Uncovering the psychoactivity of a cannabinoid from liverworts associated with a legal high

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INTRODUCTION
Cannabinoids have a wide range of actual and potential therapeutic applications, and marijuana is widely used not only for its recreational but also for its medicinal properties (1, 2). To date, the plant Cannabis sativa L. is the only known natural source of CB1 cannabinoid receptor (CB1R) activators, with Δ9-trans-tetrahydrocannabinol (Δ9-trans-THC) being the most potent partial agonist (3, 4). The THC scaffold was presumed unique to the Cannabis plant genus until the bibenzyl cis-THC, (−)-cis-perrottetinene (cis-PET) (Fig. 1), was isolated from the liverwort Radula perrottetii Gottsch ex Steph in 1994 by Asakawa and co-workers and later from Radula marginata Taylor ex Gottsch (5, 6). Further, Cullman and Becker isolated cis-PET from Radula laxiramea Steph (7). Bryophytes are a distinctive group of an early-diverged lineage of nonvascular land plants comprising more than 20,000 species (Fig. 1). This division includes mosses, hornworts, and liverworts (8). Liverworts often generate the opposite stereochemical configuration in secondary metabolite biosynthesis compared with higher plants (9). This is also the case for the cis configuration in the cyclohexene ring in cis-PET compared with Δ9-trans-THC. The absolute stereochemistry of cis-PET was fully structurally characterized in 2008 (10). Comparatively little attention has been paid to the liverworts for use in human diet or medicine to date (9). Worldwide anecdotal reports both affirm and refute the effectiveness of consuming R. marginata preparations inducing a legal cannabis-like high. However, the pharmacology of this natural product remains unknown. R. marginata is endemic to New Zealand and Tasmania, and collected dried samples are currently sold via the internet as an emerging recreational drug or as incense (see Acknowledgments). A molecular understanding of the effects of PET is essential to anticipate potential toxicological or recreational effects in humans. On the basis of a previously established stereodivergent synthesis of Δ9-THCs (11–13), we report a versatile total synthesis of natural cis-PET and its (−)-trans diastereoisomer (Figs. 2 and 3). This enabled a comparative study of PET and Δ9-THC structure-activity relationships for CB1R and CB2R activation, respectively, as well as cannabimimetic and biochemical effects in mice. To further investigate the pharmacological effects of the liverwort cannabinoid, we measured the bioavailability of cis-PET to the brain and profiled this natural product on major central nervous system (CNS) receptors. The CB1R-mediated psychoactive pharmacology is characterized by a typical central effect pattern in mice, generally referred to as the "tetrad test," which includes antinociception, hypothermia, catalepsy, and locomotion inhibition (14). A full assessment of both PET diastereoisomers on the endocannabinoid system (ECS) was performed. This study not only provides a molecular basis on which to assess the mode of action and emerging recreational use of smoked Radula liverwort preparations as legal high but also allows a direct comparison with Δ9-THC for potential therapeutic applications.

RESULTS
Stereodivergent total synthesis of cis- and trans-PET
We previously introduced stereodivergent catalysis to enable the synthesis of all stereoisomers of a target, applying it to the synthesis of Δ9-THCs (11–13). Maintaining this stereodivergent step, the strategy was adapted to synthesize sufficient amounts of PETs for biological testing and relied on a SNAr cyclization to assemble the dihydrobenzopyran core (15). Following a stereodivergent step using iridium- and amine-catalyzed aldehyde allylation (1 + 2 → 3), the SrAr precursors 4 were prepared through ring-closing metathesis, oxidative esterification, and subsequent Grignard addition (Fig. 2).
Fig. 1. Phylogenetic separation of bryophytes and angiosperms and convergent evolution of the tetrahydrocannabinoid scaffold in (−)-trans-THC and (−)-cis-PET. Both cannabinoids act as partial agonists on CB1 receptors in vitro and in vivo. Ma, mega-annum. Photo credit: S. Fischer (D-CHAB, LOC, ETH Zurich).

Fig. 2. Stereodivergent synthesis of SNAr precursors. Reagents and conditions: (a) 1.0 equiv 1, 3.0 equiv 2, 3 mol% [(Ir(cod)Cl)2], 12 mol% (S)-L, 15 mol% (S) or (R)-A, 5 mol% Zn(OTf)2, 1,2-dichloroethane (0.5 M), 25°C, 20 hours; for (S,R)-3: 59% yield, > 20:1 diastereomeric ratio (d.r.), > 99% enantiomeric excess (e.e.), for (R,R)-3: 76% yield, > 12:1 d.r., > 99% e.e.; (b) 5 mol% Grubbs II cat., CH2Cl2, 25°C, 16 hours; (c) 2.3 equiv NaClO2, 2.0 equiv NaH2PO4, 30 equiv 2-methyl-2-butene, tert-BuOH/H2O, 25°C, then 2.0 equiv Me3SiCHN2, C6H6/MeOH, 0°C, 90 min; (d) 8.0 equiv KOH, 3.9 equiv I2, MeOH, 0°C, 45 min; (e) 4.0 equiv MeMgBr, THF, 25°C, for (S,R)-4: 56% yield over three steps; see the Supplementary Materials for structures of (S)-L, (S)-A, and (R)-A, as well as for further details.
Similarly, at the human peripheral cannabinoid receptor type 2 (CB2R), the natural prod-
endocannabinoid-degrading enzymes

