

Design, Synthesis, and Biological Evaluation of Third-Generation Anticancer Tubulin Inhibitors Incorporating Beta and Unnatural Amino Acids

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Organic Chemistry**

by

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**DEPARTMENT OF CHEMISTRY
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Design, Synthesis, and Biological Evaluation of Third-Generation Anticancer Tubulin Inhibitors Incorporating Beta and Unnatural Amino Acids

A THESIS

*Submitted in partial fulfilment of the
requirements for the award of the degree*

of

Master of Science

by

Meet Nimbark



**DEPARTMENT OF CHEMISTRY
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May 2026**



INDIAN INSTITUTE OF TECHNOLOGY INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled “*Design, Synthesis, and Biological Evaluation of Third-Generation Anticancer Tubulin Inhibitors Incorporating Beta and Unnatural Amino Acids*” 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 2025 to May 2026 under the supervision of **Prof. Venkatesh Chelvam**, Professor, Indian Institute of Technology 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.

22/05/2026

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

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May 22, 2026

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ABSTRACT

Tubulysins are highly potent microtubule-depolymerizing tetrapeptides with remarkable anticancer activity; however, their complex structures, poor metabolic stability, and susceptibility to proteolytic degradation limit their therapeutic application. In this work, we report the design and synthesis of third-generation tubulin inhibitors that incorporate β -amino acids and unnatural amino acids to enhance protease resistance and improve overall peptide stability. The central fragments of thiazole-containing amino acids were synthesized using solution-phase reaction. Reductive amination was employed to access the N-terminal N-alkyl pipercolic acid derivatives. The novel tubulin inhibitors were synthesized using solid-phase peptide synthesis (SPPS) on glutamic acid and asparagine resins, followed by purification using RP-HPLC. The potency of the newly synthesized third-generation tubulin inhibitors was evaluated against the skin cancer cell line, A431, and human breast cancer cell line, MCF7, to establish detailed structure–activity relationships and identify a potential lead compound for anticancer drug development.

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SYMBOL/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, and related terms follow the guidelines of the IUPAC–IUB Commission on Biochemical Nomenclature (Pure Appl. Chem. 1974, 40, 315–331). Unless otherwise stated, all amino acids are of the L-configuration, and the standard three-letter codes are used throughout. Additional abbreviations employed in this report are listed below.

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-Fluorenylmethoxycarbonyl
Gly	Glycine
Glu	Glutamic
HCl	Hydrochloric acid
HOBt	Hydroxy benzotriazole
Ile	Isoleucine
Leu	Leucine
m.p.	Melting point
m	Multiplet
Na ₂ SO ₄	Sodium sulphate
Na ₂ CO ₃	Sodium carbonate

NMR	Nuclear magnetic resonance spectroscopy
PyBOP	Benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate
q	Quartet
s	Singlet
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
TMS	Tetramethyl silane

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 stated 9.7 million cancer-related deaths globally, with lung cancer identified as the leading cause of mortality (Figure 2) [1].

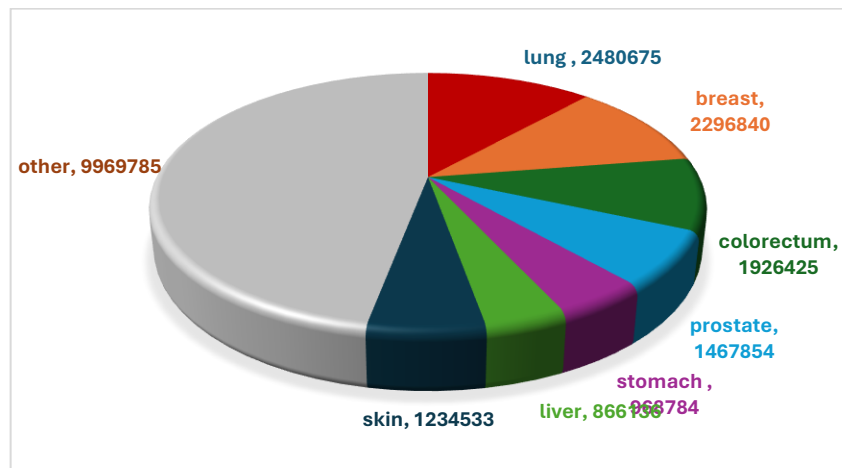


Figure 1. Number of cancer cases worldwide

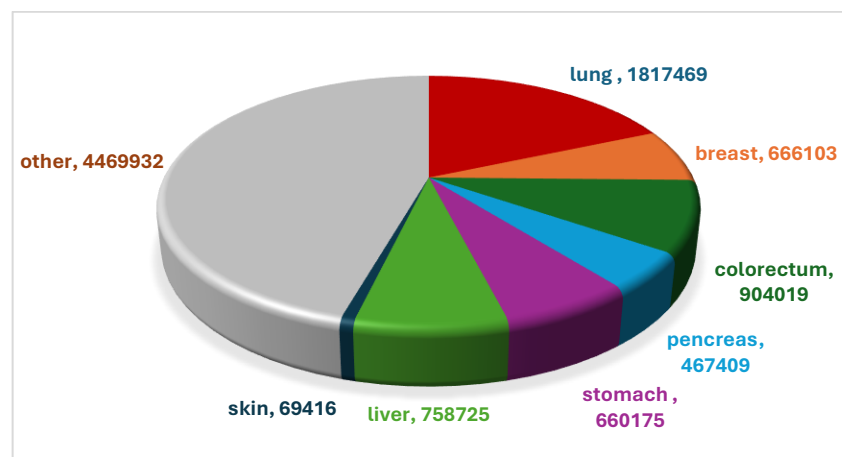


Figure 2. Number of cancer deaths worldwide

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 cylindrical polymers composed of α -tubulin and β -tubulin heterodimers, present in the cytoplasm of eukaryotic cells. They are a major component of the cytoskeleton, extending throughout the cell to maintain its shape, enable intracellular transport and signaling, and, most importantly, facilitate cell division by forming the mitotic spindle during mitosis [3]. In cancer, microtubules play a significant role, as the rapid, 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]. During mitosis, in prophase, microtubules polymerize from centrosomes to form the mitotic spindle. They elongate by polymerization to reach and attach to kinetochores on chromosomes. In metaphase, continuous polymerization and depolymerization maintain tension, keeping chromosomes aligned at the equatorial plate. During anaphase, controlled depolymerization at the kinetochore and spindle pole ends shortens the microtubules, pulling sister chromatids apart. This precise balance of assembly and disassembly ensures accurate chromosome segregation before cytokinesis [3].

1.3 Microtubule inhibitors and their mechanism of action

Microtubule inhibitors are chemotherapeutic agents that disrupt the normal dynamic behaviour of microtubules, which is essential for mitosis. Microtubules are made of α - and β -tubulin dimers that undergo polymerization (assembly) and depolymerization (disassembly). These inhibitors interfere with either process, leading to defective spindle formation and mitotic arrest. They are broadly classified into microtubule-stabilizing agents and microtubule-destabilizing agents. Stabilizing agents, such as paclitaxel (Taxol), bind to β -tubulin in

polymerized microtubules, preventing depolymerization by stabilizing the spindles. Destabilizing agents, such as vincristine, vinblastine, or colchicine, bind to tubulin heterodimers or to specific sites, such as the vinca domain for vincristine and vinblastine and the colchicine site for colchicine, on microtubules, preventing polymerization or promoting disassembly. Both mechanisms block the dynamic instability needed for chromosome alignment and segregation. This activates the spindle assembly checkpoint, keeping cells arrested in metaphase. Prolonged arrest triggers apoptosis or mitotic catastrophe, leading to cell death. These inhibitors are widely used in anticancer therapy to target rapidly dividing tumor cells; however, they also affect normal dividing cells.

1.4 Tubulysin derivatives

Tubulysins are among the most important families of natural products due to their therapeutic potential, notably in cancer treatment. The first tubulysin derivative was extracted from a 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 their unique structural features and strong cytotoxicity against various cancers, tubulysin derivatives have attracted significant interest in medicinal chemistry. These natural tetrapeptides bind to the vinca domain of β -tubulin within the $\alpha\beta$ -heterodimer, ultimately leading to microtubule depolymerization and 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].

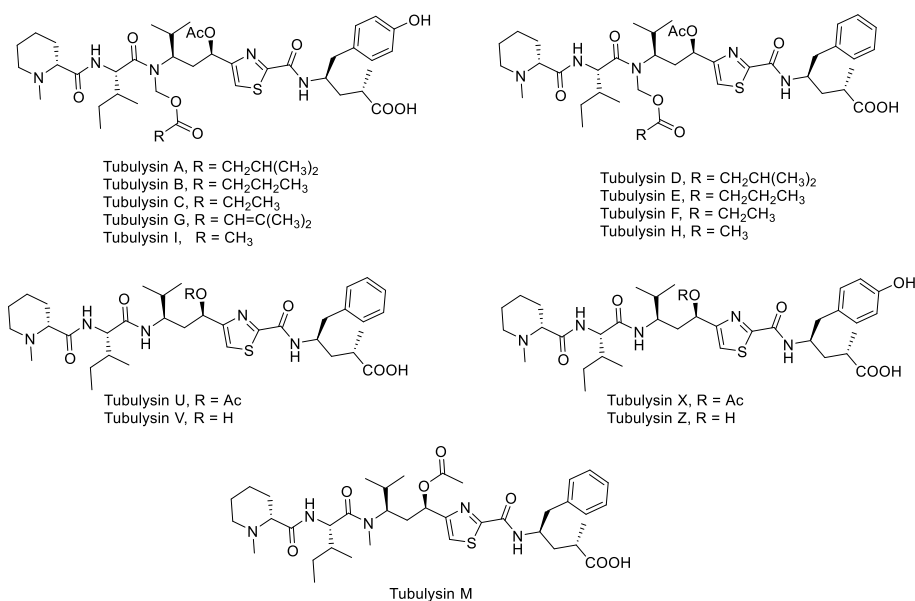


Figure 3. Structure of the naturally occurring tubulysin derivatives

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 that are resistant to proteases 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 patient-derived human cancer cell lines, including the MCF7 (breast cancer) and A431 (skin cancer).

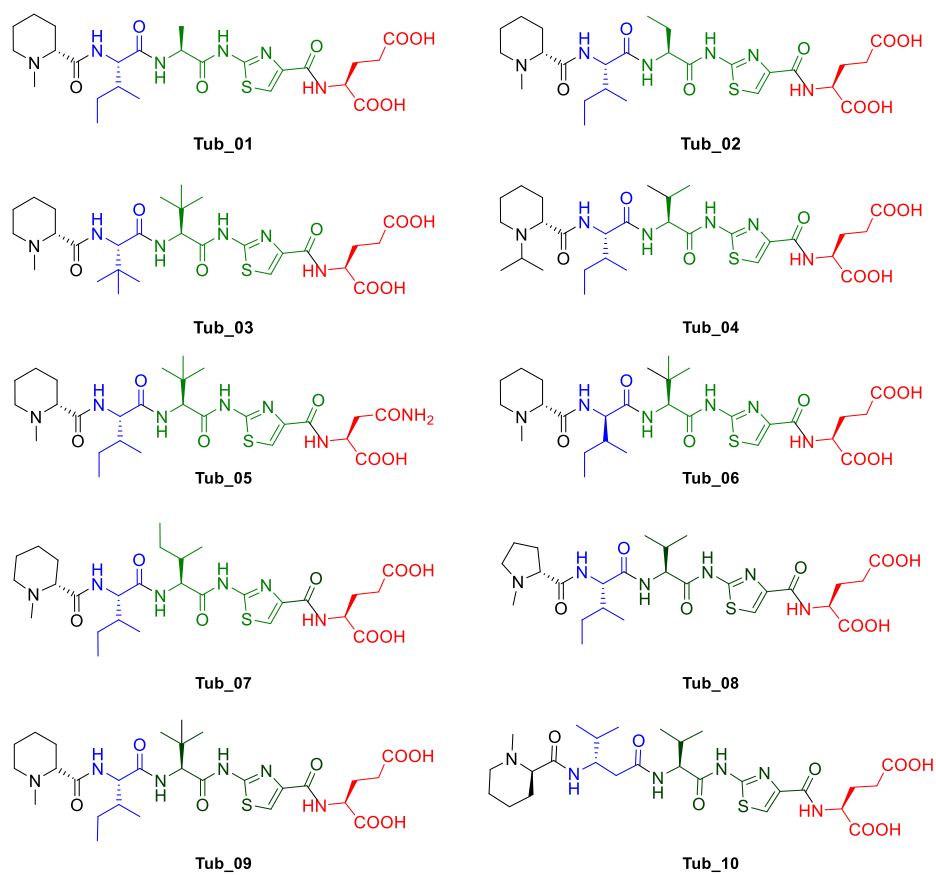


Figure 4. Structures of third-generation tubulin inhibitors incorporating unnatural α -amino acid.

CHAPTER 2

LITERATURE REVIEW

Anticancer 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 cytotoxicity [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 activity ($IC_{50} = 0.02$ nM). The chemical structure of the tubulysin M consists of *N*-methyl pipecolic acid, isoleucine, tubuvaline, and tubuphenylalanine from N to C-terminus of the tetrapeptide, respectively. However, the structural complexity of the tubuvaline and tubuphenylalanine moieties poses a significant synthetic challenge. In 2016, using a 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 instability, the synthesis of these naturally occurring anticancer peptides is further complicated. A major drawback of peptide-based anticancer agents is proteolytic degradation, as proteases can cleave the amide bonds formed by natural α -amino acids [8]. Previously, our research group reported the synthesis of several tubulysin fragments and tubulysin derivatives using solid-phase peptide synthesis [9]. In this work, we are using solid-phase peptide synthesis with β - and unnatural amino acids to prepare hitherto novel third-generation tubulysin derivatives. It is anticipated that these new tubulysin derivatives will improve the tetrapeptide's stability, performance during cancer treatment, and 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 dry solvents under an inert environment. For transferring all moisture-sensitive liquids, glass syringes were used under a nitrogen atmosphere. Air and moisture-sensitive materials were also carefully transferred under a nitrogen atmosphere. The reactions were monitored by analytical TLC (thin-layer chromatography) on Merck 60 F254 silica gel plates. TLC plates were analyzed under UV irradiation at 254 nm. UV-inactive compounds were analyzed by staining with potassium permanganate. 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 column chromatography to purify the compounds. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV 500 MHz NMR spectrometer, supported by DST-FIST, Government of India. The NMR samples were prepared in CDCl_3 , DMSO-d_6 , or MeOH-d_4 , and chemical shifts were reported in parts per million (ppm) relative to TMS as an internal reference. High-resolution mass spectra were recorded using Xevo G3 QTOF mass spectrometer.

3.2 Drying of solvents

3.2.1 Drying of DCM

The required amount of DCM was taken in a round-bottom flask, a pinch of CaH_2 was added, and the mixture was refluxed for 1 h. The solvent was distilled off under reflux and collected in a round-bottomed flask containing flame-dried 4 Å 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, and the mixture was stirred for 1 h. The solvent was

distilled under reduced pressure 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 (**2**)

2-Aminothiazole-4-carboxylic acid **1** (250 mg, 1.734 mmol) and sodium carbonate (367 mg, 3.468 mmol) were suspended in dry DMF (3 mL) in a single-neck round-bottom flask (25 mL) fitted with a rubber septum. Using a glass syringe (1 mL), 4-methoxybenzyl chloride (0.46 mL, 3.468 mmol) was added to the reaction mixture dropwise while being continuously stirred. At room temperature under an inert atmosphere, the reaction mixture was stirred for an additional 24 h. After the completion of the reaction (as determined by TLC), the reaction mixture was diluted with EtOAc (1 × 10 mL) and quenched with cold brine (1 × 10 mL). The reaction mixture was further extracted using EtOAc (3 × 25 mL), and the combined organic layer was dried over anhydrous Na₂SO₄, 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 **2** in 60% (548 mg); yield (Scheme 1). Pale yellow solid, *R*_f=0.36 (1:1 hexane-EtOAc); m.p. 202–204 °C; ¹H 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* = 8.6 Hz, 2H), 5.14 (s, 2H), 3.75 (s, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 173.62, 161.28, 159.20, 148.26, 129.91, 128.24, 114.26, 113.90, 65.39, 55.14; IR 2956–2919 (=C–H), 2849 (C–H), 1713 (C=O), 1515–1450 (C=C), 1255–1086 (C–O), 1019 (=C–H bend), 799 (C–N) cm⁻¹; HRMS (+ESI) calculated for [C₁₂H₁₂N₂O₃S + H⁺] 265.0647, found, 265.0624.

3.3.2 General procedure for the synthesis of 4-methoxybenzyl protected thiazole-Fmoc amino acid dipeptide (**4a–d**)

In a single-neck round-bottom flask (50 mL), Fmoc-protected amino acid **3a–d** (1.0 equiv.) was dissolved in dry DCM (3 mL), followed by sequential addition of EDC.HCl (2.0 equiv.), HOBt (1.0 equiv.) and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** (1.0 or 1.2 equiv.). The reaction mixture was stirred for an additional 16 h at room temperature under an inert atmosphere. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with DCM (1 × 10 mL) and quenched with 1N HCl (1 × 10 mL). The aqueous layer was further extracted with DCM (3 × 15 mL). The combined organic layer was washed with brine (5 mL) and dried over anhydrous Na₂SO₄, filtered, concentrated, and purified the crude reaction mixture through silica gel (neutral, 230–400 mesh) column chromatography using hexane-EtOAc solvent mixture as eluent to obtain **4a–d** (Scheme 3).

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

In a single-neck round-bottom flask (25 mL), Fmoc-protected alanine **3a** (294 mg, 0.945 mmol) was dissolved in dry DCM (3 mL). Next, EDC.HCl (362 mg, 1.890 mmol), HOBt (255 mg, 1.890 mmol), and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** (250 mg, 0.945 mmol) were added sequentially into the suspension at room temperature. The reaction mixture was stirred for an additional 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 (10 mL) and quenched with 1N HCl (10 mL). The aqueous layer was further extracted with DCM (3 × 15 mL). The combined organic layer was then dried over anhydrous Na₂SO₄, 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-protected Fmoc-alanine-

thiazole dipeptide **4a** (Scheme 2) in 80% (358 mg) yield (Scheme 3). Colorless gummy liquid, R_f = 0.40 (3:7 hexane-EtOAc); m.p 165–167 °C; ^1H NMR (500 MHz, CDCl_3) δ 11.55 (s, 1H), 7.99 (s, 1H), 7.64 (d, J = 7.6 Hz, 2H), 7.46 (t, J = 6.9 Hz, 2H), 7.31–7.23 (m, 4H), 7.18 (t, J = 7.0 Hz, 2H), 6.81 (d, J = 8.7 Hz, 2H), 5.98 (d, J = 5.8 Hz, 1H), 5.18 (s, 2H), 4.63–4.52 (m, 1H), 4.42–4.34 (m, 2H), 4.11 (t, J = 7.1 Hz, 1H), 3.72 (s, 3H), 1.40 (d, J = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 171.60, 162.40, 161.75, 159.87, 156.44, 144.59, 143.66, 141.36, 130.30, 127.85, 127.65, 127.15, 125.06, 123.37, 120.08, 114.10, 67.51, 66.95, 55.39, 50.70, 47.12, 18.45; IR 3275 (=C–H), 2952 (C–H), 1719 (C=O), 1682 (C=O), 1510 (C=C), 1241 (C–H bend), 1070 (C–O), 739 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{30}\text{H}_{28}\text{N}_3\text{O}_6\text{S} + \text{H}^+]$ 558.1699 found 558.1700.

3.3.2.2 Synthesis of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)butanamido)thiazole-4-carboxylate (**4b**)

In a single-neck round-bottom flask (25 mL), Fmoc-protected-2-aminobutyric acid **3b** (250 mg, 0.768 mmol) was dissolved in dry DCM (3 mL). Next, HATU (577 mg, 1.536 mmol), DIPEA (0.26 mL, 1.536 mmol), and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** (243 mg, 0.922 mmol) were added sequentially into the suspension at room temperature. The reaction mixture was stirred for an additional 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 (10 mL) and quenched with 1N HCl (10 mL). The aqueous layer was further extracted with DCM (3 $\times$ 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-protected Fmoc-aminobutyric-thiazole dipeptide **4b** in 78% (342 mg) yield (Scheme 2). Colorless gummy liquid, R_f = 0.32 (1:3 hexane-EtOAc); m.p 70–72 °C; ^1H NMR (500 MHz, CDCl_3) δ 10.81 (s, 1H), 8.06 (s, 1H), 7.74 (d, J =

7.6 Hz, 2H), 7.56 (t, J = 6.4 Hz, 2H), 7.41–7.33 (m, 4H), 7.28 (t, J = 5.9 Hz, 2H), 6.89 (d, J = 8.7 Hz, 2H), 5.50 (d, J = 7.1 Hz, 1H), 5.26 (s, 2H), 4.51 (d, J = 6.6 Hz, 2H), 4.44 (d, J = 5.2 Hz, 1H), 4.21 (t, J = 6.5 Hz, 1H), 3.81 (s, 3H), 2.03–1.90 (m, 1H), 1.81–1.70 (m, 1H), 0.96 (t, J = 8.7 Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.80, 162.10, 161.80, 159.91, 156.72, 144.79, 143.68, 141.44, 130.31, 127.91, 127.74, 127.20, 125.10, 123.41, 120.14, 114.14, 67.62, 66.94, 56.21, 55.43, 47.21, 25.84, 9.96; IR 3273 (=C–H), 2951 (C–H), 1717 (C=O), 1685 (C=O), 1512 (C=C), 1243 (C–H bend), 1072 (C–O), 740 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{31}\text{H}_{29}\text{N}_3\text{O}_6\text{S} + \text{H}^+]$ 572.1855 found 572.1882.

3.3.2.3 Synthesis of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylbutanamido)thiazole-4-carboxylate (**4c**)

In a single-neck round-bottom flask (25 mL), Fmoc-protected valine **3c** (250 mg, 0.735 mmol) was dissolved in dry DCM (3 mL). Next, HATU (537 mg, 1.470 mmol), DIPEA (0.25 mL, 1.470 mmol), and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** (190 mg, 0.735 mmol) were sequentially added to the suspension at room temperature. The reaction mixture was stirred for an additional 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 (10 mL) and quenched with 1N HCl (10 mL). The aqueous layer was further extracted with DCM (3 $\times$ 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-protected Fmoc-valine-thiazole dipeptide **4c** (Scheme 2) in 80% (345 mg) yield. Colorless gummy liquid, R_f = 0.36 (2:8 hexane-EtOAc); m.p. 69–71 $^\circ\text{C}$; ^1H NMR (500 MHz, CDCl_3) δ 11.46 (s, 1H), 8.01 (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, 120.0, 67.7, 60.4, 53.5, 52.3, 47.2, 31.6, 19.3, 18.3 (*higher intensity signals); IR 3273 (=C–H), 2951 (C–H), 1706 (C=O), 1512 (N–H bend), 1508 (C=C), 1164 (C–H bend), 1076 (C–O), 735 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{32}\text{H}_{31}\text{N}_3\text{O}_6\text{S} + \text{H}^+]$ 586.2006 found 586.2006.

3.3.2.4 Synthesis of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3,3-dimethylbutanamido)thiazole-4-carboxylate (**4d**)

In a single-neck round-bottom flask (25 mL), Fmoc-protected *tert*-leucine **3d** (250 mg, 0.710 mmol) was dissolved in dry DCM (3 mL). Next, EDC.HCl (271 mg, 1.420 mmol), HOBt (191 mg, 1.420 mmol), and 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** (224 mg, 0.850 mmol) were added sequentially into the suspension at room temperature. The reaction mixture was stirred for an additional 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 (10 mL) and quenched with 1N HCl (10 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-protected Fmoc-*tert*-leucine-thiazole dipeptide **4d** in 65% (230 mg) yield (Scheme 2). Colorless gummy liquid, $R_f = 0.43$ (1:3 hexane-EtOAc); m.p 65–68 °C; ^1H NMR (500 MHz, CDCl_3) δ 11.52 (s, 1H), 7.96 (s, 1H), 7.73 (d, $J = 7.6$ Hz, 2H), 7.57 (t, $J = 8.1$ Hz, 2H), 7.38–7.33 (m, 4H), 7.27 (t, $J = 7.5$ Hz, 2H), 6.90 (d, $J = 8.7$ Hz, 2H), 5.88 (d, $J = 10.1$ Hz, 1H), 5.25 (s, 2H), 4.62–4.52 (m, 3H), 4.24 (t, $J = 7.2$ Hz, 1H), 3.81 (s, 3H), 1.02 (s, 9H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.00, 161.82, 161.70, 159.88, 157.02, 144.94, 143.84, 141.44, 130.25, 127.86, 127.79, 127.19,

125.21, 123.17, 120.11, 114.12, 68.02, 66.85, 62.46, 55.42, 47.21, 35.30, 26.48. IR 3273 (=C–H), 2951 (C–H), 1684 (C=O), 1512 (N–H bend), 1506 (C=C), 1156 (C–H bend), 1062 (C–O), 737 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{33}\text{H}_{33}\text{N}_3\text{O}_6\text{S} + \text{Na}^+]$ 622.1988 found 622.1967.

3.3.3 General procedure for the synthesis of Fmoc-protected amino acid-thiazole fragments (**5a–d**)

In a round-bottom flask (25 mL), 4-methoxybenzyl protected thiazole-Fmoc amino acid dipeptide **4a–d** (1.0 equiv.) was dissolved in DCM. Next, a solution of 10% TFA in DCM (3 mL) was added to the reaction mixture at room temperature, and the mixture was 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 into column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain **5a–d** (Scheme 3).

