Design, Synthesis, and Biological Evaluation of Third-Generation Tubulin Inhibitors as Anti-cancer Agents

M.Sc. Thesis

By

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DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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Design, Synthesis, and Biological Evaluation of Third-Generation Tubulin Inhibitors as Anti-cancer Agents

A THESIS

Submitted in partial fulfilment of the

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by

Gobinda Sarkar

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the thesis entitled "**Design, Synthesis, and Biological Evaluation of Third-Generation Tubulin Inhibitors as Anti-cancer Agents**" in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF CHEMISTRY, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2024 of joining the M.Sc. program to May 2025 of M.Sc. thesis submission under the supervision of **Prof. Venkatesh Chelvam**, Department of Chemistry, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Gobinda Sarkar

Gobinda Sarkar

Signature of the student

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Venkatesh.C

Prof. Venkatesh Chelvam

Signature of the Supervisor

Gobinda Sarkar has successfully given his M.Sc. Oral Examination held on **May 16, 2025**

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M.Sc. Student

ABSTRACT

Tubulysins are the most active tetrapeptides against cancer found in nature. Tubulysin was extracted from Myxobacterium by Höfle and his coworkers in 2000. Tubulysin inhibits cancer growth by disrupting microtubules. It binds to the vinca domain of β-tubulin of alpha-beta heterodimer which finally results in the depolymerization of the microtubule, and consequently, the cytoskeleton has been disrupted. Due to this disruption, cell death occurs through apoptosis. Due to its strong ability to kill cancer cells, tubulysin has been used to treat various types of cancer. Tubulysins are effective against multidrug-resistant cancer cells. Although tubulysin has anticancer properties against cancer cells, there is a need for derivatives that have better serum stability, shelf life, and reduced toxicity to normal cells. In this study, we synthesized new tubulysin derivatives by structural modifications to improve their protease stability and biological activities. Tubulysin derivatives were synthesized by modifying tubuvaline and tubuphenylalanine subunits. The synthesis involves optimizing side chain functionalization, followed by purification using RP-HPLC and characterization using spectroscopic techniques such as ^{1}H and ^{13}C NMR and mass spectrometry.

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SYMBOLS/ UNITS

J	Coupling constant
δ	Delta
h	Hour
Hz/MHz	Hertz/Mega Hertz
mg	Milli gram
mL	Milliliter
R_f	Retardation factor
ppm	Parts per million

ACRONYMS

Most abbreviations for amino acids, substituents, reagents, etc. follow the guidelines set forth by the IUPAC-IUB Commission on Biochemical Nomenclature in Pure and Applied Chemistry, 40, 315-331 (1974). The L configuration is present in all amino acids. The same three-letter code represents all amino acids. Below is a list of additional abbreviations used in this report

CaH_2	Calcium Hydride
$CDCl_3$	Chloroform-d
d	Doublet
dd	Doublet of doublet
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DIPEA	N, N-Diisopropylethylamine
DMF	N, N-Dimethylformamide
EDC.HCl	1-Ethyl-3-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EtOAc	Ethyl acetate
Fmoc	9-Fluorenylmethoycarbonyl
Gly	Glycine
Glu	Glutamic
HCl	Hydrochloric acid
HOBt	Hydroxy benzotriazole
Ile	Isoleucine
Leu	Leucine
m.p.	Melting point
m	Multiplet
Na_2SO_4	Sodium sulphate
Na_2CO_3	Sodium carbonate

NMR	Nuclear magnetic resonance spectroscopy
PyBOP	Benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate
Phe	Phenylalanine
q	Quartet
s	Singlet
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
TMS	Tetramethyl silane
THF	Tetrahydro furan
Fmoc-β-Ho-Phe-OH	(*R*)-3-(Fmoc-amino)-3-phenylpropionic acid, Fmoc-L-β-homophenylalanine
Fmoc-β-Ho-Ile-OH	(3R,4S)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-methylhexanoic acid
Fmoc-L-tert-leu-OH	(S)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylbutanoic acid
Fmoc-β-Ho-Val-OH	N-(9-Fluorenylmethoxycarbonyl)-L-valine derivative

Chapter 1

Introduction

1.1 Global and Indian cancer statistics

Cancer is a major global health threat, causing millions of deaths annually and affecting people across all demographics. According to the GLOBOCAN 2022 report, approximately 20 million new cancer cases were reported worldwide in 2021. Among females, breast cancer was the most diagnosed type, while in males, lung cancer was the most prevalent (Figure 1). The report also recorded 9.7 million cancer-related deaths globally, with lung cancer identified as the leading cause of mortality (Figure 2) [1].

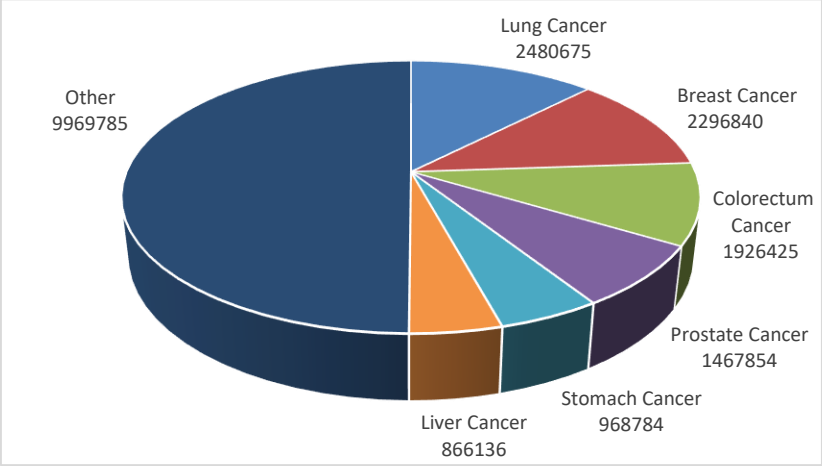


Figure 1. Number of cancer cases worldwide

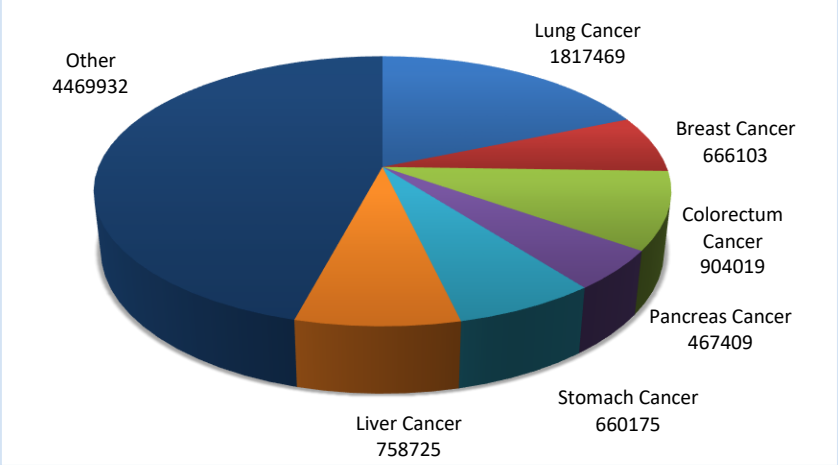


Figure 2. Number of cancer deaths worldwide

In India, the estimated number of incident cases of cancer was found to be 1,461,427 for the year 2021, where lung and breast cancers were the leading sites of cancer in males and females, respectively [2].

1.2 Microtubules

Microtubules are dynamic, filamentous structures composed of α- and β-tubulin subunits, forming a critical part of the cytoskeletal structures of a cell. They play vital roles in maintaining cell shape, intracellular transport, signaling, and, most importantly, cell division through their role in forming the mitotic spindle during mitosis [3]. In cancer, the role of microtubules is particularly significant as the rapid and uncontrolled division of cancer cells depends on efficient microtubule dynamics. Cancer cells disrupt the dynamic instability of microtubules to progress through the cell cycle rapidly; consequently, targeting microtubules has become a critical strategy in anticancer therapy [4].

1.3 Microtubule inhibitors and their mechanism of action

Microtubule inhibitors function by disrupting the dynamics of microtubules, thereby impairing cell division and motility. Their ability to disturb microtubule dynamics makes tubulin inhibitors valuable in cancer treatment, as they can effectively halt or limit the spread of cancer cells. Inhibitors such as vincristine and paclitaxel (Taxol) target microtubules to induce mitotic arrest and thereby apoptosis. Similarly, tubulysin derivatives act on the tubulin cytoskeleton through a comparable mechanism, highlighting their potential as promising therapeutic agents in oncology [5].

1.4 Tubulysin derivatives

Tubulysins are one of the most important family of natural products due to their therapeutic potential, notably in cancer treatment. The first tubulysin derivative was extracted from myxobacterium in the year 2000 by Höfle and his coworkers, followed by the discovery of

different types of tubulysin derivatives (Figure 3) [4-5]. All tubulysin derivatives share a peculiar feature at the C-termini: a tubuphenylalanine (found in tubulysin D, E, F, H, U, V, and M) or tubutyrosine (Tut) (found in tubulysin A, B, C, G, I, X, and Z) along with an odd N, O acetal. Due to these unique structural features and strong cytotoxic effects against various cancers, tubulysin derivatives have a significant interest in medicinal chemistry. These natural tetrapeptides bind to the vinca domain of β-tubulin of the αβ-heterodimer, which finally results in the depolymerization of the microtubule, leading to cell apoptosis. It has been reported that each of the tubulysin derivatives may be useful in cancer treatment since their mechanisms of action are identical, i.e., inhibiting the formation of the tubulin cytoskeleton, among which tubulysin M has the lowest IC_{50} value of 0.02 nM, making it the most potent anticancer agent among all the tubulysin derivatives [6].

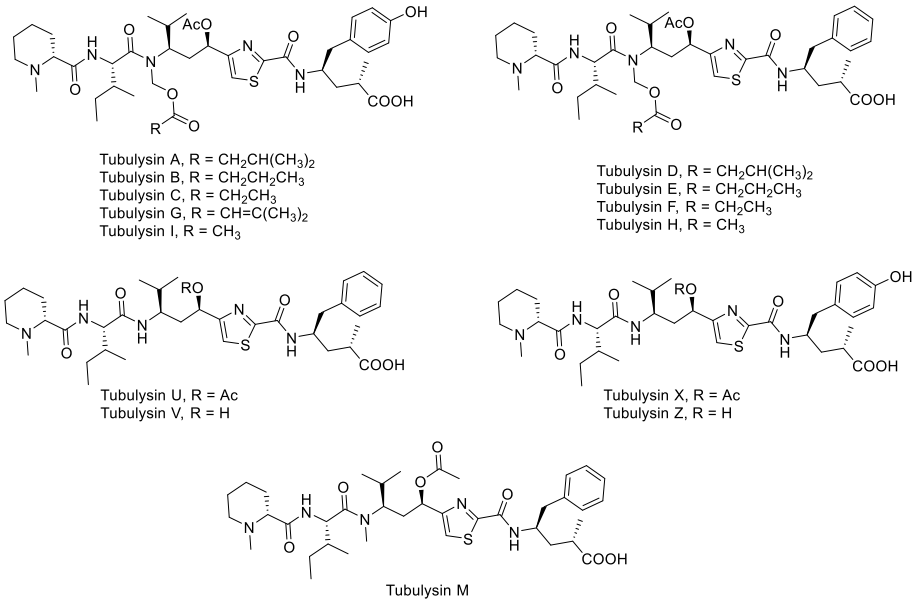


Figure 3. Structure of the naturally occurring tubulysin derivatives

1.5 Objectives of the project

The primary objective of this project is to design and develop novel third-generation tubulin inhibitors incorporating beta-amino acids and unnatural amino acids, which are resistant to protease enzymes in the body. A comprehensive Structure-Activity Relationship (SAR) study of the synthesized compounds will also be conducted to identify a lead-potent anticancer agent. The anticancer activity of the synthesized compounds will be evaluated against the MCF-7 (breast cancer), HeLa (cervical cancer), A375 (melanoma) and LNCaP (prostate cancer) patient-derived human cancer cell lines.

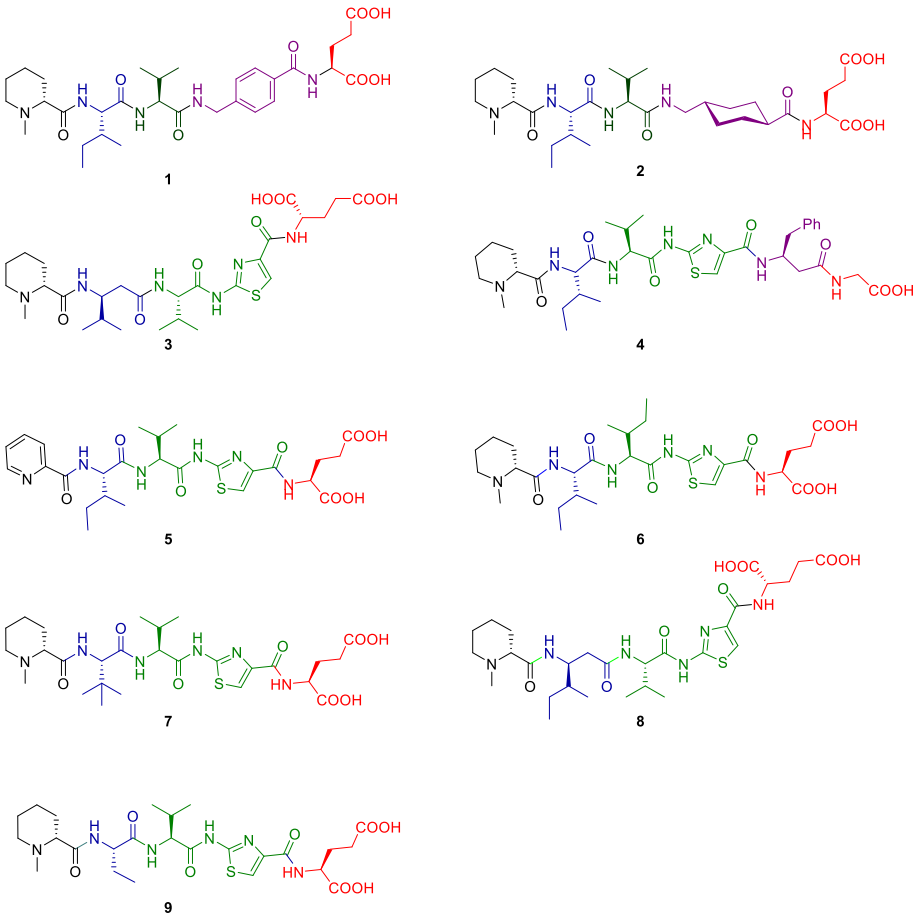


Figure 4. Structures of third-generation tubulin inhibitors incorporating beta and unnatural-alpha amino acids

Chapter 2

Literature Review

Anti-cancer peptides are short peptides, composed of 10-60 amino acids, that can inhibit tumor growth. To date, among various anticancer peptides, tubulysin is the most active tetrapeptide exhibiting remarkable cytotoxic activity [7]. Different types of tubulysin derivatives have been discovered, among which tubulysin M is the most potent microtubule-depolymerizing anticancer agent with a low nanomolar IC_{50} value (0.02 nM). The chemical structure of the tubulysin M consists of N-methyl pipecolic acid, isoleucine, tubuvaline, and tubuphenylalaine from N to C-terminus of the tetrapeptide. However, the structural complexity of the tubuvaline and tubuphenylalaine moieties poses a significant synthetic challenge. In 2016, using a lengthy multistep synthetic approach, Nicolau and coworkers reported the synthesis of a library of tubulysin derivatives with structural modification [6]. Beyond their structural difficulties, limited natural abundance, and biological instabilities further complicate the synthesis of these naturally occurring anti-cancer peptides. A major drawback of the peptide-based anticancer agents is their proteolytic degradation, as these enzymes can cleave the amide bonds formed by natural alpha amino acids [8].

Previously, our research group reported synthesizing several tubulysin fragments and tubulysin derivatives via multi-step solution and solid-phase peptide synthesis [9]. In this current work, we are working on solid-phase peptide synthesis using β- and unnatural α-amino acids to prepare hitherto unknown third-generation tubulysin derivatives. It is anticipated that these new tubulysin derivatives will improve the tetrapeptides stability, performance during cancer treatment and increase their bioavailability. The structures of newly designed tubulysin derivatives are shown in Figure 4.

Chapter 3

EXPERIMENTAL SECTION

3.1 General information and methods

The moisture-sensitive reactions were performed using oven-dried glassware in a dry solvent under an inert environment. For transferring all moisture-sensitive liquids, glass syringes were used under a nitrogen atmosphere. Under a nitrogen atmosphere, air and moisture-sensitive materials were also carefully transferred. The reactions were monitored using analytical TLC (thin layer chromatography) on Merck silica gel plates. TLC plates were analyzed using UV irradiation at 254 nm. UV inactive compounds were analyzed by staining with iodine or ninhydrin. The compounds were concentrated by evaporating the volatile solvents at 40 °C using a rotary evaporator under reduced pressure. All compounds were isolated using column chromatography. Distilled solvents were used as eluents for purifying the compounds using column chromatography ^{1}H and ^{13}C NMR spectra were recorded using the Bruker AV 500 MHz NMR spectrometer, which was supported by DST-FIST, Government of India. The NMR samples were prepared using either $CDCl_3$ or DMSO-d_6 as a solvent and chemical shifts were reported in parts per million (ppm) using TMS as an internal reference. High-resolution mass spectra were recorded using Bruker Daltonik High-Performance LC-MS (ESI-TOF) spectrometer.

3.2 Drying of solvents

For drying organic solvents, drying agents such as CaH_2 and anhydrous Na_2SO_4 were used.

3.2.1 Drying of DCM

The required amount of DCM was taken in a round-bottom flask, and a pinch of CaH_2 was added and refluxed for 1 h. The solvent was distilled off at boiling conditions and collected in a round-bottomed flask containing flame-dried 4Å molecular sieves.

3.2.2 Drying of DMF

The required amount of DMF was taken in a round-bottom flask. A pinch of CaH_2 was added to it and stirred overnight. The solvent was distilled off by vacuum distillation and collected in another round-bottom flask containing flame-dried 4Å molecular sieves.

3.3 Experimental procedures for the synthesis of fragments of tubulin inhibitors

3.3.1 Synthesis of 4-methoxybenzyl-2-Aminothiazole-4-carboxylate (11)

2-Aminothiazole-4-carboxylic acid **10** (250 mg, 1.734 mmol) and sodium carbonate (367 mg, 3.468 mmol) were suspended in dry DMF (3 mL) taken in a single-neck round-bottom flask (25 mL) fitted with a rubber septum. Using a glass syringe (1 mL), 4-methoxylbenzyl chloride (0.46 mL, 3.468 mmol) was added to the reaction mixture dropwise while being continuously stirred. At room temperature in an inert atmosphere, the reaction mixture was further stirred for 24 h. The reaction mixture was diluted with EtOAc (1 × 10 mL) and quenched with cold brine (1 × 10 mL) after the completion of the reaction (as determined by TLC). The reaction mixture was extracted using EtOAc (3 × 15 mL) and the combined organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to obtain the crude product. The crude residue was purified using a mixture of hexane-EtOAc as the eluent over silica gel (neutral 230–400 mesh) column chromatography to obtain the pure product **11** (Scheme 1) in 66% yield (300 mg); Pale yellow solid, R*f* = 0.38 (3:2 hexane-EtOAc); m.p 200–202 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.88 (s, 2H), 7.70 (s, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 6.93 (d, *J* = 8.7 Hz, 2H), 5.14 (s, 2H), 3.75 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.6, 161.3, 159.2, 148.3, 129.9, 128.2, 114.3, 113.9, 65.4, 55.1; IR 2931 (O–H), 2860 (C–H) 1706 (C=O), 1449 (C–C), 1000 (C–O), 697 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_{12}H_{12}N_2O_3S + Na^+$]: 287.0461 found 287.0503.

