New Chemical Matter from Extinct Life and for Extant Life: Paleopharmaceuticals and Bioterrorism Countermeasures

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DEDICATION

This dissertation is dedicated to my family, my friends, and my future:

to Steven Lee McDermott and Becky Lynn (McDermott) Raasch,

to Amanda Degner and Katherine Schlasner,

and to my dream of a happy and healthy life.

ABSTRACT

Fossils are a promising source of novel drug scaffolds and new drugs, referred to herein as paleopharmaceuticals. Baltic amber, or fossilized resin produced by extinct pines of the family Sciadopityaceae, has been used in traditional medicine to treat a variety of diseases. To explain the medicinal significance of Baltic amber, we report optimized extraction procedures, specifically single rounds of conservative or Soxhlet extraction with dichloromethane. We also present comprehensive surveys of the chemical compositions of Baltic amber and resin from *Sciadopitys verticillata*, the only extant Sciadopityaceae species. Our analyses using gas chromatography-mass spectrometry showed that Baltic amber and *S. verticillata* resin are primarily composed of terpenes and terpenoids; however, the identities of these compounds are different because of the chemical transformations that occur during fossilization. The only terpenoid identified in both was verticillol, and this is the first known report of verticillol as a constituent of Baltic amber. Finally, we present *in vitro* antibacterial activity data for abietane-type diterpenoids like those identified in Baltic amber. These compounds possess significant antibacterial activity against antibiotic-resistant Gram-positive bacterial strains; therefore, abietane-type diterpenoids are a promising drug scaffold for new paleopharmaceuticals.

Bacillus anthracis, the causative agent of anthrax, has been identified by the Centers for Disease Control and Prevention as a bioterrorism weapon with significant potential to be a severe threat to public health and safety. Current treatments approved by the Food and Drug Administration for anthrax infections do not target the cause of the lethality and a promising target for the discovery and development of effective anthrax therapeutics: the anthrax toxin lethal factor. While progress has been made toward their development, there

are no LF inhibitors currently approved to treat anthrax infections. Recently, we performed an experimental high-throughput screen and identified two compounds with significant LF inhibitory activity. We report the design, synthesis, and evaluation of novel small-molecule LF inhibitors based on these two compounds, increasing their solubility for structural biology studies while maintaining their predicted binding affinities and experimental biological activities.

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CHAPTER 1. Abietane-Type Diterpenoids Extracted from Baltic Amber Exhibit Antibacterial Activity

1.1 Introduction

Resin is a type of plant exudate: a viscous liquid or sticky substance secreted by a variety of florae.[1-3] Other examples of plant exudates include dyes, gums, latexes, oils, and waxes.[3] Resins are complex mixtures that are primarily composed of terpenoids, which are natural products with structures resulting from the polymerization of isoprene,[1,2,4] and are produced by plants as a physical and chemical defense mechanism against phytophagous insects and pathogenic microorganisms.[3-5] As a physical defense mechanism, resin entraps and entombs insects, resulting in asphyxiation; as part of its chemical defense mechanisms, resin has intrinsic antimicrobial activity, resulting in microbial cell growth inhibition and death.[3] Certain plant resins undergo a slow and complex fossilization process to become what is known today as amber.[2] During this process, known as amberization, exposure to oxygen, heat, and light causes oxidation of resin constituents, evaporation of volatile compounds, polymerization of specific diterpenoids via a free-radical mechanism, and hardening of the resin.[1,3,4,6-8] Ultimately, the hardened resin falls from the plant and is transported by water to fluvial or marine environments, where it is gradually buried beneath sediment and preserved for millions of years.[1,3,9] Further chemical transformations such as cyclization, isomerization, and formation of cross-linkages occur during fossilization as a result of increased temperature and pressure within the deposit site.[1,4,7] Amber, also known as resinite, is therefore fossilized plant resin and representative of both organic mineraloids and chemofossils.[2,4,7,9,10]

The largest known deposit of amber on Earth is located in the Baltic Sea region, primarily in Estonia, Latvia, Lithuania, Poland, and parts of Russia.[11,12] Amber from this region, known as Baltic amber, is further characterized by an age of approximately 45 million years;[3,13] a macromolecular structure comprising polymerized communic acid, communol, and succinic acid;[14] a succinic acid content of 3-8% by mass;[2,11,15-18] and a feature referred to as the "Baltic shoulder" in infrared spectra.[19] Baltic amber is also referred to by its mineralogical name of succinite, from the Latin word *succus* ("juice" or "sap") in reference to its resinous origin and from the term *succinum* that was used in ancient Europe to describe all classifications of amber.[8,15,17,20] The resin that became Baltic amber is generally accepted to have been produced by extinct pines of the family Sciadopityaceae.[13,21-24] One species of this family is extant: *Sciadopitys verticillata* (Thunberg) Siebold and Zuccarini, also known as the Japanese umbrella pine because it is an evergreen conifer with cladodes arranged in whorls and indigenous to Japan.[21,22,25-27] *S. verticillata* produces a resin that has been shown to exhibit antibacterial activity as well as the "Baltic shoulder" feature in infrared spectra;[13,21-23] furthermore, inclusions of cladodes characteristic of Sciadopityaceae have been found in samples of Baltic amber.[24]

Historically, Baltic amber has been used in northern European traditional medicine to treat a variety of conditions, including infectious disease.[10,28-30] Other chemofossils used medicinally include fossilized Pycnodontiform fish teeth (lapis chelidonius), echinoid spines (lapis judaicus), belemnites (lapis lincis), mammoth tusks (unicornum verum), Lepidotes/Scheenstia fish teeth (bufonite), shark teeth (glossopetra), and peat (jet).[10] Fossils are essentially a treasure trove of compounds that likely represent unexplored areas of chemical space because of the different metabolic products that may have been produced

by extinct species and the chemical transformations that occur during fossilization; therefore, fossils such as Baltic amber are a promising source of novel drug scaffolds and new drugs, which we refer to as paleopharmaceuticals.

The chemical composition of Baltic amber has been reported in several scientific studies.[31,32] However, to our knowledge, no comprehensive survey of the natural products within Baltic amber has been published, nor have the therapeutic targets of these bioactive small molecules been identified. Toward our objective of explaining the medicinal significance of Baltic amber, we report optimized methods for extracting compounds from Baltic amber and *S. verticillata* resin as well as the identities of their constituents. We also report the results of bacterial cell assays, in which abietane-type diterpenoids like those present in Baltic amber showed significant antibacterial activity against antibiotic-resistant Gram-positive bacterial strains.

1.2 Experimental

1.2.1 General Experimental Procedures

The US Stoneware Norton Table Top Jar Rolling Mill used during Baltic amber preparation was provided by the University of Minnesota Institute for Rock Magnetism. The Agilent 7200 Q-TOF GC/MS used during extract characterization was provided by the University of Minnesota Department of Chemistry Mass Spectrometry Laboratory. Abietic acid, dehydroabietic acid, and palustric acid were purchased from Toronto Research Chemicals and used without additional purification.

1.2.2 Experimental Biological Materials

Baltic amber was acquired from Lithuania in June 2017. An *S. verticillata* specimen was acquired from Avant Gardens in May 2019.

1.2.3 Baltic Amber Preparation

Baltic amber was prepared for extraction using a US Stoneware Norton Table Top Jar Rolling Mill. To a grinding jar (size 000) was added Baltic amber (25 g) and 25 grinding beads. The jar was sealed and rolled on its side for 3 h, and the resulting semi-fine powder was collected for extraction.

1.2.4 Baltic Amber Conservative Extraction

To a ground-glass Erlenmeyer flask was added ground Baltic amber (150 mg) and either dichloromethane or ethanol (60 mL). The flask was sealed with a stopper and Parafilm, and the mixture was allowed to sit for 24 h under ambient conditions. The mixture was then filtered using vacuum filtration, and the resulting extract was concentrated under reduced pressure to a volume of 0.5 mL. For the multiple-round extracts, the remaining amber was massed, and the extraction procedure was repeated with the remaining amber until the difference between the mass of the amber pre- and post-extraction was $\leq$ 1 mg. All rounds of extraction were combined and concentrated under reduced pressure to a volume of 0.5 mL.

1.2.5 Baltic Amber Decoction Extraction

To a round-bottom flask equipped with a stir bar was added ground Baltic amber (150 mg) and either dichloromethane or ethanol (60 mL). The flask was fitted with a condenser, and the mixture was heated to reflux and stirred for 3 h. The mixture was then filtered using vacuum filtration, and the resulting extract was concentrated under reduced pressure to a volume of 0.5 mL.

1.2.6 Baltic Amber Sonication Extraction

To a round-bottom flask was added ground Baltic amber (150 mg) and either dichloromethane or ethanol (60 mL). The flask was fitted with a condenser, and the mixture was sonicated for 3 h under ambient conditions. The mixture was then filtered using vacuum filtration, and the resulting extract was concentrated under reduced pressure to a volume of 0.5 mL.

1.2.7 Baltic Amber Soxhlet Extraction

To a Soxhlet apparatus was added ground Baltic amber (150 mg) and either dichloromethane or ethanol (60 mL). The solvent was heated to reflux, and the extraction was run for 3 h. The resulting extract was then concentrated under reduced pressure to a volume of 0.5 mL.

1.2.8 *Sciadopitys verticillata* Conservative Extraction

To a ground-glass Erlenmeyer flask was added either a freshly picked bundle of 10 cladodes or a freshly cut section of stem from *S. verticillata* and dichloromethane (60 mL). The flask was sealed with a stopper and Parafilm, and the mixture was allowed to sit for 24 h under ambient conditions. The mixture was then filtered using vacuum filtration, and the resulting extract was concentrated under reduced pressure to a volume of 0.5 mL.

1.2.9 *Sciadopitys verticillata* Soxhlet Extraction

To a Soxhlet apparatus was added either a freshly picked bundle of 10 cladodes or a freshly cut section of stem from *S. verticillata* and dichloromethane (60 mL). The solvent was heated to reflux, and the extraction was run for 3 h. The resulting extract was then concentrated under reduced pressure to a volume of 0.5 mL.

1.2.10 Extract Characterization

All crude extracts were characterized using an Agilent 7200 Q-TOF GC/MS equipped with a DB-5 column and EI ion source and were injected into the instrument at a volume of 1 μL without additional purification. Extracts of Baltic amber resulting from one round of extraction were run using the following temperature program: 50 °C for 2 min, 5 °C/min increase for 54 min, 320 °C for 5 min. Extracts of Baltic amber resulting from multiple rounds of conservative extraction and all extracts of *S. verticillata* resin were run using the following temperature program: 50 °C for 1 min, 10 °C/min increase for 9 min, 20 °C/min increase for 3 min, 10 °C/min increase for 3 min, 5 °C/min increase for 8 min, 10 °C/min increase for 3 min, 20 °C/min increase for 1 min, 320 °C for 2 min. Data analysis was conducted using the Qualitative Analysis Program (Version B.07.00) within the Agilent MassHunter Workstation Software, and compound identification was performed using the installed National Institute of Standards and Technology Mass Spectral Search Program (Version 2.0).

1.2.11 Antibacterial Activity Evaluation

Antibacterial activity was evaluated using minimum inhibitory concentration (MIC) assays conducted by Pharmacology Discovery Services. Abietic acid, dehydroabietic acid, and palustric acid were each dissolved in DMSO to a concentration of 6.4 mg/mL, and a two-fold serial dilution was performed, resulting in ten additional solutions of decreasing concentration for each compound. 4 μL of each solution was then added to 196 μL of broth medium seeded with either *Escherichia coli* (ATCC 10536), ciprofloxacin-resistant *Escherichia coli* (CDC AR Bank #0067), *Klebsiella pneumoniae* (ATCC 10031), carbapenem-resistant *Pseudomonas aeruginosa* (CDC AR Bank #0095), ciprofloxacin-

resistant *Pseudomonas aeruginosa* (CDC AR Bank #0251), imipenem-resistant *Pseudomonas aeruginosa* (CDC AR Bank #0237), meropenem-resistant *Pseudomonas aeruginosa* (CDC AR Bank #0252), methicillin-resistant *Staphylococcus aureus* (NRS 71), or multidrug-resistant *Streptococcus pneumoniae* (ATCC 51916) in a 96-well plate. The final compound concentrations ranged from 0.125 μg/mL to 128 μg/mL, and the final solvent concentration was 2% DMSO. Following incubation for 24 h at 36 °C, the plate was visually analyzed, each well was scored based on growth, and MIC values were reported. Each compound was evaluated in duplicate; 0.0078 μg/mL ciprofloxacin was used as the positive control for *E. coli*; 0.25 μg/mL tigecycline was used as the positive control for ciprofloxacin-resistant *E. coli*; 0.5 μg/mL amikacin was used as the positive control for *K. pneumoniae*, carbapenem-resistant *P. aeruginosa*, and meropenem-resistant *P. aeruginosa*; 0.5 μg/mL colistin was used as the positive control for ciprofloxacin-resistant *P. aeruginosa*; 1 μg/mL amikacin was used as the positive control for imipenem-resistant *P. aeruginosa*; 0.25 μg/mL vancomycin was used as the positive control for methicillin-resistant *S. aureus* and multidrug-resistant *S. pneumoniae*; and DMSO was used as the negative control for all bacterial strains.

1.3 Results and Discussion

1.3.1 GC-MS Analysis of Single-Round Baltic Amber Extracts

Toward the determination of optimal extraction conditions, ground Baltic amber of western Lithuanian provenience was extracted using all possible combinations of four extraction techniques (conservative, decoction, sonication, and Soxhlet) and two solvents (dichloromethane and ethanol). Extraction procedures were adapted from previous work,[33] which served as a starting point for the optimization of extraction conditions. Following

one round of extraction, the resulting crude extracts were filtered, concentrated, and analyzed via gas chromatography-mass spectrometry (GC-MS), which has proven successful as an analytical technique in the characterization of amber and the identification of plant resin constituents.[13,14,31-44] The aggregate mass spectrum for each peak in the gas chromatogram was then compared to mass spectra in a National Institute of Standards and Technology standard reference database to determine the most probable identities for the compounds present in each extract.[45]

GC-MS analysis resulted in a comprehensive survey of the chemical composition of Baltic amber as 94 unique compounds were identified in the eight extracts tested. **Figures 1.1-1.8** present gas chromatograms of the single-round Baltic amber extracts. The compounds identified in the single-round Baltic amber extracts as well as their acquisition times, match factors, reverse match factors, and probability values are provided in **Tables 1.1-1.8**. Match factor quantifies the comparison of the observed mass spectrum to the database mass spectrum, and reverse match factor quantifies the comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match. Probability quantifies the comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the standard reference database search output, and the compounds that returned the highest probability values within each search output are reported.[45]

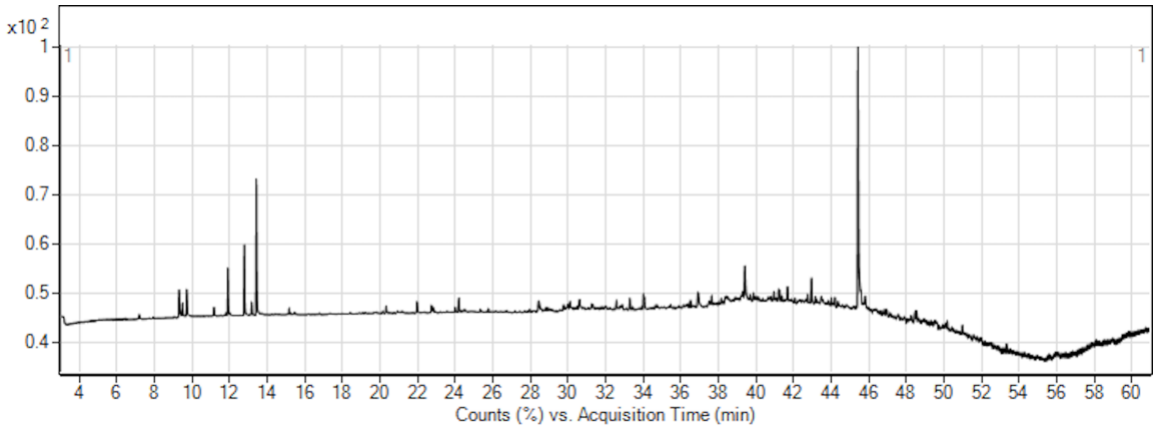


Figure 1.1. Gas chromatogram of the single-round Baltic amber extract produced via conservative extraction with dichloromethane.