3.3.3.1 Synthesis of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanamido) thiazole-4-carboxylic acid (**5a**)

In a single-neck round-bottom flask (50 mL), 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanamido)thiazole-4-carboxylate **4a** (300 mg, 0.890 mmol) was dissolved in a solution of 10% TFA in DCM (3 mL) 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 into column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain Fmoc-Ala-thiazole fragment **5a** in 98% (218 mg) yield (Scheme 3). White solid, R_f = 0.14 (2:3 hexane-EtOAc); m.p 250–252°C; ^1H NMR (500 MHz, DMSO- d_6) δ 12.63 (s, 1H), 8.06 (s, 1H), 7.89–7.83 (m, 3H), 7.71 (t, J = 8.0 Hz, 2H), 7.41 (t, J = 8.0 Hz, 2H), 7.32 (t, J = 8.7 Hz, 2H), 4.35–4.27 (m, 2H), 4.24–4.19 (m, 2H), 1.32 (d, J = 7.7 Hz, 3H); ^{13}C

NMR (125 MHz, DMSO- d_6) δ 172.78, 163.03, 162.00, 156.05, 144.79, 143.92, 140.87, 127.83, 127.27, 125.40, 123.03, 120.27, 65.88, 50.24, 46.76, 17.49; IR 3273 (=C–H), 2956 (C–H), 1682 (C=O), 1536 (C=O), 1510 (N–H bend), 1243 (C–H bend), 1185 (C–O), 737 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{22}\text{H}_{19}\text{N}_3\text{O}_5\text{S} + \text{H}^+]$ 438.1124 found 438.1122.

3.3.3.2 Synthesis of (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido)thiazole-4-carboxylic acid (**5b**)

In a single-neck round-bottom flask (50 mL), 4-methoxybenzyl (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido)thiazole-4-carboxylate **4b** (0.300 mg, 0.640 mmol) was dissolved in a solution of 10% TFA in DCM (3 mL) 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 into column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain Fmoc-Abu-thiazole fragment **5b** in 92% (218 mg) yield (Scheme 3). White solid, R_f = 0.17 (2:3 hexane-EtOAc); m.p 220–222 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 12.65 (s, 1H), 8.30 (s, 1H), 8.06 (s, 1H), 7.89 (d, J = 7.6 Hz, 2H), 7.83 (d, J = 7.5 Hz, 1H), 7.74 (t, J = 7.2 Hz, 2H), 7.42 (t, J = 7.5 Hz, 2H), 7.34 (t, J = 7.5 Hz, 2H), 4.31–4.27 (m, 2H), 4.24–4.20 (m, 2H), 1.77–1.71 (m, 1H), 1.69–1.63 (m, 1H), 0.91 (t, J = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.21, 163.19, 161.76, 156.39, 144.55, 143.92, 140.87, 127.85, 127.26, 125.44, 120.28, 120.26, 65.92, 56.20, 46.79, 24.82, 10.65; IR 3166 (=C–H), 2956 (C–H), 1682 (C=O), 1536 (C=O), 1510 (N–H bend), 1243 (C–H bend), 1185 (C–O), 737 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{23}\text{H}_{20}\text{N}_3\text{O}_5\text{S} + \text{Na}^+]$ 474.1100 found 452.1119.

3.3.3.3 Synthesis of (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutan amido)thiazole-4-carboxylic acid (5c)

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 **4c** (0.250 mg, 0.427 mmol) was dissolved in a solution of 10% TFA in DCM (3 mL) 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 into column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain Fmoc-Val-thiazole fragment **5c** in 95% (188 mg) yield (Scheme 3). White solid, R_f = 0.16 (2:3 hexane-EtOAc); m.p 213–215 °C; ^1H NMR (500 MHz, DMSO- d_6) 12.67 (s, 1H), 8.07 (s, 1H), 7.87 (d, J = 7.6 Hz, 2H), 7.81 (d, J = 8.3 Hz, 1H), 7.73 (t, J = 7.8 Hz, 2H), 7.40 (t, J = 7.8 Hz, 2H), 7.34–7.28 (m, 2H), 4.32–4.19 (m, 3H), 4.16 (t, J = 7.9 Hz, 1H), 2.10–2.04 (m, 1H), 0.92 (d, J = 7.0 Hz, 3H), 0.89 (d, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 171.75, 162.98, 161.57, 156.45, 144.74, 143.85, 140.80, 127.81, 127.20, 125.45, 123.07, 120.23, 65.94, 60.33, 46.75, 30.13, 19.10, 18.57; IR 3166 (=C–H), 2956 (C–H), 1682 (C=O), 1538 (C=O), 1511 (N–H bend), 1243 (C–H bend), 1185 (C–O), 737 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{24}\text{H}_{23}\text{N}_3\text{O}_5\text{S} + \text{Na}^+]$ 487.1251, found, 487.1426.

3.3.3.4 Synthesis of (S)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethyl butanamido)thiazole-4-carboxylic acid (5d)

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 **4d** (230 mg, 0.479 mmol) was dissolved in a solution of 10% TFA in DCM (3 mL) 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 into column chromatography and purified through silica gel (neutral, 230–400 mesh) using hexane-EtOAc as eluent to obtain Fmoc-protected *tert*-leucine-thiazole fragment **5d** in 72% (140 mg) yield (Scheme 3). White solid, R_f =0.16 (2:3 hexane-EtOAc); m.p 189–191 °C; ^1H °C; ^1H NMR (500 MHz, DMSO- d_6) 12.67 (s, 1H), 8.30 (s, 1H), 8.08 (s, 1H), 7.87 (t, J = 8.2 Hz, 2H), 7.76 (t, J = 7.6 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.32–7.28 (m, 2H), 4.33–4.29 (m, 2H), 4.25–4.21 (m, 2H), 0.99(s, 9H), ^{13}C NMR (125 MHz, DMSO- d_6) δ 170.63, 162.90, 161.28, 156.41, 144.71, 143.81, 140.73, 127.71, 127.08, 125.47, 122.91, 120.13, 79.19, 66.04, 62.46, 59.81, 46.68, 34.11, 26.45; IR 3151 (=C–H), 2958 (C–H), 1682 (C=O), 1538 (C=O), 1510 (N–H bend), 1243 (C–H bend), 1185 (C–O), 737 (=C–H bend) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_{25}\text{H}_{24}\text{N}_3\text{O}_5\text{S} + \text{Na}^+]$ 502.1413, found, 502.1397.

3.3.4 General procedure for the synthesis of N-alkyl pipecolic acids (**8a–c**)

In a double-neck round-bottom flask (25 mL), a hydrogen gas-filled bladder was fitted to one neck of the round-bottom flask through a borosilicate glass heavy-wall glass stop cock adapter. Pipecolic acid **6** (1.0 equiv.) and the corresponding aldehydes or ketones **7a–c** (2.0 equiv.) were added to the round-bottom flask, and the reactants were dissolved in methanol (2 mL). 10% Pd/C (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 expelled through a syringe needle inserted through the rubber septum, connected to a filtration pump via a silicone tube, for 2 minutes or until the solvent started bubbling. The reaction flask 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 an 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 (3×5 mL), and the filtrate was concentrated under reduced pressure to obtain N-alkyl pipecolic acids **8a–c** (Scheme 4)

3.3.4.1 Synthesis of N-methyl pipecolic acid **8a**

In a double-neck round-bottom flask (25 mL), a hydrogen gas-filled bladder was fitted to one neck of the round-bottom flask through a borosilicate glass heavy-wall glass stop cock adapter. Pipecolic acid **6** (200 mg, 1.548 mmol) and 37% formaldehyde in H₂O **7a** (0.227 mL, 3.09 mmol) were added to the round-bottom flask and dissolved in MeOH (2 mL). 10% Pd/C (33 mg, 0.309 mmol) was added slowly to the mixture, and the reaction mixture was sealed using a rubber septum. The residual air in the reaction vessel was expelled through a syringe needle inserted through the rubber septum, connected to a filtration pump via a silicone tube, for 2 minutes or until the solvent started 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 an 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 yield **8a** (99%, 222 mg) (Scheme 4). White solid; $R_f = 0.21$ (MeOH/DCM) 1:1; m.p 101–103 °C; ¹H NMR (500 MHz, MeOH-d₄) 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); ¹³C NMR (125 MHz, MeOH-d₄) δ 173.5, 70.4, 55.3, 43.2, 29.5, 24.1, 22.6; IR 2938 (O–H), 2861 (C–H) 1608 (C=O), 1320 (C–C), 1211 (C–O), 1008 (C–N) cm⁻¹; HRMS (+ESI) m/z calculated. for C₇H₁₃NO₂ [M+H]⁺, 144.1019, found, 144.1096.

3.3.4.2 Synthesis of N-ethyl pipecolic acid **8b**

In a double-neck round-bottom flask (25 mL), a hydrogen gas-filled bladder was fitted to one neck of the round-bottom flask through a borosilicate glass heavy-wall glass stop cork adapter. Pipecolic acid **6** (200 mg, 1.548 mmol) and 25% acetaldehyde in H₂O **7b** (0.52 mL, 2.322 mmol) were added to the round-bottom flask and dissolved in MeOH (2 mL). 10% Pd/C (34 mg, 0.232 mmol) was added slowly to the mixture, and the reaction mixture was sealed using a rubber septum. The residual air in the reaction vessel was expelled through a syringe needle inserted through the rubber septum, connected to a filtration pump via a silicone tube, for 2 minutes or until the solution began to bubble. 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 an 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 **8b** in 95% (173 mg) yield (Scheme 4). Yellowish green, R_f =0.25 (1:1 MeOH-DCM); m.p 98–100 °C; IR 2936 (O–H), 2862 (C–H), 1606 (C=O), 1317 (C–C), 1173 (C–O), 1004 (C–N) cm⁻¹; ¹H NMR (500 MHz, MeOH-d₄) δ 3.62–3.54 (m, 1H), 3.40–3.34 (m, 1H), 3.15–3.04 (m, 1H), 2.92 (t, J = 10.9 Hz, 1H), 2.27–2.16 (m, 1H), 1.92–1.70 (m, 5H), 1.60–1.50 (m, 1H), 1.33 (t, J = 7.4 Hz 3H); ¹³C NMR (125 MHz, MeOH-d₄) δ 173.73, 69.03, 51.47, 23.71, 22.76, 20.98, 13.88, 9.63; HRMS (+ESI) calculated for [C₈H₁₅NO₂ + H⁺] 158.1181, found, 158.1152.

3.3.4.3 Synthesis of N-isopropyl pipecolic acid **8c**

In a double-neck round-bottom flask (25 mL), a hydrogen gas-filled bladder was fitted to one neck of the round-bottom flask through a borosilicate glass heavy-wall glass stop cock adapter. Pipecolic acid **6** (200 mg, 1.548 mmol) and acetone **7c** (0.228 mL, 3.096 mmol) were added to the round-bottom flask and dissolved in MeOH (2 mL). 10% Pd/C (32 mg, 0.309 mmol) was added slowly to the mixture, and the reaction mixture was sealed using a rubber septum. The residual air in the reaction vessel was expelled through a syringe needle inserted through the rubber septum, connected to a filtration pump via a silicone tube, for 2 minutes or until the solvent began to bubble. 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 an 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 **8c** in 92% (144 mg) yield (Scheme 4). Green solid, R_f =0.27 (1:1 MeOH-DCM); m.p 90–92 °C; IR 2935 (O–H), 2862 (C–H), 1605 (C=O), 1318 (C–C), 1175 (C–O), 1002 (C–N) cm^{-1} ; HRMS (+ESI) calculated for $[\text{C}_8\text{H}_{15}\text{NO}_2 + \text{H}^+]$ 172.1338, found, 172.1338.

3.4 Solid-phase peptide synthesis of novel third-generation tubulin inhibitors Tub_01 to Tub_06

3.4.1 General procedure for solid-phase peptide synthesis

3.4.1.1 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 the peptide vessel. After draining the DCM, the beads were again

swelled with DMF (5 mL) for 15 minutes, and the process was repeated three times.

3.4.1.2 General procedure for the Kaiser test

A few resin beads from the peptide vessel were taken 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 the mixture was 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 dark blue colour designates the absence of the amine group. The test was performed after coupling each amino acid and after deprotection of the Fmoc group at each step.

3.4.1.3 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.

3.4.1.4 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 dried under nitrogen, and the crude product was further purified by RP-HPLC.

3.4.1.5 General procedure for RP-HPLC

The synthesised tubulin inhibitors **Tub_01** to **Tub_06** were purified using a Buchi Reveleris Prep RP-HPLC system. The crude tubulin inhibitors were dissolved in a mixture of CH₃CN: H₂O (1:1, 1 mL) and injected into the sample injector for elution using RP-PFP (Reverse Phase PentafluoroPhenyl) preparative column (XSelect CSH Prep Fluorophenyl 5 μ m; 19 $\times$ 150 mm). A flow rate of 10 mL/min (mobile phase, A = 0.1% TFA in H₂O and B = acetonitrile) is maintained throughout the run, and the mobile phase gradient was gradually changed from 1% B (v/v) to 35% B (v/v) over 15 minutes. The mobile phase gradient was further increased to 50% B (v/v) in 8 minutes, then to 70% B (v/v) in an additional 2 minutes, and finally to 100% B (v/v) over the next 3 minutes. The chromatograms were recorded at λ = 210, 240, and 280 nm. Pure fractions of tubulin inhibitors were collected using an automatic fraction collector, the organic solvent was evaporated under reduced pressure using a rotary evaporator, and the aqueous solution was lyophilised for 48 h to yield tubulin inhibitors **Tub_01** to **Tub_06**.

3.4.2 SPPS of novel third-generation tubulin inhibitors (**Tub_01** to **Tub_06**)

3.4.2.1 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_01**) incorporating Alanine-thiazole fragment

H-Glu(OtBu)-2-Cl-Trt resin **9** (200 mg, 0.096 mmol) was initially swelled in DCM (1 $\times$ 5 mL), followed by DMF (1 $\times$ 5 mL) for 15 min each. The solvent was drained using a filtration pump, and the beads were dried until freely flowing. Fmoc-Ala-thiazole-OH **5a** (168 mg, 0.384 mmol), PyBOP (133 mg, 0.256 mmol), DIPEA (0.223 mL, 0.128 mmol) were dissolved in DMF (0.5 mL) 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) and isopropanol (3×5 mL) for 3 min each. The solvent was drained using a filtration pump, and the 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 to obtain tripeptide **10**. Next, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL) to the resin beads for 10 min each. The resin beads were then washed with DMF (3×5 mL) and then with 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. The next amino acid, Fmoc-(*S*)-Ile-OH (91 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 0.128 mmol) were dissolved in DMF (0.5 mL) in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, and added to the peptide vessel containing resin beads and the coupling reaction was continued for 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **11**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-(*R*)-methyl pipecolic acid **8a** (37 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 0.128 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was added to the growing peptide to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O (9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_01** as a white solid, washed with ice-cold diethyl ether (3×5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product **Tub_01** obtained is a white solid with a yield of 40% (30 mg); ^1H NMR (500 MHz, DMSO- d_6) δ 12.42 (s, 1H), 9.56 (s, 1H), 8.72–8.85 (m, 2H), 8.52 (d, $J = 5.8$ Hz, 1H), 8.10 (s, 1H), 4.42 (t, $J = 6.2$ Hz, 1H), 4.33–4.26 (m, 1H), 4.42 (t, $J = 8.1$ Hz, 1H), 3.03–2.88 (m, 2H), 2.61 (s, 3H), 2.47–2.45 (m, 2H), 2.33–2.25 (m, 2H), 2.08–1.93 (m, 2H), 1.91–1.80 (m, 1H), 1.76–1.70 (m, 2H), 1.67–1.47 (m, 2H), 1.42–1.32 (m, 2H), 1.28 (d, 3H), 1.12–1.00 (m, 1H), 0.84 (d, 3H), 0.78 (t, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 174.17, 173.53, 172.03, 170.88, 168.03, 161.36, 160.83, 140.38, 127.40, 66.26, 57.22, 54.76, 52.02, 48.88, 41.88, 36.91, 30.56, 28.73, 26.18, 24.29, 22.59, 21.17, 17.45, 15.51, 11.17; HRMS (+ESI) calculated for $[\text{C}_{28}\text{H}_{44}\text{N}_6\text{O}_8\text{S}+\text{H}^+]$ 583.2550, found, 583.2532

3.4.2.2 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_02**) incorporating Abu-thiazole fragment

H-Glu(OtBu)-2-Cl-Trt resin **9** (200 mg, 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-Abu-thiazole-OH **5b** (174 mg, 0.384 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 0.128 mmol) were dissolved in DMF (0.5 mL) 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) and isopropanol (3×5 mL) for 10 min each. The solvent was drained using a filtration pump, and the 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 to obtain tripeptide **13**. Next, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL) to the resin beads for 10 min each. The resin beads were then washed with DMF (3×5 mL) and then with 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. The next amino acid, Fmoc-(*S*)-Ile-OH (91 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 0.128 mmol) were dissolved in DMF (0.5 mL) in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, and added to the peptide vessel containing resin beads, and the coupling reaction was continued for 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **14**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-Methyl-(*R*)-pipecolic acid **8a** (37 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 0.128 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was added to the growing peptide to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O

(9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_02** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained **Tub_02** is a white solid with a yield of 32% (24 mg); ¹H NMR (500 MHz, DMSO-d₆) δ 12.46 (s, 1H), 9.60 (s, 1H), 8.69 (d, *J* = 8.3 Hz, 1H), 8.64 (d, *J* = 7.6 Hz, 1H), 8.41 (d, *J* = 6.8 Hz, 1H), 8.11 (s, 1H), 4.39–4.34 (m, 1H), 4.32–4.27 (m, 1H), 4.21 (t, *J* = 7.6 Hz, 1H), 3.01–2.91 (m, 1H), 2.61 (s, 3H), 2.48–2.44 (m, 1H), 2.33–2.26 (m, 2H), 2.07–1.92 (m, 2H), 1.91–1.81 (m, 1H), 1.77–1.68 (m, 4H), 1.66–1.46 (m, 3H), 1.41–1.32 (m, 2H), 1.11–1.01 (m, 1H), 0.89–0.80 (m, 6H), 0.77 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (125 MHz, DMSO-d₆) δ 174.10, 173.48, 171.36, 171.17, 167.98, 161.30, 160.65, 140.36, 127.36, 66.24, 57.33, 54.71, 54.47, 51.99, 41.75, 36.69, 30.53, 28.67, 26.16, 24.86, 24.35, 22.55, 21.15, 15.48, 11.07, 10.49; HRMS (+ESI) calculated for [C₂₆H₄₀N₆O₈S+H⁺] 597.2706, found, 597.2683.

3.4.2.3 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_03**) incorporating *tert*-leucine-thiazole fragment

H-Glu(OtBu)-2-Cl-Trt resin **9** (150 mg, 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-*tert*-Leu-thiazole-OH **5d** (93 mg, 0.192 mmol), PyBOP (100 mg, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) were dissolved in DMF (0.5 mL) 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) and isopropanol (3×5 mL) for 10 min each. The solvent was drained using a filtration pump, and the 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 to obtain tripeptide **16**. Next, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL) to the resin beads for 10 min each. The resin beads were then washed with DMF (3×5 mL) and then with 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. The next amino acid, Fmoc-(*S*)-*tert*-Leu-OH (68 mg, 0.192 mmol), PyBOP (100 mg, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol) were dissolved in DMF (0.5 mL) in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, and added to the peptide vessel containing resin beads, and the coupling reaction was continued for 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **17**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-Methyl-(*R*)-pipecolic acid **8a** (28 mg, 0.019 mmol), PyBOP (100 mg, 0.192 mmol), and DIPEA (0.168 mL, 0.96 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was added to the growing peptide to accomplish the amide coupling,

followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O (9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_03** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained **Tub_03** is a white solid with a yield of 30% (18 mg); ¹H NMR (500 MHz, DMSO-d₆) δ 12.47 (s, 1H), 9.59 (s, 1H), 8.63 (d, *J* = 7.6 Hz, 1H), 8.58 (d, *J* = 8.5 Hz, 1H), 8.12 (s, 1H), 8.05 (d, *J* = 7.4, 1 H), 4.45–4.38 (m, 2H), 4.33–4.27 (m, 1H), 3.01–2.91 (m, 1H), 2.58 (s, 3H), 2.48–2.44 (m, 2H), 2.33–2.26 (m, 2H), 2.05–1.96 (m, 2H), 1.91–1.81 (m, 1H), 1.77–1.68 (m, 2H), 1.65–1.48 (m, 2H), 1.44–1.32 (m, 1H), 0.92 (s, 9H), 0.86 (s, 9H); ¹³C NMR (125 MHz, DMSO-d₆) δ 174.08, 173.46, 170.23, 170.04, 167.98, 161.29, 160.23, 140.42, 127.21, 66.18, 60.66, 60.12, 54.71, 51.94, 41.63, 34.46, 34.15, 30.51, 28.83, 26.64, 26.48, 26.13, 22.52, 21.20; HRMS (+ESI) calculated for [C₂₈H₄₄N₆O₈S+H⁺] 625.3019, found 625.3026.

3.4.2.4 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_04**) incorporating valine-thiazole fragment

H-Glu(OtBu)-2-Cl-Trt resin **9** (200 mg, 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-valine-thiazole-OH **5c** (119 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol) were dissolved in DMF (0.5 mL) 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 the 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 to obtain tripeptide **19**. Next, the FmocNH protecting group was cleaved by the 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. The next amino acid, Fmoc-(*S*)-Ile-OH (90 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol) were dissolved in DMF (0.5 mL) 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 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **20**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-Isopropyl-(*R*)-pipecolic acid **8c** (43 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was added to the

growing peptide to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O (9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_04** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained **Tub_04** is a white solid with a yield of 31% (20 mg); ¹H NMR (500 MHz, DMSO-d₆) δ 12.51 (s, 1H), 8.65 (m, 2H), 8.17 (d, *J* = 7.5 Hz, 1H), 8.16 (s, 1H), 4.47–4.38 (m, 2H), 4.34–4.28 (m, 2H), 2.97–2.82 (m, 2H), 2.61 (s, 2H), 2.34 (t, *J* = 7.2 Hz, 2H), 2.11–2.02 (m, 3H), 1.94–1.85 (m, 2H), 1.81–1.71 (m, 4H), 1.44–1.35 (m, 2H), 1.24 (d, *J* = 13.0 Hz, 1H), 1.16 (d, *J* = 13.0 Hz, 1H), 1.12–1.03 (m, 1H), 0.96–0.85 (m, 8H), 0.84–0.75 (m, 7H); ¹³C NMR (125 MHz, DMSO-d₆) δ *173.93, 173.34, 171.16, 170.77, *161.09, 140.26, 127.31, 58.17, 57.11, *51.83, 36.53, 36.36, *30.42, 30.24, 28.80, 26.07, 24.30, 19.02, 18.59, 18.36, 18.12, 15.37, 14.28, 10.90 (*higher intensity signals); HRMS (+ESI) calculated for [C₂₉H₄₆N₆O₈S+H⁺] 639.3176, found 639.3163.