\[ \Delta^9\text{-THC} \] was about 10 times less potent than
CB1R functional level,
\[ \text{cis} \] and \[ \text{trans} \] were altered the binding relationships at CB receptors. In
\[ \text{[35S]} \text{GTP} \gamma \text{S} \] (17 and a moderately active CB1R partial
amid hydrolase (FAAH), monoacyl glycerol lipase (MAGL), and
effects on the major endocannabinoid-degrading enzymes fatty acid
despite their nanomolar receptor binding affinities. No relevant ef-
mments using various (i.e., equipotent to
CB1R activation in mice (hypothermia, catalepsy, hypolocomotion,
and analgesia) was in the same range as seen with \[ \Delta^9\text{-trans-THC} \] at the dose of
10 mg/kg, in agreement with the different potencies measured in
vitro for CB1R activation. The pharmacological effects of PET and
THC diastereoisomers were fully abolished by pretreatment with the
CB1 receptor antagonist rimonabant (SR1) at 5 mg/kg, indicating
that the tetrad is dependent on CB1 receptor activation in the brain.

In vitro pharmacology of PET and \[ \Delta^9\text{-THC} \] isomers
on cannabinoid receptors and
endocannabinoid-degrading enzymes

At the human cannabinoid receptor type 1 (CB1R), the natural prod-
cis-PET exhibited a \( K_I = 481 \text{ nM} \), while its diastereoisomer
\[ \text{trans-PET} \] was more potent (\( K_I = 127 \text{ nM} \)) (see Table 1 and fig. S1).
Similarly, at the human peripheral cannabinoid receptor type 2
(CB2R), \( K_I \) values of 225 and 126 nM for \[ \text{cis- and trans-PET} \] were measured,
respectively. These differences parallel the lower affinity
\[ \Delta^9\text{-cis-THC} \] compared to \[ \Delta^9\text{-trans-THC} \]. \[ \Delta^9\text{-cis-THC} \] is a minor
 Cannabinoid in cannabis (16) and a moderately active CB1R partial
agonist (Table 1), in agreement with the in vivo data reported for
racemic \[ \Delta^9\text{-cis-THC} \] prior to the identification of CB receptors
(17). Thus, the benzyl substituent in PET does not fundamentally
alter the binding relationships at CB receptors. In \[ \text{[35S]} \text{GTP} \gamma \text{S} \] binding assays, both PETs were partial CB1R agonists such as the
\[ \Delta^9\text{-THCs} \], reaching 60 to 80% of the maximal efficacy of the known
full CB1R/CB2R agonist CP55,940 (see Table 1 and fig. S2). At the
CB1R functional level, \[ \text{cis-PET} \] was about 10 times less potent than
\[ \Delta^9\text{-trans-THC} \] [half maximal effective concentration (EC\(_{50}\)) = 406 nM
versus 43 nM] (fig. S2A). Similarly, \[ \Delta^9\text{-trans-THC} \] showed a lower
EC\(_{50}\) value at CB2R compared with \[ \text{cis-PET} \] (12 nM versus 167 nM
(fig. S2B). \[ \text{cis-PET} \] was slightly more potent at CB2R than
\[ \text{trans-PET} \], revealing a distinct stereoschmial bias toward CB2R over
CB1R at the functional level as compared with the corresponding
\[ \Delta^9\text{-THCs} \]. At CB2R, all cannabinoids were inefficient partial agonists
despite their nanomolar receptor binding affinities. No relevant ef-
fects on the major endocannabinoid-degrading enzymes fatty acid
amid hydrolase (FAAH), monoacyl glycerol lipase (MAGL), and
\( \alpha/\beta \) hydroxases 6 and 12 (ABHD6 and ABHD12) were observed in
vitro below 10 \( \mu \text{M} \) (table S1).

In vivo pharmacology of cis- and trans-PET

The potential cannabimimetic effects of PET diastereoisomers were
assessed in vivo in a battery of four tests typically associated with
CB1R activation in mice (hypothermia, catalepsy, hypolocomotion,
and analgesia), collectively referred to as the “tetrad” (14). Experi-
ments using various (i.e., equipotent to \[ \Delta^9\text{-trans-THC} \] doses showed that
\[ \text{cis-PET} \] (50 mg/kg) and \[ \text{trans-PET} \] (40 mg/kg) elicited the full
tetrad in BALB/c mice upon intraperitoneal injection (Fig. 4). The
magnitude of hypothermia, catalepsy, hypolocomotion, and anal-
gesia was in the same range as seen with \[ \Delta^9\text{-trans-THC} \] at the dose of
10 mg/kg, in agreement with the different potencies measured in
vitro for CB1R activation. The pharmacological effects of PET and
THC diastereoisomers were fully abolished by pretreatment with the
CB1 receptor antagonist rimonabant (SR1) at 5 mg/kg, indicating
that the tetrad is dependent on CB1 receptor activation in the brain.