Table 1.1. Compounds identified in the single-round Baltic amber extract produced via conservative extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
7.148-7.216	805	858	19.8	Camphene
9.277-9.378	894	897	27.2	*m*-Cymene
9.455-9.536	807	837	52.5	Eucalyptol
9.668-9.779	877	880	27.8	*o*-Cymene
11.126-11.193	772	777	34.1	Fenchone
11.850-11.941	820	823	27.5	Fenchol
12.738-12.825	801	802	30.4	Camphor
13.115-13.193	791	805	28.9	Borneol
13.368-13.517	827	827	44.3	Borneol
15.125-15.193	771	810	30.1	Isobornyl formate
20.290-20.402	709	759	12.3	Isolongifolol
21.923-22.000	840	907	33.9	α-Ionene
22.705-22.759	886	910	70.1	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
22.807-22.874	768	849	24.4	Calamenene
23.947-24.011	728	773	15.5	Caryophyllenol
24.153-24.230	760	777	24.4	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
28.396-28.497	810	848	58.1	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene

29.718-29.806	538	562	4.81	2-benzyl-7-isopropyl-10-methyl-1,5-dithia-Spiro[5.5]undecane oxide
29.938-30.025	630	657	9.32	Heptatriacontanol
30.545-30.680	643	694	4.18	3-ethyl-3-hydroxy-Androstan-17-one
31.223-31.290	688	749	10.6	Sclarene
32.545-32.599	774	796	56.6	18-Norabieta-8,11,13-triene
33.237-33.314	750	795	51.8	18-Norabieta-8,11,13-triene
33.972-34.083	770	784	79.4	Abieta-8,11,13-triene
36.468-36.556	669	684	18.4	3-ethyl-5-(2-ethylbutyl)-Octadecane
36.856-37.005	656	704	6.74	5-hydroxymethyl-5,8a-dimethyl-y,2-bis(methylene)-decahydro-(1α,4aβ,5α,8aα)-Naphthalenepentan-1-ol
37.443-37.544	658	706	18.9	3,5-dehydro-6-methoxy-Cholest-22-en-21-ol pivalate
37.615-37.669	690	863	5.88	Eicosanol
38.121-38.189	603	613	9.40	Ethyl cholate
39.285-39.339	654	699	8.72	17-methyl-(3α,5α,17β)-Androstane-3,17-diol
39.363-39.464	862	902	89.4	dehydro-Epiabiet-4-ol
40.928-40.972	698	707	6.71	Pentatriacont-17-ene
41.181-41.282	613	790	7.71	Oleanitrile
41.640-41.717	681	752	16.9	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
42.709-42.786	699	714	18.5	3-ethyl-5-(2-ethylbutyl)-Octadecane

42.918-42.992	700	756	12.9	5-(1-bromo-1-methylethyl)-2-methyl-Cyclohexanol
43.134-43.178	626	761	7.36	(*E*)-4-methyl-6-(2,6,6-trimethyl-cyclohex-1-enyl)-Hex-3-en-1-ol
43.424-43.512	588	602	9.99	Androst-5,7-dien-3-ol-17-one acetate
43.974-44.041	670	691	11.0	Pentatriacont-17-ene
44.170-44.224	693	703	7.21	3-ethyl-5-(2-ethylbutyl)-Octadecane
45.377-45.637	822	831	62.3	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

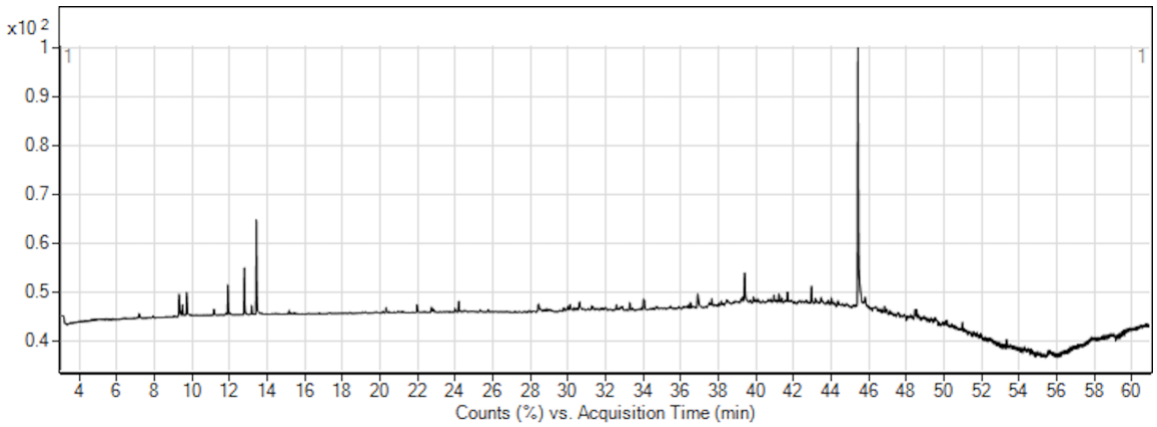


Figure 1.2. Gas chromatogram of the single-round Baltic amber extract produced via decoction extraction with dichloromethane.

Table 1.2. Compounds identified in the single-round Baltic amber extract produced via decoction extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
7.151-7.205	830	863	14.4	Camphene
9.276-9.364	894	897	26.3	*m*-Cymene
9.461-9.529	816	845	55.3	Eucalyptol
9.667-9.755	885	888	30.1	*o*-Cymene
11.128-11.192	783	796	41.8	Fenchone
11.853-11.951	831	833	33.4	Fenchol
12.737-12.838	807	808	32.7	Camphor
13.128-13.182	808	818	27.7	Borneol
13.368-13.493	829	829	48.3	Borneol
20.290-20.357	700	750	6.90	Isolongifolol
21.922-21.990	842	880	28.8	1,6,8-trimethyl-1,2,3,4-tetrahydro-Naphthalene
22.695-22.782	826	899	57.3	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
23.946-24.014	709	774	16.8	Palustrol
24.152-24.230	745	766	18.6	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
28.385-28.473	834	859	58.9	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
30.075-30.129	660	693	3.18	1-cyclopentyl-4-(3-cyclopentylpropyl)-Dodecane
30.581-30.659	714	765	12.8	7-isopropyl-1,9a-dimethyl-4-methylene-

				2,3,3a,4,6,8,9,9a,10,10a-decahydro-Dicyclopenta[a,d]cycloocten-5(1*H*)-one
31.212-31.280	689	759	11.3	Verticillol
32.535-32.612	737	786	53.1	18-Norabieta-8,11,13-triene
33.226-33.324	677	769	53.6	18-Norabieta-8,11,13-triene
33.982-34.083	744	763	64.0	Abieta-8,11,13-triene
36.486-36.545	657	681	17.5	3-ethyl-5-(2-ethylbutyl)-Octadecane
36.846-37.004	670	707	7.59	3-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(22),7(16),9,13,23,29-hexaene
37.618-37.682	650	668	5.09	Oleyl palmitoleate
38.111-38.188	695	713	11.5	Ethyl cholate
39.284-39.315	608	650	9.00	17-methyl-(3α,5α,17β)-Androstane-3,17-diol
39.352-39.463	859	900	90.2	dehydro-Epiabiet-4-ol
39.834-39.878	725	733	36.8	3-ethyl-5-(2-ethylbutyl)-Octadecane
40.917-40.981	687	703	7.81	Pentatriacont-17-ene
41.180-41.248	617	665	23.7	3-(tridec-9-enyl)-4,5,6,7-tetrahydro-Benz[z]isoxazole-5-ol-4-one
41.366-41.420	608	621	28.4	Oleic acid 2-[(*Z*)-octadec-9-enyloxy]ethyl ester
41.639-41.717	634	725	14.9	2,6,6-trimethyl-1-phenylsulfonylmethyl-Cyclohexene

42.917-43.005	659	746	11.4	5-(1-bromo-1-methylethyl)-2-methyl-Cyclohexanol
43.423-43.521	605	622	31.8	Androst-5,7-dien-3-ol-17-one acetate
43.973-44.051	621	660	11.8	Pentatriacont-17-ene
45.377-45.626	819	843	70.6	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

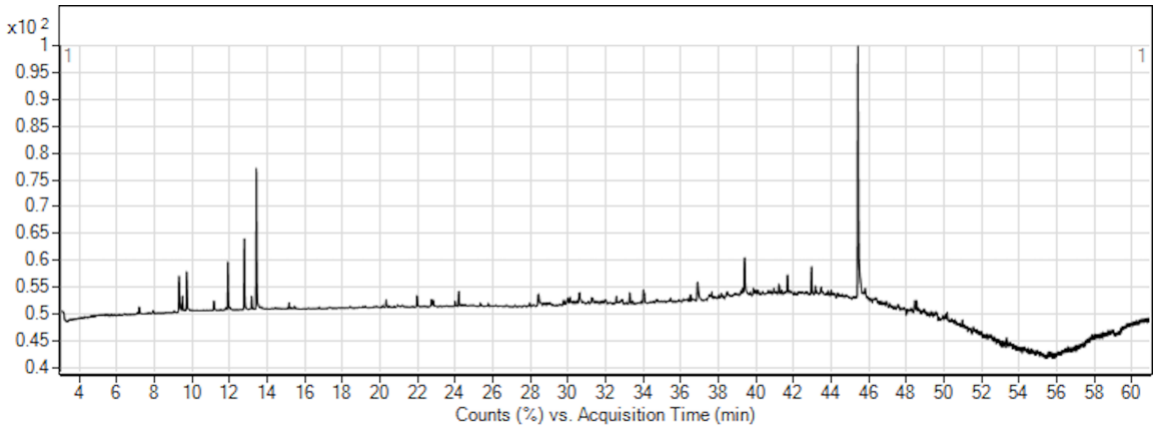


Figure 1.3. Gas chromatogram of the single-round Baltic amber extract produced via sonication extraction with dichloromethane.

Table 1.3. Compounds identified in the single-round Baltic amber extract produced via sonication extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
7.143-7.211	835	876	18.9	Camphene
9.268-9.369	880	884	21.6	*m*-Cymene
9.454-9.518	795	835	51.8	Eucalyptol
9.673-9.764	886	888	31.1	*o*-Cymene
11.124-11.191	798	808	42.1	Fenchone
11.856-11.933	825	828	30.4	Fenchol
12.736-12.827	810	811	30.8	Camphor
13.121-13.198	807	810	24.6	Borneol
13.363-13.488	814	814	38.1	Borneol
15.114-15.195	750	821	15.2	Isobornyl formate
20.292-20.346	706	752	6.54	Shyobunol
21.928-21.982	842	880	29.7	1,6,8-trimethyl-1,2,3,4-tetrahydro-Naphthalene
22.694-22.761	848	892	59.3	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
22.809-22.863	797	849	33.6	Calamenene
23.935-24.013	718	771	18.9	Caryophyllenol
24.154-24.222	759	778	22.2	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
28.381-28.492	805	843	53.3	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene

29.923-30.024	605	640	9.20	2,6,9,12,16-pentamethyl-Heptadeca-2,6,11,15-tetraene-9-carboxylic acid
30.075-30.139	648	686	3.47	1-cyclopentyl-4-(3-cyclopentylpropyl)-Dodecane
30.597-30.675	676	764	13.8	7-isopropyl-1,9a-dimethyl-4-methylene-2,3,3a,4,6,8,9,9a,10,10a-decahydro-Dicyclopenta[a,d]cycloocten-5(1*H*)-one
31.221-31.289	698	760	16.0	Biformene
32.547-32.611	758	791	55.9	18-Norabieta-8,11,13-triene
33.229-33.320	704	790	50.4	18-Norabieta-8,11,13-triene
33.974-34.085	760	778	79.2	Abieta-8,11,13-triene
36.480-36.544	657	672	15.7	3-ethyl-5-(2-ethylbutyl)-Octadecane
36.851-37.000	683	707	7.59	Pimara-7,15-dien-3-ol
37.627-37.661	643	717	3.57	Pentatriacont-17-ene
38.103-38.180	693	694	16.5	dimethoxy-Lycopene
39.344-39.459	835	880	88.3	dehydro-Epiabiet-4-ol
39.833-39.877	688	704	27.2	3-ethyl-5-(2-ethylbutyl)-Octadecane
40.913-40.990	665	694	12.3	Oleyl palmitoleate
41.179-41.257	623	635	10.4	Ethyl cholate
41.645-41.709	701	764	19.5	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
42.920-42.997	705	783	12.2	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-

				methylene-Cyclopentane carboxylate
43.129-43.183	591	765	4.41	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-Phosphonous dichloride
43.429-43.507	587	603	14.5	Androst-5,7-dien-3-ol-17-one acetate
45.379-45.608	812	842	71.4	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

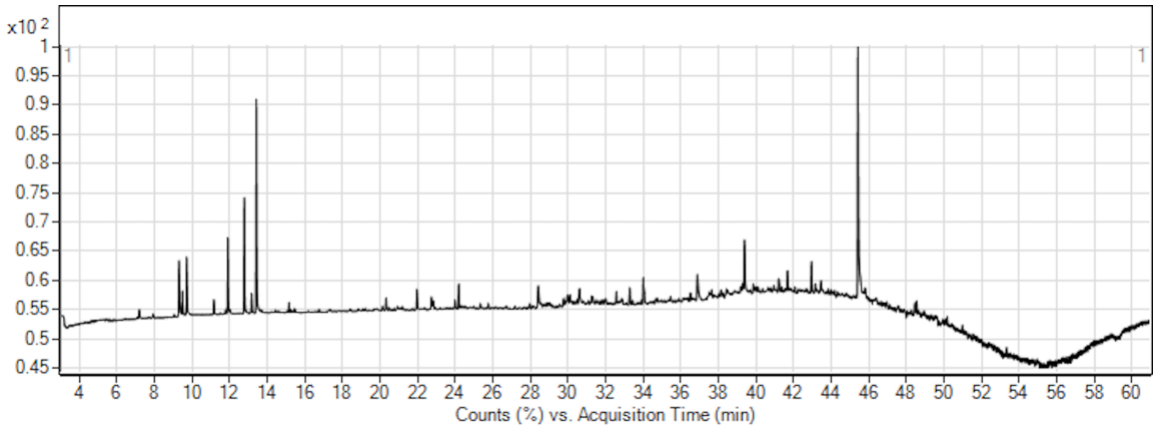


Figure 1.4. Gas chromatogram of the single-round Baltic amber extract produced via Soxhlet extraction with dichloromethane.

Table 1.4. Compounds identified in the single-round Baltic amber extract produced via Soxhlet extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
7.143-7.234	817	855	12.5	Camphene
9.268-9.393	882	886	24.7	*m*-Cymene
9.453-9.541	800	826	54.6	Eucalyptol
9.649-9.787	880	883	28.0	*o*-Cymene
11.123-11.177	795	800	38.5	Fenchone
11.855-11.923	824	826	33.2	Fenchol
12.736-12.827	812	813	36.4	Camphor
13.107-13.138	799	815	26.2	Borneol
13.363-13.488	820	820	38.1	Borneol
15.127-15.171	786	805	32.0	Isobornyl formate
20.268-20.359	689	741	5.38	Methyl 2-hydroxy-octadeca-9,12,15-trienoate
21.914-21.995	835	867	26.1	1,6,8-trimethyl-1,2,3,4-tetrahydro-Naphthalene
22.704-22.747	883	898	70.7	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
22.808-22.852	811	851	29.7	Calamenene
23.945-24.002	742	778	20.4	Palustrol
24.154-24.222	782	795	34.0	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
28.381-28.482	788	842	49.5	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene

29.727-29.791	589	799	13.9	Podocarpa-8,11,13-triene
29.936-30.024	633	661	8.49	2,6,10,15,19,23-hexamethyl-Tetracosa-2,6,14,18,22-pentaene-10,11-diol
30.074-30.128	669	725	3.46	Octadec-13-enal
30.584-30.661	699	752	13.5	7-isopropyl-1,9a-dimethyl-4-methylene-2,3,3a,4,6,8,9,9a,10,10a-decahydro-Dicyclopenta[a,d]cycloocten-5(1*H*)-one
31.221-31.289	708	773	9.68	Verticillol
31.929-32.068	669	698	25.1	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)-hexa-1,3,5-trienyl]-Cyclohex-1-en-1-carboxaldehyde
32.547-32.601	764	779	53.7	18-Norabieta-8,11,13-triene
33.232-33.333	723	729	63.5	18-Norabieta-8,11,13-triene
33.974-34.085	778	790	78.7	Abieta-8,11,13-triene
36.467-36.544	654	673	21.1	3-ethyl-5-(2-ethylbutyl)-Octadecane
36.838-36.999	674	725	11.1	5-hydroxymethyl-5,8a-dimethyl-y,2-bis(methylene)-decahydro-(1α,4aβ,5α,8aα)-Naphthalenepentan-1-ol
37.617-37.684	644	644	19.9	Oleyl palmitoleate
38.082-38.194	634	706	5.85	1(22),7(16)-diepoxy-Tricyclo[20.8.0.0(7,16)]triacontane
39.148-39.216	635	685	39.3	3-hydroxy-17-oxo-Androsta-5,7,9(11)-triene
39.253-39.320	612	663	13.2	17-methyl-(3α,5α,17β)-Androstane-3,17-diol

39.823-39.877	692	707	28.5	3-ethyl-5-(2-ethylbutyl)-Octadecane
41.169-41.270	606	666	31.4	3-(tridec-9-enyl)-4,5,6,7-tetrahydro-Benz[z]isoxazole-5-ol-4-one
41.644-41.709	690	754	14.8	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
42.920-42.997	693	770	15.2	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.129-43.183	590	636	3.96	(all-*E*)-3-bromo-2,6,10,15,19,23-hexamethyl-Tetracosa-6,10,14,18,22-pentaen-2-ol
43.419-43.510	601	612	19.3	Androst-5,7-dien-3-ol-17-one acetate
45.379-45.622	794	828	68.4	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

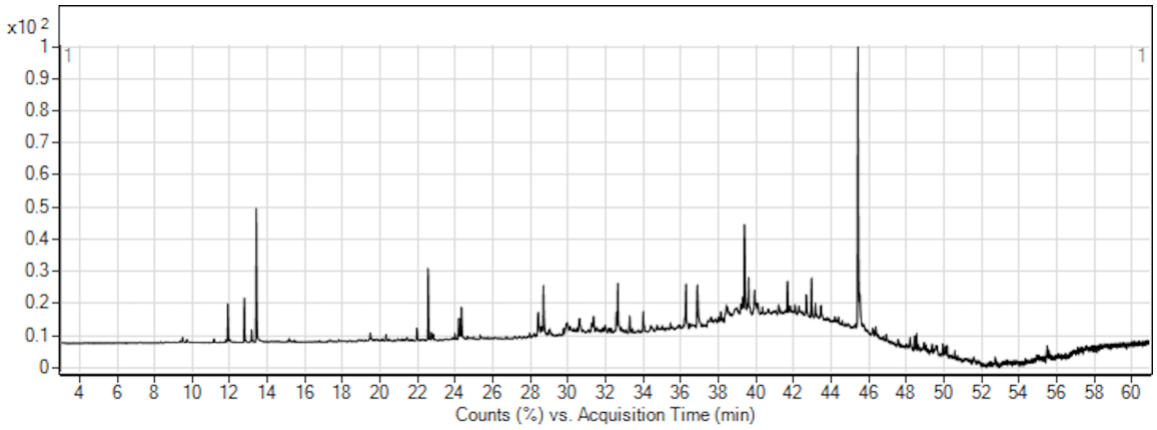


Figure 1.5. Gas chromatogram of the single-round Baltic amber extract produced via conservative extraction with ethanol.

