Isolation of Natural Products from *Casearia nigrescens*

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Abstract

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As part of the continuing work of the International Cooperative Biodiversity Group (ICBG), plant extracts were received from Madagascar. The extracts were screened for cytotoxicity using the A2780 human ovarian cancer cell line bioassay. The crude extract of *Casearia nigrescens* was fractionated and yielded five known compounds and one new compound that were cytotoxic. Mass spectrometry and 1D and 2D NMR techniques were used to determine the structure of the isolated compounds.

The dichloromethane fraction of *Casearia nigrescens* was weakly active in the A2780 human ovarian cancer cell line bioassay. Further separation of the dichloromethane fraction resulted in the isolation of five known compounds (casearlucin A, caseamenbrol A, *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene, casearlucin B, and *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene) and one new compound (casearlucin L). Based on the available literature this was the first investigation of the natural products of *Casearia nigrescens*. The structure of the known compounds was determined by comparison of the NMR, MS, optical rotation, UV, and IR data with the data found in the literature. The structure of casearlucin L was determined by NMR data and comparison with the NMR data for similar known compounds.

Acknowledgements

I would like to thank Dr. Kingston for allowing me to work in his group and learn more about the area of natural product chemistry. I would also like to thank all the members of the Kingston group for their help and guidance. Finally, I would like to thank Tom Glass and Bill Bebout for all their help with spectral analysis.

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I. Natural Product Chemistry

1.1 General Introduction

Natural product chemistry stems from the use of nature for medicinal purposes. The World Health Organization (WHO) estimates that approximately 80% of the world relies on natural sources for primary medical treatment and that the health care systems for the remaining 20% of the population also incorporate natural sources in their medical treatment.[1] Natural product chemistry can be defined as the exploration of nature in search of novel drugs or drug leads.

Some possible sources of natural products include plants, marine organisms, microbes and fungi. Of the approximately 250,000 higher species of plants it is estimated that only 5-15% have been investigated for natural products.[2] Marine organisms are abundant in the oceans, which cover more than 70% of the Earth's surface.[2] Also, research suggests that less than 1% of bacterial species and less than 5% of fungal species are currently known.[3] Therefore, it is important that natural product chemistry continues to explore natural resources in search of new natural products.

1.2 Historically Important Natural Products

Some well known and important drugs have originated from natural sources. A few examples are given to indicate what an important impact these drugs have had on medical

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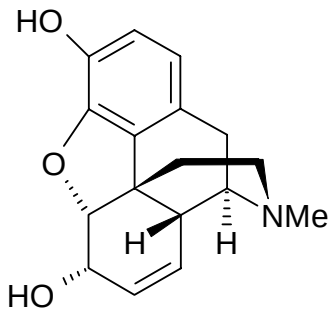
[1] Cragg, G. M. Natural Product Drug Discovery and Development: The United States National Cancer Institute Role. *Puerto Rico Health Sciences Journal* **2002**, *21*, 97-111.
[2] Cragg, G. M.; Newman, D. J. Natural Product Drug Discovery in the Next Millennium. *Pharmaceutical Biology* **2001**, *39*, S8-17.
[3] Cragg, G. M.; Newman, D. J. Nature's Bounty. *Chem. Br.* **2001**, *37*, 22-26.

treatment and disease control. It can be noted that these drugs were isolated from natural sources many of which have been used by various cultures throughout history.

1.2.1 Morphine

Morphine (**1.1**) was first isolated from the opium poppy (*Papaver somniferum*) in 1861.[2] In ancient Mesopotamia the oils of *P. somniferum* were used as an analgesic.[2] The discovery and isolation of morphine led to an increased interest in alkaloid chemistry and resulted in the development of other analgesic agents.[2] Morphine was the first commercially available pure natural product, marketed in 1826.[4]



1.1 Morphine

1.2.2 Quinine

Malaria has been and continues to be a problem in many areas around the world. The native Amerindians of the Amazon region used the bark of the *Cinchona* tree to treat malaria.[5] Quinine (**1.2**), the active component of *Cinchona* bark, was isolated in 1820 from *C. officinalis*.[1] Quinine was the first effective anti-malarial drug to be isolated.[6] Other anti-malarial drugs such as chloroquine (**1.3**) and mefloquine (**1.4**) were synthesized based on the structure of quinine.[2]

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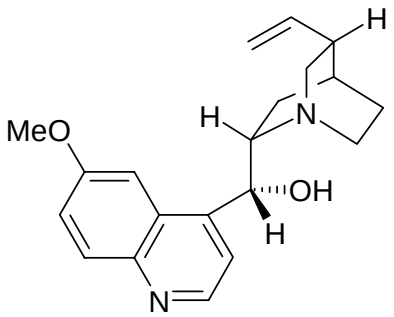
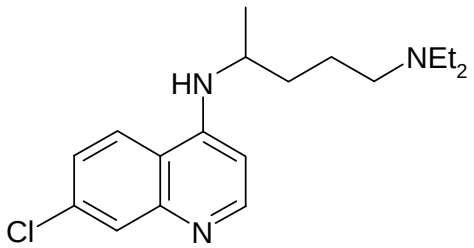
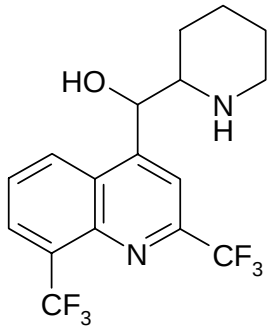
[4] Newman, D. J.; Cragg, G. M.; Snader, K. M. The Influence of Natural Products upon Drug Discovery. *Nat. Prod. Rep.* **2000**, *17*, 215-234.
[5] Clark, A. M. Natural Products as a Resource for New Drugs. *Pharm. Res.* **1996**, *13*, 1133-1141.
[6] Phillipson, J. D. Phytochemistry and Medicinal Plants. *Phytochemistry* **2001**, *56*, 237-243.



1.2 Quinine



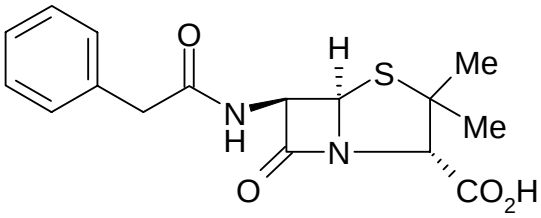
1.3 Chloroquine



1.4 Mefloquine

1.2.3 Penicillin

Penicillin (**1.5**) was first discovered in 1929 by Fleming from the fungus, *Penicillium notatum*.[2] This discovery was important to the development of antibiotics and changed medicine forever.[5] The discovery of this revolutionary drug from a natural source prompted the investigation of nature for other novel compounds.[1]

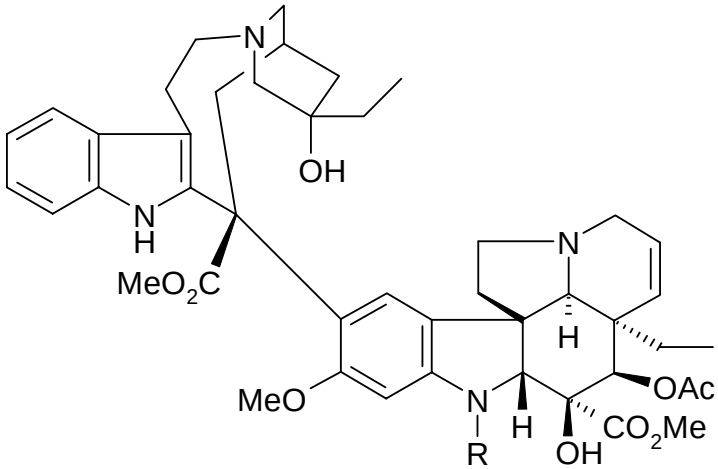


1.5 Penicillin G

1.2.4 Vinblastine and Vincristine

The plant *Catharanthus roseus*, commonly known as the Madagascar periwinkle, was used in some cultures as a folk remedy to treat diabetes.[2] However, when *C. roseus* was evaluated for hypoglycemic compounds, no such compounds were found.[5] Instead, the results led to the hypothesis that the plant extract contained a compound potentially useful for the treatment of cancer.[5] Upon further investigation of *C. roseus* for anti-cancer agents, vinblastine

(**1.6**) and vincristine (**1.7**) were isolated in 1954.[7] These drugs were developed by Eli Lily and are important agents in the treatment of cancer.

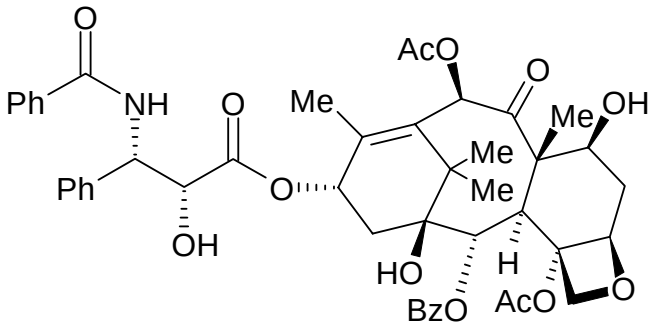


1.6 Vinblastine R = Me
1.7 Vincristine R = CHO

1.2.5 Paclitaxel

A random collection of plants by the United States Department of Agriculture (USDA) for the National Cancer Institute (NCI) yielded an extract with anti-cancer activity.[2] The extract was from the Pacific yew tree (*Taxus brevifolia*) and the active compound, paclitaxel (**1.8**), was isolated in 1969.[1] Several Native American tribes use various parts of *Taxus* trees for the treatment of a wide range of non-cancerous ailments.[2] Paclitaxel is an important anti-cancer drug in use today.

[7] Noble, R. The Discovery of the vinca alkaloids—Chemotherapeutic Agents against Cancer. *Biochem. Cell Biol.* **1990**, *68*, 1344-1351.



1.8 Paclitaxel

1.3 Natural Products in Pharmaceuticals

As the previous examples show, natural products are important pharmaceuticals. In a study of the pharmaceuticals on the market from 1981-2002, only 43% of the drugs were purely synthetic, while the remaining 57% were derived from a natural source (Figure 1.1).[8] The data shown in Figure 1.1 categorizes natural sources in the following way: biological – a peptide or protein isolated from an organism or cell line; natural product; natural product derivative – derived from a natural product usually with some semi-synthetic modifications; synthetic drug; synthetic drug with a natural product pharmacophore; vaccine.[8]

[8] Newman, D. J.; Cragg, G. M.; Snader, K. M. Natural Products as Sources of New Drugs over the Period 1981-2002. *J. Nat. Prod.* **2003**, *66*, 1022-1037.

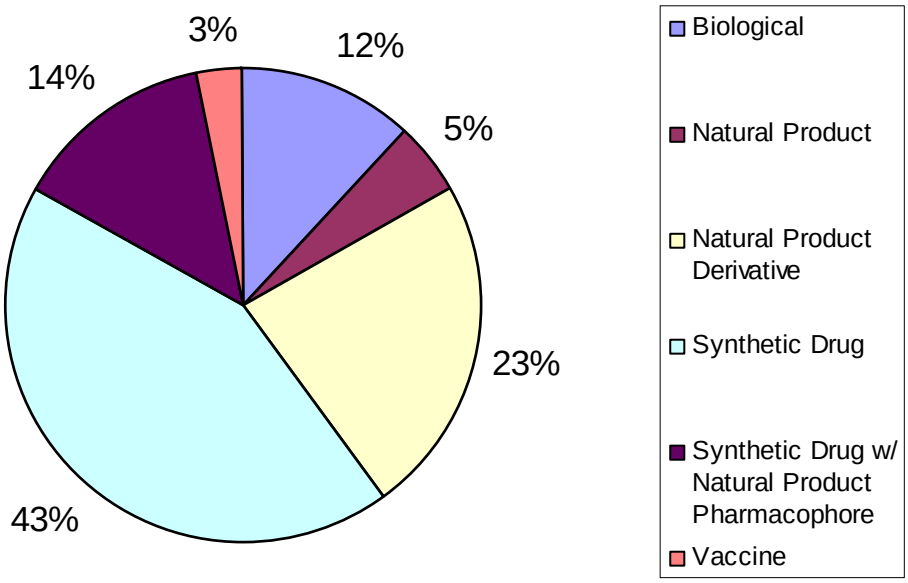


Figure 1.1 Pharmaceuticals containing natural products (1981-2002).
Adapted from Newman, Cragg, and Snader (2003).[8]

1.3.1 Cancer and the Role of Natural Products

The research presented in this paper focuses on natural products that are cytotoxic against cancer. Cancer is a devastating disease that affects millions of people each year. The American Cancer Society estimates that over 560,000 Americans will die of non-invasive cancers this year, making cancer the second leading cause of death in the United States.[9] Natural products are important in the treatment of cancer. In a study of anti-cancer drugs on the market from the 1940's-2002, only 36% of the drugs were purely synthetic while the remaining 64% were of natural origins (Figure 1.2).[8]

[9] American Cancer Society—Cancer Facts & Figures 2004. http://www.cancer.org (accessed April 2004).

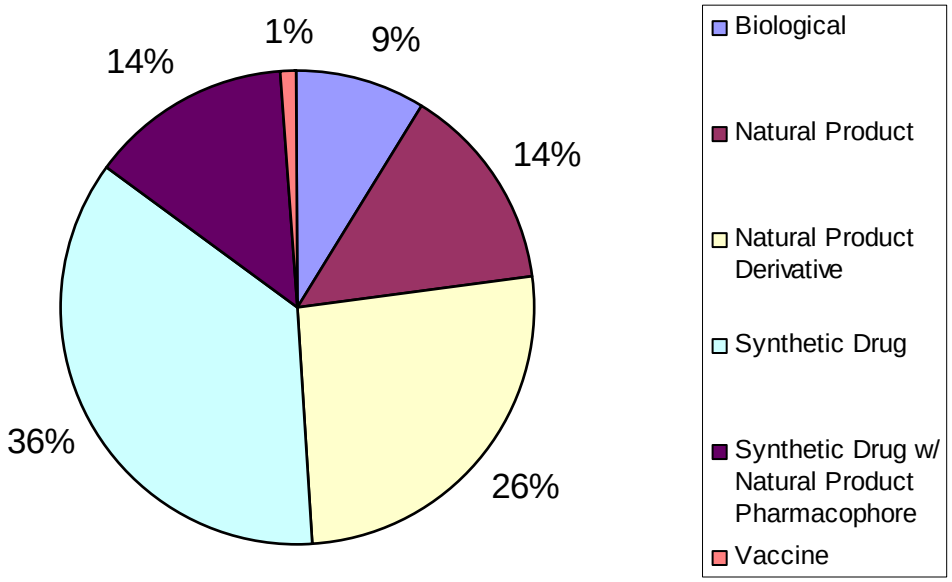


Figure 1.2 Anti-cancer drugs containing natural products (1940's-2002). Adapted from Newman, Cragg, and Snader (2003).[8]

1.4 International Cooperative Biodiversity Group (ICBG)

This research is in conjunction with the International Cooperative Biodiversity Group (ICBG). The ICBG program focuses on four main areas: drug discovery, economic development, conservation, and biodiversity.[10] The drug discovery portion of the program is carried out at Virginia Tech. Plant extracts from Madagascar are screened for bioactivity against cancer. Active components of the extract are isolated and identified; highly active compounds may then be developed for commercial use.[10] The ICBG program provides learning opportunities in the area of science, biodiversity, and conservation to the people of Madagascar.[10] Compensation is also provided to the county for the use of natural resources. With adequate compensation to Madagascar for the plant extracts that are collected and for any new compounds that are developed into commercial drugs, the ICBG program hopes to motivate

[10] International Cooperative Biodiversity Groups. http://www.fic.nih.gov/programs/icbg.html (accessed April 2004).

the country to conserve their natural resources.[10] As a final goal of the ICBG program, while extracts are collected, the biodiversity of the region is assessed.[10]

1.5 Bioassays

Bioassays are used to screen natural product extracts. Once an extract is identified as active, purification of the extract begins. The fractions that are obtained from purification are again evaluated for bioactivity. Purification followed by bioassay testing continues until pure compounds are isolated. This process is known as bioassay-guided fractionation.

1.5.1 Mechanism-Based Assays

There are two basic types of bioassays commonly used to evaluate natural extracts, mechanism-based assays and cell-based assays. Mechanism-based assays analyze extracts for activity based on interactions with an enzyme or a receptor.[11] This type of assay is highly sensitive and is useful in identifying new compounds with a specific activity.[12] However, there are drawbacks to using mechanism-based assays. Although the assay is highly specific and selective, extracts that are not active in the mechanism-based assay may be active *in vivo*.[13] This anomaly may result if the compound acts by a different mechanism or if the compound is metabolized *in vivo* to an active compound.[13] Compounds that are active in the mechanism-based assay may not be active *in vivo* if the compound does not penetrate the cell membrane.[5] Therefore, it may be beneficial to use a cell-based assay.

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[11] Houghton, P. J. Use of small scale Bioassays in the Discovery of novel drugs from Natural Sources. *Phytotherapy Research* **2000**, *14*, 419-423.

[12] Vlietenck, A. J. Screening Methods for Detection and Evaluation of Biological Activities of Plant Preparations. In *Bioassay Methods in Natural Product Research and Drug Development*; Bohlin, L., Bruhn, J. G., Eds.; Proceedings of the Phytochemical Society of Europe; Kluwer Academic Publishers: Boston, 1999; 37-52.

[13] Tulp, M. Th. M. The Use of Receptor Binding, a Very Specific, High Capacity Screening Method, in the Identification of Biologically Active Components from Natural Sources. In *Bioassay Methods in Natural Product Research and Drug Development*; Bohlin, L., Bruhn, J. G., Eds.; Proceedings of the Phytochemical Society of Europe; Kluwer Academic Publishers: Boston, 1999; 53-65.

1.5.2 Cell-Based Assays

Cell-based assays use intact cells from human or animal origins.[11] Extracts are evaluated for their cytotoxicity against the cells. Cell-based assays eliminate extracts containing compounds that are unable to enter the cell or are rapidly metabolized by the cell; characteristics which are unwanted in a drug.[12] However, the mechanism by which the compound inhibits the cell is unknown from the results of the cell-based assay. By using the two types of assays in combination, information about the mechanism by which the cytotoxic compound acts can be determined.

1.5.3 A2780 Assay

A cell-based assay is used to screen the extracts from Madagascar and bioassay-guided fractionation is used to isolate cytotoxic anti-cancer agents. Human ovarian cancer cells (A2780) are used in a microtiter plate assay. The plates are seeded with cells and the compounds (dissolved in 1:1 DMSO:H_2O) are added to the cells at specific concentrations. The plates are incubated at 37°C and 5% CO_2 for 48 hours. Then Alamar Blue (Biosource International) is added to the cells and the plates are incubated for three hours. During this time the Alamar Blue is taken up by the live cells and reduced. The reduced form of Alamar Blue is stable and fluorescent. The fluorescence of each well in the plate is measured. The fluorescence is directly proportional to the percent inhibition of the growth of the cells. The IC_{50} value is determined by plotting the data on a dose response curve of percent inhibition versus concentration. The IC_{50} value is defined as the concentration of sample necessary to produce 50% inhibition of the growth of the cells. The smaller the IC_{50} value the more active the compound.

II. Cytotoxic Clerodane Diterpenes from *Casearia nigrescens*

2.1 Introduction

As part of the continuing work of the ICBG program, plant extracts were received from Madagascar. The extracts were screened for bioactivity using the A2780 human ovarian cancer cell line assay. When screened, the crude extract of *Casearia nigrescens* was active (IC_{50} 12 μg/mL). Further evaluation of the extract resulted in the isolation of two new compounds and four known compounds.

