PREPARATION OF PEG-DERIVATIZED PPMP AS A DUAL-FUNCTIONAL CARRIER FOR ANTICANCER DRUGS

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Our goal is to synthesize PEG-derivatized PPMP as a dual-function carrier for anticancer drugs to co-deliver PPMP and classic anticancer drugs. The clinical application of anticancer drugs has been limited by low water solubility, rapid elimination, lack of tissue-specificity and toxicity. A number of macromolecular delivery systems are under investigation to circumvent these limitations and improve the potential of the anticancer drugs. However, for most delivery systems, vehicles themselves rarely possess pharmacological activity. One interesting approach in the design of a carrier is that components of the carrier system exhibit biological activity, either possessing antitumor effect or sensitizing tumor cells to loaded anticancer drugs. PPMP blocks glucosylation of ceramide, a tumor-suppressor lipid, which induces antiproliferative and apoptotic responses in cancer cells. We conjugate PPMP with PEG to increase solubility. At the same time, amphiphilic PEG_{5K}-$PPMP_2$ could self-assemble into micelles and serve as a carrier for other hydrophobic anticancer drugs. We propose that PEG_{5k}-$PPMP_2$ could be an effective and novel dual functional carrier for anticancer drugs.

In this thesis, the first aim was to examine the efficacy of co-treatment of PPMP with other anticancer drugs (camptothecin and doxorubicin) in different cancer cell lines. *In vitro* cytotoxicity studies showed more effective tumor-killing effect in the combination treatment than single drug treatment. The combination of PPMP and CPT or DOX showed synergism in all of the cancer cell lines examined. The second aim was to synthesize PEG_{5K}-$PPMP_2$ as an amphiphilic prodrug for PPMP and a carrier for other anticancer drugs. PEG_{5K}-$PPMP_2$ was successfully synthesized from Z-D-Ser-OH through a scheme with 12 steps. The products of each steps were confirmed by NMR.

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1. INTRODUCTION

1.1 Limitations of current anticancer drugs.

There are several thousand drugs with anticancer activity. However, only less than 400 anticancer drugs were approved by FDA for use in the clinic. Clinical application of anticancer drugs is limited by problems such as low water solubility, rapid elimination, lack of tissue-specificity and toxicity.[1] More than 40 percent of anticancer drugs listed in WHO are poorly water-soluble, resulting in poor absorption, low bioavailability, local toxicity and rapid elimination.[2] In addition, less than 5 percent of delivered chemotherapeutic drug reaches the tumor site when injected intravenously. The broad tissue distribution of anticancer drugs leads to serious systemic toxicity, involving myelosuppression, mucositis and alopecia.

1.2 New effective delivery systems for anticancer agents are required.

A good delivery system could not only overcome the limitations described above, but also increase the potency of anticancer agents. In the past decade, nanocarriers have emerged as an attractive research field in cancer therapy. Nanocarriers include liposomes, micelles, and nanoparticles[3]. Micelle is an aggregate of amphiphilic molecules dispersed in aqueous solution, with a hydrophilic head outside and hydrophobic tail inside the structure. Polyethylene glycol (PEG) is a safe, non-toxic and biodegradable polymer, has been widely used as the classic hydrophilic head of the micelle. Compared with liposomes or other nanoparticles, micelles have relatively small sizes (10–100 nm)[4], are easy to prepare, and can preferentially accumulate at tumor sites, thereby minimizing undesirable side effects on normal tissues. This is due to the Enhanced Permeability and Retention (EPR) effect [5, 6].

PEG-derivatised micellar system will prevent the recognition and binding of plasma protein, further preventing the rapid elimination of nanoparticles by reticuloendothelial system (RES) [4]. In addition, the versatility of PEG-derivatised micellar system enables it to solubilize and deliver different types of anticancer drugs.

1.3 Dual-functional micelles possessing pharmacological activity are more potent and promising than current biocompatible carriers.

For most current delivery systems, vehicles themselves rarely possess pharmacological activity. The use of “inert” excipients that lack therapeutic activity not only adds to the cost, but also potentially imposes safety issues. [7] One interesting approach in the design of a carrier is that components of the carrier system exhibit biological activity, either possessing antitumor effect or sensitizing tumor cells to loaded anticancer drugs. Anticancer drug encapsulation with a biologically active carrier is a more attractive strategy than simple drug combination as it could deliver multiple agents working at several signaling pathways to the tumor site at the same time.[8] By applying dual-functional carrier, we could reach the effect of counteracting the side effects caused by the loaded anticancer drugs and/or promoting synergistic effects with the incorporated drugs. [9]

1.4 Overproduction of ceramide, a p53-independent pro-death lipid, is considered as an ideal pharmacologic function of carriers.

Classic chemotherapeutic drugs directly or indirectly damage DNA, leading to cell death mostly via p53-dependent apoptosis. Tumor cells that do not have functional p53 show, at best, modest responses to p53-dependent chemotherapeutic agents. Thus, developing a

carrier, which is cytotoxic but in a p53-independent maner, offers the potential to by-pass the drug-resistance and to minimize side effects.[10]

Ceramide, also called tumor suppressor lipid, is involved in cell apoptosis, growth arrest, differentiation, and senescence. Ceremide works by targeting Cathespsin D, ceramide-activated protein phosphatases, and kinase suppressors of RAS[11]. Effects towards the overproduction of ceramide in tumor cells are one direction of cancer therapy.[12] Chemotherapeutics and radiation treatment will induce ceramide accumulation. [13-15] However, tumor cells could simultaneously up-regulate the metabolite pathways of ceramide. Glucosylceramide synthase (GCS), which clears ceramide level by incorporating it into glucosylceramide, is one major elimination pathway. [16-18]Inhibition of GCS increases ceramide response and restores sensitivity of tumor cells to the action of anticancer drugs.

PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1- propanol) is a potent inhibitor of GCS. PPMP also inhibits l-O-acylceramide synthase, another minor metabolite pathway. The combination of PPMP and other anticancer drugs such as Paclitaxel[19], 4-HPR[20], Irinotican[19] has been reported to lead to increased ceramide level and cytotoxicity on tumor cells, such as those derived from neuroblastoma and melanoma and prostate, lung, colon, breast and pancreatic cancers. Hence, co-delivery of PPMP and other anticancer agents should be able to increase the efficacy of treatment, by restoring resistance to anticancer agents.

1.5 PEG_{5k}-$PPMP_2$ micellar system is an ideal carrier to co-deliver PPMP and other anticancer drugs.

PPMP is poorly water-soluble and PEGylation is designed to improve its solubility. At the same time, PEG-PPMP conjugates are amphiphilic molecules and form micelles, which can be used to carry hydrophobic anticancer drugs. PEG-coated micelles can avoid the rapid degradation and elimination by RES. In addition, the small size of micellar system (<100nm) leads to passive targeting to the tumor sites via EPR effect and further reduces the broad distribution and off-target toxicity of the anti-cancer agent. Most importantly, PEG_{5k}-$PPMP_2$ micellar system can deliver PPMP and other anticancer drugs to the tumor cells simultaneously, thus increasing the efficacy of treatment. Hence, PPMP blocks the activity of GCS that is upregulated during cancer treatment. In addition, the mechanism of PPMP is different from the action of common anticancer drugs, thereby enabling a synergistic effect of PPMP with other anticancer agents.

1.6 Overview of thesis

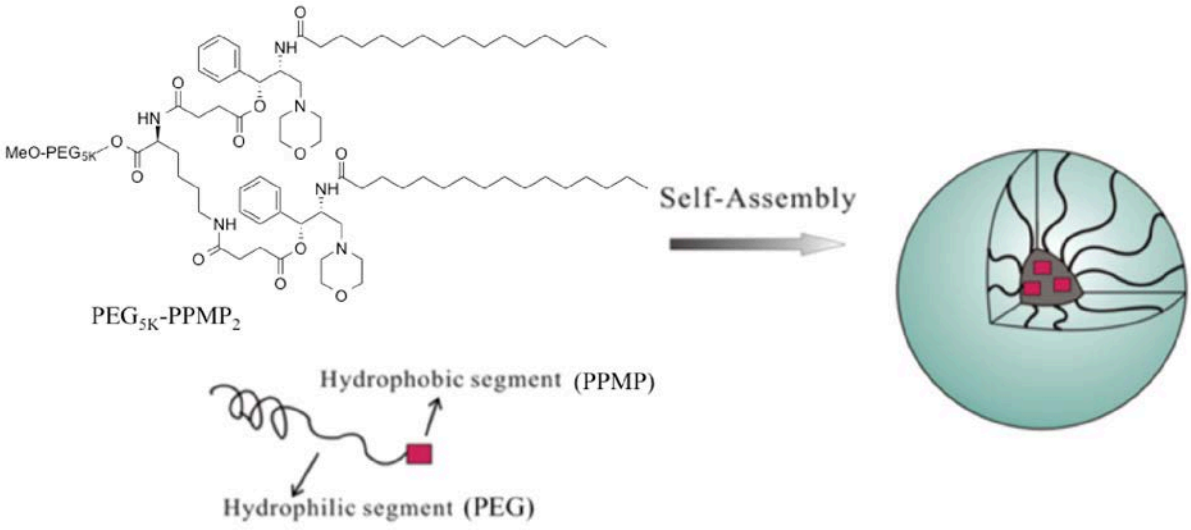


Figure 1. Overview of PEG_{5K}-$PPMP_2$ conjugate as dual-functional carrier.

In this thesis, we firstly examined whether the co-administration of PPMP and other anticancer drugs showed synergy in various cancer cell lines. Then, PEG_{5K}-$PPMP_2$ was synthesized from Z-D-Ser-OH through 12 steps to serve as the prodrug of PPMP and a carrier for other anticancer drugs.

2. MATERIALS AND METHODS

2.1 Materials

(Benzyloxy)carbonyl)-*D*-serine (Z-D-Ser-OH),1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl) and (S)-2,6-Bis-tert-butoxybonylaminohexanoic acid (Boc-lys(Boc)-OH) were purchased from GL Biochem (Shanghai, China). N,O-dimethylhydroxylamine hydrochloride was purchased from Oakwood Chemical (SC, USA). *Tert*-butyldimethylsilyl chloride and sodium carbonate were purchased from Thermo Fisher Scientific Inc (MA, USA). Phenylmagnesium bromide solution and succinic anhydride were purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). N-methyl morpholine (NMM), imidazole, L-Selectride solution, potassium sodium tartrate, methanosulfonyl chloride, morpholine, acetic acid, pyridine, sodium hydroxide, palmitic acid, poly(ethylene glycol) methyl ether (MeO−PEG−OH, MW = 5000 kDa), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT), and Dulbecco's Modified Eagle's Medium (DMEM) were all purchased from Sigma-Aldrich (MO, U. S. A.). Fetal bovine serum (FBS) and penicillin−streptomycin solution were purchased from Invitrogen (NY, U. S. A.). Dicyclohexylcarbodiimide (DCC) was purchased from Alfa Aesar (MA, U. S. A.). 4- (dimethylamino) pyridine (DMAP) was purchased from Calbiochem-Novabiochem Corporation (CA, U. S. A.). All solvents used in this study were HPLC grade.

2.2 Cell Culture

HCT-116 is a human colon carcinoma cell line. PC-3 is human prostate cancer cell line. 4T1.2 is a mouse metastatic breast cancer cell line. All cell lines were cultured in DMEM

containing 5% FBS and 1% penicillin−streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.3 Cytotoxicity Assay

HCT-116 (2000 cells/well), PC-3 (5000 cells/well) and 4T1 (1000 cells/well) were seeded in 96-well plates followed by 24 h of incubation in DMEM with 10% FBS and 1% streptomycin/penicillin. Then, various concentrations of free PPMP, free CPT or DOX, and the combination of both respectively were added in triplicate and cells were incubated for 72 h. After removing the medium in the plates, 100 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in PBS (0.5 mg/mL) was added and cells were further incubated for 4 h. The medium was then removed and MTT formazan was solubilized by DMSO. The absorbance of each well was measured by the microplate-reader, with wavelength at 550 nm and reference wavelength at 630 nm. Untreated groups was served as controls. Cell viability was calculated as $[(OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%]$.

2.4 Synthesis of PEG_{5k}-$PPMP_2$ Conjugate

Figure 2 shows the synthesis scheme of PEG_{5k}-$PPMP_2$ conjugate.