3.4.2.5 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_05**) incorporating Asn(Trt) resin

Asn(Trt) resin **22** (200 mg, 0.116 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-tert-leu-thiazole **5d** (111 mg, 0.232 mmol), PyBOP (120 mg, 0.232 mmol), and DIPEA (0.202 mL, 1.160 mmol) were dissolved in DMF (0.5 mL) 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) and isopropanol (3×5 mL) for 10 min each. The solvent was drained using a filtration pump, and the 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 to obtain tripeptide **23**. Next, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL) to the resin beads for 10 min each. The resin beads were then washed with DMF (3×5 mL) and then with 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. The next amino acid, Fmoc-(*S*)-Ile-OH (82 mg, 0.232 mmol), PyBOP (120 mg, 0.232 mmol), and DIPEA (0.202 mL, 1.160 mmol) were dissolved in DMF (0.5 mL) in a 2 mL glass vial, mixed thoroughly using a Pasteur pipette, and added to the peptide vessel containing resin beads and the coupling reaction was continued for 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **24**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-methyl-(*R*)-pipecolic acid **8a** (33 mg, 0.232 mmol), PyBOP (120 mg, 0.232 mmol), and DIPEA (0.202 mL, 1.160 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was

added to the growing peptide to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O (9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_05** as a white solid, washed with ice-cold diethyl ether (3 × 5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained **Tub_05** is a white solid with a yield of 33% (21 mg); ¹H NMR (500 MHz, DMSO-d₆) δ 12.5 (s, 1H), 8.75 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 7.5 Hz, 1H), 8.10 (s, 1H), 6.56 (s, 1H), 4.66–4.63 (m, 1H), 4.52 (d, *J* = 8.2 Hz, 1H), 4.38 (t, *J* = 8.0 Hz, 1H), 2.82 (d, *J* = 6.2 Hz, 1H), 2.79 (d, *J* = 6.2 Hz, 1H), 2.64 (d, *J* = 8.0 Hz, 1H), 2.57 (s, 2H), 2.03–1.90 (m, 1H), 1.80–1.69 (m, 3H), 1.67–1.59 (m, 1H), 1.58–1.50 (m, 1H), 1.44–1.31 (m, 2H), 1.27–1.18 (m, 1H), 1.13–1.03 (m, 2H), 0.96 (s, 9H), 0.84–0.72 (m, 6H); HRMS (+ESI) calculated for [C₂₇H₄₄N₆O₈S+H⁺] 610.3023, found 610.3028

3.4.2.6 Solid phase peptide synthesis of third-generation tubulin inhibitor (**Tub_06**) incorporating Fmoc-(R)-Isoleucine

H-Glu(OtBu)-2-Cl-Trt resin **9** (200 mg, 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-tert-leu-thiazole **5d** (122 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol) were dissolved in DMF (0.5 mL) 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) and isopropanol (3×5 mL) for 10 min each. The solvent was drained using a filtration pump, and the 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 to obtain tripeptide **16**. Next, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL) to the resin beads for 10 min each. The resin beads were then washed with DMF (3×5 mL) and then with 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. The next amino acid, Fmoc-(*R*)-Ile-OH (82 mg, 0.232 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol) were dissolved in DMF (0.5 mL) 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 6 h. The resin beads were then again 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 the 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 to obtain tetrapeptide **26**. Using the same protocol described above, the FmocNH protecting group was cleaved by adding 20% piperidine in DMF (1×4 mL; 2×3 mL). The resin beads were then again 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 the beads were dried until freely flowing by bubbling N_2 gas through the peptide vessel. The Kaiser test was performed to confirm the deprotection of the Fmoc group. Finally, a mixture of *N*-methyl-(*R*)-pipecolic acid **8a** (36 mg, 0.256 mmol), PyBOP (133 mg, 0.256 mmol), and DIPEA (0.223 mL, 1.280 mmol), dissolved in DMF (0.5 mL) in a 2 mL glass vial, was added to the growing peptide to accomplish the amide coupling, followed by acid cleavage using a cocktail composed of TFA:TIPS:H₂O

(9.5:0.25:0.25) (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 steel needle through the rubber septum. Ice-cold diethyl ether was added to precipitate the tubulin inhibitor **Tub_06** as a white solid, washed with ice-cold diethyl ether (3×5 mL), 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 RP-HPLC, acetonitrile was removed under reduced pressure, and the collected pure fractions were freeze-dried in a lyophilizer. The product obtained **Tub_06** is a white solid with a yield of 40% (40 mg); ^1H NMR (500 MHz, DMSO- d_6) δ 12.53 (s, 1H), 8.75 (d, $J = 8.0$ Hz, 1H), 8.65 (d, $J = 8.0$ Hz, 1H), 8.23–8.15 (m, 2H), 4.65 (d, $J = 8.2$ Hz, 1H), 4.38–4.30 (m, 2H), 3.89 (d, $J = 5.2$ Hz, 1H), 2.63 (s, 1H), 2.50 (s, 3H), 2.34 (t, $J = 7.5$ Hz, 3H), 2.14–2.00 (m, 1H), 1.95–1.80 (m, 3H), 1.54–1.45 (m, 1H), 1.19–1.05 (m, 2H), 1.00–0.91 (m, 15H), 0.88–0.84 (m, 4H); ^{13}C NMR (125 MHz, DMSO- d_6) δ *174.26, 173.66, 169.92, 169.06, 161.41, 160.20, 140.49, 127.94, 60.42, 56.96, *52.17, 37.57, 36.94, 34.82, 34.47, 30.75, 26.81, 26.76, 26.40, 24.04, 15.90, 15.29, 11.68, 11.49 (*higher intensity signals); HRMS (+ESI) calculated for $[\text{C}_{28}\text{H}_{44}\text{N}_6\text{O}_8\text{S} + \text{H}^+]$ 625.3019, found 625.3026

3.5 In vitro cytotoxic study

3.5.1 Culture of cancer cell lines

MCF7 and A431 cancer cell lines were grown in a T-25 Flask containing sterile-filtered RPMI 1640 medium, supplemented with foetal bovine serum, sodium pyruvate (100 mM), non-essential amino acids, and 1% penicillin streptomycin under 37 °C and 5% CO₂ 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 the cytotoxicity assay.

3.5.2 In vitro cytotoxicity assay of tubulin inhibitors Tub_01, Tub_02, Tub_03 and Tub_09 against A431 cell line (skin cancer)

5000 A431 cells/well were seeded in a 96-well plate and allowed to form a monolayer at 60% confluency over 48 h. The spent medium was then discarded, and cells were washed with 1X PBS ($1 \times 200 \mu\text{L}$). 200 μL of fresh medium containing tubulin inhibitors **Tub_01**, **Tub_02**, **Tub_03** and **Tub_09** at various concentrations (100 μM to 10 pM) was then added to 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 ($2 \times 200 \mu\text{L}$). 50 μL of MTT solution (5 mg/mL) was added to each well, plates were then carefully wrapped with aluminum foil and incubated at 37 °C and 5% CO₂ for 4 h. After incubation, the MTT solution was carefully aspirated using a pipette, and 200 μL of DMSO per well was added to dissolve the formazan crystals. The absorbance of each well containing live cells was measured using the Synergy H4 multiplate reader at 570 nm. Dose vs response curves were obtained from a plot of log[concentration] vs % cell viability, and IC₅₀ values were calculated using GraphPad Prism 8.01.

3.5.3 In vitro cytotoxicity assay of tubulin inhibitors Tub_07, Tub_08, Tub_09 and Tub_10 against MCF7 cell line (breast cancer)

5000 MCF7 cells/well were seeded in a 96-well plate and allowed to form a monolayer at 60% confluency over 48 h. The spent medium was then discarded, and cells were washed with 1X PBS ($1 \times 200 \mu\text{L}$). 200 μL of fresh medium containing tubulin inhibitors **Tub_07**, **Tub_08**, **Tub_09** and **Tub_10** at various concentrations (100 μM to 10 pM) was then added to 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 ($2 \times 200 \mu\text{L}$). 50 μL of MTT solution (5 mg/mL) was added to each well, plates were then carefully wrapped with aluminum foil and incubated at 37 °C and 5% CO₂ for 4

h. After incubation, the MTT solution was carefully aspirated using a pipette and 200 μ L of DMSO per well was added to dissolve the formazan crystals. The absorbance of each well containing live cells was measured using the Synergy H4 multiplate reader at 570 nm. Dose vs response curves were obtained from a plot of log[concentration] vs % cell viability, and IC₅₀ values were calculated using GraphPad Prism 8.01.

3.6. Synthesis of benzyl-2-aminooxazole 4-carboxylate **32**

3.6.1 Optimization of synthesis of benzyl-2-aminooxazole 4-carboxylate **32**

During the exploration of the synthesis of benzyl 2-aminooxazole-4-carboxylate **32** via transesterification of methyl 2-aminooxazole-4-carboxylate **31** (1.0 equiv.) with benzyl alcohol **29** (30.0 equiv.) in the presence of TsOH (0.1 equiv.) at 90 °C for 12 h, no formation of the desired product was observed (Entry 1). Under identical conditions of reactants and catalyst, at an elevated temperature of 120 °C, the reaction failed to afford **32** with decomposition of the methyl-aminooxazole-carboxylate **31** (Entry 2). When the catalyst, TsOH, was replaced by an organic base, triethylamine, at 90 °C for 24 h, **32** was obtained in 7% yield (Entry 3). Increasing the temperature from 90 °C to 120 °C resulted in the formation of a trace amount of **32** with decomposition of reactants (Entry 4). Switching from organic to inorganic base, when the reaction was carried out between methyl 2-aminooxazole-4-carboxylate **31** (1.0 equiv.) and benzyl alcohol **29** (50.0 equiv.) in the presence of potassium bicarbonate (2.0 equiv.) at 100 °C for 6 h, the yield of **32** was improved to 40% (Entry 5). Keeping all other reaction conditions unchanged, performing the reaction in a sealed tube resulted in an improved yield of 46% (Entry 6). Reducing the equivalents of benzyl alcohol from 50.0 to 25.0 and increasing the reaction time to 48 h further enhanced the yield to 58% (Entry 7). The optimal condition was obtained when the reaction was carried out in a sealed tube at 100 °C for 9 h, resulting in a further increase in yield to 64% (Entry 8). Increasing the base equivalent from 2.0 to 4.0 and 6.0 did not enhance

the reaction yield; rather, a decline in yield to 59–61% was observed (Entries 9–10). Furthermore, the use of alternative inorganic bases, such as sodium bicarbonate, sodium carbonate, and potassium carbonate, resulted in a significant reduction in yield to 8–10% (Entries 11–13). Using an organic base such as DIPEA resulted in a lower yield of 10% (Entry 14), whereas a strong base, DBU (Entry 15), resulted in no product formation. Overall, potassium bicarbonate (2.0 equiv.) in a sealed tube at 100 °C for 9 h was identified as the optimal condition, affording the desired benzyl-2-aminoxazole-4-carboxylate **32** in 64% yield.

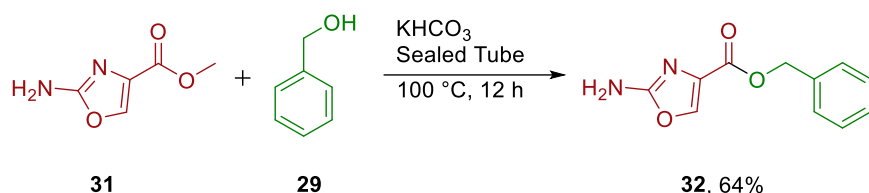


Table 1. Optimization of reaction conditions for the synthesis of benzyl-2-aminoxazole -4- carboxylate **32**

S.N	31 (equiv.)	29 (equiv.)	Reagent (equiv.)	Temperature	Reaction Time	32 Yield
1.	1.0	30.0	TsOH (0.1)	90 °C	12 h	NP
2.	1.0	30.0	TsOH (0.1)	120 °C	12 h	NP
3.	1.0	25.0	Et ₃ N (5.0)	90 °C	24 h	7%
4.	1.0	25.0	Et ₃ N (5.0)	120 °C	24 h	Trace
5.	1.0	50.0	KHCO ₃ (2.0)	100 °C	6 h	40%
6.	1.0	50.0	KHCO ₃ (2.0)	100 °C/ Sealed tube	6 h	46%
7.	1.0	25.0	KHCO ₃ (2.0)	100 °C	48 h	58%
8.	1.0	25.0	KHCO ₃ (2.0)	100 °C/ Sealed tube	12 h	64%
9.	1.0	25.0	KHCO ₃ (4.0)	100 °C/ Sealed tube	12 h	59%
10.	1.0	25.0	KHCO ₃ (6.0)	100 °C/ Sealed tube	12 h	61%

11.	1.0	25.0	NaHCO ₃ (2.0)	100 °C/ Sealed tube	24 h	11%
12.	1.0	25.0	Na ₂ CO ₃ (2.0)	100 °C/ Sealed tube	24 h	8%
13.	1.0	25.0	K ₂ CO ₃ (2.0)	100 °C/ Sealed tube	24 h	10%
14.	1.0	25.0	DIPEA (2.0)	100 °C/ Sealed tube	48 h	10%
15	1.0	25.0	DBU (2.0)	100 °C/ Sealed tube	24 h	NR

NP = No Product, NR = No reaction

3.6.2 Procedure for the synthesis of benzyl-2-aminooxazole-4-carboxylate (**32**)

Methyl-2-aminooxazole-4-carboxylate **31** (100 mg, 0.700 mmol) and sodium bicarbonate (140 mg, 1.400 mmol) were suspended in benzyl alcohol **29** (25.0 equiv.) in a sealed tube (15 mL) at room temperature. The reaction mixture was then transferred directly to a preheated silicon oil bath at 100 °C and stirred for 9 h. After the completion of the reaction (as monitored by TLC), the reaction mixture was diluted with EtOAc (1 × 10 mL) and quenched with distilled water (1 × 10 mL). The reaction mixture was further extracted using EtOAc (3 × 25 mL), and the combined organic layer was dried over anhydrous Na₂SO₄, 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 **32** in 64% (98 mg); yield (Scheme 11). Pale yellow solid, *R_f* = 0.34 (1:1 hexane-EtOAc); m.p. 183–185 °C; ¹H NMR (500 MHz, CDCl₃) δ 7.62 (s, 1H), 7.33–7.27 (m, 5H), 5.71 (brs, 2H), 5.19 (s, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 161.59, 161.21, 137.79, 135.64, 132.34, 128.74, 128.62, 66.72; IR 3362 (NH₂), 3166 (=C–H), 2956 (C–H), 1721 (C=O, ester), 1602 (C=N/NH₂ bend), 1511 (aromatic C=C), 1243 (C–O), 1185 (C–O–C), 737 (=C–H bend) cm⁻¹; HRMS (+ESI) calculated for [C₁₁H₁₀N₂O₃ + H⁺] 219.0770, found, 219.0774.

3.6.3 Synthesis of benzyl (S)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)oxazole-4-carboxylate (**33**)

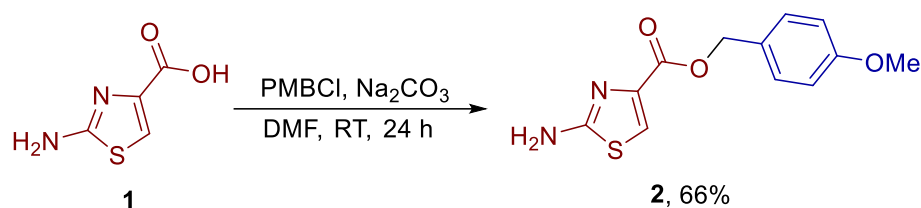
In a single-neck round-bottom flask (25 mL), Fmoc-protected valine **3c** (100 mg, 0.458 mmol) was dissolved in dry DCM (3 mL). Next, HATU (316 mg, 0.832 mmol), DMAP (50 mg, 0.416 mmol), and benzyl-2-aminooxazole-4-carboxylate **32** (140 mg, 0.458 mmol) were sequentially added to the suspension at room temperature. The reaction mixture was stirred for an additional 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 (10 mL) and quenched with 1N HCl (10 mL). The aqueous layer was further extracted with DCM (3 × 15 mL). The combined organic layer was then dried over anhydrous Na₂SO₄, filtered, concentrated, and purified through silica gel (neutral 230–400 mesh) column chromatography using hexane-EtOAc solvent mixture as eluent to obtain the intermediate benzyl-protected Fmoc-Valine-thiazole dipeptide **33** in 60% (130 mg) yield (Scheme 12). Colorless gummy liquid, *R_f*=0.36(2:5 hexane-EtOAc); m.p 156–158 °C; ¹H NMR (500 MHz, CDCl₃) δ 10.44 (s, 1H), 7.95 (s, 1H), 7.71 (d, *J* = 7.5 Hz 2H), 7.51 (t, *J* = 7.8 Hz 2H), 7.36–7.30 (m, 7H), 7.24 (t, *J* = 7.5 Hz 2H), 6.05 (d, *J* = 17.6 Hz 1H), 5.28 (s, 2H), 4.51–4.39 (m, 2H), 4.34 (t, *J* = 7.2 Hz, 1H), 4.12 (q, *J* = 7.2, 1H), 2.26–2.17 (m, 1H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.94 (d, *J* = 6.9 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 160.94, 156.96, 153.61, 143.84, 141.38, 140.83, 135.29, 132.18, 128.73, 128.70, 127.83, 127.20, 125.13, 120.07, 67.45, 67.14, 60.55, 47.16, 31.08, 21.20, 19.35, 17.80, 14.33; IR 3340 (N–H), 3060 (=C–H), 2960 (C–H), 1735 (C=O), 1692 (C=O), 1655 (C=O), 1600 (C=C), 1515 (N–H bend), 1240 (C–H bend), 1175 (C–O), 740 (=C–H bend) cm⁻¹; HRMS (+ESI) calculated for [C₃₁H₂₉N₃O₆ + H⁺] 540.2134 found 540.2115.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Synthesis of PMB-protected 2-aminothiazole-4-carboxylic acid (2)

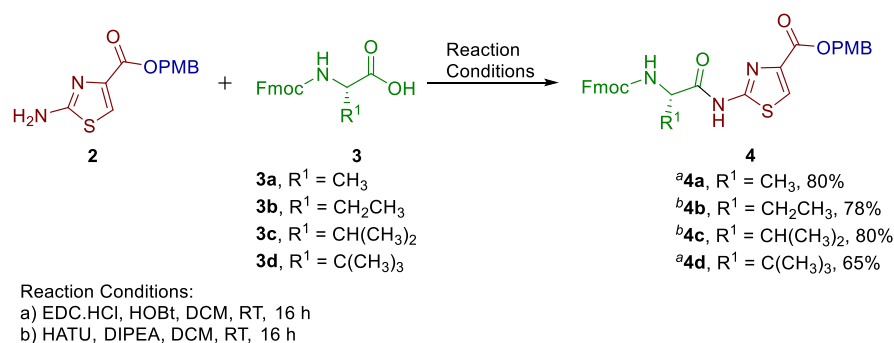
The carboxylic acid group of 2-aminothiazole-4-carboxylic acid **1** was protected with the acid-labile 4-methoxybenzyl (PMB) group. This protection was carried out by reacting 2-aminothiazole-4-carboxylic acid with 4-methoxybenzyl chloride (PMBCl) at room temperature in the presence of the inorganic base Na₂CO₃, using the polar aprotic solvent dry DMF under an inert atmosphere. The reaction was carried out for 24 h to afford 4-methoxybenzyl-2-aminothiazole-4-carboxylate **1** (Scheme 1).



Scheme 1. Synthesis of PMB-protected 2-aminothiazole-4-carboxylic acid **2**

4.2 Synthesis of Fmoc amino acid-PMB-protected thiazole dipeptides (4a–d)

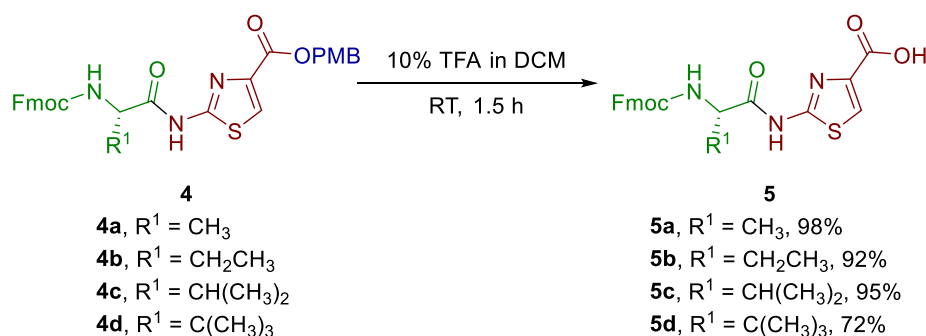
The free amine group present in the 4-methoxybenzyl-2-aminothiazole-4-carboxylate **2** was coupled with the carboxylic acid group of Fmoc-protected amino acid **3a–d** via the formation of an amide bond to obtain the desired Fmoc amino acid-PMB-protected thiazole dipeptides **4a–d**. The reaction was carried out using the coupling agent EDC.HCl along with HOBT, or HATU in the presence of DIPEA, in dry DCM for 16 h (Scheme 2).



Scheme 2. Synthesis of PMB-protected thiazole-Fmoc amino acid dipeptides **4a–d**

4.3 Synthesis of Fmoc-protected amino acid-thiazole fragments (**5a–d**)

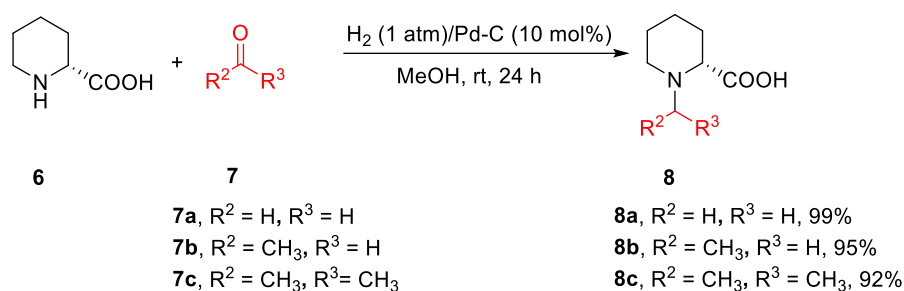
The acid-labile 4-methoxybenzyl (PMB) group of the intermediates **4a–d** was deprotected using 10% TFA in DCM at room temperature. The reaction was carried out for 1.5 h to afford Fmoc-protected amino acid-thiazole fragments **5a–d** (Scheme 3).



Scheme 3. Synthesis of Fmoc-protected amino acid-thiazole fragments **5a–d**

4.4 Synthesis of pipecolic acid derivatives (**8a–c**)

Pipecolic acid **6** was reacted with carbonyl compounds **7a–c** at room temperature under the reductive environment of hydrogen (H₂) and using the Pd/C (10% w/w) catalyst in polar protic solvent methanol. The reaction was carried out for 24 h to obtain our desired pipecolic acid derivatives **8a–c** (Scheme 4).

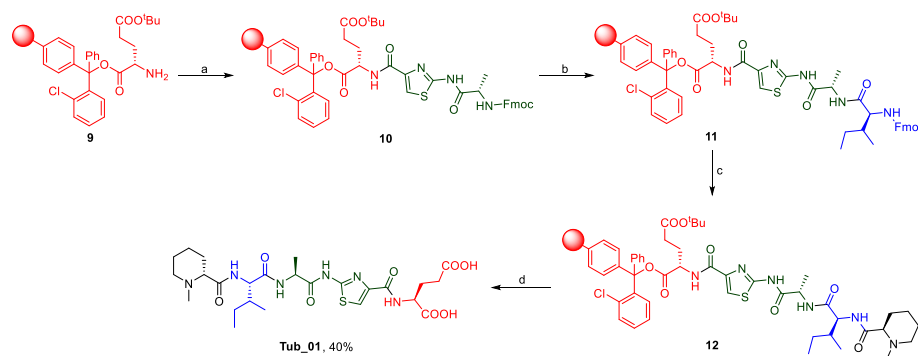


Scheme 4. Synthesis of pipecolic acid derivatives **8a–c**

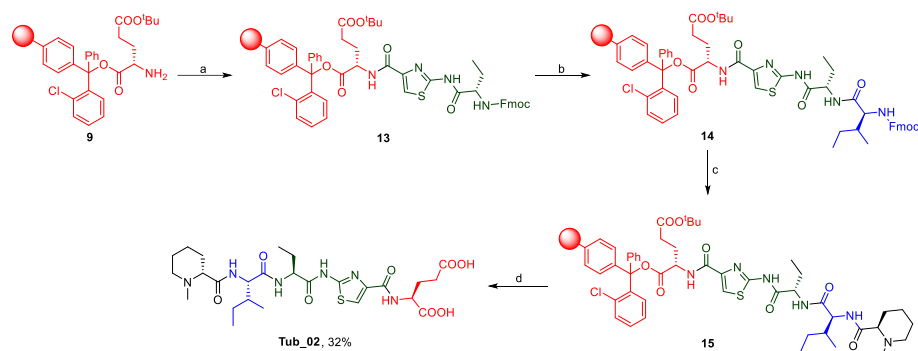
4.5 Synthesis of novel tubulin inhibitors (Tub_01 to Tub_06)

The synthesis of third-generation tubulin inhibitors was carried out by solid-phase peptide synthesis (SPPS) using H-Glu(O^tBu)-2-Cl-Trt resin **9** and Asn(Trt) resin **22**. The resins were initially swelled with DCM, followed by DMF. Next, the carboxylic acid group of Fmoc-thiazole fragments (Fmoc-Ala-thiazole fragment **5a** for tubulin inhibitor **Tub_01**; Fmoc-Abu-thiazole fragment **5b** for tubulin inhibitor **Tub_02**; Fmoc-*tert*-Leu-thiazole fragment **5d** for tubulin inhibitor **Tub_03**; Fmoc-Val-thiazole fragment **5c** for **Tub_04**; Fmoc-*tert*-Leu-thiazole fragment **5d** for **Tub_05**; Fmoc-*tert*-Leu-thiazole fragment **5d** for **Tub_06**) was coupled with the free amino group of the resin using the coupling agent PyBOP and the base DIPEA in DMF for 6 h. The resin beads were washed with DMF and isopropanol, dried, and the coupling was confirmed by the Kaiser test. The Fmoc group was then deprotected with 20% piperidine in DMF, followed by washing and drying. The deprotection is further verified by the Kaiser test. The next Fmoc-amino acids (Fmoc-(S)-Ile-OH for **Tub_01**, **Tub_02**, **Tub_04**, and **Tub_05** and Fmoc-(S)-*tert*-Leu-OH for **Tub_03** and Fmoc-(R)-Ile-OH for **Tub_06**) were then coupled to the growing peptide chain using the same procedure to build the tripeptide sequence. Subsequently, *N*-methyl-(R)-pipecolic acid **8a** for **Tub_01**, **Tub_02**, **Tub_03**, **Tub_05**, and **Tub_06** and *N*-isopropyl pipecolic acid **8c** for **Tub_04** were coupled to the resin-bound peptide and finally cleaved from the resin beads with the cleavage cocktail solution of TFA:TIPS:H₂O (9.5:0.25:0.25). The mother liquor was concentrated under reduced pressure, and the peptide

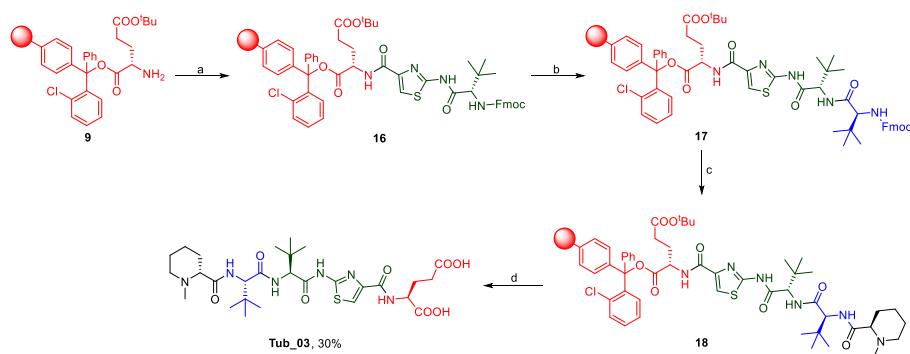
was precipitated in ice-cold diethyl ether, then purified by reverse-phase HPLC to obtain the desired peptides.



