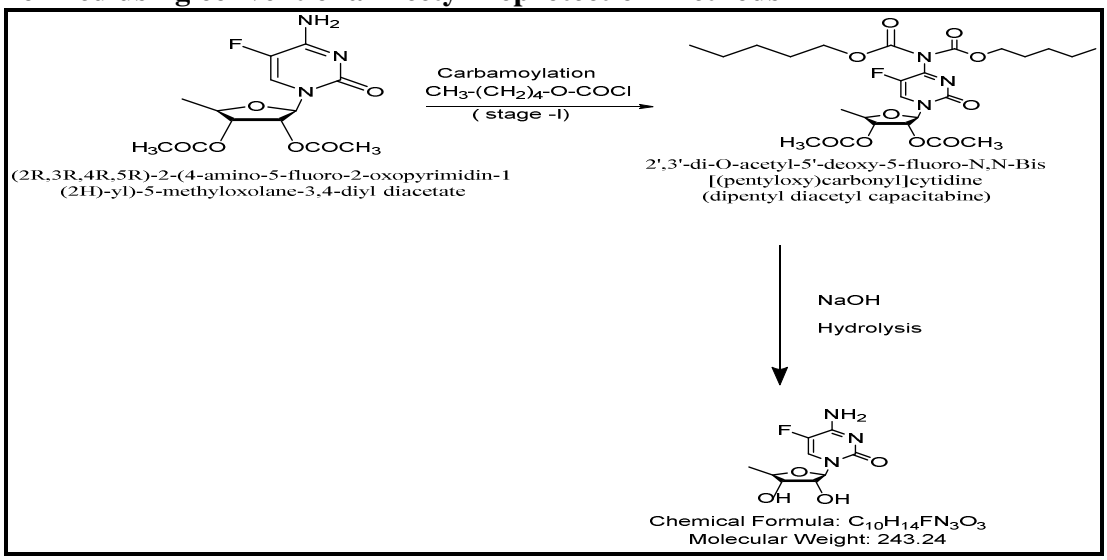


Figure No.3: By product formed during Capecitabine synthesis by chemical method Synthetic Scheme for the preparation of Di-Pentyloxycarbonyl capecitabine

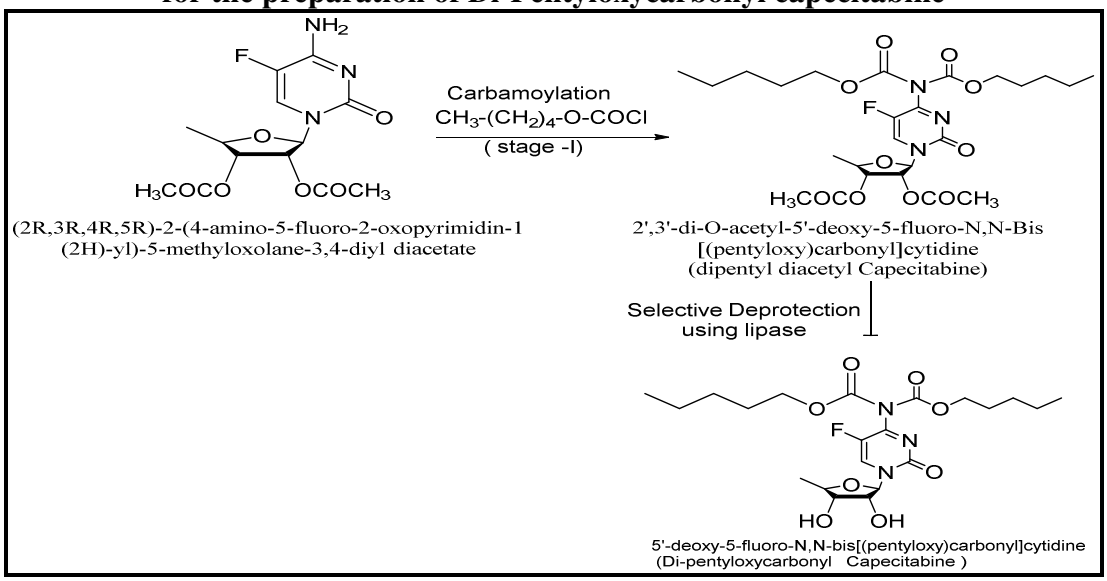


Figure No.4: Enzymatic Synthesis Scheme of Novel Di-pentyloxycarbonyl Capecitabine Characterization Data

