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Isolation and Anti-Ferroptotic Activity of the New Tetrahydrocannabilactolvarin and Cannabinol Derivatives from a Δ^9 -THCV chemotype of *Cannabis sativa* L.

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Abstract

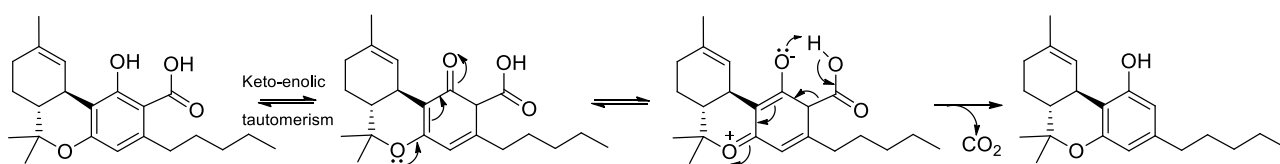
Natural acidic phytocannabinoids from *Cannabis sativa* L. are unstable compounds that undergo decarboxylation, a reaction characterized by distinct kinetics and temperature requirements for each acidic-neutral compound pair. Another critical organic reaction in the plant converts Δ^9 -tetrahydrocannabinol into cannabinol. This degradation represents a crucial issue from both pharmacological and legal standpoints. In this view, the dereplication of a Δ^9 -tetrahydrocannabivarin *C. sativa* L. chemotype, stored for ten years under controlled conditions, was performed in this study. This led to the isolation of cannabinovarinic acid and revealed different rates of decarboxylation and aromatization for Δ^9 -tetrahydrocannabinolic acid and Δ^9 -tetrahydrocannabivarinic acid. Moreover, a new tetrahydrocannabitrioxovarin derivative, named tetrahydrocannabilactolvarin, was isolated and structurally characterized. Inspired by the recently reported activity of phytocannabinoids on ferroptosis, the anti-ferroptotic potential of tetrahydrocannabilactolvarin, the cannabinolic acids stabilized as methyl esters with their neutral counterpart was investigated ascribing ferroptosis inhibition primarily to cannabinol and cannabinovarin.

Keywords

Cannabis sativa, Cannabinoids, Tetrahydrocannabiolvarin, THCLV, Δ^9 -THCV, Ferroptosis.

Introduction

Cannabis sativa L. is a plant well known for its remarkable ability to biosynthesize a wide array of secondary metabolites belonging to different categories (mainly phenolic compounds and terpenes), showing a phytochemical profile that represents a rapidly advancing field of study, driven by the emergence of novel chemotypes through crossbreeding and by continuous improvements in extraction and analytical methodologies [1, 2]. Its chemical variety is fostered not only by true biosynthetic products but also by spontaneous, non-enzymatic transformations occurring in the plant tissues. Among these, decarboxylation is the most critical of these processes, involving the conversion of acidic cannabinoids into their neutral counterparts, via the elimination of a carboxyl group as carbon dioxide (CO_2). This process proceeds via intramolecular proton transfer from the carboxylic moiety in its anti-conformation to the keto-tautomerized ortho-hydroxyl group, ultimately leading to CO_2 release (**Scheme 1**) [3].



Scheme 1. Decarboxylation process of Δ^9 -THCA: anti-conformation of the carboxylic moiety, followed by the keto-enolic tautomerization and intramolecular proton transfer to the ortho-hydroxyl group with the final release of CO_2 .

This structural change, which is facilitated by light and temperature [4,5], is characterized by different kinetics features for each pair of acidic-neutral compounds. The decarboxylation of the natural acidic form has a significant impact on the bioactivity, since the neutral form exhibits significantly enhanced bioavailability due to the higher lipophilicity and stability [4,5].

Another well-known and unique transformation engages the narcotic active principles of cannabis, Δ^9 -tetrahydrocannabinol (**1**, Δ^9 -THC), that undergoes air oxidation with the final aromatization at the menthyl moiety resulting into cannabinol (**2**, CBN). The presence of this latter cannabinoid has been proposed as a marker of time and storage-condition of cannabis samples [6, 7]. The formation of CBN has a major pharmacological and legal impact, since the aromatized derivative has a low psychoactive activity [6].

In this work, the phytochemical profile of a *C. sativa* chemotype rich in Δ^9 -tetrahydrocannabivarin (Δ^9 -THCV), stored for ten years at controlled temperature and monitored relative humidity in a sealed pack bag, was analysed, discovering different rates of decarboxylation and aromatization for Δ^9 -

tetrahydrocannabinolic acid (Δ^9 -THCA) and Δ^9 -tetrahydrocannabivarin acid (Δ^9 -THCVA), leading to the isolation of cannabinovarinic acid (**4**, CBNVA) and the new tetrahydrocannabilactolvarin (**5**, THCLV) (**Figure 1**), whose structural skeleton had never been identified in any cannabis strain, neither as C_3 nor as C_5 analogue.

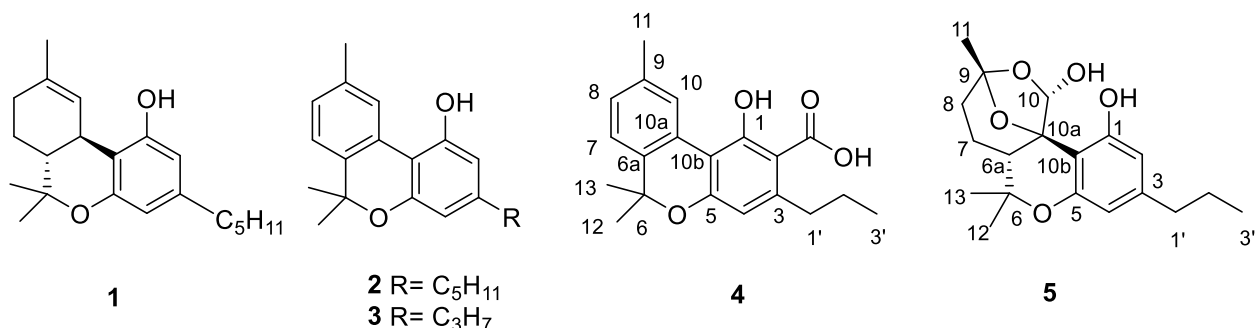


Figure 1. Structure of isolated phytocannabinoids: Δ^9 -THC (1), CBN (2), CBNV (3), CBNVA (4), THCLV (5)

Recent studies have demonstrated that phytocannabinoids can prevent ferroptotic death in hippocampal neurons [8]. Ferroptosis is a distinct and iron-dependent form of regulated cell death characterized by uncontrolled lipid peroxidation, typically arising from the excessive oxidative membrane stress or the failure of protective systems, such as glutathione peroxidase 4 (GPX4)/glutathione (GSH) and ferroptosis suppressor protein 1 (FSP1)/coenzyme Q10 (CoQ10) [9]. Since ferroptosis is involved in several pathological conditions, such as neurodegenerative diseases, liver damage and ischaemia-reperfusion injury, small molecules that can counteract this process could have significant therapeutic implications. In this context, capitalizing on the study of Liang et al. (2024) [8], we compared the anti-ferroptotic potential of the new THCLV (**5**), the cannabinolic acids stabilized as methyl esters (**9**, **12**) with their neutral counterpart (**2**, **3**).

Results and Discussion

Aerial parts belonging to a *C. sativa* chemotype rich in Δ^9 -THCV, air-dried at room temperature in 2014 and stored in a heat-sealed bag for 10 years at controlled temperature and monitored relative humidity (RH), were analysed by chromatographic techniques both for the quantification, isolation and structural elucidation of phytocannabinoids.

Chemical characterization of the plant material

To provide a complete characterization of cannabinoids in the plant material, a detailed analysis was carried out by means of UHPLC-HRMS on an ethanolic extract by comparing the experimental data of compounds (t_R , precursor and product ions) with those of analytical standards and data available in the literature [10-12]. **Table 1** lists the compounds identified in the extracts along with their corresponding mass spectral data.

CBNV (**3**) and its acidic form CBNVA (**4**) were putatively identified. Specifically, CBNV (**3**) exhibited a $[M+H]^+$ precursor ion at m/z 283.1689, with product ions at m/z 265.1582 and 223.1112. This compound showed an efficient ionization in the negative ion mode, with a $[M-H]^-$ precursor ion at m/z 281.1550 and two product ions at m/z 251.1079 and 171.0807, while its corresponding acidic form, CBNVA (**4**), showed precursor ion $[M-H]^-$ at m/z 325.1445. As expected, the extract was found to contain many additional varinic cannabinoids, such as cannabidivarinic acid (CBDVA), Δ^9 -THCVA, cannabichromevarinic acid (CBCVA) and the corresponding neutral forms cannabidivarin (CBDV), Δ^9 -THCV (**6**) and cannabichromevarin (CBCV).

Intriguingly, an unidentified compound was detected at 6.5 min, having a precursor ion $[M+H]^+$ at m/z 335.1853. The MS/MS data indicated two product ions at m/z 317.1742 and 299.1638, which could correspond to two sequential neutral losses of H_2O (-18 Da). This compound also displayed the characteristic Δ^9 -THCV fragments at m/z 165.0909 and 123.0805, where the product ion at m/z 165 could correspond to a resorcinol ring with the propyl side chain, while that at m/z 123 could arise from further fragmentation involving the loss of the propyl moiety [10-11].

In addition to the above-mentioned compounds, also pentyl-type phytocannabinoids commonly found in standard *C. sativa* chemotypes were detected, including cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabinolic acid (CBNA), Δ^9 -THCA and cannabichromenic acid (CBCA), together with their corresponding neutral counterparts cannabidiol (CBD), cannabigerol (CBG), CBN (**2**), Δ^9 -THC (**1**) and cannabichromene (CBC), were detected in the samples.

