The semisynthetic cannabinoid Hexahydrocannabinol (HHC)

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Abstract

Cannabis is a double-faced plant with uncountable therapeutic properties, on one side, and controversial psychotropic activities, on the other side, modulated by CB1 endocannabinoid receptors. \( \Delta^9 \)-Tetrahydrocannabinol (\( \Delta^9 \)-THC) has been identified as the main component responsible for the psychotropic effects, while its isomer cannabidiol (CBD) has shown completely different pharmacological properties. Based on the remarkable beneficial effects, Cannabis has spread worldwide and it is openly sold in shops and online. In order to overcome restrictions due to legal issues, nowadays semi-synthetic derivatives of CBD are often added to cannabis products, obtaining “high” effects similar to those given by \( \Delta^9 \)-THC. The first semi-synthetic cannabinoid appeared in the EU was obtained by cyclization and hydrogenation of CBD, and known as hexahydrocannabinol (HHC). At present, very little is known about HHC, its pharmacological properties, and diffusion, since it is not commonly investigated in routine toxicological assays. In the present work, the synthetic strategies aimed to obtain an excess of the active epimer of HHC were investigated and the two epimers were purified and individually tested for their cannabinomimetic activity. Lastly, a simple and fast chromatographic method with a UV detector and a high-resolution mass spectrometer was applied to identify and quantify up to ten major phytocannabinoids, as well as the HHC epimers in commercial cannabis samples.

Introduction

Cannabis is the most widely spread drug of abuse worldwide with an estimated 4% of the population aged 15–64 using it in 2019 as reported in the World Drug Report 2021\(^1\). \( \Delta^9 \)-Tetrahydrocannabinol (\( \Delta^9 \)-THC), identified in the early 1960s by Raphael Mechoulam, is the major phytocannabinoid responsible for the psychotropic activity of cannabis. The \( \Delta^9 \)-THC isomer cannabidiol (CBD) is the second most abundant phytocannabinoid identified in Cannabis and is void of any psychotropic activity. Notably, CBD is largely present in cannabis cultivated for industrial purposes (herein called hemp varieties). Other so-called “minor” phytocannabinoids have been isolated and characterized and some of them have proved a \( \Delta^9 \)-THC-like euphoriant activity, for instance the thermodynamically stable regioisomer \( \Delta^8 \)-THC and the oxidation product cannabinol (CBN). More recently, our research group identified for the first time three \( \Delta^9 \)-THC homologs, namely \( \Delta^9 \)-tetrahydrocannabiphorol (\( \Delta^9 \)-THCP)\(^2\), \( \Delta^9 \)-tetrahydrocannabihexol (\( \Delta^9 \)-THCH)\(^3\) and \( \Delta^9 \)-tetrahydrocannabutol (\( \Delta^9 \)-THCB)\(^4\), with the former having proved a cannabinomimetic activity even higher than \( \Delta^9 \)-THC in animal experiments.

After the identification of \( \Delta^9 \)-THC as the main psychotropic component of cannabis, the chemical and pharmaceutical research moved to the modification of the lead compound to obtain more potent analogues\(^5\). Moreover, the identification of the endocannabinoid receptors CB1 and CB2 has led to the discovery of new scaffolds, thus allowing the development of increasingly potent synthetic cannabinoids. However, having scarce therapeutic usefulness, such compounds are synthesised in illegal laboratories and sold for recreational use.
Over 250 synthetic compounds have been recently developed in clandestine laboratories to target the endocannabinoid system. They are generally added to products often called “Spice” or “K2” and distributed to the end users. Besides the lack of therapeutic utility, the use of synthetic cannabinoids (SCBs) has been documented as completely unsafe with a series of dangerous side effects. In this context, SCBs are different from THC, which displays a relatively large safety margin when used for recreational purposes, requiring higher doses to cause serious adverse effects.

Low THC-containing cannabis (up to 3%) has been recently legalized in the USA. These varieties can contain high levels of CBD, which has no cannabinomimetic properties and has been recognized as a powerful antiepileptic drug. Nonetheless, when CBD is treated with acids it is cyclized to $\Delta^9$-THC and $\Delta^8$-THC. Both THC isomers are under international restrictions being scheduled under schedule II of the United Nations Convention on Psychotropic Substances. Semi-synthetic derivatives of CBD seem to be the most widely employed strategy in USA to overcome the issue related to the marketing of controlled substances. The first semi-synthetic cannabinoid (SSC) reported in the EU and monitored as a novel psychoactive substance (NPS) by the EU Early Warning System since 21 October 2022 is hexahydrocannabinol, better known as HHC. Although it was described for the first time in 1944 by Adams, HHC has only recently attracted the attention of toxicology researchers and analysts. Few experimental studies demonstrated its THC-like cannabimimetic effects, thus at present it is openly sold as a “legal” alternative to illegal THC, often sprayed onto or mixed with cannabis products and marketed as “legal highs”.