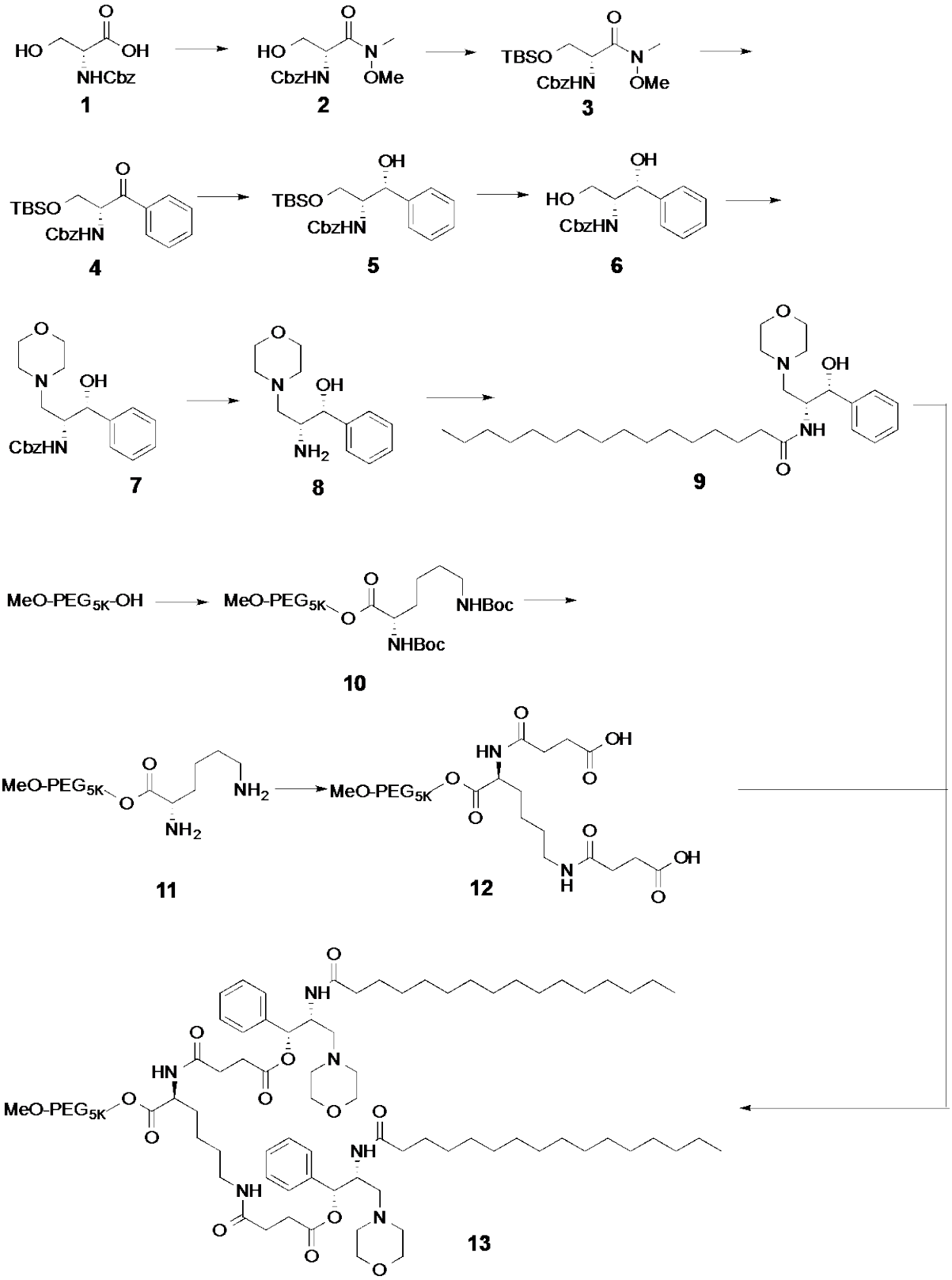


Figure 2. Synthesis scheme of PEG_{5K}-$PPMP_2$ conjugate.

2.4.1 Synthesis of PPMP

Step 1: Preparation of **Compound 2** [benzyl (*R*)-(3-hydroxy-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate]

N,O-dimethylhydroxylamine hydrochloride (1.5 g, 15.4 mmol, 1.5 eq), N-methyl morpholine (2.9 mL, 2.7 g, 26.3 mmol, 2.5 eq) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI•HCl, 2.1 g, 11.0 mmol, 1.05 eq) were added slowly to a cosuspension of ((benzyloxy)carbonyl)-*D*-serine (2.5g, 10.5 mmol, 1.0 eq) in DCM (methylene dichloride, 50 mL). The mixture was stirred for 5 hours at room temperature and quenched with HCL (40 mL, 1 M, 40 mmol). The aqueous layer was extracted with EtOAc (ethyl acetate, 300 mL). Then the organic layer was washed by saturated brine and concentrated on a rotary evaporator. The residue was chromatographed with pure EtOAc on silica gel to afford the **Compound 2** (2.4 g, 8.5mmol, 81% yield).

Step 2: Preparation of **Compound 3** [benzyl (*R*)-(3,8,8,9,9-pentamethyl-4-oxo-2,7-dioxa-3-aza-8-siladecan-5-yl)carbamate]

TBSCl (*tert*-butyldimethylsilyl chloride, 1.9 g, 12.6 mmol, 1.5 eq) was added to the solution of benzyl (*R*)-(3-hydroxy-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (2.4 g, 8.5mmol, 1.0 eq) in DCM (methylene dichloride, 40 mL) followed by imidazole (1.4 g, 21.3mmol, 2.5 eq). The mixture was stirred at room temperature for 12 hours and quenched with water (20mL). The aqueous layer was extracted with DCM (methylene dichloride, 300 mL). Then the organic layer was washed by saturated brine and concentrated on a rotary evaporator. The residue was chromatographed (1:2 EtOAc/PE) on silica gel to afford the **Compound 3** (2.5 g, 6.3 mmol, 74% yield).

Step 3: Preparation of **Compound 4** [benzyl (*R*)-(3-((*tert*-butyldimethylsilyl)oxy)-1-oxo-1-phenylpropan-2-yl)carbamate]

Benzyl (*R*)-(3,8,8,9,9-pentamethyl-4-oxo-2, 7-dioxa-3-aza-8-siladecan-5-yl) carbamate (2.5 g, 6.3 mmol, 1.0 eq) dissolved in THF (tetrahydrofuran, 22 mL) was cooled down to 0 °C. Phenylmagnesium bromide (9.5 mL of 1M solution in THF, 9.5 mmol, 1.5 eq) was added dropwise. The mixture was stirred at 0 °C for 3 h followed by stirring at room temperature overnight and quenched by hydrogen chloride (10 mL, 1M, 10 mmol). The aqueous layer was extracted with EtOAc (ethyl acetate, 300 mL). Then the organic layer was washed by saturated brine and concentrated on a rotary evaporator. The residue was chromatographed (1:9 EtOAc/PE) on silica gel to give the **Compound 4** (2.4 g, 5.8 mmol, 92% yield).

Step 4: Preparation of **Compound 5**: [benzyl ((1*R*,2*R*)-3-((*tert*-butyldimethylsilyl)oxy)-1-hydroxy-1-phenylpropan-2-yl)carbamate]

Benzyl (*R*)-(3-((*tert*-butyldimethylsilyl)oxy)-1-oxo-1-phenylpropan-2-yl)carbamate (2.4 g, 5.8 mmol, 1.0 eq) dissolved in THF (tetrahydrofuran,17 ml) was cooled down to -70 ^{0}C. L Selectride (12 mL of 1M solution in THF, 12 mmol, 2.0 eq) was added dropwise. The mixture was stirred at -70 ^{0}C for 3 h and quenched with an aqueous solution of potassium sodium tartrate (13 mL, 1M, 13 mmol). The aqueous layer was extracted with EtOAc (ethyl acetate, 300 mL). Then the organic layer was washed by saturated brine and concentrated on a rotary evaporator. The product was purified by column chromatography using 1:4 EtOAc/PE to afford **Compound 5** (1.9 g, 4.5 mmol, 78% yield).

Step 5: Preparation of **Compound 6**: benzyl ((1*R*, 2*R*)-1,3-dihydroxy-1-phenylpropan-2-yl) carbamate

Benzyl ((1*R*, 2*R*)-3-((*tert*-butyldimethylsilyl) oxy)-1-hydroxy-1-phenylpropan-2-yl) carbamate (1.9 g, 4.5 mmol) was dissolved in a 15 mL mixture of acetic acid/THF/ water (3/1/1). The mixture was stirred at room temperature overnight and quenched by potassium carbonate (20 g). The crude was evaporated and chromatographed (1:1 EtOAc/PE) on silica gel to give the **Compound 6** (1.0 g, 3.3 mmol, 75% yield).

Step 6: Preparation of **Compound 7** [benzyl ((1*R*, 2*R*)-1-hydroxy-3-morpholino-1-phenylpropan-2-yl)carbamate]

Benzyl ((1*R*, 2*R*)-1,3-dihydroxy-1-phenylpropan-2-yl)carbamate (1.0g, 3.3mmol, 1.0 eq) was dissolved in 7 mL pyridine and cooled to 0 ^{0}C. Methanosulfonyl chloride (0.26 mL, 378mg, 3.3mmol, 1.0 eq) was added to the mixture and stirred for 2 h. Then morpholine (2.9g, 33mmol, 10.0 eq) was added and the mixture was stirred at 50 ^{0}C overnight. The crude was evaporated and the residue was partitioned between EtOAc (ethyl acetate, 300 mL) and hydrogen chloride (20 mL, 12M, 120 mmol). Then the organic layer was washed by saturated brine and concentrated on a rotary evaporator. The product was purified by column chromatography using pure EtOAc on silica gel to give the **Compound 7** (990 mg, 2.7 mmol, 81% yield).

Step 7: Preparation of **Compound 8** [(1*R*,2*R*)-2-amino-3-morpholino-1-phenylpropan-1-ol]

Benzyl ((1*R*, 2*R*)-1-hydroxy-3-morpholino-1-phenylpropan-2-yl)carbamate (990mg, 2.7mmol) was dissolved in a 10 mL mixture of aqueous NaOH (40%in weight)/methanol solution. The mixture was heated to 150 ^{0}C in the microwave for about 15 minutes. The crude was evaporated and the residue was partitioned between EtOAc (ethyl acetate, 300 mL) and hydrogen chloride (20 mL, 12M, 120 mmol). After concentrating, the crude was

chromatographed using 15:4:1 DCM/MeOH/NH_4OH on silica gel to afford **Compound 8** (479 mg, 2.03 mmol, 76% yield).

Step 8: Preparation of **Compound 9** [*N*-((1*R*,2*R*)-1-hydroxy-3-morpholino-1-phenylpropan-2-yl)palmitamide]

(1*R*, 2*R*)-2-amino-3-morpholino-1-phenylpropan-1-ol (479mg, 2.0mmol, 1.0 eq), palmitic acid (1.0 g, 4.0mmol, 2.0 eq), dicyclohexylcarbodiimide (DCC, 836mg, 4.0 mmol, 2.0 eq) and DMAP (146mg, 1.2 mmol, 0.6 eq) were dissolved in DCM and stirred at room temperature overnight. The mixture was filtered through cotton and the filtrate was concentrated on a rotary evaporator. The residue was chromatographed (1:4 EtOAc/PE) on silica gel to afford the **Compound 9** (617 mg, 1.3 mmol, 65% yield).

2.4.2 Conjuation of PPMP with PEG_{5K}

Step 1: Preparation of **Compound 10** (MeO-PEG_{5k}-di-Boc-lysine)

MeO-PEG_{5k}-OH (5g, 1 mmol, 1.0 eq), (S)-2,6-Bis-tert-butoxycarbonylaminohexanoic acid (Boc-Lys(Boc)-OH) (865mg, 2.5 mmol, 2.5 eq), DCC (309 mg, 1.5 mmol, 1.5 eq) and DMAP (37mg, 0.3 mmol, 0.3 eq) were dissolved in DCM and stirred at room temperature for two days. The mixture was filtered through cotton and then purified through two cycles of dissolution/reprecipitation with DCM/ether and DCM/ethanol respectively to afford **Compound 10** (4.5 g, 0.8 mmol, 84% yield).

Step 2: Preparation of **Compound 11** [MeO-PEG_{5k}-lysine-$(NH_2)_2$]

MeO-PEG_{5k}- di-Boc-lysine (4.5g, 0.8 mmol) was dissolved in 20 mL mixture of trifluoroacetic acid (TFA) (50% in volume)/DCM. The mixture was stirred for 2 hours at room temperature and purified through two cycles of dissolution/reprecipitation with DCM/ether to give **Compound 11** (4.0 g, 0.8 mmol, 97% yield).

Step 3: Preparation of **Compound 12** [MeO-PEG_{5k}-$(COOH)_2$]

MeO-PEG_{5k}-lysine-$(NH_2)_2$ (4.0 g, 0.8 mmol, 1.0 eq), succinic anhydride (400 mg, 4.0 mmol, 5.0 eq), DMAP (488 mg, 4.0 mmol, 5.0 eq) were dissolved in 20 mL DCM and refluxed for 2 days at 60 ^{0}C. The mixture was filtered through cotton and then purified through two cycles of dissolution/reprecipitation with DCM/ether and DCM/ethanol respectively to afford **Compound 12** (3.8 g, 0.7 mmol, 91% yield).