2.1.1 Indigenous use of *Casearia* species

Casearia species grow as shrubs or small trees in tropical regions.[14] The indigenous people of these regions use preparations of *Casearia* species for medical treatment. Some common ethnobotanical uses of *Casearia* species include treatment of diarrhea, stomach ulcers, inflammation, viral infections, fevers, and snakebites.[14] Preparations are also made and applied topically to clean the skin, treat wounds and skin diseases, and ease the discomfort of insect bites.[14]

2.1.2 Bioactivity of *Casearia* species extracts

Significant research has been done to explore the healing potential of extracts from *Casearia* species. Research indicates that extracts from *Casearia* species have anti-cancer,[15, 16]

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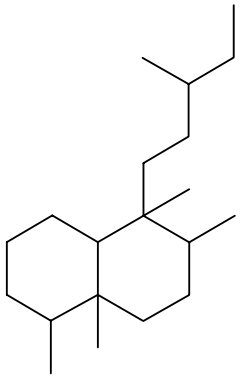
[14] Raintree Nutrition—Tropical Plant Database. http://www.rain-tree.com/guacatonga.htm (accessed May 2004).
[15] Itokawa, H.; Totsuka, N.; Takeya, K.; Watanabe, K.; Obata, E. Antitumor principles from *Casearia sylvestris* Sw. (Flacourtiaceae), structure elucidation of new clerodane diterpenes by 2-D NMR spectroscopy. *Chem. Pharm. Bull.* **1988**, *36*, 1585-1588.
[16] Prakash, C. V. S.; Hoch, J. M.; Kingston, D. G. I. Structure and Stereochemistry of New Cytotoxic Clerodane Diterpenoids from the Bark of *Casearia lucida* from the Madagascar Rainforest. *J. Nat. Prod.* **2002**, *65*, 100-107.

anti-fungal,[17] anti-ulcer,[18] anti-hyperglycemic,[19] analgesic,[20] and anti-inflammatory activities,[20] as well as the ability to neutralize several types of snake venom.[21] The majority of compounds isolated from *Casearia* species are clerodane diterpenes[15, 16, 22, 23] (**2.1**).



2.1 Clerodane diterpene skeleton

Continued research of *Casearia* species may result in the isolation of novel compounds with important medicinal properties.

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[17] Oberlies, N. H.; Burgess, J. P.; Navarro, H. A.; Pinos, R. E.; Fairchild, C. R.; Peterson, R. W.; Soejarto, D. D.; Farnsworth, N. R.; Kinghorn, A. D.; Wani, M. C.; Wall, M. E. Novel Bioactive Clerodane Diterpenoids from the leaves and twig of *Casearia sylvestris*. *J. Nat. Prod.* **2002**, *65*, 95-99.

[18] Basile, A. C.; Sertie, J. A.; Panizza, S.; Oshiro, T. T.; Azzolini, C. A. Pharmacological assay of *Casearia sylvestris*. I: Preventive anti-ulcer activity and toxicity of the leaf crude extract. *J. Ethnopharmacol.* **1990**, *30*, 185-197.

[19] Prakasam, A.; Sethupathy, S.; Pugalendi, K. V. Antihyperglycemic Effect of *Casearia esculenta* root Extracts in streptozotocin-induced Diabetic Rats. *Pharmazie*. **2002**, *57*, 758-760.

[20] Ruppelt, B. M.; Pereira, E. F.; Goncalves, L. C.; Pereira, N. A. Pharmacological Screening of Plants Recommended by Folk Medicine as Anti-snake Venom--I. Analgesic and Anti-inflammatory Activities. *Mem. Inst. Oswaldo Cruz* **1991**, *86*, 203-205.

[21] Borges, M. H.; Soares, A. M.; Rodrigues, V. M.; Oliveira, F.; Fransheschi, A. M. Rucavado, A.; Giglio, J. R.; Homsi-Brandeburgo, M. I. Neutralization of Proteases from *Bothrops* Snake Venoms by the aqueous extract from *Casearia sylvestris* (Flacourtiaceae). *Toxicon* **2001**, *39*, 1863-1869.

[22] Hayashi, K.; Nakanishi, Y.; Bastow, K. F.; Cragg, G.; Nozaki, H.; Lee, K.-H. Antitumor Agents. Part 212: Bucidarasins A-C, Three New Cytotoxic Clerodane Diterpenes from *Bucida buceras*. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 345-348.

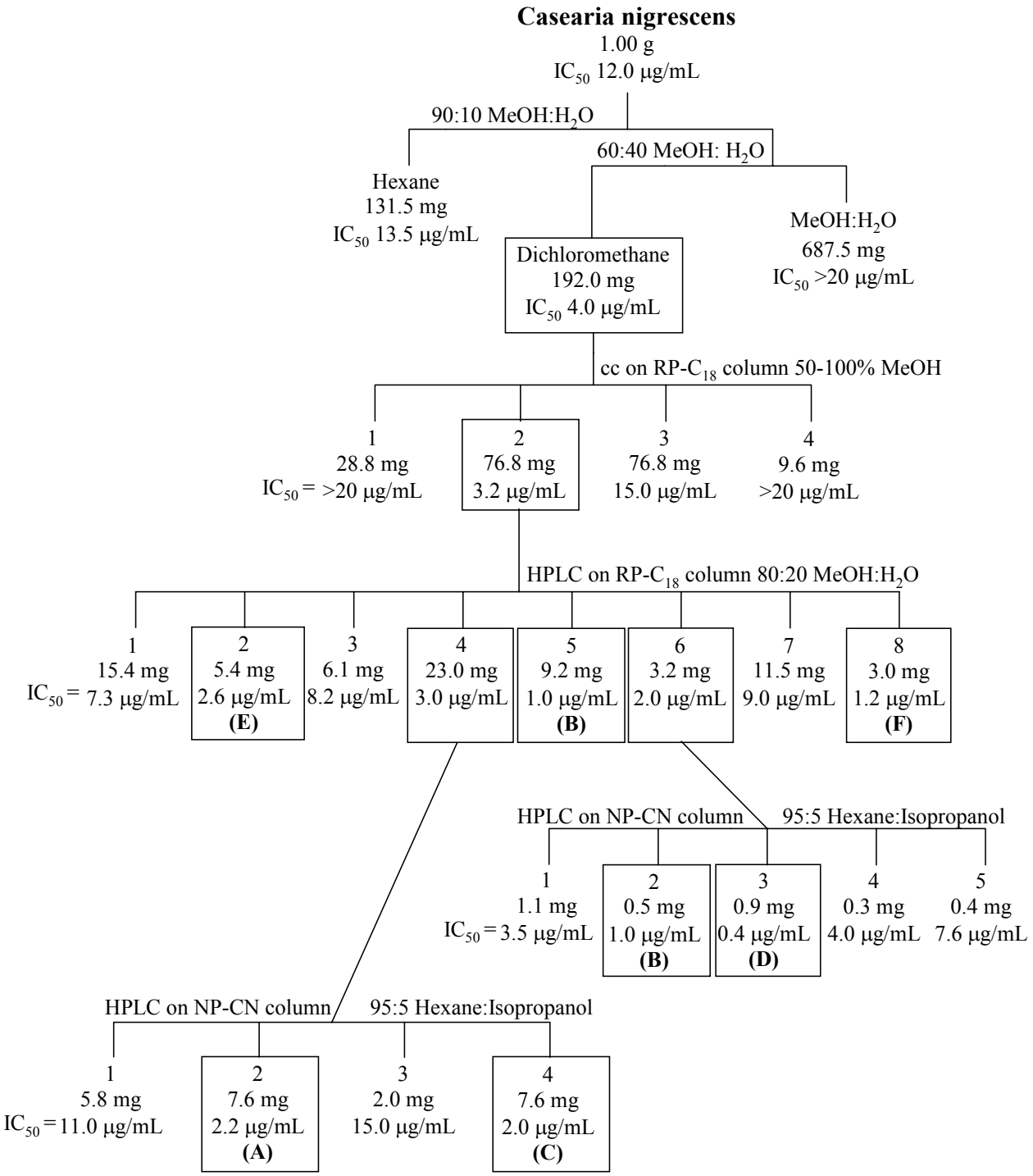
[23] Gibbons, S.; Gray, A. I.; Waterman, P. G. Clerodane diterpenes from the bark of *Casearia tremula*. *Phytochemistry* **1996**, *41*, 565-570.

2.2 Results and Discussion

2.2.1 Isolation of Compounds from *Casearia nigrescens*

The crude plant extract from *Casearia nigrescens*, a Madagascar species, was collected as part of the continuing work of the ICBG program. The crude extract was screened in house in the A2780 human ovarian cancer cell line assay. The results indicated that the crude extract was weakly active (IC_{50} 12 μg/mL). Further examination of the extract resulted in the isolation of two new compounds and four known compounds.

A portion of the crude extract (1.00 g) was subjected to fractionation (Scheme 2.1). First liquid-liquid partitioning was used. The crude extract was dissolved in aqueous methanol (90%), which was extracted with hexane. The aqueous methanol was then diluted (60%) and extracted with dichloromethane. The hexane, dichloromethane, and aqueous methanol fractions were concentrated. The fractions were tested in the A2780 assay and the dichloromethane fraction was found to be most active (IC_{50} 4.0 μg/mL). Further separation of the dichloromethane fraction on a reverse-phase C_{18} open column using a gradient elution of 50% aqueous methanol to 100% methanol resulted in the collection of four fractions. When tested in the A2780 assay the second fraction was most active (IC_{50} 3.2 μg/mL). This active fraction was then subjected to further separation on HPLC using reverse-phase C_{18} with an isocratic elution of aqueous methanol (80%). Eight fractions were collected from the HPLC separation and when tested five fractions were found to be active in the A2780 assay. When the five active fractions were further investigated three were found to be pure compounds: Fraction 2—compound **E** (IC_{50} 2.6 μg/mL), Fraction 5—compound **B** (IC_{50} 1.0 μg/mL), and Fraction 8—compound **F** (IC_{50} 1.2 μg/mL). The other two fractions, Fraction 4 (IC_{50} 3.0 μg/mL) and Fraction 6 (IC_{50} 3.2 μg/mL), required further purification. Fraction 4 was further purified using a normal phase cyano column



Scheme 2.1 Fractionation tree for *Casearia nigrescens*.

on HPLC with an isocratic elution of hexane:isopropanol (95:5). This resulted in the collection of four fractions. When tested two fractions were active in the A2780 assay and upon further investigation these two fractions were found to be pure compounds: compound **A** (IC_{50} 2.2 μg/mL) and compound **C** (IC_{50} 2.0 μg/mL). Fraction 6 was also further purified using a normal phase cyano column on HPLC with an isocratic elution of hexane:isopropanol (95:5). This resulted in the collection of five fractions, two of which were pure. One of the pure fractions resulted in the additional isolation of compound **B** (IC_{50} 1.0 μg/mL) and the other pure fraction resulted in the isolation of compound **D** (IC_{50} 0.4 μg/mL).

2.2.2 Structure Elucidation of Compound A

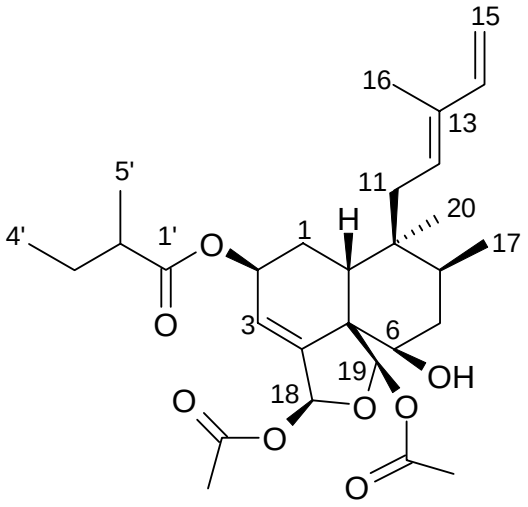
Compound **A** was isolated as a viscous oil. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 541.27765, which corresponded to the molecular formula $C_{29}H_{42}O_8$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

The ^{1}H NMR spectrum of compound **A** in $CDCl_3$ showed the presence of signals for two methyl groups (δ 0.81 s; δ 0.93 d, J = 6.7 Hz); two oxymethine protons (δ 3.80 m; δ 5.44 m); two acetal-acyloxy methine protons (δ 6.51 s; δ 6.73 t; J = 1.6 Hz); and a trisubstituted olefinic proton (δ 6.01 dd, J = 4.4 Hz, 1.8 Hz) all indicative of the basic skeleton of a clerodane diterpene. The ^{1}H NMR spectrum also contained signals for two methyl groups (δ 1.93 s; δ 2.08 s) corresponding to the methyls of two acetate units. The signals for two additional methyl groups (δ 0.97 t, J = 7.5 Hz; δ 1.18 d, J = 7.1 Hz) and one of the previously mentioned oxymethine protons (δ 5.44 m) indicated the presence of a 2-methylbutanoyloxy side chain located at C-2. Also observed in the ^{1}H NMR spectrum was a signal for an allylic methyl (δ 1.66

s); a saturated methylene (δ 1.63 m; δ 2.23 dd, J = 8.0 Hz, 16.7 Hz); a terminal unsaturated methylene (δ 4.93 d, J = 10.6 Hz; δ 5.10 d, J = 17.4 Hz); and two vinyl protons (δ 5.36 br d; δ 6.26 dd, J = 10.7 Hz, 17.1 Hz) all suggesting the presence of a six carbon diene side chain.

The ^{13}C NMR spectrum of compound **A** in $CDCl_3$ was also consistent with the structure observed in the ^{1}H NMR spectrum. The basic clerodane diterpene structure was supported by the presence of signals for two methyl groups (δ 15.7; δ 25.0); two oxymethines (δ 66.2; δ 73.0); two acetal-acyloxy methines (δ 95.7; δ 97.0); an unsaturated methine (δ 122.0); and an unsaturated quaternary carbon (δ 145.4) in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum also contained signals for two methyl groups (δ 21.3; δ 21.6) and two carbonyls (δ 169.5; δ 170.2) corresponding to two acetate units. The signals for two additional methyl groups (δ 11.7; δ 16.7); a carbonyl group (δ 176.0); and the one of the oxymethines (δ 66.2) mentioned above suggested the presence of a 2-methylbutanoyloxy chain located at C-2. Also observed in the ^{13}C NMR spectrum was a signal for an allylic methyl (δ 12.1); a saturated methylene (δ 30.4); a terminal unsaturated methylene (δ 111.2); two unsaturated methines (δ 129.0; δ 141.3); and an unsaturated quaternary carbon (δ 135.8) which were consistent with the presence of a diene side chain in the molecule.

The data from the ^{1}H NMR and the ^{13}C NMR spectra in $CDCl_3$ when compared to the literature were found to be in close agreement with the data for the known compound casearlucin A[16] (**2.2**) (also known as bucidarasin B[22]) (Table 2.1). Based on the similarity of the experimental and literature data of the ^{1}H NMR and ^{13}C NMR spectra compound **A** was determined to be casearlucin A. The identity of compound **A** was further supported by the MS, optical rotation, UV, and IR data which were in agreement with the literature values for casearlucin A[16] (bucidarasin B[22]).



2.2 Casearlucin A

Table 2.1 ^{1}H NMR and ^{13}C NMR data for casearlucin A and compound **A**.

	Casearlucin A[16, a]		Compound **A**[a]	
Position	^{13}C NMR data	^{1}H NMR data	^{13}C NMR data	^{1}H NMR data
1	26.8	1.88 m	26.8	1.89 m
2	66.2	5.43 br s	66.2	5.44 m
3	121.9	6.00 d (4.6)	122.0	6.01 dd (1.8, 4.4)
4	145.2		145.4	
5	53.6		53.6	
6	73.0	3.78 d (6.9)	73.0	3.80 m
7	37.5	1.67 m	37.5	1.62 m
				1.74 m
8	36.9	1.80 m	36.9	1.76 m
9	37.7		37.7	
10	36.9	2.37 br t (8.9)	36.8	2.37 br t (8.6)
11	30.4	1.58 m	30.4	1.63 m
		2.23 dd (8.0, 16.7)		2.23 dd (8.0, 16.7)
12	129.0	5.37 br s	129.0	5.36 br d (6.2)
13	135.8		135.8	
14	141.3	6.25 dd (10.5, 17.1)	141.3	6.26 dd (10.7, 17.0)
15	111.2	4.92 d (11.2)	111.2	4.93 d (10.6)
		5.09 d (17.6)		5.10 d (17.4)
16	12.1	1.65 s	12.1	1.66 s
17	15.7	0.92 d (7.1)	15.7	0.93 d (6.7)
18	95.8	6.72 t (1.6)	95.7	6.73 t (1.6)
19	97.1	6.50 s	97.0	6.51 s
20	25.0	0.80 s	25.0	0.81 s
1'	175.9		176.0	
2'	41.3	2.44 m	41.2	2.46 m
3'	27.2	1.69 m	27.2	1.69 m
4'	11.7	0.96 t (7.6)	11.7	0.97 t (7.3)
5'	16.7	1.17 d (6.9)	16.7	1.18 d (7.1)
Acetyl Me	21.3[b]	1.93 s	21.3[b]	1.93 s
	21.5[b]	2.06 s	21.6[b]	2.08 s
CO	169.5[c]		169.5[c]	
	170.2[c]		170.2[c]	

[a]Recorded in $CDCl_3$. [b, c] Values with the same superscript are interchangeable within their respective column.

2.2.3 Structure Elucidation of Compound B

Compound **B** was isolated as an amorphous solid. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 541.27783, which corresponded to the

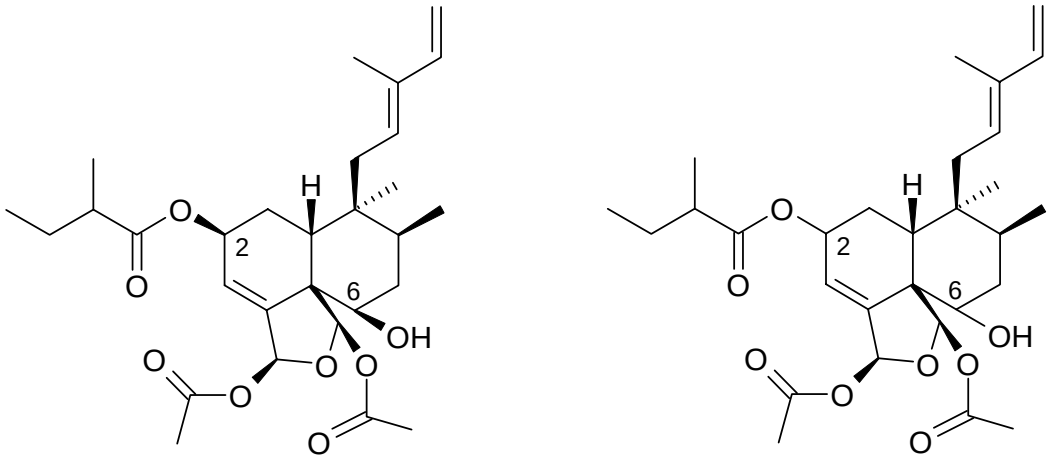
molecular formula $C_{29}H_{42}O_8$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

The ^{1}H NMR spectrum of compound **B** in $CDCl_3$ showed the presence of signals for two methyl groups (δ 0.84 s; δ 0.92 d, *J* = 6.6 Hz); two oxymethine protons (δ 4.00 m; δ 5.61 m); two acetal-acyloxy methine protons (δ 6.46 s; δ 6.70 m); and a trisubstituted olefinic proton (δ 5.89 br s) all indicative of the basic skeleton of a clerodane diterpene. The ^{1}H NMR spectrum also contained the signals for two methyl groups (δ 1.94 s; δ 2.09 s) corresponding to the methyls of two acetate units. The signals for two additional methyl groups (δ 0.92 t, *J* = 7.4 Hz; δ 1.16 d, *J* = 6.9 Hz) and one of the previously mentioned oxymethine protons (δ 5.61 m) indicated the presence of a 2-methylbutanoyloxy chain located at C-2. Also observed in the ^{1}H NMR spectrum was a signal for an allylic methyl (δ 1.65 s); a saturated methylene (δ 1.64 m; δ 2.20 dd, *J* = 8.4 Hz, 17.1 Hz); a terminal unsaturated methylene (δ 4.94 d, *J* = 10.6 Hz; δ 5.09 d, *J* = 17.4 Hz); and two vinyl protons (δ 5.37 br d, *J* = 7.8 Hz; δ 6.30 dd, *J* = 10.9 Hz, 17.2 Hz) all suggesting the presence of a six carbon diene side chain.

