Synthesis and Characterization of Inhibitor Loaded Nanoparticles for Temporal Targeting of PI3K Signalling

A thesis submitted towards partial fulfillment of

BS-MS dual degree program

by

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under the guidance of

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Certificate

This is to certify that this dissertation entitled “Synthesis and Characterization of Inhibitor Loaded Nanoparticles For Temporal Targeting of PI3K Signaling” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by “Suhas Gawali” at Indian Institute of Science Education and Research, Pune under the supervision of “Dr. Sudipta Basu”, Assistant Professor, Department of chemistry during the academic year 2012-2013.

Supervisor	Head Bio/Chem/Phy/Math Science
Date:	Date:
Place:	Place:

Dedicated to my parents …

Declaration

I hereby declare that the matter embodied in the report entitled “Synthesis and Characterization of Inhibitor Loaded Nanoparticles For Temporal Targeting of PI3K Signaling” are the results of the investigations carried out by me at the Department of Chemistry, the Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sudipta Basu and the same has not been submitted elsewhere for any other degree.

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Student

Suhas Gawali

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Contents

Abstract

The phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) signaling pathways play a pivotal role in the growth and survival of various cancers. PI103, a small molecule inhibitor targeting PI3K/AKT/mTOR pathway is a promising anti- cancer agent. Major drawback of this drug is its poor water solubility and off-target toxicity. These limitations can be overcome by harnessing nanotechnology based approaches. Traditional nano-formulations are not always compatible with the physicochemical properties of the agents, leading to poor encapsulation efficiency, sub-optimal uncontrolled drug release, reduced efficacy and vector related toxicity. To address these challenges, we have developed two novel nano-platforms, based on rational design of molecules that facilitate supramolecular self-assembly. In current study we have developed a novel vitamin D3 (cholecaciferol) and lithocholic acid nanoparticles for delivery of PI3K signalling inhibitor in cancer. These nanocarriers are characterized for size, shape and morphology using dynamic light scattering (DLS) and electronic microscopic techniques. Sustained release of bioactive drug over the period of five days is investigated. This study demonstrates the potential of vitamin D3 and lithocholic acid as promising novel nanovectors for delivery of the PI3K signaling inhibitor in cancer.

Chapter 1: Introduction

1.1 PI3K Signaling

Phosphatidylinositol 3-kinase (PI3K) and its downstream proteins are very crucial elements in cellular signal transduction pathway that play an important role in regulating important functions such as cellular behavior, growth, proliferation, survival and metabolism[1] (Fig1). The role of PI3K is to catalyze the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate(PIP2). PIP3 is generated by the class I PI3Ks, which comprise p110α, P110β and P110δ (class IA) and P110γ (class IB) that are activated by receptor tyrosine kinases, Ras and G-protein coupled receptors at cell surface.[1,2] Activated PI3K is the lipid kinase that phosphorylate the 3-hydroxy position of the inositol ring of phosphatidylinositol, which is the second messenger that recruits protein kinase B (AKT) and activates several downstream targets such as mammalian target of rapamycin (mTOR), DNA-PK, ATM, and ATR, which are pivotal in DNA damage repair.[2,3]

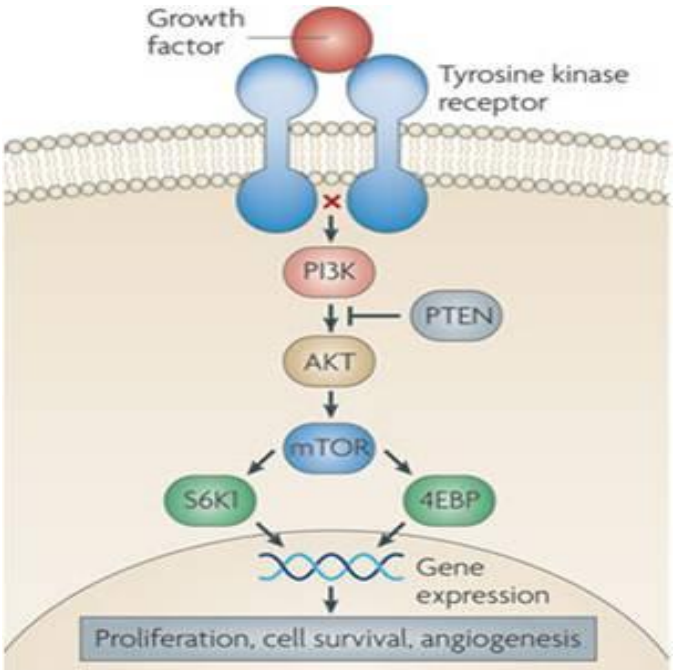


Figure.1. A simplified overview of the PI3K– AKT–mTOR pathway. mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog molecules.

Phosphatase and tensin homolog molecule (PTEN) is a negative regulator of PI3K pathway which is tyrosine phosphatase with homology to tensin.[1] PTEN inhibits PI3K function, leading to suppression of AKT and mammalian target of rapamycin (mTOR) signalling.

1.2 Importance of PI3K signaling in cancer

One pathway that is commonly deregulated and a promising target for therapies is the PI3-kinase (PI3K)-Akt pathway.[4] PI3K-AKT signaling is implicated in ovarian, breast, colon and pancreatic cancer.[5,6,7] Genetic changes such as loss of the PTEN gene and amplification or mutation of the *PIK3CA* gene activate the PI3K pathway which encodes the p110α PI3K isoform. PTEN are tumor suppressors that are functionally inactivated in various malignancies.[8] In addition loss-of-function, mutations in the gene are extremely common among PTEN sporadic glioblastomas, melanomas, prostate cancers, endometrial carcinomas, breast tumors, lung cancers, and lymphomas.[9] PI3K pathway is most frequently activated pathway in sporadic human tumors; estimates indicate that mutations in one or more PI3K pathway components account for up to 30% of all human cancers. As a result PI3K-AKT signaling pathway has emerged as important target for cancer tharapeutics.[8]

1.3 PI3K Inhibitors Over last decades, various PI3K inhibitors have evolved as cancer therapeutics. Very initially employed PI3K inhibitors were wortmannin and LY294002, which are still widely used in research. Wortmannin is a fungal metabolite, originally identified in 1987 as a potent inhibitor of the respiratory burst in neutrophils and monocytes.[10] It was found to inhibit PI3K[11] by covalent attachment with Lys 802 at ATP binding site. The synthetic flavone LY294002, derived from a broad spectrum protein kinase inhibitor quercetin, a natural product, was first reported as an inhibitor of PI3K in 1994.[12] Although their role as PI3K inhibitors is proven, these small molecules show limitations. Wortmannin is an electrophilic and thus fairly unstable whereas LY294002 has very poor potency and selectivity towards PI3Ks.[13] These disadvantages were overcome when PI103 was introduced. Detailed structural and pharmacological analyses showed that, PI103 exhibited excellent potency and selectivity towards class I PI3Ks compared to other 70 types of kinases. Also, it showed higher stability than wortmannin and LY294002.[14] PI103 has IC_{50} values against recombinant PI3K isoforms

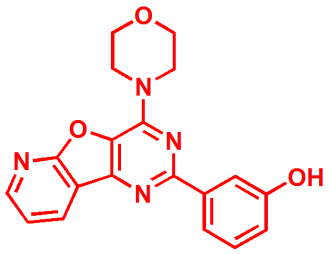


Figure.2. PI103 as Inhibitor of PI3K pathway.

in nanomolar range as compare to wortmannin and LY294002 in macro molar concentration.[14] PI103 (Fig. 2), which inhibits both PI3K/AKT and mTOR signaling, has been utilized in several preclinical tumor models.[15] PI103 has been shown to inhibit cell proliferation, tumor growth and induced apoptosis through its direct effects on the inhibition of PI3K and mTOR.[14,15,16,17] PI103 showed antiproliferative activity against a range of human cancer cell lines *in vitro* as well as significant antitumor activity in human tumor xenografts in athymic mice.[14,18]

1.4 Drug delivery using nanoparticles

Although, variety of approaches has been put forward to tackle cancer cells, crucial problem lies in drug delivery system. Effective treatment strategies need to be developed to abate the problem of off-target toxic side effects. Using Nanoparticles of very specific size as drug carriers can offer some extent of selectivity. These approaches exploit the unique conditions of leaky angiogenic tumor vasculature coupled with impaired intratumoral lymphatic drainage, contributing to an enhanced permeation and retention (EPR) of a drug in tumor region[19] Nanocarriers should have biocompatible, biodegradable, and easily functionalized with high differential uptake efficiency in the target cells over normal cell. Moreover, nanovectors should be soluble or suspension under aqueous condition, having extended circulating half life time and low aggregation rate. Indeed, currently there are 11 types of nanocarrier based drug delivery systems, clinically available to increase intratumoral drug concentration.[20] However, traditional processes for nanoformulation have limitations with controlled drug encapsulation, suboptimal drug release and often incompatible with physicochemical properties of many

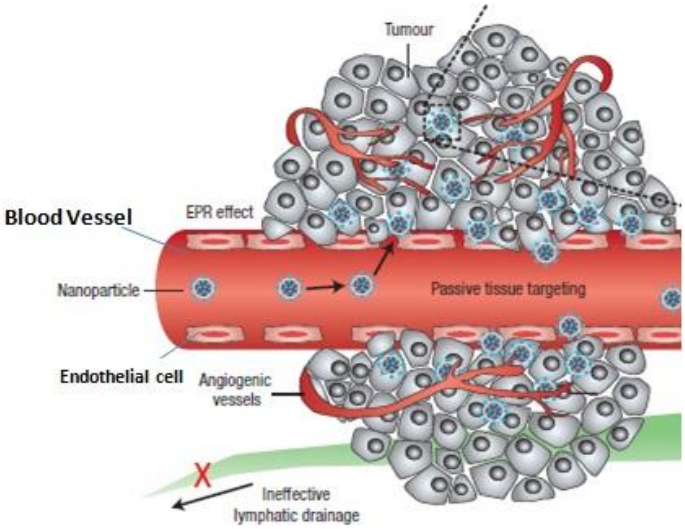


Figure.3. Enhance permeation and retention (EPR) effect

chemotherapeutic agents. To address these challenge, we developed novel lithocholic acid and vitaminD3 based nanoparticles which overcome above challenges. Lithocholic acid is a bile acid[21] and vitamin-D3 is dietary supplement which present in various food and also synthesized in our body by sunlight.

Chapter 2: Experimental Procedure

2.1 Materials and Instruments

General chemical were of the best grade available and was supplied by Sigma-Aldrich Chemicals, Avanti Polar Lipids, Inc and selleck chemicals. Dry solvents were also purchased from sigma-aldrich.

^{1}H-NMR and ^{13}C-NMR spectra were recorded using a 400 MHz Jeol NMR spectrometer in $CDCl_3$ containing a small amount of TMS as an internal standard. The absorption studies were done by a Perkin-Elmer Lambda 45 UV–visible spectrophotometer. The size determination of the NPs is carried out by dynamic light scattering (DLS), using a Nano ZS-90 apparatus utilizing a 633 nm red laser from Malvern instruments. Atomic force microscope images were recorded for drop cast samples using JPK instruments attached with Nanowizard-II setup. AFM is also attached with a Zeiss inverted optical microscope. TEM images were recorded using a Technai-300 instrument.

