SYNTHESIS OF ANTICANCER DRUGS AND FOLATE RECEPTOR TARGETED DELIVERY

M.Sc. Thesis

by

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DISCIPLINE OF CHEMISTRY

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SYNTHESIS OF ANTICANCER DRUGS AND FOLATE RECEPTOR TARGETED DELIVERY

A THESIS

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Bishnubasu Giri

Dedicated to My Mom,
Dad & Sister

Abstract

- Gallinamide A, a marine cyanobacterial natural product, is an excellent inhibitor towards human cathepsin L with IC_{50} value of 5 nM for 30 minutes pre-incubation. Cathepsin L is a lysosomal endopeptidese which helps to degrade extracellular matrix leading to metastasize. We probably synthesized cysteinyl, isoleucinyl and alanyl derivatives of the natural product by solid phase peptide synthesis. These derivatives of natural product might have better or similar type activity like Gallinamide A.
- The problem of toxicity in drug delivery arises due to uptake of drugs by healthy cells. Therefore targeted drug delivery has a vital role in therapeutic applications. Folate receptor, overexpressed on certain malignant cancer cells, is a crucial biomarker for delivery of toxic drugs during chemotherapy. It has very high affinity towards folic acid and also folate conjugates. It is very important to synthesis new folate receptor inhibitors. We have synthesized two new folate receptor inhibitors such as pteroate and hydrazidepteroate linkers and these two new inhibitors may have good affinity towards folate receptor.

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NOMENCLATURE

α	Alfa
β	Beta
γ	Gamma
δ	Delta
s	Singlet
d	Doublet
t	Triplet
m	Multiplet
M	Molar
g	Gram
h	Hour
J	Coupling constant
μM	Micromolar
nM	Nanomolar
mM	Millimolar
mL	Millilitre
dd	Doublet of doublet
Hz/MHz	Hertz/Mega Hertz
R_f	Retention factor
K_D	Equilibrium dissociation constant
IC_{50}	Inhibition constant
ppm	Parts per million
brs	Broad singlet

ACRONYMS

Abbreviations used for amino acids, resins, substituents, reagents, etc. are largely in accordance with the recommendations of the IUPAC-IUB commission on Biochemical Nomenclature, 1974, Pure and Applied Chemistry, 40, 315-331. All amino acids have L-configuration. Standard three letter coding is used for all amino acids. Additional abbreviations used in this thesis are listed below.

Ala	Alanine
Asp	Aspartic Acid
ACS	American Chemical Society
Boc	*tert*-butyloxycarbonyl
BuLi	Butyl Lithium
Cys	Cysteine
$CDCl_3$	Chloroform-d
$CHCl_3$	Chloroform
CH_3	Methyl
2-Cl-Trt	2-Chlorotrityl
DMF	*N*,*N*-Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DIPEA	Diisopropylethylamine
DCM	Dichloromethane
Dap	Diaminopropionic acid
DHP	Dihydropyran
DMAP	4-Dimethylaminopyridine
EDT	Ethanedithiol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ESI-MS	Electrospray Ionization Mass Spectrometry
EtOAc	Ethyl Acetate
Fmoc	Fluorenylmethyloxycarbonyl
FR	Folate Receptor
Glu	Glutamic Acid
GPI	Glycophosphatidylinositol

HCHO	Formaldehyde
H_2O	Water
HCl	Hydrochloric Acid
HBTU	2-(1-H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid Hexafluorophosphate
Ile	Isoleucine
Leu	Leucine
LC-MS	Liquid Chromatography-Mass Spectrometry
$LiAlH_4$	Lithium Aluminium Hydride
NMR	Nuclear Magnetic Resonance
NHMeOMe.HCl	*N,O*-Dimethylhydroxylamine Hydrochloride
$NaBH_4CN$	Sodium Cyanoborohydride
NIR	Near Infrared
NH_4OAc	Ammonium Acetate
Na_2SO_4	Sodium Sulphate
OH	Hydroxyl
PPTS	Pyridinium *p*-toluenesulphonic Acid
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinoph-osphonium Hexafluorophosphate
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SPPS	Solid Phase Peptide Synthesis
TMS	Tetramethylsilane
TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
TFA	Trifluoroacetic Acid
TIPS	Triisopropylsilyl
OtBu	*tert*-Butoxy

Chapter 1

Synthesis of Natural Product Gallinamide A and Its Derivatives

1.1. Introduction:

Cancer is the second leading cause of death next only to cardiovascular disease in the world. Many research groups are working towards finding a permanent cure for this deadly disease that results in loss of human lives in short span of time after its manifestation. One of the promising sources to discover new drugs for treating cancer is from marine. Gallinamide A (**17**), a linear depsipeptide, was first isolated by Linington and co-workers in 2008 as a secondary antimalarial metabolite from *Schizothrix* species of cyanobacteria near the reef of Piedras Gallinas (Caribbean coast of Panama) *[1]*. It was found that Gallinamide A has identical linear structure with Symplostatin 4, a natural product isolated by Luesch and co-workers from cyanobacteria of the genus *Symploca* which was collected from Key Largo (Florida Keys) *[2]*. Galinamide A and Symplostatin 4 both have aliphatic peptidic backbone and consists of dimethylated *N*-terminal amino acid moiety, (*S*)-2-hydroxyisocaproic acid unit, an unusual 4-(*S*)-amino-2-(*E*)-pentenoic acid moiety and *C*-terminal *N*-acyl pyrrolinone entity *[1]*. In Gallinamide A the absolute configuration of *N*,*N*-dimethylisoleucinyl stereocenters were not elucidated due to lack of available isolated material *[1]*. The unit, 4-(*S*)-amino-2-(*E*)-pentenoic acid, present in both Gallinamide A and Symplostatin 4, is unusual and is present only in few natural products in nature *[3-5]* and this functional moiety is responsible for the exhibited biological activities of this family of natural products *[6]*. Gallinamide A was shown to have antimalarial activity of 8.4 μM (IC_{50}) towards W2 strain *p. falciparum*. Against mammalian vero cells it shows moderate activity and most surprisingly, it does not show any cytotoxicity toward NCI-H460 human lung tumour or neuro-2a mouse neuroblastoma cell lines *[7]*, whereas Symplostatin 4 is moderately active against HeLa cervical

carcinoma and HT-29 colon adenocarcinoma cell lines with IC_{50} value of 12 and 53 nM respectively *[2]*.

The total synthesis of depsipeptide Gallinamide A, published by Conroy and his co-workers in 2011, is the only reported solution phase synthesis with 30-33% overall yield *[8]*. But the total synthesis of an identical natural product Symplostatin 4 was published by Conroy and co-workers in 2010 *[7]* and Stolze group in 2012 *[9]*. The same group has also published the solid phase synthesis of Gallinamide A in 2014 *[10]*. Solution phase peptide synthesis has several disadvantages including moderate to poor yield, requirement of chromatographic purification techniques, time consuming and expensive protocols. Also the possibility of side reactions is much greater in solution phase peptide synthesis and the reported total synthesis methods suffer from multi-step process. These reported peptide synthetic methods are generally not amenable for scale up and hence results in poor biological characterization. Recently it was reported that Gallinamide A is an excellent inhibitor towards human cathepsin L with IC_{50} value of 5 nM for 30 minutes preincubation *[11]*. Cathepsin L is a lysosomal endopeptidase which is present in most of the eukaryotic cells and it is a member of the papain-like family of cysteine proteinases *[12-13]*. Apart from regular protein degradation *[14-17]* cysteine proteases are critically involved in multistep process of tumour growth, invasion and metastasis *[18]*. Metastasis is the advanced stage of cancer wherein the disease spreads to lymph nodes and distant organs. The interaction between the oncogenic cells and surrounding normal cells promotes tumour growth and proliferation *[19]*. After this stage oncogenic cells start to invade through blood vessel and metastasise to other parts of body. Cysteine protease cathepsin L plays a major role in the spreading of cancer by degrading the extra-cellular matrix and helps to invade and metastasis *[20]*.

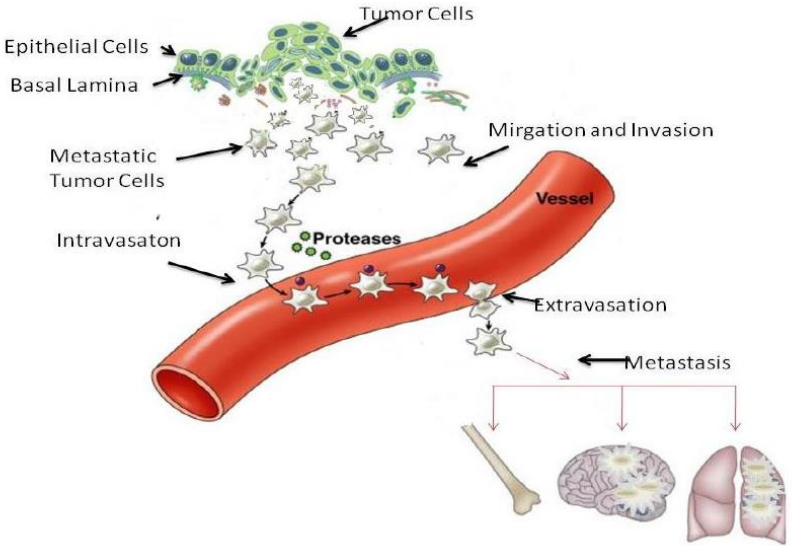


Figure 1.1: A general model of tumour cell metastasis where proteases have a major role.

In this chapter we have reported the synthesis of derivatives of natural product Gallinamide A, which might have similar biological activity like that of the natural product.