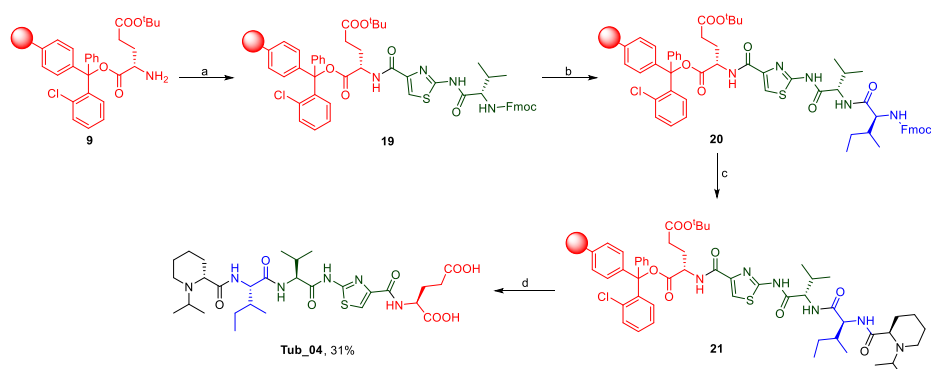
Scheme 5. Synthesis of a new tubulin inhibitor **Tub_01**. Reagents and conditions: (a) i) Fmoc-Ala-thiazole-OH **5a**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Methyl-(*R*)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, iii) Precipitate in ice cold diethyl ether, iv) Purified using RP-HPLC.



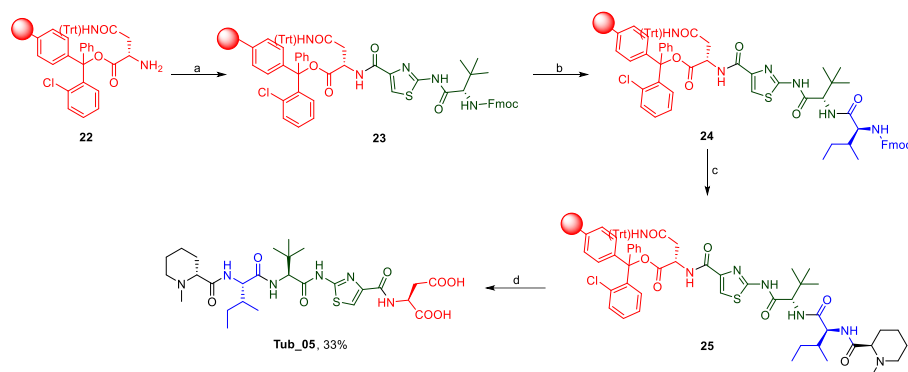
Scheme 6. Synthesis of a new tubulin inhibitor **Tub_02**. Reagents and conditions: (a) i) Fmoc-Abu-thiazole-OH **5b**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Methyl-(*R*)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, iii) Precipitate in ice cold diethyl ether, iv) Purified using RP-HPLC.



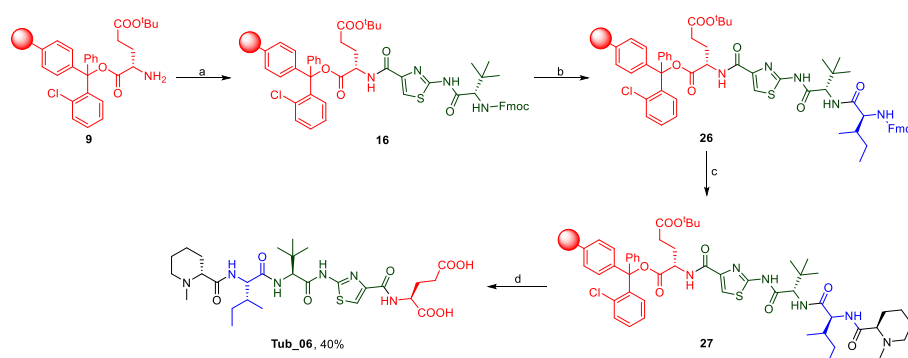
Scheme 7. Synthesis of a new tubulin inhibitor **Tub_03**. Reagents and conditions: (a) i) Fmoc-*tert*-Leu-thiazole-OH **5d**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-*tert*-Leu-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Methyl-(*R*)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, iii) Precipitate in ice cold diethyl ether, iv) Purified using RP-HPLC.



Scheme 8. Synthesis of a new tubulin inhibitor **Tub_04**. Reagents and conditions: (a) i) Fmoc-Val-thiazole-OH **5c**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Isopropyl-(*R*)-pipecolic acid **8c**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, 3) Precipitate in ice cold diethyl ether, 4) Purified using RP-HPLC.



Scheme 9. Synthesis of a new tubulin inhibitor **Tub_05**. Reagents and conditions: (a) i) Fmoc-*tert*-leu-thiazole-OH **5d**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Methyl-(*R*)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, 3) Precipitate in ice cold diethyl ether, 4) Purified using RP-HPLC.



Scheme 10. Synthesis of a new tubulin inhibitor **Tub_06**. Reagents and conditions: (a) i) Fmoc-*tert*-leu-thiazole-OH **5d**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(*S*)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) N-Methyl-(*R*)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, 3) Precipitate in ice cold diethyl ether, 4) Purified using RP-HPLC.

4.6 *In vitro* cytotoxic studies of synthesized tubulin inhibitors Tub_01 to Tub_03, and Tub_07 to Tub_10

Synthesized tubulin inhibitors Tub_01 to Tub_03 and Tub_07 to Tub_10, along with the standard drug Colchicine, were evaluated for their cytotoxic activity against the patient-derived cancer cell lines MCF7 and A431. The cells were treated with increasing concentrations of the synthesized compounds ranging from 10 pM to 100 μ M (10 pM, 50 pM, 100 pM, 500 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, 50 μ M, and 100 μ M) and incubated for 48 h.

The synthesized tubulin derivatives exhibited variable anticancer activities against skin and breast cancer cell lines. Against the A431 cell line, **Tub_01** and **Tub_02** displayed the least activity with IC₅₀ values of 478.3 nM (Figure 5) and 293.4 nM (Figure 6), respectively. Tubulin inhibitor **Tub_03** showed moderate cytotoxic activity with an IC₅₀ value of 161.6 nM (Figure 7), whereas **Tub_09** demonstrated the most potent cytotoxic activity with an IC₅₀ value of 47.7 nM (Figure 8).

Against the MCF7 cell line, **Tub_07** and **Tub_08** demonstrated moderate cytotoxic activity with IC₅₀ values of 160.6 nM (Figure 9) and 125.4 nM (Figure 10), respectively, while **Tub_09** exhibited the highest anticancer activity with an IC₅₀ value of 36.6 nM (Figure 11). **Tub_10**, however, showed weak activity with an IC₅₀ value of 796.4 nM (Figure 12).

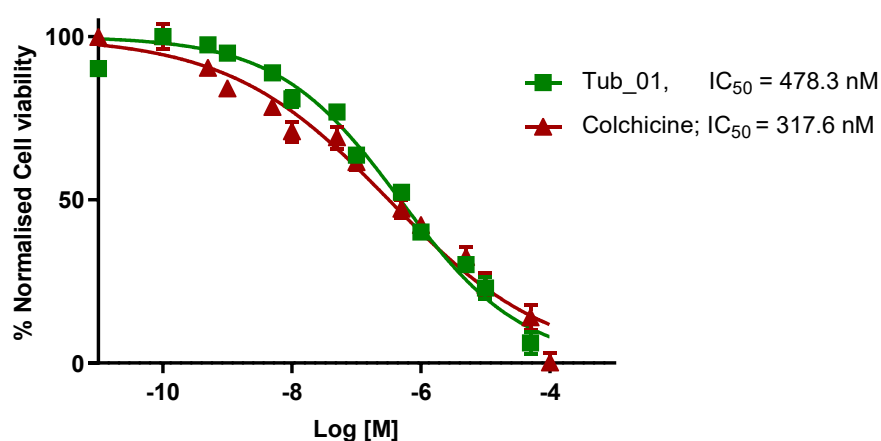


Figure 5. IC₅₀ study of tubulin inhibitor **Tub_01** with the standard drug colchicine against the skin cancer (A431) cell line

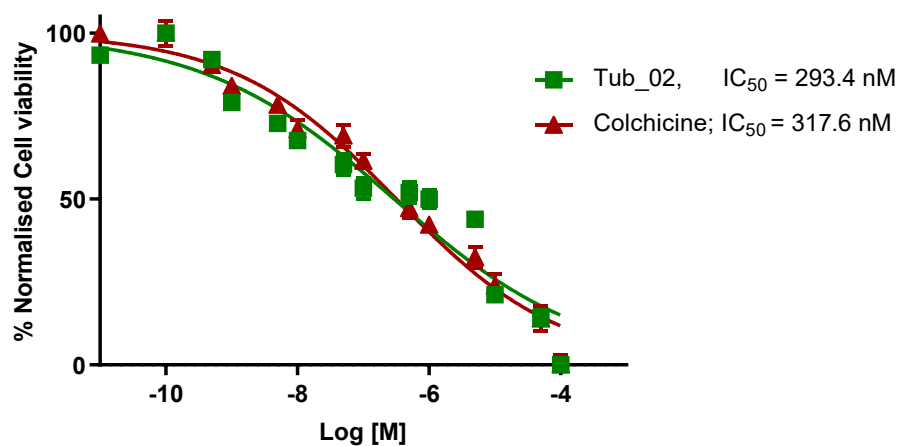


Figure 6. IC_{50} study of tubulin inhibitor **Tub_02** with the standard drug colchicine against the skin cancer (A431) cell line

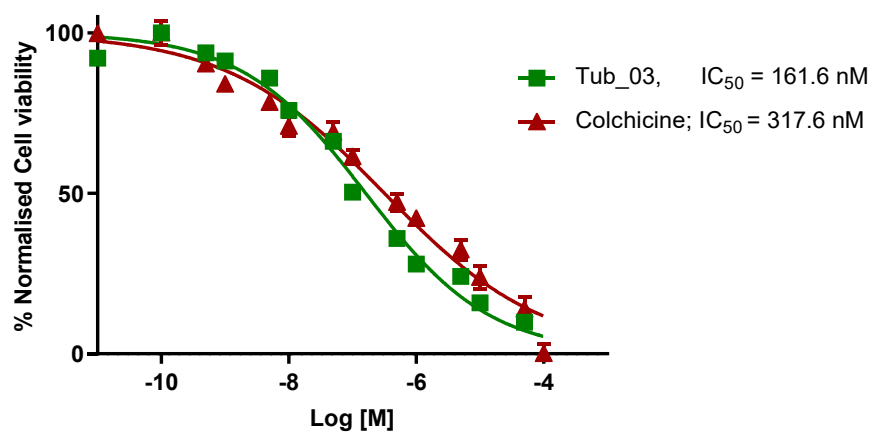


Figure 7. IC_{50} study of tubulin inhibitor **Tub_03** with the standard drug colchicine against the skin cancer (A431) cell line

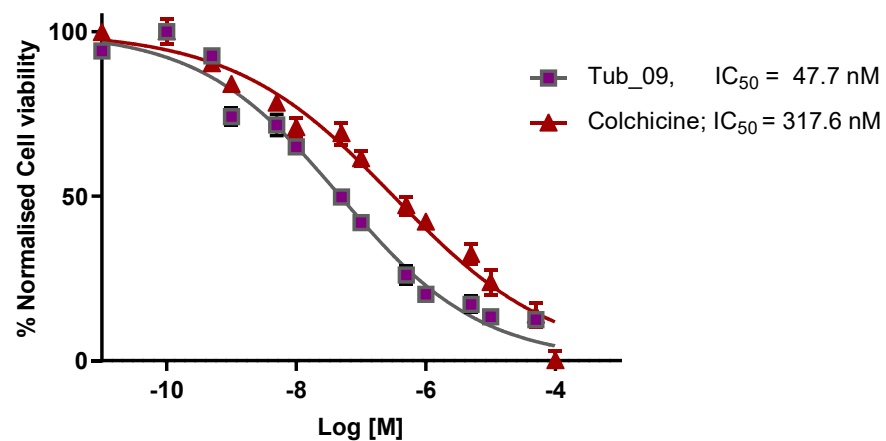


Figure 8. IC₅₀ study of tubulin inhibitor **Tub_09** with the standard drug colchicine against the skin cancer (A431) cell line

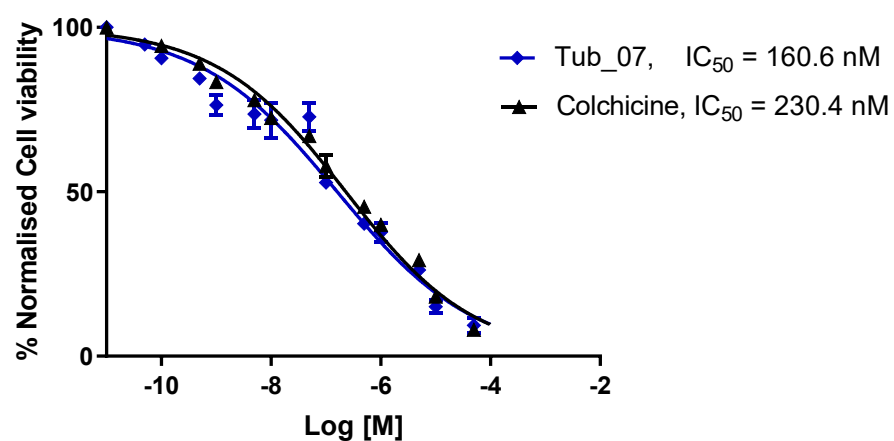


Figure 9. IC₅₀ study of tubulin inhibitor **Tub_07** with the standard drug colchicine against the breast cancer (MCF7) cell line

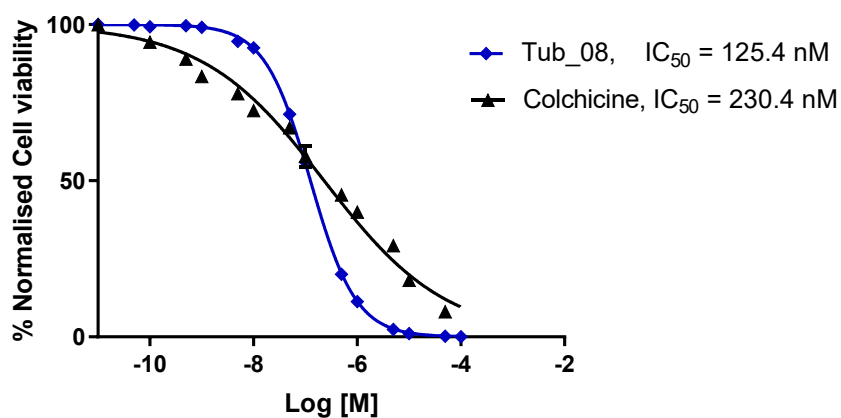


Figure 10. IC₅₀ study of tubulin inhibitor **Tub_08** with the standard drug colchicine against the breast cancer (MCF7) cell line

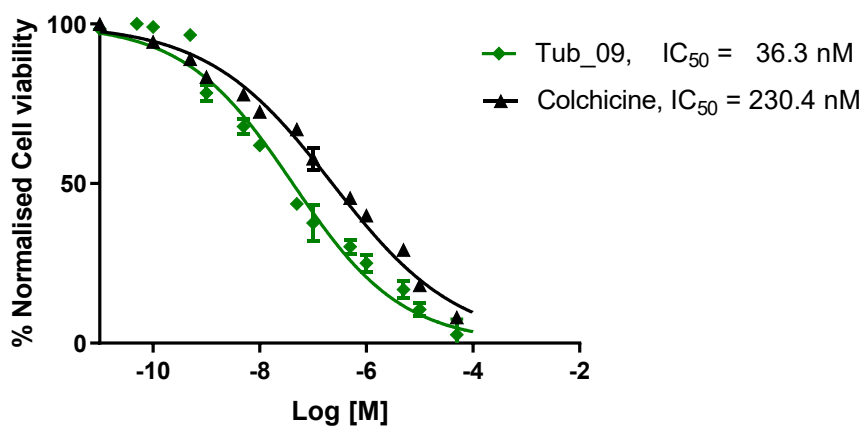


Figure 11. IC₅₀ study of tubulin inhibitor **Tub_09** with the standard drug colchicine against the breast cancer (MCF7) cell line

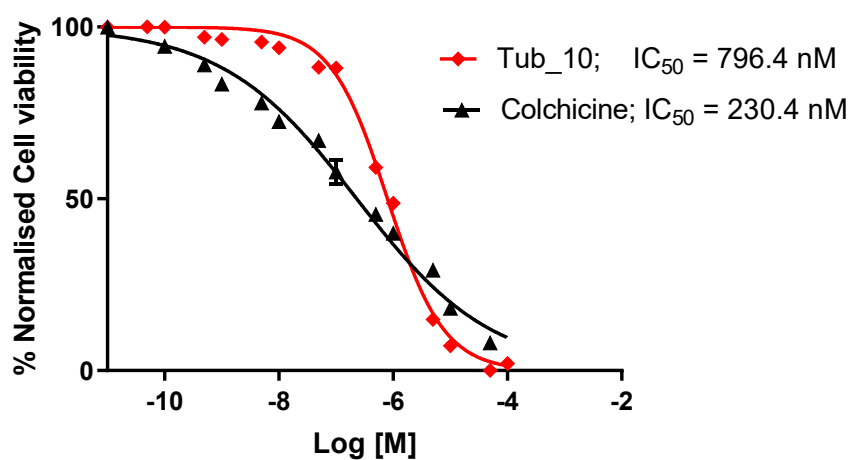


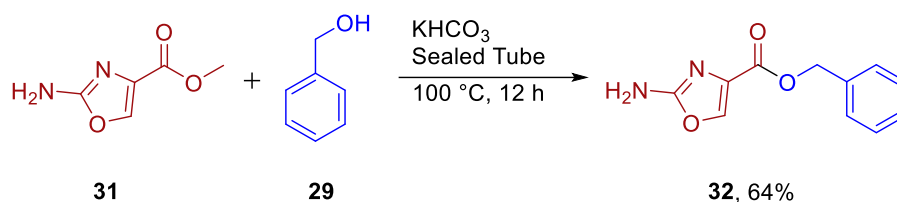
Figure 12. IC₅₀ study of tubulin inhibitor **Tub_10** with the standard drug colchicine against the breast cancer (MCF7) cell line

4.7 Synthesis of bioisostere tubulin inhibitors incorporating an oxazole ring (Tub_11)

4.7.1 Synthesis of benzyl-protected Fmoc-valine-oxazole fragment (33)

4.7.1.1 Synthesis of Benzyl 2-aminooxazole-4-carboxylate (32)

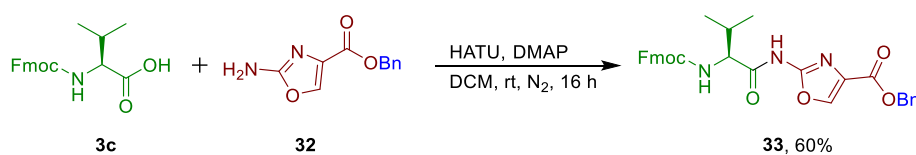
Transesterification of the methyl to benzyl group of methyl 2-aminooxazole 4-carboxylate **31** is performed using an excess amount of benzyl alcohol **29**. This reaction was carried out by reacting methyl 2-aminooxazole-4-carboxylate with benzyl alcohol at 100 °C in a sealed tube in the presence of the inorganic base potassium bicarbonate for 12 h to afford benzyl 2-aminooxazole-4-carboxylate **32** (Scheme 1).



Scheme 11. Synthesis of benzyl 2-aminooxazole-4-carboxylate **32**

4.7.1.2 Synthesis of Fmoc amino acid-benzyl-protected oxazole dipeptide (33)

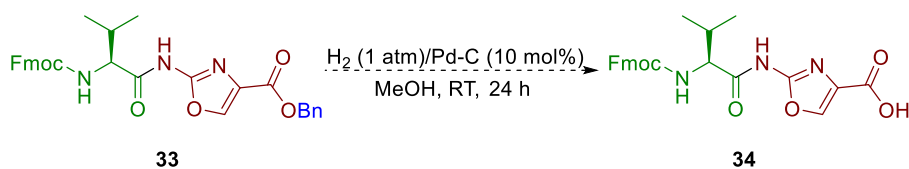
The free amine group present in the benzyl 2-aminooxazole-4-carboxylate **32** was coupled with the carboxylic acid group of Fmoc-protected amino acid **3c** via the formation of an amide bond to obtain the desired Fmoc amino acid-Benzyl-protected oxazole dipeptide **33**. The reaction was carried out using the coupling agent HATU in the presence of DMAP, in dry DCM for 16 h (Scheme 12).



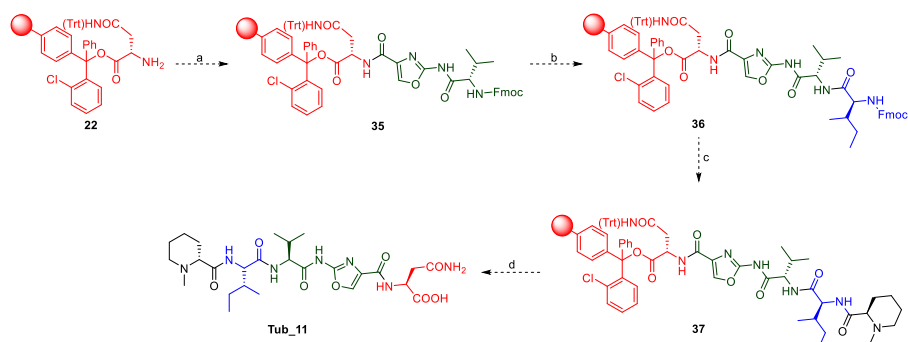
Scheme 12. Synthesis of Fmoc amino acid-benzyl-protected oxazole dipeptide **33**

4.7.2 Future Aspects: Proposed synthesis of tubulin inhibitor (Tub_11) with valine-oxazole fragment

For the synthesis of the Fmoc-valine-oxazole fragment, the debenzoylation reaction of the benzyl-protected Fmoc-valine-oxazole fragment (**33**) will be carried out, where the benzyl-protected Fmoc-valine-oxazole fragment (**33**) will be treated with hydrogen gas in the presence of activated Pd/C (Scheme 13). The fragment will be used in solid-phase peptide synthesis to produce the tubulin inhibitor **Tub_11**. The synthesis of third-generation tubulin inhibitors will be carried out using Asn(Trt) resin **22**. After swelling the beads with DCM and DMF, the carboxylic acid group of Fmoc-valine-oxazole fragment **34** will be coupled with the free amino group of the resin using the coupling agent PyBOP and the base DIPEA in DMF for 6 h. After confirming the amide bond coupling by the Kaiser test, the NHFmoc group will be deprotected by 20% piperidine in DMF. After verification of the deprotection of the Fmoc group by the Kaiser test, the next amino acid, Fmoc-(S)-Ile-OH, will be coupled with the free amine group of the growing peptide chain using the same procedure to build the tripeptide **36**. Subsequently, *N*-methyl-(R)-pipecolic acid **8a** will be coupled to the resin-bound peptide and finally cleaved from the resin beads with the cleavage cocktail solution of TFA:TIPS:H₂O (9.5:0.25:0.25). The mother liquor was concentrated under reduced pressure, and the peptide was precipitated in ice-cold diethyl ether, then purified by reverse-phase HPLC to obtain the desired tubulin inhibitor **Tub_11** (Scheme 14).



Scheme 13. Proposed synthesis of valine thiazole fragment **34**



Scheme 14. Proposed synthesis of a new tubulin inhibitor, **Tub_11**.
 Reagents and conditions: (a) i) Fmoc-valine-oxazole-OH **34**, PyBOP, DIPEA, DMF, 6 h; (b) i) 20% Piperidine, DMF, rt, 30 min; ii) Fmoc-(S)-Ile-OH, PyBOP, DIPEA, DMF, 6 h; (c) i) 20% Piperidine, DMF, rt, 30 min; ii) *N*-Methyl-(R)-pipecolic acid **8a**, PyBOP, DIPEA, DMF, 6 h; (d) i) 10 mL of TFA, TIPS, H₂O (9.50:0.25:0.25), ii) Evaporate TFA, 3) Precipitate in ice cold diethyl ether, 4) Purified using RP-HPLC.

CHAPTER 5

CONCLUSION

In medicinal chemistry, peptides are promising and have high potential to act as anticancer agents. However, peptide drugs composed of α -amino acids are unstable in biological systems and undergo proteolytic hydrolysis in blood serum, making them challenging to use clinically. In the current study, we have utilized solid-phase peptide synthesis to prepare new third-generation tubulin inhibitors using unnatural β - and 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, including ^1H and ^{13}C NMR and mass spectrometry. The synthesized tubulin derivatives showed a range of anticancer activity against the A431 cell line. Among them, **Tub_09** exhibited the highest potency with an IC_{50} value of 47.7 nM, while **Tub_03** showed moderate activity, and **Tub_01** and **Tub_02** displayed weak cytotoxicity. Against the MCF7 cell line, **Tub_09** demonstrated the strongest cytotoxicity, with an IC_{50} of 36.6 nM. **Tub_07** and **Tub_08** showed moderate activity, whereas **Tub_10** exhibited weak anticancer activity. Overall, **Tub_09** emerged as the most promising tubulin-targeting anticancer candidate among the synthesized derivatives. These findings encourage the further development of tubulin inhibitors through bioisosteric replacement of the thiazole ring with an oxazole moiety. Future studies will focus on the synthesis and biological evaluation of oxazole-based derivatives to improve anticancer potency and selectivity.