Table 1. UHPLC-HRMS data of cannabinoids detected in cannabis extracts both in the positive and negative ion mode, together with their fragmentation data

Compound	<i>t_R</i>	[M+H] ⁺	MS/MS	[M-H] ⁻	MS/MS
Unknown	6.5	335.1853	317.1742 (100), 299.1638 (39), 241.1220 (32), 165.0909 (92), 123.0805 (27)	333.1708	333.1712 (36), 315.1606 (84), 179.0706 (100), 151.0755 (78)
Cannabidivarinic acid (CBDVA)	8.4	–	–	329.1758	329.1762 (100), 311.1658 (69), 285.1864 (26), 217.1231 (75), 107.0491 (71)
Cannabidivarin (CBDV)	9.3	287.2007	287.2003 (87), 231.1379 (16), 165.0910 (100), 135.1160 (17)	–	–
Cannabidiolic acid (CBDA)	11.0	359.2213	341.2107 (100), 262.1483 (11), 219.1013 (34)	357.2073	357.2077 (100), 339.1968 (64), 313.2177 (29), 245.1548 (79), 179.1071 (37)
Cannabinovarin (CBNV)	11.1	283.1689	283.1687 (100), 265.1582 (40) 223.1112 (61)	281.1548	281.1550 (100), 251.1079 (37), 171.0807 (13)
Cannabigerolic acid (CBGA)	11.3	–	–	359.2228	359.2232 (48), 341.2124 (100), 315.2330 (37), 191.1071 (24)
Cannabigerol (CBG)	11.6	317.2475	317.2466 (4), 261.1116 (3) 193.1220 (100), 123.0440 (15)	–	–
Cannabidiol (CBD)	11.8	315.2319	315.2316 (100), 259.1690 (14), 193.1223 (40), 135.1168 (20), 123.0442 (15), 93.0703 (19)	–	–
Cannabinovarinic acid (CBNVA)	12.1	–	–	325.1445	325.1450 (56), 281.1550 (100), 251.1080 (55), 171.0809 (20)
Δ ⁹ -Tetrahydrocannabivarin (Δ ⁹ -THCV)	12.2	287.2007	287.2004 (100), 231.1380 (14), 165.0911 (40) 135.1160 (16), 123.0442 (6)	–	–
Δ ⁹ -Tetrahydrocannabivarinic acid (Δ ⁹ -THCVA)	13.2	–	–	329.1758	329.1762 (100), 285.1863 (75), 217.1231 (35), 107.0941 (10)
Cannabinol (CBN)	13.4	311.2000	311.1999 (100), 293.1895 (28), 241.1220 (18) 223.1113 (55)	309.1862	309.1862 (100), 279.1392 (35), 171.0807 (12)
Cannabichromevarinic acid (CBCVA)	13.7	–	–	329.1758	329.1763 (100), 311.1658 (26), 285.1763 (30), 353.1752 (63), 309.1864 (100) 279.133 (49), 171.0808 (21)
Cannabichromene (CBC)	14.4	–	–	353.1758	–
Δ ⁹ -Tetrahydrocannabinol (Δ ⁹ -THC)	14.5	315.2319	315.2316 (100), 259.1690 (18), 193.1223 (45), 135.1168 (21), 123.0441 (17)	–	–
Cannabichromevarin (CBCV)	15	287.2007	287.2003 (100), 231.1379 (13), 165.0910 (27), 135.1168 (14)	–	–
Cannabichromene (CBC)	15.4	315.2319	315.2316 (40), 259.1691 (34), 193.1233 (100), 135.1168 (16), 123.0442 (15)	–	–
Δ ⁹ -Tetrahydrocannabinolic acid (Δ ⁹ -THCA)	15.8	359.2213	341.2104 (100), 219.1012 (32)	357.2073	357.2079 (100), 313.2179 (56), 245.1549 (26), 191.1072 (24)
Cannabichromenic acid (CBCA)	16.5	359.2213	341.2105 (100), 219.1013 (79)	357.2073	357.2077 (100), 339.1961 (5), 313.2178 (21), 243.1392 (6), 191.1072 (33)

The quantification of phytocannabinoids in the plant material was performed using HPLC-UV/Vis. The applied extraction conditions allowed us to keep the acid and neutral cannabinoid composition as intact as possible to perform a reliable quantification, which was performed at 220 and 210 nm, respectively [13]. A representative chromatogram from the HPLC-UV/Vis analysis of the ethanolic extract from the plant material is shown in **Figure 2**.

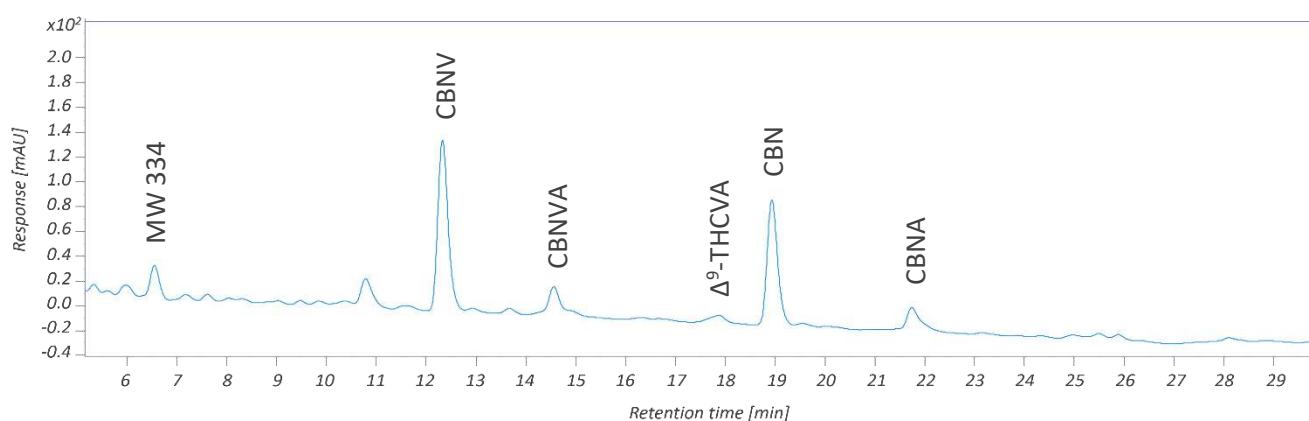


Figure 2. HPLC-UV/Vis representative chromatogram of cannabinoids in the plant extract, acquired at 210 nm.

Overall, the analysis revealed that CBNV (**3**) was the most abundant compound in the plant material (2.5 mg/g), followed closely by CBN (**2**) (2.1 mg/g). The unknown compound with molecular weight (MW) 334 was also quantified (0.6 mg/g). The acidic compounds, namely CBNVA (**4**), Δ^9 -THCVA and CBNA, were quantified at 1.2, 0.5 and 0.9 mg/g, respectively. All other compounds annotated by UHLC-HRMS were found to be below the limit of detection (LOD) or the limit of quantification (LOQ) (**Supplementary Table 4**).

Isolation and structural characterization of the unknown compound

To enable the isolation and unequivocal structural elucidation of the unknown metabolite detected during dereplication of the ethanolic extract, a larger-scale extraction was performed using acetone. Subsequent chromatographic fractionation of the resulting extract afforded compound **5** in pure form, alongside CBN (**2**, 0.86%) and CBNV (**3**, 0.75%), as the major cannabinoids. The isolation procedure also yielded a small amount of CBNVA (**4**, 0.091%), isolated for the first time, and a residual trace of Δ^9 -THC (**1**, 0.003%). The molecular formula of **4** was established as $C_{19}H_{22}O_4$ by ESI-HRMS from the $[M+H]^+$ protonated ion at m/z 327.1561, consistent with a cannabinol acid derivative bearing a propyl side chain. The ^1H NMR spectrum ($CDCl_3$, 400 MHz, **Supplementary Figure 4 and 5**) displayed the characteristic resonances of a cannabinol-type aromatic system, including a singlet aromatic proton at δ_H 6.47 (1H, s, H-4), together with aromatic signals at δ_H 8.49 (1H, s, H-10) and 7.17–7.12 (2H, overlapped, H-7 and H-8). A deshielded carboxylic acid proton at δ_H 12.76 (br s) and a carbonyl carbon resonance at δ_C 176.5 supported the presence of a cannabinoid acid functionality. The propyl side chain was clearly established by the presence of only two methylene groups at δ_H 2.99 (H-1') and 1.75–1.67 (H-2'), correlated with carbons at δ_C 38.9 and 24.6, respectively, together with the terminal methyl triplet at δ_H 1.05 (H-3'; δ_C 14.3).

Crucially, the purification process also allowed the isolation of the previously unidentified compound **5**. The molecular formula of **5** was established as C₁₉H₂₆O₅ by ESI-HRMS, accounting for seven degrees of unsaturation. The ¹H NMR spectrum (CDCl₃, **Table 2**) showed two resorcinol singlets at δ_{H} 6.36 and 6.29 ppm, the methine proton at δ_{H} 5.73, a series of multiplets between δ_{H} 1.60 and 2.45, and four methyl groups, three singlets at δ_{H} 1.31, 1.39, and 1.64 and a triplet at δ_{H} 0.92. This pattern of methyl resonances is suggestive of an oxidized THC-type skeleton, in agreement with the neutral losses of water observed in the HRMS analysis.

Table 2. ¹H (700 MHz) and ¹³C (175 MHz) data of compound **5** in CDCl₃

Pos.	δ_{C} , type	δ_{H} , mult., <i>J</i> in Hz
1	155.6, C	
2	110.4, CH	6.36, d, 1.5
3	146.6, C	
4	110.6, CH	6.29, d, 1.5
5	154.1, C	
6	77.8, C	
6a	45.2, CH	2.26, dd, 4.3, 12.7
7a	19.2, CH ₂	1.64, overlapped
7b		1.79, overlapped
8a	34.5, CH ₂	1.76, overlapped
8b		1.88, m
9	106.9, C	
10	96.8, CH	5.73, d, 11.0
10a	84.2, C	
10b	103.5, C	
11	24.7, CH ₃	1.64, s
12	21.8, CH ₃	1.31, s
13	28.6, CH ₃	1.39, s
1'	37.8, CH ₂	2.45, t, 6.8
2'	23.8, CH ₂	1.60, overlapped
3'	13.8, CH ₃	0.92, t, 7.0
1-OH		7.59, bs
11-OH		4.30, d, 11.0

Interpretation of the 2D NMR COSY and HSQC spectra defined two independent spin systems (**Figure 3**): a resorcinol-linked propyl side chain, and a C₃ moiety extending from the methylene protons H₂-8 (δ_{H} 1.76 and 1.88) to the methine H-6a. The HSQC spectrum revealed that the signal at δ_{H} 5.73 (H-10) belongs to a hemiacetal functionality (δ_{C} 96.8). The ¹³C NMR spectrum showed two further oxygenated quaternary carbons, including an acetal carbon at δ_{C} 106.9 (C-9) and an oxygenated carbon at δ_{C} 84.2 (C-10a) (**Table 2**). The presence of these groups suggested compound **5** to be a highly oxygenated derivative of Δ^9 -THCV, lacking the typical methyl double bond. Key ^{2,3}J_{H,C} cross-peaks observed in the 2D HMBC spectrum (**Figure 3a**) enabled complete structural assignment. Specifically, HMBC correlations from H₃-11 to C-9 and C-8 connected the acetal carbon C-9 to C-8, while the geminal methyl protons H₃-12 and H₃-13 showed HMBC correlations to C-6a and to the quaternary C-6. Finally, H-10 (δ_{H} 5.73) showed HMBC cross-peaks with to C-9, C-10a, C-10b, and C-6a, thus building an oxygenated seven-membered ring arising from

a rearrangement of the terpenyl moiety and incorporating a lactol functionality. In agreement with the molecular formula, an oxygen bridge must connect C-9 and C-10a (δ_c 84.2).

