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Bioactive Prenylogous Cannabinoid from Fiber Hemp (*Cannabis sativa*)

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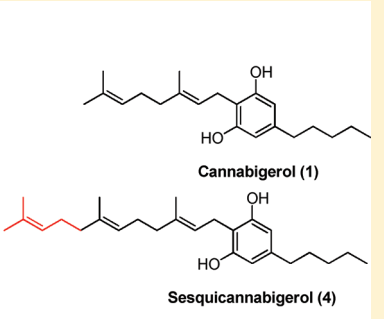
Supporting Information

ABSTRACT: The waxy fraction from the variety Carma of fiber hemp (*Cannabis sativa*) afforded the unusual cannabinoid **4**, identified as the farnesyl prenylogue of cannabigerol (CBG, **1**) on the basis of its spectroscopic properties. A comparative study of the profile of **4** and **1** toward metabotropic (CB_1, CB_2) and ionotropic (TRPV1, TRPV2, TRPM8, TRPA1) targets of phytocannabinoids showed that prenylogation increased potency toward CB_2 by ca. 5-fold, with no substantial difference toward the other end-points, except for a decreased affinity for TRPM8. The isolation of **4** suggests that *C. sativa* could contain yet-to-be-discovered prenylogous versions of medicinally relevant cannabinoids, for which their biological profiles could offer interesting opportunities for biomedical exploitation.



Cannabinoids are a class of meroterpenoids typical of *Cannabis sativa* L. (hemp, Cannabinaceae).[1] With the exception of cannabigerol (CBG, **1**), which also occurs in an African *Helichrysum* species,[2] *C. sativa* represents the sole natural source of these compounds. Cannabinoids are made up by an isoprenyl moiety bound to a resorcinyl-type polyketide unit. Due to postcoupling, mainly oxidative, modifications, there is no shortage of structural variations in the prenyl moiety of cannabinoids, but variance in the cannabinoid biogenetic building blocks has so far been reported only in the resorcinyl moiety, which can derive from either a hepta- or a hexaketide precursor, resulting in compounds with an *n*-pentyl (cannabinoid sensu stricto) or a *n*-propyl (cannabidivarin) alkyl moiety. Conversely, the isoprenyl moiety is of the monoterpene type in all the more than 100 cannabinoids characterized so far.[1]

Owing to the presence of high concentrations of the psychotropic agent Δ^9-THC (**2**), biomedical attention on *C. sativa* has so far mainly focused on varieties of this plant used for recreational purposes (marijuana), while the pharmacological potential of fiber hemp is still largely untapped. This observation is surprising, since certain biological properties of THC are shared also by nonpsychotropic cannabinoids,[1,3] suggesting that some pharmacologically useful activities of *C. sativa*, such as antibacterial, anti-inflammatory, and antipsoriasis actions, could be dissected from its psychotropic potential. Our interest in this area has focused on chemotypes derived from Carmagnola, a variety of exceptional length (up to 5−6 m) once extensively grown in Northern Italy especially for the production of naval ropes.[4] In previous work, we have characterized some unique cannabinoids and phenolics from relatively polar fractions of these hemp strains.[5] During the manipulation of crude acetone extracts from the variety Carma, a hemp chemotype characterized by the accumulation of CBG (**1**) and cannabidiol (CBD, **3**) in a ca. 6:1 ratio,[5] we observed the formation of a copious waxy precipitate, from which sesquicannabigerol (sesqui-CBG, **4**), a lipophilic cannabinoid of a novel biogenetic type, was obtained. The sesquicannabinoid **4** is a minor constituent (ca. 100 mg/kg