The molecule of HHC exists as two epimers, $9R$ and $9S$ and in vitro studies indicated that the $R$ epimer is the one exerting the cannabimimetic activity, while the $S$ epimer is either void or has less psychotropic activity. At the best of the authors’ knowledge, the only in vivo study for the evaluation of the psychotropic activity of HHC epimers was carried out in 1980 by Mechoulam et al. on rhesus monkeys that were observed for somatic and behavioural changes after administration of the single epimers of HHC. The authors observed that administration of 1 mg/kg of the epimer with the equatorial methyl substituent ($9R$-HHC) induced severe stupor and ataxia, full ptosis, immobility, crouched posture lasting for more than 3 h, and absence of reaction even when external stimuli were applied. The effects decreased at lower doses up to 0.1 mg/kg, at which only a tranquillity state was observed. On the other hand, the epimer with the axial methyl group ($9S$-HHC) induced drowsiness, decreased motor activity, occasional partial ptosis, and occasional head drop only at 2 mg/kg, although the authors stated that the compound was not pure and a 2–3% impurity of the other epimer could interfere with the results. It is clear that the potency of a cannabis product with HHC depends on the abundance of one epimer with respect to the other. Indeed, just a few months ago Casati et al. reported the presence of an excess of the $9R$ epimer in a hemp variety. It is therefore important to investigate the formation of one epimer with respect to the other starting from CBD and analyze the epimeric ratio in HHC cannabis derived products. As the acidic treatment is the key to selectively obtain either one HHC epimer or the other, the present work aims to evaluate the epimeric excess resulting from different synthetic strategies and to determine
the concentrations of both epimers in commercial HHC-containing cannabis products. To this end, the high-performance liquid chromatography technique coupled to a diode array detector and high-resolution mass spectrometry (HPLC-DAD-HRMS) was exploited. Together with HHC epimers, ten other phytocannabinoids commonly analysed in cannabis extracts were determined, including CBD, $\Delta^9$-THC, $\Delta^8$-THC, cannabigerol (CBG), cannabichromene (CBC), the corresponding carboxylated native precursors cannabidiolic acid (CBDA), $\Delta^9$-tetrahydrocannabinolic acid ($\Delta^9$-THCA), cannabigerolic acid (CBGA), and cannabichromenic acid (CBCA), respectively, and the THC oxidation product cannabinol (CBN) (Fig. 1).

Lastly, the tetrad test was performed on mice after administration of the single HHC epimers and the results were compared to those reported for $\Delta^9$-THC.

**Results**

**Synthesis of HHC epimers.** The synthesis of HHC epimers was performed starting from the acidic treatment of CBD. According to the conditions, cyclization and hydrogenation of CBD can pass through the formation of either $\Delta^9$-THC or $\Delta^8$-THC as reaction intermediates. In particular, Gaoni and Mechoulam obtained $\Delta^9$-THC by treating CBD with HCl as a catalyst for a short time (2 h) and $\Delta^8$-THC when treating the same starting material with $p$TSA for a long time (18 h)\(^{18}\). Subsequent reduction of the double bond on the terpene group afforded HHC as a mixture of the two epimers. Hydrogenation of $\Delta^9$-THC provided an excess of the $S$ epimer with respect to the $R$ epimer (about 2:1), while hydrogenation of $\Delta^8$-THC gave a 3:1 epimeric ratio in favour of the $R$ epimer\(^{18}\).

Based on this knowledge, CBD was treated either with HCl or $p$TSA for a short or long time, respectively, and subsequently subject to hydrogenation without purification of the intermediates. HHC was obtained as the product in both reactions, but with different epimeric ratios according to the reaction conditions: specifically, the reaction with HCl for 2 h afforded a 57:43 $S/R$ ratio, whereas the reaction with $p$TSA for 18 h gave a 61:39 $R/S$ ratio (Fig. 2).

The single epimers of HHC were isolated by semi-preparative LC obtaining (9$S$)-HHC and (9$R$)-HHC with a purity grade greater than 95% and 99%, respectively, which were sufficient to perform the biological tests.

Both epimers were characterized by UV, MS and NMR. Whilst the UV spectra were superimposable (Fig. 3a-b), the MS\(^2\) dimension provided similar patterns, distinguishable only by the relative abundance (RA) of one fragment at $m/z$ 193.1223 in HESI+ mode corresponding to the resorcinol group attached to one carbon atom of the reduced terpene moiety and with the oxygen atom no longer included in the pyran ring (Fig. 3c-d). This fragment had a RA of 83% in the mass fragmentation spectrum of the $S$ epimer and a RA of 20% in that of the $R$ epimer. On the other hand, the HESI- mode provided identical fragmentation patterns (Fig. 3e-f). Additionally, the $^1$H NMR showed peculiar differences indicating that the first eluted epimer on reversed phase HPLC is (9$S$)-HHC, and the second eluted epimer is (9$R$)-HHC (Fig. 3g).