Table 1.5. Compounds identified in the single-round Baltic amber extract produced via conservative extraction with ethanol.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
11.851-11.919	827	827	33.1	Fenchol
12.735-12.823	801	804	34.8	Camphor
13.116-13.157	774	817	27.4	Borneol
13.363-13.487	821	821	37.2	Borneol
19.455-19.522	740	838	4.35	Tridec-5-ene
20.291-20.345	666	738	4.44	Shyobunol
21.914-21.961	841	878	25.4	1,6,8-trimethyl-1,2,3,4-tetrahydro-Naphthalene
22.507-22.595	871	873	35.9	2,4-bis(1,1-dimethylethyl)-Phenol
22.693-22.757	866	907	69.3	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
23.944-23.998	740	771	27.6	Caryophyllenol
24.154-24.208	768	779	18.1	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
24.282-24.369	764	851	5.81	3-hexyl-Sulfolane
28.357-28.478	765	845	42.4	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
28.657-28.758	697	790	2.84	Tetradec-7-ene
31.210-31.278	693	742	11.0	Cembrene
31.315-31.382	797	870	78.2	7,9-di-*tert*-butyl-1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione

32.533-32.600	687	757	38.8	dehydro-Epiabiet-4-ol
32.614-32.681	784	833	5.01	Docosene
33.218-33.319	671	768	49.9	18-Norabieta-8,11,13-triene
33.973-34.051	752	764	73.3	Abieta-8,11,13-triene
36.176-36.220	682	705	5.50	Pentatriacont-17-ene
36.247-36.314	742	843	4.46	Docosene
36.837-36.952	664	695	8.24	3-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(22),7(16),9,13,23,29-hexaene
38.102-38.159	625	636	7.95	Dioleylglycol
39.161-39.226	641	700	42.8	3-hydroxy-17-oxo-Androsta-5,7,9(11)-triene
39.229-39.330	672	729	8.30	2,3-epoxy-(2α,3α,5α,17β)-Androstan-17-ol
39.333-39.458	856	889	90.9	dehydro-Epiabiet-4-ol
39.566-39.633	770	855	6.20	Docosene
39.880-33.957	707	736	14.6	3-hydroxy-(5β)-Androst-2-en-17-one
40.075-40.143	593	647	8.73	2,5-bis(1,1,3,3-tetramethylbutyl)-Thiophene
40.322-40.375	618	763	8.92	2,6,6-trimethyl-1-phenylsulfonylmethyl-Cyclohexene
41.168-41.256	599	608	15.3	Eicosyl oleate
41.644-41.698	724	756	29.2	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate

41.796-41.880	593	615	8.36	2,6,10,15,19,23-hexamethyl-Tetracosa-2,6,14,18,22-pentaene-10,11-diol
42.039-42.103	597	821	3.47	Phthalic acid hexyl 7-methyloct-3-yn-5-yl ester
42.629-42.720	725	759	10.3	Pentatriacont-17-ene
42.919-42.997	752	804	29.1	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.128-43.196	669	800	13.6	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.418-43.559	561	571	14.6	11-dehydroxy-9-thiocyanto-1,2-dihydro-11-oxo-Prednisolone
45.381-45.493	832	856	80.8	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

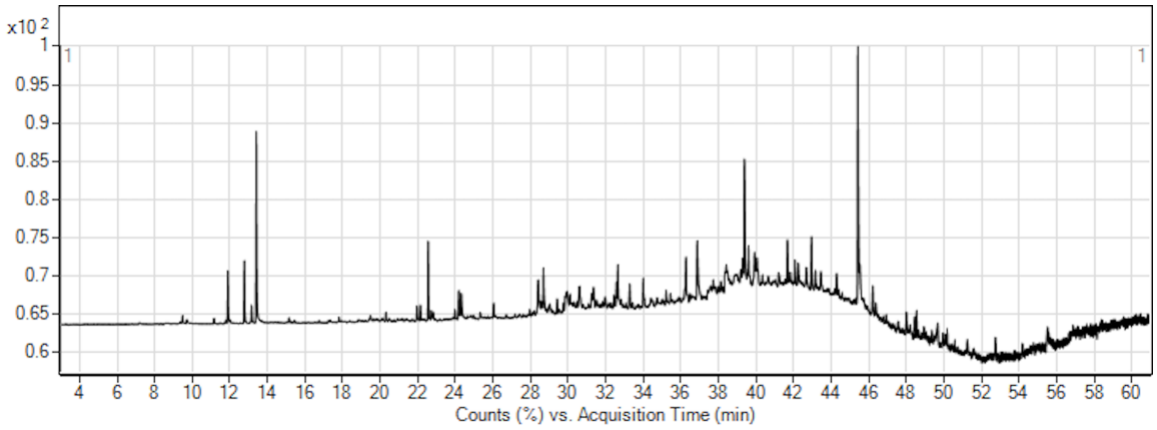


Figure 1.6. Gas chromatogram of the single-round Baltic amber extract produced via decoction extraction with ethanol.

Table 1.6. Compounds identified in the single-round Baltic amber extract produced via decoction extraction with ethanol.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
11.848-11.926	826	831	31.5	Fenchol
12.735-12.813	784	794	28.9	Camphor
13.116-13.164	797	805	20.8	Borneol
13.359-13.494	818	818	36.6	Borneol
20.278-20.355	710	763	6.14	Shyobunol
21.907-21.985	822	907	26.7	α-Ionene
22.507-22.585	880	882	35.9	2,4-bis(1,1-dimethylethyl)-Phenol
23.928-24.019	664	747	10.7	Palustrol
24.127-24.228	751	784	16.9	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
24.289-24.386	719	862	8.51	3-hexyl-Sulfolane
28.330-28.488	727	836	23.2	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
28.515-28.616	594	602	12.4	Ethyl cholate
28.654-28.741	693	740	5.23	Trogodermal
29.716-29.784	565	573	7.95	Nonacosa-10,12,14-triynoic acid
30.064-30.141	594	643	6.85	Butyl octadeca-9,12,15-trienoate
31.207-31.271	705	771	13.7	Verticillol

31.288-31.389	744	857	66.8	7,9-di-*tert*-butyl-1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione
32.546-32.600	700	721	44.1	18-Norabieta-8,11,13-triene
32.617-32.681	766	838	4.42	Docosene
33.218-33.319	655	756	41.5	18-Norabieta-8,11,13-triene
33.966-34.081	748	770	67.6	Abieta-8,11,13-triene
36.243-36.297	744	769	7.72	17-chloro-Heptadec-7-ene
36.820-36.932	701	714	20.1	Pimara-7,15-dien-3-ol
39.131-39.232	609	675	35.6	3-hydroxy-17-oxo-Androsta-5,7,9(11)-triene
39.246-39.313	680	745	7.77	2,3-epoxy-(2α,3α,5α,17β)-Androstan-17-ol
39.340-39.438	867	903	90.2	dehydro-Epiabiet-4-ol
39.569-39.623	706	735	4.52	Pentatriacont-17-ene
39.883-39.947	716	745	16.5	Pimara-7,15-dien-3-ol
41.627-41.715	715	793	36.3	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
41.776-41.867	571	586	7.69	Betulin
42.032-42.096	752	871	4.90	Phthalic acid octyl 2-propylpentyl ester
42.619-42.710	667	694	6.32	1,1-bis(4-methylcyclohexyl)-Dodecane
42.919-42.987	751	804	38.0	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.128-43.182	694	796	19.0	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-

				methylene-Cyclopentane carboxylate
43.458-43.482	578	614	13.6	Gibberellin A4
45.368-45.479	821	855	74.0	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

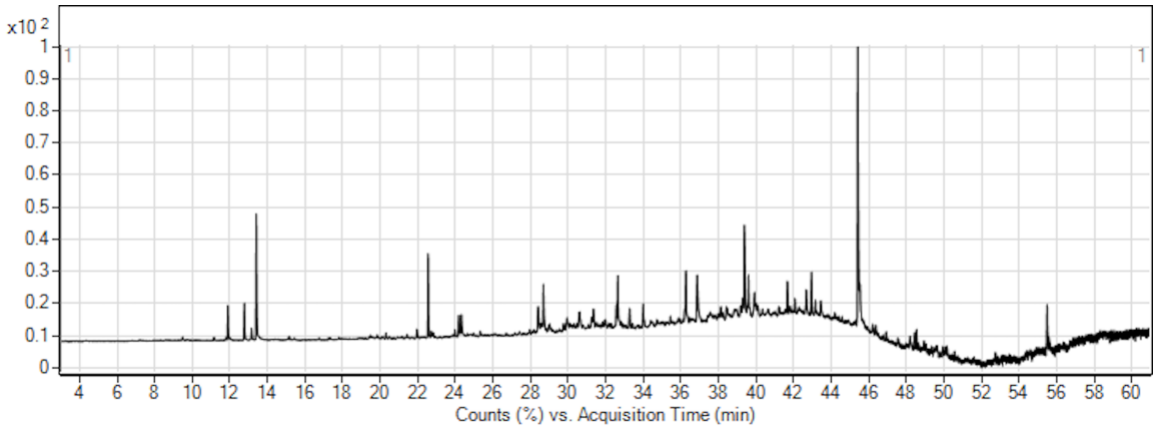


Figure 1.7. Gas chromatogram of the single-round Baltic amber extract produced via sonication extraction with ethanol.

Table 1.7. Compounds identified in the single-round Baltic amber extract produced via sonication extraction with ethanol.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
11.846-11.910	795	805	31.5	Fenchol
12.747-12.811	814	820	40.3	Camphor
13.115-13.192	738	806	17.3	Borneol
13.371-13.469	823	823	35.6	Borneol
21.905-22.007	780	872	25.8	1,6,8-trimethyl-1,2,3,4-tetrahydro-Naphthalene
22.506-22.594	877	878	36.5	2,4-bis(1,1-dimethylethyl)-Phenol
24.145-24.223	753	786	22.3	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
24.284-24.375	709	869	7.32	3-hexyl-Sulfolane
28.363-28.473	796	849	54.9	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
28.652-28.736	694	743	3.65	Trogodermal
31.192-31.280	698	775	12.1	Verticillol
31.283-31.398	719	838	62.6	7,9-di-*tert*-butyl-1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione
32.531-32.599	671	696	35.4	18-Norabieta-8,11,13-triene
32.626-32.690	788	834	4.77	Docosene
33.213-33.314	646	757	54.5	18-Norabieta-8,11,13-triene
33.565-34.066	735	754	59.1	Abieta-8,11,13-triene
36.238-36.296	750	767	6.59	17-chloro-Heptadec-7-ene

36.829-36.930	696	711	19.3	Pimara-7,15-dien-3-ol
38.063-38.178	609	632	16.6	Oleyl palmitoleate
39.116-39.203	630	702	41.6	3-hydroxy-17-oxo-Androsta-5,7,9(11)-triene
39.268-39.322	590	646	5.74	17-methyl-(3α,5α,17β)-Androstane-3,17-diol
39.335-39.426	861	893	90.7	dehydro-Epiabiet-4-ol
39.568-39.622	747	858	5.32	Docosene
39.878-39.946	703	742	10.7	Pimara-7,15-dien-3-ol
41.636-41.700	729	791	35.4	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
42.037-42.094	694	863	3.47	Phthalic acid octyl 2-propylpentyl ester
42.627-42.705	694	826	5.12	Octacosanol
42.904-42.995	722	795	26.2	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.113-43.191	617	811	7.78	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.413-43.525	594	618	21.0	Androst-5,7-dien-3-ol-17-one acetate
45.366-45.488	821	848	76.2	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by

the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

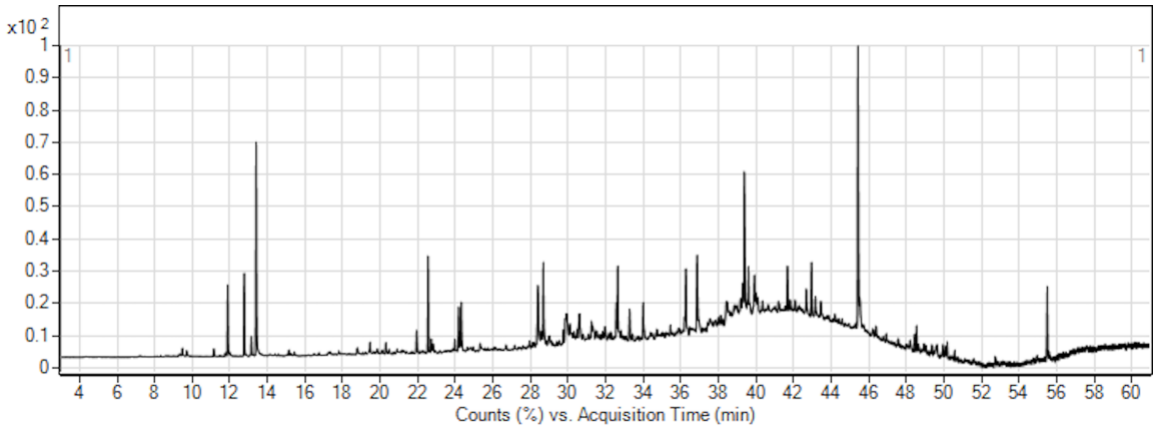


Figure 1.8. Gas chromatogram of the single-round Baltic amber extract produced via Soxhlet extraction with ethanol.

Table 1.8. Compounds identified in the single-round Baltic amber extract produced via Soxhlet extraction with ethanol.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
9.447-9.501	835	854	62.5	Eucalyptol
9.676-9.720	892	911	30.7	*m*-Cymene
11.113-11.167	802	811	38.6	Fenchone
11.839-11.903	820	822	31.7	Fenchol
12.722-12.834	801	802	32.3	Camphor
13.104-13.191	789	810	22.3	Borneol
13.357-13.478	831	831	45.6	Borneol
15.114-15.168	755	798	14.2	Isobornyl formate
18.737-18.858	735	818	24.7	3,3-dimethyl-Phthalide
19.429-19.503	785	844	4.21	Tetradec-7-ene
20.279-20.353	735	762	8.77	Shyobunol
20.437-20.528	662	689	4.13	Heptatriacontanol
21.898-21.975	843	910	36.3	α-Ionene
22.508-22.572	876	877	45.9	2,4-bis(1,1-dimethylethyl)-Phenol
22.680-22.734	877	904	72.9	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
22.795-22.839	826	851	43.9	Calamenene
22.932-23.999	740	769	32.0	Caryophyllenol
24.141-24.205	767	771	18.4	Caryophyllenol
24.279-24.367	710	816	4.28	Tetradec-7-ene
25.278-25.355	725	766	29.7	dihydro-Agarofuran

27.912-27.976	576	603	11.2	Tricosa-1,8,15,22-tetrayne
28.324-28.459	711	831	24.7	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
28.533-28.631	583	616	7.71	(*Z*)-7-methyl-Tetradec-8-en-1-ol acetate
28.648-28.783	695	721	4.64	7-bromomethyl-Pentadec-7-ene
32.534-32.598	717	728	57.3	18-Norabieta-8,11,13-triene
32.615-32.692	787	834	4.93	Docosene
33.222-33.313	733	796	67.1	18-Norabieta-8,11,13-triene
33.971-34.035	794	800	82.2	Abieta-8,11,13-triene
35.418-35.472	648	650	13.3	Astaxanthin
36.153-36.207	650	680	6.62	18-bromo-Octadecan-1-ol
36.234-36.301	736	757	5.90	17-chloro-Heptadec-7-ene
36.821-36.922	696	706	18.8	Pimara-7,15-dien-3-ol
39.155-39.199	624	659	32.0	3-hydroxy-17-oxo-Androsta-5,7,9(11)-triene
39.260-39.314	647	669	14.4	17-methyl-(3α,5α,17β)-Androstane-3,17-diol
39.341-39.415	858	881	90.8	dehydro-Epiabiet-4-ol
39.570-39.624	758	843	5.66	Docosene
39.881-39.945	733	745	18.2	Prasterone
39.972-40.015	679	693	11.9	Agathic acid
40.075-40.117	606	666	11.3	*N*-(2-hydroxyethyl)-*N*'-(1,7-phenanthrolin-6-yl)-Thiourea
40.316-40.393	610	738	14.2	2,6,6-trimethyl-1-phenylsulfonylmethyl-Cyclohexene

41.628-41.716	734	782	30.5	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-Phosphonous dichloride
41.786-41.854	591	718	8.37	2,6,6-trimethyl-1-phenylsulfonylmethyl-Cyclohexene
42.039-42.097	651	824	9.34	Phthalic acid octyl tridec-2-yn-1-yl ester
42.626-42.694	706	810	3.75	Eicosanol
42.927-42.991	747	785	21.3	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.122-43.186	709	798	28.8	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
43.409-43.497	546	554	9.80	11-dehydroxy-9-thiocyanto-1,2-dihydro-11-oxo-Prednisolone
45.376-45.477	827	853	80.0	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

Two classes of terpenes and terpenoids were primarily identified in Baltic amber: (1) monoterpenes and monoterpenoids such as borneol, camphene, camphor, *m*-cymene, *o*-cymene, eucalyptol, fenchol, and fenchone (**Figure 1.9A**) and (2) abietane-, pimarane-, and labdane-type diterpenes and diterpenoids such as abieta-8,11,13-triene, dehydro-epiabiet-4-ol, 7-isopropyl-1,9a-dimethyl-4-methylene-2,3,3a,4,6,8,9,9a,10,10a-decahydro-dicyclopenta[a,d]cyclooctene-5(1*H*)-one, 18-norabieta-8,11,13-triene, pimara-7,15-dien-3-ol, and verticillol (**Figure 1.9B**). These terpenes and terpenoids were represented by large and distinctive peaks in the gas chromatograms (**Figures 1.1-1.8**). All of the compounds reported above also returned match factors and reverse match factors of greater than 700 in at least one of the extracts tested (**Tables 1.1-1.8**); therefore, these compounds are likely present in Baltic amber. Diterpenes and diterpenoids such as agathic acid, biformene, cembrene, podocarpa-8,11,13-triene, and sclarene (**Figure 1.9C**), however, returned match factors and reverse match factors of less than 700 (**Table 1.1**, **Tables 1.3-1.5**, and **Table 1.8**), suggesting that the identities of these compounds are not present in the standard reference database but are structural analogues of the reported compounds. Overall, the significant presence of terpenes and terpenoids in Baltic amber is expected because resin is known to be composed of terpenes and terpenoids.[1,2,4] Another compound, erucamide (**Figure 1.9D**), was represented by a very large and distinctive peak in all of the gas chromatograms (**Figures 1.1-1.8**) and returned match factors and reverse match factors of greater than 790 in all of the extracts tested (**Tables 1.1-1.8**). Erucamide is a common slip agent used in the plastic manufacturing process,[46] and it is hypothesized that this compound is a contaminant originating from the plastic bag used to transport and store the Baltic amber used in this work.