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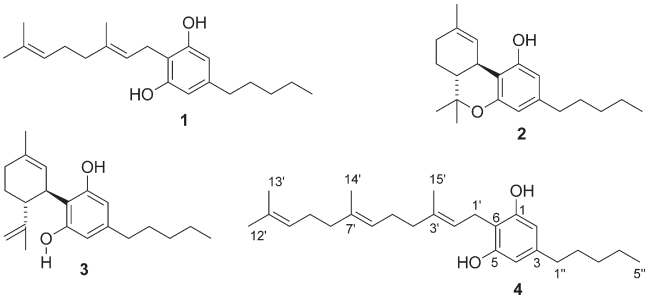
Table 1. Comparative Activity of CBG (1) and Sesqui-CBG (4) toward Various Biological End-Points of Phytocannabinoids (all EC_{50} and IC_{50} values are in μM)

	CB_1		CB_2		TRPV1			TRPV2			TRPA1			TRPM8
compound	IC_{50}	K_i	IC_{50}	K_i	efficacy[a]	EC_{50}	IC_{50}[b]	efficacy[a]	EC_{50}	IC_{50}[c]	efficacy[d]	EC_{50}	IC_{50}[e]	IC_{50}[f]
CBG (1)	3.31	0.76	3.46	0.88	33.8 ± 2.3	1.3 ± 0.5	2.6 ± 0.2	73.6 ± 1.2	1.7 ± 0.1	1.5 ± 0.2	99.9 ± 1.1	0.7 ± 0.03	13.0 ± 4.8	0.16 ± 0.02
sesqui-CBG (4)	3.27	0.75	0.71	0.18	38.8 ± 1.9	4.4 ± 1.0	5.7 ± 0.3	56.0 ± 0.3	2.7 ± 0.2	2.3 ± 0.4	164.4 ± 16.9	1.7 ± 0.9	1.2 ± 0.2	0.95 ± 0.05

[a] % ionomycin. [b] vs capsaicin 0.1 μM. [c] vs LPC (lysophosphatidylcholine) 3 μM. [d] % AITC (allyl isothiocyanate). [e] vs AITC 100 μM. [f] vs icilin 0.25 μM.

on dry weight basis), unlikely to significantly contribute to the overall bioactivity of hemp extracts, but its structure has interesting implications for *Cannabis* research, justifying its disclosure in this report.



Compound **4** was obtained as a white powder. The molecular formula, $C_{26}H_{40}O_2$, assigned to **4** by HRESIMS implied seven unsaturation degrees. The ^{1}H NMR spectrum of **4** ($CDCl_3$) was similar overall to that of CBG (**1**),[6] but showed the presence of further signals suggestive of a longer isoprenyl residue. Thus, one additional multiplet was present in the olefin region of the spectrum and one additional signal in the allylic methyl region, while the broad signals of the allylic methylenes (δ_H 1.96−2.07) integrated for eight rather than four protons. Accordingly, the ^{13}C NMR spectrum ($CDCl_3$) showed, in addition to the four phenyl resonances of the symmetrically substituted resorcinyl moiety, the presence of six further sp^2 signals from three trisubstituted carbon−carbon double bonds. The structure of **4** and the complete assignment of its ^{1}H and ^{13}C NMR signals were secured by the analysis of the 2D NMR spectra. The COSY spectrum confirmed the presence of an *n*-pentyl and a farnesyl moiety, and all proton signals were then associated with those of the corresponding carbon atoms using the HSQC spectrum. Finally, the HMBC spectrum was used to establish the connections between the spin systems. In particular, the cross-peaks of the four allylic methyls at δ_H 1.59, 160, 1.68, and 1.80 were instrumental in assembling the C_{15} terpenoid moiety, while cross-peaks of H-1′ (δ_H 3.38) with C-6 (δ_C 111.3) and with the oxygen-bearing carbon atoms C-1/C-5 (δ_C 155.4) and of H-1″ (δ_H 2.45) with C-3 (δ_C 143.0) and with the protonated C-2/C-4 (δ_C 108.5) aromatic pair showed that **4** belongs to the normal series of cannabinoids, having a *para*-relationship between prenyl and the alkyl substituents. ROESY cross-peaks of Me-15′ with H_2-1′ and of H-6′ with H_2-8′ supported the 2′*E*, 6′*E* configuration of **4**, in accordance with the chemical shift pattern of the allylic methyls.[6] The plant material from which **4** was obtained was thermally decarboxylated before extraction, and the fraction from which **4** was isolated was treated with bases to simplify its purification. Since phytocannabinoids are present in hemp mainly in carboxylated form and only in traces as their corresponding esters,[1] it seems logical to assume that the native form of **4** is the one carboxylated at C-2, as usual for phytocannabinoids.