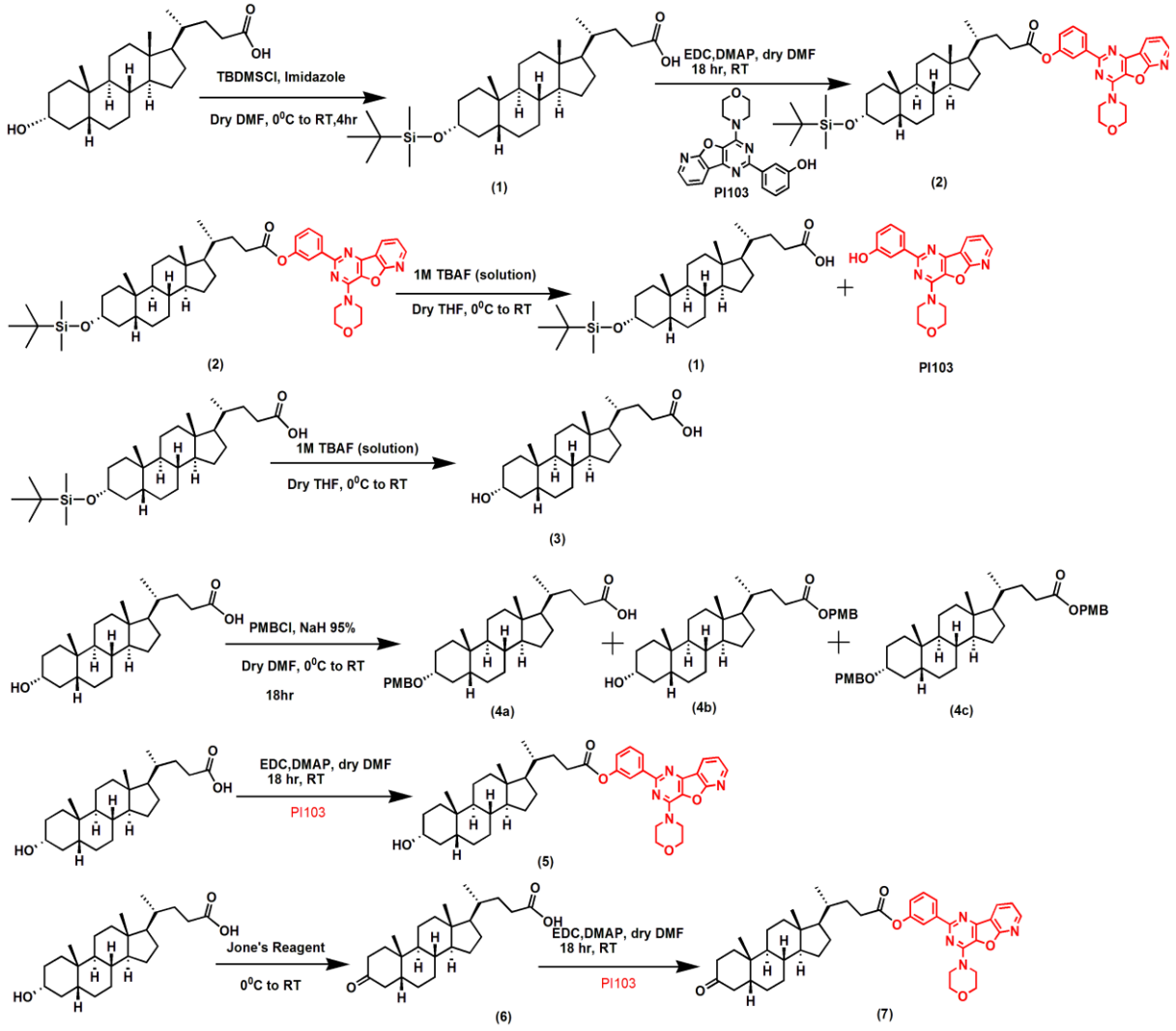
2.2 Procedures and Protocols

2.2.1 Synthesis of lithocholic acid-PI103 conjugate (scheme 1)

Synthesis of TBDMS protected-lithocholic acid (1)

Lithocholic acid (**3**) (200 mg, 0.528 mmol, 1 equiv) was dissolved in 2 mL dry dimethylformamide (DMF) under nitrogen atmosphere in round bottom flask. Into the flask, imidazole (395 mg, 5.80 mmol, 10 equiv) was added, followed by the addition of tert-. butyldimethylsilylchloride (596 mg, 3.96 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 4h. The reaction was quenched with water and organic layer was extracted using dichloromethane (1 X 30 mL). Organic layer was washed thoroughly with double distilled water (2 X 30 mL), brine (1 X 30 mL), dried over anhydrous Na_2SO_4 and then concentrated under vacuum. The crude material was purified by flash chromatography through a plug of silica gel using EtOAc:pet ether = 1:5 as the eluant. The pure product was obtained as a white solid in 80% yield (200 mg).

1H NMR ($CDCl_3$, 400 MHz): δ = 3.56-3.48 (m, 1H), 2.38-2.30 (m, 1H), 2.23-2.12 (m, 1H), 1.89-1.67 (m, 7H), 1.51-1.48 (m, 2H), 1.39-0.99 (m, 20H), 0.87-0.83 (m, 18H), 0.58 (s, 3H).



Scheme.1. Synthesis of lithocholic acid –PI103 conjugate

Synthesis of TBDMS protected lithocholic acid-PI103 conjugate (2)

TBDMS protected lithocholic acid (**1**) (5 mg, 0.010 mmol, 1 equiv) was dissolved in 2 mL dry dimethylformamide (DMF) at 0°C under inert atmosphere to a dry round-bottom flask equipped with a magnetic stir bar. Into the solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (3.83 mg, 0.02 mmol, 2 equiv) was added, followed by dimethylaminopyridine (DMAP) (1.25 mg, 0.010 mmol, 1 equiv). PI103 (4.18 mg,

0.012 mmol, 1.2 equiv) was added into the reaction mixture and stirred for 20h. The reaction was diluted with DCM and quenched with double distilled water (1 X 20 mL). The organic layer was extracted by DCM (2 X 20 mL), washed with brine (1 X 30 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated to obtain crude product. The crude product was purified by flash chromatography through a plug of silica gel using EtOAc:pet ether = 1:4 as the eluant. Pure product was obtained as a white solid in 43% yield (3.50 mg).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.60 (s, 1H), 8.59-8.57(m, 1H), 8.35-8.33(m, 1H), 7.49-7.44 (m, 2H), 7.19-7.16 (m, 1H), 4.22-4.20 (t, 4H, *J* = 4.8Hz), 3.92-3.89 (t, 4H, *J* = 5.2Hz), 3.60-3.53 (m, 1H), 2.69-2.61 (m, 1H), 2.56-2.48 (m, 1H), 1.99-1.72 (m, 6H), 1.57 (s, 9H), 1.40-1.29 (m, 9H), 1.24 (s, 6H), 1.20-1.05 (m, 8H), 1.00-0.98 (d, 3H, *J* = 6.4Hz), 0.89 (s, 3H), 0.88 (s, 6H).

Attempted deprotection of TBDMS from conjugate (2)

TBDMS-lithocholic acid-PI103 conjugate (**2**) (9 mg, 0.010 mmol, 1 equiv) was dissolved in 2 mL of tetrahyadrofuran (THF) & cooled at 0°C under inert atmosphere. Into the solution, 1 M tetra-n-butylammonium fluoride (TBAF) (50 µL) was added. Reaction was monitored by thin layer chromatography for 90 min.

Deprotection of TBDMS group from compound (1)

TBDMS-lithocholic acid (**1**) (50 mg, 0.10 mmol, 1 equiv) was dissolved in 2 mL THF and 1 M TBAF (100 µL) at 0°C under inert atmosphere for 18 h. Reaction was monitored by thin layer chromatography.

Synthesis of p-Methoxybenzyl-lithocholic acid (4a-c)

To a stirred solution of lithocholic acid (**3**) (50 mg, 0.13 mmol, 1 equiv) in anhydrous DMF (2 mL) was added NaH 95% dispersion in mineral oil (10 mg, 0.41 mmol, 3 equiv) portionwise at 0 °C under inert atmosphere. After 30 min, p-methoxybenzyl chloride (PMBCl) (54 µL, 0.40 mmol, 3 equiv) was added dropwise into the reaction mixture. The mixture was kept stirring at room temperature for 4 h. Reaction was monitored by TLC. TLC showed multiple spots with an equal intensity in reaction mixture.

Synthesis of lithocholic acid-PI103 conjugate (5)

Lithocholic acid (**3**) (5 mg, 0.013 mmol, 1 equiv) was dissolved in anhydrous DMF (2 mL) at 0°C under inert atmosphere to a dry round-bottom flask equipped with a magnetic stir bar. Into the solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (5.06 mg, 0.026 mmol, 2 equiv) was added, followed by dimethylaminopyridine (DMAP) (1.6 mg, 0.013 mmol, 1 equiv). PI103 (5.52 mg, 0.016 mmol, 1.2 equiv) was added into the reaction mixture and stirred for 20 h. Water was added (1 X 30 mL), followed by DCM (1 X 30 mL) and the layers were separated. The aqueous layer was extracted with DCM (2 X 30 mL). The combined DCM extracts were washed with brine (1 X 30 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated. Purification of the residue by silica gel chromatography (DCM:MeOH = 50:1) furnished lithocholic acid-PI103 conjugate **5** (30% yield). Yield was very poor because in chromatography PI103 & ester conjugate has same R_f value, therefore unable to separate.

HRMS(ESI): m/z: for $C_{43}H_{55}N_4O_5^+$ $[M+H]^+$:calculated = 707.4127; observed = 707.4158.

Synthesis of keto-lithocholic acid (6)

Preparation of Jone's reagent: In 5 mL glass vials 0.350 mg CrO_3, 0.350 mL concentrated H_2SO_4 was added and nice reddish brown suspension formed. After 5 min 0.9 mL water was added to the suspension in a dropwise manner. Whole addition was done in 0°C.

Prepared Jone's reagent (0.6 mL, 1 mmol, 7.7 equiv) was added dropwise to a solution of lithocholic acid (50 mg, 0.13 mmol, 1 equiv) in 6 mL HPLC grade acetone and stirred at 0°C under inert atmosphere for 1h. Reaction was monitored by TLC. After completion of reaction, isopropenol was added dropwise to destroy excess Jone's reagent, as indicated by the appearance of deep green color. The reaction mixture was then concentrated under reduced pressure and diluted with DCM (20 mL), washed with saturated $NaHCO_3$ (2 X 20 mL) solution, water (2 X 20 mL), dried over anhydrous Na_2SO_4 and concentrated. The crude material was purified by flash chromatography

through a plug of silica gel using EtOAc:pet ether = 1:4 to give compound **6** as colorless white solid (26mg, 53%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.66-2.51 (m, 1H), 2.37-2.08 (m, 5H), 1.97-1.93 (m, 3H), 1.81-1.73 (m, 4H), 1.55-1.53 (m, 1H), 1.41-1.31 (m, 7H), 1.18 (s,3H), 1.07-1.02 (m,4H), 0.95(s, 3H), 0.87-0.86 (d, 3H, *J* = 6.4Hz), 0.62 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 213.80, 180.07, 56.49, 56.01, 44.40, 42.86, 42.43, 40.79, 40.11, 37.28, 37.08, 35.60, 35.37, 34.96, 31.05, 30.81, 29.78, 28.22, 26.68, 25.84, 24.23, 22.73, 21.26, 18.33, 12.16.
HRMS (ESI): m/z: for $C_{24}H_{38}O_3Na^+$ $[M+Na]^+$:calculated = 397.2718; observed = 397.2714.

Synthesis of keto-lithocholic acid-PI103 conjugate (7)

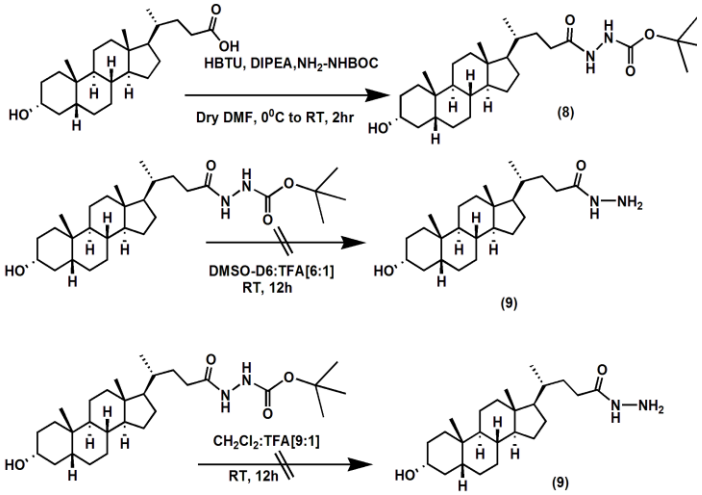
For synthesis of keto-lithocholic-PI103 conjugate (**7**), keto-lithocholic acid (5 mg, 0.013 mmol, 1 equiv) was dissolved into 2 mL dry N,N'-dimethylformamide and cooled at 0°C added under inert atmosphere. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (5.06 mg, 0.0264 mmol, 2 equiv), 4-dimethylaminopyridine (1.61 mg, 0.013 mmol, 1 equiv) were added into the solution. After 10 min, PI103 (5.09 mg, 0.015 mmol, 1.1 equiv) was added into the reaction mixture then reaction mixture was stirred at room temperature for 24 h. After 24 h, the reaction was quenched with 0.1 N HCl (1 X 5 mL) and diluted with DCM (1 X 10 mL). The organic layer was extracted with DCM (2 X 20 mL) and washed with brine solution (1 X 10 mL). The organic layer was dried anhydrous sodium sulphate (Na_2SO_4). Organic solvent was then evaporated using rotary evaporator and the crude product was purified using silica gel (100-200 mesh size) column chromatography EtOAC:Pet ether = 1:4 to obtain **7** mg (96% yield) of pure compound **7** as white solid powder.

^{1}H NMR ($CDCl_3$, 400 MHz): δ **=** 8.55-8.52 (m, 2H), 8.30-8.28 (m, 1H), 8.12-8.11 (m, 1H), 7.45-7.39 (m, 2H), 7.19-7.10 (m, 1H), 417-4.15 (m, 4H), 3.87-3.84 (m, 4H), 2.67-2.57 (m, 2H), 2.51-2.43 (m, 1H), 2.31-2.24 (m, 2H), 2.13-2.08 (m, 1H), 1.99-1.91 (m, 5H), 1.85-1.72 (m, 4H), 1.44-1.37 (m, 6H), 1.08-1.02 (m, 6H), 0.97-0.95 (m, 4H), 0.81(s, 3H)

^{13}C NMR ($CDCl_3$, 100 MHz): δ **=** 213.48, 172.77, 162.73, 158.73, 151.02, 149.64, 148.89, 139.93, 133.45, 131.86, 129.29, 125.51, 123.20, 121.23, 120.27, 115.36, 114.08, 66.95, 56.48, 56.03, 45.73, 42.85, 40.73, 40.09, 35.56, 31.93, 29.70, 29.38, 22.70, 18.39, 14.14, 12.14.