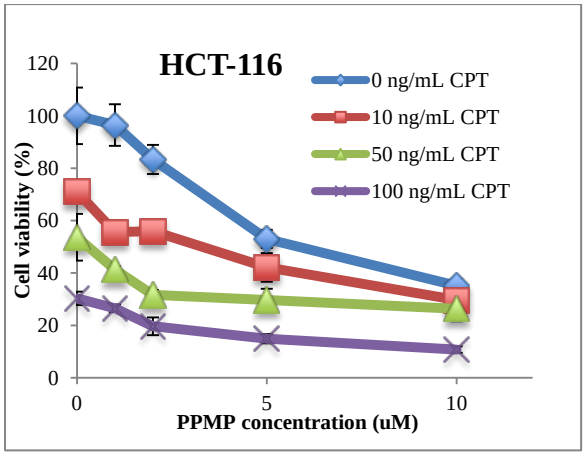
Step 4: Preparation of **Compound 13** (PEG_{5k}-$PPMP_2$)

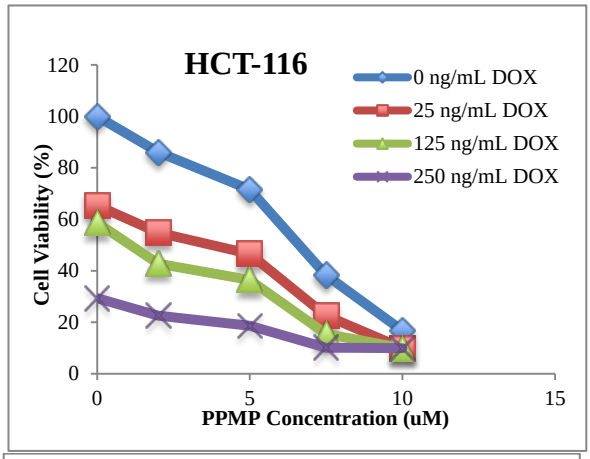
MeO-PEG_{5k}-$(COOH)_2$ (compound 12, 1.1g, 0.2 mmol), *N*-((1*R*,2*R*)-1-hydroxy-3-morpholino-1-phenylpropan-2-yl)palmitamide (PPMP, 617mg, 1.3 mmol), DCC (402 mg, 2.0 mmol) and DMAP (10 mg, 0.06 mmol) were dissolved in DCM and stirred at room temperature for two days. The mixture was filtered through cotton and then purified through two cycles of dissolution/reprecipitation with DCM/ether and DCM/ethanol respectively to afford **Compound 13** (872 mg, 0.1 mmol, 92% yield).

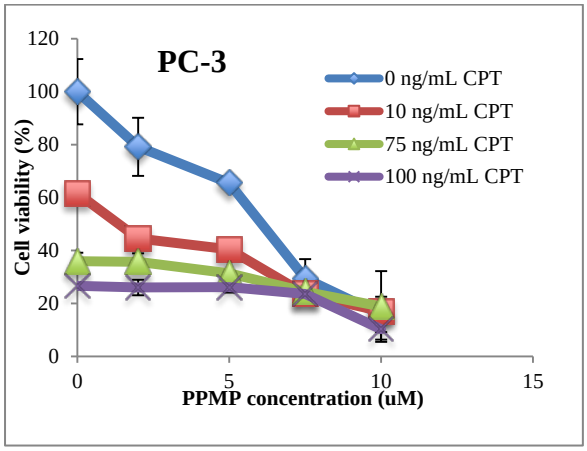
3. RESULT

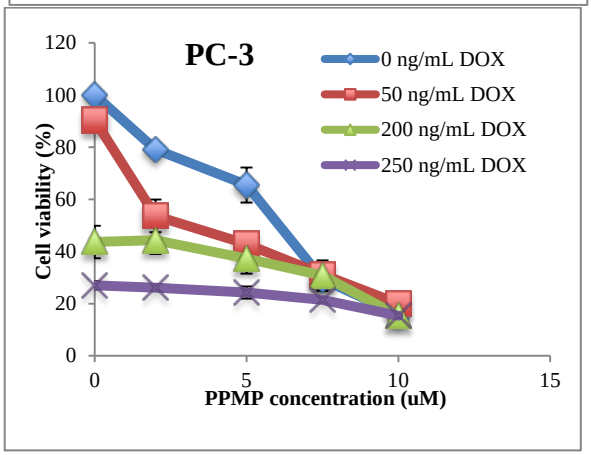
3.1 Combinational Effect of PPMP and Other Anticancer Drugs on Cancer Cell Growth

The cytotoxicity of co-treatment of PPMP and other anticancer drugs (Camptothecin or Doxorubicin) was assessed in three cancer cell lines including HCT-116 (human colon), PC-3 (human prostate) and 4T1.2 (mouse breast). When three cell lines were treated with PPMP, CPT or DOX alone, there was a dose-dependent inhibition of cell proliferation. When the concentration of CPT or DOX was fixed and the concentration of PPMP in co-treatment was gradually increased, the growth-inhibition effect was progressively increased with increasing PPMP concentration, indicating more effective tumor-killing effect in the combination treatment.









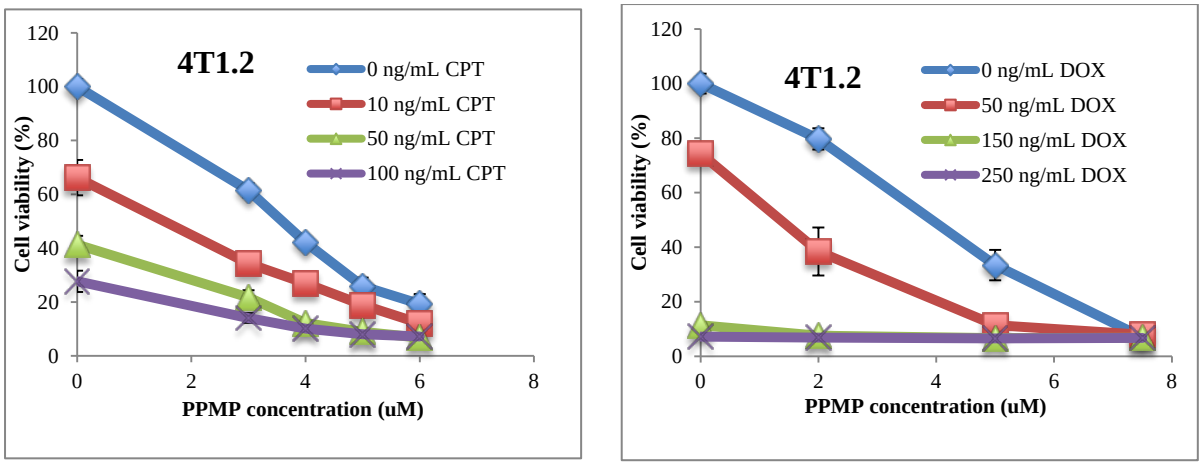


Figure 3. Synergistic action between PPMP and CPT or DOX in inhibiting the proliferation of tumor cells. Various types of tumor cells including HCT116, PC3 and 4T1.2 cells were treated with different concentrations of free PPMP, free CPT or DOX, and combination of both respectively for 96 h, and the cytotoxicity was determined by MTT assay. The experiment was performed in triplicate and repeated three times.

Table 1 compares the cytotoxicity (IC_{50}) of single treatment and that of combination treatment. It is apparent that the combination of PPMP and CPT or DOX showed significant synergy in all of the cancer cell lines examined.

Drug Combination	Cell Line	d1(μM)	D_{50}1(μM)	d2(ng/mL)	D_{50}2(ng/mL)	CI
PPMP+CPT	HCT-116	3.5	7.0	10.0	55.3	0.68
PPMP+CPT	PC-3	1.8	6.1	10.0	42.2	0.53
PPMP+CPT	4T1.2	1.7	3.7	10.0	38.1	0.72
PPMP+DOX	HCT-116	3.8	7.2	25.0	170.0	0.67
PPMP+DOX	PC-3	2.0	6.0	50.0	175.4	0.62
PPMP+DOX	4T1.2	1.3	3.8	50.0	91.2	0.89

Table 1. Combination Index (CI) of simultaneous treatment of PPMP and CPT or DOX in HCT-116, PC-3 and 4T1.2 cells. Cells were treated with a combination of PPMP and CPT or DOX and cell viability was determined by MTT assay. The CI was calculated by the formula: $CI=(d1/D_{50}1)+(d2/D_{50}2)$, where $D_{50}1$ is the concentration of PPMP required to produce 50% effect alone, and d1 is the concentration of PPMP required to produce the same 50% effect in combination with d2. $D_{50}2$ is similarly the concentration of CPT or DOX required to produce 50% effect alone, and d2 is the concentration of CPT or DOX required to produce the same 50% effect in combination with d1. The CI values are interpreted as follows: <1.0, synergism; 1.0, additive; and >1.0, antagonism. Each experiment was done in triplicate. Data are presented as means ± standard deviation.

3.2 Synthesis of PEG_{5k}-$PPMP_2$ Conjugate

Compound 2: ^{1}H NMR (400 MHz, DMSO) δ=7.2-7.4 (m, 5H), 5.1(q, 2H), 4.8 (broad s, 1H), 3.8-3.9 (m, 2H), 3.8 (s, 3H), 3.2 (s, 3H).

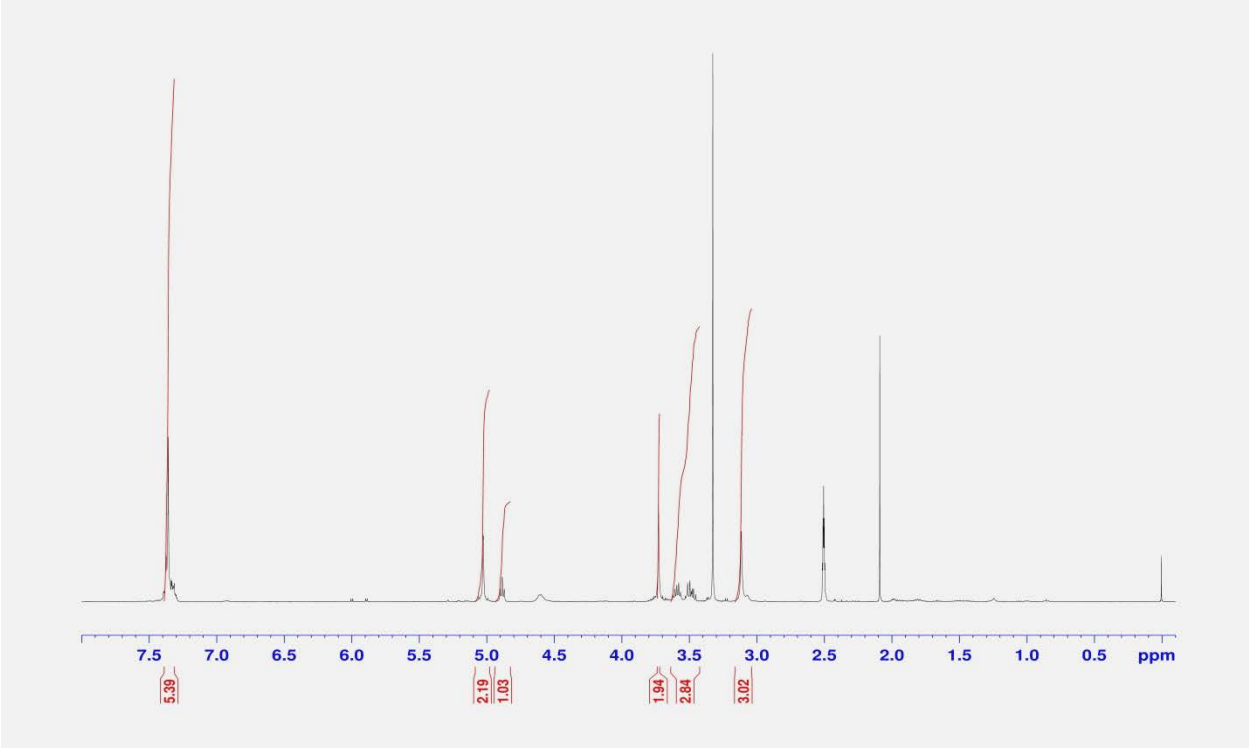


Figure 4. ^{1}H NMR of Compound 2.

Compound 3: ^{1}H NMR (400 MHz, DMSO-d6) δ=7.2-7.4 (m, 5H), 5.1(q, 2H), 4.8 (broad s, 1H), 3.7-3.8 (m, 3H), 3.6-3.7 (m, 2H), 3.2 (s, 3H), 0.9 (s, 9H), 0 (s, 6H).