APPENDIX

The ^1H and ^{13}C NMR spectra, along with the mass spectrometry data of the synthesised compounds, are listed below.

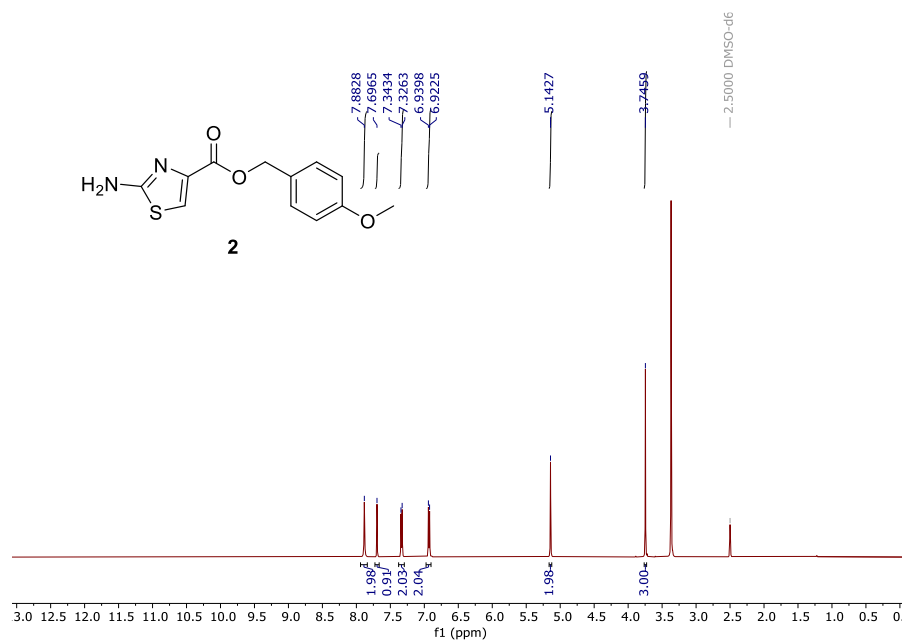


Figure 13. ^1H NMR spectrum of 4-methoxybenzyl 2-aminothiazole-4-carboxylate **2**

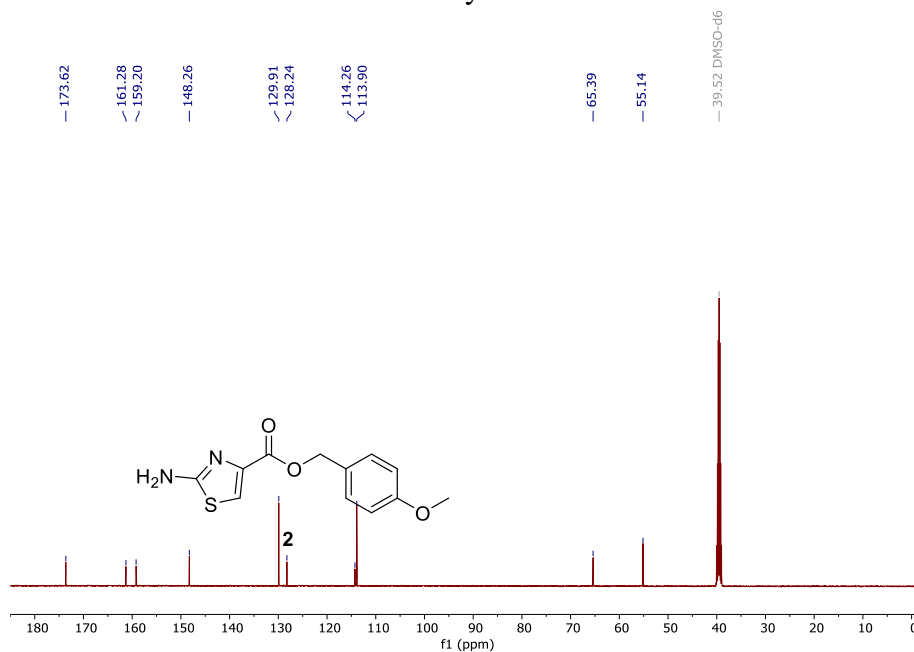


Figure 14. ^{13}C NMR spectrum of 4-methoxybenzyl 2-aminothiazole-4-carboxylate **2**

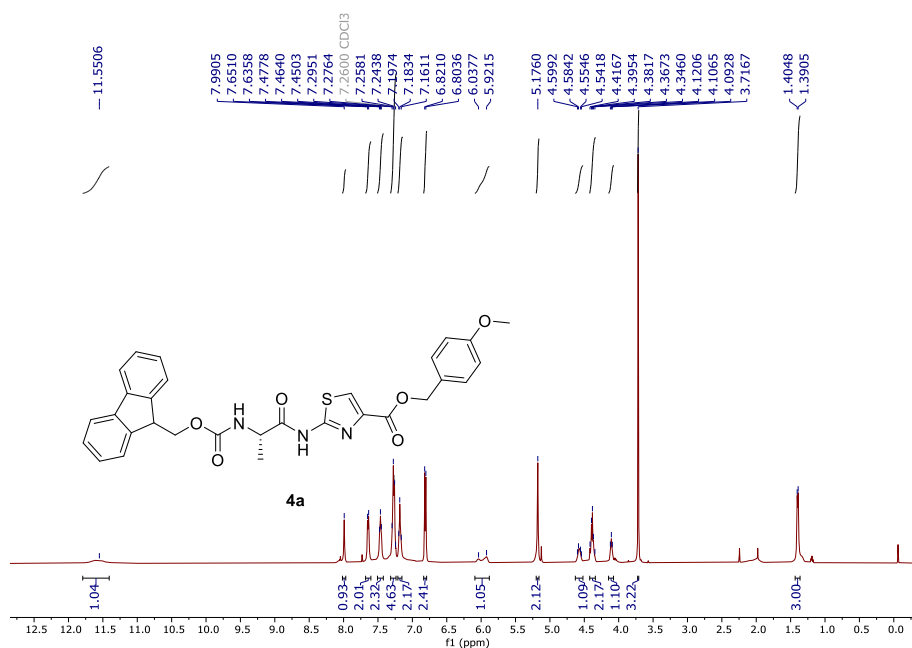


Figure 15. ¹H NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)propanamido)thiazole-4-carboxylate **4a**

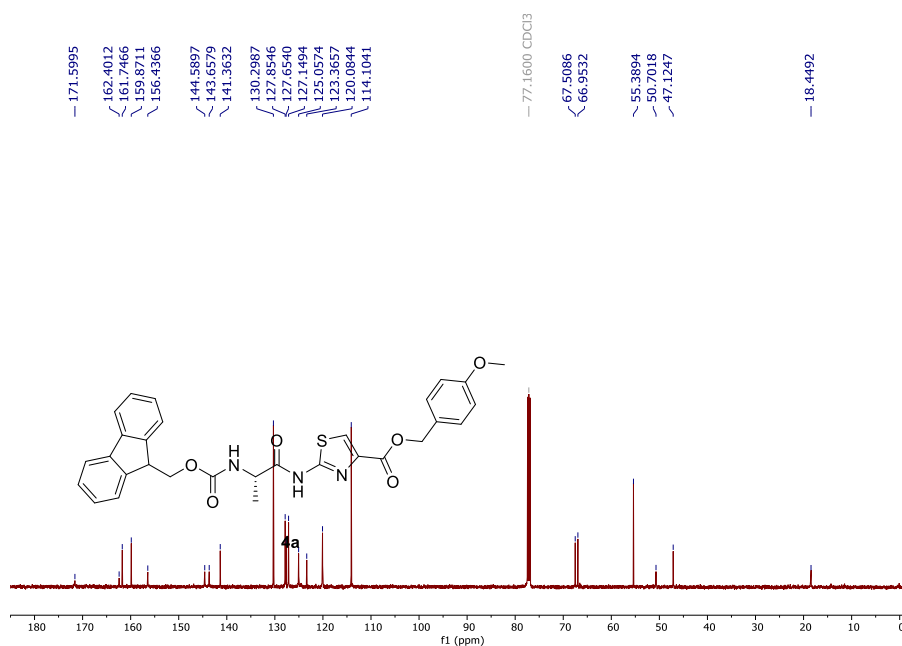


Figure 16. ¹³C NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)butanamido)thiazole-4-carboxylate **4a**

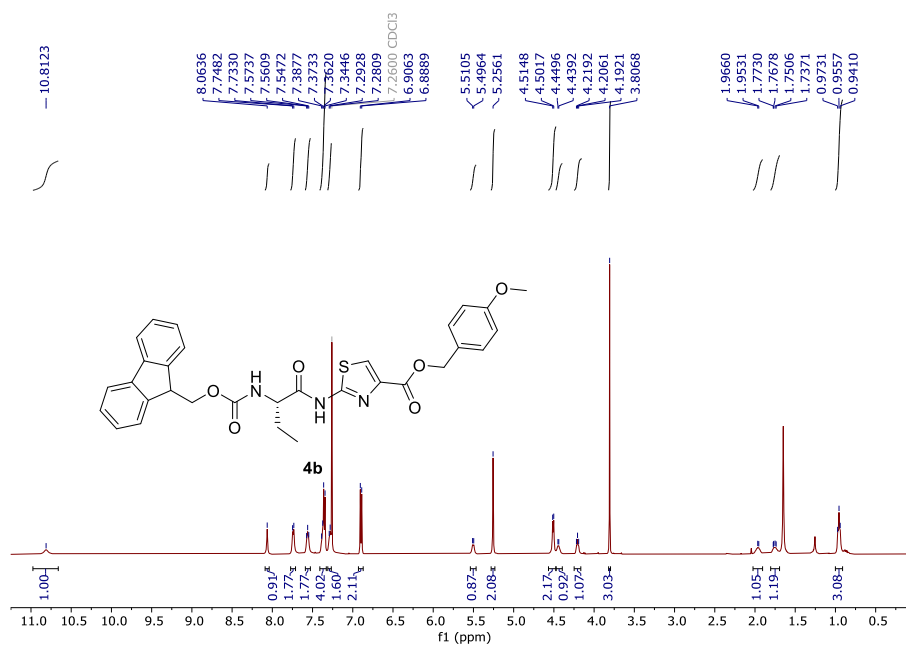


Figure 17. ¹H NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)butanamido)thiazole-4-carboxylate **4b**

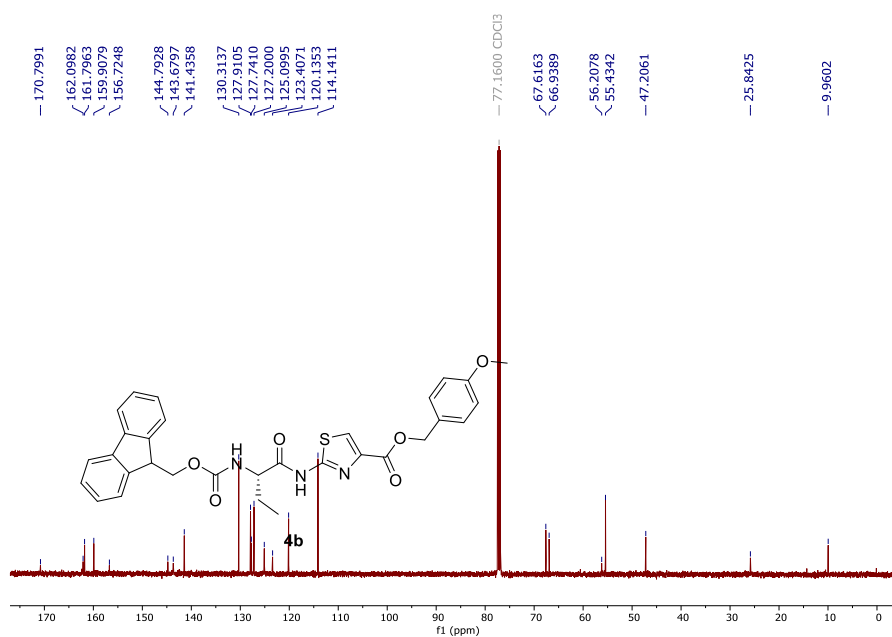


Figure 18. ¹³C NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)butanamido)thiazole-4-carboxylate **4b**

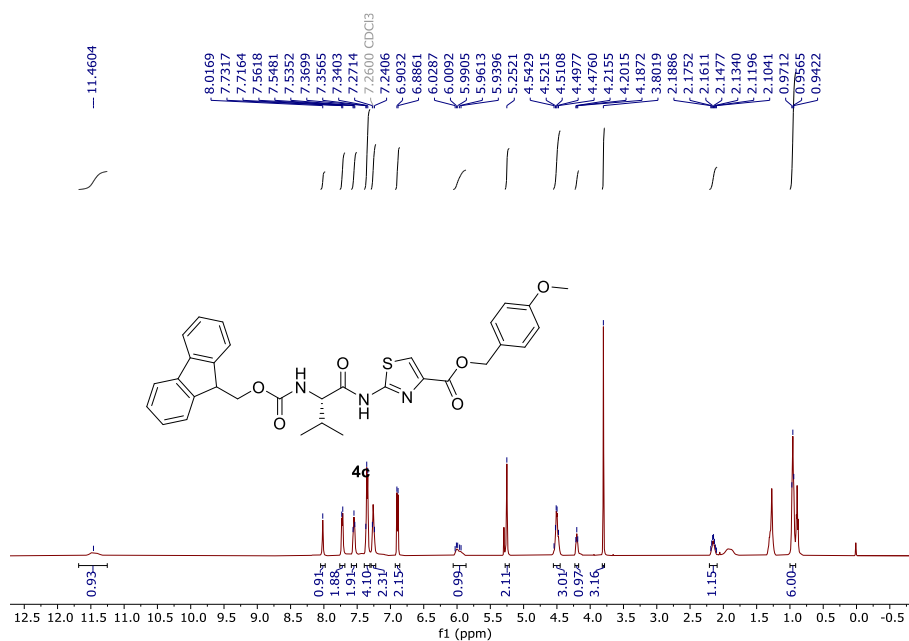


Figure 19. ¹H NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylbutanamido)thiazole-4-carboxylate **4c**

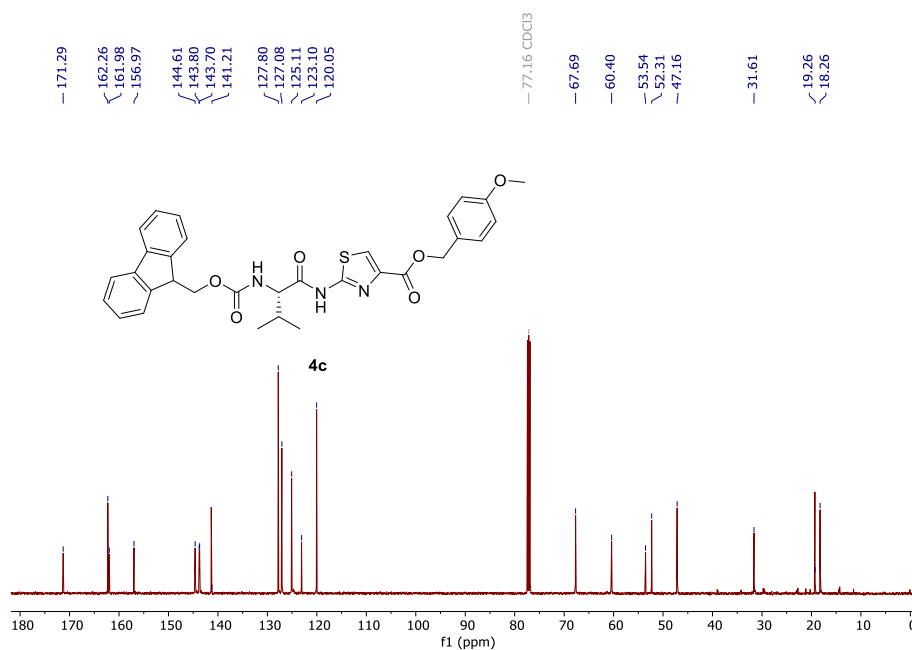


Figure 20. ¹³C NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylbutanamido)thiazole-4-carboxylate **4c**

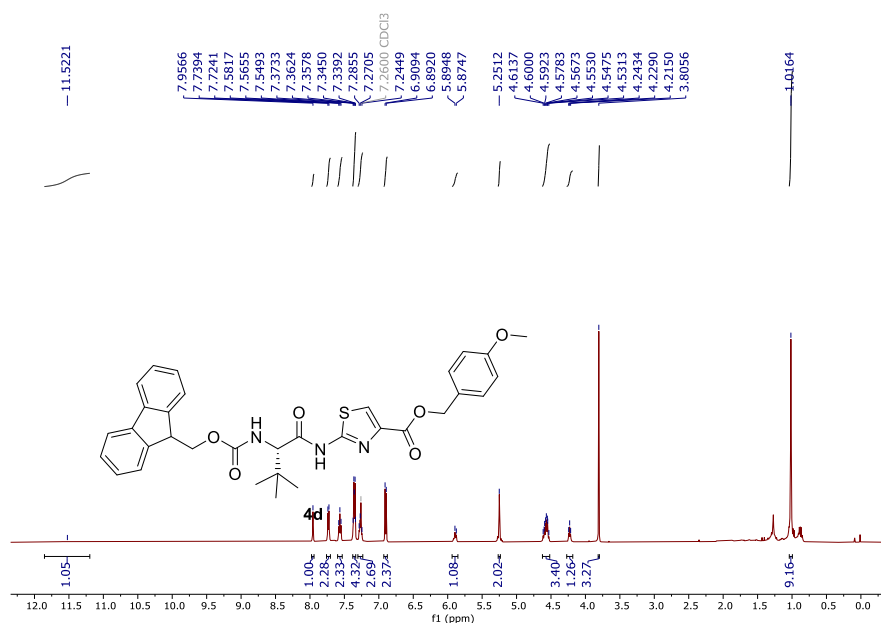


Figure 21. ¹H NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3,3-dimethylbutanamido)thiazole-4-carboxylate **4d**

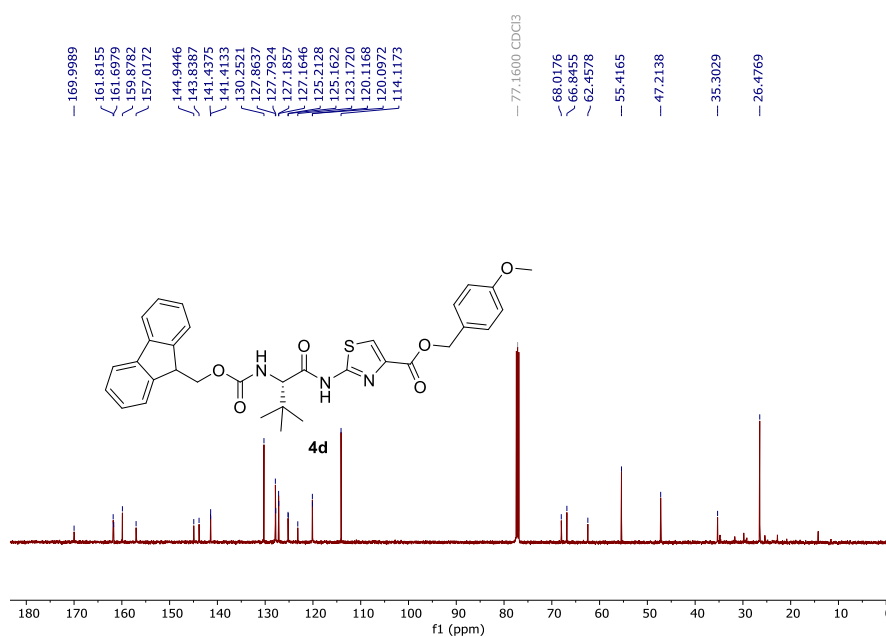


Figure 22. ¹³C NMR spectrum of 4-methoxybenzyl (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3,3-dimethylbutanamido)thiazole-4-carboxylate **4d**

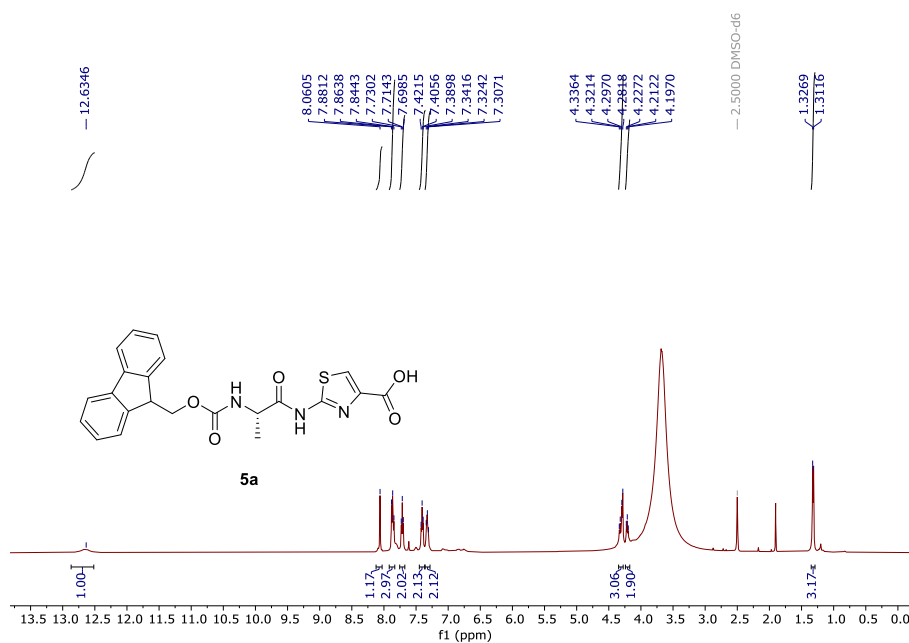


Figure 23. ¹H NMR spectrum of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido) thiazole-4-carboxylic acid

5a

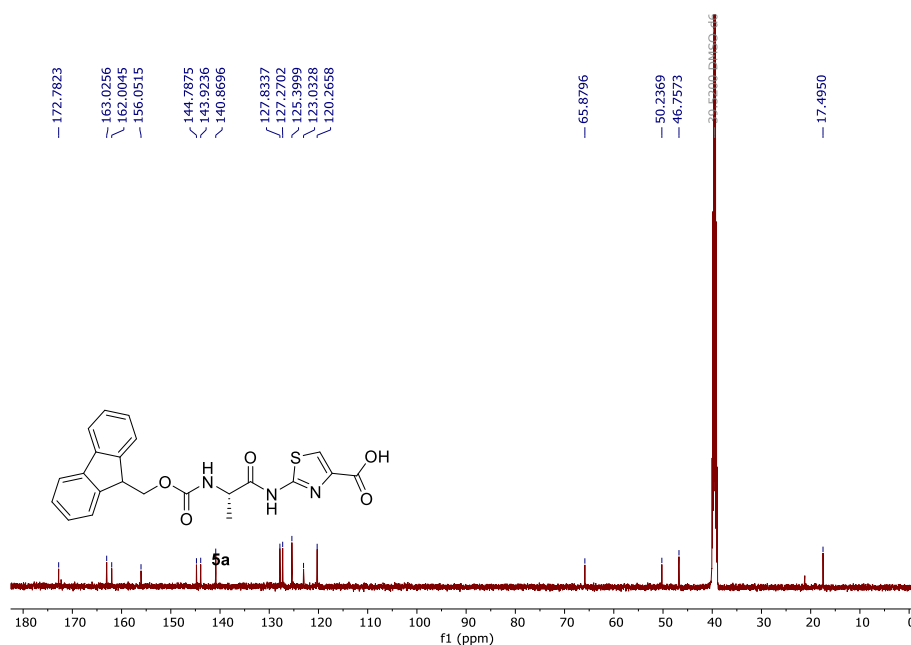


Figure 24. ¹³C NMR spectrum of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido) thiazole-4-carboxylic acid

5a

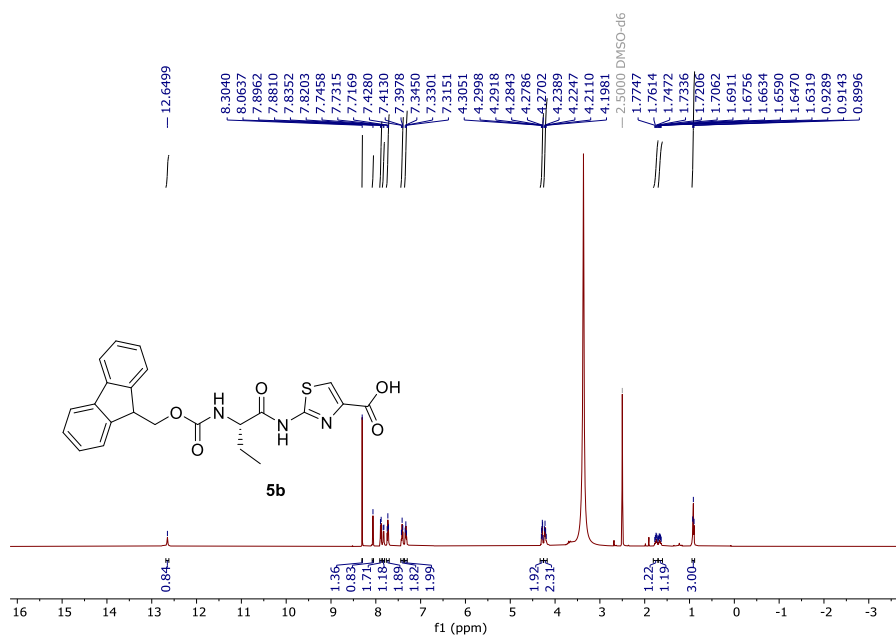


Figure 25. ¹H NMR spectrum of *(S)*-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido)thiazole-4-carboxylic acid **5b**

