

**Pair of *cis-trans* isomers as novel asteltoxin
analogs with a hydroxylated α -pyrone moiety
produced by solid-state fermentation of
Pochonia suchlasporia TAMA 87**

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NGUYEN THI KHANH NGOC

**Graduate School of
Environmental and Life Science
(Doctor's Course)
OKAYAMA UNIVERSITY**

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ABSTRACT

In our previous study, asteltoxin H, a new asteltoxin analog, was isolated from the MeOH extract of the solid-state fermentation (SSF) cultures of *Pochonia suchlasporia* TAMA 87 (*J. Pestic. Sci.* **45**, 81-85 (2020)). Asteltoxin H was identified as the first member of a new series of asteltoxin analogs having an α -pyrone moiety with a 3,5-dimethyl-2-pyrone-based structure not found in any other known asteltoxin analogs. After that, ET-4 was isolated as the second member of this series of compounds.

Furthermore, the partially purified SSF culture extract of TAMA 87 was indicated to contain more than 10 compounds with UV spectra similar to those of asteltoxin analogs in HPLC analysis with photodiode array detection. Among them, peak 1 was found to have a UV spectrum similar to that of asteltoxin H and have a much shorter HPLC retention time than asteltoxin H. This result suggested that peak 1 would be a potential candidate for new asteltoxin analogs having an α -pyrone moiety with a 3,5-dimethyl-2-pyrone based structure.

In order to produce the target compound, *P. suchlasporia* TAMA 87 was cultured by solid-state fermentation. Mycelial discs of *P. suchlasporia* TAMA 87 were inoculated into two hundred different Erlenmeyer flasks (200 mL), each containing an autoclaved rolled barley-based solid medium. The fungal culture was extracted by adding 50 mL of MeOH to each flask. The mixture was filtered and concentrated *in vacuo* to give an MeOH extract (31.5 g). This amount was continuously used for purification and structural analysis of the target compound.

The target compound was purified from the MeOH extract obtained from the production step. The MeOH extract (31.5 g) was partitioned between EtOAc and water. The EtOAc-soluble fraction was concentrated *in vacuo* to yield an EtOAc extract (13.8 g) and subsequently purified by silica gel column chromatography using hexane/EtOAc and EtOAc/MeOH to afford seven fractions (F1-F7). F4 (476 mg), which was eluted by the 10:90 (v/v) hexane/EtOAc mixture, was further purified by preparative ODS-HPLC to give peak 1 (11.22 mg). Peak 1 was detected as a single peak in ODS-HPLC analysis. However, its ^1H - and ^{13}C -NMR spectra revealed peak 1 correspond to two compounds. Therefore, different columns and conditions were evaluated to obtain a suitable separation. As a result, the separation was achieved by preparative C30-HPLC eluted with isocratic 50% aqueous MeOH to obtain peak 1-a (4.3 mg) and peak 1-b (3.3 mg).

The chemical structure of peak 1-b was determined with a combination of spectroscopic methods, including 1D, 2D NMR, HRESIMS, and UV-Vis analyses. This compound exhibited UV absorption maxima at 374, 282, and 219 nm, which were similar to those of asteltoxin H. Comparing spectroscopic data of peak 1-b to those of asteltoxin H, the structure of peak 1-b was established as a new asteltoxin that differs from asteltoxin H in its α -pyrone moiety. In detail, peak 1-b showed the similar ^1H - and ^{13}C -NMR signals as those of asteltoxin H, except that the C-22 methyl signal (δ_{H} 2.05, δ_{C} 15.2) of asteltoxin H was replaced by an oxygenated methylene signal (δ_{H} 4.49, δ_{C} 59.6). In addition, based on NOESY correlation, the relative stereochemistry of peak 1-b was determined to be identical to that of asteltoxin H. Comparison of the optical rotation value of peak 1-b with asteltoxin H indicated that the absolute configuration of peak 1-b was the same as that of asteltoxin H. Peak 1-b was a novel compound and named as asteltoxin U.

Peak 1-a showed similar UV spectrum to that of peak 1-b and its molecular formula is $C_{23}H_{30}O_7$ based on the HRESIMS, which indicated that peak 1-a was an isomer of peak 1-b. Peak 1-a showed almost the same ^1H - and ^{13}C -NMR signals as those of peak 1-b, except for those of the conjugated triene (C-9 to C-14) moiety. The proton-proton coupling constants of the olefinic protons indicated peak 1-a possess a $9E$, $11Z$, $13E$ conjugated triene moiety as that of isoasteltoxin, whereas peak 1-b was indicated to have an all-*trans* ($9E$, $11E$, $13E$) conjugated triene. The NOESY correlations of peak 1-a also indicated that its relative configuration of 2,8-dioxabicyclo[3.3.0]octane ring were also similar to those of peak 1-b except for the olefinic proton signals of the conjugated triene moiety. The absolute configuration of peak 1-a was determined by similar optical rotation value to isoasteltoxin. Therefore, the absolute configuration of peak 1-a was same as that of isoasteltoxin. Peak 1-a was a novel compound and named as asteltoxin V.

The chemical structures of peak 1-b and peak 1-a were determined by spectroscopic methods and were found to be similar to that of asteltoxin H except for the modification of α -pyrone moiety. In detail, the methyl group on the γ -position of the α -pyrone moiety in asteltoxin H was replaced by a hydroxymethyl group. In addition, peak 1-b and peak 1-a were identified as a pair of isomers differing in the geometry of the double bond between C-11 and C-12 in the conjugated triene moiety.

Furthermore, peak 1-b and peak 1-a were examined for their inhibitory activity toward the first cleavage of sea urchin embryos. Peak 1-b showed inhibitory activity with a minimum inhibition concentration value of $3.1 \mu\text{g/mL}$, whereas peak 1-a did not show inhibitory activity up to $25 \mu\text{g/mL}$. The difference between peak 1-b and peak 1-a in the inhibitory activity toward the first cleavage of sea urchin embryos indicated that the geometry of the conjugated triene in asteltoxins contributes to their biological activities.

TABLE OF CONTENTS

ACKNOWLEDGMENT	i
ABSTRACT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF SCHEMES	viii
Chapter 1 . Introduction	1
1.1. Background and History.....	1
1.2. Asteltoxins and related compounds.....	6
1.3. Objective of the study	14
Chapter 2. Solid-state Fermentation of <i>Pochonia suchlasporia</i> TAMA 87	15
2.1. Introduction	15
2.2. Materials and Methods	15
2.2.1. Fungal strain.....	15
2.2.2. Solid-state fermentation	15
2.2.3. Solid-state fermentation culture	17
2.3. Results and Discussion.....	18
Chapter 3. Purification and Isolation of peak 1-a and peak 1-b produced by <i>Pochonia suchlasporia</i> TAMA 87	20
3.1. Introduction	20
3.2. Materials and Methods	20
3.2.1. Purification of the bioactive compounds produced by <i>Pochonia suchlasporia</i> TAMA 87	20
3.2.2. Isolation of target compound by preparative ODS-HPLC	23
3.2.3. The separation of peak 1-a and peak 1-b from peak 1 by preparative C30- HPLC 26	
3.3. Results and Discussion.....	28
Chapter 4. Structural Elucidation of the Bioactive Compound peak 1-b and peak 1-a	45
4.1. Introduction	45
4.2. Materials and Methods	46
4.2.1. Mass Spectrometry (MS) analysis.....	46
4.2.2. UV spectra measurement	46
4.2.3. Optical rotation measurement	47
4.2.4. Nuclear Magnetic Resonance (NMR) measurement.....	47
4.3. Results and Discussions	48
4.3.1. Chemical structure determination of peak 1-b	48
4.3.1.1. Determination of planar structure	50

4.3.1.2.	Determination of relative configuration	53
4.3.1.3.	Determination of absolute configuration.....	55
4.3.2.	The chemical structure determination of peak 1-a	55
4.3.2.1.	Determination of planar structure	58
4.3.2.2.	Determination of relative configuration.....	61
4.3.2.3.	Determination of absolute configuration.....	63
Chapter 5.	Bioactivity of peak 1-a and peak 1-b	64
5.1.	Introduction	64
5.2.	Materials and Methods	64
5.3.	Results and Discussions	66
REFERENCES		70
APPENDIX		72

LIST OF TABLES

Table 1-1. Asteltoxin-type compounds and their bioactivities	6
Table 2-1. Composition of YTP solution	16
Table 2-2. The Oat types used to prepare the solid-state fermentation culture.....	17
Table 3-1. Condition for silica gel chromatography	21
Table 3-2. Condition for preparative ODS-HPLC	22
Table 3-3. Condition for TLC analysis	23
Table 3-4. Condition for ODS-HPLC analysis	23
Table 3-5. The sample name and weight (dissolved in 5 mL MeOH)	24
Table 3-6. Condition for preparative ODS-HPLC	24
Table 3-7. Condition for ODS-HPLC analysis	27
Table 3-8. Condition for preparative C30-HPLC.....	27
Table 3-9. The peak 1 samples name and weight (dissolved in 1 mL MeOH).....	33
Table 3-10. The peak area and purity of NN501-75-peak 1 in different wavelength	35
Table 3-11. Condition for NMR analysis.....	35
Table 3-12. Peak area and purity of NN501-94-peak 1 in different wavelength.	37
Table 3-13. Predicted and collected fractions belonging to target compounds	41
Table 3-14. Conditions for C30-HPLC analysis	42
Table 3-15. The purity and yield of NN501-107-peak 1-a	43
Table 3-16. The purity and yield of NN501-107-peak 1-b	44
Table 3-17. The yield summarization of peak 1-a and peak 1-b based on the peak area.....	44
Table 3-18. The weight predication of peak 1-a and peak 1-b.....	44
Table 4-1. Techniques for structural elucidation	45
Table 4-2. Condition for mass spectrometry (MS) analysis.....	46
Table 4-3. Condition for UV spectra measurement	46
Table 4-4. Condition for optical rotation measurement	47
Table 4-5. The ¹ H and ¹³ C-NMR and HSQC data of ET-1 (Syaefudin S. et al., 2020) and peak 1-b.....	49
Table 4-6. The COSY correlation of protons of peak 1-a and peak 1-b	50
Table 4-7. The HSQC and HMBC correlations of peak 1-b.....	51
Table 4-8. The HR-ESI-MS of peak 1-b	53
Table 4-9. The NOESY correlations of peak 1-b.....	54
Table 4-10. The ¹ H and ¹³ C-NMR data of ET-1 (Syaefudin S. et al., 2020), peak 1-a and peak 1-b.....	56
Table 4-11. UV-Vis spectrum of peak 1-a, peak 1-b, isoasteltoxin and asteltoxin.....	57
Table 4-12. The ratio of two major ε values of peak 1-a, peak 1-b, isoasteltoxin and asteltoxin	58
Table 4-13. The COSY correlation of protons of peak 1-a and peak 1-b	58
Table 4-14. The HSQC and HMBC of peak 1-a	59
Table 4-15. The HR-ESI-MS of peak 1-a	61
Table 4-16. The HSQC and HMBC correlations of peak 1-a	62
Table 5-1. Summarize the MIC value of peak 1-a, peak 1-b and ΔPLH	67
Table 5-2. Sea urchin embryo cleavage inhibition activity 120 minutes after the start of incubation in ΔPLH (positive control)	67
Table 5-3. Sea urchin embryo cleavage inhibition activity 120 minutes after the start of incubation in peak 1-b and peak 1-a.....	68

LIST OF FIGURES

Figure 1-1. The chemical structure of reported asteltoxins, ET-1 (asteltoxin H) and ET-4	2
Figure 1-2. The analytical HPLC chromatogram of fr.4.....	5
Figure 1-3. The UV spectra of peak 1 and ET-1 by LC-PDA detector.....	5
Figure 2-1. <i>P. suchlasporia</i> TAMA 87 on the rolled barley-based medium for 22 days of fermentation.	19
Figure 3-1. TLC chromatogram of the EtOAc soluble fraction and fr. 1 ~ fr. 7 resulted in the silica gel column chromatography. (Cultivated with new bought oat)	29
Figure 3-2. TLC chromatogram of the EtOAc soluble-fraction and fr. 1 ~ fr. 7 resulted in the silica gel column chromatography by using anisaldehyde-sulfuric acid in detection method.	29
Figure 3-3. The analytical HPLC chromatograms of NN501-48-fr.4, NN501-51-fr.4, NN501-52-fr.4, NN501-56-fr.4, NN501-57-fr.4, NN501-58-fr.4, NN501-59-fr.4, NN501-60-fr.4, ...	33
Figure 3-4. The HPLC chromatograms of NN501-75-peak 1 detected at various wavelength.	35
Figure 3-5. The chromatograms of NN501-94-peak 1 detected at 380 nm, 280 nm, 254 nm and 210 nm.	37
Figure 3-6. The ¹ H NMR spectrum of NN501-94-peak 1	38
Figure 3-7. The ¹³ C NMR spectrum of NN501-94-peak 1	38
Figure 3-8. Analytical HPLC chromatogram of peak 1 analyzed by InertSustain AQ-C18 column.....	39
Figure 3-9. Analytical HPLC chromatogram of peak 1 analyzed by Inertsil ODS-HL column	39
Figure 3-10. The analytical HPLC chromatogram of peak 1 analyzed by InertSustain C30 column.....	40
Figure 3-11. The preparative HPLC chromatogram of NN501-106-peak 1	41
Figure 3-12. Analytical HPLC chromatogram of peak 1-a.....	43
Figure 3-13. Analytical HPLC chromatogram of peak 1-b.....	43
Figure 4-1. The UV spectrum of peak 1-b	48
Figure 4-2. The COSY correlation of peak 1-b.....	51
Figure 4-3. The HMBC correlation of peak 1-b.....	52
Figure 4-4. The planar structure of peak 1-b.....	52
Figure 4-5. HR-ESI-MS chromatogram of peak 1-b (positive ion mode, 1 µg/mL)	52
Figure 4-6. HR-ESI-MS chromatogram of peak 1-b (negative ion mode, 1 µg/mL)	53
Figure 4-7. The NOESY correlations of peak 1-b.	54
Figure 4-8. The relative configurations of peak 1-b.....	55
Figure 4-9. The absolute configuration of peak 1-b.....	55
Figure 4-10. The UV spectrum of peak 1-a	57
Figure 4-11. The HMBC correlations of peak 1-a	60
Figure 4-12. The planar structure of peak 1-a.....	60
Figure 4-13. HR-ESI-MS chromatogram of peak 1-a (positive ion mode, 1 µg/mL).....	60
Figure 4-14. HR-ESI-MS chromatogram of peak 1-a (negative ion mode, 1 µg/mL).....	61
Figure 4-15. The NOESY correlations of peak 1-a.....	62
Figure 4-16. The relative configurations of peak 1-a.....	63
Figure 4-17. The absolute configuration of peak 1-a	63
Figure 5-1. Method for fertilizing sea urchin eggs.....	65
Figure 5-2. Serial dilution for samples and ΔPLH	66
Figure 5-3. Sea urchin fertilized eggs after 120 min treatment with peak 1-a.....	68
Figure 5-4. Sea urchin fertilized eggs after 120 min treatment with peak 1-b.....	68
Figure 5-5. Sea urchin fertilized eggs after 24 hours treatment with peak 1-a	69
Figure 5-6. Sea urchin fertilized eggs after 24 hours treatment with peak 1-b.....	69

LIST OF SCHEMES

Scheme 1-1. The purification and isolation of ET-1 and ET-4 from solid-state fermentation of <i>P. suchlasporia</i>	4
Scheme 2-1. Preparation of solid-sate fermentation culture of <i>P. suchlasporia</i> TAMA 87. ...	16
Scheme 2-2. Preparation for MeOH extract of solid-state culture of <i>P. suchlasporia</i> TAMA 87.....	18
Scheme 3-1. The purification and isolation of peak 1.....	21
Scheme 3-2. Sample preparation of NN501-59-fr.4, NN501-60-fr.4, NN501-61-fr.4 and NN501-62-fr.4 for preparative HPLC.....	24
Scheme 3-3. Fractionation of combined fraction with high purity of peak 1	25
Scheme 3-4. The combination of NN501-75-peak 1	25
Scheme 3-5. The combination of NN501-94-peak 1	26
Scheme 3-6. Combination of NN501-106-peak 1.....	26
Scheme 3-7. The separation of peak 1 through preparative C30-HPLC.....	27
Scheme 3-8. The sample preparation of NN501-75-peak 1 for NMR analysis	34
Scheme 3-9. The sample preparation of NN501-94-peak 1 for NMR analysis	36
Scheme 3-10. Combination of NN501-107-peak 1-a.....	42
Scheme 3-11. Combination of NN501-107-peak 1-a.....	42
Scheme 5-1. Method for inhibitory assay using sea urchin embryos.....	65

Chapter 1 . Introduction

1.1. Background and History

Fungal secondary metabolites, like antibiotics, pigments, growth hormones, antitumor agents, and others, are not essential for the growth and development of microorganisms, but they have provided numerous pharmaceutical agents for human and animal health (Ruiz B. *et al.*, 2010). Among the microorganisms producing the above-mentioned compounds, bacteria, including actinobacteria, and fungi produce a diverse array of bioactive small molecules with significant potential to be used in medicine (O'Brien & G. D. Wright, 2011). These bioactive compounds are mainly produced by the activation of cryptic gene clusters which are not active under normal conditions and, thus, the expression of these clusters would be helpful in the exploitation of the chemical diversity of microorganisms (R. K. Pettit, 2011), (F. Wu *et al.*, 2012.).

Fungi are known for their production of chemical and biological diversity of secondary metabolites (G. F. Bills & J. B. Gloer, 2016). There are variety of structural classes of fungal secondary metabolites which are classified based on the precursor primary metabolites used during secondary metabolism; some of these secondary metabolites maybe antimicrobial (bacterial and other fungi), toxic towards plants (phytotoxins) or human and animals (mycotoxins) (G. F. Bills & J. B. Gloer, 2016) (M. Nett *et al.*, 2009).

In our laboratory, during the screening of *Pochonia suchlasporia* TAMA 87 for bioactive compounds, the solid-state fermentation (SSF) culture of this fungus has been found to produce a novel polyhydroxylated pyrrolizidine alkaloid, pochonicine (H. Usuki *et al.*, 2009). This compound showed the potential inhibition of β -N-acetylglucisaminidase (GlcNAcase) from various organisms, including plants, mammals, fungi, and insects (Usuki *et al.*, 2009). Since GlcNAcase is an essential enzyme for the normal growth of insects, pochonicine might contribute for the insect growth regulatory activity. Therefore, the examination of SSF culture of *P. suchlasporia* TAMA 87 on the insect growth would be conducted.

Takatsuji has performed a bioassay using prepupal larvae of *Lucilia cuprina*, a green bottle fly, to evaluate insecticidal and insect growth regulatory activity. As a result, MeOH extract of the SSF culture of *P. suchlasporia* TAMA 87 was examined and found to have insecticidal activity against the blowfly (S. Syaefudin *et al.*, 2020). Moreover, the MeOH extract obtained from SSF culture of *P. suchlasporia* TAMA 87 was partitioned between EtOAc and H₂O. Biological assay investigation showed that the insecticidal activity of EtOAc-soluble fraction was much higher than that of the H₂O-soluble fraction where pochonicine was recovered, so that the active compound might belong to EtOAc soluble fraction.

Furthermore, the EtOAc extract was then purified by silica gel column chromatography to give nine fractions. Among those fractions, three fractions (fraction 3-1, fraction 3-2 and fraction 3-3) which were eluted with hexane/EtOAc (30:70, v/v) showed higher insecticidal activity compared to other fractions, especially fraction 3-1 exhibited the highest insecticidal activity (S. Syaefudin *et al.*, 2020). Then, ET-1 (90% purity, observed at 280 nm using

analytical HPLC) which was the main peak in fr.3-1 was elucidated the chemical structure using UV-Vis, NMR and optical rotation measurements.

Syaefudin continuously confirmed and determined the relative and absolute configuration of ET-1 by instrumental analyses. Chemical structure elucidation of ET-1 revealed that ET-1 is a new asteltoxin that differs from asteltoxin-type compounds in its α -pyrone moiety. These known asteltoxin-type compounds share the same α -pyrone moiety (4-methoxy-5-methyl-2-pyrone-based structure) (Figure 1-1), but ET-1 has another type of α -pyrone moiety (3,5-dimethyl-2-pyrone-based structure) indicating a new structural variation of asteltoxins. This is the first report of this unique α -pyrone moiety with no methoxy group among reported asteltoxin-type compounds. Based on the NOESY and optical rotation, the absolute configuration of ET-1 was identified to that of asteltoxin and named asteltoxin H (S. Syaefudin *et al.*, 2020). Moreover, ET-1 exhibited insecticidal activity with LD₅₀ value of 0.94 $\mu\text{g}/\text{mg}$ prepupal body weight. This compound could delay the development stage of *Lucilia sericata*, by prolonging the prepupal stage of insects, indicating insect growth regulatory activity of the ET-1.

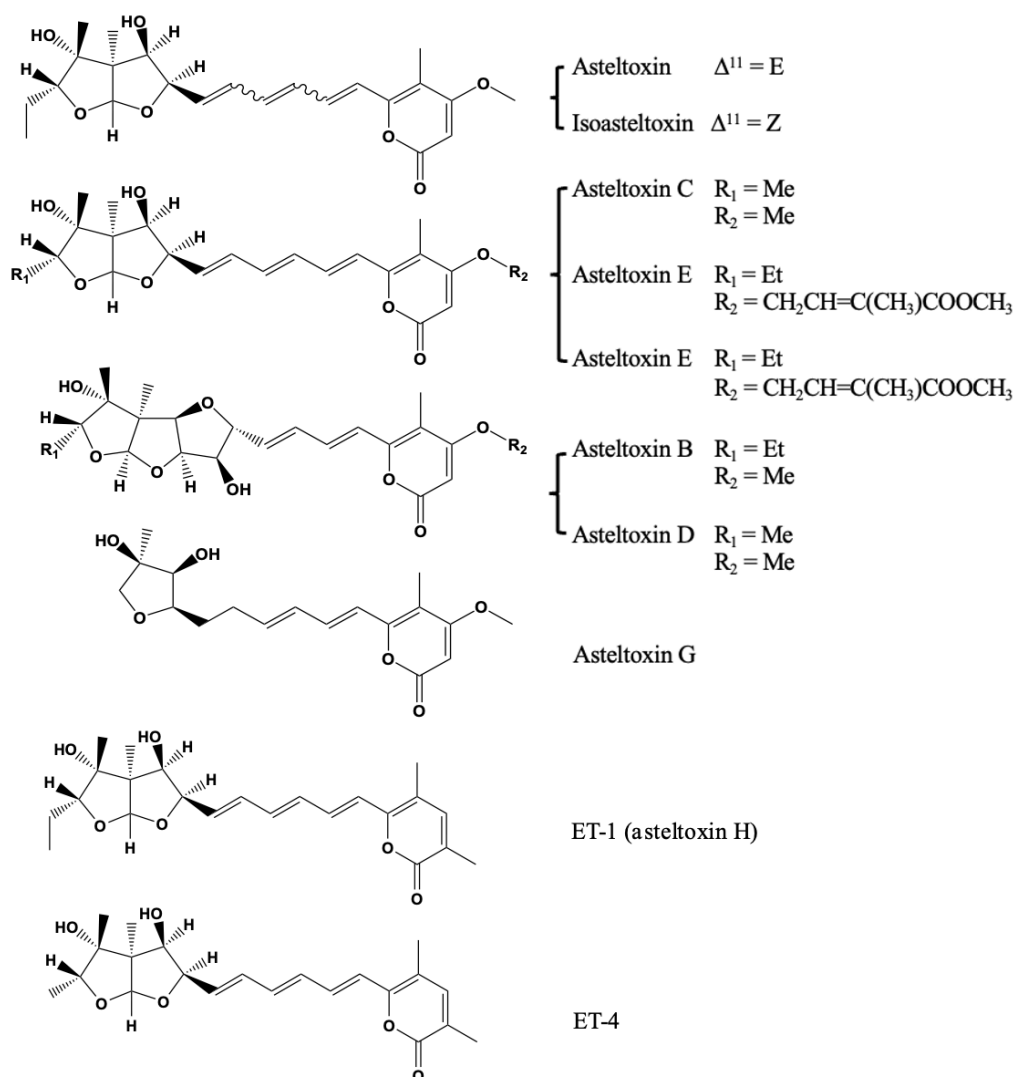
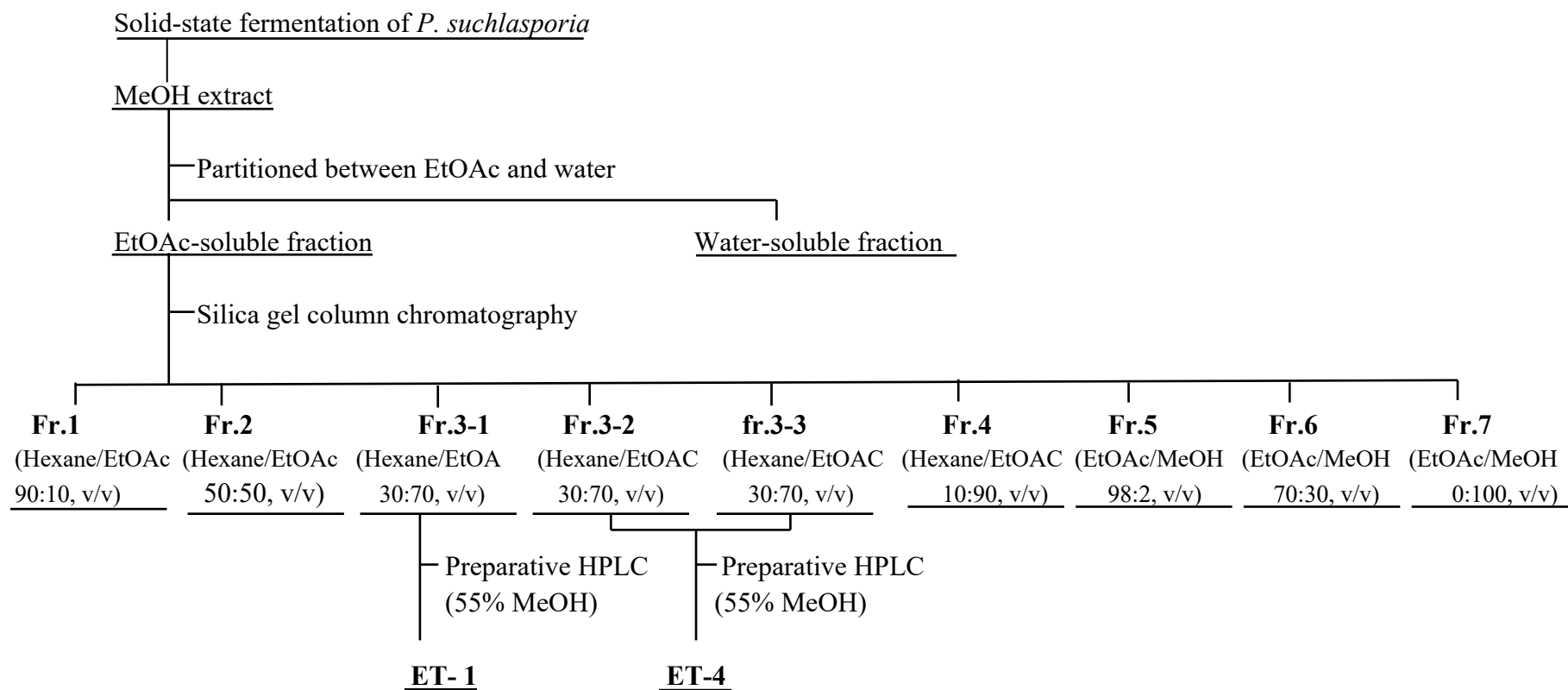


Figure 1-1. The chemical structure of reported asteltoxins, ET-1 (asteltoxin H) and ET-4

After the chemical structure determination of ET-1 in the fr.3-1, there is another compound with similar UV spectrum to ET-1, and a high peak area was observed in fractions 3-2 and 3-3 detected by analytical HPLC. The chemical structure of this compound, named ET-4, was determined by instrumental analyses, similarly to that of ET-1. The results indicate that ET-4 is a novel compound possessing the same α -pyrone moiety as ET-1, and the ethyl group at C-3 of ET-1 is replaced with a methyl group (Figure 1-1) (S. Syaefudin *et al.*, 2020), (Doctor thesis of Syaefudin, 2020) which suggests that this fungus has a novel biosynthetic pathway of asteltoxins and may synthesize asteltoxin having the same novel alpha-pyrone moiety.

The chemical structure of ET-4 supports to the presence of a new series of the asteltoxin-type compounds with an unique α -pyrone moiety.



Scheme 1-1. The purification and isolation of ET-1 and ET-4 from solid-state fermentation of *P. suchlasporia*

In the analytical HPLC chromatogram of fr.4 (Figure 1-2) obtained from silica gel column chromatography, there are at least 10 peaks which have similar UV spectra with ET-1 and ET-4.

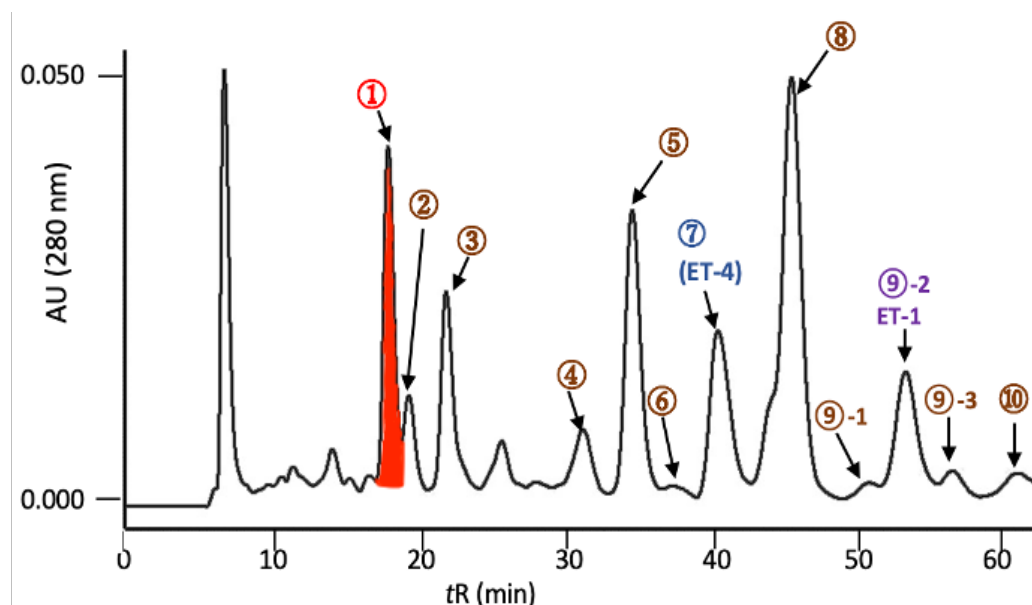


Figure 1-2. The analytical HPLC chromatogram of fr.4

In this study, based on the LC-PDA detector, peak 1 which was painted as red colour was observed to have a UV spectrum similar to that of ET-1 (Figure 1-3) and its HPLC retention time is different from that of ET-1. Therefore, peak 1 is a potential candidate for new asteltoxin analogs having an α -pyrone moiety with a 3,5-dimethyl-2-pyrone based structure (similar to that of ET-1). So, peak 1 is my target compound in this study.

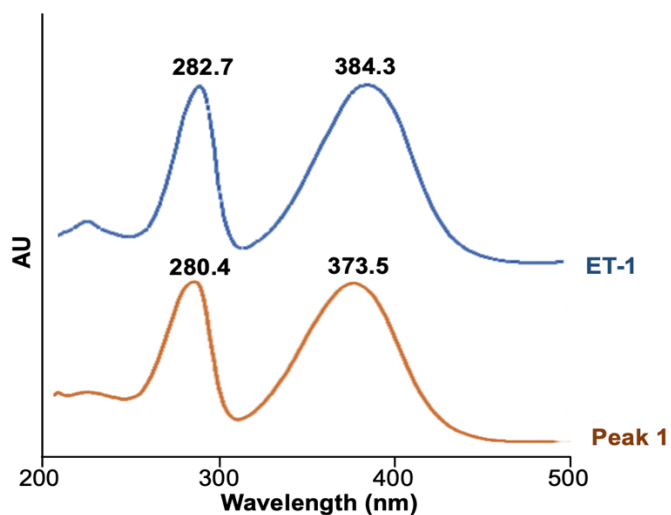
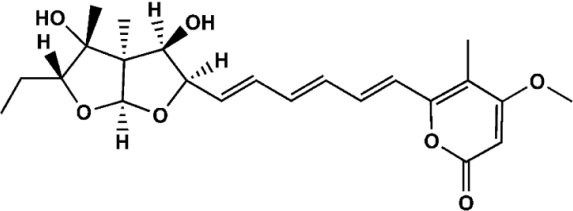
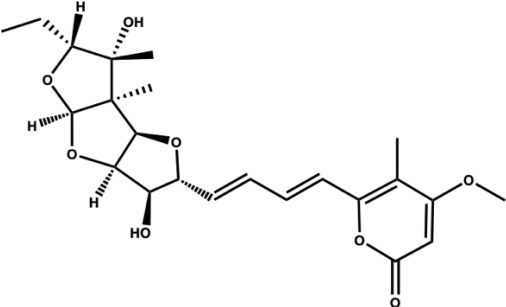
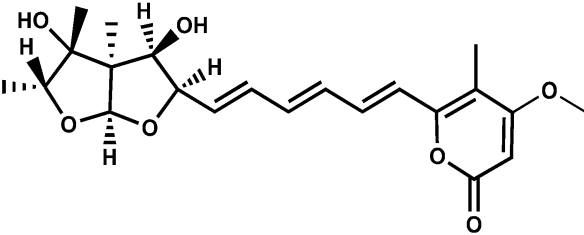


Figure 1-3. The UV spectra of peak 1 and ET-1 by LC-PDA detector.

