# Discovery of Novel Bioactive Compounds from a Rare Actinomycete *Amycolatopsis* sp. 26-4

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**Table of Contents**

# General Introduction

Natural products play an indispensable role in the drug development process. It is estimated that ca. 35% of small-molecule drugs is either natural products or their synthetic derivatives.[1] Since the discovery of penicillin in 1928 and streptomycin in 1943, so many antibiotics have been successfully discovered from microbes, such as sulfonamide, bacitracin, chloramphenicol, tetracycline, vancomycin, etc. There is no doubt that antibiotic drugs are the most successful chemotherapy, since new antibiotics saved millions of lives from infectious diseases worldwide and increased life expectancy by up to two decades in recent history.[2] Therefore, compounds with novel skeletons and biological activities have always been the hotspot of natural products research.

In natural products research, microorganisms still remains as important source of new drug compounds. So far, among the bioactive compounds that have been isolated from microbes, 45 % are produced by actinomycetes, 38 % by fungi and 17 % by unicellular eubacteria.[3] Actinomycetes continue to play the most significant role in drug discovery and development.

*Amycolatopsis*, genus of a rare actinomycete, was misidetentified as *Streptomyces* and later as *Nocardia* in the past. In 1986, *Amycolatopsis* was recognized as a unique genus by Lechevalier.[4] *Amycolatopsis* is an important actinomycete in the industry of antibiotics, which produced many potent antibiotics on the market, such as balhimycin by *Amycolatopsis balhimycina*, thiazomycins by *Amycolatopsis fastidiosa*, rifamycins by *Amycolatopsis mediterranei*, azicemicins by *Amycolatopsis sulphurea*, and vancomycin by *Amycolatopsis orientalis*.[5] Among these antibiotics, rifamycin is one of the major drugs used for clinical treatment of tuberculosis. Vancomycin is a famous antibiotics and recognized as the last line of defense against some resistant pathogenic bacteria. *Amycolatopsis* sp. exhibited the good ability of producing bioactive secondary metabolites.



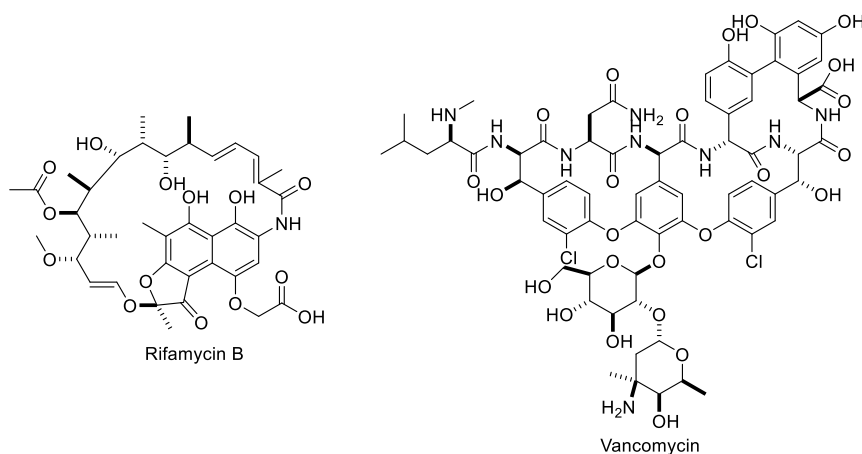**Figure 1** Structures of rifamycin B and vancomycin.

Sulfur, special element in natural products, always connected with aromatic ring as a thioether in the known compounds.[6] Thioethers in alphatic chain are rare, have never been discovered in peptides.[7] Sulfur often occurred as thiazoline or thiazole in peptides from microorganism and marine invertebrates, such as karamomycins, apratoxins,

marthiapeptides, trunkamides, bistratamides, bisebromoamide, etc.[8] Most of them exhibited cytotoxicity against human cancer cells. It is worth noting that sulfur-containing heterocycle plays a critical role in several bioactive natural products and pharmaceutical agents.
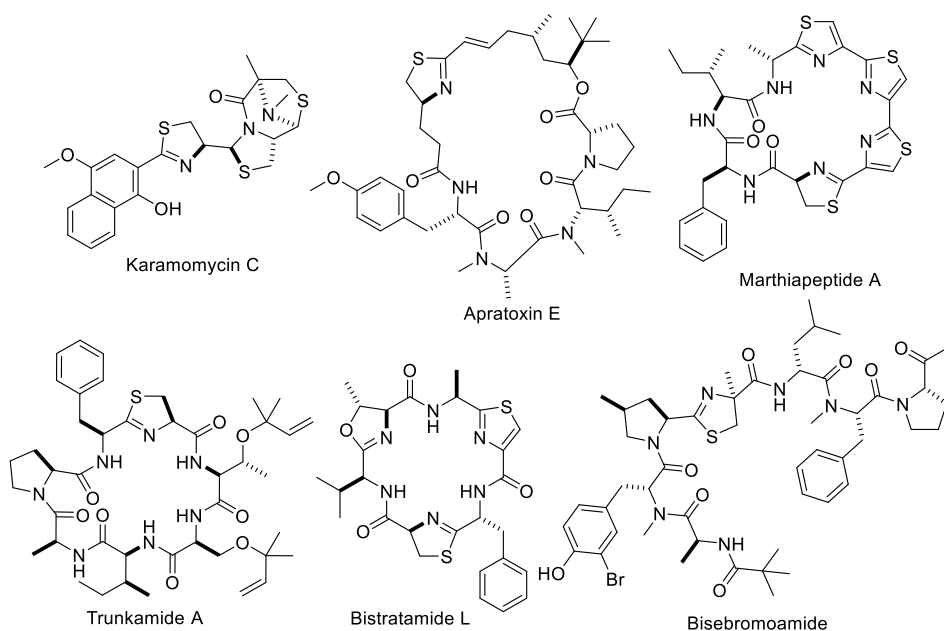


Karamomycin C

Apratoxin E

Marthiapeptide A

Trunkamide A

Bistratamide L

Bisebromoamide

**Figure 2** Structures of thiazole/thiazoline containing peptides.

Because of the unique structure and bioactivity, total synthesis of sulfur-containing peptides has been considering as an attractive research topic. Several compounds have been successfully synthesized in recent years.[9] Chemical synthesis of thiazole ring was easily accessible by various approaches and Hantzsch reaction discovered in 1889 remains one of the most reliable routes.[10] However, synthesis of thiazoline is not so easy due to the epimerization of chiral carbon. The condensation of L-cysteine and aryl nitriles or $\alpha,\alpha$-difluoroalkyl amines is an alternative way to obtain optically active thiazoline in good yields.[11] Dehydration reaction between thioamide and alcohol is commonly used in thiazoline containing peptide synthesis.[20,32]

Since the rise of antibiotic resistance, finding novel natural antibiotics to fight infection has become an urgent need.[12] In this research, I performed a systematic research for the secondry metabelites of *Amycolatopsis* sp. 26-4 derived from Iriomote island near Okinawa, Japan. Based on previous screenings for structurally unique and biologically interesting natural products, I isolated and identified a series of novel sulfur-containing heterocyclic lipopeptides named thioamycolamides from the culture broth of *Amycolatopsis* sp. 26-4. (**Chapter 1-1**). A bioinspired concise total synthesis of thioamycolamide A has been accomplished (**Chapter 1-2**). Two novel decapeptides, Amycolapeptin A and Amycolapeptin B, which were not detected in monoculture of *Amycolatopsis* sp. 26-4, have been isolated from the combined-culture broth of *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonsis* TP-B0596 (**Chapter 2**).

# Chapter 1

# Studies on Thioamycolamides Produced by *Amycolatopsis* sp. 26-4

## 1 Isolation, Structure Determination, and Cytotoxicity of Sulfur-Containing Cyclic Lipopeptides Thioamycolamides.

### 1.1 Introduction

Organosulfur compounds, such as thiols, sulfoxides, sulfones, thioesters, and thioamides, because of their reactive functions, have always been a research hotspot in organic chemistry.[13] As an essential element of organisms, sulfur also plays a critical role in primary metabolism, which has been discussed in various publications.[14] However, compared with other natural products, sulfur-containing characteristic secondary metabolites are relatively rare,[15] and we only have limited knowledge about their biological significance and biosynthetic mechanisms. Moreover, because of their novel structures and unique biological activities, unusual enzyme involved in organosulfur natural products synthesis is becoming the focus of many researchers worldwide.[16]

In natural products research, chemical screening without information of bioactivity is also an important method to discover novel metabolites.[17] From research for structurally unique and biologically interesting natural products, I investigated novel natural products from a library of rare actinomycetes derived from Iriomote island near Okinawa, Japan. Based on my preliminary screenings using physicochemical properties including both HRMS and UV spectroscopy, I discovered a series of compounds with molecular weight from 420 to 480 *m/z* from the culture broth of *Amycolatopsis* sp. 26-4. The chemical formular of these compounds were not recorded in the natural product mass databass (AntiBase), suggested that moleculars haven't been discovered before. Coumponds were subsequently isolated and identified as novel sulfur-containing heterocyclic lipopeptides named thioamycolamides A-E. Among these five metabolites with unprecedented carbon skeletons, thioamycolamide A (**1**) was produced as the major product by this Gram-positive bacterium (Figure 1.1.1).
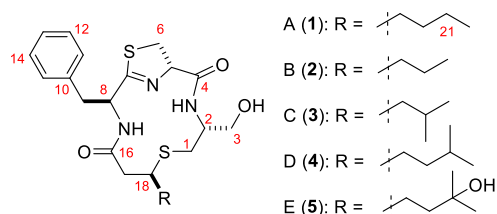


**Figure 1.1.1** Structures of thioamycolamides A-E (**1**-**5**).

## 1.2 Structure Elucidation of Thioamycolamides A-E

**Table 1.1.1** $^1$H NMR data (600 MHz) and $^{13}$C NMR data (150 MHz) of **1**–**5** in DMSO-$d_6$.

| | Thioamycolamide A (**1**) | | | Thioamycolamide B (**2**) | | | Thioamycolamide C (**3**) | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_C$, type | $\delta_H$ (*J* in Hz) | | $\delta_C$, type | $\delta_H$ (*J* in Hz) | | $\delta_C$, type | $\delta_H$ (*J* in Hz) |
| 1 | 34.9, CH$_2$ | 2.79, dd (13.0, 8.8) | 1 | 34.8, CH$_2$ | 2.80, dd (13.0, 8.8) | 1 | 34.9, CH$_2$ | 2.80, dd (12.9, 9.1) |
| | | 3.10, dd (13.0, 4.3) | | | 3.10, dd (13.0, 4.3) | | | 3.13, dd (12.8, 4.1) |
| 2 | 50.6, CH | 3.39, m | 2 | 50.6, CH | 3.39, m | 2 | 50.7, CH | 3.38, m |
| 3 | 61.9, CH$_2$ | 3.21, m | 3 | 61.9, CH$_2$ | 3.21, m | 3 | 61.9, CH$_2$ | 3.22, m |
| | | 3.51, m | | | 3.51, dt (10.5, 4.6) | | | 3.53, m |
| 4 | 170.2, C | | 4 | 170.2, C | | 4 | 170.2, C | |
| 5 | 77.7, CH | 5.08, t (6.7) | 5 | 77.7, CH | 5.08, t (6.7) | 5 | 77.7, CH | 5.08, dd (9.0, 4.4) |
| 6 | 36.8, CH$_2$ | 3.56, d (6.7) | 6 | 36.8, CH$_2$ | 3.56, d (6.9) | 6 | 36.9, CH$_2$ | 3.56, m |
| 7 | 177.7, C | | 7 | 177.6, C | | 7 | 177.7, C | |
| 8 | 54.3, CH | 4.54, m | 8 | 54.3, CH | 4.54, m | 8 | 54.2, CH | 4.55, m |
| 9 | 37.6, CH$_2$ | 2.96, dd (13.9, 9.9) | 9 | 37.6, CH$_2$ | 2.96, dd (13.9, 9.9) | 9 | 37.6, CH$_2$ | 2.96, dd (14.0, 9.9) |
| | | 3.03, dd (13.9, 5.0) | | | 3.03, dd (13.9, 5.0) | | | 3.03, dd (13.9, 5.1) |
| 10 | 137.4, C | | 10 | 137.4, C | | 10 | 137.4, C | |
| 11/15 | 129.1, CH | 7.32, d (7.9) | 11/15 | 129.1, CH | 7.32, d (7.8) | 11/15 | 129.1, CH | 7.32, d (7.2) |
| 12/14 | 128.3, CH | 7.29, t (7.6) | 12/14 | 128.3, CH | 7.29, t (7.6) | 12/14 | 128.3, CH | 7.29, t (7.6) |
| 13 | 126.6, CH | 7.22, t (7.1) | 13 | 126.6, CH | 7.22, t (7.2) | 13 | 126.6, CH | 7.22, t (7.1) |
| 16 | 171.6, C | | 16 | 171.5, C | | 16 | 171.6, C | |
| 17 | 42.5, CH$_2$ | 2.39, d(8.3) | 17 | 42.5, CH$_2$ | 2.38, m | 17 | 42.8, CH$_2$ | 2.38, m |
| 18 | 45.7, CH | 2.90, m | 18 | 45.4, CH | 2.92, m | 18 | 43.7, CH | 2.96, m |
| 19 | 36.8, CH$_2$ | 1.42, m | 19 | 39.3, CH$_2$ | 1.42, m | 19 | 46.2, CH$_2$ | 1.30, m |
| | | 1.54, m | | | 1.51, m | | | 1.37, m |
| 20 | 28.8, CH$_2$ | 1.35, m | 20 | 19.7, CH$_2$ | 1.41, m | 20 | 25.0, CH | 1.87, m |
| | | 1.45, m | | | 1.50, m | | | |
| 21 | 21.8, CH$_2$ | 1.28, m | 21 | 13.6, CH$_3$ | 0.88, t (6.8) | 21 | 22.9, CH$_3$ | 0.87, d (6.6) |
| 22 | 14.0, CH$_3$ | 0.87, t (7.3) | | | | 22 | 21.6, CH$_3$ | 0.87, d (6.6) |
| 2-NH | | 6.77, d (5.7) | 2-NH | | 6.77, d (5.7) | 2-NH | | 6.73, d (5.6) |
| 3-OH | | 4.82, s | 3-OH | | 4.82, dd (6.4, 5.1) | 3-OH | | 4.81, s |
| 8-NH | | 8.85, d (6.7) | 8-NH | | 8.85, d (6.8) | 8-NH | | 8.86, d (6.8) |

|  | Thioamycolamide D (**4**) | | | Thioamycolamide E (**5**) | |
| --- | --- | --- | --- | --- | --- |
|  | $\delta_C$, type | $\delta_H$ (*J* in Hz) |  | $\delta_C$, type | $\delta_H$ (*J* in Hz) |
| 1 | 35.0, $CH_2$ | 2.79, dd (13.0, 8.9) | 1 | 34.9, $CH_2$ | 2.79, dd (13.0, 8.8) |
|  |  | 3.10, dd (13.0, 4.3) |  |  | 3.10, dd (13.0, 4.3) |
| 2 | 50.6, CH | 3.39, m | 2 | 50.6, CH | 3.38, m |
| 3 | 61.9, $CH_2$ | 3.21, m | 3 | 61.9, $CH_2$ | 3.21, m |
|  |  | 3.51, m |  |  | 3.52, m |
| 4 | 170.2, C |  | 4 | 170.2, C |  |
| 5 | 77.7, CH | 5.08, dd (7.4, 6.0) | 5 | 77.7, CH | 5.08, t (6.7) |
| 6 | 36.8, $CH_2$ | 3.56, m | 6 | 36.8, $CH_2$ | 3.56, d (6.8) |
| 7 | 177.6, C |  | 7 | 177.6, C |  |
| 8 | 54.3, CH | 4.54, m | 8 | 54.2, CH | 4.54, m |
| 9 | 37.6, $CH_2$ | 2.95, dd (13.9, 9.9) | 9 | 37.6, $CH_2$ | 2.96, dd (13.9, 9.9) |
|  |  | 3.03, dd (13.9, 5.0) |  |  | 3.03, dd (13.9, 5.1) |
| 10 | 137.4, C |  | 10 | 137.4, C |  |
| 11/15 | 129.1, CH | 7.32, d (8.0) | 11/15 | 129.1, CH | 7.32, d (7.6) |
| 12/14 | 128.3, CH | 7.29, t (7.6) | 12/14 | 128.3, CH | 7.30, t (7.6) |
| 13 | 126.6, CH | 7.22, t (7.2) | 13 | 126.6, CH | 7.23, t (7.1) |
| 16 | 171.5, C |  | 16 | 171.5, C |  |
| 17 | 42.5, $CH_2$ | 2.40, d (8.3) | 17 | 42.6, $CH_2$ | 2.39, m |
| 18 | 46.0, CH | 2.88, m | 18 | 46.4, CH | 2.88, m |
| 19 | 35.8, $CH_2$ | 1.26, m | 19 | 32.2, $CH_2$ | 1.47, m |
|  |  | 1.38, m |  |  | 1.62, m |
| 20 | 34.9, $CH_2$ | 1.41, m | 20 | 40.9, $CH_2$ | 1.38, m |
|  |  | 1.56, m |  |  | 1.62, m |
| 21 | 27.3, CH | 1.50, m | 21 | 68.5, C |  |
| 22 | 22.7, $CH_3$ | 0.86, d (6.7) | 22 | 29.5, $CH_3$ | 1.06, s |
| 23 | 22.4, $CH_3$ | 0.85, d (6.7) | 23 | 29.3, $CH_3$ | 1.06, s |
| 2-NH |  | 6.77, d (5.7) | 2-NH |  | 6.77, d (5.7) |
| 3-OH |  | 4.82, dd (6.2, 5,2) | 3-OH |  | 4.83, s |
| 8-NH |  | 8.84, d (6.7) | 8-NH |  | 8.84, d (6.7) |
|  |  |  | 21-OH |  | 4.13, s |

*Amycolatopsis* sp. 26-4 culture broth (10 L) was filtered, which was extracted with EtOAc (2 × 10 L) and concentrated *in vacuo* to give crude extract 2.30 g. The extract was subjected to the ODS $C_{18}$ column using a gradient elution of $MeOH/H_2O$ stepwise to give 8 fractions. Each fraction was analysed by LCMS. By LCMS guided isolation, I successfully purified metabolites thioamycolamide A (22.20 mg), thioamycolamide B (2.68 mg), thioamycolamide C (4.22 mg), thioamycolamide D (2.54 mg) and thioamycolamide E (0.50 mg).

The molecular formula of thioamycolamide A (**1**) was determined to be $C_{22}H_{31}N_3O_3S_2$ by the analysis of HRMS measurement (*m/z* 450.1853 [M + H]$^+$ calcd. 450.1880). In order to elucidate the planar structure, detailed 1D & 2D NMR experiments (COSY, HMBC, and HSQC) were carried out (Table 1.1.1). The $^1$H-$^1$H COSY cross peaks of H13/H12 and H12/H11 suggested the existence of mono-substituted benzene ring. The HMBC correlations from methylene H9 to

C10 and C11 confirmed a connection between C10 and C9. The COSY signals for H9/H8 and H8/8-NH, accompanied with the HMBC cross peaks from H9, H8, and 8-NH to C7, indicated a phenylalanine moiety. The carbonyl carbon at C16 was positioned beside 8-NH, which was inferred from the strong cross peak of H8 and 8-NH to C16 in the HMBC spectrum. The presence of an aliphatic chain moiety was established by the analysis of the $^1$H-$^1$H COSY correlations; H17/H18, H18/H19, H19/H20, and H20/H21, and the HMBC data as shown in Figure 1.1.2. Moreover, from the HMBC signals, it was determined that C1 was connected with the aliphatic chain at C18 through a heteroatom. The $^1$H-$^1$H COSY spectrum also revealed correlations between H1/H2, H2/H3, H3/3-OH, and H2/2-NH, and the HMBC correlations from H2 to C4 and 2-NH to C4 corroborated linkages between C1/C2, C2/C3, C3/3-OH, 2/2-NH, and 2-NH/C4. The C5-7 moiety was confirmed to be thiazoline from $^1$H-$^1$H COSY correlations for H5/H6 and the HMBC correlations from H5 to C4/C7 and from H6 to C4/C5/C7, which would be generated by dehydration of cysteine. The heteroatom between C1 (δ 34.9 ppm) and C18 (δ 45.7 ppm) was assigned to be sulfur by the analysis of HRMS data and the chemical shifts of C1 and C18. Thus, the planar structure of thioamycolamide A was elucidated as shown in Figure 1.1.2 and included one phenylalanine, two cysteines and a lipid moiety.

The alkyl chain at C18 of each metabolite varied in terms of the number of carbon atoms and the branching pattern of the methyl groups. The carbon numbers found in the side chains of **1**-**5** were three, four and five, and the terminal structure of the side chains was normal- (without branched methyl) or iso-type. These structural moieties were confirmed by HRMS and NMR analyses (Figure 1.1.2). The congeners were named in the same way as thioamycolamides B, C, D, and E, respectively.
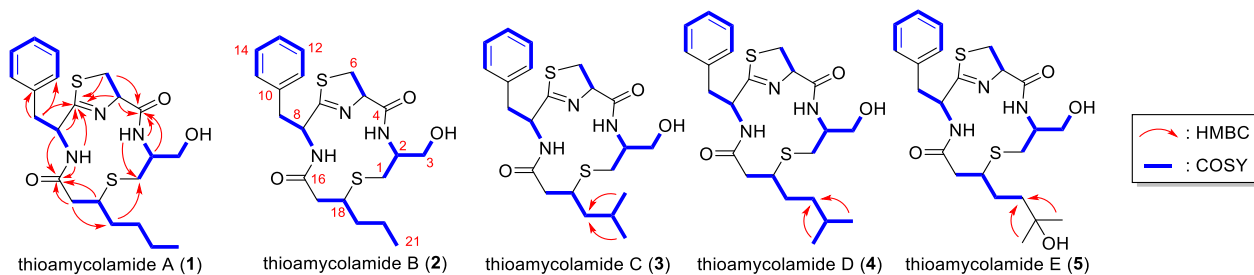


**Figure 1.1.2** Planar structures, key HMBC correlations, and COSY correlations of thioamycolamides A-E (**1**–**5**).

The absolute configurations of C5 and C8 were determined by the LC-MS based Marfey's analysis.[18] At this stage, the peptide **1** should be carefully derivatized for the chromatographic analysis because the C$_\alpha$ isomerization of peptides would often be problematic, leading to the structural misassignments.[19] Especially, the reversible isomerizations of thiazoline have been exemplified under acidic or basic conditions.[20] To suppress the isomerization, the peptide was hydrolyzed by 2 M aqueous hydrogen chloride at 90 ℃ for 4 h. Then, the hydrolysates were derivatized by L/D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucine-amide),[18] and analyzed by LC-MS. The comparison of the retention times between the standards and obtained derivertives shed light on the presence of L-Phe and D-Cys in **1** (See experimental section; Figure 1.1.5).
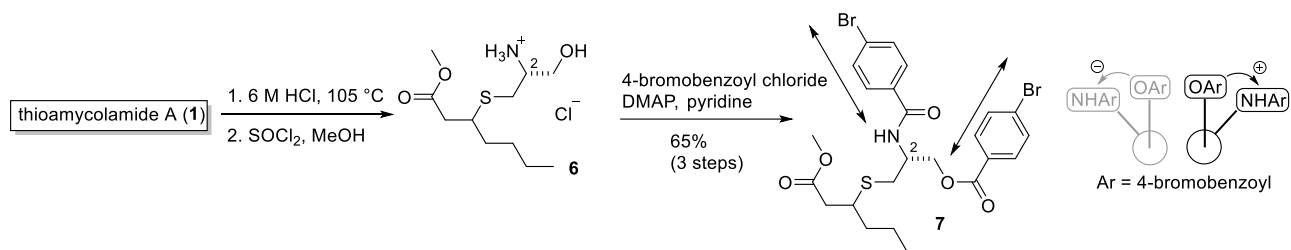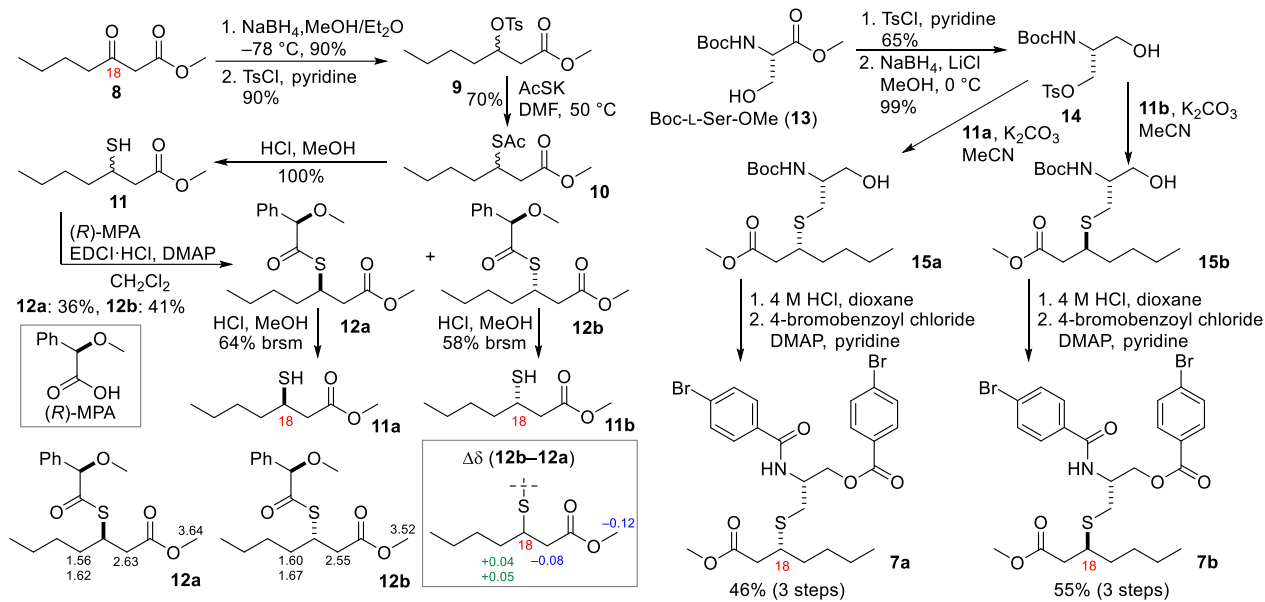
**Scheme 1.1.1** Synthesis of **7** from **1**.

To determine the configuration of C2, 4-bromobenzoate **7** was synthesized (Scheme 1.1.1). Total hydrolysis of **1** following with methyl esterification provided the key fragment **6**. Then, **6** was condensed with 4-bromobenzoyl chloride to deliver **7** in 65% yield over three steps. Bromobenzoate **7** exhibited positive exciton split CD bands at 255 nm ($\Delta\varepsilon$ 2.4) and 238 nm ($\Delta\varepsilon$ -3.5), thereby indicating *R* configuration at C2 (Figure 1.1.4).[21]

The chirality of C18 was determined by the combination of the chemical synthesis and NMR analysis, which was the most challenging task in this structural elucidation because the thioether bridge is rare in nature. The required standard samples for the spectroscopic comparison, namely, the 2*R*18*R* and 2*R*18*S* isomers of **7**, were prepared by the chemical synthesis and optical resolution (Scheme 1.1.2). The selective reduction of *β*-ketoester **8** by NaBH4 gave corresponding racemic alcohol, and then the alcohol was converted to the tosylate **9**. The nucleophilic substitution reaction between **9** and AcSK (potassium thioacetate) delivered the thioester **10**. Hydrolysis of **10** furnished thiol **11**, and then racemic thiol **11** was treated with (*R*)-MPA (2-methoxy-2-phenylacetic acid), EDCI HCl, and DMAP led to the diastereomixture of **12a**/**12b**, which could be separated by reversed-phase HPLC. Hydrolysis of thioesters **12a**/**12b** led to optically pure thiols **11a**/**11b**, respectively.

The absolute configuration of the thiols **11a** and **11b** were determined by applying Riguera's method in chiral thiols **11a**/**11b**.[22] Thioester **12b** is the enantiomer of **11a**-(*S*)-MPA, which could be considered as the equivalent compound in ^{1}H NMR. The difference of the ^{1}H NMR chemical shifts ($\Delta\delta$) between **12a** and **12b** validated the absolute configurations of C18 as depicted in **11a**/**11b**, respectively (Scheme 1.1.2).

**Scheme 1.1.2** Synthesis of **7a** and **7b**. (brsm = based on recovered starting material)

With optically pure thiols **11a** and **11b** in hand, the standard diastereomers **7a** and **7b** for the structural determination were chemically constructed (Scheme 1.1.2). At the outset, Boc-L-Ser-OMe (**13**) was transformed into corresponding tosylate, and then its methyl ester was reduced by *in situ* generated LiBH$_4$, leading to **14**. Subsequently, tosylate **14** was separately coupled with enantiomeric thiols **11a** and **11b** to furnish thioethers **15a** and **15b**, respectively. Treatment of **15a**/**15b** with 4 M HCl liberated the corresponding aminoalcohols, which were then condensed with 4-bromobenzoyl chloride to give **7a**/**7b**.

The $^1$H NMR spectra of synthesized two diastereomers **7a** and **7b** were then compared with that of authentic **7** derived from natural **1**. As charted in Figure 4, the NMR data of **7b** with C18(*S*)-configuration was identical to that of authentic **7**. Unifying the all data of our structural studies, the complete structure of **1** was elucidated as drawn in Figure 1.1.1.
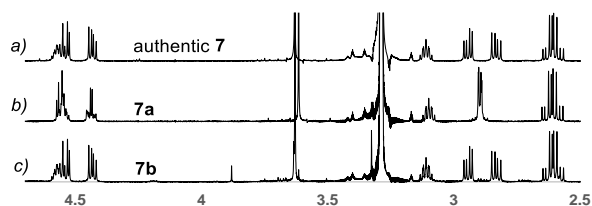


**Figure 1.1.3** $^1$H NMR spectra of authentic **7** and synthetic **7a**/**7b**.

The alkyl chain at C18 of each metabolite varied in terms of the number of carbon atoms and the branching pattern of the methyl groups. The carbon numbers found in the side chains of **1**-**5** were three, four and five, and the terminal structure of the side chains was normal- (without branched methyl) or iso-type. These structural moieties were confirmed by HRMS and NMR analyses. The congeners were named in the same way as thioamycolamides B, C, D, and E, respectively. The peptides **1**-**5** were isolated from the same strain and exhibited almost identical NMR data in the main skeleton (Table 1.1.1), which suggests that they may originate from a similar biosynthetic pathway; the optical rotation

of **1** ($[\alpha]_D^{20}$ = -75.0 ) is close to those of **2** ($[\alpha]_D^{20}$ = -91.9 ), **3** ($[\alpha]_D^{20}$ = -54.1 ), **4** ($[\alpha]_D^{20}$ = -94.1 ), and **5** ($[\alpha]_D^{20}$ = -114.1 ). Additionally, the ECD spectrum of **1** showed one positive Cotton effect at 222 nm and one negative Cotton effect at 252 nm, which were in good agreement with the other experimental curves, indicating the same absolute configurations at the chiral center of **2**-**5** (See spectra data; Figure S2).

## 1.3 Cytotoxicity

**Table 1.1.2** Cytotoxicties of **1**-**5** against human cancer cells (IC$_{50}$, $\mu$M).[a]

| Sample | HT 1080 | Hela S3 |
|---|---|---|
| Thioamycolamide A (**1**) | 11.94±1.43 | 21.22±2.57 |
| Thioamycolamide B (**2**) | 78.57±12.86 | 67.88±15.31 |
| Thioamycolamide C (**3**) | >100 | >100 |
| Thioamycolamide D (**4**) | 6.53±0.36 | 9.34±0.21 |
| Thioamycolamide E (**5**) | >100 | >100 |
| Adriamycin | 0.77±0.06 | 0.43±0.05 |

[a]IC$_{50}$ values are shown as mean ±SD (n=4).

The cytotoxicities of all isolated compounds were evaluated in two human cancer cell lines, fibrosarcoma HT1080 and cervix adenocarcinoma HeLa S3 (Table 1.1.2).[17d] Adriamycin was used as a positive control, with IC$_{50}$ values of 0.77 and 0.43 $\mu$M, respectively. Peptides **1** and **4** with a four carbon-chain length (*n*-butyl or 2-methyl-butyl) at C18 exhibited moderate cytotoxicity with IC$_{50}$ values of 6.53-21.22 $\mu$M, whereas **5** with a 2,2-dimethyl-2-butanol side chain had an IC$_{50}$ value over 100 $\mu$M, indicating the importance of the aliphatic property for their biological activities. Moreover, **2** and **3**, both of which have a three carbon-chain length at C18, showed weak (IC$_{50}$, 78.57, 67.88 $\mu$M, respectively) or no cytotoxicity even at 100 $\mu$M, respectively. These structure-activity relationships (SARs) suggest that both hydrophobicity and the length of the side chain at C18 are crucial for exhibiting the cytotoxicity presumably based on the difference of membrane permeability.

## 1.4 Proposed Biosynthetic Pathway
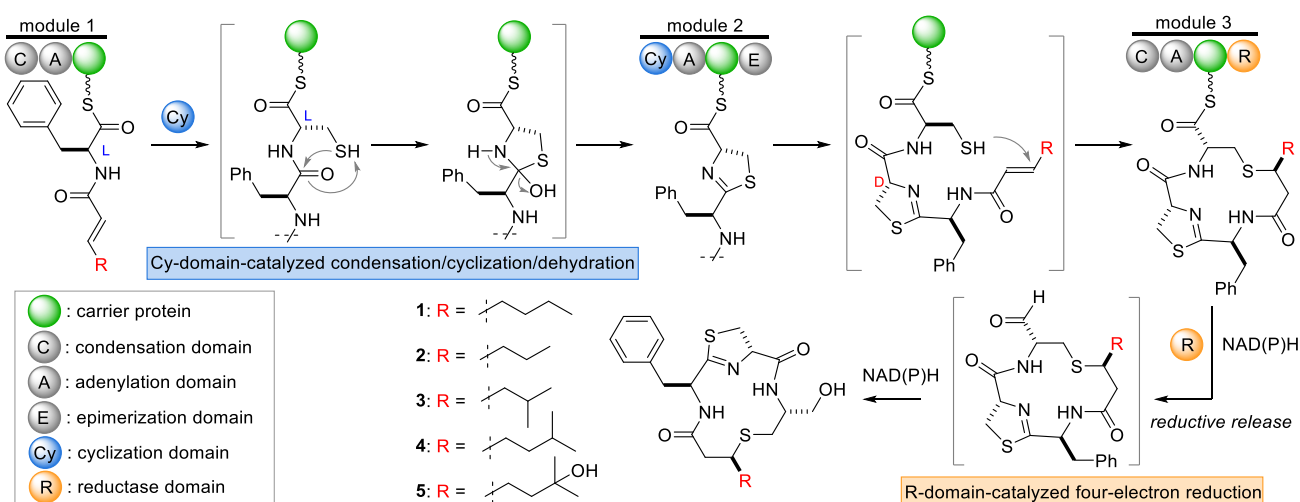


**Scheme 1.1.3** Putative biosynthetic pathway of **1**-**5**

9

Although the biosynthetic gene cluster of these peptide **1**-**5** has not been identified and the possibility of RiPPs pathway[23] can not be ruled out, based on their structural features, the biosynthesis of thioamycolamides is reasonably proposed to be assembled by non-ribosomal peptide synthetase (NRPS) using *α,β*-unsaturated fatty acids as the starter unit (Scheme 3). The putative cyclization (Cy) domain catalyzes the generation of the thiazoline ring between L-Phe and the adjacent D-Cys residues via three chemical steps (condensation, cyclization, and dehydration).[16a,24] Then, the second cysteine would be incorporated into the peptide chain, which would form the thioether linkage by Michael-type addition. In the canonical cases, a thioesterase (TE) domain is involved in the peptide release from the carrier protein. However, the C-terminus alcohol at C3 implies that these cyclic peptides would be released through reductase (R) domain-mediated four-electron reduction.[25]

## 1.5 Experimental Section

### 1.5.1 General Procedures

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AVANCE DMX 600 NMR (600 MHz for $^1$H NMR), JEOL ECA500 (500 MHz for $^1$H NMR), or a JEOL ECZ600 (600 MHz for $^1$H NMR) spectrometer. Chemical shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standard (CDCl$_3$, $^1$H δ 7.25, $^{13}$C δ 77.2, CD$_3$OD, $^1$H δ 3.31, $^{13}$C δ 49.0, DMSO-$d_6$, $^1$H δ 2.50, $^{13}$C δ 39.5). ESI-MS and LC-MS experiments were recorded on a Shimadzu LCMS-IT-TOF. Optical rotations were recorded on a JASCO P-2200 polarimeter. The IR spectra were run on a JASCO FTIR-4100 spectrometer equipped with a ZnSe ATR plate. UV spectra were recorded in MeOH on a Hitachi U-2910. CD spectra were measured on a JASCO J-715 circular dichroism spectrometer with a 1 mm path length cell. High performance liquid chromatography (HPLC) experiments were performed with a SHIMADZU HPLC system equipped with a LC-20AD intelligent pump. All reagents were used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F$_{254}$ pre-coated plates. Column chromatography was performed using 40-50 μm Silica Gel 60N (Kanto Chemical Co., Inc.).

### 1.5.2 Identification and Cultivation of the Actinomycete *Amycolaptosis sp*. 26-4

16S ribosomal RNA gene was used to identify the strain 26-4. This sequence was then used in BLAST search and strain 26-4 showed a similarity of 99.3% to sequence obtained from *Amycolatopsis vancoresmycina* strain ST101170. Therefore, strain 26-4 was identified as an *Amycolatopsis* sp. The strain was cultured on shakers (180 rpm) at 28 ℃ for 1 week in 1 L Erlenmeyer flasks containing 300 mL of liquid culture medium, composed of glucose (10.0 g/L), soluble starch (5.0 g/L), soytone (10.0 g/L), peptone (2.0 g/L), NaCl (2.0 g/L), K$_2$HPO$_4$ (0.5 g/L), MgSO$_4$ (0.5 g/L) and yeast extract (2.0 g/L).

### 1.5.3 Extraction and Isolation

10 L of the culture broth was filtered, which was extracted with EtOAc (2 × 10 L) and concentrated *in vacuo* to give crude extract (2.30 g). The extract was subjected to the ODS C$_{18}$ column using a gradient elution of MeOH/H$_2$O (10%, 30%, 50%, 70%, 100% MeOH in H$_2$O) stepwise to give 8 fractions. Every fraction was dissolved in methanol and centrifuged at 8,000 rpm for 5 min. Fraction 6 was separated by semi-preparative HPLC (Cosmosil ODS SP100 (Nacalai Tesque, Kyoto), Φ10 × 250 mm, MeCN:H$_2$O=40:60, 2 mL/min) to give compound thioamycolamide E (0.50 mg, $t_R$ 12.5

min) and compound thioamycolamide B (2.68 mg, $t_R$ 32.0 min). Fraction 7 was separated by preparative HPLC (Cosmosil ODS SP100 (Nacalai Tesque, Kyoto), $\Phi 20 \times 250$ mm, MeOH:H$_2$O=70:30, 8 mL/min) to give thioamycolamide A (22.20 mg, $t_R$ 31.0 min), thioamycolamide C (4.22 mg, $t_R$ 28.0 min) and thioamycolamide D (2.54 mg, $t_R$ 43.5 min).

Thioamycolamide A (**1**): white, powder, $[\alpha]^{20}_D$ = -75.0 (*c* 0.48, CH$_3$OH), UV/vis (CH$_3$OH) $\lambda_{max}$ (log$\varepsilon$) 205 (4.55), 252 (3.65), IR $\nu_{max}$ 3289, 2925, 2856, 1651, 1530, 1435, 1074, 1029 cm$^{-1}$, CD (*c* 1.11 $\times 10^{-3}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 222 (+10.8), 254 (-9.0), $^1$H and $^{13}$C NMR, Table 1.1.1; HRMS (ESI) calcd for C$_{22}$H$_{32}$N$_3$O$_3$S$_2^+$ [M + H]$^+$ 450.1880, found 450.1853.

Thioamycolamide B (**2**): white, powder, $[\alpha]^{20}_D$ = -91.9 (*c* 0.27, CH$_3$OH) , UV/vis (CH$_3$OH) $\lambda_{max}$ (log$\varepsilon$) 205 (4.51), 252 (3.64), IR $\nu_{max}$ 3286, 2956, 2927, 2870, 1648, 1534, 1266, 1025 cm$^{-1}$, CD (*c* 1.15 $\times 10^{-3}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 222 (+12.7), 253 (-10.7); $^1$H and $^{13}$C NMR, Table 1.1.1; HRMS (ESI) calcd for C$_{21}$H$_{30}$N$_3$O$_3$S$_2^+$ [M + H]$^+$ 436.1723, found 436.1708.

Thioamycolamide C (**3**): white, powder, $[\alpha]^{20}_D$ = -54.1 (*c* 0.42, CH$_3$OH) , UV/vis (CH$_3$OH) $\lambda_{max}$ (log$\varepsilon$) 204 (4.33), 252 (3.50), IR $\nu_{max}$ 3266, 2918, 1648, 1528, 1266, 1023 cm$^{-1}$, CD (*c* 1.11 $\times 10^{-3}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 221 (+7.3), 253 (-6.2), $^1$H and $^{13}$C NMR, Table 1.1.1, HRMS (ESI) calcd for C$_{22}$H$_{32}$N$_3$O$_3$S$_2^+$ [M+H]$^+$ 450.1880, found 450.1856.

Thioamycolamide D (**4**): white, powder, $[\alpha]^{20}_D$ = -94.1 (*c* 0.25, CH$_3$OH) , UV/vis (CH$_3$OH) $\lambda_{max}$ (log$\varepsilon$) 205 (4.73), 252 (3.81), IR $\nu_{max}$ 3296, 2922, 2867, 1655, 1541, 1455, 1366, 1029 cm$^{-1}$ , CD (*c* 1.08 $\times 10^{-3}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 221 (+15.1), 253 (-12.7), $^1$H and $^{13}$C NMR, Table 1.1.1, HRMS (ESI) calcd for C$_{23}$H$_{34}$N$_3$O$_3$S$_2^+$ [M + H]$^+$ 464.2036, found 464.2017.

Thioamycolamide E (**5**): white, powder, $[\alpha]^{20}_D$ = -114.1 (*c* 0.05, CH$_3$OH) , UV/vis (CH$_3$OH) $\lambda_{max}$ (log$\varepsilon$) 205 (4.65), 252 (3.83), IR $\nu_{max}$ 3296, 2970, 2925, 2853, 1655, 1541, 1022 cm$^{-1}$, CD (*c* 1.04 $\times 10^{-3}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 222 (+17.9), 254 (-15.5), $^1$H and $^{13}$C NMR, Table 1.1.1, HRMS (ESI) calcd for C$_{23}$H$_{34}$N$_3$O$_4$S$_2^+$ [M + H]$^+$ 480.1985, found 480.1977.

### 1.5.4 Cytotoxicity Assay

Cytotoxicity of **1**-**5** against HT1080 and HeLa S3 cell lines was evaluated by a WST-8 colorimetric assay (Cell Counting Kit-8, Dojindo). Briefly, cells were cultured in 96-well plates (3,000 cells/well) for 24 h followed by exposure to a sequential dilution of test compounds for 72 h, and then the viability was assessed by WST-8. The absorbance was measured at 450 nm using an iMark microplatereader (BIO-RAD). Adriamycin was evaluated as a positive control. IC$_{50}$ values are shown as the mean $\pm$ SD (n=4).

### 1.5.5 Synthesis of Bromobenzoate 7 from 1
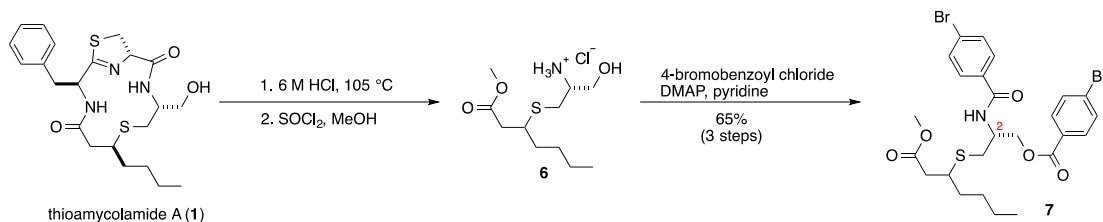
An analytical amount of **1** (1.0 mg, 2.22 μmol) was hydrolyzed with 6 M HCl (200 μL) for 12 h at 105 °C in oil bath, and then dried *in vacuo*. The obtained hydrolysate was dissolved in MeOH (100 μL), to which SOCl$_2$ (20 μL) was added at 0 °C. After 30 min the solution was dried *in vacuo*. The residue was mixed with 4-bromobenzoyl chloride (20 mg, 0.091 mmol) and DMAP (1 mg) in pyridine (0.2 mL), which was stirred at 40 °C for 12 h. The reaction was quenched with MeOH. The mixture was subjected to reversed-phase HPLC (Cosmosil ODS SP100 (Nacalai Tesque, Kyoto), Φ10 × 250 mm, H$_2$O/MeOH (10/90), 2mL/min) to yield **7** (0.89 mg, t$_R$ 24.0 min, 65% over three steps) as a white powder.

**7**: $^1$H NMR (600 MHz, CD$_3$OD): δ 7.91 (m, 2H), 7.69 (m, 2H), 7.62 (m, 4H), 4.58 (m, 1H), 4.54 (dd, *J* = 11.0, 4.5 Hz, 1H), 4.43 (dd, *J* = 11.0, 6.7 Hz, 1H), 3.63 (s, 3H), 3.11 (m, 1H), 2.94 (dd, *J* = 13.7, 6.2 Hz, 1H), 2.83 (dd, *J* = 13.7, 7.8 Hz, 1H), 2.61 (qd, *J* = 15.6, 7.1 Hz, 2H), 1.57 (m, 2H), 1.38 (m, 2H), 1.27 (m, 2H), 0.84 (t, *J* = 7.3 Hz, 3H); CD (*c* 0.96 × 10$^{-3}$ mol/L, MeOH) $\lambda_{max}$ (Δε): 238 (-3.5), 254 (+2.5), HRMS (ESI) calcd for C$_{25}$H$_{29}$NO$_5$SBr$_2$Na$^+$ [M + Na]$^+$ 636.0025, found 636.0010.
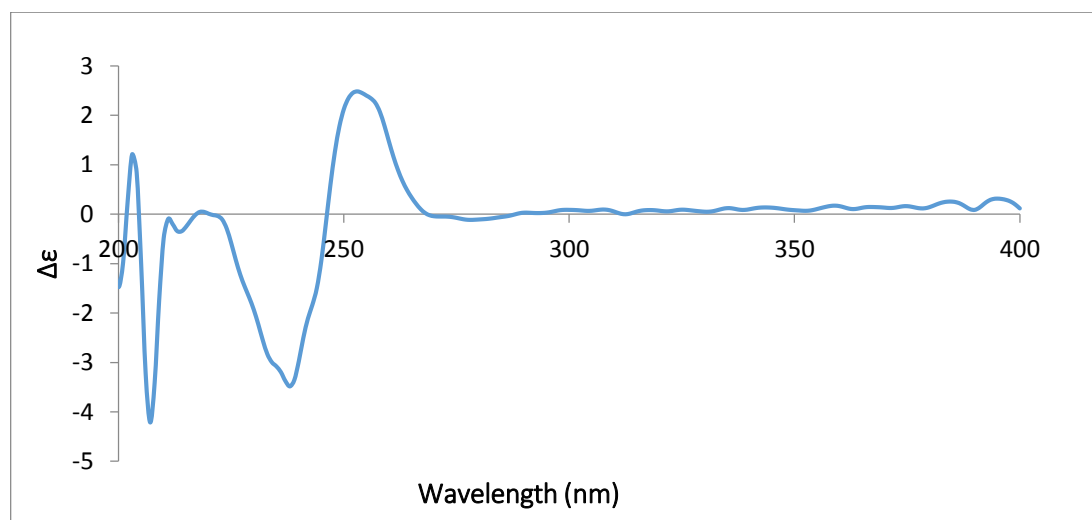


**Figure 1.1.4** Experimental ECD spectra of bromobenzoate **7** from thioamycolamide A (**1**) in MeOH.

### 1.5.6 Acid Hydrolysis of 1 and Marfey's Method

Thioamicolamide A (**1**) (0.1 mg) was hydrolyzed with 2 M HCl (100 μL) for 4 h at 90 °C in oil bath under N$_2$, and then dried in vacuo. The obtained hydrolysate was dissolved in H$_2$O (100 μL), to which 1 M NaHCO$_3$ (20 μL) was added. The hydrolysate added L- or D-FDLA (0.5% w/v in acetone, 20 μL), and the mixtures were stirred for 2 h at 50 °C under N$_2$. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL), evaporated, and then dissolved in MeCN (100 μL). The derivatives were analyzed by LC-MS; LC separation was performed on a reversed-phase column (UnisonUK-Phenyl (Imtakt Corp, Kyoto), Φ3.0 × 150 mm) with an isocratic elution system of H$_2$O/MeCN containing 0.05% TFA (40:60 for 30 min, 0.2 mL/min).

*L-FDLA product derived from natural thioamycolamide A (**1**)
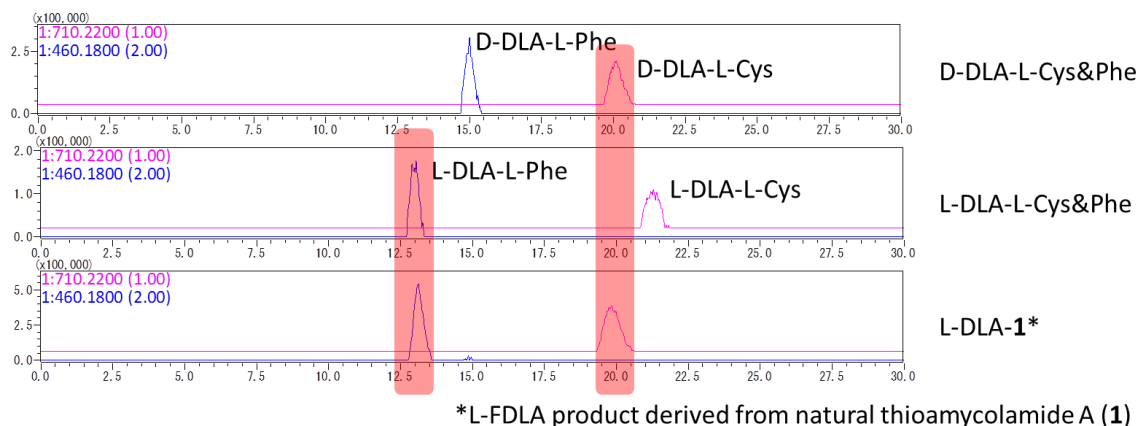
**Figure 1.1.5** LC-MS analysis of Marfey's derivatives

**1.5.7 Synthesis of Bromobenzoates 7a and 7b**



*β*-Ketoester **8** (3 mL, 18.9 mmol) was dissolved in MeOH/Et$_2$O (20/20 mL), to which NaBH$_4$ (710 mg, 18.9 mmol) was added at -78 ℃, then the reaction mixture was stirred for 10 min. The reaction was quenched by addition of aqueous solution of NH$_4$Cl (40 mL). The mixture was extracted with EtOAc (60 mL ×2). The combined organic layer was washed with brine (100 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc : hexanes =1 : 4) on silica gel to give hydroxyl ester **S1** (2.688 g, 90%) as a colorless oil.

**S1**: $^1$H NMR (500 MHz, CDCl$_3$): δ 3.91 (m, 1H), 3.60 (s, 3H), 2.41 (dd, *J* = 16.2, 3.5 Hz, 1H), 2.32 (dd, *J* = 16.2, 8.9 Hz, 1H), 1.38 (m, 3H), 1.22 (m, 3H), 0.80 (t, *J* = 7.1 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 173.3, 67.9, 51.6, 41.3, 36.3, 27.6, 22.5, 13.9; HRMS (ESI) calcd for C$_8$H$_{16}$O$_3$Na$^+$ [M + Na]$^+$ 183.0991, found 183.0973.
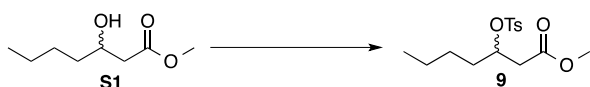


TsCl (1.65 g, 8.66 mmol) was added to an ice-cooled solution of **S1** (462.5 mg, 2.89 mmol, mixture of isomers) and in pyridine (8 mL). Stirring was continued at room temperature for 12 h. The reaction was quenched by addition of aqueous solution of NH$_4$Cl (20 mL). The mixture was extracted with EtOAc (20 mL ×2). The combined organic layer was washed with brine (40 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc : hexanes =1 : 15) on silica gel to yield tosylate **9** (816.3 mg, 90%) as a colorless oil.

**9**: $^1$H NMR (500 MHz, CDCl$_3$): δ 7.70 (d, *J* = 8.3 Hz, 2H), 7.26 (d, *J* = 8.1 Hz, 2H), 4.79 (m, 1H), 3.50 (s, 3H), 2.65 (dd, *J* = 15.8, 6.1 Hz, 1H), 2.50 (dd, *J* = 15.8, 6.7 Hz, 1H), 2.35 (s, 3H), 1.55 (m, 2H), 1.09 (m, 4H), 0.71 (t, *J* = 7.1 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.9, 144.7, 133.8, 129.6, 127.7, 79.2, 51.7, 39.4, 34.1, 26.5, 22.0, 21.4, 13.6; HRMS (ESI) calcd for C$_{15}$H$_{22}$O$_5$SNa$^+$ [M + Na]$^+$ 337.1080, found 337.1082.

13
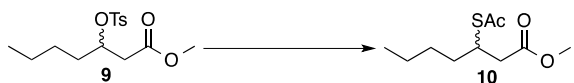
AcSK (862 mg, 7.55 mmol) was added to a solution of **9** (790 mg, 2.52 mmol) in DMF (8 mL). The mixture was heated in oil bath to 50 °C for 1 h, cooled, diluted by addition of brine (20 mL). The mixture was extracted with Et$_2$O (20 mL × 2). The combined organic layer was washed with brine (40 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane/EtOAc, 40:1) to yield thioester **10** (380 mg, 70%) as a yellowish oil.

**10**: $^1$H NMR (500 MHz, CDCl$_3$): δ 3.81 (m, 1H), 3.65 (s, 3H), 2.61 (m, 2H), 2.29 (s, 3H), 1.62 (m, 2H), 1.30 (m, 4H), 0.86 (t, $J$ = 7.1 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 195.2, 171.4, 51.7, 40.3, 39.6, 33.8, 30.6, 29.0, 22.3, 13.9; HRMS (ESI) calcd for C$_{10}$H$_{18}$O$_3$SNa$^+$ [M + Na]$^+$ 241.0868, found 241.0861.



Thioester **10** (199 mg, 0.91 mmol) and 2 M HCl in MeOH (3 mL, prepared from 1.5 mL MeOH and 1.5 mL 4 M HCl in dioxane) was stirred at room temperature for 12 h. The solvent was removed at reduced pressure to yield thiol **11** (160 mg, 100%) as a yellowish oil, which was used without further purification.

**11**: $^1$H NMR (500 MHz, CDCl$_3$): δ 3.68 (s, 3H), 3.17 (m, 1H), 2.66 (dd, $J$ = 15.9, 5.3 Hz, 1H), 2.50 (dd, $J$ = 15.8, 8.8 Hz, 1H), 1.66 (d, $J$ = 7.2 Hz, 1H), 1.61 (m, 1H), 1.46 (m, 2H), 1.32 (m, 3H), 0.88 (t, $J$ = 7.1 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 172.0, 51.8, 44.2, 38.0, 36.6, 29.3, 22.4, 14.0; HRMS (ESI) calcd for C$_8$H$_{17}$O$_2$S$^+$ [M + H]$^+$ 177.0944, found 177.0896.
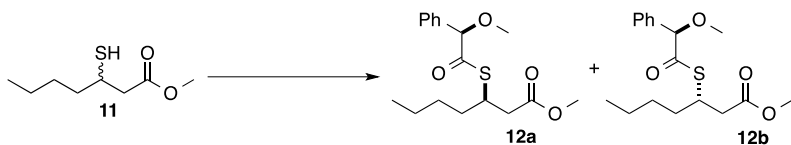


Thiol **11** (50 mg, 0.28 mmol) was mixed with (R)-MPA (2-methoxy-2-phenylacetic acid) (51.8 mg, 0.31 mmol) in dry CH$_2$Cl$_2$ (2 mL) under N$_2$, followed by EDCI·HCl (59.8 mg, 0.31 mmol) and DMAP (3.5 mg, 0.03 mmol) at 0 °C. The resulting mixture was allowed to warm up to rt and stirred 1 h. The reaction was quenched with 0.2 M HCl (3 mL). The combined organics were washed with brine (5 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was subjected to reversed-phase HPLC (CHIRALPAK IC 10 × 250 mm (Senshu Scientific Co. Ltd.), H$_2$O/MeOH (25/75)) to yield MPA derivatives **12a** (33.4 mg, t$_R$ 44.0 min, 36%) and **12b** (37.6 mg, t$_R$ 47.0 min, 41%) as a colorless oil.

**12a**: [α]$^{20}$$_D$ = 24.8 (c 0.33, MeOH); $^1$H NMR (500 MHz, CDCl$_3$): δ 7.42 (m, 2H), 7.34 (m, 3H), 4.72 (s, 1H), 3.80 (m, 1H), 3.64 (s, 3H), 3.45 (s, 3H), 2.63 (m, 2H), 1.64 (m, 1H), 1.55 (m, 1H), 1.23 (m, 4H), 0.80 (t, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 200.1, 171.6, 136.3, 128.8, 128.7, 127.1, 89.0, 58.2, 51.9, 39.8, 39.4, 34.0, 29.0, 22.4, 14.0; HRMS (ESI) calcd for C$_{17}$H$_{24}$O$_4$SNa$^+$ [M + Na]$^+$ 347.1287, found 347.1286.

**12b:** [α]$^{20}$$_D$ = -9.2 (c 0.33, MeOH); $^1$H NMR (500 MHz, CDCl$_3$): δ 7.41 (m, 2H), 7.34 (m, 3H), 4.73 (s, 1H), 3.81 (m, 1H), 3.52 (s, 3H), 3.46 (s, 3H), 2.55 (d, $J$ = 6.8 Hz, 2H), 1.68 (m, 1H), 1.59 (m, 1H), 1.31 (m, 4H), 0.86 (t, $J$ = 7.1

Hz, 3H) NMR (125 MHz, CDCl$_3$) δ 200.1, 171.5, 136.3, 128.8, 128.7, 127.1, 89.0, 58.2, 51.7, 39.9, 39.4, 34.0, 29.1, 22.5, 14.0; HRMS (ESI) calcd for C$_{17}$H$_{25}$O$_4$S$^+$ [M + H]$^+$ 325.1468, found 325.1429.



Thioester **12a** (16.7 mg, 0.05 mmol) and 2 M HCl in MeOH (1 mL, prepared from 0.5 mL MeOH and 0.5 mL 4 M HCl in dioxane) was stirred at room temperature for 24 h. The solvent was removed at reduced pressure. The residue was purified by column chromatography (silica gel, hexane/EtOAc, 10:1) to yield thiol **11a** (2.50 mg, 28%) and starting material **12a** (9.35 mg, 56% was recovered) as a yellowish oil.

**11a**: [α]$^{20}$$_D$ = -9.6 (c 0.04, MeOH); $^1$H NMR & $^{13}$C NMR signals are identical to those of **11**.



Thioester **12b** (18.8 mg, 0.06 mmol) and 2 M HCl in MeOH (1 mL, prepared from 0.5 mL MeOH and 0.5 mL 4 M HCl in dioxane) was stirred at room temperature for 24 h. The solvent was removed at reduced pressure. The residue was purified by column chromatography (silica gel, hexane/EtOAc, 10:1) to yield thiol **11b** (2.36 mg, 23%) and starting material **12b** (11.31 mg, 60% was recovered) as a yellowish oil.

**11b**: [α]$^{20}$$_D$ = 15.6 (c 0.03, MeOH); $^1$H NMR & $^{13}$C NMR signals are identical to those of **11**.
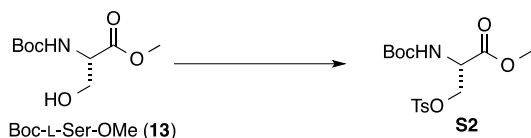


TsCl (437.8 mg, 2.30 mmol) was added to a stirred solution of Boc-L-Ser-OMe (**13**) (250 mg, 1.14 mmol) in pyridine (3 mL) at 0 ℃. The resulting mixture was allowed to warm up to rt and stirred overnight. The reaction was quenched by addition of aqueous solution of NH$_4$Cl (10 mL). The mixture was extracted with EtOAc (10 mL × 2). The combined organic layer was washed with brine (20 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc : hexanes =1 : 5) on silica gel to give tosylate **S2** (270.1 mg, 65%) as a colorless oil.

**S2**: [α]$^{20}$$_D$ = 4.1 (c 0.44, MeOH).    $^1$H NMR (500 MHz, CDCl$_3$): δ 7.72 (d, J = 6.8 Hz, 2H), 7.31 (d, J = 6.8 Hz, 2H), 5.32 (d, J = 7.4, 1H), 4.47 (d, J = 6.4, 1H), 4.35 (d, J = 10.1, 1H), 4.25 (d, J = 10.1, 1H), 3.64 (s, 3H), 2.40 (s, 3H), 1.37 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.0, 155.0, 145.2, 132.3, 130.0, 128.0, 80.5, 69.5, 53.0, 52.9, 28.2, 21.7; HRMS (ESI) calcd for C$_{16}$H$_{23}$NO$_7$SNa$^+$ [M + Na]$^+$ 396.1087, found 396.1097.

NaBH$_4$ (82 mg, 2.17 mmol) was added to the solution of tosylate **S2** (270 mg, 0.72 mmol) in THF (1 mL) at 0 ℃, followed by LiCl (92 mg, 2.17 mmol) and MeOH 3 mL. The reaction mixture was stirred at 0 ℃ for 0.5 h then allowed to warm to rt for 1h. The reaction was quenched by addition of citric acid aqueous solution (10%, w/v 10 mL). The mixture was extracted with CH$_2$Cl$_2$ (10 mL ×2). The combined organics were washed with saturated aqueous solution of NaHCO$_3$ and brine (20 mL for each), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo* to give alcohol **14** (249 mg, 99%) as a colorless oil without further purification.

**14**: $[\alpha]^{20}_D$ = 12.4 (*c* 0.61, MeOH); $^1$H NMR (500 MHz, CDCl$_3$): δ 7.76 (d, *J* = 8.0 Hz, 2H), 7.33 (d, *J* = 7.9 Hz, 2H), 5.06 (s, 1H), 4.09 (m, 2H), 3.82 (m, 1H), 3.71 (m, 1H), 3.59 (m, 1H), 2.42 (s, 3H), 1.38 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 155.6, 145.3, 132.4, 130.1, 128.0, 80.2, 68.4, 61.1, 50.9, 28.3, 21.7; HRMS (ESI) calcd for C$_{15}$H$_{23}$NO$_6$SNa$^+$ [M + Na]$^+$ 368.1138, found 368.1135.



An analytical amount of **11a** (0.48 mg, 2.72 μmol) and tosylate **14** (1.90 mg, 5.45 μmol) were added in MeCN (200 μL) under N$_2$, to which K$_2$CO$_3$ (0.80 mg, 5.79 μmol) was added at room temperature for 24 h, then filtered and concentrated *in vacuo* to give crude thioether **15a**, which was used in the next reaction without further purification.

Thioether **15a** and 4 M HCl in dioxane (0.2 mL) were stirred at room temperature for 1 h. The solvent was removed *in vacuo*. The obtained hydrolysate was mixed with 4-bromobenzoyl chloride (6.00 mg, 27.3 μmol) and catalyst DMAP (0.04 mg, 0.3 μmol) in pyridine (300 μL), which was stirred at 40 ℃ in oil bath for 24 h. The reaction was quenched with MeOH and concentrated in vauo. The residue was subjected to reversed-phase HPLC (Cosmosil ODS SP100 ID or Φ20 × 250 mm, H$_2$O/MeOH (10/90)) to yield bromobenzoate **7a** (0.77 mg, t$_R$ 30.0 min, 46% over three steps) as a white powder.

**7a**: $^1$H NMR (600 MHz, CD$_3$OD): δ 7.90 (m, 2H), 7.70 (m, 2H), 7.62 (m, 4H), 4.55 (m, 2H), 4.44 (m, 1H), 3.61 (s, 3H), 3.10 (m, 1H), 2.90 (dd, *J* = 6.7, 1.4 Hz, 2H), 2.61 (qd, *J* = 15.7, 7.2 Hz, 2H), 1.58 (m, 2H), 1.39 (m, 2H), 1.28 (m, 2H), 0.86 (t, *J* = 7.3 Hz, 3H); $^{13}$C NMR (150 MHz, CD$_3$OD) δ 173.8, 169.4, 166.9, 134.7, 133.0, 132.8, 132.4, 130.3, 130.3, 129.2, 127.2, 66.5, 52.2, 51.0, 43.7, 41.7, 35.9, 32.4, 30.2, 23.5, 14.3; HRMS (ESI) calcd for C$_{25}$H$_{30}$NO$_5$SBr$_2$$^+$ [M + H]$^+$ 614.0206, found 614.0176.
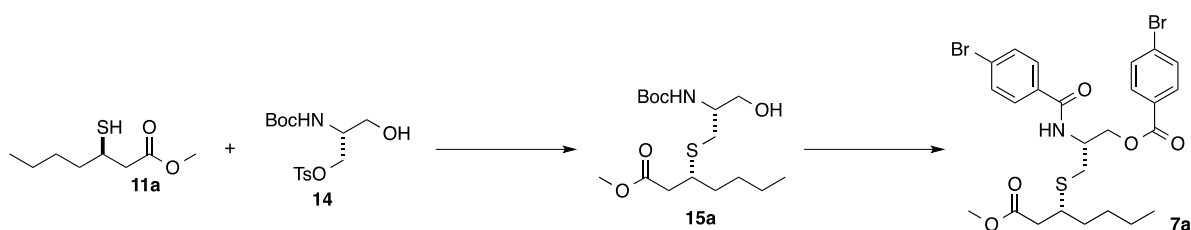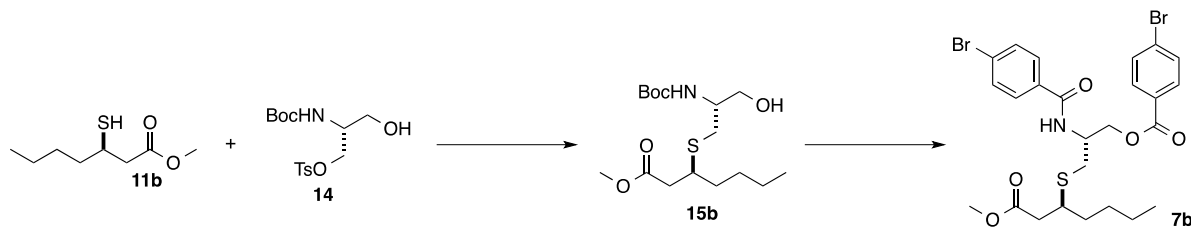
Bromobenzoate **7b** (0.91 mg, $t_R$ 30.0 min, 55% over three steps from **11b**) was synthesized by the same procedures as those of **7a**.