HRMS (ESI): m/z: for $C_{43}H_{53}N_4O_5^+$ $[M+H]^+$: calculated = 705.4017, observed = 705.4028.

Synthesis of compound 8 (Scheme 2)



Schme.2. Synthesis of lithocholic acid moiety

To a suspension of lithocholic acid (25 mg, 0.066 mmol, 1 equiv) Hydrazine-BOC (13.16 mg, 0.099 mmol, 1.5 equiv) and DIPEA (34.7 µL, 0.199 mmol, 3 equiv) in dry DMF (2 mL) were added HBTU (50.32 mg, 0.132 mmol, 2 equiv) and the mixture stirred at room temperature for 2 h under inert atmosphere. After 2 h, the reaction was quenched with 0.1 N HCl (1 X 10 mL) and diluted with DCM (1 X 30 mL). The organic layer was extracted with DCM (2 X 20 mL) and washed with brine solution (1 X 10 mL). The organic layer was dried anhydrous sodium sulphate (Na_2SO_4). Organic solvent was then evaporated using rotary evaporator and the crude product was purified using silica gel (100-200 mesh size) column chromatography EtOAC:Pet ether = 2:3, to furnish the compound **8** as white powder, 59% yield, 19 mg.

1H NMR (DMSO-D6, 400 MHz): δ **=** 9.50 (s, 1H), 8.70 (s, 1H), 4.51-4.50 (d, 1H, *J* = 4.4Hz), 3.40 (s, 6H), 2.57-2.56 (m, 1H), 2.18-1.54(m, 10H), 1.45 (s, 9H), 1.30-1.07 (m, 12H), 0.95-0.93- (m, 5H), 0.67 (s, 3H).

HRMS (ESI): m/z: for $C_{29}H_{50}N_2O_4Na^+$ $[M+H]^+$: calculated = 513.3668, observed = 513.3662.

Attempted synthesis of compound 9

Reaction condition 1(see Table.1)**,** Lithocholic-hydrazine-Boc (**8**) was dissolved in 1 mL DMSO: trifloroacetic acid (6:1) (by volume) under inert atmosphere for 12 h.

Reaction condition 2, Lithocholic-hydrazine-Boc (**8**) was dissolved in 1 mL DCM: trifloroacetic acid (9:1) (by volume) under inert atmosphere for 12 h. Reaction was monitored by TLC, which showed multiple spots. After the removal of solvent, the crude product was purified using flash chromatography with eluting solvent EtOAc:Pet ether = 1:4. Product was so less we were unable to characterize.

	Reactant	**Solvent:acid**	**Product**
1.	Lithocholic-hydrazine-Boc	DMSO-d6:TFA	No
2.	Lithocholic-hydrazine-Boc	DCM:TFA	<10%

Table.1. Attempted synthesis of compound 9.

2.2.2 Synthesis of cholesterol-PI103 conjugate (Scheme 3)

Synthesis of Cholesterol-Succinic acid conjugate (10)

In a two neck RB flask equipped with a magnetic stirring bar, cholesterol 94% pure (54 mg, 0.13 mmol, 1 equiv) was dissolved in 1 mL dry DCM and 1 mL anhydrous pyridine at 0°C under inert atmosphere. Succinic anhydride (64 mg, 0.65 mmol, 5 equiv) and 4-dimethylaminopyridine (DMAP) (15.75 mg, 0.13 mmol, 1 equiv) were added in the reaction mixture. Reaction mixture was stirred for 18 h at room temperature and monitored by TLC. The reaction was quenched with 0.2 M aq. HCl solution, washed with water (2 X 40 mL), brine (1 X 40 mL) after dissolved in DCM. Crude product was dried over anhydrous Na_2SO_4.The solvent was removed *in vaccuo* and crude mixture was purified by flash chromatography on silica gel using EtOAC:Pet ether = 1:4(v/v), to furnished the compound **10** as colorless liquid (33.8 mg, 55% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.31-5.30 (m, 1H), 4.60-4.52 (m, 1H), 2.62-2.51 (m, 4H), 2.26-2.24 (d, 2H), 1.96-1.86 (m, 2H), 1.81-1.71 (m, 3H), 1.56-1.35 (m, 7H), 1.31-

1.23 (m, 4H), 1.18 (s, 3H), 1.10-0.99 (m, 7H), 0.95 (s, 3H), 0.85-0.84 (d, 3H, *J* = 4Hz), 0.80-0.78 (dd, 6H, *J* = 4.8Hz, *J* = 1.6Hz), 0.61 (s, 3H).

HRMS (ESI): m/z: for $C_{31}H_{50}O_4Na^+$ $[M+H]^+$: calculated = 509.3606, observed = 509.3587.

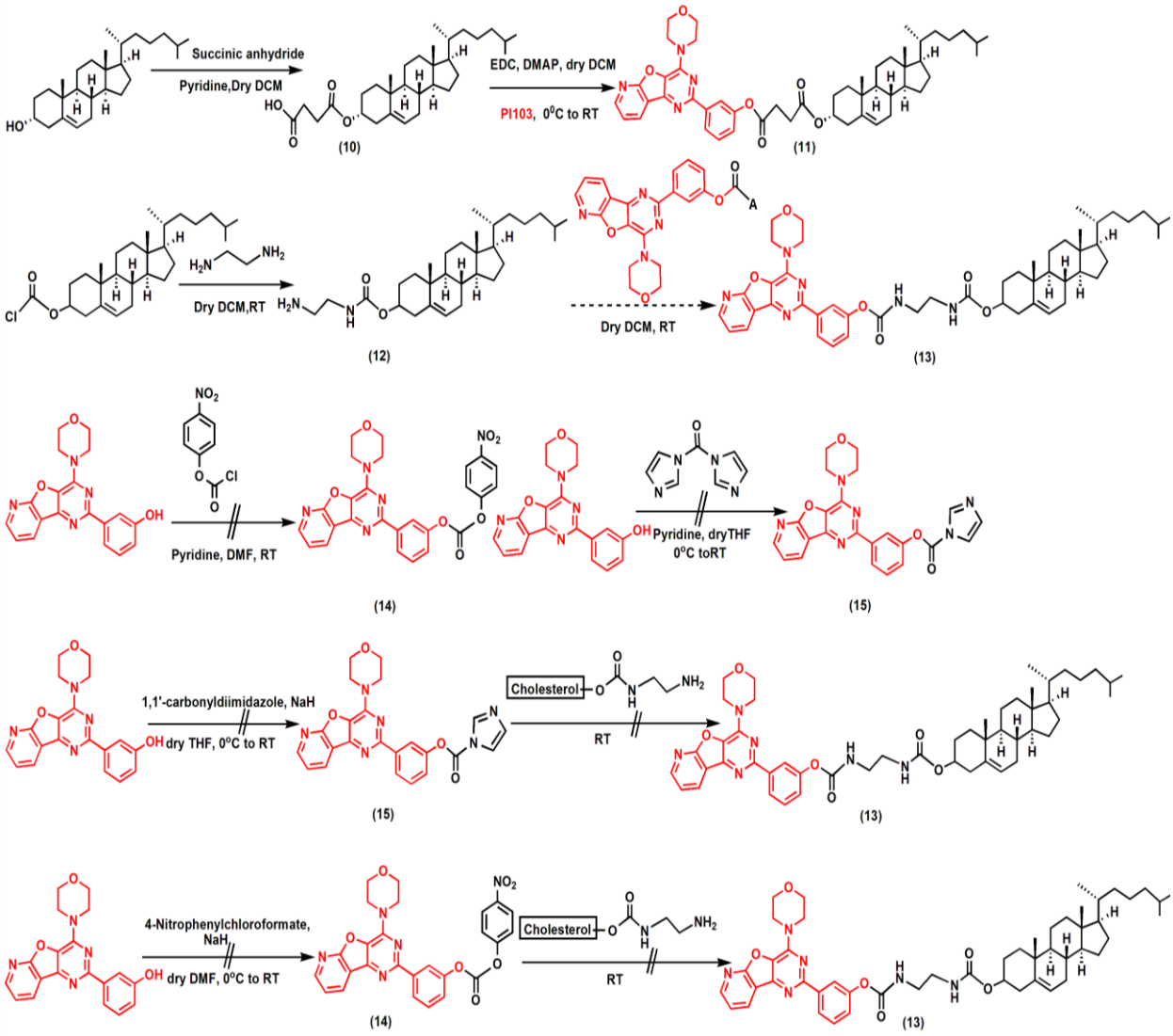
Synthesis of cholesterol-succinic acid-PI103 conjugate (11)

In a two neck RB, cholesterol-succinic acid (5 mg, 0.010 mmol, 1 equiv), N-(3-dimethylamino propyl-N'-methyl carbodiimide hydrochloride (3.91 mg, 0.020 mmol, 2 equiv), 4-dimethylamino-pyridine (1.24 mg, 0.010 mmol, 1 equiv) were dissolved in 2 mL DCM at 0°C under inert atmosphere . After ten min PI103 (4.25 mg, 0.011 mmol, 1.2 equiv) was added into the reaction mixture then reaction mixture was allowed to come at room temperature and was stirred for 18 h. The progress of the reaction was monitored by TLC with EtOAC:Pet ether = 2:5 as mobile phase. Reaction was quenched with 0.1 M aq. HCl solution washed with water (2 X 40 mL) and brine solution (1 X 30 mL). Crude product was dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and crude mixture was purified by silica gel flash chromatography (EtOAC:Pet ether = 1:5) to obtain compound **11** as white solid powder (4 mg, 48% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.54 (s, 1H), 8.53-8.52 (m, 1H), 8.30-8.28 (m, 1H), 8.13-8.12 (t, 1H, 2Hz), 7.44-7.39 (m, 2H), 7.15-7.12 (m, 1H), 5.30-5.29 (m, 1H), 4.64-4.56 (m, 1H), 4.17-4.14 (t, 4H, *J* = 5.1 Hz), 3.87-3.84 (t, 4H, *J* = 4.8Hz), 2.89-2.86 (m, 2H), 2.71-2.68 (m, 2H), 2.29-2.27 (m, 2H), 1.97-1.71 (m, 7H), 1.18 (s, 11H), 1.09-1.00 (m, 7H), 0.94 (s, 3H), 0.85-0.83 (d, 3H, *J* = 6.4Hz), 0.80-0.78 (dd, 6H, *J1* = 6.8Hz, *J2* = 2Hz), 0.59 (s, 3H)

Synthesis of compound 12

In a two neck RB flask, ethylenediamine (37.20 µL, 0.56 mmol, 1 equiv) was dissolved in 5 mL dry DCM cooled at 0°C under inert atmosphere. Cholesteryl chloroformate (27



Scheme.3. Synthesis of cholesterol-PI103 conjugate

mg, 0.056 mmol, 1 equiv) was dissolved in 2 mL dry DCM and added dropwise into the reaction mixture. Reaction mixture was stirred for 24 h. The reaction mixture was diluted in DCM (1 X 30 mL) and washed thoroughly with water (2 x 30 mL). Organic layer was dried over anhydrous Na_2SO_4 and after filtration concentrated under reduced pressure to obtain 23.5 mg (90% yield) of a white solid.

Attempted synthesis of compound 14

PI103 (5 mg, 0.0143 mmol, 1 equiv) and pyridine (2.30 µL, 0,029 mmol, 2 equiv) were dissolved in 1 mL dry DMF followed by addition of 4-nitrophenyl chloroformate (10 mg, 0.043 mmol, 1.5 equiv). This reaction mixture stirred at room temperature for 30 h. Reaction was monitored by TLC. No desired product was formed determined by TLC.

Attempted synthesis of compound 15

PI103 (5 mg, 0.0143 mmol, 1 equiv) and pyridine (2.30 µL, 0.029 mmol, 2 equiv) were dissolved in 1 mL dry THF and stirred at 0°C under inert atmosphere. After ten min, 1,1'-carbonyldiimidazole (11.6 mg, 0.071 mmol, 5 equiv) was added into the reaction mixture and stirred for 8 h at room temperature. No desired product was formed determined by TLC.

Attempted synthesis of compound 13

	Reactant-1	**Reactant-2**	**base**	**Activator**	**Product**
1.	Compound 15 (in situ)	Compound 12	NaH	1,1'-carbonyl diimidazole	No
2.	Compound 14 (in situ)	Compound 12	NaH	4-nitrophenyl chloroformate	No

Table.2. Attempted synthesis of compound 13

Reaction condition 1 (as shown in Table 2) sodium hydride 95% pure (0.27 mg, 0.011 mmol, 2 equiv) was suspended in THF (1 mL) and cooled to 0°C. PI103 (2 mg, 0.0054 mmol, 1 equiv) was added into the suspension and stirred for 30 min under inert atmosphere. Then 1,1'-carbonyl diimidazole (1.39 mg, 0.0086 mmol, 1.5 equiv) was added into the reaction mixture and stirred at room temperature for 4 h. Compound **12** (4.06 mg, 0.0086 mmol, 1.5 equiv) was added into the reaction mixture. Reaction was monitored by TLC, but no desired product was formed.