1.2. Astelt toxins and related compounds

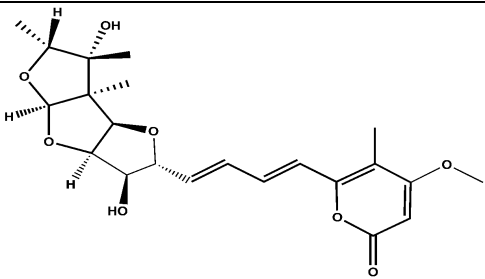
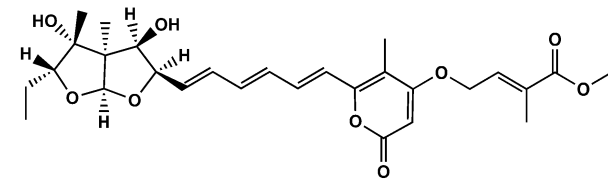
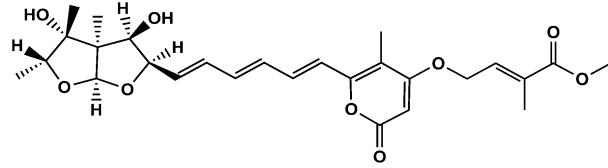
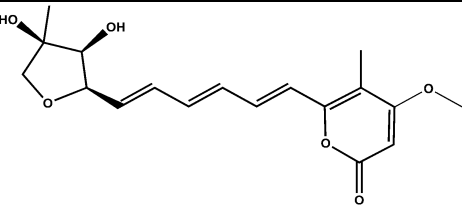
The chemical structure of previous reported astelt toxins and their bioactivity were summarized in Table 1-1.

Table 1-1. Astelt toxin-type compounds and their bioactivities

Compounds	Chemical structures	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Astelt toxin ⁴⁾		418	$[\alpha]_D^{23} +20.0$ (c 1.15, MeOH)	267 (4.47) 273 (4.47) 367 (4.52)	MeOH	Anticancer Antivirus	<i>Aspergillus stellatus</i> Curzi (G. J. Kruger <i>et al.</i> , 1979)
Astelt toxin B ¹⁾		434	$[\alpha]_D^{25} +93.1$ (c 0.1, MeOH)	248 (4.45) 343 (4.30)	MeOH	AChE inhibitor	<i>Pochonia bulbillosa</i> 8-H-28 (H. Adachi <i>et al.</i> , 2015)
Astelt toxin C ¹⁾		404	$[\alpha]_D^{27} +11.3$ (c 0.1, MeOH)	269 (4.32) 274 (4.33) 364 (4.35)	MeOH	Antiproliferative	<i>Pochonia bulbillosa</i> 8-H-28 (H. Adachi <i>et al.</i> , 2015)

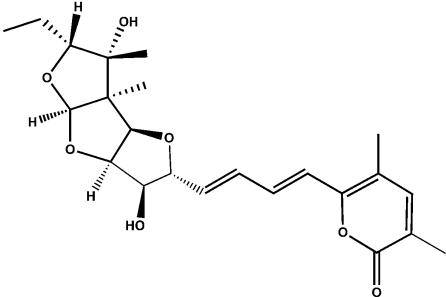
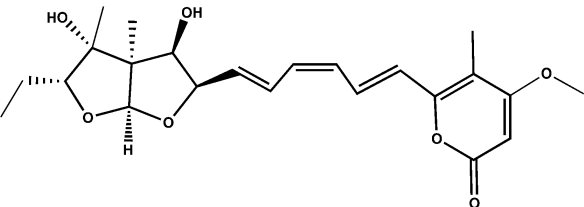
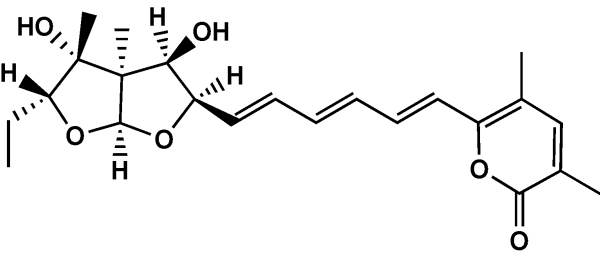
ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structure	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin D ¹⁾		420	$[\alpha]_D^{27} +127.8$ (c 0.1, MeOH)	248 (4.66) 343 (4.48)	MeOH	ND	<i>Pochonia bulbillosa</i> 8-H-28 (H. Adachi <i>et al.</i> , 2015)
Asteltoxin E ¹³⁾		516	$[\alpha]_D^{25} +21.4$ (c 0.72, MeOH)	211 (3.27) 267 (3.05) 300 (2.74) 356 (2.87)	MeOH	Antivirus	<i>Aspergillus</i> sp. SCSIO XWS02F40 (Y. Q. Tian <i>et al.</i> , 2016)
Asteltoxin F ¹³⁾		502	$[\alpha]_D^{25} +20.4$ (c 0.48, MeOH)	210 (3.26) 267 (3.11) 300 (2.53) 356 (2.90)	MeOH	Antivirus	<i>Aspergillus</i> sp. SCSIO XWS02F40 (M. Wang <i>et al.</i> , 2015)
Asteltoxin G ⁵⁾		334	$[\alpha]_D^{25} +7.75$ (c 0.20, MeOH)	N/A	MeOH	ND	<i>Aspergillus ochraceopetaliformis</i> (J. T. Liu <i>et al.</i> , 2018)

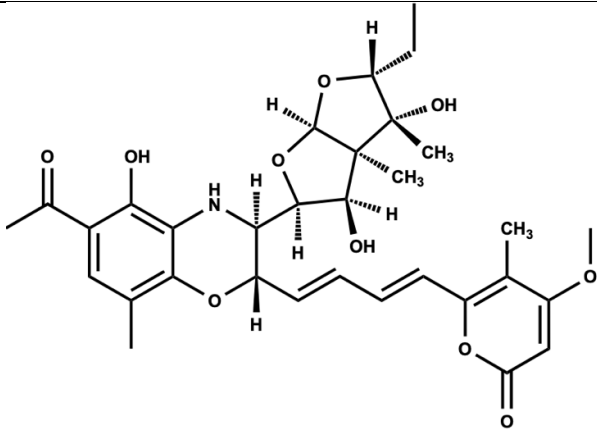
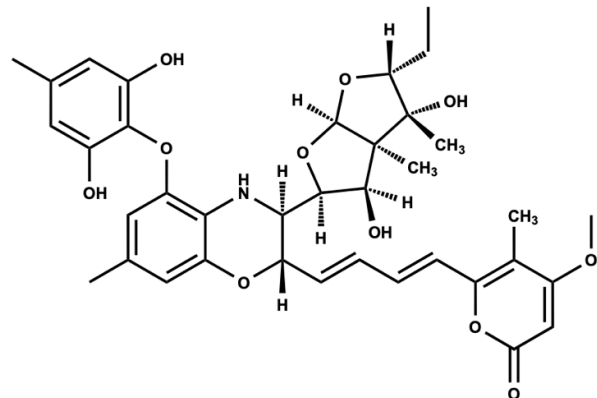
ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structure	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Mattes's Asteltoxin G		402	$[\alpha]_D^{NP} +48$ (c 0.1, MeOH)	263 358	MeOH	Anticancer	<i>Pochonia suchlasporia</i> (Mattes AO. 2019)
Isoasteltoxin ¹⁵⁾		418	$[\alpha]_D^{25} +31.4$ (c 0.2, MeOH)	221 (3.70) 268 (4.13) 273 (4.13) 365 (4.08)	MeOH	Antivirus	<i>Aspergillus ochraceopetaliformis</i> SCSIO 05702 (Y. Q. Tian <i>et al.</i> , 2016)
ET-1 (Asteltoxin H) ¹¹⁾		402	$[\alpha]_D^{19} +15.4$ (c 0.55, MeOH)	212 (3.95) 283 (4.45) 378 (4.46)	MeOH	Insecticidal activity	<i>Pochonia suchlasporia</i> var. <i>suchlasporia</i> TAMA 87 (S. Syaefudin <i>et al.</i> , 2020)

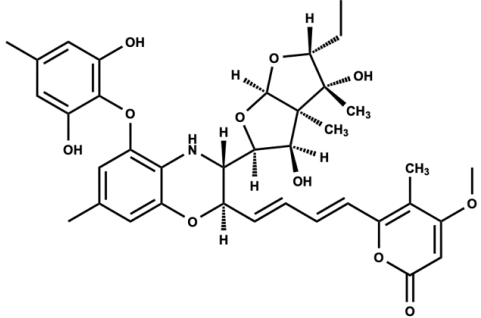
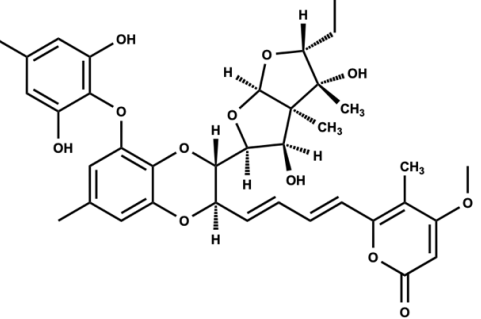
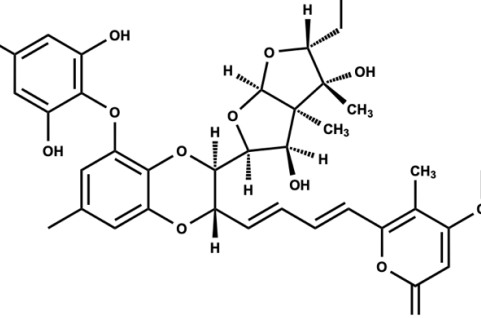
NP- Not Reported, ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structures	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin I ¹⁰⁾		619	$[\alpha]_D^{29} +107$ (c 0.1, MeOH)	242 (4.39), 285 (4.14), 342 (4.21)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation, the HCT116 human cancer cell line inhibition	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755 (C. L. Qin <i>et al.</i> , 2025)
Asteltoxin J ¹⁰⁾		700	$[\alpha]_D^{29} +108$ (c 0.1, MeOH)	205 (4.59), 245 (4.47), 344 (4.13)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755 (C. L. Qin <i>et al.</i> , 2025)

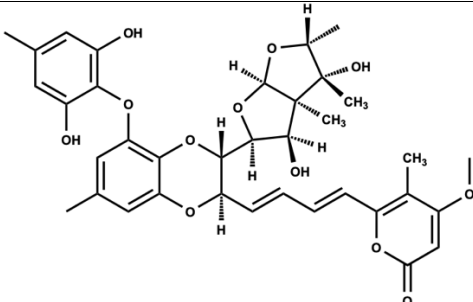
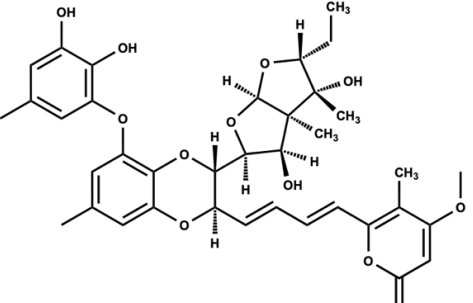
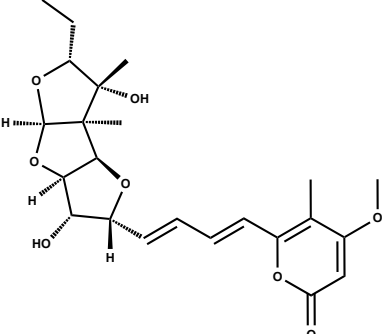
ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structures	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin K ¹⁰⁾		700	$[\alpha]_D^{29} -14$ (c 0.1, MeOH)	205 (4.64), 245 (4.52), 344 (4.18)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin L ¹⁰⁾		701	$[\alpha]_D^{29} -21$ (c 0.1, MeOH)	206 (2.96), 244 (2.72), 344 (2.43)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin M ¹⁰⁾		701	$[\alpha]_D^{29} +106$ (c 0.1, MeOH)	206 (4.58), 243 (4.35), 343 (4.03)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755

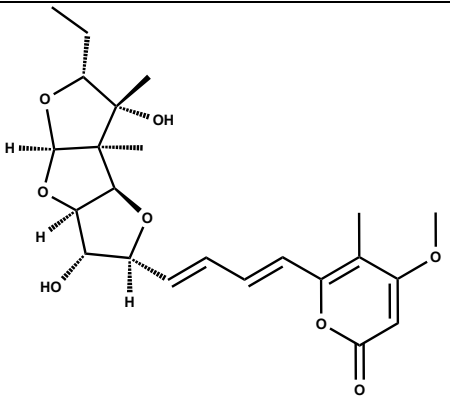
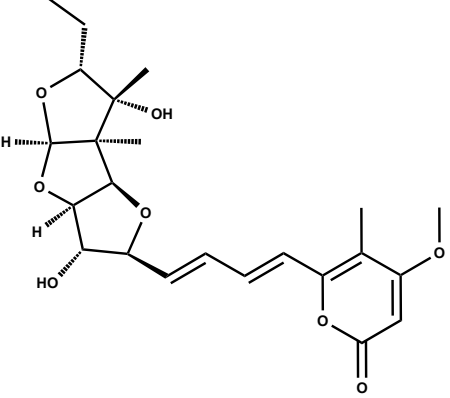
ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structure	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin N ¹⁰⁾		687	$[\alpha]_D^{29} -23$ (c 0.1, MeOH)	207 (4.50), 243 (4.34), 344 (4.03)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin O ¹⁰⁾		701	$[\alpha]_D^{29} -27$ (c 0.1, MeOH)	205 (4.45), 243 (4.26), 343 (3.98)	MeOH	Immunosuppressive against concanavalin A - induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin P ¹⁰⁾		457	$[\alpha]_D^{29} -34$ (c 0.1, MeOH)	246 (3.99), 343 (3.78)	MeOH	ND	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755

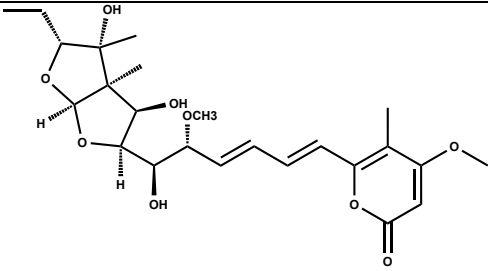
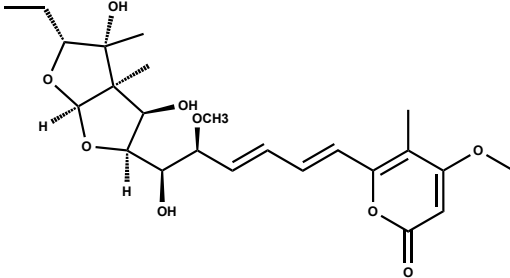
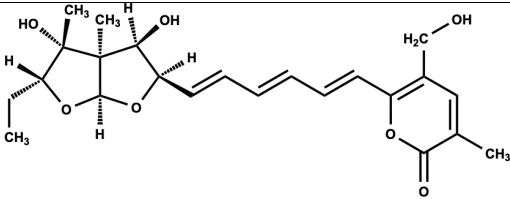
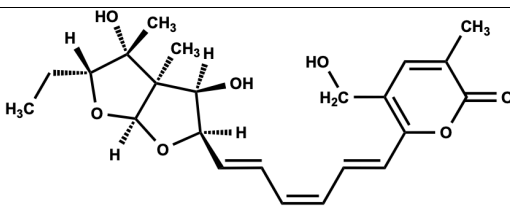
ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structure	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin Q ¹⁰⁾		443	$[\alpha]_D^{29} +55$ (c 0.1, MeOH)	247 (4.36), 342 (4.13)	MeOH	ND	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin R ¹⁰⁾		457	$[\alpha]_D^{29} +15$ (c 0.1, MeOH)	209 (4.20), 225 (4.18), 251 (3.99), 331 (3.86)	MeOH	ND	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755

ND- Not Determined

Table 1-1. Asteltoxin-type compounds and their bioactivities (continued)

Compounds	Chemical structure	MW	$[\alpha]_D$	UV spectra (nm)	Solvent	Bioactivities	Producers (Strains)
Asteltoxin S ¹⁰⁾		489	$[\alpha]_D^{29} + 3$ (c 0.1, MeOH)	248 (2.67), 343 (2.41)	MeOH	ND	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin T ¹⁰⁾		489	$[\alpha]_D^{29} + 24$ (c 0.1, MeOH)	247 (4.35), 342 (4.11)	MeOH	ND	Endophytic fungus <i>Pochonia bulbillosa</i> KNI755
Asteltoxin U (peak 1-b)		418	$[\alpha]_D^{25} + 10.7$ (c 0.15, MeOH)	374 (4.16), 282 (4.14)	MeOH	Inhibition of the first cleavage of fertilized sea urchin embryos	<i>Pochonia</i> <i>suchlasporia</i> var. <i>suchlasporia</i> TAMA 87
Asteltoxin V (peak 1-a)		418	$[\alpha]_D^{25} + 15.7$ (c 0.20, MeOH)	374 (4.13), 282 (4.16)	MeOH	ND	<i>Pochonia</i> <i>suchlasporia</i> var. <i>suchlasporia</i> TAMA 87

ND- Not Determined

1.3. Objective of the study

In this study, the MeOH extract of the SSF culture of *P. suchlasporia* TAMA 87 was purified by solvent extraction, silica gel chromatography, and preparative HPLC to obtain bioactive compounds. Extensive spectroscopic analysis was performed to elucidate their chemical structures. Their inhibitory activities toward the first cleavage of sea urchin embryos were evaluated.

Chapter 2. Solid-state Fermentation of *Pochonia suchlasporia* TAMA 87

2.1. Introduction

Searching for biologically based insect controls has increased due to public demand for more human and environment-friendly chemical compounds. One of the promising agrochemicals sources is microorganism. Interest in microbial metabolites as insecticides arises from attempts to solve a dilemma in pest control management, environmental issues and uncontrollable crop-harming insects (Y. Tanaka & S. Omura, 1993). Beside of their versatility in structure and activity, microbial metabolites are expected to become more degradable (Y. Tanaka & S. Omura, 1993). Moreover, microbial cultivation commonly needs less time compared with nurturing the plants.

Among the promising microorganisms, *Pochonia suchlasporia* TAMA 87 is expected to be a new source of chemical compounds with insecticidal activities. Previously, our laboratory reported that this fungus could produce pochonicine, a novel compound with potent inhibition toward β -N-acetylglucosaminidases (GlcNAcases) of various organisms including insects, fungi, mammals, and a plant but no inhibition against β -glucosidase of almond, α -glucosidase of yeast, or chitinase of *Bacillus* sp. (H. Usuki *et al.*, 2009). Since GlcNAcase inhibition would affect to the normal growth of insect, searching for insecticidal compounds produced by this fungus seems to be interesting. Besides, from the EtOAc soluble-fraction, ET-1, with a unique α -pyrone moiety and insecticidal activity, was isolated. Besides, the EtOAc soluble-fraction showed many compounds which show similar UV spectrum to that of ET-1 and peak 1 is one of our target compounds with much shorter retention time compared to that of ET-1. Therefore, to purification of peak 1, the cultivation and incubation of *P. suchlasporia* TAMA 87 in rolled barley-based solid medium was performed.

2.2. Materials and Methods

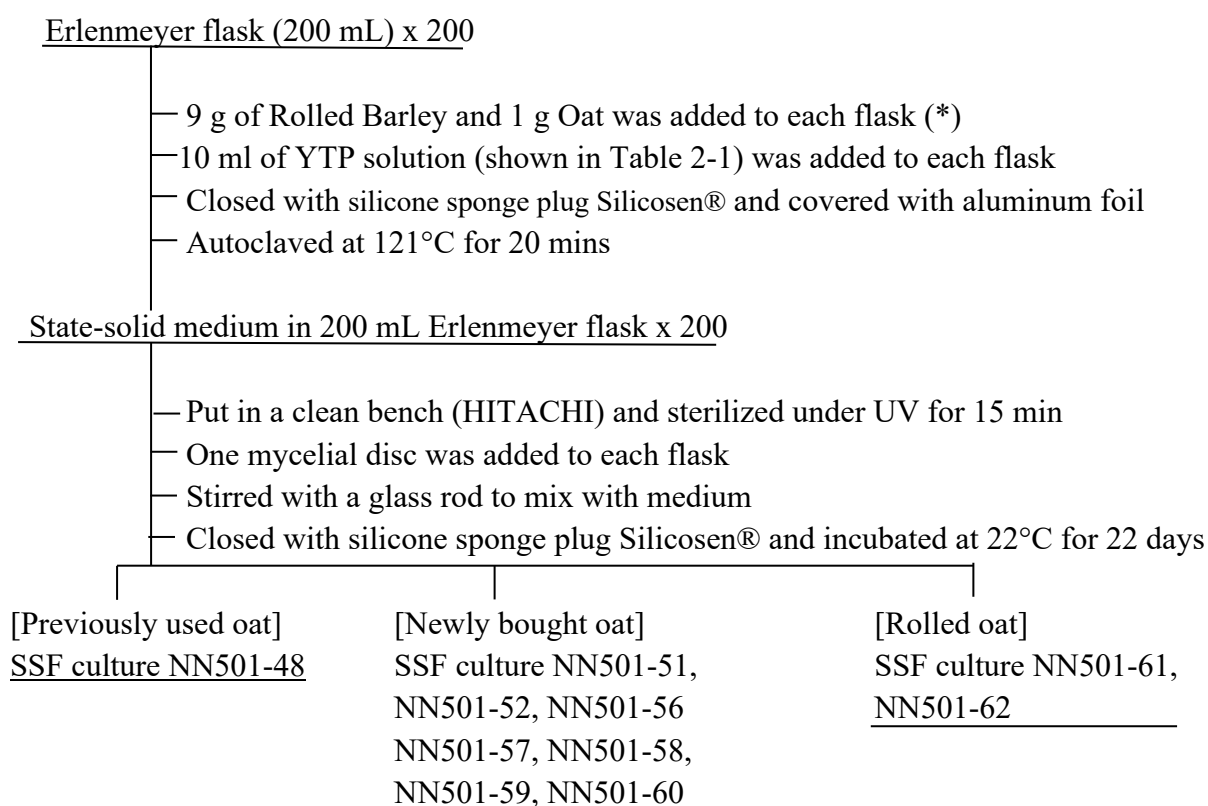
2.2.1. Fungal strain

Pochonia suchlasporia TAMA 87 was used for insecticidal assay. This fungal strain was leased from the HypaGenesis, Inc. The isolation and identification of this fungus have been reported by Usuki *et al.* (2009). *P. suchlasporia* TAMA 87 was deposited at the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan as FERM AP-21204 (H. Usuki *et al.*, 2009).

2.2.2. Solid-state fermentation

The *P. suchlasporia* TAMA 87 culture was grown on a yeast extract agar slant. An aliquot of the stock culture was sub-cultured in a Petri dish with a diameter of 9 cm that contained a malt yeast agar peptone (MYA-P) medium consisting of 1% (w/v) glucose, 1% (w/v) malt extract (Becton, Dickinson and Company, Sparks, MD, USA), 0.1% (w/v) yeast extract (Nacalai Tesque Inc., Kyoto, Japan), 0.1% (w/v) Hipolypepton (Nihon Seiyaku, Tokyo, Japan), and 2% (w/v) agar

(Nacalai Tesque Inc., Kyoto, Japan). After eight days of incubation at 22°C, discs 7 mm in diameter were cut from the growing edge of the fungal colony.



* Oat used in samples were three different types which displayed in the Table 2-2

Note: Each sample same contained 20 SSF cultures flasks, totally 10 samples.

Scheme 2-1. Preparation of solid-sate fermentation culture of *P. suchlasporia* TAMA 87.

Table 2-1. Composition of YTP solution

Distilled water	500 ml
Yeast extract	1 g
KH ₂ PO ₄	0.5 g
Sodium L-tartrate dihydrate	0.5 g

Table 2-2. The Oat types used to prepare the solid-state fermentation culture

Type of Oat			
			
Brand	Peeled oats - DoggyMan H.A Co., Ltd., Osaka, Japan	Peeled oats - DoggyMan H.A Co., Ltd., Osaka, Japan (Made in Canada)	Rolled oats – Quaker PepsiCo, Inc., NY, America
Sample	NN501-48	NN501-51, NN501-52, NN501-56, NN501-57, NN501-58, NN501-59, NN501-60	NN501-61, NN501- 62

A fungal disc was inoculated into each of twenty different Erlenmeyer flasks (200 mL), each containing an autoclaved rolled barley-based solid medium consisting of 9 g of rolled barley (Kyowa Seibaku Co., Kanagawa, Japan), 1 g of peeled oats (Table 2-2) 10 mL of water, 20 mg of yeast extract (Nacalai Tesque Inc., Kyoto, Japan), 10 mg of sodium L-tartrate dihydrate, and 10 mg of KH_2PO_4 . Fermentation was performed for 22 days in a static condition at 22°C.

2.2.3. Solid-state fermentation culture

P. suchlasporia TAMA 87 culture was extracted by adding 50 mL of MeOH to each flask, shaking the flasks well, and keeping them overnight at room temperature. A certain portion of the SSF culture was concentrated to dryness and weighed. The total yield weight was calculated out from the weight of the portion. The remaining SSF culture was then filtered by filter paper (ADVANTEC No. 2, 55 mm) and concentrated *in vacuo* to give an MeOH extract (31.5 g) in total 10 samples (20 culture flasks per sample). The MeOH extract (in MeOH solution) was kept at -25°C.

Solid-state fermentation culture of *Pochonia suchlasporia* TAMA 87

(in 200 mL Erlenmeyer flask) x 20 flasks

-
- 50 mL of MeOH was added to each flask
 - Stirred homogeneously with glass rod
 - Covered by aluminum foil and kept overnight at room temperature
 - Combined and transferred to 2 L Erlenmeyer flask (each flask was rinsed with MeOH 3 times (total 150 mL of MeOH for 20 flasks) and rinse solution was combined)
 - Vacuum filtered through double-layer filter paper (ADVANTEC® No.2, 125 mm) on a Buchner funnel (φ140 mm).
 - Rinsed with MeOH until the filtrate became almost colorless (approximate 50 mL of MeOH)
 - Concentrated in vacuum to obtained approximately 1200 mL sample

MeOH extract (approximately 1200 mL yellow-colored solution)

Scheme 2-2. Preparation for MeOH extract of solid-state culture of *P. suchlasporia* TAMA 87

2.3. Results and Discussion

P. suchlasporia TAMA 87 was inoculated onto a malt yeast agar peptone (MYA-P) medium. On MYA-P medium, the fungus grows covered the surface area after 8 days of incubation. The fungal colonies are white color with yellow reverse. A 7 mm diameter mycelial agar disc was cut from the growing edge of the fungal colony to afford a uniform amount of fungus that will be transferred into solid-state fermentation culture system. When the fungal disc will not be used directly for bioactive compound(s) production, it can be stored in a glycerol solution and then kept it at -80°C. Practically, the fungal disc can be used after 1 year of storage.

Production of bioactive compounds was done under static conditions after inoculating a fungal disc into a 200 mL of Erlenmeyer flask, containing rolled barley-based solid medium. Similar to the growth of *P. suchlasporia* TAMA 87 on MYA-P medium, all of surface areas of the rolled barley-based solid medium were covered by the mycelium of the fungus. As observed on the top side of solid medium, the fungal colonies are white, and some parts also appeared in yellow color. Meanwhile, fungal colonies are mostly yellow color if they are observed from the bottom side of solid-medium. The color of fungal colonies, either on the top side and bottom side of solid medium, becomes more yellow in longer incubation (Figure 2-1).

After 22 days of incubation, solid-state fermentation (SSF) cultures of *P. suchlasporia* TAMA 87 were extracted with MeOH. By adding 50 mL of MeOH to each flask (200 flasks in total), shaking the flasks well, and keeping them overnight at room temperature, chemical compounds in the SSF culture were extracted. Microbial metabolites-containing MeOH solvent

was then filtered and concentrated to afford a MeOH extract with a yield of 31.5 g from 10 samples (20 flasks of SSF each sample).

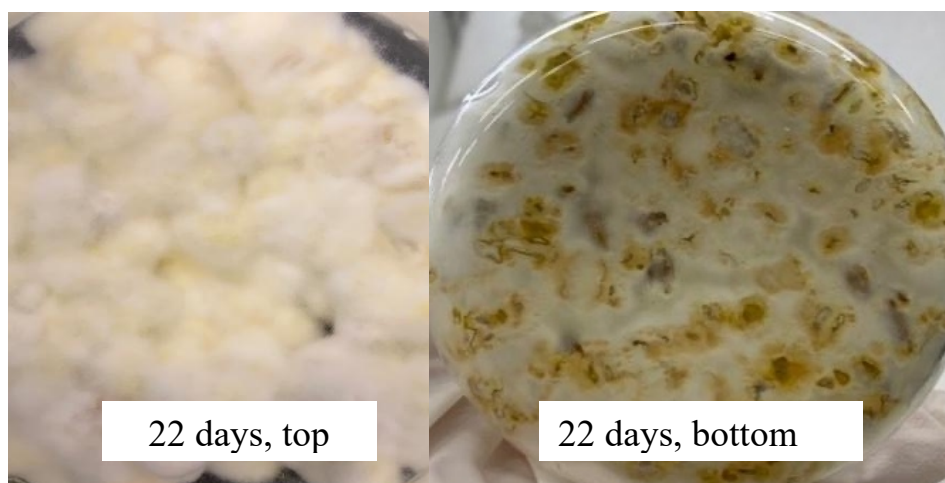


Figure 2-1. *P. suchlasporia* TAMA 87 on the rolled barley-based medium for 22 days of fermentation.

Chapter 3. Purification and Isolation of peak 1-a and peak 1-b produced by *Pochonia suchlasporia* TAMA 87

3.1. Introduction

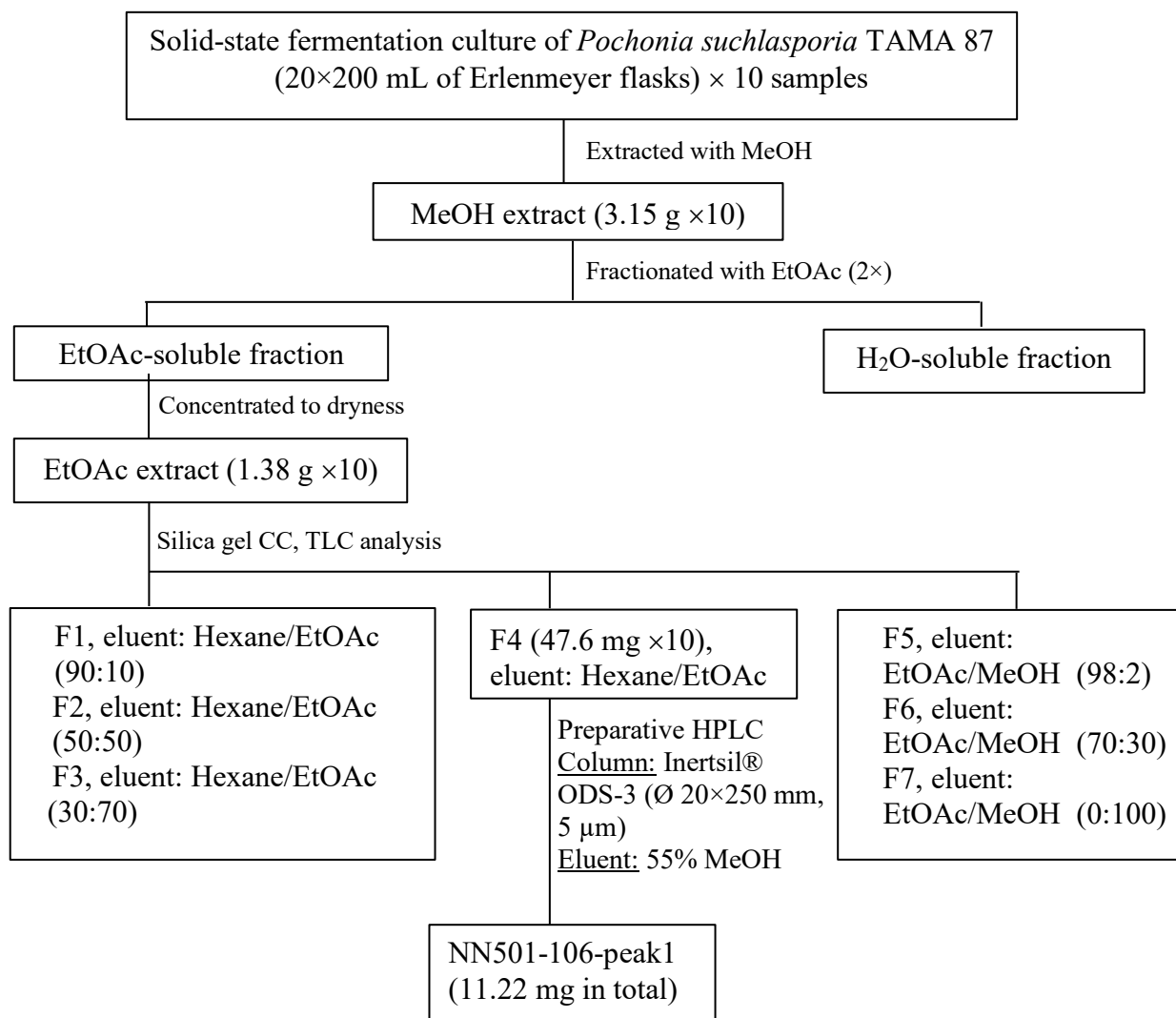
Bioactive compounds are most often present in complex mixtures that may contain multiple active constituents with different modes of action, or with synergistic and/or antagonistic effects occurring simultaneously. To analyze the biological activity of such mixtures and identify active compounds, various strategies have been developed. One of the most successful approaches is bioassay-guided purification, in which compounds are isolated step by step based on their biological activity until a pure bioactive compound is obtained. Related to this study, a preliminary study revealed that the EtOAc extract obtained from the purification of fermentation cultures. Based on the result from LC-MS/MS analyzed in chapter 1, the target compound of this study is peak 1, belongs to fraction 4, which has a high possibility of possessing the same α -pyrone moiety as ET-1.

Some purification techniques, silica gel column chromatography (silica gel CC), thin layer chromatography (TLC analysis), analytical high-performance liquid chromatography (analytical HPLC) and preparative high-performance liquid chromatography (preparative HPLC), were performed to target compound(s). Detailed purification methods, including isolation of the target compound(s) and purity analysis, are described in this chapter.