**7b**: ^{1}H NMR (600 MHz, CD$_3$OD): δ 7.91 (m, 2H), 7.69 (m, 2H), 7.62 (m, 4H), 4.58 (m, 1H), 4.54 (dd, $J$ = 11.1, 4.5 Hz, 1H), 4.43 (dd, $J$ = 11.1, 6.7 Hz, 1H), 3.63 (s, 3H), 3.11 (m, 1H), 2.94 (dd, $J$ = 13.7, 6.2 Hz, 1H), 2.83 (dd, $J$ = 13.7, 7.9 Hz, 1H), 2.61 (qd, $J$ = 15.7, 7.2 Hz, 2H), 1.56 (m, 2H), 1.37 (m, 2H), 1.26 (m, 2H), 0.84 (t, $J$ = 7.3 Hz, 3H); ^{13}C NMR (150 MHz, CD$_3$OD) δ 173.9, 169.3, 167.0, 134.9, 133.0, 132.7, 132.4, 130.3, 130.3, 129.2, 127.0, 63.8, 53.3, 52.2, 43.1, 41.7, 35.9, 32.4, 30.2, 23.5, 14.3; HRMS (ESI) calcd for $C_{25}H_{30}NO_5SBr_2^+$ [M + H]$^+$ 614.0206, found 614.0140.

# 2 Bioinspired Concise Total Synthesis of Thioamycolamide A

## 2.1 Introduction

As described in chapter 1-1, rare sulfur-containing cycliclipopeptide thioamycolamide A (**1**, Figure 1.1.1) along with the minor analogues thioamycolamides B-E (**2**-**5**) were isolated.[26] The peptide **1** shows moderate inhibitory activities against several human cancer cell lines, and chemical structure of **1** was established by the combination of spectroscopic analyses and chemical synthesis of their partial structure. Its cyclic skeletal structure bears a D-configured thiazoline, a thioether bridge, a fatty acid-side chain, and a reduced C-terminus. Although its biosynthetic gene cluster has not been identified, these structural features suggest that the cytotoxin **1** was assembled by a nonribosomal peptide synthetase (NRPS) as illustrated in Scheme 1.1.3.

The highly modified structure of **1** would gain much attention from chemists, though the reported biosynthetically unusual structures are sometimes turned out to be structural missassignments, and therefore its unprecedented structure demands to be confirmed by chemical synthesis before launching programs on the biosynthetic study. I completed the concise total synthesis of **1** based on its putative biosynthetic pathway. Furthermore, the highly sensitive labeling reagent was efficiently utilized for the structural confirmation of natural **1** and synthetic compounds.

The major obstacle in the total synthesis of **1** was arose from the highly epimerizable nature of thiazoline moiety (Scheme 1.2.1a).[20,27] In addition, although the late stage thiazoline formation (Scheme 1.2.1b) seemed to be a plausible alternative, the synthesis of thioamide **25** is a puzzle due to the formation of thiazolinone **30** in the peptide chain elongation (Scheme 1.2.1c).[28] Moreover, another approach for preventing the above thiazolinone formation, namely, thioamidation after the peptide elongation requires site-selective thioamidation. Accordingly, in this study, linear peptide **16** was planned to be synthesized with limited reactions which suppress the isomerization of thiazoline. On the other hand, linear peptide **16** would be cyclized to **1** without isomerization through a thio-Michael addition.
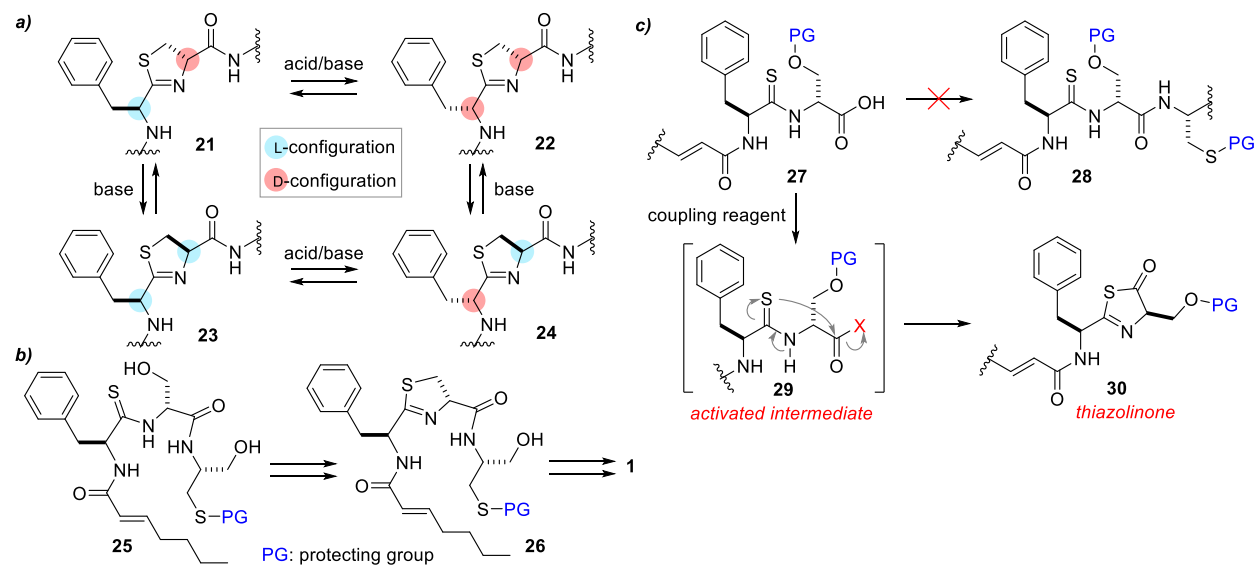


**Scheme 1.2.1** (a) Epimerization of thiazoline; (b) An alternate approach; (c) Thiazolinone formation.

## 2.2 Retrosynthesis of 1

The retrosynthesis of **1** is summarized in Scheme 1.2.2. The chemical synthesis based on the putative biosynthesis often gives efficiency in its synthesis and insights into the biosynthesis. Consequently, in light of its plausible biosynthesis, the macrolactam **1** was retrosynthetically acyclized to **16**. The linear peptide **16** would be synthesized by the assembly of the components **17**-**20** with the isomerization-suppressed procedures.
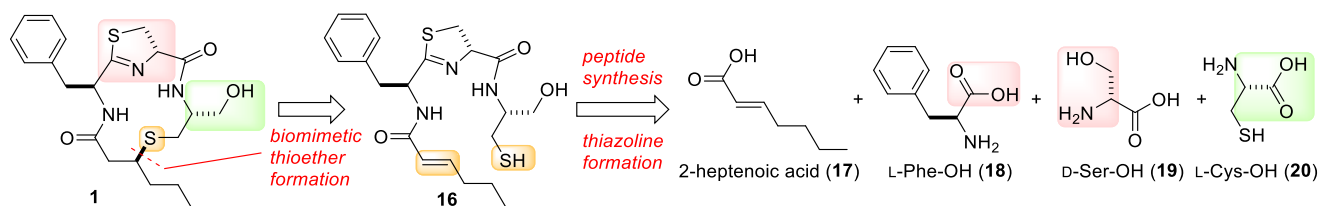


**Scheme 1.2.2** Retrosynthesis of **1**.

## 2.3 Total Synthesis of 1

The total synthesis of **1** started from the chemical construction of thiazoline moiety using Wipf's cyclodehydration[20] of $\beta$-thioamide as a key reaction (Scheme 1.2.3). Initially, Boc-L-Phe-OH (**31**) was amidated with **32** to give **33**. The hydroxy function of **33** was protected by TBS group to form **34**, and then dipeptide **34** was transformed into corresponding thioamide **35** by the action of Lawesson's reagent.[29] In order to avoid the acid-promoted epimerization of thiazoline (Scheme 1.2.1a), the fatty acid was attached at this timing, namely, Boc group of **35** was selectively removed in the presence of TBS group by the treatment of TMSOTf,[30] and then liberated amine **36** was condensed with acid **17**. At this stage, we then attempted to the transformation of thioamide into thiazoline to prevent the thiazolinone formation (Scheme 1.2.1c). The undesired 1,4-additions to $\alpha,\beta$-unsaturated amide[31] of **37** should also be avoided. TBS group of **37** was cleaved by TBAF/AcOH, followed by the cyclodehydration utilizing DAST (diethylaminosulfur trifluoride) successfully led to thiazoline **39**. Finally, **39** was converted to carboxylic acid **40** by Nicolaou's method[32] without no significant thiazoline isomerization in the reaction. However, it is noteworthy that the epimerization of acid **40** was observed during silica gel column chromatography. Therefore, **40** had to be used immediately without chromatographic purification.
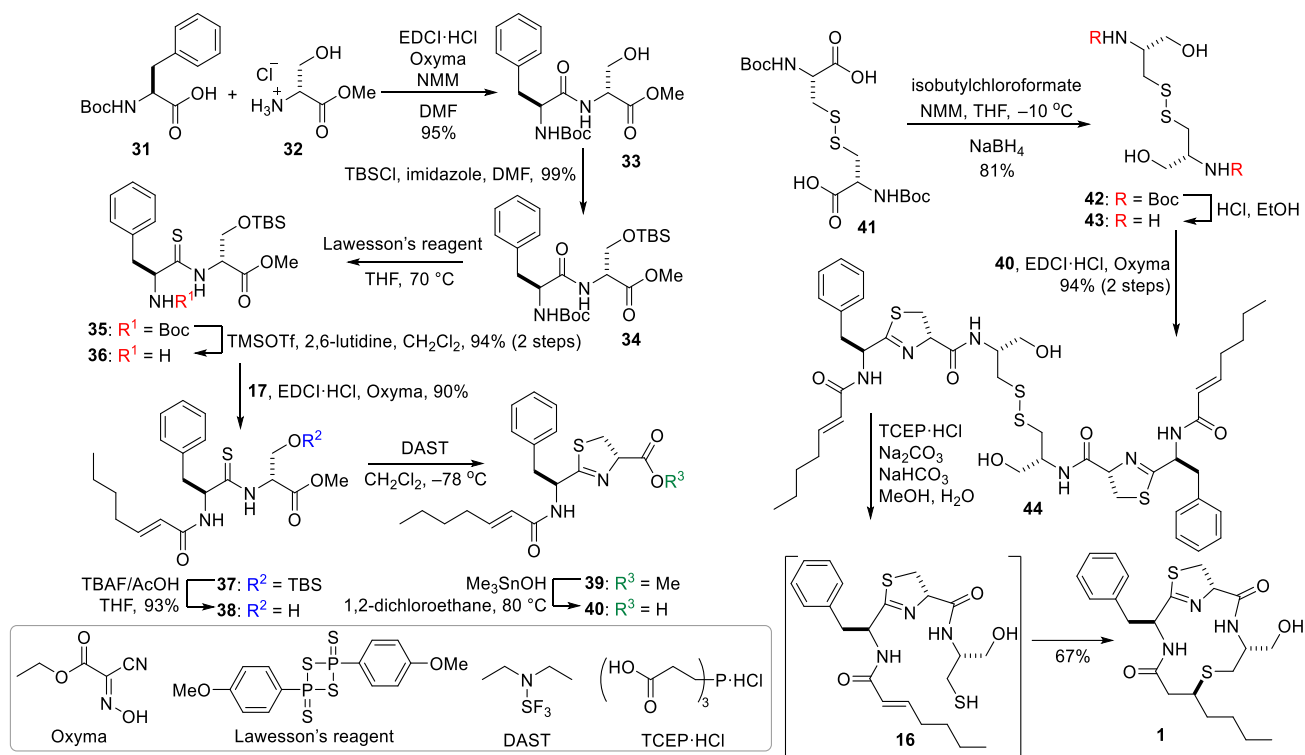
**Scheme 1.2.3** Total synthesis of **1** via one-pot reduction/cyclization.

With stereochemically pure **40** in hand, total synthesis of **1** was accomplished by the peptide chain elongation followed by the bioinspired cyclization (Scheme 1.2.3). In this study, the dimeric cystine, which can release active thiol function under mild reductive conditions, was used as an S-protectred cysteine. Boc-L-cystine (**41**) was converted to the active ester, which was in situ reduced to alcohol **42** by NaBH$_4$. Boc group of **42** was removed by HCl, and then another building block **40** was attached to its N-terminus under the EDCI·HCl/Oxyma[33] conditions. After scrutinizing the reaction conditions (Table 1.2.3) of the one-pot reduction/cyclization, the linear peptide **16** was successfully bridged by thioether in pH 9 Na$_2$CO$_3$/NaHCO$_3$ buffer which contains TCEP·HCl as a reductant. After reversed-phase ODS HPLC purification, **1** was obtained in 67% yield from **44**. The highly modified cyclic peptide **1** was synthesized in only 10 longest linear sequence from commercially available starting materials **31** and **32**.

**Table 1.2.1** $^1$H NMR data (600 MHz) and $^{13}$C NMR data (150 MHz) for natural **1** and synthetic **1** in DMSO-$d_6$.

| pos | natural **1** $\delta_C$, type | $\delta_H$ ($J$ in Hz) | pos | synthetic **1** $\delta_C$, type | $\delta_H$ ($J$ in Hz) |
|---|---|---|---|---|---|
| 1 | 34.9, CH$_2$ | 2.79, dd (13.0, 8.8) | 1 | 34.9, CH$_2$ | 2.79, dd (13.1, 8.8) |
| | | 3.10, dd (13.0, 4.3) | | | 3.10, dd (13.0, 4.3) |
| 2 | 50.6, CH | 3.39, m | 2 | 50.6, CH | 3.39, m |
| 3 | 61.9, CH$_2$ | 3.21, m | 3 | 61.9, CH$_2$ | 3.22, m |
| | | 3.51, m | | | 3.51, d (10.1) |
| 4 | 170.2, C | | 4 | 170.3, C | |
| 5 | 77.7, CH | 5.08, t (6.7) | 5 | 77.7, CH | 5.08, m |
| 6 | 36.8, CH$_2$ | 3.56, d (6.7) | 6 | 36.8, CH$_2$ | 3.56, m |
| 7 | 177.7, C | | 7 | 177.7, C | |
| 8 | 54.3, CH | 4.54, m | 8 | 54.3, CH | 4.54, m |
| 9 | 37.6, CH$_2$ | 2.96, dd (13.9, 9.9) | 9 | 37.6, CH$_2$ | 2.95, dd (13.9, 10.0) |
| | | 3.03, dd (13.9, 5.0) | | | 3.03, dd (13.9, 5.0) |
| 10 | 137.4, C | | 10 | 137.4, C | |
| 11/15 | 129.1, CH | 7.32, t (7.9) | 11/15 | 129.1, CH | 7.32, t (7.8) |
| 12/14 | 128.3, CH | 7.29, t (7.6) | 12/14 | 128.3, CH | 7.30, t (7.6) |
| 13 | 126.6, CH | 7.22, t (7.1) | 13 | 126.7, CH | 7.22, m |
| 16 | 171.6, C | | 16 | 171.6, C | |
| 17 | 42.5, CH$_2$ | 2.39, d (8.3) | 17 | 42.5, CH$_2$ | 2.39, m |
| 18 | 45.7, CH | 2.90, m | 18 | 45.8, CH | 2.90, m |
| 19 | 36.8, CH$_2$ | 1.42, m | 19 | 36.9, CH$_2$ | 1.42, m |
| | | 1.54, m | | | 1.55, m |
| 20 | 28.8, CH$_2$ | 1.35, m | 20 | 28.8, CH$_2$ | 1.35, m |
| | | 1.45, m | | | 1.45, m |
| 21 | 21.8, CH$_2$ | 1.28, m | 21 | 21.9, CH$_2$ | 1.28, m |
| 22 | 14.0, CH$_3$ | 0.87, t (7.3) | 22 | 14.0, CH$_3$ | 0.87, t (7.3) |
| 2-NH | | 6.77, d (5.7) | 2-NH | | 6.77, d (5.7) |
| 3-OH | | 4.82, s | 3-OH | | 4.82, s |
| 8-NH | | 8.85, d (6.7) | 8-NH | | 8.84, d (6.8) |

## 2.4 Structural Confirmation of 1

Although the NMR spectra of synthetic **1** were well agreed to those of natural **1**, the spectroscopic data of peptides vary in the conditions (e.g. concentration, pH, and temperature), which often arise structural confusion of peptitidic natural product.[34] Consequently, having developed a rapid synthetic entry to **1**, we then turned our attention to the structural validation of chemically constructed **1**.

The essential task at this stage is the structural confirmation of chemically constructed **1** because thio-Michael addition in the present synthesis potentially gives **1** as a diastereomeric mixture, and the thiazoline moiety might be isomerized not only in the chemical conversions but also in the purification steps. In addition, another important notice

in the peptide chemistry is that the diastereomers of peptidic middle-sized molecules often exhibit similar properties (e.g. bioactivities and NMR spectra), and their separation are often laborious. In order to establish the efficiency of our bioinspired total synthesis, we had to show the purity of synthesized **1**, which would be readily isomerized as with natural **1**.

In chapter 1-1, natural **1** was carefully hydrolyzed (2 M HCl, 90 °C, 4 h) to determine the stereochemistry of C5 by Marfey's method. Then the absolute configuration of C2 and C18 were evaluated by the combination of the spectroscopic analyses (CD and NMR) and chemical synthesis. A multi-milligram scale of **1**, which was totally consumed by the chemical degradation, was required for CD and NMR analyses.

Recently, highly sensitive labeling reagent (1-fluoro-2,4-dinitrophenyl-5-L-valine-*N*,*N*-dimethylethylenediamine-amide, FDVDA) was developed for structural evaluation of peptidic compounds in our group.[35] In FDVDA, *N*,*N*-dimethylethane-1,2-diamine was condensated with valine moiety, which could enhance sensitivity in mass spectrometry analysis (Scheme 1.2.4b). The improved reagents enabled the detection of component amino acids resulting from under milder chemical degradation using smaller amounts of peptide, which would be useful for the structural analysis of peptide **1**.

In this study (Scheme 1.2.4b), acid hydrolysates of natural/synthetic **1** were derivatized with labeling reagent L-FDVDA, and the derivatives were chromatographically compared with the chemically synthesized standard samples. A minute amount (<0.1 mg) of **1** were enough for the LC-MS-based chromatographic comparison of DVDA derivatives (Figure 1.2.2).
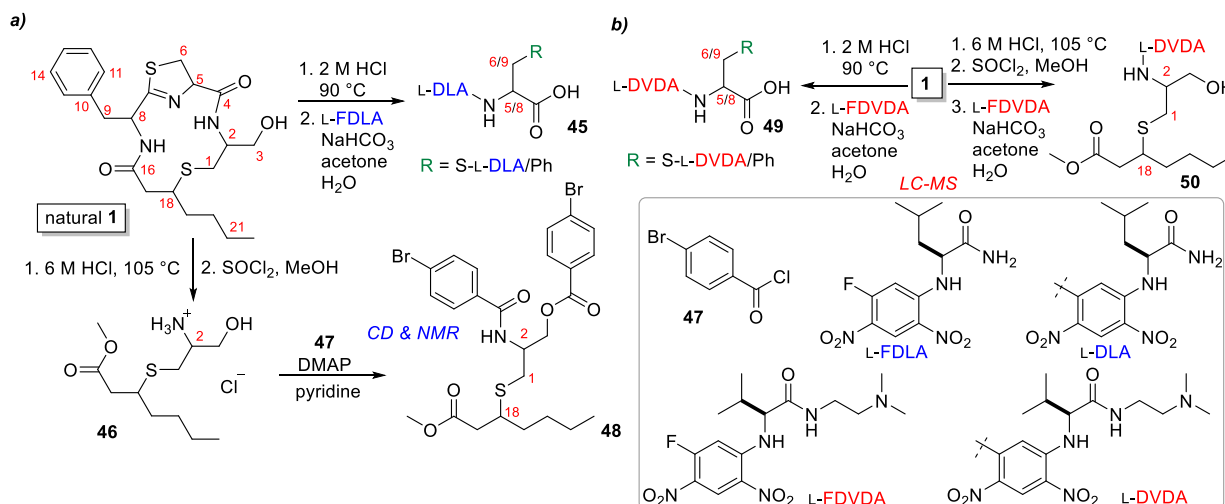


**Scheme 1.2.4** (a) Structural studies on **1** in chapter 1-1; (b) Structural studies on **1** in this work.

The synthesis of all four possible isomers of **50** is summarized in Scheme 1.2.5. Treatment of previously synthesized thioethers **54a**/**54b** with HCl liberated corresponding amines, which were labeled with L-FDVDA by nucleophilic aromatic substitution (S$_N$Ar) reaction, leading to **50a**/**50b**. On the other hand, in the first step for the synthesis of **50c**/**50d**, the hydroxy function of **51** was protected with TBS group, which can be cleaved simultaneously with Boc group by HCl.

Second, reduction of methyl ester of **55**, followed by the tosylation of generated primary alcohol furnished **56**. Then, **56** was converted to **50c**/**50d** by the same fashion as **50a**/**50b**.
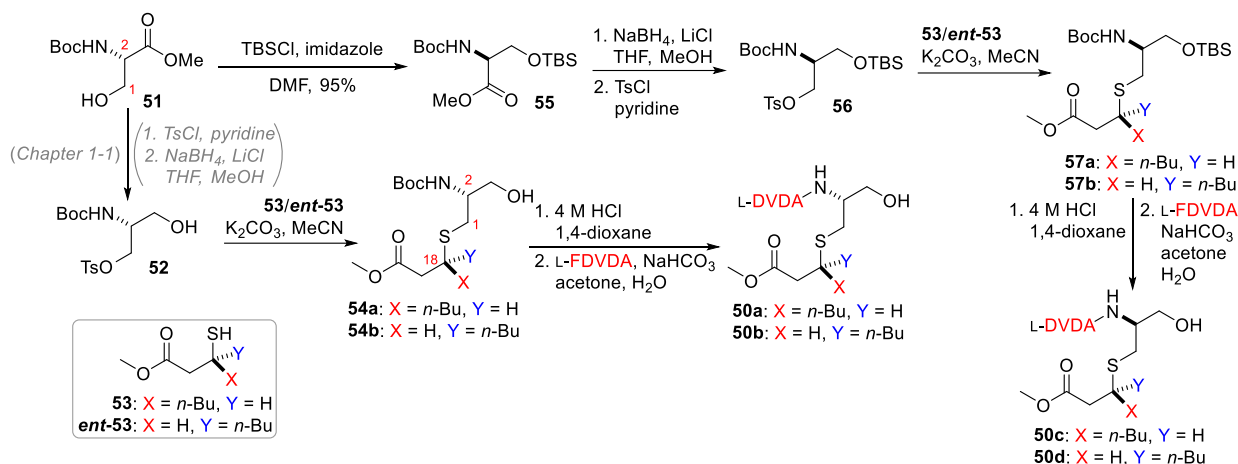


**Scheme 1.2.5** Synthesis of four possible isomers of **50**.

Finally, the key labeled thioether **50** derived from natural/synthetic **1** were chromatographically compared with the standard thioethers **50a**-**d** by LC-MS experiments. The retention time of authentic **50** was matched with that of **50a**, confirming the structure of natural and synthetic thioamycolamide A as depicted in **1** (Figure 1.2.1). Moreover, no detectable peaks of **50b**-**50d** were observed in the LC-MS experiments of authentic samples even in the use of the highly sensitive labeling reagent, showing the stereochemical purity of **1**. The thioether bridge have been stereoselectively constructed in this study, which strongly supports our proposed thio-Michael addition pathway for its thioether biosynthesis.
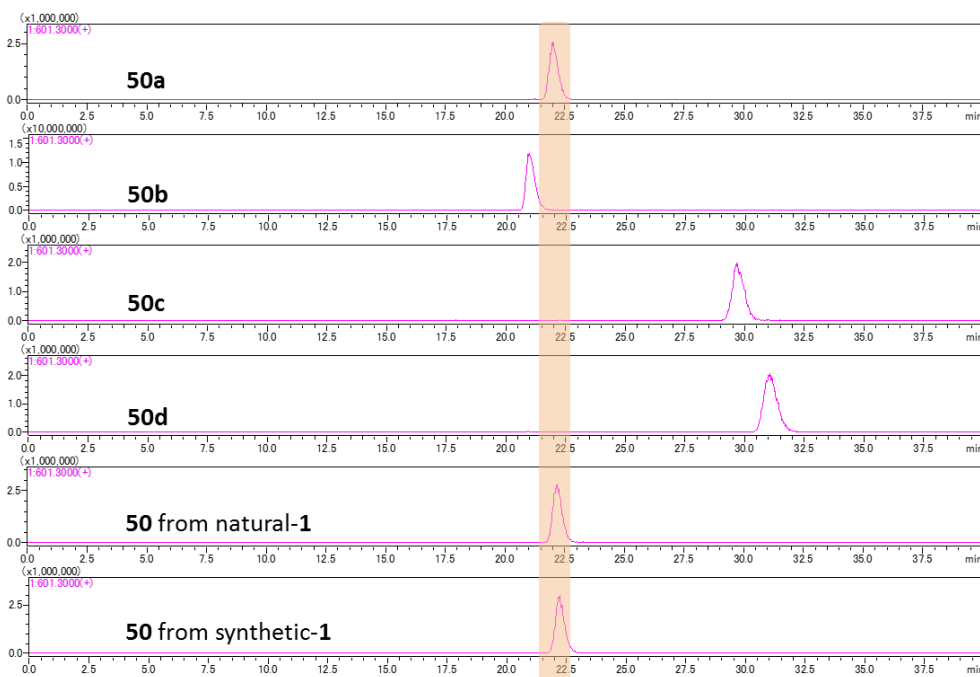


**Figure 1.2.1** LC-MS analysis of L-DVDA derivatives **50a**-**50d** and authentic **50**.

23

## 2.5 Cytotoxicity

A preliminary bioassay of synthetic **1** was carried out using two human cancer cell lines, HT1080 and HeLa S3, which displayed well-agreed bioactivities between synthetic and natural **1**.

**Table 1.2.2** Cytotoxic activities (IC$_{50}$) of natural and synthetic **1**

| Sample | Cytotoxic activity ($\mu$M) | |
|---|---|---|
| | HT 1080 | Hela S3 |
| natural **1** | 9.60±1.03 | 15.47±3.54 |
| synthetic **1** | 11.61±1.44 | 14.74±5.16 |
| Adriamycin | 0.30±0.05 | 0.37±0.06 |

## 2.6 Experimental Section

### 2.6.1 General Procedures

See 1.5.1

### 2.6.2 Cytotoxicity Assay

Cytotoxicity of compounds against HT1080 and HeLa S3 cell lines was evaluated by a WST-8 colorimetric assay (Cell Counting Kit-8, Dojindo). Briefly, cells were cultured in 96-well plates (3000 cells/well) for 24 hours followed by exposure to natural and synthetic **1** for 72 hours, and then the viability was assessed by WST-8. The absorbance was measured at 450 nm using an iMark microplatereader (BIO-RAD). Adriamycin was evaluated as a positive control. IC$_{50}$ values are shown as the mean ±SD (n=4).

### 2.6.3 Total Synthesis of 1
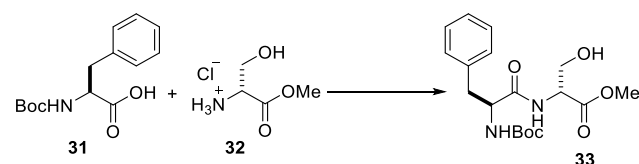


To a solution of Boc-L-Phe-OH (**31**) (1 g, 3.77 mmol) and H-D-Ser-OMe hydrochloride (**32**) (645 mg, 4.15 mmol) in DMF (15 mL) were added *N*-methylmorpholine (467.6 μL, 4.17 mmol), Oxyma (589 mg, 4.14 mmol), and EDCI·HCl (795 mg, 4.15 mmol) at 0 ℃. After being stirred at room temperature overnight, saturated aqueous NH$_4$Cl (20 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (40 mL ×2). The combined organic layer was washed with saturated aqueous NaHCO$_3$ (50 mL) and brine (50 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes = 1 : 1) on silica gel to give dipeptide **33** (1.31 g, 95%) as a white foam: $[\alpha]^{20}_{D} = -6.5$ (*c* 0.14, MeOH); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.32 (t, *J* = 7.3 Hz, 2H), 7.25 (m, 3H), 6.72 (d, *J* = 7.3 Hz, 1H), 5.12 (d, *J* = 6.5 Hz, 1H), 4.57 (m, 1H), 4.38 (d, *J* = 6.3 Hz, 1H), 3.78 (m, 1H), 3.74 (s, 3H), 3.06 (d, *J* = 7.2 Hz, 2H), 1.40 (s, 9H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 171.3, 170.7, 155.8, 136.7, 129.4, 128.9, 127.3, 80.8, 62.7, 56.3, 54.8, 52.9, 38.8, 28.4; HRMS (ESI) calcd for C$_{18}$H$_{26}$N$_2$O$_6$Na$^+$ [M + H]$^+$ 389.1683, found 389.1703.
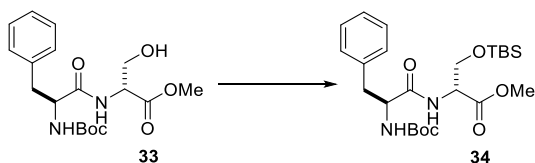
To a solution of **33** (760 mg, 2.07 mmol) in DMF (15 mL) were added TBSCl (469 mg, 3.11 mmol) and imidazole (424 mg, 6.23 mmol) at 0 ℃. After being stirred at room temperature for 30min, saturated aqueous NH₄Cl (20 mL) was added to the reaction mixture. The mixture was extracted with EtOAc (20 mL × 2). The combined organic layer was washed with brine (40 mL × 2), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes =1 : 4) on silica gel to yield **34** (990.6 mg, 99%) as a white foam: $[\alpha]^{20}_D = -17.1$ (c 0.63, MeOH); $^1$H NMR (500 MHz, CDCl₃) δ 7.21 (m, 2H), 7.14 (m, 3H), 6.82 (d, J = 7.4 Hz, 1H), 5.23 (d, J = 7.7 Hz, 1H), 4.56 (d, J = 7.7 Hz, 1H), 4.44 (d, J = 7.2 Hz, 1H), 3.93 (d, J = 9.7 Hz, 1H), 3.63 (s, 3H), 3.57 (d, J = 7.7 Hz, 1H), 3.10 (dd, J = 13.8, 6.5 Hz, 1H), 2.96 (dd, J = 13.4, 7.2 Hz, 1H), 1.32 (s, 9H), 0.78 (s, 9H), -0.06 (s, 3H), -0.07 (s, 3H); $^{13}$C NMR (125 MHz, CDCl₃) δ 171.0, 170.5, 155.3, 136.8, 129.2, 128.4, 126.7, 79.8, 63.2, 55.5, 54.0, 52.2, 38.3, 28.1, 25.6, 18.0, -5.6, -5.9; HRMS (ESI) calcd for $C_{24}H_{40}N_2O_6SiNa^+$ [M + H]$^+$ 503.2548, found 503.2541.
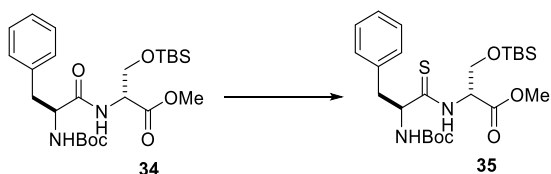


To a solution of **34** (984.3 mg, 2.05 mmol) in THF (15 mL) was added Lawesson's reagent (498.0 mg, 1.23 mmol) at room temperature. After being stirred at 70 ℃ for 1 h, the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, hexanes:EtOAc, 10:1) to give thioamide **35** as a light yellow oil (1.02 g, 100%): $[\alpha]^{20}_D = -20.3$ (c 0.69, MeOH); $^1$H NMR (400 MHz, CDCl₃) δ 8.17 (d, J = 7.8 Hz, 1H), 7.24 (m, 2H), 7.18 (m, 3H), 5.34 (d, J = 5.2 Hz, 1H), 5.07 (d, J = 4.3 Hz, 1H), 4.65 (d, J = 6.3 Hz, 1H), 3.95 (d, J = 9.9 Hz, 1H), 3.68 (s, 3H), 3.52 (m, 1H), 3.21 (dd, J = 13.3, 7.2 Hz, 1H), 3.10 (m, 1H), 1.35 (s, 9H), 0.79 (s, 9H), -0.05 (s, 6H); $^{13}$C NMR (100 MHz, CDCl₃) δ 203.3, 169.4, 155.0, 136.8, 129.2, 128.6, 126.9, 80.1, 62.5, 62.2, 59.2, 52.5, 42.1, 28.2, 25.6, 18.1, -5.5, -5.8; HRMS (ESI) calcd for $C_{24}H_{40}N_2O_5SiSNa^+$ [M + H]$^+$ 519.2319, found 519.2301.
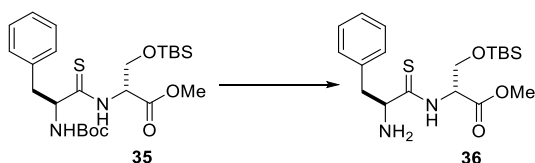


To a solution of **35** (1.02 g, 2.05 mmol) in dry CH₂Cl₂ (20 mL) 2,6-lutidine (1.40 mL, 12.1 mmol) was added at room temperature, followed by dropwise addition of TMSOTf (1.46 mL, 8.08 mmol). The mixture was stirred at room temperature for 8 h, then quenched by addition of MeOH (5 mL). The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, CH₂Cl₂ : MeOH, 200:1, 0.05% triethylamine) to give amine **36** as a light yellow oil (763 mg, 94%): $[\alpha]^{20}_D = -16.1$ (c 0.33, MeOH); $^1$H NMR (500 MHz, CDCl₃) δ 7.30

25

(m, 2H), 7.22 (m, 3H), 5.25 (s, 1H), 4.11 (dd, $J$ = 10.3, 2.6 Hz, 1H), 4.06 (dd, $J$ = 10.1, 3.7 Hz, 1H), 3.97 (dd, $J$ = 10.3, 3.1 Hz, 1H), 3.75 (s, 3H), 3.60 (dd, $J$ = 13.8, 3.7 Hz, 1H), 2.60 (dd, $J$ = 13.8, 10.1 Hz, 1H), 0.83 (s, 9H), 0.00 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 205.6, 169.7, 138.1, 129.2, 128.7, 126.9, 63.7, 62.3, 58.9, 52.4, 43.6, 25.6, 18.0, -5.6, -5.7; HRMS (ESI) calcd for C$_{19}$H$_{33}$N$_2$O$_3$SSi$^{++}$ [M + H]$^+$ 397.1976, found 397.1990.
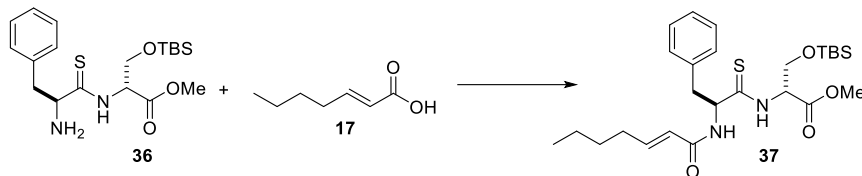


To a solution of **36** (147.8 mg, 0.37 mmol) and **17** (52.5 mg, 0.41 mmol) in DMF (3 mL) were added Oxyma (58.3 mg, 0.41 mmol) and EDCI HCl (78.6 mg, 0.41 mmol) at 0 ℃. After being stirred at room temperature for overnight, saturated aqueous NH$_4$Cl (10 mL) was added to the reaction mixture. The mixture was extracted with EtOAc (10 mL × 2). The combined organic layer was washed with saturated aqueous NaHCO$_3$ (20 mL) and brine (20 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes = 1 : 5) on silica gel to give **37** (170.6 mg, 90%) as a colorless oil.

[$\alpha$]$^{20}$$_D$ = -29.5 (*c* 0.12, MeOH); $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.15 (d, $J$ = 7.7 Hz, 1H), 7.23 (m, 2H), 7.18 (m, 3H), 6.80 (dt, $J$ = 15.1, 6.9 Hz, 1H), 6.64 (d, $J$ = 7.9 Hz, 1H), 5.77 (d, $J$ = 15.3 Hz, 1H), 5.03 (m, 2H), 3.90 (dd, $J$ = 10.2, 2.6 Hz, 1H), 3.67 (s, 3H), 3.43 (dd, $J$ = 10.2, 3.4 Hz, 1H), 3.25 (dd, $J$ = 13.4, 6.2 Hz, 1H), 3.08 (dd, $J$ = 13.4, 8.1 Hz, 1H), 2.14 (td, $J$ = 8.1, 1.2 Hz, 2H), 1.39 (m, 2H), 1.29 (m, 2H), 0.87 (t, $J$ = 7.3 Hz, 3H), 0.79 (s, 9H), -0.05 (s, 6H) ppm; $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 203.0, 169.3, 165.3, 145.8, 136.7, 129.3, 128.6, 127.0, 123.2, 62.2, 60.7, 59.4, 52.5, 42.1, 31.8, 30.3, 25.6, 22.3, 18.1, 13.9, -5.5, -5.8; HRMS (ESI) calcd for C$_{26}$H$_{43}$N$_2$O$_4$SSi$^+$ [M + H]$^+$ 507.2707, found 507.2724.



To a solution of **37** (169 mg, 0.33 mmol) in THF (3 mL) were added AcOH (22.6 μL, 0.4 mmol) and TBAF (0.4 mL, 0.4 mmol, 1 M in THF) at 0 ℃. After being stirred at room temperature for 3 h, the reaction mixture was diluted with EtOAc (10 mL) and washed with saturated aqueous NH$_4$Cl and brine (10 mL for each). The combined organic layer was dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes = 1 : 2) on silica gel to give **38** (121.8 mg, 93%) as a white foam: [$\alpha$]$^{20}$$_D$ = −0.9 (*c* 0.30, MeOH); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.95 (d, $J$ = 7.3 Hz, 1H), 7.20 (m, 5H), 6.80 (d, $J$ = 8.3 Hz, 1H), 6.73 (dt, $J$ = 15.3, 6.9 Hz, 1H), 5.77 (dt, $J$ = 15.3, 1.3 Hz, 1H), 5.18 (dt, $J$ = 8.4, 6.7 Hz, 1H), 5.00 (m, 1H), 3.82 (dd, $J$ = 11.8, 3.2 Hz, 1H), 3.66 (s, 3H), 3.56 (dd, $J$ = 11.9, 3.1 Hz, 1H), 3.21 (dd, $J$ = 13.1, 6.4 Hz, 1H), 3.06 (dd, $J$ = 13.1, 8.6 Hz, 1H), 2.12 (m, 2H), 1.37 (m, 2H), 1.28 (m, 2H), 0.87 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 203.4, 169.7, 165.8, 146.4, 136.6, 129.6, 128.5, 127.0, 123.0, 61.5, 60.4, 60.1, 52.8, 42.5, 31.8, 30.2, 22.3, 13.9; HRMS (ESI) calcd for C$_{20}$H$_{28}$N$_2$O$_4$SNa$^+$ [M + Na]$^+$ 415.1662, found 415.1661.

26
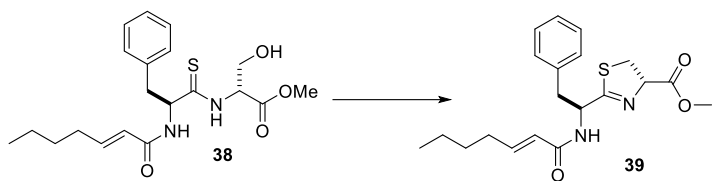
To a solution of **38** (115 mg, 0.29 mmol) in CH$_2$Cl$_2$ (3 mL) was added DAST (104 μL, 0.80 mmol) at −78 ℃. After being stirred at −78 ℃ for 1 h, the reaction mixture was poured to ice-water (10 mL) and extracted with EtOAc (10 mL ×2). The combined organic layer was washed with brine (10 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure to afford thiazoline **39** (107.1 mg, 98%) as a white powder, which was used without further purification: [α]$^{20}$$_D$ = −70.4 (c 0.11, MeOH); $^1$H NMR (500 MHz, CDCl$_3$) δ 7.23 (m, 3H), 7.17 (m, 2H), 6.84 (dt, J = 15.2, 6.9 Hz, 1H), 6.14 (d, J = 7.5 Hz, 1H), 5.74 (dt, J = 15.3, 1.4 Hz, 1H), 5.21 (dd, J = 12.3, 6.1 Hz, 1H), 5.01 (m, 1H), 3.78 (s, 3H), 3.58 (dd, J = 11.2, 9.0 Hz, 1H), 3.51 (dd, J = 11.2, 9.5 Hz, 1H), 3.25 (dd, J = 14.0, 6.2 Hz, 1H), 3.12 (dd, J = 14.0, 5.7 Hz, 1H), 2.15 (m, 2H), 1.41 (m, 2H), 1.32 (m, 2H), 0.89 (t, J = 7.3 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 175.3, 171.0, 165.5, 146.2, 135.9, 129.7, 128.5, 127.1, 123.0, 77.6, 52.9, 52.7, 39.3, 35.7, 31.9, 30.4, 22.3, 14.0; HRMS (ESI) calcd for C$_{20}$H$_{27}$N$_2$O$_3$S$^+$ [M + H]$^+$ 375.1737, found 375.1736.



To a solution of **39** (27.5 mg, 73.4 μmol) in 1,2-dichloroethane (1 mL) was added trimethyltin hydroxide (40 mg, 0.22 mmol) at room temperature. The reaction mixture was heated at 80 ℃ for 1 h and then cooled to room temperature and concentrated. The residue was dissolved in EtOAc (10 mL), washed with 0.01 M KHSO$_4$ aqueous solution (5 mL × 5) and brine (10 mL), dried over MgSO$_4$, filtered and concentrated to afford **40** (26.4 mg, 100%) as a white foam, which was used without further purification: [α]$^{20}$$_D$ = −22.9 (c 0.04, MeOH); $^1$H NMR (500 MHz, MeOD-$d_4$) δ 7.25 (m, 4H), 7.18 (m, 1H), 6.73 (dt, J = 15.2, 7.0 Hz, 1H), 5.88 (d, J = 15.4 Hz, 1H), 5.17 (t, J = 9.1 Hz, 1H), 5.08 (dd, J = 9.6, 5.1 Hz, 1H), 3.57 (dd, J = 9.1, 3.7 Hz, 2H), 3.27 (dd, J = 14.1, 5.1 Hz, 1H), 3.00 (dd, J = 14.1, 9.7 Hz, 1H), 2.17 (m, 2H), 1.42 (m, 2H), 1.33 (m, 2H), 0.92 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (125 MHz, MeOD-$d_4$) δ 179.0, 173.6, 168.4, 146.9, 138.3, 130.3, 129.4, 127.8, 123.9, 79.1, 54.7, 40.1, 35.7, 32.7, 31.5, 23.2, 14.2; HRMS (ESI) calcd for C$_{19}$H$_{25}$N$_2$O$_3$S$^+$ [M + H]$^+$ 361.1580, found 361.1568.
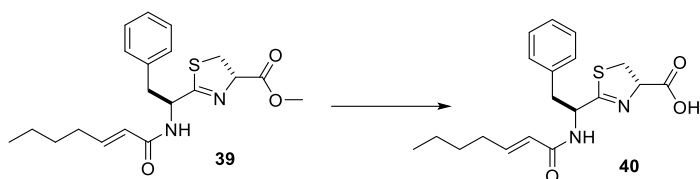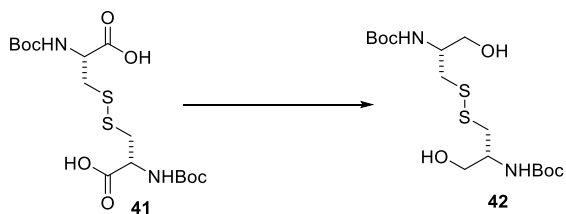


To a solution of **41** (2.98 g, 6.8 mmol) in THF (40 mL) were added N-methylmorpholine (1.64 mL, 14.9 mmol) and isobutyl chloroformate (1.95 mL, 14.8 mmol) at −10 ℃. After being stirred for 15 min, NaBH$_4$ (1022 mg, 27.0 mmol)

was added to the solution. After being stirred for further 15 min at −10 ℃, saturated aqueous NH₄Cl (40 mL) was added to the reaction mixture. The mixture was extracted with EtOAc (50 mL × 2). The combined organic layer was washed with brine (80 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes = 2 : 1) on silica gel to give **42** (2.27 g, 81%) as a white powder: $[\alpha]^{20}_D = 85.1$ (*c* 0.77, MeOH); $^1$H NMR (500 MHz, MeOD-*d₄*) $\delta$ 6.55 (d, *J* = 8.3 Hz, 1H), 3.85 (m, 1H), 3.62 (dd, *J* = 11.1, 4.9 Hz, 1H), 3.57 (dd, *J* = 11.1, 5.5 Hz, 1H), 2.97 (dd, *J* = 13.7, 5.7 Hz, 1H), 2.82 (dd, *J* = 13.7, 7.8 Hz, 1H), 1.45 (s, 9H); $^{13}$C NMR (125 MHz, MeOD-*d₄*) $\delta$ 158.0, 80.2, 63.9, 53.4, 41.5, 28.8; HRMS (ESI) calcd for $C_{16}H_{32}N_2O_6S_2Na^+$ [M + Na]$^+$ 435.1594, found 435.1587.
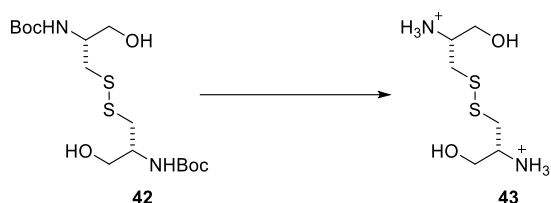


To **42** (283 mg, 0.69 mmol) was added 2 M HCl in ethanol (3 mL) at room temperature. After being stirred for 1 h, the reaction mixture was concentrated to afford **43** (196 mg, 100%) as a white foam, which was used without further purification: $[\alpha]^{20}_D = -100.4$ (*c* 0.29, MeOH); $^1$H NMR (500 MHz, MeOD-*d₄*) $\delta$ 3.88 (dd, *J* = 11.8, 3.7 Hz, 1H), 3.76 (dd, *J* = 11.8, 5.4 Hz, 1H), 3.60 (m, 1H), 3.12 (dd, *J* = 14.4, 6.6 Hz, 1H), 3.05 (dd, *J* = 14.4, 7.1 Hz, 1H); $^{13}$C NMR (125 MHz, MeOD-*d₄*) $\delta$ 61.2, 53.3, 37.0; HRMS (ESI) calcd for $C_6H_{17}N_2O_2S_2^+$ [M + H]$^+$ 213.0726, found 213.0730.
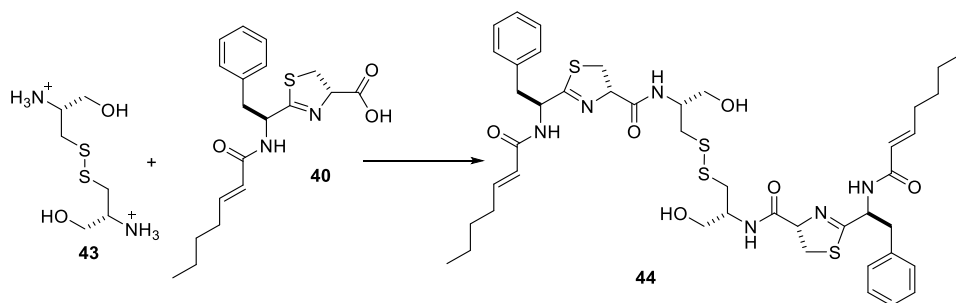


To a solution of **43** (8.6 mg, 30.2 μmol) and **40** (24 mg, 66.6 μmol) in DMF (0.5 mL) were added *i*-Pr₂NEt (11.6 μL, 66.6 μmol), Oxyma (9.5 mg, 66.9 μmol), and EDCI·HCl (12.8 mg, 66.8 μmol) at 0 ℃. After being stirred at 0 °C for 2 h and at room temperature for 2 h, saturated aqueous NH₄Cl (5 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (5 mL × 2). The combined organic layer was washed with saturated aqueous NaHCO₃ (10 mL) and brine (10 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (CHCl₃ : MeOH = 50 : 1) on silica gel to give **44** (25.6 mg, 94%) as a white foam: $[\alpha]^{20}_D = -86.3$ (*c* 0.50, MeOH); $^1$H NMR (500 MHz, MeOD-*d₄*) $\delta$ 7.26 (m, 4H), 7.20 (m, 1H), 6.75 (dt, *J* = 15.3, 7.0 Hz, 1H), 5.91 (dt, *J* = 15.4, 1.4 Hz, 1H), 5.09 (t, *J* = 8.9 Hz, 1H), 4.95 (dd, *J* = 9.3, 5.6 Hz, 1H), 4.22 (m, 1H), 3.68 (dd, *J* = 11.4, 4.8 Hz, 2H), 3.59 (m, 2H), 3.52 (dd, *J* = 11.2, 8.2 Hz, 1H), 3.31 (m, 1H), 3.06 (dt, *J* = 13.9, 7.7 Hz, 1H), 2.92 (dd, *J* = 13.8, 8.1 Hz, 1H), 2.18 (m, 2H), 1.43 (m, 2H), 1.34 (m, 2H), 0.92 (t, *J* = 7.3 Hz, 3H); $^{13}$C NMR (125 MHz, MeOD-

*d₄*) δ 178.6, 173.1, 168.7, 147.0, 138.5, 130.3, 129.5, 127.8, 124.0, 80.0, 63.4, 55.2, 52.3, 40.6, 39.7, 36.6, 32.8, 23.3, 14.2; HRMS (ESI) calcd for $C_{44}H_{61}N_6O_6S_4^+$ [M + H]$^+$ 897.3530, found 897.3527.
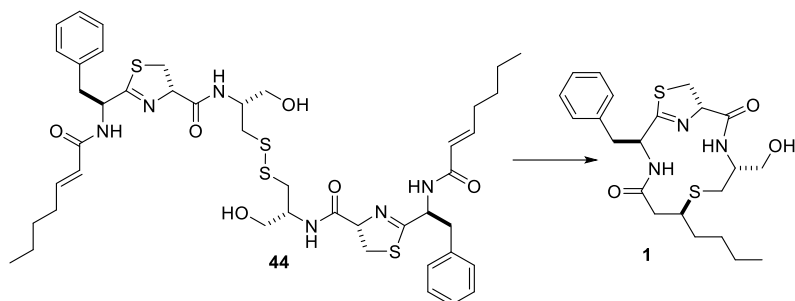


To a solution of **44** (23 mg, 25.6 μmol) in MeOH/aqueous buffer (pH 9, Na₂CO₃/NaHCO₃) (10 mL/10 mL) was added TCEP·HCl (22 mg, 76.8 μmol) at room temperature. The reaction mixture was stirred for 30 min and then diluted with pH 9 Na₂CO₃/NaHCO₃ buffer (20 mL). The reaction mixture was allowed to room temperature for an additional 2 days. The mixture was extracted with EtOAc (50 mL ×2). The combined organic layer was washed with brine (80 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by reversed-phase HPLC (Cosmosil ODS SP100 20 ×250 mm, H₂O/MeOH (30/70), 210 nm) to afford **1** (15.4 mg, $t_R$ 31.0 min, 67%) as a white foam: $[\alpha]^{20}_D = -70.4$ (*c* 0.39, MeOH); ¹H NMR (600 MHz, DMSO-*d₆*) δ 8.84 (d, *J* = 6.8 Hz, 1H), 7.31 (m, 4H), 7.22 (t, *J* = 7.1 Hz, 1H), 6.77 (d, *J* = 5.7 Hz, 1H), 5.08 (dd, *J* = 7.1, 6.2 Hz, 1H), 4.82 (s, 1H), 4.54 (td, *J* = 9.3, 5.6 Hz, 1H), 3.56 (d, *J* = 6.8 Hz, 2H), 3.51 (d, *J* = 10.1 Hz, 1H), 3.39 (m, 1H), 3.21 (t, *J* = 7.1 Hz, 1H), 3.10 (dd, *J* = 13.0, 4.3 Hz, 1H), 3.03 (dd, *J* = 13.9, 5.1 Hz, 1H), 2.95 (dd, *J* = 13.9, 9.9 Hz, 1H), 2.90 (m, 1H), 2.79 (dd, *J* = 13.1, 8.8 Hz, 1H), 2.39 (m, 2H), 1.55 (m, 1H), 1.41 (m, 3H), 1.27 (m, 2H), 0.87 (t, *J* = 7.3 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d₆*) δ 177.7, 171.6, 170.3, 137.4, 129.1, 128.3, 126.7, 77.7, 61.9, 54.3, 50.6, 45.8, 42.5, 37.6, 36.9, 36.8, 34.9, 28.8, 14.0; HRMS (ESI) calcd for $C_{22}H_{32}N_3O_3S_2^+$ [M + H]$^+$ 450.1880, found 450.1892.

The solvent of cyclization step from **44** to **1** was investigated. Each entry was tested at same concentration (**44**, 1 mM) and room temperature for 48 h, the reaction mixture was analyzed by HPLC (Table 1.2.3). When the reaction was performed in the presence of pH 9 NaHCO₃/Na₂CO₃ buffer, the reaction speed could be significantly improved, while at pH 10 buffer, the base condition would cause the epimerization in thiazoline and effect the yield of **1**. Finally, reduction of the disulfide by using TCEP and then followed by a Michael addition/cyclization in pH 9 Na₂CO₃/NaHCO₃ buffer to afford **1** in a good yield.

**Table 1.2.3** Optimization of the cyclization condition of **44**

| Entry | Solvent | Yield (%)[a] |
|---|---|---|
| 1 | 0.1M PBS buffer, pH 8, 20% MeOH | 5% |
| 2 | MeOH, 5% TEA | <1% |
| 3 | 0.1M NaHCO$_3$ solution pH 8.3, 20% MeOH | 45% |
| 4 | 0.1M Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 9, 20% MeOH | 71% |
| 5 | 0.1M Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 10, 20% MeOH | 48% |

[a] Estimated by HPLC

### 2.6.4 Structural Confirmation of 1

Natural **1** (0.1 mg) and synthetic **1** (0.1 mg) were hydrolyzed with 2 M HCl (100 µL) for 4 h at 90 ℃ under N$_2$ and then dried *in vacuo*, respectively. The each hydrolysate was dissolved in H$_2$O (100 µL), and then 1 M NaHCO$_3$ (20 µL) was added to the solution. To the resulting solution was added L-FDVDA (0.5% w/v in acetone, 20 µL), and then stirred for 2 h at 50 ℃. The solution was cooled to room temperature, neutralized with 2 M HCl (20 µL), evaporated, and then dissolved in MeCN (100 µL), filtered through a membrane filter (SHIMADZU, TORAST$^{TM}$ DISC, PTFE 0.22 µm), and then analyzed by LC-MS [column: Cadenza CD-C18, 3.0 × 150 mm; eluent: MeCN/H$_2$O/formic acid = 50/50/0.1 to 100/0/0.1 (0–30 min), 0.2 mL/min; detection: ESI-positive].
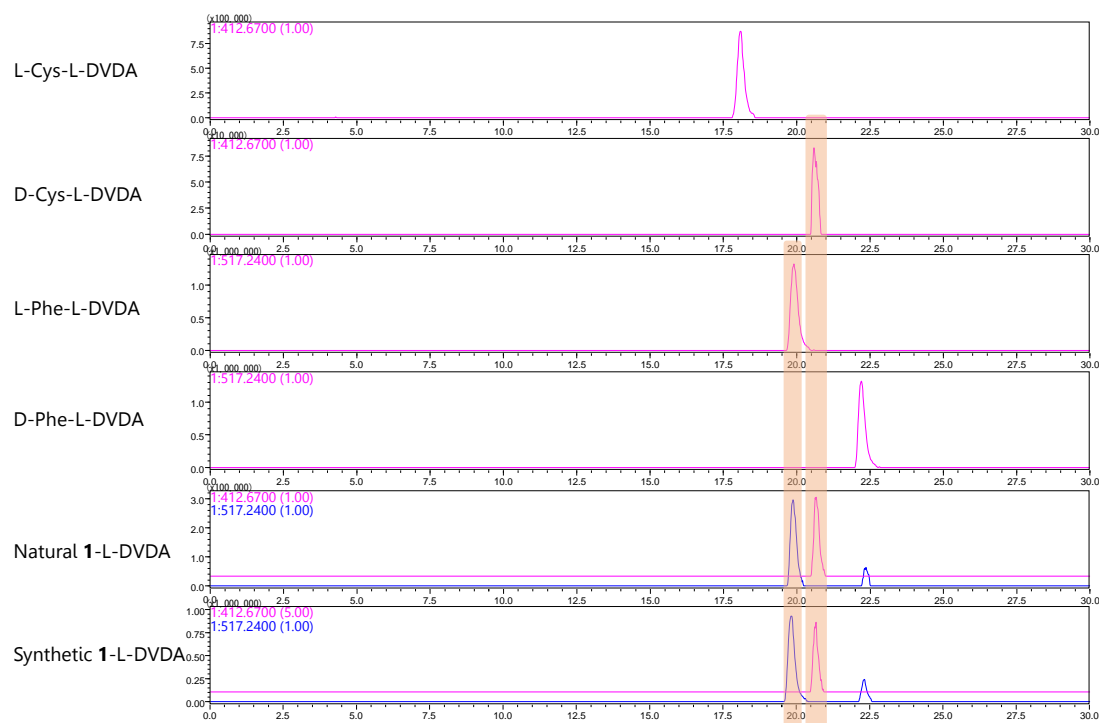


**Figure 1.2.2** LC-MS analysis of L-DVDA derivatives

To a solution of Boc-L-Ser-OMe (**51**) (500mg, 2.28 mmol) in DMF (10 mL) were added imidazole (466 mg, 6.84 mmol) and TBSCl (515.6 mg, 3.42 mmol) at 0 ℃. After being stirred for 0.5 h at roomtemperature, saturated aqueous NH$_4$Cl (20 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (30 mL ×2). The combined organic layer was washed with brine (50 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : Hexanes =1 : 20) on silica gel to afford **55** (718.8 mg, 95%) as a colorless oil: $[\alpha]^{20}{}_D$ = 7.0 (*c* 2.22, MeOH); $^1$H NMR (500MHz, CDCl$_3$) δ 5.33 (d, *J* = 8.5 Hz, 1H), 4.34 (dt, *J* = 8.8, 2.7 Hz, 1H), 4.03 (dd, *J* = 10.0, 2.6, 1H), 3.81 (dd, *J* = 10.0, 3.0, 1H), 3.73 (s, 3H), 1.45 (s, 9H), 0.85 (s, 9H), 0.02 (s, 3H), 0.01 (s, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 171.4, 155.6, 80.2, 63.9, 55.7, 52.4, 28.5, 25.8, 18.3, -5.4, -5.5; HRMS (ESI) calcd for C$_{15}$H$_{31}$NO$_5$SiNa$^+$ [M + Na]$^+$ 356.1864, found 356.1845.



To a solution of **55** (700 mg, 2.10 mmol) in THF (2 mL) were added NaBH$_4$ (238 mg, 6.30 mmol), LiCl (267 mg, 6.30 mmol), and MeOH (6 mL) at 0 ℃. After being stirred at 0 ℃ for 0.5 h and at roomtemperature for 1 h, aqueous citric acid solution (10%, w/w, 20 mL) was added to the reaction mixture. The resulting solution was extracted with CH$_2$Cl$_2$ (20 mL x 2). The combined organic layer was washed with saturated aqueous NaHCO$_3$ (40 mL) and brine (40 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure to give **S3** (664 mg) as a colorless oil, which was used in the next reaction without further purification: $[\alpha]^{20}{}_D$ =17.8 (*c* 1.02, MeOH); $^1$H NMR (500MHz, CDCl$_3$) δ 5.13 (d, *J* = 6.3 Hz, 1H), 3.69 (m, 3H), 3.60 (m, 2H), 3.17 (s, 1H), 1.39 (s, 9H), 0.84 (s, 9H), 0.01 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 156.1, 79.5, 63.6, 63.4, 52.8, 28.4, 25.9, 18.3, -5.5; HRMS (ESI) calcd for C$_{14}$H$_{31}$NO$_4$SiNa$^+$ [M + Na]$^+$ 328.1915, found 328.1919.
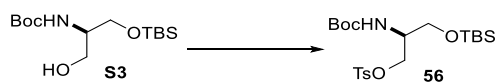


To a stirred solution of **S3** (500 mg, 1.64 mmol) in pyridine (10 mL) was added TsCl (650 mg, 3.41 mmol) at 0 ℃. After being stirred at room temperature overnight, saturated aqueous NH$_4$Cl (40 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (40 mL ×2). The combined organic layer was washed with brine (80 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : Hexanes =1 : 10) on silica gel to afford **56** (581.2 mg, 77%) as a colorless oil: $[\alpha]^{20}{}_D$ = −2.2 (*c* 0.05, MeOH); $^1$H NMR (400MHz, CDCl$_3$) δ 7.73 (d, *J* = 8.1 Hz, 2H), 7.29 (d, *J* = 8.1 Hz, 2H), 4.75 (d, *J* = 8.3, 1H), 4.03 (m, 2H), 3.80 (s, 1H), 3.62 (dd, *J* = 10.0, 3.7 Hz, 1H), 3.48 (dd, *J* = 10.0, 6.2 Hz, 1H), 2.39 (s, 3H), 1.36 (s, 9H), 0.77 (s, 9H), -0.04 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 155.1, 145.0, 132.6, 130.0, 128.0, 79.8, 67.9, 60.9, 50.4, 28.3, 25.8, 21.7, 18.1, 14.2, 5.6; HRMS (ESI) calcd for C$_{21}$H$_{37}$NO$_6$SiSNa$^+$ [M + Na]$^+$ 482.2003, found 482.1961.
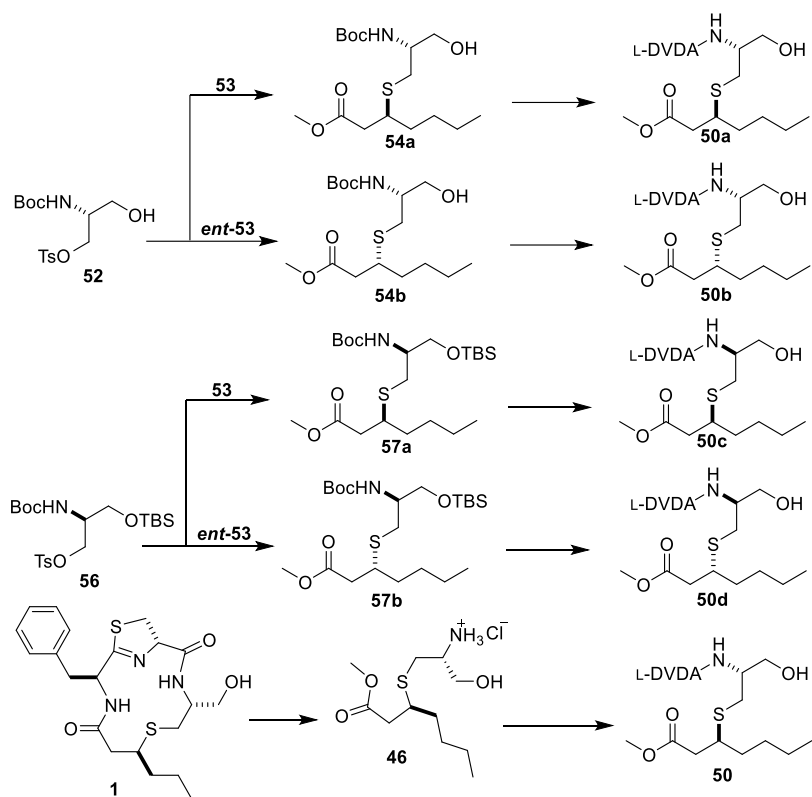
To a solution of **53** (0.6 mg, 3.40 μmol) and **52** (1.53 mg, 4.43 μmol) in MeCN (100 μL) was added $K_2CO_3$ (1mg) at room temperature. After being stirred for 24 h, the mixture was filtered and concentrated under reduced pressure. To the residue was added 4 M HCl in dioxane (0.2 mL). After being stirred at room temperature for 1 h, the solvent was removed under reduced pressure. The obtained hydrolysate was mixed with L-FDVDA (5mg/mL in acetone, 40 μL) and 1 M $NaHCO_3$ aqueous solution (20 μL) in $H_2O$ (100 μL), which was stirred at 50 °C for 2 h. The reaction was quenched with 2 M HCl (20 μL), evaporated, dissolved in MeCN (100 μL), filtered through a membrane filter (SHIMADZU, TORAST™ DISC, PTFE 0.22 μm). Diastereomers **50b-50d** were prepared by same way.

Natural **1** (0.1 mg) and synthetic **1** (0.1 mg) were hydrolyzed with 6 M HCl (200 μL) for 12 h at 105 °C, and then dried *in vacuo*. The obtained hydrolysate was dissolved in MeOH (100 μL), to which $SOCl_2$ (10 μL) was added at 0°C. After 30 min the solution was dried *in vacuo*. The obtained mixture was dissolved in $H_2O$ (50 μL), to which 1 M $NaHCO_3$ (20 μL) was added. The hydrolysate added L-FDVDA (0.5% w/v in acetone, 20 μL), and the mixtures were stirred for 2 h at 50 ℃. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL), evaporated, in MeCN (100 μL), filtered through a membrane filter (SHIMADZU, TORAST™ DISC, PTFE 0.22 μm), and then analyzed by LC-MS with **50a-50d** [column: Cadenza CD-C18, 3.0 × 150 mm; eluent: MeCN/$H_2O$/formic acid = 60/40/0.1 (isocratic min), 0.2 mL/min; detection: ESI-positive] (Figure 1.2.1).

# Chapter 2

# Studies on Combined-Culture of *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonsis* TP-B0596.

## 1 Introduction

In 2011, Onaka reported mycolic acid-containing bacteria *Tsukamurella pulmonis* TP-B0596 can influence the biosynthesis of cryptic natural products in *Streptomyces lividans* TK23. The subsequent research showed *Tsukamurella pulmonis* TP-B0596 is an activator strain which could change secondary metabolism in ~90% of *Streptomyces* species in combined-culture compared with single culture.[36] This new coculture method was called "combined-culture". Although their biological roles in the complex microbial communities are still largely unknown, in recent years, "combined-culture" fermentation method has been proved as a simple and powerful tool to search potential metabolites. Several novel bioactive compounds produced by the combined-culture were discovered such as arcyriaflavin E, chojalactones, dracolactams, catenulobactins, niizalactams, etc.[37]
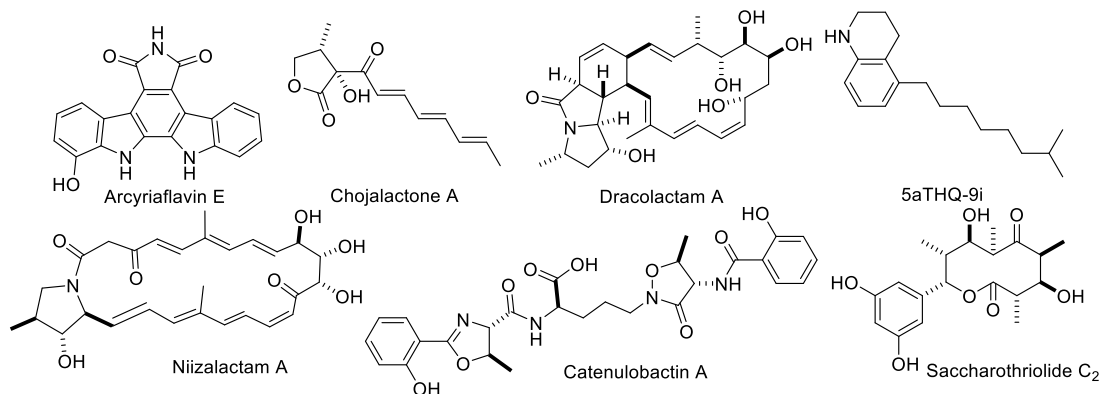


**Figure 2.1** Structures of novel compounds produced by the combined-culture.

By applying combined-culture method, our laboratory priviously discovered several novel 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs) from the combined-culture broth of *Streptomyces nigrescens* and *Tsukamurella pulmonis* TP-B0596; saccharothriolide $C_2$ from *Saccharothrix* sp. A1506 and *Tsukamurella pulmonis* TP-B0596.[17c,37f]

*Amycolatopsis* sp. 26-4 exhibited the ability of producing structurally unique secondary metabolites in the thioamicolamides research. In order to activate silent cryptic biosynthetic gene clusters and obtain more novel skeleton metabolites, combined-culture method was applied to strain *Amycolatopsis* sp. 26-4 with *Tsukamurella pulmonis* TP-B0596. Two novel nonapeptides, amycolapeptins A (**58**) and B (**59**), which were not produced in a single culture of *Amycolatopsis* sp. 26-4, isolated from the combined-culture broth, with 22-membered depsipeptide, *β*-hydroxytyrosine, and highly modified tryptophan moieties. The chemical structures including the absolute configuration were elucidated by spectroscopic analysis, chemical synthesis of fragments, a highly sensitive Marfey's method, and CD spectroscopy.

