

Isolation and identification of the bioactive natural product A-26771B from an endophytic

***Penicillium expansum* isolate**

by

Kirstyn A. Forgrave

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Supervisor: Dr. Christopher A. Gray, Department of Biological Sciences and
Department of Chemistry

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ABSTRACT

Natural products are an important source of bioactive compounds and endophytic fungi derived from marine algae represent an under-investigated source of natural products. The objective of this research was to isolate and confirm that an extract from an endophytic *Penicillium expansum* contained the natural product A-26771B. The natural product was tentatively detected, using high-resolution electrospray ionization mass spectroscopy data, in a methanolic extract from the endophyte. Despite repeated rounds of high performance liquid chromatography and multiple fermentations of the endophyte, the compound was isolated in sub-milligram quantities and identified using proton nuclear magnetic resonance data. However, prior to obtaining carbon nuclear magnetic resonance data that would confirm the identification of A-26771B the natural product degraded. Future work should include isolating more of the molecule A-26771B to determine the bioactivity of the molecule against *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.

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First and foremost, I would like to express my deepest gratitude to Dr. Christopher A. Gray and Dr. John A. Johnson for their guidance, support, and feedback throughout my project. I would also like to thank the past and current members of the Natural Product Research Group at the University of New Brunswick. Furthermore, I would like to thank Dr. Hilary Jenkins at the NMR Facility at McMaster University for attempting to obtain an NMR for my sample.

STATEMENT OF RESEARCH CONTRIBUTION

An endophytic *Penicillium expansum* was previously isolated from the brown alga *Desmarestia viridis*. A methanolic extract of the endophyte was assessed against a suite of organisms and inhibited the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* and was analyzed using high-resolution electrospray ionization mass spectroscopy. The high-resolution electrospray ionization mass spectroscopy data provided a mass-to-charge ratio that indicated the bioactive natural product A-26771B could be in the extract. My contribution began with a fermentation (10 L) of the *Penicillium expansum* isolate to generate an extract. In an attempt to isolate A-26771B, the fractionation of the extract was guided by nuclear magnetic resonance spectroscopy. After multiple fractionation steps, sub-milligram quantities of the molecule were isolated.

Table of Contents

ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
STATEMENT OF RESEARCH CONTRIBUTION	iv
Table of Contents	v
List of Tables	vi
List of Figures.....	vii
List of Symbols or Abbreviations.....	xi
1. Introduction	1
2. Results and Discussion	4
3. Experimental.....	13
3.1 General experimental procedures	13
3.2 Liquid culture fermentation and extraction	14
3.3 Fractionation of extract.....	14
References	16
Appendix	25

List of Tables

Table 1: A comparison of the experimental ^1H NMR spectral data (400 MHz, CD_3OD) with spectral data reported for the molecule A-26771B. ⁸³	8
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List of Figures

Figure 1: Normalized bioactivity for an extract generated from the endophytic <i>Penicillium expansum</i> isolate from the marine alga <i>Desmarestia viridis</i> against a suite of organisms (<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Mycobacterium smegmatis</i> , <i>Mycobacterium tuberculosis</i> , <i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Candida albicans</i> , and <i>Saccharomyces cerevisiae</i>). ⁵⁴	3
Figure 2: The structure of the macrocyclic natural product A-26771B. ^{79,80}	3
Figure 3: ¹ H NMR spectrum (CDCl ₃) of A-26771B reported in literature ⁸⁴ (top) compared to ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the extract prior to fractionation (bottom).	5
Figure 4: ¹ H NMR spectra (400 MHz, CD ₃ OD) for the reverse-phase flash column fractions seven (top), eight, and nine (eluting at 40 % H ₂ O; 60 % ACN, 30 % H ₂ O; 70 % ACN, and 20 % H ₂ O; 80 % ACN respectively) compared to the ¹ H NMR spectrum of A-26771B obtained from the second round of high-performance liquid chromatography (bottom).	6
Figure 5: ¹ H NMR spectra (400 MHz, CD ₃ OD) of the reverse-phase flash column fraction seven (eluting at 40 % H ₂ O; 60 % ACN; top), the corresponding reverse-phase high-performance liquid chromatography fraction referred to as 7c (middle), and the molecule A-26771B obtained from the second round of high-performance liquid chromatography referred to as 7c1(bottom).	6
Figure 6: ¹ H NMR spectra (400 MHz, CD ₃ OD) of the reverse-phase flash column fraction eight (eluting at 30 % H ₂ O; 70 % ACN; top), the corresponding reverse-phase high-performance liquid chromatography fraction referred to as 8c (middle), and the molecule A-26771B obtained from the second round of high-performance liquid chromatography referred to as 8c1(bottom).	7

Figure 7: ¹H NMR spectrum (CDCl₃) of A-26771B reported in literature⁸⁴ (top) compared to ¹H NMR spectra (400 MHz, CD₃OD) of the high-performance liquid chromatography fractions identified to be A-26771B.9

Figure 8: ¹H NMR spectrum (CDCl₃) of A-26771B reported in literature⁸⁴ (top) compared to ¹H NMR spectrum (600 MHz, CDCl₃) of the high-performance liquid chromatography fraction identified to be A-26771B after the molecule degraded (bottom)..... 11

Figure A1: Schematic representation of the fractionation protocol followed for the extract generated from an endophytic *Penicillium expansum* isolate.25

Figure A2: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 100 % H₂O and referred to as fraction 1.26

Figure A3: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 90 % H₂O; 10 % ACN and referred to as fraction 2.....26

Figure A4: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 80 % H₂O; 20 % ACN and referred to as fraction 3.....27

Figure A5: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 70 % H₂O; 30 % ACN and referred to as fraction 4.....27

Figure A6: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 60 % H₂O; 40 % ACN and referred to as fraction 5.....28

Figure A7: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 50 % H₂O; 50 % ACN and referred to as fraction 6.....28

Figure A8: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 10 % H₂O; 90 % ACN and referred to as fraction 10.....29

Figure A9: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the reverse-phase flash column fraction eluting at 100 % ACN and referred to as fraction 11.	29
Figure A10: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the reverse-phase flash column fraction eluting at 100 % EtOAc and referred to as fraction 12.	30
Figure A11: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7a.....	30
Figure A12: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7b.	31
Figure A13: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7d.	31
Figure A14: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7e.....	32
Figure A15: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7c2.....	32
Figure A16: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7c3.....	33
Figure A17: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 7c4.....	33
Figure A18: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8a.....	34
Figure A19: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8b.	34

Figure A20: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8d.	35
Figure A21: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8e.....	35
Figure A22: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8f.	36
Figure A23: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8g.	36
Figure A24: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8c2.....	37
Figure A25: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8c3.....	37
Figure A26: ¹ H NMR spectrum (400 MHz, CD ₃ OD) of the high-performance liquid chromatography fraction referred to as 8c4.....	38

List of Symbols or Abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
^1H NMR	Proton nuclear magnetic resonance spectroscopy
CDCl_3	Deuterated chloroform
CD_3OD	Deuterated methanol
ACN	Acetonitrile
δ	Chemical shift in ppm
d	Doublet
dd	Doublet of doublets
EtOAc	Ethyl acetate
H	Hydrogen
H_2O	Water
HPLC	High-performance liquid chromatography
HRESIMS	High-resolution electrospray ionization mass spectroscopy
Hz	Hertz
J	Coupling constant
m	Multiplet
MeOH	Methanol
MHz	Megahertz
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance spectroscopy
ppm	Parts per million
RPFC	Revere-phase flash column
rpm	Revolutions per minute
s	Singlet
S/N	Signal-to-noise ratio
TLC	Thin layer chromatography
t	Triplet

1. Introduction

Natural products are organic compounds that are not essential for the growth and development of the producing organism.¹⁻⁴ Investigation of natural products began more than 200 years ago and over 300,000 natural products have been isolated.^{5,6} As more natural products are isolated it becomes increasingly evident that they represent an important source of biologically active molecules.^{1,3,7-11} Natural products, as an important source of biologically relevant molecules, have influenced the development of many fields, perhaps most significantly in the development of chemical therapeutics.⁷⁻¹² To date, most of the natural products isolated have been sourced from plants, however, in an attempt to obtain more bioactive natural products, isolation efforts have shifted to include more sources of these molecules.¹³⁻⁶² One source of particular interest is microbes associated with other organisms.⁵²⁻⁶²

Endophytic fungi, or endophytes, represent an important source of bioactive natural products.^{52-54,57,63} Endophytes live within the tissues of plants without causing apparent harm to the host.^{4,53,56,63-65} Previous studies have indicated that endophytes may be ubiquitous in plants⁵⁷ and that the population of endophytes within a plant may vary with the tissue type,⁶⁶⁻⁷⁰ as well as the geographic and temporal distributions of the plant.^{68,71-75} This provides the immense biodiversity of endophytes which allows access to a plethora of chemical entities available for investigation.

Much of the research on endophytic fungi has focused on terrestrial plants, however, endophytes derived from marine macroalgae are a source of bioactive natural products.^{13,52-54}

The possibility that fungi could inhabit marine macroalgae was not considered until the late nineteenth century.⁷⁶ Since then, few studies have isolated natural products from endophytes derived from marine macroalgae. Using the database Scopus® and searching "marine" AND "alga*" AND "endophyt*" resulted in the search of the terms marine, algae, alga, endophyte(s), and endophytic which yielded 168 articles, whereas, searching for "endophyt*" resulted in the search of the terms endophyte(s) and endophytic which yielded 16,538 articles.⁷⁷ These values indicate that only about one percent of articles on endophytes have focused on endophytes derived from marine algae.⁷⁷ Despite being relatively unexplored, the few macroalgal-derived endophytes that have been investigated have shown themselves to be an important source of biologically active molecules,^{13,52-54,62,78} therefore, further investigation into endophytes derived from marine macroalga is warranted.

The endophyte used for this thesis was isolated from the brown alga, *Desmarestia viridis* and was identified to be a *Penicillium expansum* isolate.⁵⁴ A methanolic extract of the *Penicillium expansum* isolate was tested against a suite of organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Candida albicans*, and *Saccharomyces cerevisiae*) and displayed broad-spectrum bioactivity against *E. coli*, *P. aeruginosa*, *M. smegmatis*, *M. tuberculosis*, and *S. aureus* (Figure 1).⁵⁴ High-resolution electrospray ionization mass spectroscopy (HRESIMS) data was generated for a methanolic extract of the endophyte and was used to tentatively detect the macrocyclic lactone A-26771B (Figure 2).

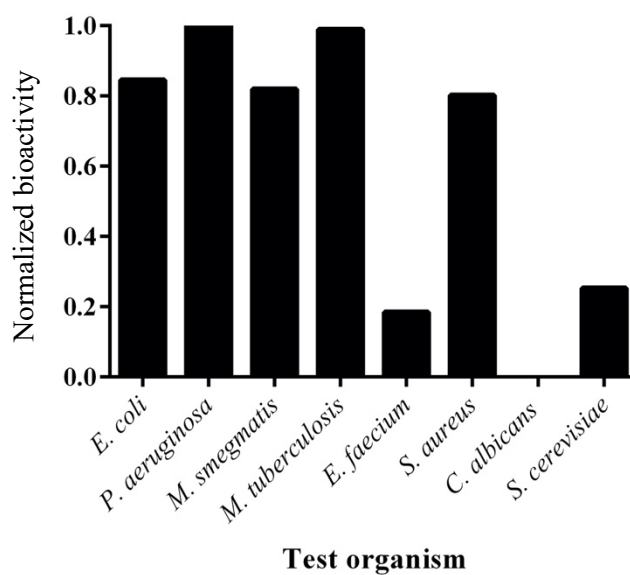


Figure 1: Normalized bioactivity for an extract generated from the endophytic *Penicillium expansum* isolate from the marine alga *Desmarestia viridis* against a suite of organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Candida albicans*, and *Saccharomyces cerevisiae*).⁵⁴

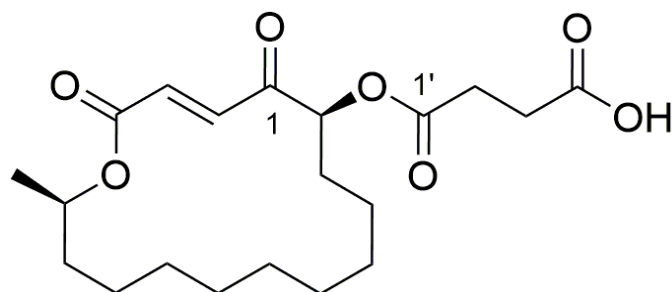


Figure 2: The structure of the macrocyclic natural product A-26771B.^{79,80}

The natural product A-26771B was first isolated in 1975 from a *Penicillium turbatum* isolate and since has been isolated from a co-culture of *Penicillium fuscum* and *Penicillium camemberti*.^{81–83} The biological activity of the natural product A-26771B has been reported against a range of organisms, however, the activity of the molecule against mycobacteria was

never established.^{81,82} The previously derived methanolic extract from the endophytic *Penicillium expansum* isolate, that was tentatively identified to contain the macrocyclic lactone A-26771B, inhibited the growth of *M. smegmatis* and *M. tuberculosis*. The work described in this thesis aimed to determine whether the presence of the molecule A-26771B was responsible for the biological activity against mycobacteria displayed by the methanolic extract.