3.2. Materials and Methods

3.2.1. Purification of the bioactive compounds produced by *Pochonia suchlasporia* TAMA 87

EtOAc-H₂O partition of MeOH extract was described in Scheme 2.2. Each sample was obtained from 20 SSF flasks, total 10 samples. Approximately 1.2 L Erlenmeyer flask per sample containing about 200 mL H₂O (rolled barley solid medium of 20 flasks (10 mL medium x 20 flasks)). Therefore, MeOH was removed until the amount of residual aqueous solution was less than 200 mL. The MeOH extract (3.15 g) obtained from the production step was then partitioned between EtOAc and water. The EtOAc-soluble fraction was concentrated *in vacuo* to yield an EtOAc extract (1.38 mg), which was subsequently purified by silica gel column chromatography using hexane/EtOAc and EtOAc/MeOH to afford seven fractions (Fr1 - Fr7). The procedure for the purification was summarized in Figure 3-1. Meanwhile, condition for the silica gel column chromatography was summarized in Table 3-1. F4 (4.76 mg), which was eluted by the 10:90 (v/v) hexane/EtOAc mixture, was further purified by preparative HPLC to obtain peak 1. Peak 1 was then combined together in 10 samples to get NN501-106-peak 1 (11.22 mg). Conditions for the preparative HPLC were summarized in Table 3-2.



Scheme 3-1. The purification and isolation of peak 1

Table 3-1. Condition for silica gel chromatography

Sample volume	NN501-48-EtOAc soluble fraction (1.26 g in 20 mL MeOH) NN501-51-EtOAc soluble fraction (1.32 g in 20 mL MeOH) NN501-52-EtOAc soluble fraction (1.33 g in 20 mL MeOH) NN501-56-EtOAc soluble fraction (1.40 g in 20 mL MeOH) NN501-57-EtOAc soluble fraction (1.36 g in 20 mL MeOH) NN501-58-EtOAc soluble fraction (1.34 g in 20 mL MeOH) NN501-59-EtOAc soluble fraction (1.43 g in 20 mL MeOH) NN501-60-EtOAc soluble fraction (1.35 g in 20 mL MeOH) NN501-61-EtOAc soluble fraction (1.33 g in 20 mL MeOH) NN501-62-EtOAc soluble fraction (1.34 g in 20 mL MeOH)
Sample adsorbent	cellulose powder A (ADVANTEC [®] , 100-200 mesh)
Silica gel	Wakogel C-100 (particle size 150 ~ 425 µm, 10% (w/v)) Silica gel activation by Ngoc

Eluent and fractionation (*)	Hexane: EtOAc = 90:10	300	mL	3	beds/Fr.1
	Hexane: EtOAc = 50:50	300	mL	3	beds/Fr.2
	Hexane: EtOAc = 30:70	100	mL	3×1	beds/Fr.3-1~3-3
	Hexane: EtOAc = 10:90	300	mL	3	beds/Fr.4
	EtOAc: MeOH = 98:2	300	mL	3	beds/Fr.5
	EtOAc: MeOH = 70:30	300	mL	3	beds/Fr.6
	MeOH = 100	300	mL	3	beds/Fr.7

(*) For a bed, it needs eluent about (volume in cm³ or mL):

1 bed = $\pi \times (\text{diameter of column (in cm)}/2)^2 \times h$; h: height of silica gel (in cm)

Table 3-2. Condition for preparative ODS-HPLC

Pump	Waters 616 (Nihon Waters, Tokyo, Japan)
Controller	Waters 600 (Nihon Waters, Tokyo, Japan)
Column	Inertsil® ODS-3, Ø 20 × 250 mm, 5 µm (GL Sciences, Tokyo, Japan)
Column oven	Waters CHM (Nihon Waters, Tokyo, Japan)
Column temperature	30°C
Integrator	HITACHI D-2500 (Hitachi, Tokyo, Japan)
Detector	Waters 486 (Nihon Waters, Tokyo, Japan)
Detection wavelength	254 nm
Eluent	55% MeOH
Flowrate	10 mL/min
Fraction size	12 sec/fraction

Fractions obtained from the silica gel column chromatography were then analyzed for their purities by thin-layer chromatography (TLC) and analytical high-performance liquid chromatography (analytical HPLC). Analytical HPLC was also used to determine the purity of fraction 4 obtained from silica gel column chromatography and the purity of compounds after the preparative HPLC purification step. Detailed experiments for TLC and HPLC were as follows.

This method was performed to investigate the separation quality of the silica gel column chromatography. In addition, the purity of the active compounds from the obtained fractions could also be determined. This method can also be used to optimize the solvent system that would be applied for column chromatography. However, the solvent system for the TLC and silica gel column chromatography has been optimized by Takatsuji (Master Thesis of Takatsuji 2015).

To achieve the purposes, fractions from the silica gel column chromatography must be evaporated to remove all the solvents and then dissolved in MeOH. Each fraction was spotted on the TLC plate with a capillary pipette and developed with the 20:80 (v/v) hexane/EtOAc mixture in the chamber under saturated conditions. The presence of bioactive compounds was observed under the short-wave (254 nm) and long-wave (352 nm) UV light exposure. As a control, EtOAc extract of the solid-state fermentation culture (sample subjected to the silica gel column

chromatography) was used. Condition for the TLC analysis was summarized in Table 3-3. Ratio of the distance the analyte (spot) travelled versus the distance the eluent travelled was determined as a retardation factor (Rf).

Table 3-3. Condition for TLC analysis

Sample	EtOAc soluble fraction
	Fr. 1 ~ Fr. 7, @ 2 μ L
TLC plate	Silica gel 60 F254 (Merck Ltd., JPN, 1.05554) Pre-washed with MeOH: EtOAc = 1:1 (v/v)
Detection	UV ₂₅₄ () UV ₃₅₂ []
Developing solvent	Hexane: EtOAc = 20:80 (v/v)
Visualizing	Anisaldehyde-sulfuric acid

3.2.2. Isolation of target compound by preparative ODS-HPLC

Analytical HPLC was employed to determine the purity of the fractions, either obtained from the silica gel column chromatography or the preparative HPLC. The analytical HPLC was carried out using an Inertsil® ODS-3 column ($\varnothing$ 4.6 $\times$ 250 mm, 5 μ m) (GL Sciences, Tokyo, Japan) and a HITACHI system (Hitachi, Tokyo, Japan) equipped with an L-2300 column oven, L-7455 diode array detector, and an L-2130 pump. The solvent used was 55% MeOH, using an isocratic system. This condition was summarized in Table 3-4.

Table 3-4. Condition for ODS-HPLC analysis

Pump	HITACHI L-2130
Column	Inertsil® ODS-3, $\varnothing$ 4.6 $\times$ 250 mm, 5 μ m
Column oven	HITACHI L-2300
Column temperature	30°C
Detector	HITACHI L-7455
Detection wavelength	200-500 nm, chromatogram detected at 280 nm
Eluent	55% MeOH
Flowrate	0.529 mL/min

Preparative HPLC fractions with high purity of compounds (purity: >95%) were then combined and fractionated with EtOAc (spectroscopic grade) to obtain purified peak 1. The purified peak 1 was dissolved in MeOH (spectroscopic grade) and kept at -25°C. The entire steps for this method were described in Figure 3-2.

Based on the analytical HPLC chromatogram of NN501-48-fr.4, NN501-51-fr.4, NN501-52-fr.4, NN501-56-fr.4, NN501-57-fr.4, NN501-58-fr.4, NN501-59-fr.4, NN501-60-fr.4, NN501-61-fr.4, and NN501-62-fr.4, fractions were collected from the 15th-minute with 10 seconds per fraction to isolate peak 1, peak 2 and peak 3. Sample preparations and conditions for preparative HPLC are described in Scheme 13-6.

Fr. 4 samples (in Table 3-5)*

- Concentrated to dryness (Evaporator, EYELA NVC-1100)
- Dissolved in 1235 μ L MeOH
- Added with 665 μ L deionized H₂O

Fr.4 in 65% MeOH (1.9 mL)

- Centrifuged (KUBOTA 3740, 10000 rpm, 10 min, 4°C)
- Preparative HPLC (condition was summarized in Table 3-6)
- Kept at -25°C

* Fr. 4 samples were purified separately (totally 10 samples).

Scheme 3-2. Sample preparation of NN501-48-fr.4, NN501-51-fr.4, NN501-52-fr.4, NN501-56-fr.4, NN501-57-fr.4, NN501-58-fr.4, NN501-59-fr.4, NN501-60-fr.4, NN501-61-fr.4 and NN501-62-fr.4 for preparative HPLC

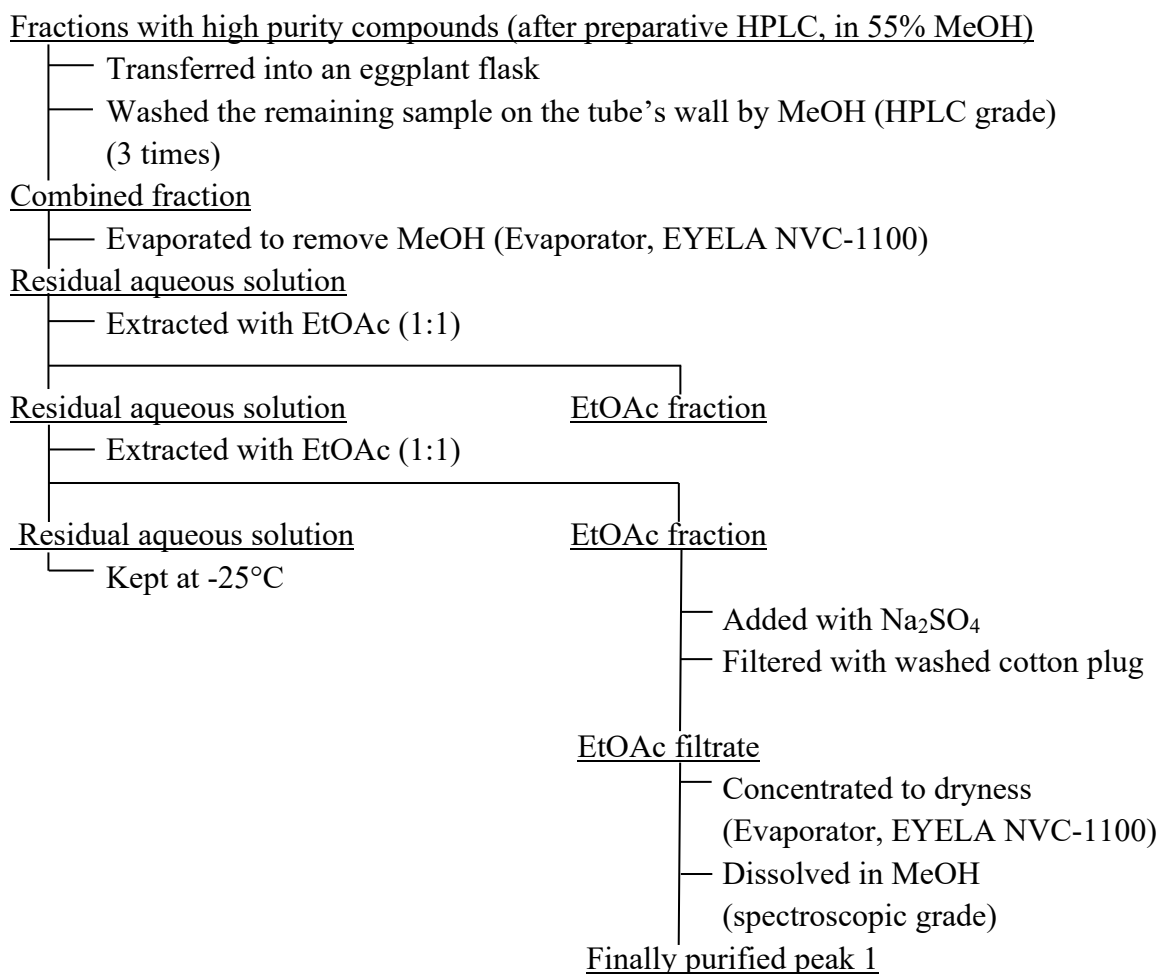
Table 3-5. The sample name and weight (dissolved in 5 mL MeOH)

Sample	NN501-48-fr.4	NN501-51-fr.4	NN501-52-fr.4	NN501-56-fr.4	NN501-57-fr.4
Weight (mg/5 mL MeOH)	45.14	56.79	54.40	49.75	51.25

Sample	NN501-58-fr.4	NN501-59-fr.4	NN501-60-fr.4	NN501-61-fr.4	NN501-62-fr.4
Weight (mg/5 mL MeOH)	47.33	51.65	49.91	47.41	49.87

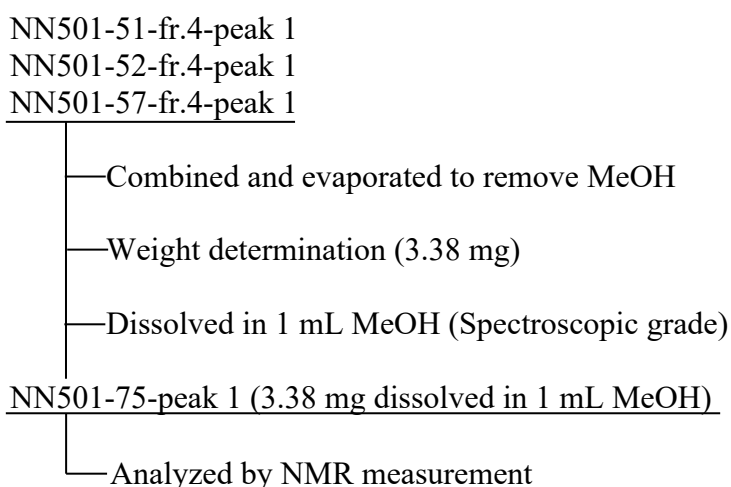
Table 3-6. Condition for preparative ODS-HPLC

Pump	Waters 616
Controller	Waters 600
Column	Inertsil ODS-3, $\varnothing$ 20 $\times$ 250 mm
Column oven	Waters CHM
Column temperature	30°C
Integrator	HITACHI D-2500
Detector	Waters 486
Detection wavelength	254 nm
Eluent	55% MeOH
Flowrate	10 mL/min
Injection volume	1.9 mL
Sample	NN501-48-fr.4 (45.14 mg), NN501-51-fr.4 (56.97 mg), NN501-52-fr.4 (54.40 mg), NN501-56-fr.4 (49.75 mg), NN501-57-fr.4 (51.25 mg), NN501-58-fr.4 (47.41 mg), NN501-59-fr.4 (51.65 mg), NN501-60-fr.4 (49.91 mg), NN501-61-fr.4 (47.41 mg), NN501-62-fr.4 (49.87 mg),
Fraction size	10 seconds/fraction (1.67 mL/fr)

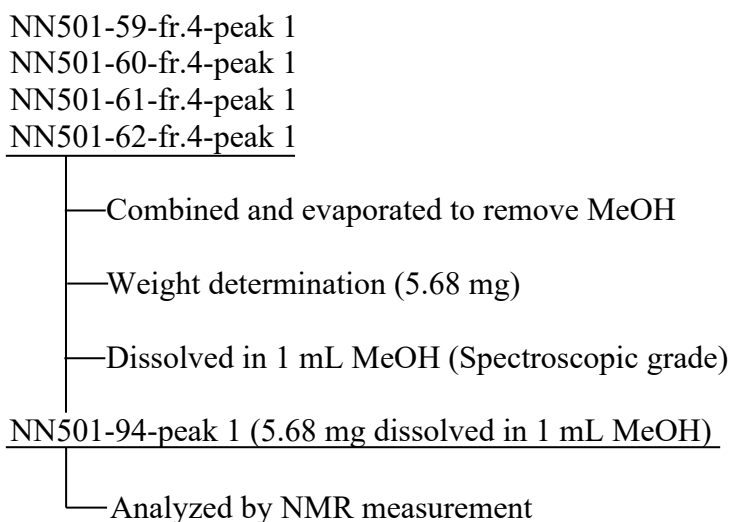


Scheme 3-3. Fractionation of combined fraction with high purity of peak 1 (repeated at 10 times with ten obtained fr.4 samples)

After obtained peak 1, peak 1 was analysed by NMR measurement. Due to the many signals in the NMR spectra of peak 1 sample NN501-75-peak 1, the other peak 1 sample NN501-94-peak 1 was analyzed again. Sample NN501-75-peak 1 and NN51-94-peak 1 were obtained as the Scheme 3-3 and 3-4.



Scheme 3-4. The combination of NN501-75-peak 1



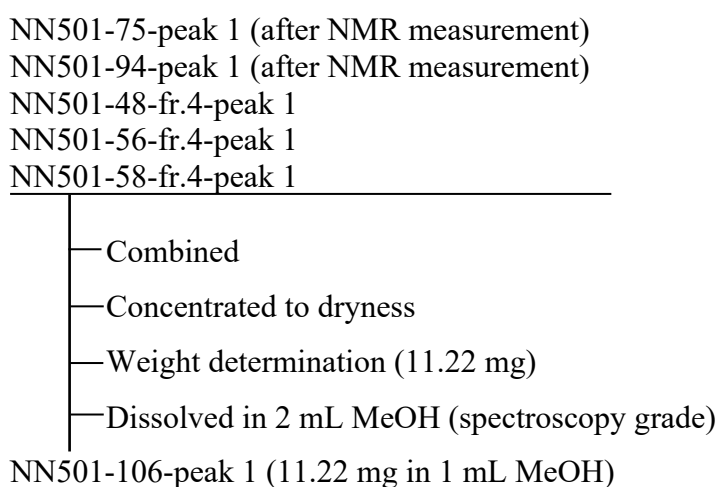
Scheme 3-5. The combination of NN501-94-peak 1

Since the ^1H and ^{13}C NMR spectra of both NN501-75-peak 1 and NN501-94-peak 1 showed signals corresponding to the mixture of two compounds. Therefore, peak 1 was continuously tried to find out the column which could separate the two mixture compounds.

3.2.3. The separation of peak 1-a and peak 1-b from peak 1 by preparative C30-HPLC

To isolate two compounds from peak 1, four different analytical HPLC columns were used to try to separate them. The columns used to analyzed sample peak 1 were Inertsil ODS-3 (Ø 4.6×250 mm), InertSustain AQ-C18 (Ø 4.6×250 mm), Inertsil ODS-HL (Ø 4.6×250 mm) and InertSustain C30 (Ø 4.6×250 mm).

After determining the columns could be used to separate two compounds from peak 1, peak 1 sample was combined from ten samples of peak 1 to obtain 11.22 mg NN501-106-peak 1 (Scheme 3-6).



Scheme 3-6. Combination of NN501-106-peak 1

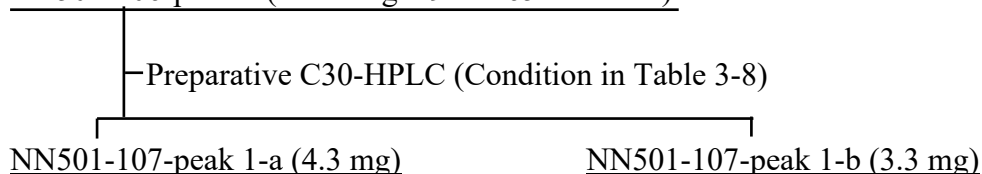
Table 3-7. Condition for ODS-HPLC analysis

Pump	HITACHI L-2130
Column	Inertsil ODS-3, InertSustain AQ-C18 Inertsil ODS-HL, InertSustain C30 (Ø 4.6×250 mm)
Column oven	HITACHI L-2300
Column temperature	30°C
Detector	HITACHI L-7455
Detection wavelength	200-500 nm, chromatogram detected at 280 nm
Eluent	55% MeOH
Flowrate	1.0 mL/min
Injection volume	5 µL

After determining the columns could be used to separate two compounds from peak 1, peak 1 sample was combined from samples NN501-75-peak 1, NN501-94-peak 1, NN501-48-fr.4-peak 1 and NN501-58-peak 1 to obtain 11.22 mg NN501-106-peak 1 (Scheme 3-6). Then, NN501-106-peak 1 was analyzed by analytical HPLC as the conditions in Table 3-8.

After analyzed peak 1 with different columns, peak 1 was observed to separate into two compounds by the InertSustain C30 column. Therefore, InertSustain C30 column was used to isolate two compounds by preparative HPLC (Scheme 3-7).

NN501-106-peak 1 (11.22 mg 1.9 mL 65% MeOH)

**Scheme 3-7.** The separation of peak 1 through preparative C30-HPLC**Table 3-8.** Condition for preparative C30-HPLC

Pump	Waters 616
Controller	Waters 600
Column	Inertsubstain C30, Ø 10×250 mm
Column oven	Waters CHM
Column temperature	30°C
Integrator	HITACHI D-2500
Detector	Waters 486
Detection wavelength	254 nm
Eluent	55% MeOH
Flowrate	5 mL/min
Injection volume	1.8 mL
Sample	NN501-106-peak 1 (11.22 mg)
Fraction size	12 seconds/fraction (1 mL/fr)

3.3. Results and Discussion

The obtained fractions were then subjected to TLC analysis to investigate the separation quality of the silica gel column chromatography. Under short-wave UV light (254 nm), some compounds appeared as dark violet spots on a bright green background. Meanwhile, under long-wave UV light (352 nm) exposure, some bright spots (dark green fluorescence) on a blue dark background appeared (Figure 3-5). Ratio of the distance of the analyte (spot) travelled versus the distance the eluent travelled was determined as a retardation factor (Rf).

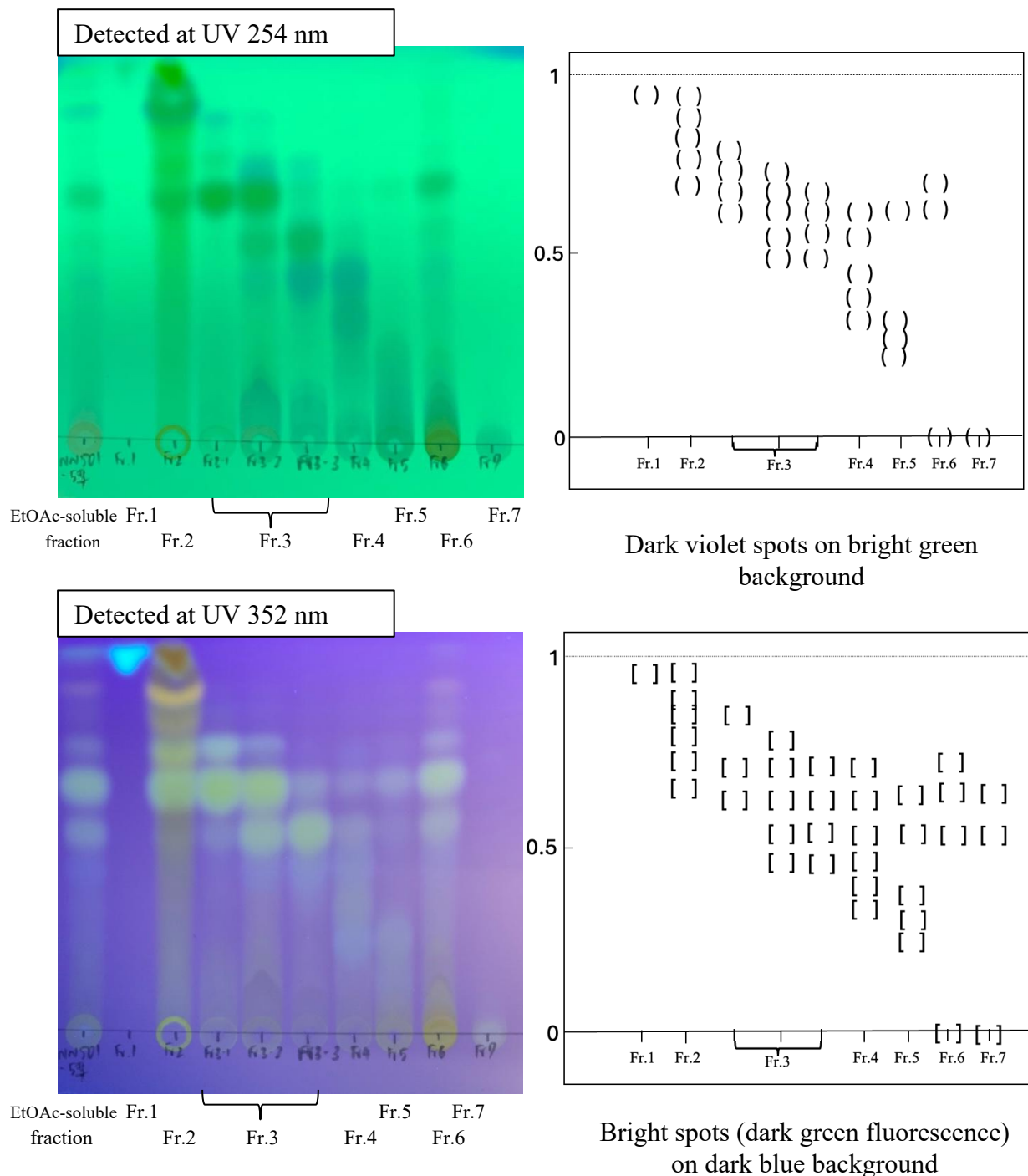


Figure 3-1. TLC chromatogram of the EtOAc soluble fraction and fr. 1 ~ fr. 7 resulted in the silica gel column chromatography. (Cultivated with new bought oat)
 TLC plate : Silica gel 60 F254 (Merck Ltd., Japan, 1.05554).
 Developing solvent : hexane: EtOAc = 20:80, 5 mL

After analyzing by TLC, the observation indicated that in there are several compounds containing in fraction 4 and their spots belonged same *R_f* which indicated that the purification through the silica gel column chromatography showed high effectively.

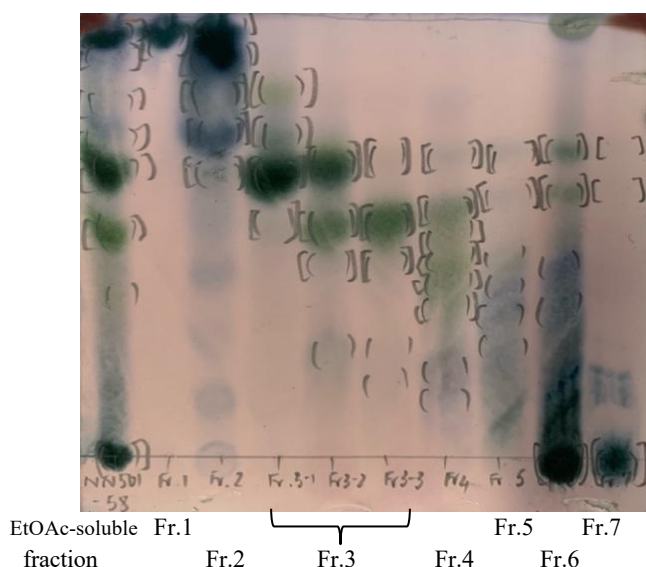
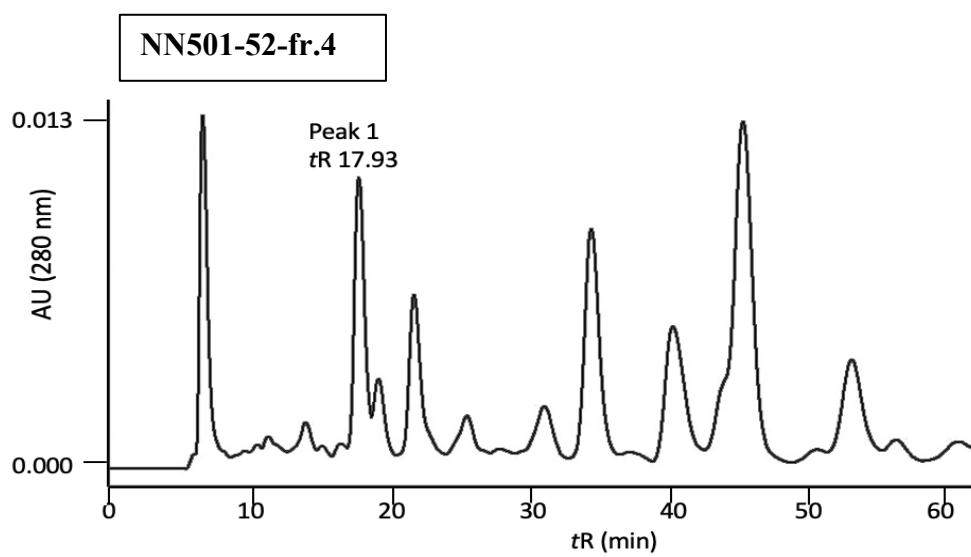
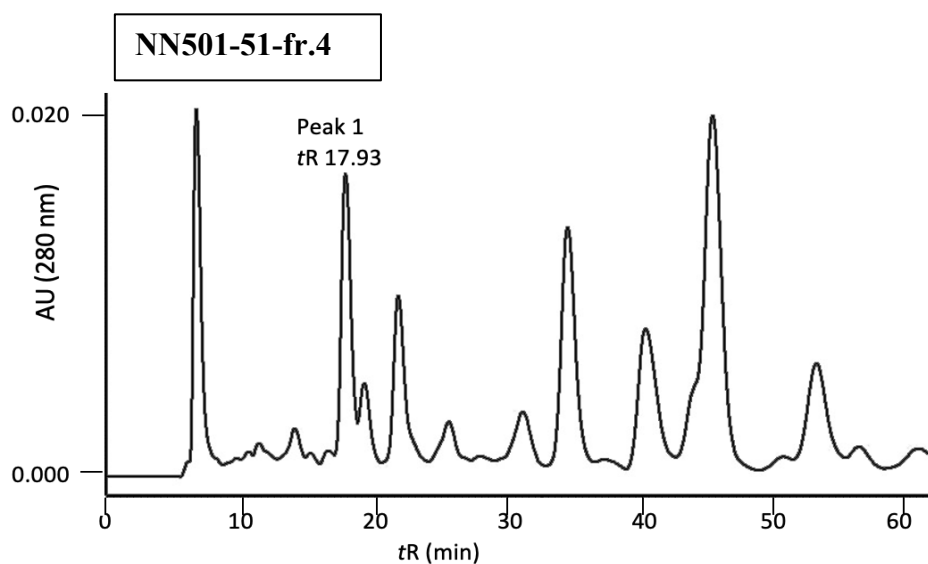
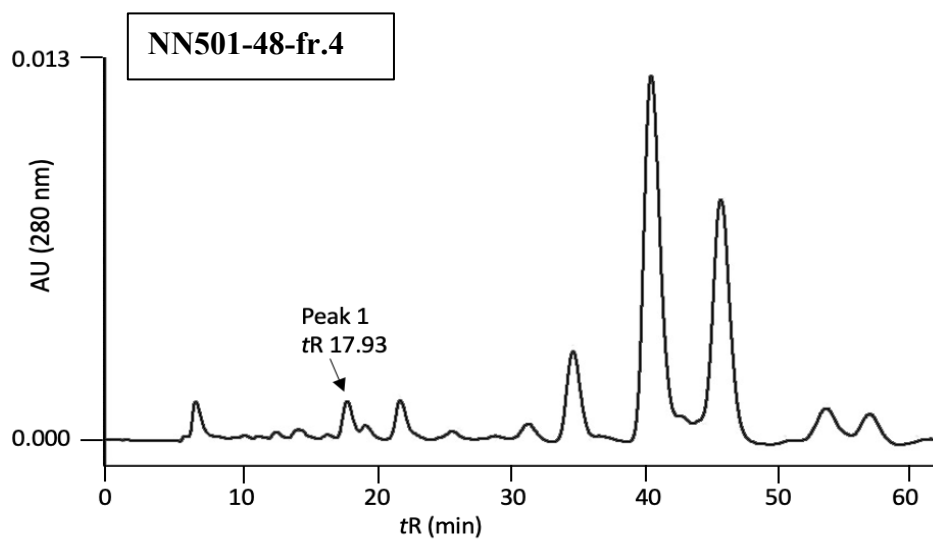
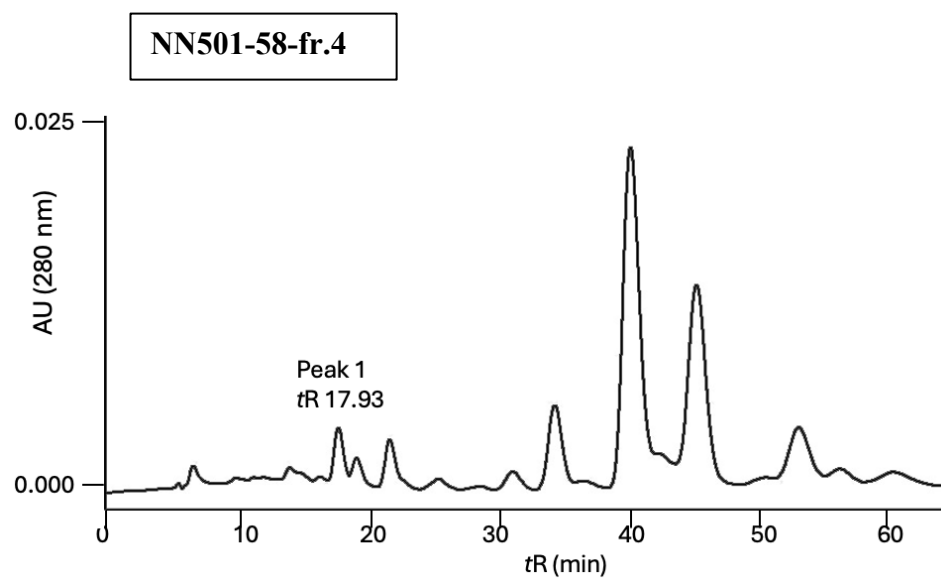
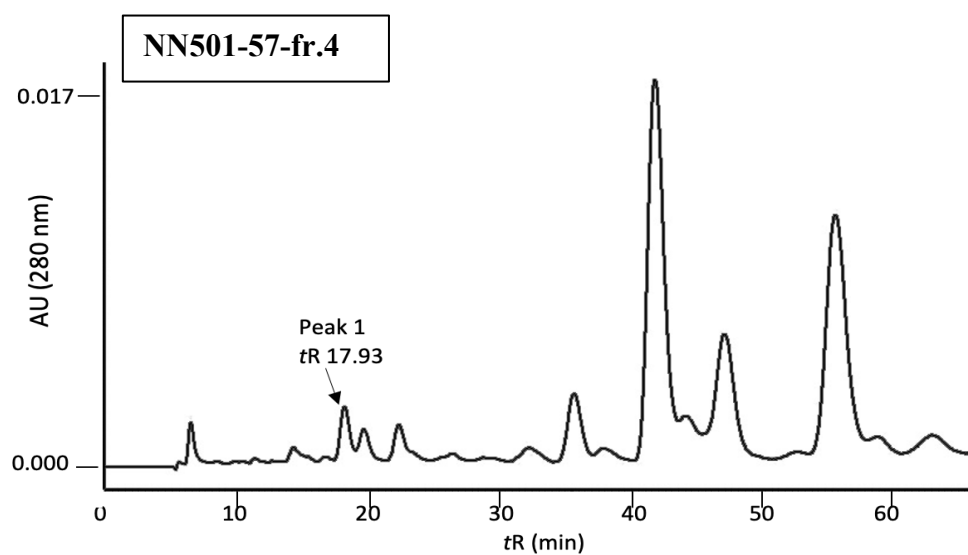
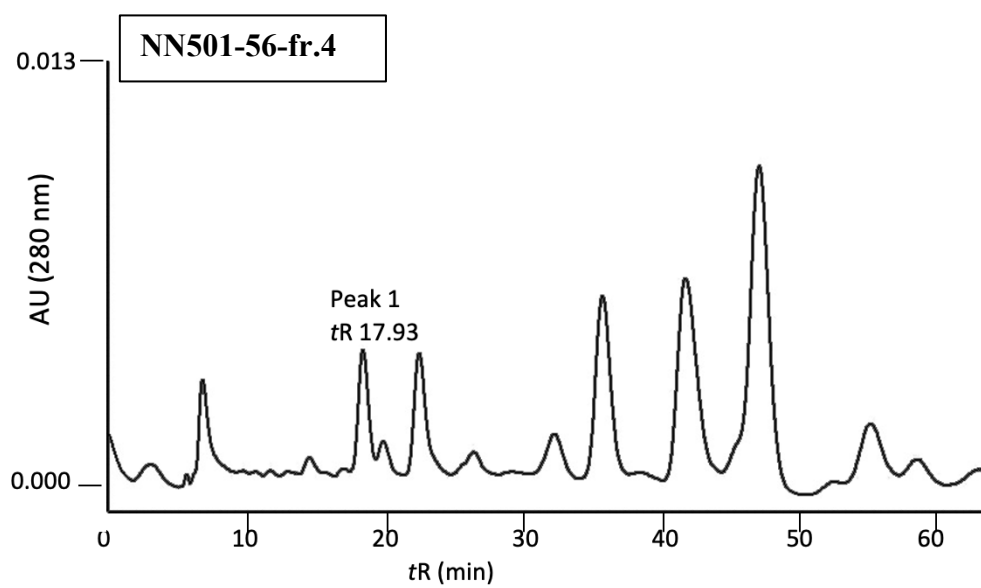


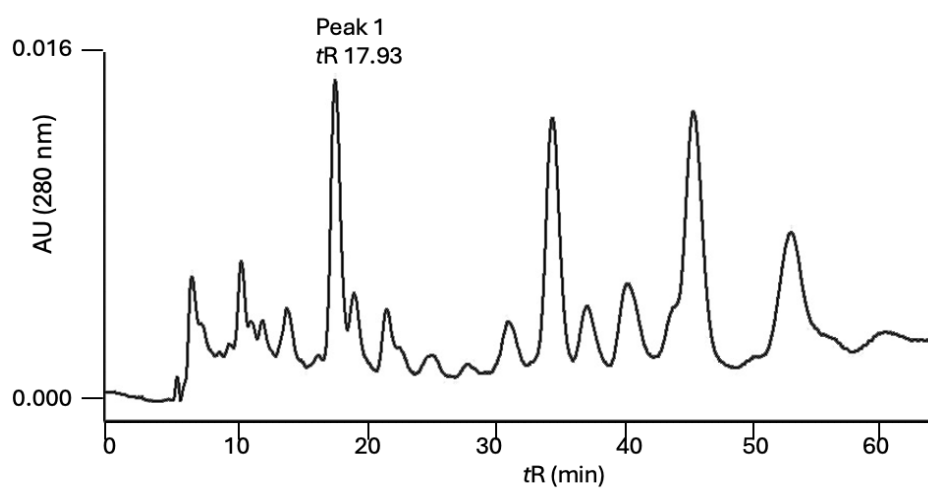
Figure 3-2. TLC chromatogram of the EtOAc soluble-fraction and fr. 1 ~ fr. 7 resulted in the silica gel column chromatography by using anisaldehyde-sulfuric acid in detection method.
 TLC plate: Silica gel 60 F254 (Merck Ltd., Japan, 1.05554)
 Developing solvent: hexane: EtOAc = 20:80 in 5 mL

In the Figure 3-6, TLC chromatogram of fractions resulted from the silica gel column chromatography then visualized by using anisaldehyde-sulfuric acid. The visualization of the TLC results, the spots corresponding to each compound in the fractions were displayed. The dark-green spots were displayed clearly and strongly in the *R_f* 0.62-0.63 (ET-1) and 0.51-0.53 (ET-4) which were two main compounds with high concentration, other spots were also clear. Therefore, the separation of silica gel column chromatography satisfied with the guideline of separation.

