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Terpene Hydroperoxides in Allergic Contact Dermatitis: Synthesis, Lipid Peroxidation, and Immunological Implications

A thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy
(PhD)

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Under the supervision of

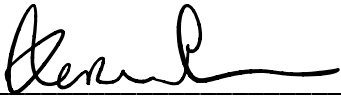
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Declaration

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"If we don't feel stupid it means we aren't really trying"

Schwartz, M. A. (2008). The importance of stupidity in scientific research. *Journal of Cell Science*, 121(11), 1771-1771.

Abstract

Allergic contact dermatitis (ACD) is a type IV hypersensitivity reaction mediated by T cells. Contact allergens are typically too small to elicit an immune response on their own and must bind to larger macromolecules, classically proteins, to exert immunogenic effects. However, the potential role of lipids in facilitating these effects has remained largely unexplored. This work reports the successful synthesis and NMR characterisation of non-commercially available contact allergen hydroperoxides: limonene-2-hydroperoxide (Lim-2-OOH), linalool-6/7-hydroperoxides (Lin-6/7-OOH), and geraniol-6/7-hydroperoxides (Ger-6/7-OOH). Lipidomic analysis of HaCaT keratinocytes treated with Lim-2-OOH and Lin-6/7-OOH revealed remodeling of the lipidome, with upregulation of plasmalogen/plasmenyl phospholipids (O-/P-PC and O-/P-PE), sphingolipids (HexnCer), and triacylglycerols, alongside a decrease in polyunsaturated phospholipids (PC and PE). Incubation of phosphatidylcholine 16:0/18:1 (PLPC) liposomes with the synthesised hydroperoxides under biomimetic conditions induced oxidation predominantly yielding long-chain products, with lower levels of electrophilic short-chain products. The degree of lipid peroxidation was equal to or greater than that induced by hydrogen peroxide (H_2O_2), with Lim-2-OOH causing significantly more oxidation than all other hydroperoxides tested. Immunological studies demonstrated that Ger-6/7-OOH differentially modulated CD1 protein responses. CD1a tetramers loaded with Ger-6/7-OOH showed reduced tetramer-positive events, whereas CD1d tetramers loaded with Ger-6/7-OOH showed a marked increase in tetramer-positive T cells, comparable to the positive control α -GalCer. Functional assays further revealed that terpenes and their hydroperoxides exhibited diverse modes of interaction with CD1 proteins: some acted as agonists, eliciting T cell activation and cytokine responses; others acted as antagonists or displaced endogenous ligands, reducing CD1-TCR binding and functional outputs. Overall, this work demonstrates that terpene hydroperoxide contact allergens can modify lipids under biomimetic conditions by promoting lipid peroxidation, and can also directly engage CD1-mediated pathways to initiate immunogenic effects independently of classical haptenation mechanisms. These findings highlight a potential role for unconventional T cells in ACD and expand our understanding of lipid-driven immunogenicity.

Keywords: skin, allergy, terpene hydroperoxides, lipidomics, oxidation, CD1 proteins, T cells, inflammation

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List of Abbreviations

AA	Arachidonic acid
AAPH	2,2'azobis(2-amidinopropane)dihydrochloride
ACD	Allergic contact dermatitis
ACN	Acetonitrile
AD	Atopic dermatitis
AdA	Adrenic acid
ADRP	Adipose differentiation related protein
AP	Adaptor protein
APC	Antigen presenting cell
BC	Benzalkonium chloride
BuOH	Butanol
CD	Contact dermatitis
CD1	Cluster of differentiation 1
CE	Cholesterol ester
CER	Ceramide
CHOL	Cholesterol
CHOLSF	Cholesterol sulfate
CL	Cardiolipin
COSY	Correlation spectroscopy
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DCM	Dichloromethane
dDC	dermal dendritic cell
DEPT-135	Distortionless enhancement by polarization transfer at 135°
DG	Diacylglycerol
DLN	Draining lymph node
DMAPP	Dimethylallyl pyrophosphate
DMEM	Dubelcco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMSO- <i>d</i> ₆	Deuterated dimethyl sulfoxide
DNFB	2,4-Dinitrofluorobenzene
EC3	Estimated concentration to cause a stimulation index of 3
EO	Essential oil
eoxPL	Enzymatically oxidized phospholipid
ESI	Electrospray ionization
EtOAc	Ethyl acetate
FA	Fatty acid
FBS	Foetal bovine serum
FFA	Free fatty acid
FPP	Farnesyl phosphate
FSC	Forward scatter
GBA1	β-Glucocerebrosidase
Ger-6-OOH	Geraniol-6-hydroperoxide
Ger-7-OOH	Geraniol-7-hydroperoxide
GGPP	Geranylgeranyl pyrophosphate
GL	Glycerolipid

GlcCer	Glucosylcermide
GPL	Glycerophospholipid
GPP	Geranyl phosphate
GSL	Glycosphingolipid
HaCaT	Immortalized human keratinocytes
Hex _n Cer	Hexosyl ceramide
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
ICD	Irritant contact dermatitis
IL	Interleukin
iNKT	Invariant natural killer T (cell)
IPA	Isopropanol
IPP	Isopentyl pyrophosphate
KC	Keratinocyte
LA	Linoleic acid
LC	Langerhans cell
LC-MS	Liquid chromatography mass spectrometry
Lim-2-OOH	Limonene-2-hydroperoxide
Lin-6-OOH	Linalool-6-hydroperoxide
Lin-7-OOH	Linalool-7-hydroperoxide
LLNA	Local lymph node assay
LOX	Lipoxygenase
LPC	Lyso phosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPS	Lipopolysaccharide
LTQ	Linear ion trap quadrupole
MeOH	Methanol
MEP	Methylerythritol 4-phosphate
MG	Monoacylglycerol
MHC	Major histocompatibility complex
MM-LDL	Minimally modified low-density lipoprotein
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFA	Monounsaturated fatty acid
MVA	Mevalonate
NB-UVB	Narrowband ultraviolet B
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
OA	Oleic acid
oxPL	Oxidized phospholipid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC O-	Plasmany PC (alkyl-acyl)
PC P-	Plasmenyl PC (alkenyl-acyl)
PE O-	Plasmany PE (alkyl-acyl)
PE P-	Plasmenyl PE (alkenyl-acyl)
PG	Phosphatidylglycerol

PI	Phosphatidylinositol
PIM	Phosphatidylinositol mannose
PL	Phospholipid
PLPC	1-palmitoyl-2-(linoleoyl)-sn-glycero-3-PC
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
QQQ	Triple quadrupole
Q-TOF	Quantum time of flight
SAP	Saposin
SB	Stratum basale
SC	Stratum corneum
SDS	Sodium dodecyl sulphate
SG	Stratum granulosum
SL	Sphingolipid
SM	Sphingomyelin
SS	Stratum spinosum
SSC	Side scatter
T _C	Cytotoxic T cell
T _C 1	Type 1 cytotoxic T cell
TCR	T cell receptor
T _{EFF}	Effector T cell
TG	Triacylglycerol
T _H	Helper T cell
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOF-SIMS	Time of flight secondary ion mass spectroscopy
T _{REG}	Regulatory T cell
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
α-GalCer	α-Galactosylceramide

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Chapter 1: Introduction

1.1 Overview of Skin Components

Allergic contact dermatitis (ACD) is a form of dermatitis/eczema caused by contact with an allergen. It causes the skin to become itchy, blistered, or swollen. To investigate the underlying mechanisms of ACD, it is necessary to have an extensive knowledge of the skin components.

Mammalian skin fulfils many functions, one of the most important being its role as a barrier preventing excessive water loss as well as attacks by xenobiotics and microbial agents [1]. It also contributes to heat and tactile sensing, thermoregulation, ultraviolet (UV) protection, and immune surveillance [2-5]. Human skin thickness varies depending on body location, but it is composed of three distinct layers: the hypodermis, dermis, and epidermis (Fig. 1.1). The hypodermis is rich in fat and attached to the underlying muscle tissue, providing cushioning and insulation. The dermis (1-2 mm in thickness) contains networks of collagen and elastin fibres within an extrafibrillar matrix of glycosaminoglycans and proteoglycans, which provide strength and elasticity. It also contains cutaneous receptors, nerve endings, blood vessels, sweat glands, sebaceous glands, and hair follicle roots [6]. The epidermis is divided into four sublayers, the innermost being the stratum basale (SB), then the stratum spinosum (SS), followed by the stratum granulosum (SG), with the outermost being the stratum corneum (SC). The three most inner layers are referred to collectively as the “viable epidermis” (50-100 μm in thickness), whereas the term “full-thickness epidermis” includes both viable epidermis and SC (10-20 mm in thickness) [7]. The epidermis lacks a vascular blood supply and is mainly composed of keratinocytes (KC), which migrate from the SB; differentiating as they move through a calcium (Ca^{2+}) gradient towards the SC [8]. During this journey, they undergo profound changes in structure and composition, until they are terminally transformed into flattened, keratin-rich, non-viable cells called corneocytes. These corneocytes are arranged into 10–25 layers, with the outermost layers continually shed in a process called desquamation, which is balanced by ongoing cell renewal [7]. Each corneocyte is surrounded by a crosslinked protein layer called the cornified envelope, to which a lipid monolayer is covalently attached [9]. This “lipid envelope” serves as an interface between the hydrophilic corneocyte surface and the lipophilic extracellular lipid matrix that surrounds them [10]. Late in KC differentiation,

specialized organelles called lamellar bodies appear in granular cells. These contain lipids (phospholipids [PLs], glucosylceramide [GlcCers], sphingomyelin [SM], and cholesterol [CHOL]), lipid hydrolases (β -glucocerebrosidase [GBA1], acidic sphingomyelinase, secretory phospholipase A₂, and neutral lipases), and proteases (kallikreins and cathepsins), which generate the barrier lipids of the SC [11]. As KCs approach the SG-SC interface, the lamellar bodies fuse with the plasma membrane and secrete their contents into the intercellular space. Glycosphingolipids (GSLs) are then hydrolysed to form ceramides (CERs), while PLs are converted into free fatty acids (FFAs) [12]. These lipids assemble into lamellar structures surrounding the corneocytes. The intercellular lamellae are oriented parallel to the corneocyte surface, with the lipid envelope acting as a template for the organization of the extruded lipids. If the enzymatic processes that generate these intercellular lipids are altered, the resulting lipid composition and organization are disturbed, leading to impaired skin barrier function, something which is associated with psoriasis and atopic dermatitis (AD) [13, 14], and may increase the chances of ACD [15]. In the SC, the impermeable cornified envelope forces the passage of xenobiotic substances through the intercellular pathway between corneocytes. Thus, lipids play a central role in maintaining the skin's barrier properties.

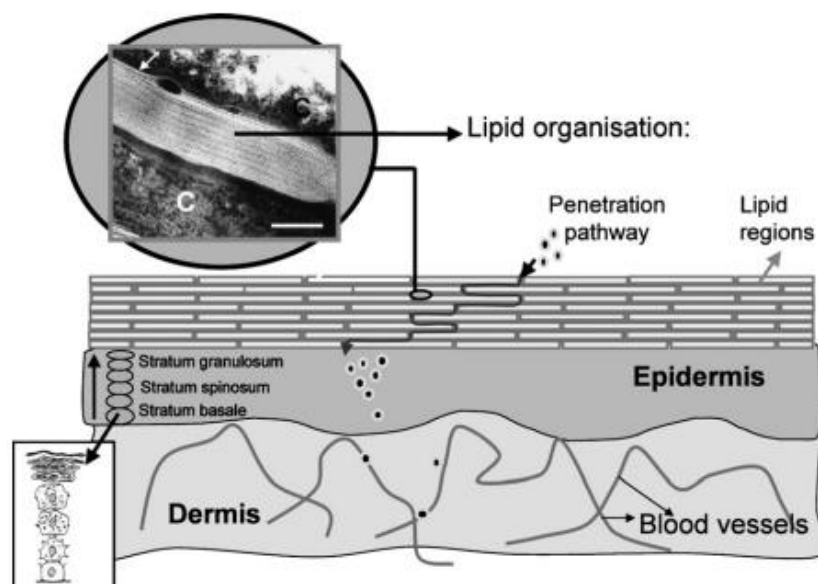


Figure 1.1. A schematic drawing of a skin cross-section.

The skin consists of three main layers: the hypodermis, dermis, and epidermis. Within the SB layer of the epidermis, cells actively proliferate. As they leave this layer, they begin to differentiate and migrate toward the skin surface. Terminal differentiation takes place at the SG-SC interface, where viable cells transform

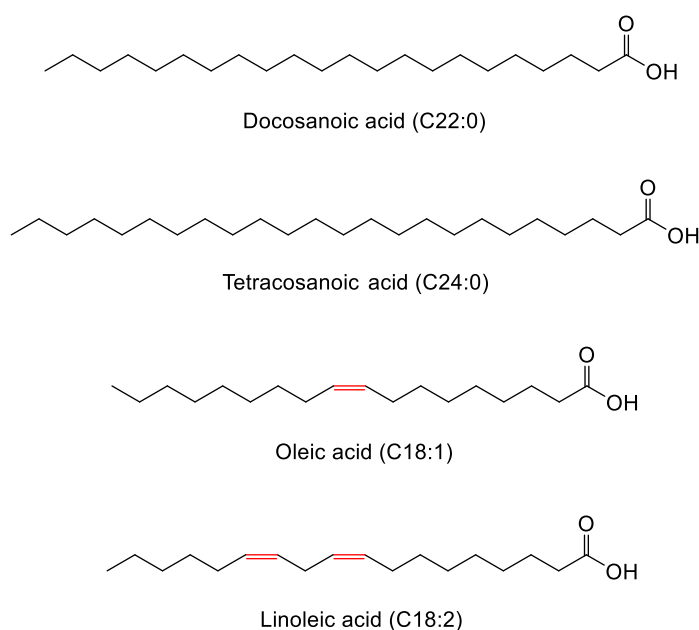
into non-viable, keratin-filled corneocytes. These corneocytes are encased by a protein-rich cell envelope with a covalently bound lipid layer. In the SC, corneocytes are embedded within lamellar lipid structures that are aligned parallel to their surface.

1.2 Overview of Epidermal Lipids

As previously mentioned, KCs undergo terminal differentiation as they migrate outward through the epidermal layers. In the viable epidermis, undifferentiated KCs in the SB layer display a lipid profile comparable to that of most mammalian cells, with PLs (essential for bilayer membrane integrity) and triacylglycerols (TGs; serving as energy stores) predominating. By contrast, the transition into the SC is marked by profound lipid remodelling.

In the SC, the lipid composition is highly specialized to support barrier function. On a total lipid mass basis, human SC consists of approximately 50% CERs, 25% CHOL, and 15% FFAs [16]. Together, these extracellular lipids form the lamellar bilayers that constitute the “mortar” in the classic “brick-and-mortar” model of the skin barrier. PLs are progressively degraded during differentiation to generate FFAs, which in this case are predominantly long-chain and unbranched (C22-C24) species (Fig. 1.2A) [16]. Importantly, these FFAs also contribute to the acidification of the SC, with the skin surface pH typically ranging between 5.0 and 5.5; a mildly acidic environment that regulates the activity of many SC enzymes [17-19]. CHOL is the major free sterol present in the SC, while cholesterol sulfate (CHOLSF) is only a minor component (Fig. 1.2B), with a ratio of approximately 95:5 in the SC [20-22].

A.



B.

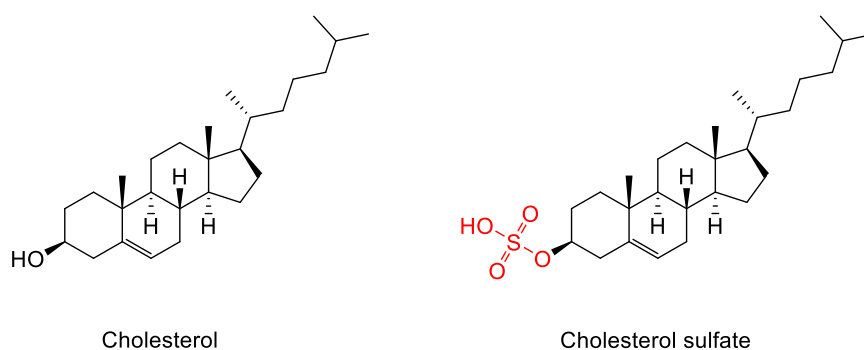


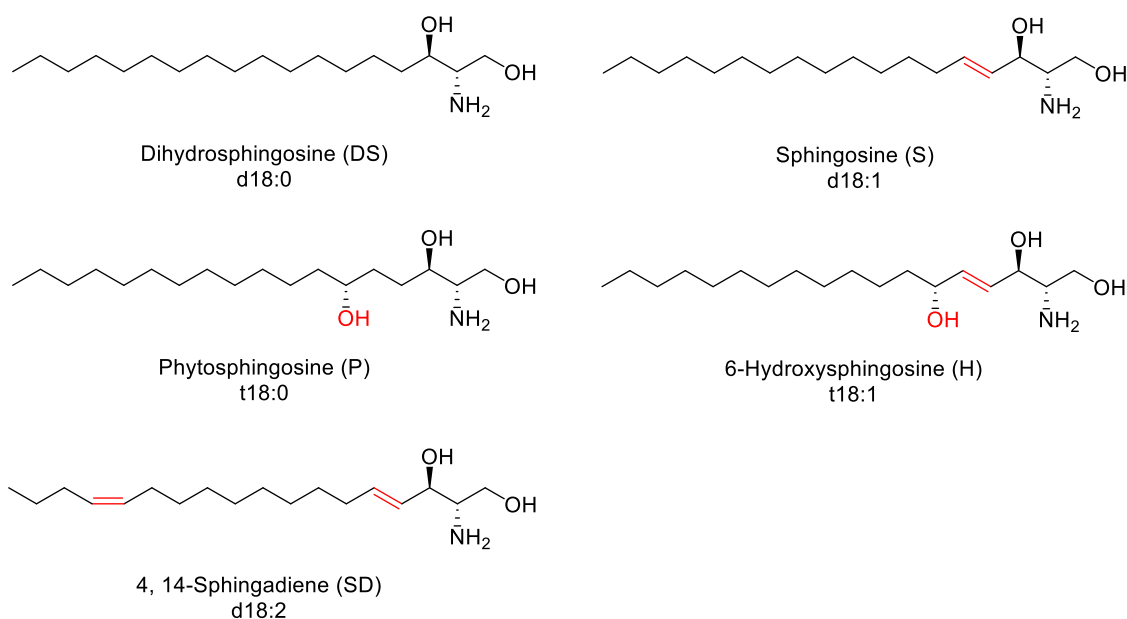
Figure 1.2. Representative chemical structures of SC lipids.

(A) FFAs including saturated long-chain species (C22 docosanoic acid, C24 tetracosanoic acid), monounsaturated FA (MUFA) C18 oleic acid (OA), and polyunsaturated FA (PUFA) C18 linoleic acid (LA). **(B)** Sterol components: CHOL and its minor derivative, CHOLSF.

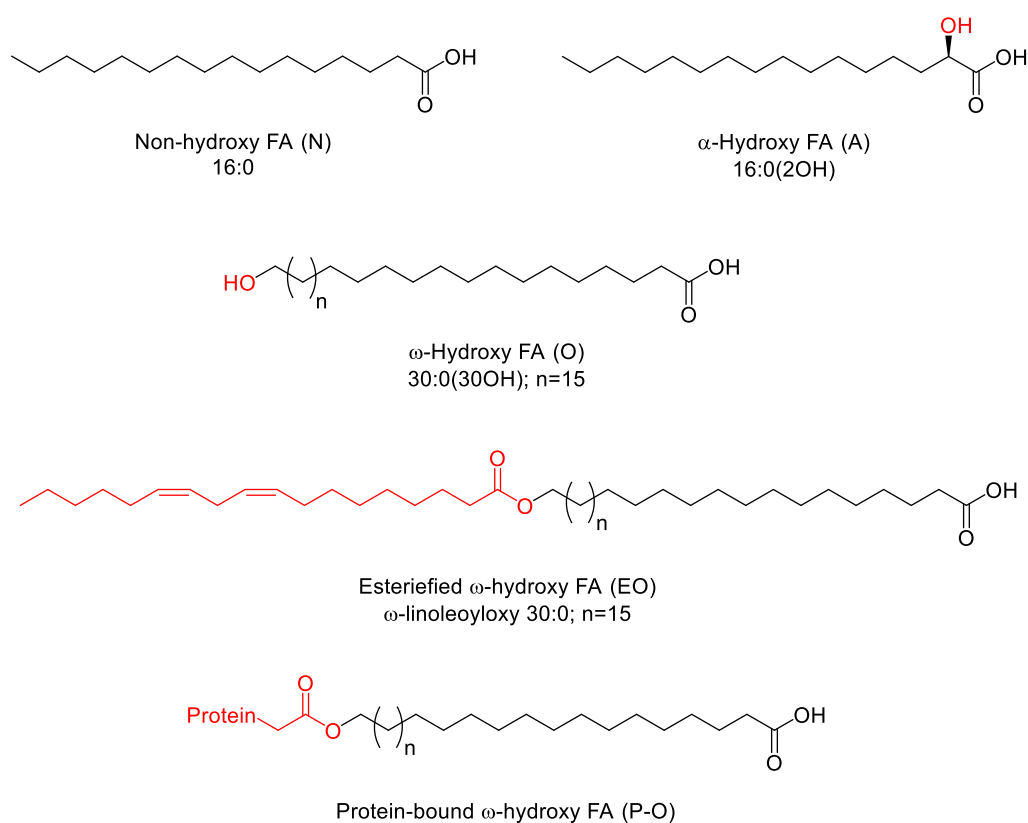
Among SC lipids, CERs are uniquely diverse and central to barrier architecture (Fig. 1.3A). Their abundance is ensured by differentiation-dependent activation of neutral and acid sphingomyelinases, and most importantly by GBA1 activity [23]. Interestingly, commensal bacteria such as *Staphylococcus epidermidis* can further enhance CER availability by secreting sphingomyelinase, which hydrolyses SM into CERs [24]. The structural diversity of SC CERs arise from variations in sphingoid base and fatty acid (FA) chain lengths. Differentiation of KCs activates biosynthetic pathways for the sphingoid bases phytoceramide and 6-hydroxysphingosine, in addition to the more common

sphingosine and dihydrosphingosine species. Sphingoid base lengths also shift toward longer forms (C20-C22), which can become as abundant as (or exceed) conventional C18 bases [25]. A particularly striking feature is the biosynthesis of acylceramides with highly hydrophobic esterified ω -hydroxy FAs (Fig. 1.3B). These span across adjacent lamellar membranes, creating a scaffold critical for barrier integrity. Acylceramides also serve as precursors of protein-bound ω -hydroxyceramides, which are covalently attached to involucrin, forming the corneocyte lipid envelope that anchors free lipids within lamellae [26, 27]. Notably, acylceramides typically incorporate linoleic acid (LA), and when essential FA deficiency forces replacement with oleic acid (OA), significant abnormalities in barrier function arise [28-30].

A.



B.



C.

FAs \ LCBs	Non-hydroxy (N)	α -Hydroxy (A)	ω -Hydroxy (O)	Esterified ω -hydroxy (EO)	Protein-bound ω -hydroxy (P-O)
Dihydrosphingosine (DS)	NDS	ADS	ODS	EODS	P-ODS
Sphingosine (S)	NS	AS	OS	EOS	P-OS
Phytosphingosine (P)	NP	AP	OP	EOP	P-OP
6-Hydroxy-sphingosine (H)	NH	AH	OH	EOH	P-OH
4,14-Sphingadiene (SD)	NSD	ASD	OSD	EOSD	P-OSD

Figure 1.3. Structures and nomenclature of human CER classes.

(A) Sphingoid base structures, (B) representative FAs, and (C) their combinations, which generate the diverse CER species found in human skin. The chain lengths and degrees of unsaturation shown in (A) and (B) are examples; actual CERs may vary in both parameters.

1.3 Allergic Contact Dermatitis (ACD)

ACD accounts for approximately 20% of all contact dermatitis (CD) cases [31]. The overall prevalence of CD in the general population ranges between 1.7% and 9.8% [32, 33]. Together with irritant contact dermatitis (ICD) - the most prevalent form of CD - they make up around 90% of occupational skin disorders [33]. From an immunotoxicology point of view, this makes ACD the most prevalent manifestation of immunotoxicity in humans. More than 4000 chemicals are identified as skin sensitizers [34]. Occupation is the most significant risk factor, with hairdressers, food handlers, healthcare workers, builders and metal workers among the groups at highest risk of developing ACD. It can substantially impair quality of life, reduce workplace productivity, and contribute to socioeconomic burden at both the individual and societal levels [35]. Clinically, the hallmark of acute ACD is eczematous dermatitis, characterized by erythema, edema, papules, vesicles or bullae, oozing, and crusting. Although lesions can appear anywhere on the body, the hands are most frequently affected. Diagnosis relies on patch testing (the gold standard method) while treatment typically involves topical or systemic corticosteroids. The primary goal of management is avoidance of the offending chemical; failure to do so often leads to chronic dermatitis with significant disability and impaired quality of life.

Pathophysiologically, ACD develops in two phases: sensitization and elicitation (Fig. 1.4). The sensitization phase begins when a chemical allergen penetrates the skin at a dose sufficient to activate the immune system, leading to immunological priming and sensitization. At this stage, the skin has a normal looking appearance. Upon subsequent exposure to the same allergen a more robust secondary response occurs, resulting in the elicitation phase. This phase manifests clinically as the cutaneous inflammatory reaction recognized as ACD - a Type IV hypersensitivity reaction, meaning both sensitization and elicitation are orchestrated by T lymphocytes [36].

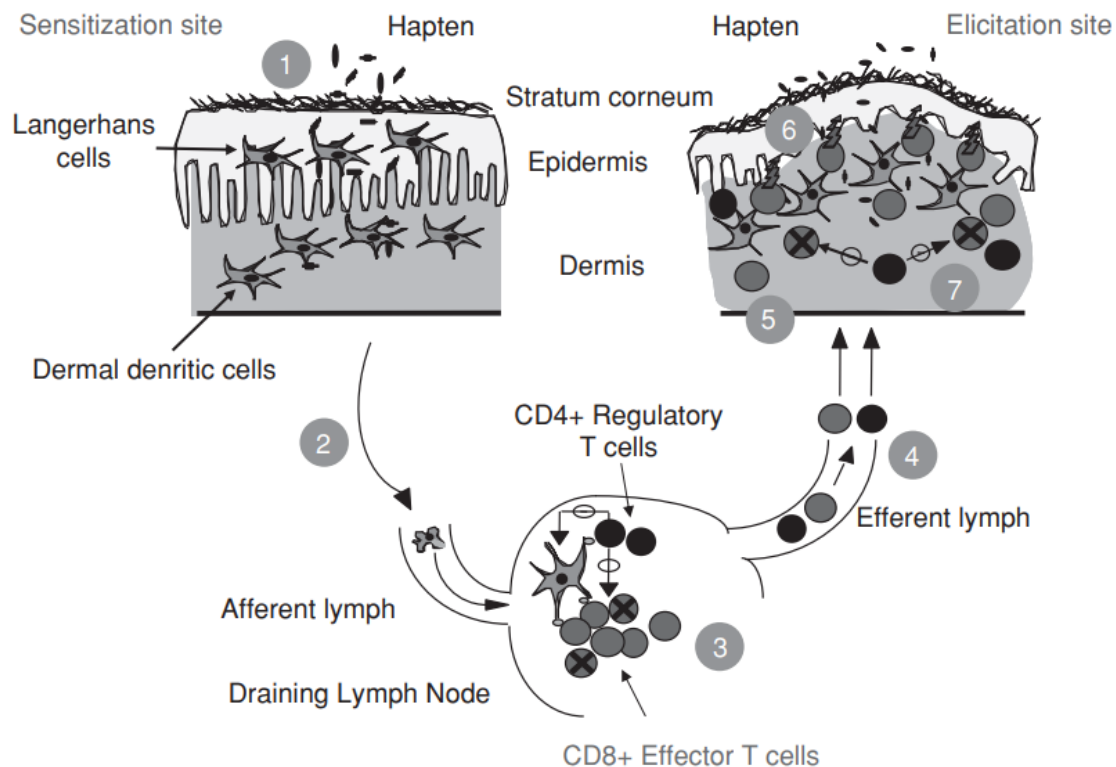


Figure 1.4. Schematic representation of the sensitization and elicitation phases of ACD. During sensitization, a chemical penetrates the SC and haptens an endogenous protein. The resulting hapten–protein adduct is internalized by cutaneous dendritic cells (DCs) (step 1). Hapten-induced inflammation promotes DC maturation and migration to the skin draining lymph node (DLN) (step 2), where hapten–peptide epitopes are presented to naïve T cells, inducing their activation and clonal expansion (step 3). Activated T cells then enter systemic circulation (step 4). Upon re-exposure, memory T cells migrate to the site of allergen contact (step 5), where they trigger KC apoptosis and cytokine/chemokine release (step 6), leading to leukocyte recruitment (including regulatory T cells) and the formation of characteristic skin lesions.

1.3.1 Contact Allergens

Contact allergens are haptens, meaning they are too small to trigger an immune response on their own and must bind to larger macromolecules to become immunogenic [37]. The prevailing dogma has long held that these macromolecules are proteins [38]. Nucleophilic amino-acid side chains in proteins are generally thought to covalently bind electrophilic haptens [39]. However, many contact allergens are not electrophilic, and only through their transformation via air exposure (oxidation) prior to skin contact (prehaptens) [40], or via enzymes they encounter in the skin (prehaptens), do these otherwise innocuous chemicals gain the electrophilic character required for 2-electron binding reactions [41, 42]. However, it is now known that some contact allergens - void of any electrophilic character - may have the ability to form hapten-

protein adducts directly via free radical reactions [43]. Once covalent bonds with protein carriers are formed, cutaneous dendritic cells (DCs) recognize them as modified-self (neo-antigens) and present them as hapten-peptide epitopes within the peptide-binding grooves of major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) for subsequent recognition by antigen-specific T cells [44].

1.3.2 Sensitization

Sensitization requires delivery of a sufficient allergen dose, which depends not only on the allergen's intrinsic potency but also on its local concentration per unit area of skin. Below a critical threshold, the immunogenic signal is inadequate to induce clinically relevant sensitization [45]. For a cutaneous immune response to be induced, a contact allergen must first penetrate the SC. This requires appropriate physicochemical properties that allow passage through the lipid lamellae. Once in the viable epidermis, the ability of the hapten to induce sensitization relies on: (i) their proinflammatory properties, thereby activating the skin's innate immune response and delivers danger signals which are able to induce recruitment, maturation, and migrations of cutaneous DCs [46-48]; and (ii) their ability to haptenate proteins and be presented as hapten-peptides in the groove of MHC classes I and II molecules at the cell surface of epidermal Langerhans cells (LCs) and dermal DCs (dDCs). Hapten-bearing DCs migrate from the skin to the draining lymph node (DLN) where they present the antigenic determinant to specific T cell precursors. Migration and maturation of these cutaneous DCs is driven initially by epidermal cytokines and further guided by chemokine receptor expression [49-52]. Epidermal cytokines such as tumour necrosis factor (TNF), interleukin (IL)-1 β , and IL-18 promote LC mobilization [53], whereas IL-10 has been shown to exert the opposite effect [54]. DCs presenting allergen-derived epitopes to T cell receptors (TCRs) activate naïve T cells to proliferate and differentiate. This results in clonal expansion of allergen-reactive T cells.

There is a direct correlation between the proliferative responses induced in skin DLN by topically applied allergens and the degree of sensitization that develops [55]. Thus, the relative potency of contact allergens can be defined as a function of the magnitude of DLN cell proliferation. Hapten-specific T cells proliferate and leave the DLN and enter blood circulation, recirculating between the lymphoid organs and the skin. Thus, at the

end of the sensitization phase of ACD, specific T cells that have been activated by hapten-bearing DCs are present in the DLN (these are central memory T cells), in the blood and in the skin (these are effector and memory T cells) [56].

1.3.3 Elicitation and Effector T Cells

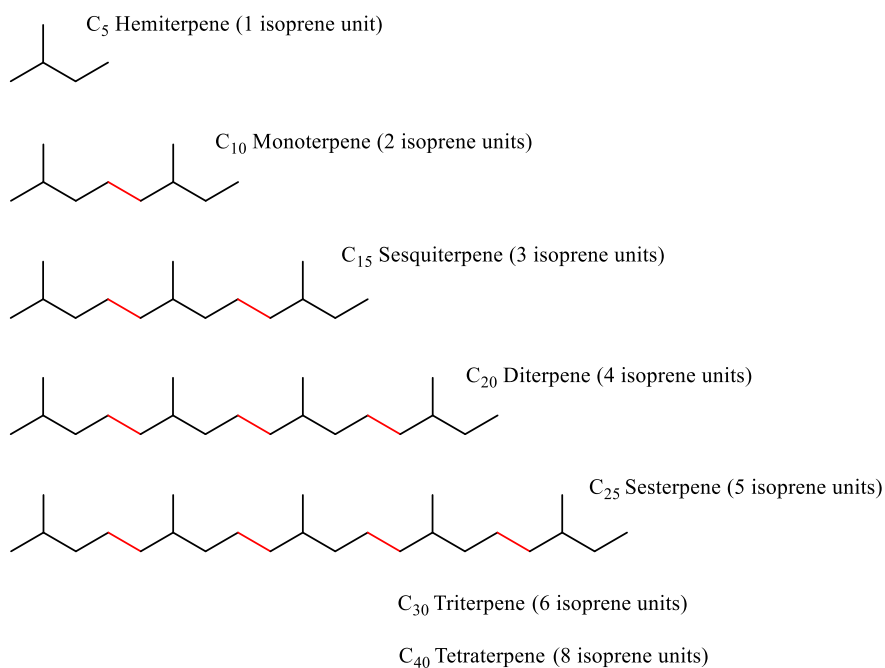
Once an individual has become sensitized, re-exposure to the offending allergen triggers allergen-specific memory T-cells recruitment to the site of contact, where interactions between T-cells and APCs can take place directly in the epidermis, thus initializing the inflammatory process [43]. Differentiation of primed CD4⁺ helper T cell (T_H) and CD8⁺ cytotoxic T cell (T_C) precursors is mainly dependent on the cytokine profile secreted during DC and T cell interactions [57]. IL-12, IL-4 or a cocktail of IL-6/IL-21/IL-23/TGF- β or IL-1, drive the differentiation of naïve T cells into type 1 cells (producing IFN- γ , IL-2 and TNF), type 2 cells (producing IL-2, IL-5, IL-10, and IL-13), and type 17 cells (producing IL-17A, IL-17F, IL-21 and IL-22), respectively [56]. In ACD, type 1 CD8⁺ T cells (T_C1) are not only cytotoxic killers but also major producers of IFN- γ , contributing substantially to the inflammatory milieu [58]. Current evidence suggests that while both CD4⁺ and CD8⁺ T cells are important, CD8⁺ T_C1 cells may act as the key effector (T_{EFF}) population driving tissue damage in ACD [59]. Their activation in the skin triggers the recruitment of leucocytes which contribute to the development of eczematous lesion and also the accompaniment of down-regulatory cells found in the CD4⁺ T cell subset (T_{REG}) [59, 60]. In conclusion, a variety of T cell subsets contribute to the elicitation and regulation of ACD. One compound class which has attracted considerable attention for their potential contribution to ACD, along with their derivatives, is the terpenes.

1.4 Terpenes

Terpenes consist of five carbon (C₅) isoprene units (2-methylbuta-1,3-diene). The number of isoprene units is responsible for the structural diversity of terpenes (Fig. 1.5A). Hemiterpenes are formed by one isoprene unit (C₅), monoterpenes by two (C₁₀), sesquiterpenes by three (C₁₅), diterpenes by four (C₂₀), sesterpenes by five (C₂₅), triterpenes by six (C₃₀) and tetraterpenes by eight (C₄₀). The biosynthesis of terpenes is dependent on the two isoprene (C₅) precursors: isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These are synthesised via either the

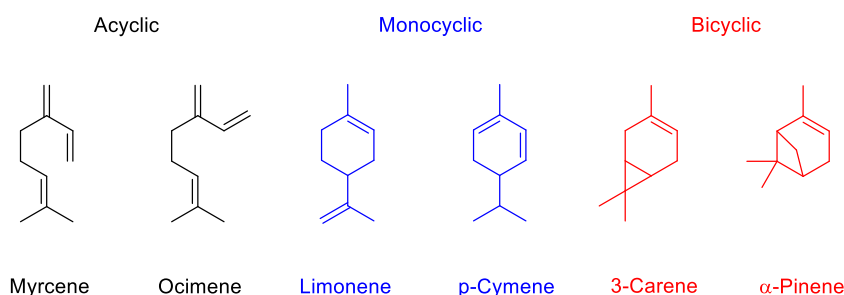
methylethylerythritol 4-phosphate (MEP) pathway or the mevalonate-dependent (MVA) pathway. Prenyltransferase enzymes catalyse the initial condensation reaction between IPP and GPP to give C₁₀-geranyl phosphate (GPP) – the universal precursor of monoterpenes. Subsequent additions of IPP give C₁₅-farnesyl pyrophosphate (FPP), and C₂₀-geranylgeranyl pyrophosphate (GGPP). These terpene precursors are then processed by monoterpene synthases/cyclases to produce a vast array of terpene and terpenoid structures [61, 62]. Terpenes are simple hydrocarbon structures, whereas terpenoids are a modified class of terpenes which contain the insertion of oxygen (Fig. 1.5B). The predominant source of terpenes is plants, which have both a cytosolic MVA and plastidial MEP pathway [63]. Essential oils (EOs) are concentrated hydrophobic liquids extracted from a variety of plants and contain a high percentage of terpenes, with monoterpenes (C₁₀) being the most representative molecule in EOs, constituting 90% [64]. The perfume and fragrance industry uses essential oils and isolated terpenes as key ingredients.

A.



B.

Monoterpenes



Monoterpenoids

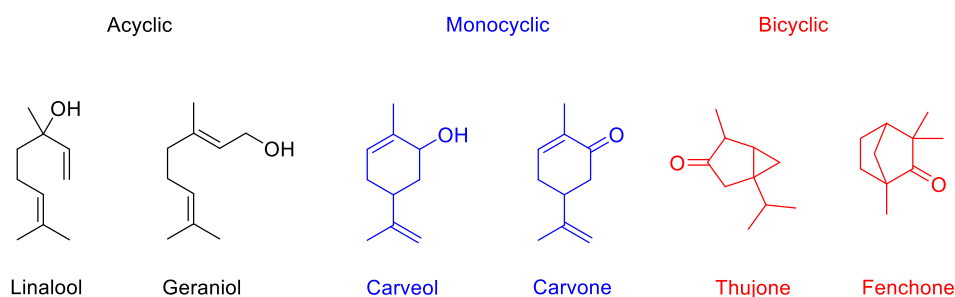


Figure 1.5. Classification and structural diversity of terpenes and monoterpenoids.

(A) Classification of terpenes based on the sequential addition of isoprene units, with the connecting bonds highlighted in red. (B) Chemical structures of representative monoterpenes and monoterpenoids, subdivided into acyclic (black), monocyclic (blue), and bicyclic (red) classes.

1.4.1 Terpenes in Consumer Products

Limonene, linalool and geraniol are commonly used in cosmetics, personal care and household products for their floral scent. In the European market, approximately one-third of cosmetic products (29.6%) contain limonene, over a quarter (26.3%) contain linalool, and just over one tenth contain geraniol (12.9%) [65]. Limonene consists of two enantiomers: *R*-(+)-limonene (or *d*-(+)-limonene) and *S*-(-)-limonene (or *l*-(-)-limonene) (Fig. 1.6). Dipentene is the racemic mixture of *R*-(+)-limonene and *S*-(-)-limonene and is offered for sale as a solvent for the paint industry. *S*-(-)-Limonene is found in turpentine (75%) [66], whereas *R*-(+)-limonene is the main constituent of several oils of the genus *Citrus* (oranges, lemons, limes etc.). (*R*)-(+)–Limonene is mainly produced from the citrus peel by pressing and distillation, and the peel oil generally consists of 95% *d*-limonene [67]. There are two enantiomeric forms of linalool: (*R*)-(-)-linalool and (*S*)-(+)-linalool.

The distribution of these two enantiomers varies in different plants. Pure (*R*)-(-)-linalool is observed in bergamot and basil oils from different locations, and lavender and lavandin oils contain >95% (*R*)-(-)-linalool. Plants showing an excess of (*S*)-(+)-linalool include false acacia (100%) and jasmine concrete or absolute (>85%) [68]. Geraniol (*trans*-3,7-dimethyl-2,6-octadiene-1-ol) occurs naturally in Palmarosa oil (80%) [69], ninde oil (66%) [70], and rose oil (44%) [71]. Terpenes have also been used as penetration enhancers for transdermal drug delivery systems [72].

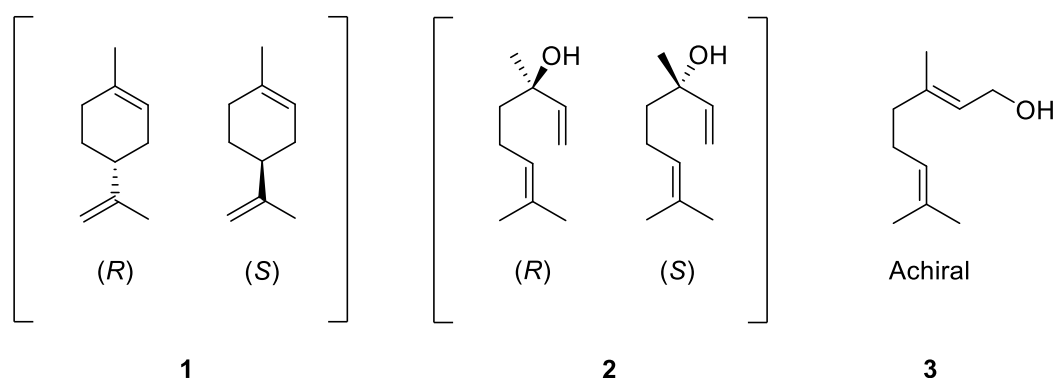


Figure 1.6. Stereochemical forms of selected monoterpenes and monoterpenoids.

The monoterpene limonene (**1**) in its two enantiomeric forms, (*R*)-(+)-limonene and (*S*)-(-)-limonene; the monoterpenoid linalool (**2**) in its two enantiomeric forms, (*R*)-(-)-linalool and (*S*)-(+)-linalool; and geraniol (**3**), an achiral monoterpenoid.

1.4.2 Autoxidation of Terpenes

Most organic compounds can undergo autoxidation, a reaction which involved the direct insertion of oxygen into a C-H bond forming a hydroperoxide (ROOH). Autoxidation is a free radical chain reaction (Fig. 1.7). This is a slow reaction made possible by the biradical character of ground-/triplet-state oxygen. The oxygen acts as a fast and efficient radical quencher, resulting in the formation of the peroxy radical (ROO[•]). The hydrogen abstraction in the propagation step is the rate limiting step in this reaction, which leads to the formation of the hydroperoxide (ROOH) and a new radical (R[•]) that feeds the chain reaction. The hydrogen abstraction step is selective, generally favouring benzylic, allylic, and tertiary hydrogens, or those next to heteroatoms (O and N), as these yield particularly stable radicals [43].

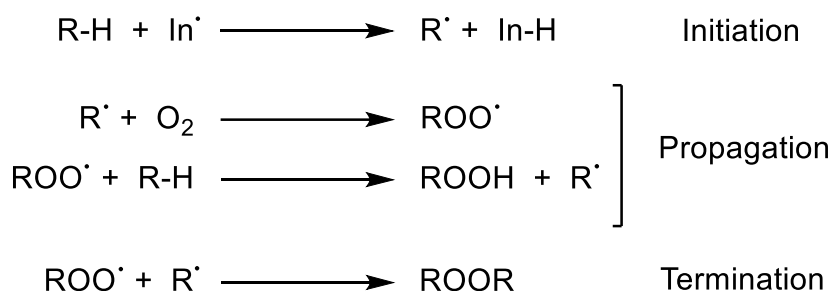


Figure 1.7. General mechanism for autoxidation.

An initiator compound ($\text{In}^\bullet$) abstracts a proton from a molecule (R-H), generating a free radical ($\text{R}^\bullet$) (initiation). Molecular oxygen rapidly reacts with this free radical to form a peroxy radical ($\text{ROO}^\bullet$), which then abstracts a proton from another molecule (R-H), producing a hydroperoxide (ROOH) and a new free radical ($\text{R}^\bullet$) that propagates the chain reaction (propagation). The process continues until two radicals react with each other, terminating the reaction (termination).

1.4.2.1 Autoxidation of (*R*)-(+)-Limonene

(*R*)-(+)-Limonene can form a number of allylic hydroperoxides in relation to its most substituted double bond. However, not all of these theoretically possible hydroperoxides have been identified in autoxidation mixtures of (*R*)-(+)-limonene (Fig. 1.8). Typically, only two of the theoretical hydroperoxides are isolated from autoxidation mixtures [73, 74], with these autoxidation mixtures containing only 5-6% of limonene-1-hydroperoxides [Lim-1-OOH (**4**)] and limonene-2-hydroperoxide [Lim-2-OOH (**5**)] after 10 weeks of air exposure [75]. Other identified oxidation products include carvone (**6**), limonene epoxide (**7**), and carveol (**8**).

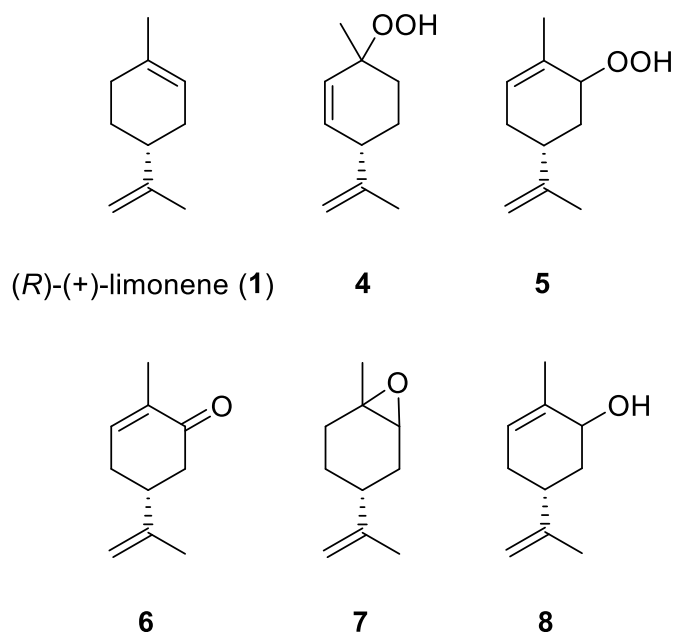


Figure 1.8. Chemical structures of autoxidation products identified from (*R*)-(+)-limonene (**1**) exposed to air for 10 weeks [76].

1.4.2.2 Autoxidation of Linalool

Linalool (**2**) is known to form various oxidation products (**9-17**) (Fig. 1.9). Like (*R*)-(+)-limonene, linalool can theoretically form a variety of hydroperoxide derivatives, however, in air-exposed linalool, two hydroperoxides, linalool-6-hydroperoxide (Lin-6-OOH) (**9**) and linalool-7-hydroperoxide (Lin-7-OOH) (**10**), were present in concentrations of 15% and 4%, respectively, after 45 weeks of air exposure [77, 78]. A third hydroperoxide (**11**) is probably formed through the degradation of (**14**). No epoxides were detected in the oxidation mixture, instead, non-allergenic pyran (**12**) and furan derivatives (**13**) were identified, possible originating from an epoxide derived from Lin-6-OOH (**9**) [78].

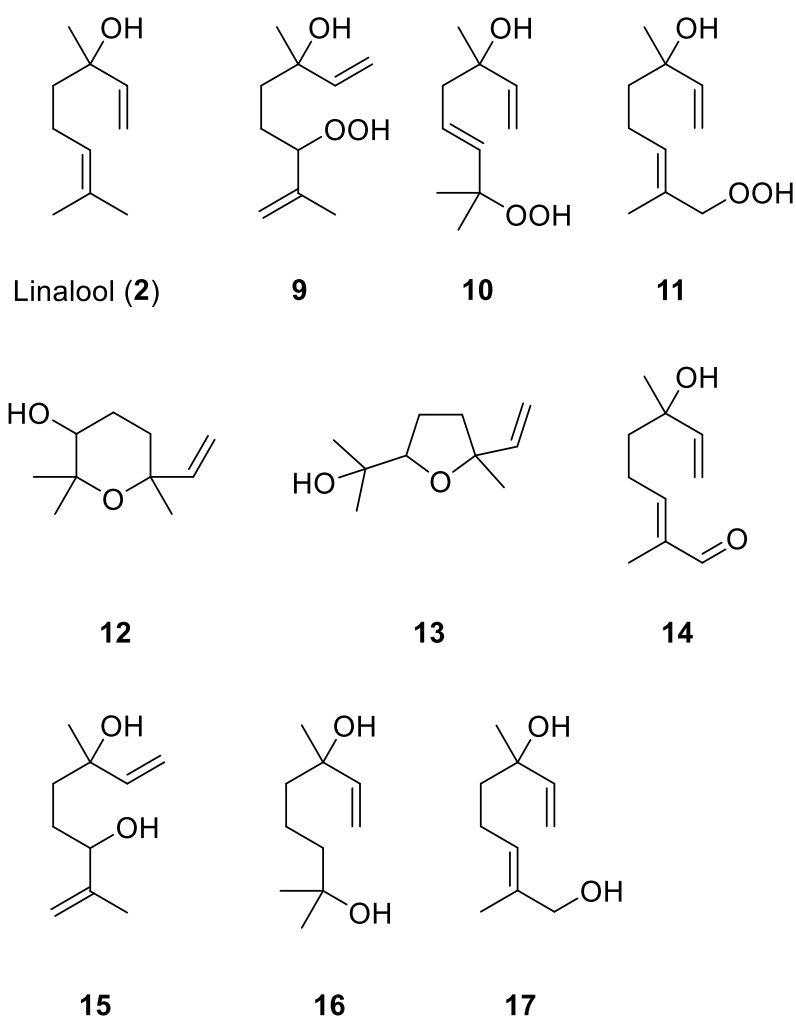


Figure 1.9. Chemical structures of autoxidation products identified from linalool (2) exposed to air for 45 weeks [77].

1.4.2.3 Autoxidation of Geraniol

The autoxidation pattern of geraniol is different from limonene and linalool. All carbons in the geraniol structure have the potential to bear a hydroperoxide. It is theorised that the C1 (Fig. 1.10) is the most likely carbon to bear a hydroperoxide at the radical generated from hydrogen abstraction as it could be stabilised by both a double bond and the adjacent hydroxyl group. However, such a hydroperoxide was not detected, the reason believed to be due to its further oxidation into the more stable products geranial (**19**) and neral (**20**). Also, the theoretical geraniol-6-hydroperoxide (Ger-6-OOH) (**24**) was not detected in the autoxidation mixture. The only detected geraniol derived hydroperoxide was geraniol-7-hydroperoxide (Ger-7-OOH) (**18**), present as 1.2% of the oxidation mixture after 10 weeks of air exposure [79].

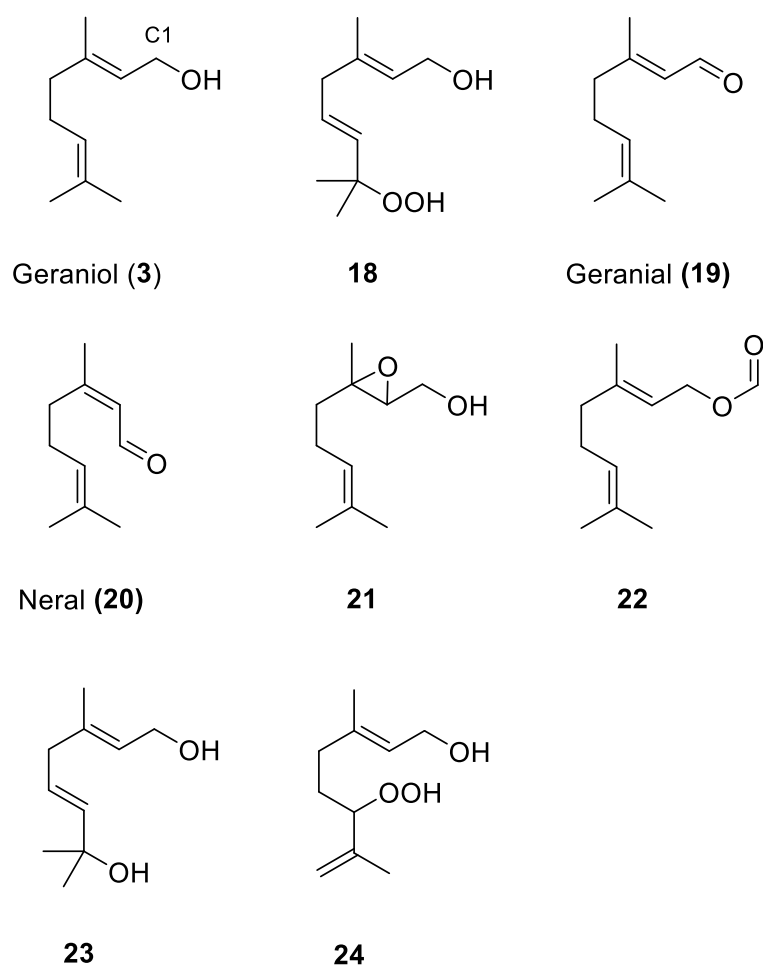


Figure 1.10. Chemical structures of autooxidation products identified from geraniol (3**) exposed to air for 10 weeks [79].**

The theoretical Ger-6-OOH (**24**) was not identified in the oxidation mixture but has been included in the diagram for completeness.

1.4.3 Sensitizing Potential of Terpenes and their Hydroperoxides

The local lymph node assay (LLNA) is an established method for identifying chemicals with the potential to induce skin sensitization and ACD. The assay is based on the principle that contact sensitization involves the stimulation of lymphocyte proliferation in skin DLNs following exposure to chemical allergens. Chemicals that elicit a ≥ 3 -fold increase in cell proliferation relative to vehicle controls are classified as skin sensitizers. The relative potency of a sensitizer is expressed as the EC3 value, defined as the estimated concentration required to induce this 3-fold proliferative response. [80]. The characterisation of contact allergens on the basis of relative skin sensitisation potency can be divided up into weak [≥ 10 EC3 ≤ 100 % (w/v)], moderate [≥ 1 EC3 < 10 % (w/v)],

strong [≥ 0.1 EC3 < 1 % (w/v)], or extreme [EC3 < 0.1 % (w/v)] [81]. Weak allergens are mostly devoid of skin toxicity, display minor proinflammatory activity, and cannot activate innate immunity, therefore, allergenicity to these compounds is seen in very few people, and in those cases it is by people who frequently manipulate their usage (e.g., occupational workers) [82]. However, the pathophysiology to some weak allergens (α -hexylcinnamaldehyde, eugenol, and hydroxycitronellol) was demonstrated to be the same as strong haptens, involving both CD8⁺ T_{EFF} cells and CD4⁺ T_{REG} cells, however differences lie in the activity of CD4⁺ T_{REG} cells during priming; in strong haptens CD4⁺ T_{REG} cells did not prevent but participate in the resolution of skin inflammation where with weak allergens, the presence of CD4⁺ T_{REG} cells abrogates the CD8⁺ T cell priming. Although ACD could not be developed in response to three weak fragrance allergens in normal mice, those mice with depleted with CD4⁺ T_{REG} cells developed a typical ACD reaction [83]. *R*-(+)-Limonene itself is a weak allergen with an EC3 value of 30% (w/v) (Table 1.1) [84]. However, its primary oxidation product Lim-2-OOH (Fig. 1.11A) is a strong contact sensitizer with an EC3 value of 0.83% (w/v) [85]. For comparison, although nickel is the most common cause of ACD, it exhibits relatively low skin-sensitizing potency, being classified as a moderate sensitizer with an EC3 value of 3.5% (w/v) [86]. Purified linalool is also demonstrated to be a weak allergen, with an EC3 value of 55% (w/v) [87]. However, two of its primary oxidation products, the structural isomers Lin-6-OOH and Lin-7-OOH (Fig. 1.11B) have been shown to be moderate skin sensitizers. When tested as a 5:4 mixture of the respective isomers, they exhibited an EC3 value of 1.6% (w/v). [78]. Pure geraniol is considered a weak allergen, with an EC3 value of 22.4% (w/v). However, its primary autooxidation product Ger-7-OOH (Fig. 1.11C), and synthesised theoretical oxidation product Ger-6-OOH, have been identified as moderate skin sensitizers. When tested as a 3:5 mixture of the respective isomers, they exhibited an EC3 value of 1.4% (w/v) [79].

Table 1.1. List of contact allergens with their respective EC3 values (% w/v).

Allergen	EC3 (% w/v)
(R)-(+)-Limonene	30
Linalool	55
Geraniol	22.4
Lim-2-OOH	0.83
Lin-6-OOH and Lin-7-OOH (5:4)	1.6
Ger-6-OOH and Ger-7-OOH (5:3)	1.4
Nickel	3.5

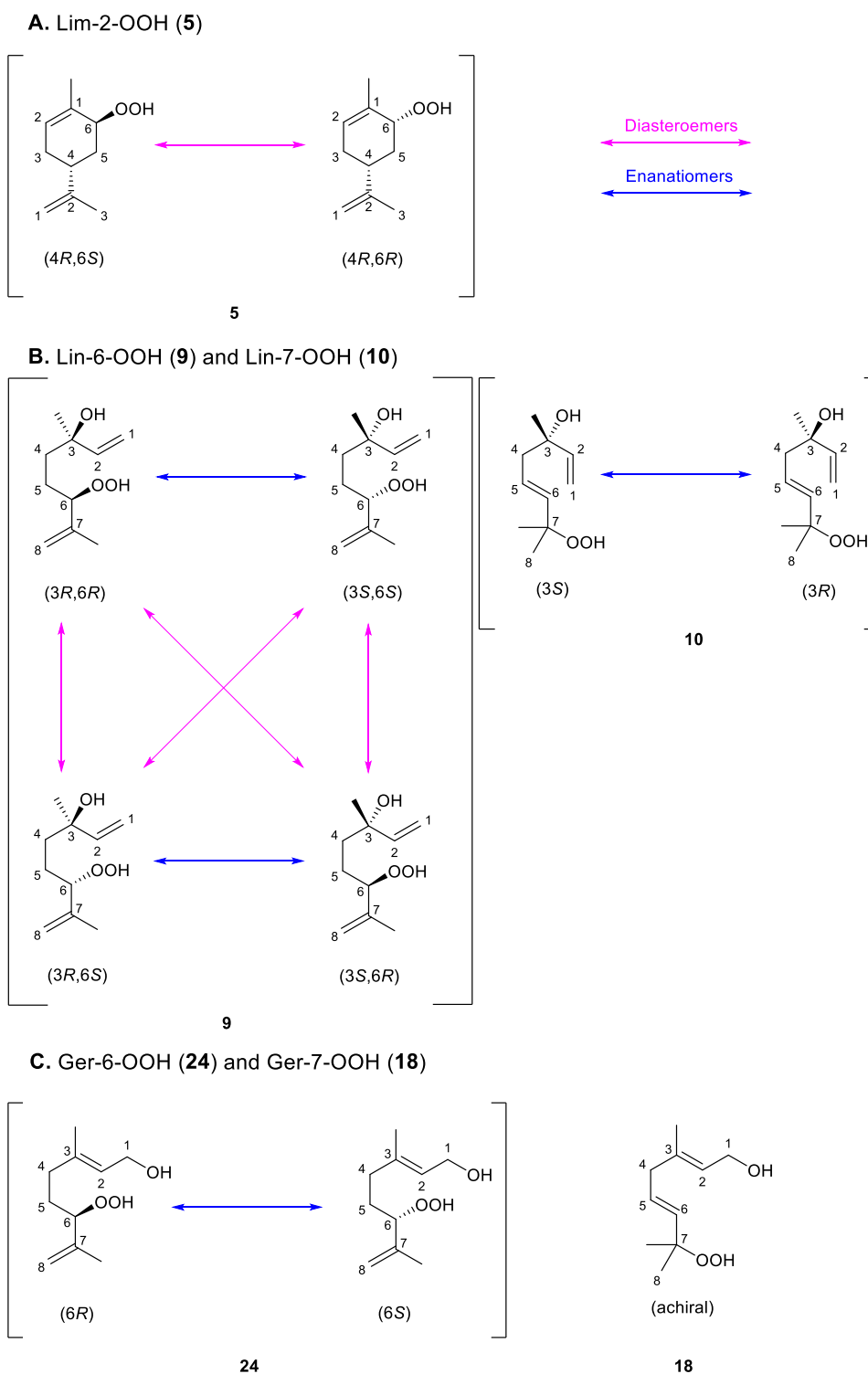


Figure 1.11. Structural isomers and stereochemical variants of allergenic terpene hydroperoxides.

Allergenic terpene hydroperoxides derived from (*R*)-(+)-limonene (**1**), showing Lim-2-OOH (**5**) as a pair of diastereomers; from linalool (**2**), showing structural isomers of Lin-6-OOH (**9**) (present as four unique configurations, of which there are four diastereomeric pairs and two enantiomeric pairs), and Lin-7-OOH (**10**) present as a pair of enantiomers; and from geraniol (**3**), showing structural isomers Ger-6-OOH (**24**) as a pair of enantiomers, and Ger-7-OOH (**18**) as an achiral compound.

1.4.4 Mechanism of Action of Terpene Hydroperoxides in ACD

To exhibit their immunogenic effects, terpene hydroperoxides must first haptenate to larger macromolecules. There has been several reports in recent years which have discussed the potential for these otherwise non-electrophilic compounds to undergo free radical reactions via the homolytic cleavage of the O-O and O-H bonds due to their relatively weak bond dissociation energies (approximately 42 and 88 kcal.mol⁻¹, respectively), and this has been demonstrated using a combination of liquid chromatography-mass spectrometry (LC-MS), tandem MS (MS/MS), and spin trapping studies [88-90]. Such cleavages lend themselves to the generation of alkoxyl (RO•) and peroxy (ROO•) radicals, which can subsequently undergo intramolecular cyclization, fragmentation, or hydrogen abstraction to afford carbon centered radicals (R•) [91]. It is believed that these oxygen-centered or carbon-centered radicals could be pivotal in the haptenation processes associated with these contact allergens. This has been demonstrated with the acyclic tertiary-allylic hydroperoxide contact allergen Lin-7-OOH which, in the presence of ferrous salts, formed several carbon centered radicals (allylic, oxiranylcarbonyl, and methyl) which were all reactive towards histidine (used as a model peptide), whereas the cyclic secondary-allylic hydroperoxide contact allergen Lim-2-OOH had much fewer possibilities of rearrangement, but was also capable of forming adducts with histidine [91]. Such experiments highlight the relevance of these contact allergens in terms of the number of possible radical intermediates they can form under biomimetic systems depending on their parent structures, which could generate different potentially immunogenic protein chemical modifications; protein modifications which could ultimately be responsible for skin sensitization.

1.4.5 Major Aims of PhD Thesis

The results generated as part of this thesis are presented in experimental chapters 2 to 5. The aims of which are as follows:

- Chapter 2 - to synthesise terpene hydroperoxides contact allergens that would be used in subsequent experimental chapters.
- Chapter 3 - to investigate the ability of terperene hydroperoxide contact allergens to modulate the HaCaT cell lipidome.

- Chapter 4 - to investigate the ability of terpene hydroperoxide contact allergens to mediate lipid peroxidation of phospholipid vesicles under biomimetic conditions.
- Chapter 5 - to investigate the ability of terpene hydroperoxide contact allergens to potentiate CD1a/d-restricted T cell binding and subsequent CD1a/d-restricted T cell activation (for an introduction to CD1 proteins, see Chapter 5).

Chapter 2: Terpene Hydroperoxide Synthesis

2.1 Introduction

Terpene hydroperoxide contact allergens, commonly found in cosmetic products, have emerged as a focal point due to their high allergenic potency despite lacking intrinsic electrophilic character. These compounds are generated via air oxidation of their parent terpenes, a process facilitated by the presence of easily abstractable allylic protons. However, terpene hydroperoxides are inherently unstable, containing O–O and O–H bonds with low dissociation energies, rendering them susceptible to homolytic cleavage in the presence of ferrous salts. This cleavage initiates a cascade of oxygen- and carbon-centered radical species. This property is of particular relevance to this project, as the skin is rich in lipids that may undergo free radical reactions and oxidative modification.

Although the SC is predominantly composed of CERs, FFAs, and CHOL, a notable proportion of monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) is also present - particularly LA, which is essential for the formation of acylceramides [92]. Deficiency in LA is associated with significant skin barrier abnormalities. In contrast, the viable epidermis contains a higher proportion of PLs, many of which incorporate acylated PUFAs.

Current research on ACD and haptenation primarily focuses on protein targets. However, the potential contribution of lipids as sources of immunogenic modification products remains underexplored. This PhD project therefore aimed to investigate the generation of modified skin lipids and their potential immunogenicity, with a particular focus on free radical-mediated modifications. Given their strong propensity to generate radical cascades, terpene hydroperoxides were chosen as model allergens.

The terpene hydroperoxides Lim-2-OOH (**5**), Lin-6/7-OOH (**9** and **10**), and Ger-6/7-OOH (**24** and **18**) were selected as model allergens due to the widespread presence of their parent terpenes in cosmetic and household products (Fig. 2.1). As these hydroperoxides are not commercially available - and air autoxidation yields only small amounts amid complex mixtures - they had to be synthesized in the laboratory. The goal of this chapter was therefore to synthesise pure compounds, enabling the experiments designed to address the central research questions outlined above. The compounds were prepared

via selective chemical oxidation, followed by careful purification and comprehensive nuclear magnetic resonance (NMR) characterization, providing the high-purity materials essential for subsequent investigations into lipid modification and immunogenicity.

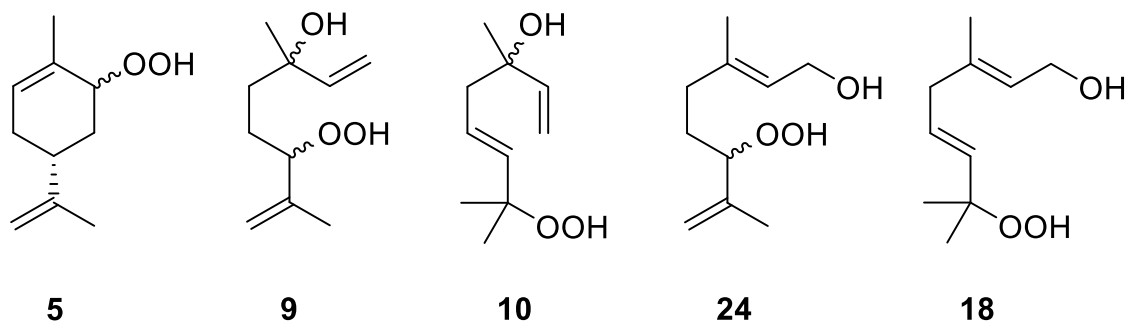


Figure 2.1. Chemical structures of the terpene hydroperoxides, the synthesis of which was the goal of this chapter.

2.2 Materials and Methods

2.2.1 Materials

Organic solvents (dichloromethane [DCM], ethyl acetate [EtOAc], hexane, and butanol [BuOH]) were purchased from Fisher Scientific (Leicestershire, UK). Hydrogen peroxide (30 % w/v) was purchased from Merck (Darmstadt, Germany). (*R*)-(+)-Limonene, (±)-linalool, geraniol, sodium dodecyl sulphate (SDS), and sodium molybdate were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2.2 Synthesis of Limonene-2-Hydroperoxide (Lim-2-OOH)

Using methods developed by Arnau and co-workers [91], a catalytic amount of concentrated sulfuric acid (3-4 drops) and (-)-carveol (97%, 6.0 g, 38.2 mmol, 1 equivalent) were added to an aqueous solution of hydrogen peroxide (30%, 142 mL, 1.25 mol, 33 equivalents) maintained at 0 °C. The reaction mixture was stirred at ambient temperature for 48 hours. Reaction progress was monitored periodically by thin layer chromatography (TLC) and ¹H NMR spectroscopy. Aliquots (1 mL) were withdrawn at various time points, subjected to a mini workup involving extraction with hexane (10 mL) and water (9 mL), vigorous shaking in a 50 mL separatory funnel, followed by separation of the organic layer. The organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. TLC analysis employed UV illumination for compound

visualization; where compounds were not UV active, staining with ammonium molybdate reagent was used. The stain was prepared by dissolving 12 g ammonium molybdate in 190 mL distilled water, followed by addition of 0.5 g ceric (IV) sulfate and 10 mL concentrated sulfuric acid. The reaction mixture was extracted with hexane (5 × 200 mL). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated under vacuo. Crude samples (5-20 mg) were dissolved in 1.5 mL deuterated dimethyl sulfoxide (DMSO-*d*₆) and analyzed by ¹H and ¹³C NMR spectroscopy. Purification was performed via column chromatography using a glass column (55 mm internal diameter) packed with 20 cm silica gel (wet-packed with hexane). Approximately 200 mg of crude Lim-2-OOH, dissolved in 0.5 mL DCM, was loaded onto the column. Initial elution with 50 mL hexane removed residual DCM, followed by gradient elution with hexane/EtOAc mixtures, starting at 1% EtOAc and gradually increasing to 5%. The EtOAc concentration did not exceed 5% to prevent co-elution of impurities. Elution was monitored by TLC with UV detection and ammonium molybdate staining as needed. Multiple spotting of concentrated fractions was employed to confirm purity and absence of co-elution. Fractions containing pure product were pooled and solvents removed under vacuo. This gave a ~35% yield as a pair of diastereomers, (4*R*,6*R*)-*cis* and (4*R*,6*S*)-*trans*, in a ratio of approximately 8:1 in favour of the *trans* isomer. Purified samples were subsequently prepared for comprehensive NMR characterization:

(4*R*,6*S*)-6-Hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene (Isomer 1):

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.31 (m, 1 H), 1.72 (s, 3 H), 1.74 (s, 3 H), 1.77 (m, 1 H), 2.06 (m, 1 H), 2.21 (m, 1 H), 2.27 (m, 1 H), 4.16 (m, 1 H), 4.73 (m, 2 H), 5.68 (s, 1 H), 11.48 (s, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 20.8, 21.1, 30.5, 30.8, 34.8, 80.8, 109.1, 128.2, 129.8, 148.9.

(4*R*,6*R*)-6-Hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene (Isomer 2):

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.64 (m, 1 H), 1.64 (s, 3 H), 1.72 (s, 3 H), 1.86 (m, 1 H), 2.01 (m, 1 H), 2.07 (m, 1 H), 2.23 (m, 1 H), 4.38 (m, 1 H), 4.73 (m, 2 H), 5.54 (s, 1 H), 11.17 (s, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 19.1, 20.3, 30.3, 32.7, 39.9, 82.2, 109.1, 125.3, 133.7, 148.6.

2.2.3 Synthesis of Linalool-6/7-Hydroperoxides (Lin-6/7-OOH)

Using methods developed by Arnau and co-workers [91], a microemulsion was prepared by the gradual, dropwise addition of an aqueous sodium molybdate solution (Na_2MoO_4 , 3.38 g in 18 mL distilled water) into a suspension of SDS (27.9 g) in a solvent mixture of BuOH (34 mL) and DCM (166 mL). The mixture was stirred continuously, resulting in a clear microemulsion within 5 minutes. Subsequently, ($\pm$)-linalool (3.0 g, 19.4 mmol, 1 equivalent) was introduced into the microemulsion, followed by the incremental addition of hydrogen peroxide (30%, total volume 15 mL, 132 mmol, 8.2 equivalents). The initial addition (1 mL) caused the microemulsion to turn red, which gradually shifted to yellow after 15 minutes. The remaining portions of hydrogen peroxide were added sequentially at 10-minute intervals. The resulting light yellow reaction mixture was stirred overnight at ambient temperature. Following completion, solvents were removed under reduced pressure, and the crude product was dissolved in DCM (900 mL). The suspension was stirred for 48 hours and then filtered to remove insoluble material. The filtrate was partially concentrated under vacuum and washed with distilled water (3×100 mL). The combined aqueous phases were extracted twice with DCM (2×100 mL). Due to the persistence of SDS, complete removal during aqueous washing proved challenging, as evidenced by occasional cloudiness upon shaking. Therefore, a limited number of washes were employed, with residual SDS removed during subsequent flash chromatography. The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Purification of the crude Lin-6/7-OOH mixture was conducted via column chromatography on silica gel (glass column, 55 mm internal diameter, 20 cm length, wet-packed with hexane). Approximately 200 mg of crude product, dissolved in 0.5 mL DCM, was loaded onto the column. Initial elution with 50 mL hexane removed residual DCM, followed by gradient elution using hexane/EtOAc mixtures starting at 10% EtOAc, gradually increasing to 20%. The EtOAc concentration did not exceed 20% to prevent co-elution of impurities. Elution was monitored by TLC using ammonium molybdate staining. Multiple spotting of concentrated fractions ensured the absence of co-elution. Pure fractions were combined and solvents removed under vacuum. This gave Lin-6/7-OOH (**9** and **10**) in ~85% yield as two structural isomers: Lin-6-OOH (**9**), present as four unique configurations (four diastereomeric pairs and two enantiomeric pairs), and Lin-

7-OOH (**6**), present as a pair of enantiomers. The purified samples were then prepared for comprehensive NMR characterization (as described previously for the synthesis of Lim-2-OOH):

6-Hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol (Lin-6-OOH):

^1H NMR (400 MHz, DMSO- d_6) δ 1.12 (s, 6 H), 1.28 (m, 2 H), 1.30 (m, 2 H), 1.40 (m, 2 H), 1.41 (m, 2 H), 1.62 (s, 6 H), 4.08 (t, 2 H), 4.44 (s, 2 H), 4.85 (s, 2 H), 4.87 (s, 2 H), 4.94 (t, 2 H), 5.13 (m, 2 H), 5.81 (m, 2 H), 11.24 (s, 2 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 16.69, 16.72, 24.90, 24.94, 27.74 (2C), 37.95, 38.01, 71.16 (2C), 88.36 (2C), 110.90 (2C), 113.04, 113.09, 144.55, 144.57, 145.95 (2C).

(E)-7-Hydroperoxy-3,7-dimethylocta-1,5-dien-3-ol (Lin-7-OOH):

^1H NMR (400 MHz, DMSO- d_6) δ 1.11 (s, 3 H), 1.21 (s, 6 H), 2.13 (m, 2 H), 4.52 (s, 1 H), 4.93 (t, 1 H), 5.10 (m, 1 H), 5.55 (m, 1 H), 5.56 (m, 1 H), 5.87 (m, 1 H), 10.79 (s, 1 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 24.62, 24.64, 26.84, 45.29, 71.41, 80.25, 111.01, 125.27, 137.16, 146.03.

2.2.4 Synthesis of Geraniol-6/7-Hydroperoxides (Ger-6/7-OOH)

Using methods developed by Arnau and co-workers [91], a microemulsion was prepared by the gradual, dropwise addition of an aqueous sodium molybdate solution (Na_2MoO_4 , 3.38 g in 18 mL distilled water) into a suspension of SDS (27.9 g) in a solvent mixture of BuOH (34 mL) and DCM (166 mL). The mixture was stirred continuously, resulting in a clear microemulsion within 5 minutes. Subsequently, geraniol (3.0 g, 19.4 mmol, 1 equivalent) was introduced into the microemulsion, followed by the incremental addition of hydrogen peroxide (30%, total volume 15 mL, 132 mmol, 8.2 equivalents). The initial addition (1 mL) caused the microemulsion to turn red, which gradually shifted to yellow after 15 minutes. The remaining portions of hydrogen peroxide were added sequentially at 10-minute intervals. The resulting light yellow reaction mixture was stirred overnight at ambient temperature. Following completion, solvents were removed under reduced pressure, and the crude product was dissolved in DCM (900 mL). The suspension was stirred for 48 hours and then filtered to remove insoluble material. The filtrate was partially concentrated under vacuum and washed with distilled water (3×100 mL). The combined aqueous phases were extracted twice with DCM (2

× 100 mL). Due to the persistence of SDS, complete removal during aqueous washing proved challenging, as evidenced by occasional cloudiness upon shaking. Therefore, a limited number of washes were employed, with residual SDS removed during subsequent flash chromatography. The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Purification of the crude Ger-6/7-OOH mixture was conducted via column chromatography on silica gel (glass column, 55 mm internal diameter, 20 cm length, wet-packed with hexane). Approximately 200 mg of crude product, dissolved in 0.5 mL DCM, was loaded onto the column. Initial elution with 50 mL hexane removed residual DCM, followed by gradient elution using hexane/EtOAc mixtures starting at 5% EtOAc, gradually increasing to 20%. The EtOAc concentration did not exceed 20% to prevent co-elution of impurities. Elution was monitored by TLC using ammonium molybdate staining. Multiple spotting of concentrated fractions ensured the absence of co-elution. Pure fractions were combined and solvents removed under vacuum. This gave Ger-6/7-OOH (**24** and **18**) in ~23% yield as two structural isomers: Ger-6-OOH (**24**), present as a pair of enantiomers, and Ger-7-OOH (**18**), an achiral compound. The purified samples were then prepared for comprehensive NMR characterization as described previously:

(E)-6-Hydroperoxy-3,7-dimethylocta-2,7-dien-1-ol (Ger-6-OOH):

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.45 (m, 1 H), 1.57 (s, 3 H), 1.60 (m, 1 H), 1.67 (s, 3 H), 1.91 (m, 2 H), 3.93 (m, 2 H), 4.14 (m, 1 H), 4.44 (m, 1 H), 4.89 (m, 1 H), 4.92 (m, 1 H), 5.26 (m, 1 H), 11.30 (s, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 16.6, 17.3, 29.0, 35.4, 58.1, 87.8, 113.7, 126.0, 135.5, 144.9.

(2E,5E)-7-Hydroperoxy-3,7-dimethylocta-2,5-dien-1-ol (Ger-7-OOH):

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.23 (s, 6 H), 1.56 (s, 3 H), 2.69 (m, 2 H), 3.95 (m, 2 H), 4.47 (m, 1 H), 5.29 (m, 1 H), 5.51 (m, 1 H), 5.59 (m, 1 H), 10.85 (s, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 16.5, 25.1 (2 C), 42.3, 58.0, 80.7, 126.5, 127.1, 134.9, 137.0.

2.3 Results and Discussion

2.3.1 Synthesis and Characterisation of Lim-2-OOH (5) Diastereomers

Lim-2-OOH can be synthesised via S_N1 and/or S_N2 nucleophilic substitution mechanisms. Both of these methods use (-)-carveol as the starting material, but the S_N2 type mechanism requires two reaction steps. For an S_N2 mechanism to take place, the alcohol functionality is first converted into a good leaving group (achieved by reacting the starting material with methane sulfonyl chloride under basic conditions), followed by substitution by the hydroperoxide via a silver ion-assisted reaction of the alcohol-corresponding halide (Fig. 2.2).

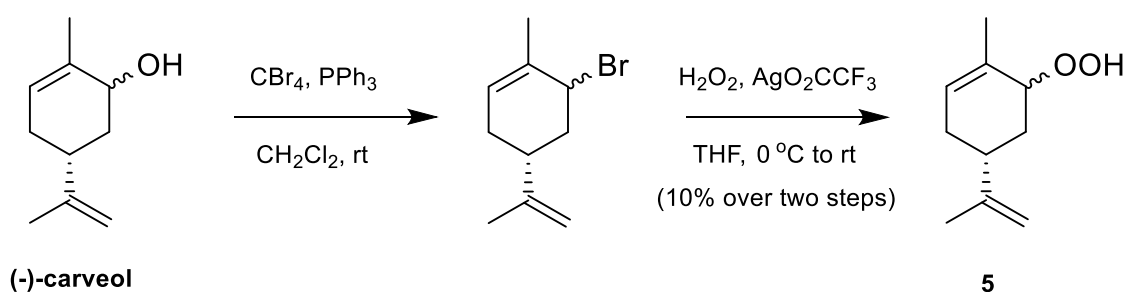
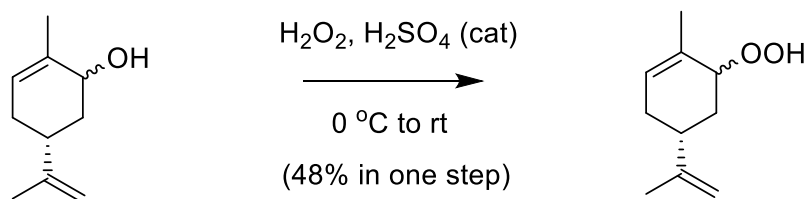


Figure 2.2. Two-step synthesis of Lim-2-OOH via sequential substitution reactions.

The synthesis involves two substitution steps. First, the hydroxyl group is converted to a bromide intermediate using the Appel reaction, which proceeds through an S_N2 -like displacement mechanism. Next, the bromide undergoes nucleophilic substitution with a hydroperoxide source, introducing the hydroperoxide ($-OOH$) group via a classical S_N2 pathway.

Lim-2-OOH can also be synthesised in a single step and with higher yield by quenching a secondary carbocation - generated via protonation of the alcohol functionality of (-)-carveol, followed by loss of water - with hydrogen peroxide (Fig. 2.3). Pioneering studies on hydroperoxide synthesis by Davies and co-workers in the 1950s demonstrated that alkyl-oxygen bond heterolysis proceeds predominantly via an S_N1 mechanism, with minor contribution from an S_Ni pathway [93]. This was the primary method employed in the present work.

A. General reaction



B. $\text{S}_{\text{N}}1$ reaction mechanism

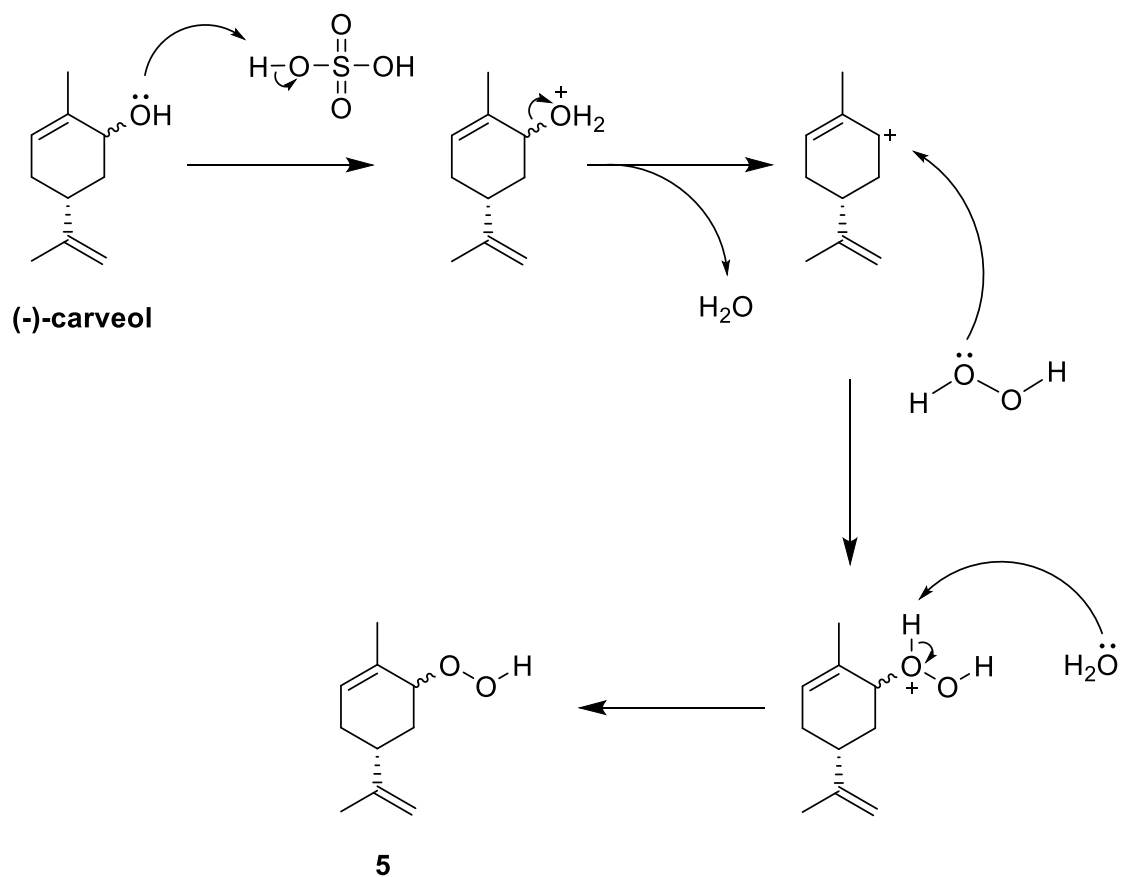


Figure 2.3. One-step acid-catalysed synthesis of Lim-2-OOH (5) from (-)-carveol via an $\text{S}_{\text{N}}1$ mechanism.

The reaction mixture was stirred at room temperature for 48 h, however, ^1H NMR monitoring indicated no further reaction after 32 h. A substantial amount of unreacted (-)-carveol remained, as evidenced by the intensity of the lowest spot on the TLC plate (Fig 7) and the persistence of vinylic proton signals in the ^1H NMR spectrum (Fig. 2.4). TLC revealed two additional products: the least polar ($R_f = 0.48$, hexane/EtOAc 95:5), visible under UV light and migrating highest on the plate, and a second product ($R_f = 0.29$, hexane/EtOAc 95:5), which was not UV active and required ammonium molybdate staining for visualisation. ^1H NMR confirmed the formation of new signals, most notably two vinylic proton resonances in the 5.00 - 6.00 ppm range (approximate ratio 8:1) and two hydroperoxy proton resonances in the 10.50-12.00 ppm range (also in an approximate ratio of 8:1) (Fig. 2.5). Of the 200 mg of crude Lim-2-OOH subjected to purification, 70 mg of pure Lim-2-OOH was isolated as a colourless oil, corresponding to a 35% yield.

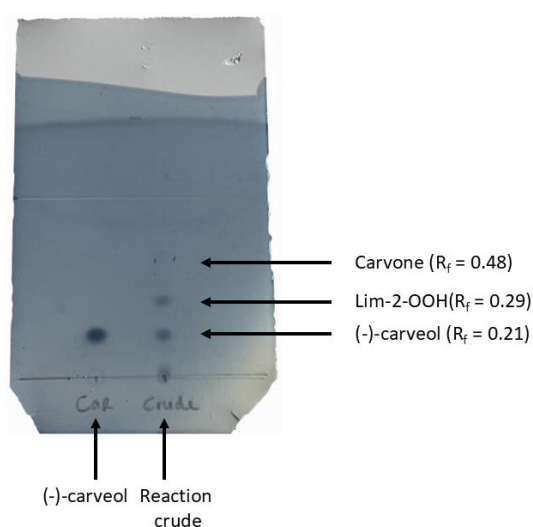


Figure 2.4. TLC analysis of the synthesis of Lim-2-OOH.

TLC plate showing starting material (-)-carveol (left lane) and the crude reaction mixture from the synthesis of Lim-2-OOH (right lane). From bottom to top, the crude mixture displays spots corresponding to unreacted (-)-carveol ($R_f = 0.21$), Lim-2-OOH ($R_f = 0.29$), and carvone ($R_f = 0.40$). The mobile phase was hexane/EtOAc (95:5, v/v), and spots were visualised using ammonium molybdate staining.

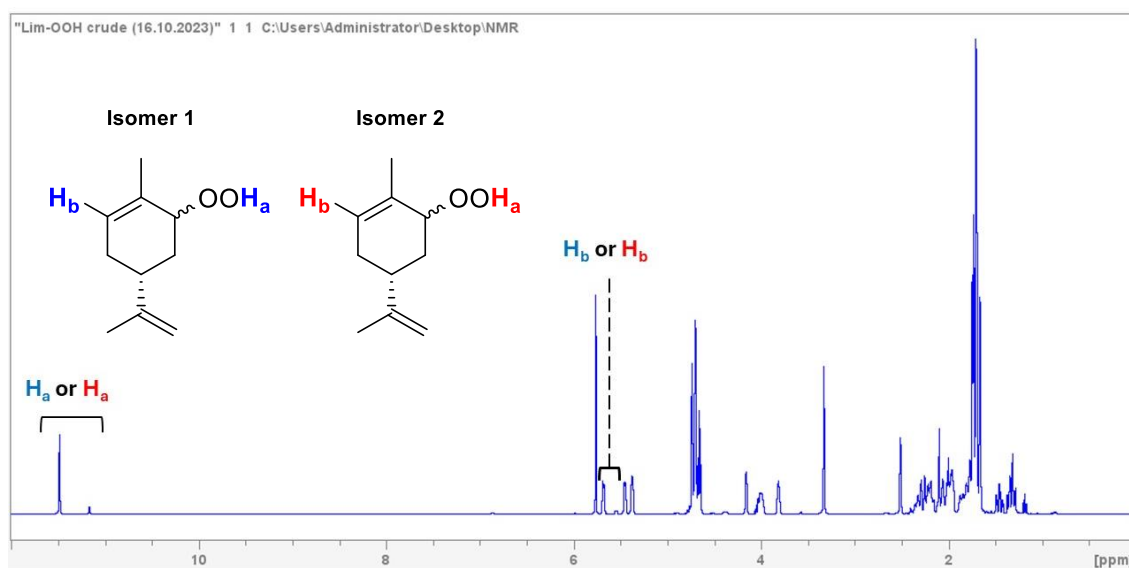


Figure 2.5. ^1H NMR spectrum of the Lim-2-OOH reaction mixture.

Distinct resonances corresponding to hydroperoxy protons appear at 10.50-12.00 ppm, while vinylic protons resonate at 5.00-6.00 ppm. The presence of duplicate sets of chemical shifts indicates the formation of diastereomers, with an approximate 8:1 ratio favouring one diastereomer.

The observed 8:1 ratio of newly formed vinylic and hydroperoxy proton signals suggests that the reaction favoured the formation of one Lim-2-OOH diastereomer over the other, as well as selective consumption of one diastereomer of the (-)-carveol starting material (Fig. 2.6). Analysis of the ^1H NMR spectra of the starting material and the crude Lim-2-OOH reaction mixture indicates that the (1*S*,5*R*)-*trans*-(-)-carveol diastereomer is preferentially consumed during the reaction. This assignment was supported by derivative NMR experiments, including Nuclear Overhauser Effect Spectroscopy (NOESY) of the two (-)-carveol isomers, which revealed correlations between protons on carbons 1 and 5 in the *trans* isomer but not in the *cis* isomer. This data strongly suggests that the alcohol and isopropenyl substituents are positioned on opposite faces of the ring system (Fig. 2.7).

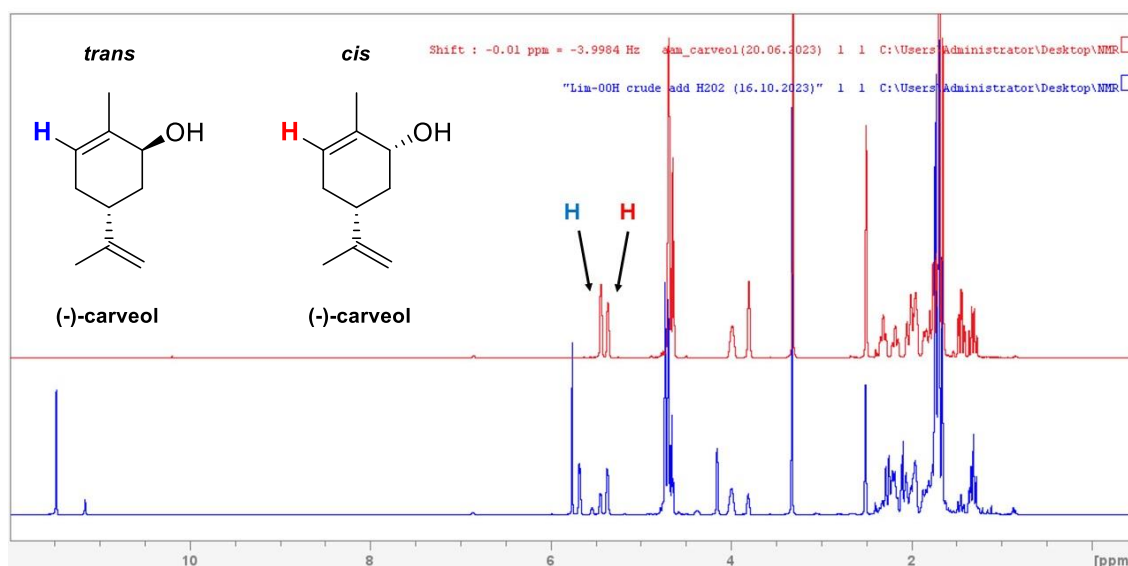


Figure 2.6. ^1H NMR spectra showing changes in the (-)-carveol *trans/cis* isomer ratio during Lim-2-OOH synthesis.

Stacked ^1H NMR spectra of (-)-carveol (top) and the Lim-2-OOH crude reaction mixture (bottom). The predominant isomer ratio shifts from 57:43 (*trans*:*cis*) in the starting material to 32:68 (*trans*:*cis*) in the crude mixture, demonstrating stereoselective consumption of (-)-carveol diastereomers during product formation.

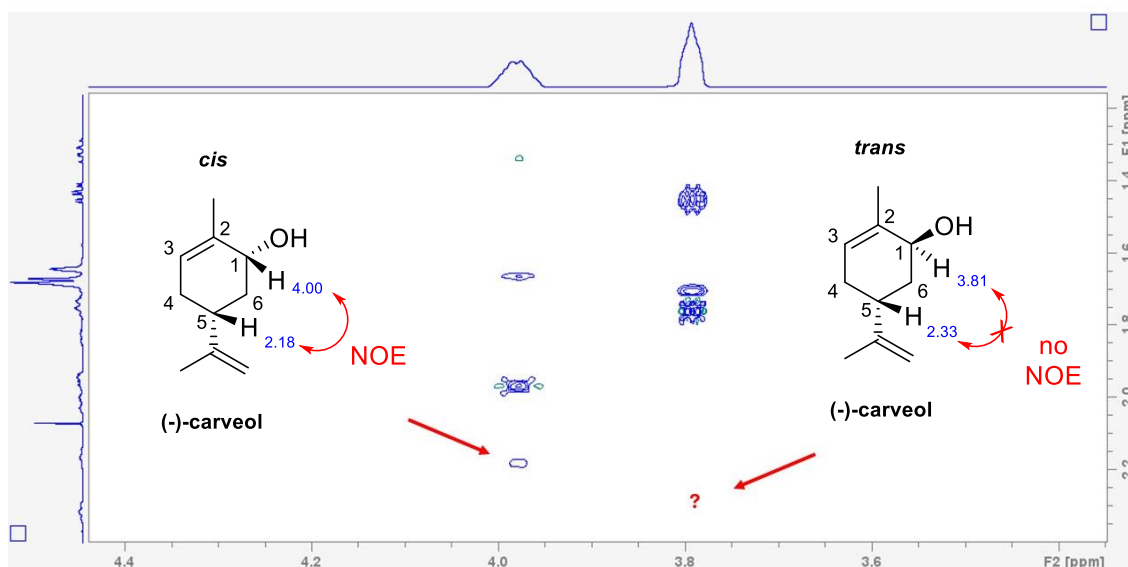


Figure 2.7. NOESY analysis distinguishing (-)-carveol *trans* and *cis* isomers.

The pure (-)-carveol sample shows a *trans*:*cis* isomer ratio of 57:43 based on ^1H NMR integration. NOE correlations were observed between proton 1 (4.00 ppm) and proton 5 (2.18 ppm) in one isomer, indicating these protons are spatially close and therefore on the same face of the ring. This implies that the alcohol and isopropenyl groups are on the same side, consistent with the *cis* isomer. In contrast, the other isomer shows no NOE between proton 1 (3.81 ppm) and proton 5 (2.33 ppm), suggesting these protons are on opposite faces of the ring and supporting a *trans* configuration for the alcohol and isopropenyl groups.

Regarding product characterization of Lim-2-OOH, even with purified material, conventional ^1H NMR provides limited information beyond the identification of hydroperoxy and vinylic protons and their relative integrals, as previously discussed. The presence of diastereomeric mixtures further complicates spectral interpretation, particularly for proton assignments. Signal duplication due to diastereomers significantly hinders the resolution and assignment of protons in the downfield region (3-12 ppm). Furthermore, proton resonances in the upfield region (0-3 ppm) are severely convoluted by overlapping chemical shifts and complex multiplicities, effectively arising from two distinct stereoisomeric species within the sample, rendering unambiguous assignments nearly impossible.

The ^{13}C NMR spectrum of the purified Lim-2-OOH (Fig. 2.8) exhibited 20 distinct carbon resonances, indicating the presence of two C10 monoterpene derivatives in the sample. The relative intensities of these signals reflected the diastereomeric mixture, with approximately 10 peaks showing higher intensity and 10 peaks of lower intensity. This pattern is consistent with the differing abundances of the two isomers, as previously suggested by the ^1H NMR data.

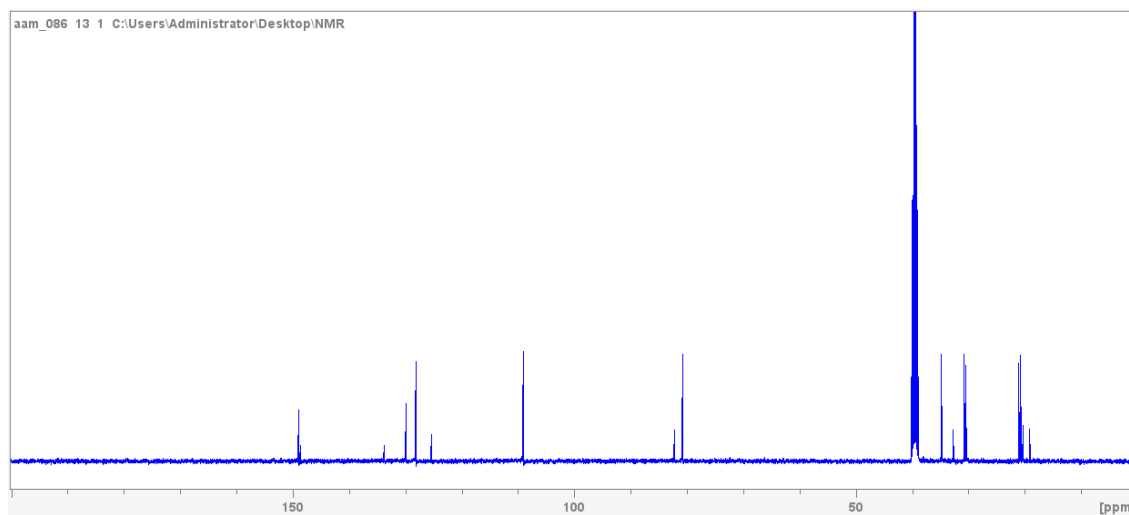


Figure 2.8. ^{13}C NMR evidence of diastereomeric composition in purified Lim-2-OOH.

The ^{13}C NMR spectrum of purified Lim-2-OOH displays 20 distinct carbon resonances, corresponding to two C10 monoterpene derivatives. Approximately half of these peaks exhibit higher intensities, while the other half are noticeably weaker, reflecting the differing abundances of the two diastereomers. The close chemical shift proximity of the corresponding high- and low-intensity signals further supports their similar chemical environments and confirms their isomeric relationship.

By combining the ^{13}C NMR spectrum with a distortionless enhancement by polarization transfer at 135° (DEPT-135) experiment, each carbon atom in the molecule could be classified according to its substitution pattern: methyl ($-\text{CH}_3$), methylene ($-\text{CH}_2-$), methine ($>\text{CH}-$), or quaternary ($>\text{C}<$). In the DEPT-135 spectrum, signals corresponding to methyl and methine carbons appear in positive phase (upright), methylene carbons appear in negative phase (inverted), and quaternary carbons are absent due to the lack of directly attached protons (Fig. 2.9).

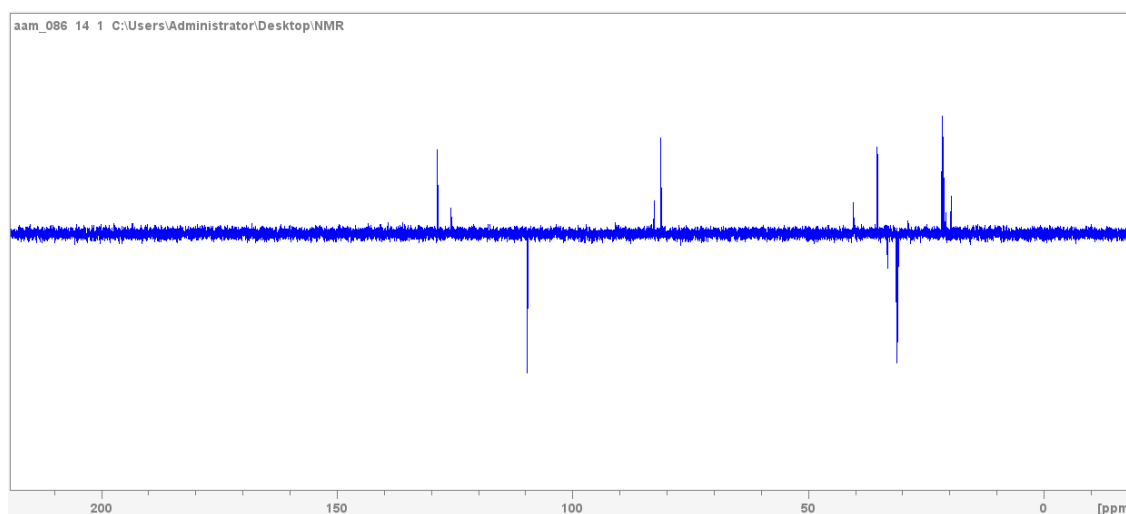
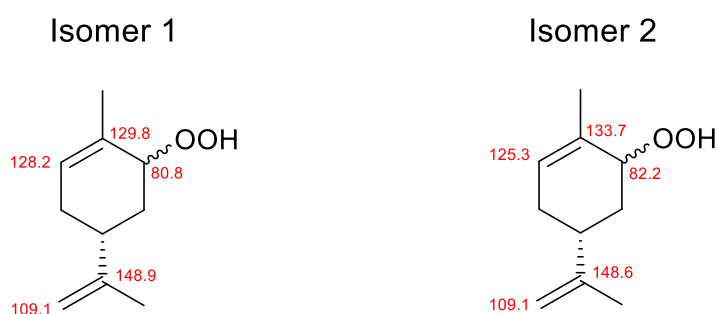


Figure 2.9. DEPT-135 Spectrum of Purified Lim-2-OOH.

The DEPT-135 spectrum of purified Lim-2-OOH enables differentiation of carbon types based on signal phase and chemical shift.

Using data obtained from the ^{13}C NMR spectrum and DEPT-135, along with the fortuitous difference in signal intensities between diastereomers, it was possible to partially assign carbon resonances to the stereoisomers present in the purified Lim-2-OOH sample (Fig. 2.10). However, beyond the assignment of carbons exhibiting downfield chemical shifts, the high degree of similarity among the remaining methine ($>\text{CH}-$), methylene ($-\text{CH}_2-$), and methyl ($-\text{CH}_3$) carbon signals precluded unambiguous allocation to specific positions (as illustrated in the preceding figure). To resolve these ambiguities and enable subsequent proton assignments, a combination of two-dimensional nuclear magnetic resonance (2D NMR) techniques was employed: Heteronuclear Single Quantum Coherence (HSQC) to detect ^{13}C - ^1H correlations over one bond, Heteronuclear Multiple Bond Correlation (HMBC) to identify ^{13}C - ^1H correlations over two to four bonds, ^1H - ^1H Correlation Spectroscopy (^1H - ^1H COSY) to map scalar

couplings between adjacent protons, and NOESY to probe spatial proximity between protons.



5

Figure 2.10. Partial ¹³C NMR signal assignment for the two putative diastereomers of purified Lim-2-OOH.

Carbons exhibiting downfield chemical shifts were more readily assigned due to their association with distinct structural features (e.g., terminal olefinic carbons versus olefinic carbons within a ring system). Assignment was further guided by differences in signal intensities corresponding to the major and minor diastereomers.