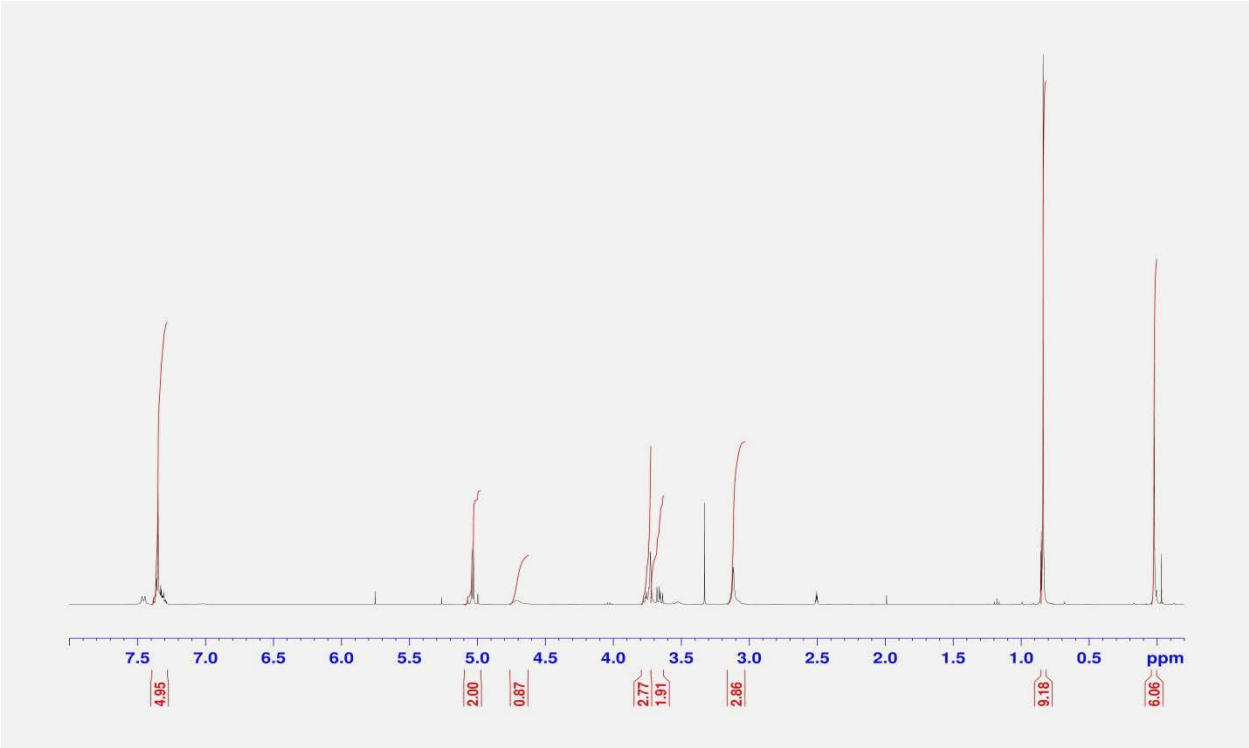


Figure 5. ^{1}H NMR of Compound 3.

Compound 4: ^{1}H NMR (400 MHz, DMSO) δ=7.2-8.0 (m, 10H), 5.3 (m, 1H), 5.1 (s, 2H), 4.0-4.2 (m, 2H), 0.9 (s, 9H), 0 (s, 6H).

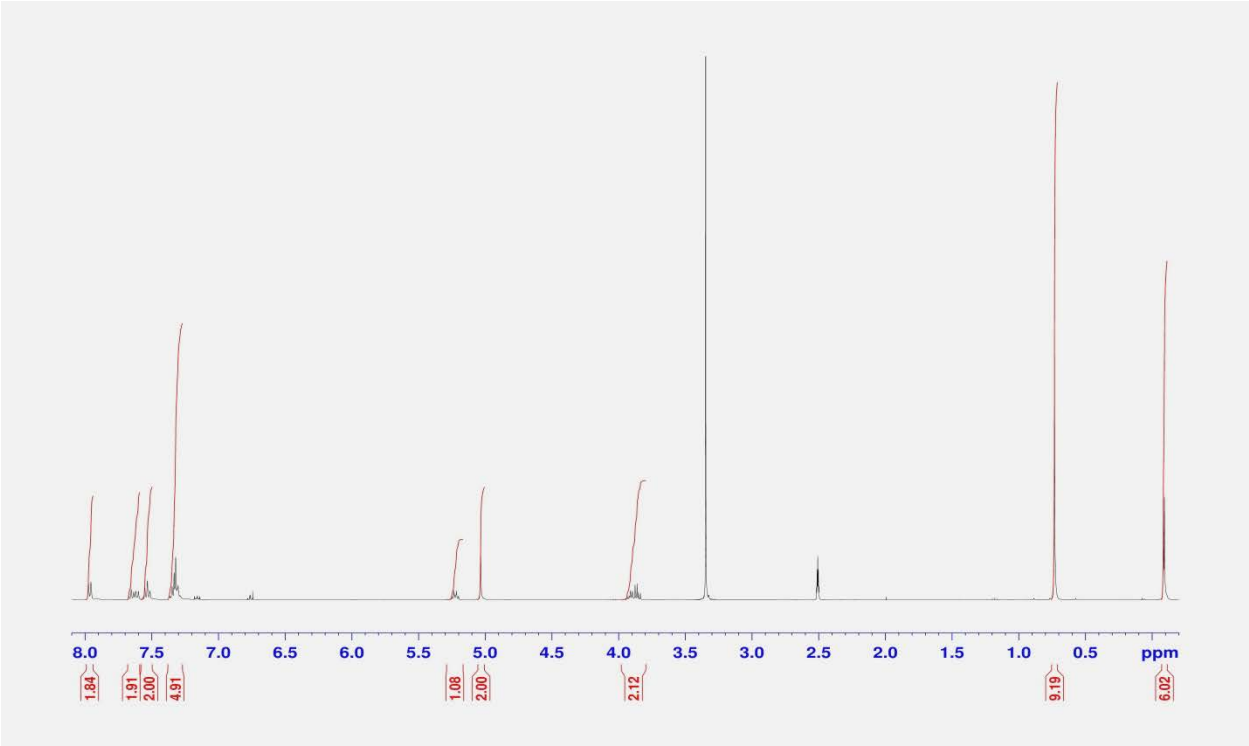


Figure 6. ^{1}H NMR of Compound 4.

Compound 5: ^{1}H NMR (400 MHz, DMSO-d6) δ= 7.2-7.4 (m, 10H), 5.4 (d, 1H), 5.0 (d, 2H), 4.9 (broad s, 1H), 3.6-3.8 (m, 2H), 3.5 (broad s, 1H), 0.9 (s, 9H), 0 (s, 6H).

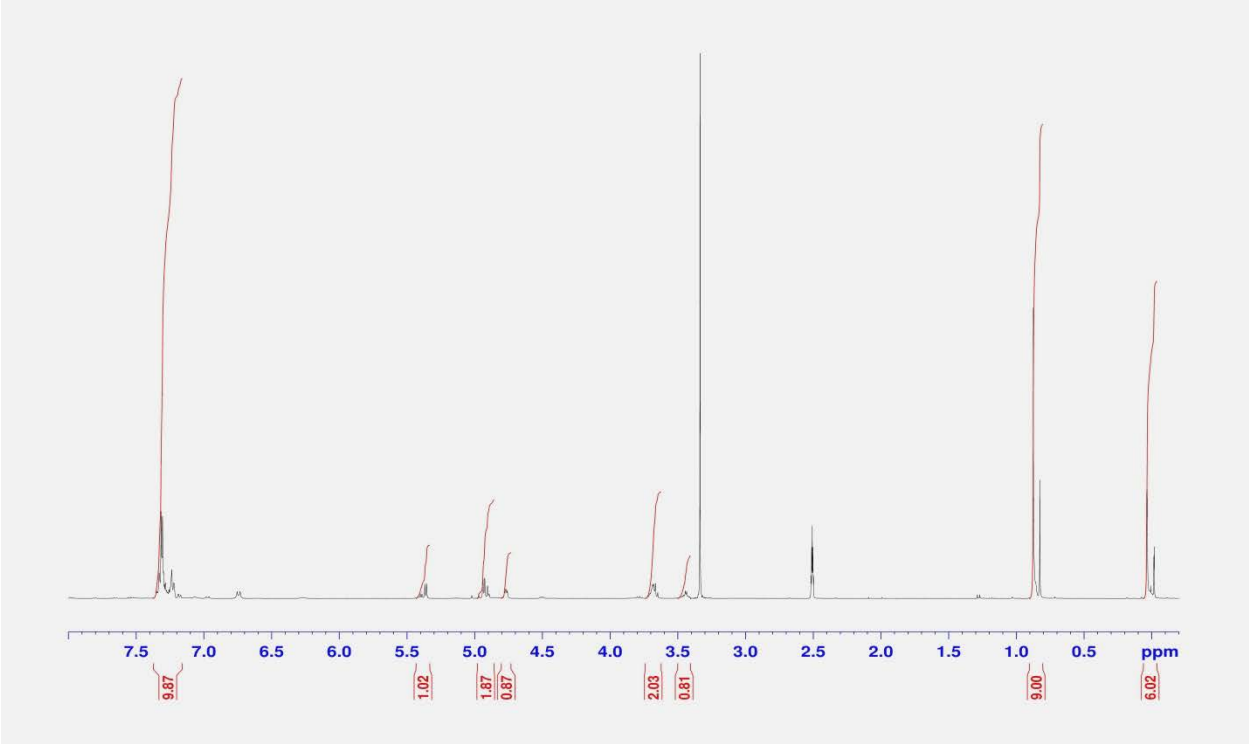


Figure 7. ^{1}H NMR of Compound 5.

Compound 6: ^{1}H NMR (400 MHz, DMSO-d6) δ= 7.2-7.4 (m, 10H), 5.4 (d, 1H), 5.0 (d, 2H), 4.9 (broad s, 1H), 4.8 (s, 1H), 3.6-3.8 (m, 2H), 3.5 (broad s, 1H).

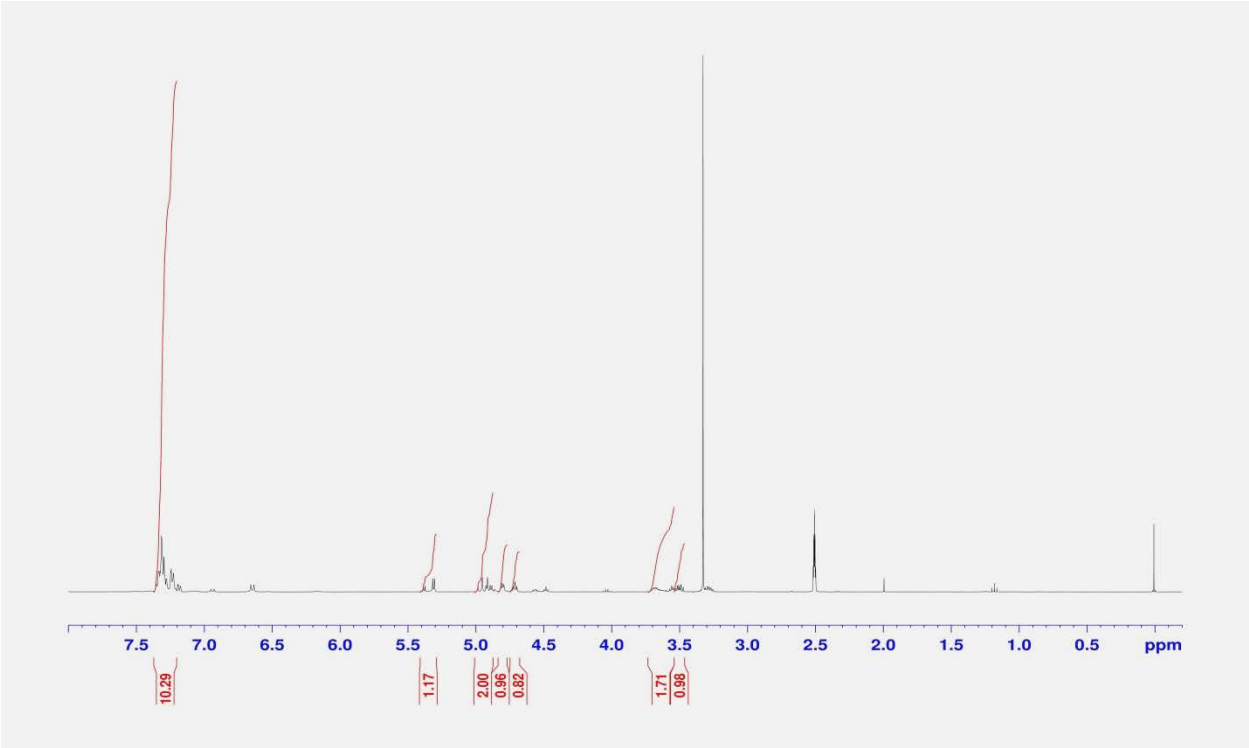


Figure 8. ^{1}H NMR of Compound 6.

Compound 7: ^{1}H NMR (400 MHz, DMSO-d6) δ=7.2-7.4 (m, 10H), 5.2 (s, 1H), 5.0 (d, 2H), 3.8-3.9 (t, 4H), 3.6-3.7 (t, 4H), 3.3-3.4 (m, 2H).