LC-MS/MS quantification of cis- and trans-perrottetinene
PET, endocannabinoids, prostaglandins, and arachidonic
acid levels in mouse brain

We developed a liquid chromatography–tandem mass spectrom-
etry (LC-MS/MS) method to quantify \[ \Delta^9\text{-trans-THC} \], cis-PET, and
\[ \text{trans-PET} \] in the brain. Endocannabinoid levels and other
N-acylethanolamines (NAEs), arachidonic acid (AA), prostaglandin
\( D_2 \) (PGD\(_2\)), and prostaglandin \( E_2 \) (PGE\(_2\)) were quantified to evalu-
ate potential biochemical changes upon cannabinoid exposure (Fig. 5
and fig. S3). We measured \[ \Delta^9\text{-trans-THC} \], cis-PET, and \[ \text{trans-PET} \]
concentrations of 0.24, 1.64, and 0.80 nmol/g, respectively, in total
brain 1 hour after injection (Fig. 5A). This corresponds to a concentra-
tion of 197 nM for \[ \Delta^9\text{-trans-THC} \], 1350 nM for \[ \text{cis-PET} \], and
662 nM for \[ \text{trans-PET} \], in line with the bioactive concentrations for
CB1R activation (Table 1). Correlation between brain penetration
and behavioral pharmacology was also observed for \[ \Delta^9\text{-trans-THC} \]
(Fig. 5A), in line with the literature (18). In plasma, the concentra-
tions of \[ \Delta^9\text{-trans-THC} \], cis-PET, and \[ \text{trans-PET} \] were 0.47, 1.79, and
1.56 nmol/ml, respectively (fig. S4). These results show that
\[ \Delta^9\text{-trans-THC} \] and \[ \text{PET diastereoisomers rapidly and efficiently accu-
mulate in the brain with } C_{\text{brain}}/C_{\text{plasma}} \text{ ratios (} K_P \text{ values) of 0.51 for }
\[ \Delta^9\text{-trans-THC} \], 0.91 for cis-PET, and 0.51 for \[ \text{trans-PET} \]. Animals
treated with \[ \Delta^9\text{-trans-THC} \], cis-PET, and \[ \text{trans-PET} \] did not display
any significant modulation of the endocannabinoids anandamide
(AEA) and 2-arachidonoyl glycerol (2-AG) levels in the brain as
compared with vehicle (Fig. 5, B and C). Similarly, the brain levels
of AA were not affected by the treatments (Fig. 5D). Unexpectedly,
cis- and \[ \text{trans-PET} \] significantly reduced the basal levels of PGD\(_2\)
and PGE\(_2\) in the brain in a CB1 receptor–dependent manner (Fig. 5,
E and F). In sharp contrast, no significant acute effects on
prostaglandins were observed in \[ \Delta^9\text{-trans-THC} \]–treated animals. Given the
wide-reaching importance of prostaglandins in the brain (19, 20),
these pharmacological effects notably differentiate PET from
\[ \Delta^9\text{-trans-THC} \].

cis-PET pharmacology on major CNS targets

To characterize the in vitro pharmacology of cis-PET, we assessed
this novel cannabinoid in a screening panel of 44 CNS targets, includ-
ing G protein–coupled receptors (GPCRs), ion channels, nuclear
receptors, membrane transporters, and enzymes. At the concentration of 10 μM (blue line), cis-PET showed the highest binding affinity toward CB1R and CB2R (≥90%), followed by serotonin receptors 5-HT2A (86%) and 5-HT2B (85%), cholecystokinin receptor (CCK1; 84%), dopamine transporter (DAT; 83%), and L-type Ca²⁺ channel (80%) (Fig. 6). At a concentration of 1 μM, cis-PET still binds significantly to CB1R (68%) and CB2R (75%), while it markedly lost affinity for 5-HT2A (5%), 5-HT2B (30%), CCK1 (32%), DAT (27%), and L-type Ca²⁺ channel (9%) (Fig. 6, orange line). This is in agreement with the full CB1R dependence of the cis-PET effects in vivo obtained in the behavioral tests (Fig. 4, tetrad) and modulation of prostaglandin levels in mouse brain (Fig. 5, E and F). Last, these data are also in perfect agreement with the measured brain concentration of cis-PET, which reached approximately 1.4 μM at the dose.
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of 50 mg/kg (Fig. 5A). \( \Delta^9 \)-trans-THC has been shown to interact with other targets beyond CB receptors at concentrations between 1 and 10 \( \mu \)M, including 5-HT2A receptors, DAT, Ca\(^{2+} \) channels, Na\(^+ \) channels, Kv channels, and monoamino oxidase (MAO) (21–23). In addition, \( \Delta^9 \)-trans-THC inhibited 5-HT transporter (SERT) and norepinephrine transporter (NAT) at concentrations <1 \( \mu \)M (21, 22).

To directly compare \( \text{cis-PET} \) and \( \Delta^9 \)-trans-THC, the latter was also assessed in the screening panel of 44 CNS targets at 1 \( \mu \)M (Fig. 6). The results indicate specific binding to CB1R (95%) and CB2R (90%), with only minor effects on CCK1 (40%), DAT (22%), and histamine 1 receptor (H1; 20%). Neither \( \Delta^9 \)-trans-THC nor \( \text{cis-PET} \) inhibited cyclooxygenase-2 (COX-2) at physiological concentrations (Fig. 6 and fig. S5).

