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Iodine-Promoted Aromatization of *p*-Menthane-Type Phytocannabinoids

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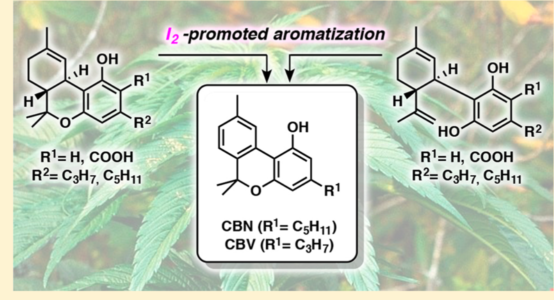
Supporting Information

ABSTRACT: Treatment with iodine cleanly converts various *p*-menthane-type phytocannabinoids and their carboxylated precursors into cannabinol (CBN, **1a**). The reaction is superior to previously reported protocols in terms of simplicity and substrate range, which includes not only tricyclic tetrahydrocannabinols such as Δ^9-THC (**2a**) but also bicyclic phytocannabinoids such as cannabidiol (CBD, **3a**). Lower homologues from the viridin series (**2c** and **3c**, respectively) afforded cannabivarin (CBV), a non-narcotic compound that, when investigated against a series of ionotropic (thermo-TRPs) biological end-points of phytocannabinoids, retained the submicromolar TRPA1-activating and TRPM8-inhibiting properties of CBN, while also potently activating TRPV2. Treatment with iodine provides an easy access to CBN (**1a**) from crude extracts and side-cuts of the purification of Δ^9-THC and CBD from respectively narcotic *Cannabis sativa* (marijuana) and fiber hemp, substantially expanding the availability of this compound and, in the case of fiber hemp, dissecting it from narcotic phytocannabinoids.



Cannabinol (CBN, **1a**) was first isolated by Easterfield at Cambridge University in 1896[1] and remained for almost four decades the only phytocannabinoid available in pure form. Its tricyclic dibenzopyrane nature was first proposed by Cahn,[2] and the full structure was eventually elucidated by Adams at Urbana−Champaign in 1940.[3] Adams also recognized its artifact nature and its close relationship with the unstable and more hydrogenated narcotic principle of marijuana, elusive at that time and only identified two decades later as Δ^9-tetrahydrocannabinol (Δ^9-THC, **2a**) by Mechoulam in Jerusalem.[4] CBN is a partial agonist of the two cannabinoid receptors (CB_1 and CB_2), retaining ca. 10% of the potency of Δ^9-THC for both these end-points.[5] It was long considered of minor interest in terms of bioactivity and mostly relevant as a marker for the identification of narcotic *Cannabis sativa* L. (Cannabaceae) in archeological plant samples due to its exceptional stability and relationship with Δ^9-THC.[6]

On the other hand, CBN shows a pharmacology that goes substantially beyond its affinity for cannabinoid receptors. Thus, CBN shows antibacterial activity on multi-drug-resistant *Staphylococcus aureus* (MDRSA) comparable to that of Δ^9-THC (**2a**) and cannabidiol (CBD, **3a**),[7] potently activates and desensitizes TRPA1 in a noncovalent fashion,[8] and in animal experiments induces a remarkable prolongation of sleeping time.[9] Despite its recognized pharmacological potential[5] and its availability in pure form from over a century, CBN (**1a**) remains paradoxically underinvestigated in terms of bioactivity,[5] while the biological profile of its lower homologue cannabivarin (CBV, **1b**) is substantially unexplored.

CBN (**1a**) shows similar polarity and chromatographic behavior with Δ^9-THC, and its purification from aged and partially degraded marijuana samples is therefore complicated. For this reason, CBN is more conveniently obtained by aromatization of Δ^9-THC and mixtures of hydrocannabinols. The reaction was first reported by Adams using sulfur as the oxidant under relatively harsh conditions (heating neat at ca. 250 °C).[10] More recently, chloranyl (tetrachloro-1,4-benzoquinone) was found to selectively oxidize Δ^9-THC to CBN while leaving other isomeric tetrahydrocannabinols unaffected,[11] and a dehydrogenation protocol based on refluxing a chloroform solution of Δ^9-THC with selenium dioxide and trimethylsilyl polyphosphate (prepared from P_4O_{10} and hexamethyldisiloxane) was also described.[12] We have developed a mild protocol to convert *p*-menthane phytocannabinoids, including cannabidiol (CBD, **3a**), to CBN, extending its availability to non-narcotic cannabis biomasses, as well as to crude extracts, side-cuts, and mother liquors from the purification of the major medicinal phytocannabinoids (Δ^9-THC and CBD).