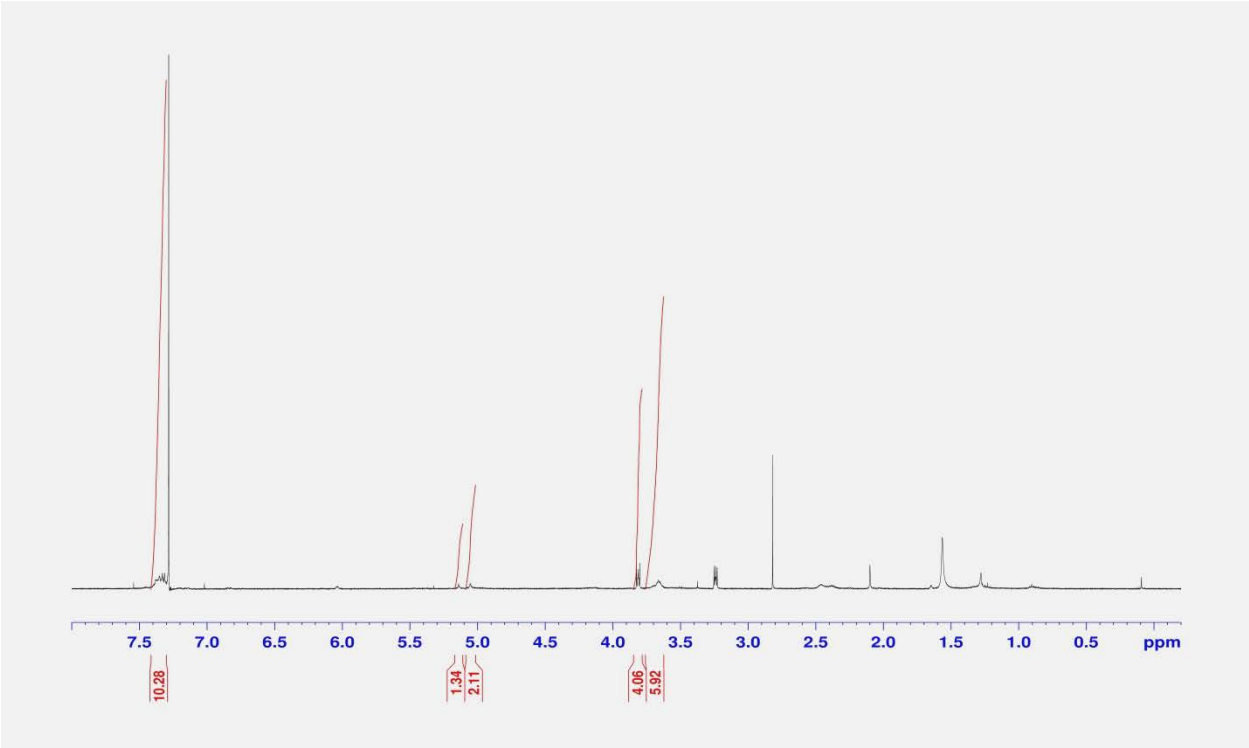


Figure 9. ^{1}H NMR of Compound 7.

Compound 8: ^{1}H-NMR (400 MHz, $CDCl_3$) δ= 7.3-7.6 (m, 5H), 4.9-5.0 (m, 1H), 4.0-4.2 (m, 1H), 3.9-4.0 (m, 4H), 3.3-3.8 (m, 6H).

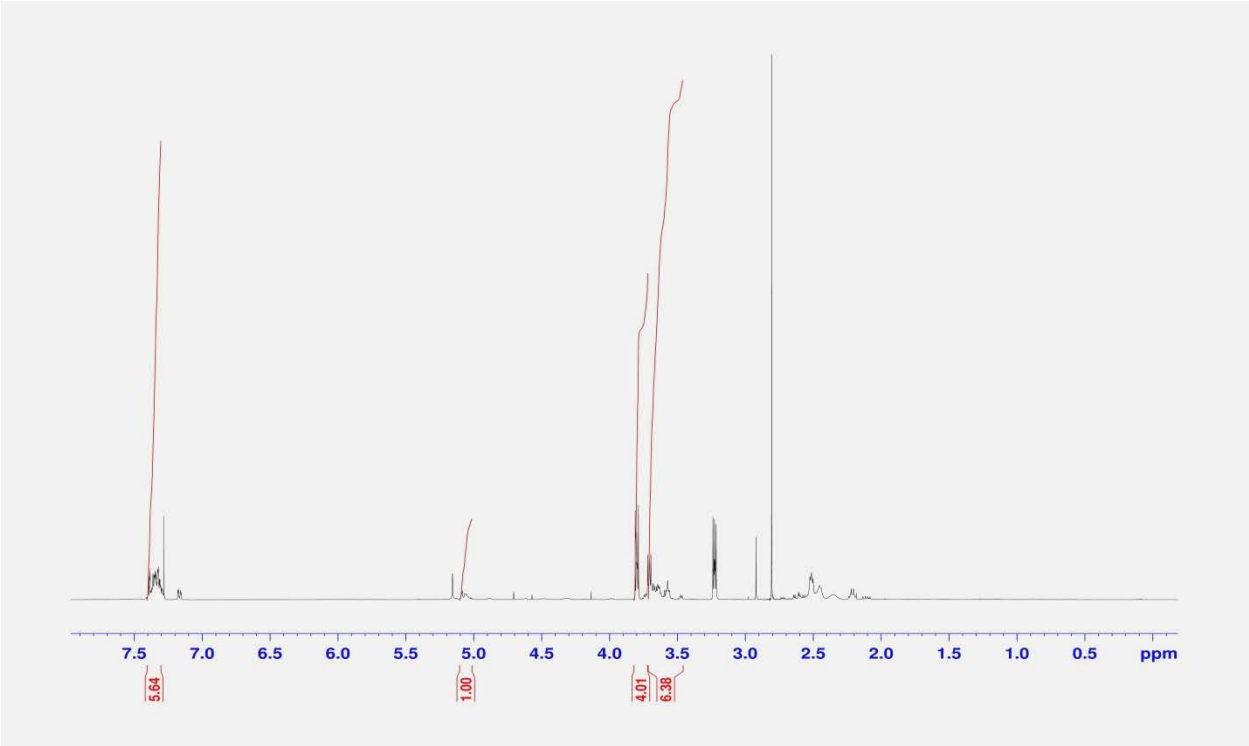


Figure 10. ^{1}H NMR of Compound 8.

Compound 9: ^{1}H-NMR (400 MHz, $CDCl_3$) δ=7.35-7.22 (m, 5H), 5.77 (d, 1H,), 4.84 (d, 1H,), 4.25 (dt, 1H), 3.66 (t, 4H), 2.58-2.42 (m, 6H), 2.16-2.04 (m, 2H), 1.53 (t, 2H,), 1.26 (broad s, 25H), 0.88 (t, 3H).

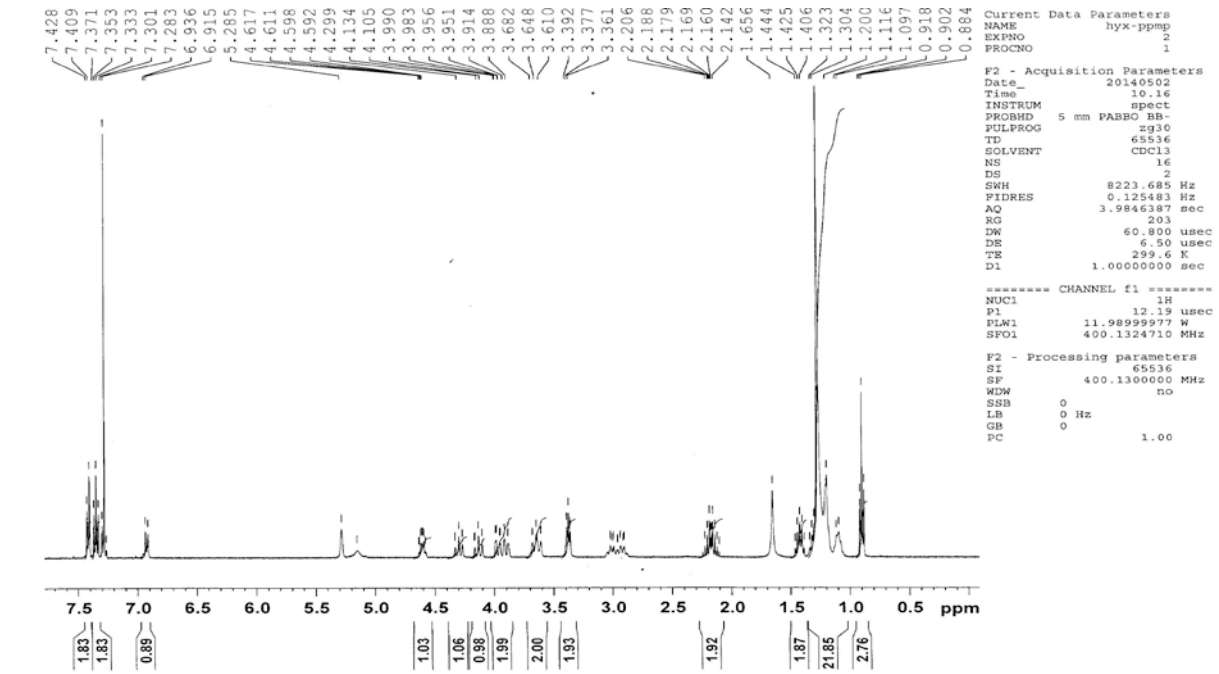


Figure 11. ^{1}H NMR of Compound 9.

Compound 10: ^{1}H-NMR (400 MHz, $CDCl_3$) δ=3.6-3.7 (s, 463 H), 3.4 (s, 3H), 1.5 (s, 18H).

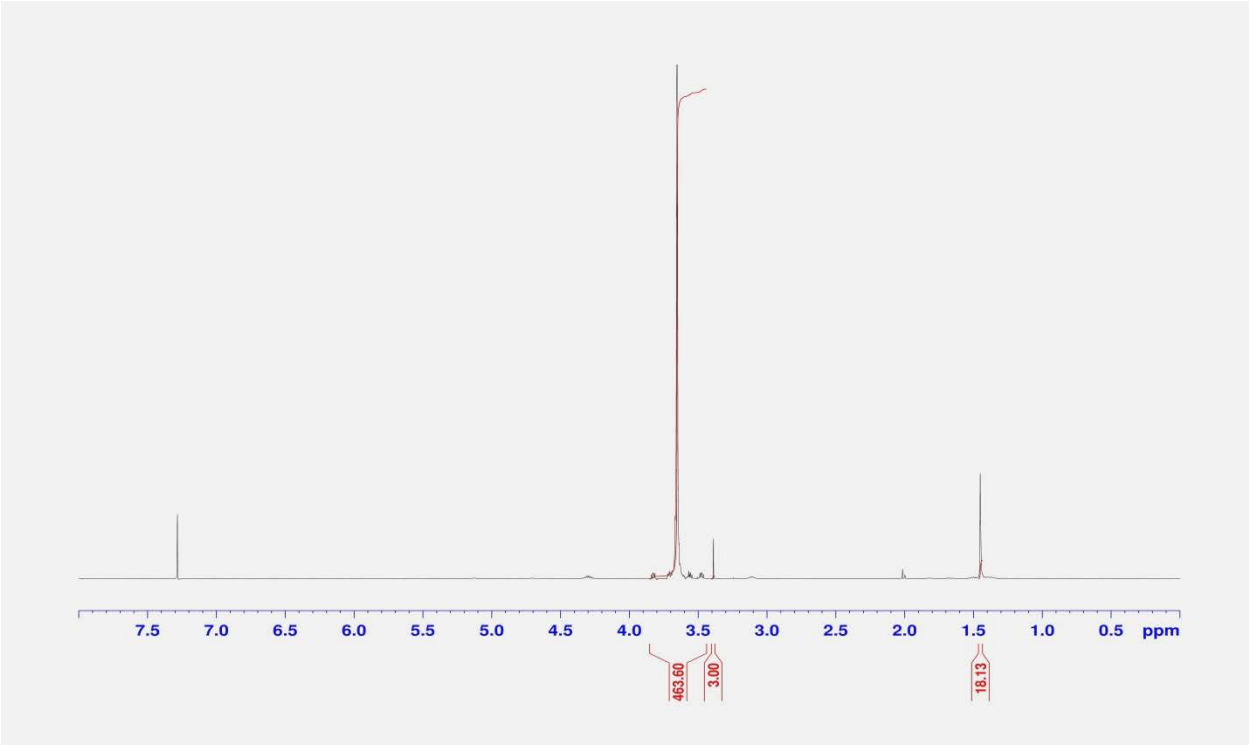


Figure 12. ^{1}H-NMR of Compound 10.

Compound 11: ^{1}H-NMR (400 MHz, $CDCl_3$) δ=3.6-3.7 (s, 482 H), 3.4 (s, 3H).