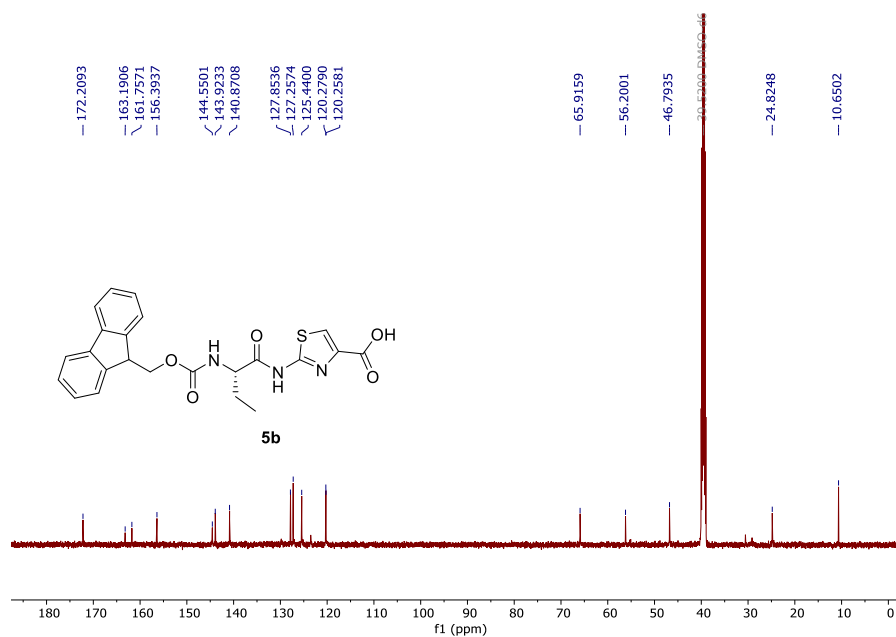


Figure 26. ¹³C NMR spectrum of *(S)*-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido)thiazole-4-carboxylic acid **5b**

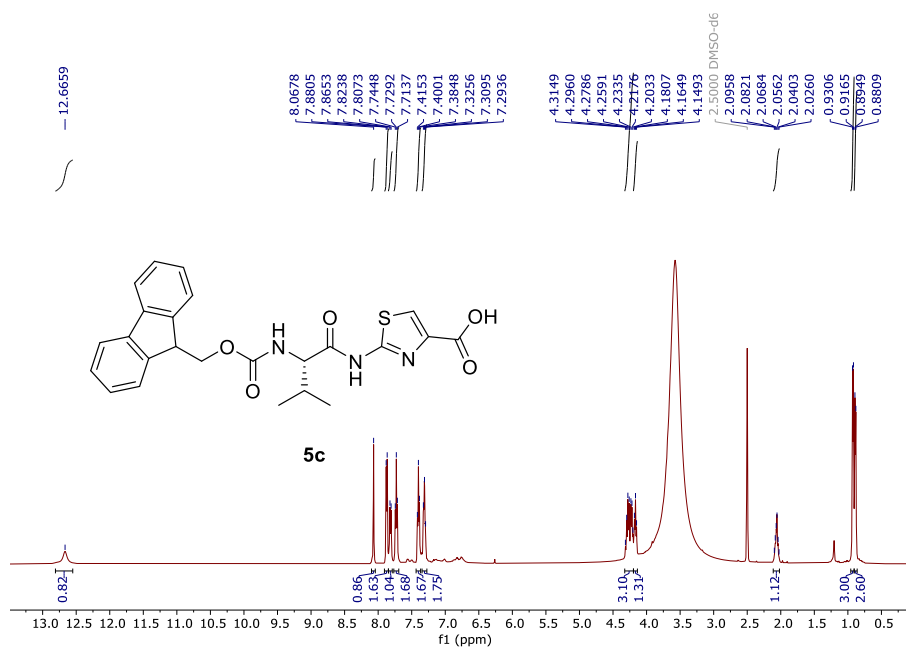


Figure 27. ¹H NMR spectrum of (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methyl butanamido) thiazole-4-carboxylic acid **5c**

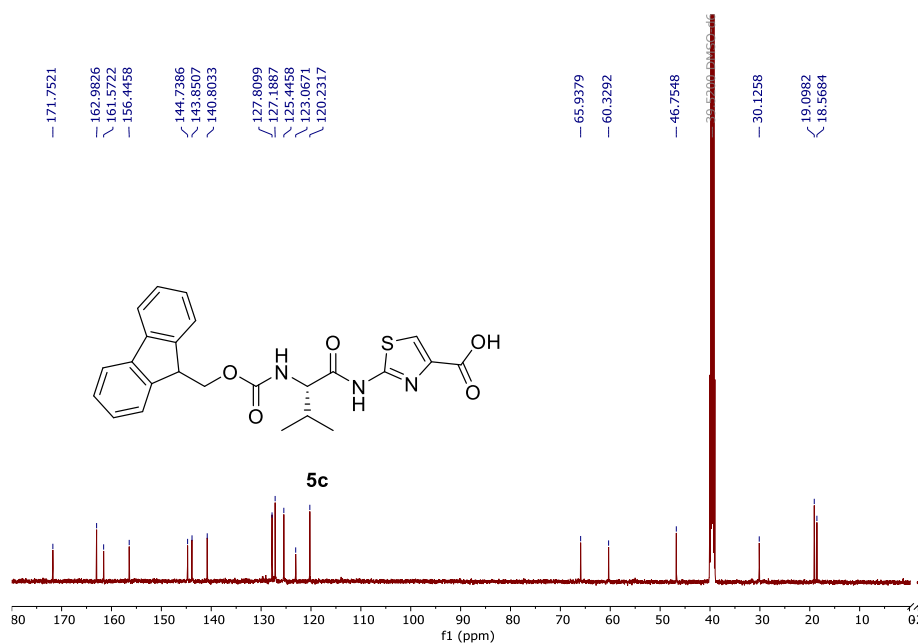


Figure 28. ¹³C NMR spectrum of (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methyl butanamido) thiazole-4-carboxylic acid **5c**

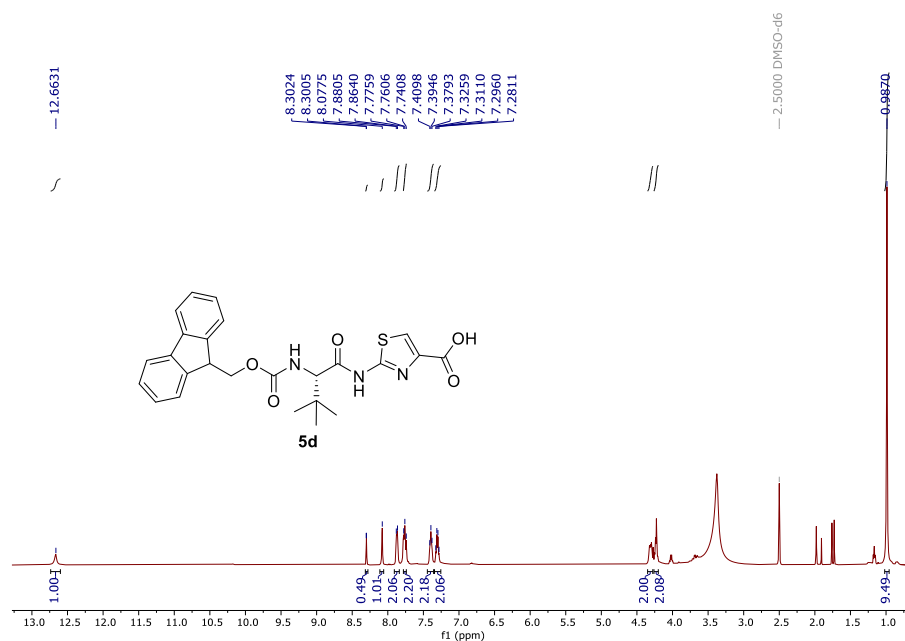


Figure 29. ¹H NMR spectrum of (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylbutanamido)thiazole-4-carboxylic acid **5d**

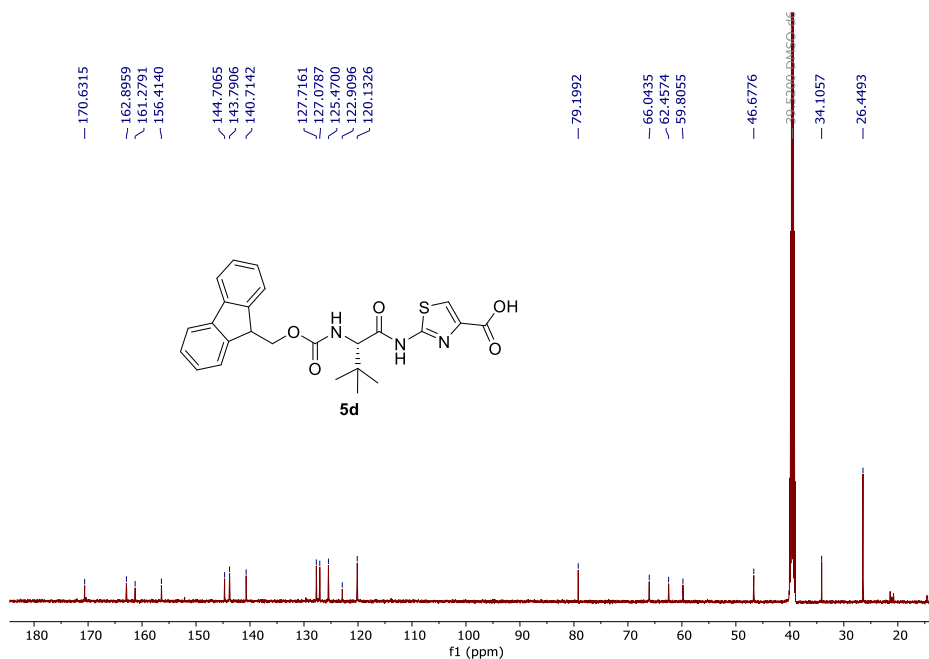


Figure 30. ¹³C NMR spectrum of (*S*)-2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylbutanamido)thiazole-4-carboxylic acid **5d**