3.3.2 General procedure for the synthesis of 4-methoxybenzyl protected thiazole-Fmoc amino acid dipeptide (13a-b)

In a round-bottom flask (25 mL), Fmoc-protected amino acid **12a-b** (1 equiv.) was dissolved in dry DCM and EDC.HCl (2 equiv.), HOBt (2 equiv.) and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **11** (1.2 equiv.) were added sequentially into the suspension at room temperature. The reaction mixture was then stirred for 16 h under an inert atmosphere. After the completion of the reaction (as monitored by TLC), the reaction mixture was quenched with milli-Q water (5 mL) and extracted with DCM (3 × 15 mL). The combined organic layer was washed with brine (5 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was then purified through silica gel (neutral, 230–400 mesh) column chromatography using hexane-EtOAc as eluent to obtain **13a-b** (Scheme 2).

3.3.2.1 Synthesis of 4-methoxybenzyl (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylbutanamido)thiazole-4-carboxylate (13a)

In a single-neck round bottom flask (25 mL), Fmoc-protected valine **12a** (500 mg, 1.473 mmol) was dissolved in dry DCM (4 mL). Next, EDC.HCl (564 mg, 2.94 mmol), HOBt (3.98 mg, 2.94 mmol), and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **11** (467 mg, 1.767 mmol) were added sequentially into the suspension at room temperature. The reaction mixture was then further stirred for 16 h at room temperature under inert atmosphere. After the completion of the reaction (as monitored by TLC), the reaction mixture was diluted with DCM (5 mL) and quenched with water (5 mL). The aqueous layer was further extracted with DCM (3 × 15 mL). The combined organic layer was then dried over anhydrous Na_2SO_4, filtered, concentrated, and purified through silica gel (neutral 230–400 mesh) column chromatography using hexane-EtOAc solvent mixture as eluent to obtain the intermediate 4-methoxybenzyl (S)-2-(2-((((9H-fluoren-9 yl)methoxy) carbonyl)amino)-3-methylbutanamido)thiazole-4-carboxylate **13a**

(Scheme 2) in 85% yield (730 mg); White solid, R*f* = 0.32 (3:1 hexane-EtOAc); m.p 300–303 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.46 (s, 1H), 8.02 (s, 1H), 7.72 (d, *J* = 7.7 Hz, 2H), 7.55 (t, *J* = 6.9 Hz, 2H), 7.37–7.34 (m, 4H), 7.26 (t, *J* = 6.9 Hz, 2H), 6.89 (d, *J* = 7.7 Hz, 2H), 6.03–5.94 (m, 1H), 5.25 (s, 2H), 4.54–4.48 (m, 3H), 4.20 (t, *J* = 7.0 Hz, 1H), 3.80 (s, 3H), 2.18–2.10 (m, 1H), 0.97–0.94 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.3, 162.3*, 162.0, 157.0, 144.6, 143.8, 143.7, 141.2*, 127.8, 127.1*, 125.1, 123.1, 120.1, 67.7, 60.4, 53.5, 52.3, 47.2, 31.6, 19.3, 18.3 (*higher intensity signals); IR 3276 (N–H), 2954-2871 (=C–H), 2434 (C–H), 1689 (C=O), 1541 (C=O), 1515 (C=O), 1207 (C–O), 1094 (=C–H bend), 737 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_{32}H_{31}N_3O_6S + H^+$]: 586.2006 found 586.2006.

3.3.2.2 Synthesis of 4-methoxybenzyl 2-((2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylpentanamido)thiazole-4-carboxylate (13b)

In a single-neck round bottom flask (25 mL), Fmoc protected isoleucine **12b** (766 mg, 2.17 mmol) was dissolved in dry DCM (4 mL). Next, EDC.HCl (833 mg, 4.35 mmol), HOBt (588 mg, 4.35 mmol) and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **11** (690 mg, 2.610 mmol) were added sequentially to the mixture at room temperature. The reaction mixture was then further stirred for 16 h at room temperature under an inert atmosphere. After the completion of the reaction (as monitored by TLC), the reaction mixture was diluted with DCM (5 mL) and quenched with water (5 mL). The aqueous layer was further extracted with DCM (3 × 15 mL). The combined organic layer was then dried over anhydrous Na_2SO_4, filtered, concentrated, and purified through silica gel (neutral 230–400 mesh) column chromatography using hexane-EtOAc solvent mixture as eluent to obtain the intermediate 4-methoxybenzyl-2-((2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylpentanamido)thiazole-4-carboxylate **13b** (Scheme 2) in 80% yield (1031 mg); R*f* = 0.25 (7:3 hexane-EtOAc); m.p 295–297 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.60 (s, 1H), 7.83 (s, 1H), 7.74 (d, *J* = 7.7, 2H), 7.56 (t, *J* = 7.0, 2H), 7.37 (t, *J* = 7.3, 2H), 7.34 (d, *J* = 8.2,

2H), 7.28 (t, *J* = 8.4, 2H), 6.86 (d, *J* = 8.2, 2H), 5.50 (d, *J* = 8.4, 1H), 5.27 (s, 2H), 4.53–4.50 (m, 1H), 4.42–4.41 (m, 2H), 4.19 (t, *J* = 6.7, 1H), 3.79 (s, 3H), 1.46–1.42 (m, 1H), 1.26 (s, 1H), 1.17–1.11 (m, 1H), 0.94 (d, *J* = 6.8, 3H), 0.88 (t, *J* = 7.8, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 161.4, 159.9, 157.9, 156.6, 143.7, 141.5, 141.4, 130.6, 127.9, 127.7, 127.2, 125.1, 122.8, 120.1, 114.1, 67.4, 67.2, 60.0, 55.4, 47.3, 37.5, 24.8, 15.7, 11.6; IR 3276 (N–H), 2954-2871 (=C–H), 2434 (C–H), 1689 (C=O), 1541 (C=O), 1515 (C=O), 1207 (C–O), 1095 (=C–H bend), 737 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_{33}H_{33}N_3O_6S$ + H^+]: 600.2163 found 600.2102.

3.3.3 General procedure for the synthesis of Fmoc-protected amino acid-thiazole fragments (14a-b)

In a round-bottom flask (25 mL), 4-methoxybenzyl protected thiazole-Fmoc amino acid dipeptide **13a** or **13b** was dissolved in DCM. Next, a solution of 20% TFA in DCM (5 mL) was added to the reaction mixture at room temperature and stirred for 1.5 h. After the completion of the reaction (as monitored by TLC), excess TFA was evaporated under reduced pressure. The crude reaction mixture was then transferred directly for column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain **14a** or **14b** (Scheme 3).

3.3.3.1 Synthesis of (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methyl butanamido) thiazole-4-carboxylic acid (14a)

In a single-neck round bottom flask (50 mL), 4-methoxybenzyl (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)thiazole-4-carboxylate **13a** (500 mg, 0.835 mmol) was dissolved in DCM (5 mL). Next, a solution of 20% TFA in DCM (5 mL) was added to the reaction mixture at room temperature and stirred for 1.5 h. After the completion of the reaction (as monitored by TLC), excess TFA was evaporated under reduced pressure. The crude reaction mixture was then transferred directly for column

chromatography (Silica gel 230–400 mesh) purification using hexane-ethyl acetate solvent mixture as eluent to obtain Fmoc-Val-thiazole-OH fragment **14a** (Scheme 3) with a yield of 96% (380 mg); white solid, R*f* = 0.1 (3:2 hexane-EtOAc); m.p 175–177 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.63 (s, 1H), 8.03 (s, 1H), 7.88–7.64 (m, 5H), 7.32 (dt, *J* = 45.6, 7.7 Hz, 4H), 4.34–4.07 (m, 5H), 2.03 (q, *J* = 8.4, 7.6 Hz, 1H), 0.87 (dd, *J* = 17.9, 6.7 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.8, 163.0, 161.6, 156.5, 144.7, 143.9, 140.8, 127.8, 127.2, 125.5, 123.1, 120.2, 65.9, 60.3, 46.8, 30.1, 19.1, 18.6; IR 3299 (N–H), 2956–2872 (=C–H), 2465 (C–H), 1682 (C=O), 1536 (C=O), 1510 (C=O), 1243 (C–O), 1185 (=C–H bend), 737 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_{24}H_{23}N_3O_5S$ + Na^+]: 488.1251 found 488.1426.

3.3.3.2 Synthesis of 4-methoxybenzyl 2-((2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylpentanamido)thiazole-4-carboxylic acid (14b)

In a single-neck round bottom flask (50 mL), 4-methoxybenzyl-2-((2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylpentanamido)thiazole-4-carboxylate **13b** (567 mg, 0.945 mmol) was dissolved in DCM (5 mL). Next, a solution of 20% TFA in DCM (5 mL) was added to the reaction mixture at room temperature and stirred for 1.5 h. After the completion of the reaction (as monitored by TLC), excess TFA was evaporated under reduced pressure. The crude reaction mixture was then purified through silicagel column chromatography (230–400 mesh) using hexane-ethyl acetate solvent mixture as eluent to obtain Fmoc-isoleucine-thiazole fragment **14b** (Scheme 3) in 88% yield (400 mg); R*f* = 0.08 (3:2 hexane-EtOAc); m.p 191–193 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.60 (s, 1H), 7.99 (s, 1H), 7.87 (d, *J* = 7.6 Hz, 2H), 7.79–7.71 (m, 3H), 7.41–7.37 (m, 2H), 7.31 (t, *J* = 7.7 Hz, 2H), 4.33–4.15 (m, 4H), 1.87–1.85 (m, 1H), 1.97–1.91 (m, 1H), 1.52–1.47 (m, 1H), 0.87–83 (m, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.1, 170.4, 162.4, 143.8, 140.74, 140.71, 127.72, 127.65, 127.1, 125.4, 120.2, 120.1, 59.8, 46.7, 38.3, 20.8, 15.3, 14.1, 10.6; IR 2956 (=C–H), 1686 (C=O),

1527 (C=O), 1452 (C=O), 1238 (C–O), 1094 (=C–H bend), 603 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_{25}H_{25}N_3O_5S + Na^+$]: 502.1407 found 502.1414.

3.3.4 Synthesis of N-methyl pipecolic acid (16)

In a double-neck round bottom flask (25 mL), a hydrogen-filled bladder was fitted to one neck of the round bottom flask through a borosilicate glass heavy-wall glass stop cock adapter. Pipecolic acid **15** (300 mg, 2.32 mmol) and formaldehyde (370 µL, 4.65 mmol%) were added to the round bottom flask and dissolved in methanol (2 mL). 10% Pd/C (24.7 mg, 10 mol%) was added slowly to the mixture and the reaction mixture was sealed using a rubber septum. The residual air in the reaction vessel was expunged through a syringe needle inserted through the rubber septum that is connected to a filtration pump through a silicone tube for 2 minutes or until the solution starts bubbling. The reaction mixture is now filled with hydrogen gas from the bladder via a stop cock and allowed to stir at room temperature for 24 h. After the completion of the reaction as monitored by TLC using MeOH and DCM (1:1) as eluent, the reaction mixture was diluted with MeOH (15 mL) and filtered through a celite powder filled sintered Buchner funnel with inner joint fitted to a round bottom flask (50 mL) to remove the palladium/charcoal with the help of a suction pump. The celite pad was washed with MeOH (3 × 3 mL) and the filtrate was concentrated under reduced pressure to obtain desired N-methyl pipecolic acid **16** (Scheme 4); Yield 98% (325 mg); White solid; R_f = 0.21 (MeOH/DCM) 1:1; m.p 101–103 °C; ^{1}H NMR (500 MHz, MeOH-d_4) δ 3.47–3.37 (m, 2H), 3.01 (t, *J* = 8.3 Hz, 1H), 2.88 (s, 3H), 2.23 (d, *J* = 13.4 Hz, 1H), 1.89–1.83 (m, 2H), 1.79–1.74 (m, 2H), 1.59–1.52 (m, 1H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 173.6, 70.4, 55.3, 43.2, 29.5, 24.1, 22.7; IR 2929 (O–H), 2861 (C–H) 1696 (C=O), 1315 (C–C), 1173 (C–O), 995 (C–N) cm^{-1}; HRMS (+ESI) calculated for [$C_7H_{13}NO_2 + H^+$]: 144.1019 found 144.1096.

3.4 General procedure for solid phase peptide synthesis

3.4.1 Resin swelling

The resin beads used in solid phase synthesis were first swelled with DCM (5 mL) for 30 minutes by bubbling nitrogen through the beads in peptide vessel. After draining DCM, the beads were again swelled with DMF (5 mL), repeating the process for 15 minutes (three times).

3.4.2 General procedure for the Kaiser test

A few resin beads from the peptide vessel were taken out from the peptide vessel and placed in a test tube (10 mL). Then, 2 drops of each ninhydrin, phenol, and 0.1% potassium cyanide were added to the resin beads and heated at 110 °C in a sand bath for 2 minutes. The appearance of dark blue colour indicates the presence of free amine groups in the resin beads, while the absence of the dark blue colour designates the absence of the amine group. The test was performed after coupling each amino acid as well as after the deprotection of Fmoc group at each step.

3.4.3 General procedure for Fmoc deprotection

The Fmoc group of the N-terminus of the growing peptide chain was deprotected using 20% piperidine in DMF (10 mL) by bubbling nitrogen gas through the resin beads for 10 minutes in each step of Fmoc deprotection. The procedure was repeated three times (1 × 4 mL; 2 × 3 mL) to ensure the complete deprotection of the Fmoc group.

3.4.4 General procedure for peptide cleavage from resin beads

The peptide was cleaved from the resin beads using 10 mL of cleavage cocktail (a mixture of 9.5 mL trifluoroacetic acid, 0.25 mL triisopropylsilane, and 0.25 mL water) by bubbling nitrogen gas through the resin. First, 5 mL of the cocktail was added to the resin, and nitrogen gas was bubbled for 30 minutes, followed by the addition of 2.5 mL of the cocktail twice, with nitrogen bubbling for 5 minutes each time. The collected mother liquor after peptide cleavage was evaporated under reduced pressure and the concentrated solution was precipitated in ice-

cold diethyl ether. The precipitate was then dried using nitrogen gas and the obtained crude product was further purified using HPLC.

3.5. Solid-phase peptide synthesis of novel third-generation tubulin inhibitors (1-9)

3.5.1 Solid phase peptide synthesis of third-generation tubulin inhibitor (1) incorporating 4-aminomethyl benzoic acid

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.096 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump, and the beads were dried until freely flowing. Fmoc-aminomethyl benzoic acid (0.071 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain dipeptide **18**. After that Fmoc-protected amino acid, Fmoc-Val-OH (0.065 g, 0.019 mmol), PyBOP (0.099g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were added to the peptide vessel. The same steps were repeated to obtain tripeptide **19** as described above for amide coupling and deprotection of the Fmoc group. The next amino acid, Fmoc-Ile-OH (0.067 g, 0.019

mmol), PyBOP (0.099g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **20**. Finally, a mixture of N-Methyl pipecolic acid **16** (0.027 g, 0.019 mmo), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **20** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round-bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **1** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL), and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 40% (23.7 mg) (Scheme 5). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.41 (s, 1H), 9.65 (s, 1H), 8.76 (d, *J* = 8.4 Hz, 1H), 8.59 (t, *J* = 6.1 Hz, 1H), 8.56 (d, *J* = 7.7 Hz, 1H), 8.08 (d, *J* = 8.9 Hz, 1H), 7.80 (d, *J* = 8.1 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 4.40–4.33 (m, 2H), 4.30–4.27 (m, 2H), 4.15 (t, *J* = 8.3 Hz, 1H), 3.81–3.77 (m, 1H), 3.32 (d, *J* = 12.3 Hz, 1H), 3.05–2.98 (m, 1H), 2.63 (s, 3H), 2.33 (t, *J* = 7.6, 2H), 2.11–2.00 (m, 2H), 1.97–1.89 (m, 2H), 1.78–1.73 (m, 3H), 1.67–1.53 (m, 2H), 1. 43–1.35 (m, 2H), 1.13–1.04 (m, 1H), 0.84–0.77 (m, 12H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.1, 173.6, 171.1, 170.6, 167.9, 166.5, 143.2, 132.5, 127.6, 127.1, 66.2, 58.3, 57.4, 54.6, 52.1, 41.9, 41.5, 36.6, 30.64, 30.59, 28.6, 26.1, 24.4, 22.5,

21.1, 19.4, 18.5, 15.5, 11.0; HRMS (+ESI) calculated for [$C_{31}H_{47}N_5O_8$ + H^+]: 618.3497 found 618.2358.

3.5.2 Solid phase peptide synthesis of third-generation tubulin inhibitor (2) incorporating trans-4-aminomethyl cyclohexanecarboxylic acid

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.096 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Trans-4-(Fmoc-aminomethyl)cyclohexanecarboxylic acid (0.072 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using a filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using a suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain dipeptide **22**. After that Fmoc-protected amino acid, Fmoc-Val-OH (0.065 g, 0.019 mmol), PyBOP (0.099 g, 0.192 mmol), DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were added to the peptide vessel. The same steps were repeated to obtain tripeptide **23** as described above for amide coupling and deprotection of the Fmoc group. The next amino acid, Fmoc-Ile-OH (0.067 g, 0.019 mmol), PyBOP (0.099 g, 0.192 mmol), DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5

mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **24**. Finally, a mixture of N-methyl pipecolic acid **17** (0.027 g, 0.019 mmol), PyBOP (0.099 g, 0.192 mmol), DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **24** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round-bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **2** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 50% (30 mg) (Scheme 6). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.67 (s, 1H), 8.78 (d, *J* = 8.2, 1H), 8.01 (d, *J* = 8.9, 1H), 7.96–7.93 (m, 2H), 4.27 (t, *J* = 7.7, 1H), 4.19–4.14 (m, 1H), 4.10 (t, *J* = 8.3, 1H), 3.83–3.79 (m, 1H), 3.35 (d, *J* = 12.1, 1H), 3.07–3.00 (m, 1H), 2.94–2.85 (m, 2H), 2.65 (s, 3H), 2.28–2.23 (m, 2H), 2.10 (t, *J* = 12.1, 1H), 2.04 (d, *J* = 14.1, 1H), 1.98–1.88 (m, 2H), 1.78–1.68 (m, 9H), 1.65–1.54 (m, 2H), 1.43–1.35 (m, 2H), 1.34–1.20 (m, 3H), 1.14–1.05 (m, 1H), 0.83–0.78 (m, 12H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.4, 173.8, 173.5, 170.6, 170.2, 167.7, 66.0, 58.1, 57.3, 54.4, 50.9, 44.6, 43.7, 41.4, 37.0, 36.4, 30.1, 29.6, 28.8, 28.6, 28.5, 26.3, 24.2, 22.3, 21.0, 19.2, 18.3, 15.3, 10.9; HRMS (+ESI) calculated for [$C_{31}H_{53}N_5O_8$ + H^+]: 624.3967 found 624.2761.

3.5.3 Solid-phase peptide synthesis of third-generation tubulin inhibitor (3) incorporating β-homo-valine

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.128 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Val-thiazole-OH **14a** (0.119 g, 0.256 mmol), PyBOP (0.133 g, 0.256 mmol), and DIPEA (0.23 mL, 1.28 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain tripeptide **26**. The next amino acid, Fmoc-β-Ho-Val-OH (0.0905 g, 0.256 mmol), PyBOP (0.133 g, 0.256 mmol), and DIPEA (0.23 mL, 1.28 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **27**. Finally, a mixture of N-methyl pipecolic acid **16** (0.0367 g, 0.256 mmol), PyBOP (0.133 g, 0.256 mmol), and DIPEA (0.23 mL, 1.28mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **27** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25)

(1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **3** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 38% (22 mg) (Scheme 7). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.48 (s, 1H), 9.60 (s, 1H), 8.65 (d, *J* = 7.7 Hz, 1H), 8.41 (d, *J* = 9 Hz, 1H), 8.28 (d, *J* = 7.8 Hz, 1H), 8.19 (s, 1H), 4.41–4.34 (m, 2H), 4.06–4.00 (m, 1H), 3.66–3.61 (m, 1H), 3.33 (d, *J* = 12.2 Hz, 2H), 3.03–2.96 (m, 1H), 2.66 (d, *J* = 4.2 Hz, 3H), 2.49–2.46 (m, 1H), 2.37–2.29 (m, 3H), 2.12–1.99 (m, 2H), 1.95–1.88 (m, 2H), 1.78–1.71 (m, 2H), 1.65–1.58 (m, 2H), 1.48–1.43 (m, 1H), 1.38–1.32 (m, 1H), 0.92–0.85 (m, 12H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.8*, 173.2*, 171.0, 170.5, 167.0, 139.9, 127.3, 66.2, 58.0, 54.2, 51.7, 51.6, 41.4, 37.3, 31.5, 30.3, 30.2, 28.2, 26.0, 22.3, 21.0, 19.2, 19.0, 18.5, 17.9 (*higher intensity signals); HRMS (+ESI) calculated for [$C_{27}H_{42}N_6O_8S$ + H^+]: 611.2858. found: 611.2880.