1.2. Experimental Section:

1.2.1. General Information and Methods:

All 2-chlorotrityl resins (H-Ala-2-ClTrt resin, H-Ile-2-ClTrt resin, H-Cys(Trt)-2-ClTrt resin), amino acids and coupling agents, used in solid phase peptide synthesis (SPPS) were purchased from Iris Biotech GmbH, Sigma Aldrich and NovaBiochem. ACS grade DMF, isopropanol, DMSO and biotech. grade DIPEA were purchased from Sigma Aldrich and used in SPPS without further purification. All peptide synthesis was performed manually by using peptide vessels (Chemglass) and standard peptide coupling procedures.

^{1}H NMR data were recorded by using Bruker AV 400 MHz NMR spectrometer with TMS as internal reference. Deuterated solvents like $CDCl_3$, DMSO-d_6 were used as solvent for recording NMR spectrum. Mass data were recorded on BrukermicroTOF-Q II by positive and negative mode electrospray ionization method. Reactions were

monitored by TLC and all compounds were purified by column chromatography. Column chromatography was performed using 100-200 or 320-400 mesh silica-gel. DMF was dried by vacuum distillation method and THF was dried by distillation method using sodium metal. Distilled hexane and distilled ethyl acetate were used as eluents in column chromatography.

1.2.1.1. Resin Swelling:

All resins used in solid phase peptide synthesis were swelled initially with 5 mL DCM for 30 minutes by bubbling nitrogen and after draining DCM, the resin was swelled once again with 5 mL DMF thrice for 15 minutes each.

1.2.1.2. Kaiser Test:

Few resin beads were taken in a test-tube and 2 drops each of ninhydrin, phenol and 0.1% potassium cyanide solution were added to the test-tube and heated for 2 minutes at 110 °C through sea sand kept in a beaker. The presence of free amine group was confirmed by the appearance of blue coloured resin beads in the test tube. The test was performed after coupling each amino acid to the growing peptide chain by the aforementioned procedure.

1.2.1.3. Fmoc Deprotection:

The Fmoc protected amino group in the growing peptide chain was deprotected using 20% piperidine in DMF (7 mL) by bubbling nitrogen for 10 minutes through the swelled resin beads in the peptide synthesizer vessel. The procedure was repeated twice to ensure complete deprotection of Fmoc protecting group from the amino acid.

1.2.1.4. Resin Cleavage:

A mixture of 9.25 mL TFA, 0.25 mL TIPS, 0.25 mL H_2O and 0.25 mL EDT was prepared and 5 mL of this cocktail solution was added to resin beads and nitrogen was bubbled through the suspension for 30 minutes. The cleaved peptide solution was collected from the peptide vessel in a RB. The residual peptide in the resin was treated with the cocktail solution (2 x 5 mL) and the same procedure was

repeated as mentioned above. Portions of the cleaved peptide solution were collected together in RB.

1.2.2. Synthesis of *N-Fmoc-4-(S)-amino-2-(E)-pentenoicacid* (2):

1.2.2.1. Synthesis of *(S)-(9H-fluoren-9yl)methyl (1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (Weinrab amide):*

Fmoc-Ala-OH (5 g, 16.06 mmol) and HBTU (8.5 g, 22.48 mmol) was taken in a 100 mL single neck round bottom flask (RB) and 15 mL dry DMF was added followed by drop wise addition of *N*,*N*-diisopropylethylamine (7 mL, 40.15 mmol) under inert atmosphere and stirred for 15 minutes at room temperature. In an another 25 mL single neck RB, *N*,*O*-dimethylhydroxylamine hydrochloride (1.6 g, 16.06 mmol) was taken and dissolved with 5 mL of dry DMF under inert atmosphere and subsequently it was added drop wise to the reaction mixture. The reaction mixture was further stirred for 16 h at room temperature and cooled to 0 °C. The yellowish oily crude mixture was purified through column chromatography using 10% ethyl acetate and hexane. The pure compound was obtained as white solid. (5.3 g, 93% yield). R_f = 0.50 [1:1 v/v Hexane/EtOAc]; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.28 (d, 2H, *J* = 8 Hz), 4.14 (t, 1H, *J* = 8 Hz), 4.68 (brs, 1H, *α*-H), 3.69 (s, 3H, CH_3), 3.13 (s, 3H, CH_3), 1.29 (d, 3H, *J* = 8 Hz, CH_3), 7.68 (d, 2H, *J* = 10.76 Hz), 7.52 (t, 2H, *J* = 8 Hz), 7.31 (t, 2H, *J* = 8 Hz), 7.23 (t, 2H, *J* = 8 Hz); Mass (ESI) m/z 377.1 [(M+Na)+].

1.2.2.2. Synthesis of *(S, E)-tert-butyl-4-((((9H-fluoren-9yl)methoxy) carbonyl)amino)-pent-2-enoate:*

The Weinrab amide (1 g, 2.82 mmol) was taken in a 100 mL double neck RB 5 mL THF was added to it and stirred at -20 °C under stirring condition. After 15 minutes, lithium aluminium hydride ($LiAlH_4$) (3 mL, 1 M solution in THF, 3.1 mmol) was added drop wise at -20 °C and stirred for 30 minutes. The reaction mixture was subsequently partitioned between ethyl acetate (EtOAc) (20 mL) and 0.1 M HCl (10 mL). The resulting solution was then washed with brine

(20 mL x 3) and EtOAc (50 mL). The organic layer was dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated under vacuum using rotavapor. Without purification, the resulting residue was used for the next step where *tert*-butyl(triphenylphosphoranylidene)acetate (1 g, 2.71 mmol) was added to it followed by the addition of 5 mL THF. The reaction mixture was stirred overnight at room temperature and diluted with ethyl acetate (20 mL) and washed with brine solution (20 mL x 3). The organic layer was dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated using rotavapor under reduced pressure. The crude mixture was purified by column chromatography using 7% ethyl acetate and hexane. The pure product was obtained as white solid (0.769 g, 69.3% yield). R_f = 0.50 [3:1 v/v Hexane/EtOAc]; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H, *J* = 8.4 Hz), 7.61 (d, 2H, *J* = 7.04 Hz), 7.42 (t, 2H, *J* = 7.28 Hz), 7.34 (t, 2H, *J* = 7.28 Hz), 6.79 (dd, 1H, CH, *J* = 4.28 Hz, 15.56 Hz), 5.85 (d, 1H, CH, *J* = 15.56 Hz), 4.83 (d, 1H, *J* = 6.52), 4.45 (d, 3H, *J* = 6.28), 4.23 (t, 1H, *J* = 6.52) 1.51 (s, 9H), 1.31 (d, 3H, *J* = 6.28); Mass (ESI) m/z 416.1 [(M+Na)+].

1.2.2.3. Synthesis of the Intermediate *N-Fmoc-4(S)-amino-2(E)-pentenoicacid:*

(*S*,*E*)-tert-butyl-4-((((9H-fluoren-9yl)methoxy)carbonyl)amino) -pent-2-enoate (**10**) (0.769 g, 1 mmol) was taken in a 100 mL RB followed by addition of 3 mL DCM. 6 mL of 50% TFA in DCM was added dropwise to the solution and stirred at room temperature and the reaction was monitored by TLC. After 3 h, the *tert*-butyl group is completely deprotected. The reaction mixture was then concentrated using rotavapor under reduced pressure and solvent-exchange with $CHCl_3$ (5 mL) was performed five times. The crude mixture was purified by silica-gel column chromatography using 50% ethyl acetate and hexane. The product was obtained as white solid (0.586 g, 88.8% yield). R_f = 0.40 [100% EtOAc]; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (d, 2H, *J* = 7.2 Hz), 7.7 (d, 2H, *J* = 7.0 Hz), 7.5 (d, NH, *J* = 7.5 Hz), 7.4 (t, 2H, *J* = 7.04 Hz), 7.33 (t, 2H, *J* = 7.04 Hz), 6.74 (dd, 1H,

CH, *J* = 3.7 Hz, 15.5 Hz), 5.75 (d, 1H, CH, *J* = 15.56 Hz), 4.2 (m, 4H), 1.17 (d, 3H, *J* = 6.28); Mass (ESI) m/z 360.1 [(M+Na)+].

1.2.3. Synthesis of Alanyl Derivative of Gallinamide A:

H-Ala-2-ClTrt resin (0.100 g, 0.041 mmol) was swelled in DCM and DMF as mentioned in 1.2.1.1 of experimental section. Then *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid (0.0346 g, 0.1025 mmol), PyBOP (0.0533 g, 0.1025 mmol) and DIPEA (0.018 mL, 0.1025 mmol) in 0.5 mL DMF was added to peptide vessel and the coupling reaction was continued overnight. The resin was washed with DMF (5 mL x 3) followed by the washing with isopropanol (3 mL x 3). Completion of reaction was confirmed by performing Kaiser test according to section 1.2.1.2. Then a solution of 20% piperidine in DMF was added to the peptide vessel to cleave the Fmoc protecting group according to the section 1.2.1.3. Resins were washed with DMF (3 mL x 3) and isopropanol (3 mL x 3). The formation of free amine was confirmed by the Kaiser test. After swelling the resin in DMF, Fmoc-(*L*)-Leu-OH (0.0348 g, 0.1025 mmol), PyBOP (0.0533 g, 0.1025 mmol) and DIPEA (0.018 mL, 0.1025 mmol) in 0.5 mL DMF was added to the resin and the mixture was bubbled with nitrogen overnight. Resin was washed with DMF (3 mL x 3) followed by washing with isopropanol (3 mL x 3). After confirming the completion of reaction by Kaiser test, 20% piperidine in DMF was added to resin to deprotect Fmoc protected amine and the formation of amine was assessed by the Kaiser test. The resin was washed with DMF followed by isopropanol as before. A solution of (*S*)-2-hydroxyisocaproic acid (0.0135 g, 0.1025 mmol), PyBOP (0.0533 g, 0.1025 mmol) and DIPEA (0.018 mL, 0.1025 mmol) in 0.5 mL DMF was added to the resin and the reaction continued overnight. The reaction was ensured by Kaiser Test. Fmoc-(*L*)-Ile-OH, HATU and DIPEA in 0.5 mL dry DMF was added to the peptide vessel under inert atmosphere and swelled it overnight. The resin was washed with DMF and isopropanol respectively. After deprotection of Fmoc group by 20% piperidine in DMF, a solution of sodium cyanoborohydride (0.0129 g, 0.205 mmol)

and formaldehyde (0.5 mL) in DMSO was added to the resin and reacted for 48 h. The resin was washed with DMSO (3 mL x 5) followed by hot water (3 mL x 5). Resins were then transferred to a filter paper and washed with water and DMSO. After completely washing, the resins were again transferred to peptide vessel and washed with DMF and isopropanol. Finally the resin was cleaved using cocktail solution mentioned in section 1.2.1.4. The crude mixture was concentrated under vacuum to provide a brown liquid.