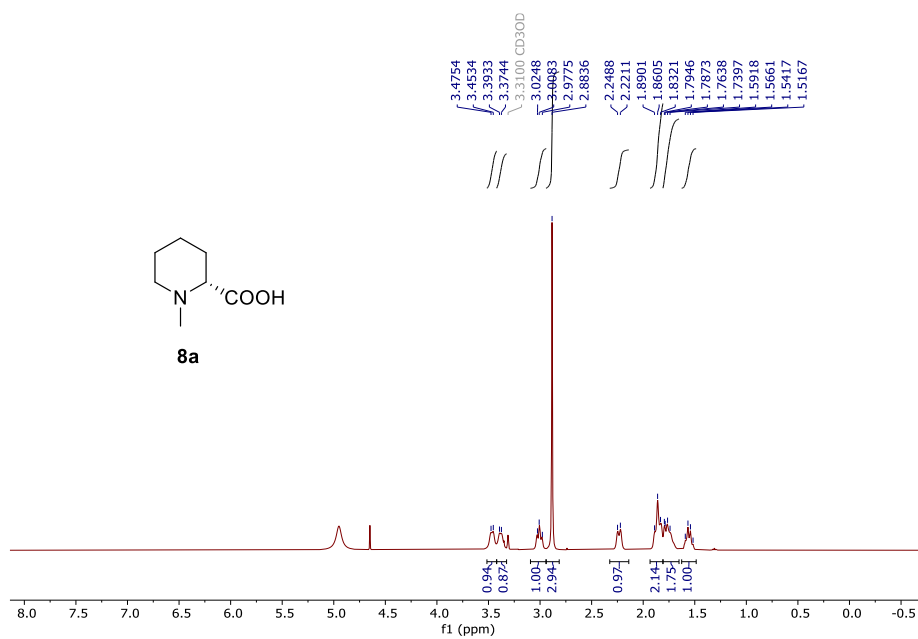


Figure 31. ¹H NMR spectrum of (*R*)-1-methylpiperidine-2-carboxylic acid **8a**

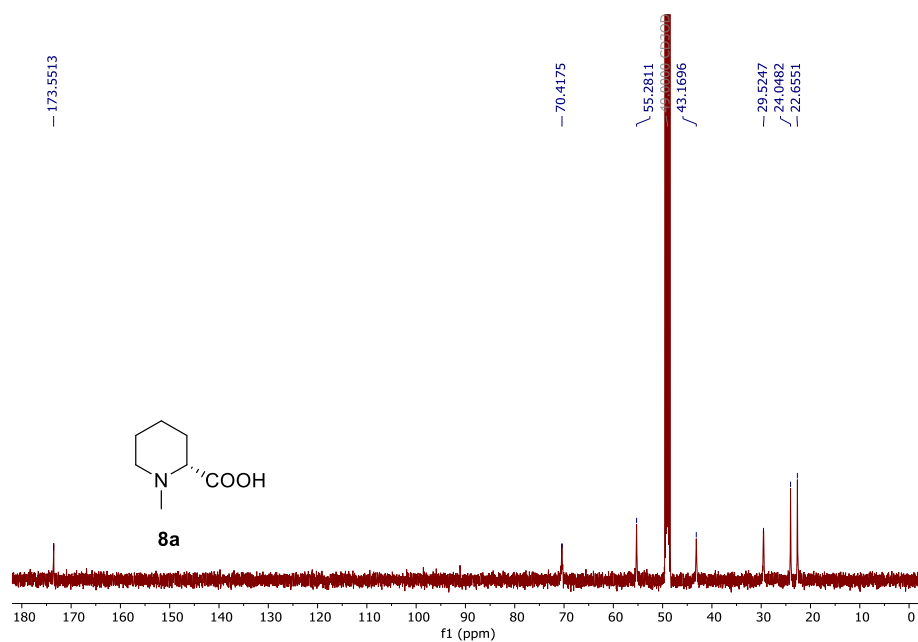


Figure 32. ¹³C NMR spectrum of (*R*)-1-methylpiperidine-2-carboxylic acid **8a**

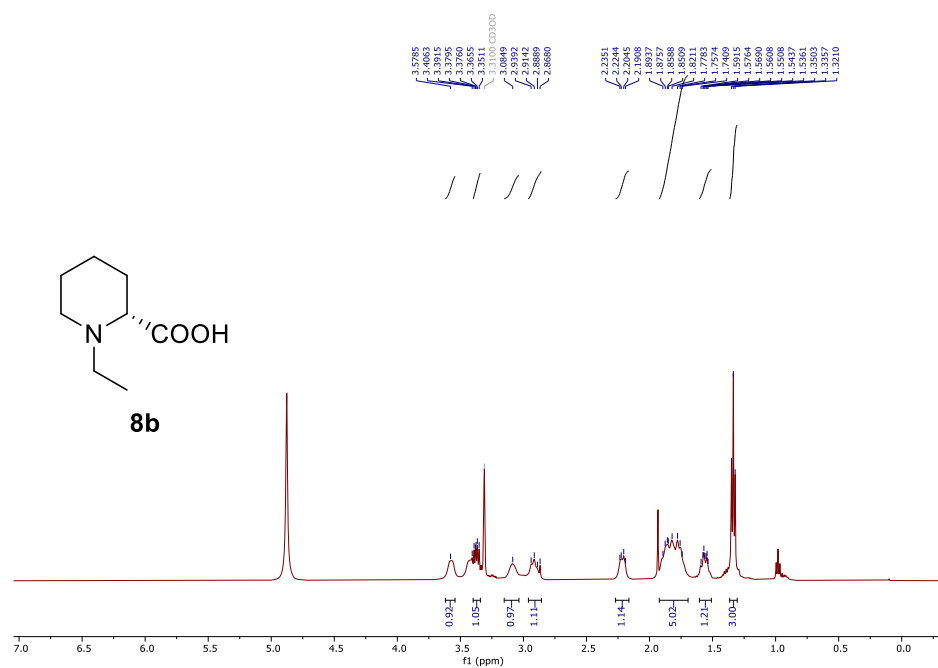


Figure 33. ¹H NMR spectrum of (*R*)-1-ethylpiperidine-2-carboxylic acid **8b**

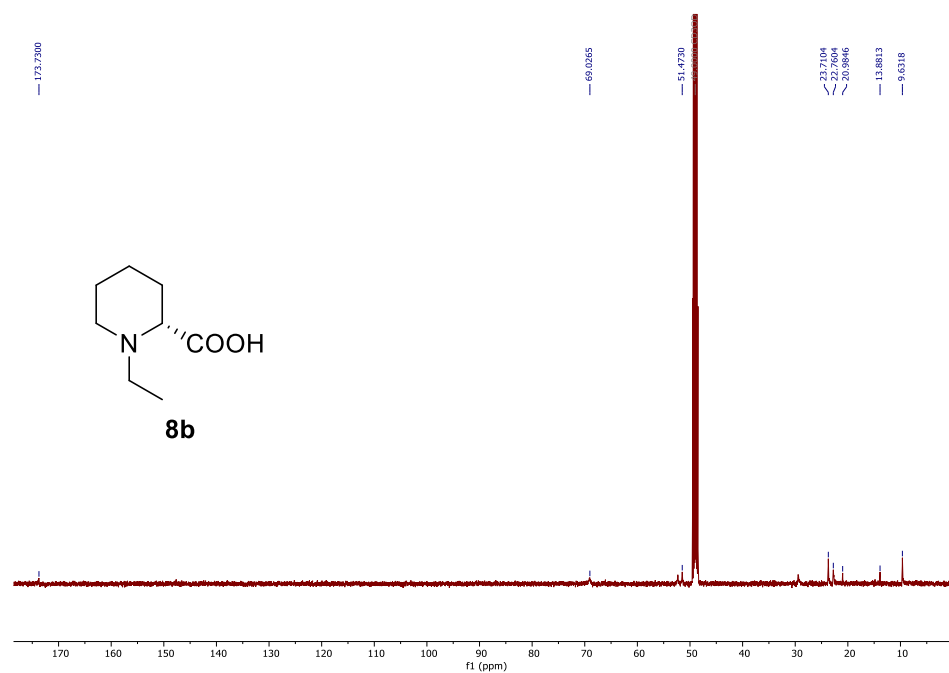


Figure 34. ¹³C NMR spectrum of (*R*)-1-ethylpiperidine-2-carboxylic acid **8b**

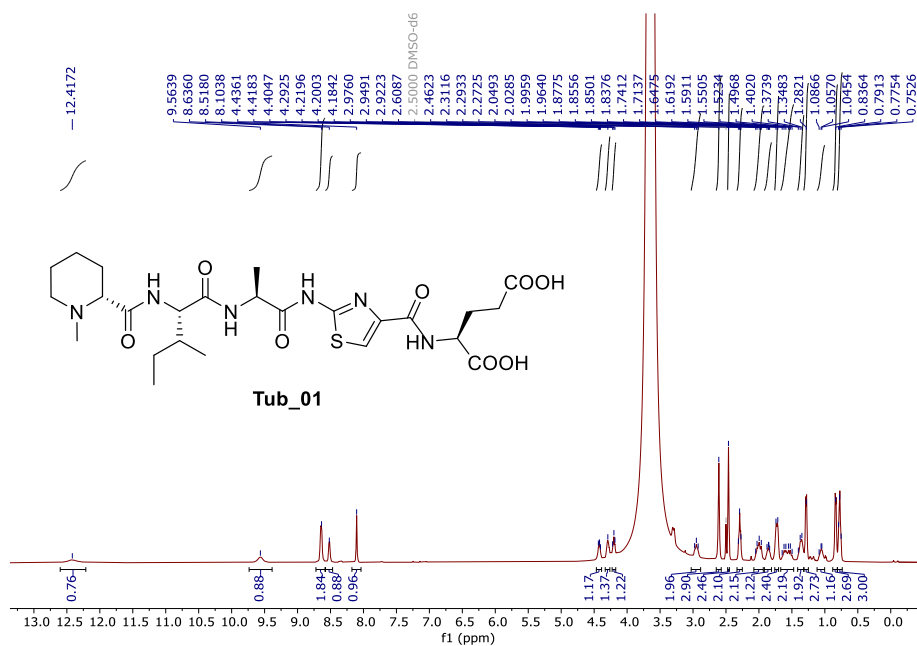


Figure 35. ^1H NMR spectrum of tubulin inhibitor **Tub_01**

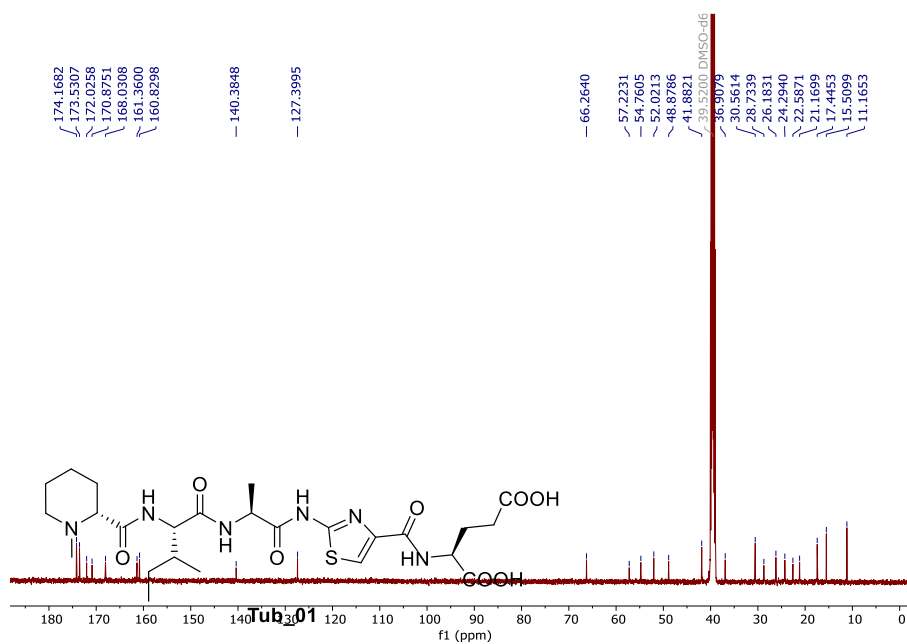


Figure 36. ^{13}C NMR spectrum of tubulin inhibitor **Tub_01**

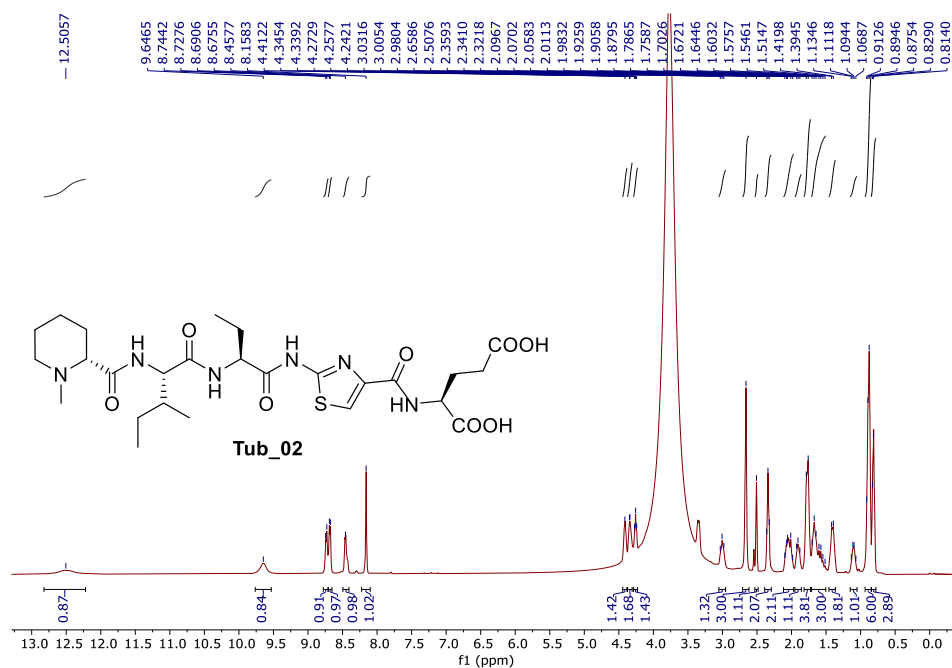


Figure 37. ¹H NMR spectrum of tubulin inhibitor **Tub_02**

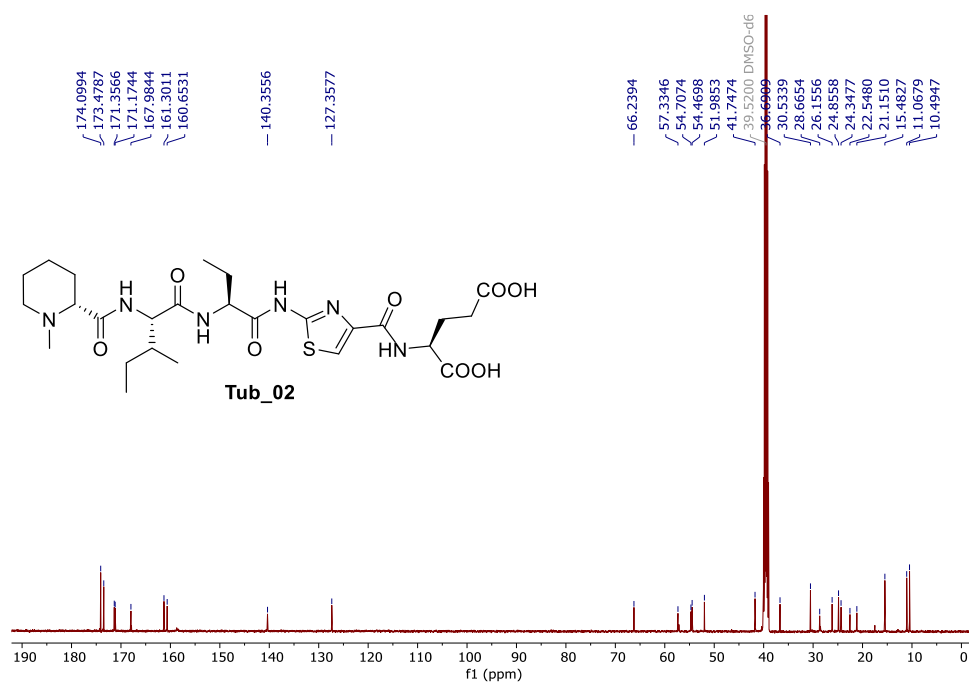


Figure 38. ¹³C NMR spectrum of tubulin inhibitor **Tub_02**

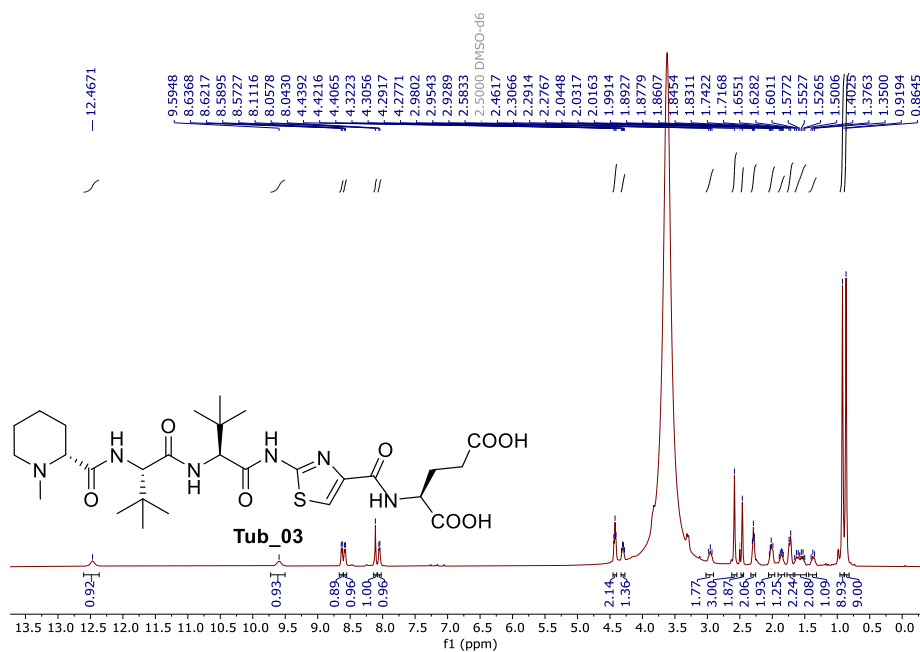


Figure 39. ¹H NMR spectrum of tubulin inhibitor **Tub_03**

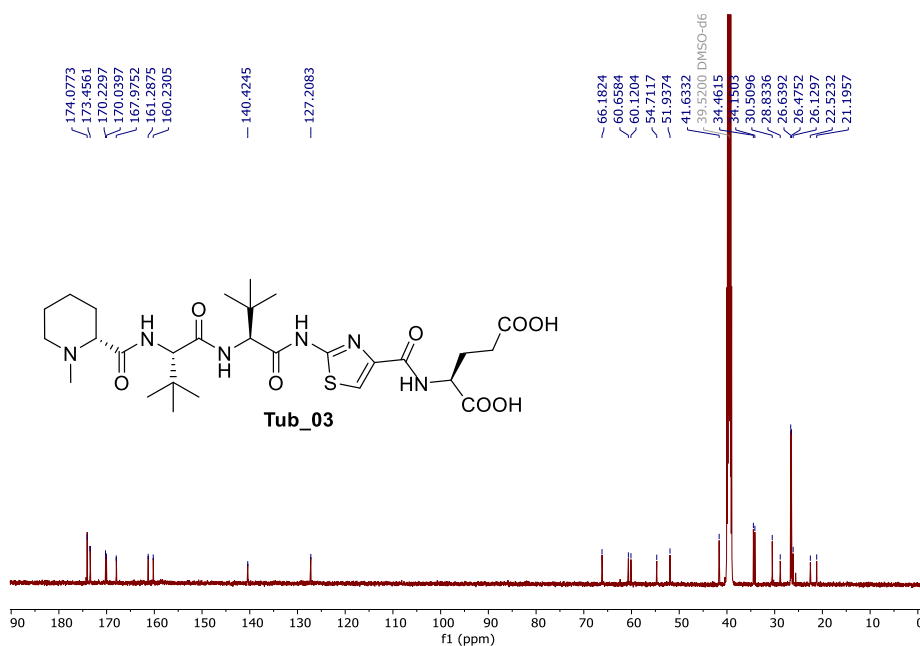


Figure 40. ¹³C NMR spectrum of tubulin inhibitor **Tub_03**

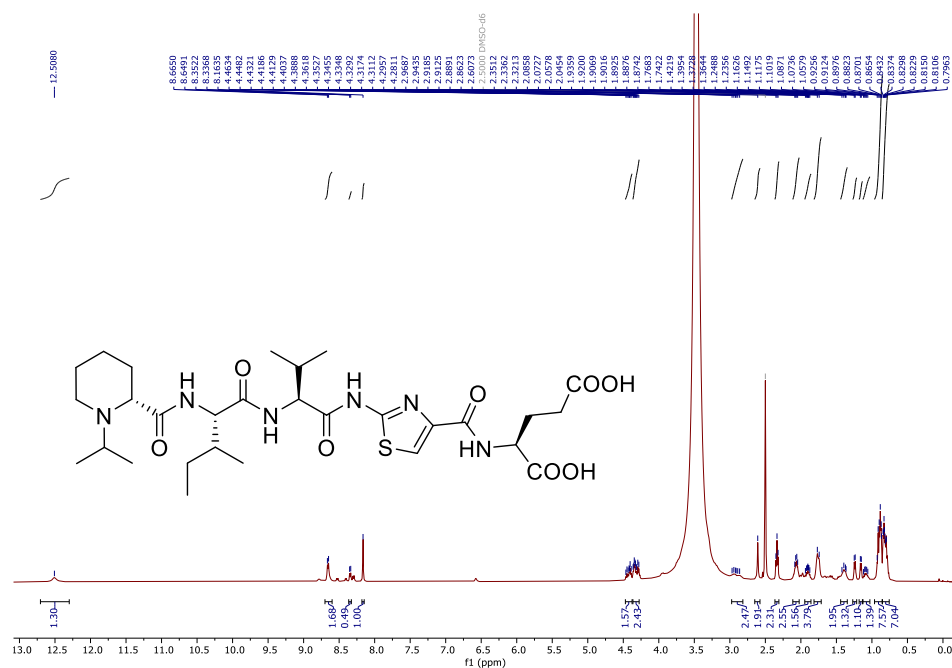


Figure 41. ¹H NMR spectrum of tubulin inhibitor Tub_04

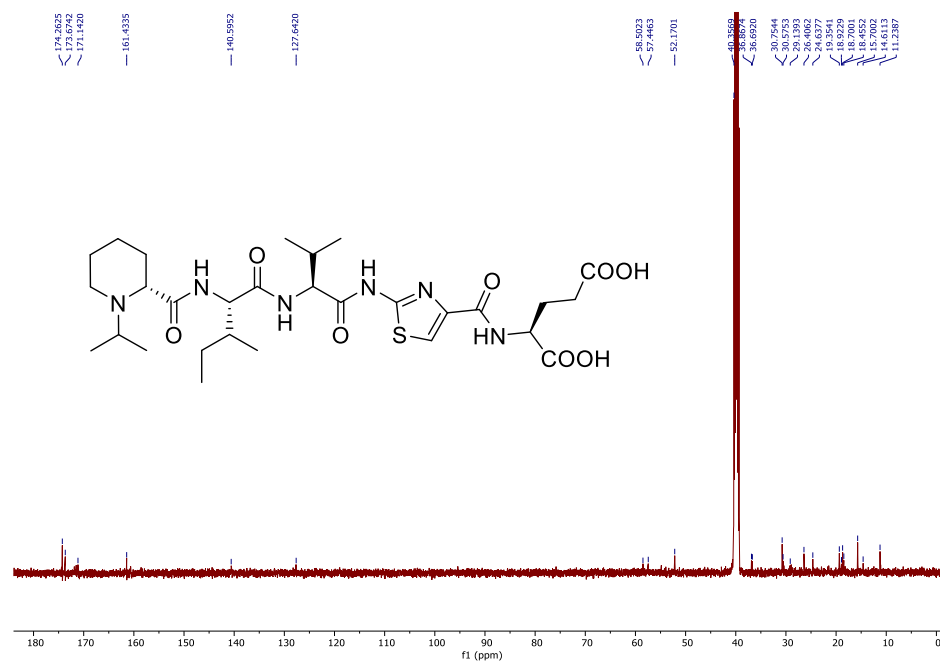


Figure 42. ¹³C NMR spectrum of tubulin inhibitor Tub_04

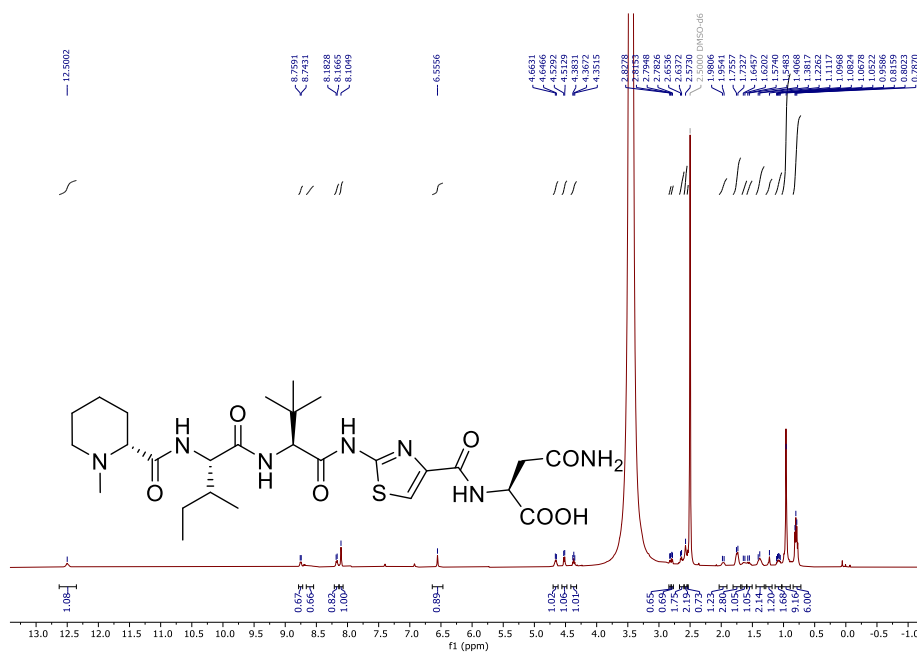


Figure 43. ¹H NMR spectrum of tubulin inhibitor **Tub_05**

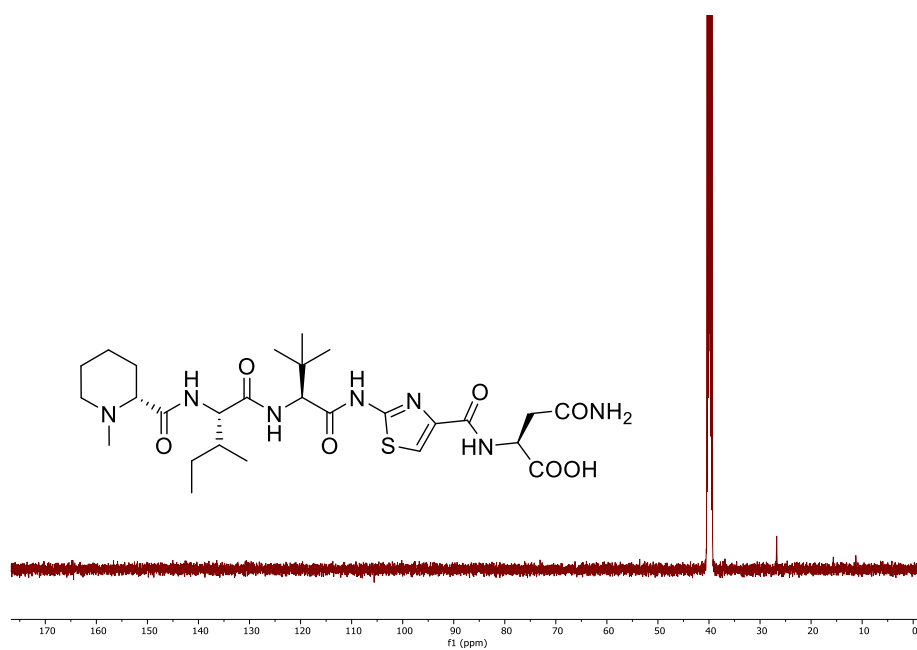


Figure 44. ¹³C NMR spectrum of tubulin inhibitor **Tub_05**

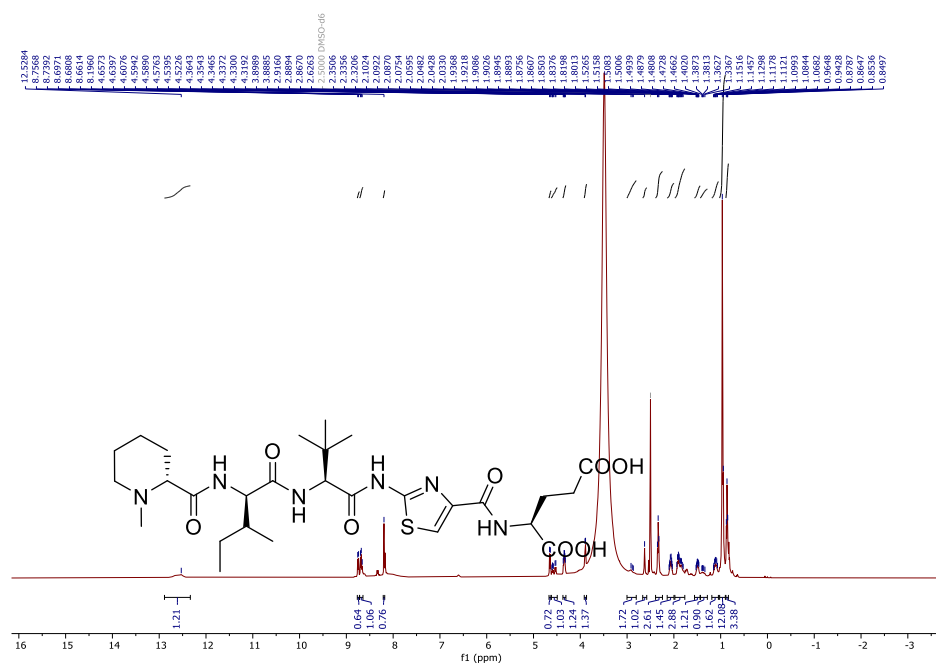


Figure 45. ^1H NMR spectrum of tubulin inhibitor **Tub_06**

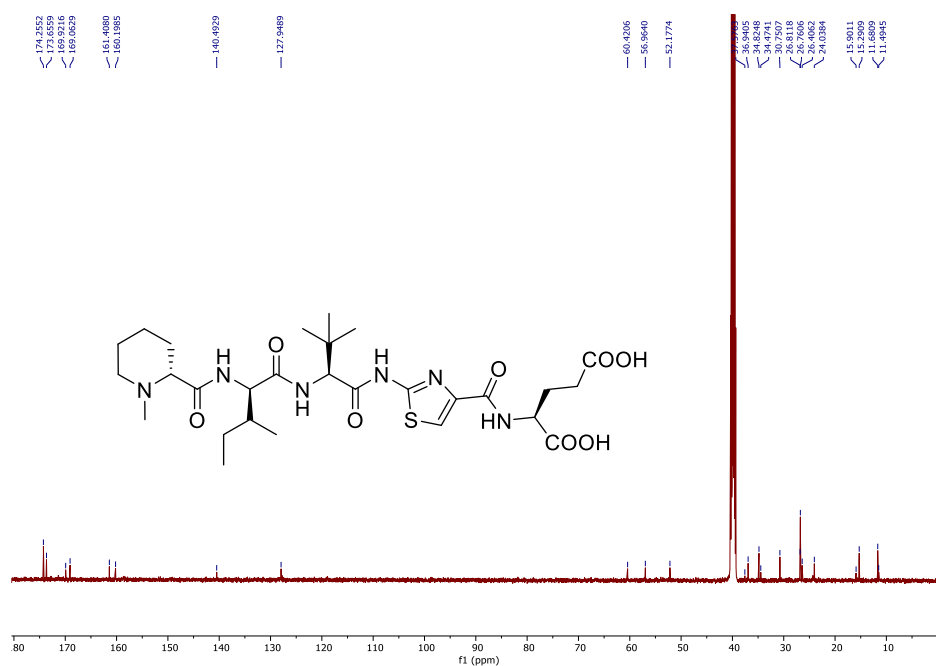


Figure 46. ^{13}C NMR spectrum of tubulin inhibitor **Tub_06**

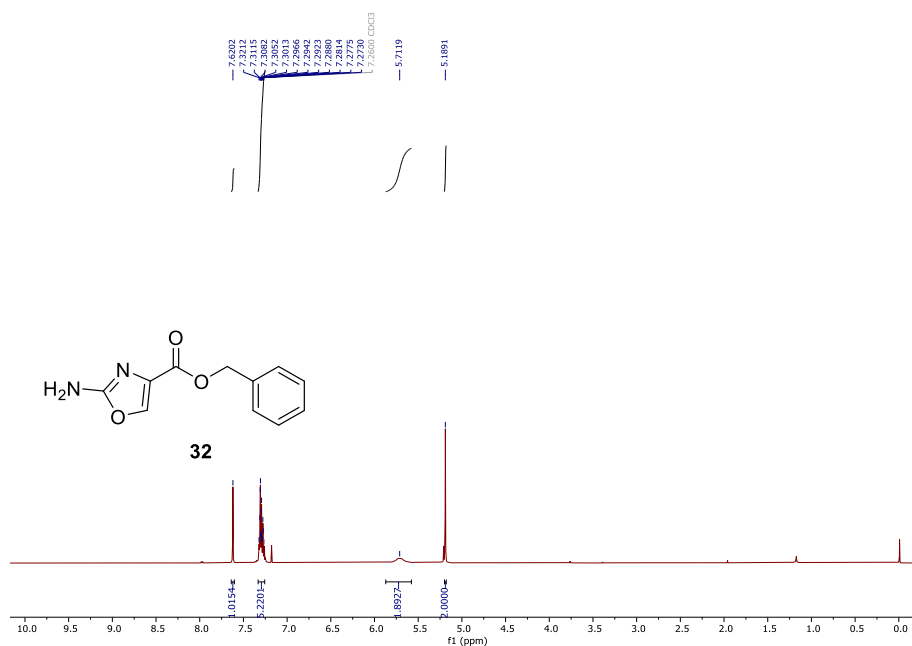


Figure 47. ¹H NMR spectrum of benzyl 2-aminooxazole-4-carboxylate **32**

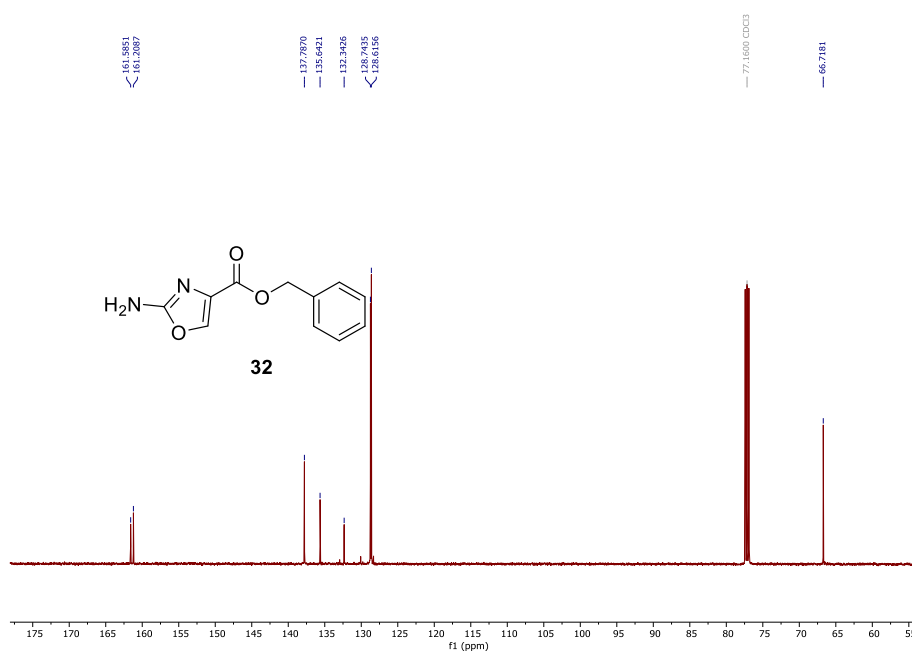


Figure 48. ¹³C NMR spectrum of benzyl 2-aminooxazole-4-carboxylate **32**

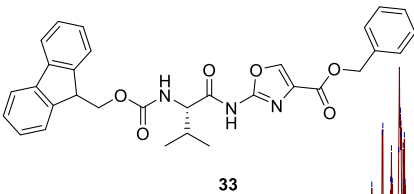


Figure 49. ¹H NMR spectrum of benzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)oxazole-4-carboxylate **33**

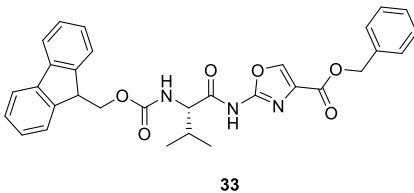


Figure 50. ^{13}C NMR spectrum of benzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)oxazole-4-carboxylate **33**

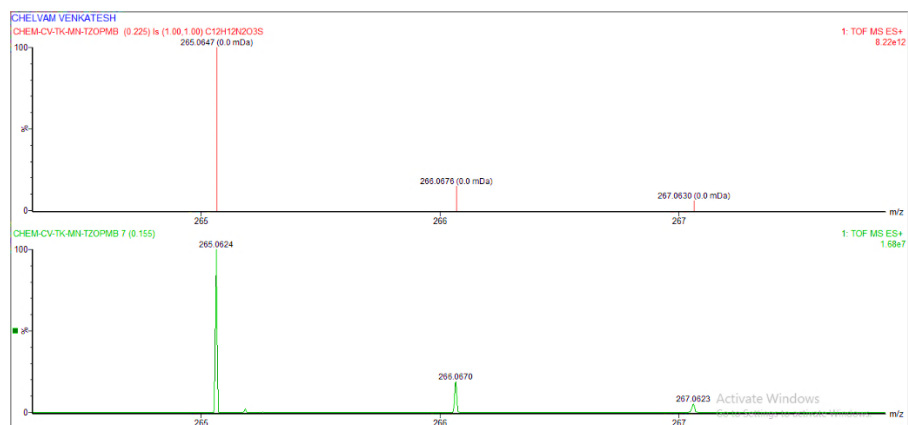


Figure 51. HRMS of 4-methoxybenzyl 2-aminothiazole-4-carboxylate **2**

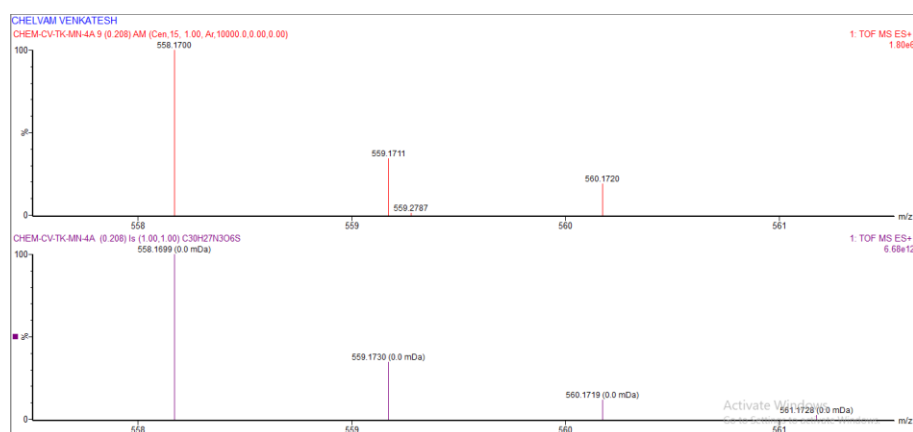


Figure 52. HRMS of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)propanamido)thiazole-4-carboxylate **4a**