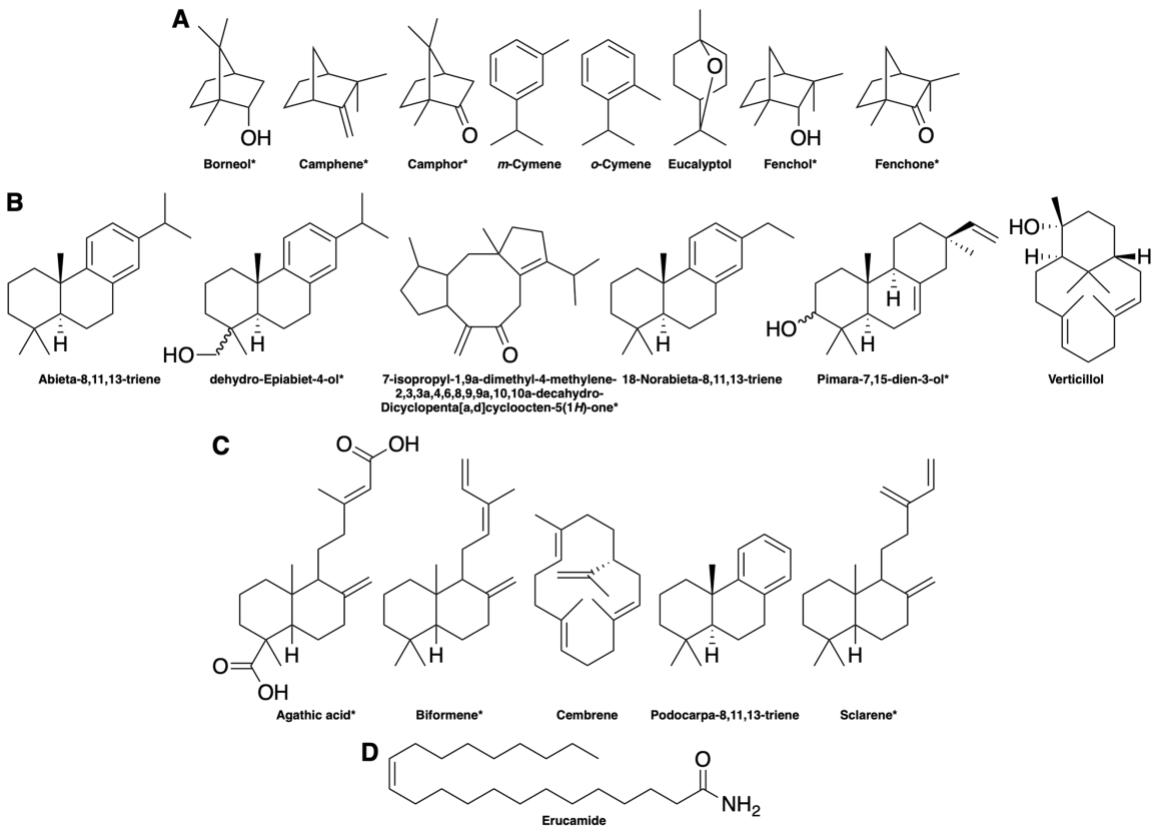


Figure 1.9. Compounds identified in at least one of the single-round Baltic amber extracts and represented by large peaks in the corresponding gas chromatograms: (A) monoterpenes and monoterpenoids; (B) abietane-, pimarane-, and labdane-type diterpenes and diterpenoids with match factors and reverse match factors greater than 700; (C) diterpenes and diterpenoids with match factors and reverse match factors less than 700; and (D) erucamide. *Stereochemistry to be determined.

Other compounds identified in Baltic amber represent several other structural classes: sesquiterpenes and sesquiterpenoids such as calamenene, caryophyllenol, isolongifolol, palustrol, and shyobunol; phenols such as 2,4-bis(1,1-dimethylethyl)-phenol; and hydrocarbons such as α-ionene, 3-ethyl-5-(2-ethylbutyl)-octadecane, 1,1,4,5,6-pentamethyl-2,3-dihydro-1H-indene, and 1,6,8-trimethyl-1,2,3,4-tetrahydro-naphthalene (**Figure 1.10**). Interestingly, many steroids and steroid-like compounds were also identified, most of which returned match factors and/or reverse match factors of less than 700, and in some cases less than 600, which represents a very poor match (**Tables 1.1-1.8**).[45] These compounds require further purification and characterization to determine their identities. Succinic acid, which is present in the insoluble macromolecular structure of amber, was identified in none of the eight extracts tested because no derivatization (e.g., treatment with diazomethane[13] or hexamethyldisilazane[14]) was performed.

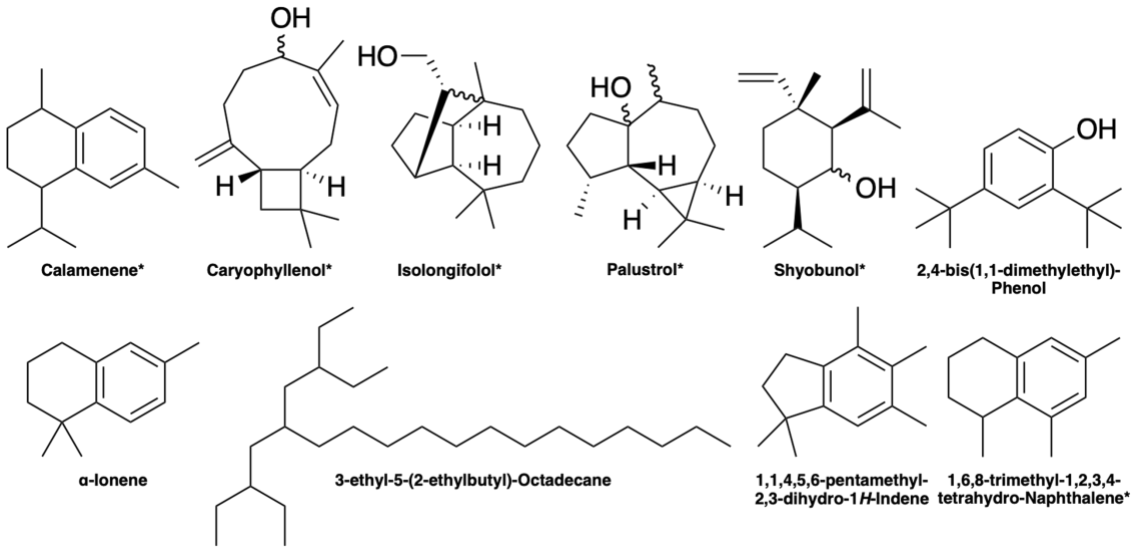


Figure 1.10. Compounds identified in at least one of the single-round Baltic amber extracts and represented by small peaks in the corresponding gas chromatograms: sesquiterpenes and sesquiterpenoids, phenols, and hydrocarbons. *Stereochemistry to be determined.

Based on these data, the optimal extraction conditions were determined to be the conservative and the Soxhlet extraction techniques using dichloromethane as a solvent because (1) the conservative and the Soxhlet extraction techniques were less destructive to the amber than the decoction and the sonication extraction techniques and (2) dichloromethane extracted more of the terpenes and terpenoids reported above than ethanol (**Tables 1.1-1.8**).

1.3.2 GC-MS Analysis of Multiple-Round Baltic Amber Extracts

Following GC-MS analysis of the single-round Baltic amber extracts, extracts resulting from multiple successive rounds of conservative extraction using the same Baltic amber samples were analyzed to test additional extraction conditions. Based on the results of the single-round Baltic amber extracts, the GC-MS temperature program was modified to optimize peak resolution. This program was used for the remainder of the studies reported. Multiple successive rounds of extraction allowed the identification of various hydrocarbons (**Figures 1.11-1.12** and **Tables 1.9-1.10**), which are poorly soluble in dichloromethane and ethanol and unable to be fully extracted in a single round. No additional compounds of potential medicinal interest were identified via multiple successive rounds of extraction; therefore, one round of extraction was determined to be sufficient for future studies.

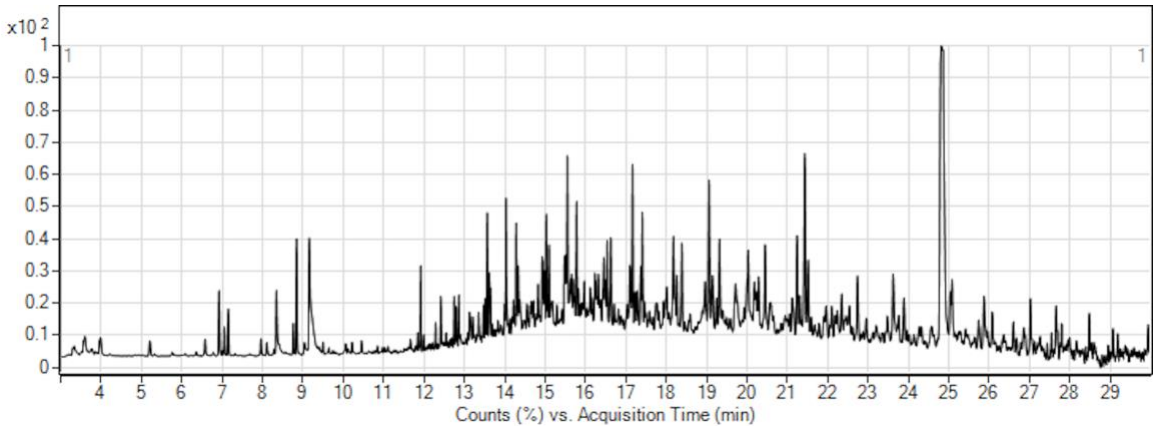


Figure 1.11. Gas chromatogram of the multiple-round Baltic amber extract produced via conservative extraction with dichloromethane.

Table 1.9. Compounds identified in the multiple-round Baltic amber extract produced via conservative extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
3.283-3.360	861	877	27.5	Spiro[2.4]hepta-4,6-diene
3.546-3.630	677	700	18.4	Vinyl methacrylate
3.941-4.012	769	793	14.5	3-methyl-3-Nitrobut-1-ene
5.175-5.233	762	826	86.2	1,1,2,2-tetrachloro-Ethane
6.899-6.943	856	876	22.0	*m*-Cymene
7.034-7.068	828	830	55.5	Eucalyptol
7.125-7.162	889	893	32.7	*o*-Cymene
7.941-7.972	772	798	32.3	Fenchone
8.086-8.124	794	840	10.9	Undecyne
8.323-8.377	824	827	27.0	Fenchol
8.822-8.866	823	824	35.4	Camphor
9.014-9.058	798	802	28.0	Borneol
9.136-9.274	834	834	45.4	Borneol
9.476-9.507	736	767	13.0	Isothujol
10.036-10.073	776	783	18.1	Isobornyl acetate
10.198-10.235	711	728	17.0	8a-methyl-1,2,3,5,8,8a-hexahydro-Naphthalene
10.438-10.468	776	827	59.5	Pelargonic acid
11.827-11.854	727	771	3.24	Heptadecan-9-ol
11.902-11.932	834	847	3.88	4-methyl-Undec-1-ene
11.979-12.003	775	828	6.75	Heptacosane

12.269-12.300	738	754	8.54	Shyobunol
12.394-12.428	710	816	2.89	Docosene
12.732-12.758	636	801	59.6	Tridecan-3-one
12.779-12.806	804	825	6.34	2,6,10,14-tetramethyl-Heptadecane
12.853-12.873	855	889	25.9	α-Ionene
13.109-13.163	722	771	18.0	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
13.200-13.221	806	850	6.03	Sulfurous acid pentadecyl hexyl ester
13.335-13.366	650	650	5.97	3-ethyl-5-(2-ethylbutyl)-Octadecane
13.457-13.477	720	777	5.08	1-bromo-4-bromomethyl-Decane
13.497-13.521	722	799	3.88	Tetradec-7-ene
13.548-13.578	797	823	7.12	Octadecanesulfonyl chloride
13.605-13.632	685	685	5.58	*tert*-Hexadecanethiol
13.639-13.656	731	740	11.8	Palustrol
14.017-14.037	780	842	6.03	Nonadec-9-ene
14.266-14.300	704	753	39.3	Pentadecan-3-one
14.320-14.340	717	770	29.7	1,2,3-trimethyl-4-propenyl-Naphthalene
14.813-14.833	812	829	5.24	2-methyl-Octadecane
14.914-14.954	711	763	10.3	Fenchol
14.971-14.991	761	849	5.41	Allyl octadecyl oxalate
15.015-15.045	811	866	4.25	Nonadecane
15.083-15.106	789	806	5.05	2-methyl-Nonadecane
15.531-15.562	862	874	7.59	Eicosanol

15.754-15.798	758	779	65.1	Heptadecan-3-one
16.304-16.327	687	843	5.54	Isoheneicosane
16.439-16.459	731	785	5.81	1-(4-bromobutyl)-Piperidin-2-one
16.520-16.547	843	865	4.80	Heptacosane
16.601-16.638	800	855	7.08	2-bromo-Tetradecane
17.046-17.107	818	820	6.36	3-methyl-Eicosane
17.144-17.177	849	868	6.27	Eicosanol
17.346-17.376	728	732	84.3	Abieta-8,11,13-triene
17.390-17.420	694	741	43.0	Methyl 2-oxo-octadecanoate
18.000-18.024	778	809	7.98	2-methyl-Hexacosane
18.152-18.196	694	742	11.3	2-methyl-Tetradec-4-ene
18.243-18.274	877	882	9.64	Heptacosane
18.355-18.405	791	808	4.82	2,21-dimethyl-Docosane
18.948-18.972	656	772	5.41	Heptacosane
19.036-19.080	761	847	6.55	eicosyl-Cyclopentane
19.117-19.161	713	786	4.06	Cetyl glycidyl ether
19.289-19.350	625	738	19.5	Pentadecan-3-one
20.014-20.041	797	830	82.6	Oleamide
20.159-20.210	733	766	37.5	Isobutyl stearate
20.264-20.301	736	743	5.42	11-pentan-3-yl-Heneicosane
20.426-20.470	701	748	11.0	3-ethyl-5-(2-ethylbutyl)-Octadecane
20.537-20.615	828	868	88.7	dehydro-Epiabiet-4-ol
21.100-21.161	786	822	7.87	2-methyl-Hexacosane
21.215-21.262	852	854	7.11	Octacosanol

21.401-21.448	752	843	80.0	Oleanitrile
21.492-21.525	639	657	8.33	8,8-diheptyl-Pentadecane
22.713-22.763	795	795	6.05	Hexatriacontane
23.587-23.644	800	830	8.02	Octacosanol
23.867-23.907	615	628	18.5	3-ethyl-Tetracosane
24.757-24.916	826	859	82.9	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

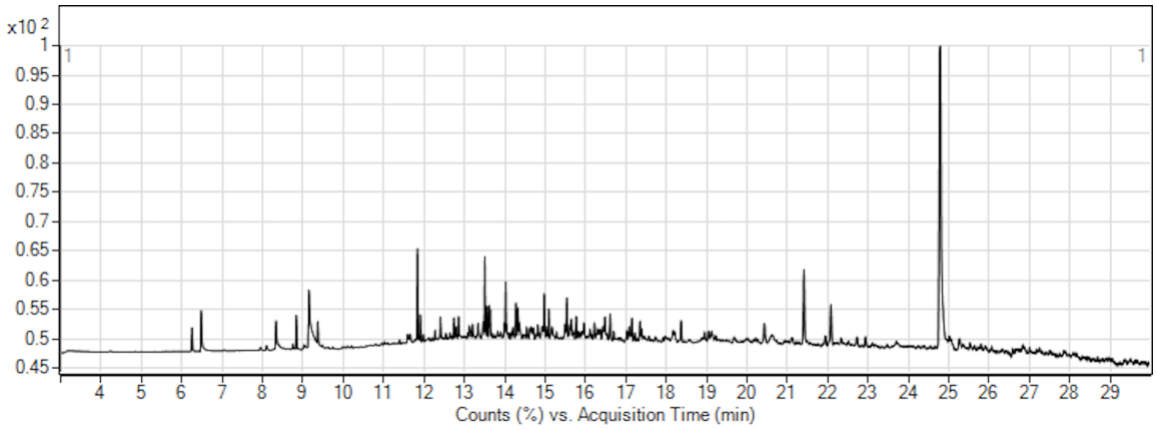


Figure 1.12. Gas chromatogram of the multiple-round Baltic amber extract produced via conservative extraction with ethanol.