As the signal intensity and spectral resolution in the 2D NMR experiments were more pronounced for Isomer 1 compared to Isomer 2, the initial characterization focused on the predominant isomer. HSQC revealed a one-bond correlation between the methine carbon at 128.2 ppm and its proton at 5.68 ppm (Fig. 2.11). The α -carbon bearing the hydroperoxy group (80.8 ppm) showed an HSQC correlation with the proton at 4.16 ppm (Appendix A, Fig. A1). The terminal vinylic carbon at 109.1 ppm correlated with protons at 4.73 ppm (Appendix A, Fig. A2).

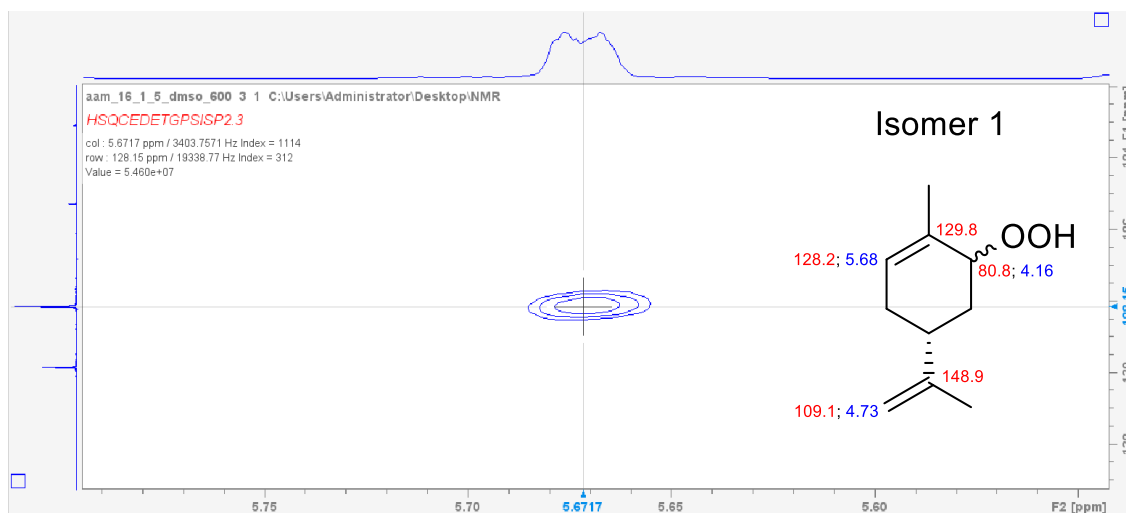


Figure 2.11. HSQC correlation identifying a vinylic carbon-proton pair in the predominant Lim-2-OOH isomer.

HSQC spectrum showing a correlation between the carbon resonance at 128.2 ppm (y-axis) and the proton resonance at 5.68 ppm (x-axis), consistent with a vinylic CH environment. The inset depicts the partially elucidated structure of the predominant Lim-2-OOH isomer, incorporating three confirmed HSQC correlations.

HMBC experiments were performed to detect long-range (2-4 bond) heteronuclear correlations. Targeting the previously assigned vinylic proton at 4.73 ppm, a correlation was observed with a carbon resonance at 34.8 ppm, consistent with a three-bond coupling (Appendix A, Fig. A3). Subsequent HSQC analysis confirmed a direct C-H correlation between this carbon and a proton at 2.21 ppm (Appendix A, Fig. A4). HMBC targeting of the quaternary carbon at 148.9 ppm revealed a strong two-bond correlation with methyl protons at 1.72 ppm (Fig. 2.12). HSQC data then established that these methyl protons correlate directly to the carbon at 20.8 ppm (Appendix A, Fig. A5).

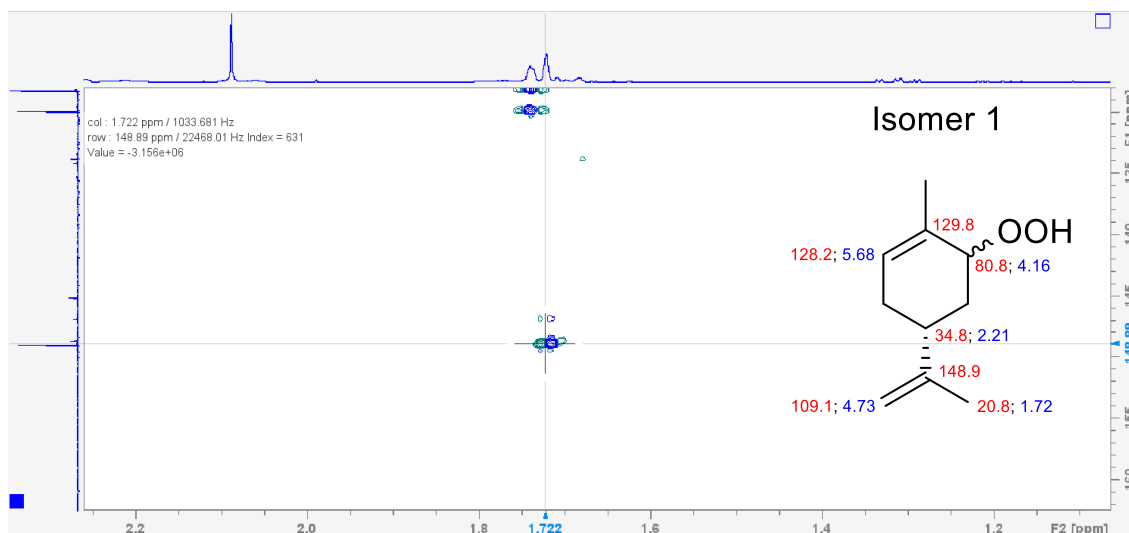


Figure 2.12. Long-range HMBC correlation supporting partial structural assignment of the predominant Lim-2-OOH isomer.

The spectrum shows a correlation between the quaternary carbon at 148.9 ppm and the methyl protons at 1.72 ppm. The inset displays the updated partial structural assignment of the predominant isomer based on this correlation and others described.

The quaternary alkene carbon at 129.8 ppm exhibited a HMBC correlation over three bonds with methyl protons at 1.74 ppm (Appendix A, Fig. A6). These methyl protons displayed a HSQC correlation with a carbon at 21.1 ppm (Appendix A, Fig. A7). To identify the methylene carbon adjacent to the vinylic carbon at 128.2 ppm, HMBC analysis was performed by targeting the corresponding vinylic proton at 5.68 ppm. This revealed a correlation with a carbon at 30.5 ppm, assigned as the methylene carbon (Appendix A, Fig. A8). Subsequent HSQC analysis established direct correlations with protons at 2.06 and 1.77 ppm (Appendix A, Fig. A9), an assignment further supported by H-H COSY data (Fig. 2.13).

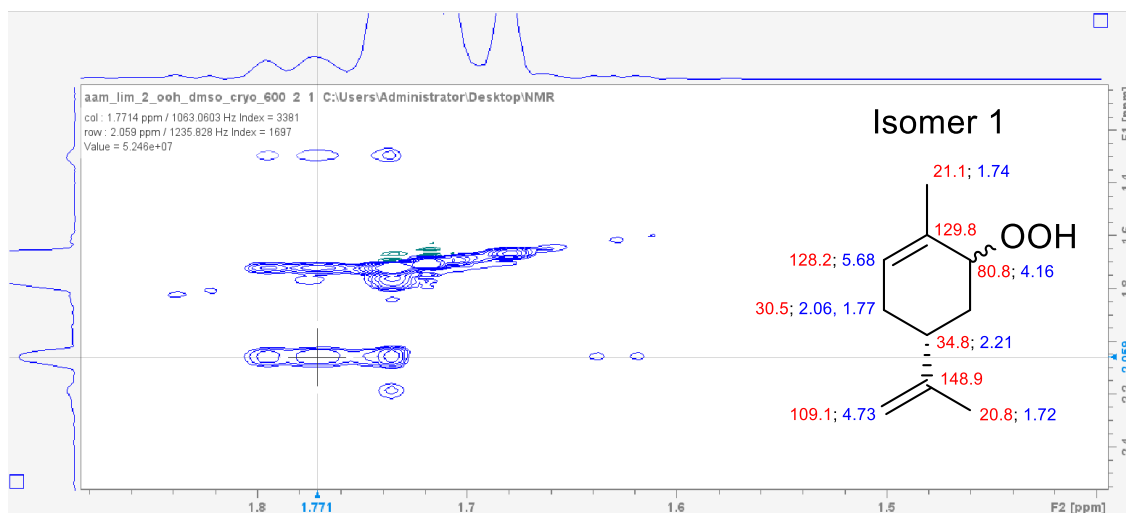


Figure 2.13. ^1H - ^1H COSY correlation of geminal methylene protons in the predominant Lim-2-OOH isomer.

^1H - ^1H COSY spectrum showing correlations from the geminal methylene protons at 1.77 ppm and 2.06 ppm. The inset illustrates the updated partial structural assignment of the predominant isomer, incorporating these correlations into the cumulative NMR-based elucidation.

No HMBC correlation was observed between the proton at 4.16 ppm, assigned to the hydroperoxy-bearing carbon, and the adjacent methylene carbon. Similarly, no HMBC correlation was detected for the proton at 2.21 ppm, attached to the isopropenyl-bearing carbon. Consequently, HMBC was employed to probe the connectivity by targeting the hydroperoxy-bearing carbon at 80.8 ppm. This approach revealed a distinct correlation with a single proton resonance at 2.27 ppm (Fig. 2.14), assigned to one of the protons on the previously unassigned methylene carbon. Subsequent HMBC and HSQC analyses enabled the assignment of the methylene carbon at 30.8 ppm (Appendix A, Fig. A10) and the corresponding methylene proton at 1.31 ppm (Appendix A, Fig. A11), respectively.

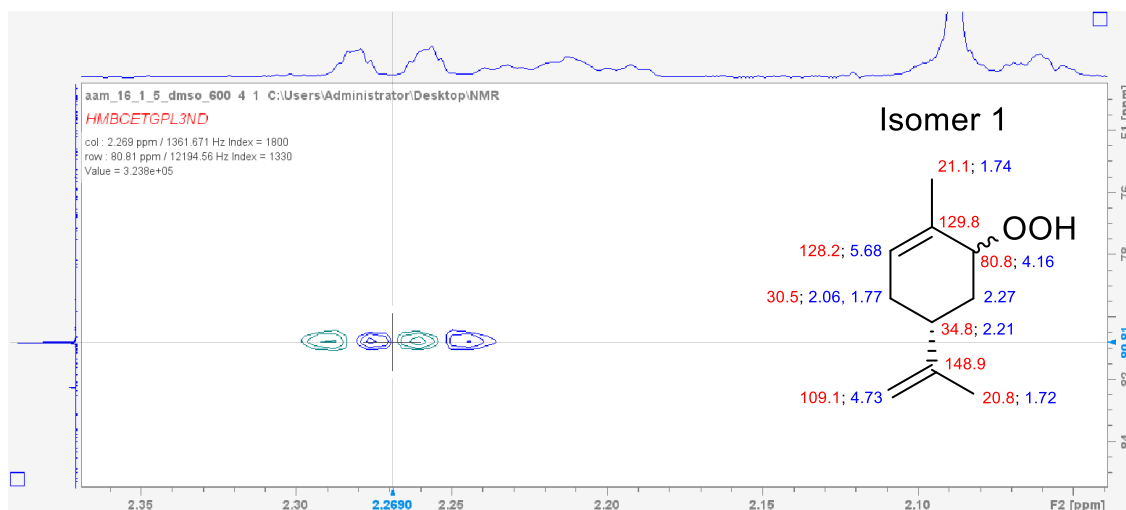
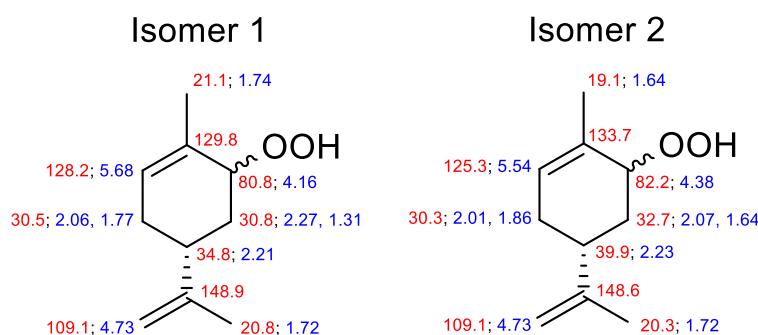


Figure 2.14. HMBC correlation between the hydroperoxy-bearing carbon and adjacent methylene proton.

HMBC spectrum showing a correlation between the hydroperoxy-bearing carbon at 80.8 ppm and a proton at 2.27 ppm on the adjacent methylene carbon. The inset depicts the predominant isomer with near-complete carbon and proton assignments achieved at this stage of the structural elucidation.

Assignment of carbon and proton chemical shifts for Isomer 2 proved more challenging due to its lower abundance relative to Isomer 1, which resulted in reduced signal intensity and necessitated increased scan times to improve spectral resolution. Despite these constraints, comprehensive and accurate assignments were achieved (Appendix A, Fig. A12-24), and the structural characterisation of both isomers was considered nearly complete based on the available spectroscopic data (Fig. 2.15).



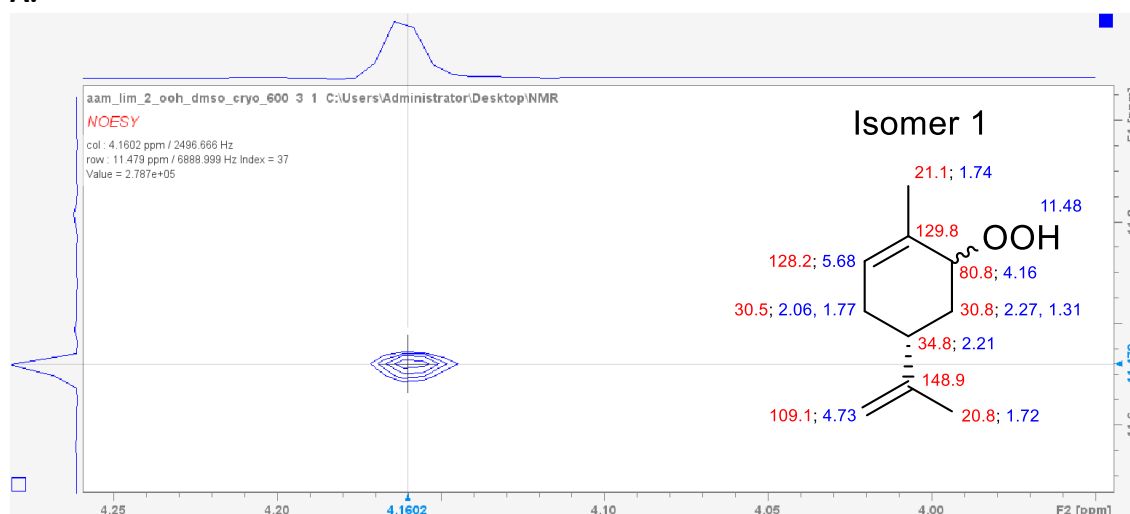
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Figure 2.15. Comparative carbon and proton assignments for major and minor isomers of Lim-2-OOH.

Structural representations of Isomer 1 (major) and Isomer 2 (minor) showing the NMR-derived carbon and proton chemical shift assignments elucidated up to this stage of the analysis.

Two protons, tentatively assigned as hydroperoxy protons resonating at 11.48 and 11.17 ppm, did not exhibit correlations in the HMBC spectra. Instead, NOESY, which probes through-space H-H interactions, was employed to detect spatial correlations. In Isomer 1, a NOESY correlation was observed between the proton at 4.16 ppm and the hydroperoxy proton at 11.48 ppm (Fig. 2.16A). Similarly, in Isomer 2, a correlation was detected between the proton at 4.38 ppm and the hydroperoxy proton at 11.17 ppm (Fig. 2.16B).

A.



B.

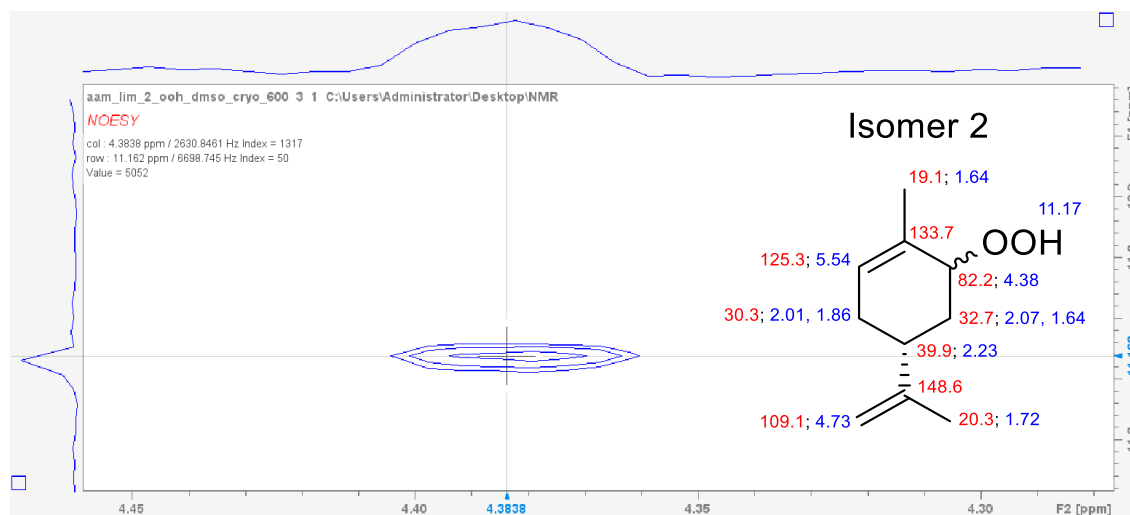
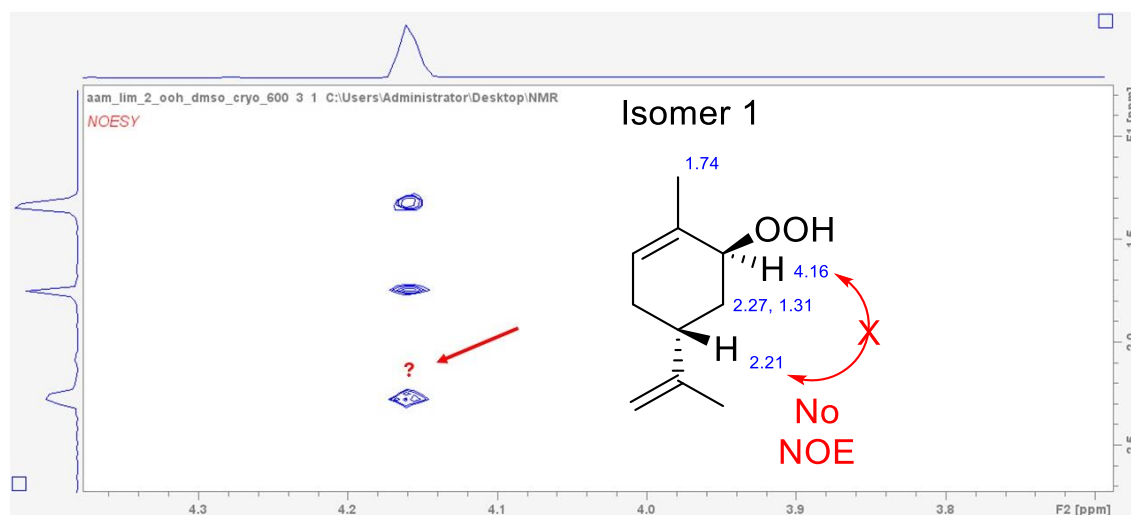


Figure 2.16. NOESY correlations of hydroperoxy protons and adjacent protons within individual Lim-2-OOH diastereomers.

This figure presents NOESY spectra showing through-space correlations between the protons attached to the hydroperoxy-bearing carbons and their respective hydroperoxy protons within Isomer 1 (Panel A) and Isomer 2 (Panel B). Insets depict the corresponding partially assigned structures for each isomer, highlighting the assigned hydroperoxy protons.

To elucidate the absolute configuration of the two isomers, NOESY experiments were employed to identify through-space proton-proton correlations involving the protons attached to the hydroperoxy-bearing carbon (4.16 ppm for Isomer 1 and 4.38 ppm for Isomer 2) and the protons attached to the isopropenyl-bearing carbon (2.21 ppm for Isomer 1 and 2.23 ppm for Isomer 2). For Isomer 1, the proton at 4.16 ppm exhibited NOE interactions with protons at 1.31 ppm, 1.74 ppm, and 2.27 ppm, but no NOE correlation was observed with the proton at 2.21 ppm (Fig. 2.17A). In contrast, despite lower signal intensity due to its reduced abundance, Isomer 2 showed NOE correlations between the proton at 4.38 ppm and protons at 1.64 ppm, 2.07 ppm, and notably 2.23 ppm (Fig. 2.17B).

A.



B.

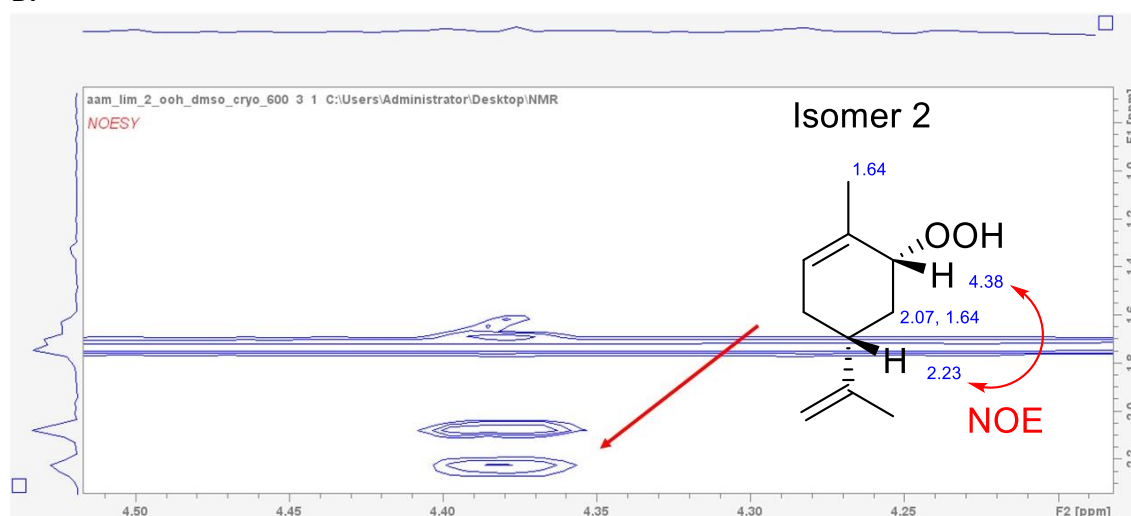
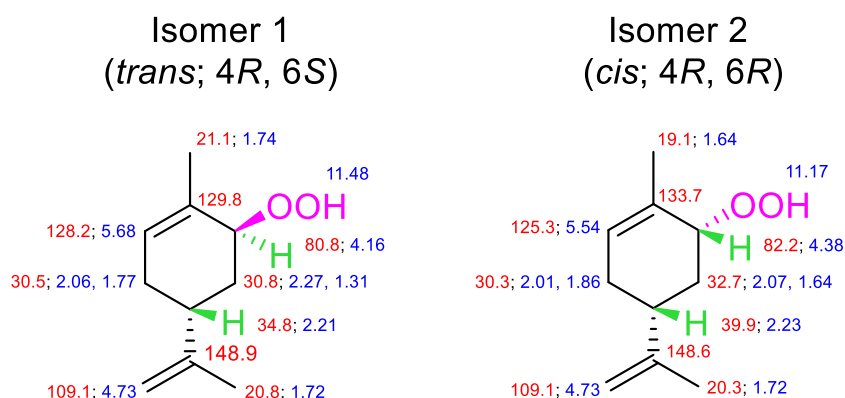


Figure 2.17. NOESY analysis of through-space proton-proton correlations in hydroperoxy-bearing isomers.

NOESY spectra highlighting through-space correlations between the proton attached to the hydroperoxy-bearing carbon and adjacent protons within each isomer. **(A)** Isomer 1 demonstrates NOE interactions between the hydroperoxy proton and neighbouring protons, with an absence of NOE observed between the hydroperoxy proton and the proton attached to the isopropenyl-bearing carbon. Conversely, **(B)** Isomer 2 exhibits a distinct NOE correlation between these two protons, indicating closer spatial proximity in this isomer. These NOE patterns provide critical insights into the stereochemical relationships of the substituents within each isomer.

The absence of NOE between the 4.16 ppm and 2.21 ppm protons in Isomer 1, contrasted with the presence of NOE between the 4.38 ppm and 2.23 ppm protons in Isomer 2, indicates that in Isomer 1 these protons are spatially further apart and thus positioned on opposite sides of the ring (*trans* configuration), whereas in Isomer 2 they are in closer spatial proximity on the same side of the ring (*cis* configuration). Based on this analysis, the absolute configurations can be confidently assigned as (4*R*,6*S*)-6-hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene for Isomer 1 and (4*R*,6*R*)-6-hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene for Isomer 2 (Fig. 2.18).

A.



5

B.

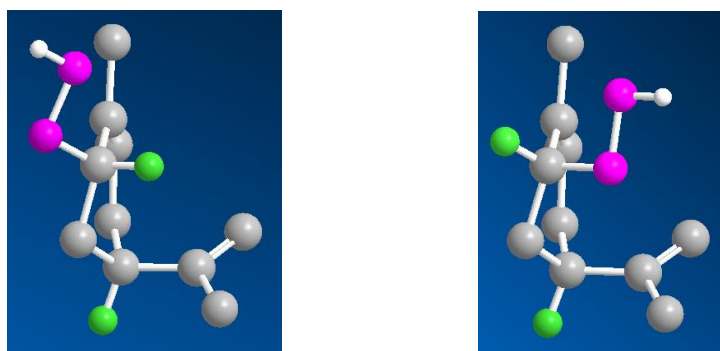


Figure 2.18. Comprehensive structural characterization and stereochemical assignment of Lim-2-OOH diastereomers.

(A) Complete ^1H and ^{13}C NMR chemical shift assignments for both Lim-2-OOH diastereomers, integrating all spectroscopic data including HSQC, HMBC, and NOESY correlations. The cumulative NMR evidence strongly supports the stereochemical assignment of the predominant isomer (Isomer 1) as the *trans* diastereomer, while the minor isomer (Isomer 2) corresponds to the *cis* configuration. **(B)** Corresponding 3D ball-and-stick molecular models for each isomer, with hydroperoxy groups highlighted in magenta and the protons attached to the hydroperoxy-bearing and isopropenyl-bearing carbons highlighted in green, matching the colour scheme utilized in panel A for clear correlation between spectral data and molecular structure.

2.3.2 Synthesis and Characterisation of Lin-6-OOH (9) and Lin-7-OOH (10) Structural Isomers

To synthesise the monoterpene hydroperoxides of linalool (Lin-7-OOH and Lin-6-OOH) the Schenck reaction was used. In its simplest form (Fig. 2.19), the Schenck reaction is

the preparation of hydroperoxides generated from alkenes containing allylic hydrogens from singlet oxygen ($^1\text{O}_2$).

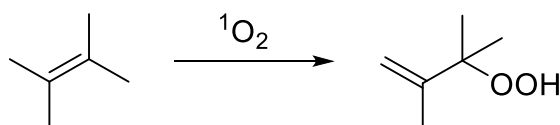


Figure 2.19. | General reaction scheme of the 'ene' or 'Schenck' reaction.

The reaction involves the insertion of singlet oxygen across a double bond with a subsequent intramolecular allylic proton abstraction.

The proposed mechanism involves initial interaction between singlet oxygen and the alkene double bond, potentially facilitated by a small dipole moment in singlet oxygen. Nucleophilic attack of the π -electrons from the alkene onto singlet oxygen generates a transient zwitterionic intermediate, in which the tertiary carbon bears a formal positive charge, while one oxygen atom carries a formal negative charge due to cleavage of the O-O π -bond. The lone pair on the oxygen atom covalently bound to the carbon can delocalize to partially stabilize the carbocation. Subsequent allylic hydrogen abstraction leads to the formation of a new C=C bond, with the oxygen atom recovering its lone pair to yield the neutral allylic hydroperoxide product (Fig. 2.20) [94].

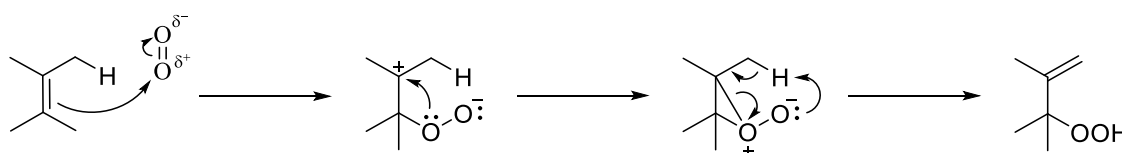


Figure 2.20. Proposed mechanistic pathway for allylic hydroperoxide formation in the Schenck reaction of linalool.

The position of hydroperoxide incorporation in the Schenck rearrangement is dictated by both stereoselectivity and regioselectivity. In trisubstituted alkenes bearing a geminal dimethyl substituent, the allylic hydrogens on the bulkier alkyl substituent generally exhibit higher reactivity than those associated with the smaller substituent [95]. Consequently, for linalool, it was anticipated that among the possible Lin-6-OOH and Lin-7-OOH structural isomers (Fig. 2.21), formation of Lin-7-OOH would be favoured over Lin-6-OOH, as this pathway positions the hydroperoxide group on the more substituted carbon centre.

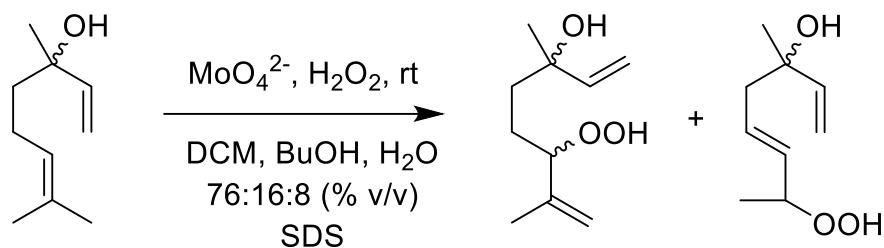


Figure 2.21. Formation of Lin-6-OOH and Lin-7-OOH structural isomers via the Schenck rearrangement of Linalool.

The scheme depicts the allylic hydroperoxides generated from the Schenck rearrangement of racemic linalool (**2**). Two distinct structural isomers are obtained: Lin-6-OOH (**9**), present as four unique configurations, of which there are four diastereomeric pairs and two enantiomeric pairs; and Lin-7-OOH (**10**), possessing a single chirality centre and therefore existing as an enantiomeric pair.

Following reaction workup, TLC analysis indicated a clean transformation, with complete consumption of linalool starting material ($R_f = 0.76$, hexane/EtOAc 70:30) and the appearance of a single, more polar product spot with lower mobility ($R_f = 0.16$) (Fig. 2.22). None of the compounds were UV-active; therefore, visualization required ammonium molybdate staining. Although the reaction was expected to yield a mixture of two linalool hydroperoxide structural isomers, the observation of a single product spot suggests that the isomers possess nearly identical polarity and, consequently, very similar R_f values, making them a challenge to separate by TLC or flash chromatography.

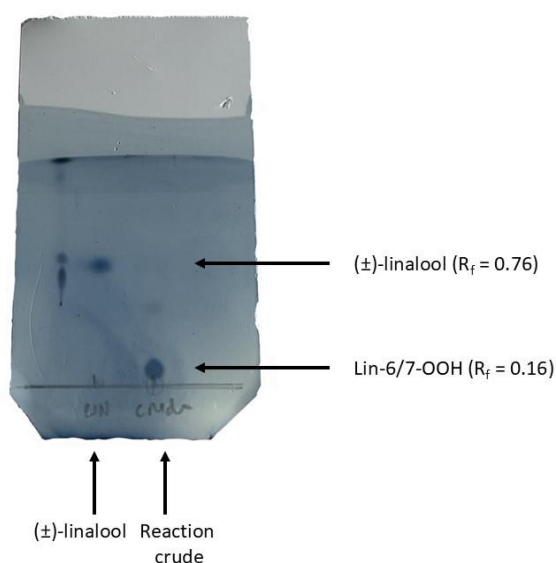


Figure 2.22. TLC analysis of the Lin-6/7-OOH reaction mixture.

TLC (hexane/EtOAc 70:30) comparing ($\pm$)-linalool starting material (left lane, $R_f = 0.76$) with the crude reaction mixture (right lane) containing Lin-6/7-OOH structural isomers ($R_f = 0.16$). The absence of the starting material spot in the crude mixture indicates complete conversion under the reaction conditions.

^1H NMR spectroscopy was employed to assess the crude reaction mixture for hydroperoxy proton resonances, expected in the 10.50-12.00 ppm region (Fig. 2.23). The crude spectrum displayed a relatively clean profile, consistent with TLC observations. Comparison of spectra from the crude material and the sample purified via flash chromatography revealed near-identical profiles, indicating minimal impurity removal during purification. Flash chromatography was performed primarily to eliminate residual SDS from the reaction workup, which imparted a cloudy appearance to the crude sample but was not detectable by TLC. From 200 mg of crude Lin-6/7-OOH subjected to purification, 170 mg of colourless oil was obtained, corresponding to an approximate 85% yield (although residual SDS in the crude may have influenced the calculated yield). The most diagnostically significant feature in both spectra was the presence of two resonances in the 10.50-12.00 ppm region, consistent with hydroperoxy protons. Closer examination of the purified sample, recorded at 600 MHz (compared to 400 MHz for the crude), revealed that the most downfield signal was in fact a doublet. This is consistent with the Lin-6-OOH isomer possessing two chirality centres, giving rise to four stereoisomers with unique configurations i.e., (3*R*,6*R*), (3*R*,6*S*), (3*S*,6*R*), and (3*S*,6*S*), from which four diastereomeric- and two enantiomeric-relationships exist. Integration of these hydroperoxy resonances indicated an approximate 5:4 ratio in favour of Lin-6-OOH, providing further evidence for the coexistence of Lin-6/7-OOH structural isomers in the sample.

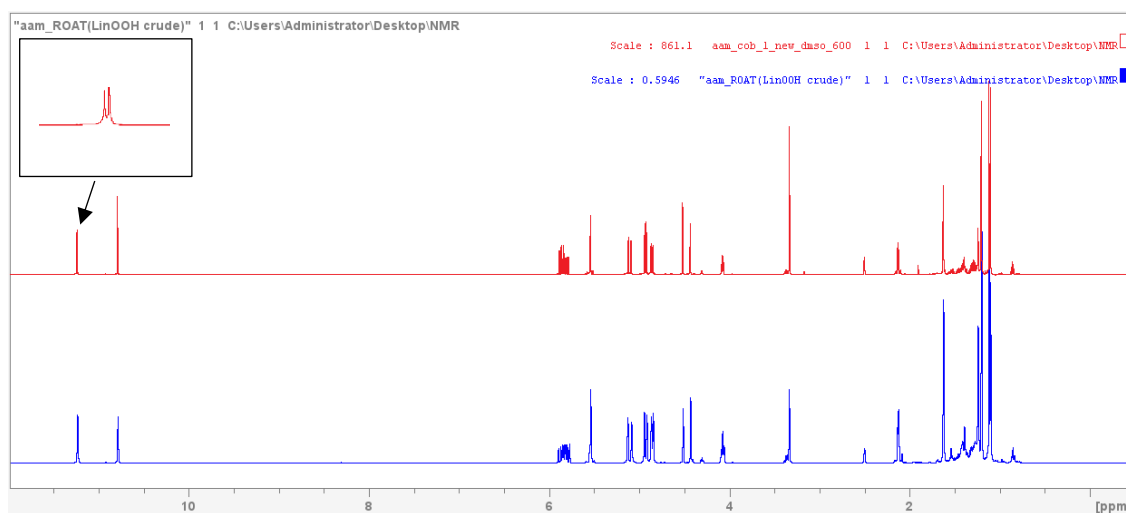


Figure 2.23. ^1H NMR spectra of crude and purified Lin-6/7-OOH.

^1H NMR spectra of the crude reaction mixture (bottom) and purified Lin-6/7-OOH (top) showing hydroperoxy proton resonances in the 10.50-12.00 ppm region. Initial analysis reveals two major signals, but higher-resolution spectra (600 MHz, inset) of the purified sample resolve three distinct resonances. This splitting corresponds to the presence of the Lin-6-OOH structural isomer as a mixture of diastereomers, while the single resonance corresponds to the Lin-7-OOH structural isomer.

The presence of hydroperoxy proton resonances provided substantial evidence that the sample comprised the desired Lin-6/7-OOH structural isomers. However, unambiguous assignment of proton chemical shifts in the downfield region (approximately 3-12 ppm) remained challenging, and spectral overlap rendered attribution in the upfield region (approximately 0-3 ppm) infeasible - an inherent limitation when analyzing isomeric mixtures. To reduce ambiguity and avoid premature assignments, further structural elucidation was undertaken using ^{13}C NMR combined with DEPT-135 experiments (Fig. 2.24). This approach enabled differentiation of carbon environments by identifying methyl ($-\text{CH}_3$), methylene ($=\text{CH}_2$), methine ($>\text{CH}-$), and quaternary ($>\text{C}<$) carbons. A total of 30 carbon resonances of comparable intensity were observed, consistent with the coexistence of Lin-6-OOH diastereomeric pairs (20 carbons total, reflecting two monoterpenoid derivatives) alongside the Lin-7-OOH enantiomeric pair (10 carbons), thereby supporting the proposal of Lin-6/7-OOH structural isomers.

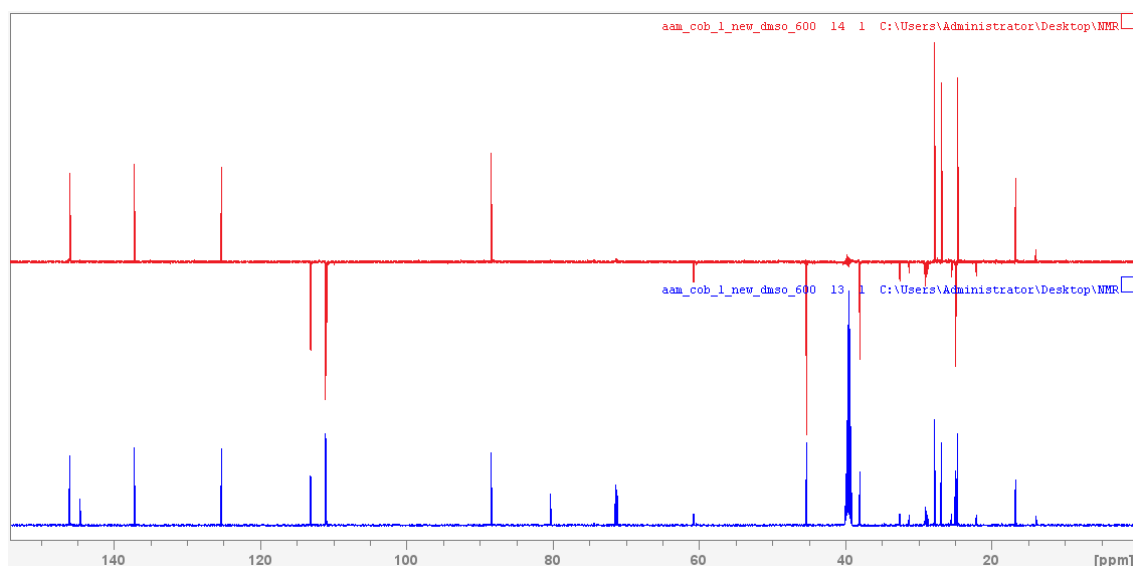


Figure 2.24. Stacked ^{13}C NMR and DEPT-135 spectra of purified Lin-6/7-OOH mixture.

^{13}C NMR (bottom) and DEPT-135 (top) spectra of the purified Lin-6/7-OOH sample. Detailed examination reveals that several peaks appearing as singlets are actually closely spaced doublets, differing by approximately 0.02 ppm. This subtle splitting is indicative of the presence of diastereomeric Lin-6-OOH species within the sample, consistent with their distinct but closely related carbon environments.

Since Lin-6-OOH and Lin-7-OOH are structural isomers rather than stereoisomers, a substantial number of carbon resonances can be confidently assigned without recourse to additional 2D NMR experiments. This is attributed to the distinct rearrangements of the double bond following allylic hydrogen abstraction during synthesis - specifically, the formation of terminal versus internal double bonds - resulting in unique carbon chemical environments proximal to the rearranged double bond and hydroperoxy substituent. However, carbon environments in regions of structural similarity between the isomers exhibit minimal chemical shift differences (Fig. 2.25).

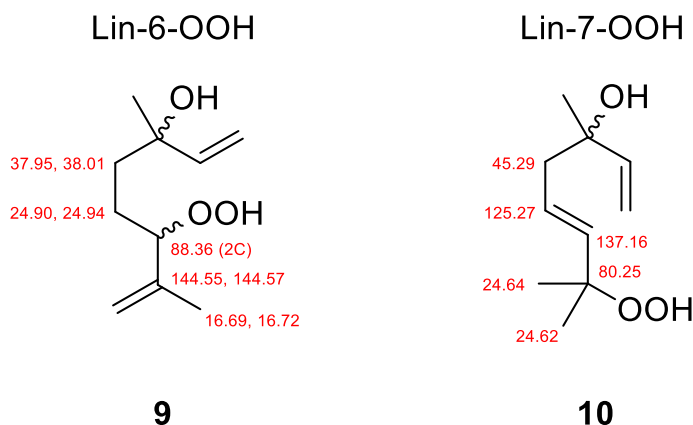


Figure 2.25. Partial carbon chemical shift assignment of Lin-6-OOH (9) diastereomers and Lin-7-OOH (10) enantiomers.

Building upon the carbon assignments, a combination of HSQC and HMBC experiments was employed to assign the directly correlated proton resonances. Focusing initially on the Lin-6-OOH diastereomers, HSQC revealed correlations between the methyl carbons at 16.69 and 16.72 ppm and protons resonating at 1.62 ppm (Appendix A, Fig.A25). Subsequent HMBC analysis of these methyl protons (1.62 ppm) enabled the identification of the terminal olefinic carbons located three bonds away, observed at 113.04 and 113.09 ppm (Fig. 2.26). Further HSQC data permitted the assignment of protons at 4.87 and 4.85 ppm to these terminal olefinic carbons, confirming their connectivity (Appendix A, Fig.A26).



Figure 2.26. HMBC correlation between methyl protons and terminal olefinic carbons in Lin-6-OOH (9) diastereomers.

HMBC spectrum illustrating the long-range correlation between methyl protons resonating at 1.62 ppm (x-axis) and the terminal olefinic carbons at 113.04 and 113.09 ppm (y-axis) corresponding to the Lin-6-

OOH diastereomers. The inset depicts the partially elucidated molecular structure based on these spectral assignments.

HSQC spectroscopy was subsequently employed to establish direct one-bond proton-carbon correlations, revealing connectivity between the hydroperoxy-bearing carbons resonating at 88.36 ppm and the proton signals at 4.08 ppm (Appendix A, Fig. A27). Additional correlations were observed between methylene carbons at 24.90 and 24.94 ppm and protons at 1.40 and 1.30 ppm (Appendix A, Fig. A28), as well as between methine carbons at 37.95 and 38.01 ppm and the protons resonating at 1.41 and 1.28 ppm (Fig. 2.27), thereby facilitating detailed proton and carbon assignment within the molecular framework.

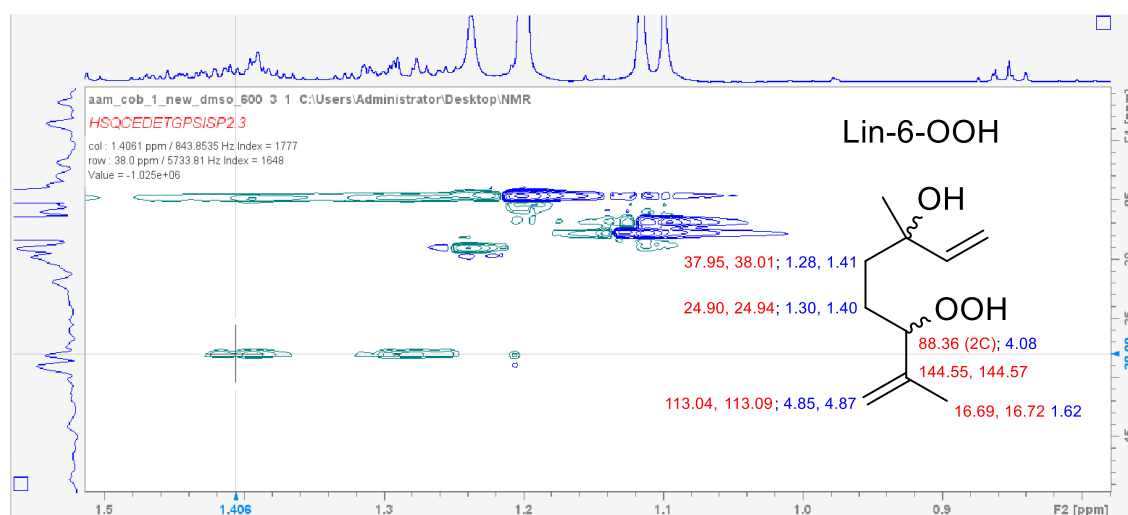


Figure 2.27. HSQC correlations of methylene carbons and protons in Lin-6-OOH (9) diastereomers.

This figure presents HSQC correlations between methylene carbons resonating at 37.95 and 38.01 ppm and the associated protons at 1.28 and 1.41 ppm within the Lin-6-OOH diastereomeric mixture. The inset illustrates the partial assignment of proton and carbon resonances determined up to this stage, prior to analysis of the structural region shared with the Lin-7-OOH isomer.

Characterization of these structural isomers becomes increasingly challenging with increasing distance from the site(s) of oxygen insertion into the terpene backbone. This is due to the convergence of their molecular frameworks in these distal regions, where the deshielding influence of the hydroperoxy group is attenuated, resulting in only subtle chemical shift differences between the isomers. To address this, HMBC was employed to exploit long-range ^1H - ^{13}C correlations (2–4 bonds) from the most recently assigned methylene protons. This analysis revealed a correlation between the methylene protons

at 1.41 ppm and the hydroxyl-bearing carbons at 71.16 ppm (2C) (Appendix A, Fig. A29). Subsequently, a reverse HMBC approach was undertaken, using the hydroxyl-bearing carbon(s) as the reference point to identify previously unassigned protons within 2–4 bonds. This strategy established correlations with the vinylic proton(s) at 5.81 ppm, the terminal vinylic protons at 5.13 and 4.94 ppm, and the hydroxyl proton(s) at 4.44 ppm (Fig. 2.28).

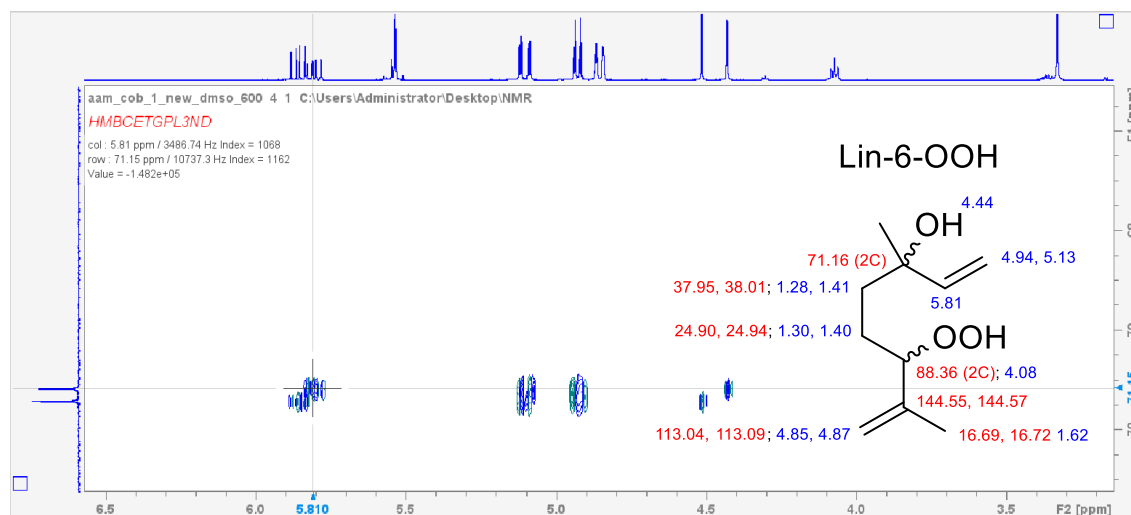


Figure 2.28. HMBC correlations between the hydroxy-bearing quaternary carbons and proximate proton resonances in Lin-6-OOH (9) diastereomers.

Two-dimensional HMBC spectra showing correlations between the hydroxy-substituted quaternary carbons at 71.16 ppm (y-axis) and neighbouring methine protons at 5.81 ppm, terminal olefinic protons at 4.94 and 5.13 ppm, and hydroxyl protons at 4.44 ppm (x-axis).

An additional HMBC correlation, not depicted in the previous figure, was observed between the hydroxy-substituted quaternary carbon and the methyl protons on the adjacent carbon at 1.12 ppm (Appendix A, Fig. A30). Subsequent HSQC analysis completed the structural characterisation of the Lin-6-OOH isomer by establishing direct H-C correlations for these newly identified protons. Specifically, the methyl protons at 1.12 ppm were assigned to carbons at 27.74 ppm (Appendix A, Fig. A31), the vinylic protons at 5.81 ppm were assigned to quaternary carbons at 145.95 ppm (Appendix A, Fig. A32), and the terminal olefinic protons were assigned to carbons at 110.90 ppm (Appendix A, Fig. A33).

As previously noted, ^1H NMR analysis at 600 MHz revealed two closely spaced resonances at 11.24 ppm, the duplication of which was attributed to the presence of a

diastereomeric pair, suggesting that these hydroperoxy protons were associated with the Lin-6-OOH isomer. HMBC analysis confirmed this assignment through the observed correlation between the hydroperoxy proton at 11.24 ppm and the hydroperoxy-bearing carbons at 88.36 ppm (Lin-6-OOH), as well as between the hydroperoxy proton at 10.78 ppm and its corresponding hydroperoxy-bearing carbon at 80.25 ppm (Lin-7-OOH) (Fig. 2.29).

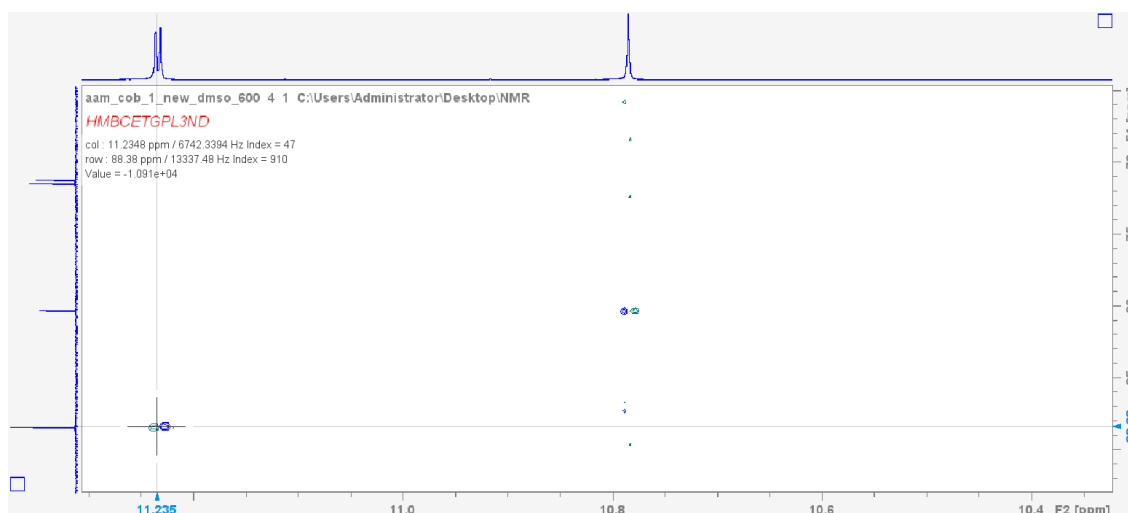


Figure 2.29. HMBC correlations between hydroperoxy-bearing carbons and hydroperoxy protons in Lin-6-OOH (9) and Lin-7-OOH (10).

HMBC spectra illustrating long-range correlations between the hydroperoxy-bearing carbon at 88.36 ppm and the hydroperoxy proton at 11.24 ppm for Lin-6-OOH, and between the hydroperoxy-bearing carbon at 80.25 ppm and the hydroperoxy proton at 10.79 ppm for Lin-7-OOH.

In contrast to the second isomer of Lim-2-OOH described earlier, the assignment of ^1H and ^{13}C resonances for Lin-7-OOH proved less challenging, as its abundance was comparable to that of Lin-6-OOH. Comprehensive assignments were achieved (Appendix A, Fig.A34-45), and, based on the data presented thus far, the structural characterisations of both Lin-6-OOH and Lin-7-OOH were effectively complete (Fig. 2.30).

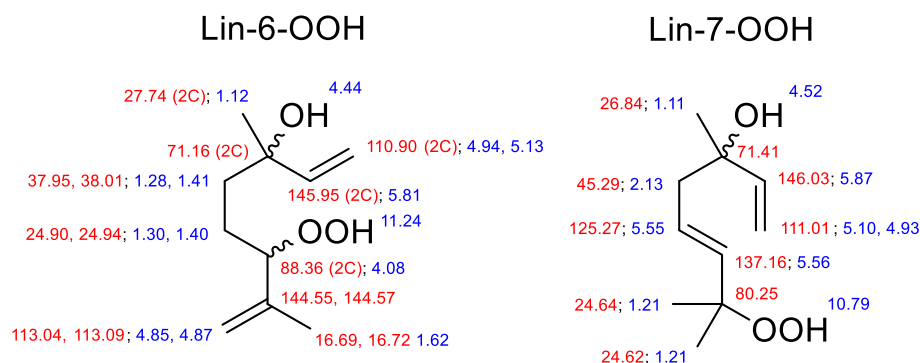


Figure 2.30. Comprehensive ^1H and ^{13}C NMR assignments for Lin-6-OOH (9) diastereomers and Lin-7-OOH (10) enantiomers.

Complete ^1H and ^{13}C NMR chemical shift assignments for the two pairs of diastereomers corresponding to Lin-6-OOH (20 distinct carbon resonances distinguishable to two decimal places and 36 proton resonances, where the protons associated with the diastereomeric carbons are not spectroscopically distinguishable) and for the enantiomeric pair of Lin-7-OOH (10 distinct carbon resonances and 18 proton resonances).

2.3.3 Synthesis and Characterisation of Ger-6-OOH (24) and Ger-7-OOH (18)

Structural Isomers

The synthesis of the Ger-6-OOH and Ger-7-OOH structural isomers was carried out under Schenck reaction conditions, following a similar approach to that previously described for the synthesis of Lin-6-OOH and Lin-7-OOH. The Schenck reaction has already been outlined in the preceding section, they will not be reiterated here. The key distinction in this case lies in the use of geraniol as the parent terpene, rather than linalool, which results in two notable structural differences. First, geraniol contains allylic protons adjacent to one of its double bonds, whereas the corresponding double bond in linalool does not possess allylic protons in relation to its terminal alkene. Second, the number and distribution of chirality centres differ between the starting materials and products. Geraniol is an achiral *trans*-alkene with no chirality centres, whereas the oxidation products Ger-6-OOH and Ger-7-OOH contain one and zero chirality centres, respectively. Consequently, Ger-6-OOH is obtained as a racemic mixture of enantiomers, while Ger-7-OOH remains an achiral compound (Fig. 2.31).

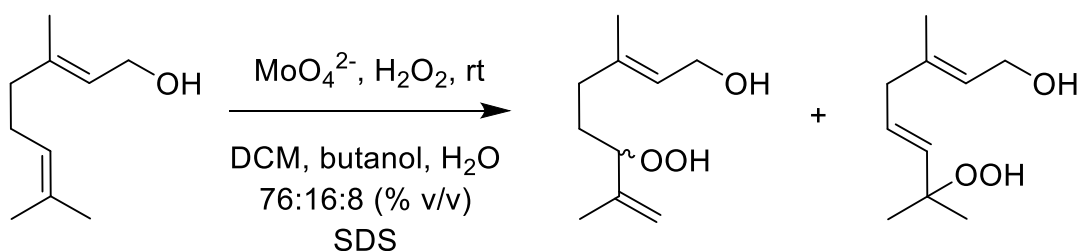


Figure 2.31. Formation of Ger-6-OOH (24) and Ger-7-OOH (18) structural isomers via the Schenck rearrangement of geraniol (3).

The scheme depicts the allylic hydroperoxides generated from the Schenck rearrangement of geraniol (3). Two distinct structural isomers are obtained: Ger-6-OOH (24), bearing one chirality centre, consequently existing as a pair of enantiomers; and Ger-7-OOH (18), an achiral compound.

In contrast to the synthesis of Lin-6/7-OOH using the Schenck reaction method, the ammonium molybdate-stained TLC of crude Ger-6/7-OOH indicated the formation of a complex reaction mixture (Fig. 2.32). This can be explained by the presence of additional allylic protons in geraniol compared to linalool, thereby increasing the likelihood of generating more hydroperoxide species. A substantial amount of unreacted geraniol remained ($R_f = 0.74$, hexane/EtOAc 70:30), along with two prominent spots that were more polar than geraniol and of similar intensity, suggesting the formation of hydroperoxide species with inserted oxygen atoms. The most prominent of these spots ($R_f = 0.15$) was purified by flash chromatography, yielding 45 mg of a colourless oil (0.242 mmol, 23% yield) from 200 mg of crude material applied to the silica. As with the Lin-6/7-OOH reaction workup, the removal of trace amounts of SDS proved challenging; therefore, the 23% yield is an approximate value.

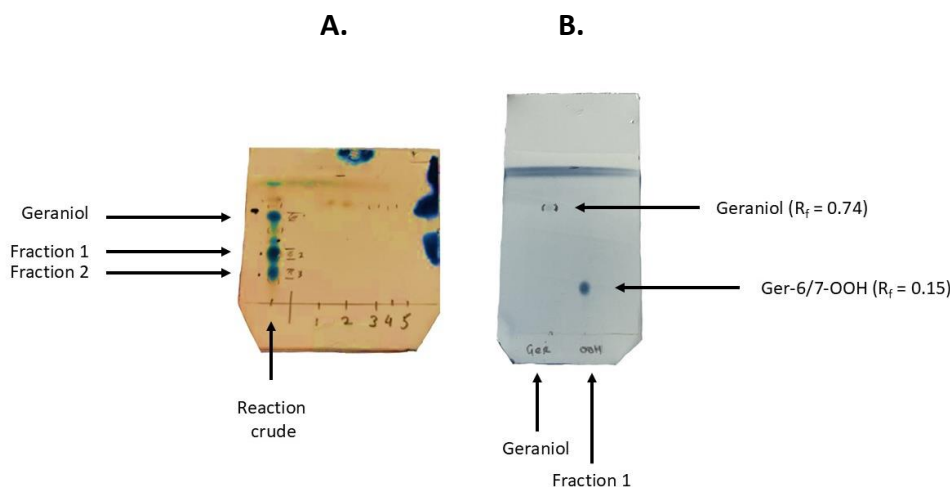


Figure 2.32. TLC analysis of the Ger-6/7-OOH synthesis.

(A) TLC of the crude reaction mixture, showing unreacted geraniol migrating closest to the solvent front, along with two prominent, more polar product spots below. (B) TLC comparison of pure geraniol (left lane) and purified fraction 1 (right lane). The mobile phase was hexane/EtOAc (70:30 v/v), and the plate was visualised using ammonium molybdate staining.

^1H NMR analysis of the crude reaction mixture revealed characteristic hydroperoxy proton resonances in the 10.50-12.00 ppm range (Fig. 2.33). Although this initially appeared to correspond to two hydroperoxy protons, magnification of this spectral region revealed the presence of multiple distinct signals. This observation was subsequently confirmed by flash chromatography and fractionation of the crude Ger-6/7-OOH reaction mixture. The additional signals cannot be attributed to diastereomers of Ger-6-OOH, as this oxidation product of geraniol contains only a single chirality centre and therefore does not exist as a pair of diastereomers, unlike Lin-6-OOH. Consequently, only one hydroperoxy proton resonance is expected for Ger-6-OOH. Since the hydroperoxy proton signals associated with fraction 1 represented the predominant species in the crude reaction mixture - as determined after purification of fractions 1 and 2 (Fig. 2.34) - fraction 1 was selected for further analysis. The higher recovery of material from fraction 1 facilitated subsequent ^{13}C NMR and related spectroscopic studies; thus, detailed characterisation in this work is focused exclusively on fraction 1.

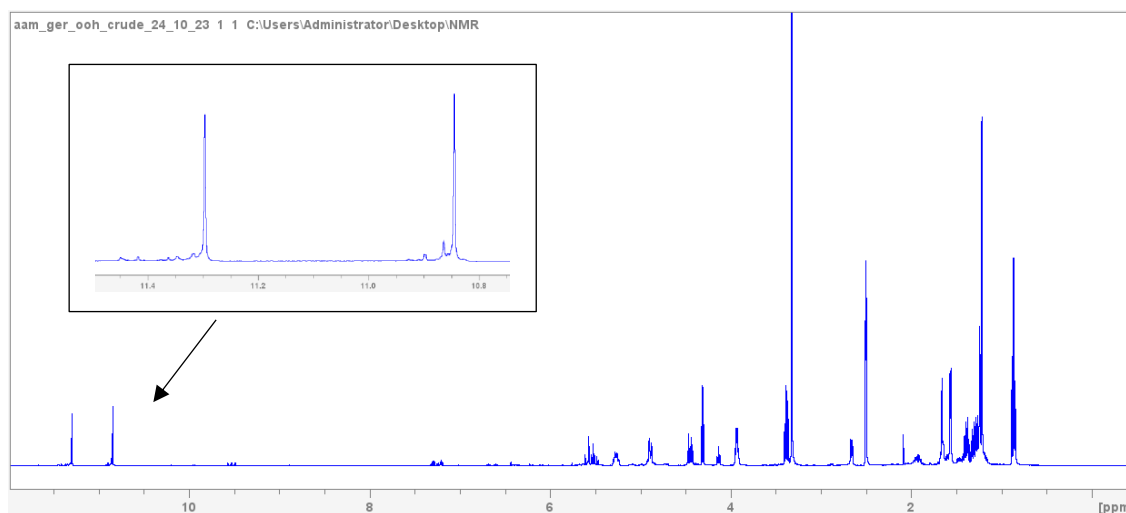


Figure 2.33. ^1H NMR analysis of the crude reaction mixture upon Schenck rearrangement of geraniol.

^1H NMR spectrum of the crude Ger-6/7-OOH reaction mixture showing characteristic hydroperoxy proton resonances in the 10.50-12.00 ppm region. The magnified inset reveals multiple distinct downfield signals, indicating the presence of more than two hydroperoxide-containing species.

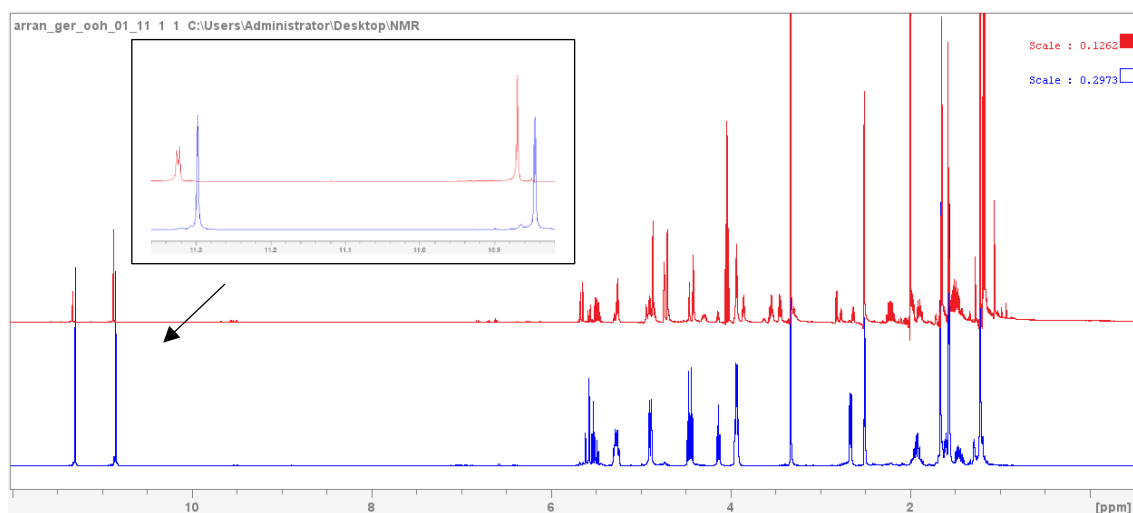


Figure 2.34. Comparative ^1H NMR analysis of purified fractions 1 and 2 from the Ger-6/7-OOH reaction mixture.

Stacked ^1H NMR spectra of purified fraction 1 (bottom) and fraction 2 (top), calibrated to the residual $\text{DMSO-}d_6$ signal at 3.33 ppm. The hydroperoxy proton resonances in the 10.50–12.00 ppm region display non-overlapping chemical shifts between the two fractions, indicating distinct hydroperoxide species. Other signals (<6 ppm) were also very different).

^{13}C NMR spectroscopy, combined with DEPT-135 analysis, was used to determine the number of carbon resonances, their chemical shifts, and the classification of each carbon as methyl ($-\text{CH}_3$), methylene ($-\text{CH}_2-$), methine ($>\text{CH}-$), or quaternary ($>\text{C}<$) (Fig. 2.35). A total of 20 carbon resonances of comparable intensity were observed, consistent with the presence of two monoterpene derivatives: Ger-6-OOH enantiomers (10 carbons) and Ger-7-OOH (10 carbons).

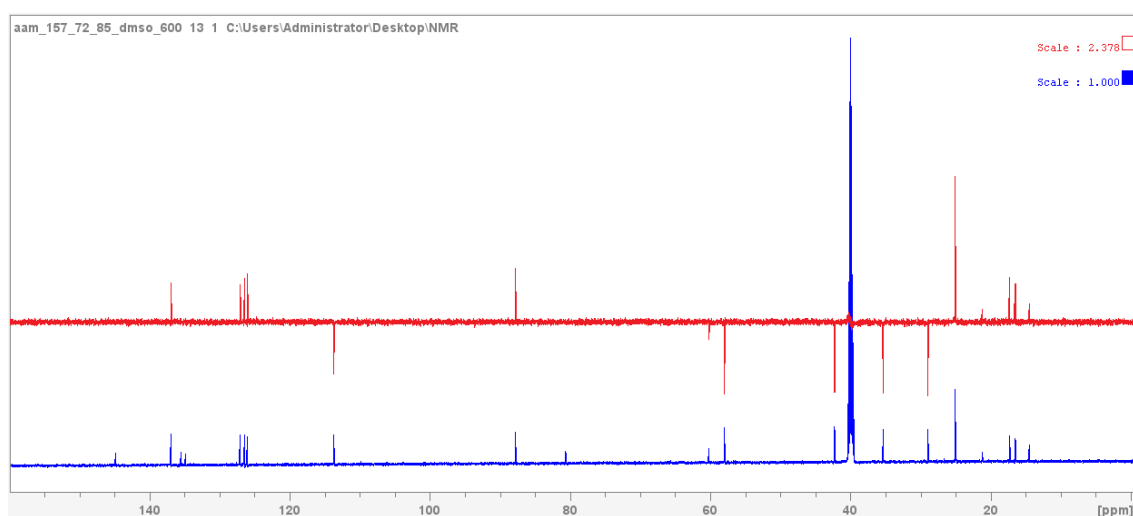


Figure 2.35. Stacked ^{13}C NMR and DEPT-135 spectra of purified fraction 1 from the Ger-6/7-OOH reaction mixture.

^{13}C NMR spectrum (bottom) and the corresponding DEPT-135 spectrum (top) for purified fraction 1 obtained from the Ger-6/7-OOH reaction mixture.

Given that Ger-6-OOH and Ger-7-OOH are structural isomers, with Ger-6-OOH existing as a pair of enantiomers due to its single chirality centre and Ger-7-OOH being achiral, a significant number of carbon resonances can be assigned without further 2D NMR experiments. This assignment relies on the expected differences in double bond positioning (terminal versus internal) and the nature of the hydroperoxy-bearing carbon (secondary versus tertiary), which generate distinct chemical environments (Fig. 2.36).

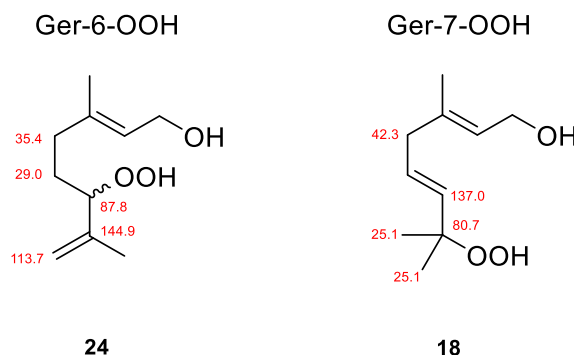


Figure 2.36. Partial ^{13}C NMR chemical shift assignment of Ger-6/7-OOH structural isomers. Partial assignment of carbon chemical shifts corresponding to the Ger-6-OOH (**24**) enantiomers (10 carbons) and the achiral Ger-7-OOH (**18**) compound (10 carbons). Assignments were made conservatively, focusing on chemical shifts exhibiting clear differentiation between the two structural isomers.

Following the initial carbon assignments, a combination of HSQC and HMBC experiments was employed to assign the directly correlated proton resonances. Starting with the hydroperoxy-bearing carbon in the putative Ger-6-OOH isomer (87.8 ppm), HSQC revealed a correlation to a proton resonance at 4.14 ppm (Fig. 2.37).

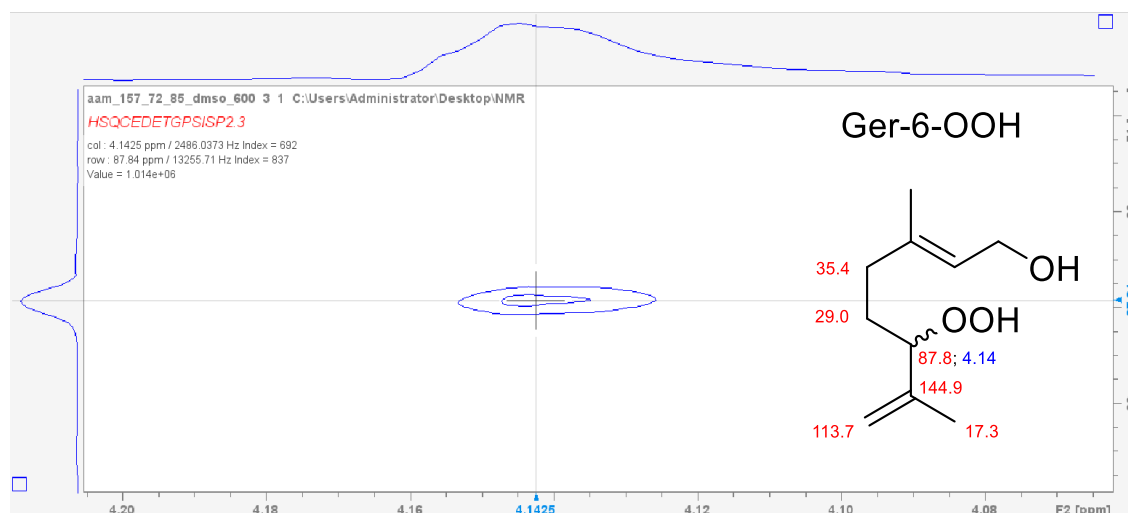


Figure 2.37. HSQC Correlation of hydroperoxy-bearing carbon and proton in Ger-6-OOH (24**).**

HSQC correlation between the hydroperoxy-bearing carbon resonating at 87.8 ppm and its directly bonded proton at 4.14 ppm. The inset illustrates the updated structural assignment of carbon and proton chemical shifts for the Ger-6-OOH isomer.

Additional HSQC correlations were observed between the terminal alkene carbon (113.7 ppm) and protons resonating at 4.92 and 4.89 ppm (Appendix A, Fig. A46). HMBC experiments further identified a correlation between the quaternary carbon at 144.9 ppm and methyl protons located two bonds away at 1.67 ppm (Appendix A, Fig. A47), with subsequent HSQC analysis linking these methyl protons to a carbon resonance at 17.3 ppm (Appendix A, Fig. A48). HSQC experiments were employed to identify the geminal protons attached to the methylene carbon resonating at 29.0 ppm, which were assigned to chemical shifts at 1.60 and 1.45 ppm (Appendix A, Fig. 49). Subsequently, HSQC analysis of the methylene carbon at 35.4 ppm revealed correlation with a single proton resonance at 1.91 ppm, indicating equivalent chemical environments for both geminal protons on this carbon (Appendix A, Fig. 50). These protons at 1.91 ppm were then used as probes in HMBC experiments to establish long-range correlations with previously unassigned carbons. HMBC revealed connectivities to a methyl carbon three bonds removed at 16.6 ppm (Appendix A, Fig. 51), a methine carbon three bonds away at 126.0 ppm (Appendix A, Fig. 52), and a quaternary carbon two bonds distant at 135.5 ppm (Fig. 2.38).

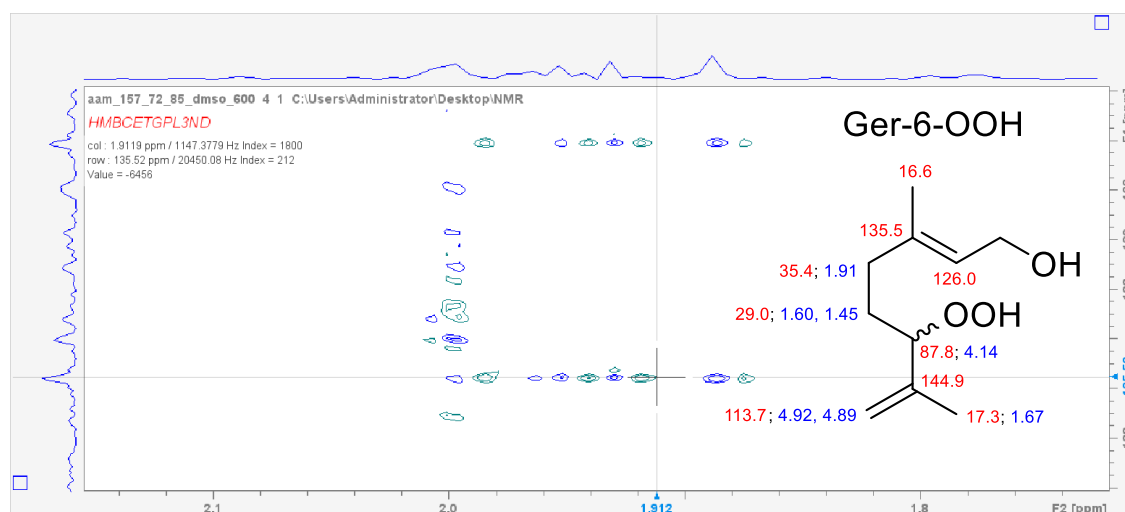


Figure 2.38. HMBC correlations from methylene protons at 1.91 ppm (x-axis) and the quaternary carbon at 135.5 ppm (y-axis).

This figure illustrates the utility of the methylene protons at 1.91 ppm in identifying the quaternary carbon at 135.5 ppm and in elucidating the distal region of the Lin-6-OOH isomer. The inset highlights additional

carbons identified through correlations with these protons, including the methyl carbon at 16.6 ppm and the methine carbon at 126.0 ppm.

HSQC was then employed to assign protons to the newly identified carbons, revealing a correlation between the methyl carbon at 16.6 ppm and protons resonating at 1.57 ppm (Appendix A, Fig. A53), as well as between the methine carbon at 126.0 ppm and a proton at 5.26 ppm (Appendix A, Fig. A54). HMBC further established correlations between the methine carbon at 126.0 ppm and the methylene protons at 3.93 ppm (notably appearing as a single chemical shift for two protons), in addition to the hydroxy proton at 4.44 ppm (Fig. 2.39). Subsequent HSQC analysis identified the carbon at 58.1 ppm as directly bonded to the methylene protons at 3.93 ppm, thereby confirming the carbon-proton connectivity in this region of the molecule (Appendix A, Fig. A55).

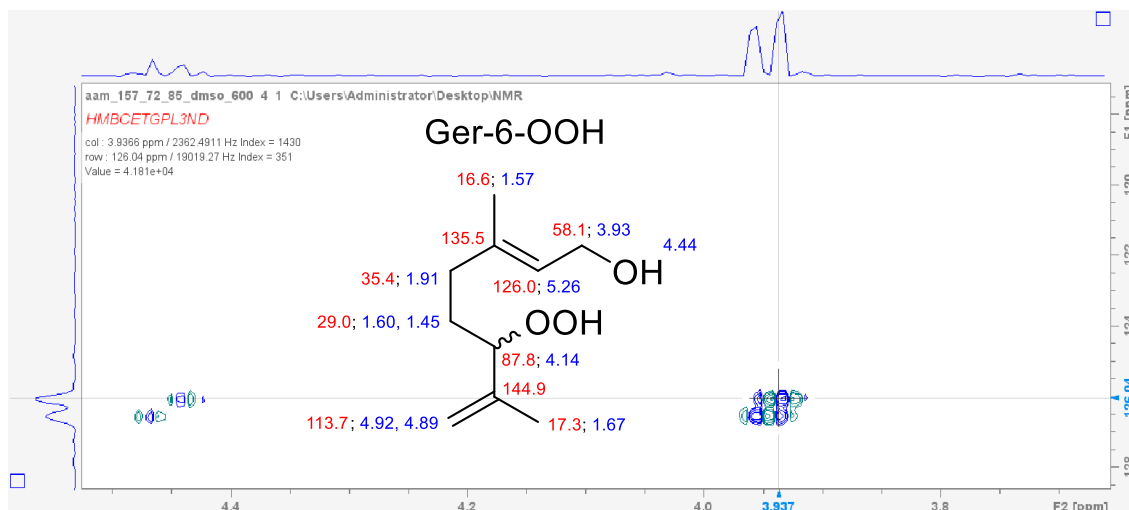


Figure 2.39. HMBC correlation of methine carbon (126.0 ppm) with methylene protons (3.93 ppm) and structural elucidation of distal Ger-6-OOH regions.

HMBC correlation between the methine carbon at 126.0 ppm (y-axis) and the methylene protons at 3.93 ppm (x-axis). The inset summarizes the combined application of HMBC and HSQC techniques to assign protons and carbons within the distal region of the Lin-6-OOH isomer relative to the site of oxygen insertion.

In addition to the near-complete characterization of Ger-6-OOH, the assignment of carbon and proton chemical shifts for Ger-7-OOH was also accomplished (Appendix A, Fig. A56-66), with both isomer characterizations approaching completion based on the data presented (Fig. 2.40). Although no HMBC correlations were detected between the hydroperoxy protons and their corresponding hydroperoxy-bearing carbons, the distinct chemical environments of these protons - secondary carbon for Ger-6-OOH and tertiary

carbon for Ger-7-OOH - together with analogous correlations observed for Lin-6/7-OOH hydroperoxy protons, support the assignment of the proton signals at 11.30 ppm and 10.85 ppm to Ger-6-OOH and Ger-7-OOH, respectively. This comprehensive structural elucidation, alongside parallels drawn from the Lin-6/7-OOH isomers, provides compelling evidence that the purified fraction 1 obtained from the crude reaction mixture corresponds to the Ger-6/7-OOH structural isomers. Integration of the hydroperoxy resonances indicated an approximate 1:1 ratio of Ger-6-OOH and Ger-7-OOH structural isomers.

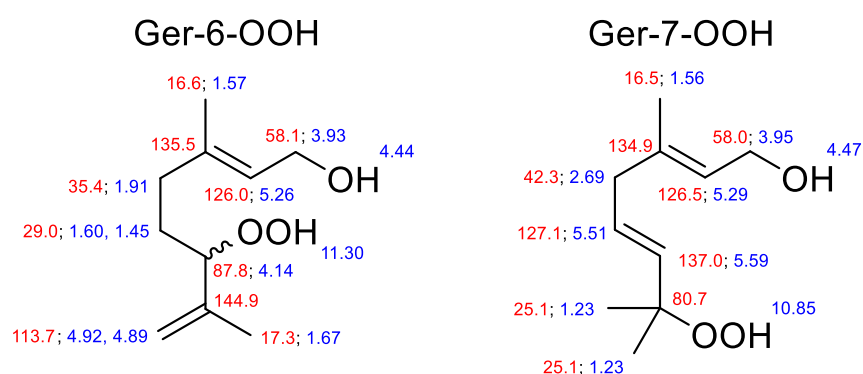


Figure 2.40. Comprehensive NMR characterization of Ger-6-OOH (24) and Ger-7-OOH (18) structural isomers.

Complete assignment of carbon and proton chemical shifts for the structural isomers Ger-6-OOH and Ger-7-OOH, based on combined NMR analyses.

2.3.4 Conclusions

Lim-2-OOH (**5**) was synthesised in ~35% yield as a pair of diastereomers, (*4R,6R*)-*cis* and (*4R,6S*)-*trans*, in a ratio of approximately 8:1 in favour of the *trans* isomer. Previous studies reported a 3:1 ratio using an alternative synthetic route, without specifying the predominant isomer [73]. The proton and carbon chemical shifts obtained here were consistent with literature values [96].

Lin-6/7-OOH (**9** and **10**) was synthesised in ~85% yield as two structural isomers: Lin-6-OOH (**9**), present as four unique configurations (four diastereomeric pairs and two enantiomeric pairs), and Lin-7-OOH (**6**), present as a pair of enantiomers. The ratio of Lin-6-OOH to Lin-7-OOH was 5:4, closely matching the reported 6:4 ratio [91]. NMR assignments for both isomers aligned with previously published data.

Ger-6/7-OOH (**24** and **18**) was obtained in ~23% yield as two structural isomers: Ger-6-OOH (**24**), present as a pair of enantiomers, and Ger-7-OOH (**18**), an achiral compound. The isomer ratio (1:1) differed slightly from the literature-reported 5:3 ratio in favour of Ger-7-OOH [79], likely due to differences in singlet oxygen generation (chemical oxidation herein vs. photooxidation in previous studies). NMR data agreed with published values.

Collectively, this chapter established robust and selective synthetic routes to the target terpene hydroperoxide isomers, with complete structural verification by NMR spectroscopy. These compounds, purified and stored under N₂ at -80 °C, represent critical intermediates for the functional and mechanistic studies described in Chapters 3-5. The work presented here was therefore foundational to the experimental design and interpretation of the subsequent research in this thesis.

Chapter 3: A Lipidomic Approach Towards Identifying the Effects of Fragrance Hydroperoxides on Keratinocytes

The work presented in this chapter is based on the following publication:

Moore, A. I., Moreira, A. S., Guerra, I. M., Goracci, L., Domingues, P., Melo, T., Domingues, M.R., & O'Boyle, N. M. (2025). A lipidomic approach towards identifying the effects of fragrance hydroperoxides on keratinocytes. *Contact Dermatitis*, 92(3), 176-186.

3.1 Introduction

For many years, the skin was considered primarily a physical barrier with a passive role in immune responses, with key immunological events thought to occur in the DLNs or circulation [97]. This view changed with the observation that LCs reside in close proximity to infiltrating T cells in ACD, providing the first evidence that the epidermis contains immunocompetent cells [98]. Since then, LCs have been recognised as pivotal players in cutaneous immunity, with phenotypic characterisation revealing constitutive expression of MHC class II and CD1a, thereby providing the capacity to process and present a diverse array of antigens [99-102]. KCs, the most abundant epidermal cell type, constituting approximately 90% of the epidermis, also play an active role in ACD by producing a wide range of cytokines and chemokines (e.g., IL-1, IL-6, IL-8, GM-CSF, TNF, MCAF, TGF- α , TGF- β) that regulate immune cell activation and recruitment while maintaining the primary structural barrier [103-106]. In recent years, a strong connection has been established between innate immune signalling processes and the regulation of lipid metabolism [107-109].

Lipids not only serve a structural role in membranes and function as a source of energy, but they also modulate cellular signalling process, and the involvement of lipids as mediators in immunological responses has been well documented [110-115]. The main lipids in mammalian cell membranes are PLs, SLs and CHOL, with FAs being the main building blocks of lipid synthesis and contributing towards their diversity [116]. The glycerolipids (GLs), TGs, are used for energy storage (Fig. 3.1). Innate immune receptors on KCs can sense pathogens, thereby bridging the gap between environmental signals and the underlying immune cells [117, 118]. Therefore, it is of utmost importance to study the cross-talk between KC-mediated immune activation and disruptions to lipid homeostasis triggered by contact allergens.

Changes in the KC lipid profile have been previously explored by lipidomic approaches. Calderón et al. investigated the effects of the pentacyclic triterpenoid betulin (a compound known for possessing wound healing properties) on KCs by using untargeted ultra-high performance liquid chromatography (UHPLC)-ESI-Q-TOF-MS/MS [119], showing an upregulation in glycerophospholipids (GPLs), sphingolipids (SLs) and diacylglycerols (DGs), and a downregulation of cholesterol esters (CEs) and TGs. Reich et

al. also identified changes in the KC lipidome after exposure to narrowband ultraviolet B (NB-UVB) via shotgun lipidomics using an LTQ-Orbitrap and Q-TOF, with results showing an upregulation of TGs, alkyl-acyl PCs (PC O-), alkyl-acyl PEs (PE O-) and CERs, and a downregulation of PIs, SMs and PCs with short chain FA moieties [120]. The effects of the anticancer agent 2-deoxyglucose on the lipid metabolism of KCs demonstrated the upregulation of CER and GlcCer lipid species using untargeted liquid chromatography with high resolution MS [121].

Lipidomic analyses of KCs in the context of allergen exposure have not been thoroughly investigated. One of the most significant contributions was by Santinha et al., where alterations in the PL profile of cultured HaCaT cells upon exposure to non-allergenic immune stimulator lipopolysaccharide (LPS, 1 µg/mL), experimental contact allergen 2,4-dinitrofluorobenzene (DNFB, 5 µM), and skin irritant benzalkonium chloride (BC, 2 µM) were investigated using a combination of LXQ linear ion trap, Q-TOF and QQQ analysis methods [122]. There were similarities and differences between the effects of DNFB and BC depending on the PL subtype. For example, both chemicals caused the downregulation of PE (18:1/18:1 and 18:0/18:2) and PE (18:4). In contrast, there was discrimination between the allergen and irritant effects on PC (18:1/18:1) (increased upon exposure to DNFB and LPS, but decreased by BC) and PS (18:0/18:1) (increased by DNFB and LPS, but decreased by BC). Hence, it may be possible to distinguish allergens from irritants by appropriate selection of lipid species for monitoring. Such lipidomic analyses are made possible through the advances made in MS equipment, as demonstrated by Knox et al., who recently studied the effects of metal contact allergens on ex vivo skin using ToF-SIMS [123]. A decrease in CHOL, monoacylglycerols (MGs) and DGs in the viable epidermis of nickel-treated skin samples was noted, in contrast to an increase in MGs and DGs in the viable epidermis and SC for cobalt treated skin samples. Lipidomics is useful for the identification and quantification of lipid biomarkers in health and disease. This information allows one to deduce distinct metabolites whose levels change in response to a stimulus, but lipidomic approaches have not been utilised for other contact allergens, such as fragrances.

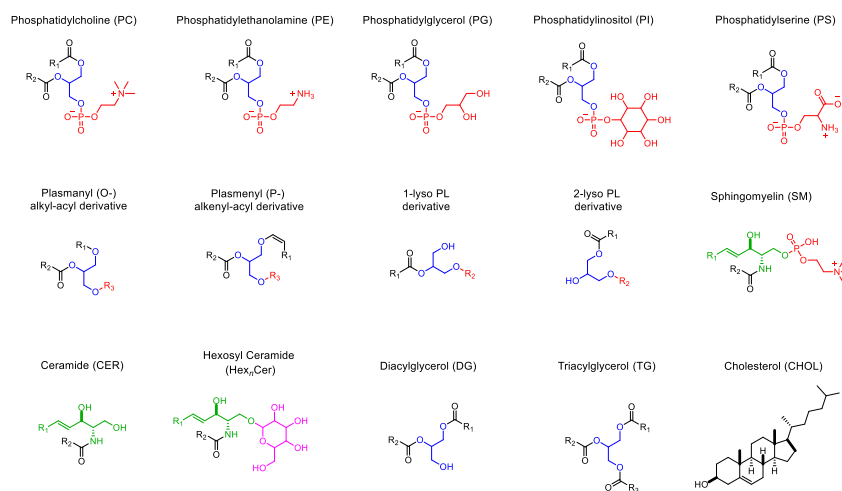


Figure 3.1. Lipid classes most commonly found in mammalian cell membranes.

In red are various PL head groups. In blue are lipids which contain a glycerol backbone. Green highlights lipids with a sphingoid backbone. Purple represents the sugar units in Hex_nCers. Black represents fatty acid chains which are found in all the above lipids except CHOL (unless esterified).

(*R*)-(+)-Limonene and linalool are commonly used in cosmetics, personal care and household products for their floral scent. Their proclivity to undergo autoxidation has already been discussed in detail, but briefly, the primary oxidation products of (*R*)-(+)-limonene and linalool are Lim-2-OOH and Lin-6/7-OOH, respectively (Fig. 3.2). It has been mentioned that these allylic hydroperoxides haptenate via free radical mechanisms through the homolytic cleavage of their O-O and/or O-H bonds [124]. Following this cleavage, a cascade of radical intermediates has been reported [91, 125]. Previous in vitro studies have shown covalent adduct formation with model peptides [126, 127] and oxidation of thiols on the cell surface of THP-1 cells [128]. Together with their protein reactivity in KCs and activation of immune networks, their effects on lipid metabolism are of significant interest.

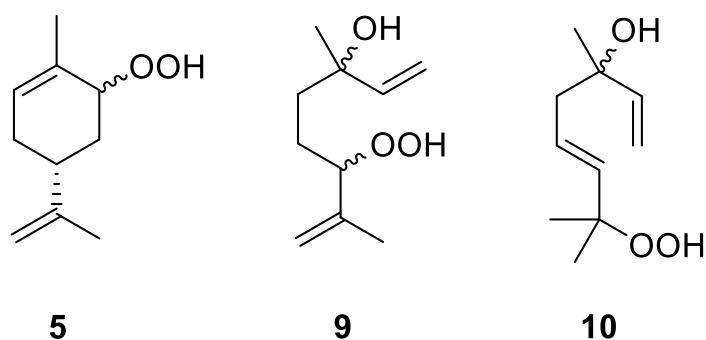


Figure 3.2. Structures of limonene and linalool hydroperoxide contact allergens.

Limonene-2-hydroperoxide (Lim-2-OOH) (**5**), linalool-6-hydroperoxide (Lin-6-OOH) (**9**) and linalool-7-hydroperoxide (Lin-7-OOH) (**10**).

The aim of this chapter was to use a lipidomics approach to identify changes in the lipidome of HaCaT cells (an immortalised human KC cell line [129]) in response to treatment with Lim-2-OOH and Lin-6/7-OOH using high-resolution C18 LC–MS and MS/MS. DNFB was also included for two reasons, firstly, it is a well-known experimental contact allergen with an EC3 value of 0.03% (w/v) [130], and secondly, it is believed that DNFB reacts with proteins via conventional two-electron mechanisms, rather than the free radical mechanisms associated with terpene hydroperoxides [131]. By investigating the relationship between these immune activators and subsequent changes in lipid metabolism, it may be possible to learn more about how these contact allergens cause inflammation and ultimately ACD.

3.2 Materials and Methods

3.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Microbiology grade DMSO and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNFB was purchased from Acros. Organic solvents (DCM, methanol [MeOH], acetonitrile [ACN] and isopropanol [IPA]) were purchased from Fisher Scientific (Leicestershire, UK), were HPLC grade, and used without further purification. Perchloric acid (HClO_4 , 70%) was purchased from Chem-Lab NV (Zedelgem, Belgium) and ammonium molybdate tetrahydrate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) from Panreac (Barcelona, Spain), ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) from VWR International (Leicestershire, UK) and sodium dihydrogen phosphate dihydrate

NaH₂PO₄·2H₂O) from Riedel-de Haën (Seelze, Germany). Milli-Q water was used, filtered through a 0.22 µm filter in a Milli-Q Millipore system (Milli-Q plus 185). Skin sensitising compounds, Lim-2-OOH, Lin-6-OOH and Lin-7-OOH in a 5:4 ratio (Lin-6/7-OOH), were synthesised according to previously mentioned methods (see Chapter 1, section 2.2). The lipid standards, N-heptadecanoyl-D-erythro-sphingosine (Cer[18:1;O2/17:0]), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM[18:1;O2/17:0]), 1-nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC[19:0]), 1,2-dimyristoyl-sn-glycero-3-phospho-(10-*rac*-glycerol) (dMPG), 1,2-dipalmitoyl-sn-glycero-3-phospho-(10-*myo*-inositol) (dPPI), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (dMPS), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (dMPC) and (1,3-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol) [CL(14:0)₄], were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

3.2.2 Cell Culture

The human keratinocyte cell line (HaCaT) was acquired from CLS Cell Lines Service (Germany). The cell line was confirmed to be free from mycoplasma. The cells were cultured in DMEM (high glucose) supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cell morphology was monitored under a microscope to detect any changes. The cells were grown until 70% confluency.

3.2.3 Determination of Cell Viability by the alamarBlue Assay

HaCaT cells were treated with compounds for 24 h in a dose response experiment and analysed for their viability through the reduction of the weakly fluorescent probe (resazurin) to the highly fluorescent derivative (resorufin) [132]. Briefly, HaCaT cells were seeded at a density of 0.2×10^5 cells/well (96-well plate) and incubated overnight at 37°C in 200 µL of media. The next day, stock solutions of compounds were freshly prepared in microbiology grade DMSO, old media was removed and different working concentrations of Lim-2-OOH and Lin-6/7-OOH ([10, 50, 100, 250 and 500 µM] in 200 µL of media) or DNFB ([2.5, 5 and 10 µM] in 200 µL of media) were added. DMSO (0.1% v/v) was used as a vehicle control. After 24 h, the treatments were removed and alamarBlue (10% v/v in DMEM, 200 µL) was added to each well and incubated for a further 3 h. Fluorescence intensities of resorufin were read using a FLUOstar OPTIMA micro-plate reader (BMG Labtech, Germany) with an excitation wavelength of 544 nm

and emission wavelength of 590 nm. Percentage viability was calculated relative to the vehicle control. Based on cell viability results, a concentration of 10 μ M was chosen for lipid extraction studies. Experiments were performed in technical triplicates on three independent occasions.

3.2.4 Chemical Treatment of HaCaT Cells for Lipid Extraction

HaCaT cells (1×10^7 cells) were cultured in 150 cm² T flasks and treated with contact allergens for 24 h at 37 °C. Stock solutions of allergens (solubilised in microbiology grade DMSO) were added to obtain a final concentration of 10 μ M DNFB, 10 μ M Lin-2-OOH and 10 μ M Lin-6/7-OOH. The final concentration of DMSO did not exceed 0.01% (v/v). Control experiments consisted of a vehicle (DMSO [0.01%]). Experiments were performed on five independent occasions.

3.2.5 Lipid Extraction

After 24 h treatment times, untreated cells and treated cells were washed twice with ice-cold phosphate buffered saline (PBS), scraped in 5 mL of ice-cold PBS, and pelleted by centrifugation at 1000 rpm for 4 min. The pellet was solubilised in 1 mL of ultra-pure water (Milli-Q H₂O). Milli-Q H₂O was used in the preparation of all samples and standard solutions and was obtained using a Milli-Q Millipore system (Milli-Q plus 185). Lipids were then extracted using the Bligh and Dyer method [133], as previously reported [122]. Briefly, 3.75 mL of an ice-cold 1:2 (v/v) DCM:MeOH solution was added to the solubilised pellet, vortexed and agitated gently on ice for 30 min under agitation (75 rpm) using an orbital shaker (Stuart Reciprocating Shaker SSL2, Stuart, UK), with vortexing every 5 min. An additional volume of DCM (1.25 mL) was added to the homogenate, then vortexed, followed by an additional volume of Milli-Q H₂O (1.25 mL), and then vortexed again. Samples were then centrifuged for 10 min at 2000 rpm to separate the aqueous and organic phases. The total lipid extract was recovered from the organic phase (bottom phase), transferred into a new glass tube, and dried under a stream of nitrogen gas. After drying, the lipid extracts were resuspended in 300 μ L of DCM, vortexed and transferred to an amber glass vial. The extract was then dried again under a stream of nitrogen gas and stored at -4 °C. Chemical treatments were carried out for five biological repeats in order to verify the reproducibility of the results.

3.2.6 Quantification of Phospholipids

In order to determine the total amount of PL in each lipid extract, the phosphorous assay was performed according to Bartlett and Lewis [134], as previously reported [122]. Briefly, lipid extracts were resuspended in 200 μL of CHCl_3 , thereafter, 10 μL of each sample was added to a separate glass tube. The transferred extracts were dried under nitrogen gas. Afterwards, 125 μL of 70% perchloric acid was added to each tube and incubated in a heating block (Stuart, Staffordshire, UK) at 180 $^{\circ}\text{C}$ for 60 min (clear samples indicated that the reaction had finished). Once cooled to room temperature, 825 μL of Milli-Q H_2O and 125 μL of $\text{NaMoO}_4\cdot\text{H}_2\text{O}$ (2.5% w/v in Milli-Q H_2O) were added to the lipid samples and then vortexed. Following this, 125 μL of ascorbic acid (10% w/v in Milli-Q H_2O) was added to each of the lipid samples. Samples were vortexed, incubated in a water bath at 100 $^{\circ}\text{C}$ for 10 min, and placed in cold water. The absorbance was measured at 797 nm using a Multiskan G01.0038 Microplate Spectrophotometer (Thermo Scientific, Hudson, NH, USA). The phosphorous content was calculated by means of a calibration curve that was constructed by carrying out the same procedure (omitting the heating block step) with standards between 0.1 and 2.0 μg of phosphorous, prepared from a stock solution of $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$ (100 $\mu\text{g}/\text{mL}$ of phosphorous). The total amount of PL was then estimated by multiplying the total phosphorous values by 25, as previously done [135].

3.2.7 C18 LC-MS and MS/MS

The total lipid extracts were analysed by reverse-phase liquid chromatography on a Dionex Ultimate 3000 (Thermo Fisher Scientific, Bremen, Germany) using an Ascentis[®] Express C18 column (Sigma-Aldrich, 2.1 $\times$ 100 mm, 2.7 μm) coupled to the Q-Exactive[®] hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), as previously done [135, 136]. Mobile phase A was water/ACN (40:60) with 10 mM ammonium formate and 0.1% formic acid. Mobile phase B was IPA/ACN (90:10) with 10 mM ammonium formate and 0.1% formic acid. The following gradient was applied: 32% B (0 min), 45% B (2.5 min), 52% B (4 min), 58% B (5 min), 66% B (8 min), 70% B (11 min), 85% B (14 min), 97% B (18 min), 97% B (25 min), 32% B (25.01 min) and 32% B (33 min). A volume of 5 μL of each sample, containing 10 μg of lipid extract (in 10 μL of DCM), 82 μL of a solvent system composed of IPA/MeOH 50:50, and 8 μL of a

mixture of phospholipid standards (dMPC – 0.04 µg, Cer 18:1;O2/17:0 – 0.04 µg, SM 18:1;O2/17:0 – 0.04 µg, dMPE – 0.04 µg, LPC (19:0) – 0.04 µg, dPPI - 0.08 µg, CL (14:0)₄ - 0.16 µg, dMPG – 0.024 µg and dMPS - 0.08 µg) was injected into the column at 55 °C and at a flow rate of 260 µL/min. The mass spectrometer operated in simultaneous positive mode [electrospray ionization (ESI) 3.0 kV] and negative mode (ESI -2.7 kV). The capillary temperature was 320 °C and the sheath gas flow 35 AU. Data acquisition was performed in full-scan mode with a high resolution of 70,000 and automatic gain control (AGC) target of 3×10^6 , in a *m/z* range of 200-1,600, with two microscans, and a maximum injection time (IT) of 100 ms. MS/MS were obtained with a resolution of 17,500, AGC target of 1×10^5 , 1 microscan, maximum IT of 100 ms. The cycles consisted of a full-scan mass spectrum and 10 data-dependent MS/MS scans, which were repeated continuously throughout the experiments with a dynamic exclusion of 30 s and an intensity threshold of 8×10^4 . The normalised collision energy (NCE) ranged between 20, 24 and 28 eV in the negative mode and 25 and 30 eV in the positive mode. Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Bremen, Germany).

3.2.8 Data Processing

The LC-MS data was processed using Lipostar software (Molecular Discovery Ltd., version 2.1.5) [137]. This software was used for raw data import, peak detection and identification. Lipid assignment and identification were performed against a database created from LIPID MAPS structure database (version February 2024). The database was fragmented using DB Manager Module of Lipostar, following Lipostar Fragmentation rules. The raw files were directly imported and aligned using settings described by Lange et al. [138]. Automatic peak picking was carried out with the semi-parametric differential abundance analysis (SDA) smoothing level set to low and a minimum signal-to-noise ratio of 3 and a signal filtering threshold of 600,000 in the positive mode and 5000 for the negative mode. Automatic isotope clustering settings were set to 0 ppm with a retention time tolerance of 0.2 min. The MS/MS filter was applied to retain only features with MS/MS spectra for identification. Lipid identification was based on the following parameters: 5 ppm precursor ion mass tolerance and a 20 ppm product ion mass tolerance. An automatic approval process was applied to retain structures with a

confidence level of 2-4 stars. The approved annotations from Lipostar were confirmed, considering the typical fragmentation pattern of each lipid class (Appendix B, Fig. B1-8). Normalisation of the data was performed by sum of the areas.

3.2.9 Statistical Analysis

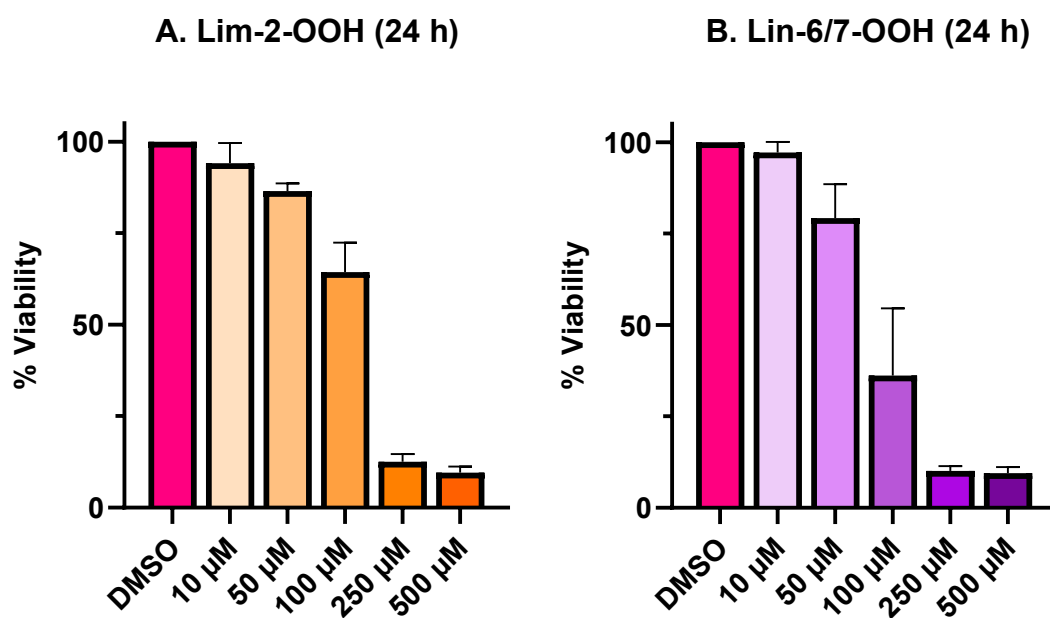
Multivariate and univariate statistical analyses were performed using R version 3.6.2 [139] in RStudio version 1.2.5 [140]. The R package MetaboAnalyst was used for imputation (1/5 of min) and log transformation (base 10) of the C18 LC-MS data [141]. EigenMS was also used for normalisation of LC-MS data [142]. The R packages FactoMineR [143] and factoextra [144] were used to perform principal component analysis (PCA). Heatmaps were created from autoscaled data using the R package pheatmap [145], using 'Euclidean' as clustering distance and 'ward.D' as the clustering method. Data normality and variance homogeneity were assessed using Shapiro-Wilk and Levene's tests, respectively. Based on these tests, data were analysed using ANOVA if assumptions were met, or Kruskal-Wallis test if not. Significant differences identified by ANOVA were further examined by Tukey's Honest Significant Differences (HSD) test, while Dunn's test was used for the Kruskal-Wallis post-hoc comparisons. *p*-values were corrected for multiple testing using a Benjamin-Hochberg method for the false discovery rate (FDR, *q*-values). All univariate analyses were performed using the *r* package rstatix [146], with a significance threshold of $p < 0.05$. All graphics and boxplots were created using the R package ggplot2 [147].