The objective of this research was to confirm that an extract of the endophytic *Penicillium expansum* isolate, derived from the marine alga *Desmarestia viridis*, contained the macrocyclic lactone A-26771B.

2. Results and Discussion

The endophytic *Penicillium expansum* isolate was fermented and extracted to generate an extract that was analyzed by proton nuclear magnetic resonance (¹H NMR). When the ¹H NMR for the extract was compared to the ¹H NMR reported for the molecule A-26771B,⁸⁴ resonances indicative of A-26771B were partially obscured which indicated the complexity of the extract (Figure 3). Purification of the extract involved loading the extract onto a C-18 silica column and eluted with water (H₂O), methanol (MeOH), and ethyl acetate (EtOAc). The MeOH fraction was selected for further investigation since the high-resolution electrospray ionization mass spectroscopy (HRESIMS) data for the previous methanolic extract derived from the endophytic *Penicillium expansum* isolate was tentatively detected to contain A-26771B. The MeOH fraction was further separated using a reverse-phase flash column (RPFC) that afforded twelve fractions (a schematic representation of the fractionation protocol is given in Figure A1). The twelve RPFC fractions were analyzed using ¹H NMR spectroscopy to identify which fractions contained

the macrocyclic lactone A-26771B. The ^1H NMR of the RPFC fractions seven, eight, and nine contained resonances indicative of A-26771B, the most noticeable being the two doublets (d) at 7.31 ppm and 6.71 ppm (Figure 4), and were subjected to reverse-phase high-performance liquid chromatography (HPLC; 35 % H_2O ; 65 % ACN). The quantity of material in the RPFC fraction nine was exhausted during the process of reverse-phase HPLC. One fraction that was collected from each of the RPFC fractions 7 and 8, with the same retention time on the reverse-phase HPLC column, was identified to contain A-26771B by analyzing the ^1H NMR spectra, and was referred to as 7c and 8c in the schematic representation of the fractionation protocol (Figure 5, Figure 6, and Figure A1). HPLC was successful in further purifying A-26771B, however, the samples still contained impurities. This was evident in the ^1H NMR by the two doublets at 6.78 ppm and 6.14 ppm.

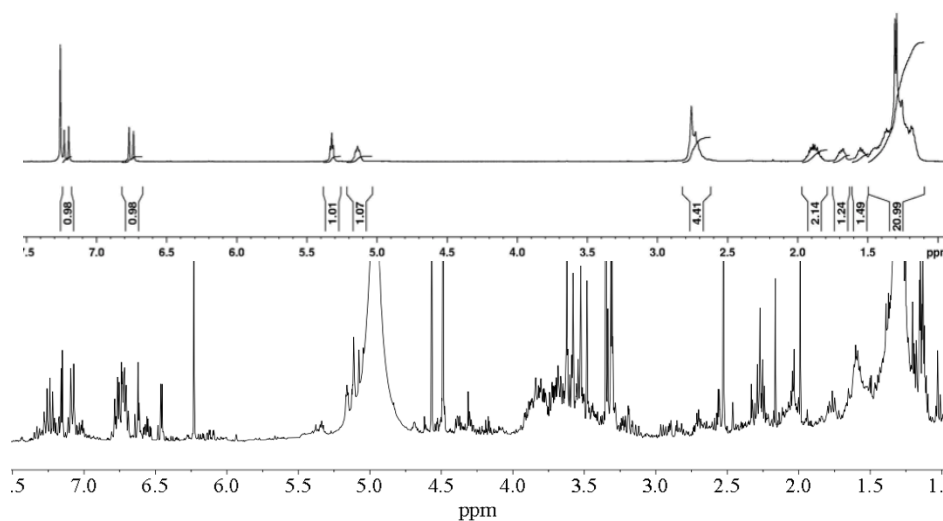


Figure 3: ^1H NMR spectrum (CDCl_3) of A-26771B reported in literature⁸⁴ (top) compared to ^1H NMR spectrum (400 MHz, CD_3OD) of the extract prior to fractionation (bottom).

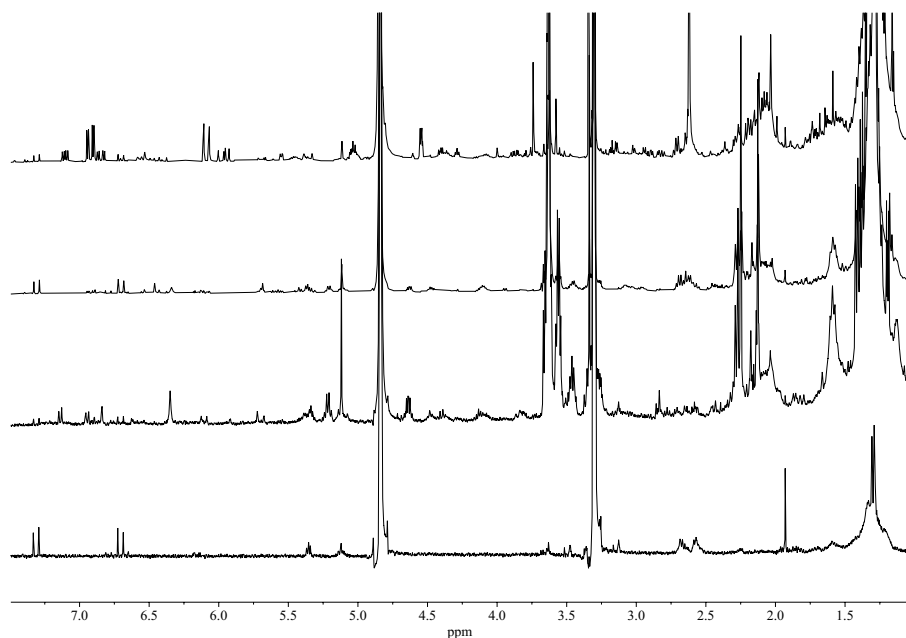


Figure 4: ^1H NMR spectra (400 MHz, CD_3OD) for the reverse-phase flash column fractions seven (top), eight, and nine (eluting at 40 % H_2O ; 60 % ACN, 30 % H_2O ; 70 % ACN, and 20 % H_2O ; 80 % ACN respectively) compared to the ^1H NMR spectrum of A-26771B obtained from the second round of high-performance liquid chromatography (bottom).

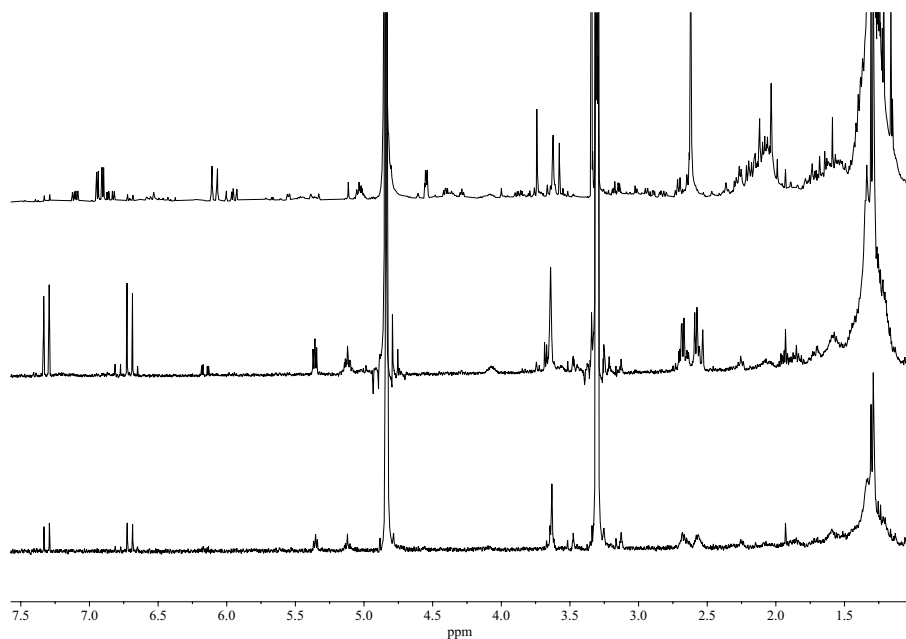


Figure 5: ^1H NMR spectra (400 MHz, CD_3OD) of the reverse-phase flash column fraction seven (eluting at 40 % H_2O ; 60 % ACN; top), the corresponding reverse-phase high-performance liquid chromatography fraction referred to as 7c (middle), and the molecule A-26771B obtained from the second round of high-performance liquid chromatography referred to as 7c1 (bottom).

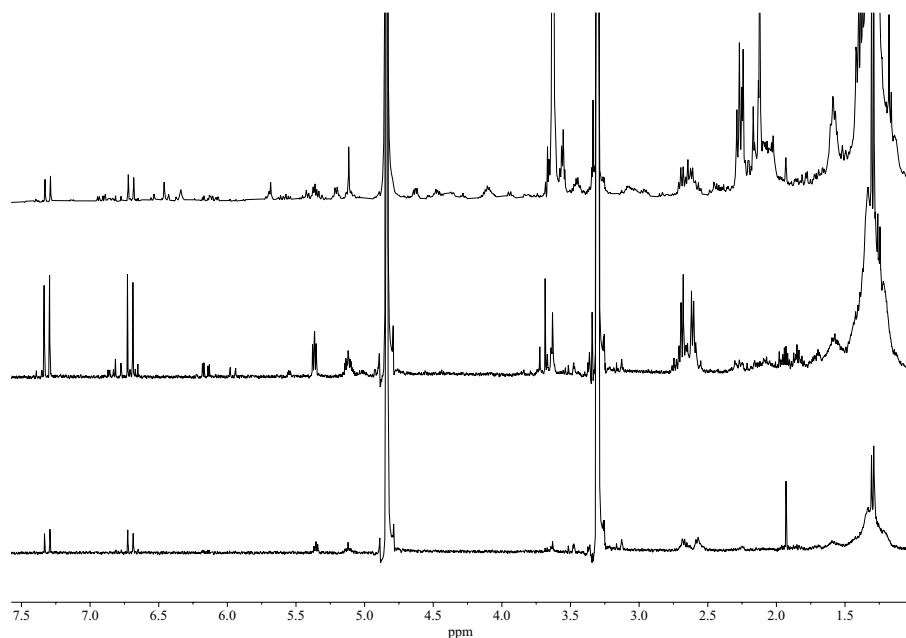


Figure 6: ^1H NMR spectra (400 MHz, CD_3OD) of the reverse-phase flash column fraction eight (eluting at 30 % H_2O ; 70 % ACN; top), the corresponding reverse-phase high-performance liquid chromatography fraction referred to as 8c (middle), and the molecule A-26771B obtained from the second round of high-performance liquid chromatography referred to as 8c1 (bottom).

Since impurities were present in the reverse-phase HPLC fractions containing A-26771B, the compatibility of the fractions with normal-phase HPLC was assessed using thin layer chromatography (TLC), however, no suitable solvent conditions were found. Since normal-phase HPLC conditions were not obtained, reverse-phase HPLC conditions were revisited. The reverse-phase HPLC fractions identified to contain A-26771B were further separated using the revised reverse-phase HPLC conditions (33 % H_2O ; 67 % ACN). One fraction was collected from each impure fraction suspected to contain A-26771B, with the same retention time on the reverse-phase HPLC column, and was referred to as 7c1 and 8c1 in the schematic representation of the fractionation protocol (Figure A1). The ^1H NMR spectra for the two reverse-phase HPLC fractions matched the reported chemical shifts,^{83,84} thus identifying them to be A-26771B (0.8 mg and 0.2 mg respectively; Table 1 and Figure 7).