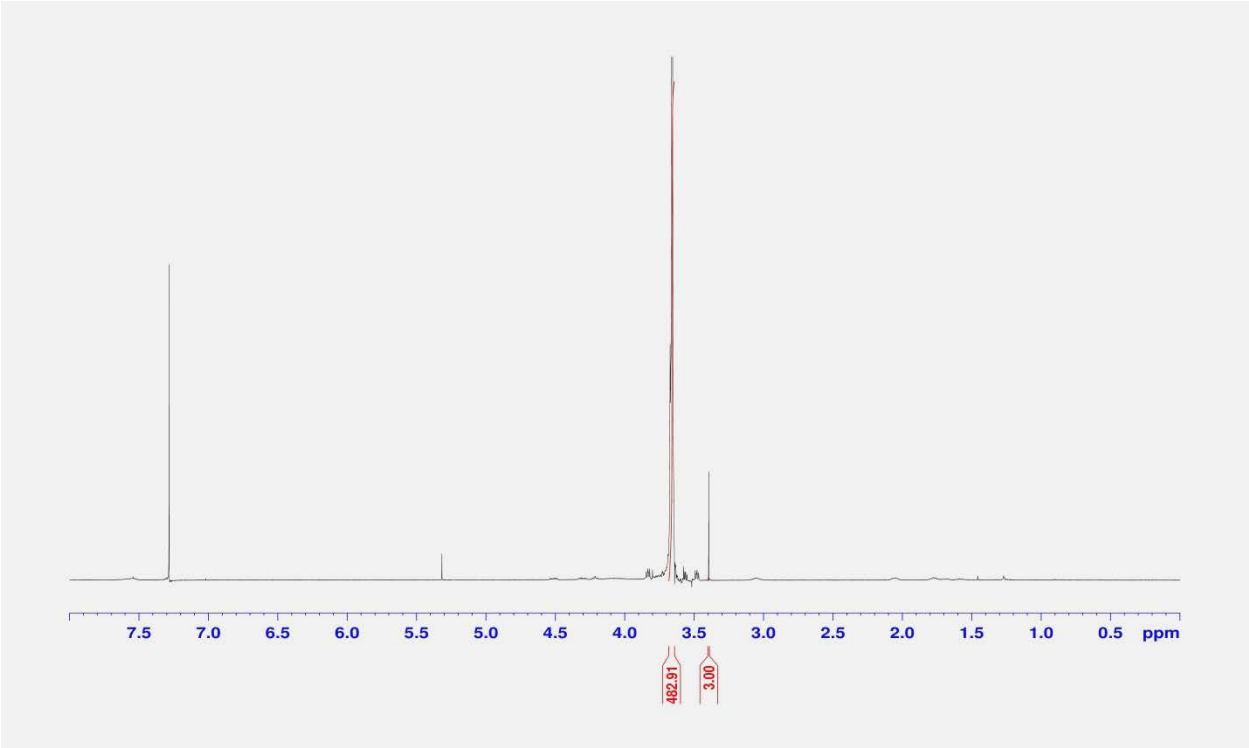


Figure 13. ^{1}H-NMR of Compound 11.

Compound 12: ^{1}H-NMR (400 MHz, $CDCl_3$) δ=3.6-3.7 (s, 451 H), 3.4 (s, 3H), 2.4 (m, 8H).

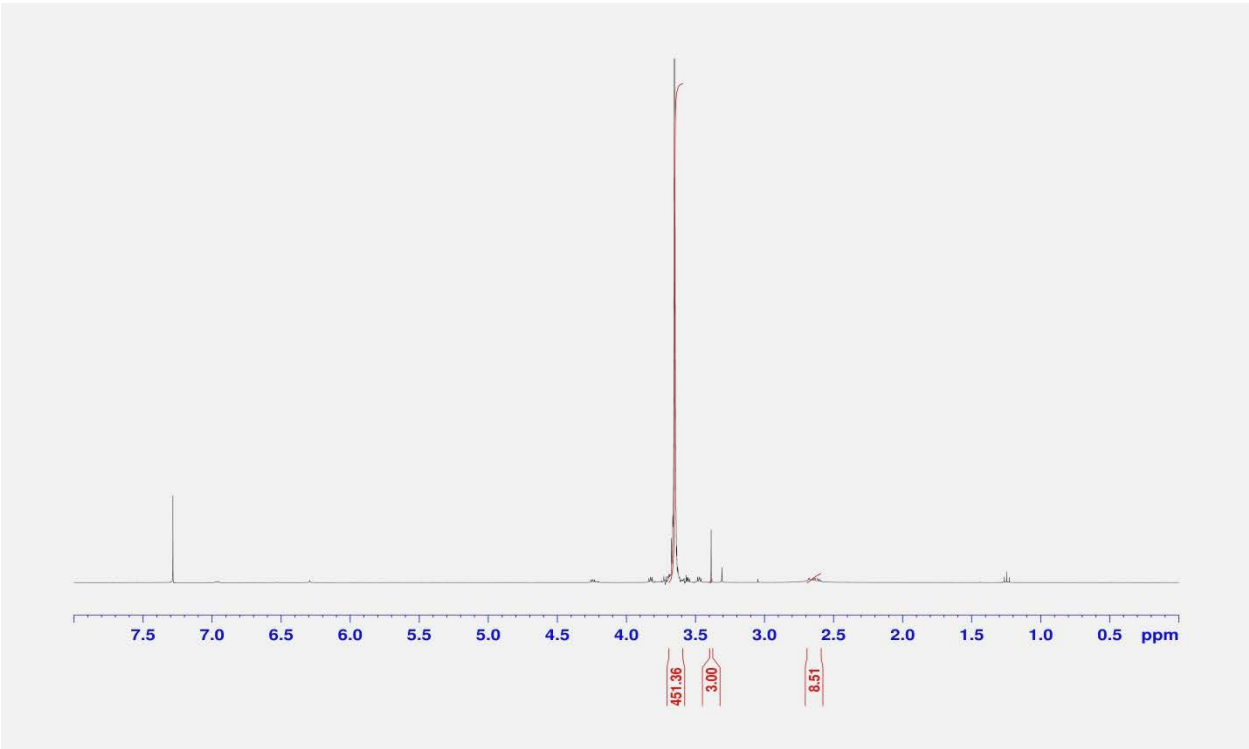


Figure 14. ^{1}H-NMR of Compound 12.

Compound 13: ^{1}H-NMR (400 MHz, $CDCl_3$) δ=3.6-3.7 (s, 444 H), 3.4 (s, 3H), 0.9 (s, 6H).

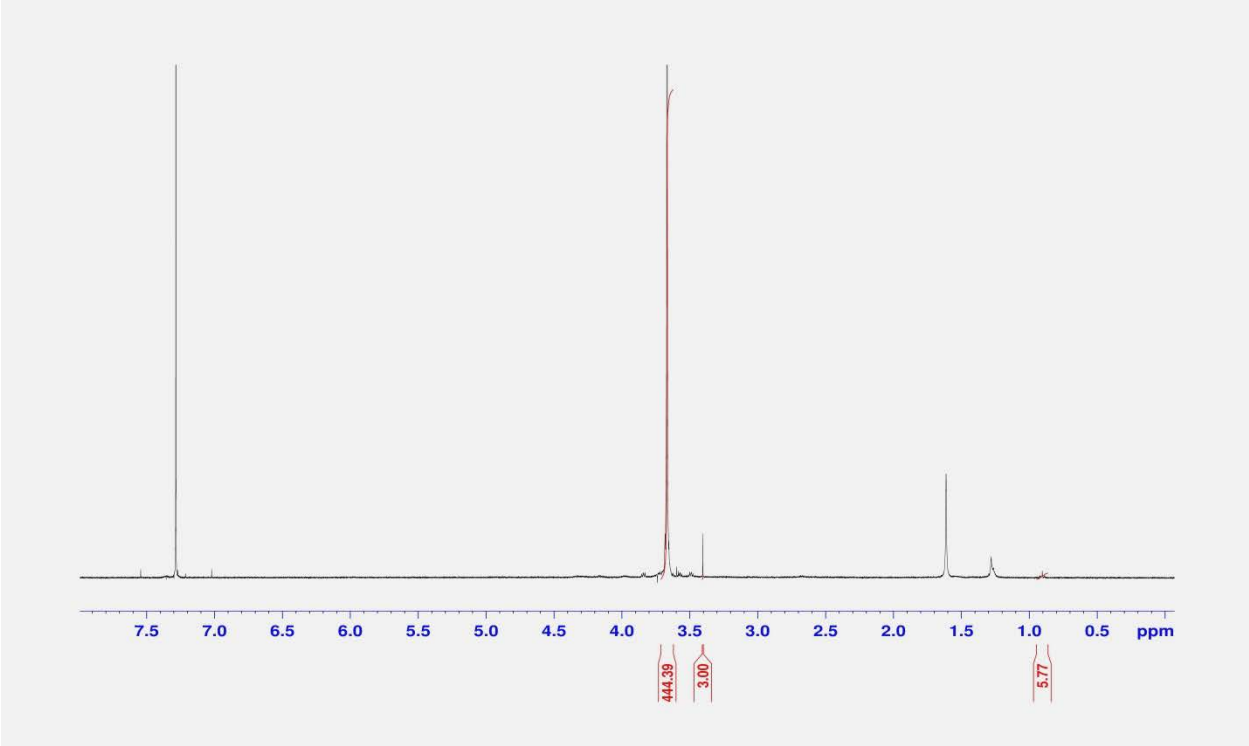


Figure 15. ^{1}H-NMR of Compound 13.

4. DISCUSSION

4.1 Cytotoxicity of Combination of PPMP with Other Anticancer Drugs

The combination index (CI) calculated after co-administration of PPMP and CPT or DOX assessed whether the combination could confer synergistic, additive, or antagonistic effects (Table 1). CI was calculated by the equation CI = $(d1/D_{50}1)+(d2/D_{50}2)$, with $D_{50}1$ being the concentration of PPMP producing 50% cell-killing effect in single treatment and d1 being the PPMP concentration required to achieve the same 50% killing effect with d2 in co-treatment. Similarly, D502 is the concentration of CPT or DOX producing 50% killing effect in single treatment, and d2 is the CPT or DOX concentration required to obtain the same 50% cell-killing effect in combination with d1. A CI of <1, =1 and >1 is suggestive of synergism, additive effect, and antagonism, respectively. Our data suggested that PPMP and CPT or DOX showed significant synergy in all of the cancer cell lines examined.

4.2 Synthesis of PEG_{5K}-$PPMP_2$

4.2.1 Step 1 in 2.4.1

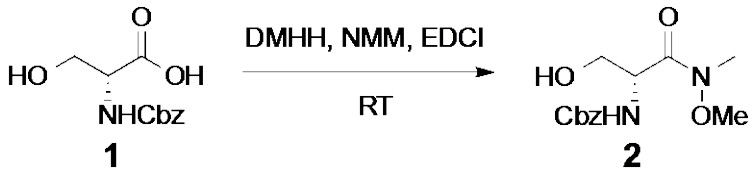


Figure 16. Step 1 in 2.4.1.

Step 1 was designed to form Weinreb-Nahm amide from compound 1, which was subsequently treated with a Grignard reagent in step 3 to generate Weinreb-Nahm ketone. The Weinreb–Nahm ketone synthesis is a chemical reaction used to make carbon-carbon

bonds. The original reaction involved two consecutive nucleophilic acyl substitutions: the conversion of an carboxylic acid into an N,O-dimethylhydroxyamide, known as a Weinreb–Nahm amide, and subsequent treatment of this species with an organometallic reagent.

The major advantage of this method over addition of organometallic reagents to more typical acyl compounds is that it avoids the common problem of over-addition. The underlying mechanism was that the tetrahedral intermediate formed as a result of nucleophilic acyl substitution by the organometallic reagent is stabilized by chelation from the methoxy group.

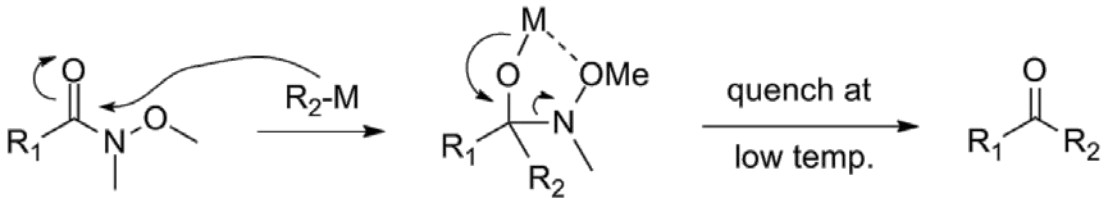


Figure 17. Mechanism of forming Weinreb-Nahm amide and ketone.

N,*O*-Dimethylhydroxylamine hydrochloride was used in this amide coupling reactions to form Weinreb amide. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC or EDCI) is a coupling agent or dehydration agent. It was used as a carboxyl-activating agent for compound 1 to couple *N*,*O*-Dimethylhydroxylamine to yield amide bond of Compound 2. To improve the yield and decrease side reactions, *N*-Hydroxysuccinimide (NHS) or Hydroxybenzotriazole (HOBt) could be also added. Moreover, EDC could be substituted by other carbodiimides, such as DCC (N,N'-dicyclohexylcarbodiimide) or DIC (N,N'-diisopropylcarbodiimide). *N*-Methylmorpholine, an organic base of intermediate strength, was used to neutralize the hydrochloride of *N*,*O*-Dimethylhydroxylamine. Hence, hydrochloride was used to quench the reaction.

4.2.2 Step 2 in 2.4.1

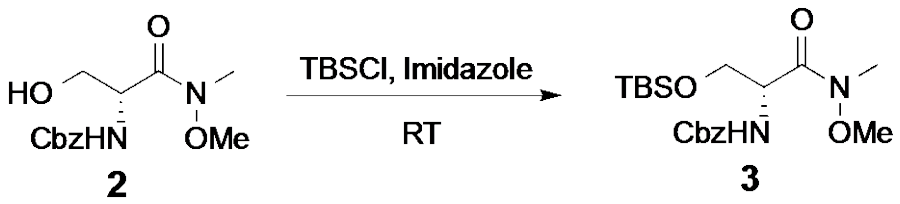


Figure 18. Step 2 in 2.4.1.