**DISCUSSION**

The isolation of the liverwort-derived bibenzyl cannabinoid \( \text{cis-PET} \) was first reported in 1994 (5), although in 1988, Crombie and co-workers already predicted \( \text{cis-PET} \) as a natural product (24). Possibly because of a lack of isolated pure substance, no research was conducted on its biological activity. In recent years, reports on *Radula* as legal high have accumulated. Our study shows that \( \text{cis-PET} \) is a moderately potent but efficacious psychoactive cannabinoid identified outside the *Cannabis* genus, in agreement with the reported recreational use of *R. marginata*, which is endemic to New Zealand and Tasmania. Like \( \Delta^9 \)-trans-THC, \( \text{cis-PET} \) occurs in the plant as acid (5, 6), which is progressively decarboxylated upon drying or smoking. Dried *R. marginata* collected in the wild is currently sold in the internet as legal high, making reference to \( \text{cis-PET} \) being structurally similar to THC (see Acknowledgments). The online community reporting about legal highs both affirms and refutes the anecdotes regarding the cannabis-like effects of smoked *R. marginata*. Given the uncertain amounts of \( \text{cis-PET} \) in the purchasable preparations and the lack of information regarding the pharmacology of \( \text{cis-PET} \), we synthesized both PET diastereoisomers and tested them in vitro and in mice. We show that \( \text{cis-PET} \) is readily bioavailable to the brain in mice (\( K_p \) value of 0.91) and selectively binds to CB1 and CB2 cannabinoid receptors CB1R and CB2R at nanomolar concentrations in vitro, without affecting the other components of the ECS and 44 CNS targets including GPCRs, ion channels, nuclear receptors, membrane transporters, and enzymes. In vivo, \( \text{cis-PET} \) induced analgesia, catalepsy, hypolocomotion, and hypothermia (collectively called tetrad) in a CB1R-dependent manner similarly to \( \Delta^9 \)-trans-THC. As in the case of the THC scaffold, the \( \text{cis-PET} \) isomer is a less potent CB1R partial agonist than the \( \text{trans-PET} \). Unexpectedly, the PET diastereoisomers differed pharmacologically from \( \Delta^9 \)-trans-THC as it significantly reduced basal PGD\(_2\) and E\(_2\) levels in the brain in a CB1R-dependent manner, potentially limiting its adverse effects (20) and reducing neuroinflammation (19, 25). The interplay between CB1R activation by cannabinoids

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Fig. 5. Brain concentrations of \( \Delta^9 \)-trans-THC and PET diastereoisomers and induced biochemical changes. LC-MS/MS quantification of (A) \( \Delta^9 \)-trans-THC (red), \( \text{cis-PET} \) (green), and \( \text{trans-PET} \) (blue); (B) AEA; (C) 2-AG; (D) AA; (E) PGD\(_2\); and (F) PGE\(_2\) in the brain of male BALB/c mice 1 hour after intraperitoneal injection. The effects of PET diastereoisomers on PGD\(_2\) and PGE\(_2\) were inhibited by the CB1 receptor antagonist rimonabant (SR1). Doses are expressed in mg/kg. Data show means ± SD. Groups were compared with the vehicle-treated control group using a one-way ANOVA following Tukey’s post hoc test \( n = 5 \) to 15 mice per group. **\( P < 0.01 \), *\( P < 0.05 \) versus vehicle or as reported by the arches. ns, not significant.
and modulation of PGE₂ has been emphasized previously. Activation of CB₁R by Δ⁹-trans-THC and CP55,940 was shown to induce COX-2 expression in different cell types, including neurons and astrocytes (20, 26, 27). Chen and colleagues (20) reported that Δ⁹-trans-THC acutely increases COX-2 expression in the brain in a CB₁R-dependent mechanism by specifically recruiting a Gᵢ subunit-mediated intracellular signaling, while 2-AG led to CB₁R-dependent COX-2 suppression via the Gᵢ subunit. Moreover, in vivo, Δ⁹-trans-THC (10 mg/kg) strongly increased basal PGE₂ levels in the hippocampal region, giving rise to central adverse effects (20). Using the same dose of Δ⁹-trans-THC, in our experiments we could not detect a significant change of basal PGD₂ and PGE₂ levels in mouse whole brain (Fig. 5, E and F). Conversely, PET diastereomers specifically reduced basal PGD₂ and PGE₂ levels without affecting COX-2 activity. It remains to be determined whether PET recruits the CB₁R-mediated transduction pathways reported for 2-AG, which also reduces basal prostaglandin levels (19, 28). Recent studies have provided strong evidence that low-dose Δ⁹-trans-THC treatment may still be beneficial with reduced central side effects (29, 30). Thus, the less potent PET diastereoisomers showing additional effects on brain prostaglandin levels exhibit pharmacological characteristics of potential phytocannabinoids. The inhibition of constitutive prostaglandin production is not associated with toxic effects in the brain, while in the periphery, it may induce an impairment of the renal plasma flow, possibly leading to kidney injury (31–33). Although the moderate but significant inhibition of brain prostaglandin levels induced by cis-PET is not comparable to the pharmacological blockage of COX-2 activity elicited by nonsteroidal anti-inflammatory drugs, the reduction of brain prostaglandins may contribute to the neuropharmacology of this new cannabinoid (30). Our data also provide additional important information about the functional consequences of Δ⁹-THC side-chain modifications (34, 35). Using stereodivergent total synthesis, after more than 20 years from the first report of cis-PET, this study finally uncovers the underlying molecular mechanism of action. This gives a rationale for the recreational use of Radula liverworts and provides a molecular basis on which to assess the prospective toxicity and usefulness of the plant material.