Specifically, a peculiar shift in the NMR spectrum was observed for the hydrogen attached to the C10α
(from 2.85 ppm in (S)-HHC to 3.03 ppm in (R)-HHC) and for the hydrogen atom of the C10a (from 2.67 ppm to 2.49–2.37 ppm in the S and R epimers, respectively)\textsuperscript{19}.

**HPLC-UV-HRMS analysis.** A previously reported and validated HPLC-UV-HRMS method\textsuperscript{20} was adapted for the determination of ten phytocannabinoids and the HHC epimers. A C18 stationary phase with the core-shell technology was employed for the separation of all phytocannabinoids and an isocratic elution program at 70% ACN for 20 min allowed to obtain a good baseline resolution of all peaks. Figure 4 shows the UV traces of all cannabinoid standards at the concentration of 10 µg/mL at 228 nm.

The method was applied to analyse all phytocannabinoids and HHC epimers in ethanolic extracts of two hemp biomass samples (HHC-1 and HHC-2), one HHC hashish sample (HHC-3), and in a pure HHC sample (HHC-4) bought on the online market (Figure S5, Supporting Information). Extraction of the analytes was carried out in ethanol, as previous works demonstrated its superior ability to extract this class of compounds\textsuperscript{2–4,21–23}. The chromatograms of the extracts were checked for interfering compounds by HRMS, which confirmed the absence of other cannabinoids at the retention time of the analytes. All extracts were found to contain both epimers with an excess of the \textit{R} form, which was about twice the amount of the \textit{S} form. In the sample claimed to be pure HHC, the two epimers were in a 58:42 mixture. In the three analysed extracts the concentrations for the \textit{S} epimer ranged from 8.40–11.66% (on dry weight), while those of the \textit{R} epimer were found in the range 17.13–26.46%.

All extracts showed a strong presence of CBDA and CBD together with lower amounts of CBGA, which indicated that the inflorescence came from industrial hemp. In particular, CBDA ranged from 2.8–7.18% and CBD ranged from 1.20–2.60%. CBGA was also found in low concentrations from 0.06–0.26%.

Additionally, CBN was always present in all samples and the pure HHC sample in concentrations variable from 0.05–0.21%. All samples showed the presence of both \(\Delta^9\)-THC and \(\Delta^8\)-THC, with a prevalent amount of the former. THCA and CBCA were present in trace.

Isomers of HHC could be putatively identified in all extracts by HRMS, along with other oxidation derivatives of HHC. As no standard was available for these compounds, only the molecular formula could be hypothesized (Figure S5). Interestingly, the sample from pure HHC showed the presence of a unique peak at 12.96 min with precursor ion [M + H]\textsuperscript{+} at m/z 319.2629 and [M-H]\textsuperscript{−} at m/z 317.2488 with higher intensity in positive ionization mode (Figure S6). This compound with predicted molecular formula C\textsubscript{21}H\textsubscript{34}O\textsubscript{2} showed a characteristic fragment at m/z 139.1480, which differs by two hydrogen atoms from that of HHC (137.1324) and four hydrogen atoms from CBD- and THC-like cannabinoids (137.1174). This fragment could be likely attributed to the terpene moiety. Considering the higher lipophilicity given by the shorter retention time compared to HHC and THC isomers and the HRMS fragmentation pattern, a CBD-like structure with all saturated C-C bonds on the terpene moiety could be hypothesized. This could be derived from the hydrogenation of the residual CBD starting material in the reaction mixture.

**In vivo determination of the cannabinoid profile of (9\textit{R})-HHC and (9\textit{S})-HHC.** The cannabimimetic activity of (9\textit{R})-HHC and (9\textit{S})-HHC was evaluated by the tetrad of behavioral tests on mice, which
consisted of the assessment of spontaneous activity, immobility index (catalepsy), analgesia and changes in rectal temperature (Fig. 5a). Cannabinoid activity is physiologically manifested by a decrease in locomotor activity, catalepsy, analgesia and hypotermia. After intraperitoneal (i.p.) administration, \( (9R) \)-HHC at 10 mg/kg significantly reduced the spontaneous activity of mice in the open field (1476.750 ± 159.842 cm, \( p = 0.0183 \)), as compared to the vehicle-treated group (3009.000 ± 272.840 cm), whereas \( (9S) \)-HHC did not affect the locomotion (3469.750 ± 833.532 cm, \( p = 0.7392 \)) (Figs. 5b and 5f). Moreover, \( (9R) \)-HHC administration induced an increase in the latency for moving from the catalepsy bar (Fig. 5c) (14.250 ± 7.642 s, \( p = 0.3097 \)), which was though not statistically significant as compared to the vehicle-treated group (0.250 ± 0.250 s). In the hot plate test, \( (9R) \)-HHC induced a significant antinociceptive effect (Fig. 5d) (22.200 ± 3.040 s, \( p = 0.0204 \)) as compared to the vehicle-treated group (13.768 ± 2.367 s). \( (9S) \)-HHC produced neither catalepsy (0.500 ± 0.289 s, \( p = 0.6259 \)) nor analgesia (13.250 ± 3.146 s, \( p = 0.9934 \)). \( (9R) \)-HHC showed a trend in the induction of decrease in the body temperature (Fig. 5e) (-2.125 ± 0.808°C, \( p = 0.1553 \)) greater than \( (9S) \)-HHC (-0.850 ± 0.435°C, \( p = 0.1137 \)), which however was not statistically significant as compared to vehicle-treated mice (0.575 ± 0.354°C).