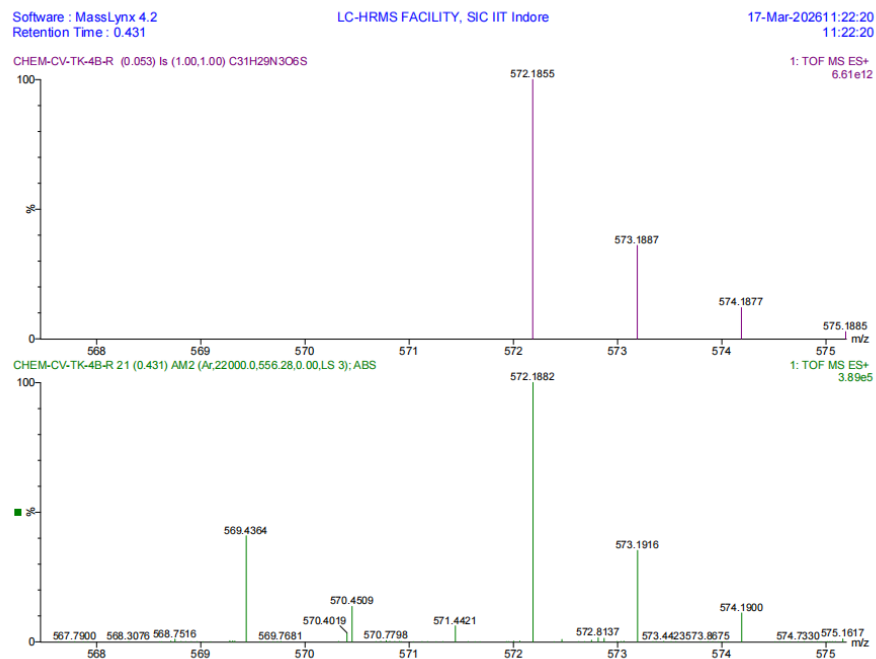


Figure 53. HRMS of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)butanamido)thiazole-4-carboxylate **4b**

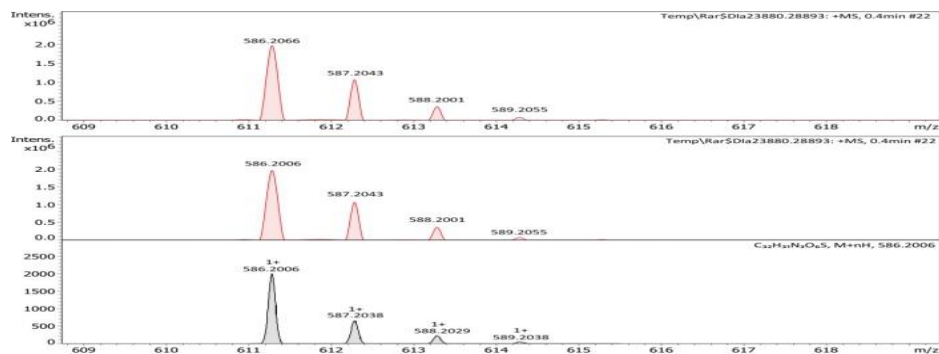


Figure 54. HRMS of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3-methylbutanamido)thiazole-4-carboxylate **4c**

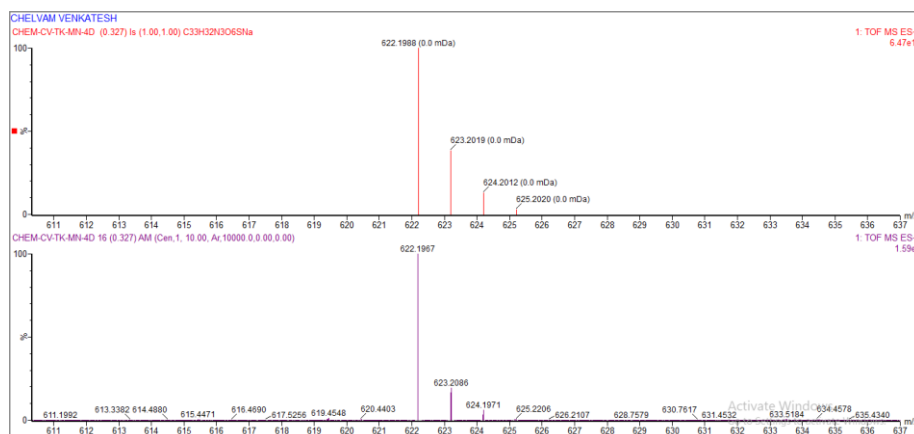


Figure 55. HRMS of 4-methoxybenzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-3,3-dimethylbutanamido)thiazole-4-carboxylate **4d**

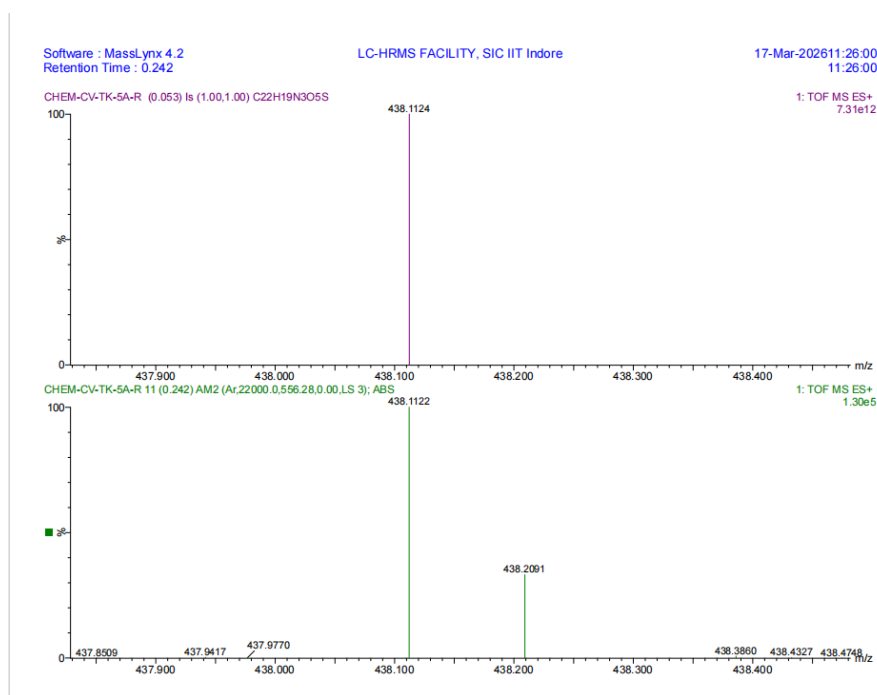


Figure 56. HRMS of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido) thiazole-4-carboxylic acid **5a**

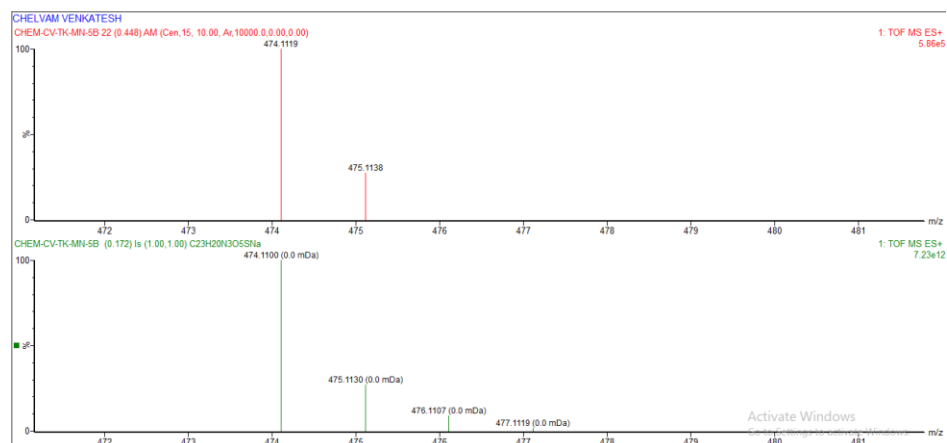


Figure 57. HRMS of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)butanamido) thiazole-4-carboxylic acid **5b**

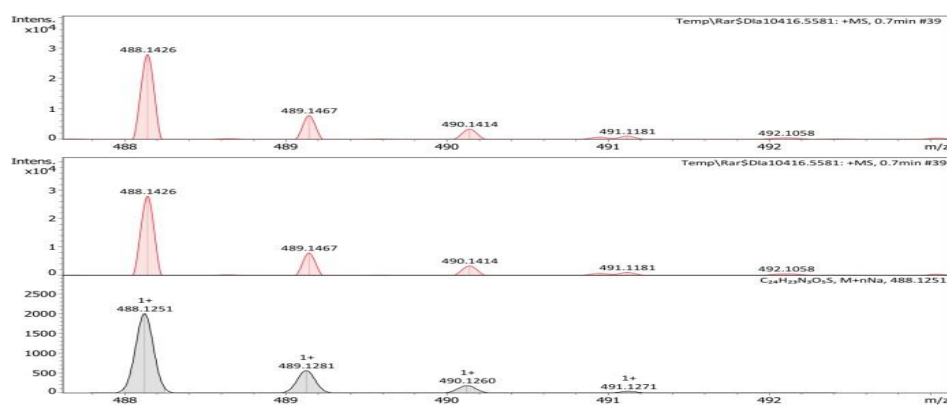


Figure 58. HRMS of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutan amido)thiazole-4-carboxylic acid **5c**

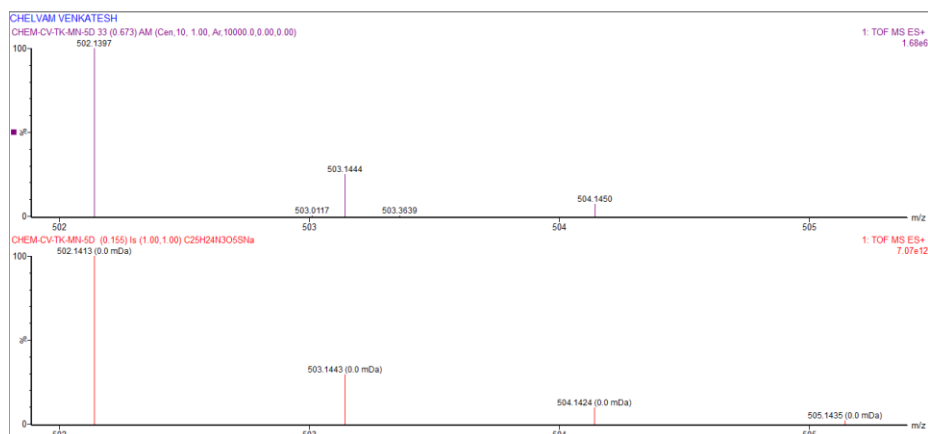


Figure 59. HRMS of (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylbutanamido)thiazole-4-carboxylic acid **5d**

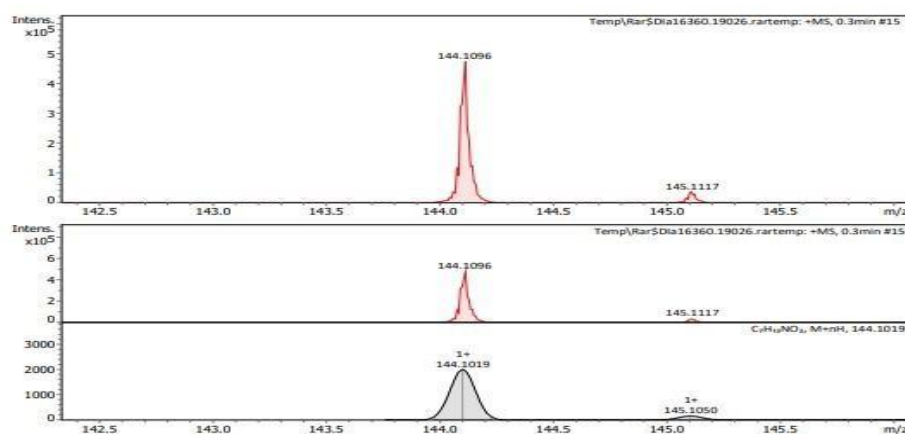


Figure 60. HRMS of (*R*)-1-Methylpiperidine-2-carboxylic acid **8a**

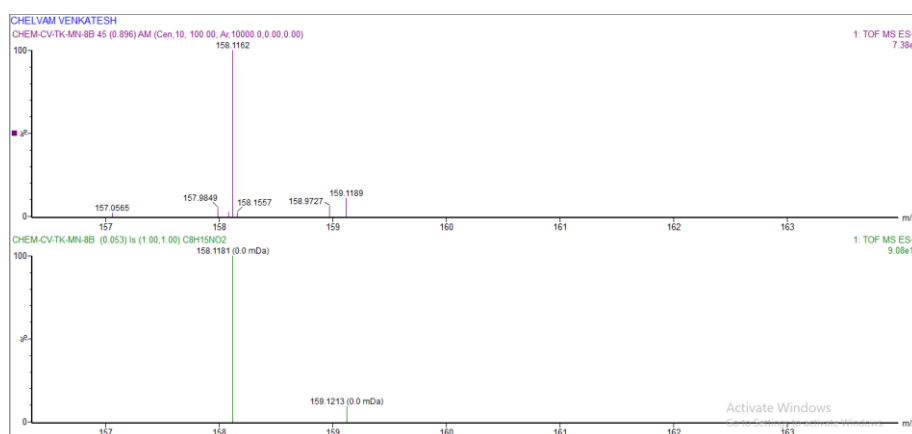


Figure 61. HRMS of (*R*)-1-ethylpiperidine-2-carboxylic acid **8b**

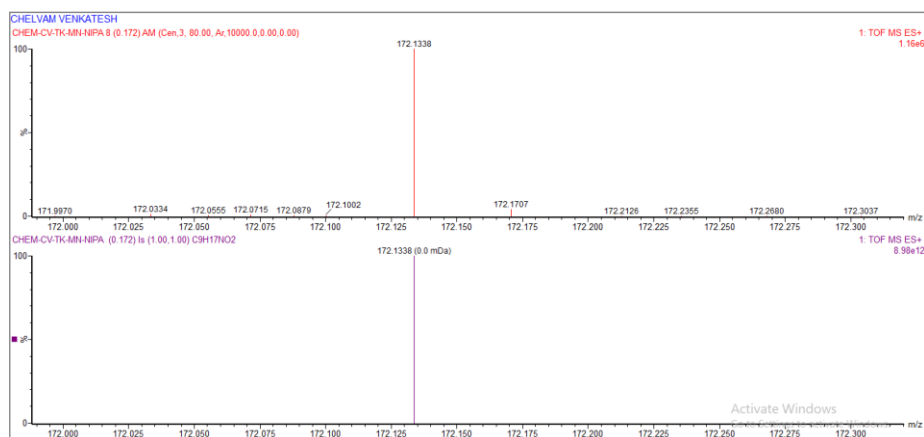


Figure 62. HRMS of (*R*)-1-isopropylpiperidine-2-carboxylic acid **8c**

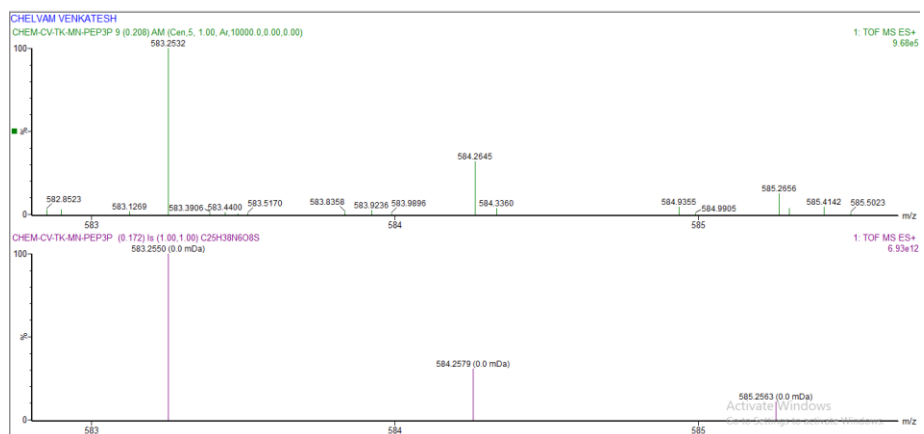


Figure 63. HRMS of tubulin inhibitor **Tub_01**

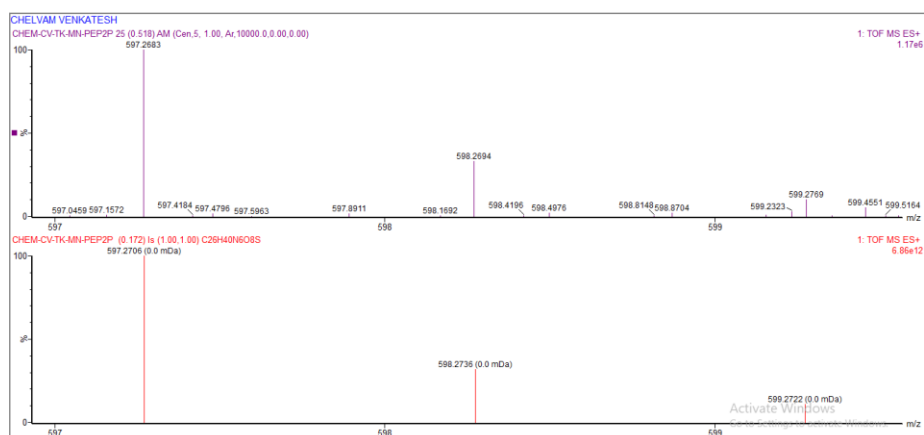


Figure 64. HRMS of tubulin inhibitor **Tub_02**

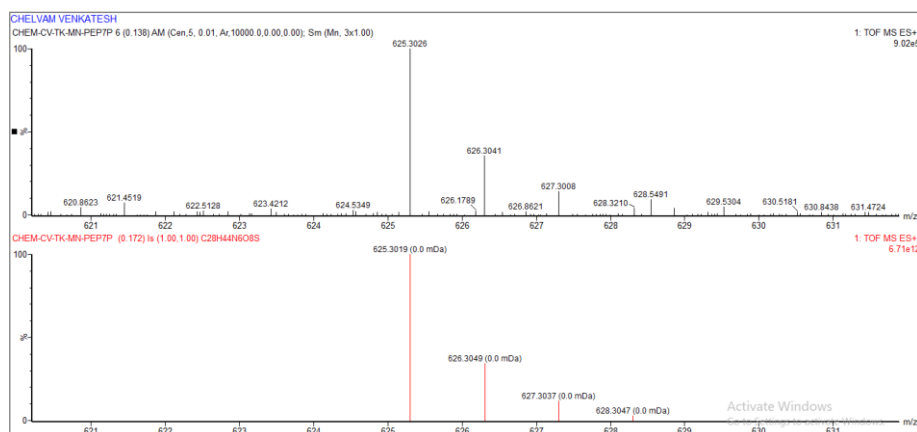


Figure 65. HRMS of tubulin inhibitor **Tub_03**

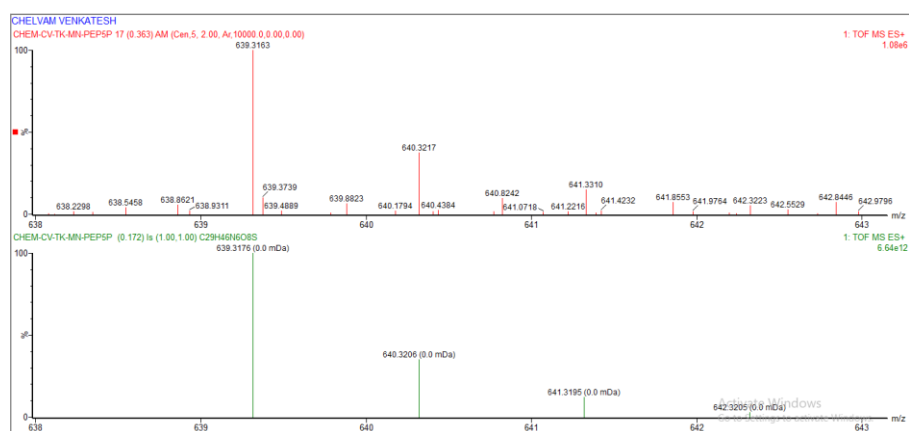


Figure 66. HRMS of tubulin inhibitor **Tub_04**

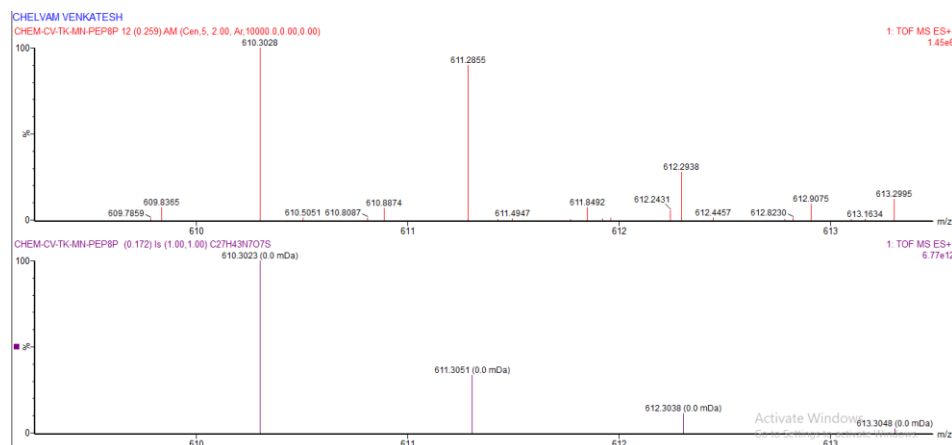


Figure 67. HRMS of tubulin inhibitor Tub_05

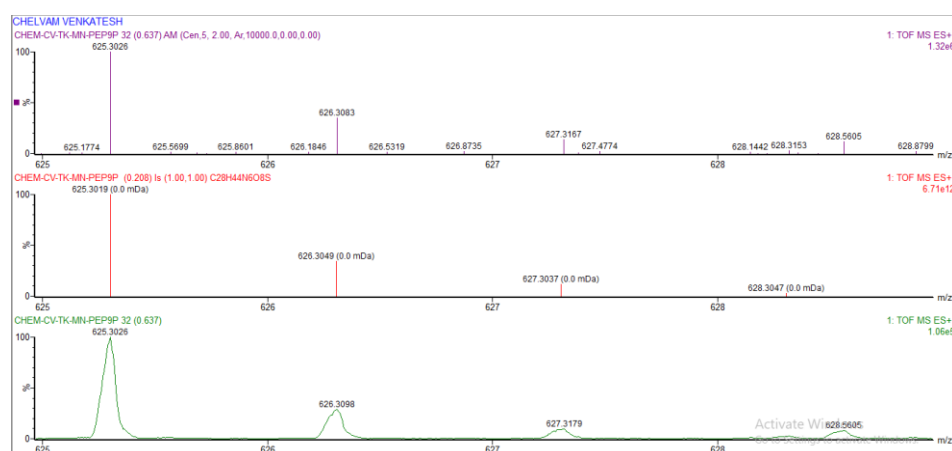


Figure 68. HRMS of tubulin inhibitor Tub_06

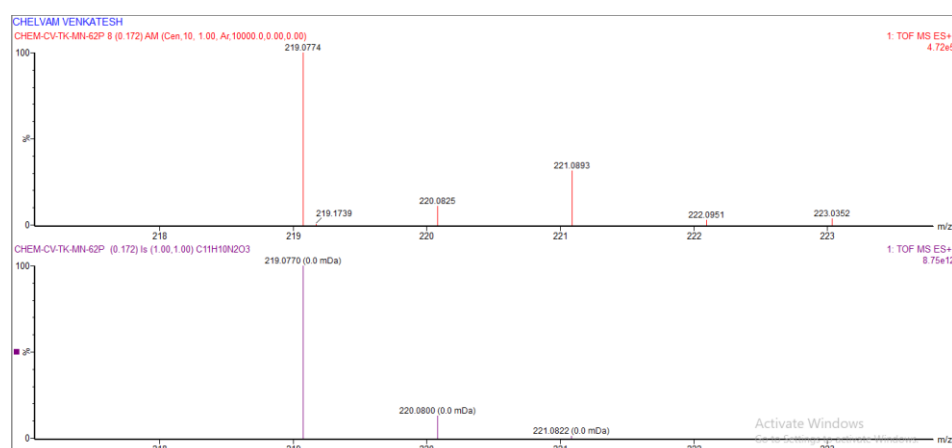


Figure 69. HRMS of benzyl-2-aminooxazole-4-carboxylate 32

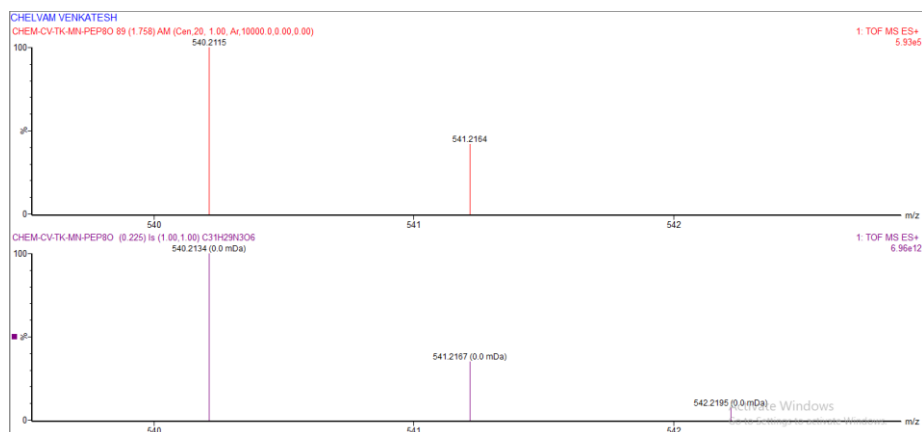


Figure 70. HRMS of benzyl (*S*)-2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)oxazole-4-carboxylate **33**

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