The effect of prenylogation on the biological profile of phytocannabinoids is unknown, and we have therefore compared the action of sesqui-CBG **4** toward various end-points known to be affected by phytocannabinoids, namely, the two cannabinoid receptors (CB_1, CB_2) and the four thermo-TRPs (TRPV1, TRPV2, TRPM8, and TRPA1), which have been suggested to act as ionotropic phytocannabinoid receptors.[7] The profile of CBG (**1**) is characterized by low affinity for both CB_1 and CB_2, ca. 500-fold lower than that of Δ^9-THC (**2**), a significant agonistic activity toward TRPV1 and TRPA1, and a potent inhibitory activity toward TRPM8 (Table 1). With the exception of a minor affinity for TRPM8, the activity profile of sesqui-CBG (**4**) (Table 1) was similar toward the "ionotropic" phytocannabinoid receptors and CB_1, while the affinity at CB_2 was potentiated by a factor of ca. 5-fold. A series of cannabinoid-like meroterpenoids was described recently from an alpine *Rhododendron* species (*R. ferrugineum* L.).[8] These compounds share with **4** a sesquiterpenoid moiety, but were devoid of activity at CB_1, CB_2, and TRPV1, presumably because of the oxygenation of the terminal prenyl moiety and/or the presence of a short (methyl) alkyl substitution on the resorcinyl core.[1] This observation suggests strict structure−activity relationships for sesquicannabinoids, the activity of which on the biological end-points of phytocannabinoids requires lipophilic moieties on the two alkyl residues of the aromatic ring.[9] Taken together, these observations suggest that sesquicannabinoids might be interesting compounds for targeting topical end-points of phytocannabinoids, since both CB_2 and thermo-TRPs are highly expressed in skin tissues, and their modulation has potential for the treatment of skin diseases such as psoriasis, atopic dermatitis, and itch.[3]

The presence of polyketide homologues in *C. sativa* is not surprising, since polyketide synthases are cassette-type enzymes that show broad tolerance in terms of starters.[10] On the other hand, the biosynthesis of monoterpenes and sesquiterpenes is largely compartimentalized, taking place from different precursors in different cellular locations, namely, the cytoplasm for sesquiterpenoids and the chloroplasts for monoterpenoids.[11] Despite a certain exchange of isoprenoid building blocks between the two compartments, meroterpenoids are generally homogeneous in terms of prenylation. In this context, it would therefore be interesting to investigate the intracellular biogenetic origin of **4** and define the enzymology of its biosynthesis. Cannabigerol (**1**), the precursor of all cannabinoids,[12] is formed by the action of a prenyl transferase on olivetol, and is next converted into CBD or Δ^9-THC by two distinct oxidative terpene cyclases.[13] Sesqui-CBG (**4**) might be formed by an independent prenyltransferase, or, alternatively, the prenyl transferase responsible for the geranylation step could show loose substrate specificity, accepting also farnesyl pyrophosphate in addition to geranyl