3.3 Results

3.3.1 Cell Viability Assays

AlamarBlue cell viability assays were performed on HaCaT cells prior to lipidomic analyses. Contact allergens were tested over a 24 h incubation period at concentrations ranging from 10 to 500 μM (Fig. 3.3). A clear dose-dependent decrease in cell viability was observed relative to the vehicle control (DMSO, 0.1% w/v). Lim-2-OOH and Lin-6/7-OOH produced similar reductions in viability across this range, whereas DNFB was markedly more potent at lower concentrations. Although the tested range was not optimal for DNFB, the data indicated that 84% of cells remained viable following 10 μM treatment for 24 h. This level of mild cytotoxicity is considered sufficient to elicit the

danger signals that initiate skin sensitization [148]. Lim-2-OOH and Lin-6/7-OOH showed minimal effects at 10 μ M, with cell viabilities of 94% and 97%, respectively. For consistency, 10 μ M was selected as the working concentration for all contact allergens in subsequent lipidomic analyses.



C. DNFB (24 h)

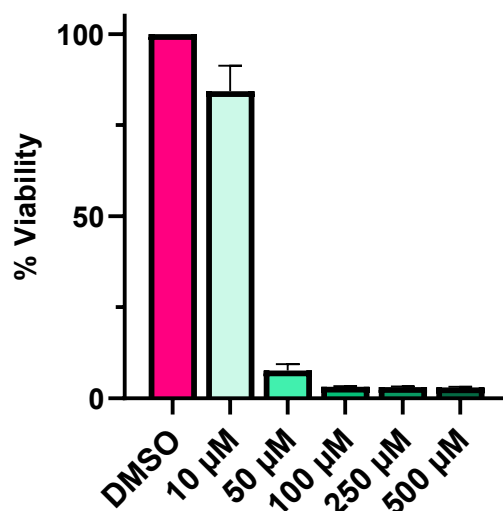


Figure 3.3. alamarBlue cell viability assays of HaCaT cells treated with contact allergens (24 h).

Cell viability assays were performed at a concentration range between 10 and 500 μ M of (A) Lim-2-OOH, (B), Lin-6/7-OOH, and (C) DNFB, over a 24 h period, and calculated relative to the vehicle control (DMSO, 0.1% v/v).

3.3.2 Lipid Extraction and Phosphorus Assay

Approximately 1×10^7 HaCaT cells from each sample pellet were subjected to lipid extraction using the Bligh and Dyer method. Quantification of the extracted lipids was performed using a phosphorus assay, based on the premise that PLs constitute the predominant lipid class in mammalian cells. This quantification enabled normalization of sample preparation for downstream lipidomic analyses. A phosphorus calibration curve was generated by measuring the absorbance of sodium phosphate monobasic dihydrate standards at 797 nm (Fig. 3.4).

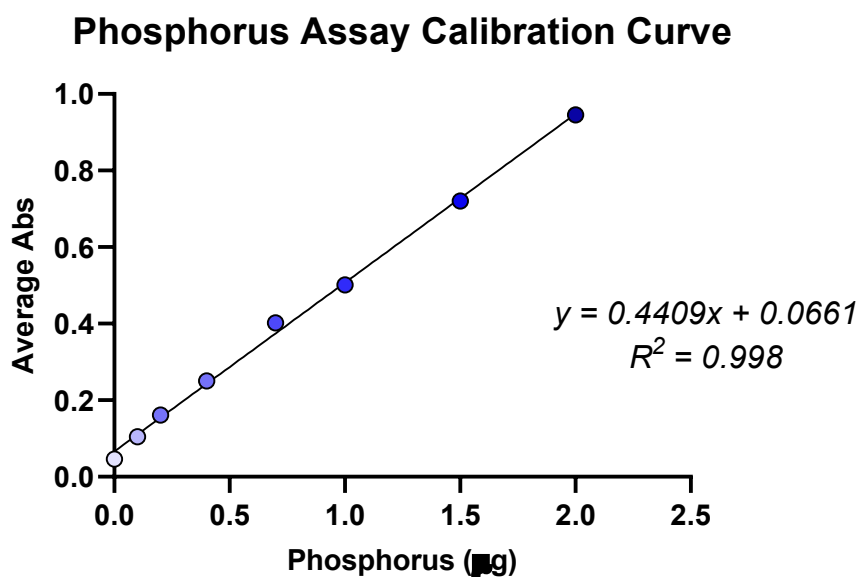


Figure 3.4. Calibration curve for the phosphorus assay.

Calibration curve generated using sodium phosphate monobasic dihydrate standards containing between 0.1 and 2.0 µg phosphorus, prepared from a 100 µg/mL phosphorus stock solution.

The calibration exhibited excellent linearity ($R^2 = 0.998$), enabling accurate conversion of absorbance values to phosphorus content. Phosphorus mass values (µg) were subsequently multiplied by 25 to estimate total PL mass, reflecting the fact that phosphorus constitutes only ~4% of the molecular weight of an average mammalian PL (molar mass assumed: 774 g/mol; phosphorus atomic mass: 30.97 g/mol). As an example, in the first of five biological replicates, PL content across untreated and treated samples ranged from 81.88 to 170.7 µg (Table 3.1). For subsequent lipidomic analysis, dried lipid extracts (stored at $-4\text{ }^{\circ}\text{C}$) were reconstituted to 1 µg/µL, and 10 µg of each sample was subjected to high-resolution mass spectrometry.

Table 3.1. Example of PL content extracted from HaCaT cells exposed to various experimental conditions.

Samples (n=1)	Avg. Abs 797 nm	Phosphorus (µg)	Extracted PL (µg)
Cells only	0.1565	0.2049	102.5
Vehicle (DMSO, 0.01% v/v)	0.1881	0.2767	138.3
DNFB (10 µM)	0.2166	0.3416	170.7
Lim-2-OOH (10 µM)	0.1383	0.1638	81.9
Lin-6/7-OOH (10 µM)	0.1652	0.2248	112.4

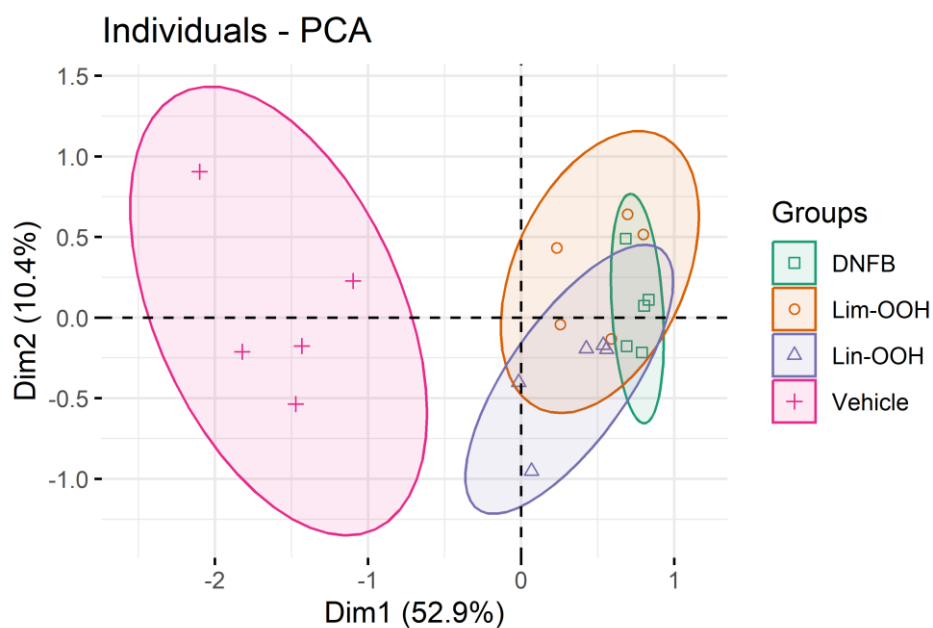
3.3.3 C18 LC-MS and MS/MS with Lipostar Data Processing Identifies the HaCaT Cell Lipidome

The lipidome of HaCaT cells treated with fragrance hydroperoxides, DNFB, and the vehicle control was identified and semi-quantified (relative to appropriate internal standards) using high-resolution C18 LC-MS and MS/MS. A total of 328 lipid species were detected, spanning 10 lipid classes (Table 3.2). The classes identified were PC, comprising of diacyl, monoacyl (LPC), alkyl acyl (PC O-) and alkenyl-acyl (PC P-) species; PE, comprising of diacyl, monoacyl (LPE), alkyl-acyl (PE O-) and alkenyl-acyl (PE P-) species; PG; PI, comprising of diacyl, alkyl-acyl (PI O-) and alkenyl-acyl (PI P-) species; PS; SM; Cer; Hex_nCer; DG and TG.

3.3.4 Multivariate Analysis Using Principal Component Analysis (PCA)

To assess differences between allergen-treated cell groups and the vehicle control group, multivariate analysis using PCA was performed on the LC-MS analysis dataset. The PCA score plot of the first two dimensions (Fig. 3.5A) showed a discrimination between fragrance hydroperoxide-treated cells (Lim-2-OOH [orange] and Lin-6/7-OOH [blue]) and the vehicle control group (red), based on changes in their total lipidomes. Minimal discrimination was observed among the cell groups subjected to allergen treatment, including DNFB. The model accounted for 63.3% of the total variance, PC1 (Dim1) accountable for 52.9% and PC2 (Dim2) accountable for 10.4% of the variance seen. All three allergen-treated cell groups and the vehicle control group were separated along the Dim1 (x-axis). According to loading values, the highest influence on Dim1 was PC O- 36:0, followed by TG 58:3, TG 49:2 and DG 40:5 (Fig. 3.6). The lipidome of untreated cells was also compared to the vehicle control (DMSO[0.01% v/v]). Differences between untreated and DMSO-treated cells were noted even at this low concentration (Fig. 3.5B), but the minimal separation among the allergen-treated cell groups was maintained.

A.



B.

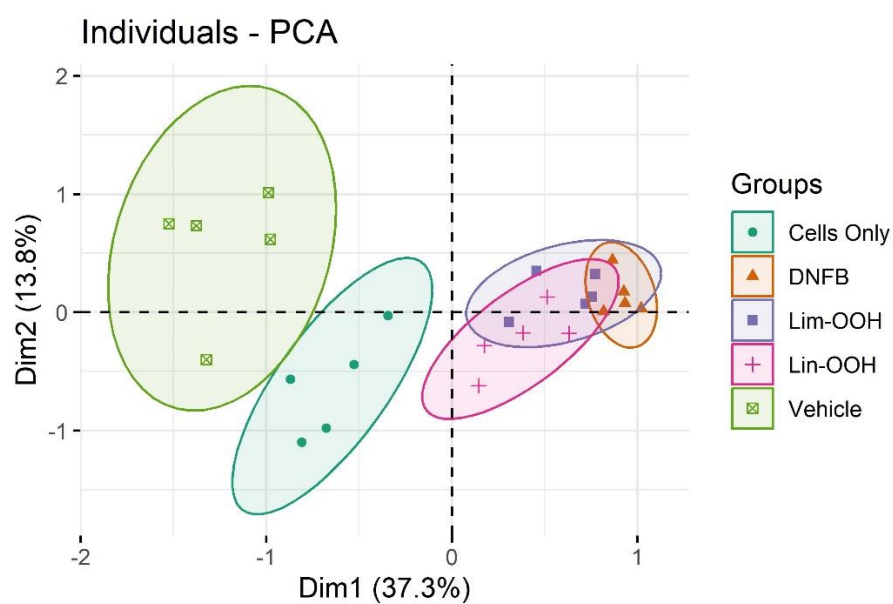


Figure 3.5. PCA score plots of lipid profiles from allergen-treated HaCaT cells.

(A) PCA score plots derived from C18 LC-MS/MS analysis of total lipid extracts from HaCaT cells treated with Lim-2-OOH (10 μ M), Lin-6/7-OOH (10 μ M), DNFB (10 μ M), or vehicle control (0.01% v/v DMSO) for 24 h, followed by lipid extraction and analysis. **(B)** PCA score plots with the addition of the untreated “cells only”.

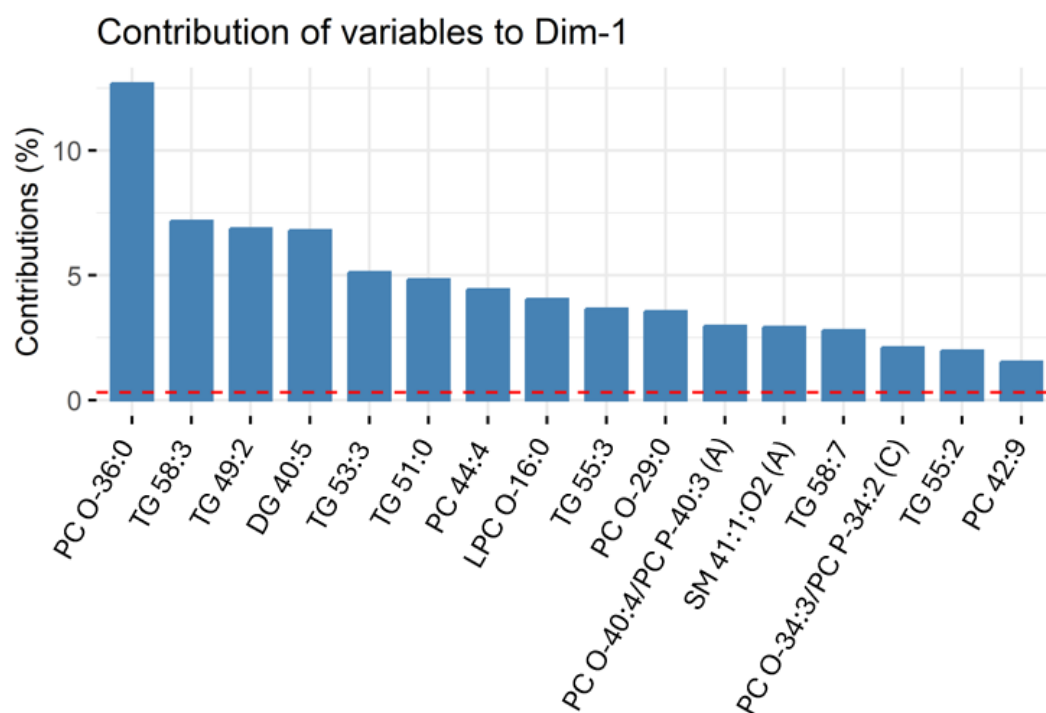


Figure 3.6. Variables ranked by their contribution (%) to Dimension 1 (PC1) in the principal component analysis (PCA) of the lipid species dataset.

Dim-1 accounted for 52.9% of the total variance.

Table 3.2. Neutral and polar lipids identified by C18-LC-MS and MS/MS of total lipid extracts obtained from treated and untreated HaCaT cells (mass error < 5 ppm).^a

Lipid species	Theoretical <i>m/z</i>	Observed <i>m/z</i>	Error (ppm)	FA chain(s)	Retention time (mins)	Formula
DG identified as [M+H]⁺						
DG 34:3	591.4983	591.496	3.9	16:0/18:3	13.84	C37H66O5
DG 36:3	619.5296	619.5272	4	*	14.86	C39H70O5
DG 36:4	617.514	617.5116	3.8	16:0/20:4	13.89	C39H68O5
DG 38:4	645.5453	645.543	3.5	18:0/20:4	14.88	C41H72O5
DG 38:5	643.5296	643.5274	3.5	18:1/20:4	13.93	C41H70O5
DG 40:5	671.5609	671.5593	2.3	18:1/22:4	14.86	C43H74O5
TG identified as [M+NH₄]⁺						
TG 44:0	768.7076	768.7072	0.5	14:0/14:0/16:0	17.53	C47H90O6
TG 46:0	796.7389	796.7384	0.6	14:0/16:0/16:0	18.01	C49H94O6
TG 46:1	794.7232	794.7225	0.8	14:0/16:0/16:1	17.54	C49H92O6
TG 47:0	810.7545	810.7532	1.7	15:0/16:0/16:0	18.22	C50H96O6
TG 48:0	824.7702	824.7697	0.6	14:0/16:0/18:0	18.45	C51H98O6
TG 48:1	822.7545	822.754	0.6	16:0/16:0/16:1	18	C51H96O6
TG 48:2	820.7389	820.7385	0.4	14:0/16:1/18:1	17.54	C51H94O6
TG 49:0	838.7858	838.7851	0.9	16:0/16:0/17:0	18.64	C52H100O6
TG 49:1	836.7702	836.7698	0.5	15:0/16:0/18:1	18.21	C52H98O6
TG 49:2	834.7545	834.754	0.7	16:0/16:1/17:1	17.78	C52H96O6
TG 50:0	852.8015	852.8008	0.8	16:0/16:0/18:0	18.86	C53H102O6
TG 50:1	850.7858	850.7855	0.4	16:0/16:0/18:1	18.43	C53H100O6
TG 50:2	848.7702	848.7695	0.8	16:0/16:1/18:1	17.99	C53H98O6
TG 50:3	846.7545	846.754	0.6	16:1/16:1/18:1	17.55	C53H96O6
TG 51:0	866.8171	866.8165	0.7	16:0/17:0/18:0	18.99	C54H104O6
TG 51:1	864.8015	864.8002	1.5	16:0/17:0/18:1	18.63	C54H102O6

TG 51:2	862.7858	862.785	0.9	16:0/17:1/18:1	18.2	C54H100O6
TG 52:0	880.8328	880.8322	0.6	16:0/18:0/18:0	19.25	C55H106O6
TG 52:1	878.8171	878.8165	0.7	16:0/18:0/18:1	18.84	C55H104O6
TG 52:2	876.8015	876.801	0.5	16:0/18:1/18:1	18.42	C55H102O6
TG 52:3	874.7858	874.785	1	16:1/18:1/18:1	18	C55H100O6
TG 52:4	872.7702	872.7693	1	16:1/18:1/18:2	17.65	C55H98O6
TG 53:1	892.8328	892.8315	1.4	17:0/18:0/18:1	19.01	C56H106O6
TG 53:3	888.8015	888.8016	0.2	17:1/18:1/18:1	18.19	C56H102O6
TG 54:0	908.8641	908.8632	0.9	18:0/18:0/18:0	19.61	C57H110O6
TG 54:1	906.8484	906.8479	0.6	18:0/18:0/18:1	19.22	C57H108O6
TG 54:2	904.8328	904.832	0.8	18:0/18:1/18:1	18.82	C57H106O6
TG 54:3	902.8171	902.8163	0.9	18:1/18:1/18:1	18.41	C57H104O6
TG 54:5	898.7858	898.785	0.9	18:1/18:2/18:2	17.65	C57H100O6
TG 55:2	918.8484	918.8473	1.2	18:0/18:1/19:1	18.99	C58H108O6
TG 55:3	916.8328	916.8317	1.2	18:1/18:1/19:1	18.58	C58H106O6
TG 56:1	934.8797	934.8788	1	18:0/18:1/20:0	19.58	C59H112O6
TG 56:4	928.8328	928.8307	2.2	18:0/18:1/20:3	18.5	C59H106O6
TG 56:6	924.8015	924.8004	1.2	16:0/18:1/22:5	17.87	C59H102O6
TG 58:1	962.911	962.9101	0.9	18:0/18:1/22:0	19.91	C61H116O6
TG 58:2	960.8954	960.8944	1	18:1/18:1/22:0	19.52	C61H114O6
TG 58:3	958.8797	958.8788	0.9	18:1/18:1/22:1	19.15	C61H112O6
TG 58:7	950.8171	950.8166	0.6	18:1/18:1/22:5	17.85	C61H104O6
TG 60:3	986.911	986.9104	0.6	18:1/20:1/22:1	19.49	C63H116O6
(Hex)Cer identified as [M+H] ⁺						
Cer 32:1;O2	510.4881	510.4873	1.5	18:1;O2/14:0	9.95	C32H63NO3
Cer 34:0;O2	540.535	540.5345	1	18:0;O2/16:0	11.91	C34H69NO3
Cer 34:1;O2	538.5194	538.5192	0.3	18:1;O2/16:0	11.33	C34H67NO3
Cer 40:1;O2	622.6133	622.6135	0.4	18:1;O2/22:0	15.08	C40H79NO3
Cer 41:1;O2	636.6289	636.6296	1.1	18:1;O2/23:0	15.45	C41H81NO3

Cer 42:0;O2	652.6602	652.6608	0.8	18:0;O2/24:0	16.05	C42H85NO3
Cer 42:1;O2 (A)	650.6446	650.6451	0.9	18:0;O2/24:1	15.33	C42H83NO3
Cer 42:1;O2 (B)	650.6446	650.645	0.6	18:1;O2/24:0	15.79	C42H83NO3
Cer 42:2;O2	648.6289	648.6294	0.7	18:1;O2/24:1	15.01	C42H81NO3
Cer 44:1;O2	678.6759	678.6782	3.4	18:1;O2/26:0	16.38	C44H87NO3
Cer 44:2;O2	676.6602	676.6621	2.7	18:1;O2/26:1	15.71	C44H85NO3
HexCer 34:1;O2	700.5722	700.5721	0.1	18:1;O2/16:0	9.9	C40H77NO8
HexCer 40:1;O2	784.6661	784.6656	0.6	18:1;O2/22:0	13.94	C46H89NO8
HexCer 42:1;O2 (A)	812.6974	812.6969	0.6	18:1;O2/24:0	14.86	C48H93NO8
HexCer 42:2;O2 (A)	810.6817	810.6815	0.3	18:1;O2/24:1	13.85	C48H91NO8
HexCer 42:2;O2 (B)	810.6817	810.6816	0.2	18:1;O2/24:1	14.04	C48H91NO8
HexCer 44:1;O2	840.7287	840.7287	0.1	18:1;O2/26:0	15.56	C50H97NO8
HexCer 42:1;O2 (B)	828.6923	828.6886	4.5	18:1;O2/24:0(2OH[R])	14.62	C48H93NO9
Hex2Cer 34:1;O2	862.625	862.6242	1	18:1;O2/16:0	9.3	C46H87NO13
Hex2Cer 40:1;O2	946.7189	946.7185	0.4	18:1;O2/22:0	13.25	C52H99NO13
Hex2Cer 42:1;O2	974.7502	974.7495	0.7	18:1;O2/24:0	14.33	C54H103NO13
Hex2Cer 42:2;O2	972.7346	972.734	0.6	18:1;O2/24:1	13.15	C54H101NO13
Hex2Cer 44:1;O2	1002.7815	1002.7805	1	18:1;O2/26:0	15.11	C56H107NO13
Hex2Cer 44:2;O2	1000.7659	1000.7654	0.5	18:1;O2/26:1	14.23	C56H105NO13
Hex3Cer 34:1;O2	1024.6778	1024.6774	0.4	18:1;O2/16:0	8.88	C52H97NO18
Hex3Cer 42:1;O2	1136.803	1136.8024	0.6	18:1;O2/24:0	13.91	C60H113NO18
Hex3Cer 42:2;O2	1134.7874	1134.7861	1.1	18:1;O2/24:1	12.59	C60H111NO18
Hex(3)-HexNAc-Cer 42:1;O2	1339.8824	1339.8816	0.6	18:1;O2/24:0	13.37	C68H126N2O23
Hex(3)-HexNAc-Cer 42:2;O2	1337.8668	1337.8653	1.1	18:1;O2/24:1	11.97	C68H124N2O23
SM identified as [M+H] ⁺						
SM 32:0;O2	677.5592	677.561	2.7	*	8.64	C37H77N2O6P
SM 32:1;O2	675.5436	675.5453	2.6	*	8.12	C37H75N2O6P
SM 33:1;O2	689.5592	689.5584	1.2	*	8.77	C38H77N2O6P

SM 34:0;O2	705.5905	705.5899	0.8	*	9.94	C39H81N2O6P
SM 34:1;O2 (A)	703.5749	703.5747	0.2	18:1;O2/16:0	9.4	C39H79N2O6P
SM 34:1;O2 (B)	703.5749	703.5744	0.6	*	9.92	C39H79N2O6P
SM 34:2;O2	701.5592	701.5575	2.5	*	8.3	C39H77N2O6P
SM 36:0;O2	733.6218	733.622	0.3	*	11.29	C41H85N2O6P
SM 36:1;O2	731.6062	731.6058	0.4	*	10.73	C41H83N2O6P
SM 36:2;O2	729.5905	729.5912	0.9	*	9.6	C41H81N2O6P
SM 38:1;O2	759.6375	759.6374	0	*	12.16	C43H87N2O6P
SM 40:0;O2	789.6844	789.6843	0.2	*	14.1	C45H93N2O6P
SM 40:1;O2 (A)	787.6688	787.6691	0.4	*	12.69	C45H91N2O6P
SM 40:1;O2 (B)	787.6688	787.6685	0.3	*	13.59	C45H91N2O6P
SM 40:2;O2 (A)	785.6531	785.6528	0.4	*	12.07	C45H89N2O6P
SM 40:2;O2 (B)	785.6531	785.654	1.1	*	12.31	C45H89N2O6P
SM 41:1;O2 (A)	801.6844	801.6849	0.6	*	13.39	C46H93N2O6P
SM 41:1;O2 (B)	801.6844	801.6848	0.5	*	14.2	C46H93N2O6P
SM 41:2;O2	799.6688	799.6692	0.5	*	12.81	C46H91N2O6P
SM 42:1;O2 (A)	815.7001	815.6988	1.6	*	14	C47H95N2O6P
SM 42:1;O2 (B)	815.7001	815.6997	0.5	*	14.67	C47H95N2O6P
SM 42:2;O2	813.6844	813.6842	0.3	*	13.47	C47H93N2O6P
SM 42:3;O2	811.6688	811.6666	2.6	*	12.22	C47H91N2O6P
SM 43:1;O2	829.7157	829.7166	1	*	15.12	C48H97N2O6P
SM 43:2;O2 (A)	827.7001	827.7008	0.9	*	13.87	C48H95N2O6P
SM 43:2;O2 (B)	827.7001	827.7004	0.4	*	14.08	C48H95N2O6P
SM 44:1;2O	843.7314	843.7308	0.7	*	15.54	C49H99N2O6P
SM 44:2;2O	841.7157	841.7151	0.7	*	14.59	C49H97N2O6P
PC identified as [M+H] ⁺ (determination of fatty acyl chains by MS/MS of [M+CHOO] ⁻)						

PC 29:0	692.5225	692.5236	1.6	*	8.87	C37H74NO8P
PC 30:0 (A)	706.5381	706.5374	1.1	14:0/16:0	9.48	C38H76NO8P
PC 30:0 (B)	706.5381	706.5394	1.8	*	9.92	C38H76NO8P
PC 30:1	704.5225	704.5225	0.1	*	8.38	C38H74NO8P
PC 31:0 (A)	720.5538	720.5529	1.2	*	9.91	C39H78NO8P
PC 31:0 (B)	720.5538	720.5533	0.7	*	10.13	C39H78NO8P
PC 31:1	718.5381	718.5379	0.3	*	9.01	C39H76NO8P
PC 32:0	734.5694	734.5691	0.5	16:0/16:0	10.75	C40H80NO8P
PC 32:1	732.5538	732.5535	0.4	16:0/16:1	9.62	C40H78NO8P
PC 32:2 (A)	730.5381	730.5377	0.5	16:1/16:1	8.52	C40H76NO8P
PC 32:2 (B)	730.5381	730.5355	3.6	*	9.25	C40H76NO8P
PC 33:0 (A)	748.5851	748.5844	0.9	16:0/17:0	11.19	C41H82NO8P
PC 33:0 (B)	748.5851	748.5846	0.6	*	11.46	C41H82NO8P
PC 33:1 (A)	746.5694	746.5686	1.2	*	9.96	C41H80NO8P
PC 33:1 (B)	746.5694	746.569	0.5	16:0/17:1	10.23	C41H80NO8P
PC 33:2	744.5538	744.5529	1.1	16:1/17:1	9.13	C41H78NO8P
PC 34:0	762.6007	762.6000	0.9	16:0/18:0	12.17	C42H84NO8P
PC 34:1	760.5851	760.5846	0.6	16:0/18:1	10.81	C42H82NO8P
PC 34:2 (A)	758.5694	758.5688	0.9	16:1/18:1	9.7	C42H80NO8P
PC 34:2 (B)	758.5694	758.5688	0.8	*	9.9	C42H80NO8P
PC 34:3 (A)	756.5538	756.5534	0.6	16:1/18:2	8.78	C42H78NO8P
PC 34:3 (B)	756.5538	756.553	1	*	9.35	C42H78NO8P
PC 34:4	754.5381	754.5368	1.8	*	8.42	C42H76NO8P
PC 35:0	776.6164	776.6153	1.4	*	12.59	C43H86NO8P
PC 35:1 (A)	774.6007	774.6000	1	*	11.26	C43H84NO8P
PC 35:1 (B)	774.6007	774.6004	0.5	17:0/18:1	11.52	C43H84NO8P
PC 35:2	772.5851	772.5842	1.2	17:1/18:1	10.31	C43H82NO8P
PC 35:3 (A)	770.5694	770.5682	1.7	*	9.38	C43H80NO8P

PC 35:3 (B)	770.5694	770.5676	2.4	*	9.94	C43H80NO8P
PC 35:3 (C)	770.5694	770.5673	2.8	*	11.34	C43H80NO8P
PC 35:4	768.5538	768.5501	4.7	*	10.22	C43H78NO8P
PC 35:5	766.5381	766.538	0.2	*	10.38	C43H76NO8P
PC 36:0	790.632	790.6309	1.4	*	13.62	C44H88NO8P
PC 36:1	788.6164	788.6164	0	18:0/18:1	12.23	C44H86NO8P
PC 36:2	786.6007	786.5996	1.4	18:1/18:1	10.9	C44H84NO8P
PC 36:3 (A)	784.5851	784.584	1.4	18:1/18:2	9.95	C44H82NO8P
PC 36:3 (B)	784.5851	784.5839	1.5	16:0/20:3	10.57	C44H82NO8P
PC 36:3 (C)	784.5851	784.5831	2.5	*	12.58	C44H82NO8P
PC 36:4	782.5694	782.5676	2.4	16:0/20:4	9.64	C44H80NO8P
PC 36:5	780.5538	780.5530	1	*	8.54	C44H78NO8P
PC 36:5	780.5538	780.5532	0.7	16:0/20:5	8.84	C44H78NO8P
PC 37:1 (A)	802.632	802.6312	1	*	12.6	C45H88NO8P
PC 37:1 (B)	802.632	802.6319	0.2	*	12.97	C45H88NO8P
PC 37:2	800.6164	800.6156	0.9	18:1/19:1	11.54	C45H86NO8P
PC 37:3	798.6007	798.5981	3.2	*	10.56	C45H84NO8P
PC 37:4	796.5851	796.5836	1.8	*	9.67	C45H82NO8P
PC 37:5 (A)	794.5694	794.5689	0.6	*	9.14	C45H80NO8P
PC 37:5 (B)	794.5694	794.5660	4.4	*	10.3	C45H80NO8P
PC 37:5 (C)	794.5694	794.5705	1.4	*	10.58	C45H80NO8P
PC 38:1	816.6477	816.6471	0.8	*	13.64	C46H90NO8P
PC 38:2	814.6320	814.6310	1.2	18:1/20:1	12.21	C46H88NO8P
PC 38:3 (A)	812.6164	812.6145	2.4	18:1/20:2	11.15	C46H86NO8P
PC 38:3 (B)	812.6164	812.6157	0.9	18:0/20:3	11.49	C46H86NO8P
PC 38:4	810.6007	810.5994	1.6	*	10.59	C46H84NO8P
PC 38:5	808.5851	808.5837	1.8	18:1/20:4	9.71	C46H82NO8P
PC 38:6 (A)	806.5694	806.5715	2.6	18:1/20:5	8.93	C46H80NO8P

PC 38:6 (B)	806.5694	806.5693	0.2	16:0/22:6	9.32	C46H80NO8P
PC 38:6 (C)	806.5694	806.5660	4.2	*	10.18	C46H80NO8P
PC 38:7	804.5538	804.5533	0.6	*	8.21	C46H78NO8P
PC 39:2	828.6477	828.6472	0.5	*	12.95	C47H90NO8P
PC 39:3	826.632	826.6306	1.7	*	11.8	C47H88NO8P
PC 39:4	824.6164	824.618	1.9	*	10.89	C47H86NO8P
PC 39:5	822.6007	822.5977	3.6	*	9.96	C47H84NO8P
PC 39:6	820.5851	820.5825	3.2	*	9.92	C47H82NO8P
PC 40:1	844.679	844.6785	0.5	*	14.7	C48H94NO8P
PC 40:2	842.6633	842.6624	1.1	18:1/22:1	13.61	C48H92NO8P
PC 40:3	840.6477	840.6475	0.2	*	12.56	C48H90NO8P
PC 40:4 (A)	838.632	838.6319	0.2	*	11.53	C48H88NO8P
PC 40:4 (B)	838.632	838.6312	1	*	11.88	C48H88NO8P
PC 40:5 (A)	836.6164	836.614	2.8	18:0/22:5	10.92	C48H86NO8P
PC 40:5 (B)	836.6164	836.6143	2.5	*	12.56	C48H86NO8P
PC 40:6 (A)	834.6007	834.599	2	18:1/22:5	9.76	C48H84NO8P
PC 40:6 (B)	834.6007	834.5989	2.2	18:0/22:6	10.55	C48H84NO8P
PC 40:7 (A)	832.5851	832.5836	1.8	18:1/22:6	9.39	C48H82NO8P
PC 40:7 (B)	832.5851	832.5810	4.9	*	10.24	C48H82NO8P
PC 40:7 (C)	832.5851	832.5827	2.9	*	10.89	C48H82NO8P
PC 40:8	830.5694	830.5684	1.2	*	8.49	C48H80NO8P
PC 41:7	846.6007	846.5989	2.2	*	9.94	C49H84NO8P
PC 42:1	872.7103	872.7095	0.9	*	15.58	C50H98NO8P
PC 42:10	854.5694	854.5686	1	*	8.19	C50H80NO8P
PC 42:2	870.6946	870.6938	1	18:1/24:1	14.66	C50H96NO8P
PC 42:3	868.679	868.6784	0.7	*	13.74	C50H94NO8P
PC 42:4	866.6633	866.6645	1.3	*	12.78	C50H92NO8P
PC 42:5	864.6477	864.6474	0.3	*	11.65	C50H90NO8P

PC 42:6	862.632	862.6305	1.8	*	10.91	C50H88NO8P
PC 42:7 (A)	860.6164	860.6141	2.6	*	9.94	C50H86NO8P
PC 42:7 (B)	860.6164	860.6147	2	*	10.55	C50H86NO8P
PC 42:9	856.5851	856.5843	0.9	*	8.62	C50H82NO8P
PC 44:1	900.7416	900.7407	1	*	16.31	C52H102NO8P
PC 44:2	898.7259	898.7251	1	*	15.5	C52H100NO8P
PC 44:3	896.7103	896.7093	1.1	*	14.66	C52H98NO8P
PC 44:4	894.6946	894.6945	0.2	*	13.92	C52H96NO8P
PC 46:2	926.7572	926.7563	1	*	16.22	C54H104NO8P
PC O-29:0	678.5432	678.5428	0.6	*	9.69	C37H76NO7P
PC O-30:0	692.5589	692.558	1.3	(O-16:0/14:0) and (O-14:0/16:0)	10.36	C38H78NO7P
PC O-31:0 (A)	706.5745	706.5717	4	*	10.75	C39H80NO7P
PC O-31:0 (B)	706.5745	706.5727	2.5	*	10.99	C39H80NO7P
PC O-31:1/PC P-31:0	704.5589	704.5571	2.5	*	10.75	C39H78NO7P
PC O-32:0	720.5902	720.59	0.3	(O-16:0/16:0)	11.75	C40H82NO7P
PC O-32:1/PC P-32:0	718.5745	718.5739	0.8	(O-16:0/16:1) and (O-16:1/16:0) and (O-14:0/18:1) and (O-18:1/14:0) and (P-18:0/14:0) and (P-14:0/18:0)	10.55	C40H80NO7P
PC O-33:0 (A)	734.6058	734.6052	0.9	*	12.19	C41H84NO7P
PC O-33:0 (B)	734.6058	734.6057	0.1	*	12.56	C41H84NO7P
PC O-33:1	732.5902	732.5896	0.8	(O-16:0/17:1) and (O-17:1/16:0)	11.14	C41H82NO7P
PC O-33:2/PC P-33:1	730.5745	730.5728	2.3	*	10.82	C41H80NO7P
PC O-34:0	748.6215	748.621	0.7	(O-18:0/16:0) and (O-16:0/18:0)	13.31	C42H86NO7P
PC O-34:3/PC P-34:2 (A)	742.5745	742.5741	0.6	*	10.35	C42H80NO7P

PC O-34:3/P-34:2 (B)	742.5745	742.5731	1.9	(O-16:1/18:2) and (O-18:2/16:1) and (P-16:0/18:2) and (P-18:2/16:0) and (P-18:1/16:1) and (P-16:1/18:1)	10.49	C42H80NO7P
PC O-34:3/PC P-34:2 (C)	742.5745	742.5727	2.4	*	12.59	C42H80NO7P
PC O-35:1/PC P-35:0 (A)	760.6215	760.621	0.7	*	12.25	C43H86NO7P
PC O-35:1/PC P-35:0 (B)	760.6215	760.6215	0.1	*	12.58	C43H86NO7P
PC O-35:2/PC P-35:1 (A)	758.6058	758.6054	0.5	*	11.2	C43H84NO7P
PC O-35:2/PC P-35:1 (B)	758.6058	758.6048	1.4	*	11.97	C43H84NO7P
PC O-35:2/PC P-35:1 (C)	758.6058	758.6053	0.7	*	12.23	C43H84NO7P
PC O-36:0	776.6528	776.6522	0.7	*	14.57	C44H90NO7P
PC O-36:1	774.6371	774.6366	0.7	(O-16:0/20:1) and (O-20:1/16:0) and (O-18:0/18:1) and (O-18:1/18:0)	13.37	C44H88NO7P
PC O-36:2/PC P-36:1	772.6215	772.6212	0.4	(O-18:1/18:1) and (O-20:1/16:1) and (O-16:1/20:1) and (P-18:0/18:1) and (P-18:1/18:0) and (P-16:0/20:1) and (P-20:1/16:0)	13.04	C44H86NO7P
PC O-36:3/PC P-36:2 (A)	770.6058	770.6026	4.2	(O-18:1/18:2) and (O-18:2/18:1) and (P-18:0/18:2) and (P-18:2/18:0)	10.74	C44H84NO7P
PC O-36:3/PC P-36:2 (B)	770.6058	770.6049	1.2	*	11.05	C44H84NO7P
PC O-36:3/PC P-36:2 (C)	770.6058	770.6041	2.3	(O-16:0/20:3) and (O-20:3/16:0) and (O-18:1/18:2) and (O-18:2/18:1) and (P-18:1/18:1)	11.59	C44H84NO7P
PC O-36:4	768.5902	768.5887	1.9	(O-16:0/20:4)	10.5	C44H82NO7P
PC O-36:5 (A)	766.5745	766.5735	1.3	(O-16:0/20:5)	9.63	C44H80NO7P

PC O-36:5/PC P-36:4 (B)	766.5745	766.5724	2.7	*	9.93	C44H80NO7P
PC O-37:4/PC P-37:3	782.6058	782.604	2.3	*	11.17	C45H84NO7P
PC O-38:1/PC P-38:0	802.6684	802.6665	2.4	*	14.56	C46H92NO7P
PC O-38:2/PC P-38:1	800.6528	800.6513	1.8	*	13.46	C46H90NO7P
PC O-38:3/PC P-38:2	798.6371	798.636	1.3	*	12.56	C46H88NO7P
PC O-38:4/PC P-38:3 (A)	796.6215	796.6188	3.4	*	11.11	C46H86NO7P
PC O-38:4/PC P-38:3 (B)	796.6215	796.6202	1.6	*	11.47	C46H86NO7P
PC O-38:4 (C)	796.6215	796.6198	2.1	(O-18:0/20:4)	11.86	C46H86NO7P
PC O-38:5/PC P-38:4	794.6058	794.6041	2.1	(O-18:1/20:4) and (P-18:0/20:4)	10.54	C46H84NO7P
PC O-38:6/PC P-38:5 (A)	792.5902	792.5883	2.4	*	9.67	C46H82NO7P
PC O-38:6 (B)	792.5902	792.5888	1.7	(O-16:0/22:6)	10.23	C46H82NO7P
PC O-40:4/PC P-40:3 (A)	824.6528	824.6508	2.4	*	12.6	C48H90NO7P
PC O-40:4/PC P-40:3 (B)	824.6528	824.6507	2.5	*	13.33	C48H90NO7P
PC O-40:5/PC P-40:4	822.6371	822.6351	2.4	*	11.9	C48H88NO7P
PC O-40:6/PC P-40:5 (A)	820.6215	820.6193	2.6	*	10.57	C48H86NO7P
PC O-40:6 (B)	820.6215	820.6202	1.5	(O-18:0/22:6)	11.44	C48H86NO7P
PC P-30:0/PC O-30:1	690.5432	690.5423	1.4	*	10.13	C38H76NO7P
PC P-32:0/PC O-32:1	718.5745	718.5744	0.1	(P-16:0/16:0) and (O-16:1/16:0) and (O-16:0/16:1)	11.49	C40H80NO7P
PC P-32:1/PC O-32:2	716.5589	716.5581	1	(P-16:0/16:1) and (P-16:1/16:0) and (O-16:1/16:1)	10.24	C40H78NO7P
PC P-34:0/PC O-34:1	746.6058	746.6053	0.7	(P-18:0/16:0) and (P-16:0/18:0) and (O-16:0/18:1) and (O-18:1/16:0)	11.81	C42H84NO7P

PC P-34:1/PC O-34:2 (A)	744.5902	744.5892	1.3	(P-18:0 /16:1) and (P-16:1/18:0) and (O-18:1/16:1) and (O-16:1/18:1)	10.55	C42H82NO7P
PC P-34:1/PC O-34:2 (B)	744.5902	744.5893	1.2	(P-18:0/16:1) and (P-16:1/18:0) and (O-18:1/16:1) and (O-16:1/18:1)	10.72	C42H82NO7P
PC P-34:1/PC O-34:2 (C)	744.5902	744.5897	0.6	(P-16:0 /18:1) and (P-18:1/16:0) and (O-16:1/18:1) and (O-18:1/16:1)	11.54	C42H82NO7P
PC P-35:2/PC O-35:3	756.5902	756.5883	2.5	*	10.9	C43H82NO7P
PC P-36:1/PC O-36:2	772.6215	772.6203	1.5	(P-18:0/18:1) and (P-18:1/18:0) and (O-18:1/18:1)	11.87	C44H86NO7P
PC P-36:4/PC O-36:5	766.5745	766.5736	1.2	(P-16:0/20:4) and (O-16:1/20:4)	10.21	C44H80NO7P
PC P-36:5/PC O-36:6 (A)	764.5589	764.5577	1.5	*	9.38	C44H78NO7P
PC P-36:5/PC O-36:6 (B)	764.5589	764.5581	1.1	*	11.04	C44H78NO7P
PC P-38:6/PC O-38:7 (A)	790.5745	790.5724	2.7	*	9.44	C46H80NO7P
PC P-38:6/PC O-38:7 (B)	790.5745	790.5731	1.8	(P-16:0/22:6) and (O-16:1/22:6)	9.89	C46H80NO7P
PC P-40:6/PC O-40:7 (A)	818.6058	818.6039	2.4	P(P-18:0/22:6) and (O-18:22:6)	10.16	C48H84NO7P
PC P-40:6/PC O-40:7 (B)	818.6058	818.6036	2.7	*	11.12	C48H84NO7P
LPC identified as [M+H]⁺ (determination of fatty acyl chains by MS/MS of [M+CHOO] ⁻)						
LPC 16:0	496.3398	496.3397	0.2	*	2.85	C24H50NO7P
LPC 18:0	524.3711	524.3712	0.3	*	3.86	C26H54NO7P

LPC O-16:0	482.3605	482.3605	0.1	*	3.33	C24H52NO6P
PE identified as [M - H]⁻ (confirmation of polar head group by MS/MS of [M+H] ⁺)						
PE 32:0	690.5079	690.5083	0.5	16:0/16:0	11.08	C37H74NO8P
PE 32:1	688.4923	688.492	0.4	16:0/16:1	9.93	C37H72NO8P
PE 33:1	702.5079	702.5082	0.4	16:0/17:1	10.54	C38H74NO8P
PE 34:1	716.5236	716.5227	1.2	16:0/18:1	11.19	C39H76NO8P
PE 36:4	738.5079	738.5072	1	16:0/20:4	9.96	C41H74NO8P
PE 35:2	728.5236	728.5228	1.1	17:1/18:1	10.63	C40H76NO8P
PE 36:1	744.5549	744.5547	0.3	18:0/18:1	12.58	C41H80NO8P
PE 38:1	772.5862	772.5862	0	18:0/20:1	13.87	C43H84NO8P
PE 40:4	794.5705	794.5699	0.8	18:0/22:4	12.2	C45H82NO8P
PE 40:6	790.5392	790.5407	1.9	18:0/22:6	10.86	C45H78NO8P
PE 36:2	742.5392	742.5388	0.6	18:1/18:1	11.26	C41H78NO8P
PE 36:3	740.5236	740.523	0.8	18:1/18:2	10.27	C41H76NO8P
PE 38:2	770.5705	770.5701	0.6	18:1/20:1	12.55	C43H82NO8P
PE 38:3 (A)	768.5549	768.5546	0.4	18:1/20:2	11.45	C43H80NO8P
PE 38:3 (B)	768.5549	768.5542	0.9	18:1/20:2	11.82	C43H80NO8P
PE 38:4	766.5392	766.538	1.6	18:1/20:3	10.57	C43H78NO8P
PE 38:5	764.5236	764.5212	3.2	18:1/20:4	10.04	C43H76NO8P
PE 40:1	800.6175	800.6175	0	18:1/22:0	14.82	C45H88NO8P
PE 40:2	798.6018	798.6019	0.2	18:1/22:1	13.82	C45H86NO8P
PE 40:7	788.5236	788.5228	1	18:1/22:6	9.7	C45H76NO8P
PE 42:1	828.6488	828.6493	0.7	24:0/18:1	15.53	C47H92NO8P
PE P-32:0/PE O-32:1	674.513	674.5132	0.3	(P-16:0/16:0) and (O-16:1/16:0) and (O-16:0/16:1)	11.84	C37H74NO7P
PE P-32:1/PE O-32:2	672.4974	672.4975	0.2	(P-16:0/16:1) and (P-16:1/16:0) and (O-16:1/16:1)	10.59	C37H72NO7P

PE P-33:1/PE O-33:2	686.513	686.513	0.1	(P-16:0 /17:1) and (P-17:1 /16:0) and (O-16:1 /17:1) and (O-17:1 /16:1)	11.24	C38H74NO7P
PE P-34:1/PE O-34:2	700.5287	700.5292	0.7	(P-16:0 /18:1) and (P-18:1/16:0) and (O-16:1/18:1) and (O-18:1/16:1)	11.92	C39H76NO7P
PE P-36:1/PE O-36:2	728.56	728.5596	0.5	(P-16:0/20:1) and (P-20:1/16:0) and (P-18:0/18:1) and (P-18:1/18:0) and (O-16:1/20:1) and (O-20:1/16:1) and (O-18:1/18:1)	13.35	C41H80NO7P
PE P-36:3/PE O-36:4	724.5287	724.5291	0.6	(P-16:0 /20:3) and (O-16:1/20:3)	11.15	C41H76NO7P
PE P-36:4/PE O-36:5	722.513	722.5124	0.9	(P-16:0 /20:4) and (O-16:1/20:4)	10.58	C41H74NO7P
PE P-36:5/PE O-36:6	720.4974	720.4964	1.3	(P-16:0/20:5) and (P-16:1/20:5)	9.73	C41H72NO7P
PE P-38:6/PE O-38:7	746.513	746.5124	0.8	(P-16:0/22:6) and (O-16:1/22:6)	10.2	C43H74NO7P
PE P-34:0/PE O-34:1	702.5443	702.5451	1.2	(P-18:0/16:0) and (P-16:0/18:0) and (O-18:1/16:0) and (O-16:1/18:0)	13.28	C39H78NO7P
PE P-38:3/PE O-38:4	752.56	752.5589	1.4	(P-18:0/20:3) and (O-18:1/20:3)	12.57	C43H80NO7P
PE P-38:4/PE O-38:5	750.5443	750.5435	1	(P-18:0/20:4) and (O-18:1/20:4)	11.93	C43H78NO7P
PE P-38:5/PE O-38:6	748.5287	748.5277	1.3	(P-18:0/20:5) and (O-18:1/20:5)	11.01	C43H76NO7P

PE P-40:4/PE O-40:5	778.5756	778.5751	0.6	(P-18:0/22:4) and (O-18:1/22:4)	12.94	C45H82NO7P
PE P-40:6/PE O-40:7	774.5443	774.5438	0.6	(P-18:0/22:6) and (O-18:1/22:6)	11.5	C45H78NO7P
PE P-40:7/PE O-40:8	772.5287	772.5277	1.2	(P-18:1/22:6) and (O-18:2/22:6)	10.27	C45H76NO7P
LPE identified as [M - H]⁻ (confirmation of polar head group by MS/MS of [M+H] ⁺)						
LPE 18:0	480.3096	480.31	0.9	18:0	480.31	C23H48NO7P
PI identified as [M-H]⁻						
PI 32:1	807.5029	807.5027	0.3	16:0/16:1	8.21	C41H77O13P
PI 34:1	835.5342	835.533	1.4	16:0/18:1	9.36	C43H81O13P
PI 36:4	857.5186	857.5179	0.8	16:0/20:4	8.28	C45H79O13P
PI 34:2	833.5186	833.5178	0.9	16:1/18:1	8.3	C43H79O13P
PI 35:1	849.5499	849.5487	1.4	17:0/18:1	9.93	C44H83O13P
PI 36:1	863.5655	863.5646	1.1	18:0/18:1	10.5	C45H85O13P
PI 38:1	891.5968	891.5955	1.4	18:0/20:1	11.62	C47H89O13P
PI 38:2	889.5812	889.5802	1.1	18:0/20:2	10.66	C47H87O13P
PI 38:3	887.5655	887.5653	0.2	18:0/20:3	9.92	C47H85O13P
PI 38:4	885.5499	885.5485	1.5	18:0/20:4	9.44	C47H83O13P
PI 40:4 (A)	913.5812	913.581	0.2	18:0/22:4	10.24	C49H87O13P
PI 40:6 (B)	909.5499	909.5496	0.3	18:0/22:6	9.13	C49H83O13P
PI 36:2	861.5499	861.5485	1.5	18:1/18:1	9.41	C45H83O13P
PI 38:5	883.5342	883.5335	0.7	18:1/20:4	8.36	C47H81O13P
PI O-38:6	867.5393	867.5388	0.5	(O-16:0/22:6)	8.61	C47H81O12P
PI O-38:4	871.5706	871.5666	4.5	(O-18:0/20:4)	10.1	C47H85O12P
PI O-40:6	895.5706	895.5693	1.5	(O-18:0/22:6)	9.77	C49H85O12P
PI P-38:4/PI O-38:5	869.5549	869.5549	0	(P-18:0/20:4) and (O-18:1/20:4)	8.98	C47H83O12P

PS identified as [M-H] ⁻						
PS 30:3	700.4195	700.4166	4.1	12:0/18:3	7.07	C36H64NO10P
PS 34:1	760.5134	760.5125	1.2	16:1/18:0	9.65	C40H76NO10P
PS 36:1	788.5447	788.5444	0.4	18:0/18:1	10.82	C42H80NO10P
PS 38:3	812.5447	812.5431	2	18:0/20:3	10.2	C44H80NO10P
PS 40:6	834.5291	834.5286	0.6	18:0/22:6	9.39	C46H78NO10P
PS 36:2	786.5291	786.5284	0.9	18:1/18:1	9.7	C42H78NO10P
PS 36:2	786.5291	786.5286	0.6	18:1/18:1	9.91	C42H78NO10P
PS 38:1	816.576	816.5755	0.7	18:1/20:0	12.15	C44H84NO10P
PS 40:1	844.6073	844.6069	0.5	18:1/22:0	13.51	C46H88NO10P
PS 40:2	842.5917	842.5905	1.4	18:1/22:1	12.09	C46H86NO10P
PS 40:4	838.5604	838.5574	3.5	20:0/20:4	10.54	C46H82NO10P
PS 40:5	836.5447	836.5427	2.4	20:0/20:5	9.74	C46H80NO10P

^aNeutral and polar lipids identified by C18-LC-MS and MS/MS of total lipid extracts obtained from treated and untreated HaCaT cells (mass error < 5 ppm). Observed m/z values and respective errors were checked for all samples. Lipid species are labelled as follows: AA xx:n (AA=lipid class abbreviation; xx=number of carbon atoms in fatty acid(s); n=number of double bonds). The 'O-' prefix is used for plasmalogen species to indicate the presence of an alkyl-ether substituent, whereas the 'P-' prefix is used for plasmalogen species to indicate the alk-1-enyl ether substituent. Molecular species with known fatty acyl constituents are labelled using (sn-1/sn-2) nomenclature, considering that in animals the sn-1 position generally contains smaller and saturated fatty acids, while fatty acids with a higher unsaturation degree are located at sn-2. *, Lipid species identified by retention time, mass accuracy and confirmation of polar head group by MS/MS of [M+H]⁺ ions. The suffix "A", "B" or "C" etc., is used to differentiate isomers with distinct elution times. Lipid class abbreviations: DG, diacylglycerols; CER, ceramide; Hex_nCer, hexosylceramide; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; and TG, triacylglycerol.

3.3.5 Univariate Analysis Using Analysis of Variance (ANOVA)

Univariate analysis showed that 137 out of 328 identified lipid species had significant differences (q -value < 0.05) (Appendix B, Table B1). The results of the ANOVA were used to create a heatmap (double dendrograms) with two-dimensional hierarchical clustering analysis of the 50 lipid species with the lowest q -values (q -value < 0.01), which were responsible for distinction between allergen treated cell groups and the vehicle control (Fig 3.7). As illustrated by the hierarchical dendrogram at the top of the heatmap, the samples were clustered into two independent groups: one cluster representing the vehicle control group (pink) and the other representing the allergen-treated cell groups, including DNFB (green), Lim-2-OOH (red) and Lin-6/7-OOH (purple). The separation of the allergen-treated cell groups from the vehicle control group followed a similar trend to the one previously observed in the PCA score plot (Fig. 3.5A). Based on similarity changes in lipid composition, the clustering of the individual lipid species led to the formation of two distinct clusters. The first group, occupying the top seven rows of the dendrogram (Fig. 3.7), contained two DG, one HexCer, three PC and one PE, which were more abundant in the vehicle control group. The remaining rows included 43 lipid species that were more abundant in cells treated with the fragrance hydroperoxides or DNFB, namely 1 DG, 1 HexCer, 1 Hex₃Cer, 8 PC, 13 ether-linked PC (3 O- and 10 O-/P-), 1 ether-linked PE (O-/P-), 5 SM and 13 TG.

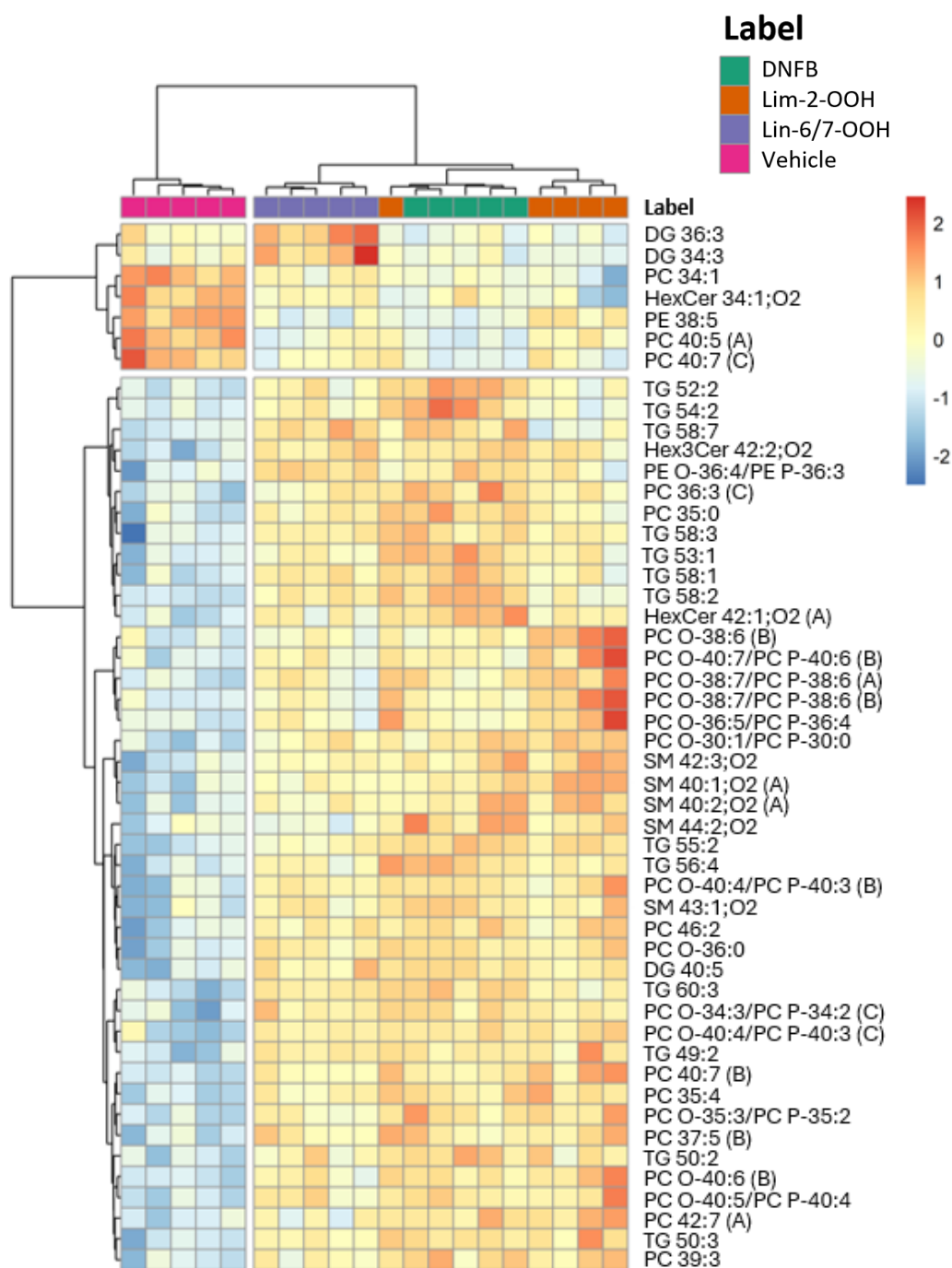
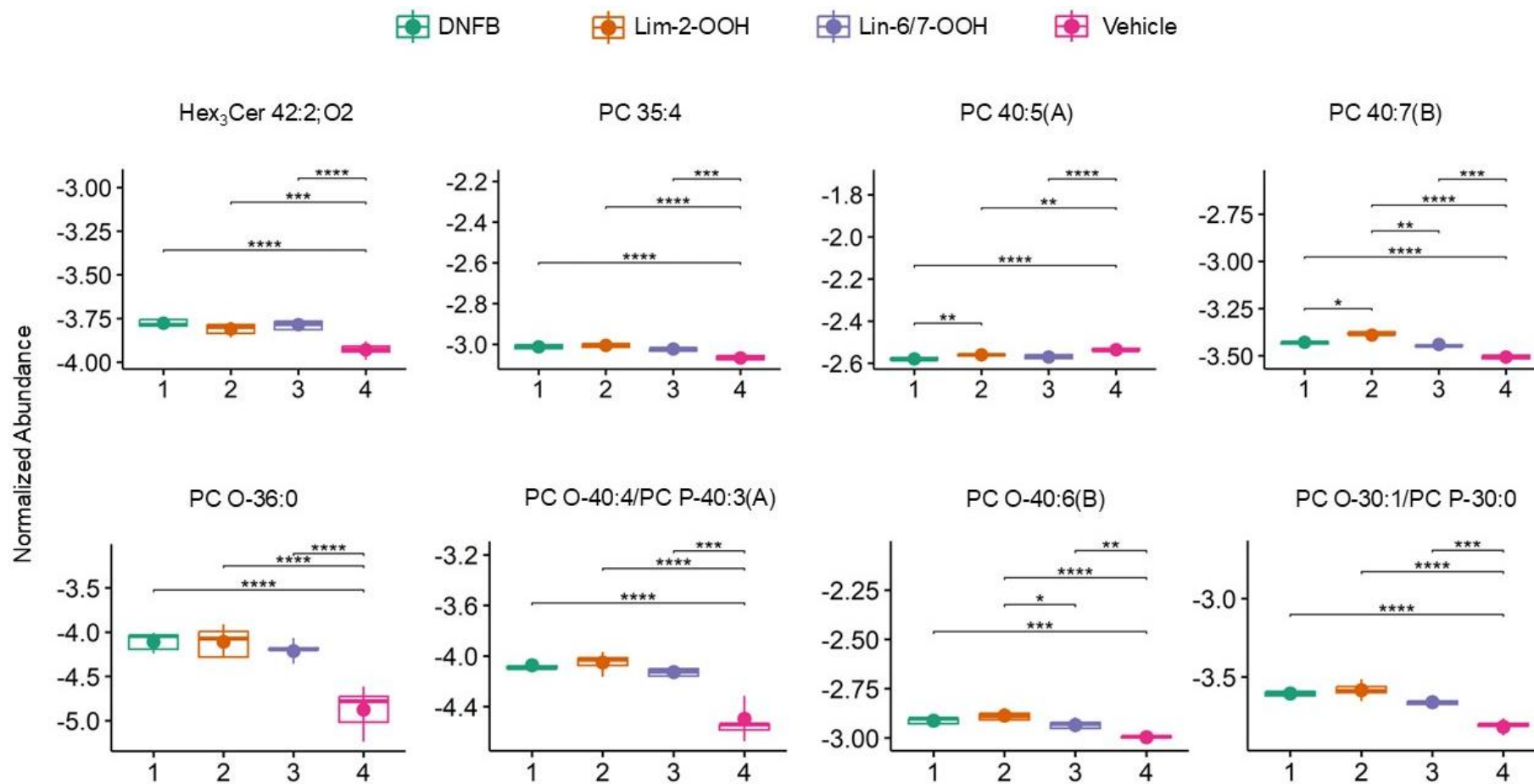


Figure 3.7. Two-dimensional hierarchical clustering heatmap of the 50 most discriminative lipid species between HaCaT cells treated with terpene hydroperoxides, DNFB and the vehicle control.

The relative abundance levels are depicted on the red-yellow-blue scale, with numbers indicating the fold change from the overall mean. The red colour indicates high abundance, while blue indicates low abundance (yellow represents null values). The clustering of the allergen-treated cell groups and the vehicle control is represented by the dendrogram at the top (horizontal), while the clustering of individual lipid species is represented by the dendrogram on the left (vertical). The lipid species are labelled as follows: AA xx:n (AA=lipid class abbreviation; xx=number of carbon atoms in FA; n=number of double bonds). The prefix 'O-' is used for plasmalogen species to indicate the presence of an alkyl ether substituent, whereas the prefix 'P-' is used for plasmalogen species to indicate the alk-1-enyl ether substituent. The suffix "A", "B", or "C" is used to differentiate isomers with distinct elution times. The abbreviations of lipid classes are as follows: DG, diacylglycerol; Hex_nCer, hexosylceramides; PC, phosphatidylcholine; PE,

phosphatidylethanolamine; SM, sphingomyelin; and TG, triacylglycerol. The fatty acyl chain composition of each lipid species can be found in Supplementary Table S1. HaCaT cells were treated with Lim-2-OOH (10 μ M), Lin-6/7-OOH (10 μ M), DNFB (10 μ M) or vehicle control (DMSO 0.01% v/v) for 24 hr followed by lipid extraction and analysis (n=5).

Univariate analysis was performed, and box plots of the 16 most significant lipid species with the lowest *q*-values from ANOVA (Appendix B, Table B1) annotated using Tukey's HSD test were plotted (Fig. 3.8). Among the 16 lipid species, comparison of the fragrance hydroperoxide/DNFB-treated cells and the vehicle control identified 16 lipid species that were upregulated in all three allergen-treated samples. These included five ether-linked PC species, two PC species, four TG species and one Hex_nCer species. Additionally, one PC species (PC 40:5 [A]) and one PE species (PE 38:5) were significantly downregulated in all three allergen treated cell groups when compared to the vehicle control group. The FA composition of the 16 upregulated species was predominantly PUFAs with respect to PC and PE species (e.g., 22:5, 22:6 and 20:4), and monounsaturated FAs (NUFA) with respect to HexCer (24:1) and TG species (e.g., 16:1, 18:1, 19:1 and 22:1) (FA compositions are provided in Table 3.2). Among the 16 upregulated lipid species, there were significant differences between Lim-2-OOH and Lin-6/7-OOH. Four PCs and one TG were significantly increased by treatment with Lim-2-OOH compared to Lin-6/7-OOH, with only one significant increase for TG 59:7 in favour of Lin-6/7-OOH. Between Lim-2-OOH and DNFB, there were significant increases seen in PC 40:5 (A), PC 40:7 (B), PC O-35:3/P-35:2, and PE 38:5 in favour of Lim-2-OOH, but significant increases in TG 58:2 and TG 58:7 for DNFB. Between DNFB and Lin-6/7-OOH, there was one significant increase seen in TG 55:3 for DNFB.



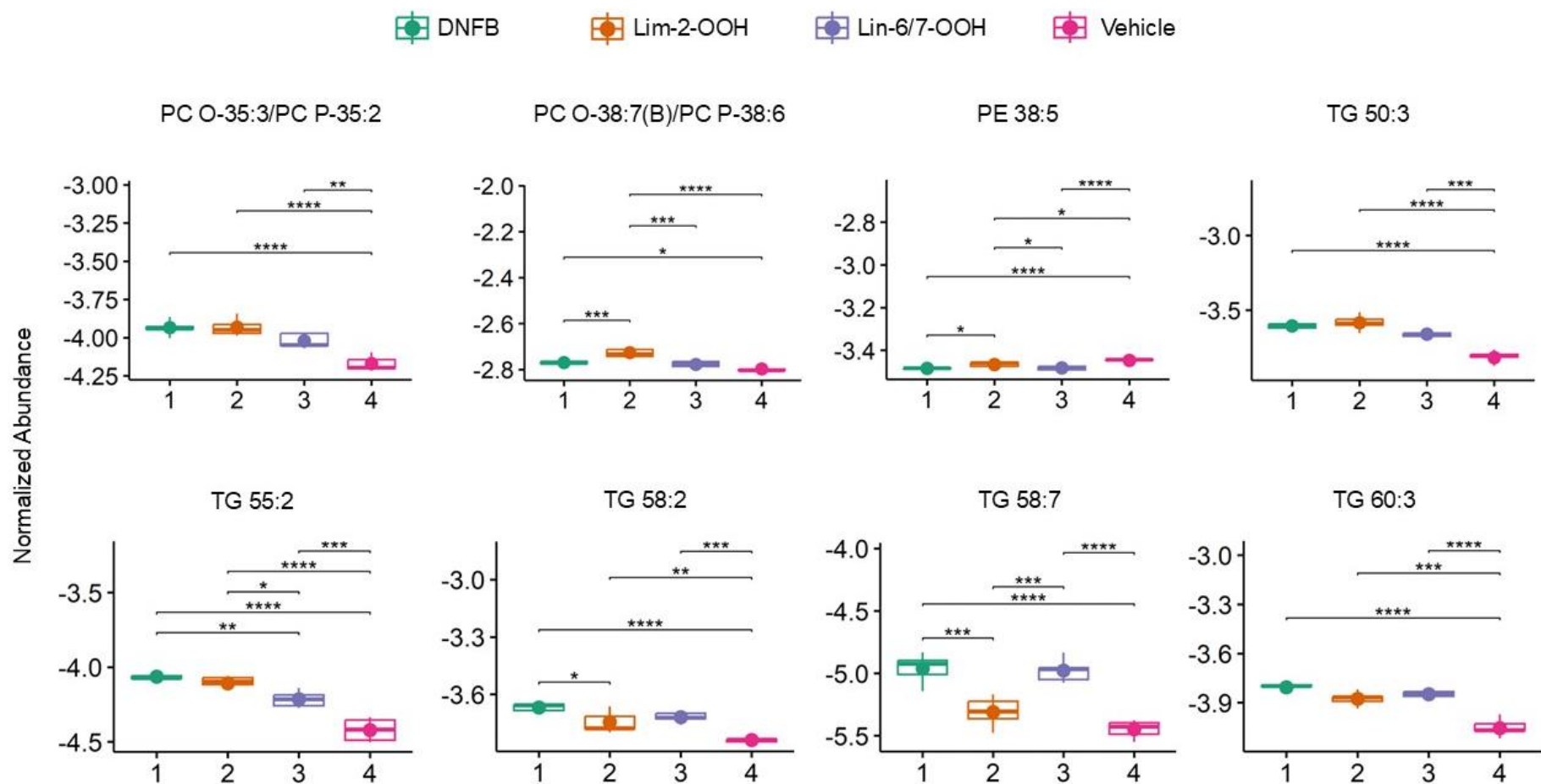


Figure 3.8. Boxplots of the 16 most significant lipid species identified through univariate analysis between HaCaT cells treated with fragrance hydroperoxides, DNFB and the vehicle control.

HaCaT cells were treated with Lin-2-OOH (10 μ M), Lin-6/7-OOH (10 μ M), DNFB (10 μ M) or vehicle control (DMSO 0.01% v/v) (n=5).

3.4 Discussion

Lipids play a structural role in cells and also modulate cellular signalling processes. Changes in the lipidome of the plasma membrane can modify mechanical properties, such as curvature and fluidity, which alter several membrane-dependent events, such as phagocytosis [149], ion channel gating [150], and signal transduction [151]. The dysregulation of skin lipid homeostasis has not been thoroughly investigated in the context of ACD. In this chapter, the lipidome of HaCaT cells exposed to Lim-2-OOH and Lin-6/7-OOH is compared alongside DNFB, and C18 LC-MS/MS is used to reveal changes at the species level. Investigating the differences in lipid species in allergen-treated HaCaT cells may allow us to better understand the lipid profile of KCs associated with ACD.

The multivariate PCA analysis of the total lipid profile (Fig. 3.5) revealed distinct clustering between the vehicle control and cells exposed to fragrance hydroperoxides (Lim-2-OOH and Lin-6/7-OOH). Clustering of DNFB was also distinct from the vehicle control, with some overlap with the hydroperoxides. The differences between the untreated cells and vehicle-treated cells could be attributable to the known oxidising properties of DMSO. From the univariate analysis (Fig. 3.7), there was upregulation in 13 alkyl-ether (plasmanyl) and/or vinyl-ether (plasmenyl/plasmalogen) PCs (PC O-/P-) and 1 alkyl-ether (plasmanyl) or vinyl-ether (plasmenyl/plasmalogen) PE (PE O-/P-) lipid species. There were both similarities and differences between the effects of Lim-2-OOH and Lin 6/7-OOH as shown in the results. Plasmalogens have a vinyl-ether bond in the sn-1 position, whereby the adjacent hydrogens have relatively low dissociation energies compared to diacylglycerophospholipids, therefore sparing the oxidation of PUFAs in the sn-2 position [152]. The oxidation products of plasmalogens are unable to propagate lipid peroxidation; thus, plasmalogens are often regarded as endogenous antioxidants [153]. The contact allergens used in this experiment are known to trigger the release of reactive oxygen species (ROS) [128, 154], and an upregulation in the synthesis of these lipids may be a direct response of the cells to a disruption in redox homeostasis. Interestingly, plasmanyl and plasmenyl lipids are known to play an active role in ferroptosis, with plasmanyls believed to induce ferroptosis and plasmenyls believed to abrogate it [155, 156]. Although the capability of these contact allergens to induce

ferroptosis in KCs is presently unknown, it has been demonstrated that ferroptosis plays a significant role in initiating UVB-induced inflammation in KCs [157].

There was also a significant decrease in PE 38:5 (18:1/20:4) seen for all allergen-treated cells. As these allergens are known to cause the oxidation of cell surface thiols [128, 158], it is plausible that the decrease in PE 38:5 is a result of an altered GSH/GSSG ratio, which could lead to an increase in oxidative stress followed by lipid peroxidation [159]. In fact, di- and tri-oxygenated PEs containing arachidonic acid (AA) [such as the detected PE 38:5 (18:1/20:4)] and adrenic acid (AdA) chains are known to be the most common ferroptotic signalling molecules [160]. Unlike the immunogenically silent apoptosis, ferroptosis is immunogenic as affected cells release damage-associated molecular patterns (DAMPs), which promote a series of reactions related to inflammation [161]. In a similar way, there were also decreases seen for PC 40:5 and PC 40:7, two lipid species with PUFAs at the sn-2 position. This decrease may be due to oxidative degradation of these PC species bearing PUFAs. While the connection between the oxidative and sensitising properties of contact allergens has not been investigated in this chapter, it is well known that some allergens possess both oxidising and sensitising properties. The relevance of this to manifestation of clinical symptoms of ACD is unknown. It is possible that allergens that are redox-active can putatively also cause lipid peroxidation, contributing to inflammation and DAMPs as discussed above.

Interestingly, there was an increase in 13 TG lipid species exposed to fragrance hydroperoxides or DNFB (Fig. 3.7). Skin irritants and contact allergens both caused an increase in the expression of adipose differentiation related protein (ADRP) in human epidermal cell lines [162]. As a lipid-storage-droplet associated protein, ADRP governs the deposition and release of lipids from cytosolic droplets. Lipid droplets can be a source of FA for signalling/gene regulation, or TGs for membrane PL synthesis. A concentration-dependent accumulation of TGs was previously demonstrated following treatment with the skin irritant SDS [163]. If contact allergens damage lipid bilayers and allow an influx of extracellular calcium, triggering the activation of gene expression for ADRP, this could result in the accumulation of TGs.

There was also an increase in several long chain SL exposed to Lim-2-OOH and Lin-6/7-OOH, including five SM with FA compositions of between C20 and C26, and two GSLs

with FA compositions of C24 in allergen-treated cells. Cell membranes are mainly composed of PL, SL, CHOL and membrane-associated proteins. These molecules are non-homogenously distributed in membranes, leading to the formation of membrane lipid microdomains (lipid rafts) that are rich in CHOL and SL [164]. Such domains organise signalling platforms involved in normal or pathological conditions. It has been shown that cultured human KCs respond to UVB light by increasing SL synthesis, primarily through the increase in both LCB2 mRNA and protein levels leading to increased serine palmitoyltransferase (SPT) activity [165]. This enzyme was upregulated in the epidermis as part of the homeostatic repair in response to permeability barrier perturbation by acetone in a murine model [166]. It is unknown at present if contact allergens have a direct effect on SPT activity. SM has been shown to act as a T cell antagonist when loaded onto CD1a antigen-presenting molecules [167]. Although CD1a is expressed in normal keratinocytes [168], its expression level is lower than in professional antigen-presenting cells such as LCs [100]. In contrast, GSLs are known T cell agonists when presented by CD1d molecules [169]. CD1d is expressed in keratinocytes under normal conditions and is upregulated in inflammatory skin pathologies [170]. A previous study examined whether contact allergens could activate CD1a- and CD1d-restricted T cells. It found that the upregulation of inflammatory mediators was not due to individual allergens acting as CD1-restricted T cell agonists. Instead, it was postulated that compounds such as DNCB, and other contact allergens, directly influenced the formation of CD1-self-antigen complexes [171]. Therefore, the observation in this study that contact allergens increased the abundance of GSLs HexCer 42:1;O2 (A) and Hex3Cer 42:2;O2 in KCs is of significant interest and warrants further investigation.

3.5 Conclusions

In summary, this chapter is the first to evaluate the plasticity of the HaCaT cell lipidome in response to allylic hydroperoxide allergens Lim-2-OOH and Lin-6/7-OOH, together with the experimental contact allergen DNFB. Significant changes in the lipidome were observed for allergen-treated cells at 24-h, with increases in 9 plasmalyl/plasmenyl PL (PC O-/P- and PE O-/P-), SL (SM and Hex Cer) and TG lipid species, and conversely, a decrease in some PUFA PE and PUFA-PC lipid species. Further studies will elucidate the significance of changes in lipids, lipid peroxidation and lipid metabolism in ACD.

During biological lipid peroxidation, only a small fraction of the available substrate undergoes oxidation. Previous estimates suggest that the ratio of non-oxidized substrate to oxidized products, even in diseased states, typically ranges from 50:1 to 10,000:1 [172]. In LC-MS, ion suppression can sometimes be an issue depending on the sample matrix, whereby a decrease in an analyte ions signal can be caused by other co-eluting compounds present in the sample [173, 174]. ESI involves competition for charge, meaning that high concentrations of native (non-oxidized) lipids can suppress the ionization of low-abundance oxidized species, reducing their detection efficiency. Another major challenge in identifying oxidized lipids by LC–MS is the limited availability of authentic standards. Without standards, the chromatographic and fragmentation characteristics of these compounds remain uncertain, which partly explains why software tools such as MSDial and Lipostar perform well for native lipids but struggle to accurately annotate oxidized species, as their fragmentation libraries remain underdeveloped. Given these challenges, the HaCaT cell model presents considerable complexity, prompting the need for a more simplified model, which is explored in the next chapter.

The work presented in this chapter is based on the following publication:

Moore, A. I., Moreira, A. S., Guerra, I. M., Goracci, L., Domingues, P., Melo, T., Domingues, M.R., & O'Boyle, N. M. (2025). A lipidomic approach towards identifying the effects of fragrance hydroperoxides on keratinocytes. *Contact Dermatitis*, 92(3), 176-186.

Chapter 4: Terpene Hydroperoxides as Lipid Peroxidation Inducers: Biomimetic and HaCaT Cell Studies

The work presented in this chapter is based on the following publication:

Moore, A. I., Moreira, A. S., Conde, T., Melo, T., Domingues, P., O'Boyle, N. M., & Domingues, M. R. (2025). Terpene Hydroperoxides as Lipid Peroxidation Inducers: Biomimetic and HaCaT Cell Studies in Allergic Contact Dermatitis. *Contact Dermatitis*, 93(1), 16-30.

4.1 Introduction

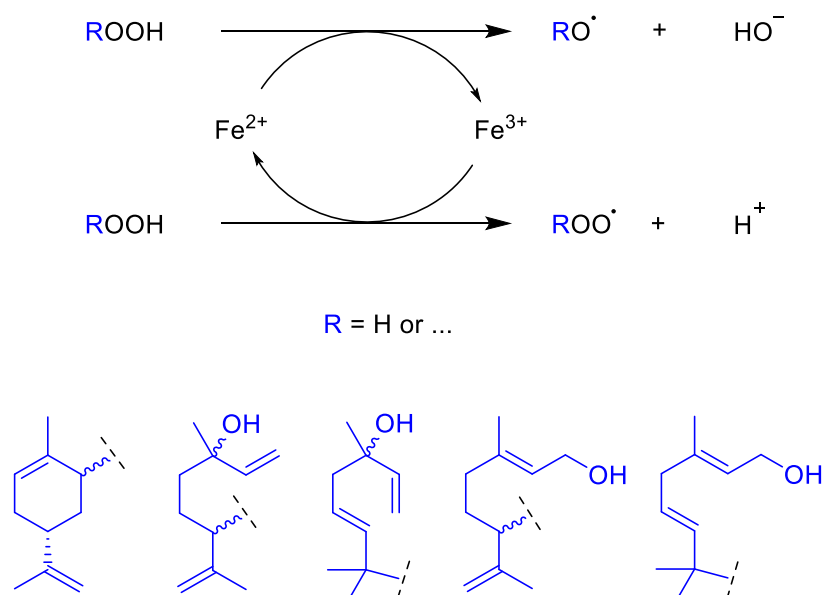
PLs are well-known targets of oxidation, as esters of PUFAs. PLs can undergo oxidation through enzymatic or non-enzymatic/metal-catalysed mediated processes [175, 176]. The resulting PL oxidation products (oxPLs) encompass a diverse array of species, including long-chain oxidation products, characterised by the insertion of multiple oxygen atoms into the unsaturated fatty acyl chain(s) [177]. These products include hydroperoxy, hydroxy, keto, and epoxy derivatives. However, long-chain oxidation products can further degrade via β -cleavage of C-C bonds, which in turn generate a variety of short-chain products, such as free and esterified aldehydes, keto-aldehydes, hydroxy-aldehydes, carboxylic acids, keto-carboxylic acids, and hydroxy-carboxylic acids [178]. Due to the complexity of identifying radical-induced oxPLs in vivo, most current knowledge on oxPL identification and their biological effects comes from studies using induced oxidation of liposomes via biomimetic systems like the Fenton reaction [179-182], 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) [183, 184], photosensitization [185, 186], or by enzymatic oxidation mediated by lipoxygenases (LOX) [187, 188]. The diversity of structurally different compounds is commonly analysed using LC-MS and MS/MS [189-191].

These structurally different oxidation products may have a plethora of biological effects, which contribute towards several physiological and pathophysiological processes [192]. OxPLs are known to possess pro-inflammatory effects. For example, they are active components in minimally modified low-density lipoprotein (MM-LDL) and are capable of inducing chemokine expression and monocyte adhesion to endothelial cells, contributing significantly to the pathogenesis of atherosclerosis [193]. Also, oxPLs have been shown to act as high-affinity ligands for macrophage scavenger receptor CD36, causing enhanced platelet activity, a contributing factor to dyslipidaemia [194]. Enzymatically oxidised PLs (eoxPLs) are known to facilitate enhanced coagulation factor activities on membrane surfaces and have been shown to restore homeostasis in human and murine conditions of pathological bleeding [195]. Paradoxically, some oxPLs possess anti-inflammatory properties; for example, oxPLs have been shown to be endogenous inhibitors of Toll-like receptor 4 (TLR4) produced in response to oxidative stress [196]. The role of oxPLs in allergy is largely unexplored to date, but it has been suggested that

oxidised PCs (oxPCs) are mechanistic effectors of oxidised stress in asthma and may contribute towards asthma pathology by promoting airway narrowing and inducing a pro-inflammatory phenotype and contraction of airway smooth muscle [197]. While the role of oxPLs in ACD has not yet been established, there is a strong precedent for environmental toxins causing skin lipid damage [198, 199]. Contact allergen-induced inflammation plays a critical role in both the sensitisation and elicitation phases of ACD [47, 200]. Therefore, it is conceivable that oxPLs could be generated through hydroperoxide allergens and their radical intermediates, thus serving as an inflammatory stimulus necessary for skin sensitisation.

Hydrogen peroxide (H_2O_2) in the presence of metal cations is able to induce oxidation of biomolecules such as lipids due to its propensity to form free radicals through the cleavage of its labile O-O bond, catalysed in the presence of transition metals (Fenton reaction). Therefore, terpene hydroperoxide contact allergens can potentially induce the radical oxidation of biomolecules, such as membrane PLs, in a way that mirrors Fenton chemistry (Fig. 4.1).

A.



B.

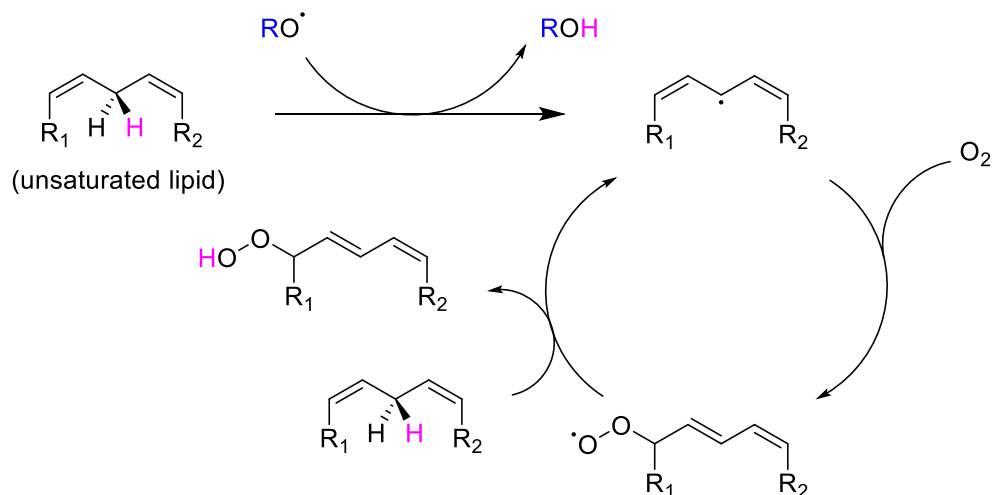


Figure 4.1. Schematic overview of the Fenton reaction and its role in lipid peroxidation.

(A) Hydrogen peroxide in the presence of ferrous salts can generate alkoxy and peroxy radicals. **(B)** These radicals can initiate lipid peroxidation chain reactions by abstracting a labile hydrogen atom from a lipid, leading to oxygen insertion and formation of lipid peroxy radicals. The resulting peroxy radical can then abstract another hydrogen atom from a neighbouring lipid, terminating itself as a hydroperoxide while propagating a new lipid radical.

Thus, the goal of this chapter was to use LC-MS and MS/MS to investigate if terpene hydroperoxide contact allergens (Lim-2-OOH [5], Lin-6/7-OOH [9 and 10], and Ger-6/7-OOH [24 and 18]) could promote oxidation of PLs, using 1-palmitoyl-2-(linoleoyl)-sn-glycero-3-PC (PLPC) liposomes as a model of cell membranes (Fig. 4.2), and compare with the well-known biomimetic system of lipid oxidation that uses H₂O₂ (positive control) in the presence of Fe²⁺ (Fenton reaction). Additionally, using a human epidermal keratinocyte cell line (HaCaT), the C11-BODIPY^{581/591} ratio-fluorescence assay was used to compare the oxidising potential of terpene hydroperoxides to that of H₂O₂. The C11-BODIPY^{581/591} ratio-fluorescence probe is a lipophilic indicator, whose oxidation by peroxy radicals can be analytically monitored [201]. Other probes used to measure oxidation in cellular membranes only contain fluorophores that can be measured in their non-oxidised forms [202], leading to inaccurate quantification due to the variations in cellular uptake of the probe [203]. The C11-BODIPY^{581/591} probe uses ratio-fluorescence and is a popular choice for ratio-imaging of oxidation processes in living cells [204-206].

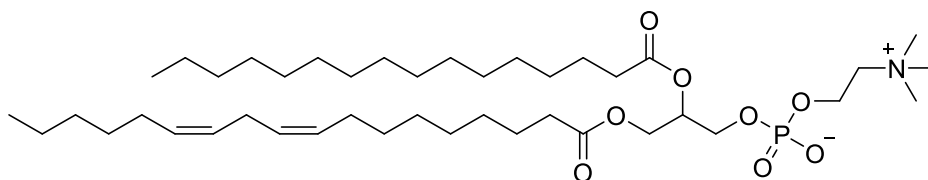


Figure 4.2. Structure of 1-palmitoyl-2-linoeloyl-phosphatidylcholine 16:0/18:2 (PLPC).

4.2 Materials and Methods

4.2.1 Materials

DMEM and FBS were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Microbiology grade DMSO and trypsin were purchased from Sigma Aldrich (St. Louis, MO, USA). Organic solvents (DCM, MeOH, ACN, and IPA) were purchased from Fisher Scientific (Leicestershire, UK), were HPLC grade and used without further purification. Milli-Q water was used, filtered through a 0.22 µm filter in a Milli-Q Millipore system (MilliQ plus 185). Skin sensitising compounds, Lim-2-OOH ([5*R*]-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide), a mixture of linalool hydroperoxides (6-hydroperoxy-3,7- dimethylocta-1,7-diene-3-ol [Lin- 6- OOH] and 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol [Lin-7-OOH]) in a 5:4 ratio [Lin-6/7-OOH]), and a mixture of geraniol hydroperoxides (6-hydroperoxy-3,7- dimethylocta-2,7-dien- 1-ol [Ger-6-OOH] and 7-hydroperoxy-3,7- dimethylocta-2,5-dien-1-ol [Ger- 7- OOH]) in a ratio of 1:1, were synthesised as previously described (see section 2.2). Lim-2-OOH was obtained as a mixture of two diastereomers in a ratio of approx. 8:1 as determined by integration of the proton NMR signals. Lim-1-OOH was not evaluated in this study. The lipids 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) and 1,2-dimyristoyl-sn-glycero-3- phosphocholine (DMPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The C11- BODIPY^{581/591} (Lipid Peroxidation Sensor) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium bicarbonate, iron (II) chloride tetrahydrate, and hydrogen peroxide (30% w/w) were purchased from Merck (Darmstadt, Germany).

4.2.2 Preparation of PLPC Liposomes and Oxidation by Fenton Reaction

PLPC (125 µg, 0.165 µmol) and internal standard DMPC (42 µg, 0.062 µmol) were added to methanol- washed Eppendorf tubes from stock solutions (1 mg/mL) and then dried under a nitrogen stream. DMPC was used as an internal standard since it has saturated

fatty acids (PC 14:0/14:0) which are not prone to oxidation. Terpene hydroperoxides (Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH) were added to Eppendorfs (25 μ L, 500 mM solution in dichloromethane) containing the lipids and dried under a nitrogen stream. Ammonium bicarbonate buffer (5 mM, pH 7.4) was then added to the eppendorfs containing the lipids and hydroperoxides (223 μ L). For the positive control, H₂O₂ was added to a separate eppendorf (25 μ L, 500 mM) containing the lipids and buffer. For the negative control, only buffer was added to a separate Eppendorf (248 μ L) containing the lipids. FeCl₂ solution (2 μ L, 5 mM) was then added to the Eppendorfs to make up a final reaction volume of 250 μ L, where the final concentrations of lipid, hydroperoxide (ROOH), and Fe(II) were 0.66 mM, 50 mM (0.84%–0.93% w/v of terpene hydroperoxides), and 0.04 mM, respectively. The final molar ratio of lipid/hydroperoxide/Fe(II) was 1: 76: 0.06, respectively. Liposomes were formed by vortexing and sonication. All reaction mixtures were then incubated at 37°C for 24 h under stirring. To have a final 5 mM concentration of hydroperoxides (0.084%–0.093% w/v of terpene hydroperoxides), the same procedure was done using 50 mM stock solution of each hydroperoxide and 0.5 mM stock solution of FeCl₂ solution. The phospholipid oxidation products were extracted using the Folch method with dichloromethane (666 μ L)/ methanol (333 μ L) (2:1, v/v) [207]. The extent of oxidation was monitored using C18 LC- MS and MS/MS for five independent experiments (n = 5).

4.2.3 C18 LC-MS and MS/MS Analysis

The total lipid extracts were analysed by reverse-phase liquid chromatography on a Dionex Ultimate 3000 (Thermo Fisher Scientific, Bremen, Germany) using an Ascentis Express C18 column (Sigma-Aldrich, 2.1 $\times$ 150 mm, 2.7 μ m) coupled to the Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher, Scientific, Bremen, Germany), as previously done [135, 136]. Mobile phase A was water/acetonitrile (40:60) with 10 mM ammonium formate and 0.1% formic acid. Mobile phase B was isopropanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid. The following gradient was applied: 32% B (0 min), 45% B (1.5 min), 52% B (4 min), 58% B (5 min), 66% B (8 min), 70% B (11 min), 85% B (14 min), 97% B (18 min), 97% B (25 min), 32% B (25.01) min and 32% B (33 min). A volume of 10 μ L of each sample, containing 10 μ g of lipid extract (in 10 μ L of dichloromethane), and 90 μ L of a solvent

system composed of isopropanol/methanol (50:50), was injected into the column at 50°C and at a flow rate of 260 $\mu\text{L min}^{-1}$. The mass spectrometer operated in simultaneous positive mode (ESI 3.0 kV) and negative mode (ESI -2.7 kV). The capillary temperature was 320°C and the sheath gas flow was 35 arbitrary units. Data acquisition was performed in full scan mode with a resolution of 70,000 and an automatic gain control (AGC) target of 3×10^6 , in an m/z range of 200-1600, with 2 microscans, and a maximum injection time (IT) of 100 ms. Tandem mass spectra (MS/MS) were obtained with a resolution of 17,500, AGC target of 1×10^5 , 1 microscan, and a maximum IT of 100 ms. The cycles consisted of a full-scan mass spectrum and 10 data-dependent MS/MS scans, which were repeated continuously throughout the experiments with a dynamic exclusion of 30 s and an intensity threshold of 8×10^4 . The normalised collision energy (NCE) ranged between 20, 24 and 28 eV in negative mode and 25 and 30 eV in positive mode. Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Bremen, Germany).

4.2.4 Data Processing

Identification of PLPC oxidation products (mass accuracy and MS/MS interpretation) was done based on previous works that have identified PLPC oxidation products formed by the Fenton reaction [181, 182, 189, 207, 208]. The LC- MS data was processed using Lipostar software (Molecular Discovery Ltd., version 2.1.5) [137]. This software was used for raw data import, peak detection, and relative quantification. Automatic peak picking was carried out with the semi-parametric differential abundance analysis (SDA) smoothing level set to low and a minimum signal-to-noise ratio of 3 and a signal filtering threshold of 10,000 in the positive mode and 10,000 for the negative mode. Automatic isotope clustering settings were set to 0 ppm with a retention time tolerance of 0.2 min. The MS/MS filter was applied to retain only features with MS/MS spectra for identification. Lipid identification was based on the following parameters: a 5 ppm precursor ion mass tolerance and a 20 ppm product ion mass tolerance. Normalisation of the data was performed by dividing the peak area of each lipid species by the area of the internal standard (DMPC).

4.2.5 Determination of Cell Viability by the alamarBlue Assay

HaCaT cells were treated with compounds for 3 h in a dose-response experiment and analysed for their viability through the reduction of the weakly fluorescent probe (resazurin) to the highly fluorescent derivative (resorufin) [132]. Briefly, HaCaT cells were seeded at a density of 0.2×10^5 cells/well (96-well plate) and incubated overnight at 37°C in 200 µL of media. The next day, stock solutions of compounds were freshly prepared in microbiology grade DMSO, old media was removed, and different working concentrations of Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH ([10, 50, 100, 250 and 500 µM] in 200 µL of media) were added. DMSO (0.5% v/v) was used as a vehicle control. After 3 h, the treatments were removed, and alamarBlue (10% v/v in DMEM, 200 µL) was added to each well and incubated for a further 3 h. Fluorescence intensities of resorufin were read using a FLUOstar OPTIMA microplate reader (BMG labtech, Germany) with an excitation wavelength of 544 nm and emission wavelength of 590 nm. Percentage viability was calculated relative to the vehicle control. Based on cell viability results, the same concentration ranges were used for LPO studies. Experiments were performed in technical triplicates in 3 independent experiments (n = 3).

4.2.6 C11- BODIPY^{581/591} Ratio-Fluorescence Assay (Microplate Reader)

HaCaT cells (0.2×10^5 cells/well) were cultured in 96 well plates and incubated overnight. The cells were washed twice with HBSS. Cells were stained with 100 µL of C11-BODIPY^{581/591} probe (10 µM made up in HBSS) and incubated for 40 min at 37°C. The probe was removed, and the treatments of H₂O₂ and terpene hydroperoxides (10, 50, 100, 250, and 500 µM) were added in triplicate. The plate was covered in aluminium foil and incubated for 3 h at 37°C. The treatments were then removed, and the wells were filled with 200 µL of HBSS. Fluorescence intensities of the oxidised probe (λ_{Ex} 485 nm, λ_{Em} 520 nm) and unoxidized probe (λ_{Ex} 570 nm, λ_{Em} 610 nm) were then read using a BioTek SYNERGY H1 fluorescence microplate reader with bottom optics. The final concentration of the vehicle (DMSO) did not exceed 0.5% (v/v). Control experiments consisted of cells only and a vehicle [DMSO (0.5%)]. Experiments were performed in technical triplicates in 4 independent experiments (n = 4).

4.2.7 C11- BODIPY^{581/591} Ratio-Fluorescence Assay (Confocal Microscopy)

HaCaT cells (0.1×10^5 cells/well) were cultured in an 8 well plate and incubated overnight. The cells were washed twice with HBSS. Cells were stained with 100 μ L of C11-BODIPY^{581/591} probe (10 μ M made up in HBSS) and incubated for 40 min at 37°C. The probe was removed, and the treatments of H₂O₂ and terpene hydroperoxides (100 μ M) were added in triplicate. The plate was covered in aluminium foil and incubated for 3 h at 37°C. The treatments were then removed, and the wells were filled with 200 μ L of HBSS. Confocal microscopy images were captured at ambient temperature using a Leica SP8 Gated STED spectrofluorometer (Mannheim, Germany). Fluorescence intensities of C11-BODIPY^{581/591} were measured by simultaneous acquisition of the green (485/520 nm) and red signals (570/610 nm). Images are representative of 3 independent biological replicates.

4.2.8 Statistics

Data was analysed using one-way ANOVA. Post hoc Tukey's multiple comparisons test was used to determine values between individual data groups. Analyses were performed using GraphPad Prism (version 8.4.2) for Windows, GraphPad Software (Boston, Massachusetts USA).

4.3 Results

4.3.1 PL Oxidation in Biomimetic Fenton Reaction Conditions with H₂O₂ or Terpene Hydroperoxides Monitored by LC-MS/MS

PLPC liposomes were subjected to oxidation using terpene hydroperoxides, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH, in biomimetic Fenton reaction conditions, as well as with H₂O₂/ Fe²⁺ (Fenton reaction, used as a positive control). The resulting oxidation products of PLPC were analysed by LC-MS and MS/MS (Appendix C, Fig. C1-17).

The analysis allowed the identification of 14 PLPC oxidation products that were common across all experimental conditions (Table 4.1). These included long-chain products with 1-4 oxygen atoms inserted into the *sn*2 chain, as well as short-chain products such as aldehydes, dicarboxylic acids, and lyso-PC compounds. The formation of these products aligns with findings previously reported in the literature for PLPC oxidation under Fenton conditions [177, 207, 208]. LPO products were absent in the control (Ctrl; ammonium

bicarbonate buffer only). The reconstructed ion chromatograms (RICs) of the major oxidation products identified in Table 4.1 show clear distinctions in retention times between long- and short-chain products, with long-chain products eluting between 4.3 and 7.8 min (Appendix C, Fig. C16.A-F) and short-chain products predominantly eluting between 2.0 and 4.0 min (Appendix C, Fig. C17A-I). With respect to long-chain oxidation products, it is evident that as the number of oxygens inserted into the *sn*2 acyl chain is increased, there is an associated increase in the polarity of the products, characterised by shorter retention times, along with an increase in the number of isomers formed, as demonstrated by the increased number of chromatographic peaks (Appendix C, Fig. C16.D-F). This is also in agreement with previous reports for oxidised PLPC (oxPLPC) [208].

After 24 h of exposure to 50 mM concentrations of H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH, under Fenton reaction conditions, the relative abundances of unmodified PLPC, calculated as a percentage of the total sum of PLPC and its oxidised products (after normalisation to DMPC, used as an internal standard), were approximately 94%, 94%, 93%, and 80%, respectively (data not shown). This highlights Lim-2-OOH as being the most potent oxidant among the tested compounds. The statistical significance of PLPC consumption was demonstrated by considering the normalised area of unmodified PLPC relative to DMPC (PLPC/DMPC ratio) and comparing it to the negative control (Ctrl; ammonium bicarbonate buffer only) (Fig. 4.3). DMPC was used as the internal standard since it is esterified with saturated fatty acids (PC 14:0/14:0) and is not prone to oxidation. One-way ANOVA confirmed significant reductions in PLPC levels due to oxidative degradation under treatments with H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH at 50 mM compared to control conditions, highlighting the impact of these oxidants. The most pronounced PLPC degradation occurred after a 24-h incubation with Lim-2-OOH at 50 mM.

Table 4.1. PLPC oxidation products identified by C18-LC-MS and MS/MS in different radical induced oxidation conditions using H₂O₂ and terpene hydroperoxides (Lin-6/7-OOH, Ger-6/7-OOH, or Lim-2-OOH) in biomimetic Fenton reaction systems.

Classification type	Compound	Retention time (mins)	Molecular formula	Positive Mode			Negative Mode		
				Observed mass [M+H] ⁺	Theoretical mass [M+H] ⁺	Actual error (ppm)	Observed mass [M+CHOO] ⁻	Theoretical mass [M+CHOO] ⁻	Actual error (ppm)
Unoxidized PC	PLPC	10.1	C42H80NO8P	758.5684	758.5670	1.85	802.5586	802.5598	-1.50
Long chain products (polyoxygenated)	PLPC+14	6.5	C42H78NO9P	772.5486	772.5492	-0.78	816.5381	816.5391	-1.22
	PLPC+16	7.8	C42H80NO9P	774.5646	774.5649	-0.39	818.5542	818.5547	-0.61
	PLPC+32	6.6	C42H80NO10P	790.559	790.5598	-1.01	834.5485	834.5496	-1.32
	PLPC+48	5.3	C42H80NO11P	806.553	806.5547	-2.11	850.5434	850.5446	-1.41
	PLPC+64	3.1 – 5.8	C42H80NO12P	822.5478	822.5496	-2.19	866.5384	866.5394	-1.15
Short chain products (aldehydes)	1-palmitoyl-2-(8-oxo-octanoic acid) PC	3.5	C32H62NO9P	636.4234	636.4240	-0.94	680.4138	680.4139	-0.15
	1-palmitoyl-2-(9-oxo-nonanoic acid) PC	3.7	C33H64NO9P	650.4392	650.4396	-0.61	694.4293	694.4295	-0.29
	1-palmitoyl-2-(11-oxo-9-undecenoic acid) PC	4.0	C35H66NO9P	676.4557	676.4553	0.59	720.445	720.4452	-0.28
	1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC	3.5	C35H66NO10P	692.4484	692.4503	-2.74	736.4401	736.4401	0.00

	1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC	3.3	C36H66NO10P	704.4503	704.4503	0.00	748.4386	748.4401	-2.00
	1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC	3.3	C36H68NO10P	706.4643	706.4659	-2.26	750.4551	750.4557	-0.80
Short chain products (dicarboxylic acids)	1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC	3.2	C36H68NO11P	722.4589	722.4608	-2.63	766.4493	766.4507	-1.83
lyso-PCs	1-palmitoyl-lyso PC	3.0	C24H50NO7P	496.3395	496.3403	-1.61	540.3301	540.3301	0.00
	2-linoleoyl-lyso PC	2.5	C26H50NO7P	520.3397	520.3403	-1.15	564.3300	564.3301	-0.18

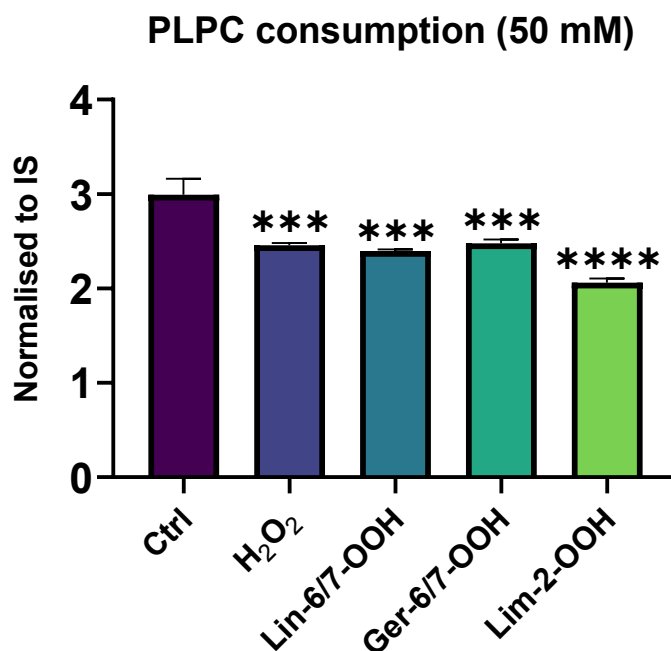


Figure 4.3. PLPC consumption induced by hydroperoxides (50 mM) determined using LC-MS. Relative abundances of unmodified PLPC after a 24-h incubation at 37°C in a biomimetic Fenton reaction system with H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH (all 50 mM). The relative abundances were calculated by normalising the PLPC values to the internal standard (IS; DMPC) and comparing to the negative control (Ctrl; ammonium bicarbonate buffer only). Significant differences relative to each hydroperoxide are indicated as follows: *** $p \leq 0.001$, **** $p \leq 0.0001$. Statistical significance was determined using one-way ANOVA, with normalised data derived from five independent experiments ($n = 5$).