Moreover, the findings on the pharmacological effects of cis-PET provide a marked example of convergent evolution of bioactive plant secondary metabolites in the plant kingdom. Despite the existence of more than 20,000 bryophyte species globally, cis-PET is the first psychoactive constituent in this group of plants. Recently, putative genes involved in the biosynthesis of cis-PET were described (8). The abuse potential of Radula species is probably low, given the relatively moderate potency and low abundance of cis-PET (0.7 to 7% in an extract) (5, 6) as compared with marijuana, with Δ⁹-trans-THC contents typically higher than 10% (36). Nevertheless, cis-PET could be an interesting lead for drug development as it might have fewer side effects than Δ⁹-trans-THC and reduces prostaglandins. The likely relevance of signaling-specific CB₁R pathways (37), in particular related to the reduced prostaglandin biosynthesis observed with the bibenzyl PET scaffold, will need to be studied in preclinical disease models.
Infrared spectra were measured neat on a PerkinElmer UATR Two Chemistry at the Eidgenössische Technische Hochschule Zürich (ETHZ). The NMR spectrometry service operated by the Laboratory of Organic Chemistry at the ETHZ on a Bruker AV 101-MHz spectrometer and are reported in parts per million (ppm), with the solvent resonance used as the internal standard (CDCl₃ at 7.26 ppm). Peaks are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, or m = multiplet, coupling constant(s) in Hz, integration. ¹³C NMR spectra were recorded with ¹H decoupling on a Bruker AV 101-MHz or Bruker AV 151-MHz spectrometer and are reported in parts per million, with the solvent resonance used as the internal standard (CDCl₃ at 77.16 ppm). Peaks are reported as follows: s = singlet, d = doublet, coupling constant(s) in Hz. ¹³F NMR spectra were recorded with ¹H decoupling on a Bruker AV 377-MHz or Bruker AV 471-MHz spectrometer and are reported in parts per million. Peaks are reported as follows: s = singlet, d = doublet, coupling constant(s) in Hz. Unless otherwise noted, all NMR measurements were conducted at room temperature. NMR service measurements were obtained at the NMR spectrometry service operated by the Laboratory of Organic Chemistry at the Eidgenössische Technische Hochschule Zürich (ETHZ). Infrared spectra were measured neat on a PerkinElmer UATR Two Spectrometer. The tubes were pre-extracted with a mixture of dry ice and methanol. After extraction, the tubes were washed with 70% ethanol and 100% methanol. The final step was to dry the tubes at 100°C for 30 min. The tubes were then ready for use.

**Radioligand binding assays on cannabinoid receptors**

The assay was performed as previously described (40). Briefly, 15 µg of membrane preparation obtained from CHO cells stably transfected with hCB1 or hCB2 receptors were resuspended in 300 µl of binding buffer [50 mM tris-HCl, 2.5 mM EDTA, 5 mM MgCl₂, and fatty acid–free bovine serum albumin (BSA; 0.5 mg/ml) (pH 7.4)] in silanized glass tubes and coincubated with the tested compounds at different concentrations (1 nM to 100 nM). The mixture was kept on ice until the binding reaction was started by adding the tested compound, vehicle (negative control), or CP55,940 (positive control). Nonspecific binding was measured in the presence of 100 nM WIN55,512. After the incubation time, membrane suspensions were rapidly filtered through a 0.5% polyethyleneimine presoaked 96-well microplate bonded with GF/B glass fiber filters (UniFilter-96 GF/B, PerkinElmer Life Sciences) under vacuum and washed 12 times with 150 µl of ice-cold washing buffer. Filters were added to 45 µl of MicroScint-20 scintillation liquid, and radioactivity was measured with a 1450 MicroBeta Trilux top counter. Data were collected from at least three independent experiments performed in triplicate, and the nonspecific binding was subtracted. Results were expressed as [¹³H]CP55,940 bound as percentage of binding in vehicle-treated CHO cells. The Cheng-Prusoff equation was used to calculate the inhibition constant (Kᵢ) values. Data were collected from at least three independent experiments performed in triplicate, and the nonspecific binding was subtracted. Results were expressed as [¹³H]CP55,940 bound as percentage of binding in vehicle-treated CHO cells. The Cheng-Prusoff equation was used to calculate the inhibition constant (Kᵢ) values. Data were collected from at least three independent experiments performed in triplicate, and the nonspecific binding was subtracted. 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**[¹³S]GTPγS binding assay**