Table 1.10. Compounds identified in the multiple-round Baltic amber extract produced via conservative extraction with ethanol.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
7.026-7.063	645	843	29.2	Eucalyptol
7.930-7.970	698	806	22.7	Fenchone
8.071-8.132	747	807	5.80	3-Nonen-1-ol
8.314-8.382	829	838	32.8	Fenchol
8.814-8.854	812	817	37.3	Camphor
9.009-9.056	790	820	21.9	Borneol
9.134-9.286	832	833	43.8	Borneol
9.353-9.384	769	788	3.66	Tridec-5-ene
11.812-11.860	841	854	6.19	1,4-dimethyl-Cyclooctane
11.893-11.924	809	855	3.85	Nonadecane
11.974-11.995	695	818	7.34	Isoheneicosane
12.264-12.281	780	799	18.9	Ledol
12.393-12.420	700	801	2.44	Heptadec-8-ene
12.774-12.797	782	844	5.51	2,6-dimethyl-Heptadecane
12.841-12.868	872	905	46.6	α-Ionene
13.104-13.135	729	794	27.1	1,1,4,5,6-pentamethyl-2,3-dihydro-1*H*-Indene
13.189-13.209	724	793	6.65	Isoheneicosane
13.330-13.351	759	836	6.52	Isoheneicosane
13.448-13.475	689	729	5.18	Octadecyl vinyl ether
13.489-13.516	824	839	4.56	Tetradec-7-ene

13.539-13.570	734	801	10.3	Octadecanesulfonyl chloride
13.597-13.620	741	820	6.21	2,6,10,15-tetramethyl-Heptadecane
13.631-13.651	732	752	14.8	2,2,4a,7a-tetramethyl-decahydro-1*H*-Cyclobuta[e]inden-5-ol
13.975-13.991	740	806	5.72	2-ethyl-2-methyl-Tridecanol
14.005-14.032	724	835	4.49	Hexadecanol
14.261-14.292	668	745	27.7	Pentadecan-3-one
14.309-14.339	711	756	27.9	1,2,3-trimethyl-4-propenyl-Naphthalene
14.356-14.373	700	707	35.6	1,2-diethyl-4-phenyl-Benzene
14.524-14.551	802	830	52.2	1,4-dimethyl-2-(2,5-dimethylphenyl)-Benzene
14.804-14.821	810	847	7.20	Isoheneicosane
14.902-14.949	680	691	3.39	2-methyl-Hexadecanol
14.963-14.983	817	852	6.39	Docosene
15.071-15.105	816	848	5.20	Stearyl Iodide
15.486-15.499	744	813	6.08	Stearyl Iodide
15.520-15.550	815	842	7.04	Eicosanol
15.634-15.655	703	842	4.63	Phthalic acid isobutyl tridec-2-yn-1-yl ester
15.749-15.779	744	786	45.2	Heptadecan-3-one
15.945-15.975	719	749	27.4	7-isopropyl-1,9a-dimethyl-4-methylene-2,3,3a,4,6,8,9,9a,10,10a-decahydro-Dicyclopenta[a,d]cycloocten-5(1*H*)-one
16.204-16.241	712	724	38.6	Heptatriacontanol

16.467-16.494	740	839	3.73	Docosene
16.586-16.623	793	843	6.14	Heptacosane
17.139-17.162	778	840	6.86	Eicosanol
17.331-17.371	773	780	87.8	Abieta-8,11,13-triene
17.382-17.405	604	621	12.9	1-[1-methyl-2-(pentadecyloxy)ethoxy]-octadecane
18.134-18.184	648	688	5.89	7,8-epoxy-2-methyl-Nonadecane
18.191-18.225	703	730	30.0	Arachidic acid
18.343-18.394	788	831	5.23	Heptacosane
19.028-19.058	795	832	5.39	Docosene
19.102-19.146	726	742	36.2	Heptatriacontanol
19.213-19.240	611	652	12.4	Behenyl chloride
21.379-21.446	759	856	73.0	Oleanitrile
22.054-22.104	861	872	7.25	Phthalic acid 2-methylbutyl octyl ester
22.320-22.350	751	784	56.3	Gondamide
22.694-22.742	736	783	9.37	Heptacosane
22.917-22.954	793	803	38.4	1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl-3-methylene-Cyclopentane carboxylate
24.735-24.887	852	879	84.4	Erucamide

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by

the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

1.3.3 GC-MS Analysis of *Sciadopitys verticillata* Extracts

To compare the chemical compositions of *S. verticillata* resin and Baltic amber, bundles of *S. verticillata* cladodes and sections of *S. verticillata* stems were extracted using the conservative and Soxhlet extraction techniques with dichloromethane as the solvent. Extraction procedures were adapted from previous work.[21,33] Both cladodes and stems were extracted because resin composition can vary between different tissues within the same plant.[9] Following one round of extraction, the resulting crude extracts were filtered, concentrated, and analyzed via GC-MS as previously described for the Baltic amber extracts.

GC-MS analysis resulted in a comprehensive survey of the chemical composition of *S. verticillata* resin as 49 unique compounds were identified in the four extracts tested. **Figures 1.13-1.16** present gas chromatograms of the *S. verticillata* resin extracts. The compounds identified in the *S. verticillata* extracts as well as their acquisition times, match factors, reverse match factors, and probability values are provided in **Tables 1.11-1.14**.

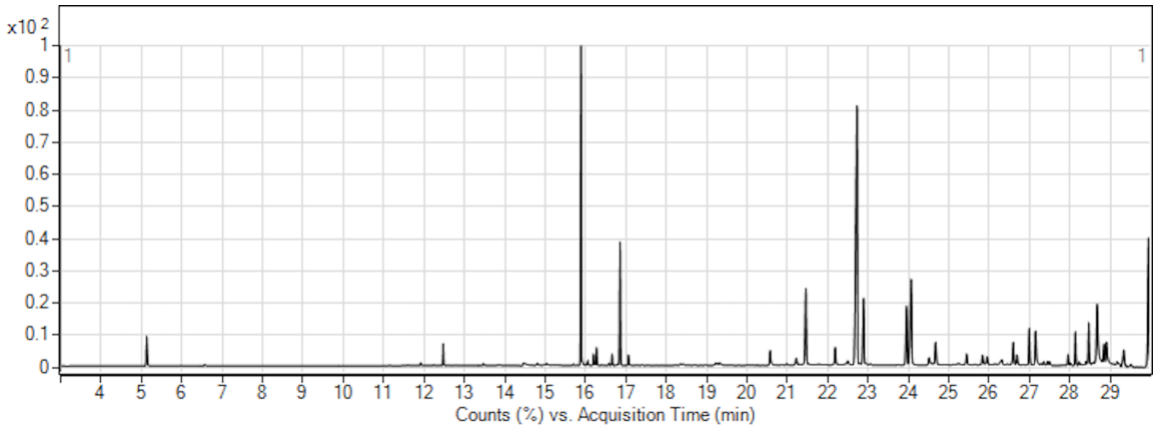


Figure 1.13. Gas chromatogram of the *Sciadopitys verticillata* cladode resin extract produced via conservative extraction with dichloromethane.

Table 1.11. Compounds identified in the *Sciadopitys verticillata* cladode resin extract produced via conservative extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
5.111-5.151	844	844	11.1	β-Ocimene
12.468-12.488	838	854	16.9	Germacrene D
15.861-15.905	824	824	22.8	Verticillol
16.050-16.067	789	791	19.6	Verticillol
16.182-16.209	806	806	19.0	Biformene
16.256-16.290	845	845	32.3	Verticillol
16.644-16.668	852	863	43.3	Kaurene
16.819-16.883	857	860	61.9	Kaurene
17.045-17.086	712	734	11.8	Squalene
20.550-20.597	669	714	15.7	Methyl retinoate
21.194-21.259	685	718	12.3	(5β)-Cholest-23-ene
21.424-21.481	592	630	9.33	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
22.152-22.210	746	746	12.8	3,17-diacetoxy-Androstan-1-one
22.642-22.749	619	626	24.4	3-acetoxy-Pregna-5,14-diene-3,20-diol-18-carboxylic acid lactone
22.851-22.915	594	616	13.3	Methyl (3β)-acetoxy-23,24-bisnor-(5β)-chol-5-enoate
23.913-23.984	602	614	21.6	16,17-epoxy-Pregnenolone

24.014-24.095	685	692	10.6	3-ethyl-3-hydroxy-Androstan-17-one
24.490-24.547	611	631	20.3	Gibberellin A1
24.649-24.696	608	642	9.87	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
25.411-25.475	657	675	8.14	Androsta-1,4-diene-3,11,17-trione
25.806-25.863	858	872	87.3	δ-Tocopherol
25.930-25.974	677	708	14.1	3-ethyl-3-hydroxy-Androstan-17-one
26.565-26.625	816	852	6.08	Eicos-9-en-1-ol
26.676-26.716	895	895	49.6	β-Tocopherol
26.963-27.020	684	834	55.6	Nonadecan-10-one
27.121-27.192	779	868	9.55	Germacrane
27.931-27.971	688	797	8.47	Trogodermal
28.123-28.157	825	828	17.0	Triacontane-1,30-diol
28.444-28.484	796	840	8.46	Octadec-17-ynoic acid
28.636-28.727	706	729	13.1	2-hexadecyl-1,1'-Bicyclopentane
28.821-28.862	657	747	4.56	Linoleoyl chloride
28.879-28.936	757	804	4.39	Octadec-17-ynoic acid
29.297-29.375	651	690	4.49	Hexadec-9-enal
29.897-29.982	766	831	6.32	Octadec-17-ynoic acid

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect

match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

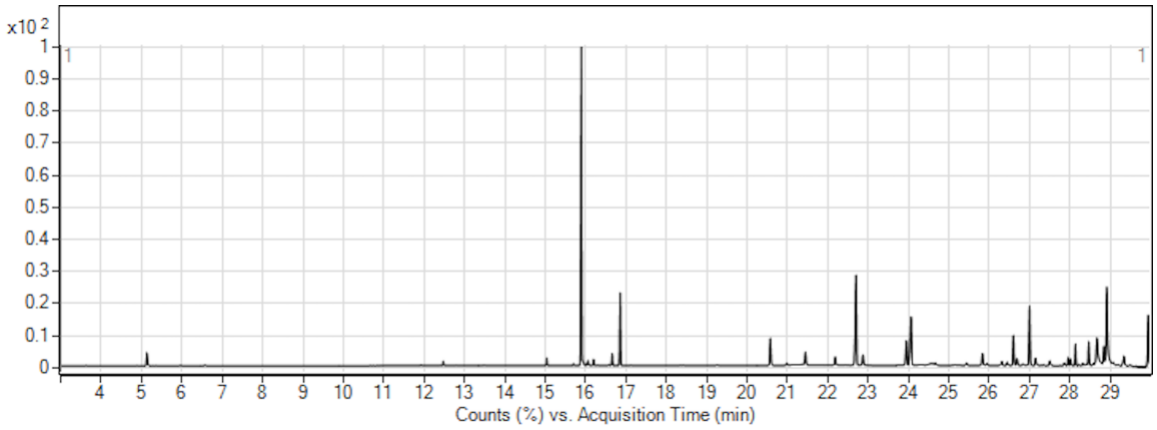


Figure 1.14. Gas chromatogram of the *Sciadopitys verticillata* cladode resin extract produced via Soxhlet extraction with dichloromethane.

Table 1.12. Compounds identified in the *Sciadopitys verticillata* cladode resin extract produced via Soxhlet extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
5.113-5.170	846	846	11.4	β-Ocimene
15.017-15.057	846	881	69.4	Phytone
15.860-15.914	826	826	25.6	Verticillol
16.042-16.099	804	807	26.3	Biformene
16.177-16.217	803	804	19.6	Biformene
16.629-16.690	858	867	43.9	Kaurene
16.828-16.875	854	856	60.4	Kaurene
20.549-20.603	666	711	16.2	Methyl retinoate
21.426-21.476	603	636	9.75	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
22.161-22.215	749	750	13.9	3,17-diacetoxy-Androstan-1-one
22.654-22.724	613	620	23.1	3-acetoxy-Pregna-5,14-diene-3,20-diol-18-carboxylic acid lactone
22.846-22.890	589	608	14.5	Methyl (3β)-acetoxy-23,24-bisnor-(5β)-chol-5-enoate
23.912-23.976	593	604	19.2	16,17-epoxy-Pregnenolone
24.010-24.087	679	685	10.6	3-ethyl-3-hydroxy-Androstan-17-one
25.814-25.848	881	881	91.2	δ-Tocopherol
26.287-26.341	735	836	9.79	Trogodermal

26.573-26.631	822	859	6.75	Eicos-9-en-1-ol
26.664-26.712	901	901	51.6	β-Tocopherol
26.958-27.035	689	840	59.8	Nonadecan-10-one
27.130-27.177	759	861	11.5	Germacrane
27.477-27.548	771	790	35.6	α-Tocopherol
27.936-27.977	743	825	11.7	Hexadec-9-enal
28.004-28.027	743	814	4.47	epoxy-Cyclodecane
28.112-28.162	829	832	17.3	Triacontane-1,30-diol
28.439-28.486	781	852	6.20	14-methyl-Hexadec-8-yn-1-ol
28.631-28.722	706	739	6.39	2-hexadecyl-1,1'-Bicyclopentane
28.823-28.857	632	749	3.90	Octadec-9-en-1-ol
28.881-28.962	754	786	3.84	(*Z*,*Z*)-Octadeca-2,13-dien-1-ol
29.296-29.366	726	814	5.51	Hexadec-9-enal
29.903-29.974	740	792	6.01	Octadec-17-ynoic acid

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

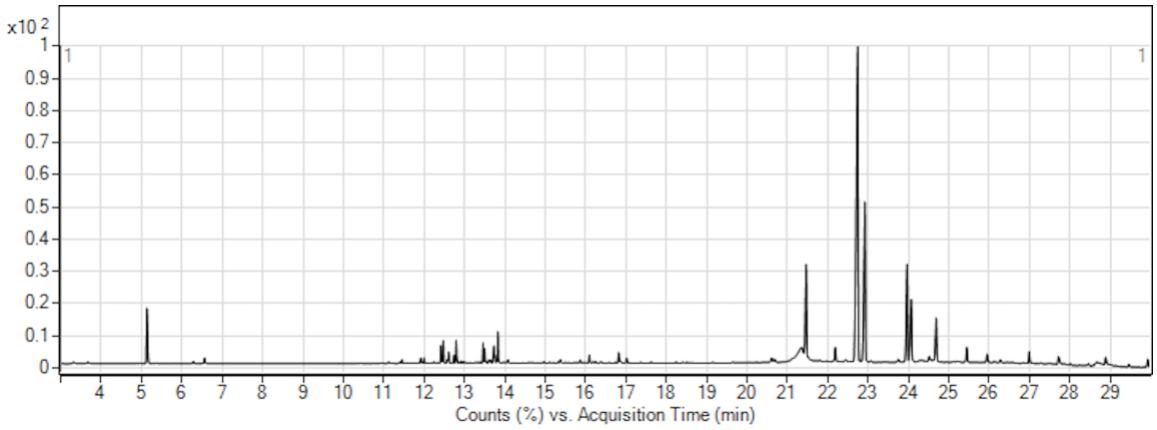


Figure 1.15. Gas chromatogram of the *Sciadopitys verticillata* stem resin extract produced via conservative extraction with dichloromethane.

Table 1.13. Compounds identified in the *Sciadopitys verticillata* stem resin extract produced via conservative extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
5.109-5.160	859	861	13.8	Car-3-ene
12.396-12.423	876	880	16.8	γ-Muurolene
12.470-12.490	834	851	15.9	Germacrene D
12.598-12.622	879	881	17.7	α-Muurolene
12.783-12.810	832	832	53.9	δ-Cadinene
13.448-13.472	825	826	30.6	α-Eudesmol
13.492-13.519	772	797	11.8	β-Ionone
13.708-13.741	840	861	28.8	Cadinol
13.806-13.839	832	835	23.6	Cadinol
16.801-16.852	860	865	4.20	Hexadecanol
21.314-21.399	729	781	3.32	(*Z*,*Z*)-Octadeca-2,13-dien-1-ol
21.426-21.490	615	652	9.59	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
22.164-22.205	742	743	13.3	3,17-diacetoxy-Androstan-1-one
22.657-22.765	618	626	21.2	3-acetoxy-Pregna-5,14-diene-3,20-diol-18-carboxylic acid lactone
22.866-22.937	587	612	17.0	Methyl (3β)-acetoxy-23,24-bisnor-(5β)-chol-5-enoate
23.908-23.996	603	616	21.1	16,17-epoxy-Pregnenolone acetate

24.013-24.097	690	697	11.6	3-ethyl-3-hydroxy-Androstan-17-one
24.640-24.718	620	656	11.0	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
25.416-25.464	658	674	9.05	Androsta-1,4-diene-3,11,17-trione
25.922-25.980	683	706	11.1	3-ethyl-3-hydroxy-Androstan-17-one
26.975-27.022	611	808	31.0	Nonadecan-10-one
27.697-27.767	566	630	8.09	Limonen-6-ol pivalate
29.903-29.960	745	853	31.4	Sitostenone

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

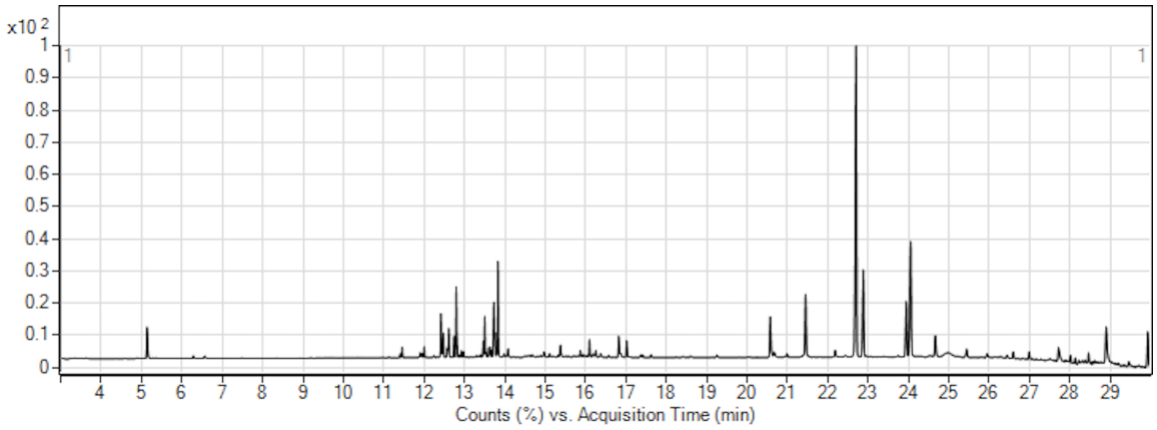


Figure 1.16. Gas chromatogram of the *Sciadopitys verticillata* stem resin extract produced via Soxhlet extraction with dichloromethane.