**Table 2.1** ^{1}H NMR data (600 MHz) and ^{13}C NMR data (150 MHz) for amycolapeptin A (**58**) and B (**59**) in CD$_3$OH.

| units | pos | amycolapeptin A (**58**) $\delta_C$, type | $\delta_H$ ($J$ in Hz) | pos | amycolapeptin B (**59**) $\delta_C$, type | $\delta_H$ ($J$ in Hz) |
|---|---|---|---|---|---|---|
| Val | 1 | 170.9, C | | 1 | 170.9, C | |
| | 2 | 57.2, CH | 4.65, dd (7.2, 3.0) | 2 | 57.3, CH | 4.64, dd (7.1, 2.9) |
| | 3 | 33.6, CH | 2.06, m | 3 | 33.6, CH | 2.05, m |
| | 4 | 19.3, CH$_3$ | 0.80, d (6.8) | 4 | 19.2, CH$_3$ | 0.80, d (6.8) |
| | 5 | 17.7, CH$_3$ | 0.70, d (6.8) | 5 | 17.8, CH$_3$ | 0.70, d (6.8) |
| | NH | | 6.69, d (7.2) | NH | | 6.74, d (7.2) |
| Pro/4-Me-Pro | 1 | 173.1, C | | 1 | 173.0, C | |
| | 2 | 62.8, CH | 3.80, m | 2 | 63.4, CH | 3.83, m |
| | 3 | 32.9, CH$_2$ | 1.67, m | 3 | 40.6, CH$_2$ | 1.28, m |
| | | | 1.86, m | | | 1.94, dd (12.2, 5.8) |
| | 4 | 23.0, CH$_2$ | 1.80, m | 4 | 31.4, CH | 2.25, m |
| | | | 1.87, m | | | |
| | 5 | 47.7, CH$_2$ | 3.46, m | 5 | 54.5, CH$_2$ | 2.96, m |
| | | | 3.68, m | | | 3.80, m |
| | | | | 6 | 17.2, CH$_3$ | 1.02, d (6.3) |
| *N*-Me-Phe | 1 | 173.7, C | | 1 | 173.6, C | |
| | 2 | 55.6, CH | 5.50, dd (8.3, 7.5) | 2 | 55.3, CH | 5.49, m |
| | 3 | 36.8, CH$_2$ | 3.02, dd (13.4, 7.0) | 3 | 36.8, CH$_2$ | 3.02, dd (13.3, 6.9) |
| | | | 3.22, m | | | 3.22, m |
| | 4 | 137.4, C | | 4 | 137.4, C | |
| | 5/9 | 130.0, CH | 7.18, d (7.3) | 5/9 | 130.0, CH | 7.18, d (7.2) |
| | 6/8 | 130.0, CH | 7.28, t (7.3) | 6/8 | 130.0, CH | 7.29, m |
| | 7 | 128.2, CH | 7.23, m | 7 | 128.2, CH | 7.23, m |
| | *N*-Me | 32.2, CH$_3$ | 3.44, s | *N*-Me | 32.1, CH$_3$ | 3.43, s |
| Trp | 1 | 174.4, C | | 1 | 174.4, C | |
| | 2 | 53.0, CH | 4.61, m | 2 | 53.0, CH | 4.60, m |
| | 3 | 26.6, CH$_2$ | 3.09, m | 3 | 26.6, CH$_2$ | 3.09, m |
| | | | 3.25, dd (14.8, 11.2) | | | 3.25, dd (15.6, 12.3) |
| | 4 | 111.0, C | | 4 | 111.0, C | |
| | 5 | 128.0, C | | 5 | 128.0, C | |
| | 6 | 118.6, CH | 7.49, d (7.8) | 6 | 118.6, CH | 7.48, d (7.8) |
| | 7 | 119.8, CH | 6.99, m | 7 | 119.9, CH | 6.99, m |
| | 8 | 122.4, CH | 7.06, m | 8 | 122.4, CH | 7.06, m |
| | 9 | 112.5, CH | 7.32, d (8.0) | 9 | 112.4, CH | 7.31, d (8.0) |
| | 10 | 138.0, C | | 10 | 138.0, C | |
| | 11 | 124.9, CH | 6.83, s | 11 | 124.9, CH | 6.82, s |
| | 2-NH | | 9.01, d (6.5) | 2-NH | | 8.99, d (6.4) |
| | 11-NH | | 10.17, s | 11-NH | | 10.17, s |
| 4-OH-Pro | 1 | 171.3, C | | 1 | 171.3, C | |
| | 2 | 61.7, CH | 3.79, m | 2 | 61.8, CH | 3.79, m |

| | 3 | 36.8, CH$_2$ | 0.45, m | 3 | 36.8, CH$_2$ | 0.45, m |
|---|---|---|---|---|---|---|
| | | | 2.10, dd (11.7, 6.7) | | | 2.09, dd (11.5, 6.6) |
| | 4 | 68.6, CH | 3.60, m | 4 | 68.6, CH | 3.60, dt (14.5, 7.3) |
| | 5 | 52.6, CH$_2$ | 2.46, dd (12.7, 8.0) | 5 | 52.5, CH$_2$ | 2.46, dd (12.6, 8.0) |
| | | | 3.12, m | | | 3.12, m |
| *N*-Me-Tyr | 1 | 173.8, C | | 1 | 173.8, C | |
| | 2 | 58.5, CH | 5.11, m | 2 | 58.5, CH | 5.11, m |
| | 3 | 36.9, CH$_2$ | 2.93, m | 3 | 36.9, CH$_2$ | 2.93, m |
| | | | 3.09, m | | | 3.09, m |
| | 4 | 127.7, C | | 4 | 127.7, C | |
| | 5/9 | 131.9, CH | 6.98, d (8.2) | 5/9 | 131.9, CH | 6.97, d (8.1) |
| | 6/8 | 116.9, CH | 6.71, d (8.9) | 6/8 | 116.9, CH | 6.71, d (8.9) |
| | 7 | 157.8, C | | 7 | 157.8, C | |
| | *N*-Me | 33.4, CH$_3$ | 3.08, s | *N*-Me | 33.3, CH$_3$ | 3.08, s |
| Thr | 1 | 171.9, C | | 1 | 171.9, C | |
| | 2 | 54.7, CH | 5.00, s | 2 | 54.7, CH | 5.00, m |
| | 3 | 71.8, CH | 5.61, q (6.5) | 3 | 71.8, CH | 5.61, q (6.5) |
| | 4 | 17.0, CH$_3$ | 1.05, d (6.5) | 4 | 17.1, CH$_3$ | 1.04, d (6.5) |
| | NH | | 7.73, d (9.2) | NH | | 7.72, d (8.9) |
| *β*-OH-Tyr | 1 | 172.0, C | | 1 | 172.0, C | |
| | 2 | 60.5, CH | 4.88, m | 2 | 60.5, CH | 4.88, m |
| | 3 | 73.6, CH | 5.13, m | 3 | 73.6, CH | 5.13, m |
| | 4 | 131.8, C | | 4 | 131.8, C | |
| | 5/9 | 129.0, CH | 7.23, d (8.1) | 5/9 | 129.0, CH | 7.23, m |
| | 6/8 | 116.2, CH | 6.73, d (8.9) | 6/8 | 116.2, CH | 6.73, d (8.9) |
| | 7 | 158.5, C | | 7 | 158.5, C | |
| | NH | | 8.09, d (8.4) | NH | | 8.08, d (8.1) |
| Imhsa | 1 | 177.6, C | | 1 | 177.6, C | |
| | 2 | 77.9, C | | 2 | 77.9, C | |
| | 3 | 36.3, CH$_2$ | 3.21, m | 3 | 36.3, CH$_2$ | 3.21, m |
| | | | 3.31, m | | | 3.31, m |
| | 4 | 109.3, C | | 4 | 109.2, C | |
| | 5 | 129.5, C | | 5 | 129.5, C | |
| | 6 | 119.8, CH | 7.61, d (7.9) | 6 | 119.8, CH | 7.61, d (7.8) |
| | 7 | 119.8, CH | 7.01, m | 7 | 119.7, CH | 7.01, m |
| | 8 | 122.1, CH | 7.08, m | 8 | 122.1, CH | 7.08, m |
| | 9 | 112.1, CH | 7.32, d (8.0) | 9 | 112.1, CH | 7.31, d (8.0) |
| | 10 | 137.7, C | | 10 | 137.7, C | |
| | 11 | 125.9, CH | 7.21, s | 11 | 125.9, CH | 7.21, s |
| | 12 | 44.5, CH$_2$ | 2.68, d (15.3) | 12 | 44.4, CH$_2$ | 2.68, d (15.1) |
| | | | 2.82, d (15.3) | | | 2.82, d (15.1) |
| | 13 | 173.3, C | | 13 | 173.3, C | |
| | 11-NH | | 10.28, s | 11-NH | | 10.27, s |

## 2 Structure Elucidation of Amycolapeptins A and B

Fifteen liter of the combined-culture broth was filtered, extracted with EtOAc and concentrated *in vacuo* to give crude extract (0.63 g). The extract was separated by ODS $C_{18}$ column and semi-preparative HPLC to give two novel compounds amycolapeptin A (**58**, 37.7 mg) and B (**59**, 11.9 mg).
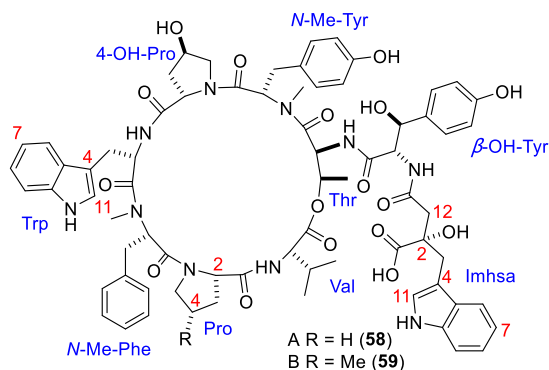


**Figure 2.2** Structures of amycolapeptins A and B (**58**, **59**).

The molecular formula of amycolapeptin A (**58**) was determined to be $C_{72}H_{82}N_{10}O_{17}$ by the analysis of HRMS data ($m/z$ 1359.6012 $[M + H]^+$ calcd. 1359.5932). The $^{13}C$ NMR spectrum in **58** showed the presence of 10 amide carbonyl signals from 170.9 to 177.5 ppm, suggest that compound **58** is a peptide, and a large number of carbon signals from 109.3 to 158.5 ppm revealed that several aromatic amino acid units in **58**. The $^1H$ NMR spectrum of **58** displayed signals for two *N*-Me groups ($\delta_H$ 3.44, 3.08). The UV spectrum showed an absorption band at $\lambda_{max}$ 280 nm and amino proton signals at $\delta_H$ 10.17, 10.28, indicating the existence of two tryptophan (Trp) moieties. The carbon signals at $\delta_C$ 158.5, 157.8, and $\delta_C$ 117.0, 116.2, showed two tyrosine (Tyr) moieties in **58**.

Detailed analysis of the 1D NMR and 2D NMR spectra including COSY, HMQC, HMBC in $CD_3OH$ allowed for the complete spectral assignment of eight amino acids: four normal amino acid residues consisting of proline (Pro), valine (Val), threonine (Thr), and tryptophan (Trp), together with four nonproteinogenic amino acid residues: *β*-hydroxytyrosine (*β*-OH-Tyr), *N*-methylphenylalanine (*N*-Me-Phe), *N*-methyltyrosine (*N*-Me-Tyr) and 4-hydroxyproline (4-OH-Pro). In addition, a 2-((1*H*-indol-3-yl)methyl)-2-hydroxysuccinic acid (Imhsa) moiety was also established (Figure 2.2). NMR assignments for amycolapeptin A (**58**) are listed in Table 2.1.

The amino acid sequence of **58** was assigned from ROESY correlations between *α*-protons and neighboring residue NH or *N*-Me protons (Figure 2.3a), with the following correlations observed: the Pro H5/*N*-Me-Phe H2, the *N*-Me-Phe *N*-Me/Trp H2, the Trp NH/4-OH-Pro H2, 4-OH-Pro H5/*N*-Me-Tyr H2, *N*-Me-Tyr *N*-Me /Thr H2, Thr NH/*β*-OH-Tyr H2, and the *β*-OH-Tyr NH/Imhsa H12. The presence of an ester bond between the carbonyl group of Val and the hydroxy group of Thr was suggested by chemical shift of the *β*-methine proton H-3 ($\delta_H$ 5.61 upfield shifted) and confirmed by the presence of an HMBC correlation between this proton and the Val carbonyl carbon C-1 ($\delta_C$ 170.9).
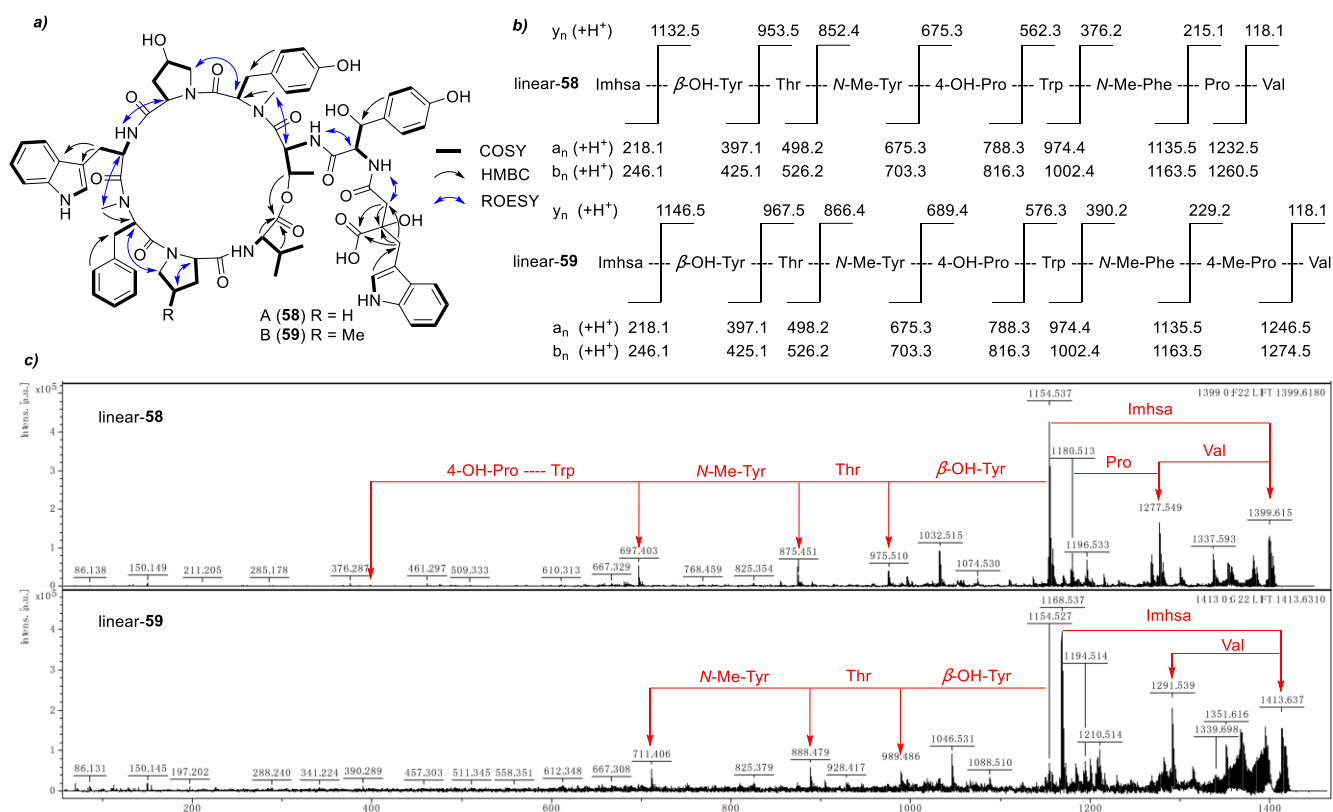
**Figure 2.3** (a) Key COSY, HMBC, and ROESY correlations for amycolapeptin A (**58**) and B (**59**); (b) The characteristic product ions were assigned as a-, b-, and y-series of amino acid fragmentation; (c) MS/MS analysis of linear **58** and **59**.

Furthermore, the amino acid sequence of **58** was determined by MS/MS analysis. Alkali hydrolysis to cleave the ester bond of **58**. The sequence of **58** from the N-terminal was determined to be the following: Imhsa - $\beta$-OH-Tyr - Thr - *N*-Me-Tyr - 4-OH-Pro - Trp - *N*-Me-Phe - Pro - Val (Figure 2.3b, c).

The absolute configurations of the amino acid constituents of **58** were determined by acid hydrolysis and application of the highly sensitive Marfey's method. The comparison of the retention times between the standards and obtained derivertives shed light on the presence of L-Val, L-Pro, L-*N*-Me-Phe, L-Trp, L-4-OH-Pro, L-*N*-Me-Tyr, and L-Thr in **58** (Figure 2.7). However, I did not detect $\beta$-OH-Tyr, which was destroyed by hydrolysis. This property has been reported in litereture.[38]

In order to determine the absolute configuration of $\beta$-OH-Tyr unit, amycolapeptin A (**58**) was submitted to ozonolysis, followed by hydrogen peroxide workup, transforms into the acid-stable $\beta$-OH-Asp.[38] The oxidized amycolapeptin A (**58**) was hydrolyzed, derivatized with L-FDAA. Standards of $\beta$-OH-Asp were prepared from *trans*-epoxysuccinic acid and *cis*-epoxysuccinic acid, respectively, according to literature protocols.[39] Although synthetic $\beta$-OH-Asp were mixture of isomers (Scheme 2.1), previous research has been established that the elution order with $C_{18}$ reversed phase conditions for L-FDAA-$\beta$-OH-Asp diastereomers is 2*R*,3*R* (**61a**) → 2*S*,3*S* (**61b**) → 2*R*,3*S* (**61c**) → 2*S*,3*R* (**61d**).[39a,40] The subsequent LC-MS analysis revealed the presence of (2*S*,3*R*)-$\beta$-OH-Asp (**61d**) in the hydrolysate, and thus (2*S*,3*S*)-$\beta$-OH-Tyr in amycolapeptin A (**58**) (Figure 2.4).
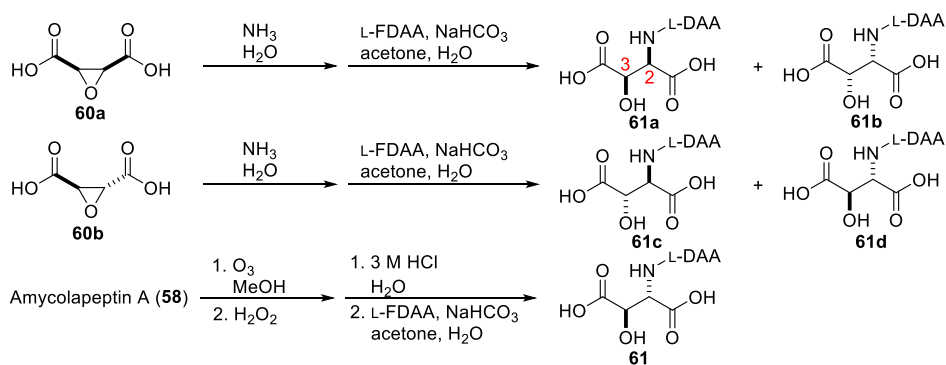
**Scheme 2.1** Synthesis of β-OH-Asp derivatives
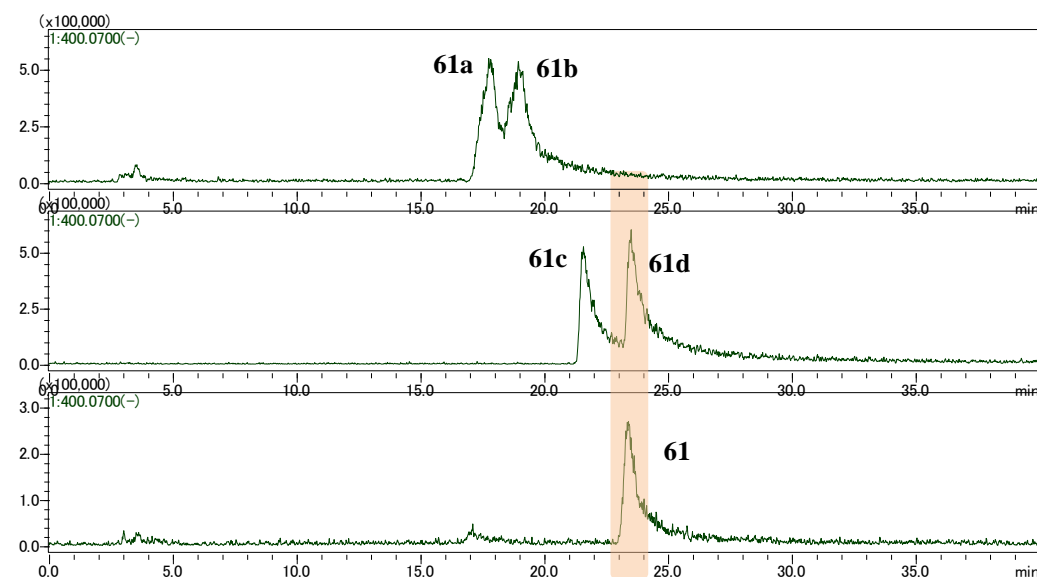


**Figure 2.4** LC-MS analysis of β-OH-Asp Marfey's derivatives
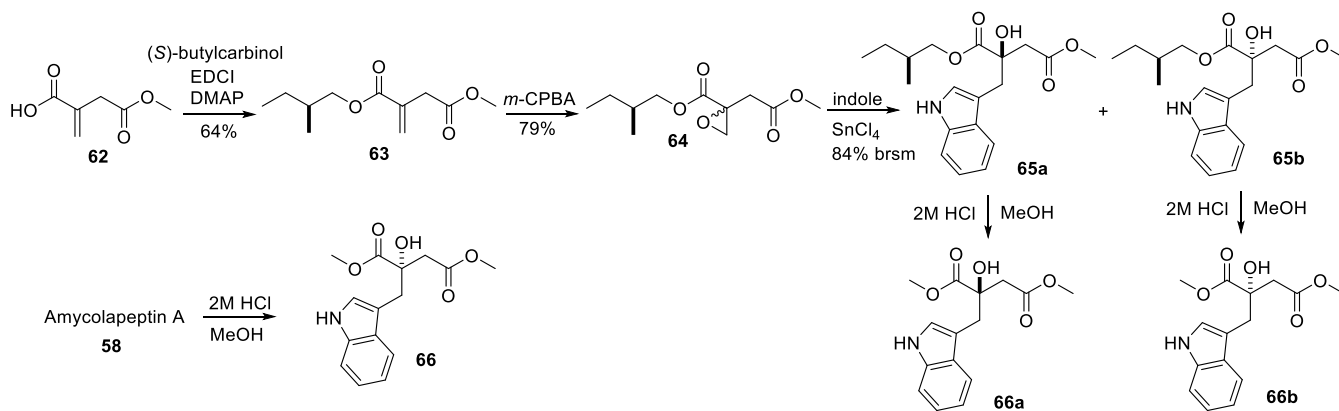


**Scheme 2.2** Synthesis of **66** isomers.

The chirality of C2 in Imhsa moiety was determined by the combination of the chemical synthesis and LC-MS analysis. Hydrolysis of amycolapeptin A (**58**) yield methy ester of Imhsa moiety (**66**). The required standard samples for the comparison, namely, the 2*R* and 2*S* enantiomers of **66**, were prepared by the chemical synthesis and optical resolution

(Scheme 2.2). Monomethyl itaconate (**62**) was treated with (*S*)-butylcarbinol, EDCI·HCl, and DMAP led to the ester **63**. The epoxidation by *m*-CPBA (*meta*-Chloroperoxybenzoic acid) furnished mixture of diastereomers **64**. The coulping reaction between indole and **64** delivered diastereomixture of **65a**/**65b**, which could be separated by reversed-phase HPLC.[41]
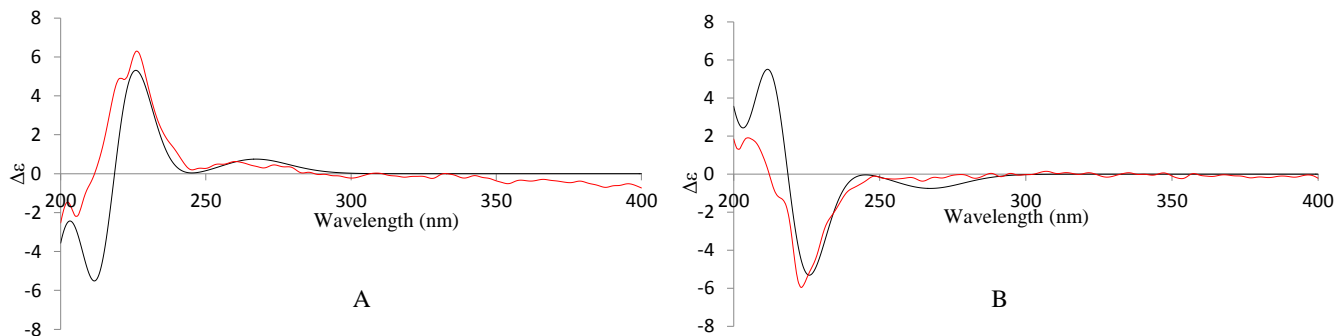


**Figure 2.5** A: m062x/def2tzvp calculated ECD spectra (σ = 0.2 eV) of 2*R* of **65** (black), and the experimental ECD spectrum (red) for **65a**. B: calculated ECD spectra (σ = 0.2 eV) of 2*S* of **65** (black), and the experimental ECD spectrum (red) for **65b**.

The absolute configuration of the **65a** and **65b** were defined by comparison of the quantum chemical calculated and experimental ECD spectra (Figure 2.5). Hydrolysis of **65a**/**65b** led to optically pure **66a**/**66b**, respectively. Finally, the methy ester **66** derived from amycolapeptin A (**58**) was chromatographically compared with the standard compounds **66a** and **66b** by LC-MS, LC separation was performed on a chirality column. The retention time of **66** was matched with that of **66b**, confirming *S* configuration at C2 (Figure 2.8).

Amycolapeptin B (**59**) was isolated from the same fraction and exhibited almost same NMR data in the main skeleton (Table 2.1). The amino acid sequence of **59** was also determined by ROESY spectra and MS/MS analysis (Figure 2.3), only the Pro unit in **58** was changed to 4-Me-Pro, which suggests that they may originate from a similar biosynthetic pathway. The optical rotation of **58** ([*α*]$_D^{20}$ = -40.9 ) is close to those of **59** ([*α*]$_D^{20}$ = -30.2 ). The ECD spectrum of **58** showed one positive Cotton effect at 230 nm and two negative Cotton effects at 215 and 274 nm, which were in good agreement with the experimental curve of analogue **59**, indicating the same absolute configurations at the chiral center of **59** (Figure S118). Therefore, 4-Me-Pro unit in **59** was confirmed to L type. The ROESY across peak of H2 (δ$_H$ 3.83)/H4 (δ$_H$ 2.25) indicated that H2 and H4 were located at the same side of the molecule, suggest the configuration of 4-Me-Pro in **59** was assigned as 2*S*,4*S*.

## 3 Cytotoxicity

The cytotoxicities of amycolapeptins A (**58**) and B (**59**) were evaluated with HeLa S3 cells. Adriamycin was used as a positive control. Both of them showed no significant cytotoxicity even at 100 *μ*M, respectively.

## 4 Exprimental Section

### 4.1 General Procedures

See 1.5.1

## 4.2 Cultivation of the *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonsis* TP-B0596

*Amycolatopsis* sp. 26-4 was cultured on shakers (180 rpm) at 28 °C for 4 days in 500 mL Erlenmeyer flasks containing 200 mL of seed culture medium (ISP2), composed of glucose (4.0 g/L), malt extract (10.0 g/L) and yeast extract (4.0 g/L). *Tsukamurella pulmonsis* TP-B0596 was cultured on shakers (180 rpm) at 28 °C for 2 days in 500 mL Erlenmeyer flasks containing 200 mL of seed culture medium (ISP2), composed of glucose (4.0 g/L), malt extract (10.0 g/L) and yeast extract (4.0 g/L). 1 mL *Amycolatopsis* sp. 26-4 and 1 mL *Tsukamurella pulmonsis* TP-B0596 seed culture broth were inoculated in 1 L Erlenmeyer flasks containing 400 mL of culture medium (ISP3), composed of oatmeal (20.0 g/L), $FeSO_4 \cdot 7H_2O$ (1.0 mg/L), $MnCl_2 \cdot 4H_2O$ (1.0 mg/L) and $ZnSO_4 \cdot 7H_2O$ (1.0 mg/L), shaken 180 rpm at 28 °C for 5 days. As controls, two strains were cultured at the same condition individually.
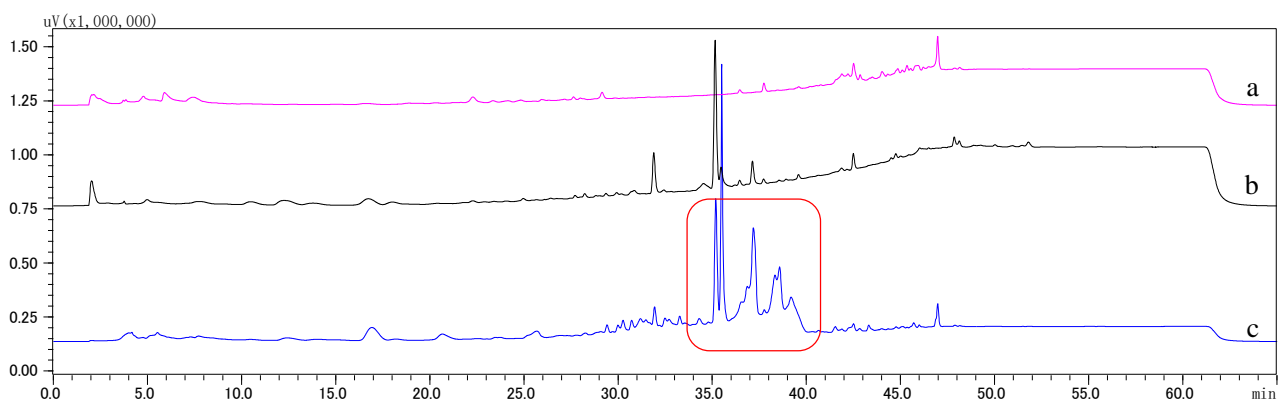


**Figure 2.6** HPLC data comparison of EtOAC extracts of each single culture and combined-culture broths. (a) *Tsukamurella pulmonsis* TP-B0596; (b) *Amycolatopsis* sp. 26-4; (c) *Amycolatopsis* sp. 26-4 + *Tsukamurella pulmonsis* TP-B0596 (Cosmosil PEGASIL ODS SP100 Φ 4.6 × 250mm, 20-100% MeOH 40min + 100% MeOH 15min + 20% MeOH 10min, 1.0 mL/min, 210nm)

## 4.3 Extraction and Isolation

Fifteen liter of the combined-culture broth was filtered, which was extracted with EtOAc (2 × 15 L) and concentrated *in vacuo* to give crude extract (0.63 g). The extract was subjected to the ODS $C_{18}$ column using a gradient elution of $MeOH/H_2O$ (10%, 30%, 50%, 70%, 100% MeOH in $H_2O$) stepwise to give 7 fractions. Every fraction was dissolved in methanol and centrifuged at 8,000 rpm for 5 min. Fractions 5 and 6 was separated by semi-preparative HPLC (Cosmosil π-nap (Nacalai Tesque, Kyoto), Φ20 × 250 mm, $MeCN:H_2O=45:55$, 0.05%TFA, 8 mL/min) to give compound amycolapeptin A (37.7 mg, $t_R$ 36.0 min) and compound amycolapeptin B (11.9 mg, $t_R$ 46.0 min).

Amycolapeptin A (**58**): white, powder, $[\alpha]^{20}_D$ = -40.9 (*c* 0.07, $CH_3OH$), UV/vis ($CH_3OH$) $\lambda_{max}$ (log$\varepsilon$) 200 (4.99), 221 (4.85), 279 (4.01) IR $\nu_{max}$ 3293, 2926, 2853, 1734, 1627, 1517, 1455, 1228, 1211, 1094 cm$^{-1}$, CD (*c* 2.87 × 10$^{-4}$ mol/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$): 216 (-10.9), 231 (+11.6), 275 (-2.4), $^1$H and $^{13}$C NMR, Table 2.1; HRMS (ESI) calcd for $C_{72}H_{83}N_{10}O_{17}^+$ [M + H]$^+$ 1359.5932, found 1359.6012

Amycolapeptin B (**59**): white, powder, $[\alpha]^{20}_D$ = -30.2 (*c* 0.29, $CH_3OH$) , UV/vis ($CH_3OH$) $\lambda_{max}$ (log$\varepsilon$) 200 (4.98), 221 (4.87), 280 (4.05) IR $\nu_{max}$ 3293, 2966, 2932, 1727, 1627, 1517, 1452, 1239, 1204, 1094 cm$^{-1}$ , CD (*c* 2.33 × 10$^{-4}$

mol/L, MeOH) $\lambda_{\max}$ ($\Delta\varepsilon$): 216 (-12.2), 231 (+10.7), 275 (-1.7), $^1$H and $^{13}$C NMR, Table 2.1; HRMS (ESI) calcd for $C_{73}H_{85}N_{10}O_{17}^+$ [M + H]$^+$ 1373.6089, found 1373.6208

## 4.4 Cytotoxicity Assay

Cytotoxicity of **58** and **59** against HeLa S3 cell lines was evaluated by a WST-8 colorimetric assay (Cell Counting Kit-8, Dojindo). Briefly, cells were cultured in 96-well plates (3,000 cells/well) for 24 h followed by exposure to a sequential dilution of test compounds for 72 h, and then the viability was assessed by WST-8. The absorbance was measured at 450 nm using an iMark microplatereader (BIO-RAD). Adriamycin was evaluated as a positive control. $IC_{50}$ values are shown as the mean $\pm$ SD (n=4).

## 4.5 Alkali hydrolysis

Amycolapeptins **58** and **59** (100 μg) were dissolved in 1 mL of a mixture of 50% methanol, 45% water and 5% $NH_3$, and incubated at room temperature for 48 h and evaporated. The obtained linear peptides were analyzed by LC-MS.

## 4.6 Acid Hydrolysis of 58 and highly sensitive Marfey's Method

Amycolapeptin A (**58**) (0.1 mg) was hydrolyzed in 3 M HCl (100 μL) with 0.1% phenol for 6 h at 100 ℃ under $N_2$, and then dried *in vacuo*. The obtained hydrolysate was dissolved in $H_2O$ (100 μL), to which 1 M $NaHCO_3$ (20 μL) was added. The hydrolysate added L-FDVDA (0.5% w/v in acetone, 20 μL), and the mixtures were stirred for 2 h at 50 °C. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL), evaporated, and then dissolved in MeCN (100 μL). The derivatives were analyzed by LC-MS; LC separation was performed on a reversed-phase column (Cadenza CD-C18, 3.0 $\times$ 150 mm) with a gradient elution system of $H_2O$/MeCN containing 0.1% FA (20% MeCN 10 min, 20-90% MeCN 20 min, 90% MeCN 10 min, 0.2 mL/min).
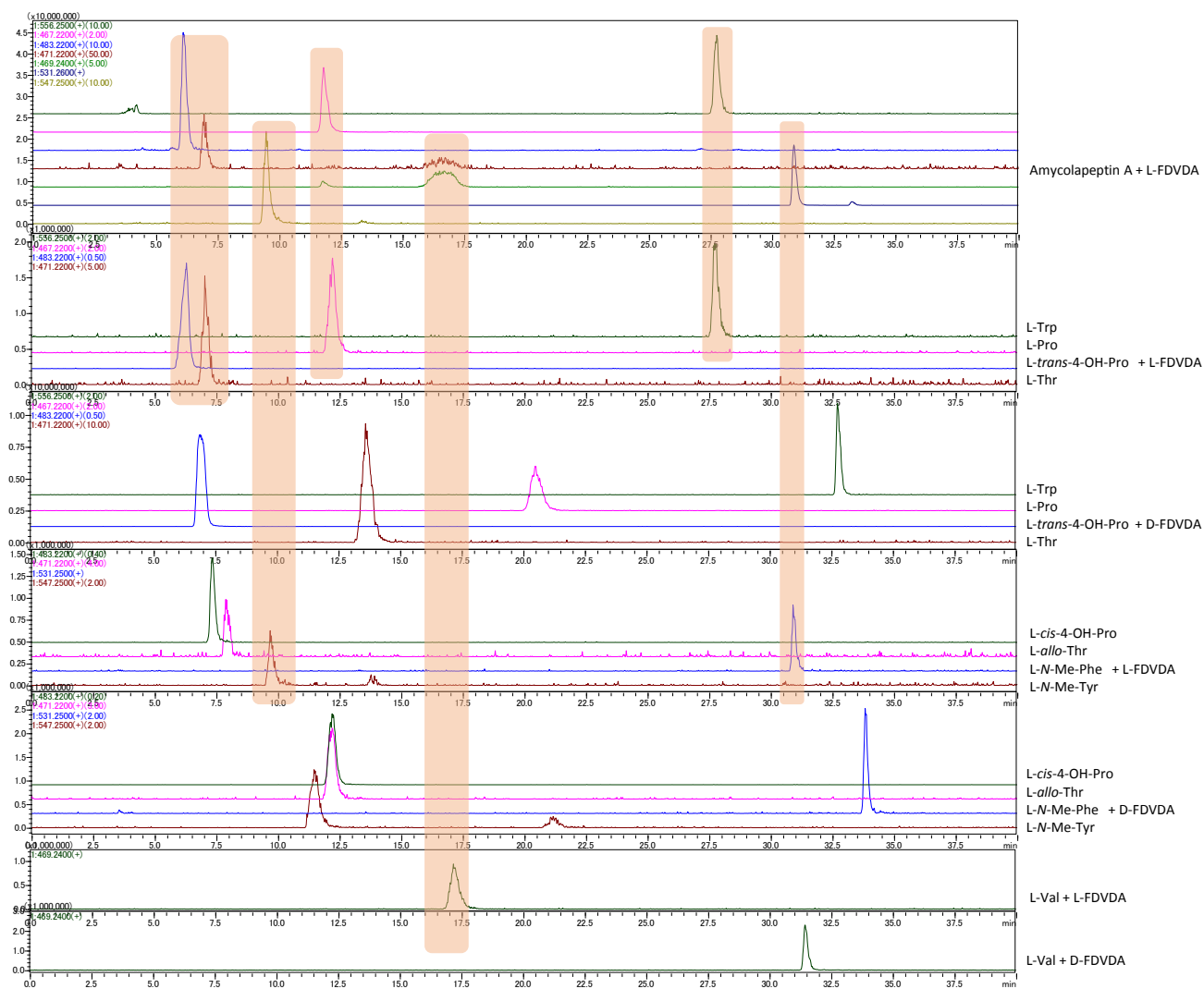
**Figure 2.7** LC-MS analysis of Marfey's derivatives

### 4.7 Synthesis of β-OH-Asp Diastereomer

Diastereomeric mixtures of *β*-OH-Asp were prepared by treatment of *cis*-epoxysuccinic acid (**60a**, 1 mg) and *trans*-epoxysuccinic acid (**60b**, 1 mg) with 100 μL of 28% ammonia water. Reactions were allowed to proceed for 24 h at 50 ℃. Reaction mixtures were dried *in vacuo*. This material was used as standards without further purification.

### 4.8 Ozonolysis of β-hydroxy tyrosine in amycolapeptin A

Amycolapeptin A (**58**) (0.5 mg) was dissolved in MeOH (1 mL) at 0 ℃. A stream of ozone was bubbled for 10 min through the solution. Subsequently 200 μL of $H_2O_2$ (30%) were added to the reaction mixture which was then allowed to stand at room temperature for 1 h. The solvent was removed *in vacuo*. The resiue was hydrolyzed in 3 M HCl (100 μL) for 6 h at 100 ℃ under $N_2$, and then dried *in vacuo*. The obtained hydrolysate and standards were dissolved in $H_2O$ (100 μL), to which 1 M $NaHCO_3$ (20 μL) was added. The hydrolysate added L-FDAA (0.5% w/v in acetone, 20 μL), and the mixtures were stirred for 2 h at 50 ℃. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL),

42

evaporated, and then dissolved in MeCN (100 μL). The derivatives were analyzed by LC-MS; LC separation was performed on a reversed-phase column (Cosmosil 5C$_{18}$-AR-II, 2.0 × 250 mm) with a gradient elution system of H$_2$O/MeCN containing 0.1% FA (10-30% MeCN 30 min, 30% MeCN 10min, 0.2 mL/min).

## 4.9 Synthesis of 66 isomers
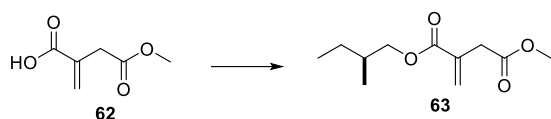


Monomethyl itaconate **62** (500 mg, 3.47 mmol) was mixed with (*S*)-butylcarbinol (411 μL, 3.82 mmol) in dry CH$_2$Cl$_2$ (15 mL) under N$_2$, followed by EDCI·HCl (732 mg, 3.82 mmol) and DMAP (42.4 mg, 0.35 mmol) at 0 ℃. The resulting mixture was allowed to warm up to rt and stirred overnight. The reaction was quenched with 0.2 M HCl (15 mL). The combined organics were washed with brine (15 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc : hexanes =1 : 4) on silica gel to yield easter **63** (473.5 mg, 64%) as a colorless oil.

**63**: [*α*]$^{20}$$_D$ = 3.3 (*c* 1.54, MeOH); $^1$H NMR (600 MHz, CDCl$_3$): δ 6.26 (s, 1H), 5.63 (s, 1H), 3.98 (dd, *J* = 10.8, 5.9 Hz, 1H), 3.89 (dd, *J* = 10.8, 6.6 Hz, 1H), 3.61 (s, 3H), 3.27 (s, 3H), 1.66 (m, 1H), 1.36 (m, 1H), 1.16 (m, 1H), 0.85 (m, 6H); $^{13}$C NMR (150 MHz, CDCl$_3$) δ 171.2, 166.2, 134.0, 128.4, 69.6, 52.0, 37.6, 34.2, 26.0, 16.4, 11.2; HRMS (ESI) calcd for C$_{11}$H$_{18}$O$_4$Na$^+$ [M + Na]$^+$ 237.1097, found 237.1115.
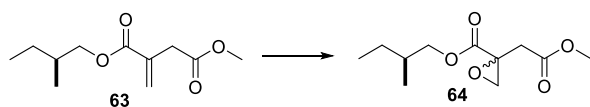


*m*-CPBA (75%) (513 mg, 2.23 mmol) was added to a solution of ester **63** (398 mg, 1.86 mmol) in CHCl$_3$ (3 mL). The mixture was heated in oil bath to 70 ℃ for 12 h, cooled, diluted by addition of CHCl$_3$ (10 mL). The mixture was washed with aqueous solution of Na$_2$SO$_3$ (10%, 10 mL), NaHCO$_3$ (10 mL) and brine (10 mL), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc, 5:1) to yield epoxy **64** (337.5 mg, 79%) as a colorless oil.

**64**: $^1$H NMR (600 MHz, CDCl$_3$): δ 4.02 (m, 1H), 3.94 (m, 1H), 3.68 (s, 3H), 3.16 (d, *J* = 5.7 Hz, 1H), 2.92 (dd, *J* = 17.0, 4.7 Hz, 1H), 2.87 (d, *J* = 5.7 Hz, 1H), 2.79 (dd, *J* = 17.0, 4.2 Hz, 1H), 1.70 (m, 1H), 1.37 (m, 1H), 1.15 (m, 1H), 0.87 (m, 6H); $^{13}$C NMR (150 MHz, CDCl$_3$) δ 169.9, 169.5, 70.7, 70.6, 53.7, 52.2, 52.0, 37.4, 34.1, 25.9, 16.4, 16.3, 11.3, 11.2; HRMS (ESI) calcd for C$_{11}$H$_{18}$O$_5$Na$^+$ [M + Na]$^+$ 253.1046, found 253.1062.
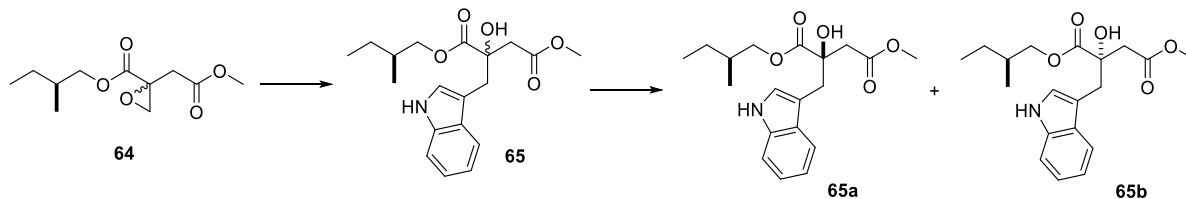


To a solution of **64** (111.6 mg, 0.48 mmol) and indole (62.5 mg, 0.53 mmol) in CCl$_4$ (5 mL) were added SnCl$_4$ (62.4 μL, 0.53 mmol) in 0.5 mL CCl$_4$ at 0 ℃. After being stirred at room temperature for 1 h, saturated aqueous NaHCO$_3$ (10

mL) was added to the reaction mixture. The mixture was extracted with EtOAc (10 mL ×2). The combined organic layer was washed with brine (20 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc : hexanes =1 : 2) to yield **65** (9.0 mg, 5%) as a yellow foam and starting material **64** (104.5 mg, 94% was recovered).

**65**: $^1$H NMR (600 MHz, CD$_3$OD): δ 7.50 (d, $J$ = 7.9 Hz, 1H), 7.32 (d, $J$ = 8.1 Hz, 1H), 7.12 (s, 1H), 7.07 (t, $J$ = 7.5 Hz, 1H), 6.99 (t, $J$ = 7.2 Hz, 1H), 3.88 (dd, $J$ = 10.5, 6.1 Hz, 0.5H), 3.80 (dd, $J$ = 10.6, 6.5 Hz, 0.5H), 3.71 (dd, $J$ = 10.7, 5.7 Hz, 0.5H), 3.64 (dd, $J$ = 10.6, 6.5 Hz, 0.5H), 3.62 (s, 3H), 3.21 (m, 1H), 3.13 (d, $J$ = 14.5 Hz, 1H), 3.08 (m, 1H), 2.73 (d, $J$ = 16.0 Hz, 1H), 1.49 (m, 1H), 1.25 (m, 1H), 1.04 (m, 1H), 0.81 (m, 3H), 0.75 (m, 3H); $^{13}$C NMR (150 MHz, CD$_3$OD) δ 176.1, 172.6, 137.8, 129.4, 125.4, 122.2, 119.7, 119.6, 112.2, 109.4, 77.4, 77.3, 71.1, 71.0, 52.1, 43.8, 43.7, 36.7, 36.6, 35.1, 35.0, 26.9, 16.7, 16.5, 11.6, 11.5; HRMS (ESI) calcd for C$_{19}$H$_{26}$O$_5$N$^+$ [M + H]$^+$ 348.1805, found 348.1825.

One mg of **65** was subjected to reversed-phase HPLC (CHIRALPAK OD-RH 4.6 ×150 mm (Daicel Chemical Co. Ltd.), H$_2$O/MeOH (35/65)) to yield **65a** (0.22 mg, $t_R$ 35.0 min, 22%) and **65b** (0.19 mg, $t_R$ 37.5 min, 19%).

**65a**: [α]$^{20}_D$ = 17.5 ($c$ 0.02, MeOH); $^1$H NMR (600 MHz, CD$_3$OD): δ 7.50 (d, $J$ = 8.0 Hz, 1H), 7.32 (d, $J$ = 7.9 Hz, 1H), 7.12 (s, 1H), 7.07 (t, $J$ = 7.2 Hz, 1H), 6.99 (t, $J$ = 7.6 Hz, 1H), 3.88 (dd, $J$ = 10.5, 6.1 Hz, 1H), 3.64 (dd, $J$ = 10.6, 6.5 Hz, 1H), 3.62 (s, 3H), 3.21 (d, $J$ = 14.5 Hz, 1H), 3.13 (d, $J$ = 14.5 Hz, 1H), 3.08 (d, $J$ = 16.0 Hz, 1H), 2.73 (d, $J$ = 16.2 Hz, 1H), 1.49 (m, 1H), 1.25 (m, 1H), 1.03 (m, 1H), 0.81 (t, $J$ = 7.5 Hz, 3H), 0.76 (d, $J$ = 6.6 Hz, 3H).

**65b**: [α]$^{20}_D$ = −10.0 ($c$ 0.02, MeOH); $^1$H NMR (600 MHz, CD$_3$OD): δ 7.50 (d, $J$ = 7.7 Hz, 1H), 7.32 (d, $J$ = 8.0 Hz, 1H), 7.12 (s, 1H), 7.07 (t, $J$ = 7.5 Hz, 1H), 6.99 (t, $J$ = 7.5 Hz, 1H), 3.81 (dd, $J$ = 10.5, 6.6 Hz, 1H), 3.71 (dd, $J$ = 10.6, 5.6 Hz, 1H), 3.62 (s, 3H), 3.21 (d, $J$ = 14.5 Hz, 1H), 3.14 (d, $J$ = 14.5 Hz, 1H), 3.08 (d, $J$ = 16.2 Hz, 1H), 2.73 (d, $J$ = 16.1 Hz, 1H), 1.49 (m, 1H), 1.26 (m, 1H), 1.05 (m, 1H), 0.82 (t, $J$ = 7.5 Hz, 3H), 0.75 (d, $J$ = 6.7 Hz, 3H).
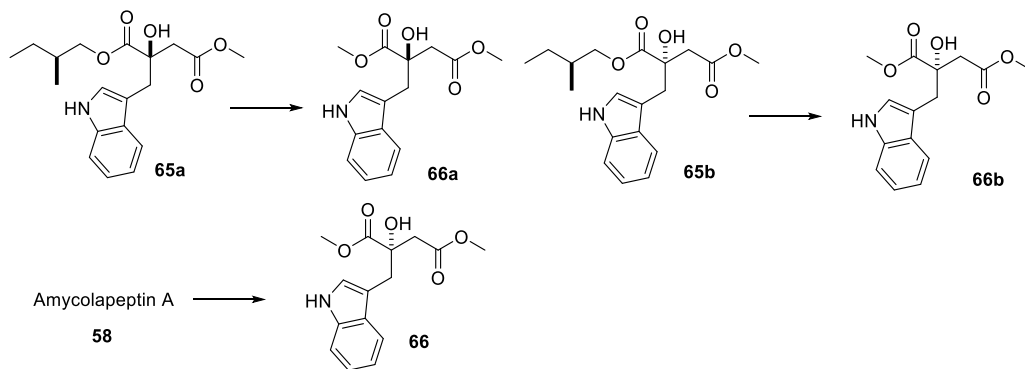


**65a** (0.01 mg) and 2 M HCl in MeOH (200 μL, prepared from 100 μL MeOH and 100 μL 4 M HCl in dioxane) was stirred at room temperature for 12 h. The solvent was removed at reduced pressure to yield **66a**, which was used for further LC-MS analysis. Enantiomer **66b** were prepared by same way.

Amycolapeptin A (0.1 mg) was hydrolyzed with 2 M HCl in MeOH (200 μL, prepared from 100 μL MeOH and 100 μL 4 M HCl in dioxane) for 12 h at 90 °C under N$_2$, and then dried *in vacuo*. The obtained hydrolysate was dissolved in MeOH (100 μL), and then analyzed by LC-MS with **66a/66b** (column: CHIRALPAK OD-RH, 4.6 ×150 mm; eluent: MeOH/H$_2$O = 75/25, 0.4 mL/min; detection: ESI-positive).

44

**66a/b**: ^{1}H NMR (600 MHz, CD$_3$OD): δ 8.18 (s, 1H), 7.58 (d, $J$ = 7.8 Hz, 1H), 7.33 (d, $J$ = 8.1 Hz, 1H), 7.16 (t, $J$ = 7.9 Hz, 1H), 7.10 (t, $J$ = 7.5 Hz, 1H), 7.07 (d, $J$ = 2.2 Hz, 1H), 3.66 (s, 3H), 3.65 (s, 3H), 3.24 (d, $J$ = 14.5 Hz, 1H), 3.13 (d, $J$ = 14.5 Hz, 1H), 3.09 (d, $J$ = 16.1 Hz, 1H), 2.80 (d, $J$ = 16.0 Hz, 1H); ^{13}C NMR (150 MHz, CD$_3$OD) δ 175.3, 171.5, 136.0, 128.0, 124.1, 122.1, 119.7, 119.1, 111.3, 109.0, 76.2, 53.0, 52.1, 42.9, 35.4; HRMS (ESI) calcd for C$_{15}$H$_{18}$O$_5$N$^+$ [M + H]$^+$ 292.1179, found 292.1186.
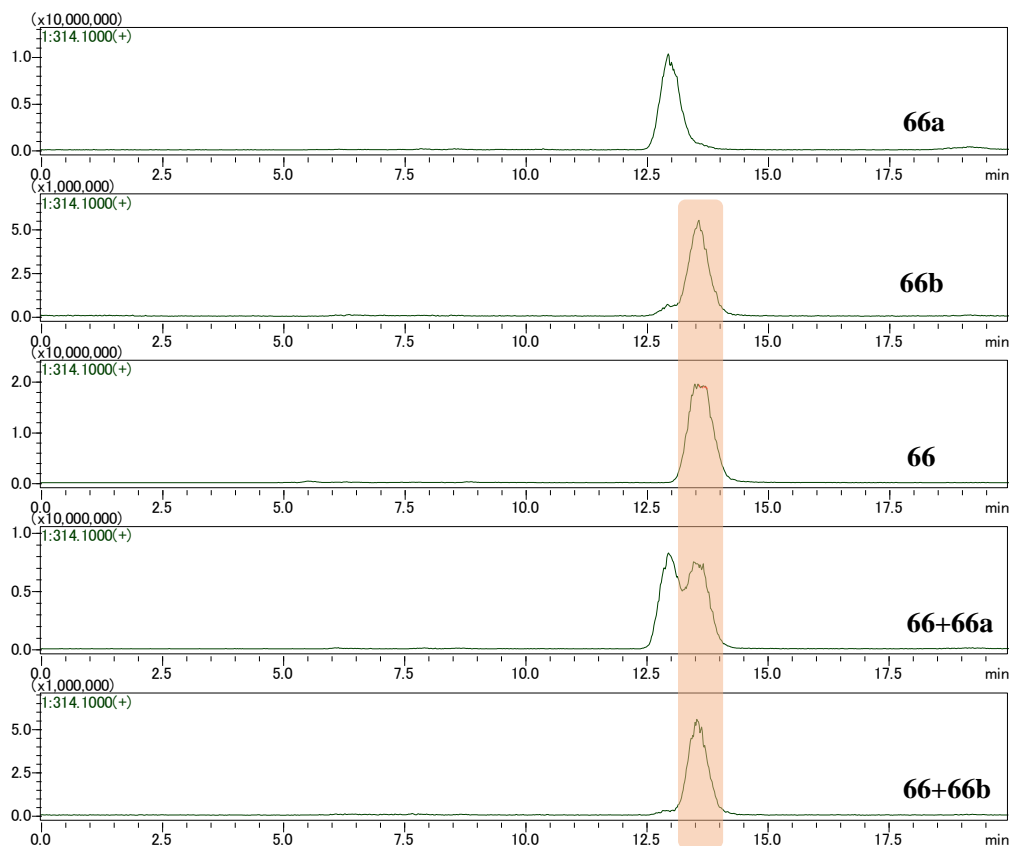


**Figure 2.8** LC-MS analysis of **66** and isomers **66a/66b**

## 4.10 Computation Section

The absolute configurations of **65a**/**65b** were determined by simulation of the ECD spectrum. The minimum energy geometries of two conformers were optimized using DFT calculations at the m062x/def2tzvp level in the gas phase by the GAUSSIAN 09.[42] Then Time-dependent DFT calculations were performed on the lowest-energy conformations for each configuration using 30 excited states and under the CH$_3$OH solution. The calculated ECD curves were drawn by using SpecDis with a σ of 0.2 eV.[43] The calculated ECD spectrum for 2*R* showed a negative Cotton effect at 210 nm and two positive Cotton effect at 230 and 280 nm which is identical to the **65a** experimental ECD spectra. The calculated ECD spectrum for 2*S* showed an opposite curve which is identical to the **65a** experimental ECD spectra (Figure 2.5). Therefore, the absolute configurations of **65a** were determined as 2*R*, **65b** was determined as 2*S*.

# Conclusions

In this thesis, I summarized the complete research of novel cyclic sulfur-containing lipopeptides, thioamycolamides, including isolation, structure elucidation, absolute configuration determination, total synthesis, biological evaluation and plausible biosynthetic pathway. Moreover, two novel nonapeptides, amycolapeptins A and B, which were not produced by a single culture of *Amycolatopsis* sp. 26-4, were successfully isolated from the combined-culture broth. The structures of amycolapeptins were also confirmed.

**In Chapter 1-1**, I isolated the sulfur-containing cytotoxic cycliclipopeptidyl metabolites, thioamycolamides A–E from the culture broth of actinomycete *Amycolatopsis* sp. 26-4. Their unprecedented structures were precisely elucidated by the combination of extensive spectroscopic analyses, Marfey's method and chemical synthesis of fragments. Putative biosynthetic pathway of thioamycolamides is proposed to be assembled by non-ribosomal peptide synthetase (NRPS) using α,β-unsaturated fatty acids, phenylalanine, cysteine as components. These compounds exhibited varying cytotoxicities toward the growth of two human cancer cells. The differences in the activities seemed to be related to the length and methylation/hydroxylation patterns of the C18 side chain, suggest that the length of side chain are crucial for exhibiting the cytotoxicity presumably based on the difference of membrane permeability.

**In Chapter 1-2**, the first total synthesis of the cytotoxic cyclic lipopeptide thioamycolamide A (**1**) was accomplished. The peptide chain was elongated by an isomerization-suppression procedure and the thioether linkage was constructed by the bioinspired thio-Michael addition. Thioamycolamide A (**1**) was successfully synthesized in 10 steps in a linear sequences from readily available starting materials. The structural identity of natural and synthetic **1** was confirmed by LC-MS experiments using improved labeling reagent. In the LC-MS experiments, the stereochemical purity of chemically constructed **1** was verified, which corroborates the efficiency of bioinspired synthesis and provides insight into the proposal that the thioether bridge can be stereoselectively formed by thio-Michael addition.

**In Chapter 2**, combined-culture method was applied to strain *Amycolatopsis* sp. 26-4 with the mycolic acid-containing bacterium (MACB) *Tsukamurella pulmonis* TP-B0596. Two novel nonapeptides, amycolapeptins A and B, were isolated from the combined-culture broth. The chemical structures were elucidated by spectroscopic analysis, chemical synthesis of the fragment, a highly sensitive Marfey's method and CD spectroscopy. Combined-culture research and discovery of amycolapeptins proved the potential of actinomycete *Amycolatopsis* sp. 26-4 for producing other unprecedented structures.

The differences in the activities of thioamycolamides A-E suggests that further SARs study in the C18 side chain would be helpful for possible anticancer development of this class of compounds. The biological roles of strains *Amycolatopsis* sp. 26-4 and *Tsukamurella pulmonis* TP-B0596 in the microbial communities are unknown, further studies on combined-culture may provide answers to these interesting questions. The biosynthetic mechanisms of thioamycolamides and amycolapeptins in the producing organism are also of considerable interest, because of their highly modified skeletons. I believe that my results in my thesis would be helpful for drug development, microorganism biosynthesis and microbial communication research in the near future.

# References

1. Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2016**, *79*, 629.

2. Martens, E.; Demain, A. L. *J. Antibiot.* **2017**, *70*, 520.

3. Selvameenal, L.; Radhakrishnan, M.; Balagurunathan, R. *Indian J. Pharm. Sci.* **2009**, *71*, 499.

4. Lechevalier, M. P.; Prauser, H.; Labeda, D. P.; Ruan, J.-S. *Int. J. Syst. Bacteriol.* **1986**, *36*, 29.

5. (a) Zhang, C.; Zink, D. L.; Ushio, M.; Burgess, B.; Onishi, R.; Masurekar, P.; Barrett, J.; Singh, S. B. *Bioorgan. Med. Chem.* **2008**, *16*, 8818. (b) Nadkarni, S. R.; Patel, M. V.; Chatterjee, S.; Vijayakumar, E. K. S.; Desikan, K. R.; Blumbach, J.; Ganguli, B. N. *J. Antibiot.* **1994**, *47*, 334. (c) Tsuchida, T.; Sawa, R.; Takahashi, Y.; Iinuma, H.; Sawa, T.; Naganawa, H.; Takeuchi, T. *J. Antibiot.* **1995**, *48*, 217. (d) Riva, S.; Silvestri, L. G. *Annu. Rev. Microbiol.* **1972**, *26*, 199. (e) Cetinkaya, Y.; Falk, P.; Mayhall, C. G. *Clin. Microbiol. Rev.* **2000**, *13*, 686.

6. (a) Cai, S.; King, J. B.; Du, L.; Powell, D. R.; Cichewicz, R. H. *J. Nat. Prod.* **2014**, *77*, 2280. (b) Ferreira-Lima, N.; Vallverdú-Queralt, A.; Meudec, E.; Mazauric, J. P.; Sommerer, N.; Bordignon-Luiz, M. T.; Cheynier, V.; Le Guernevé, C. *J. Nat. Prod.* **2016**, *79*, 2211. (c) Daengrot, C.; Rukachaisirikul, V.; Tansakul, C.; Thongpanchang, T.; Phongpaichit, S.; Bowornwiriyapan, K.; Sakayaroj, J. *J. Nat. Prod.* **2015**, *78*, 615. (d) Yuan, W. H.; Teng, M. T.; Yun, Y. F.; Jiang, N.; Ma, L.; Sun, S. S.; Zhang, P. *J. Nat. Prod.* **2020**, *83*, 1716.

7. Wang, X.; Shaaban, K. A.; Elshahawi, S. I.; Ponomareva, L. V.; Sunkara, M.; Zhang, Y.; Copley, G. C.; Hower, J. C.; Morris, A. J.; Kharel, M. K.; Thorson, J. S. *J. Nat. Prod.* **2013**, *76*, 1441.

8. (a) Shaaban, K. A.; Shaaban, M.; Rahman, H.; Grün-Wollny, I.; Kämpfer, P.; Kelter, G.; Fiebig, H.; Laatsch, H. *J. Nat. Prod.* **2019**, *82*, 870. (b) Urda, C.; Fernández, R.; Rodríguez, J.; Pérez, M.; Jiménez, C.; Cuevas, C. *Mar. Drugs*. **2017**, *15*, 209. (c) Zhou, X.; Huang, H.; Chen, Y.; Tan, J.; Song, Y.; Zou, J.; Tian, X.; Hua, Y.; Ju, J. *J. Nat. Prod.* **2012**, *75*, 2251. (d) Teruya, T.; Sasaki, H.; Fukazawa, H.; Suenaga, K. *Org. Lett.* **2009**, *11*, 5062. (e) Matthew, S.; Schupp, P. J.; Luesch, H. *J. Nat. Prod.* **2008**, *71*, 1113. (f) Carroll, A. R.; Coll, J. C.; Bourne, D. J.; MacLeod, J. K.; Zabriskie, T. M.; Ireland, C. M.; Bowden, B. F. *Aust. J. Chem.* **1996**, *49*, 659.

9. (a) Kojima, K.; Yakushiji, F.; Katsuyama, A.; Ichikawa, S. *Org. Lett.* **2020**, *22*, 4217. (b) Guo, Y.; Zhou, J.; Gao, B.; Zhao, M.; Yan, J. L.; Xu, Z.; Choi, S.; Ye, T. *Org. Lett.* **2019**, *21*, 5471. (c) Zhang, Y.; Islam, M. A.; McAlpine, S. R. *Org. Lett.* **2015**, *17*, 5149. (d) Shapiro, J. A.; Morrison, K. R.; Chodisetty, S. S.; Musaev, D. G.; Wuest, W. M. *Org. Lett.* **2018**, *20*, 5922.

10. Metwally, M. A.; Abdel-Latif, E.; Amer, F. A.; Kaupp, G. *J. Sulfur Chem.* **2004**, *25*, 63.

11. (a) Fukuhara, T.; Hasegawa, C.; Hara, S. *Synthesis*. **2007**, 1528. (b) Maltsev, O. V.; Walter, V.; Brandl, M. J.; Hintermann, L. *Synthesis*. **2013**, *45*, 2763.

12. Chen, S.; Wu, Q.; Shen, Q.; Wang, H. *ChemBioChem*. **2016**, *17*, 119.

13. (a) Yang, X.; Davison, R. T.; Nie, S.; Cruz, F. A.; McGinnis, T. M.; Dong, V. M. *J. Am. Chem. Soc.* **2019**, *141*, 3006. (b) Gómez, J. E.; Cristòfol, À.; Kleij, A. W. *Angew. Chem. In. Ed.* **2019**, *58*, 3903.

14. (a) Fontecave, M.; Ollagnier-de-Choudens, S.; Mulliez, E. *Chem. Rev.* **2003**, *103*, 2149. (b) Mueller, E. G. *Nat. Chem. Biol.* **2006**, *2*, 185.

15. (a) Bae, M.; An, J. S.; Bae, E. S.; Oh, J.; Park, S. H.; Lim, Y.; Ban, Y. H.; Kwon, Y.; Cho, J. C.; Yoon, Y. J.; Lee, S. K.; Shin, J.; Oh, D. C. *Org. Lett.* **2019**, *21*, 3635. (b) N'Nang, E. O.; Bernadat, G.; Mouray, E.; Kumulungui, B.; Grellier, P.; Poupon, E.; Champy, P.; Beniddir, M. A. *Org. Lett.* **2018**, *20*, 6596.

16. (a) Dunbar, K. L.; Scharf, D. H.; Litomska, A.; Hertweck, C. *Chem. Rev.* **2017**, *117*, 5521. (b) Maini, R.; Kimura, H.; Takatsuji, R.; Katoh, T.; Goto, Y.; Suga, H. *J. Am. Chem. Soc.* **2019**, *141*, 20004. (c) Dong, L. B.; Rudolf, J. D.; Kang, D.; Wang, N.; He, C. Q.; Deng, Y.; Huang, Y.; Houk, K. N.; Duan, Y.; Shen, B. *Nat. Commun.* **2018**, *9*, 2362. (d) Leisinger, F.; Burn, R.; Meury, M.; Lukat, P.; Seebeck, F. P. *J. Am. Chem. Soc.* **2019**, *141*, 6906.