Table 1: A comparison of the experimental ^1H NMR spectral data (400 MHz, CD_3OD) with spectral data reported for the molecule A-26771B.⁸³

No.	Experimental data in CD_3OD	Reported data in CD_3OD
	δ_{H} ppm, int., multi., (J in Hz)	δ_{H} ppm, int., multi., (J in Hz)
2	6.71, 1H, d, (15.9)	6.7, 1H, d, (15.9)
3	7.31, 1H, d, (15.9)	7.3, 1H, d, (15.9)
5	5.35, 1H, t, (5.4)	5.37, 1H, dd, (5.9, 4.9)
6	1.93, 2H, m	1.95, 1H, m
		1.87, 1H, m
7	1.13-1.71, 2H, m	1.34, 2H, m
8	1.13-1.71, 2H, m	1.34, 2H, m
9	1.13-1.71, 2H, m	1.34, 2H, m
10	1.13-1.71, 2H, m	1.34, 2H, m
11	1.13-1.71, 2H, m	1.34, 2H, m
12	1.13-1.71, 2H, m	1.34, 2H, m
13	1.13-1.71, 2H, m	1.34, 2H, m
14	1.13-1.71, 2H, m	1.72, 1H, m
		1.58, 1H, m
15	5.12, 1H, m	5.13, 1H, m
16	1.30, 3H, d (6.4)	1.30, 3H, d, (6.4)
2'	2.69, 2H, m	2.70, 2H, m
3'	2.58, 2H, m	2.62, 2H, m

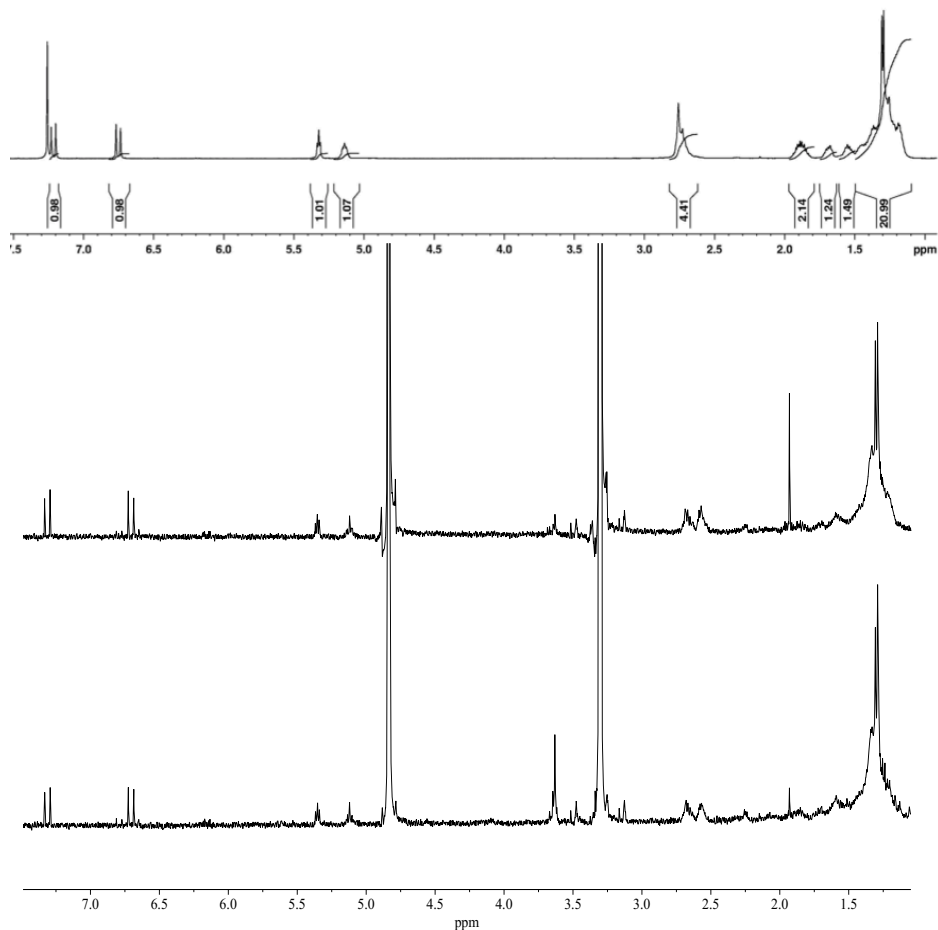


Figure 7: ^1H NMR spectrum (CDCl_3) of A-26771B reported in literature⁸⁴ (top) compared to ^1H NMR spectra (400 MHz, CD_3OD) of the high-performance liquid chromatography fractions identified to be A-26771B.

Carbon nuclear magnetic resonance spectroscopy (^{13}C NMR) data would confirm the isolation of A-26771B, however, with the limited quantity of material in the HPLC fractions identified to be A-26771B, the signal-to-noise ratio (S/N) was too low to obtain interpretable data using the available 400 MHz spectrometer. To increase the S/N, one can increase the sample concentration, increase the magnetic field strength, or decrease the temperature of the probe.⁸⁵ The initial attempt to obtain ^{13}C NMR spectral data involved the fermentation (2×10 L) of the endophytic *Penicillium expansum* isolate to generate more extract, however, due to time

constraints the natural product A-26771B was not isolated from the extracts as the fractionation protocol was not completed. Using the sub-milligram quantities of A-26771B, all three solutions to increase the S/N were attempted. One of the reverse-phase HPLC fractions identified to be A-26771B, was sent to the NMR Facility at McMaster University for data acquisition using a Bruker Avance Neo 600 NMR equipped with a prodigy cryoprobe. The use of the 600 MHz NMR instead of a 400 MHz NMR increased the S/N by a factor of two⁸⁵ and the use of a prodigy cryoprobe further increased the S/N by a factor of four,⁸⁶ thus, the instruments allowed for an eight-fold increase in the S/N. At the NMR Facility, located at McMaster University, the sample was run in a 3 mm NMR tube instead of a 5 mm NMR tube which increased the concentration of the sample and further increased the S/N. Unfortunately, prior to the acquisition of the ¹³C NMR spectrum for A-26771B, the compound degraded. This became evident upon receiving the ¹H NMR spectrum from the NMR facility at McMaster University. When comparing the experimental ¹H NMR to the spectrum reported for the molecule A-26771B,⁸⁴ several resonances characteristic of A-26771B were absent, including the two doublets at 7.31 ppm and 6.71 ppm (Figure 8).

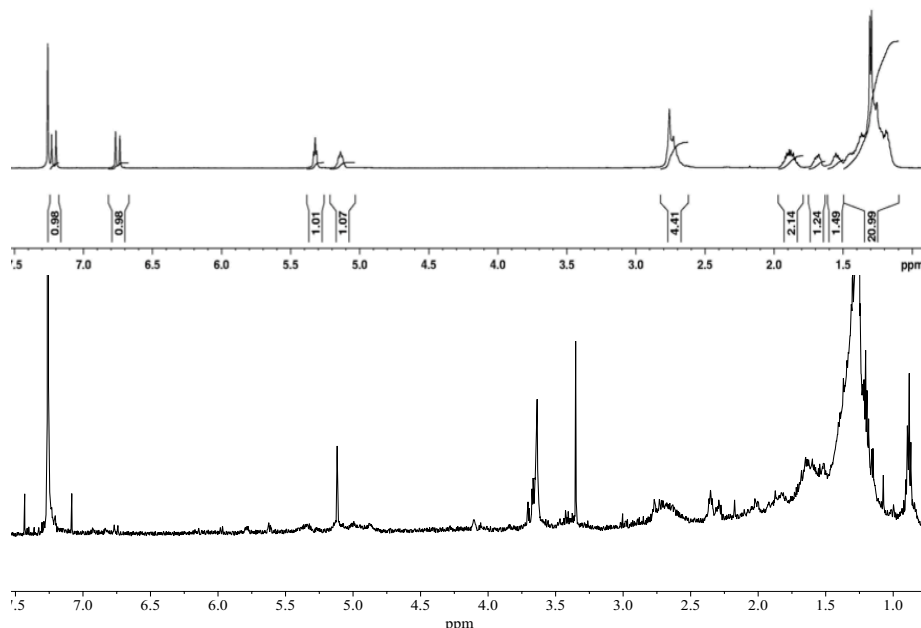


Figure 8: ^1H NMR spectrum (CDCl_3) of A-26771B reported in literature⁸⁴ (top) compared to ^1H NMR spectrum (600 MHz, CDCl_3) of the high-performance liquid chromatography fraction identified to be A-26771B after the molecule degraded (bottom).

Several factors could have contributed to the degradation of A-26771B. Natural products are prone to degradation during storage leading to the loss of the desired product.⁸⁷ The longer the natural product is stored the likelihood of the molecule degrading increases.⁸⁷ Thus, time could have played a role in the degradation of A-26771B. Deuterated chloroform (CDCl_3) was used to dissolve the sample at McMaster University which could have also contributed to the degradation of A-26771B, as chloroform is decomposed by the water in the air to form hydrochloric acid.⁸⁸ The acid could have reacted with the natural product to degrade the desired product.

The objective of this research was to confirm that an extract of the endophytic *Penicillium expansum* isolate, derived from the marine alga *Desmarestia viridis*, contained the macrocyclic lactone A-26771B. During this project, the natural product A-26771B was successfully isolated from the extract after several fractionation steps, however, the molecule degraded before the structure could be confirmed and before the bioactivity of the molecule could be assessed against *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. In the future, in order to confirm the planar structure, determine the stereochemistry, and determine the bioactivity of the molecule against *M. tuberculosis* and *M. smegmatis*, a larger quantity of A-26771B would be required. Revisions to the fractionation protocol would be recommended to increase the efficiency of isolating A-26771B since the molecule A-26771B is produced in sub-milligram quantities by the endophyte. Modifications of the current HPLC conditions could allow for better separation of the constituents, thus negating the need for multiple rounds of HPLC. For example, hydrophilic interaction liquid chromatography has been shown to increase the separation efficiency for some samples compared to reverse-phase and normal-phase HPLC.⁸⁹ Another technique that could greatly benefit the fractionation protocol would be to introduce size-exclusion chromatography, which would add a new dimension to the fractionation protocol by allowing the extract to be separated by atomic mass as well as polarity.⁹⁰

3. Experimental

3.1 General experimental procedures

Fermentations of the endophytic *Penicillium expansum* isolate were conducted using Bacto™ malt extract broth (Becton Dickinson, Mississauga, Ontario). Solvents for extraction and isolation were ACS certified or high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Ottawa, Ontario). Deuterated solvents for nuclear magnetic resonance (NMR) spectroscopy were purchased from Sigma-Aldrich (Oakville, Ontario). Normal-phase thin layer chromatography (TLC) was performed on SiliaPlate aluminum backed TLC plates (200 µm thickness, Silicycle, Quebec City, Quebec). TLC plates were visualized by charring with a potassium permanganate stain (1.0 g potassium permanganate, 2.0 g sodium carbonate and 100 ml of water). Solid-phase extraction was performed using Sep-Pak® C18 Cartridges (55-105 µm, 125 Å, 2 g; Waters, NA, USA). Reverse-phase flash chromatography (RPFC) was performed using a Biotage Flash+ chromatography system fitted with C18 Silicycle cartridges (40-63 µm, 60 Å, 25 g; Silicycle, Quebec). Reverse-phase high-performance liquid chromatography (HPLC) was carried out using a Waters 600 pump, a Phenomenex C18 Luna silica column (10 µm, 100 Å, 250 × 10 mm) and a Waters 2485 dual wavelength detector (190 nm & 254 nm) at a flow rate of 4 mL/min. ¹H NMR spectra were recorded in deuterated methanol (CD₃OD) on an Agilent 400-MR DD2 NMR spectrometer at 400 MHz and in deuterated chloroform (CDCl₃) on a Bruker Avance Neo 600 NMR spectrometer at 600 MHz equipped with a prodigy cryoprobe. Samples were prepared in a 5 mm NMR tube for the 400 MHz NMR and in a 3mm NMR tube for the 600 MHz NMR. The

spectra were processed using MNova® 6 software (Mestrelab Research® S.L.) and calibrated to residual protonated solvent resonances (3.310 ppm CD₃OD for and 7.260 ppm for CDCl₃).