Across all oxidants, the most abundant oxidation product formed was PLPC + 32 Da (addition of 2 oxygens), followed by PLPC + 16 Da (addition of 1 oxygen) and PLPC + 14 Da (addition of 1 oxygen and loss of 2 hydrogens) (Fig. 4.4). Polyoxygenated long-chain products, such as PLPC + 48 Da (addition of 3 oxygens) and PLPC + 64 Da (addition of 4 oxygens), were less abundant. Short-chain oxidation products were the least abundant, each contributing less than 6.5%, regardless of the oxidant used (see insets of Fig. 4.4).

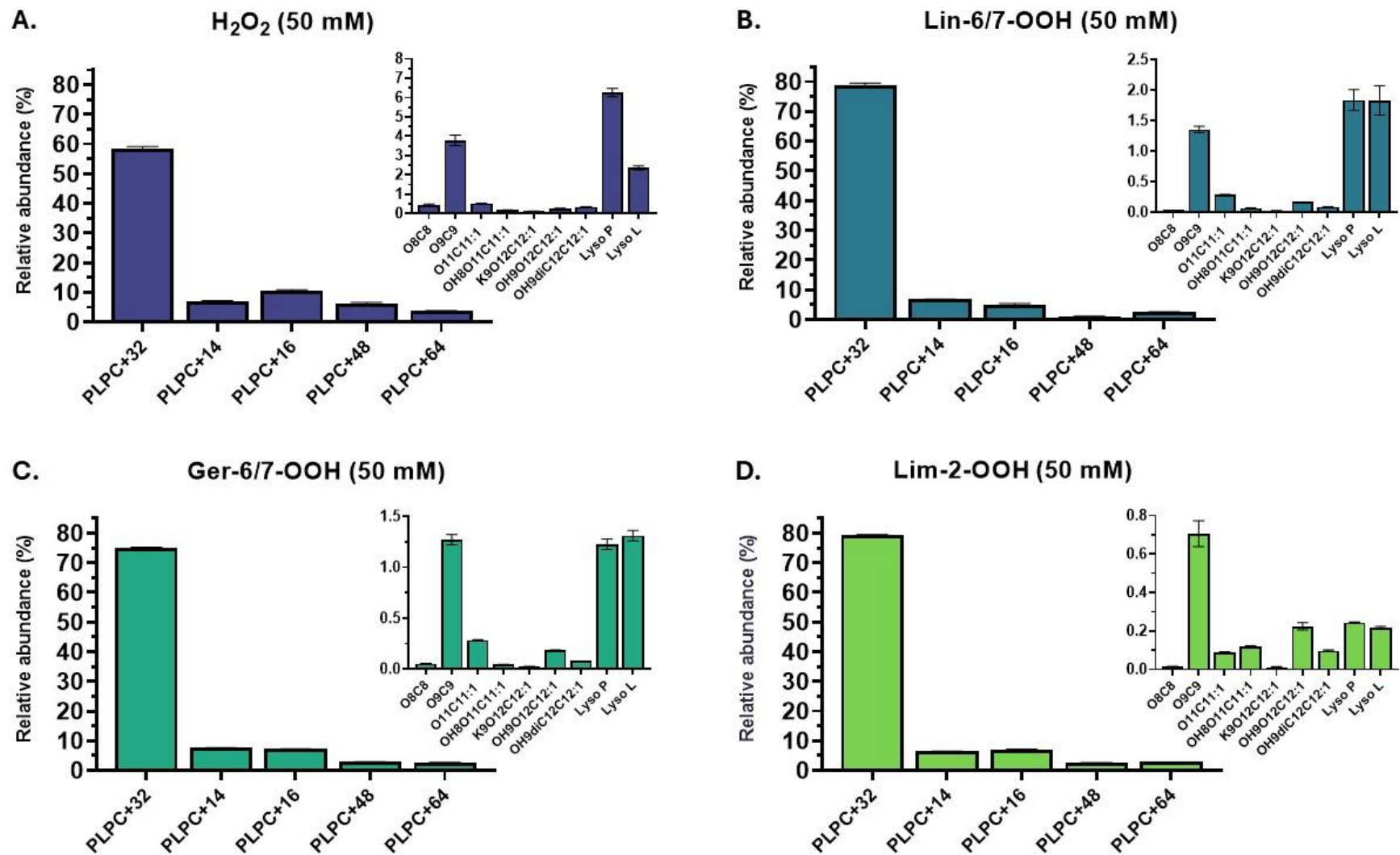
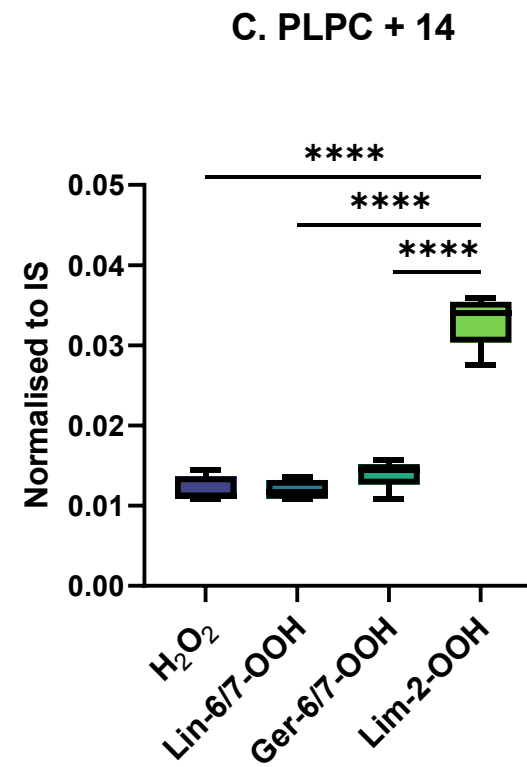
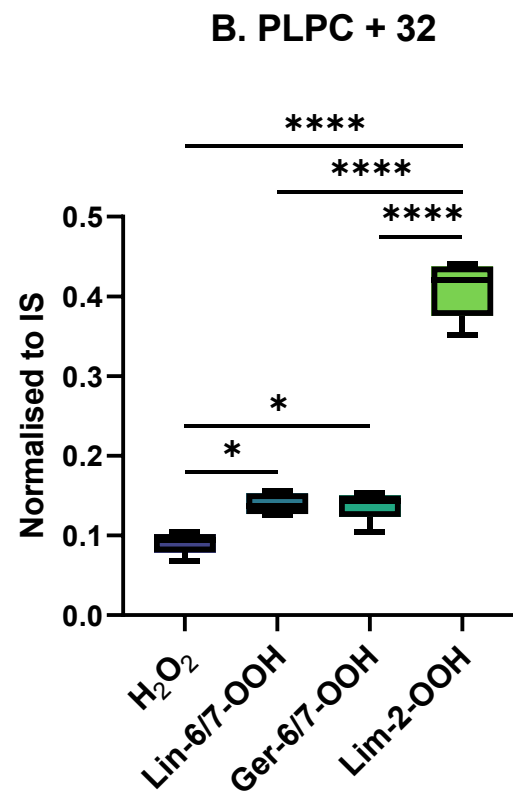
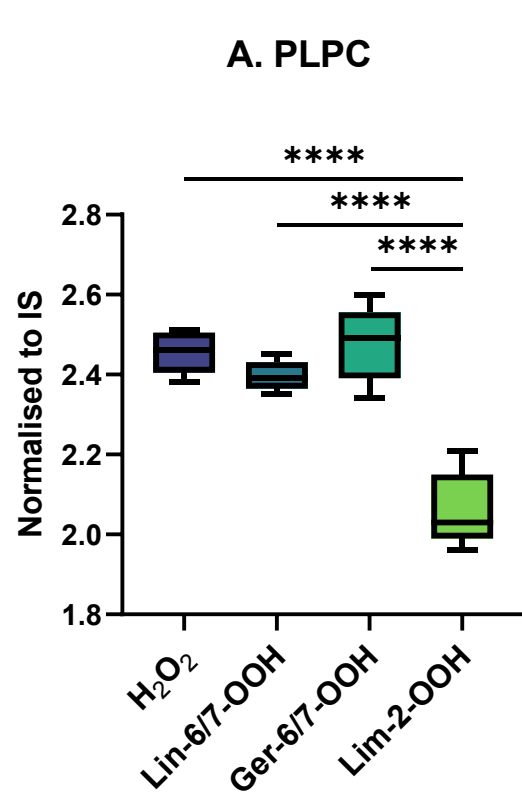
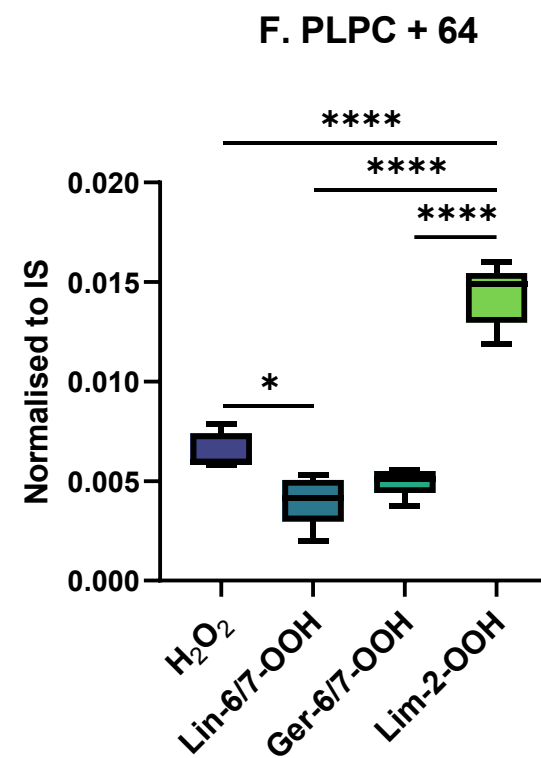
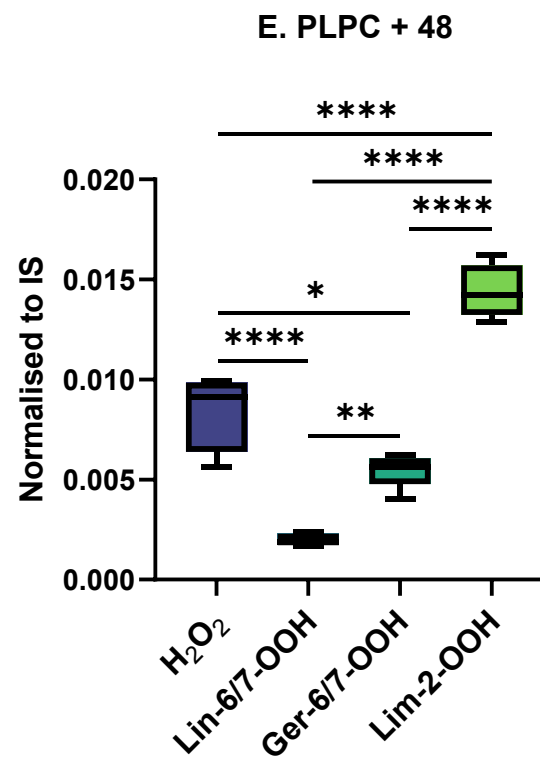
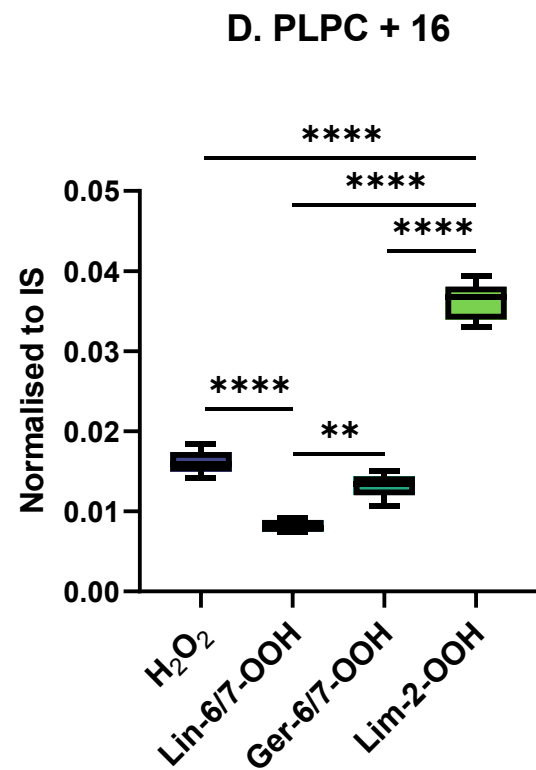


Figure 4.4. OxPLPC product profiles induced by hydroperoxides (50 mM) determined using LC-MS.

Relative abundances (%) of long- and short-chain oxidation products (oxPLPC) after a 24-h incubation at 37°C in a biomimetic Fenton reaction system with (A) H₂O₂, (B) Lin-6/7-OOH, (C) Ger-6/7-OOH, and (D) Lim-2-OOH (all 50 mM). The relative abundances were calculated by dividing the peak area of each oxPLPC product by the area sum for all oxPLPC products. Values represent the mean ± SD from five independent experiments (n = 5). Short-chain product abbreviations: O8C8, 1-palmitoyl-2-(8-oxo-octanoic acid) PC; O9C9, 1-palmitoyl-2-(9-oxo-nonanoic acid) PC; O11C11:1, 1-palmitoyl-2-(11-oxo-9-undecenoic acid) PC; OH8O11C11:1, 1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC; K9O12C12:1, 1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC; OH9O12C12:1, 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC; OH9diC12C12:1, 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC; Lyso P, 1-palmitoyl-lyso PC; and Lyso L, 2-linoleoyl-lyso PC.

The variation in the abundance of oxPLPC products induced by different terpene hydroperoxides at 50 mM concentrations was assessed using univariate analysis. One-way ANOVA, followed by Tukey's multiple comparisons test, was performed to identify significant differences between the hydroperoxides tested. The results indicated significantly higher PLPC oxidation, as indicated by the decrease in the unmodified PLPC ion, induced by Lim-2-OOH compared to H₂O₂, Lin-6/7-OOH, and Ger-6/7-OOH (Fig. 4.5A). A significant concurrent increase was observed in the formation of the most abundant oxPLPC product (PLPC + 32 Da) when exposed to Lim-2-OOH compared to the other hydroperoxides (Fig. 4.5B). Additionally, Lin-6/7-OOH and Ger-6/7-OOH also showed significant increases in the formation of PLPC + 32 Da compared to H₂O₂. There were also significantly higher relative abundances in the formation for PLPC + 14 Da and PLPC + 16 Da when treated with Lim-2-OOH compared to the other hydroperoxides (Fig. 4.5C,D), as well as higher abundances of PLPC + 16 Da seen for H₂O₂ and Ger-6/7-OOH relative to Lin-6/7-OOH. Significantly higher levels of PLPC + 48 Da and PLPC + 64 Da were also measured when tested with Lim-2-OOH compared to the other hydroperoxide compounds (Fig. 4.5E,F). For the short chain oxidation products, 1-palmitoyl-2-(9-oxo-nonanoic acid) PC, a different trend was noted as there were higher relative abundances after incubations with H₂O₂ compared to Lin-6/7-OOH, Ger-6/7-OOH, but no significant differences between H₂O₂ and Lim-2-OOH (Fig. 4.5G). However, there were still significant increases in the formation of this short-chain product following Lim-2-OOH treatments relative to Lin-6/7-OOH and Ger-6/7-OOH. 1-Palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC was most abundant following exposure to Lim-2-OOH compared to the other hydroperoxides tested (Fig. 4.5H).





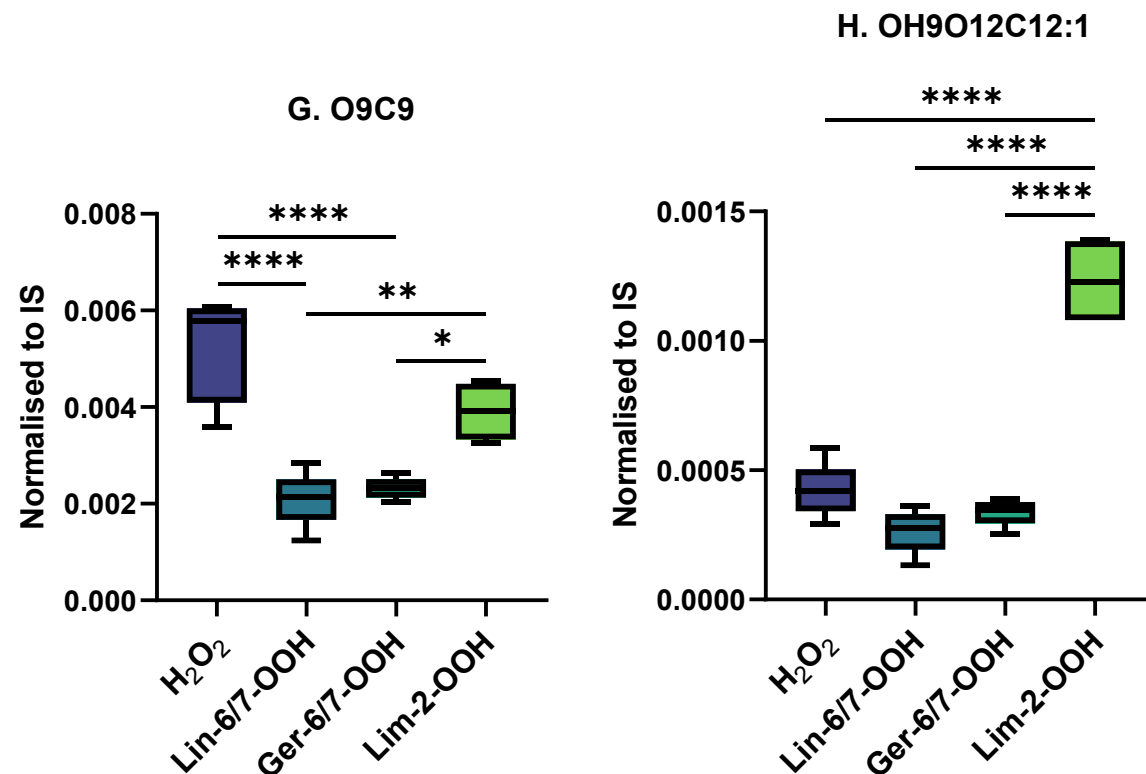


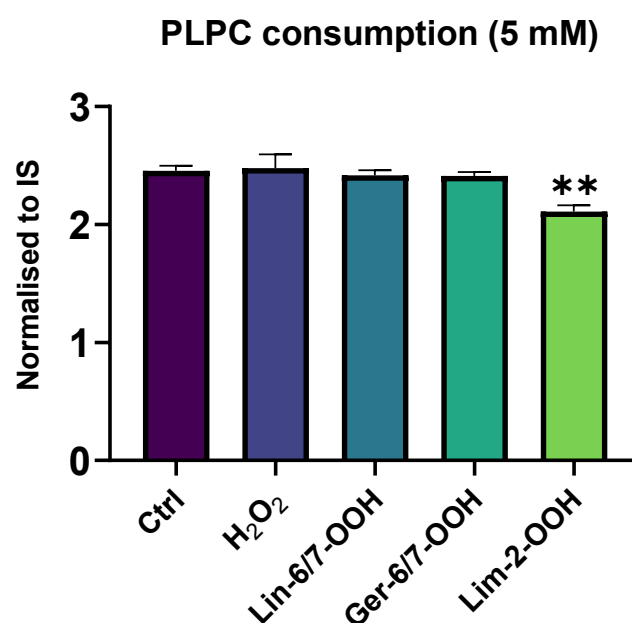
Figure 4.5. Consumption of PLPC and concomitant formation of oxPLPC products when exposed to different hydroperoxides (50 mM) determined using LC-MS.

Normalised areas of **(A)** unoxidized PLPC (m/z 758.57), **(B)** PLPC + 32 Da (m/z 790.56), **(C)** PLPC + 14 Da (m/z 772.55), **(D)** PLPC + 16 Da (m/z 774.56), **(E)** PLPC + 48 Da (m/z 806.55), **(F)** PLPC + 64 Da (m/z 822.55), **(G)** O9C9 (1-palmitoyl-2-(9-oxo-nonanoic acid) PC) (m/z 650.44), and **(H)** OH9O12C12:1 (1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC) (m/z 706.46), as their $[M+H]^+$ ions, after 24-h incubation periods at 37°C with hydroperoxides H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH at 50 mM concentrations. Short-chain product abbreviations: O9C9, 1-palmitoyl-2-(9-oxo-nonanoic acid) PC; and OH9O12C12:1, 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic

acid) PC. Significant differences relative to each hydroperoxide are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$. Statistical significance was determined using one- way ANOVA followed by Tukey's multiple comparison tests, with normalised data derived from five independent experiments ($n = 5$).

The extent of oxidation was also evaluated at lower concentrations of hydroperoxides (5 mM). At the lower concentration of 5 mM, there was only significant oxidation seen for Lim-2-OOH (Fig. 4.6A), once again supporting the previous findings that Lim-2-OOH is a potent oxidizer. This consumption of PLPC resulted in a concomitant formation of oxPLPC products that resembled the product profile at 50 mM (Fig. 4.6B), although the abundances of the oxPLPC products were expectedly lower than previously seen (Fig. 4.4D).

A.



B.

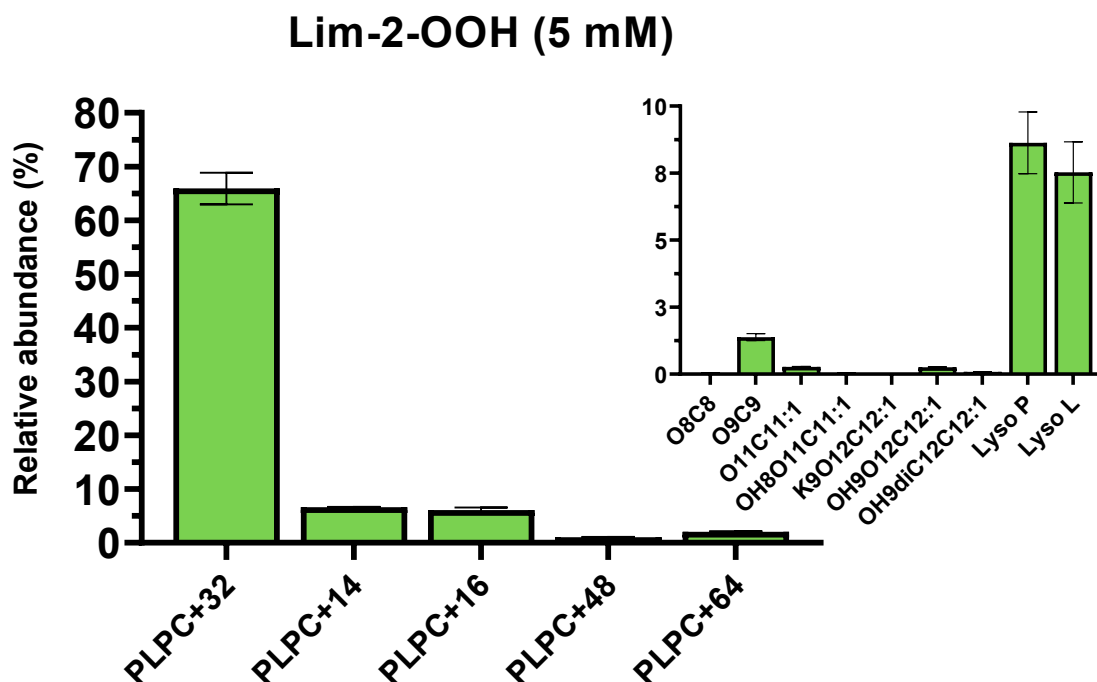


Figure 4.6. PLPC consumption and oxPLPC product profile induced by hydroperoxides (5 mM) determined using LC- MS.

(A) Relative abundances of unmodified PLPC after a 24-h incubation at 37°C in a biomimetic Fenton reaction system with H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH (all 5 mM). The relative abundances were calculated by normalising the PLPC values to the IS (DMPC) and comparing to the negative control (Ctrl; ammonium bicarbonate buffer only). Significant differences relative to each hydroperoxide are indicated as follows: ** $p \leq 0.01$. Statistical significance was determined using one-way ANOVA, with data derived from five independent experiments ($n = 5$). **(B)** Relative abundances (%) of long- and short-chain oxidation products after a 24-h incubation at 37°C in a biomimetic Fenton reaction system with Lim-2-OOH (5 mM). The relative abundances were calculated by dividing the peak area for each oxPLPC product by the area sum for all oxPLPC products. Values represent the mean $\pm$ SD from five independent experiments ($n = 5$). Short-chain product abbreviations: O8C8, 1-palmitoyl-2-(8-oxo-octanoic acid) PC; O9C9, 1-palmitoyl-2-(9-oxo- nonanoic acid) PC; O11C11:1, 1-palmitoyl-2-(11-oxo-9-undecenoic acid) PC; OH8O11C11:1, 1-palmitoyl-2- (8-hydroxy-11-oxo-9-undecenoic acid) PC; K9O12C12:1, 1-palmitoyl-2-(9-keto-12-oxo-10- dodecenoic acid) PC; OH9O12C12:1, 1-palmitoyl-2-(9-hydroxy-12-oxo-10- dodecenoic acid) PC; OH9diC12C12:1, 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC; Lyso P, 1-palmitoyl-lyso PC; and Lyso L, 2- linoleoyl-lyso PC.

4.3.2 Lipid Peroxidation In Vitro in HaCaT Cells

AlamarBlue cell viability assays were performed on HaCaT cells prior to C11-BODIPY^{581/591} ratio-fluorescence measurements. Contact allergens were tested at concentrations ranging from 10 to 500 μ M over 3 h (Fig. 4.7). A clear dose-dependent decrease in cell viability was observed relative to the vehicle control (DMSO, 0.5% v/v), even within this short incubation period. Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH produced comparable reductions across most concentrations, except at 500 μ M where Lim-2-OOH reduced viability to 41%. At lower concentrations, only mild cytotoxicity was observed, and this range was therefore selected for subsequent BODIPY^{581/591} assays. H₂O₂ was tested only at 100 μ M to support planned confocal microscopy experiments.

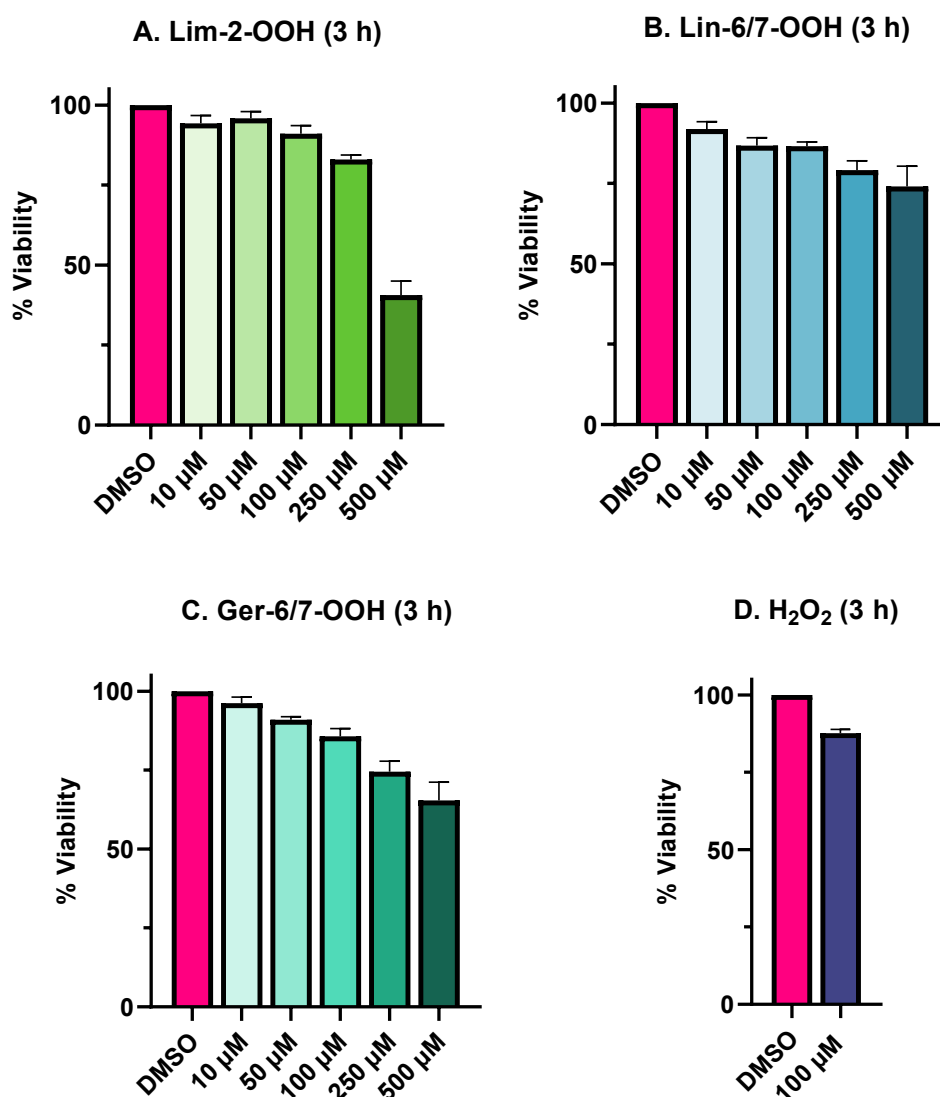
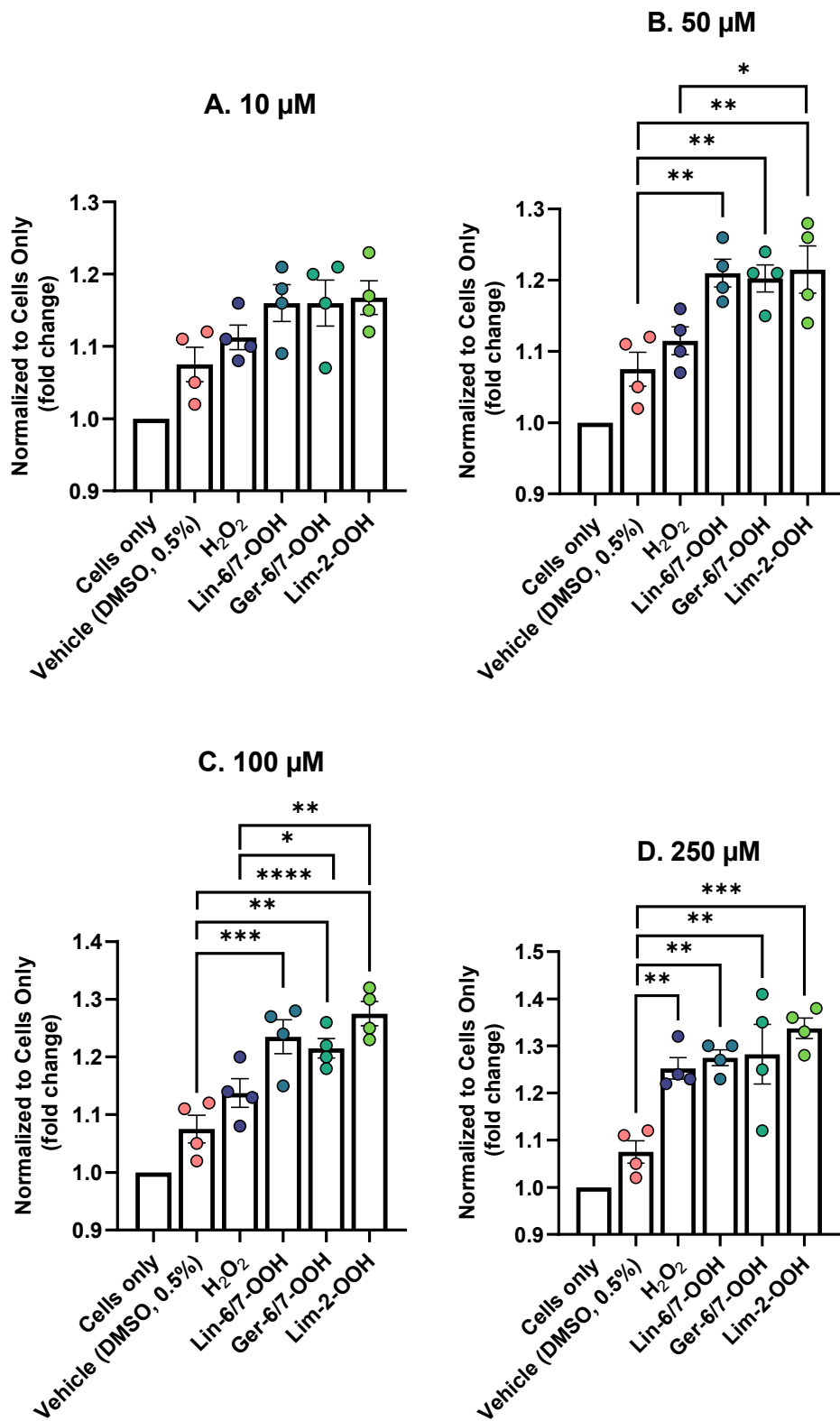


Figure 4.7. alamarBlue cell viability assays of HaCaT cells treated with contact allergens (3 h).

Cell viability assays were performed at a concentration range between 10 and 500 μM of (A) Lim-2-OOH, (B), Lin-6/7-OOH, and (C) Ger-6/7-OOH, over a 3 h period, and calculated relative to the vehicle control (DMSO, 0.5% v/v). (D) H_2O_2 was tested only at a 100 μM concentration over 3 h and also compared to the vehicle.

The ratio-fluorescence assay C11-BODIPY^{581/591} was performed on the HaCaT cell line to quantify relative LPO levels in vitro as the next step to establishing the relevance of LPO in keratinocytes. The lipidome of the HaCaT cell line was previously characterised by this group and was shown to contain high levels of PC lipids with unsaturated *sn2* acyl chains, which are known to be susceptible to radical-induced oxidation (previously shown in Table 3.2). Univariate analysis was performed using one-way ANOVA to examine the effects of the hydroperoxides (H_2O_2 , Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH) at a range of concentrations (10, 50, 100, 250, and 500 μM) on LPO, followed by post hoc analyses using Tukey's multiple comparison tests. After 3 h of incubation, the ratio of fluorescence showed that the vehicle (0.5% DMSO) had no significant impact on LPO relative to the cells only (without DMSO) (Fig. 4.8). The lower concentration range (10–100 μM) of terpene hydroperoxides (Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH) showed a dose-dependent increase in LPO when compared to the vehicle control (DMSO, 0.5%), with significant differences established at concentrations as low as 50 μM for Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH ($p \leq 0.01$). In the case of treatment with H_2O_2 , there was no dose-dependent increase at this concentration range, however, there were statistically significant increases in LPO seen at 250 ($p \leq 0.01$) and 500 μM ($p \leq 0.001$). Interestingly, of the hydroperoxides tested, Lim-2-OOH caused the highest significant increase in LPO at 100 μM ($p \leq 0.0001$), 250 μM ($p \leq 0.001$), and 500 μM ($p \leq 0.0001$), relative to the vehicle control, however, Tukey's multiple comparison tests showed only select cases of significant differences between the hydroperoxides tested, that was between H_2O_2 and Lim-2-OOH at 50 μM ($p \leq 0.05$), H_2O_2 and Lin-6/7-OOH at 100 μM ($p \leq 0.05$), and H_2O_2 and Lim-2-OOH at 100 μM ($p \leq 0.01$).



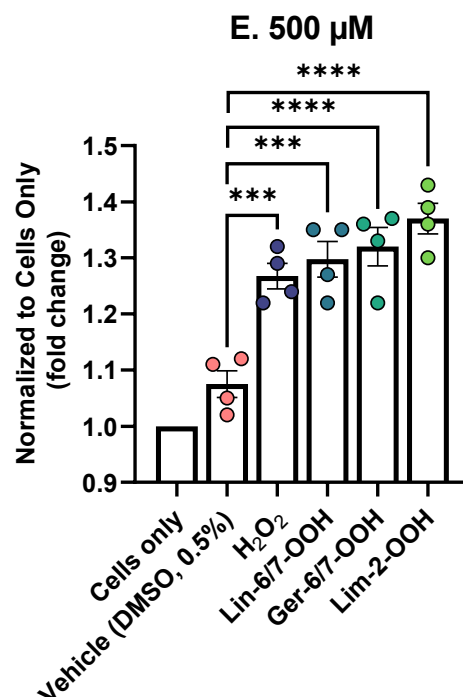


Figure 4.8. Relative LPO in HaCaT cells after hydroperoxide treatments determined using the C11-BODIPY^{581/591} ratio-fluorescence assay.

HaCaT cells were stained with C11-BODIPY^{581/591} and then incubated with hydroperoxides (H₂O₂, Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH) at concentrations of (A) 10, (B) 50, (C) 100, (D) 250, and (E) 500 μ M at 37°C for 3 h. C11-BODIPY_{UNOX}: λ_{EX} = 570 nm, λ_{EM} = 610 nm, and C11-BODIPY_{OX}: λ_{EX} = 485 nm, λ_{EM} = 520 nm. Significant differences are relative to the vehicle control and are indicated as follows: * p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001, **** p $\leq$ 0.0001. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison tests, with data derived from four independent experiments (n = 4).

Confocal microscopy was used to obtain additional spatial insights into the oxidative effects of hydroperoxide compounds (100 μ M) on cultured cells following a 3 h incubation period at 37°C. Low levels of green fluorescence intensity (oxidised probe) revealed minimal oxidation after 3 h in untreated cells (Fig. 4.9A - bottom panel) and vehicle-treated cells (0.5% DMSO) (Fig. 4.9B - bottom panel), consistent with the previously reported quantitative data. In contrast, a noticeable increase in green fluorescence was observed primarily at the cell periphery after treatment with H₂O₂ (Fig. 4.9C - bottom panel), indicating an increase in LPO. This increase in green fluorescence appeared even more pronounced in cells treated with the terpene hydroperoxides Lin-6/7-OOH (Figure 9D - bottom panel), Ger-6/7-OOH (Fig. 4.9E - bottom panel), and Lim-2-OOH (Fig. 4.9F - bottom panel), with some green fluorescence spreading throughout the cells, further supporting the earlier quantitative findings. In contrast, red

fluorescence intensities (unoxidized probe) showed no visually apparent concomitant decrease in hydroperoxide-treated cells.

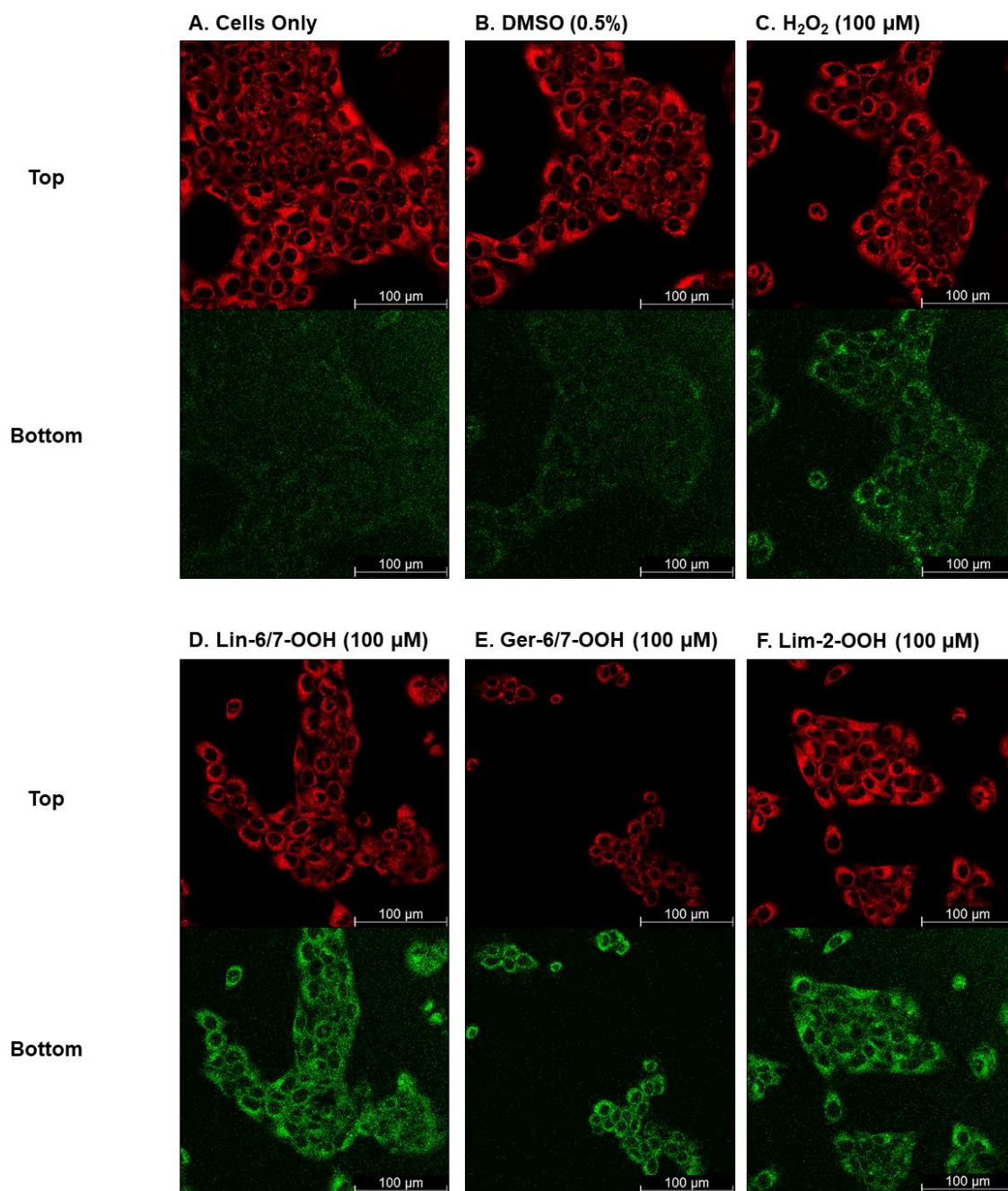


Figure 4.9. Confocal microscopy images of HaCaT cells loaded with C11-BODIPY^{581/591} and exposed to 100 μM concentrations of hydroperoxide compounds.

The top panels (red fluorescence) depict the probe in its unoxidized state, while the bottom panels (green fluorescence) show the oxidised probe (indicating LPO). Treatments were: (A) cell media alone, (B) vehicle control (0.5% DMSO), (C) H₂O₂, (D) Lin-6/7-OOH, (E) Ger-6/7-OOH, and (F) Lim-2-OOH. C11-BODIPY_{UNOX} (red): $\lambda_{EX} = 570$ nm, $\lambda_{EM} = 610$ nm, and C11-BODIPY_{OX} (green): $\lambda_{EX} = 485$ nm, $\lambda_{EM} = 520$ nm. Images are representative of three independent biological replicates.

4.4 Discussion

This study utilised LC-MS and MS/MS to demonstrate that terpene hydroperoxides can cause significant LPO in biomimetic systems of PLPC liposomes, and the C11-BODIPY^{581/591} ratio-fluorescence assay to monitor their contribution to LPO in HaCaT cells. Oxidative stress refers to an imbalance in the body between ROS and antioxidants that can lead to cell and tissue damage. Among the hydroperoxides tested, Lim-2-OOH exhibited the greatest oxidising potential, significantly depleting PLPC and generating oxPLPC at both 50 mM (0.84% w/v) and 5 mM (0.084% w/v). Interestingly, Lim-2-OOH is also the terpene hydroperoxide with the lowest EC3 value (0.83% w/v) in the LLNA (of those tested in this work). In contrast, Lin-6/7-OOH and Ger-6/7-OOH could only induce LPO at 50 mM (0.93% w/v), aligning with their higher EC3 values (1.6% w/v and 1.4% w/v, respectively). It could be expected that H₂O₂ (positive control) would induce the greatest degree of LPO, given that hydroxyl radicals are highly reactive and non-specific. However, the most extensive oxidation was associated with Lim-2-OOH, the most potent skin sensitizer evaluated. The oxPLPC profile was similar irrespective of the hydroperoxide. However, terpene hydroperoxides are well known to be specific skin sensitizers with little cross reactivity [209], suggesting that they are not simply unspecific oxidising agents. The role of LPO in skin sensitization requires further investigation, but it is possible that they contribute towards an oxidative imbalance in the skin, contributing to inflammation.

To initiate adaptive immune responses, a strong inflammatory signal is required [47]. Contact allergens have been found to generate ROS in skin cells, leading to a pro-inflammatory response via an indirect activation of NF- κ B, as ROS degrades hyaluronic acid (HA) in the extracellular matrix, which in turn activates TLR2 and TLR4, leading to increased expression of inactive pro-interleukin-1 β (pro-IL-1 β) and pro-IL-18 [210]. Contact allergen-induced ROS also indirectly activates the NLRP3 inflammasome by promoting ATP release, which binds to the purinergic receptor P2X7R, triggering inflammasome activation, which leads to caspase-1 activity and the subsequent production of activated IL-1 β and IL-18 [211, 212]. HA fragmentation is one example of a damage-associated molecular pattern (DAMP) generated by ROS that activates TLRs. However, the potential role of oxPLPC products as DAMPs in the context of ACD remains

unexplored, despite the recognition of oxPL products as pro-inflammatory mediators in other diseases [213, 214]. Thus, the generation of oxPLPC products by terpene hydroperoxides suggests a potential link between oxidation potential and a pro-inflammatory response in sensitisation events.

Although exploring the mechanism(s) underlying the upregulation of LPO among the hydroperoxides tested was beyond the scope of this chapter, it is likely related to the identity and quantity of the radicals formed. Structural differences among the terpene hydroperoxides may lead to the formation of distinct radical intermediates, with some radicals being more reactive than others [215]. Previous spin-trapping studies have characterised such phenomena [91, 216, 217], suggesting that the innate ability of each hydroperoxide to form oxygen- or carbon-centered radicals in the presence of ferrous ions may contribute to their reactivity and oxidising potential, which may explain the varying degrees of LPO observed across the hydroperoxides tested.

Another possible explanation for the higher LPO observed with Lim-2-OOH is its greater lipophilicity. While all hydroperoxides in this study are non-polar oils, Lin-6/7-OOH and Ger-6/7-OOH exhibit higher polarity relative to Lim-2-OOH, evidenced by the higher proportion of polar solvent required for their purification via flash chromatography [91]. This suggests that Lim-2-OOH's greater lipophilicity enhances its ability to integrate into lipid membranes, increasing its access to unsaturated fatty acyl chains, thus exhibiting stronger oxidative effects.

LC-MS identified the most abundant oxPLPC products as long-chain products containing direct insertions of oxygen atoms into the sn2 fatty acyl chains. Upon further oxidation, rearrangement of alkoxyl radicals with carbon-carbon cleavages resulted in the formation of smaller relative abundances of short-chain products (< 6.5% of total oxPLPC products). For lipid classes such as phospholipids, there is a lack of commercially available oxidised standards. For this reason, only relative abundances have been discussed. However, previous insights into oxidised PLPC products have been obtained from in vitro studies using PC (16:0/18:2) liposomes in biomimetic systems, allowing the determination of m/z values for both long- and short-chain precursor ions [208, 218]. The most prominent short-chain product across all conditions tested was 1-palmitoyl-2-(9-oxo-nonanoic acid) PC, suggesting that the favoured location for oxygen insertion was

at carbon 9 in the sn2 acyl chain, which subsequently fragmented to yield this short-chain product. Such oxPLs, containing carbonyl groups formed by cleavages, may react with proteins in the local environment, forming adducts which have the potential to be immunogenic [219].

Hapten-protein adduct formation is considered a fundamental step in skin sensitization, whereby low molecular weight compounds bind to proteins to cause their immunogenic effect [220]. Terpene hydroperoxides do not inherently possess electrophilic properties characteristic of many contact allergens. Consequently, it has been shown that these hydroperoxides form protein adducts via free radical mechanisms or undergo further oxidation to become electrophiles and then react to form protein adducts by conventional two-electron mechanisms [126, 221]. However, this study highlights an additional possibility: terpene hydroperoxides are capable of generating oxidative stress by acting as ROS. This oxidative stress leads to direct chemical modifications of PUFA-containing phospholipids (and theoretically any other lipid class containing PUFA acyl chains), resulting in the formation of lipid hydroperoxides. Upon further degradation, these lipid hydroperoxides yield short-chain electrophilic oxidation products which could induce electrophilic stress [222, 223]. Degradation of PL hydroperoxides can also generate small electrophilic aldehydes, such as 4-hydroxynonenol (4-HNE) and malondialdehyde (MDA), which are known to be toxic [224, 225]. While deciphering the immunogenicity of oxPLPC products was not the aim of this paper, the identification of oxidation products, particularly the formation of further oxidised electrophilic short-chain oxPLPC species, mediated by terpene hydroperoxides, raises possibilities regarding hapten-protein conjugation in ACD that merit further investigation.

The C11-BODIPY^{581/591} ratio fluorescence assay demonstrated that terpene hydroperoxides were able to induce dose-dependent LPO at concentrations ranging from 10 to 500 μ M in HaCaT cells, with statistically significant LPO occurring for Lin-6/7-OOH ($p \leq 0.01$), Ger-6/7-OOH ($p \leq 0.01$), and Lim-2-OOH ($p \leq 0.01$) at 50 μ M. In previous work, it has been demonstrated that terpene hydroperoxides can oxidise cell surface thiols, causing a reduction in the GSH/GSSG ratio [128], and it is possible that an increase in oxidative stress contributed to LPO in this study, but the direct interactions of the hydroperoxide allergens on polyunsaturated PLs in the cell membrane causing the C11-

BODIPY^{581/591} probe to become oxidised cannot be dismissed. It is likely that a combination of both cellular stress response and direct hydroperoxide-PL interactions causes this LPO in HaCaT cells. Since terpene hydroperoxides are relatively non-polar compounds, their potential to interact with and penetrate into the lipid-rich environment of keratinocytes is high, and a contributing factor to why higher levels of LPO were observed in HaCaT cells when compared to H₂O₂.

Confocal microscopy revealed minimal oxidation (green fluorescence) in cells containing only media or vehicle (DMSO, 0.5%). Treatment with hydroperoxide compounds (100 µM) increased green fluorescence, particularly with H₂O₂ and even more so with terpene hydroperoxides, though differences between them were difficult to discern. Notably, red fluorescence intensity (non-oxidised probe) did not visibly decrease, suggesting incomplete probe oxidation-possibly due to the absence of Fe²⁺ or hemin, which are known to drive radical formation [203]. Thus, any observed LPO likely relied on transition metals present in the cell culture media (trace elements that serve as cofactors for various enzymatic and cellular processes) or intracellular transition metals [226]. Also, it is possible that the high concentration of phenol red and tyrosine in the cell culture media (DMEM) may have inhibited the oxidation processes to some extent [227].

4.5 Conclusions

In summary, the aim of this chapter was to determine if allergenic terpene hydroperoxides (Lin-6/7-OOH, Ger-6/7-OOH, and Lim-2-OOH) could cause LPO under biomimetic Fenton reaction conditions. LC-MS/MS demonstrated that the terpene hydroperoxide allergens oxidised PLPC liposomes to a degree comparable (or higher) to H₂O₂ (positive control). Furthermore, the terpene hydroperoxide allergen Lim-2-OOH caused significantly greater increases in the formation of oxPLPC products compared to all other hydroperoxides tested. The C11-BODIPY^{581/591} ratio-fluorescence assay demonstrated that terpene hydroperoxide allergens induce significant LPO in the HaCaT keratinocyte cell line at a concentration of 50 µM over a 3 h period, with the most significant increases observed for Lim-2-OOH at each concentration. Radical initiation

using Fe^{2+} confirmed that the generation of these LPO products has the potential to occur in vivo (in the epidermis) once the intact terpene hydroperoxides have penetrated into the skin. This work establishes a link between terpene hydroperoxide allergens and their ability to cause LPO. Further studies are needed to explore the potential role of these oxidised products as mechanistic effectors in ACD.

The next objective of this project was initially to investigate the immunogenic potential of the identified compounds via CD1 proteins. However, the project was redirected to focus on testing the immunogenic potential of the terpene hydroperoxides themselves, as well as their parent terpenes, as potential ligands and/or activators of CD1-restricted T cells.

The work presented in this chapter is based on the following publication:

Moore, A. I., Moreira, A. S., Conde, T., Melo, T., Domingues, P., O'Boyle, N. M., & Domingues, M. R. (2025). Terpene Hydroperoxides as Lipid Peroxidation Inducers: Biomimetic and HaCaT Cell Studies in Allergic Contact Dermatitis. *Contact Dermatitis*, 93(1), 16-30.

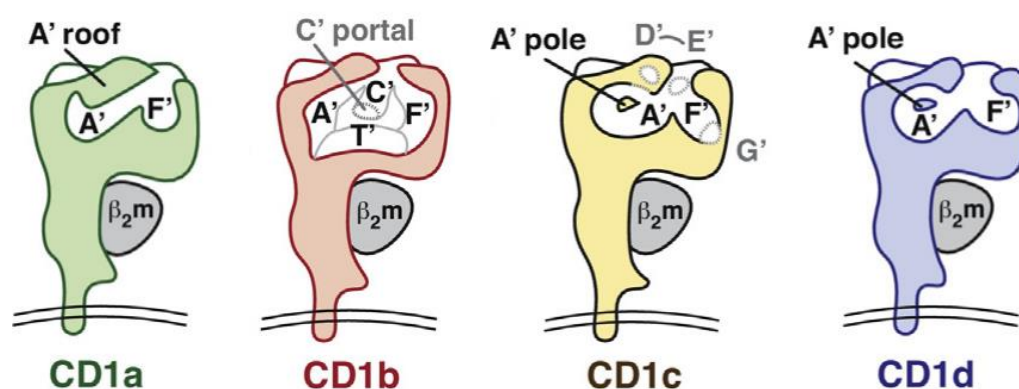
Chapter 5: Terpenes and their Hydroperoxide Derivatives as Ligands for CD1 Molecules and their T Cell Responses

5.1 Cluster of Differentiation 1 (CD1) Proteins

It has long been understood that the immune system distinguishes between self and non-self and between dangerous and harmless primarily through the presentation of peptide fragments by MHC proteins. More recently, another family of antigen-presenting molecules has been identified: the cluster of differentiation 1 (CD1) proteins, which specialize in presenting lipid-based antigens - including foreign and self-derived lipids TCRs. A major difference between MHC and CD1 proteins lies in their genetic variability. MHC molecules are highly polymorphic, with numerous alleles encoding each class I or class II MHC protein, allowing for immense diversity in antigen presentation between individuals. In contrast, CD1 proteins are relatively monomorphic. While there are multiple isoforms - CD1a, CD1b, CD1c, CD1d, and CD1e - they are encoded by a limited number of genes with little allelic variation [228]. A possible reason for this is that lipids are not encoded by genes, thus, they are not readily modified by mutations, therefore, they are likely to present conserved structures to the immune system. As a result of this limited polymorphism, CD1 molecules are more conserved across individuals and even across species. For example, CD1d molecules from humans can often present antigens to mouse TCRs and vice versa - a degree of cross-species reactivity is not typically observed with MHC molecules, which require a matched host context for effective TCR recognition. Based on sequence analysis, the different CD1 isoforms are divided into three groups. Group 1 includes CD1a, CD1b, and CD1c. These isoforms present both microbial and self-derived mammalian lipids to TCRs and are associated with a high diversity in the TCRs that recognize them. Group 2 consists of CD1d. T cells that recognize CD1d are known as natural killer T (NKT) cells. A major subset of these cells express an invariant TCR α -chain (invariant NKT or iNKT cells), which allows them to bridge innate and adaptive immunity. Other CD1d-restricted T cells with diverse TCRs have been described (termed type 2 NKT cells). Group 3 consists of CD1e. Unlike the other isoforms, CD1e does not present antigens directly to T cells. Instead, it is thought to assist in the intracellular processing and loading of lipids onto other CD1 molecules, thereby facilitating their antigen presentation function. CD1 molecules present a diverse range of lipid antigens to T cells, spanning from unique microbial lipids to widely occurring self-lipids of mammalian origin.

5.1.1 Structure of CD1 Proteins

CD1 proteins share sequence homology and an overall domain structure with MHC class I molecules. Like MHC I, they consist of a heavy chain containing three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$), which associate non-covalently with $\beta 2$ -microglobulin ($\beta 2m$) (Fig. 5.1) [229]. However, unlike MHC class I molecules, CD1 proteins present lipid antigens instead of fragmented peptides, whereby hydrophobic alkyl chains position themselves deep inside the hydrophobic pockets located beneath the CD1 surface. The polar head groups of these lipids extend outward through the openings of these pockets on the membrane surface of the CD1 protein, where they are stabilized by hydrogen bonds. These interactions also assist in properly orienting the lipid antigen. Compared to MHC class I molecules, CD1 proteins feature larger binding pockets, and the architecture of these pockets varies across the different CD1 isoforms, enabling each to recognize distinct lipid antigens. CD1a specifically is highly expressed on Langerhans cells, therefore its antigen presentation properties could be of significant interest in the context of ACD [167].



	CD1a	CD1b	CD1c	CD1d
Pockets	2	4	2	2
Cleft volume	1690 Å ³	2220 Å ³	2060 Å ³	1760 Å ³
Approximate carbon (C) chain length	C38.3	C65.3	C47.3	C38.8

Figure 5.1. CD1 antigen binding cleft architecture.

CD1 proteins contain antigen-binding pockets (black labels) and surface portals (grey labels). All CD1 isoforms feature an F' portal that enables antigen head groups to protrude for TCR engagement. Some isoforms also possess accessory portals that may permit lateral antigen protrusion. Notably, CD1c contains

multiple accessory portals (D', E', and G') which confer structural flexibility by allowing the α 1- α 2 superdomain to rearrange upon ligand binding. While most CD1 molecules have two pockets, CD1b is unique with four (A', F', C', and T') and possesses the largest internal volume, enabling it to accommodate exceptionally long lipid ligands (image adapted from Moody and Cotton 2017)[229].

5.1.2 Diversity of Lipids Presented by CD1 Proteins

5.1.2.1 Foreign Lipid Antigens

Many of the unique lipids found in *Mycobacterium tuberculosis* and other mycobacteria can be presented by CD1a, CD1b, and CD1c molecules to activate clonally diverse T cells. For example, didehydroxymycobactin and sulfatide have been shown to activate CD1a-restricted T cells (Fig. 5.2A) [230, 231], while phosphatidylinositol mannosides have been shown to activate CD1d-restricted T cells (Fig. 5.2B) [232]. However, mycobacterial lipids are generally not known for their iNKT cell stimulatory activity, so they are not typically associated with CD1d presentation. Research on iNKT cells has long focused on the synthetic glycolipid α -galactosylceramide (α -GalCer), a potent activator of these cells via CD1d (Fig. 5.2C) [233]. This discovery prompted the search for naturally occurring analogs. α -Glucuronosylceramides, structurally similar to α -GalCer, were identified in *Sphingomonas* spp. and shown to stimulate semi-invariant NKT cells (Fig. 5.2D) [234]. Another class of α -linked lipids capable of semi-invariant NKT cell stimulation is α -galactosyldiacylglycerol found in the spirochete *Borrelia burgdorferi* (Fig. 5.2E) [235].

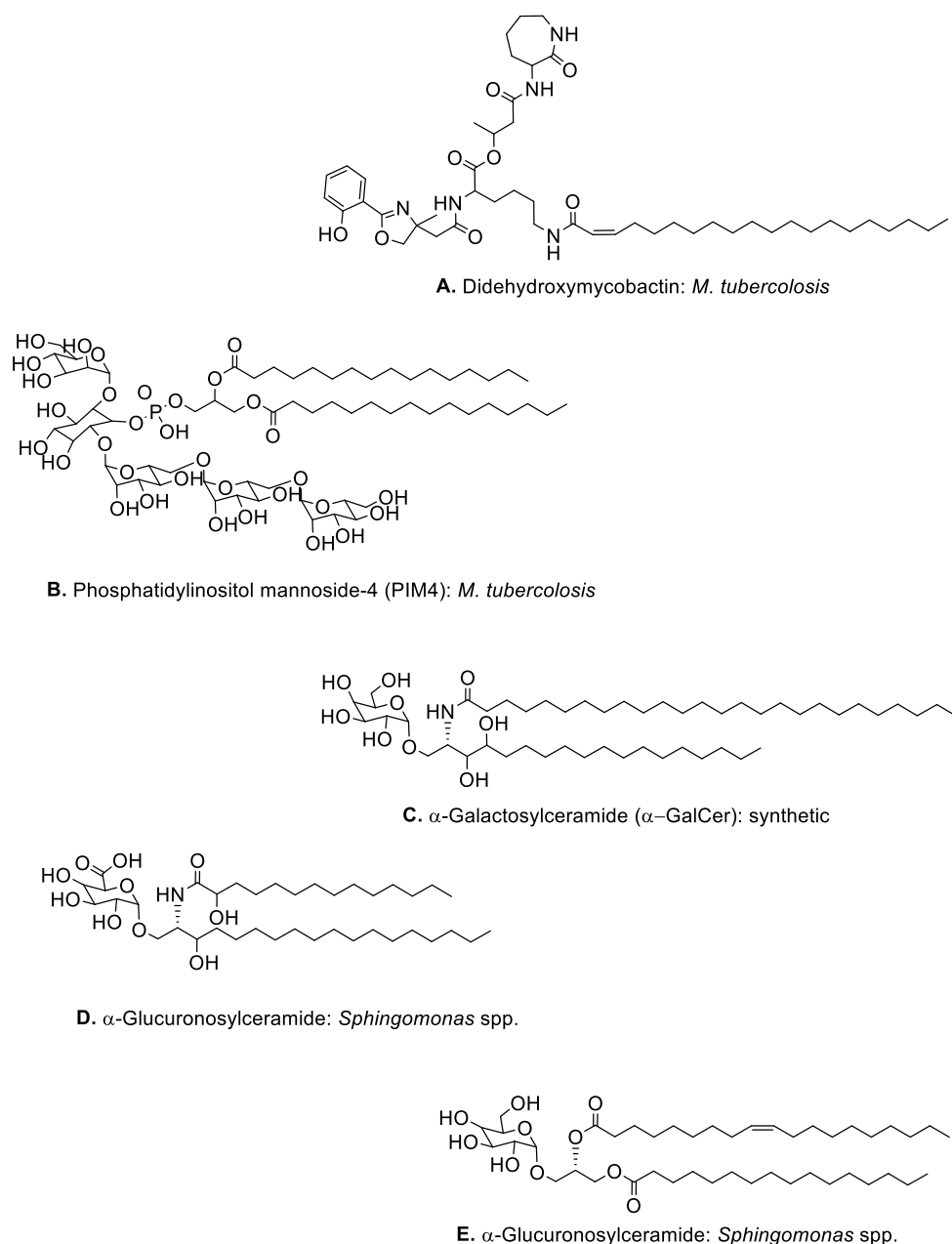


Figure 5.2. Structures of microbial lipids presented by CD1 molecules.

5.1.2.2 Mammalian Self-Lipid Antigens

The first identified examples of group 1 CD1-restricted human T cell responses were directed against self-lipid antigens [236]. In cell-free assays, CD1d molecules loaded with mammalian lipids such as phosphatidylinositol (Fig. 5.3A), phosphatidylglycerol (Fig. 5.3B), and phosphatidylethanolamine (Fig. 5.3C) were able to stimulate NKT cell populations, although their agonistic strength was significantly lower compared to α -GalCer [237]. Sulphatide (Fig. 5.3D), a major component of myelin, was identified as a broadly reactive self-lipid antigen that could be presented by CD1a, CD1b, CD1c, and

CD1d, activating clonally restricted human T cells [238]. In addition, glycosphingolipids such as the ganglioside GD3 have been shown to stimulate iNKT cells through presentation by CD1d (Fig. 5.3E) [239]. More recently, it has been found that the squalene, which accumulates naturally in the epidermis and sebum, functions as an autoantigen presented by CD1a (Fig. 5.3F) [167].

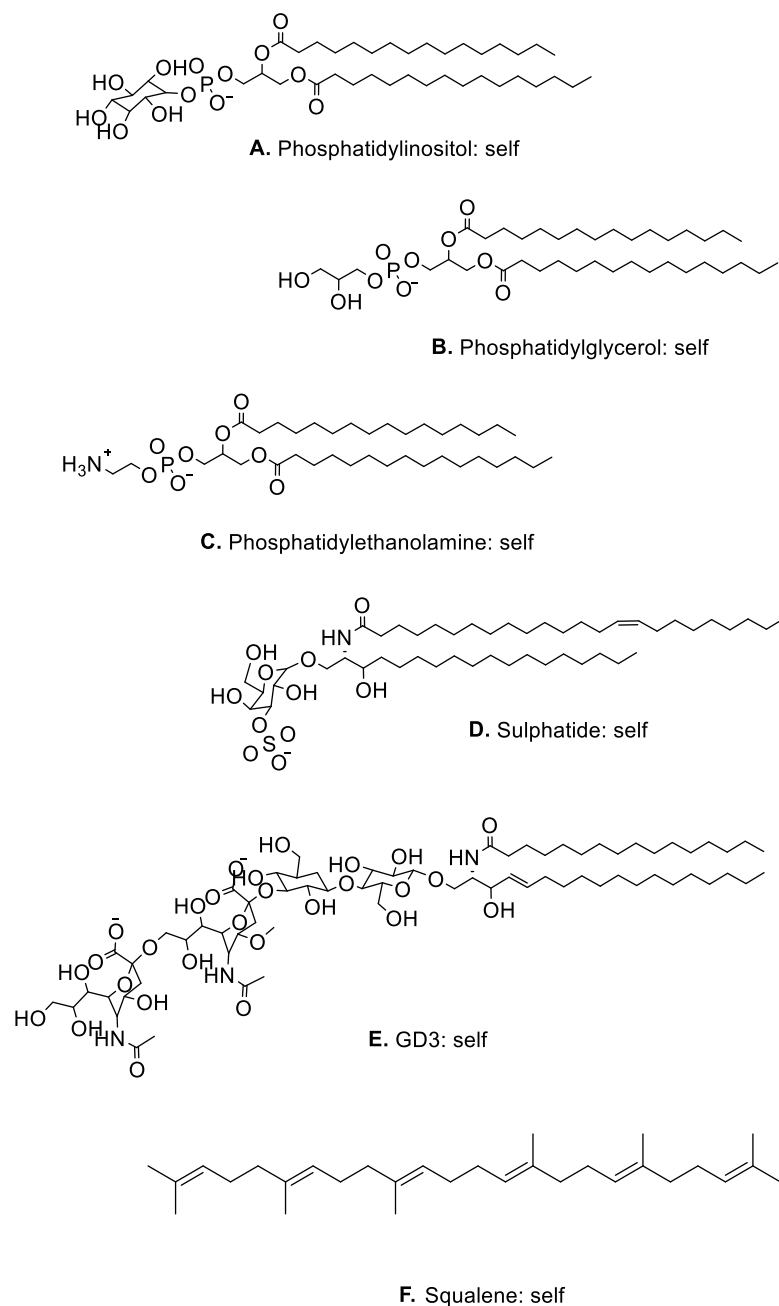


Figure 5.3. Structures of mammalian self-lipids presented by CD1 molecules.

5.1.3 CD1 Trafficking in the Secretory Pathway

When CD1 proteins are first synthesised, they have a signal sequence which tells the cell to send them to the endoplasmic reticulum (ER). After translation into the lumen of the ER, they become glycosylated by the addition of N-linked oligosaccharides [240]. These sugars help attract protein folding chaperones calnexin and calreticulin [241]. Another helper protein, ERp57, helps CD1 form disulphide bonds which are important for maintaining its structure (similar to how MHC class I molecules are folded and stabilized) [242]. CD1 molecules bind self (endogenous) lipids in the ER, and a small protein called β_2m in order to leave the ER and reach the cell surface [243]. Lipid ligands had previously been eluted from CD1 isoforms and identified using MS [167, 244, 245], however, quantitative methods for enumerating endogenous ligands or systematically comparing them across CD1 isoforms was lacking until a recent groundbreaking study by Huang and co-workers, which solved lipidomes for the four human CD1 antigen-presenting molecules, thereby providing a map of self-lipid display [246]. The study identified at least 1,300 unique lipids from the four isoforms, with partially overlapping lipidomes. Overcapture analysis which involved comparing cellular lipidomes to CD1 lipidomes found many matches, suggestive of lipids being presented in a chemically unmodified form (unlike peptide antigen processing in the context of MHC). The study proposed three general capture mechanisms dependent of whether the clefts match (CD1a and CD1d), partially match (CD1c), or universally mismatch (CD1b) lipid anchor (i.e., carbon chain length) size. Endogenous lipids from CD1a and CD1d showed an optimal anchor length of C38, which matches the cleft of CD1a (1,690 Å³) and CD1d (1,760 Å³) and calculated cleft capacities of C38.3 or C38.8, respectively. The size match, low volume variance among crystal structures, and lack of spacer lipids from crystal structures point towards a 1:1 CD1:lipid stoichiometry as the most likely cellular mechanism of loading endogenous lipid capture. Furthermore, the ligands eluted from CD1a and CD1d molecules clustered in similar patterns in over capture analysis, showing preferences for SL and PL capture. CD1d-SL capture occurred regardless of the chain length, but CD1a-SL capture was exclusively very long chain (>C36) species. It is known that SMs inhibit T cell responses via CD1a and CD1d through negative blocking effects [247, 248]. CD1c is known to have a large cleft volume variance [249], which matched the variance seen in

eluted lipids: PC and SM species had alkyl chains between C30-C46, with no favoured length. The mean lipid anchor length was C39 - smaller than the predicted cleft volume (C47.3) - but did not exceed high variance of the cleft that was discussed (C32-C60). Due to the partial size mismatch, there is occasional use of diversely positioned spacer lipids. For the CD1b molecule, the cleft capacity was approximately C65 (2,220 Å), however, there was no size overlap with other CD1 molecules in terms of eluted lipids (C30-42). Essentially, there was a 2 fold mismatch between cleft size and lipid length, leading towards strong evidence for dual lipid capture. CD1b crystal structure work showed a 2:1 lipid:CD1b stoichiometry with high reproducibility in terms of lipid positioning within the pockets. Given a lack of self-lipids with >C65 chain length, it is proposed that dual lipid capture is the normal mechanism of self-lipid capture, with antigenic lipids positioned in the upper chamber and spacer lipids (which do not contact a TCR) are in the lower chamber to prop up the antigenic lipids. Although this study was comprehensive, approximately 1,300 events that were deemed to be CD1 ligands were not identifiable and therefore yet to be discovered as antigens or TCR-blocking lipids.

5.1.4 CD1 Trafficking in the Endocytic Pathway

CD1a and CD1c readily capture exogenous lipid antigens at the cell surface, but CD1b and CD1d do this to a much lesser degree [229]. After reaching the plasma membrane, CD1 molecules seem to undergo internalization primarily through a dominant, likely constitutive, pathway into endosomes. Internalization of CD1 from the plasma membrane is crucial for enabling antigen sampling within the endocytic system (Fig. 5.4). There is a correlation between the degree of recycling through the endosomal compartments and the acidic pH required for efficient lipid antigen capture. Adaptor protein complexes (AP1, AP2, AP3 and AP3) control intracellular sorting by binding to amino-acid residues of cytoplasmic tails from transmembrane proteins, causing their transition to migrate into transport vehicles. CD1a has short cytoplasmic tails which lack any recycling motifs, meaning that they don't bind to Aps (Fig. 5.4; CD1a = green) [250]. However, CD1a localizes to juxtanuclear compartments that express the transferrin receptor, as well as to clathrin-coated vesicles and specialized sorting endosomes known as Birbeck granules, which are characteristic of LCs [250, 251]. The cytoplasmic tails of human CD1c and CD1d bind to AP2 which controls their sorting to late endosomes (Fig.

5.4; CD1c = orange; and CD1d = blue) [252, 253]. The tail motif is important for antigen presentation as deletion of the sequence results in the failure to activate iNKT cells, reflecting the failure of the CD1d to traffic through the low pH environment of the endosomal compartments [254]. CD1b binds to AP2 and AP3 [255], and is the isoform which penetrates furthest into the endosomal network, reaching late endosomes, lysosomes and MHC II compartments (Fig. 5.4; CD1c = red) [256, 257]. These isoform-specific trafficking pathways enable CD1 proteins to access distinct intracellular compartments, each providing a unique lipid environment that supports the selective loading of lipid antigens.

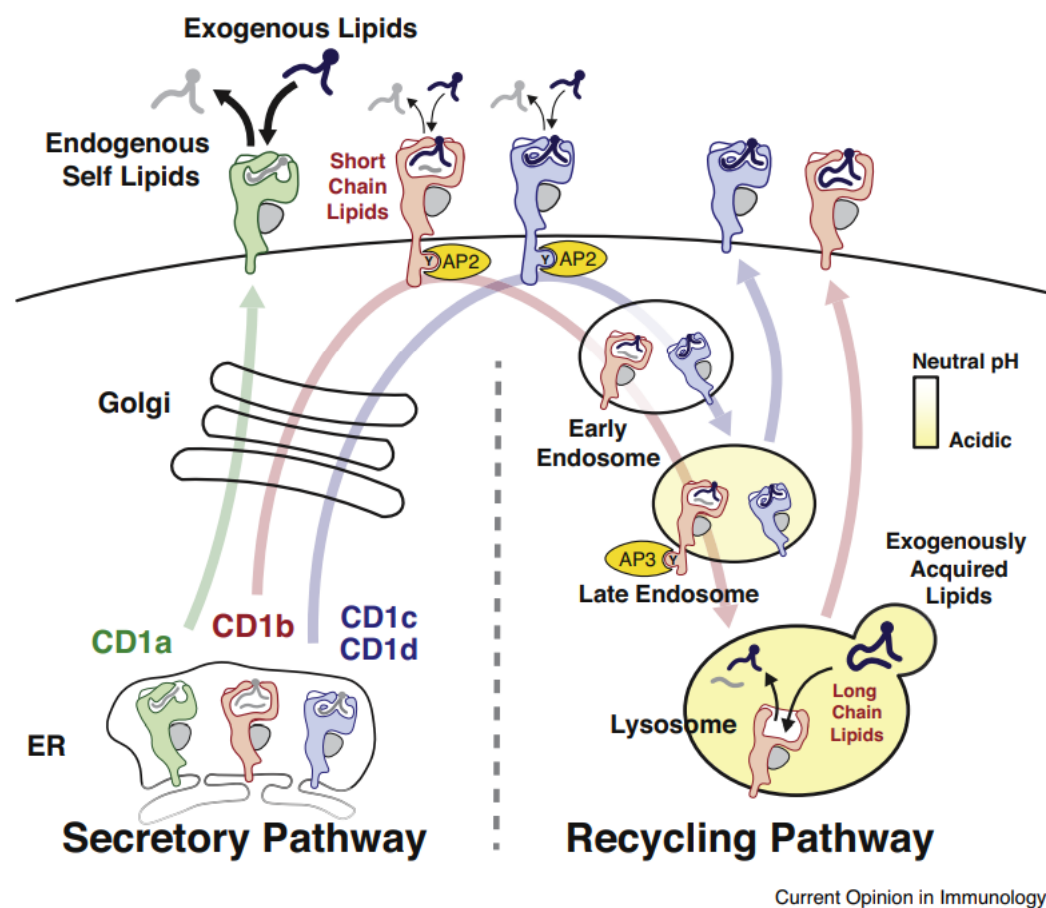


Figure 5.4. Subcellular pathways of lipid antigen capture by human CD1 proteins [229].

5.1.5 Lipid Trafficking

Cells take up lipids from the extracellular environment for their metabolic needs. One way in which this can occur is when lipid complexes with apolipoprotein E (apoE) in the blood to form lipid-transport particles. In this case, low density lipoprotein (LDL) receptors (LDLRs) bind apoE leading to the internalization of the lipid-transport particles into endosomes [258] (Fig. 5.5). Other mechanisms of lipid uptake may involve scavenger receptors (SR), such as SR-A, SR-BI, LOX1, and CD36, which bind modified forms of LDL and apoptotic cells [259], which may render them susceptible to the uptake of foreign lipids from infected cells. C-type lectins are another way that lipids can be taken up by cells. These receptors bind carbohydrate moieties and a well-known one is the macrophage mannose receptor (MR) expressed primarily by macrophages and dendritic cells. It has been shown to play a role in the uptake of the bacterial lipoglycan lipoarabinomannan (LAM) and its transport to the endocytic pathway where it is subsequently presented to CD1b [260]. Another possible route of lipid uptake in LCs is through the endocytic receptor Langerin, which internalizes potential lipid antigens and accumulates in Birbeck granules - specialized subdomains of the endosomal recycling compartment. These antigens are then trafficked for processing and presentation, likely via CD1a [261]. One other way in which exogenous lipid matter can be taken up by cells is through phagocytosis, either through the uptake of pathogenic bacteria, apoptotic cells or other large particles, in a process requiring actin to form a cup around the particle that eventually closes to form a phagosome [262]. Once formed, phagosomes mature via the endocytic system.

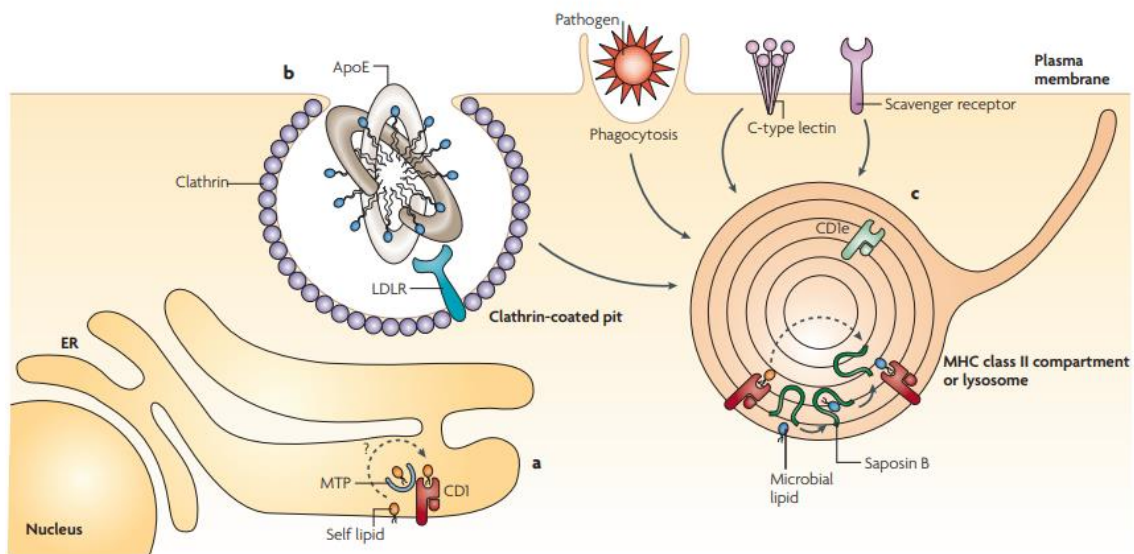


Figure 5.5. Lipid loading and exchange in the endocytic pathway.

Self-lipids shown in orange are loaded onto CD1 molecules in the ER. Possible mechanism of exogenous lipid uptake involve: clathrin-dependent uptake of apoE-lipid complexes bound to LDLR; phagocytosis of external lipid mater which could be infected cell material or apoptotic cells; C-type lectins which bind carbohydrate moieties on glycolipids; and scavenger receptors which can bind modified (e.g., oxidized) form of LDL or apoptotic cells [263].

5.1.6 Lipid Exchange in the Endocytic Pathway

Several lines of evidence indicate that microbial and self-antigens may be predominantly loaded onto CD1 molecules in the late endosomal or lysosomal compartments [260, 264]. Because antigenic lipids are hydrophobic and membrane-bound CD1 proteins are exposed to the hydrophilic environment, there must be mediators that operate to facilitate the loading of lipids onto CD1. Molecules which have been shown to take up this mediator role are saposins (SAPs) – enzymatically inactive cofactors found in lysosomes which promote the degradation of SLs by hydrolysis (Fig. 5.6) [265, 266]. It has been demonstrated that SAP-C has a dual function: it extracts lipid antigens from membrane structure, and it binds to the antigen-presenting molecule CD1b, thus promoting the loading of antigenic lipids [267]. It was also demonstrated that SAPs were indispensable for the binding of an exogenous lipid antigen, α -GalCer, to CD1d in the endocytic pathway [268, 269]. It has also been mentioned that CD1e, the only isoform that is not expressed at the cell surface of APCs, could play a role in facilitating the loading of exogenous lipids onto other CD1 isoforms, as was shown for the α -mannosidase-dependent processing of phosphatidylinositol mannose 6 (PIM₆) and PIM₂ [270].

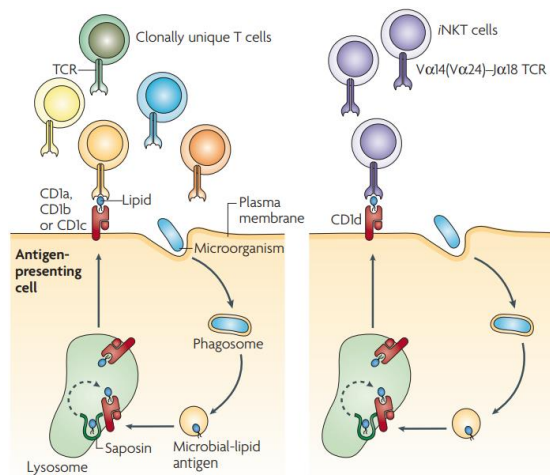


Figure 5.6. Lipid exchange in the endocytic pathway.

Saposin (SAP)-mediated disruption of lipid membranes within lysosomal compartments facilitates the displacement of self-lipids and promotes the loading of exogenous lipids into CD1 clefts prior to egression to the cell surface once again for antigen presentation to CD1-restricted T cells [263].

5.1.7 Functions of CD1-Restricted T cells

As previously mentioned, group 1 CD1 (a, b and c) and group 2 CD1(d) are able to present both microbial- and self-lipid antigens to CD1-restricted T cells. Pathogen recognition can be achieved by TCR contact with any one of the many foreign lipids produced during infection, thus, the use of TCRs with diverse α - and β -chains to detect many types of foreign lipids is a central feature of their function as effector T cells (Table 5.1). However, group 1 CD1-restricted T cells lack any specific surface markers or genetic means of deletion, therefore, most knowledge on group 1 CD1-restricted T cell function is derived from observations of long-term T cell clones [271].

A particular subset of CD1d-restricted T cells which has an invariant α -chain (V α 24/J α 18 in humans) paired with a semi-variant β -chain are iNKT cells [236]. This invariant TCR serves as a defining surface marker that allows effector functions to be tracked and measured on a single-cell or population basis. Such studies have concluded that iNKT cells are preprimed memory T cells that secrete large amounts of multiple cytokines, including IFN- γ , TNF, IL-4 and IL-13, and work through direct modulation of NK cells, B cells, macrophages and dendritic cells (DCs) to control infection [272]. However, for CD1d-restricted T cells which do not possess the invariant α -chain, they also lack defined surface markers or genetic means and are therefore far less understood compared to iNKT cells. Although iNKT cells have nearly the same TCR α sequence, they can comprise

of CD4⁺, CD8⁺ and/or double negative NKT cells. CD8⁺ and double negative iNKT cells are more likely to be cytotoxic and produce T_H1 cytokines, whereas CD4⁺ are more likely to produce T_H2-type cytokines [273-275]. Although iNKT cells are mostly associated with innate immunity, they bridge the gap between innate and adaptive immunity through their ability to provide help to B cells, influencing antibody production [276].

Table 2.1 Characteristics of CD1-restricted T cells [263].

	Group 1 CD1-restricted T cells	CD1d-restricted iNKT cells	CD1d-restricted diverse NKT cells
Antigens	Microbial and self-lipids	Microbial and self-lipids	Unknown
T cell population	Clonally diverse	Canonical TCR α but polyclonal	Clonally diverse
TCR	TCR α : diverse; TCR β : diverse	TCR α : invariant (V α 24/J α 18 in humans) ; TCR β : limited V β with diverse CDR3	TCR α : diverse; TCR β : diverse
Precursor frequency	One per thousands, unique specificity for single antigen	<1% of T cells in humans (respond <i>en masse</i> to a single antigen)	Unknown
Memory	Yes	No	Unknown
Immunity	Adaptive, slow	Innate-like, rapid (hours to few days)	Unknown

5.1.8 Contact Allergens in CD1-Restricted T cell Responses

A clear division has been proposed so far in terms of lipid antigens presented by CD1 molecules: microbial lipids or (mammalian) self-lipids. However, in recent years, contact allergens associated with ACD have been studied for their possible role in CD1-mediated T cell activation, as ACD is a Type IV hypersensitivity reaction (T cell mediated). Since most contact allergens are non-lipidic small molecules, it was believed that they need to bind to larger macromolecules in order to exert their immunogenic effect, a process referred to as haptenation. Although there is evidence that certain drugs, such as sulfamethoxazole, lidocaine, and penicillins which can trigger a T cell response through haptenation of MHC or TCR, a TCR epitope has never been discovered in relation to a ACD. Also, many contact allergens lack electrophilic sites which make them unattractive

targets for electron-rich amino acid residues from proteins, thus T cell reactivity to these compounds must come from alternative mechanisms (one such method mentioned previously was via free radical reactions). Dinitrochlorobenzene (DNCB) is a textbook contact allergen, and it was used to explore its effect on CD1a- and CD1d-restricted T cell responses [171]. The study postulated that DNCB had a direct effect in the formation of CD1-self-antigen complexes, probably through increased synthesis of endogenous lipid-antigens in some cell types, with the effect of DNCB being lost when endogenous lipids were replaced with exogenous lipids e.g., sulfatide. It was also found that other contact sensitizers induced activation of the same T cell clones, thus indicating that multiple sensitizers shared this capability, and the results were not contact sensitizer specific. It has also been shown that plant-derived lipophilic contact sensitizer urushiol triggered a CD1a-dependent skin inflammation driven by CD4⁺ helper T cells that produced the cytokines IL-17 and IL-22 (T_H17 cells) [100]. Crystal structure analysis of the CD1a-urushiol complex showed that the ligand bound in a non-covalent way in the CD1a antigen-binding cleft, 80% of the urushiol was buried in the CD1a cleft, thus CD1a presented urushiol with 20% of the molecular surface potentially exposed for direct TCR contact (Fig. 5.7). However, further studies are necessary to elucidate the crystal structure of the CD1a-urushiol-TCR ternary complex. This went against the commonly held belief that urushiol was a pro-hapten whose catechol group is oxidized to quinone that presumably then reacts with an endogenous protein to exert its immunogenic effect [210]. However, considerable evidence of urushiol as a CD1a ligand and CD1a-restricted T cell activator doesn't necessarily debunk the pro-hapten theory, and it is possible that a combination of both mechanisms may work in some synergistic way.

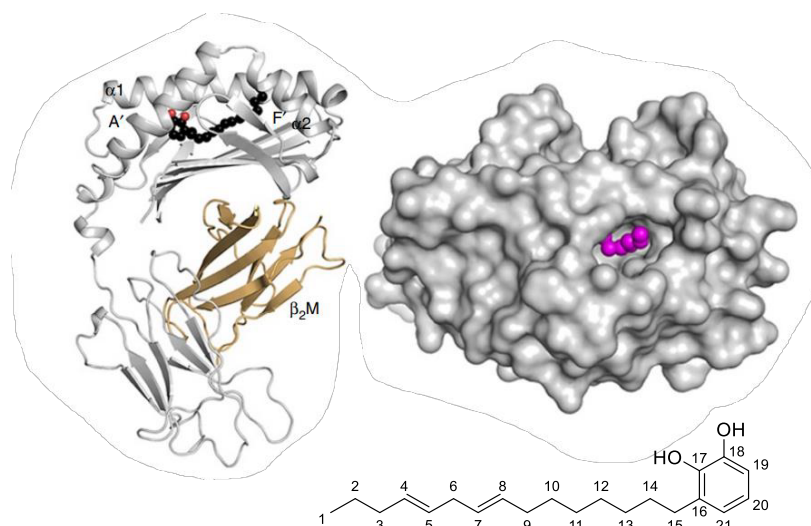


Figure 5.7. Crystal structure of CD1a-urushiol complex.

Binary crystal structure of CD1a-urushiol C15:2, and top view of the molecular surface of the $\alpha 1$ and $\alpha 2$ domains of CD1a (grey) and urushiol (magenta) [100].

The known repertoire of CD1a contact allergens was extended through the discovery of farnesol, benzyl benzoate, benzyl cinnamate and coenzyme Q-compounds [277]. In this study, balsam of Peru is a tree oil-derived contact allergen which showed a significant CD1a-restricted T cell response using an $\alpha\beta$ T cell line known as BC2. Efforts to identify the offending chemicals within the balsam of Peru extract led to the discovery of benzyl cinnamate and benzyl benzoate as the CD1a-presented antigens, but crystal structures could not be acquired (personal communication). The same study then focused on testing chemical structures with different rings, unsaturations and molecular size to identify subsequent contact allergens which activate CD1a-restricted T cells. The approach was guided by the fact that squalene, benzyl benzoate, and benzyl cinnamate have ringed or unsaturated structures which are CD1a-restricted T cell activators. This led to the discovery that farnesol and coenzyme Q2 were CD1a antigens. Subsequently, elution studies using MS analysis in tandem with crystallography of the CD1a-farnesol complex revealed that farnesol is able to displace endogenously loaded lipids and bind deep within the CD1a cleft whereby it is out of reach of any TCR (Fig. 5.8), which supports the “absence of interference” theory, whereby it removes inhibitory ligands with polar headgroups and when it does so it reveals a part of the CD1a protein that is detected by the CD1a-restricted T cells [167, 278].

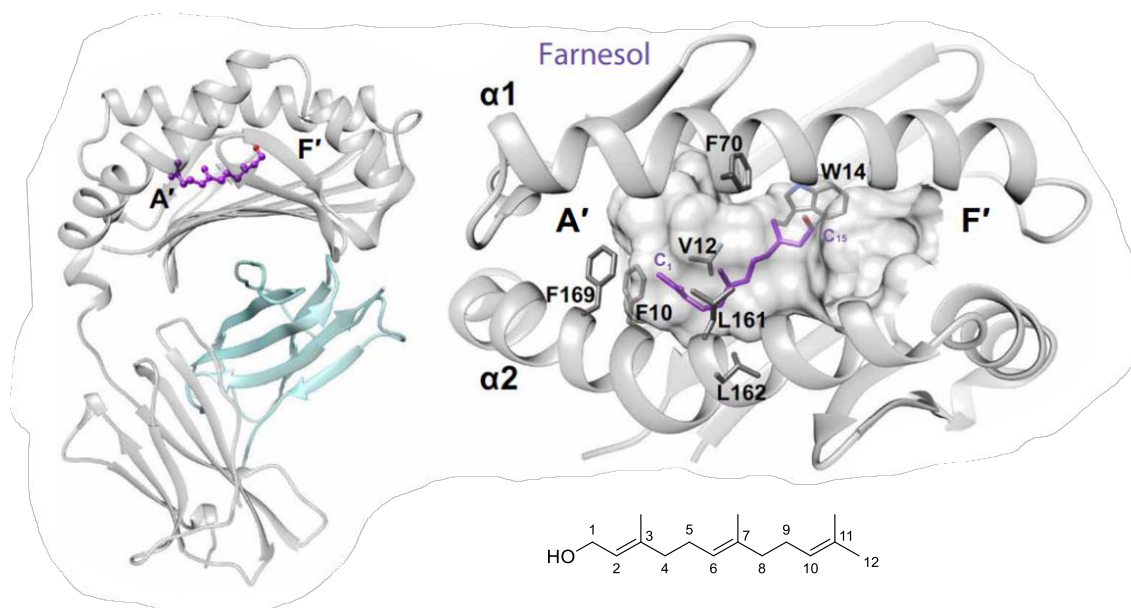


Figure 5.8. Crystal structure of CD1a-farnesol complex.

Binary crystal structure of CD1a-farnesol, and molecular interactions between farnesol (purple) and the hydrophobic residues within the CD1a binding cleft (grey) [277].

This chapter set out to investigate: (i) the ability of terpene hydroperoxide contact allergens (Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH), and their non-oxidized parent terpenes ((*R*)-(+)-limonene, linalool, and geraniol), to influence the ability of CD1 tetramers to bind with T cells from the peripheral blood mononuclear cells (PBMCs) of 6 healthy donor controls (ctrl) and 6 fragrance allergy donor samples (F^+) using cell-free assays; and (ii) using mock and CD1-transfectant APCs (C1R-mock, C1R-CD1a, HeLa-mock, and HeLa-CD1d), challenged with terpenes and their hydroperoxides, and incubated with the PBMCs of Ctrl and F^+ groups, to access their potential to cause T cell activation using TNF, IFN- γ , and IL-2 production as readouts.

5.2 Materials and Methods

5.2.1 Lipid Sources

(*R*)-(+)-limonene (183164), linalool (L2602), geraniol (163333), and farnesol (277541) were obtained from Sigma-Aldrich (St Louis, MO, USA). α -GalCer was acquired from Avanti Research (Alabaster, AL, USA). Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH were synthesised according to methods described previously (section 2.2).

5.2.2 CD1 Tetramer Sources

Unloaded CD1a and CD1d tetramers were provided by the National Institutes of Health (NIH) Tetramer Core Facility (Emory University, Atlanta, GA).

5.2.3 Antibody Sources

Eflour 506-conjugated fixable viability dye (65-0866-14) was acquired from Thermo Fisher Scientific (Waltham, MO, USA). Purified anti-CD36 (678702), OKT3 (317302), CD3-PacificBlue (300431), CD4-FITC (344604), CD8-APC/Cy7 (344714), CD14-PE/Cy7 (367112), CD19-PerCP/Cy5.5 (152406), TNF-PE (502909), IFN- γ -PE/Cy7 (502528), and IL-2-PerCP (500350) were obtained from BioLegend (San Diego, CA, USA). FcR blocking agent (130-059-901) was obtained from (Miltenyi Biotec, Bergisch Gladbach, Germany).

5.2.4 PBMC Preparation

Healthy donor control (Ctrl) blood was collected from the National Blood Centre on the St James' Hospital campus (Dublin, Ireland). Under sterile conditions, 10 mL of blood from a blood pack was diluted 1:5 in PBS. In two universal tubes, 7.5 mL of Lymphoprep was added to each, followed by the slow addition of 25 mL diluted blood per tube at a 45° angle. Tubes were centrifuged at 400 g for 30 minutes with the brake and accelerator turned off. The plasma layer was discarded, and the PBMC-containing buffy coat was carefully collected using a sterile plastic pipette, avoiding red blood cells. The buffy coat was washed with 20 mL PBS and centrifuged at 1500 rpm for 8 minutes (brake and accelerator set to 9). After discarding the supernatant, the cell pellet was resuspended in 3–5 mL of complete RPMI. PBMCs were counted using a haemocytometer after mixing 10 μ L of the cell suspension with 190 μ L of trypan blue. For cryopreservation, unused PBMCs were pelleted, resuspended in sterile-filtered FCS containing 10% DMSO, and aliquoted into 3–6 cryovials. Vials were placed in a Mr Frosty container containing isopropanol and stored at –80 °C until use. PBMCs were also prepared from ~10 mL of blood collected from fragrance-allergic patients at Tallaght Hospital (Dublin, Ireland). These patients were clinically diagnosed by patch testing. Unlike control blood, patient samples were processed on the same day of collection and were less concentrated; thus, a 1:2 dilution with 10 mL of PBS was performed. If PBMCs were used on the day of isolation (or after thawing), they were resuspended at 2×10^6 cells/mL in complete RPMI

supplemented with 1 ng/mL IL-15. For overnight culture, 6 mL of the cell suspension ($\sim 1.2 \times 10^7$ cells) was combined with 6 μ L IL-15 (6 ng total) in a T25 flask and incubated at 37 °C.

5.2.5 CD1 Tetramer Assays

5.2.5.1 Lipid Loading

To a glass amber vial, 8 μ g of lipid [farnesol (1 μ g/ μ L in DCM, 36.98 nmol), α -GalCer (1 μ g/ μ L in DMSO, 5.83 nmol), limonene (1 μ g/ μ L in DCM, 58.72 nmol), linalool (1 μ g/ μ L in DCM, 51.86 nmol), geraniol (1 μ g/ μ L in DCM, 51.86 nmol), Lim-2-OOH (1 μ g/ μ L in DCM, 47.62 nmol), Lin-6/7-OOH (1 μ g/ μ L in DCM, 43.01 nmol), or Ger-6/7-OOH (1 μ g/ μ L in DCM, 43.01 nmol)] was added. The final volume was adjusted to 45 μ L using PBS containing 0.5% CHAPS, followed by vortexing for 30 seconds. Next, 3.85 μ L of CD1a (1.3 mg/mL; 5.005 μ g; 9.43 pmol) or CD1d (1.3 mg/mL; 5.005 μ g; 10.13 pmol) monomer was added, along with 1 μ L of 0.5% sodium azide. Vials were sealed with plastic caps, wrapped in parafilm, vortexed again for 30 seconds, and incubated at 37 °C for 2 hours. Loaded tetramers were stored at 4 °C until use.

5.2.5.2 Tetramer Staining

PBMCs were harvested and counted following overnight incubation (section 5.2.4). A total of 0.5×10^6 cells were transferred into flow cytometry tubes, washed with 1 mL PBS, and centrifuged at 1500 rpm for 8 minutes (standard wash conditions used throughout). After discarding the supernatant, cells were resuspended in 100 μ L of fixable viability dye (efluor 506, diluted 1:1000 in PBS), vortexed, and incubated in the dark at room temperature for 15 minutes. Cells were then washed with FACS buffer (PBS + 2% FCS), resuspended in 50 μ L FACS buffer containing 0.5 μ g anti-CD36, and incubated on ice for 15 minutes. After washing, cells were resuspended in 24 μ L FACS buffer and incubated with 1 μ L of lipid-loaded tetramer for 30 minutes at room temperature in the dark. OKT3 (1 mg/mL) was diluted 1:100 in FACS buffer, and 10 μ L (0.1 μ g) was added per tube. Samples were vortexed and incubated at 37 °C for 10 minutes, then washed with 1 mL FACS buffer. A surface staining cocktail was prepared containing 2.5 μ L each of CD3-PacificBlue, CD4-FITC, CD8-APC/Cy7, CD14-PE/Cy7, and CD19-PerCP/Cy5.5, made up to 50 μ L with cold FACS buffer. This cocktail was added to each tube and incubated on ice for 20 minutes in the dark. Cells were washed twice with cold FACS

buffer and fixed with 250 μ L of 4% PFA for 10 minutes at room temperature in the dark. After a final wash, cells were resuspended in 333 μ L FACS buffer and acquired on the flow cytometer.

5.2.5.3 Flow Cytometry (Acquisition)

Flow cytometric analysis was performed using a BD FACSCanto II cytometer with FACSDiva software. The following fluorochrome-conjugated antibodies were used: CD3–Pacific Blue, CD4–FITC, CD8–APC/Cy7, CD14–PE/Cy7, and CD19–PerCP/Cy5.5. Data were acquired for forward scatter (FSC), side scatter (SSC), and the corresponding fluorescence channels. Appropriate compensation was performed using single-stained controls, with the unstained control serving as baseline. Single-stained compensation beads were acquired for each fluorochrome-conjugated antibody. Bead populations were gated using FSC and SSC (Fig. 5.9A), and fluorescence signals were verified to be on scale and maximally detected in their designated channels (Fig. 5.9B). Detector voltages were adjusted as required, and 5,000 events were collected per compensation control. After acquiring and recording all compensation controls, the compensation matrix was calculated and applied to the experiment. The absence of any warning messages indicated that there was no significant spectral overlap, and that the compensation was correctly set up. Next, within the experimental layout, each antibody was assigned to its respective fluorochrome channel. During sample acquisition, lymphocyte populations were identified based on FSC and SSC parameters, and gates were set accordingly (Fig. 5.9C). The acquisition dashboard was configured to acquire and record 100,000 events per sample.

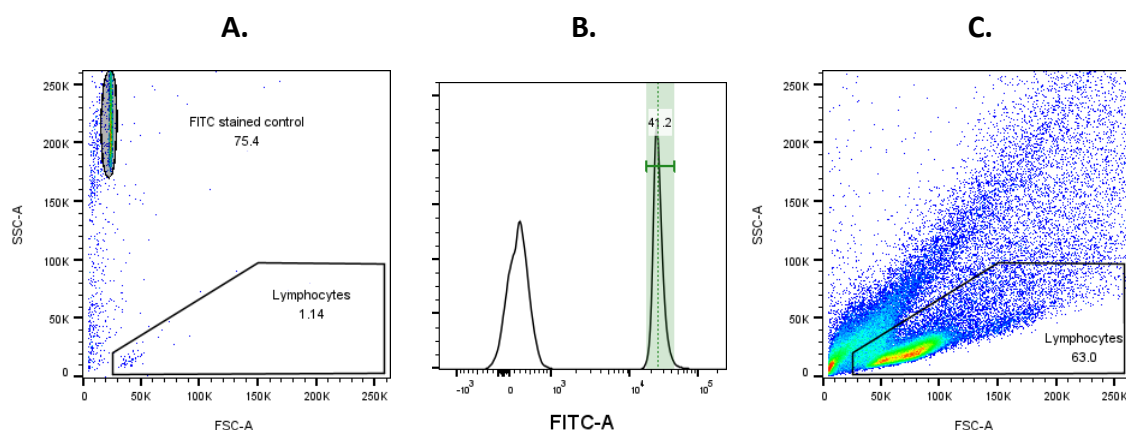


Figure 5.9. FSC-Area vs SSC-Area dot plots of compensation beads and PBMCs during compensation set-up for acquisition.

(A) FITC-labelled compensation beads were acquired by flow cytometry and an oval gate was drawn around the FITC-labelled compensation beads in the top left corner of the dot plot. (B) A histogram of the FITC-labelled events gated in (A) showing FITC staining intensity confined to the FITC channel and within scale, confirming appropriate detector voltage settings. (C) PBMCs were acquired by flow cytometry where lymphocytes are located predominantly in the bottom left quadrant with a polygon gate drawn around them.

5.2.5.4 FlowJo Gating Strategy (Analysis)

FCS files exported from BD FACSDiva were imported into FlowJo v10 for analysis. Compensation controls were re-gated using the Compensation Wizard found in the Cytometry toolbar, and the resulting compensation matrix was applied to all samples within the group. Using a “CD1d_α-GalCer” dataset as an example (i.e., PBMCs from one donor incubated with α-GalCer-loaded CD1d tetramer), a polygon gate was drawn on the FSC-A vs. SSC-A dot plot to identify the lymphocyte population. This gate was placed primarily in the bottom-left quadrant and extended across to the bottom-right quadrant to include both typical lymphocytes and any CD1d-restricted T cells that may have increased in size due to tetramer binding or activation (Fig. 5.10A). From the gated lymphocyte population, a doublet exclusion step was performed; the y-axis was changed from SSC-A to FSC-H, and a gate was drawn to include only single cells, excluding doublets or cell aggregates (Fig. 5.10B). To exclude dead cells, the x-axis parameter was changed to the viability dye channel (e.g., eFluor 506), and a gate was drawn to include only viability dye-negative events, thus viability dye-positive events, corresponding to dead cells, were excluded from further analysis (Fig. 5.10C). From the resulting population, the x-axis and y-axis parameters were set to CD19 and CD14, respectively. A gate was then drawn around the CD19-negative and CD14-negative population to

exclude B cells and monocytes from further analysis (Fig. 5.10D). From the remaining population, the x-axis and y-axis were set to CD3 and tetramer, respectively. A quadrant gate was applied to distinguish four populations: CD3-positive/tetramer-negative T cells (bottom right), CD3-negative/tetramer-positive T cells (top left), CD3-negative/tetramer-negative cells (bottom left), and CD3-positive/tetramer-positive T cells (top right) (Fig. 5.10E).