**Discussion**

Since the discovery in the early ‘60s by Prof. Mechoulam of \( \Delta^9 \)-THC as the active ingredient of cannabis responsible for the euphoriant activity, strong efforts have been devoted by medicinal chemists to the modification of the chemical structure to increase the pharmacological properties. Very few if even none of these derivatives reached the market as a drug. On the other hand, for about fifteen years \( \Delta^9 \)-THC derivatives and new compounds with very little structural similarity with the lead compound have been produced and marketed illegally as synthetic cannabinoids (SCs) or “spice”. To date, we can count over 250 different SCs as drugs of abuse that the authorities are trying to put under control by adding them to the scheduled substances. More recently, the cultivation of cannabis for industrial purposes has made a comeback thanks to its high concentrations of CBD which includes various beneficial properties. Unlike THC, CBD is void of the euphoriant effects and exerts several valuable pharmacological activities and is currently used in medicinal specialties as a drug against some types of infantile epilepsy. During the 40th Expert Committee on Drug Dependence (ECDD) meeting, the WHO suggested that CBD should not be added to the scheduled substances as it is void of the intoxicating properties typical of THC or other SCs. Moreover, the recreational use of industrial hemp inflorescence as a substitute of high-THC cannabis has now become a widespread phenomenon called “light cannabis” in some European countries. Basically, hemp inflorescence, hashish and other hemp derivatives are sold as smoking products. Whilst with the 2018 Farm Bill Act the USA have allowed the legal marketing of all cannabis products with THC levels below 0.3%, the European legislation is still unclear and such business is in a grey area of the law. Along with the SCs, the SSCs appeared in the USA a few years ago. Such compounds derive from chemical modifications of compounds extracted from cannabis. In particular, HHC, the hydrogenation product of \( \Delta^9 \)-THC and \( \Delta^8 \)-THC, has met with great success. The removal of the double bond in either the C9 or C8 position generates a new stereogenic centre with the possibility to
obtain either the $R$ or $S$ epimer. Prof. Mechoulam had already suggested that it is possible to obtain an excess of the $R$ epimer starting from $\Delta^8$-THC and an excess of the $S$ epimer starting from $\Delta^9$-THC\textsuperscript{18}.

*In vitro* studies suggested that the $R$ epimer is the one with the highest affinity for the CB1 receptor, while the $S$ epimer has a very poor affinity\textsuperscript{17}. This implies that different epimeric mixtures with different intensities of the biological effects can be obtained based on the synthetic procedure. It is known from the literature that CBD can isomerize to either $\Delta^9$-THC or $\Delta^8$-THC under acidic catalysis according to the reaction time, type of catalyst and reaction conditions\textsuperscript{2,12,18}. Starting from this knowledge, the production of $\Delta^8$-THC and $\Delta^9$-THC as semi-synthetic products of CBD extracted from hemp has rapidly increased\textsuperscript{14}. However, both THCs are under control and thus not legally marketed. Therefore, the subsequent reduction of the double bond leading to the formation of the semi-synthetic HHC has overcome the legality issue. Moreover, many users have reported on online forums effects similar to those obtained with THC\textsuperscript{14}. At present, HHC is sprayed onto hemp products and openly sold to offer the well-known “high” effects of cannabis.