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	R_1
1a	n-C_5H_{11}
1b	n-C_3H_7

	R_1	R_2		R_1	R_2
2a	H	n-C_5H_{11}	**3a**	H	n-C_5H_{11}
2b	COOH	n-C_5H_{11}	**3b**	COOH	n-C_5H_{11}
2c	H	n-C_3H_7	**3c**	H	n-C_3H_7
2d	COOH	n-C_3H_7	**3d**	COOH	n-C_3H_7

A combination of iodine and dichlorodicyanobenzoquinone (DDQ) was recently reported to aromatize *p*-menthadienes or *p*-menthene alcohols to their corresponding fully aromatic *p*-cymene derivatives via an iterative addition−elimination mechanism and a final oxidative aromatization.[13] The high yields of the reaction prompted us to evaluate the possibility of applying these conditions also to the aromatization of Δ^9-THC (**2a**). As a *p*-menthene derivative, **2a** lacks one double bond or double-bond equivalent to achieve aromatization via the addition−elimination mechanism operating in *p*-menthadienes and *p*-menthene alcohols. Nevertheless, Δ^9-THC shows an inherent tendency to aromatize, which we hoped to foster through the agency of the iodine−DDQ pair. In the event, DDQ turned out to be redundant, and refluxing in toluene with two equivalents of iodine was sufficient for the spot-to-spot conversion of Δ^9-THC (**2a**) into CBN (**1a**). After workup and chromatographic purification, a rewarding 70% yield was obtained.

The reaction was also successful with the acidic precursor of Δ^9-THC (THCA-A or pre-THC, **2b**), with decarboxylation, sluggish at this temperature in solvent phase, being presumably accelerated by the formation of HI in the dehalogenative step.[14] Since cannabidiol (**3a**) is turned by acids into a mixture of isomeric tetrahydrocannabinols,[15] we next wondered if the aromatization could be directly applied also to CBD (**3a**). In the event, an excellent yield was obtained from both CBD (**3a**) (72%) and its acidic precursor (**3b**), telescoping into a single-step decarboxylation, cyclization, and aromatization. Starting from the viridins **2c** and **3c**, the lower homologues of respectively Δ^9-THC and CBD, cannabivarin (CBV, **1b**), the C_3 lower homologue of CBN (**1a**), was obtained. This compound was first isolated by Markus from a Nepalese sample of hashish in 1971,[16] but has been substantially overlooked in the literature in terms of both spectroscopic characterization and bioactivity profile. The paucity of bioactivity data is surprising, given the current interest in divarinol (5-propylresorcinol)-type phytocannabinoids as negative modulators of the endocannabinoid system.[17] CBN has been reported to retain a significant (10%) activity in binding assays on CB_1 and CB_2 (EC_{50} = 75 nM and 73 nM, respectively),[18] while CBV was only marginally active (EC_{50} = 565 nM and 4780 nM, respectively) in these assays.[18] On the other hand, nothing is known on the activity of CBV on thermo-TRPs, another major class of phytocannabinoid targets,[8] and it was therefore interesting to compare its activity to that of CBN, a potent, sub-micromolar, noncovalent activator of TRPA1 (EC_{50} = 0.18 ± 0.02 μM)[8] and inhibitor of TRPM8 (IC_{50} = 0.21 ± 0.05 μM).[8] The activity against these two targets was fully retained by its lower homologue CBV (**1b**) (EC_{50} = 0.20 ± 0.09 μM for the activation of TRPA1 and IC_{50} = 0.50 ± 0.05 μM for the inhibition of TRPM8). On the other hand, activity on TRPV2 was significantly increased (EC_{50} = 3.1 ± 0.10 μM for CBV vs 19 ± 3.7 μM for CBN) and, to a lesser extent, also that on TRPV4 (EC_{50} = 7.3 ± 0.50 μM for CBV vs 16.1 ± 4.5 μM for CBN). These observations are interesting, since potent noncovalent activators of TRPA1 are rare,[19] while few modulators of TRPV2, a clinically validated cardiovascular target,[20] have been reported within both natural and synthetic products.[21] Differences for the modulation of TRPV3 were less significant (EC_{50} = 3.5 ± 0.60 μM for CBV vs 5.3 ± 2.7 μM for CBN), while both CBN and CBV showed only marginal activity against the capsaicin receptor TRPV1 (EC_{50} = 6.6 ± 0.10 μM for CBV vs 6.2 ± 3.7 μM for CBN). The spectroscopic characterization of CBV is reported in the Experimental Section. Apart from having two less carbons in the alkyl chain, no other significant spectroscopic difference with CBN[21] was detected.