17. (a) Kishimoto, S.; Nishimura, S.; Hattori, A.; Tsujimoto, M.; Hatano, M.; Igarashi, M.; Kakeya, H. *Org. Lett.* **2014**, *16*, 6108. (b) Tsunematsu, Y.; Nishimura, S.; Hattori, A.; Oishi, S.; Fujii, N.; Kakeya, H. *Org. Lett.* **2015**, *17*, 258. (c) Sugiyama, R.; Nishimura, S.; Ozaki, T.; Asamizu, S.; Onaka, H.; Kakeya, H. *Org. Lett.* **2015**, *17*, 1918. (d) Lu, S.; Nishimura, S.; Hirai, G.; Ito, M.; Kawahara, T.; Izumikawa, M.; Sodeoka, M.; Shin-ya, K.; Tsuchida, T.; Kakeya, H. *Chem. Commun.* **2015**, *51*, 8074. (e) Kakeya, H. *Nat. Prod. Rep.* **2016**, *33*, 648. (f) Lu, S.; Nishimura, S.; Takenaka, K.; Ito, M.; Kato, T.; Kakeya, H. *Org. Lett.* **2018**, *20*, 4406.

18. Harada, K. I.; Fujii, K.; Mayumi, T.; Hibino, Y.; Suzuki, M.; Ikai, Y.; Oka, H. *Tetrahedron Lett.* **1995**, *36*, 1515.

19. Kuranaga, T.; Mutoh, H.; Sesoko, Y.; S. Goto,; Matsunaga, M.; Inoue, M. *J. Am. Chem. Soc.* **2015**, *137*, 9443.

20. (a) Wipf, P.; Fritch, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 12358. (b) Wipf, P.; Fritch, P. C. *Tetrahedron Lett.* **1994**, *35*, 5397.

21. (a) Matsunaga, S.; Nishimura, S.; Fusetani, N. *J. Nat. Prod.* **2001**, *64*, 816. (b) Nakanishi, K.; Berova, N.; Woody, R. W. *Circular Dichroism, Principles and Applications*; VCH Publishers Inc.: New York, **1994**.

22. Porto, S.; Seco, J. M.; Ortiz, A.; Quiñoá, E.; Riguera, R. *Org. Lett.* **2007**, *9*, 5015.

23. Ortega, M. A.; van der Donk, W. A. *Cell Chem. Biol.* **2016**, *23*, 31.

24. Hur, G. H.; Vickery, C. R.; Burkart, M. D. *Nat. Prod. Rep.* **2012**, *29*, 1074.

25. Du, L.; Lou, L. *Nat. Prod. Rep.* **2010**, *27*, 255.

26. Pan, C.; Kuranaga, T.; Liu, C.; Lu, S.; Shinzato, N.; Kakeya, H. *Org. Lett.* **2020**, *22*, 3014.

27. (a) Boden, C. D. J.; Pattenden, G.; Ye, T. *Synlett* **1995**, 417. (b) Konigsberg, W.; Hill, R. H.; Craig, L. C. *J. Org. Chem.* **1961**, *26*, 3867. (c) Hirotsu, Y.; Shiba, T.; Kaneko, T. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 1870. (d) Yonetani, K.; Hirotsu, Y.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 3302.

28. Burkhart, J. L.; Kazmaier, U. *Synthesis* **2011**, 4033.

29. (a) Thomsen, I.; Clausen, K.; Scheibye, S.; Lawesson, S.-O. *Org. Synth.* **1984**, *62*, 158. (b) Ozturk, T.; Ertas, E.; Mert, O. *Chem. Rev.* **2007**, *107*, 5210.

30. Sakaitani, M.; Ohfune, Y. *J. Org. Chem.* **1990**, *55*, 870.

31. Yamashita, T.; Matoba, H.; Kuranaga, T.; Inoue, M. *Tetrahedron* **2014**, *70*, 7746.

32. Nicolaou, K. C.; Estrada, A. A.; Zak, M.; Lee, S. H.; Safina, B. S. *Angew. Chem., Int. Ed.* **2005**, *44*, 1378.

33. Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. *Chem.-Eur. J.* **2009**, *15*, 9394.

34. (a) Zou, B.; Long, K.; Ma, D. *Org. Lett.* **2005**, *7*, 4237. (b) Sugiyama, H.; Watanabe, A.; Teruya, T.; Suenaga, K. *Tetrahedron Lett.* **2009**, *50*, 7343. (c) Ma, B.; Litvinov, D. N.; He, L.; Banerjee, B.; Castle, S. L. *Angew. Chem., Int. Ed.* **2009**, *48*, 6104. (d) Kuranaga, T.; Sesoko, Y.; Sakata, K.; Maeda, N.; Hayata, A.; Inoue, M. *J. Am. Chem. Soc.* **2013**, *135*, 5467.

35. (a) Kuranaga, T.; Minote, M.; Morimoto, R.; Pan, C.; Ogawa, H.; Kakeya, H. *ACS Chem. Biol.* **2020**, in press. (b) Ogawa, H. *Master thesis*. Kyoto University, **2019**.

36. (a) Onaka, H.; Mori, Y.; Igarashi, Y.; Furumai, T. *Appl. Environ. Microbiol.* **2011**, *77*, 400. (b) Onaka, H. *J. Antibiot.* **2017**, *70*, 865.

37. (a) Hoshino, S.; Wakimoto, T.; Onaka, H.; Abe, I. *Org. Lett.* **2015**, *17*, 1501. (b) Hoshino, S.; Zhang, L.; Awakawa, T.; Wakimoto, T.; Onaka, H.; Abe, I. *J. Antibiot.* **2015**, *68*, 342. (c) Hoshino, S.; Okada, M.; Awakawa, T.; Asamizu, S.; Onaka, H.; Abe, I. *Org. Lett.* **2017**, *19*, 4992. (d) Hoshino, S.; Ozeki, M.; Awakawa, T.; Morita, H.; Onaka, H.; Abe, I. *J. Nat. Prod.* **2018**, *81*, 2106. (e) Hoshino, S.; Okada, M.; Wakimoto, T.; Zhang, H.; Hayashi, F.; Onaka, H.; Abe, I. *J. Nat. Prod.* **2015**, *78*, 3011. (f) Jiang, Y.; Lu, S.; Hirai, G.; Kato, T.; Onaka, H.; Kakeya, H. *Tetrahedron Lett*. **2019**, *60*, 1072.

38. (a) Zampella, A.; D'Orsi, R.; Sepe, V.; Casapullo, A.; Monti, M. C.; D'Auria, M. V. *Org. Lett.* **2005**, *7*, 3585. (b) Schubert, V.; Di Meo, F.; Saaidi, P. L.; Bartoschek, S.; Fiedler, H. P.; Trouillas, P.; Süssmuth, R. D. *Chem. Eur. J.* **2014**, *20*, 4948. (c) Lin, Z.; Falkinham III, J. O.; Tawfik, K. A.; Jeffs, P.; Bray, B.; Dubay, G.; Cox, J.; Schmidt, E. W. *J. Nat. Prod.* **2012**, *75*, 1518.

39. (a) Robertson, A. W.; McCarville, N. G.; MacIntyre, L. W.; Correa, H.; Haltli, B.; Marchbank, D. H.; Kerr, R. G. *J. Nat. Prod.* **2018**, *81*, 858. (b) Jones, C. W., III; Leyden, D. E.; Stammer, C. H. *Can. J. Chem.* **1969**, *47*, 4363.

40. Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K. I. *Anal. Chem.* **1997**, *69*, 3346.

41. (a) Deechongkit, S.; You, S. L.; Kelly, J. W. *Org. Lett.* **2004**, *6*, 497. (b) Shin, I.; Ambler, B. R.; Wherritt, D.; Griffith, W. P.; Maldonado, A. C.; Altman, R. A.; Liu, A. *J. Am. Chem. Soc.* **2018**, *140*, 4372.

42. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci,B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, O.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*, Revision A.1; Gaussian, Inc.: Wallingford, CT, **2009**.

43. Bruhn, T.; Schaumlöffel, A.; Hemberger, Y. SpecDis, version 1.63. *University of Wuerzburg* **2015**, Germany.

# Spectral data

## Chapter 1-1
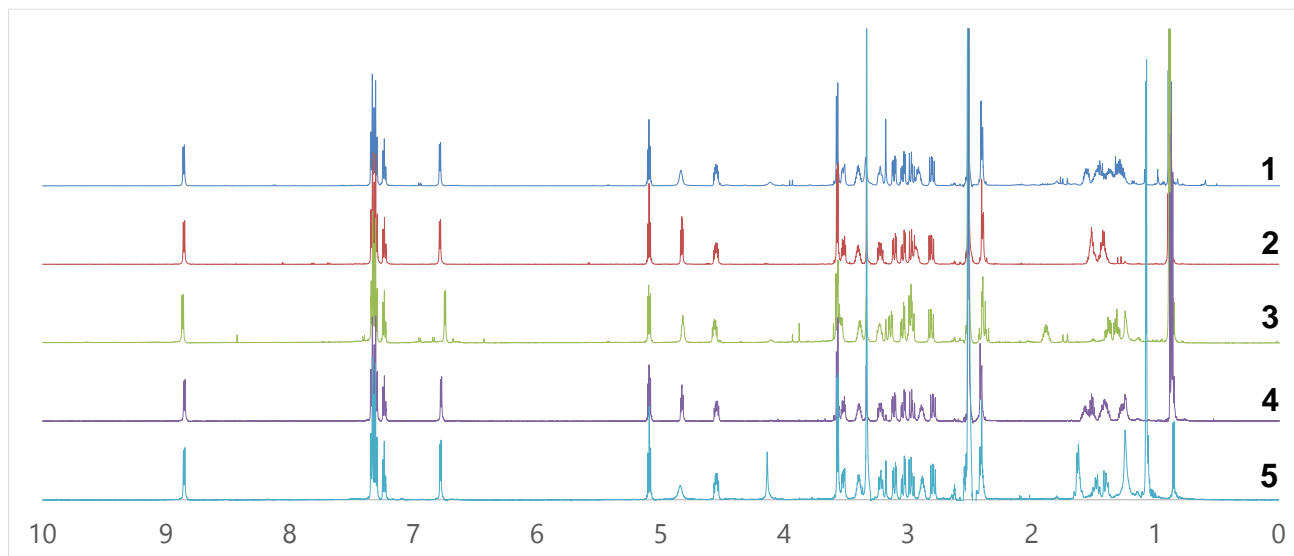


Figure S1. Comparison of the $^1$H NMR spectra of **1**-**5** in DMSO-$d_6$ (600 MHz).
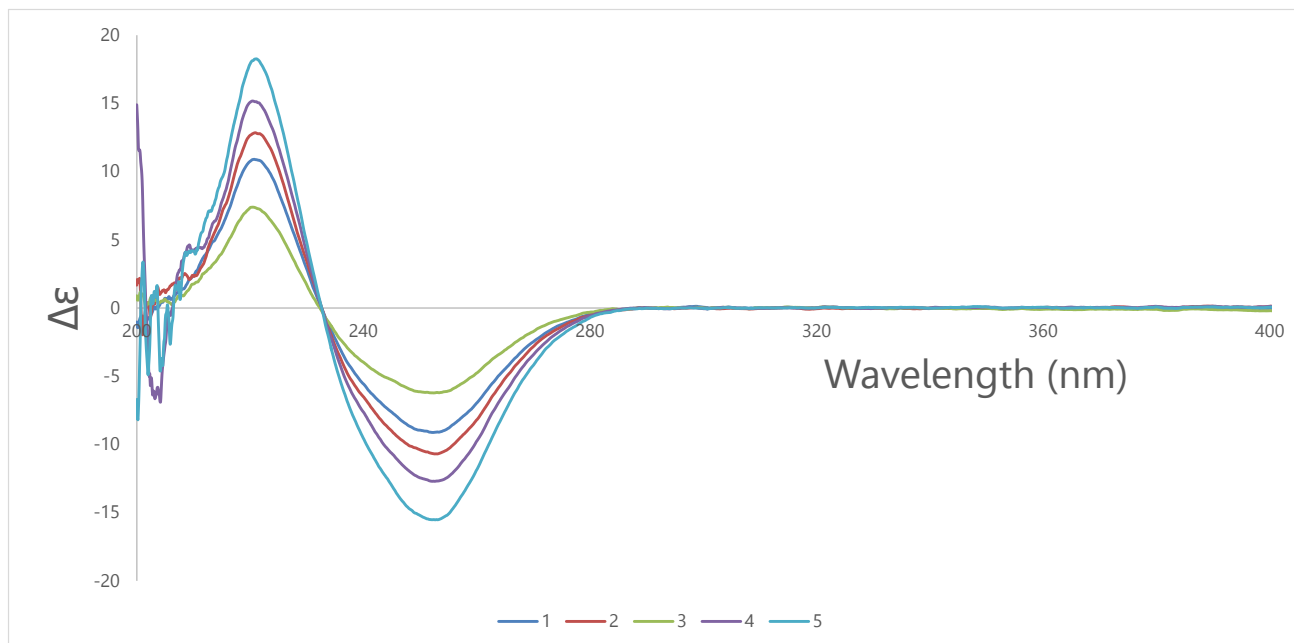


Figure S2. Comparison of the CD spectra of **1**-**5** in MeOH.
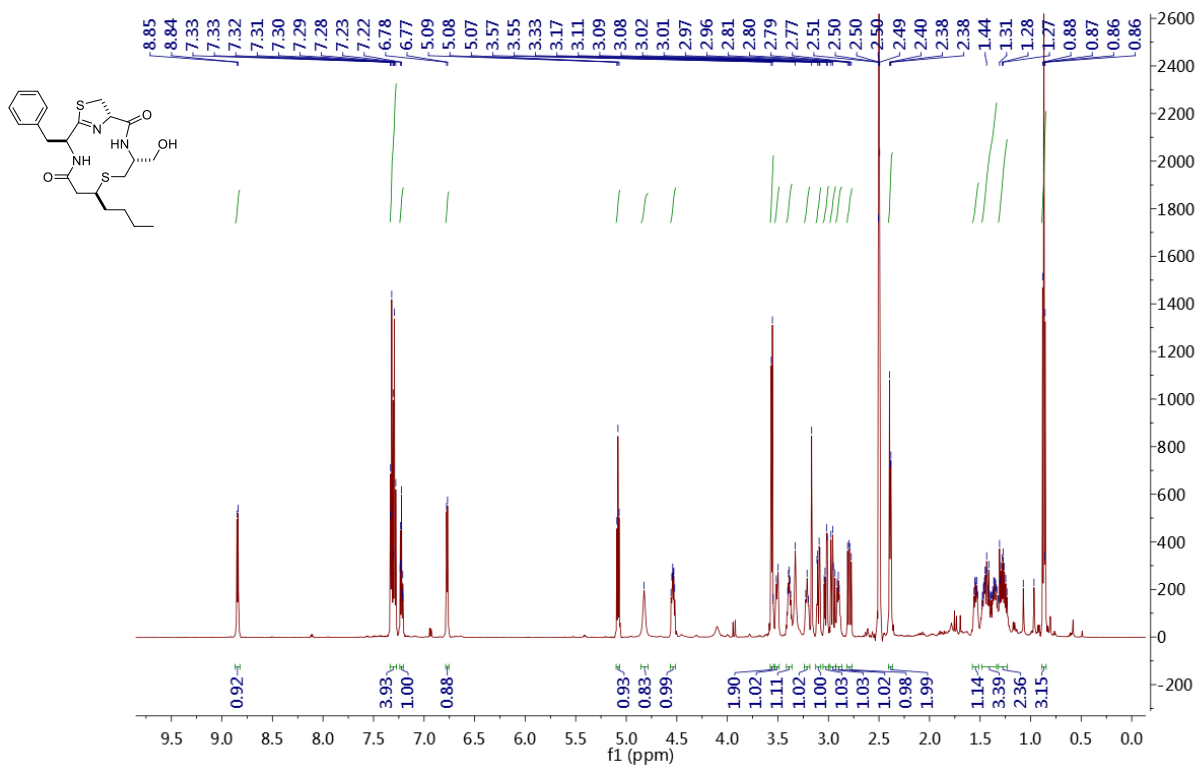
Figure S3. $^1$H NMR spectrum (600 MHZ) of **1** in DMSO-$d_6$.
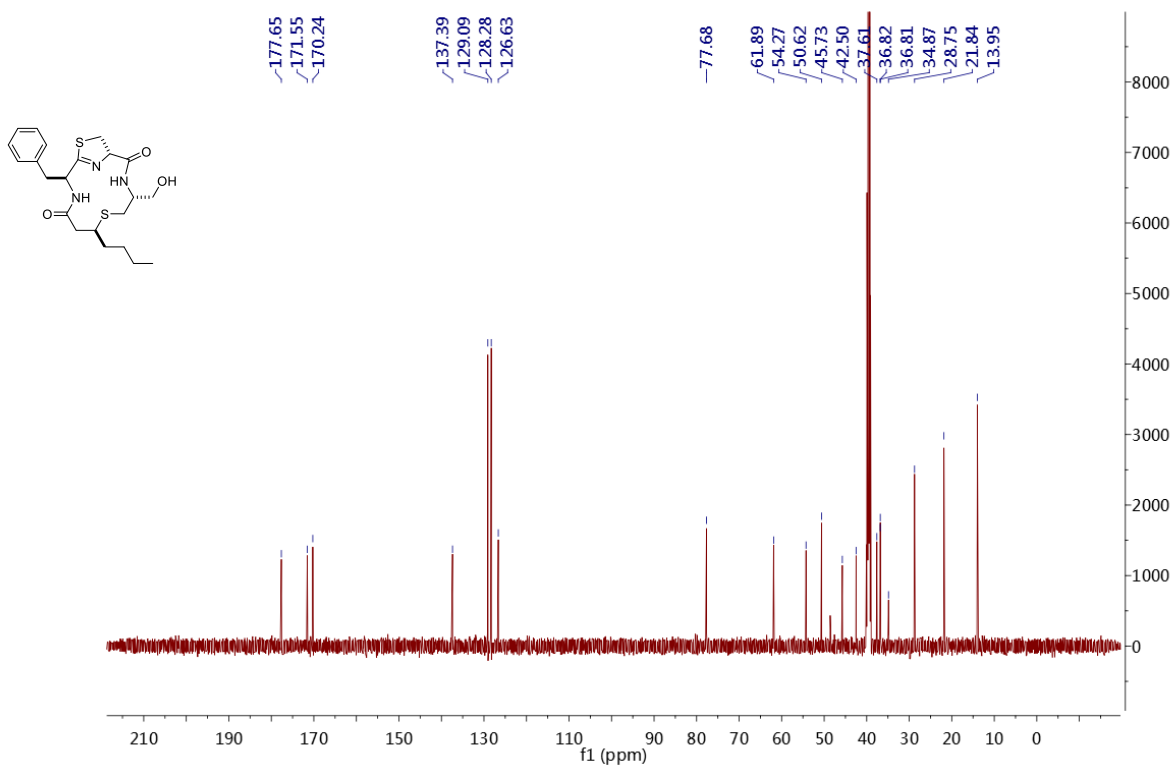


Figure S4. $^{13}$C NMR spectrum (150 MHz) of **1** in DMSO-$d_6$.
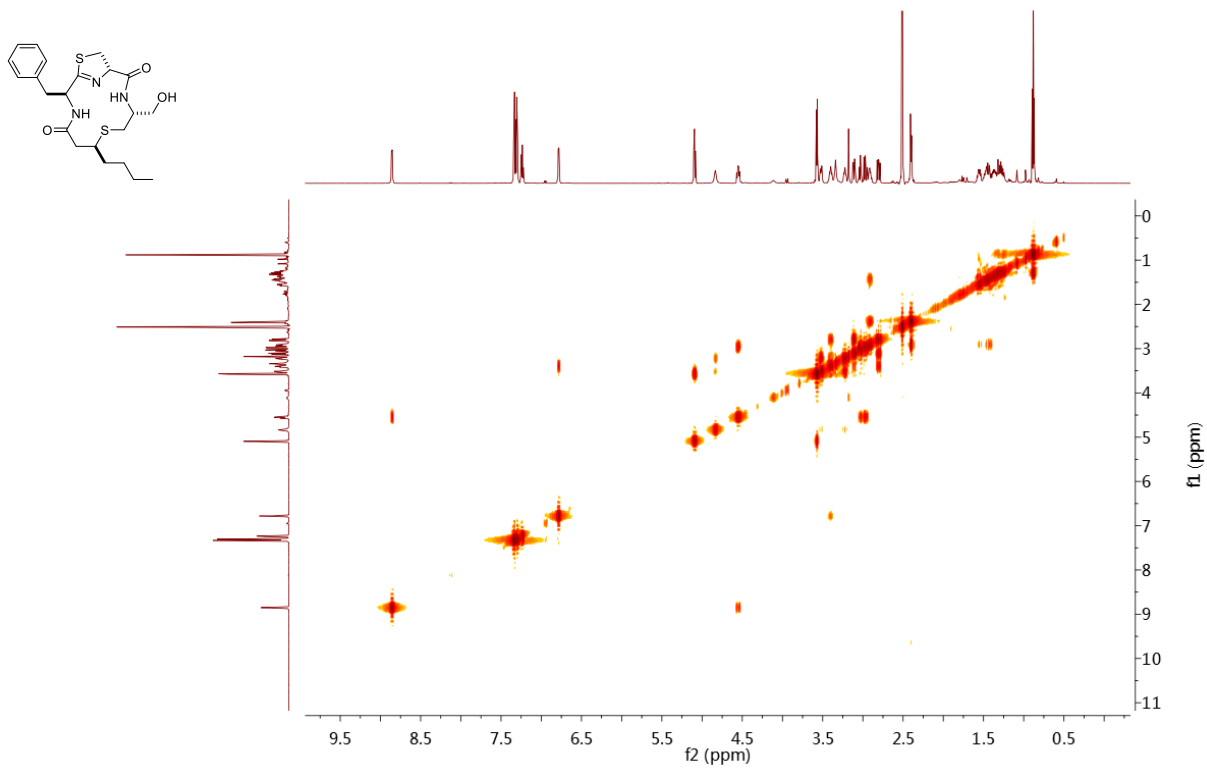
Figure S5. COSY spectrum (600 MHz) of **1** in DMSO-$d_6$.
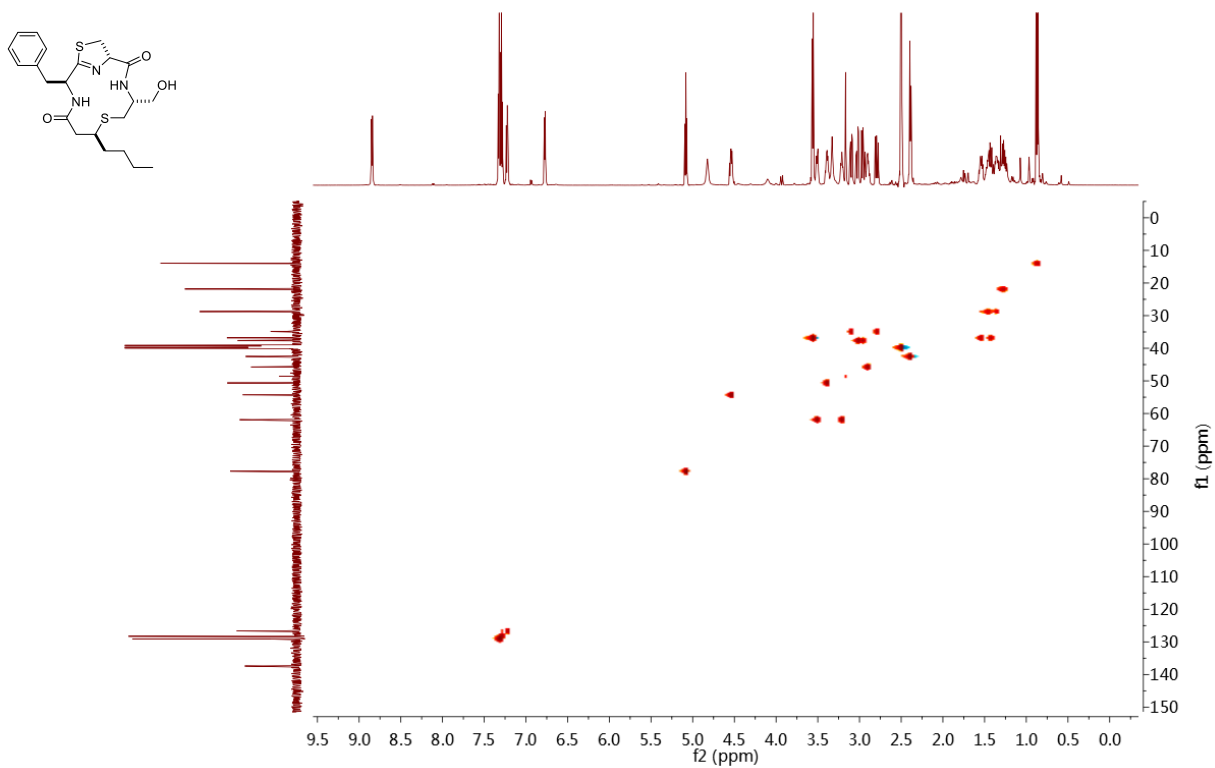


Figure S6. HSQC spectrum (600 MHz) of **1** in DMSO-$d_6$.
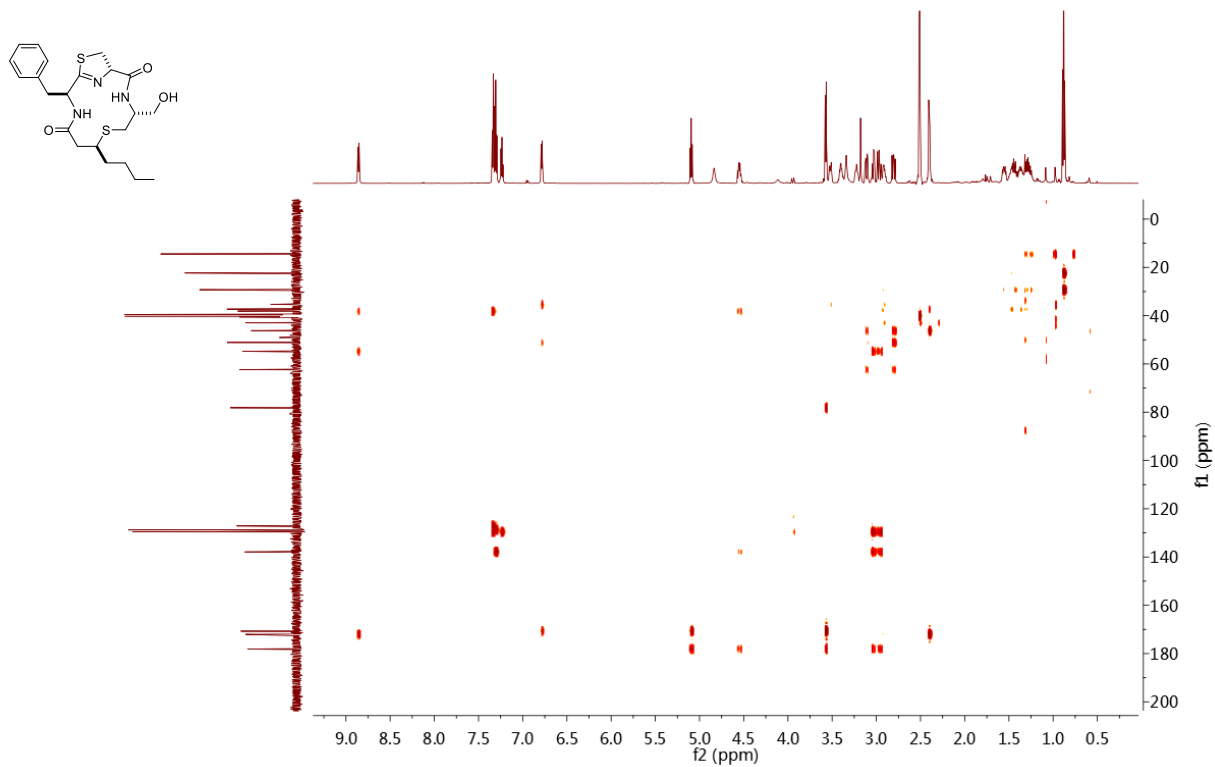
Figure S7. HMBC spectrum (600 MHz) of **1** in DMSO-$d_6$.
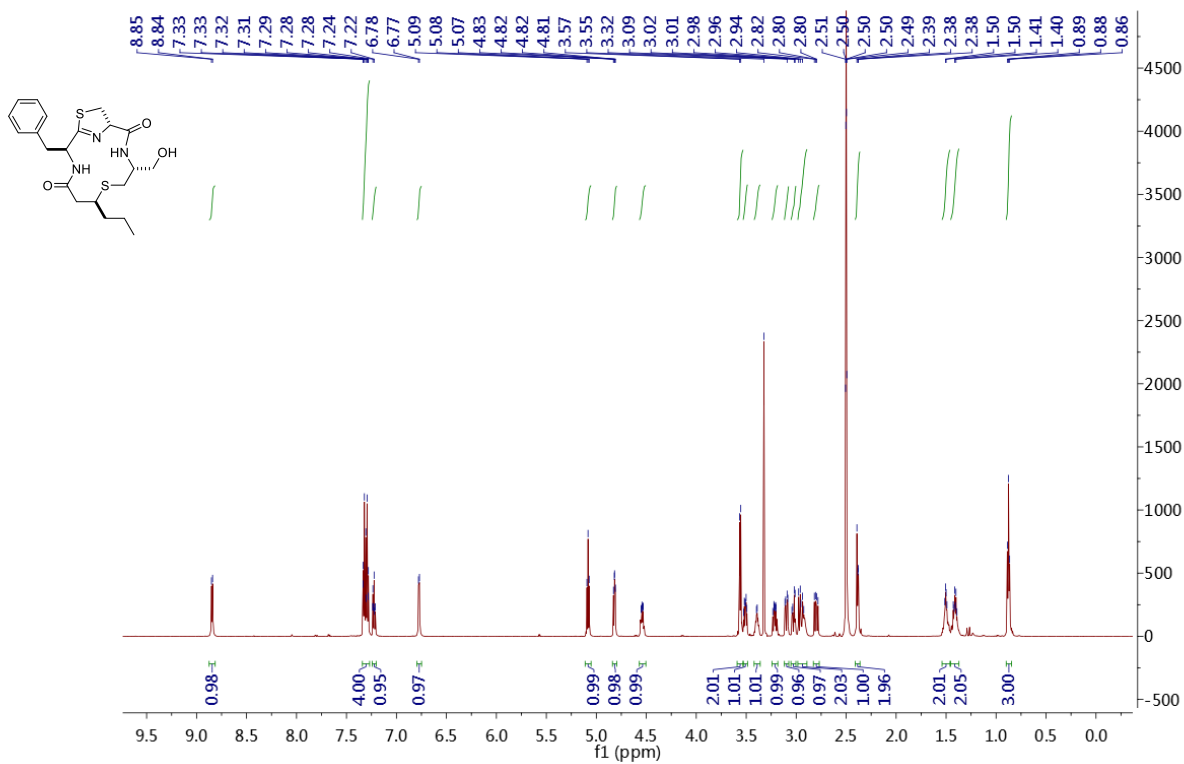


Figure S8. $^1$H NMR spectrum (600 MHz) of **2** in DMSO-$d_6$.
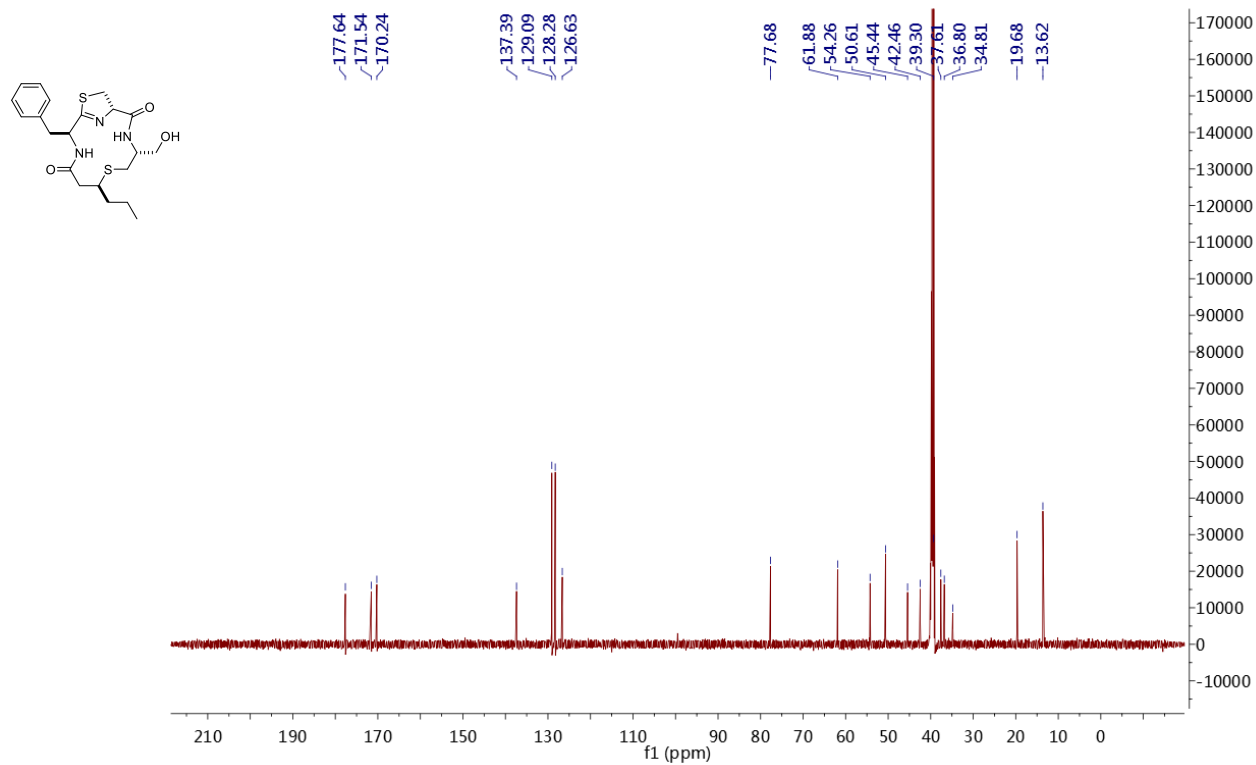
Figure S9. $^{13}$C NMR spectrum (150 MHz) of **2** in DMSO-$d_6$.
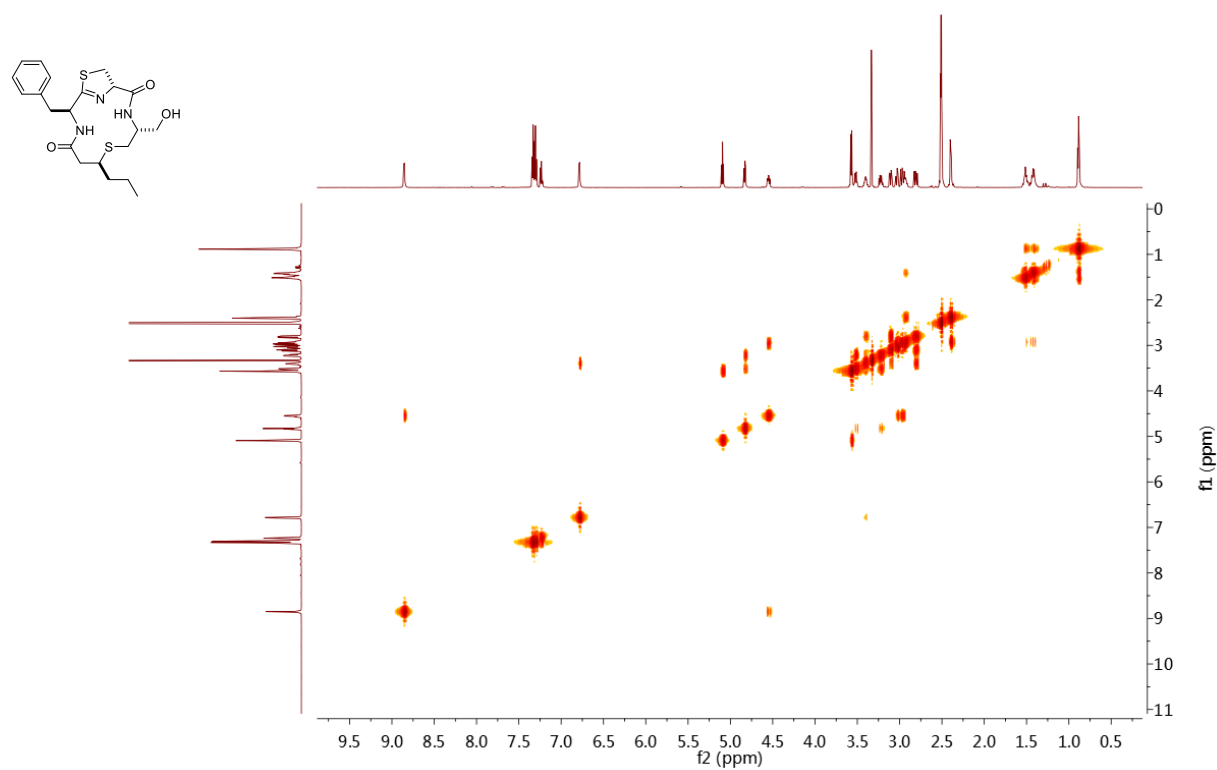


Figure S10. COSY spectrum (600 MHz) of **2** in DMSO-$d_6$.

54
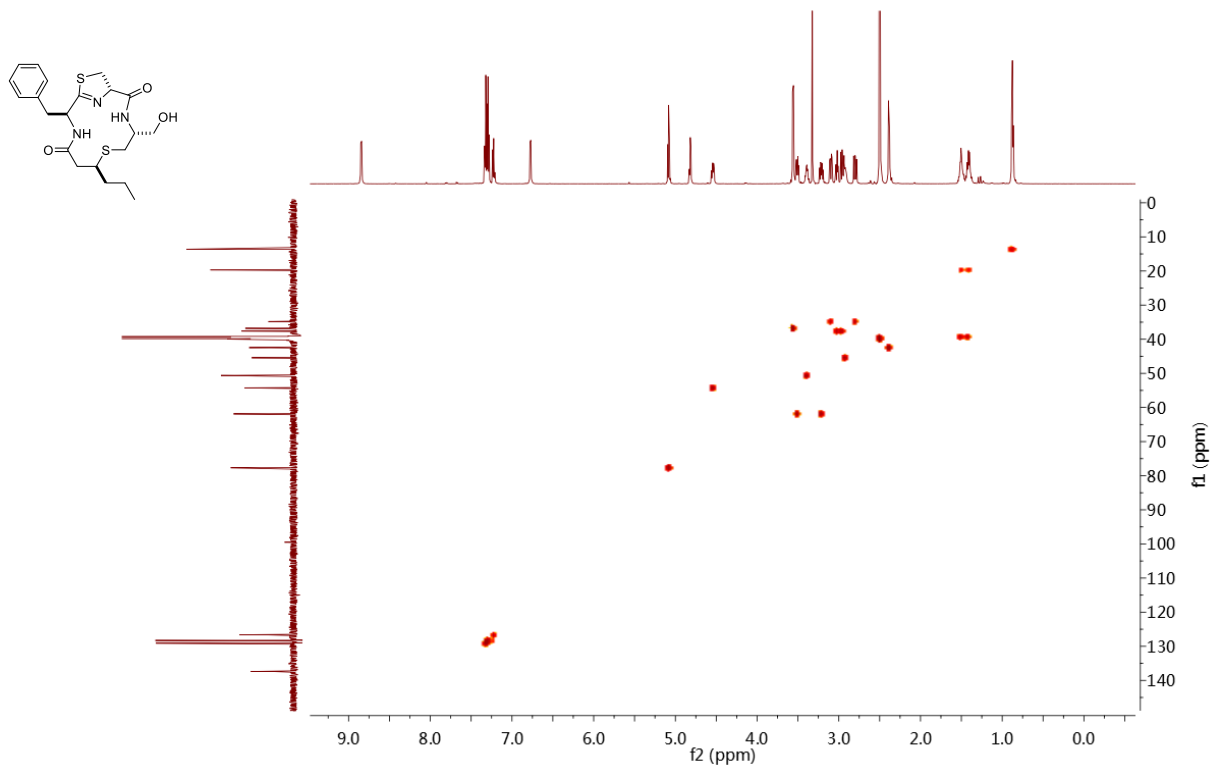
Figure S11. HSQC spectrum (600 MHz) of **2** in DMSO-$d_6$.
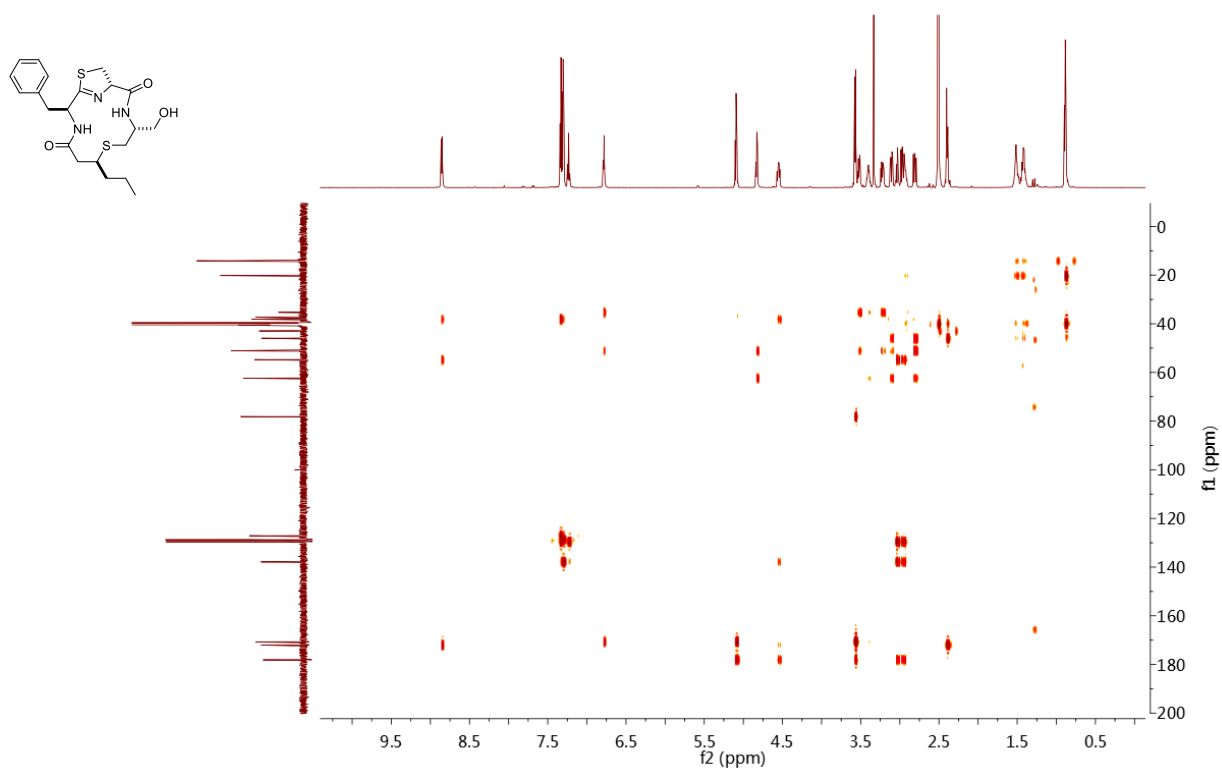


Figure S12. HMBC spectrum (600 MHz) of **2** in DMSO-$d_6$.
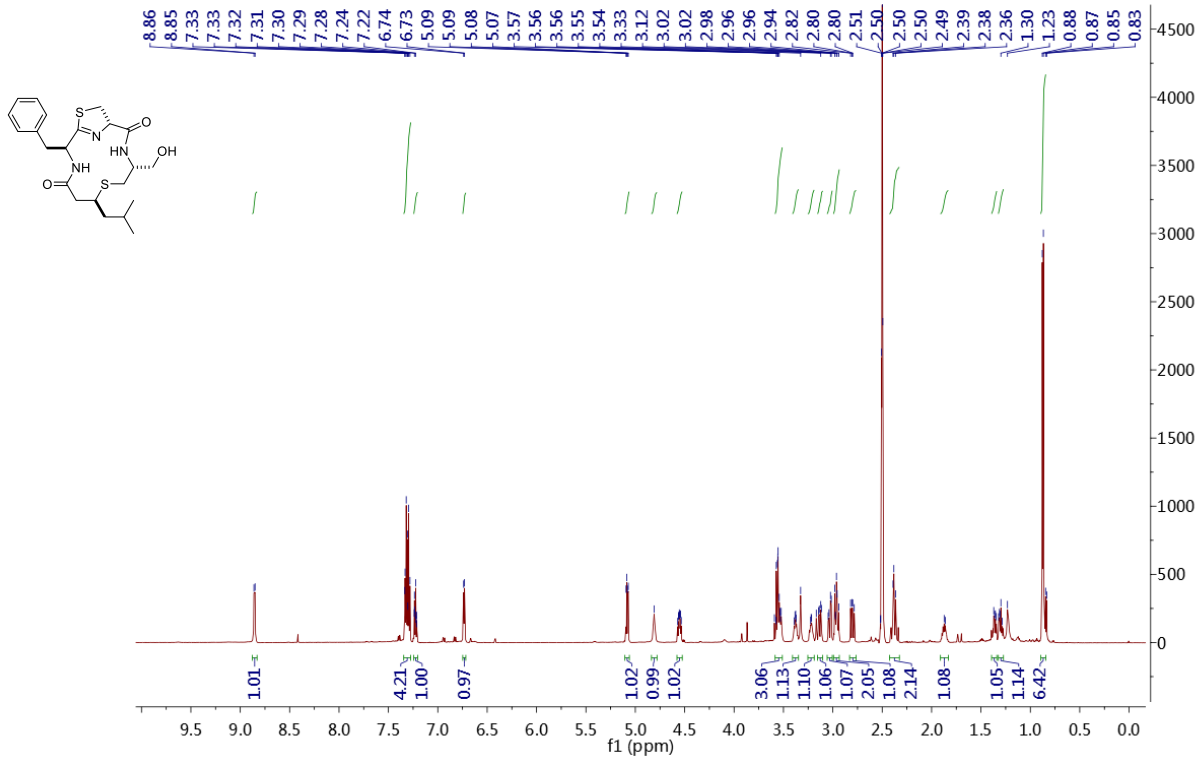
Figure S13. $^1$H NMR spectrum (600 MHz) of **3** in DMSO-$d_6$.
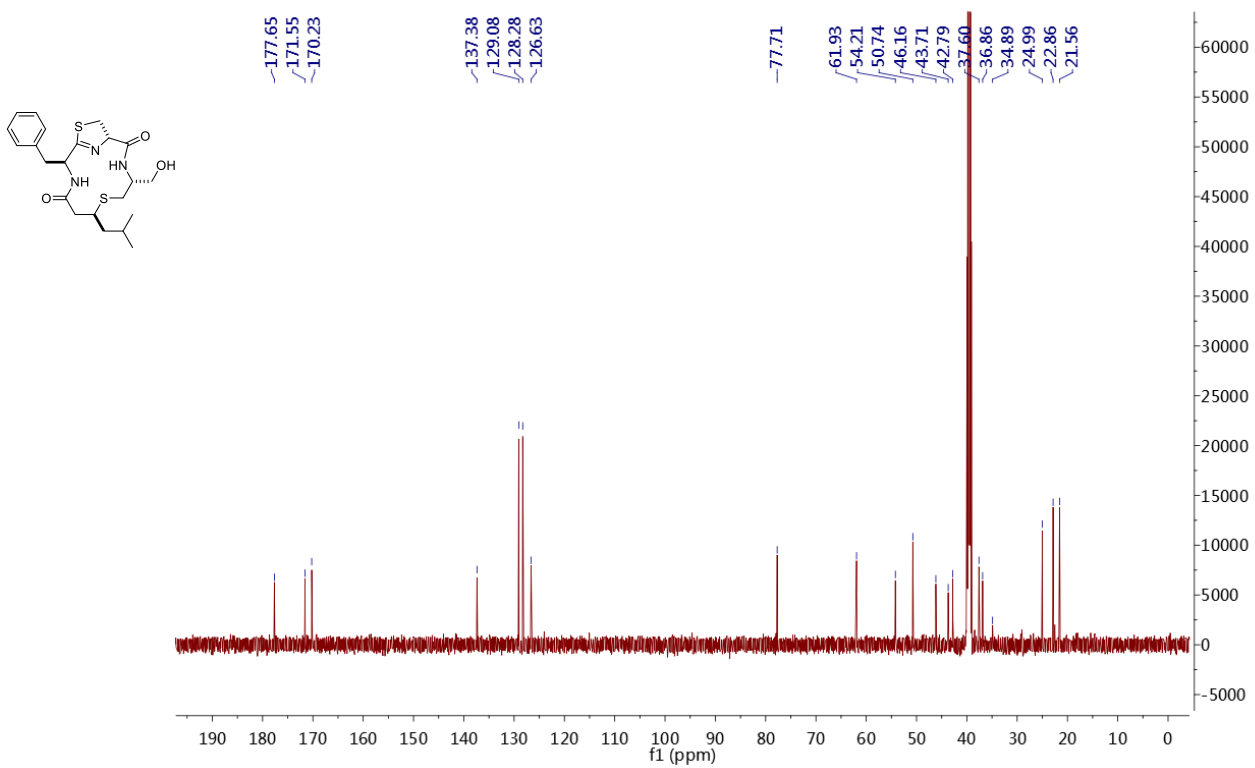


Figure S14. $^{13}$C NMR spectrum (150 MHz) of **3** in DMSO-$d_6$.
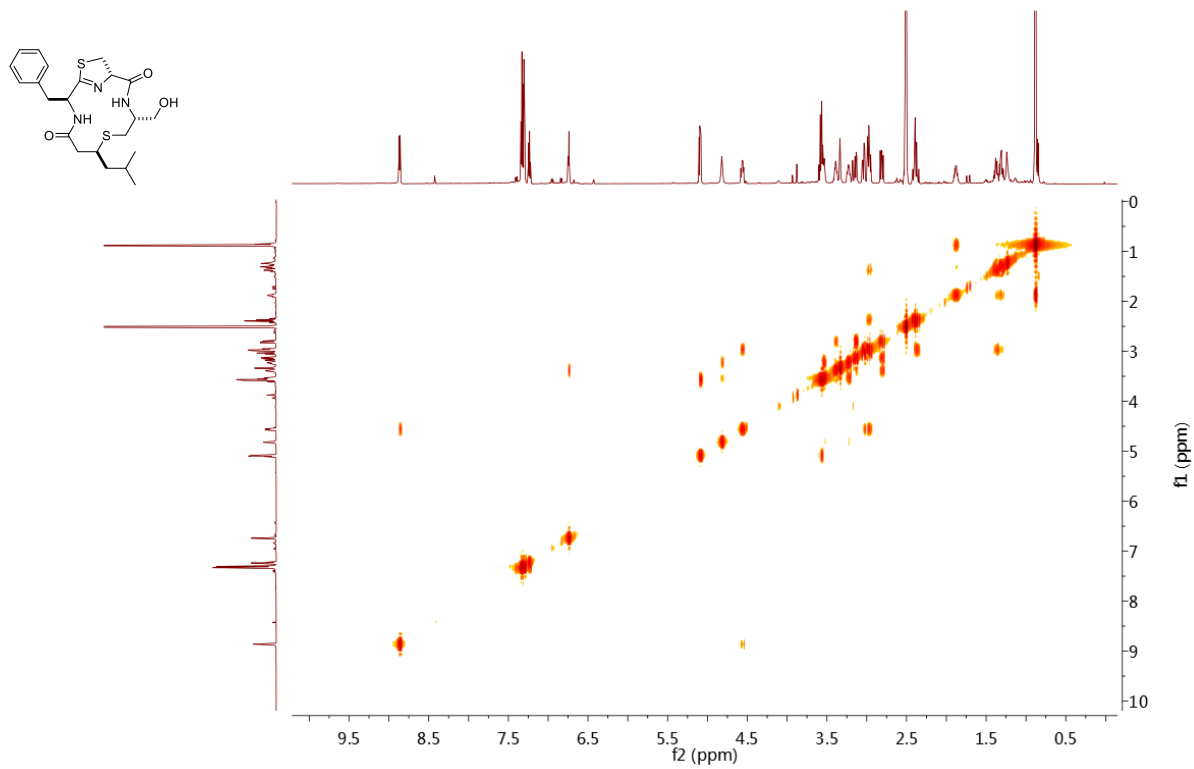
Figure S15. COSY spectrum (600 MHz) of **3** in DMSO-$d_6$.
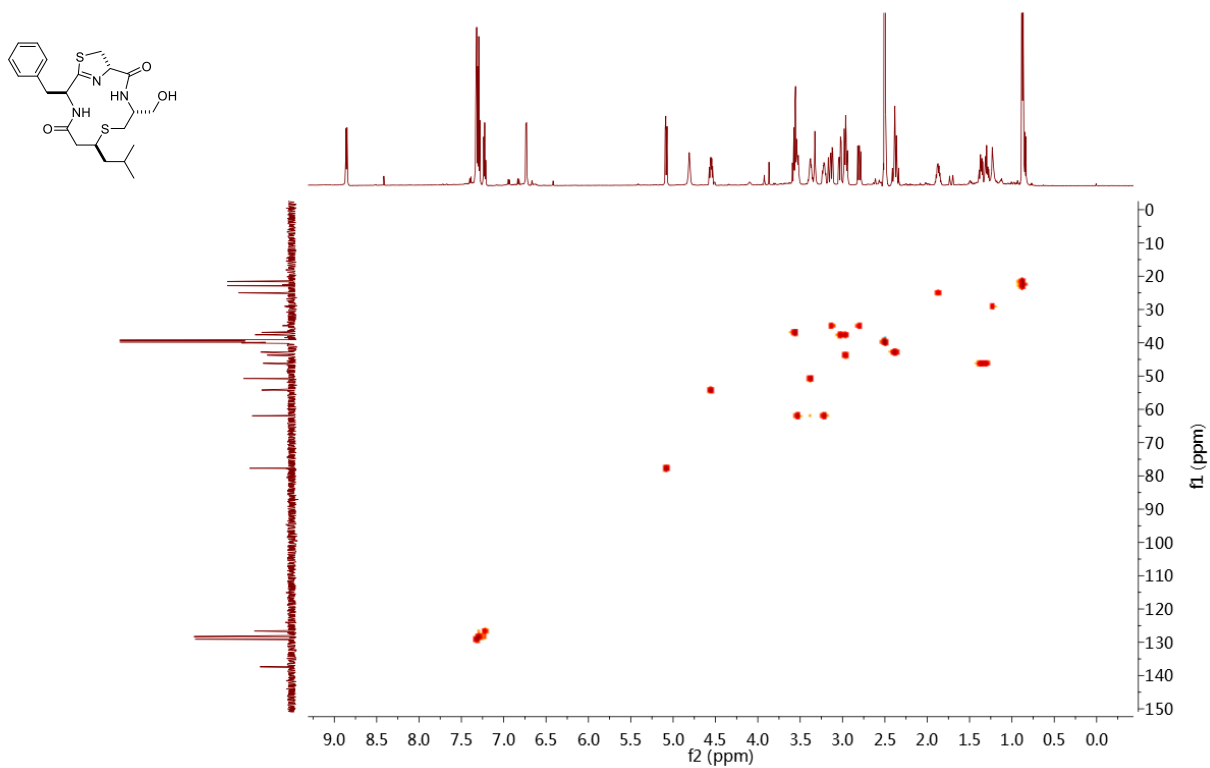


Figure S16. HSQC spectrum (600 MHz) of **3** in DMSO-$d_6$.

Figure S17. HMBC spectrum (600 MHz) of **3** in DMSO-$d_6$.



Figure S18. $^1$H NMR spectrum (600 MHz) of **4** in DMSO-$d_6$.
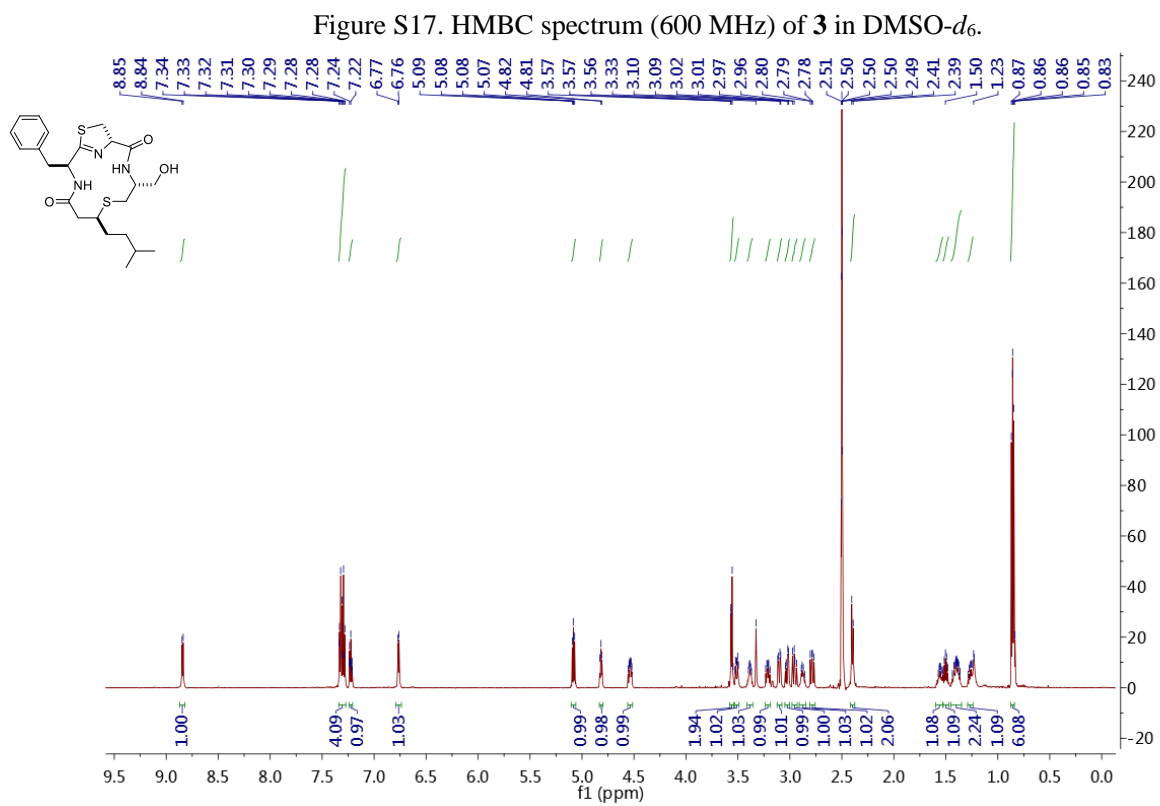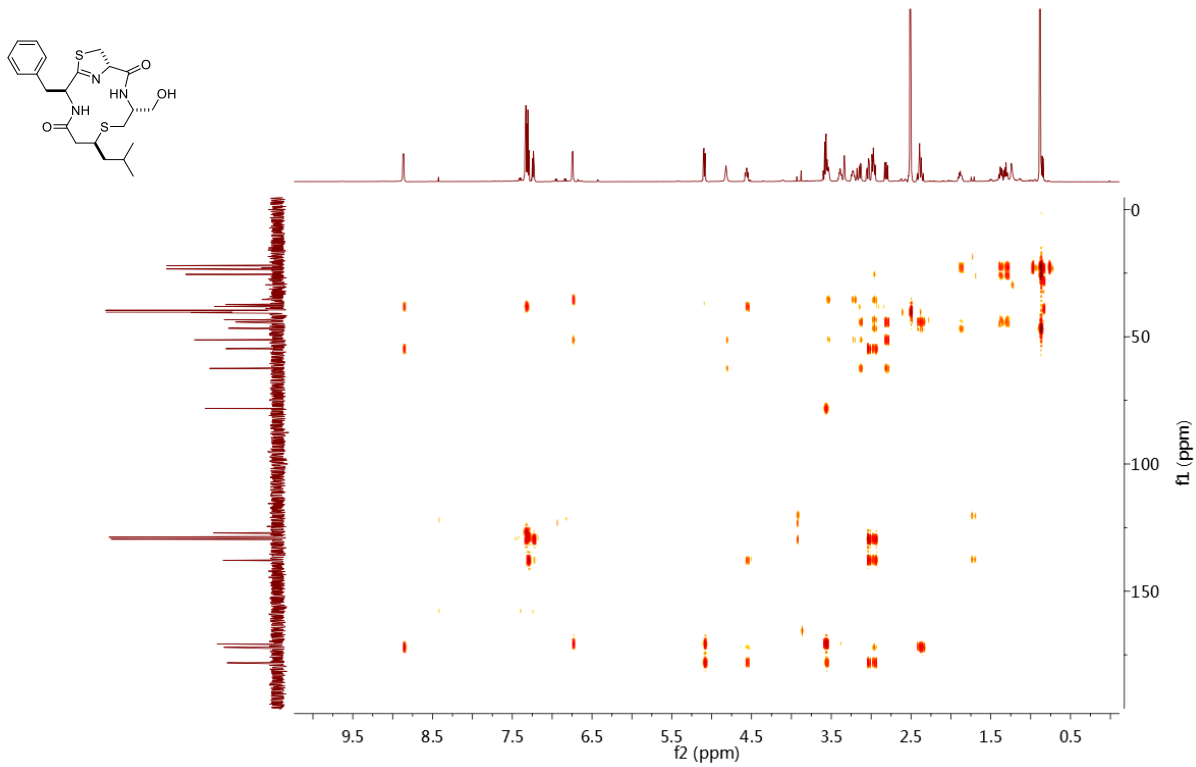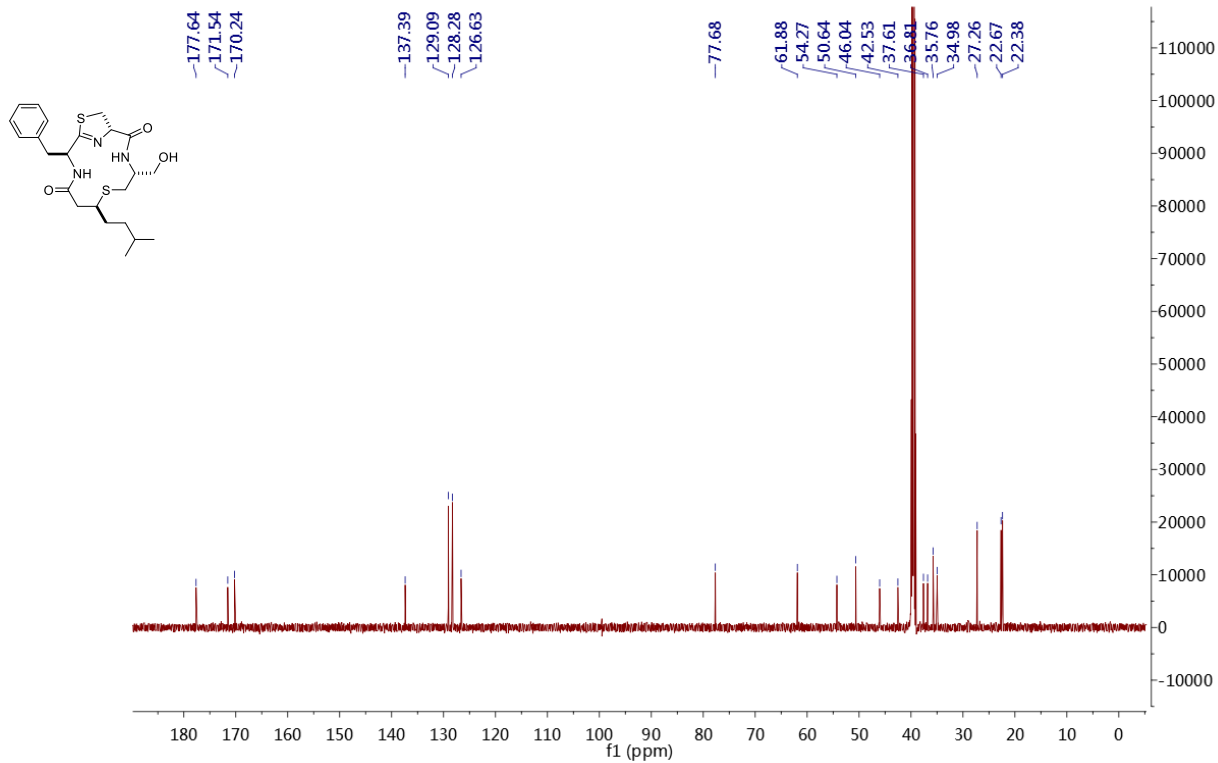
Figure S19. $^{13}$C NMR spectrum (150 MHz) of **4** in DMSO-$d_6$.
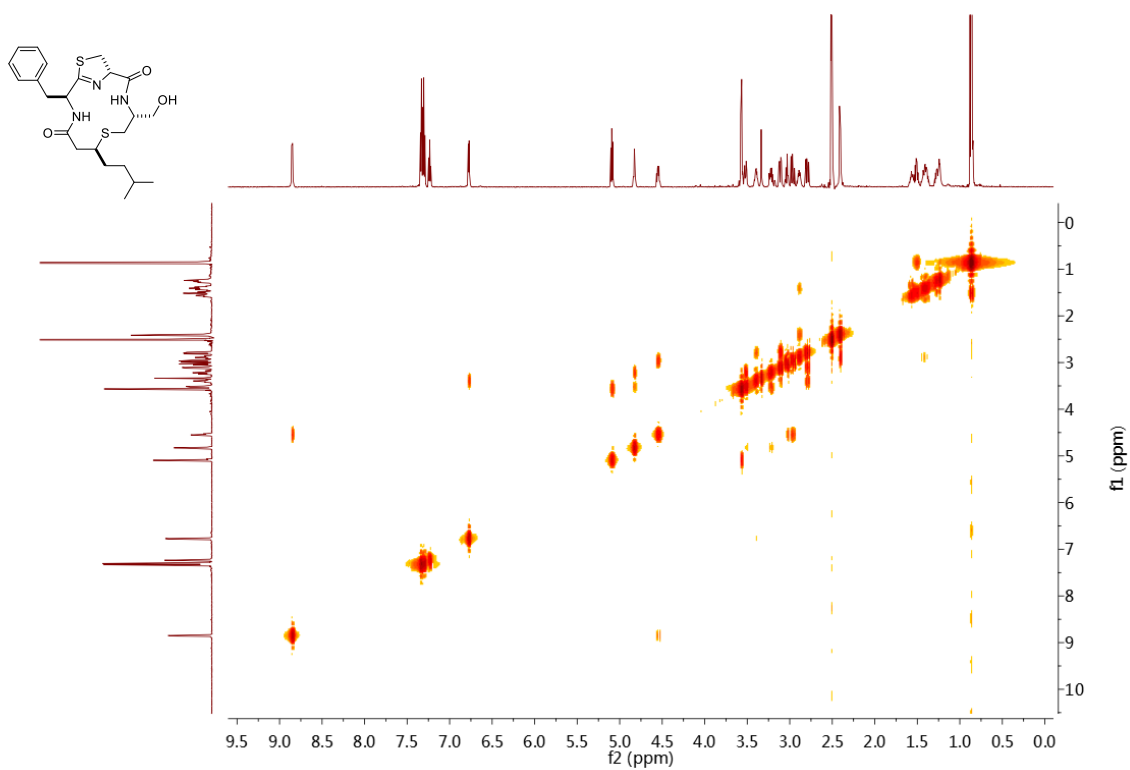


Figure S20. COSY spectrum (600 MHz) of **4** in DMSO-$d_6$.