The ^{13}C NMR spectrum of compound **B** in $CDCl_3$ was also consistent with the structure observed in the ^{1}H NMR spectrum. The HSQC data for compound **B** was helpful in determining the number and type of carbons present in the molecule as well as assigning which protons were associated with which carbons. The basic clerodane diterpene structure was supported by the presence of signals for two methyl groups (δ 15.7; δ 25.1); two oxymethines (δ 70.5; δ 74.2); two acetal-acyloxy methines (δ 95.2; δ 96.7); an unsaturated methine (δ 124.4); and an unsaturated quaternary carbon (δ 144.3) in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum also contained signals for two methyl groups (δ 21.3; δ 21.7) and two carbonyl groups (δ 169.6; δ 170.2) corresponding to two acetate units. The signals for two additional methyl groups (δ 11.8;

δ 16.6); a carbonyl group (δ 176.9); and one of the oxymethines (δ 70.5) mentioned above suggested the presence of a 2-methylbutanoyloxy chain located at C-2. Also observed in the ^{13}C NMR spectrum was a signal for an allylic methyl (δ 12.0); a saturated methylene (δ 30.1); a terminal unsaturated methylene (δ 111.2); two unsaturated methines (δ 128.8; δ 141.2); and an unsaturated quaternary carbon (δ 135.9) which were constant with the presence of a diene side chain in the molecule.

When the ^{1}H NMR data and the ^{13}C NMR data for compound **B** was compared to the spectral data for casearlucin A (compound **A**), many similarities were observed (Figure 2.1). However, there were two significant differences observed in both the proton and carbon spectra for these two compounds (Table 2.2). In casearlucin A the 2-methylbutanoyloxy chain was β, with the signal for the oxymethine proton of C-2 at δ 5.44 ppm and the carbon at δ 66.2 ppm. The alcohol was also β in casearlucin A with the signal for the oxymethine proton of C-6 at δ 3.80 ppm and the carbon at δ 73.0 ppm. In compound **B** the signal for the oxymethine proton of C-2 was at δ 5.61 ppm and the carbon was at δ 70.5 ppm, while the signal for the oxymethine proton of C-6 was at δ 4.00 ppm and the carbon was at δ 74.2 ppm. These differences indicated a possible difference in stereochemistry of these two compounds particularly at C-2 and C-6.



Casearlucin A
(Compound **A**)

Compound **B**

Figure 2.1 Comparison of casearlucin A (compound **A**) and compound **B**.

Table 2.2 ^{1}H NMR and ^{13}C NMR data for casearlucin A (compound **A**) and compound **B**.

	Casearlucin A (Compound **A**)[a]		Compound **B**[a]	
Position	^{13}C NMR data	^{1}H NMR data	^{13}C NMR data	^{1}H NMR data
1	26.8	1.89 m	26.2	2.14 m
2	**66.2**	5.44 m	70.5	5.61 m
3	122.0	6.01 dd (1.8, 4.4)	124.4	5.89 br s
4	145.4		144.3	
5	53.6		53.5	
6	**73.0**	3.80 m	74.2	4.00 m
7	37.5	1.62 m	37.8	1.64 m
		1.74 m		1.76 m
8	36.9	1.76 m	36.9	1.84 m
9	37.7		38.5	
10	36.8	2.37 br t (8.6)	41.5	2.37 m
11	30.4	1.63 m	30.1	1.64 m
		2.23 dd (8.0, 16.7)		2.20 dd (8.4, 17.1)
12	129.0	5.36 br d (6.2)	128.8	5.37 br d (7.8)
13	135.8		135.9	
14	141.3	6.26 dd (10.7, 17.0)	141.2	6.30 dd (10.9, 17.2)
15	111.2	4.93 d (10.6)	111.2	4.94 d (10.6)
		5.10 d (17.4)		5.09 d (17.4)
16	12.1	1.66 s	12.0	1.65 s
17	15.7	0.93 d (6.7)	15.7	0.92 d (6.6)
18	95.7	6.73 t (1.6)	95.2	6.70 m
19	97.0	6.51 s	96.7	6.46 s
20	25.0	0.81 s	25.1	0.84 s
1'	176.0		176.9	
2'	41.2	2.46 m	41.2	2.37 m
3'	27.2	1.69 m	26.9	1.68 m
				1.49 m
4'	11.7	0.97 t (7.3)	11.8	0.92 t (7.4)
5'	16.7	1.18 d (7.1)	16.6	1.16 d (6.9)
Acetyl Me	21.3	1.93 s	21.3	1.94 s
	21.6	2.08 s	21.7	2.09 s
CO	169.5		169.6	
	170.2		170.2	

[a]Recorded in $CDCl_3$.

To ensure that the basic structure of compound **B** was correct, the COSY and HMBC data for compound **B** were obtained. The COSY data for compound **B** obtained in $CDCl_3$ indicated correlations between H-10/H-1; H-1/H-2; H-2/H-3; H-6/H-7; H-7/H-8; and H-8/H-17, which supported the basic clerodane diterpene structure. Correlation H-8/H-17 showed that one of the methyl groups was attached to C-8. Other correlations in the COSY spectra (H-2'/H-3', H-5'; H-3'/H-4') supported the presence of the 2-methylbutanoyloxy chain. The presence of the diene side chain was supported by the correlations between H-11/H-12 and H-14/H-15. All the expected COSY correlations for compound **B** were observed (Figure 2.2).

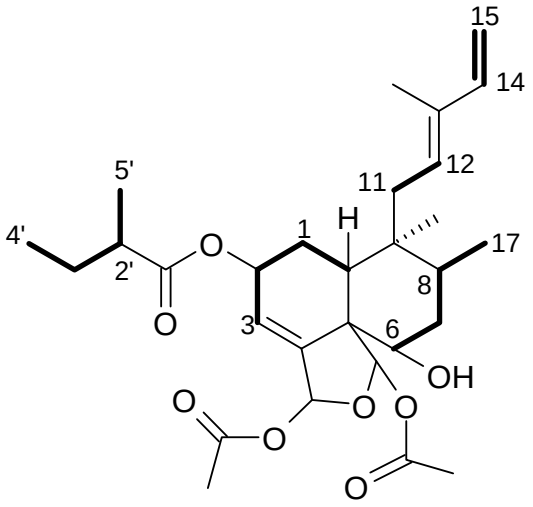


Figure 2.2 COSY correlations observed for compound **B** shown in bold. (Recorded in $CDCl_3$.)

The results from the HMBC spectrum (Figure 2.3) for compound **B** further supported the findings of the ^{1}H NMR, ^{13}C NMR, and COSY spectra. The HMBC data also helped identify the position of attachment of the side chains. The basic clerodane diterpene structure was supported by the following HMBC correlations: H-1/C-2; H-2/C-3; H-3/C-5; H-7/C-6, C-8; H-10/C-1, C-9. The COSY data indicated that one methyl group was attached to the ring at C-8; the HMBC correlation H-20/C-9 supported the attachment of a second methyl group to the ring at C-9. As suggested by the proton and carbon spectra, two acetate units were identified. The presence of

the acetate unit was supported by two H-MeCO/C-COO HMBC correlations. The connectivity of the acetate units to the ring was supported by the H-18/C-COO and H-19/C-COO correlations in the HMBC spectra. The HMBC correlations H-2'/C-1', C-5'; H-3'/C-2', C-4'; H-4'/C-3'; H-5'/C-2' supported the presence of the 2-methylbutanoyloxy side chain and the HMBC correlation H-2/C-1' supports the connectivity of the 2-methylbutanoyloxy side chain to C-2. The presence of the diene side chain was supported by the correlations H-11/C-12; H-12/C-14; H-14/C-13; H-15/C-14; H-16/C-12. The HMBC correlation H-11/C-9 supported the connection of the diene side chain to the ring at C-9.

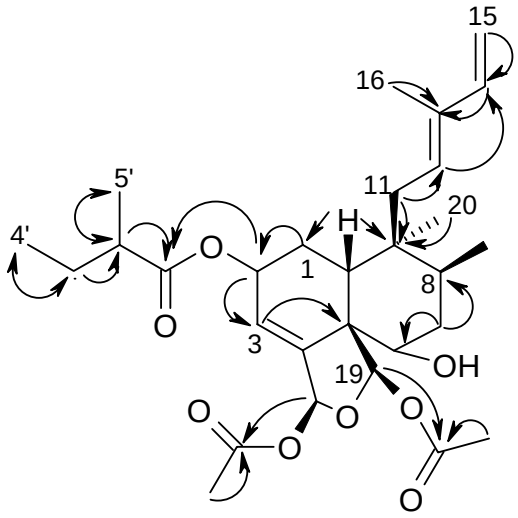


Figure 2.3 HMBC correlations observed for compound **B**. (Recorded in $CDCl_3$.)

The MS, ^{1}H NMR, ^{13}C NMR, COSY, and HMBC data supported the proposed structure for compound **B**; however, the stereochemistry of the compound was unclear. NOESY correlations would give an indication of the stereochemistry in the molecule. To obtain a better idea of the expected NOESY correlations Spartan calculations were done. In order to execute the Spartan calculations stereochemistry must be assigned for each compound. Based on the previously discussed spectral results in which casearlucin A and compound **B** differed in the chemical shifts of the protons and carbons at position 2 and 6 two of the three possible structures

were proposed for compound **B**, compound **B-1** and compound **B-2**. Compound **B-1** was assigned the β-C-2 and α-C-6 stereochemistry, while compound **B-2** was assigned α-C-2 and β-C-6 stereochemistry. The three compounds differed in stereochemistry at C-2 and C-6 (Figure 2.4).

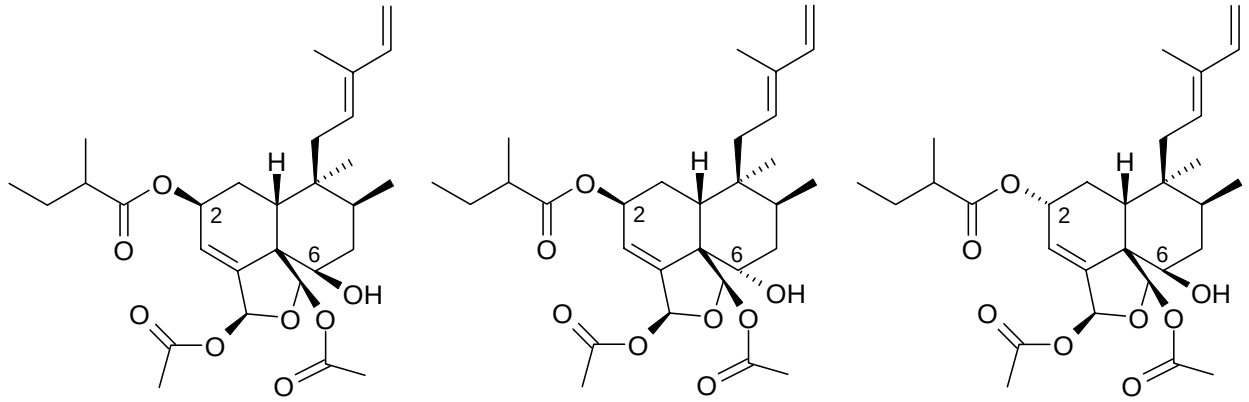


Figure 2.4 Stereochemistry in casearlucin A, compound **B-1**, and compound **B-2** analyzed using Spartan.

The equilibrium conformer at the ground state and the conformation distribution for each compound was found using MMFF calculations in Spartan. The low energy conformers for each compound were analyzed. NOESY data shows a correlation for pairs of protons that are close in space. Therefore, the conformers that were found using Spartan were analyzed for pairs of protons that were less than 3Å apart in space (Figure 2.5).

From the Spartan calculations for all three compounds there was an expected correlation between H-10 and H-12, since the stereochemistry was expected to be the same for all three compounds, specifically H-10 is β and the diene side chain at C-9 is also β. There was also an expected correlation between H-11 and H-19 for all three compounds, since the stereochemistry for the diene side chain at C-9 is β and H-19 is α for all three compounds. The Spartan calculations suggested a NOESY correlation between H-6 and H-8 in casearlucin A and

compound **B-2** since these protons were diaxial in these two compounds. However, in the structure of compound **B-1** the alcohol group was α. Therefore, H-6 and H-8 cannot be diaxial and thus were too far apart to show a NOESY correlation as suggested by the Spartan calculations. Spartan calculations suggested that only in compound **B-2** was there a NOESY correlation between H-2 and H-10 since the 2-methylbutanoyloxy side chain at C-2 was α, making H-2 and H-10 diaxial. This correlation was only seen in compound **B-2** because it was the only one of the three compounds in which the 2-methylbutanoyloxy side chain at C-2 was α.

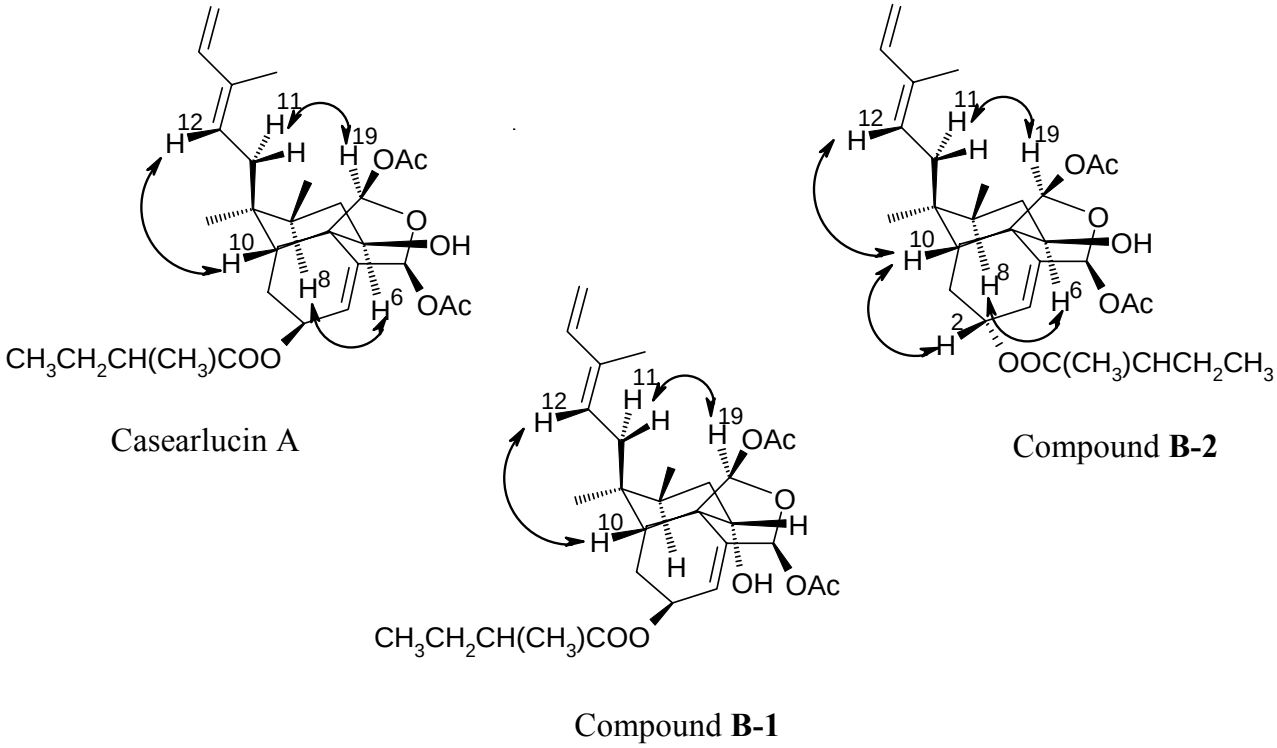


Figure 2.5 Correlations suggested by Spartan calculations.

Knowing the results from the Spartan calculations, the NOESY data for compound **B** was obtained in $CDCl_3$. The NOESY data revealed important information about the attachment of side chains and the stereochemistry in compound **B** (Figure 2.6). The relative stereochemistry of H-10 in compound **B** was assigned as β. The H-10/H-12 NOESY correlation for compound **B**

suggested that the diene side chain attached to C-9 was also β in orientation. The NOESY correlations H-11/H-16; H-12/H-14; H-14/H-15; H-15/H-15; H-15/H-16 supported the connectivity of the diene side chain and the correlation H-12/H-14 indicated that the conformation of the diene side chain was E. Other correlations (H-11/H-17; H-12/H-20) were consistent with the presence of two methyl groups in the molecule. The H-11/H-17 correlation indicated that the methyl group attached to C-8 was β since the diene side chain was β in orientation. The H-12/H-20 correlation indicated that the methyl group attached to C-9 was α since the diene side chain was β in orientation. The correlation between H-11 and H-19, which was expected from Spartan calculations, indicated that H-19 must be α, since the diene side chain at C-9 was β. The NOESY data showed a correlation between H-2 and H-3; however, this correlation did not give conclusive evidence of the stereochemistry at C-2 since H-3 was a vinyl proton and therefore was planar. NOESY data was also obtained for casearlucin A (compound **A**) for comparison purposes. The NOESY data indicated a strong correlation between H-6/H-8. This was consistent with the structure in which the alcohol group was β and H-6 and H-8 were diaxial. When the NOESY data for casearlucin A (compound **A**) was compared to the NOESY data for compound B a clear H-6/H-8 correlation was not readily observed. A 1D NOESY experiment in which H-6 was excited indicated a strong correlation with H-7, but still gave only a questionable correlation to H-8. The lack of similarity between the NOESY data for casearlucin A (compound **A**) and compound **B** as well as the very questionable H-6/H-8 NOESY correlation for compound **B** led to the following conclusions. If there is not a H-6/H-8 correlation, it may be because the protons were diaxial and the correlation did not significantly show up in the NOESY spectrum, or it may be because the protons were not diaxial in which case no correlation would be expected.

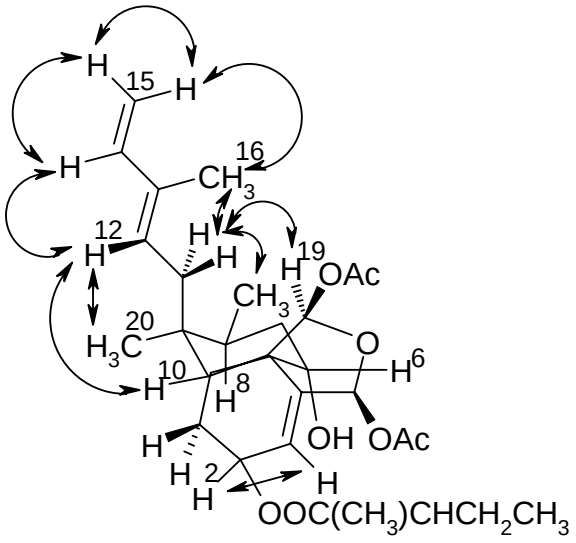


Figure 2.6 NOESY correlations observed for compound **B**. (Recorded in $CDCl_3$.)

To further examine the stereochemistry at C-6 in compound **B** a reaction was proposed (Figure 2.7).

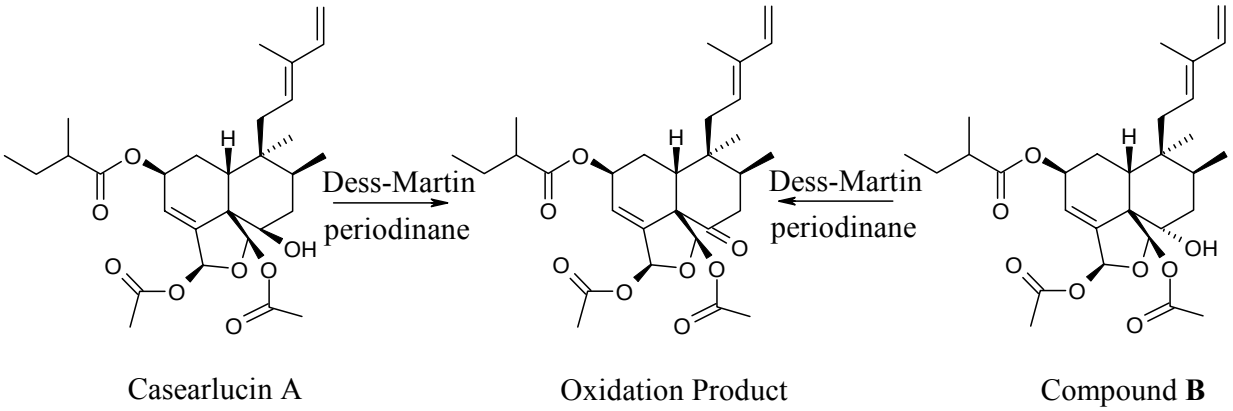


Figure 2.7 Proposed oxidation reaction of casearlucin A and compound **B** using Dess-Martin periodinane.