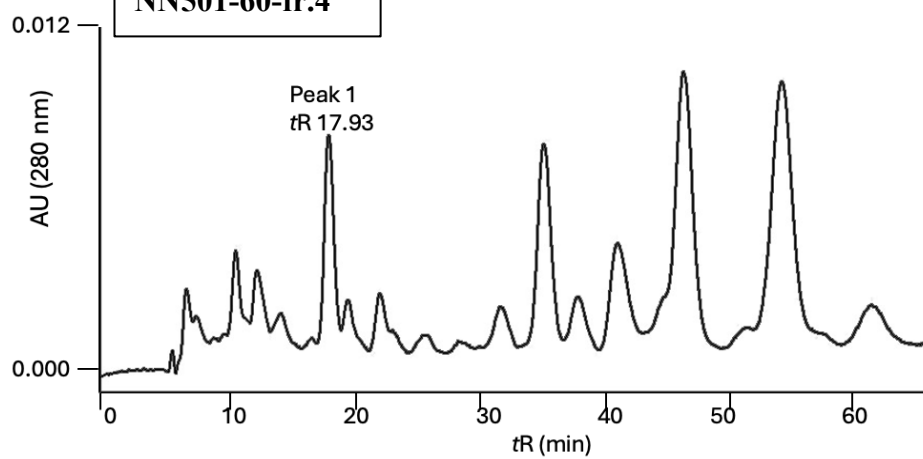




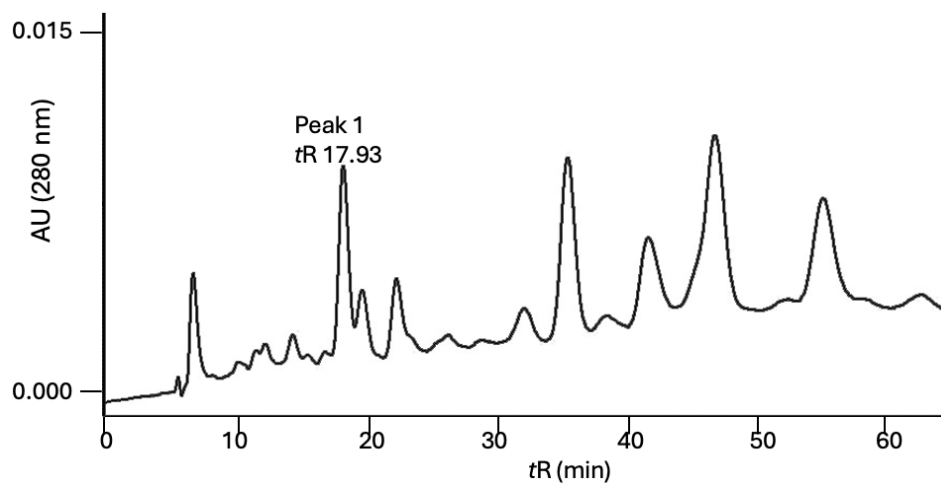
NN501-59-fr.4



NN501-60-fr.4



NN501-61-fr.4



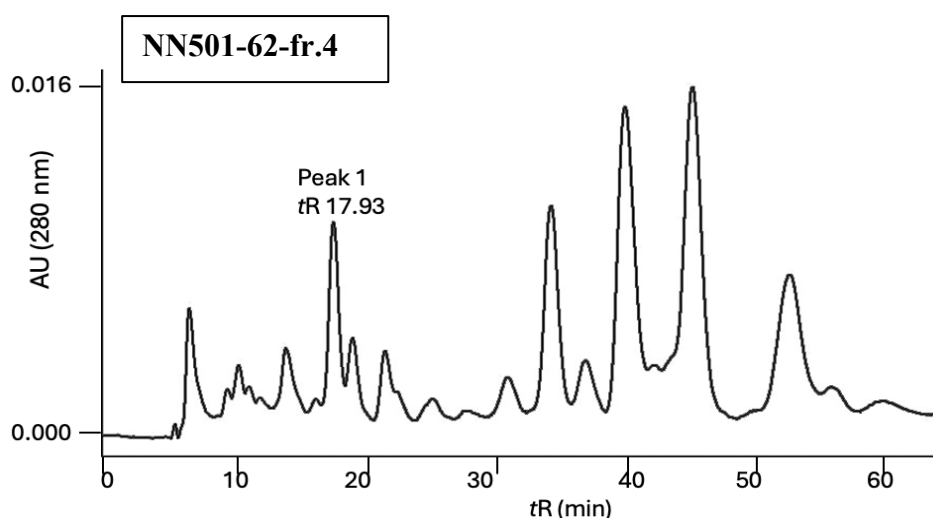


Figure 3-3. The analytical HPLC chromatograms of NN501-48-fr.4, NN501-51-fr.4, NN501-52-fr.4, NN501-56-fr.4, NN501-57-fr.4, NN501-58-fr.4, NN501-59-fr.4, NN501-60-fr.4, NN501-61-fr.4, NN501-62-fr.4.

After isolated by preparative ODS-HPLC, 10 samples of peak 1 were obtained as Table 3-9 below.

Table 3-9. The peak 1 samples name and weight (dissolved in 1 mL MeOH)

Sample	NN501-48-fr.4-peak 1	NN501-51-fr.4-peak 1	NN501-52-fr.4-peak 1	NN501-56-fr.4-peak 1	NN501-57-fr.4-peak 1
Weight (mg/mL MeOH)	1.21	1.27	1.24	0.96	1.02

Sample	NN501-58-fr.4-peak 1	NN501-59-fr.4-peak 1	NN501-60-fr.4-peak 1	NN501-61-fr.40-peak 1	NN501-62-fr.4-peak 1
Weight (mg/mL MeOH)	1.51	1.13	0.93	1.16	1.74

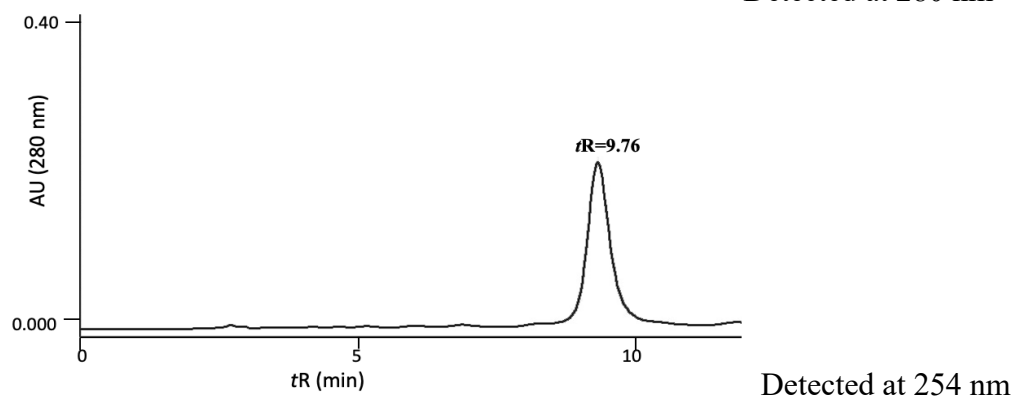
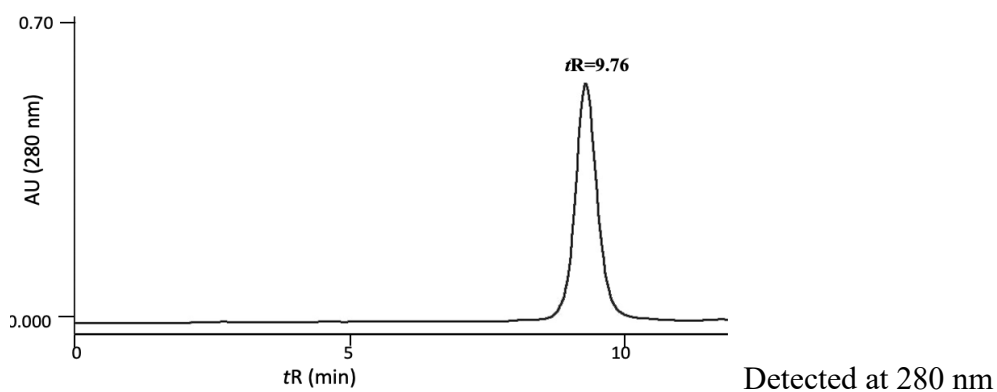
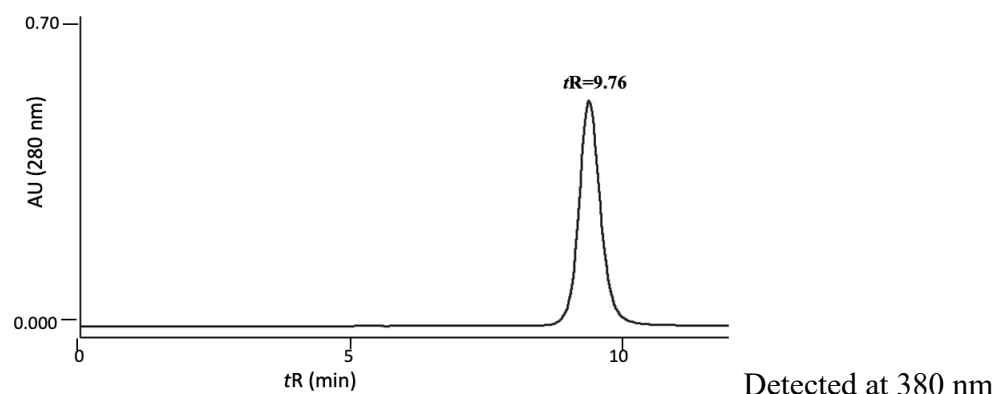
The obtained peak 1 samples were combined and check its purity again before measuring analytical instruments. The NMR measurements of peak 1 were performed in two times due to the complex of peaks in NMR spectra. Two samples (NN501-75-peak 1 and NN501-94-peak 1) used to analyzed was summarized in Scheme 3-8 and Scheme 3-9.

NN501-75- peak 1 (3.44 mg in 1 mL MeOH)

- Placed into a vacuum desiccator and dried
- Dissolved in 0.8 mL CDCl₃ (purity 99.8%)
- NMR measurement (conditions were summarized in Table 3-11)

Scheme 3-8. The sample preparation of NN501-75-peak 1 for NMR analysis

After combination, NN501-75-peak 1 were obtained with 3.44 mg totally. Then the purity of this sample in many different wavelengths was analyzed again by analytical HPLC before analyzing by NMR spectroscopy.



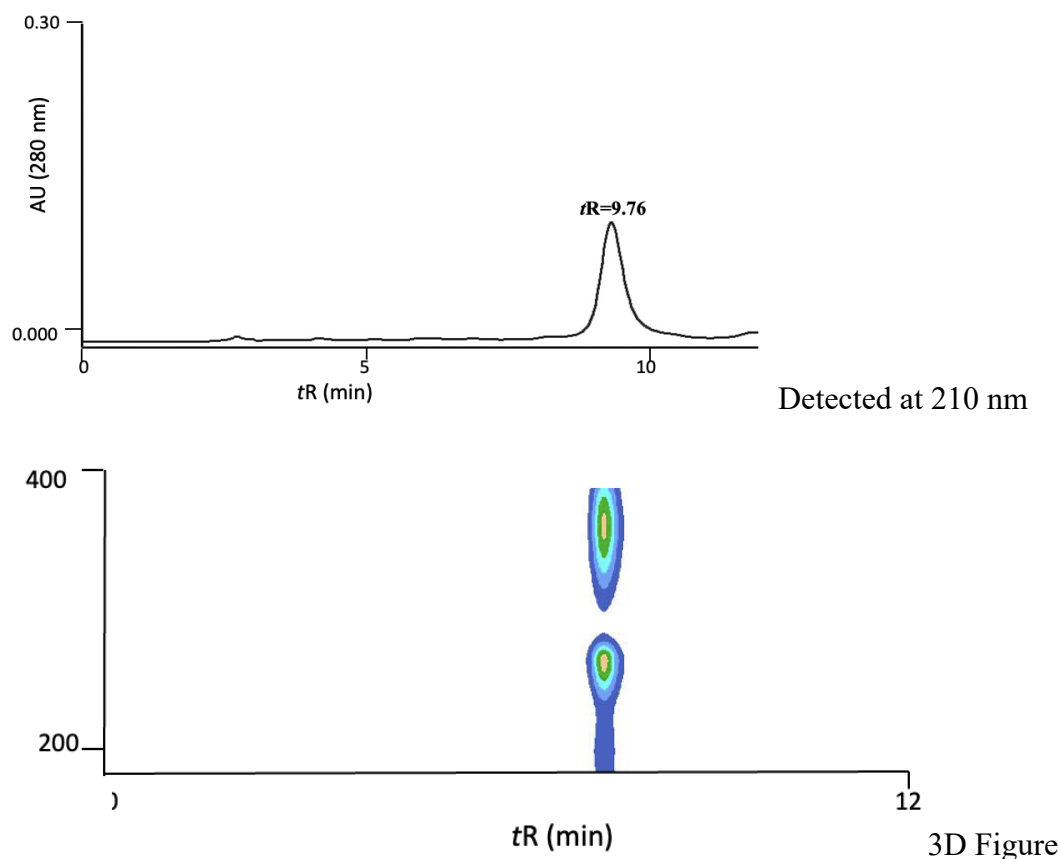


Figure 3-4. The HPLC chromatograms of NN501-75-peak 1 detected at various wavelength.

The peak area and purity of NN501-75-peak 1 were recorded in Table 3-10 below:

Table 3-10. The peak area and purity of NN501-75-peak 1 in different wavelength

NN501-75-peak 1	Peak area	Purity (%)
Detected at 380 nm	7148812	99.57
Detected at 280 nm	7937774	99.22
Detected at 254 nm	3197464	97.24
Detected at 210 n	2731671	95.99

Sample NN501-75-peak 1 with 3.44 mg and 99.57% purity (at 280 nm) was used to NMR measurement.

Table 3-11. Condition for NMR analysis

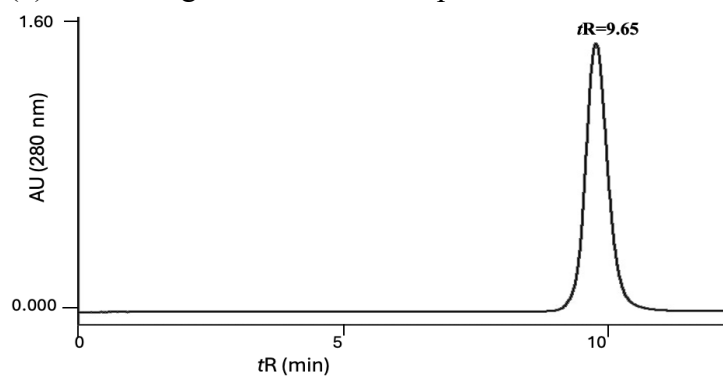
Sample	NN501-75-peak 1 (3.44 mg), NN501-75-peak 1 (5.68 mg)
Instrument	Varian NMR System 6000
Solvent	CDCl_3 0.8 mL
Type of measurement	^1H , ^{13}C , COSY, HSQC, HMBC, NOESY

NN501-94-peak 1 (5.68 mg in 1 mL MeOH)

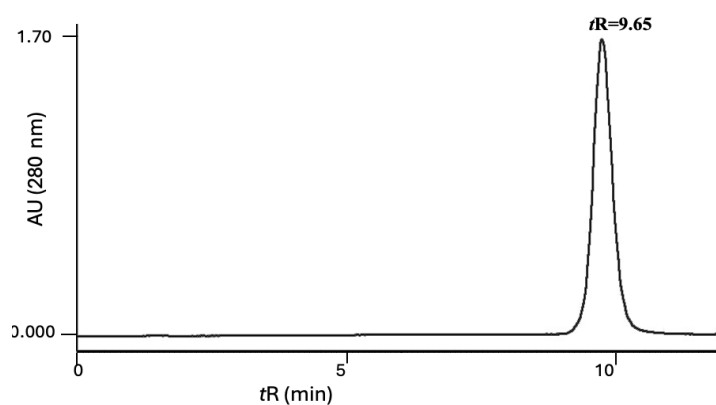
- Placed into a vacuum desiccator and dried
- Dissolved in 0.8 mL CDCl₃ (purity 99.8%)
- NMR measurement (conditions were summarized in Table 3-11)

Scheme 3-9. The sample preparation of NN501-94-peak 1 for NMR analysis

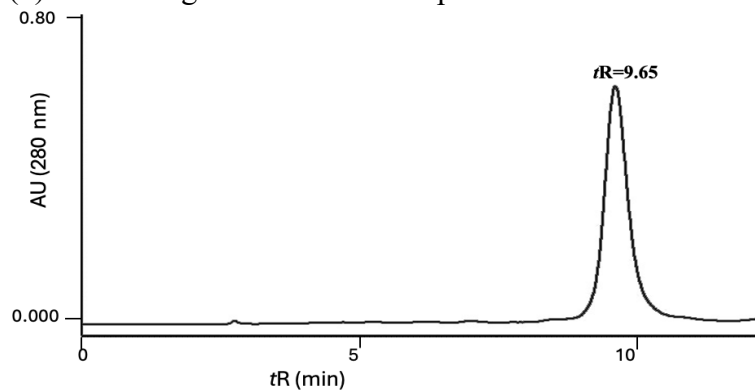
(1) Chromatogram of NN501-94-peak 1 detected at 380 nm



(2) Chromatogram of NN501-94-peak 1 detected at 280 nm



(3) Chromatogram of NN501-94-peak 1 detected at 254 nm



(4) Chromatogram of NN501-94-peak 1 detected at 210 nm

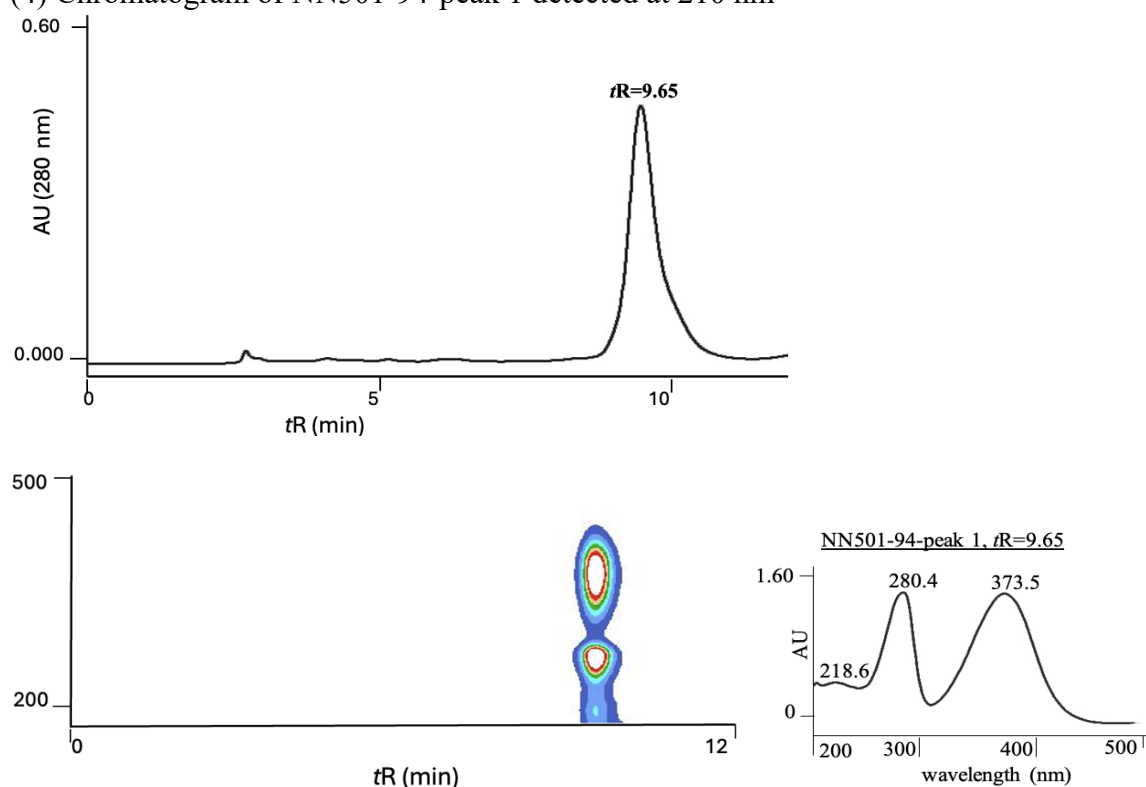


Figure 3-5. The chromatograms of NN501-94-peak 1 detected at 380 nm, 280 nm, 254 nm and 210 nm.

The peak area and purity of NN501-94-peak 1 were recorded in table 3-12 below:

Table 3-12. Peak area and purity of NN501-94-peak 1 in different wavelength.

NN501-94-peak 1	Peak area (in 5 μ L)	Purity (%)
Detected at 380 nm	20668377	99.62
Detected at 280 nm	22440819	98.65
Detected at 254 nm	9744483	96.69
Detected at 210 nm	8496798	95.82

Sample NN501-94-peak 1 with 5.68 mg and 98.65% purity (at 280 nm) was used to NMR measurement.

The NMR spectra of two samples (NN501-75-peak 1 and NN501-94-peak 1) exhibited the double-like signals (Figure 3-10 and Figure 3-11). Therefore, there would contain two compounds mixing in peak 1. Next the separation of two compounds from peak 1 was performed.

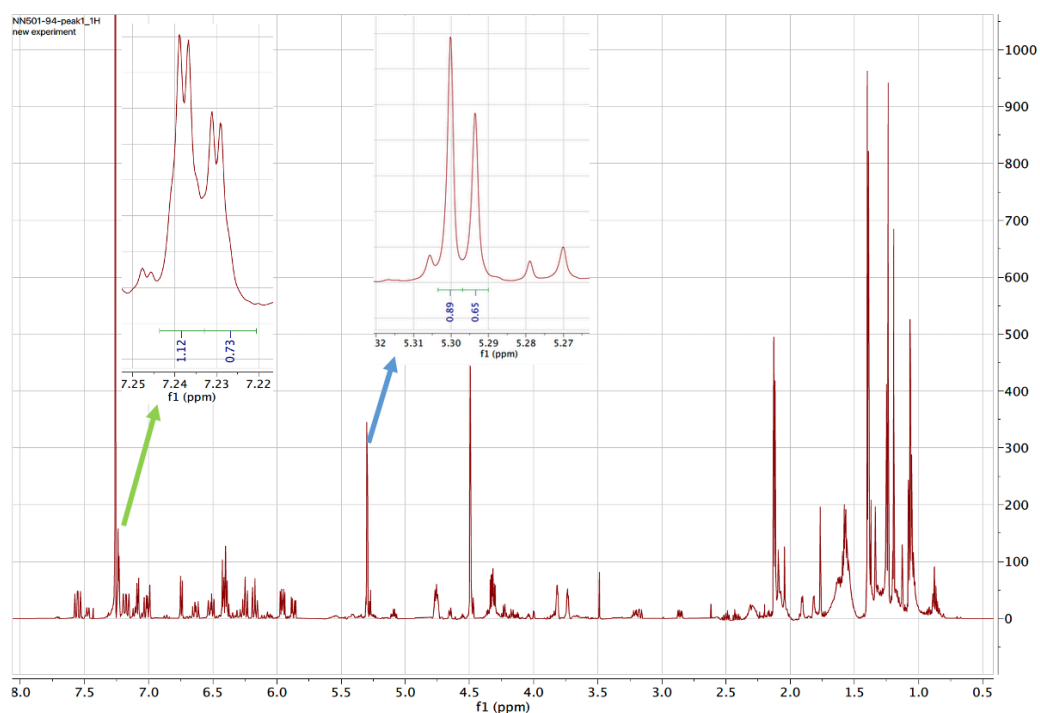


Figure 3-6. The ^1H NMR spectrum of NN501-94-peak 1

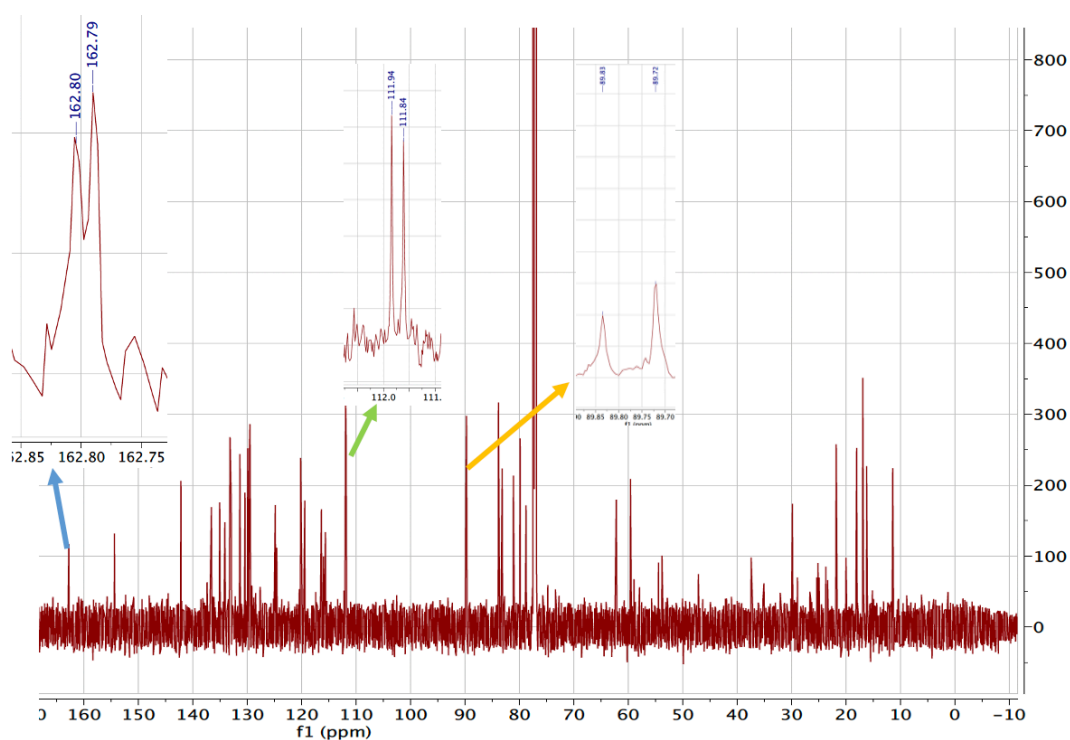


Figure 3-7. The ^{13}C NMR spectrum of NN501-94-peak 1

Due to the column ODS-3 could not use to detect and separate peak 1 into two different peaks, therefore, I tried to separate by three different columns. The Figure 3-12 to 3-14 showed the analytical HPLC chromatograms, 3D Figures and UV spectrum of peak 1.