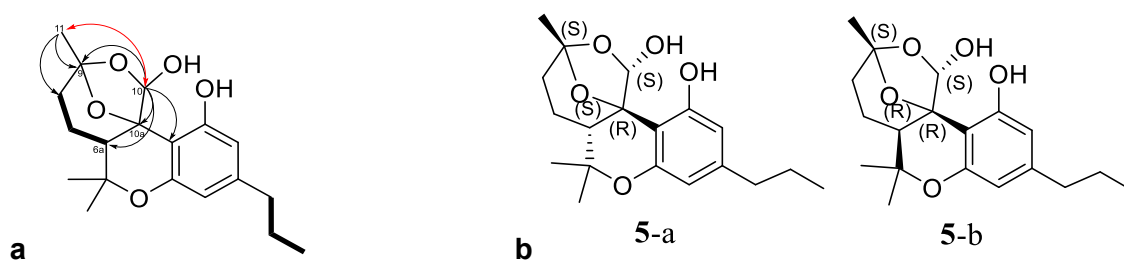


Figure 3. Structural and stereochemical characterization of the new cannabinoid THCLV (5): **a)** diagnostic 2D NMR COSY (bold), HMBC (arrows) and NOESY (red arrow) correlations of compound **5**, **(b)** structures of the two diastereoisomers **5-a** (SSSR/RRRS) and **5-b** (RSSR/SRRS) used for ^{13}C NMR calculation.

In summary, the planar structure of **5**, named tetrahydrocannabilactolvarin (THCLV) features a cannabinoid scaffold fused to a rigid oxabicyclic framework including an oxepane ring alongside ketal and lactol functionalities. No detectable interconversion was observed for **5** after 48 h in MeOH/H₂O solution, indicating configurational stability at C-10, likely ascribable to intramolecular hydrogen bonding.

The structure of compound **5** includes four stereogenic centers (C-6a, C-9, C-10, and C-10a) and definition of the relative configuration needed the combined analysis of the NOESY spectrum and a computationally assisted prediction of ^{13}C NMR data.

The key NOESY cross-peak of H-10 with H₃-11 defined the relative arrangement of two stereogenic acetal carbons. The rigid bicyclic framework fixes the bridging oxygen, between C-9 and C-10a, on the same face of the molecule, thus determining the relative arrangement of C-9 and C-10a. On these bases, only the two diastereomers **5-a** (SSSR/RRRS) and **5-b** (SRRS/RSSR) (**Figure 3b**) are possible. The stereochemical assignment of compound **5** was obtained by comparison of experimental and DFT-calculated ^{13}C NMR chemical shifts.

A preliminary conformational search using Simulated Annealing (INSIGHT II package), followed by DFT calculations with Gaussian09, revealed that for each diastereomer only two conformers account for nearly the entire population (**Figure 4, Supplementary Table 1**).

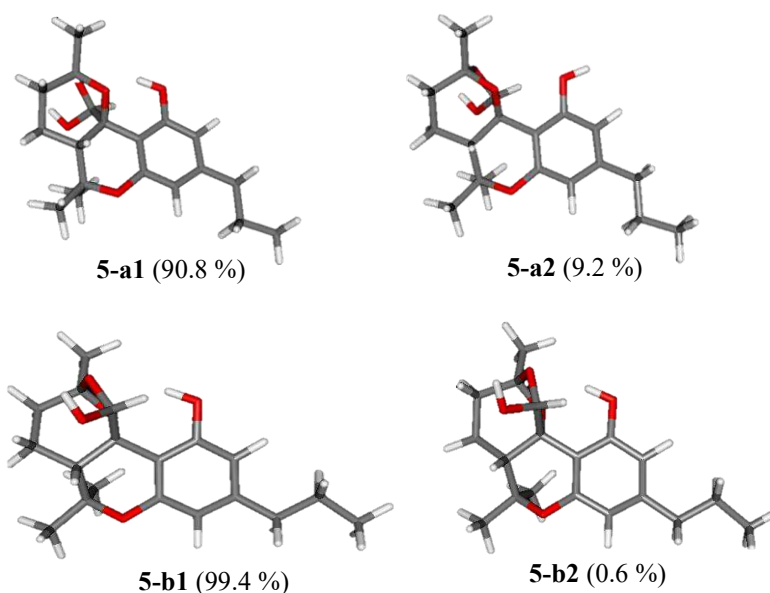


Figure 4. Two main conformers (and their relative population) for the two possible diastereomers of **5**.

GIAO ^{13}C calculations were performed using the mPW1PW91 functional and the 6-31G (d, p) basis set, employing as input the geometries previously optimized at the mPW1PW91/6-31G (d, p) level. The chemical shift calculations considered exclusively the two most populated conformers for each diastereomer (**Supplementary Table 3**). After performing a Boltzmann-weighted averaging, DP4+ probabilities were calculated.

The DP4+ statistical analysis clearly indicated that the **5-a** model is the one most compatible with the experimental NMR data (DP4+ = 100%, **Supplementary Table 2**), whereas the **5-b** model is highly unlikely (DP4+ = 0%). Based on these results, the relative configuration of compound **5**, corresponding to the diastereomer **5-a**, was unambiguously assigned.

Although the evident relationship with Δ^9 -THCV was strongly suggestive of the absolute configuration of **5**, we decided to use the **5-a** model for TDDFT calculations, performed using the CAM-B3LYP functional and the def2TZVP basis sets, considering at least 50 excited states in all cases and employing the IEF-PCM model for MeOH. The rotatory strength values were summed after Boltzmann statistical weighting, and the $\Delta\epsilon$ values were calculated by forming sums of Gaussian functions centered at the wavelengths of the respective electronic transitions and multiplied by the corresponding rotatory strength values. The ECD spectra were obtained for **5-a** (SSSR) and its enantiomer (**Figure 5**). The similarity of the **5-a** (SSSR) spectrum with the

experimental ECD spectrum allowed the unambiguous assignment of the absolute configuration to **5**.

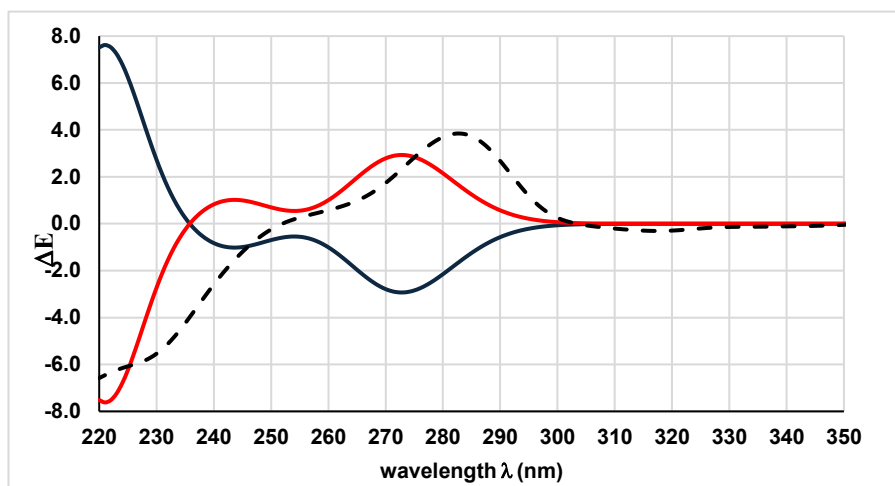
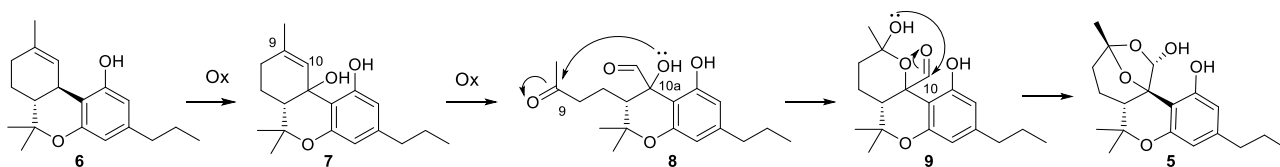


Figure 5. Experimental ECD spectrum of compound **5** (black); TDDFT-calculated curves of model **5-a** (SSSR) (blue) and its enantiomer (red).

Proposed biogenetic origin of tetrahydrocannabilactolvarin (THCLV)

The newly isolated compound **5** could plausibly arise from a sequential oxidation on Δ^9 -THCV (**6**) (**Scheme 2**). The typical aromatization of Δ^9 -THC (**1**) to CBN (**2**) is known to involve successive oxidative steps, leading to the formation of a mixture including the hydroxylated intermediate 10a-OH- Δ^9 -THC [14]. The parallel pattern from Δ^9 -THCV (**6**) obviously involves the formation of 10a-OH- Δ^9 -THCV (**7**). Starting from intermediate **7**, oxidative cleavage of $\Delta^{9,10}$ could generate a keto-aldehyde intermediate (**8**). Intramolecular nucleophilic attack of the C-10a hydroxyl group at C-9 would afford a fused δ -lactol ring. This intermediate may subsequently undergo a second intramolecular nucleophilic addition to the C-10 aldehyde, ultimately forming the additional O-bridged ring observed in **5**.