Scientific studies on HHC are sparse, thus it is necessary to undertake a more in-depth investigation of this topic. Our work demonstrates that it is possible to obtain HHC from CBD and an excess of one epimer can be selectively obtained according to the reaction conditions. The application of a validated analytical method for the separation of the two epimers has allowed to identify the presence of this SSC in two commercial samples of hemp biomass (HHC-1 and HHC-2) and one hemp hashish sample (HHC-3). The concentrations of the $R$ epimer were 2-fold higher than those of the $S$ epimer in all cases. The hemp biomass also presented high concentrations of other phytocannabinoids, in particular CBDA and CBD, thus indicating that the sample derived from industrial hemp. On the other hand, $\Delta^9$-THC and $\Delta^8$-THC were found in amounts lower than 0.5% in the two biomass extracts, as well as in the pure HHC mixture, and around 2% in the HHC hashish sample. They could probably be residues of the HHC synthesis, even though a natural origin cannot be ruled out. Even though HHC is reported to be present in trace amounts in cannabis inflorescence, its levels were far higher than those reached by CBD and THC, thus suggesting its exogenous origin. Additionally, since the $R$ epimer was found in excess with respect to the $S$ epimer, HHC was most likely obtained starting from CBD with $\Delta^9$-THC as the reaction intermediate rather than $\Delta^9$-THC. The analysis of the pure HHC sample (HHC-4) showed no trace of CBDA or CBD and the presence of CBN, $\Delta^8$-THC and $\Delta^9$-THC as impurities besides the main component HHC. The $R$ epimer was in slight excess, suggesting that the same synthetic route was followed.

After clarifying the synthetic process to obtain HHC, it seemed important to evaluate the cannabinomimetic activity of this compound with the tetrad test in mice. Considering the poor availability of the two epimers, only one dose (10 mg/kg) was tested, which is the one employed for THC in the tetrad test. The results showed that at least two of the four behaviours of the tetrad were significantly affected by $(9R)$-HHC, but not by $(9S)$-HHC administration. In particular, hypolocomotion and analgesia were increased by $(9R)$-HHC, findings which are in line with a CB1-mediated mechanism of action.
In summary, a possible origin has been proposed for the first SSC appeared in the European market and the biological tests elucidated that its cannabinomimetic activity lies in the $R$ epimer, while the $S$ epimer has little activity, thus confirming the \textit{in vitro} binding studies on CB1 receptor. It will be extremely important to evaluate whether or not HHC should be added to the scheduled narcotic substances.

**Methods**

**Chemicals and reagents.** Ethanol 96%, acetonitrile (ACN), and formic acid (FA) were all LC-MS grade and were bought from Sigma Aldrich (USA). Ultrapure water was obtained with a water purification system (Direct-Q 3UV, Merck Millipore, Milan, Italy). Chemicals and solvents employed in the synthetic process were reagent grade and used without further purification. The following abbreviations for common organic solvents were adopted: diethyl ether (Et$_2$O); dichloromethane (CH$_2$Cl$_2$); cyclohexane (CE); chloroform (CDCl$_3$); ethanol (EtOH). Stock solutions (1 mg/mL) of certified reference cannabinoid standards of CBDA, $\Delta^9$-THCA, CBGA, CBCA, CBD, CBG, CBC, CBN, as well as of $\Delta^9$-THC and $\Delta^8$-THC (500 $\mu$g/mL) were bought from Cayman Chemical (Ann Arbor, Michigan, USA). Stock solutions (1 mg/mL) of CBDH, $\Delta^9$-THCH, (9$R$)-HHC and (9$S$)-HHC were obtained by properly diluting the pure compounds in-house synthesized.

**Synthetic procedure a for HHC.** CBD (0.7 mmol) was dissolved in 20 mL of absolute EtOH containing 0.05% HCl and refluxed for 2 h. The resulting crude was neutralized with a saturated solution of Na$_2$CO$_3$ and subject to hydrogenation with an H-Cube ThalesNano flow reactor (Budapest, Hungary) according to the following experimental conditions: 3 mm 10% Pd/C cartridge, 30°C, 20 bar, 1 mL/min. The crude product mixture showed a 57:43 $S/R$ HHC mixture along with other by-products. The solvent was evaporated and the crude of the reaction was purified with semipreparative HPLC-UV (Column Luna 5 $\mu$m C18, 100 Å, 250 x 10 mm-Phenomenex, Bologna, Italy). An isocratic elution was employed with mobile phase 80% ACN (with 0.1% FA) and 20% MilliQ water (with 0.1% FA) at a flow rate of 7.5 mL/min. The UV trace was followed at 228 nm and the compounds of interest were obtained with a purity higher than 95% (12 mg for (9$R$)-HHC and 8 mg for (9$S$)-HHC).

**Synthetic procedure b for HHC.** To a solution of CBD (0.7 mmol) in 15 mL of anhydrous CH$_2$Cl$_2$, pTSA (10 mg, 10% mol) was added at room temperature, under a nitrogen atmosphere. The reaction was stirred in the same conditions for 48 h. After that, the mixture was diluted with Et$_2$O and washed with a saturated solution of NaHCO$_3$. The organic layer was collected, washed with brine, dried over anhydrous Na$_2$SO$_4$ and concentrated. The obtained crude was subject to hydrogenation as described for the synthetic procedure b to give a crude product with a 61:39 $R/S$ HHC mixture. The two epimers were purified with semi-preparative HPLC as reported above (11 mg for (9$S$)-HHC and 8 mg for (9$R$)-HHC).