Casearlucin A (compound **A**) and compound **B** were proposed to differ only in the stereochemistry at C-6. If the alcohol at C-6 was oxidized in both of these compounds the same product should be obtained from each. This would indicate that the proposed structure of compound **B** was correct, giving positive evidence that the alcohol group of compound **B** is α. However, if the reaction product for these two compounds is not the same, then these two

compounds must differ in stereochemistry at another position. The oxidation reaction was done using Dess-Martin periodinane. The reactants and products were analyzed using ^{1}H NMR spectra. Prior to oxidation the ^{1}H NMR spectrum for casearlucin A showed a peak at δ 3.80 ppm, which corresponded to α-H-6. Following oxidation the peak at δ 3.80 ppm was gone, indicating that oxidation of the alcohol occurred. The ^{1}H NMR spectrum of the oxidation product of casearlucin A also showed a peak at δ 5.43 ppm corresponding to α-H-2. For compound **B** the ^{1}H NMR spectrum prior to oxidation showed a peak at δ 4.00 ppm, which corresponded to β-H-6. Following oxidation of compound **B**, the peak at δ 4.00 ppm in the ^{1}H NMR spectrum was gone indicating that oxidation of the compound took place. Also in the ^{1}H NMR spectrum of the oxidation product of compound **B** there was a peak at δ 5.53 ppm, which corresponded to H-2. Since the ^{1}H NMR spectrum for the oxidation products for these two compounds were not the same, and since the major difference occurred in the shift of the peak corresponding to H-2, it was hypothesized that these two compounds differed in their stereochemistry at C-2 as well as at C-6. The NOESY data was re-evaluated and a correlation between H-2 and H-10 for compound **B** was noted, which indicated that H-2 and H-10 must be diaxial, making the 2-methylbutanoyloxy side chain at C-2 α. Based on the data obtained a structure was initially proposed for compound **B** (Figure 2.8).

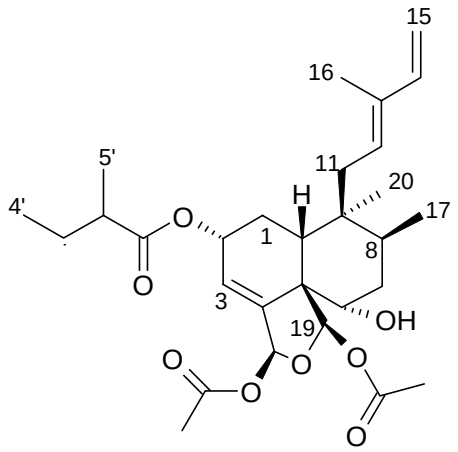
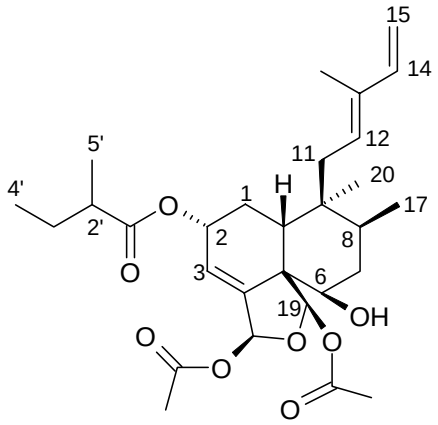


Figure 2.8 Initially proposed structure of compound **B**.

Following this work an additional search of the literature yielded a recent publication of a similar structure, caseamenbrols (**2.3**).[24]



2.3 Caseamenbrol A
(Compound **B**)

When the data for compound **B** and caseamenbrol A were compared the data was found to be in close agreement (Table 2.3). The alcohol group of caseamenbrol A was assigned β stereochemistry based on a NOESY correlation between H-6/H-8.[24] Further review of the 1D and 2D NOESY spectra for compound **B** resulted in the conclusion that there was a NOESY correlation between H-6/H-8. Therefore compound **B** was determined to be caseamenbrol A

[24] Shen, Y.-C.; Wang, L.-T.; Wang, C.-H.; Khalil, A. T.; Guh, J.-H. Two New Cytotoxic Clerodane Diterpenoids from *Casearia membranacea*. *Chem. Pharm. Bull.* **2004**, *52*, 108-110.

(**2.3**). The identity of compound **B** was further supported by the MS, optical rotation, UV, and IR data which were in agreement with the literature values for caseamenbrol A.[24]

Table 2.3 ^{1}H NMR and ^{13}C NMR data for caseamenbrol A and compound **B**.

	Caseamenbrol A[24, a]		Compound **B**[a]	
Position	^{13}C NMR data	^{1}H NMR data	^{13}C NMR data	^{1}H NMR data
1	26.2	2.18 m	26.2	2.14 m
2	70.5	5.61 m	70.5	5.61 m
3	124.2	5.88 br s	124.4	5.89 br s
4	144.3		144.3	
5	53.5		53.5	
6	74.1	3.99 dd (12.2, 6.6)	74.2	4.00 m
7	37.6	1.64 m	37.8	1.64 m
		1.74 m		1.76 m
8	36.8	1.76 m	36.9	1.84 m
9	38.4		38.5	
10	41.5	2.42 dd (14.2, 2.4)	41.5	2.37 m
11	30.1	1.68 m	30.1	1.64 m
		2.25 m		2.20 dd (8.4, 17.1)
12	128.9	5.33 d (5.1)	128.8	5.37 br d (7.8)
13	135.9		135.9	
14	141.3	6.29 dd (10.7, 17.3)	141.2	6.30 dd (10.9, 17.2)
15	111.1	4.92 d (10.7)	111.2	4.94 d (10.6)
		5.08 d (17.3)		5.09 d (17.4)
16	12.0	1.64 s	12.0	1.65 s
17	15.6	0.91 d (6.2)	15.7	0.92 d (6.6)
18	95.2	6.68 br s	95.2	6.70 m
19	96.7	6.46 s	96.7	6.46 s
20	25.1	0.83 s	25.1	0.84 s
1'	176.6		176.9	
2'	41.2	2.37 m	41.2	2.37 m
3'	26.8	1.52 m	26.9	1.68 m
		1.23 m		1.49 m
4'	11.7	0.95 t (7.0)	11.6	0.92 t (7.4)
5'	16.6	1.14 d (7.0)	16.6	1.16 d (6.9)
Acetyl Me	21.3	1.93 s	21.3	1.94 s
	21.7	2.08 s	21.7	2.09 s
CO	169.5		169.6	
	170.2		170.2	

[a]Recorded in $CDCl_3$.

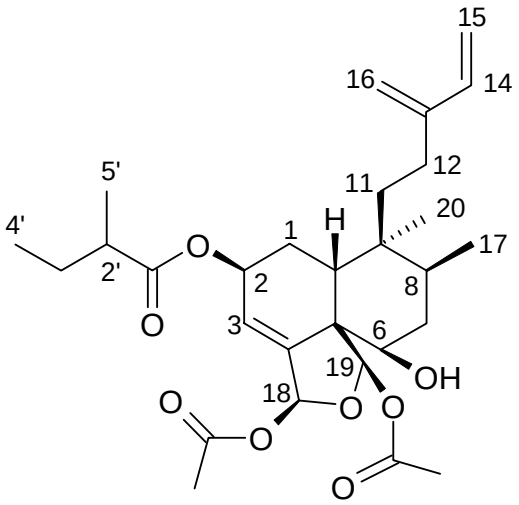
2.2.4 Structure Elucidation of Compound C

Compound **C** was isolated as a viscous oil. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 541.2766, which corresponded to the molecular formula $C_{29}H_{42}O_8$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

Comparison of the ^{1}H NMR and ^{13}C NMR data of compound **C** to casearlucin A (compound **A**) indicated that the two compounds were identical except for the configuration of the double bonds in the diene side chain (Table 2.4 & Table 2.5). The ^{1}H NMR spectrum of compound **C** in $CDCl_3$ showed the presence of signals for two saturated methylenes (δ 1.26 m & δ 1.50 m; δ 2.08 m); two terminal unsaturated methylenes (δ 4.93 s & δ 5.04 s ; δ 5.02 d, J = 11.00 Hz & δ 5.16 d, J = 17.40 Hz); and one vinyl protons (δ 6.44 dd, J = 10.75 Hz, 17.65 Hz) all suggesting the presence of a six carbon diene side chain, but with a different double bond configuration than was observed in casearlucin A (compound **A**). The ^{13}C NMR spectrum of compound **C** in $CDCl_3$ was also consistent with the observed differences in the double bond configuration of the diene side chain as compared to casearlucin A (compound **A**). The ^{13}C NMR spectrum contained signals for two saturated methylenes (δ 23.8; δ 28.0); two terminal unsaturated methylenes (δ 112.3; δ 115.6); one unsaturated methine (δ 140.5); and an unsaturated quaternary carbon (δ 145.1).

The data from the ^{1}H NMR and the ^{13}C NMR spectra in $CDCl_3$ when compared to the literature were found to be in close agreement with the data for the known compound *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene[16, 23] (**2.4**) (Table 2.4 & Table 2.5). Based on the similarity of the experimental and literature data of the ^{1}H NMR and ^{13}C NMR spectra

compound **C** was determined to be *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene. The identity of compound **C** was further supported by the MS, optical rotation, UV, and IR data which were in agreement with the literature values for *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene.[23]



2.4 *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene

Table 2.4 ^{1}H NMR data for casearlucin A, compound **C**, and *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene.

	Casearlucin A (Compound **A**)[a]	Compound **C**[a]	*rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene[23, a]
Position	^{1}H NMR data	^{1}H NMR data	^{1}H NMR data
1	1.89 m	1.92 m	1.90 m
2	5.44 m	5.42 m	5.40 br s
3	6.01 dd (1.8, 4.4)	5.98 d (4.2)	5.96 d (4.1)
6	3.80 m	3.80 dd (3.9, 11.9)	3.78 br t (8.4)
7	1.62 m	1.72 m	1.70 m
	1.74 m		
8	1.76 m	1.77 m	1.75 m
10	2.37 br t (8.6)	2.33 m	2.31 dd (6.1, 10.8)
11	1.63 m	1.26 m	1.25 m
	2.23 dd (8.0, 16.7)	1.50 m	1.48 m
12	5.36 br d	2.08 m	2.06 m
14	6.26 dd (10.7, 17.0)	6.44 dd (10.8, 17.7)	6.42 dd (11.0, 17.7)
15	4.93 d (10.6)	5.02 d (11.0)	4.95 d (10.3)
	5.10 d (17.4)	5.16 d (17.4)	5.14 br d (17.7)
16	1.66 s	4.93 s	4.93 s
		5.04 s	5.01 s
17	0.93 d (6.7)	0.92 d (7.0)	0.90 d (6.0)
18	6.73 t (1.6)	6.72 m	6.69 br s
19	6.51 s	6.46 s	6.46 br s
20	0.81 s	0.91 s	0.89 s
2'	2.46 m	2.44 m	2.42 m
3'	1.69 m	1.65 m	1.63 m
4'	0.97 t (7.3)	0.96 t (7.5)	0.94 t (7.4)
5'	1.18 d (7.1)	1.17 d (6.9)	1.15 d (7.0)
Acetyl Me	1.93 s	1.87 s	1.85 s
	2.08 s	2.05 s	2.03 s

[a]Recorded in $CDCl_3$.

Table 2.5 ^{13}C NMR data for casearlucin A, compound **C**, and *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene.

Position	Casearlucin A (Compound **A**)[a] ^{13}C NMR data	Compound **C**[a] ^{13}C NMR data	*rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene[23, a] ^{13}C NMR data
1	26.8	26.9	26.9
2	66.2	66.2	66.3
3	122.0	122.0	121.8
4	145.4	145.4	145.7
5	53.6	53.8	53.9
6	73.0	73.2	73.0
7	37.5	37.3	37.0
8	36.9	37.6	37.6
9	37.7	37.5	37.5
10	36.8	36.5	36.5
11	30.4	28.0	28.0
12	129.0	23.8	23.9
13	135.8	145.1	145.2
14	141.3	140.5	140.6
15	111.2	112.3	112.2
16	12.1	115.6	115.6
17	15.7	15.8	15.8
18	95.7	95.6	95.7
19	97.0	97.8	98.1
20	25.0	25.5	25.5
1'	176.0	175.9	176.0
2'	41.2	41.2	41.3
3'	27.2	27.1	27.1
4'	11.7	11.7	11.7
5'	16.7	16.7	16.7
Acetyl Me	21.3	21.3	21.3
	21.6	21.5	21.5
CO	169.5	169.8	169.8
	170.2	170.1	170.2

[a]Recorded in $CDCl_3$.

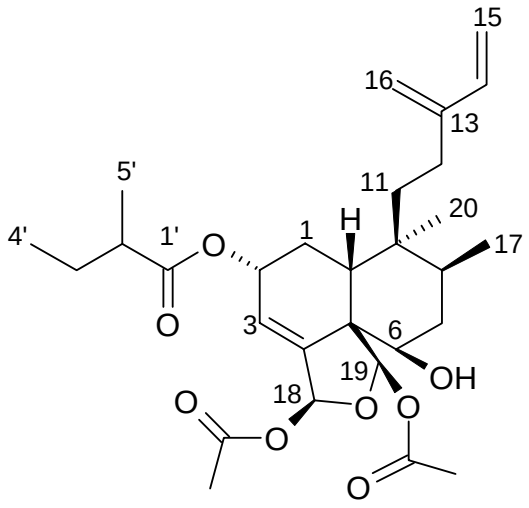
2.2.5 Structure Elucidation of Compound D

Compound **D** was isolated as an amorphous solid. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 541.27386, which corresponded to the molecular formula $C_{29}H_{42}O_8$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

Due to the small quantity of material isolated a ^{1}H NMR spectrum was the only type of NMR data obtained for this compound. Based on the ^{1}H NMR data a novel structure was proposed for this compound. Comparison of the ^{1}H NMR data of compound **D** to *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) suggested that these two compounds were identical except for the stereochemistry at C-2 and C-6. In *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) the 2-methylbutanoyloxy chain was β, with the signal for the oxymethine proton of C-2 at δ 5.42 ppm. The alcohol was also β in *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) with the signal for the oxymethine proton of C-6 at δ 3.80 ppm. In compound **D** the signal for the oxymethine proton of C-2 was at δ 5.60 ppm, while the signal for the oxymethine proton of C-6 was at δ 4.01 ppm. The ^{1}H NMR data of compound **D** was also compared to the ^{1}H NMR data of caseamenbrol A (compound **B**), paying particular attention to the chemical shifts of the peaks corresponding to the protons of C-2 and C-6. In caseamenbrol A (compound **B**) the 2-methylbutanoyloxy chain was α, with the signal for the oxymethine proton of C-2 at δ 5.61 ppm. The alcohol was β in caseamenbrol A (compound **B**) with the signal for the oxymethine proton of C-6 at δ 4.00 ppm. The similarity in chemical shift

of the peaks corresponding to the protons of C-2 and C-6 in caseamenbrol A (compound **B**) and compound **D** suggested that the stereochemistry of the 2-methylbutanoyloxy chain was α and the stereochemistry of the alcohol was β in compound **D**.

Based on the ^{1}H NMR data a novel structure was proposed for compound **D** (**2.5**). This novel compound was named casearlucin L. The proposed structure of compound **D** was supported by comparison of the ^{1}H NMR data to the ^{1}H NMR data for *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) and caseamenbrol A (compound **B**) (Table 2.6).



2.5 Casearlucin L

Table 2.6 ^{1}H NMR data for *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**), compound **D**, and caseamenbrol A (compound **B**).

	rel-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (Compound **C**)[a]	Casearlucin L (Compound **D**)[a]	Caseamenbrol A (Compound **B**)[a]
Position	^{1}H NMR data	^{1}H NMR data	^{1}H NMR data
1	1.92 m	2.15 m	2.14 m
2	5.42 m	5.60 m	5.61 m
3	5.98 d (4.2)	5.87 br s	5.89 br s
6	3.80 dd (3.9, 11.9)	4.01 m	4.00 m
7	1.72 m	1.72 m	1.64 m
			1.76 m
8	1.77 m	1.80 m	1.84 m
10	2.33 m	2.37 m	2.37 m
11	1.26 m	1.50 m	1.64 m
	1.50 m		2.20 dd (8.4, 17.1)
12	2.08 m	2.08 m	5.37 br d (7.8)
14	6.44 dd (10.8, 17.7)	6.42 dd (10.7, 17.8)	6.30 dd (10.9, 17.2)
15	5.02 d (11.0)	5.04 d (10.4)	4.94 d (10.6)
	5.16 d (17.4)	5.21 d (17.9)	5.09 d (17.4)
16	4.93 s	4.91 s	1.65 s
	5.02 s	5.04 s	
17	0.92 d (7.0)	0.92 d (6.9)	0.92 d (6.6)
18	6.72 m	6.70 m	6.70 m
19	6.46 s	6.41 s	6.46 s
20	0.91 s	0.91 s	0.84 s
2'	2.44 m	2.37 m	2.37 m
3'	1.65 m	1.66 m	1.68 m
			1.49 m
4'	0.96 t (7.5)	0.94 t (7.6)	0.92 t (7.4)
5'	1.17 d (6.9)	1.16 d (7.1)	1.16 d (6.9)
Acetyl Me	1.87 s	1.89 s	1.94 s
	2.05 s	2.07 s	2.09 s

[a]Recorded in $CDCl_3$.

2.2.6 Structure Elucidation of Compound E

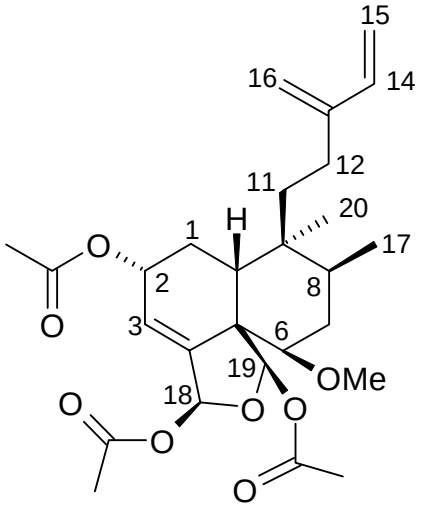
Compound **E** was isolated as a viscous oil. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 513.24579, which corresponded to the molecular formula $C_{27}H_{38}O_8$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

Comparison of the MS, ^{1}H NMR data, and ^{13}C NMR data of compound **E** to *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) (Table 2.4 & Table 2.5) indicated that compound **E** did not contain a 2-methylbutanoyloxy chain, but instead contained an additional acetate unit as well as a methoxy group instead of an alcohol. The ^{1}H NMR spectrum of compound **E** in $CDCl_3$ showed the presence of signals for three methyl groups that were part of acetate units (δ 1.87 s; δ 2.06 s; δ 2.09 s) and a methyl group corresponding to a methoxy group (δ 3.33 s). Based on the chemical shifts of H-2 (δ 5.58 m) and H-6 (δ 3.49 dd, *J* = 4.00 Hz, 12.25 Hz) it appeared that the additional acetate unit was attached to C-2 and the methoxy group was attached to C-6.

The ^{13}C NMR spectrum of compound **E** in $CDCl_3$ was also consistent with the presence of an additional acetate unit as well as the presence of a methoxy group instead of an alcohol as compared to *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) (Table 2.4 & Table 2.5). The ^{13}C NMR spectrum of compound **E** contained signals for three methyl groups (δ 21.3; δ 21.4; δ 21.8) and three carbonyl groups (δ 169.9; δ 170.2; δ 171.0) that were part of acetate units and a methyl group corresponding to a methoxy group (δ 57.6). The ^{13}C NMR data

also supported the attachment of the additional acetate unit to C-2 (δ 71.0) and the methoxy group to C-6 (δ 83.1) based on the chemical shifts of C-2 and C-6.

The data from the ^{1}H NMR and the ^{13}C NMR spectra in $CDCl_3$ when compared to the literature were found to be in close agreement with the data for the known compound casearlucin B[16] (**2.6**) (Table 2.7). Based on the similarity of the experimental and literature data of the ^{1}H NMR and ^{13}C NMR spectra compound **E** was determined to be casearlucin B. The identity of compound **E** was further supported by the MS, optical rotation, UV, and IR data which were in agreement with the literature values for casearlucin B.[16]



2.6 Casearlucin B

Table 2.7 ^{1}H NMR and ^{13}C NMR data for casearlucin B and compound **E**.