59
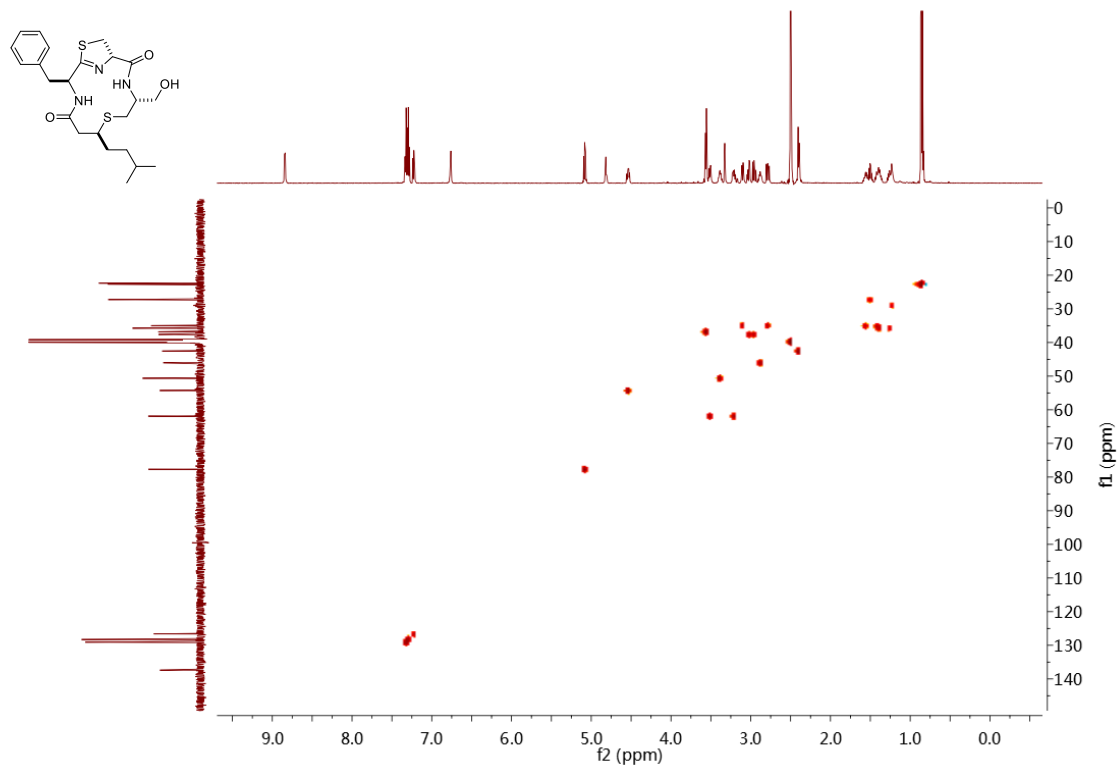
Figure S21. HSQC spectrum (600 MHz) of **4** in DMSO-$d_6$.
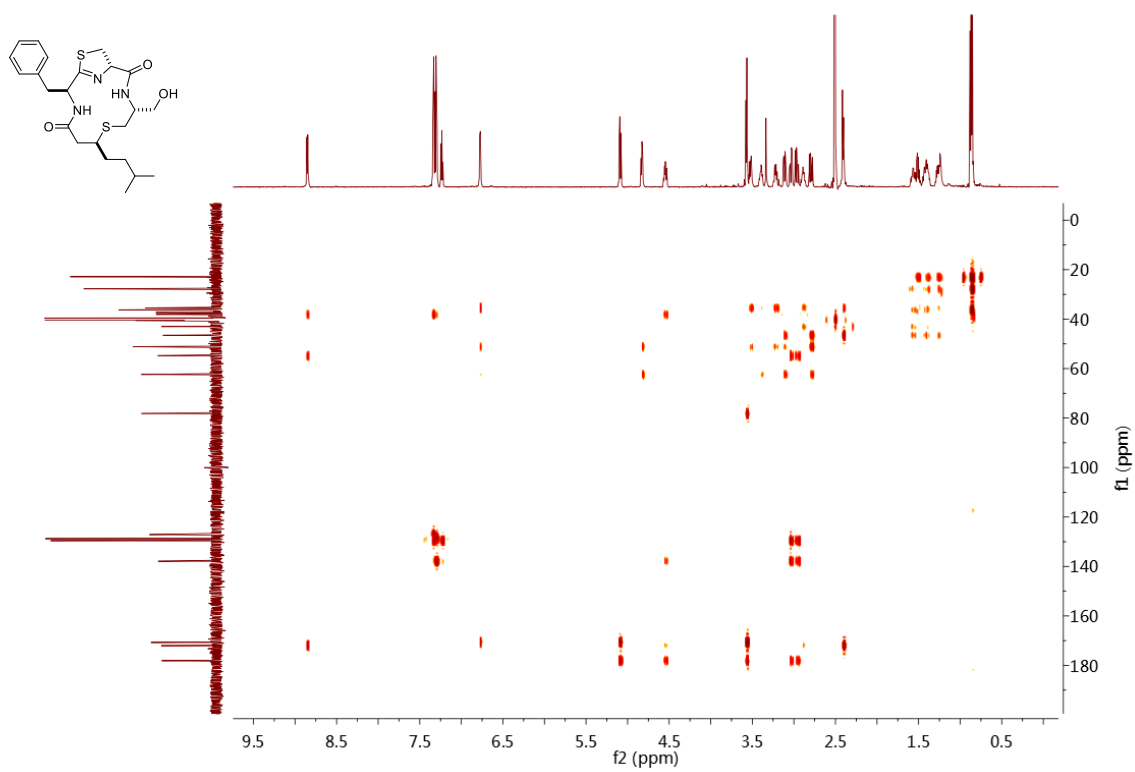


Figure S22. HMBC spectrum (600 MHz) of **4** in DMSO-$d_6$.
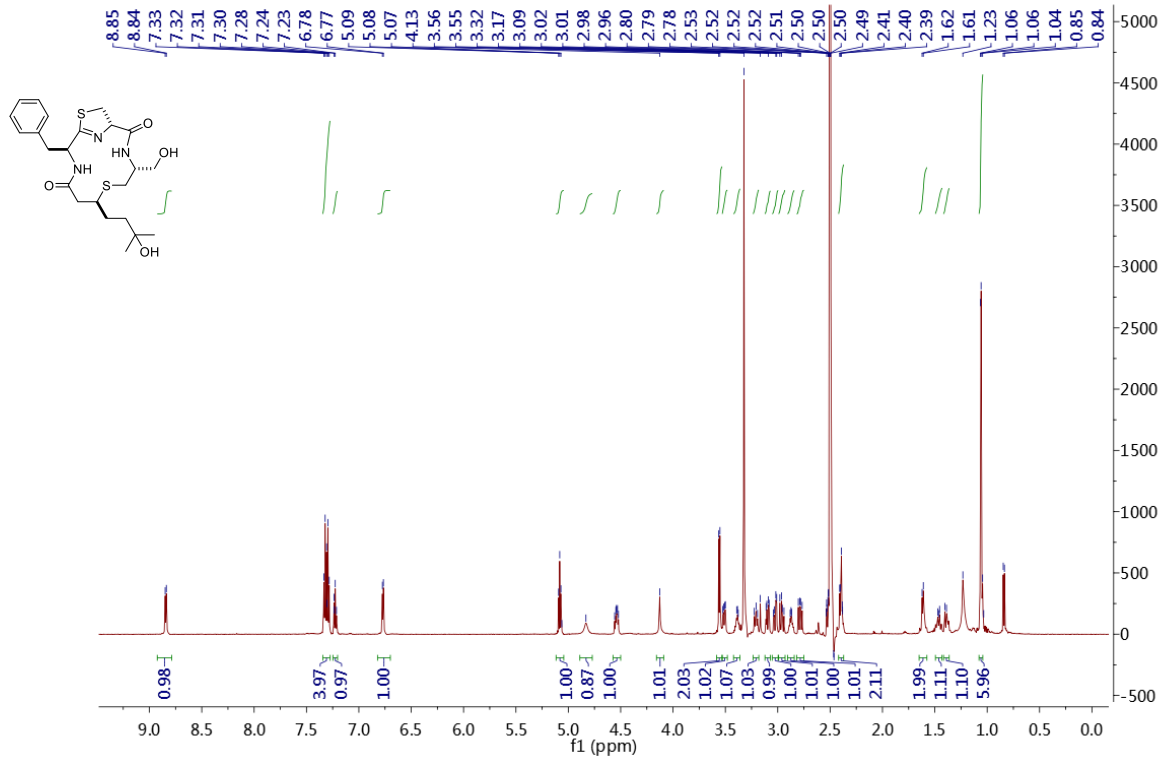
Figure S23. $^1$H NMR spectrum (600 MHz) of **5** in DMSO-$d_6$.
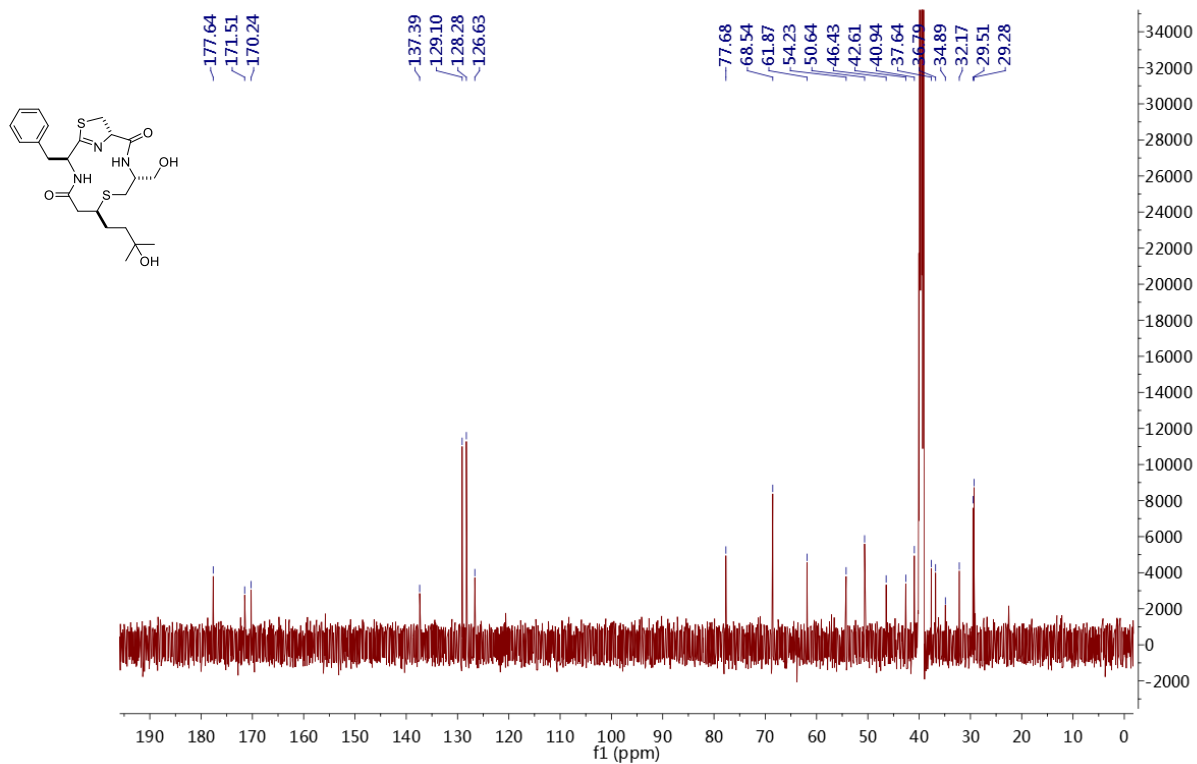


Figure S24. $^{13}$C NMR spectrum (150 MHz) of **5** in DMSO-$d_6$.
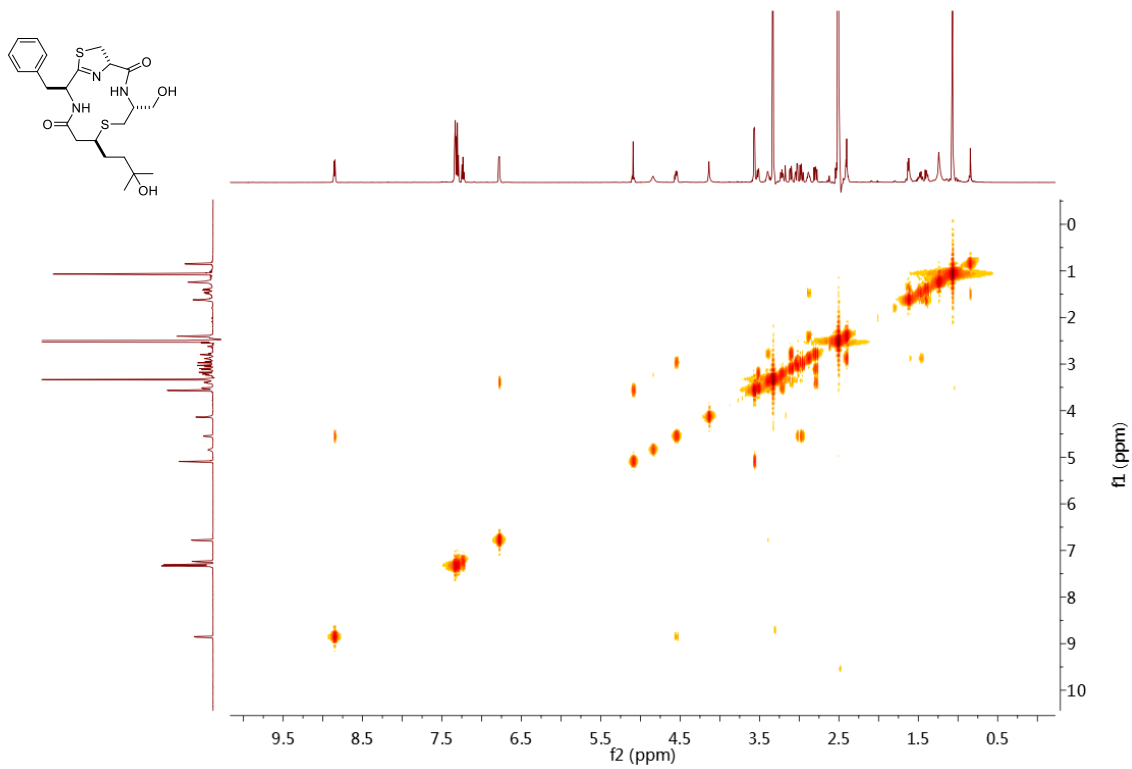
Figure S25. COSY spectrum (600 MHz) of **5** in DMSO-$d_6$.
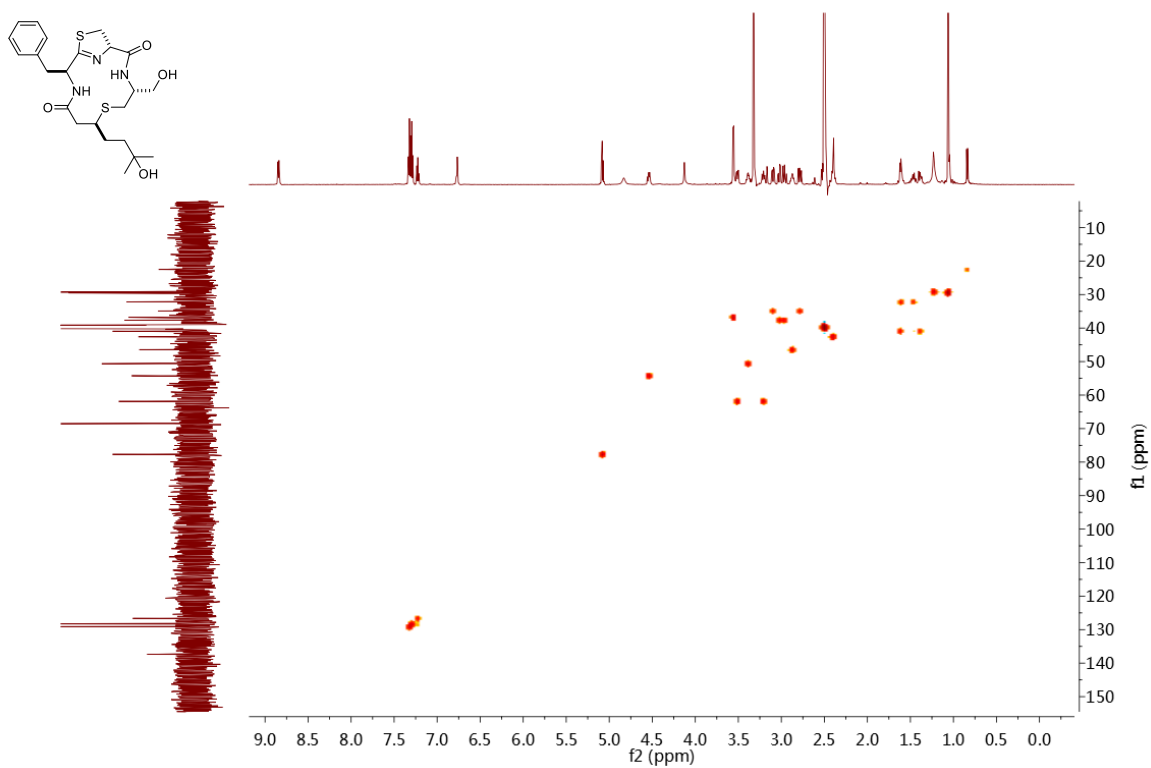


Figure S26. HSQC spectrum (600 MHz) of **5** in DMSO-$d_6$.
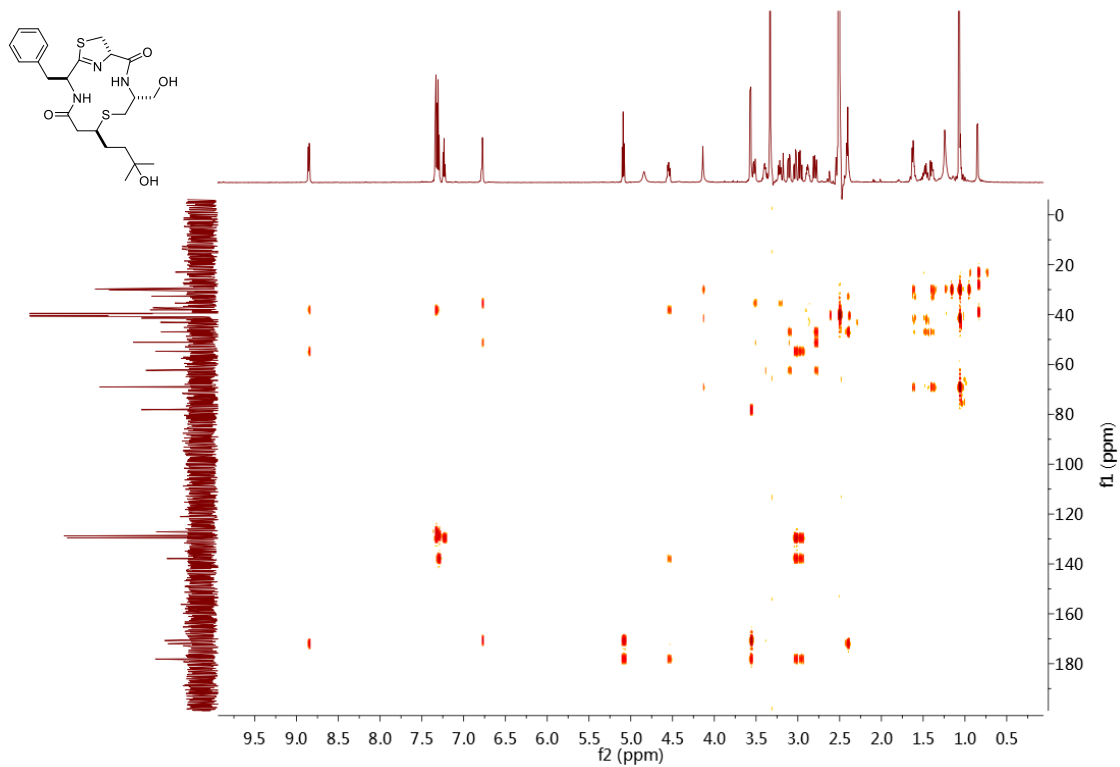
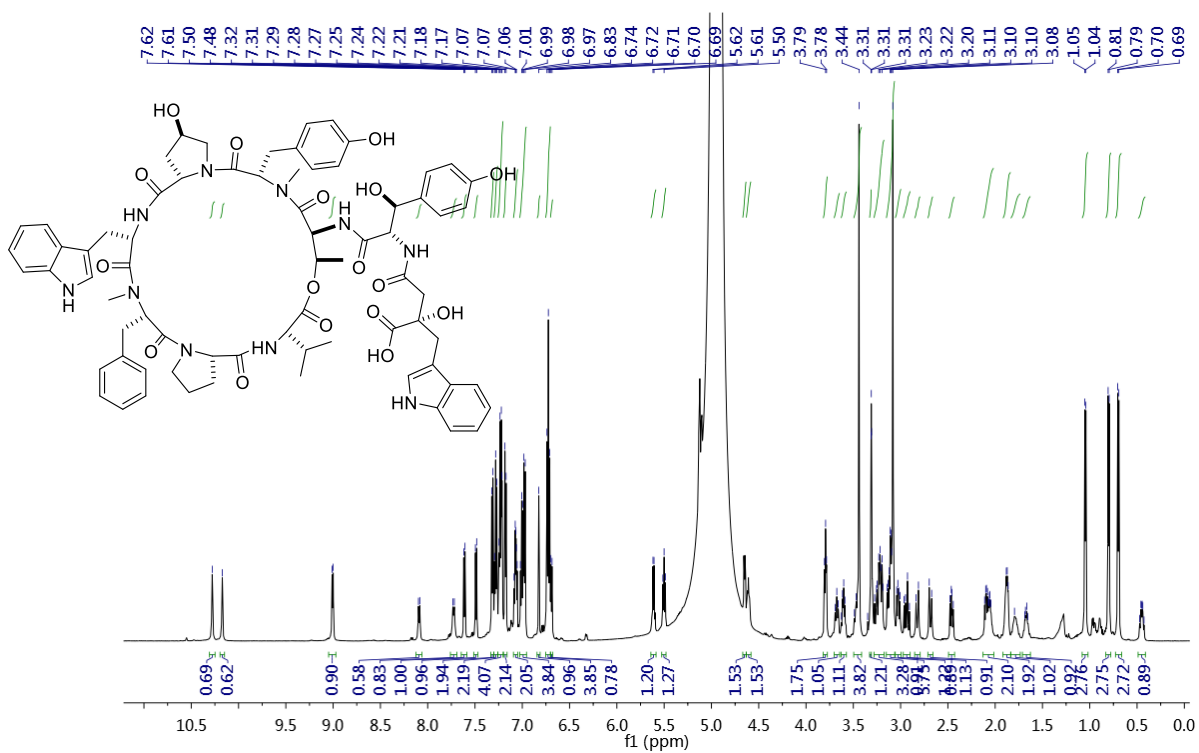Figure S27. HMBC spectrum (600 MHz) of **5** in DMSO-$d_6$.
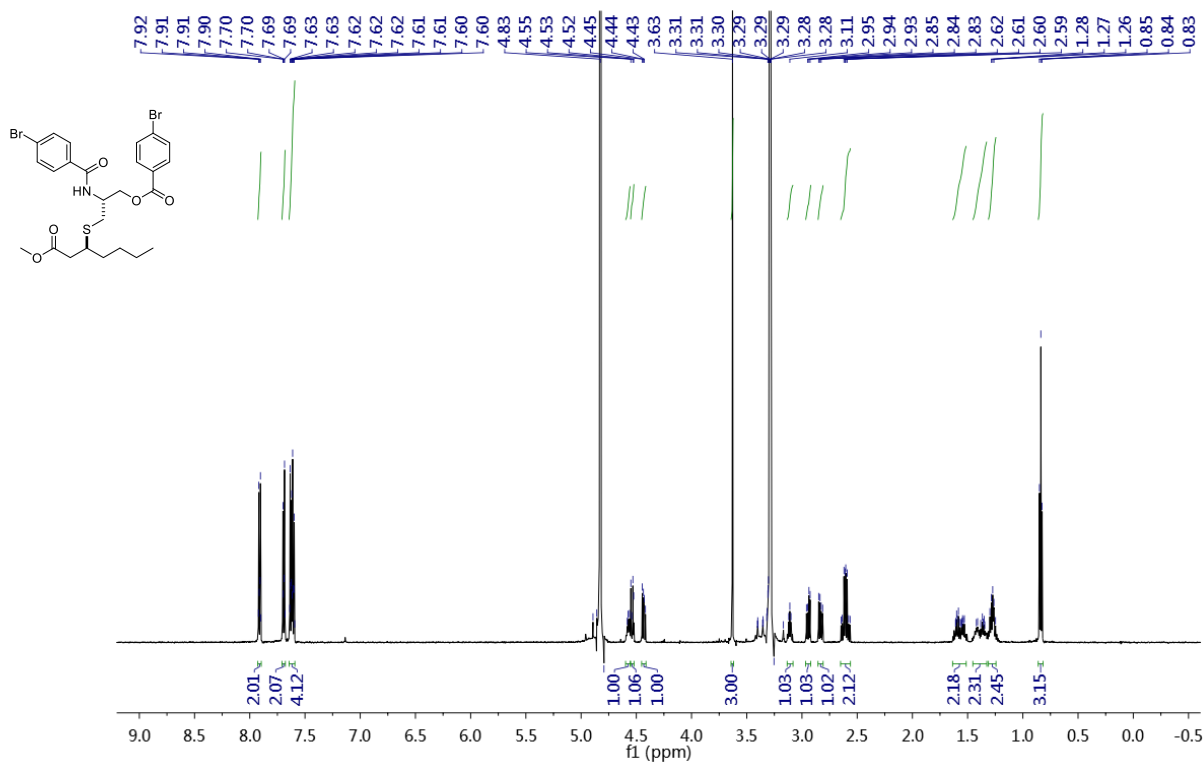


Figure S28. $^1$H NMR spectrum (600 MHz) of **7** in CD$_3$OD.
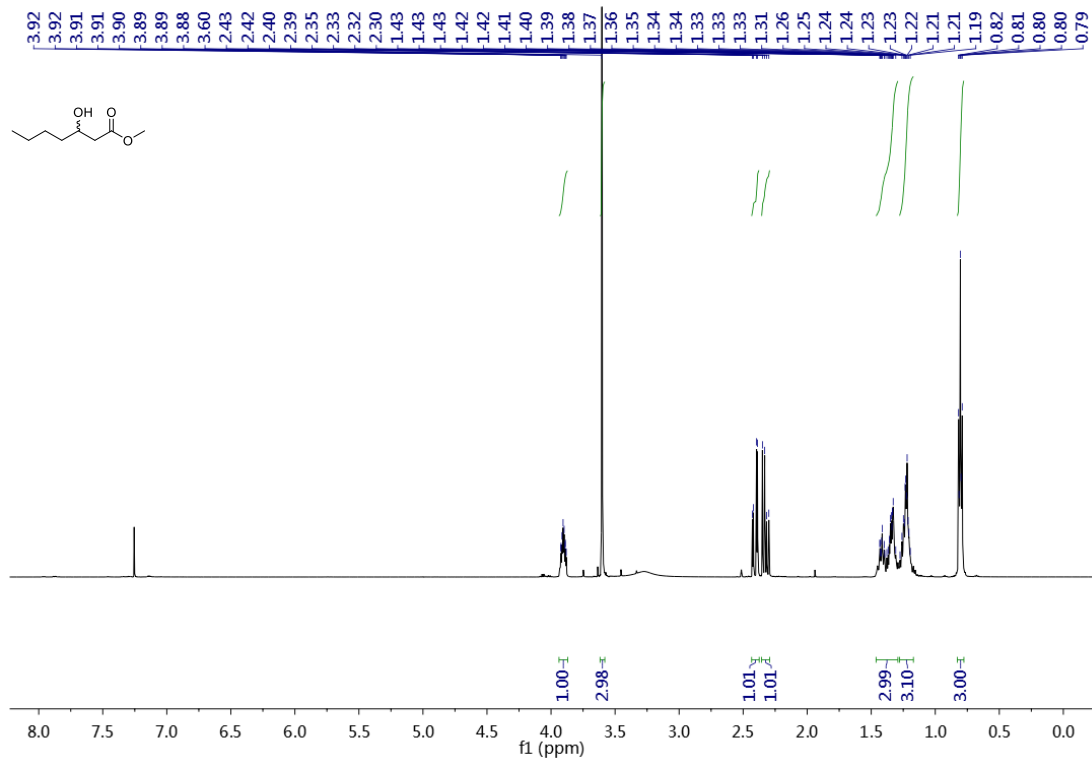
Figure S29. $^1$H NMR spectrum (500 MHz) of **S1** in CDCl$_3$.
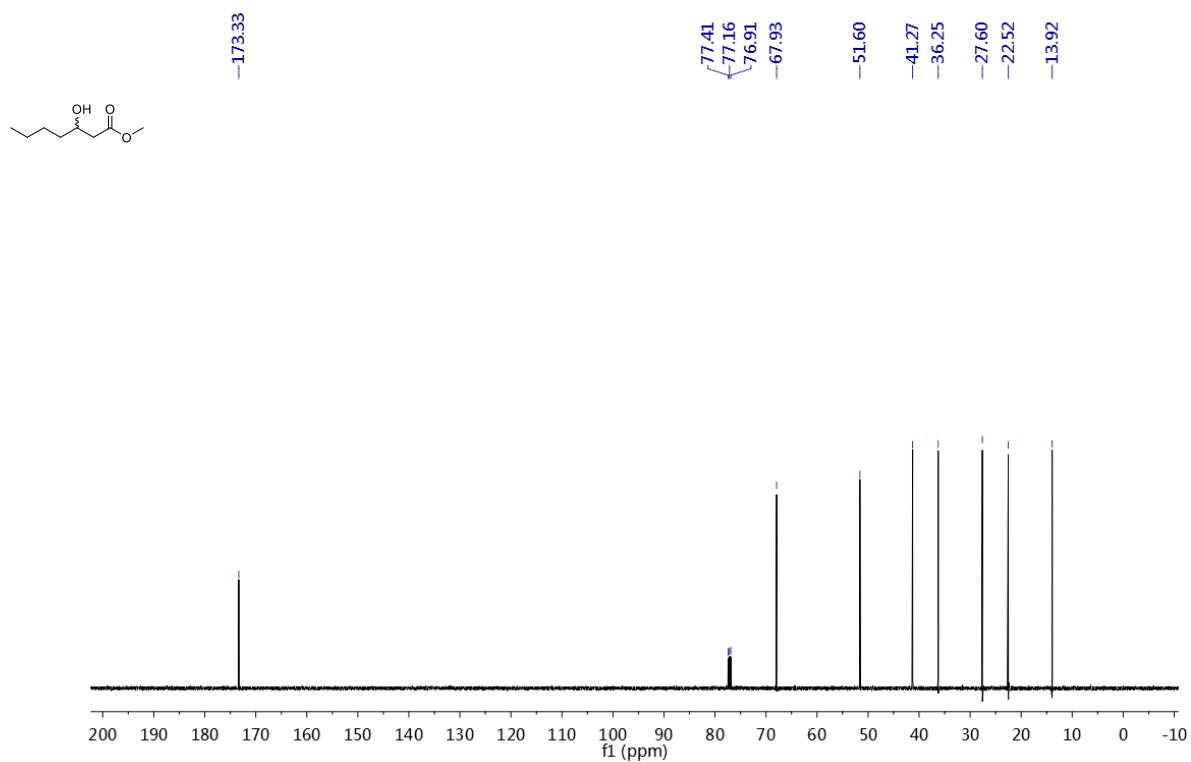


Figure S30. $^{13}$C NMR spectrum (125 MHz) of **S1** in CDCl$_3$.
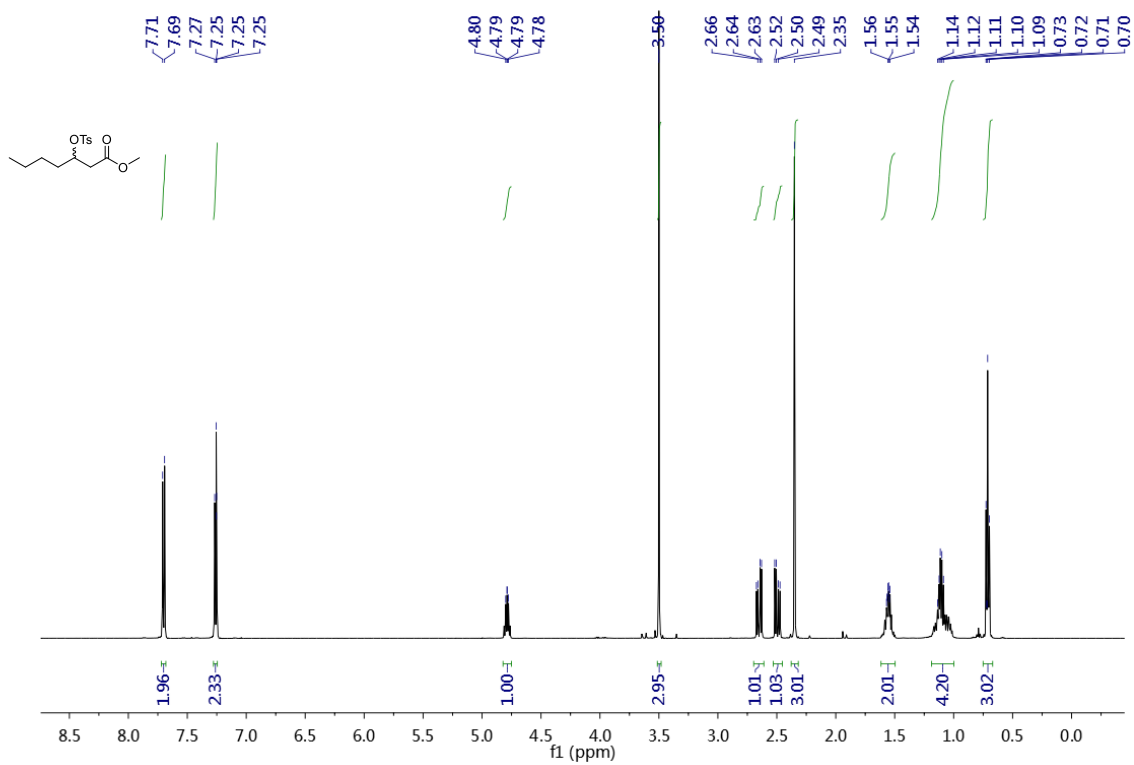
Figure S31. $^1$H NMR spectrum (500 MHz) of **9** in CDCl$_3$.
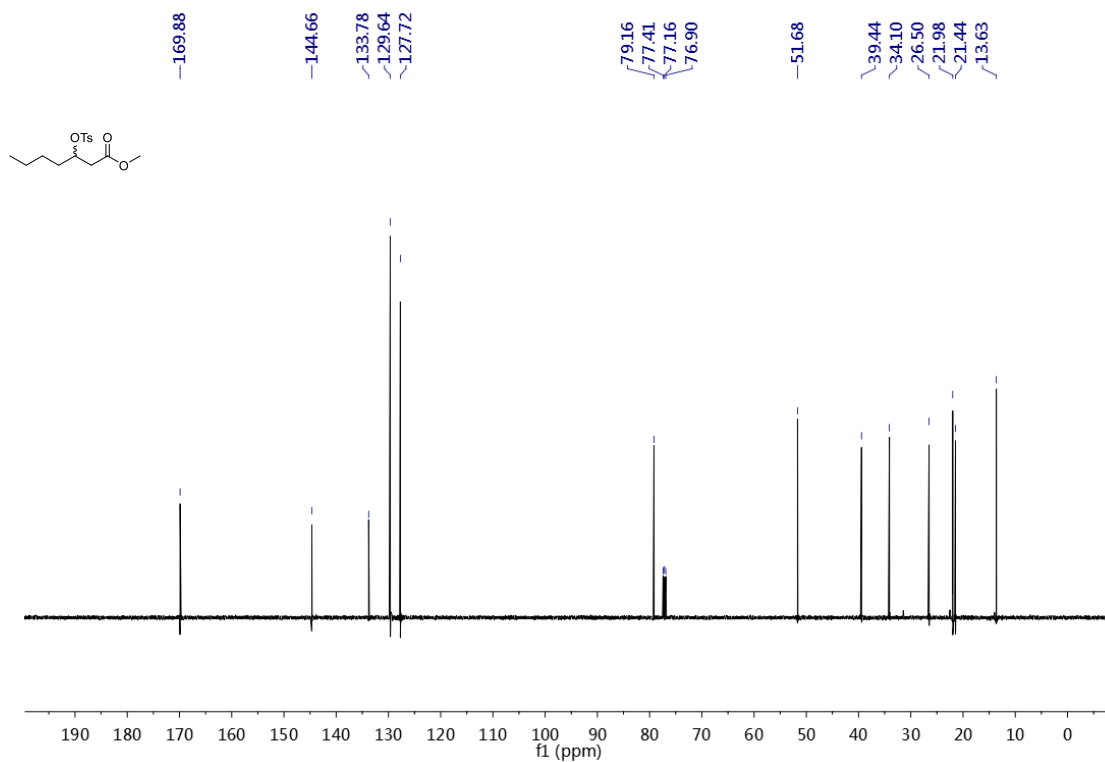


Figure S32. $^{13}$C NMR spectrum (125 MHz) of **9** in CDCl$_3$.
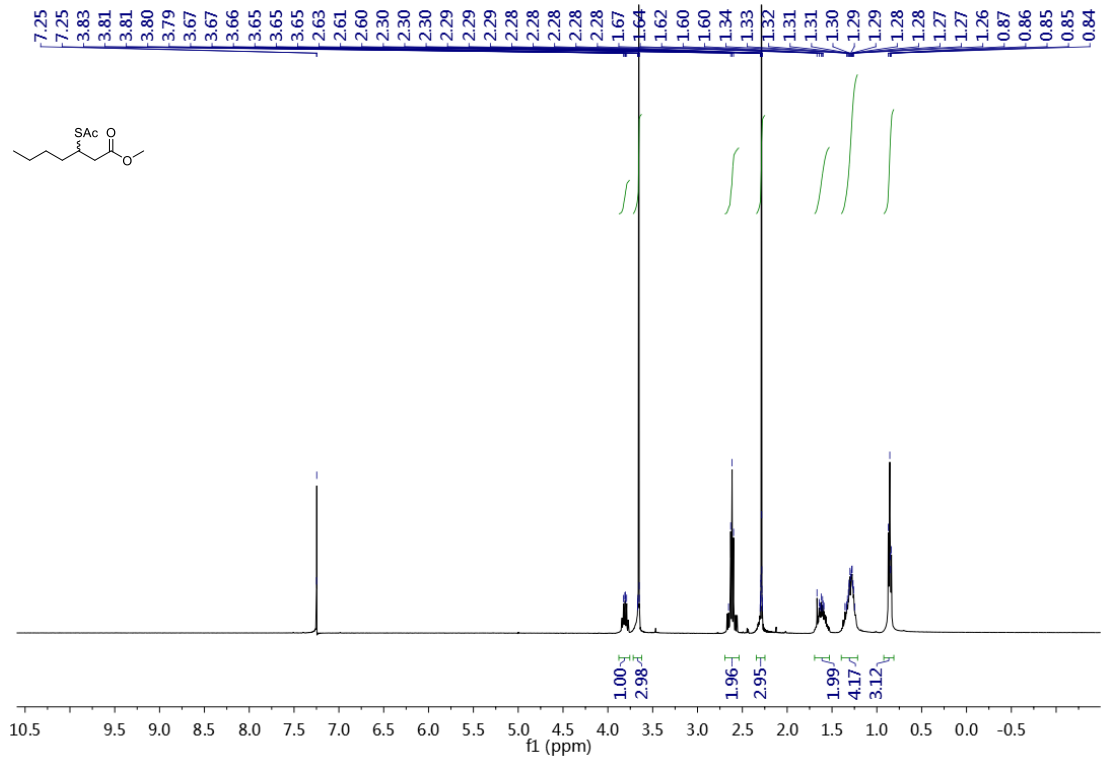
Figure S33. ¹H NMR spectrum (500 MHz) of **10** in CDCl₃.
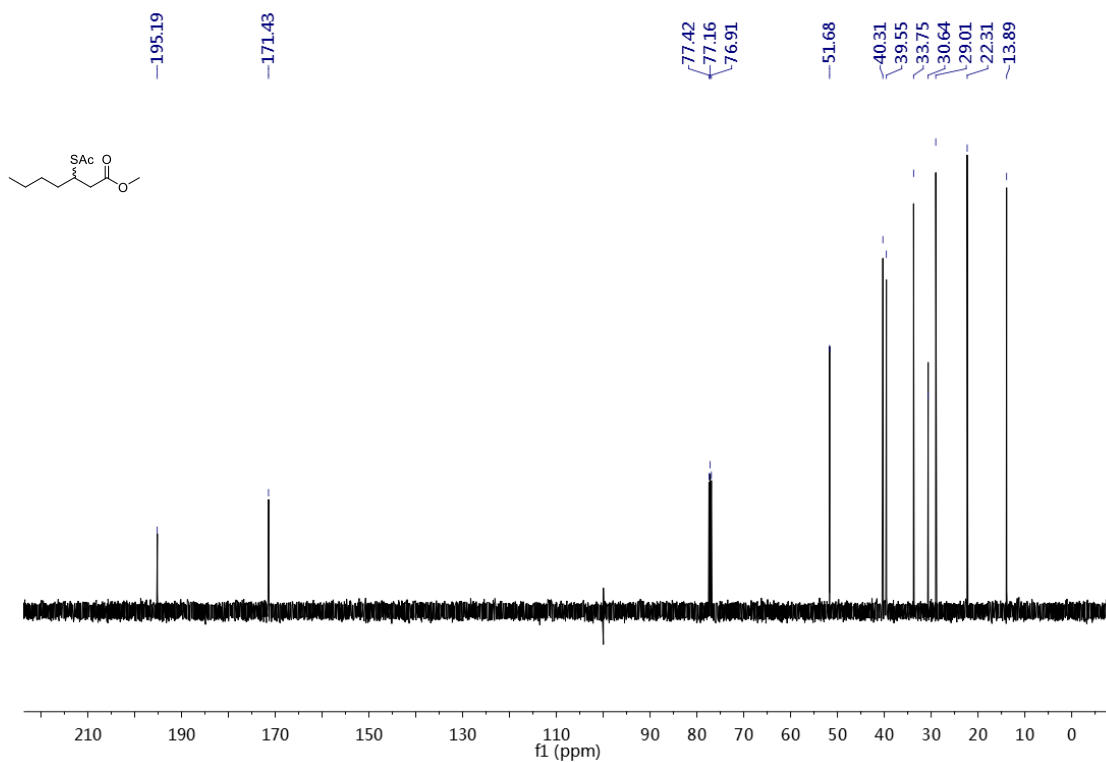


Figure S34. ¹³C NMR spectrum (125 MHz) of **10** in CDCl₃.
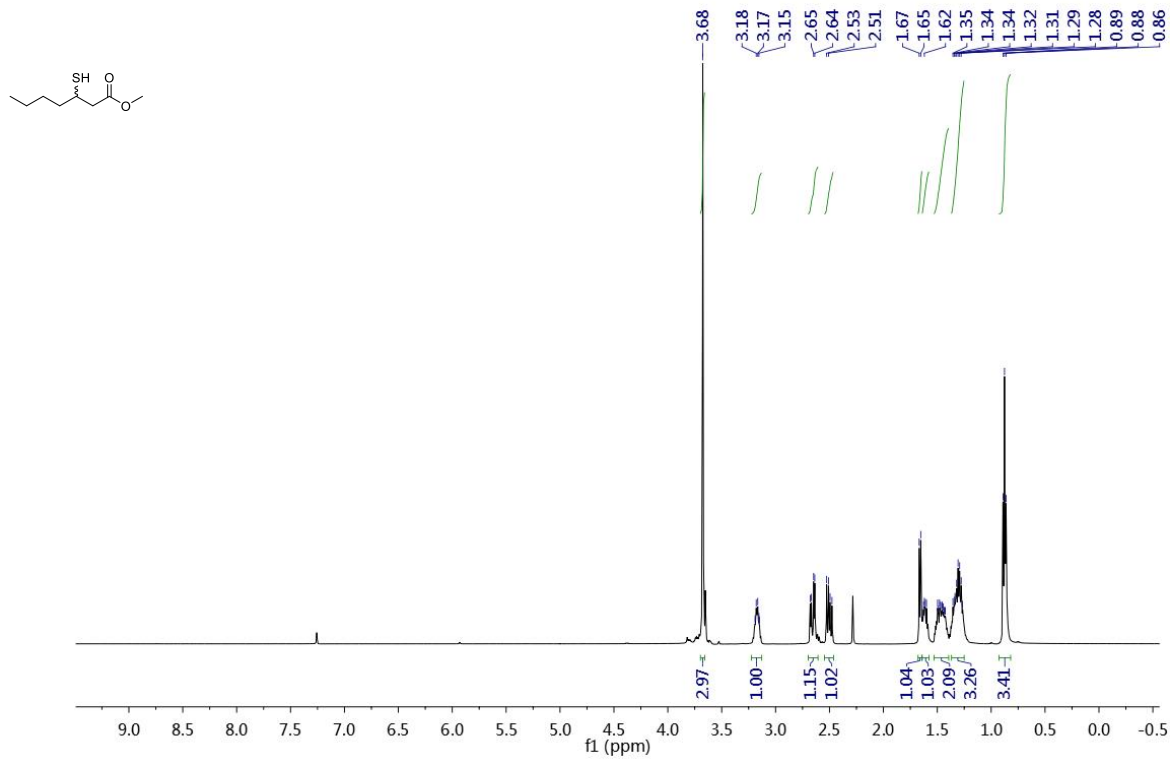
Figure S35. $^1$H NMR spectrum (500 MHz) of **11** in CDCl$_3$.
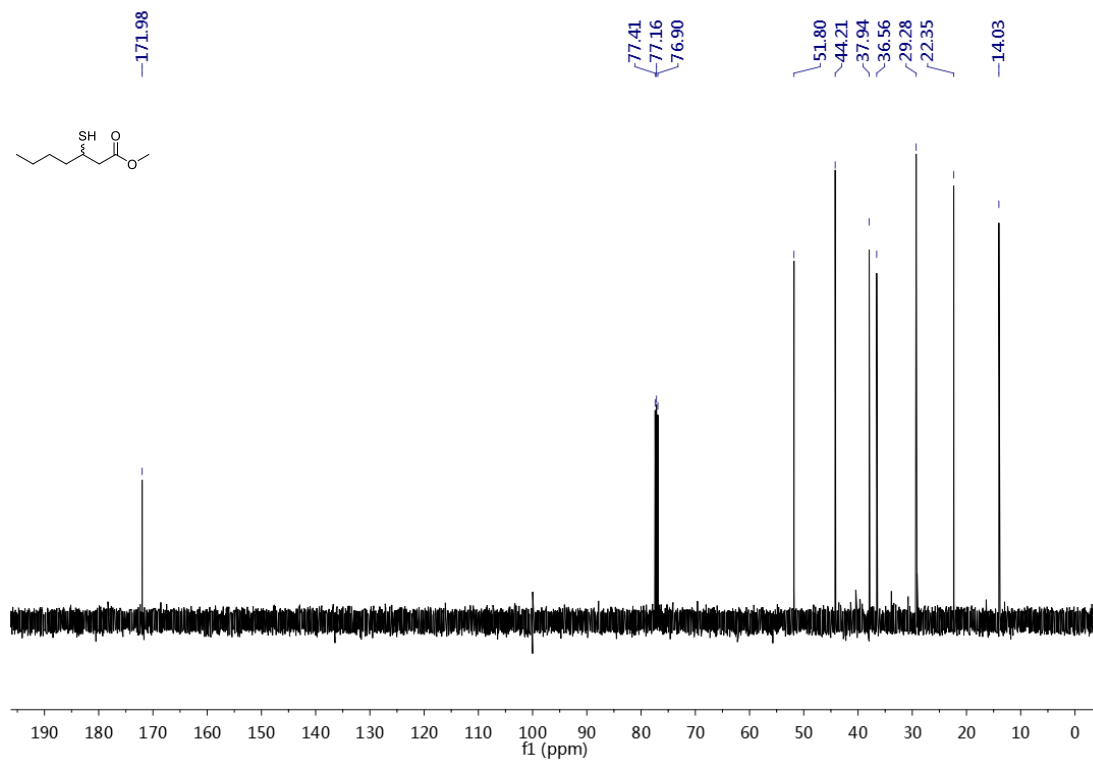


Figure S36. $^{13}$C NMR spectrum (125 MHz) of **11** in CDCl$_3$.
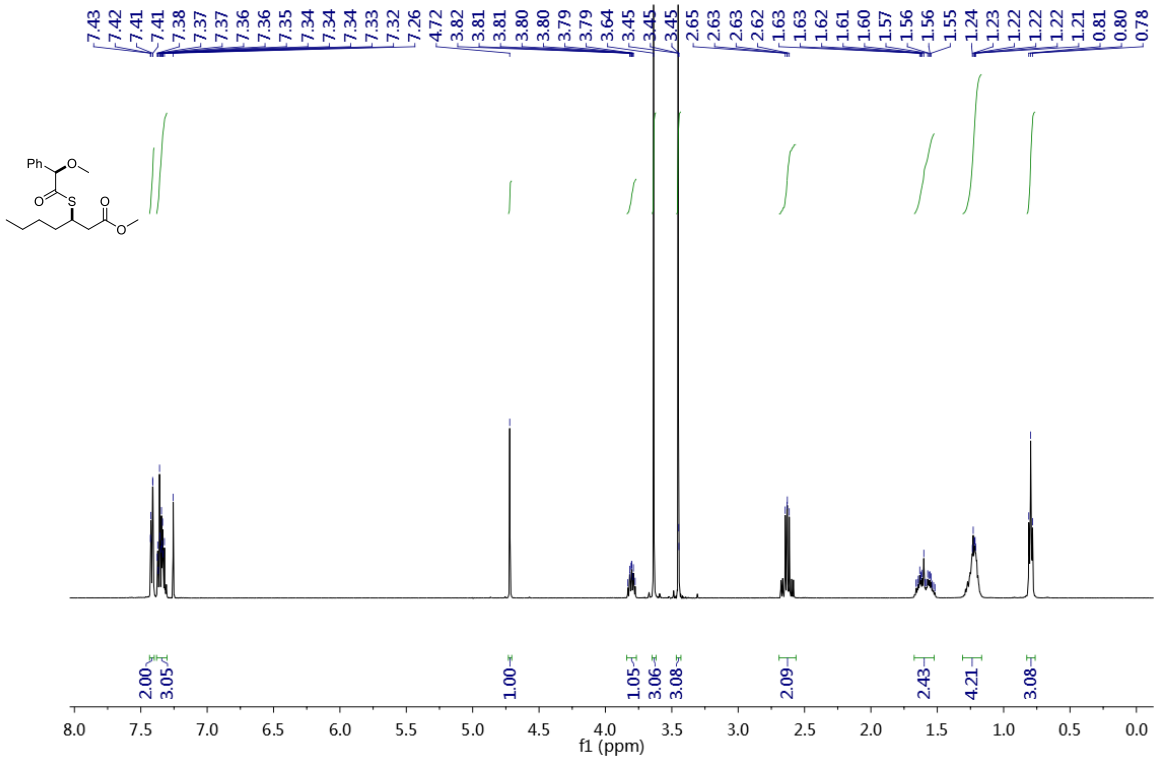
Figure S37. $^1$H NMR spectrum (500 MHz) of **12a** in CDCl$_3$.
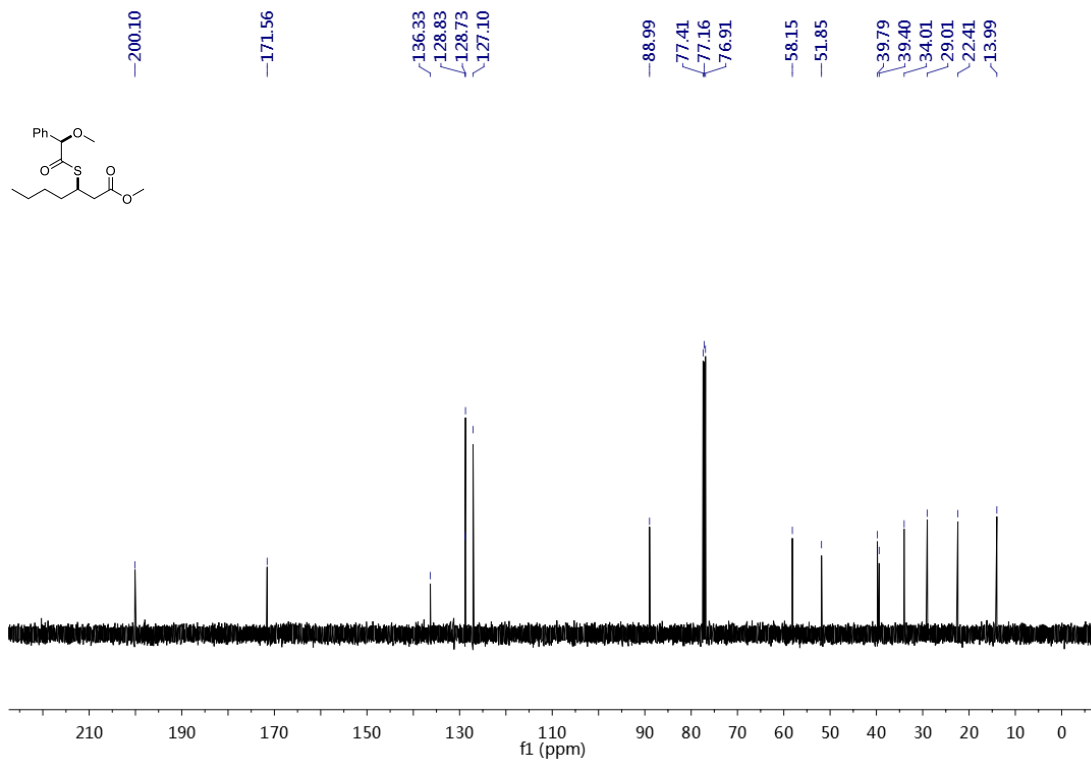


Figure S38. $^{13}$C NMR spectrum (125 MHz) of **12a** in CDCl$_3$.
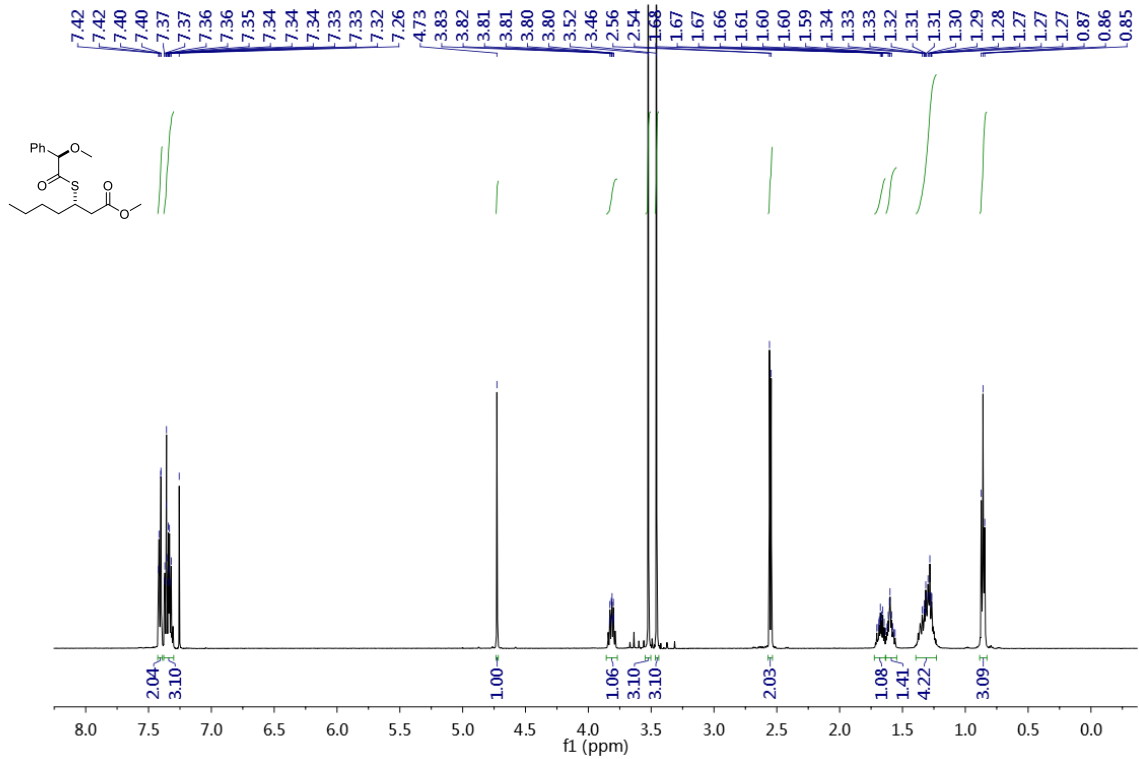
Figure S39. $^1$H NMR spectrum (500 MHz) of **12b** in CDCl$_3$.
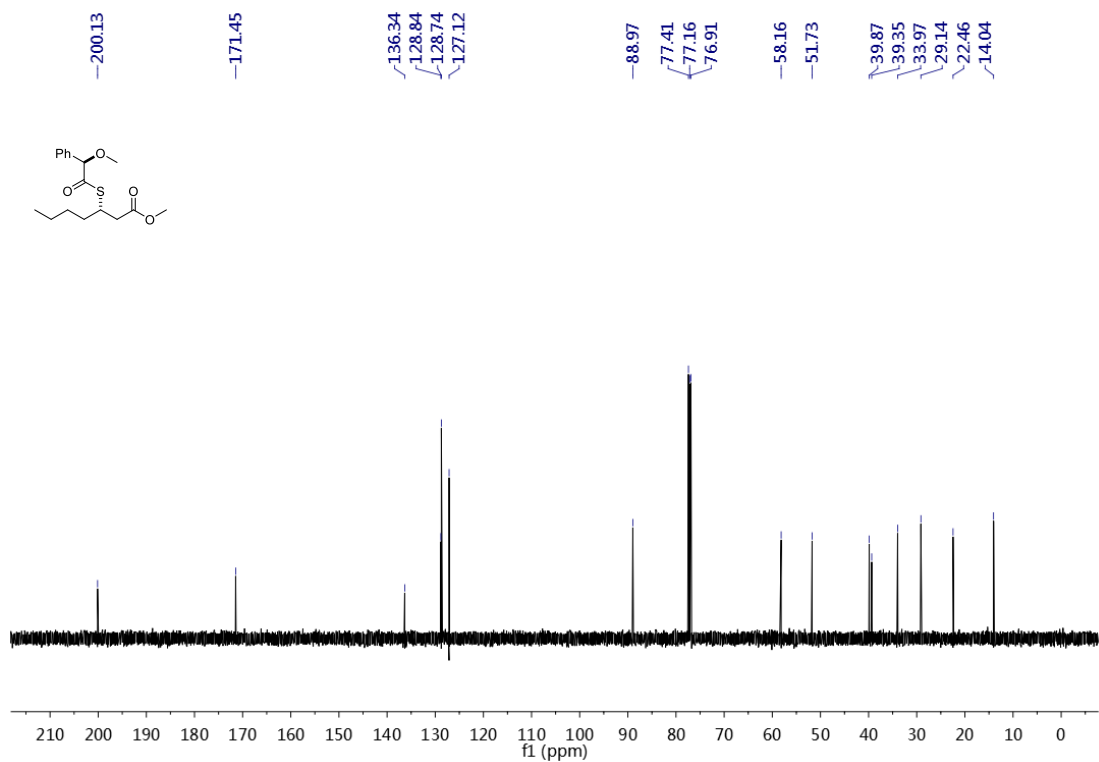


Figure S40. $^{13}$C NMR spectrum (125 MHz) of **12b** in CDCl$_3$.
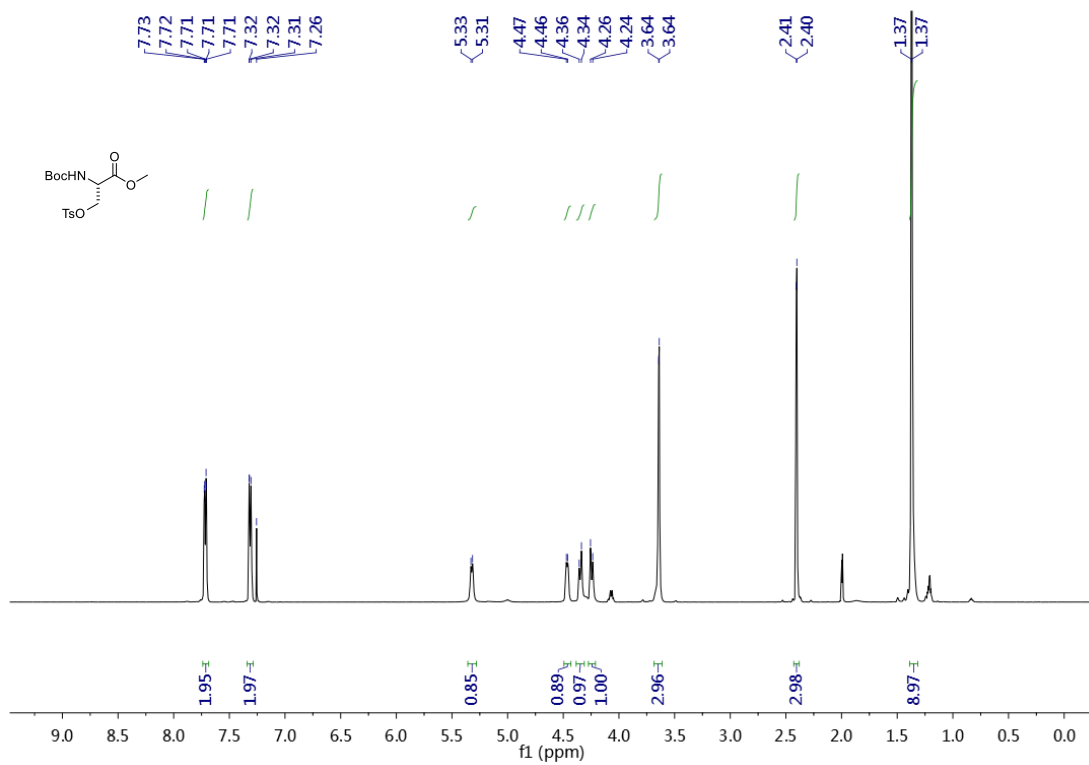
Figure S41. $^1$H NMR spectrum (500 MHz) of **S2** in CDCl$_3$.
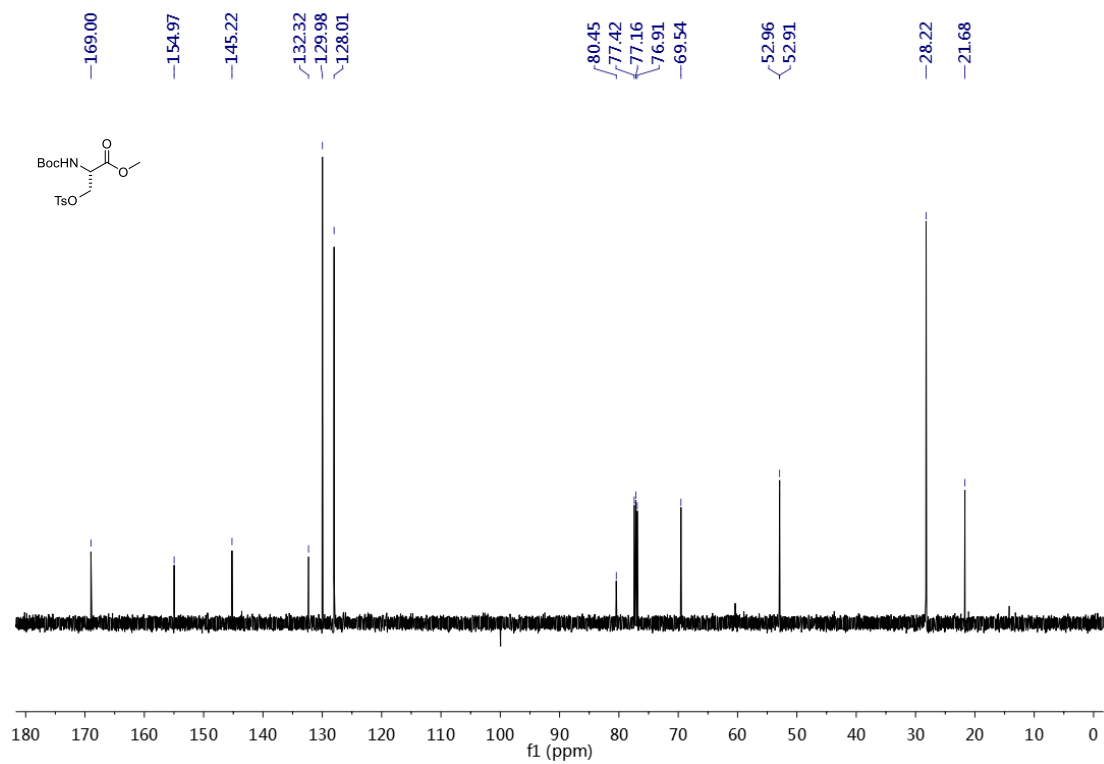