Table 1.14. Compounds identified in the *Sciadopitys verticillata* stem resin extract produced via Soxhlet extraction with dichloromethane.

Acquisition Time (min)	Match Factor[a]	Reverse Match Factor[b]	Probability[c] (%)	Compound Name
5.115-5.162	847	848	10.6	Car-3-ene
12.401-12.425	874	877	17.5	γ-Muurolene
12.465-12.486	843	866	17.6	Germacrene D
12.600-12.620	884	887	20.8	α-Muurolene
12.725-12.752	848	850	11.5	γ-Cadinene
12.779-12.813	828	829	51.8	δ-Cadinene
13.484-13.518	770	774	11.4	Alloaromadendrene oxide
13.713-13.740	844	858	26.6	Cadinol
13.804-13.845	839	843	23.9	Cadinol
16.803-16.844	872	884	6.18	Docosene
20.554-20.608	683	726	18.2	Methyl retinoate
21.421-21.472	597	631	9.66	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
22.639-22.727	615	622	20.5	3-acetoxy-Pregna-5,14-diene-3,20-diol-18-carboxylic acid lactone
22.841-22.906	589	608	13.7	Methyl (3β)-acetoxy-23,24-bisnor-(5β)-chol-5-enoate
23.911-23.982	594	604	17.8	16,17-epoxy-Pregnenolone acetate
24.009-24.079	682	688	11.0	3-ethyl-3-hydroxy-Androstan-17-one

24.639-24.693	620	655	15.9	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-Naphthalen-2(1*H*)-one
28.863-28.934	814	826	50.3	Clionasterol
29.895-29.952	723	801	11.7	Sitostenone

[a]Comparison of the observed mass spectrum to the database mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [b]Comparison of the database mass spectrum to the observed mass spectrum, with 999 representing a perfect match and 700 representing a fair match.[45] [c]Comparison of the match factor returned by the reported compound to the match factors returned by the other compounds listed in the search output; the compounds that returned the highest probability values within each search output are reported.[45]

Two classes of compounds were primarily identified in *S. verticillata* resin: (1) diterpenes and diterpenoids such as biformene, kaurene, and verticillol (**Figure 1.9** and **Figure 1.17**) and (2) steroids such as clionasterol, 3,17-diacetoxy-androstan-1-one, and sitostenone (**Figure 1.17**). These compounds were represented by large and distinctive peaks in the gas chromatograms (**Figures 1.13-1.16**). All of the compounds reported above also returned match factors and reverse match factors of greater than 700 in at least one of the extracts tested (**Tables 1.11-1.14**); therefore, these compounds are likely present in *S. verticillata* resin. Some steroids, however, returned match factors and reverse match factors of less than 700, and in some cases less than 600 (**Tables 1.11-1.14**). These compounds likely require further purification and characterization to determine their identities.

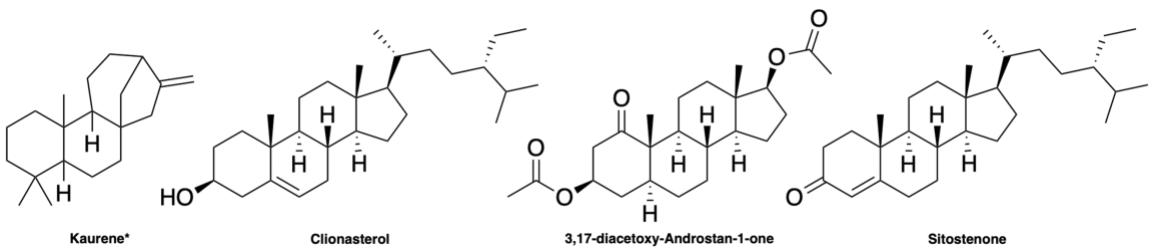


Figure 1.17. Compounds identified in at least one of the *Sciadopitys verticillata* resin extracts and represented by large peaks in the corresponding gas chromatograms: kaurene and steroids. *Stereochemistry to be determined.

Regarding the diterpenes and diterpenoids that are likely present in *S. verticillata* resin, biformene and kaurene are hypothesized to transform during the fossilization process into the abietane- and pimarane-type diterpenoids identified in Baltic amber extracts. Interestingly, the diterpenoid verticillol is the only compound identified in both *S. verticillata* cladode resin and at least one of the Baltic amber extracts (**Table 1.2**, **Table 1.4**, **Tables 1.6-1.7**, and **Tables 1.11-1.12**) and represented by large and distinctive peaks in the corresponding gas chromatograms (**Figure 1.2**, **Figure 1.4**, **Figures 1.6-1.7**, and **Figures 1.13-1.14**). Verticillol, and the verticillane scaffold, are unique to the species *S. verticillata*, and to our knowledge, this is the first report of verticillol as a possible constituent of Baltic amber, which would provide further evidence that the resin that became Baltic amber was produced by an extinct member of the family Sciadopityaceae.[47-49]

Other compounds identified in *S. verticillata* resin represent several other structural classes: monoterpenes such as car-3-ene and β-ocimene; sesquiterpenes and sesquiterpenoids such as alloaromadendrene oxide, γ-cadinene, δ-cadinene, cadinol, α-eudesmol, germacrane, germacrene D, α-muurolene, and γ-muurolene; the triterpene squalene; vitamin E forms such as α-tocopherol, β-tocopherol, and δ-tocopherol; aldehydes and ketones such as β-ionone, phytone, and trogodermal; and various hydrocarbons (**Figure 1.18**). Succinic acid was not identified in the extracts tested, so it is unknown whether the succinic acid in Baltic amber is a metabolism or fossilization product.

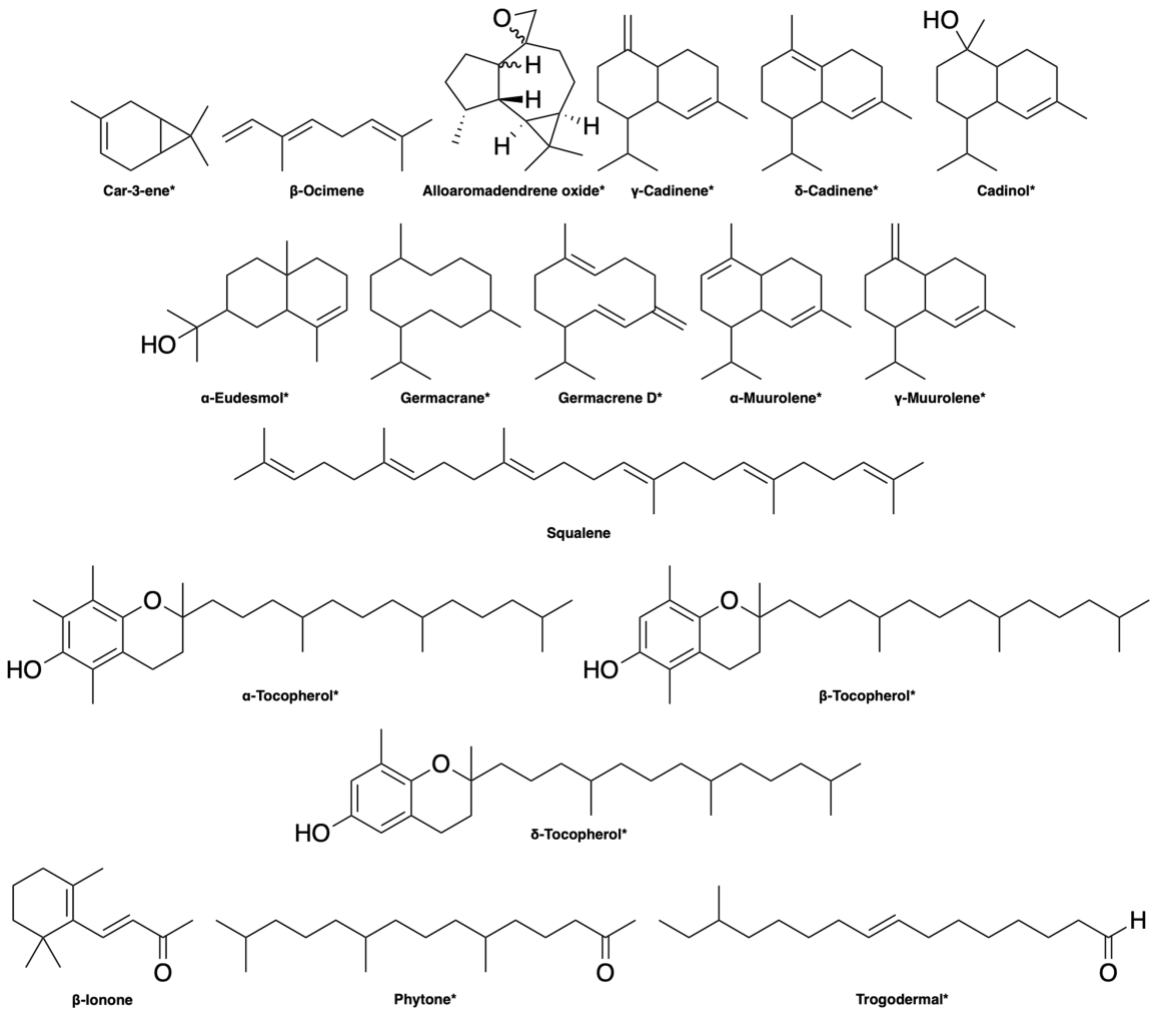


Figure 1.18. Compounds identified in at least one of the *Sciadopitys verticillata* resin extracts and represented by small peaks in the corresponding gas chromatograms: monoterpenes, sesquiterpenes and sesquiterpenoids, squalene, vitamin E forms, and aldehydes and ketones. *Stereochemistry to be determined.

Based on these data, the chemical compositions of *S. verticillata* resin and Baltic amber are very different; however, the identification of the diterpenes biformene and kaurene in *S. verticillata* resin as well as the diterpenoid verticillol in both *S. verticillata* resin and Baltic amber further supports the hypothesis that an extinct ancestor of *S. verticillata* produced the resin that fossilized and became Baltic amber.

1.3.4 Antibacterial Activity of Abietane-Type Diterpenoids

Toward the determination of the bioactivities of the constituents of Baltic amber, three of the most common abietane-type diterpenoids—abietic acid, dehydroabietic acid, and palustric acid—were purchased (**Figure 1.19**),[49] and their antibacterial activity was evaluated. The antibacterial activity of these compounds was specifically evaluated because plants produce resin as a chemical defense mechanism against pathogenic microorganisms and Baltic amber has been used in northern European traditional medicine to treat bacterial infections.[3,10,28-30] Standard MIC assays were performed by Pharmacology Discovery Services to evaluate the *in vitro* antibacterial activity of these compounds against 9 bacterial strains. MIC values represent the lowest concentration at which a compound completely inhibits visible growth of a bacterial colony.

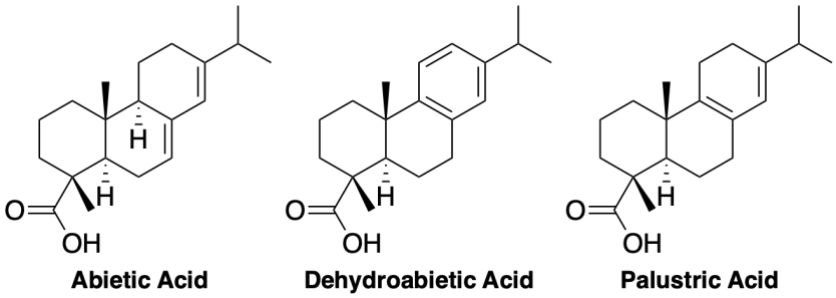


Figure 1.19. Abietic acid, dehydroabietic acid, and palustric acid.

These data show that abietic acid, dehydroabietic acid, and palustric acid are active against Gram-positive bacteria and not Gram-negative bacteria (**Table 1.15**), which means the composition of the bacterial cell membrane is important for the activity of these compounds. Previous studies suggest that abietic acid is incorporated into phospholipid membranes due to its amphipathic properties, thereby increasing membrane fluidity and permeability, disturbing membrane-bound proteins, and ultimately inhibiting bacterial cell growth.[50] This could explain the observed antibacterial activity of these compounds as Gram-positive bacteria have one phospholipid membrane whereas Gram-negative bacteria have two phospholipid membranes, which could protect them from these inhibitory effects. Furthermore, these data show that abietic acid, dehydroabietic acid, and palustric acid are active against antibiotic-resistant strains. This is significant because the treatment of bacterial infections has become more challenging due to the development of antibiotic resistance.

Table 1.15. *In vitro* antibacterial activity of abietane-type diterpenoids against various bacterial strains.

Bacterial Strain	Abietic Acid MIC (μg/mL)	Dehydroabietic Acid MIC (μg/mL)	Palustric Acid MIC (μg/mL)
Escherichia coli	>128	>128	>128
Escherichia coli, ciprofloxacin-resistant	>128	>128	>128
Klebsiella pneumoniae	>128	>128	>128
Pseudomonas aeruginosa, carbapenem-resistant	>128	>128	>128
Pseudomonas aeruginosa, ciprofloxacin-resistant	>128	>128	>128
Pseudomonas aeruginosa, imipenem-resistant	>128	>128	>128
Pseudomonas aeruginosa, meropenem-resistant	>128	>128	>128
Staphylococcus aureus, methicillin-resistant	32	64	32
Streptococcus pneumoniae, multidrug-resistant	128	128	128

1.4 Conclusion

Thus, chemical matter extracted from Baltic amber may be the first of many paleopharmaceuticals, specifically abietane-type diterpenoids for the treatment of infections caused by Gram-positive bacteria, including antibiotic-resistant strains. Furthermore, the different chemical compositions of resin and amber supports the study of chemofossils for novel drug scaffolds and new drugs. Ongoing work includes further exploration of extraction conditions such as the ratio of sample to solvent, solvent (e.g., acetone, benzene, hexanes, and methanol), and time; analysis via liquid chromatography-mass spectrometry; isolation of individual compounds from crude extracts via high-performance liquid chromatography; characterization of pure compounds; and biological activity studies to explain the medicinal significance of Baltic amber.

CHAPTER 2. Optimization of Anthrax Toxin Lethal Factor Inhibitors via Bioisosteric Replacement

2.1 Introduction

Bacillus anthracis is a Gram-positive, rod-shaped, sporulating bacterium and the causative agent of anthrax, a lethal infectious disease.[51] The Centers for Disease Control and Prevention have identified *B. anthracis* as a bioterrorism weapon with significant potential to be a severe threat to public health and safety because of the high mortality rates associated with anthrax infections.[52,53] *B. anthracis* has two plasmids, pXO1 and pXO2, which are required for the virulence of anthrax.[53] pXO2 encodes the polysaccharide capsule that protects the bacteria from phagocytosis by macrophages.[54,55] pXO1 encodes the tripartite anthrax exotoxin, which consists of the edema factor (EF), lethal factor (LF), and protective antigen (PA).[56] Mortality among patients with anthrax ultimately results from toxemia caused by the anthrax toxin.[53]

Current treatments approved by the Food and Drug Administration for anthrax infections include antibiotics such as ciprofloxacin, doxycycline, levofloxacin, and penicillin G, which inhibit bacterial cell growth but not the action of the anthrax toxin.[57] Therefore, antibiotics are only effective early in the pathology of the disease when diagnosis is challenging as there are multiple routes of exposure with non-specific signs and symptoms.[53] Monoclonal antibodies such as obiltoxaximab and raxibacumab and polyclonal antibodies such as Anthrasil® are also approved to treat anthrax infections by targeting PA, one component of the anthrax toxin;[57] however, antibody-based treatments present several limitations, including pharmacokinetic liabilities, rare but serious adverse

reactions, and high costs.[58,59] There is one vaccine currently approved for the prophylactic treatment of anthrax infections: BioThrax®, which stimulates the immune system to produce antibodies that target PA.[57] Unfortunately, BioThrax® is only recommended for populations at high risk of exposure to *B. anthracis* because of the vigorous vaccination schedule of five doses administered over 18 months followed by annual booster doses.[60] Thus, a key unmet need exists to discover and develop effective therapeutics that directly target the anthrax toxin, especially for use as bioterrorism countermeasures.

The mechanism of action of the anthrax toxin starts when PA binds to an anthrax toxin receptor on the surface of a host cell.[61] This binding event signals the proteolysis of PA,[62] which then forms heptamers capable of binding EF and/or LF monomers.[63] Following endocytosis into the host cell,[64] this complex undergoes a pH-dependent conformational change into a channel that translocates EF and LF into the cytoplasm where they exhibit their enzymatic activity.[65] EF is an adenylate cyclase,[66] and LF is a zinc metalloproteinase that catalyzes the proteolysis of mitogen-activated protein kinase kinases (MAPKKs) in macrophages,[67,68] thereby compromising host immune defense mechanisms and allowing the bacteria to replicate and produce more anthrax toxin.[69-73] LF also induces hypoxia late in the pathology of the disease, resulting in circulatory shock and ultimately host death.[74,75] Overall, LF is important for the lethality of anthrax infections and is therefore a promising target for the discovery and development of effective anthrax therapeutics.