Reaction condition 2 sodium hydride 95% pure (0.27 mg, 0.011 mmol, 2 equiv) was suspended in THF (1 mL) and cooled to 0°C. PI103 (2 mg, 0.0054 mmol, 1 equiv) was added into the suspension and stirred for 30 min under inert atmosphere. Then 4-nitrophenyl chloroformate (1.73 mg, 0.0086 mmol, 1.5 equiv) was added into the reaction mixture and stirred at room temperature for 4 h. Compound **12** (4.06 mg, 0.0086 mmol, 1.5 equiv) was added into the reaction mixture. Reaction was monitored by TLC, but no desired product was formed.

2.2.3 Synthesis of vitamin D3-succinic acid-PI103 conjugate (Scheme 4)

Synthesis of vitamin D3-succinic acid (17)

Vitamin D3 (**16**) (50 mg, 0.13 mmol, 1 equiv) was dissolved in (2 mL) anhydrous pyridine cooled at 0°C under inert atmosphere and then succinic anhydride (65 mg, 0.65 mmol, 5 equiv) and DMAP (7.93 mg, 0.065 mmol, 0.5 equiv) were added into the reaction mixture. The reaction mixture was stirred for 24 h at room temperature. Reaction was monitored by TLC where R_f value of product = 0.80 (in EtOAC:Pet ether = 1:5). After the removal of the solvent, the residue was dissolved in (1 X 40 mL) of DCM. The solution was washed with 0.1 M aq. HCL (2 X 40 mL) & brine (1 X 40 mL) & dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and crude mixture was purified by silica gel flash column chromatography (AcOEt:petether = 1:5) to obtain compound **17** as a colorless viscous liquid (46 mg, 75% yield).

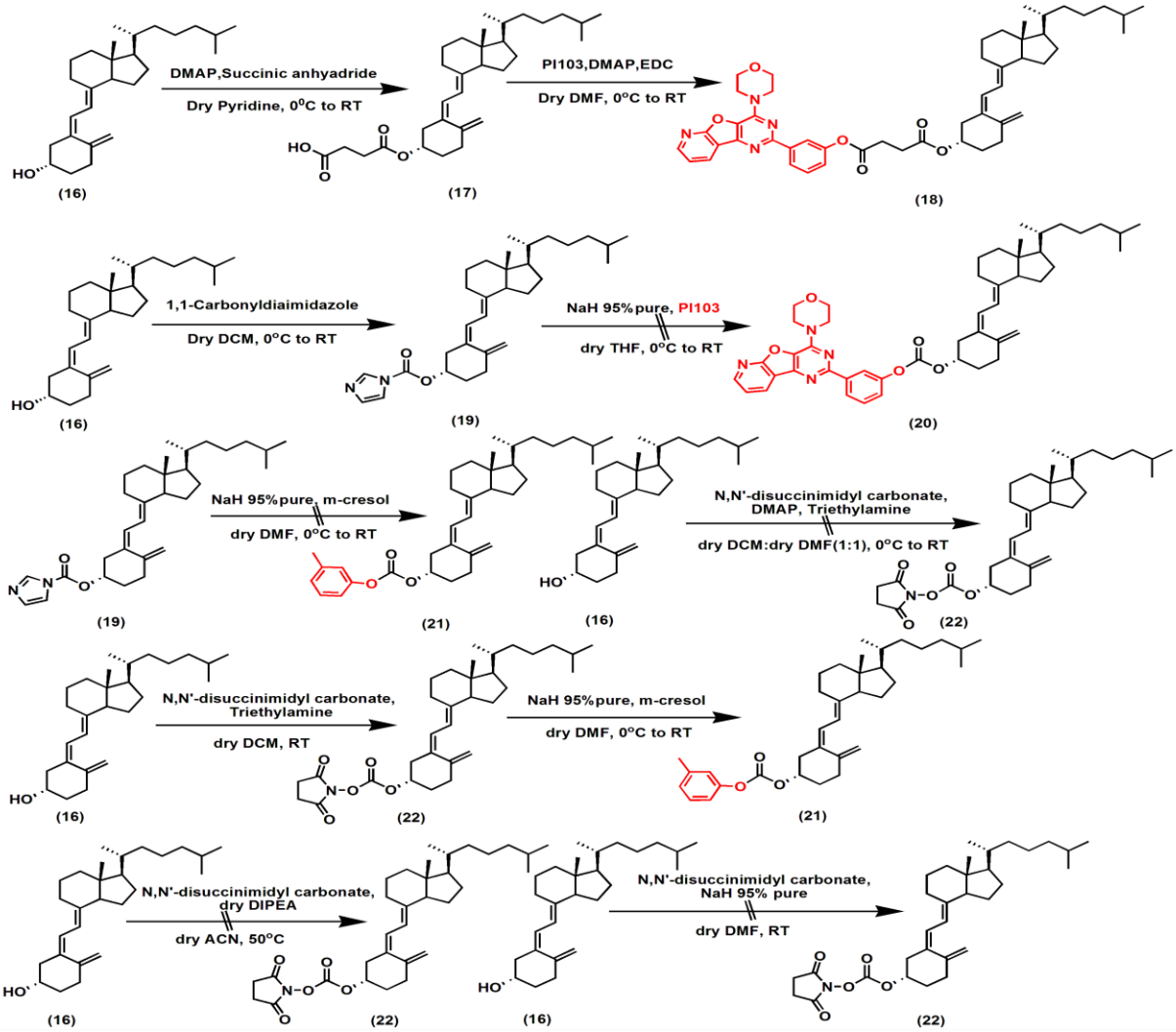
^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.22-6.19 (d, 1H, *J* = 12 Hz), 6.03-6.01 (d, 1H, *J* = 8 Hz), 5.056-5.050 (d, 1H, *J* = 2.4 Hz), 5.00-4.94 (m, 1H), 4.835-4.829 (d, 1H, *J* = 2.4Hz), 2.68-2.66 (m, 2H), 2.62-2.60 (m, 2H), 2.42-2.33 (m, 2H), 2.02-1.92 (m, 4H), 1.54-1.44 (m, 4H), 1.35-1.25 (m, 14H), 1.17-1.07 (m, 5H), 0.92-0.91 (d, 3H, *J* = 4 Hz), 0.87-0.86 (d, 3H, *J* = 4 Hz), 0.855-0.851 (d, 3H, *J* = 1.6 Hz).

^{13}C NNMR (100 MHz, $CDCl_3$): δ = 177.9, 171.7, 144.6, 142.7, 134.2, 122.7, 117.6, 112.9, 72.4, 56.7, 56.5, 46.0, 42.2, 40.7, 39.6, 36.3, 32.2, 31.9, 29.8, 29.3, 29.2, 29.1, 28.1, 27.8, 24.0, 23.7, 22.9, 22.7, 22.4, 18.9, 12.1.

HRMS (ESI): m/z: for $C_{31}H_{49}O_4^+$ $[M+H]^+$: calculated = 485.3553, observed = 385.3632 and for $C_{31}H_{48}O_4Na^+$ $[M+Na]^+$: calculated = 507.3450; observed = 507.3445.

Synthesis of vitamin-succinic-PI103 conjugate (18)

Vitamin D3-succinic acid **17** (10 mg, 0.0206 mmol, 1 equiv) was added under inert tmosphere to 2 mL dry N,N dimethylformamide and cooled at 0°C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (7.91 mg, 0.041 mmol, 2 equiv) and 4-dimethylaminopyridine (DMAP) (2.5 mg, 0.0206 mmol, 1 equiv) were added into the solution. After 10 min PI103 (8.60 mg, 0.024 mmol, 1.2 equiv) was added into the reaction mixture then the reaction mixture was stirred at room temperature for 24 h. The



Scheme.4. Synthesis of vitaminD3-PI103 conjugate

progress of the reaction was checked by silica thin layer chromatography (TLC) with EtOAC:Pet ether = 2:5 ether as mobile phase. Reaction mixture was quenched with 0.1 M aq. HCl solution washed with water (2 X 40 mL) and brine solution (1 X 30 mL). Crude product dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and crude mixture was purified by silica gel flash chromatography (EtOAC:Pet ether = 1:4) to give compound **18** as white solid powder (8.90 mg, 53% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.61-8.59 (m, 2H), 8.37-8.35 (m, 1H), 8.19-8.18 (m, 1H), 7.48-7.43 (m, 1H), 7.21-7.20 (m, 1H), 7.19-7.18 (m, 1H), 5.05-5.02 (m, 1H), 5.01-4.94 (m, 1H), 4.83-4.81 (m, 1H), 4.23-4.21 (t, 4H, *J* = 4.6 Hz), 3.93-3.90 (t, 4H, *J* = 4.8 Hz), 2.95-2.92 (t, 2H, *J* = 6.6 Hz), 2.78-2.75 (t, 2H, *J* = 6.8 Hz), 2.61-2.56 (m, 1H), 2.44-2.34 (m, 1H), 2.04-1.92 (m, 2H), 1.62-1.52 (m, 10H), 1.28-1.19 (m, 15H), 0.91-0.85 (m, 9H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.9, 162.4, 158.7, 150.9, 149.6, 148.8, 147.2, 144.5, 142.6, 139.9, 134.1, 133.4, 131.9, 129.3, 125.6, 123.1, 122.6, 121.2, 120.3, 117.4, 115.4, 112.8, 72.4, 72.0 66.9, 56.6, 56.5, 45.9, 45.8, 42.1, 40.5, 39.5, 36.1, 32.1, 32.0, 29.7, 29.6, 28.8, 28.02, 23.9, 23.7, 22.8, 22.6, 22.3, 18.8, 14.0, 11.9.

HRMS (ESI): m/z: for $C_{50}H_{63}N_4O_6$ $[M+H]^+$: calculated = 815.4748, observed = 815.4752.

Synthesis of compound 19

To the solution of vitaminD3 **(16)** (25 mg, 0.0643 mmol, 1 equiv) in dry DCM (2 mL) and 1,1'-carbonyl diimidazole (105 mg, 0.64 mmol, 1 equiv), was added. The reaction mixture was stirred at room temperature for 24 h. Reaction mixture was washed with water (2 X 40 mL) and brine solution (1 X 30 mL) after diluting in dichloromethane. Crude product was dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and product was purified by silica gel flash chromatography (EtOAC:Pet ether = 1:10) to obtain compound **19** as white solid powder (16 mg, 52% yield).

Attempted synthesis of compound 20

To the suspension of sodium hydride 95% pure (1 mg, 0.008 mmol, 1equiv) in dry THF (2 mL) PI103 (2.91 mg, 0.0083 mmol, 1 equiv) was added. The reaction mixture was stirred at 0°C for 30 min. Subsequently, compound **19** (4 mg, 0.008 mmol, 1 equiv) was added to the reaction mixture with continuous stirring at room temperature for 24 h. No product was formed determined by TLC.

Attempted synthesis of compound 21

Reaction condition 1 (Table.3)

	Reactant 1	**Reactant 2**	**Base**	**Product**
1.	Compound 19	m-cresol	NaH	No
2.	Compound 22	m-cresol	NaH	17%

Table.3. Synthesis of compound 21

To the suspension of sodium hydride 95% pure (1.1 mg, 0.046 mmol, 2 equiv) in dry DMF (2 mL) and m-cresol (2.4 µL, 0.023 mmol, 1 equiv) was added. The reaction mixture was stirred at 0°C for 30 min. Subsequently, compound **19** (11 mg, 0.023 mmol, 1 equiv) was added to the reaction mixture with continuous stirring at room temperature for 24 h. No product was formed determined by TLC.

Reaction condition 2 To the suspension of sodium hydride (2.75 mg, 0.114 mmol, 10 equiv) in dry DMF (2 mL), m-cresol (1.3 µL, 0.014 mmol, 1 equiv) was added. The reaction mixture was stirred at 0°C for 30 min. Subsequently, compound **22** (6 mg, 0.014 mmol, 1 equiv) was added to the reaction mixture with continuous stirring at room temperature for 24 h. Reaction mixture was monitored by TLC which it showed that complete conversion didn't occur. Reaction mixture was washed with water (2 X 20 mL) after diluting with DCM (1 X 20 mL) and brine solution (1 X 30 mL). Crude product was dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and crude mixture was purified by silica gel flash chromatography (EtOAC:Pet ether = 1:50) to give compound **21** as white solid powder (1 mg, 17% yield).

HRMS (ESI): m/z: for $C_{35}H_{50}O_3Na^+$ $[M+Na]^+$: calculated = 541.3657, observed = 541.3657.