pyrophosphate as a prenylating agent. With both alternatives (loose substrate specificity of the cannabinoid enzymatic machinery or the presence of enzymes with distinct prenylogous specificity), it does not seem unreasonable to assume that also the prenylogues of Δ^9-THC (**2**) and CBD (**3**) occur in *C. sativa*. These more lipophilic analogues of the pharmacologically important phytocannabinoids are as yet unknown, and represent a still unexplored region of their pharmacophore space.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). Homonuclear ^{1}H connectivities were determined by the COSY experiment. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with the HSQC experiment. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 250 ms. Two- and three-bond ^{1}H−^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for $^{2,3}J$ = 9 Hz. Low- and high-resolution ESIMS were obtained on a LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (70−230 mesh) was used for gravity column chromatography. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or spraying with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Flash chromatography was carried out on Biotage SP-1 equipment, using homemade alumina cartridges prepared from column chromatography neutral alumina (Brockmann activity 1) from Macherey-Nagel.

Plant Material. *Cannabis sativa* var. Carma was derived from a greenhouse cultivation at CRA-CIN, Rovigo (Italy), where a voucher specimen is kept. The plant material was collected in November 2010 and was supplied by Dr. Gianpaolo Grassi (CRA-CIN, Rovigo, Italy). The manipulation of the plant was done in accordance with its legal status (Authorization SP/101 of the Ministero della Salute, Rome, Italy).

Extraction and Isolation. Dried, powdered flowered aerial parts were heated at 120 °C for 2.5 h to decarboxylate precannabinoids and then extracted exhaustively with acetone (2 × 9 L) in a shaker. Removal of the solvent left a black resinous residue (74 g, 7.4%), which was dissolved in MeOH (30 mL/g of extract) and filtered through RP18 silica gel for the removal of pigments. During the filtration, formation of a dark green, waxy precipitate (8.5 g, 0.85%) was observed on top of the paper filter used to protect the surface of the filtration bed. When analyzed by ^{1}H NMR, this material showed only signals typical of waxes and polyprenols. On the other hand, TLC analysis (petroleum ether−EtOAc, 9:1) showed also the presence of some UV-absorbing spots. To simplify purification, the waxy precipitate was hydrolyzed by suspension in 100 mL of 4% methanolic KOH and stirring 1 h at room temperature. After dilution with water (100 mL water), removal of methanol, and addition of brine (50 mL), the basic water phase was extracted with CH_2Cl_2, affording 3.40 g of a semisolid waxy residue. The latter was further purified by gravity column chromatography on silica gel (75 g, petroleum ether−EtOAc, 95:5, as eluant) to afford 149 mg of a compound showing ^{1}NMR signals and color reactions typical of a phytocannabinoid. Final purification was achieved by flash chromatography on neutral alumina, affording 98 mg (0.0098%) of **4**.

Sesquicannabigerol (sesqui-CBG, 4): colorless oil; ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.25 (2H, s, H-2/H-4), 5.27 (1H, bt, J = 6.2 Hz, H-2′), 5.09 (1H, m, H-6′), 5.05 (1H, m, H-10′), 3.38 (2H, d, J = 6.2 Hz, H_2-1′), 2.45 (2H, t, J = 7.5 Hz, H_2-1″), 2.07 (4H, overlapped m, H_2-5′ and H_2-9′), 2.05 (2H, overlapped m, H_2-4′), 1.96 (2H, m, H_2-8′), 1.80 (3H, bs, H_3-15′), 1.68 (3H, bs, H_3-12′), 1.60 (3H, bs, H_3-14′), 1.59 (3H, bs, H_3-13′), 1.56 (2H, m, H_2-2″), 1.30−1.27 (4H, m, H_2-3″ and H_2-4″), 0.87 (3H, t, J = 7.2 Hz, H_3-5″); ^{13}C NMR (175 MHz, $CDCl_3$) δ 155.4 (C-1/C-5), 143.0 (C-3), 139.5 (C-3′), 135.3 (C-7′), 132.2 (C-11′), 124.6 (C-6′), 124.2 (C-10′), 122.2 (C-2′), 111.3 (C-6), 108.5 (C-2/C-4), 40.0 (C-4′, C-8′), 35.8 (C-1″), 31.6 (C-3″), 31.1 (C-2″), 27.1 (C-5′), 27.0 (C-9′), 26.0 (C-12′), 23.0 (C-4″), 22.5 (C-1′), 18.1 (C-13′), 16.6 (C-15′), 16.4 (C-14′), 14.0 (C-5″); ESIMS (negative ion): m/z 383 $[M - H]^-$; HRESIMS m/z 383.2939, calcd for $C_{26}H_{39}O_2$, 383.2950.