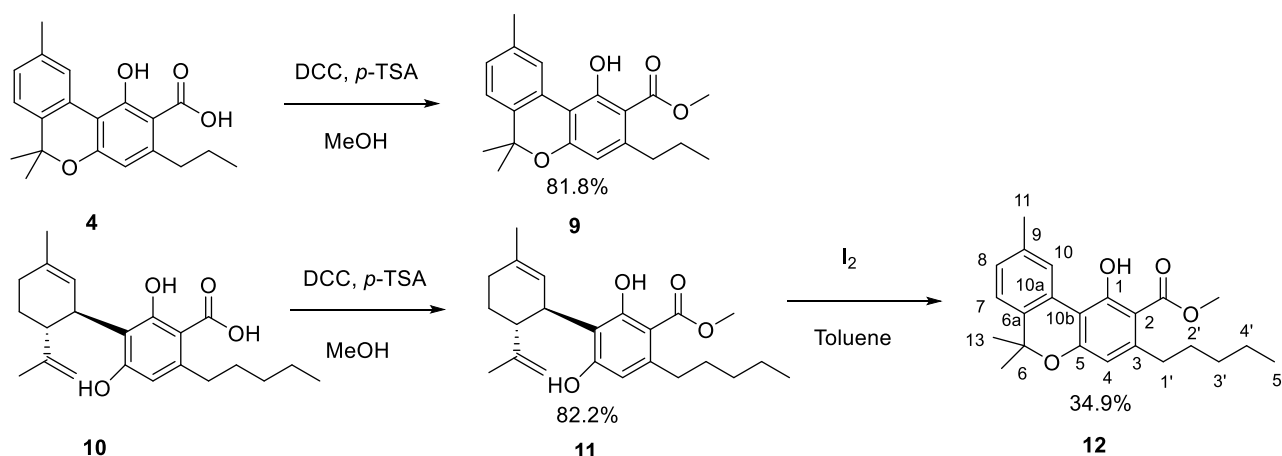


Scheme 2. Postulated biogenetic origin of **5:** formation of 10a-OH- Δ^9 -THCV (**7**) from Δ^9 -THCV (**6**), generation of a keto-aldehyde intermediate (**8**). Intramolecular nucleophilic attack of the C-10a hydroxyl group at C-9 to afford a fused δ -lactol ring with a second intramolecular nucleophilic addition to the C-10 aldehyde forming the additional O-bridged ring observed in THCLV (**5**).

According to the scientific literature, the decarboxylation process is strictly temperature and time-dependent: while low temperatures slow the process, they cannot entirely prevent it over extended periods [15]. In this case, the ten-year storage of the plant material was sufficient for the complete decarboxylation of Δ^9 -THCA, leaving only barely detectable traces and leading to the formation of **1**. Regarding the varin analogue Δ^9 -THCVA, the oxidative degradation evidently competed with or preceded its complete decarboxylation, resulting in the accumulation of CBNVA (**4**) [6, 16]. As previously described by Fućak et al. (2023) [3], the chromatographic isolation of a residual CBNVA (**4**), alongside the detection of CBNA, suggests a potential difference in the degradation rates between Δ^9 -THCVA and Δ^9 -THCA. This discrepancy can be speculatively attributed to the varying length of the aliphatic side chain. Mechanistically the decarboxylation process requires the stabilisation of the electron pair resulting from the cleavage of the C-C bond of aromatic carboxylic acid. The absence of two methylene moieties in the propyl chain of Δ^9 -THCVA likely reduces the electron-donating effect towards the aromatic ring compared to the pentyl side chain of Δ^9 -THCA. Consequently, a lower electron density on the ring could slow down the decarboxylation rate, thereby favoring the competitive oxidative degradation of Δ^9 -THCVA in CBNVA (**4**).

Anti-ferroptotic activity

Driven by these chemical insights and the successful isolation of both known and novel varin derivatives, we sought to explore their biological relevance. Inspired by the recent study of Liang et al. (2024) [8], who have described CBN (**2**) to be a unique inhibitor of oxytosis/ferroptosis presenting a fragment-based investigation of the cannabinoid chemical space, we decided to investigate the anti-ferroptotic potential of structurally related derivatives. A primary objective of this biological screening was to spotlight the newly discovered compound THCLV (**5**). Due to its unique, oxidized THC-type skeleton, THCLV (**5**) represents a crucial addition to the cannabinoid structure-activity relationship (SAR) landscape, allowing us to evaluate how an increased degree of oxidation impacts anti-ferroptotic activity. To systematically map this SAR, our study compared the anti-ferroptotic potential of CBN (**2**) and CBNV (**3**) with their corresponding acidic precursors. To ensure chemical stability and facilitate comparative analysis, the acidic precursors were tested as their methyl esters, i.e. CBNVA- and CBNA-methyl ester (**9** and **12**). The esterification procedure followed the method established by Tamburello et al. for CBDA (2023) [17]. Since CBNA could not be isolated in the plant material, its methyl ester was semi-synthesised from CBDA-methyl ester (**11**) with the procedure proposed by Pollastro et al. (2017) [18] (**Scheme 3**). This panel, combining the stabilized acidic forms, their neutral counterparts, and the uniquely oxidized THCLV (**5**), aimed to elucidate how specific structural features modulate cellular protection against ferroptosis.



Scheme 3. Reaction of esterification of CBNVA (**4**) to CBNVA-methyl ester (**9**). Reaction of esterification of CBDA (**10**) to CBDA-methyl ester (**11**) and its conversion to CBNA-methyl ester (**12**).

The anti-ferroptotic potential of the newly identified THCLV (**5**) was evaluated and compared with the reference cores CBN (**2**), CBNV (**3**), alongside their acidic and esterified derivatives. In the initial cellular screening, THCLV (**5**) demonstrated a concentration-dependent protective profile against ferroptosis. At higher concentrations (45 μ M) THCLV (**5**) showed pronounced protection against ferroptosis, matching the strong protection observed for CBN (**2**), CBNV (**3**) and as well as the free acid CBNVA (**4**), whereas methyl-esters **9** and **12** were not active (**Figure 6a**). However, this protective effect was lost at lower concentrations (4.5 μ M): while the two neutral derivatives CBN (**2**) and CBNV (**3**) displayed particularly strong anti-ferroptotic effects, conferring almost complete protection, THCLV (**5**) was inactive and CBNA-methyl ester (**12**) provided only partial protection. This trend was further reflected in detailed concentration-dependent studies, which identified THCLV (**5**) as the least active derivative among the protective cannabinoids, yielding an EC₅₀ of 7.5 μ M compared to CBN (**2**) (EC₅₀ = 0.23 μ M), the most potent derivative, followed by CBNVA (**4**) (EC₅₀ = 3.75 μ M) (**Figure 6b**). The exemplarily tested cannabinoids CBN (**2**) and THCLV (**5**) did not exhibit iron-binding capacity (**Figure 6c**), and they displayed only weak DPPH radical scavenging activity (**Figure 6d**).

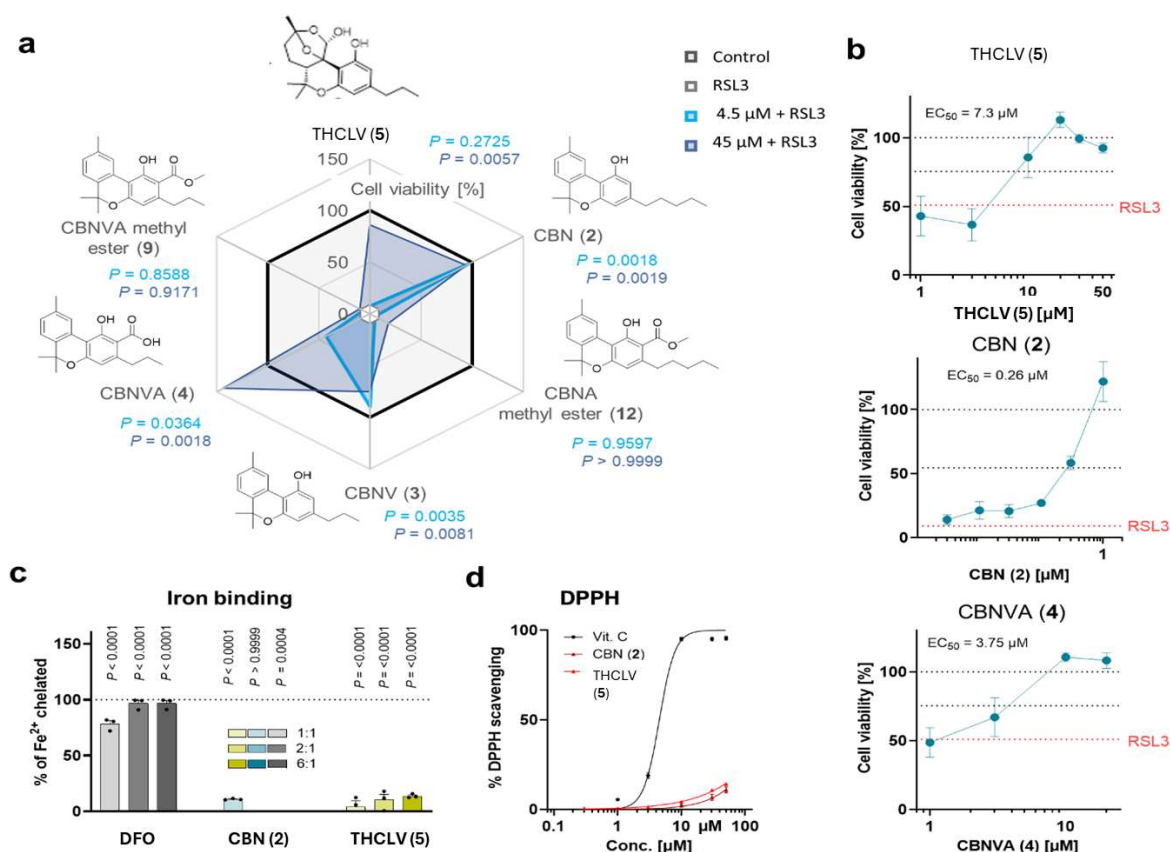


Figure 6. Anti-ferroptotic activity of the isolated cannabinoids: **a)** The radar plot shows the percentage changes in cell viability (MTT assay) of HepaRG cells treated with RSL3 (0.8 μ M) in the presence of tested compounds (4.5 and 45 μ M) for 48 h relative to vehicle (DMSO, 0.5%). Data are shown as mean values of $n = 4$ independent experiments. P values vs. RSL3, mixed-effects models (REML) ANOVA **(b)** Anti-ferroptotic activity of CBN (2), CBNVA (4) and THCLV (5) in HepaRG cells treated with RSL3 (0.4 μ M) for 48 h, determined by MTT assay. Means $\pm$ SEM of $n = 3$ independent experiments. **(c)** Iron-binding capacity was measured using ferene at iron-to-compound ratios of 1:1, 1:2, 1:6. DFO was used as positive control. P values vs. vehicle control, multiple comparison two-way ANOVA with Tukey's *post-hoc* test. Means and individual values $\pm$ SEM of $n = 3$ independent experiments. **(c)** Radical scavenging activity of CBN (2) and THCLV (5) was evaluated using the DPPH assay. Vitamin C (Vit. C) was used as a positive control. Means $\pm$ SEM of $n = 3$ independent experiments.