Attempted synthesis of compound 22

Reaction condition 1 (Table.4)

To the solution of vitaminD3 (**16**) (50 mg, 0.129 mmol, 1 equiv) in dry DCM (4 mL) triethylamine (21.5 μL, 0.155 mmol, 1.2 equiv), N,N'-disuccinimidyl carbonate (40 mg, 0.155 mmol, 1.2 equiv) were added. Reaction mixture was stirred at room temperature for 24 h. The reaction was monitored by TLC which it showed that complete convergen of vitaminD3 didn't occur. Reaction mixture washed with water (2 X 40 mL) and brine solution (1 X 30 mL) after diluting in DCM. Crude product dried over anhydrous Na_2SO_4. The solvent was removed *in vaccuo* and crude mixture was purified by silica gel flash chromatography (EtOAC:Pet ether = 1:5) to give compound **22** as white solid powder (7 mg, 10% yield).

^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 6.20-6.17 (d, 1H, *J* = 11.2Hz), 5.96-5.93 (d, 1H, *J* = 11.2 Hz), 5.024-5.020(d, 1H, *J* = 1.6Hz), 4.92-4.87(m, 1H), 4.81-4.80(d, 1H, *J* = 2Hz), 2.74 (s, 3H), 2.63-2.59 (dd, 1H, *J1* = 13.2, *J2* = 4Hz), 2.48-2.43 (m, 1H), 2.39-2.33 (m, 1H), 2.19-2.10 (m, 1H), 2.02-1.76 (m, 4H), 1.62-1.39 (m, 8H), 1.18 (s, 9H), 1.07-1.03 (m, 2H), 0.86-0.84 (d, 3H *J* = 6.4Hz), 0.81-0.80 (d, 3H, *J* = 2Hz), 0.79 (d, 3H, *J* = 1.6Hz), 0.47 (s, 3H).

HRMS (ESI): m/z: for $C_{32}H_{47}NO_5Na^+$ $[M+Na]^+$: calculated = 548.3351, observed = 548.3354.

	Reactant-1	**Solvent**	**base**	**Activator**	**Product**
1.	Vitamin D3 (**16)**	DCM	Triethylamine	N,N'-disuccinimidyl carbonate	10%
2.	Vitamin D3 (**16)**	ACN	DIPEA	N,N'-disuccinimidyl carbonate	No
3.	Vitamin D3 **(16)**	DMF	NaH 95% pure	N,N'-disuccinimidyl carbonate	No
4.	Vitamin D3 **(16)**	DCM:DMF	Triethylamine	N,N'-disuccinimidyl carbonate	No

Table.4. Attempted synthesis of compound 22

Reaction condition 2 To the solution of vitaminD3 (**16**) (10 mg, 0.028 mmol, 1 equiv) in dry ACN (5 mL) dry DIPEA (10.8 µL, 0.068 mmol, 2.4 equiv) and N,N'-disuccinimidyl carbonate (16 mg, 0.068 mmol, 2.4 equiv) were added. Reaction mixture was stirred at 50°C for 12 h. Reaction was monitored by TLC. No product was formed determined by TLC.

Reaction condition 3 To the suspension of sodium hydride 95% pure (1.5 mg, 0.062 mmol, 2.4 equiv) in dry DMF (5 mL) and vitaminD3 (**16**) (10 mg, 0.026 mmol, 1 equiv) was added. The reaction mixture was stirred at 0°C for 30 min. Subsequently, N,N'-disuccinimidyl carbonate (16 mg, 0.068 mmol, 2.4 equiv) was added and reaction mixture was stirred at room temperature. No product was formed as determined by TLC.

Reaction condition 4 To the solution of vitaminD3 (**16**) (10 mg, 0.0259 mmol, 1 equiv) in 4 mL dry DCM: DMF (1:1) triethylamine (7.2 µL, 0.052 mmol, 2 equiv), 4-dimethylaminopyridine (1.57 mg, 0.013 mmol, 2 equiv) and N,N'-disuccinimidyl carbonate (13.3 mg, 0.052mmol, 2 equiv). Reaction mixture was stirred at room temperature for 24 h. No product was formed as determined by TLC.

2.2.4 Standard calibration curve of PI103 by UV-Vis spectrophotometry

A calibration curve was plotted in the concentration range of 2.5 to 25 µM by diluting the 1 mM standard stock solution with dimethyl sulfoxide (DMSO). The absorbance was measured at 293 nm (which is characteristic λ_{max} value for PI103)

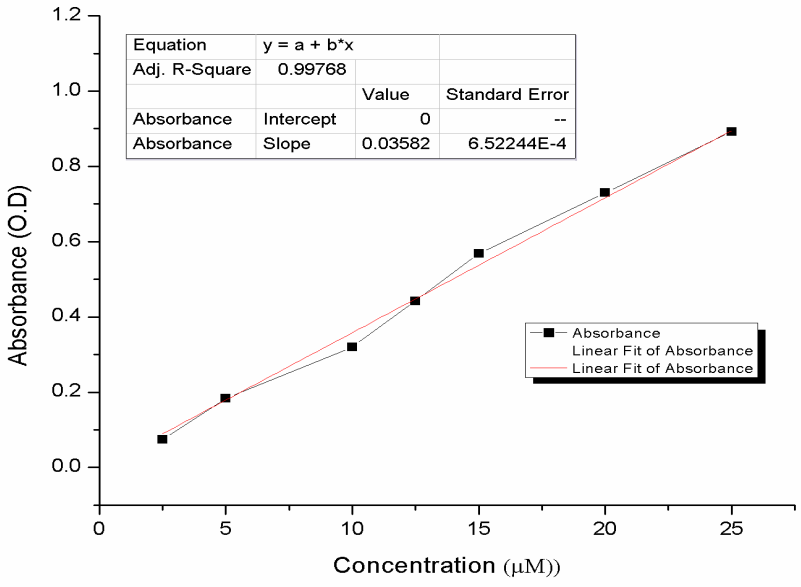


Figure.4. Standard calibration curve of PI103 by UV-Vis spectrophotometry

Concentration (μM)	2.5	5	10	12.5	15	20	25
Absorbance (O.D)	0.0748	0.1835	0.3201	0.4427	0.568	0.73	0.8927

Table.5. Absorbance of PI103 in different concentration

against the corresponding solvent blank. The linearity was plotted for absorbance (A) against concentration (C) with R^2 value 0.9976 and with the slope equation y = 0.0358x.

2.2.5 Synthesis of NPs

Synthesis of keto-lithocholic acid-PI103 NPs

The general procedure of synthesizing self-assembled keto-lithocholic acid-PI103 is as follows: 10.0 mg of L-α-phosphotidyl choline, 5.0 mg keto-lithocholic acid-PI103 conjugate (**7**), and 1.0 mg of DSPE-PEG_{2000} were dissolved in 10.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film using a rotary evaporator. The lipid-drug film was then hydrated with 1.0 mL H_2O for 1 h at 60°C. The hydrated nanoparticles looked white. This mixture was passed although Sephadex G-25 column, and extruded at 65°C to obtain sub-200 nm particles. Final active drug loading of 98.18 μM in NPs was achieved.

Synthesis of vitamin D3-succinic acid-PI103 NPs

The general procedure of synthesizing self-assembled vitamin D3-succinic acid-PI103 is as follows: 10.0 mg of L-α-phosphotidyl choline, 5.0 mg vitamin D3-succinic acid-PI103 conjugate (**18**), and 1.0 mg of DSPE-PEG_{2000} were dissolved in 10.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film using a rotary evaporator. The lipid-drug film was then hydrated with 1.0 mL H_2O for 1 h at 60°C. The hydrated nanoparticles looked white. This mixture was passed although Sephadex G-25 column, and extruded at 65°C to obtain sub-200 nm particles. Final active drug loading of 185.57 μM in NPs was achieved.

Determination of drug loading by UV-VIS spectrophotometry

Prepared NPs were dissolved in spectroscopy grade of dimethyl sulfoxide with three different dilution 5%, 10%, 15% and respective amount of blank were prepared water in dimethyl sulfoxide. In order to check drug loading in NPs absorbance value at 293nm were compared with calibration curve of PI103.

2.2.5 Release kinetics of inhibitor loaded NPs at different pH

Concentrated drug-loaded NPs were suspended in buffer (pH = 5.5 and 7.4) and sealed in a dialysis membrane (MWCO = 500 Da); The dialysis bags were incubated in 10 mL PBS buffer at room temperature with gentle shaking. A 500-µL portion of the aliquot was collected from the incubation medium at predetermined time intervals. Aliquots were concentrated on speed *vaccuo* and diluted in 250 µL spectroscopic grade DMSO and the released drug was quantified by UV-VIS spectrophotometer.

2.3.1 Instrumentation

Atomic Force Microscopy

One droplet of NPs solution of 1 (around 5 µM) was drop-casted with micropipette (5 µL) on to freshly cleaved mica surface. The solution was allowed to dry (evaporation) in air for approximately 30 min before AFM measurement.

Field-Emission Scanning Electron Microscope (FESEM) Five micro litter of prepared NPs (after 20 times diluted original solution) were drop casted onto silicon wefer and sample were allowed to dry in air and images were obtained using Ultra-55, Zeiss NTS GmbH, at an operating voltage of 4.0 KV. Multiple images taken at different locations were used for image analysis.

Transmission Electron Microscopy (TEM) Five micro litter of prepared NPs (after 20 times diluted original solution) were drop casted onto copper grid and sample were allowed to dry in air and then imaged using a high resolution (300KV) TEM images. TEM analysis was recorded using a Tecnai-300 instrument.

Chapter 3: Results and Discussion

3.1 Synthesis of drug conjugated vectors:

For delivering PI103 specifically to cancer tissue to inhibit PI3K signaling, we explored there different nanovectors based on their biocompatibility and biodegradability. We chose lithocholic acid, vitamin D3 and cholesterol for this purpose. Lithocholic acid is a component of bile acid in our body. Vitamin D3 is also synthesized in our body by sunlight and digested in liver and starch. Finally cholesterol is an important compound in our cell membrane.

First, free -OH group of lithocholic acid was protected with tert-butyldimethylsilyl chloride in presence of imidazole in anhydrous DMF to give the TBDMS protected lithocholic acid **1** (Scheme1). This acid **1** was coupled with PI103 in DMF using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and catalytic amount 4-dimethylaminopyridine (DMAP) to afford product **2** in overall 35% yield. The second step was the deprotection of compound **2** with 1 M tetra-n-butylammonium fluoride (TBAF) solution. Unexpectedly product **1** and PI103 where reformed by breaking of ester linkage instead of deprotection of protecting group. This reaction was completed in 1.5 h. Where deprotection of TBDMS protected lithocholic acid was completed in 18 h. Compound **3** shows that deprotection of TBDMS protected lithocholic acid are slower than breaking of ester linkage. Hence we tried to use different protecting group strategy. We protected the free -OH of lithocholic acid by using p-methoxybenzyl chloride yielded multiple products, due to the presence of –OH and –COOH on lithocholic acid. Hence we tried to conjugate PI103 directly with lithocholic acid. Compound **5** was synthesized using direct coupling of lithocholic acid with PI103 drug in DMF using EDC & DMAP. Purification of compound **5** from the reaction mixture was very hard because of same polarity of PI103 and compound **5** in EtOAc/Pet ether and methenol/DCM eluant system. Then we converted the free 2'-OH group of lithocholic acid to the corresponding ketone by Jone's reaction to obtain compound **6** in 53% yield. We then conjugate PI103 with compound **6** by using EDC and DMAP as coupling reagent to obtain compound **7** in 96% yield.

In order to get temporal drug release profile we aimed to synthesize different bonding connectivity. Compound **8** was synthesized by amide coupling of the carboxylic acid of lithocholic acid with N-Boc hydrazine using the coupling reagent HBTU with diisopropylethyl amine (DIPEA) as base. For deprotection step we used DMSO solvent for the reaction with trifluoroacetic acid (6:1), but reaction didn't occur. Compound **8** was recovered from last reaction using normal workup and again reaction kept in DCM solvent with trifluroacetic acid (9:1). Product yield was too less because lithocholic acid was poorly soluble in DCM.

We also explored vitamin-D3 as a novel nanovector for PI103 delivery in cancer. Vitamin-D3 is biocompatible, biodegradable and important component in many food products. First, vitamin-D3 was reacted with succinic anhydride using pyridine as a base, to afford compound **17** in good yield (75% yield). Further compound **17** was coupled with PI103 using EDC and DMAP, resulted compound **18** in moderate yield (53% yield). In order to make different bonding connectivity for temporal drug release we aimed to synthesize carbonate linkage. Vitamin-D3 treated with 1,1'-carbonyldiaimidazole in DCM solution, but reaction didn't occur. Synthesis of compound **22** was done using vitamin-D3 and N,N'-disuccinimidyl carbonate to a basic solution of triethylamine to isolate compound **22** in overall 10% yield. All our attempts to synthesize vitamin D3-PI103 conjugate with carbonate linkage did not give us expected product. So we synthesized Nanoparticles from vitamin D3-PI103 conjugate having ester linkage.