From a mechanistic standpoint, the aromatization of Δ^9-THC, a 2π-system, is somewhat surprising, owing to its low unsaturation.[13] The reaction presumably involves the formation of an intermediate 4π-system by addition of iodine and the elimination of two molecules of hydrogen iodide (Scheme 1).

Scheme 1. Possible Mechanism for the Iodine-Induced Aromatization of Δ^9-THC (2a) to CBN (1a) (R = C_5H_{11})

2a → (+ I_2) → 4 → (- 2 HI) → 5 → (+ I_2) → 6 → (- 2 HI) → 1a

This generates a diene (**5**) that could then undergo conjugate 1,4-addition of iodine, affording the allyl diiodide **6**, which eventually aromatizes by loss of two further units of HI. The generation of a strong acid is presumably also responsible for the cyclization of CBD to THC via a shift of the exocyclic double bond to an endocyclic location, a reaction similar to that reported for the conversion of limonene into *p*-cymene.[13] Remarkably, under these acidic conditions, reversible retro-Friedel-Craft fragmentation of CBD did not take place,[15] and the *para*-relationship between the alkyl and the isoprenyl substituents of the resorcinyl core was maintained. Retro-Friedel-Craft reaction is, indeed, responsible for scrambling of the relationship between the alkyl and the terpenyl residues, with the formation of abnormal cannabinoids during the acidic treatment of the native phytocannabinoids.[23] After partition between petroleum ether and acetonitrile to remove fats and waxes, the aromatization reaction could also be applied to crude extracts from fiber hemp without any previous isolation of purified phytocannabinoids (see Experimental Section). The reaction with iodine makes it therefore possible to obtain CBN from non-narcotic *C. sativa* sources, overcoming the regulatory

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issues associated with obtaining this compound from aged marijuana extracts or from Δ^9-THC. Hopefully, this could foster studies on the pharmacology and clinical potential of CBN (**1a**), the first phytocannabinoid to be isolated and elucidated structurally.

■ EXPERIMENTAL SECTION

General Experimental Procedures. IR spectra were registered on an Avatar 370 FT-IR Techno-Nicolet apparatus. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on Varian INOVA NMR spectrometers. Chemical shifts were referenced to the residual solvent signal (CD_3OD: δ_H = 3.34, δ_C = 49.0). Homonuclear ^{1}H connectivities were determined by the COSY experiment. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with the HSQC experiment. Two- and three-bond ^{1}H−^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for a $^{2,3}J$ = 9 Hz. Low- and high-resolution ESIMS were obtained on an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer.

Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates and visualized by staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Chemical reagents and solvents were purchased form Sigma-Aldrich (Germany) and were used without any further purification unless stated otherwise. Petroleum ether with a boiling point of 40−60 °C was used. Silica gel 60 (70−230 mesh) used for gravity column chromatography (GCC) and RP C_{18} (Nucleodur C_{18} HTec) used for vacuum filtration were purchased from Macherey-Nagel. Flash chromatography was carried out on Biotage SP-1 equipment, using Biotage Snap Cartrige KP-C_{18}-HS, 60 g (particle size 25 μm). For all fractions obtained with Biotage SP-1, a liquid−liquid partition between EtOAc/brine and anhydrification with Na_2SO_4 was applied before complete evaporation.

Plant Material. The CBDV (**3c**)-rich *Cannabis sativa* strain used in this study was obtained from a greenhouse cultivation at CRA-CIN, Rovigo (Italy), where a voucher specimen is kept, and was supplied by Dr. Gianpaolo Grassi (CRA-CIN, Rovigo, Italy). The manipulation of the plant material and of the narcotic phytocannabinoids was done in accordance with their legal status (Authorization SP/101 of the Ministero della Salute, Rome, Italy).

Isolation of Cannabidivarin (3c, CBDV). Dried aerial parts of *C. sativa* (flowers and leaves, 156 g) were extracted with acetone (3 × 2 L) in a 10 L vertical percolator. Evaporation of the solvent left a black gum (11.6 g, 7.4%). The latter was then dissolved with heating (40 °C) in the minimal amount of methanol and then charged on a bed of 35 g of RP-C_{18} (ratio extract/RP-C_{18} silica gel 1:3) packed with methanol in a sintered filtration funnel (9 × 15 cm) with vacuum side arm. Washing with methanol (200 mL) under a vacuum gave 9.8 g of a dark green gum, which was decarboxylated by heating at 130 °C under stirring. Fractionation by GCC on silica gel (400 g, 20 mL fractions) by using a petroleum ether/EtOAc gradient (from 90:10 to 20:80) gave 2.71 g of crude CBDV, which was purified by flash column chromatography on a Snap Cartrige KP-C_{18}-HS (60 g, 15 mL volume fractions) using a methanol/acidic water (phosphate buffer, pH = 3) gradient, from 70:30 to 95:05. Crystallization with ether afforded **3c** (963 mg) as a white powder.[24]