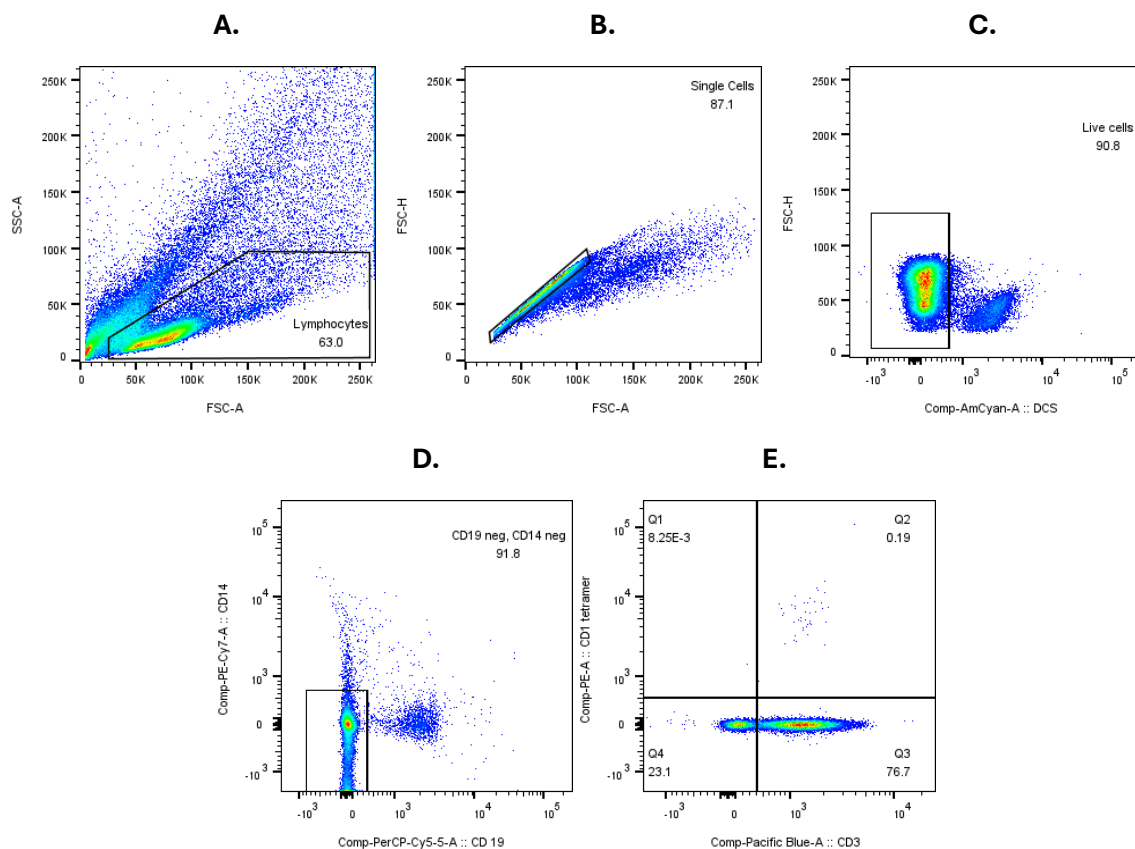


Figure 5.10. Gating strategy used for identifying CD1 tetramer-positive T cells.

PBMCs were incubated with an α -GalCer-loaded CD1d tetramer and antibodies specific for cell surface markers and acquired by flow cytometry. Dot plots show events and gates which have been drawn to include (A) lymphocytes, (B) single cells, (C) viable cells, (D) CD14-negative and CD19-negative cells, and (E) the top right quadrant represents CD1 tetramer-positive T cells.

5.2.5.5 Statistical Analysis

The percentage of tetramer-positive T cells in each sample was calculated by dividing the number of events in the CD3-positive/tetramer-positive quadrant (top right) by the total number of CD3-positive events (i.e., the sum of the top right and bottom right quadrants). Comparisons between unloaded and loaded tetramers were made based on

the percentage of positive T cells. A normality test was applied to assess the distribution of the paired differences between conditions across experiments. Depending on the outcome, either paired parametric tests (paired t-test) or non-parametric tests (Wilcoxon signed-rank test) were used. To enable comparison across independent experiments, data were normalized by expressing results as fold change relative to the unloaded tetramer condition.

5.2.6 CD1 Functional Assays

5.2.6.1 Cell Culture

Four cell lines were used in the cytokine functional assays: C1R mock-transfected cells (C1R-mock), C1R cells transfected with CD1a (C1R-CD1a), HeLa mock-transfected cells (HeLa-mock), and HeLa cells transfected with CD1d (HeLa-CD1d). Cell lines were kindly provided by Dr. Steven Porcelli (Albert Einstein College of Medicine, New York). C1R-mock and C1R-CD1a cells were cultured in complete RPMI (cRPMI) medium consisting of RPMI supplemented with 0.05 mM L-glutamine, 10% HyClone fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B (Fungizone), and 25 mM HEPES. Cells were maintained in T75 flasks and split three times per week at a 1:3 ratio. HeLa-mock and HeLa-CD1d cells were cultured in complete DMEM (cDMEM), which included DMEM supplemented with the same components as cRPMI. These cells were also maintained in T75 flasks and passaged three times per week at a 1:3 ratio. On days when cells were passaged or seeded for functional assays, 3.5 mL of pre-warmed (37 °C) trypsin was added to detach the adherent HeLa cells. Flasks were incubated at 37 °C for 3–5 minutes until detachment was observed.

5.2.6.2 Preparation of APCs and Lipid Loading

Target cell lines (C1R-mock, C1R-CD1a, HeLa-mock, HeLa-CD1d) were removed from the CO₂ incubator. HeLa cells were trypsinised as previously described to detach them from the T75 flasks. C1R cells (mock and CD1a-transfected) were resuspended by pipetting up and down several times using a pipette boy, then transferred to separate universal tubes using different pipettes. All cell suspensions were centrifuged at 1500 rpm for 8 minutes. HeLa cells (mock and CD1d-transfected) were each resuspended in 20 mL of cDMEM to neutralise residual trypsin, mixed thoroughly, transferred to separate universal tubes using different pipettes, and centrifuged at 1500 rpm for 8 minutes. Following

centrifugation, C1R and HeLa cells were resuspended in 10 mL of their respective media (cRPMI for C1R cells and cDMEM for HeLa cells). To determine cell concentration, 10 μ L of each suspension was mixed with 190 μ L trypan blue, and viable cells were counted using a haemocytometer. Based on the cell counts, suspensions were diluted to a final concentration of 1.0×10^6 cells/mL. From these suspensions, 100 μ L of each cell line was added per well of a 96-well plate, corresponding to 0.1×10^6 cells per well. Lipids were prepared at concentrations of 1 mg/mL in DMSO and stored in amber glass vials at -80°C . Prior to use, the vials were brought to room temperature and vortexed for 1 minute. Under sterile conditions, 2 μ L of each lipid stock solution was transferred into individual Eppendorf tubes containing 198 μ L of complete RPMI (for C1R cell assays) or 198 μ L of complete DMEM (for HeLa cell assays). This 1:100 dilution yielded a working concentration of 10 $\mu\text{g/mL}$ (equivalent to 1 $\text{ng}/\mu\text{L}$). From each lipid working solution, 2 μ L was added to the designated wells of a 96-well plate. This corresponds to a final lipid amount of 20 ng per well, or 196.1 ng/mL in a total well volume of 102 μL . The final DMSO concentration in the assay was 0.0198%. For unloaded controls, 0.0198% DMSO alone was added. The plate was incubated for 24 hours at 37°C and 5% CO_2 .

5.2.6.3 Incubation of PBMCs with APCs

PBMCs were retrieved from the incubator after being prepared as previously described (Section 4.2.4). The cells were counted, and sufficient cells were set aside to seed all wells, including a surplus for pipetting error. The cells were pelleted by centrifugation at 1500 rpm for 8 minutes and resuspended in complete RPMI (cRPMI) at a density of 2.0×10^6 cells/mL. Then, 100 μL (containing 0.2×10^6 cells) was added to each of the labelled wells in the 96-well plate (Fig. 5.11). One well was designated as unstimulated PBMCs, while another was designated as stimulated PBMCs and supplemented with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (1 $\mu\text{g/mL}$). cRPMI was added to each well to bring the final volume to 200 μL . Finally, 1 μL of Brefeldin A (2 mg/mL in DMSO; final concentration 10 $\mu\text{g/mL}$) was added to each well. The plate was incubated at 37°C with 5% CO_2 for 12 hours.

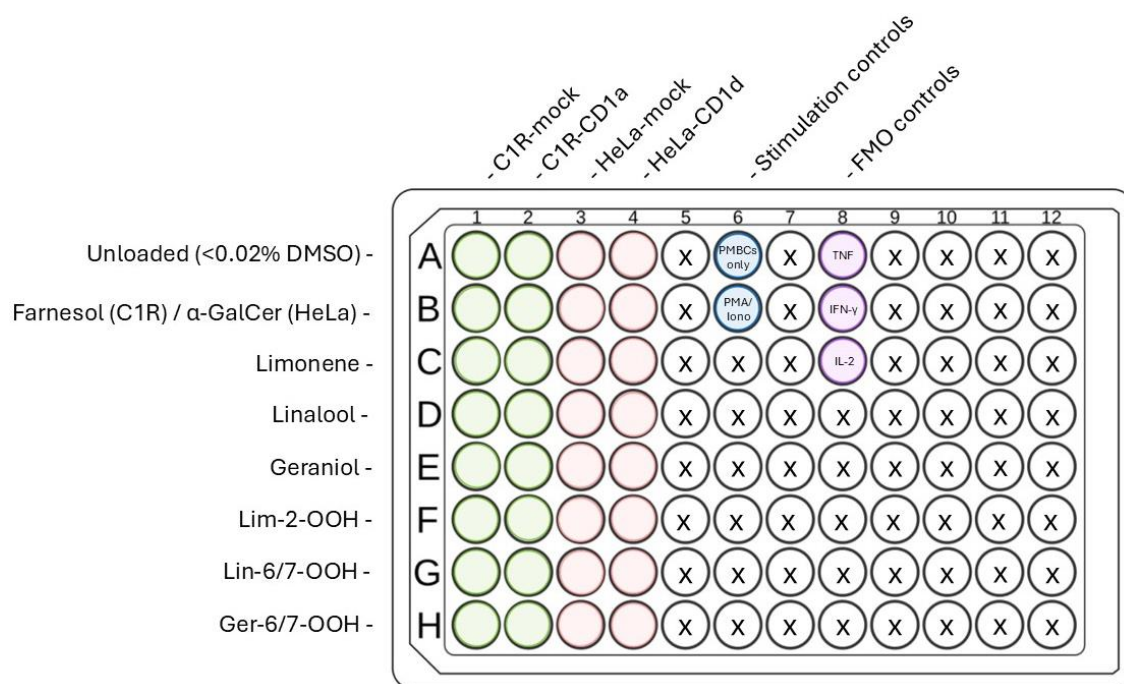


Figure 5.11. 96-well plate layout for CD1 functional assays PBMCs added.

Layout used for functional assays involving C1R-mock and C1R-CD1a cell lines (green), as well as HeLa-mock and HeLa-CD1d cell lines (pink). Lipids were added across rows, with their identities indicated to the left of the plate. PBMCs were added to every well except those marked with an 'X'. Stimulation controls (blue) included unstimulated PBMCs (well A6) and PBMCs stimulated with PMA and ionomycin (well B6). Additional wells (purple) were seeded with PBMCs for FMO controls. Wells marked with an 'X' were intentionally left empty.

5.2.6.4 Staining

After stimulating the PBMCs, cells were resuspended by pipetting up and down three times using a 200 μ L pipette and transferred to labelled FACS tubes. PBS (1 mL) was added to each tube, mixed by vortexing, and centrifuged at 1500 rpm for 8 minutes. Supernatants were discarded, and 100 μ L of dead cell stain (eFluor 506-conjugated fixable viability dye, diluted 1:1000 in PBS) was added to each tube. Tubes were vortexed and incubated in the dark at room temperature for 15 minutes. Next, 1 mL of FACS buffer was added to each tube, followed by vortexing and centrifugation at 1500 rpm for 8 minutes. A Fc block solution was prepared by mixing 3 μ L of Fc block with 47 μ L of FACS buffer for each tube. Fifty microliters of this Fc block solution was added to each tube, followed by vortexing and incubation in the dark at room temperature for 15 minutes. Cells were washed with 1 mL of FACS buffer, vortexed, and centrifuged at 1500 rpm for 8 minutes. A cell surface antibody cocktail was prepared by mixing CD3-Pacific Blue (1.25 μ L), CD4-FITC (1.25 μ L), CD8-APC/Cy7 (1.25 μ L), and FACS buffer (46.25 μ L) per tube. Fifty microliters of this cocktail was added to each tube, vortexed, and incubated in the

dark at room temperature for 20 minutes. FMO controls were occasionally prepared by including all antibodies except one in the panel. After staining, 1 mL of FACS buffer was added to each tube, followed by vortexing and centrifugation at 1500 rpm for 8 minutes. Supernatants were discarded, and 100 μ L of intracellular fixation buffer was added to each tube, vortexed, and incubated in the dark at room temperature for 20 minutes. Permeabilization buffer (10X) was diluted 1:10 with deionized water to prepare 1X working solution. One millilitre of this 1X permeabilization buffer was added to each tube, vortexed, and centrifuged at 1500 rpm for 8 minutes. An intracellular antibody cocktail was prepared by mixing TNF-PE (1.25 μ L), IFN- γ (1.25 μ L), IL-2 (1.25 μ L), and 1X permeabilization buffer (46.25 μ L) per tube. Fifty microliters of this cocktail was added to each tube, vortexed, and incubated in the dark at room temperature for 30 minutes. Cells were washed with 1 mL of FACS buffer, vortexed, and centrifuged at 1500 rpm for 8 minutes. After discarding the supernatant, cells were resuspended in 333 μ L of FACS buffer and prepared for acquisition by flow cytometry.

5.2.6.5 Flow Cytometry (Acquisition)

Using the BD FACSCanto II flow cytometer and FACSDiva software, a new experiment was created with the fluorochromes used: CD3-Pacific Blue, CD4-FITC, CD8-APC/Cy7, TNF-PE, IFN- γ -PE/Cy7, and IL-2-PerCP. Aside from the updated fluorochrome panel, the flow cytometry acquisition method was the same as previously described (section 5.2.5.3).

5.2.6.6 FlowJo Gating Strategy (Analysis)

FCS files exported from FACSDiva were imported into FlowJo v10 for analysis. Compensation controls were re-gated using the compensation tool in the Cytometry toolbar, and the resulting matrix was applied to all samples within the group. Using examples involving a C1R cell type (mock or transfectant) and a HeLa cell type (mock or transfectant) i.e., PBMCs from one donor which were incubated with one of these cells types, a polygon gate was drawn around the lymphocyte population on the FSC-A vs. SSC-A plot, encompassing the bottom-left quadrant and extending into the bottom-right quadrant (Fig. 5.12.A,E) This broader gate was used to include both resting and potentially activated T cells, which may exhibit altered size due to lipid binding events. A higher frequency of events can be seen in Fig. 5.6A relative to Fig. 5.6B because the

C1R cells are non-adherent and get harvested with the PBMCs, thus appearing as a separate population on FSC-A vs SSC-A. From the gated lymphocyte populations, the y-axis was changed from SSC-A to FSC-H to identify and include only single cells, excluding doublets or cell clusters (Fig. 5.12.B,F). By changing the x-axis parameter to the dead cell stain, a gate was drawn around the negative population to include only viable cells, while dead cell-positive events were excluded (Fig. 5.12.C,G). From the remaining viable lymphocyte population, the x-axis was set to CD3 and the y-axis to one of the inflammatory cytokines of interest (TNF, IFN- γ , or IL-2). A quadrant gate was applied to define four populations: cytokine-negative/CD3-positive T cells (bottom right), cytokine-positive/CD3-negative T cells (top left), cytokine-negative/CD3-negative (bottom left), and cytokine-positive/CD3-positive T cells (top right) (Fig. 5.12.D,H).

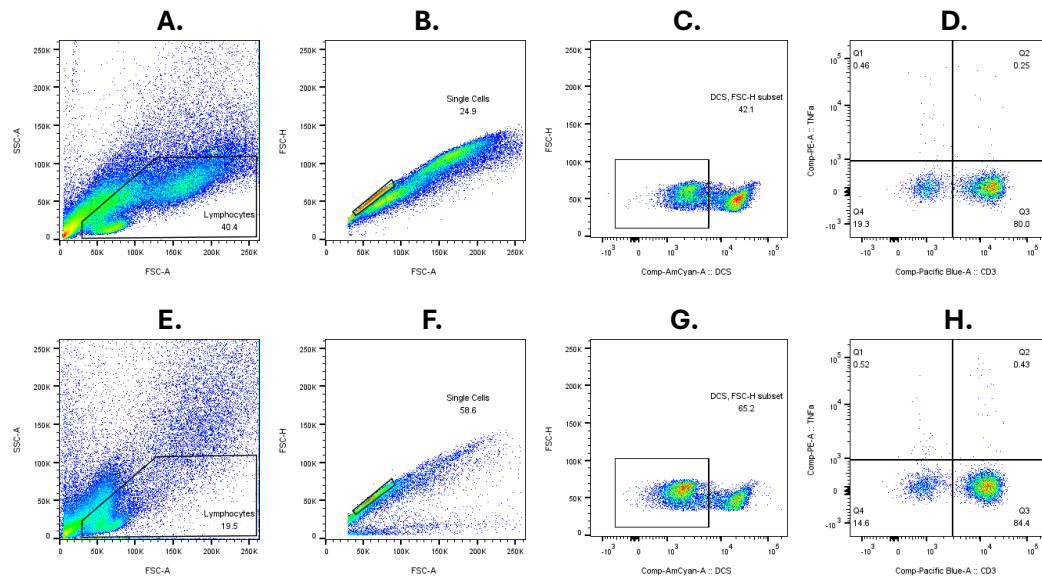


Figure 5.12. Gating strategy used for identifying cytokine-positive T cells.

Flow cytometric gating strategy for PBMCs co-cultured with C1R (A–D) or HeLa (E–H) cells. PBMCs were incubated with target cells and stained with antibodies against cell surface markers and intracellular cytokines. (A, E) Lymphocytes were identified by drawing a polygon gate in the lower left quadrant, extending into the lower right quadrant to capture potential activated T cells. (B, F) Single cells were selected by applying a polygon gate, excluding doublets and aggregates. (C, G) Live cells (viability dye-negative) were gated using a rectangular gate, excluding dead cells (viability dye-positive). (D, H) Quadrant gating was applied to distinguish four populations: cytokine⁺/CD3[−] (top left), cytokine[−]/CD3[−] (bottom left), cytokine[−]/CD3⁺ (bottom right), and cytokine⁺/CD3⁺ (top right).

5.3 Results

5.3.1 CD1a Tetramer Assays

CD1a tetramers were loaded with specific lipids, followed by incubation with PBMCs and stained with cell surface antibodies before acquisition by flow cytometry. The population of interest comprised events that were double-positive for CD1a tetramer and CD3 (T cells), representing tetramer-T cell complexes, and this population was located in the top right quadrant of the dot plots (Fig. 5.13). The dot plots shown are representative of the results observed across all experiments ($n = 12$).

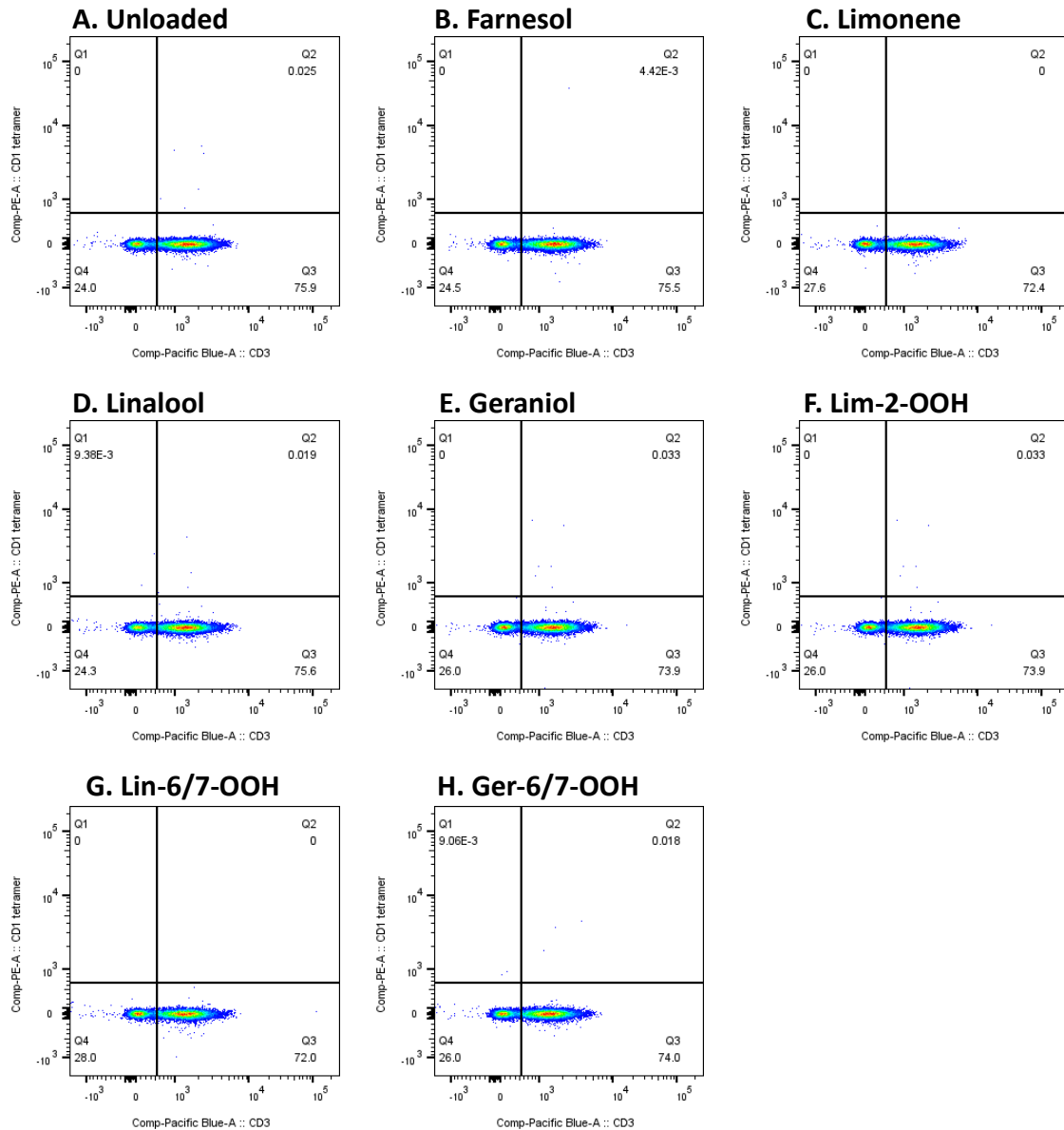
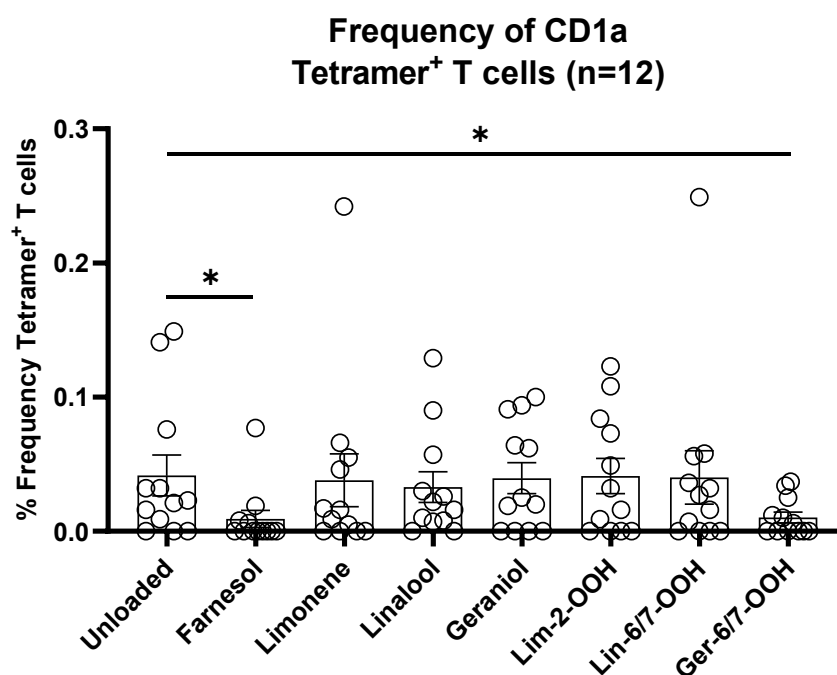


Figure 5.13. Flow cytometric analysis of CD1a tetramer⁺ T cells.

CD1a tetramers were loaded with individual lipids, stained together with cell surface markers, and analysed by flow cytometry. Representative dot plots are shown for each lipid-loaded CD1a tetramer. The top right quadrant indicates CD1a tetramer⁺/CD3⁺ T cell events.

Statistical analyses were performed to determine if there were significant differences in the percentage frequency of tetramer⁺/CD3⁺ complexes between unloaded and loaded tetramers. The mean frequency of tetramer⁺/CD3⁺ events for unloaded CD1a tetramers was 0.042% and results showed two statistically significant decreases in these events when CD1a tetramers were loaded with farnesol ($p = 0.0137$; mean frequency = 0.009%) and Ger-6/7-OOH ($p = 0.0137$; mean frequency = 0.010%) (Fig. 5.14A). For the other lipids tested, there were no clear increases or decreases in the frequency of tetramer⁺/CD3⁺ events. Frequency data was then normalised to the unloaded tetramers with the results then expressed in terms of their fold change to account for any inter-experimental variability. Statistical analyses revealed significant decreases in fold change for farnesol ($p = 0.0215$) and Ger-6/7-OOH ($p = 0.0137$), thereby confirming what was initially seen in frequency analyses (Fig. 5.14.B).

A.



B.

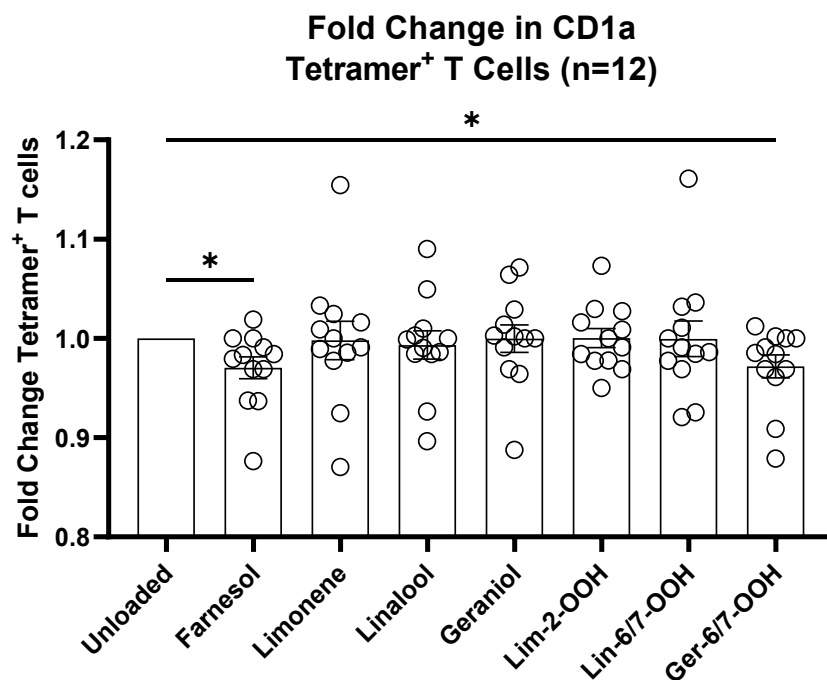


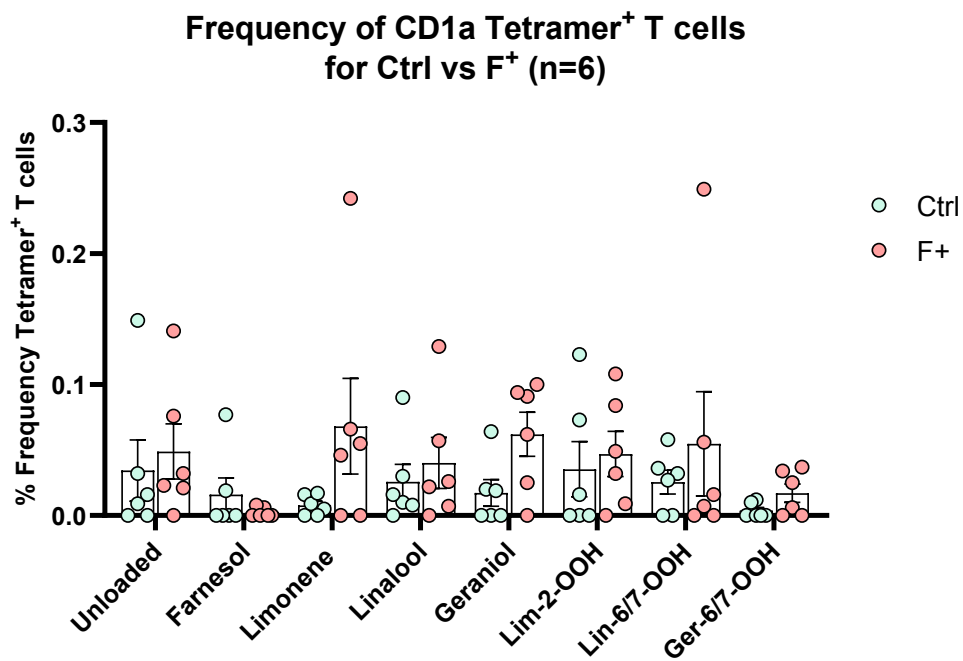
Figure 5.14. Frequency and normalization of CD1a tetramer-positive T cells.

Fluorescently labelled CD1a tetramers were loaded with different lipids, incubated with donor-derived PBMCs ($n = 12$), stained with cell surface marker antibodies, and analyzed by flow cytometry. **(A)** Percentage of CD1a tetramer-positive T cells for unloaded and loaded CD1a tetramers. **(B)** Frequency data normalized to unloaded CD1a tetramers, with loaded tetramer frequencies expressed as fold change. Statistical comparisons were performed relative to unloaded CD1a tetramers. Significant differences are indicated as: * $p \leq 0.05$. Statistical tests included paired t-tests or Wilcoxon matched-pairs signed-rank tests, as appropriate based on data normality, across twelve independent experiments ($n = 12$).

The pooled samples ($n = 12$) were then divided into their respective healthy control (Ctrl; $n = 6$) and fragrance allergy patient (F^+ ; $n = 6$) cohorts, and the frequency of tetramer⁺/CD3⁺ complexes was assessed (Fig. 5.15.A). The same trends that were observed for farnesol and Ger-6/7-OOH remained when comparing Ctrl and F^+ cohorts separately, however, none of the results reached the statistical significance seen when the samples were pooled ($n=12$). Interestingly, in the F^+ cohort, there appears to be a modest upward trend in tetramer⁺/CD3⁺ complexes for limonene and geraniol (not statistically significant, but not mirrored in the Ctrl cohort). The results were normalized to their respective unloaded tetramers for both Ctrl and F^+ cohorts, and the frequency of CD1a tetramer⁺/CD3⁺ complexes was expressed as fold change (Fig. 5.15.B). Consistent with the frequency data, a downward trend was observed for farnesol and

Ger-6/7-OOH in both cohorts, approaching but not reaching statistical significance in F⁺ ($p = 0.0683$ and $p = 0.0951$, respectively), along with the upward trends that were noted for limonene and geraniol in the F⁺ cohort, but without statistical significance.

A.



B.

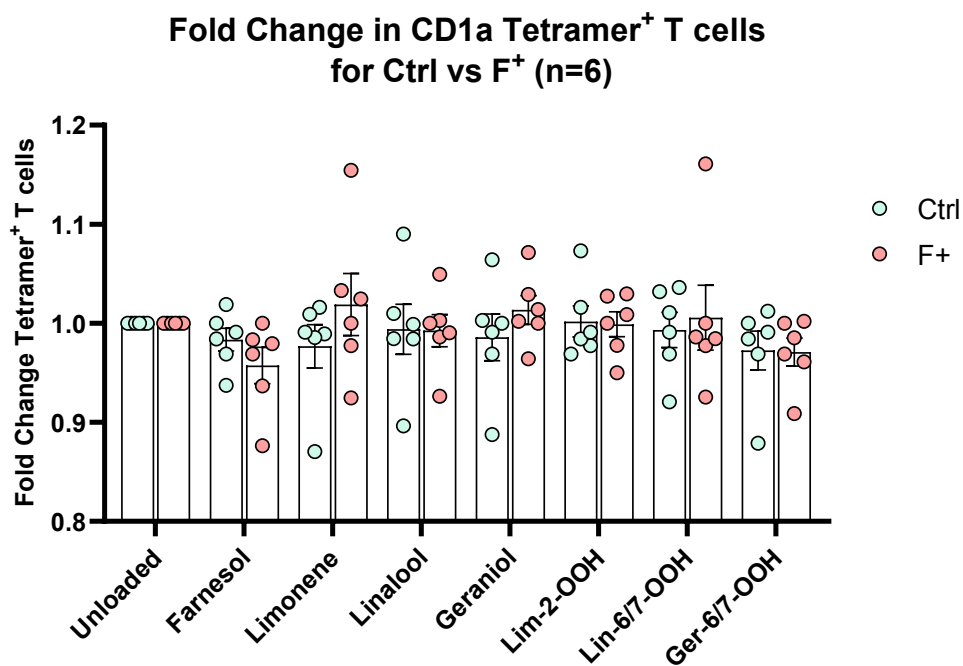
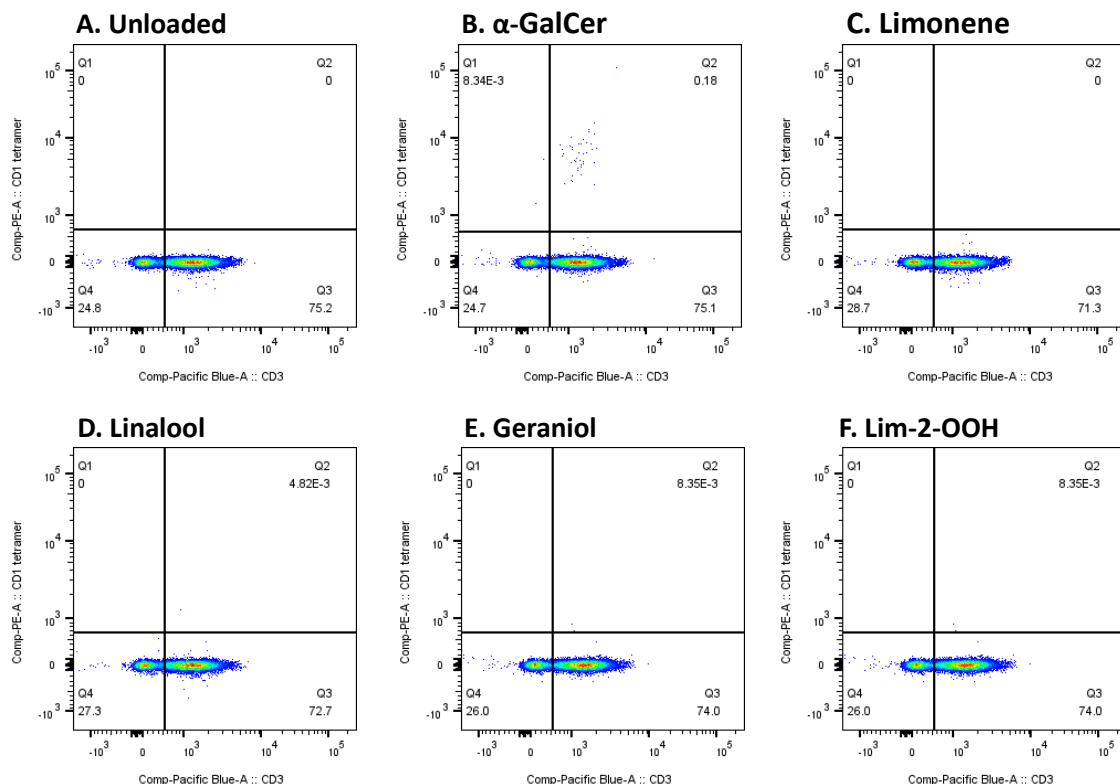


Figure 5.15. Comparison of CD1a tetramer-positive T cell frequencies between Ctrl and F⁺ cohorts.

Pooled data were divided into Ctrl (green; n = 6) and F⁺ (red; n = 6) cohorts. (A) Frequency of CD1a tetramer-positive T cells. (B) Fold change in CD1a tetramer-positive T cells. Differences were assessed between Ctrl and F⁺ groups. Statistical significance was assessed using paired t-tests or Wilcoxon matched pairs signed-rank tests, depending on data normality, for six independent experiments (n = 6).

5.3.2 CD1d Tetramer Assays

CD1d tetramers were loaded with specific lipids, followed by incubation with PBMCs and stained with cell surface antibodies before acquisition by flow cytometry. The population of interest was events that were double-positive for CD1d tetramer and CD3 (T cells), representing CD1d tetramer-T cell complexes located in the top right quadrant of the dot plots (Fig. 5.16). The dot plots shown are representative of the results observed across all experiments (n = 12).



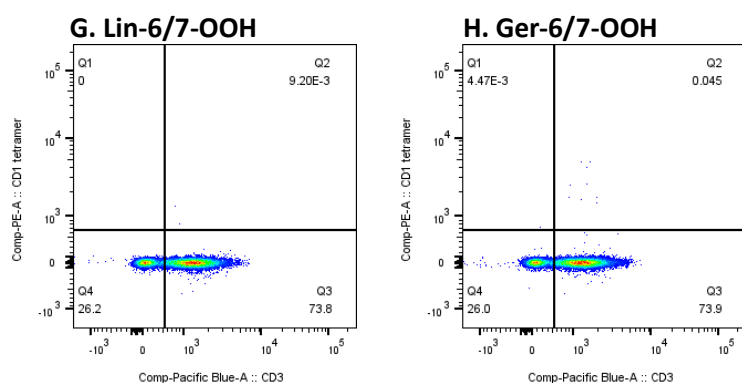
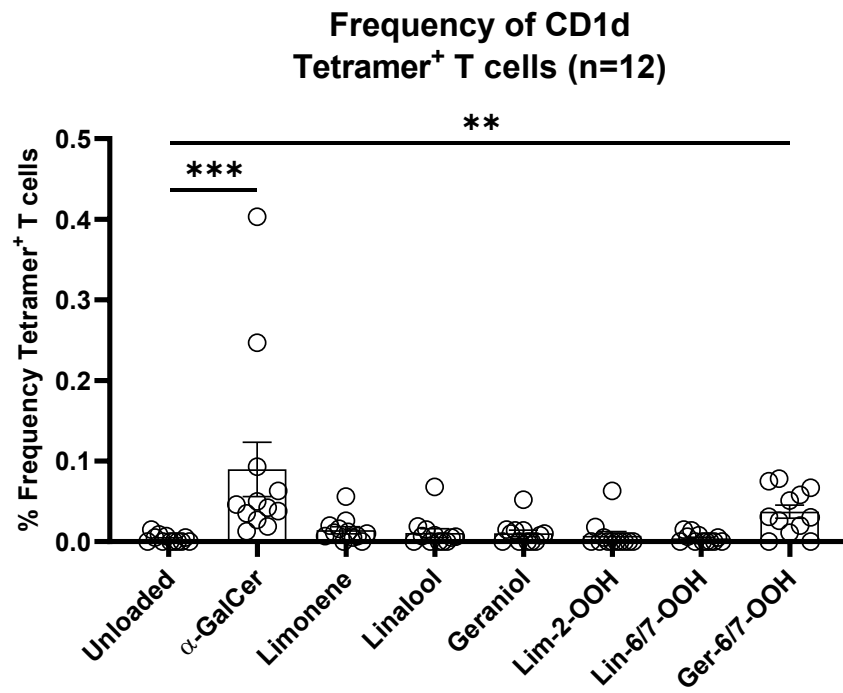


Figure 5.16. Flow cytometry analysis of CD1d tetramer⁺ T cells.

CD1d tetramers were loaded with individual lipids, stained together with cell surface markers, and analyzed by flow cytometry. Representative dot plots are shown for each lipid-loaded CD1d tetramer. The top right quadrant indicates CD1d tetramer⁺/CD3⁺ T cell events.

Statistical analyses were conducted to assess differences in the percentage of tetramer⁺/CD3⁺ events between unloaded and loaded tetramers. The mean frequency of tetramer⁺/CD3⁺ events for unloaded CD1d tetramers was 0.003%. Loading CD1d tetramers with α -GalCer (positive control) or Ger-6/7-OOH resulted in statistically significant increases in these events, with mean frequencies of 0.090% ($p = 0.0005$) and 0.037% ($p = 0.0012$), respectively (Fig. 5.17). Limonene showed a modest upward trend (mean frequency = 0.014%), but this did not reach statistical significance ($p = 0.0637$). No clear changes in tetramer⁺/CD3⁺ frequency were observed for the remaining lipids tested. These frequency results were then normalised to the unloaded tetramers, with the results then expressed in terms of their fold change (Fig. 5.17.B). Statistical analyses revealed significant increases in fold change for α -GalCer ($p = 0.0005$) and Ger-6/7-OOH ($p = 0.0012$), thereby confirming what was initially seen in frequency analyses.

A.



B.

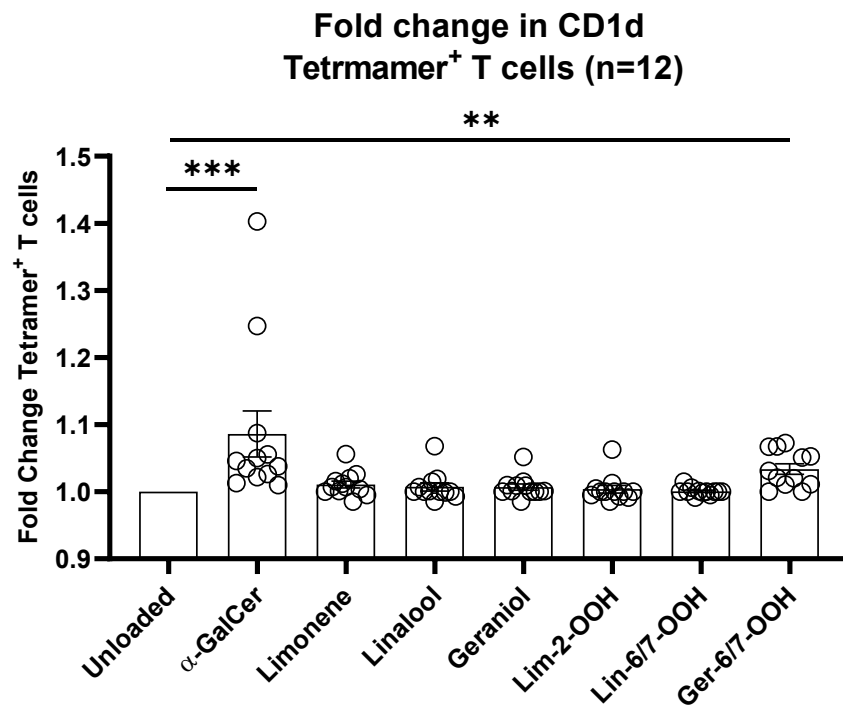


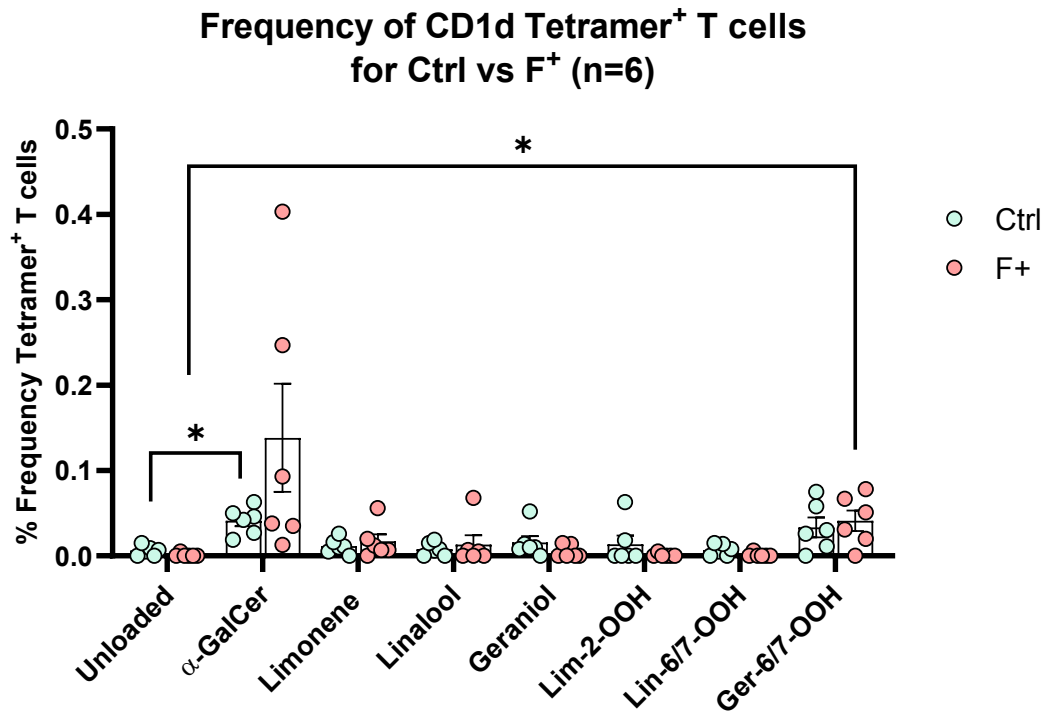
Figure 5.17. Frequency and fold change of CD1d tetramer-positive T cells following lipid loading.

Fluorescently labelled CD1d tetramers were loaded with various lipids, incubated with PBMCs from different donors (n = 12), stained with cell surface marker antibodies, and analyzed by flow cytometry. **(A)** Frequencies of CD1d tetramer-positive T cells. **(B)** Fold change in CD1d tetramer-positive T cells

normalized to unloaded CD1d tetramers. Significant differences relative to each lipid are indicated as: ** $p \leq 0.01$ and *** $p \leq 0.001$. Statistical significance was determined using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, across twelve independent experiments ($n = 12$).

The pooled samples ($n = 12$) were then divided into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts, and the frequency of tetramer⁺/CD3⁺ complexes was assessed (Fig. 5.18.A). The increases in CD1d tetramer-positive T cells observed for α -GalCer and Ger-6/7-OOH were also seen when the cohorts were analyzed separately. In the Ctrl, α -GalCer induced a significant increase in tetramer⁺/CD3⁺ events ($p = 0.0051$), while in the F⁺, the increase Unloaded fell short of statistical significance ($p = 0.0828$). Ger-6/7-OOH produced a statistically α -GalCer significant increase in the F⁺ ($p = 0.0176$) but fell short of statistical significance in the Ctrl ($p = 0.0556$). In both cohorts, limonene exhibited a modest upward trend in tetramer⁺/CD3⁺ frequency, but this did not reach significance. Results from Ctrl and F⁺ were normalized to their respective unloaded CD1d-tetramer frequencies and the frequencies of lipid-loaded CD1d tetramer⁺/CD3⁺ complexes were expressed as fold change relative to unloaded tetramers (Fig. 5.18.B). Consistent with the frequency data, α -GalCer induced a significant increase in the control cohort ($p = 0.0052$) and approached significance in the patient cohort ($p = 0.0829$). Ger-6/7-OOH elicited a significant increase in the F⁺ samples ($p = 0.0175$) but fell short of statistical significance in the Ctrl samples ($p = 0.0557$). The modest increase observed for limonene in the frequency data was also reflected in the fold change for the F⁺ samples, although this did not reach significance ($p = 0.1198$).

A.



B.

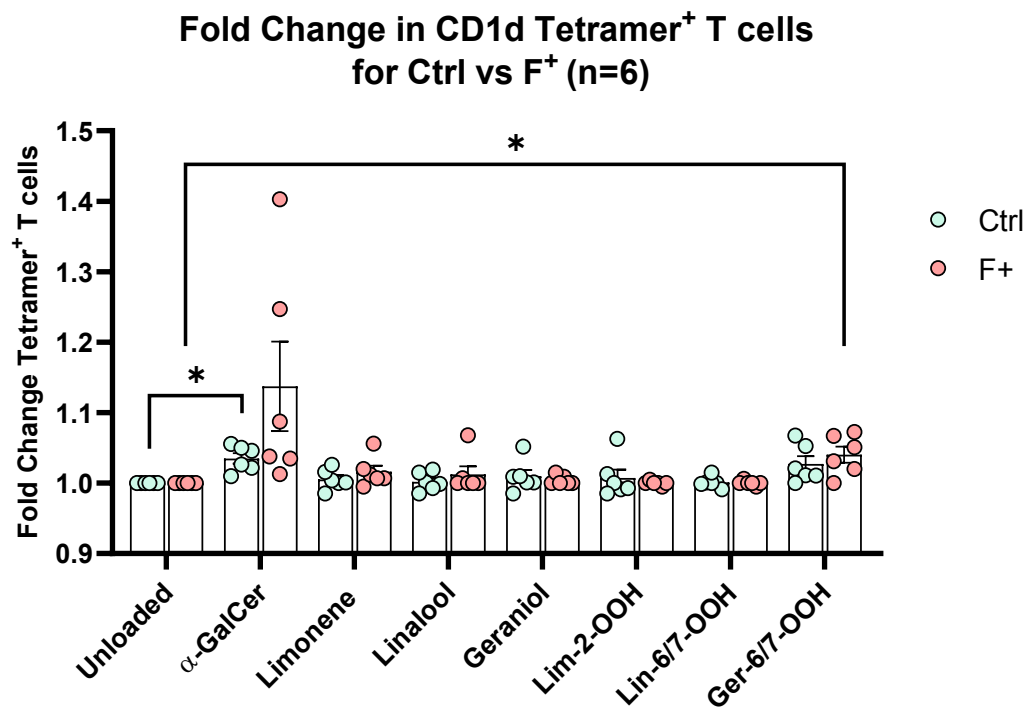


Figure 5.18. Comparison of CD1d tetramer-positive T cells between Ctrl and F⁺ cohorts.

(A) Frequency of CD1d tetramer-positive T cells in Ctrl and F⁺ groups. **(B)** Fold change in CD1d tetramer positive T cells, with Ctrl and F⁺ frequencies normalized to their respective unloaded CD1d tetramer conditions. Significant differences relative to unloaded tetramers are indicated as follows: * $p \leq 0.001$.

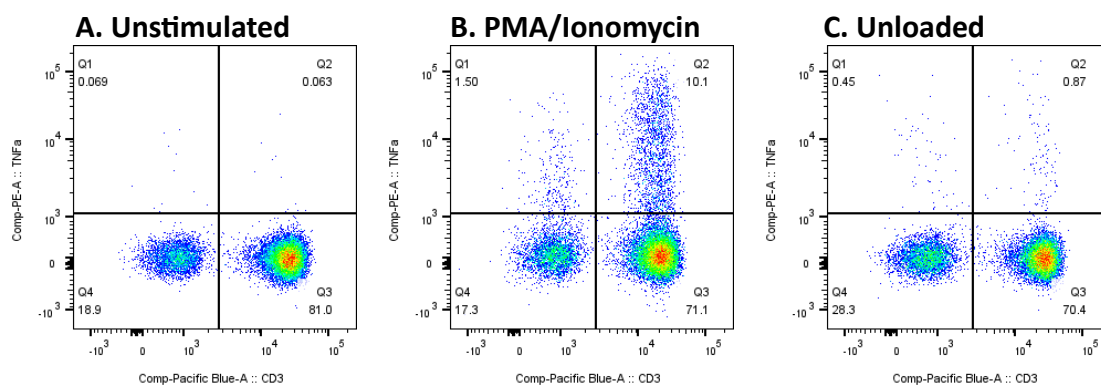
Non-significant results are left unmarked. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for six independent experiments (n = 6).

5.3.3 CD1a Functional Assays

C1R-mock and C1R-CD1a cells were loaded with various lipids and co-cultured with PBMCs from the same donors described in CD1 tetramer assays. Following incubation, cells were harvested and stained with cell-surface and intracellular cytokine antibodies. Flow cytometric analysis was performed, and cytokine⁺/CD3⁺ events were quantified.

5.3.3.1 TNF

Representative dot plots of TNF⁺/CD3⁺ events (Fig. 5.19) illustrate consistent patterns observed across all experiments (n = 12). Control samples included unstimulated PBMCs (Fig. 5.19.A) and PBMCs stimulated with PMA/ionomycin in the absence of C1R-CD1a cells (Fig. 5.19.B). Unstimulated PBMCs exhibited negligible TNF⁺ T cell frequencies, whereas PMA/ionomycin stimulation induced a robust TNF⁺ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.19.C) or lipid-loaded C1R-CD1a cells (Fig. 5.19.D-J) resulted in a moderate increase in TNF⁺ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.



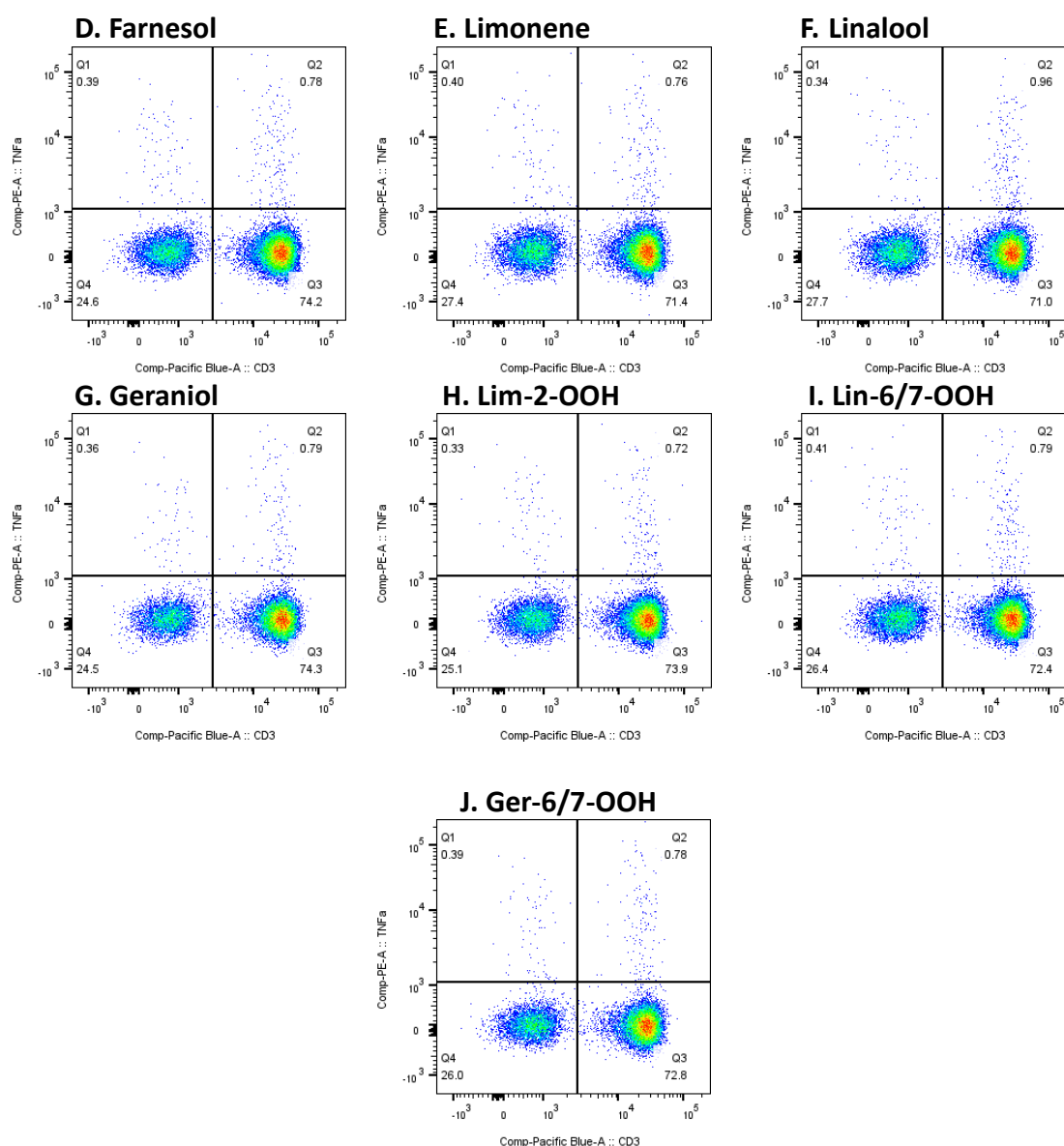


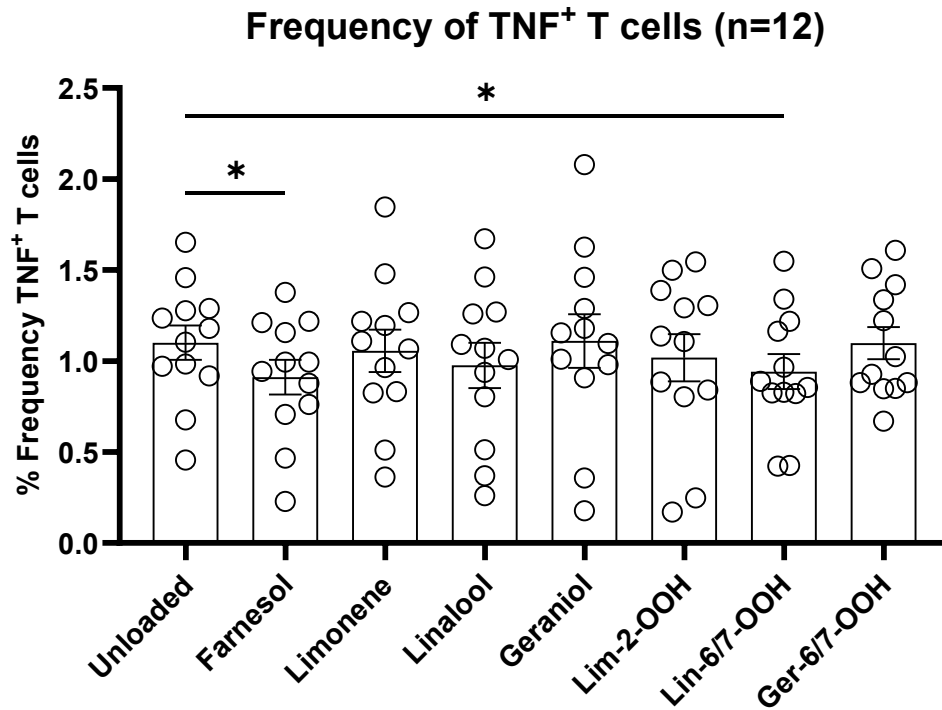
Figure 5.19. Flow cytometry analysis of TNF⁺ T cells.

C1R-CD1a cells were loaded with various lipids and co-cultured with PBMCs. Cells were then stained with cell-surface and intracellular antibodies and analyzed by flow cytometry. Representative dot plots are shown for (A) unstimulated PBMCs (without C1R-CD1a cells), (B) PBMCs stimulated with PMA/ionomycin (without C1R-CD1a cells), (C) PBMCs incubated with each unloaded C1R-CD1a cells, and (D-J) PBMCs incubated with lipid-loaded C1R-CD1a cells. The top-right quadrant indicates TNF⁺/CD3⁺ T cell events.

Statistical analyses were performed to assess differences in the frequency of TNF⁺/CD3⁺ events between unloaded and lipid-loaded conditions. The mean frequency of TNF⁺/CD3⁺ events in unloaded samples was 1.100% (Fig. 5.20.A). Significant reductions were observed for farnesol (mean: 0.911%, $p = 0.0162$) and Lin-6/7-OOH (mean: 0.942%, $p = 0.0149$). To account for any inter-experimental variability, frequency data was normalized and represented in fold changes relative to unloaded conditions (Fig. 5.20.B).

The significant reductions for farnesol and Lin-6/7-OOH were maintained ($p = 0.0170$ and $p = 0.0095$, respectively).

A.



B.

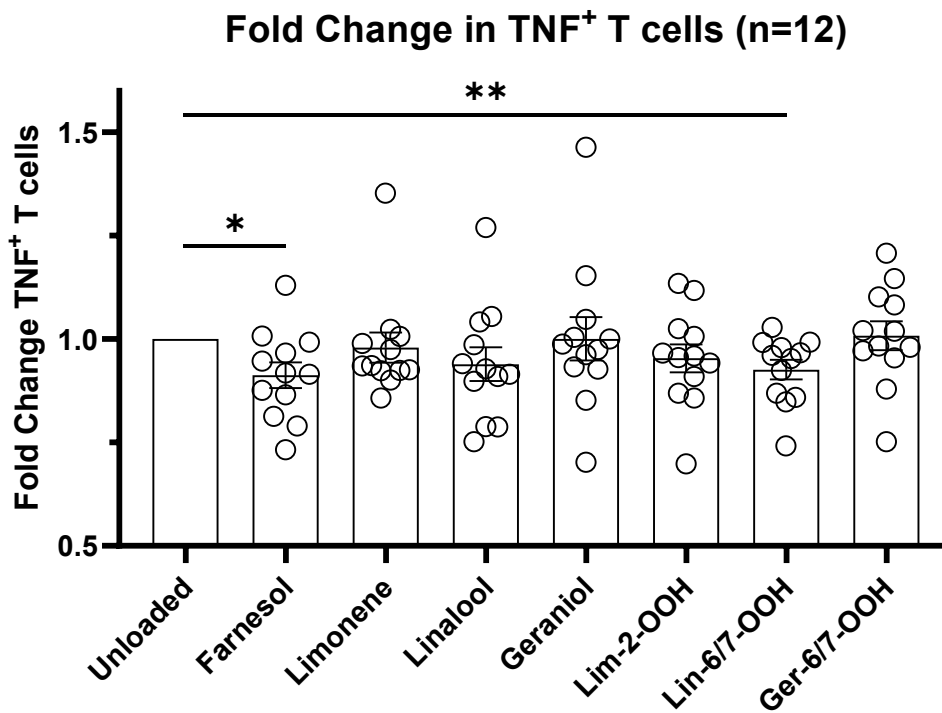


Figure 5.20. TNF⁺ T cell responses to lipid-loaded C1R-CD1a cells.

C1R-CD1a cells were loaded with lipids, co-cultured with PBMCs, stained with cell surface antibodies, and analyzed by flow cytometry. (A) Frequencies of TNF⁺ T cells for unloaded and lipid-loaded C1R-CD1a cells. (B) Fold change in TNF⁺ T cell frequencies normalized to unloaded C1R-CD1a conditions. Significant differences relative to unloaded tetramers are indicated as follows: * $p \leq 0.05$ and ** $p \leq 0.01$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for six independent experiments (n = 12).

As a control, C1R-mock cells were co-cultured with PBMCs to assess whether changes in TNF⁺ T cell frequency were CD1a-dependent. TNF⁺/CD3⁺ frequencies from C1R-mock and C1R-CD1a cells were compared across 12 donors (Fig. 5.21). The mean frequency in unloaded C1R-mock cells (0.659%) was significantly lower than in unloaded C1R-CD1a cells (1.100%; $p = 0.0002$). This increase in C1R-CD1a cells likely reflects autoreactivity driven by presentation of endogenously loaded self-lipids, providing a baseline or “background” level of reactivity. When lipid-loaded conditions were analysed, the relative difference between C1R-mock and C1R-CD1a cells was used to infer whether exogenous lipids displaced or interacted with endogenous ligands. For farnesol, TNF⁺ T cell frequency in C1R-CD1a cells remained significantly higher than in C1R-mock cells ($p = 0.0197$), but the magnitude of this difference was smaller than in the unloaded comparison. This reduction suggests that farnesol interacted with, or displaced, endogenous lipids. This interpretation is consistent with Fig. 5.20, where farnesol loading altered TNF⁺ T cell frequencies in C1R-CD1a cells. By contrast, limonene, linalool, geraniol, and Ger-6/7-OOH also produced significant increases in TNF⁺ T cell frequencies between C1R-CD1a and C1R-mock cells, but the magnitude of these differences closely mirrored those of the unloaded controls. This suggests that the observed increases likely reflect presentation of endogenous self-lipids rather than specific effects of these compounds, in line with the lack of changes in TNF⁺ frequencies observed in Fig. 5.20. Likewise, Lin-6/7-OOH produced a significant increase in TNF⁺ T cell frequency when comparing C1R-CD1a and C1R-mock cells ($p = 0.0206$). However, the magnitude of this difference was again lower than in the unloaded control, suggesting that Lin-6/7-OOH may displace or interfere with endogenous ligands. This is consistent with Fig. 5.20, where Lin-6/7-OOH loading led to a significant reduction in TNF⁺ T cell frequency relative to unloaded C1R-CD1a conditions.

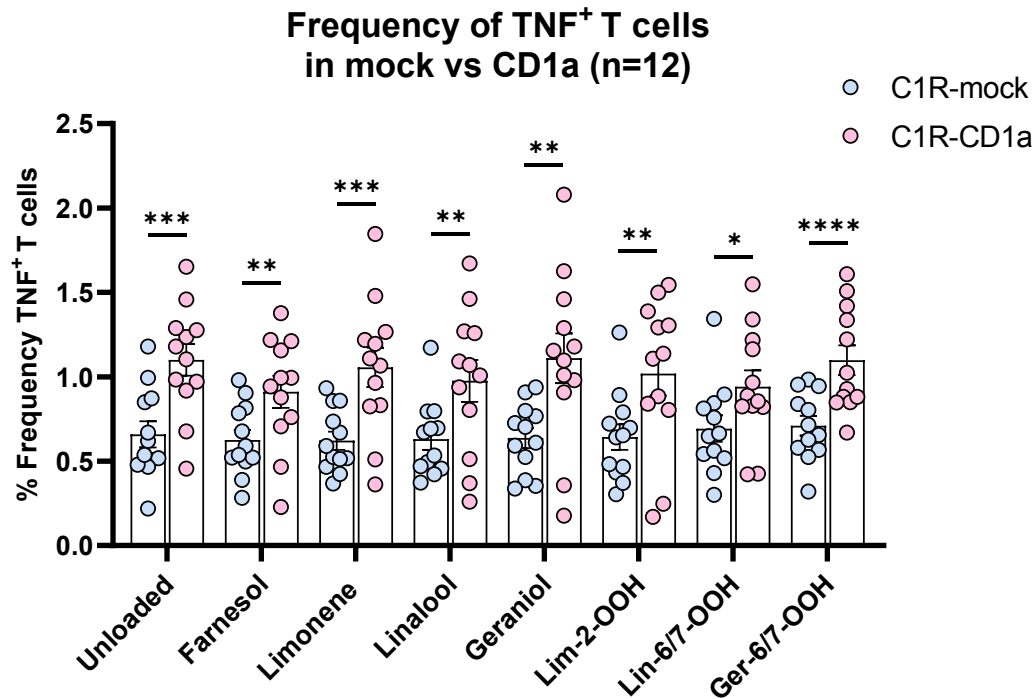
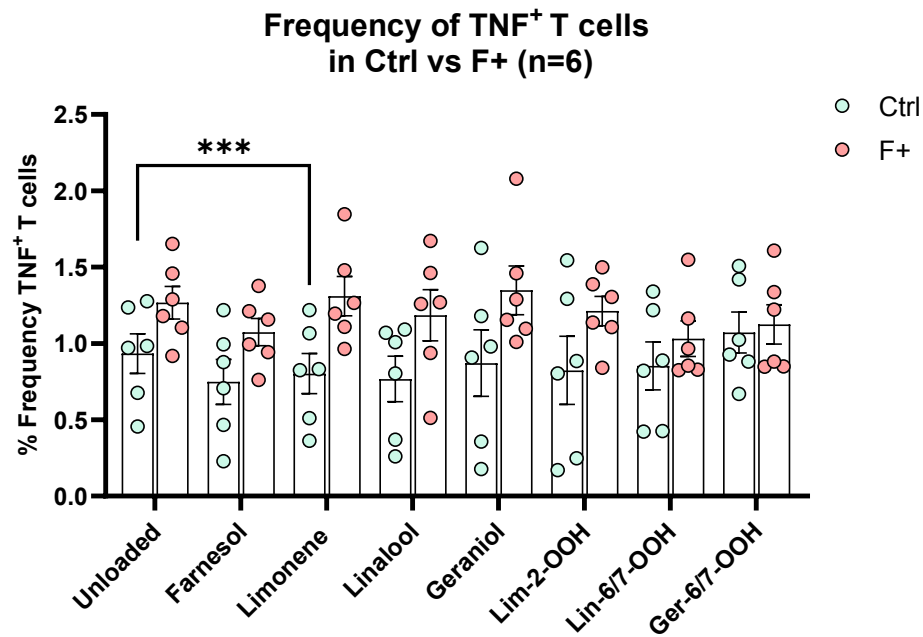


Figure 5.21. TNF⁺ T cell responses to lipid-loaded C1R-mock and C1R-CD1a cells.

Frequency of TNF⁺ T cells following co-culture with C1R-mock (blue) or C1R-CD1a (pink) cells loaded with various lipids. Significant differences between C1R-mock and C1R-CD1a are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

The pooled dataset ($n = 12$) was divided into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts to compare TNF⁺/CD3⁺ T cell responses to unloaded and lipid-loaded C1R-CD1a cells. Overall, the F⁺ cohort exhibited higher TNF⁺ frequencies across all conditions, with mean frequencies in unloaded C1R-CD1a cells of 0.933% for Ctrl and 1.267% for F⁺ (Fig. 5.22.A). The only statistically significant decrease was observed for limonene in the Ctrl cohort ($p = 0.0009$), while farnesol showed a downward trend that did not reach significance ($p = 0.0517$). Other lipid conditions displayed modest increases or decreases between cohorts, none of which were statistically significant. When frequencies were expressed as fold changes relative to unloaded C1R-CD1a cells (Fig. 5.22.B), farnesol showed a non-significant downward trend in the Ctrl cohort ($p = 0.0681$). Limonene again exhibited a significant decrease in Ctrl ($p = 0.0010$) but not in F⁺ ($p = 0.7084$). Conversely, Lin-6/7-OOH caused a significant decrease in F⁺ ($p = 0.0454$) that was absent in Ctrl ($p = 0.1325$).

A.



B.

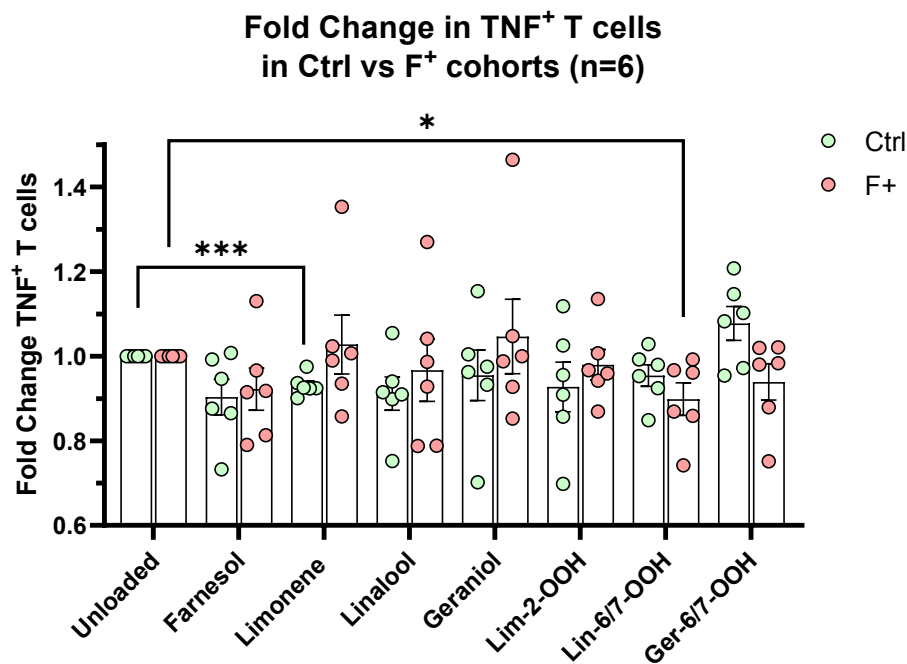
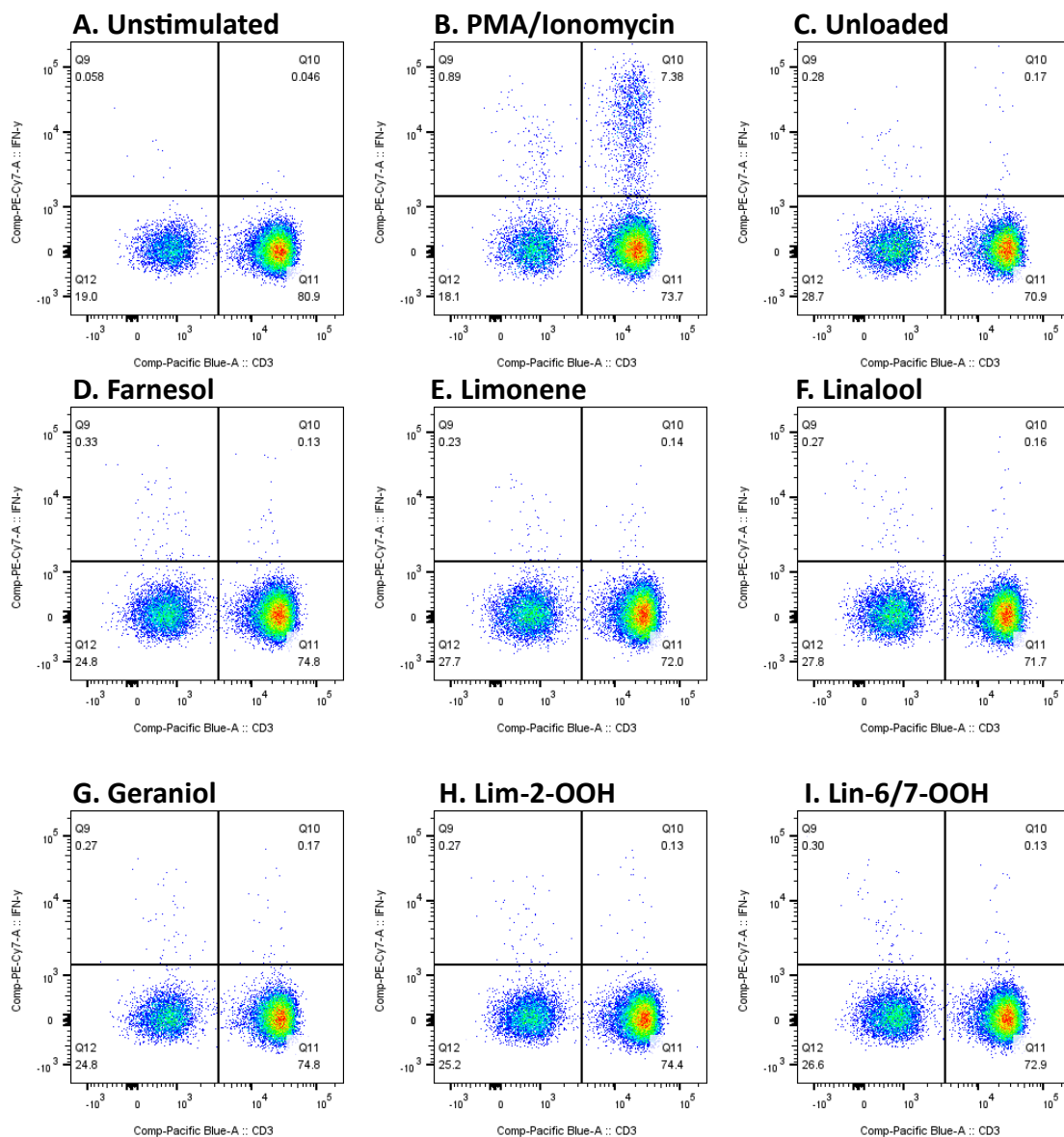


Figure 5.22. TNF⁺ T cell responses in Ctrl and F⁺ subsets to lipid-loaded C1R-CD1a cells.

(A) Differences in TNF⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets (n = 6) following co-culture with lipid-loaded C1R-CD1a cells, expressed relative to unloaded conditions. (B) Differences in TNF⁺ T cell frequencies between Ctrl and F⁺ subsets (n = 6) expressed as fold changes normalized to their respective unloaded C1R-CD1a conditions are indicated as follows: * $p \leq 0.05$ and *** $p \leq 0.001$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments (n = 12).

5.3.3.2 IFN- γ

Representative dot plots of IFN- γ^+ /CD3 $^+$ (Fig. 5.23) illustrate consistent patterns observed across all experiments (n = 12). Control samples included unstimulated PBMCs (Fig. 5.23.A) and PBMCs stimulated with PMA/ionomycin in the absence of C1R-CD1a cells (Fig. 5.23.B). Unstimulated PBMCs exhibited negligible IFN- γ^+ T cell frequencies, whereas PMA/ionomycin stimulation induced a strong IFN- γ^+ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.23.C) or lipid-loaded C1R-CD1a cells (Fig. 5.23.D-J) resulted in a moderate increase in IFN- γ^+ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.



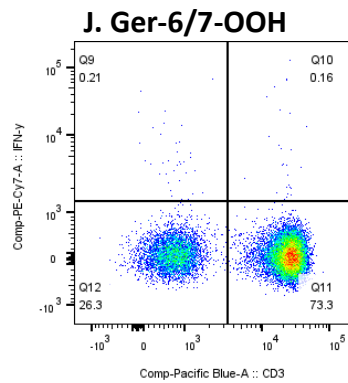
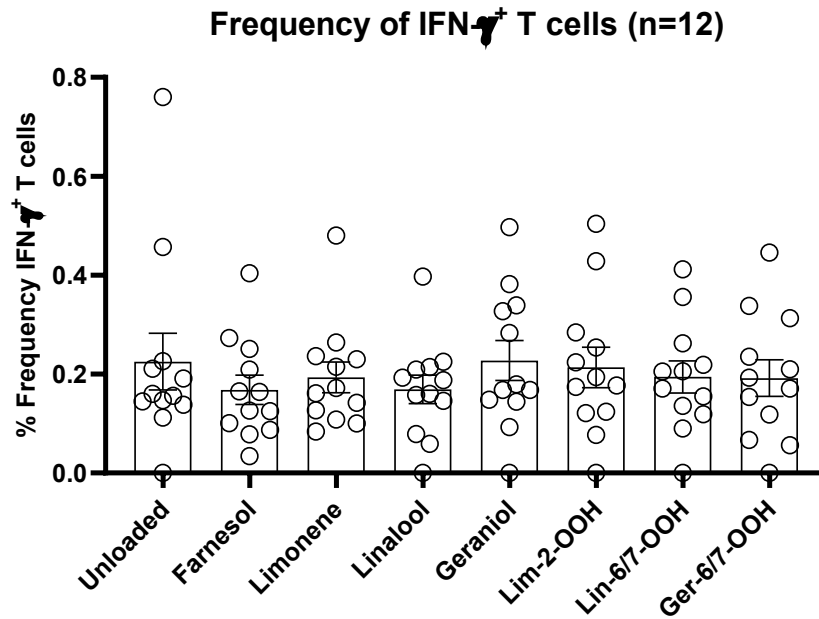


Figure 5.23. Flow cytometric analysis of IFN- γ ⁺ T cells.

Stained with cell surface markers and an intracellular IFN- γ antibody, cells were then analysed by flow cytometry. Representative dot plots are shown for **(A)** unstimulated PBMCs (without C1R-CD1a cells), **(B)** PBMCs stimulated with PMA/ionomycin (without C1R-CD1a cells), and **(C–J)** PBMCs incubated with each lipid-loaded C1R-CD1a condition. IFN- γ ⁺/CD3⁺ T cells are located in the top-right quadrant.

Statistical analyses were performed to assess differences in IFN- γ ⁺/CD3⁺ T cell frequencies between unloaded and lipid-loaded C1R-CD1a cells. The mean frequency of IFN- γ ⁺/CD3⁺ events in unloaded samples was 0.225% (Fig. 5.24.A). Several lipid-loaded conditions exhibited downward trends in IFN- γ ⁺/CD3⁺ frequencies, although none reached statistical significance. To account for inter-experimental variability, frequencies were normalized to the unloaded C1R-CD1a condition and expressed as fold changes (Fig. 5.24.B). This normalization revealed a significant decrease for Lin-6/7-OOH ($p = 0.0095$).

A.



B.

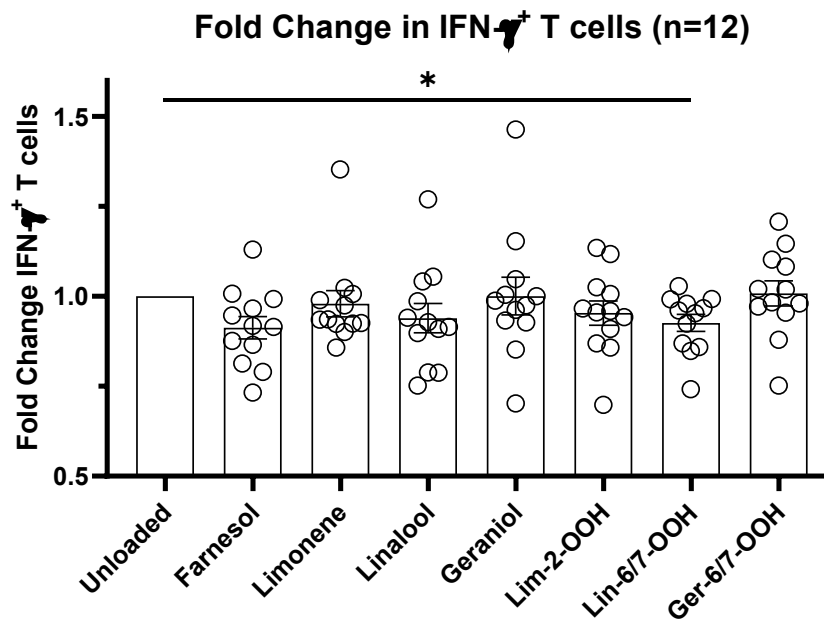


Figure 5.24. IFN- γ ⁺ T cell responses to lipid-loaded C1R-CD1a cells.

C1R-CD1a cells were loaded with various lipids and co-cultured with PBMCs. Following incubation, cells were stained with cell-surface antibodies and intracellular IFN- γ antibody and analysed by flow cytometry. **(A)** Frequencies of IFN- γ ⁺/CD3⁺ events. **(B)** IFN- γ ⁺/CD3⁺ frequencies normalized to unloaded C1R-CD1a cells and expressed as fold changes. Significant differences relative to unloaded cells are indicated as follows: * $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, across twelve independent experiments (n = 12).

As a control, C1R-mock cells were co-cultured with PBMCs to assess whether changes in IFN- γ ⁺ T cell frequency were CD1a-dependent. IFN- γ ⁺/CD3⁺ frequencies from C1R-mock and C1R-CD1a cells were compared across 12 donors (Fig. 5.25). The mean frequency in unloaded C1R-mock cells (0.176%) was lower than in unloaded C1R-CD1a cells (0.225%), and this trend persisted across all lipid-loaded conditions. Overall, consistent with the IFN- γ ⁺/CD3⁺ frequency data for C1R-CD1a cells, no significant differences were observed between C1R-mock and C1R-CD1a cells under most conditions. The exception was limonene loading, where a significant increase was detected between mock and transfectants. However, as shown in Fig. 5.24, IFN- γ ⁺ T cell frequencies in C1R-CD1a cells were actually lower upon limonene loading compared with the unloaded condition. Therefore, the apparent significance observed in this comparison is more likely attributable to a reduction in IFN- γ ⁺ T cell frequencies in limonene-treated C1R-mock cells, rather than a genuine increase in the transfected cells.

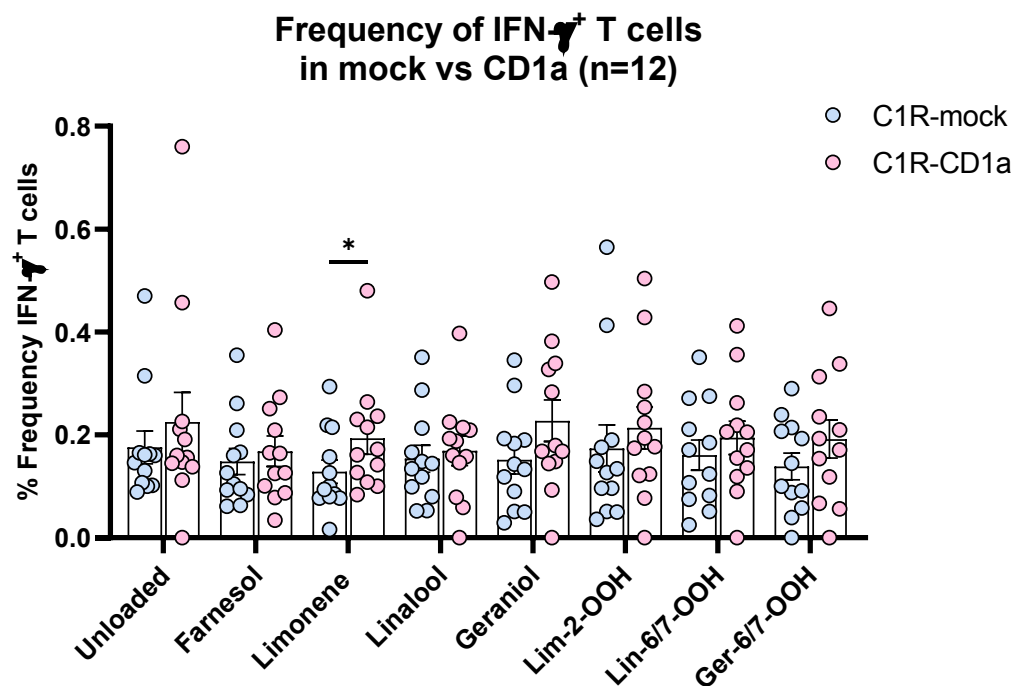
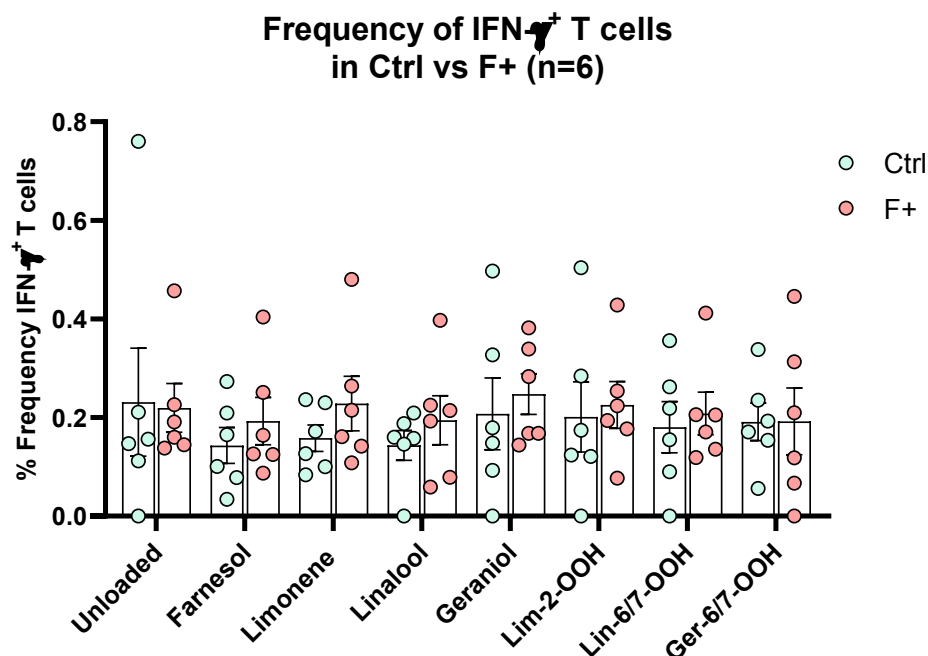


Figure 5.25. IFN- γ ⁺ T cell responses to lipid-loaded C1R-mock and C1R-CD1a cells.

Frequency of IFN- γ ⁺ T cells following co-culture with C1R-mock (blue) or C1R-CD1a (pink) cells loaded with various lipids. Significant differences relative to unloaded cells are indicated as follows: * $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments (n = 12).

The pooled dataset ($n = 12$) was stratified into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts to evaluate IFN- γ ⁺/CD3⁺ T cell responses to unloaded and lipid-loaded C1R-CD1a cells. Overall, both cohorts exhibited comparable IFN- γ ⁺ frequencies across all conditions, with mean frequencies in unloaded C1R-CD1a cells of 0.220% for Ctrl and 0.231% for F⁺ (Fig. 5.26.A). Farnesol induced a downward trend in IFN- γ ⁺ T cell frequency within the Ctrl cohort (mean = 0.143%), although this did not reach statistical significance ($p = 0.0835$). Frequency data were subsequently normalized to the unloaded C1R-CD1a condition for each cohort and expressed as fold changes (Fig. 5.26.B). A significant reduction in IFN- γ ⁺ T cells was observed in the Ctrl cohort for limonene ($p = 0.0010$) and in the F⁺ cohort for Lin-6/7-OOH ($p = 0.0454$). Farnesol exhibited non-significant downward trends in both cohorts ($p = 0.0681$ and $p = 0.1783$, respectively). Notably, Ger-6/7-OOH in the Ctrl cohort produced a distinct upward trend in IFN- γ ⁺ frequency compared with all other lipid conditions; however, the data was not normally distributed, and this effect was not confirmed as statistically significant by Wilcoxon matched-pairs signed-rank testing.

A.



B.

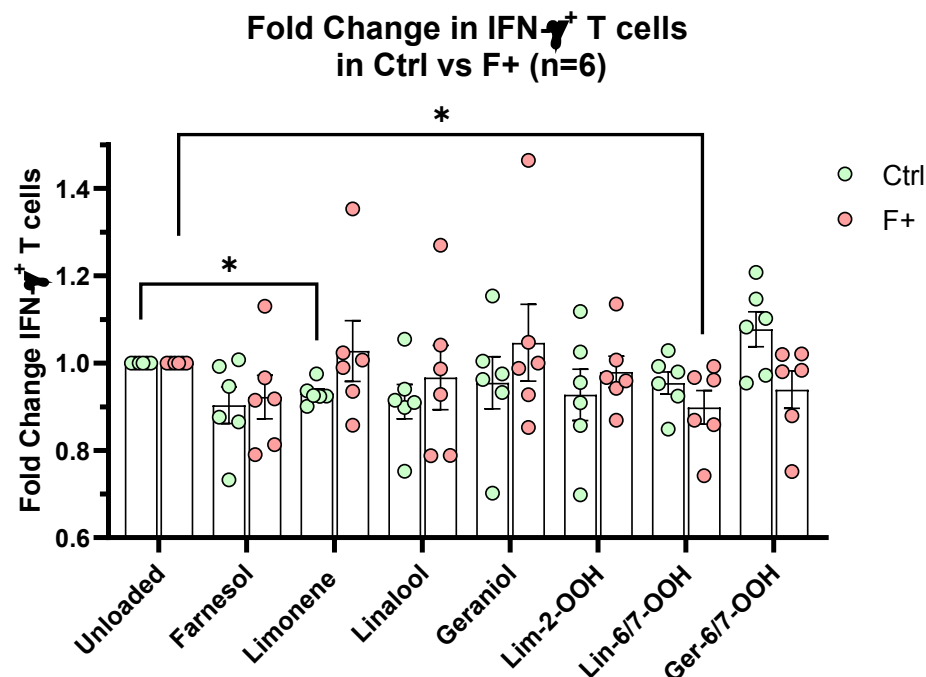


Figure 5.26. IFN- γ ⁺ T cell responses in Ctrl and F⁺ subsets to lipid-loaded C1R-CD1a cells.

(A) Differences in IFN- γ ⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets ($n = 6$) following co-culture with lipid-loaded C1R-CD1a cells, expressed relative to unloaded conditions. **(B)** Differences in IFN- γ ⁺ T cell frequencies between Ctrl and F⁺ subsets ($n = 6$) expressed as fold changes normalized to their respective unloaded C1R-CD1a conditions. Significant differences relative to unloaded cells are indicated as follows: * $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

5.3.3.3 IL-2

Representative dot plots of IL-2⁺/CD3⁺ (Fig. 5.27) illustrate consistent patterns observed across all experiments ($n = 12$). Control samples included unstimulated PBMCs (Fig. 5.27.A) and PBMCs stimulated with PMA/ionomycin in the absence of C1R-CD1a cells (Fig. 5.27.B). Unstimulated PBMCs exhibited negligible IL-2⁺ T cell frequencies, whereas PMA/ionomycin stimulation induced a robust IL-2⁺ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.27.C) or lipid-loaded C1R-CD1a cells (Fig. 5.27.D–J) resulted in a moderate increase in IL-2⁺ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.

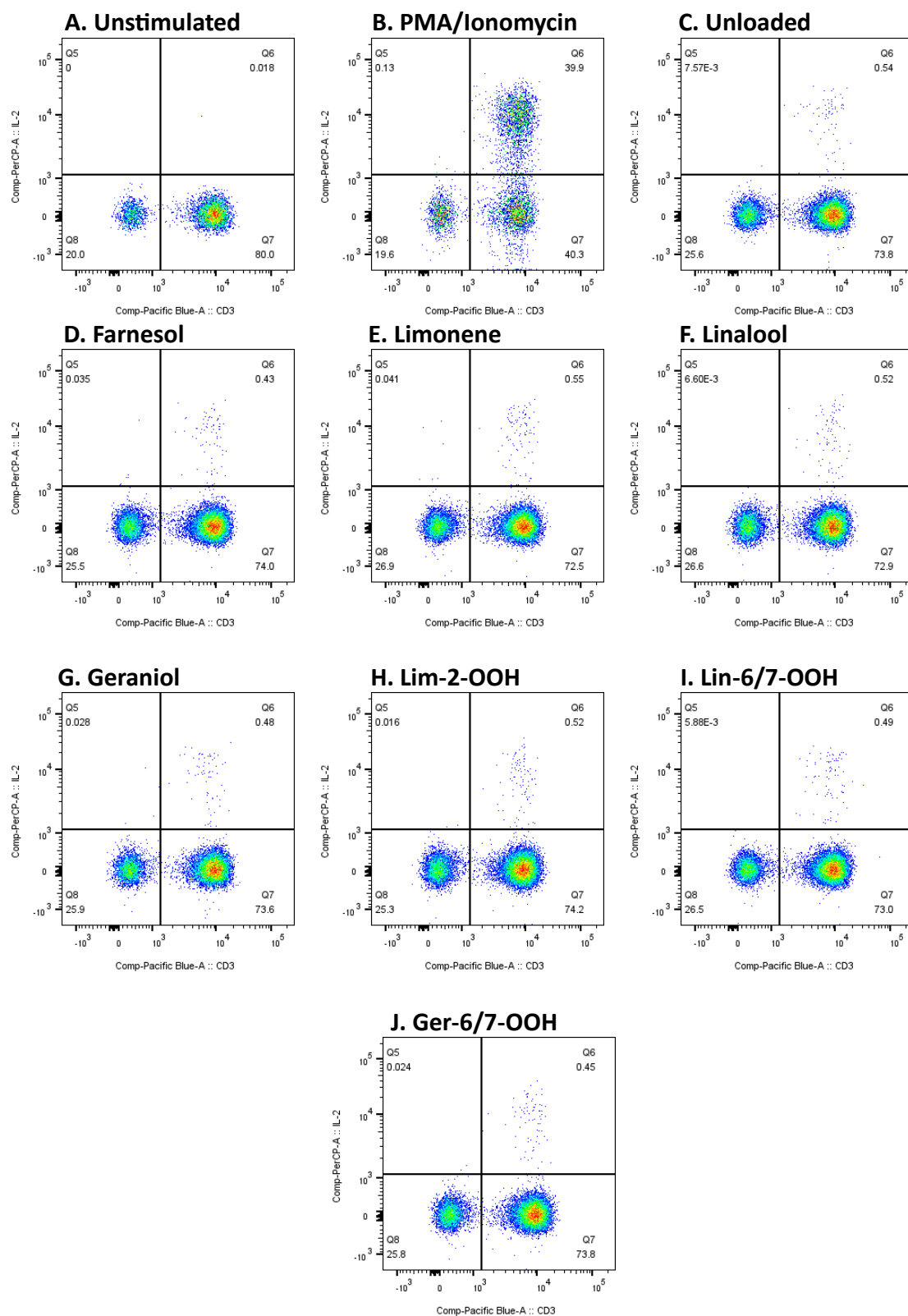
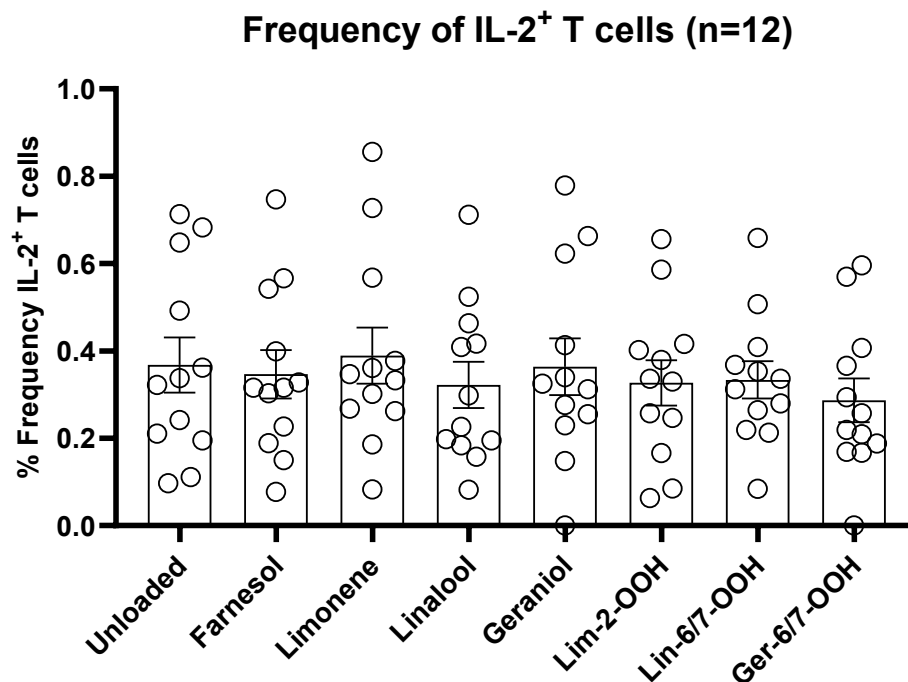


Figure 5.27. Flow cytometric analysis of IL-2⁺ T cells.

Stained with cell surface markers and an intracellular IL-2 antibody, cells were then analysed by flow cytometry. Representative dot plots are shown for **(A)** unstimulated PBMCs (without C1R-CD1a cells), **(B)** PBMCs stimulated with PMA/ionomycin (without C1R-CD1 cells), and **(C–J)** PBMCs incubated with each lipid-loaded C1R-CD1a condition. IL-2⁺/CD3⁺ T cells are located in the top-right quadrant.

Statistical analyses were performed to assess differences in the frequency of IL-2⁺/CD3⁺ events between unloaded and lipid-loaded conditions. The mean frequency of IL-2⁺/CD3⁺ events in unloaded samples was 0.368% (Fig. 5.28.A). There was ultimately no lipid loading conditions which caused a significant difference in the frequency of IL-2⁺ T cells relative to the unloaded C1R-CD1a cells. To account for inter-experimental variability, IL-2⁺ T cell frequencies were normalized to their respective unloaded conditions and expressed as fold changes (Fig. 5.28.B). Across all tested lipids, fold-change values demonstrated minimal deviation, indicating that lipid loading of C1R-CD1a cells did not markedly alter IL-2⁺ T cell responses.

A.



B.

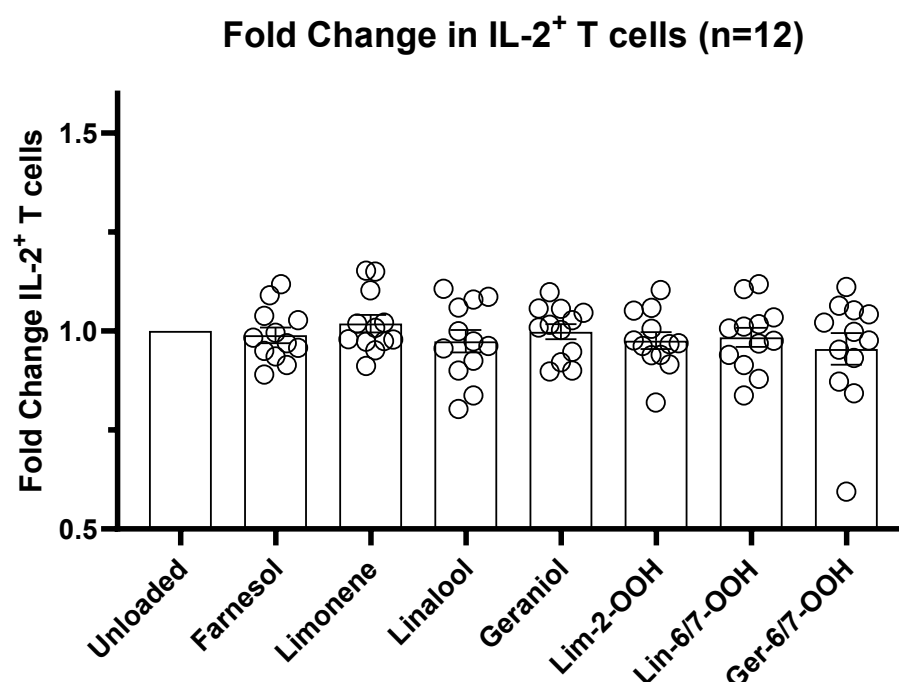


Figure 5.28. IL-2⁺ T cell responses to lipid-loaded C1R-CD1a cells.

C1R-CD1a cells were loaded with various lipids and co-cultured with PBMCs. Following incubation, cells were stained with cell-surface antibodies and intracellular IL-2 antibody and analysed by flow cytometry. **(A)** Frequencies of IL-2⁺/CD3⁺ events. **(B)** IL-2⁺/CD3⁺ frequencies normalized to unloaded C1R-CD1a cells and expressed as fold changes. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, across twelve independent experiments (n = 12).

The frequencies of IL-2⁺ T cells induced by lipid-loaded C1R-CD1a cells were compared with those induced by the corresponding lipid-loaded C1R-mock cells to evaluate CD1a-specific effects (Fig. 5.29). In the unloaded condition, C1R-mock cells induced a mean IL-2⁺ T cell frequency of 0.178%, whereas C1R-CD1a cells induced 0.368%, representing a statistically significant increase ($p = 0.0005$). As observed previously with TNF⁺ T cells, this increase is most likely due to autoreactivity triggered by endogenously loaded lipids. Consequently, increases observed across other loaded conditions may partly reflect the presence of undisplaced endogenous ligands. Notably, limonene loading resulted in a stronger effect, with a highly significant increase between C1R-mock and C1R-CD1a cells ($p = 0.0001$). This is consistent with the trend seen in Fig. 5.28, where limonene induced a marginal, though not statistically significant, increase in IL-2⁺ T cells relative to unloaded C1R-CD1a cells. Interestingly, Ger-6/7-OOH was the only lipid condition that did not elicit a significant difference between C1R-mock and C1R-CD1a cells ($p = 0.2561$).

In fact, Ger-6/7-OOH-loaded C1R-CD1a cells induced lower IL-2⁺ T cell frequencies than unloaded C1R-CD1a cells (Fig. 5.29), suggesting that the absence of a significant increase in this comparison may be CD1a-dependent.