1.2.4. Synthesis of Isoleucinyle Derivative of Gallinamide A:

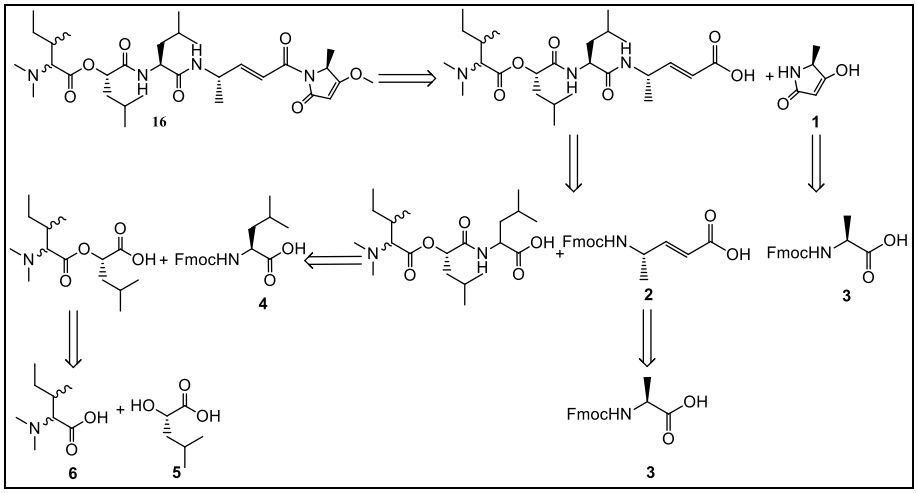
H-Ile-2-ClTrt resin (0.2 g, 0.08 mmol) was swelled according to section 1.2.1.1 and the coupling procedure was repeated as mentioned before in section 1.2.3. The crude mixture was concentrated to give the product as brown liquid.

1.2.5. Synthesis of Cysteinyl Derivative of Gallinamide A:

H-Cys(Trt)-2-ClTrt resin (0.2 g, 1 mmol) was swelled according to section 1.2.1.1 and the coupling procedure was repeated as mentioned before in section 1.2.3. The crude mixture was concentrated to give the product as brown liquid.

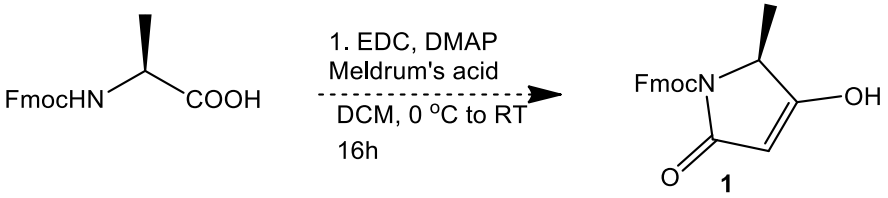
1.3. Results and Discussion:

Because solution phase synthesis suffers from many drawbacks, a new solid phase synthetic strategy is proposed in the present project. A simple retrosynthetic analysis of Gallinamide A is depicted in Scheme 1 by disconnecting the parent compound into five different components, labelled as **1**, **2**, **4**, **5** and **6**. The various components such as pyrrolinone derivative (**1**), pentenoic acid derivative (**2**) and other simple building blocks like **4, 5** and **6** could be synthesised in laboratory following reported or modified procedures.



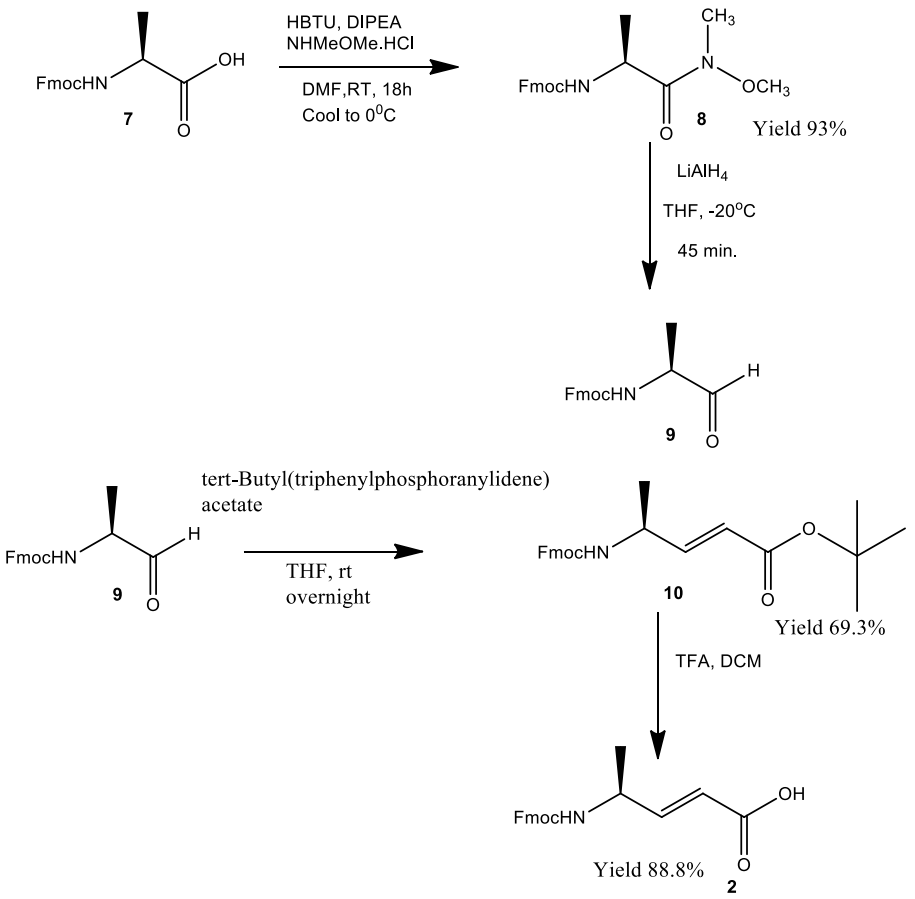
Scheme 1.1: Retrosynthetic analysis of Gallinamide A.

As shown Scheme 1.1, intermediates **1** and **2** could be synthesized from Fmoc protected alanine amino acid. Compound **3** could be condensed with meldrum's acid in presence of EDC and *N*,*N*-dimethylaminopyridine to give **1** *[7]*, shown in Scheme 1.2.



Scheme 1.2: Synthesis of N-Fmoc-pyrrolinone derivative.