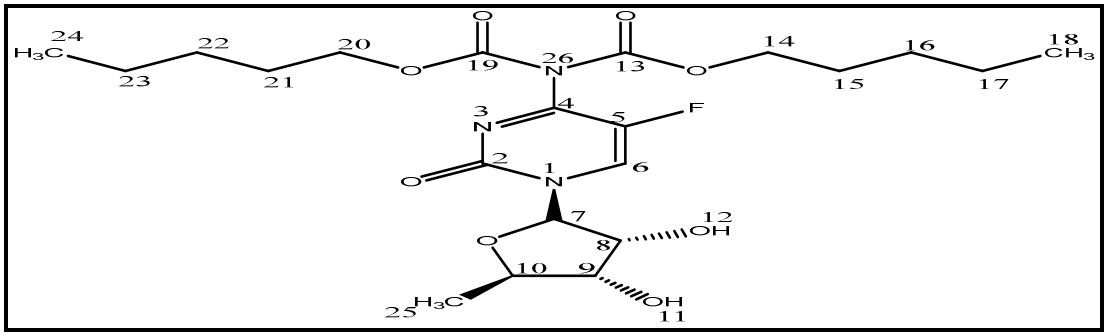
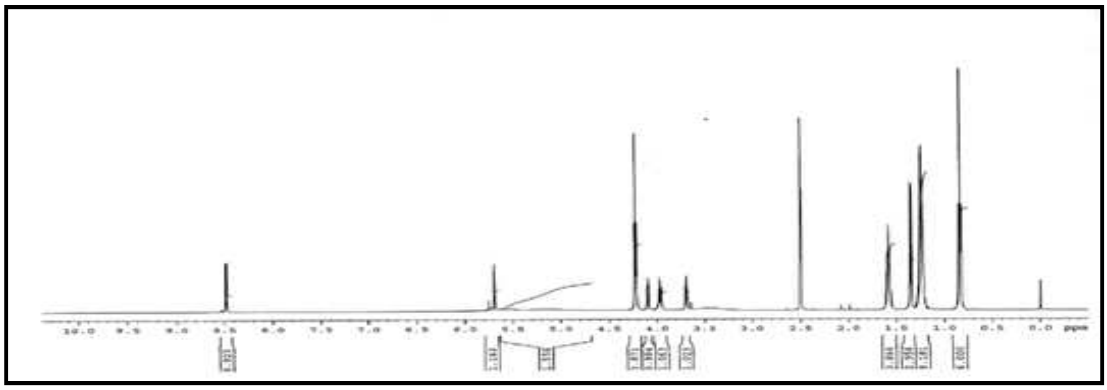


Figure No.5: Di-pentyloxycarbonyl Capecitabine



HPLC Purity ~98%; Mass: ES$^+$ 474.21
Figure No.6: ^{1}H NMR of the Di-Pentyloxycarbonyl Capecitabine

CONCLUSION

It is clear from above study that the new analogue, 5'-deoxy-5-fluro-N, N-bis[(pentyloxy) carbonyl] cytidine (Di-pentyloxycarbonyl Capecitabine) which is a related substance of active pharmaceutical ingredient namely capecitabine has been prepared through enzymatic hydrolysis, as a potential related compound of capecitabine (active pharmaceutical ingredient) this preparation will have a greater importance in regulatory query aspects and getting approval of drug master file.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

BIBLIOGRAPHY

1. Novozymes, 'Lipases for Biocatalysis: for smarter chemical synthesis' 1-6.
2. Sethi M K, *et al.* 'Enantioselective synthesis of (S)-1-Boc-3-Hydroxypiperidine using enzymes and whole cell biocatalysts', *Der Pharma Chemica,* 8(7), 2016. 112-117.
3. Annobom D, *et al.* 'From Structure to Catalysis: Recent Development in the Biotechnological Applications of Lipases', *Biomed Research International,* 2014, Article ID 684506, 2014, 11.
4. Andualema B and Gessesse A. 'Microbial Lipases and their industrial applications: Review', *Biotechnology,* 11(3), 2012, 100-118.
5. Carvalho, *et al.* 'Recent Advances in Lipase mediated preparation of Pharmaceuticals and their intermediates', *Int. J. Mol. Sci,* 16(12), 2015, 29682-29716.
6. Chen X *et al.* 'Efficient synthesis of the chiral alcohol intermediate of Crizotinib using dual-enzyme@$CaHPO_4$ hybrid nanoflowers assembled by mimetic biomineralization', *J. Chemical Technology and Biotechnology,* 94(1), 2019, 236-243.
7. Singh A, *et al.* 'Lipase catalyzed kinetic resolution for the production of (S)-3-[5-(4-fluorophenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one: An intermediate for the synthesis of ezetimibe' *J. Mol. Catal. B,* 85-86, 2013, 99-104.

8. Yamashita S, *et al*. 'Chemoenzymatic total synthesis and determination of the absolute configuration of (S)-nebracetam' *Tetrahedron,* 19(18), 2008, 2115-2118.
9. Hiriyanna S G, Basavaiah K. 'Impurity Profile Study of Capecitabine', *Acta Chromatographica,* 20(4), 2008, 609-624.
10. 'Method for synthesizing Capecitabine Impurities', CN103910773A, 2014.
11. Kadaboina Rajasekhar, *et al*. 'Preparation of Capecitabine, WO2010/066586 A2, 2010.

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