**NMR characterization of HHC.** NMR spectra were recorded either on a Bruker 400 (working at 400.134 MHz for $^1$H and 100.62 MHz for $^{13}$C) or a Bruker 600 spectrometer (working at 600.130 MHz for $^1$H and 150.902 MHz for $^{13}$C). Monodimensional spectra were acquired with a spectral width of 8278 Hz (for $^1$H
NMR) and 23.9 kHz (for $^{13}$C NMR), a relaxation delay of 1 s and a number of transients of 32 and 1024 for $^1$H NMR and $^{13}$C NMR, respectively. NMR spectra were acquired in CDCl$_3$ and chemical shifts ($\delta$) were registered in ppm with respect to that of the residual solvent ($\delta$ = 7.26 ppm for $^1$H and $\delta$ = 77.20 ppm for $^{13}$C); coupling constants are reported in Hz, splitting patterns are expressed as singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), quintet (qnt), multiplet (m), broad signal (b).

(9 S)-6,6,9-trimethyl-3-pentyl-6a,7,8,9,10,10a-hexahydro-6$^H$-benzo[c]chromen-1-ol ((9 S)-HHC). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.25 (s, 1H, C4), 6.07 (s, 1H, C2), 4.61 (s, 1H, OH), 2.92–2.79 (m, 1H, C10$^\alpha$), 2.67 (td, $J$ = 11.4, 2.9 Hz, 1H, C10a), 2.46–2.38 (m, 2H, C1'), 2.11 (m, 1H, C9), 1.68–1.61 (m, 2H, C8-C7), 1.58–1.52 (m, 2H, C2'), 1.46–1.44 (m, 1H, C6a) 1.36 (s, 3H, C12), 1.33–1.28 (s, 6H, C4'-C3'-C10$^\beta$), 1.13 (d, $J$ = 7.2 Hz, 3H, C11), 1.09 (s, 3H, C13), 0.88 (t, $J$ = 7.0 Hz, 3H, C5$'^\prime$). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 155.38, 154.77, 142.65, 110.63, 110.19, 107.79, 77.03, 50.09, 36.33, 35.59, 32.40, 31.77, 30.76, 29.49, 28.06, 27.76, 27.10, 23.26, 22.73, 19.30, 18.98, 14.20.

(9 R)-6,6,9-trimethyl-3-pentyl-6a,7,8,9,10,10a-hexahydro-6$^H$-benzo[c]chromen-1-ol ((9 R)-HHC). $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 6.25 (s, 1H, C4), 6.08 (s, 1H, C2), 4.64 (s, 1H, OH), 3.03 (d, $J$ = 12.9 Hz, 1H, C10$^\alpha$), 2.49–2.37 (m, 3H, C1'-C10a), 1.87–1.80 (m, 2H, C8-1H, C7-1H), 1.67–1.60 (m, 1H, C9), 1.59–1.52 (m, 2H, C2'), 1.46–1.44 (m, 1H, C6a), 1.36 (s, 3H, C12), 1.34–1.24 (m, 4H, C4'-C3'), 1.16–1.07 (m, 2H, C8-1H, C7-1H), 1.06 (s, 3H, C13) 0.94 (d, $J$ = 6.6 Hz, 3H, C11), 0.88 (t, $J$ = 7.0 Hz, 3H, C5$'^\prime$), 0.78 (m, 1H, C10$^\beta$). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 155.15, 154.83, 142.74, 110.44, 110.23, 107.77, 77.14, 49.30, 39.16, 35.71, 35.58, 33.03, 31.75, 30.76, 28.24, 27.94, 22.78, 22.73, 19.22, 14.21.

**HPLC-UV-HRMS analysis.** A Vanquish Core system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with binary pump, vacuum degasser, thermostated autosampler and column compartment, and diode array detector (DAD) was interfaced to an Exploris 120 Orbitrap mass analyzer with a heated electrospray ionization source (HESI). The chromatographic separation was achieved on a Poroshell 120 EC C18 (100 × 3.0 mm, 2.7 µm) (Agilent, Milan, Italy) with an isocratic elution at 70% ACN for 20 min and a washing step at 98% ACN for 3 min. The column was re-equilibrated at 70% ACN for further 3 min for a total run time of 26 min at a constant flow rate of 0.5 mL/min.