*** Column InertSustain AQ-C18**

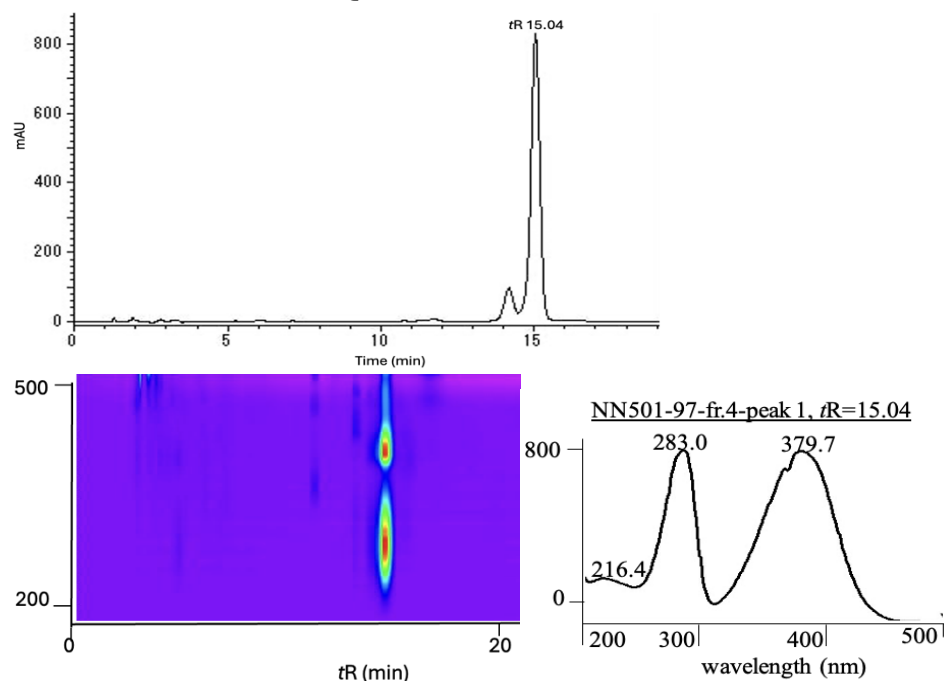


Figure 3-8. Analytical HPLC chromatogram of peak 1 analyzed by InertSustain AQ-C18 column

*** Column Inertsil ODS-HL**

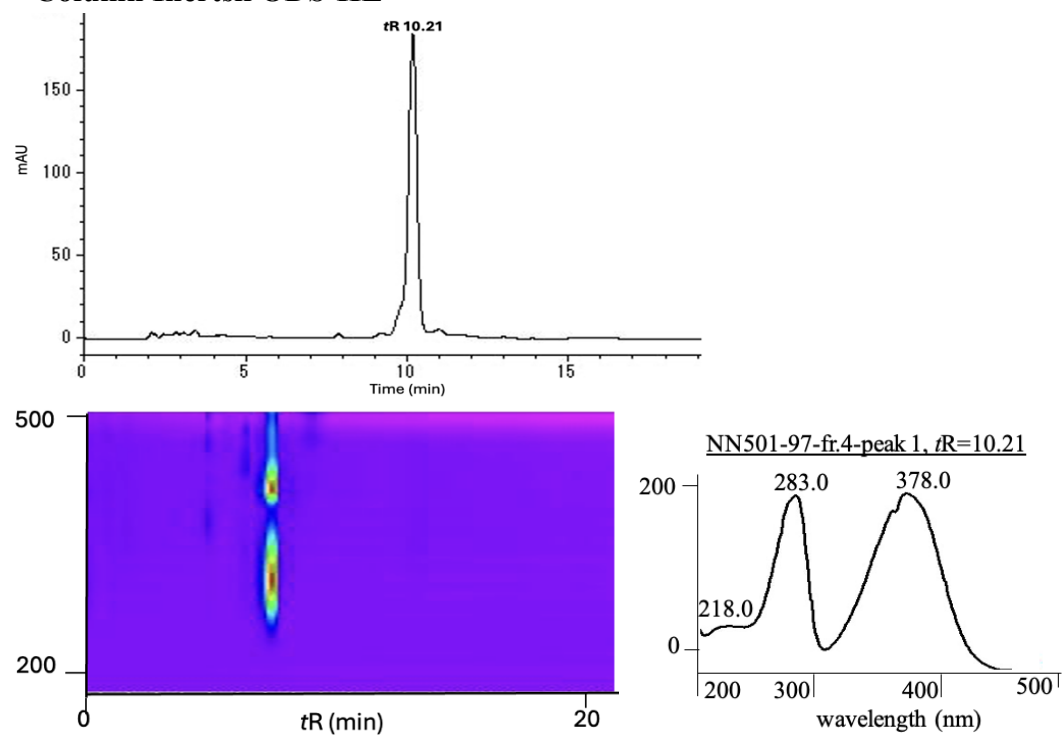


Figure 3-9. Analytical HPLC chromatogram of peak 1 analyzed by Inertsil ODS-HL column

* Column InertSustain C30

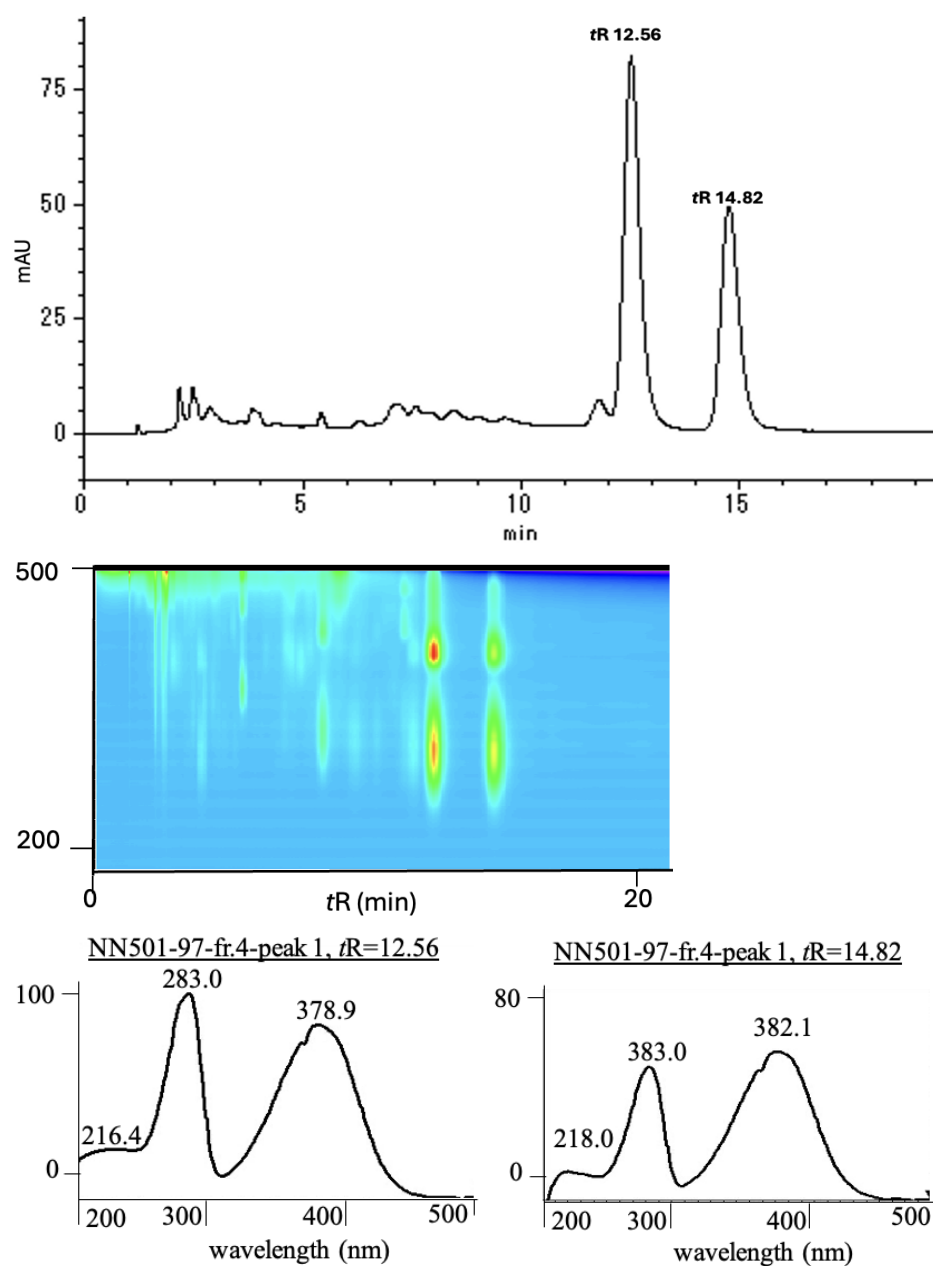


Figure 3-10. The analytical HPLC chromatogram of peak 1 analyzed by InertSustain C30 column

Through the Figure 3-12 to 3-15, the analytical HPLC chromatogram of peak 1 analyzed by InertSustain C30 can separate peak 1 into two different compounds. Therefore, two compounds will be tried to isolate from peak 1 by InertSustain C30 column.

The chromatogram of preparative HPLC of sample NN501-106-peak 1 is shown in the Figure 2 and explained in the Table 3-15. The t_R of target compounds is later than in the analytical HPLC two times.

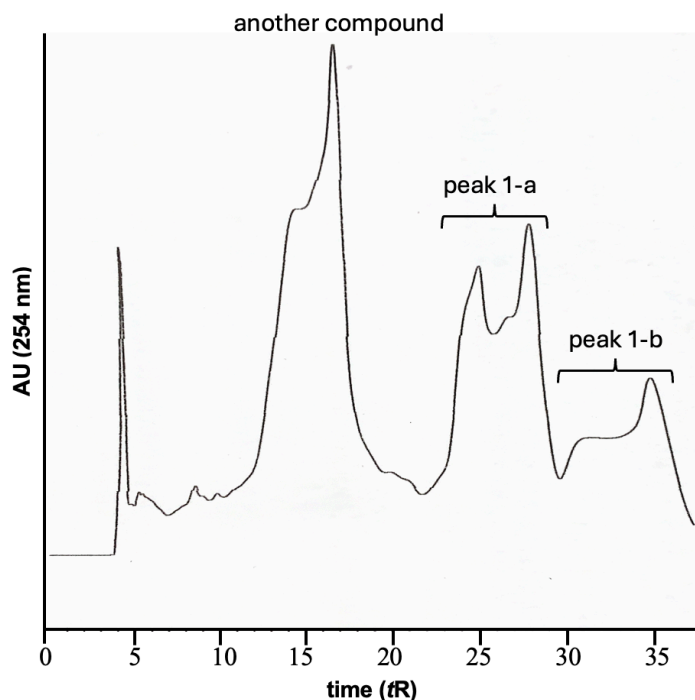


Figure 3-11. The preparative HPLC chromatogram of NN501-106-peak 1

The retention times of peaks 1-a and 1-b in the preparative run were later than those observed by analytical HPLC (13 min for peak 1-a and 18 min for peak 1-b). The apparent splitting in the preparative chromatogram can be attributed to the presence of two isomers, which are partially resolved under preparative conditions. Furthermore, the large column bed and gradient delay volume of the preparative system may enhance this resolution, causing the two isomers to appear as split or overlapping peaks. The purity of the two compounds obtained from preparative HPLC was further confirmed by analytical HPLC.

Table 3-13. Predicted and collected fractions belonging to target compounds

	NN501-106-peak 1	
	Peak 1-a	Peak 1-b
<i>t</i> R (min)	22-30	30-37
Predicted fractions	85-125	125-160
Collected fractions to combination	95-119	130-154

Sample were collected from 85-95 and 154-160 has a very small amount and low purity (< 80%) of peak 1-a and peak 1-b. Therefore, fractions were obtained and store in -25°C freezer.

After collected target fractions, the combinations of them were performed as Scheme 3-10 and 3-11.

Peak 1-a (fr. 95-119)

- Collected in 50 mL Erlenmeyer flask (23.2 mL)
- Injected in analytical HPLC (Condition in Table 3-14)
- Concentrated to remove MeOH
- Partitioned between EtOAc and H₂O

EtOAc-soluble fraction

- Concentrated to dryness
- Dissolved in 1 mL MeOH (spectroscopy grade)

NN501-107-peak 1-a

Scheme 3-10. Combination of NN501-107-peak 1-a

Peak 1-b (fr. 130-154)

- Collected in 50 mL Erlenmeyer flask (21.1 mL)
- Injected in analytical HPLC (Condition in Table 3-14)
- Concentrated to remove MeOH
- Partitioned between EtOAc and H₂O

EtOAc-soluble fraction

- Concentrated to dryness
- Dissolved in 1 mL MeOH (spectroscopy grade)

NN501-107-peak 1-b

Scheme 3-11. Combination of NN501-107-peak 1-a

Table 3-14. Conditions for C30-HPLC analysis

Pump	HITACHI L-2130
Column	Inertsil ODS-3, Ø 4.6×250 mm
Column oven	HITACHI L-2300
Column temperature	35°C
Detector	HITACHI L-2450
Detection wavelength	200-500 nm, chromatogram detected at 280 nm.
Eluent	50% MeOH
Flowrate	1.06 mL/min
Injection volume	5 µL
Sample	Combination of peak 1-a (23.2 mL), peak 1-b (21.2 mL)

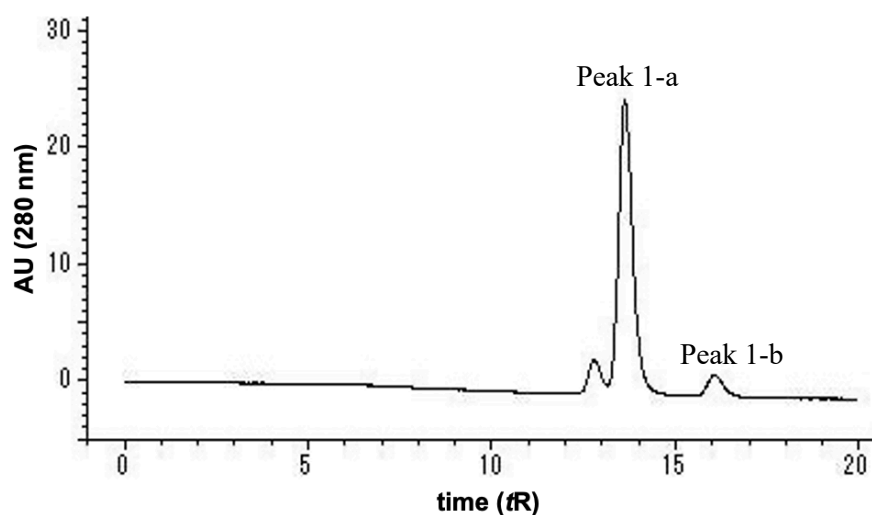


Figure 3-12. Analytical HPLC chromatogram of peak 1-a

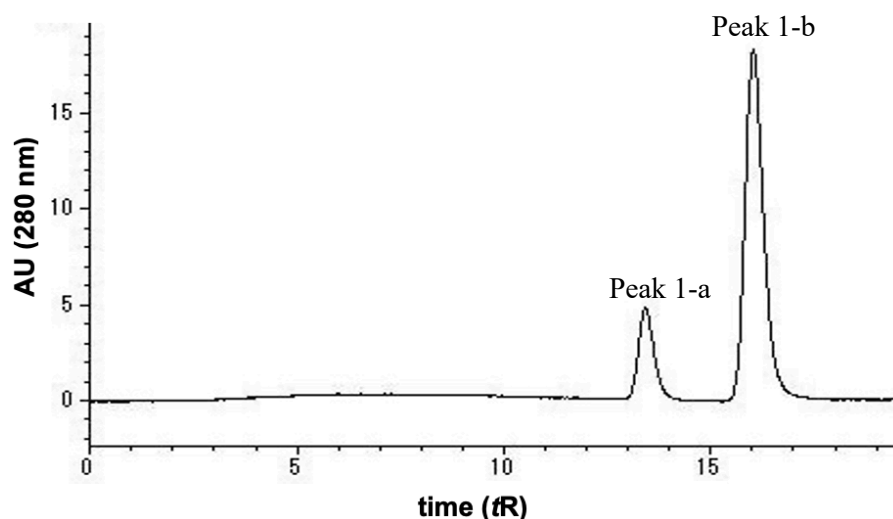


Figure 3-13. Analytical HPLC chromatogram of peak 1-b

Combination of three sample peak 1-a, peak 1-b and mixer was analyzed by analytical HPLC as Figure 3-16, 3-17 and summarized in Table 3-26, 3-27 and 3-28.

Table 3-15. The purity and yield of NN501-107-peak 1-a

	Before preparative HPLC NN501-106-peak 1(11.22 mg) Dilute 100 times		After combination Peak 1-a - From fr. 95-119 (23.2 mL)		Purity (%)	Yield (%)
	Peak area ($\times 10^5$) per injection	Peak area in total ($\times 10^9$)	Peak area ($\times 10^5$) per injection	Peak area in total ($\times 10^9$)		
Peak 1-a	77.1	15.4	25.4	11.8	88.81	76.62
peak 1-b	55.3	11.1	1.64	0.76	6.34	6.84

Table 3-16. The purity and yield of NN501-107-peak 1-b

	Before preparative HPLC NN501-106-peak 1(11.22 mg) Dilute 100 times		After combination Peak 1-b - From fr. 130-154 (21.1 mL)		Purity (%)	Yield (%)
	Peak area (x 10 ⁵) per injection	Peak area in total (x 10 ⁹)	Peak area (x 10 ⁵) per injection	Peak area in total (x 10 ⁹)		
Peak 1-a	77.1	15.4	4.04	1.70	15.74	11.04
peak 1-b	55.3	11.1	21.6	9.12	84.26	82.16

Table 3-17. The yield summarization of peak 1-a and peak 1-b based on the peak area

	Before preparative HPLC (NN501-106-peak 1)	Yield (%)		Total yield
		NN501-107-peak 1-a	NN501-107-peak 1-b	
Peak 1-a	100 %	76.62	11.04	87.82%
Peak 1-b	100 %	6.84	82.16	97.38%

The predication of weight of peak 1-a and peak 1-b was in Table 3-18. The total weight of peak 1 before preparative HPLC is 11.22 mg. Therefore, the weights of NN501-107-peak 1-a and NN501-107-peak 1-b were predicted as the ratio between peak area.

Table 3-18. The weight predication of peak 1-a and peak 1-b.

	Total peak area (x 10 ⁹) – before preparative HPLC	Peak area (x 10 ⁹) of main sample	Weight predication (mg)	Weight determination (mg)
Peak 1-a	15.4	11.8	5.00	4.33
Peak 1-b	11.1	9.12	3.86	3.29

Therefore, 4.3 mg of peak 1-a and 3.3 mg of peak 1-b were obtained after purification process.

Chapter 4.

Structural Elucidation of the Bioactive Compound peak 1-b and peak 1-a

4.1. Introduction

Purification methods afforded some bioactive compounds. Enough of the isolated compound(s), as well as high purity of the purified compounds are required in order to perform successful structural elucidation. According to Hanson J. R., 2003 structure elucidation can be divided into four stages: preliminary characterization by determining physical properties, molecular weight, and functional groups; structural simplification by identifying carbon skeleton; relative stereochemistry by investigating functional groups and their relative configuration in space; and absolute configuration.

To achieve structural information, some techniques have been proposed (D. L. Pavia *et al.*, 2015; D. A. Dias *et al.*, 2016) (Table 4-1). Most of the time, chemical structure determination is carried out by the joint analysis of those several techniques. Sometimes, those techniques are not enough for elucidating the compound. Therefore, chemical modification, e.g. derivatization, hydrolysis, degradation or oxidation of the sample must be done to get structural information.

Table 4-1. Techniques for structural elucidation

Technique(s)	Structural information
Fourier-transform infrared spectroscopy (FTIR)	Functional groups
UV spectroscopy	Chromophores (e.g. conjugated system)
Mass spectrometry (MS)	Molecular weight, elemental composition
MS ⁿ (tandem mass spectrometry)	Building blocks or repeating unit (e.g. amino acids, carbohydrates)
Nuclear Magnetic Resonance (NMR)	Relative stereochemistry, functional groups
Circular dichroism (CD)	Absolute configuration
Specific optical rotation $[\alpha]_D^T$	
X-ray crystallography	

In this study, bioactive compounds produced by *Pochonia suchlasporia* TAMA 87 were determined by a combination of spectroscopic analyses, including 1D NMR (¹H-, ¹³C-NMR), 2D (¹H-¹H Correlation Spectroscopy/COSY, ¹H-¹³C Heteronuclear Single Quantum Coherence Spectroscopy/HSQC, ¹H-¹³C Heteronuclear Multiple Bond Correlation Spectroscopy/HMBC, Nuclear Overhauser Effect Spectroscopy/NOESY), High Resolution Electrospray Ionization Mass Spectrometry/HRESIMS, UV spectroscopy, and optical rotation. Data from those measurements were then compared to the literature. Literature searches were done using scientific-web search, e.g. SciFinder, WoS (Web of Science), and Google Scholar.

In this study, instrumental analysis (UV-Vis, MS, NMR) and optical rotation of peak 1-b were performed, and its absolute configuration were elucidated. Due to NMR data of peak 1-

b are similar to those of ET-1 (S. Syaefudin *et al.*, 2020), therefore, this chapter described the procedures for elucidating the structures of peak 1-b.

4.2. Materials and Methods

4.2.1. Mass Spectrometry (MS) analysis

The objective of this experiment was performed to determine molecular formula and molecular weight of the compounds. To achieve this objective, low- and high- resolutions of MS analysis was employed using a Bruker Compact mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Table 4-2 described condition for the MS analysis.

Table 4-2. Condition for mass spectrometry (MS) analysis

Instrument	Bruker Compact mass spectrometer
Concentration of sample	1 µg/mL
Solvent	95% MeOH
Source type	Electrospray ionization (ESI)
Ion polarity	Positive and Negative
Scan	50-1000 (<i>m/z</i>)
Focus	Active
Set capillary	4500 V
Set end plate offset	-500 V
Set nebulizer	0.4 Bar
Set dry heater	180°C
Set dry gas	4.0 L/min

4.2.2. UV spectra measurement

Preliminary information of the UV spectra was obtained by observing UV profile appeared on DAD HPLC's system for the related peak. Later on, UV spectra of the compounds were determined by employing a UV-Vis 2550 spectrophotometer (Shimadzu, Tokyo, Japan). Detailed condition of this experiment was showed in Table 4-3.

Table 4-3. Condition for UV spectra measurement

Instrument	Shimadzu UV-2550 spectrophotometer
Solvent	MeOH (spectroscopic grade)
Wavelength	190-500 nm
Sampling period	2.0 nm
Sample amount	3 mL
Sample cell	Material: quartz Inside width: 10 mm Pathlength: 10 mm

4.2.3. Optical rotation measurement

Optical rotation was performed to determine specific rotation of the compounds. Optical activity was measured with a P-2200 polarimeter (JASCO, Tokyo, Japan). In this experiment, compound was measured at the sodium (D-line) wavelength 589 nm with MeOH (spectroscopic grade) used as a blank. Condition for the measurement was summarized in Table 4-4. The specific rotation value $[\alpha]$ of the compound in degree at a specific temperature (T) and wavelength (λ) is determined by following formula.

$$[\alpha]_D^T = \frac{100 \cdot \alpha}{c \cdot l}$$

Where: $[\alpha]_D^T$ = specific rotation (°)

α = observed rotation = angle of rotation (°)

l = Length of polarimeter tube (0.5 dm)

c = Concentration of the compound (g/100 mL)

Table 4-4. Condition for optical rotation measurement

Instrument	JASCO P-2200 polarimeter
Solvent	MeOH (spectroscopic grade)
Number of measurements	12*
Sample Integration time	5 sec
Cell size	Ø 3.5×50 mm
Sample	NN501-107-peak 1-b (3.3 mg/2 mL MeOH) NN501-107-peak 1-a (4.3 mg/2 mL MeOH)

*minimum and maximum values were removed, and the average of the remaining 10 values were used

4.2.4. Nuclear Magnetic Resonance (NMR) measurement

NMR spectra were recorded on a Varian NMR System 600 spectrometer (Varian, Palo Alto, CA, USA) operating at 600 MHz for ^1H and 150 MHz for ^{13}C NMR. The solvent used was deuterated chloroform. The observed chemical shift values (δ) were given in ppm (parts per million) and the coupling constants (J) in Hertz (Hz). The chemical shifts (δ) are reported with reference to residual proton and carbon signals of CDCl_3 (δ 7.26 in ^1H -NMR and 77.16 ppm in ^{13}C -NMR). Structure elucidation of the compounds was carried out in detail by various one- and two-dimension NMR spectral studies such as ^1H - ^1H COSY, HSQC, HMBC and NOESY. In this research, 3.3 mg of peak 1-b and 4.3 mg of peak 1-a (NN501-107) was used for the NMR measurements. These samples (NN501-107-peak 1-b and NN501-107-peak 1-a) showed a purity of 83% (observed at 280 nm using analytical HPLC) and it was dissolved in 0.8 mL of CDCl_3 (isotopic purity: 99.8 atom %D). However, it should be noted that usually ± 1 mg of high purity compound is required for ^1H -NMR measurement. Meanwhile, at least 2 mg of high purity compound is needed to measure ^{13}C -NMR.

4.3. Results and Discussions

4.3.1. Chemical structure determination of peak 1-b

Since peak 1-b have a yellow color, its chemical structure might have conjugated double bond that would be sensitive to the light. Therefore, this compound should be handled carefully to prevent direct exposure of the light, such as covering the sample bottle by aluminum foil during storage. The presence of conjugated double bond was proved by UV spectrophotometry analysis with their maximum absorption (Figure 4-1).

Figure 4-1 depicted UV spectrum of peak 1-b resulted. The UV spectrum of peak 1-b showed absorption maxima at λ_{max} (MeOH) nm (log ϵ): 374 (4.16), 282 (4.14) which indicated that peak 1-b possesses conjugated double bond

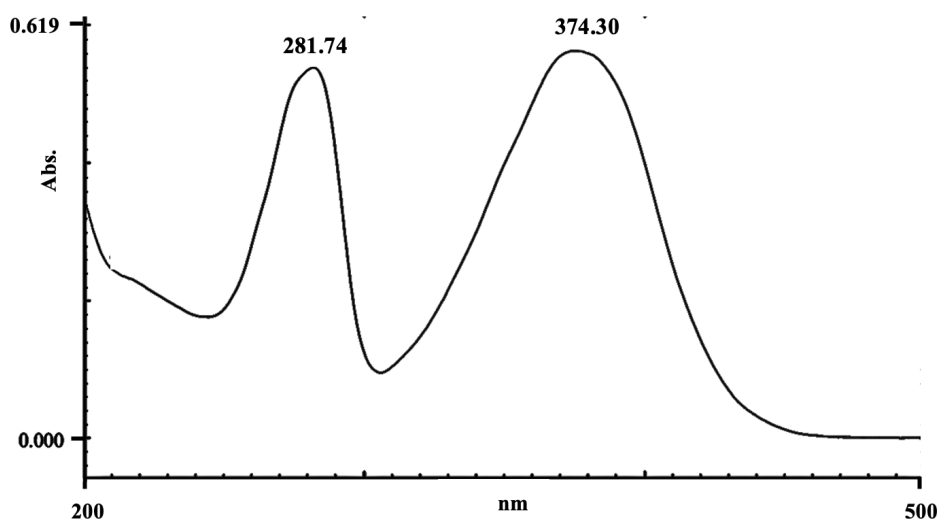


Figure 4-1. The UV spectrum of peak 1-b

Besides, the ^1H - and ^{13}C -NMR spectra of peak 1-b and ET-1 are very similar, only different in the α -pyrone moiety signals. In detail, the carbon skeleton of the α -pyrone moiety of ET-1 and peak 1-b are similar, but there is a methylene substituent in peak 1-b instead of 1 methyl substituent in ET-1. It suggests that the chemical structure of peak 1-b and ET-1 are similar, only different in the substituent in the α -pyrone moiety.

Based on the NMR analysis of peak 1-b (^1H , ^{13}C), the table recorded the NMR analysis results comparing to those of ET-1.

Table 4-5. The ^1H and ^{13}C -NMR and HSQC data of ET-1 (Syaefudin S. et al., 2020) and peak 1-b

Position	ET-1 (500 MHz, CDCl_3)			Peak 1-b (600 MHz, CDCl_3)	
	δ_{C}	δ_{H} (mult., J , int)		δ_{C}	δ_{H} (mult., J , int)
1	11.4	1.06 (t, 7.5, 3H)		11.4	1.07 (t, 7.5, 3H)
2	21.7	1.56 (m, 2H)		21.8	1.55 (m, 2H)
3	89.8	4.31 (dd, 8.1, 4.8, 1H)		89.8	4.30 (dd, 8.1, 4.7, 1H)
4-OH		1.65 (brs, 1H)			1.59 (brs, 1H)
4	81.1			81.1	
5	62.3			62.3	
6	111.8	5.29 (s, 1H)		111.9	5.29 (s, 1H)
7-OH		1.79 (d, 4.4, 1H)			1.72 (d, 4.7, 1H)
7	78.7	3.73 (dd, 3.5, 2.6, 1H)		78.8	3.73 (dd, 4.7, 2.9, 1H)
8	83.1	4.74 (brs, 1H)		83.1	4.75 (brs, 1H)
9	128.7	5.85 (dd, 15.3, 4.8, 1H)		129.6	5.87 (dd, 15.4, 5.0)
10	134.3	6.64 (dd, 15.3, 10.8, 1H)		134.1	6.65 (ddd, 15.4, 10.9, 1.5, 1H)
11	135.6	6.48 (dd, 14.8, 10.8, 1H)		136.6	6.52 (dd, 14.8, 10.9, 1H)
12	133.3	6.40 (dd, 14.8, 11.1, 1H)		133.0	6.41 (dd, 14.8, 11.1, 1H)
13	133.4	7.11 (dd, 15.0, 11.1, 1H)		135.1	7.20 (dd, 15.0, 11.1, 1H)
14	120.5	6.29 (d, 15.0, 1H)		119.4	6.40 (d, 15.0, 1H)
15	152.9			154.5	
16	112.8			116.0	
17	144.5	6.98 (d, 15.0, 1H)		142.2	7.22 (q, 1.3, 1H)
18	124.3			124.7	
19	163.1			162.7	
20	18.0	1.39 (s, 3H)		18.1	1.39 (s, 3H)
21	16.2	1.19 (s, 3H)		16.1	1.20 (s, 3H)
22	15.2	2.05 (s, 3H)		59.6	4.49 (s, 2H)
23	16.8	2.09 (s, 3H)		16.9	2.13 (d, 1.3, 3H)

Based on the ^1H and ^{13}C -NMR data of ET-1 compared to those of peak 1-b. ET-1 and peak 1-b had similar chemical structure, only different α -pyrone moiety.

4.3.1.1. Determination of planar structure

The planar configuration of peak 1-b was determined and confirmed by 1D and 2D NMR data and HR-ESI-MS data. Since the NMR data of peak 1-b were similar to those of ET-1, therefore, the chemical structure of peak 1-b was determined according to the chemical structure of ET-1. Based on Table 5-6, the chemical structures of peak 1-b and ET-1 are similar, only different in the methylene group belonging to α -pyrone moiety of peak 1-b (C-22) instead of methyl group (C-22) belonging to α -pyrone moiety of ET-1.

The COSY correlation of peak 1-b were summarized in Table 4-6.

Table 4-6. The COSY correlation of protons of peak 1-a and peak 1-b

Peak 1-b (600 MHz, CDCl ₃)	
δ_H (mult., <i>J</i> , int)	COSY correlation (δ_H)
1.07 (t, 7.5, 3H)	1.55
1.20 (s, 3H)	
1.39 (s, 3H)	
1.55 (m, 2H)	1.07, 4.31
1.59 (brs, 1H)	
1.72 (d, 4.7, 1H)	3.73
2.13 (d, 1.3, 3H)	7.22
3.73 (dd, 5.3, 2.9, 1H)	1.72, 4.76
4.31 (dd, 8.1, 4.7, 1H)	1.55
4.50 (s, 2H)	
4.75 (brs, 1H)	3.73, 5.87
5.30 (s, 1H)	
5.87 (dd, 15.4, 4.9)	4.75, 6.65
6.41 (dd, 14.9, 11.2, 1H)	6.52, 7.20
6.52 (dd, 14.9, 10.8, 1H)	6.41, 6.65
6.41 (d, 14.9, 1H)	
6.65 (ddd, 15.4, 10.8, 1.5, 1H)	5.87, 6.52
7.20 (dd, 14.9, 11.2, 1H)	
7.22 (q, 1.3, 1H)	2.13

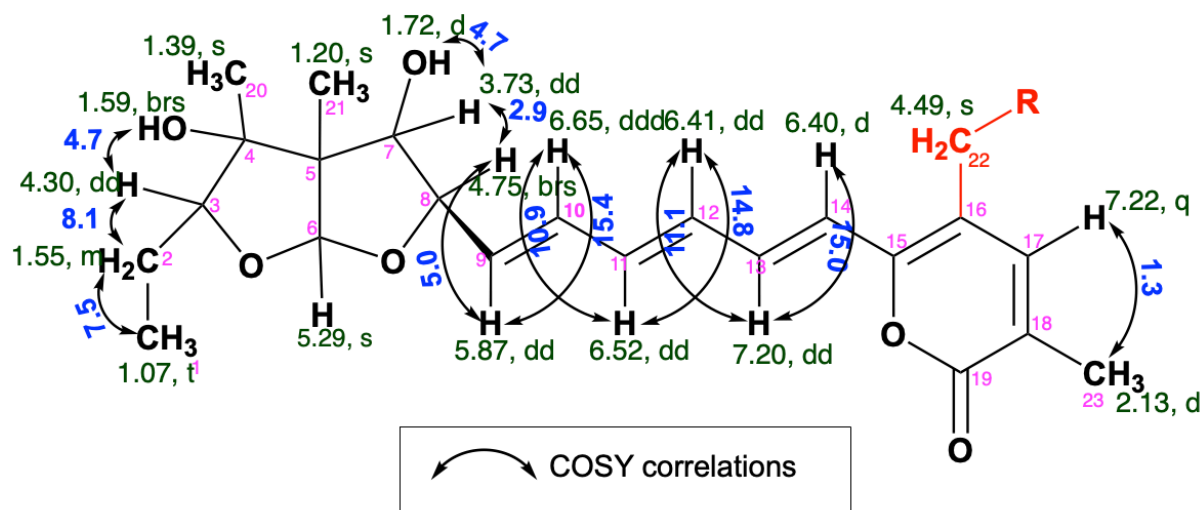


Figure 4-2. The COSY correlation of peak 1-b.

The COSY correlations indicated the connections of H-1/H-2/H-3, H-7/H-8/H-9/H-10/H-11/H-12/H-13/H-14 and H-17/H-23 as Figure 4-2.

Table 4-7. The HSQC and HMBC correlations of peak 1-b

δ_C	δ_H (mult., J , int)	HMBC correlations (δ_C)
11.4	1.07 (t, 7.5, 3H)	21.8, 89.8
16.1	1.20 (s, 3H)	62.3, 78.8, 81.1, 111.8
16.9	2.13 (d, 1.3, 3H)	124.7, 142.2, 162.7
18.1	1.39 (s, 3H)	62.3, 89.8, 81.1
21.8	1.55 (m, 2H)	
59.6	4.50 (s, 2H)	111.8, 142.2, 154.5
62.3		
78.8	3.73 (dd, 4.8, 2.9, 1H)	81.1
81.1		
83.1	4.76 (brs, 1H)	
89.8	4.32 (dd, 8.4, 4.4, 1H)	
111.9	5.30 (s, 1H)	16.1, 83.1, 89.8
116.0		
119.4	6.40 (d, 15.0, 1H)	154.5, 162.7
124.7		
129.6	5.87 (dd, 15.4, 4.9)	
133.0	6.41 (dd, 14.8, 11.2, 1H)	
134.1	6.65 (ddd, 15.4, 10.8, 1.5, 1H)	
135.1	7.20 (dd, 15.0, 11.2, 1H)	154.5
136.6	6.52 (dd, 14.8, 10.8, 1H)	
142.2	7.22 (q, 1.3, 1H)	16.9, 59.6, 154.5, 162.7
154.5		
162.7		

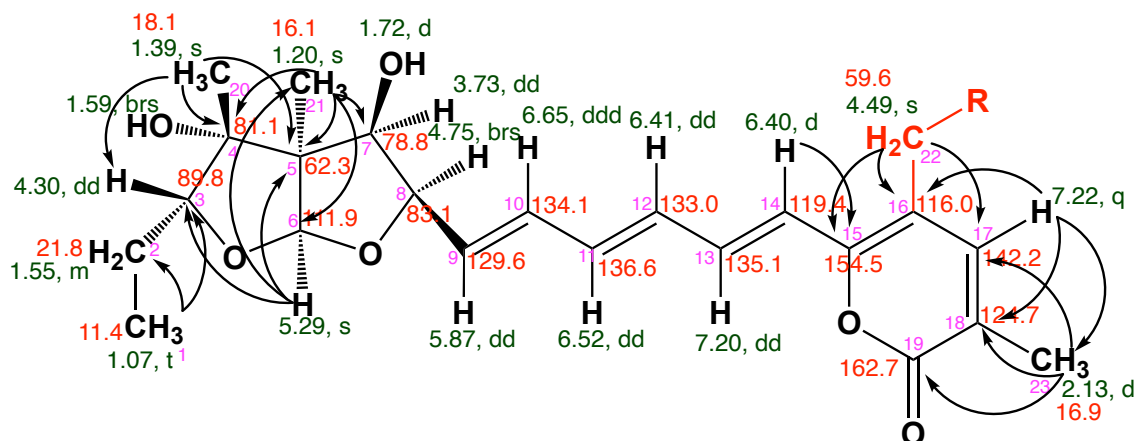


Figure 4-3. The HMBC correlation of peak 1-b

The chemical shift of methylene group (δ_C 59.6, δ_H 4.49) indicated that this methylene is an oxygenated carbon. Besides, peak 1-b possessed same molecular formula was determined to be $C_{23}H_{30}O_7$ on the basis of the HR-ESI-MS ion peak at m/z 441.1878 $[M+Na]^+$, while the HR-ESI-MS of ET-1 showed m/z 425.1933 $[M+Na]^+$, respectively. Therefore, there was a hydroxyl group attached to the methylene group since the difference in the molecular formula of peak 1-b was higher than that of ET-1 16 Da.