Finally, we explored cholesterol as nano vector. To synthesis of lipid using ester bonding connectivity with cholesterol, we synthesized compound **10** using cholesterol with succinic anhydride in the presence of pyridine base as well as solvent. Then acid group of compound 12 conjugated with drug PI103 using two equivalents of EDC and DMAP (in catalytic amount). We also tried to synthesize cholesterol-PI103 conjugate having carbamate linkage. To this end we synthesized first cholesterol-ethylene diamine conjugate **12**. But our attempt to conjugate PI103 with compound **12** did not give us expected product.

3.2 Synthesis and Characterization of NPs

So finally for synthesis of NPs, we got two drug loaded conjugate **7** and **18** with good yield and less number of steps. This two conjugates were used for further study.

3.2.1. Synthesis and characterization of NPs (compound 7) (Fig 5)

We engineered the NPs from the compound **7** (keto-lithocholic acid-PI103), L-α-phosphatidylcholine (PC) and 1,2-distearoyl-sn-glycero-3-phos-phoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE- PEG_{2000}) in 1:2:0.2 weight ratio using a lipid-film hydration self assembly method.[22] The morphology of NPs was determined by Atomic force microscopy (AFM). AFM image clearly shows the spherical shaped nanostructure (Fig. 5a and 5c). AFM graph shows the curve surface which is spherical geometry with diameter roughly 120 nm (Fig. 5b). DLS data also supports this information and reveals the size distribution of NPs 90 nm to 290 nm with 127 nm mean hydrodynamic diameter as Z-average (Fig. 5d).

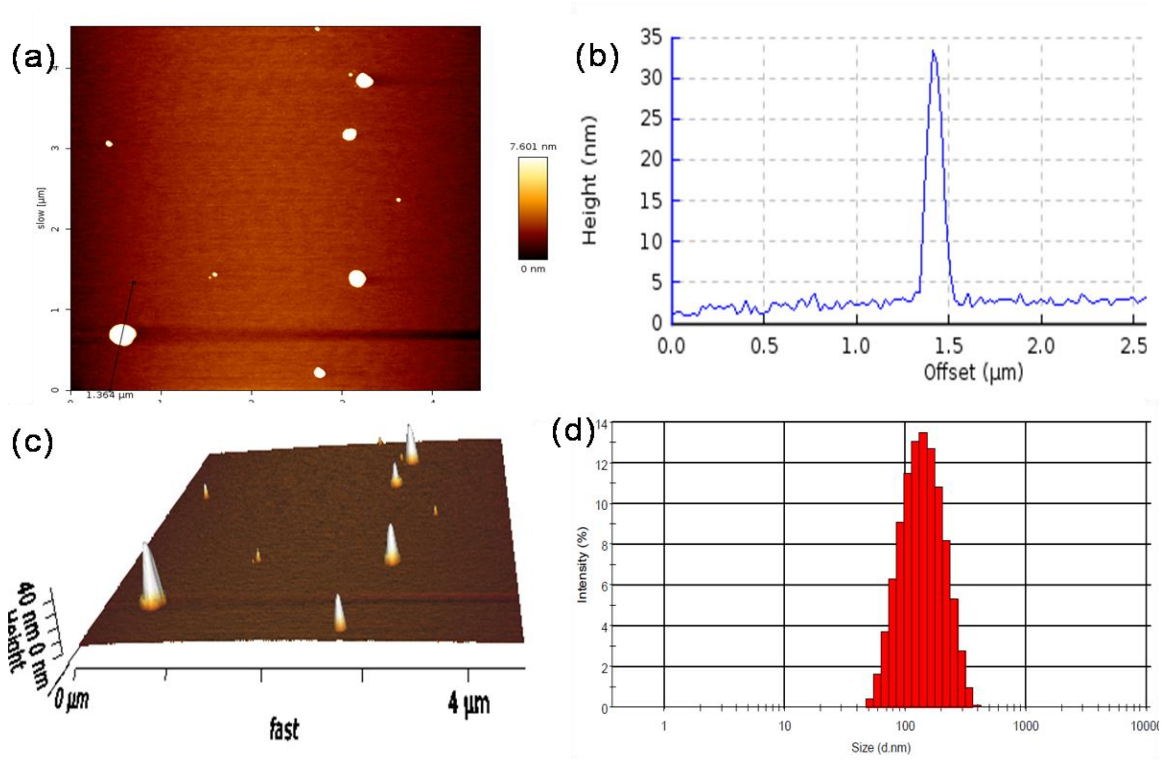


Figure.5. (a-c) Atomic force microscopy image, d) Dynamic light scattering (DLS) histogram (compound **7**)

3.2.2. Synthesis and characterization of NPs (compound 18) (Fig. 6)

We engineered the NPs from the compound **18** (vitaminD3-succinic acid-PI103), phosphatidylcholine (PC) and 1,2-distearoyl-sn-glycero-3-phos-phoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE- PEG_{2000}) in 1:2:0.2 weight ratio using a lipid-film hydration self assembly method. The morphology of the NPs was examined by Field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) (Fig. 6a and 6b). TEM images showed well dispersed, round-shaped NPs that correlated with dynamic light scattering (DLS) measurements. DLS analysis revealed that all these NPs had size distribution 90 to 295 nm with (z-average) mean hydrodynamic diameter of NPs around 150 nm (Fig. 6c). AFM image also supported the existence of spherical shaped NPs (Fig. 6d).

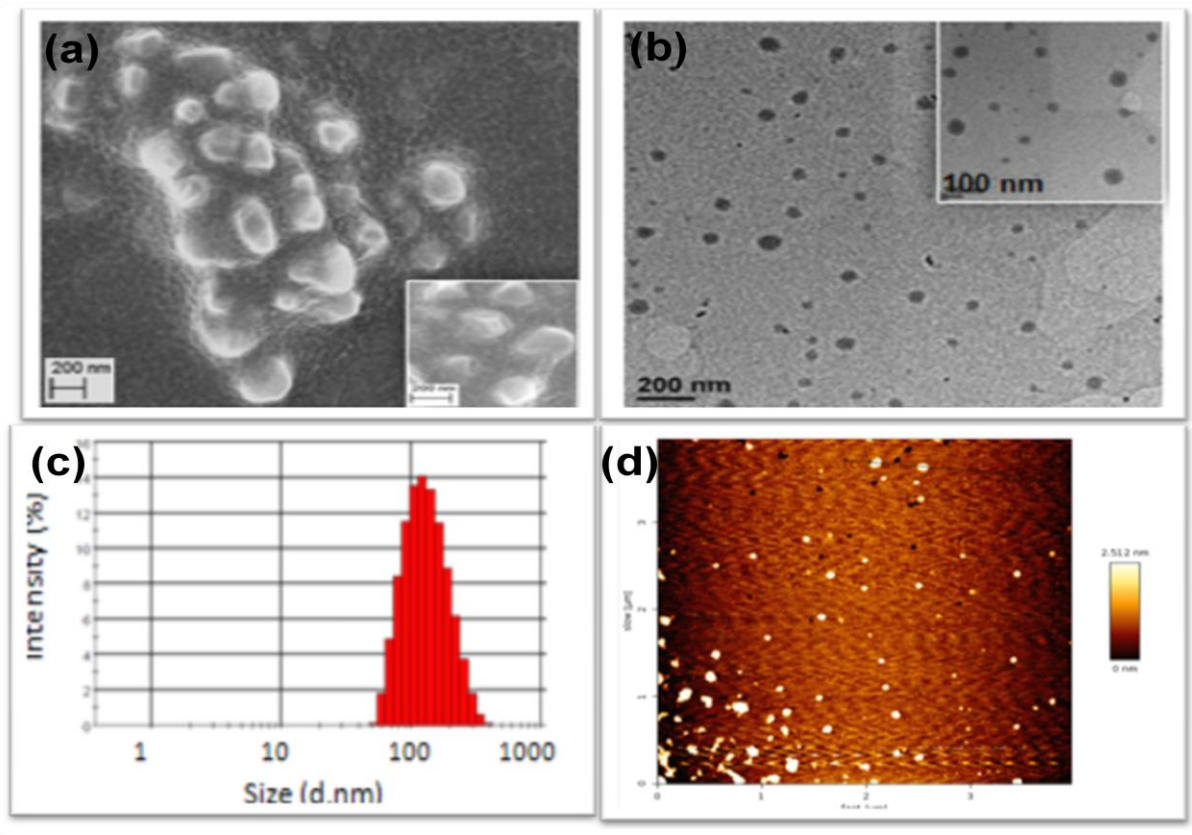


Figure.6.a) FESEM image b) Transmission electron microscopy (TEM) image c) Dynamic light scattering (DLS) histogram d) Atomic force microscopy image of NPs (compound **18**)

3.3 Evaluation of PI103 loading in NPs

For PI103 loading, prepared NPs were dissolved in spectroscopic grade DMSO in 5%, 10% and 15% dilution. Absorbance was measured at 293 nm against the corresponding solvent blank in 400 µL quartz cuvette. Drug loading in NPs of compound **7** and **18** were obtained as follows:

NPs were synthesized using compound **7** and compound **18**, drug loading was 98.18± 11.33 µM, 165.43±18.72 µM respectively.

	NPs from compound **7**	NPs from compound **18**
5%	86.05 µM	148.79 µM
10%	99.99 µM	185.72 µM
15%	108.50 µM	161.79 µM
Mean	98.18 µM	165.43 µM
Std. dev.	11.33 µM	18.72 µM

Table.5. Drug loading in NPs

3.4 Release kinetic studies of PI103 drug from NPs

The *in vitro* release profile of PI103 was obtained at 37°C by representing the percentage released PI103 with respect to the amount of PI103 encapsulated in NPs. The sustained release of PI103 from NPs synthesized from compound **7** and **18** was demonstrated over the period of 112 and 93 h respectively.

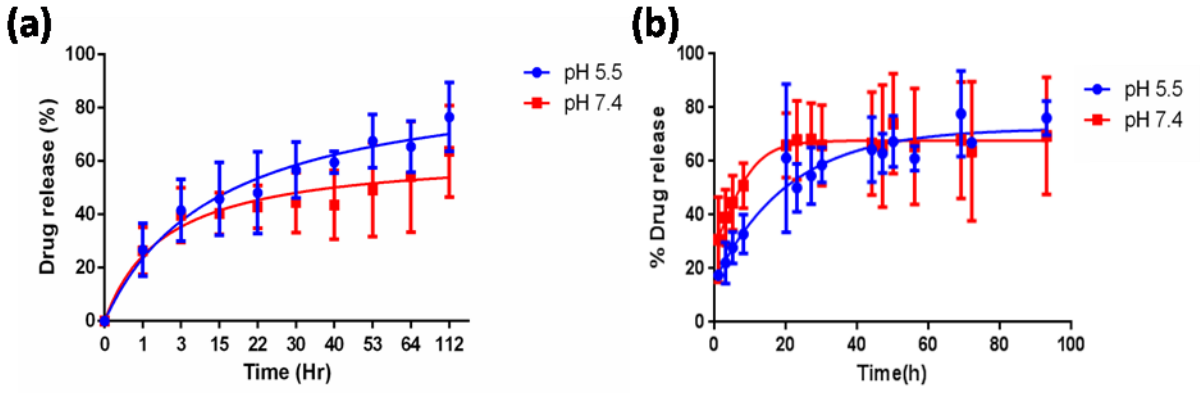


Figure.7. Release kinetics of PI103 drug from NPs, a) compound **7** b) compound **18**

We evaluated the PI103 release profile in two different pH buffer (pH = 7.4 and pH = 5.5). pH = 5.5 and pH 7.4, which mimics the acidic environment, lysosomal component and physiological condition respectively. In pH = 5.5, Keto-lithocholic acid-PI103 NPs showed a sustained release of active PI103 over 112 h, having 63.69 ± 9.89 % (SEM, n=3) release of PI103 at 112 h, whereas at pH = 7.4 similar amount 76.03 ± 7.44 % (SEM, n=3) PI103 was release at 112 h **(Fig. 7a)**. On the other hand, vitaminD3-succinic acid-PI103-NP showed a sustained release of active PI103 over 93h, having 77.74 ± 9.2 % (SEM, n=3) release of PI103 at 69h, whereas at pH = 7.4 similar amount 74.03 ± 10.7 % (SEM, n=3) PI103 was release at 50h (**Fig. 7b**). From the release kinetics it is clear that PI103 was released from the NPs in slow and sustained manner over period of time.

Conclusion

This is the first study wherein, we have successfully demonstrated the synthesis of NPs using biocompatible, biodegradable and non-toxic vitamin-D3 and lithocholic acid. This study serves as a novel nano-platform for synthesis of self-assembled NPs. The size, shape and morphology of the synthesized nanoparticles are compatible with EPR (Enhanced Permeability and Retention) effect for tumor homing. The time dependent release profile in the pH conditions mimicking tumor microenvironment and physiological conditions demonstrates the pH dependent sustained release of the active drug for temporal of PI3K signaling. Biological studies are needed to demonstrate the applicability of these nanocarriers in cancer therapeutics.