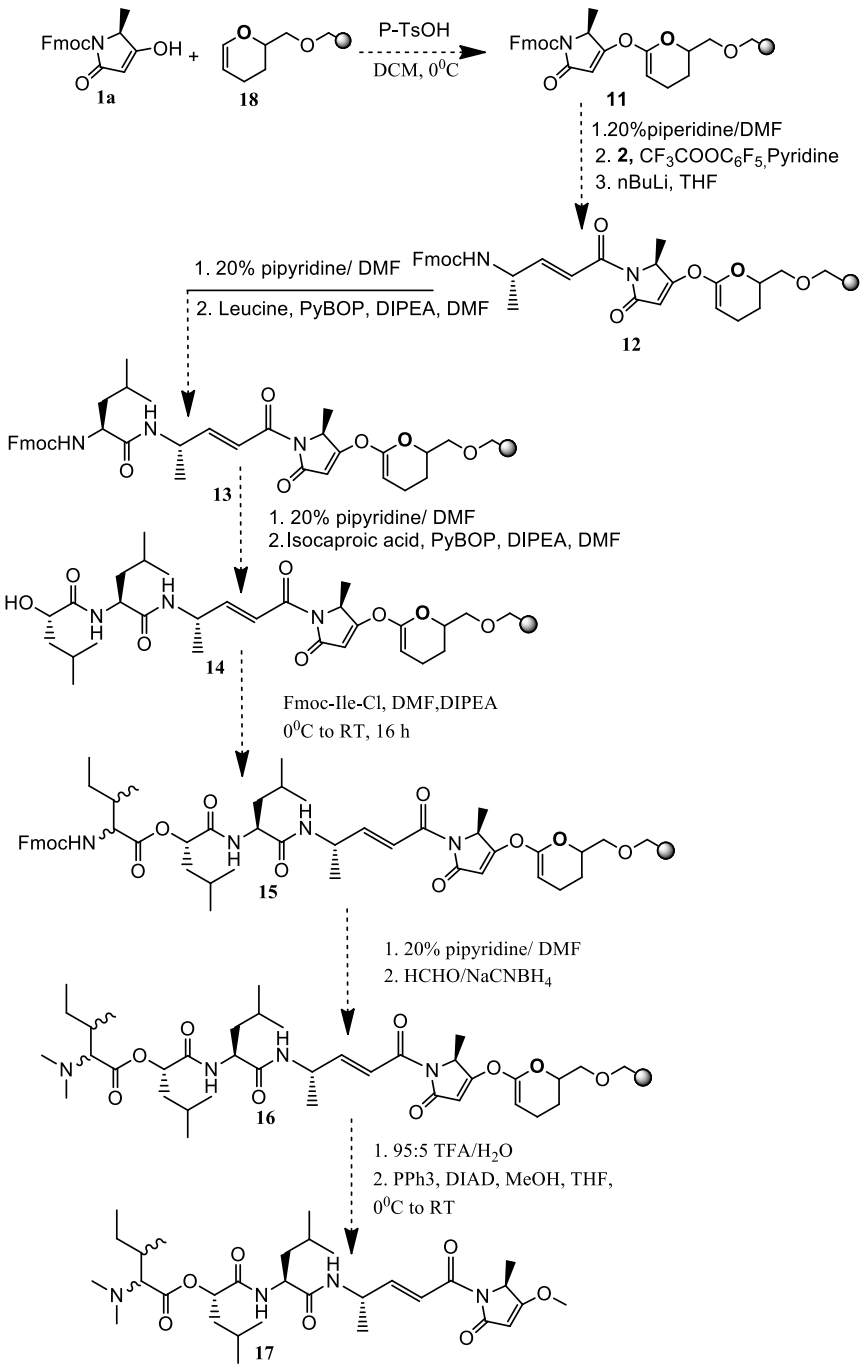
Fmoc-Ala-OH (**3**) is coupled with *N*,*O*-dimethylhydroxylamine hydrochloride (NHMeOMe.HCl) in presence of HBTU and diisopropylethylamine to give Weinrab amide (**8)** which was reduced to Fmoc-alanal by lithium aluminium hydride ($LiAlH_4$) to give **9**. Compound **9** undergoes Wittig reaction in presence of *tert*-butyl(triphenylphosphoranylidene)acetate to give (*S*,*E*)-*tert*-butyl 4-((((9H-fluoren-9yl)methoxy)carbonyl)amino)-pent-2-enoate (**10**) *[21]*, which was transformed to the intermediate *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid by the deprotection of *tert* butyl group with TFA as shown in Scheme 1.3.



Scheme 1.3: Synthesis of N-Fmoc protected 4-(S)-amino-2-(E)-pentenoic acid.

Compound **1** could be attached with DHP resin (**18**) through acetal formation in presence of PPTs in 1, 2-dichloroethane to give **6** *[22]*. After deprotection of Fmoc, compound **6** could be coupled with **2** in presence of pentafluorophenyltrifluoroacetate and *n*-BuLi to give compound **12** *[7]* which after deprotection of Fmoc could undergo condensation reaction with L-leucine amino acid (**4**) to give **13** in presence of HBTU. Now, Fmoc deprotected compound **13** could be coupled with (*S*)-2-hydroxyisocaproic acid (**5**) in presence of PyBOP to give **14** which could undergo esterification with **3** to give **15**. After deprotection of Fmoc, Compound **15** could undergo reductive methylation in presence of HCHO and $NaBH_4CN$ to give **16**. At the end of the synthesis the attached DHP resin could be cleaved by 95:5 trifluoroacetic acid and water at 60 °C *[22]* followed by methylation of

hydroxyl group in the C-terminal to give the natural product, which is shown in the Scheme 1.4.



Scheme 1.4: Synthesis of Gallinamide A

In this project we have successfully synthesized the Weinrab amide (**8**), *tert*-butyl(triphenylphosphoranylidene)acetate and (*S,E*)-*tert*-butyl 4-((((9H-fluoren-9 yl)methoxy)carbonyl)amino)-pent-2-enoate. In ^{1}H

NMR spectrum shown in figure 1.2, the peaks from 7.68 ppm to 7.23 ppm are due to the eight protons of Fmoc group, broad singlet at 4.68 for one α-H, doublet at 4.28 ppm is due to Fmoc methylene proton, triplet at 4.14 ppm is due to Fmoc-CH proton, sharp singlet at 3.69 ppm is due to methoxy protons, a sharp singlet at 3.13 is due to methyl protons, and doublet at 1.29 ppm for three alanyl methyl protons. The presence of above mentioned peaks in the reported spectrum as per literature confirms the formation of Weinrab amide.

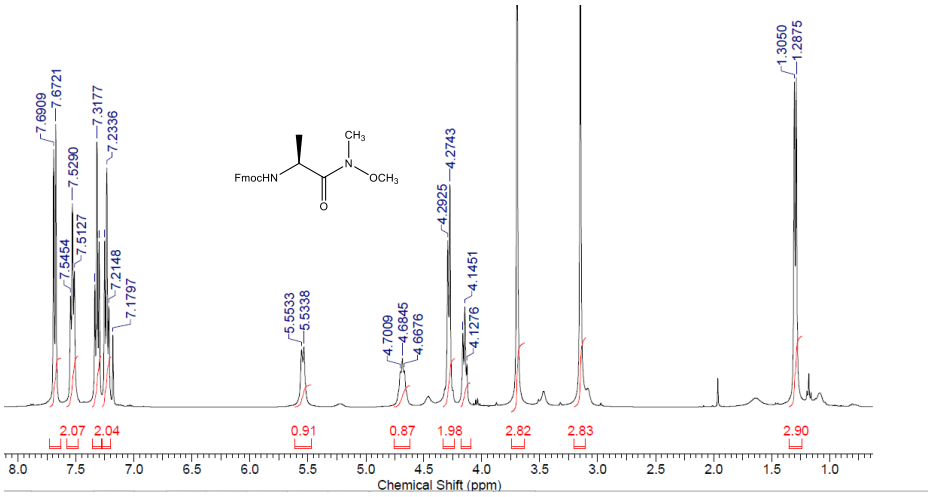


Figure 1.2: ^{1}H NMR spectrum of Weinrab amide.

Mass spectrum analysis show m/z at 377.1 [(M+Na)+] which confirms the formation of Weinrab amide.

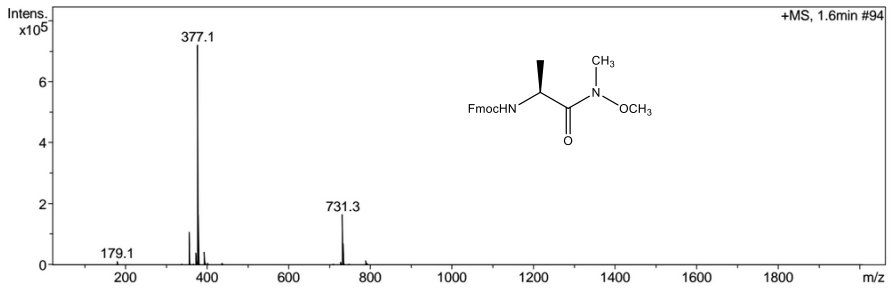


Figure 1.3: Mass spectra of Weinrab amide.

In ^{1}H NMR spectrum ($CDCl_3$) shown in figure 1.4, the peaks from 7.78 ppm to 7.34 ppm are due to the eight protons of Fmoc nucleus. Doublet of doublet at 6.79 ppm is for vinylic photon and the coupling constant 15.56 and 4.28 confirms the formation of trans double bond.

The doublet at 5.85 ppm is due to the other vinylic proton. The NH proton comes as a doublet at 4.83 ppm. One doublet at 4.45 ppm is due to Fmoc methylene proton and alanyl proton. The Fmoc-CH proton comes as a triplet at 4.23 ppm. 9 Hs of *tert*-butyle group comes as a sharp singlet at 1.51 ppm. The doublet at 1.31 ppm is due to the alanyl methyl protons. Also in mass spectrum the peak at m/z 416.2 [(M+Na)+] (Figure 1.5) confirms the formation of the compound (*S*,*E*)-tert-butyl 4-((((9H-fluoren-9yl)methoxy)carbonyl)amino)- pent-2-enoate (**10**).

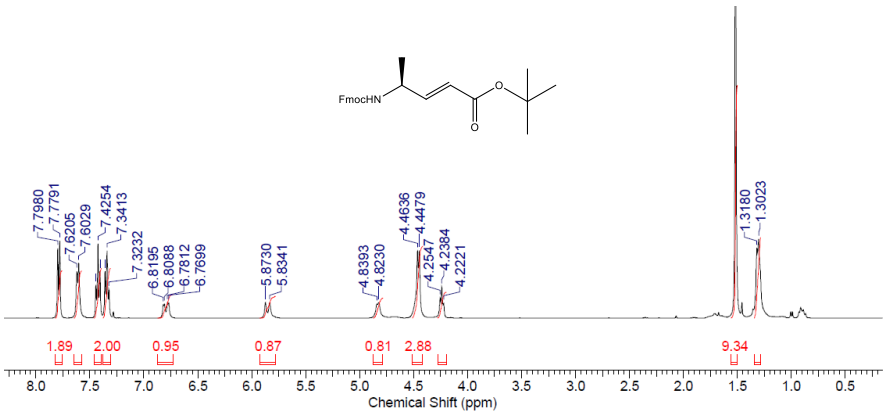


*Figure 1.4: ^{1}H NMR of compound **10**.*

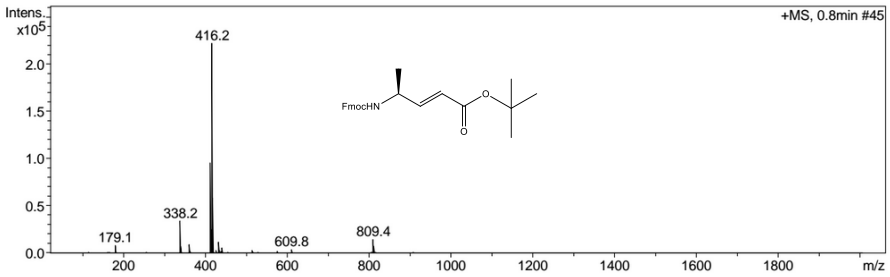


*Figure 1.5: Mass spectra of compound **10**.*

The *tert*-butyl group of compound **10** is successfully deprotected with 50% TFA in DCM to give *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid, the formation of which is confirmed by ^{1}H NMR spectrum (DMSO-d_6). The peaks at 7.88, 7.7, 7.4, and 7.3 ppm are due to the Fmoc nucleus protons. NH proton shows a doublet at 7.5 ppm and a multiplate at 4.2 ppm is due to methylene protons, alanyl proton and the acidic proton of Fmoc nucleus as shown in figure 1.6. Doublet of doublet at 6.79 ppm is for vinylic photon and the coupling constant

15.5 and 3.7 confirms the formation of trans double bond. The doublet at 5.75 ppm is due to the other vinylic proton. The sharp peaks at 3.3 and 2.5 ppm are due to moisture and DMSO respectively. In mass spectrum the peak at m/z 360.1 [(M+Na)+] confirms the formation of *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid (**2**) (Figure 1.7).