TRPV1, TRPV2, TRPM8, and TRPA1 Receptor Assays. HEK-293 cells stably overexpressing recombinant rat TRPA1, rat TRPM8, rat TRPV2, or human TRPV1 were selected by G-418 (Geneticin; 600 μg mL^{-1}), grown on 100 mm diameter Petri dishes as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal bovine serum, and 2 mM glutamine and maintained under 5% CO_2 at 37 °C. Stable expression of each channel was checked by quantitative PCR. The effect of the substances on $[Ca^{2+}]_i$ was determined using Fluo-4, a selective intracellular fluorescent probe for Ca^{2+}. Toward this aim, on the day of the experiment, cells overexpressing the TRP channels were loaded for 1 h at room temperature with the methyl ester Fluo4-AM (4 μM in dimethyl sulfoxide containing 0.02% Pluronic F-127, Invitrogen) in minimum essential medium without fetal bovine serum. After the loading, cells were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in Tyrode's buffer, and transferred (50−60 000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B; PerkinElmer Life and Analytical Sciences, Waltham, MA) under continuous stirring. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence at 25 °C (λ_{EX} = 488 nm, λ_{EM} = 516 nm). Curve fitting (sigmoidal dose−response variable slope) and parameter estimation were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e., half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}), calculated by using GraphPad. The efficacy of the agonists was first determined by normalizing their effect to the maximum Ca^{2+} influx effect on $[Ca^{2+}]_i$ observed with application of 4 μM ionomycin (Sigma). The effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allyl isothiocyanate (AITC). In the case of TRPM8 the experiments were carried out at 22 °C with a Fluorescence Peltier System (PTP-1, Perkin-Elmer). Antagonist/desensitizing behavior was evaluated against capsaicin (0.1 μM) for TRPV1, icilin (0.25 μM) for TRPM8, AITC (100 μM) for TRPA1, or lysophosphatidylcholine (LPC) (3 μM) for TRPV2, by adding the compounds in the quartz cuvette 5 min before stimulation of cells with agonists. Data are expressed as the concentration exerting a half-maximal inhibition of agonist $[Ca^{2+}]_i$-increasing effect (IC_{50}), which was calculated again using GraphPad Prism software. The effect on $[Ca^{2+}]_i$ exerted by the agonist alone was taken as 100%. All determinations were performed at least in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by Bonferroni's test.

CB_1 and CB_2 Receptor Binding Assays. Membranes harvested from human recombinant CB_1 (Bmax = 2.5 pmol/mg protein) or CB_2 (Bmax = 4.7 pmol/mg protein) receptor transfected HEK-293 cells were incubated with the high-affinity ligand [^{3}H]-CP-55,940 (0.14 nM, K_d = 0.18 or 0.084 nM, K_d = 0.31 nM, respectively, for CB_1 and CB_2), and displaced with 10 μM of the heterologous competitor for nonspecific binding WIN 55212-2 (K_i values 9.2 and 2.1 nM, respectively, for the CB_1 and CB_2). All compounds were assayed according to the manufacturer's (Perkin-Elmer, Milano, Italy) instructions. Increasing concentrations of compounds were incubated with [^{3}H]-CP-55,940 for 90 min at 30 °C to generate displacement curves. IC_{50} values of the test compounds for the displacement of the bound radioligand were

obtained by GraphPad Prsim and used to calculate K_i values via the Cheng−Prusoff equation. Data are represented as means ± SEM of at least $n = 3$ experiments.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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