The assay was performed as previously described (40). Briefly, 5 µg of clean membrane prepared in-house from CHO-hCB2 and CHO-hCB1 cells was diluted in silanized plastic tubes with 200 µl of GTPγS binding buffer [50 mM tris-HCl, 3 mM MgCl₂, and 100 mM NaCl (pH 7.4)] supplemented with 0.5% of fatty acid–free BSA in the presence of 10 µM GDP and 0.1 nM [³⁵S]GTPγS (1250 Ci/mmol). The mixture was kept on ice until the binding reaction was started by adding the tested compound, vehicle (negative control), or CP55,940 (positive control). Nonspecific binding was measured in the presence of 100 nM GTPγS (Sigma). The tubes were incubated at 30°C for 90 min under shaking, and then, they were put on ice to stop the reaction. An aliquot (185 µl) of the reaction mixture was rapidly filtered through a 96-well microplate bonded with GF/B glass fiber filters (UniFilter-96 GF/B, PerkinElmer Life Sciences) previously precoated with ice-cold washing buffer [50 mM
Enzyme inhibition assays for FAAH, MAGL, ABHD6, ABHD12, and COX-2

The enzyme inhibition assays were performed as previously published (40). Briefly, for AEA and 2-OG hydrolysis, different concentrations of cis-PET, trans-PET, and Δ⁹-trans-THC were preincubated with 200 μg of pig brain homogenate in tris-HCl buffer (pH 7.6) supplemented with 0.1% of fatty acid-free BSA for 20 min at 37°C. Then, 0.1 μM AEA or 1 μM 2-OG containing trace amounts of [ethanolamine-1-3H]-AEA (1 nM) or [glycerol-1,2,3-3H]-2-OG (1 nM), respectively, were incubated with the mixtures for another 15 min at 37°C. The reaction was stopped by adding two volumes of ice-cold CHCl₃:MeOH mixture (1:1) and centrifuged at 16,000g for 10 min at 4°C. The aqueous phase was collected, and the radioactivity was measured by liquid scintillation spectroscopy.

hABHD6 and hABHD12 activity was determined using cell homogenates from hABHD6 and hABHD12 stably transfected human embryonic kidney (HEK) 293 cells. Different concentrations of cis-PET, trans-PET, and Δ⁹-trans-THC were preincubated with 40 μg of cell homogenate for 30 min at 37°C in assay buffer [1 mM tris, 10 mM EDTA, 0.1% BSA (pH 7.6)]. Dimethyl sulfoxide (DMSO) was used as vehicle control, and 10 μM WWL70 or 20 μM tetrahydropyrrolactin (THL) as positive controls. Then, 10 μM 2-OG was added and incubated for 5 min at 37°C. The reaction was stopped by the addition of 400 μl of ice-cold CHCl₃:MeOH (1:1). The samples were vortexed and centrifuged (16,000g, 10 min, 4°C). Aliquots (200 μl) of the aqueous phase were assayed for tritium content by liquid scintillation spectroscopy. Blank values were recovered from tubes containing no enzyme.

Basal 2-OG hydrolysis occurring in nontransfected HEK293 cells was subtracted. Inhibition of human recombinant COX-2 was assessed using an in-house validated COX fluorescent inhibitor screening assay kit from Cayman Chemical Europe as previously published (40).

In vitro pharmacological screening
cis-PET and Δ⁹-trans-THC were profiled in a Cerep (Eurofins Pharma Discovery Services) screening panel of 44 targets including GPCRs, ion channels, nuclear receptors, membrane transporters, and enzymes mostly expressed in the CNS. cis-PET was tested at 1 and 10 μM, while Δ⁹-trans-THC was tested only at 1 μM. Data presented were obtained from two independent experiments performed in duplicate. The GPCRs tested were as follows: α₁A adrenergic receptor, α₂A adrenergic receptor, α₂B adrenergic receptor, β₁ adrenergic receptor, β₂ adrenergic receptor, CB₁R, CB₂R, D₁ dopamine receptor type 1, D₂ dopamine receptor type 2, H₁ histamine receptor type 1, H₂ histamine receptor type 2, delta-type opioid receptor, mu-type opioid receptor, kappa-type opioid receptor, 5-HT₁A serotonin receptor, 5-HT₁B serotonin receptor, 5-HT₂A serotonin receptor, 5-HT₂B serotonin receptor, 5-HT₃ serotonin receptor, M₁ muscarinic receptor, M₂ muscarinic receptor, M₃ muscarinic receptor, A₂A adenosine receptor, ETA endothelin receptor, CCK₁ cholecystokinin A receptor, and V₁A vasopressin/oxytocin receptor. Ion channels were as follows: GABAₐ channel at the benzodiazepine (BZD), α₄β₂ acetylcholine ion channel, Cav₁.2 (l-type) calcium ion channel (dihydropyridine site), Kv voltage-gated potassium ion channel, Na⁺ voltage-gated sodium channel, human ether-a-go-go-related gene (hERG) potassium channels, N-methyl-d-aspartate (glutamate) receptor. Nuclear receptors were as follows: androgen receptor and glucocorticoid receptor. Enzymes were as follows: MAO-A, acetylcholinesterase, lymphocyte-specific protein tyrosine kinase phosphodiesterase 3A, phosphodiesterase 4D2, COX-1, and COX-2. Membrane neurotransmitter transporters were as follows: noradrenaline transporter, DAT, and serotonin transporter.