Step 2 was to protect primary alcohol of **Compound 2** to form TBS ether by reaction with TBSCl (*tert*-butyldimethylsilyl chloride) in DCM. A widely used method of protecting hydroxyl groups is to turn them into silyl ethers. TBSCl is one kind of tri-alkyl-silyl-chloride reagents to form silyl ether. Imidazole is required for this reaction as both a catalyst and base. On the one hand, for the very bulky silyl reagents, a catalytic amount of imidazole is needed to speed up their reaction with the hydroxyl. This increase in rate is due to the formation of a reactive intermediate. On the other hand, imidazole will neutralize the hydrochloride produced during etherification.

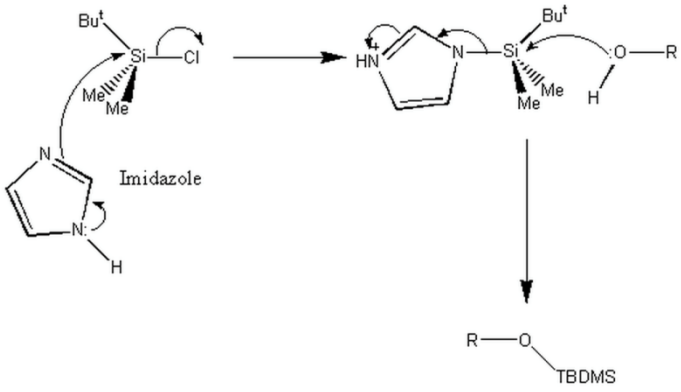


Figure 19. Mechanism of imidazole as a catalyst.

One extremely reliable and rapid procedure to form silyl ether is the Corey protocol in which the alcohol is reacted with a silyl chloride and imidazole in DMF. However, DMF is hard to be removed completely, even though DMF would be miscible and extracted by water. If

DMF is replaced by dichloromethane, the reaction will be somewhat slower, but the purification of the compound will be simplified. Hence, we replaced DMF with DCM as the solvent. Nonetheless, a relatively high yield was achieved after overnight reaction. In addition, water was used to quench the reaction, since imidazole is soluble in water. It would be better if the reaction was quenched with acidulous ammonium chloride solution.

4.2.3 Step 3 in 2.4.1

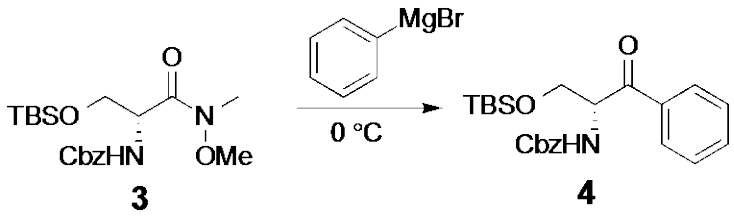


Figure 20. Step 3 in 2.4.1.

Step 3 was to synthesize Weinreb ketone through the treatment of a Grignard reagent as mentioned above, in which only one benzyl ring was added to compound 3. At the beginning, the temperature was kept at 0 °C for 3 hours, since it was an exothermic reaction. Then, the reaction was quenched with hydrochloride (1 M) to remove the excess amount of phenylmagnesium bromide. It would be better if the acidulous ammonium chloride solution was used to quench the reaction.

4.2.4 Step 4 in 2.4.1

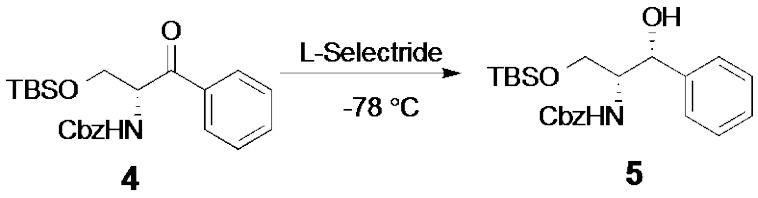


Figure 21. Step 4 in 2.4.1.

Step 4 was to reduce the ketone group of **Compound 4** to hydroxyl group. L-Selectride (1.0 M lithium tri-*sec*-butylborohydride in THF) was used as the reducing agent. L-Sectride has been regarded as an excellent, mild and highly selective reducing agent. Compared to $NaBH_4$ (sodium tetrahydridoborate), the selectivity of L-selectride comes from three aspects: (1) There is only one hydride in one L-selectride molecule so that the amount of reducing agent could be tightly controlled. (2) L-selectride could selectively reduce the ketone groups with less steric hindrance, owing to the bulky structure with greater steric hindrance from three isobutyl groups. (3) The reducing capacity of L-selectride is affected by temperature. In other words, ketone groups could be reduced at low temperature, therefore other groups in Compound **4**, such as silyl ether, will not be reduced. Hence, the temperature was set strictly to -78 °C to ensure the selective reduction of the ketone group.

With regard to the quenching of the reaction, potassium sodium tartrate was used instead of ammonium chloride. At the beginning, ammonium chloride was used to remove excess amount of L-selectride. However, L-selectride was not be eliminated completely and still conjugated with the product, resulting in the mixture of several spots as shown in by TLC after column purification twice. Then potassium sodium tartrate was used to remove L-selectride more efficiently than ammonium chloride. Potassium sodium tartrate is a quardentate chelator, with two hydroxyl groups and two carboxyl groups, which could chelate with L-selectride other than broking the carbon-boron bond by the acidity of

ammonium chloride. After stirring overnight, L-selectride could be removed from the product, leading to pure **Compound 5**.

4.2.5 Step 5 in 2.4.1

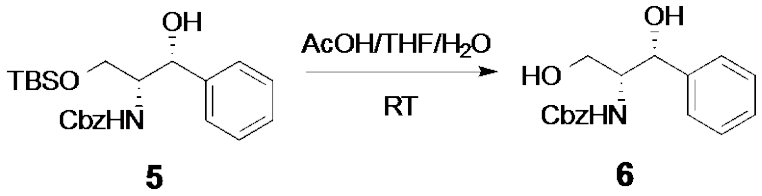


Figure 22. Step 5 in 2.4.1.

Step 5 was to deprotect TBS group by acetic acid. *Tert*-Butyldimethysilyl ethers could be converted back to the alcohols under acidic conditions. THF, which is miscible to water, was added to increase the solubility of **Compound 5**. Hydrogen chloride, trifluoroacetic acid (TFA) and tetra-n-butylammonium fluoride (TBAF) could also be used to deprotect TBS group. Methanol, which is also miscible to water, could substitute THF in this reaction.
To quench the reaction, excess amount of acetic acid was neutralized by adding potassium carbonate until there was no bubble (CO_2) produced.

4.2.6 Step 6 in 2.4.1

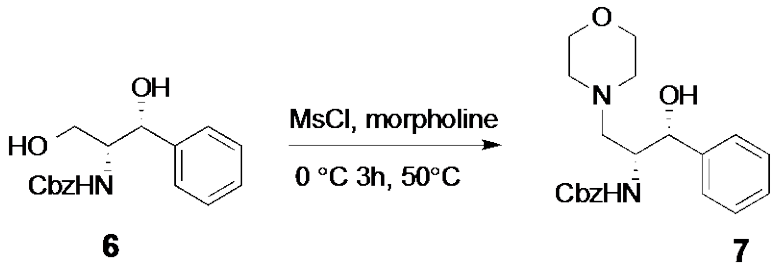


Figure 23. Step 6 in 2.4.1.

Step 6 was to substitute the hydroxyl group of **Compound 6** by morpholine ring. In fact, step 6 included two consecutive steps, the sulfonylation of hydroxyl group by methanosulfonyl cholride and the substitution of methanesulfonate by morpholine. The hydroxide anion OH^- was a particularly poor leaving group, as its negative charge cannot be stabilized. However, a hydroxy group may be altered through esterification by the sulfonic acid to serve as a better leaving group, since sulfonic acid derivatives possess substituents that considerably stabilize the negative charge by resonance stabilization.

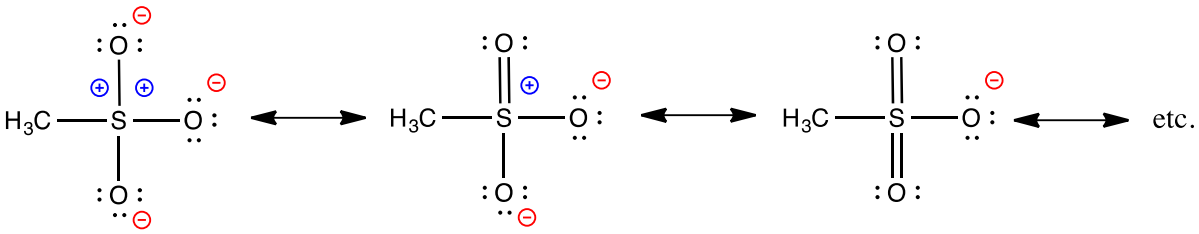


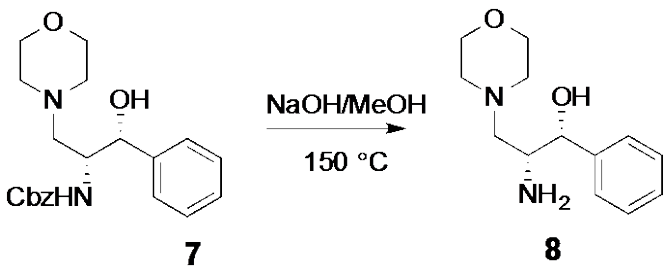
Figure 24. Mechanism of sulfonic acid stabilizing the negative charge by resonance stabilization.

The temperature of MsCl protection was set to 0°C since it was an exothermic reaction. After 3 hours reaction, excess amount of morpholine was added to the mixture and reacted at 50°C overnight to ensure the completion of morpholine substitution.

Pyridine was used as both the solvent and the base to neutralize the hydrogen chloride during MsCl protection. To quench the reaction, pyridine, which is miscible to water, was removed by evaporation at 60°C at first. Then hydrogen chloride (12 M) was added to remove excess amount of base, including 9 equivalent of morpholine and pyridine residue.

However, pyridine was a toxic solvent with unpleasant smell. In addition, the boiling point of pyridine was 115.2 °C so that it was hard to evaporate and remove the pyridine. Therefore, it would be better if organic base, such as triethylamine or N-methyl morpholine, was included in DCM as the solvent.

4.2.7 Step 7 in 2.4.1



7 **8**

Figure 25. Step 7 in 2.4.1.

Step 7 was to deprotect Cbz group and expose the free amines of **Compound 7**. There are two types of methods to deprotect Cbz group, involving the hydrolysis by strong base and the hydrogenolysis. In step 7, hydrolysis was chosen since it only took 15 minutes in microwave to complete the reaction. Methanol was served as the solvent to dissolve both **Compound 6** and sodium hydroxide. However, the limitations of this reaction came from two aspects: 1) The volume of special reaction tubes for microwave reactor (Biotage) was limited (5 mL), hence only small amount of **Compound 6** could be handled each time. 2) The mixture after reaction was so viscous that the methanol could not be evaporated and removed completely, which led to low yield.

The alternative for this step was hydrogenolysis, which required 24 hours for reaction while the post-treatment of the reaction was convenient. Benzyl L-threo-1 -hydroxy-3-morpholino-1-phenylpropan-2-yl)carbamate (Compound 6, 990 mg, 2.7 mmol) was dissolved in 20 mL methanol at room temperature followed by addition of Pd-C 10 % (540 mg). The mixture was satured with hydrogen and stirred for 24 h at room temperature under hydrogen atmosphere (balloon). The catalyst Pd-C was removed by filtration on celite and the solution was evaporated to remove methanol. The crude product was purified by column chromatography on silica as mentioned in 2.4.1.

4.2.8 Step 8 in 2.4.1

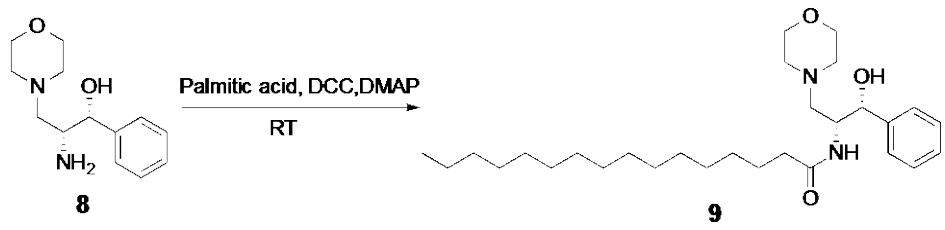


Figure 26. Step 8 in 2.4.1.

Step 8 was to couple a 16-carbon chain to compound 8. Palmitic acid was a sixteen-carbon saturated fatty acid, which was condensed with the amino group of **Compound 8** to form amide bond. DCC was used as the coupling agent, while DMAP was used as catalyst. DCC was firstly reacted with palmitic acid to form reactive intermediate. Then, DCC hydrates to form dicyclohexylurea (DCU), a compound that is insoluble in most organic solvents and in water, therefore, leading to quenching of the reaction. DCU was removed by filtration. DCC was one of the first carbodiimides developed. DCC has achieved popularity mainly because of its high efficiency in facilitating amide coupling reactions and the fact that it is inexpensive.