However, CBN (2) significantly suppressed lipid peroxidation in artificial membranes starting from 2 μ M and reaching 50% inhibition at 20 μ M (**Figure 7a**), classifying it as a moderate lipophilic antioxidant [19]. In contrast, THCLV (5) was less effective, inhibiting lipid peroxidation by 30% at 20 μ M (**Figure 7b**). These differences in direct antioxidant activity were not reflected in the cellular assays, where both cannabinoids inhibited membrane peroxidation by 53-56% at a concentration of 30 μ M (2 h of pre-treatment) (**Figure 7c**). Interestingly, while CBN (2) retained its ability to prevent

lipid peroxidation (44%), THCLV (**5**) largely lost its inhibitory effect on lipid peroxidation upon 24 h pre-incubation prior to RSL3 stimulation (**Figure 7c, d**).

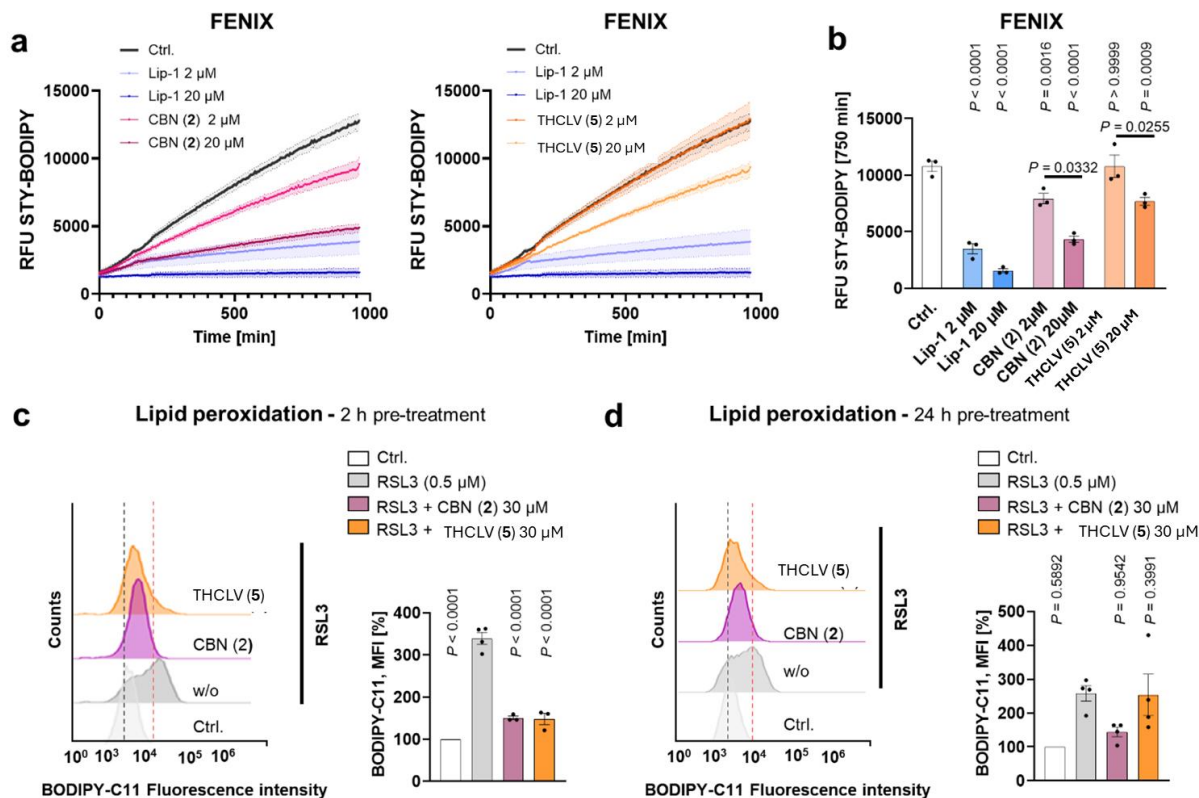


Figure 7. CBN (2) and THCLV (5) suppress phospholipid peroxidation in artificial membranes and attenuate RSL3-induced lipid peroxidation in HepaRG cells: (a, b) Inhibition of lipid peroxidation in liposomes treated with vehicle (DMSO, 1%, 'Ctrl.'), CBN (**2**) or THCLV (**5**) (at 2 and 20 μM, each), determined by the FENIX assay. Lip-1 (2 and 20 μM) was used as positive control. (a) Time-course analysis of phosphatidylcholine peroxidation in liposomes treated with **2** or **5** (2 μM and 20 μM) or lipoxstatin-1 (Lip-1; 2 μM and 20 μM). RFU, relative fluorescence intensity. (b) Bar graph showing the peroxidation levels at 750 min, based on the data presented in (a). Means ± SEM with a shaded error band (line graphs) or means and individual values ± SEM (bar charts) of $n = 3$ independent experiments. P values vs. vehicle control, repeated measures one-way ANOVA with Dunnett's *post-hoc* tests or two-tailed paired Student's t test for pairwise comparisons as indicated by bars. (c, d) Lipid peroxidation in HepaRG cells (2×10^5 cells/well) was evaluated by BODIPY-C11 staining and flow cytometric analysis. Cells were pre-treated with vehicle (DMSO, 0.25%) or CBN (**2**) or THCLV (**5**) for 2 h (c) or 24 h (d), followed by exposure to vehicle (DMSO, 0.25%) or RSL3 (0.5 μM) for 2 h. Left panels: representative histograms. Right panels: Quantitative analysis of mean intensities of BODIPY-C11-stained cells. Means and individual values ± SEM of $n = 3$ (c) or $n = 4$

(d) independent experiments. *P* values vs. RSL3, repeated measures one-way ANOVA with Dunnett's *post-hoc* tests.

Conclusion

The phytochemical analysis of a Δ^9 -THCV rich chemotype of *C. sativa*, stored for 10 years in controlled conditions, led to the quantification and the isolation of CBNV (**3**) and CBNVA (**4**), along with residual trace of Δ^9 -THC (**1**) and its aromatic derivative CBN (**2**). A difference in the degradation rates between Δ^9 -THCA and Δ^9 -THCVA can be speculatively attributed to the different length of their aliphatic side chain. Another major compound of the extract is the new tetrahydrocannabitrioxovarin derivative, THCLV (**5**), which features a phytocannabinoid scaffold fused to a rigid oxabicyclic framework including an oxepane ring and in summary, the planar structure of **5**, named tetrahydrocannabilactolvarin (THCLV) features a cannabinoid scaffold fused to a rigid oxabicyclic framework including an oxepane ring alongside a ketal and lactol functionalities. Notably, the corresponding pentyl analogue of THCLV has never been previously described or detected in cannabis samples. Capitalizing on recent studies on the anti-ferroptotic activity of phytocannabinoids, we compared the anti-ferroptotic potential of THCLV (**5**), the methyl ester-stabilized cannabinolic acids (**9**, **12**), and their neutral counterparts (**2**, **3**). The results ascribe ferroptosis inhibition primarily to CBN (**2**) and CBNV (**3**), and to a lesser extent to THCLV (**5**) and CBNVA (**4**). Mechanistically, these phytocannabinoids directly suppress lipid peroxidation, as demonstrated in artificial membranes and hepatocytes using CBN (**2**) and THCLV (**5**) as representative models. However, their cellular efficacy varied significantly over time: while both derivatives provided comparable short-term protection in hepatocytes, CBN (**2**) maintained a prolonged anti-peroxidative effect, whereas THCLV (**5**) exhibited a time-dependent loss of activity, highlighting the critical role of structural details on the cannabinoid skeleton on intracellular stability. In particular, while compounds **2** and **3** share the resorcinol ring, the less active derivative **5** lacks the additional benzenoid ring resulting from aromatization of the *p*-menthane-derived moiety. The higher activity displayed by CBN (**2**) and CBNV (**3**) compared with the rearranged analogue THCLV (**5**) points to the importance of the fully aromatic dibenzopyran framework.

Methods

General material and methods

Silica gel 60 (70-230 mesh), silica flash (50 μ m), reversed-phase (RP) C₁₈ silica gel (25 μ m) and Celite® 545 (particle size 0.02-0.1 mm), used for low-pressure chromatography (LPC), flash chromatography and vacuum chromatography, was purchased from Macherey-Nagel (Düren, Germany). Flash chromatography Isolera One with DAD (Uppsala, Sweden) and HPLC JASCO with UV-vis detector-2075 plus (Oklahoma, Japan) were used for further purification and analysis.

Purifications were monitored by TLC on 60 F254 (0.25 mm) plates purchased from Merck (Darmstadt, Germany), visualised by staining with 5% H₂SO₄ in EtOH and heating.