3.2 Liquid culture fermentation and extraction

Portions of the endophytic *Penicillium expansum* isolate (5 mm × 5 mm) were transferred into 250 mL Erlenmeyer flasks containing 2.0% Bacto™ malt extract broth (5 L; 50 × 100 ml batches), stoppered with foam baffles, and shaken (150 rpm) at 21 °C (± 2 °C), in the dark, for two weeks. Following fermentation, the cultures were sonicated for 30 seconds to lyse the cells and the resulting cell debris were removed by filtration through cotton wool. The filtrate was extracted with ethyl acetate (3 x 1.6 L EtOAc per 5 L of broth) and concentrated in vacuo to give an extract. The extract from two fermentations were combined to give the extract (326 mg). The extract was stored at -20 °C.

3.3 Fractionation of extract

An extract (326 mg) obtained from combining two separate fermentations (5L) was loaded onto C-18 silica (32 g) and eluted first with water (H₂O) (224 mL) then methanol (MeOH; 224 mL) then ethyl acetate (EtOAc; 224 mL). The MeOH fraction was separated by reverse-phase flash column chromatography (RPFC) employing a stepwise gradient of water to acetonitrile (ACN; 10% increments, 150 mL per elution) followed by a column wash of EtOAc (150 mL) that resulted in twelve fractions. Fractions seven (40 % H₂O; 60 % ACN), eight (30 % H₂O; 70 % ACN), and nine (20 % H₂O; 80 % ACN) contained ¹H NMR resonances indicative of A-26771B. Fractions seven (21 mg) and eight (13 mg) were individually separated by two

rounds of isocratic reverse-phase high-performance liquid chromatography (HPLC; 35 % H₂O; 65 % ACN then 33 % H₂O; 67 % ACN) resulting in the isolation of sub-milligram quantities of A-26771B (0.8 mg and 0.2 mg respectively). Fraction 9 was identified to contain A-26771B, however, material was exhausted after the first round of HPLC.

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Appendix

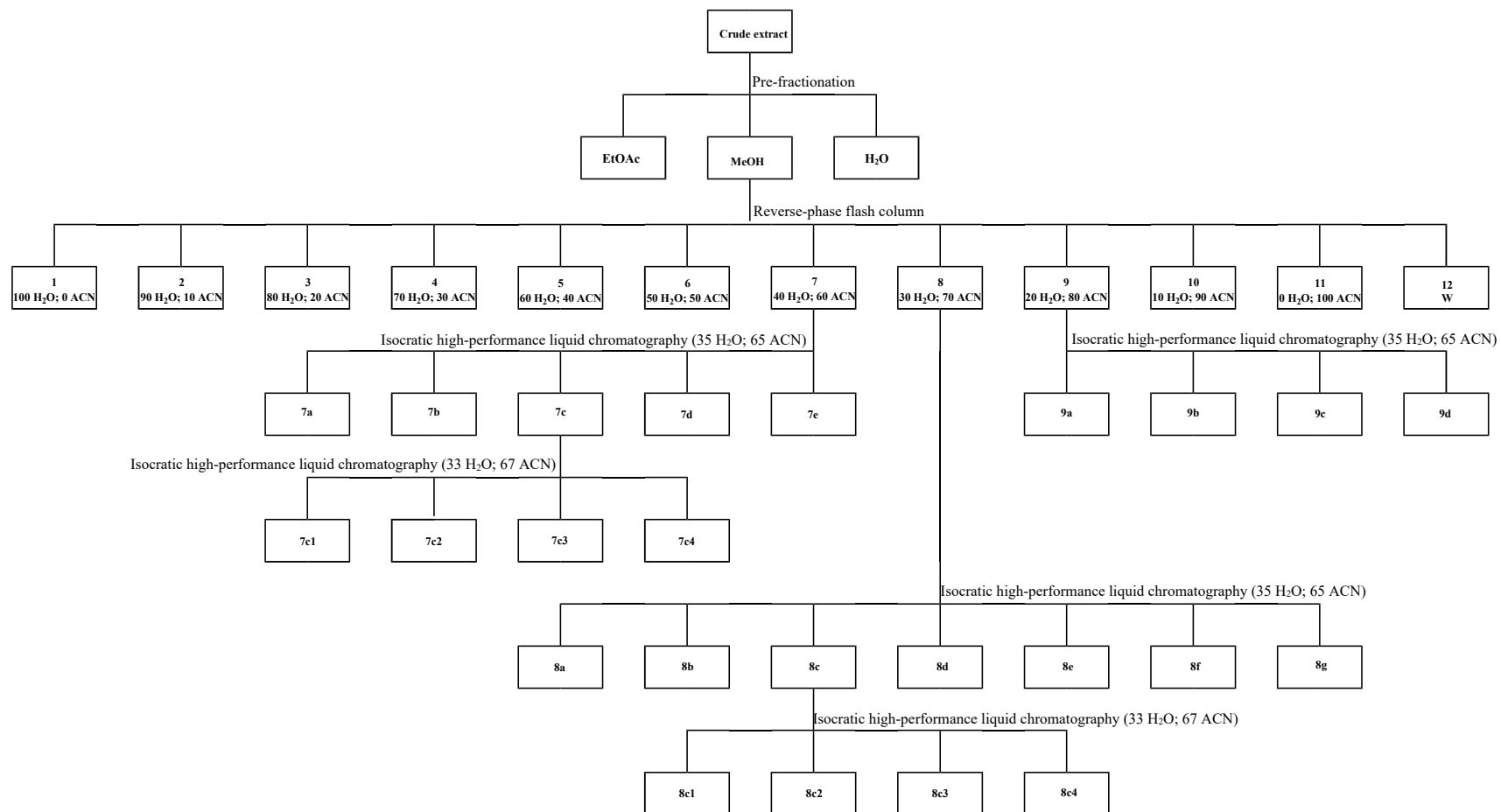


Figure A1: Schematic representation of the fractionation protocol followed for the extract generated from an endophytic *Penicillium expansum* isolate.

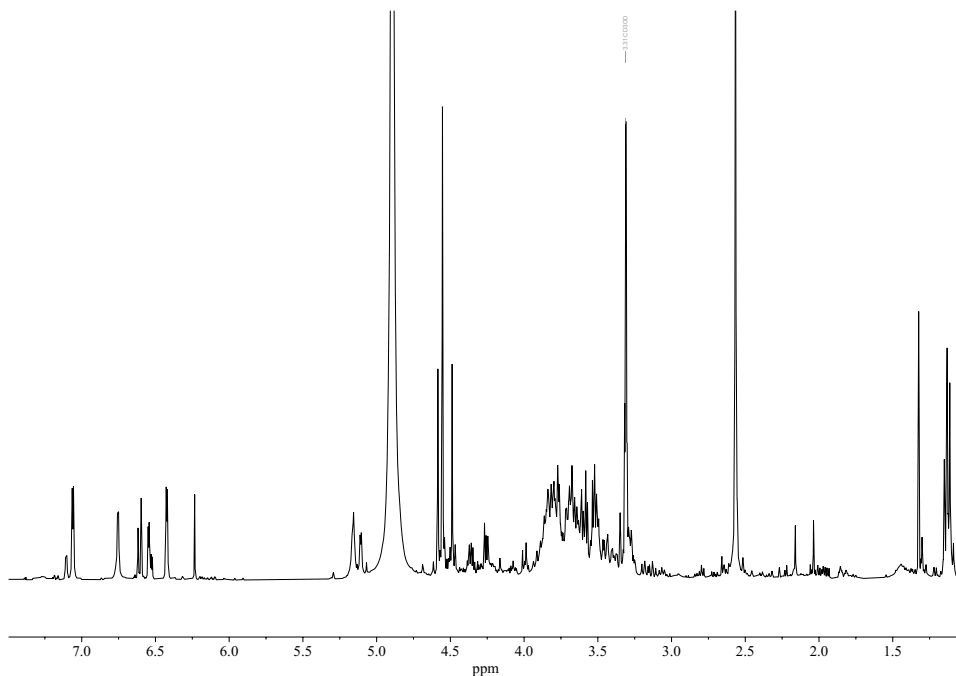


Figure A2: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 100 % H₂O and referred to as fraction 1.

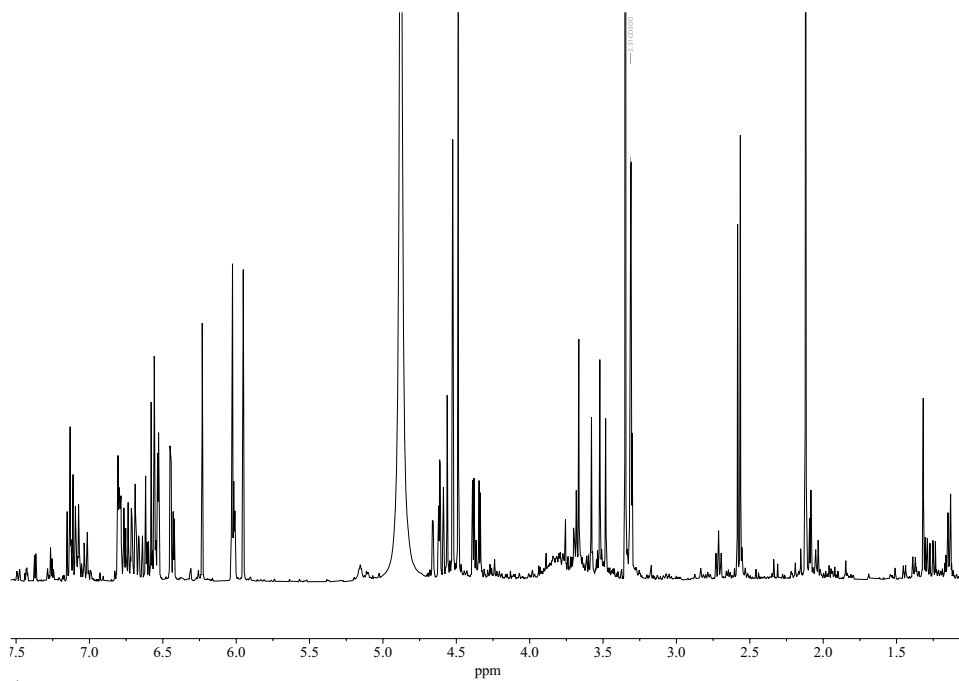


Figure A3: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 90 % H₂O; 10 % ACN and referred to as fraction 2.

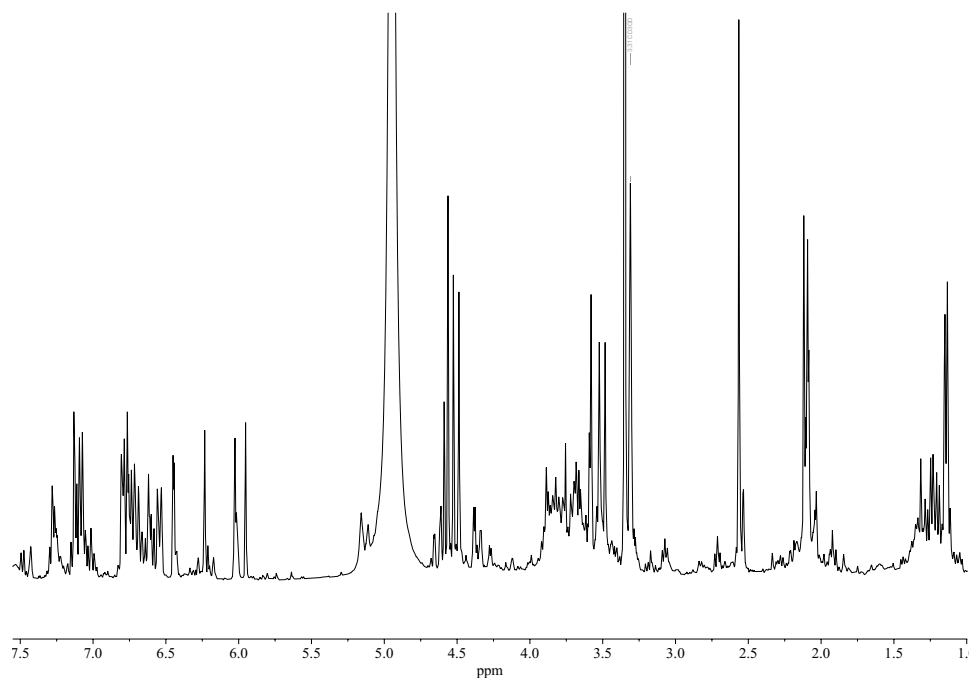


Figure A4: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 80 % H₂O; 20 % ACN and referred to as fraction 3.