	Casearlucin B[16, a]		Compound **E**[a]	
Position	^{13}C NMR data	^{1}H NMR data	^{13}C NMR data	^{1}H NMR data
1	26.7	1.66 m	26.6	1.66 m
		2.14 m		2.15 m
2	71.0	5.58 br t (6.9)	71.0	5.58 m
3	123.6	5.82 br s	123.6	5.82 br s
4	145.4		145.1	
5	53.2		53.1	
6	83.2	3.48 dd (3.9, 12.1)	83.1	3.49 dd (4.0, 12.3)
7	31.4	1.72 m	31.4	1.72 m
8	37.1	1.86 m	37.1	1.87 m
9	41.3		41.2	
10	38.4	2.34 dd (4.6, 13.0)	38.3	2.34 dd (3.0, 14.0)
11	27.6	1.26 m	27.5	1.26 m
		1.46 m		1.46 m
12	23.8	2.08 m	23.8	2.08 m
13	145.9		145.5	
14	140.4	6.42 dd (11.0, 17.6)	140.4	6.42 dd (11.0, 17.6)
15	112.6	5.01 d (10.8)	112.6	5.02 d (10.6)
		5.18 d (17.6)		5.20 d (17.9)
16	115.6	4.90 s	115.6	4.91 s
		5.02 s		5.03 s
17	15.9	0.92 d (6.8)	16.0	0.93 d (6.7)
18	95.8	6.62 t (1.6)	95.8	6.62 t (1.6)
19	98.0	6.27 s	98.0	6.37 s
20	25.7	0.93 s	25.7	0.94 s
Acetyl Me	21.3[b]	1.86 s	21.3[b]	1.87 s
	21.4[b]	2.06 s	21.4[b]	2.06 s
	21.6[b]	2.09 s	21.8[b]	2.09 s
CO	169.8[c]		169.9[c]	
	170.2[c]		170.2[c]	
	170.3[c]		171.0[c]	
OMe	57.6	3.30 s	57.6	3.33 s

[a]Recorded in $CDCl_3$. [b, c] Values with the same superscript are interchangeable within their respective column.

2.2.7 Structure Elucidation of Compound F

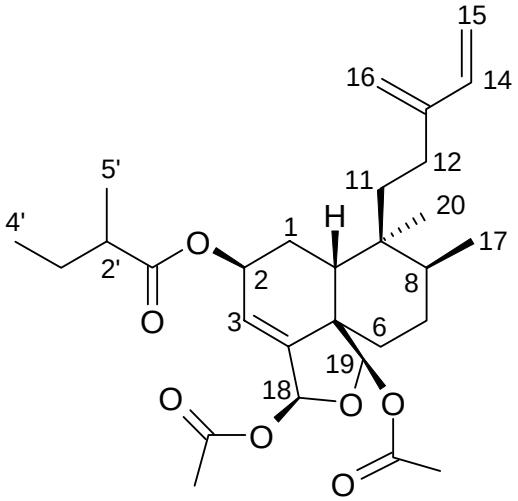
Compound **F** was isolated as a viscous oil. The HRFAB mass spectrum gave a molecular ion formed as a sodium ion adduct of *m/z* 525.28088, which corresponded to the molecular formula $C_{29}H_{42}O_7$ + Na. This molecular formula indicated nine units of unsaturation in the molecule.

Comparison of the MS, ^{1}H NMR, and ^{13}C NMR data of compound **F** to *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) (Table 2.4 & Table 2.5) indicated that compound **F** did not contain an alcohol group. The ^{1}H NMR spectrum of compound **F** in $CDCl_3$ showed the presence of a signal for a saturated methylene (δ 1.53 m; δ 1.72 m) instead of an oxymethine proton (δ 3.80 dd, *J* = 3.90 Hz, 11.90 Hz) as observed in *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**).

The ^{13}C NMR spectrum of compound **F** in $CDCl_3$ was also consistent with the absence of an alcohol group as compared to *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) (Table 2.4 & Table 2.5). The ^{13}C NMR spectrum of compound **F** contained a signal for a saturated methylene (δ 29.4) instead of an oxymethine (δ 73.2) as observed in *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**).

The data from the ^{1}H NMR and the ^{13}C NMR spectra in $CDCl_3$ when compared to the literature were found to be in close agreement with the data for the known compound *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-

3,13(16),14-triene[16, 23] (**2.7**) (Table 2.9). Based on the similarity of the experimental and literature data of the ^{1}H NMR and ^{13}C NMR spectra compound **F** was determined to be *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene. The identity of compound **F** was further supported by the MS, optical rotation, UV, and IR data which were in agreement with the literature values for *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene.[23]



2.7 *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene

Table 2.8 ^{1}H NMR and ^{13}C NMR data for *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene and compound **F**.

	rel-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene[23, a]		Compound **F**[a]	
Position	^{13}C NMR data	^{1}H NMR data	^{13}C NMR data	^{1}H NMR data
1	26.2	1.90 m	26.2	1.90 m
2	66.4	5.34 br s	66.4	5.36 br s
3	120.5	5.87 dd (1.4, 4.6)	120.5	5.87 d (4.1)
4	145.4		145.3	
5	49.4		49.4	
6	29.4	1.53 m	29.4	1.53 m
		1.71 m		1.72 m
7	27.3	1.45 m	27.3	1.46 m
8	37.3	1.60 m	37.3	1.64 m
9	37.3		37.4	
10	34.3	2.20 dd (5.7, 11.9)	34.3	2.21 dd (4.4, 11.5)
11	28.2	1.25 m	28.2	1.25 m
		1.46 m		1.47 m
12	23.7	2.07 m	23.7	2.06 m
13	147.3		147.2	
14	140.5	6.40 dd (10.8, 17.6)	140.5	6.43 dd (10.8, 17.6)
15	112.1	4.99 d (10.8)	112.2	4.97 d (10.8)
		5.15 d (17.6)		5.15 d (17.6)
16	115.4	4.91 s	115.5	4.93 s
		5.01 s		5.02 s
17	15.8	0.85 d (6.7)	15.8	0.86 d (6.8)
18	94.5	6.65 br d (1.4)	94.5	6.67 br d (1.6)
19	99.6	6.27 br s	99.6	6.29 s
20	26.1	0.91 s	26.2	0.91 s
1'	175.9		176.0	
2'	41.3	2.42 m	41.3	2.42 m
3'	27.1	1.66 m	27.1	1.68 m
4'	11.7	0.94 t (7.4)	11.7	0.92 t (7.2)
5'	16.7	1.15 d (7.0)	16.7	1.16 d (6.8)
Acetyl Me	21.2[b]	1.86 s	21.3[b]	1.88 s
	21.3[b]	2.03 s	21.4[b]	2.01 s
CO	169.9[c]		170.1[c]	
	170.2[c]		170.3[c]	

[a]Recorded in $CDCl_3$. [b, c] Values with the same superscript are interchangeable within their respective column.

2.2.8 Biological Evaluation of Isolated Compounds

As previously mentioned extracts from *Casearia* species showed biological activity as anti-cancer, anti-fungal, anti-ulcer, anti-hyperglycemic, analgesic, and anti-inflammatory agents, and were able to neutralize several types of snake venom.[25] The clerodane diterpenes that were isolated from *Casearia nigrescens* were tested for anti-cancer activity in the A2780 assay. The compounds had IC_{50} values ranging from 0.4 to 2.6 μg/mL (Table 2.9). However, there are only slight differences in the structures of these compounds; therefore, the activity of the compound can not be assigned on the basis of the chemical structure.

Table 2.9 IC_{50} values for compounds **A-F** tested in the A2780 assay.

Casearlucin A (compound **A**)	IC_{50} = 2.2 μg/mL
Caseamenbrol A (compound **B)**	IC_{50} = 1.0 μg/mL
rel-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**)	IC_{50} = 2.0 μg/mL
Casearlucin L (compound **D)**	IC_{50} = 0.4 μg/mL
Casearlucin B (compound **E**)	IC_{50} = 2.6 μg/mL
rel-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **F**)	IC_{50} = 1.2 μg/mL

2.3 Experimental Section

2.3.1 General Experimental Procedures

High resolution FABMS for all compounds was obtained using a JEOL HX-110 instrument. 1D NMR and 2D NMR data were obtained on the JEOL Eclipse instrument at 500 MHz for proton and 125 MHz for carbon and on the Varian Inova instrument at 400 MHz for proton and 100 MHz for carbon. The optical rotation was obtained using a Perkin-Elmer 241 polarimeter. A Shimadzu UV-1201 spectrophotometer was used to obtain UV spectra. IR

[25] Chapter II Section 2.1.2 Bioactivity of *Casearia* species extracts.

spectra were obtained using a Midac M-Series FTIR spectrophotometer. Fractions were separated using a Supelco Discovery DSC-18 6 mL tube (1 g) followed by further separation using a Shimadzu LC-10AT Liquid Chromatogram with a Varian Dynamax C-18 column (250 x 10.0 mm) or a Varian Dynamax CN column (250 x 10.0 mm).

2.3.2 Cytotoxic Bioassay

All compounds were tested for cytotoxicity in the A2780 human ovarian cancer cell line assay as discussed previously.[26]

2.3.3 Plant Material

The root and bark of *Casearia nigrescens* Tul. (Flacourtiaceae) were collected by N. M. Andrianjafy and others in Toamasina, Madagascar (Coordinates 17.45.28S 048.45.40E; Elevation 815 m) in November 2001. The extracts were collected from two trees, one 10 m tall with a diameter at breast height (DBH) of 16 cm and the other 12 m tall with a DBH of 16 cm. The collection was assigned collection numbers Andrianjafy 254 and Andrianjafy 257. A root and bark extract were taken from each tree and assigned ICBG numbers MG1147 (root), MG1149 (bark), MG1156 (root), and MG1158 (bark). A voucher specimen was deposited at the Missouri Botanical Garden, St. Louis, MO.

2.3.4 Extract Preparation

The dried plant material was ground and extracted overnight at room temperature with a 1:1 mixture of dichloromethane and methanol. The solvent was removed from the plant material. The plant material was then extracted with methanol for 30 minutes. The 1:1 dichloromethane:methanol and methanol fractions were combines and concentrated on a rotary

[26] Chapter I Section 1.5.3 A2780 assay.

evaporator below 40º C to give a thick concentrate. The concentrate was dried overnight under high vacuum to give the crude extract.

2.3.5 Bioassay Guided Fractionation and Isolation of Compounds

A portion of the crude extract (1.00 g) from *Casearia nigrescens* was subjected to fractionation. First liquid-liquid partitioning was used. The crude extract was dissolved in aqueous methanol (90%), which was extracted with hexane. The aqueous methanol was then diluted (60%) and extracted with dichloromethane. The hexane, dichloromethane, and aqueous methanol fractions were concentrated. The fractions were tested in the A2780 assay and the dichloromethane fraction was found to be most active (IC_{50} 4.0 μg/mL). Further separation of the dichloromethane fraction on a reverse-phase C_{18} open column using a gradient elution of 50% aqueous methanol to 100% methanol resulted in the collection of four fractions. When tested in the A2780 assay the second fraction was most active (IC_{50} 3.2 μg/mL). This active fraction was then subjected to further separation on HPLC using reverse-phase C_{18} with an isocratic elution of aqueous methanol (80%). Eight fractions were collected from the HPLC separation and when tested five fractions were found to be active in the A2780 assay. When the five active fractions were further investigated three were found to be pure compounds: Fraction 2—casearlucin B (compound **E**) [5.4 mg; IC_{50} 2.6 μg/mL], Fraction 5—caseamenbrol A (compound **B)** [9.2 mg; IC_{50} 1.0 μg/mL], and Fraction 8—*rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **F**) [3.0 mg; IC_{50} 1.2 μg/mL]. The other two fractions (Fraction 4—IC_{50} 3.0 μg/mL and Fraction 6—IC_{50} 3.2 μg/mL) required further purification. Fraction 4 was further purified using a normal phase cyano column on HPLC with an isocratic elution of hexane:isopropanol (95:5). This resulted in the collection of four fractions. When tested two fractions were active in the A2780

assay and upon further investigation these two fractions were found to be pure compounds: casearlucin A (compound **A)** [7.6 mg; IC_{50} 2.2 μg/mL] and *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C)** [7.6 mg; IC_{50} 2.0 μg/mL]. Fraction 6 was also further purified using a normal phase cyano column on HPLC with an isocratic elution of hexane:isopropanol (95:5). This resulted in the collection of five fractions, two of which were pure. One of the pure fractions resulted in the additional isolation of novel caseamenbrol A (compound **B)** [0.5 mg; IC_{50} 1.0 μg/mL] and the other pure fraction resulted in the isolation of novel casearlucin L (compound **D)** [0.9 mg; IC_{50} 0.4 μg/mL].

Casearlucin A (compound A) (2.2): viscous oil; $[\alpha]_D^{25.0°}$ +26.3 (*c* 0.08, $CHCl_3$); $[\alpha]_D^{25.0°}$ +32.5 (*c* 0.08, MeOH); [literature[16]: $[\alpha]_D$ +10.6 (*c* 0.52, MeOH); literature[22]: $[\alpha]_D$ +38.7 (*c* 3.2, MeOH)]; UV (MeOH) λ_{max} 223 nm (ε 10250); [literature[16]: UV (MeOH) λ_{max} 226 nm (ε 12400); literature[22]: UV (MeOH) λ_{max} 228 nm (ε 11748)]; IR ν_{max} 3459.0, 2959.6, 1746.8, 1727.6, 1449.6, 1363.4, 1229.1, 965.5 cm^{-1}; [literature[16]: IR ν_{max} 3455, 2960, 1755, 1735, 1645, 1453, 1128, 1065, 753 cm^{-1}; literature[22]: IR (neat) ν_{max} 3449, 1754, 1734, 1373, 1229 cm^{-1}]; ^1H NMR data, see Table 2.1; ^{13}C NMR data, see Table 2.1; HRFABMS *m/z* 541.27765 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_8$ + Na, 541.27786).

Caseamenbrol A (compound B) (2.3): amorphous solid; $[\alpha]_D^{23.5}$ -56.9 (*c* 0.197, $CHCl_3$); [literature[24]: $[\alpha]_D^{25}$ -8.3 (*c* 0.38, MeOH)]; UV (MeOH) λ_{max} 229 nm (ε 10789); [literature[24]: UV (MeOH) λ_{max} 234 nm (shoulder)]; IR ν_{max} 3496.4, 2968.6, 1756.2, 1727.7, 1454.3, 1368.2, 1224.2, 1176.3, 960.5 cm^{-1}; [literature[24]: IR (neat) ν_{max} 3433, 2967, 2934, 1735, 1720, 1645

cm^{-1}]; ^{1}H NMR data, see Table 2.2; ^{13}C NMR data, see Table 2.3; HRFABMS *m/z* 541.27783 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_8$ + Na, 541.27786).

***rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound C) (2.4):** viscous oil; $[\alpha]_D^{25.0}$ +14.0 (*c* 0.129, $CHCl_3$); [literature[23]: $[\alpha]_D$ +13 (*c* 0.24, $CHCl_3$)]; UV (MeOH) λ_{max} 223 nm (ε 7127); IR ν_{max} 3501.4, 2964.5, 2940.6, 1746.8, 1727.6, 1459.0, 1373.0, 1219.5, 1066.2, 999.0 cm^{-1}; [literature[23]: IR ν_{max} (KBr) 3300-3695 broad OH, 3008, 2970, 2938, 2879, 1757, 1726, 1639, 1463, 1374, 1227, 1151, 1082, 1053, 979, 945, 601 cm^{-1}]; ^{1}H NMR data, see Table 2.4; ^{13}C NMR data, see Table 2.5; HRFABMS *m/z* 541.2766 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_8$ + Na, 541.27786).

Casearlucin L (compound D) (2.5): amorphous solid; $[\alpha]_D^{23.5}$ -54.2 (*c* 0.024, $CHCl_3$); UV (MeOH) λ_{max} 223 nm (ε 8464); IR ν_{max} 3463.0, 2945.3, 1746.8, 1727.6, 1454.5, 1373.0, 1224.2, 1109.2, 998.9 cm^{-1}; ^{1}H NMR data, see Table 2.6; HRFABMS *m/z* 541.27386 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_8$ + Na, 541.27786).

Casearlucin B (compound E) (2.6): viscous oil; $[\alpha]_D^{25.0}$ -54.5 (*c* 0.123, $CHCl_3$); $[\alpha]_D^{25.0}$ -25.2 (*c* 0.123, MeOH); [sample[27]: $[\alpha]_D^{26.0}$ -27.0 (*c* 0.063, MeOH)]; UV (MeOH) λ_{max} 223 nm (ε 9700); [literature[16]: UV (MeOH) λ_{max} 232 nm (ε 11310)]; IR ν_{max} 2954.9, 1751.5, 1732.6, 1723.0, 1459.0, 1219.5, 1095.0, 1023.1, 888.8 cm^{-1}; [literature[16]: IR ν_{max} 2956, 1753, 1728, 1723, 1642,

[27] Casearlucin B was previously isolated by another group member. A sample of the compound was obtained in order to measure the optical rotation of casearlucin B as a point of comparison.

1451, 1120, 1065, 748 cm^{-1}]; ^{1}H NMR data, see Table 2.7; ^{13}C NMR data, see Table 2.7; HRFABMS *m/z* 513.24579 $[M+Na]^+$ (calcd for $C_{27}H_{38}O_8$ + Na, 513.24654).

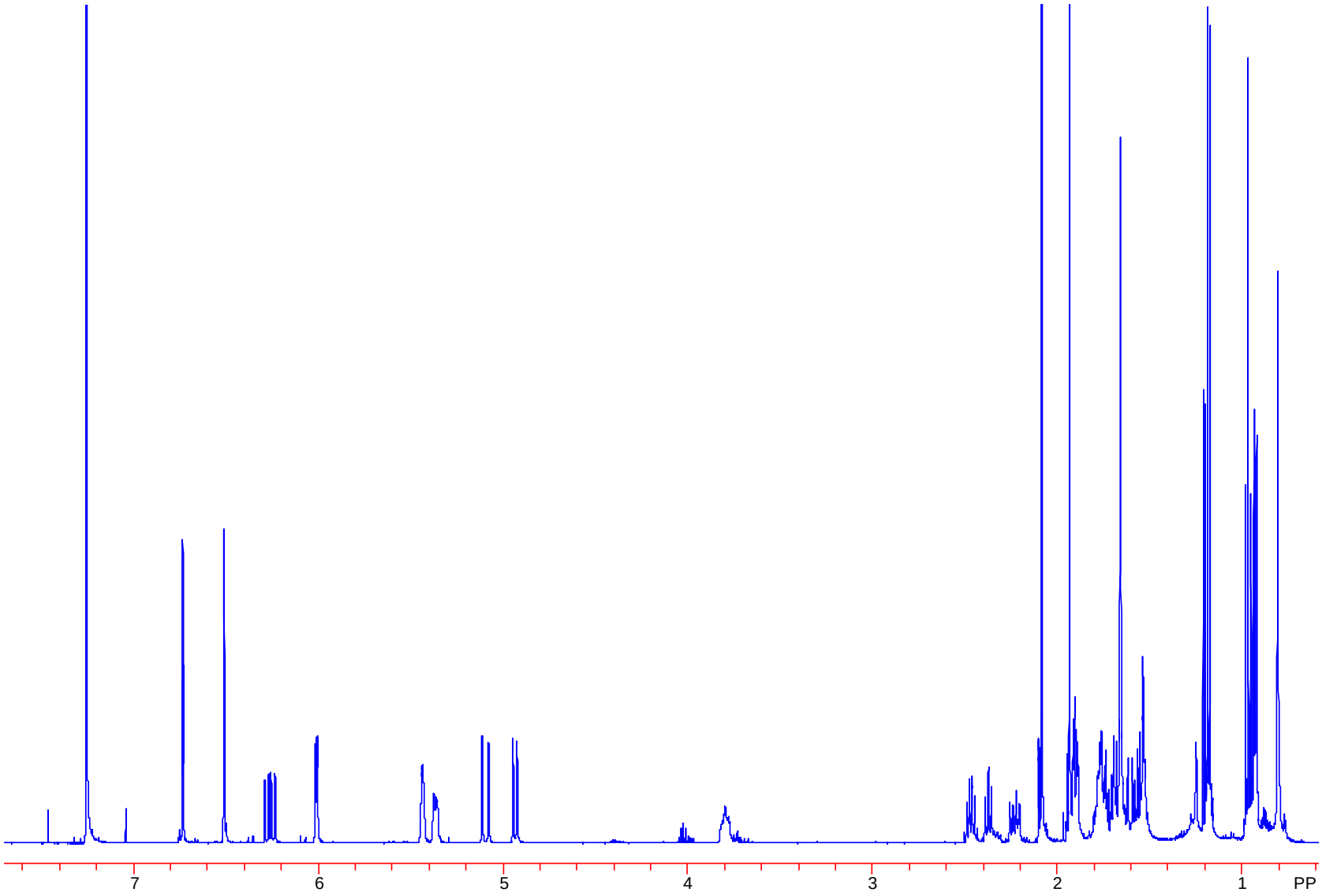
***rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound F) (2.7):** viscous oil; $[\alpha]_D^{25.0}$ -5.2 (*c* 0.058, $CHCl_3$); [literature[23]: $[\alpha]_D$ -6 (*c* 0.14, $CHCl_3$)]; UV (MeOH) λ_{max} 222 nm (ε 8067); IR ν_{max} 2959.6, 2921.4, 2854.2, 1751.5, 1727.6, 1459.2, 1373.0, 1224.4, 1056.6, 941.4, 888.8 cm^{-1}; [literature[23]: IR ν_{max} (KBr) 2968, 2939, 2878, 1757, 1730, 1692, 1618, 1462, 1375, 1229, 1114, 1067, 1005, 958, 933, 888, 601, 406 cm^{-1}]; ^{1}H NMR data, see Table 2.8; ^{13}C NMR data, see Table 2.8; HRFABMS *m/z* 525.28088 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_7$ + Na, 525.28296).