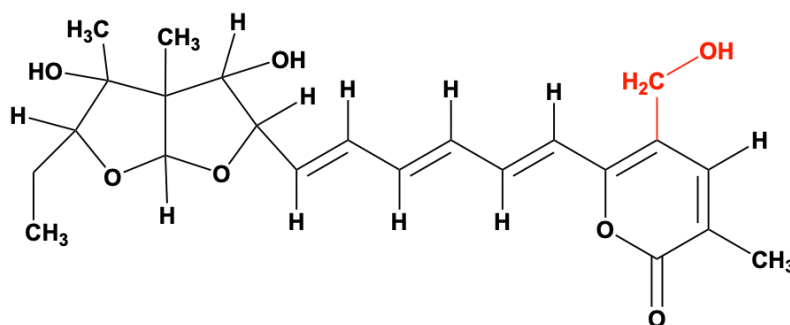


Figure 4-4. The planar structure of peak 1-b

The planar configuration of peak 1-b was determined by 1D and 2D NMR data and confirmed by HR-ESI-MS data as shown in Figure 4-5 and 4-6.

+MS, 4.6min, #273

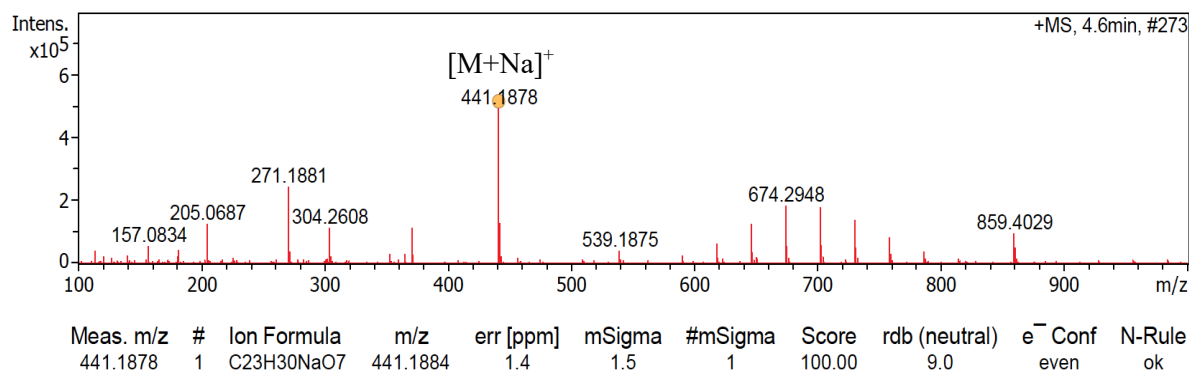


Figure 4-5. HR-ESI-MS chromatogram of peak 1-b (positive ion mode, 1 μ g/mL)

-MS, 4.7min, #274

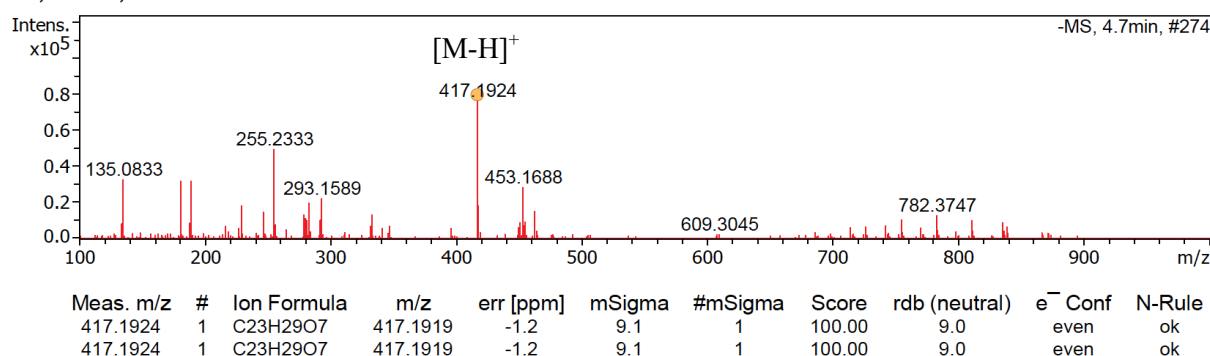


Figure 4-6. HR-ESI-MS chromatogram of peak 1-b (negative ion mode, 1 µg/mL)

Table 4-8. The HR-ESI-MS of peak 1-b

Mode	Sample	Found	Calculated	Calc. Formula	Molecular Formula
Positive	Peak 1-b	<i>m/z</i> 441.1878	<i>m/z</i> 441.1884	C ₂₃ H ₃₀ O ₇ Na	C ₂₃ H ₃₀ O ₇
Negative		<i>m/z</i> 417.1924	<i>m/z</i> 417.1919	C ₂₃ H ₂₉ O ₇	

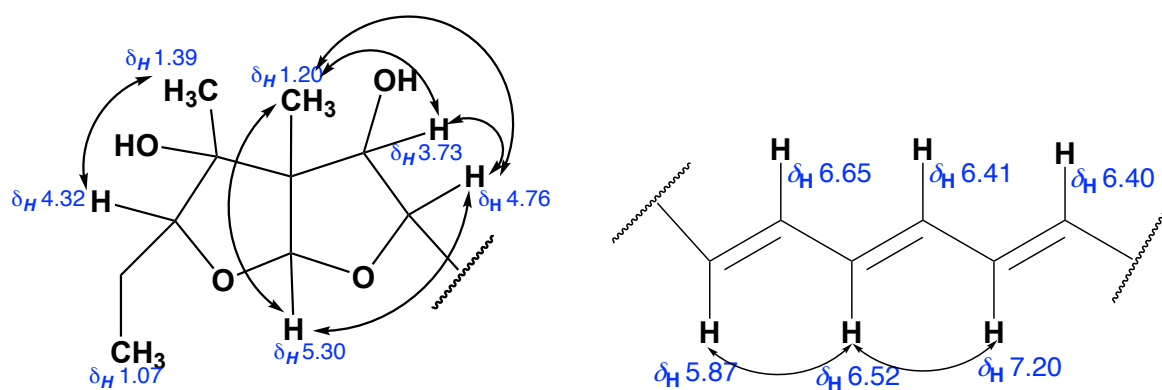
The HR-ESI-MS of peak 1-b (Figure 4-5 and 4-6) indicated that the molecular formula of peak 1-b is C₂₃H₃₀O₇, this formula also confirmed with the planar structure of peak 1-b in Figure 4-4.

4.3.1.2. Determination of relative configuration

The relative configuration of peak 1-b was determined by NOESY correlations. Key correlations were observed between H-6/H-8 and H-21, and H-7/H-8, indicating H-6, H-7, H-8 and H-21 are oriented on the same side of the 2,8-dioxabicyclo[3.3.0]octane ring. There was a NOESY correlation of H-3/H-20, but no significant correlation was observed between H-2 and H-20 as well as H-20 and H-21. It was indicated that H-3 and H-20 are on the opposite side of H-6, H-7, H-8 and H-21. These data indicated that the relative configuration of peak 1-b was identical to that of ET-1.

Table 4-9. The NOESY correlations of peak 1-b

δ_C	δ_H (mult., J , int)	NOESY correlations (δ_H)
11.4	1.07 (t, 7.5, 3H)	
16.1	1.20 (s, 3H)	3.73, 4.76, 5.30
16.9	2.13 (d, 1.3, 3H)	
18.1	1.39 (s, 3H)	4.32
21.8	1.55 (m, 2H)	
59.6	4.50 (s, 2H)	
62.3		
78.8	3.73 (dd, 4.8, 2.9, 1H)	1.20, 4.76
81.1		
83.1	4.76 (brs, 1H)	1.24, 5.30
89.8	4.32 (dd, 8.4, 4.4, 1H)	1.39
111.9	5.30 (s, 1H)	1.20, 4.76
116.0		
119.4	6.40 (d, 15.0, 1H)	6.41
124.7		
129.6	5.87 (dd, 15.4, 4.9)	
133.0	6.41 (dd, 14.9, 11.2, 1H)	6.40
134.1	6.65 (ddd, 15.4, 10.8, 1.5, 1H)	6.41
135.1	7.20 (dd, 14.9, 11.2, 1H)	6.52
136.6	6.52 (dd, 14.9, 10.8, 1H)	5.87
142.2	7.22 (q, 1.3, 1H)	
154.5		
162.7		

**Figure 4-7.** The NOESY correlations of peak 1-b.

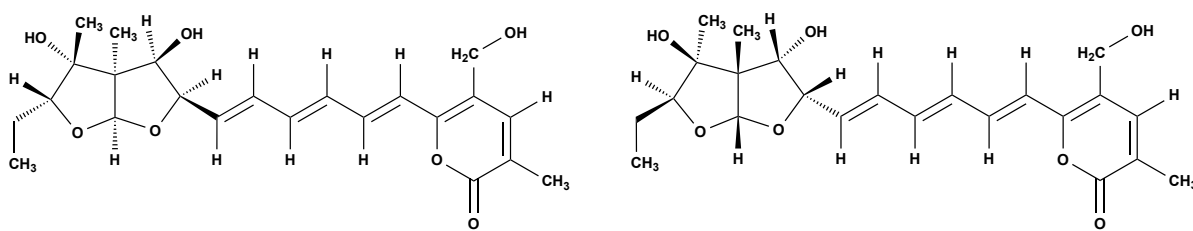


Figure 4-8. The relative configurations of peak 1-b

4.3.1.3. Determination of absolute configuration

Absolute structure of peak 1-b was determined by comparing the specific rotation value of astetoxin H. Peak 1-a shows positive specific optical rotation with an $[\alpha]_D^{25}$ value of +10.7 (c 0.15, MeOH), similar to that of ET-1, which has an $[\alpha]_D^{19}$ value of +15.4 (c 0.55, MeOH) (S. Syaefudin *et al.*, 2020). Based on these data, the absolute configuration of peak 1-a was concluded as the same as ET-1. Furthermore, peak 1-b is considered to be a novel compound and named as asteltoxin U.

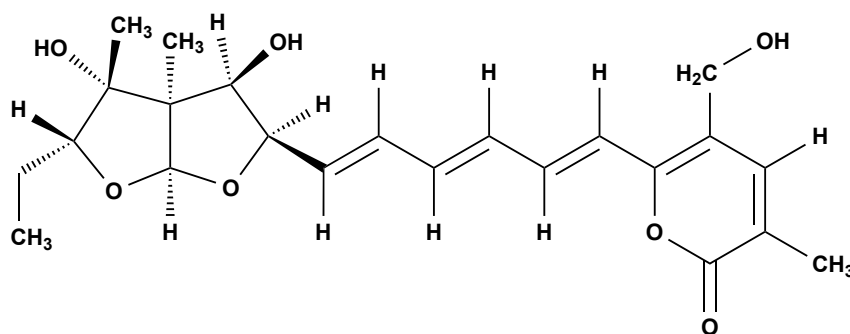


Figure 4-9. The absolute configuration of peak 1-b

4.3.2. The chemical structure determination of peak 1-a

After determined the chemical structure of peak 1-b, the chemical structure of peak 1-a was continuously elucidated by NMR spectra, UV-Vis spectrum, HR-ESI-MS and optical rotation activity.

The ^1H - and ^{13}C -NMR spectra of peak 1-a comparing to those of peak 1-b and ET-1. The comparison indicated that peak 1-a and peak 1-b tend to be different in the conjugated double bonds.

Table 4-10. The ^1H and ^{13}C -NMR data of ET-1 (Syaefudin S. et al., 2020), peak 1-a and peak 1-b

Position	ET-1 (500 MHz, CDCl_3)		δ_{C}	δ_{H} (mult., <i>J</i> , int)	δ_{C}	δ_{H} (mult., <i>J</i> , int)	δ_{C}	δ_{H} (mult., <i>J</i> , int)
	δ_{C}	δ_{H} (mult., <i>J</i> , int)						
1	11.4	1.06 (t, 7.5, 3H)	11.4	1.07 (t, 7.5, 3H)	11.4	1.07 (t, 7.5, 3H)	11.4	1.07 (t, 7.5, 3H)
2	21.7	1.56 (m, 2H)	21.8	1.57 (m, 2H)	21.8	1.57 (m, 2H)	21.8	1.55 (m, 2H)
3	89.8	4.31 (dd, 8.1, 4.8, 1H)	89.7	4.32 (dd, 8.4, 4.3, 1H)	89.8	4.30 (dd, 8.1, 4.7, 1H)	89.8	4.30 (dd, 8.1, 4.7, 1H)
4-OH		1.65 (brs, 1H)		1.61 (brs, 1H)		1.59 (brs, 1H)		1.59 (brs, 1H)
4	81.1		81.1		81.1		81.1	
5	62.3		62.2		62.3		62.3	
6	111.8	5.29 (s, 1H)	112.0	5.30 (s, 1H)	111.9	5.29 (s, 1H)	111.9	5.29 (s, 1H)
7-OH		1.79 (d, 4.4, 1H)		1.81 (d, 5.2, 1H)		1.72 (d, 4.7, 1H)		1.72 (d, 4.7, 1H)
7	78.7	3.73 (dd, 3.5, 2.6, 1H)	79.9	3.82 (dd, 5.2, 3.0, 1H)	78.8	3.73 (dd, 4.7, 2.9, 1H)	78.8	3.73 (dd, 4.7, 2.9, 1H)
8	83.1	4.74 (brs, 1H)	83.8	4.77 (brs, 1H)	83.1	4.75 (brs, 1H)	83.1	4.75 (brs, 1H)
9	128.7	5.85 (dd, 15.3, 4.8, 1H)	131.4	5.96 (dd, 15.4, 6.3, 1H)	129.6	5.87 (dd, 15.4, 5.0)	129.6	5.87 (dd, 15.4, 5.0)
10	134.3	6.64 (dd, 15.3, 10.8, 1H)	129.7	7.01 (dd, 15.4, 11.2, 1H)	134.1	6.65 (ddd, 15.4, 10.9, 1.5, 1H)	134.1	6.65 (ddd, 15.4, 10.9, 1.5, 1H)
11	135.6	6.48 (dd, 14.8, 10.8, 1H)	133.1	6.25 (dd, 11.2, 11.1, 1H)	136.6	6.52 (dd, 14.8, 10.9, 1H)	136.6	6.52 (dd, 14.8, 10.9, 1H)
12	133.3	6.40 (dd, 14.8, 11.1, 1H)	129.5	6.18 (dd, 11.8, 11.1, 1H)	133.0	6.41 (dd, 14.8, 11.1, 1H)	133.0	6.41 (dd, 14.8, 11.1, 1H)
13	133.4	7.11 (dd, 15.0, 11.1, 1H)	130.0	7.56 (dd, 14.9, 11.8, 1H)	135.1	7.20 (dd, 15.0, 11.1, 1H)	135.1	7.20 (dd, 15.0, 11.1, 1H)
14	120.5	6.29 (d, 15.0, 1H)	120.2	6.41 (d, 14.9, 1H)	119.4	6.40 (d, 15.0, 1H)	119.4	6.40 (d, 15.0, 1H)
15	152.9		154.4		154.5		154.5	
16	112.8		116.4		116.0		116.0	
17	144.5	6.98 (d, 15.0, 1H)	142.1	7.23 (q, 1.3, 1H)	142.2	7.22 (q, 1.3, 1H)	142.2	7.22 (q, 1.3, 1H)
18	124.3		124.9		124.7		124.7	
19	163.1		162.7		162.7		162.7	
20	18.0	1.39 (s, 3H)	18.1	1.40 (s, 3H)	18.1	1.39 (s, 3H)	18.1	1.39 (s, 3H)
21	16.2	1.19 (s, 3H)	16.2	1.24 (s, 3H)	16.1	1.20 (s, 3H)	16.1	1.20 (s, 3H)
22	15.2	2.05 (s, 3H)	59.6	4.50 (s, 2H)	59.6	4.49 (s, 2H)	59.6	4.49 (s, 2H)
23	16.8	2.09 (s, 3H)	16.9	2.13 (d, 1.3, 3H)	16.9	2.13 (d, 1.3, 3H)	16.9	2.13 (d, 1.3, 3H)

The NMR data in Table 4-10, peak 1-a showed almost the same ^1H - and ^{13}C -NMR signals as those of peak 1-b, except for those of the conjugated triene (C-9 to C-14) moiety. It suggested that peak 1-a and peak 1-b could be considered as the double bond isomers similar to that of isoasteltoxin and asteltoxin.

Figure 4-10 depicted UV spectrum of peak 1-a resulted. The result indicated that chemical structure of peak 1-a also possess conjugated double bond. The UV spectrum of peak 1-b showed absorption maxima at λ_{max} (MeOH) nm (log ϵ): 374 (4.13), 282 (4.16).

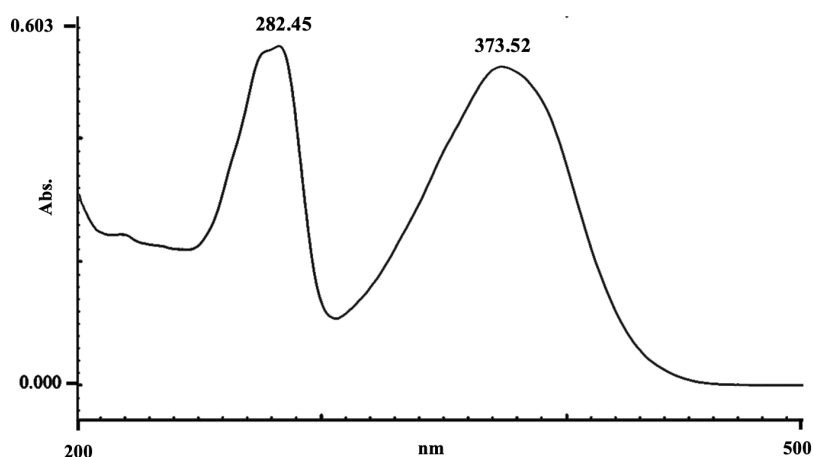


Figure 4-10. The UV spectrum of peak 1-a

Table 4-11. UV-Vis spectrum of peak 1-a, peak 1-b, isoasteltoxin and asteltoxin

Compound	λ_{max} (nm)	Abs.	ϵ	log ϵ
Peak 1-a	373.52	0.515	1.36×10^4	4.13
	282.45	0.548	1.45×10^4	4.16
	219.45	0.244	0.65×10^4	3.81
Peak 1-b	374.30	0.563	1.43×10^4	4.16
	281.74	0.539	1.37×10^4	4.14
	219.39	0.231	0.59×10^4	3.76
ET-1 (S. Syaefudin <i>et al.</i> , 2020)	378.4	0.597	2.91×10^4	4.46
	283.2	0.581	1.83×10^4	4.45
	212.2	0.184	0.90×10^4	3.95
Isoasteltoxin (J. Wang <i>et al.</i> , 2016)	365	N/A	1.20×10^4	4.08
	273		1.35×10^4	4.13
	268		1.35×10^4	4.13
	221		0.50×10^4	3.70
Asteltoxin (H. Adachi <i>et al.</i> , 2015)	385	N/A	N/A	3.47
	363			3.47
	272			
Asteltoxin (G. J. Kruger <i>et al.</i> , 1979)	367	N/A	3.31×10^4	4.52
	272.5		2.95×10^4	4.47
	267		2.95×10^4	4.47

N/A: Not available

The $\lambda_{\max}$ of peak 1-a and peak 1-b tend to similar. However, the ratios of two major ϵ values of two compounds were different. These ratios between ϵ values of two maximum absorptions are similar to those of isoasteltoxin and asteltoxin (Table 4-12).

Table 4-12. The ratio of two major ϵ values of peak 1-a, peak 1-b, isoasteltoxin and asteltoxin

Compounds	$\lambda_{\max}$ (nm)	ϵ	Ratio*
Peak 1-a	374	1.36×10^4	0.94
	282	1.45×10^4	
Peak 1-b	374	1.43×10^4	1.04
	282	1.37×10^4	
Isoasteltoxin	365	1.20×10^4	0.89
	273	1.35×10^4	
Asteltoxin	367	3.31×10^4	1.12
	273	2.95×10^4	

* The ratio was calculated by the division between the ϵ values of two $\lambda_{\max}$ wavelengths.

For example:

$$\text{Ratio of peak 1-a} = \epsilon (\text{at } 374 \text{ nm}) / \epsilon (\text{at } 282 \text{ nm}) = (1.36 \times 10^4) / (1.45 \times 10^4) = 0.94$$

4.3.2.1. Determination of planar structure

The planar structure of peak 1-a was determined and confirmed by 1D and 2D NMR (^1H , ^{13}C , COSY, HSQC and HMBC) and HR-ESI-MS results.

The COSY correlation of peak 1-a and peak 1-b were summarized in Table 4-13.

Table 4-13. The COSY correlation of protons of peak 1-a and peak 1-b

Peak 1-a (600 MHz, CDCl_3)	
δ_{H} (mult., J , int)	COSY correlation (δ_{H})
1.07 (t, 7.5, 3H)	1.57
1.24 (s, 3H)	
1.40 (s, 3H)	
1.57 (m, 2H)	1.07, 4.30
1.58 (brs, 1H)	4.30
1.81 (d, 5.3)	3.82
2.13 (d, 1.3, 3H)	7.23
3.82 (dd, 5.3, 3.0, 1H)	1.81, 4.77
4.30 (dd, 8.4, 4.4, 1H)	1.57
4.50 (s, 2H)	
4.77 (brs, 1H)	3.82, 5.96
5.30 (s, 1H)	
5.96 (dd, 15.4, 6.3, 1H)	4.77, 7.01
6.18 (dd, 11.8, 11.1, 1H)	6.25, 7.56
6.25 (dd, 11.2, 11.1, 1H)	6.18, 7.01
6.41 (d, 14.9, 1H)	7.56
7.01 (dd, 15.4, 11.2, 1H)	5.96, 6.25
7.23 (q, 1.3, 1H)	2.13
7.56 (dd, 14.9, 11.8, 1H)	6.18, 6.41

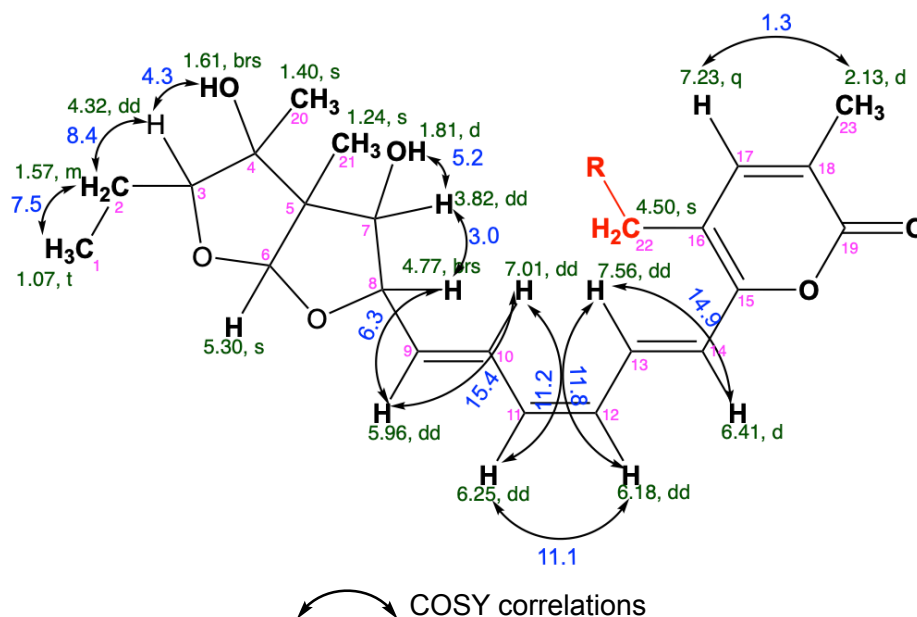


Figure 4-11. The COSY correlation of peak 1-a

Table 4-14. The HSQC and HMBC of peak 1-a

δ_C	δ_H (mult., J , int)	HMBC correlations (δ_C)
11.4	1.07 (t, 7.5, 3H)	21.8, 89.7
16.2	1.24 (s, 3H)	62.2, 79.9, 81.1, 112.0
16.9	2.13 (d, 1.3, 3H)	124.9, 142.1, 162.8
18.1	1.40 (s, 3H)	62.2, 89.7, 81.1
21.8	1.57 (m, 2H)	
59.6	4.50 (s, 2H)	111.9, 142.1, 154.4
62.2		
79.9	3.82 (dd, 4.7, 3.1, 1H)	81.1
81.1		
83.8	4.77 (brs, 1H)	
89.7	4.32 (dd, 8.4, 4.4, 1H)	
111.9		
112.0	5.30 (s 1H)	16.2, 83.8, 89.7
120.2	6.41 (d, 14.9, 1H)	154.4, 162.7
124.9		
129.5	6.18 (dd, 11.8, 11.1, 1H)	
129.7	7.01 (dd, 15.4, 11.2, 1H)	
130.0	7.56 (dd, 14.9, 11.8, 1H)	154.4
131.4	5.95 (dd, 15.4, 6.3, 1H)	
133.1	6.25 (dd, 11.2, 11.1, 1H)	
142.1	7.23 (q, 1.3, 1H)	16.9, 59.6, 154.4, 162.7
154.4		
162.7		

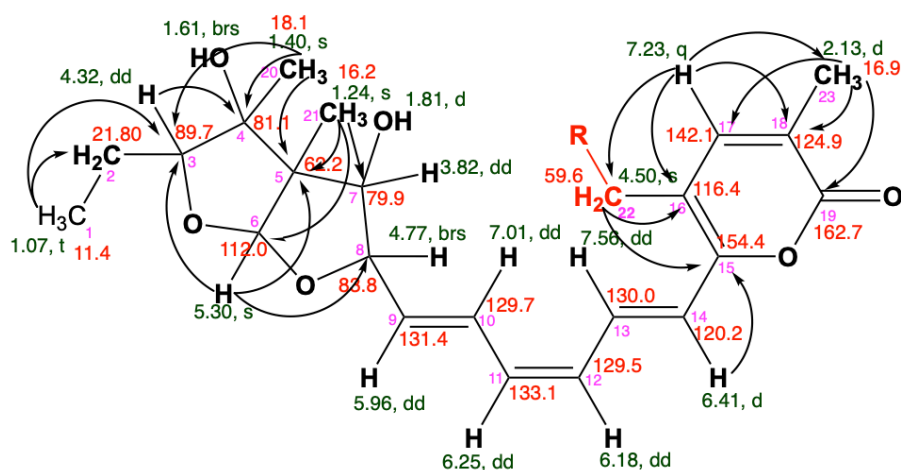


Figure 4-11. The HMBC correlations of peak 1-a

The chemical shift of methylene group (δ_C 59.6, δ_H 4.50) indicated that this methylene is an oxygenated carbon. Besides, peak 1-a and peak 1-b possessed same NMR signals in the α -pyrone moiety. Therefore, there was a hydroxyl group attached to the methylene group.

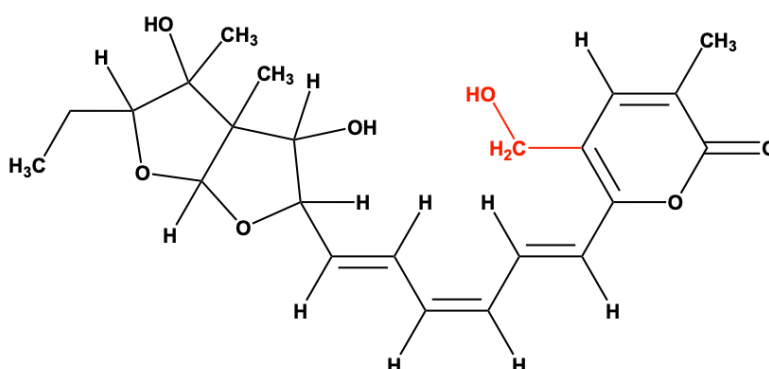


Figure 4-12. The planar structure of peak 1-a

The mass spectra of NN501-110-peak 1-a was measured HR-ESI-MS in positive ion mode and negative ion mode as shown in Figure 4-14 and 4-15.

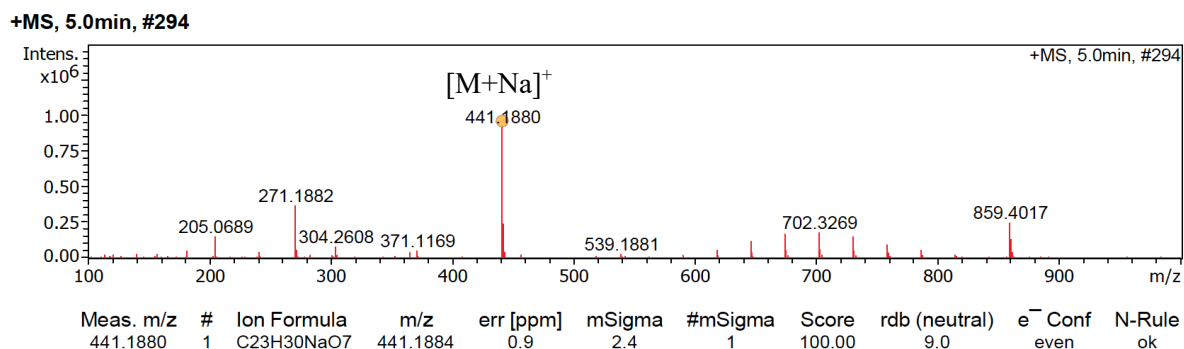


Figure 4-13. HR-ESI-MS chromatogram of peak 1-a (positive ion mode, 1 μ g/mL)

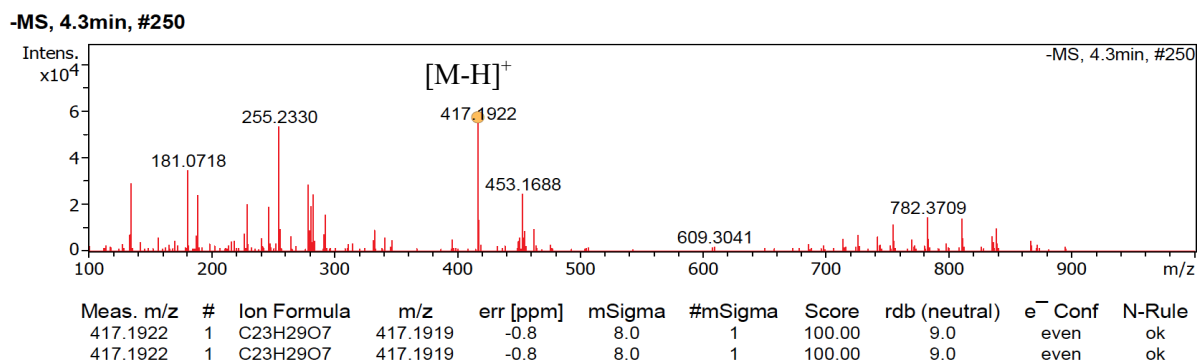


Figure 4-14. HR-ESI-MS chromatogram of peak 1-a (negative ion mode, 1 µg/mL)

Table 4-15. The HR-ESI-MS of peak 1-a

Mode	Sample	Found	Calculated	Calc. Formula	Molecular Formula
Positive	Peak 1-a	<i>m/z</i> 441.1880	<i>m/z</i> 441.1884	C ₂₃ H ₃₀ O ₇ Na	C ₂₃ H ₃₀ O ₇
Negative		<i>m/z</i> 417.1922	<i>m/z</i> 417.1919	C ₂₃ H ₂₉ O ₇	

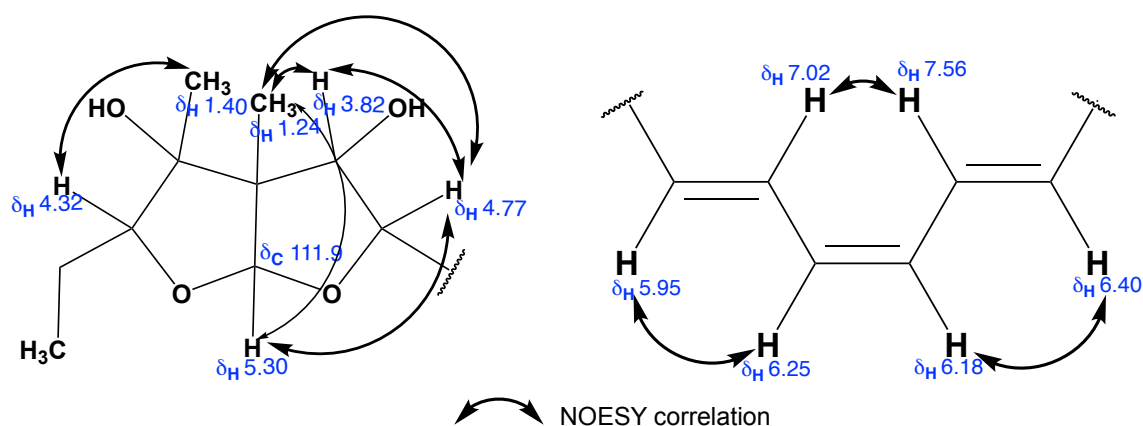
Through the results of HR-ESI-MS (Table 4-15), the molecular formula of peak 1-a is similar to that of peak 1-b. This planar structure agreed with the high-resolution mass spectrometry data of peak 1-a, which indicated the molecular formula of peak 1-a to be C₂₃H₃₀O₇.