Figure S42. $^{13}$C NMR spectrum (125 MHz) of **S2** in CDCl$_3$.
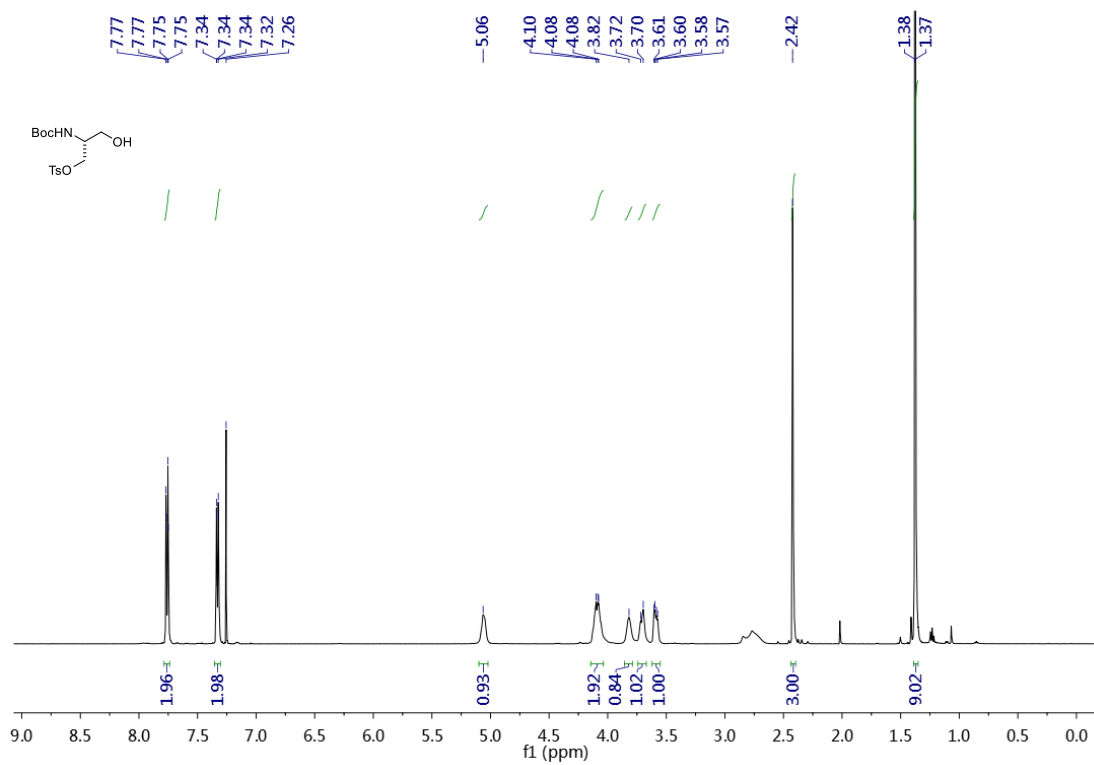
Figure S43. $^1$H NMR spectrum (500 MHz) of **14** in CDCl$_3$.
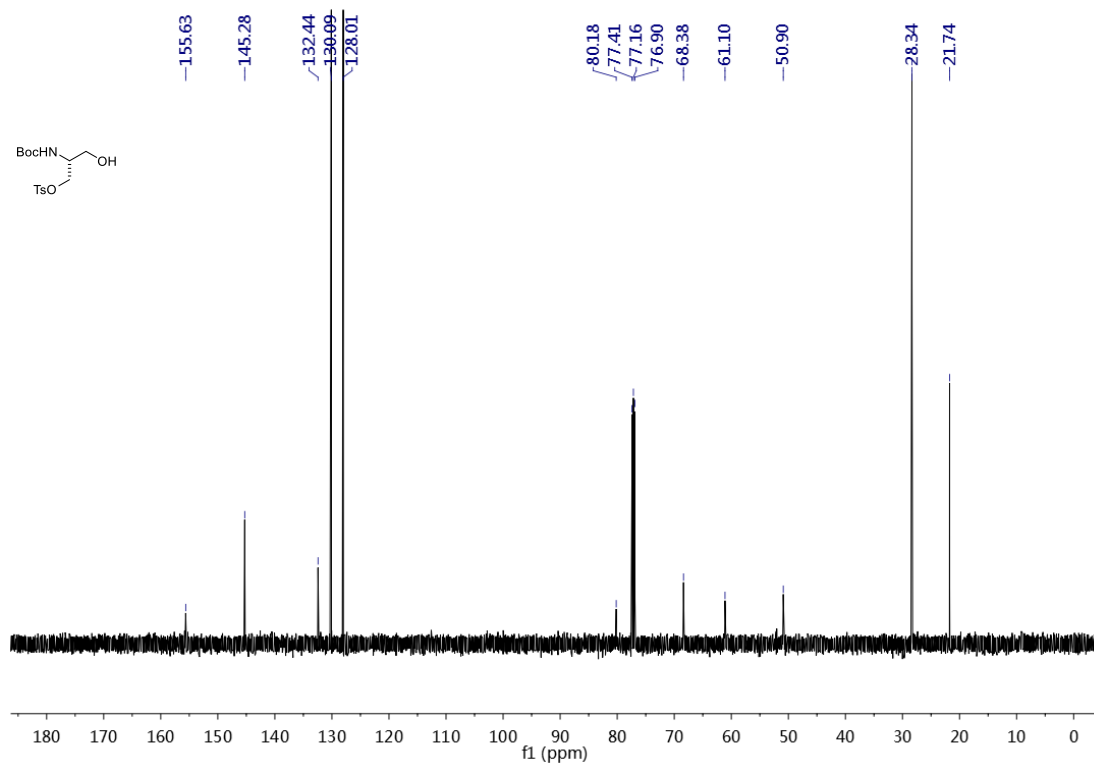


Figure S44. $^{13}$C NMR spectrum (125 MHz) of **14** in CDCl$_3$.
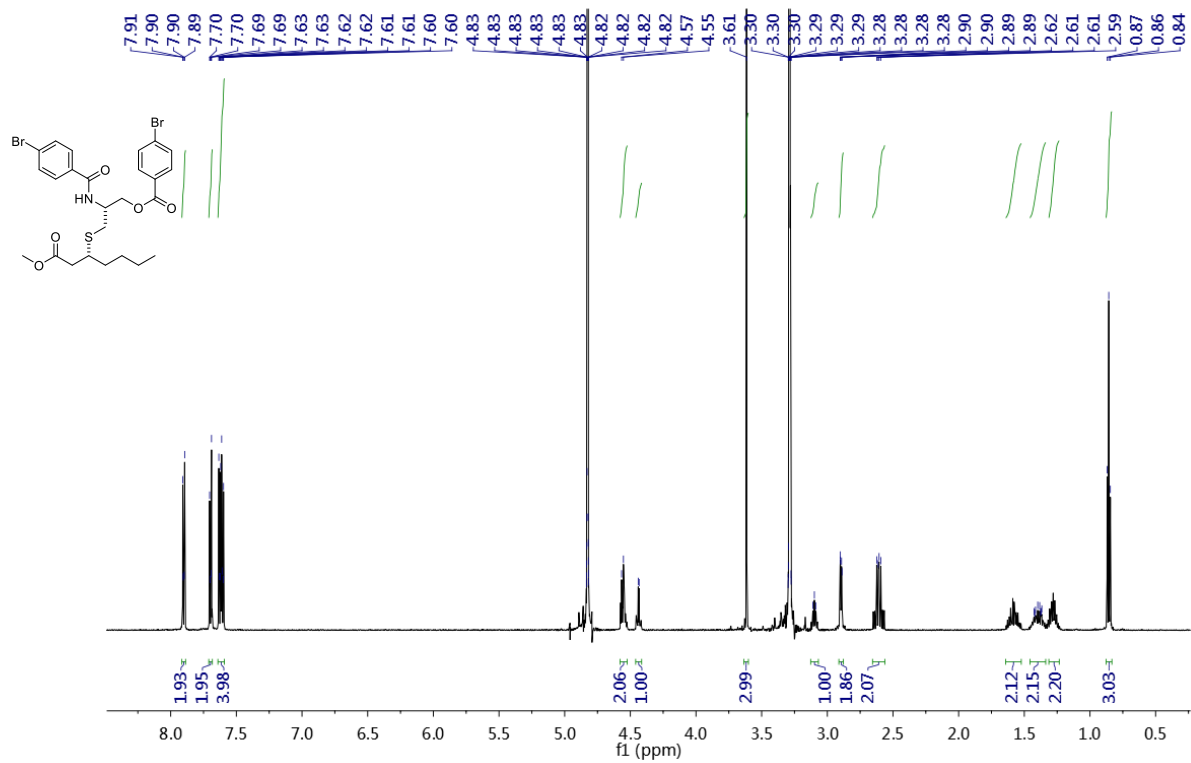
Figure S45. $^1$H NMR spectrum (600 MHz) of **7a** in CD$_3$OD.
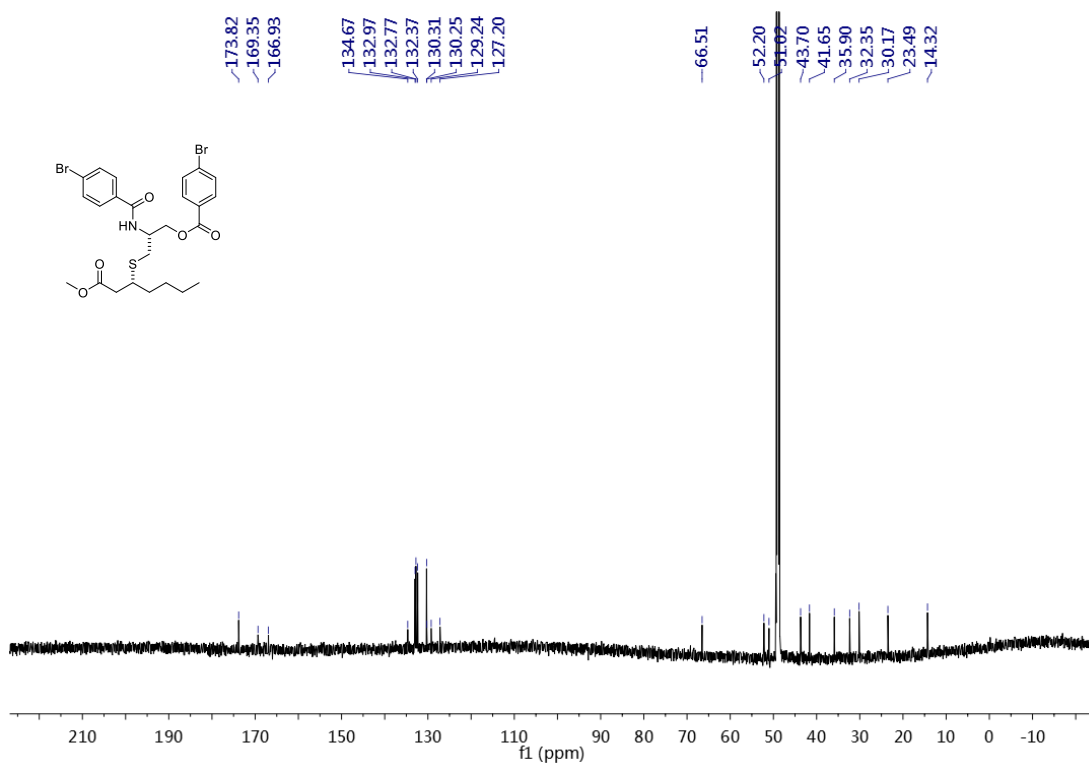


Figure S46. $^{13}$C NMR spectrum (150 MHz) of **7a** in CD$_3$OD.
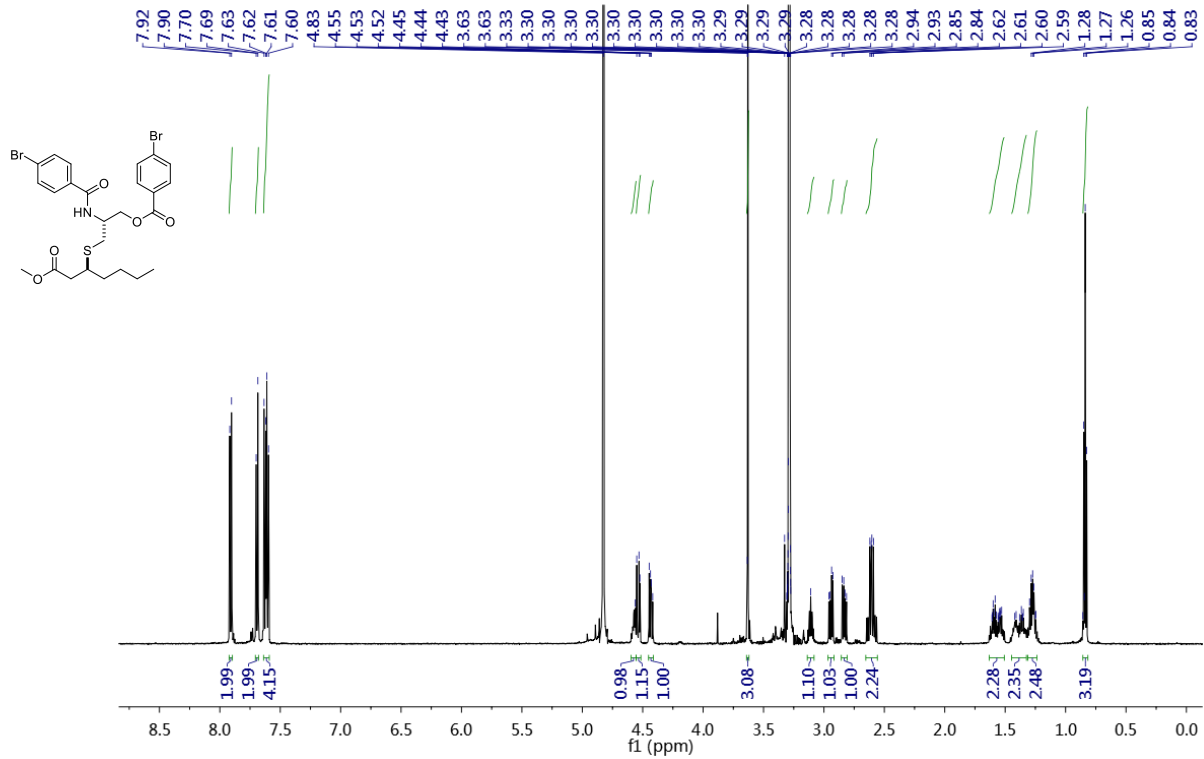
Figure S47. $^1$H NMR spectrum (600 MHz) of **7b** in CD$_3$OD.
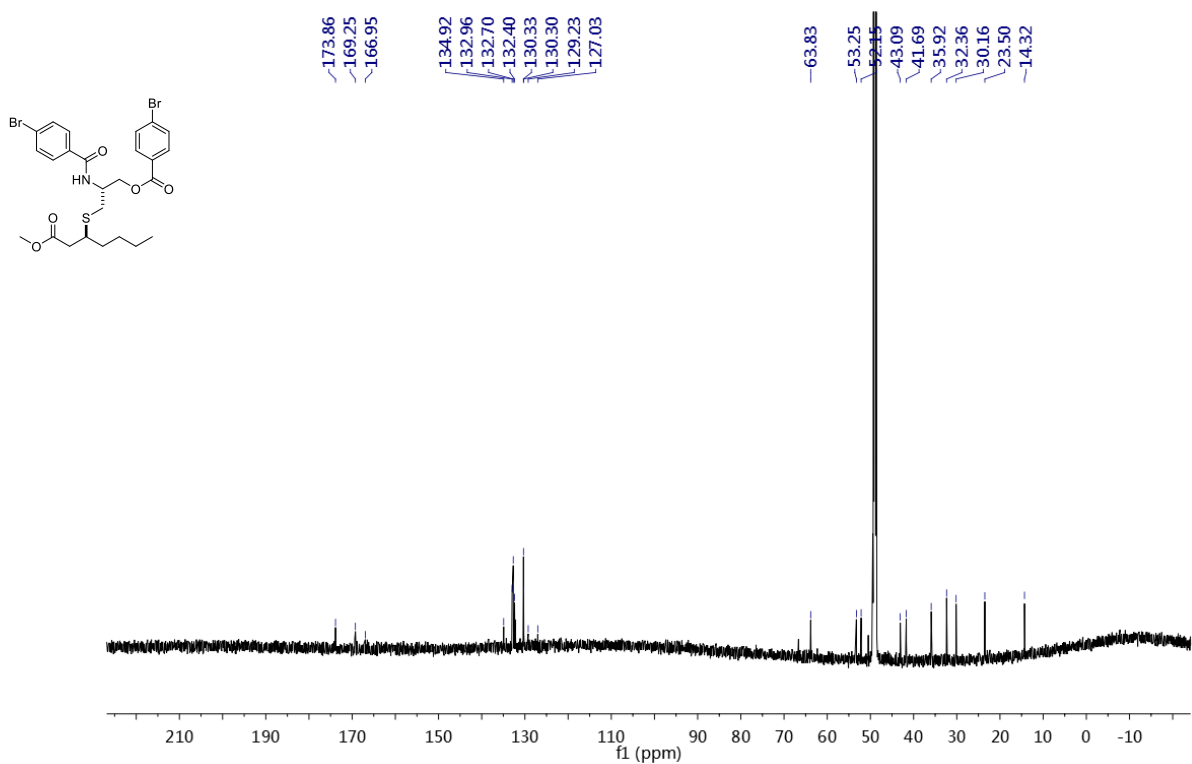


Figure S48. $^{13}$C NMR spectrum (150 MHz) of **7b** in CD$_3$OD.

73

Figure S49. HRMS spectrum of **1**.
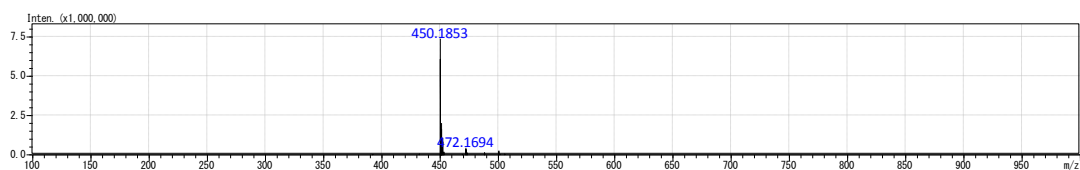


Figure S50. HRMS spectrum of **2**.
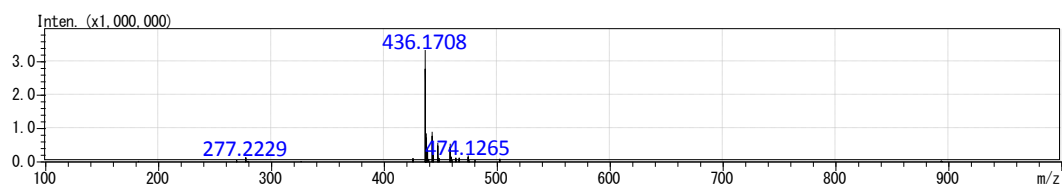


Figure S51. HRMS spectrum of **3**.
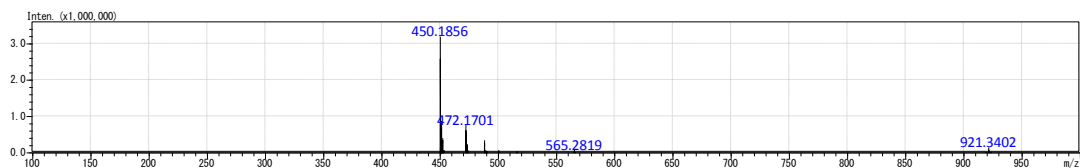


Figure S52. HRMS spectrum of **4**.
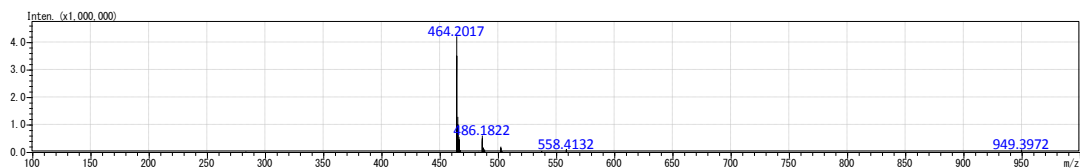


Figure S53. HRMS spectrum of **5**.
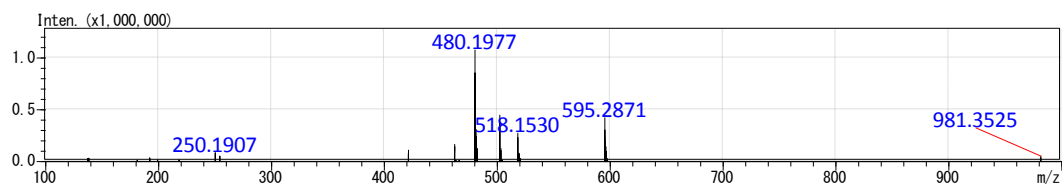


Figure S54. HRMS spectrum of **7**.
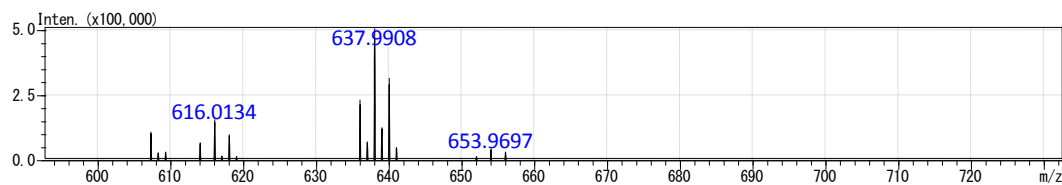


Figure S55. HRMS spectrum of **S1**.
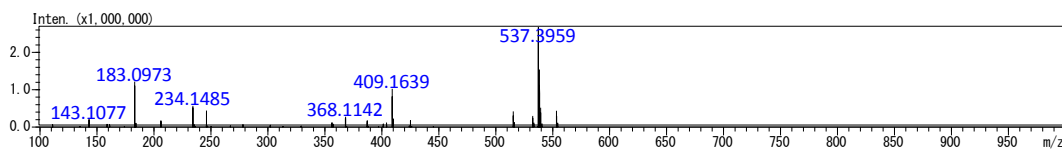
Figure S56. HRMS spectrum of **9**.
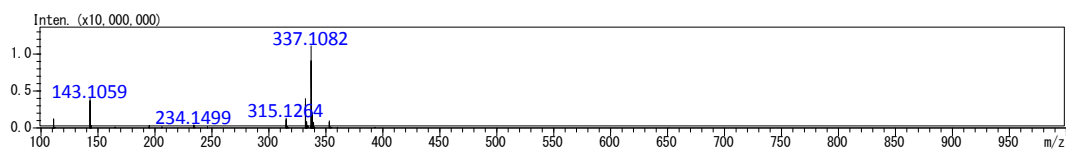


Figure S57. HRMS spectrum of **10**.
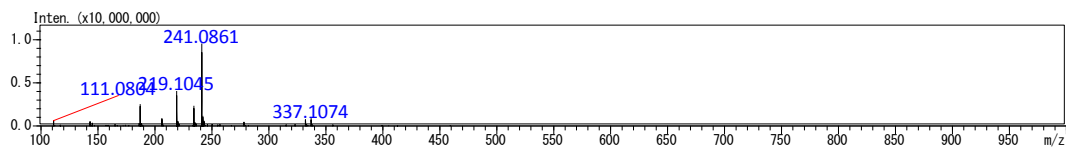


Figure S58. HRMS spectrum of **11**.
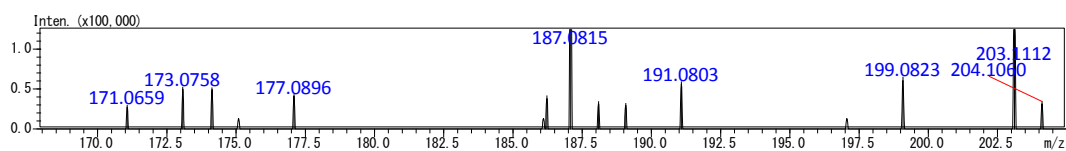


Figure S59. HRMS for compound **12a**.
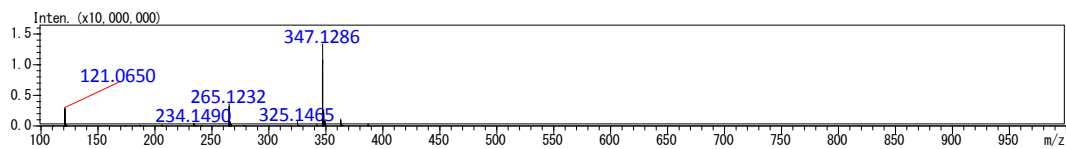


Figure S60. HRMS for compound **12b**.
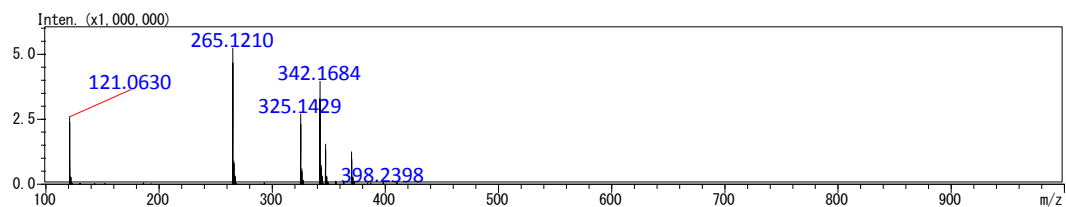


Figure S61. HRMS for compound **S2**.
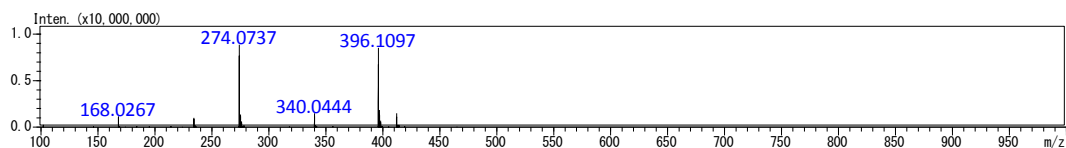


Figure S62. HRMS for compound **14**.
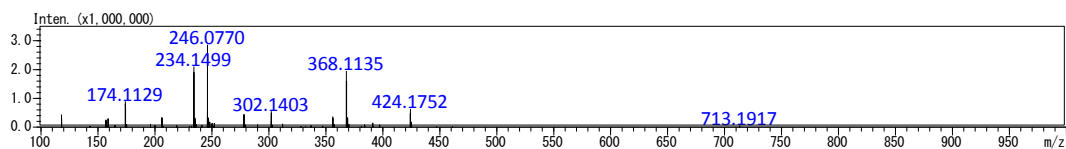


Figure S63. HRMS for compound **7a**.
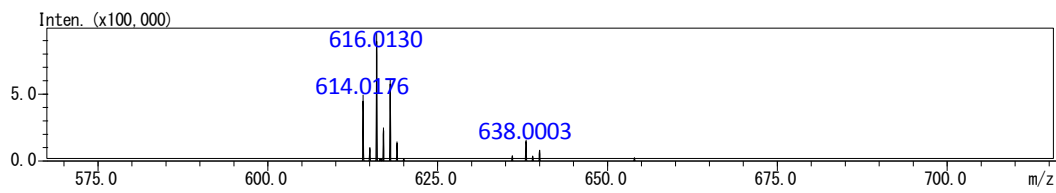
Figure S64. HRMS for compound **7b**.
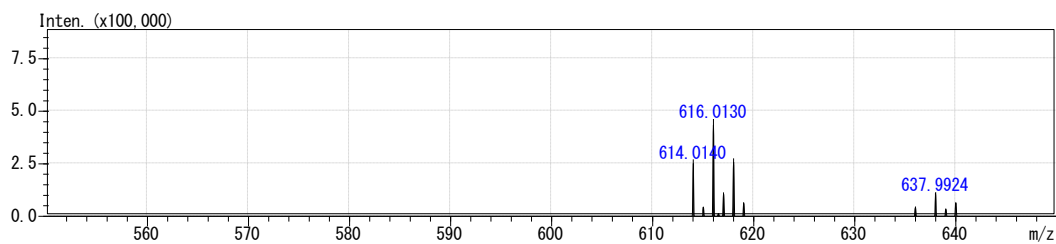
**Chapter 1-2**
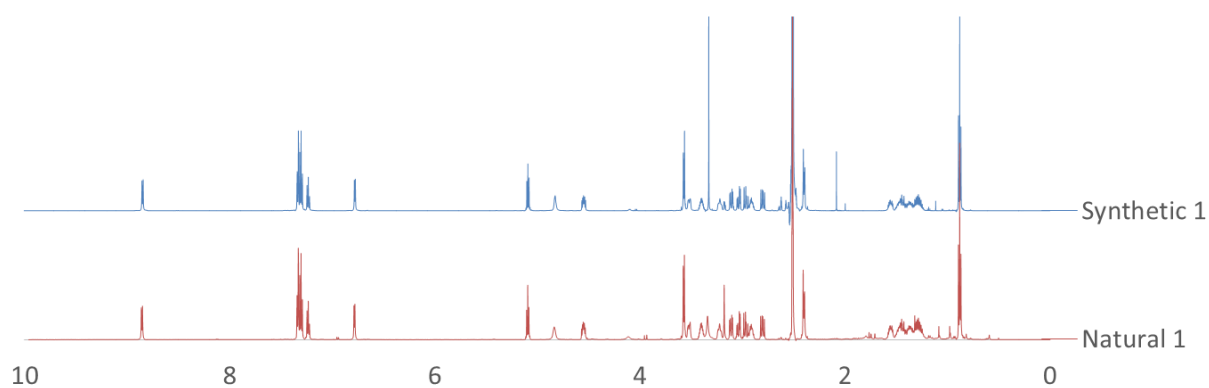


Figure S65. Comparison of the $^1$H NMR spectra of natural **1** and synthetic **1**.
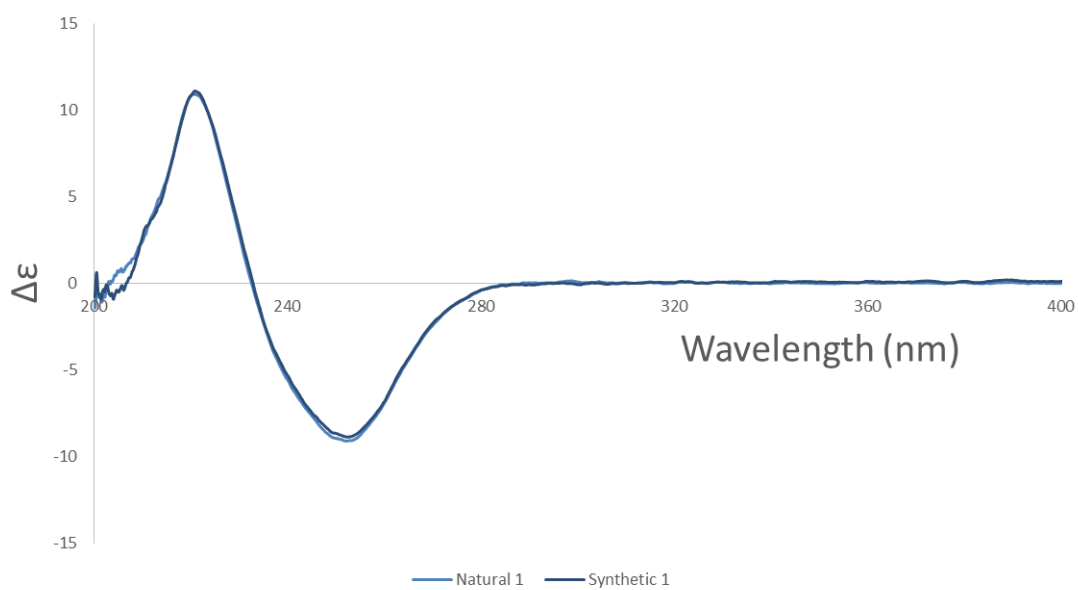


Figure S66. Comparison of the CD spectra of natural **1** and synthetic **1**.
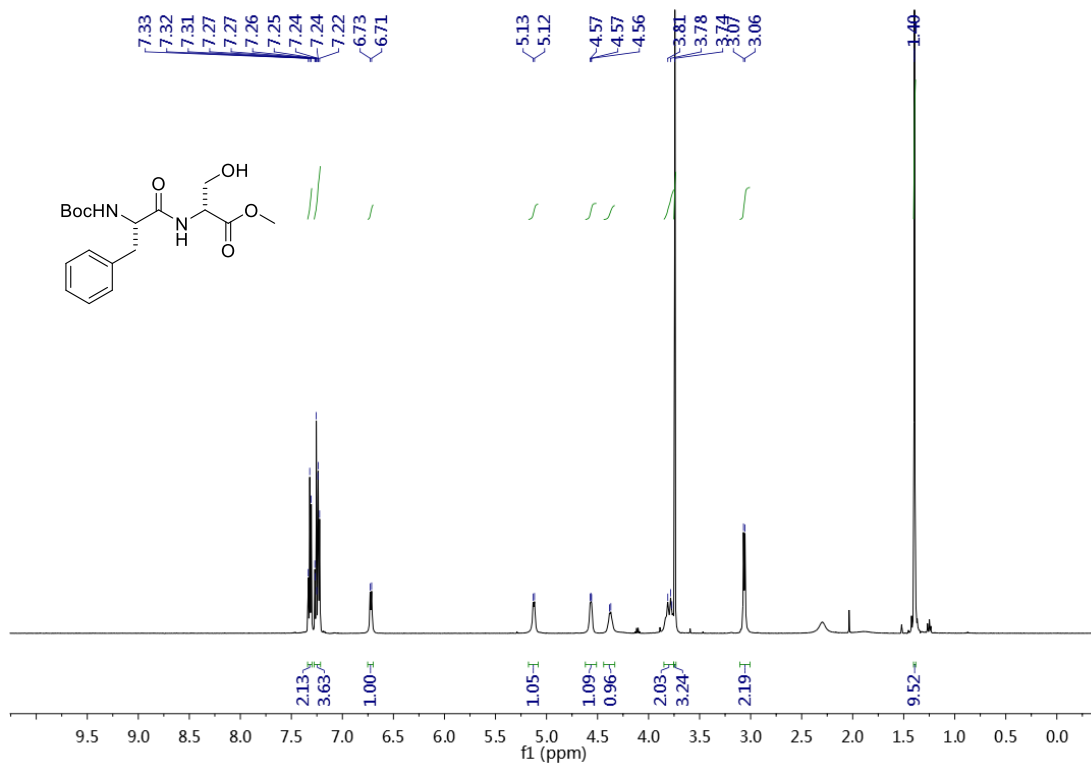
Figure S67. $^1$H NMR spectrum (500 MHz) of **33** in CDCl$_3$.
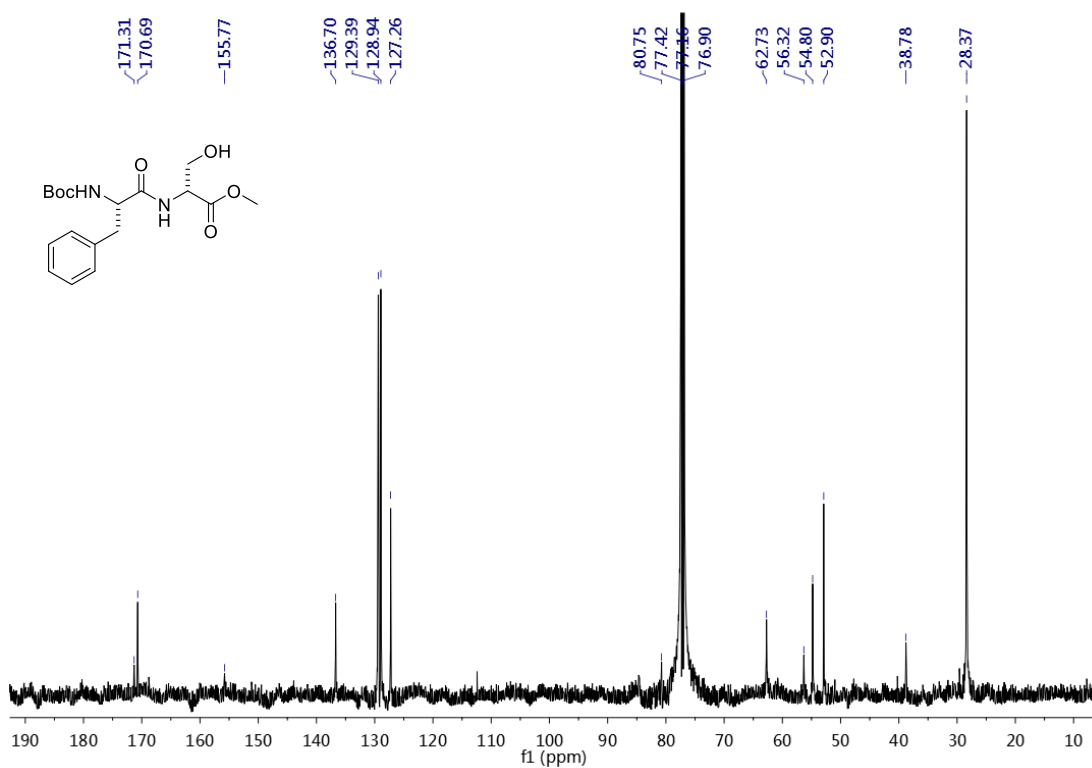


Figure S68. $^{13}$C NMR spectrum (125 MHz) of **33** in CDCl$_3$.
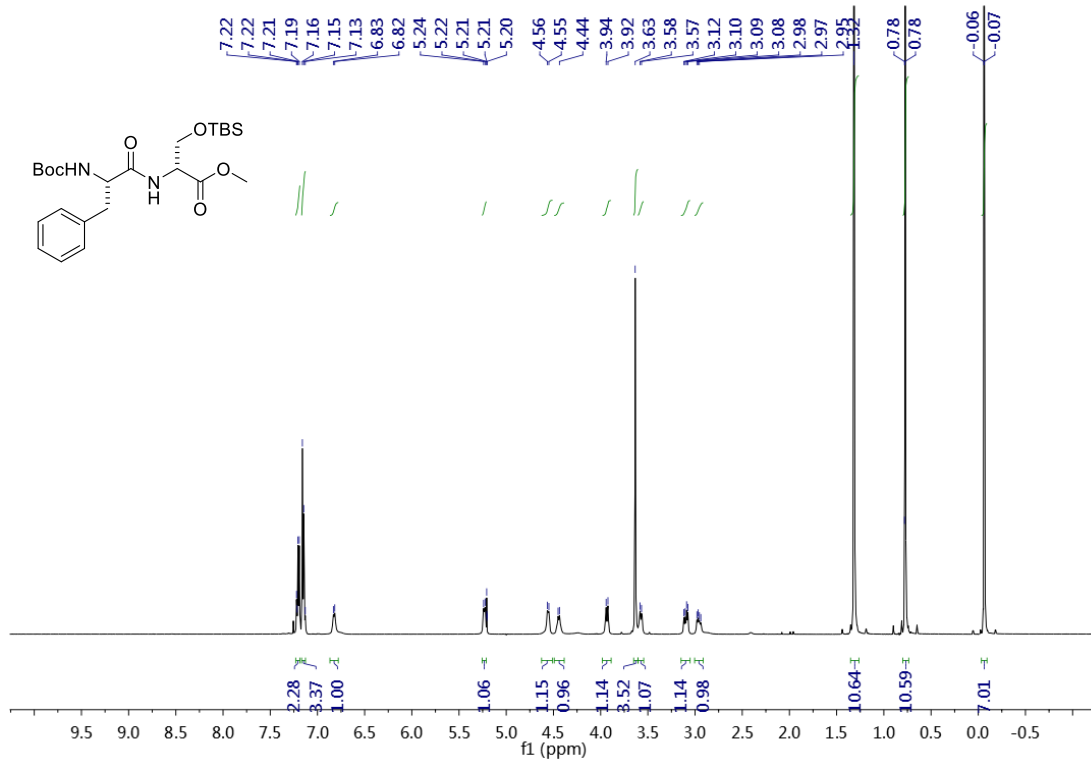
Figure S69. $^1$H NMR spectrum (500 MHz) of **34** in CDCl$_3$.
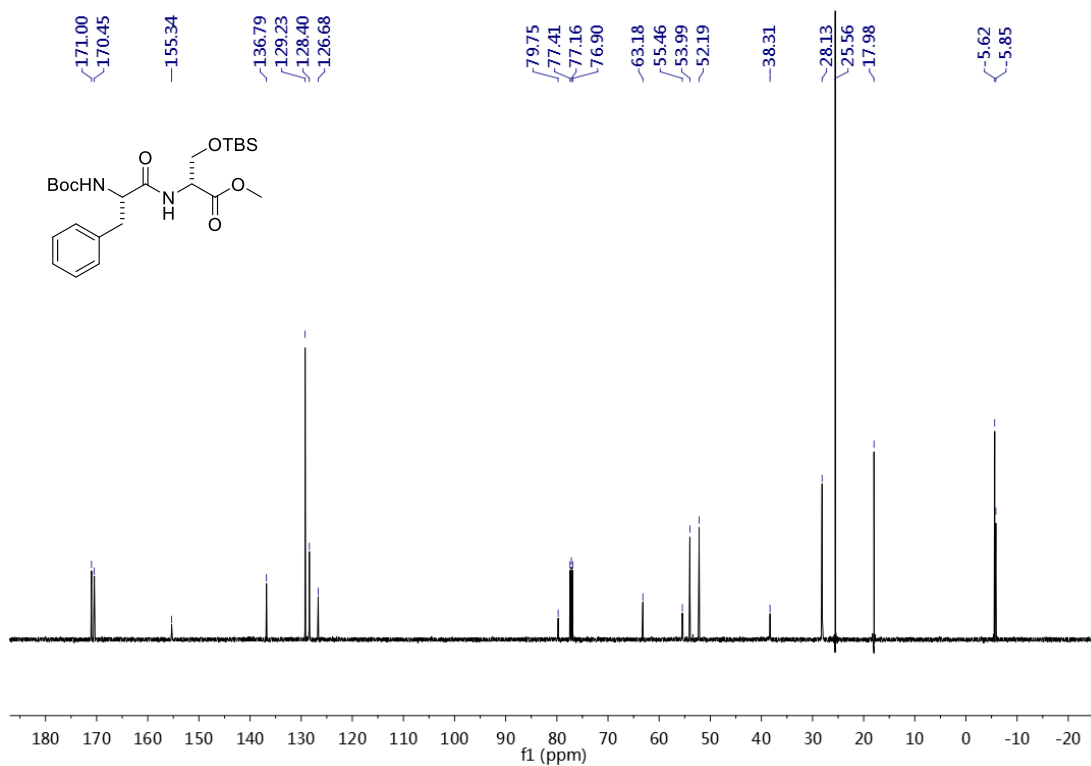


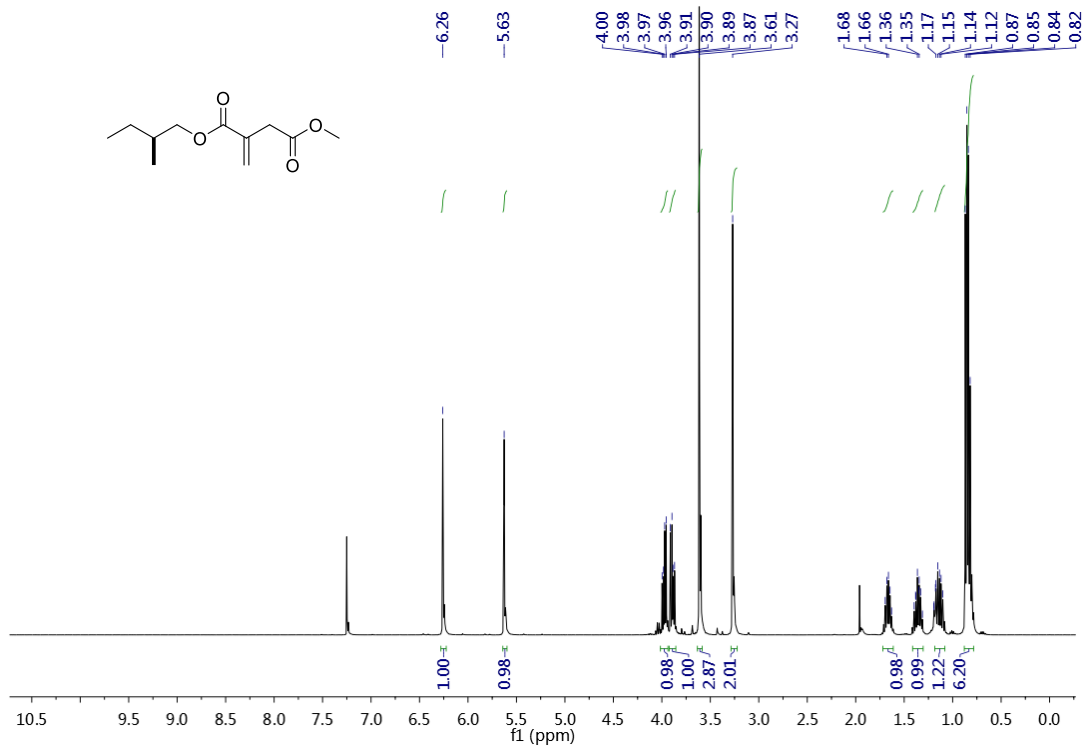Figure S70. $^{13}$C NMR spectrum (125 MHz) of **34** in CDCl$_3$.
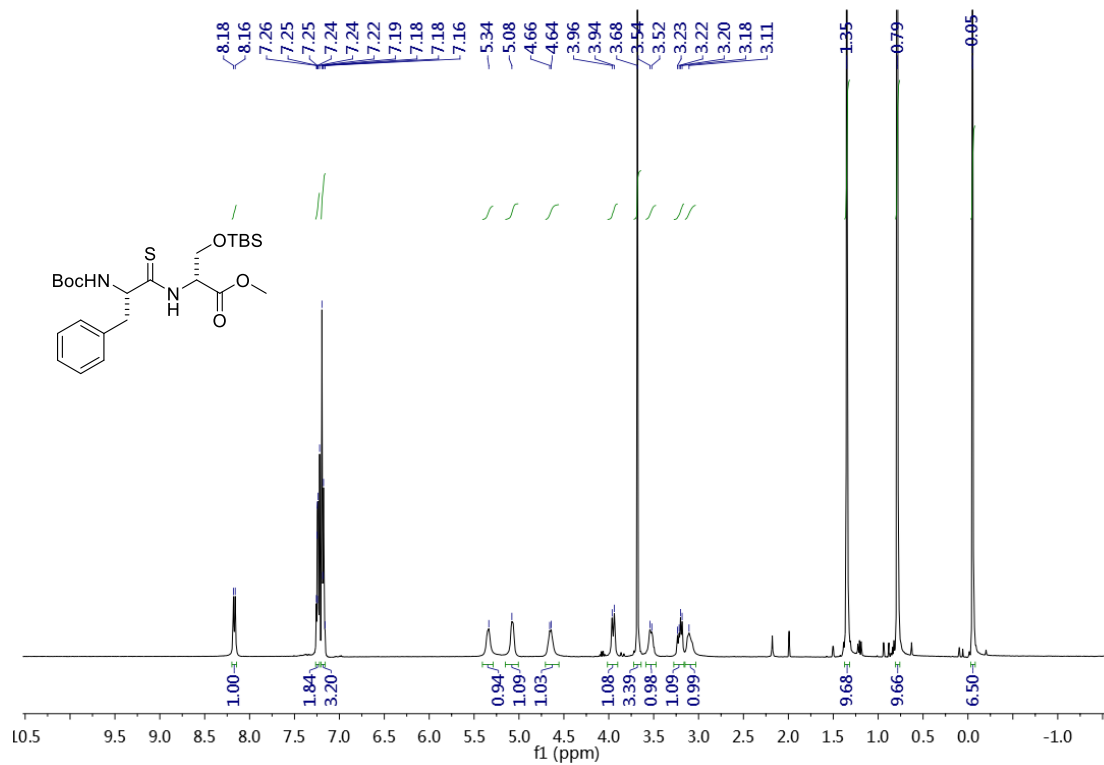
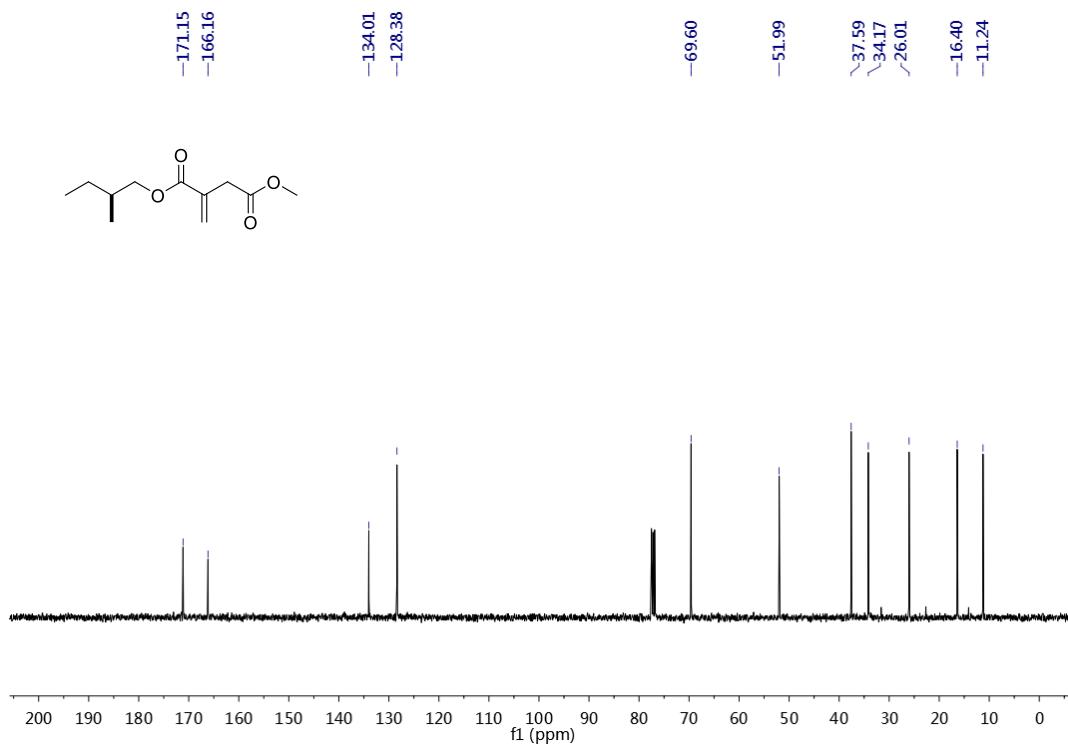Figure S71. ¹H NMR spectrum (500 MHz) of **35** in CDCl₃.
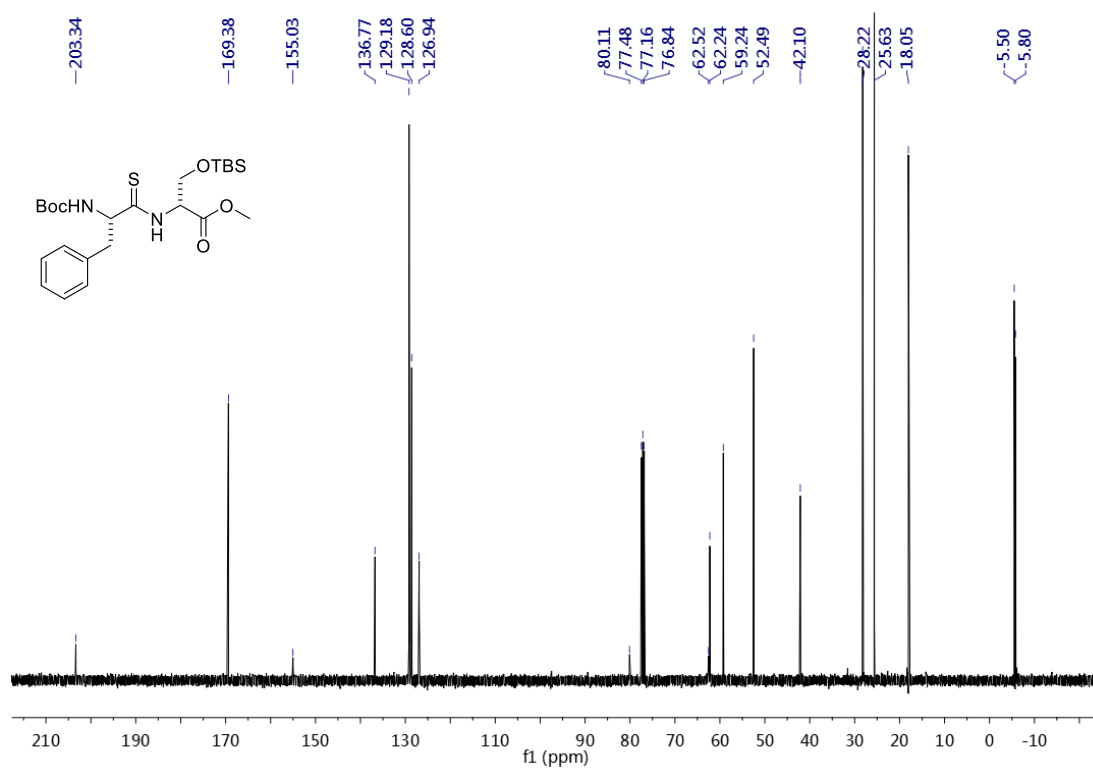

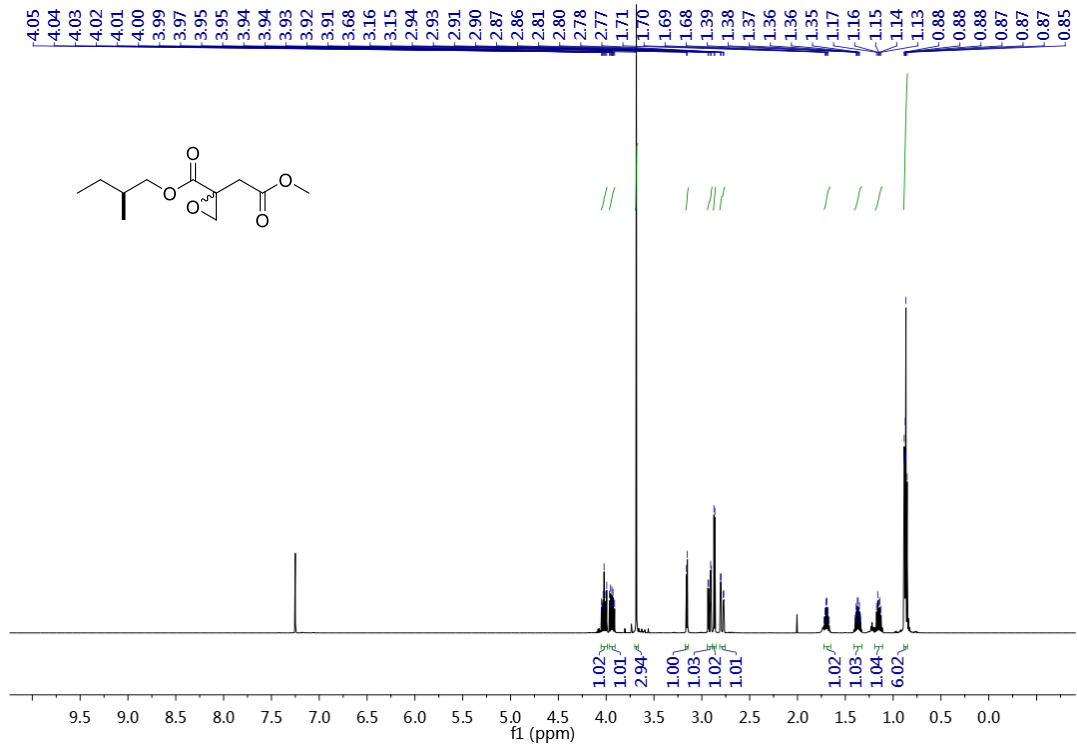
Figure S72. ¹³C NMR spectrum (125 MHz) of **35** in CDCl₃.
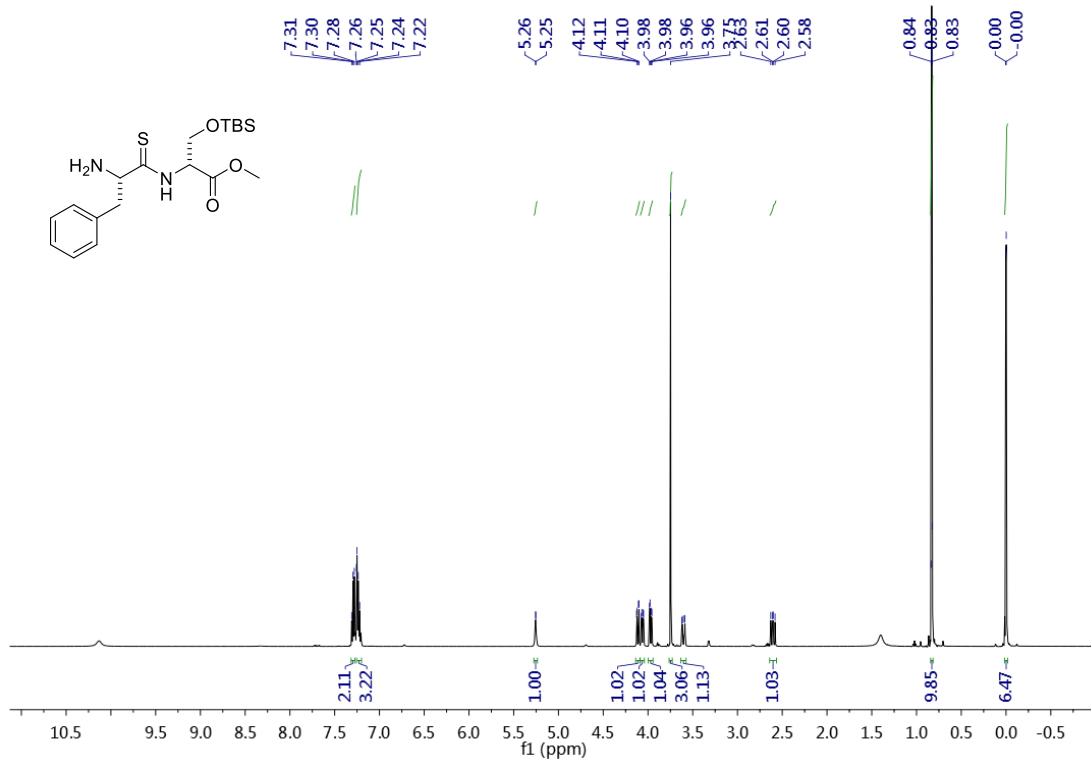
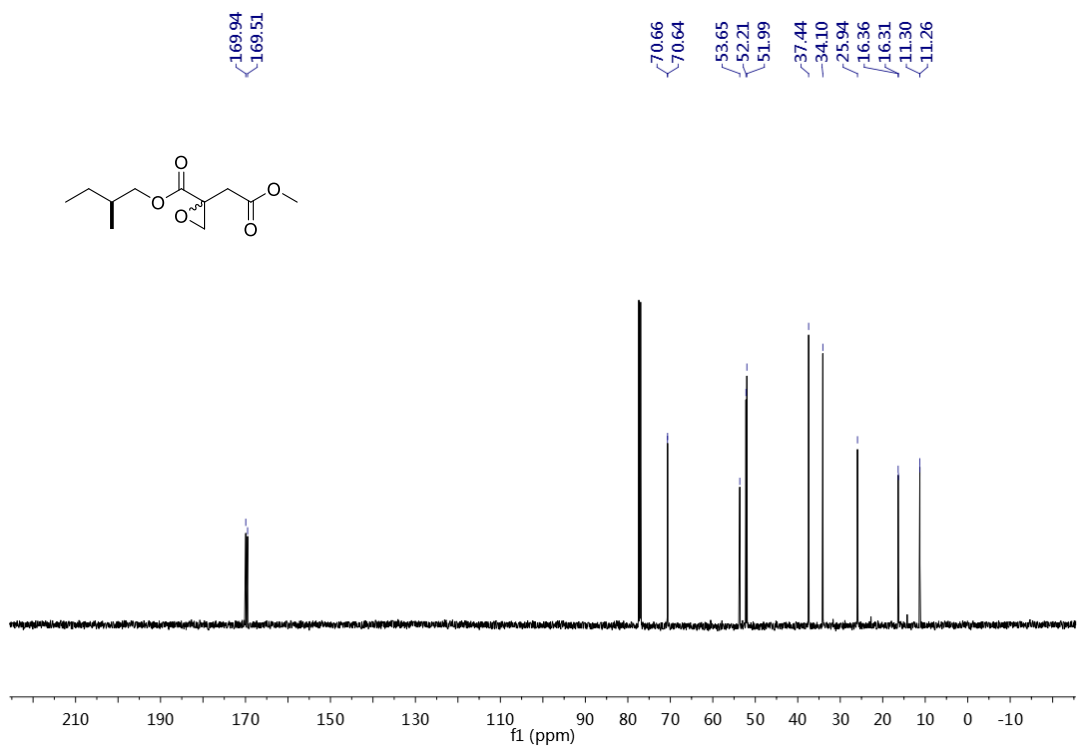Figure S73. $^1$H NMR spectrum (500 MHz) of **36** in CDCl$_3$.
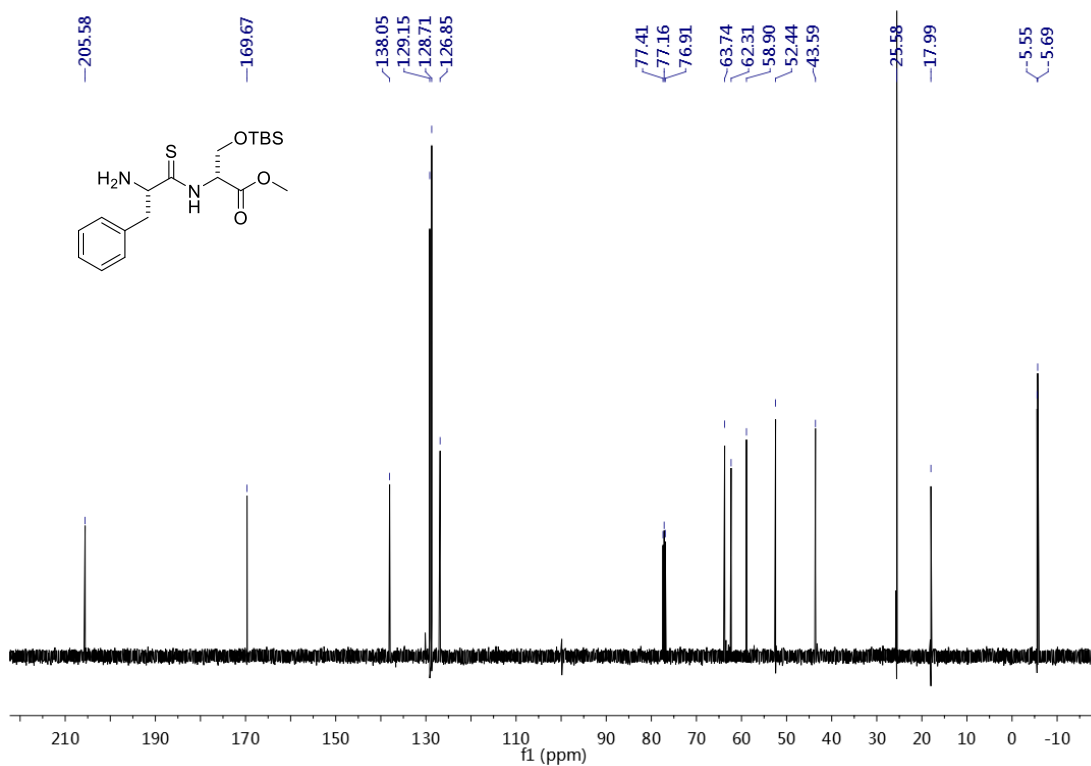


Figure S74. $^{13}$C NMR spectrum (125 MHz) of **36** in CDCl$_3$.
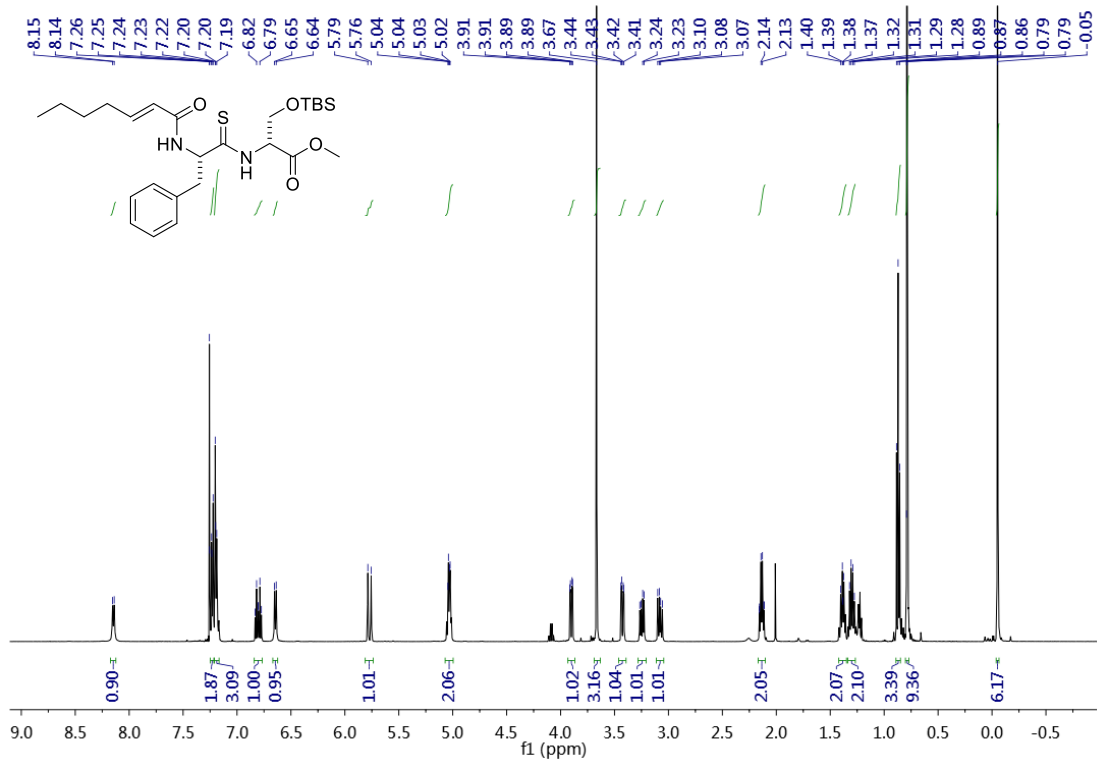
Figure S75. $^1$H NMR spectrum (500 MHz) of **37** in CDCl$_3$.
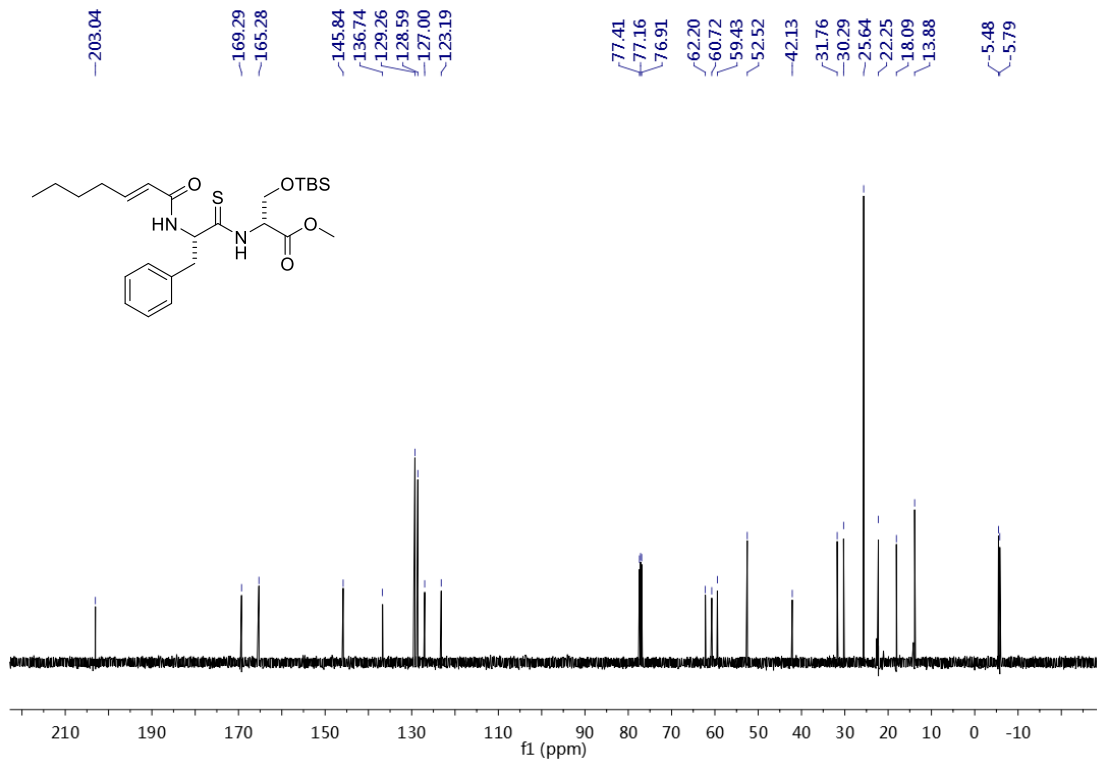