Aromatization of *p*-Menthane Cannabinoids: Reaction of CBD (3a) and with a CBDV (3c)-Rich Crude Extract as Exemplificative. *Reaction with CBD (3a).* To a solution of CBD (**3a**, 100 mg, 0.32 mmol) in toluene (20 mL) was added iodine (162 mg, 0.64 mmol, 2 molar equiv). The solution was refluxed, following its course by TLC (petroleum ether/EtOAc, 9:1, R_f **3a** = 0.55, R_f **1a** = 0.45). After 60 min, the reaction was worked up by cooling to room temperature and sequentially washed with 5% $Na_2S_2O_3$ and brine. After drying, the organic phase was evaporated, and the residue was purified by GCC on silica gel with petroleum ether as eluant to afford 72 mg (72%) of **1a**[22] as a pale yellow oil.

Reaction with a CBDV (3c)-Rich Extract. The plant material was extracterd and defatted as described for the isolation of CBDV. The crude extract (2 g) containing CBDVA (**3d**) as its major phytocannabinoid was dissolved in toluene (100 mL), and iodine (200 mg) was added. The solution was then refluxed, following the course of the reaction by TLC (petroleum ether/EtOAc, 9:1; R_f **3d** = 0.05, R_f **1b** = 0.35). Additional amounts of iodine (100 and 150 mg) were added after respectively 30 and 40 min. After refluxing for 15 min from the last addition, the reaction was worked up as described for the reaction with CBD (**3a**). Purification by GCC on silica gel with petroleum ether as eluant afforded 25 mg of CBN (**1a**)[22] and 150 mg of cannabivarin (**1b**).

Cannabivarin (1b): orange oil; ^{1}H NMR (CD_3OD, 500 MHz) δ_H 8.33 (1H, s, H-10), 7.12 (1H, d, J = 7.8 Hz, H-7), 7.01 (1H, d, J = 7.8 Hz, H-8), 6.35 (1H, s, H-4), 6.26 (1H, s, H-2), 2.44 (2H, t, J = 7.5 Hz, H-1′), 2.34 (3H, s, H-11), 1.62 (2H, sextet, J = 7.5 Hz, H-2′), 1.53 (6H, s, H-12, H-13), 0.94 (3H, t, J = 7.5 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 155.0 (C-1), 154.4 (C-4a), 143.9 (C-3), 143.6 (C-10a), 136.4 (C-6a), 136.0 (C-9), 126.9 (C-8), 126.7 (C-10), 121.8 (C-7), 109.3 (C-4), 108.9 (C-2), 108.6 (C-10b), 76.6 (C-6), 37.4 (C-1′), 26.0 (C-12, C-13), 24.1 (C-2′), 20.1 (C-11), 12.7 (C-3′); ESIMS m/z 305 $[M + Na]^+$; HRESIMS m/z $[M + Na]^+$ 305.1519 ($C_{19}H_{22}O_2Na$ requires 305.1517).

Thermo-TRPs (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, TRPA1) Receptor Assays. HEK-293 cells stably overexpressing recombinant rat TRPA1, TRPM8, TRPV2-4, and TRPM8 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 real-time PCR. The effect of the substances on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) 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 in the dark 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 (about 100 000 cells) to the quartz cuvette of the spectrofluorimeter (PerkinElmer LS50B; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. $[Ca^{2+}]_i$ 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, USA). 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 increases in fluorescence in wild-type HEK293 cells (i.e., not transfected with any construct) were used as a baseline and subtracted from the values obtained from transfected cells. 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, PerkinElmer). Antagonist/desensitizing behavior was evaluated against capsaicin (0.1 μM) for TRPV1, icilin (0.25 μM) for TRPM8, AITC (100 μM) for TRPA1, lysophosphatidylcholine (3 μM) for TRPV2, and 10 nM GSK1016790A for TRPV4 by adding the compounds in the quartz cuvette 5 min before stimulation of cells with agonists. In the case of TRPV3, rat TRPV3-expressing HEK-293 cells were first sensitized with the nonselective agonist 2-aminoethoxydiphenyl borate (100 μM). Antagonist/desensitizing behavior was evaluated against thymol (100 μM). 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 at least performed in triplicate.

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Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by Bonferroni's test.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00946.

^{1}H and ^{13}C NMR spectra for CBV (**1b**) and efficacy, potency, and IC_{50} values for the activity of **1b** toward thermo-TRPs (PDF)

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Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to Dr. Susan Band Horwitz, of Albert Einstein College of Medicine, Bronx, NY, for her pioneering work on bioactive natural products.

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