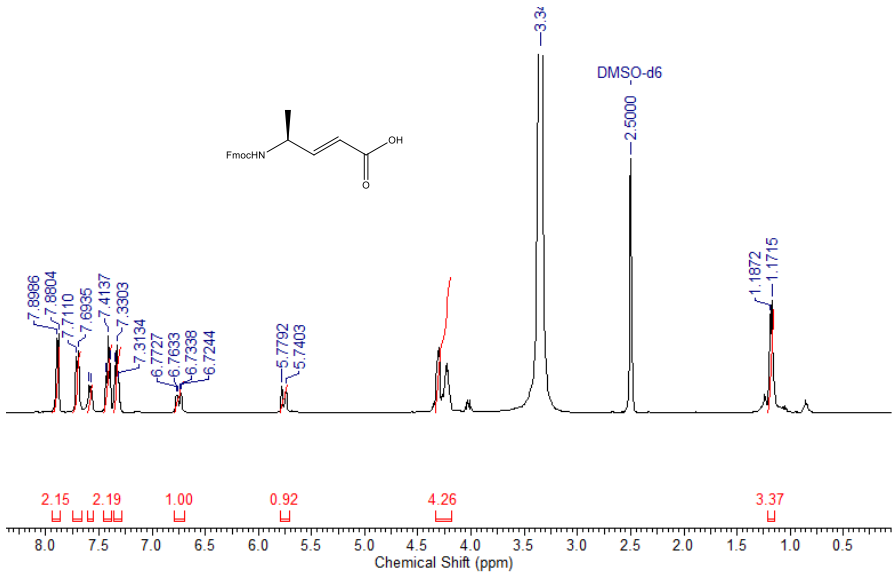
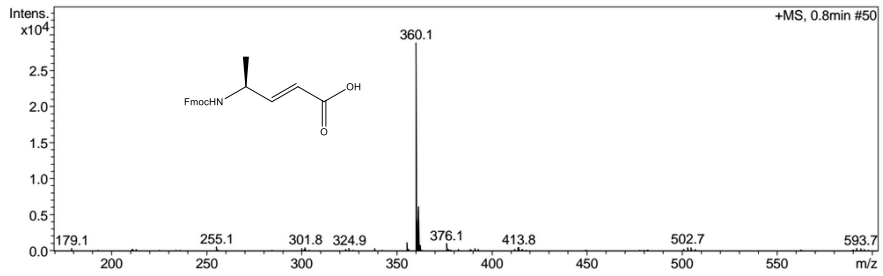


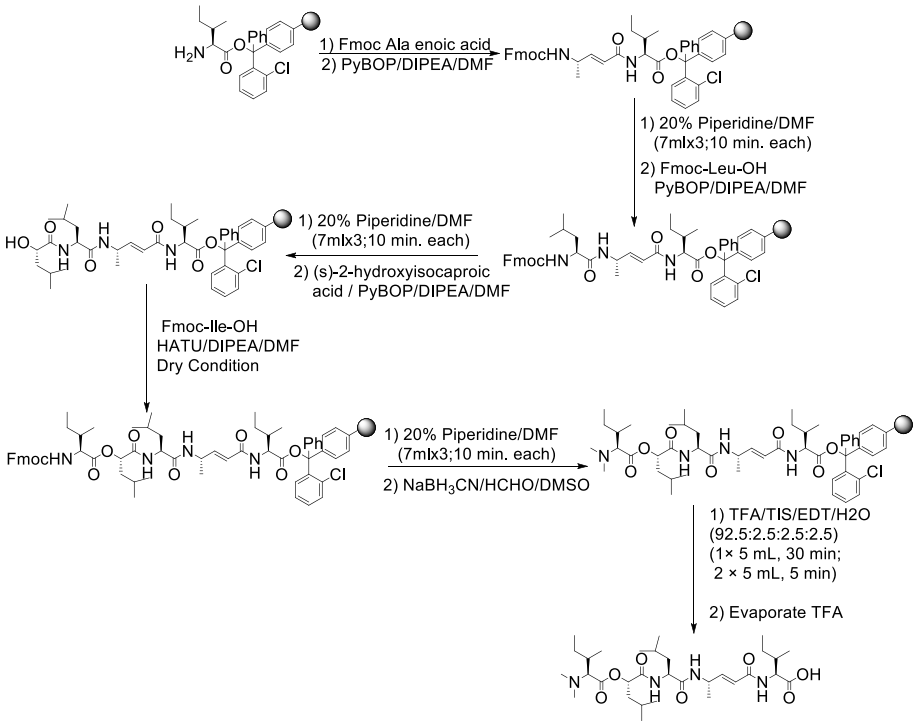
Figure 1.6: [1]H NMR of intermediate N-Fmoc-4-(S)-amino-2-(E)-pentenoicacid (***2*).**



Figire 1.7: Mass spectrum of compound ***2***

The free amine in H-Ile-2-ClTrt resin was coupled with *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid using PyBOP as coupling agent and DIPEA as base. After de-protection of Fmoc group, Fmoc-Leu-OH was coupled with the *N*-terminal enoic acid using the coupling agent PyBOP and base DIPEA. Next, (*S*)-2-hydroxyisocapric acid was coupled with *N*-terminal amino acid in a similar manner. The esterification between OH group of (*S*)-2-hydroxyisocapric acid and the COOH group of Fmoc-Ile-OH was performed using the coupling

agent HATU, DIPEA in dry DMF. After the removal of *N*-terminal Fmoc group, dimethylation was performed by reductive methylation using HCHO and $NaBH_4CN$. After the completion of the reaction, the resin was cleaved by a mixture of TFA, TIPS, EDT and H_2O cocktail solution as shown in scheme 1.5.



Scheme 1.5: Synthesis of isoleucinyl derivative of Gallinamide A.

LC-MS (negative mode) data has confirmed the formation of isoleucinyl derivative as shown in figure 1.8. The molecular peak at 595.49 (m/z) with retention time 10.2 minutes is due to the presence of desired product.