Chemical reagents and solvents were from Aldrich (Milan, Italy) and were used without any further purification, unless stated otherwise. Ammonium formate was provided from Fluka (Charlotte, NC, USA). Water (H₂O) was purified using a Milli-Q® Advantage 10 system from Millipore (Milan, Italy). The standard solution of cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), cannabinol (CBN), Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and tetrahydrocannabivarin (Δ^9 -THCV) were purchased from LGC standards (Milan, Italy). A cannabinoic acids mixture, composed of cannabichromenic acid (CBCA), cannabidiolic acid (CBDA), cannabidivarinic acid (CBDVA), cannabigerolic acid (CBGA), cannabinolic acid (CBNA), Δ^9 -tetrahydrocannabinolic acid (Δ^9 -THCA) and tetrahydrocannabivarinic acid (Δ^9 -THCVA) were purchased from Merck Life Science s.r.l. (Milan, Italy). CBN-type ($\geq 97\%$) compounds, namely cannabinovarinic acid (CBNVA), cannabinovarin (CBNV) and tetrahydrocannabiolvarin (THCLV), were isolated in this work. ¹H and ¹³C NMR spectra were recorded with Bruker 400 spectrometer (Bruker®, Billerica, MA, USA). Chemical shifts were referenced to the residual solvent signal (CDCl₃: δ_H = 7.25, δ_C = 77.0). UV and ECD spectra were registered on a JASCO J-710 instrument. High-resolution ESIMS data were obtained on an LTQ OrbitrapXL (Thermo Scientific, Waltham, MA, USA) mass spectrometer.

Plant material

Cannabis sativa L. aerial parts (inflorescences and leaves), belonging to a Δ^9 -THCV chemotype, were provided by Doc. Gianpaolo Grassi (CREA – Research Centre for Cereal and Industrial Crops of Rovigo, Italy). The vegetable material was air-dried at room temperature in 2014 and stored in a heat-sealed tetra Pak bag at controlled temperature (25 °C) and monitored relative humidity for 10 years.

Sample preparation for UHPLC and HPLC analysis

According to a previous method [13, 20], a portion of 50 mg of plant material was weighed and submitted to a dynamic maceration under magnetic stirring with 4 mL of ethanol (EtOH) for 15 min. The extract was filtered with a paper filter. The extraction was repeated twice with 4 mL and 1 mL of the extraction solvent, respectively. The extracts were pooled and adjusted to a final volume of 5 mL with EtOH. The solution was filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) filter into an HPLC vial. The sample preparation was performed in duplicate for all the samples considered in this study. The samples were stored at 4 °C until analysis was performed

UHPLC-HRMS analysis

The qualitative analysis of cannabinoids was performed on a Thermo Scientific (Massachusetts, United States) UHPLC Ultimate 3000 equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electro-spray ionization (HESI) source (Thermo Scientific, Massachusetts, United States). An Ascentis® Express C18 column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used for the separation of the target compounds. According to a method previously described [9] with slight modifications, the chromatographic conditions for the qualitative analysis of cannabinoids consisted of a binary gradient elution by using 0.1% HCOOH in H₂O (solvent A) and 0.1% HCOOH in ACN (solvent B) as the mobile phase. The gradient elution program was set up as follows: the initial conditions were 50% B then raised to 67% B in 2 min, held at 67% B for 4 min and then raised to 90% B until 10 min, kept at 90% B until 14 min, decreased to 50% B over the next min, and held at 50% B until 20 min for re-equilibration of the system. A flow rate of 0.3 mL/min was used. The injection volume was 3 µL. The column temperature was set at 30 °C. As for MS acquisition, the HESI source was operated both in the positive and the negative ion mode. The MS source parameters were set as follows: sheath gas flow rate (N₂) 37, auxiliary gas flow rate (N₂) 28, auxiliary gas temperature: 290 °C capillary temperature: 320 °C, electrospray voltage: 3.4 kV (+) and 2.9 kV (–). MS was operated in the full MS mode, followed by data-dependent MS/MS mode. Data acquisition in full MS mode was performed at 70,000 resolution, and the AGC target was set to 1×10^6 with a maximum ion injection time (IT) of 243 ms. The scan range was 100–1000 *m/z* for all acquisition events. Data acquisition in data-dependent MS/MS (dd-MS/MS) mode was performed at 17,500 resolution with an isolation window of 3 *m/z*. To study the response of the major product ions of cannabinoids with energy, the samples were analysed in the range of 20, 30, 50 NCE levels.

HPLC-UV/Vis analysis

The HPLC-UV/Vis analysis for the quantification of cannabinoids was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II Vialsampler system, consisting of a quaternary pump, an autosampler injection and a UV variable wavelength detector. Compounds were separated on an Ascentis® Express C₁₈ column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of a 2 mM ammonium formate solution and 0.1% HCOOH in H₂O (solvent A) and 0.1% HCOOH in ACN (solvent B). According to a previous work [11], the gradient was set as follows: 0–20 min from 70% to 90% B, which was held for 5 min with a 10 min post-running time. Flow rate and injection volume were set at 0.2 mL/min and 3 µL, respectively. Chromatograms were recorded at the wavelength of 210 and 220 nm for the detection of neutral cannabinoids and cannabinoid acids, respectively. Standard solutions for acidic cannabinoids and neutral cannabinoids were diluted with EtOH to reach a stock solution of 100 µg/mL. A five-point calibration curve was generated covering the concentration range of 5–100

µg/mL, while THCLV, CBNVA, CBNA Δ^9 -THCVA calibration curves were in the concentration range of 2.5–50 µg/mL. Three injections were performed for each standard solution, and two injections for each sample. The limit of detection (LOD) values obtained for acidic cannabinoids ranged from 0.3 to 1.5 µg/mL, while those for neutral cannabinoids ranged from 0.2 to 0.8 µg/mL. The limit of quantification (LOQ) values was in the range of 1.1–2.6 µg/mL for acidic cannabinoids and 0.6–2.6 µg/mL for neutral cannabinoids.

Extraction and isolation

The plant material (319 g, a reference standard with code Cs/THCV14, is stored in the laboratory of phytochemistry in Novara), were extracted with acetone (plant material/acetone ratio 1:10 w/v, 2 × 12 h) in a 10 L vertical stainless-steel percolator at room temperature, filtered to avoid vegetal material residue and evaporated at reduced pressure (556 mbar, 40 °C) to finally obtain a dark green syrup (19.1 g, 5.6% yield). The raw acetonetic extract was dissolved at 40 °C in 60 mL of methanol (MeOH) (raw extract/MeOH ratio 1:3 w/v) and stored at –8 °C to foster the condensation of fatty acids and waxes. After 12 h, the liquid fraction was vacuum filtered in a sintered funnel (9 × 15 cm) with a side arm for vacuum on a layer of stratified Celite® protected by a paper filter to obtain the methanolic fraction (14.7 g). Depigmentation occurred on this latter portion and, for this purpose, the fraction was vacuum filtered on 45 g of RP C₁₈ (raw extract/stationary phase ratio of 1:3 w/w) in a sintered funnel (8 × 11 cm) with a side arm for vacuum to obtain the depigmented fraction (12.9 g) after evaporation at reduced pressure (337 mbar, 40 °C).

This latter fraction was purified with LPC on silica gel (390 g, petroleum ether (PE)–ethyl acetate (EtOAc) gradient from 100:0 to 20:80) to afford five fractions (I–V). Fraction II (630 mg) was further purified on silica gel (30 g, PE–EtOAc gradient from 100:0 to 80:20) to afford 10 mg of Δ^9 -THC (**1**) [21] and 443.5 mg of CBN (**2**) [21]. Fraction III (6.5 g) was fractionated with LPC on silica gel (180 mL, PE–EtOAc gradient from 95:5 to 70:30) to obtain 2.3 g of **2** and 2.4 g of CBNV (**3**) [22]. Fraction IV (3.5 g) is purified by flash chromatography on RP C₁₈ (30 g, H₂O 0.3% formic acid/MeOH 0.3% formic acid, gradient from 50:50 to 0:100) to afford four subfractions each of them have been further purified with HPLC on silica gel leading to obtain respectively 290.3 mg of CBNVA (**4**), 135.3 mg of THCLV (**5**). All the isolated natural compounds were identified according to ¹H NMR previously described in the literature except for CBNA and THCLV. For NMR spectra see **Supplementary Figures 1-9**.

CBNVA (4): white powder; ¹H NMR (CDCl₃, 400 MHz): δ = 8.49 (1H, dd, J = 12.3, 12.3 Hz, H-10), 7.17-7.12 (2H, dd, J = 8.5, 17.9 Hz, H-7, H-8), 6.47 (1H, s, H-4), 2.99 (2H, t, J = 4.7 Hz, H-1'), 2.43 (3H, s, H-11), 1.75-1.67 (2H, tt, J = 14.2, 13.7 Hz, H-2'), 1.67 (6H, s, H-12, H-13), 1.05 (3H, t, J = 7.1 Hz, H-3'). ¹³C-NMR (100 MHz; CDCl₃): δ = 176.5 (COOH), 163.6 (C-1, C), 159.5 (C-5, C), 148.8 (C-3, C), 137.1 (C-9, C), 135.8 (C-6a, C), 128.1 (C-8, CH), 127.2 (C-7, CH), 126.6 (C-10b, C), 122.3 (C-10, CH), 113.2 (C-4, CH), 109.1 (C-6a, C), 104.0 (C-2, C), 78.6 (C-6, C), 38.9 (C-1', CH₂), 27.5 (C-

12, C-13, CH₃), 24.6 (C-2',CH₂), 21.6 (C-11, CH₃), 14.3 (C-3', CH₃); HR-ESIMS (*m/z*): [M + H]⁺ calcd. for C₂₀H₂₃O₄, 327.1596; found, 327.1561.

THCLV (5): white powder; [α]_D - 95 (c 0.1, CH₂Cl₂); ¹H NMR (CDCl₃, 400 MHz) and ¹³C-NMR (CDCl₃, 200 MHz) (**Table 1**); HR-ESIMS (*m/z*): [M + H]⁺ calcd. for C₁₉H₂₇O₅, 335.1847; found, 335.1845.

Computational study

A preliminary conformational search for the compound **5** models was conducted using simulated annealing in the INSIGHT II package. The solution phase in CHCl₃ was simulated by setting the corresponding dielectric constant. Conformational energy minimization was achieved through steepest descent, followed by the quasi-Newton-Raphson method (VA09A). Restrained simulations were run for 500 ps using the CVFF force field, as implemented in Discover software (Accelrys, San Diego, CA). The simulation began at 1000 K, and the temperature gradually decreased to 300 K. A final energy minimization step was performed to refine the obtained structures, using successive applications of steepest descent and quasi-Newton-Raphson (VA09A) algorithms. Both dynamic and mechanical calculations were conducted with flat-well distance restraints set to 1 (kcal/mol)/Å². A total of one hundred structures were generated.