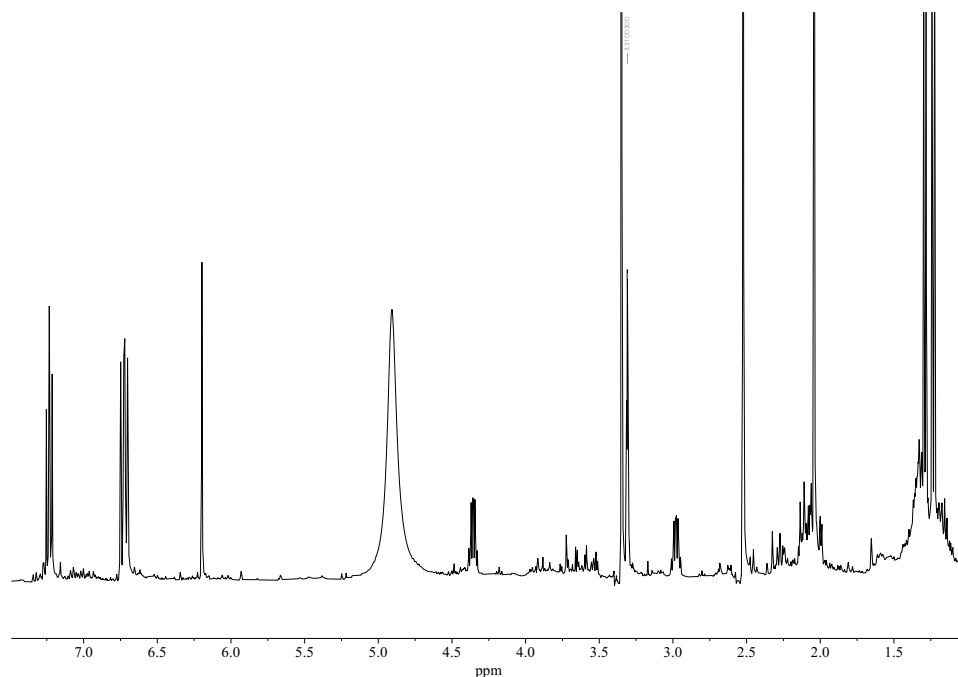


Figure A5: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 70 % H₂O; 30 % ACN and referred to as fraction 4.

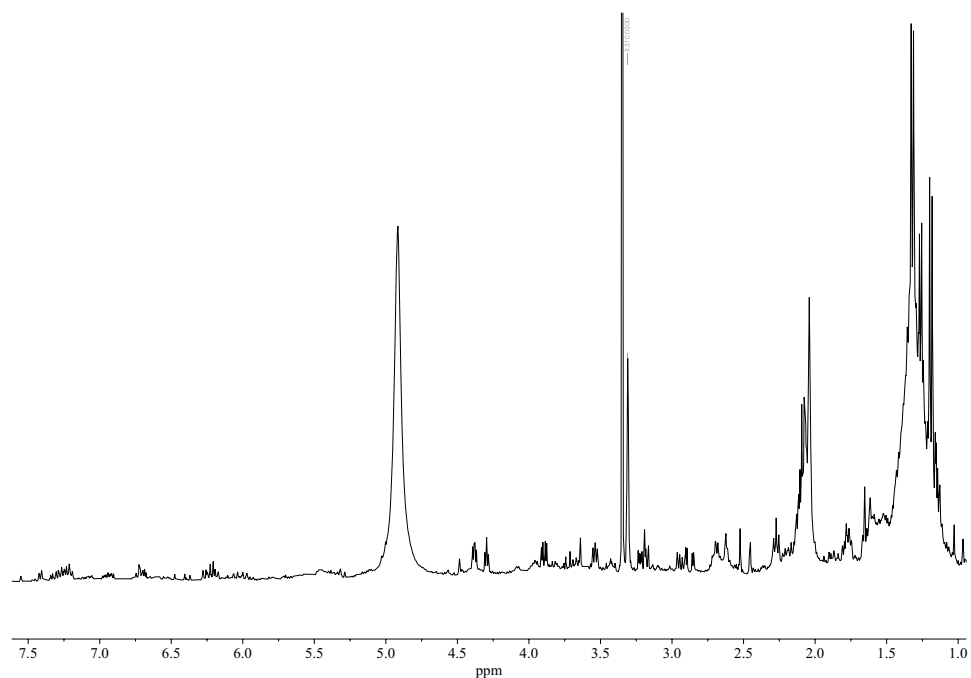


Figure A6: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 60 % H₂O; 40 % ACN and referred to as fraction 5.

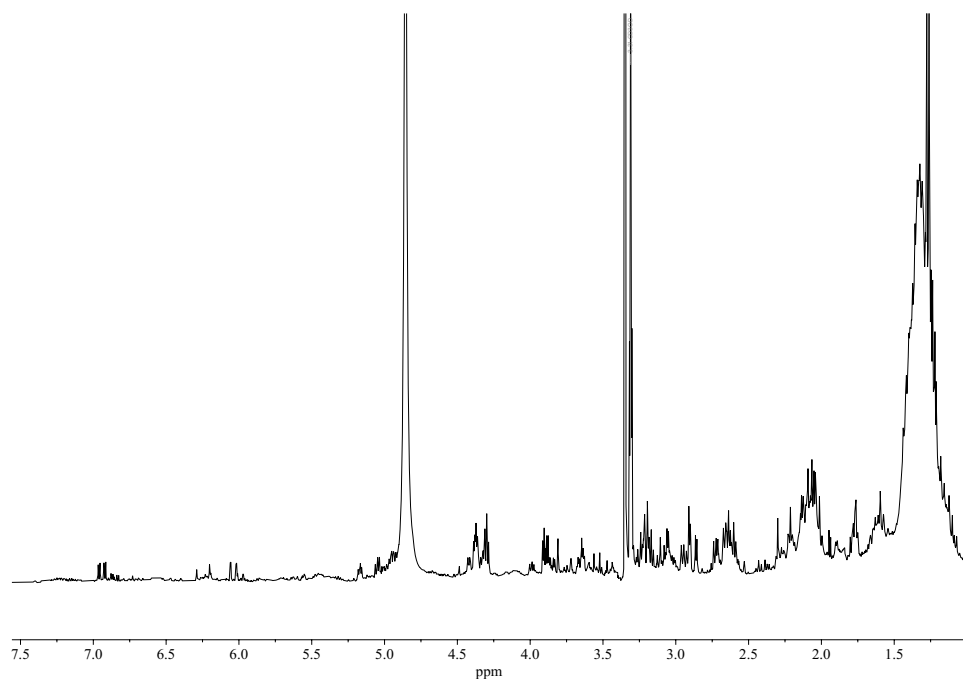


Figure A7: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 50 % H₂O; 50 % ACN and referred to as fraction 6.

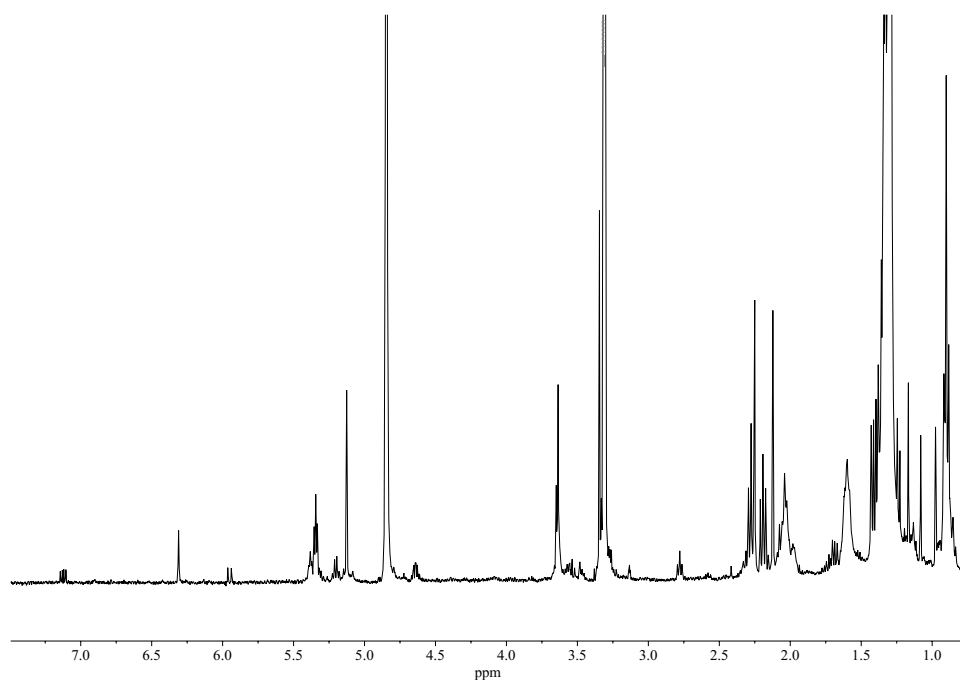


Figure A8: ^1H NMR spectrum (400 MHz, CD_3OD) of the reverse-phase flash column fraction eluting at 10 % H_2O ; 90 % ACN and referred to as fraction 10.

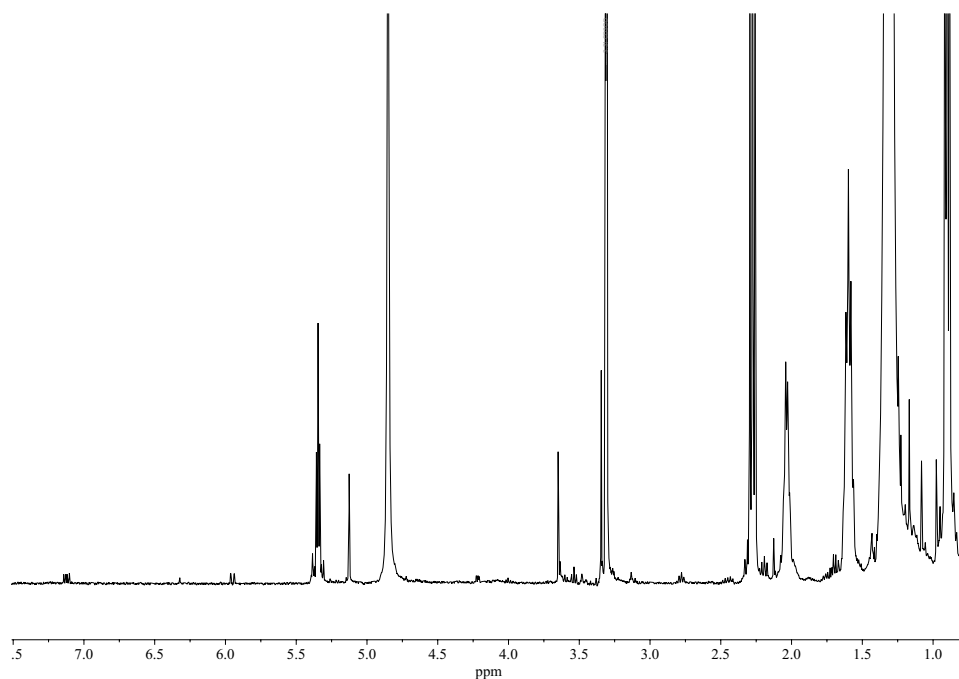


Figure A9: ^1H NMR spectrum (400 MHz, CD_3OD) of the reverse-phase flash column fraction eluting at 100 % ACN and referred to as fraction 11.