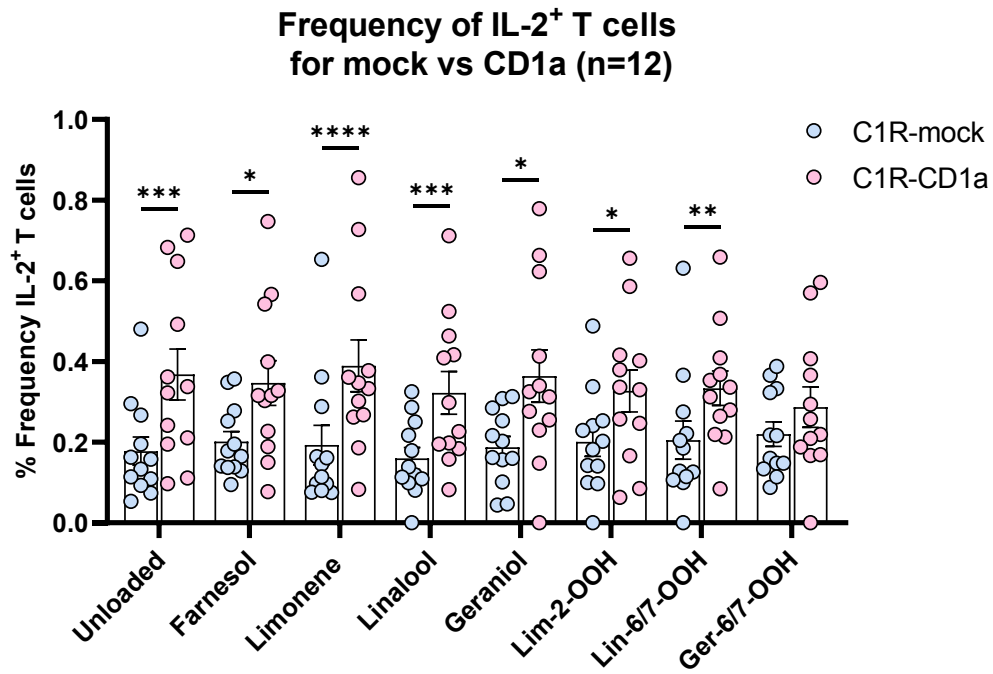


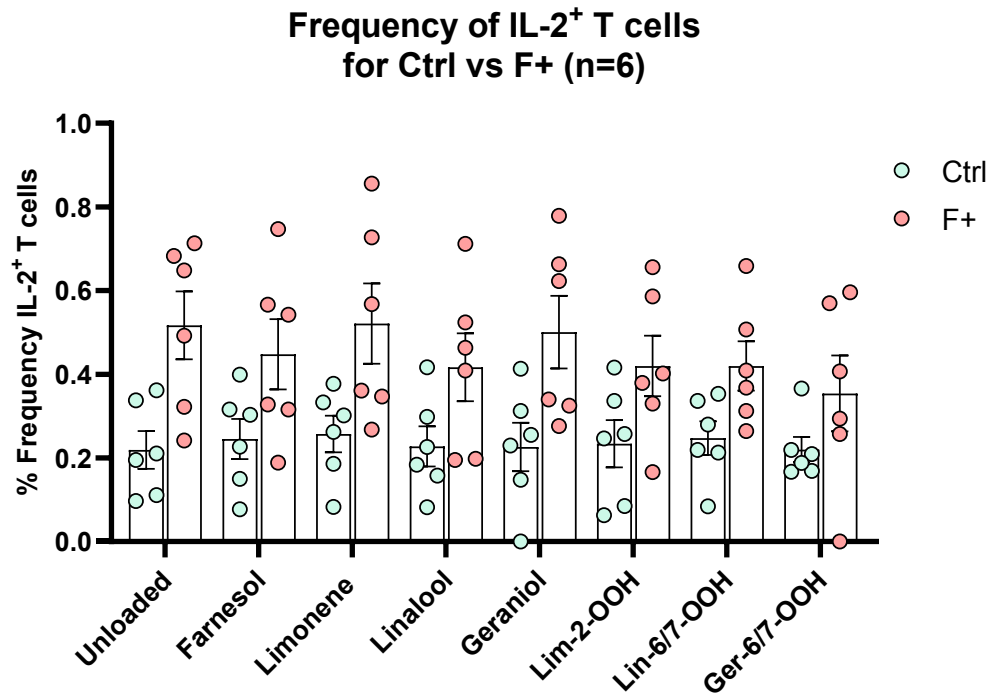
Figure 5.29. IL-2⁺ T cell responses to lipid-loaded C1R-mock and C1R-CD1a cells.

Frequency of IL-2⁺ T cells following co-culture with C1R-mock (blue) or C1R-CD1a (pink) cells loaded with various lipids. Non-significant results are left unmarked. Significant differences between C1R-mock and C1R-CD1a are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

The pooled dataset ($n = 12$) was stratified into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts to assess IL-2⁺/CD3⁺ T cell responses to unloaded and lipid-loaded C1R-CD1a cells. The F⁺ cohort exhibited markedly higher baseline IL-2⁺ T cell frequencies compared with the Ctrl cohort, with mean frequencies of 0.512% and 0.219%, respectively, in unloaded C1R-CD1a cells - a trend that was consistently observed across all lipid-loaded conditions (Fig. 5.30.A). Subtle reductions in mean IL-2⁺ frequencies were noted within the F⁺ cohort that were not evident in Ctrl; however, these decreases were modest and did not approach statistical significance. Frequency data were subsequently normalized to the unloaded C1R-CD1a condition for each cohort and expressed as fold changes (Fig. 5.30.B). When analysed in this manner, the reduction in IL-2⁺ T cell responses associated

with the F⁺ cohort became more apparent, but remained minor and statistically non-significant.

A.



B.

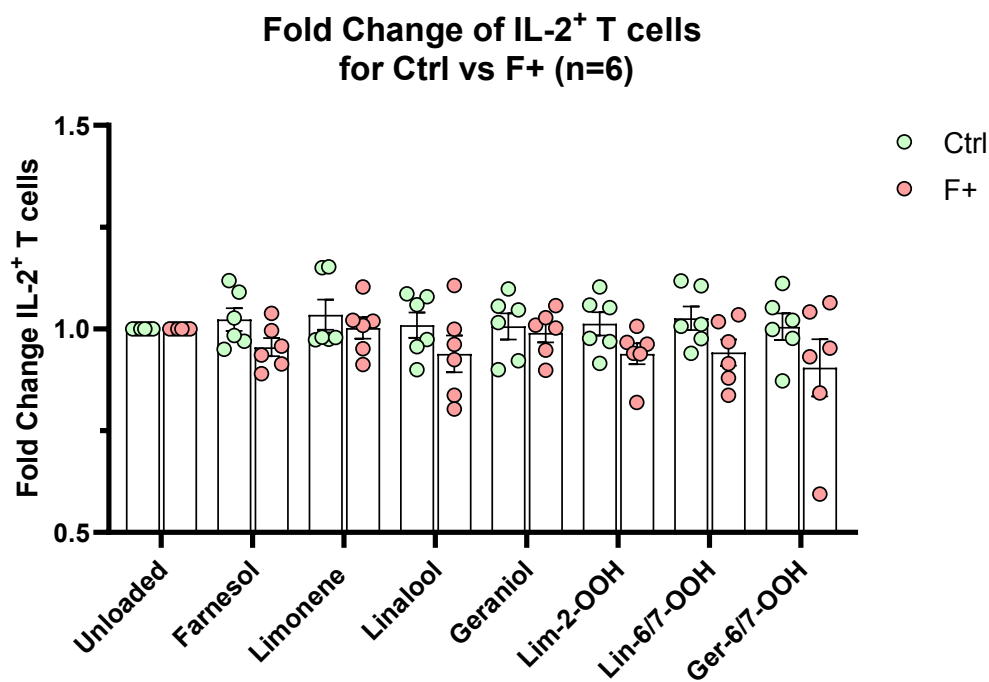


Figure 5.30. IL-2⁺ T cell responses in Ctrl and F+ subsets to lipid-loaded C1R-CD1a cells.

(A) Differences in IL-2⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets (n = 6) following co-culture with lipid-loaded C1R-CD1a cells, expressed relative to unloaded conditions. **(B)** Differences in IL-

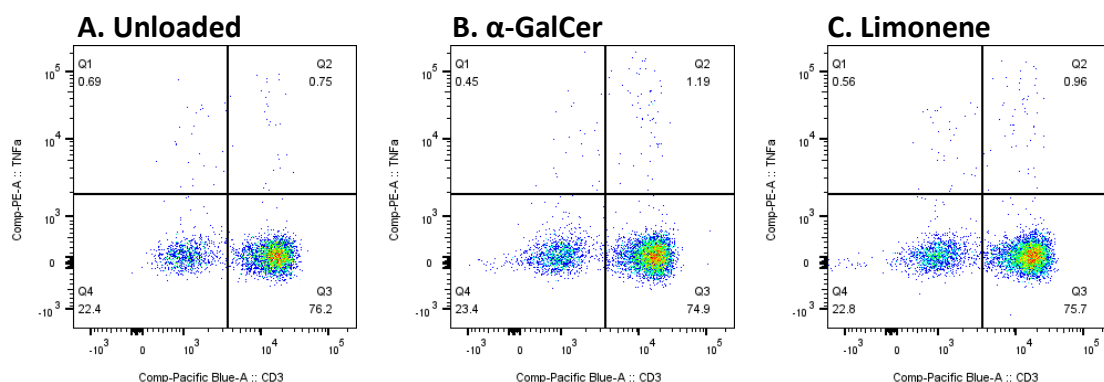
2⁺ T cell frequencies between Ctrl and F⁺ subsets (n = 6) expressed as fold changes normalized to their respective unloaded C1R-CD1a conditions. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments (n = 12).

5.3.4 CD1d Functional Assays

HeLa-mock and HeLa-CD1d cells were loaded with various lipids and co-cultured with PBMCs from the same donors described in CD1 tetramer assays. Following incubation, cells were harvested and stained with cell-surface and intracellular cytokine antibodies. Flow cytometric analysis was performed, and cytokine⁺/CD3⁺ events were quantified

5.3.4.1 TNF

Representative dot plots of TNF⁺/CD3⁺ (Fig. 5.31) illustrate consistent patterns observed across all experiments (n = 12). Control samples included unstimulated PBMCs (shown previously in Fig. 19.A) and PBMCs stimulated with PMA/ionomycin in the absence of C1R-CD1a cells (shown previously in Fig. 5.19.B). Unstimulated PBMCs exhibited negligible TNF⁺ T cell frequencies, whereas PMA/ionomycin stimulation induced a robust TNF⁺ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.31.A) or lipid-loaded C1R-CD1a cells (Fig. 5.31.B–H) resulted in a moderate increase in TNF⁺ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.



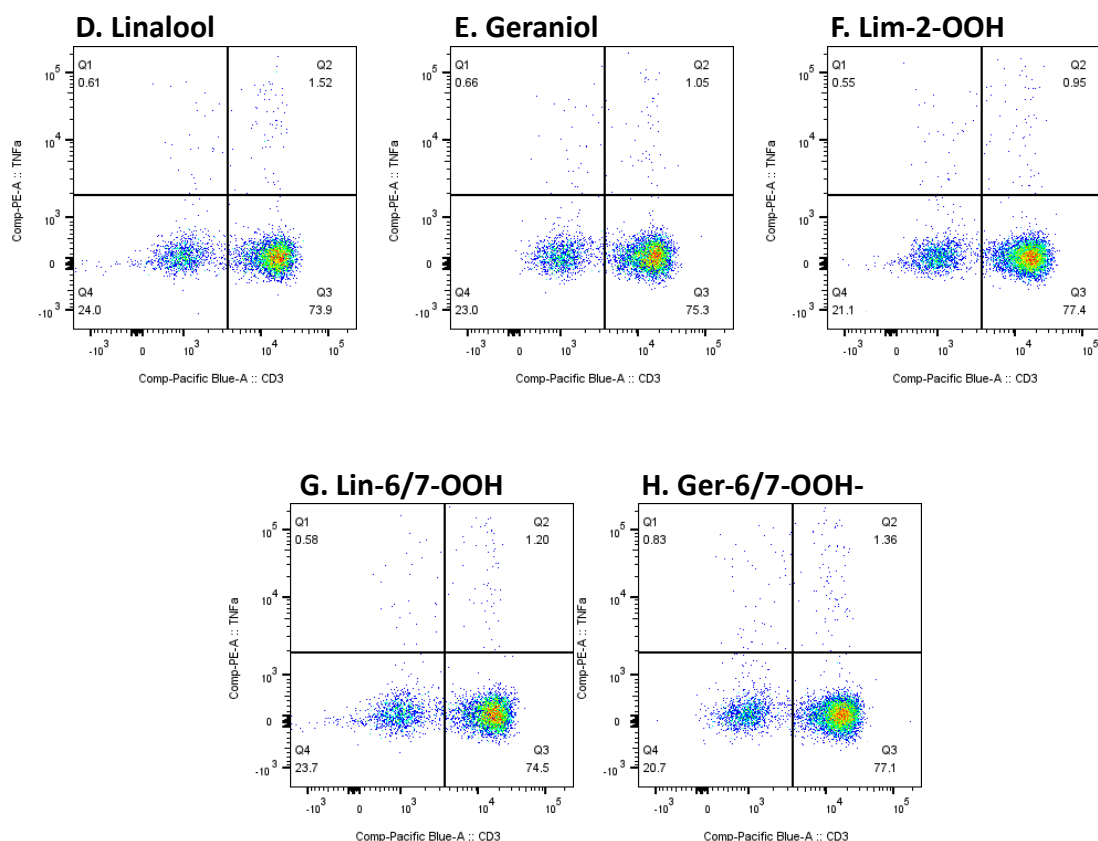
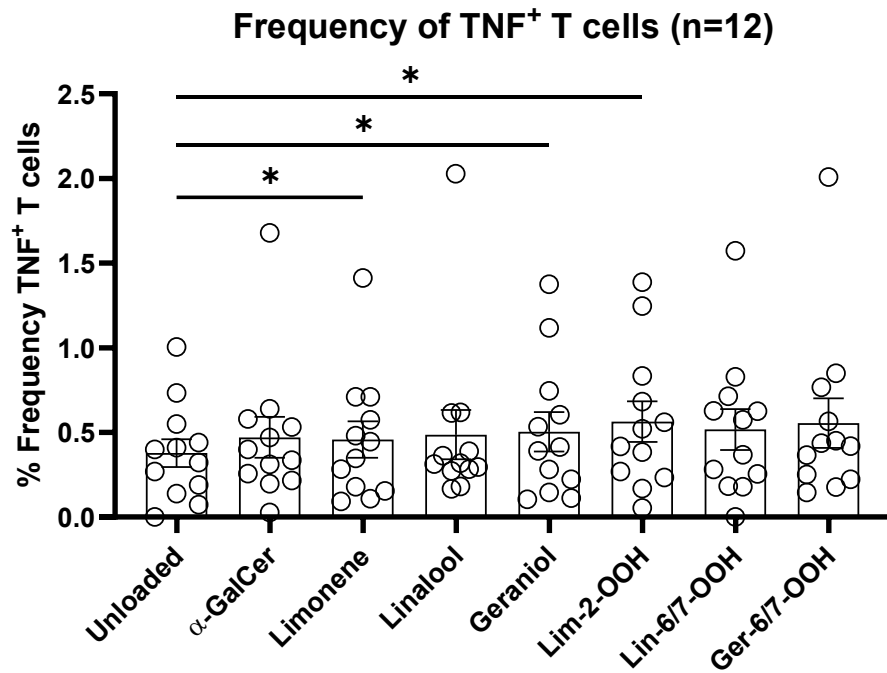


Figure 5.31. Flow cytometry analysis of TNF⁺ T cells.

Stained with cell surface markers and an intracellular TNF antibody, PBMCs were then analysed by flow cytometry. Representative dot plots are shown for **(A)** PBMCs incubated with unloaded HeLa-CD1d cells, and **(B-H)** lipid-loaded HeLa-CD1d conditions. TNF⁺/CD3⁺ T cells are located in the top-right quadrant.

Statistical analyses were conducted to evaluate differences in TNF⁺/CD3⁺ T cell frequencies between unloaded and lipid-loaded conditions. The mean frequency of TNF⁺/CD3⁺ events in unloaded samples was 0.378% (Fig. 5.32.A). Significant increases were detected upon loading with limonene (mean: 0.458%, $p = 0.0122$), geraniol (mean: 0.504%, $p = 0.0112$), and Lim-2-OOH (mean: 0.563%, $p = 0.0452$). To control for potential inter-experimental variability and mitigate spurious statistical significance, frequencies were normalized to unloaded HeLa-CD1 cells. Following normalization, the significant differences observed in the raw frequency data were maintained (Fig. 5.32.B).

A.



B.

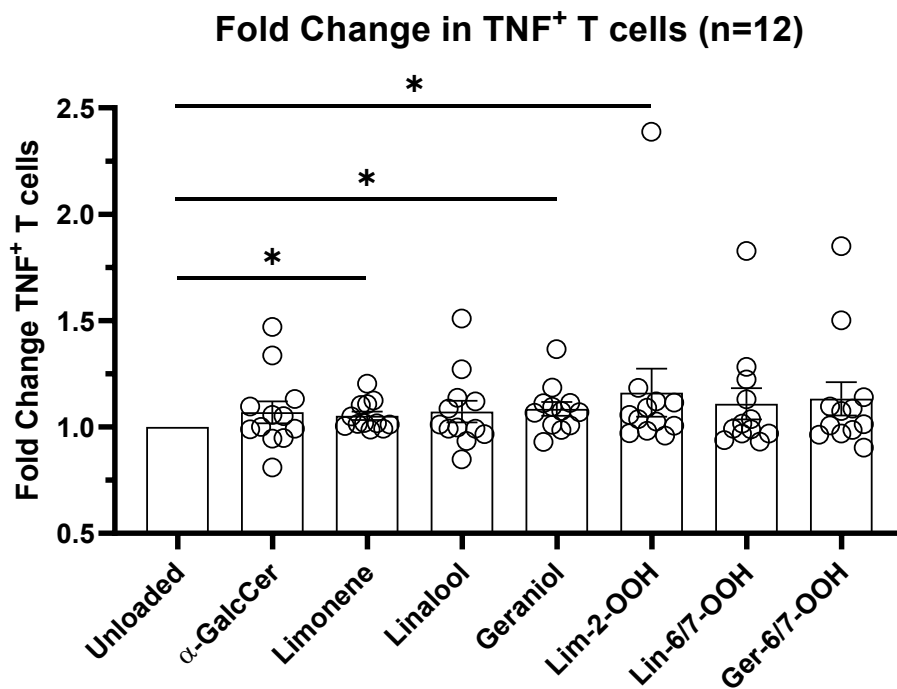


Figure 5.32. TNF⁺ T cell responses to lipid-loaded HeLa-CD1d cells.

HeLa-CD1d cells were loaded with lipids, co-cultured with PBMCs, stained with cell surface antibodies, and analyzed by flow cytometry. **(A)** Frequencies of TNF⁺ T cells for unloaded and lipid-loaded HeLa-CD1d cells. **(B)** Fold change in TNF⁺ T cell frequencies normalized to unloaded HeLa-CD1d conditions. Significant differences relative to unloaded tetramers are indicated as follows: * $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for six independent experiments ($n = 12$).

As a control, HeLa-mock cells were co-cultured with PBMCs to assess whether changes in TNF⁺ T cell frequency were specifically CD1d-dependent. TNF⁺/CD3⁺ frequencies from HeLa-mock and HeLa-CD1d cells were compared across 12 donors (Fig. 5.33). The mean frequency in unloaded HeLa-mock cells (0.212%) was lower than in unloaded HeLa-CD1d cells (0.378%), although this difference did not reach statistical significance ($p = 0.0739$). Across all loaded conditions, increases in TNF⁺ T cell frequency were consistently observed in HeLa-CD1d relative to HeLa-mock cells. Statistically significant increases were detected for the positive control α -GalCer ($p = 0.0361$), as well as for linalool ($p = 0.0434$), geraniol ($p = 0.0079$), and Lim-2-OOH ($p = 0.0069$). These findings corroborate earlier observations (Fig. 5.32), where loading HeLa-CD1d cells with geraniol or Lim-2-OOH led to significant increases relative to unloaded HeLa-CD1d cells. Together, these results support the conclusion that the observed increase in TNF⁺ T cells was CD1d-dependent.

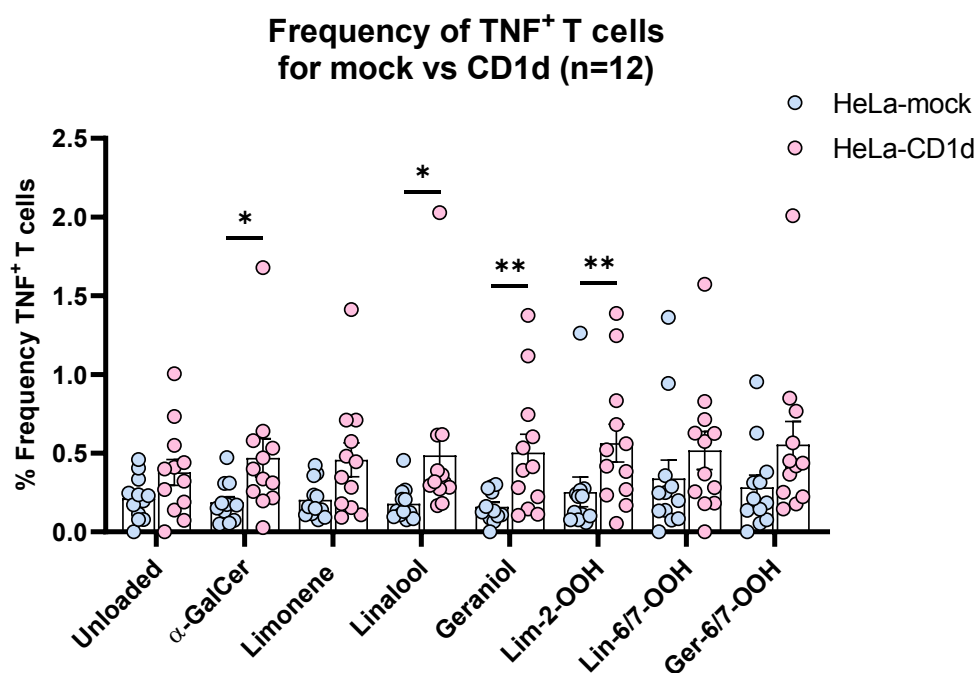
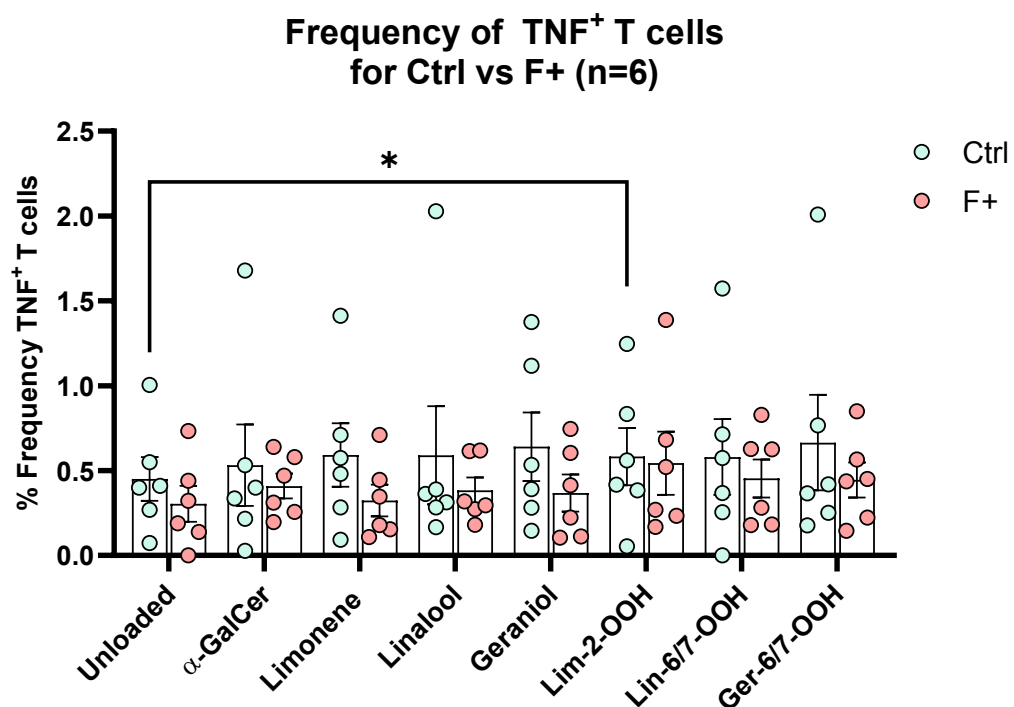


Figure 5.33. TNF⁺ T cell responses to lipid-loaded HeLa-mock and HeLa-CD1d cells.

Frequency of TNF⁺ T cells following co-culture with HeLa-mock (blue) or HeLa-CD1d (pink) cells loaded with various lipids. Non-significant results are left unmarked. Significant differences between HeLa-mock and HeLa-CD1d are indicated as follows: * $p \leq 0.05$ and ** $p \leq 0.01$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

The pooled dataset ($n = 12$) was divided into Ctrl ($n = 6$) and F^+ ($n = 6$) cohorts to compare $TNF^+/CD3^+$ T cell responses to unloaded and lipid-loaded HeLa-CD1d cells. Overall, the Ctrl cohort exhibited higher TNF^+ frequencies across all conditions, with mean frequencies in unloaded HeLa-CD1d cells of 0.451% for Ctrl and 0.304% for F^+ (Fig. 5.34.A). The only statistically significant differences among the two cohorts from the frequency data was an increase in TNF^+ T cells when HeLa-CD1d cells were loaded with Lim-2-OOH (mean frequency = 0.583%; $p = 0.0463$). To control for potential inter-experimental variability and mitigate chance statistical significance, frequencies were normalized to unloaded HeLa-CD1 cells. Following normalization, the significant differences observed were in agreement with the raw frequency data and extended to limonene ($p = 0.0369$) in the Ctrl (Fig. 5.34.B). A visual increase was also noticeable for geraniol in the Ctrl but fell short of being statistically significant ($p = 0.0943$).

A.



B.

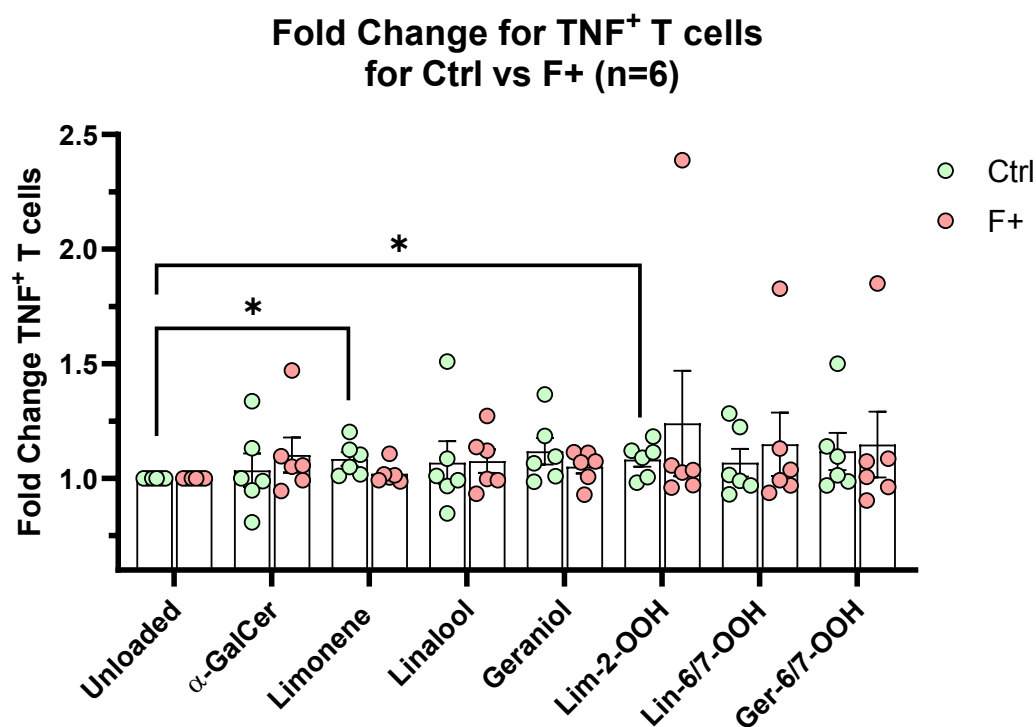


Figure 5.34. TNF⁺ T cell responses in Ctrl and F⁺ subsets to lipid-loaded HeLa-CD1d cells.

(A) Differences in TNF⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets (n = 6) following co-culture with lipid-loaded C1R-CD1a cells, expressed relative to unloaded conditions. (B) Differences in TNF⁺ T cell frequencies between Ctrl and F⁺ subsets (n = 6) expressed as fold changes normalized to their respective unloaded C1R-CD1a conditions.

5.3.4.2 IFN- γ

Representative dot plots of IFN- γ ⁺/CD3⁺ (Fig. 5.35) illustrate consistent patterns observed across all experiments (n = 12). Control samples included unstimulated PBMCs (previously shown in Fig. 5.23.A) and PBMCs stimulated with PMA/ionomycin in the absence of HeLa-CD1d cells (previously shown in Fig. 5.23.B). Unstimulated PBMCs exhibited negligible IFN- γ ⁺ T cell frequencies, whereas PMA/ionomycin stimulation induced a strong IFN- γ ⁺ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.35.A) or lipid-loaded C1R-CD1a cells (Fig. 5.35.B-H) resulted in a moderate increase in IFN- γ ⁺ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.

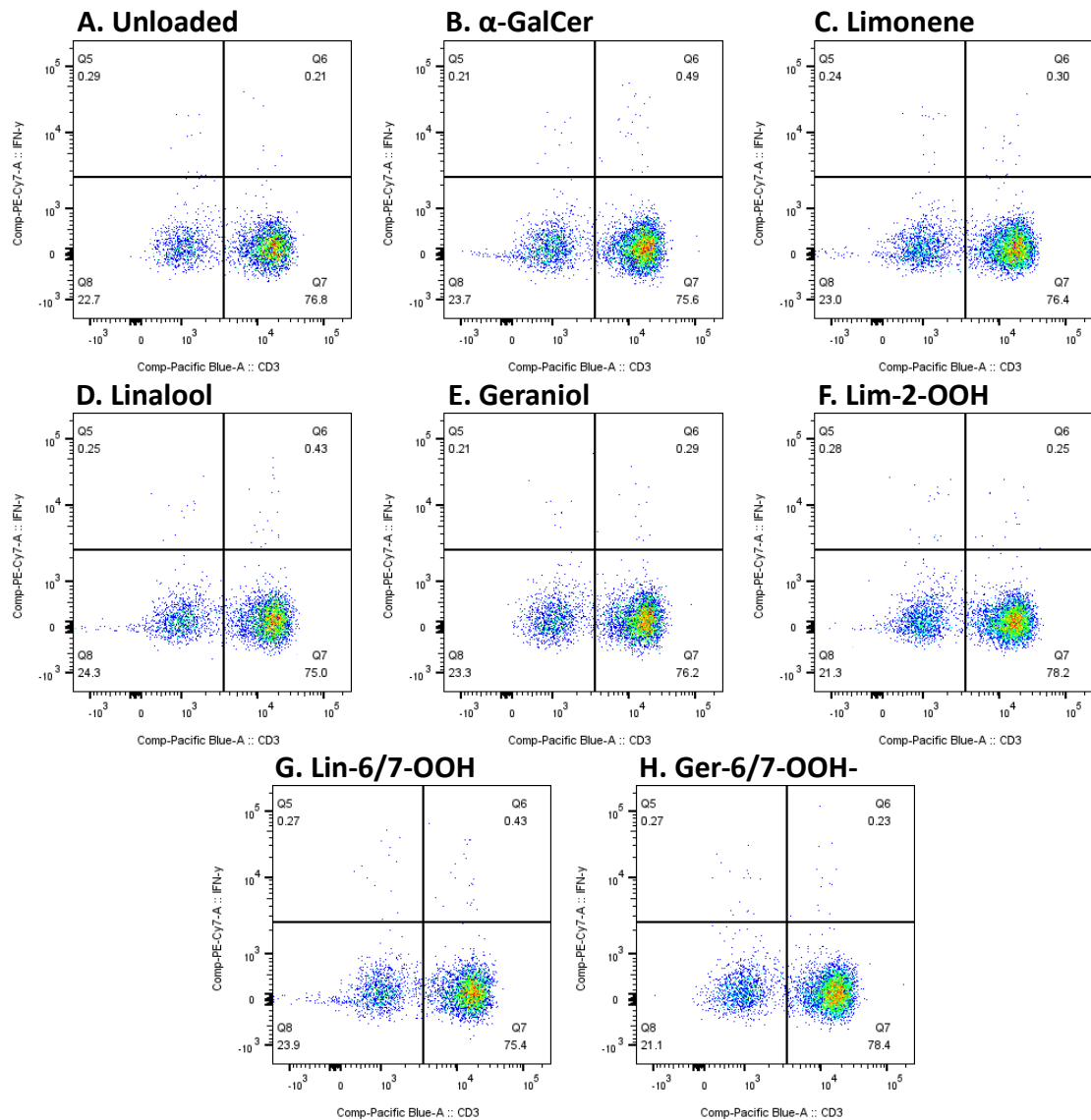


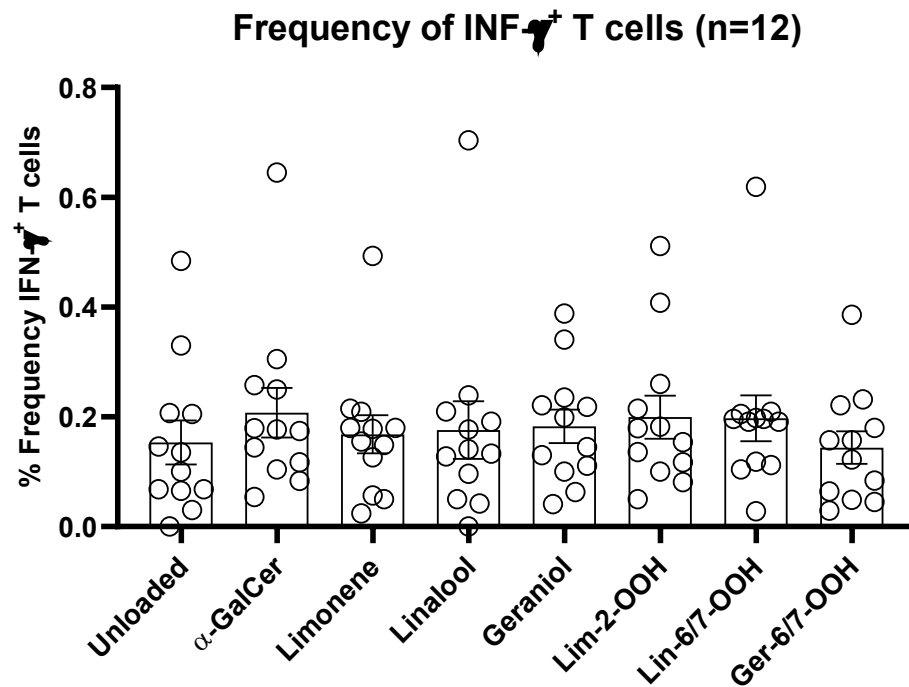
Figure 5.35. Flow cytometric analysis of IFN- γ ⁺ T cells.

HeLa-CD1d cells were loaded with the indicated lipids and co-cultured with PBMCs, followed by staining with cell surface markers and intracellular IFN- γ antibody, then analysed by flow cytometry. Representative dot plots are shown for (A) PBMCs incubated with unloaded HeLa-CD1d cells, and (B-H) lipid-loaded HeLa-CD1d conditions. IFN- γ ⁺/CD3⁺ T cells are located in the top-right quadrant.

Statistical analyses were conducted to evaluate differences in the frequency of IFN- γ ⁺/CD3⁺ events between unloaded and lipid-loaded HeLa-CD1d cells. The mean frequency of IFN- γ ⁺/CD3⁺ events in unloaded samples was 0.153% (Fig. 5.36.A). No statistically significant differences were detected between unloaded HeLa-CD1d cells and those loaded with any of the tested lipids. To account for inter-experimental variability, frequency data were normalized to the unloaded HeLa-CD1d condition, and changes were expressed as fold differences (Fig. 5.36.B). Modest increases in fold change were observed across most lipid-loaded conditions, with the exception of Ger-6/7-OOH,

which exhibited a slight decrease relative to unloaded cells; however, none of these changes reached statistical significance.

A.



B.

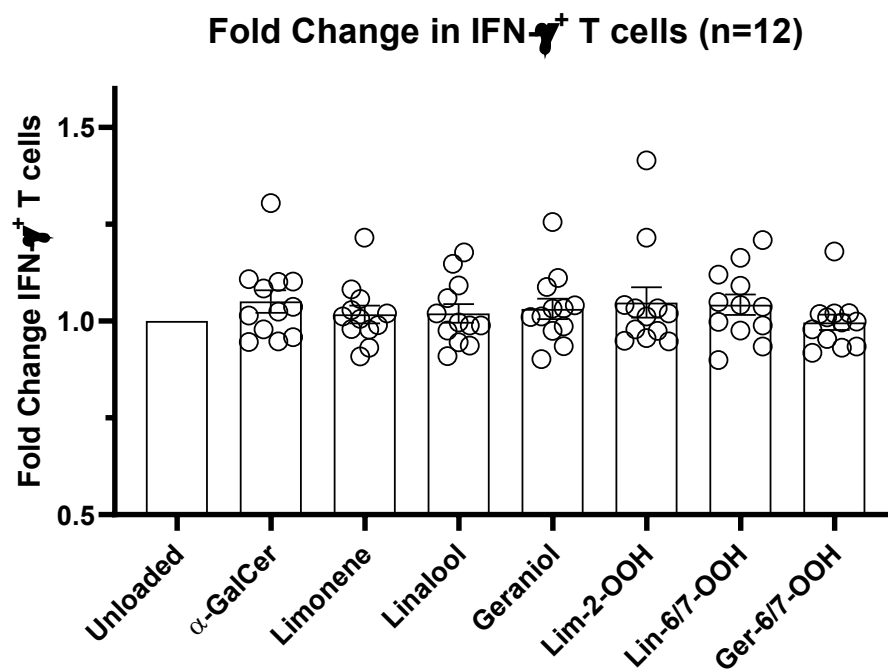


Figure 5.36. IFN- γ ⁺ T cell responses in Ctrl and F+ subsets to lipid-loaded HeLa-CD1d cells.

(A) Differences in IFN- γ^+ T cell frequencies between Ctrl (green) and F $^+$ (red) subsets ($n = 6$) following co-culture with lipid-loaded HeLa-CD1d cells, expressed relative to unloaded conditions. **(B)** Differences in IFN- γ^+ T cell frequencies between Ctrl and F $^+$ subsets ($n = 6$) expressed as fold changes normalized to their respective unloaded HeLa-CD1d conditions. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

As a control, HeLa-mock cells were co-cultured with PBMCs to assess whether changes in IFN- γ^+ T cell frequency were CD1d-dependent. IFN- γ^+ /CD3 $^+$ frequencies from HeLa-mock and HeLa-CD1d cells were compared across 12 donors (Fig. 5.37). The mean frequency in unloaded HeLa-mock cells (0.077%) was lower than in unloaded HeLa-CD1d cells (0.153%), and this trend persisted across all lipid-loaded conditions. Although the difference between unloaded mock and unloaded CD1d cells did not reach statistical significance, significant increases were observed in several loaded conditions, including α -GalCer ($p = 0.0132$), geraniol ($p = 0.0139$), Lin-6/7-OOH ($p = 0.0006$), and Ger-6/7-OOH ($p = 0.0275$). However, if these increases were truly CD1d-dependent, one would also expect significant differences between unloaded and lipid-loaded HeLa-CD1d cells for the same lipids. While marginal increases were observed (previously seen in Fig. 5.36), none reached statistical significance. Moreover, in the case of Ger-6/7-OOH, IFN- γ^+ T cell frequencies were lower than those seen with unloaded HeLa-CD1d cells. These findings suggest that the apparent differences between mock and transfected cells may instead reflect variability in standard deviation, with tighter values in certain HeLa-mock conditions and broader variation in the unloaded HeLa-mock group.

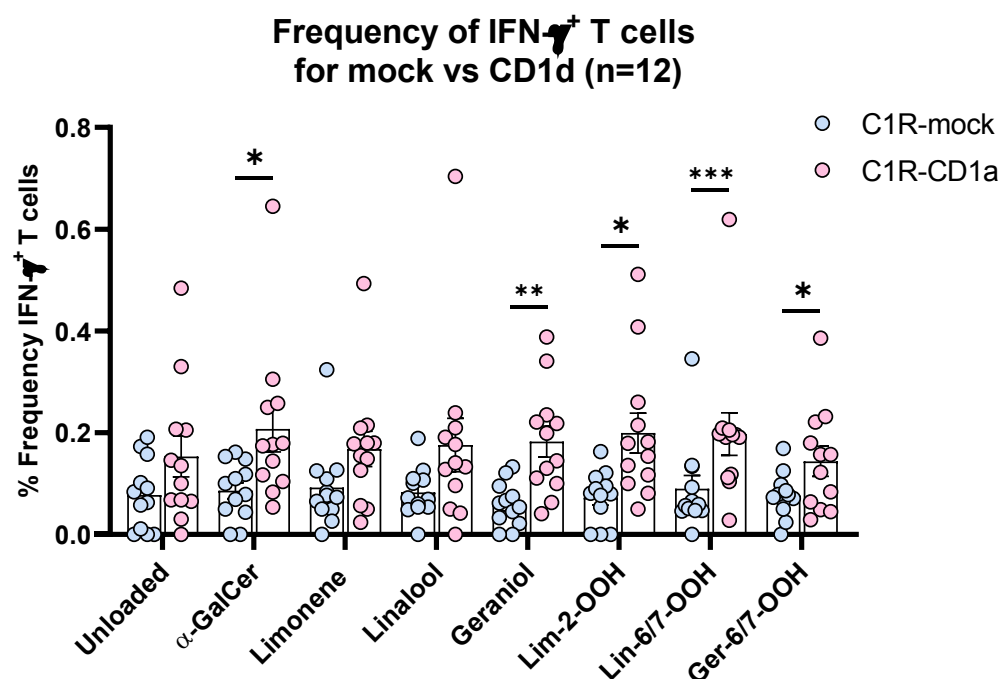


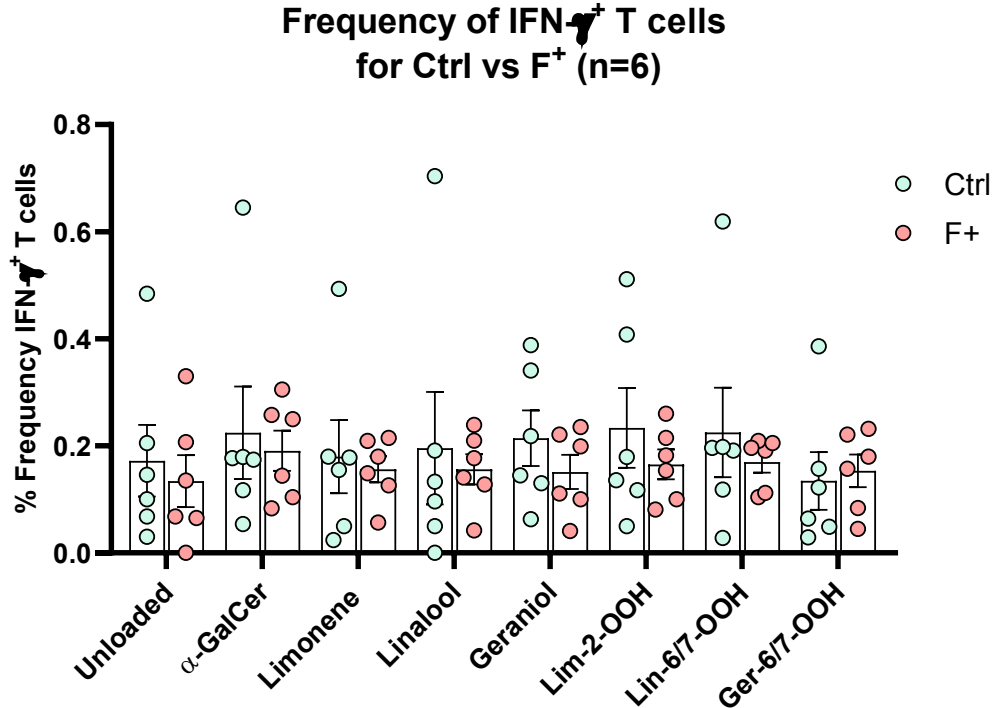
Figure 5.37. IFN- γ ⁺ T cell responses to lipid-loaded C1R-mock and HeLa-CD1d cells.

Frequency of IFN- γ ⁺ T cells following co-culture with HeLa-mock (blue) or HeLa-CD1d (pink) cells loaded with various lipids. Non-significant results are left unmarked. Significant differences between HeLa-mock and HeLa-CD1d are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

The pooled dataset ($n = 12$) was stratified into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts to evaluate IFN- γ ⁺/CD3⁺ T cell responses to unloaded and lipid-loaded HeLa-CD1d cells. Overall, both cohorts exhibited comparable IFN- γ ⁺ frequencies across all conditions, with mean frequencies in unloaded HeLa-CD1d cells of 0.172% for Ctrl and 0.134% for F⁺ (Fig. 5.38.A). Subtle increases and decreases were observed in lipid-loaded conditions; however, none reached statistical significance, and the relatively high standard deviations limited the ability to visually discern strong trends. To account for inter-experimental variability, frequency data were normalized to their respective unloaded HeLa-CD1d conditions and expressed as fold changes (Fig. 5.38.B). Normalization reduced variability and revealed marginal increases in α -GalCer, geraniol, and Lin-6/7-OOH in both cohorts, though these changes were not statistically significant. Interestingly, Ger-6/7-OOH was the only lipid to induce a reduction in IFN- γ ⁺ T cell fold

change within the Ctrl cohort, and this effect approached but did not reach statistical significance ($p = 0.0958$).

A.



B.

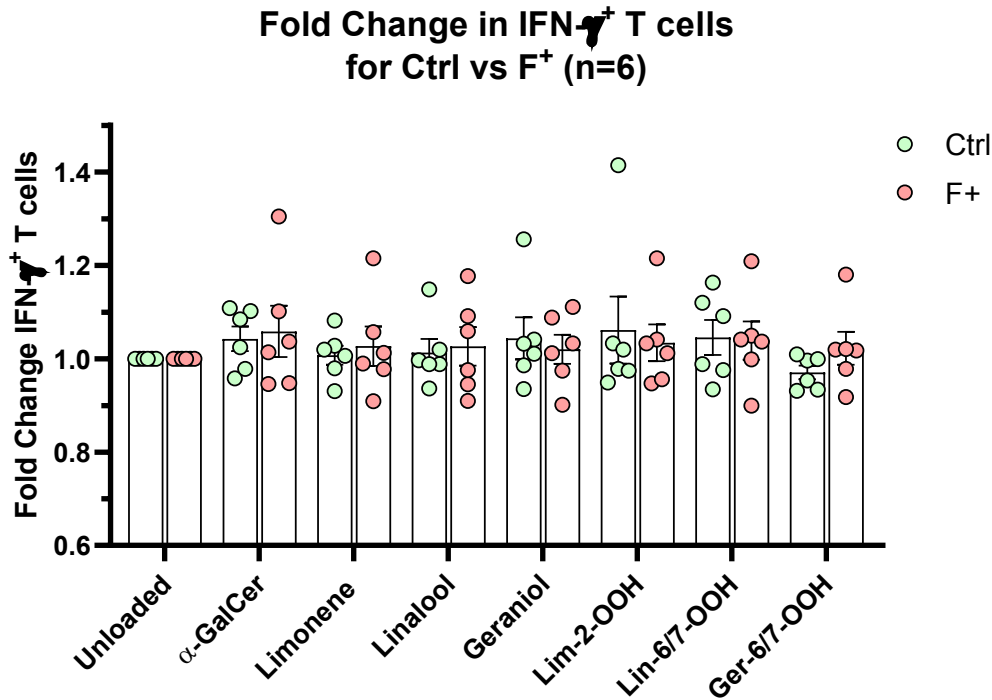


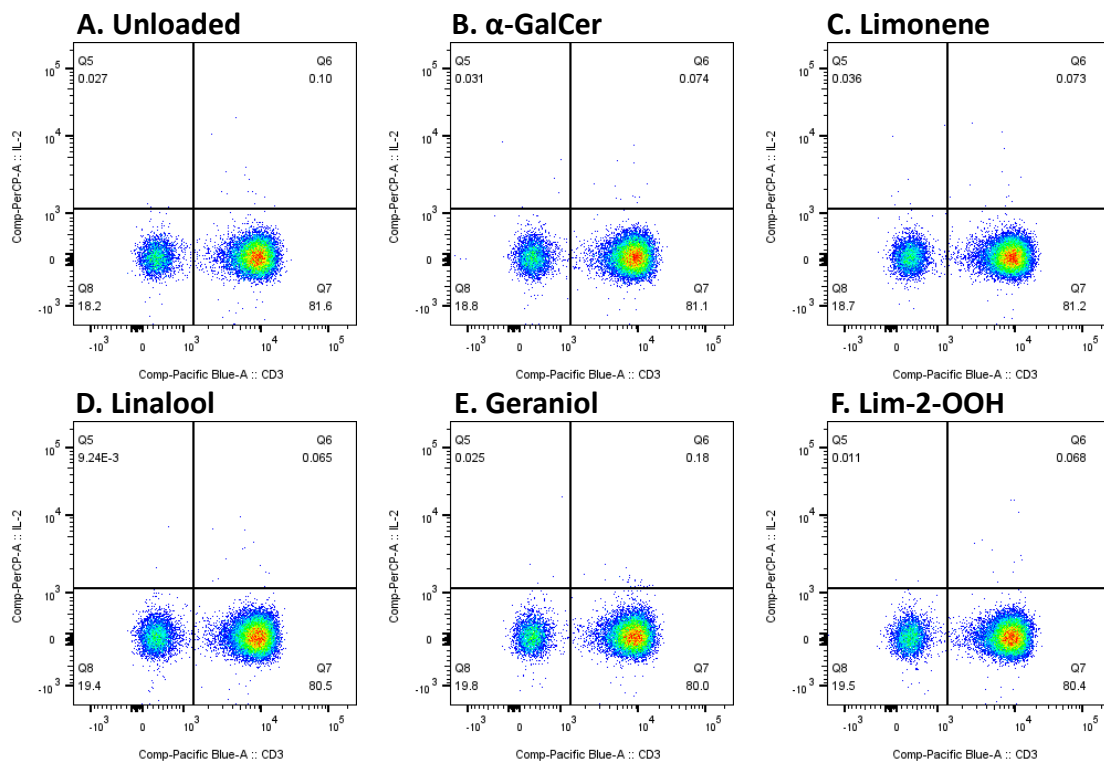
Figure 5.38. IFN- γ ⁺ T cell responses in Ctrl and F⁺ subsets to lipid-loaded HeLa-CD1d cells.

(A) Differences in IFN- γ ⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets (n = 6) following co-culture with lipid-loaded HeLa-CD1d cells, expressed relative to unloaded conditions. **(B)** Differences in

IFN- γ ⁺ T cell frequencies between Ctrl and F⁺ subsets (n = 6) expressed as fold changes normalized to their respective unloaded HeLa-CD1d conditions. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments (n = 12).

5.3.4.3 IL-2

Representative dot plots of IL-2⁺/CD3⁺ (Fig. 5.39) illustrate consistent patterns observed across all experiments (n = 12). Control samples included unstimulated PBMCs (Fig. 5.27.A) and PBMCs stimulated with PMA/ionomycin in the absence of C1R-CD1a cells (Fig. 5.27.B). Unstimulated PBMCs exhibited negligible IL-2⁺ T cell frequencies, whereas PMA/ionomycin stimulation induced a robust IL-2⁺ T cell response. Co-culture of PBMCs with either unloaded (Fig. 5.39.A) or lipid-loaded C1R-CD1a cells (Fig. 5.39.B–H) resulted in a moderate increase in IL-2⁺ T cells compared to unstimulated controls, but responses remained substantially lower than those induced by PMA/ionomycin. Visual differences between the various lipid-loading conditions were minimal.



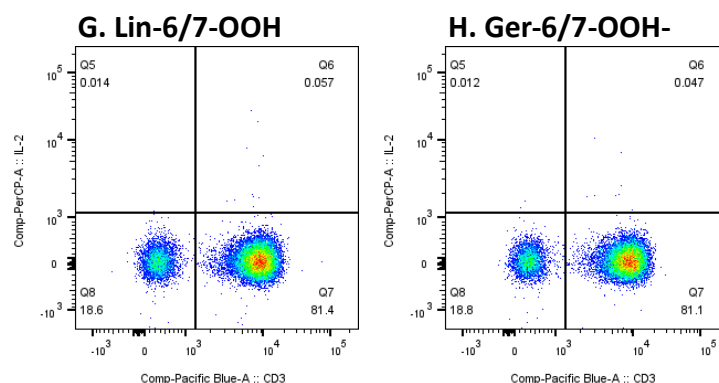
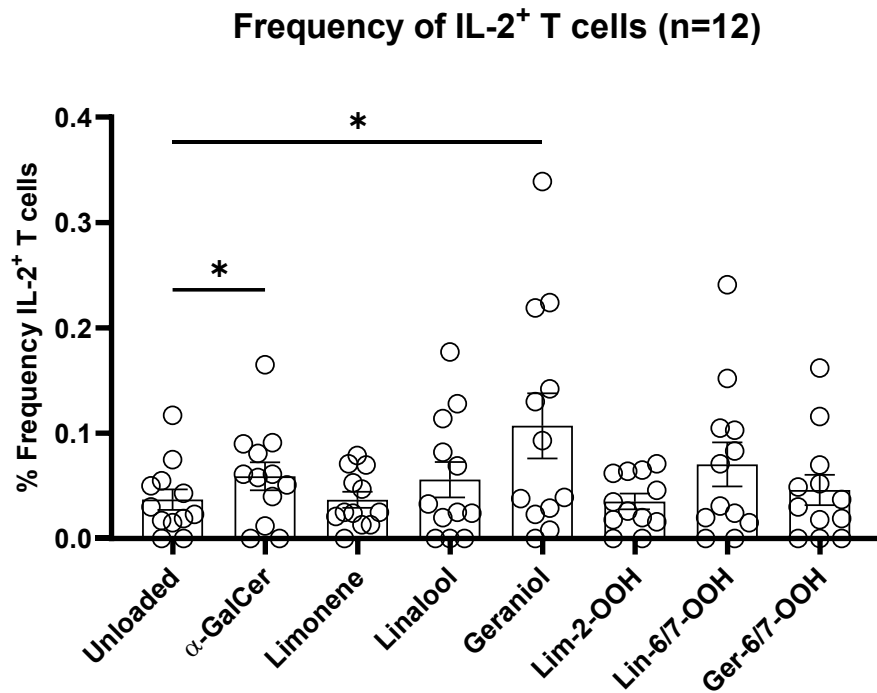


Figure 5.39. Flow cytometric analysis of IL-2⁺ T cells.

HeLa-CD1d cells were loaded with the indicated lipids and co-cultured with PBMCs, followed by staining with cell surface markers and intracellular IL-2 antibody, then analysed by flow cytometry. Representative dot plots are shown for (A) PBMCs incubated with unloaded HeLa-CD1d cells, and (B-H) lipid-loaded HeLa-CD1d conditions. IL-2⁺/CD3⁺ T cells are located in the top-right quadrant.

Statistical analyses were performed to assess differences in the frequency of IL-2⁺/CD3⁺ events between unloaded and lipid-loaded conditions. The mean frequency of IL-2⁺/CD3⁺ events in unloaded samples was 0.370% (Fig. 5.40.A). There were upward trends seen in lipid-loaded HeLa-CD1d cells, with statistically significant increases observed for both α -GalCer (mean frequency = 0.059%; $p = 0.0262$) and gerniol (mean frequency = 0.107%; $p = 0.0269$). Lin-6/7-OOH also showed an upward trend, but failed to reach statistical significance (mean frequency = 0.070%; $p = 0.1198$). To account for inter-experimental variability, IL-2⁺ T cell frequencies were normalized to their respective unloaded conditions and expressed as fold changes (Fig. 5.40.B).

A.



B.

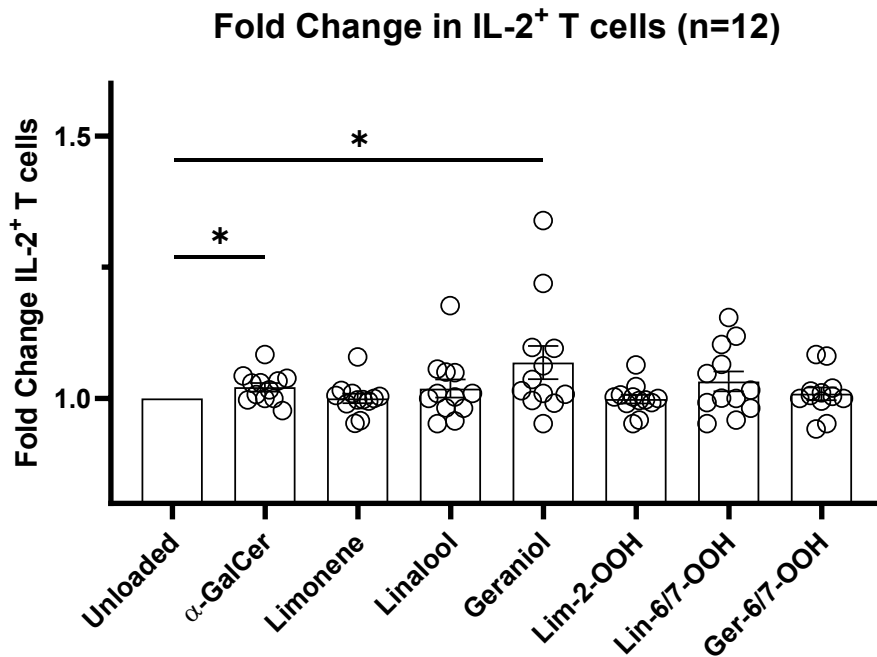


Figure 5.40. IL-2⁺ T cell responses to lipid-loaded HeLa-CD1d cells.

HeLa-CD1d cells were loaded with various lipids and co-cultured with PBMCs. Following incubation, cells were stained with cell-surface antibodies and intracellular IL-2 antibody and analysed by flow cytometry. **(A)** Frequencies of IL-2⁺/CD3⁺ events. **(B)** IL-2⁺/CD3⁺ frequencies normalized to unloaded HeLa-CD1d cells and expressed as fold changes. Significant differences relative to unloaded cells are indicated as follows:

* $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, across twelve independent experiments ($n = 12$).

The frequencies of IL-2⁺ T cells induced by lipid-loaded HeLa-CD1d cells were compared with those induced by the corresponding lipid-loaded HeLa-mock cells to assess CD1d-specific effects (Fig. 5.41). Although unloaded HeLa-mock cells (0.046%) and unloaded HeLa-CD1d cells (0.037%) showed similar IL-2⁺ T cell frequencies, the trends observed in lipid-loaded HeLa-CD1d cells were not reproduced in the HeLa-mock controls. In particular, statistically significant increases were detected between mock and transfected cells when CD1d was loaded with geraniol ($p = 0.0194$) and Lin-6/7-OOH ($p = 0.0312$). The effect of geraniol is likely CD1d-dependent, as a significant increase was also observed between unloaded HeLa-CD1d and geraniol-loaded HeLa-CD1d cells (previously seen in Fig. 5.40).

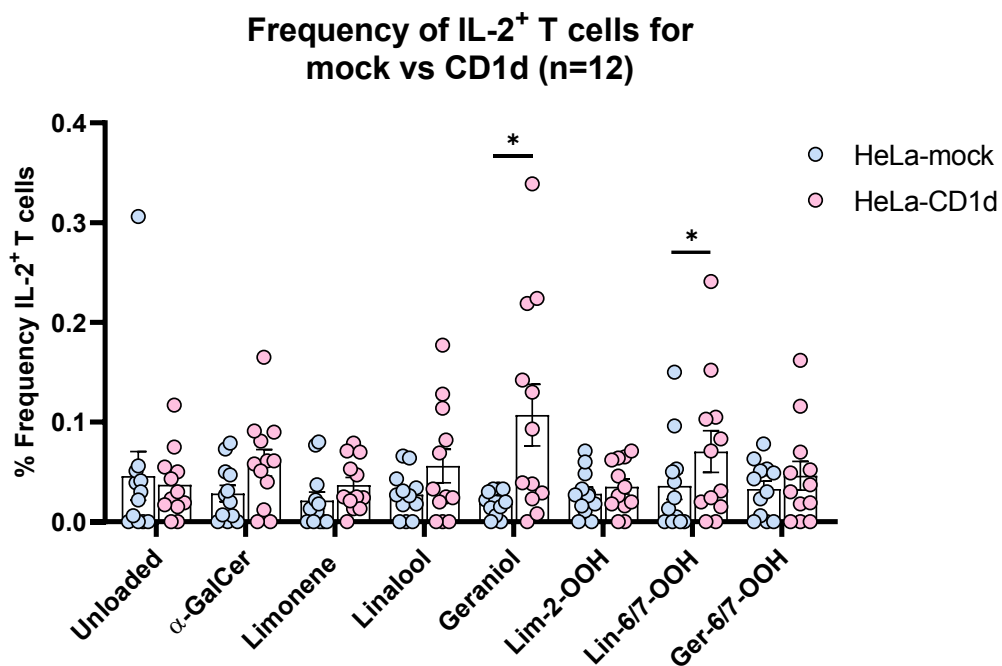


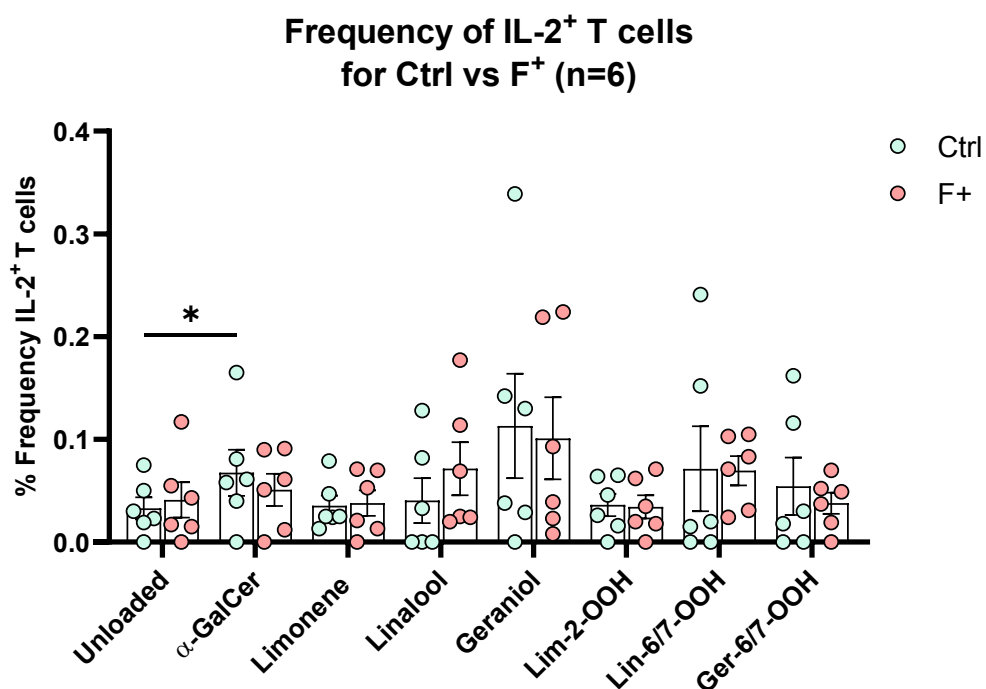
Figure 5.41. IL-2⁺ T cell responses to lipid-loaded C1R-mock and HeLa-CD1d cells.

Frequency of IL-2⁺ T cells following co-culture with HeLa-mock (blue) or HeLa-CD1d (pink) cells loaded with various lipids. Significant differences between HeLa-mock and HeLa-CD1d are indicated as follows: * $p \leq 0.05$. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments ($n = 12$).

The pooled dataset ($n = 12$) was stratified into Ctrl ($n = 6$) and F⁺ ($n = 6$) cohorts to evaluate IL-2⁺/CD3⁺ T cell responses to unloaded and lipid-loaded HeLa-CD1d cells.

Baseline IL-2⁺ frequencies were comparable between cohorts, with mean values of 0.033% for Ctrl and 0.041% for F⁺ in unloaded HeLa-CD1d cells (Fig. 5.42.A). A statistically significant increase was observed for α -GalCer in the Ctrl cohort (mean frequency = 0.068%; $p = 0.0383$), while a similar but non-significant trend was seen in F⁺. Geraniol induced the largest increase in mean frequency across both cohorts; however, this was accompanied by higher inter-donor variability, and the increase did not reach statistical significance. Frequency data were subsequently normalized to their respective unloaded conditions and expressed as fold changes (Fig. 5.42.B). In this analysis, the α -GalCer-induced increase in the Ctrl cohort approached statistical significance ($p = 0.0625$) and was consistently observed across both cohorts. Geraniol and Lin-6/7-OOH also showed upward trends in both Ctrl and F⁺ cohorts, with geraniol again demonstrating the highest mean increase, though variability limited statistical significance.

A.



B.

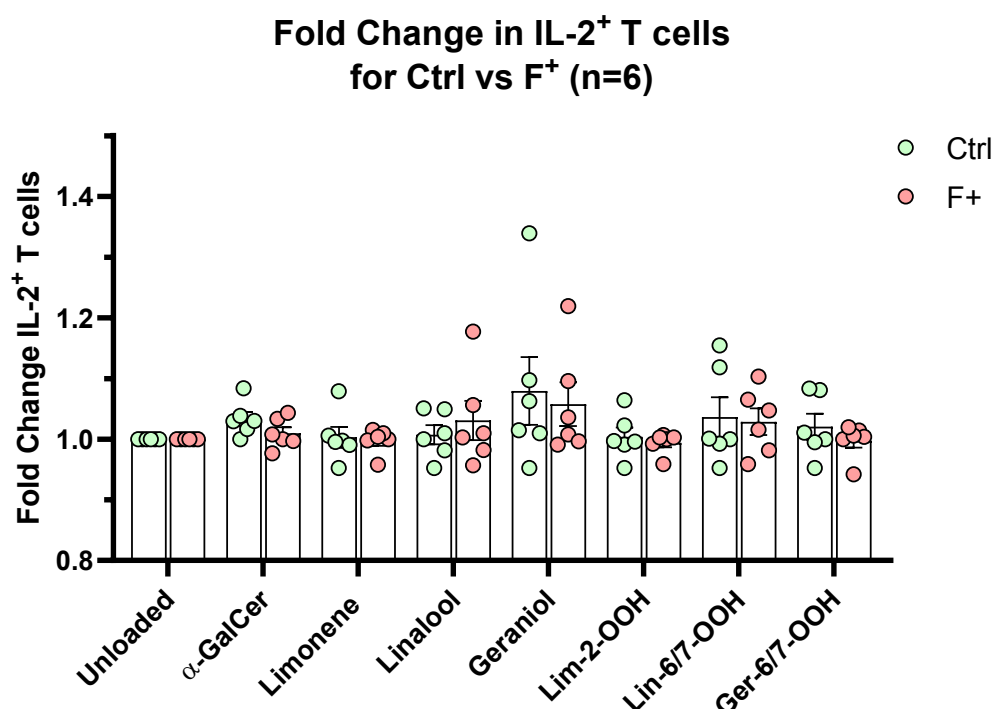


Figure 5.42. IL-2⁺ T cell responses in Ctrl and F⁺ subsets to lipid-loaded HeLa-CD1d cells.

(A) Differences in IL-2⁺ T cell frequencies between Ctrl (green) and F⁺ (red) subsets (n = 6) following co-culture with lipid-loaded HeLa-CD1d cells, expressed relative to unloaded conditions. (B) Differences in IL-2⁺ T cell frequencies between Ctrl and F⁺ subsets (n = 6) expressed as fold changes normalized to their respective unloaded HeLa-CD1d conditions. Significant differences relative to unloaded cells are indicated as follows: * p ≤ 0.001. Statistical significance was assessed using paired t-tests or Wilcoxon matched-pairs signed-rank tests, depending on data normality, for twelve independent experiments (n = 12).

5.4 Discussion

CD1 proteins are non-polymorphic relatives of MHC class I and II molecules that present lipid antigens to TCRs. Unlike classical MHC molecules, which primarily present peptide fragments, CD1 molecules specialize in the presentation of exogenous lipids (e.g., mycobacterial) and endogenous self-lipids. ACD is a type IV hypersensitivity reaction mediated by T cells. While many contact allergens require haptentation to become immunogenic, not all are electrophilic, thus some may elicit T cell responses via alternative mechanisms. Recent studies have demonstrated that CD1a can present certain lipophilic contact allergens, such as urushiol and farnesol, to CD1a-restricted T cells. Furthermore, CD1d has been shown to bind small, non-lipophilic drug-like compounds (PPBFs) and activate CD1d-restricted T cells which do not possess the invariant α-chain (group 2 NKT cells) [279]. Building upon these findings, this chapter

investigates potential CD1-restricted T cell responses to the strong contact allergen Lim-2-OOH, moderate allergens Lin-6/7-OOH and Ger-6/7-OOH, and weak allergens limonene, linalool, and geraniol, as possible mechanisms of T cell activation in ACD. These studies employed CD1a and CD1d tetramers and CD1-transfected cell lines (C1R-CD1a and HeLa-CD1d) co-cultured with PBMCs from healthy controls (Ctrl) and fragrance-allergic patients (F⁺), followed by cell-surface and intracellular cytokine staining and flow cytometric analysis.

5.4.1 Effects of α -GalCer on CD1d Tetramer Staining and Functional Assays

Of all CD1 isoforms, CD1d is the most extensively studied, in part because the invariant TCR α -chain expressed by iNKT cells serves as a reliable surface marker, facilitating their identification and experimental use. This enabled the discovery of α -galactosylceramide (α -GalCer) as a potent CD1d ligand and the gold-standard positive control in CD1d tetramer assays. Structural analyses have demonstrated that the fatty acyl chains of α -GalCer are buried within the hydrophobic pockets of CD1d, while its polar headgroup protrudes to engage the TCR [280]. In this study, α -GalCer-loaded CD1d tetramers produced significantly higher T cell staining compared with unloaded tetramers. In human peripheral blood, iNKT cells range from undetectable to >1% of circulating T cells in rare individuals, with a median frequency of approximately 0.05% [281-283]. Interestingly, a higher frequency of T cell staining was observed in the F⁺ group compared to the Ctrl group, suggesting that F⁺ patients may possess more iNKT cells than Ctrl donors. Higher frequencies of iNKT cells have been reported in other allergic conditions, but there is limited data in the context of ACD [284]. Literature reports indicate that CD1d-restricted iNKT cells are necessary for the development of contact hypersensitivity [285], and that iNKT cell-derived IL-4 is required for B-1 cells to initiate contact hypersensitivity [286, 287]. Thus, the higher frequency of iNKT cells observed in the F⁺ cohort may result from repeated allergen exposure leading to selective expansion of these cells. In functional assays, HeLa-CD1d cells loaded with α -GalCer showed significant increases in IL-2⁺ T cells in pooled cohorts. iNKT cells are often described as ‘bridging the gap between innate and adaptive immunity’, and IL-2 is a key growth factor for T cells [288]. It is therefore possible that iNKT-derived IL-2 could enhance the clonal expansion of other T cells, including allergen-specific CD4⁺ and CD8⁺ T cells, during the

sensitization phase, with IL-2 acting as an early amplifier to increase the pool of activated T cells that mediate the elicitation phase of ACD. Alternatively, this IL-2 production may also have a regulatory function, as IL-2 is critical for the survival and expansion of T_{REG} cells - which express high levels of the interleukin-2 receptor (IL-2R) - and early IL-2 availability could facilitate T_{REG}-mediated suppression [289].

5.4.2 Effects of Farnesol on CD1a Tetramer Staining and Functional Assays

Compared to CD1d, CD1a is less extensively studied due to the lack of a defined surface marker and the absence of a well-established experimental ligand equivalent to α -GalCer, which serves as the gold standard for CD1d research. Farnesol, a sesquiterpene structurally related to the terpenes investigated in this chapter, is a moderate contact allergen (EC3 value: 4.8%) [290, 291], and has been previously utilized in CD1a-related studies. In this study, farnesol was included as a putative positive control for CD1a tetramer staining and C1R-CD1a functional assays. CD1a tetramer assays conducted in this study demonstrated a significant decrease in T cell staining when CD1a tetramers were loaded with farnesol. This was initially unexpected, as farnesol has previously been reported to activate CD1a-restricted T cells, and its interaction with CD1a has been structurally confirmed through crystallographic analysis, establishing farnesol as a bona fide CD1a ligand. However, further investigation revealed that farnesol-loaded CD1a tetramers failed to induce T cell staining of a CD1a-restricted T cell clone (BC2) above the background levels observed with farnesol-loaded CD1b tetramers, which were used as a negative control. Surface plasmon resonance (SPR), a more sensitive technique than tetramer staining, has demonstrated that CD1a–farnesol complexes exhibit low binding affinity for the BC2 TCR. This likely reflects a sensitivity limitation of CD1 tetramer staining rather than a true lack of ligand allergenicity. It is plausible that, under the lipid-loading conditions employed, farnesol effectively displaced endogenous CD1a ligands but did not subsequently engage TCRs with sufficient affinity to sustain staining or activation in PBMCs. This mechanism may also explain the observed reduction in TNF⁺ T cell frequencies following C1R-CD1a loading with farnesol. The decrease is unlikely to represent an active suppression of TNF production by farnesol but rather a reduction in baseline CD1a autoreactivity caused by displacement of endogenous agonistic ligands and their replacement with a lower-affinity farnesol complex. Previous studies reporting

farnesol-induced activation of BC2 clones used IFN- γ as a readout, rather than in bulk PBMCs from multiple donors. Therefore, it remains possible that, following isolation and expansion of a farnesol-responsive CD1a-restricted T cell population, robust activation and inflammatory cytokine production could be observed under clonal conditions.

5.4.3 Effects of Limonene on CD1a/d Tetramer Staining and Functional Assays

Limonene is a cyclic monoterpene and classified as a weak contact allergen. ACD to limonene is generally associated with occupational exposure and misuse rather than typical consumer use. In CD1a tetramer assays, limonene produced negligible staining, whereas in CD1d tetramer assays a modest increase in T cell staining was observed, although this effect was markedly lower than that induced by the positive control α -GalCer. Structurally, limonene does not conform to the canonical features of a CD1 ligand: it is a 10-carbon molecule, significantly below the approximate lipid chain capacity of CD1 isoforms; it lacks acyl chains necessary for optimal hydrophobic pocket engagement; and it contains no heteroatom-bearing functional groups that could mediate polar or hydrogen-bonding interactions with residues in the CD1 binding groove. However, the presence of unsaturated bonds may permit weak interactions (as was shown for the crystal structure of CD1a loaded with farnesol [277]). Moreover, small hydrophobic compounds such as farnesol, despite being below the typical size threshold, have been shown to sequester within the CD1 hydrophobic pocket without directly engaging the TCR; in such cases, the displacement of endogenous ligands can reveal previously hidden epitopes capable of TCR recognition. It remains unclear whether the responding population comprises invariant NKT (iNKT) cells or group 2 NKT cells.

Functional assays revealed an increase in TNF⁺ T cells when HeLa-CD1d cells were loaded with limonene. The increase in TNF⁺ T cells could reflect a CD1d-mediated effect, consistent with the modest increase in CD1d tetramer staining. Nevertheless, this result does not fully align with previously reported immunosuppressive effects of limonene on TNF production. Importantly, the prior studies were not conducted in a contact hypersensitivity (CHS) model, and thus the effects of limonene on CD1d-transfected HeLa cells and their interactions with PBMCs may differ in this experimental context.

5.4.4 Effects of Linalool on CD1a/d Tetramer Staining and Functional Assays

Linalool showed no discernible differences in either tetramer or functional assays. This is notable given that, apart from farnesol and α -GalCer, the compounds tested in these assays were monoterpenes, monoterpenoids, or their oxidation products. Subtle structural variations among these molecules appear to influence interactions with CD1a- or CD1d-restricted T cells. The impact of stereochemistry on CD1-mediated antigen presentation is perhaps most clearly illustrated by the differential activities of α -GalCer and β -GalCer, with α -GalCer being a potent iNKT cell activator and β -GalCer acting as a non-agonistic ligand. Therefore, the results suggest that T cell recognition of these compounds involves highly specific structural interactions.

5.4.5 Effects of Geraniol on CD1a/d Tetramer Staining and Functional Assays

Geraniol, a structural isomer of linalool, showed minimal activity in CD1a or CD1d tetramer staining assays but induced significant increases in the frequency of TNF⁺ and IL-2⁺ T cells when loaded onto HeLa-CD1d cells. Geraniol has previously been reported to possess anti-inflammatory properties, including the ability to reduce IL-2 and TNF production in activated T cells [292]; however, these findings were not investigated in the context of unconventional T cells, such as CD1-restricted T cells. The reported effects of geraniol on CD1-restricted T cells is limited. One study examined CD1a-geraniol complexes and their ability to activate CD1a-restricted T cells using IFN- γ as a readout, but no increase was observed. It should be noted, however, that this study employed a different cytokine (IFN- γ), a BC2 T cell clone, and CD1a-transfected K562 antigen-presenting cells, which may account for the discrepancies. While clonal models offer the advantage of providing high numbers of CD1-restricted T cells for activation studies, they do not recognize all allergens. Therefore, it is possible that geraniol-specific T cells are present within PBMCs, and sorting and expanding CD1d-geraniol⁺ T cells could help elucidate their functional relevance. Although the crystal structure of CD1-geraniol complexes has been solved, it has not yet been published (personal communication).

5.4.6 Effects of Lim-2-OOH on CD1a/d Tetramer Staining and Functional Assays

Lim-2-OOH did not induce discernible changes in T cell staining in CD1 tetramer assays but elicited a modest increase in TNF⁺ T cells when presented by HeLa-CD1d cells. Most

reported CD1 ligands are native lipids, either derived from mammalian self-lipids or from mycobacterial sources, whereas the role of lipid oxidation products as CD1 agonists remains largely unexplored, representing an area with substantial future research potential. Structural studies with farnesol have demonstrated that hydroxyl functional groups can facilitate non-covalent interactions within the CD1 binding groove. Thus, it is plausible that oxidation of limonene, a hydrocarbon lacking heteroatoms, to its primary oxidation product Lim-2-OOH, imparts chemical features that enhance non-covalent interactions with CD1 molecules. However, no crystal structure has yet been reported demonstrating the accommodation of a hydroperoxide within either the CD1a or CD1d binding cleft.

5.4.7 Effects of Lin-6/7-OOH on CD1a/d Tetramer Staining and Functional Assays

As previously noted, linalool did not produce discernible effects in any of the assays; however, its oxidation to a mixture of Lin-6/7-OOH structural isomers resulted in a reduction of TNF⁺ and IFN- γ ⁺ T cells when presented by C1R-CD1a cells. Although Lin-6/7-OOH didn't show any statistically significant reduction in CD1a tetramer-positive T cell staining, functional assays suggest that Lin-6/7-OOH, like farnesol, may displace endogenous CD1a-associated lipids, thereby lowering the baseline frequency of cytokine-producing T cells. Interestingly, these lipids are classified as moderate contact allergens, yet their effect appears to be suppressive rather than stimulatory with respect to inflammatory cytokine output. This raises the possibility that CD1a-restricted T cells may exert a regulatory function under certain conditions. Given that CD1a naturally accommodates a range of endogenous lipids with either inhibitory or pro-inflammatory properties, it is plausible that Lin-6/7-OOH acts as a natural antagonist, suppressing TCR engagement and subsequent T cell activation. However, in the context of a T cell mediated inflammatory reaction; having a known contact allergen which reduces T cell activation, albeit through a subset of T cells, was not expected and warrants further investigation.

5.4.8 Effects of Ger-6/7-OOH on CD1a/d Tetramer Staining and Functional Assays

Ger-6-OOH and Ger-7-OOH are structural isomers of each other and also share structural similarity with Lin-6/7-OOH. Despite this, they exhibited markedly different effects. Notably, when CD1a tetramers were loaded with Ger-6/7-OOH, a significant decrease in

CD1a tetramer-positive T cells was observed of a similar degree to farnesol. However, when CD1d tetramer were loaded with Ger-6/7-OOH, a significant increase was observed in CD1d tetramer staining assays to a similar degree to the positive control α -GalCer. It remains unclear whether the T cells stained by Ger-6/7-OOH loaded CD1d tetramers were invariant NKT (iNKT; type I) cells or group 2 NKT cells lacking the invariant α -chain. Nevertheless, the evidence strongly supports Ger-6/7-OOH as a ligand for CD1d, particularly as the tetramer staining intensity was of a similar magnitude to that observed with α -GalCer, a well-characterized iNKT agonist. Interestingly, Ger-6/7-OOH did not elicit significant changes in any of the functional assays performed. This raises an important question: does Ger-6/7-OOH act primarily as a binding ligand that stabilizes the CD1d–TCR interaction, or does it actively trigger T cell activation and downstream cytokine responses?

5.5 Conclusions

In summary, the ligands evaluated in this chapter demonstrated diverse modes of interaction with CD1 proteins. Some seemed to behave as agonists, eliciting measurable T cell activation and cytokine responses, while others either functioned as antagonists or, through displacement of endogenous lipids, reduced the overall binding affinity of the CD1–TCR interaction, and resulted in decreased tetramer staining and lower functional readouts. Lin-6/7-OOH behaved similarly to farnesol in both tetramer and functional assays, causing reduced tetramer staining and diminished cytokine production, suggesting a possible antagonistic or low-affinity effect. In contrast, Ger-6/7-OOH exhibited strong CD1d–TCR binding, comparable to α -GalCer in tetramer staining, yet this did not translate into T cell activation, highlighting the distinction between ligand affinity and functional agonism. Across most ligands, only marginal differences were observed in cytokine-positive T cell frequencies or selective tetramer staining between Ctrl and F⁺ cohorts. However, one notable observation was that F⁺ donors exhibited higher frequencies of iNKT cells when CD1d tetramers were loaded with α -GalCer, suggesting a potential expansion or enrichment of these cells in the F⁺ cohort. Importantly, this chapter represents the first investigation of these specific monoterpenes and their primary oxidation products - terpene hydroperoxides, which are moderate to strong contact allergens - as CD1a and CD1d ligands, providing initial

insights into their possible contributions to CD1a/d-restricted T cell activation mechanisms in ACD. These findings require further validation to substantiate and extend the observed results.

Chapter 6: Summary of PhD Project and Future Work

6.1 Summary of PhD Project

This PhD set out to investigate a possible link between terpene hydroperoxide contact allergens, the lipid modifications they induce, the potential of these modified lipids to act as CD1 ligands, their activation of CD1-ligand-restricted T cells, and the possibility of such T cells mediating the clinical manifestation of allergic ACD. The work made progress in all of these areas, although not always along the linear pathway originally envisioned.

The terpene hydroperoxides Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH were successfully synthesised using published methods. Their NMR characterisations closely matched previous reports. An interesting observation was that Lim-2-OOH synthesis favoured one stereoisomer product over the other, with evidence strongly suggesting the (4*R*,6*S*) isomer. Attempts were made to selectively protect the structural isomers of the acyclic hydroperoxides Lin-6/7-OOH to enable separation by flash chromatography. Although successful in one instance (data not shown), the method proved time-consuming and was not essential for subsequent steps.

To explore potential terpene hydroperoxide mediated lipid modifications, a lipidomic study was undertaken. Treatment of HaCaT cells with the contact allergens Lim-2-OOH, Lin-6/7-OOH, and the experimental contact allergen DNFB resulted in similar lipidomic changes. There was an upregulation of several plasmalogen/plasmenyl phospholipids (O-/P-PC and O-/P-PE), sphingolipids (HexnCer), and triglycerides (TGs), accompanied by a decrease in some PUFA-containing phospholipids (PC and PE). This work served as an introduction for the PhD candidate in using mass spectrometry for detecting lipid composition changes, and the output resulted in a publication in *Contact Dermatitis*. However, it focused more on lipid metabolism and changes in the expression of native lipids classes induced by terpene hydroperoxides, rather than the generation of immunogenic non-native lipids) and was ultimately a hypothesis-generating study that could serve as a useful springboard for future projects.

To explore the potential of terpene hydroperoxide contact allergens to mediate lipid peroxidation, liposomes of PLPC were created and incubated with the terpene hydroperoxides under biomimetic conditions. Lim-2-OOH, Lin-6/7-OOH, and Ger-6/7-OOH

were all able to induce significant oxidation of PLPC, to a comparable or greater degree than H_2O_2 . Long-chain PLPC oxidation products predominated, and upon further oxidation, low levels of shorter-chain, more electrophilic products were detected. Lim-2-OOH proved to be the most potent oxidizer. These results raise the question of whether lipid peroxidation products may act as damage-associated molecular patterns (DAMPs), contributing to the danger signals required to drive strong inflammatory responses during sensitization. This work therefore established a link between terpene hydroperoxides and their ability to oxidize lipids, and the results were published in *Contact Dermatitis*.

The isolation of the individual terpene hydroperoxide-mediated oxidized PLPC products proved challenging. These difficulties meant that testing individual oxidized species as CD1 ligands was not feasible, and even testing oxidized mixtures, despite fractionation, would have added multiple layers of complication in an already challenging area (CD1 proteins), one where the PhD researcher lacked prior expertise. To maintain clarity of interpretation, remain focused on the link between terpene hydroperoxides, CD1 proteins, and ACD, and provide the novelty expected of a PhD project, the decision was made to test terpene hydroperoxides and their parent terpenes directly as potential CD1a/d ligands. Their ability to mediate CD1a/d-restricted T cell recognition was examined using fluorescently labelled CD1a/d tetramers, together with functional assays employing PBMCs incubated with CD1-transfected cell lines. The terpenes and their hydroperoxide derivatives demonstrated diverse modes of interaction with CD1 proteins. Lin-6/7-OOH behaved similarly to farnesol in both tetramer and functional assays, reducing tetramer staining and diminishing cytokine production, consistent with a low-affinity or antagonistic effect. In contrast, Ger-6/7-OOH showed strong CD1d-TCR binding, comparable to α -GalCer in tetramer staining, but this did not translate into T cell activation-highlighting the important distinction between ligand affinity and functional agonism. A notable observation was that F^+ donors displayed higher frequencies of iNKT cells when CD1d tetramers were loaded with α -GalCer, suggesting a possible expansion or enrichment of these cells in the F^+ cohort. Importantly, this work represents the first investigation of terpene hydroperoxide contact allergens as CD1a and CD1d ligands, offering initial insights into their potential contributions to T cell activation mechanisms in ACD.

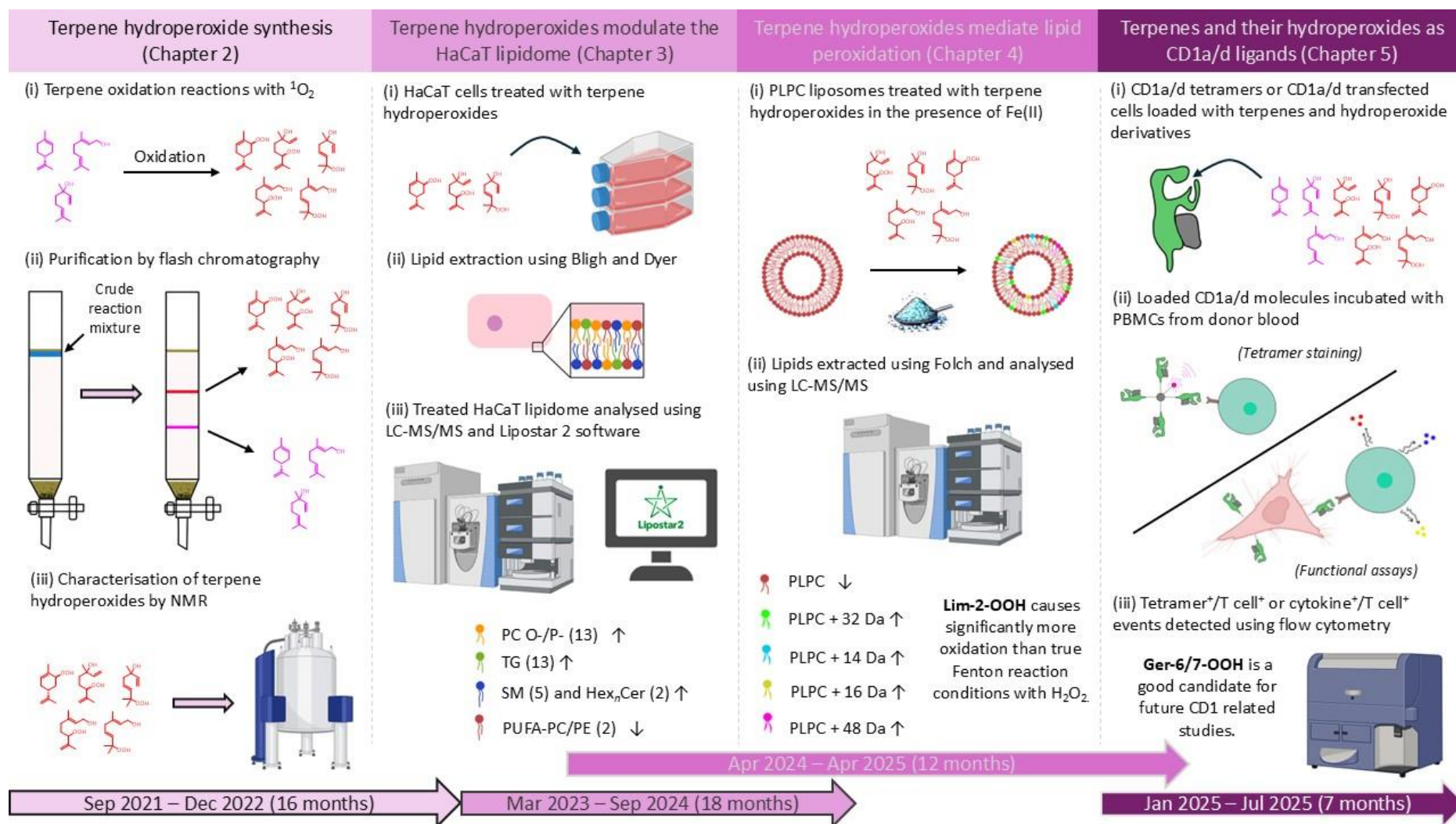


Figure 6.1. PhD concept image.

Experimental work with the steps, methods of analysis, some key findings, and the timeframe of which they occurred during the PhD project.

6.2 Future Project Work

Although isolating specific isomers was not the primary focus of this PhD project, the role of stereochemistry in biological processes is of clear importance. In the context of T cell responses mediated by CD1d and α -GalCer, it is well established that subtle stereochemical differences can have major functional consequences. Building on the observation that Ger-6/7-OOH, present as a 1:1 mixture of structural isomers, was able to induce tetramer staining of PBMCs, it would be valuable to isolate and test the individual isomers to determine whether Ger-6-OOH, Ger-7-OOH, or both contribute to this effect. This becomes especially relevant in light of experimental evidence showing that autoxidation of geraniol yielded only one detectable hydroperoxide isomer (Ger-7-OOH). For CD1a to have a role in facilitating T cell responses to geraniol hydroperoxides in ACD, it seems likely that CD1 should display some degree of specific binding to Ger-7-OOH.

As a follow-up to the HaCaT lipidomic study, further work could explore whether the lipid changes observed represent an antioxidant response. Previous studies have shown that contact allergens can activate antioxidant gene elements, and here, exposure to contact allergens upregulated several plasmalogen and/or plasmalogen phospholipid species—lipids often referred to as endogenous antioxidants. Another noteworthy observation was the upregulation of selected glycosphingolipids (HexnCer). Since keratinocytes are known to express CD1d, with expression upregulated in diseased states, and given that certain glycosphingolipids are potent activators of CD1d-restricted T cells, one possibility is that contact allergens activate keratinocytes in a way that alters endogenous lipid loading in the ER, thereby making them targets for CD1d-restricted T cells.

The PLPC liposome oxidation experiments were designed with future CD1 studies in mind. The goal was to identify modified lipids generated by terpene hydroperoxides and to test them individually as ligands in CD1 tetramer staining and functional assays. This idea was inspired by LC-MS studies of hapten–peptide adducts using model peptides such as histidine. In the present study, lipid oxidation products common to all terpene hydroperoxides were observed. Interestingly, Lim-2-OOH produced an additional product with an m/z greater than PLPC itself, consistent with the formation of a potential adduct containing a phosphatidylcholine fragment. This product was reproducible, not seen with

other hydroperoxides, and represents a lead worth pursuing. However, given the complexity of such work and the high level of LC-MS and lipid expertise required, it was not possible to fully investigate this during the PhD. The study of oxidized lipids as CD1 ligands remains largely unexplored, and progress is hampered by the limited availability of oxidized lipid standards. Because isolating or synthesizing individual oxPLPC products was not feasible at the scale used here, an alternative approach would have been to test oxidized fractions of PLPC and PAPC induced by Lim-2-OOH, followed by LC-MS characterization if functional activity was detected. This strategy, while attractive, would require subsequent identification and isolation of the active species—work that is more tractable with free linoleic acid as the substrate than with complex phospholipids. Ultimately, the number of steps and associated risks meant that this project shifted focus toward testing the parent terpenes and their hydroperoxides directly as ligands. Nonetheless, the ideas developed here remain viable and could form the basis of a dedicated future PhD project.

In the context of CD1-related future work, several changes could be implemented to help validate and extend the existing results. For example, the staining of CD1d tetramer-positive T cells when CD1d tetramers were loaded with Ger-6/7-OOH was an encouraging finding, but it does not prove that Ger-6/7-OOH was binding directly to CD1d. A useful validation step would be to run CD1d–Ger-6/7-OOH “loaded” tetramers on IEF gels; a shift in the bands would strongly suggest ligand binding. This approach could serve both as confirmation of Ger-6/7-OOH binding and as a first step for evaluating future ligands of interest before moving into more resource-intensive tetramer staining experiments.

The next stage of future work would involve isolating, sorting, and expanding CD1 tetramer-positive T cells specific for allergen-loaded tetramers. This would initially yield polyclonal T cell populations with different TCR gene segments, which could then be expanded into T cell lines (e.g., 30 cells per well). From these lines, further sorting could generate monoclonal T cells (1 cell per well), creating defined T cell clones. These clones would allow TCR sequencing and the subsequent use of CRISPR-mediated homology-directed repair (HDR) to transfer TCRs into recipient cells.

Jurkat cells, as an immortalised T cell line, could be engineered into HDR Jurkats, which are well-suited for assays of T cell stimulation such as NF- κ B and CD69 upregulation. Primary cells could also be used to generate HDR primary T cells, which would enable more physiologically relevant functional assays, such as LegendPlex profiling of Th1 versus Th2 cytokine responses to allergen- or lipid-loaded CD1 proteins. If such functional assays produce strong immunostimulatory data, the corresponding lipid or allergen would become a candidate for structural studies of CD1–lipid complexes, or ideally, CD1–lipid–TCR ternary structures.

This proposed workflow aligns with approaches used in immunology laboratories studying CD1-mediated T cell responses. Narrowing the scope to a single CD1 protein would be advisable, as the workload required to cover multiple isoforms would be prohibitive. Strong collaboration would also be essential—both to gain expertise in methods such as HDR T cell engineering and to ensure realistic goal-setting given available resources. Most published studies focus on HDR Jurkats, with far fewer using HDR primary cells, and only a handful of groups have experience generating T cell clones. Collaboration will therefore be critical to determining what is achievable and where to focus efforts.

Overall, this remains an exciting field, with growing interest in the role that CD1 proteins may play in T cell responses during allergic contact dermatitis.