III. Conclusion

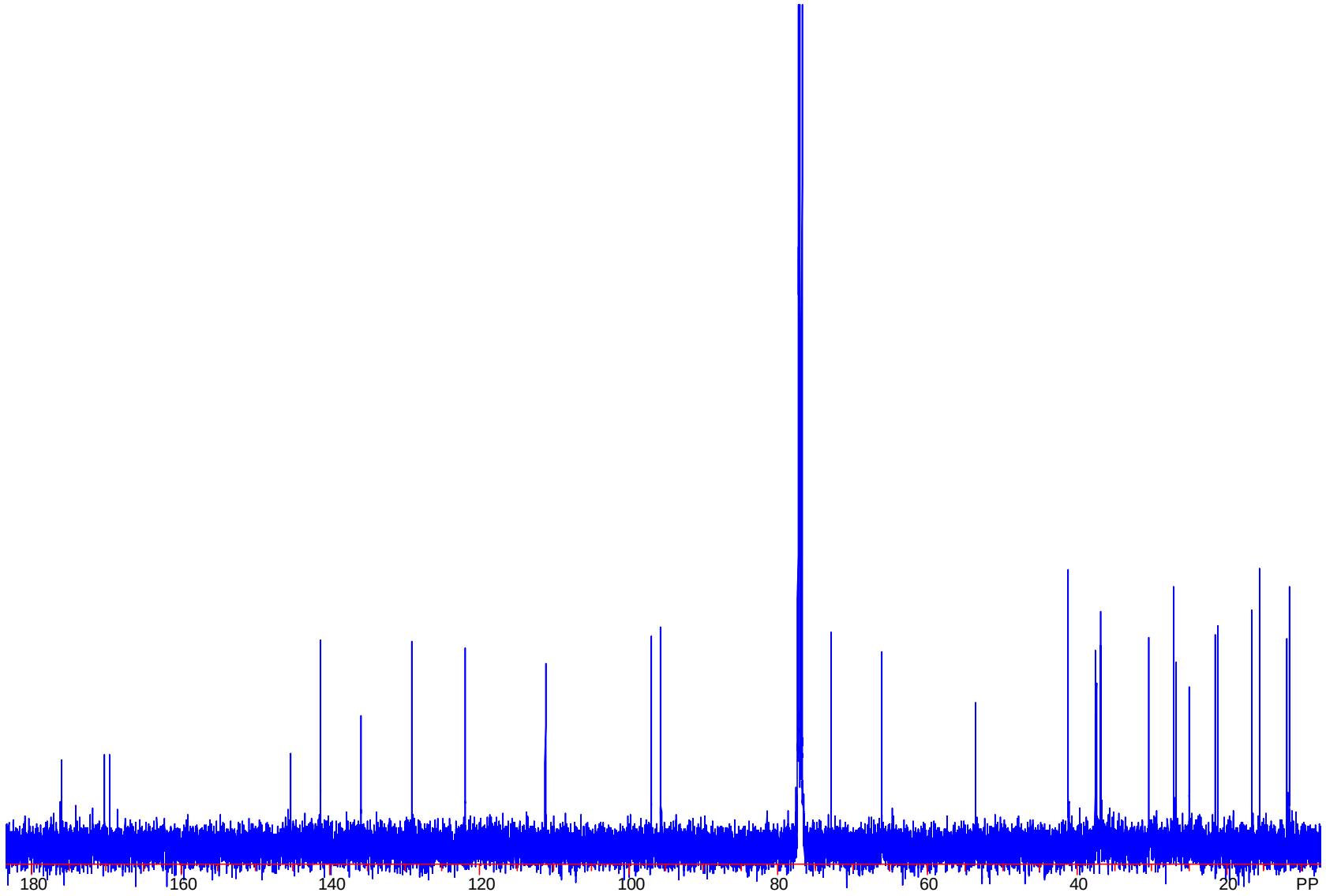
As part of the continuing work of the International Cooperative Biodiverisity Group (ICBG) in search of anti-cancer compounds the plant extract from *Casearia nigrescens* was found to be cytotoxic in the A2780 human ovarian cancer cell line assay. The crude extract of *Caseaia nigrescens* was fractionated and yielded five known compounds (casearlucin A, caseamenbrol A, *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene, casearlucin B, and *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene) and one new compound (Casearlucin L) that were responsible for the cytotoxicity of the extract. The structure of these compounds was elucidated using 1D and 2D NMR techniques.

Appendix

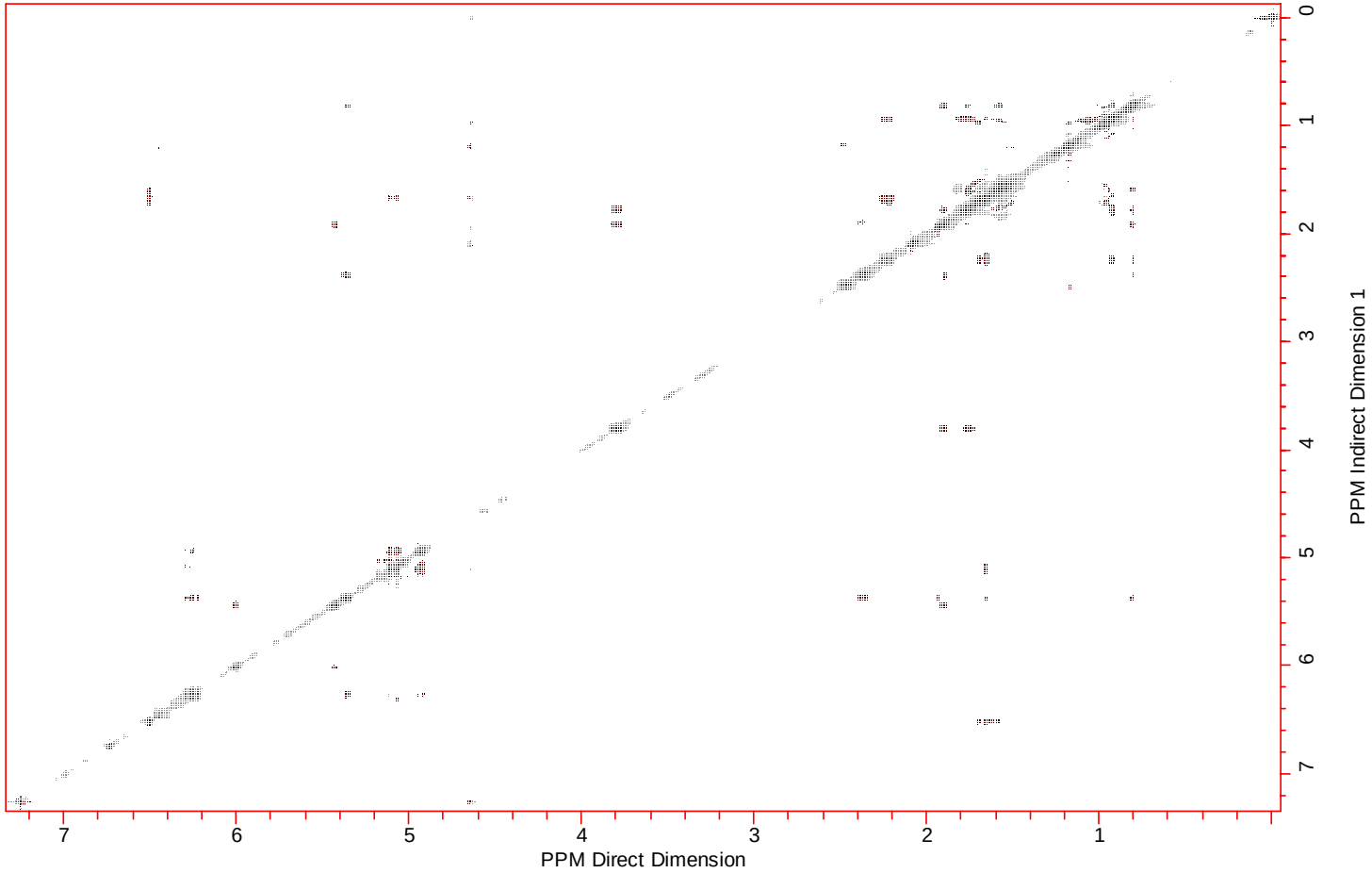
^{1}H NMR spectrum of casearlucin A (compound **A**) in $CDCl_3$.



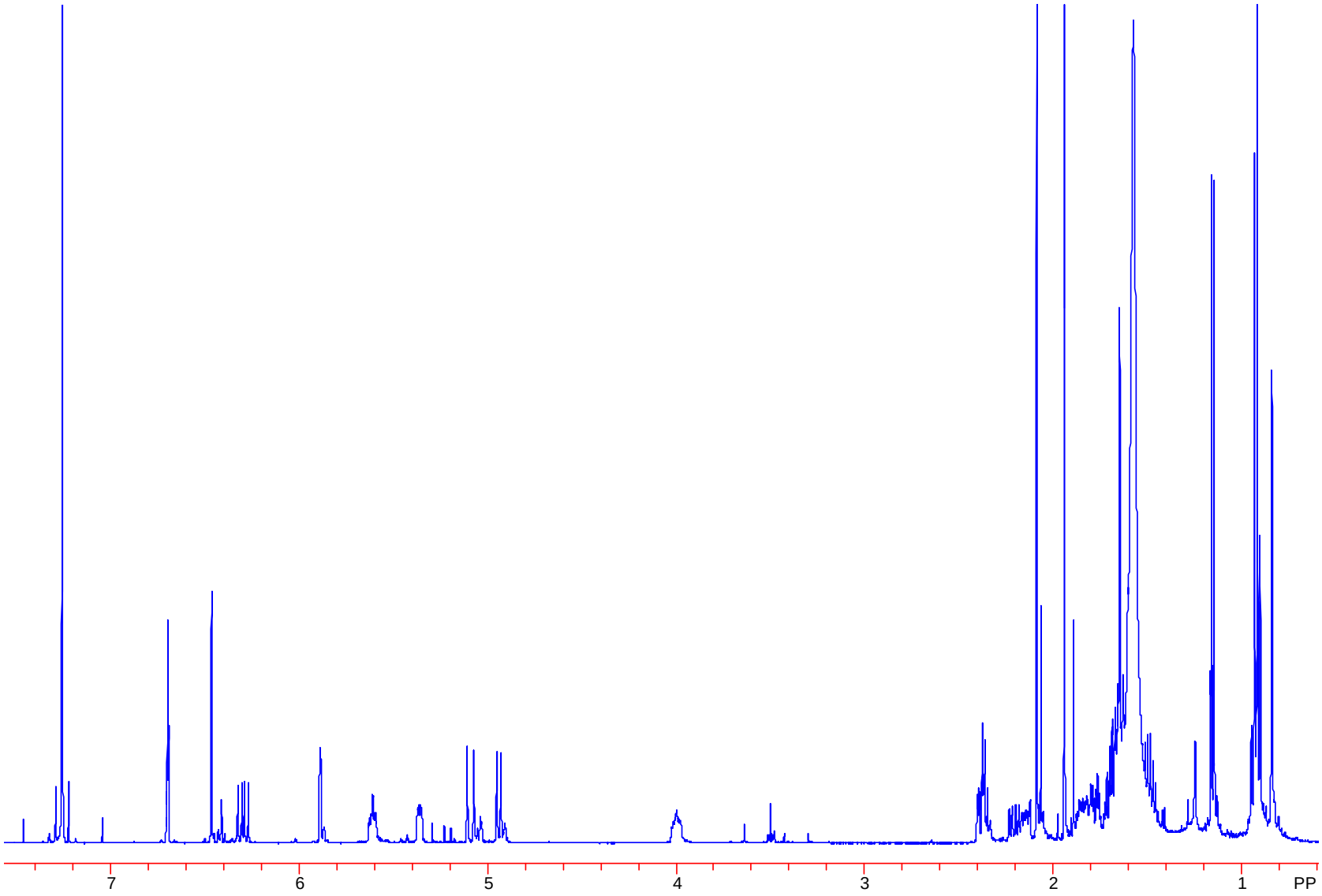
^{13}C NMR spectrum of casearlucin A (compound **A**) in $CDCl_3$.



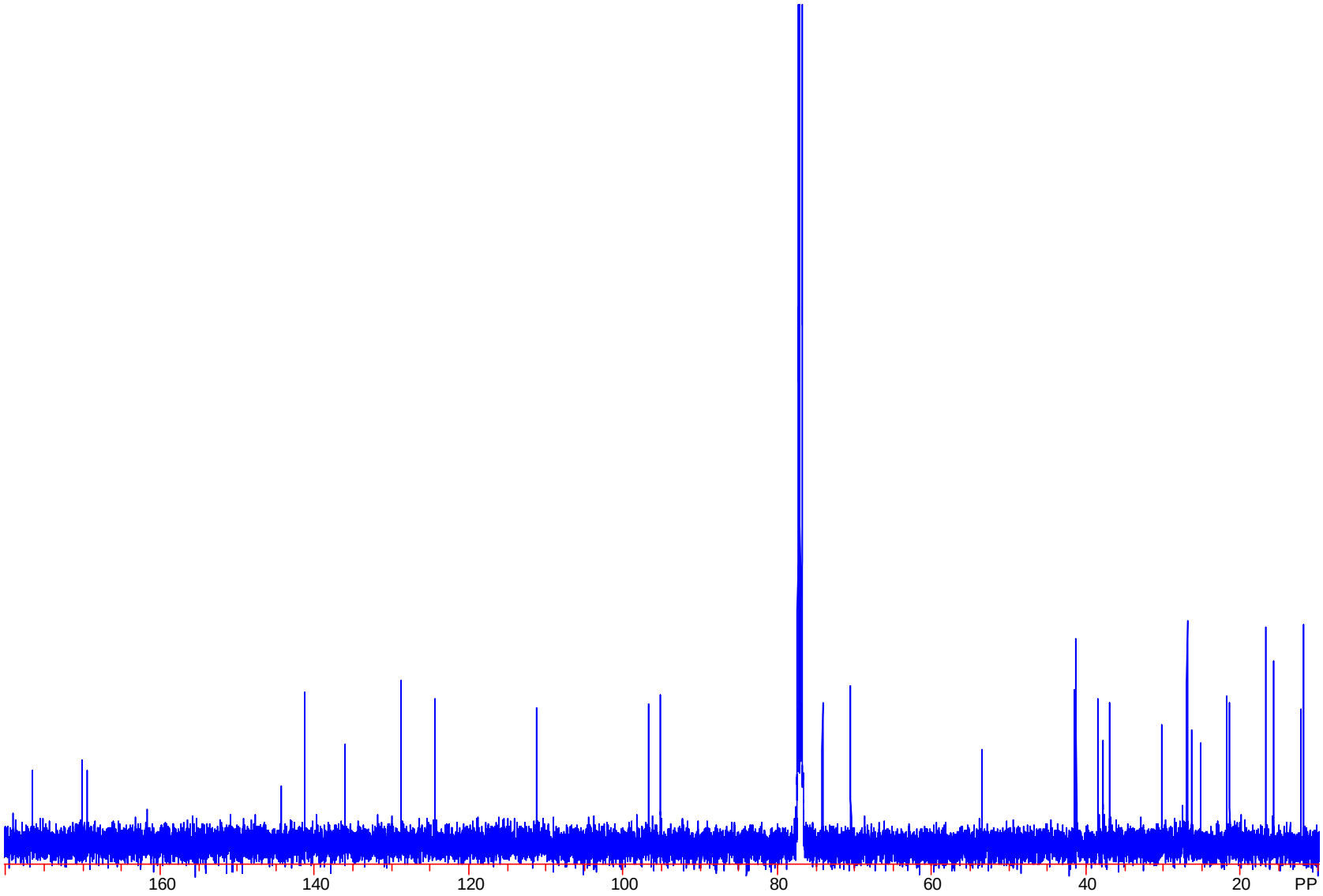
NOESY spectrum of casearlucin A (compound **A**) in $CDCl_3$.



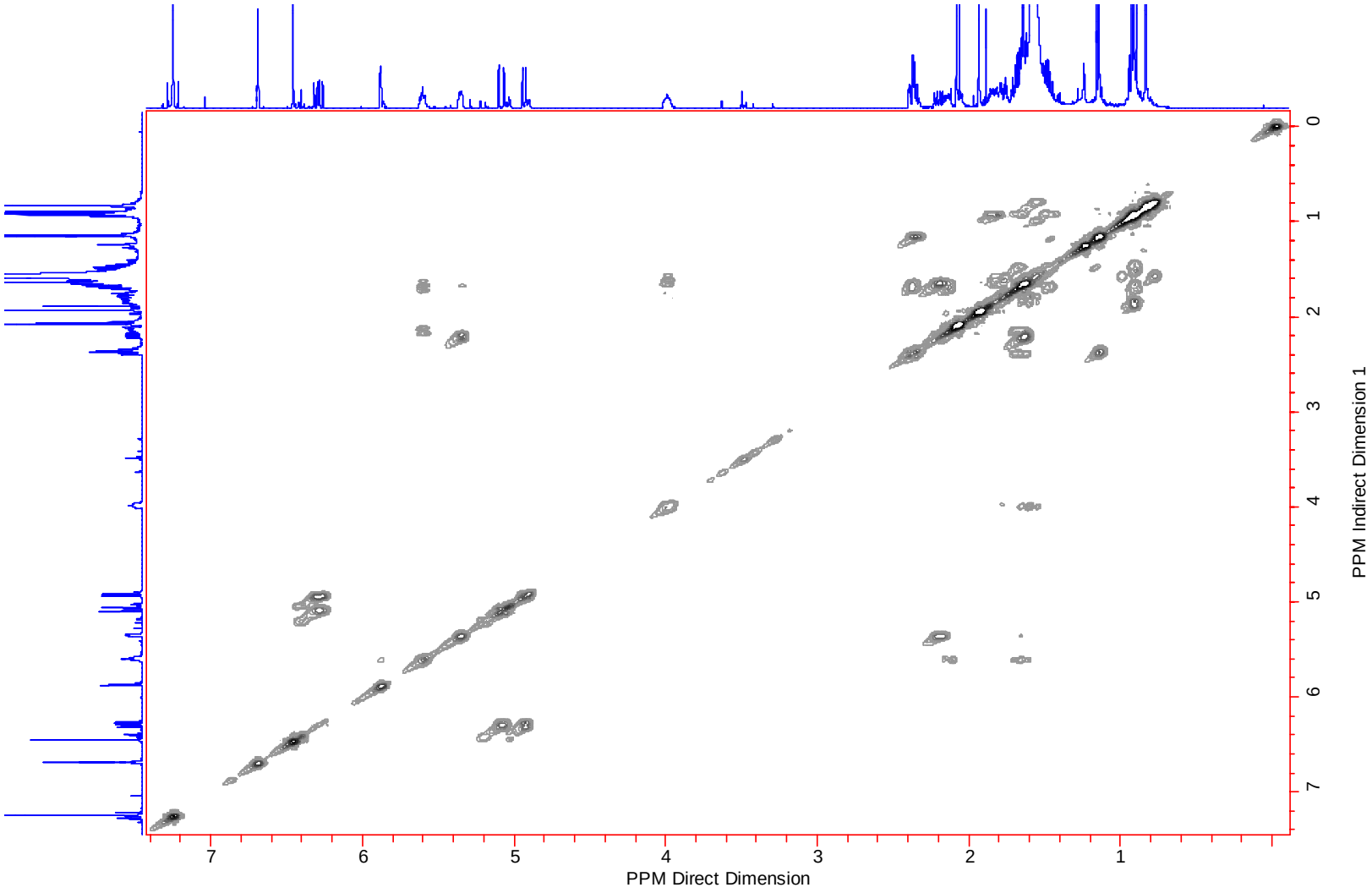
^{1}H NMR spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