The HESI parameters were optimized in previous works for cannabinoids: spray voltage 4200 kV and 3800 kV for HESI+ and HESI- mode respectively, sheath gas 70 au, auxiliary gas 5 au, sweep gas 0.5 au, ion transfer tube temperature 390°C and vaporizer temperature 150°C$^{23,25}$. The mass analyzer was operated in both full scan (FS) and data-dependent acquisition (DDA) mode. In FS mode the resolution was set at 60,000 FWHM (full width at half maximum), the RF lens level at 70%, the maximum injection time 54 ms, the $m/z$ range at 150–750, and the isolation window at $m/z$ 1.2. In DDA mode the resolution was set at 30,000 FWHM, the maximum injection time at 22 ms, the $m/z$ range at 50–750, the isolation window at $m/z$ 1.2, and the stepped normalized collision energy (NCE) at 20-40-100$^{23,25}$. The injection volume was 5 µL. The analyses were acquired with Xcalibur 3.0 and processed using Chromelon 7 for the UV traces and TraceFinder 54.0 for the MS traces (all from Thermo Fisher Scientific).
Calibration standards and sample preparation and cannabinoids quantification. Calibration solutions of all phytocannabinoid standards were prepared by diluting the stock solutions with ACN to get the final concentrations indicated in Table S1. Each dilution was run in triplicate and the calibration curves were built using both UV and MS data. Area of the peaks for each analyte was plotted against nominal concentration and the back-calculated concentration was checked to be within 15% of the nominal value. Samples of hemp inflorescence and HHC hashish were extracted using the method reported in the German Pharmacopoeia for the extraction of phytocannabinoids from cannabis inflorescence\textsuperscript{21}. The extracts HHC-1 and HHC-2 from hemp inflorescence were analysed after 100× dilution with mobile phase, while the extract HHC-3 from HHC hashish was 1000× diluted. The sample of pure HHC (HHC-4) was injected at the concentration of 10 µg/mL obtained by dissolving 10 mg of the sample in 1 mL of ACN and preparing serial 10× dilutions with mobile phase up to the desired concentration.

Quantification of cannabinoids was accomplished with both UV and MS data. The UV chromatograms were extracted at 228 nm. The exact \textit{m}/\textit{z} of the precursor ion in both HESI+ and HESI- mode was extracted with a 5-ppm error from the HRMS TIC and used for calibration.

\textbf{Tetrad Test.} Male C57BL6/J mice (7 weeks old; n = 4) received (9\textit{R})-HHC (10 mg/kg) or vehicle (1:1:18; EtOH:Kolliphor EL:0.9% saline) by i.p. administration. Hypomotility (open field test), hypothermia (body temperature), antinociceptive (hot plate test), and cataleptic (bar test) effects were evaluated using the procedures reported by Metna-Laurent et al.\textsuperscript{25}. The same animals were used in all four behavioral tests. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test.

\textit{Body temperature.} After immobilization of the mouse the probe was gently inserted 1 cm into the rectum and the temperature was registered after stabilization. The probe was cleaned with 70% EtOH and dried with a paper towel in between each mouse.

\textit{Open field.} Behavioral assays of locomotor activity were performed 30 min after drug (or vehicle) injection. The apparatus was cleaned before each behavioral session with a 70% EtOH solution. Naïve mice were randomly assigned to a treatment group. Behaviors were recorded, stored, and analyzed using an automated behavioral tracking system (Any-maze, Video-tracking software by Ugo Basile). Mice were placed in an open field test (OFT) arena (l×w×h: 44×44×30 cm) and their ambulatory activity (total distance travelled in centimeters) was recorded for 15 min and analyzed.

\textit{Bar test.} The bar consisted of a 40 cm in length and 0.4 cm in diameter glass rod, which was horizontally elevated by 5 cm above the surface. Both forelimbs of the mouse were positioned on the bar and its hind legs on the floor of the cage, ensuring that the mouse was not lying down on the floor. The chronometer was stopped when the mouse descended from the bar (i.e., when the two forepaws touched the floor) or after 5 min (cut-off time). Catalepsy was measured as the time duration each mouse held the elevated bar by both its forelimbs (latency for moving in seconds).
**Hot plate.** Each mouse was placed on a hot plate (Ugo Basile), which was kept at the constant temperature of 52°C. Licking of the hind paws or jumping were considered as a nociceptive response (NR) and the latency was measured in s 85 min after drug or vehicle administration, taking a cut-off time of 30 s to prevent tissue damage.

**Animals.** Male C57BL/6 mice (Charles River, Italy) weighing 18–20 g were used for the tetrad experiments. A 12 h light/dark cycle starting light at 6:00 A.M., a constant temperature of 20–22°C, and a 55–60% humidity were maintained for at least 1 week before the beginning of the experiments. Each cage housed three mice with chow and tap water available *ad libitum*. The experimental procedures employed for the work presented herein were approved by the Animal Ethics Committee of the University of Campania “L. Vanvitelli” (Naples, Italy). Animal care and welfare were entrusted to adequately trained personnel in compliance with Italian (D.L. 116/92) and European Commission (O.J. of E.C. L358/1, 18/12/86) regulations on the protection of animals used for research purposes. All efforts were made to minimize animal numbers and avoid unnecessary suffering during the experiments.

**Declarations**

**Acknowledgments**

The authors acknowledge Giovanna Negro and Giulio Guarini for their help in the extraction and sample analysis.