Animals
In vivo experiments were performed in accordance with the Swiss Federal guidelines, which agree with the Institutional Animal Care and Use Committee (IACUC) guidelines. Male BALB/c mice (8 to 10 weeks old) were provided by Janvier Labs (St Berthevin, France). Mice were housed in groups of five per cage in a specific pathogen-free unit under controlled 12-hour light/12-hour dark cycle (ambient temperature, 21° ± 2°C; humidity, 40 to 50%) with free access to standard rodent chow and water. The mice were acclimatized to the animal house for 1 week before the experiments.

Tetrad test
Compounds were dissolved in pure DMSO and administered intraperitoneally at different doses using five to eight mice for each treatment group. cis-PET, trans-PET, and Δ⁹-trans-THC were administered 1 hour before assessing locomotion, catalepsy, body temperature, and analgesia (collectively referred as tetrad test). The rectal temperature was measured before (basal) and 1 hour after injection with a thermocouple probe (1 to 2 cm; Testo AG, Switzerland), and the change in rectal temperature was expressed as the difference between basal and postinjection temperatures. Catalepsy was measured using the bar test, where mice were retained in an imposed position with forelimbs resting on a bar 4 cm high; the end point of catalepsy was considered when both front limbs were removed or remained over 120 s. Locomotion was determined using the rotarod test; animals were placed on the rotarod (Ugo Basile, Italy) at 6 rpm, and the latency to fall was measured with a cutoff time of 120 s. Catalepsy and locomotion were measured in three trials. The hot plate test was performed to evaluate analgesia using a 54°C to 56°C hot plate (Thermo Scientific, Waltham, Massachusetts, USA) with a Plexiglas cylinder. The latency to the first nociceptive response (paw lick or foot shake) was measured.

LC-MS/MS quantifications of PET, THC, AEA, 2-AG, PEA, LEA, OEA, AA, PGD₂, and PGE₂ in mouse brain tissue
LC-MS/MS analyses were performed on a Shimadzu UFLC coupled to a TripleQuad 4000 QTRAP mass spectrometer (ABSciex Concord, Ontario, Canada). AEA, 2-AG, OEA, PEA, LEA, AA, PGE₂, and PGD₂ were quantified in mouse brains using our recently published method (40). For the analysis of cis-PET, trans-PET, and Δ⁹-trans-THC, a Reprosil Fluosil 100 PFP (particle size, 3 μm; 2 mm by 50 mm; Dr. A. Maisch, Germany) column was used in reversed-phase mode with gradient elution starting with 95% of phase A (0.1% formic acid and 2 mM ammonium acetate in water) and 5% of phase B (0.1% formic acid in acetonitrile). The amount of phase B was linearly increased to 40% at 3 min, then to 58% at 8 min and kept for 1 min, and then increased linearly again to 80% at 10 min;
the column was flushed for 3.5 min with 99% of phase B with subsequent reequilibration at 5% for a further 3 min. The total analysis time was 17 min at 40°C, the flow rate was 0.3 ml/min, and the injection volume was 10 μl. These LC conditions provided the chromatographic resolution of cis- and trans-PET prior to MS/MS (see fig. S6 for the chromatogram), where both isomers ([M+H]+ of 349 m/z) showed the same fragmentation pattern (m/z = 293, 267, 227). The TurboLion Spray interface was operated in positive mode. The MS parameters of the ESI source were as follows: capillary voltage, 4.5 kV; temperature, 600°C; nebulizer pressure, 50 psi. Peaks were integrated using the Analyst software version 1.5 (AB Sciex Concord, Ontario, Canada). Because of the lack of a deuterated version for interest molecules and the chemical similarity between Δ⁹-trans-THC and both PET isomers, we used Δ⁸-trans-THC as internal standard (IS) for cis- and trans-PET, whereas cis-PET was used as IS for Δ⁹-trans-THC. The ratio of peak area of analyte to peak area of IS was used to ensure linearity of the calibrations. The slope, intercept, and regression coefficient of those calibration lines were determined. The concentrations of PET diastereoisomers and Δ⁹-trans-THC in the brain and plasma were calculated applying a model previously described (40). The ratios between brain and plasma concentrations (Kp) of Δ⁹-trans-THC, cis-PET, and trans-PET were calculated as previously reported (40).