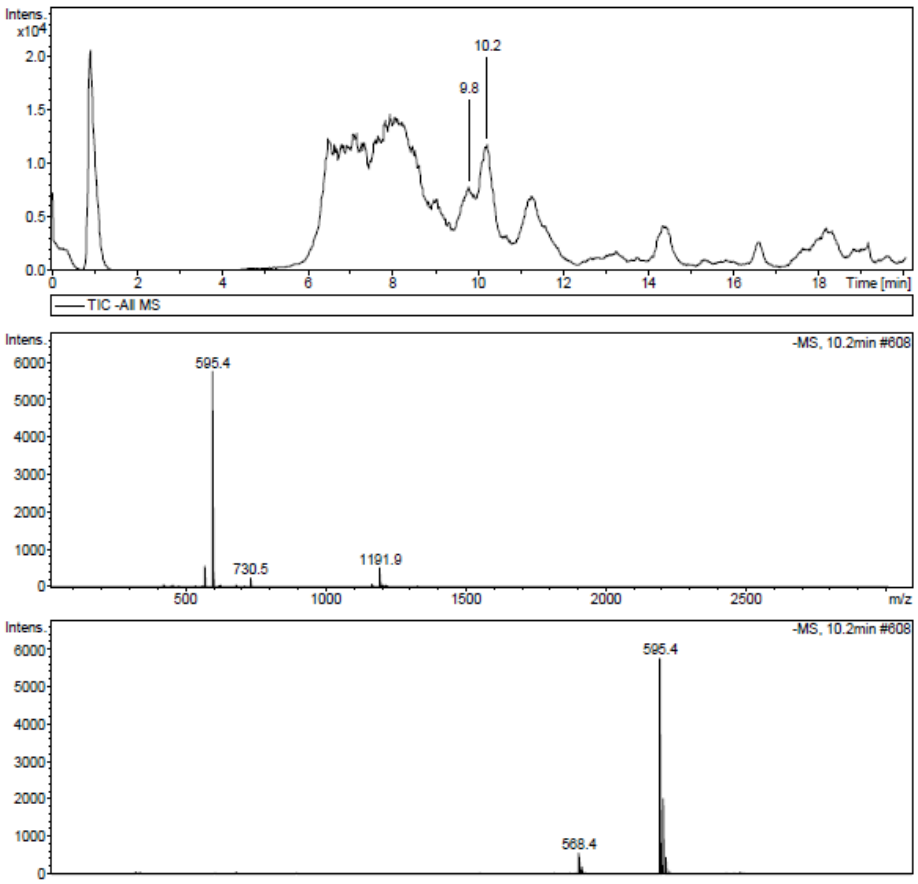
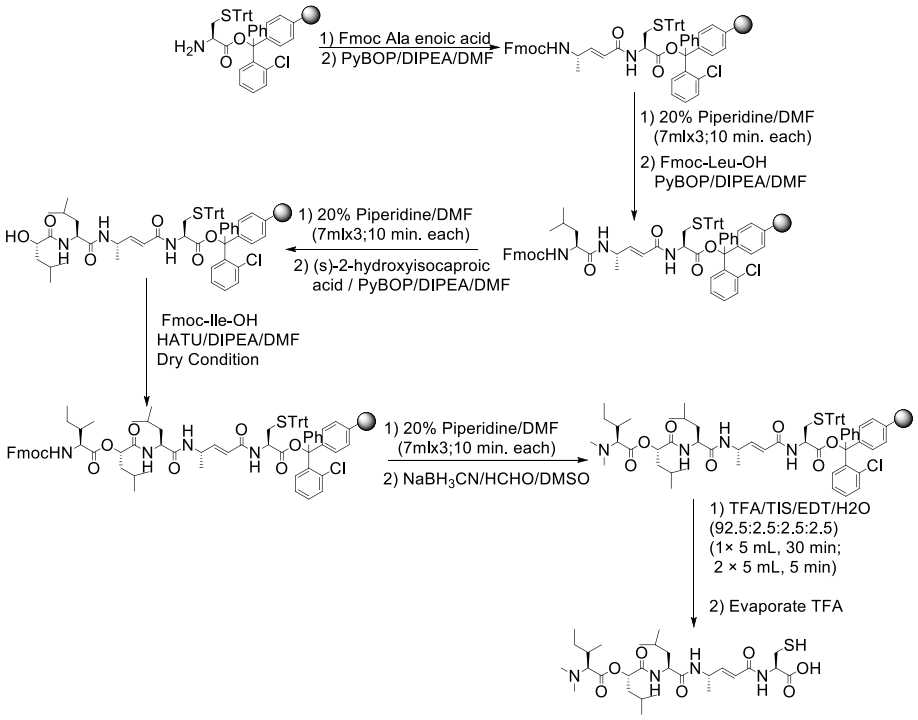
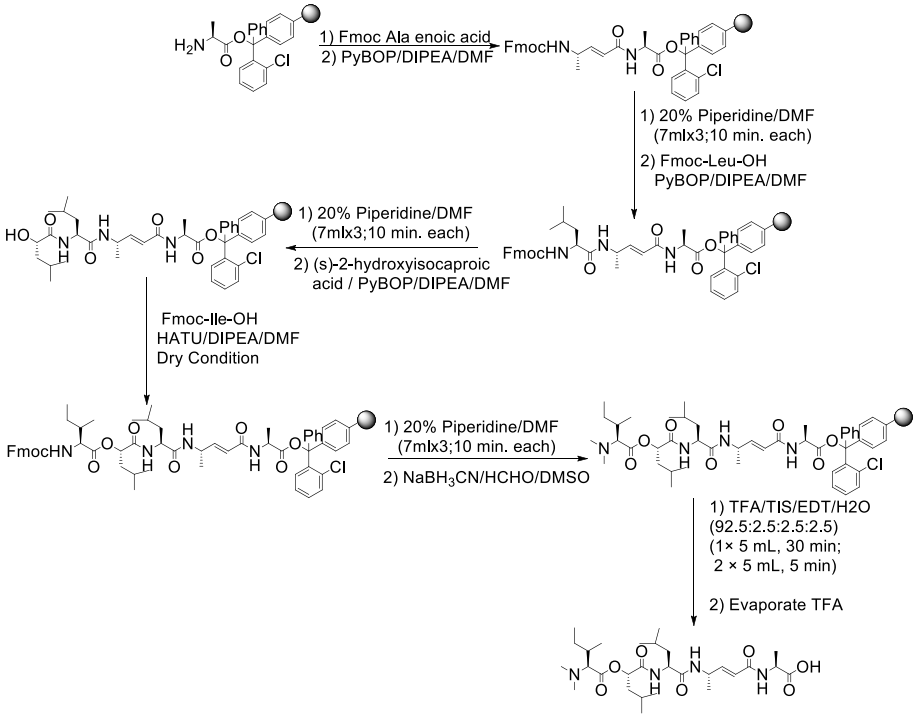


Figure 1.8: LC-MS of isoleucinyl derivative.

Similarly, alanyl and cysteinyl derivatives of Gallinamide A were synthesized as shown in schemes 1.6 and 1.7 respectively. The compounds (cysteinyl derivative) are identified by crude ^{1}H NMR (Figure A1) and yet to be purified.



Scheme 1.6: Synthesis of cysteinyl derivative of Gallinamide A.



Scheme 1.7: Synthesis of alanyl derivative of Gallinamide A.

1.4. Conclusion:

We have successfully synthesized Fmoc protected Weinrab amide (**8),** (*S*,*E*)-tert-butyl-4-((((9H-fluoren-9-yl)methoxy)carbonyl)-amino)-pent-2-enoate (**10**) and *N*-Fmoc-4-(*S*)-amino-2-(*E*)-pentenoicacid (**2**) with good yield *[21]*. We have also synthesized cysteinyl, isoleucinyl and alanyl derivatives of Gallinamide A. Cysteinyl and isoleucinyl derivatives are partially characterised by crude ^{1}H NMR and LC-MS spectrum respectively. Biological activity of the newly synthesized compounds will be performed in future.

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Chapter 2

Folate Receptor Targeted Drug Delivery

2.1. Introduction:

One of the major problems of drug delivery for therapeutic applications of pathological disease state is toxicity caused by uptake of drugs in healthy cells. To overcome this problem researchers have found special biomarkers or proteins over-expressed on abnormal cells by which drugs can be delivered selectively avoiding toxicity to normal and healthy cells or tissues. One such target is folate receptor (FR), a glycosylphosphatidyinositol-linked protein that transport its ligands into the cell via receptor mediated endocytosis *[1, 2]*. It is over-expressed and selectively upregulated on many malignant cancer cells *[3]* and activated macrophages *[4]*. Dr. Christopher P. Leamon and Prof. Philip S. Low first discovered that folate receptor can be used for diagnostic and therapeutic purpose by attaching vitamin folate or folic acid to a cargo to form "folate-conjugate" *[5]*. Folic acid is a vital nutrient which is required by all the living cells for biosynthesis of nucleotide and for the proper metabolic maintenance of thymidine synthesis and DNA formation *[6]*. It has very high affinity towards the folate receptor (K_D = 10^{-10} M) and the binding affinity is not compromised even when diagnostic and therapeutic cargos are attached to folic acid *[7]*. Since mammalian cells cannot synthesis folate, fortification from external sources, such as nutrients are absolutely necessary. Cellular uptake of folate is generally mediated by two types of surface proteins expressed on cells. One is reduced folate carrier which is a low affinity (K_D ~ 1-5 µM) membrane spanning protein and other is GPI anchored high affinity protein (K_D ~ 1 nM) which is known as folate binding protein or folate receptor (FR) *[8, 9]*.

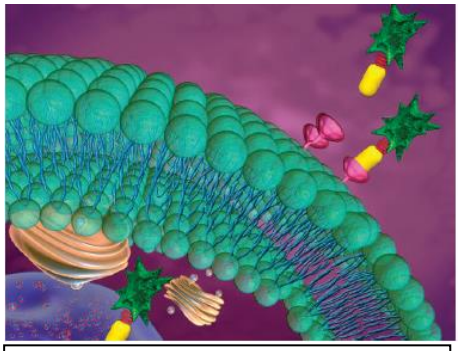


Figure 2.1: Folate receptor mediated endocytosis

Human folate receptor (h-FR) is encoded by several genes like FR-α, FR-β, FR-γ, and FR-δ, though FR-δ positive tissue is not found till now *[10]*. It has been shown that folate receptors have very limited distribution towards normal cells whereas FR-α and FR-β are highly over-expressed in cancer cells and activated macrophages respectively *[10]*. FR-α is found to be over-expressed specially in cancers of ovary, kidney, brain, breast, lung, endometrium, colon and hematopoietic cells of myelogenous origin *[11]*. FR-β is found to be over-expressed in different types of inflammatory diseases such as rheumatoid arthritis, psoriasis, Crohn's disease, atherosclerosis, systemic lupus erythematosus, diabetes, osteoarthritis, ulcerative colitis, glomerulonephritis, and sarcoidosis. In this chapter we have synthesized FR targeting molecules such as pteroate linker, hydrazide pteroate linker and one more well-established targeting molecule EC-20. We would like to explore these two hitherto unknown small molecule inhibitors to deliver either therapeutic warheads or functional imaging agents to cancers expressing folate receptors.

2.2. Experimental Section:

2.2.1 General Information and Methods:

H-Cys(Trt)-2-ClTrt resin, amino acids [Fmoc-Asp(OtBu)-OH, Boc-Dap(Fmoc)-OH and *N*-Fmoc(*α*-OtBu)-Glu-OH.HCl] and coupling agent PyBOP, used in solid phase peptide synthesis (SPPS) were purchased from Iris Biotech GmbH, Sigma Aldrich and NovaBiochem. N_{10}-(trifluoroacetyl)pteroic acid was purchased from Sigma Aldrich. ACS grade DMF, isopropanol, DMSO and biotech grade DIPEA were purchased from sigma Aldrich and used in SPPS without further purification. All peptide synthesis were performed manually by using peptide vessels (Chemglass) and standard peptide coupling procedures. Mass spectrometric data were recorded on BrukermicroTOF-Q II by positive mode electrospray ionization method. ^{1}H NMR data was recorded by using Bruker AV 400 MHz NMR spectrometer with TMS as internal reference. Deuterated solvents like DMSO-d_6, $CDCl_3$ were used for recording NMR spectrum.