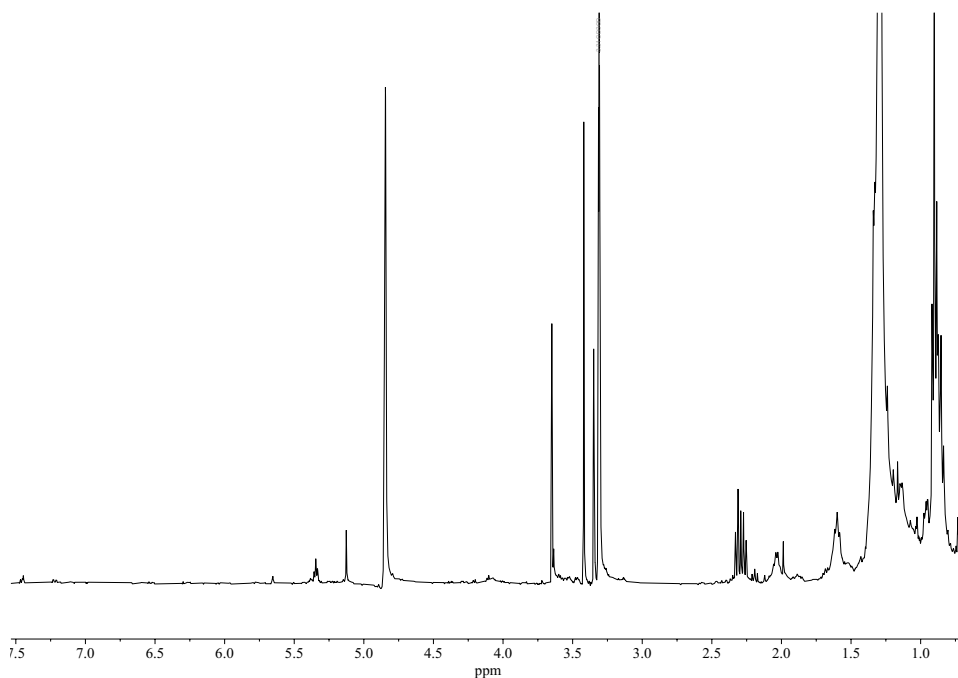


Figure A10: ¹H NMR spectrum (400 MHz, CD₃OD) of the reverse-phase flash column fraction eluting at 100 % EtOAc and referred to as fraction 12.

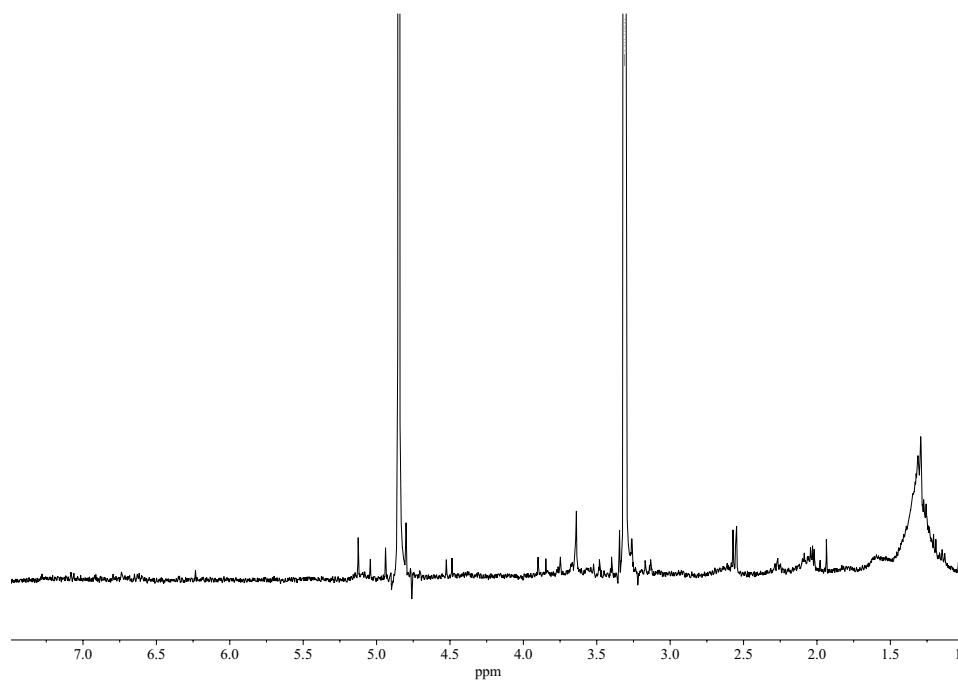


Figure A11: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7a.

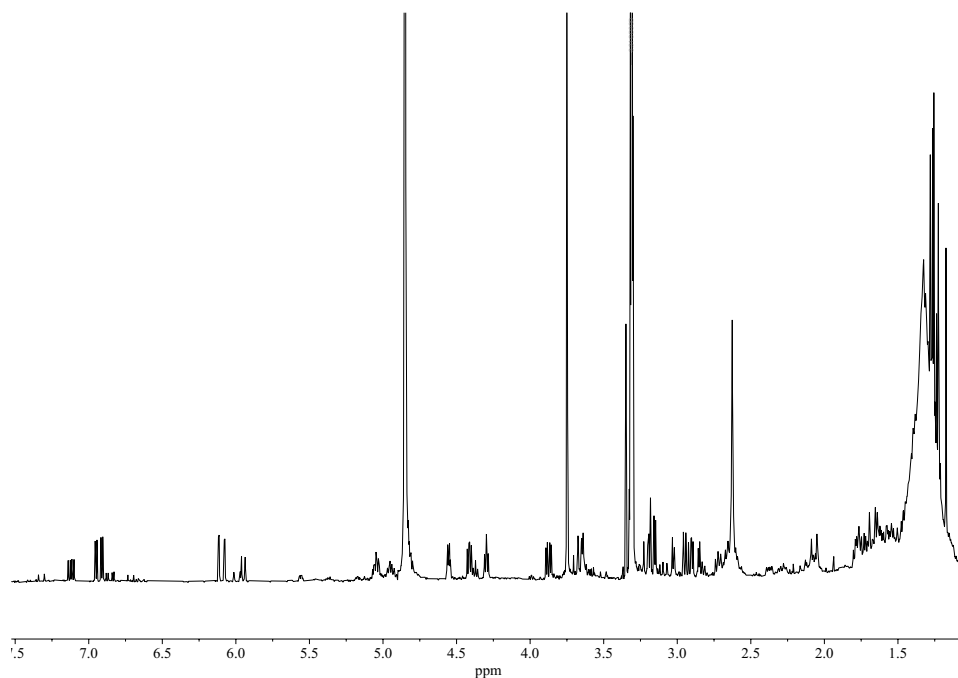


Figure A12: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7b.

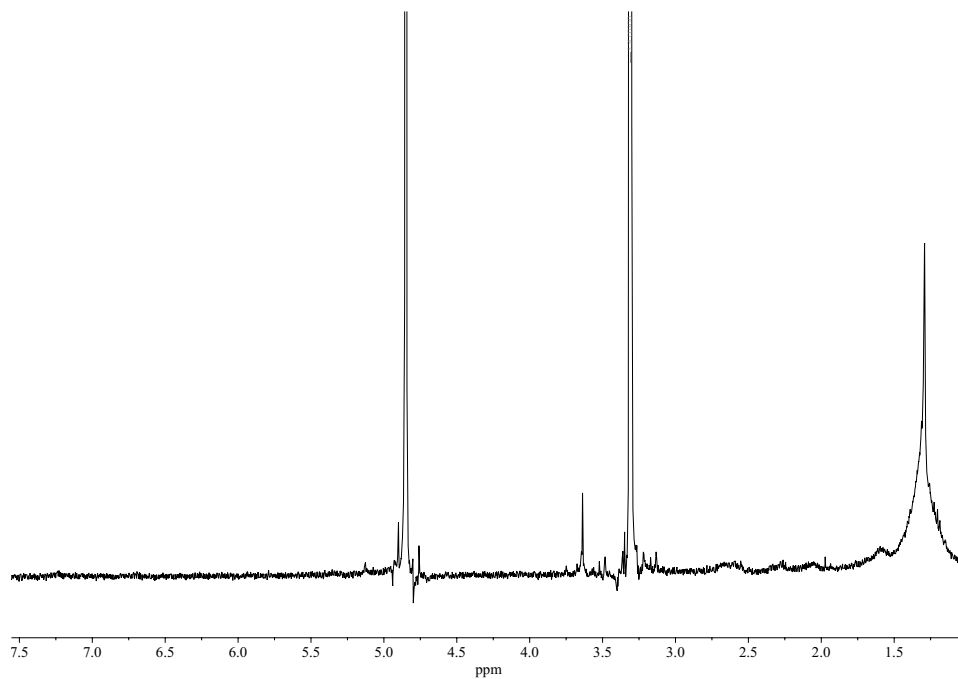


Figure A13: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7d.

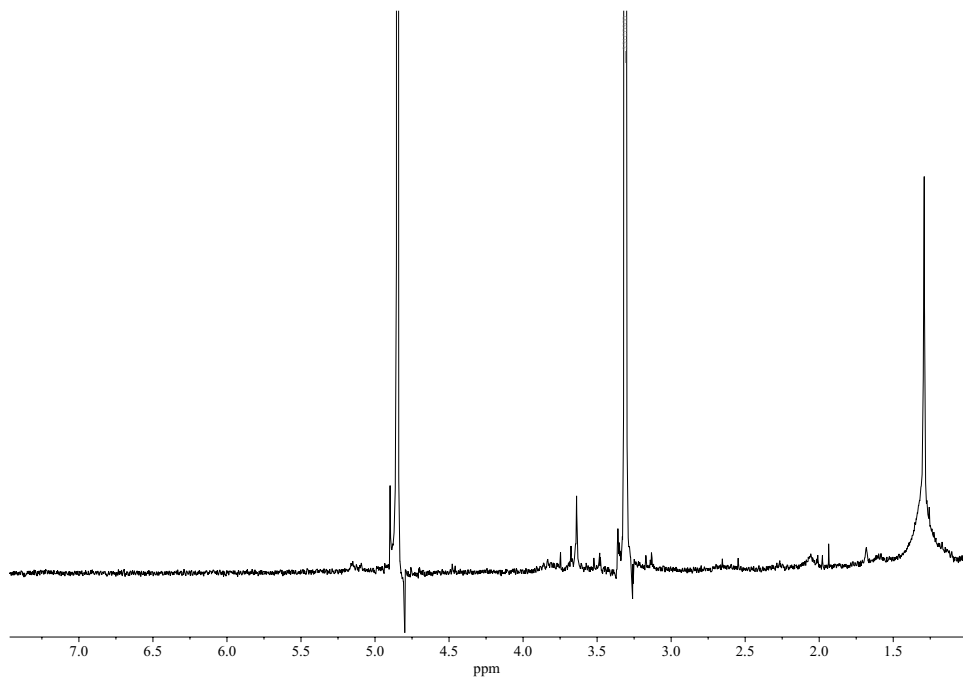


Figure A14: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7e.

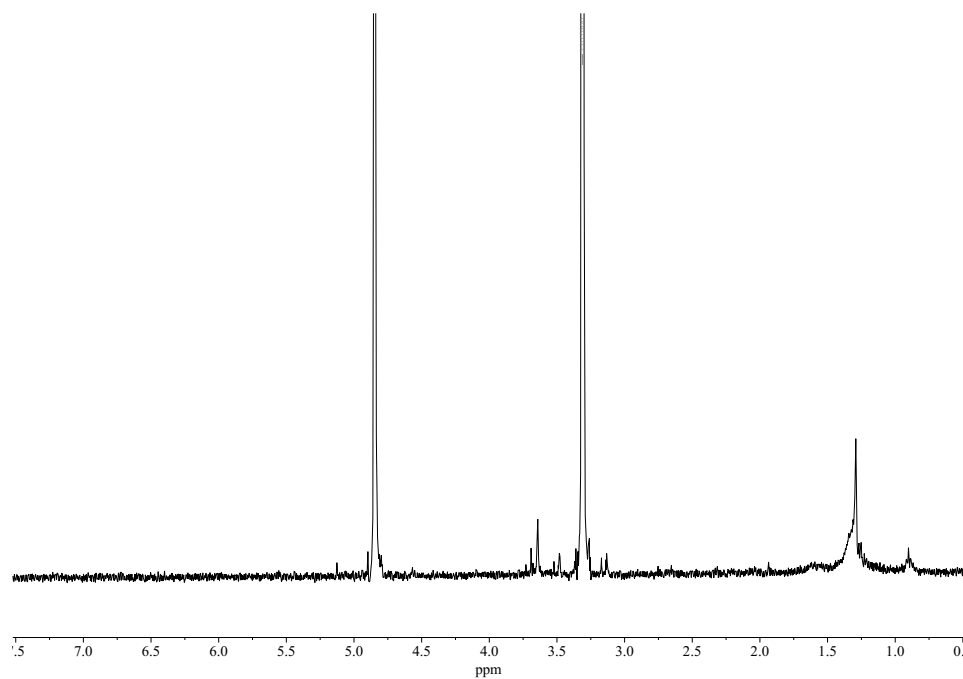


Figure A15: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7c2.