Figure S76. $^{13}$C NMR spectrum (125 MHz) of **37** in CDCl$_3$.
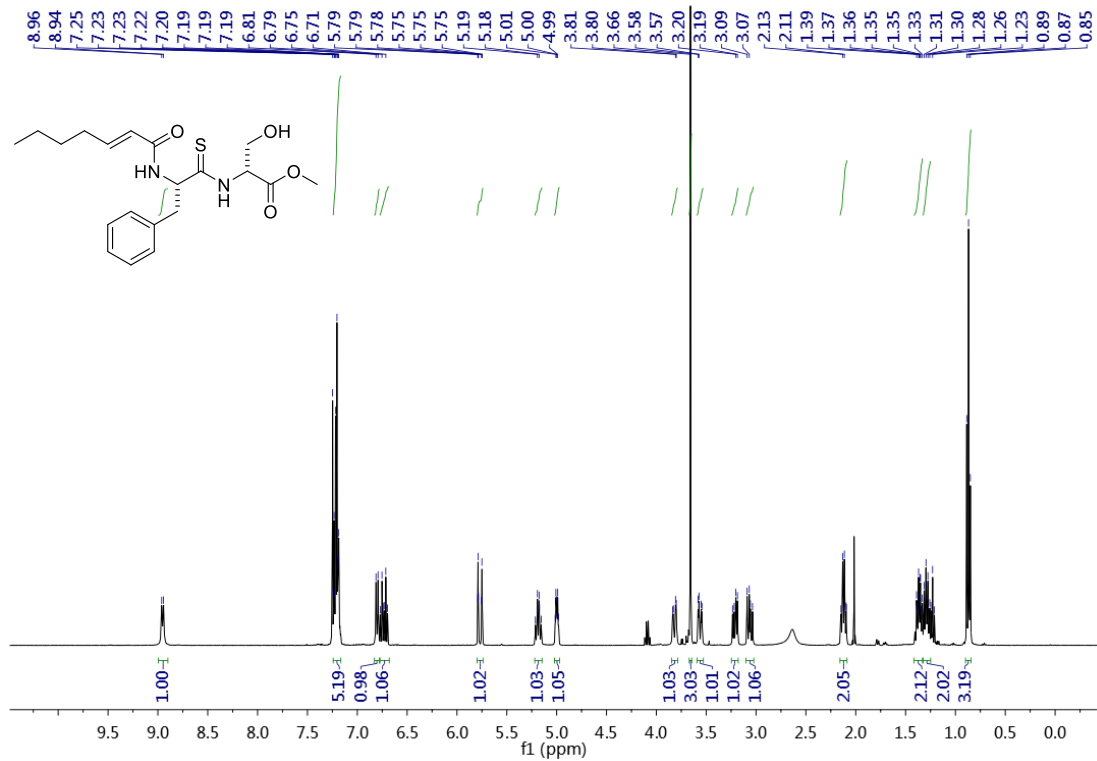
Figure S77. $^1$H NMR spectrum (500 MHz) of **38** in CDCl$_3$.
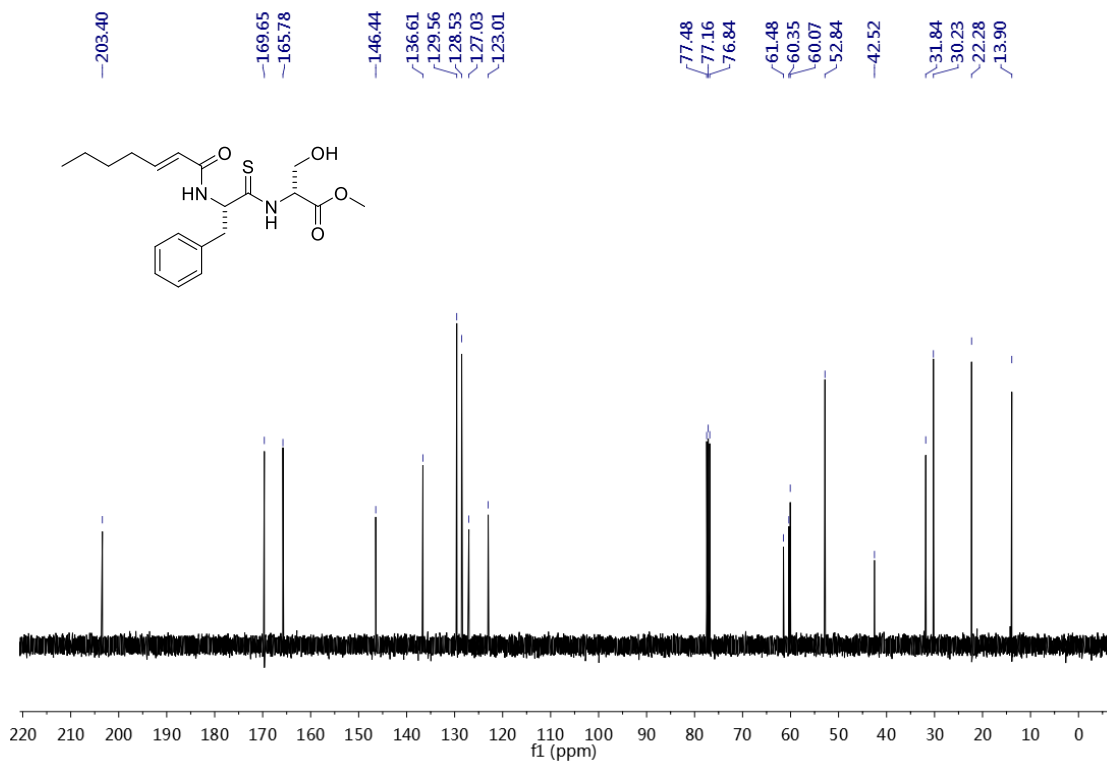


Figure S78. $^{13}$C NMR spectrum (125 MHz) of **38** in CDCl$_3$.
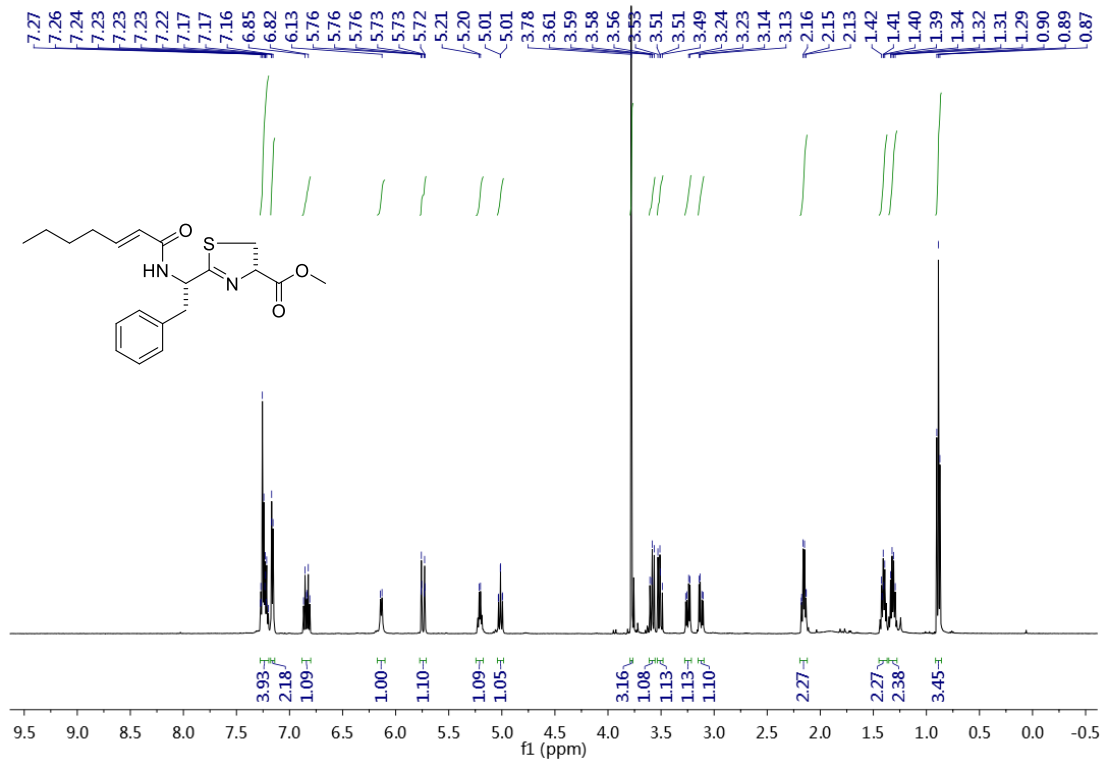
Figure S79. $^1$H NMR spectrum (500 MHz) of **39** in CDCl$_3$.
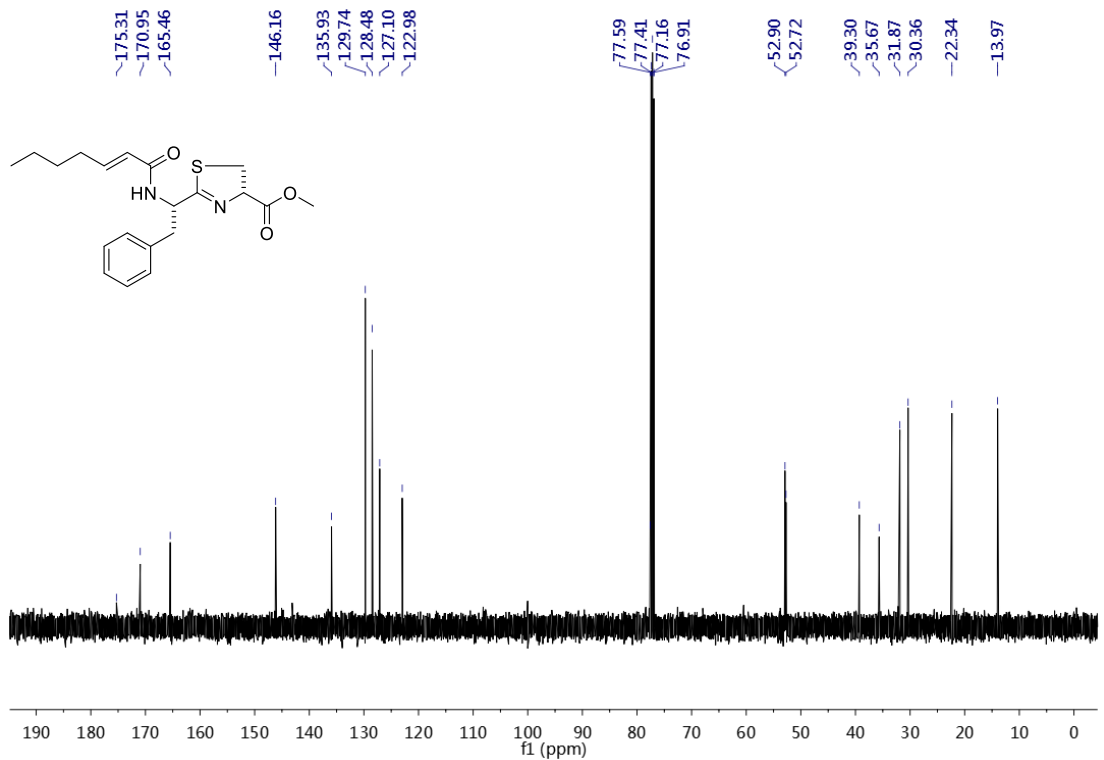


Figure S80. $^{13}$C NMR spectrum (125 MHz) of **39** in CDCl$_3$.
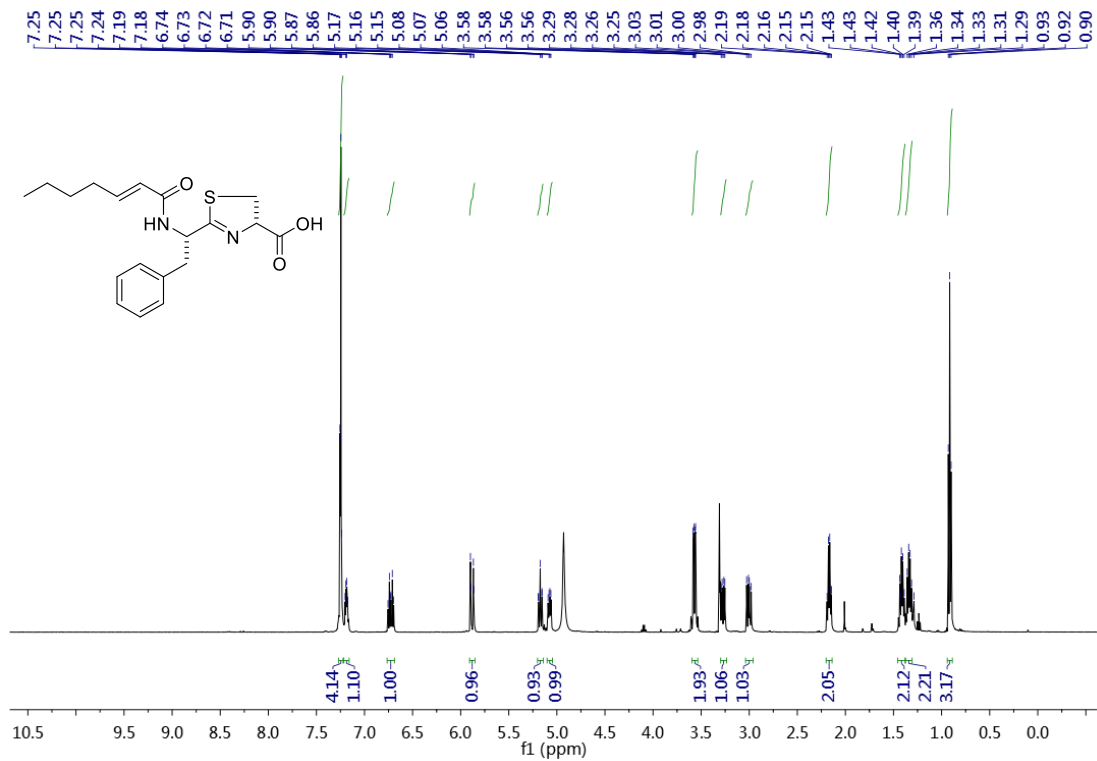
Figure S81. $^1$H NMR spectrum (500 MHz) of **40** in CD$_3$OD.
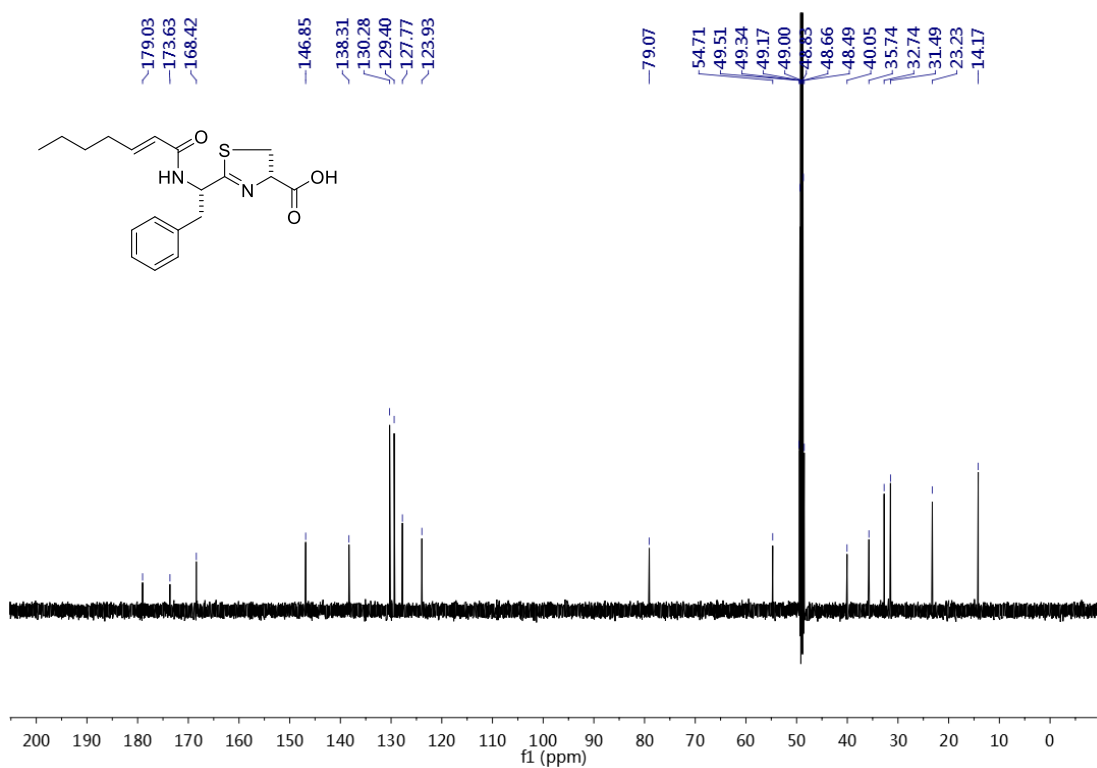


Figure S82. $^{13}$C NMR spectrum (125 MHz) of **40** in CD$_3$OD.
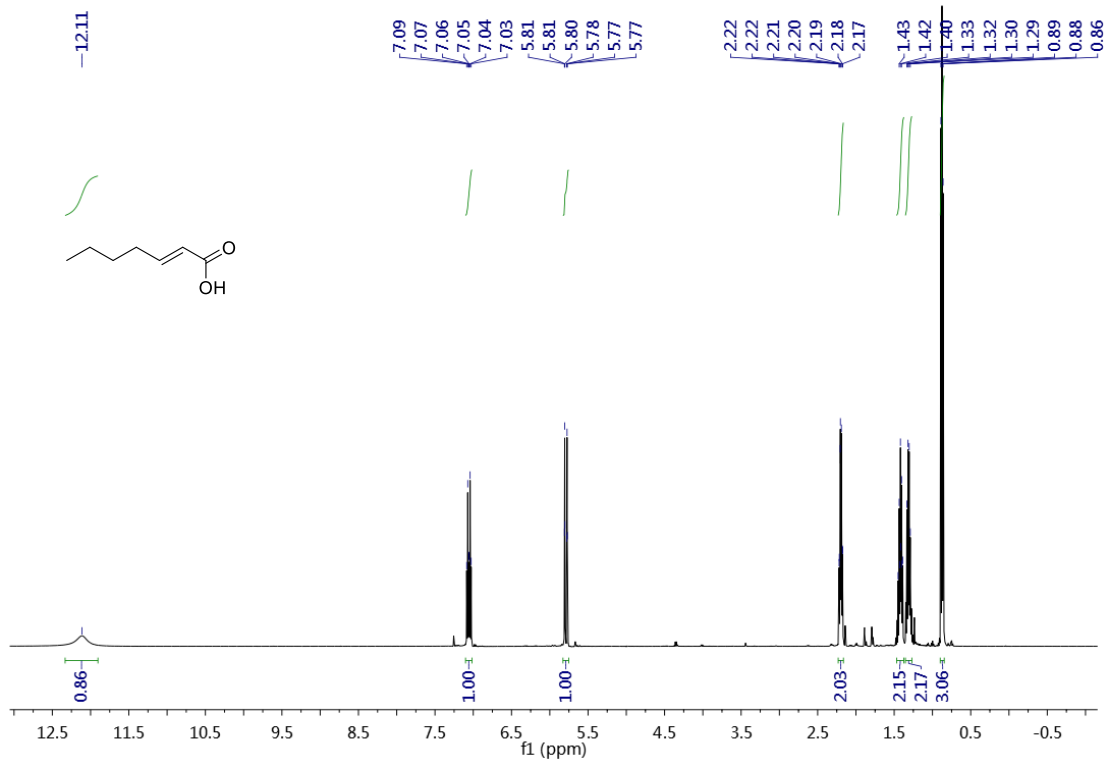
Figure S83. $^1$H NMR spectrum (500 MHz) of **17** in CDCl$_3$.
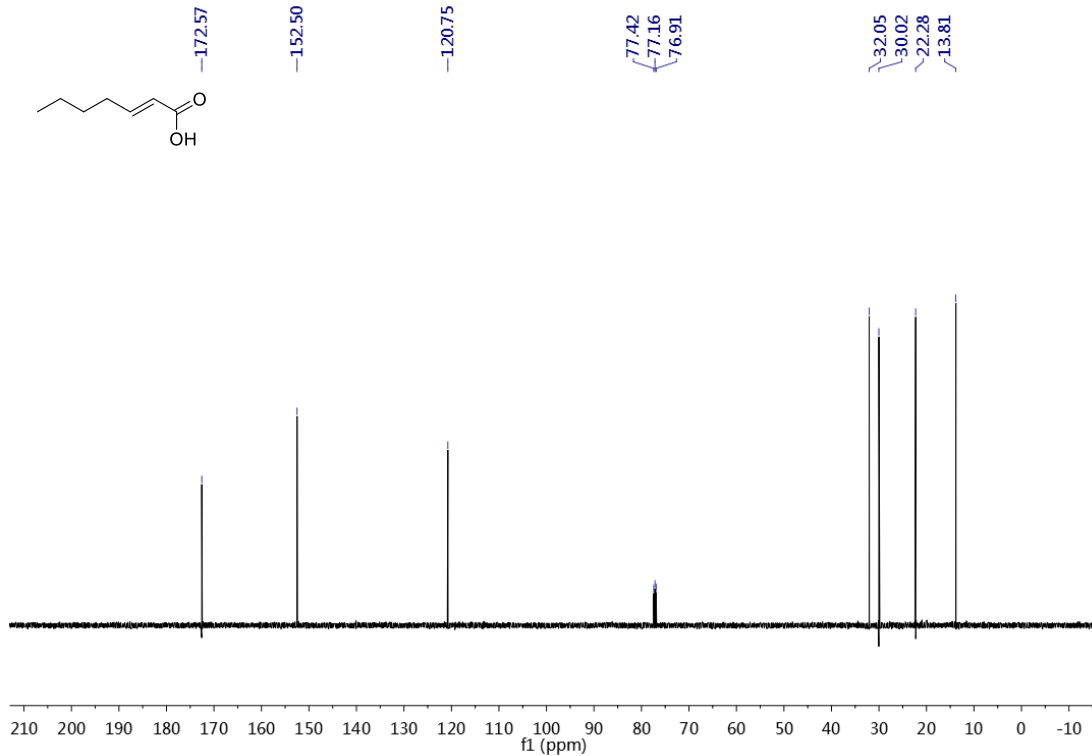


Figure S84. $^{13}$C NMR spectrum (125 MHz) of **17** in CDCl$_3$.
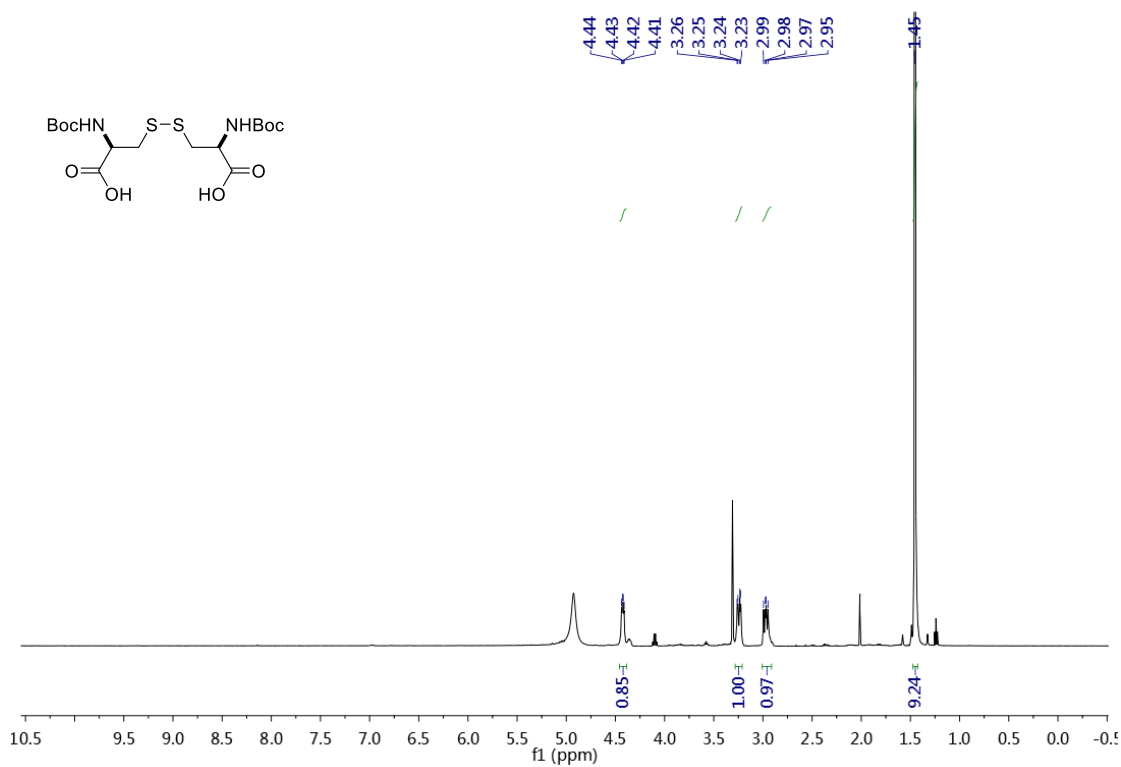
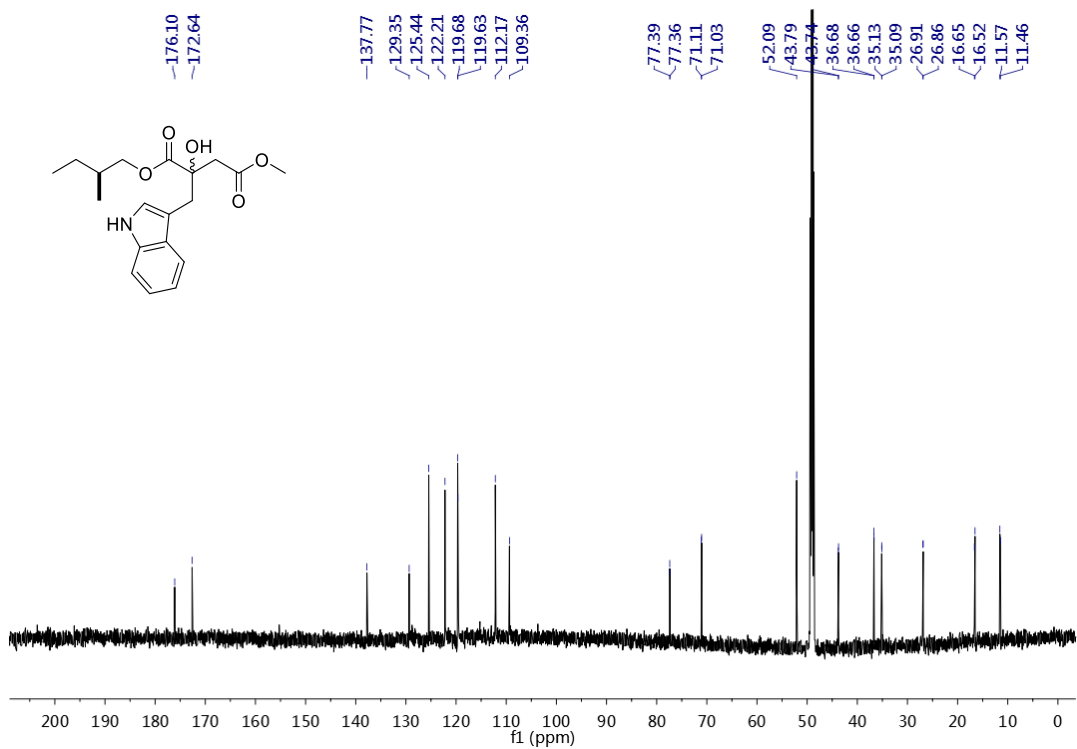Figure S85. $^1$H NMR spectrum (500 MHz) of **41** in CD$_3$OD.
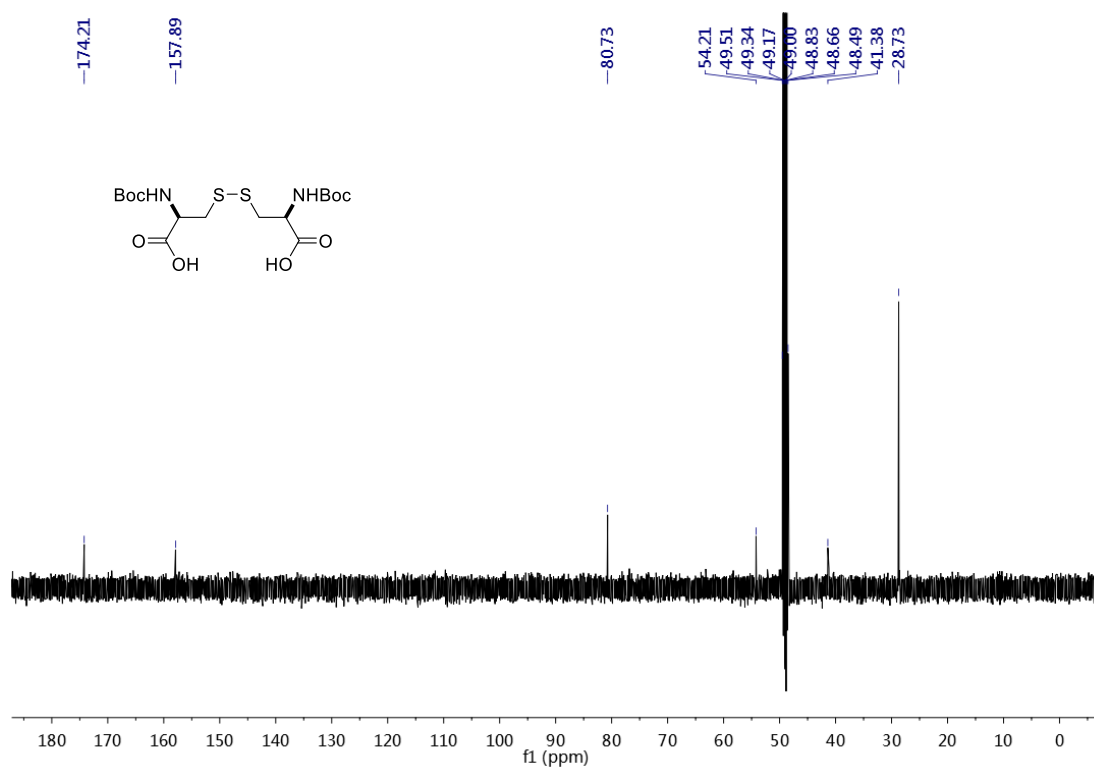


Figure S86. $^{13}$C NMR spectrum (125 MHz) of **41** in CD$_3$OD.
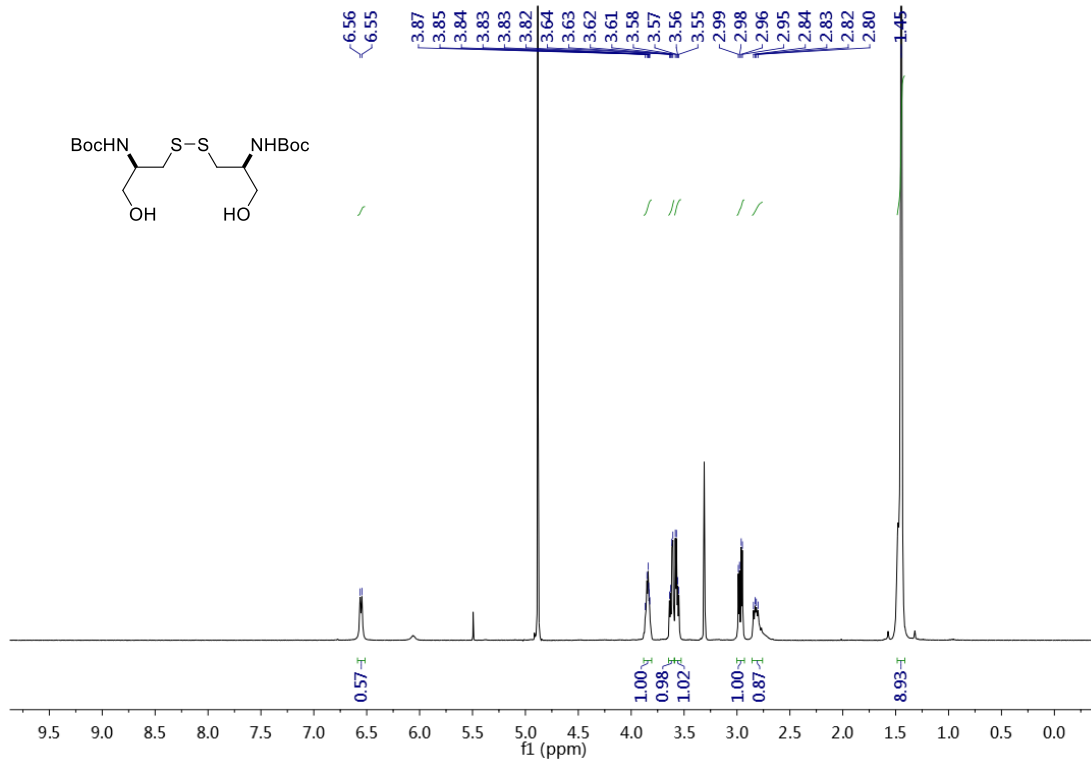
Figure S87. $^1$H NMR spectrum (500 MHz) of **42** in CD$_3$OD.
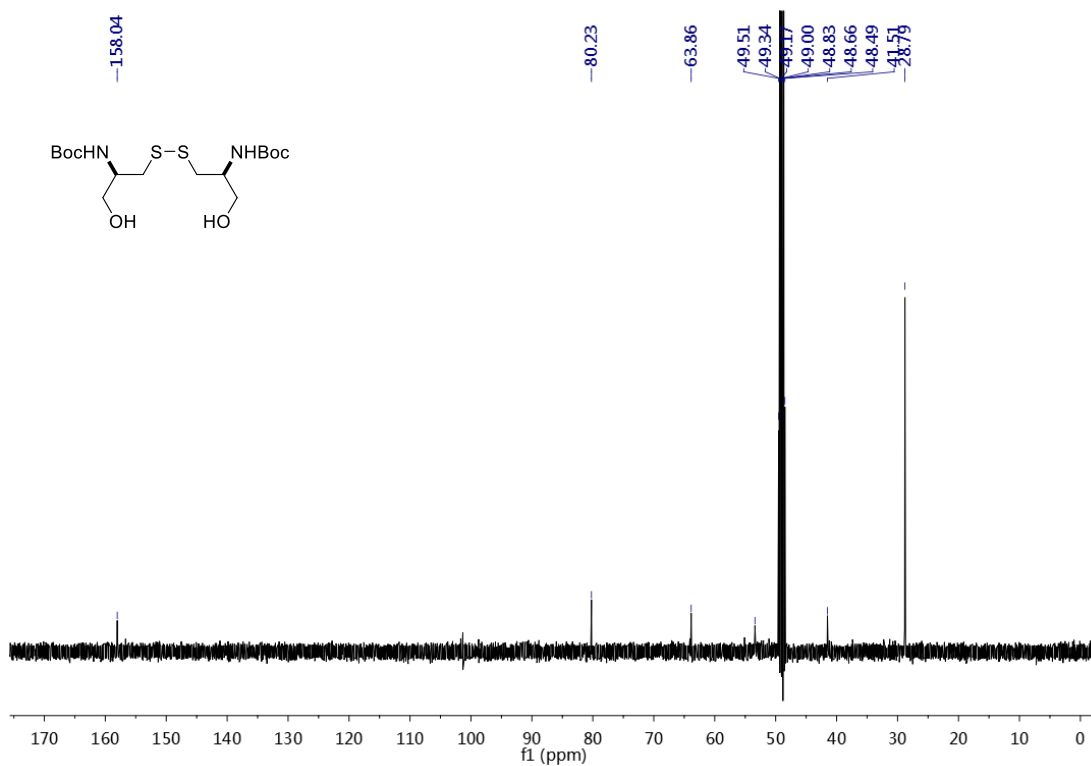


Figure S88. $^{13}$C NMR spectrum (125 MHz) of **42** in CD$_3$OD.
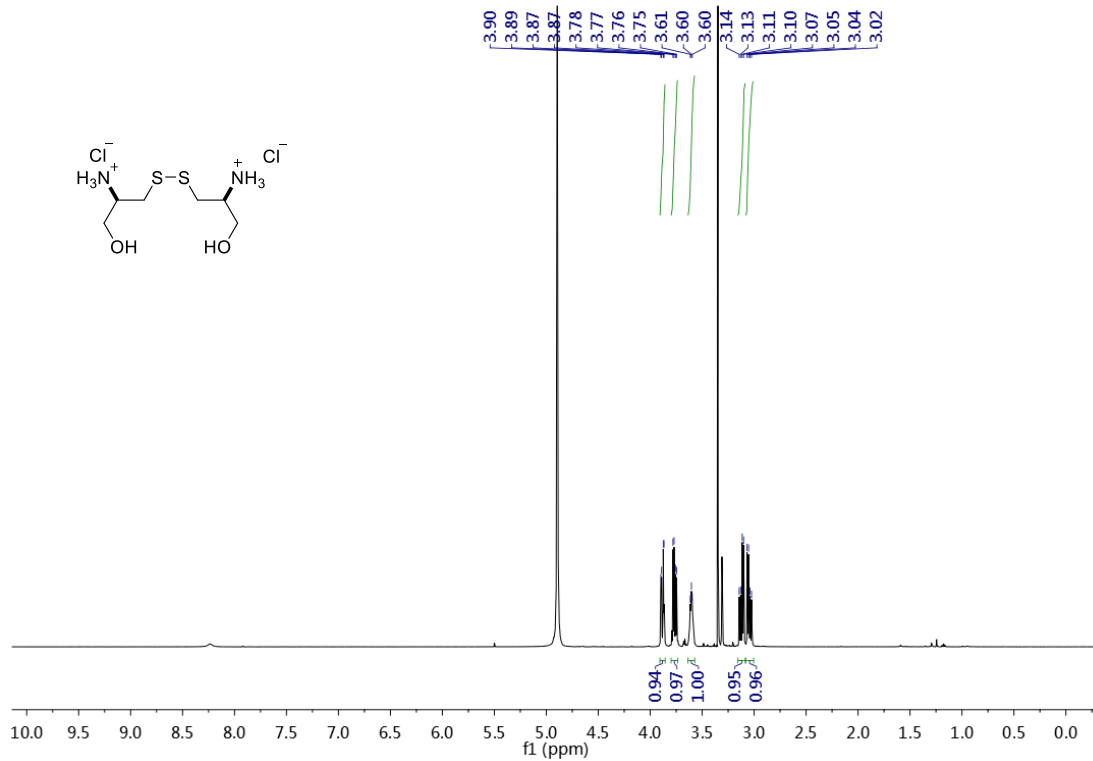
Figure S89. $^1$H NMR spectrum (500 MHz) of **43** in CD$_3$OD.
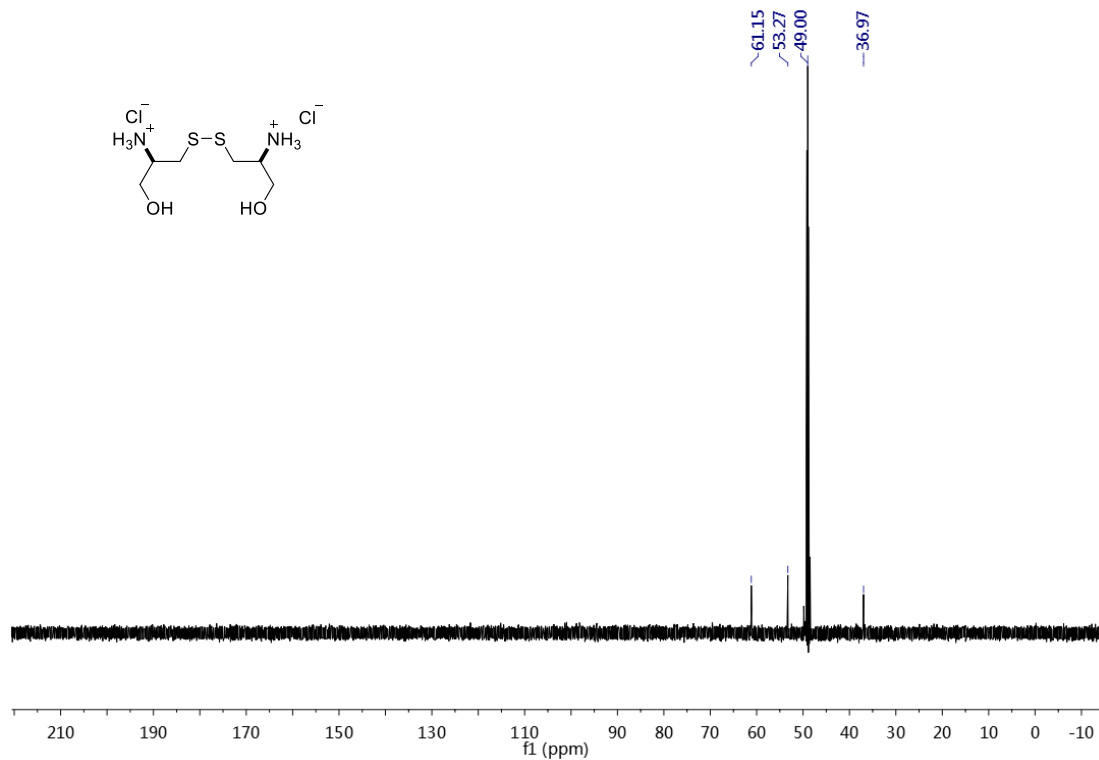


Figure S90. $^{13}$C NMR spectrum (125 MHz) of **43** in CD$_3$OD.
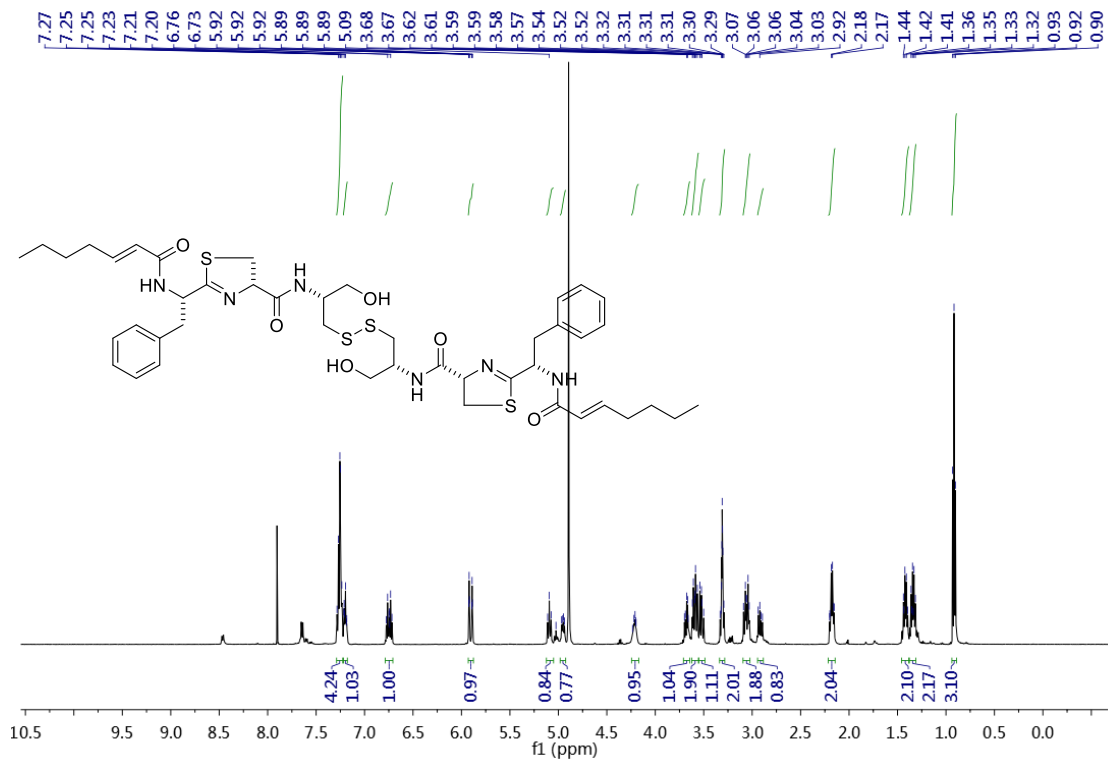
Figure S91. $^1$H NMR spectrum (500 MHz) of **44** in CD$_3$OD.
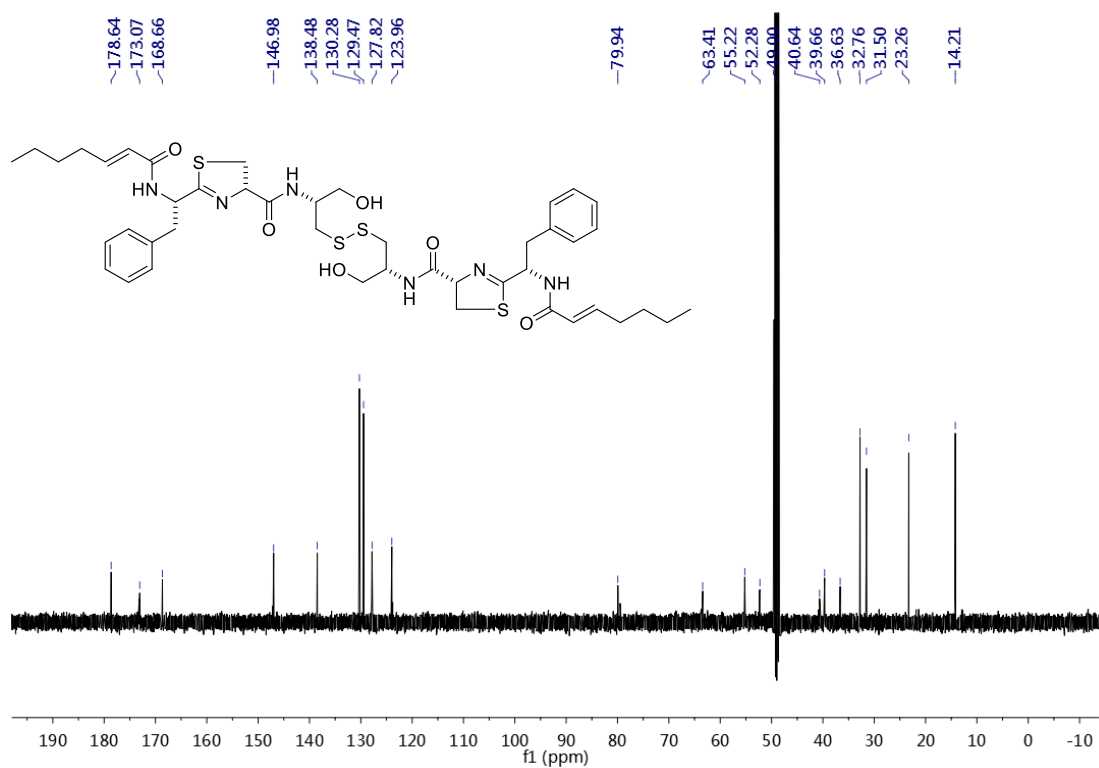


Figure S92. $^{13}$C NMR spectrum (125 MHz) of **44** in CD$_3$OD.
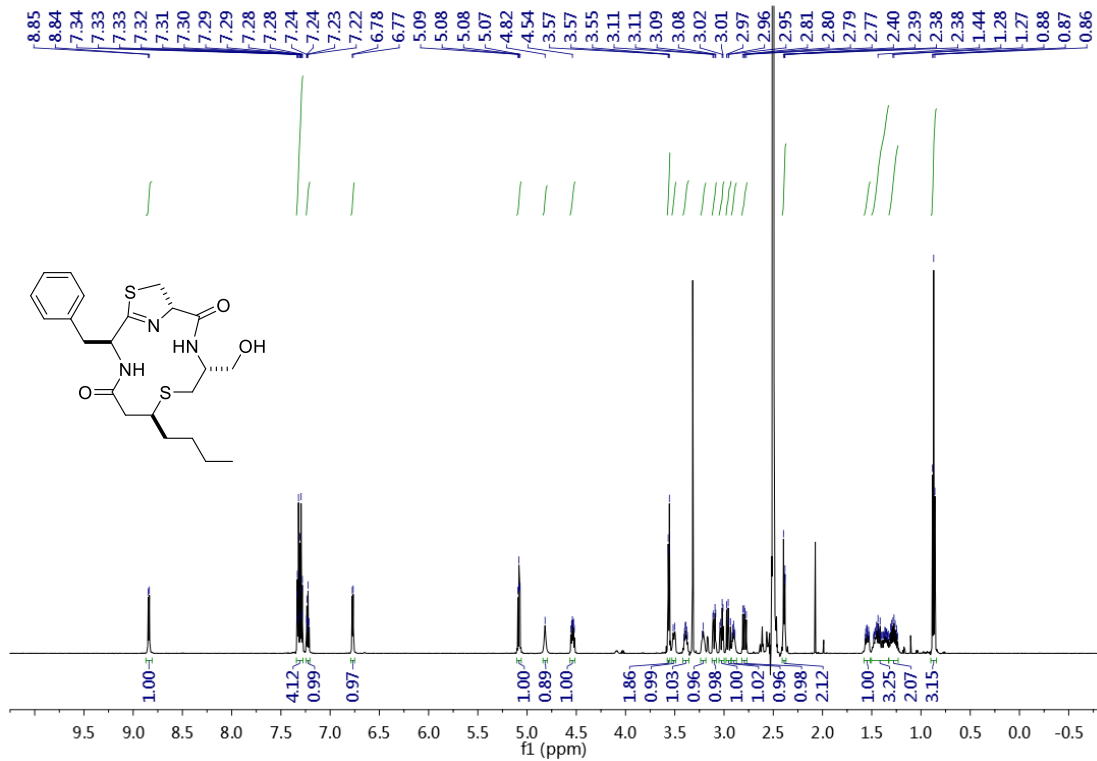
Figure S93. $^1$H NMR spectrum (600 MHz) of synthetic **1** in DMSO-$d_6$.
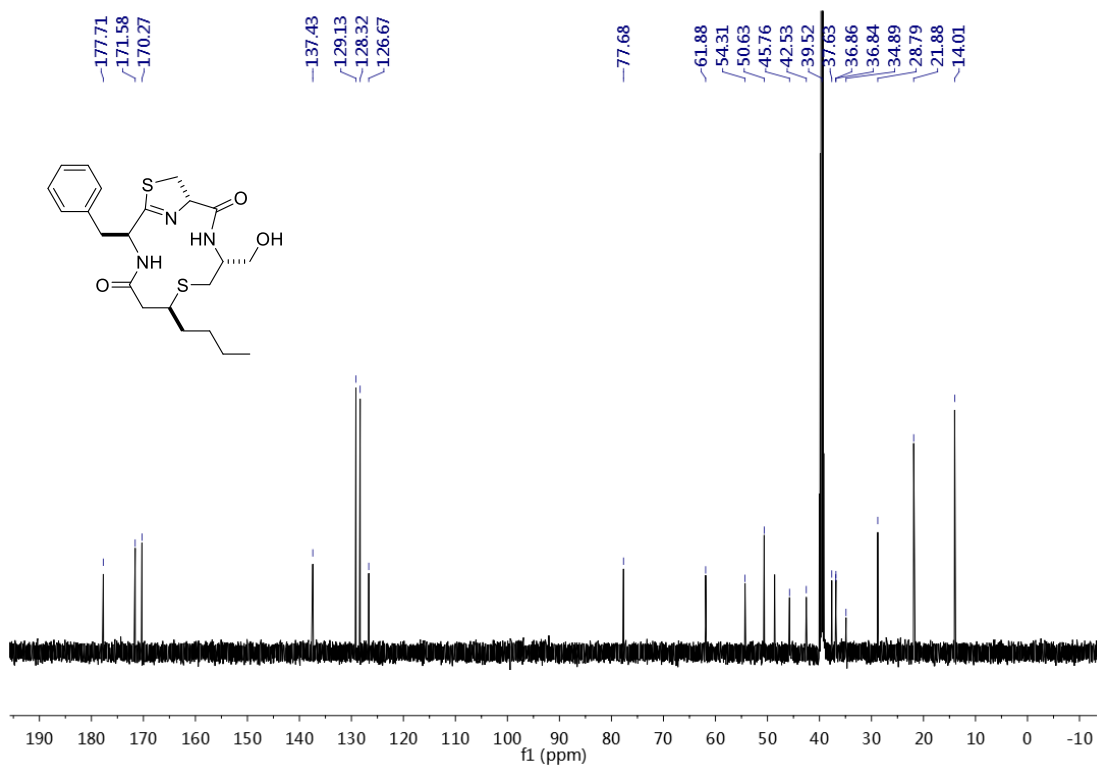


Figure S94. $^{13}$C NMR spectrum (150 MHz) of synthetic **1** in DMSO-$d_6$.
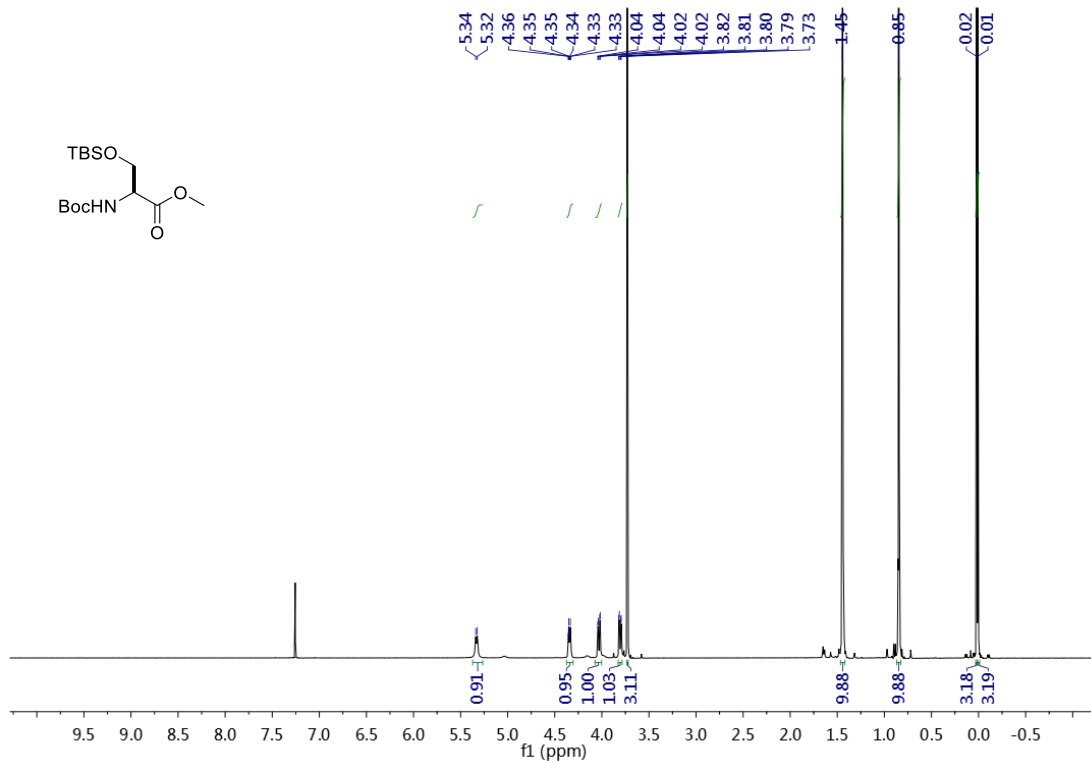
Figure S95. $^1$H NMR spectrum (500 MHz) of **55** in CDCl$_3$.
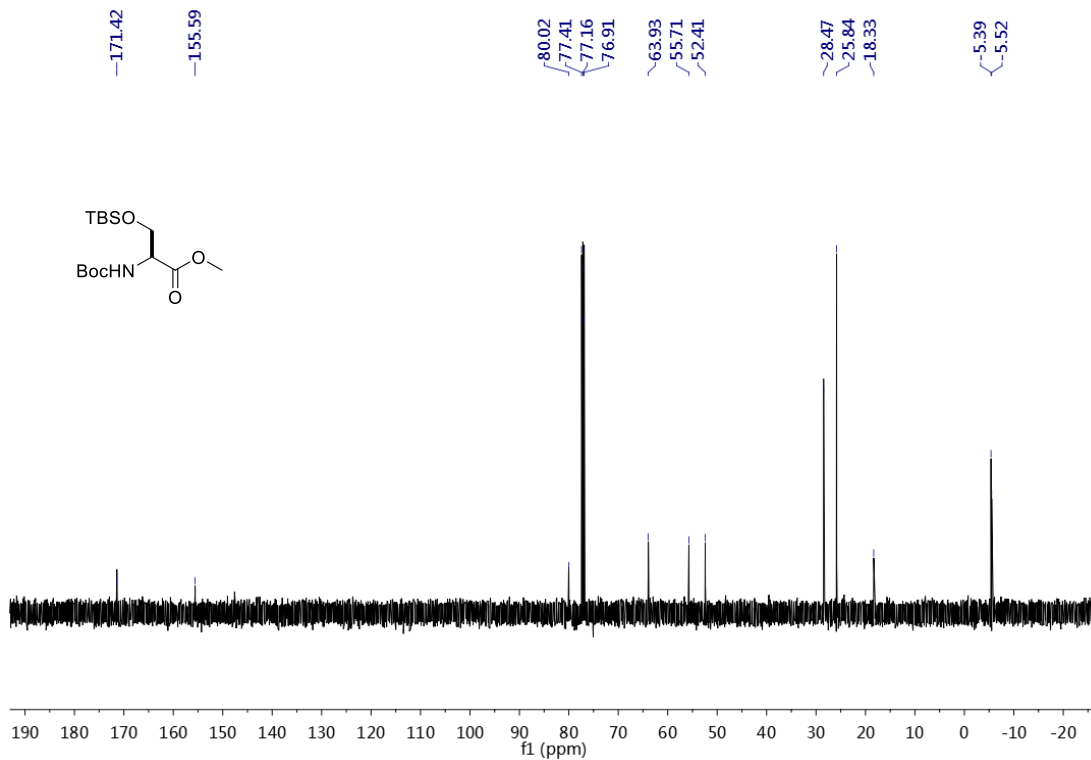


Figure S96. $^{13}$C NMR spectrum (125 MHz) of **55** in CDCl$_3$.

Figure S97. $^1$H NMR spectrum (500 MHz) of **S3** in CDCl$_3$.



Figure S98. $^{13}$C NMR spectrum (125 MHz) of **S3** in CDCl$_3$.
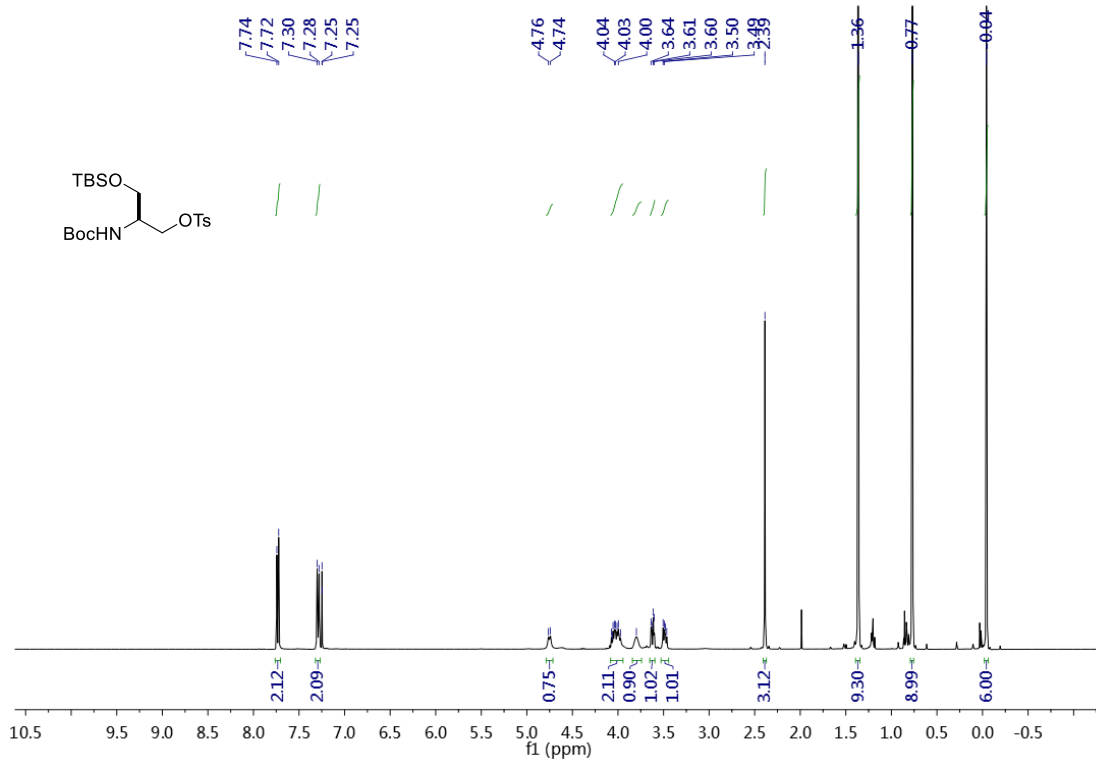
Figure S99. $^1$H NMR spectrum (500 MHz) of **56** in CDCl$_3$.
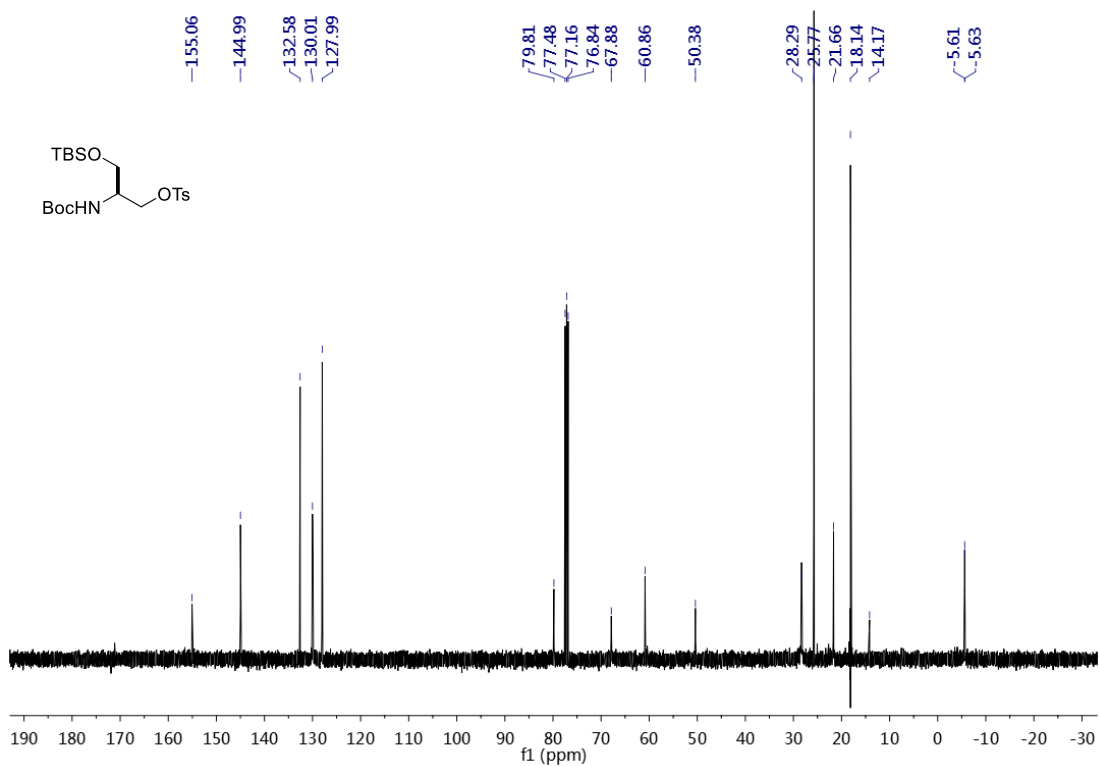


Figure S100. $^{13}$C NMR spectrum (125 MHz) of **56** in CDCl$_3$.
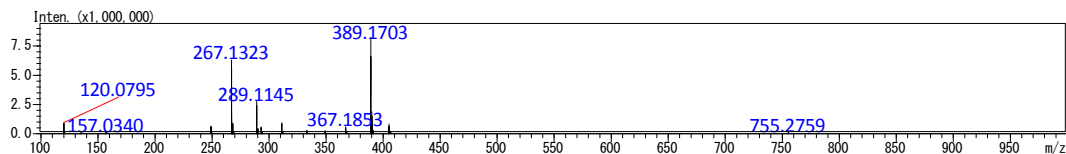
Figure S101. HRMS for compound **33**.
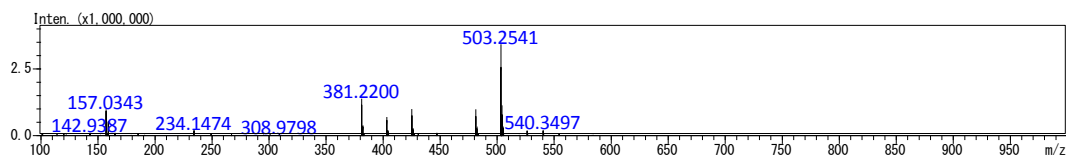


Figure S102. HRMS for compound **34**.
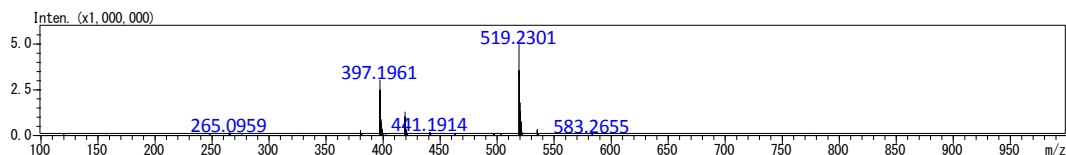


Figure S103. HRMS for compound **35**.
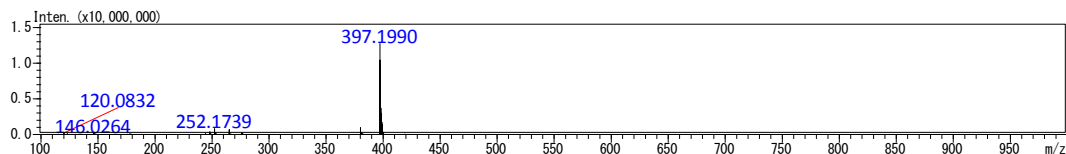


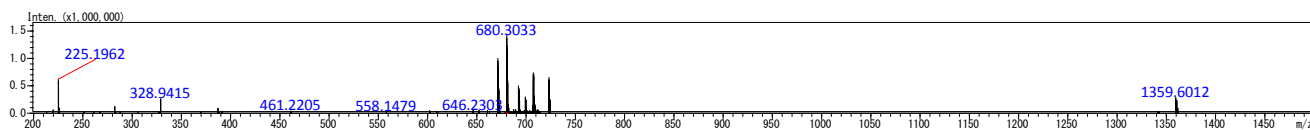Figure S104. HRMS for compound **36**.
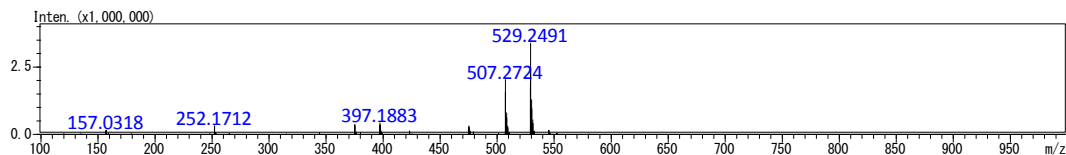


Figure S105. HRMS for compound **37**.
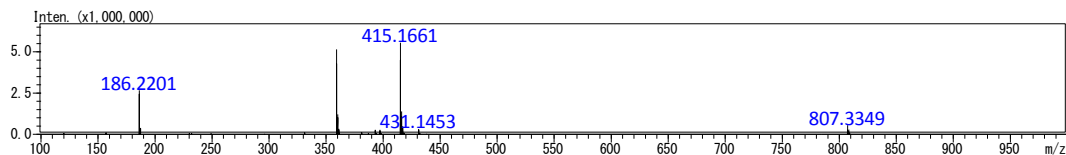


Figure S106. HRMS for compound **38**.
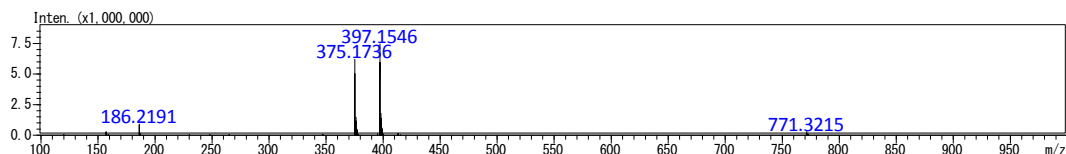


Figure S107. HRMS for compound **39**.
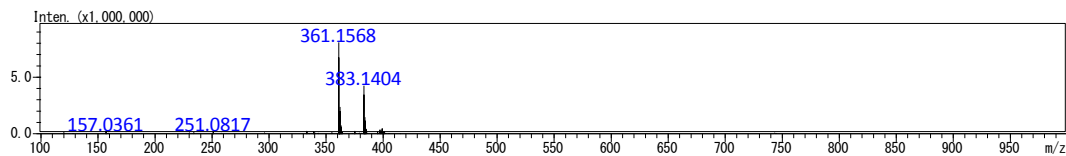
Figure S108. HRMS for compound **40**.
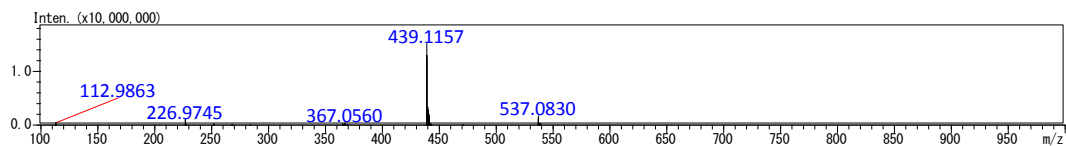


Figure S109. HRMS for compound **41**.
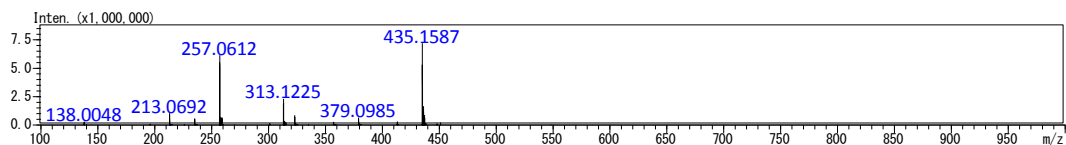


Figure S110. HRMS for compound **42**.
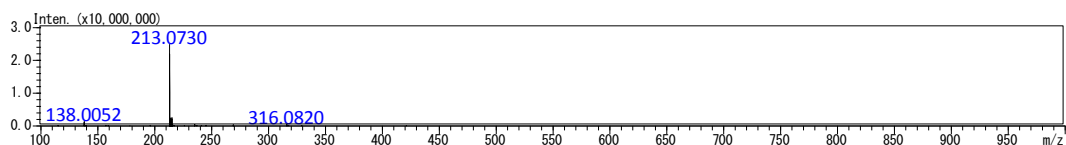


Figure S111. HRMS for compound **43**.
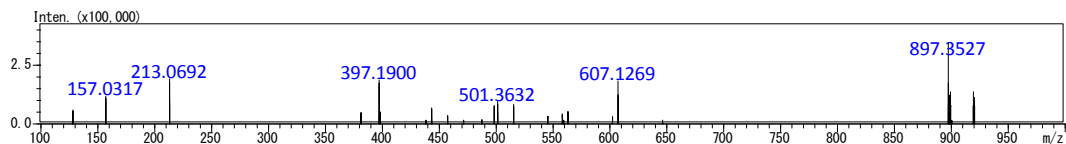


Figure S112. HRMS for compound **44**.
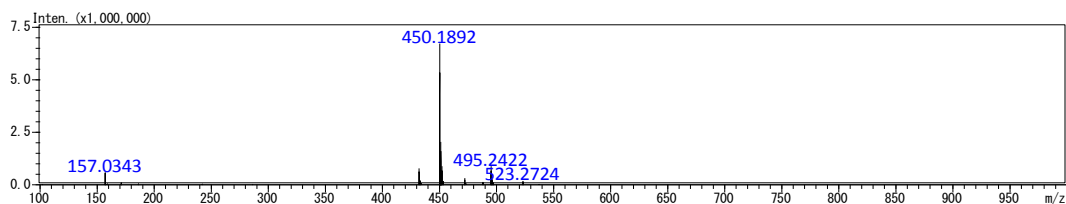


Figure S113. HRMS for synthetic **1**.
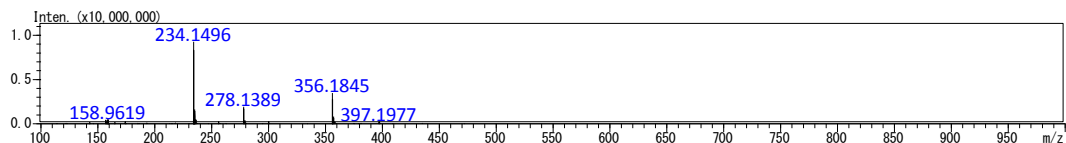


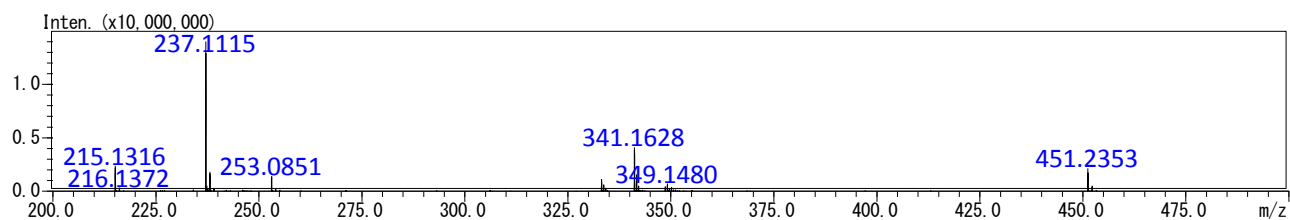Figure S114. HRMS for compound **45**.
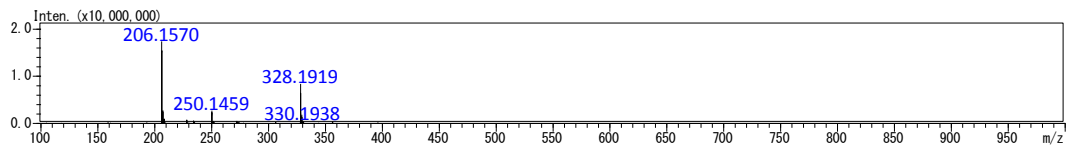
Figure S115. HRMS for compound **S3**.
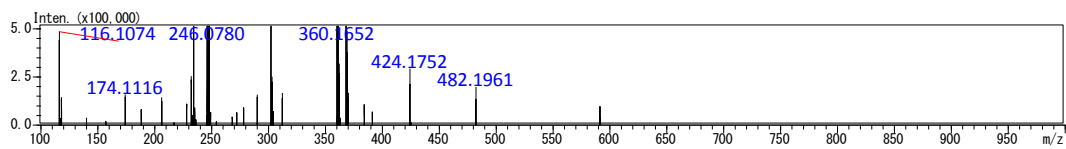


Figure S116. HRMS for compound **56**.
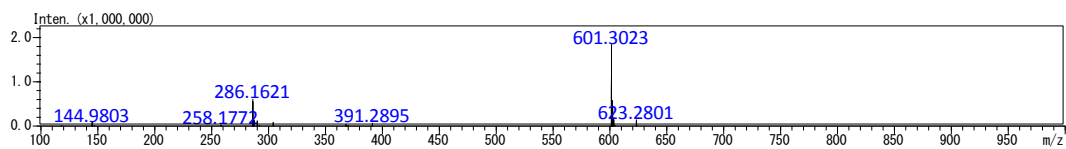


Figure S117. HRMS for compound **50**.