2.2.1.1. Resin Swelling:

H-Cys(Trt)-2-ClTrt resin used in solid phase peptide synthesis was swelled initially with 5 mL DCM for 30 minutes by bubbling nitrogen and after draining DCM, the resin is swelled once again with 5 mL DMF thrice for 15 minutes each.

2.2.1.2. Kaiser Test:

Few resin beads were taken in a test-tube and 2 drops each of ninhydrin, phenol and 0.1% potassium cyanide solution were added to the test-tube and heated for 2 minutes at 110 °C through sea sand kept in a beaker. The presence of free amine group is confirmed by the appearance of blue coloured resin beads in the test tube. The test is performed after coupling each amino acid by the aforementioned procedure.

2.2.1.3. Fmoc Deprotection:

The Fmoc protected amino group in the growing peptide chain is deprotected using 20% piperidine in DMF (7 mL) by bubbling nitrogen for 10 minutes through the swelled resin beads in the peptide synthesizer vessel. The procedure is repeated twice to ensure complete deprotection of Fmoc protecting group from the amino acid.

2.2.1.4. Resin Cleavage:

A mixture of 9.25 mL TFA, 0.25 mL TIPS, 0.25 mL H_2O and 0.25 mL ethane dithiol was prepared and 5 mL of this cocktail solution was added to resin beads and nitrogen was bubbled through the solution for 30 minutes. The cleaved peptide solution was collected from the resin in a RB. The residual peptide in the resin was mixed with cocktail solution twice and same procedure was repeated as above. The remaining cleaved peptide solution was collected together in RB and the mother liquor was distilled under vacuum using rotavapor to obtain a viscous liquid from which the required peptide chelating linker was precipitated out using ice cold ether. The precipitated peptide was dried under nitrogen atmosphere while being protected from light using aluminium foil. The crude peptide can be

purified by RP-HPLC using buffer solution (20 mM NH_4OAc, pH = 5.0) and acetonitrile.

2.2.2 Synthesis of Pteroate Linker:

H-Cys(Trt)-2-ClTrt resin (0.150 g, 0.093 mmol) was swelled according to section 2.2.1.1. Then Fmoc-Asp(OtBu)-OH (0.095 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to peptide vessel and bubbled overnight. The resin was washed with DMF (5 mL x 3) followed by the washing with isopropanol (3 mL x 3). Completion of reaction was confirmed by performing the Kaiser test according to section 2.2.1.2. Then a solution of 20% piperidine in DMF was added to the peptide vessel to cleave the Fmoc protecting group according to the section 2.2.1.3. Resins were washed with DMF (3 mL x 3) and isopropanol (3 mL x 3). The completion of reaction was confirmed by the Kaiser test. After swelling the resin in DMF, Boc-Dap(Fmoc)-OH (0.099 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to the resin and bubbled with nitrogen overnight. Resin was washed with DMF (3 mL x 3) followed by the washing with isopropanol (3 mL x3). The completion of reaction was confirmed by Kaiser test. After deprotection of Fmoc group with 20% piperidine in DMF, the formation of free amine was assessed by the Kaiser test. The resin was washed with DMF followed by isopropanol like aforementioned procedure and bubbled properly with DMF. N_{10}-(trifluoroacetyl)pteroic acid (0.043 g, 0.14 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMSO was added to the peptide vessel and bubbled overnight. The peptide vessel was rapped with aluminium foil. Then resin was washed with DMF and isopropanol. The completion of reaction was confirmed by the Kaiser test. 1% hydrazine in DMF was added to resins thrice, 2 mL each for 2 minutes. After the deprotection of trifluoroacetyl group resin was washed properly with DMF. Finally the resin was cleaved and the compound was collected according to section 2.2.1.4.

2.2.3 Synthesis of HydrazidePteroate Linker:

H-Cys(Trt)-2-ClTrt resin (0.150 g, 0.093 mmol) was swelled according to section 2.2.1.1. Then Fmoc-Asp(OtBu)-OH (0.095 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to peptide vessel and bubbled overnight. The resin was washed with DMF (5 mL x 3) followed by the washing with isopropanol (3 mL x 3). Completion of reaction was confirmed by performing the Kaiser test according to section 2.2.1.2. A solution of 20% piperidine in DMF was added to the peptide vessel to cleave the Fmoc protecting group according to the section 2.2.1.3. Resins were washed with DMF (3 mL x 3) and isopropanol (3 mL x 3). The completion of reaction was confirmed by the Kaiser test. After swelling the resin in DMF, Boc-Dap(Fmoc)-OH (0.099 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to the resin and bubbled with nitrogen overnight. Resin was washed with DMF (3 mL x 3) followed by the washing with isopropanol (3 mL x 3). The completion of reaction was confirmed by the Kaiser test. After deprotection of Fmoc group with 20% piperidine in DMF, the formation of free amine was assessed by the Kaiser test. Resin was washed with DMF followed by isopropanol like aforementioned procedure and bubbled properly with DMF. N_{10}-(trifluoroacetyl)pteroicacid (0.043 g, 0.14 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMSO was added to the peptide vessel and bubbled overnight. The peptide vessel was rapped with aluminium foil. Resin was washed with DMF and isopropanol. The completion of reaction was confirmed by the Kaiser test. 1% hydrazine in DMF was added to resins thrice, 2 mL each for 10 minutes. After that resin was washed properly with DMF. Finally the peptide was cleaved and the compound was collected according to section 2.2.1.4.

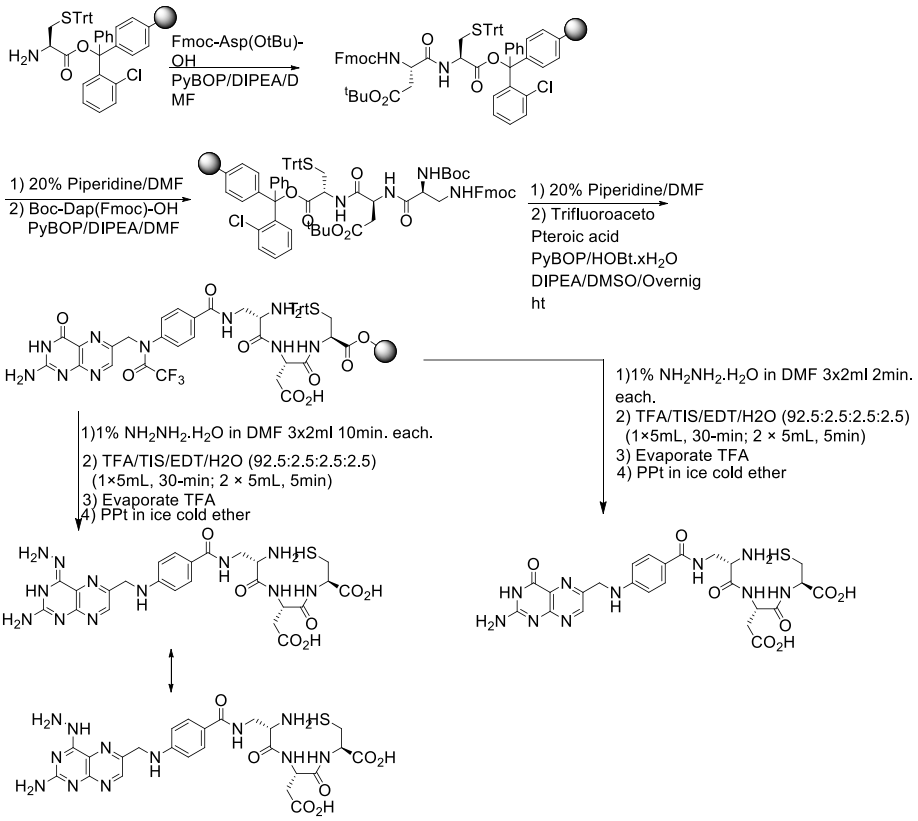
2.2.4 Synthesis of EC-20:

H-Cys(Trt)-2-ClTrt resin (0.150 g, 0.093 mmol) was swelled according to section 2.2.1.1. Then Fmoc-Asp(OtBu)-OH (0.095 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to peptide vessel and bubbled overnight. The resin was washed with DMF (5 mL x 3) followed by the washing with isopropanol (3 mL x 3). Completion of reaction was confirmed by performing the Kaiser test according to section 2.2.1.2. A solution of 20% piperidine in DMF was added to the peptide vessel to cleave the Fmoc protecting group according to the section 2.2.1.3. Resins were washed with DMF (3 mL x 3) and isopropanol (3 mL x 3). The completion of reaction was confirmed by the Kaiser test. After swelling the resin in DMF, Boc-Dap(Fmoc)-OH (0.099 g, 0.233 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMF was added to the resin and bubbled with nitrogen overnight. Resin was washed with DMF (3 mL x 3) followed by the washing with isopropanol (3 mL x 3). The reaction was confirmed by the Kaiser test. After deprotection of Fmoc group with 20% piperidine in DMF, the formation of free amine was assessed by Kaiser test. Resin was washed with DMF followed by isopropanol like aforementioned procedure and bubbled properly with DMF. N-Fmoc-(α-OtBu)-Glu-OH (0.197 g, 0.465 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) was added to the peptide vessel and bubbled it overnight. After completion of the reaction, confirmed by Kaiser test, 20% piperidine was added to resin to form free amine which was comfirmed by the Kaiser test. Resin was washed with DMF and isopropanol. N_{10}-(trifluoroacetyl)pteroicacid (0.043 g, 0.14 mmol), PyBOP (0.121 g, 0.233 mmol) and DIPEA (0.041 mL, 0.233 mmol) in 0.3 mL DMSO was added to the peptide vessel and bubbled overnight. The peptide vessel was rapped with aluminium foil. Resin was washed with DMF and isopropanol. The completion of reaction was confirmed by the Kaiser test. 1% hydrazine in DMF was added to resins thrice, 2 mL each for 10 minutes. After that resin was