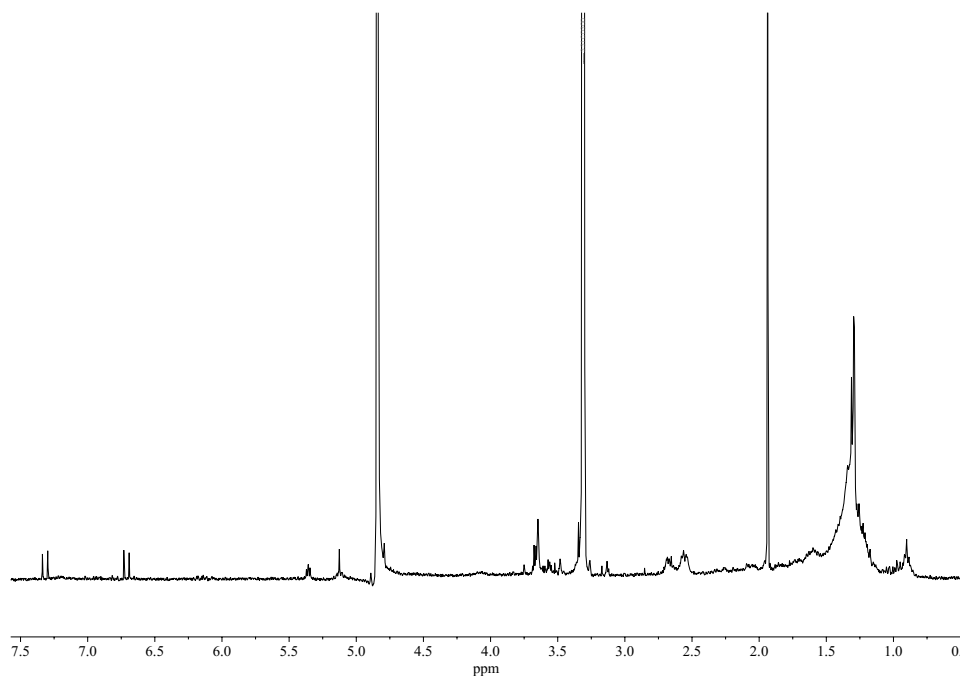


Figure A16: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7c3.

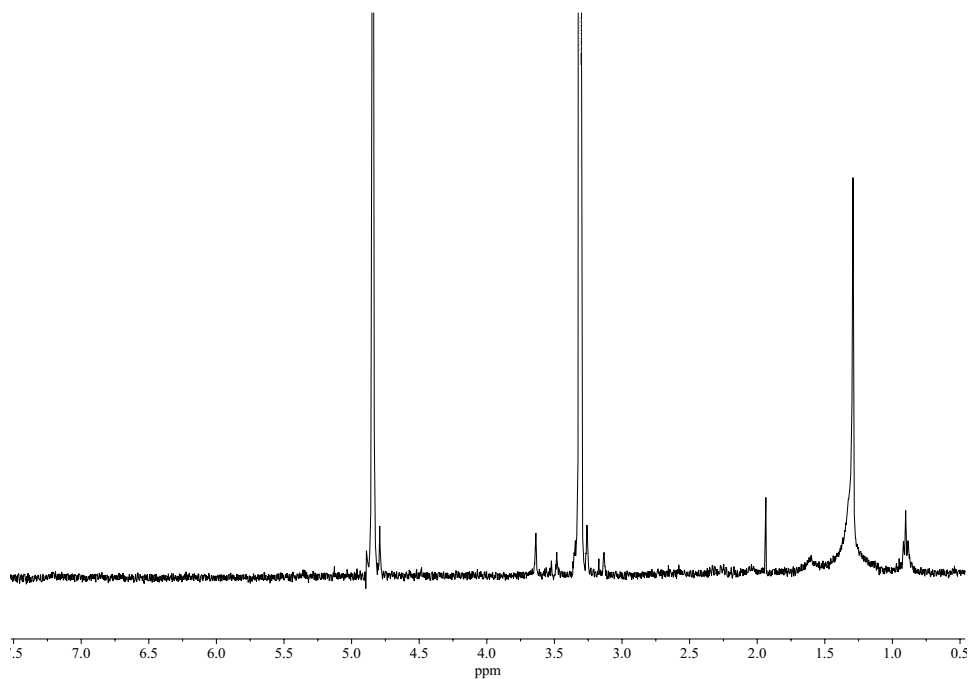


Figure A17: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 7c4.

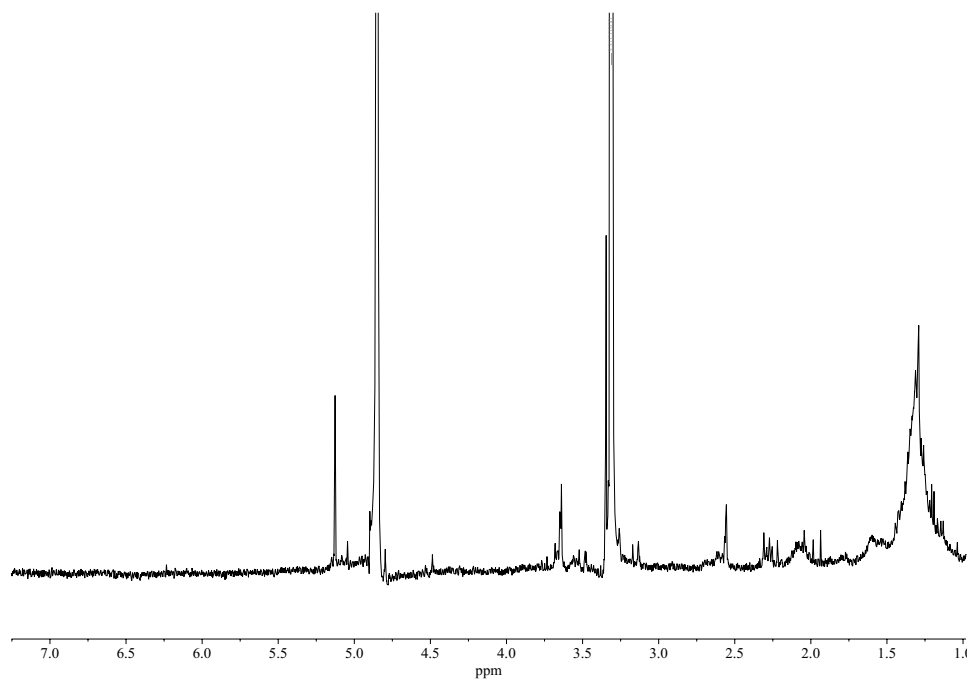


Figure A18: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8a.

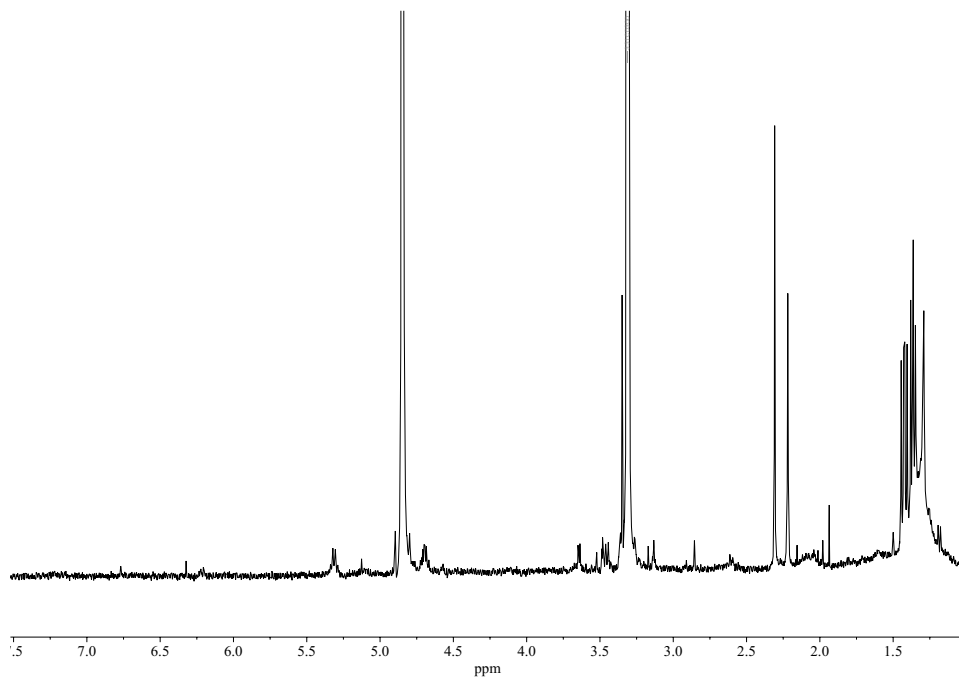


Figure A19: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8b.

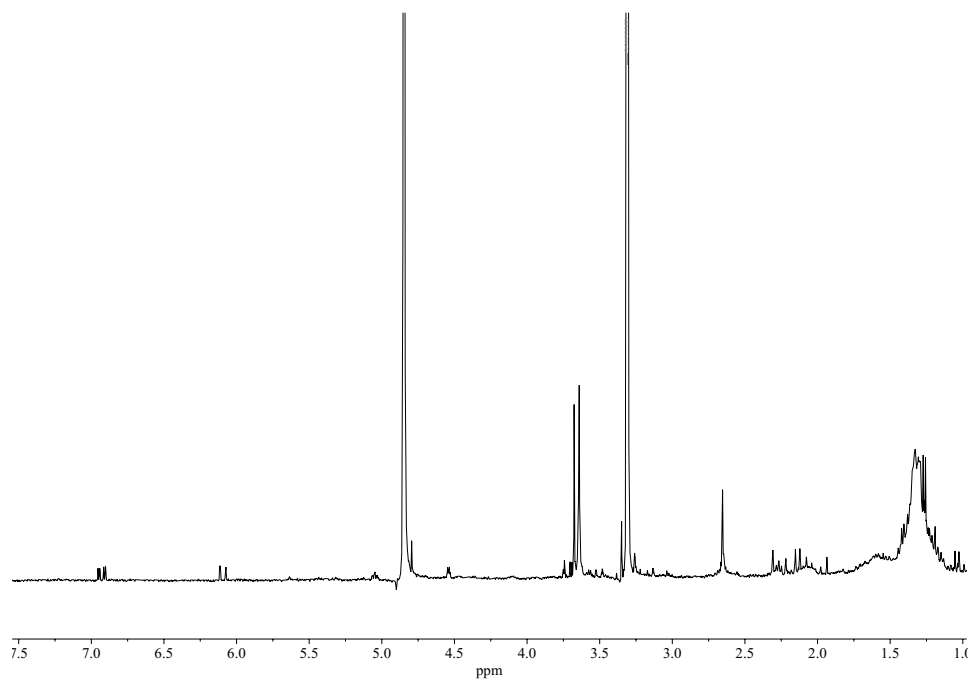


Figure A20: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8d.

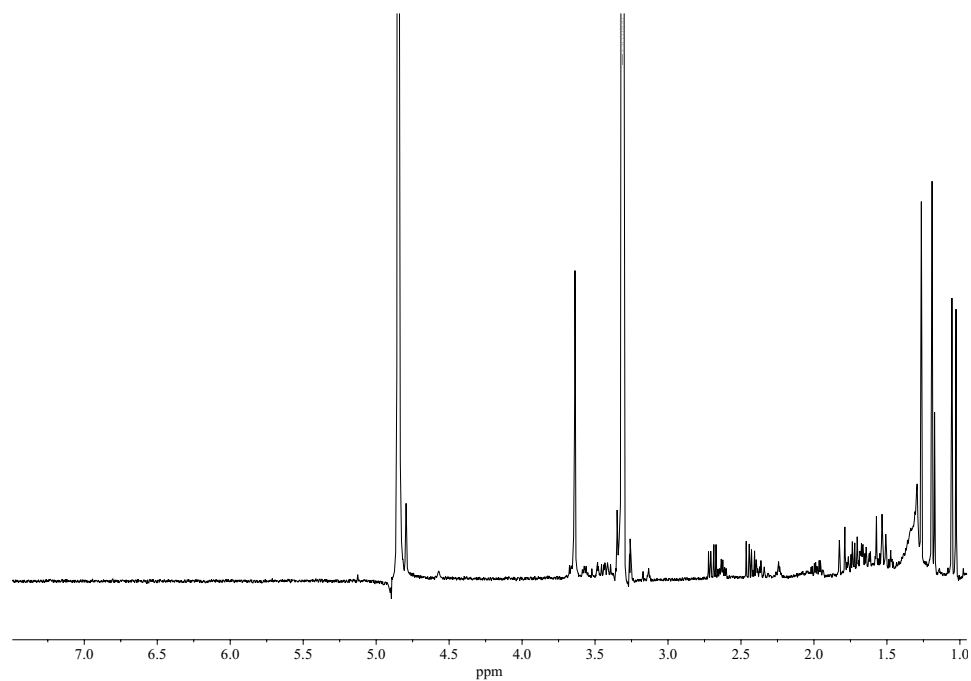


Figure A21: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8e.