3.5.4 Solid phase peptide synthesis of third-generation tubulin inhibitor (4) incorporating β-homo-phenylalanine

H-Gly-2-Cl-Trt resin **29** (0.150 g, 0.166 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-β-Ho-Phe-OH (0.133g, 0.332 mmol), PyBOP (0.172 g, 0.332 mmol), and DIPEA (0.29 mL, 1.6 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and

the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain dipeptide **30**. After that Fmoc-protected amino acid, Fmoc-Val-thiazole-OH **14a** (0.154 g, 0.332 mmol), PyBOP (0.172g, 0.332 mmol), and DIPEA (0.29 mL, 1.6 mmol) in 0.5 mL were added to the peptide vessel. The same steps were repeated to obtain tetrapeptide **31** as described above for amide coupling and deprotection of the Fmoc group. The next amino acid, Fmoc-Ile-OH (0.108 g, 0.332 mmol), PyBOP (0.172 g, 0.332 mmol), and DIPEA (0.29 mL, 1.6 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before, to afford pentapeptide **32**. Finally, a mixture of N-methyl pipecolic acid **16** (0.047 g, 0.332 mmol), PyBOP (0.172 g, 0.332 mmol), and DIPEA (0.29 mL, 1.6 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the pentapeptide **32** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle

through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **4** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in lyophilizer. The product obtained is a white solid with a yield of 37% (32 mg) (Scheme 8). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.46 (brs, 1H), 9.58 (brs, 1H), 8.69 (d, *J* = 8.1 Hz, 1H), 8.35–8.33 (m, 2H), 8.27 (*t*, J = 5.8 Hz, 1H), 7.96 (s, 1H), 7.24–7.13 (m, 5H), 4.42–4.33 (m, 3H), 4.24 (t, *J* = 7.8 Hz, 1H), 3.34–3.31 (m, 2H), 3.00–2.95 (m, 1H), 2.89–2.85 (m, 1H), 2.79–2.74 (m,1H), 2.62 (m, 3H), 2.45–2.36 (m, 3H), 2.07–1.98 (m, 2H), 1.77–1.73 (m, 3H), 1.65–1.54 (m. 2H), 1.44–1.32 (m, 2H), 1.10–1.02 (m, 1H), 0.88 (d, J = 6.6 Hz, 3H), 0.85 (d, J = 6.6 Hz, 3H), 0.81 (d, J = 6.8 Hz, 3H), 0.78 (t, J = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.2, 171.0, 170.5*, 167.6, 160.0, 159.7, 139.0, 138.6, 129.0, 128.0, 127.6, 126.0, 65.9, 58.0, 57.1, 54.3, 48.5, 41.3, 40.5, 39.9, 36.1, 29.9, 28.2, 24.0, 22.1, 20.7, 18.7, 18.1, 15.0, 10.6 (*higher intensity signal); HRMS (+ESI) calculated for [$C_{34}H_{49}N_7O_7S + H^+$]: 700.3487 found 700.3232.

3.5.5 Solid-phase peptide synthesis procedure for novel third-generation tubulin inhibitor (5) incorporating 2-nicotinic acid

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.096 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Val-thiazole-OH **14a** (0.068 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.17 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was

drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using a suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain tripeptide **26**. The next amino acid, Fmoc-Ile-OH (14c) (0.067 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.17 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **35**. Finally, a mixture of pyridine-2-carboxylic acid (0.024 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.17 mL, 0.96 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **35** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **5** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in lyophilizer. The product obtained is a white solid with a yield of 45% (26 mg) (Scheme 9). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.54 (s, 1H), 8.67 (d, J = 4.8 Hz, 1H),

8.57 (d, *J* = 9.5 Hz, 1H), 8.49 (d, *J* = 7.5 Hz, 1H), 8.06–8.04 (m, 1H), 8.02 (dd, *J* = 7.5 Hz, *J* = 1.8 Hz, 1H), 7.99 (d, *J* = 8.1 Hz, 1H), 7.83 (s, 1H), 7.64–7.62 (m, 1H), 4.60–4.56 (m, 1H), 4.49–4.44 (m, 1H), 4.40 (t, *J* = 7.3 Hz, 1H), 2.34–2.23 (m, 2H), 2.17–2.04 (m, 2H), 1.99–1.93 (m, 1H), 1.90–1.84 (m, 1H), 1.48–1.43 (m, 1H), 1.11–1.02 (m, 1H), 0.92 (d, *J* = 6.7 Hz, 3H), 0.90–0.88 (m, 6H), 0.82 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.8, 173.1, 171.3, 170.9, 163.2, 160.6, 157.5, 149.3, 148.7, 144.0, 138.2, 126.9, 122.0, 118.2, 58.3, 56.5, 51.4, 37.7, 30.11, 30.07, 26.6, 24.4, 19.0, 18.5, 15.5, 11.0; HRMS (+ESI) calculated for [$C_{26}H_{34}N_6O_8S + H^+$]: 591.2232. found 591.2288

3.5.6 Solid-phase peptide synthesis procedure for novel third-generation tubulin inhibitor (6) incorporating Isoleucine-thiazole fragments

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.096 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Isoleucine-thiazole-OH **14b** (0.092 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using a suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to

confirm the deprotection of the Fmoc group to obtain tripeptide **37**. The next amino acid, Fmoc-Ile-OH (0.067 g, 0.019 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **38**. Finally, a mixture of N-methyl pipecolic acid **16** (0.027 g, 0.019 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) in DMF (0.5 mL) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **38** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **6** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 38% (22.7 mg) (Scheme 10). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.48 (s, 1H), 9.63 (s, 1H), 8.73 (d, *J* = 8.2 Hz, 1H), 8.62 (d, *J* = 7.8 Hz, 1H), 8.36 (d, *J* = 7.8 Hz, 1H), 8.13 (s, 1H), 4.41 (t, *J* = 8.2 Hz, 1H), 4.33–4.29 (m, 1H), 4.24 (t, *J* = 7.8 Hz, 1H), 3.31 (d, *J* = 12.3 Hz, 1H), 3.00–2.99 (m, 1H), 2.62 (s, 3H), 2.47–2.45 (m, 1H), 2.30 (t, *J* = 7.6 Hz, 1H), 2.07–2.00 (m, 2H), 1.90–1.79 (m, 2H), 1.75–1.69 (m, 3H), 1.64–1.51 (m, 2H), 1.37–1.32 (m, 2H), 1.19–1.11 (m, 1H), 1.08–1.02 (m, 1H), 0.81–0.74 (m, 12H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.8, 173.3, 171.0, 170.9, 167.8, 161.0, 160.2, 140.2, 127.2, 66.0, 57.1, 57.0, 54.4, 51.8, 41.4, 36.4, 36.1, 30.4, 28.5, 26.0, 24.5, 24.3, 22.4,

21.0, 15.3, 15.2, 10.8, 10.7; HRMS (+ESI) calculated for [$C_{27}H_{42}N_6O_8S$ + H^+]: 625.3014 found: 625.2856.

3.5.7 Solid-phase peptide synthesis procedure for novel third generation tubulin inhibitor (7) incorporating unnatural amino acid, *tert*-Leucine

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.100 g, 0.064 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Val-thiazole-OH **14a** (0.060 g, 0.128 mmol), PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using a suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain tripeptide **26**. The next amino acid, Fmoc-*tert*-leu-OH **(14b)** (0.045 g, 0.128 mmol), PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **40**. Finally, a mixture of N-Methyl pipecolic acid **16** (0.018 g, 0.128 mmol),

PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **40** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **7** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 38% (15 mg) (Scheme 11); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.52 (s, 1H), 8.63 (d, *J* = 8.8 Hz, 1H), 8.29 (d, *J* = 7.4 Hz, 1H), 7.97 (d, *J* = 8.1 Hz, 1H), 8.84 (s, 1H), 4.49–4.39 (m, 3H), 3.88 (d, *J* = 10.4 Hz, 1H), 3.01 (t, *J* =9.9 Hz, 1H), 2.65 (s, 3H), 2.34–2.23 (m, 2H), 2.16–2.04 (m,3H), 1.99–1.91 (m,1H), 1.77 (d, *J* = 11.2), 1.71–1.55 (m, 2H), 1.44–1.36 (m, 1H), 0.93–0.89 (m, 15H); HRMS (+ESI) calculated for [$C_{27}H_{42}N_6O_8S$ + H^+]: 611.2858. found: 611.2882.

3.5.8 Solid-phase peptide synthesis procedure for novel third-generation tubulin inhibitor (8) incorporating β-homo-isoleucine

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.150 g, 0.096 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Val-thiazole-OH **14a** (0.089 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.17 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h

by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using a suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain tripeptide **26**. The next amino acid, Fmoc-β-Ho-Ile-OH (0.071 g, 0.192 mmol), PyBOP (0.099 g, 0.192 mmol), and DIPEA (0.16 mL, 0.96 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20% piperidine in DMF as mentioned before to afford tetrapeptide **42**. Finally, a mixture of N-Methyl pipecolic acid **17** (0.028 g, 0.192 mmol), PyBOP (0.099 g, 0.192mmol), and DIPEA (0.16 mL, 0.96 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **42** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **8** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and

the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 58% (35 mg) (Scheme 12). HRMS (+ESI) calculated for [$C_{28}H_{44}N_6O_8S + H^+$]: 625.3014 found 625.3109.

3.5.9 Solid-phase peptide synthesis procedure for novel third generation tubulin inhibitor (9) incorporating unnatural amino acid, aminobutyric acid

H-Glu(O^{t}Bu)-2-Cl-Trt resin **17** (0.100 g, 0.064 mmol) was initially swelled in DCM (1 × 5 mL) followed by DMF (1 × 5 mL) for 15 min each. The solvent was drained using a filtration pump and the beads were dried until freely flowing. Fmoc-Val-thiazole-OH **14a** (0.060 g, 0.128 mmol), PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was carried out for 6 h by bubbling a stream of nitrogen gas through the resin beads in the peptide vessel. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL) for 10 min each, the solvent was drained using a filtration pump and beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The amide coupling was confirmed by performing the Kaiser test. Next, the FmocNH protecting group was cleaved by addition of 20% piperidine in DMF (1 × 4 mL; 2 × 3 mL) for 10 min each to the resin beads. The resin beads were then washed with DMF (3 × 5 mL) followed by isopropanol (3 × 5 mL), the solvent was drained using suction pump and the beads were dried until freely flowing by bubbling N_2 gas through the resin beads in the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group to obtain tripeptide **26**. The next amino acid, Fmoc-Abu-OH (0.041 g, 0.128 mmol), PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) were taken in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, added to the peptide vessel containing resin beads and the coupling reaction was continued for 6h. The FmocNH protecting group was cleaved by addition of 20%

piperidine in DMF as mentioned before to afford tetrapeptide **44**. Finally, a mixture of N-Methyl pipecolic acid **17** (0.018 g, 0.128 mmol), PyBOP (0.067 g, 0.128 mmol), and DIPEA (0.11 mL, 0.64 mmol) in DMF (0.5 mL) taken in a 2 mL glass vial was added to the tetrapeptide **45** to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H_2O (9.50:0.25:0.25) (1 × 5 mL; 2 × 2.5 mL) as described earlier. The mother liquor was collected in a 25 mL round bottom flask from the peptide vessel, then transferred to a 15 mL centrifuge tube and fitted with a septum. The crude peptide solution was concentrated under reduced pressure to evaporate TFA by inserting a 19 Gauge stainless needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **9** as a white solid, washed with ice cold diethyl ether (3 × 5 mL) and dried by passing a stream of nitrogen gas for 15 minutes and purified by reverse-phase high performance liquid chromatography (RP-HPLC). After purification through HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained is a white solid with a yield of 43% (16 mg) (Scheme 13). HRMS (+ESI) calculated for [$C_{25}H_{38}N_6O_8S$ + H^+]: 583.2545. found: 583.2297.

3.6 *In vitro* cytotoxic study

3.6.1 Culture of cancer cell lines

MCF7 and HeLa cancer cell lines were grown in T-25 Flask containing sterile filtered RPMI 1640 medium, supplemented with fetal bovine serum, sodium pyruvate (100 mM), non-essential amino acid, and 1% penicillin streptomycin under 37 °C and 5% CO_2 to form a monolayer until 60% confluency. The cells were then trypsinized using 0.25% trypsin-EDA, collected and centrifuged at 800 rpm for 5 min. The obtained cell pellet was resuspended in fresh medium and then used for cytotoxicity assay.

3.6.2 *In vitro* cytotoxicity assay of tubulin inhibitors 1-4

5000 MCF7 and HeLa cells/well were seeded in a 96-well plate and allowed to form a monolayer of 60% confluency over a period of 48 h. The spent medium was then discarded, and cells were washed with 1X PBS (1 × 200 µL). 200 µL of fresh medium containing tubulin inhibitors **1-4** with various concentrations (100 µM−10 pM) was then added into the well in triplicate. The cells were then incubated for 48h. After incubation, the spent medium was discarded, and the cells were washed carefully with 1X PBS (2 × 200 µL). 50 µL of MTT solution (5 mg/mL) was added in each well, plates were then carefully wrapped with aluminum foil and incubated at 37 °C and 5% CO_2 for 4 h. After incubation, MTT solution was carefully aspirated using pipette and 200 µL of DMSO per well was added to dissolve the formazan crystals. The absorbance from each well corresponding to live cells was measured using Synergy H4 multiplate reader at a wavelength of 570 nm. Dose vs response curves were obtained from a plot of log[concentration] vs % cell viability and IC_{50} values were calculated using GraphPad Prism 8.01.

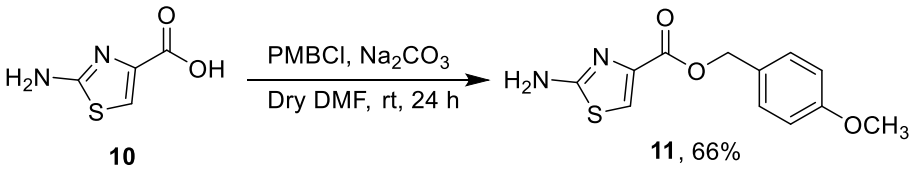
Chapter 4

Results and Discussion

4.1 Reaction schemes

4.1.1 Synthesis of 4-methoxybenzyl 2-aminothiazole-4-carboxylate (11)

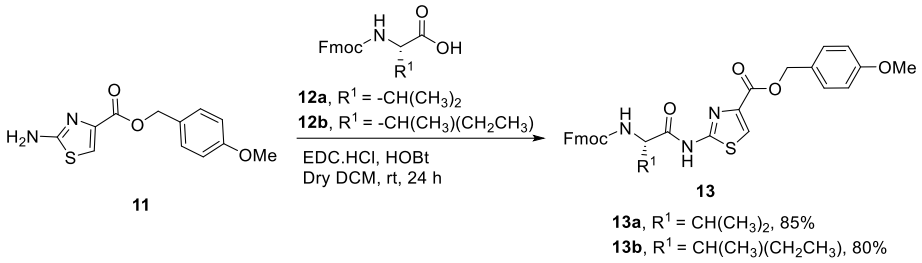
The carboxylic acid group of 2-aminothiazole-4-carboxylic acid **10** is protected with the acid-labile para-methoxybenzyl (PMB) group. This protection was achieved by reacting 2-aminothiazole-4-carboxylic acid with 4-methoxybenzyl chloride (PMBCl) at room temperature in the presence of the inorganic base Na_2CO_3, using the polar aprotic solvent dry DMF under inert atmosphere. The reaction was carried out for 24 hours to afford 4-methoxybenzyl-2-Aminothiazole-4-carboxylate **11**.



Scheme 1. Synthesis of 4-methoxybenzyl-2-aminothiazole-4-carboxylate **11**

4.1.2 Synthesis of 4-methoxybenzylcarboxyl protected thiazole-Fmoc amino acid dipeptide (13a-b)

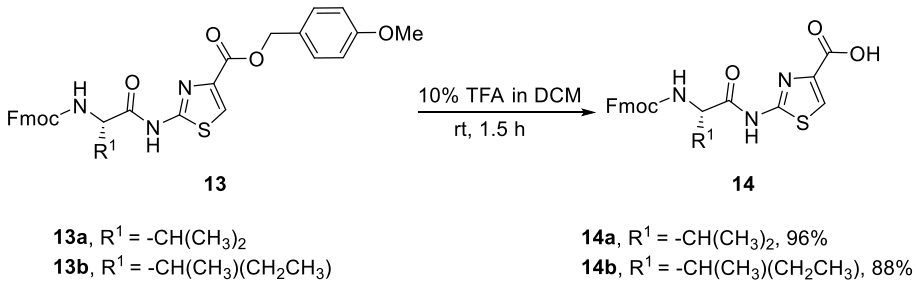
The free amine group present in the 4-methoxybenzyl-2-aminothiazole-4-carboxylate **11** was coupled with the carboxylic acid group of Fmoc-protected amino acid **12a-b** via the formation of an amide bond to obtain the product **13a-b.** The reaction was carried out using the coupling agent EDC.HCl, along with HOBt as an additive to prevent racemization in dry DCM for 24 h, to yield the desired product.



Scheme 2. Synthesis of 4-methoxybenzyl protected thiazole-Fmoc amino acid dipeptide **13a-b**

4.1.3 Synthesis of Fmoc-amino acid-thiazole fragment (14a-b)

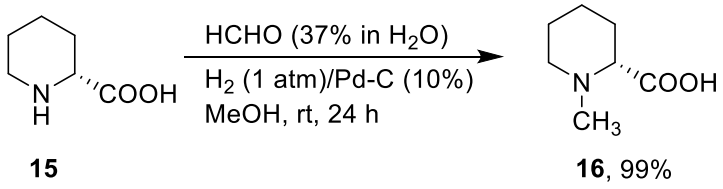
The acid-labile p-methoxybenzyl (PMB) group of the intermediate **13a-b** was deprotected by 10% TFA in DCM at room temperature. The reaction was carried out for 1.5 h to afford **14a-b**.



Scheme 3. Synthesis of Fmoc-amino acid-thiazole fragment **14a-b**

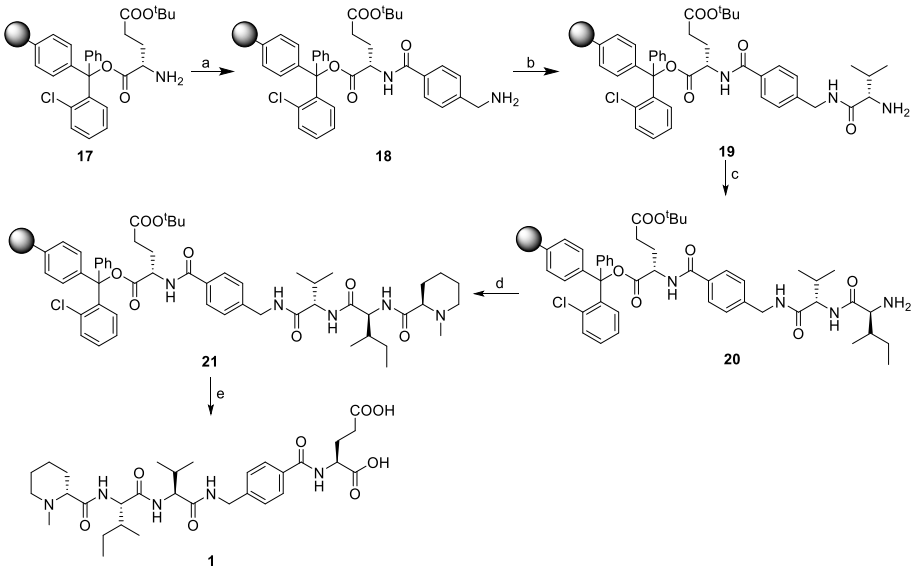
4.1.4 Synthesis of N-methyl pipecolic acid (16)

Pipecolic acid **15** is reacted with formaldehyde (37% in water) at room temperature in the presence of H_2/Pd-C (10%) and using the polar solvent methanol. The reaction was carried out for 24 h to obtain our desired product N-methyl pipecolic acid **16**.