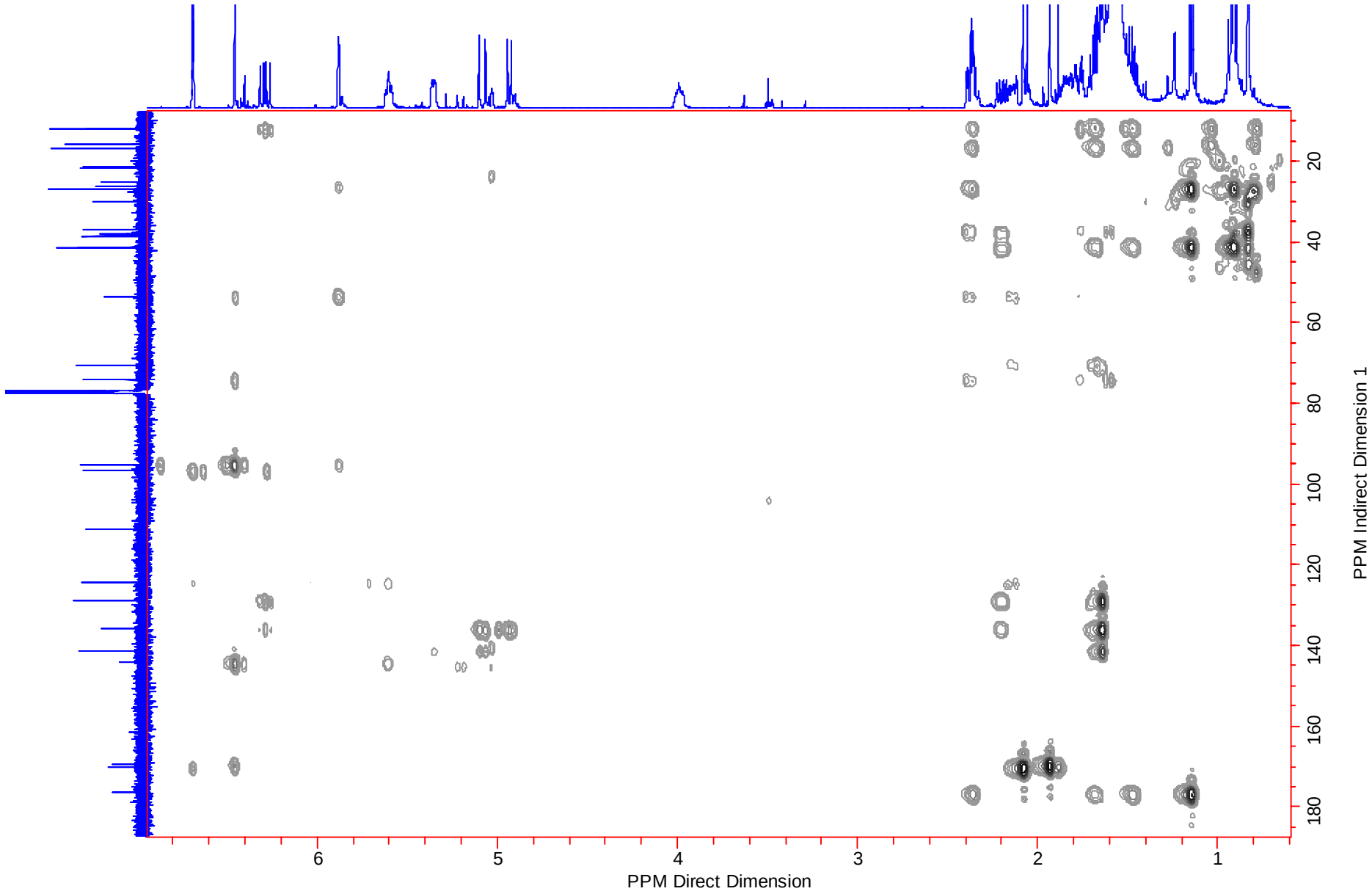
^{13}C NMR spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



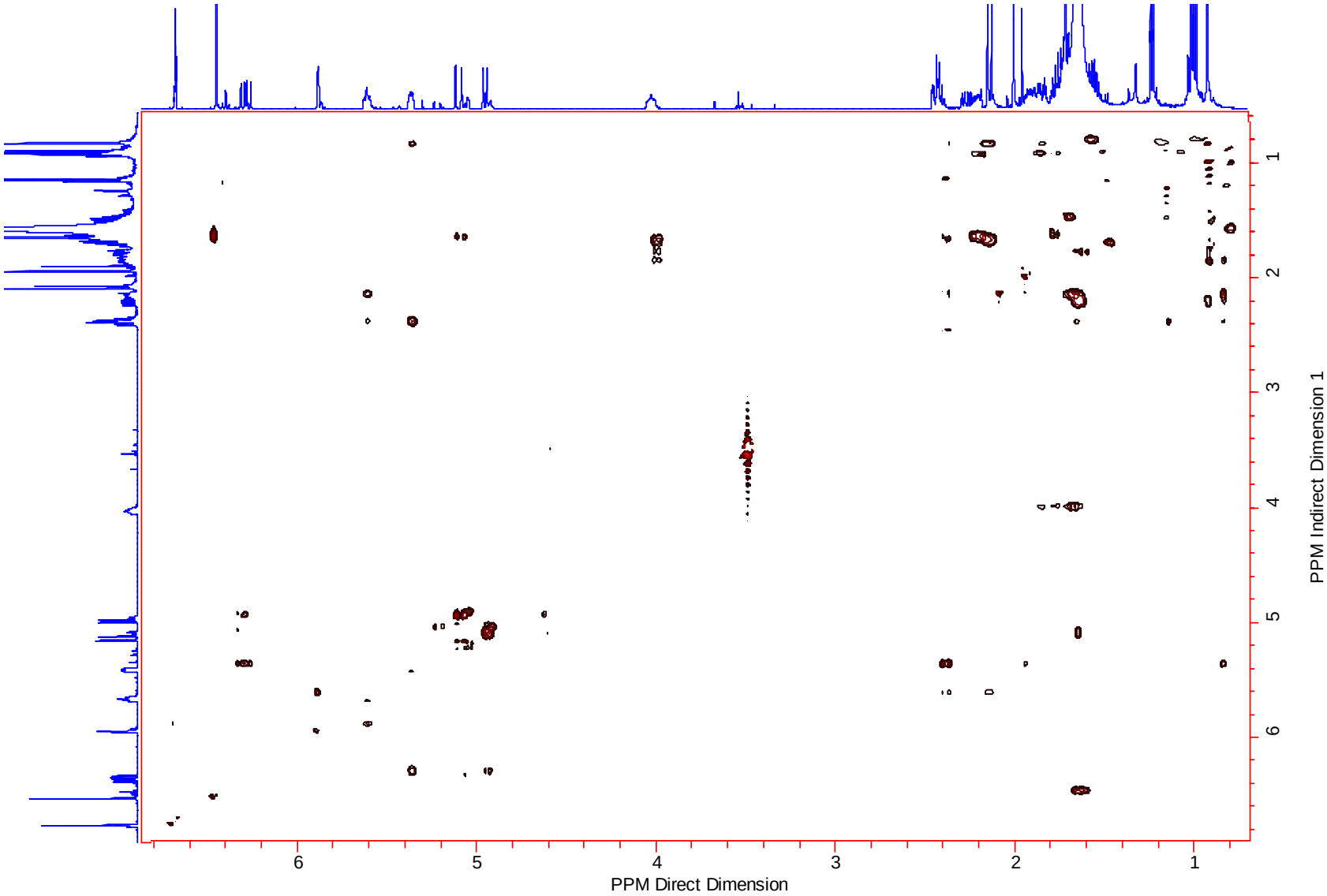
COSY spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



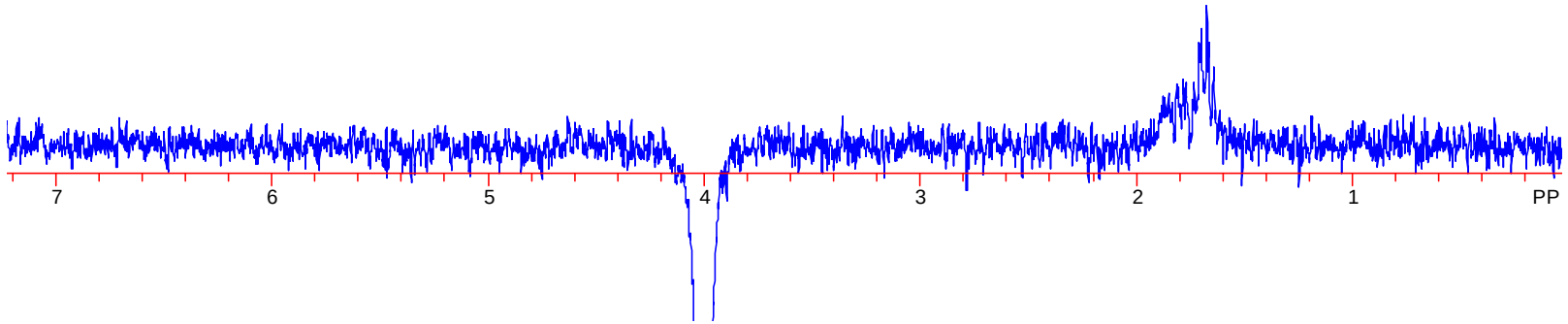
HMBC spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



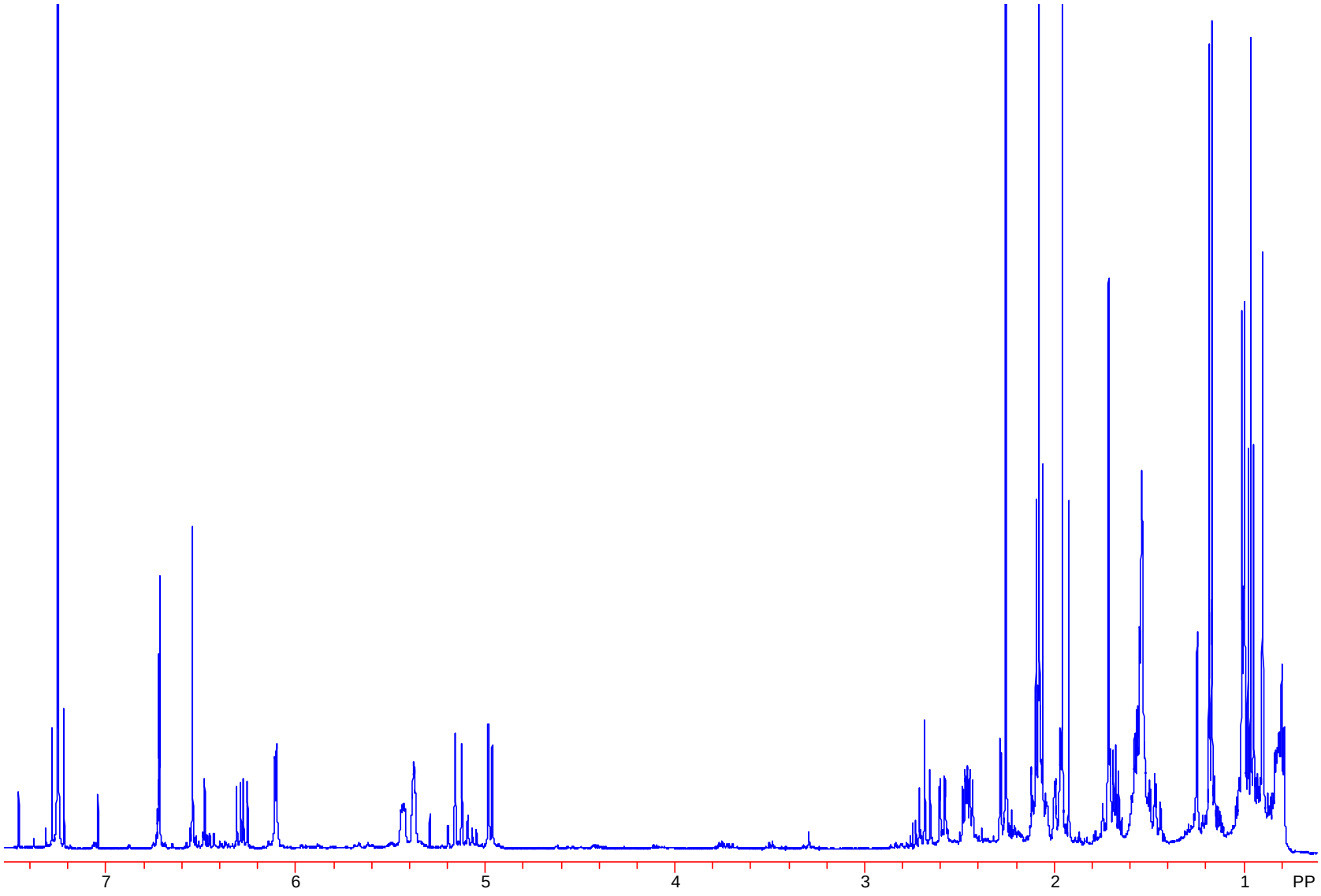
NOESY spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



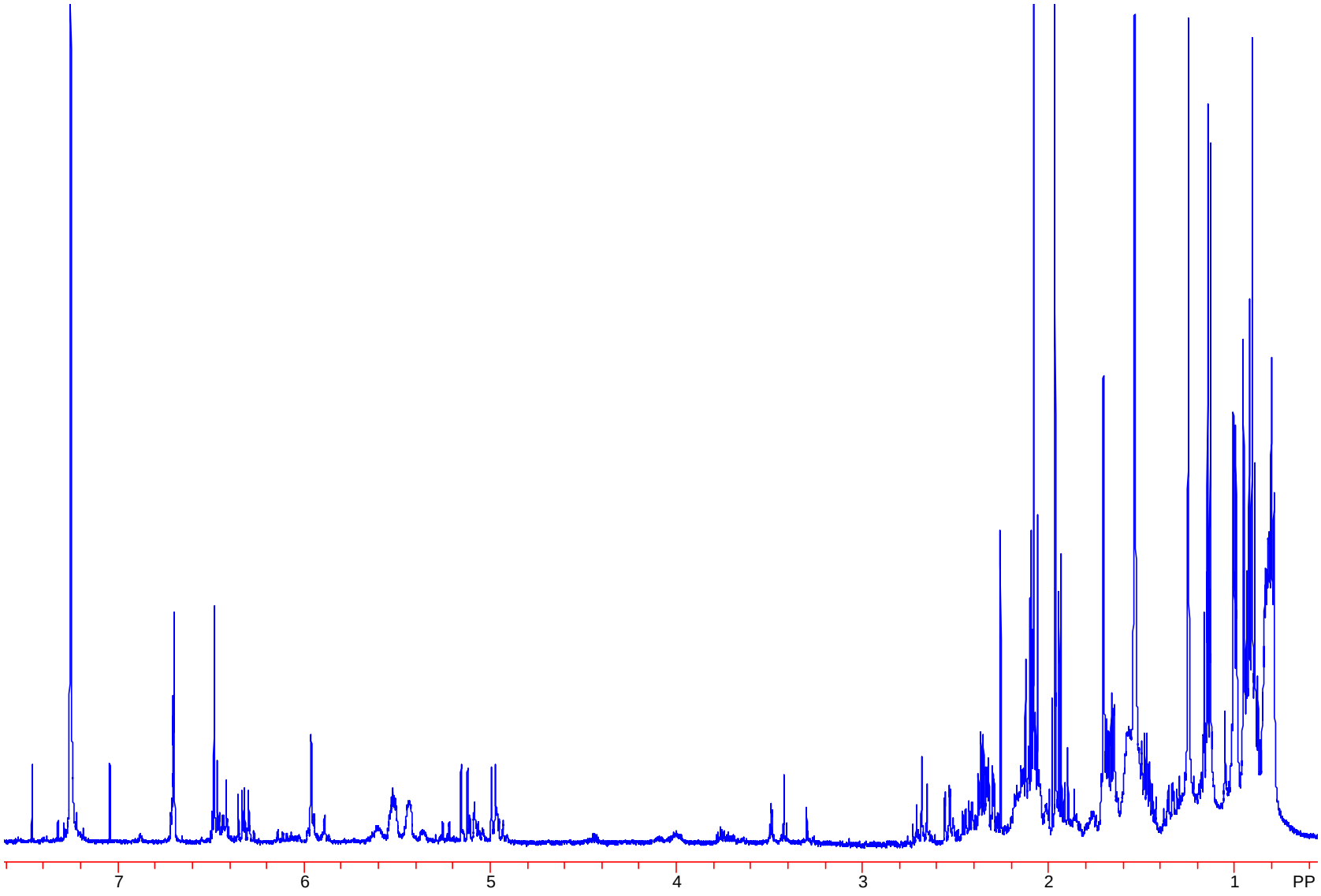
1D NOESY spectrum of caseamenbrol A (compound **B**) in $CDCl_3$.



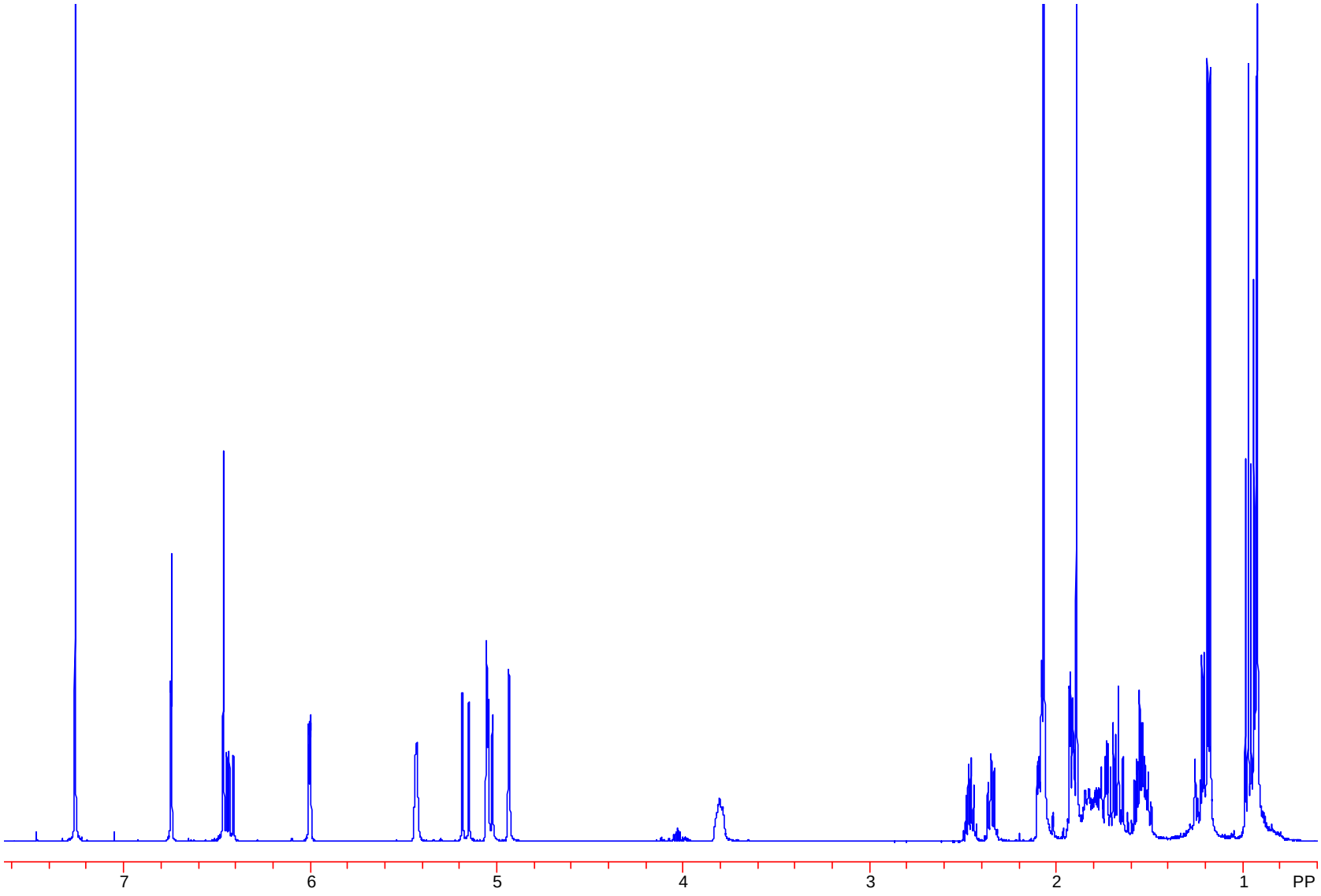
^{1}H NMR spectrum of oxidation product of casearlucin A (compound **A**) in $CDCl_3$.