4.3.2.2. Determination of relative configuration

The relative configuration of peak 1-a was determined by the NOESY correlations. In detail, key correlations were observed between H-6/H-8 and H-21, and H-7/H-8, indicating H-6, H-7, H-8 and H-21 are oriented on the same side of the 2,8-dioxabicyclo[3.3.0]octane ring. There was a NOESY correlation of H-3/H-20, but no significant correlation was observed between H-2 and H-20 as well as H-20 and H-21. It was indicated that H-3 and H-20 are on the opposite side of H-6, H-7, H-8 and H-21.

Table 4-16. The HSQC and HMBC correlations of peak 1-a

δ_C	δ_H (mult., <i>J</i> , int)	NOESY correlations (δ_H)
11.4	1.07 (t, 7.5, 3H)	
16.2	1.24 (s, 3H)	3.82, 4.77, 5.30
16.9	2.13 (d, 1.3, 3H)	
18.1	1.40 (s, 3H)	4.32
21.8	1.57 (m, 2H)	
59.6	4.50 (s, 2H)	
62.2		
79.9	3.82 (dd, 4.7, 3.1, 1H)	1.24, 4.77
81.1		
83.8	4.77 (brs, 1H)	1.24, 5.30
89.7	4.32 (dd, 8.4, 4.4, 1H)	1.40
111.9		
112.0	5.30 (s 1H)	1.24, 4.77
120.2	6.41 (d, 14.9, 1H)	
124.9		
129.5	6.18 (dd, 11.8, 11.1, 1H)	6.25, 6.41
129.7	7.01 (dd, 15.4, 11.2, 1H)	7.56
130.0	7.56 (dd, 14.9, 11.8, 1H)	7.02
131.4	5.95 (dd, 15.4, 6.3, 1H)	6.25
133.1	6.25 (dd, 11.2, 11.1, 1H)	6.18, 5.9
142.1	7.23 (q, 1.3, 1H)	
154.4		
162.7		

**Figure 4-15.** The NOESY correlations of peak 1-a

The key NOESY correlations of the 2,8-dioxabicyclo[3.3.0]octane ring in peak 1-a were also similar to those of peak 1-b, except for the olefinic proton signals of the conjugated triene moiety. Furthermore, the double bond between C-11 and C-12 possessed the *Z*-geometry, which was also confirmed by the NOESY correlation of H-10/H-13.

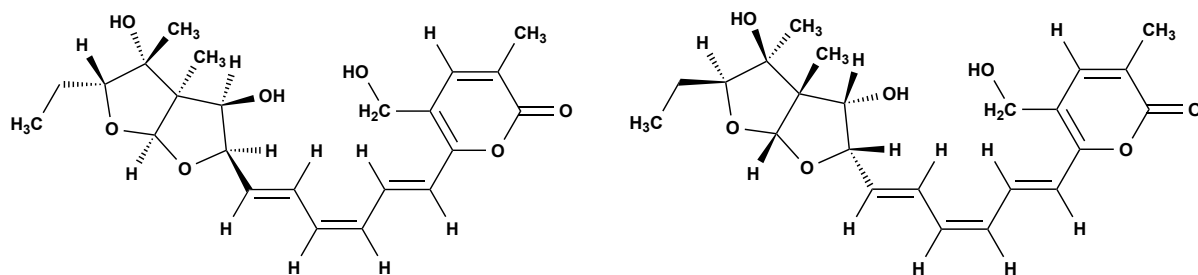


Figure 4-16. The relative configurations of peak 1-a

4.3.2.3. Determination of absolute configuration

Absolute structure of peak 1-a was determined by comparing the specific rotation value of isoasteltoxin since it possesses similar conjugated double bond configuration. Peak 1-a shows positive optical rotation with an $[\alpha]_D^{25} +15.7$ (c 0.20, MeOH) which was the same sign as that of isoasteltoxin $[\alpha]_D^{25} +31.4$ (c 0.20, MeOH) (J. Wang *et al.*, 2016), suggesting that the absolute configuration of peak 1-a was concluded as the same as isoasteltoxin as well as peak 1-b and ET-1.

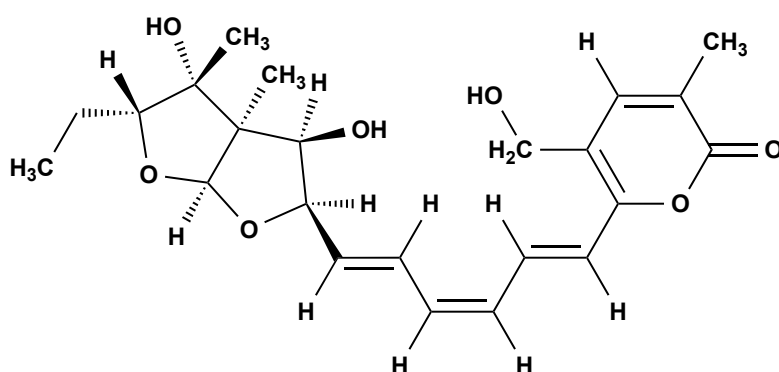


Figure 4-17. The absolute configuration of peak 1-a

Chapter 5. Bioactivity of peak 1-a and peak 1-b

5.1. Introduction

Due to the small amount of peak 1-a (4.3 mg) and peak 1-b (3.3 mg), peak 1-a and peak 1-b cannot be used to evaluate their insecticidal activities, which normally requires about 9 mg/sample. Therefore, two compounds were examined for preliminary information on their toxicity to animal cells. Particularly, their effects on the cell division of sea urchin embryos were evaluated.

The inhibition of early embryonic cleavage in sea urchins has emerged as a sensitive and reliable indicator of the biological activity of natural products and synthetic compounds. Among the earliest and most critical stages of development, the first cleavage is highly susceptible to chemical disruption. Bioactive compounds that interfere with this process may target essential cellular mechanisms such as microtubule polymerization, spindle formation, DNA synthesis, or cell cycle progression (Pagano G. *et al.*, 1986; Kobayashi *et al.*, 2008). Consequently, the sea urchin embryo assay offers a valuable platform for screening compounds with potential cytotoxic, antiproliferative, or mitotic inhibitory properties. In particular, natural products derived from marine or microbial sources have been frequently evaluated using this model to assess their embryotoxic or antimetabolic effects (Morroni L. *et al.*, 2016). The transparency of embryos, rapid developmental timeline, and ethical advantages of using non-vertebrate models make this assay especially suitable for mechanism-oriented and high-throughput bioactivity screening. The inhibition of the first cleavage serves not only as an endpoint for toxicity evaluation but also provides insight into the mode of action of bioactive compounds at the cellular and molecular level.

5.2. Materials and Methods

In order to evaluate the bioactivity of peak 1-a and peak 1-b, an inhibitory activity assay was performed towards sea urchin embryos, *Hemicentrotus pulcherrimus*. The eggs and sperm of sea urchin *Hemicentrotus pulcherrimus* were provided by Tateyama Marine Laboratory, Marine and Coastal Research Center, Ochanomizu University, Japan. The first cleavage of sea urchin embryos was blocked when treated at doses higher than MIC (minimum inhibitory concentration).

Bioactivity of peak 1-a and peak 1-b have been compared to dehydrophenylhistin (Δ PLH). Δ PLH was used as a positive control since it has been known to exhibit potent cytotoxic activity and higher than that of the known anticancer drugs, such as taxol, vinblastine, and vincristine (H. Kanzaki *et al.*, 2002). To conduct this experiment, sea urchin eggs were fertilized with sea urchin sperm (by mixing them gently). This fertilization step was described in Figure 5-1. The fertilized eggs were then used for the experiment.

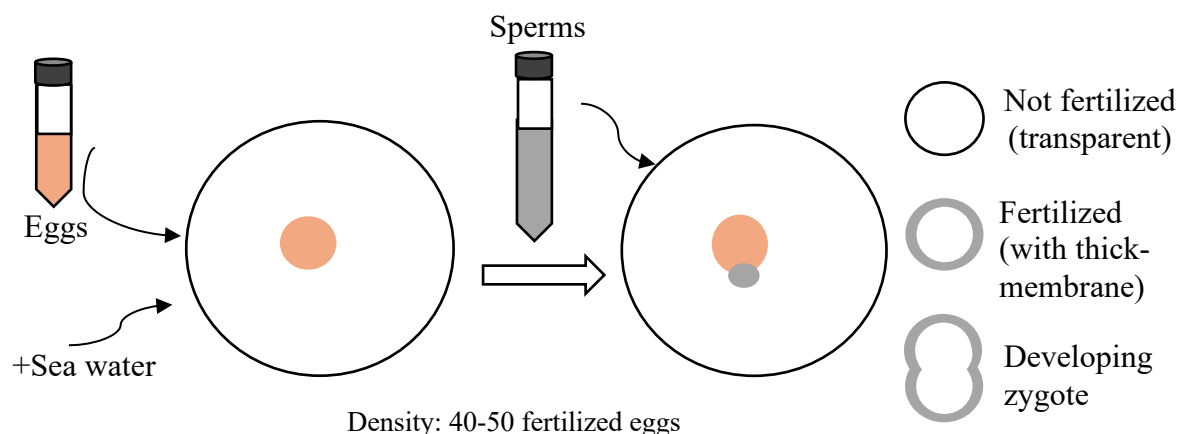


Figure 5-1. Method for fertilizing sea urchin eggs.

Meanwhile, appropriately 50 μg of sample (except for ΔPLH : 6.25 $\mu\text{g}/\text{mL}$ in MeOH, which was prepared by Kato) was dissolved in MeOH and then prepared for the experiment. The amount of each sample and volume of MeOH added to each sample was summarized in Figure 5-2.

Sample(s) with certain amount

- Transferred into the first well of 24-well tissue culture plate (flat bottom, FALCON)
- Added with MeOH (HPLC grade) until the final volume of 200 μL

Approx. 50 μg of sample (in 200 μL of MeOH)

- Diluted with MeOH (HPLC grade) into several concentration
- Evaporated to remove MeOH solvent
- Added with 20 μL of the DMSO/MeOH (1:1) solution, 1 mL of sea water, and 1 drop of the fertilized eggs (at room temperature)
- Observed motility and cell division of the sea urchin embryos by using a microscope (OLYMPUS IX70)

Scheme 5-1. Method for inhibitory assay using sea urchin embryos

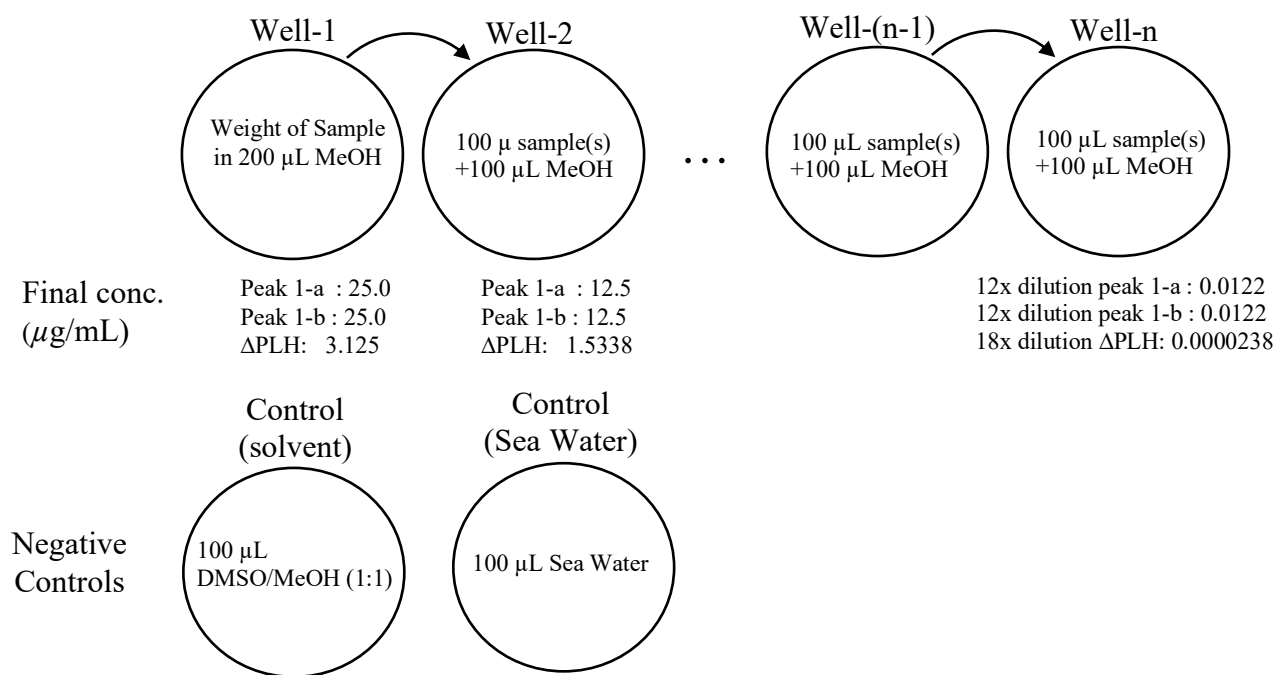


Figure 5-2. Serial dilution for samples and ΔPLH

The inhibitory activity was evaluated after the cleavage period by observation under the microscope. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of compounds that completely inhibited the first cleavage of fertilized sea urchin embryos.

5.3. Results and Discussions

The developing stage activity (cleavage activity) of sea urchin embryos was evaluated at 120 min after starting the culture due to the low temperature (about 15°C). The first cleavage of sea urchin embryos was blocked when treated at doses higher than MIC (minimum inhibitory concentration). Later on, cleavage inhibition activity and motility of the embryo were also evaluated at 24 h after starting the treatment.

Before assay, there is no inhibition for the cell division activity of sea urchin embryos treated in DMSO/MeOH (1:1), DMSO, and seawater after 120 min of treatment. In addition, the embryos also could swim freely in all the negative controls after 24 h of treatment. The results indicated that fertilized eggs developed normally, and there was no effect of the DMSO/MeOH (1:1) on the sea urchin embryos. Therefore, the fertilized eggs and solvents could be used for this experiment.

Table 5-1. Summarize the MIC value of peak 1-a, peak 1-b and Δ PLH (15°C, Feb 26th, 2025)

Compound	MIC ($\mu\text{g/mL}$)
Peak 1-b	3.1
Peak 1-a	> 25.0
Δ PLH	3.1×10^{-3}

When the swimming of sea urchin embryos was observed 24 hours after fertilization, no swimming was observed in the concentration areas that showed activity for tested samples, but swimming was observed in the concentration areas that showed no activity. Furthermore, in the concentration areas where partial inhibition was confirmed, swimming was observed, although the number of swimming was smaller than in the concentration areas that showed no activity, except for peak 1-a. With peak 1-a, swimming was not confirmed up to the second hole, but in the third and fourth holes where partial activity was confirmed, swimming was observed, even with few amounts.

Table 5-2. Sea urchin embryo cleavage inhibition activity 120 minutes after the start of incubation in Δ PLH (positive control)

Δ PLH	Concentration ($\mu\text{g/mL}$)					
	3.1	1.6	0.78	0.39	0.20	0.098
Cleavage inhibition	+	+	+	+	+	+

Δ PLH	Concentration ($\mu\text{g/mL}$)					
	0.049	0.024	0.012	6.1×10^{-3}	3.1×10^{-3}	1.5×10^{-3}
Cleavage inhibition	+	+	+	+	+	± 50

Δ PLH	Concentration ($\mu\text{g/mL}$)					
	7.6×10^{-4}	3.8×10^{-4}	1.9×10^{-4}	9.5×10^{-5}	5.0×10^{-5}	2.5×10^{-5}
Cleavage inhibition	-	-	-	-	-	-

+: Completely inhibits cleavage

$\pm X$: Inhibits X% of cleavage

-: Does not inhibit cleavage

(February 26, 2025, room temperature around 15°C)

Table 5-3. Sea urchin embryo cleavage inhibition activity 120 minutes after the start of incubation in peak 1-b and peak 1-a

Compounds	Concentration ($\mu\text{g/mL}$)					
	25	13	6.5	3.1	1.6	0.78
Peak 1-b	+	+	+	+	± 80	± 41
Peak 1-a	± 22	± 15	-	-	-	-

Compounds	Concentration ($\mu\text{g/mL}$)					
	0.39	0.20	0.098	0.049	0.024	0.012
Peak 1-b	-	-	-	-	-	-
Peak 1-a	-	-	-	-	-	-

+: Completely inhibits cleavage

$\pm X$: Inhibits X% of cleavage

-: Does not inhibit cleavage

(February 26, 2025, room temperature around 15°C)

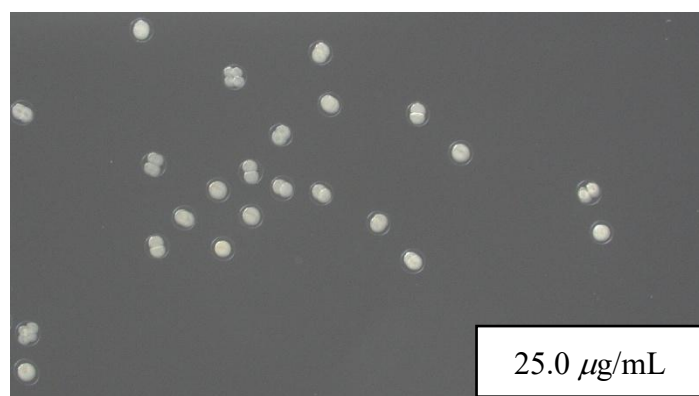


Figure 5-3. Sea urchin fertilized eggs after 120 min treatment with peak 1-a

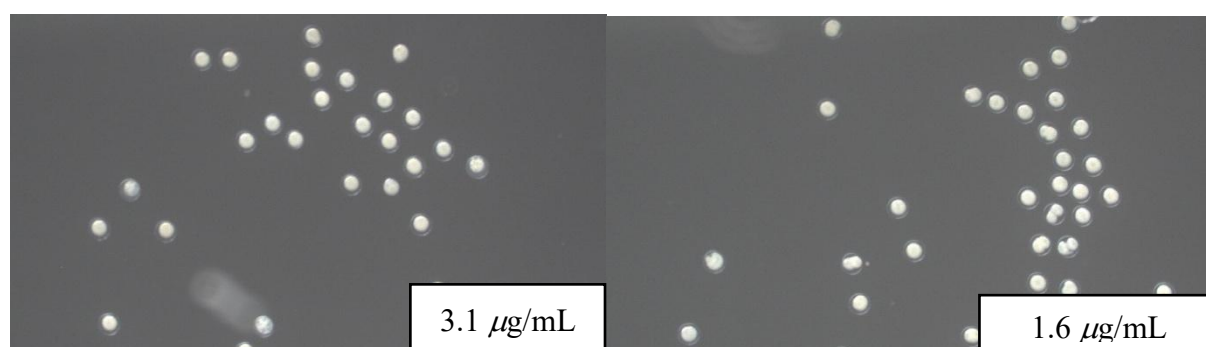


Figure 5-4. Sea urchin fertilized eggs after 120 min treatment with peak 1-b

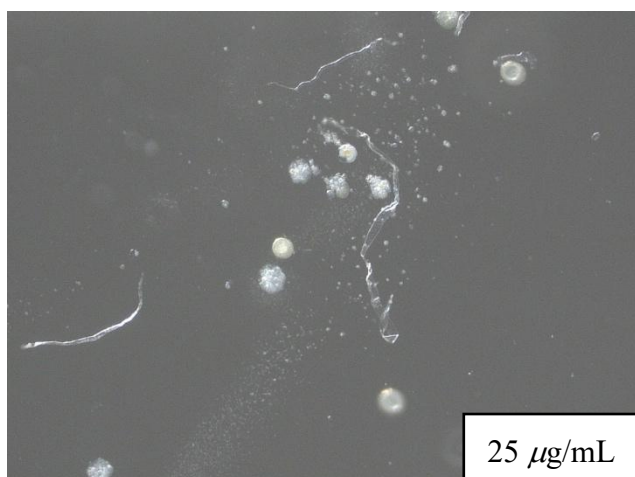


Figure 5-5. Sea urchin fertilized eggs after 24 hours treatment with peak 1-a

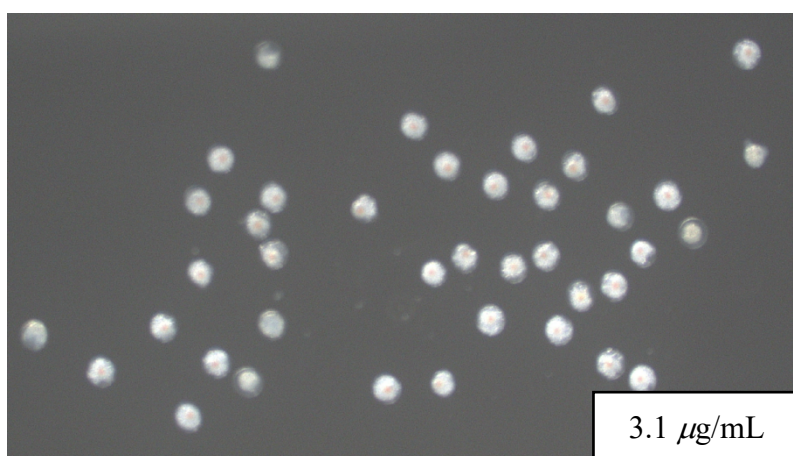


Figure 5-6. Sea urchin fertilized eggs after 24 hours treatment with peak 1-b

Peak 1-b and peak 1-a were evaluated for their inhibitory activity toward the first cleavage of sea urchin embryos. Peak 1-b having an all-*trans* conjugated triene inhibited the first cleavage of sea urchin embryos with an MIC value of 3.1 µg/mL, whereas its 11-*cis* isomer, peak 1-a, did not show inhibitory activity up to the concentration of 25 µg/mL. J. Wang *et al.* evaluated the antiviral activity of asteltoxin and its 11-*cis* isomer, isoasteltoxin, by determining the inhibition of the cytopathic effect of influenza virus, finding that the IC₅₀ values of asteltoxin and isoasteltoxin against H1N1/H3N2 influenza viruses were $0.54 \pm 0.06/0.84 \pm 0.02$ µM, and $0.23 \pm 0.05/0.66 \pm 0.09$ µM, respectively (J. Wang *et al.*, 2016). These results indicated that the geometry of the conjugated triene in asteltoxins is considered to contribute to their biological activities.

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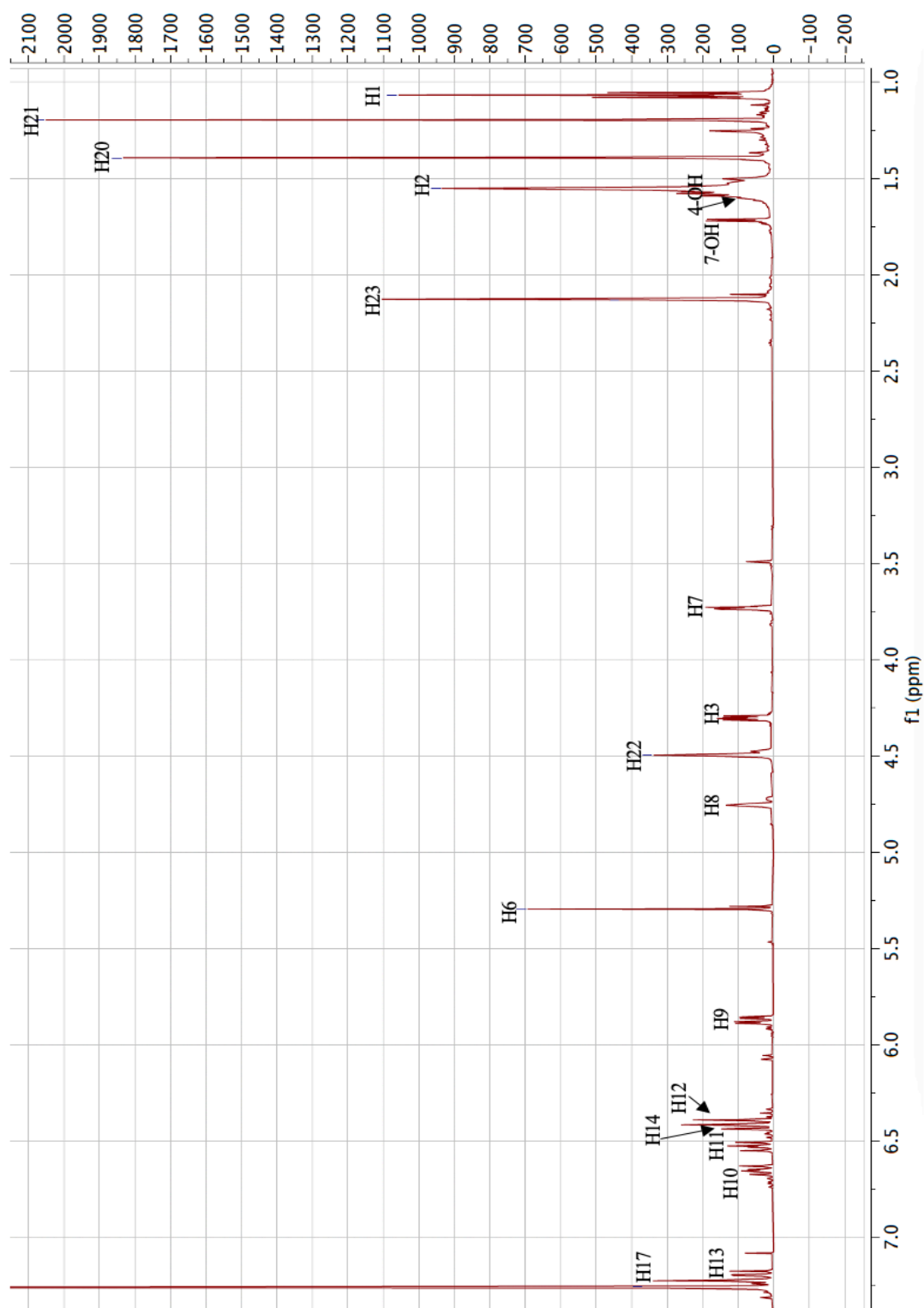
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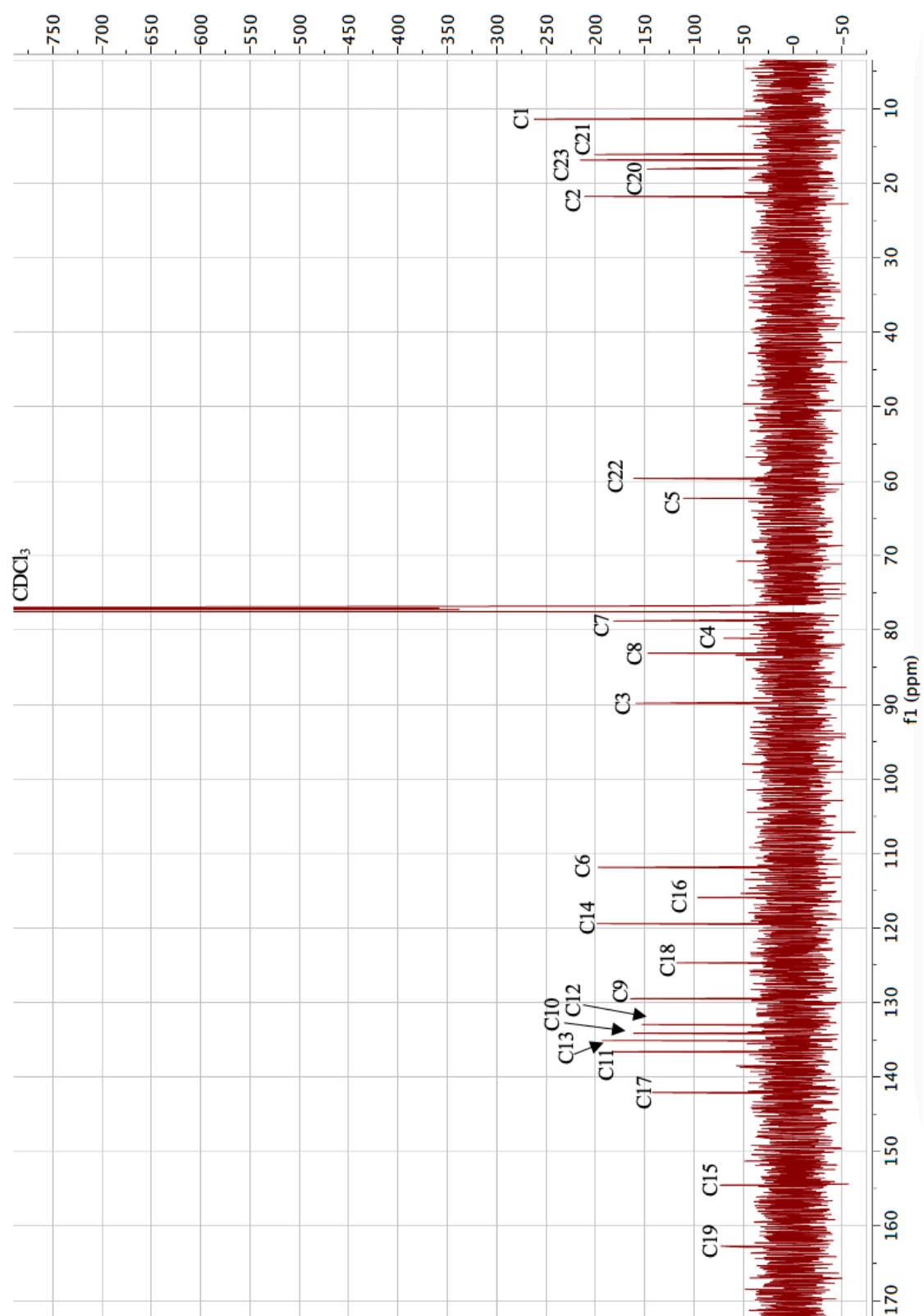
APPENDIX

Contents

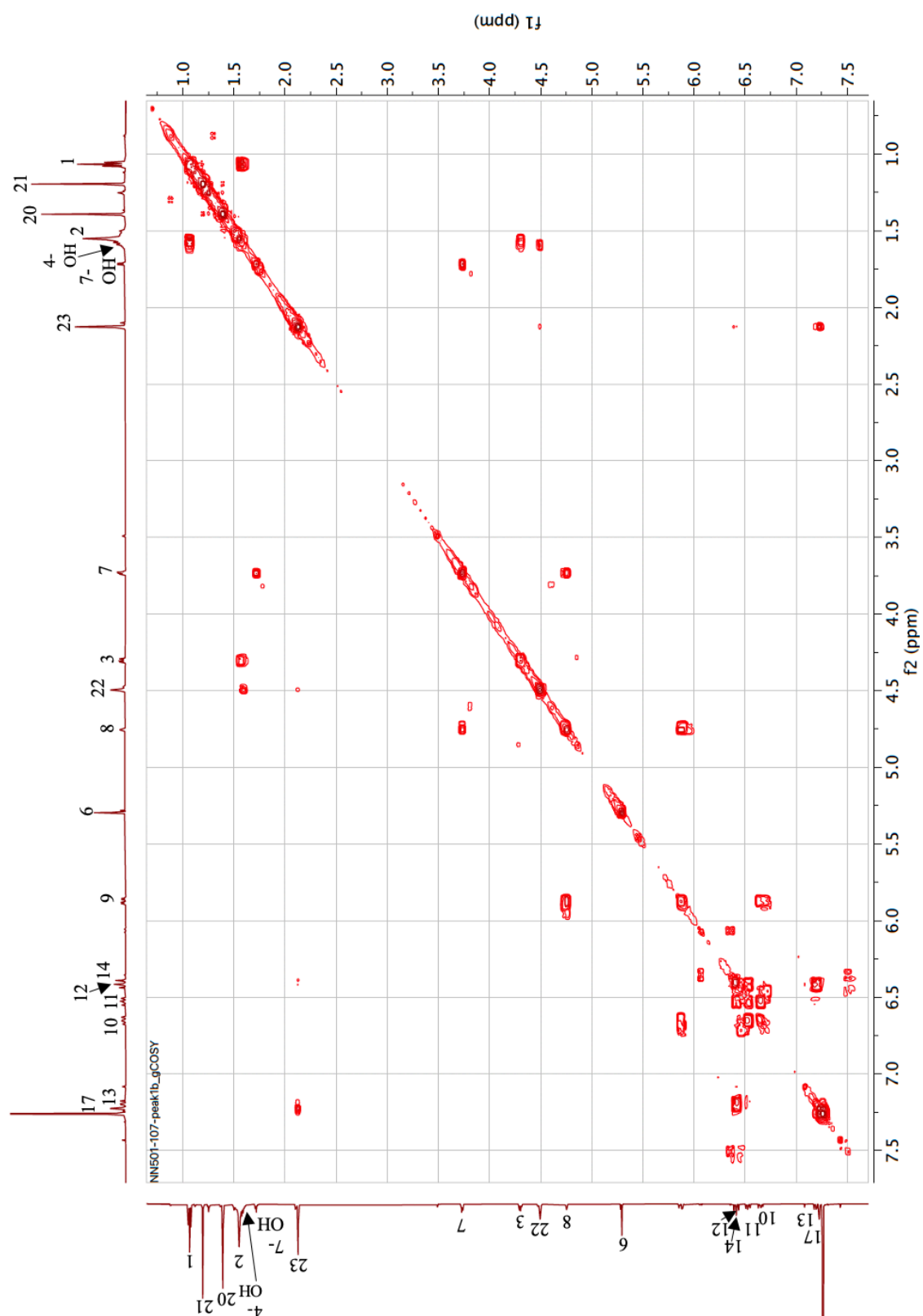
Supplemental Fig. S1. ^1H NMR (600 MHz, CDCl_3) spectrum of peak 1-b	S1
Supplemental Fig. S2. ^{13}C NMR (150 MHz, CDCl_3) spectrum of peak 1-b	S2
Supplemental Fig. S3. COSY spectrum of peak 1-b	S3
Supplemental Fig. S4. HSQC spectrum of peak 1-b	S4
Supplemental Fig. S5. HMBC spectrum of peak 1-b	S5
Supplemental Fig. S6. NOESY spectrum of peak 1-b	S6
Supplemental Fig. S7. ^1H NMR (600 MHz, CDCl_3) spectrum of peak 1-a	S7
Supplemental Fig. S8. ^{13}C NMR (150 MHz, CDCl_3) spectrum of peak 1-a	S8
Supplemental Fig. S9. COSY spectrum of peak 1-a	S9
Supplemental Fig. S10. HSQC spectrum of peak 1-a	S10
Supplemental Fig. S11. HMBC spectrum of peak 1-a	S11
Supplemental Fig. S12. NOESY spectrum of peak 1-a	S12