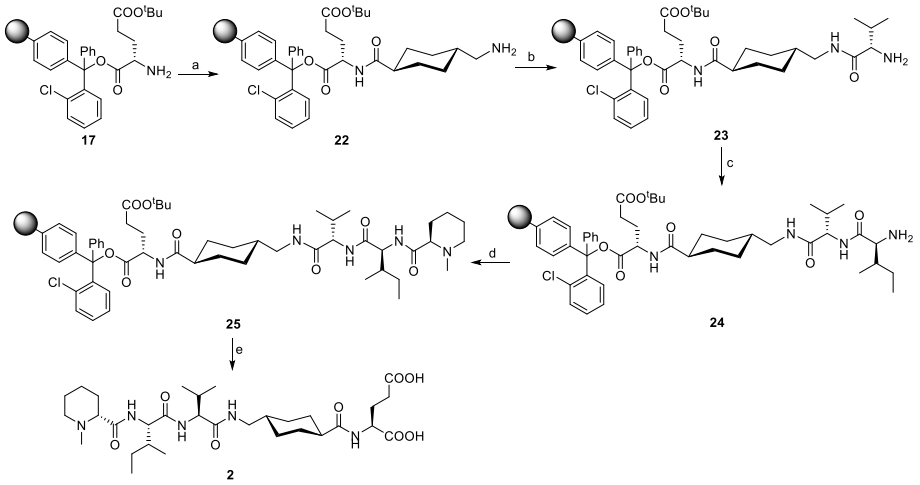
Scheme 4. Synthesis of N-methyl pipecolic acid **16**

4.1.5 Solid phase peptide synthesis of third-generation tubulin inhibitor (1) incorporating 4-aminomethyl benzoic acid



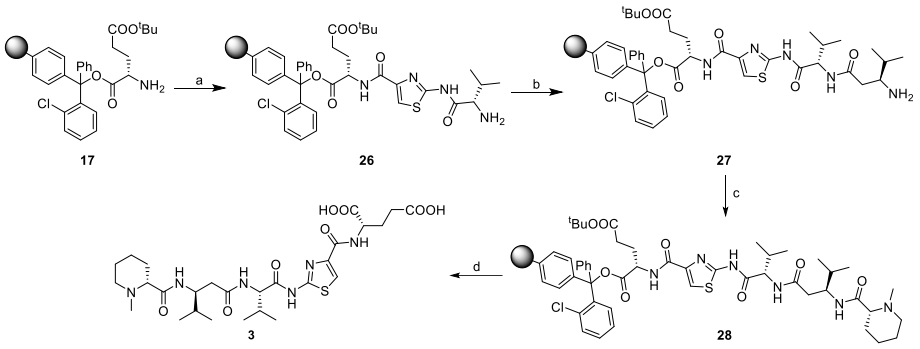
Scheme 5. Synthesis of third-generation tubulin inhibitor **1** incorporating 4-aminomethylbenzoic acid. Reagents and conditions: (a) i) Fmoc-aminomethyl benzoic acid, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; (b) i) Fmoc-Val-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; (c) i) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; d) (N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (e) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.1.6 Solid phase peptide synthesis of third generation tubulin inhibitor (2) incorporating trans-4-aminomethyl cyclohexane carboxylic acid



Scheme 6. Synthesis of third-generation tubulin inhibitor **2** incorporating trans-4-aminomethyl cyclohexane carboxylic acid. Reagents and conditions: (a) i) Trans-4-(Fmoc-aminomethyl)cyclohexane carboxylic acid, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; b) i) Fmoc-Val-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; (c) i) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; d) (N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (e) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

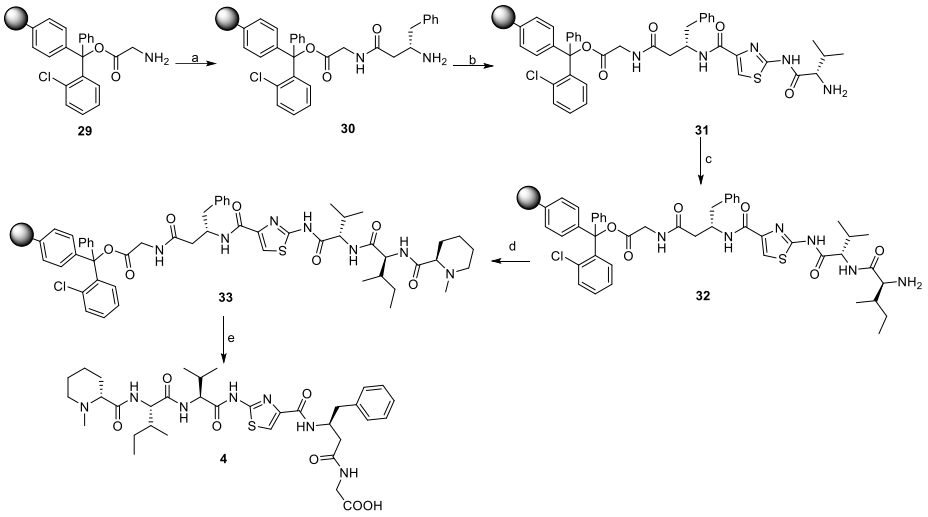
4.1.7 Solid-phase peptide synthesis of third-generation tubulin inhibitor (3) incorporating β-homo-valine



Scheme 7. Synthesis of third-generation tubulin inhibitor **3** incorporating unnatural amino acid, β-homo-valine. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **14a**, PyBOP, DIPEA, DMF, 6

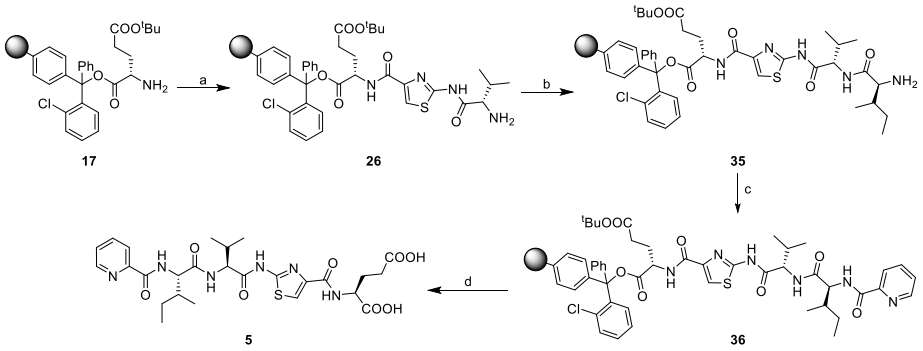
h; ii) 20% Piperidine in DMF, rt, 30 min; (b) i) Fmoc-β-homo-val-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (c) N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (d) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.1.8 Solid phase peptide synthesis of third-generation tubulin inhibitor (4) incorporating β-homo-phenylalanine



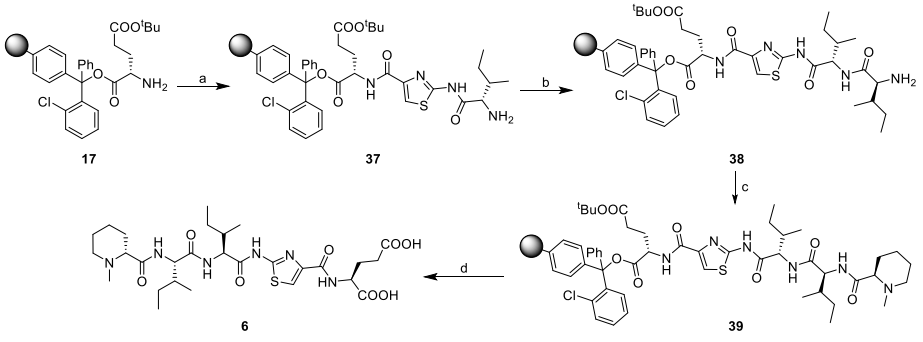
Scheme 8. Synthesis of third-generation tubulin inhibitor **4** incorporating β-homo-phenylalanine. Reagents and conditions: (a) i) Fmoc-β-Phe-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (b) i) Fmoc-Val-thiazole-OH **14a**, PyBOP, DIPEA, DMF, 6 h; (ii) 20% Piperidine in DMF, rt, 30 min; (c) (i) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (ii) 20% Piperidine in DMF, rt, 30 min; (d) N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (e) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.1.9 Solid-phase peptide synthesis of third-generation tubulin inhibitor (5) incorporating 2-nicotinic acid



Scheme 9. Synthesis of third-generation tubulin inhibitor **5** incorporating 2-nicotinic acid. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **14a**, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (b) i) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (c) 2-Nicotinic acid, PyBOP, DIPEA, DMF, 6 h; (d) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

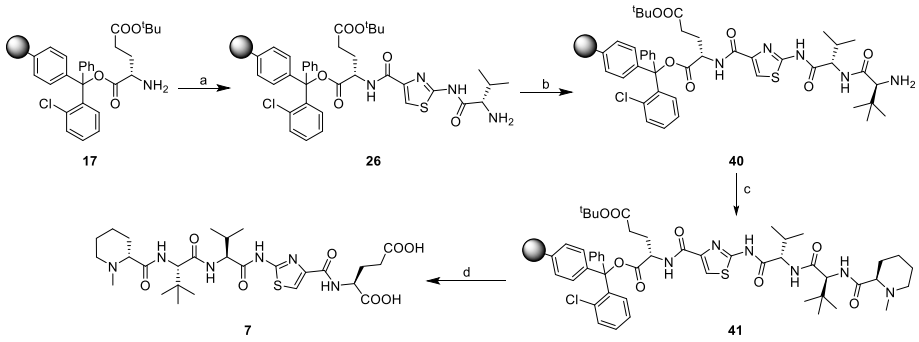
4.1.10 Solid phase peptide synthesis of third-generation tubulin inhibitor (6) incorporating Isoleucine-thiazole fragment



Scheme 10. Synthesis of third-generation tubulin inhibitor **6** incorporating isoleucine-thiazole fragment. Reagents and conditions: (a) i) Fmoc-Ile-thiazole-OH **14b**, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; (b) i) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine, DMF, rt, 30 min; (c) N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h: (d) i) TFA/TIPS/H_2O

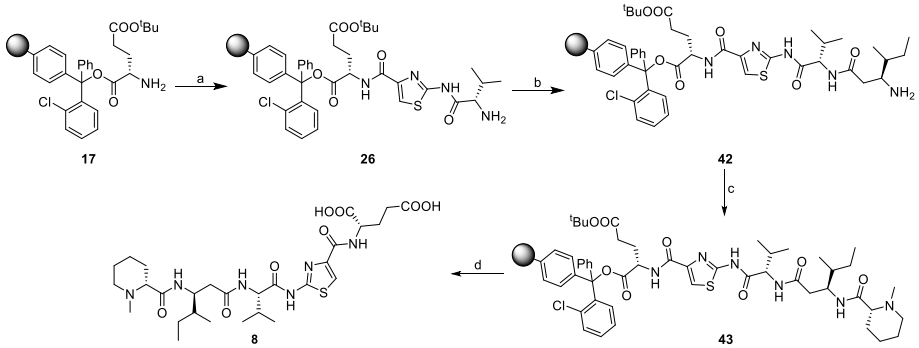
(9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.1.11 Solid-phase peptide synthesis of third-generation tubulin inhibitor (7) incorporating *tert*-leucine



Scheme 11. Synthesis of third-generation tubulin inhibitor **7** incorporating *tert*-leucine. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **14a**, PyBOP, DIPEA, DMF, 6 h; (ii) 20% Piperidine in DMF, rt, 30 min; (b) (i) Fmoc-*tert*-Leu-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (c) N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (d) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

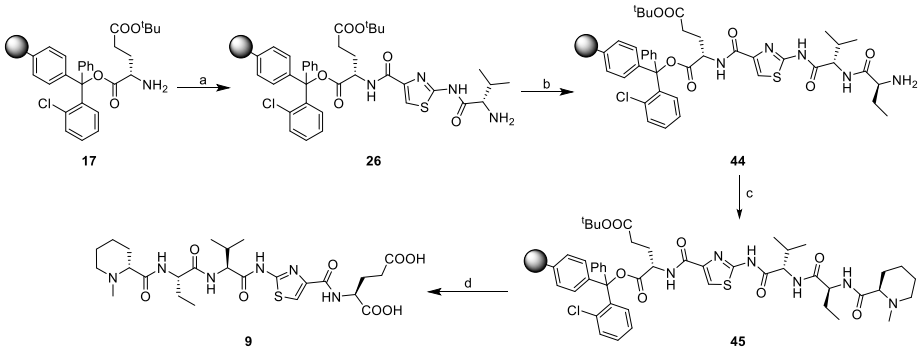
4.1.12 Solid-phase peptide synthesis of third-generation tubulin inhibitor (8) incorporating β-homo-Isoleucine



Scheme 12. Synthesis of third-generation tubulin inhibitor **8** incorporating β-homo-isoleucine. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **15a**, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in

DMF, rt, 30 min; (b) i) Fmoc-β-homo-Ile -OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (c) N-Methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (d) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.1.13 Solid-phase peptide synthesis of third-generation tubulin inhibitor (9) incorporating aminobutyric acid



Scheme 13. Synthesis of third-generation tubulin inhibitor **9** incorporating aminobutyric acid. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **14a**, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (b) i) Fmoc-Abu-OH, PyBOP, DIPEA, DMF, 6 h; ii) 20% Piperidine in DMF, rt, 30 min; (c) N-methyl pipecolic acid **16**, PyBOP, DIPEA, DMF, 6 h; (d) i) TFA/TIPS/H_2O (9.50:0.25:0.25) (1 × 5 mL, 15 min; 2 × 5 mL, 15 min); ii) evaporate TFA; iii) precipitate in ice-cold diethyl ether; iv) Purified using HPLC

4.2 *In vitro* cytotoxic studies of synthesised tubulin inhibitors 1-4

Four newly synthesised tubulin inhibitors **1-4**, along with a standard drug, colchicine, were screened for their cytotoxicity against two patient-derived cancer lines, MCF7 (breast cancer) and HeLa (cervical cancer). The cancer cells were treated with increasing concentrations of the tubulin inhibitors **1-4** from 10 pM to 100 μM (10 pM, 50 pM, 100 pM, 500 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM) and incubated for 48 h. During *in vitro* cytotoxicity studies, the synthesized tubulin inhibitors **1** and **2** exhibited

half-maximal inhibitory concentration (IC_{50}) values of 91.7 nM and 222.1 nM, respectively, against MCF-7 cells compared to the standard drug colchicine, which showed an IC_{50} of 228.4 nM (Figure 5). In contrast, tubulin inhibitors **3** and **4** showed IC_{50} values of 1.42 µM and 2.29 µM, respectively, against HeLa cells, whereas colchicine had the IC_{50} of 349.5 nM under the same conditions (Figure 6).

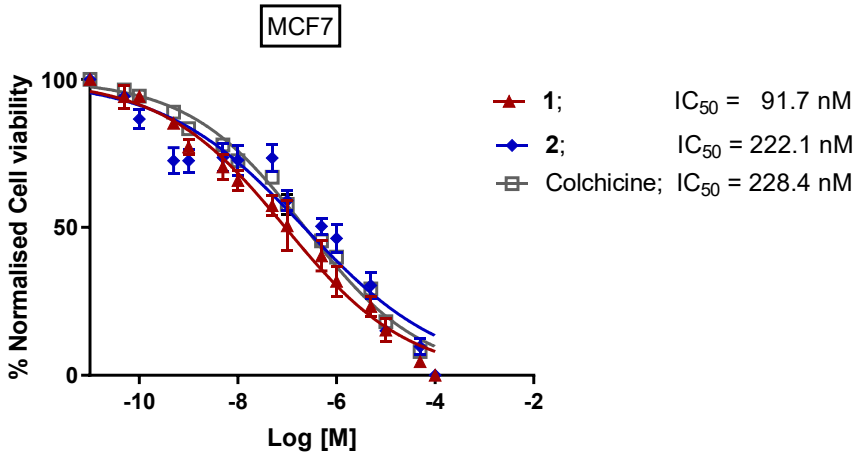


Figure 5. IC_{50} study of tubulin inhibitors **1** and **2** with the standard drug colchicine against breast cancer (MCF7) cell line

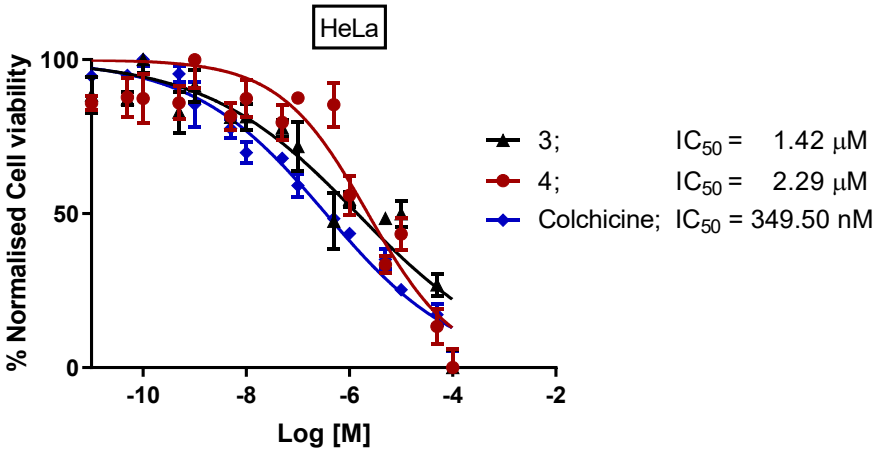


Figure 6. IC_{50} study of tubulin inhibitors **3** and **4** with the standard drug colchicine against cervical cancer (HeLa) cell line.

Chapter 5

Conclusion

In medicinal chemistry, peptides are promising and have high potential to act as anticancer agents. However, the peptide drugs are unstable under biological systems and undergo hydrolysis in the blood serum due to the presence of proteases and challenging for clinical use. In the current study, we have utilized solid-phase peptide synthesis to prepare new third-generation tubulin inhibitors using β- and unnatural α-amino acids. The designed peptides are more biologically stable and effective, improving current peptide-based cancer treatments. In this work we have synthesized various fragments required for the synthesis of new third-generation tubulin inhibitors, followed by the synthesis of designed third-generation tubulin inhibitors using solid-phase peptide synthesis, using the methodology developed in our laboratory. The synthesized compounds were successfully characterized using spectroscopic techniques such as ^{1}H and ^{13}C NMR and mass spectrometry. The *in vitro* studies of the newly designed tubulin inhibitors **1-4** have been performed against breast and cervical cancer cells and found to be in the range of 91 nM to 2.29 μM.