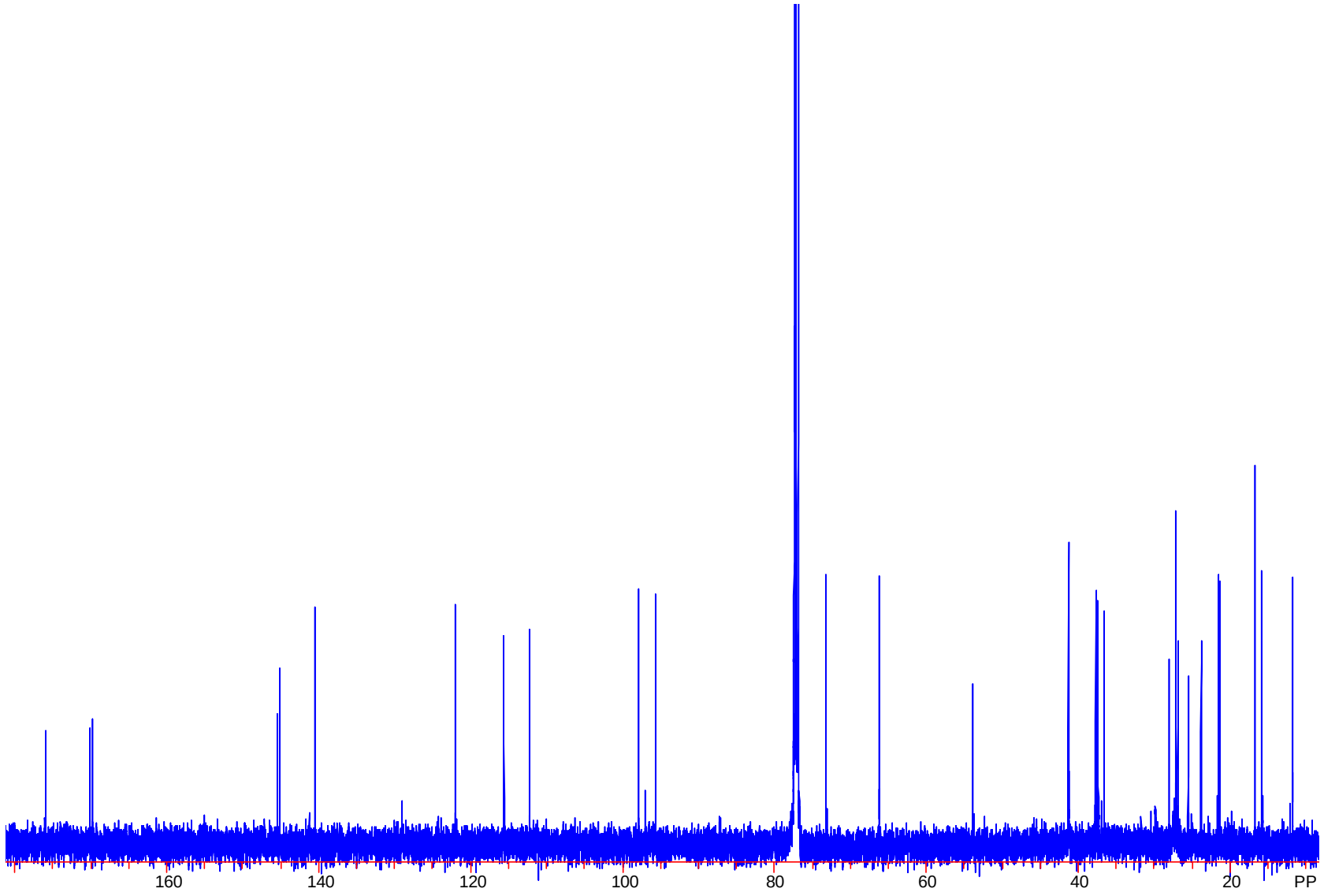
^{1}H NMR spectrum of oxidation product of caseamenbrol A (compound **B**) in $CDCl_3$.



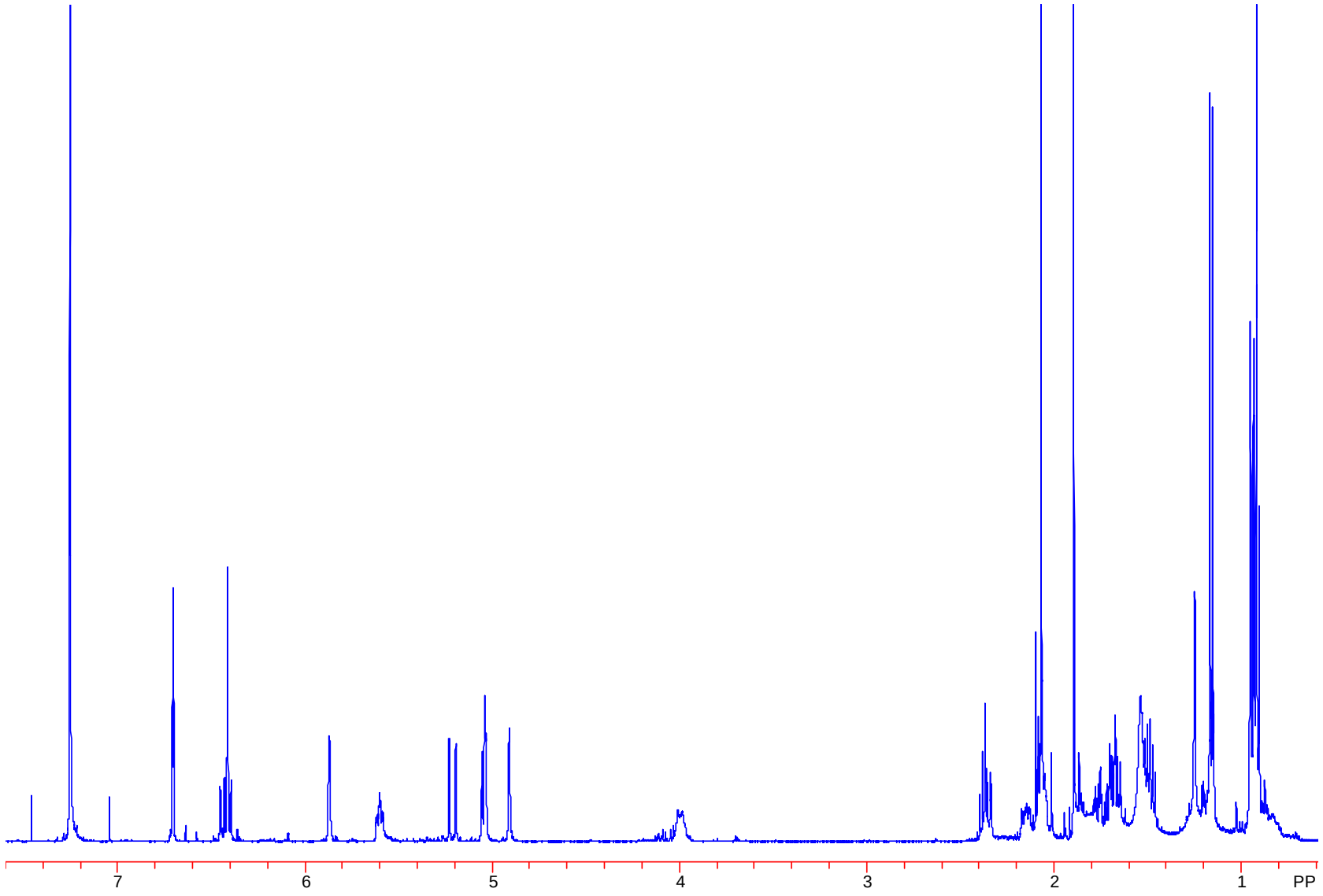
^{1}H NMR spectrum of *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) in $CDCl_3$.



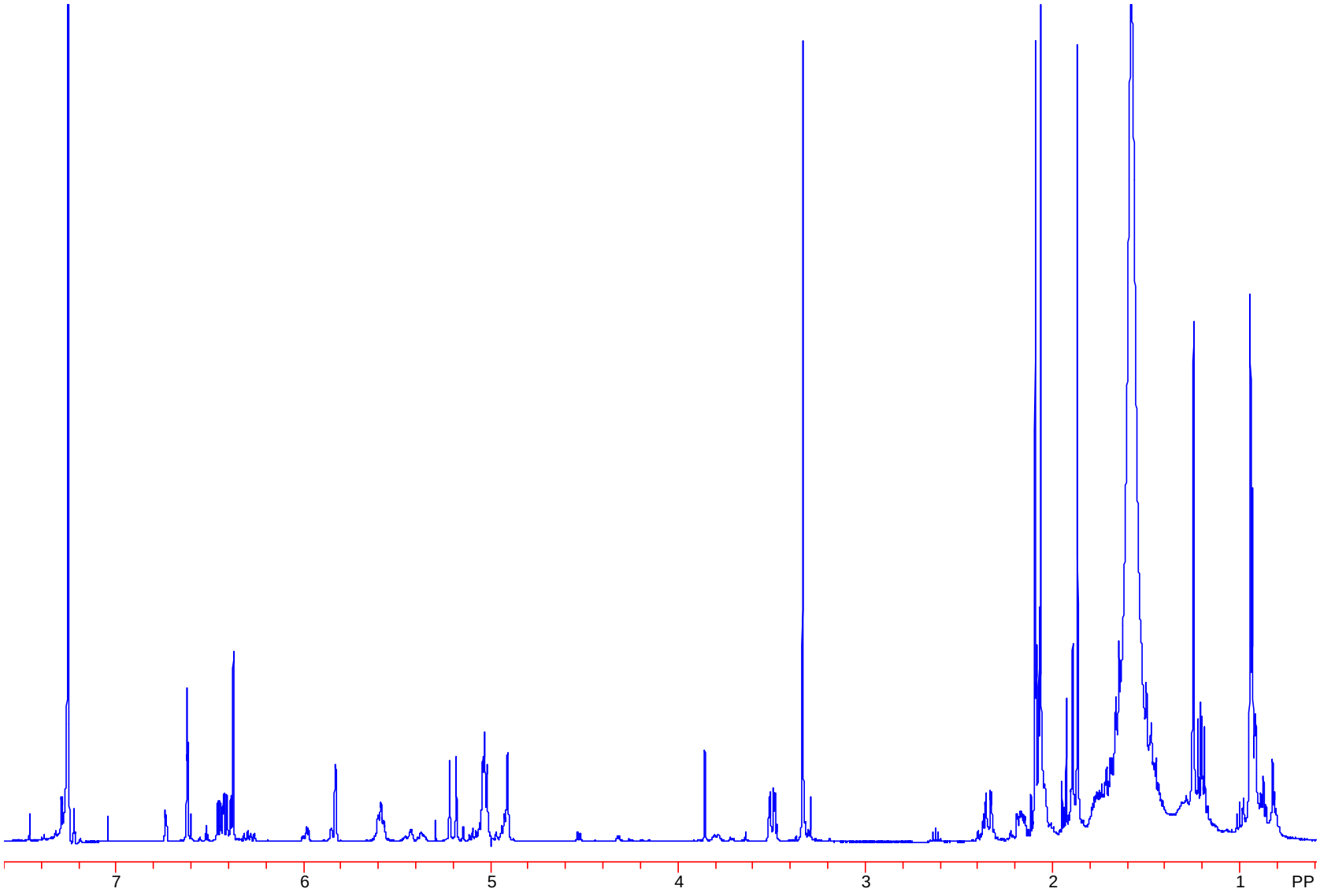
^{13}C NMR spectrum of *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-6(R)-hydroxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **C**) in $CDCl_3$.



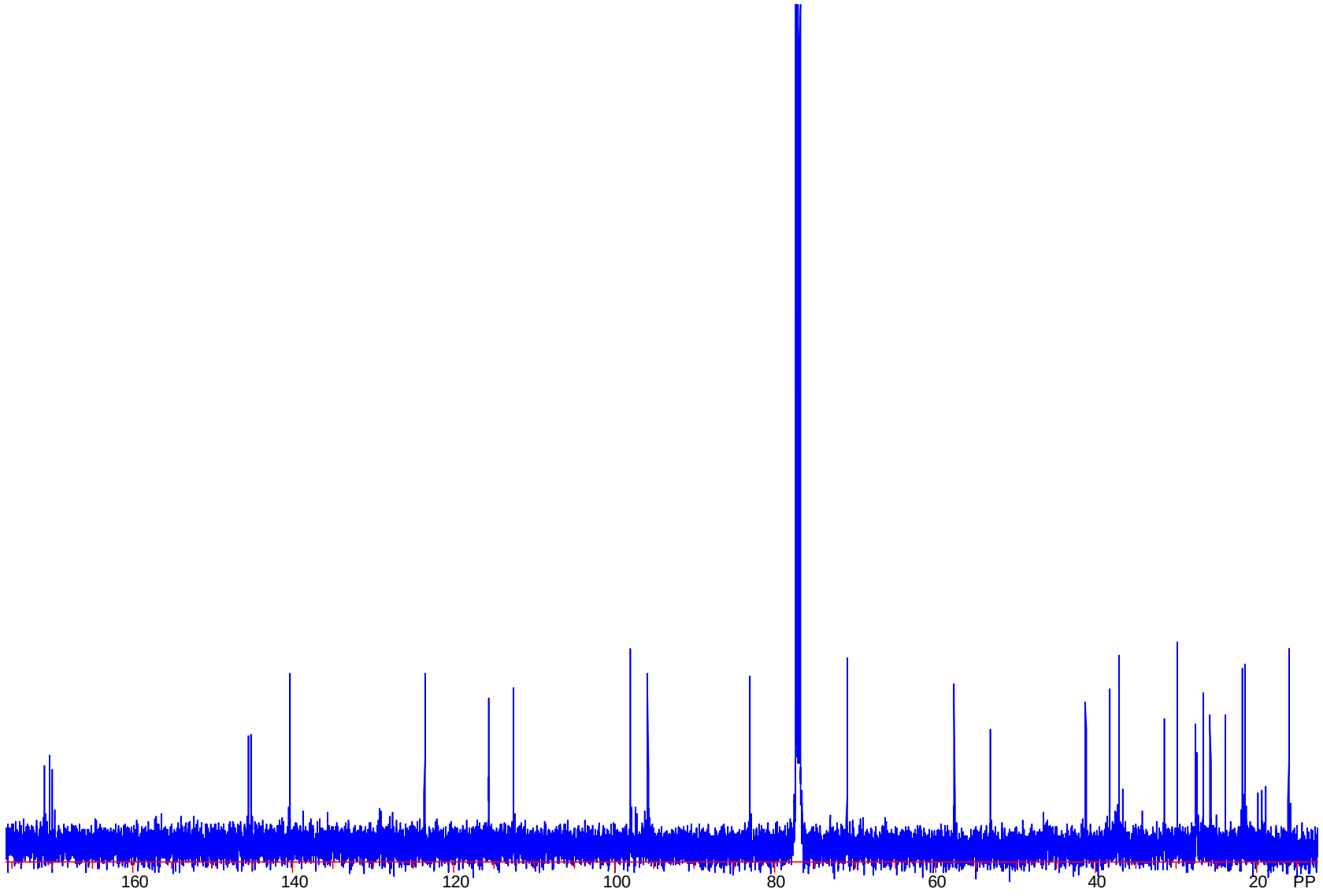
^{1}H NMR spectrum of casearlucin L (compound **D**) in $CDCl_3$.



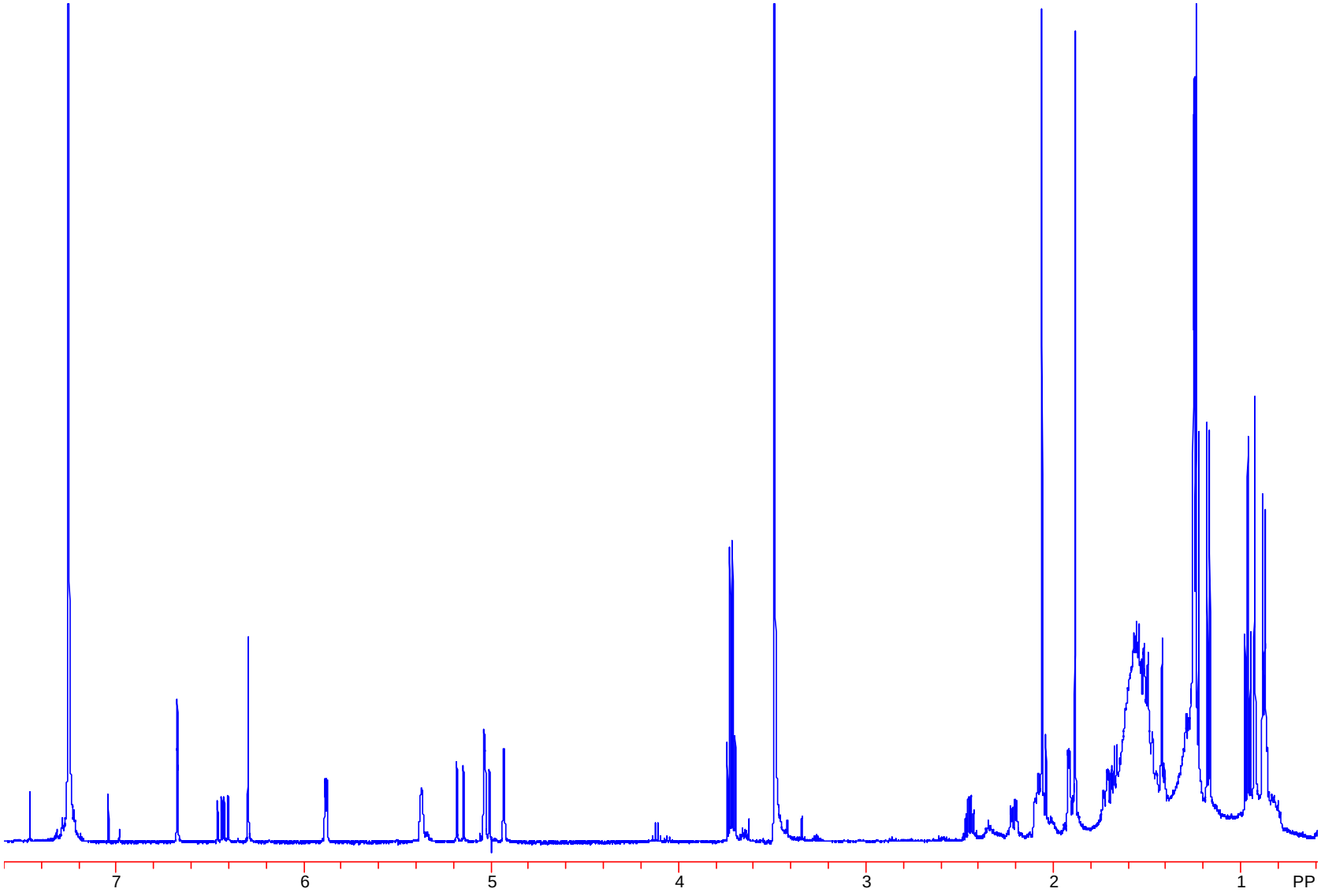
^{1}H NMR spectrum of casearlucin B (compound **E**) in $CDCl_3$.



^{13}C NMR spectrum of casearlucin B (compound **E**) in $CDCl_3$.



^{1}H NMR spectrum of *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **F**) in $CDCl_3$.



^{13}C NMR spectrum of *rel*-18(S),19(R)-diacetoxy-18,19-epoxy-2(S)-(2ξ-methylbutanoyloxy)-5(R),8(S),9(S),10(R)-cleroda-3,13(16),14-triene (compound **F**) in $CDCl_3$.