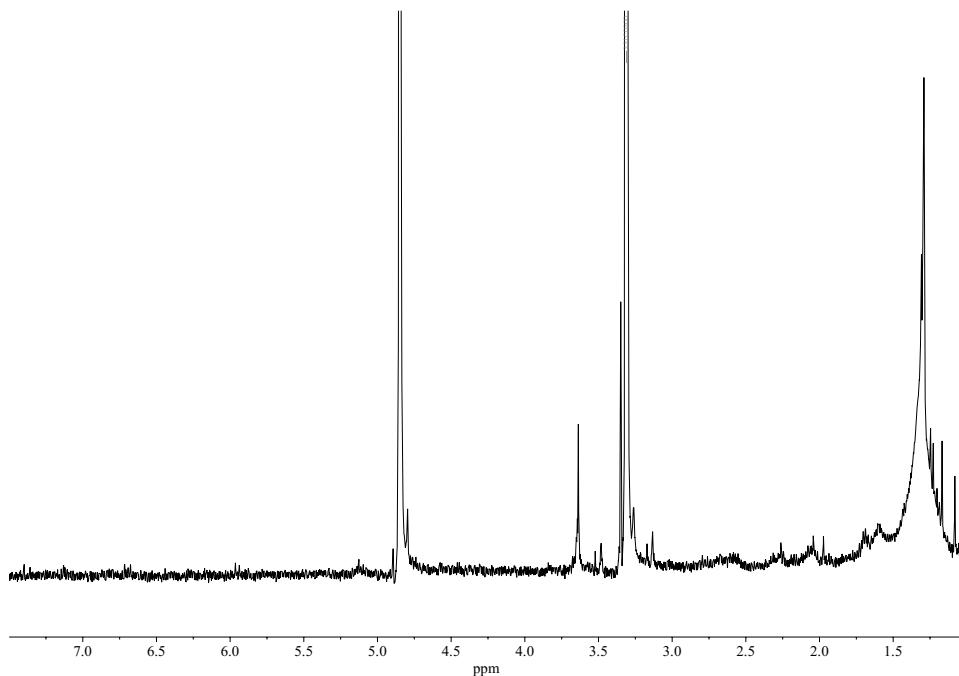


Figure A22: ^1H NMR spectrum (400 MHz, CD_3OD) of the high-performance liquid chromatography fraction referred to as 8f.

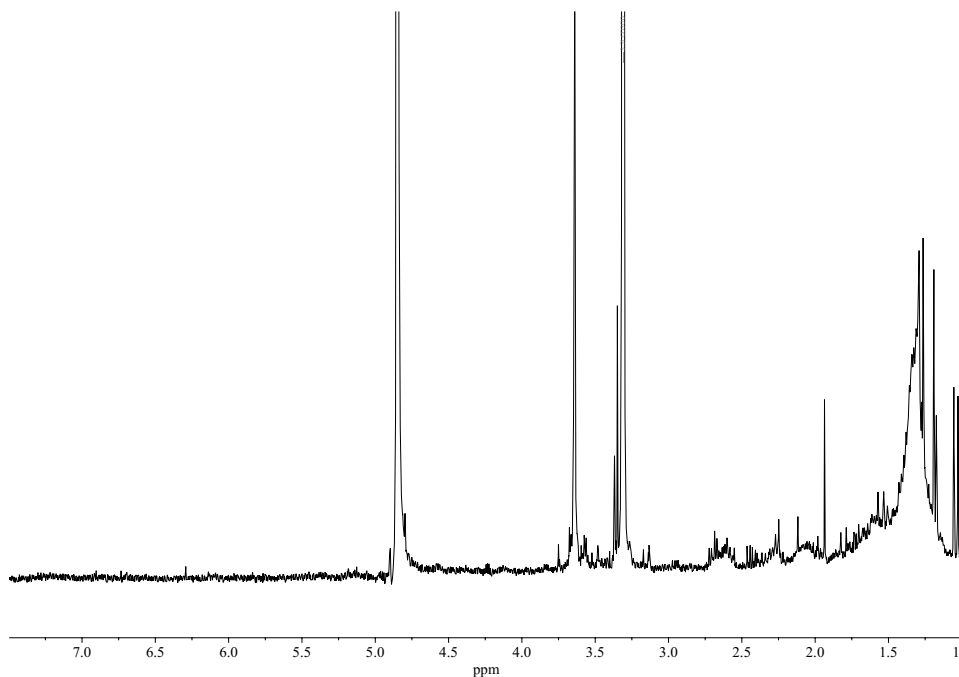


Figure A23: ^1H NMR spectrum (400 MHz, CD_3OD) of the high-performance liquid chromatography fraction referred to as 8g.

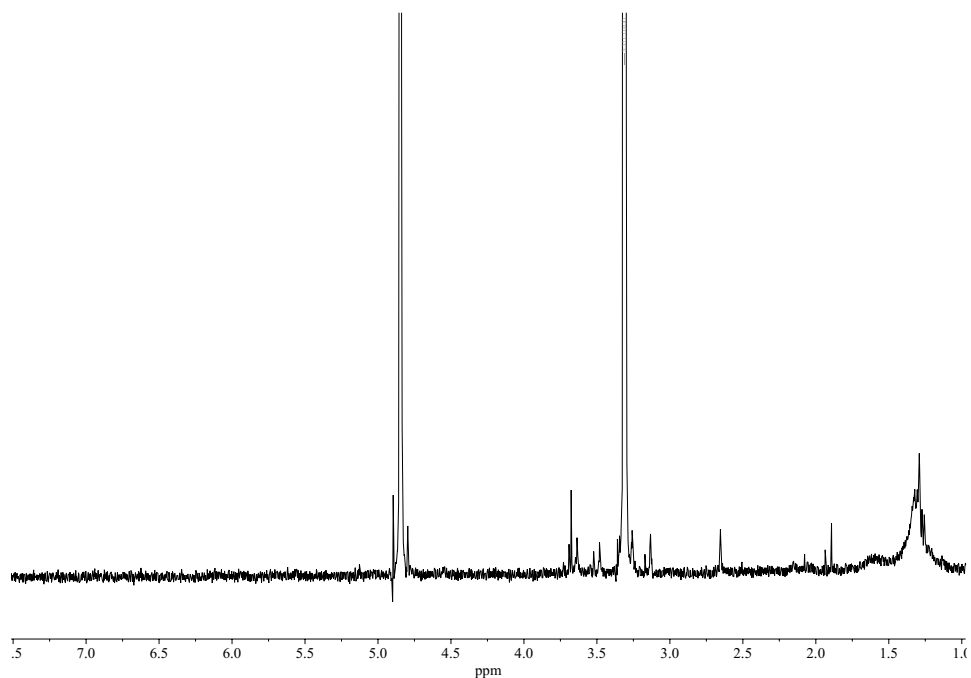


Figure A24: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8c2.

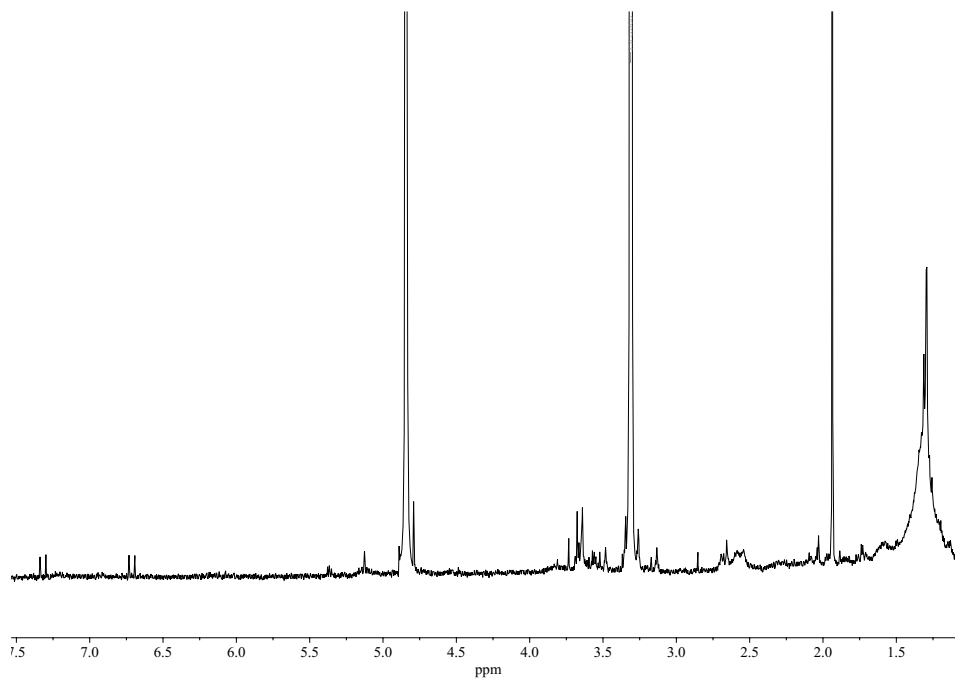


Figure A25: ¹H NMR spectrum (400 MHz, CD₃OD) of the high-performance liquid chromatography fraction referred to as 8c3.

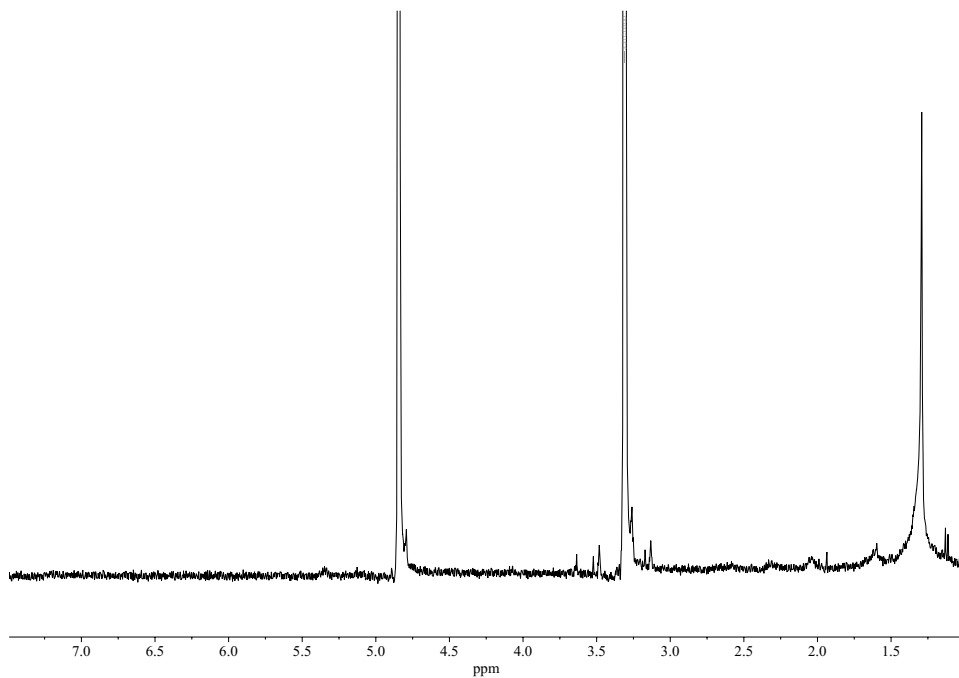


Figure A26: ^1H NMR spectrum (400 MHz, CD_3OD) of the high-performance liquid chromatography fraction referred to as 8c4.