Appendix A

^{1}H and ^{13}C NMR Spectra and Mass spectrometry of the synthesised compounds are listed below

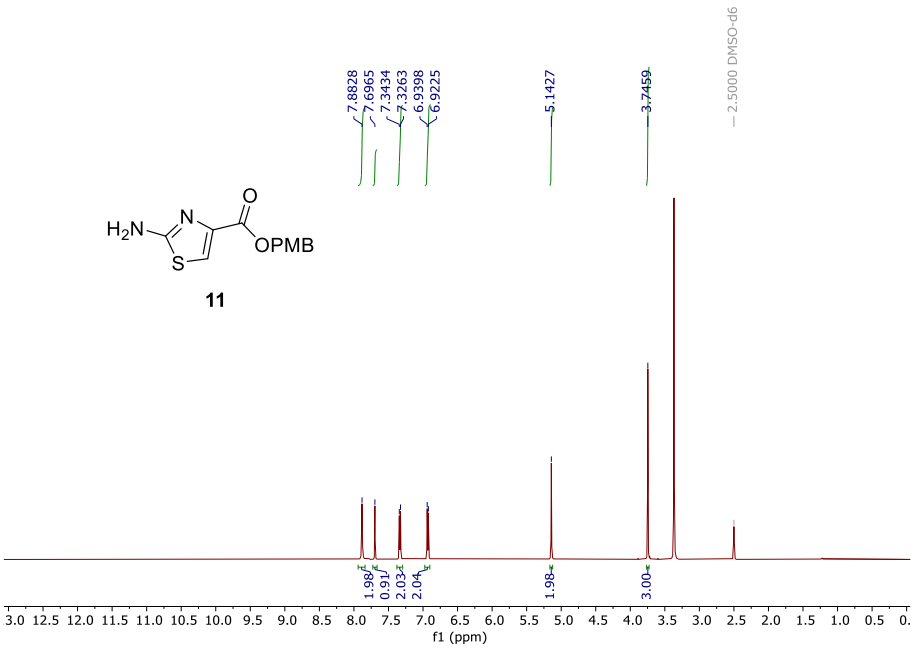


Figure 7. ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of **11**

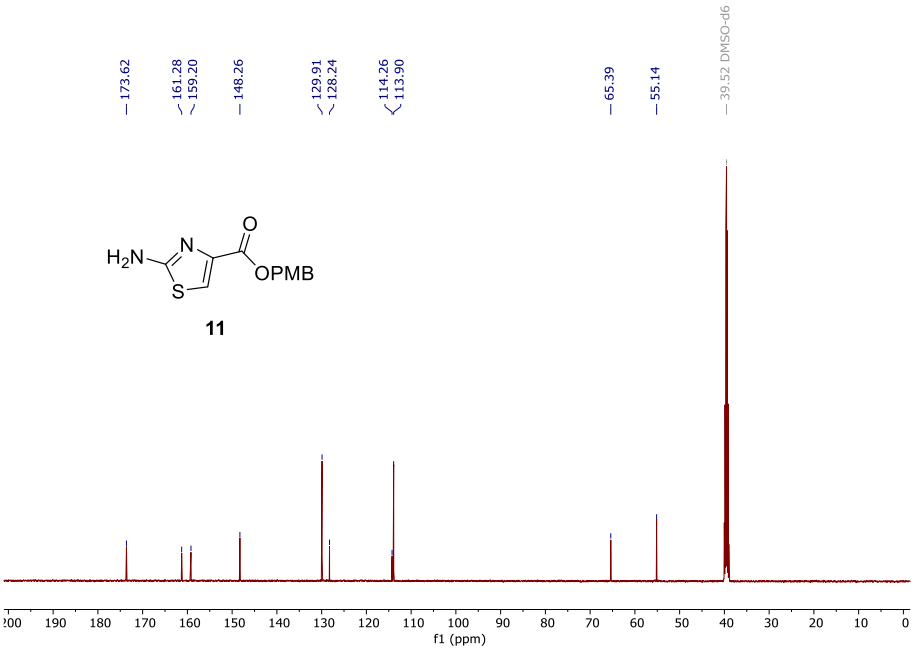


Figure 8. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of **11**

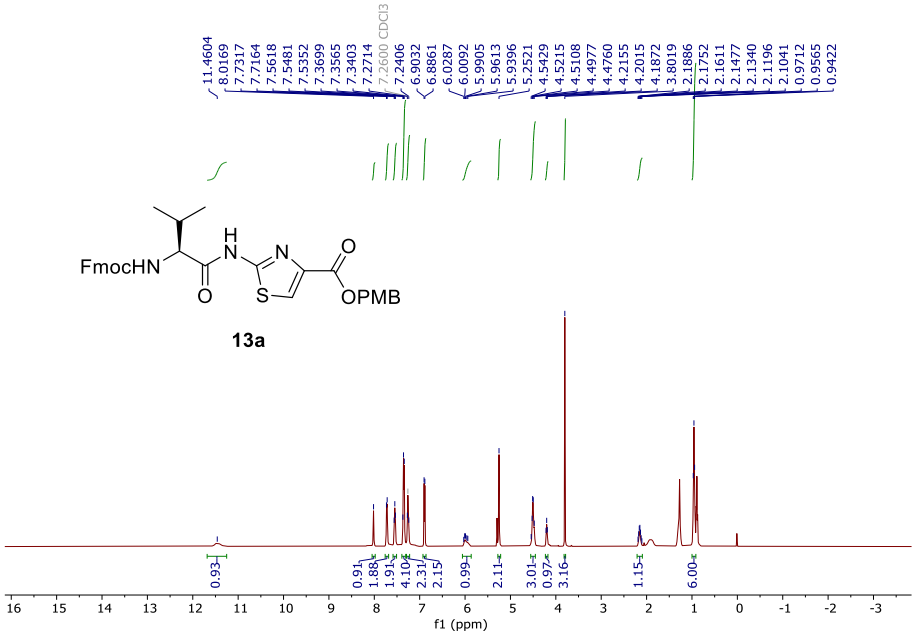


Figure 9. ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of **13a**

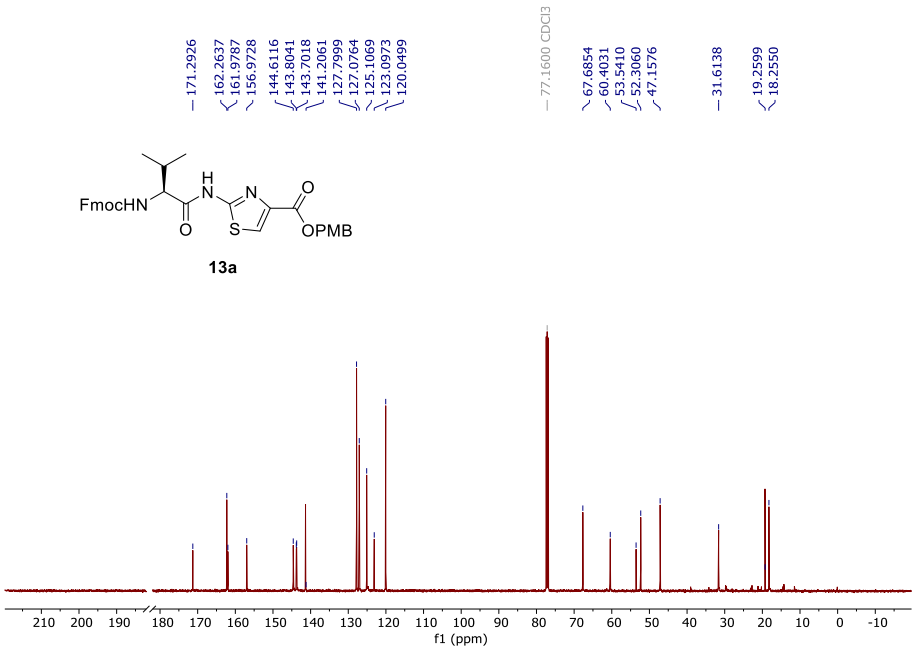


Figure 10. ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of **13a**

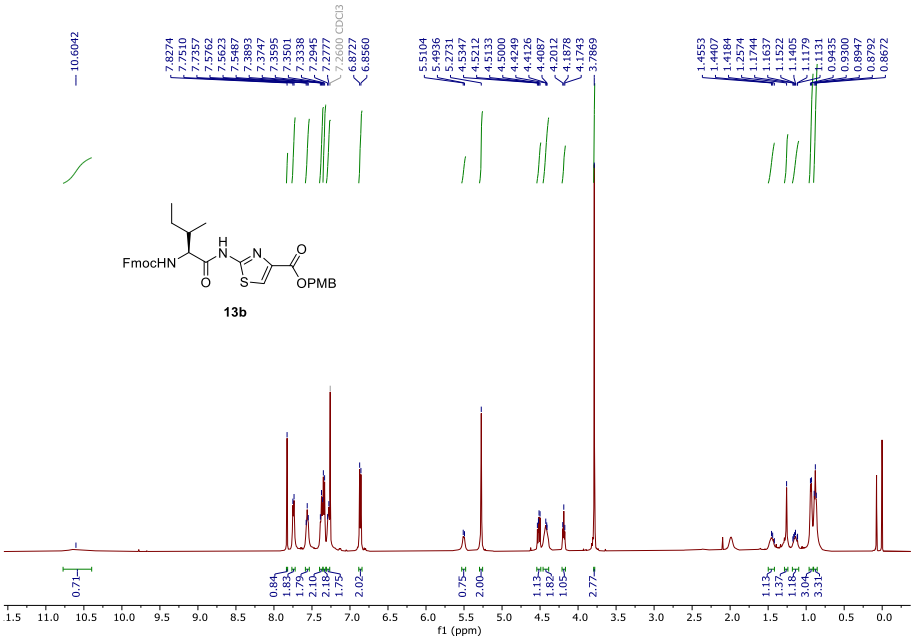


Figure 11. ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of **13b**

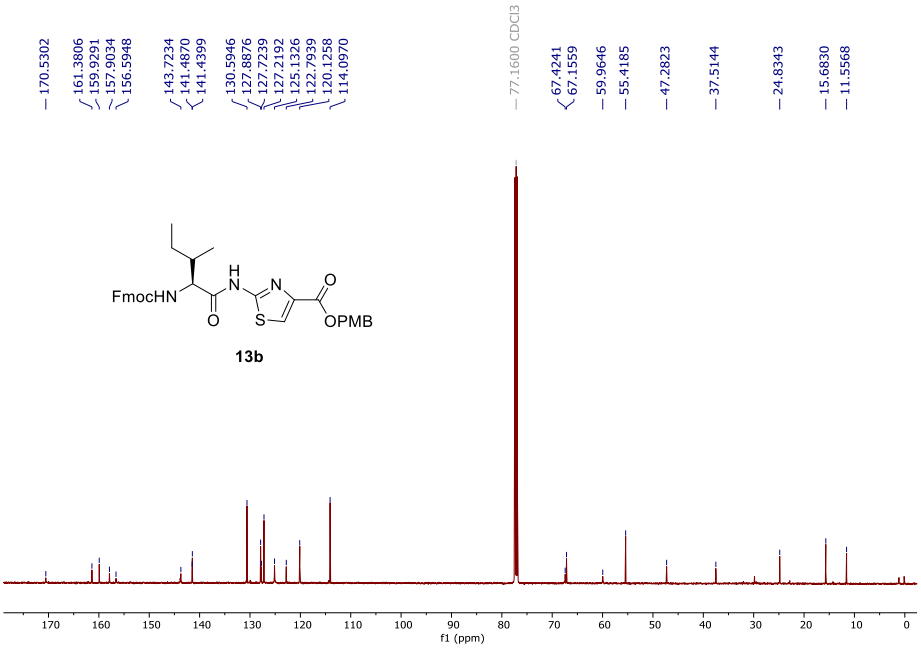


Figure 12. ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of **13b**

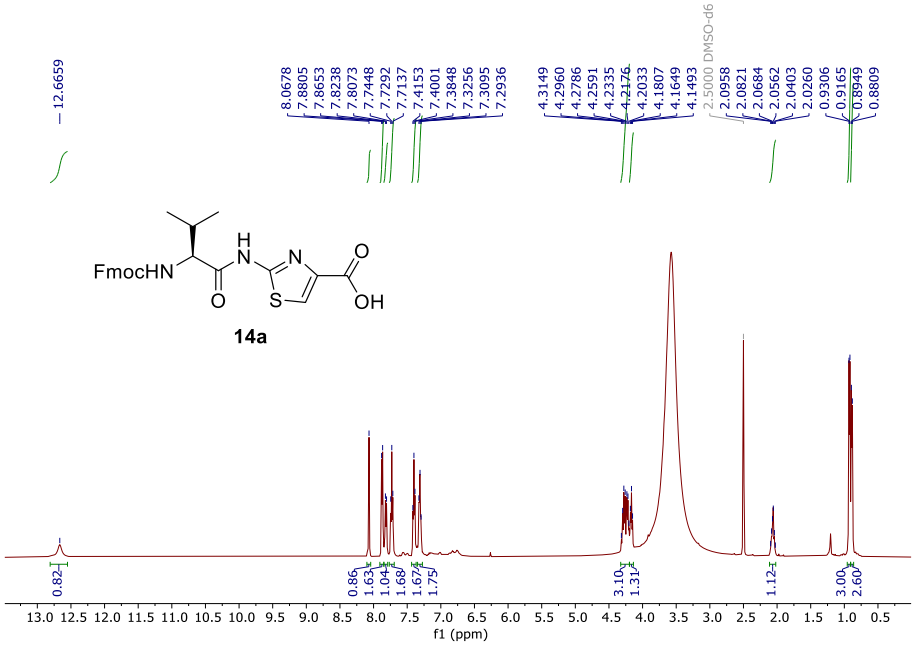


Figure 13. ^{1}H NMR (500 MHz, DMSO-d$_6$) spectrum of **14a**

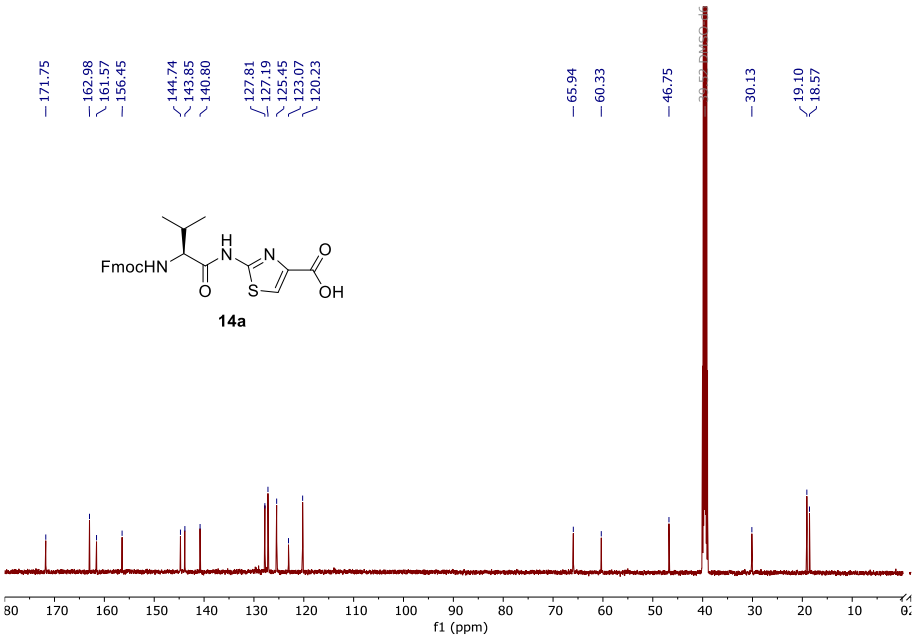


Figure 14. ^{13}C NMR (125 MHz, DMSO-d$_6$) spectrum of **14a**

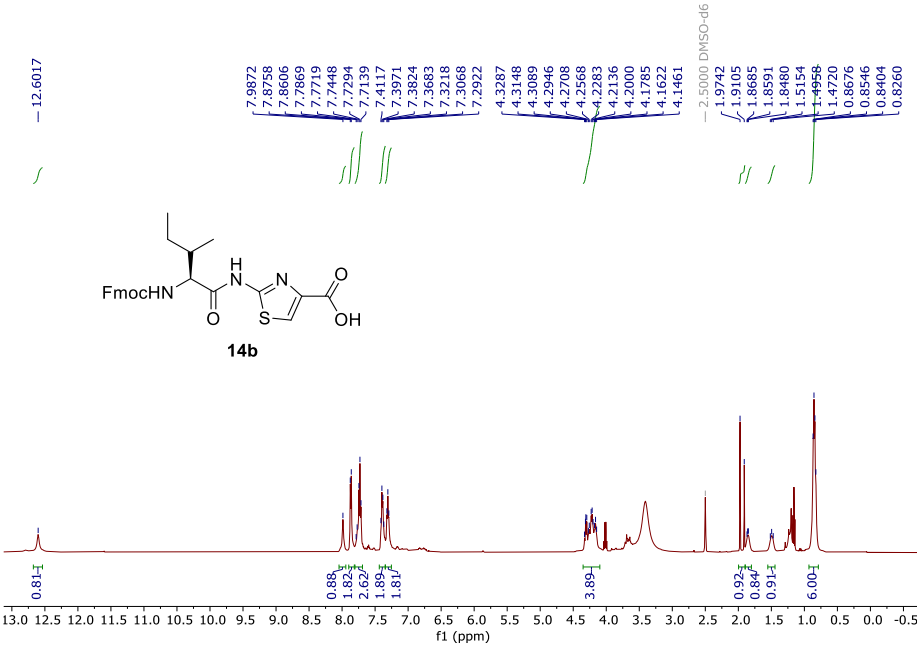


Figure 15. ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of **14b**