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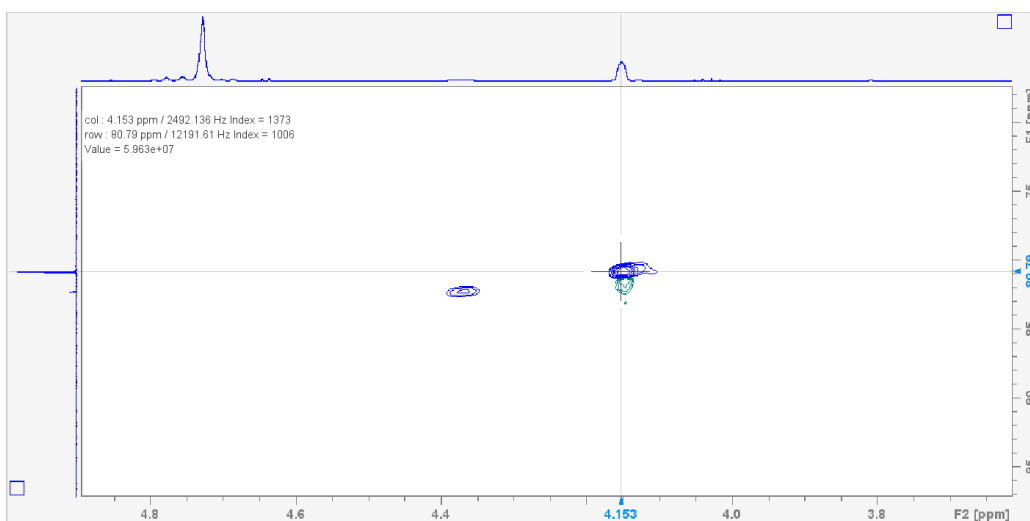
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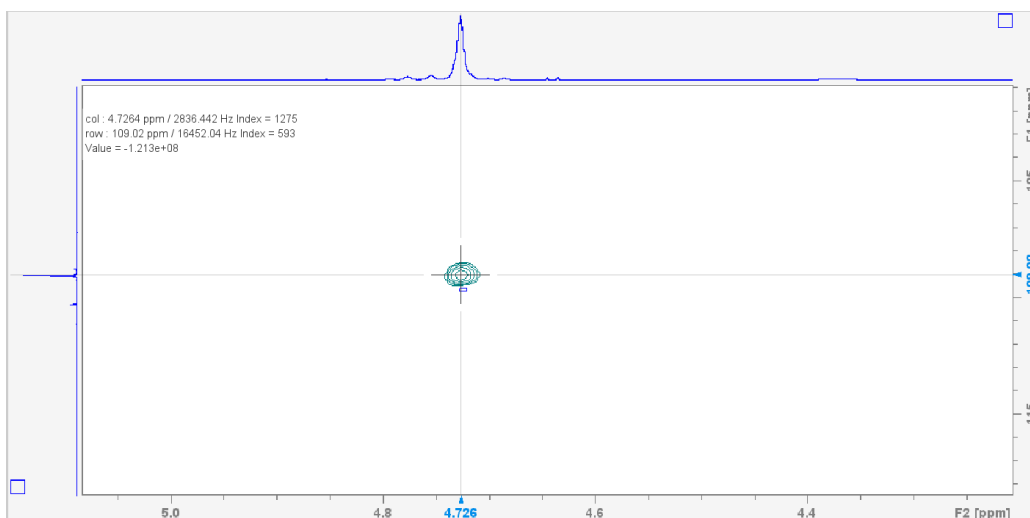
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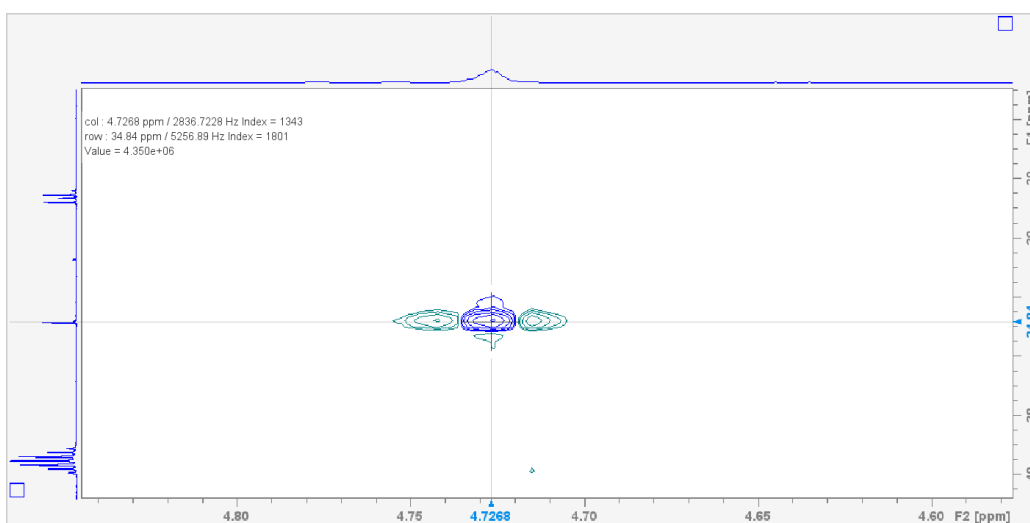
Appendix A: NMR Characterisation Data (Chapter 2)



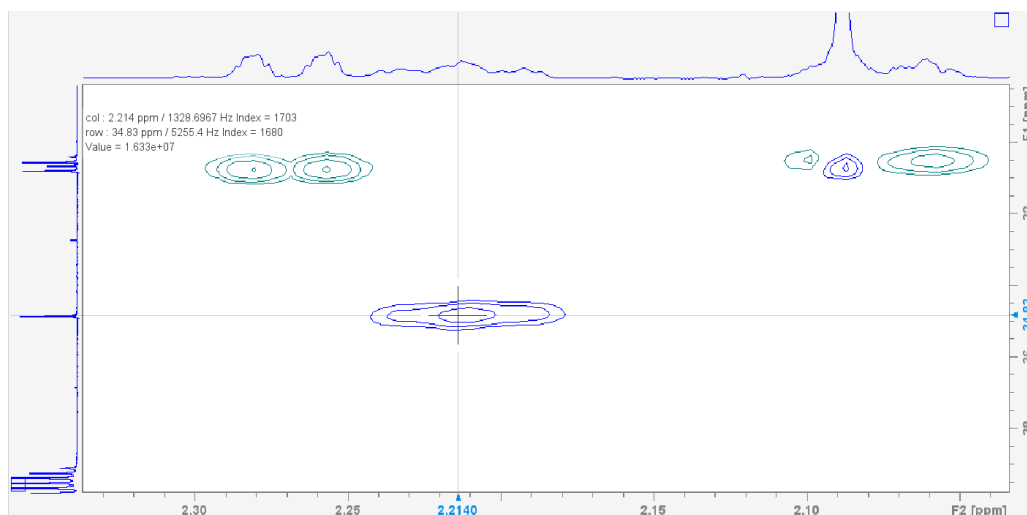
Appendix Figure A 1. HSQC 80.8 > 4.16 (Isomer 1)



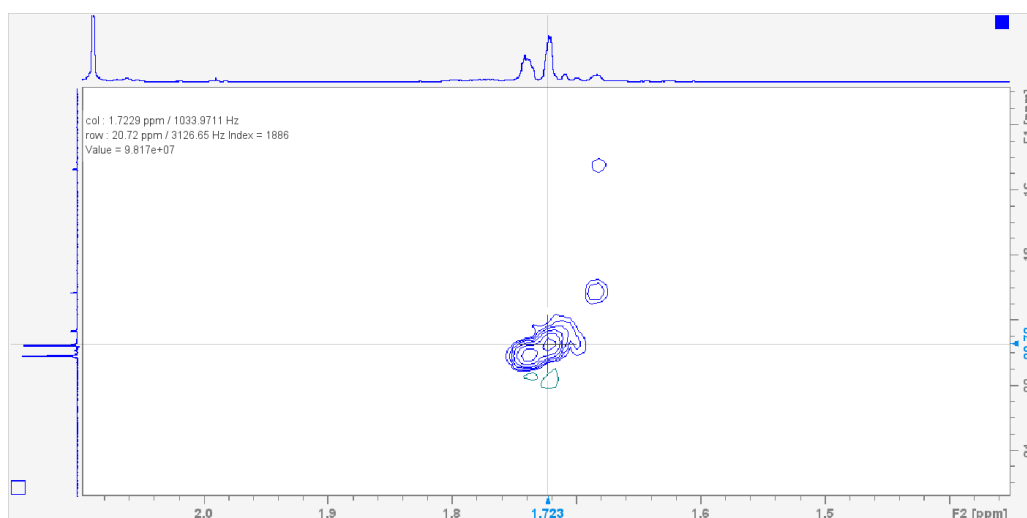
Appendix Figure A 2. HSQC 109.1 > 4.73 (Isomer 1)



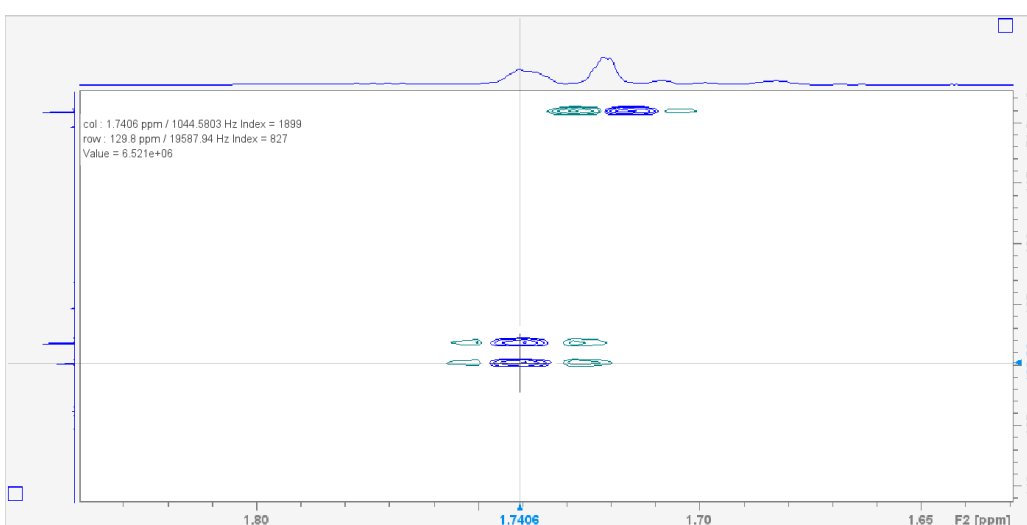
Appendix Figure A 3. HMBC 4.73 > 34.8 (Isomer 1)



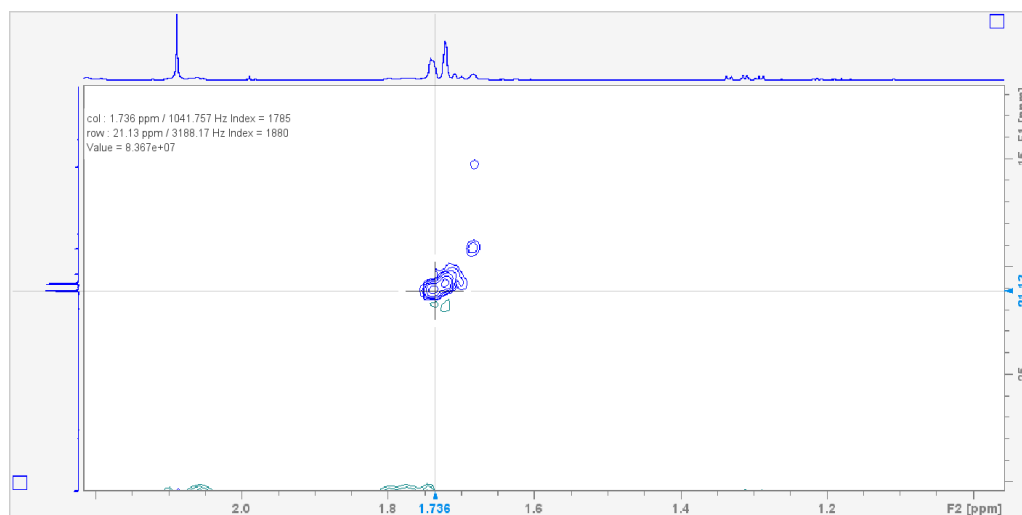
Appendix Figure A 4. HSQC 34.8 > 2.21 (Isomer 1)



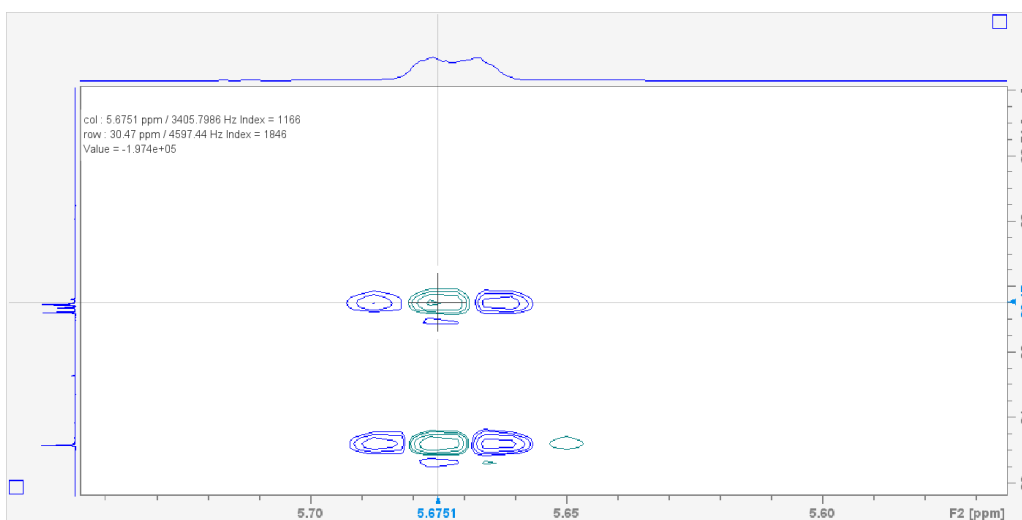
Appendix Figure A 5. HSQC 1.72 > 20.8 (Isomer 1)



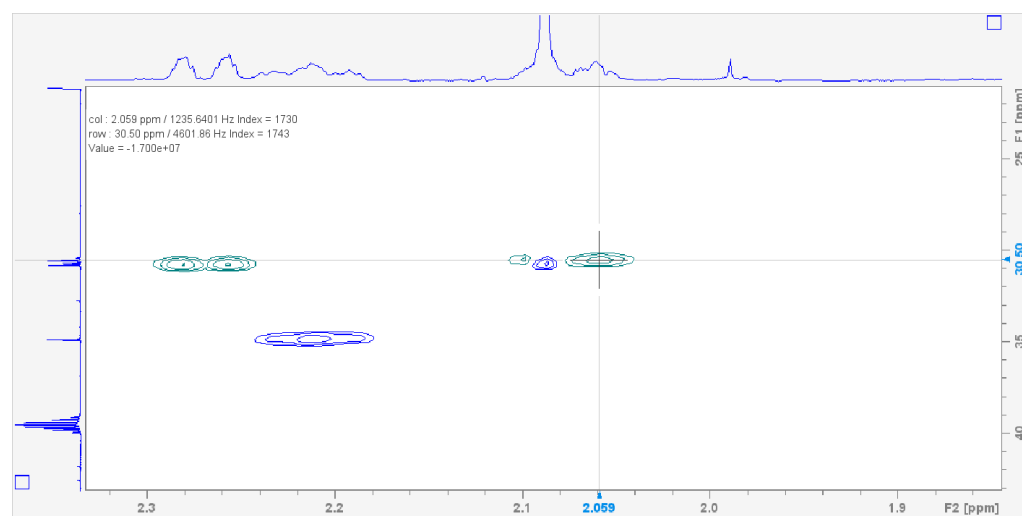
Appendix Figure A 6. HMBC 129.8 > 1.74 (Isomer 1)



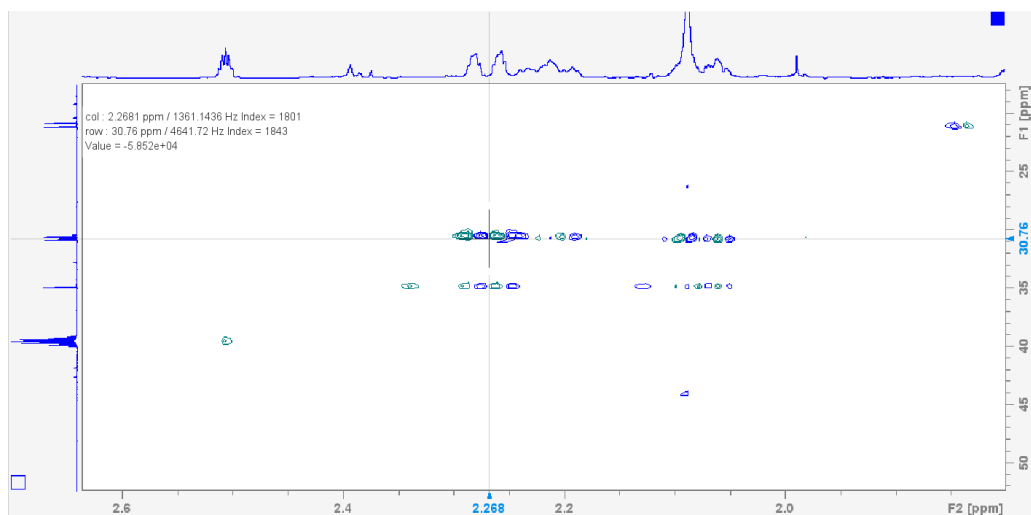
Appendix Figure A 7. HSQC 1.74 > 21.1 (Isomer 1)



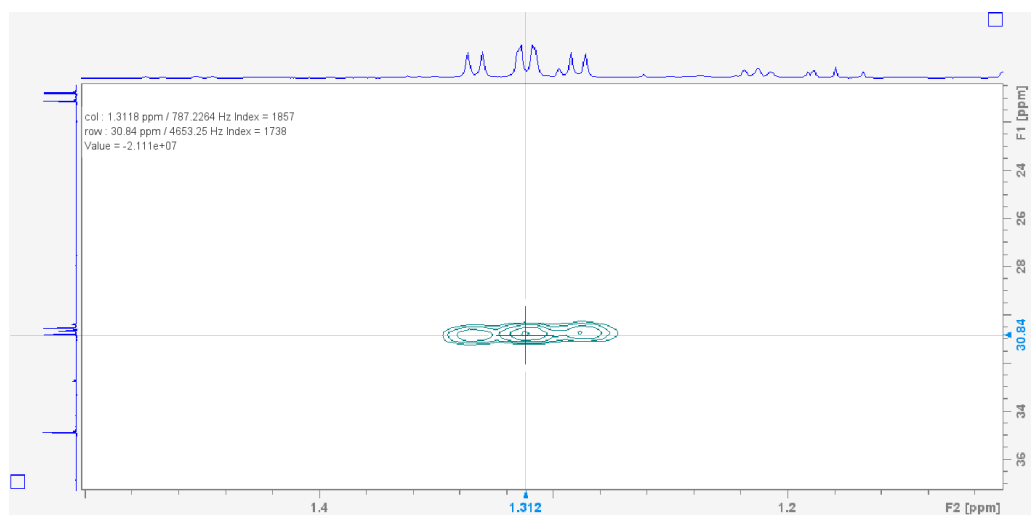
Appendix Figure A 8. HMBC 5.68 > 30.5 (Isomer 1)



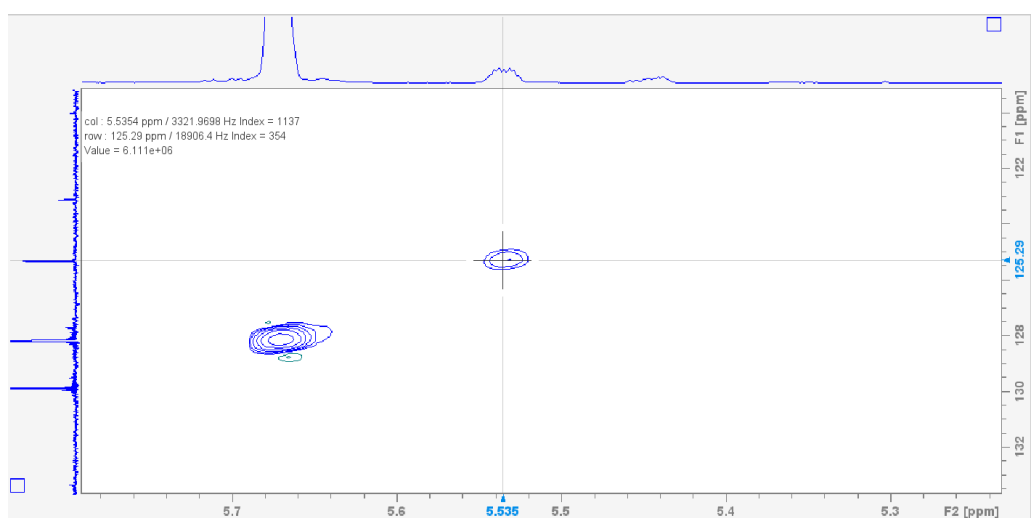
Appendix Figure A 9. HSQC 30.5 > 2.06 and 1.77 (Isomer 1)



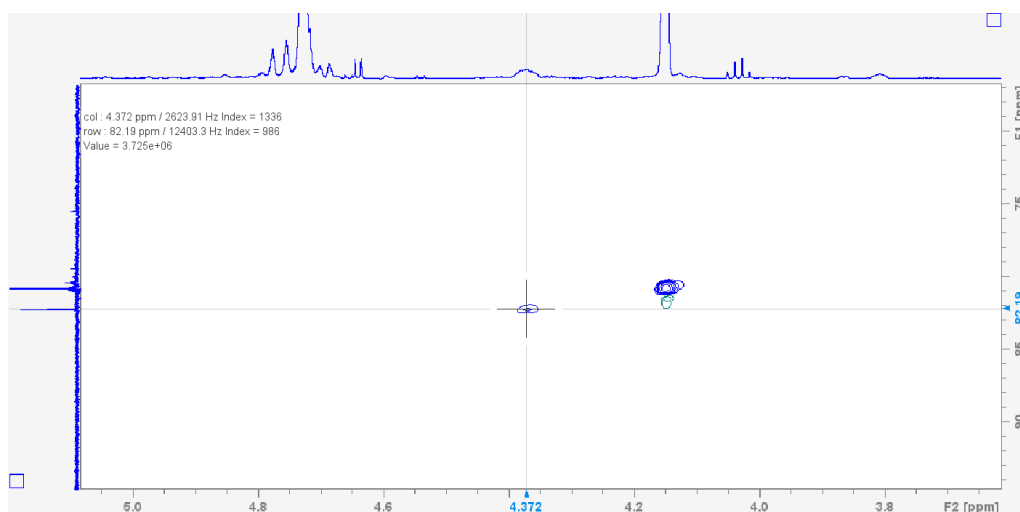
Appendix Figure A 10. HMBC 2.27 > 30.8 (Isomer 1)



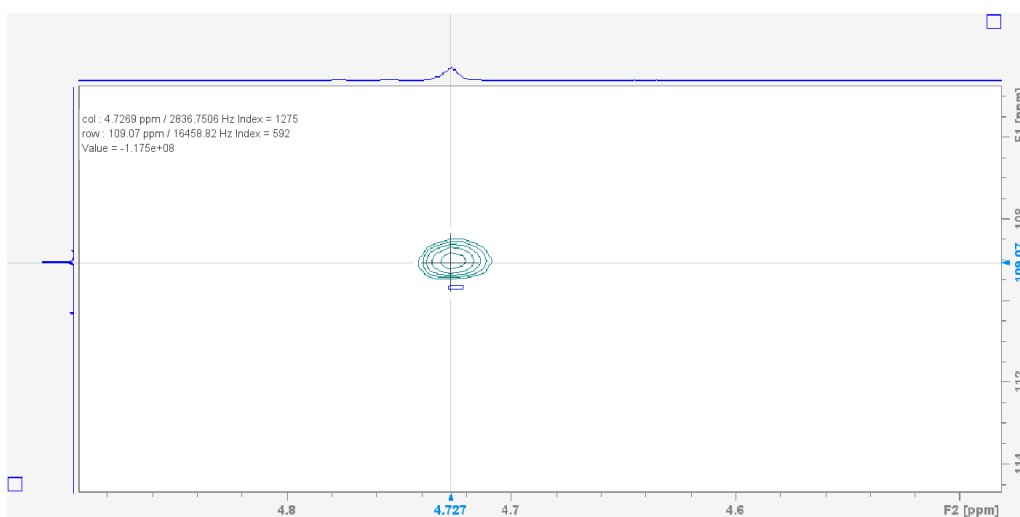
Appendix Figure A 11. HSQC 30.8 > 1.31 (Isomer 1)



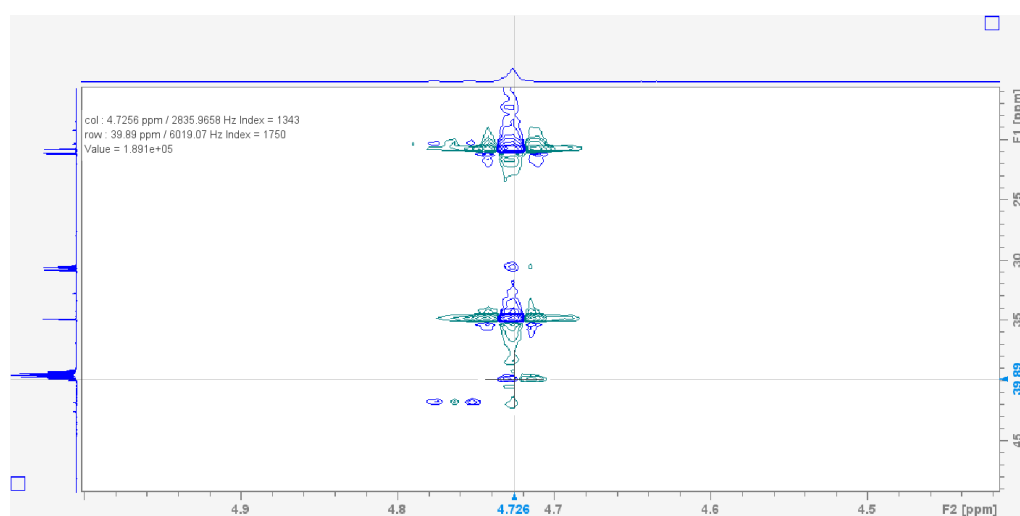
Appendix Figure A 12. HSQC 125.3 > 5.54 (Isomer 2)



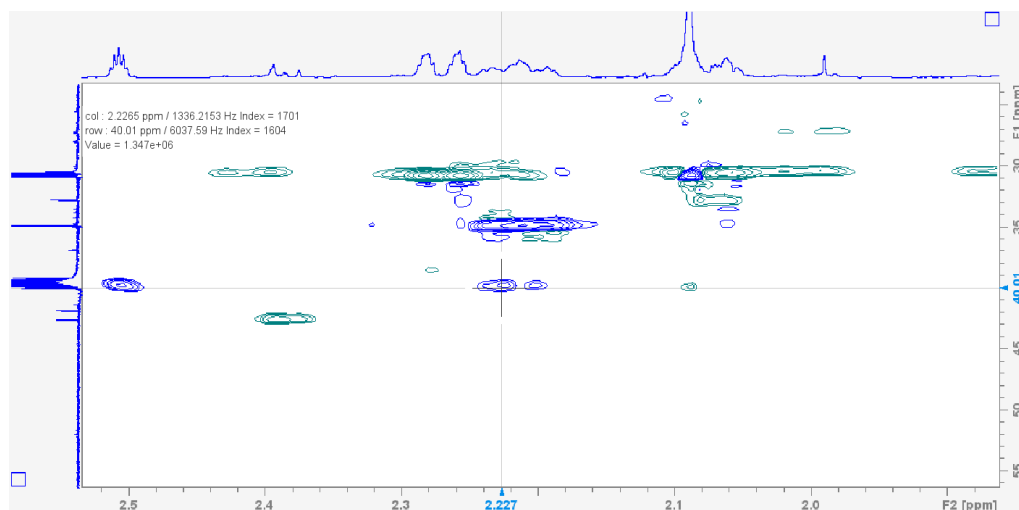
Appendix Figure A 13. HSQC 82.2 > 4.38 (Isomer 2)



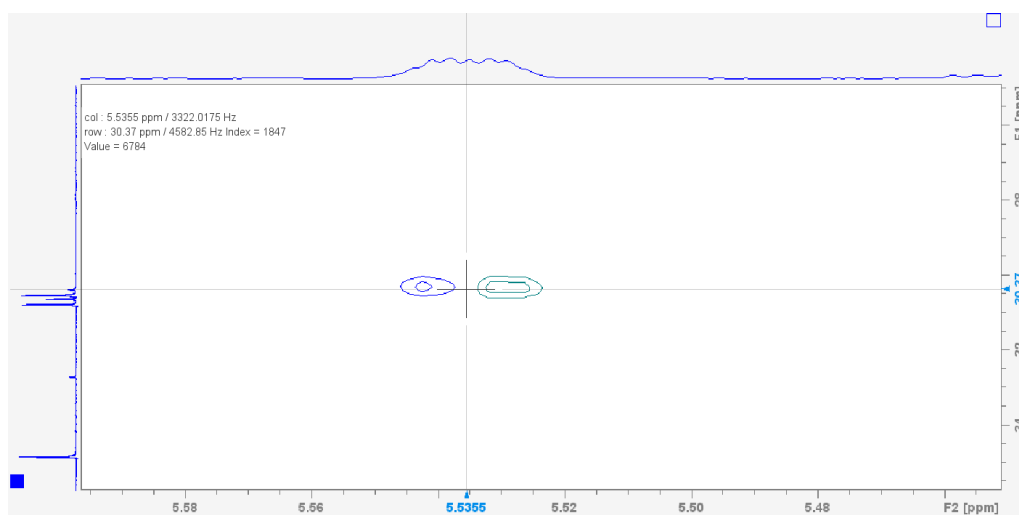
Appendix Figure A 14. HSQC 109.1 > 4.73 (Isomer 2)



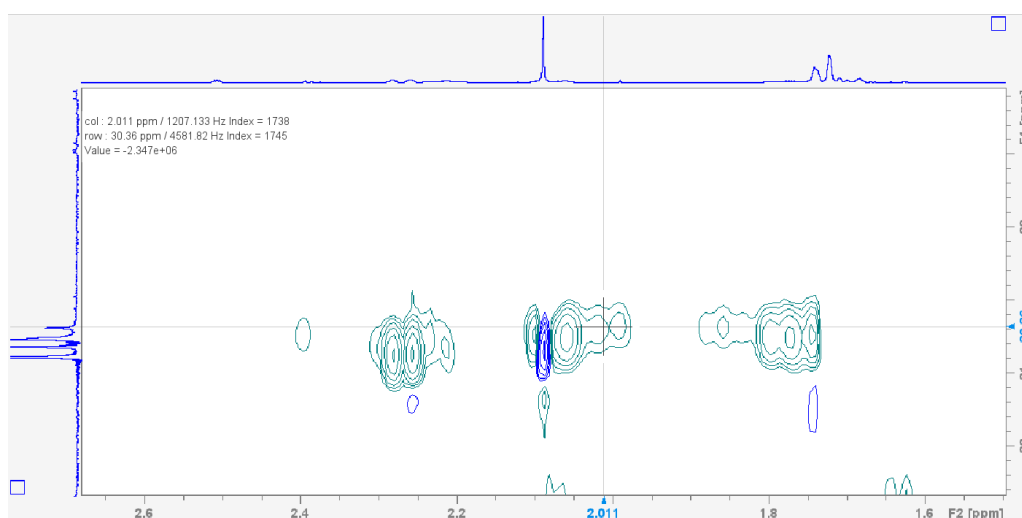
Appendix Figure A 15. HMBC 4.73 > 39.9 (Isomer 2)



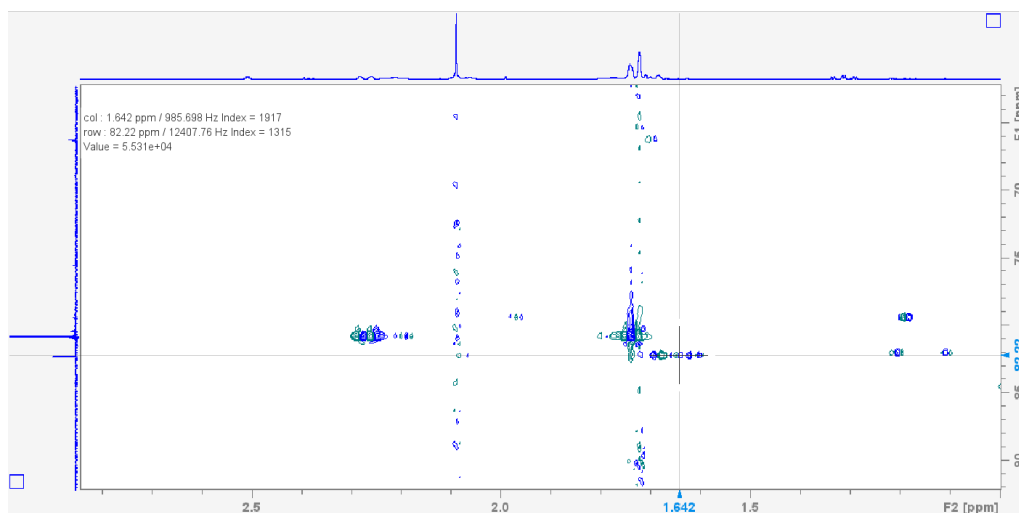
Appendix Figure A 16. HSQC 39.9 > 2.23 (Isomer 2)



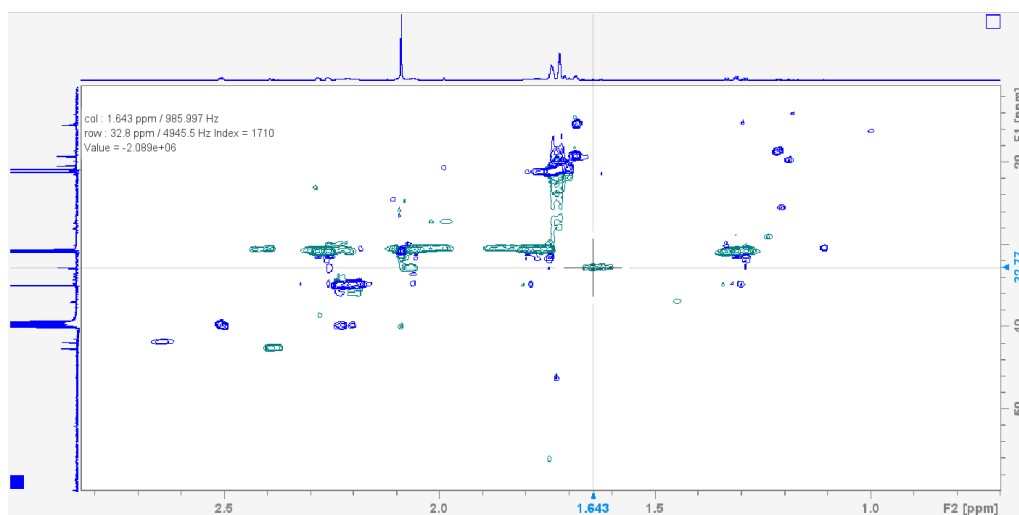
Appendix Figure A 17. HMBC 5.54 > 30.3 (Lim-2-OOH: Isomer 2)



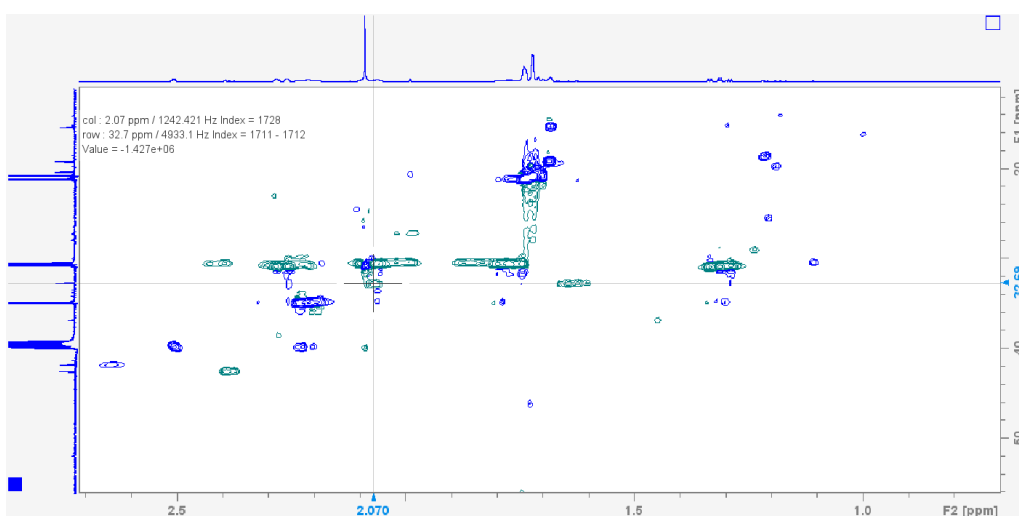
Appendix Figure A 18. HSQC 30.3 > 2.01 and 1.86 (Lim-2-OOH: Isomer 2)



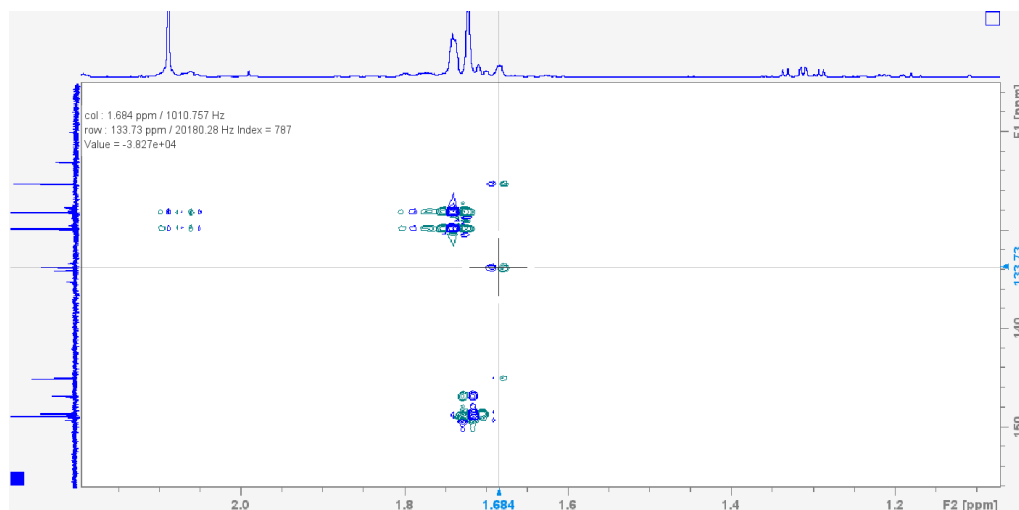
Appendix Figure A 19. HMBC 82.2 > 1.64 (Lim-2-OOH: Isomer 2)



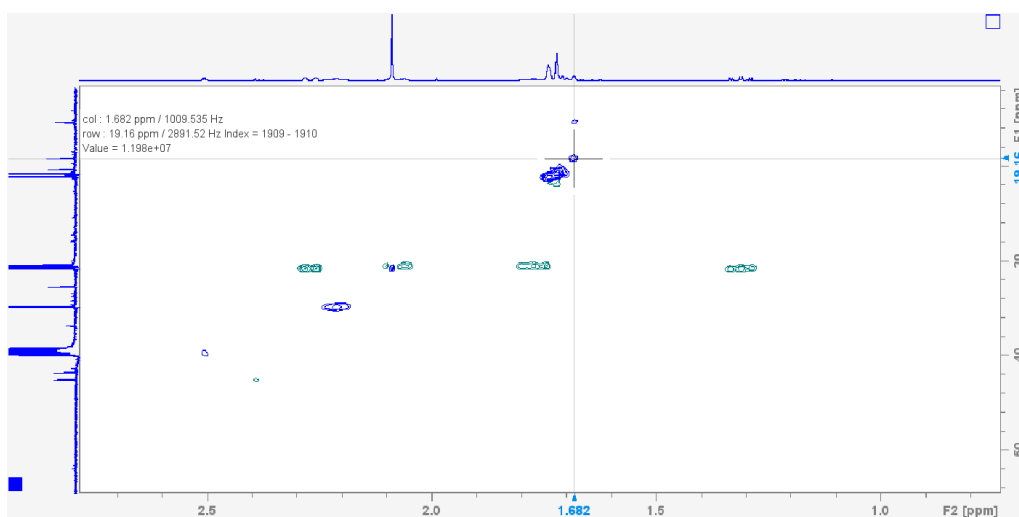
Appendix Figure A 20. HSQC 1.64 > 32.7 (Lim-2-OOH: Isomer 2)



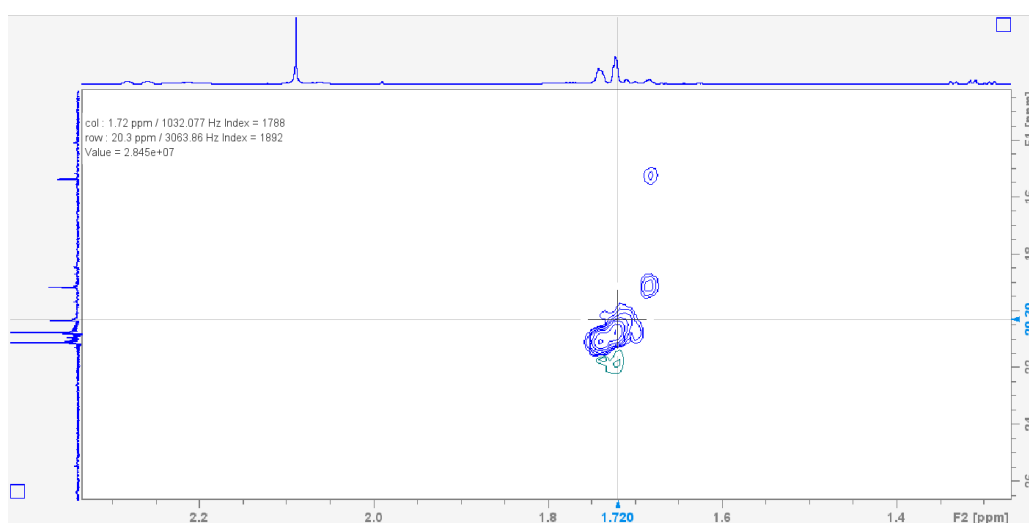
Appendix Figure A 21. HSQC 32.7 > 2.07 (Lim-2-OOH: Isomer 2)



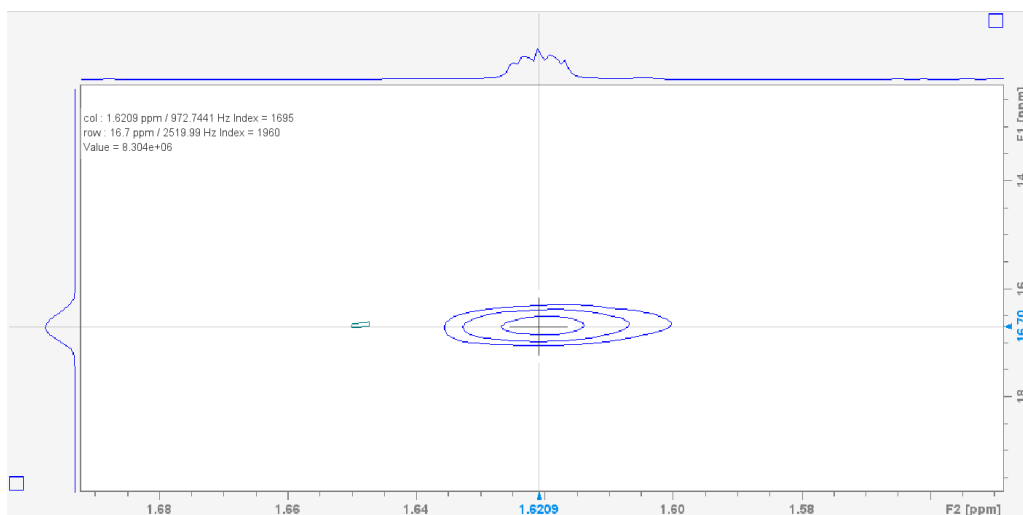
Appendix Figure A 22. HMBC 133.7 > 1.68 (Lim-2-OOH: Isomer 2)



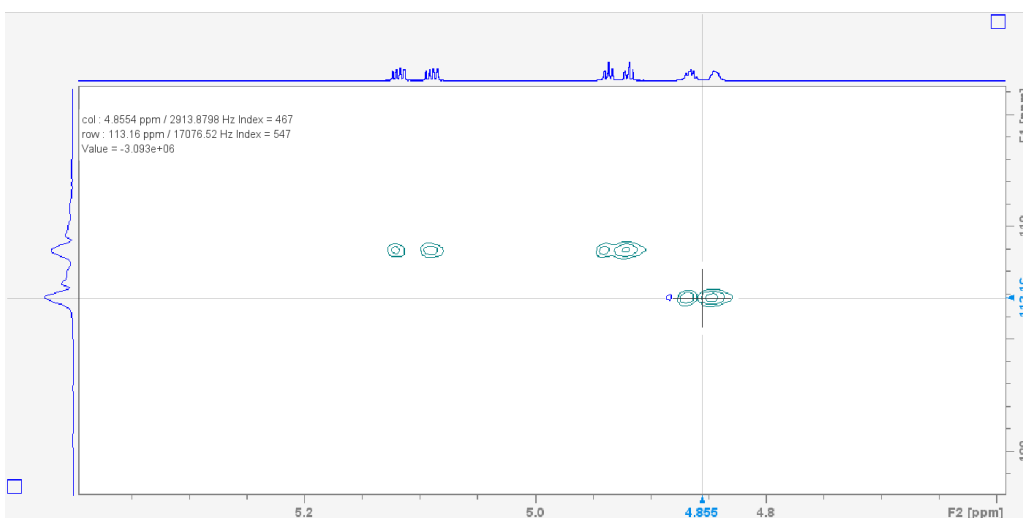
Appendix Figure A 23. HSQC 1.68 > 19.1 (Lim-2-OOH: Isomer 2)



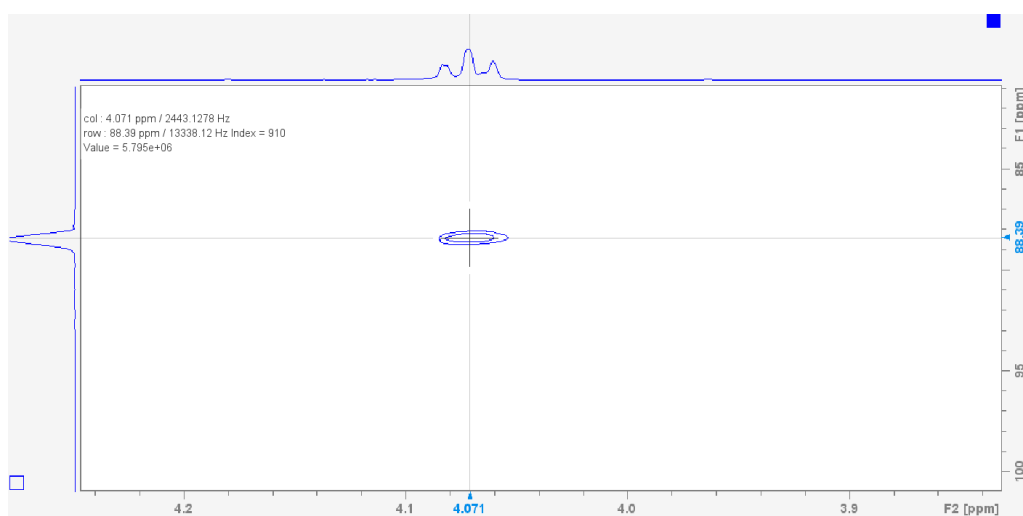
Appendix Figure A 24. HSQC 20.3 > 1.72 (Lim-2-OOH: Isomer 2)



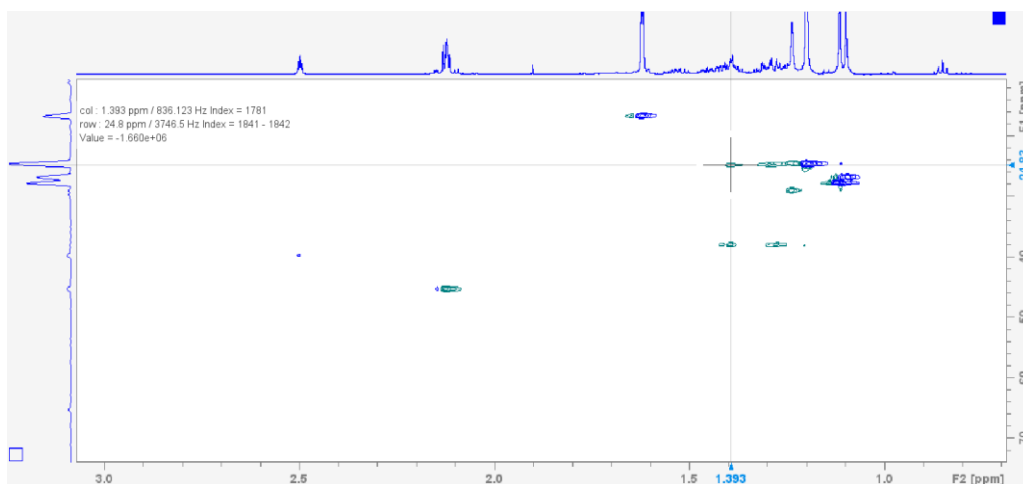
Appendix Figure A 25. HSQC 16.69 and 16.72 > 1.62 (Lin-6-OOH)



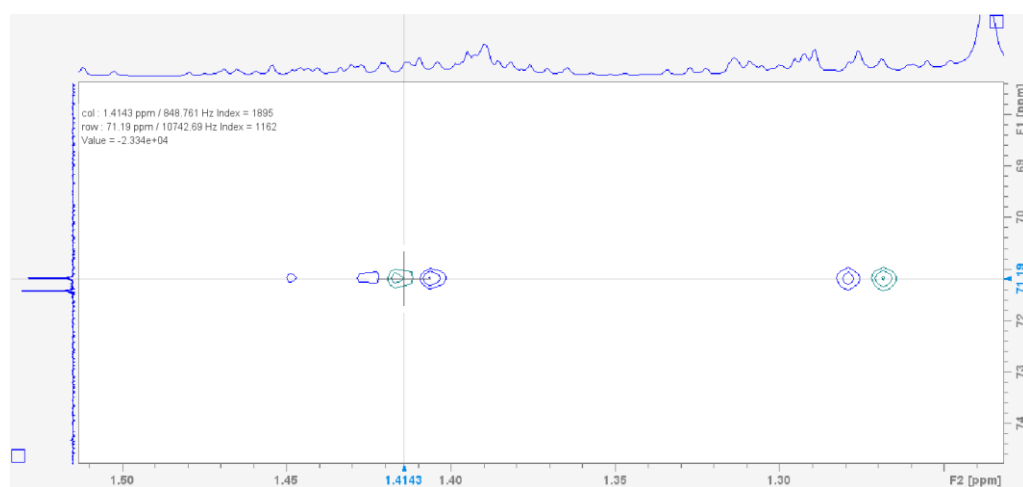
Appendix Figure A 26. HSQC 133.04 and 113.09 > 4.85 and 4.87 (Lin-6-OOH)



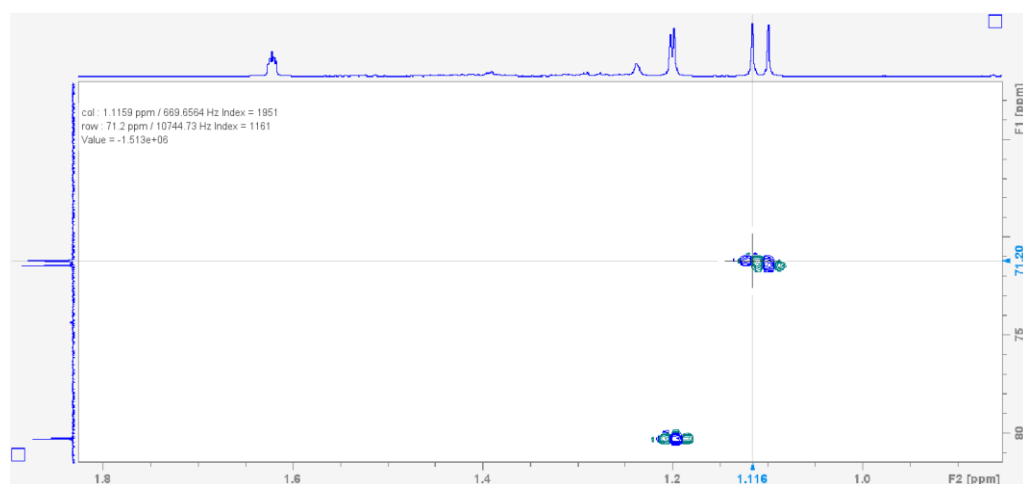
Appendix Figure A 27. HSQC 88.36 > 4.08 (Lin-6-OOH)



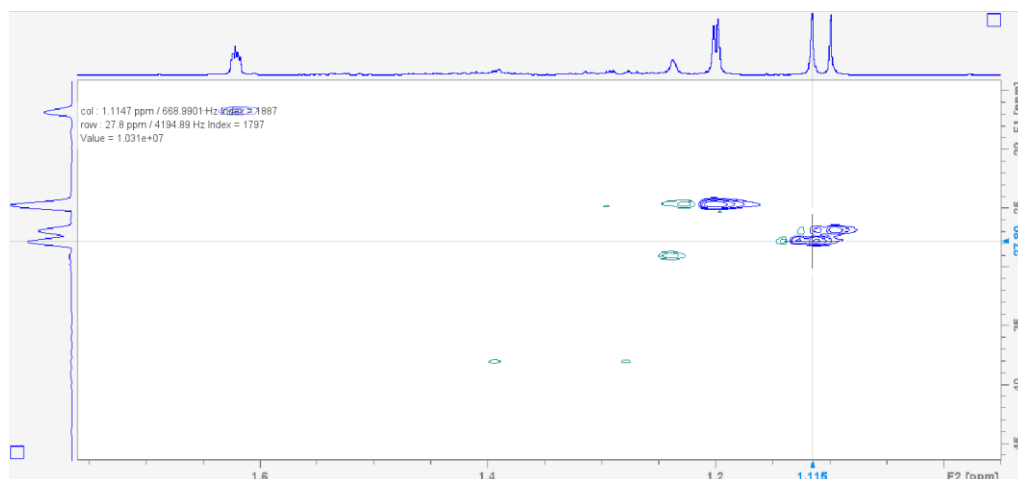
Appendix Figure A 28. HSQC 24.90 and 24.94 > 1.40 and 1.30 (Lin-6-OOH)



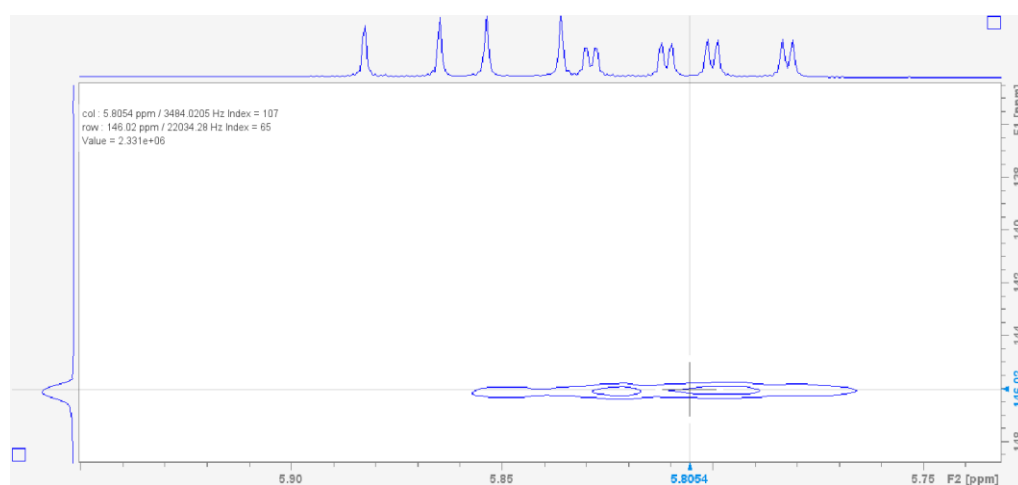
Appendix Figure A 29. HMBC 1.74 > 71.16 (Lin-6-OOH)



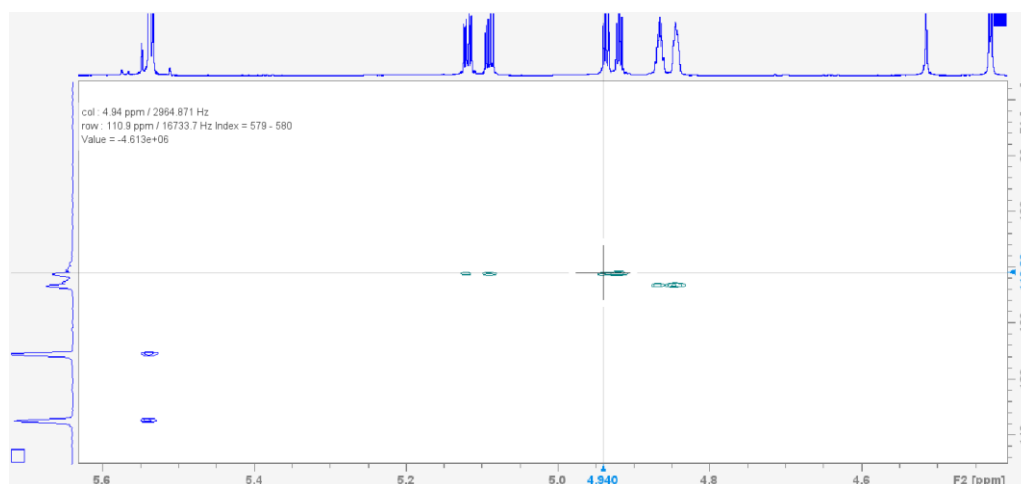
Appendix Figure A 30. HMBC 71.16 > 1.12 (Lin-6-OOH)



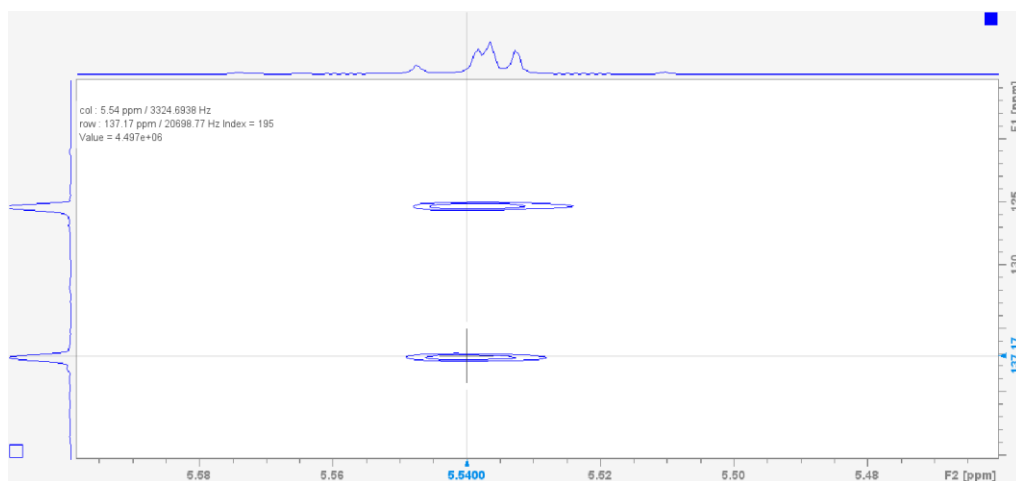
Appendix Figure A 31. HSQC 1.12 > 27.74 (Lin-6-OOH)



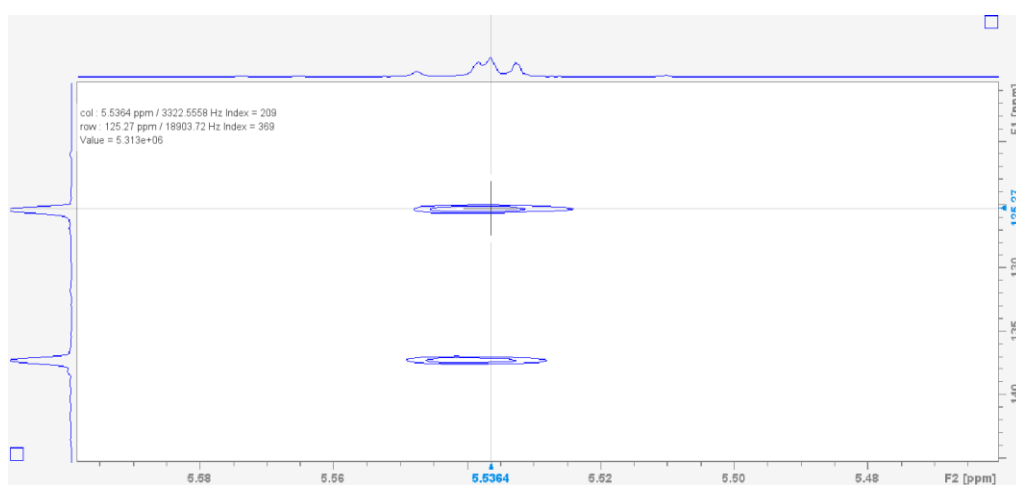
Appendix Figure A 32. HSQC 145.95 > 5.81 (Lin-6-OOH)



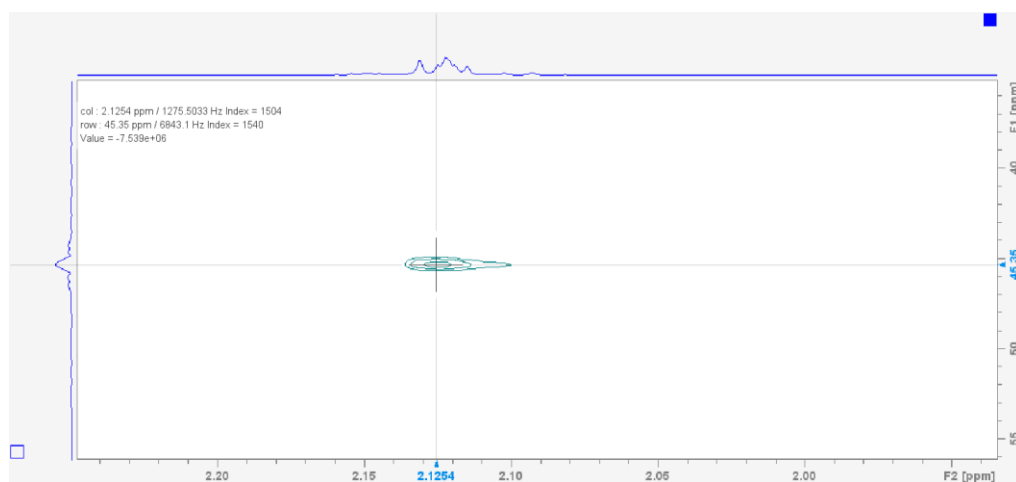
Appendix Figure A 33. HSQC 4.94 and 5.13 > 110.90 (Lin-6-OOH)



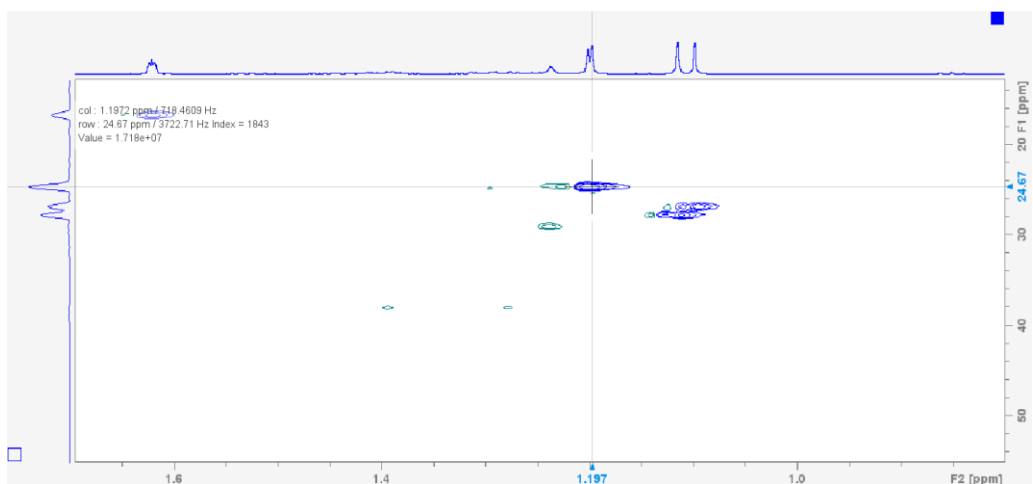
Appendix Figure A 34. HSQC 137.16 > 5.56 (Lin-7-OOH)



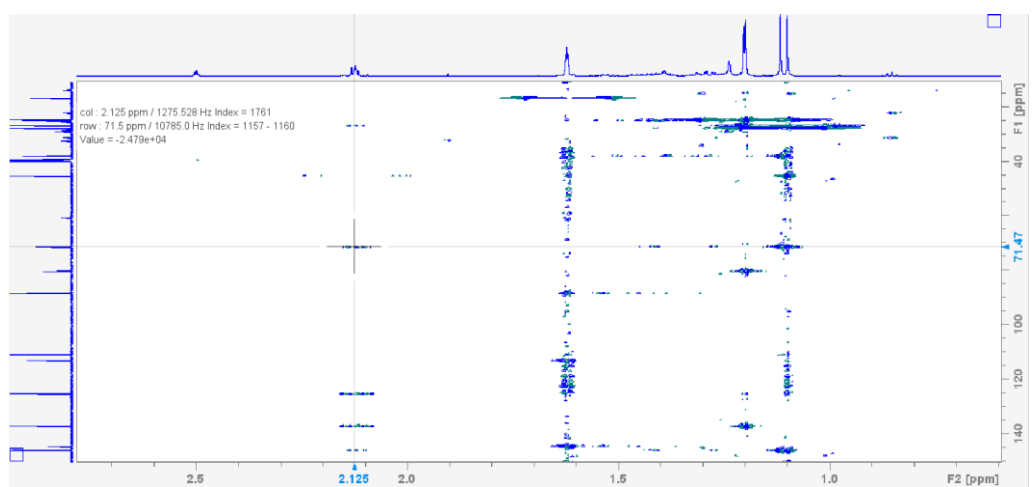
Appendix Figure A 35. HSQC 125.27 > 5.54 (Lin-7-OOH)



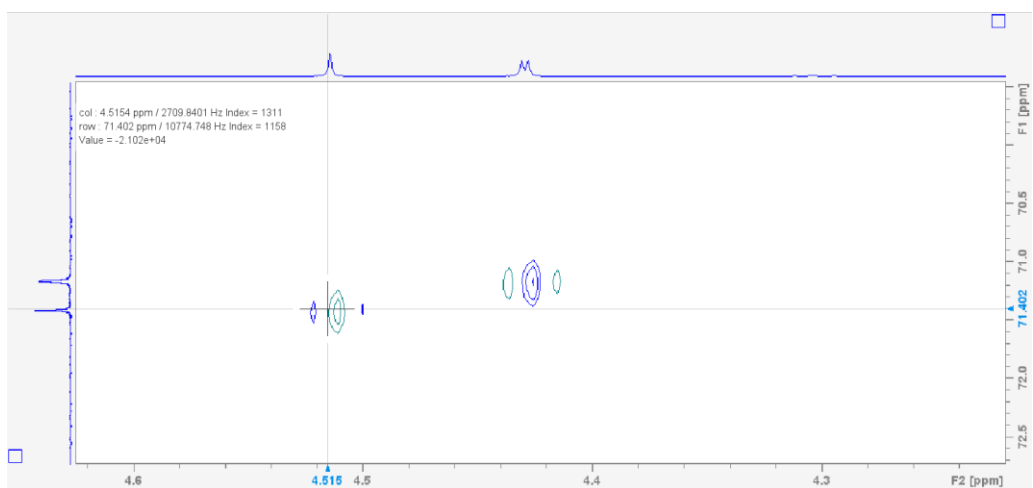
Appendix Figure A 36. HSQC 45.29 > 2.13 (Lin-7-OOH)



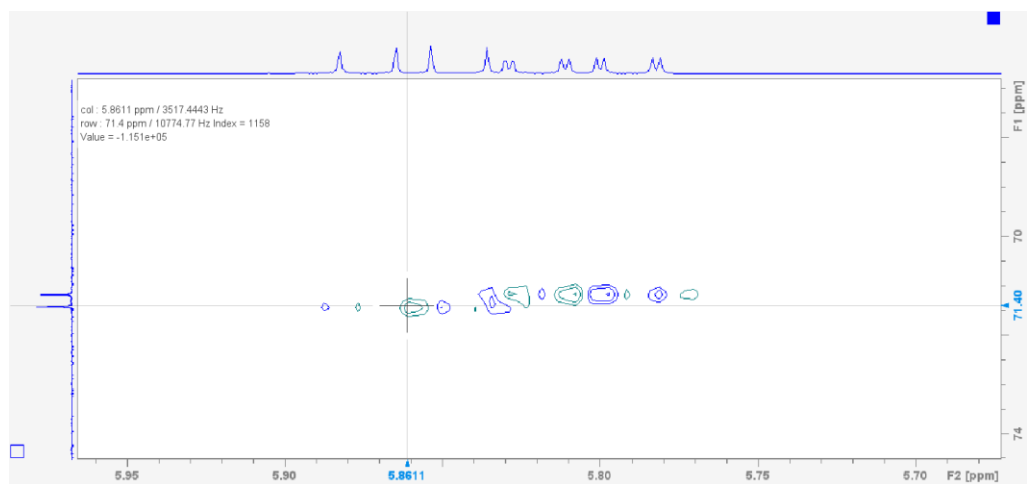
Appendix Figure A 37. HSQC 24.62 and 24.64 > 1.21 (Lin-7-OOH)



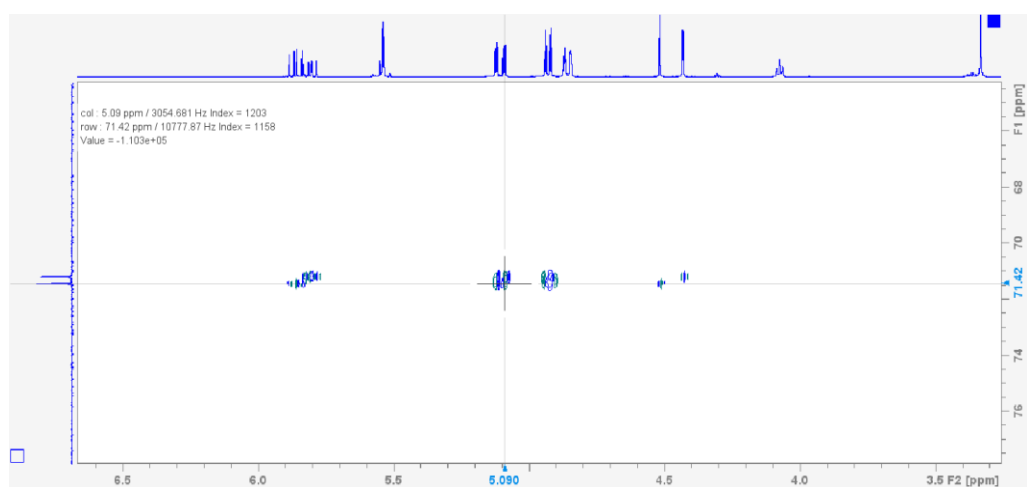
Appendix Figure A 38. HMBC 2.13 > 71.41 (Lin-7-OOH)



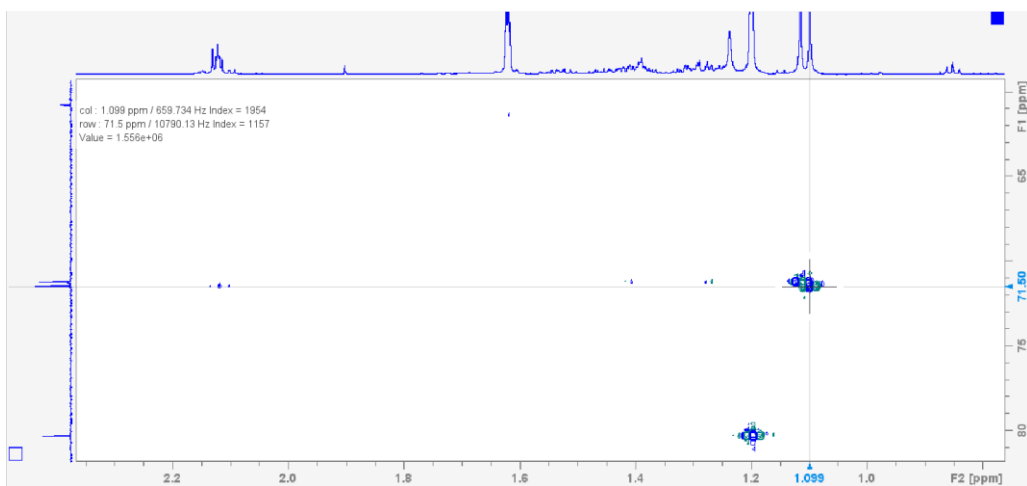
Appendix Figure A 39. HMBC 71.41 > 4.52 (Lin-7-OOH)



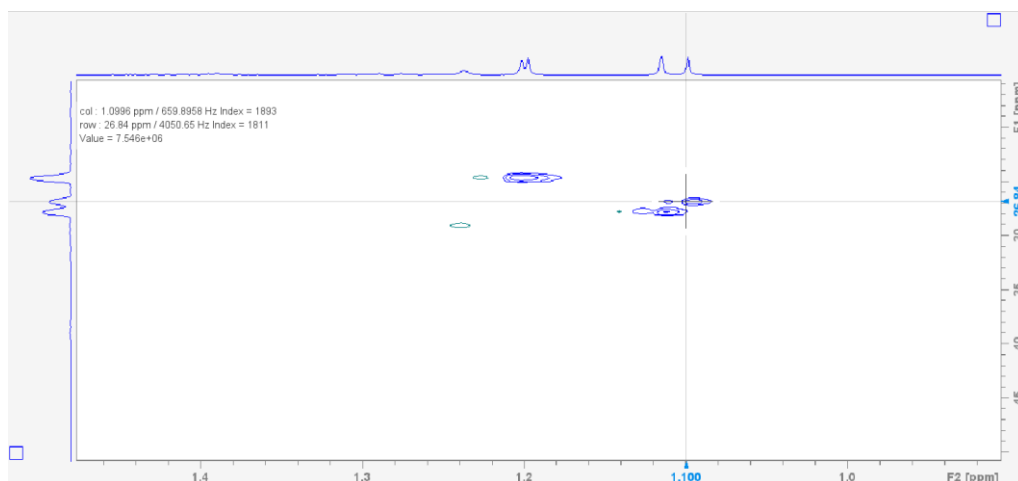
Appendix Figure A 40. HMBC 71.41 > 5.87 (Lin-7-OOH)



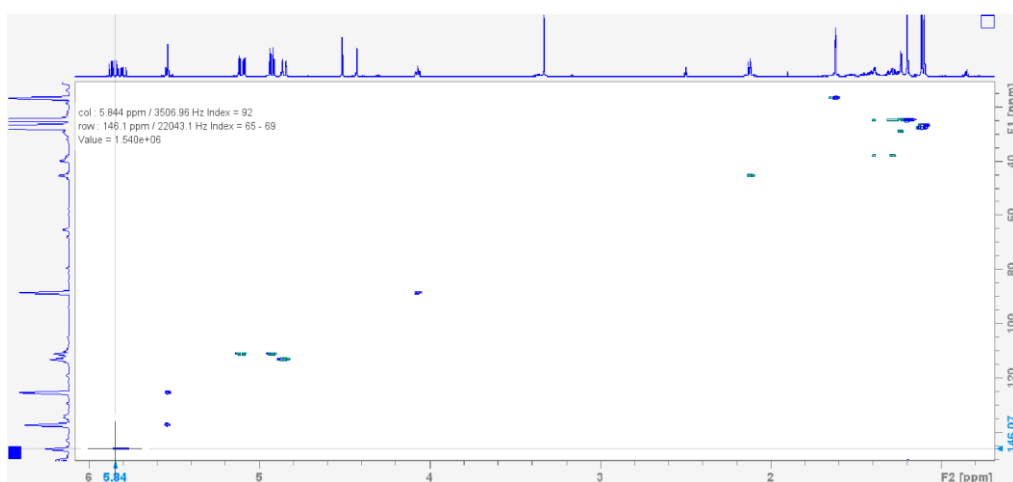
Appendix Figure A 41. HMBC 71.41 > 5.10 and 4.93 (Lin-7-OOH)



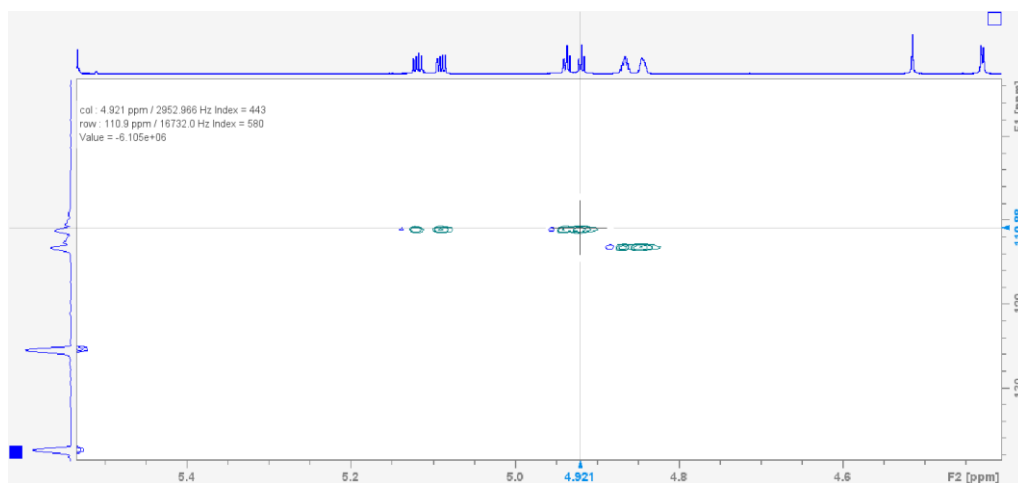
Appendix Figure A 42. HMBC 71.41 > 1.11 (Lin-7-OOH)



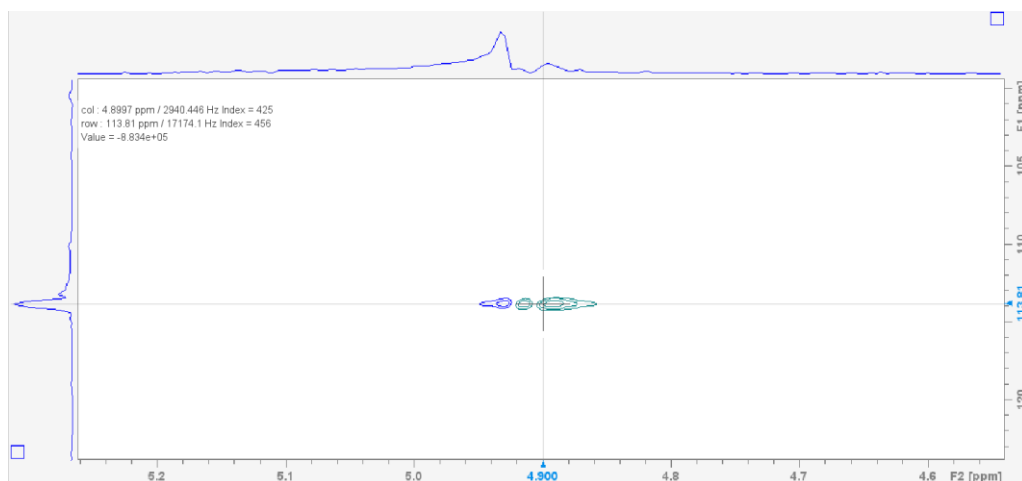
Appendix Figure A 43. HSQC 1.11 > 26.84 (Lin-7-OOH)



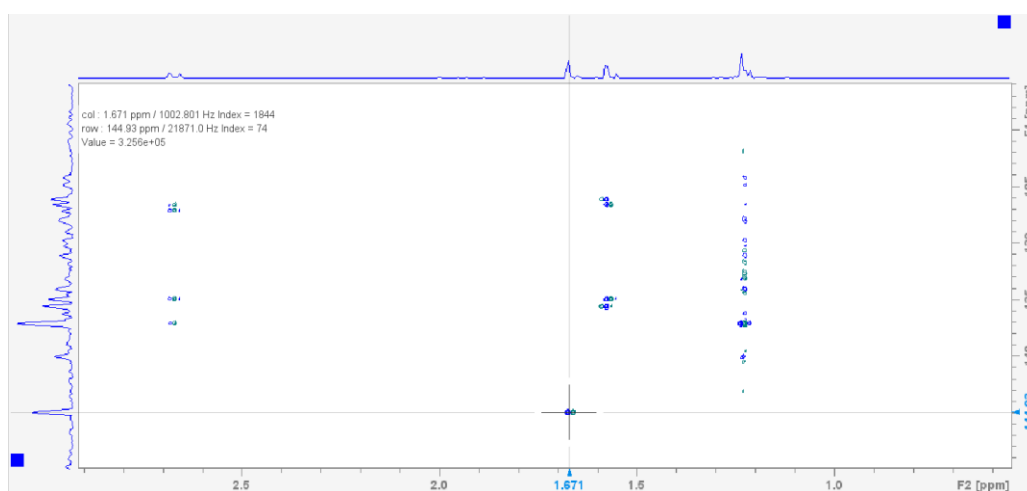
Appendix Figure A 44. HSQC 5.87 > 146.03 (Lin-7-OOH)



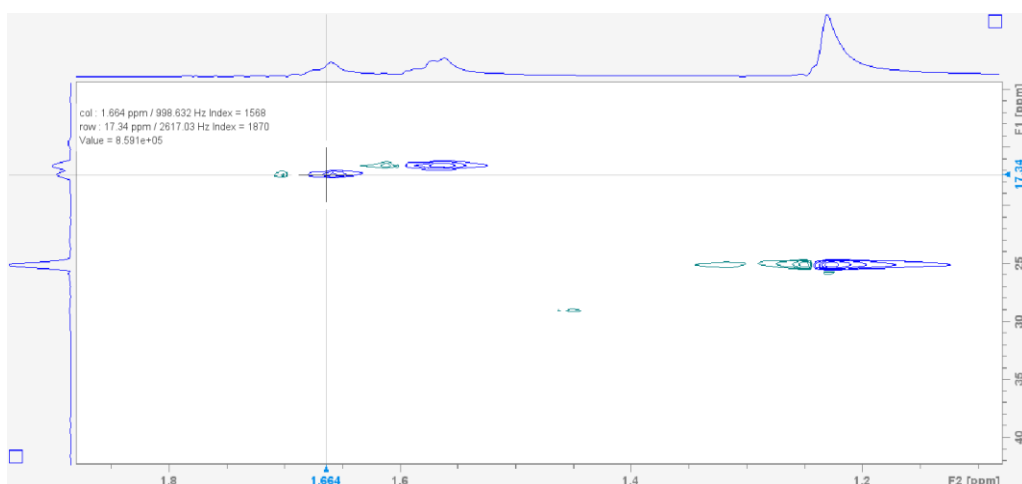
Appendix Figure A 45. HSQC 4.93 and 5.10 > 111.01 (Lin-7-OOH)



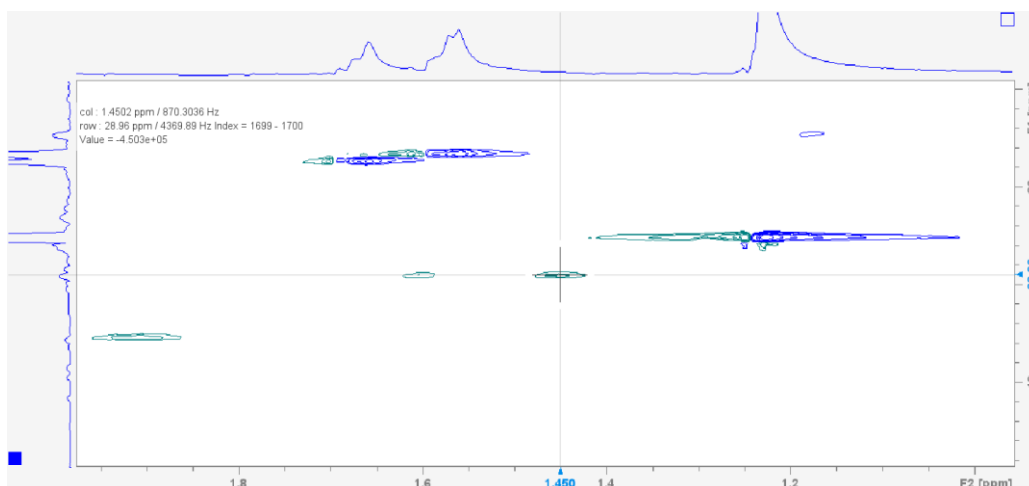
Appendix Figure A 46. HSQC 113.7 > 4.92 and 4.89 (Ger-6-OOH)



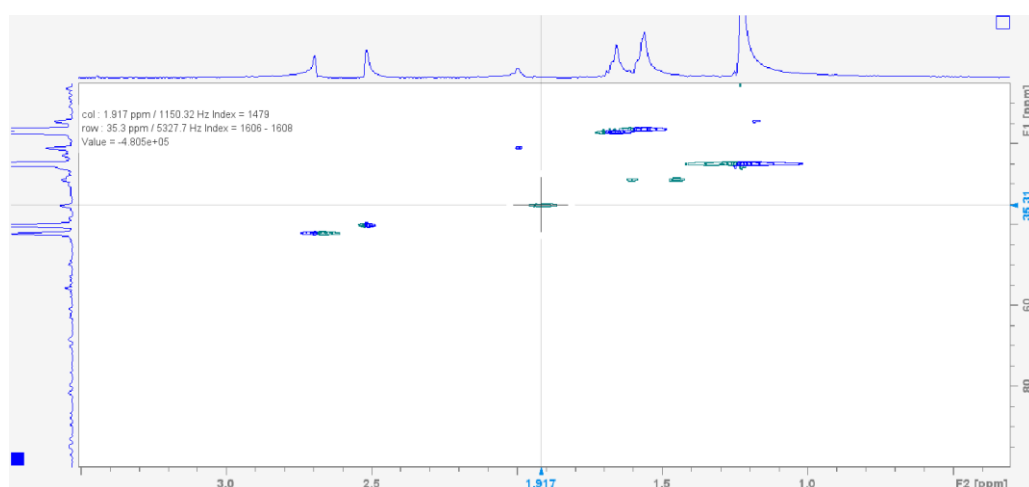
Appendix Figure A 47. HMBC 144.9 > 1.67 (Ger-6-OOH)



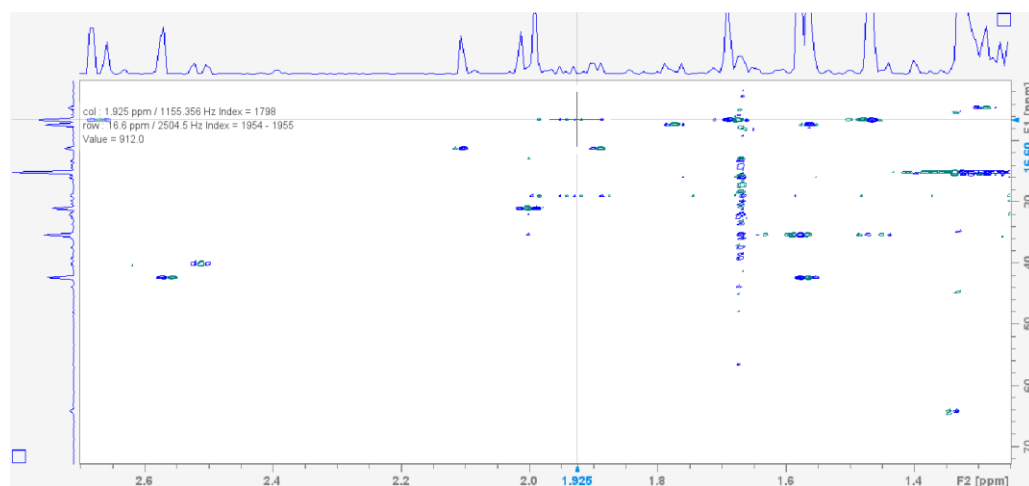
Appendix Figure A 48. HSQC 1.67 > 17.3 (Ger-6-OOH)



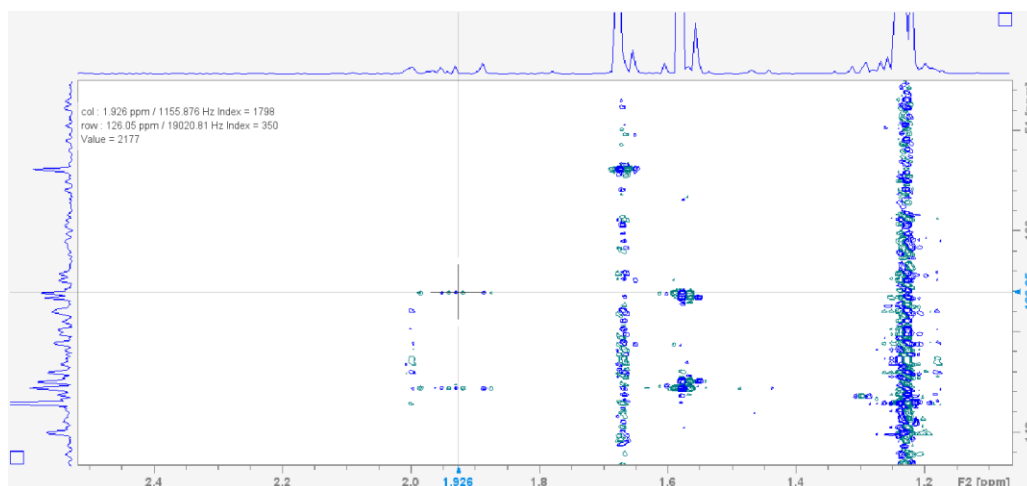
Appendix Figure A 49. HSQC 29.0 > 1.45 and 1.60 (Ger-6-OOH)



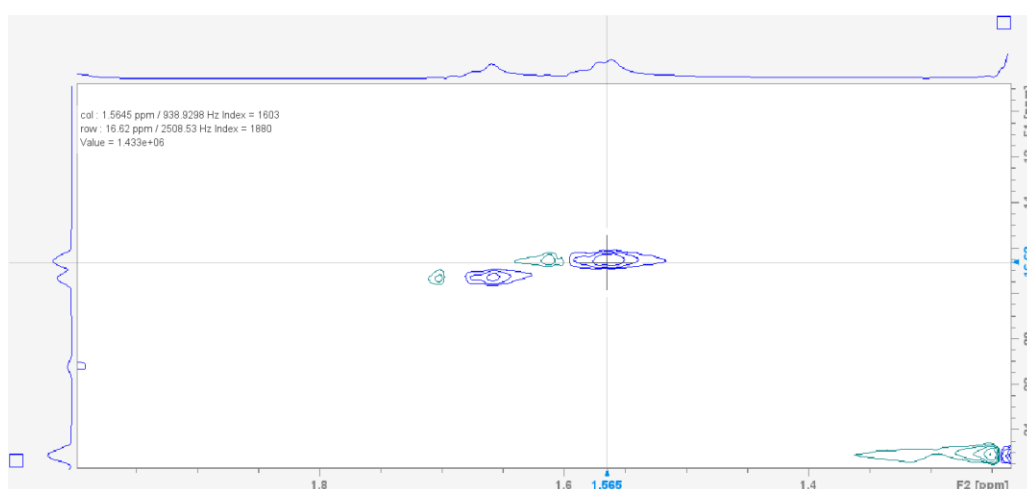
Appendix Figure A 50. HSQC 35.4 > 1.91 (Ger-6-OOH)



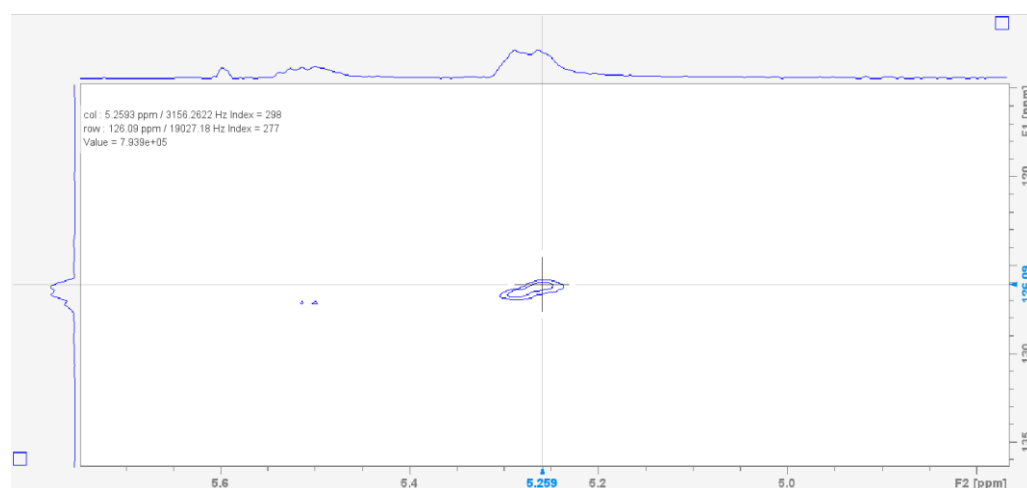
Appendix Figure A 51. HMBC 1.91 > 16.6 (Ger-6-OOH)



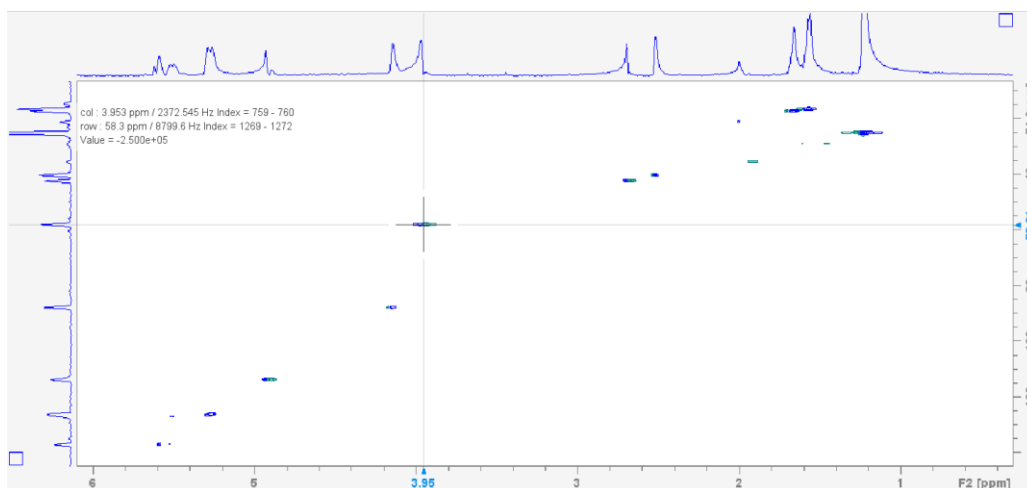
Appendix Figure A 52. HMBC 1.91 > 126.0 (Ger-6-OOH)



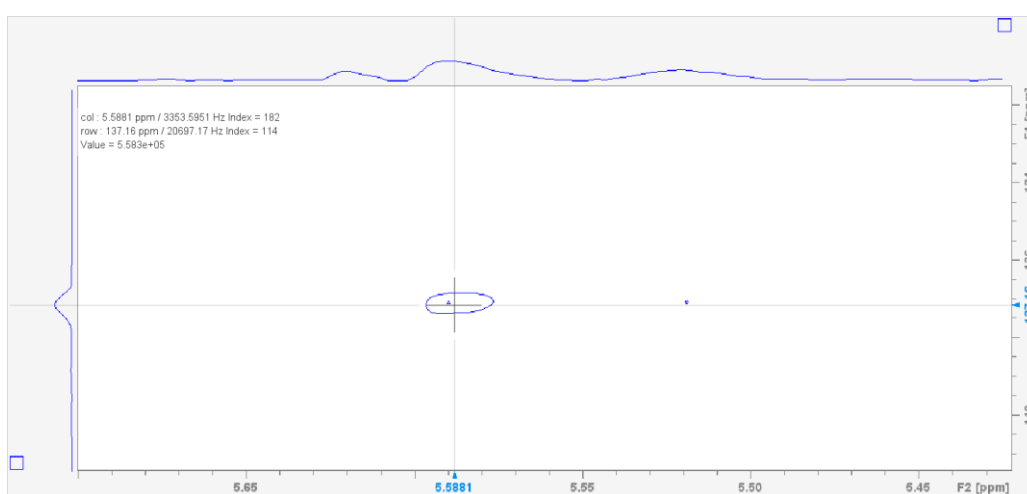
Appendix Figure A 53. HSQC 16.6 > 1.57 (Ger-6-OOH)



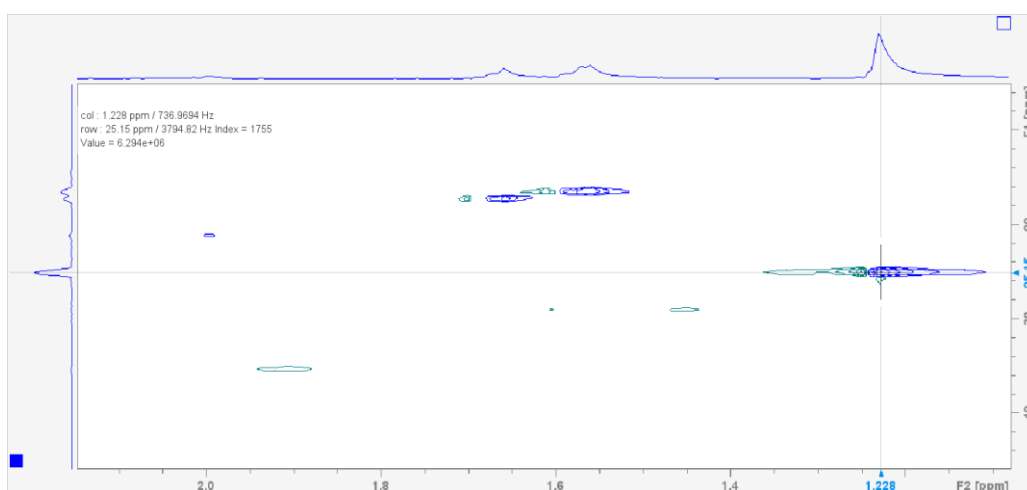
Appendix Figure A 54. HSQC 126.0 > 5.26 (Ger-6-OOH)



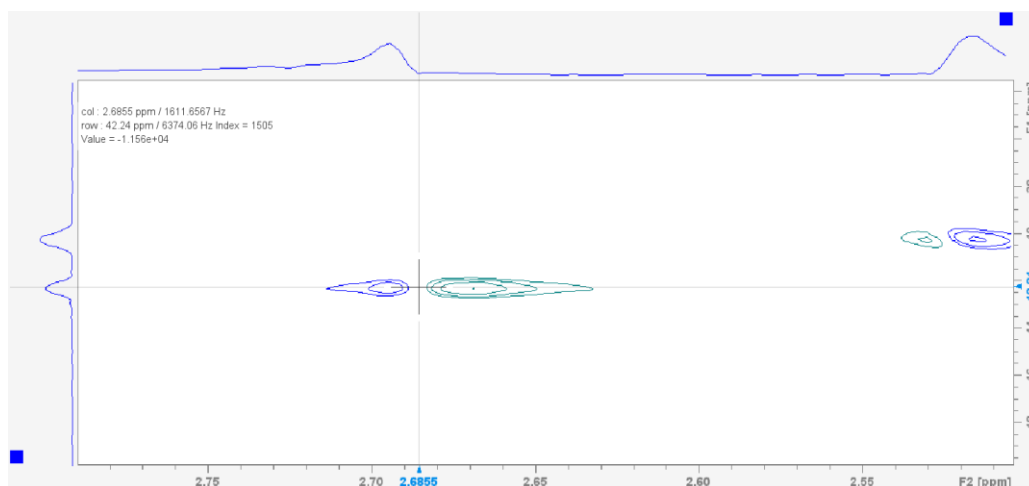
Appendix Figure A 55. HSQC 58.1 > 3.93 (Ger-6-OOH)



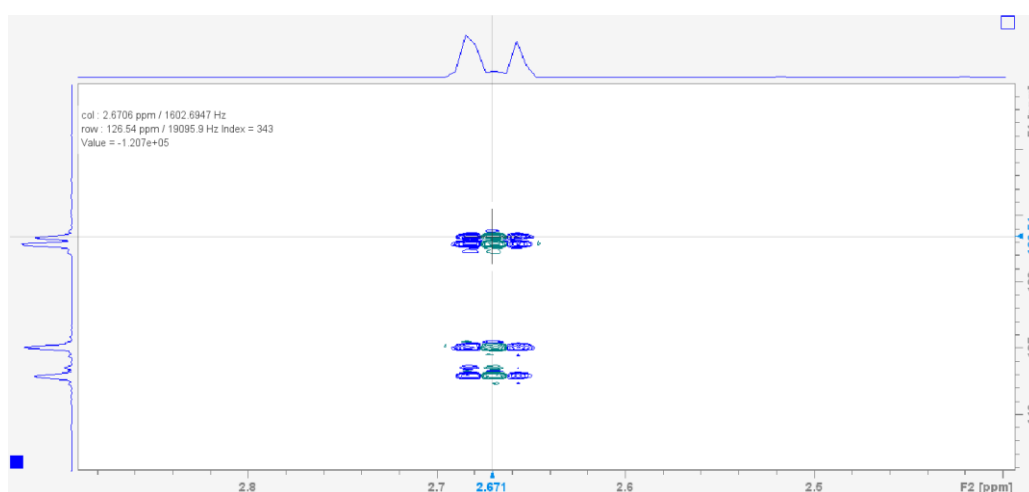
Appendix Figure A 56. HSQC 137.0 > 5.59 (Ger-7-OOH)



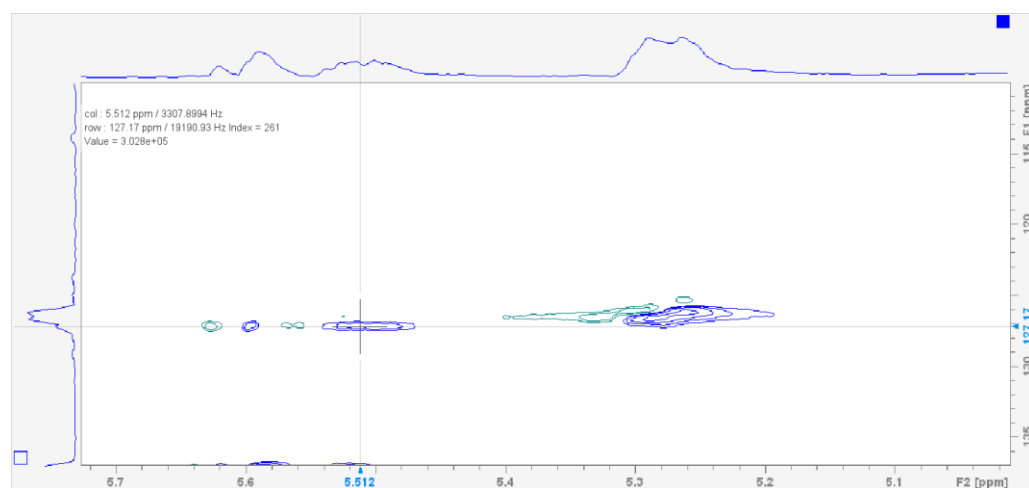
Appendix Figure A 57. HSQC 25.1 > 1.23 (Ger-7-OOH)



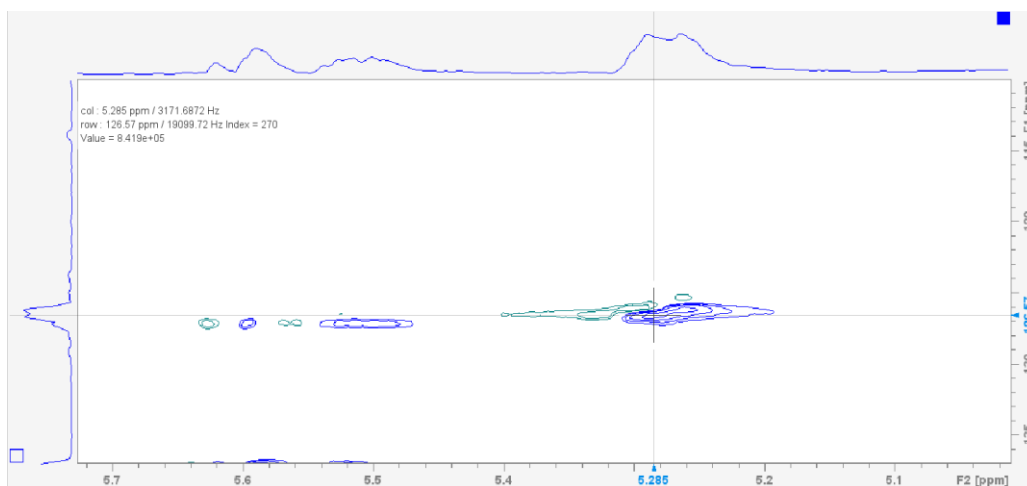
Appendix Figure A 58. HSQC 42.3 > 2.69 (Ger-7-OOH)



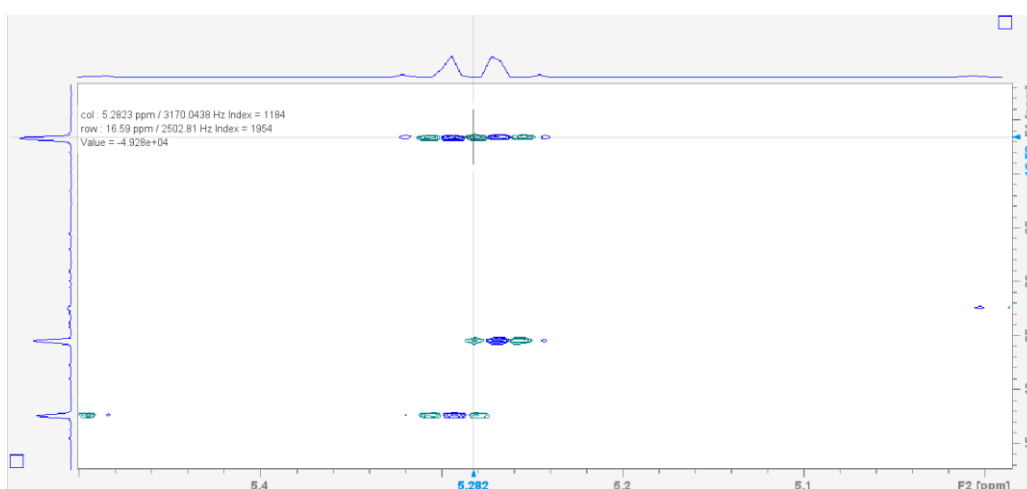
Appendix Figure A 59. HMBC 2.69 > 126.5, 127.1, and 134.9 (Ger-7-OOH)



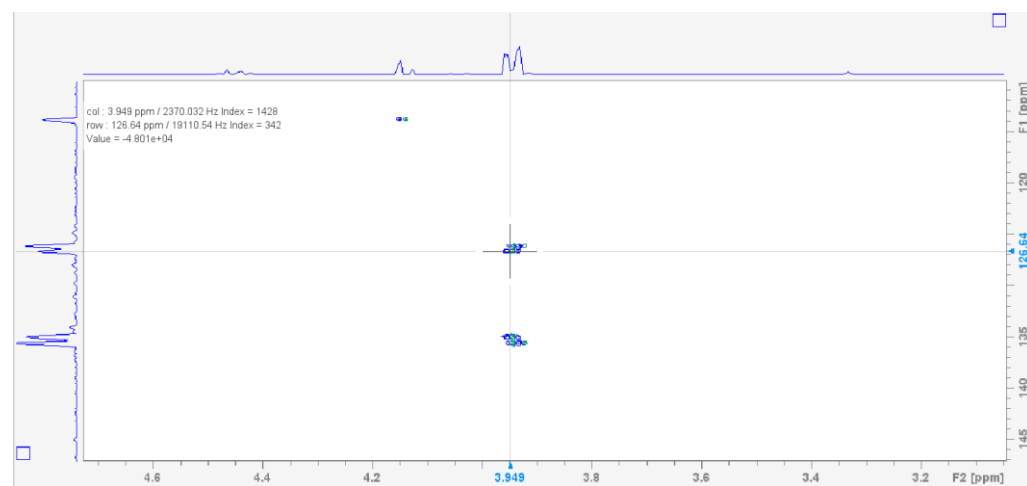
Appendix Figure A 60. HSQC 127.3 > 5.51 (Ger-7-OOH)



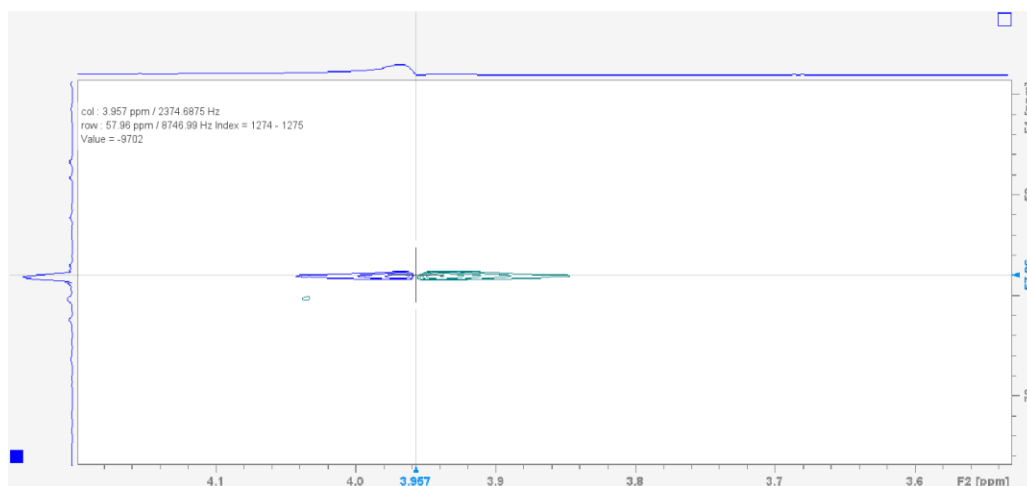
Appendix Figure A 61. HSQC 126.5 > 5.29 (Ger-7-OOH)



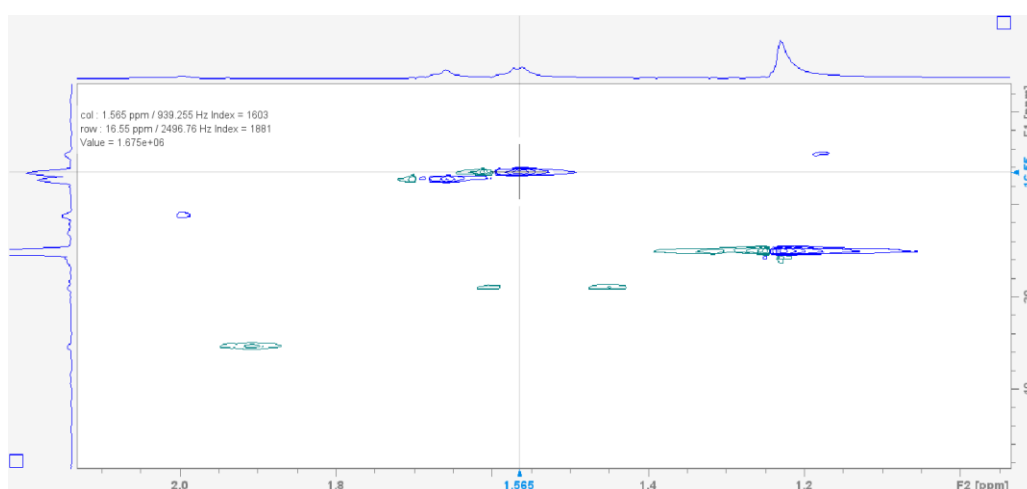
Appendix Figure A 62. HMBC 5.29 > 16.5 (Ger-7-OOH)



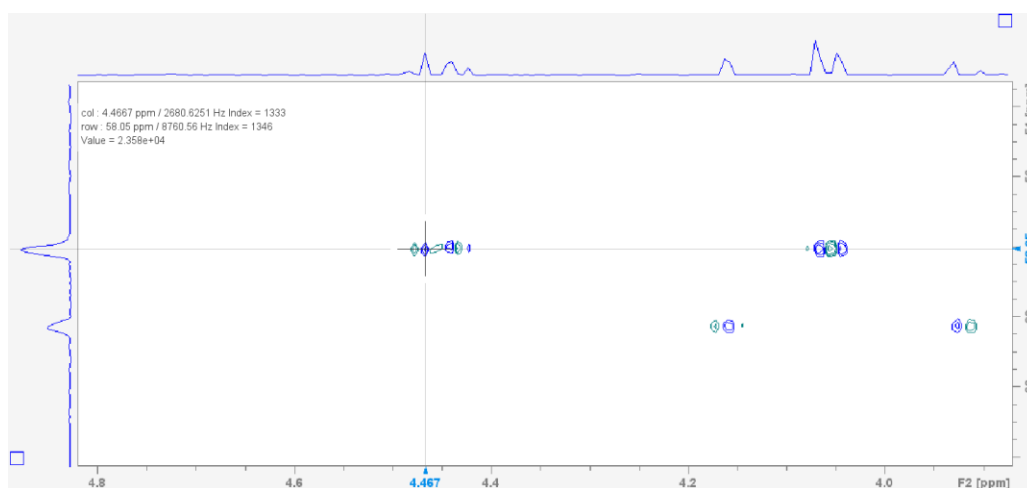
Appendix Figure A 63. HMBC 126.5 > 3.95 (Ger-7-OOH)



Appendix Figure A 64. HSQC 3.95 > 58.0 (Ger-7-OOH)



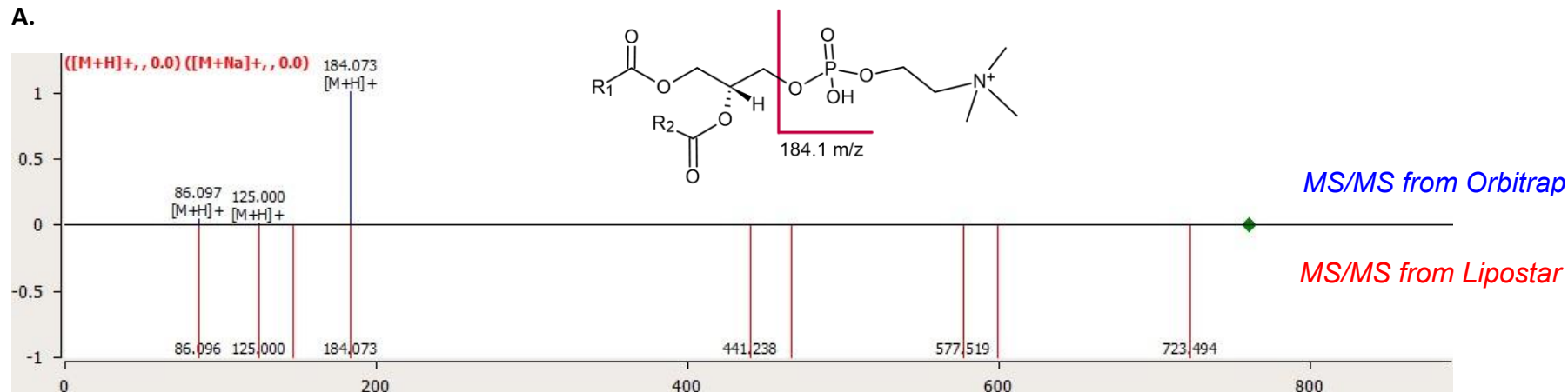
Appendix Figure A 65. HSQC 16.5 > 1.56 (Ger-7-OOH)



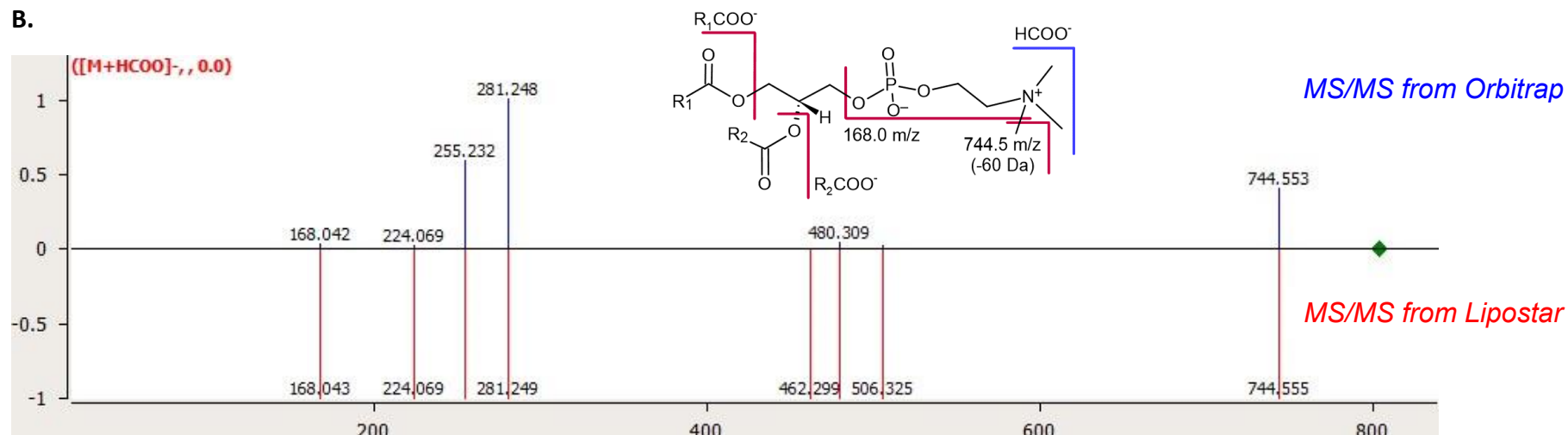
Appendix Figure A 66. HMBC 58.0 > 4.47 (Ger-7-OOH)

Appendix B: LC-MS/MS Fragmentation Data (Chapter 3)

A.