**Chapter 2**
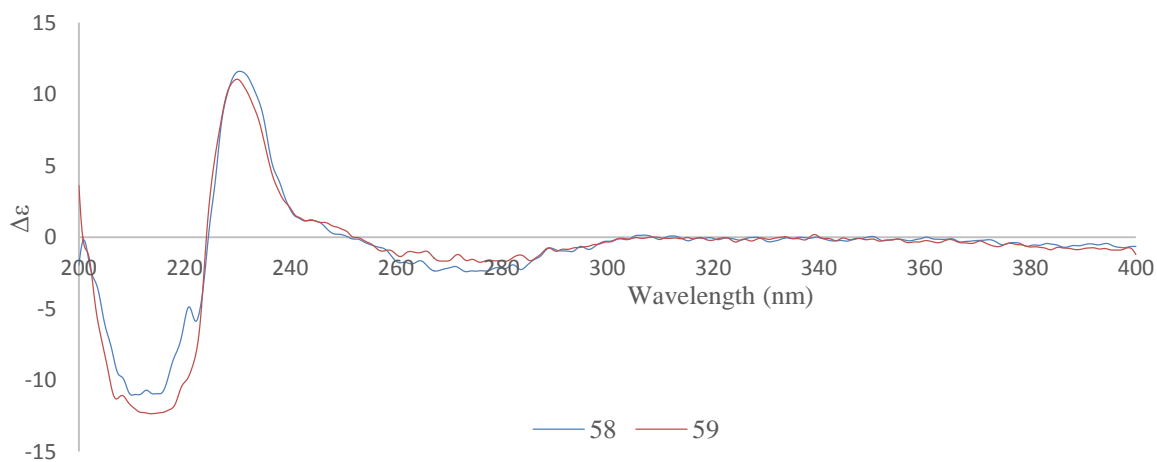


Figure S118. Comparison of the CD spectra of **58**, **59** in MeOH.



Figure S119. ¹H NMR spectrum (600 MHZ) of **58** in CD₃OH.

98
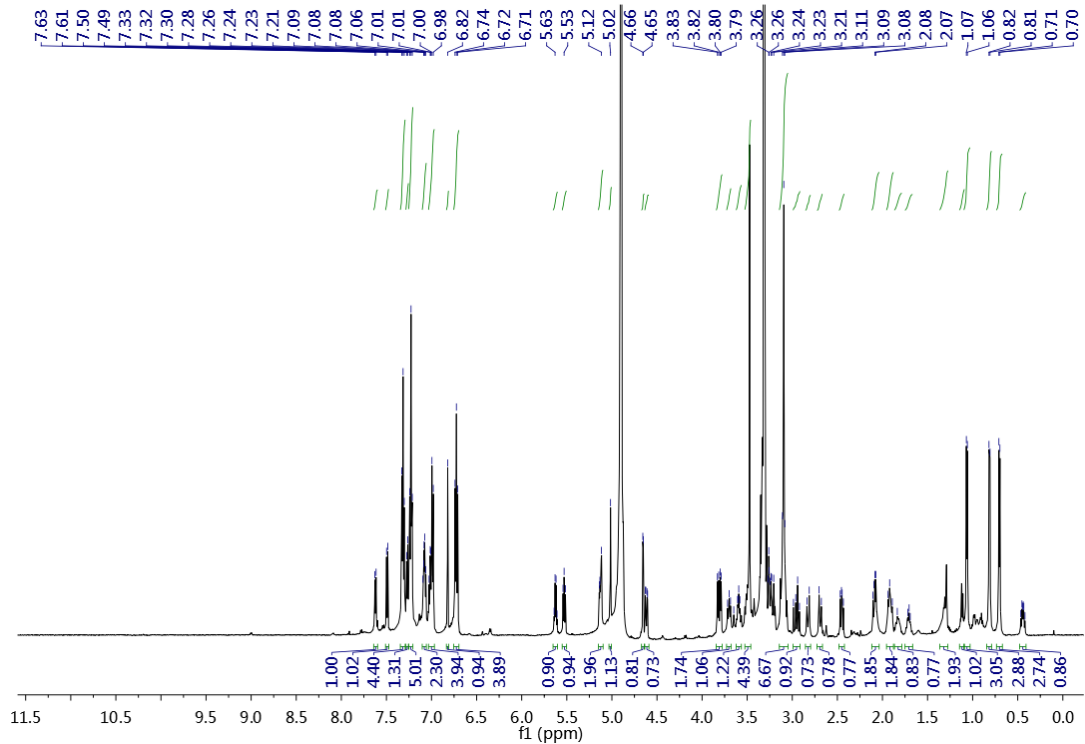
Figure S120. $^1$H NMR spectrum (600 MHZ) of **58** in CD$_3$OD.
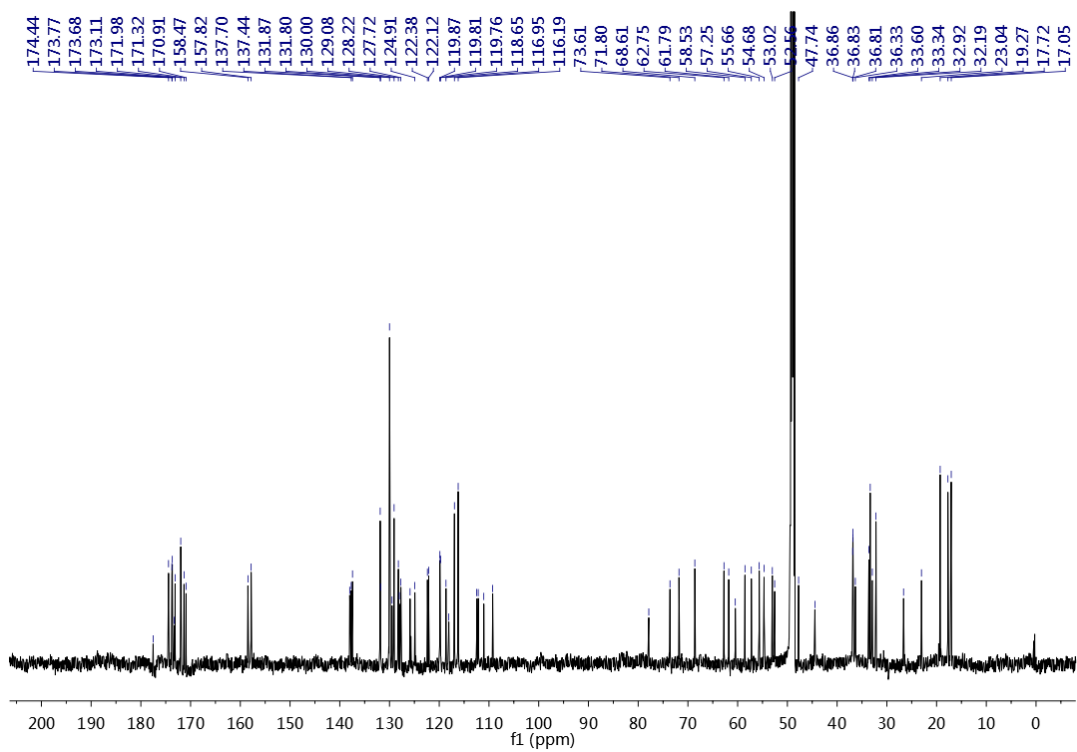


Figure S121. $^{13}$C NMR spectrum (150 MHz) of **58** in CD$_3$OH.
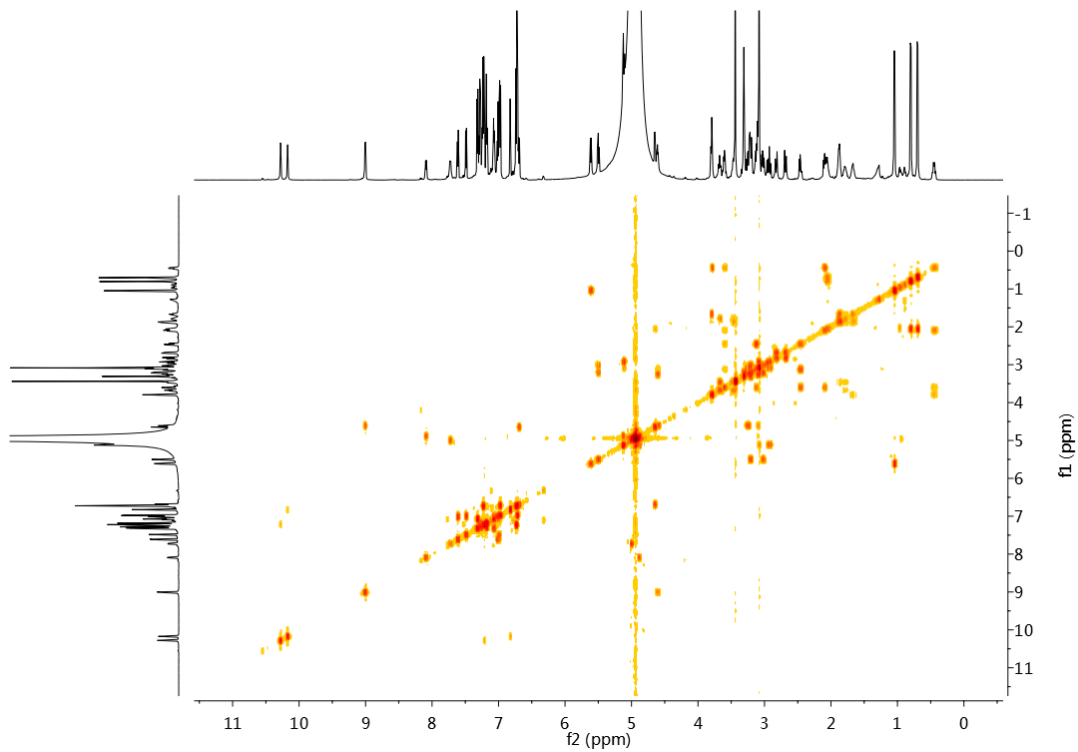
Figure S122. COSY spectrum (600 MHz) of **58** in CD$_3$OH.
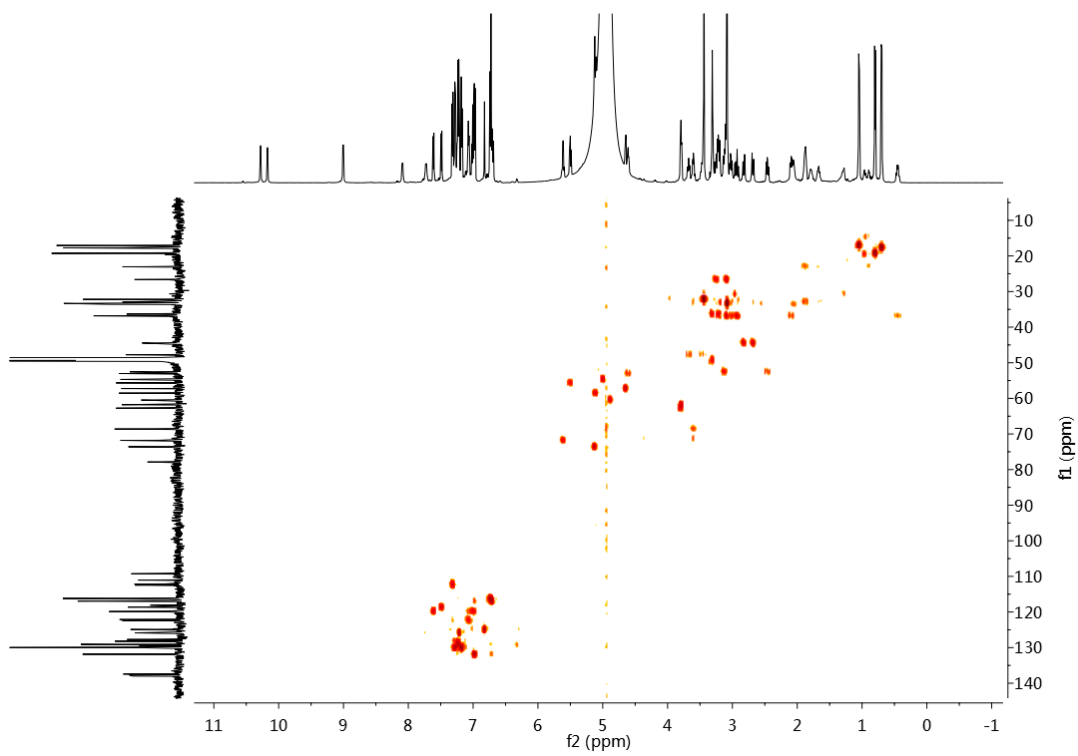


Figure S123. HMQC spectrum (600 MHz) of **58** in CD$_3$OH.
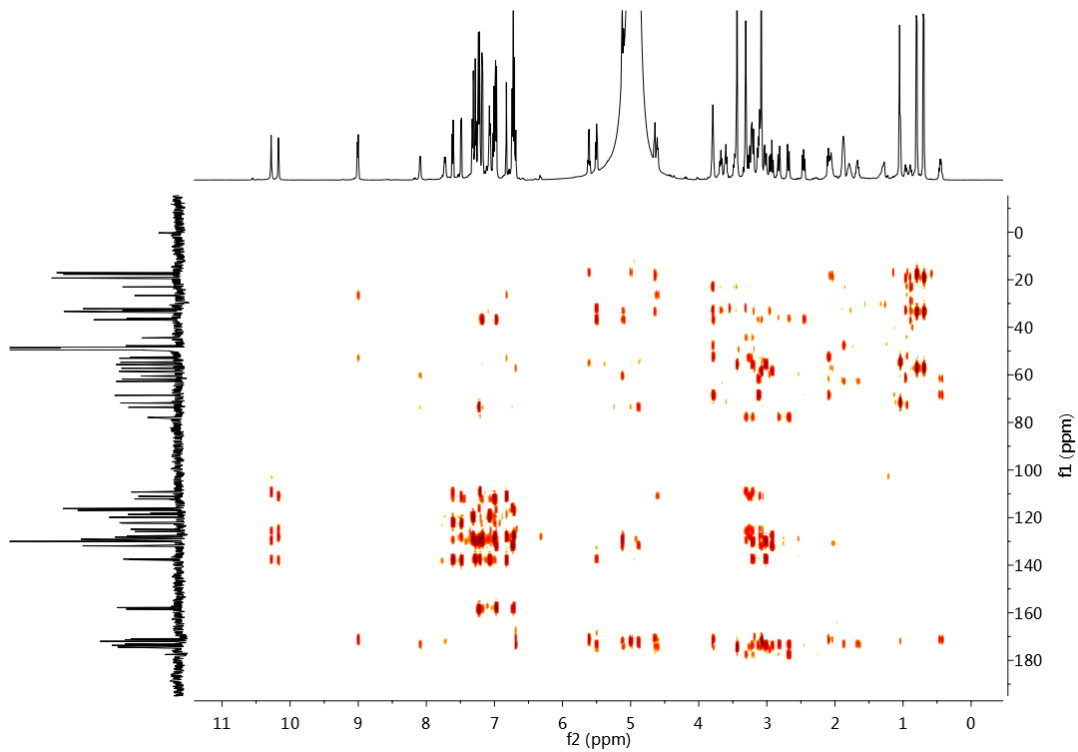
Figure S124. HMBC spectrum (600 MHz) of **58** in CD$_3$OH.
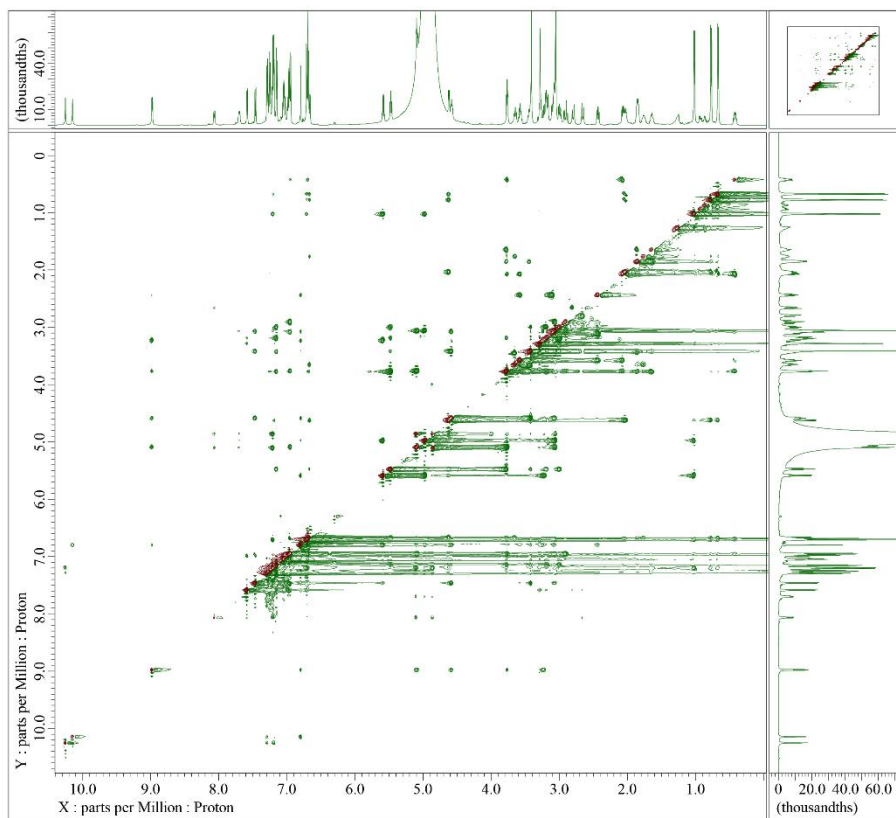


Figure S125. ROESY spectrum (600 MHz) of **58** in CD$_3$OH.

101
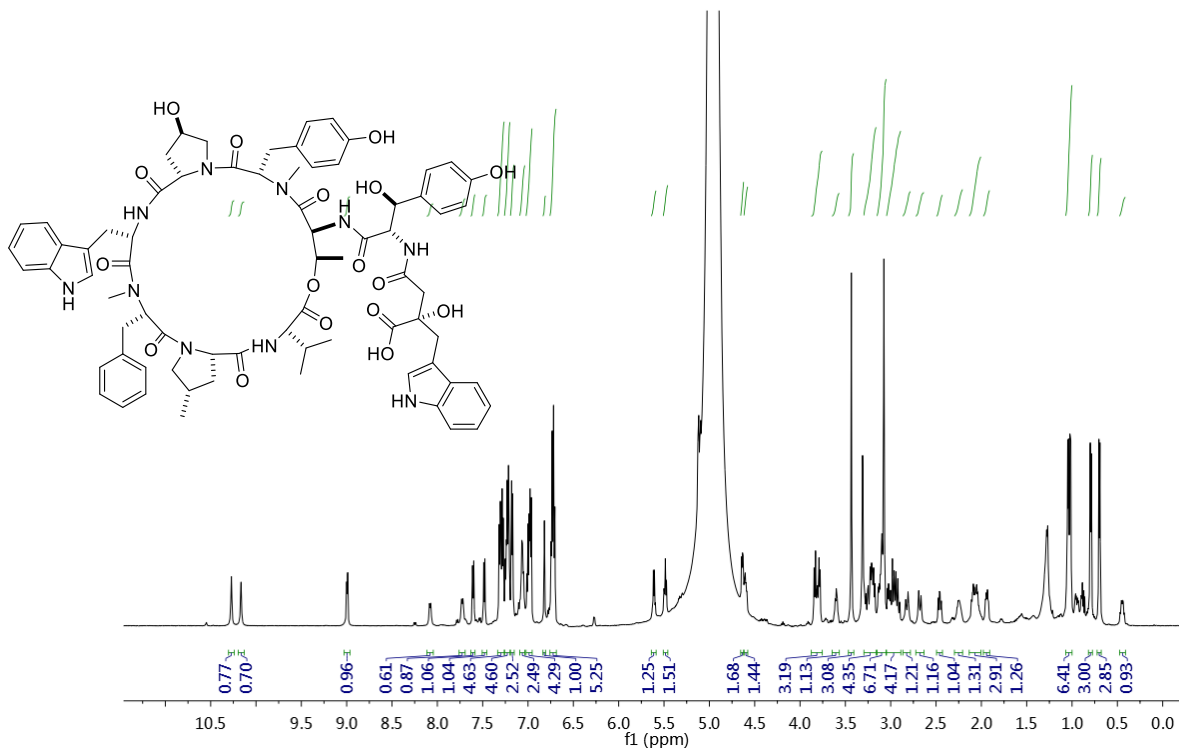
Figure S126. $^1$H NMR spectrum (600 MHZ) of **59** in CD$_3$OH.
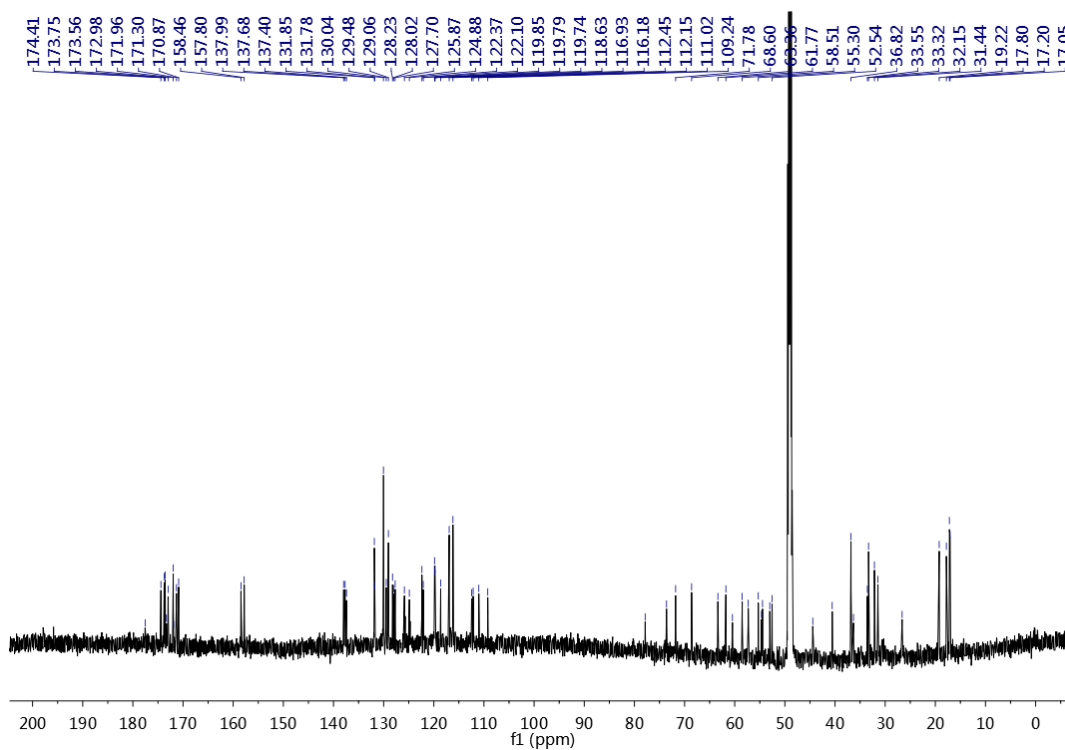


Figure S127. $^{13}$C NMR spectrum (150 MHz) of **59** in CD$_3$OH.
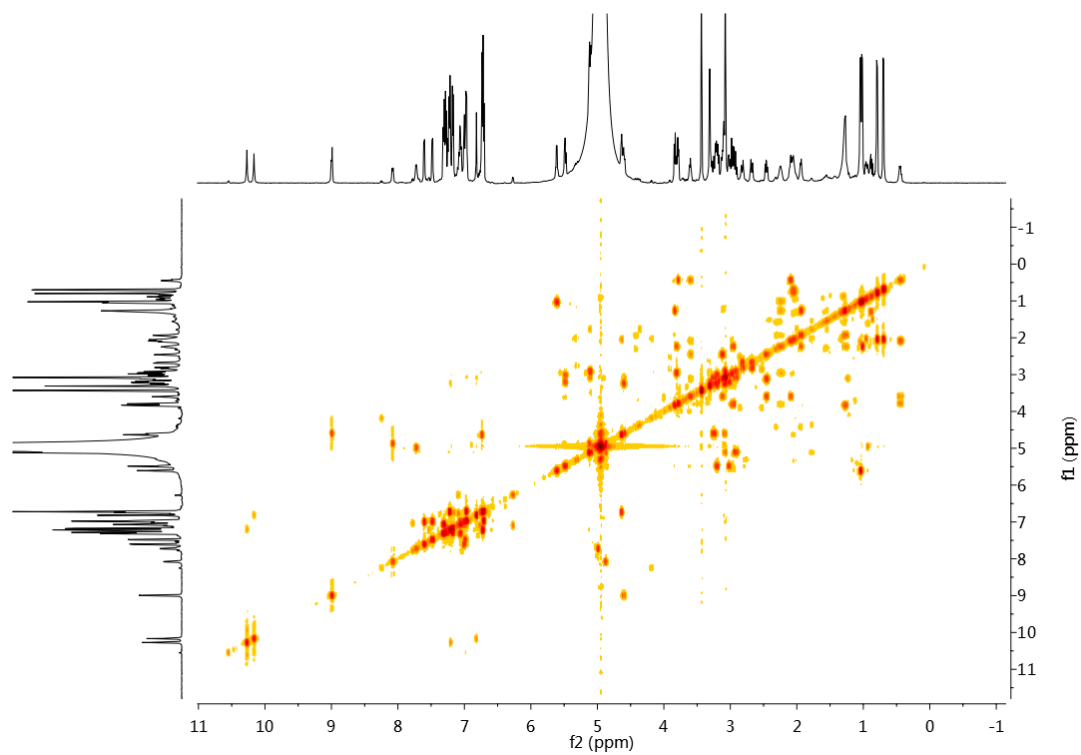
Figure S128. COSY spectrum (600 MHz) of **59** in CD$_3$OH.
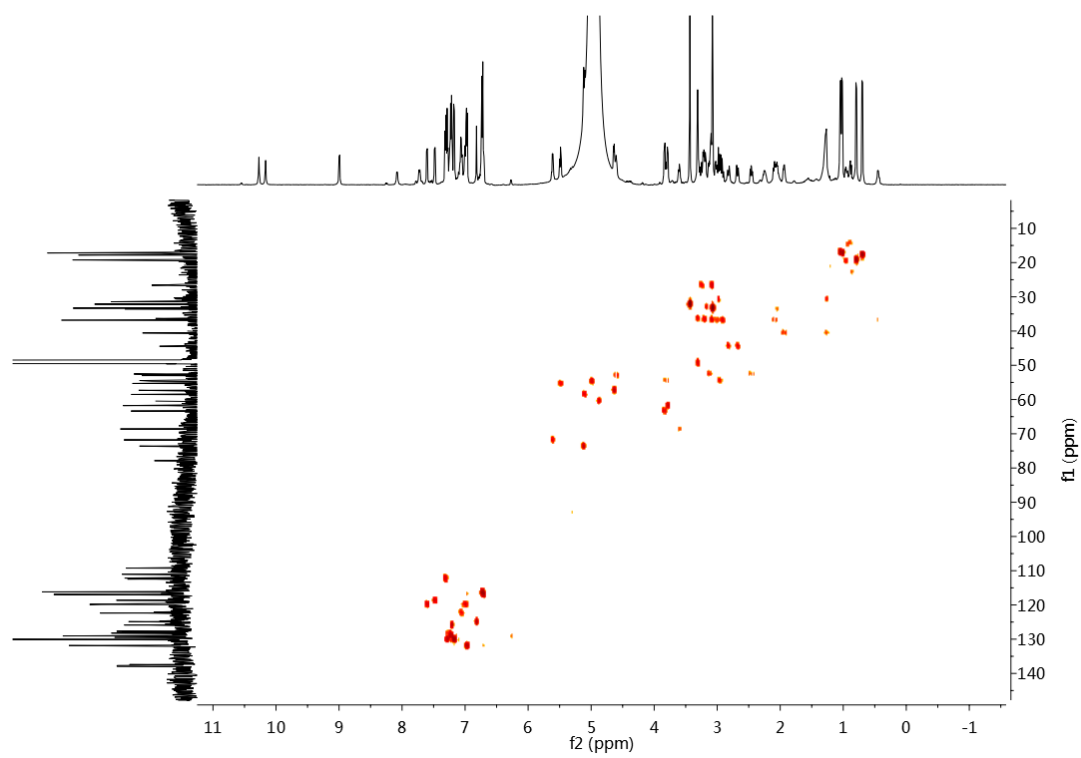


Figure S129. HMQC spectrum (600 MHz) of **59** in CD$_3$OH.

103
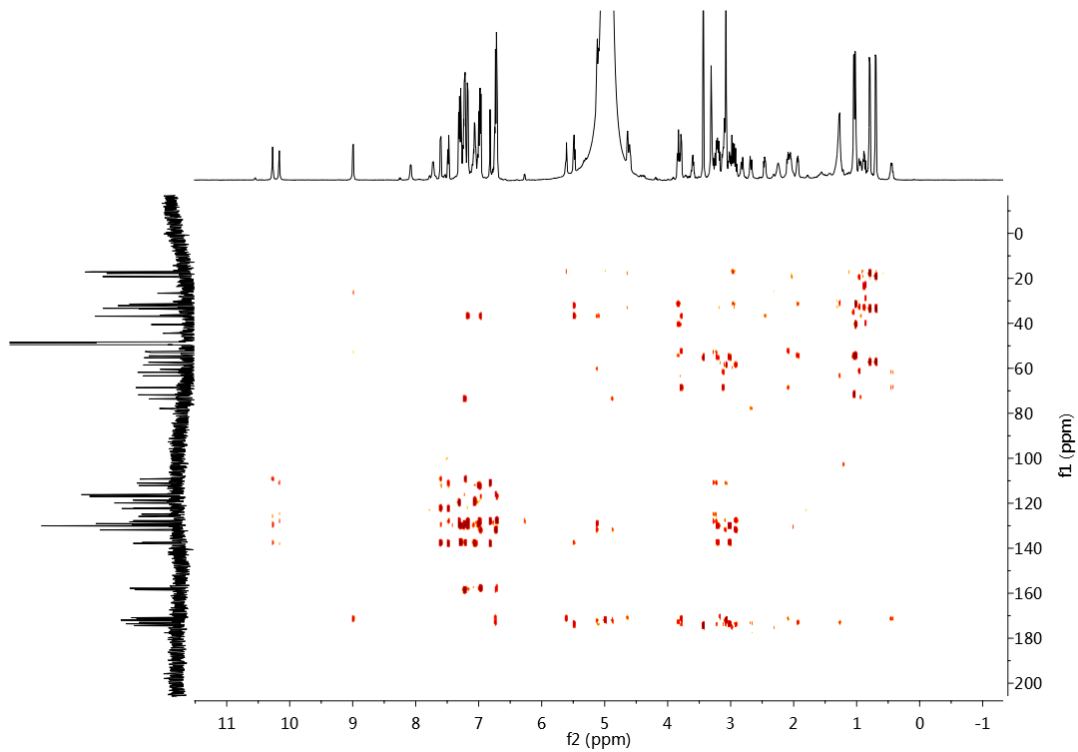
Figure S130. HMBC spectrum (600 MHz) of **59** in CD₃OH.
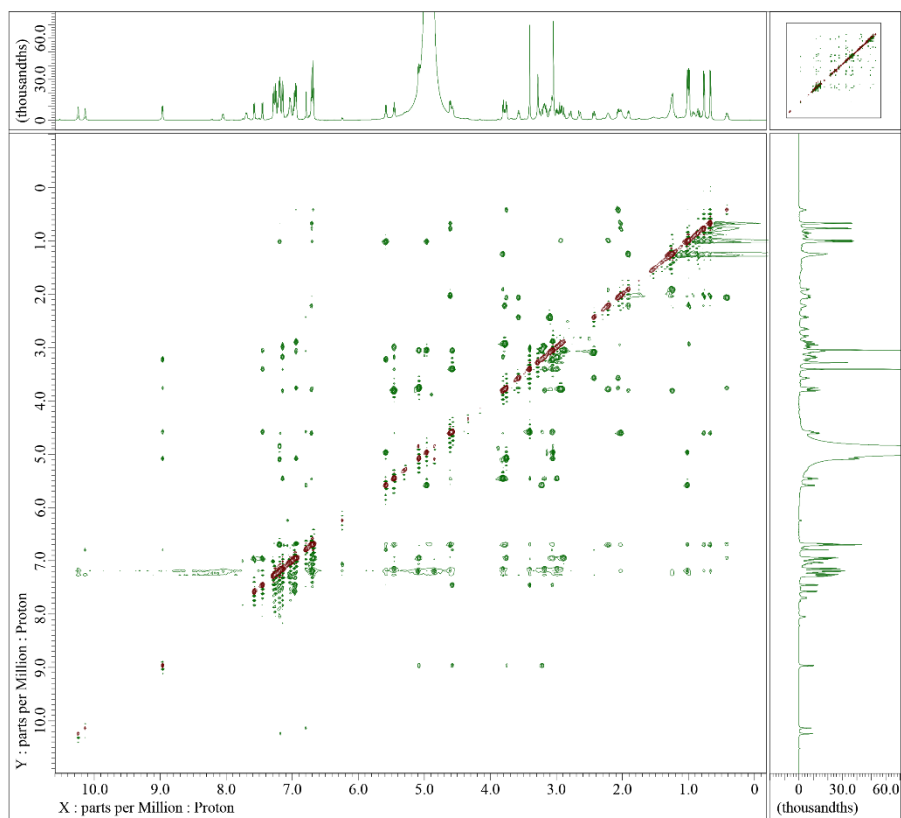


Figure S131. ROESY spectrum (600 MHz) of **59** in CD₃OH.

Figure S132. $^1$H NMR spectrum (600 MHz) of **63** in CDCl$_3$.



Figure S133. $^{13}$C NMR spectrum (150 MHz) of **63** in CDCl$_3$.

Figure S134. $^1$H NMR spectrum (600 MHz) of **64** in CDCl$_3$.



Figure S135. $^{13}$C NMR spectrum (150 MHz) of **64** in CDCl$_3$.

106
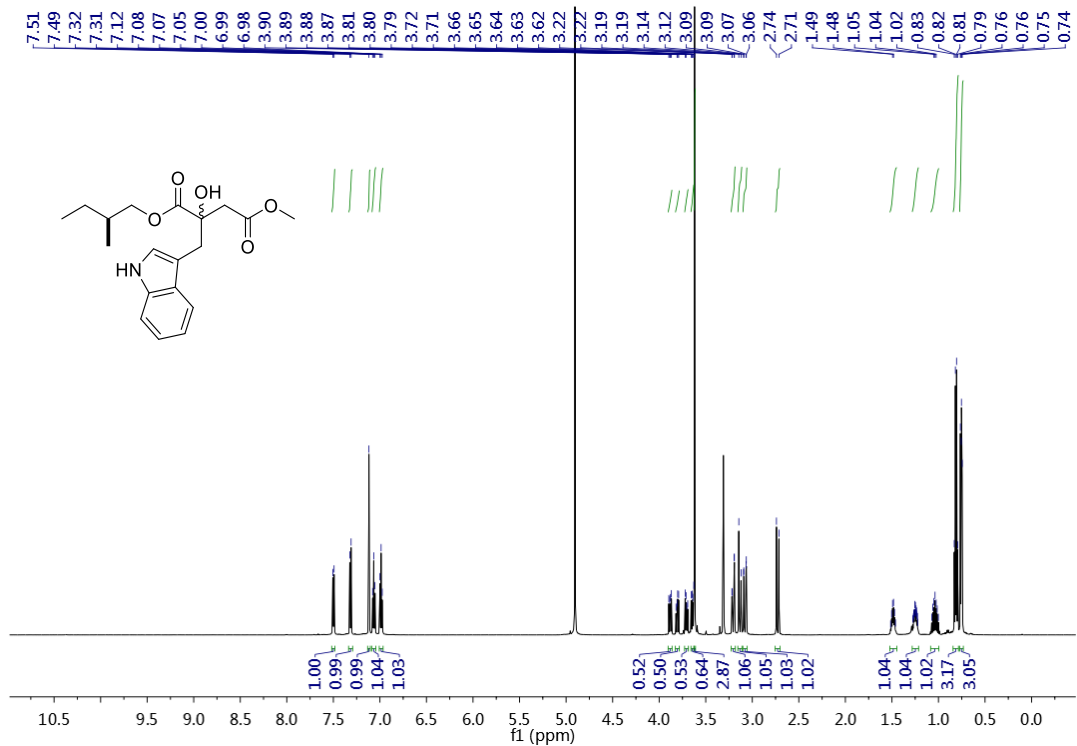
Figure S136. ¹H NMR spectrum (600 MHz) of **65** in CD₃OD.



Figure S137. ¹³C NMR spectrum (150 MHz) of **65** in CD₃OD.
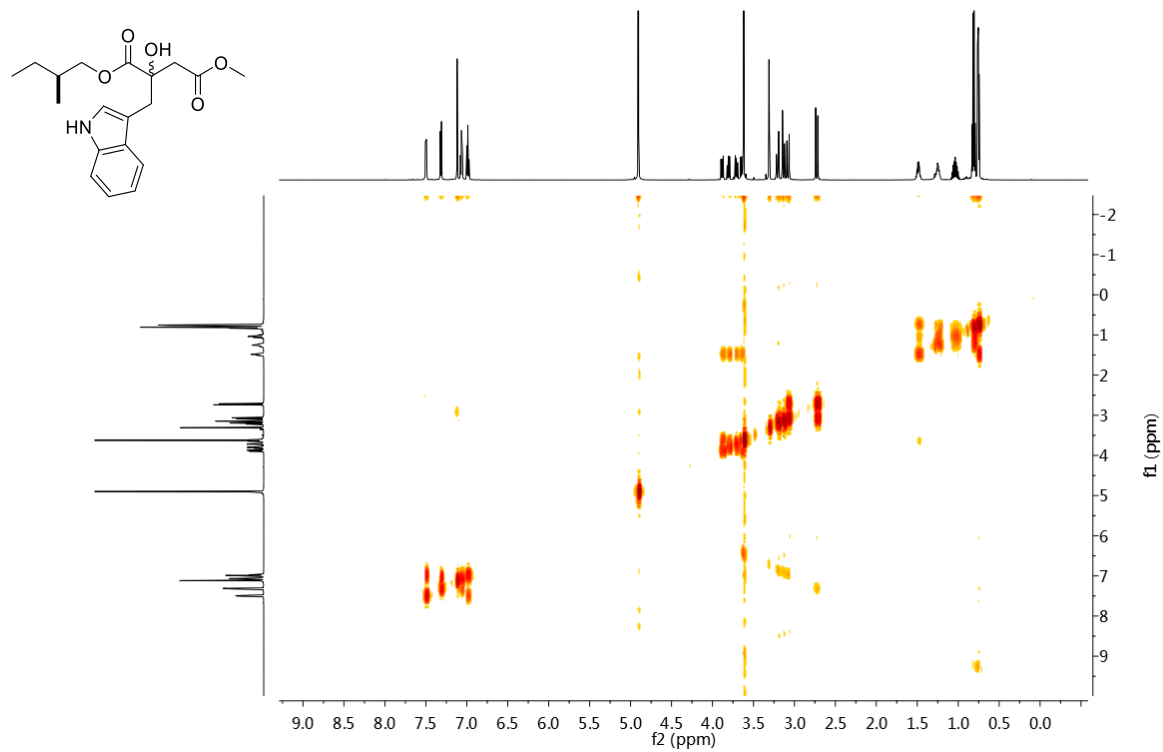
Figure S138. COSY spectrum (600 MHz) of **65** in CD$_3$OD.
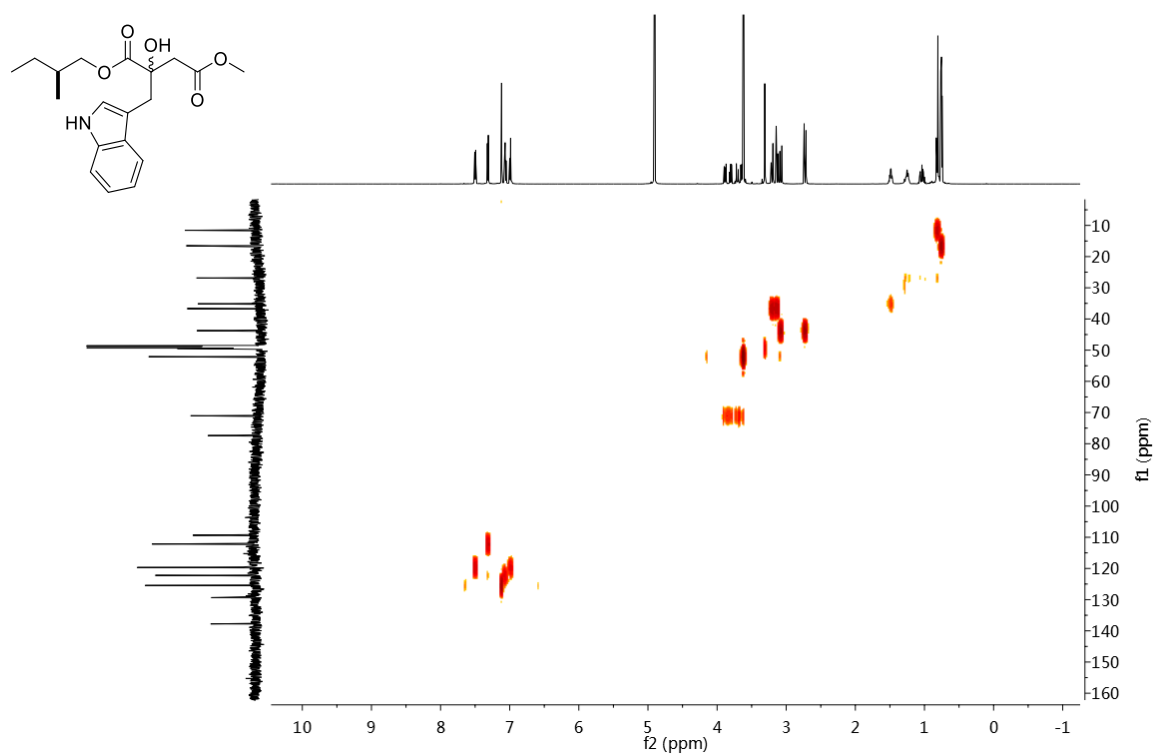


Figure S139. HMQC spectrum (600 MHz) of **65** in CD$_3$OD.
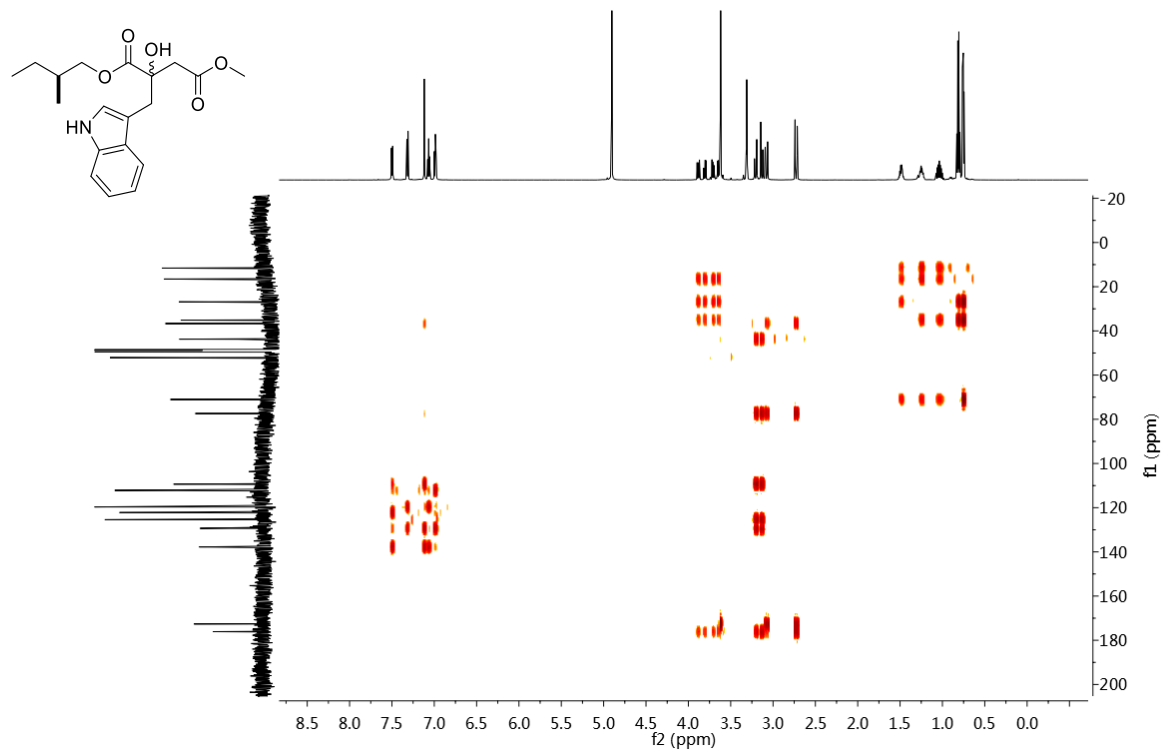
Figure S140. HMBC spectrum (600 MHz) of **65** in CD₃OD.
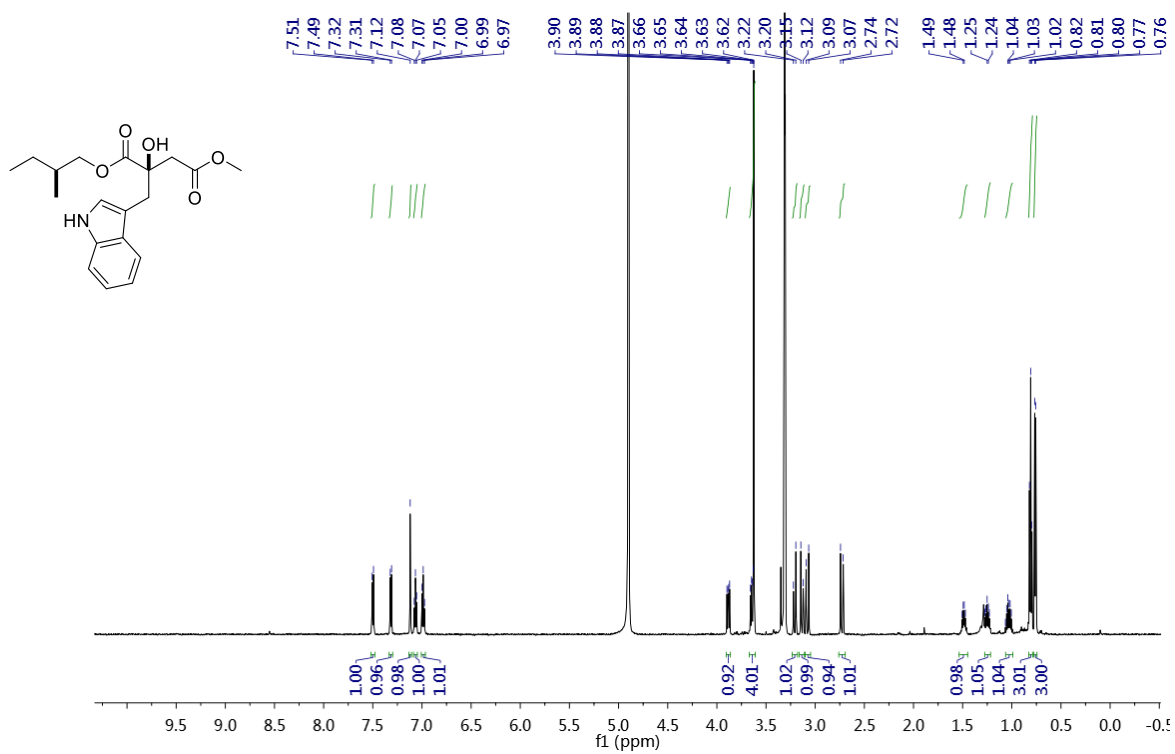


Figure S141. ¹H NMR spectrum (600 MHz) of **65a** in CD₃OD.
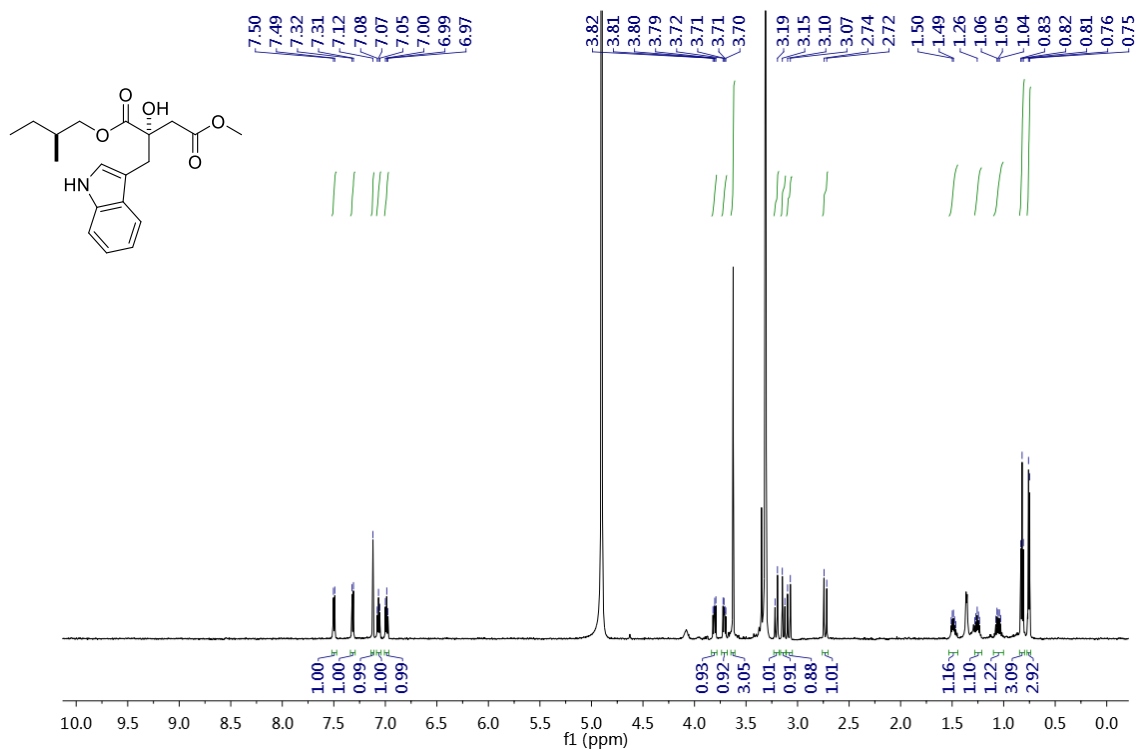
Figure S142. $^1$H NMR spectrum (600 MHz) of **65b** in CD$_3$OD.
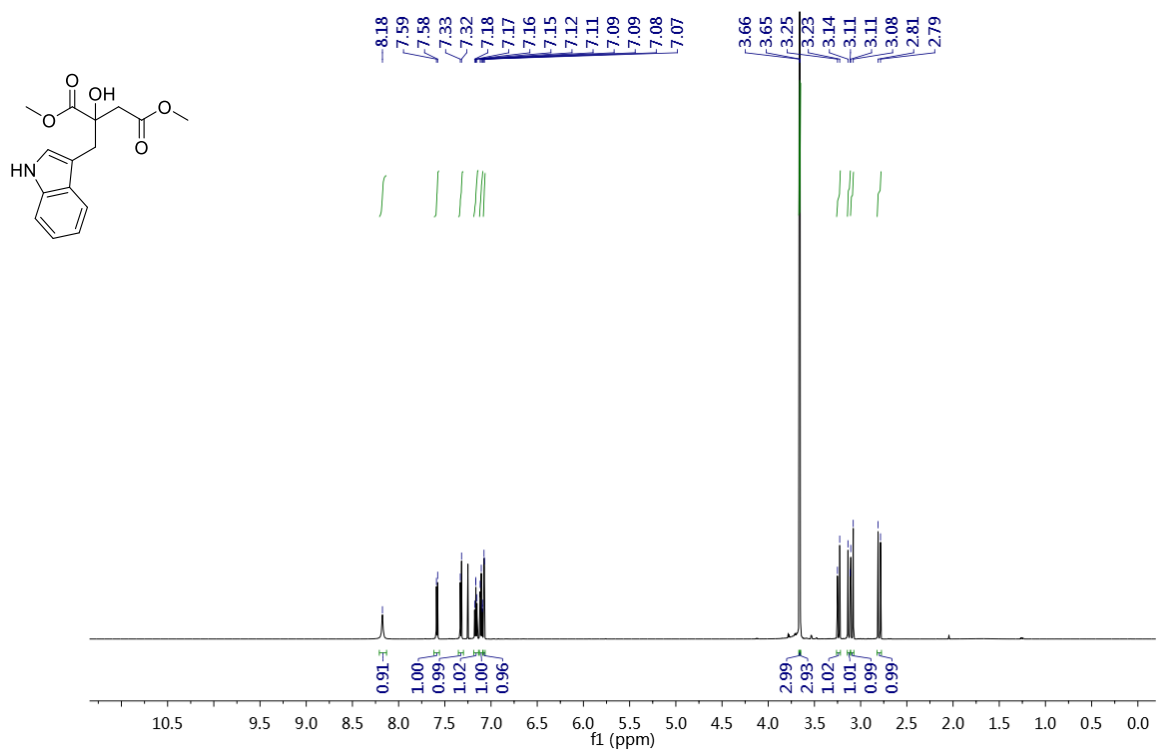


Figure S143. $^1$H NMR spectrum (600 MHz) of **66a/b** in CDCl$_3$.
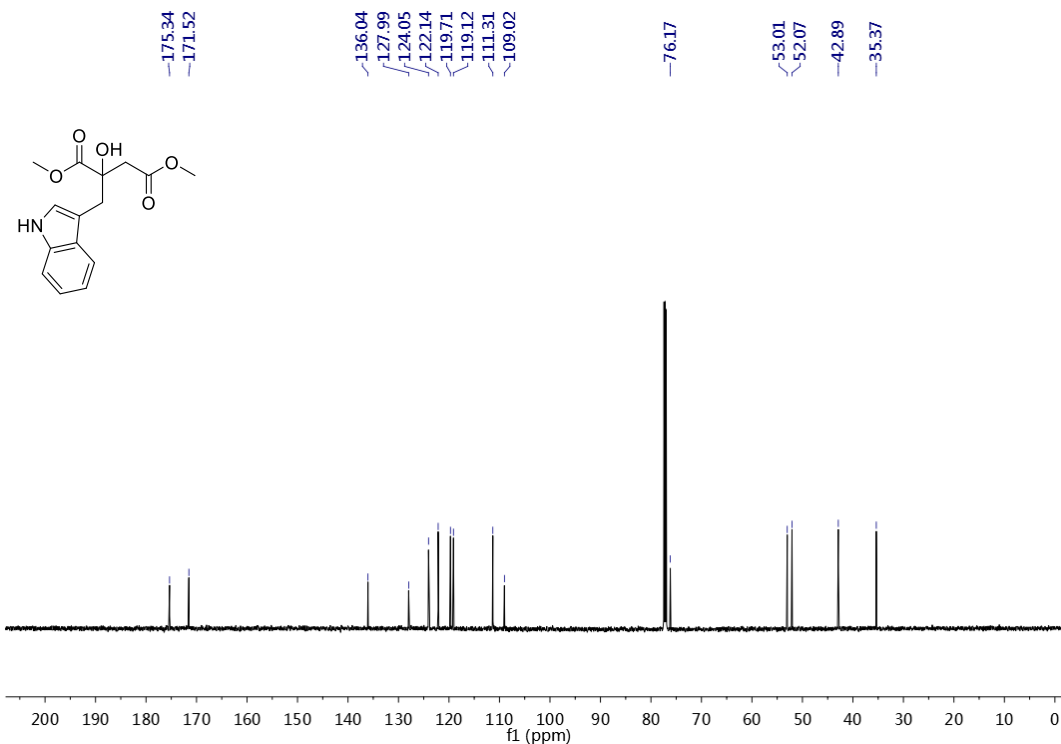
Figure S144. $^{13}$C NMR spectrum (150 MHz) of **66a/b** in CDCl$_3$.



Figure S145. HRMS for compound **58**.
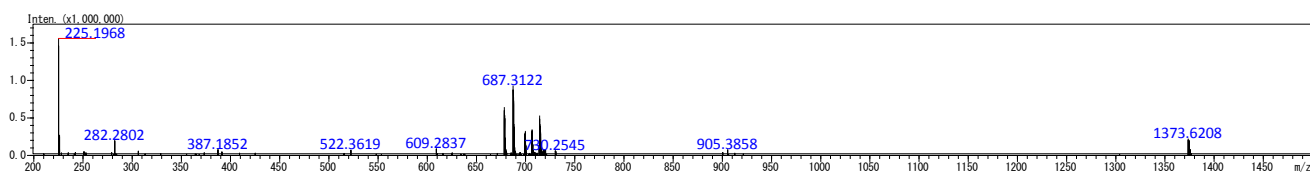


Figure S146. HRMS for compound **59**.



Figure S147. HRMS for compound **63**.
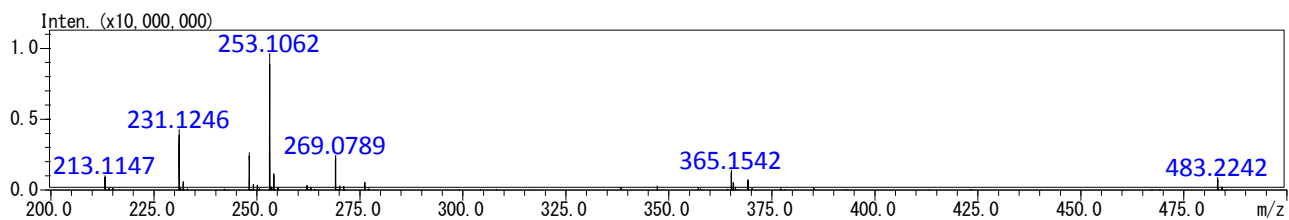
Figure S148. HRMS for compound **64**.
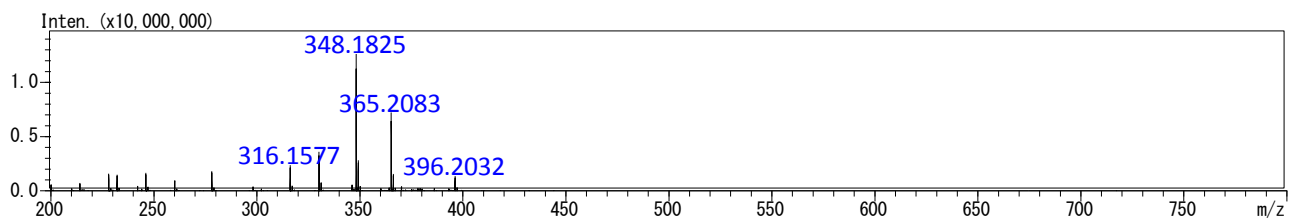


Figure S149. HRMS for compound **65**.
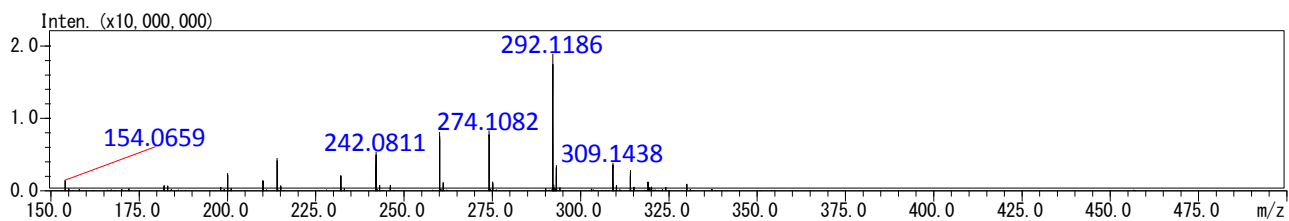


Figure S150. HRMS for compound **66**.

# Acknowledgements