Several scientific studies have been reported regarding the discovery and development of small-molecule LF inhibitors as potential anthrax therapeutics.[76-78,80-104] The first LF inhibitors were peptides designed to imitate the endogenous MAPKK substrate and interact with the catalytic zinc ion via a hydroxamic acid functional group, resulting in the

discovery of a compound with a modest inhibition constant (K_i): GM6001 (K_i = 2.1 mM) (**Figure 2.1**).[76] Subsequent studies of non-peptidic sulfonamide hydroxamates produced one of the most potent LF inhibitors currently known: MK-702 (K_i = 24 nM) (**Figure 2.1**).[77,78] The hydroxamic acid functional group, however, presents several limitations, including pharmacokinetic liabilities and selectivity issues.[79,80] Therefore, recent studies have been focused on the discovery of non-hydroxamate small molecules.[81-104] While progress has been made toward their development, there are no LF inhibitors currently approved to treat anthrax infections. Thus, our overall objective is to design novel, potent, selective, druglike small-molecule LF inhibitors.

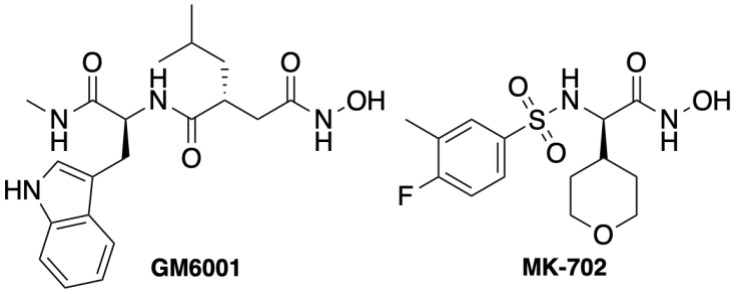


Figure 2.1. GM6001 and MK-702.

To achieve this objective, we have conducted and published several scientific studies regarding structural biology, molecular modeling, and high-throughput screening work.[105-111] LF is composed of four domains: (I) a binding domain that interacts with PA, (II) a large central domain, (III) a small helical domain that moves when a ligand binds to the enzyme, and (IV) a catalytic domain that interacts with the endogenous MAPKK substrate as well as small-molecule inhibitors.[108,110] We have identified four ligand-induced conformational states for the LF active site: the bioactive, open, tight, and tunnel conformations.[108,110] The LF active site is composed of a zinc ion coordinated to three residues (H686, H690, and E735) and three subsites: the large and solvent-exposed S1-S2 subsite, the small and hydrophobic S1' subsite, and the dynamic and solvent-exposed S2' subsite.[108,110] We have shown that a monodentate zinc-binding group and interactions with residues in at least two of the three subsites are sufficient for enzyme inhibition.[105] Furthermore, we have demonstrated that interactions with residues in the S2' subsite do not significantly impact biological activity.[111] We have also generated a validated, comprehensive pharmacophore map, which includes important features for the design of LF inhibitors,[106] as well as validated, optimized parameters for virtual screening via docking and scoring, topomer searching, and pharmacophore mapping.[107,109] Finally, we have performed a virtual high-throughput screen of approximately 35 million compounds and identified a series of dibenzylamines as prospective small-molecule LF inhibitors.[105] The results of these structural biology, molecular modeling, and high-throughput screening studies constitute important preliminary data toward achievement of our overall objective.

Recently, to identify novel small-molecule LF inhibitors, we performed an experimental high-throughput screen of approximately 228,000 compounds maintained by

the University of Minnesota Institute for Therapeutics Discovery and Development using two validated and optimized *in vitro* biochemical assays: a Förster Resonance Energy Transfer (FRET) assay and an orthogonal Mobility Shift Assay (MSA).[112] This resulted in the identification and confirmation of two compounds with significant LF inhibitory activity: **1** (GPHR-00194983, MSA $IC_{50} = 4.36 \pm 0.31$ μM) and **2** (GPHR-00220772, MSA $IC_{50} = 3.90 \pm 0.94$ μM) (**Figure 2.2**).[112] We then hoped to determine the enzyme-inhibitor complexes of these two compounds bound to LF via structural biology studies; however, both compounds proved too insoluble in the solutions required for crystallization. Therefore, our current objective is to design, synthesize, and evaluate potent, selective, druglike small-molecule LF inhibitors based on **1** and **2**, increasing the solubility of these two compounds for structural biology studies while maintaining their predicted binding affinities and experimental biological activities. Herein, we report efforts to optimize these compounds via bioisosteric replacement.

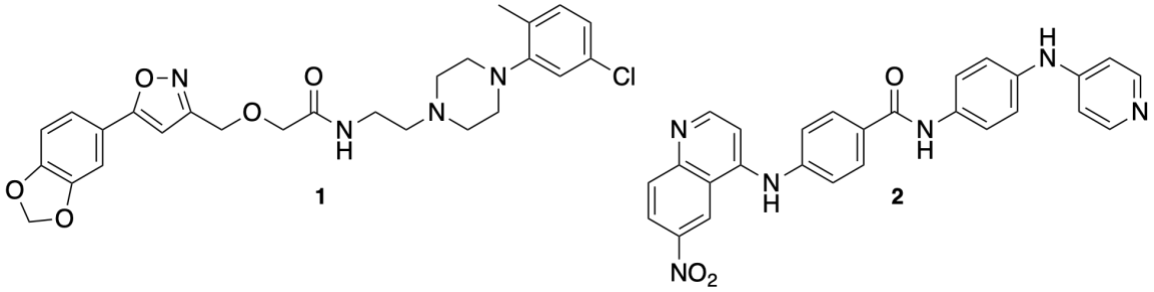


Figure 2.2. 1 (GPHR-00194983) and **2** (GPHR-00220772).

2.2 Experimental

2.2.1 General Experimental Procedures

Molecular modeling studies were carried out on Minnesota Supercomputing Institute workstations running CentOS Linux Release 6.10 (Final). The Labcyte Echo 550 liquid handler, Molecular Devices SpectraMax M2e microplate reader, and Caliper Life Sciences LabChip 3000 used during LF inhibitory activity evaluation were provided by the University of Minnesota Institute for Therapeutics Discovery and Development. Chemicals were purchased from commercial sources and used without additional purification.

2.2.2 Experimental Biological Materials

B. anthracis BH450/pSJ115 was acquired from Dr. Stephen H. Leppla, and LF was expressed from this strain. MAPKKide Peptide Substrate (o-Abz/Dnp) was synthesized by the University of Minnesota Genomics Center, and MAPKKide Peptide Substrate (DABCYL/FITC) was purchased from Celtek Peptides.

2.2.3 Library Generation, Ligand Preparation, and Physicochemical Property Prediction

Structures were sketched in ChemDraw Professional 16.0.1.4 (PerkinElmer Informatics, Inc.), and virtual compound libraries based on the sketched structures but with bioisosteric replacements were generated in Pipeline Pilot 9.0.2.1 (Accelrys Software, Inc.), using the SD Reader, Enumerate Bioisosteres, Remove Duplicate Molecules, 3D Coordinates, Add Hydrogens, Minimize Molecule, and SD Writer components, in that order. Ligand preparation was carried out using LigPrep in Maestro 10.4.017 (Schrödinger, Inc.): MMFFs was set as the force field, ionization states at pH 7.4 ± 1.0 were generated using Epik, metal binding states were added, and all stereoisomers were generated.

Physicochemical property prediction was carried out using QikProp in Maestro 10.4.017 (Schrödinger, Inc.). Defaults were used for all unspecified parameters.

2.2.4 Protein Preparation and Grid Generation

Protein preparation was carried out using the Protein Preparation Wizard in Maestro 10.4.017 (Schrödinger, Inc.): bond orders were assigned, hydrogens were added, zero-order bonds to metals were created, disulfide bonds were created, missing side chains were filled in using Prime, all waters were deleted, hydrogens of altered species were minimized using PROPKA at pH 7.4, and all hydrogens were minimized. Grid generation was carried out using Glide in Maestro 10.4.017 (Schrödinger, Inc.): the co-crystallized ligand was excluded and selected as the centroid for docking, ligands with length ≤ 25 Å were docked, and S655 and Y728 were selected as rotatable groups. Defaults were used for all unspecified parameters.

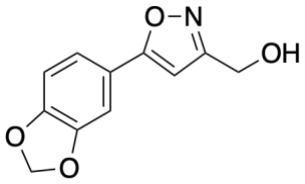
2.2.5 Virtual Screening

Ligand docking was carried out using Glide in Maestro 10.4.017 (Schrödinger, Inc.): extra precision (XP) was set as the precision, and the number of poses per ligand written in the output was set to 10. Defaults were used for all unspecified parameters.

2.2.6 Synthesis

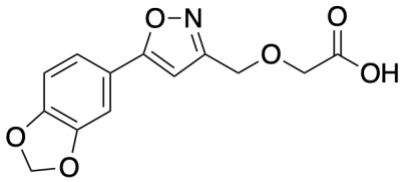
Synthesized compounds were characterized using a Bruker 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million and normalized to deuterated chloroform (7.26 ppm).

(5-(benzo[*d*][1,3]dioxol-5-yl)isoxazol-3-yl)methanol (1.17b)



To a round-bottom flask equipped with a stir bar was added 1 M $LiAlH_4$ (2.8 mL, 2.8 mmol). The solution was cooled to 0 °C, and a solution of **1.17a** (330 mg, 1.4 mmol) in tetrahydrofuran (THF, 5.2 mL) was added. The mixture was stirred for 90 min and allowed to warm to ambient temperature. The mixture was then cooled to 0 °C. Ethyl acetate (15 mL) was added, and then a saturated solution of Na_2SO_4 (30 mL) was slowly added. The solid was filtered, rinsed with diethyl ether (3 × 15 mL), and washed with a saturated solution of NaCl (2 × 15 mL). The combined organic layers were dried over $MgSO_4$, filtered using vacuum filtration, and concentrated under reduced pressure. The crude product was purified using flash chromatography to yield the desired product as a yellow oil (88.8 mg, 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (dd, 1H), 7.24 (s, 1H), 6.90 (d, 1H), 6.47 (s, 1H), 6.06 (s, 2H), 4.81 (s, 2H).

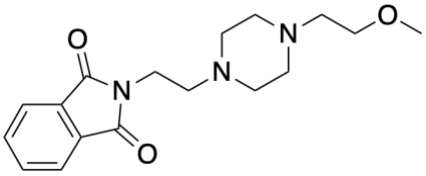
2-((5-benzo[*d*][1,3]dioxol-5-yl)isoxazol-3-yl)methoxy)acetic acid (1.17c)



To a round-bottom flask equipped with a stir bar was added THF (5 mL), and the solvent was cooled to 0 °C. NaH (60% dispersion in mineral oil, 46.5 mg, 1.16 mmol) was added, and then a solution of bromoacetic acid (59.3 mg, 0.43 mmol) in THF (0.5 mL) was added. The mixture was stirred for 30 min and allowed to warm to ambient temperature. A solution of **1.17b** (85 mg, 0.39 mmol) in dimethylformamide (DMF, 1.5 mL) was added dropwise,

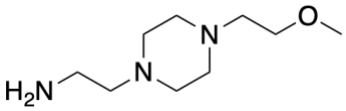
and then the mixture was stirred for 18 h at ambient temperature. H_2O (10 mL) was added to quench the reaction, which was then extracted with diethyl ether (3 × 10 mL). The aqueous layer was acidified to pH 2-3 and then extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried over $MgSO_4$, filtered using vacuum filtration, and concentrated under reduced pressure to yield the desired product as a pale-yellow solid (80.8 mg, 75%). ^{1}H NMR (440 MHz, $CDCl_3$) δ 7.38 (dt, 1H), 7.30 (t, 1H), 6.94 (d, 1H), 6.74 (s, 1H), 6.04 (d, 2H), 4.70 (s, 2H), 4.15 (s, 2H).

2-(2-(4-(2-methoxyethyl)piperazin-1-yl)ethyl)isoindoline-1,3-dione (1.17e)



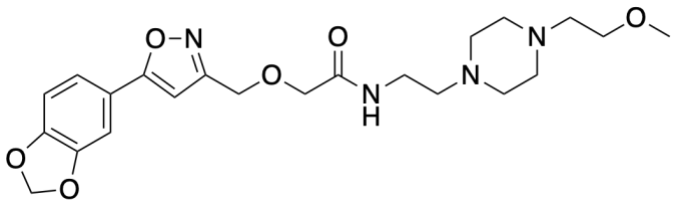
To a round-bottom flask equipped with a stir bar were added DMF (25 mL) and *N*-(2-bromoethyl)phthalimide (1.057 g, 4.2 mmol). **1.17d** (500 mg, 3.5 mmol) and K_2CO_3 (719 mg, 5.2 mmol) were then added, and the mixture was heated to 90 °C and stirred for 6 h. The mixture was allowed to cool to ambient temperature, and H_2O (30 mL) was then added. The mixture was extracted with ethyl acetate (4 x 25 mL) and then washed with a saturated solution of NaCl (4 x 30 mL). The combined organic layers were dried over $MgSO_4$, filtered using vacuum filtration, and concentrated under reduced pressure. The crude product was purified using flash chromatography to yield the desired product as a yellow-orange oil (172.5 mg, 16%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (m, 2H), 7.71 (m, 2H), 3.81 (t, 2H), 3.48 (t, 2H), 3.33 (s, 3H), 2.57 (m, 12H).

2-(4-(2-methoxyethyl)piperazin-1-yl)ethan-1-amine (1.17f)



To a round-bottom flask equipped with a stir bar were added ethanol (5 mL) and **1.17e** (172.5 mg, 0.54 mmol). Hydrazine monohydrate (0.075 mL) was then added, and the mixture was heated to 70 °C and stirred for 3 h. The mixture was then allowed to cool to ambient temperature. Volatiles were evaporated, and ethyl acetate (5 mL) was added. This organic layer was filtered using vacuum filtration and concentrated under reduced pressure to yield the desired product as a pale-yellow oil (90.9 mg, 89%). ^{1}H NMR (440 MHz, $CDCl_3$) δ 3.51 (t, 2H), 3.35 (d, 3H), 2.79 (t, 2H), 2.50 (m, 12H), 1.83 (s, 2H).

2-((5-(benzo[*d*][1,3]dioxol-5-yl)isoxazol-3-yl)methoxy)-*N*-(2-(4-(2-methoxyethyl)piperazin-1-yl)ethyl)acetamide (1.17)



To a round-bottom flask equipped with a stir bar were added dichloromethane (10 mL), **1.17f** (76 mg, 0.40 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (103.7 mg, 0.54 mmol), and 4-dimethylaminopyridine (82.6 mg, 0.68 mmol). **1.17c** (75 mg, 0.27 mmol) was then added, and the mixture was stirred for 20 h under ambient conditions. A saturated solution of $NaHCO_3$ (15 mL) was added to quench the reaction, which was then extracted with dichloromethane (3 × 15 mL) and washed with a saturated solution of NaCl (3 × 15 mL). The combined organic layers were dried over $MgSO_4$, filtered using vacuum filtration, and concentrated under reduced pressure. The crude product was purified using flash chromatography to yield the desired product as an oily

yellow solid (64.5 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$). δ 7.31 (dd, 1H), 7.22 (s, 1H), 7.02 (s, 1H), 6.89 (d, 1H), 6.43 (s, 1H), 6.04 (s, 2H), 4.68 (s, 2H), 4.06 (s, 2H), 3.45 (t, 2H), 3.38 (t, 2H), 3.32 (s, 3H), 2.50 (m, 12H).

2.2.7 Lethal Factor Inhibitory Activity Evaluation

LF inhibitory activity was evaluated using a FRET assay. Compounds were each dissolved in DMSO to a concentration of 10 mM, and 2 nL of each solution was added to 198 nL of DMSO in a 384-well microplate using a Labcyte Echo 550 liquid handler. An approximately three-fold serial dilution was then performed, resulting in seven additional solutions of decreasing concentration for each compound and an eight-point dose-response curve. The final compound concentrations ranged from 43.53 nM to 100.0 μM, and the final solvent concentration was 1% DMSO. 10 μL of 100 nM LF in 40 mM HEPES-KOH (pH 8.0) with 0.02% Triton X-100 was added to each well, resulting in final concentrations of 50 nM LF, 20 mM HEPES-KOH (pH 8.0), and 0.01% Triton X-100. Following pre-incubation for 15 min at 37 °C, 9.8 μL of 15 μM MAPKKide Peptide Substrate (o-Abz/Dnp) was added to each well to initiate the reaction, resulting in a final concentration of 7.35 μM MAPKKide Peptide Substrate (o-Abz/Dnp). Following incubation for 5 min at 37 °C, 5 μL of 50 mM EDTA was added to each well to terminate the reaction. Fluorescence was measured at excitation and emission wavelengths of 320 nm and 420 nm, respectively, using a Molecular Devices SpectraMax M2e microplate reader. The data was normalized to the negative controls, and IC_{50} values were calculated using Prism 5 (GraphPad, Inc.). Each compound was evaluated in duplicate, 43.53 nM to 100.0 μM GM6001 and 6.219 nM to 10.00 μM MK-702 were used as the positive controls, and DMSO with and without LF were used as the negative controls.