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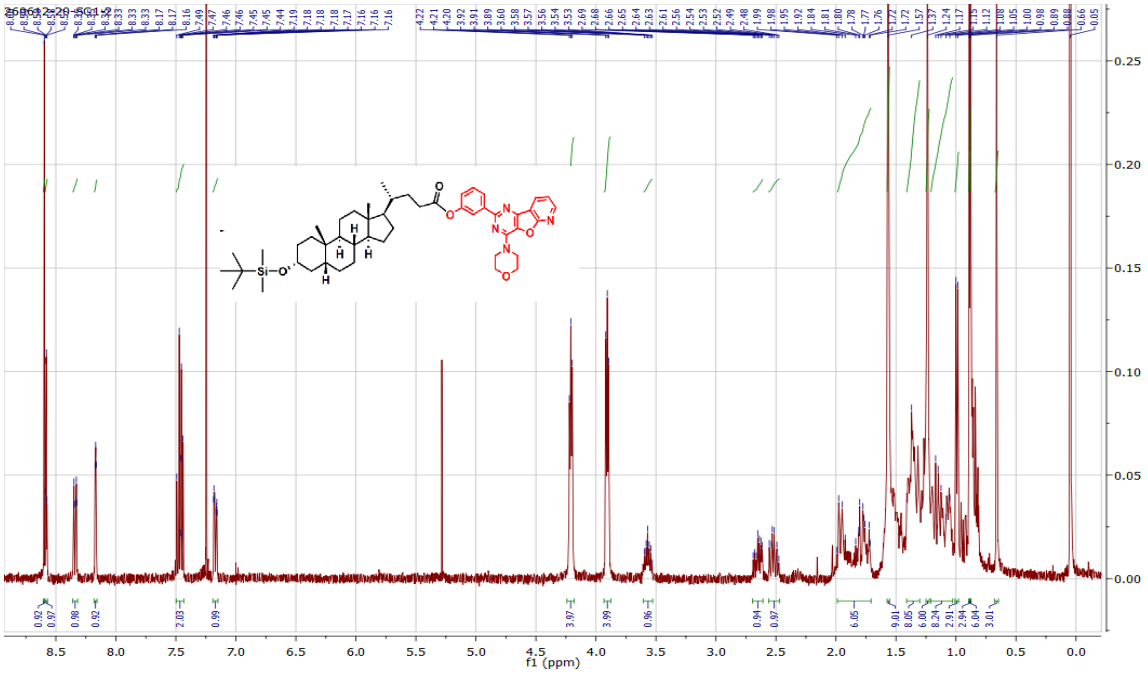
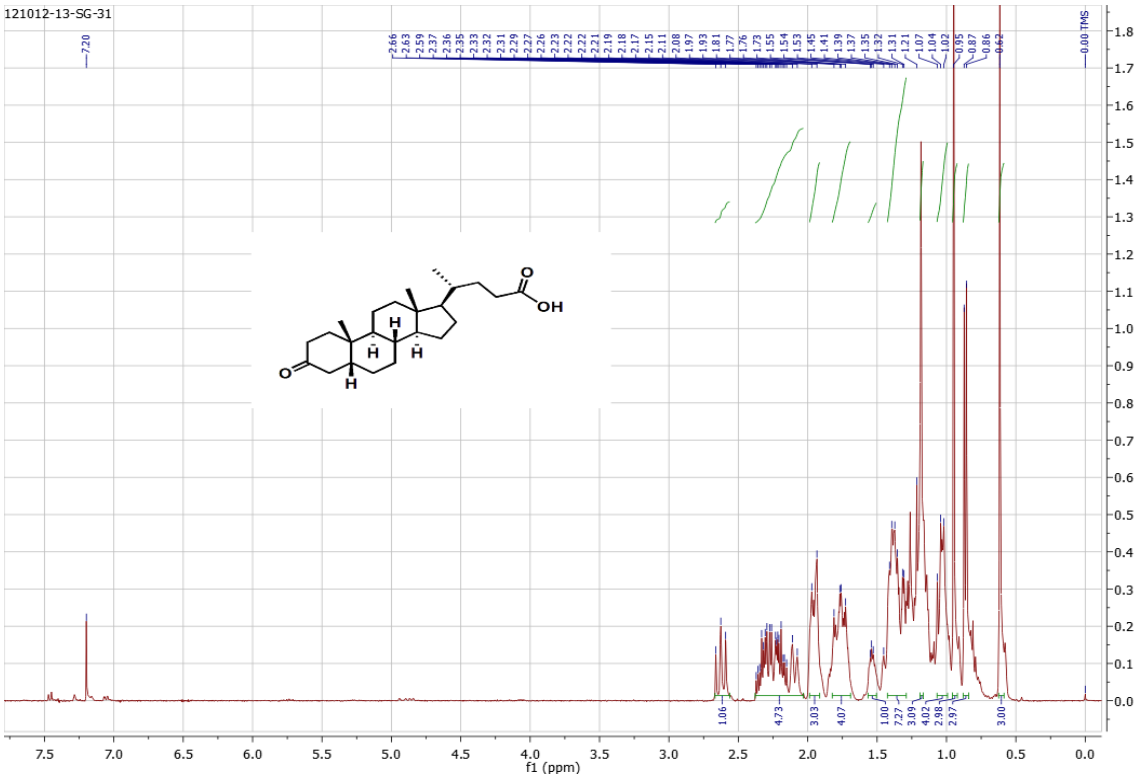
Appendix: Selected Spectrum

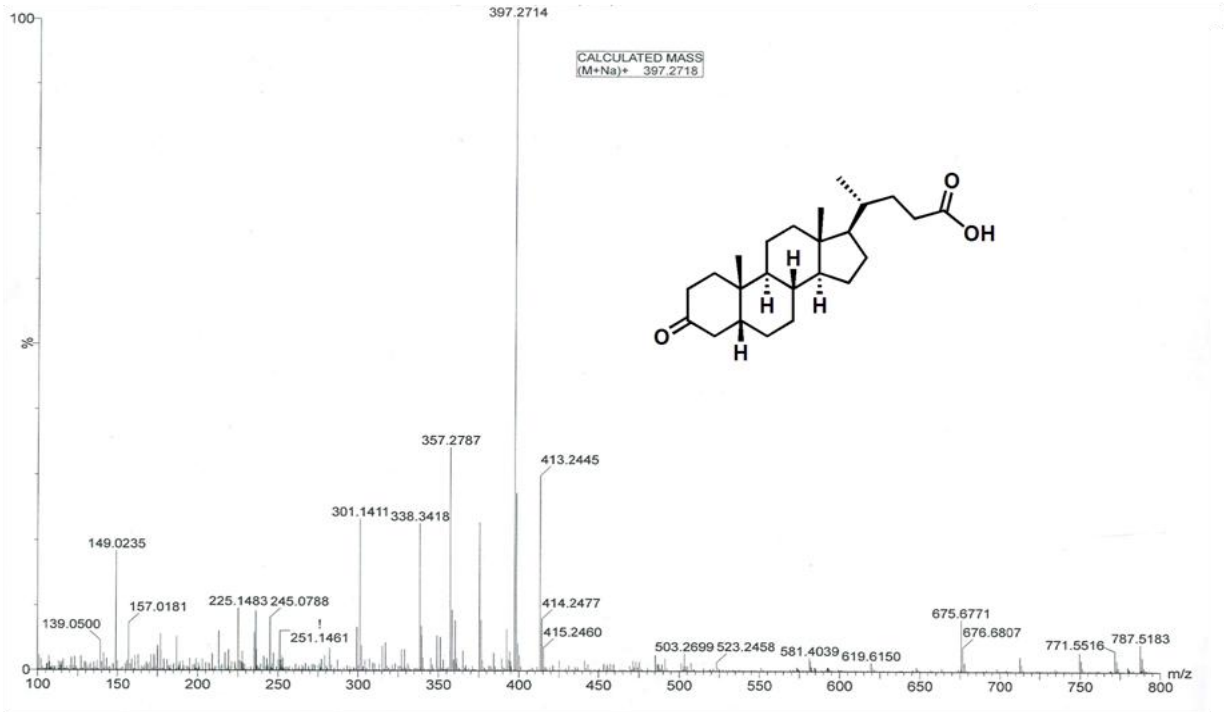
^{1}H NMR (400 MHz, $CDCl_3$)



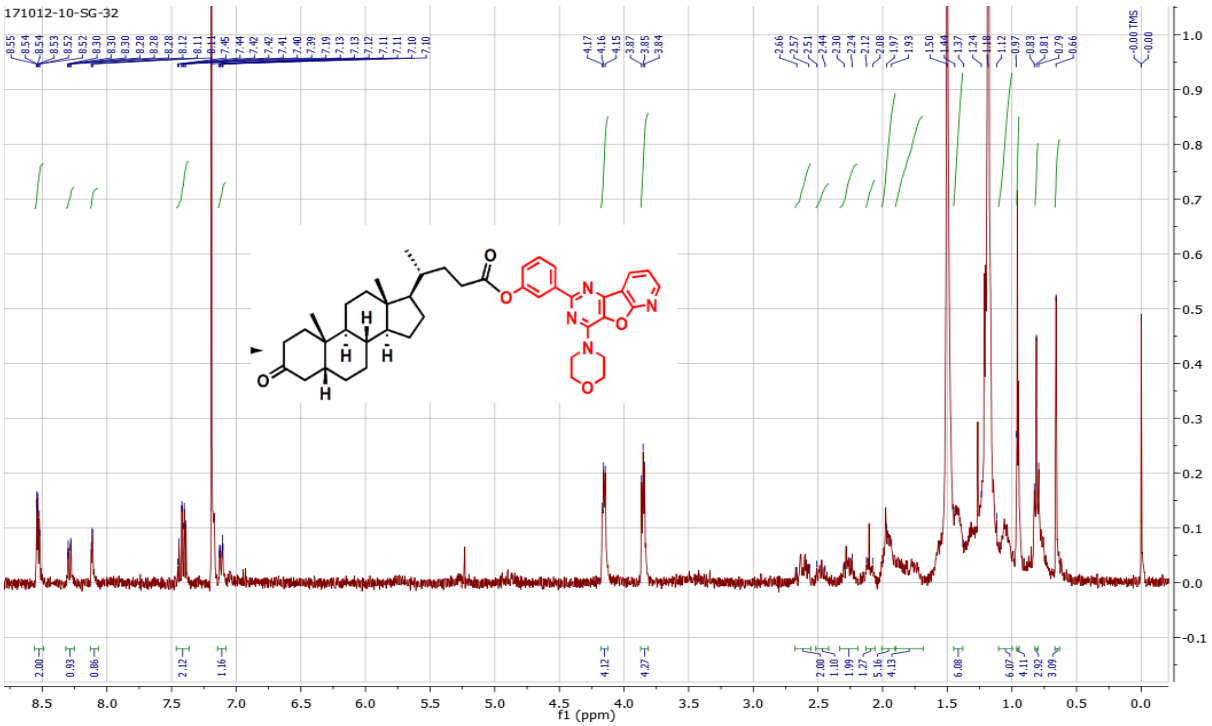
^{1}H NMR (400 MHz, $CDCl_3$)



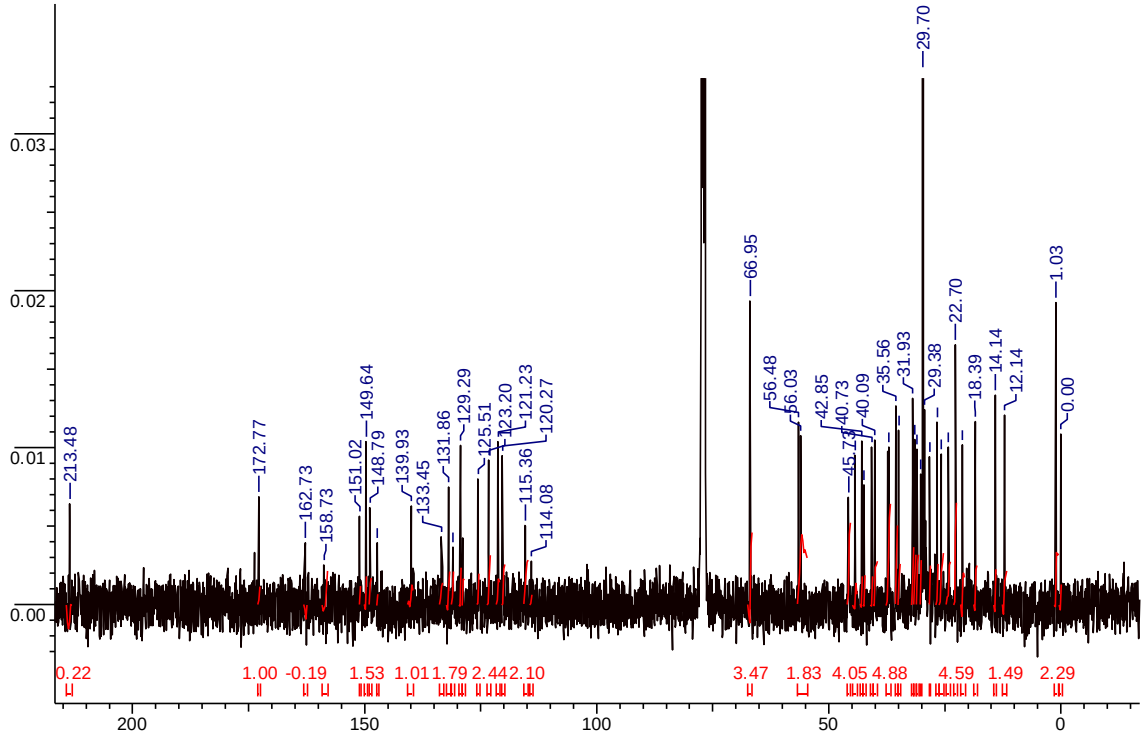
HRMS (ESI)