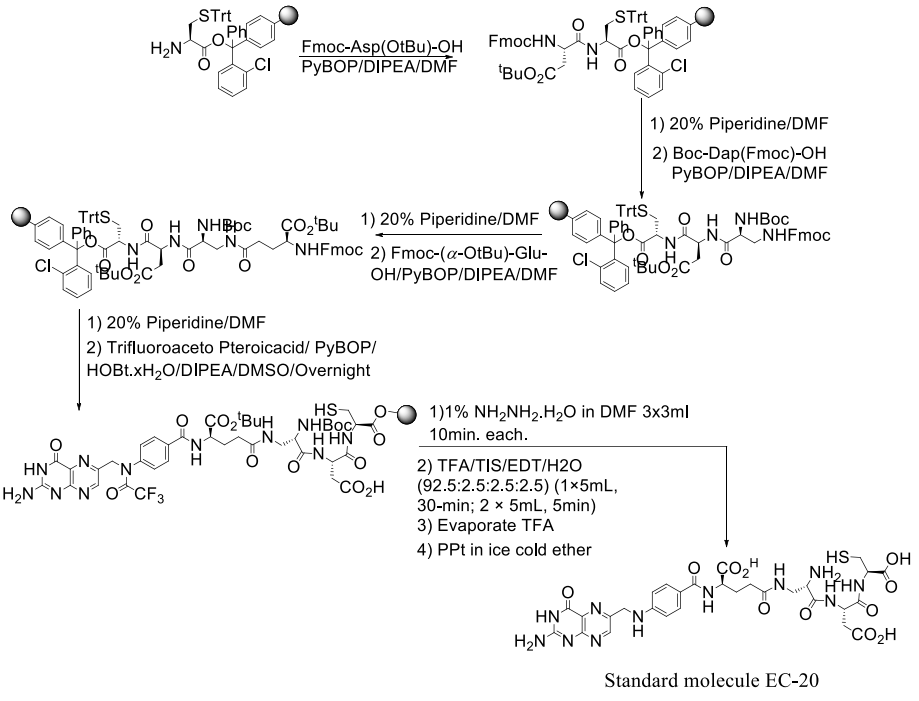
washed properly with DMF. Finally the peptide was cleaved and compound was collected according to section 2.2.1.4.

2.3. Results and Discussion:

An inhibitor of folate receptor is a magic bullet which finds its target and very specifically binds to it. Due to over-expression of FR protein on malignant cancer cells a number of FR inhibitors are discovered to deliver drugs or imaging agents selectively to the cancer cells. EC-20, a molecule synthesized by coupling of folic acid and a chelating linker, is such an inhibitor which is already in clinical trial. The pteridine moiety of folic acid binds to the FR protein and enters to the cell by receptor mediated endocytosis. So, pteridine nucleus is an essential moiety for the FR targeting. We have newly synthesized pteroate, hydrazidepteroate linkers as probable folate inhibitors and a well-established FR targeting molecule EC-20 by reported procedure to compare the newly synthesized inhibitors *[12]*. The resin H-Cys(Trt)-2-ClTrt was attached to second amino acid Fmoc-Asp(OtBu)-OH using PyBOP as coupling agent and DIPEA as base. After de-protection of Fmoc group, the free amine group was coupled with Boc-Dap(Fmoc)-OH using PyBOP and DIPEA. Next, de-protection of Fmoc group from diaminopropanoic acid is carried out followed by coupling of N_{10}-(trifluoroacetyl)pteroicacid to the free amine of *N*-terminal amino acid. The trifuoroacetyl group is deprotected using 1% hydrazine solution in DMF for a short reaction time of 2 minutes for the preparation of pteroate linker whereas the deprotection reaction using hydrazine is continued for longer time to react with the pteridine nucleus of pteroic acid for the preparation of hydrazidepteroate linker. The pteroate small molecule inhibitors were finally detached from the resin using a cocktail mixture of TFA, TIPS, EDT and H_2O in appropriate ratio. The cleaved inhibitors were precipitated out in ice cold ether and it could be purified by HPLC chromatography for further evaluation in FR expressing cancer cell lines.



Scheme 2.1: Synthesis of pteroate and hydrazidepteroate inhibitor.



Scheme 2.2: Synthesis of standard molecule EC-20

Mass, and RP-HPLC spectrum, shown in figure 2.2 and figure 2.3 respectively, has proved the formation of pteroate linker and characterisation of its hydrazide derivative and EC-20 is under process.

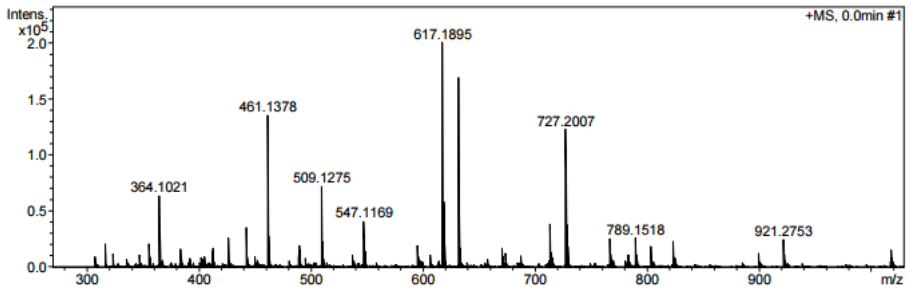


Figure 2.2: Mass spectrum of pteroate linker.

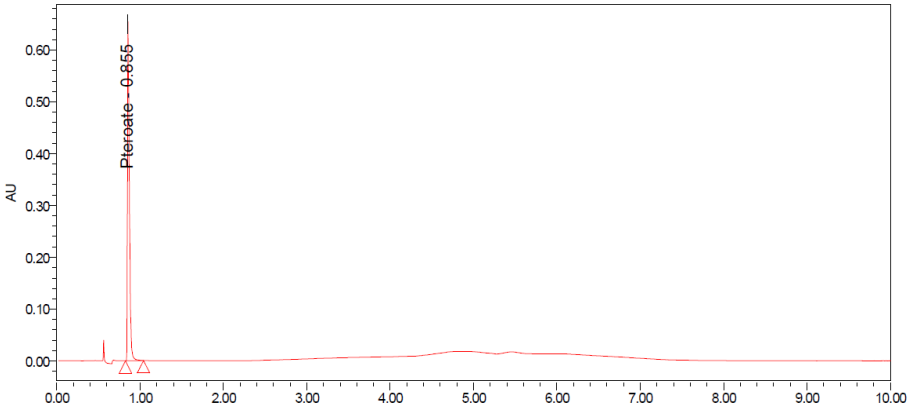


Figure 2.3: RP-HPLC spectrum of pteroate linker.

2.4. Conclusion:

In this chapter we have synthesized pteroate and hydrazidepteroate linkers for targeting folate receptor. After purification by RP-HPLC we can perform cell study to measure the binding affinity constants by attaching NIR fluorescent dyes or radioisotopes. We would like to do the comparative study with EC-20, a well-established folate targeting ligand reported elsewhere.

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APPENDIX-A

Characterization data of cysteinyl Gallinamide A derivative: Chapter 1

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1. ^{1}H crude NMR:

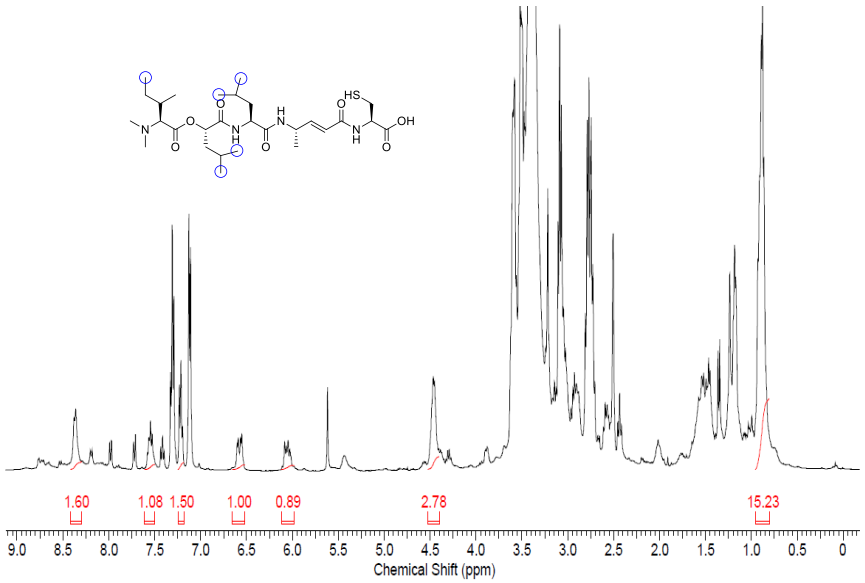

Figure A1: Crude ^{1}H NMR of cysteinyl Gallinamide A derivative. The sharp singlet near 1 ppm is due to 15 marked methyl Hs. One doublet of doublet at 6.5 ppm and one doublet at 6 ppm is due to vinylic Hs. NH protons are at the region of 8.5 to 7 ppm.