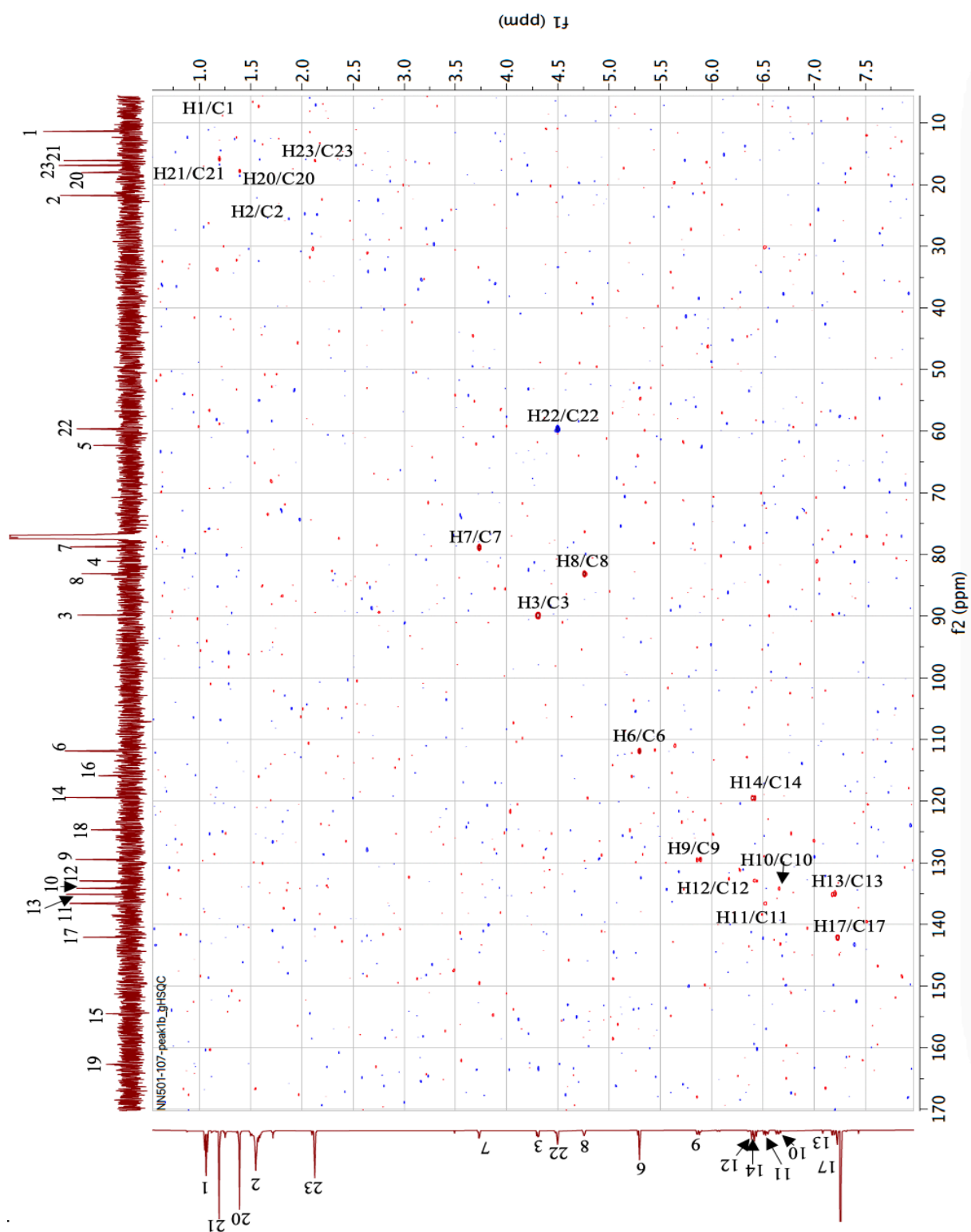


Supplemental Fig. S1. ^1H NMR (600 MHz, CDCl_3) spectrum of peak 1-b

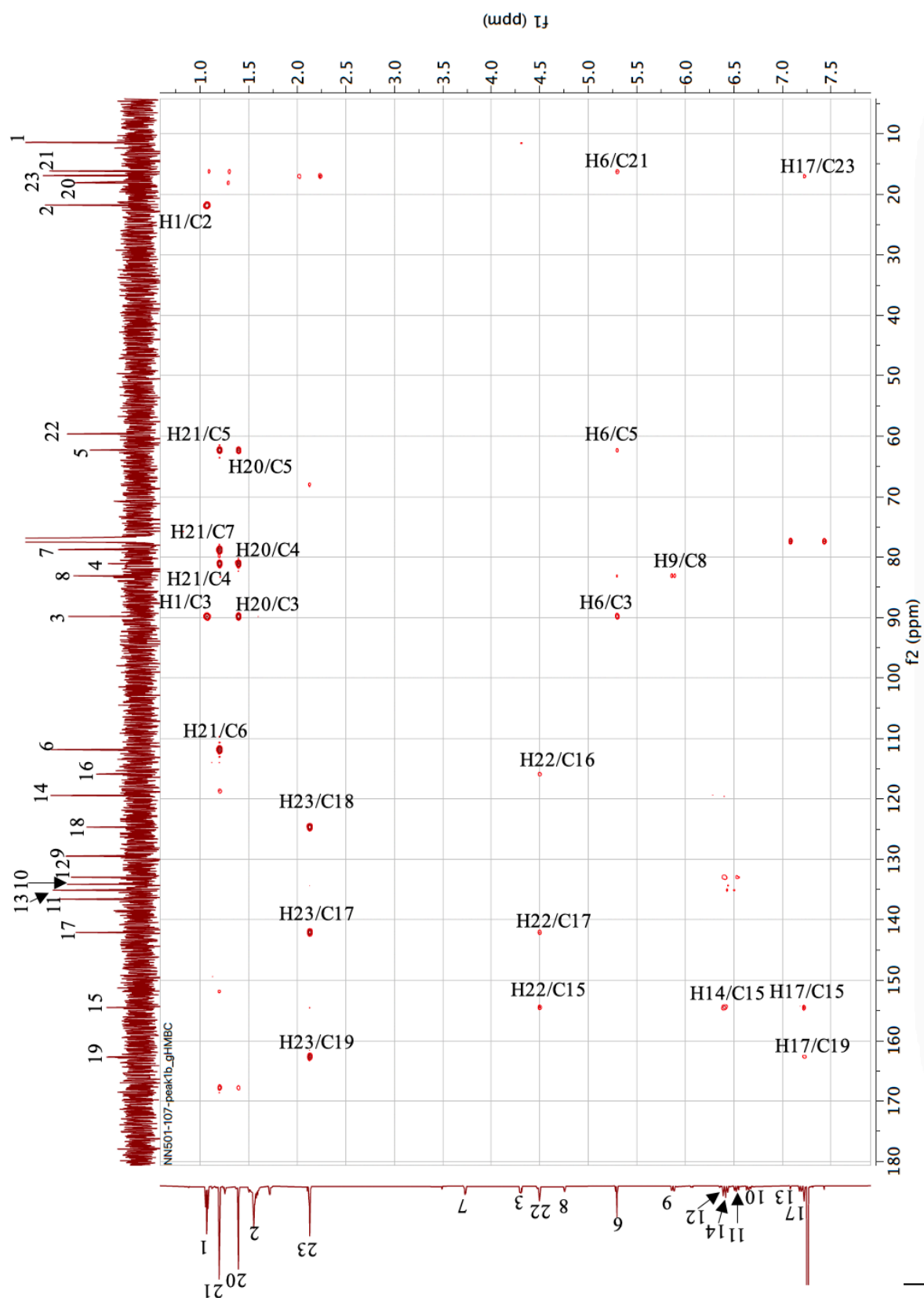


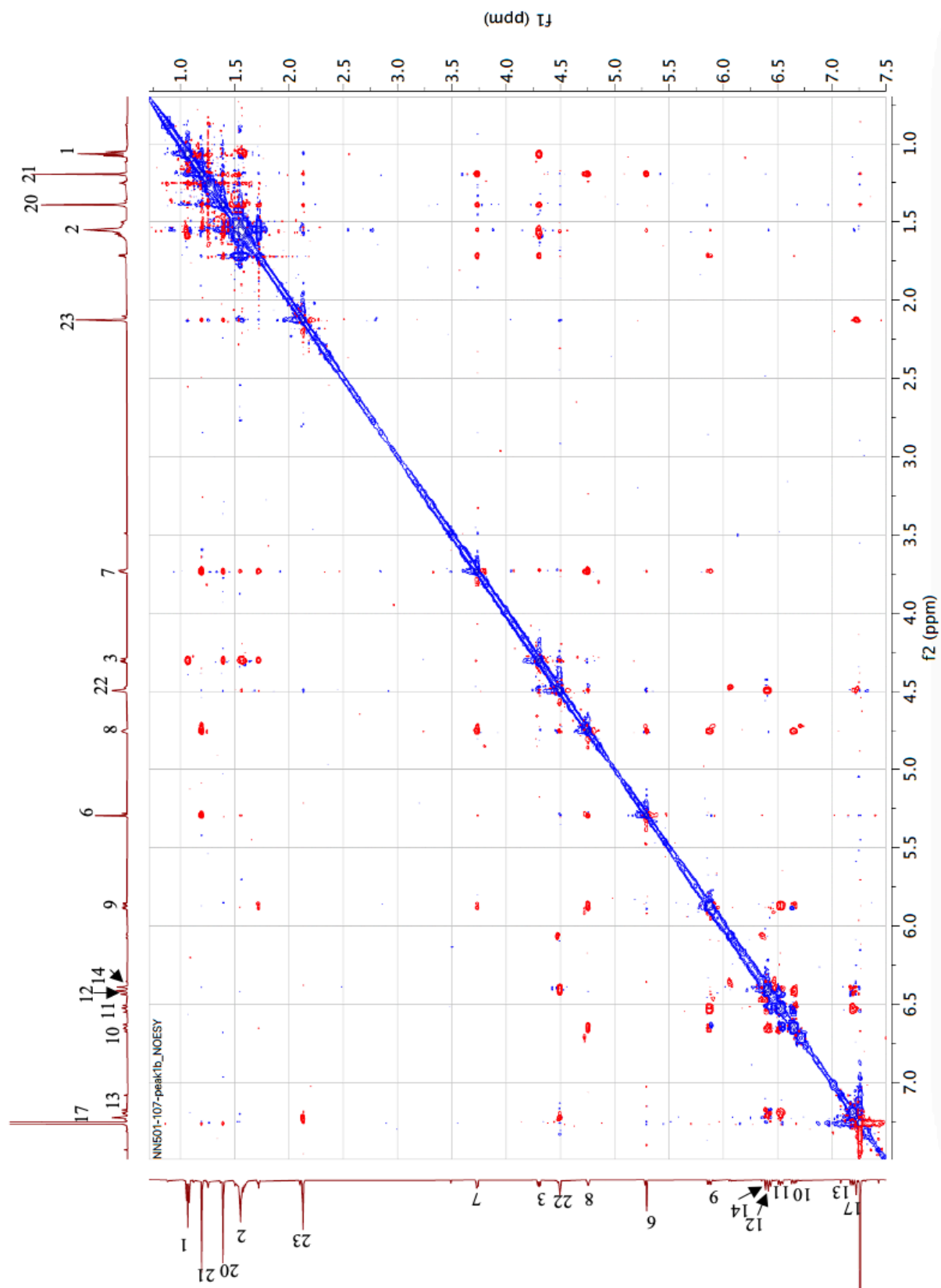
Supplemental Fig. S2 ^{13}C NMR (150 MHz, CDCl_3) spectrum of peak 1-b



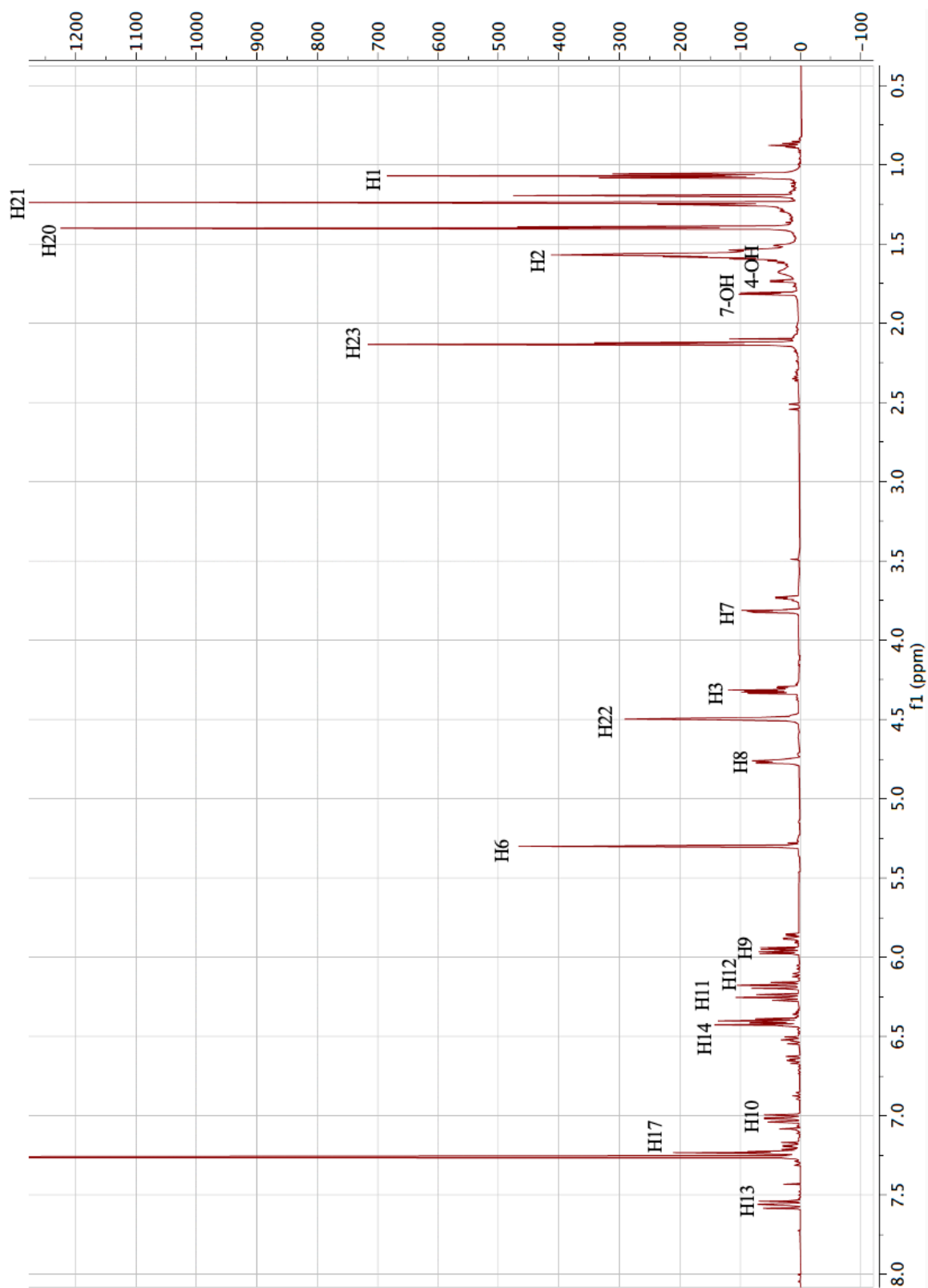


Supplemental Fig. S4. HSQC spectrum of peak 1-b

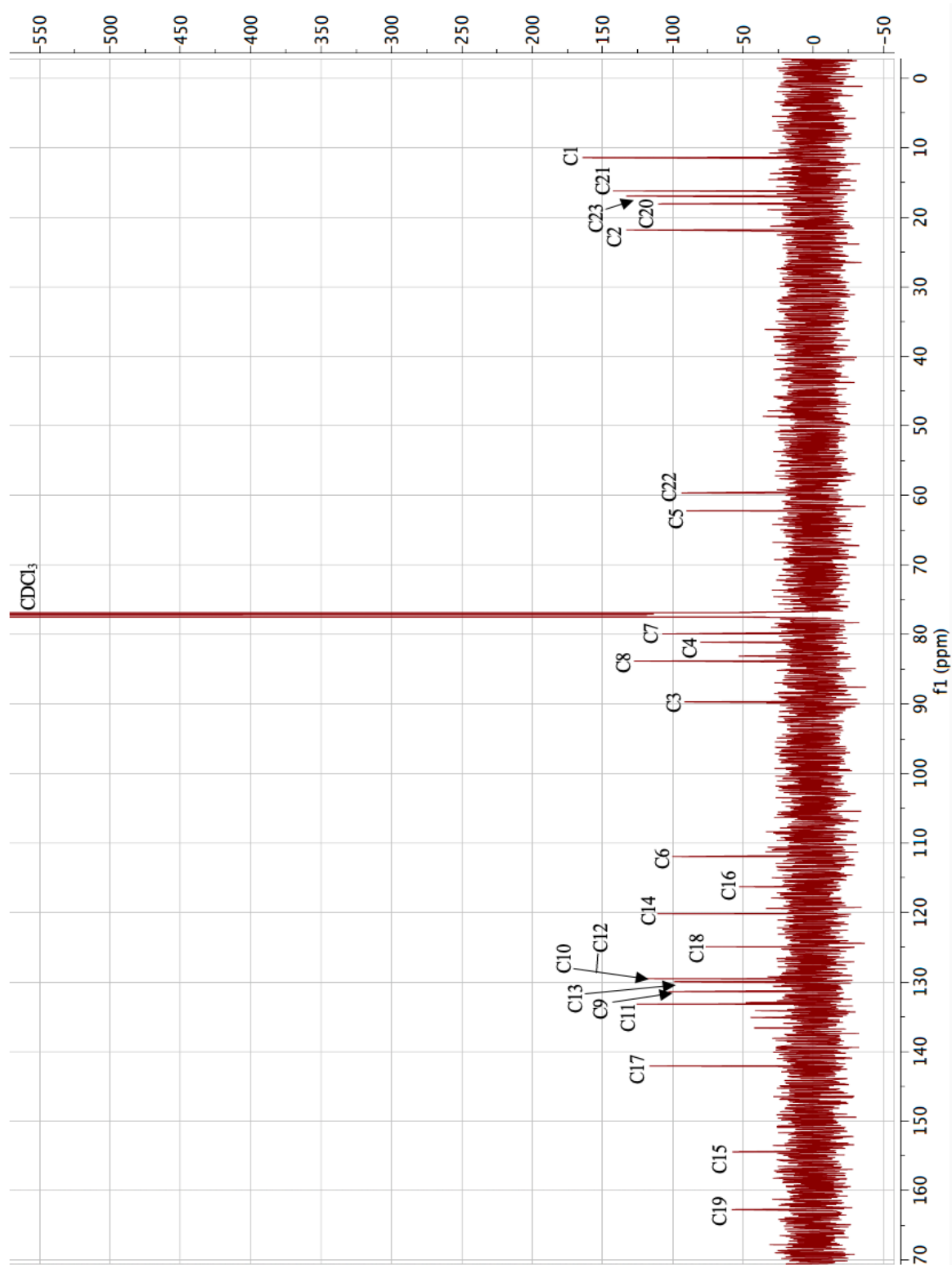




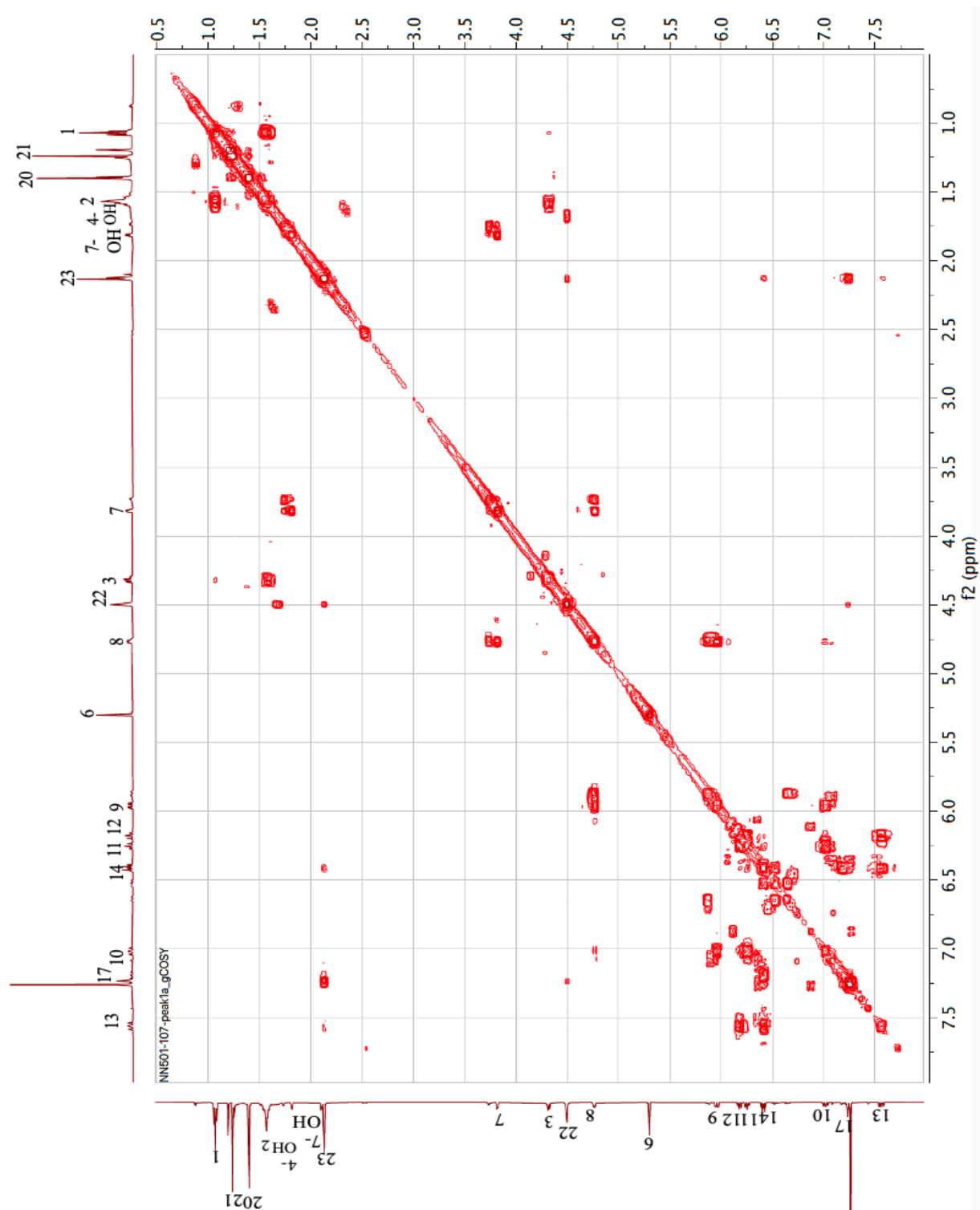
Supplemental Fig. S6. NOESY spectrum of peak 1-b

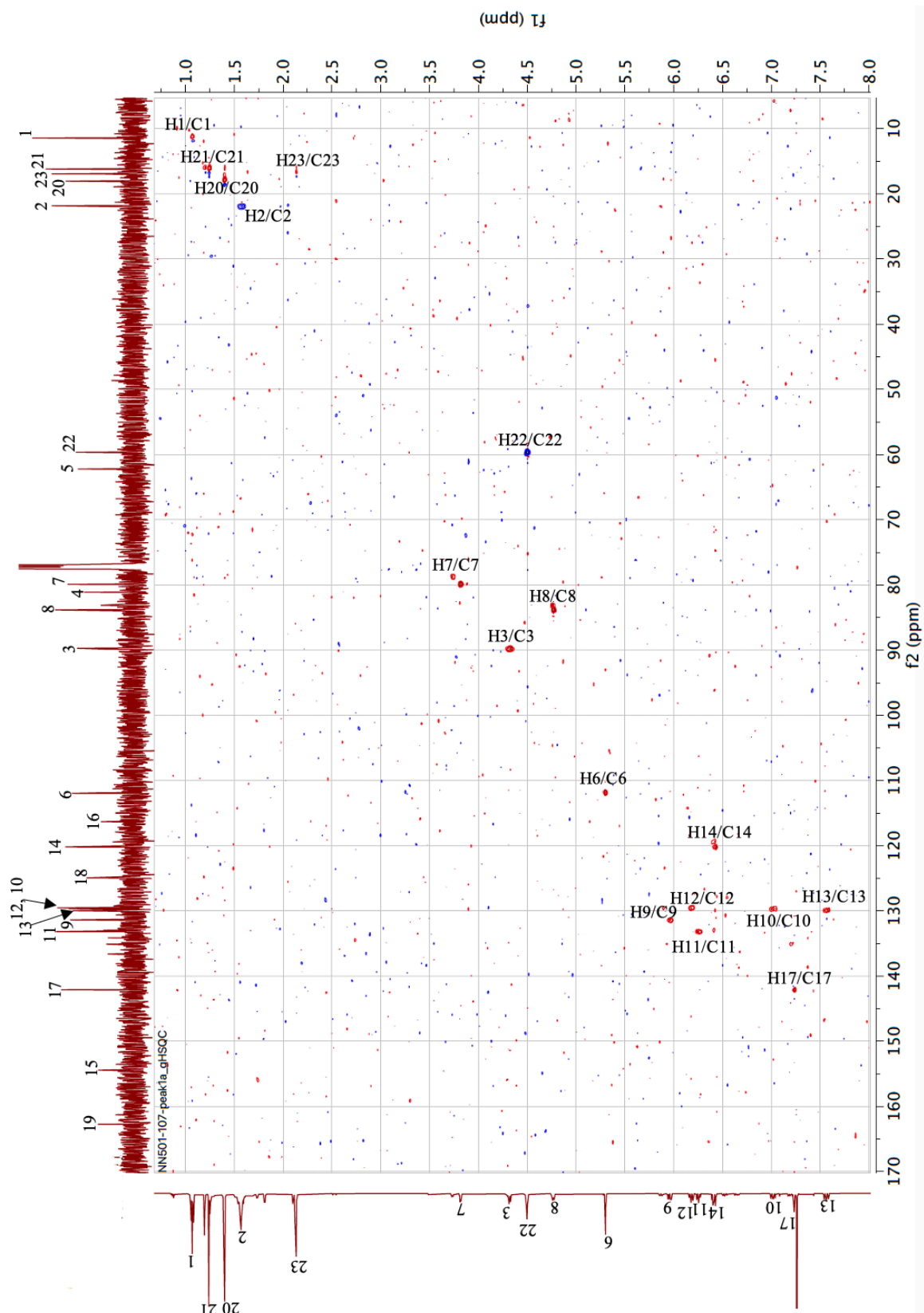


Supplemental Fig. S7. ^1H NMR (600 MHz, CDCl_3) spectrum of peak 1-a

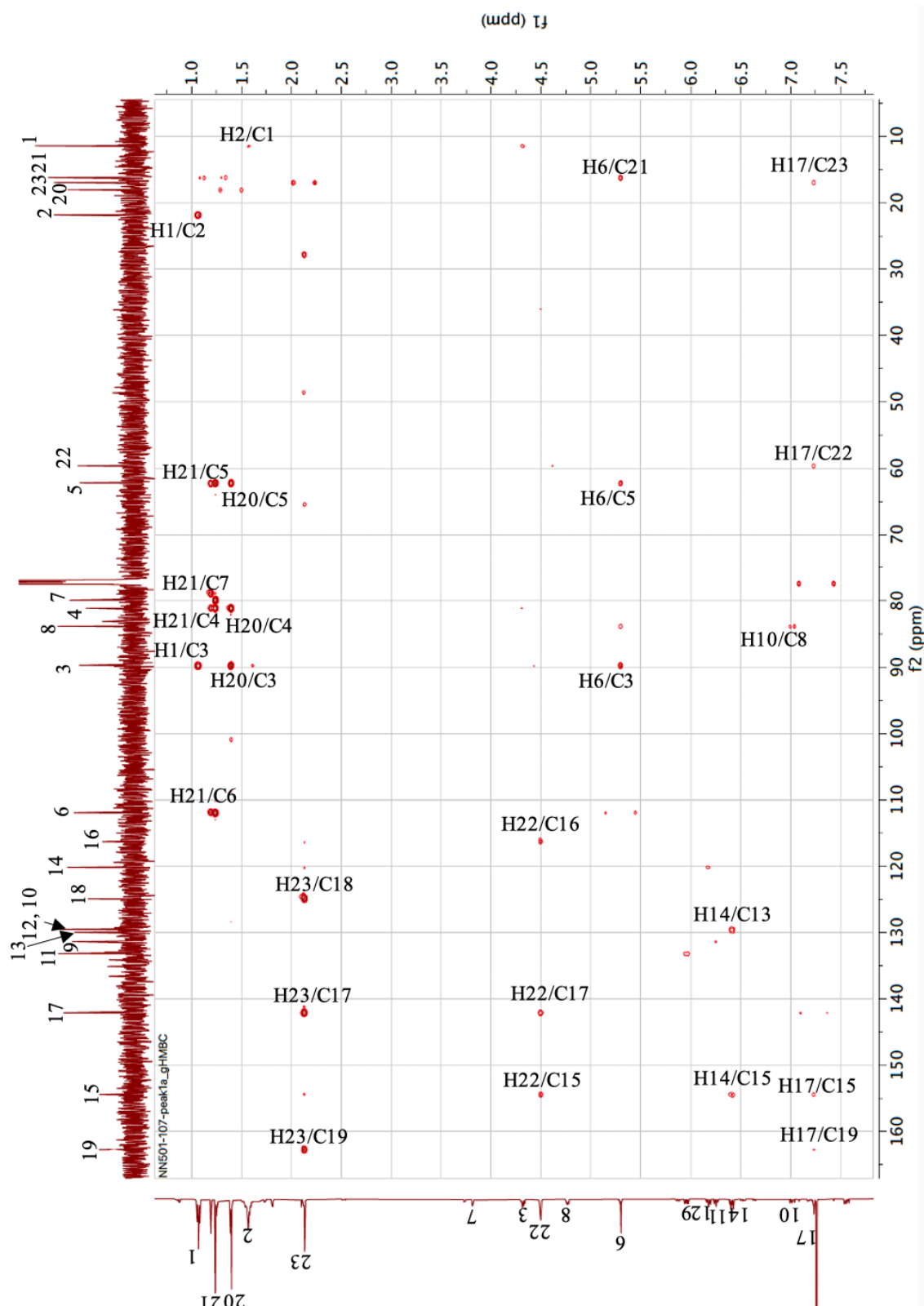


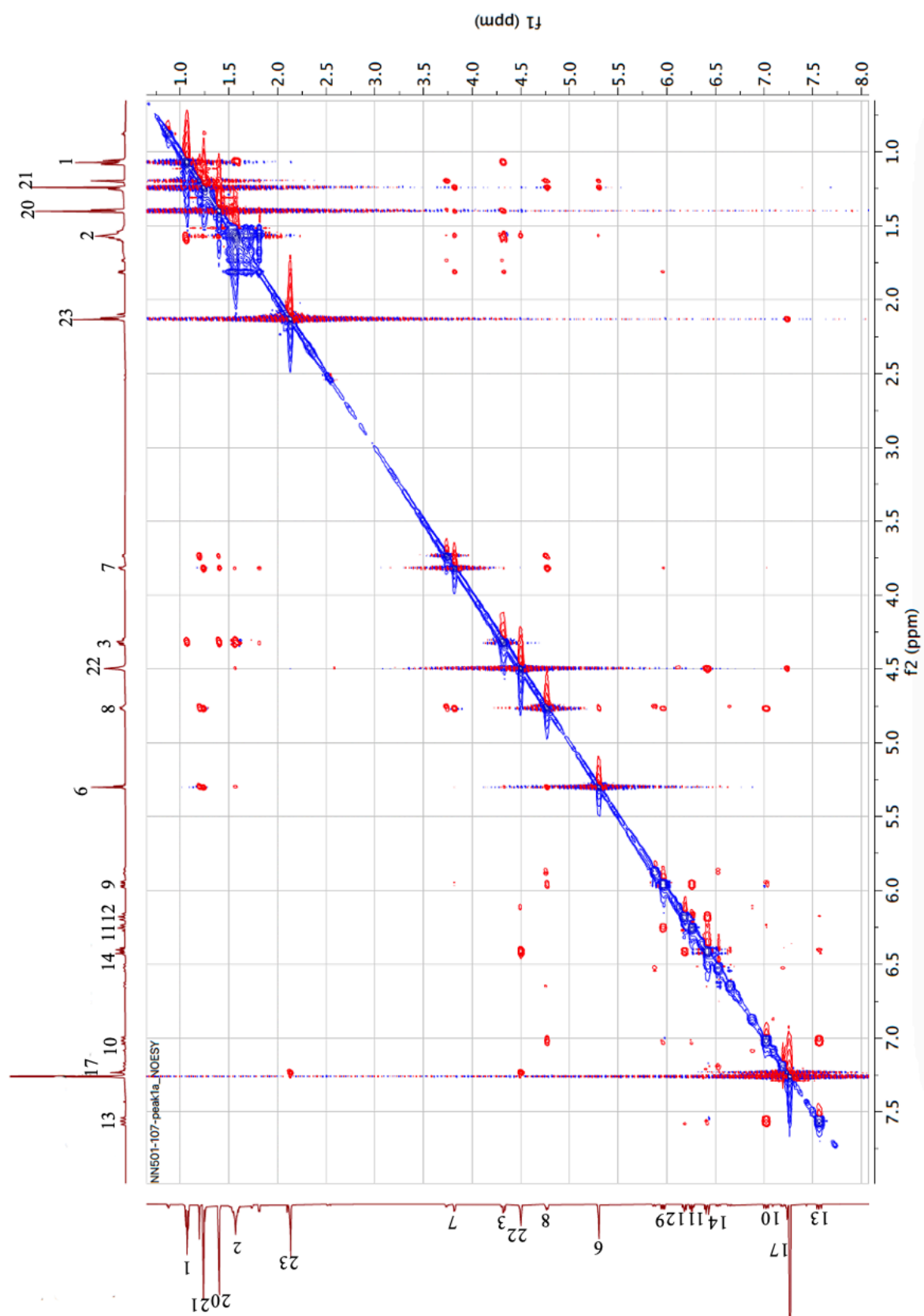
Supplemental Fig. S8. ¹³C NMR (150 MHz, CDCl₃) spectrum of peak 1-a





Supplemental Fig. S10. HSQC spectrum of peak 1-a





Supplemental Fig. S12. NOESY spectrum of peak 1-a