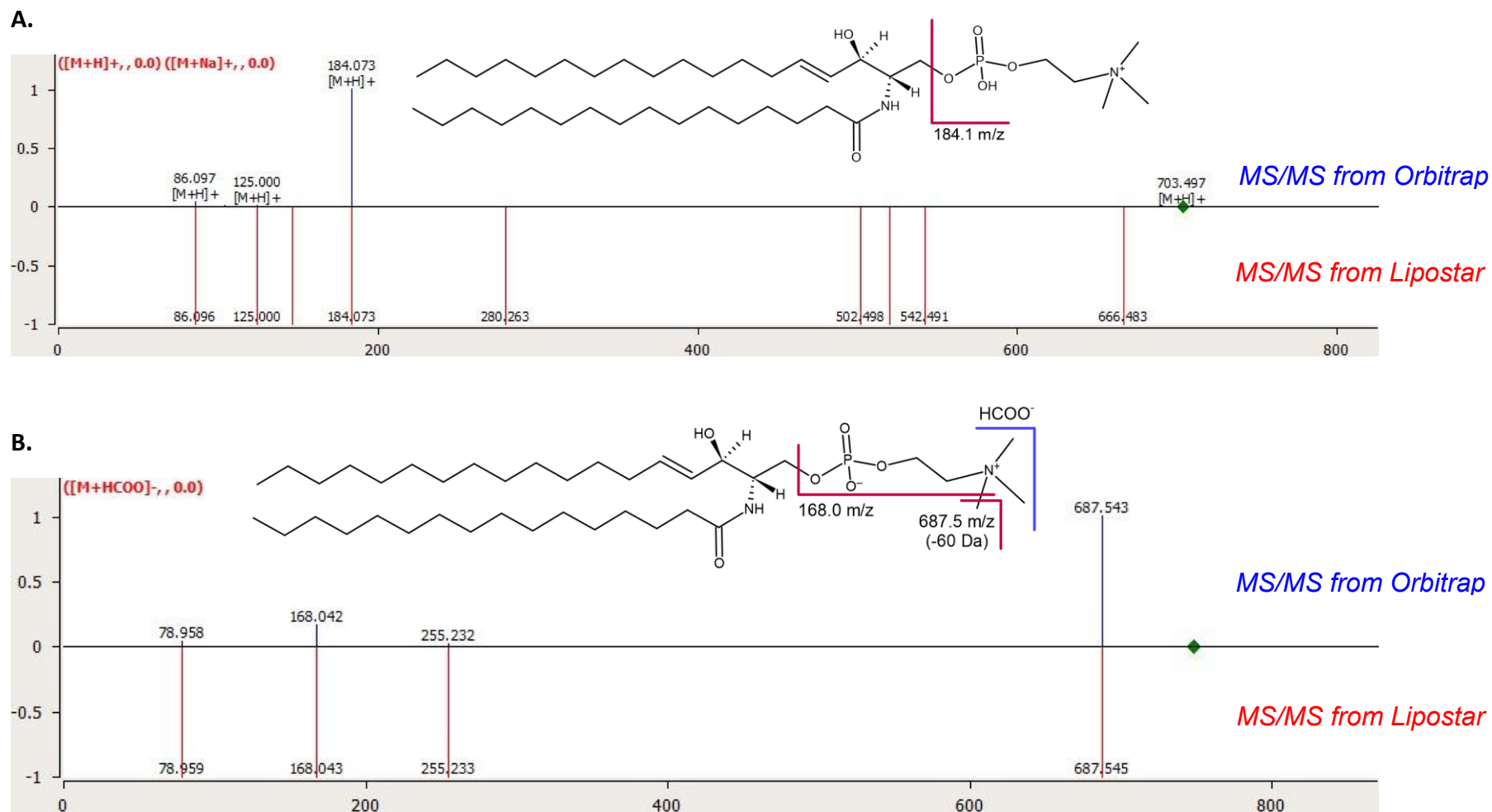


B.



Appendix Figure B 1. Representative MS/MS spectra of phosphatidylcholine (PC) lipid species.

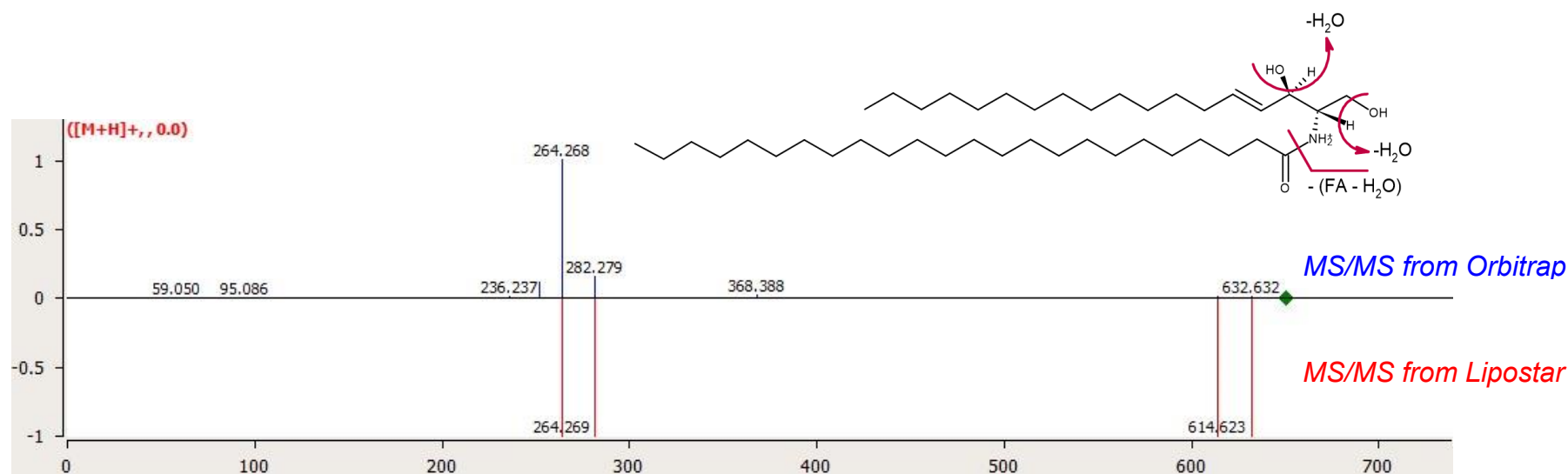
(A) Orbitrap and Lipostar MS/MS spectra of the lipid species PC (34:1) observed in positive mode as $[M + H]^+$ ion at m/z 760.5846. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. (B) Orbitrap and Lipostar MS/MS spectra of the lipid species PC (34:1) observed in negative mode as $[M + HCOO]^-$ ion at m/z 804.5756. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at m/z 255.2 and 281.2, corresponding to fatty acyl carboxylate anions 16:0 (R_1COO^-) and 18:1 (R_2COO^-) allowed to identify the fatty acyl composition of PC (16:0/18:1). Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 168.0 (formula: $C_4H_{11}NO_4P$; exact mass: 168.0425), corresponding to the phosphocholine polar head without a methyl group, and by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate. For LPC, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.



Appendix Figure B 2. Representative MS/MS spectra of sphingomyelin (SM) lipid species.

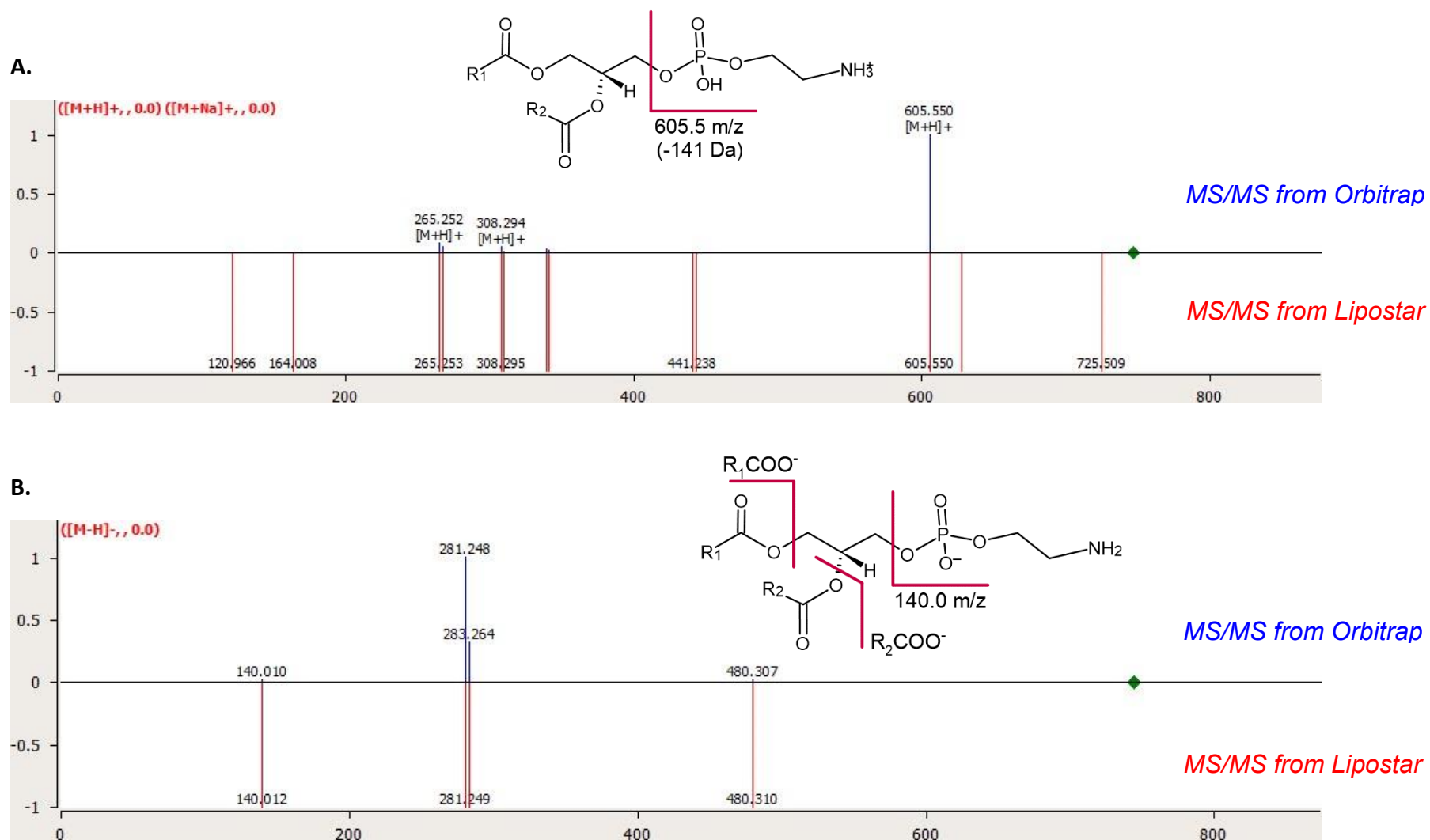
(A) Orbitrap and Lipostar MS/MS spectra of the lipid species SM(d34:1;2O) observed in positive mode as $[M + H]^+$ ion at m/z 703.5747. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head; **(B)** Orbitrap and Lipostar MS/MS spectra of the lipid species SM(d34:1;2O) observed in negative mode as $[M + HCOO]^-$ ion at m/z 747.5650. Confirmation of phospholipid class

was achieved by the identification of the product ion at m/z 168.0 (formula: $C_4H_{11}NO_4P$; exact mass: 168.0425), corresponding to the phosphocholine polar head without a methyl group, and by observing the characteristic neutral loss 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate.



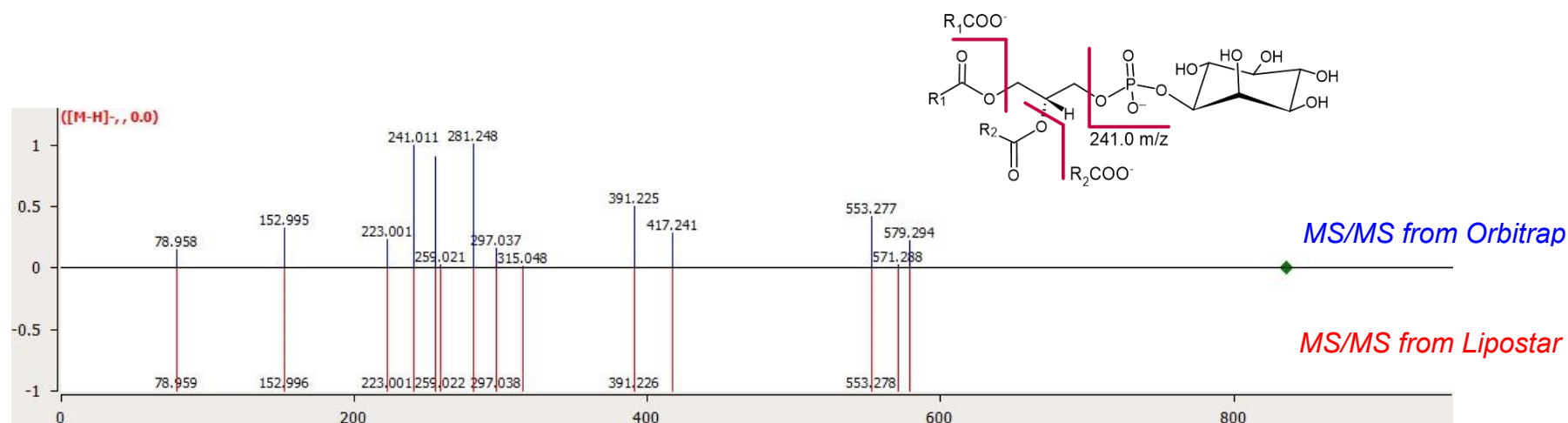
Appendix Figure B 3. Representative MS/MS spectra of Ceramide (Cer) lipid species.

Orbitrap and Lipostar MS/MS spectra of the lipid species Cer(d42:1;20) observed in positive mode as $[M + H]^+$ ion at m/z 650.6450. Confirmation of phospholipid class was achieved by the identification of the abundant product ions of the sphingoid base at m/z 264.3 and 282.3, corresponding to $[Sphingosine\ d18:1-2H_2O+H]^+$ and $[Sphingosine\ d18:1-H_2O+H]^+$, respectively. The mass difference of 350 Da between the product ions at m/z 614.6 ($[M-2H_2O+H]^+$) and 264.2, plus 18 Da, allowed to infer the presence of 24:0 as the fatty acyl amide substituent.



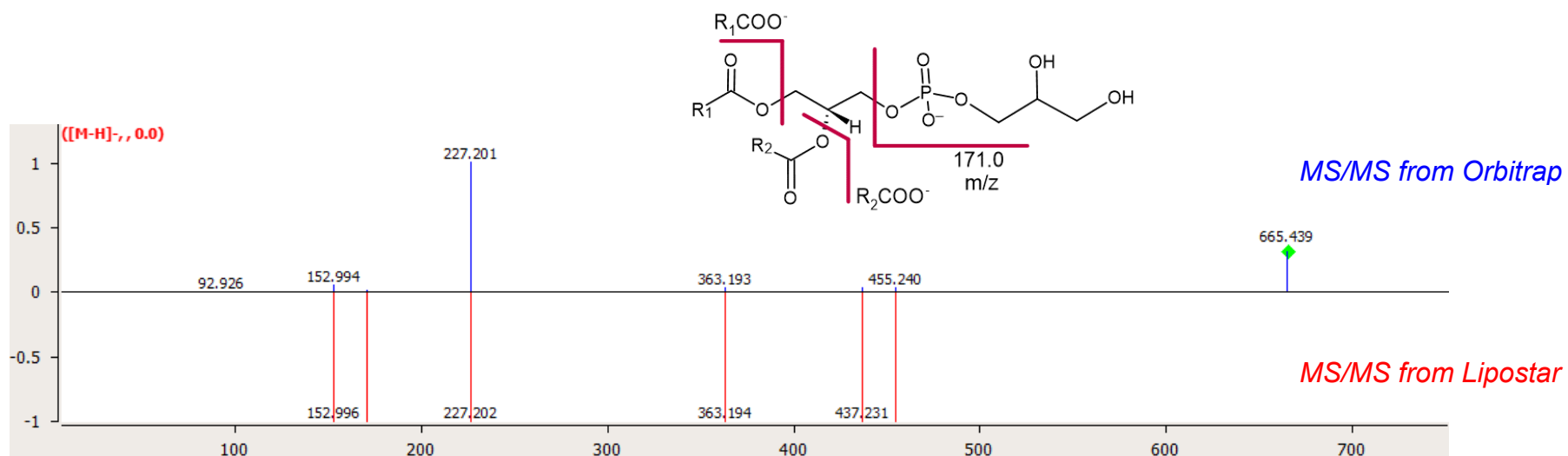
Appendix Figure B 4. Representative MS/MS spectra of phosphatidylethanolamine (PE) lipid species.

(A) Orbitrap and Lipostar MS/MS spectra of the lipid species PE (36:1) observed in positive mode as $[M + H]^+$ ion at m/z 746.5670. Confirmation of phospholipid class was achieved by the identification of the neutral loss of 141 Da (formula: $C_2H_8NO_4P$; exact mass: 141.0191), corresponding to phosphoethanolamine polar head. **(B)** Orbitrap and Lipostar MS/MS spectra of the lipid specie PE (36:1) observed in negative mode as $[M - H]^-$ at m/z 744.5547. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at m/z 283.2 and 281.2, corresponding to fatty acyl carboxylate anions of 18:0 (R_1COO^-) and 18:1 (R_2COO^-), allowed to identify the fatty acyl composition of PE (18:0/18:1). The confirmation as PE species was also achieved by observing the product ion at m/z 140.0 (formula: $C_2H_7NO_4P$; exact mass: 140.0113), corresponding to phosphoethanolamine polar head. For LPE, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.



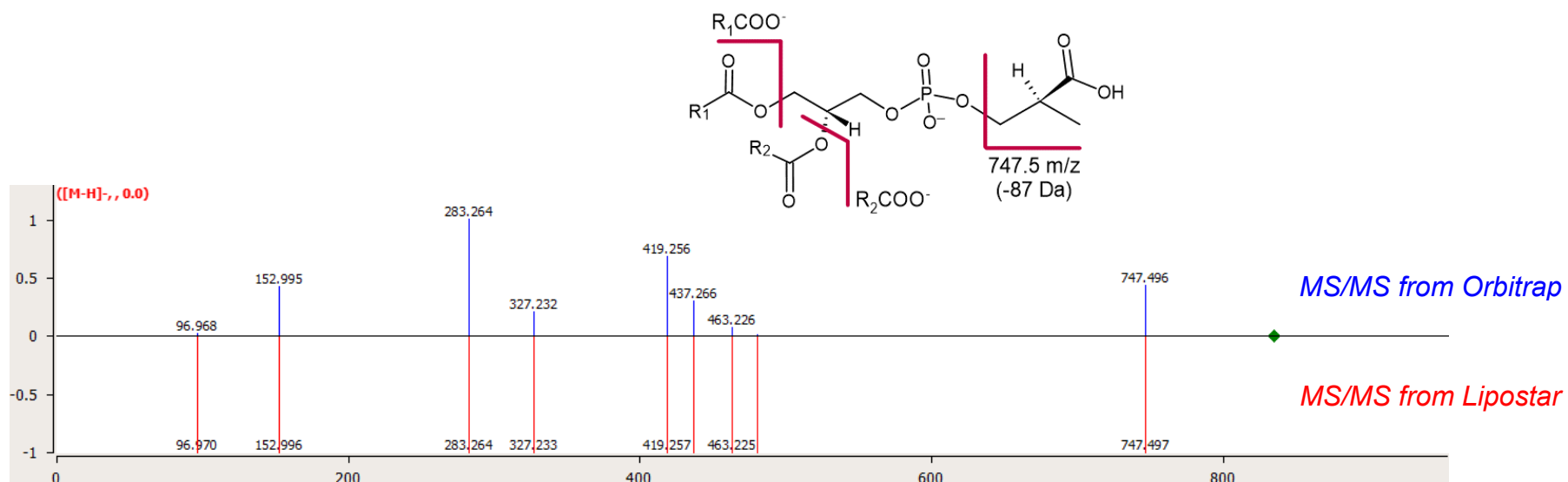
Appendix Figure B 5. Representative MS/MS spectra of phosphatidylinositol (PI) lipid species.

Orbitrap and Lipostar MS/MS spectra of the lipid species PI (34:1) observed in positive mode as $[M - H]^-$ ion at m/z 835.5530. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 241.0 (formula: $C_6H_{10}O_8P$; exact mass: 241.0113), corresponding to the inositol head group. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at m/z 255.2 and 281.2, corresponding to fatty acyl carboxylate anions of 16:0 (R_1COO^-) and 18:1 (R_2COO^-) allowed to identify the fatty acyl composition of PI (16:0/18:1).



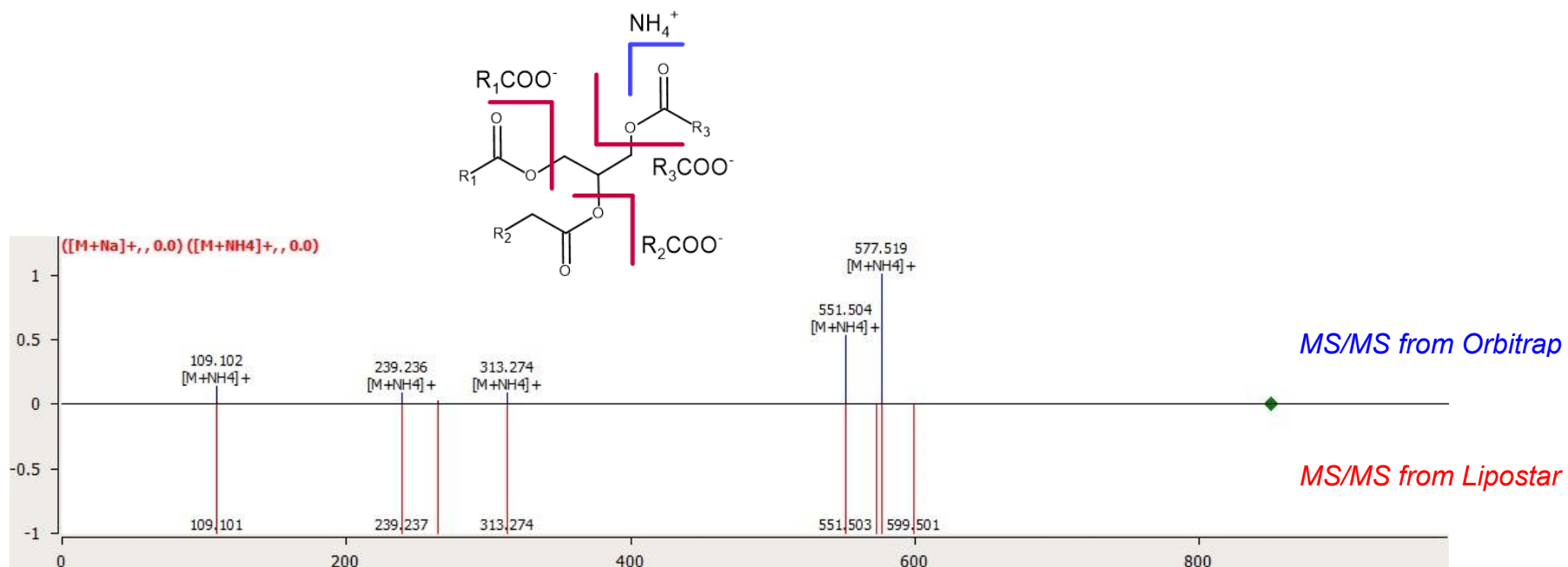
Appendix Figure B 6. Representative MS/MS spectra of phosphatidylglycerol (PG) lipid species.

Orbitrap and Lipostar MS/MS spectra of the lipid species PG (28:0) observed in negative mode as $[M-H]^-$ ion at m/z 655.4403. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 171.0 (formula: $C_3H_8O_6P$; exact mass: 171.0059), corresponding to phosphoglycerol polar head. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ion(s) observed at m/z 227.2, corresponding to fatty acyl carboxylate anions of 14:0 (R_1COO^-) and 14:0 (R_2COO^-), allowed to identify the fatty acyl composition of PG (14:0/14:0).



Appendix Figure B 7. Representative MS/MS spectra of phosphatidylserine (PS) lipid species.

Orbitrap and Lipostar MS/MS spectra of the lipid species PS (40:6) observed in positive mode as [M-H]⁻ ion at m/z 834.5286. Confirmation of phospholipid class was achieved by the identification of a neutral loss of 87 Da, corresponding to the serine head group. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as [RCOO]⁻. The product ions observed at m/z 283.2 and 327.2, corresponding to fatty acyl carboxylate anions of 18:0 (R₁COO⁻) and 22:6 (R₂COO⁻) allowed to identify the fatty acyl composition of PI (18:0/22:6).



Appendix Figure B 8. Representative MS/MS spectra of triacylglycerol (TG) lipid species.

Orbitrap and Lipostar MS/MS spectra of the lipid species TG (50:1) observed in positive mode as [M + NH₄]⁺ ion at *m/z* 850.7855. Confirmation of lipid class was achieved by the identification of the distinct FA moieties (NL of RCOOH + NH₃). The product ions at *m/z* 551.5 and 577.5 resulted from the combined loss of NH₃ (-17 Da) and each fatty acyl chain 18:1 (-282 Da) and 16:0 (-256) from the precursor ion, respectively. These product ions allowed to identify the fatty acyl composition of TG (16:0/16:0/18:1). The acylium ions (RCO⁺) of FA can also be observed.

Appendix Table B 1. The 137 lipid species showing significant variation between allergen-treated cell groups (DNFB, Lim-2-OOH, and Lin-6/7-OOH) and the vehicle control group.

Lipid Species (C:N)	FDR, <i>q</i> -value	Statistical significance level
TG 55:2	2.54E-05	****
PC 40:7 (B)	8.91E-05	****
PC 35:4	1.20E-04	***
TG 60:3	1.20E-04	***
TG 58:7	1.20E-04	***
TG 50:3	1.20E-04	***
PC 40:5 (A)	1.20E-04	***
TG 58:2	1.47E-04	***
Hex3Cer 42:2 (2O)	1.71E-04	***
PC O- 36:0	1.71E-04	***
PC P-38:6/PC O-38:7 (B)	1.71E-04	***
PC O-40:6 (B)	1.86E-04	***
PC O-40:4/PC P-40:3 (A)	2.07E-04	***
PC P-30:0/PC O-30:1	2.45E-04	***
PC P-35:2/PC O-35:3	2.54E-04	***
TG 52:2	2.80E-04	***
TG 49:2	3.06E-04	***
PC P-36:4/PC O-36:5	3.79E-04	***
DG 34:3	4.18E-04	***
DG 40:5	4.18E-04	***
TG 56:4	4.18E-04	***
PC 36:3 (C)	4.65E-04	***
SM 44:2;O2	5.42E-04	***
PC 34:1	5.42E-04	***
PC O-38:6 (B)	5.68E-04	***
TG 58:1	5.68E-04	***
PC 35:0	5.72E-04	***
PC 42:7 (A)	6.32E-04	***
PC O-40:5/PC P-40:4	6.61E-04	***
PC 39:3	7.90E-04	***
TG 50:2	8.34E-04	***
PC 46:2	8.95E-04	***
TG 58:3	1.11E-03	**
PE P-36:3/PE O-36:4	1.11E-03	**
TG 54:2	1.15E-03	**
SM 40:2;O2 (A)	1.15E-03	**
PC 37:5 (B)	1.18E-03	**
SM 43:1;O2	1.40E-03	**
SM 42:3;O2	1.46E-03	**
TG 51:2	1.59E-03	**

PE P-36:4/PE O-36:5	1.84E-03	**
PC P-32:1/PC O-32:2	1.91E-03	**
TG 54:3	1.93E-03	**
PC 34:4	2.06E-03	**
PC 40:3	2.29E-03	**
PC O-38:3/PC P-38:2	2.44E-03	**
PE 38:4	2.53E-03	**
PC O-29:0	2.53E-03	**
PE P-34:1/PE O-34:2	2.59E-03	**
TG 44:0	3.05E-03	**
PE 38:3 (B)	3.05E-03	**
PE P-34:0/PE O-34:1	3.05E-03	**
PS 40:6	3.05E-03	**
LPC O-16:0	3.16E-03	**
PE 40:6	3.17E-03	**
PC 38:6 (B)	3.35E-03	**
Cer 41:1;O2	3.35E-03	**
PC O-38:1/PC P-38:0	3.36E-03	**
PC P-34:1/PC O-34:2 (C)	3.86E-03	**
PC 42:1	4.10E-03	**
PC 40:5 (B)	4.10E-03	**
PC 38:6 (C)	4.10E-03	**
PC 39:5	4.10E-03	**
PE 40:7	4.10E-03	**
TG 51:0	4.10E-03	**
PC 36:4	4.10E-03	**
PC O-35:2/PC P-35:1 (A)	4.10E-03	**
PC 42:4	7.63E-03	**
PC 39:2	7.63E-03	**
SM 41:1;O2 (A)	7.63E-03	**
PC 37:1 (A)	7.63E-03	**
SM 36:2;O2	1.09E-02	**
TG 52:1	1.09E-02	*
PS 40:5	1.35E-02	*
TG 52:4	1.35E-02	*
Hex3Cer 34:1;O2	1.35E-02	*
TG 52:0	1.35E-02	*
TG 54:1	1.59E-02	*
PC 40:6 (B)	1.59E-02	*
PC 42:6	1.59E-02	*
TG 53:3	1.59E-02	*
PC O-40:6/PC P-40:5 (A)	1.79E-02	*
PC O-35:1/PC P-35:0 (B)	1.79E-02	*
PC 42:3	1.79E-02	*
PC O-37:4/PC P-37:3	1.79E-02	*
PC 37:3	2.05E-02	*

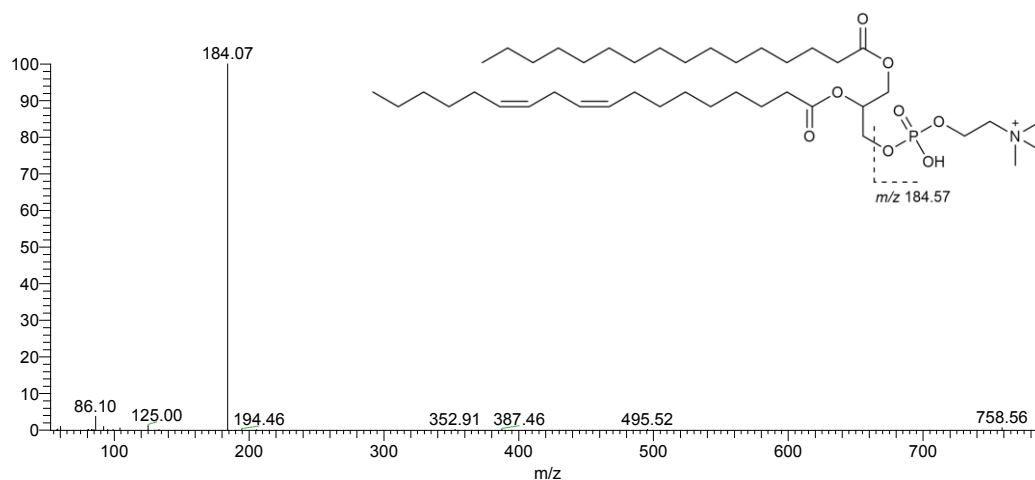
PC 42:7 (B)	2.26E-02	*
PC O-33:0 (B)	2.26E-02	*
PC 38:3 (B)	2.26E-02	*
PC O-35:2/PC P-35:1 (B)	2.26E-02	*
PC 38:6 (A)	2.48E-02	*
TG 56:6	2.48E-02	*
PC O-35:2/PC P-35:1 (C)	2.69E-02	*
PC 40:8	2.69E-02	*
PC P-32:0/PC O-32:1	2.69E-02	*
PC P-36:5/PC O-36:6 (A)	2.93E-02	*
PC 38:5	3.10E-02	*
PC 31:0 (A)	3.10E-02	*
PC 38:2	3.10E-02	*
PC 37:4	3.10E-02	*
PC 44:3	3.31E-02	*
HexCer 42:1;O2 (A)	3.45E-02	*
PC 40:7 (C)	3.45E-02	*
PC P-40:6/PC O-40:7 (B)	3.45E-02	*
TG 53:1	3.45E-02	*
PC P-38:6/PC O-38:7 (A)	3.45E-02	*
SM 41:2;O2	3.45E-02	*
PC O-40:4/PC P-40:3 (B)	3.45E-02	*
PE 38:5	3.45E-02	*
SM 40:1;O2 (A)	3.45E-02	*
HexCer 34:1;O2	3.45E-02	*
PC 39:6	3.45E-02	*
DG 36:3	3.45E-02	*
HexCer 42:1;O2 (B)	3.48E-02	*
PC 31:0 (B)	3.48E-02	*
PC 39:4	3.48E-02	*
PC P-36:1/PC O-36:2	3.67E-02	*
PC O-36:4	3.67E-02	*
TG 56:1	3.72E-02	*
Hex3Cer 42:1;O2	3.75E-02	*
PC 36:2	3.75E-02	*
TG 49:0	3.75E-02	*
TG 55:3	3.86E-02	*
TG 50:1	3.88E-02	*
PC O-34:3/PC P-34:2 (C)	3.88E-02	*
PC 37:2	4.07E-02	*
PC 42:2	4.22E-02	*
PC 44:4	4.27E-02	*
SM 34:1;O2 (A)	4.36E-02	*
PC 44:1	4.36E-02	*
PS 38:3	4.36E-02	*
PC 35:3 (B)	4.36E-02	*

SM 42:2;O2	4.52E-02	*
PE P-32:1/PE O-32:2	4.52E-02	*
PE P-38:3/PE O-38:4	4.65E-02	*
PC O-38:4 (C)	4.84E-02	*
PC 42:5	5.00E-02	*

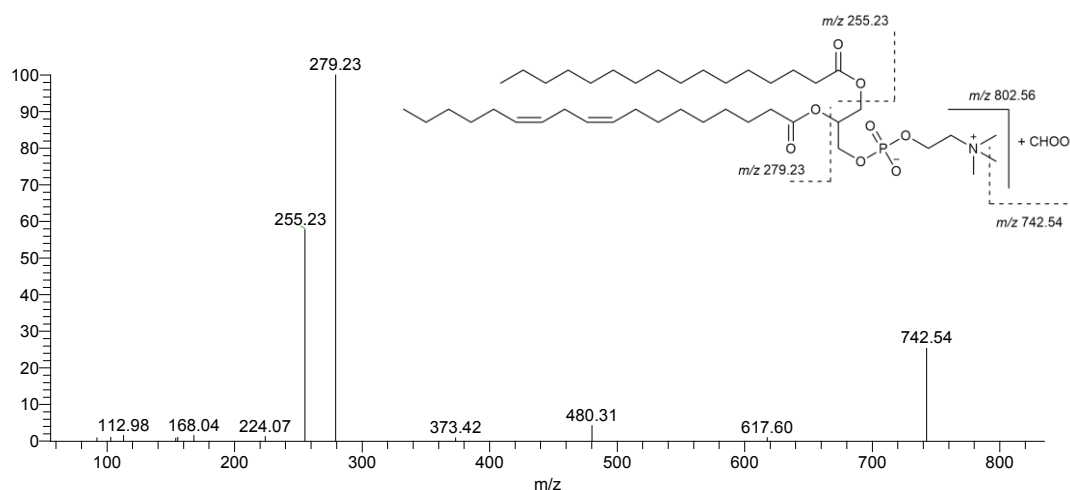
^aThe 137 lipid species showing significant variation between allergen-treated cell groups (DNFB, Lim-OOH, and Lin-OOH) and the vehicle control group, ordered by increasing values of *q*-value (* if *q*-value < 0.05, ** if *q*-value < 0.01, *** if *q*-value < 0.001 and **** if *q*-value < 0.0001); Lipid species are labelled as follows: AA xx:n (AA=lipid class abbreviation; xx=number of carbon atoms in fatty acid(s); n=number of double bonds). The 'O-' prefix is used for plasmalogen species to indicate the presence of an alkyl ether substituent, whereas the 'P-' prefix is used for plasmalogen species to indicate the alk-1-enyl ether substituent. The suffix "A" or "B" is used to differentiate isomers with distinct elution times. Lipid class abbreviations: DG, diacylglycerols; Hex_nCer, hexosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; and TG, triacylglycerol.

Appendix C: LC-MS and MS/MS of oxPLPC Products (Chapter 4)

A.



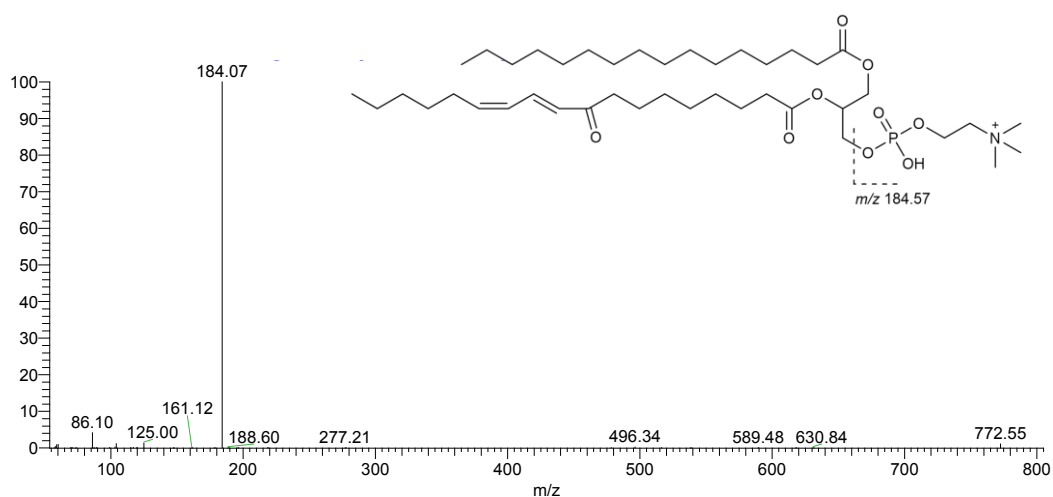
B.



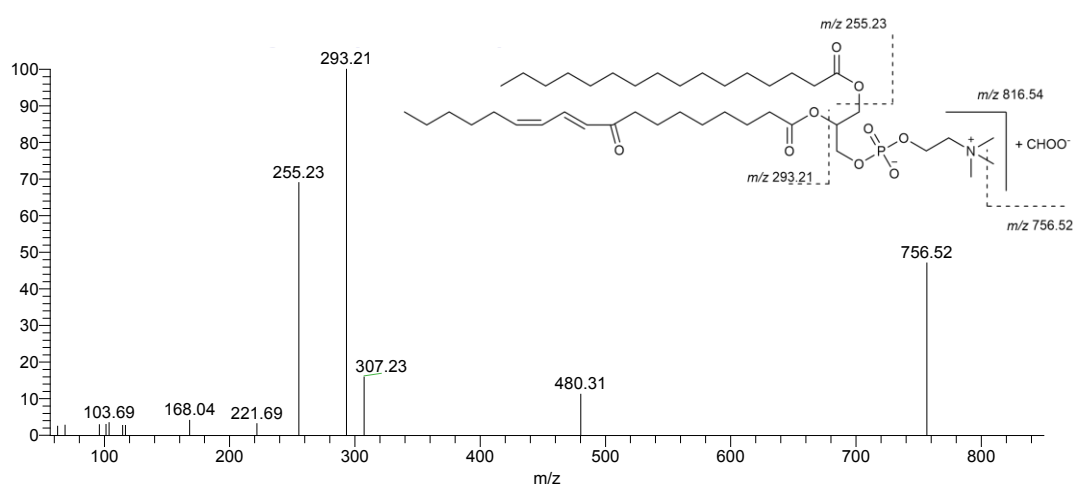
Appendix Figure C 1. MS/MS spectra of unoxidized PLPC.

(A) Orbitrap MS/MS spectrum of unoxidized PLPC in positive mode as $[\text{M} + \text{H}]^+$ ion at m/z 758.5684. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $\text{C}_5\text{H}_{15}\text{NO}_4\text{P}$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of unoxidized PLPC in negative mode as $[\text{M} + \text{HCOO}]^-$ ion at m/z 802.5586. The product ions observed at m/z 255.23 and 279.23, corresponding to fatty acyl carboxylate anions 16:0 ($[\text{R1COO}]^-$) and 18:2 ($[\text{R2COO}]^-$). Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $\text{C}_2\text{H}_4\text{O}_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 742.54) and the product ion at m/z 168.04 (formula: $\text{C}_4\text{H}_{11}\text{NO}_4\text{P}$; exact mass: 168.0426), corresponding to the de-methylation of the phosphocholine headgroup.

A.



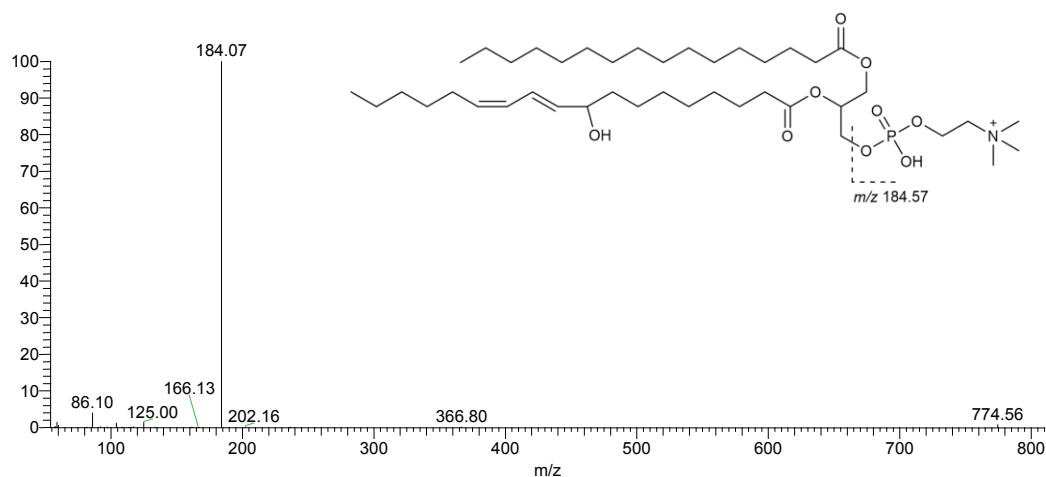
B.



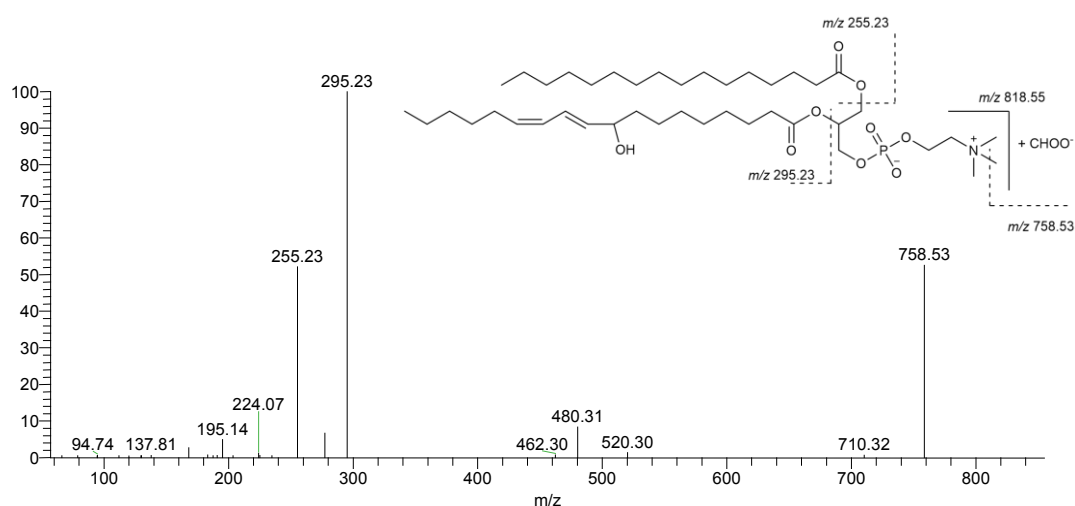
Appendix Figure C 2. MS/MS spectra of PLPC + 14 Da.

(A) Orbitrap MS/MS spectrum of PLPC + 14 Da observed in positive mode as $[M + H]^+$ ion at m/z 772.5486. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of PLPC + 14 Da observed in negative mode as $[M + HCOO]^-$ ion at m/z 816.5381. The product ions observed at m/z 255.23 and 293.21, corresponding to fatty acyl carboxylate anions 16:0 ($[R1COO]^-$) and $[R2COO+O-2H]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 756.52) and the product ion at m/z 168.04 (formula: $C_4H_{11}NO_4P$; exact mass: 168.0426), corresponding to the de-methylation of the phosphocholine headgroup.

A.



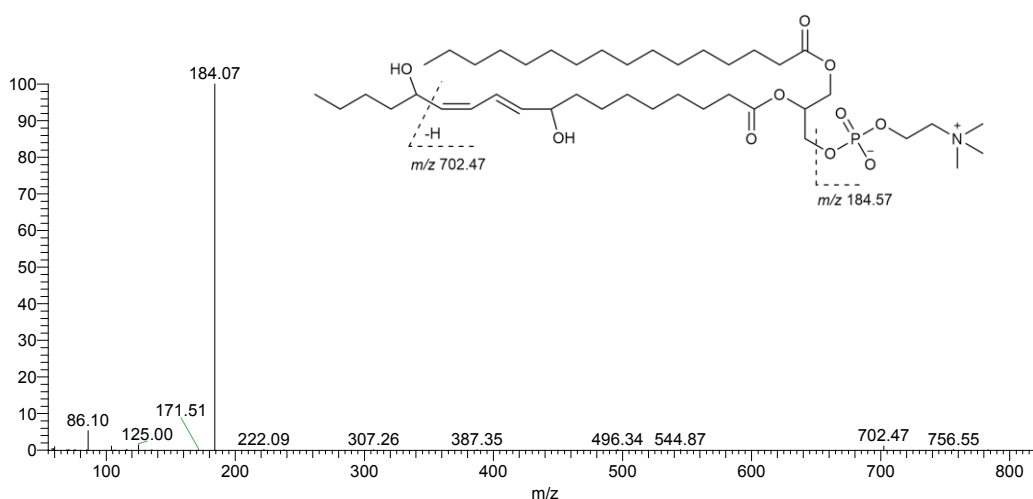
B.



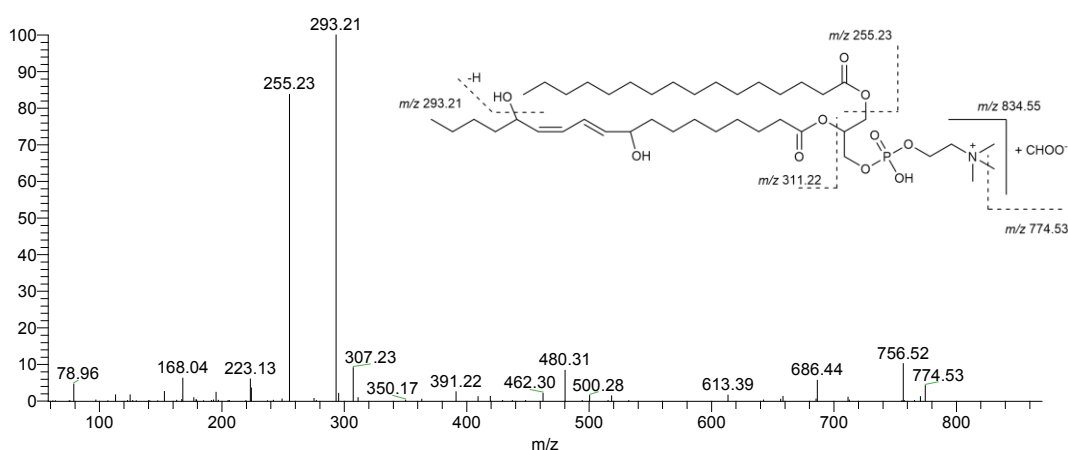
Appendix Figure C 3. MS/MS spectra of PLPC + 16 Da.

(A) Orbitrap MS/MS spectrum of PLPC + 16 Da observed in positive mode as $[M + H]^+$ ion at m/z 774.5646. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of PLPC + 16 Da observed in negative mode as $[M + HCOO]^-$ ion at m/z 818.5542. The product ions observed at m/z 255.23 and 295.23, corresponding to fatty acyl carboxylate anions 16:0 ($[R1COO]^-$) and $[R2COO+O]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 758.53).

A.



B.

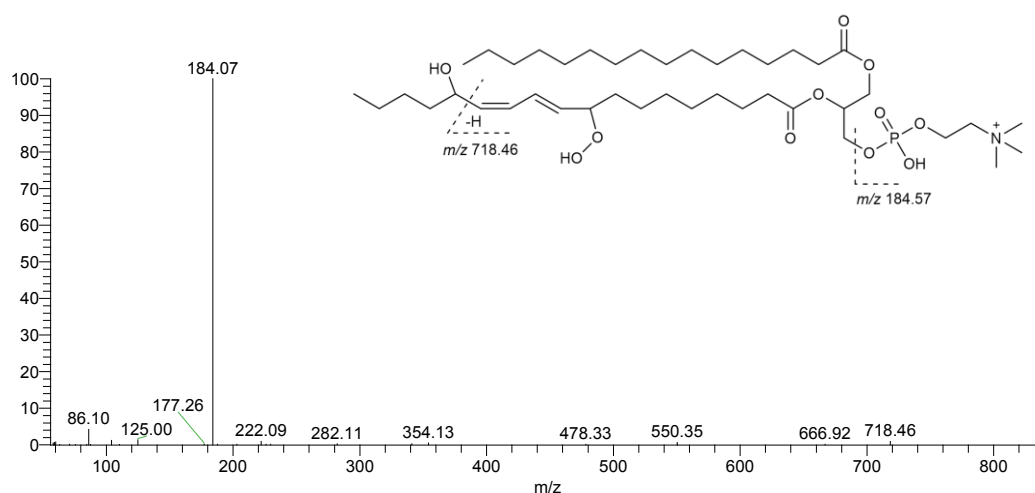


Appendix Figure C 4. MS/MS spectra of PLPC + 32 Da.

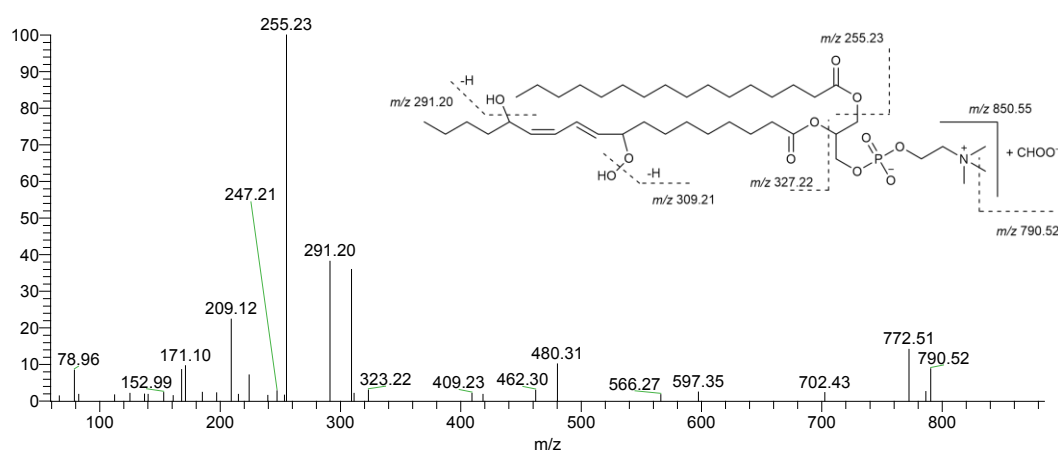
(A) Orbitrap MS/MS spectrum of PLPC + 32 Da observed in positive mode as $[M + H]^+$ ion at m/z 790.5590. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. Confirmation of the ion as a 1-palmitoyl-2-(9,14-dihydroxyoctadeca-10,12-dienoic acid) PC derivative was achieved by identification of the product ion at m/z 702.47 (formula $C_{37}H_{69}NO_9P$; exact mass 702.4710).

(B) Orbitrap MS/MS spectrum of PLPC + 32 Da observed in negative mode as $[M + HCOO]^-$ ion at m/z 834.5485. The product ions observed at m/z 255.23 and 293.21, corresponding to fatty acyl carboxylate anions 16:0 ($[R_1COO]^-$) and $[R_2COO + 2O - H_2O]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 774.53) and the product ion at m/z 168.04 (formula: $C_4H_{11}NO_4P$; exact mass: 168.0426), corresponding to the de-methylation of the phosphocholine headgroup.

A.



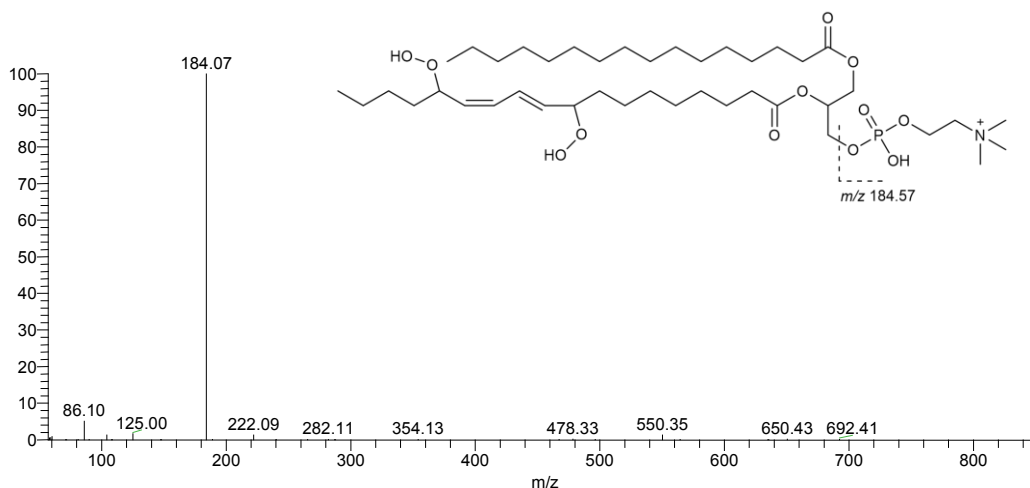
B.



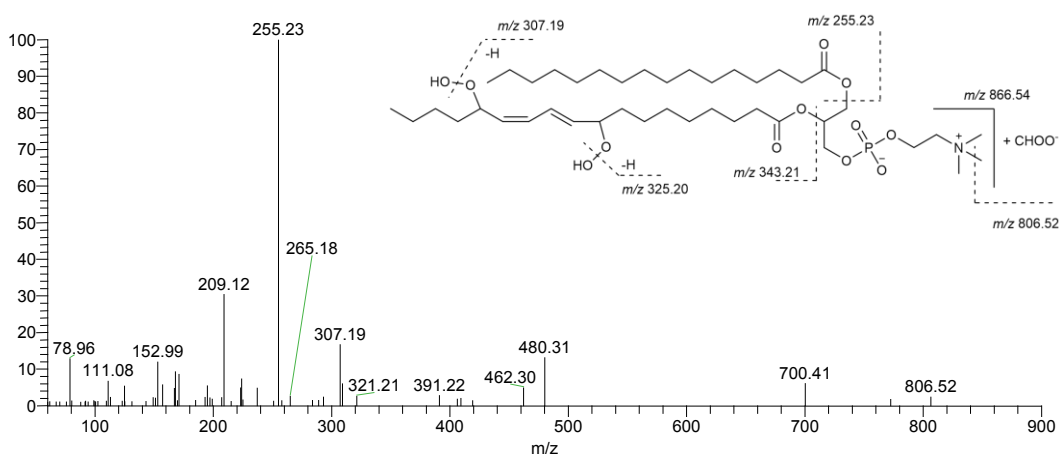
Appendix Figure C 5. MS/MS spectra of PLPC + 48 Da.

(A) Orbitrap MS/MS spectrum of PLPC + 48 Da observed in positive mode as $[M + H]^+$ ion at m/z 806.5530. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. Confirmation of the ion as a 1-palmitoyl-2-(9-hydroperoxy-14-hydroxyoctadeca-10,12-dienoic acid) PC derivative was achieved by identification of the product ion at m/z 718.46 (formula $C_{37}H_{69}NO_{10}P$; exact mass 718.4659). **(B)** Orbitrap MS/MS spectrum of PLPC + 48 Da observed in negative mode as $[M + HCOO]^-$ ion at m/z 850.5434. The product ions observed at m/z 255.23, 309.21, and 291.20 corresponding to fatty acyl carboxylate anions 16:0 ($[R1COO]^-$), $[R2COO+3O-H_2O]^-$, and $[R2COO+3O-2H_2O]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 790.52).

A.



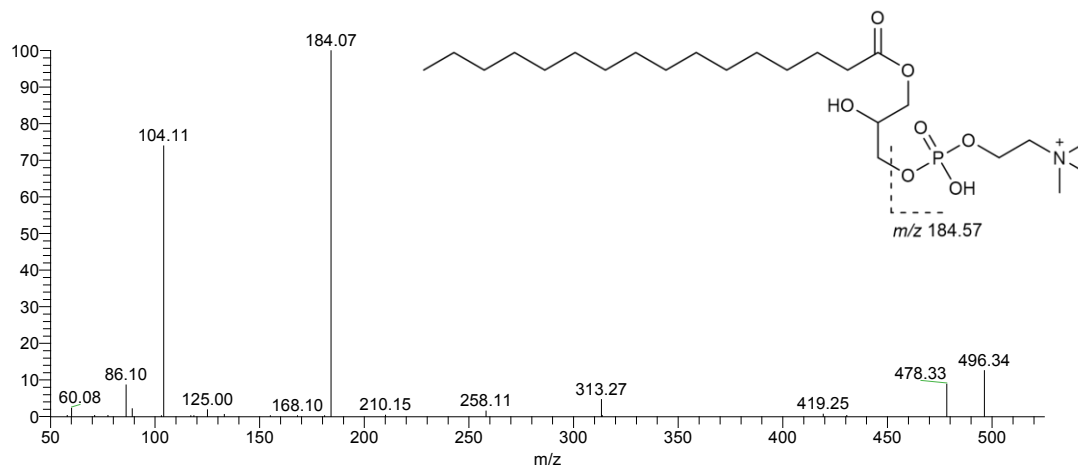
B.



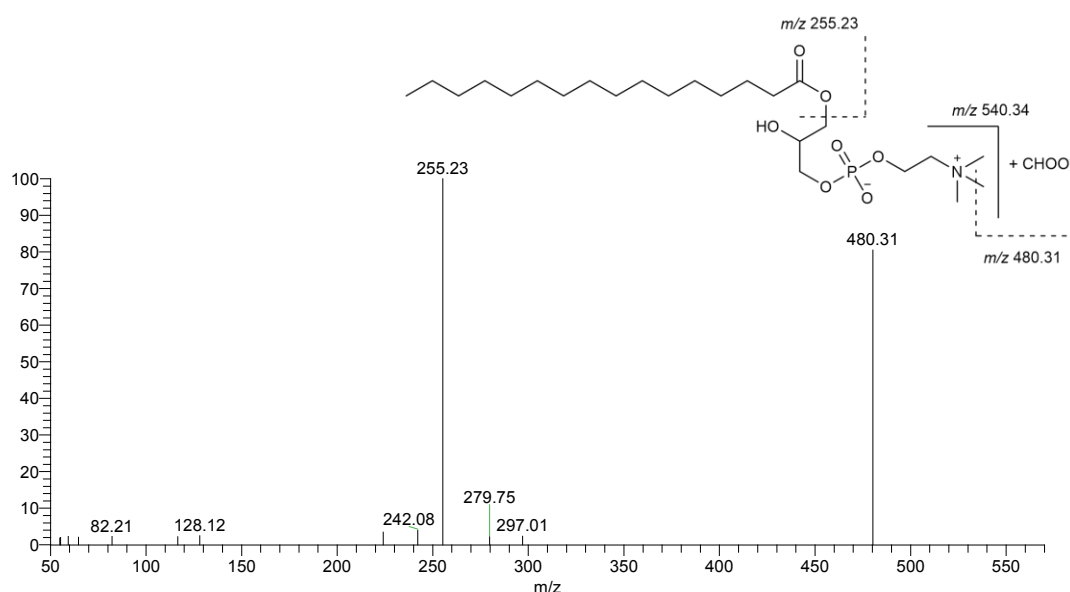
Appendix Figure C 6. MS/MS spectra of PLPC + 64 Da.

(A) Orbitrap MS/MS spectrum of PLPC + 64 Da observed in positive mode as $[M + H]^+$ ion at m/z 822.5478. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of PLPC + 64 Da observed in negative mode as $[M + HCOO]^-$ ion at m/z 866.5384. The product ions observed at m/z 255.23 and 307.19 corresponding to fatty acyl carboxylate anions 16:0 ($[R1COO]^-$) and $[R2COO+4O-2H_2O]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 806.52).

A.



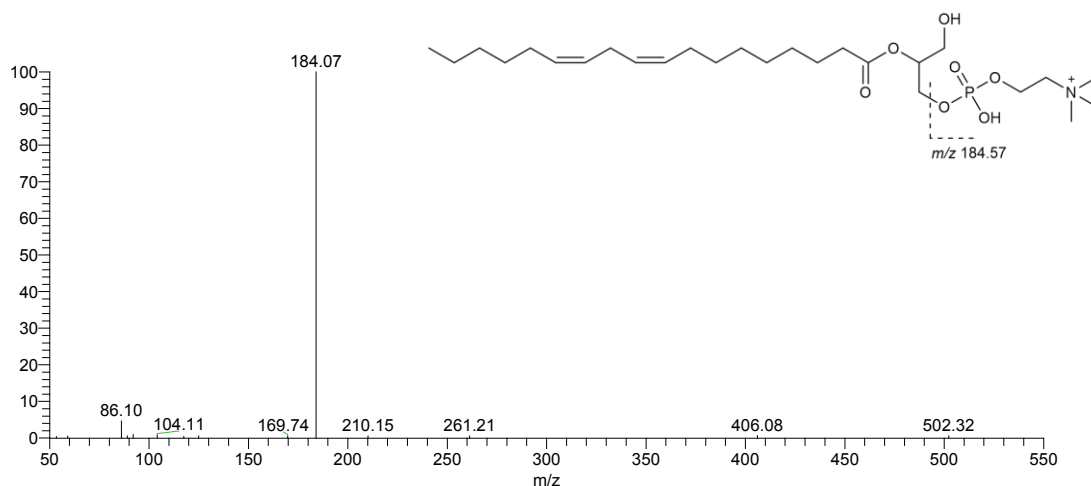
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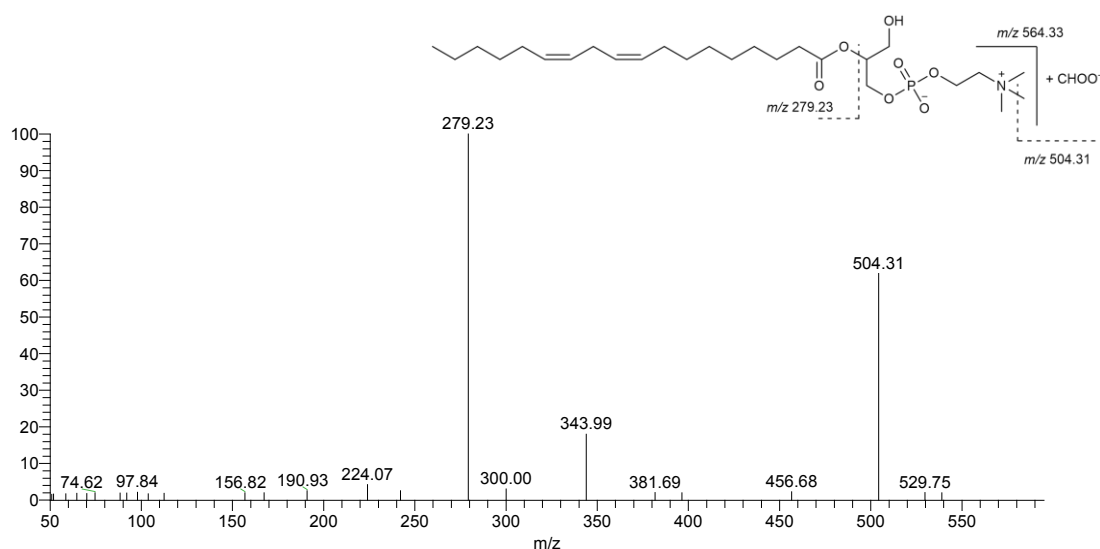
Appendix Figure C 7. MS/MS spectra of 1-palmitoyl lyso PC.

(A) Orbitrap MS/MS spectrum of 1-palmitoyl lyso PC observed in positive mode as $[M + H]^+$ ion at m/z 496.3395. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of 1-palmitoyl lyso PC observed in negative mode as $[M + HCOO]^-$ ion at m/z 540.3301. The product ion observed at m/z 255.23 corresponds to the fatty acyl carboxylate anion 16:0 ($[R1COO]^-$). Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 480.31).

A.

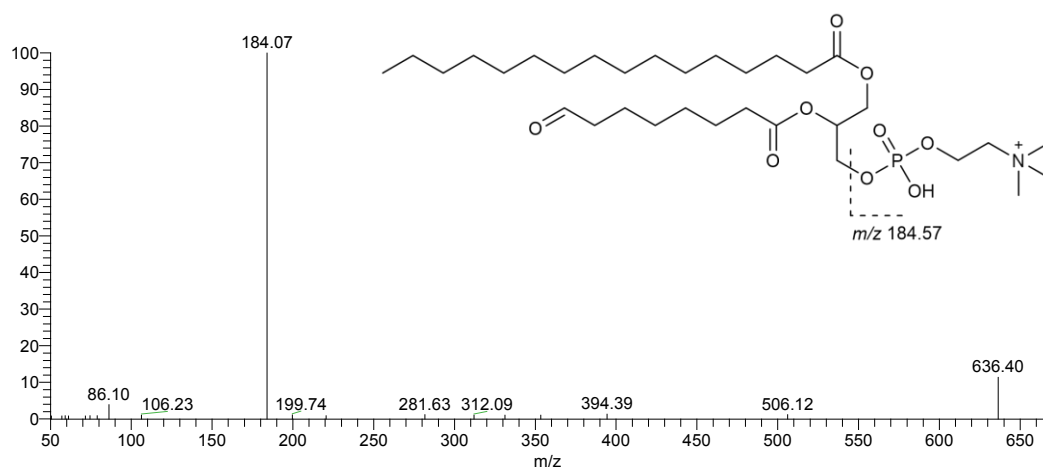


B.



Appendix Figure C 8. MS/MS spectra of 2-linoleoyl lyso PC.

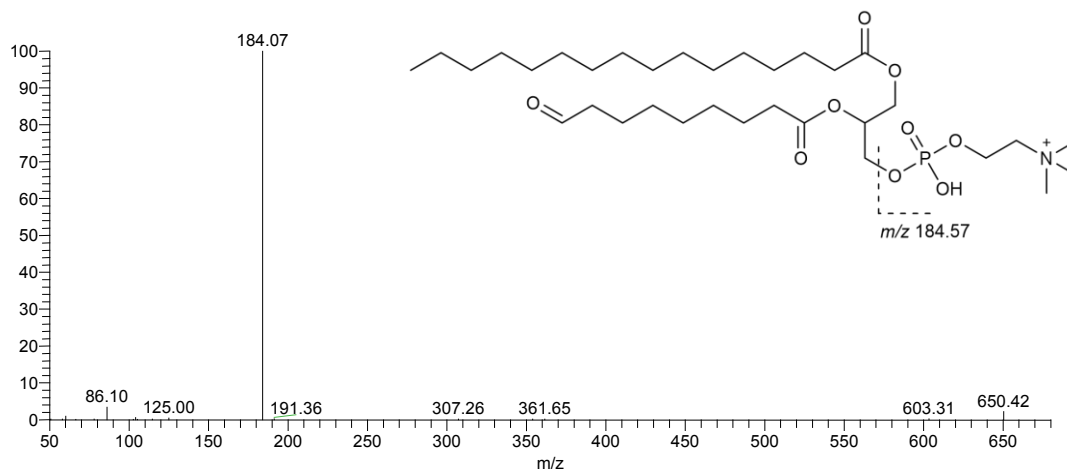
(A) Orbitrap MS/MS spectrum of 2-linoleoyl lyso PC observed in positive mode as $[M + H]^+$ ion at m/z 520.3397. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of 2-linoleoyl lyso PC observed in negative mode as $[M + HCOO]^-$ ion at m/z 564.3300. The product ion observed at m/z 279.23 corresponds to the fatty acyl carboxylate anion 18:2 ($[R2COO]^-$). Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $C_2H_4O_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 504.31).



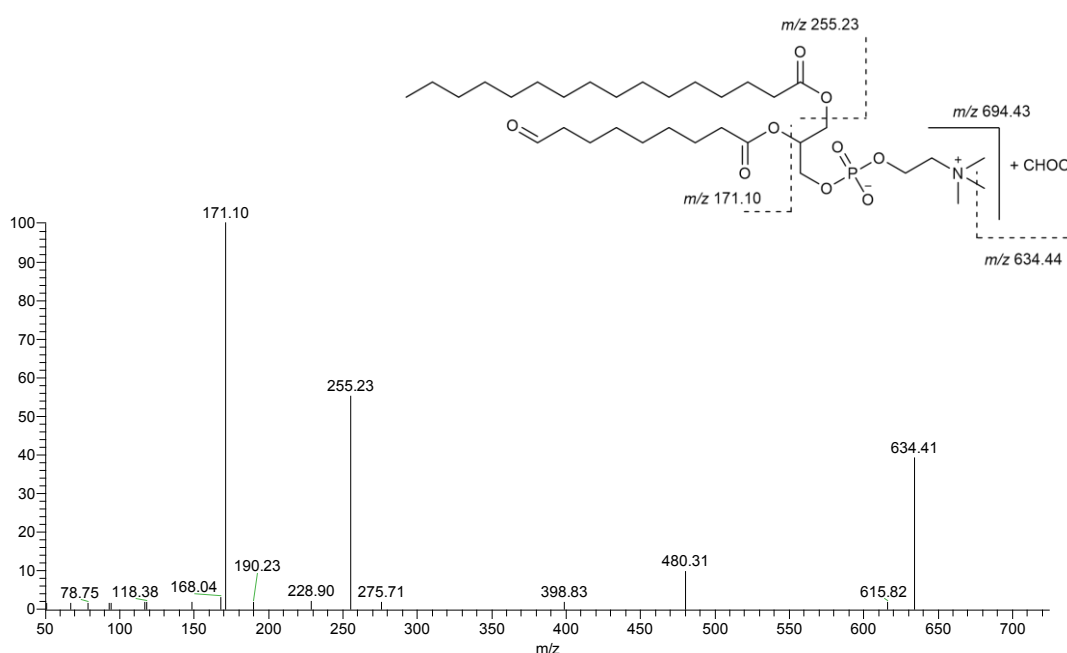
Appendix Figure C 9. MS/MS spectra of 1-palmitoyl-2-(8-oxo-octanoic acid) PC.

Orbitrap MS/MS spectrum of 1-palmitoyl-2-(8-oxo-octanoic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 636.4234. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head.

A.

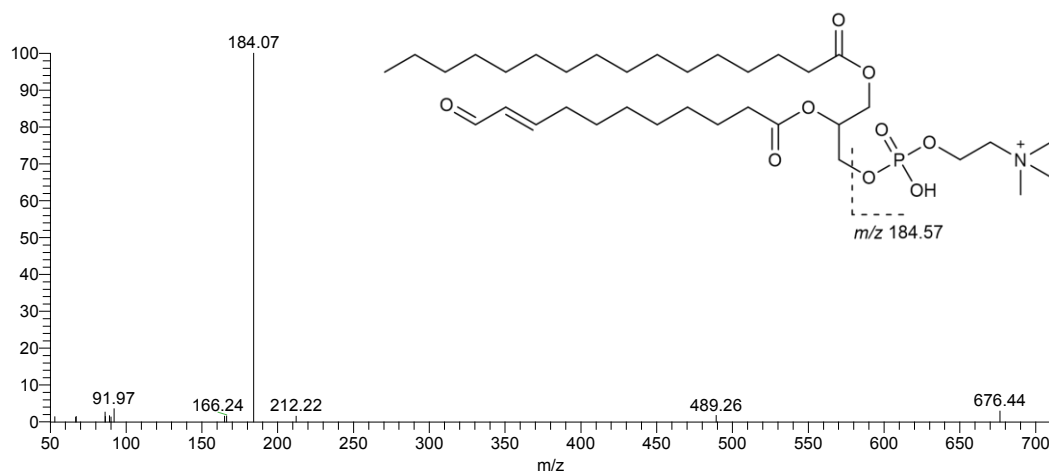


B.



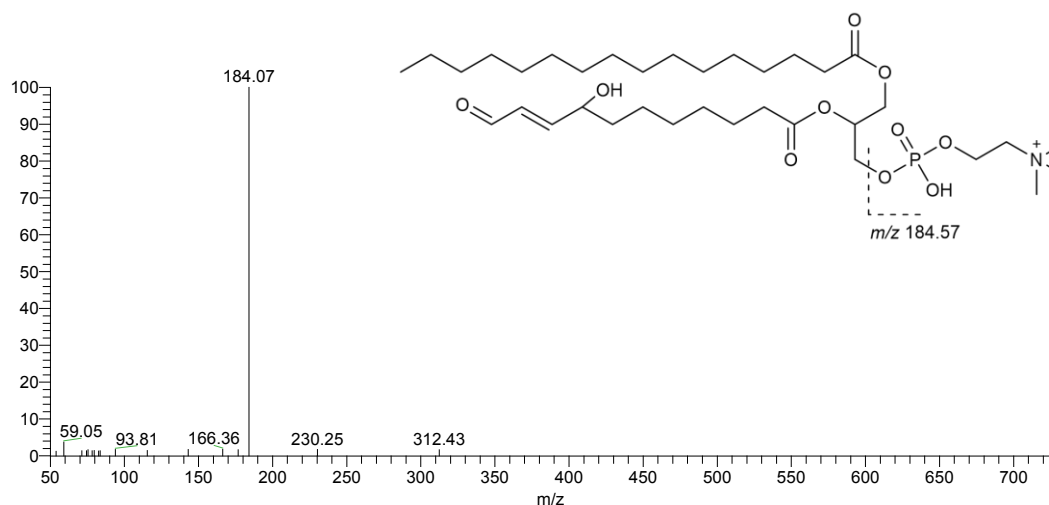
Appendix Figure C 10. MS/MS spectra of 1-palmitoyl-2-(9-oxo-nonanoic acid) PC

(A) Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-oxo-nonanoic acid) PC observed in positive mode as $[\text{M} + \text{H}]^+$ ion at m/z 650.4392. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $\text{C}_5\text{H}_{15}\text{NO}_4\text{P}$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-oxo-nonanoic acid) PC observed in negative mode as $[\text{M} + \text{HCOO}]^-$ ion at m/z 694.4293. The product ion observed at m/z 255.23 corresponds to the fatty acyl carboxylate anion 16:0 ($[\text{R1COO}]^-$), and the product ion observed at m/z 171.10 corresponds to carboxylate anion $[\text{R2COO} + \text{O-C}_9\text{H}_{16}]^-$. Confirmation of phospholipid class was achieved by observing the characteristic neutral loss of 60 Da (formula: $\text{C}_2\text{H}_4\text{O}_2$; exact mass: 60.0211), corresponding to the loss of methyl formate (m/z 634.44).



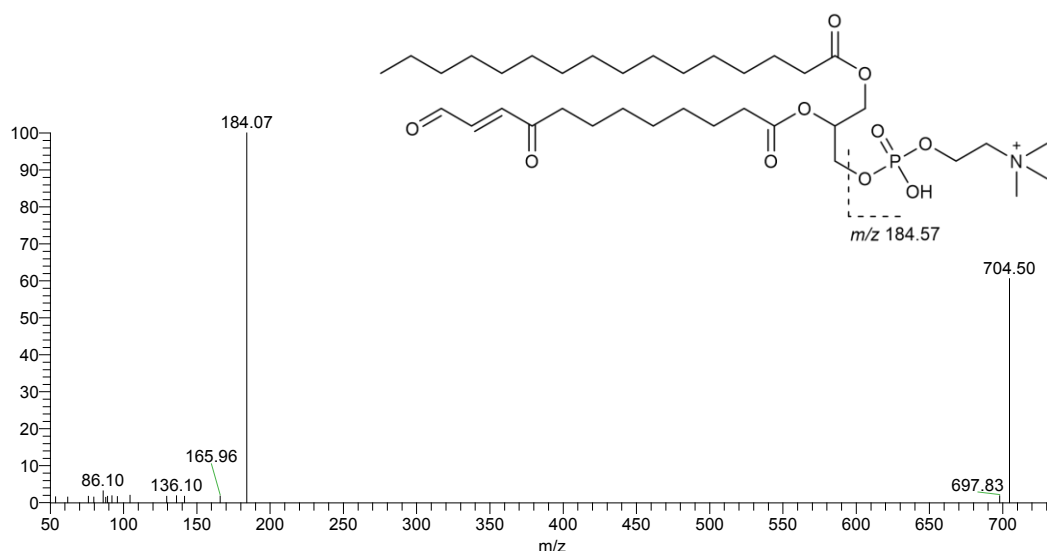
Appendix Figure C 11. MS/MS spectra of 1-palmitoyl-2-(11-oxo-9-undecenoic acid) PC.

Orbitrap MS/MS spectrum of 1-palmitoyl-2-(11-oxo-9-undecenoic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 676.4557. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head.

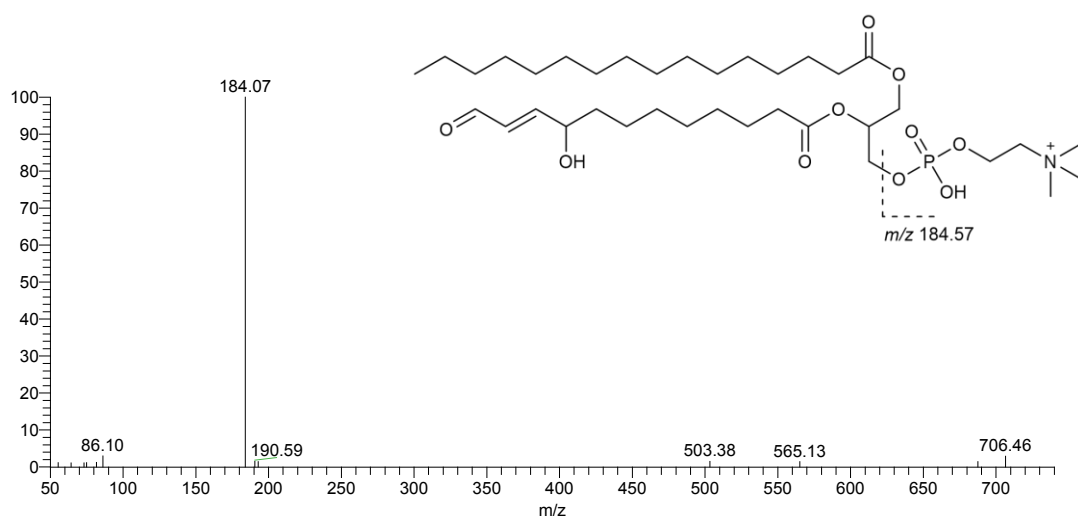


Appendix Figure C 12. MS/MS spectra of 1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC.

Orbitrap MS/MS spectrum of 1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 692.4484. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head.



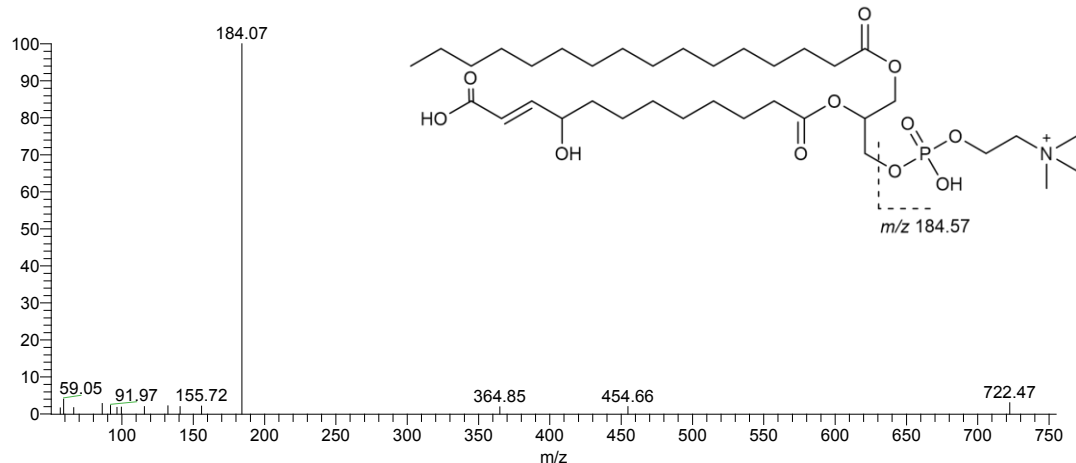
Appendix Figure C 13. MS/MS spectra of 1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC. Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 704.4503. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head.



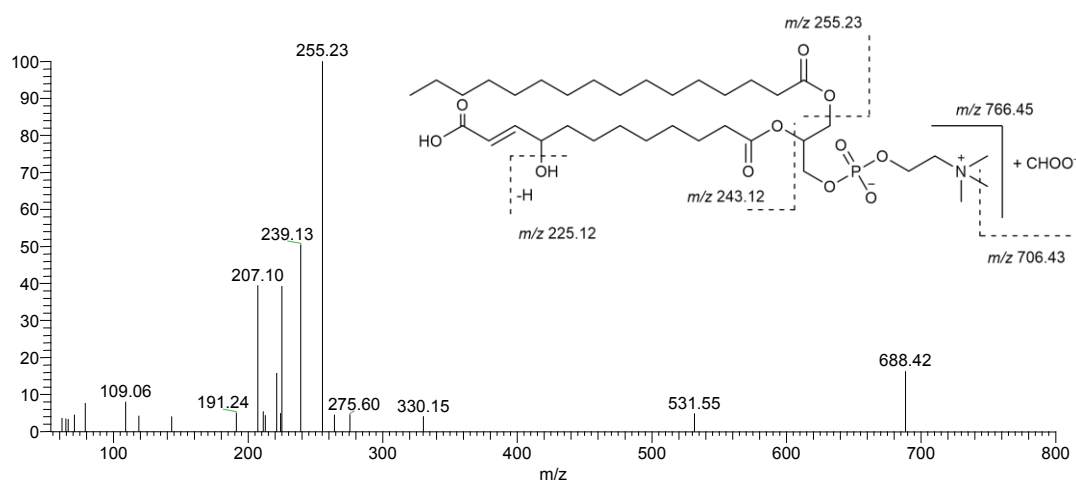
Appendix Figure C 14. MS/MS spectra of 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC.

Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 706.4643. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head.

A.



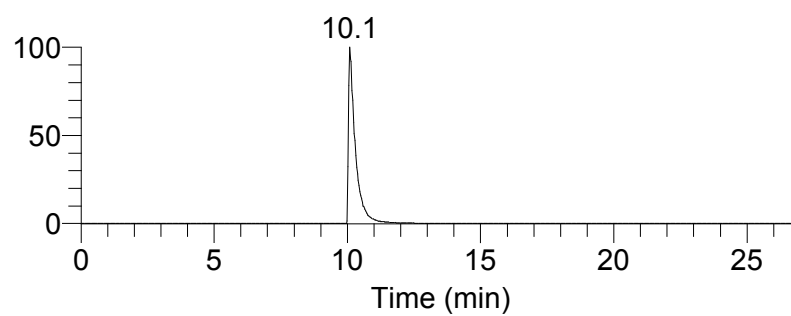
B.



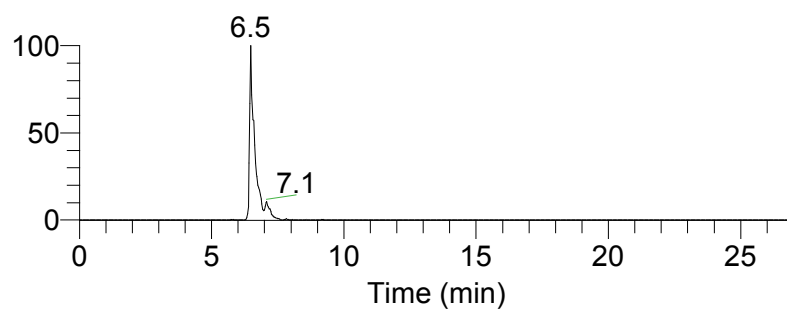
Appendix Figure C 15. MS/MS spectra of 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC.

(A) Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC observed in positive mode as $[M + H]^+$ ion at m/z 722.4589. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.07 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **(B)** Orbitrap MS/MS spectrum of 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC observed in negative mode as $[M + HCOO]^-$ ion at m/z 766.4493. The product ion observed at m/z 255.23 corresponds to the fatty acyl carboxylate anion $[R_1COO]^-$, and the product ion observed at m/z 225.11 corresponds to carboxylate anion $[R_2COO-H_2O]^-$, and the product ion observed at 207.10 corresponds to the carboxylate anion $[R_2COO-2H_2O]^-$.

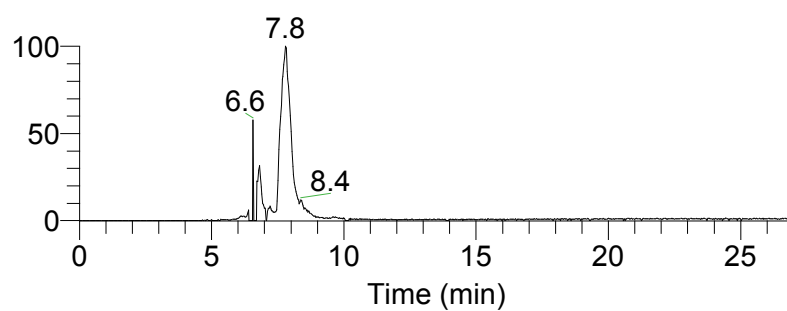
A. (unmodified) PLPC m/z 758



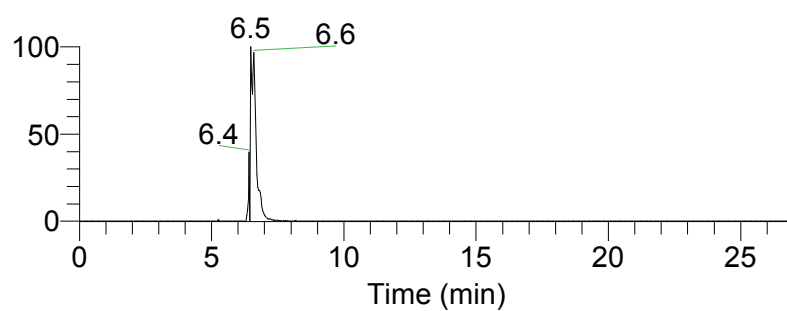
B. PLPC + 14 m/z 772



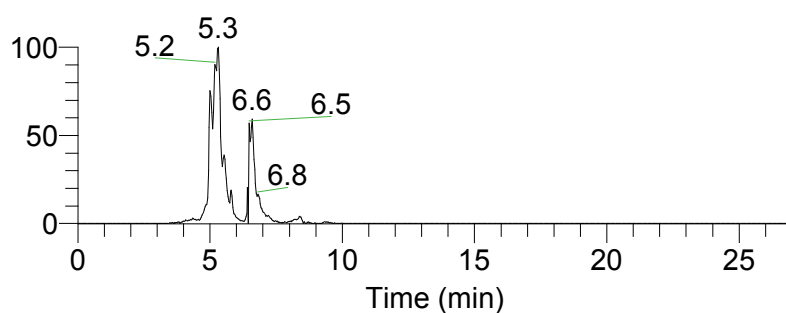
C. PLPC + 16 m/z 774



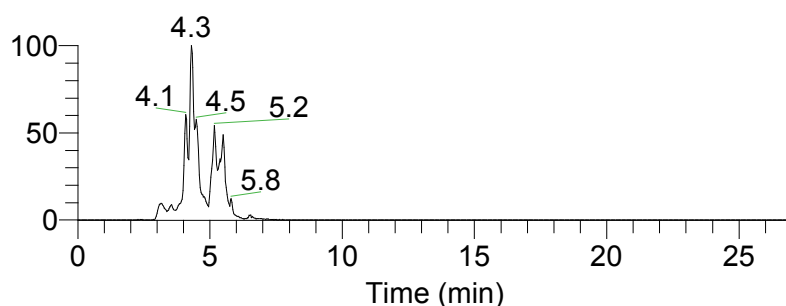
D. PLPC + 32 m/z 790



E. PLPC + 48 m/z 806



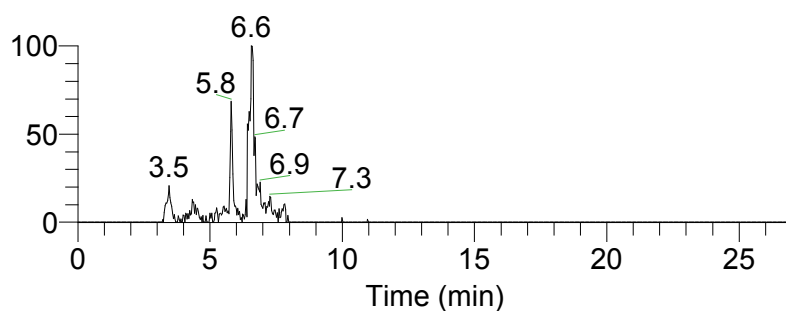
F. PLPC + 64 m/z 822



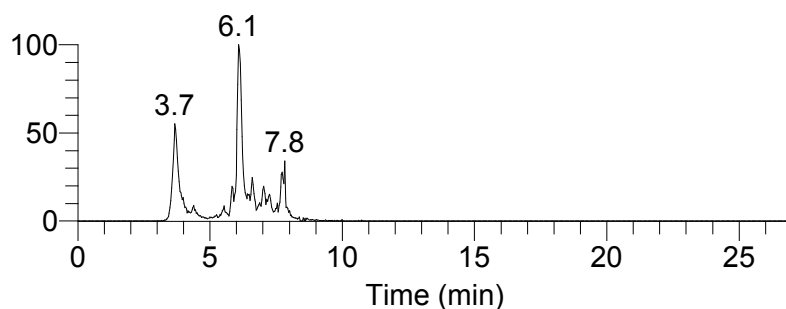
Appendix Figure C 16. Reconstructed ion chromatograms (RICs) of long-chain oxPLPC products identified by LC-MS/MS after 24-hour incubation of PLPC under oxidative conditions.

(A) (unmodified) PLPC m/z 758, **(B)** PLPC + 14 m/z 772, **(C)** PLPC + 16 m/z 774, **(D)** PLPC + 32 m/z 790, **(E)** PLPC + 48 m/z 806, and **(F)** PLPC + 64 m/z 822.

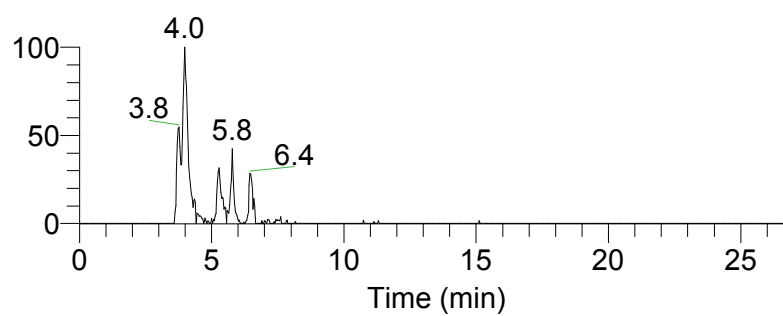
A. 1-Palmitoyl-2-(8-oxo-octanoic acid) PC m/z 636 (RT 3.5)



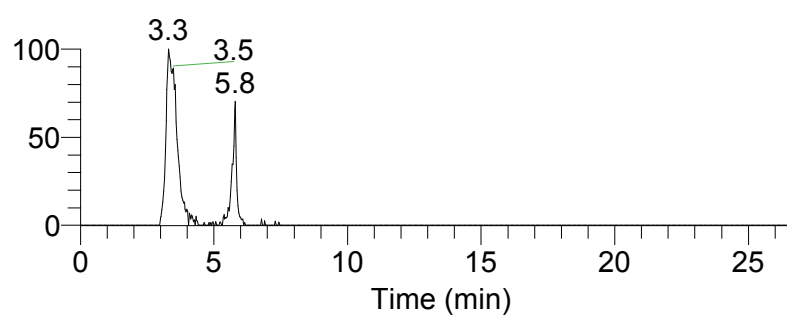
B. 1-Palmitoyl-2-(9-oxo-nonanoic acid) PC m/z 650 (RT 3.7)



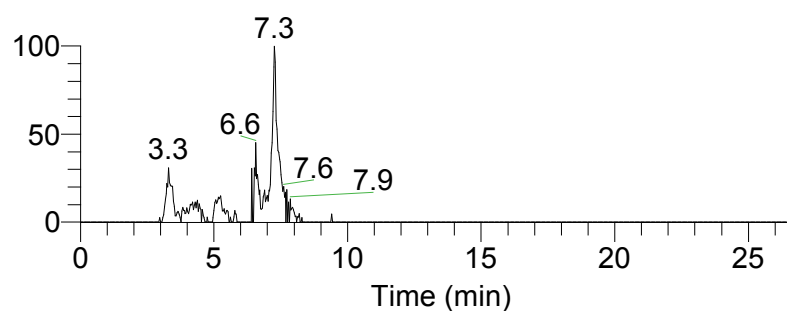
C. 1-Palmitoyl-2-(11-oxo-9-undecenoic acid) PC m/z 676 (RT 4.0)



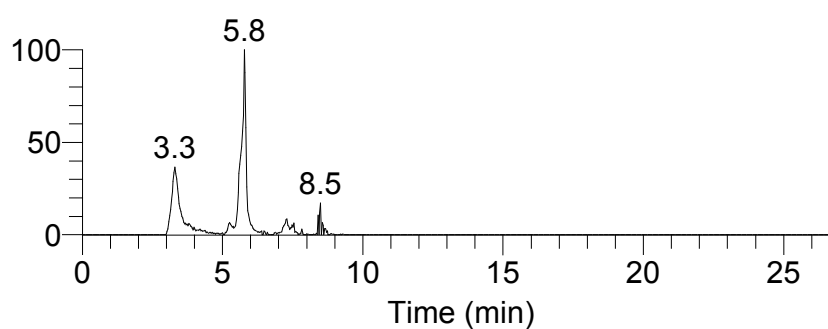
D. 1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC m/z 692 (RT 3.5)



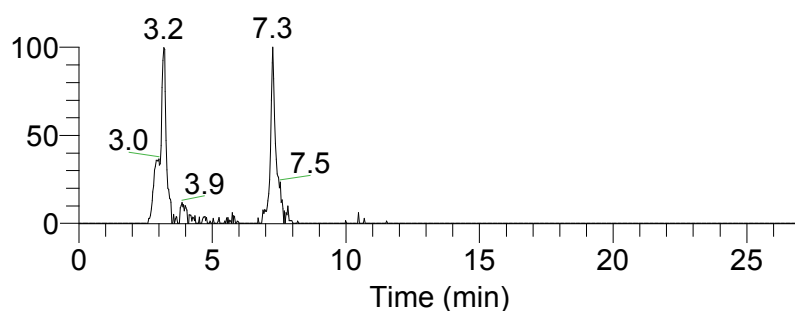
E. 1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC m/z 704 (RT 3.3)



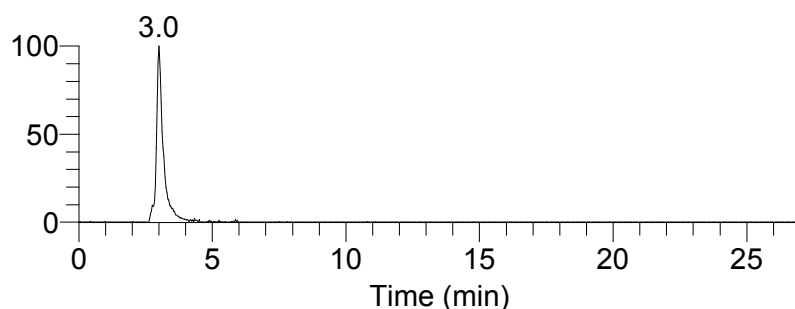
F. 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC m/z 706 (RT 3.3)



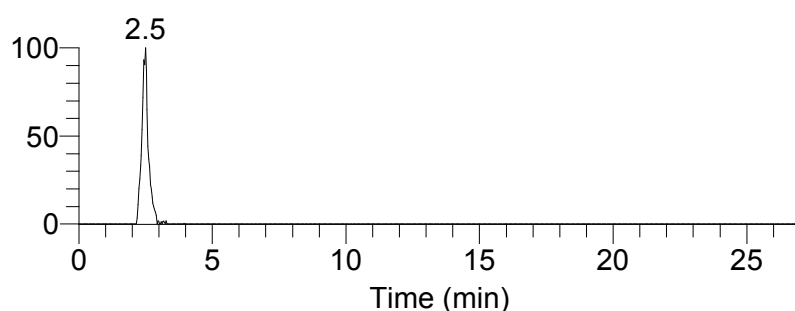
G. 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC m/z 722 (RT 3.2)



H. 1-palmitoyl-lyso PC m/z 496



I. 2-linoleoyl-lyso PC m/z 520



Appendix Figure C 17. Reconstructed ion chromatograms (RICs) of short-chain oxPLPC products identified by LC-MS/MS after 24-hour incubation of PLPC under oxidative conditions.

(A) 1-Palmitoyl-2-(8-oxo-octanoic acid) PC m/z 636, (B) 1-Palmitoyl-2-(9-oxo-nonanoic acid) PC m/z 650, (C) 1-Palmitoyl-2-(11-oxo-9-undecenoic acid) PC m/z 676, (D) 1-palmitoyl-2-(8-hydroxy-11-oxo-9-undecenoic acid) PC m/z 692, (E) 1-palmitoyl-2-(9-keto-12-oxo-10-dodecenoic acid) PC m/z 704, (F) 1-palmitoyl-2-(9-hydroxy-12-oxo-10-dodecenoic acid) PC m/z 706, (G) 1-palmitoyl-2-(9-hydroxy-10-dodecendioic acid) PC m/z 722, (H) 1-palmitoyl-lyso PC m/z 496, and (I) 2-linoleoyl-lyso PC m/z 520.