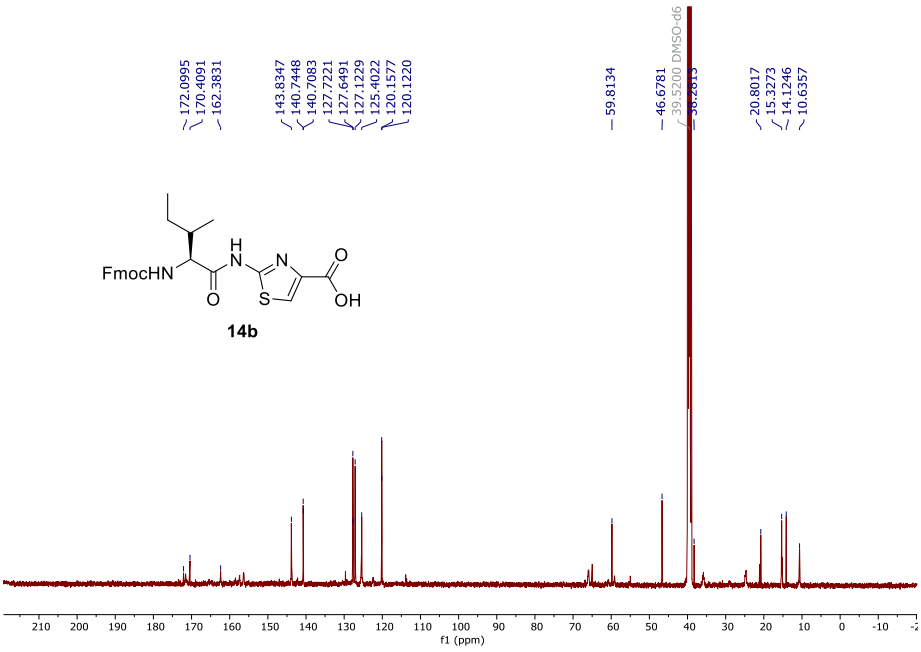


Figure 16. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of **14b**

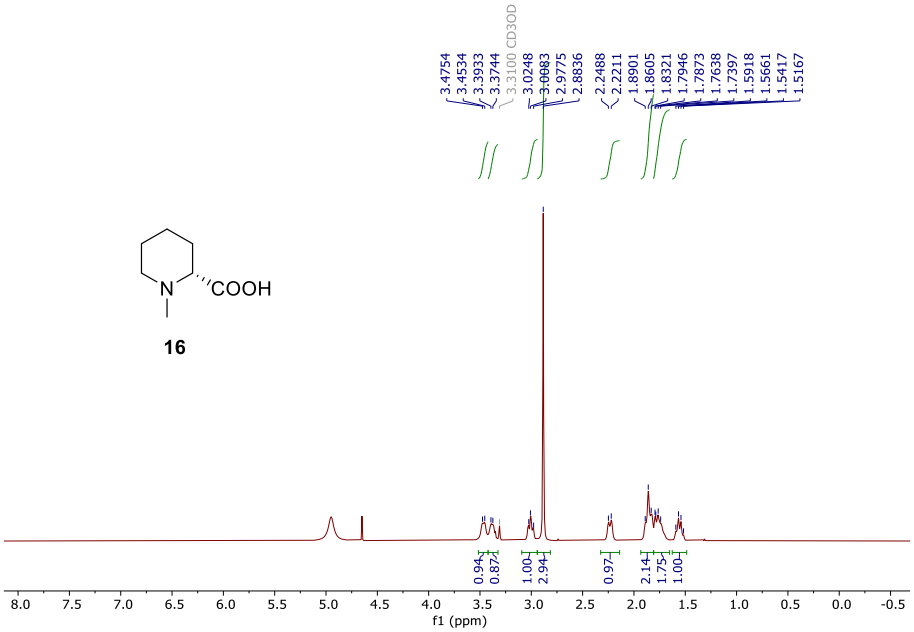


Figure 17. ^{1}H NMR (500 MHz, MeOH-d$_4$) spectrum of **16**

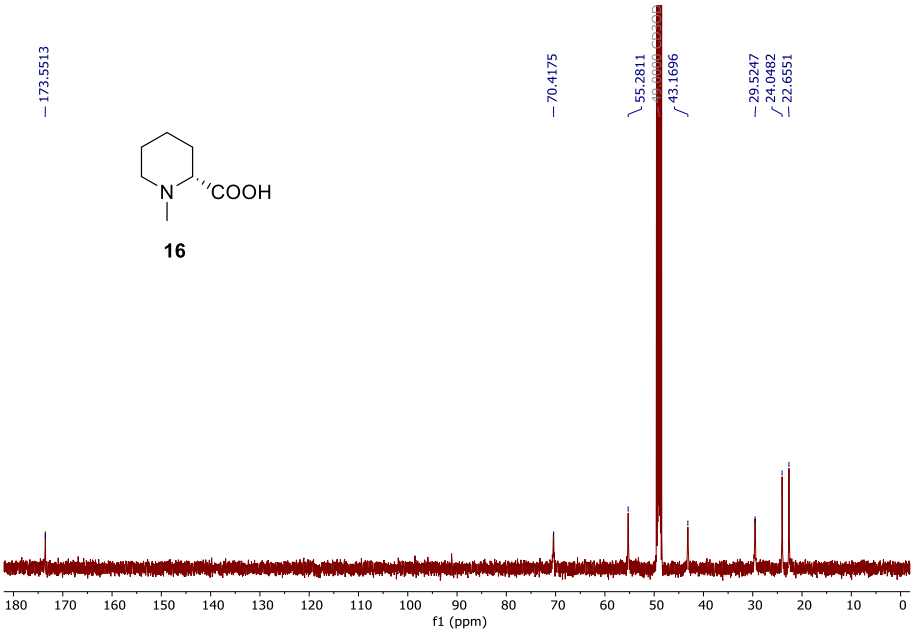


Figure 18. ^{13}C NMR (125MHz, MeOH-d$_4$) spectrum of **16**

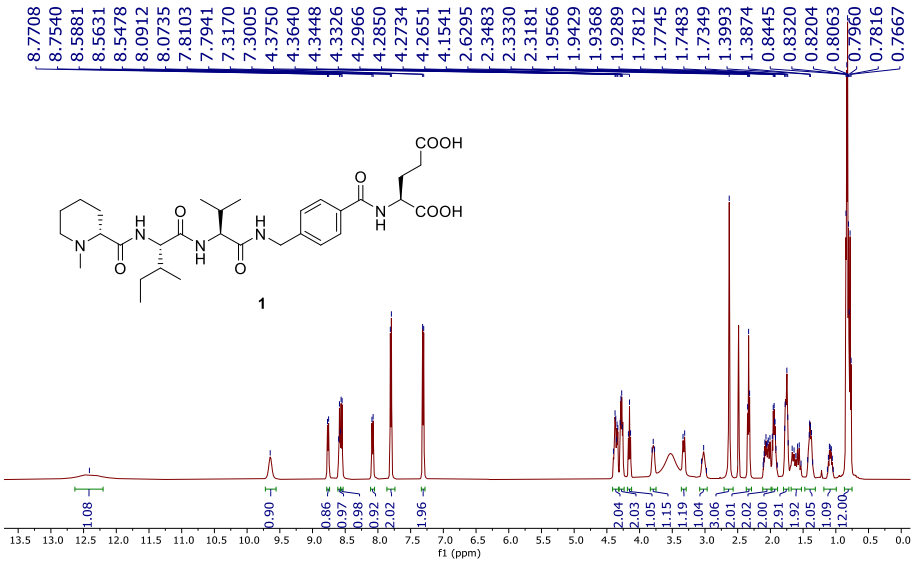


Figure 19. ^{1}H NMR (500 MHz, DMSO-d_6) of tubulin inhibitor **1**

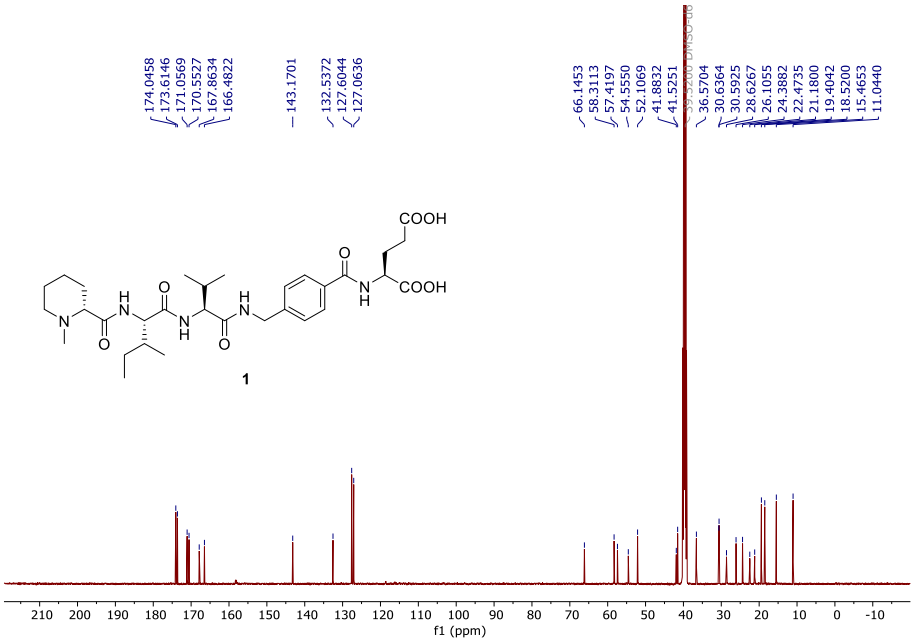


Figure 20. ^{13}C NMR (125 MHz, DMSO-d_6) of tubulin inhibitor **1**

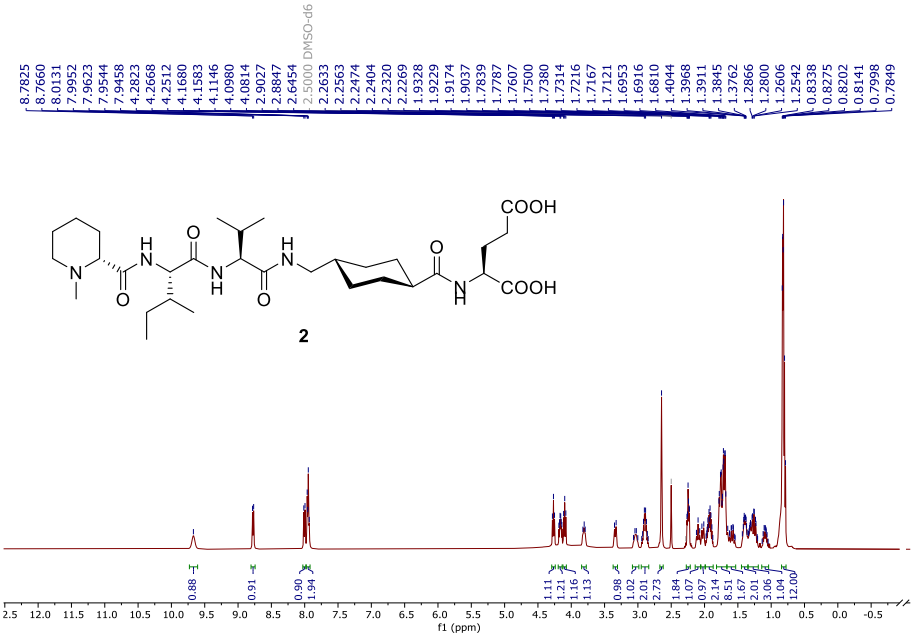


Figure 21. ^{1}H NMR (500 MHz, DMSO-d_6) of tubulin inhibitor **2**

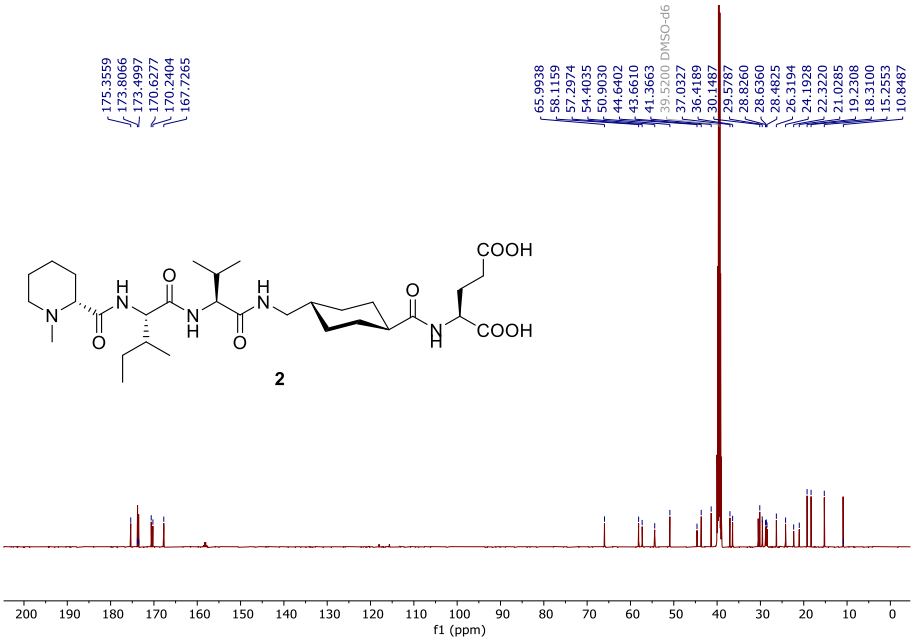


Figure 22. ^{13}C NMR (125 MHz, DMSO-d_6) of tubulin inhibitor **2**

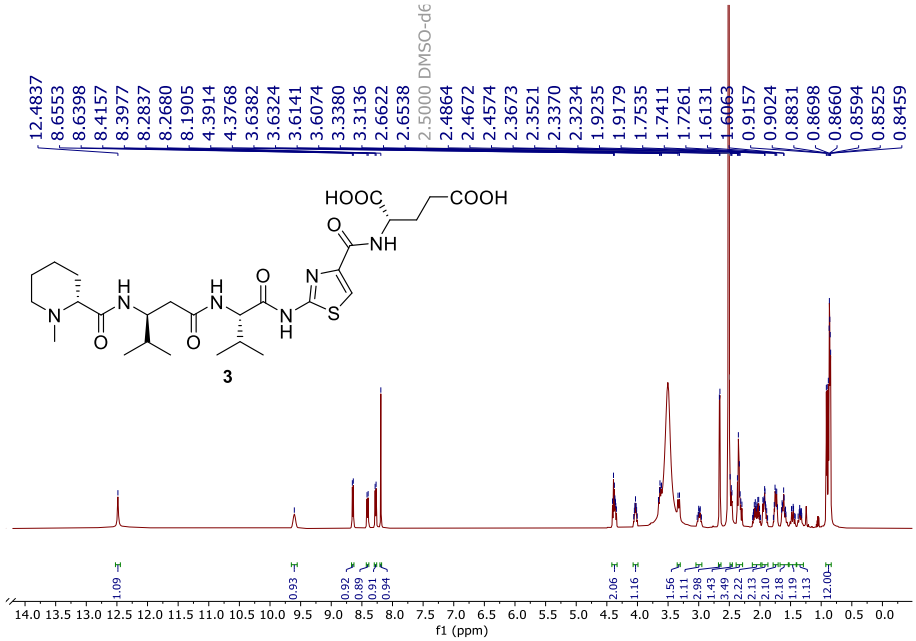


Figure 23. ^{1}H NMR (500 MHz, DMSO-d$_6$) of tubulin inhibitor **3**

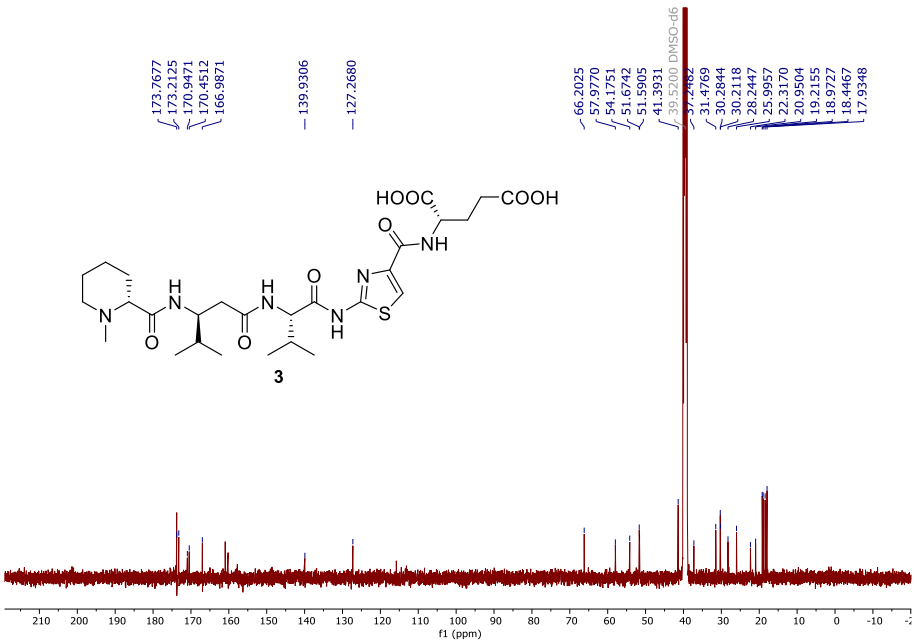


Figure 24. ^{13}C NMR (125 MHz, DMSO-d$_6$) of tubulin inhibitor **3**

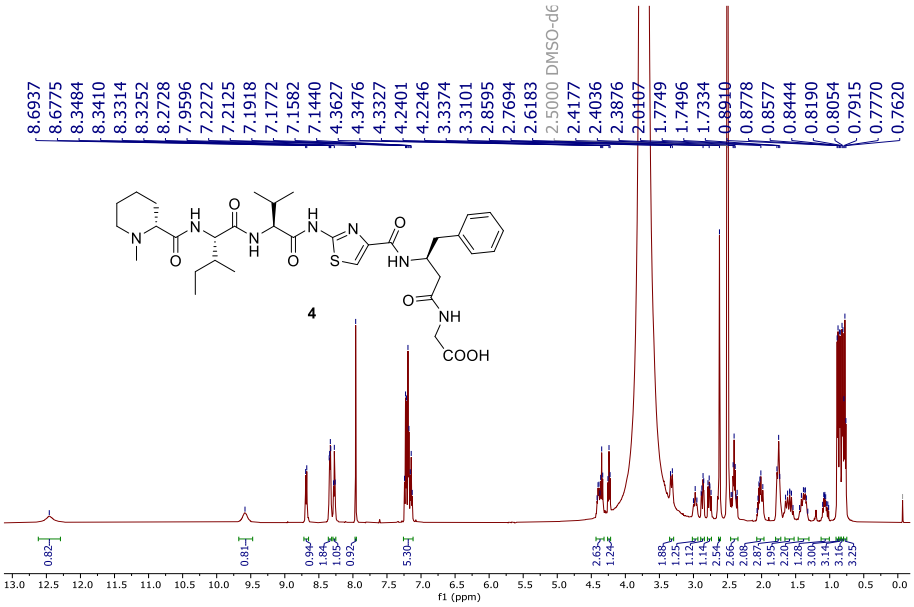