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**Data availability**

All data generated or analysed during this study are included in this published article and its supplementary information files.

**Author Contributions**

G.C. and C.C. developed and supervised the project, conceived the experiments plan and drafted the manuscript; M.S. carried out the synthesis and purification of HHC epimers; F.Russo carried out the spectroscopic characterization of HHC epimers and optimized the HPLC-UV-HRMS method; L.L., M.P., F.Ricciardi and S.M. performed the *in vivo* tetrad tests; A.G., A.L. and A.L.C. performed the HPLC-UV-HRMS analyses, M.A.V. and G.B. performed the statistical analysis; L.C. and E.P. performed the extraction of the samples; G.G. provided the instrumental and material resources. All authors reviewed the manuscript.
Additional Information

Competing interests

The authors declare no competing interests.

References

12. EMCDDA. (European Monitoring Centre for Drugs and Drug Addiction, Lisbon, 2022).


### Tables

**Table 1.** Concentration of phytocannabinoids and HHC epimers in the three extracts and in the commercial HHC mixture. Values are expressed in % (w/w) as mean±SD (n=3).
<table>
<thead>
<tr>
<th></th>
<th>HHC-1</th>
<th>HHC-2</th>
<th>HHC-3</th>
<th>HHC-4</th>
</tr>
</thead>
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<tr>
<td>CBDA</td>
<td>7.54±0.51</td>
<td>4.38±0.26</td>
<td>2.91±0.13</td>
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</tr>
<tr>
<td>CBGA</td>
<td>0.12±0.03</td>
<td>0.29±0.04</td>
<td>0.11±0.07</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>CBG</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>CBD</td>
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<td>1.51±0.12</td>
<td>2.83±0.33</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>CBN</td>
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<td>&lt;LOQ</td>
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<td>0.13</td>
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<td>Δ⁹-THC</td>
<td>0.35±0.02</td>
<td>0.26±0.01</td>
<td>1.97±0.12</td>
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</tr>
<tr>
<td>Δ⁸-THC</td>
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<td>&lt;LOD</td>
<td>0.24±0.01</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>CBC</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
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<tr>
<td>(9S)-HHC</td>
<td>9.36±0.43</td>
<td>8.78±0.54</td>
<td>11.61±0.08</td>
<td>4.15±0.07</td>
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<tr>
<td>(9R)-HHC</td>
<td>18.71±0.97</td>
<td>17.73±0.86</td>
<td>26.50±0.07</td>
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<td>Δ⁹-THCA</td>
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<td>&lt;LOQ</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
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<tr>
<td>CBCA</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

**Figures**

![Chemical structures](image1)

**Figure 1**
Chemical structure of the analyzed cannabinoids.

Figure 2

**Synthesis of HHC.** Step 1a-b: Cyclization of CBD. Step 2: Hydrogenation of the crude reaction mixture.
Figure 3

HPLC-UV-HRMS/MS and NMR characterization of the isolated (9S)-HHC and (9R)-HHC. HPLC-UV trace of (9S)-HHC (a) and (9R)-HHC (b) with the respective UV spectrum in the boxes; HRMS/MS pattern in HESI+ mode of (9S)-HHC (c) and (9R)-HHC (d) (the discriminant fragment is circled in blue and red for (9S)-HHC and (9R)-HHC respectively); HRMS/MS pattern in HESI- mode of (9S)-HHC (e) and (9R)-HHC (f); discriminant chemical shifts in the $^1$H NMR spectra of (9S)-HHC (blue) and (9R)-HHC (red) (g).
Figure 4

HPLC-UV chromatogram of a cannabinoid standard mixture. HPLC-UV chromatogram of a standard mixture containing ten phytocannabinoids (CBDA, CBGA, CBG, CBD, CBN, Δ⁹-THC, Δ⁸-THC, CBC, THCA, and CBCA) and the two HHC epimers at the concentration of 10 µg/mL.
Figure 5

Dose-dependent effects of (9R)-HHC and (9S)-HHC administration (10mg/kg, i.p.) on the tetrad behavioural tests in mice in comparison to vehicle. Time schedule of the tetrad tests in min from (9R)-HHC, (9S)-HHC or vehicle administration (a). Locomotion decrease induced by (9R)-HHC administration in the open field test (b,f). Decrease of body temperature after (9R)-HHC administration in the open field test (e); the values are expressed as the difference between the basal temperature (i.e., taken before (9R)-HHC or vehicle administration) and the temperature measured after (9R)-HHC or vehicle administration. Increase in the latency for moving from the catalepsy bar after (9R)-HHC administration (c). Increase in the latency after the first sign of pain shown by the mouse in the hot plate test following (9R)-HHC administration (d). Data are represented as the mean±SEM of 4 mice per group. * indicates significant differences compared to vehicle injection, *p<0.05 Tukey’s test.

Supplementary Files
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