DFT calculations were performed on an Intel(R) Core (TM) i5-4440 processor at 3.0 GHz using the Gaussian16W package (Multiprocessor). GIAO 13C calculations were performed using the mPW1PW91 functional and 6-31G (d, p) basis set using the geometry previously optimized at the mPW1PW91/6-31G (d, p) level as input. DP4+ probabilities were computed using the available DP4+ Toolbox (Excel file). For these calculations, the IEF-PCM solvent continuum model, as implemented in Gaussian (MeOH solvent). The rotatory strength values for electronic transitions from the ground state to singly excited states for the two conformers of 5-a were calculated using TDDFT at the CAM-B3LYP/def2TZVP level with Gaussian 16W, including at least 50 excited states in each case and using the IEF-PCM model for MeOH. The rotatory strength values were summed after a Boltzmann statistical weighting and Δε values were calculated by forming sums of Gaussian functions centered at the wavelengths of the respective electronic transitions and multiplied by the corresponding rotatory strengths. The resulting ECD spectra were then compared to the experimental spectra using SpecDis 1.71 software.

Synthesis and semi-synthesis of cannabinoids

CBNVA-methyl ester (9). CBNVA (**4**, 50 mg, 0.22 mmol) was dissolved in MeOH (10 mL), *N,N'*-dicyclohexylcarbodiimide (DCC, 63 mg, 2 equiv/mmol) and *p*-toluene sulfonic acid (*p*-TSA, cat.) were added. The reaction was stirred at room temperature and followed by TLC (silica, PE-EtOAc 80:20), monitoring the disappearance of **4**. After 1 h the MeOH was evaporated at reduced pressure (337 mbar, 40 °C), dissolved in toluene (10 mL), and placed at 0 °C for 4 h to remove by filtration on Celite® the *N,N'*-dicyclohexylurea (DCU). After solvent evaporation at reduced pressure (77 mbar,

40 °C), the final residue was purified by HPLC on silica (PE-ethyl acetate isocratic 95:5) to afford CBNVA-methyl ester (**9**) as white powder (43.2 mg, 0.18 mmol, 81.8% based on the conversion of **4**). The structure of the compound was elucidated with NMR in **Supplementary Figure 10 and 11**.

CBNVA-methyl ester (**9**): white powder; ¹H-NMR (400 MHz; CDCl₃): δ 12.83 (1H, s, OH-1), 8.47 (1H, s, H-10), 7.13 (2H, dd, *J* = 8.0 Hz, *J* = 1.1 Hz, H-7, H-8), 6.41 (1H, s, H-4), 3.99 (3H, s, OCH₃), 2.88 (2H, t, *J* = 7.7 Hz, H-1'), 2.43 (3H, s, H-11), 1.64 (6H, s, H-12, H-13), 1.60-1.58 (2H, m, H-2'), 1.00 (3H, t, *J* = 7.3 Hz, H-3'). ¹³C-NMR (100 MHz; CDCl₃): δ 172.8 (COOH), 162.4 (C-1, C), 158.3 (C-5, C), 147.1 (C-3, C), 137.0 (C-9, C), 135.9 (C-6a, C), 127.9 (C-8, CH), 127.1 (C-7, CH), 126.9 (C-10b, C), 122.3 (C-10, CH), 112.8 (C-4, CH), 109.0 (C-10a, C), 105.5 (C-2, C), 78.3 (C-6, C), 52.0 (OCH₃), 39.0 (C-1',CH₂), 27.4 (C-12, C-13, CH₃), 24.7 (C-2',CH₂), 21.6 (C-11, CH₃), 14.3 (C-3', CH₃); HR-ESIMS (*m/z*): [M + H]⁺ calcd. for C₂₁H₂₅O₄, 314.1753; found, 341.1747.

CBDA-methyl ester (11). CBDA-methyl ester (**11**) was obtained following the procedure described by Tamburello et al. (2023) [17] starting from CBDA (**10**) purified at that time. CBDA (**10**, 300 mg, 0.836 mmol) was dissolved in MeOH (15 mL), DCC (345 mg, 2 equiv/mmol) and a catalytic quantity of *p*-TSA were added. The reaction was stirred at room temperature and followed by TLC (silica, PE-EtOAc 80:20), monitoring the disappearance of **10**. After 1 h the MeOH was evaporated at reduced pressure (337 mbar, 40 °C), dissolved in toluene (15 mL), and placed at 0 °C for 4 h to remove by filtration on Celite® the DCU. After solvent evaporation at reduced pressure (77 mbar, 40 °C), the final residue was purified by HPLC on silica (PE-ethyl acetate isocratic 95:5) to afford CBDA-methyl ester (**12**) as pale oil (256 mg, 0.687 mmol, 82.2% based on the conversion of **10**). The structure of the compound was elucidated with ¹H NMR in **Supplementary Figure 14** and was identified according to data described in scientific literature [17].

CBNA-methyl ester (12). CBNA-methyl ester (**12**) was semi-synthesized following the procedure reported by Pollastro et al. (2017) [18]. CBDA-methyl ester (**11**, 256 mg, 0.687 mmol) was dissolved in toluene (15 mL) and iodine (I₂, 697 mg, 4 equiv/mmol) was added. The reaction was stirred at room temperature for 1 h and followed by TLC (silica, PE-EtOAc 95:5), monitoring the disappearance of **11**. After 1 h, the reaction was quenched with Na₂SO₄ 5% aqueous solution, anhydried with Na₂SO₄ and the solvent evaporated at reduced pressure (77 mbar, 40 °C). The residue was by HPLC on silica (PE-EtOAc gradient from 95:5 to 80:20) to afford CBNA-methyl ester (**12**) as white powder (64.7 mg, 0.241 mmol, 34.9% based on the conversion of **12**). The structure of the compound was elucidated with NMR in **Supplementary Figure 12 and 13**.

CBNA-methyl ester (**12**): white powder; ¹H NMR (400 MHz, CDCl₃): δ 12.83 (1H, s, OH-1), 8.46 (1H, dd, *J* = 12.3, 12.3 Hz, H-10), 7.13 (2H, dd, *J* = 8.5, 17.9 Hz, H-7, H-8), 6.43 (1H, s, H-4), 3.99 (3H, s, H-14), 2.87 (2H, t, *J* = 4.7 Hz, H-1'), 2.42 (3H, s, H-11), 1.64 (6H, s, H-12, H-13), 1.58 (2H, tt, *J* = 14.2, 13.7 Hz, H-2'), 1.40-1.36 (4H, m, H-3', H-4'), 0.94 (3H, t, *J* = 7.1 Hz, H-5'). ¹³C-NMR (100 MHz; CDCl₃): δ 172.8 (COOH), 162.4 (C-1, C), 158.3 (C-5, C), 147.4 (C-3, C), 137.0 (C-9, C), 135.9 (C-

6a, C), 127.9 (C-8, CH), 127.1 (C-7, CH), 126.9 (C-10b, C), 122.2 (C-10, CH), 112.6 (C-4, CH), 109.0 (C-10a, C), 105.5 (C-2, C), 78.3 (C-6, C), 51.9 (OCH₃), 37.0 (C-1',CH₂), 32.0 (C-4',CH₂), 31.2 (C-3',CH₂), 27.3 (C-12, C-13, CH₃-CH₃), 22.5 (C-2',CH₂), 21.5 (C-11, CH₃), 14.0 (C-5', CH₃); HR-ESIMS (*m/z*): [M + H]⁺ calcd. for C₂₃H₂₉O₄, 369.2066; found, 369.2061.

Cell culture

Human HepaRG liver cells were purchased from Biopredic International (Rennes, France, #HPR101) and routinely cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were passaged every 3–5 days at 60–80% confluence using trypsin-EDTA (Merck, #59418C), and experiments were conducted with cells between passages 18 and 40. Cultures were maintained in William's E medium (Sigma-Aldrich, W4128) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, #0001662318), 2 mM L-glutamine (Sigma-Aldrich, R8758), 5 µg/mL human insulin (Sigma-Aldrich, I9278), and 37 µM hydrocortisone 21-hemisuccinate (Cayman, H2270).

Cell viability assay

HepaRG cells were plated in 96-well plates at 10,000 cells per well and allowed to attach for 24 h at 37 °C under 5% CO₂. Treatments included vehicle (0.5% DMSO), or the compounds combined with the ferroptosis inducer RSL3 (0.8 µM, **Figure 6a**; 0.4 µM, **Figure 6b**; Cayman, Cay19288) and were applied for 48 h. Cell viability was measured based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by oxidoreductases to form insoluble formazan. Following treatment, 20 µL of 5 mg/mL MTT in phosphate-buffered saline (PBS, pH 7.4, sterile) was added to each well, and plates were incubated for 3 h at 37 °C and 5% CO₂. Formazan crystals were then solubilized by adding 100 µL of 10% SDS in 20 mM HCl (pH 4.5) and incubating for 16 h in the dark on an orbital shaker (60–80 rpm). Absorbance was recorded at 570 nm using a Hidex Sense microplate reader (Hidex, Turku, Finland). Values were normalized to the vehicle-treated wells (set to 100%) after correcting for background from ethanol controls.

BODIPY 581/591 C11 staining for cellular lipid peroxidation

HepaRG cells were plated in 12-well plates at 2 × 10⁵ cells per well and allowed to adhere for 24 h at 37 °C in a 5% CO₂ atmosphere. Cells were then treated with vehicle control (DMSO, 0.25%) or compounds (30 µM) for 2 h under the same conditions. Ferroptosis was induced by adding 0.5 µM RSL3, and after 2 h, 2 µM C11-BODIPY (Cayman, #27086) was added for 30 min at 37 °C in the dark at 37 °C and 5% CO₂. Following staining, cells were washed with PBS (pH 7.4), detached using 0.25% trypsin-EDTA, neutralized with growth medium, and collected along with the wash fractions. Samples were centrifuged at 1,200 × g for 5 min at 4 °C, washed twice with HBSS, and resuspended in 500 µL HBSS. Flow cytometry was performed on a CytoFLEX instrument (Beckman, Austria; λ_{ex}/λ_{em} = 488/550 nm), acquiring 10,000 events per sample within a gate defined for HepaRG cells

[23]. Data were analyzed using FlowJo v10.10.0 with a consistent gating strategy that excluded debris.