Figure 25. ^{1}H NMR (500 MHz, DMSO-d_6) of tubulin inhibitor **4**

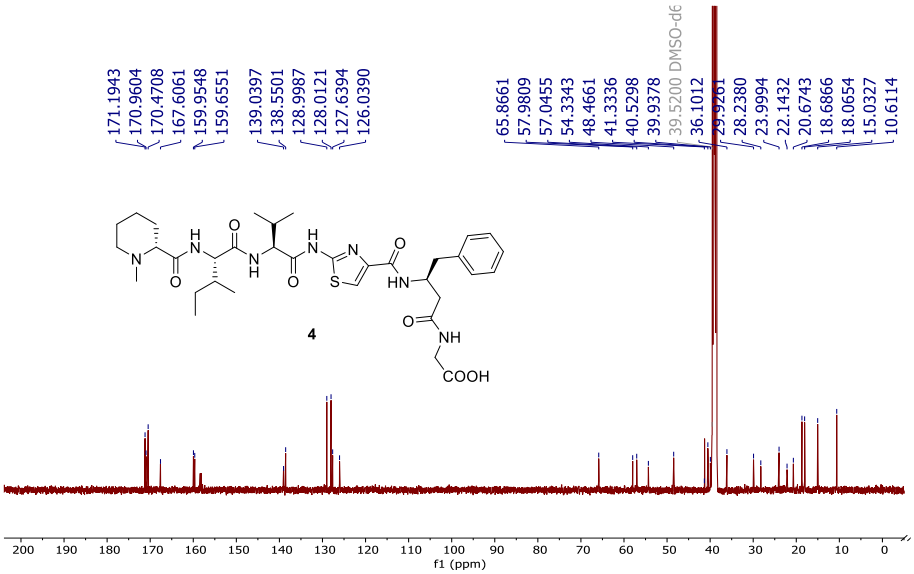


Figure 26. ^{13}C NMR (125 MHz, DMSO-d_6) of tubulin inhibitor **4**

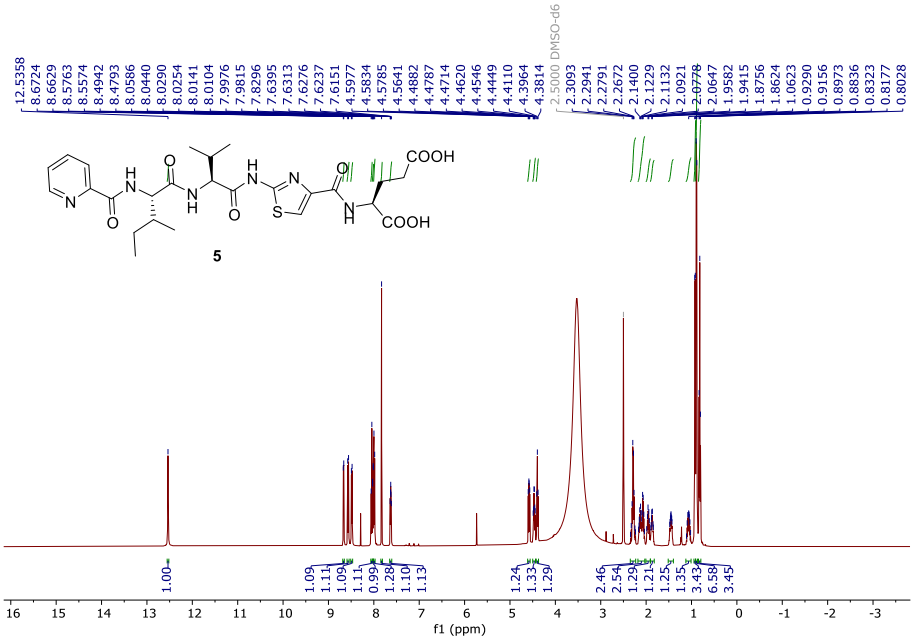


Figure 27. ^{1}H NMR (500 MHz, DMSO-d_6) of tubulin inhibitor **5**

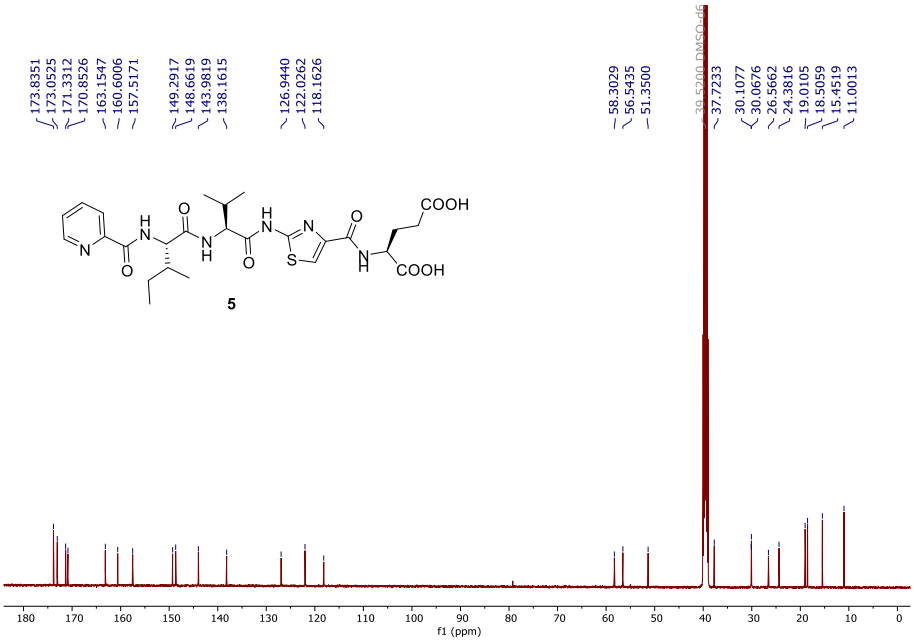


Figure 28. ^{13}C NMR (125 MHz, DMSO-d_6) of tubulin inhibitor **5**

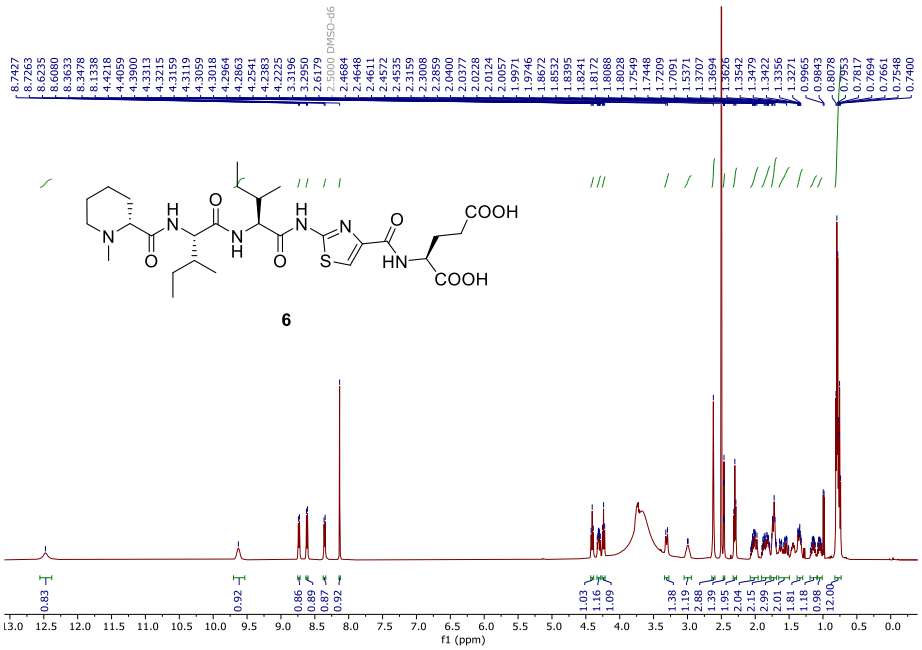


Figure 29. ^{1}H NMR (500 MHz, DMSO-d$_6$) of tubulin inhibitor **6**

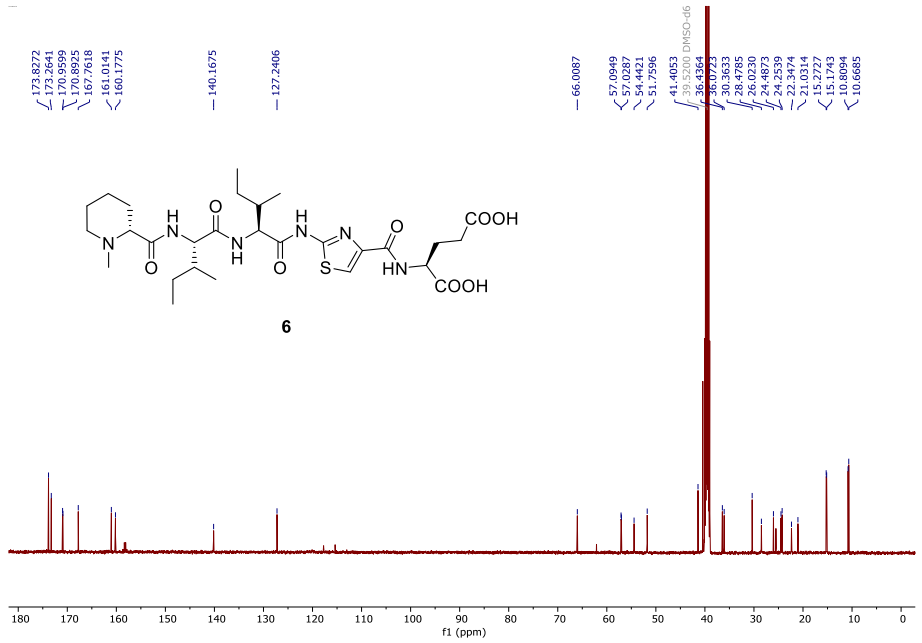


Figure 30. ^{13}C NMR (125 MHz, DMSO-d$_6$) of tubulin inhibitor **6**

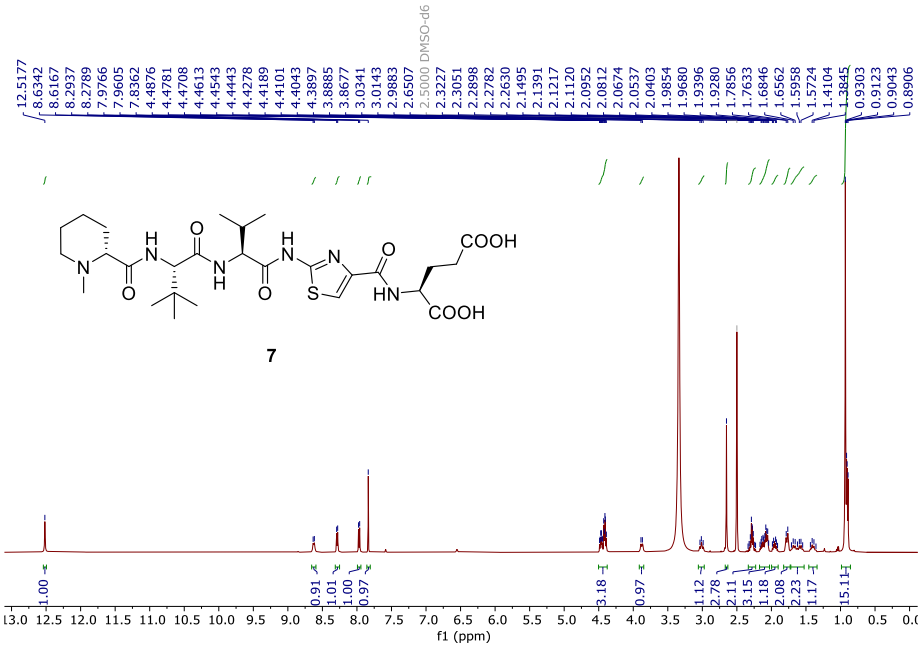


Figure 31. ^{1}H NMR (125 MHz, DMSO-d_6) of tubulin inhibitor **7**

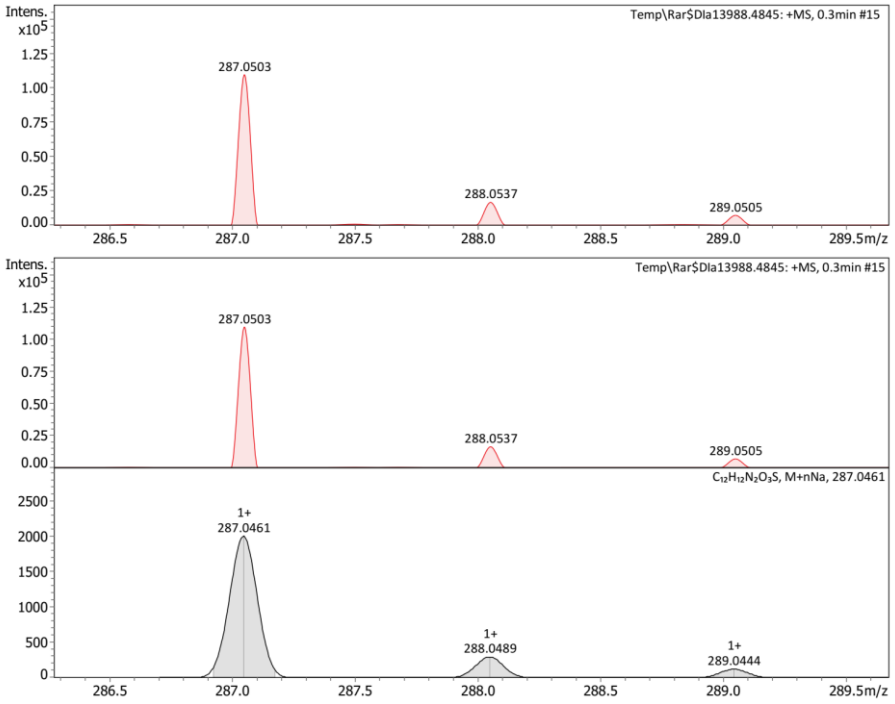


Figure 32. Mass spectrum of **11** in MeOH

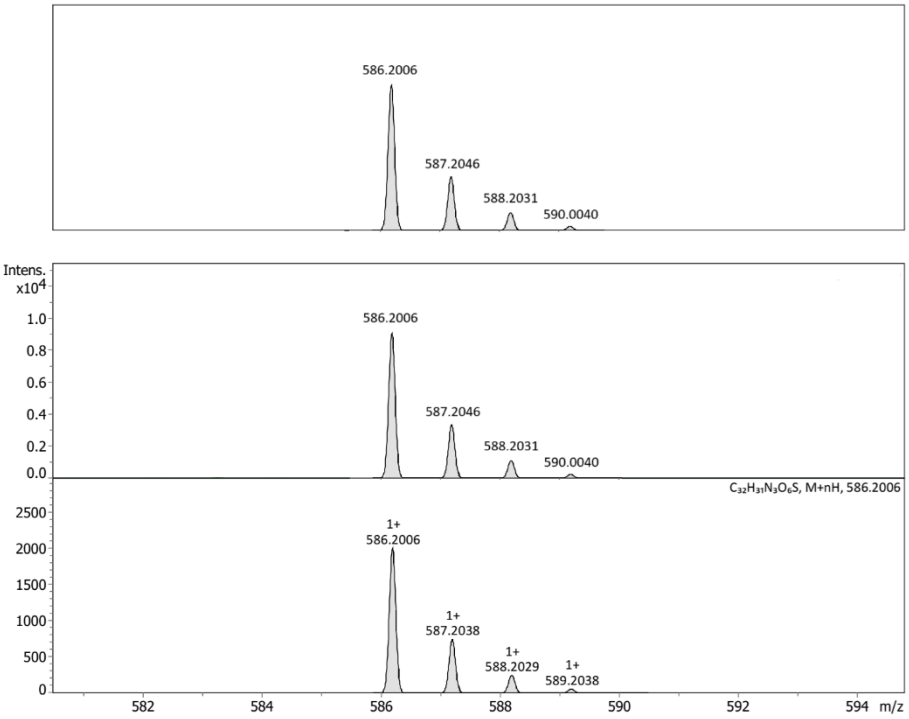


Figure 33. Mass spectrum of **13a** in MeOH

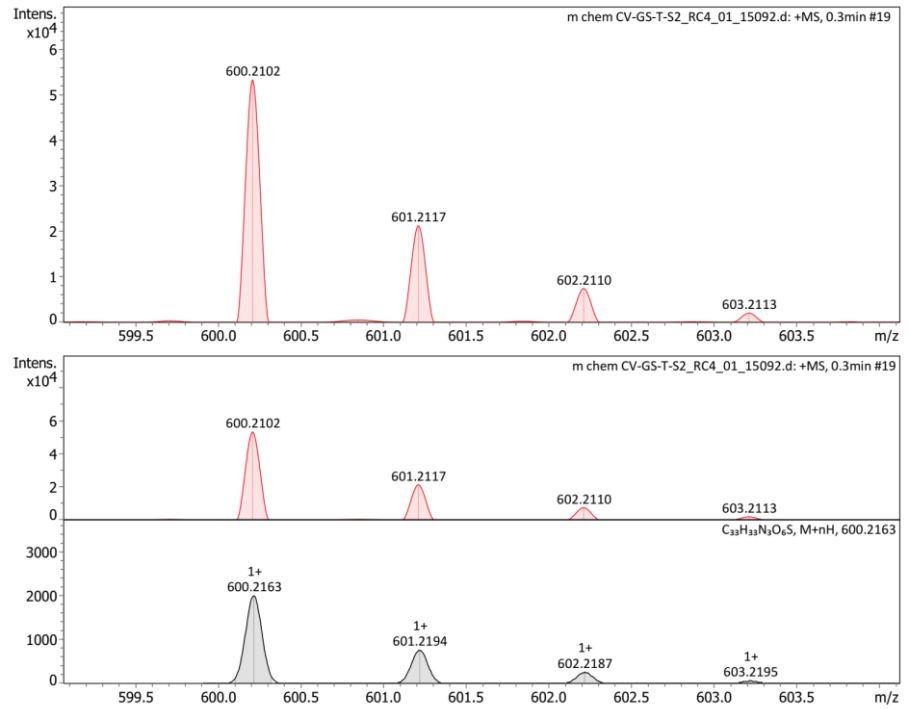


Figure 34. Mass spectrum of **13b** in MeOH

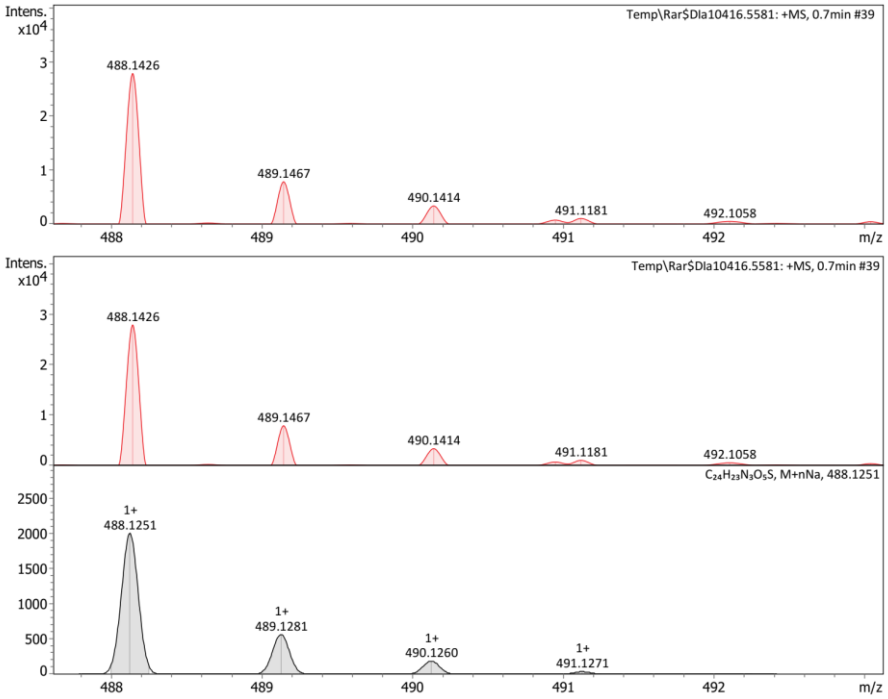


Figure 35. Mass spectrum of **14a** in MeOH

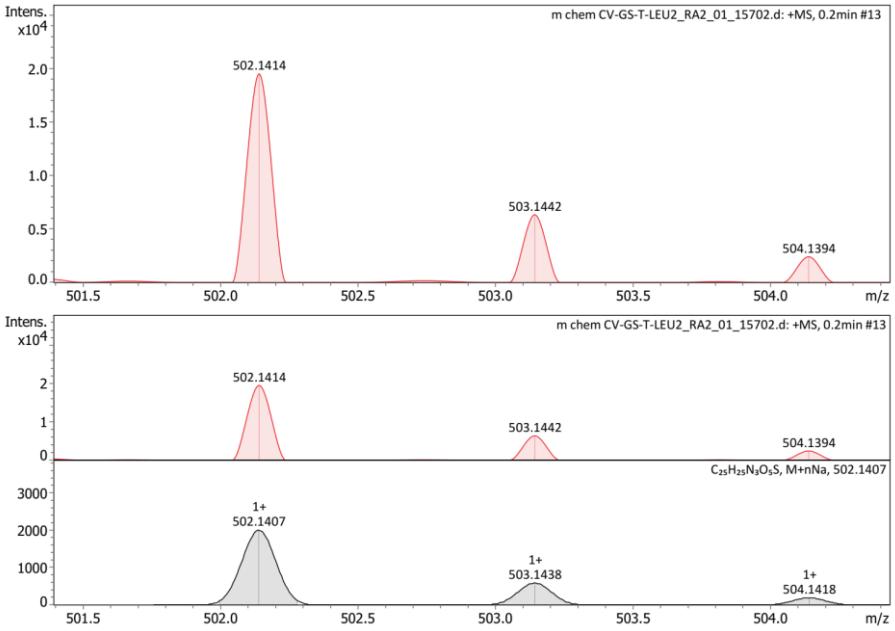