LF inhibitory activity was also evaluated using an MSA. Compounds were each dissolved in DMSO to a concentration of 10 mM, and 2 nL of each solution was added to 198 nL of DMSO in a 384-well microplate using a Labcyte Echo 550 liquid handler. An approximately three-fold serial dilution was then performed, resulting in seven additional solutions of decreasing concentration for each compound and an eight-point dose-response curve. The final compound concentrations ranged from 43.53 nM to 100.0 μM, and the final solvent concentration was 1% DMSO. 10 μL of 100 nM LF in 40 mM HEPES-KOH (pH 8.0) with 0.02% Triton X-100 was added to each well, resulting in final concentrations of 50 nM LF, 20 mM HEPES-KOH (pH 8.0), and 0.01% Triton X-100. Following pre-incubation for 15 min at 37 °C, 9.8 μL of 8 μM MAPKKide Peptide Substrate (DABCYL/FITC) was added to each well to initiate the reaction, resulting in a final concentration of 3.92 μM MAPKKide Peptide Substrate (DABCYL/FITC). Following incubation for 10 min at 37 °C, 4 μL of a 32.5 μM EDTA and 500 μM phenanthroline solution was added to each well to terminate the reaction. Fluorescence was measured and percent conversion was calculated using a Caliper Life Sciences LabChip 3000. The data was normalized to the negative controls, and IC_{50} values were calculated using Prism 5 (GraphPad, Inc.). Each compound was evaluated in duplicate, 43.53 nM to 100.0 μM GM6001 and 6.219 nM to 10.00 μM MK-702 were used as the positive controls, and DMSO with and without LF were used as the negative controls.

2.3 Results and Discussion

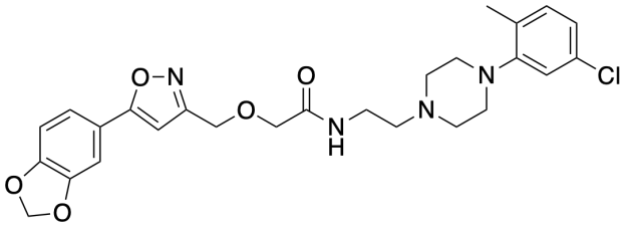
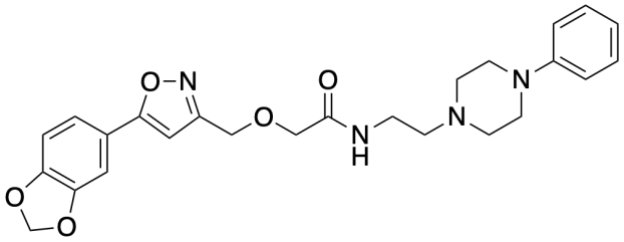
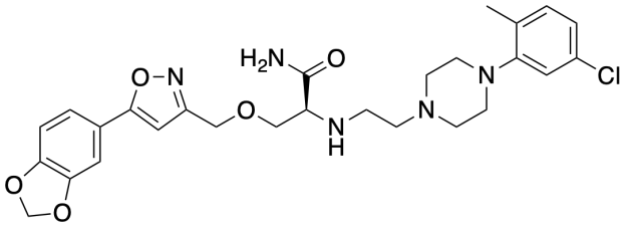
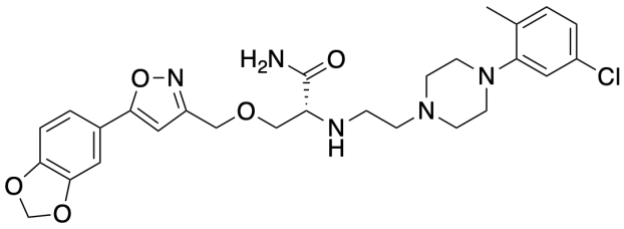
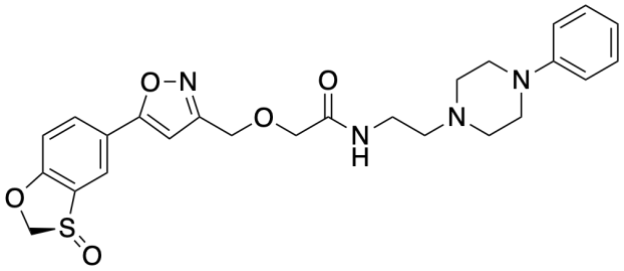
2.3.1 Optimization of GPHR-00194983 via Bioisosteric Replacement

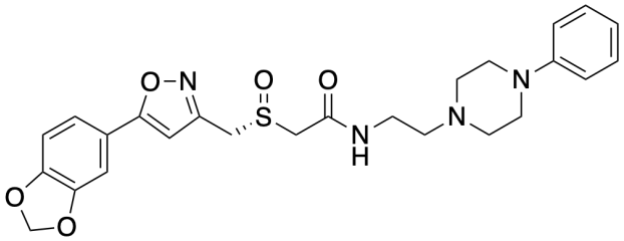
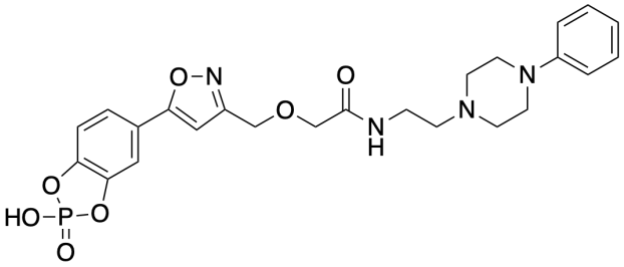
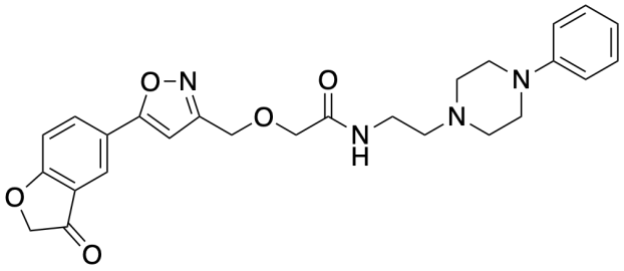
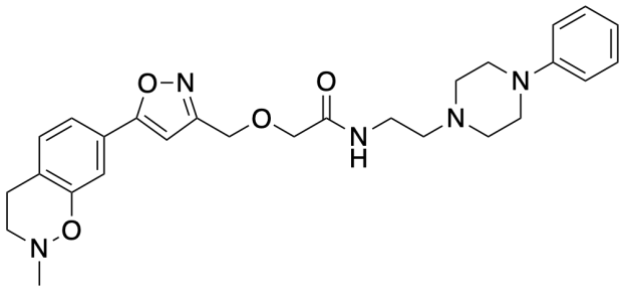
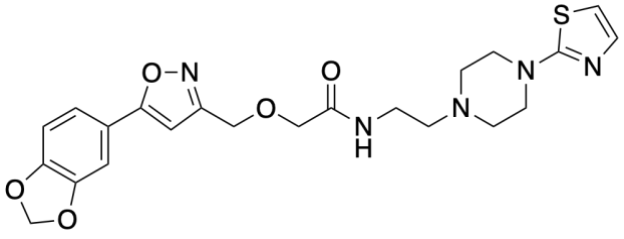
To increase the solubility of **1** for structural biology studies while maintaining its predicted binding affinity and experimental biological activity, a virtual library of

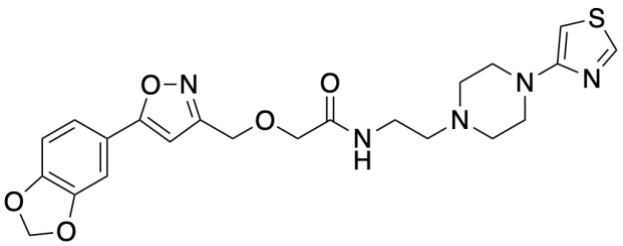
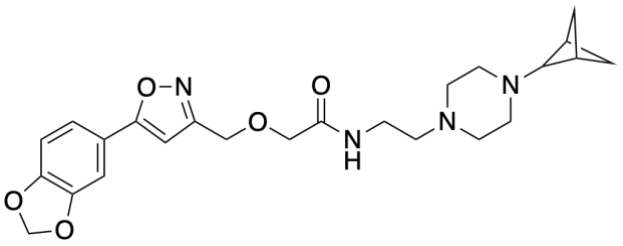
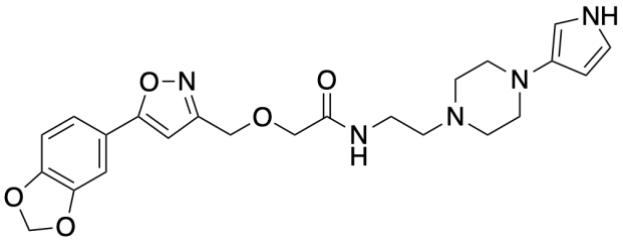
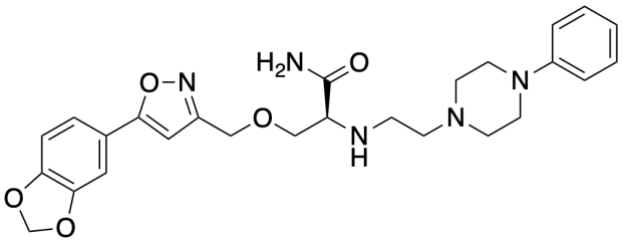
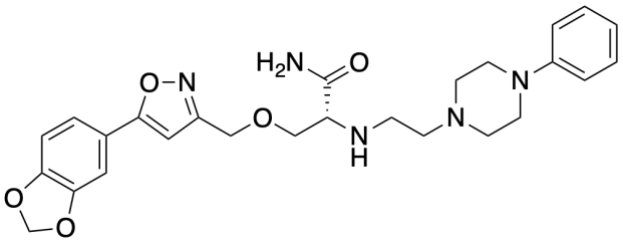
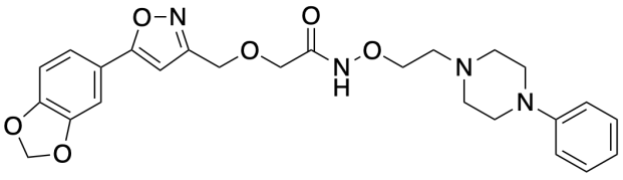
compounds based on **1** and **1.1**, an unsubstituted derivative (**Table 2.1**), was generated using the "Enumerate Bioisosteres" component within Pipeline Pilot. Bioisosteric enumeration resulted in a virtual library of 150 new compounds: 68 based on **1** and 82 based on **1.1**. Following library generation, these compounds were prepared for molecular modeling studies in Schrödinger Maestro. Values for conformation-independent aqueous solubility (CIQPlogS) were calculated using QikProp in Maestro to predict the solubility of these compounds for structural biology studies, and docking and scoring was carried out using Glide in Maestro to predict the affinity of these compounds for the LF active site as represented by 1YQY.pdb.[77,113-115]

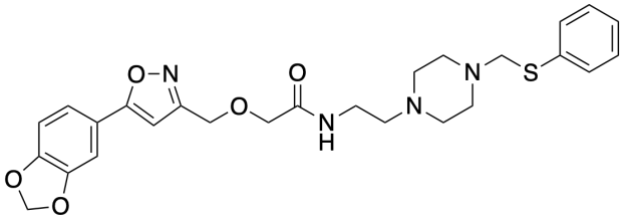
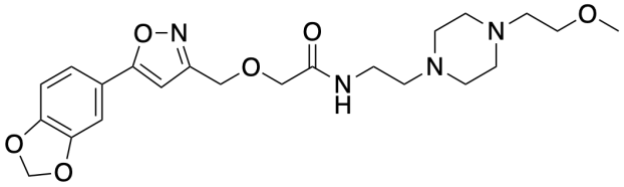
Based on the results of the molecular modeling studies, **1** returned a CIQPlogS value of -5.064 and a docking score of -6.389; therefore, to identify compounds with increased solubility and maintained predicted binding affinity, the hit criteria were defined as a CIQPlogS value > -4.000 and a docking score < -6.000. **Table 2.1** presents the compound IDs, structures, CIQPlogS values, and docking scores of the identified hits. In comparison to **1**, **1.1** has a more favorable CIQPlogS but a less favorable docking score, meaning the methyl group and the chlorine atom on the phenyl ring decrease the predicted solubility but increase the predicted binding affinity of **1**. As a result, only 2 of the 16 hit compounds are based on **1** while the remaining hit compounds are based on **1.1**.

Table 2.1. Identified hit compounds based on **1**.

Compound ID	Structure	CIQPlogS	Docking Score[a]
1		-5.064	-6.389
1.1		-4.085	-5.403
1.2		-3.954	-8.003
1.3		-3.954	-6.395
1.4		-3.384	-7.099

1.5		-3.830	-6.043
1.6		-2.909	-6.085
1.7		-3.811	-6.858
1.8		-3.370	-6.569
1.9		-3.754	-6.477

1.10		-3.754	-6.115
1.11		-2.153	-6.007
1.12		-2.585	-6.561
1.13		-3.015	-8.033
1.14		-3.015	-7.190
1.15		-3.705	-7.084

1.16		-3.343	-6.471
1.17		-1.213	-6.761

[a]Docking scores for the highest scoring conformations for each compound are reported.

Ultimately, **1.17** was prioritized for synthesis because of its high predicted solubility, similar predicted binding affinity to **1**, uniformity amongst its predicted binding modes, and synthesizability. **1.17** was synthesized using the convergent synthetic route outline in **Figure 2.3**.

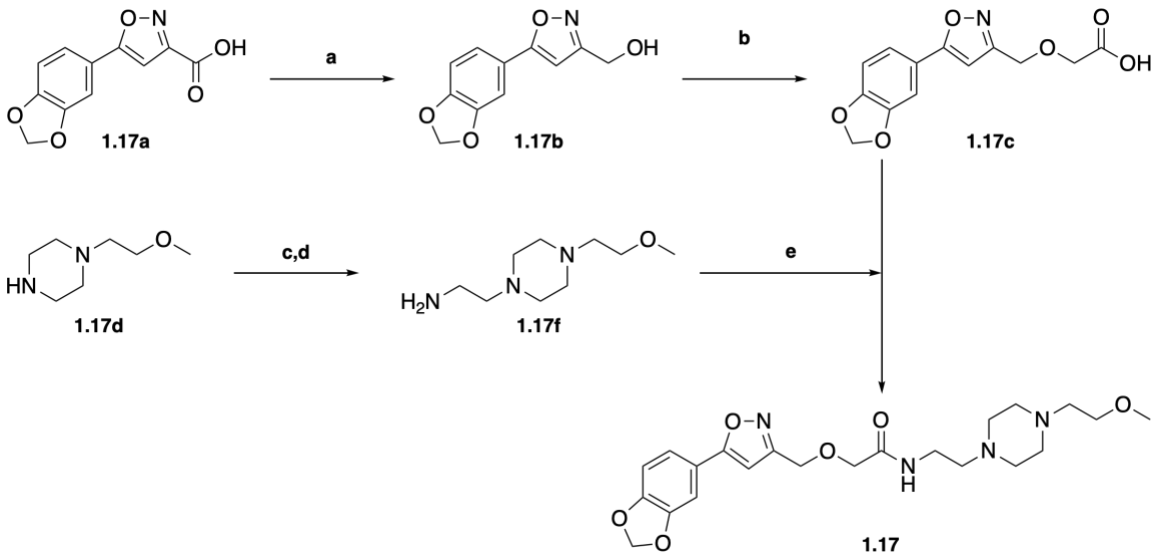


Figure 2.3. Synthesis of **1.17**. Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C (29%); (b) NaH, bromoacetic acid, DMF/THF (3:11), room temperature (75%); (c) *N*-(2-bromoethyl)phthalimide, K_2CO_3, DMF, 90 °C (16%); (d) hydrazine monohydrate, ethanol, 70 °C (89%); (e) EDC·HCl, DMAP, DCM, room temperature (53%).

The *in vitro* inhibitory activities of **1.17** and **1.17a-f** against LF were evaluated using a validated FRET assay, and IC_{50} values were calculated based on the resulting eight-point dose-response curves. IC_{50} values represent the concentration at which a compound exhibits half of its maximal inhibitory effect. Based on the results of the FRET assay, the LF inhibitory activity of **1.17** could not be evaluated because it is autofluorescent. The *in vitro* inhibitory activities of **1.17** and **1.17a-f** against LF were then evaluated using an orthogonal and validated MSA, and IC_{50} values were calculated based on the resulting eight-point dose response curves. Based on the results of the MSA, **1.17** is inactive. Therefore, the substituted phenyl ring is involved in a significant interaction with the LF active site and replacing it with a methoxyethyl chain abolishes the LF inhibitory activity of **1**. However, based on work by Elbek Kurbanov,[112] **1** is a false positive and should not be prosecuted further.

2.3.2 Optimization of GPHR-00220772 via Bioisosteric Replacement

To increase the solubility of **2** for structural biology studies while maintaining its predicted binding affinity and experimental biological activity, a virtual library of compounds based on **2** and an unsubstituted derivative was generated using the "Enumerate Bioisosteres" component within Pipeline Pilot. Bioisosteric enumeration resulted in a virtual library of 104 new compounds: 55 based on **2** and 49 based on its unsubstituted derivative. Following library generation, these compounds were prepared for molecular modeling studies in Schrödinger Maestro. Values for CIQPlogS were calculated using QikProp in Maestro to predict the solubility of these compounds for structural biology studies. Unfortunately, the highest CIQPlogS value returned by the compounds in this library is -5.919, which is less than **1**, a compound already known to be insoluble in the

solutions required for crystallization. Therefore, bioisosteric replacement alone is not sufficient for increasing the solubility of **2**.

2.4 Conclusion

Thus, **1** is a false positive, and bioisosteric replacement is not sufficient to increase the solubility of **2** for structural biology studies. Ongoing work includes further design, synthesis, and evaluation of small-molecule LF inhibitors based on **1** and **2** via structure-based design; identification of functional groups that bind to the LF active site via biophysical fragment-based screening and structural biology studies; and lead optimization toward the discovery and development of novel, potent, selective, druglike small-molecule inhibitors as effective anthrax therapeutics, especially for use as bioterrorism countermeasures.

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