Radical scavenging assay

The radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Briefly, 100 μ L of the compound at different concentrations (0.3, 1, 3, 10, 30, and 50 μ M in EtOH) was mixed with 100 μ L of 0.1 mM DPPH solution in ethanol [24]. The mixtures were incubated in the dark for 30 min on an orbital shaker (130 rpm, POS 300, Grantbio, The Netherlands). Absorbance was measured at 520 nm using a Hidex Sense microplate reader. The percentage of DPPH radical scavenging was calculated using the formula: % DPPH scavenging = $(1 - A_{\text{sample}})/A_0 \times 100$. A_{sample} : absorbance of the sample, A_0 : absorbance of the control (DPPH solution). Vitamin C at equivalent concentrations (1–50 μ M) was included as a positive control.

Iron-binding assay

The iron-chelating ability of the test compounds was evaluated by mixing 5 μ L of with 5 μ L of 0.5 mM ferrous sulfate heptahydrate ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$) in 40 μ L of HEPES buffer (50 mM, pH 3–6) at compound-to- Fe^{2+} molar ratios of 1:1, 3:1, or 6:1 [24]. The mixtures were incubated for 2 min, after which 50 μ L of 5 mM ferrene (in 5 mM HEPES) was added, followed by an additional 2 min of incubation in the dark. Formation of the Fe^{2+} –ferrene complex was quantified by measuring absorbance at 595 nm using a Hidex Sense microplate reader. Deferoxamine (DFO) was included as a positive control. Chelation efficiency was calculated and expressed as the percentage of ferrous iron bound by the test compound.

FENIX assay

Artificial membranes were prepared from egg phosphatidylcholine, as described previously [25]. In brief, phosphatidylcholine was dissolved in a minimal volume of chloroform (CHCl_3) to ensure complete solubilization, and the solvent was evaporated under a gentle argon stream to form a uniform lipid film on the inner wall of a glass vial. Residual solvent was removed by leaving the vial under argon for an additional 30 min. The dried lipid film was then rehydrated with phosphate-buffered saline (PBS pH 7.4) to a final concentration of 20 mM phosphatidylcholine. Liposomes were generated by subjecting the suspension to ten freeze–thaw cycles (4 min on ice, 4 min at room temperature), with 4 min of sonication after each thaw (Sonorex Super Rx 2554, Germany). The suspension was then extruded 20–25 times through a 100 nm polycarbonate membrane using a mini-extruder (Avanti Research™ – A Croda Brand). Only liposome preparations with a mean diameter of ~100 nm and a polydispersity index of 0.2–0.3, as determined by dynamic light scattering (Zetasizer Advance Ultra, Malvern Panalytical, The Netherlands), were used for subsequent experiments. Liposomes were stored at 4 °C and used within two weeks. Phospholipid peroxidation

was measured using a modified fluorescence-enabled inhibited autoxidation (FENIX) assay [26]. Egg phosphatidylcholine liposomes (1 mM in PBS pH 7.4) were combined with 1 μ M STY-BODIPY (Cayman, #Cay27089) in black 96-well polypropylene plates (Greiner, #655087) and incubated with either liproloxstatin-1 (Lip-1, 2 and 10 μ M) or the compounds (2 and 20 μ M) for 10 min at 37 °C, followed by 5 min of vigorous mixing. Autoxidation was initiated by adding 2 μ M DTUN (Cayman, #Cay32742). Control wells contained 1% DMSO, 1 μ M STY-BODIPY, and DTUN. After a brief 5-min mixing step and 10-min equilibration at 37 °C, fluorescence ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 485/535$ nm) was recorded over 960 minutes in bottom-read mode using a Hidex Sense microplate reader.

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Authors contributions

S. S.: Investigation, Formal analysis, Data curation. **G. C.:** Investigation, Formal analysis, Data curation. **L. B.** Investigation, Formal analysis. **A. C.:** Investigation, Formal analysis. **M. M.:** Formal analysis. **D. N.:** Formal analysis. **Paolo Luciano:** Formal analysis. **O. T. S.:** Supervision, Data curation. **A. K.:** Formal analysis, Data curation, Supervision. **S. C. K.:** Writing - original draft, Methodology, Investigation, Data curation, Conceptualization. **F. P.:** Writing - original draft, Methodology, Investigation, Data curation, Conceptualization, Supervision. **F. P.:** Writing - original draft, Methodology, Investigation, Data curation, Conceptualization, Supervision.

Competing interest

All authors declare no competing interesting.

Data availability

The NMR data of the isolated and semi synthetic cannabinoids, table of conformational analysis and quantitative analysis of acidic and neutral cannabinoids by HPLC-UV/Vis are provided in the Supplementary Information.

References

- 1 Mazzara, E. *et al.* A comprehensive phytochemical analysis of terpenes, polyphenols and cannabinoids, and micromorphological characterization of 9 commercial varieties of *Cannabis sativa* L. *Plants* **11**(7), 891 (2022).
- 2 Ingvarlsen, C. R., & Brinch-Pedersen, H. Challenges and potentials of new breeding techniques in *Cannabis sativa*. *Frontiers in Plant Science*, **14**, 1154332 (2023).
- 3 Fućak, T., Kreft, S., Svedružić, Ž. M., & Tavčar, E. Mechanism and kinetics of CBDA decarboxylation into CBD in hemp. *Journal of Plant Biochemistry and Biotechnology*, **32**(3), 608-621 (2023).
- 4 Moreno, T., Dyer, P., & Tallon, S. Cannabinoid decarboxylation: a comparative kinetic study. *Industrial & Engineering Chemistry Research*, **59**(46), 20307-20315 (2020).
- 5 Filer, C. N. Acidic cannabinoid decarboxylation. *Cannabis and Cannabinoid Research*, **7**(3), 262-273 (2022).
- 6 Maioli, C., Mattoteia, D., Amin, H. I. M., Minassi, A., & Caprioglio, D. Cannabinol: history, syntheses, and biological profile of the greatest “minor” cannabinoid. *Plants*, **11**(21), 2896 (2022).
- 7 Khouchlaa, A. *et al.* Health benefits, pharmacological properties, and metabolism of cannabinol: A comprehensive review. *Industrial Crops and Products*, **213**, 118359 (2024).
- 8 Liang, Z. *et al.* Fragment-based drug discovery and biological evaluation of novel cannabinol-based inhibitors of oxytosis/ferroptosis for neurological disorders. *Redox biology*, **72**, 103138 (2024).
- 9 Zheng, J., & Conrad, M. Ferroptosis: when metabolism meets cell death. *Physiological Reviews*, **105**(2), 651-706 (2025).
- 10 Berman, P. *et al.* A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in *Cannabis*. *Scientific Reports*, **8**, 14280 (2018).
- 11 Brighenti, V. *et al.* A new HPLC method with multiple detection systems for impurity analysis and discrimination of natural versus synthetic cannabidiol. *Analytical and Bioanalytical Chemistry*, **416**, 4555-4569 (2024).

- 12 Ferri, E. *et al.* Single trichome phytocannabinomics of two different cannabis varieties. *Journal of Pharmaceutical and Biomedical Analysis* **261**, 116836 (2025).
- 13 Brighenti, V., Pellati, F., Steinbach, M., Maran, D., & Benvenuti, S. Development of a new extraction technique and HPLC method for the analysis of non-psychoactive cannabinoids in fibre-type *Cannabis sativa* L. (hemp). *Journal of Pharmaceutical and Biomedical Analysis*, **143**, 228-236 (2017).
- 14 Taschwer, M., & Schmid, M. G. Determination of the relative percentage distribution of THCA and Δ^9 -THC in herbal cannabis seized in Austria—Impact of different storage temperatures on stability. *Forensic Science International*, **254**, 167-171 (2015).
- 15 Harvey, D. J. Stability of cannabinoids in dried samples of cannabis dating from around 1896–1905. *Journal of Ethnopharmacology*, **28**(1), 117-128 (1990).
- 16 Turner C. E.; Elsohly M.A. Constituents of cannabis sativa L. XVI. A possible decomposition pathway of Δ^9 -tetrahydrocannabinol to cannabinol. *Journal of Heterocyclic Chemistry*, **16**, 1667-1668 (1979).
- 17 Tamburello, M. *et al.* Antiviral activity of cannabidiolic acid and its methyl ester against SARS-CoV-2. *Journal of Natural Products*, **86**(7), 1698-1707 (2023).
- 18 Pollastro, F. *et al.* Iodine-promoted aromatization of p-menthane-type phytocannabinoids. *Journal of Natural Products*, **81**(3), 630-633 (2017).
- 19 Mallais, M., Hanson, C. S., Giray, M., & Pratt, D. A. General approach to identify, assess, and characterize inhibitors of lipid peroxidation and associated cell death. *ACS Chemical Biology*, **18**(3), 561-571 (2023).
- 20 Pellati, F. *et al.* New methods for the comprehensive analysis of bioactive compounds in *Cannabis sativa* L. (hemp). *Molecules*, **23**, 2639 (2018).
- 21 Choi, Y. H. *et al.* NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of *Cannabis sativa*. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, **15**(6), 345-354 (2004).
- 22 Merkus, F. W. Cannabivarin and tetrahydrocannabivarin, two new constituents of hashish. *Nature*, **232**(5312), 579-580 (1971).
- 23 Walzl, L. *et al.* Context-dependent Activities of Mitrephorone Link Lipid Redirection, Anti-inflammatory Action, and Ferroptosis Control to Hepatocyte Protection. *Theranostics*, **16**(10), 5406 (2026).

24 Plangger, I. *et al.* Development of a Synthetic Platform for Ent-Pimaranes Reveals their Potential as Novel Non-Redox Active Ferroptosis Inhibitors. *Chemistry–A European Journal*, **31**(7), e202403811 (2025).

25 Su, F., Descher *et al.* Iron (III)-salophene catalyzes redox cycles that induce phospholipid peroxidation and deplete cancer cells of ferroptosis-protecting cofactors. *Redox Biology*, **75**, 103257 (2024).

26 Shah, R., Farmer, L. A., Zilka, O., Van Kessel, A. T. M., & Pratt, D. A. Beyond DPPH: use of fluorescence-enabled inhibited autoxidation to predict oxidative cell death rescue. *Cell Chemical Biology*, **26**, 1594-1607 (2019).

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