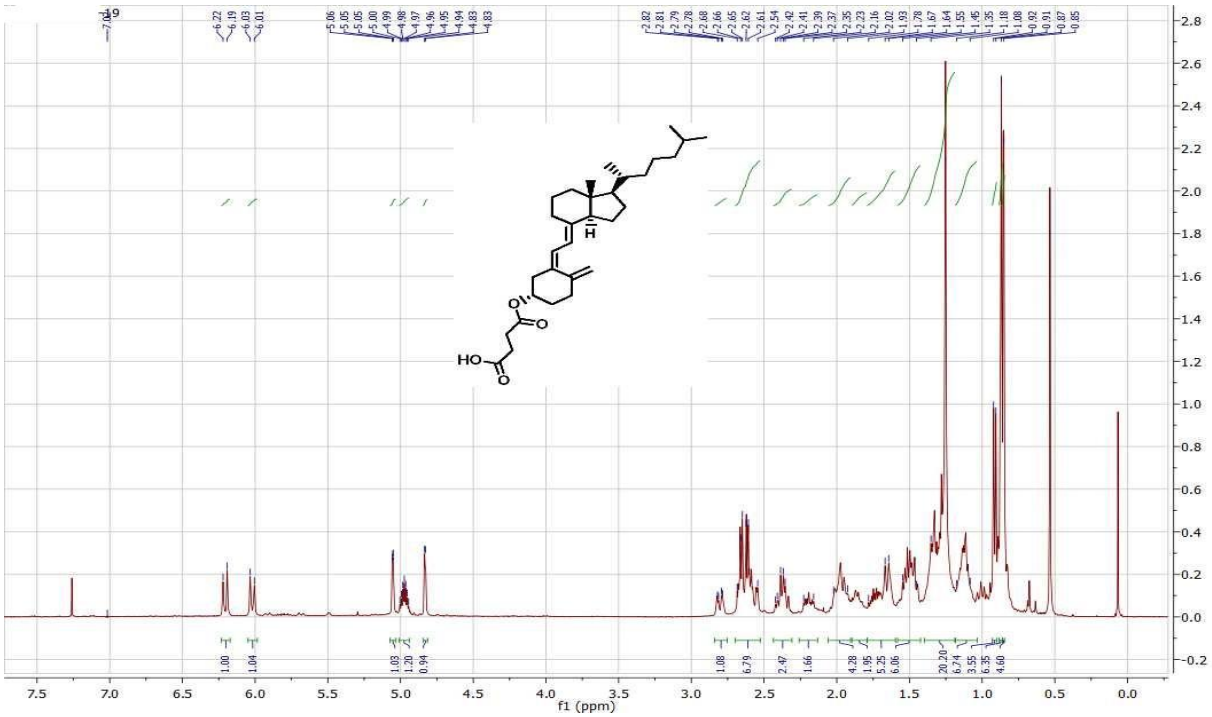
^{1}H NMR (400 MHz, $CDCl_3$)



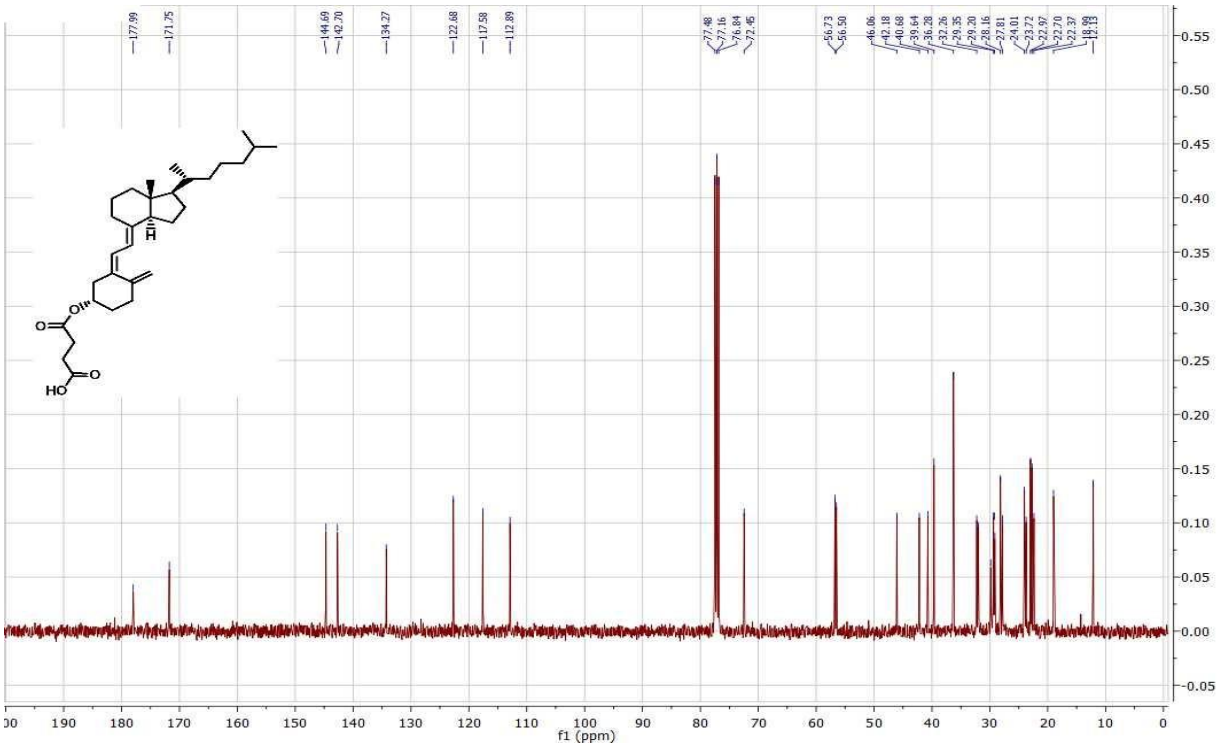
^{13}C NMR (400 MHz, $CDCl_3$) Compound 7 (scheme 1)



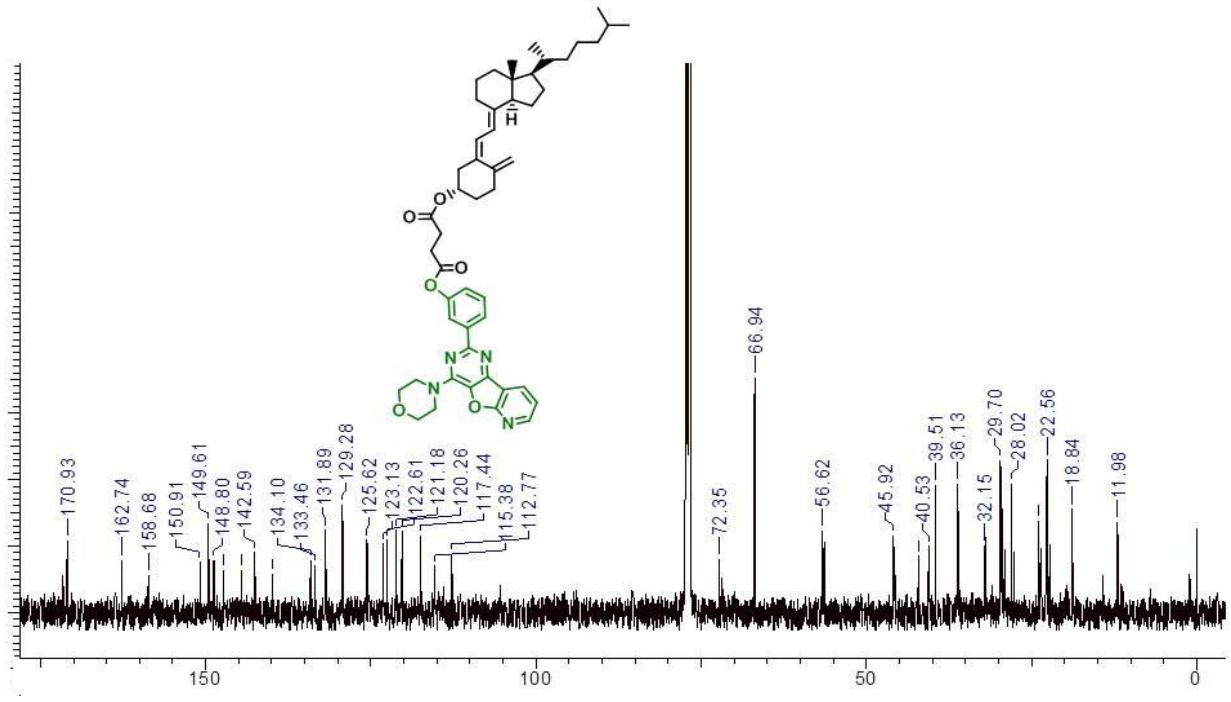
^{1}H NMR (400 MHz, $CDCl_3$)



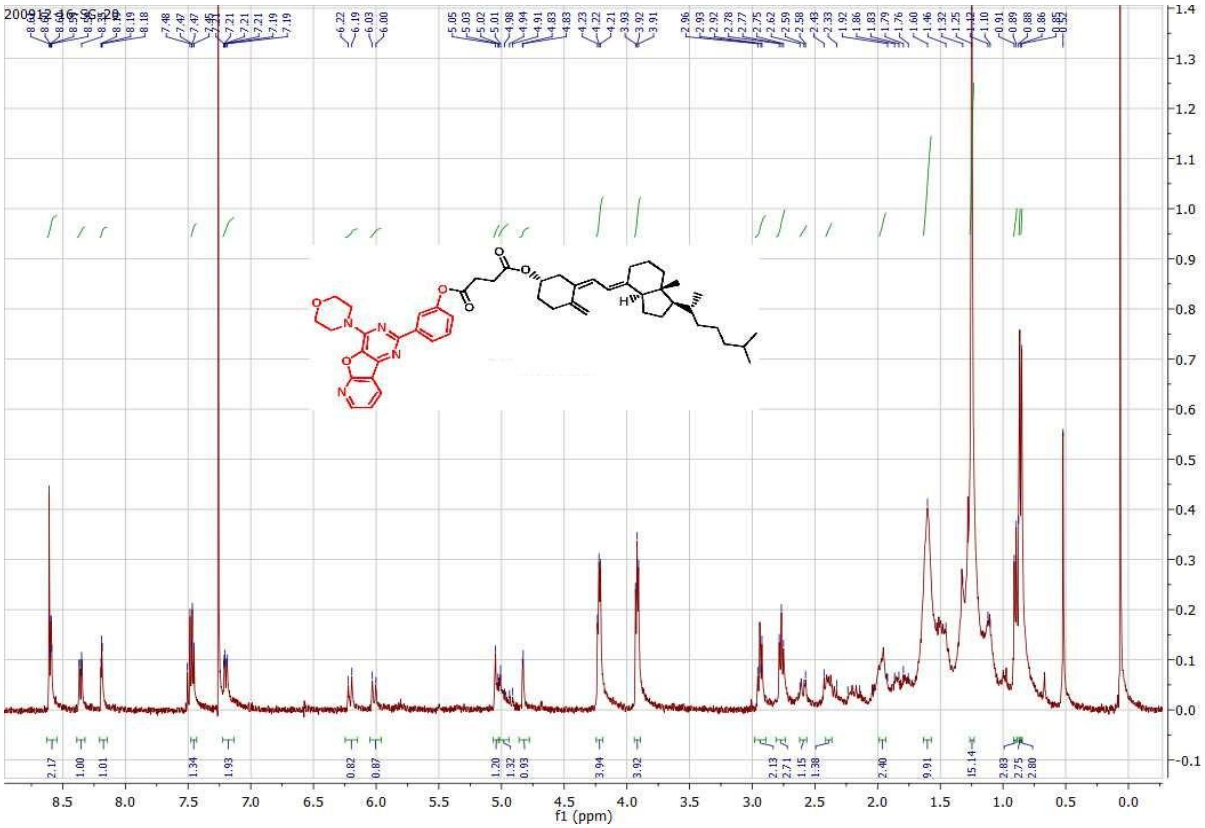
^{13}C NMR (400 MHz, $CDCl_3$)



^{13}C NMR (100 MHz, $CDCl_3$)



^{1}H NMR (400 MHz, $CDCl_3$)



HRMS (ESI)