Figure 36. Mass spectrum of **14b** in MeOH

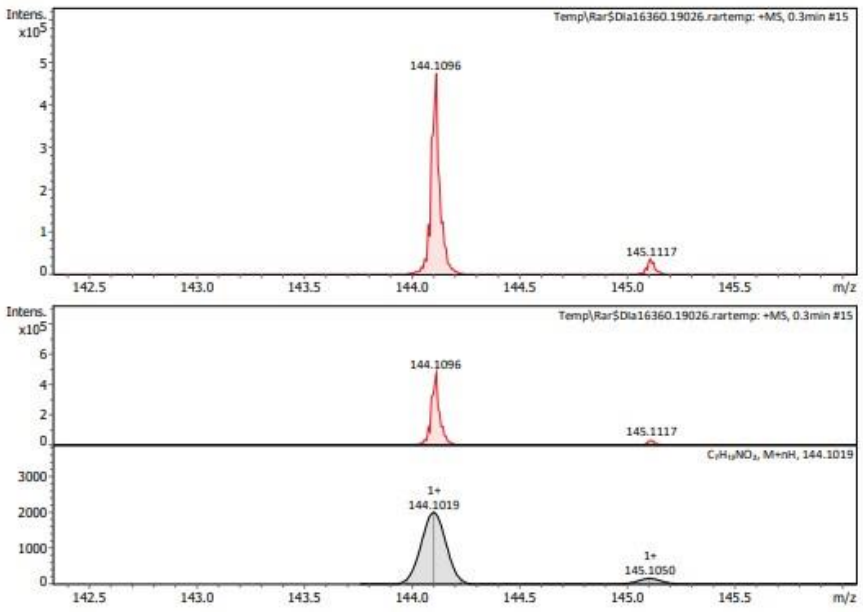


Figure 37. Mass spectrum of **16** in MeOH

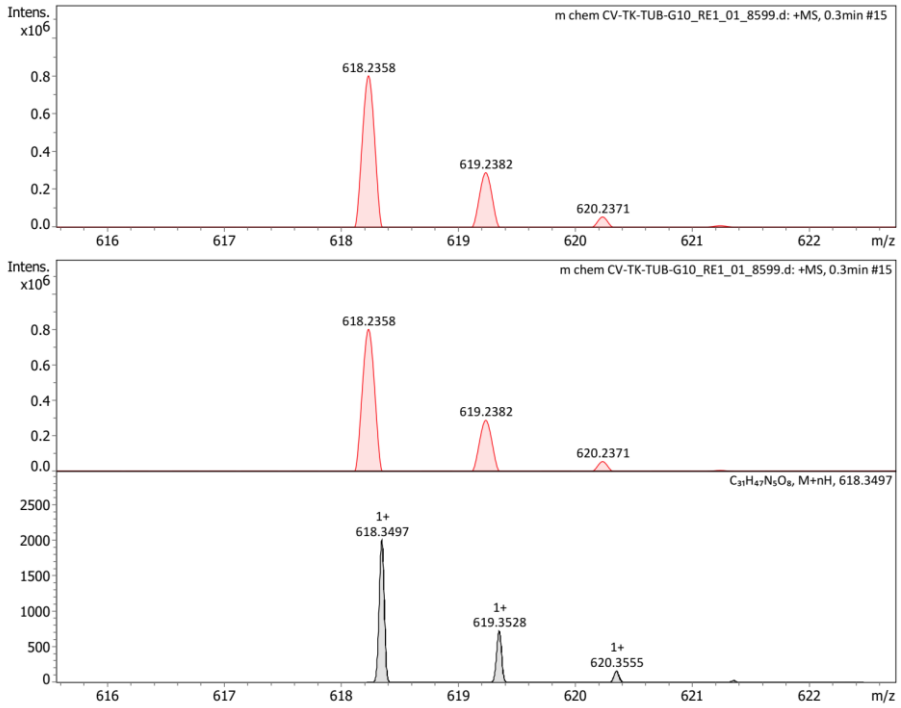


Figure 38. Mass spectrum of tubulin inhibitor **1** in MeOH

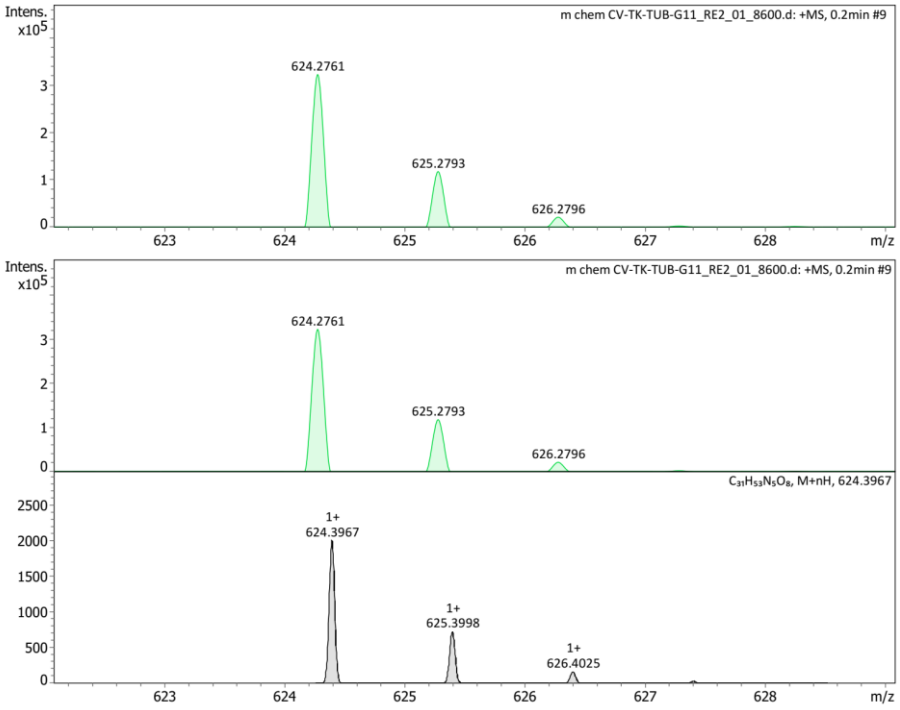


Figure 39. Mass spectrum of tubulin inhibitor **2** in MeOH

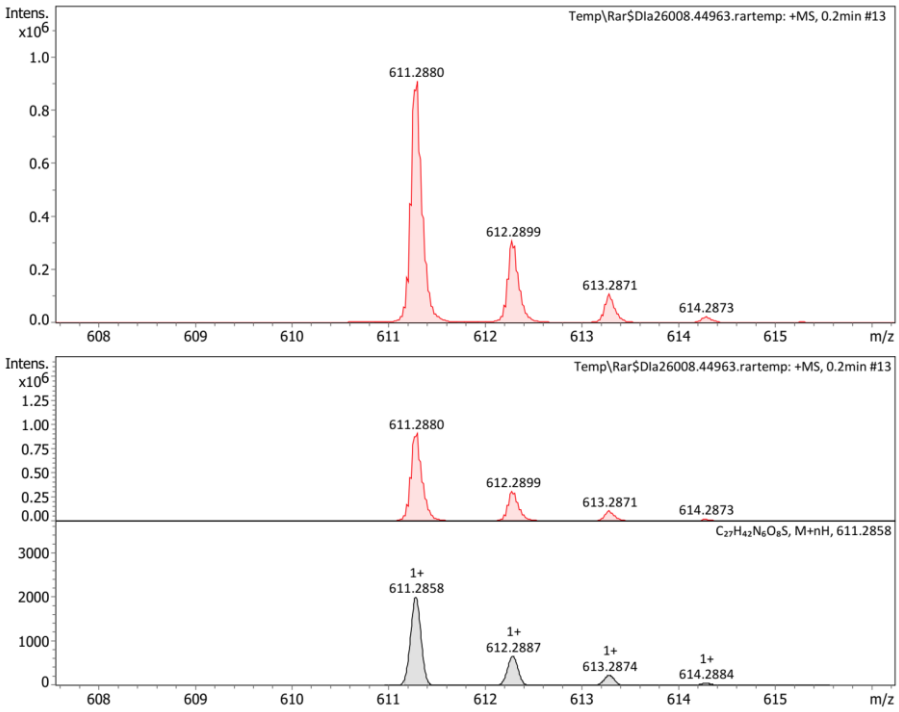


Figure 40. Mass spectrum of tubulin inhibitor **3** in MeOH

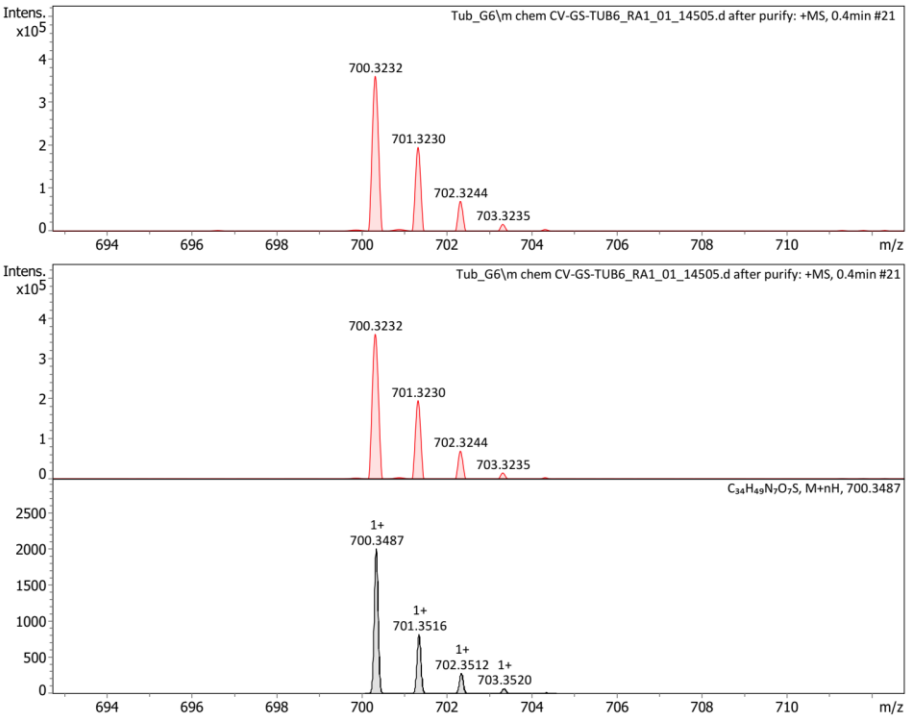


Figure 41. Mass spectrum of tubulin inhibitor **4** in MeOH

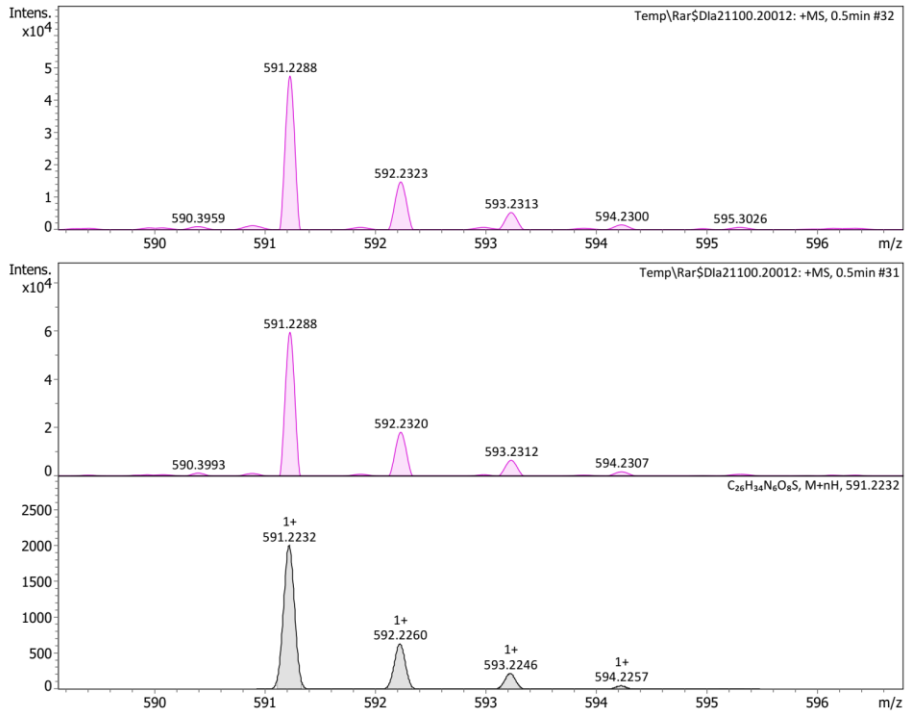


Figure 42. Mass spectrum of tubulin inhibitor **5** in MeOH

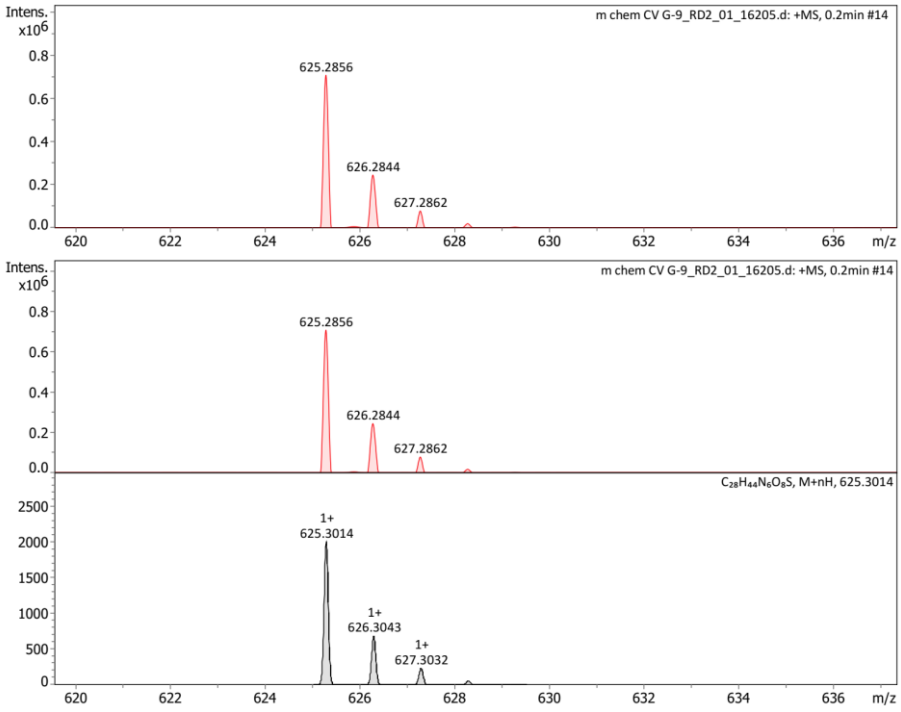


Figure 43. Mass spectrum of tubulin inhibitor **6** in MeOH

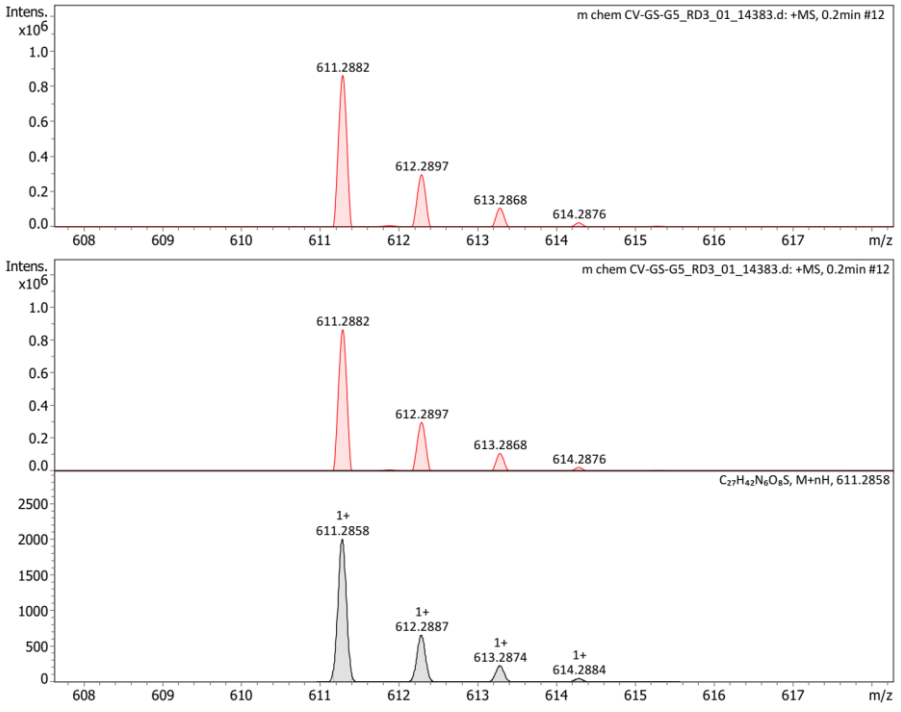


Figure 44. Mass spectrum of tubulin inhibitor **7** in MeOH

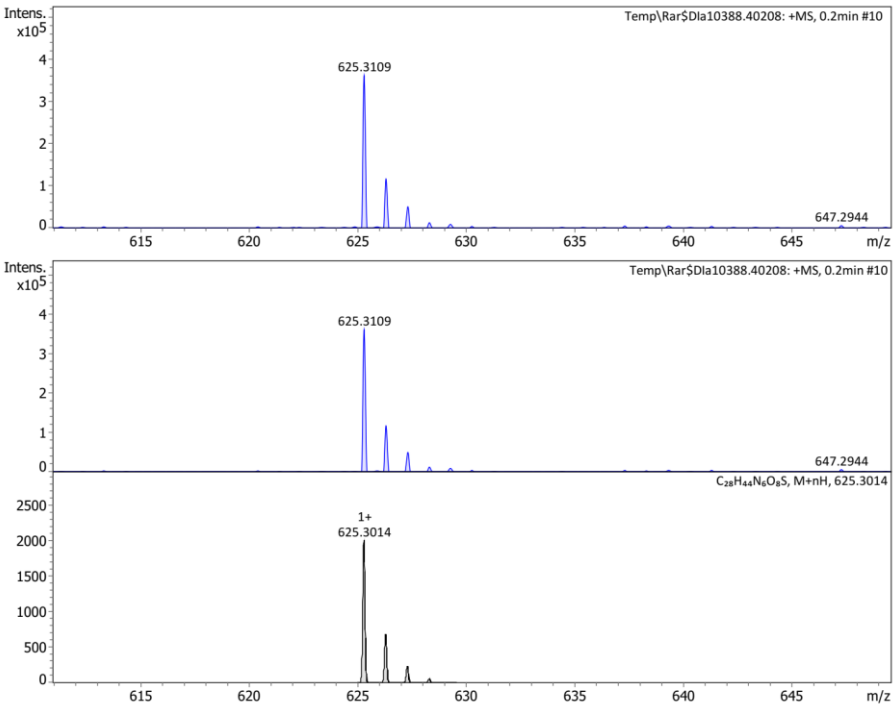


Figure 45. Mass spectrum of tubulin inhibitor **8** in MeOH

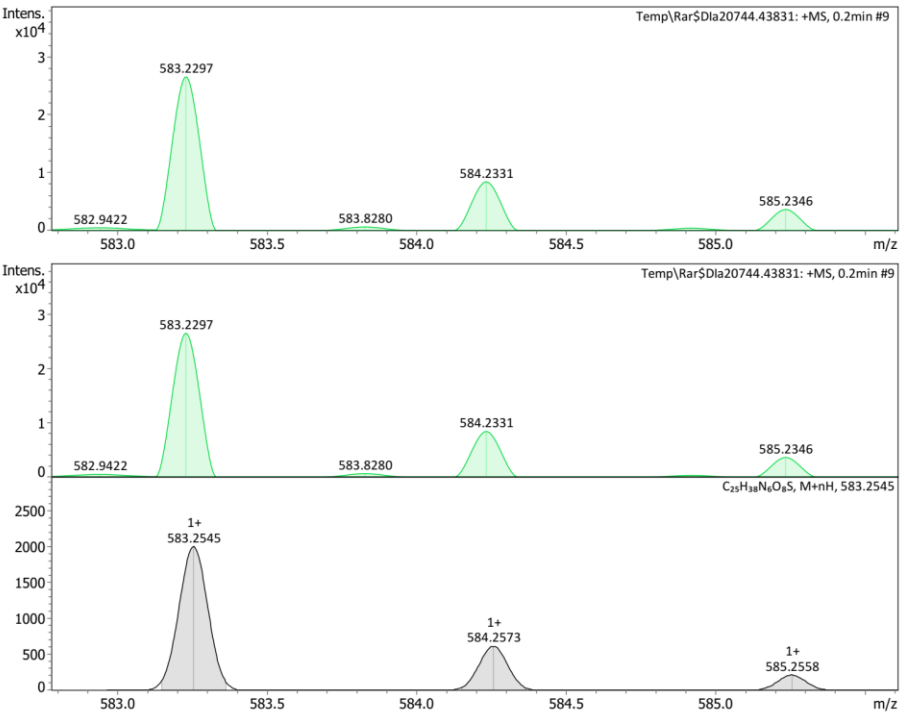


Figure 46. Mass spectrum of tubulysin derivative **9** in MeOH

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