Sample preparation
Mouse brains were divided into two sagittal hemispheres, and one hemisphere was used for LC-MS/MS analyses. Half brains were weighted when still frozen and transferred to 2-ml microcentrifuge vials (XXTuff Microvials, BioSpec, Oklahoma, USA) with three chrome-steel beads (diameter, 2.3 mm; BioSpec, Oklahoma, USA) and 1.5 ml of 0.1 M formic acid. Samples were homogenized using a 24-position Mini bead beater (BioSpec Products Inc., Bartlesville, OK, USA) for the extraction of administered molecules (cis-PET, trans-PET, and Δ⁹-trans-THC), as well as for endogenous molecules AEA, 2-AG, PEA, OEA, LEA, PGE₂, PGD₂, AA, corticosterone, and progesterone using a previously described extraction protocol (40). For the quantification of cis-PET, trans-PET, and Δ⁸-trans-THC, a different extraction method was used. Briefly, 200 μl of brain homogenate or plasma was rapidly transferred to a silanized Eppendorf tube containing 400 μl of acetone and 400 ng/ml of the designed internal standard. Samples were centrifuged at 13,200 rpm for 10 min at 4°C to precipitate proteins, and the supernatant was further extracted with 1 ml of ethyl acetate:hexane (9:1) 0.1% formic acid strongly vortexed for 30 s and sonicated (Sonicator Merck-Gruppe) in a cold bath for 5 min. Samples were centrifuged at 13,200 rpm for 10 min at 4°C and kept for 1 hour at −20°C to freeze the lower aqueous phase. The upper organic phase was recovered in plastic tubes and dried into a speed vacuum. The extracts were reconstituted in 50 μl of acetonitrile with 20% of water, and 10 μl was injected in the LC-MS/MS system.

Statistical analyses
Data were collected from at least three independent experiments each performed in triplicate. Results are expressed as mean values and standard error deviation. The statistical significance difference among groups was determined by the Student’s t test (paired, one-tailed, or two-tailed t test) or one-way ANOVA followed by a Tukey’s post hoc test. Statistical differences between the treated and control groups were considered as significant if p < 0.05. GraphPad 5.0 software was used to fit the concentration-dependent curves and for the statistical analysis.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/10/eaat2166/DC1
Fig. S1. Radioligand displacement assay using [³H]CP55,940 and membranes from CHO cells stably transfected with human CB1R and CB2R, respectively.
Fig. S2. [¹⁴C]GTPγS binding curves for THC and PET stereoisomers on human cannabinoid receptors.
Fig. S3. LC-MS/MS quantifications of the NAEs OEA, PEA, and LEA in the brain of BALB/c male mice 1 hour after intraperitoneal injection of cannabinoids.
Fig. S4. LC-MS/MS quantification of Δ⁹-thc, cis-PET, and trans-PET in plasma of BALB/c male mice 1 hour after intraperitoneal injection of cannabinoids.
Fig. S5. Inhibition of COX-2 activity.
Fig. S6. Representative LC-MS/MS reaction monitoring chromatogram of reference standards in mouse brain tissue showing LC separation of cis- and trans-PET.
Table S1. Profiling of THC and PET diastereoisomers on endocannabinoid-degrading enzymes in vitro.

Synthesis and characterization of products
Preparation of allylic alcohol 1 Preparation of (+)-cis-PET (1R)-PET Preparation of (−)-trans-PET (1R)-PET SFC traces

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Acknowledgments: This paper is dedicated to Prof. Raphael Mechoulam, the father of modern cannabinoid research, on the occasion of his 86th birthday and to Prof. Yoshinori Asakawa, pioneer in phytochemical studies of liverworts, on the occasion of his 77th birthday. The recreational R. marginata material was found at www.botanicalspirit.com/radula-marginata and www.fastincense.com/Remarkable-Herbs-Radula-Marginata-10g.aspx (internet sites accessed on 06 September 2018). We would like to acknowledge C. Arena and P. Schenker for performing some replicates of the [35S]GTPγS assays, M. Dalghi Gen for performing the ABHD measurements, D. Pellegrato for optimizing analytical methods, and R.-P. Charles and J. M. V. Paredes for support with animal experiments and dissections. We are grateful to G. Appendino for bringing this topic to our attention. S. Fischer is acknowledged for the picture of R. perrottetii. Funding: J.G. thanks the Forschungsstiftung University of Bern and NCCR TransCure. E.M.C. and J.G. thank the Swiss National Foundation for support of this work in part (200020_1522898 and 163359). Author contributions: J.G. and E.M.C. conceived the idea. J.G., A.C., E.M.C., and M.A.S. designed the study and led the writing and revision of the manuscript. E.M.C. led and M.A.S. and R.E. performed the syntheses and prepared cannabinoids. A.C. and J.G. led the in vitro and in vivo pharmacological experiments. A.C. performed the receptor assays, and I.R.-M. and V.P. carried out the pharmacokinetic measurements. I.R.-M. performed the behavioral tests, and A.C. led the statistical analyses. A.C. performed the receptor assays, and I.R.-M. and V.P. carried out the pharmacokinetic measurements. I.R.-M. performed the behavioral tests, and A.C. led the statistical analyses. All coauthors revised the several versions of the manuscript and contributed to their improvement. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data are available from authors upon request.

Submitted 13 June 2018
Accepted 19 September 2018
Published 24 October 2018

10.1126/sciadv.aat2166

Citation: A. Chicca, M. A. Schafroth, I. Reynoso-Moreno, R. Erni, V. Petrucci, E. M. Carreira, J. Gertsch, Uncovering the psychoactivity of a cannabinoid from liverworts associated with a legal high. Sci. Adv. 4, eaat2166 (2018).
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Sci Adv 4 (10), eaat2166.
DOI: 10.1126/sciadv.aat2166