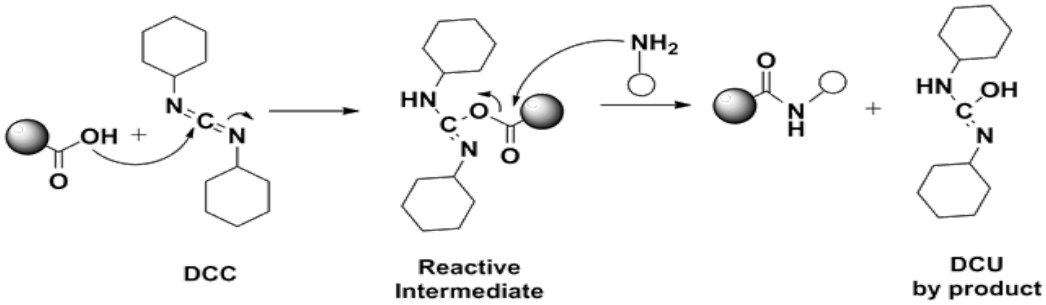


Figure 27. Mechanism of DCC as the coupling agent for amide coupling.

4.2.9 Step 1 in 2.4.2

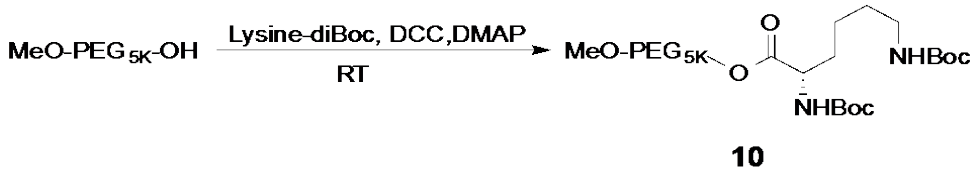


Figure 28. Step 1 in 2.4.2.

Step 1 in 2.4.2 was to couple lysine-di-Boc to MeO-PEG_{5K}-OH via an ester bond. This esterification was named as Steglich esterification. The Steglich esterification is a variation of an esterification with DCC as a coupling reagent and DMAP as a catalyst. The reaction was first described by Wolfgang Steglich in 1978. DMAP suppresses the side reaction towards DCU, acting as an acyl transfer-reagent. The reaction mechanism is described below:

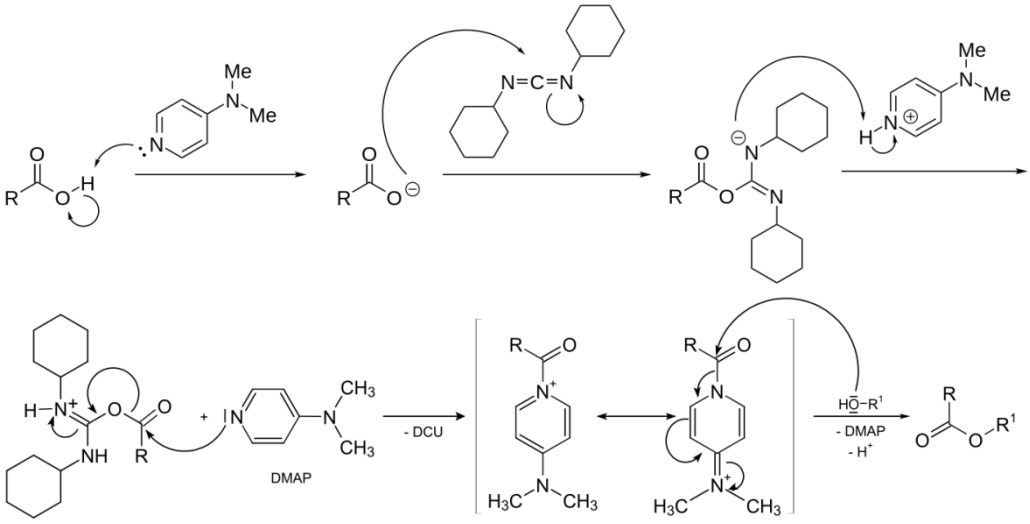


Figure 29. Mechanism of Steglich esterification.

4.2.10 Step 2 in 2.4.2

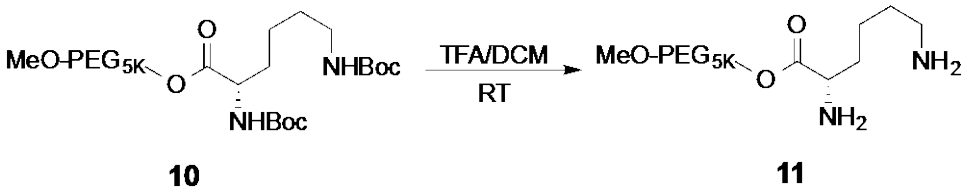


Figure 30. Step 2 in 2.4.2.

Step 2 in 2.4.2 was to remove Boc group from PEG_{5K}-lysine-di-Boc. The mechanism of reaction was described as below: Firstly, the tert-butyl carbamate becomes protonated. Then,

the tert-butyl cation is lost resulting in formation of a carbamic acid. Next, decarboxylation of the carbamic acid results in the free amine. Finally, protonation of amine under the acidic conditions yields the product as the TFA salt. Hence, prior to the next step, TFA salt should be removed by triethylamine (TEA) to produce naked amino group. It is worth mentioning that the reaction time was limited to 2 hours, which was sufficient to remove Boc group while ensuring that PEG chain would not be cleaved.

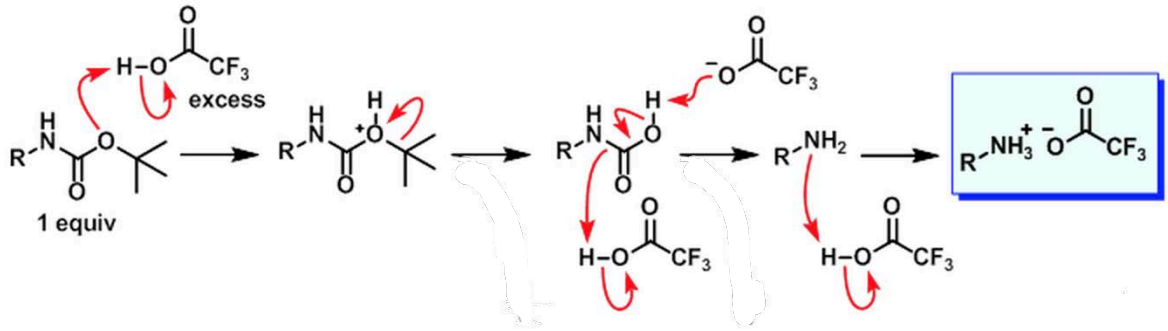


Figure 31. Mechanism of removing Boc group by TFA.

4.2.11 Step 3 in 2.4.2

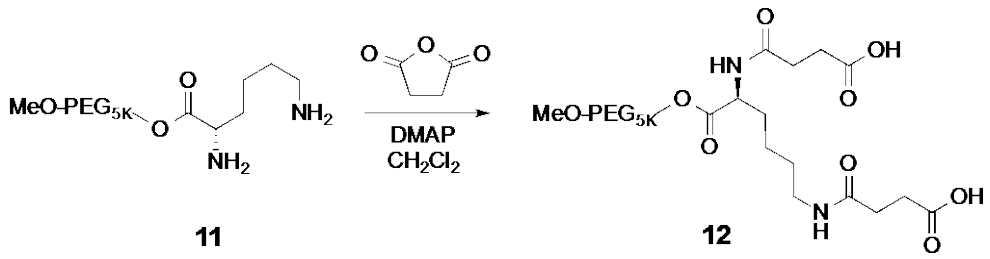


Figure 32. Step 3 in 2.4.2.

Step 3 in 2.4.2 was to convert amino group to carboxyl group through the acylation of the amino group by succinic anhydride. Since PPMP has a hydroxyl group, which was not able to conjugate with amino group of PEG_{5K}-lysine directly, it is necessary to convert the amino group to the carboxyl group. DMAP was used as a catalyst. The temperature was set at 60°C to accelerate the reaction.

4.2.12 Step 4 in 2.4.2

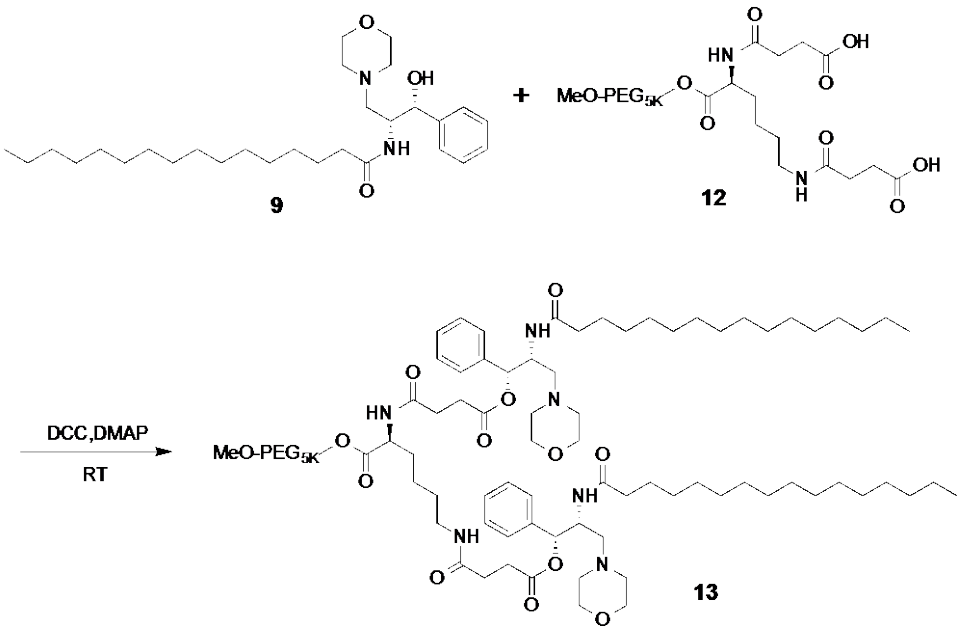


Figure 33. Step 4 in 2.4.2.

Step 4 in 2.4.2 was to couple PPMP to PEG-lysine-$(COOH)_2$. The underlying mechanism was similar to that mentioned in 4.2.9. Six fold of PPMP was added to ensure that every carboxyl group has three equivalent PPMP to afford complete conjugation. NMR of **Compound 13** showed two end methyl groups of the two PPMP, which should be integrated to six hydrogen in δ=0.9. Hence, the conjugation efficiency of step 4 in 2.4.2 could be considered as 100%. In conclusion, we successfully obtained pure PEG_{5K}-$PPMP_2$.

5. PERSPECTIVE

In this thesis, we successfully synthesized PEG_{5K}-$PPMP_2$ to serve as the prodrug of PPMP and a carrier for other anticancer drugs. In the future, we will continue this study as followed:

5.1 Develop PEG_{5K}- $PPMP_2$ micellar system to co-deliver PPMP and DOX.

We will choose DOX as the model drug of our micellar system. DOX loaded and empty PEG_{5K}-$PPMP_2$ micelles will be prepared. The characterization of our micellar system will be made, involving shape, critical micellar concentration (CMC), drug loading efficiency, release and hemolytic effect.

5.2 Test the efficacy of DOX loaded PEG_{5K}-$PPMP_2$ micelles in normal and drug-resistant cancer cells lines.

First, it has been reported that drug resistance of cancer cells was correlated with overexpression of glucosylceramide synthase (GCS), so we will compare the GCS gene expression level (by real-time PCR) of KB8 (parental epidermal carcinoma cell line) and KB85 (multidrug-resistant epidermal carcinoma cell line). Next, we will compare the efficacy of empty PEG_{5K}-$PPMP_2$ micelles, DOX•HCl solution and DOX loaded PEG_{5K}-$PPMP_2$ micelles. Efficacy studies will include measures of cytotoxicity (by MTT assay), ceramide level (by LC-MS) and GCS gene expression level. These three studies will be done on HCT-116, PC-3, 4T1.2, MCF-7, KB8 and KB85 cell lines.

5.3 Examine the efficacy and safety of DOX loaded PEG_{5K}-$PPMP_2$ micelles in vivo.

The in vivo therapeutic activity of DOX formulated in PEG_{5K}-$PPMP_2$ micelles will be investigated in a syngeneic murine breast cancer model (4T1.2). Female BALB/c mice will be randomly divided into five groups and administered i.v. injection with PBS (control), free PEG_{5K}-$PPMP_2$ micelles, DOX•HCl solution(5 mg DOX/ kg), PEG_{5K}-$PPMP_2$/DOX(5 mg DOX/kg), and PEG_{5K}-$PPMP_2$/DOX(10 mg DOX/kg), respectively. Tumor size and body weight will be measured. Furthermore, biodistribution and plasma pharmacokinetics of PEG_{5K}-$PPMP_2$ micelles/DOX will be measured via NIRF optical imaging and HPLC in tumor-bearing mice.

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